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Indoloquinolizidine–Peptide Hybrids as Multiple Agonists for D_1 and D_2 Dopamine Receptors

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Multiple-specificity ligands are considered promising pharmacological tools that may show higher efficacy in the treatment of diseases for which the modulation of a single target is therapeutically inadequate. We prepared a set of novel ligands for D_1 and D_2 dopamine receptors by combining two indolo[2,3-*a*]quinolizidine scaffolds with various tripeptide moieties. The binding and functional properties of these molecules were determined by radioligand binding studies in brain striatum membranes and by intracellular cAMP production assays in cells expressing different dopamine receptor subtypes. Some indoloquinolizidine–peptide hybrids, mainly with the *trans* configuration, showed dual agonist activity at both D_1 and D_2 dopamine receptors and may therefore be useful for testing the therapeutic potential of multivalent drugs on these targets.

Introduction

Multi-target ligand approaches constitute a new trend in pharmacological research, with potential applications in disorders for which the alteration of a single receptor is insufficient to elicit the desired therapeutic effect.[1] The development of ligands that simultaneously activate different dopamine receptor subtypes is one of the most successful therapeutic approaches for the treatment of Parkinson's disease (PD).[2,3] However, with the exception of levodopa, most multiple dopamine agonists in current use interact at D_2 dopamine receptors (D_2R) and D_3 dopamine receptors (D_3R), but bind poorly to the D_1 dopamine receptor (D_1R) subtype, a known target for antiparkinsonian agents.[2] Molecules that combine agonist activity at D_1R and D_2R may significantly induce motor activation on degenerated nigrostriatal dopaminergic systems. The simultaneous activation of the direct pathway (via D_1R) and repression of the inhibitory indirect pathway (via D_2R) may restore the effects of dopamine depletion on malfunctioning striatonigral and striatopallidal GABAergic neurons, respectively.[4] The synthesis of chemical entities that combine rigid heterocyclic scaffolds with small peptide moieties has been validated as a useful approach for the discovery of hits that interact at multiple G-protein-coupled receptors (GPCRs). Previous work by our research group on the synthesis of ergolene peptides (ergopeptides) led to the identification of multiple ligands for both adenosine and dopamine receptors.[5] Whereas the ergolene scaffold provides an interesting platform for the discovery of multiple ligands, the insolubility problems inherent to its structure cause a significant limitation to any further application. The more water-soluble indolo[2,3-*a*]quinolizidine structure **1** (Figure 1) resembles the indoloazecine and tetrahydro-β-carboline systems, whose affinities for some dopamine and serotonin receptors have already been reported,[6,7,8] and constitutes a novel and synthetically accessible scaffold. Synthetic routes to afford indolo[2,3-*a*]quinolizidines do not exceed five steps, are based on reliable chemistry, and provide the possibility to evaluate the influence of some stereochemical diversity on the biological properties. Taking these considerations into account, we envisaged the potential application of novel alkaloid–peptide hybrids that combine the indolo[2,3-*a*]quinolizidine structure with diverse peptide moieties. Therefore, a 20-member library of indoloquinolizidine–peptide hybrids was constructed, and their activity at D_1R and D_2R was evaluated. The incorporation of chemical diversity in both the indoloquinolizidine

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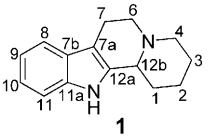


Figure 1. General indolo[2,3-*a*]quinolizidine structure.

core—using two stereochemically distinct structures—and the peptide units may provide general information about the interactions that favor the multiple activation of these receptors.

Results and Discussion

Library design and synthesis

To evaluate the potential of the indoloquinolizidine scaffold and its application in the development of dopamine ligands, we designed the synthesis of indoloquinolizidine–peptide hybrids of general structure **2** (Figure 2 a). This set of compounds combines two different diastereomeric carboxylic acids and nine different tripeptide moieties (Figure 2 b). The selection of the tripeptides was performed on the basis of our previous work,[5] thus choosing those peptides which may enhance the affinity of the heterocyclic core for the dopamine receptors.

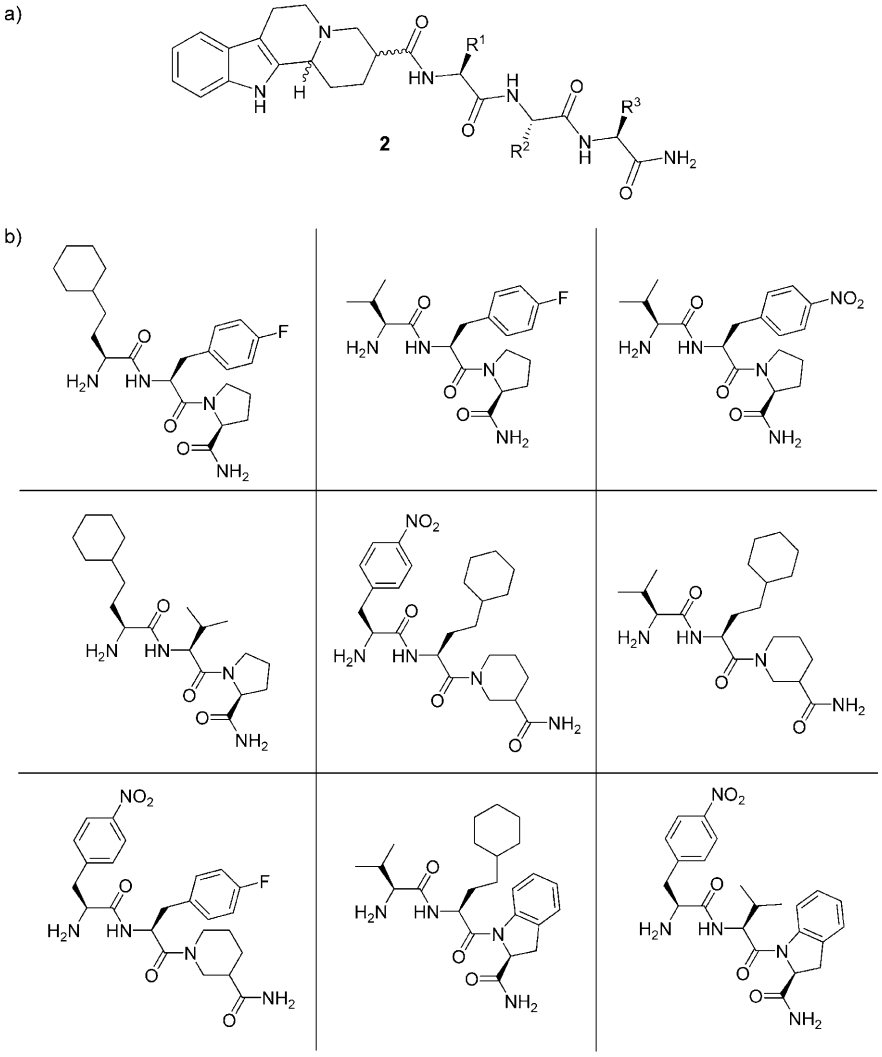


Figure 2. a) General structure of the indoloquinolizidine–peptide hybrids;, b) structures of the nine tripeptides used for construction of the library.

Acids **3** and **4** (Figure 3) present different relative stereochemistry at C3 and C12b (*cis* and *trans*, respectively), allowing distinct indolo[2,3-*a*]quinolizidine configurations to influence the biological properties of the indoloquinolizidine–peptide hybrids. On the other hand, the use of a racemic mixture for both carboxylic acids can be justified, considering this first set of indoloquinolizidine–peptide hybrids as probes for an exploratory analysis. The synthesis of carboxylic acids **3** and **4** was based on the protocol of Wenkert et al. (acid cyclization of *N*-trypthophyl-1,4-dihydropyridines to afford indoloquinolizidines).[9]

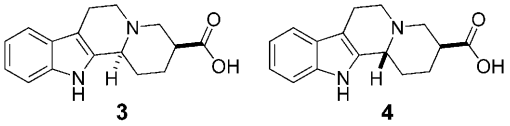
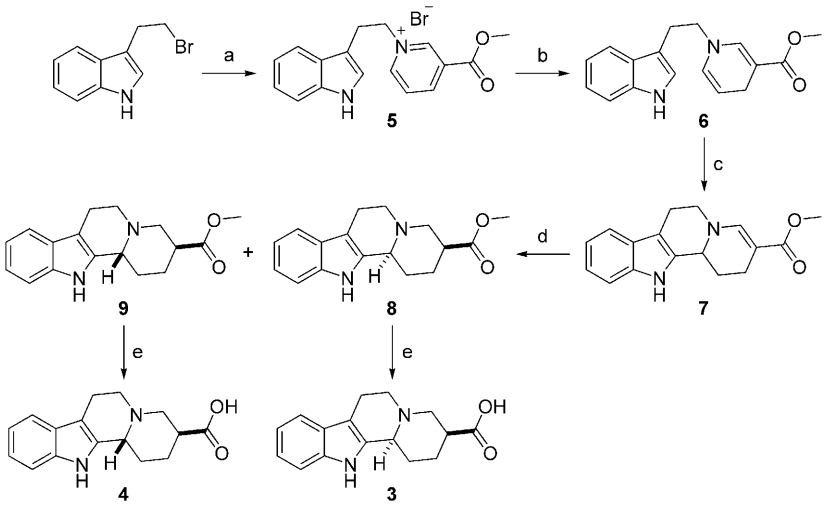


Figure 3. (3*RS*,12b*RS*)- and (3*RS*,12b*SR*)-1,2,3,4,6,7,12,12b-octahydroindolo-[2,3-*a*]quinolizin-3-carboxylic acids and general structure of indoloquinolizidine–peptide hybrids. Compounds **3** and **4** give rise to *cis* and *trans* indoloquinolizidine–peptide hybrids, respectively.

The first step in the synthesis of **3** and **4** (Scheme 1) consisted of the formation of the pyridinium salt **5**.[10] The reaction of tryptophyl bromide with methyl nicotinate in anhydrous methanol afforded the desired salt **5** in good yield (72%). Reduction of *N*-alkylpyridinium salts can lead to 1,2- or 1,4-dihydropyridines, depending on the reaction conditions used.[11,12] To selectively generate the 1,4 regioisomer, this transformation was carried out with sodium dithionite in buffered media and satisfactorily yielded dihydropyridine **6** (81%). This labile intermediate **6** was immediately cyclized by treatment with concentrated HCl in methanol to yield the corresponding tetrahydropyridine **7** (85%) as a racemic mixture in a process resembling the Pictet–Spengler reaction, which has been reported as an efficient way to obtain the tetrahydro-β-carboline ring system.[13,14] Next, the enamine reduction to afford the corresponding amino esters **8** and **9** was studied. This transformation involved the generation of a second stereogenic center, and in order to control the stereochemistry, two different re-



Scheme 1. Synthesis of **3** and **4**. Reagents and conditions: a) methyl nicotinate in MeOH, b) $Na_2S_2O_4/NaHCO_3$, CH_2Cl_2/H_2O, c) HCl (conc) in MeOH, d) $NaBH_3CN$ in AcOH, e) H_2O/CH_3CN, Δ.

duction methods were assayed: H_2–Pd/C and sodium cyanoborohydride in acid media.[15,16] Although both reactions proceeded with good yields (70–82%), the catalytic hydrogenation showed higher stereoselectivity, leading to the *trans* isomer **9** as the major product (*cis*/*trans* 8:1), whereas reduction with sodium cyanoborohydride in acetic acid slightly favored the *cis* isomer **8** (*cis*/*trans* 3:4). To explore various indoloquinolizidine stereochemistries, reduction with sodium cyanoborohydride was chosen as the most convenient method, allowing the isolation of the required indoloquinolizidines **8** and **9** after one stereochemically divergent reaction from a common precursor and standard purification by flash chromatography. Remarkably, hydrolysis of **8** and **9** under neutral conditions (water/acetonitrile, Δ, 3–5 days) led to the corresponding carboxylic acids **3** and **4** in a convenient manner. Although this hydrolysis is slower than what would result from using basic conditions (aqueous potassium hydroxide), it allows the isolation of the carboxylic acids rather than the corresponding carboxylates by simply removing the solvent.[17]

Once the synthesis of **3** and **4** was completed, the nine tripeptides were synthesized on solid phase using Rink–PS resin as a polymeric support, under standard peptide-coupling conditions (DIPCDI and HOBt as an additive) and a final cleavage with trifluoroacetic acid/water (95:5) to release the free N^{α}-terminal tripeptides with good yields (compounds **10**–**18**, table 1 in Supporting Information (SI)). Individual coupling of **3** and **4** to the nine different peptide moieties **10**–**18** using DIPCDI and HOAt resulted in the generation of 18 indoloquinolizidine–peptide hybrids. Purification by semipreparative RP-HPLC afforded the final products (compounds **19**–**36**, table 2 in SI). To check the binding properties of the indolo[2,3-*a*]quinolizidine scaffold itself, the corresponding primary amides of **3** and **4** were also synthesized. In this case, direct coupling of **3** and **4** to the Rink–PS resin (DIPCDI, HOAt), subsequent cleavage with TFA/H_2O (95:5), and purification by semipreparative RP-HPLC yielded the respective primary amides **37** and **38** (Figure 4).

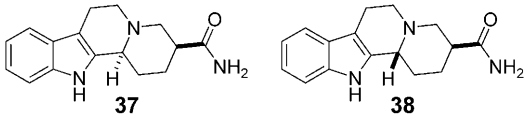


Figure 4. (3*RS*,12b*RS*)- and (3*RS*,12b*SR*)-1,2,3,4,6,7,12,12b-octahydroindolo-[2,3-*a*]quinolizin-3-carboxamides **37** and **38**, respectively.

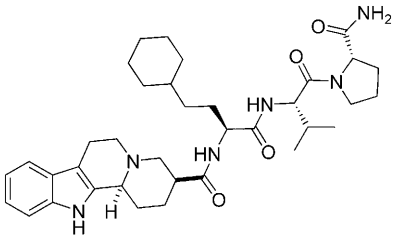
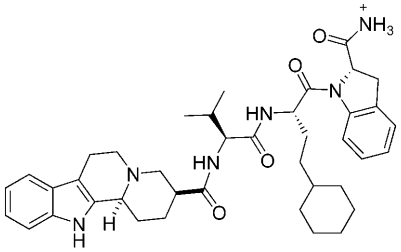
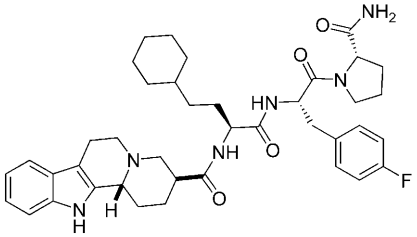
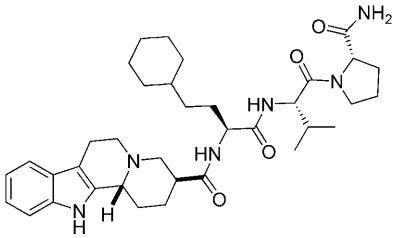
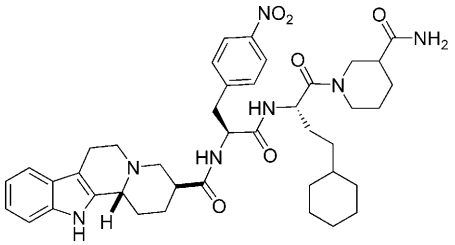
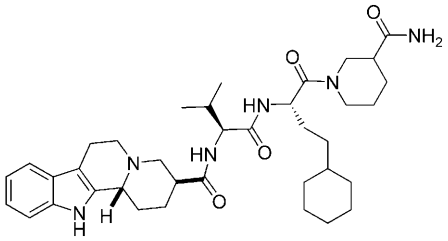
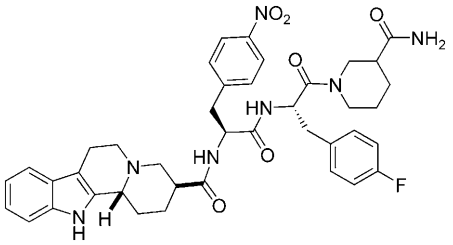
Biological assays: binding affinities of indoloquinolizidine–peptide hybrids

The binding properties of **19**–**38** were initially assayed by competitive radioligand binding experiments with D_1R and D_2R, using the radioligands [^{3}H]SCH 23390 (for D_1R) or [^{3}H]YM 09151-2 (for D_2R) and the indoloquinolizidine–peptide hybrids (at 50 μM; see Experimental Section). Because most of the compounds were able to displace more than 50% of the specific radioligand binding, further screenings at 5 μM were performed (figure 1 in SI). Interestingly, several indoloquinolizidine–peptide hybrids exhibited higher affinities than dopamine, especially at D_2R, and a strong influence of the indoloquinolizidine stereochemistry was observed in the interaction with both receptors: whilst *cis* derivatives **19**–**27** showed low or moderate affinities, stronger interactions were detected for *trans* derivatives **28**–**36**. To confirm these preliminary results, seven indoloquinolizidine–peptide hybrids (**22**, **26**, **28**, **31**, **32**, **33**, and **34**) similarly affecting D_1R and D_2R binding were selected to determine their affinity constants (K_D) for the receptors studied. K_D values (Table 1) reflect that the *trans*-indoloquinolizidine–peptide hybrids **28**, **31**, **32**, **33**, and **34** interact with greater affinities than the *cis* derivatives, and this selectivity is especially enhanced for the binding at D_1R.

Because the affinities of the tripeptides for the dopamine receptors were proven to be marginal,[1] these data suggest that the indoloquinolizidine scaffold may be responsible for the interaction at the binding site of the dopamine receptors, showing a stronger affinity in the case of the *trans* relative configuration. The tripeptides would therefore interact with the amino acids surrounding the binding site, and would lead to a better or worse accommodation of the heterocycle, explaining the binding and selectivity variations amongst the indoloquinolizi-

[1] *See Ref. [5]. Radioligand binding experiments with the N^{α}-acetylated tripeptides (50 μM) at D_1R and D_2R did not show any significant interaction of any of the tripeptides at these receptors.*

Table 1. Structures of selected indoloquinolizidine–peptide hybrids and their corresponding K_D values.

Compd	Structure	K_D [μM] D_1R	D_2R
22		17 ± 2	6 ± 2
26		27 ± 2	10 ± 2
28		0.35 ± 0.03	1.5 ± 0.2
31		1.7 ± 0.1	6 ± 1
32		4.5 ± 0.7	3.0 ± 0.3
33		7 ± 1	5 ± 1
34		0.51 ± 0.05	3.7 ± 0.5

dine–peptide hybrids. Additional binding studies at other GPCRs expressed in the brain striatum (A_1 adenosine or H_3 histamine receptors, table 3 in SI), performed as described in the Experimental Section, revealed that the indoloquinolizidine–peptide hybrids show a potentially useful selectivity profile as dual ligands for D_1R and D_2R.

Biological assays: identification of agonist/antagonist behavior

To characterize the agonist/antagonist behavior of the indoloquinolizidine–peptide hybrids at D_1R and D_2R, two *cis* derivatives (**22** and **26**, both with K_D values in the micromolar range) and two *trans* compounds (**28** and **33**, both with K_D values in the low micromolar range) were tested by evaluating intracellular cAMP levels derived from the activation or inhibition of adenylate cyclase in two cell lines: a D_2R-CHO stable cell line, and the Flp-In T-REx-293-D_1R cell line, which expresses human D_1R after induction with tetracycline.

D_2R activation leads to coupling with $G_{\alpha i}$ proteins, resulting in the inhibition of adenylate cyclase, and thus to a decrease in cAMP levels.[18] D_2R agonism therefore produces a decrease in the increased cAMP levels induced by forskolin. All compounds induced a significant decrease in cAMP levels (Figure 5), as is the case for the selective D_2R agonist (±)-2-(*N*-phenethyl-*N*-propyl)amino-5-hydroxytetralin (PPHT), indicating that they behave as D_2R agonists. Combined administration of PPHT and the four indoloquinolizidine–peptide hybrids did not diminish the capacity of PPHT to decrease forskolin-induced cAMP levels, thereby indicating that indoloquinolizidine–peptide hybrids do not act as D_2R antagonists.

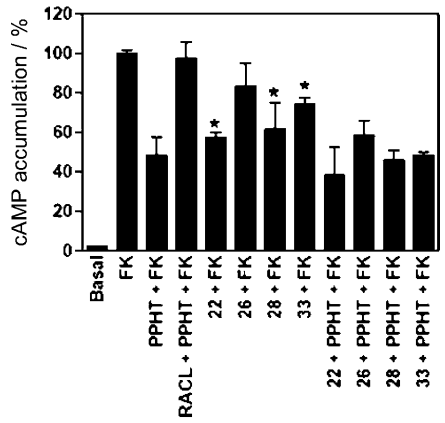


Figure 5. cAMP determination in CHO cells stably expressing the human D_2R. Cells were treated with 5 μM forskolin (FK) in the absence or presence of: 30 nM D_2R agonist PPHT, 30 μM D_2R antagonist raclopride (RACL) + 30 nM PPHT, 50 μM indoloquinolizidine–peptide hybrids, 50 μM indoloquinolizidine–peptide hybrids + 30 nM PPHT, as indicated in the Experimental Section. Results are presented as the percentage of cAMP accumulation obtained in the presence of FK (mean ± SD of 3–6 determinations, significant differences with respect to FK-treated cells were calculated by Student's *t* test for unpaired samples $^*p < 0.01$).

On the other hand, D_1R couple with $G_{\alpha s}$ proteins, and their activation induces an increase in cAMP levels.[19] Interestingly, when tested in the Flp-In T-REx-293-D_1R cell line, all four indoloquinolizidine–peptide hybrids increased cAMP levels to a similar extent as the selective D_1R agonist (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol (SKF 38393), showing that they behave as D_1R agonists and not as D_1R antagonists, as they do not diminish the capacity of SKF 38393 to increase cAMP levels (Figure 6). These results clearly indicate that the dual activation of D_1R and D_2R does not depend on the configuration of the indoloquinolizidine scaffold, as both *cis* and *trans* derivatives behave as agonists for both receptors. Moreover, this pharmacological profile is not significantly different in compounds showing K_D values in a different range (such as **28** and **33**). Therefore, these data support our above-mentioned hypothesis about the binding mode for the indoloquinolizidine–peptide hybrids, in which the heterocyclic core is primarily responsible for the interaction at the binding sites of D_1R and D_2R.

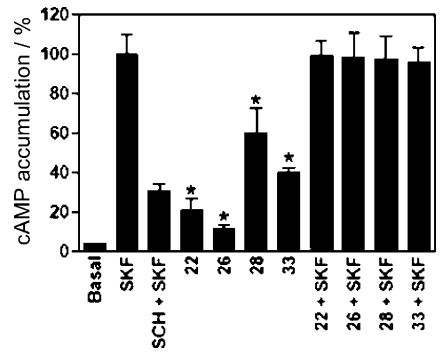


Figure 6. cAMP determination in Flp-In T-REx-293-D_1R cells. Cells were treated with 20 nM D_1R agonist SKF 38393, 50 μM D_1R antagonist SCH 23390 + 20 nM SKF 38393, 50 μM indoloquinolizidine–peptide hybrids, 50 μM indoloquinolizidine–peptide hybrids + 20 nM SKF 38393, as indicated in the Experimental Section. Results are presented as the percentage of cAMP accumulation obtained in the presence of SKF 38393 (mean ± SD of 3–6 determinations, significant differences with respect to basal levels were calculated by Student's *t* test for unpaired samples $^*p < 0.01$).

Conclusions

The chemical combination of the novel and synthetically available indolo[2,3-*a*]quinolizidine structure with various linear peptide moieties has led to the first indoloquinolizidine–peptide hybrids reported to date. These new compounds behave as multiple ligands for D_1R and D_2R, showing an enhanced activity relative to the unmodified heterocycle and notable selectivity versus other GPCR subtypes, such as adenosine or histamine. Interestingly, the binding mode of indoloquinolizidine–peptide hybrids at D_1R or D_2R is highly dependent on the indoloquinolizidine relative configuration, with stronger interactions observed for the *trans* derivatives **28**–**36** than for the *cis* derivatives **19**–**27**. Furthermore, analysis of the intracellular cAMP levels in cells expressing D_1R and D_2R indicates that these hybrids have a relevant pharmacological profile owing to their behavior as multiple dopamine agonists, with no dependence on either the peptide sequences or the indoloquinolizidine configuration. Making use of the above-mentioned ligands, we are currently working on the synthesis of novel indoloquinolizidine–peptide hybrids that show enhanced affinity for a number of dopamine receptor subtypes.

Experimental Section

Materials and equipment

All Fmoc-amino acids were purchased from Neosystem (Strasbourg, France) and Fmoc-Rink–PS resin was supplied by Calbiochem-Novabiochem AG. 1,3-Diisopropylcarbodiimide (DIPCDI) was obtained from Fluka Chemika (Buchs, Switzerland) and 1-hydroxy-(1*H*)-benzotriazole (HOBt) from Albatross Chem, Inc. (Montréal, Canada). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid (TFA) was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals of the highest commercially available purity were purchased from Aldrich (Milwaukee, WI, USA). All commercial reagents and solvents were used as received.

Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco BRL (Paisley, Scotland, UK). α-MEM without nucleosides, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, blasticidin, hygromycin B, geneticin (G418), and the Flp-In T-REx System kit were purchased from Invitrogen (Paisley, Scotland, UK). The iProof and iTaq kits were supplied by Bio-Rad (Hercules, CA, USA). Adenosine deaminase (EC 3.5.4.4) was purchased from Roche (Basel, Switzerland). [^{3}H](*R*)-PIA and the [^{3}H]cAMP assay system were supplied by Amersham Biosciences (Buckinghamshire, UK). Raclopride, dopamine, $MgCl_2$, DPCPX, PPHT, forskolin, SCH 23390 and methylhistamine were purchased from Sigma (St. Louis, MO, USA). SKF 38393 and zardaverine were supplied by Tocris Biosciences (Avonmouth, UK). [^{3}H]SCH 23390, [^{3}H]YM 09151-2 and [^{3}H]methylhistamine were supplied by PerkinElmer (Boston, MA, USA). Ecoscint H scintillation cocktail was purchased from National Diagnostics (Atlanta, GA, USA).

Analytical RP-HPLC–MS was performed with a 2795 Waters Alliance LC instrument (Milford, MA, USA) coupled with a Micromass ZQ mass spectrometer and a 996 PDA detector. Semipreparative RP-HPLC was performed on a 2767 Waters chromatography system with a Micromass ZQ mass spectrometer. Multiple sample evaporation was carried out in a Discovery SpeedVac ThermoSavant (Waltham, MA, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury 400 spectrometer (Unitat de RMN, Serveis Cientifico-Tècnics, University of Barcelona); chemical shifts (δ) are reported in ppm, and coupling constants are given in Hz. Radioligand binding experiments were performed using a Brandel (Gaithersburg, MD, USA) cell harvester and a Packard 1600 TRI-CARB scintillation counter.

Synthesis

1-[2-(indol-3-yl)ethyl]-3-methoxycarbonylpyiridinium bromide (5). Tryptophyl bromide (3.43 g, 15.3 mmol) and methyl nicotinate (1.90 g, 13.9 mmol, 0.9 equiv) were dissolved in anhydrous MeOH (15 mL). The mixture was stirred under Ar atmosphere at room temperature. The reaction was monitored by TLC (AcOH/MeOH, 2:1). After 48 h, the solvent was removed in vacuo, and the crude was washed with *tert*-butyl methyl ether (5×30 mL), obtaining **5** as a yellow solid after decantation and in vacuo removal of the solvent (4.02 g, 72%): R_f=0.41 (AcOH/MeOH, 2:1, *v/v*); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.98 (s, 1H, NH), 9.40 (s, 1H, Ar), 9.12 (d, J=6.1 Hz, 1H, Ar), 8.92 (d, J=7.8 Hz, 1H, Ar), 8.17 (dd, J=7.8, 6.4 Hz, 1H, Ar), 7.52 (d, J=7.9 Hz, 1H, Ar), 7.33 (d, J=8.1 Hz, 1H, Ar), 7.08 (dd, J=8.0, 7.2 Hz, 1H, Ar), 7.06 (s, 1H, Ar), 6.95 (dd, J=7.7, 7.2 Hz, 1H, Ar), 4.97 (t, J=7.1 Hz, 2H, CH$_2$), 3.92 (s, 3H, CH$_3$), 3.32 ppm (t, J=7.0 Hz, 2H, CH$_2$); ^{13}C NMR (400 MHz, [D$_6$]DMSO): δ=162.7, 148.6, 146.5, 145.7, 136.8, 130.1, 128.6, 127.3, 124.9, 122.0, 119.3, 118.8, 112.2, 108.9, 62.4, 54.1, 27.5 ppm; MS (ESI) *m/z* [*M*+H]$^+$: 282.3.

1-[2-(indol-3-yl)ethyl]-1,4-dihydropyridine-3-carboxylic acid methyl ester (6). Compound **5** (4.02 g, 11.1 mmol) was dissolved in H$_2$O/CH$_2$Cl$_2$ (200 mL, 2:1, *v/v*). The resulting suspension was cooled over an ice bath, and NaHCO$_3$ (3.73 g, 44.4 mmol, 4 equiv) and Na$_2$S$_2$O$_4$ (9.78 g, 55.5 mmol, 5 equiv) were slowly added. After the addition of both reagents, the ice bath was removed, and the mixture was stirred under Ar atmosphere at room temperature. The reaction was monitored by TLC (AcOH/MeOH, 2:1). After 18 h, TLC indicated that reactant **5** had been consumed. The organic phase was extracted, washed with a solution of Na$_2$CO$_3$ (3×150 mL), dried over anhydrous MgSO$_4$, and concentrated in vacuo to yield **6** as a yellow solid (2.51 g, 82%): R_f=0.33 (hexane/EtOAc/DEA, 6:4:0.1, *v/v*); ^{1}H NMR (400 MHz, CDCl$_3$): δ=8.21 (s, 1H, Ar), 7.56 (d, J=7.9 Hz, 1H, Ar), 7.35 (d, J=8.1 Hz, 1H, Ar), 7.20 (dd, J=8.0, 7.2 Hz, 1H), 7.13 (dd, J=8.0, 7.2 Hz, 1H), 7.00 (s, 1H), 6.96 (s, 1H), 5.66 (d, J=8.0 Hz, 1H, CH), 4.72 (dt, J=7.9, 3.5 Hz, 1H, CH), 3.65 (s, 3H, CH$_3$), 3.36 (t, J=7.2 Hz, 2H, CH$_2$), 3.11 (d, J=3.4 Hz, 2H, CH$_2$), 2.98 ppm (t, J=7.2 Hz, 2H, CH$_2$); ^{13}C NMR (400 MHz,CDCl$_3$): δ=169.2, 142.1, 136.6, 128.7, 127.3, 122.7, 122.3, 119.7, 118.6, 112.1, 111.6, 104.8, 96.7, 54.8, 51.1, 26.4, 22.3 ppm.

(±)-1,2,6,7,12,12b-hexahydroindolo[2,3-*a*]quinolizin-3-carboxylic acid methyl ester (7). Compound **6** (2.51 g, 8.9 mmol) was dissolved in anhydrous MeOH (35 mL), and concentrated HCl (37%, 3 mL) was added to the solution. The mixture was stirred under Ar atmosphere at room temperature. After 5 h, the solvent was removed in vacuo, and a saturated solution of Na$_2$CO$_3$ was added until pH 10 was reached. The resulting crude was diluted in CH$_2$Cl$_2$, and the organic layer was washed with a solution of Na$_2$CO$_3$ (3×50 mL), dried over anhydrous MgSO$_4$, and concentrated in vacuo to yield the racemic mixture **7** as a yellow solid (2.14 g, 85%): R_f=0.50 (hexane/EtOAc/DEA, 2:8:0.1, *v/v*); ^{1}H NMR (400 MHz, CDCl$_3$): δ=8.24 (s, 1H, NH), 7.51 (s, 1H, Ar), 7.47 (d, J=7.6 Hz, 1H, Ar), 7.34 (d, J=8.0 Hz, 1H, Ar), 7.17 (dd, J=7.6, 7.2 Hz, 1H, Ar), 7.15 (dd, J=8.0, 7.2 Hz, 1H, Ar), 4.46 (d, J=10.2 Hz, 1H, CH), 3.70 (s, 1H, CH$_2$), 3.61 (m, 2H, CH$_2$), 2.90 (m, 1H, CH$_2$), 2.78 (dd, J=14.8, 1.6 Hz, 1H, CH$_2$), 2.56 (m, 1H, CH$_2$), 2.39 (m, 2H, CH$_2$), 1.78 ppm (m, 1H, CH$_2$); ^{13}C NMR (400 MHz, CDCl$_3$): δ=169.2, 146.6, 136.4, 133.1, 127.1, 122.3, 120.0, 118.4, 111.2, 108.7, 95.0, 52.1, 51.1, 50.9, 28.6, 22.2, 20.6 ppm; MS (ESI) *m/z* [*M*+H]$^+$: 283.1.

(3*RS*,12b*RS*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxylic acid methyl ester (8) and (3*RS*,12b*SR*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxylic acid methyl ester (9). Compound **7** (2.14 g, 7.6 mmol) was dissolved in AcOH, and NaBH$_3$CN (3.56 g, 56.6 mmol, 7.5 equiv) was added over an ice bath. After the addition, the ice bath was removed, and the mixture was stirred under Ar atmosphere at room temperature. After 5 h, the solvent was removed in vacuo, and an aqueous solution of NH$_3$ was added until pH 10 was reached. The resulting crude was diluted in CH$_2$Cl$_2$, and the organic layer was washed with a saturated solution of NaHCO$_3$ (3×50 mL), dried over anhydrous MgSO$_4$, and concentrated in vacuo to yield a yellow solid. Flash chromatography (hexane/EtOAc/DEA) gave rise to diastereomers **8** and **9**.

*Ester **8*** (410 mg, 19%): R_f=0.23 (hexane/EtOAc/DEA, 2:3:0.02, *v/v*); ^{1}H NMR (400 MHz, CDCl$_3$): δ=7.75 (br s, 1H, NH), 7.46 (d, J=7.6 Hz, 1H, Ar), 7.31 (d, J=8.0 Hz, 1H, Ar), 7.12 (t, J=8.0 Hz, 1H, Ar), 7.07 (t, J=7.6 Hz, 1H, Ar), 3.67 (s, 3H, CH$_3$), 3.48 (m, 1H, CH$_2$), 3.34 (m, 1H, CH), 3.07 (m, 1H, CH), 2.96 (m, 1H, CH$_2$), 2.76 (m, 1H, CH$_2$), 2.70 (m, 1H, CH$_2$), 2.65 (m, 1H, CH$_2$), 2.16 (m, 1H, CH$_2$), 1.98 (m, 1H, CH$_2$), 1.93 (m, 1H, CH$_2$), 1.70 ppm (m, 2H, CH$_2$); ^{13}C NMR (400 MHz, CDCl$_3$): δ=174.4, 136.1, 134.4, 127.6, 121.6, 119.6, 118.3, 111.0, 108.4, 58.7, 54.9, 53.1, 52.0, 40.6, 27.3, 24.6, 20.8 ppm; MS (ESI) *m/z* [*M*+H]$^+$: 285.0.

*Ester **9*** (610 mg, 29%): R_f=0.59 (hexane/EtOAc/DEA, 2:3:0.02, *v/v*); ^{1}H NMR (400 MHz, CDCl$_3$): δ=7.75 (br s, 1H, NH), 7.48 (d, J=7.6 Hz, 1H, Ar), 7.31 (d, J=8.0 Hz, 1H, Ar), 7.14 (t, J=8.0 Hz, 1H, Ar), 7.09 (t, J=7.6 Hz, 1H, Ar), 3.71 (s, 3H, CH$_3$), 3.29 (m, 1H, CH$_2$), 3.26 (m, 1H, CH), 3.11 (m, 1H, CH$_2$), 2.99 (m, 1H, CH$_2$), 2.81 (m, 1H, CH$_2$), 2.71 (m, 1H, CH$_2$), 2.68 (m, 1H, CH$_2$), 2.50 (t, J=9.6 Hz, 1H, CH$_2$), 2.21 (m, 1H, CH$_2$), 2.15 (m, 1H, CH$_2$), 1.68 (m, 1H, CH$_2$), 1.61 ppm (m, 1H, CH$_2$); ^{13}C NMR (400 MHz, CDCl$_3$): δ=174.5, 136.3, 134.6, 127.6, 121.7, 119.7, 118.4, 111.0, 108.5, 59.7, 57.3, 53.4, 52.0, 42.0, 29.3, 27.3, 21.9 ppm; MS (ESI) *m/z* [*M*+H]$^+$: 285.0.

(3*RS*,12b*RS*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxylic acid (3). Compound **8** (410 mg, 1.4 mmol) was dissolved in CH$_3$CN (15 mL) and diluted with H$_2$O (250 mL). The mixture was stirred vigorously at 100 °C, and the reaction was monitored by RP-HPLC. After 3 days, the reactant had been consumed entirely. The reaction mixture was cooled to room temperature, filtered, and lyophilized to yield **3** as a yellow solid (213 mg, 55%): R_f=0.60 (AcOH/MeOH, 2:1, *v/v*); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ=10.69 (s, 1H, OH), 7.32 (d, J=7.8 Hz, 1H, Ar), 7.26 (d, J=8.0 Hz, 1H, Ar), 6.99 (dd, J=7.4, 7.4 Hz, 1H, Ar), 6.92 (dd, J=7.4, 7.4 Hz, 1H, Ar), 3.12 (m, 2H, CH+CH$_2$), 2.97 (m, 1H, CH$_2$), 2.75 (m, 1H, CH$_2$), 2.55 (m, 4H, CH+3CH$_2$), 2.13 (m, 1H, CH$_2$), 2.04 (m, 1H, CH$_2$), 1.60 ppm (m, 1H, CH$_2$); ^{13}C NMR (400 MHz, [D$_6$]DMSO): δ=175.7, 136.7, 136.0, 127.3, 121.1, 119.0, 118.1, 111.7, 106.7, 60.0, 56.1, 53.3, 42.1, 27.2, 25.2, 21.7 ppm; MS (ESI) *m/z* [*M*+H]$^+$: 271.2.

(3*RS*,12b*SR*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxylic acid (4). Compound **9** (610 mg, 2.1 mmol) was dissolved in CH_3CN (20 mL) and diluted with H_2O (300 mL). The mixture was stirred vigorously at 100 °C, and the reaction was monitored by RP-HPLC. After 5 days, the reactant had been consumed entirely. The reaction mixture was cooled to room temperature, filtered, and lyophilized to give **4** as a yellow solid (289 mg, 51%): R_f = 0.74 (AcOH/MeOH, 2:1, *v*/*v*); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 10.70 (s, 1H, OH), 7.33 (d, J = 7.6 Hz, 1H, Ar), 7.26 (d, J = 8.0 Hz, 1H, Ar), 6.99 (dd, J = 7.2, 7.6 Hz, 1H, Ar), 6.92 (dd, J = 7.2, 7.6 Hz, 1H, Ar), 3.13 (m, 2H, CH + CH_2), 3.00 (m, 1H, CH_2), 2.76 (m, 1H, CH_2), 2.56 (m, 2H, CH + CH_2), 2.34 (m, 2H, $2CH_2$), 2.09 (m, 1H, CH_2), 1.50 (m, 1H, CH_2), 1.37 ppm (m, 1H, CH_2); ^{13}C NMR ([D_6]DMSO): δ = 175.6, 136.7, 136.2, 127.3, 121.0, 119.0, 118.2, 111.6, 106.9, 60.0, 57.5, 53.3, 42.1, 29.4, 27.7, 22.1 ppm; MS (ESI) *m*/*z* [*M*+H]$^+$: 271.2.

Solid-phase peptide synthesis: general procedure. Peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, coupling, and subsequent deprotection steps were carried out with *N*,*N*-dimethylformamide (DMF; 5×1 min) and CH_2Cl_2 (5×1 min) using 10 mL of solvent per gram of resin each time. Peptide syntheses were carried out with Fmoc-Rink–PS resin using an Fmoc/*t*Bu protecting group solid-phase strategy. Fmoc-AA-OH (3 equiv) were coupled using DIPCDI (3 equiv) and HOBt (3 equiv) in CH_2Cl_2/DMF (1:1) for 2–4 h at room temperature. After each coupling, the resin was washed with DMF (5×1 min) and CH_2Cl_2 (5×1 min). Reaction completion was monitored by Kaiser or chloranil tests.

Fmoc protecting group removal: 1) DMF (5×1 min), 2) piperidine/DMF (2:8, 1×1 min + 2×15 min), 3) DMF (5×1 min).

Synthesis of tripeptides 10–18. Each tripeptide was synthesized starting from Fmoc-Rink–PS resin (500 mg, loading: 0.7 mmol g^{-1}). Upon synthesis of the tripeptides as described above, the resins were washed with DMF, CH_2Cl_2, and MeOH (5×1 min each). Cleavages were performed at room temperature with a solution of TFA/H_2O (95:5, *v*/*v*) for 1 h. The free N$^{\alpha}$-terminal tripeptides were evaporated under pressure and characterized by analytical RP-HPLC–MS using a reversed-phase Symmetry C_{18} (3.9×150 mm^2) 5 μm column at a flow rate of 1 mL min^{-1} (HPLC data included in table 1 in SI).

Synthesis of *cis*-indoloquinolizidine–peptide hybrids 19–27. Half the amount obtained for each crude peptide **10**–**18** was dissolved in DMF (0.5 mL) and treated with *N*,*N*-diisopropylethylamine (DIEA; 4–30 μL, 1 equiv). Compound **3** (6–33 mg, 1 equiv) was activated in DMF by HOBt (5–26 mg, 1 equiv). The resulting suspensions and DIPCDI (5–27 μL, 1 equiv) were added to each crude peptide. Couplings were performed overnight at room temperature, and the crude products were evaporated in vacuo. The crudes were purified by semipreparative RP-HPLC using a reversed-phase Symmetry C_{18} (3×10 cm^2) 5 μm column. Elution system: A: H_2O/HCOOH, 99.9:0.1; B: CH_3CN/HCOOH, 99.9:0.1. Flow rate: 25 mL min^{-1}. Purification gradient: 0→60% B over 30 min.

Synthesis of *trans*-indoloquinolizidine–peptide hybrids 28–36. Half the amount obtained for each crude peptide **10**–**18** was dissolved in DMF (0.5 mL) and treated with DIEA (4–30 μL, 1 equiv). Compound **4** (6–33 mg, 1 equiv) was activated in DMF by HOBt (5–26 mg, 1 equiv). The resulting suspensions and DIPCDI (5–27 μL, 1 equiv) were added to each crude peptide. Couplings were performed overnight at room temperature, and the crude products were evaporated in vacuo. The crudes were purified by semipreparative RP-HPLC using a reversed-phase Symmetry C_{18} (3×10 cm^2) 5 μm column. Elution system: A: H_2O/HCOOH, 99.9:0.1; B: CH_3CN/HCOOH, 99.9:0.1. Flow rate: 25 mL min^{-1}. Purification gradient: 0→50% B over 30 min.

Synthesis of (3*RS*,12b*RS*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxamide (37). Fmoc-Rink–PS resin (100 mg, loading: 0.7 mmol g^{-1}) was swollen in DMF, and the Fmoc group was removed as mentioned above. Compound **3** (57 mg, 3 equiv) and 7-aza-1-hydroxy-(1*H*)-benzotriazole (HOAt; 29 mg, 3 equiv) were dissolved in DMF. The solution obtained and DIPCDI (35 μL, 3 equiv) were added to the resin, and the coupling was carried out overnight at room temperature. Afterward, the resin was washed with DMF, CH_2Cl_2, and MeOH (5×1 min each). Cleavage was performed at room temperature with a solution of TFA/H_2O (95:5, *v*/*v*) for 1 h. The resulting crude was purified by semipreparative RP-HPLC using a reversed-phase Symmetry C_{18} (3×10 cm^2) 5 μm column. Elution system: A: H_2O/HCOOH, 99.9:0.1; B: CH_3CN/HCOOH, 99.9:0.1. Flow rate: 25 mL min^{-1}. Purification gradient: 0→50% B over 30 min.

Synthesis of (3*RS*,12b*SR*)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-3-carboxamide (38). Fmoc-Rink–PS resin (100 mg, loading: 0.7 mmol g^{-1}) was swollen in DMF, and the Fmoc group was removed as mentioned above. Compound **4** (57 mg, 3 equiv) and HOAt (29 mg, 3 equiv) were dissolved in DMF. The solution obtained and DIPCDI (35 μL, 3 equiv) were added to the resin, and the coupling was carried out overnight at room temperature. Afterward, the resin was washed with DMF, CH_2Cl_2, and MeOH (5×1 min each). Cleavage was performed at room temperature with a solution of TFA/H_2O (95:5, *v*/*v*) for 1 h. The resulting crude was purified by semipreparative RP-HPLC using a reversed-phase Symmetry C_{18} (3×10 cm^2) 5 μm column. Elution system: A: H_2O/HCOOH, 99.9:0.1; B: CH_3CN/HCOOH, 99.9:0.1. Flow rate: 25 mL min^{-1}. Purification gradient: 0→50% B over 30 min.

Sample characterization. The purified compounds **19**–**38** were characterized by analytical RP-HPLC–MS, using a reversed-phase Symmetry C_{18} (3.9×150 mm^2) 5 μm column at a flow rate of 1 mL min^{-1} in two separate elution systems.

19 (yield: 28%): 33.7 mg, *m*/*z* [*M*+H]$^+$: 699.3, 99%.

20 (yield: 25%): 27.3 mg, *m*/*z* [*M*+H]$^+$: 631.2, 96%.

21 (yield: 70%): 80.1 mg, *m*/*z* [*M*+H]$^+$: 658.2, 96%.

22 (yield: 31%): 33.8 mg, *m*/*z* [*M*+H]$^+$: 633.3, 99%.

23 (yield: 24%): 30.6 mg, *m*/*z* [*M*+H]$^+$: 740.3, 99%.

24 (yield: 33%): 36.9 mg, *m*/*z* [*M*+H]$^+$: 647.3, 99%.

25 (yield: 29%): 37.6 mg, *m*/*z* [*M*+H]$^+$: 738.3, 95%.

26 (yield: 24%): 28.2 mg, *m*/*z* [*M*+H]$^+$: 681.3, 97%.

27 (yield: 5%): 6.1 mg, *m*/*z* [*M*+H]$^+$: 706.3, 87%.

28 (yield: 13%): 15.4 mg, *m*/*z* [*M*+H]$^+$: 699.3, 99%.

29 (yield: 12%): 12.8 mg, *m*/*z* [*M*+H]$^+$: 631.2, 99%.

30 (yield: 43%): 49.8 mg, *m*/*z* [*M*+H]$^+$: 658.2, 98%.

31 (yield: 15%): 16.7 mg, *m*/*z* [*M*+H]$^+$: 633.3, 97%.

32 (yield: 6%): 7.6 mg, *m*/*z* [*M*+H]$^+$: 740.3, 99%.

33 (yield: 8%): 9.2 mg, *m*/*z* [*M*+H]$^+$: 647.3, 99%.

34 (yield: 6%): 7.9 mg, *m*/*z* [*M*+H]$^+$: 738.3, 99%.

35 (yield: 3%): 3.5 mg, *m*/*z* [*M*+H]$^+$: 681.3, 92%.

36 (yield: 4%): 5.0 mg, *m/z* $[M+H]^+$: 706.3, 91%.

37 (yield: 25%): 4.7 mg, *m/z* $[M+H]^+$: 270.0, 78%.

38 (yield: 65%): 12.3 mg, *m/z* $[M+H]^+$: 270.0, 85%.

The purities obtained using each of the two elution systems are specified in table 2 (SI). HRMS data were recorded for compounds for which the pharmacological profile was determined through cAMP production assays.

22: HRMS *m/z*: found $[M+H]^+$: 633.4129, calculated $[M+H]^+$: 633.4123.

26: HRMS *m/z*: found $[M+H]^+$: 681.4122, calculated $[M+H]^+$: 681.4123.

28: HRMS *m/z*: found $[M+H]^+$: 699.4029, calculated $[M+H]^+$: 699.4029.

33: HRMS *m/z*: found $[M+H]^+$: 647.4294, calculated $[M+H]^+$: 647.4279.

Biological assays

Radioligand binding experiments: general procedure. Membrane suspensions of lamb striatum were obtained following previously described methods.[20,21] Radioligand binding assays of membrane suspensions (0.5 (mg protein) mL^{-1} determined with bicinchoninic acid kits) were carried out at 22 °C in 50 mM Tris-HCl buffer, pH 7.4 (see conditions used for each receptor below). After radioligand incubation, free and membrane-bound ligand were separated by rapid filtration of 500 μL aliquots in a cell harvester through Whatman GF/C filters embedded in polyethylenimine.[20] Nonspecific binding was determined with unlabeled ligands at the concentration indicated below. In all cases, the filters were incubated with 10 mL Ecoscint H scintillation cocktail overnight at room temperature. Radioactivity counts in the vials were determined in the counter with 60% efficiency.

Screening of the indoloquinolizidine–peptide hybrid library. Binding experiments of the whole library were initially performed at a concentration of 50 μM for compounds **19**–**38**.

Dopamine D_1 receptor. Membranes were incubated with 1.2 nM [^{3}H]SCH 23390 (85 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) for 1.5 h. Nonspecific binding was measured in the presence of 50 μM SCH 23390.

Dopamine D_2 receptor. Membranes were incubated with 1.7 nM [^{3}H]YM 09151-2 (85.5 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) for 2 h. Nonspecific binding was measured in the presence of 50 μM raclopride.

The whole library was then tested at 5 μM using the same procedure:

Dopamine D_1 receptor. Membranes were incubated with 1.9 nM [^{3}H]SCH 23390 (85 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) for 1.5 h. Nonspecific binding was measured in the presence of 5 μM SCH 23390.

Dopamine D_2 receptor. Membranes were incubated with 1.4 nM [^{3}H]YM 09151-2 (85.5 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) for 2 h. Nonspecific binding was measured in the presence of 5 μM raclopride.

Binding experiments at adenosine A_1R and histamine H_3R were carried out following the same procedure described above. For the A_1R evaluation, membranes were incubated with 7.6 nM [^{3}H](*R*)-PIA (37 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$ and 0.2 U mL^{-1} ADA for 1 h. Nonspecific binding was measured in the presence of DPCPX. Regarding H_3R, membranes were incubated with 1.5 nM [^{3}H]methylhistamine (24 Ci $mmol^{-1}$) in 50 mM Tris-HCl buffer (pH 7.4) for 2 h. Nonspecific binding was measured in the presence of methylhistamine.

K_D determination. Competition experiments were performed by incubating membranes under the same conditions as described above, in the absence or presence of increasing concentrations of indoloquinolizidine–peptide hybrids. Nonspecific binding was determined as previously outlined. Radioligand displacement curves were analyzed by nonlinear regression using the commercial program GraFit (Erithacus Software, Surrey, UK) by fitting the total binding data to the displacement models with one or two affinity sites.[22,23] Data are the mean ±SD of 3–6 determinations, and the differences with respect to controls were tested for significance (two-tailed; $p<0.05$) using the Student's *t* test for unpaired samples. Goodness of fit was tested following the reduced χ^2 value given by the nonlinear regression program in GraFit. A modified *F* test was used to analyze whether the fit to the two-site model significantly improved on the fit to the one-site model, and $p<0.05$ was taken as a criterion of significance; when no significant improvement over the one-site model was detected, the *p* values were >0.30.

Generation of a stable cell line expressing the human D_1R. The Flp-In T-REx System was used to generate a stable mammalian cell line exhibiting tetracycline-inducible expression of human D_1R from a specific genomic cDNA location. The Flp-In T-REx-293 cells used in this system contain a single integrated FRT (Flp recombination target) site, stably express the Tet repressor, and allow one to directly proceed to the generation of a stable cell line. The human dopamine D_1 receptor cDNA was amplified using sense and antisense primers and the iProof kit, as indicated by the manufacturer. An adenine nucleotide was added to the 3′ end of the amplified fragment using the iTaq kit for cloning into pcDNA5/FRT/TO-TOPO vector, as indicated by the manufacturer, to generate the pcDNA5/FRT/TO-TOPO-D_1R construct. Flp-In T-REx-293 cells were co-transfected with the pcDNA5/FRT/TO-TOPO-D_1R construct and the pOG44 plasmid to allow integration of the expression vector pcDNA5/FRT/TO-TOPO-D_1R, under the control of a tetracycline-inducible promoter, into the genome via Flp recombinase-mediated DNA recombination at the FRT site. A polyclonal selection of isogenic cell lines was performed using 15 μg mL^{-1} blasticidin and 200 μg mL^{-1} hygromycin B, as recommended by manufacturer. Expression of human D_1R was induced by overnight incubation of Flp-In T-REx-293-D_1R cells with various concentrations of tetracycline and tested by binding experiments and Western blotting.

Cell culture. Flp-In T-REx-293-D_1R cells were cultured in DMEM supplemented with 4.5 mg mL^{-1} glucose, 0.11 mg mL^{-1} sodium pyruvate, 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine, 15 μg mL^{-1} blasticidin and 200 μg mL^{-1} hygromycin B at 37 °C in a humidified atmosphere of 5% CO_2. CHO cells were stably transfected with human D_{2L} (long-form) receptor cDNA. D_2R CHO cells were maintained in α-MEM without nucleosides, containing 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine at 37 °C in a humidified atmosphere of 5% CO_2. Geneticin (G418; 400 μg mL^{-1}) was used as a specific selector for D_2R expression.

cAMP determination. Flp-In T-REx-293-D_1R cells were grown in six-well plates to 80% confluence and incubated with 15 ng mL^{-1} tetracycline for 16 h in serum-free medium before the experiment.

D_2R CHO cells were grown in 25 cm^2 flasks to 80% confluence and incubated for 16 h in serum-free medium before the experiment. Cells were harvested washed twice in Hank's balanced salt solution (HBSS) containing 10 mM glucose and suspended in HBSS supplemented with 10 mM glucose and 10 mM $MgCl_2$ to a final volume of 1 mL in plastic tubes. Cells were pre-incubated with 50 μM zardaverine as phosphodiesterase inhibitor for 10 min at 37 °C and treated for 10 min with 30 nM PPHT, 20 nM SKF 38393, or 50 μM indoloquinolizidine–peptide hybrids in the presence or absence of 5 μM forskolin. When indicated, D_2R antagonist raclopride (30 μM), the D_1R antagonist SCH 23390 (50 μM), or indoloquinolizidine–peptide hybrids (50 μM) were added and pre-incubated 5 min before the addition of the agonist. To stop the reaction, cells were placed on ice and centrifuged at 2500 *g* for 5 min at 4 °C. The pellet was washed with ice-cold HBSS with 10 mM glucose and resuspended with 200 μL ice-cold $HClO_4$ (4%, *v/v*) for 30 min, and 1.5 M KOH was added to reach neutral pH. Samples were centrifuged at 15000 *g* for 30 min at 4 °C, and the supernatant was frozen at −20 °C. The accumulation of cAMP in the samples was measured by a [^{3}H]cAMP assay system as described in the manual from the manufacturer. Data are the mean ± SD of 3–6 determinations, and the differences with respect to controls were tested for significance (two-tailed; $p < 0.01$) using the Student's *t* test for unpaired samples.

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