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Research paper

Novel multitarget-directed ligands targeting acetylcholinesterase and σ_1 receptors as lead compounds for treatment of Alzheimer's disease: Synthesis, evaluation, and structural characterization of their complexes with acetylcholinesterase

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ABSTRACT

Pleiotropic intervention may be a requirement for effective limitation of the progression of multifactorial diseases such as Alzheimer's Disease. One approach to such intervention is to design a single chemical entity capable of acting on two or more targets of interest, which are accordingly known as Multi-Target Directed Ligands (MTDLs). We recently described donecopride, the first MTDL able to simultaneously inhibit acetylcholinesterase and act as an agonist of the 5-HT_4 receptor, which displays promising activities *in vivo*. Pharmacomodulation of donecopride allowed us to develop a novel series of indole derivatives possessing interesting *in vitro* activities toward AChE and the σ_1 receptor. The crystal structures of complexes of the most promising compounds with *Torpedo californica* AChE were solved in order to further understand their mode of inhibition.

1. Introduction

Along with the observed increase of life expectancy, senile dementia is becoming increasingly prevalent. As many of 46 million cases of dementia were reported worldwide in 2015 [1]. The most common dementia, Alzheimer's disease (AD), is characterized by a progressive and irreversible decline in memory and cognitive function as a consequence of neuronal dysfunction. According to the cholinergic hypothesis this decline is linked to a decrease in the levels of acetylcholine released at cholinergic synapses in certain areas of the brain. Indeed, until now, with one exception, the drugs authorized by the FDA for treatment of AD are acetylcholinesterase (AChE) inhibitors that provide symptomatic relief in the early stages of the disease [2]. However, with progression of AD, these drugs, including **1** [donepezil (DPZ)], rivastigmine and galanthamine, lose their efficacy. One of the greatest challenges facing pharmacologists and medicinal chemists is the discovery and development of treatments that could exert a disease-modifying effect on AD [3]. To this end many studies have been directed towards clarifying the molecular origins of the disease. AD is principally characterized by the formation in the brain of amyloid plaques whose main component is the Aβ peptide, and of neurofibrillary

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tangles (NFTs), which are aggregates of hyperphosphorylated tau protein [4]. Since the multifactorial origin of AD has been clearly established, most efforts have been directed towards a search for potent and agents that could interfere with the formation of the amyloid plaques and the NFTs, or limit the inflammation of the brain associated with the disease [5]. However, to date, these efforts have failed to identify novel therapeutics. These failures have been ascribed to difficulties in patient stratification or selection, as well as to lack of appropriate transgenic mice models [6]. It has also been suggested that the clinical ineffectiveness of the drug candidates is due to their high selectivity for a single target, implying that pleiotropic intervention will be needed due to the multifactorial origin of AD [7].

Pleiotropic intervention could of course be realized through use of a cocktail of drugs, as is currently the case in some ongoing clinical trials, in which AChE inhibitors (AChEIs), including DPZ and rivastigmine, are associated with other candidates [8,9]. Another strategy involves development of single compounds capable of simultaneously acting on several targets, which are known as Multi-Target Directed Ligands (MTDLs) [10–12]. Several activities have been associated in single compounds in order to act on several pathogenic pathways mainly those targeting the formation of the Aβ plaques, the NFTs or the neuroinflammation associated with AD. Recent examples include compounds able to simultaneously act on MAO-B inhibition and oxidative stress [13], GSK-3β and β-secretase inhibition [14], or to inhibit Aβ aggregation, act as metal chelators and serve as antioxidant [15]. Due to the diversity of chemical scaffolds among the AChEIs, and to their clinical efficacy, one of the most commonly adapted strategies has been to graft on to an AChEI a moiety displaying another pharmacological activity useful for treating AD. Several examples have been recently published associating cholinesterase inhibition properties with MAO-B inhibition [16], antioxidant properties [17] or combining several activities [18]. Based on the amyloid hypothesis, several leads with promising *in vivo* activities have been developed that both inhibit AChE and limit the formation of senile plaques [19,20]. These studies took advantage of the demonstration by Inestrosa and coworkers that ligands binding at the peripheral anionic site (PAS) [21] of AChE can retard aggregation of Aβ [22]. Many studies have then been devoted to the development of Dual Binding Site (DBS) AChEIs, which can bind simultaneously to the anionic subsite of the active site and to the PAS [23–25]. In addition to the classical targets considered for treatment of AD, the serotonergic system has also shown promise in this context, in particular modulation of 5-HT_4 and 5-HT_6 receptors [26,27]. 5-HT_4 receptors (5-HT_4R) control brain functions such as learning and memory, feeding and mood behavior. In the context of AD, activation of 5-HT_4R can promote the non-amyloidogenic cleavage of Amyloid Protein Precursor (APP) leading to the formation of a neurotrophic protein, sAPPα [28,29]. Indeed, administration of a specific 5-HT_4R agonist, RS67333, in a transgenic mouse model of AD retarded amyloidogenesis and reduced behavioral deficits [30].

In this context we have initiated a drug discovery program to assess the pharmacological benefits of generating an MTDL by combining RS67333 (**2)** and DPZ (**1),** and testing the activity of this MTDL in an object recognition test that had been used earlier to assess the synergy of RS67333 and DPZ administered together [31]. Several chemical series [32] were developed with the objectives of combining the two functions in a single compound which resulted in the recent identification of a promising MTDL candidate for AD treatment: **3** (donecopride), a 5-HT_4R agonist (*Ki* for *h*5-HT_4R 10.4 nM; IC_{50} for *h*AChE 16 nM) (Fig. 1) [33]. Thanks to its double mechanism of action and to its good bioavailability **3** was able to improve the memory performance of mice in an object recognition test, and to reverse the memory deficit induced by scopolamine in the Y-maze spontaneous alternation test [34]. **3** appears to be selective for its targets, though it also displays lower affinity for 5-HT_{2B}R and for σ receptors; among the latter σ_1R appears to be an interesting off-target due to its implication in the pathogenesis of AD [35]. Several σ_1R ligands have recently been disclosed that show promise for the treatment of AD, whether alone [36] or combined with additional drugs [37]. **1** itself has nanomolar affinity for σ_1R [38] and was proved to possess anti-amnesic and neuroprotective effects involving interaction with σ_1R [39], which could explain the affinity of its structural analog **3** for this target. Combining AChEI and σ_1R affinities shows promise for AD treatment, so we decided to continue our modulation of **3** in order to improve its affinity for this novel target. To this end, we first docked **3** into the active-site gorge of *h*AChE [34]. In this study the pose of **3** was seen to be similar to that of **1,** involving interaction of the charged nitrogen of the piperidine ring with Tyr337, an H-bond between the carbonyl of **3** and the main-chain NH of Phe295, and, more interestingly, a π-stacking interaction between the aromatic ring of **3** positioned and the indole ring of Trp286 in the PAS (Fig. 2).

We postulated that replacement of the benzene ring of **3** by an indole residue (Fig. 1) should increase the interaction of the ligand with the PAS, thus resulting in increased inhibition of βA aggregation. In addition, the influence of this substitution on other targets, such as 5-HT_4R and σ-$_1$R, as well as upon bioavailability, could thus be assessed. Moreover, it has been demonstrated that haloperidol-inspired molecules with an indole ring and a piperidine chain could act as potent σ_1R ligands with neuroprotective effects [40].

2. Results

2.1. Chemistry

A first assessment of our strategy was obtained by synthesis of the indole analog of **3** bearing the same methoxy and chlorine substituents. The supplementary pyrrole cycle was obtained using a Larock heteroannulation synthesis starting from ortho-halogenated aniline (Scheme 1). An iodine atom was introduced at position 3 of the aromatic using iodine in AcOH at room temperature to afford **4a** in quantitative yield. **4a** was engaged in a Sonogashira cross-coupling with ethynyltrimethylsilane and a mixture of $Pd(PPh_3)_4$, XPhos and K_2CO_3 in refluxing THF [41]. The cleavage of the trimethylsilyl group of the resulting intermediate **5a** under acidic conditions yielded the target compound **6a** in two steps with a 71% yield.

On the basis of the promising *in vitro* activities displayed by **6a** we decided to develop a more convergent route to obtain structural analogs. Indolic derivatives **6a-g** were synthesized by the synthetic route shown in Scheme 2, from acids **7a-c** (see Supporting Information). A convergent synthesis was designed with late stage construction of the indole ring, except for **6h**.

Compounds **9a-c** were synthesized in a three-step sequence through formation of β-keto ester derivatives by reaction between acids **7a-c,** activated *in situ* with CDI, and potassium 3-ethoxy-3-oxopropanoate [42,43]. Subsequent alkylation with a previously synthesized piperidine chain, and followed by reaction with KOH in an EtOH/H_2O mixture provided a saponification-decarboxylation reaction to afford the corresponding derivatives **9a-c** [44]. Then compounds **4a-g** were obtained by deprotection of the Boc group before alkylating the nitrogen of piperidine by various alkyl chains. Finally, the indolic ring system was constructed *via* a Larock palladium-catalyzed annulation using ethynyltrimethylsilane, a terminal alkyne, and a mixture of $Pd(PPh_3)_4$, XPhos and K_2CO_3 in refluxing THF [41]. Then a Sonogashira cross coupling-cyclization reaction, and cleavage of the trimethylsilyl group under acidic

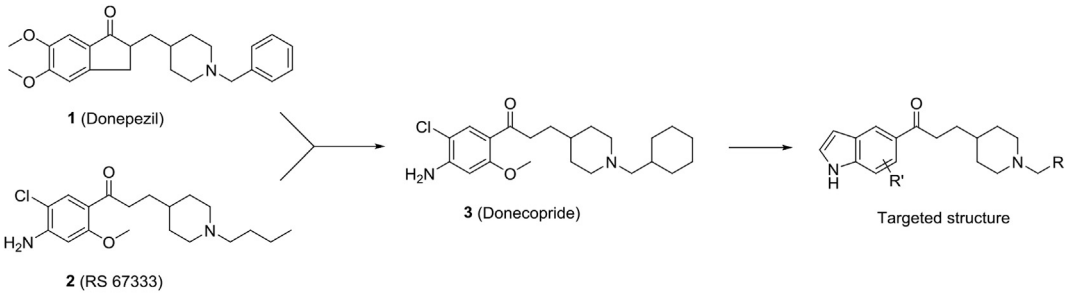


Fig. 1. Chemical structure of Donepezil **1**, RS67,333 **2** and Donecopride **3**.

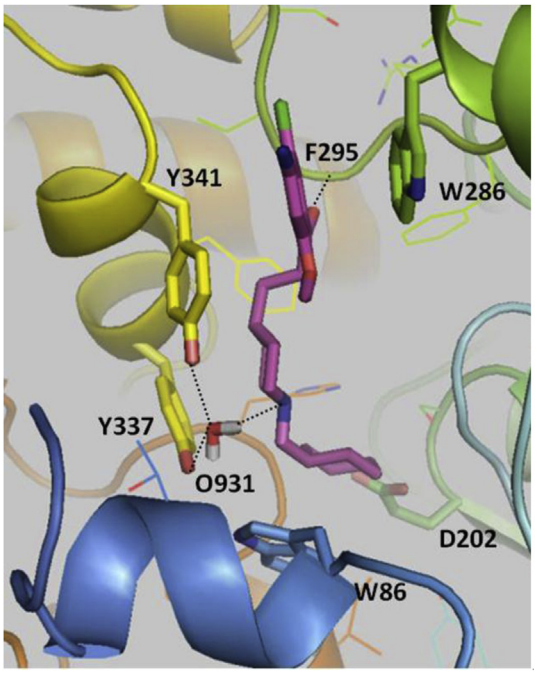


Fig. 2. Docking of **3**, donecopride, within the active-site gorge of *h*AChE [34].

conditions yielded the target compounds **6a-g**. In order to study their influence on the biological results, different groups with various electronic effects have been introduced on the nitrogen of the indole moiety. Consequently, compounds **10a-d** were obtained by alkylation of the nitrogen with a methyl and a benzyl (alkyl- and aromatic electron donating groups) or a benzenesulfonyl (electron withdrawing group), and compound **10c**, obtained as an oil, was reacted with fumaric acid in isopropanol at 50 °C to give the corresponding fumarate salt for biological evaluation.

To synthesize compound **6h**, dehalogenation of chlorinated **6a** was attempted with ammonium formate and palladium, but the reaction did not lead to the expected product, probably due to the presence of the basic nitrogen of piperidine which does not permit the insertion of palladium in the carbon-halogen bond. We decided to synthesize the indole ring directly from ester **11** (see the Supporting Information) under the same conditions as previously described to obtain compounds **6a-g from 4a-g**. Compound **13** was obtained by dehalogenation of chlorinated compound **12** in the presence of ammonium formate and Pd/C in refluxing MeOH, and was then reacted with NaOH in EtOH to give acid **14**. Finally, the target compound **6h** was obtained in a five-step sequence by the same method used to prepare **4a-g** from **7a-c**.

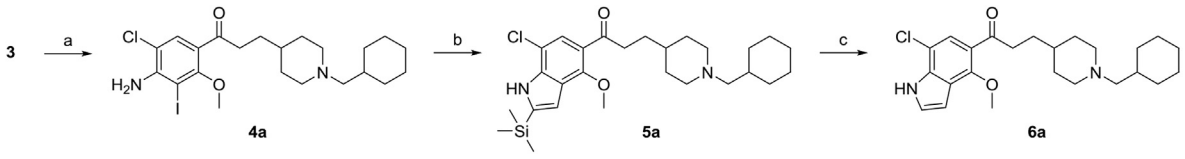
2.2. In vitro evaluation

All the synthesized compounds were evaluated for their capacity to inhibit *h*AChE and to bind to guinea pig (*gp*)5-HT$_4$R (Table 1). The most promising one were further tested for their capacity to bind to σ_1R. In these tests, DPZ was used as both an AChEI and a control ligand for σ_1R, and **3** was used both as a ligand for 5-HT$_4$R and as an AChEI control [33].

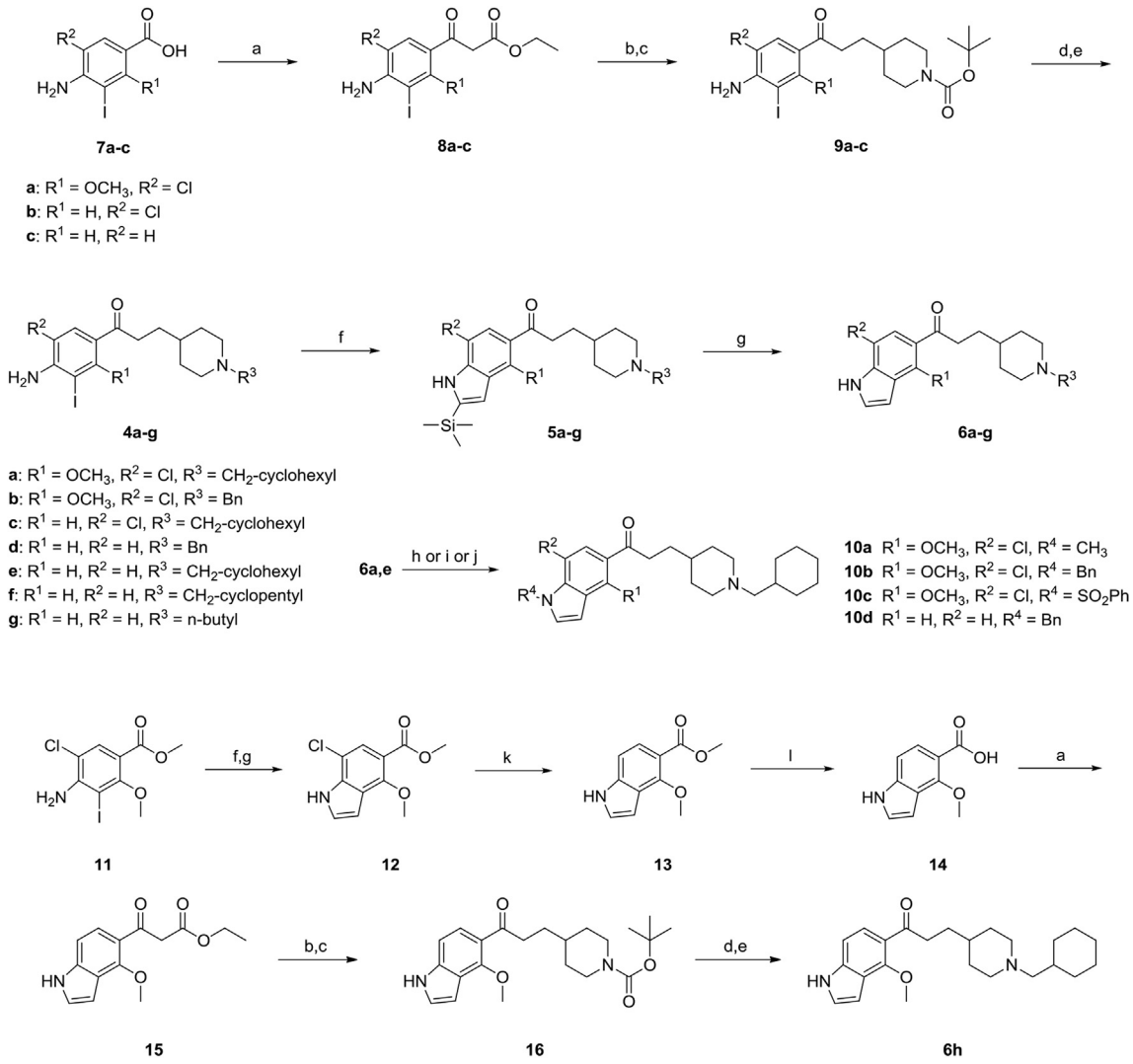
Finally, kinetic measurements were performed at three concentrations of **6a** in order to determine its mode of inhibition of *h*AChE (Fig. 3). The kinetic data clearly show that **6a** acts as a non-competitive inhibitor of the enzyme.

2.3. In vivo evaluation

σ_1R Agonists are potent anti-amnesic drugs. In particular, they significantly prevent the amnesia induced in rodents by blockade of the NMDA receptor [45,46]. Compound **6c** was therefore chosen on the basis of its *in vitro* activities towards the three targets for a preliminary *in vivo* confirmation of its σ_1R activity. Animals were tested for spontaneous alternation performance in the Y-maze, an index of spatial working memory, and for passive avoidance response, an index of long-term non-spatial memory [37,45,46]. As shown in Fig. 4, compound **6c** failed to attenuate dizocilpine-induced spontaneous alternation deficit in the dose range tested, 0.1–1 mg/kg IP (Fig. 4a), but the lowest dose very significantly attenuated dizocilpine-induced alteration in passive avoidance response (Fig. 4b).



Scheme 1. Synthesis of indole analog **6a**
[a]Reagents, conditions and yields: (a) I_2, AcOH, rt, 3 h, quant.; (b) ethynyltrimethylsilane, $Pd(PPh_3)_4$, XPhos, K_2CO_3, dry THF, reflux, 18 h, 71%; (c) TFA, DCM, rt, 2 h, quant.



Scheme 2. Synthetic route for target compounds 6a-h and 10a-c[a]

[a]Reagents, conditions and yields: (a) CDI, dry THF, rt, 5–7 h, then potassium 3-ethoxy-3-oxopropanoate, $MgCl_2$, 40 °C, 14–48 h, 57–70%; (b) *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate, K_2CO_3, DMF, rt, 24–48 h; (c) KOH, EtOH/H_2O (5:1), reflux, 3–5 h, 37–76% over two steps; (d) TFA, DCM, rt, 30min; (e) alkyl-halogenated derivatives, K_2CO_3, DMF, 110 °C, 5–15 h, 52–75% over two steps; (f) ethynyltrimethylsilane, $Pd(PPh_3)_4$, XPhos, K_2CO_3, dry THF, reflux, 17–24 h; (g) TFA, DCM, rt, 1–2 h, 26–73%; (h) CH_3I, K_2CO_3, DMF, rt, overnight, 77%; (i) BnBr, K_2CO_3, DMF, rt, overnight, 83%; (j) $PhSO_2Cl$, NaOH aq., $(nBu)_4NHSO_4$, rt, 2 h, 84%; (k) ammonium formate, Pd/C, MeOH, reflux, overnight, 82%; (l) 1N NaOH aq., EtOH, rt, overnight, 83%.

3. Discussion

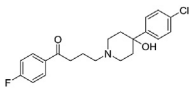
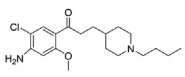
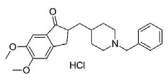
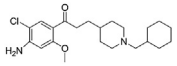
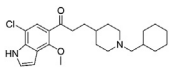
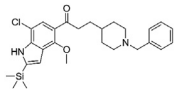
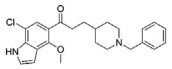
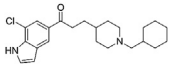
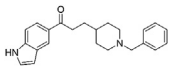
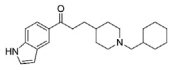
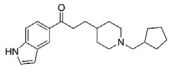
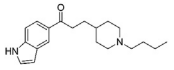
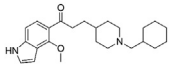
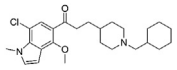
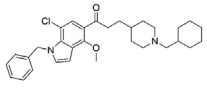
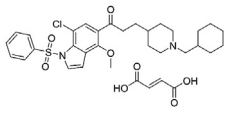
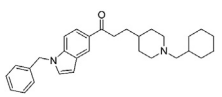
Relative to donecopride (Ki = 9.5 nM), its indole analogs showed overall a decrease in affinity for 5-HT_4R affinity, **6f** being the most potent (Ki = 25 nM). As already observed for the donecopride series [33], a cycloalkyl or an alkyl substituent on the piperidine ring appears favor affinity for 5-HT_4R activity relative to an *N*-benzyl ring. The chloro and methoxy substituents on the indole ring do not influence the activity. However, the introduction of the novel aromatic ring on the scaffold has a strong influence on the profile of the ligand. Indeed tested in a cellular functional assay using cAMP quantification, compound **6a** appears to be a potent antagonist for the 5-HT_4R (see Supporting Information) whereas donecopride is a partial agonist for this receptor.

On the contrary, in most cases, the inhibitory capacity towards AChE was maintained and compounds **6b-e** and **6h** are strong AChEI (IC_{50} = 13–51 nM) similar in affinity to donecopride (IC_{50} = 16 nM) and, for **6d** particularly, as DPZ (IC_{50} = 6 nM). Opposite to what was seen for affinity for the 5-HT_4R, and similarly to what was seen for the donecopride series, a *N*-benzyl substituent on the piperidine ring greatly enhanced inhibition of AChE relative to a cycloalkyl or an alkyl substituent. Once again, the chloro and methoxy substituents on the indole ring have little influence on affinity, whereas an *N*-substituent on the aromatic ring dramatically decreases it.

As suggested by the kinetic study performed on **6a**, these compounds could behave as non-competitive AChEIs, suggesting that they could interact with the PAS, as well as with the anionic subsite of the active site (Fig. 3). This potential dual binding site inhibition of AChE was confirmed by solution of the crystal structures of complexes obtained by soaking into *Tc*AChE crystals (Fig. 5a and b). The structures were solved at resolutions of 2.6 Å and 2.0 Å, respectively, for the **6a** and **6b** complexes (see Supplementary Table S1), and show how both compounds bind in a very similar way, as was to be expected from their chemical structures.

Both **6a** and **6b** span the whole length of the active-site gorge. The indole moiety interacts with Trp_{279} in the PAS. Neither the methoxy nor the chlorine substituents on the indole group seem to

Table 1
Inhibition of *h*AChE, and binding to *gp*5-HT_4R and σ_1R of indole derivatives.

Compound	Structure	*h*AChE		(*gp*)5-HT_4R		σ_1R
		%inh 10^{-6} M	IC_{50} (nM)	%inh 10^{-6} M	*Ki* (nM)	*Ki* (nM)
Haloperidol		–	–	–	–	1.6 ± 0.7 *n* = 2
RS67,333		–	–	100%	9.33 ± 5 *n* = 3	–
Donepezil		98%	6.0 ± 0.6, *n* = 5	10%	–	–
Donecopride		96%	16 ± 5, *n* = 2*	100%	9.5 ± 5 *n* = 2*	51 ± 6.6, *n* = 2
6a		95%	91.4 ± 12.7, *n* = 2	100%	40.9 ± 4 *n* = 3	5.1 ± 1.7, *n* = 2
5b		78%	275 ± 12, *n* = 2	62%	–	–
6b		97%	20.4 ± 0.8, *n* = 2	96%	150 ± 45 *n* = 3	5.7 ± 1.4, *n* = 2
6c		95%	28.8 ± 2.4, *n* = 2	100%	37.5 ± 15 *n* = 3	5.1 ± 1.8, *n* = 2
6d		98%	13.3 ± 0.4, *n* = 2	90%	169 ± 23 *n* = 3	3.3 ± 0.7, *n* = 2
6e		93%	51 ± 1.9, *n* = 2	100%	37.5 ± 15 *n* = 3	
6f		92%	94 ± 2.6, *n* = 2	100%	25 ± 1.6 *n* = 3	–
6g		55%	716 ± 28, *n* = 2	97%	49.6 ± 23 *n* = 3	–
6h		96%	40 ± 1, *n* = 2	100%	51 ± 15 *n* = 3	–
10a		97%	84.4 ± 13.4, *n* = 2	–	–	–
10b		87%	260.6 ± 31.5, *n* = 2	–	–	–
10c		74%	559 ± 8.4, *n* = 2	62%	–	–
10d		93%	133 ± 22, *n* = 2	42%	–	–

The inhibitory effect against *h*AChE was established according to the Ellman's method. *IC_{50} obtained from Rochais *et al.* [34].
-: not tested.

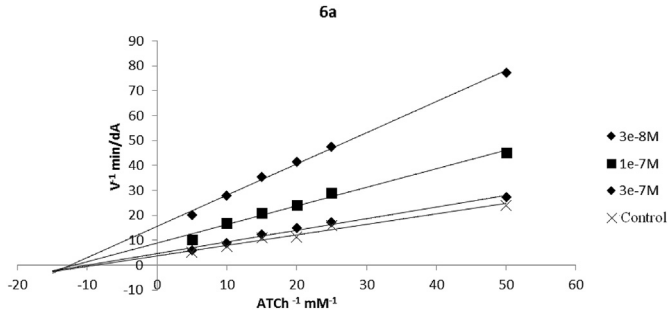


Fig. 3. Lineweaver Burk plots for inhibition of *h*AChE by **6a** (Donepezil has been used as control).

contribute to the binding of the ligands to the enzyme. Conversely, the cyclic moiety at the other extremity of both molecules, either a benzyl or a methylcyclohexyl group, is well suited to stack against Trp_{84} in the active-site of the enzyme. The benzyl of **6b**, in particular, allows formation of a π-π interaction. Midway down the gorge, the piperidine moiety is stabilized by a cation-π interaction between the nitrogen atom and residue Phe_{330}, already described in the literature for other AChEIs [47].

Comparison of the complexes of **6a** and **6b** with that of the *Tc*AChE/DPZ complex (pdb acces code 1eve), reveals that **6a** and **6b** bind higher up the gorge, as can be seen from their mode of interaction at the PAS. Other than that, the three compounds occupy the gorge in a very similar fashion.

Concerning the σ-$_1$R, the affinity of some members of the **6** series was dramatically enhanced; thus, compounds **6a-d** are very potent ligands with nanomolar affinities ($K_i = 3.3–5.7$ nM) compared to donecopride ($K_i = 51$ nM), to DPZ ($IC_{50} = 14.6$ nM). Presence of the indole ring appears crucial for interaction with σ-$_1$R activity, whatever the substituents on the aromatic ring or the piperidine moiety.

Based on its global pharmacological profile, compound **6c** ($IC_{50\,[AChE]} = 28.8$ nM, $K_i[5\text{-}HT_4R] = 37.5$ nM, $K_i[\sigma_1R] = 5.1$ nM) was evaluated *in vivo* in the dizocilpine-induced amnesia assay in mice [45,46]. Dizocilpine-treated mice showed highly significant memory impairments in both spontaneous alternation performance and passive avoidance tests. Compound **6c** was initially tested in the 0.1–1 mg/kg dose–range and did not attenuated the dizocilpine-induced spontaneous alternation performance deficit. However, **6c** at the lower dose tested dose significantly enhanced attenuated the passive avoidance deficit (40% protection). The compound therefore presented an *in vivo* efficacy coherent with its pharmacological profile and further studies must address lower doses and analyses in presence of NE-100.

4. Conclusion

Starting from donecopride, a dual AChEI/5-HT_4R agonist, pharmacomodulations led to a series of thirteen novel indole derivatives. Some of them displayed potent nanomolar inhibition of *h*AChE, and affinity for the σ-$_1$R affinity associated with a lower affinity for the 5-HT_4R. This pharmacological profile suggested a potential therapeutic approach to the treatment of AD. This, in turn, has been supported by preliminary *in vivo* experiments in which **6c** displayed a protecting effect against dizocilpine-induced impairment in the passive avoidance test in mice that correlated with its *in vitro* potent σ-$_1$R affinity. These results have to be strengthened by additional *in vivo* experiments in order to precise the pharmacological potential of **6c**.

5. Experimental section

5.1. Chemistry

All commercially available compounds were used without further purification. Melting points were determined on a Köfler apparatus. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} on aluminium plates (Merck) and visualized with UV light (254 nm). Flash chromatography was conducted on a VWR SPOT II Essential instrument with silica gel 60

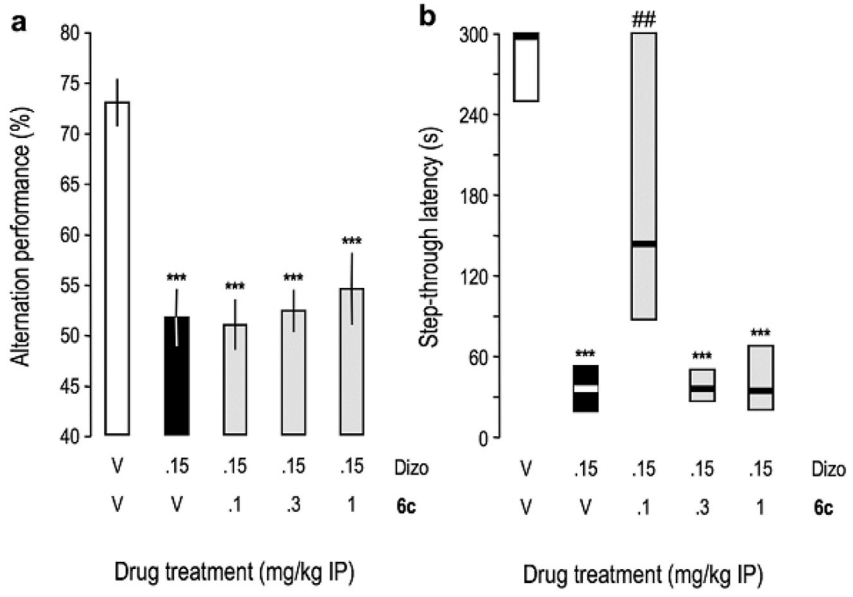


Fig. 4. Effect of **6c** on dizocilpine-induced learning impairments in mice. (a) spontaneous alternation performance and (b) Step-through latency. Animals received **6c** (0.1–1 mg/kg ip), 10 min before dizocilpine (Dizo, 0.15 mg/kg ip), 20 min before the Y-maze test session. In (a), data show mean ± SEM of n = 12–16 per group; ANOVA: $F_{(4,66)} = 13.8$, $p < 0.0001$. In (b), data show median and interquartile range of n = 12–15 per group; Kruskal-Wallis ANOVA: H = 40.9, $p < 0.0001$, in (b). ***$p < 0.001$ vs. (V + V)-treated group; ##$p < 0.01$ vs. (V + Dizo)-treated group; Dunnett's test in (a), Dunn's test in (b).

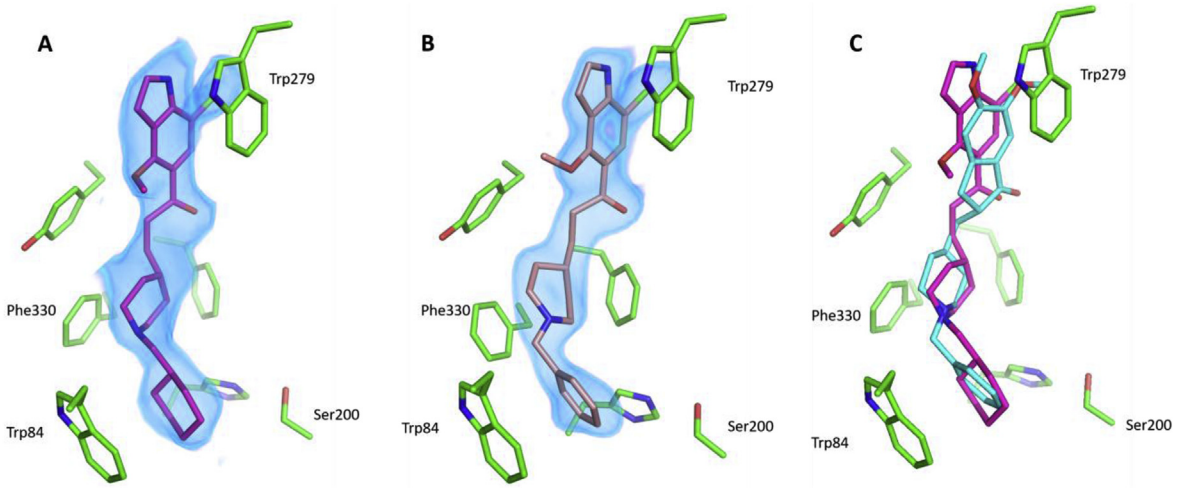


Fig. 5. Feature-enhanced electron density maps at 1σ level for ligands inside the gorge of acetylcholinesterase. (**A**) View of the active-site gorge in the crystal structure of the *Tc*AChE-**6a** complex (pdb access code 6EZH), at a resolution of 2.6 Å (**B**) View of the active-site gorge in the crystal structure of the *Tc*AChE-**6b** complex (pdb access code 6EZG) at a resolution of 2.0 Å, (**C**) superimposition between the crystal structure of the *Tc*AChE-**6a** complex and the crystal structure of the *Tc*AChE-**DPZ** complex (1eve). Donepezil is shown in cyan. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(40–63 μm). Column size and flow rate followed the manufacturer's recommendations. NMR spectra were recorded at 295 K, at 400 or 500 MHz (Bruker Avance III 400/500 MHz) for ^{1}H NMR and at 100 or 126 MHz for ^{13}C NMR in chloroform-*d*, methanol-d_4 or DMSO-d_6 with chemical shifts (δ) given in parts per million (ppm) relative to TMS as internal standard and recorded. The following abbreviations are used to describe peak splitting patterns when appropriate: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, dt = doublet of triplet. Coupling constants *J* are reported in hertz units (Hz). Infrared spectra (IR) were obtained on a PERKIN-ELMER FT-IR spectrometer and are reported in terms of frequency of absorption (cm^{-1}) using KBr discs. High-resolution mass spectra (HRMS) were obtained by electronic impact (HRMS/EI), or by electrospray (HRMS/ESI) on a Bruker maXis mass spectrometer. LC-MS (ESI) analyses were realized with Waters Alliance 2695 as separating module using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00min. This ratio was hold during 1.50 min before return to initial conditions in 0.50 min. Initial conditions were then maintained for 2.00 min (A = H_2O, B = CH_3CN; each containing HCOOH: 0.1%; column XBridge C18 2.5 μm/4.6 × 50 mm; flow rate 0.8 mL/min). MS were obtained on a SQ detector by positive ESI. Mass spectrum data are reported as m/z.

Representative procedure of method A for the synthesis of 8a-c and 15. To a solution of benzoic acid derivatives (1.0 eq.) in dry THF (10 mL/mmol) was added CDI (1.1 eq.) and the resulting mixture was stirred at room temperature for 5–7 h. Then potassium 3-ethoxy-3-oxopropanoate (1.2 eq.) and $MgCl_2$ (1.2 eq.) were added portion-wise. The reaction mixture was stirred at 40 °C for 14–48 h. After removal of the solvent, the residue was dissolved with EtOAc, and washed with a saturated aqueous $NaHCO_3$ followed by brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Chromatographic separation gave the title compounds.

Ethyl 3-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-oxo-propanoate (8a). The compound was prepared from 4-amino-5-chloro-3-iodo-2-methoxy-benzoic acid **7a** (2.5 g, 7.63 mmol) according to method A, stirring the reaction for 5 h at room temperature and then for 48 h at 40 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 80:20), to give **8a** as a pale yellow solid (63% yield); mp 87 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (s, 1H), 5.18 (br s, 2H), 4.17 (q, ^{3}J = 7.2 Hz, 2H), 3.95 (s, 2H), 3.78 (s, 3H), 1.22 (t, ^{3}J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 190.5 (CO), 168.1 (CO), 160.2 (C_q), 149.1 (C_q), 131.8 (CH), 121.1 (C_q), 113.5 (C_q), 81.3 (C_q), 62.7 (CH_3), 61.3 (CH_2), 48.2 (CH_2), 14.1 (CH_3); IR (neat, cm^{-1}) ν 3431, 3335, 2983, 2939, 1723, 1653, 1615, 1567, 1391, 1150; HRMS (ESI) calcd. for $C_{12}H_{14}ClINO_4$ $[M+H]^+$ 397.9651, found 397.9648.

Ethyl 3-(4-amino-3-chloro-5-iodo-phenyl)-3-oxo-propanoate (8b). The compound was prepared from 4-amino-3-chloro-5-iodo-benzoic acid **7b** (860 mg, 2.90 mmol) according to method A, stirring the reaction for 6 h at room temperature and then for 14 h at 40 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 80:20), to give **8b** as a white solid (66% yield); mp 84 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.15 (d, ^{4}J = 2.0 Hz, 1H), 7.86 (d, ^{4}J = 2.0 Hz, 1H), 5.10 (br s, 2H), 4.21 (q, ^{3}J = 7.3 Hz, 2H), 3.86 (s, 2H), 1.26 (t, ^{3}J = 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 188.8 (CO), 167.6 (CO), 148.0 (C_q), 139.0 (CH), 130.6 (CH), 128.0 (C_q), 117.1 (C_q), 82.0 (C_q), 61.7 (CH_2), 45.5 (CH_2), 14.2 (CH_3); IR (neat, cm^{-1}) ν 3455, 3351, 2986, 2938, 2638, 1723, 1603.5, 1319, 1275, 1181; HRMS (ESI) calcd. for $C_{11}H_{12}ClINO_3$ $[M+H]^+$ 367.9545, found 367.9542.

Ethyl 3-(4-amino-3-iodo-phenyl)-3-oxo-propanoate (8c). The compound was prepared from 4-amino-3-iodo-benzoic acid **7c** (3.30 g, 12.5 mmol) according to method A, stirring the reaction for 7 h at room temperature and then for 14 h at 40 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAC, gradient 100:0 to 80:20) to give **8c** as a yellow solid (70% yield); mp 88 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.25 (d, ^{4}J = 2.0 Hz, 1H), 7.73 (dd, ^{3}J = 8.5 Hz, ^{4}J = 2.0 Hz, 1H), 6.70 (d, ^{3}J = 8.3 Hz, 1H), 4.71 (br s, 2H), 4.20 (q, ^{3}J = 7.1 Hz, 2H), 3.87 (s, 2H), 1.25 (t, ^{3}J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 189.6 (CO), 168.0 (CO), 151.7 (C_q), 140.8 (CH), 130.8 (CH), 128.0 (C_q), 113.3 (CH), 82.7 (C_q), 61.6 (CH_2), 45.6 (CH_2), 14.2 (CH_3); IR (neat, cm^{-1}) ν 3451, 3459, 2976, 2934, 2905, 2642, 1736, 1611, 1584, 1330, 1204, 1029. HRMS (ESI) calcd. for $C_{11}H_{13}INO_3$ $[M+H]^+$ 333.9935, found 333.9931.

Ethyl 3-(4-methoxy-1*H*-indol-5-yl)-3-oxo-propanoate (15). The compound was prepared from 4-methoxy-1*H*-indole-5-carboxylic acid **14** (320 mg, 1.67 mmol) according to method A, stirring the reaction for 6 h at room temperature and then for 17 h at 40 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 70:30), to give **15** as

a colorless oil (57% yield); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.56 (br s, 1H), 7.75 (d, ^3J = 8.6 Hz, 1H), 7.20 (m, 1H), 7.08 (d, ^3J = 8.6 Hz, 1H), 6.79 (m, 1H), 4.21 (q, ^3J = 7.2 Hz, 2H), 4.21 (s, 3H), 4.03 (s, 2H), 1.26 (t, ^3J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 193.4 (CO), 169.1 (CO), 155.9 (C_q), 141.6 (C_q), 125.0 (CH), 124.4 (CH), 120.1 (C_q), 118.8 (C_q), 106.7 (CH), 103.0 (CH), 61.1 (CH_3), 60.6 (CH_2), 50.7 (CH_2), 14.2 (CH_3); IR (neat, cm^{-1}) ν 3348, 2984, 2941, 2844, 1735, 1650, 1603, 1359, 1231, 1081, 730; HRMS (ESI) calcd. for $C_{14}H_{16}NO_4$ $[M+H]^+$ 262.1074, found 262.1074.

Representative procedure of method B for the synthesis of 9a-c, 16. To a solution of β-keto ester derivatives **8a-c** and **15** (1.0 eq.) in DMF (10 mL/mmol) were added K_2CO_3 (2.0 eq.) and *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate (1.2 eq.). The resulting mixture was stirred at room temperature for 24–48 h then concentrated *in vacuo*. The residue was dissolved with EtOAc and washed with brine. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. To a stirred solution of residue (1.0 eq.), used without any purification, in a mixture of EtOH/H_2O 5:1 (24 mL/mmol) was added KOH (4.5 eq.) and the resulting mixture was refluxed for 3–5 h. After removal of the solvent, EtOAc was added. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by chromatography on silica gel to give the title compounds **9a-c** and **16**.

***Tert*-butyl 4-[3-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-oxo-propyl]piperidine-1-carboxylate (9a).** The compound was prepared from ethyl 3-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-oxo-propanoate **8a** (1.63 g, 4.10 mmol) according to method B, stirring for 48 h under argon atmosphere at room temperature for the first step, and then refluxing for 5 h for the second step. The residue was purified by chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 80:20) to give **9a** as a yellow oil (37% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.67 (s, 1H), 5.05 (br s, 2H), 4.07 (m, 2H), 3.78 (s, 3H), 2.96 (m, 2H), 2.65 (m, 2H), 1.68–1.59 (m, 4H), 1.44 (s, 9H), 1.41 (m, 1H), 1.10 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.2 (CO), 159.5 (CO), 155.1 (C_q), 148.2 (C_q), 131.4 (CH), 122.8 (C_q), 113.4 (C_q), 82.2 (C_q), 79.4 (C_q), 62.7 (CH_3), 44.0 (2*CH_2), 38.9 (CH_2), 35.7 (CH_2), 32.1 (2*CH_2), 31.1 (CH), 28.5 (3*CH_3); IR (neat, cm^{-1}) ν 3461, 3369, 2929, 2852, 1682, 1603, 1408, 1160; HRMS (ESI) calcd. for $C_{20}H_{29}ClIN_2O_4$ $[M+H]^+$ 523.0855, found 523.0854.

***Tert*-butyl 4-[3-(4-amino-3-chloro-5-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate (9b).** The compound was prepared from ethyl 3-(4-amino-3-chloro-5-iodo-phenyl)-3-oxo-propanoate **8b** (690 mg, 1.88 mmol), by method B, stirring for 48 h under argon atmosphere at room temperature for the first step, and then refluxing for 3 h for the second step. The residue was purified by chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 90:10) to give **9b** as a yellow oil (48% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.16 (d, ^4J = 1.9 Hz, 1H), 7.87 (d, ^4J = 1.9 Hz, 1H), 5.01 (br s, 2H), 4.09 (m, 2H), 2.86 (t, ^3J = 7.6 Hz, 2H), 2.67 (m, 2H), 1.70–1.63 (m, 4H), 1.45 (m, 10H), 1.13 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 196.5 (CO), 155.1 (CO), 147.4 (C_q), 138.4 (CH), 130.1 (CH), 129.0 (C_q), 117.1 (C_q), 82.1 (C_q), 79.5 (C_q), 44.4 (CH_2), 43.6 (CH_2), 35.7 (CH_2), 35.1 (CH_2), 32.0 (2*CH_2), 30.8 (CH), 28.6 (3*CH_3); IR (neat, cm^{-1}) ν 3471, 3366, 2976, 2924, 2846, 1681, 1607, 1276, 1161; HRMS (ESI) calcd. for $C_{19}H_{27}ClIN_2O_3$ $[M+H]^+$ 493.0749, found 493.0746.

***Tert*-butyl 4-[3-(4-amino-3-iodo-phenyl)-3-oxo-propyl] piperidine-1-carboxylate (9c).** The compound was prepared from ethyl 3-(4-amino-3-iodo-phenyl)-3-oxo-propanoate **8c** (1.50 g, 4.5 mmol) by method B, stirring for 48 h under argon atmosphere at room temperature for the first step, and refluxing for 5 h for the second step. The residue was purified by flash chromatography on silica gel (CH_2Cl_2/EtOAc, gradient 100:0 to 95:5) to give **9c** as a yellow solid (76% yield over 2 steps); mp 129 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, ^4J = 1.9 Hz, 1H), 7.72 (dd, ^3J = 8.3 Hz, ^4J = 1.9 Hz, 1H), 6.69 (d, ^3J = 8.5 Hz, 1H), 4.72 (br s, 2H), 4.05 (m, 2H), 2.83 (t, ^3J = 7.6 Hz, 2H), 2.64 (m, 2H), 1.67–1.58 (m, 4H), 1.42 (m, 10H), 1.09 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 197.4 (CO), 155.0 (CO), 151.2 (C_q), 140.1 (CH), 130.1 (CH), 128.7 (C_q), 113.2 (CH), 82.7 (C_q), 79.3 (C_q), 44.2 (CH_2), 43.6 (CH_2), 35.6 (CH_2), 34.9 (CH_2), 32.0 (2*CH_2), 30.9 (CH), 28.5 (3*CH_3); IR (neat, cm^{-1}) ν 3459, 3336, 2976, 2927, 2857, 1662, 1625, 1584, 1430, 1283, 1162; HRMS (ESI) calcd. for $C_{19}H_{28}IN_2O_3$ $[M+H]^+$ 459.1139, found 459.1134.

***Tert*-butyl 4-[3-(4-methoxy-1*H*-indol-5-yl)-3-oxo-propyl] piperidine-1-carboxylate (16).** The compound was prepared from ethyl 3-(4-methoxy-1*H*-indol-5-yl)-3-oxo-propanoate **15** (370 mg, 1.42 mmol) by method B, stirring for 24 h under argon atmosphere at room temperature for the first step, and refluxing for 5 h for the second step. The residue was purified by chromatography on silica gel (CH_2Cl_2/EtOAc, gradient 100:0 to 90:10) to give **16** as a white solid (55% yield over 2 steps); mp 149 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.84 (br s, 1H), 7.58 (d, ^3J = 8.7 Hz, 1H), 7.20 (m, 1H), 7.10 (d, ^3J = 8.6 Hz, 1H), 6.76 (m, 1H), 4.16 (s, 3H), 4.09 (m, 2H), 3.08 (m, 2H), 2.68 (m, 2H), 1.73–1.65 (m, 4H), 1.45 (m, 10H), 1.12 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 203.1 (CO), 155.1 (CO), 154.6 (C_q), 140.7 (C_q), 124.5 (CH), 124.3 (CH), 122.1 (C_q), 119.8 (C_q), 106.9 (CH), 102.2 (CH), 79.4 (C_q), 61.2 (CH_3), 44.5 (CH_2), 43.9 (CH_2), 40.7 (CH_2), 35.9 (CH_2), 32.2 (2*CH_2), 31.4 (CH), 28.5 (3*CH_3); IR (neat, cm^{-1}) ν 3263, 2990, 2971, 2934, 2842, 1655, 1432, 1288, 1161, 966, 858, 740; HRMS (ESI) *m/z* calcd. for $C_{22}H_{31}N_2O_4$ $[M+H]^+$ 387.2278, found 387.2278.

Representative procedure of method C for the synthesis of 4a-g, 6h. To a stirred solution of *tert*-butyl piperidine-1-carboxylate derivatives **9a-c** and **16** (1.0 eq.) in CH_2Cl_2 (20 mL/mmol) was added TFA (2 mL/mmol). The resulting mixture was stirred at room temperature for 30 min. Removal of the solvent under vacuum afforded the crude product, which was directly engaged in the next step. The residue obtained (1.0 eq.) was dissolved in DMF (10 mL/mmol) and alkyl-halogenated derivatives (1.1–1.25 eq.) and K_2CO_3 (10.0 eq.) were added. The resulting mixture was stirred at 110 °C for 5–15 h, and then concentrated *in vacuo*. EtOAc was added, the organic layer was washed several times with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The crude was purified by chromatography on silica gel column and concentrated under reduced pressure to afford the corresponding alkylated compounds **4a-g** and **6h**.

1-(4-Amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-(1-benzyl-4-piperidyl)propan-1-one (4a). The compound was prepared from *tert*-butyl 4-[3-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9a** (500 mg, 0.96 mmol) and bromomethylbenzene (126 μL, 1.06 mmol) according to method C, with stirring for 6 h at 110 °C. The residue was purified by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 60:40 to 0:100) to give **4a** as a yellow oil (62% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66 (s, 1H), 7.31–7.23 (m, 5H), 5.04 (br s, 2H), 3.77 (s, 3H), 3.48 (s, 2H), 2.95 (m, 2H), 2.87 (m, 2H), 1.91 (m, 2H), 1.67–1.58 (m, 5H), 1.27 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.6 (CO), 159.4 (C_q), 148.1 (C_q), 138.7 (C_q), 131.4 (CH), 129.5 (2*CH_2), 128.3 (2*CH_2), 127.1 (CH), 122.9 (C_q), 113.4 (C_q), 82.2 (C_q), 63.6 (CH_2), 62.7 (CH), 53.9 (2*CH_2), 39.2 (CH_2), 35.5 (CH), 32.3 (2*CH_2), 31.3 (CH_2); IR (neat, cm^{-1}) ν 3471, 3373, 3026, 2923, 1670, 1602, 1387, 699; HRMS (ESI) *m/z* calcd. for $C_{22}H_{27}ClIN_2O_2$ $[M+H]^+$ 513.0800, found 513.0795.

1-(4-Amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl] propan-1-one (4b). The compound was prepared from *tert*-butyl 4-[3-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9a** (255 mg, 0.488 mmol) and bromomethylcyclohexane (84 μL, 0.61 mmol) according to method C, with stirring for 12 h at 110 °C. The residue was purified by chromatography on deactivated silica gel column (cyclohexane/EtOAc, gradient 100:0 to 80:20) to give **4b** as a brown oil (68% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 500 MHz)

δ 7.66 (s, 1H), 5.05 (br s, 2H), 3.77 (s, 3H), 2.99 (m, 2H), 2.95 (m, 2H), 2.22 (d, ^3J = 6.6 Hz, 2H), 1.99 (m, 2H), 1.76 (m, 2H), 1.70–1.60 (m, 7H), 1.54 (m, 1H), 1.40 (m, 2H), 1.31 (m, 1H), 1.24–1.10 (m, 3H), 0.89 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 199.2 (CO), 159.3 (C_q), 148.1 (C_q), 131.3 (CH), 122.8 (C_q), 113.3 (C_q), 82.2 (C_q), 65.6 (CH_2), 62.7 (CH_3), 54.2 (2*CH_2), 39.1 (CH_2), 35.2 (CH), 34.9 (CH), 32.2 (2*CH_2), 31.4 (2*CH_2), 31.0 (CH_2), 26.7 (CH_2), 26.2 (2*CH_2); IR (neat, cm^{-1}) ν 3470, 3359, 2924, 2851, 1666, 1602, 1568, 1444, 1390, 1193, 961; HRMS (EI) *m/z* calcd for $C_{22}H_{32}ClIN_2O_2$ $[M]^+$. 518.1197, found 518.1218.

1-(4-Amino-3-chloro-5-iodo-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (4c). The compound was prepared from *tert*-butyl 4-[3-(4-amino-3-chloro-5-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9b** (450 mg, 0.91 mmol) and bromomethylcyclohexane (140 μL, 1.0 mmol) by method C, with stirring for 5 h at 110 °C. The residue was purified by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 60:40) to give **4c** as a pale yellow oil (70% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.14 (d, ^4J = 1.9 Hz, 1H), 7.84 (d, ^4J = 1.9 Hz, 1H), 4.99 (br s, 2H), 2.82 (m, 4H), 2.05 (d, ^3J = 7.1 Hz, 2H), 1.79 (m, 2H), 1.74–1.60 (m, 9H), 1.45 (m, 1H), 1.28–1.11 (m, 6H), 0.83 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 196.6 (CO), 147.1 (C_q), 138.2 (CH), 130.0 (CH), 128.9 (C_q), 117.0 (C_q), 82.1 (C_q), 66.3 (CH_2), 54.5 (2*CH_2), 35.6 (CH), 35.4 (CH_2), 35.3 (CH), 32.3 (2*CH_2), 32.2 (2*CH_2), 31.1 (CH_2), 26.9 (CH_2), 26.3 (2*CH_2); IR (neat, cm^{-1}) ν 3463, 3368, 2924, 2851, 1669, 1606, 1447, 1396, 1266, 1199; HRMS (ESI) *m/z* calcd. for $C_{21}H_{31}ClIN_2O$ $[M+H]^+$ 489.1164, found 489.1159.

1-(4-Amino-3-iodo-phenyl)-3-(1-benzyl-4-piperidyl) propan-1-one (4d). The compound was prepared from *tert*-butyl 4-[3-(4-amino-3-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9c** (350 mg, 0.76 mmol) and bromomethylbenzene (100 μL, 0.84 mmol) by method C, with stirring for 5 h at 110 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 60:40) to give **4d** as a pale yellow solid (75% yield over 2 steps); mp 106 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.26 (d, ^4J = 2.0 Hz, 1H), 7.75 (dd, ^3J = 8.5 Hz, ^4J = 2.0 Hz, 1H), 7.31 (m, 4H), 7.26–7.22 (m, 1H), 6.70 (d, ^3J = 8.5 Hz, 1H), 4.58 (br s, 2H), 3.48 (s, 2H), 2.87 (m, 2H), 2.84 (m, 2H), 1.93 (m, 2H), 1.69–1.62 (m, 4H), 1.31–1.25 (m, 3H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 197.6 (CO), 150.9 (C_q), 140.2 (CH), 138.6 (C_q), 130.1 (CH), 129.4 (2*CH), 129.1 (C_q), 128.2 (2*CH), 127.0 (CH), 113.3 (CH), 82.8 (C_q), 63.6 (CH_2), 53.9 (2*CH_2), 35.5 (CH), 35.4 (CH_2), 32.3 (2*CH_2), 31.3 (CH_2); IR (neat, cm^{-1}) ν 3458, 3341, 3219, 3028, 2921, 2858, 2797, 2750, 1663, 1616, 1581, 1330, 1196, 698; HRMS (ESI) calcd. for $C_{21}H_{26}IN_2O$ $[M+H]^+$ 449.1084, found 449.1080.

1-(4-Amino-3-iodo-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (4e). The compound was prepared from *tert*-butyl 4-[3-(4-amino-3-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9c** (410 mg, 0.90 mmol) and bromomethylcyclohexane (140 μL, 0.99 mmol), by method C, with stirring for 15 h at 110 °C. The residue was purified by chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 60:40) to give **4e** as a pale yellow solid (61% yield over 2 steps); mp 116 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.27 (d, ^4J = 2.0 Hz, 1H), 7.77 (dd, ^3J = 8.4 Hz, ^4J = 2.0 Hz, 1H), 6.71 (d, ^3J = 8.4 Hz, 1H), 4.56 (br s, 2H), 2.85 (m, 4H), 2.07 (d, ^3J = 7.0 Hz, 2H), 1.83–1.60 (m, 11H), 1.47 (m, 1H), 1.28–1.12 (m, 6H), 0.85 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 197.9 (CO), 150.9 (C_q), 140.3 (CH), 130.2 (CH), 129.3 (C_q), 113.3 (CH), 82.8 (C_q), 66.4 (CH_2), 54.6 (2*CH_2), 35.7 (CH), 35.4 (CH_2), 35.3 (CH), 32.3 (2*CH_2), 32.2 (2*CH_2), 31.3 (CH_2), 26.9 (CH_2), 26.3 (2*CH_2); IR (neat, cm^{-1}) ν 3538, 3388, 3314, 3202, 2939, 2844, 1659, 1635, 1585, 1403, 1211, 1144, 670; HRMS (ESI) calcd. for $C_{21}H_{32}IN_2O$ $[M+H]^+$ 455.1554, found 455.1552.

1-(4-Amino-3-iodo-phenyl)-3-[1-(cyclopentylmethyl)-4-piperidyl]propan-1-one (4f). The compound was prepared from *tert*-butyl 4-[3-(4-amino-3-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9c** (350 mg, 0.76 mmol) and iodomethylcyclopentane (110 μL, 0.84 mmol) by method C, with stirring for 6 h at 110 °C. The residue was purified by flash chromatography on silica gel (cyclohexane/EtOAc, gradient 100:0 to 60:40) to give **4f** as a pale yellow solid (54% yield over 2 steps); mp 99 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.27 (d, ^4J = 2.0 Hz, 1H), 7.76 (dd, ^3J = 8.4 Hz, ^4J = 2.0 Hz, 1H), 6.71 (d, ^3J = 8.5 Hz, 1H), 4.56 (br s, 2H), 2.91 (m, 2H), 2.85 (m, 2H), 2.23 (m, 2H), 2.04 (m, 1H), 1.86 (m, 2H), 1.77–1.71 (m, 2H), 1.69–1.61 (m, 4H), 1.60–1.55 (m, 2H), 1.52–1.48 (m, 2H), 1.29 (m, 3H), 1.20–1.13 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 197.7 (CO), 150.8 (C_q), 140.2 (CH), 130.1 (CH), 129.2 (C_q), 113.3 (CH), 82.8 (C_q), 65.3 (CH_2), 54.4 (2*CH_2), 37.7 (CH), 35.7 (CH), 35.4 (CH_2), 32.4 (2*CH_2), 31.9 (2*CH_2), 31.3 (CH_2), 25.4 (2*CH_2); IR (neat, cm^{-1}) ν 3454, 3341, 3219, 2929, 2862, 1665, 1618, 1582, 1333, 1205, 1188, 666; HRMS (ESI) *m/z* calcd. for $C_{20}H_{30}IN_2O$ $[M+H]^+$ 441.1397, found 441.1393.

1-(4-Amino-3-iodo-phenyl)-3-(1-butyl-4-piperidyl)propan-1-one (4g). The compound was prepared from *tert*-butyl 4-[3-(4-amino-3-iodo-phenyl)-3-oxo-propyl]piperidine-1-carboxylate **9c** (350 mg, 0.76 mmol) and 1-iodobutane (96 μL, 0.84 mmol) by method C, with stirring for 5 h at 110 °C. The residue was purified by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 60:40) to give **4g** as a pale yellow solid (60% yield over 2 steps); mp 69 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.26 (d, ^4J = 1.9 Hz, 1H), 7.76 (dd, ^3J = 8.5 Hz, ^4J = 1.9 Hz, 1H), 6.70 (d, ^3J = 8.5 Hz, 1H), 4.57 (br s, 2H), 2.92 (m, 2H), 2.86 (m, 2H), 2.28 (m, 2H), 1.86 (m, 2H), 1.72–1.62 (m, 4H), 1.50–1.43 (m, 2H), 1.34–1.25 (m, 5H), 0.90 (m, 3H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 197.6 (CO), 150.9 (C_q), 140.2 (CH), 130.1 (CH), 129.2 (C_q), 113.3 (CH), 82.8 (C_q), 59.1 (CH_2), 54.1 (2*CH_2), 35.6 (CH), 35.3 (CH_2), 32.3 (2*CH_2), 31.2 (CH_2), 29.4 (CH_2), 21.1 (CH_2), 14.2 (CH_3); IR (neat, cm^{-1}) ν 3460, 3341, 3223, 2919, 2845, 1665, 1619, 1582, 1331, 1199, 667; HRMS (ESI) *m/z* calcd. for $C_{18}H_{28}IN_2O$ $[M+H]^+$ 415.1241, found 415.1237.

3-[1-(Cyclohexylmethyl)-4-piperidyl]-1-(4-methoxy-1*H*-indol-5-yl)propan-1-one (6h). The compound was prepared from *tert*-butyl 4-[3-(4-methoxy-1*H*-indol-5-yl)-3-oxo-propyl]piperidine-1-carboxylate **16** (255 mg, 0.66 mmol) and benzyl bromide (101 μL, 0.73 mmol) by method C. The residue was purified by flash chromatography on silica gel column (CH_2Cl_2/MeOH, gradient 100:0 to 95:5) to give **6h** as a yellow oil (52% yield over 2 steps); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.48 (br s, 1H), 7.58 (d, ^3J = 8.6 Hz, 1H), 7.20 (m, 1H), 7.11 (d, ^3J = 8.6 Hz, 1H), 6.76 (m, 1H), 4.16 (s, 3H), 3.06 (t, ^3J = 7.6 Hz, 2H), 2.94 (m, 2H), 2.18 (m, 2H), 1.92 (m, 2H), 1.77–1.64 (m, 9H), 1.52 (m, 1H), 1.35–1.12 (m, 6H), 0.88 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 203.0 (CO), 154.6 (C_q), 140.5 (C_q), 124.5 (CH), 124.3 (CH), 122.4 (C_q), 119.9 (C_q), 106.7 (CH), 102.3 (CH), 66.0 (CH_2), 61.3 (CH_3), 54.5 (2*CH_2), 40.9 (CH_2), 35.6 (CH), 35.1 (CH), 32.1 (4*CH_2), 31.4 (CH_2), 26.8 (CH_2), 26.3 (2*CH_2); IR (neat, cm^{-1}) ν 3301, 2922, 2849, 1656, 1606, 1449, 1355, 1218, 1066, 733; LC-MS (ESI) t_R = 3.48 min; *m/z* $[M+H]^+$ 383.62; HRMS (ESI) *m/z* calcd. for $C_{24}H_{35}N_2O_2$ $[M+H]^+$ 383.2693, found 383.2694.

Representative procedure of method D for the synthesis of 6a-g. To a stirred solution of iodo-aniline derivatives **4a-g** (1.0 eq.), $Pd(PPh_3)_4$ (10 mol%), XPhos (20 mol%) and K_2CO_3 (2.0 eq.) in dry THF (15 mL/mmol) was added alkyne (3.0 eq.) under argon atmosphere and the reaction mixture was refluxed for 17–24 h. After evaporation *in vacuo* to remove THF, the residue was dissolved in EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. To a stirred solution of previous crude **5a-g** (1.0 eq.) *(except the compound **5b** which was isolated by flash chromatography on silica gel column)* in CH_2Cl_2 (12 mL/mmol) was added TFA (1.25 mL/mmol). The resulting mixture was stirred at room temperature for 1–2 h, and then concentrated *in vacuo*. The crude was purified by flash

chromatography on silica gel column and concentrated under reduced pressure to afford the corresponding derivatives **6a-g** (26–73% isolated yields).

1-(7-Chloro-4-methoxy-1*H*-indol-5-yl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (6a). The compound was prepared from 1-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one **4a** (65 mg, 0.13 mmol) according to method D, refluxing for 18 h under argon atmosphere for the first step, and then stirring for 2 h at room temperature for the second step. The residue was purified by chromatography on silica gel column (CH_2Cl_2/MeOH, gradient 100:0 to 90:10) to give **6a** as a pale yellow solid (52% yield); mp 156 °C; ^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.49 (s, 1H), 7.35 (d, ^3J = 3.3 Hz, 1H), 6.86 (d, ^3J = 3.3 Hz, 1H), 4.19 (s, 3H), 3.51 (m, 2H), 3.12 (t, ^3J = 7.0 Hz, 2H), 2.91 (m, 4H), 1.99 (m, 2H), 1.82 (m, 1H), 1.80–1.70 (m, 7H), 1.65 (m, 1H), 1.52 (m, 2H), 1.40–1.18 (m, 3H), 1.03 (m, 2H); ^{13}C NMR (MeOD-d_4, 100 MHz) δ 202.8 (CO), 155.2 (C_q), 138.9 (C_q), 127.1 (CH), 123.0 (CH), 122.7 (C_q), 122.4 (C_q), 112.8 (C_q), 103.8 (CH), 64.4 (CH_2), 61.7 (CH_3), 54.4 (2*CH_2), 41.3 (CH_2), 34.6 (CH), 34.3 (CH), 31.9 (2*CH_2), 31.5 (CH_2), 30.4 (2*CH_2), 27.0 (CH_2), 26.6 (2*CH_2); IR (neat, cm^{-1}) ν 3436, 2927, 2852, 1651, 1604, 1484, 1404, 1351, 1204, 1134, 728; LC-MS (ESI) t_R = 3.71 min; *m/z* $[M+H]^+$ 417.58/419.60; HRMS (ESI) *m/z* calcd. for $C_{24}H_{34}ClN_2O_2$ $[M+H]^+$ 417.2303, found 417.2304.

3-(1-Benzyl-4-piperidyl)-1-(7-chloro-4-methoxy-2-trimethylsilyl-1*H*-indol-5-yl)propan-1-one (5b). To a stirred solution of 1-(4-amino-5-chloro-3-iodo-2-methoxy-phenyl)-3-(1-benzyl-4-piperidyl)propan-1-one **4b** (260 mg, 0.51 mmol, 1.0 eq.), $Pd(PPh_3)_4$ (59 mg, 0.051 mmol, 10 mol%), XPhos (48 mg, 0.10 mmol, 20 mol%) and K_2CO_3 (141 mg, 1.02 mmol, 2.0 eq.) in dry THF (8 mL) was added ethynyltrimethylsilane (215 µL, 1.52 mmol, 3.0 eq.) under argon atmosphere and the reaction mixture was refluxed for 23 h. After evaporation *in vacuo* to remove THF, the residue was purified by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 100:0 to 90:10) and concentrated under reduced pressure to give **5b** as a pale yellow solid (45% yield); mp 162 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.37 (br s, 1H), 7.58 (s, 1H), 7.31 (m, 4H), 7.24 (m, 1H), 6.91 (d, ^4J = 2.3 Hz, 1H), 4.15 (s, 3H), 3.49 (s, 2H), 3.03 (t, ^3J = 7.6 Hz, 2H), 2.88 (m, 2H), 1.94 (m, 2H), 1.69–1.63 (m, 4H), 1.28 (m, 3H), 0.38 (s, 9H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 201.8 (CO), 153.2 (C_q), 139.9 (2*C_q), 138.7 (C_q), 129.4 (2*CH), 128.3 (2*CH), 127.0 (CH), 123.4 (CH), 122.9 (C_q), 122.0 (C_q), 111.9 (CH), 111.4 (C_q), 63.7 (CH_2), 61.5 (CH_3), 54.0 (2*CH_2), 41.0 (CH_2), 35.7 (CH), 32.4 (3*CH_2), −1.0 (3*CH_3); IR (neat, cm^{-1}) ν 3323, 3031, 2938, 2926, 2854, 2757, 1648, 1596, 1301, 838; LC-MS (ESI) t_R = 4.23 min; *m/z* $[M+H]^+$ 483.57/485.59; HRMS (ESI) *m/z* calcd. for $C_{27}H_{36}ClN_2O_2Si$ $[M+H]^+$ 483.2229, found 483.2226.

3-(1-Benzyl-4-piperidyl)-1-(7-chloro-4-methoxy-1*H*-indol-5-yl)propan-1-one (6b). To a stirred solution of 3-(1-benzyl-4-piperidyl)-1-(7-chloro-4-methoxy-2-trimethylsilyl-1*H*-indol-5-yl) propan-1-one **5b** (85 mg, 0.18 mmol, 1.0 eq.) in DCM (2.5 mL) was added TFA (250 µL). The resulting mixture was stirred at room temperature for 1 h and then concentrated *in vacuo*. The residue was dissolved with EtOAc, and washed with a saturated $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$. The crude was purified by flash chromatography on silica gel column (cyclohexane/EtOAc, gradient 50:50 to 80:20) and concentrated under reduced pressure to give **6b** as a pale brown solid (81% yield); mp 148 °C; ^{1}H NMR (MeOD-d_4, 500 MHz) δ 7.48 (br s, 6H), 7.34 (d, ^3J = 3.3 Hz, 1H), 6.85 (d, ^3J = 3.3 Hz, 1H), 4.24 (s, 2H), 4.18 (s, 3H), 3.42 (m, 2H), 3.09 (t, ^3J = 7.1 Hz, 2H), 2.94 (m, 2H), 1.98 (m, 2H), 1.69 (m, 2H), 1.64 (m, 1H), 1.46 (m, 2H); ^{13}C NMR (MeOD-d_4, 126 MHz) δ 202.7 (CO), 155.2 (C_q), 138.9 (C_q), 132.2 (2*CH), 131.1 (CH), 131.0 (C_q), 130.3 (2*CH), 127.1 (CH), 123.0 (CH), 122.6 (C_q), 122.4 (C_q), 112.8 (C_q), 103.8 (CH), 61.6 (CH_3), 61.5 (CH_2), 53.5 (2*CH_2), 41.3 (CH_2), 34.5 (CH), 31.4 (CH_2), 30.4 (2*CH_2); IR (neat, cm^{-1}) ν 3428, 2953, 2930, 2852, 1677, 1602, 1202, 1134, 721; LC-MS (ESI) t_R = 3.59 min; *m/z* $[M+H]^+$ 411.53/413.55; HRMS (ESI) *m/z* calcd. for $C_{24}H_{28}ClN_2O_2$ $[M+H]^+$ 411.1834, found 411.1831.

1-(7-Chloro-1*H*-indol-5-yl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (6c). The compound was prepared from 1-(4-amino-3-chloro-5-iodo-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one **4c** (275 mg, 0.56 mmol) and ethynyltrimethylsilane (237 µL, 1.68 mmol) according to method D, refluxing for 17 h under argon atmosphere for the first step, and then stirring for 1 h at room temperature for the second step. The residue was purified by flash chromatography on silica gel column (cyclohexane/EtOAc 50:50 to CH_2Cl_2/MeOH 95:5) to give **6c** as a white solid (64% yield); mp 172 °C; ^{1}H NMR (MeOD-d_4, 500 MHz) δ 8.29 (d, ^4J = 1.4 Hz, 1H), 7.79 (d, ^4J = 1.3 Hz, 1H), 7.42 (d, ^3J = 3.2 Hz, 1H), 6.70 (d, ^3J = 3.2 Hz, 1H), 3.56 (m, 2H), 3.14 (t, ^3J = 7.2 Hz, 2H), 2.92–2.86 (m, 4H), 2.02 (m, 2H), 1.83 (m, 1H), 1.80–1.69 (m, 7H), 1.65 (m, 1H), 1.52 (m, 2H), 1.38–1.30 (m, 2H), 1.26–1.21 (m, 1H), 1.03 (m, 2H); ^{13}C NMR (MeOD-d_4, 126 MHz) δ 201.1 (CO), 137.2 (C_q), 131.1 (C_q), 130.6 (C_q), 128.6 (CH), 122.5 (CH), 121.4 (CH), 118.3 (C_q), 105.6 (CH), 64.6 (CH_2), 54.6 (2*CH_2), 36.1 (CH_2), 34.4 (CH), 34.0 (CH), 31.7 (2*CH_2), 31.4 (CH_2), 30.5 (2*CH_2), 26.9 (CH_2), 26.5 (2*CH_2); IR (neat, cm^{-1}) ν 3382, 3202, 2933, 2854, 1675, 1452, 1202, 1131, 719; LC-MS (ESI) t_R = 3.74 min; *m/z* $[M+H]^+$ 387.61/389.58; HRMS (ESI) *m/z* calcd. for $C_{23}H_{32}ClN_2O$ $[M+H]^+$ 387.2198, found 387.2194.

3-(1-Benzyl-4-piperidyl)-1-(1*H*-indol-5-yl)propan-1-one (6d). The compound was prepared from 1-(4-amino-3-iodo-phenyl)-3-(1-benzyl-4-piperidyl)propan-1-one **4d** (245 mg, 0.55 mmol) and ethynyltrimethylsilane (233 µL, 1.65 mmol) according to method D, refluxing for 18 h under argon atmosphere for the first step, and then stirring for 1 h at room temperature for the second step. The residue was purified by flash chromatography on silica gel column (cyclohexane 100% to CH_2Cl_2/MeOH 95:5) to give **6d** as a white solid (53% yield); mp 165 °C; ^{1}H NMR (MeOD-d_4, 500 MHz) δ 8.33 (m, 1H), 7.80 (dd, ^3J = 8.7 Hz, ^4J = 1.6 Hz, 1H), 7.50 (br s, 5H), 7.44 (d, ^3J = 8.7 Hz, 1H), 7.34 (d, ^3J = 3.2 Hz, 1H), 6.60 (dd, ^3J = 3.1 Hz, ^4J = 0.6 Hz, 1H), 4.28 (s, 2H), 3.49 (m, 2H), 3.14 (t, ^3J = 7.1 Hz, 2H), 2.98 (m, 2H), 2.05 (m, 2H), 1.73 (m, 2H), 1.68 (m, 1H), 1.45 (m, 2H); ^{13}C NMR (MeOD-d_4, 126 MHz) δ 202.6 (CO), 140.5 (C_q), 132.3 (2*CH), 131.3 (CH), 130.4 (C_q, 2*CH), 129.9 (C_q), 129.1 (C_q), 127.6 (CH), 123.7 (CH), 122.4 (CH), 112.2 (CH), 104.3 (CH), 61.8 (CH_2), 53.8 (2*CH_2), 36.1 (CH_2), 34.5 (CH), 31.7 (CH_2), 30.6 (2*CH_2); IR (neat, cm^{-1}) ν 3308, 2996, 2945, 2745, 2575, 1690, 1666, 1607, 1200, 1126, 718; LC-MS (ESI) t_R = 3.35 min; *m/z* $[M+H]^+$ 347.56; HRMS (ESI) *m/z* calcd. for $C_{23}H_{27}N_2O$ $[M+H]^+$ 347.2118, found 347.2117.

3-[1-(Cyclohexylmethyl)-4-piperidyl]-1-(1*H*-indol-5-yl) propan-1-one (6e). The compound was prepared from 1-(4-amino-3-iodo-phenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl] propan-1-one **4e** (160 mg, 0.35 mmol) and ethynyltrimethylsilane (148 µL, 1.05 mmol) according to method D, refluxing for 23 h under argon atmosphere for the first step, and then stirring for 1 h at room temperature for the second step. The residue was purified by flash chromatography on silica gel column (cyclohexane 100% to EtOAc 100%) to give **6e** as a colorless oil (73% yield); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.61 (br s, 1H), 8.32 (m, 1H), 7.87 (dd, ^3J = 8.6 Hz, ^4J = 1.6 Hz, 1H), 7.41 (d, ^3J = 8.6 Hz, 1H), 7.28 (m, 1H), 6.66 (m, 1H), 3.06 (t, ^3J = 7.4 Hz, 2H), 2.90 (m, 2H), 2.12 (d, ^3J = 7.0 Hz, 2H), 1.87 (m, 2H), 1.77–1.64 (m, 9H), 1.50 (m, 1H), 1.34 (m, 3H), 1.28–1.11 (m, 3H), 0.87 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 200.9 (CO), 138.6 (C_q), 129.7 (C_q), 127.6 (C_q), 125.9 (CH), 122.7 (CH), 122.2 (CH), 111.2 (CH), 104.3 (CH), 65.8 (CH_2), 54.3 (2*CH_2), 35.8 (CH_2), 35.5 (CH), 35.0 (CH), 32.2 (2*CH_2), 31.8 (2*CH_2), 31.4 (CH_2), 26.8 (CH_2), 26.3 (2*CH_2); IR (neat, cm^{-1}) ν 3428, 2922, 2851, 1660, 1612, 1449, 730; LC-MS (ESI) t_R = 3.56 min; *m/z* $[M+H]^+$ 353.60; HRMS (ESI) *m/z* calcd. for $C_{23}H_{33}N_2O$ $[M+H]^+$ 353.2587, found 353.2585.

3-[1-(Cyclopentylmethyl)-4-piperidyl]-1-(1*H*-indol-5-yl) propan-1-one (6f). The compound was prepared from 1-(4-amino-3-iodo-phenyl)-3-[1-(cyclopentylmethyl)-4-piperidyl]propan-1-one **4f** (160 mg, 0.36 mmol) and ethynyltrimethylsilane (155 μL, 1.1 mmol) according to method D, refluxing for 17 h under argon atmosphere for the first step, and then stirring for 1 h at room temperature for the second step. The residue was purified by flash chromatography on silica gel column (cyclohexane 100% to EtOAc 100%) to give **6f** as a pale yellow solid (26% yield); mp 151 °C, ^{1}H NMR (MeOD-d_4, 500 MHz) δ 8.33 (m, 1H), 7.80 (dd, ^3J = 8.7 Hz, ^4J = 1.7 Hz, 1H), 7.44 (d, ^3J = 8.7 Hz, 1H), 7.34 (d, ^3J = 3.2 Hz, 1H), 6.61 (dd, ^3J = 3.2 Hz, ^4J = 0.9 Hz, 1H), 3.10 (m, 2H), 3.03 (m, 2H), 2.40 (d, ^3J = 7.0 Hz, 2H), 2.13–2.05 (m, 3H), 1.85–1.79 (m, 4H), 1.69 (m, 2H), 1.66–1.61 (m, 2H), 1.58–1.54 (m, 2H), 1.41 (m, 1H), 1.38–1.28 (m, 2H), 1.23–1.16 (m, 2H); ^{13}C NMR (MeOD-d_4, 126 MHz) δ 203.4 (CO), 140.5 (C_q), 130.0 (C_q), 129.0 (C_q), 127.5 (CH), 123.7 (CH), 122.5 (CH), 112.2 (CH), 104.3 (CH), 65.9 (CH_2), 55.2 (2*CH_2), 38.3 (CH), 36.6 (CH_2), 36.4 (CH), 32.9 (2*CH_2), 32.7 (CH_2), 32.5 (2*CH_2), 26.1 (2*CH_2); IR (neat, cm^{-1}) ν 3301, 2924, 2863, 1663, 1610, 1574, 1453, 1328, 1126, 729; LC-MS (ESI) t_R = 3.38 min; *m/z* $[M+H]^+$ 339.58; HRMS (ESI) *m/z* calcd. for $C_{22}H_{31}N_2O$ $[M+H]^+$ 339.2431, found 339.2430.

3-(1-Butyl-4-piperidyl)-1-(1*H*-indol-5-yl)propan-1-one (6g). The compound was prepared from 1-(4-amino-3-iodo-phenyl)-3-(1-butyl-4-piperidyl)propan-1-one **4g** (175 mg, 0.42 mmol) and ethynyltrimethylsilane (178 μL, 1.26 mmol) according to method D, refluxing for 18 h under argon atmosphere for the first step, and then stirring for 1 h at room temperature for the second step. The residue was purified by flash chromatography on silica gel column (cyclohexane 100% to EtOAc 100%) to give **6g** as a yellow oil (33% yield); ^{1}H NMR (MeOD-d_4, 500 MHz) δ 8.34 (m, 1H), 7.80 (dd, ^3J = 8.7 Hz, ^4J = 1.7 Hz, 1H), 7.44 (d, ^3J = 8.6 Hz, 1H), 7.34 (d, ^3J = 3.2 Hz, 1H), 6.61 (dd, ^3J = 3.2 Hz, ^4J = 0.8 Hz, 1H), 3.56 (m, 2H), 3.15 (t, ^3J = 7.2 Hz, 2H), 3.05 (m, 2H), 2.91 (m, 2H), 2.05 (m, 2H), 1.76–1.67 (m, 5H), 1.51–1.37 (m, 4H), 0.99 (t, ^3J = 7.4 Hz, 3H); ^{13}C NMR (MeOD-d_4, 126 MHz) δ 202.6 (CO), 140.5 (C_q), 129.9 (C_q), 129.0 (C_q), 127.6 (CH), 123.7 (CH), 122.4 (CH), 112.2 (CH), 104.3 (CH), 58.1 (CH_2), 54.1 (2*CH_2), 36.1 (CH_2), 34.6 (CH), 31.7 (CH_2), 30.8 (2*CH_2), 27.1 (CH_2), 20.9 (CH_2), 13.9 (CH_3); IR (neat, cm^{-1}) ν 3435, 2959, 2927, 2857, 1679, 1206, 1130, 736; LC-MS (ESI) t_R = 3.22 min; *m/z* $[M+H]^+$ 313.55; HRMS (ESI) *m/z* calcd. for $C_{20}H_{29}N_2O$ $[M+H]^+$ 313.2274, found 313.2272.

1-(7-Chloro-4-methoxy-1-methyl-indol-5-yl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (10a). To a solution of **6a** (56 mg, 0.13 mmol, 1.0 eq.) in DMF (1.8 mL) were added CH_3I (10 μL, 0.15 mmol, 1.1 eq.) and K_2CO_3 (74 mg, 0.54 mmol, 4 eq.) and the reaction mixture was stirred at room temperature overnight. After removal of DMF, the residue was dissolved with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and the residue was purified by chromatography on silica gel (EtOAc/MeOH gradient 100:0 to 90:10) to give **10a** as a brown solid (77% yield); mp 90 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.55 (s, 1H), 6.98 (d, ^3J = 3.2 Hz, 1H), 6.67 (d, ^3J = 3.2 Hz, 1H), 4.15 (s, 3H), 4.08 (s, 3H), 3.11–3.00 (m, 2H), 3.06 (t, ^3J = 7.4 Hz, 2H), 2.35–2.23 (m, 2H), 2.16–2.01 (m, 2H), 1.86–1.62 (m, 10H), 1.59–1.48 (m, 2H), 1.45–1.35 (m, 1H), 1.31–1.10 (m, 3H), 1.01–0.86 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 200.9 (CO), 153.3 (C_q), 135.5 (C_q), 131.6 (CH), 124.3 (CH), 124.1 (C_q), 122.6 (C_q), 112.5 (C_q), 100.9 (CH), 65.4 (CH_2), 61.9 (CH_3), 54.1 (2*CH_2), 40.3 (CH_2), 36.7 (CH_3), 35.0 (CH), 34.7 (CH), 32.0 (2*CH_2), 31.1 (CH_2), 30.9 (2*CH_2), 26.5 (CH_2), 26.0 (2*CH_2); IR (neat, cm^{-1}) ν 2924, 2848, 2804, 2766, 1662, 1589, 1487, 1452, 1284, 1011, 729; LC-MS (ESI) t_R = 4.04 min; *m/z* $[M+H]^+$ 431.60/433.62; HRMS (ESI) *m/z* calcd. for $C_{25}H_{36}ClN_2O_2$ $[M+H]^+$ 431.2460, found 431.2458.

1-(1-Benzyl-7-chloro-4-methoxy-indol-5-yl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (10b). To a solution of **6a** (63 mg, 0.15 mmol, 1.0 eq.) in DMF (2 mL) were added benzyl bromide (20 μL, 0.17 mmol, 1.1 eq.) and K_2CO_3 (83 mg, 0.60 mmol, 4.0 eq.) and the reaction mixture was stirred at room temperature overnight. After removal of DMF, the residue was dissolved with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and the residue was purified by chromatography on silica gel (EtOAc/MeOH gradient 100:0 to 90:10), to give **10b** as a white solid (83% yield); mp 83 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.46 (s, 1H), 7.28–7.17 (m, 3H), 7.01 (d, ^3J = 3.3 Hz, 1H), 6.99–6.94 (m, 2H), 6.69 (d, ^3J = 3.3 Hz, 1H), 5.69 (s, 2H), 4.02 (s, 3H), 2.98 (t, ^3J = 7.5 Hz, 2H), 2.93–2.83 (m, 2H), 2.17–2.07 (m, 2H), 1.95–1.83 (m, 2H), 1.75–1.56 (m, 10H), 1.52–1.43 (m, 1H), 1.33–1.25 (m, 2H), 1.16–1.07 (m, 3H), 0.89–0.76 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 201.0 (CO), 153.2 (C_q), 138.2 (C_q), 135.0 (C_q), 131.0 (CH), 128.8 (2*CH), 127.7 (CH), 126.3 (2*CH), 124.9 (CH), 124.4 (C_q), 123.0 (C_q), 112.2 (C_q), 101.9 (CH), 65.7 (CH_2), 62.0 (CH_3), 54.3 (2*CH_2), 51.7 (CH_2), 40.4 (CH_2), 35.3 (CH), 34.9 (CH), 32.1 (2*CH_2), 31.6 (CH_2), 31.0 (2*CH_2), 26.6 (CH_2), 26.1 (2*CH_2); IR (neat, cm^{-1}) ν 2918, 2851, 2804, 2763, 1656, 1589, 1481, 1449, 1329, 1288, 732; LC-MS (ESI) t_R = 4.27 min; *m/z* $[M+H]^+$ 507.63/509.60; HRMS (ESI) *m/z* calcd. for $C_{31}H_{40}ClN_2O_2$ $[M+H]^+$ 507.2773, found 507.2780.

1-[1-(Benzenesulfonyl)-7-chloro-4-methoxy-indol-5-yl]-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (10c). To a solution of **6a** (28 mg, 0.068 mmol, 1.0 eq.) in CH_2Cl_2 (1 mL) were added phenylsulfonyl chloride (14 μL, 0.074 mmol, 1.1 eq.), an aqueous solution of NaOH (60% weight, 0.6 mL) and $(n\text{Bu})_4NHSO_4$ (1 mg, 0.0034 mmol, 5 mol%). The reaction mixture was stirred at room temperature for 2 h then diluted with water. The organic layer was separated, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by chromatography on silica gel (EtOAc/MeOH, gradient 100:0 to 90:10), to give **7c** as a yellow oil (84% yield); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.87 (d, ^3J = 3.8 Hz, 1H), 7.77–7.74 (m, 2H), 7.57–7.54 (m, 1H), 7.47–7.43 (m, 2H), 7.44 (s, 1H), 6.84 (d, ^3J = 3.8 Hz, 1H), 3.96 (s, 3H), 3.32–3.22 (m, 4H), 2.95 (t, ^3J = 7.3 Hz, 2H), 2.58–2.50 (m, 2H), 1.80–1.72 (m, 3H), 1.71–1.63 (m, 7H), 1.62–1.56 (m, 2H), 1.49–1.43 (m, 1H), 1.22–1.16 (m, 3H), 1.01–0.91 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 200.0 (CO), 152.0 (C_q), 139.7 (C_q), 134.9 (C_q), 133.9 (CH), 130.4 (CH), 129.3 (2*CH), 128.0 (C_q), 127.8 (CH), 127.1 (2*CH), 126.7 (C_q), 114.4 (C_q), 105.2 (CH), 70.5 (CH_2), 62.7 (CH_3), 53.4 (2*CH_2), 39.7 (CH_2), 34.0 (CH), 33.6 (CH), 31.9 (2*CH_2), 29.9 (CH_2), 28.9 (2*CH_2), 25.9 (CH_2), 25.6 (2*CH_2); IR (neat, cm^{-1}) ν 3455, 3351, 2986, 2938, 2638, 1723, 1603.5, 1319, 1275, 1181; LC-MS (ESI) t_R = 4.25 min; *m/z* $[M+H]^+$ 557.59/559.60; HRMS (ESI) calcd. for $C_{30}H_{38}ClN_2O_4S$ $[M+H]^+$ 557.2234, found 557.2235.

1-(1-Benzylindol-5-yl)-3-[1-(cyclohexylmethyl)-4-piperidyl] propan-1-one (10d). To a solution of **6e** (100 mg, 0.28 mmol, 1.0 eq.) in DMF (4 mL) were added benzyl bromide (38 μL, 0.31 mmol, 1.1 eq.) and K_2CO_3 (155 mg, 1.12 mmol, 4.0 eq.) and the reaction mixture was stirred at room temperature overnight. After removal of DMF, the residue was dissolved with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, concentrated *in vacuo* and the residue was purified by chromatography on silica gel (EtOAc/MeOH gradient 100:0 to 60:40), to give **10d** as a white solid (86% yield); mp 98 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.32 (m, 1H), 7.85 (dd, ^3J = 8.7 Hz, ^4J = 1.7 Hz, 1H), 7.33–7.27 (m, 4H), 7.19 (d, ^3J = 3.2 Hz, 1H), 7.10 (m, 2H), 6.66 (dd, ^3J = 3.2 Hz, ^4J = 0.8 Hz, 1H), 5.35 (s, 2H), 3.04 (m, 2H), 2.86 (m, 2H), 2.08 (d, ^3J = 7.0 Hz, 2H), 1.84 (m, 2H), 1.77–1.64 (m, 9H), 1.48 (m, 1H), 1.34–1.11 (m, 6H), 0.86 (m, 2H); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 200.7 (CO), 138.9 (C_q), 137.0 (C_q), 129.9 (CH), 129.7 (C_q), 129.0 (2*CH), 128.4 (C_q), 128.0 (CH), 126.9 (2*CH), 123.0 (CH), 122.2 (CH), 109.7 (CH), 103.8 (CH), 66.4 (CH_2), 54.7 (2*CH_2), 50.5 (CH_2), 36.0 (CH_2), 35.9 (CH), 35.5 (CH), 32.5 (2*CH_2), 32.3 (2*CH_2), 31.7 (CH_2), 27.0 (CH_2), 26.4 (2*CH_2); IR (neat, cm^{-1}) ν 3031, 2919, 2850, 1675, 1452, 1133, 730, 718; LC-MS (ESI)

$t_R = 4.16$ min; *m/z* $[M+H]^+$ 443.75; HRMS (ESI) *m/z* calcd. for $C_{30}H_{39}N_2O$ $[M+H]^+$ 443.3057, found 443.3054.

Methyl 7-chloro-4-methoxy-1*H*-indole-5-carboxylate (12). To a stirred solution of methyl 4-amino-5-chloro-3-iodo-2-methoxy-benzoate **11** (1.5 g, 4.4 mmol, 1.0 eq.), $Pd(PPh_3)_4$ (508 mg, 0.44 mmol, 10 mol%), XPhos (420 mg, 0.88 mmol, 20 mol %) and K_2CO_3 (1.22 g, 8.8 mmol, 2.0 eq.) in dry THF (60 mL) was added ethynyltrimethylsilane (1.87 mL, 13.2 mmol, 3.0 eq.) under argon atmosphere and the reaction mixture was refluxed for 24 h. After evaporation *in vacuo* to remove THF, the residue was dissolved with EtOAc and washed with brine. The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. To a stirred solution of previous crude in CH_2Cl_2 (60 mL) was added TFA (6 mL). The resulting mixture was stirred at room temperature for 1 h, and then concentrated *in vacuo*. The crude was purified by flash chromatography on silica gel column (cyclohexane/DCM 50:50 to DCM/EtOAc 95:5) and concentrated under reduced pressure to afford **12**, as a brown solid (45% yield); mp 109 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.75 (br s, 1H), 7.71 (s, 1H), 7.24 (m, 1H), 6.80 (m, 1H), 4.10 (s, 3H), 3.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.6 (CO), 154.5 (C_q), 137.1 (C_q), 125.1 (CH), 124.1 (CH), 122.7 (C_q), 114.6 (C_q), 111.4 (C_q), 103.2 (CH), 62.1 (CH_3), 52.2 (CH_3); IR (neat, cm^{-1}) ν 3366, 3019, 2993, 2948, 2841, 1690, 1606, 1435, 1312, 1242, 974, 770; HRMS (ESI) *m/z* calcd. for $C_{11}H_{11}ClNO_3$ $[M+H]^+$ 240.0422, found 240.0421.

Methyl 4-methoxy-1*H*-indole-5-carboxylate (13). To a stirred solution of methyl 7-chloro-4-methoxy-1*H*-indole-5-carboxylate **12** (290 mg, 1.21 mmol, 1.0 eq.) in MeOH (15 mL) was added ammonium formate (763 mg, 12.1 mmol, 10.0 eq.) and then 10% Pd/C (129 mg, 10 mol%) was added under nitrogen. The resulting mixture was refluxed overnight, the catalyst was filtered through celite and the filtrate was concentrated under vacuum. The residue was dissolved with ethyl acetate, and washed with brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to afford **13**, as a white solid (82% yield); mp 150 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.51 (br s, 1H), 7.70 (d, $^3J = 8.6$ Hz, 1H), 7.20 (m, 1H), 7.13 (d, $^3J = 8.5$ Hz, 1H), 6.77 (m, 1H), 4.12 (s, 3H), 3.92 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.7 (CO), 155.6 (C_q), 140.4 (C_q), 125.5 (CH), 124.5 (CH), 121.6 (C_q), 113.7 (C_q), 106.7 (CH), 101.9 (CH), 61.9 (CH_3), 52.0 (CH_3); IR (neat, cm^{-1}) ν 3335, 3127, 2990, 2943, 2832, 1705, 1607, 1431, 1349, 1277, 1192, 1055, 763; HRMS (ESI) *m/z* calcd. for $C_{11}H_{12}NO_3$ $[M+H]^+$ 206.0812, found 206.0812.

4-Methoxy-1*H*-indole-5-carboxylic acid (14). To a stirred solution of methyl 4-methoxy-1*H*-indole-5-carboxylate **13** (430 mg, 2.10 mmol, 1.0 eq.) in EtOH (20 mL) was added a 1N NaOH solution (21 mL, 21 mmol, 10.0 eq.) under nitrogen atmosphere at room temperature. The resulting mixture was stirred overnight at room temperature, and then concentrated *in vacuo* to remove EtOH. The residue was diluted with water. The aqueous layer was acidified by addition of a HCl solution until acidic pH, and extracted several times with AcOEt. The combined organic extract was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure to give **14** as a pale yellow solid (83% yield); mp 163 °C; ^{1}H NMR (MeOD-d_4, 400 MHz) δ 7.69 (d, $^3J = 8.8$ Hz, 1H), 7.29 (d, $^3J = 3.2$ Hz, 1H), 7.18 (d, $^3J = 8.8$ Hz, 1H), 6.74 (d, $^3J = 3.3$ Hz, 1H), 4.17 (s, 3H); ^{13}C NMR (MeOD-d_4, 100 MHz) δ 170.6 (CO), 156.3 (C_q), 142.9 (C_q), 126.6 (CH), 125.9 (CH), 121.5 (C_q), 112.5 (C_q), 108.2 (CH), 101.8 (CH), 62.1 (CH_3); IR (neat, cm^{-1}) ν 3211, 3156, 3016, 2964, 2847, 1706, 1606, 1362, 1223, 720; HRMS (ESI) *m/z* calcd. for $C_{10}H_{10}NO_3$ $[M+H]^+$ 192.0655, found 192.0651.

5.2. In vitro evaluation

5.2.1. Pharmacological characterization of drugs on human 5-HT$_4$R

The method was validated by saturation studies: six concentrations of [^{3}H]GR113808 were used to give final concentrations of 0.0625–2 nM, and nonspecific binding of [^{3}H]GR113808 was defined in the presence of 30 μM serotonin to determine the Kd and the Bmax. For competition studies, 2.5 μg of proteins (5-HT_{4B} membrane preparations, HTS110M, Millipore. Millipore's 5-HT_{4B} membrane preparations are crude membrane preparations made by use of their proprietary stable recombinant cell lines to ensure high-level of GPCR surface expression.) were incubated in duplicate at 25 °C for 60 min in the absence or the presence of 10^{-6} or 10^{-8}M of each compound together with 0.2 nM [^{3}H]-GR 113808 (VT 240, ViTrax) in 25 mM Tris buffer (pH 7.4, 25 °C). At the end of the incubation, homogenates were filtered through Whatman GF/C filters (Alpha Biotech) presoaked with 0.5% polyethylenimine using a Brandel cell harvester. Filters were subsequently washed three times with 4 mL of ice-cold 25 mM Tris buffer (pH 7.4, 4 °C). Non-specific binding was evaluated in parallel, in the presence of 30 μM serotonin.

For some of these compounds, affinity constants were calculated from five-point inhibition curves using the EBDA-Ligand software and expressed as Ki ± SD.

5.2.2. Functional assay: cyclic AMP radioimmunoassay

For measurement of intracellular cyclic AMP accumulation, stably transfected cells were grown to confluence and were incubated with serum-free medium 4 h before the beginning of the assay. Then, the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline, 10 mM pargyline and 1 mM GR127935 in CHO cells to block the activity of endogenous 5-HT_{1B} receptors. 5-HT or **6a** were then added for an additional 15 min. The reaction was stopped by aspiration of the medium and addition of 500 ml of ice-cold ethanol. After 30 min incubation at room temperature, the ethanol fraction was collected and evaporated under vacuum. The pellet was reconstituted and cyclic AMP was quantified using a radio-immunoassay kit (cyclic AMP competitive radioimmunoassay, Immunotech, Marseille, France). Student's t-tests were performed using the QuickTTest software according to the CEREP cellular functional assay [48].

5.2.3. In vitro measurement of AChE activity

Inhibitory capacity of the novel ligands synthesized towards AChE biological activity was evaluated spectrometrically by the Ellman method [49]. Acetylthiocholine-iodide (ATC) and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. AChE purified from human erythrocytes (buffered aqueous solution, ≥500 units/mg protein (BCA), Sigma Aldrich) was diluted in 0.1% Triton X-100/20 mM HEPES, pH 8, to yield an enzyme stock solution with 0.25 unit/mL activity. In the procedure, 100 μL aliquots of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 were dispended into a 96 wells plate followed by 50 μL of an appropriate dilution of the test compound solution and 50 μL of the enzyme stock. After 5 min preincubation at 25 °C, the reaction was initiated by the injection of 50 μL of 10 mM ATC solution. The hydrolysis of ATC was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of ATC, at 412 nm using a 96-well microplate plate reader (TECAN Infinite M200, Lyon, France). Test compounds were dissolved in analytical grade DMSO. Donepezil was used as a reference standard. The absorbance at 412 nm was monitored at 1-min intervals for 10 min. Assays were performed with a blank containing all components except acetylthiocholine, in order to correct for non-enzymatic hydrolysis. The percent inhibition due to the presence of the test compounds was calculated by the following expression: 100 - (v_i/v_0 x 100) where v_i is the rate calculated in the presence of the inhibitor, and v_0 is the activity in its absence.

Initial screening for inhibitory capacity was performed at 10^{-6} or 10^{-5} M concentrations of compounds studied. For those displaying ≥50% inhibition, IC_{50} values were determined graphically, by plotting % inhibition versus the logarithms of six inhibitor concentrations tested, using Origin software.

5.2.4. In vitro tests of hAChE kinetic studies

Kinetic studies of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman. Acetylthiocholine iodide, lyophilized human Acetylcholinesterase and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. AChE was dissolved in 0.2 M phosphate buffer pH 7.4 such as to have enzyme solutions stock with 0.25 units/ml AChE activity. In the procedure, 100 μL of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4 were added into the 96 wells plate followed by 50 μL of test compound solution and 50 μL of enzyme solution. After 5 min of preincubation, the reaction was then initiated by the addition of 50 μL of different concentrations of acetylthiocholine iodide solution (from 0.02 to 0.2 mM). The hydrolysis of acetylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine, at a wavelength of 412 nm every minute for 10 min using a 96-well microplate plate reader (TECAN Infinite M200, Lyon, France). Test compounds were dissolved in analytical grade DMSO.

The kinetic studies were performed using four concentrations of inhibitor (0–1 μM).

5.2.5. Sigma-1 receptor affinity

The binding assays were performed by CEREP (Poitiers, France), according to Ganapathy [50]. The Sigma-1 receptor binding assay was carried out by incubating Jurkat cell membranes (10–20 mg protein per tube) with [^{3}H](+)-pentazocine (15 nM) and a range of concentrations of test ligands, at 37 °C for 2 h, in 5 mM Tris/HCl buffer (pH = 7.4). Bound radioactivity was measured using liquid scintillation counting. Inhibition constants (K*i*) were calculated from the IC_{50} values according to the method of Cheng and Prusoff [51].

5.3. X-ray crystallography

Torpedo californica acetylcholinesterase (*Tc*AChE) was purified by Lilly Toker in the Silman laboratory, as described [52].

Cholinesterase inhibitors **6a** and **6b** were synthesized by the CERMN in Caen (France).

5.3.1. Crystallization of TcAChE and soaking procedure

Orthorhombic crystals (spacegroup P212121) of *Tc*AChE were grown at 4 °C with the hanging drop vapor diffusion method using 36% PEG200/150 mM MES pH 5.8, and a protein concentration of 10 mg/ml. After 5–10 days, orthorhombic crystal plates had grown to a size of around 300 × 50 μm.

Crystals were soaked for 24–36 h in the mother liquor containing 5 mM **6a** or **6b**, with a final concentration of 10% DMSO. They were then mounted on a standard cryo-loop and flash cooled in liquid nitrogen.

Data collection was performed at the ESRF in Grenoble on beamlines ID14-4 and id23-1. Data collection information and statistics are displayed in Table S1. Data were processed using XDS/XSCALE, and amplitude factors were generated using XDSCONV. Molecular replacement was performed with PhaserMR, taking as a model the crystal structure of native *Tc*AChE in the orthorhombic spacegroup (pdb entry 1W75). The ligand was modeled using Phenix.elbow [53] with the corresponding SMILES string as input and fitted into the density using Phenix.ligand_fit. Crystal structures could be obtained from the pdb for both structure **6a** (pdb entry 6EZH) and **6b** (pdb entry 6EZH).

5.4. In vivo evaluation

5.4.1. Animals

Male Swiss mice, 6 weeks old and weighing 32 ± 2 g, from JANVIER (Saint Berthevin, France), were kept for housing and experiments taken place within the animal facility building of the University of Montpellier (CECEMA, Office of Veterinary Services agreement # B-34-172-23). Animals were housed in groups with access to food and water ad libitum, except during behavioral experiments. They were kept in a temperature and humidity controlled animal facility on a 12 h/12 h light/dark cycle (lights off at 07:00 p.m.). Mice were numbered by marking their tail using permanent markers. All animal procedures were conducted in strict adherence to the European Union Directive of September 22, 2010 (2010/63/UE).

5.4.2. Experimental design

Groups of 12–16 mice each were included in this study. Compounds were solubilized in DMSO 10% in saline at the stock solution concentration (5 mg/ml), then diluted in saline. Control vehicle solution was DMSO 2% in saline. Compounds or the vehicle solution was injected intraperitoneally (IP), 10 min before dizocilpine (0.15 mg/kg) or physiological saline, IP, 20 min before the behavioral test (Y-maze test session or passive avoidance training session).

5.4.3. Spontaneous alternation performances

All animals were tested for spontaneous alternation performance in the Y-maze, an index of spatial working memory. The Y-maze is made of grey polyvinylchloride. Each arm is 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converging at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8 min session. The series of arm entries, including possible returns into the same arm, were checked visually. An alternation is defined as entries into all three arms on consecutive occasions. The number of maximum alternations is therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/maximum alternations) x 100. Parameters included the percentage of alternation (memory index) and total number of arm entries (exploration index) [45,54]. Animals that showed an extreme behavior (Alternation percentage <20% or >90% or number of arm entries < 10) were discarded. In the present study, 8 animals were discarded accordingly, corresponding to 8.3% attrition. Data were analyzed using a parametric ANOVA, followed by a Dunnett's *post-hoc* test for multiple comparison.

5.4.4. Passive avoidance test

The apparatus is a two-compartments (15 × 20 × 15 cm high) box with one illuminated with white polyvinylchloride walls and the other darkened with black polyvinylchloride walls and a grid floor. A guillotine door separates each compartment. A 60 W lamp positioned 40 cm above the apparatus lights up the white compartment during the experiment. Scrambled footshocks (0.3 mA for 3 s) could be delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, USA). The guillotine door was initially closed during the training session. Each mouse was placed into the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was closed and the footshock delivered for 3 s. The step-through latency, that is, the

latency spent to enter the darkened compartment, and the number of vocalizations were recorded. The retention test was carried out 24 h after training. Each mouse was placed again into the white compartment. After 5 s, the door was raised. The step-through latency was recorded up to 300 s. As an upper cut-off latency was set, data were non-parametric and analyzed using a Kruskal-Wallis ANOVA, followed by a Dunn's *post-hoc* test for multiple comparison [55].

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Abbreviations

AD, Alzheimer's disease; ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; CAS, catalytic anionic site; DBS, dual-binding site; DPZ, donepezil; MTDL, multitarget directed ligands; PAS, peripheral anionic site.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2018.10.064.

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