Inverse agonism and neutral antagonism at wild-type and constitutively active mutant delta opioid receptors

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Running title:
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Text: 35 pages
Tables: 2
Figures: 5
References: 40
Abstract: 224 words
Introduction: 740 words
Discussion: 1366 words

Abbreviations
BNTX, 7-benzylidene-naltrexone; CAM, constitutively active mutant; CPM, cyclopropylmethyl, Dmt-Tic, 2’,6’-dimethylyrosine-1,2,3,4-tetrahydroquinoline-3-carboxylate; GPCR, G-protein-coupled receptor; GTPγS, guanosine 5’-O-(3-thio)triphosphate; HEK, human embryonic kidney; ICI 174864, N,N-diallyl-Tyr-Aib-Aib-Phe-Leu; NLX, naloxone; NTB, nalttriben; NTI, nalttrindole; SEAP, SEcreated Alcalin Phosphatase; SNC 80, (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide; TIPP, H-Tyr-Tic-Phe-Phe-OH; TICP(Ψ), H-Tyr-TicΨ[CH2N]Cha-Phe-OH; WT, wild-type.

Section option: Neuropharmacology
Abstract

The delta opioid receptor modulates nociceptive and emotional behaviors. This receptor has been shown to exhibit measurable spontaneous activity. Progress in understanding the biological relevance of this activity has been slow, partly due to limited characterization of compounds with intrinsic negative activity. Here we have used Constitutively Active Mutant (CAM) delta receptors in two different functional assays ([35S]GTPγS binding and a reporter gene assay) to test potential inverse agonism of fifteen delta opioid compounds, originally described as antagonists. These include the classical antagonists naloxone, naltrindole, 7-benzylidene-naltrexone and naltriben, a new set of naltrindole derivatives, TIPP and TICP(Ψ) as well as three Dmt-Tic peptides. A reference agonist (SNC 80) and inverse agonist (ICI 174864) were also included. In a screen using wild-type and CAM M262T delta receptors, NTI and close derivatives were mostly inactive, TIPP behaved as an agonist while Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2 showed inverse agonism. The two latter compounds showed negative activity across twenty-seven CAM receptors, suggesting that this activity was independent from the activation mechanism. These two compounds also exhibited nanomolar potencies in dose-response experiments performed on wild-type, M262T, Y308H and C328R CAM receptors. TICP(Ψ) exhibited strong inverse agonism at the Y308H receptor. We conclude that (i) the stable N,N(CH3)2-Dmt-Tic-NH2 compound represents a useful tool to explore the spontaneous activity of delta receptors and (ii) NTI and novel derivatives behave as neutral antagonists.
Evidence that a number of G Protein Coupled Receptors (GPCRs) show spontaneous activity has accumulated in the past years and the existence of high basal activity has been proposed for more than sixty GPCRs (Seifert and Wenzel-Seifert, 2002). Key tools to investigate receptor basal signaling are ligands that block this activity, the so-called inverse agonists (Milligan, 2003). In contrast to neutral antagonists, which compete with agonists but are devoid of biological activity per se, inverse agonists are endowed with intrinsic negative activity (Strange, 2002). Differential activity of inverse agonists and neutral antagonists was reported in recombinant systems, and was also evidenced in physiological situations (de Ligt et al., 2000).

The concept of functional inverse agonism was pioneered with delta opioid receptors, which belong to the opioid receptor family comprising also mu, kappa and ORL receptors (Kieffer, 1997). The finding that \( N,N \)-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) (Cotton et al., 1984), originally considered as delta antagonist, was able to decrease basal GTPase activity in NG108-15 neuroblastoma cells, while other antagonists had no effect in this functional assay, was the first demonstration of negative intrinsic activity at an endogenously expressed GPCR (Costa and Herz, 1989). The existence of delta receptor spontaneous activity was further confirmed using different functional assays, in cell lines expressing either the endogenous (Szekeres and Traynor, 1997) or the recombinant delta receptor expressed in HEK 293 (Chiu et al., 1996; Labarre et al., 2000; Zaki et al., 2001), CHO (Hosohata et al., 1999), rat-1 fibroblast (Merkouriis et al., 1997; Mullaney et al., 1996), GH3 (Liu and Prather, 2002) or C6 glioma cells (Neilan et al., 1999). From all these studies ICI 174864 appeared to show reliable and potent negative activity, and was taken as the reference molecule for delta inverse agonism. ICI 174864 however is a peptidic ligand, which shows low potency and limited delta selectivity towards mu and kappa opioid receptors (K_i ratios 1/155 and 1/344,
(Corbett et al., 1993). The identification of novel stable agonists, with improved pharmacological properties, is therefore highly desirable to study the biological activity of delta receptors in physiological context. (Filliol et al., 2000; Kieffer and Gavériaux-Ruff, 2002).

Basal activity of GPCRs is not easily measurable and the search for inverse agonists has been facilitated by the construction of Constitutively Active Mutant (CAM) receptors where potency and efficacy of inverse agonists are enhanced (Rossier et al., 1999). In a previous study, we have used a random mutagenesis approach to identify human delta CAM receptors (Decaillot et al., 2003). Here we have investigated the in vitro pharmacological activity of a series of delta antagonists from distinct structural families at these CAM receptors. Using two different functional assays, we have examined properties of a number of commercially available delta antagonists.

We have tested four alkaloid opioids that have been classically defined as antagonist compounds, and are widely used in pharmacological studies. These compounds are NLX, considered the reference opioid antagonist, as well as NTI, NTB and BNTX that exhibit reasonable delta selectivity (Corbett et al., 1993) and are structurally related. We also have included delta compounds that have been developed more recently in the search for compounds with better delta selectivity (see Fig. 1). Among them was a new series of NTI derivatives (Schmidhammer et al., 1998) including HS-378, HS-414, HS-464, HS-510A, HS-531 and HS-595. TIPP (Schiller et al., 1992), which was the first peptide from a new class of highly delta selective antagonists, and TICP(Ψ) a more hydrophobic and stable derivative (Schiller et al., 1999) were also tested. Further we have studied three compounds from the Dmt-Tic family where 2’, 6’ methylation of the N-terminal tyrosine residue (Dmt) is combined to the heteroaromatic residue Tic, leading to a class of pseudo-dipeptides with high delta selectivity and antagonist properties (Bryant et al., 2003; Bryant et al., 1998; Salvadori
et al., 1995). Importantly we have included the prototypic delta agonist SNC 80, as well as the reference inverse agonist ICI 174864 in all experiments.

This is the first study that compares the functional activity of a wide range of delta compounds using a set of several CAM delta receptors. Our data show that the highly delta selective ligand $N,N$ (CH$_3$)$_2$-Dmt-Tic-NH$_2$ is most potent and efficacious at all the tested mutant receptors, and seems to be a better inverse agonist than the reference ICI 174864 compound. TICP($\Psi$) also showed inverse agonism, mainly at one mutant receptor. NTI derivatives were otherwise inactive, leaving NTI and closely related analogues as neutral antagonists.
Material and Methods

**Opioid ligands.**[^3H]Diprenorphine was obtained from PerkinElmer (Life Sciences, 50 Ci/mmol). Commercial delta opioid compounds were as follows: SNC 80, ICI 174864, and naloxone (NLX) (all from Sigma, RBI), naltriben (NTB), naltrindole (NTI) and 7-benzylidene-naltrexone (BNTX, Tocris), and H-Tyr-Tic-Phe-Phe-OH (TIPP) where Tic is 1, 2, 3, 4 tetrahydroisoquinoline (Phoenix Pharmaceuticals Inc.). The delta compound H-Tyr-TicΨ[CH2N]Cha-Phe-OH (TICPΨ) was prepared as described in (Schiller et al., 1996). Dmt-Tic molecules were synthesized as reported in (Salvadori et al., 1995). The naltrindole derivatives were synthesized as described (Schutz et al., 2002).

**Expression of wild-type and mutant receptors.** For opioid ligand and [35S]GTPγS binding assays, the wild-type human delta receptor (WT), the CAM M262T and the CAM C328R human delta receptors (Decaillot et al., 2003) were stably expressed in Human Embryonic Kidney (HEK) 293 cells (American Type Culture Collection). Cells were transfected at about 70% confluency with WT or mutated delta receptor expressing vectors (pcDNA3, 8 µg / 10 cm dish) using the Jet-PEI reagent (Polytransfection, Strasbourg, France) according to the manufacturer’s protocol. Forty eight hours after transfection the culture medium, DMEM (Invitrogen) supplemented with 10 % Fetal Calf Serum (Invitrogen) and gentamycin (Invitrogen), was replaced by fresh medium containing the selecting agent G418 (Invitrogen) at a 0.5 mg/ml concentration. Cells were grown at 37 °C in the presence of 5% CO₂. Resistant colonies appeared after two weeks of G418 application. Cellular clones were amplified and screened for receptor expression by [3H]diprenorphine binding on whole cells (0.75 and 1.5 nM, 2 x 10⁵ cells / well). Clones with highest expression levels were amplified for further experiments. The Y308H mutant receptor was expressed transiently in HEK 293
cells. Transfection was performed in DMEM 10% FCS with antibiotics and cells harvested after forty eight hours. For the SEcreted Alkaline Phosphatase (SEAP) assay, all WT and CAM receptors were expressed transiently in HEK 293 cells as previously described (Befort et al., 2001b) with a slightly modified procedure. Briefly, HEK 293 cells were plated in 96 well plates (Biocoat, Falcon) at a density of 35 000 cells / well on the day before transfection. Cells were co-transfected at about 70 % confluency with the reporter gene pCRE-SEAP (Clontech, 1 µg / well) and WT or mutated delta receptor plasmid DNA (0.15 µg / well) using Jet-PEI reagent. Thirty-two hours after transfection, cells were serum-starved overnight and subjected to the SEAP test.

**Ligand binding assay.** HEK 293 cells expressing WT and CAM receptors stably or transiently were harvested for membrane preparation according to the described procedure (Befort et al., 2001b). Membranes were resuspended in 50 mM Tris HCl pH 7.4, 0.32 M sucrose buffer, aliquoted and stored at –80°C until use. Protein concentration was assayed by the Bradford method (Bio-Rad). For saturation experiments 5 µg of membrane proteins were diluted in 50 mM Tris HCl pH 7.4, in a final volume of 250 µl and incubated with variable concentrations (0.02 to 3 nM) of [3H]diprenorphine for 1 h at 25°C. Non specific binding was determined in the presence of 10 µM naloxone. For competition studies 5 µg of membrane proteins were incubated with 0.7 nM (C328R), 1 nM (WT and M262T) or 7nM (Y308H) [3H] diprenorphine, in the presence of variable concentrations (1 pM to 100 µM) of competing opioid ligand for 1h at 25°C. Membranes were washed with cold 50 mM Tris HCl pH 7.4 buffer by filtration through 0.1% polyethyleneimine-presoaked microplate filters (Unifilter GF/B) using a Filtermate Harvester (PerkinElmer). Microplates were dried, then wells were covered by 40 µl scintillation cocktail (PerkinElmer) and counted in a Microplate Scintillation/Luminescence Counter (Packard) using the TopCount-NXT software. Assays
were performed in triplicates for saturation experiments and in duplicates for competition studies. Kd and Ki values of opioid ligands were calculated using the Prism software (GraphPad, San Diego, CA).

**[^35S]GTPγS binding assay.** Opioid ligand-stimulated[^35S]GTPγS binding was performed using the same membranes as above.[^35S]GTPγS binding was carried out essentially as described previously (Befort et al., 1999). Briefly, 10 µg of membrane proteins were incubated for 1 h at 25 °C without (basal) or with opioid ligands (3 pM to 10 µM) in the assay buffer containing 50 mM Hepes, pH 7.6, 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin, 30 µM GDP and 0.2 nM[^35S]GTPγS. For background determination 10 µM non-radioactive GTPγS was used. Incubation mixture was filtered as above (ligand binding section) except that microplate filters were presoaked in water. Plates were dried and radioactivity was counted as above. Assays were performed in triplicates. EC₅₀ values of the various opioid ligands were determined with the Prism software using non-linear regression and one site competition. For each experiment ligand-stimulated[^35S]GTPγS binding values were normalized to the basal value (absence of ligand) defined as 100%.

**SEcreted Alkaline Phosphatase (SEAP) assay.** Cells were co-transfected using receptor and pCRE-SEAP expression vectors as described above (see section "Expression of wild-type and mutant receptors"). Thirty-two hours after transfection cells were serum-starved (0.1 %, instead of 10 % FCS) overnight. Forty-eight hours after transfection cells were stimulated with forskolin alone (FK 10 µM), or with forskolin and the opioid ligand to be tested. Treatments with all ligands were performed in the presence of 0.1% FCS for 5 to 6 h. Aliquots of culture medium supernatants (15 µl) were transferred into black 96-well plate,
diluted into 150 mM NaCl, 40 mM Tris-HCl, pH7.2 buffer and heat-inactivated at 65 °C for
20 min. The enzymatic reaction was performed in the presence of assay buffer (666 mM
diethanolamine, 3.3 mM MgCl₂, 6.6 mM L-homoarginine, pH 9.8), Enhancer solution
(Tropics), and 0.4 mM SEAP chemiluminescent substrate (Tropics) in a final volume of 150
µl. Luminescence was quantified 15 min after substrate addition (TopCount-NXT, Packard).
To compare data from different experiments for each compound and to allow comparison
across mutant receptors, the SEAP signals obtained in the presence of FK and opioid
compounds were normalized relative to signal obtained in the presence of FK alone, defined
as 100%. A prototypic agonist (SNC 80) and the reference inverse agonist (ICI 174864) were
included in each experiment. Dose response experiments were performed with agonist or
inverse agonist concentrations ranging from (3 pM to 100 µM). Assays were performed in
triplicate and EC₅₀ values were calculated using the Prism software as above.

Statistics. In opioid ligand screening experiments (Fig. 2) one-way ANOVA with PLSD
Fischer posteriori test was used to evaluate significant effects of the compounds (p < 0.05).
Results

Screening for inverse agonism at WT and M262T mutant delta receptors. In our previous search for CAM delta receptors, we have identified thirty point-mutated receptors with high spontaneous activity (Decaillot et al., 2003). The mutant M262T exhibited highest responsivity to ICI 174864 in the reporter gene assay, and was therefore chosen as a screening tool in this study. Also, because the mutation is located on the cytoplasmic face of the 6th transmembrane domain and is therefore distant from the binding site, we reasoned that ligand binding should be preserved for most compounds under study. First, we established stable cell lines expressing the WT or M262T receptors. Receptor expression levels were determined by Scatchard analysis of [$^3$H]diprenorphine binding. $B_{max}$ values were 4.38 ± 0.91 pmol / mg for the WT receptor ($n = 2$) and 1.57 ± 0.35 pmol / mg for the M262T receptor ($n = 2$). Basal [$^{35}$S]GTP$\gamma$S binding at the mutant receptor was consistently higher than WT (142.1 ± 7.8 % of WT, $n = 3$) despite a 2.7 fold lower expression level, confirming that M262T is a CAM receptor (Decaillot et al., 2003). [$^3$H]Diprenorphine affinity was unchanged at this mutant receptor (Table 1). In order to examine whether the M262T mutation alters binding affinities of the delta ligands under study, we determined Ki values for each compound (Table 1). Affinity changes never exceeded four-fold, and with the exception of ICI 174864, all compounds exhibited similar nanomolar affinity for WT and M262T mutant receptors. Functional activity of all the compounds was therefore tested.

We first used the classical [$^{35}$S]GTP$\gamma$S binding assay which measures receptor-mediated G protein activation occurring at an early stage of the signaling cascade. In this assay [$^{35}$S]GTP$\gamma$S binding is increased in the presence of agonist and decreased in the presence of inverse agonist. We treated membrane preparations from the two stable cell lines (WT and M262T) with each delta opioid ligand and results are shown on Fig. 2 (top panel).
The agonist SNC 80 increased basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding about two-fold at both WT and mutant receptors. As expected, ICI 174864, the reference delta inverse agonist, decreased the basal signal. In this set of experiments the negative activity of ICI 174864 was low at WT and significant at the M262T mutant receptor, consistent with the expected higher efficacy of inverse agonists at CAM receptors (Decaillot et al., 2003). No significant modification of basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding could be detected for NLX, NTI, BNTX, NTB and TIPP at WT and M262T receptors. Naltrindole derivatives (HS compounds) showed no inverse agonist activity at any receptor, and even showed a trend toward agonism at the WT receptor. Finally the compounds TICP(Ψ), Dmt-Tic-OH and \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) showed significant inverse agonism at preparations expressing the M262T receptor. The three compounds also showed detectable—although not always significant—negative activity at the WT receptor. Altogether therefore the three latter compounds appeared as the most efficacious inverse agonists in this assay.

To further examine bioactivity of the delta opioid compounds, we used the more sensitive high-throughput SEAP reporter gene assay, where the end-point measurement is performed downstream from G Protein activation along the cAMP pathway. In this assay, both a cAMP-responsive reporter gene and the receptor are transiently co-expressed in HEK 293 cells. Forskolin (FK) stimulation directly activates the reporter gene, and the amount of gene product (Secreted Alkaline Phosphatase or SEAP) is measured using a chemiluminescent substrate. Typically, an opioid agonist will decrease the FK-stimulated reporter gene activity (SEAP activity) while an inverse agonist will enhance this activity (Decaillot et al., 2003). Data are shown in Fig. 2 (bottom panel). The delta opioid agonist, SNC 80 decreased the SEAP signal at levels corresponding to 50% (WT) and 70% (M262T) of levels observed in the absence of agonist. ICI 174864 increased the SEAP signal at cells expressing both WT (120% above basal) and M262T (130% above basal) receptors. As in the
[35S]GTPγS binding assay, this activity better detected at the mutant receptor. NLX, BNTX, Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2 exhibited significant inverse agonist activity at the M262T receptor, while only N,N(CH3)2-Dmt-Tic-NH2 markedly increased SEAP activity at the WT receptor. TICP(Ψ), which showed inverse agonism in the [35S]GTPγS assay was inactive in the SEAP assay. Conversely TIPP, which was inactive in [35S]GTPγS assay, exhibited strong agonism in the SEAP test at both receptors. Finally, no significant effect of HS compounds could be observed at WT and M262T receptors, except for a slight agonist effect for HS-595. NTI and NTB were inactive at both receptors as well.

Taken together, screening data from both functional assays and from both WT and M262T CAM receptors led to the following conclusions: (i) TIPP and HS-595 show either no activity or agonist activity, (ii) NTI, NTB and the other HS derivatives show no significant activity in any test, despite their high affinity for the receptors, (iii) NLX and BNTX show inverse agonism in one test and for one receptor only and (iv) as for ICI 174864, Dmt-Tic-OH, N,N(CH3)2-Dmt-Tic-NH2 and TICP(Ψ) behave as inverse agonists. The latter compounds were therefore studied further.

Inverse agonist activity of TICP(Ψ), Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2 across a series of CAM receptors. We examined the negative functional activity of ICI 174864, TICP(Ψ), Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2. First, a control experiment was performed where “empty” HEK 293 cells (transfected with the empty pcDNA3 plasmid) were subjected to these compounds (10-5M) in both [35S]GTPγS binding and SEAP assays. All compounds were inactive (not shown), indicating that their activity is mediated by delta receptors.

We investigated whether ICI 174864, TICP(Ψ), Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2, which show inverse agonist activity at WT and M262T mutant receptors, would
also inhibit the high spontaneous activity of other CAM delta receptors independently from the position of the mutation. To test the activity of the compounds at a large set of mutant receptors, we used the SEAP assay that does not require membrane preparations. We expressed twenty-seven CAM delta receptors from our previous study (Decaillot et al., 2003), and measured the modulation of SEAP responses after TICP(Ψ), Dmt-Tic-OH and \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) stimulation. Each compound was used at a saturating concentration (1 µM) and systematically compared to ICI 174864 (10 µM). Maximal effects are shown in Fig 3. As in our first experiment (Fig 2), the most active inverse agonist was \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\). This compound enhanced the FK-induced SEAP signal at all other CAMs (Fig. 3, top panel). For the stronger effects, the rank of order was M262T > T213S = T134A = Y308H > C328R = E323K = V283A. This effect was more important than the effect of ICI 174864 at most CAM receptors, suggesting that \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) is a more efficacious inverse agonist. As for this compound, the closely related compound Dmt-Tic-OH behaved as a delta inverse agonist at most CAM receptors (Fig. 3, middle panel). For Dmt-Tic-OH, highest levels of activation were obtained at mutant receptors T134A, T213S, V283A and C328R. When data from this ligand were directly compared with those of \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\), the overall efficacy appeared lower (not shown). Also the inverse agonist activity of Dmt-Tic-OH was lower than the reference compound ICI 174864 for mutants D21G, N169S, W274R and E323K, which was not the case for \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\). Dmt-Tic-OH therefore seemed to be a less efficient inverse agonist than \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\). TICP(Ψ) was inactive at most mutants (not shown). The compound showed detectable activity at mutant receptors M262T and C328R. Interestingly the inverse agonist effect of TICP(Ψ) was remarkably active at the Y308H receptor, reaching 220 % of the FK–stimulated signal (Fig. 3, bottom panel). This was the highest value observed across all mutants and for all ligands.
Together, these data show that ICI 174864, TICP(Ψ), Dmt-Tic-OH and \(N,N(CH_3)_2\)Dmt-Tic-NH\(_2\) all exhibit inverse agonism at several CAM delta receptors. This observation confirms that these compounds are endowed with robust negative intrinsic activity, which favors the inactive receptor conformation, even though distinct activation mechanisms are likely to be involved in the different mutants (Decaillot et al., 2003). The four compounds show variable efficacies depending on the CAM receptors. At this stage, comparing the compounds is difficult because these differences could be due either to distinct intrinsic functional activities, or because mutations alter binding affinities of the compounds to variable extents. We therefore focused our attention on the three CAM receptors M262T, Y308H and C328R, which exhibited the highest basal activities (see our previous work, Decaillot et al., 2003), and where inverse agonists showed best activities in this study (Fig. 3).

**Dose-responses to Dmt-Tic compounds at WT, M262T, Y308H and C328R mutant receptors.** To fully characterize agonism and inverse agonism of the most active delta compounds, we first determined their binding affinities at the different receptors (Table 1). We have shown above that opioid binding at M262T delta receptor is comparable to WT receptor. Similarly, Ki values were unchanged at the C328R mutant, consistent with the notion that M262 and C328 residues are located on the cytoplasmic face of the receptor and unlikely involved in ligand binding (Decaillot et al., 2003). In contrast binding affinities were substantially decreased at the Y308H receptor. This was expected because this residue is centrally located within the ligand binding pocket and known to influence opioid affinity (Befort et al., 1996; Befort et al., 1999). Affinities to Y308H, however, remained measurable and dose-responses were also performed for this mutant.

Further, we determined potencies and efficacies using the two functional assays at the selected receptors. For \(^{35}\text{S}\)GTP\(\gamma\)S binding assays, we used stable expression for WT (B\(_{\text{max}}\))
4.38 ± 0.91 pmol / mg protein, n=2), M262T (B<sub>max</sub> 1.57 ± 0.35 pmol / mg protein, n=2), C328R mutants (B<sub>max</sub> 5 pmol / mg protein) and transient expression for Y308H receptor (B<sub>max</sub> 7.99 ± 1.26 pmol / mg protein, n = 2). For the SEAP assay receptors were expressed transiently, as before.

The agonist SNC 80 increased [35S]GTP<sub>γ</sub>S binding and decreased the SEAP response at WT and M262T receptors, as in our previous experiments. In the [35S]GTP<sub>γ</sub>S assay potencies (EC<sub>50</sub>) of SNC 80 were similar at WT (EC<sub>50</sub> 2.79 ± 0.85 nM, n = 3) and M262T (EC<sub>50</sub> 0.48 ± 0.14 nM, n=4) receptors. In the SEAP assay also, EC<sub>50</sub> values were comparable at WT (EC<sub>50</sub> 0.17 ± 0.06 nM, n=5) and M262T mutant (EC<sub>50</sub> 0.16 ± 0.01 nM, n=4) receptors. In the [35S]GTP<sub>γ</sub>S binding assay maximal responses (E<sub>max</sub>) were similar for the two receptors (WT, E<sub>max</sub> 187.8 ± 6%; M262T, E<sub>max</sub> 174.5 ± 5.9% from basal binding). In the SEAP assay, the agonist efficacy was higher at the WT receptor (E<sub>max</sub> 48.6 ± 4.3 % from basal) compared to M262T receptor (E<sub>max</sub> 71.0 ± 4% from basal). At the Y308H mutant SNC 80 activity was low in the SEAP assay (E<sub>max</sub> 85.3 ± 1.1 % from basal, EC<sub>50</sub> 100.41 ± 62.21 nM; n=3) and undetectable in the [35S]GTP<sub>γ</sub>S binding test, partly due to the fact that the Y308H mutation strongly impairs SNC 80 binding (46-fold decrease in affinity, see table 1) and that [35S]GTP<sub>γ</sub>S binding is less sensitive compared to SEAP assay. SNC 80 was also inactive on mutant C328R (not shown), although in this case, SNC 80 binding affinity was not impaired (see table 1). From our previous data (Decaillot et al., 2003) it is likely that the strong spontaneous activity of both Y308H and C328R mutants limits further modulation by an agonist. Whether other delta opioid agonists are able to activate these mutant receptors remains to be tested. Representative dose-responses for SNC 80 activity at WT, M262T and Y308H mutants are shown in Fig. 4.

The reference inverse agonist ICI 174864 decreased [35S]GTP<sub>γ</sub>S binding and increased SEAP responses at WT and mutant receptors (Fig. 4 and 5). In the [35S]GTP<sub>γ</sub>S assay, ICI
174868 efficacies (E_{\text{max}}) were 76.9 ± 2.4 % (WT, n = 2), 73.9 ± 2.4 % (M262T, n = 4), 92.8 ± 2.2 % (Y308H, n = 2), 68.5 ± 2.6 % (C328R, n = 2) of basal values. E_{\text{max}} in the SEAP test were 147.5 ± 21.5 % (WT, n = 3), 170.1 ± 9.5 (M262T, n = 5), 174.2 ± 12.7 % (Y308H, n = 3) and 159.5 ± 21.8 % (C328R, n = 3) of FK-stimulated signal. Potencies could not be determined accurately, reflecting the extremely low affinity of ICI 174864 compound toward WT and mutant delta receptors (> 400 nM, Table 1) compared to the other delta compounds.

Potencies and efficacies of N,N(CH_{3})_{2}-Dmt-Tic-NH_{2} are shown in Table 2. Inverse agonism of this compound was significant at the WT receptor in the two functional assays. Efficacies were consistently higher at CAM receptors compared to WT, reaching 63.8 ± 2.5 % and 248.5 ± 16.7 % of basal values in the[^35S]GTP\gammaS binding and SEAP assays, respectively (see Y308H receptor). Potencies paralleled affinities (Table 1) for all the receptors, and were in the low nanomolar range. The inverse agonist activity of N,N(CH_{3})_{2}-Dmt-Tic-NH_{2} is illustrated by representative dose response curves displayed in Fig 4, where functional activity of this compound is compared to those of the reference agonist SNC 80 and inverse agonist ICI 174864.

The closely related Dmt-Tic-OH compound showed similar properties (Table 2). Potencies and efficacies were comparable to those of N,N(CH_{3})_{2}-Dmt-Tic-NH_{2} at the WT receptor, but tended to be lower at mutant receptors particularly in the SEAP assay. Together with our previous observation of an overall lower efficacy across the twenty-seven mutant receptors (Fig 3), it seemed that inverse agonism was slightly less robust for this compound. Adding to this, results from Dmt-Tic-OH were generally less reliable across experiments (not shown), probably due to lower stability of the compound which forms a diketopiperazine (Balboni et al., 1997; Capasso et al., 1995) and subsequently loses activity (Balboni et al., 1997). N,N(CH_{3})_{2}-Dmt-Tic-NH_{2} remains therefore the best candidate for future inverse agonist studies, particularly in physiological preparations.
Finally we tested whether the inverse agonist activity of \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) could be reversed by a neutral antagonist. In a new set of experiments we observed that SNC 80 (1 \(\mu\text{M}\)) and \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) (1 \(\mu\text{M}\)) respectively decreased and increased the FK-induced SEAP signal at the M262T receptor (64 \(\pm\) 11% and 193 \(\pm\) 2 %, \(n=2\)), as earlier. Both these activities were inhibited in the presence of NTI (1 \(\mu\text{M}\)), with SEAP signal values close to values obtained with FK alone (102 \(\pm\) 9% and 104 \(\pm\) 2 %, \(n=2\), respectively). Similar results were obtained for the WT receptor (data not shown).

**Dose-responses of TICP(Ψ) at WT, M262T, Y308H and C328R mutant receptors.**

Although inverse agonist activity was detected at saturating concentrations (Fig 2 and 3), and although affinities at all the receptors were in the nanomolar range (Table 2), we could not obtain reproducible EC\(_{50}\) values in \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding dose response experiments at WT, M262T and C328R receptors (not shown). However, E\(_{\text{max}}\) values were consistent across experiments (WT, 79.1 \(\pm\) 1.9 %, \(n=2\); M262T, 77.3 \(\pm\) 1.8 %, \(n=7\); C328R, 73.8 \(\pm\) 3.6 %, \(n=3\)), demonstrating negative effect of this compound at these mutants. Interestingly TICP(Ψ) was extremely active at the Y308H receptor (Fig. 5). In the \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assay, TICP(Ψ) decreased the basal signal by 66.2 \(\pm\) 5.6 % (\(n=5\)). In the SEAP assay, the enhancement of FK activation reached 227.7 \(\pm\) 12.3 % (\(n=4\)) of basal level. Therefore in both functional assays, maximal effects of TICP(Ψ) at this mutant were comparable to those obtained with \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\), and corresponded to maximal changes that could be obtained under our experimental conditions. EC\(_{50}\) values were 20.7 \(\pm\) 5.6 nM (\([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding) and 17.4 \(\pm\) 2.2 nM (SEAP), and were comparable to the Ki value at this receptor (Table 1). In conclusion, TICP(Ψ) showed spectacular inverse agonism at the Y308H receptor, and modest inverse agonism at the WT and other CAM receptors.
Discussion

Inverse agonism at delta opioid receptors was previously demonstrated in various cell lines expressing either native or wild-type recombinant receptor. Here we have used a collection of mutant receptors with high basal activity, as another approach to examine inverse agonist properties of delta compounds. We have examined delta ligands from distinct structural families and our data show that (i) \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\) exhibits consistent and potent inverse agonism at WT and mutant receptors, (ii) TICP(Ψ) displays negative activity in a receptor-dependent fashion and (iii) NTI and related derivatives are essentially neutral compounds.

An innovative aspect of this study is the use of CAM delta receptors to better assess inverse agonism of delta opioid compounds. Indeed, when the fifteen compounds were screened for negative activity, stronger activities were detected at the M262T mutant compared to WT receptor. This demonstrates that, as was shown for other GPCRs (Behan and Chalmers, 2001; Rossier et al., 1999), a CAM delta receptor is a better detector of inverse agonism than the WT receptor. Confirming this, inverse agonist efficacies of the various compounds were generally comparable at the WT receptor, whereas partial and full inverse agonism could be distinguished at the several CAM delta receptors, when full dose-responses were performed (see Fig. 4 and 5).

The inverse agonist activity of ICI 174864 at the WT receptor appeared modest in our expression system, compared to previous studies that have used other cellular models (see introduction and references therein). Replacing Na\(^+\) by K\(^+\) ions for example, which successfully increased ICI 174864 activity in the case of excitable cells such as NG108-15 (Costa and Herz, 1989; Szekeres and Traynor, 1997) or GH3 cells (Liu and Prather, 2002)
was ineffective in our HEK 293 cells. When HEK 293 cells are used as host cells, CAM receptors therefore represent a clear advantage for the study of inverse agonism.

Another interesting finding was that the negative activity of the Dmt-Tic compounds was significant at many distinct CAM receptors, independently from the localization of the mutation. Evidence that different mutations may produce multiple active conformations has been suggested by us and others on the basis of signaling or trafficking studies (see Decaillot et al., 2003 or Whistler et al., 2002 among others). Our finding that Dmt-Tic compounds are active at all the tested CAM receptors, with mutations distributed throughout the receptor proteins, strongly suggests that these compounds are able to inhibit the several potentially distinct active conformations (Decaillot et al., 2003). Dmt-Tic molecules should therefore represent reliable and potent inverse agonists in distinct experimental contexts.

The three pseudopeptides from the Dmt-Tic family (Bryant et al., 2003) all showed negative activity, in the $[^{35}S]GTP\gamma S$ binding screening experiment. The reason why inverse agonism of Dmt-Tic-NH$_2$ was not confirmed in the SEAP assay, despite a better sensitivity of this assay, remains open. Dmt-Tic-OH, and $N,N(CH_3)_2$-Dmt-Tic-NH$_2$ otherwise remained strong inverse agonists throughout the study, the latter exhibiting consistently slightly better activity. $N,N(CH_3)_2$-Dmt-Tic-NH$_2$ therefore appears as a full inverse agonist, with an efficacy comparable to that of ICI 174864 and with a much higher potency (nanomolar instead of micromolar). Labarre and collaborators, compared functional activity of the same three Dmt-Tic compounds in a $[^{35}S]GTP\gamma S$ binding study using a HEK 293 cell line stably expressing the WT delta receptor (Labarre et al., 2000). They showed neutral antagonism, partial agonism and full inverse agonism for Dmt-Tic-NH$_2$, Dmt-Tic-OH and $N,N(CH_3)_2$-Dmt-Tic-NH$_2$, respectively. Both their data and our study therefore put forward $N,N(CH_3)_2$-Dmt-Tic-NH$_2$ as the more active delta inverse opioid agonist. As many other Dmt-Tic opioids, this compound is delta selective ($K_i$ delta 0.31 ± 0.05 nM; $K_i$ mu 511.4 ± 74.6 nM, (Salvadori et
al., 1995). In addition, because the N-methylation augments hydrophobicity and prevents spontaneous cyclization (Bryant et al., 2003), \(N,N(CH_3)_2\)-Dmt-Tic-NH$_2$ is considered a stable compound, (Bryant et al., 2003). Altogether therefore \(N,N(CH_3)_2\)-Dmt-Tic-NH$_2$ appears the best pharmacological tool to explore the spontaneous activity of delta receptors.

Peptides from the TIPP family, showed unanticipated activities. TIPP was originally developed as a delta opioid antagonist (Schiller et al., 1992). Consistent with this observation, TIPP was inactive in our \([^{35}S]GTP\gamma S\) binding assay. However in more recent studies, Martin and collaborators reported that TIPP could show agonistic properties in several cell lines using cAMP measurements (Martin et al., 2001). In addition, the latter authors showed that, in GH3 cells, TIPP exhibited inverse agonist or agonist properties, depending on whether the end-point measurement was performed early (receptor binding) or late (cAMP response) along the signaling cascade (Martin et al., 2002). In line with these observations, we found agonistic properties for TIPP in the SEAP reporter gene assay which is sensitive to cAMP levels. Together with the study by Martin and collaborators therefore, our data suggest that TIPP may activate receptors to a small extent, and that the agonistic nature of TIPP is revealed after signal amplification only. This activity however may not be easily detectable since Pineyro and collaborators showed no TIPP-induced modifications of cAMP levels when the delta receptor was expressed in HEK 293 cells (Pineyro et al., 2001). Differences between the study of Pineyro and our study may arise from distinct assay conditions. Whether TIPP behaves as agonist or inverse agonist under physiological conditions remains to be explored.

TICP(Ψ) was derived from TIPP by reduction of the peptide bound between Tic$^2$ and Phe$^3$ and saturation of the Phe$^3$ aromatic ring, leading to a chemically stable pseudopeptide with high delta selectivity (K$_i$ delta 0.259 nM; K$_i$ mu 1050 nM, (Schiller et al., 1996). Like TIPP, TICP(Ψ) was reported to be a potent antagonist (Schiller et al., 1999). In their study assessing functional activities of TIPP -see above- Pineyro et al. also examined TICP(Ψ) and
reported that TICP(Ψ) acted as full inverse agonist (similar to ICI 174864) for the cAMP response (Pineyro et al., 2001). In our study TICP(Ψ) showed only modest negative activity at the WT delta receptor, although we used a similar expression system and a similar end-point measurement. Clearly the detection of negative activity is dependent on the exact assay conditions.

TICP(Ψ) nevertheless exhibited inverse agonism at the mutant Y308H receptor in our study. In fact, not only TICP(Ψ) but also the two Dmt-Tic compounds behaved as full inverse agonists at this receptor, while ICI 174864 activity was low. The Y308H receptor, therefore, seemed particularly responsive to peptides from the TIPP and Dmt-Tic families. The reason why TICP(Ψ) showed strong inverse agonism at this receptor, and more generally, why the pseudopeptides were highly active at the Y308H receptor remains unexplained. In contrast to mutations M262T and C328R, this mutation is located centrally within the binding pocket and drastically modifies opioid binding (Befort et al., 1996; Befort et al., 1999). Specific atomic interactions may take place between these inverse agonists and the signaling core of the receptor (Tm 3 / Tm 6 / Tm 7), (Decaillot et al., 2003) that strongly favor the inactive receptor conformation. 3D-Modeling studies, in the future, may clarify specific structural features within the TICP(Ψ) - Y308H receptor complex.

Labarre et al. showed weak partial inverse agonism for the NTI derivative HS-378 (Labarre et al., 2000). Our data showed no activity for this compound, as well as for a set of five closely related HS compounds, at the CAM M262T receptor. We even observed weak partial agonism at the WT receptor for most of them. Together, it seems that NTI and NTI derivatives (HS compounds) are weakly active at the delta receptor, and this activity could be either slightly negative (Labarre et al., 2000), neutral or slightly positive (this study) depending on the experimental system. Compared to ICI 174864 and the two active Dmt-Tic compounds, these compounds could therefore be considered as neutral ligands.
In conclusion, we have investigated the functional activity of several peptidic and non-peptidic delta opioid compounds across delta receptors with variable levels of spontaneous activity. Our data indicate that the $N,N'(CH_3)_2$-Dmt-Tic-NH$_2$ compound represents a useful inverse agonist to modulate the spontaneous activity of delta receptors in recombinant and possibly physiological situations. Our data also show that TIPP peptides are endowed with intriguing properties that deserve further exploration in different experimental conditions. NTI derivatives remain essentially neutral in our experiments and the most selective compounds (HS ligands) may serve as neutral antagonists in future experiments.
Acknowledgements

We wish to thank Christophe Iderne, Mathieu Ballié and Gabriel Recht for their important contribution. We are grateful to Dr. Severo Salvadori for its generous gift of Dmt-Tic compounds and to Dr. Dominique Massotte for critical reading of the manuscript.
References


Footnotes

a)

This work was supported by the Human Frontier Science Program, the INSERM, the Université Louis Pasteur, the Association de la Recherche pour le Cancer, the Institut UPSA de la Douleur, the Mission Interministérielle de Lutte contre la Drogue et la Toxicomanie and NIDA (NIH-NIDA #DA05010).

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Legends for figures

**Fig. 1. Ligands and receptors.** Structures of delta compounds studied as potential inverse agonists. These include the classical antagonists NLX, NTI, BNTX and NTB, a set of NTI derivatives, TIPP and TICP[Ψ] from the TIPP family (Schiller et al., 1999), as well as three compounds from the Dmt-Tic pseudopeptide family (Bryant et al., 2003). INSET. CAM delta receptors used for the inverse agonist screen (M262T), as well as for dose-response curves (M262T, Y308H and C328R). Black dots indicate the position of activating mutations on the receptor, displayed in a serpentine representation. The twenty-four other CAM receptors used to test inverse agonist activity of the most potent compounds have been detailed earlier (Decaillot et al., 2003).

**Fig. 2. Screening for inverse agonist activity.** Fifteen delta compounds were tested on WT (left panels) and M262T mutant (right panels) receptors. Delta opioids include from left to right: the reference agonist SNC 80 (left black bars), the reference inverse agonist ICI 174864 (right black bars), commercial alkaloids (NLX, NTI, BNTX, NTB) and peptide (TIPP), the TIPP analogue TICP[Ψ], three Dmt-Tic compounds and six novel NTI derivatives (grey bars). Top panel. Ligand-induced $[^{35}\text{S}]{\text{GTP}}\gamma{S}$ binding. Ligand concentrations were 10 µM (except SNC 80 at 1 µM). $[^{35}\text{S}]{\text{GTP}}\gamma{S}$ binding activities were normalized to basal activity (without ligand) defined as 100 % (horizontal line). Data are means ± s.e.m. from 3 to 5 experiments performed in triplicates, and using at least two independent membrane preparations. Bottom panel. Ligand-induced SEAP activation. Ligand concentrations were at 1 µM (except ICI 174864 at 10 µM) in the presence of 10 µM forskolin (FK). SEAP activities were normalized to signal obtained with FK alone, defined as 100% and indicated by a horizontal line. Data are means ± s.e.m. from 3 to 9 independent transfection experiments.
performed in triplicates. Activities of the tested compounds were compared to basal values using the Student t test. One star: $p < 0.05$, two stars: $p < 0.01$, three stars: $p < 0.001$ (One-way ANOVA).

**Fig. 3. Inverse agonist activity of delta compounds across CAM delta receptors.**

$N,N(CH_3)_2$-Dmt-Tic-NH$_2$ (Top panel), Dmt-Tic-OH (Middle panel) and TICP(Ψ) (bottom panel) were tested at a 1 µM concentration and compared to ICI 174864 (10 µM) in each experiment. Activating mutations are distributed all along the receptor sequence, and mutant receptors are presented from N-terminal (left) to C-terminal (right) mutations. Structural and functional properties of these mutants have been described earlier (Decaillot et al., 2003). Ligand activities were quantified by the SEAP reporter gene assay. SEAP activities were normalized to the forskolin (FK) signal alone, defined as 100 % and represented by an horizontal line. Histograms of 3 to 4 independent experiments with triplicate transfections are shown.

**Fig. 4. Dose responses for inverse agonist activity of $N,N(CH_3)_2$-Dmt-Tic-NH$_2$.** Dose response curves were established on WT, M262T, Y308H and C328R CAM receptors (top to bottom) using $[^{35}\text{S}]$GTP$\gamma$S binding (left) or the SEAP reporter gene assay (right). Responses to the reference agonist and inverse agonist (SNC 80 and ICI 174864) are displayed together with responses to $N,N(CH_3)_2$-Dmt-Tic-NH$_2$. For purpose of clarity only one representative experiment is show in each experimental condition. EC$_{50}$ and efficacy values from several experiments are indicated in Table 2 and in text. In the $[^{35}\text{S}]$GTP$\gamma$S binding assay, membranes were incubated with the ligand at concentrations ranging from 3,16 pM to 100 µM. $[^{35}\text{S}]$GTP$\gamma$S binding activities were normalized to basal activity (absence of ligand), defined as 100 %. For the SEAP assay, transfected cells were incubated for 5 hours with forskolin.
alone (FK, 10 µM) or with various concentrations of the ligand (3 pM to 100 µM). SEAP activity values were normalized to FK signal (100%). SNC 80 was ineffective at the C328R mutant.

**Fig. 5. Dose responses for inverse agonist activity of TICP(Ψ) at the Y308H receptor.**

TICP(Ψ) activity was measured in [³⁵S]GTPγS binding experiment (left) and in the SEAP assay (right). Responses to the reference agonist and inverse agonist (SNC 80, ICI 174864) are also represented for comparison. [³⁵S]GTPγS binding activities as well as SEAP responses were normalized to basal activity (absence of ligand, = 100 %). EC₅₀ and efficacy values from 4 ([³⁵S]GTPγS) and 4 (SEAP) independent experiments are indicated in the result section.
Table 1. Binding affinities of delta compounds to WT and mutant delta receptors. Competition experiments were carried out on HEK 293 cell membrane preparations expressing receptors either stably (WT, M262T, C328R) or transiently (Y308H), and using variable concentrations of unlabelled ligands to displace [³H]diprenorphine. Data are means ± s.e.m. from n experiments performed in triplicates (Kd) or in duplicates (Ki) using at least two different membrane preparations.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>WT</th>
<th>M262T</th>
<th>Y308H</th>
<th>C328R</th>
</tr>
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<tr>
<td></td>
<td>Ki (nM)</td>
<td>n</td>
<td>Ki (nM)</td>
<td>n</td>
</tr>
<tr>
<td>[³H]Diprenorphine (Kd)</td>
<td>0.39 ± 0.06</td>
<td>2</td>
<td>0.72 ± 0.21</td>
<td>2</td>
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<tr>
<td>SNC 80</td>
<td>9.41 ± 3.07</td>
<td>3</td>
<td>9.62 ± 2.59</td>
<td>3</td>
</tr>
<tr>
<td>ICI 174864</td>
<td>434 ± 61</td>
<td>4</td>
<td>1308 ± 247</td>
<td>3</td>
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<tr>
<td>NLX</td>
<td>66.20 ± 0.71</td>
<td>3</td>
<td>59.11 ± 2.92</td>
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<tr>
<td>NTI</td>
<td>0.30 ± 0.15</td>
<td>3</td>
<td>0.12 ± 0.02</td>
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<tr>
<td>BNTX</td>
<td>3.22 ± 0.58</td>
<td>3</td>
<td>6.10 ± 1.48</td>
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<td>NTB</td>
<td>0.10 ± 0.02</td>
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<td>0.23 ± 0.04</td>
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<td>TiPP</td>
<td>2.90 ± 0.66</td>
<td>3</td>
<td>7.03 ± 3.05</td>
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<td>TICP[^Ψ]</td>
<td>4.62 ± 0.64</td>
<td>3</td>
<td>3.91 ± 0.27</td>
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<tr>
<td>H-Dmt-Tic-OH</td>
<td>13.13 ± 1.71</td>
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<td>5.20 ± 1.85</td>
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<td>H-Dmt-Tic-NH₂</td>
<td>9.41 ± 1.13</td>
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<td>9.43 ± 1.16</td>
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<tr>
<td>N, N(CH₃)₂-Dmt-Tic-NH₂</td>
<td>2.50 ± 0.76</td>
<td>3</td>
<td>1.81 ± 0.66</td>
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<td>HS-378</td>
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<td>0.90 ± 0.03</td>
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<td>HS-414</td>
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<td>HS-464</td>
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<td>0.40 ± 0.09</td>
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<td>HS-510A</td>
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<td>HS-531</td>
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<td>HS-595</td>
<td>0.22 ± 0.01</td>
<td>2</td>
<td>0.41 ± 0.05</td>
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Table 2. Potencies and efficacies of Dmt-Tic compounds to enhance the FK-stimulated SEAP signal and decrease basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding at WT and mutant delta receptors. EC 50 and maximal activation values are calculated from dose response curves for each ligand in both functional assays. Representative experiments for $\text{N, N(CH}_3)_2\text{-Dmt-Tic-NH}_2$ are shown in Fig. 4. Maximal responses to the compounds were normalized to basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding or FK-stimulated SEAP response obtained in the absence of ligand, defined as 100%. Values are means ± s.e.m. from n separate experiments performed in triplicates.

<table>
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<tr>
<th></th>
<th>$\text{N, N(CH}_3)_2\text{-Dmt-Tic-NH}_2$</th>
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<td>EC 50 [nM]</td>
<td>Efficacy [%]</td>
</tr>
<tr>
<td>$[^{35}\text{S}]\text{GTP} \gamma \text{S}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>4.60 ± 3.48</td>
<td>86.45 ± 4.49</td>
</tr>
<tr>
<td>M262T</td>
<td>2.30 ± 0.62</td>
<td>75.91 ± 3.53</td>
</tr>
<tr>
<td>Y308H</td>
<td>70.04 ± 13.44</td>
<td>63.81 ± 2.53</td>
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<tr>
<td>C328R</td>
<td>0.68 ± 0.25</td>
<td>68.94 ± 3.46</td>
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<td>SEAP</td>
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<td></td>
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<tr>
<td>WT</td>
<td>2.73 ± 0.72</td>
<td>134.01 ± 9.51</td>
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<tr>
<td>M262T</td>
<td>6.70 ± 2.41</td>
<td>173.72 ± 21.80</td>
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<tr>
<td>Y308H</td>
<td>65.54 ± 15.76</td>
<td>248 ± 17</td>
</tr>
<tr>
<td>C328R</td>
<td>3.55 ± 0.85</td>
<td>185 ± 13</td>
</tr>
</tbody>
</table>
Alkaloid compounds

HS 378 : R1 = CPM, R2 = Et, R3 = Me, X = NH
HS 414 : R1 = allyl, R2 = Et, R3 = H, X = NH
HS 464 : R1 = CPM, R2 = Et, R3 = H, X = NH
HS 510A : R1 = CPM, R2 = Et, R3 = H, X = O
HS 531 : R1 = allyl, R2 = Me, R3 = H, X = NMe

CPM = cyclopropylmethyl,

Peptide compounds

TIP : H-Tyr-Tic-Phe-Phe-OH

TICP[Ψ] : H-Tyr-Tic[Ψ][CH2NH]Cha-Phe-OH

Dmt-Tic-OH: R1=H, R2=OH
Dmt-Tic-NH2: R1=H, R2=NH2
N,N(CH3)2-Dmt-Tic-NH2: R1=(CH3)2, R2=NH2
This article has not been copyedited and formatted. The final version may differ from this version.
\[ ^{35}\text{S} \text{GTP} \gamma \text{S} \]

\begin{align*}
\text{WT} & \quad \text{SEAP} \\
\text{M262T} & \quad \text{ } \\
\text{Y308H} & \quad \text{ } \\
\text{C328R} & \quad \text{ } \\
\end{align*}

\[
\text{log (ligand) [M]} \quad \text{log (ligand) [M]}
\]

\begin{align*}
\% \text{basal } ^{35}\text{S} \text{GTP} \gamma \text{S binding} & \quad \% \text{FK activation} \\
\% \text{basal } ^{35}\text{S} \text{GTP} \gamma \text{S binding} & \quad \% \text{FK activation} \\
\% \text{basal } ^{35}\text{S} \text{GTP} \gamma \text{S binding} & \quad \% \text{FK activation} \\
\% \text{basal } ^{35}\text{S} \text{GTP} \gamma \text{S binding} & \quad \% \text{FK activation} \\
\end{align*}

- SNC 80
- ICI 174864
- \(N,N(\text{CH}_3)_2\)-Dmt-Tic-NH\(_2\)
SEAP % FK activation

\[ \log (\text{ligand}) [\text{M}] \]

% basal \[^{35}\text{S}]\text{GTP}\gamma \text{S}

\[ \log (\text{ligand}) [\text{M}] \]

\[ \text{SNC 80} \quad \text{ICI 174864} \quad \text{TICP}[\Psi] \]