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Brief Article

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Synthesis of Tripeptides Containing D-Trp Substituted at the Indole Ring, Assessment of Opioid Receptor Binding and in Vivo Central Antinociception

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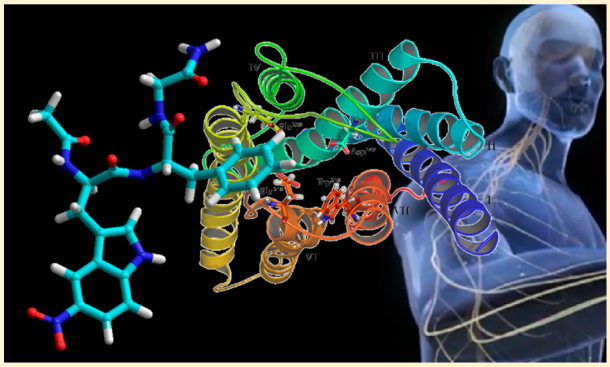
Supporting Information

ABSTRACT: The noncationizable tripeptide Ac-D-Trp-Phe-GlyNH$_2$ was recently proposed as a novel minimal recognition motif for μ-opioid receptor. The introduction of different substituents (methyl, halogens, nitro, etc.) at the indole of D-Trp significantly influenced receptor affinities and resulted in serum stability and in a measurable effect on central antinociception in mice after ip administration.



INTRODUCTION

The discovery in the mammalian brain of the endogenous opioid peptides endomorphin-1 (EM1: H-Tyr-Pro-Trp-PheNH$_2$) and endomorphin-2 (EM2: H-Tyr-Pro-Phe-PheNH$_2$)[1] aroused great expectations: the opportunity to develop painkillers devoid of the side effects of long-term usage of morphine and other μ-opioid receptor (MOR) agonists (acute tolerance, physical dependence, respiratory depression, nausea, and other gastrointestinal effects). EMs immediately attracted much attention for high MOR affinity and exceptional selectivity.[1,2] Given intrathecally, they displayed potent antinociception toward forms of pain which are resistant to morphine such as neuropathic pain.[3] The increasing interest spurred the development of modern green chemistry protocols for the production of large quantities of EMs.[4] However, their clinical use remains unrealistic due to poor metabolic stability, inability to cross the BBB, and efficient efflux.[2,5,6]

To enhance metabolic stability,[7] EM structures have been the object of extensive modifications by the peptidomimetic approach,[8] resulting in some cases in central antinociception (for selected examples, see Supporting Information). Other strategies were used to improve BBB penetration, including cationization, lipidation, glycosylation, vector-mediated transport, and halogenation (see also Supporting Information).[9,10] In this contest, a cyclic analogue of EM1 *c*[Tyr-D-Pro-D-Trp-Phe-Gly][11] showed 200 times weaker affinity than EM1, correlated to the lack of the amino group at Tyr,[1] which plays a relevant role in receptor interaction and signal transduction.[12] Nevertheless, the cyclopentapeptide was still a selective (partial) agonist of MOR by the cAMP functional assay and produced significant antinociception in a model of visceral pain.[13]

The derivative *c*[Tyr-Gly-D-Trp-Phe-Gly] (**1**), Figure 1, showed highly improved affinity and maintained the agonists

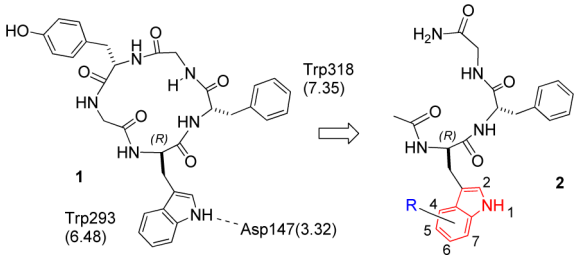


Figure 1. Opioid peptides **1** and **2a** and sketch of selected interactions of **1** with residues of MOR (Ballesteros Weinstein numbering system).

profile at MOR,[14] and the tripeptide Ac-D-Trp-Phe-GlyNH$_2$ (**2a**, R = H, Figure 1) was recognized as the minimalist MOR-active structure (Table 1).[15] Very recently, the closely correlated cyclotetrapeptide *c*[D-Pro-Phe-Trp-Phe], CJ-15,208, a naturally occurring metabolite of a fungus, revealed unexpected antinociceptive activity as a κ-opioid receptor (KOR)/MOR antagonist,[16] confirming that these lipophilic peptides might constitute promising candidates for the development of new analgesics.

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Table 1. In Vitro OR Affinities of Peptides 2 and Reference Compounds for Human ORs,[a] Peptide Stability (PS) of 1, 2a, 2m, and 2o, in Mouse Serum after 3 h, and Calculated Physicochemical Indicators of 1, 2a, 2b, 2l, 2m, and 2o

compd	sequence	purity (%)[b]	K_i MOR (nM)	K_i DOR (nM)	K_i KOR (nM)	PS (%)	clogP[c]	tPSA[c]	LLE[d]	LELP[e]
DAMGO	H-Tyr-D-Ala-Gly-*N*MePhe-Glyol		1.5 ± 0.1							
DPDPE	H-Tyr-*c*[D-Pen-Gly-Phe-D-Pen]OH			3.30 ± 0.05						
U50,488	nonpeptide				2.90 ± 0.04					
1	*c*[Tyr-Gly-D-Trp-Phe-Gly][f]	97	3.6 ± 0.3	>10^5	>10^5	90 ± 5	0.52	182	7.92	2.04
2a	Ac-D-Trp-Phe-GlyNH_2[g]	97	5.6 ± 0.2	>10^5	>10^5	59 ± 6	0.20	146	8.05	0.59
3	Ac-D-Trp-PheNH_2[g]	98	15.5 ± 0.5	24 ± 9[h]	>10^5					
2b	Ac-2-Me-D-Trp-Phe-GlyNH_2	96	314 ± 7	>10^5	>10^5		0.59	146	5.91	2.26
2c	Ac-5-Me-D-Trp-Phe-GlyNH_2	98	426 ± 16[i]	>10^5	>10^5					
2d	Ac-7-Me-D-Trp-Phe-GlyNH_2	96	>10^5							
2e	Ac-5-F-D-Trp-Phe-GlyNH_2	98	>10^5							
2f	Ac-6-F-D-Trp-Phe-GlyNH_2	96	5.7 ± 1.5[i]							
2g	Ac-5-Cl-D-Trp-Phe-GlyNH_2	96	20.8 ± 8.2[i]							
2h	Ac-6-Cl-D-Trp-Phe-GlyNH_2	96	>10^5							
2i	Ac-5-Br-D-Trp-Phe-GlyNH_2	95	114 ± 17	>10^5	>10^5					
2l	Ac-7-Br-D-Trp-Phe-GlyNH_2	96	190 ± 20	>10^5	>10^5		0.92	146	5.80	3.41
2m	Ac-5-NO_2-D-Trp-Phe-GlyNH_2	97	51.9 ± 7.4	>10^5	>10^5	93 ± 4	−0.73	192	8.01	−2.65
2n	Ac-5-Cl-2-Me-D-Trp-Phe-GlyNH_2	95	>10^5							
2o	Ac-7-Br-2-Me-D-Trp-Phe-GlyNH_2	98	4.03 ± 0.3	>10^5	>10^5	77 ± 4	1.32	146	7.07	4.04

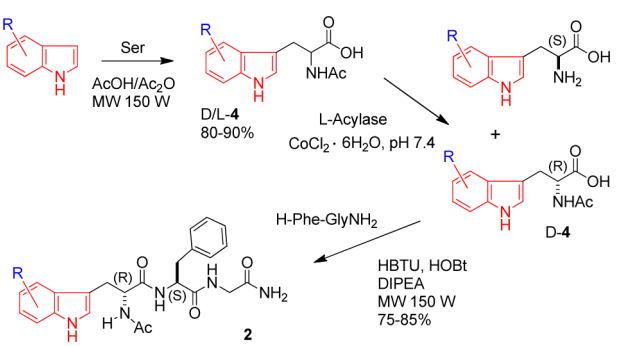
[a]Mean of 4−6 determinations ± SE. [b]Determined by RP-HPLC. [c]OSIRIS Property Explorer. [d]LLE = pK_i − clogP. [e]LELP = clogP/LE, LE = $\Delta G/N$, N = number of non-hydrogen atoms, $\Delta G = -RT \ln K_i$. [f]Reference 14. [g]Reference 15. [h][^{3}H]Diprenorphine displacement ≪50%. [i][^{3}H]DAMGO displacement ≪50%.

Conformational analysis and molecular docking led to a plausible pharmacophoric model;[14] the most relevant contacts in the message-recognition site of the receptor involve D-Trp (Figure 1). We supposed that the introduction in **2a** of EWGs or EDGs at various positions of the indole should impact H-bond and π-stacking interactions, as well as peptide stability and bioavailability. In this paper, we assess receptor affinity, central analgesia in vivo, and stability of novel compounds with substituted D-Trp in comparison to parent peptides **1** and **2a**.

RESULTS AND DISCUSSION

Very few procedures allow preparing optically pure D-configured Trp carrying substituents at different positions.[17,18] In this work, substituted and disubstituted Ac-D-Trp (**4**) have been prepared very conveniently by enzymatic resolution of the racemic mixtures,[19,20] prepared in turn from Ser and indoles in AcOH/Ac_2O (Scheme 1). The racemic acetyl-tryptophans D/L-**4** were treated with porcine kidney acylase and $CoCl_2(6H_2O)$ in a buffer pH 7.4 at 37 °C; it is well-known that this enzyme selectively deacetylates L-configured amino acids.[19,21] The reactions were monitored by RP-HPLC-ESI and continued until the peak areas of the Ac-**4** and *N*-deprotected tryptophans became nearly equivalent (for a representative example, see Supporting Information, Figure S1); in any case, the reactions were stopped after 3 days. The Ac-Trp **4** enriched in D-enantiomer and L-Trp were separated by extraction of the aqueous mixture with EtOAc at pH 3.

Scheme 1. Synthesis of Ac-Tripeptides 2b−o Containing Substituted D-Trp (R Groups, See Table 1)



The crude D-**4** were used for the reaction with H-Phe-GlyNH_2 in solution under MW irradiation, using HOBt/HBTU as coupling reagents (Scheme 1), giving the Ac-tripeptides **2** (Table 1) with R = methyl (**b**−**d**), fluoro (**e**,**f**), chloro (**g**,**h**), bromo (**i**,**l**), nitro group (**m**) at different positions, and combinations of methyl and halogens (**n**, **o**). Not unexpectedly,[19] the RP-HPLC (for a representative example, see Supporting Information, Figure S2) and NMR analyses of **2b**−**o** revealed the presence of variable amounts (5−10%) of epimers containing L-Trp. The stereochemistry of the major and minor epimer was confirmed by comparison with the HPLC and NMR analyses of **2a** and its epimer.[15] Finally, **2b**−**o** were obtained >95% pure by semipreparative RP-HPLC (e.g., Supporting Information, Figure S2).

To evaluate compounds' affinity, displacement binding assays were performed in HEK-293 cells expressing the cloned human MOR and using [^{3}H]DAMGO as specific radioligand.[14,15] The calculated K_i values are reported in Table 1. The reference compound DAMGO showed K_i in the nM range (Table 1), as expected (Supporting Information). The tripeptide **2a** was previously shown to selectively bind to MOR with nM affinity; the substitution of D-Trp with L-Trp hampered its ability to bind MOR.[15] The dipeptide Ac-D-Trp-PheNH_2 (**3**) maintained a good MOR affinity but with reduced selectivity (Table 1).[15]

Regarding the new compounds, the methyl substituted peptides 2-Me-**2b** and 5-Me-**2c** displayed affinity in the 10^{-7} M range, the latter displacing less than 50% of [^{3}H]DAMGO; on the other hand, 7-Me-**2d** did not show any significant affinity (Table 1). 6-Fluoro-**2f** showed some affinity for MOR (K_i = 5.7 ± 1.5 nM), whereas its 5-F-isomer **2e** did not. For the chloro-

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tripeptides, the scenario was reversed; the 5-Cl-**2g** a MOR ligand (K_i = 20.8 ± 8.2) and 6-Cl-**2h** were devoid of any affinity. However, both **2f** and **2g** displaced less than 50% of [^{3}H]DAMGO from MOR. 5-Br and 7-Br groups conferred the peptides **2h** and **2l** affinities in the 10^{-7} M range, while 5-nitro-**2m** showed a significant affinity to MOR (K_i = 51.9 ± 7.4 nM). Finally, **2n** and **2o** presented two substituents each at the indole: 5-Cl-2-Me-**2n** showed no receptor affinity, whereas 7-Br-2-Me-**2o** was an excellent MOR ligand (K_i = 4.03 ± 0.27 nM) despite of the presence of the two sterically demanding groups and the absence of any cationic group to bind the receptor by strong ionic interaction. It is noteworthy that individual 2-methyl (**2b**; K_i = 314 ± 7 nM) or 7-bromo substitutions (**2l**; K_i = 190 ± 20 nM) gave inferior affinities compared to the simultaneous presence of both groups (**2o**; K_i = 4.03 ± 0.27 nM).

In summary, it appeared that indole substitution had a strong impact on receptor affinities, possibly by tuning fundamental π-stacking and H-bond interactions with the receptor. Steric requirements seemed less important, as suggested for instance by the comparison of the different results for groups of similar size at the same position 5 (Table 1). The quantitative investigation of these interactions and the plausible impact on experimentally observed affinities is currently in progress in our group.

Affinity to the cloned human δ-opioid receptor (DOR) o KOR was also determined as previously reported (Supporting Information)[14,15] for those compounds which displayed a displacement of [^{3}H]DAMGO from MOR greater than 50%, using [^{3}H]diprenorphine and [^{3}H]U69,593 as DOR and KOR specific radioligands, respectively. The affinities of the reference compounds DPDPE and U50,488 were in agreement with the literature (see references in Supporting Information). None of the tested compounds displayed affinity to either DOR or KOR (Table 1), confirming their high MOR selectivity.

Previous experiments assessed the partial agonist behavior of the parent cyclopeptide **1**[14] and correlated tripeptide **2a**,[15] as they both inhibited forskolin-stimulated cAMP accumulation in HEK-293 cells stably expressing MOR, albeit to a lesser extent than morphine. This result was not unexpected because also EMs have been proposed as partial agonists in different experiments.[22,23] The lipophilic nature of agonists **1** and **2a**, the presence of a D-residue, and the cyclic structure (for **1**) led us to presume a potential ability to penetrate across the BBB, acting as analgesics in the CNS. Moving from these considerations, the central antinociceptive effect of **1** and **2a** was estimated in mice by the tail flick test (Figure 2). MOR best-binding compounds **2m** and **2o** were also assayed in the same analgesimetric test to investigate the impact of indole substitution, as it is well-known that the addition of alkyl groups or halogens to a peptide drug can improve bioavailability and therapeutic efficacy.[9,24] Dipeptide **3**, a good MOR ligand but devoid of agonist activity,[15] was employed as a negative reference.

The tail flick test was performed as previously described:[13] the tail of the animal was immersed in hot water and the latency to withdrawal measured in both vehicle- and compound-treated animals. The increase in the latency to response was expressed as the % of maximal possible effect (MPE%) with a cutoff of 10 s. The curves MPE versus time for the peptides ip administered at the dose of 20 mg/kg are shown in Figure 2A. The cyclopeptide **1** determined a mild analgesic effect, which was significant at 15 min (Figure 2A, 22% MPE) and peaked at 30 min of exposure (32% MPE). Tripeptide **2a** showed a similar analgesic profile (MPE of 19% and 37% at 15 and 30 min). As expected from its in vitro characterization, **3** did not differ from vehicle (Figure 2A). Antinociception mediated by 7-Br-2-Me-**2o** was already maximal at 15 min of exposure (33% MPE, Figure 2A), while at 30 min it showed an effect slightly reduced compared to that elicited by **2a** (32%) MPE. Finally, 5-nitro-**2m** displayed the highest analgesic effect among the tested compounds at 30 min (46% Figure 2A). Similarly to **2o**, also **2m** determined a higher antinociception at 15 min as compared to both **2a** and **1** (Figure 2A), thus highlighting a possible role of indole substitution in the faster onset of the analgesic effect, whereas antinociceptive potency was not increased. Interestingly, **2m** was comparatively more potent in vivo than **2o**, albeit displaying a slightly reduced receptor affinity (Table 1). The MPE versus time curves of the peptides ip administered at the dose of 10 mg/kg are reported in Supporting Information, Figure S3. In general, the peptides displayed a lower analgesic effect at 15 and 30 min after administration as compared to their effects when administered at 20 mg/kg. On the other hand, at the dose of 40 mg/kg (ip), **2m** produced an effect superimposable to that elicited by the dose of 20 mg/kg (MPE was maximal at 30 min after ip injection and reached 48%). A dose–response curve for the analgesic effect of **2m** at 30 min (10, 20, 40 mg/kg, ip) is reported in Figure 2B, showing a dose-releted antinociceptive response that was maximal at both 20 and 40 mg/kg.

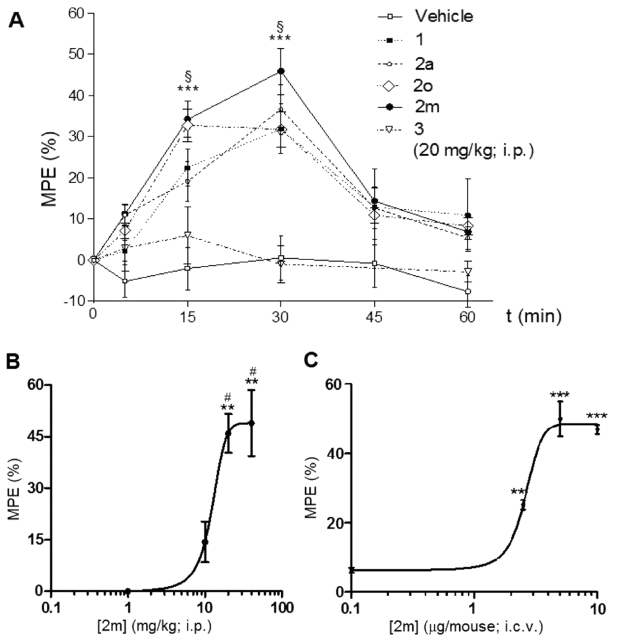

Figure 2. Antinociception produced by the tested compounds in the mouse tail flick assay. (A) Effect elicited by ip administered **1**, **2a**, **2m**, **2o**, and **3** (20 mg/kg); *** $p < 0.001$ vs vehicle; § $p < 0.05$ comparing **2m** with **2a**, **2o**, and **1**. (B) Dose–response curve of the antinociceptive response induced by ip administered **2m**; # $p < 0.05$ vs10 mg/kg; ** $p < 0.01$ vs vehicle. (C) Dose–response curve of the antinociceptive response induced by icv administered **2m**; ** $p < 0.01$ vs vehicle, *** $p < 0.001$ vs vehicle.

The antinociceptive profile of these compounds seems to be indicative of their action as partial opioid agonists. To confirm this idea, the most active opioid ligand **2m** was administered icv to circumvent any limited delivery to the CNS that could influence its antinociceptive activity after peripheral admin-

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istration. As shown in Figure 2C, icv administered **2m** resulted in a dose-related antinociceptive response in the tail-flick assay. Maximal antinociception peaked 10 min after injection, reaching 25%, 49%, and 47% MPE at the dose of 2.5, 5, and 10 μg/mouse, respectively. Apparently, compound **2m** displayed a partial antinociceptive activity when ip or icv administered, thus adding evidence that it acts as a partial MOR agonist. Analgesic activity observed after peripheral administration of **2m** seems to be not a consequence of a reduced passage through the BBB or due to its blood degradation. However, any contribution of active metabolites of **2m** to its in vivo activity deserves to be explored in further studies.

Antinociception elicited by these novel compounds would not affect behavioral responses; in fact, these compounds, administered at the above-mentioned doses, did not cause any significant alteration of spontaneous locomotor activity or circling behavior, Straub tail, or grooming (data not shown).

The complete characterization of the analgesic profile of the peptides is beyond the scope of this brief article. Albeit modest in a general perspective, the in vivo efficacies observed are not trivial, considering that linear peptides generally show very poor in vivo stability.[7,8] Previous experiments from our group and the literature showed that native EM1 was rapidly degraded and did not induce any significant elevation of the nociceptive threshold after ip administration.[2,8,25−27]

On the contrary, the tested compounds showed a good enzymatic and chemical stability (Table 1), as reported for other EM1 analogues.[28] The stability of peptides **1**, **2a**, **2m**, and **2o** was estimated by incubation in mouse serum for 3 h (see Supporting Information). Several studies carried out on novel opioid peptides have investigated the stability in serum or plasma because the peptides should resist proteolysis in the circulatory system to transverse the BBB and reach the brain.[28−30] In particular, 5-nitro-**2m** displayed high stability, being hydrolyzed only to a very modest extent (<10%) comparable to that of cyclic **1**, while 7-Br-2-Me-**2o** was slightly less stable (degraded ∼20%).

It can be perceived that compounds with apparently different structural properties showed similar onset of analgesic effects in the tail flick test in mice (Figure 2A). To correlate the in vivo efficacy to molecular properties, we calculated and analyzed relevant physicochemical indicators[31] of **1**, **2a**, **2m**, and **2o** (Table 1). The calculated log *P* (clogP), commonly utilized to estimate the octanol−water partition coefficient log *P*, is used as an index of molecular lipophilicity, which is a fundamental structural feature correlated to BBB penetration.[32] The topological polar surface area (tPSA), defined as the sum of surface contributions of polar atoms in a molecule, is another commonly used metric which has been shown to correlate well with drug transport properties.[33]

The ranking of clogP values[31] was: **2o**, 1.32; **1**, 0.52; **2a**, 0.20; **2m**, −0.73. The calculated tPSA30 values were: **2o**, 146; **2a**, 146; **1**, 182; **2m**, 192 (Table 1). It is generally accepted that low clogP and/or higher tPSA correspond to poor BBB permeation.[32,33] These calculated values accounted only partially for the experimental efficacy in the mouse tail flick assay (Figure 2A). In particular, **2m** showed significantly higher antinociception compared to the other compounds despite its clearly less favorable clogP and tPSA. Plausibly, this inconsistency is because the in vivo efficacy is also governed by intrinsic clearance and efflux transport in addition to BBB permeability.[34] A high BBB permeability enables rapid establishment of a distribution equilibrium between plasma and brain compartments, but it does not necessarily translate into sufficient unbound drug concentration in the brain for achieving in vivo efficacy.[35] At present, the recommended strategy to identify drug candidates with optimal CNS exposure is to increase permeability and solubility, reduce efflux transport, and minimize metabolism and systemic clearance.[35]

In this respect, we analyzed the lipophilic efficiency indices LLE and LELP (Table 1), which have been recently suggested to support balanced optimization of potency and ADMET profile.[35] The ligand lipophilicity efficiency, LLE = pK_i − clogP, represents the activity of a ligand without the contribution of its lipophilicity.[36,37] The optimal LLE scores range from ∼5−7 or greater. These values consent high potency while maintaining moderate lipophilicity. Aiming at enhancing drug potency, a common trend in drug discovery has emerged of placing too heavy a focus on advancing candidate molecules with higher overall lipophilicity.[34−37] The ligand efficiency-dependent lipophilicity index, LELP = clogP/LE, LE (kcal/mol) = $\Delta G/N$, $\Delta G = -RT \ln K_i$, N = number of non-hydrogen atoms, has been recently proposed to combine lipophilicity, molecular size, and potency into one composite descriptor.[37] The typical LELP scores are between −10 and 10; the lower the LELP, the more drug-like the compound.

The calculated LLE values were: **2a**, 8.05; **2m**, 8.01; **1**, 7.92; **2o**, 7.07. For LELP: **2m**, −2.65 (LELP can be less reliable for compounds with clogP <1);[37] **2a**, 0.59; **1**, 2.04; **2o**, 4.04. These rankings nicely correlated to compounds efficacy in the mouse tail flick assay. Clearly, the large LLE (>7) and the low LELP calculated for **2m** agreed with its comparatively higher efficacy in the mouse tail flick assay. Likely, the highly stable (Supporting Information), small, nonionic peptide **2m** might reach and diffuse into the CNS via the transcellular route in sufficient amount to elicit appreciable analgesia. Nevertheless, the possibility of BBB transfer via saturable transport systems, as proposed for opioid peptides including EMs,[2,38] cannot be ruled out. As for 7-Br-2-Me-**2o**, the comparison with the structurally correlated 2-Me-**2b** and 7-Br-**2l** recognizes the halogen as the major contributor to the lipophilicity indices of **2o** (Table 1). Possibly, the favorable clogP and tPSA support that **2o** is likely to cross the BBB. Accordingly, **2o** peaked already at 15 min (33% MPE, the same as **2m**), while the other tested compounds showed their maximal effect at 30 min (Figure 2A). However, the efficacy of **2o** rapidly declined (MPE at 30 min 32% vs 46% for **2m**), and this could be correlated to its lower enzymatic stability respect to **2m** (Supporting Information) and to its modest lipophilicity efficiency indices. The higher lipophilicity of **2o** can lead to nonproductive high nonspecific binding to lipids and proteins, rapid metabolism, intrinsic clearance, efflux transport, and subsequent low unbound drug concentration in the brain.[34,35,39]

CONCLUSIONS

In this work we have presented the expedient synthesis and opioid receptor affinities of a mini-library of tripeptides Ac-D-Trp-Phe-GlyNH_2 deprived of cationizable amino group, carrying methyl, nitro, and halo-substituents at various positions of the indole ring. Substitution had a strong impact on MOR affinities, which ranged from inactive to nanomolar. We also observed that the different groups at D-Trp influenced stability and lipophilicity. Peptides 5-nitro-**2m** and 7-Br-2-Me-**2o** showed moderately faster analgesia as compared to the cyclopeptide **1** and unsubstitued **2a** in mouse tail flick assay.

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The antinociceptive efficacy profiles nicely correlated to the compounds lipophilicity efficiency indices. These results support the optimality of the minimal three-residue motif as atypical, MOR-selective sequence.[40]

EXPERIMENTAL SECTION

General Methods. Chemicals were obtained from commercial sources. Mouse serum and porcine kidney acylase were purchased from Sigma-Aldrich; HEK-293 cells from ATCC, USA; plasmid pcDNA3.1+OPRM1 from UMR cDNA Research Center, USA; EXGEN 500 from Fermentas, USA. The MW-assisted synthesis was performed using a MicroSYNTH microwave lab station equipped with a built-in ATC-FO advanced fiber optic automatic temperature control. Purities were determined to be >95% by analytical RP-HPLC, performed on an C18 column (3 mm × 100 mm, 3.0 μm particle size, 110 Å pore diameter, DAD 210 nm, from a 9:1 H_2O/CH_3CN to a 2:8 H_2O/CH_3CN) at a flow rate of 1.0 mL/min. The RP-HPLC of compounds **4** were performed as described, with 0.1% TFA in the mobile phase and DAD at 254 nm. Semipreparative RP-HPLC was performed on a C18 column (21.2 mm × 150 mm, 7 μm particle size, from 8:2 H_2O/CH_3CN to 100% CH_3CN) at a flow rate of 12 mL/min. Elemental analyses were performed using a Thermo Flash 2000 CHNS/O analyzer. Mass analysis was done by ESI. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, in 5 mm tubes, at rt, 2D gCOSY were recorded in the phase sensitive mode and processed using a 90°-shifted, squared sinebell apodization. Chemical shifts are reported as δ values relative to the solvent peak.

*Preparation of Ac-D/L-tryptophans (**4**).* D/L-**4** was prepared as described in the literature.[19,20] Briefly, a mixture of Ser (1.06 g, 10.3 mmol) and indole (5.13 mmol) in AcOH (12.0 mL) and Ac_2O (4.0 mL) was stirred under MW irradiation (80 °C) for 30 min. For full details and workup, see Supporting Information. The crude D/L-**4** (80−90%, 70−80% pure by analytical RP-HPLC) were utilized without further purifications.

*Preparation of D-**4**.* The enzymatic resolution of D/L-**4** was performed as described.[19−21] Briefly, a suspension of D/L-**4** (0.41 mmol) in phosphate buffer pH 7.4 (20 mL) was shaken for 24−72 h at 37 °C with lyophilized porcine kidney acylase, grade II, 500−1500 units/mg (40 mg), and $CoCl_2(6H_2O)$ (0.20 mg). For full details and workup, see Supporting Information. The crude D-**4** (55−62%, 85−90% pure by analytical RP-HPLC) was utilized without further purification.

*Preparation of Ac-tripeptides (**2**).* The peptides were obtained by standard in-solution peptide synthesis from crude D-**4** (1.0 mmol) and H-Phe-GlyNH_2 (1.1 mmol) in 4:1 DCM/DMF (5 mL) under MW irradiation (80 °C) in the presence of HOBt/HBTU (1.1 mmol) and DIPEA (2.2 mmol). For full details and workup, see Supporting Information. The peptides **2** (75−85%) were isolated as single stereoisomers by semipreparative RP-HPLC (95−98% pure by analytical RP-HPLC).

Receptor Binding to Cloned Human MOR. HEK-293 cells were transfected with the human MOR encoding plasmid pcDNA3.1+OPRM1 using EXGEN 500. Cells stably expressing MOR were used for displacement binding assays as previously described,[14,15] with [^{3}H]DAMGO as MOR-specific radioligand. Compounds were assayed in the range of concentrations 10^{-12}−10^{-4} M. For full details and statistical analysis of data, see Supporting Information.

Tail Flick Test. The tail flick test was performed as previously described.[13] Briefly, a mouse's tail was immersed in hot water (52 ± 0.5 °C) and the latency to withdrawal was measured as a indicator of pain perception. Prior to being treated, each mouse was tested and the latency to tail flick was recorded (control latency, CL). Animals not flicking their tails within 5 s were not used (6% of mice). Responding animals were then ip injected with either vehicle or one of the compounds. Latency to withdrawal was measured at 5, 15, 30, 45, and 60 min after drug administration and defined as the test latency (TL), with a cutoff point of 10 s. The antinociceptive response was expressed as the percentage of MPE = 100 × (TL − CL)/(10 − CL). Statistical analysis was performed by two-way ANOVA with repeated measures and showed significant interactions ($p < 0.001$) between time and ligands' effects at 15 and 30 min.

Peptide Stability in Mouse Serum. Enzymatic degradation studies of the peptides and EM1 were carried out with mouse serum as described.[13] The amount of intact peptides was determined by RP-HPLC analyses (see Supporting Information).

ASSOCIATED CONTENT

Supporting Information

Syntheses and spectroscopic characterizations; binding assays to ORs; in vivo assays; peptide stability in mouse serum; references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; BBB, blood−brain barrier ; MW, microwave; DIPEA, diisopropylethylamine; HOBt, hydroxybenzotriazole; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; RP, reversed phase ; SE, standard error; EWG, electron withdrawing groups; EDG, electron donating group; ip, intraperitoneal; icv, intracerebroventricular

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