The Opioid μ Agonist/δ Antagonist DIPP-NH_2[Ψ] Produces a Potent Analgesic Effect, No Physical Dependence, and Less Tolerance than Morphine in Rats

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Opioid compounds with mixed μ agonist/δ antagonist properties are expected to be analgesics with low propensity to produce tolerance and dependence. In an effort to strengthen the μ agonist component of the mixed μ agonist/δ antagonist H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2), analogues containing structurally modified tyrosine residues in place of Tyr[1] were synthesized. Among the prepared compounds, H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2; Dmt = 2′,6′-dimethyltyrosine) and H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[Ψ]) retained a mixed μ agonist/δ antagonist profile, as determined in the guinea pig ileum and mouse vas deferens assays, whereas H-Tmt-Tic-Phe-Phe-NH_2 (Tmt = *N*,2′,6′-trimethyltyrosine) was a partial μ agonist/δ antagonist and H-Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2 was a μ antagonist/δ antagonist. DIPP-NH_2-[Ψ] showed binding affinities in the subnanomolar range for both μ and δ receptors in the rat brain membrane binding assays, thus representing the first example of a *balanced* μ agonist/δ antagonist with high potency. In the rat tail flick test, DIPP-NH_2[Ψ] given icv produced a potent analgesic effect (ED_{50} = 0.04 μg), being about 3 times more potent than morphine (ED_{50} = 0.11 μg). It produced less acute tolerance than morphine but still a certain level of chronic tolerance. Unlike morphine, DIPP-NH_2[Ψ] produced no physical dependence whatsoever upon chronic administration at high doses (up to 4.5 μg/h) over a 7-day period. In conclusion, DIPP-NH_2[Ψ] fulfills to a large extent the expectations based on the mixed μ agonist/δ antagonist concept with regard to analgesic activity and the development of tolerance and dependence.

Introduction

The development of tolerance and physical dependence induced by chronic administration of opioids, such as morphine, limits their use in the treatment of pain. A report by Abdelhamid et al.[2] first described that selective δ opioid receptor blockade with the δ antagonist naltrindole in mice greatly reduced the development of morphine tolerance and dependence in both the acute and chronic models. In a subsequent study, it was shown that continuous infusion (icv) of the highly selective δ opioid antagonist TIPP[Ψ] in parallel with continuous delivery of morphine (sc) to rats also attenuated the development of morphine tolerance and dependence to a large extent.[3] These results suggest the possibility of the combined use of a μ type opioid analgesic and a δ opioid antagonist in chronic pain treatment. Alternatively, the development of a single opioid compound acting as an agonist at the μ receptor and as an antagonist at the δ receptor might be of benefit in the management of chronic pain. Such a mixed μ agonist/δ antagonist would be expected to be an analgesic with low propensity to produce analgesic tolerance and physical dependence.

The first known compound with mixed μ agonist/δ antagonist properties was the tetrapeptide amide H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2).[4] This compound showed modest μ agonist potency in the guinea pig ileum (GPI) assay (IC_{50} = 1.70 ± 0.22 μM) and quite high δ antagonist potency in the mouse vas deferens (MVD) assay against the δ agonist DPDPE (K_e = 18.0 ± 2.2 nM). In agreement with the bioassay data, TIPP-NH_2 displayed relatively low affinity for μ receptors (K_i^{μ} = 78.8 ± 7.1 nM) and high affinity for δ receptors (K_i^{δ} = 3.0 ± 1.5 nM) in the rat brain membrane binding assays, indicating that it was quite δ-selective (K_i^{μ}/K_i^{δ} = 26.3). It showed no affinity for κ receptors in the guinea pig brain membrane assay at concentrations up to 10 μM. Furthermore, TIPP-NH_2 was shown to undergo spontaneous degradation via diketopiperazine formation in organic solvents but not in aqueous solution.[5]

In this report, we describe attempts to strengthen the μ agonist component of TIPP-NH_2 without compromising its δ antagonist properties. Since replacement of the N-terminal tyrosine residue in opioid peptides with 2′,6′-dimethyltyrosine (Dmt) has been known to produce a more dramatic binding affinity increase at the μ receptor than at the δ receptor,[6,7] the Dmt[1] analogue of TIPP-NH_2, H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2), was prepared. Furthermore, we synthesized the pseudopeptide analogue H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[Ψ]), containing a reduced peptide bond between the Tic[2] and Phe[3] residues, to exclude the possibility of chemical degradation via diketopiperazine formation.[5,8] Analogues of DIPP-NH_2 and DIPP-NH_2[Ψ] containing *N*,2′,6′-trimethyltyrosine (Tmt) in place of Dmt[1] were also prepared, since it was expected that methylation of the N-terminal amino group would render these

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Table 1. Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays of TIPP-NH$_2$ Analogues

compd	GPI IC_{50}, nM[a]	MVD K_e, nM[a]	
		DPDPE	deltorphin I
H-Dmt-Tic-Phe-Phe-NH$_2$ (DIPP-NH$_2$)	18.2 ± 1.8	0.209 ± 0.037	0.260 ± 0.064
H-Dmt-TicΨ[CH$_2$NH]Phe-Phe-NH$_2$ (DIPP-NH$_2$[Ψ])	7.71 ± 0.31	0.537 ± 0.026	0.486 ± 0.058
H-Tmt-Tic-Phe-Phe-NH$_2$	PA (38%)[b]	0.235 ± 0.032	0.233 ± 0.010
H-Tmt-TicΨ[CH$_2$NH]Phe-Phe-NH$_2$	antagonist[c]	22.1 ± 3.5	54.4 ± 6.0
H-Tyr-Tic-Phe-Phe-NH$_2$ (TIPP-NH$_2$)	1700 ± 220	18.0 ± 2.2	14.4 ± 2.2

[a] Mean of three to six determinations ± SEM. [b] Partial agonist; value in parentheses indicates maximal inhibition of the contractions (%) obtained at high concentrations. [c] K_e = 64.5 ± 5.8 nM (determined against TAPP).

peptides more lipophilic and would improve their ability to cross the blood−brain barrier (BBB). In the present paper, we report on the syntheses and in vitro opioid activity profiles of these analogues. We also describe the analgesic activity of the most promising compound, DIPP-NH$_2$[Ψ], and its propensity to produce physical dependence and tolerance.

Chemistry

Peptides were synthesized by the solid-phase method using *tert*-butyloxycarbonyl (Boc)-protected amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. Introduction of the reduced peptide bond between the Tic[2] and Phe[3] residues required a reductive alkylation reaction[9] between 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde and the amino group of the resin-bound H-Phe-Phe dipeptide. 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde was synthesized via preparation of 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-(*N*-methoxy-*N*-methylamide) as described in the literature.[8,10] The Boc derivative of commercially available D,L-2′,6′-dimethyltyrosine was prepared and incorporated into the peptides in racemic form. Boc-D,L-*N*,2′,6′-trimethyltyrosine was obtained by N-methylation of Boc-D,L-2′,6′-dimethyltyrosine and was also used in racemic form in the peptide synthesis. Peptides were cleaved from the resin by HF/anisole treatment in the usual manner. Peptide purification and separation of the diastereomeric peptides were achieved by preparative reversed-phase HPLC. The stereochemical assignment of the diastereomeric peptides was based on analysis of their acid hydrolysates by chiral TLC.

Biology

Opioid Receptor Binding Assays and in Vitro Bioassays. Binding affinities for μ and δ opioid receptors were determined by displacing, respectively, [^{3}H]-DAMGO and [^{3}H]DSLET from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 from guinea pig brain membrane binding sites. For the determination of their in vitro opioid activities, analogues were tested in bioassays based on inhibition of electrically evoked contractions of the GPI and MVD. The GPI assay is usually considered as being representative for μ receptor interactions, even though the ileum does also contain κ receptors. In the MVD assay opioid effects are primarily mediated by δ receptors, but μ and κ receptors also exist in this tissue. δ Antagonist potencies in the MVD assay were determined against the δ agonists DPDPE and deltorphin I, and the highly selective μ agonist TAPP (H-Tyr-D-Ala-Phe-Phe-NH$_2$)[11] was used for μ antagonist potency determinations in the GPI assay.

Analgesic Testing and Assessment of Tolerance and Dependence Development. Drugs were administered to rats by the intracerebroventricular (icv) route. Analgesic potencies (ED_{50}'s) were determined using the rat tail flick test. To assess the development of acute tolerance, rats were infused (icv) with the opioid drug at two different high-dose levels and the period during which the animals stayed analgesic was determined. The development of chronic tolerance was assessed by determining the ED_{50} at the end of a 7-day period during which DIPP-NH$_2$[Ψ] or morphine had been injected (icv) twice daily at a high dose. To determine the development of physical dependence, the same rats that had been used in the acute tolerance study were chronically infused (icv) with the opioid compound at three different high-dose levels for 7 days. At the end of the drug treatment, the abstinence syndrome was precipitated by subcutaneous (sc) injection of naloxone and withdrawal symptoms were assessed quantitatively by measuring the amount of time spent teeth chattering and writhing and by determining the frequency of jumps and wet dog shakes.

Results and Discussion

Substitution of Dmt for Tyr[1] in the TIPP-NH$_2$ parent peptide resulted in a compound, H-Dmt-Tic-Phe-Phe-NH$_2$ (DIPP-NH$_2$), which showed a 93-fold potency increase in the GPI assay (Table 1). This effect was reversed by naloxone at low concentration (K_e = 2.42 ± 0.27 nM), indicating that it was mediated by μ opioid receptors and not by κ receptors.[12] In comparison with the parent peptide, DIPP-NH$_2$ also displayed 86- and 55-fold increased δ antagonist potency against the δ agonists DPDPE and deltorphin I, respectively, in the MVD assay. In agreement with the results observed in the functional assays, DIPP-NH$_2$ showed corresponding increases in μ and δ receptor affinities in the rat brain membrane binding assays (Table 2). The binding data indicate that this compound is still somewhat δ receptor-selective, as indicated by the ratio of the binding inhibition constants (K_i^{μ}/K_i^{δ} = 10.1).

Reduction of the peptide bond between Tic[2] and Phe[3] of DIPP-NH$_2$ produced a compound, H-Dmt-TicΨ-[CH$_2$NH]Phe-Phe-NH$_2$ (DIPP-NH$_2$[Ψ]), which showed further enhanced μ agonist potency in the GPI assay (IC_{50} = 7.71 ± 0.31 nM). This effect was again μ receptor-mediated, as indicated by the low K_e value (1.25 ± 0.14 nM) determined for naloxone as antagonist. In the MVD assay, DIPP-NH$_2$[Ψ] turned out to be a δ antagonist with about one-half the potency of DIPP-NH$_2$, but showing K_e values against the two δ agonists that were still in the subnanomolar range. At high concentrations (>1 μM) DIPP-NH$_2$[Ψ] produced a partial agonist effect (20% inhibition of the electrically evoked contractions)

Table 2. Binding Affinities of TIPP-NH_2 Analogues at μ and δ Receptors in Rat Brain Homogenates

compd	[^{3}H]DAMGO		[^{3}H]DSLET		
	K_i^{μ},[a] nM	rel potency[b]	K_i^{δ},[a] nM	rel potency[b]	K_i^{μ}/K_i^{δ}
H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2)	1.19 ± 0.11	7.92 ± 0.73	0.118 ± 0.016	21.4 ± 2.9	10.1
H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[Ψ])	0.943 ± 0.052	10.0 ± 0.6	0.447 ± 0.007	5.66 ± 0.09	2.11
H-Tmt-Tic-Phe-Phe-NH_2	4.08 ± 0.30	2.31 ± 0.17	0.393 ± 0.070	6.44 ± 1.15	10.4
H-Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2	76.2 ± 2.0	0.124 ± 0.003	61.0 ± 17.9	0.0415 ± 0.0122	1.25
H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2)	78.8 ± 7.1	0.120 ± 0.011	3.0 ± 0.15	0.843 ± 0.043	26.3
[Leu^5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	3.73

[a] Mean of three determinations ± SEM. [b] Potency relative to that of [Leu^5]enkephalin.

in this assay. This effect was fully reversible with naloxone (100 nM) but not at all reversible with the highly δ-selective opioid antagonist TIPP[Ψ][8] (100 nM), indicating that it was entirely mediated by μ opioid receptors which are also present in the MVD aside from the predominant δ receptors. This result indicates that DIPP-NH_2[Ψ] has no δ agonist properties. In the receptor binding assays, DIPP-NH_2[Ψ] displayed subnanomolar affinity for both μ and δ receptors and a K_i^{μ}/K_i^{δ} selectivity ratio of 2.11. Taken together, these data indicate that DIPP-NH_2[Ψ] represents the first known example of a *balanced* mixed μ agonist/δ antagonist with high potency.

Replacement of Tyr^1 in TIPP-NH_2 with Tmt led to a compound, H-Tmt-Tic-Phe-Phe-NH_2, which behaved as a partial μ agonist in the GPI assay (Table 1). The maximal inhibition of the electrically evoked contractions that could be achieved with this compound amounted to 38%. In the receptor binding assay it displayed high μ receptor affinity, being about 20 times more potent than TIPP-NH_2. These results and the fact that the ileum preparation contains a large number of spare μ receptors[13] indicate that H-Tmt-Tic-Phe-Phe-NH_2 is a partial μ agonist with low intrinsic efficacy. In the MVD assay, this analogue showed 60−75 times higher δ antagonist potency against the two δ agonists, as compared to the parent peptide. In agreement with the latter result, its δ receptor affinity was also found to be high (K_i^{δ} = 0.393 ± 0.070 nM). Interestingly, the corresponding analogue with a reduced peptide bond, H-Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2, turned out to be a moderately potent μ antagonist (K_e = 64.5 ± 5.8 nM) against the highly specific μ agonist TAPP in the GPI assay. This compound also showed only moderate δ antagonist potency, being somewhat less potent than the TIPP-NH_2 parent peptide. Its moderate μ and δ antagonist potencies are in agreement with the relatively low μ and δ receptor affinities determined in the binding assays. All five peptides showed K_i^{κ} values > 1 μM in the binding assay based on displacement of [^{3}H]U69,593 from guinea pig brain membranes and, thus, did not have any significant affinity for κ opioid receptors.

On the basis of its high potency, balanced μ agonist/δ antagonist properties, and stability, DIPP-NH_2[Ψ] was selected for a determination of its analgesic potency and propensity to produce tolerance and dependence. The analgesic potencies of DIPP-NH_2[Ψ] and morphine given icv were determined in the rat tail flick test. Tail flick latencies were measured and % MPE (maximum possible effect) scores were calculated. The area under the curve (AUC) score was determined for each rat, and a log dose−response (AUC) curve was constructed (Figure 1). For DIPP-NH_2[Ψ] an ED_{50} of 0.04 μg was determined from the log dose−response curve (Table 3).

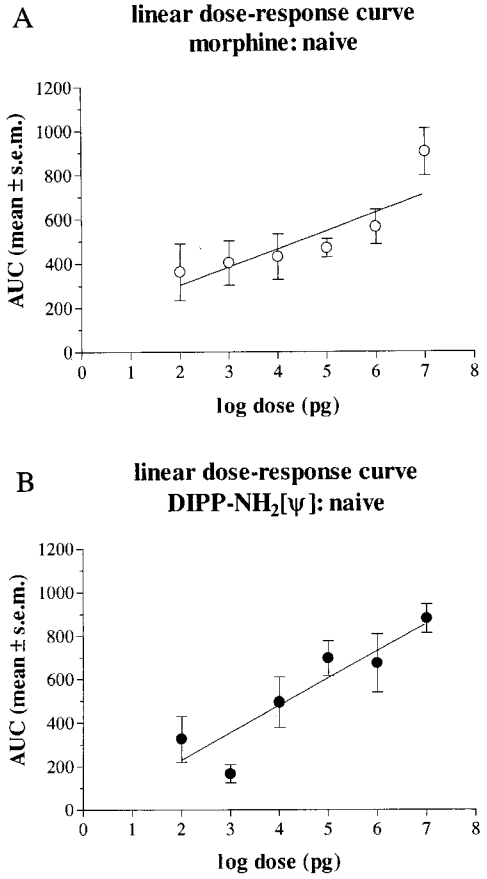

Figure 1. Log dose−response curves of morphine (A) and DIPP-NH_2[Ψ] (B) determined in the rat tail flick test.

Table 3. Analgesic Potency of DIPP-NH_2[Ψ] in Comparison with Morphine

compd	ED_{50}, μg (95% CI)[a]
DIPP-NH_2[Ψ]	0.04 (0.006−0.25)
morphine	0.11 (0.01−3.24)

[a] Maximal AUC = 1100.

This ED_{50} is about 3 times lower than that determined for morphine (ED_{50} = 0.11 μg) in the same assay system, indicating that the mixed μ agonist/δ antagonist has potent analgesic properties. The high analgesic potency of DIPP-NH_2[Ψ] is in agreement with its high μ receptor affinity and high μ opioid agonist potency determined in the in vitro assays.

To assess the development of acute analgesic tolerance, rats were continuously infused icv with DIPP-NH_2-[Ψ] at a dose of 0.15 μg [(ED_{50} × 3.7)/h] or with morphine at a dose of 0.3 μg [(ED_{50} × 2.7)/h] and the mean time after initiation of drug treatment for tail flick latencies to return to baseline levels was determined. As shown in Figure 2, the DIPP-NH_2[Ψ]-treated rats stayed

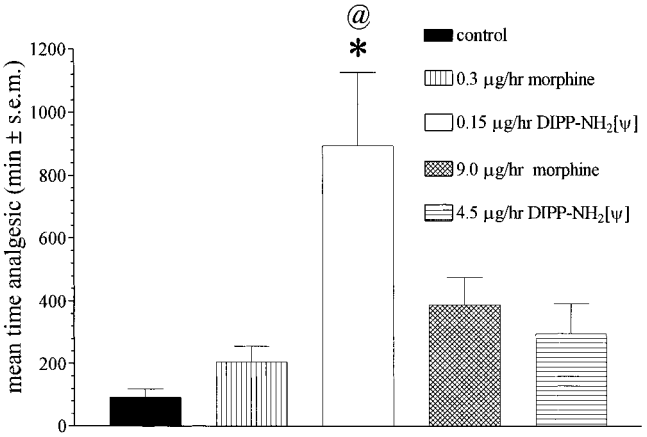

Figure 2. Mean time intervals of analgesic efficacy of morphine and DIPP-NH$_2$[Ψ] at two different doses administered icv, as determined in the rat tail flick test. Planned comparisons revealed that rats given DIPP-NH$_2$[Ψ] at a dose of 0.15 μg/h were analgesic for a significantly longer period of time than control rats ($F = 11.78_{(1,65)}$, $p < 0.05$). Planned comparisons revealed that rats given DIPP-NH$_2$[Ψ] (0.15 μg/h) were analgesic for a significantly longer period of time than rats given morphine at a dose of 0.3 μg/h ($F = 6.674_{(1,65)}$, $p < 0.05$). *Significant difference from 0.3 μg/h determined for morphine. @Significant difference from control ($p < 0.05$, post-hoc LSD *t*-tests).

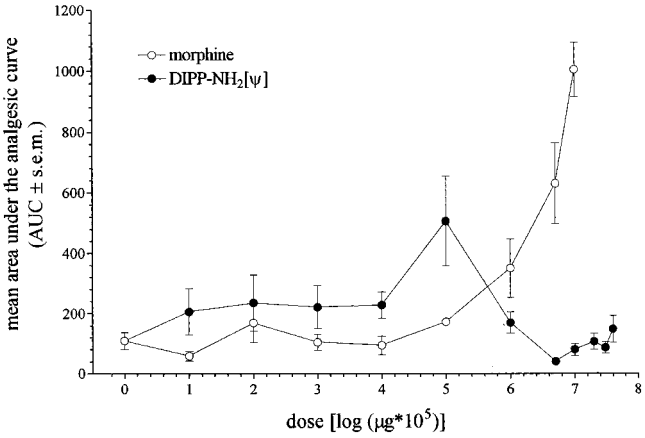

Figure 3. AUC scores determined in the rat tail flick test of rats that had been injected icv twice daily for 7 days with either morphine or DIPP-NH$_2$[Ψ] at respective doses of 0.3 and 0.15 mg/h and then were given various doses (icv) of either morphine or DIPP-NH$_2$[Ψ]. An ED$_{50}$ of 48.5 μg ($R^2 = 0.37$) was calculated from the AUC scores for rats treated with morphine. At a dose of 1 μg the AUC score of DIPP-NH$_2$[Ψ] was higher than that of morphine, but at higher doses DIPP-NH$_2$[Ψ] produced seizures which prevented the calculation of an ED$_{50}$.

analgesic for a significantly longer time interval (15 h) than the morphine-treated rats (3 h), indicating that DIPP-NH$_2$[Ψ] produced less acute tolerance at this dose level than morphine. However, when the doses of infused DIPP-NH$_2$[Ψ] and morphine were increased 30-fold to 4.5 μg [(ED$_{50}$ × 112)/h] and 9 μg [(ED$_{50}$ × 82)/h], respectively, both drugs showed a very short time interval of analgesic efficacy. Thus, at this very high dose level both DIPP-NH$_2$[Ψ] and morphine produced acute tolerance to a similar degree.

The propensities of DIPP-NH$_2$[Ψ] and morphine to produce chronic tolerance were determined by injecting the drugs icv at doses of 0.15 and 0.3 μg, respectively (corresponding to the ED$_{50}$ × 3.7 and ED$_{50}$ × 2.7, respectively), twice daily for 7 days. At the end of the drug treatment, various doses of either DIPP-NH$_2$[Ψ] or morphine were given icv and tail flick latencies were measured at 10-min intervals for 2 h. A plot of the mean AUC as a function of drug dose (Figure 3) revealed that neither drug produced a significant analgesic effect in the dose range 0.0001−0.1 μg. A significant analgesic effect was observed with DIPP-NH$_2$[Ψ] at a dose of 1 μg but not with morphine at that same dose. Thus, the results obtained at this particular dose level indicated that DIPP-NH$_2$[Ψ] had produced less chronic tolerance than morphine. However, at higher doses (10−400 μg) DIPP-NH$_2$[Ψ] produced seizures and it was no longer possible to accurately determine tail flick latencies. Therefore, it was not possible to determine an ED$_{50}$ for DIPP-NH$_2$[Ψ]. The nature of the DIPP-NH$_2$[Ψ]-induced seizures needs to be further investigated. No seizures were observed with the morphine-treated rats in the higher dose range, and an ED$_{50}$ (48.5 [7.52−1130] μg) could be calculated by performing a linear regression analysis on the AUC scores.

To assess the development of dependence, rats were infused icv either with DIPP-NH$_2$[Ψ] or with morphine at three different high-dose levels. Morphine doses were as follows: 0.3 μg [(ED$_{50}$ × 2.7)/h], 4.5 μg [(ED$_{50}$ × 41)/h], and 9.0 μg [(ED$_{50}$ × 82)/h]; DIPP-NH$_2$[Ψ] doses were as follows: 0.15 μg [(ED$_{50}$ × 3.7)/h], 2.25 μg [(ED$_{50}$ × 56)/h], and 4.5 μg [(ED$_{50}$ × 112)/h]. At the end of the drug treatment, the abstinence syndrome was precipitated by injection of naloxone (1 mg/kg, sc). Abstinence symptoms that were quantitatively assessed included teeth chattering, writhing, jumps, wet dog shakes, ptosis, lacrimation, eye twitch, and scream on touch. Rats in each of the three treatment groups, which had been administered morphine chronically at the three different dose levels, all spent a large amount of time teeth chattering and writhing (Figure 4a). Thus, even at the lowest dose used in the chronic treatment, morphine produced an intense withdrawal syndrome. On the other hand, DIPP-NH$_2$[Ψ]-treated animals in all three treatment groups spent no more time in withdrawal than control animals that had received saline during the 7-day period. This result indicates that chronically administered DIPP-NH$_2$[Ψ] had no effect on time spent in withdrawal, even at the highest dose level used. Similar observations were made when the mean frequency of jumps and wet dog shakes combined was determined in each treatment group (Figure 4b). Again, a very significant increase in the frequency of counted symptoms was observed in the case of the morphine-treated rats, whereas the DIPP-NH$_2$[Ψ]-treated animals showed no increase in the frequency of these symptoms as compared to control rats. Finally, the average severity of ptosis, lacrimation, eye twitch, and scream on touch was significantly increased in the case of the morphine-treated rats as compared to control rats, but not at all in the case of the DIPP-NH$_2$[Ψ]-treated rats (data not shown). Taken together, these results indicate that, in sharp contrast to morphine, DIPP-NH$_2$[Ψ] produced no physical dependence, even at the highest dose [(ED$_{50}$ × 112)/h] used in the 7-day drug treatment.

Conclusions

The performed structural modifications of the parent peptide TIPP-NH$_2$ resulted in mixed μ agonist/δ an-

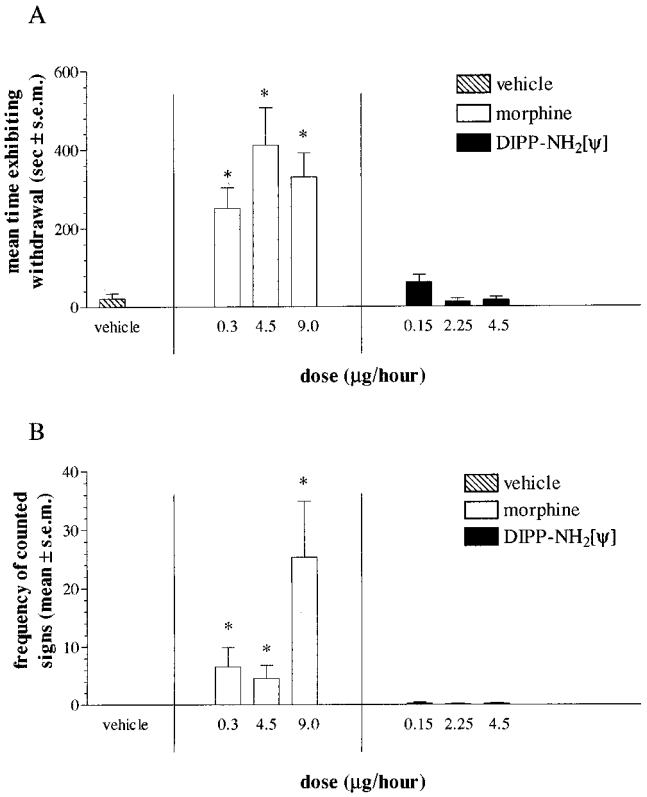

Figure 4. Evaluation of withdrawal symptoms of rats that had been infused (icv) at three different dose levels with either morphine (0.3, 4.5, and 9.0 μg/h) or DIPP-NH_2[Ψ] (0.15, 2.25, and 4.5 μg/h) for 7 days and then received naloxone (1 mg/kg, sc). (A) Mean time spent in withdrawal (teeth chattering and writhing combined) during the 40-min withdrawal period. ANOVA indicated a significant dose effect for morphine ($F_{(3,40)} = 9.16$, $p < 0.01$). There was no effect of DIPP-NH_2[Ψ] on time spent in withdrawal ($F_{(3,44)} = 2.84$, $p = 0.05$). *Significant difference from the vehicle control group (post-hoc LSD test). (B) Mean frequency of counted symptoms (jumps and wet dog shakes combined) during the 40-min withdrawal period. Kruskal−Wallis ANOVA for nonparametric data indicated a significant dose effect for morphine ($H_{(3,44)} = 23.47$, $p < 0.01$). There was no effect of DIPP-NH_2[Ψ] on the frequency of counted symptoms ($H_{(3,48)} = 2.99$, $p < 0.05$). *Significant difference from the vehicle control group (Mann−Whitney U-test).

tagonists with increased μ agonist potency, a partial μ agonist/δ antagonist, and a μ antagonist/δ antagonist. DIPP-NH_2[Ψ] was found to be a potent μ agonist and a potent δ antagonist in the functional in vitro assays and displayed subnanomolar affinity for both μ and δ receptors in the rat brain membrane binding assays. Thus, DIPP-NH_2[Ψ] turned out to be the first known opioid compound with balanced μ agonist/δ antagonist properties. Furthermore, this compound is highly stable against chemical and enzymatic degradation, as demonstrated for the structurally analogous δ antagonist TIPP[Ψ].[8]

In the rat tail flick test, DIPP-NH_2[Ψ] produced a potent analgesic effect when given icv. It was about 3 times more potent than morphine in this test system. DIPP-NH_2[Ψ] produced less acute tolerance than morphine upon continuous infusion at the lower of the two dose levels used. It did produce a certain level of chronic tolerance which, however, appeared again to be somewhat less pronounced than in the case of morphine. In the dependence studies, rats having been infused with DIPP-NH_2[Ψ] (icv) for 7 days at very high dose levels showed no withdrawal symptoms upon injection of naloxone at the end of the treatment period. Thus, unlike morphine, DIPP-NH_2[Ψ] produced no physical dependence whatsoever after chronic administration at doses much higher than needed for producing analgesia.

The results of the analgesic test and of the tolerance and dependence studies indicate that DIPP-NH_2[Ψ], to a large extent, fulfills the expectations based on the mixed μ agonist/δ antagonist concept with regard to analgesic activity and the development of tolerance and dependence. However, an effort will have to be made to develop DIPP-NH_2[Ψ] analogues with a further reduced propensity to produce acute and chronic tolerance. This could possibly be achieved by varying the ratio of μ agonist potency to δ antagonist potency through appropriate structural modifications. Other types of mixed μ agonist/δ antagonists developed in our laboratory, including analogues of the cyclic β-casomorphin peptide H-Tyr-c[-D-Orn-2-Nal-D-Pro-Gly-][14] and of the dipeptide derivative H-Dmt-Tic-NH-$(CH_2)_3$-Ph,[15] are also in the process of being examined for their analgesic properties and propensities to produce tolerance and dependence.

Experimental Section

General Methods. Precoated plates (silica gel 60 F_{254}, 250 μm; Merck, Darmstadt, FRG) were used for ascending TLC in the following solvent systems (all v/v): (I) EtOAc/hexane (2:1), (II) CH_3Cl/MeOH (9:1), (III) CH_3Cl/MeOH/AcOH (85:10:5), (IV) *n*-BuOH/AcOH/H_2O (4:1:1). The HPLC system GOLD (Beckman) consisting of a programmable solvent module 126, and a diode array detector module 168 was used for the purification and the purity control of the peptides. Recording and quantification were accomplished using the GOLD software.

Preparative reversed-phase HPLC was performed on a Vydac 218-TP column (22 × 250 mm) with a linear gradient of 30−50% MeOH in 0.1% TFA at a flow rate of 13 mL/min, absorption being measured at 216 and 280 nm. Analytical reversed-phase HPLC chromatography was carried out on a Vydac 218-TP column (4.6 × 250 mm) using a linear gradient of 15−50% acetonitrile in 0.1% TFA at a flow rate of 1.0 mL/min. Proton magnetic resonance spectra were recorded at 25 °C on a Varian VXR-400S spectrometer using tetramethylsilane as internal standard. Molecular weights of compounds were determined by FAB mass spectrometry on an MS-50 HMTCTA mass spectrometer interfaced to a DS-90 data system (Dr. M. Evans, Department of Chemistry, University of Montreal).

Amino Acids and Derivatives. Boc amino acids were purchased from Bachem Bioscience. A sample of optically pure H-L-Dmt-OH was a gift from Dr. Henry Mosberg, University of Michigan. Racemic H-D,L-Dmt-OH was obtained from Synthelec, Lund, Sweden. 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde was synthesized as described.[8]

Preparation of Boc-D,L-Dmt(Boc)-OH. To a solution of H-D,L-Dmt-OH (639 mg, 3 mmol) in 20 mL of THF/H_2O (1:1, v/v) were added di-*tert*-butyl dicarbonate (1.57 g, 7.2 mmol), triethylamine (834 μL, 7.2 mmol), and (dimethylamino)-pyridine (36 mg, 0.30 mmol). The solution was stirred at room temperature for 18 h. After solvent evaporation, the crude product was dissolved in 30 mL of EtOAc, and the solution was washed with 5% citric acid (3 × 30 mL) and H_2O (3 × 30 mL) and was dried over $MgSO_4$. After filtration and solvent evaporation, the residue was crystallized from EtOAc/hexane to yield 768 mg (65%) Boc-D,L-Dmt(Boc)-OH: TLC R_f 0.42 (II); ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 9H, $C(CH_3)_3$), δ 1.46 (s, 9H, $C(CH_3)_3$), δ 2.26 (s, 6H, CH_3 ar), δ 2.85−3.05 (m, 2H, CH_2), δ 3.99−4.05 (m, 1H, CH), δ 6.78 (s, 2H, ar), δ 7.21 (d, 1H, NH); FAB-MS *m/e* 410.

Preparation of Boc-D,L-Tmt(Boc)-OH. N^{α}-Methylation was performed according to a published procedure.[16] Boc-D,L-Dmt(Boc)-OH (409 mg, 1 mmol) and iodomethane (512 μL, 8 mmol) were dissolved in 10 mL of THF/DMF (9:1,v/v). The solution was cooled to 0° C, and sodium hydride (48 mg, 1.2 mmol; 60% dispersion in mineral oil) was added slowly. The suspension, protected from the atmosphere by a drying tube, was stirred at room temperature for 24 h. The reaction was terminated by addition of 20 mL of EtOH, and then water was added drop by drop to destroy the excess of sodium hydride. After solvent evaporation, the oily residue was partitioned between ether (20 mL) and H_2O (30 mL). The organic phase was washed with three 20-mL portions of aqueous $NaHCO_3$ (10%), and the combined aqueous extracts were acidified to pH 3 with aqueous citric acid (5%). The precipitated product was extracted with three 20-mL portions of EtOAc; the extract was washed with water (3 × 20 mL), dried over $MgSO_4$, and evaporated. The crude product was crystallized from EtOAc/hexane to yield 381 mg (90%) of Boc-D,L-Tmt(Boc)-OH: TLC R_f 0.70 (II); ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 9H, $C(CH_3)_3$), δ 1.54 (s, 9H, $C(CH_3)_3$), δ 2.32 (s, 6H, CH_3 ar), δ 2.65 (s, 3H, NCH_3), δ 3.08−3.27 (m, 2H, CH_2), δ 4.26−4.44 (m, 1H, CH), δ 6.89 (s, 2H, ar); FAB-MS *m/e* 424.

Peptide Synthesis. Peptide synthesis was performed by the manual solid-phase technique using a *p*-methylbenzhydrylamine resin (1% cross-linked, 100−200 mesh, 0.54 mequiv/g of titratable amine) obtained from Bachem Bioscience. Peptides were assembled using Boc-protected amino acids and 1,3-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling agents. The side chains of Dmt and Tmt were also Boc-protected. Optically pure Boc-L-Dmt(Boc)-OH was used for the preparation of H-Dmt-Tic-Phe-Phe-NH_2, whereas racemic Boc-D,L-Dmt(Boc)-OH and Boc-D,L-Tmt(Boc)-OH were used in the syntheses of the other peptides. The following steps were performed in each cycle: (1) addition of Boc amino acid in CH_2Cl_2 (2.5 equiv); (2) addition of HOBt (2.5 equiv); (3) addition of DIC (2.5 equiv) and mixing for 2−3 h; (4) washing with CH_2Cl_2 (3 × 1 min); (5) washing with EtOH (1 min); (6) monitoring completion of the reaction with the ninhydrin test; (7) Boc deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min); (8) washing with CH_2Cl_2 (5 × 1 min); (9) neutralization with 10% (v/v) DIEA in CH_2Cl_2 (2 × 5 min); and (10) washing with CH_2Cl_2 (5 × 1 min).

To introduce the reduced peptide bond between the Tic[2] and Phe[3] residues, a reductive alkylation reaction[9] between 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde and the amino group of the resin-bound H-Phe-Phe dipeptide was performed as follows. After two washes with DMF, 2-Boc-1,2,3,4-tetrahydroisoquinoline-3-aldehyde (2.5 equiv) in DMF containing 1% AcOH was added to the resin. Sodium cyanoborohydride (5.0 equiv) was then added portionwise over a period of 40 min, and the reaction was allowed to continue for 3 h. The resin was then washed with DMF (2 × 1 min) and CH_2Cl_2 (3 × 1 min). Deprotection and coupling of Boc-Dmt(Boc) or Boc-Tmt(Boc)-OH were then performed according to the protocol described above.

After final deprotection with 50% (v/v) TFA in CH_2Cl_2 (30 min), the resin was washed with CH_2Cl_2 (3 × 1 min) and EtOH (3 × 1 min) and was dried in a desiccator. Peptides were cleaved from the resin by treatment with HF for 60 min at 0° C (20 mL of HF plus 1 mL of anisole/g of resin). After evaporation of the HF, the resin was extracted three times with Et_2O and, subsequently, three times with glacial AcOH. The crude peptide was obtained in solid form through lyophilization of the acetic acid extract.

Crude peptides were purified by preparative RP-HPLC. In the case of H-D,L-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2, H-D,L-Tmt-Tic-Phe-Phe-NH_2, and H−Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2, separation of the diastereoisomers was achieved using various gradients of MeOH in 0.1% TFA. For the stereochemical assignments the peptide isomers (0.2 mg) were hydrolyzed in 6 N HCl (0.5 mL) containing a small amount of phenol for 24 h at 110 °C in deaerated tubes and the hydrolysates were run on HPTLC-Chir plates (Merck) using the solvent system (v/v) acetonitrile/MeOH/H_2O (6:1:1): L-Dmt, R_f 0.75; D-Dmt, R_f 0.63; L-Tmt, R_f 0.76; D-Tmt, R_f 0.64. Each peptide was 98% pure as assessed by analytical RP-HPLC and by TLC. Molecular weights were confirmed by FAB-MS.

H-Dmt-Tic-Phe-Phe-NH_2: HPLC *K′* 4.75; TLC R_f 0.17 (III), R_f 0.67 (IV); FAB-MS *m/e* 662 (M^+).

H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2: HPLC *K′* 3.37; TLC R_f 0.24 (III), R_f 0.40 (IV); FAB-MS *m/e* 648 (M^+).

H-Tmt-Tic-Phe-Phe-NH_2: HPLC *K′* 4.54; TLC R_f 0.25 (III), R_f 0.57 (IV); FAB-MS *m/e* 676 (M^+).

H-Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2: HPLC *K′* 4.52; TLC R_f 0.24 (III), R_f 0.29 (IV); FAB-MS *m/e* 662 (M^+).

In Vitro Bioassays and Receptor Binding Assays. The GPI[17] and MVD[18] bioassays were carried out as reported in detail elsewhere.[19,20] A dose−response curve was determined with [Leu[5]]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure.[21] K_e values for antagonists were determined from the ratio of IC_{50} values obtained with an agonist in the presence and absence of a fixed antagonist concentration.[22] δ Antagonist K_e values of all compounds were determined in the MVD assay against the δ agonists DPDPE and deltorphin I using antagonist concentrations ranging from 0.5 to 100 nM. The μ antagonist K_e value of H-Tmt-TicΨ[CH_2NH]Phe-Phe-NH_2 was determined in the GPI assay against the μ agonist TAPP with an antagonist concentration of 500 nM.

Opioid receptor binding studies were performed as described in detail elsewhere.[19] Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA) and [^{3}H]DSLET (Multiple Peptide Systems) from rat brain membrane binding sites, and κ opioid receptor affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2 h at 0° C with [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,-593 at respective concentrations of 0.72, 0.78, and 0.80 nM. IC_{50} values were determined from log dose−displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff,[23] using values of 1.3, 2.6, and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET, and [^{3}H]U69,593, respectively.

In Vivo Testing. Male Long Evans rats (280−350 g) were used for analgesic testing and studies on the development of tolerance and dependence. Rats were anesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals; 60 mg/kg, ip), and 23-gauge stainless steel cannulae were implanted stereotaxically in the lateral ventricle (AP = −1.3 mm and L = −1.8 mm from bregma, and V = −3.8 mm from the skull surface for chronic infusion and −3.0 mm for acute injections with the injector extending 1.5 mm beyond).[24] For the acute tolerance and dependence studies, the cannulae were attached to model 2001 Alzet osmotic mimipumps (ALZA Corp., Palo Alto, CA) filled with one of the compound solutions or saline.

Analgesic potencies of compounds administered icv to rats were determined in the radiant heat tail flick test. Baseline tail flick latencies were measured for each rat prior to drug injection. Following icv administration of increasing doses of DIPP-NH_2[Ψ] (*n* = 5−6/dose) or morphine sulfate (*n* = 4−8/dose), tail flick latencies were measured for 2 h at 10-min intervals. A percent maximum possible effect (% MPE) score for each tail flick latency measurement following drug injection was calculated using the following formula: % MPE = [(test latency − baseline latency)/(cutoff latency − baseline latency)] × 100. A cutoff latency of 10 s was used to prevent tissue injury. The area under the curve (AUC) for the % MPE scores over the 2-h test period was calculated for each rat. ED_{50} values were calculated by performing regression analysis on AUC scores and interpolating from the regression equation to find the agonist dose which produced a half-maximal AUC. ED_{50} values are presented in Table 3 along with their 95% confidence intervals (CI).

For the assessment of the development of acute tolerance, compounds were infused icv at two different dose levels

(morphine: 0.3 μg/h (n = 10) and 9.0 μg/h (n = 14); DIPP-$NH_2[\Psi]$: 0.15 μg/h (n = 16) and 4.5 μg/h (n = 14)) continuously for 4 days. Tail flick latencies were measured every 15 min during the first 2 h, every 1 h during the next 6 h, and then every 12 h for the rest of the infusion period. The mean time in minutes after the initiation of drug treatment for tail flick latencies to return to baseline levels (≤20% MPE), also referred to as the mean time interval of analgesic efficacy, was then determined from the collected data. To assess the development of chronic tolerance, morphine (n = 60) and DIPP-$NH_2[\Psi]$ (n = 88) were injected icv at a dose of 0.3 and 0.15 μg, respectively, twice daily for 7 days. At the end of the drug treatment, rats were given icv injections of various doses of morphine sulfate (n = 5−8/dose) or DIPP-$NH_2[\Psi]$ (n = 7−12/dose) and tail flick latencies, % MPEs, AUC scores, and, if possible, ED_{50} values were determined as described above.

To determine the propensities to produce dependence, compounds were infused icv for 7 days at three different dose levels. Morphine doses were as follows: 0.3 μg/h (n = 9), 4.5 μg/h (n = 10), and 9.0 μg/h (n = 10); DIPP-$NH_2[\Psi]$ doses were as follows: 0.15 μg/h (n = 13), 2.25 μg/h (n = 10), and 4.5 μg/h (n = 10); saline (n = 15). Precipitated abstinence symptoms were assessed on the seventh day of treatment after injection of naloxone (1 mg/kg, sc). For 10 min before and 40 min after naloxone injection, the amount of time spent teeth chattering and writhing (combined) and the frequency of jumps and wet dog shakes (combined) were determined. The average severity of other checked signs, including ptosis, lacrimation, eye twitch, and scream on touch, during the 40-min withdrawal period was rated on a 4-point scale, where 0 = absent and 3 = severe.

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(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373. The other abbreviations are as follows: AUC, area under the curve; BBB, blood−brain barrier; Boc, *tert*-butoxycarbonyl; DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; DIC, 1,3-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DIPP-NH_2, H-Dmt-Tic-Phe-Phe-NH_2; DIPP-$NH_2[\Psi]$, H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2; Dmt, 2′,6′-dimethyltyrosine; DPDPE, H-Tyr-c[-D-Pen-Gly-Phe-D-Pen]-OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB-MS, fast atom bombardment mass spectrometry; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; icv, intracerebroventricular; MPE, maximum possible effect; MVD, mouse vas deferens; sc, subcutaneous; TAPP, H-Tyr-D-Ala-Phe-Phe-NH_2; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP-NH_2, H-Tyr-Tic-Phe-Phe-NH_2; Tmt, *N*,2′,6′-trimethyltyrosine; U69,593, (5α, 7α, 8β)-(−)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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