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Novel Multi-Target Directed Ligands (MTDLs) with acetylcholinesterase (AChE) inhibitory and serotonergic subtype 4 receptor (5-HT₄R) agonist activities as potential agents against Alzheimer's disease: the design of donecopride.

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KEYWORDS

Alzheimer's Disease; Acetylcholinesterase; 5-HT₄ Receptors; Donecopride; MTDL

ABSTRACT

In this work, we describe the synthesis and *in vitro* evaluation of a novel series of multi-target directed ligands (MTDL) displaying both nanomolar dual-binding site (DBS) acetylcholinesterase inhibitory effects and partial 5-HT_{1R} agonist activity, among which donecopride was selected for further *in vivo* evaluations in mice. The latter displayed procognitive and anti-amnesic effects and enhanced sAPP α release, accounting for a potential symptomatic and disease-modifying therapeutic benefit in the treatment of Alzheimer's disease.

Introduction

Alzheimer's disease (AD), the most common form of dementia among the elderly, is a chronic, progressive and degenerative disorder of the brain featuring impairments in cognitive functions, especially memory processes.¹ As of 2013, there were an estimated 44.4 million people with dementia worldwide.² By 2050, it is projected that this figure will have increased to over 115 million. Not only are the numerous reasons for concern, but AD and dementia have an enormous impact on societies.³ The total estimated worldwide costs of dementia were US \$604 billion in 2010. Given that the pathogenesis of AD is complex and related to the abnormality and dysfunction of multisystems, the prevention and treatment of AD has remained elusive to date. Most of the current treatments produce only symptomatic benefits through the catalytic inhibition of acetylcholinesterase (AChE).⁴ So far, investigational new drugs that have reached clinical trials have been targeting amyloid aggregation or neurofibrillary tangle production linked to the abnormality of protein tau phosphorylation. Nevertheless, many of these trials recently failed and some of these failures have been attributed to the extreme selectivity of the tested drugs against a single target, disregarding the fact that neurodegenerative syndromes involve

multiple pathogenic factors.^{5,6} Consequently, efficient treatments should associate several drugs whose actions are oriented towards several targets involved in the pathogenesis. These drugs could be combined in a cocktail (multiple-medication therapy) or in a single formulation (multiple-compounded medication). However, these approaches might be disadvantageous for patients with respect to compliance difficulties, drug drug interactions, problems of bioavailability, and metabolism;

These issues are difficult to solve when the drugs present different pharmacokinetic profiles.^{7,8} Today, a new concept is emerging; also called "Hybride Molecules", "Designed Multiple Ligands", or "Multi-Target Directed Ligands" (MTDLs), it describes those compounds that are effective in treating complex diseases because of their ability to interact with the multiple targets thought to be responsible for the disease pathogenesis.⁹⁻²² Besides reducing the pharmacokinetic and interaction problems linked to multiple-medication therapy, MTDLs generally show a synergistic effect. They could be particularly useful in the treatment of neurodegenerative diseases like AD.²³⁻³⁴ Recently, hybrid agents with a dual mode of action against AD have been described. One of the most widely adopted approaches in the field has been to modify the molecular structure of an AChE inhibitor (AChEI) in order to complement it with additional biological activities that are useful for treating AD, like M2 receptor antagonism,³⁵ direct inhibition of amyloid beta peptide (A β) aggregation,³⁶⁻⁴¹ AChE-dependent amyloid aggregation inhibition (dual-binding site [DBS] inhibitors),⁴²⁻⁴⁶ or providing it with antioxidant properties⁴⁷⁻⁵⁰ linked to MAO-B inhibition,⁵¹⁻⁵⁴ calcium channel blockage,^{55,56} or control oxidative damage properties.⁵⁷ Within this scope, we very recently described the design of **1** (donecopride), a novel drug candidate exhibiting, for the first time, both *in vitro* DBS AChE inhibitory activity and a serotonergic subtype 4 receptor (5-HT₄R) partial agonist effect, promoting the secretion of the

neurotrophic protein sAPP α in an *in vitro* cellular model and finally leading to an *in vivo* procognitive effect in mice.⁵⁸ Donecopride was conceived as a structural compromise between donepezil (DPZ), an AChEI drug, and **2** (RS67333)⁵⁹, a 5-HT₄R agonist (Figure 1).

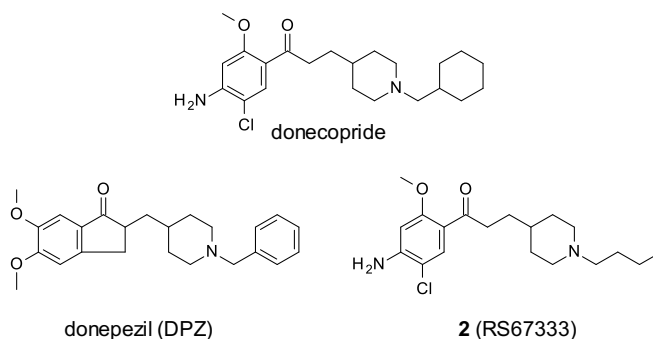


Figure 1. Structure of donecopride, DPZ, and **2** (RS67333).

This MTDL profile seems to confer to donecopride the ability to display both improvements in cholinergic neurotransmission (through the catalytic inhibition of AChE and the 5-HT₄R-dependant release of ACh) and in the inhibition of A β accumulation and aggregation. This second effect would be possible through the nonamyloidogenic 5-HT₄R-dependant cleavage of the precursor of A β and the consequent promotion of the neurotrophic protein, sAPP α , as well as through the interaction of donecopride with the peripheral anionic site (PAS) of AChE, which is able to counteract the chaperone role played by the latter in A β aggregation. Finally, these complementary activities could exert both symptomatic and disease-modifying effects for AD treatment (Figure 2).⁶⁰

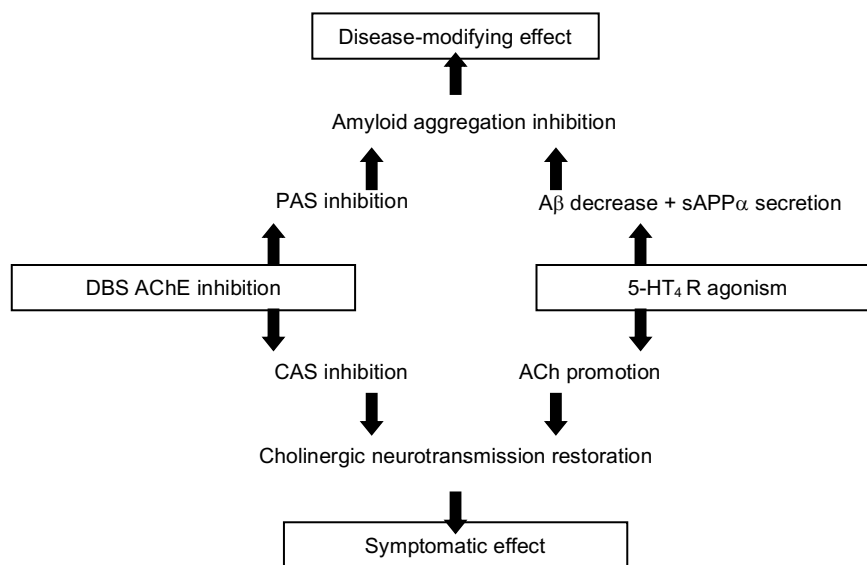


Figure 2. Pleiotropic interest of DBS AChEI/5-HT₄R agonists.

The present article aims to describe the discovery of donecopride in the context of the chemical series from which it was selected in order to establish some structure-activity relationships (SARs) as well as to describe additional *in vivo* experiments useful for precisely determining its pharmacological profile.

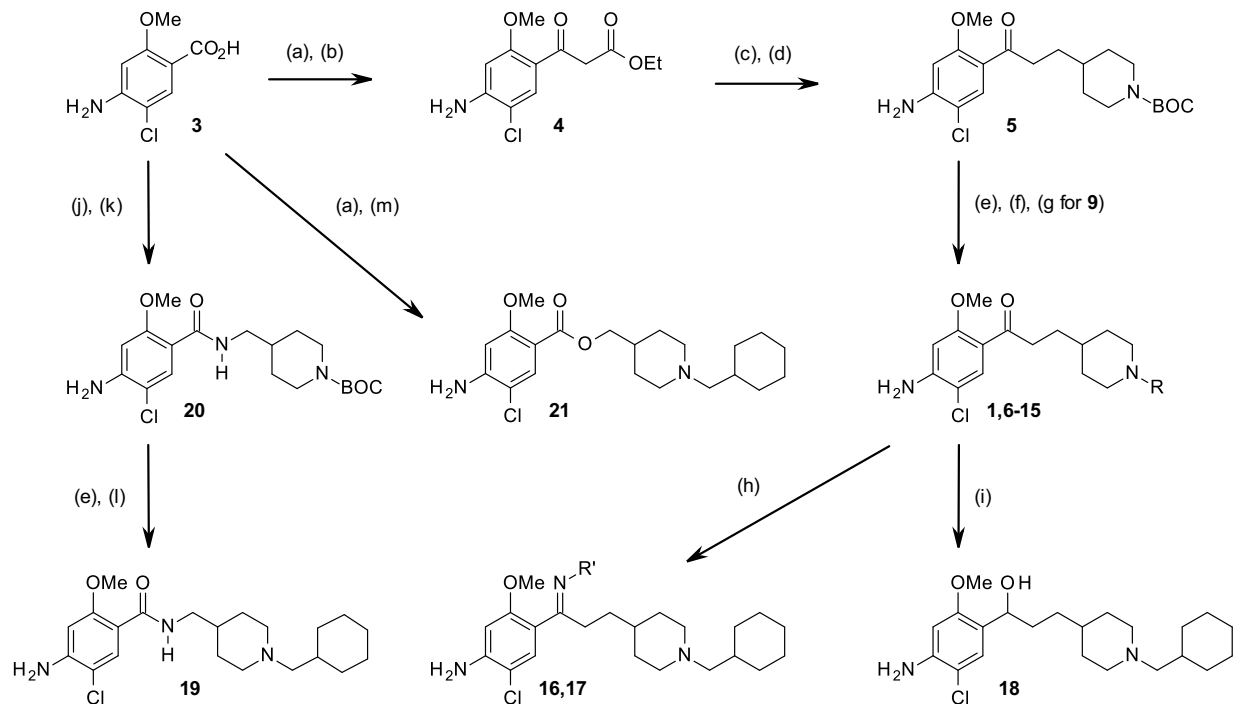
Results

Chemistry

Donecopride was obtained according to a previously reported multistep sequence⁵⁸ starting from 4-amino-5-chloro-2-methoxybenzoic acid (**3**) (Scheme 1). The sequence starts with the synthesis of a β -ketoester (**4**) with a 62% yield, which took place after the carbonyldiimidazole (CDI) activation of the carboxylic acid group of **3**. The methylpiperidine moiety was then installed through a nucleophilic substitution using *N*-Boc 4-(iodomethyl)piperidine carboxylate at room temperature to avoid the risk of a double

substitution, immediately followed by a saponification–decarboxylation sequence with hydroalcoholic potassium hydroxide which gave **5** in an 86% yield. **1** was finally obtained in 44% yield through the TFA-*N*-deprotection of **5**, immediately followed by another nucleophilic substitution with bromomethylcyclohexane. Starting from **5** and using various alkyl- and arylmethylbromides according to these experimental conditions, numerous other *N*-substituted derivatives (**6-15**) were obtained. Finally, the oxime (**16**) and methyloxime (**17**) of donecopride were selectively obtained in excellent yield under their *E* form (according to their X-ray structure) under treatment using hydroxylamine and methoxylamine respectively. The reduction of its carbonyl group, using sodium borohydride, afforded the hydroxyl derivative (**18**) in an 62% yield.

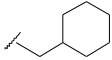
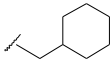
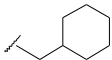
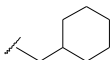
The anisamide **19**, an analog of donecopride, was issued from a peptidic coupling between **3** and *N*-Bocaminomethylpiperidine and affording **20** which was then *N*-deprotected and substituted with a methylcyclohexyl group under treatment with bromomethylcyclohexane. Finally the ester **21** was synthesized in an 31% yield through the esterification of **3** using the commercially available *N*-methylcyclohexyl-4-hydroxymethylpiperidine.



Scheme 1. Synthetic pathways for access to compounds **1**, **6-21**. Conditions and reagents: (a) CDI, THF; (b) $\text{KO}_2\text{CCH}_2\text{CO}_2\text{Et}$, MgCl_2 , THF; (c) *N*-Boc 4-iodomethylpiperidine, K_2CO_3 , DMF; (d) KOH, EtOH/ H_2O ; (e) TFA, DCM; (f) RBr, K_2CO_3 , DMF; (g) HCl/EtOH; (h) $\text{H}_2\text{NOR}'$, HCl, pyridine; (i) NaBH_4 , MeOH; (j) TEA, DMF; (k) HOBT, EDCl.HCl, *N*-Boc 4-aminomethylpiperidine, DMF; (l) CyHex CH_2Br , K_2CO_3 , DMF; (m) *N*-Boc 4-hydroxymethylpiperidine, NaH, THF.

In vitro results

All the synthesized compounds were evaluated as potential inhibitors of human AChE and equine BuChE as well as potential ligands for guinea pig 5-HT₄R (Table 1). In these tests, DPZ was used as an AChEI control and **2** as both a 5-HT₄R ligand and an AChEI control since we recently reported its MTDL pharmacological profile.⁵⁸

17	C=N- OCH ₃	CH ₂		33%	7,420	105 ± 19 n = 3
18	CHOH	CH ₂		1,030 ± 57 n = 2	3,640	122 ± 71 n = 3
19	C=O	NH		45%	ND	2.25 ± 0.28 n = 3
21	C=O	O		2,720 ± 71 n = 2	4,100	0.95 ± 0.3 n = 3

*Results reported from Lecoutey *et al.*⁵⁸

Table 1. (*h*)AChE inhibitory activity and selectivity versus (*eq*)BuChE and (*gp*)5-HT₄R affinity for DPZ, **2** and compounds **1,6–21**.

The results of these *in vitro* evaluations are reported in a double-entry table where we also reported the results concerning **2** and DPZ (Table 2). Among the 16 tested compounds, two of them (compounds **17**, **18**) were found to be almost inactive against the two designed targets. One of them (compound **16**) is highly inhibitor of AChE, but it is almost devoid of affinity for 5-HT₄R, as is the case for DPZ. Conversely, four compounds (**6**, **7**, **19**, **21**) appear to be good ligands for 5-HT₄R, but they do not show any AChE inhibitory effects. Finally, nine compounds (**1,8–10**, **11–15**) could be considered as MTDL since they exhibit both a $K_{i(5-HT_4R)} \leq 20$ nM and an $IC_{50(AChE)} \leq 400$ nM, as was found with **2**. These compounds further showed a good selectivity towards AChE versus BuChE.

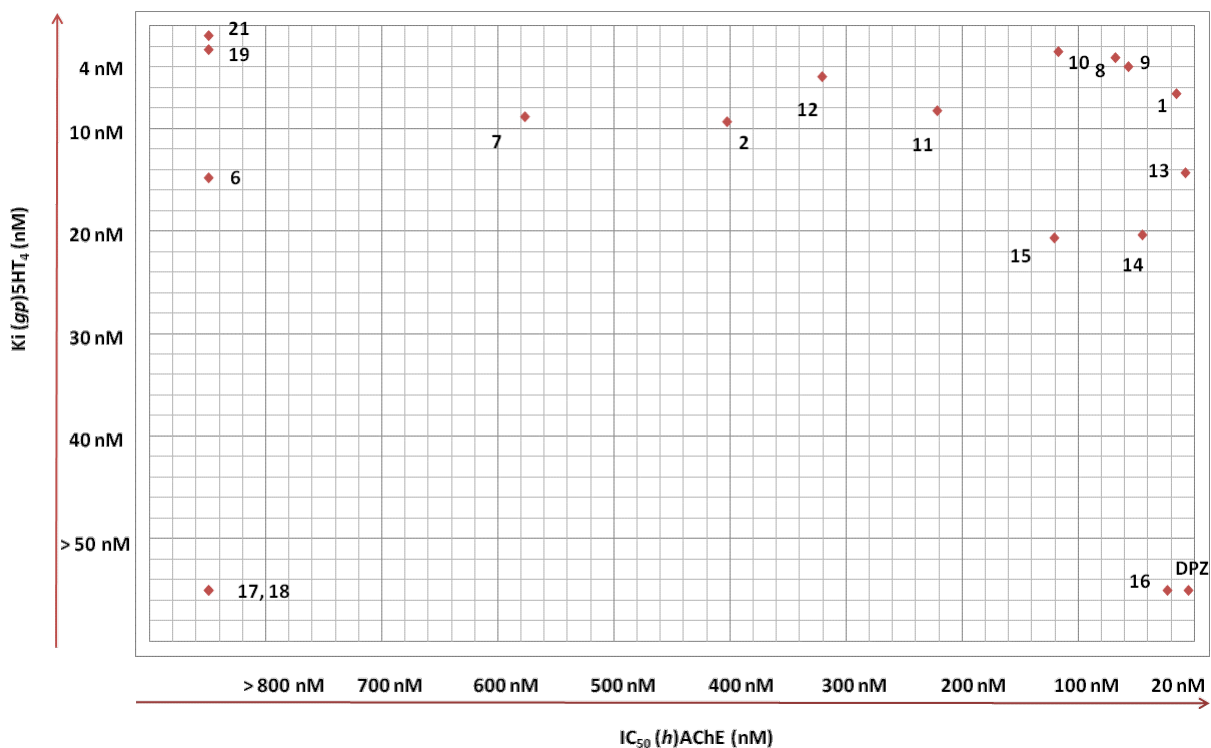


Table 2. 2D representation of (gp)5-HT₄R affinity and (h)AChE inhibitory activity for compounds **1**, **6–21**, DPZ and **2**.

The most potent compounds (**1,8–10,13**) were further evaluated to establish their affinity and their pharmacological profile with regard to their potential agonist effects towards human 5-HT₄R and their capacity to bind to the PAS of electric eel AChE using the propidium iodide displacement test (Table 3).

	(h)5-HT ₄ R			Propidium iodide displacement from (ee)AChE PAS % inhibition at 10 ⁻⁵ M
	Control ligand displacement		% control agonist response	
	% inhibition at 10 ⁻⁶ M	% inhibition at 10 ⁻⁸ M		
DPZ	–	–	–	21.5% ± 1.5% n = 2

1	100%	73%	48.3% ± 0.9% n = 3 ^a	24% ± 2% n = 2 ^a
2	–	–	48.7 ± 5.2 n = 3 ^a	–
8	100%	58%	48%	20%
9	99%	46%	58%	20%
10	100%	69%	20%	30%
13	100%	41%	65%	13%

* Results reported from Lecoutey *et al.*⁵⁸

Table 3. (*h*)5-HT₄eR binding assay (displacement of **22** [³H]GR113808^{6a}), (*h*)5-HT₄eR agonist activity, and propidium iodide competition assay for compounds **1, 8–10, 13**.

By virtue of its *in vitro* activities and selectivity towards AChE and 5-HT₄R, as well as its good druggability parameters (water solubility, blood-brain barrier [BBB] cross-membrane penetration, P-gp inhibition, mutagenicity, cytotoxicity, hERG affinity and so on),⁵⁸ compound **1** was selected for *in silico* and *in vivo* studies aiming to evaluate its potential in AD treatment. We named it donecopride.

***In silico* results**

Ahead of the *in silico* study of donecopride, its three-dimensional structure was identified via the X-ray diffraction technique. The analysis carried out on the donecopride crystal showed that two molecules are present in the asymmetric unit, which differs principally in the position of the cyclohexane ring. As the crystal space group is a centrosymmetric one, another conformer, related to the asymmetric unit by a symmetric center, is present in the crystal for both molecules of the asymmetric unit (Figure 3). Interestingly, the carbonyl group and the methoxy substituent of the benzene ring point in opposite directions in all solved structures. The four conformers

observed in the crystal were used for the docking studies, and the opposite mutual orientation of the carbonyl and methoxy groups was used as a criterion for the selection of docking poses.

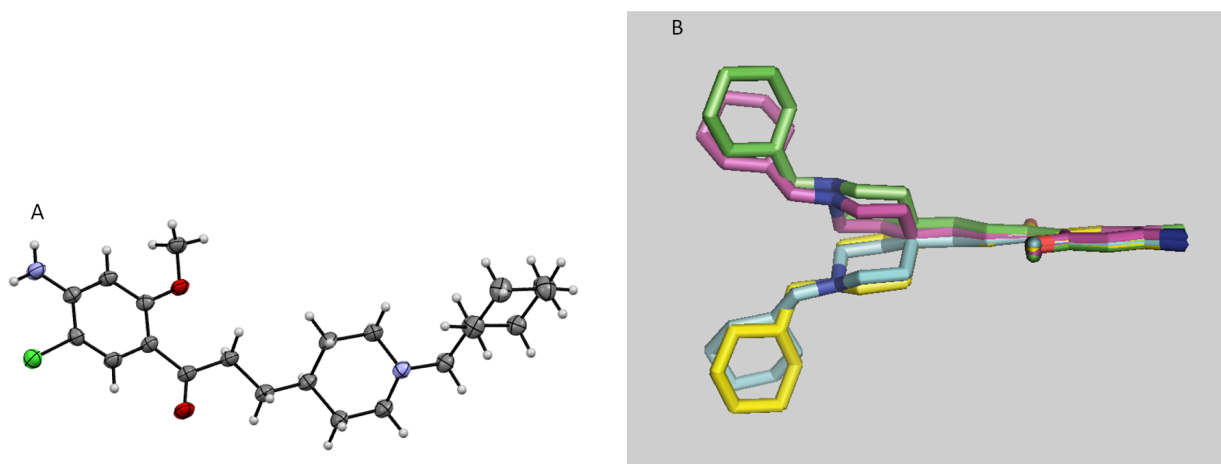


Figure 3. ORTEP diagram of donecopride (A) and superposition of its four conformations in the crystal (B).

Docking studies of donecopride were then performed in a human AChE structure (PDB ED: 4EY7)⁶² as well as in a human 5-HT₄R model, as previously reported by us.⁶³

First, the four conformers of donecopride from the crystal structure (protonated on the piperidine ring) were docked separately into the (*h*)5-HT₄R homology model that was built using the β_2 -adrenergic receptor crystal structure as a template (PDB ED: 2RH1).^{65,64} In the selected pose (Figure 4A mean ChemPLP fit = 78.61), the basic piperidine nitrogen interacts with Asp₁₀₀ (consistent with the constraint used during docking) and an additional polar interaction could be engaged through this basic nitrogen and the Tyr₃₀₂ hydroxyl group. The benzene ring is oriented towards the transmembrane helix 5 (TM5) (in yellow in Figure 4A), and the amino substituent on the benzene ring forms a hydrogen bond with the Ser₁₉₇ hydroxyl group. In this position the benzene ring is surrounded by several aromatic residues, oriented perpendicularly to it.

During the docking of donecopride conformers into (*h*)AChE, a water molecule interacting with the protonated piperidine ring of DPZ was conserved (residue number 931). In the selected scoring pose (Figure 4B: mean ChemPLP Score = 109.59), the donecopride orientation was closed to DPZ and reproduced its interactions: (1) the charged nitrogen of the piperidine ring was oriented in a position that was suitable for interaction with the water molecule in the proximity of Tyr₃₃₇ and Tyr₃₄₁; (2) the donecopride carbonyl formed a hydrogen bond with NH of the Phe₂₉₅ backbone; (3) the benzene ring was positioned in a parallel manner to the Trp₂₈₆ indole ring of the PAS at a 3.5 Å distance to favor a π -stacking interaction (which is in accordance with the result of the propidium displacement test results); and (4) the cyclohexane ring occupied the DPZ benzyl ring space (in the neighboring area of Trp₈₆).

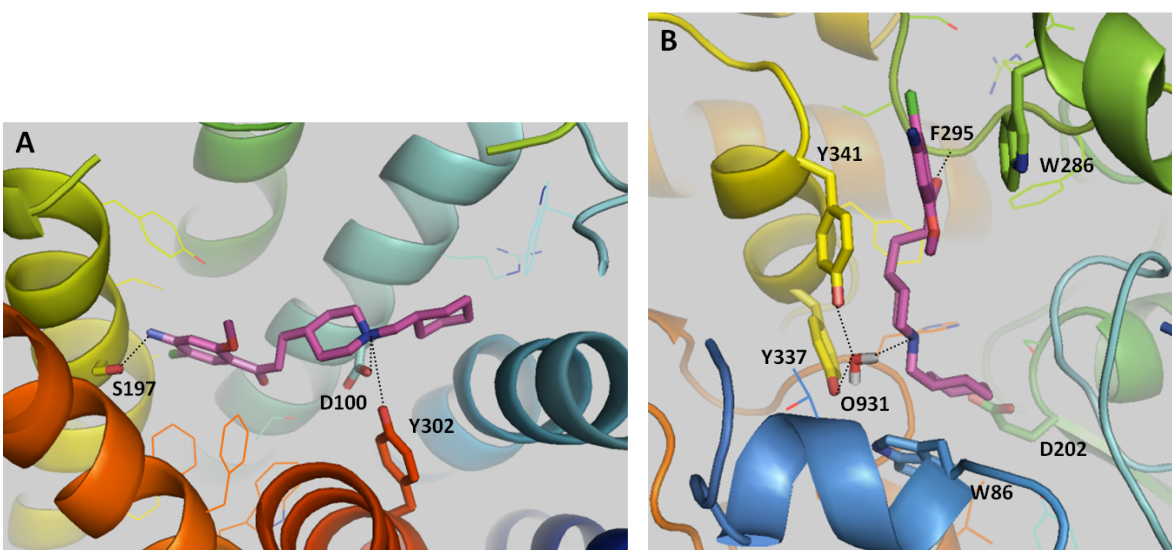


Figure 4. Donecopride positioned in the (*h*)5-HT₄R (A) and (*h*)AChE (B) binding sites from docking studies. The compound and the side chains of the binding site residues are represented by the stick-like shapes, and the protein is represented in ribbon. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

***In vivo* results**

The data concerning the preliminary toxicological and pharmacological screening of donecopride (Table 4) showed no symptoms at subtoxic doses (around sevenfold less than the approximative LD₅₀).

Compound	Doses (mg/kg)	LD ₅₀ (mg/kg)	Symptoms (subtoxic doses)	Symptoms (toxic doses)
Donecopride	1–10	75	No symptoms	
	100		Hypoactivity Passivity	Convulsions
Amphetamine	2		Hyperactivity Exophthalmy Irritability	
Chlorpromazine	10		Hypoactivity Ataxia Sleep	

Table 4. Pharmacological and toxicological properties of donecopride.

Thus, subsequent behavioral studies were realized at the maximum dose of 10 mg/kg. At the tested doses, donecopride demonstrated no effect on spontaneous locomotor activity (Figure 5).

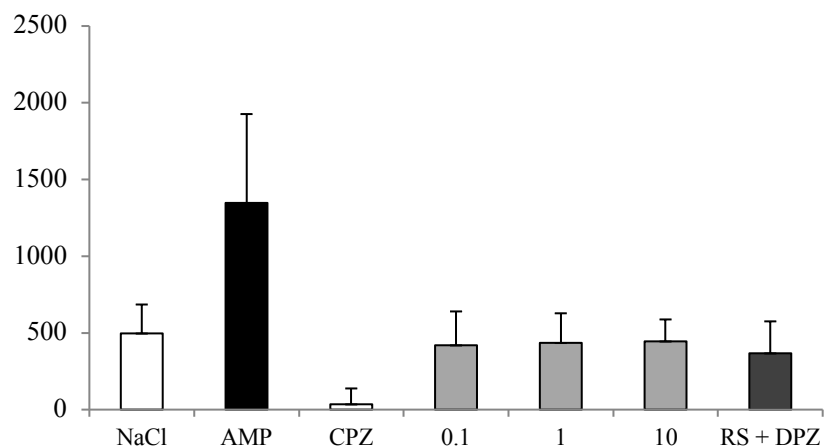


Figure 5. Effect of donecopride on spontaneous locomotor activity. Data are expressed as the mean \pm standard deviation (n=9–10). Drugs were administered intraperitoneally (IP) 30 minutes before placing mice in the actimeter, except for DPZ which was administered subcutaneously (SC) 20 minutes before the test. Donecopride: 0.1–1–10 mg/kg; AMP: amphetamine 2 mg/kg; CPZ: chlorpromazine 10 mg/kg; RS+DPZ: **2** 0.1 mg/kg + DPZ 0.3mg/kg. (*p<0.05 NaCl, SNK test).

We already showed that donecopride exhibited a procognitive effect with an improvement in memory performances, which was observed at 0.3 mg/kg and 1 mg/kg on the object recognition test.⁵⁸ Moreover, in regards to scopolamine-induced spontaneous alternation deficit, an anti-amnesic effect was observed at doses starting from 0.3 mg/kg (Figure 6). Interestingly, a comparable effect has been obtained with the association of **2** to DPZ and with DPZ alone (1 mg/kg).

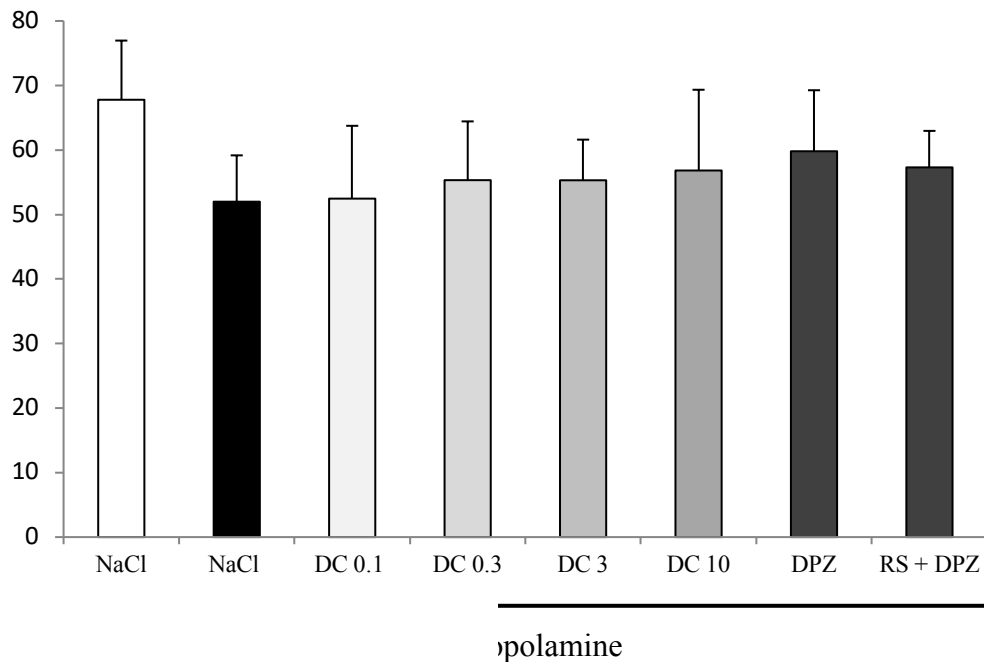


Figure 6. Effect of donecopride on scopolamine-induced impairment during the spontaneous alternation test. Data are expressed as the mean \pm standard deviation (n= 10–12). Drugs were administered IP 30 minutes before the test, except for DPZ, and scopolamine was administered SC 30 minutes and 20 minutes, respectively. DC (donecopride): 0.1, 0.3, 3, and 10 mg/kg; DPZ: 1 mg/kg; RS+DPZ: 2 0.1 mg/kg + DPZ 0.3mg/kg; Scopolamine: 0.5 mg/kg. (* $p < 0.05$ versus 50%; univariate t-test).

Finally, a decreased time of immobility was observed during the forced swimming test for the 0.3 mg/kg dose of donecopride, suggesting a slight antidepressant-like effect (Figure 7).

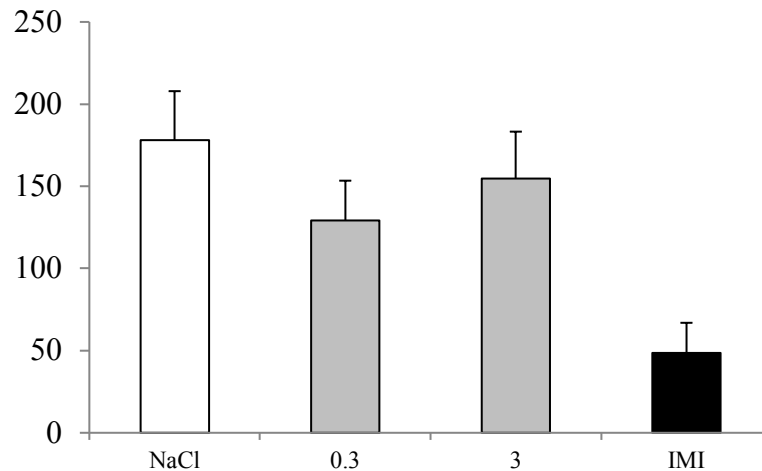


Figure 7. Effect of donecopride on time immobility during the forced swimming test. Data are expressed as the mean \pm standard deviation (n=10–12). Drugs were administered IP 30 minutes before the test. Donecopride: 0.3, 3 mg/kg; imipramine (IMI) 16 mg/kg (* $p < 0.05$ NaCl, Student–Newman–Keuls [SNK] test).

On the other hand, we have previously demonstrated that donecopride is able to increase sAPP α release *in vitro* in COS-7 cells transiently expressing 5-HT $_4$ R ($EC_{50} = 11.3$ nM, $E_{max} = 22.5$ percent of the maximum 5-HT response over the basal response).⁸ In the present work, we administered one dose of donecopride (1 mg/kg) IP to C57BL/6 mice and collected cerebrospinal fluid (CSF) 90 minutes after the injection. Quantification of sAPP α in the samples showed a slight increase in the neuroprotective APP fragment which is nonsignificant in these conditions (Figure 8). Further experiments taking into account the biodistribution of donecopride are necessary to adjust the time point of CSF collection and to unequivocally conclude that donecopride promotes *in vivo* sAPP α secretion.

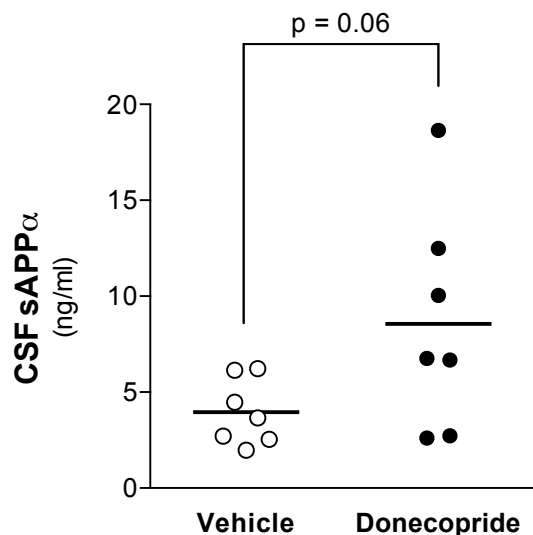


Figure 8. Acute *in vivo* treatment with donecopride shows a tendency to increase sAPP α release in the CSF of 2-month-old C57BL/6 mice. sAPP α was quantified via enzyme-linked immunosorbent assay (ELISA). Each dot represents an individual mouse; the horizontal lines show the means of each group. The p-value is indicated (unpaired Student's *t* test).

Discussion

If we first try to establish some SAR from these results, we can point out that replacing the *n*-butyl group of **2** ($K_{i[5-HT_4R]} = 9.33$ nM and $IC_{50[AChE]} = 403$ nM) with a methyl-cyclopropyl group results in a dramatic decrease in AChE inhibition (see compound **6** $IC_{50[AChE]} = 937$ nM), while its 5-HT $_4$ R affinity is almost maintained ($K_{i[5-HT_4R]} = 14.30$ nM). Substituting the piperidine moiety by a methylcyclobutyl group (compound **7**) allows us to recover the 5-HT $_4$ R affinity of **2**, but it does not totally restore its AChE inhibitory effect ($IC_{50[AChE]} = 577$ nM). A larger methylcycloalkyl group, however, highly increases the activities of compounds **8**, **1** and **9** towards the two targets ($IC_{50[AChE]} < 100$ nM; $K_{i[5-HT_4R]} < 10$ nM), thus rendering them as excellent MTDLs according to our criteria. The apparent degrees of freedom allowed for the

nature of these alkyl groups are however quickly exceeded since replacing them by either an ethylcyclohexyl group (compound **11**) or a methyl *ortho*-methylcyclohexyl group (compound **12**) results in an important decrease in the AChE inhibitory activity, while the 5-HT₄R affinity is maintained.

On the other hand, substituting the *n*-butyl group of **2** by a benzyl group, similar to those of DPZ, results in a dramatic increase in the AChE inhibition exerted by compound **13** ($IC_{50[AChE]} = 8.5$ nM), while its 5-HT₄R affinity is slightly decreased ($K_{i[5-HT_4R]} = 14.30$ nM). This compound also appears to be as good an MTDL against the considered targets. Replacing its phenyl group by a *meta* (compound **14**) or *para* (compound **15**) pyridine group, however leads to a decrease in 5-HT₄R affinity ($K_{i[5-HT_4R]} > 20$ nM) which appears to be particularly linked to the basic nature of the intracyclic nitrogen, since the methylpiperidine substituent in compound **9** dramatically restores it ($K_{i[5-HT_4R]} = 2.50$ nM).

We can also point out the apparent importance of the carbonyl group in these structures for 5-HT₄R affinity since compounds **16–18** do not bind to this target. However, the oxime **16** remains an excellent AChEI, almost as potent as DPZ. Conversely, the benzamide (**19**) and the benzoate (**21**) exhibit excellent 5-HT₄R affinities in a low nanomolar range while they are both almost devoid of the AChE inhibitory effect. The structure of **19** has been docked into the active site of (*h*)AChE. While, its conformation appeared to be similar to donecopride, the rigidity of **19** appears to be strengthened by the mesomeric effect due to its carboxamide group. Consequently, **19**, contrarily to donecopride, seems not able to bind in the same time through its carbonyl group and its piperidine nitrogen atom with Phe₂₉₅ and Water₉₃₁ of (*h*)AChE respectively (Figure 9). This might also explain the inactivity of **21** against AChE.

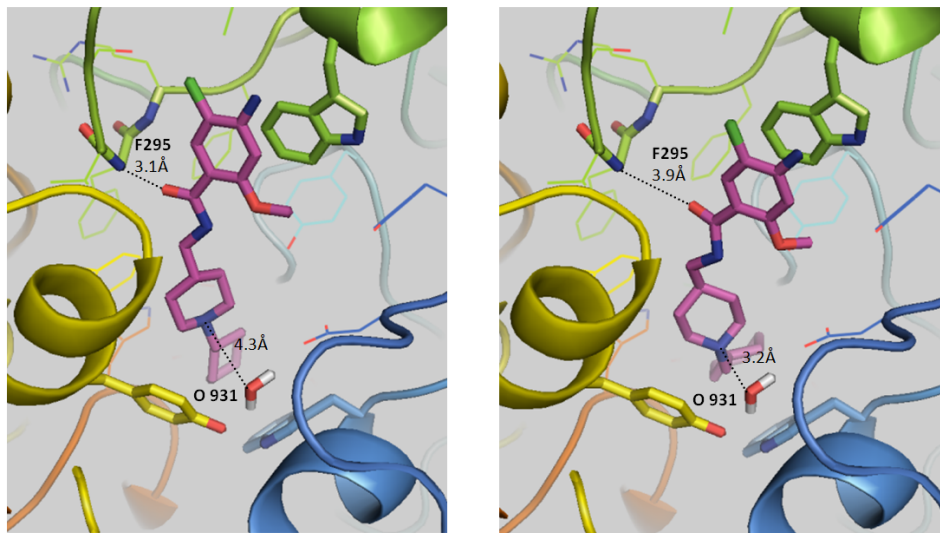


Figure 9. Two representative docking poses of compound **19** into (*h*)AChE. The compounds and the side chains of the binding site residues are in stick and the protein in ribbon representation. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

In summary, in the structural system derived from **2** as an MTDL hit, 5-HT₄R affinity seems to be linked to the presence, on the nitrogen atom of the piperidine ring, of a not-too-hindering methylcycloalkyl group, eventually bearing an intracyclic nitrogen. On the other hand, the catalytic AChE inhibition appears to require such a methylcycloalkyl group, but can also be displayed by a benzyl, a methylpyridine or a methylpiperidine substituent. It is on the basis of these results and considering our objective, that we selected compounds **1, 8–10**, and **13** for additional evaluations.

All the tested compounds displayed also high human 5-HT₄R affinities at 10⁻⁸M (Table 2) confirming the relevance of the use (*gp*)5-HT₄R to select potential (*h*)5-HT₄R ligands.⁶⁵

Concerning their pharmacological profile, while compounds **1, 8, 9**, and **13** were found to be 5-HT₄R partial agonists (in a similar manner as **2**), compound **10** acted as a very partial

agonist. This profile is in accordance with our objective, because prolonged exposure of G protein-coupled receptors to an agonist can result in receptor desensitization, which is often dependent on the degree of intrinsic activity.⁶⁶ An opposite relationship seems to have been established between these results and the $K_{i(5-HT_4R)}$ values of the tested compounds, since it appears that the higher their affinity, the more partial their agonist effect. This result appears to be concordant with that reported in the literature.⁶⁷

At last, concerning the capacity of the tested compound to displace propidium iodide from the PAS of AChE, compounds **1**, **8**, and **9** acted in a similar manner as DPZ (20%–24%), while **13** displaced propidium less (13%) and **10** displaced it slightly more (30%). The results displayed by **13** and **10** could be correlated in an opposite relation with their $IC_{50(AChE)}$ values since the most potent AChEI in the series (**13**) weakly interacted with the PAS while the less potent inhibitor (**10**) interacted more strongly. The weak AChE catalytic inhibitory effect displayed by the latter compound might appear to be inconsistent with its docking in the enzyme-active site. Indeed, the cationic nitrogen atom of the middle piperidine ring of **10** (protonated at physiologic pH, as well as the nitrogen atom of the terminal piperidine) appears able to bind to the same water molecule as donecopride (mean ChemPLP Score Fit = 104.02). At the same time, the second nitrogen atom on the terminal piperidine ring is located near Glu₂₀₂, which unusually appeared protonated using the ProPKA software (Figure 10A).^{68,69} However, in these conditions, a second pose was also generated by the docking study in which it was the terminal piperidine that interacted with the water molecule (mean ChemPLP Score Fit = 87.61; see Figure 10B). According to this hypothesis, the phenyl ring of compound **10** covers more Trp₂₈₆ whereas its interaction with Trp₈₆ would be weaker. Despite the lower score, the second pose seems to be in

better agreement with experimental results, as it features the worst binding affinity and the greatest propidium displacement for compound **10** with respect to donecopride.

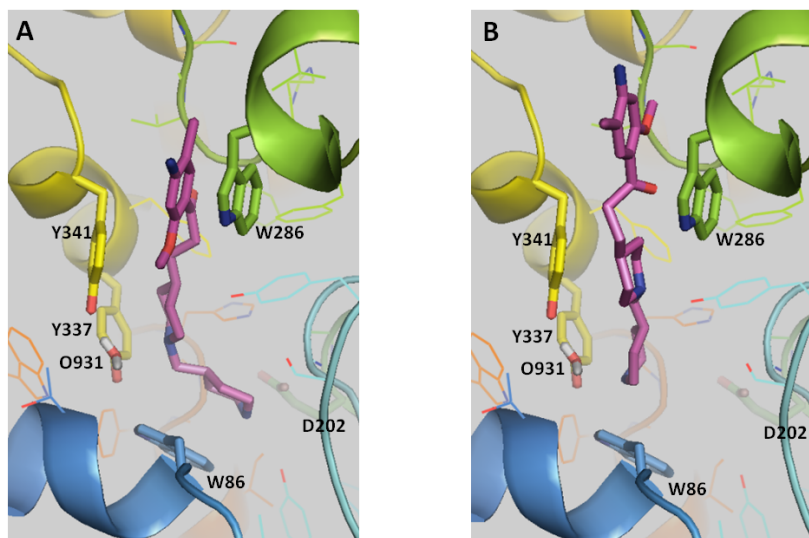


Figure 10. Two representative docking poses of compound **10** into (*h*)AChE. The compounds and the side chains of the binding site residues are represented by the stick-like shapes and the protein is represented by ribbon. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

Concerning *in vivo* investigations, we have shown that donecopride has an anti-amnesiant effect in a scopolamine-induced deficit model of working memory. Together with our previous results showing the positive effect of donecopride in recognition memory performance in mice⁸⁸ (testing recognition memory), this result confirms the high therapeutic potential of this molecule in the treatment of the cognitive disorders associated with AD. Of note, with respect to the dose (0.3 mg/kg) that revealed a beneficial effect on the two memory and behavioral tests (antiamnesiant and promnesiant effects), an interesting additional antidepressant-like effect was demonstrated. Furthermore, these behavioral effects were associated with an increased release of

the neuroprotective sAPP α fragment. Such observations are in agreement with data from the literature, showing, for instance, better memory performance in transgenic mice that overexpress MMP9, which consequently display an increased secretion of sAPP α .⁷⁰ Herein, we were only able to highlight a tendency of an increased release of the nonamyloidogenic APP fragment in the CSF 90 minutes following a systemic injection. However, that sAPP α increased upon 5-HT₄R activation is transient,⁷¹ and it is consequently highly difficult to catch the peak of sAPP α in CSF. Kinetics studies using different collection times or intracerebral microdialysis would be necessary to precisely determine the acute action of donecopride in living animals. Moreover, it should be interesting to monitor ACh levels, sAPP α , and A β in the CSF or in the brain tissue at the same time. Chronic administration of 5-HT₄R agonists reduces amyloid pathology and prevents cognitive deficits in AD mouse models.^{71,72} Further experiments exploring chronic treatments of AD transgenic mice with donecopride, in comparison with DPZ and pure 5-HT₄R agonists, would produce valuable insights regarding the potential of donecopride to exert a disease-modifying effect.

Conclusion

In conclusion, we have developed a novel series of MTDL that displayed both DBS AChE inhibitory effects and partial 5-HT₄R agonist activity *in vitro* in nanomolar ranges. Donecopride, which appears to be the lead of this family, further exhibited *in vivo* procognitive and anti-amnesic effects in NMRI mice, and it also appeared able to promote the release of sAPP α in C57BL/6 mice *in vivo*. These results – which appear to gather in a single compound both the activities of DPZ and **2** – seem likely to confer a symptomatic and disease-modifying effect to donecopride, thus designing it as a potentially promising drug candidate in AD.

Experimental section

1- Chemistry.

1-1- General material and methods:

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a Kofler melting point apparatus. ^1H and ^{13}C NMR spectra were recorded on a BRUKER AVANCE III 400MHz with chemical shifts expressed in parts per million (in chloroform-*d*, methanol-*d*, or DMSO-*d*,) downfield from TMS as an internal standard and coupling in Hertz. IR spectra were recorded on a Perkin-Elmer BX FT-IR apparatus using KBr pellets. High resolution mass spectra (HRMS) were obtained by impact electronic on a Jeol JMS GCMate spectrometer (compound **1**) or by electrospray on a Bruker maXis (compounds **4**, **7**, **8**, **13 – 19**) or on a Q-ToF micro Waters (compounds **6**, **9 – 12**, **21**). The purities of all tested compounds were analyzed by LC-MS, with the purity all being higher than 95%. Analyses were performed using a Waters Alliance 2695 as separating module (column XBridge C18 2.5 μM /4.6x50 mM) using the following gradients: A (95%)/B (5%) to A (5%)/B (95%) in 4.00 min. 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%). MS were obtained on a SQ detector by positive ESI.

1-2- General procedure for the deprotection and *N*-alkylation of *tert*-butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxo-propyl]piperidine-1-carboxylate **5** (**1**; **6-9**; **11-15**; **17**).

tert-Butyl 4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxo-propyl]piperidine-1-carboxylate **5** was dissolved in DCM/TFA (1/1). The mixture was then stirred 15 min at rt. After concentration

under reduced pressure, the resulting intermediate was dissolved in DMF or 1,4-dioxane (for compounds **13-15**). K_2CO_3 (10 eq) and halogenoalkyle (1.3 eq) were added and the mixture was warmed to 110 °C for 4 h. After cooling, DCM was added and the mixture was washed 4 times with brine. The organic layer was then dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude residue was purified on deactivated silica gel (gradient of elution: DCM to DCM/MeOH (98/2)).

1-2-1- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one (1).^{s8} Pale yellow powder (44%): mp 153 - 155 °C; 1H NMR (400 MHz, $CDCl_3$): δ 7.79 (s, 1H), 6.48 (s, 1H), 4.44 (br s, 2H), 3.85 (s, 3H), 2.89 (m, 2H), 2.84 (m, 2H), 2.08 (d, $J = 6.8$ Hz, 2H), 1.68 (m, 11H), 1.47 (m, 1H), 1.20 (m, 6H), 0.85 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.3, 159.5, 147.7, 132.2, 119.0, 111.2, 97.6, 66.3, 55.6, 54.5 (2C), 41.0, 35.8, 35.3, 32.3 (2C), 32.2 (2C), 31.4, 26.8, 26.2 (2C); LC-MS: $[M+H]^+ = 393.58 - 395.59$; IR (KBr): ν cm^{-1} 3485, 3330, 2921, 2850, 1641, 1623, 1575, 1452, 1421, 1214, 1177; HRMS (m/z) calcd for $C_{22}H_{33}ClN_2O_2$ $[M]^+ 392.22303$, found 392.22399.

1-2-2- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclopropylmethyl)-4-piperidyl]propan-1-one (6). Pale yellow powder (38%): mp 161 - 163 °C; 1H NMR (400 MHz, $CDCl_3$): δ 7.75 (s, 1H), 6.23 (s, 1H), 4.46 (s, 2H), 3.81 (s, 3H), 3.03 (m, 2H), 2.87 (m, 2H), 2.19 (d, $J = 6.8$ Hz, 2H), 1.88 (m, 2H), 1.67 (m, 2H), 1.56 (m, 2H), 1.32 (m, 1H), 1.27 (m, 2H), 0.83 (m, 1H), 0.47 (m, 2H), 0.06 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.2, 159.5, 147.7, 132.2, 118.9, 111.2, 97.5, 64.2, 55.6, 54.0 (2C), 40.9, 35.6, 32.2 (2C), 31.4, 8.5, 4.0 (2C); LC-MS: $[M+H]^+ =$

351.49 – 353.45; IR (KBr): ν cm^{-1} 3466, 3350, 2924, 2849 1644, 1621, 1586, 1465, 1420, 1215, 1178; HRMS (m/z) calcd for $\text{C}_{19}\text{H}_{28}\text{ClN}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ 351.1839, found 351.1841.

1-2-3- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclobutylmethyl)-4-piperidyl]propan-1-one (7). Yellow powder (45%): mp 142 - 144 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.77 (s, 1H), 6.25 (s, 1H), 4.41 (br s, 2H), 3.84 (s, 3H), 2.88 (t, $J = 8.0$ Hz, 2H), 2.81 (m, 2H), 2.54 (m, 1H), 2.37 (d, $J = 6.6$ Hz, 2H), 2.06 (m, 2H), 1.78 (m, 8H), 1.57 (m, 2H), 1.25 (m, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 199.1, 159.4, 147.6, 132.2, 118.9, 111.2, 97.5, 65.9, 55.6, 54.0 (2C), 40.9, 35.4, 34.2, 32.2 (2C), 31.3, 28.3 (2C), 18.8; LC-MS: $[\text{M}+\text{H}]^+ = 365.53 - 367.54$; IR (KBr): ν cm^{-1} 3485, 3327, 2923, 2852, 1642, 1624, 1575, 1453, 1421, 1214, 1175; HRMS (m/z) calcd for $\text{C}_{20}\text{H}_{30}\text{ClN}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ 365.199032, found 365.198665.

1-2-4- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclopentylmethyl)-4-piperidyl]propan-1-one (8). Yellow powder (35%): mp 141 - 143 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.79 (s, 1H), 6.26 (s, 1H), 4.44 (br s, 2H), 3.85 (s, 3H), 2.90 (m, 4H), 2.24 (d, $J = 7.1$ Hz, 2H), 2.05 (m, 1H), 1.86 (m, 2H), 1.76 (m, 2H), 1.60 (m, 6H), 1.50 (m, 2H), 1.21 (m, 5H); ^{13}C NMR (100 MHz, CDCl_3): δ 199.2, 159.4, 147.5, 132.2, 119.0, 111.2, 97.5, 65.2, 55.6, 54.3 (2C), 40.9, 37.5, 35.7, 32.2 (2C), 31.8 (2C), 31.3, 25.2 (2C); LC-MS: $[\text{M}+\text{H}]^+ = 379.57 - 381.56$; IR (KBr): ν cm^{-1} 3486, 3332, 2924, 2860, 1644, 1623, 1574, 1453, 1422, 1214, 1175; HRMS (m/z) calcd for $\text{C}_{21}\text{H}_{32}\text{ClN}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ 379.214682, found 379.214484.

1-2-5- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cycloheptylmethyl)-4-piperidyl]propan-1-one (9). Yellow powder (42%): mp 147 - 149 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.78 (s, 1H), 6.27 (s, 1H), 4.49 (br s, 2H), 3.85 (s, 3H), 2.87 (m, 2H), 2.81 (m, 2H), 2.02 (d, $J = 7.3$ Hz, 2H),

1.57 (m, 17H), 1.22 (m, 3H), 1.08 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 198.2, 158.5, 146.7, 131.2, 117.8, 110.2, 96.5, 65.3, 54.6, 53.3 (2C), 39.9, 35.4, 34.6, 31.9 (2C), 31.0 (2C), 30.3, 27.5 (2C), 25.5 (2C); LC-MS: $[\text{M}+\text{H}]^+ = 407.53 - 409.52$; IR (KBr): ν cm^{-1} 3481, 3247, 2920, 2850, 1638, 1622, 1582, 1454, 1419, 1214, 1176; HRMS (m/z) calcd for $\text{C}_{23}\text{H}_{36}\text{ClN}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ 407.2465, found 407.2461.

1-2-6- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(4-piperidylmethyl)-4-piperidyl]propan-1-one dihydrochloride (10). Following the previous general procedure the intermediate *tert*-butyl 4-[[4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxo-propyl]-1-piperidyl]methyl]piperidine-1-carboxyl ate is obtained. Pale yellow powder (24%): mp 78 - 80 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.76 (s, 1H), 6.23 (s, 1H), 4.44 (br s, 2H), 4.03 (m, 2H), 3.82 (s, 3H), 2.87 (t, J = 7.8 Hz, 2H), 2.80 (m, 2H), 2.65 (m, 2H), 2.10 (d, J = 6.8 Hz, 2H), 1.83 (m, 2H), 1.58 (m, 7H), 1.41 (s, 9H), 1.23 (m, 3H), 1.03 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 199.1, 159.5, 155.0, 147.6, 132.3, 119.0, 111.3, 97.6, 79.2, 65.1, 55.7, 54.6 (2C), 43.8 (2C), 40.9, 35.7, 33.8, 32.3 (2C), 31.3, 30.9 (2C), 28.4 (3C); LC-MS: $[\text{M}+\text{H}]^+ = 494.53 - 496.55$, $[\text{M}+\text{H}-\text{BOC}]^+ = 394.49 - 396.48$; IR (KBr): ν cm^{-1} 3479, 3347, 2922, 2850, 1688, 1674, 1622, 1587, 1466, 1452, 1247, 1215, 1175; HRMS (m/z) calcd for $\text{C}_{26}\text{H}_{40}\text{ClN}_3\text{O}_4$ $[\text{M}+\text{H}]^+$ 494.2786, found 494.2766. To a solution of *tert*-butyl 4-[[4-[3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxo-propyl]-1-piperidyl]methyl]piperidine-1-carboxylate (40 mg, 0.09 mmol) in EtOH (2 mL) was added HCl 37% (300 μL). The mixture was stirred at rt for 3 h and then concentrated under reduced pressure. The residue was then stirred into Et₂O and filtered to afford 30 mg of compound **9**. Yellow powder (80%): mp > 260 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 10.23 (br s, 1H), 9.09 (br s, 1H), 8.96 (br s, 1H), 7.53 (s, 1H), 6.46 (s, 1H), 3.99 (s, 3H), 3.45 (m, 2H), 3.22 (m, 2H), 2.91 (m, 2H), 2.81

(m, 6H), 2.11 (m, 1H), 1.96 (m, 2H), 1.76 (m, 2H), 1.63 (m, 2H), 1.46 (m, 3H), 1.38 (m, 2H); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$): δ 196.6, 159.6, 150.1, 131.1, 115.8, 109.3, 97.1, 60.4, 55.6, 52.3 (2C), 42.3 (2C), 39.7, 32.7, 30.2, 28.4 (2C), 28.3, 26.6 (2C); LC-MS: $[\text{M}+\text{H}]^+$ = 394.38 – 396.42; IR (KBr): ν cm^{-1} 3390, 3296, 3194, 2938, 2732, 1660, 1625, 1593, 1449, 1417, 1212, 1180; HRMS (m/z) calcd for $\text{C}_{21}\text{H}_{33}\text{ClN}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 394.2261, found 394.2269.

1-2-7- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclohexylethyl)-4-piperidyl]propan-1-one (11). Yellow powder (40%): mp 169 - 171 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.76 (s, 1H), 6.23 (s, 1H), 4.42 (br s, 2H), 3.82 (s, 3H), 2.89 (m, 4H), 2.28 (m, 2H), 1.84 (m, 2H), 1.61 (m, 9H), 1.36 (m, 2H), 1.20 (m, 7H), 0.90 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 199.1, 159.5, 147.6, 132.2, 118.9, 111.2, 97.5, 57.0, 55.6, 54.0 (2C), 40.8, 36.5, 35.5, 34.4, 33.4 (2C), 32.0 (2C), 31.2, 26.6, 26.3 (2C); LC-MS: $[\text{M}+\text{H}]^+$ = 407.41 – 409.52; IR (KBr): ν cm^{-1} 3486, 3324, 2918, 2847, 1637, 1621, 1571, 1453, 1304, 1212, 1173; HRMS (m/z) calcd for $\text{C}_{23}\text{H}_{36}\text{ClN}_2\text{O}_2$ $[\text{M}+\text{H}]^+$ 407.2465, found 407.2468.

1-2-8- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-[(2-methylcyclohexyl)methyl]-4-piperidyl]propan-1-one (12). Powder (49%): ^1H NMR (400 MHz, CDCl_3): δ 7.75 (s, 1H, 1H*), 6.23 (s, 1H, 1H*), 4.43 (br s, 2H, 2H*), 3.81 (s, 3H, 3H*), 2.87 (m, 2H, 3H*), 2.81 (m, 2H), 2.74 (m, 1H*), 2.32 (dd, $J = 11.8$ Hz, $J = 3.2$ Hz, 1H*), 2.06 (m, 2H), 2.00 (m, 1H*), 1.92 (m, 1H*), 1.81 (m, 3H), 1.60 (m, 7H, 11H*), 1.38 (m, 6H, 3H*), 1.21 (m, 3H, 4H*), 0.87 (d, $J = 5.8$ Hz, 3H*), 0.80 (d, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 199.3 (C, C*), 159.5 (C, C*), 147.6 (C, C*), 132.2 (C, C*), 119.0 (C, C*), 111.3 (C, C*), 97.6 (C, C*), 63.7 (C*), 62.0 (C), 56.1 (C*), 55.7 (C, C*), 54.8 (C), 54.5 (C), 53.2 (C*), 41.0 (C), 41.0 (C*), 37.2 (C, C*), 36.6

(C*), 35.9 (C), 35.9 (C*), 33.0 (C), 32.4 (2C*), 32.4 (2C), 32.3 (C*), 31.5 (C*), 31.4 (C), 31.0 (C), 26.4 (C*), 26.4 (C), 26.3 (C*), 25.6 (C), 21.9 (C, C*), 20.5 (C*), 13.6 (C); LC-MS: [M+H]⁺ = 407.54 – 409.63; HRMS (m/z) calcd for C₂₃H₂₆ClN₃O₂ [M+H]⁺ 407.2465, found 407.2458. The ratio of these 2 diastereoisomers was obtained in proportions 3/1* according to the ¹H NMR spectra.

1-2-9- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-(1-benzyl)-4-piperidylpropan-1-one (13).

¹H NMR (400 MHz, CDCl₃): pale yellow oil (23%); ¹H NMR (400 MHz, CDCl₃): δ 7.71 (s, 1H), 7.19 (m, 5H), 6.18 (s, 1H), 4.39 (s, 2H), 3.76 (s, 3H), 3.45 (s, 2H), 2.81 (m, 4H), 1.90 (br t, J = 10.2 Hz, 2H), 1.61 (br d, J = 9.2 Hz, 2H), 1.52 (q, J = 6.9 Hz, 2H), 1.22 (m, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 199.2, 159.5, 147.7, 137.7, 132.3, 129.5 (2C), 128.2 (2C), 127.1, 118.9, 111.3, 97.5, 63.3, 55.6, 53.7 (2C), 40.8, 35.4, 31.9 (2C), 31.4; HRMS (m/z) calcd for C₂₂H₂₈ClN₂O₂ [M+H]⁺ 387.183382, found 387.183120.

1-2-10- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(3-pyridylmethyl)-4-piperidyl]propan-

1-one (14). Pale yellow oil (40%); ¹H NMR (400 MHz, CDCl₃): δ 8.51 (d, J = 2.0 Hz, 1H), 8.50 (dd, J = 1.6, 4.8 Hz, 1H), 7.78 (s, 1H), 7.69 (dt, J = 1.6, 7.6 Hz, 1H), 7.25 (dd, J = 4.8, 8.0 Hz, 1H), 6.25 (s, 1H), 4.48 (s, 2H), 3.83 (s, 3H), 2.87 (m, 4H), 1.99 (br t, J = 10.2 Hz, 2H), 1.68 (br d, J = 9.2 Hz, 2H), 1.58 (q, J = 6.9 Hz, 2H), 1.24 (m, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 199.3, 159.7, 150.7, 148.8, 147.9, 137.2, 133.7, 132.4, 123.6, 119.0, 111.4, 97.7, 60.5, 55.7, 53.8 (2C), 40.9, 35.4, 32.0 (2C), 31.2; HRMS (m/z) calcd for C₂₁H₂₇ClN₃O₂ [M+H]⁺ 388.178631, found 388.178595.

1-2-11- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(4-pyridylmethyl)-4-piperidyl]propan-1-one (15). Pale yellow oil (45%); ¹H NMR (400 MHz, CDCl₃): δ 8.52 (d, J = 6.0 Hz, 2H), 7.78 (s, 1H), 7.26 (d, J = 6.0 Hz, 2H), 6.25 (s, 1H), 4.50 (s, 2H), 3.83 (s, 3H), 3.47 (s, 2H), 2.89 (t, J = 7.6 Hz, 2H), 2.83 (br d, J = 11.2 Hz, 2H), 1.97 (br t, J = 10.8 Hz, 2H), 1.68 (br d, J = 9.2 Hz, 2H), 1.59 (q, J = 6.9 Hz, 2H), 1.26 (m, 3H); ¹³C NMR (100 MHz, CDCl₃): δ 199.3, 159.7, 149.8 (2C), 148.2, 147.9, 132.4, 124.2 (2C), 119.0, 111.4, 97.6, 62.2, 55.7, 54.1 (2C), 40.9, 35.4, 32.2 (2C), 31.3; HRMS (m/z) calcd for C₂₁H₂₇ClN₃O₂ [M+H]⁺ 388.178631, found 388.178502.

1-2-12- 4-Amino-5-chloro-N-[[1-(cyclohexylmethyl)-4-piperidyl]methyl]-2-methoxybenzamide (19). Following the previous general procedure **18** is obtained starting from *tert*-Butyl 4-[[[4-amino-5-chloro-2-methoxybenzoyl]amino]methyl]piperidine-1-carboxylate (**19**). White powder (38%): mp 154 - 156°C; ¹H NMR (400 MHz, CDCl₃): δ 8.07 (s, 1H), 7.73 (t, J = 6.0 Hz, 1H), 6.27 (s, 1H), 4.39 (br s, 2H), 3.87 (s, 3H), 3.29 (m, 2H), 2.90 (m, 2H), 2.13 (d, J = 7.0 Hz, 2H), 1.90 (m, 2H), 1.66 (m, 8H), 1.48 (m, 1H), 1.36 (m, 2H), 1.16 (m, 3H), 0.84 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 164.6, 157.4, 146.6, 133.1, 112.0, 111.6, 97.9, 65.9, 56.2, 54.0 (2C), 45.1, 36.0, 35.1, 32.0 (2C), 29.7, 26.7 (2C), 26.1 (2C); LC-MS: [M+H]⁺ = 394.59 – 396.58; IR (KBr): ν cm⁻¹ 3400, 2925, 2854, 1622, 1593, 1536, 1498, 1256; HRMS (m/z) calcd for C₂₁H₃₃ClN₃O₂ [M+H]⁺ 394.225581, found 394.225427.

1-3- General procedure for the preparation of oximes (16 – 17)

To a solution of compound **1** in pyridine was added alkoxyamine hydrochloride (3.6 eq) in 3 portions every 2 h. The mixture was stirred at rt for 24 h. After dilution with water, the aqueous layer was extracted 3 times with AcOEt. The organic layers were combined, washed 5 times with

brine, dried over MgSO₄ and concentrated under reduced pressure. The crude residue was purified on deactivated silica gel (gradient: DCM to DCM/AcOEt (9/1)) to provide the desired product.

1-3-1- (E) 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-one oxime (16). White powder (35%): mp 194 - 196 °C; ¹H NMR (400 MHz, CD₃OD): δ 6.99 (s, 1H), 6.47 (s, 1H), 3.75 (s, 3H), 2.93 (m, 2H), 2.71 (m, 2H), 2.23 (d, J = 6.8 Hz, 2H), 2.02 (m, 2H), 1.72 (m, 8H), 1.55 (m, 1H), 1.28 (m, 7H), 0.93 (m, 2H); ¹³C NMR (100 MHz, CD₃OD): δ 159.8, 157.3, 145.9, 129.8, 115.8, 109.4, 98.2, 65.4, 54.6, 53.9 (2C), 35.2, 34.4, 31.8, 31.6 (2C), 30.8 (2C), 26.1, 25.7 (2C), 25.3; IR (KBr): ν cm⁻¹ 3368, 2925, 2850, 2815, 1620, 1508, 1461, 1449, 1412, 1336, 1254, 1214, 992; LC-MS: [M+H]⁺ = 408.53 – 410.51; HRMS (m/z) calcd for C₂₂H₃₅ClN₃O₂ [M+H]⁺ 408.241231, found 408.240998.

1-3-2- 2-Chloro-4-[(E)-C-[2-[1-(cyclohexylmethyl)-4-piperidyl]ethyl]-N-methoxy-carbonimidoyl]-5-methoxyaniline (17). Yellow oil (42%); ¹H NMR (400 MHz, CDCl₃): δ 7.12 (s, 1H), 6.25 (s, 1H), 4.10 (br s, 2H), 3.89 (s, 3H), 3.72 (s, 3H), 2.79 (m, 2H), 2.63 (m, 2H), 2.04 (d, J = 6.8 Hz, 2H), 1.65 (m, 9H), 1.44 (m, 1H), 1.22 (m, 8H), 0.83 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 159.9, 157.1, 144.2, 130.4, 117.0, 110.5, 98.5, 66.2, 61.5, 55.5, 54.5 (2C), 36.1, 35.2, 32.4, 32.1 (2C), 32.0 (2C), 29.7, 26.5, 26.2 (2C); LC-MS: [M+H]⁺ = 422.54 – 424.53; IR (KBr): ν cm⁻¹ 2921, 2850, 1621, 1506, 1464, 1450, 1411, 1338, 1258, 1213, 1051; HRMS (m/z) calcd for C₂₃H₃₈ClN₃O₂ [M+H]⁺ 422.256882, found 422.256616.

1-3-3- 1-(4-Amino-5-chloro-2-methoxyphenyl)-3-[1-(cyclohexylmethyl)-4-piperidyl]propan-1-ol (18). To a solution of compound **1** (33 mg, 0.084 mmol) in MeOH was added NaBH₄ (25 mg, 0.673 mmol). The mixture was stirred for 3 h. After concentration, the residue was dissolved in DCM, washed with brine, dried over MgSO₄ and then evaporated under reduced pressure. The crude product was purified on deactivated silica gel (gradient of elution: DCM to DCM/MeOH) to afford 20 mg of desired product. Pale yellow powder (62%): mp: 136 - 138 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.12 (s, 1H), 6.26 (s, 1H), 4.69 (dd, J = 7.5 Hz, J = 5.8 Hz, 1H), 4.00 (br s, 2H), 3.75 (s, 3H), 2.82 (m, 2H), 2.06 (d, J = 7.0 Hz, 2H), 1.79 (m, 2H), 1.67 (m, 10H), 1.45 (m, 1H), 1.36 (m, 1H), 1.19 (m, 6H), 0.83 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 156.2, 142.5, 127.5, 124.0, 110.6, 98.8, 70.0, 66.2, 55.4, 54.5 (2C), 35.8, 35.2, 34.4, 32.9, 32.3 (2C), 32.1 (2C), 26.8, 26.2 (2C); IR (KBr): ν cm⁻¹ 3393, 2920, 2850, 1622, 1580, 1504, 1466, 1412, 1209; LC-MS: [M+H]⁺ = 395.64 – 397.64. HRMS (m/z) calcd for C₂₂H₂₇ClN₂O₂ [M+H]⁺ 395.245983, found 395.245734.

1-3-4- [1-(Cyclohexylmethyl)-4-piperidyl]methyl 4-amino-5-chloro-2-methoxybenzoate (21). To a suspension of 4-amino-5-chloro-2-methoxybenzoic acid (101 mg, 0.5 mmol) in dry THF (3 mL) was added CDI (89 mg, 0.55 mmol). The mixture was stirred at rt for 24 h and then a solution of (1-(cyclohexylmethyl)piperidin-4-yl)methanol (106 mg, 0.50 mmol) in dry THF (2 mL) and NaH 60% (21 mg, 0.55 mmol) were added. This new mixture was stirred 3 days at rt and then concentrated under reduced pressure. The residue was dissolved in AcOEt, washed with water, dried over MgSO₄ and concentrated under *in vacuo*. The crude product was then purified on silica gel (gradient of elution: DCM (100%) to AcOEt (100%)). White powder (31%): mp 128 - 130 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.81 (s, 1H), 6.29 (s, 1H), 4.45 (br s, 2H), 4.08 (d, J =

6.0 Hz, 2H), 3.84 (s, 3H), 2.88 (m, 2H), 2.10 (d, $J = 7.1$ Hz, 2H), 1.86 (m, 2H), 1.69 (m, 7H), 1.48 (m, 1H), 1.39 (m, 2H), 1.19 (m, 4H), 0.86 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ 164.6, 160.2, 147.6, 133.2, 110.0, 109.9, 98.2, 68.9, 66.1, 56.0, 53.9 (2C), 35.6, 35.2, 32.0 (2C), 29.1 (2C), 26.8, 26.2 (2C); LC-MS: $[\text{M}+\text{H}]^+ = 395.59 - 397.56$; IR (KBr): ν cm^{-1} 3473, 3330, 2920, 2850, 1695, 1621, 1599, 1449, 1234, 1109; HRMS (m/z) calcd for $\text{C}_{21}\text{H}_{32}\text{ClN}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 395.2101, found 395.2109.

1-3-5- Ethyl 3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropanoate (4). To a suspension of 4-amino-5-chloro-2-methoxybenzoic acid (603 mg, 2.99 mmol) in dry THF (30 mL) was added portion-wise CDI (533 mg, 3.29 mmol). The mixture was stirred at rt for 6 h and, then, potassium 3-ethoxy-3-oxopropanoate (611 mg, 3.59 mmol) and MgCl_2 (342 mg, 3.59 mmol) were added portion-wise. The mixture was stirred at 40 °C for 2 days. After evaporation of the solvent, the residue was diluted with DCM, washed successively with water, saturated aqueous NaHCO_3 and brine, dried over MgSO_4 and evaporated *in vacuo*. The residue was then purified on silica gel (gradient of elution: $\text{CH}_2\text{Cl}_2/\text{AcOEt}$ 8/2 to 7/3) to afford 603 mg of product. White powder (62%): mp 124 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.92 (s, 1H), 6.23 (s, 1H), 4.56 (br s, 2H), 4.17 (qd, $J = 7.3$ Hz, 2H), 3.87 (s, 2H), 3.82 (s, 3H), 1.24 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3): δ 189.6, 168.6, 159.9, 148.7, 132.5, 117.3, 111.6, 97.0, 60.8, 55.4, 50.5, 14.2; LC-MS: $[\text{M}+\text{H}]^+ = 272.43 - 274.43$; IR (KBr): ν cm^{-1} 3463, 3362, 3323, 2983, 1725, 1648, 1622, 1573, 1469, 1423, 1326, 1262, 1221, 1155; HRMS (m/z) calcd for $\text{C}_{12}\text{H}_{15}\text{ClNO}_4$ $[\text{M}+\text{H}]^+$ 272.068412, found 272.068374.

1-3-6- *tert*-Butyl 4-(3-(4-amino-5-chloro-2-methoxyphenyl)-3-oxopropyl)piperidine-1-carboxylate (5).⁷³ To a solution of compound **3** (2.3 g, 8.5 mmol) in DMF (20 mL) were added *tert*-butyl 4-(iodomethyl)piperidine-1-carboxylate (3.05 g, 9.5 mmol) and K₂CO₃ (2.3 g, 17.1 mmol) The mixture was stirred at rt for 48 h. The reaction mixture was diluted with water and the product was extracted with AcOEt. Combined organic layer was washed with water and brine and then dried over MgSO₄. Evaporation of the solvent gave a crude mixture (4.8 g, 10.3 mmol), which was directly dissolved in EtOH (500 mL). H₂O (100 mL) and KOH (2.65 g, 47.3 mmol) were added to the solution. The reaction mixture was refluxed for 3 h. EtOH was then evaporated and the resulting residue was diluted with H₂O and extracted with AcOEt. The organic layer was washed with H₂O and brine. Evaporation of the solvent provided a crude product, which was purified by column on silica (gradient of elution: DCM to DCM/AcOEt 6/4) to provide compound **2** (2.9 g). White powder (86%): mp 135 - 137 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.71 (s, 1H), 6.23 (s, 1H), 4.63 (br s, 2H), 4.01 (m, 2H), 3.76 (s, 3H), 2.84 (t, J = 7.5 Hz, 2H), 2.61 (m, 2H), 1.61 (m, 2H), 1.53 (m, 2H), 1.39 (s, 9H), 1.35 (m, 1H), 1.03 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 198.7, 159.5, 154.8, 148.0, 131.9, 118.3, 111.0, 97.3, 79.1, 55.5, 43.8 (2C), 40.5, 35.6, 31.9 (2C), 31.0, 28.3 (3C); LC-MS: [M+H]⁺ = 397.60 – 399.60; IR (KBr): ν cm⁻¹ 3470, 3353, 2975, 2930, 2851, 1673, 1624, 1588, 1421, 1366, 1311, 1250, 1216.

1-3-7- *tert*-Butyl 4-[[[4-amino-5-chloro-2-methoxybenzoyl]amino]methyl]piperidine-1-carboxylate (20).⁷⁴ To a solution of 4-amino-5-chloro-2-methoxybenzoic (94 mg, 0.467 mmol) in DMF (3 mL) were added Et₃N (64 μL, 0.467 mmol) and *tert*-butyl 4-(aminomethyl)piperidine -1-carboxylate (100 mg, 0.467 mmol). The mixture was cooled to -5°C

and HOBT (63 mg, 0.467 mmol) and EDCl.HCl (90 mg, 0.467 mmol) were then added. The mixture was then stirred for 24 h. After dilution with water, the solution was extracted 3 times with AcOEt. The organic layers were combined, washed 4 times with water, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude residue was purified on silica gel (gradient of elution: DCM to DCM/AcOEt 7/3) to provide 155 mg of desired product. White powder (85%): mp 106 - 108 °C; ¹H NMR (400 MHz, CDCl₃): δ 8.08 (s, 1H), 7.77 (t, J = 5.9 Hz, NH), 6.27 (s, 1H), 4.37 (br s, 2H), 4.09 (m, 2H), 3.72 (s, 3H), 3.30 (m, 2H), 2.66 (m, 2H), 1.70 (m, 3H), 1.42 (s, 9H), 1.16 (m, 2H); ¹³C NMR (100MHz, CDCl₃): δ 164.7, 157.4, 154.9, 146.8, 133.1, 112.4, 111.6, 97.8, 79.4, 56.2, 45.0, 43.9 (2C), 36.5, 29.9 (2C), 28.5 (3C); LC-MS: [M+H]⁺ = 398.53 – 400.52; IR (KBr): ν cm⁻¹ 3405, 3339, 2976, 2928, 2852, 1680, 1630, 1595, 1542, 1501, 1425, 1315, 1252, 1173, 1144.

2- X-Ray Crystallography.

Single crystals of donecopride suitable for X-ray crystallographic analysis were obtained by slow evaporation. Data for crystal structures analysis were collected at 296 K with a Bruker–Nonius Kappa CCD area detector diffractometer with graphite–monochromatized Mo K_α radiation (λ=0.71073 Å). The structure was solved using direct methods and refined by full-matrix least-squares analysis on *F*². Program(s) used to solve structure: SHELXS–97.⁷⁵ Program(s) used to refine structure: SHELXL–2014.⁷⁶ Software used to prepare material for publication: SHELXL–2014. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre, CCDC. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (+44-1223-336408; E-mail: deposit@ccdc.cam.ac.uk or <http://www.ccdc.cam.ac.uk>).

3- In vitro biological studies.

3-1- Pharmacological characterization of drugs on guinea pig 5-HT₄R.

Binding to native 5-HT₄R from guinea pig was determined using the method of Grossman.⁶¹ For membrane preparations male guinea pigs (300-350 g, Charles River) were subjected to euthanasia by cervical dislocation and decapitated. Brains were rapidly removed at 4°C and striatal regions carefully dissected and pooled. The tissues were then suspended in 10 volumes of HEPES buffer 50 mM pH 7.4 at 4°C. After homogenization at 4°C (Ultra-Turrax, maximal speed, 15 sec), and ultracentrifugation (23,000 x g, 60 min, 4°C), the pellet was resuspended in 10 volumes of HEPES buffer 50 mM pH 7.4 at 4°C in order to obtain a tissue concentration of about 100 mg protein/mL. The protein concentration was determined by the method of Lowry⁷⁷ using bovine serum albumin as standard.

For radioligand binding studies, 600 µg of membrane were incubated in duplicate at 37°C for 30 min with **22** (Perkin Elmer), fixed concentration of compound and HEPES buffer 50 mM pH 7.4 at 37°C. Incubation was terminated by rapid vacuum filtration through 0.5% polyethylenimine-pres soaked Whatman GF/B filters (Alpha Biotech) using a Brandel Cell Harvester. Filters were subsequently washed three times with 4 ml of HEPES buffer 50 mM pH 7.4 at 4°C.

The method was validated from saturation studies: 6 concentrations of **22** were used to give final concentrations of 0.02-0.8 nM, non-specific binding of **22** was defined in the presence of 30 µM serotonin to determine the K_d and the B_{max}.

For competition studies, **22** was used to give a final concentration of 0.1 nM. Percentages of inhibition of the binding of **22** were obtained for concentrations of 10⁻⁶ and 10⁻⁸ M of the

ligands tested. For some of these compounds, affinity constants were calculated from 5-point inhibition curves using the EBDA-Ligand software, and expressed as $K_i \pm SD$.

3-2- Pharmacological characterization of drugs on human 5-HT₄R.

The method was validated from saturation studies: six concentrations of **22** were used to give final concentrations of 0.0625-2 nM, and nonspecific binding of **22** was defined in the presence of 30 μ M serotonin to determine the K_d and the B_{max} . For competition studies, 2.5 μ g of proteins (5-HT_{4B} membrane preparations, HTS110M, Millipore. Millipore's 5-HT_{4B} membrane preparations are crude membrane preparations made from 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 10⁻⁸M of each drug and 0.2 nM **22** (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 μ M serotonin.

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

3-3- Determination of cAMP production.

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% dialyzed fetal calf serum (dFCS) and antibiotics. Cells were transiently transfected with plasmid encoding HA-tagged 5-HT₄R, then seeded in 24-well plates (70,000 cells/well). 24 hrs after transfection, cells were exposed to the indicated concentrations of 5-HT₄R ligands in the

presence of 0.1 mM L-ascorbic acid and 0.1 mM of the phosphodiesterase inhibitor RO-20-1724, at 37°C in 250 µl of HBS (20 mM HEPES; 150 mM NaCl; 4.2 mM KCl; 0.9 mM CaCl₂; 0.5 mM MgCl₂; 0.1% glucose; 0.1% BSA). After 10 min, cells were then lysed by addition of the same volume of Triton-X100 (0.1%). Quantification of cAMP production was performed by HTRF[®] by using the cAMP Dynamic kit (Cisbio Bioassays) according to the manufacturer's instructions.

3-4- *In vitro* tests of AChE and BuChE biological activity.

Inhibitory capacity of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman.⁷⁸ Acetyl- or butyrylthiocholine iodide and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. Lyophilized BuChE from equine serum (Sigma Aldrich) was dissolved in 0.2 M phosphate buffer pH 7.4 such as to have enzyme solutions stock with 2.5 units/mL enzyme activity. AChE from human erythrocytes (buffered aqueous solution, ≥500 units/mg protein (BCA), Sigma Aldrich) was diluted in 20 mM HEPES buffer pH 8, 0.1% Triton X-100 such as to have enzyme solution with 0.25 unit/mL enzyme 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 (0.05 U final). After 5 min of preincubation at 25°C, the reaction was then initiated by the injection of 50 µL of 10 mM acetyl- or butyrylthiocholine iodide solution. The hydrolysis of acetyl- or butyrylthiocholine 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 acetyl- or butyrylthiocholine, at a wavelength of 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 rate of absorbance increase at 412 nm was followed every minute for 10 min. Assays were performed

with a blank containing all components except acetyl- or butyrylthiocholine, in order to account for non-enzymatic reaction. The reaction slopes were compared and the percent inhibition due to the presence of test compounds was calculated by the following expression: $100 - (v_i/v_0 \times 100)$ where v_i is the rate calculated in the presence of inhibitor and v_0 is the enzyme activity.

First screening of AChE and BuChE activity was carried out at a 10^{-6} or 10^{-5} M concentration of compounds under study. For the compounds with significant inhibition ($\geq 50\%$), IC_{50} values were determined graphically by plotting the % inhibition versus the logarithm of six inhibitor concentrations in the assay solution using the Origin software.

3-5- Propidium competition assay.

Propidium exhibits an increase in fluorescence on binding to AChE PAS, making it a useful probe for competitive ligand binding to the enzyme.⁷⁹ Fluorescence was measured in a Tecan Infinite M200 plate reader. Measurements were carried out in 200 μ L final in 96-well plates. 5 U eeAChE in 1 mM Tris/HCl, pH 8.0 buffer was incubated for 6 h at 25°C, with a 150 μ L 10^{-5} M solution of tested compounds. One micromolar propidium iodide 50 μ L solution was added 10 min before fluorescence measurement. The excitation wavelength was 535 nm, and that of emission, 595 nm. Each assay was repeated two different times.

4- Docking studies.

For each docked compound a preliminary calculation on its protonation state at pH 7.4 was carried out using standard tools of the ChemAxon Package (<http://www.chemaxon.com/>) and the majority microspecies protonated on one or two nitrogen atoms at this pH was used for docking studies.

The homology model of the (*h*)5-HT₄R was built using the high-resolution (2.4 Å) crystal structure of the (*h*)β₂ adrenergic receptor-T4 lysozyme fusion protein bound to carazolol (PDB: 2RH1) under the procedure described.⁶³

The crystallographic coordinates of (*h*)AChE used for docking studies were obtained from X-Ray structure of the DPZ/AChE complex (PDB ID 4EY7, a structure refined to 2.35 Å with an R factor of 17.7 %).⁸⁰

The docking of donecopride into the (*h*)5-HT₄R was carried out with the GOLD program (v5.0) using the default parameters.^{81,82} This program applies a genetic algorithm to explore conformational spaces and ligand binding modes. To evaluate the proposed ligand positions, the ChemPLP fitness function was applied in these docking studies. The binding site in the 5-HT₄R model was defined as a 10Å sphere centered on the aspartic acid residue Asp₁₀₀. Because the mutagenesis studies have shown that the interaction between the positively ionisable amine of ligands and Asp₁₀₀ of 5-HT₄R is crucial for ligand binding, a hydrogen bond constraint between positively ionisable amine ligand and OD atom of Asp₁₀₀ was used during the docking. Furthermore, special attention was paid during the docking procedure to the following amino acids in the binding site, which were kept flexible: Arg₉₆, Asp₁₀₀, Thr₁₀₄, Tyr₁₉₂, Ser₁₉₇ and Trp₂₉₄.

Docking of donecopride into (*h*)AChE was carried out also by means of the GOLD program with the default parameters. To evaluate the proposed ligand poses, the ChemPLP fitness function was applied. The binding site in the AChE model was defined as a 7 Å sphere from the co-crystallized ligand, DPZ and a water molecule interacting with protonated piperidine ring of DPZ was conserved during the docking (residue number 931).

5- In vivo biological studies.

5-1- Animals.

Adult male NMRI mice (3 months old, weighing 35-40g) from Janvier labs (Le Genest-Saint-Isle, France) were used to perform experiments. Mice were housed by ten in standard polycarbonate cages in standard controlled conditions ($22\pm 2^{\circ}\text{C}$, $55\pm 10\%$ humidity) with a reversed 12h light/dark cycle (light on at 7pm). Food and water were available *ad libitum* in the home cage. All experiments were conducted (between 9am and 3pm) during the active - dark - phase of the cycle and were in agreement with the European Directives and French law on animal experimentation (personal authorization n° 14-17 for MB and 14-60 for TF).

5-2- CNS-activity and acute toxicity test.

Behavioral and neurological changes induced by graded doses (1, 10, 100 mg/kg) of the tested derivatives were evaluated in mice, in groups of four, by a standardized observation technique at different times (30 min, 3 and 24 h) after intraperitoneal administration.^{s3} Major changes of behavioral data (for example, hypo- or hyperactivity, ataxia, tremors, convulsion, etc.) were noted in comparison to the control group. The approximate DL_{50} of the compounds were also calculated through the quantification of mortality after 24 h. Amphetamine (2 mg/kg), chlorpromazine (10 mg/kg) were used as stimulant and depressive references, respectively.

5-3- Locomotor activity.

Locomotion of mice was measured using an actimeter (Imetronic®) through infrared detection. Eight individual removable polycarbonate cages (21cm length, 7cm wide and 12cm high), where

each mouse was placed, are disposed in the actimeter. Locomotor activity was measured by recording the number of interruption of beams of the red light over a period of 30min through an attached recording system to the actimeter. Donecopride was tested at 1, 3 and 10 mg/kg. Amphetamine (2 mg/kg), chlorpromazine (10 mg/kg) and association **2** (0.1 mg/kg)-DPZ (0.3 mg/kg) were used as stimulant, depressive and pharmacological references, respectively.⁸⁴

5-3- Spatial working memory.

Anti-amnesiant activity of tested compounds was evaluated by reversal of scopolamine (0.5 mg/kg) - induced deficit on spontaneous alternation behavior in the Y maze test.⁸⁵ The Y maze made of grey plastic consisted of three equally spaced arms (21-cm long, 7-cm wide with walls 15-cm high). The mouse was placed at the end of one of the arms and allowed to move freely through the maze during a 5 min session while the sequence of arm entries was recorded by an observer. An arm entry was scored when all four feet crossed into the arm. An alternation was defined as entries into all three arms on a consecutive occasion. The number of possible alternation is thus the total number of arm entries minus two; the percentage of alternation was calculated as (actual alternation/ possible alternation) 100. Donecopride was tested at 0.1, 0.3, 3, and 10 mg/kg. Different additional groups (DPZ (0.3 mg/kg) and association **2** (0.1 mg/kg) + DPZ (0.3 mg/kg)) were tested as clinical and pharmacological references, respectively.

5-4- Forced swimming test.

The FST⁸⁶ was carried out in mice individually forced to swim during 6 min in an open cylindrical container (diameter 12cm, height 20 cm) with a water depth of 13 cm at 23-24°C.⁸⁷ The duration of immobility, after a delay of 2 min, was measured during the last 4 min. Each mouse was

judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. Animals were not pre-tested. Donecopride was tested at 0.3 and 3 mg/kg. Imipramine (16 mg/kg) was used as clinical reference.

5-5- Pharmacological treatments.

2 and DPZ hydrochloride were purchased from Tocris[®] (Cookson, UK). Amphetamine (+)- α -Methylphenethylamine hemisulfate, chlorpromazine hydrochloride, imipramine hydrochloride and scopolamine hydrobromide were purchased from Sigma (France). All those pharmacological compounds were dissolved in NaCl 0.9% as the vehicle were administered intraperitoneally 30min before tests, except DPZ and scopolamine which were subcutaneously administered 20 and 30 min respectively before tests.

5-6- Statistical analysis.

Results were expressed as mean \pm SD and were analyzed by one way analysis of variance (ANOVA), with Statview[®] software. In case of significance, a SNK (Student-Newman-Keuls) *post hoc* test was realized. Additionally, for the spontaneous alternation test, the percentage of alternation was compared to a theoretical 50% value (random alternation) by an univariate *t-test*. Differences were considered as statistically significant if the *p* value was strictly under 0.05.

5-7- Cerebrospinal fluid (CSF) collection and sAPP α quantification.

To explore the effect of acute injection of donecopride on sAPP α release *in vivo*, two different groups of WT C57BL/6 mice (n = 7/group) received one intraperitoneal (i.p.) injection of vehicle

(0.9 % w/v NaCl; 0.2 % dimethyl sulfoxide in water) or donecopride (1 mg/kg). 90 min post-injection, mice were anesthetized and mounted onto a stereotaxic instrument. The neck skin was cut and subcutaneous tissue and muscles separated with the help of micro-retractors (Fine Science Tools, Heidelberg, Germany). Mice were then laid down so that the head formed an angle of about 135° with the body.^{*} A capillary tube (Borosilicate glass, B100-75-10, Sutter Instruments, Novato, California, USA) was used to punch the dura mater of the cisterna magna. CSF was collected by capillary action and transferred to 0.5 mL microtubes, immediately frozen on dry ice and stored at -80°C until use. Once thawed, samples were heated at 60°C for 5 min as described in literature⁸⁹ and analyzed without further freezing-thawing cycles. ELISA kits from IBL International (Hamburg, Germany) for the dosage of sAPP α (mouse/rat sAPP α assay kit, #27415) were used according to the manufacturer's instructions. Reactions were read at 620 nm and 450 nm using an Infinite 2000 luminescence counter. The sAPP α ELISA kits enable the precise and selective quantification of sAPP α *versus* sAPP β .

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Author Contributions

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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, multi-target directed ligands; PAS, peripheral anionic site; P-gp, P-glycoprotein.

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