Development of Novel Potential Pleiotropic Compounds of Interest in Alzheimer’s Disease Treatment through Rigidification Strategy

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Abstract: The development of Multi-Target Directed Ligand is of clear interest for the treatment of multifactorial pathology such as Alzheimer’s disease (AD). In this context, acetylcholinesterase (AChE) inhibitors have been modulated in order to generate novel pleiotropic compounds targeting a second protein of therapeutic interest in AD. Among them, donecopride was the first example of a dual acetylcholinesterase inhibitor and 5-HT$_4$ receptor agonist. In order to explore the structural diversity around this preclinical candidate we have explored the preparation of novel constrained analogs through late-stage rigidification strategy. A series of phenylpyrazoles was prepared in a late-stage functionalization process and all compounds were evaluated in vitro towards AChE and 5-HTRs. A docking study was performed in order to better explain the observed SAR towards AChE, 5-HT$_4$R and 5-HT$_6$R and this study led to the description of novel ligand targeting both AChE and 5-HT$_6$R.

Keywords: Alzheimer’s disease; acetylcholinesterase; 5-HT$_6$R; donecopride; MTDL

1. Introduction

In the field of neurodegenerative diseases, the prototype of multifactorial pathologies, the development of Multi-Target Directed Ligands (MTDLs) offered great promise and therapeutic opportunities [1]. Indeed, these compounds are rationally designed to display multiple activities by modulating several biological targets of interest in the treatment of a specific disease. Chosen to demonstrate a therapeutic synergy, the association of these biological targets needs to be rigorously determined [2]. In the field of Alzheimer’s disease (AD), multiple in vitro or in vivo MTDL candidates have been generated over the last years and could lead to preclinical or clinical opportunities [3]. Because of its validated interest in the treatment of AD symptoms, acetylcholinesterase (AChE) inhibitors, such as donepezil, have been chosen as a starting point to develop novel analogs with a second biological target such as receptors [4]. As an example, we recently described the preclinical candidate donecopride, the first MTDL able to demonstrate AChE inhibition and activating serotoninergic receptors 5-HT$_4$ (Figure 1) [5–7].

This secondary target was chosen because of the ability of 5-HT$_4$R agonists to favor the “non-amyloidogenic” cleavage of the amyloid precursor protein (APP) by $\alpha$-secretase, inducing then a decrease in amyloid pathology. [8] This positive effect has already been demonstrated with the use of RS67333 a reference 5-HT$_4$R agonist, in primary neurons, [9] and led to the in vivo improvement of memory in several animal models of AD [10]. Indeed, in the context of AD, the activation of 5-HT$_4$Rs with agonist has led to the increase in neurotransmitter release as well as a clear impact of amyloid load in transgenic mice models after chronic administration. Several 5-HT$_4$R agonists have been studied in clinical...
trials in recent years to demonstrate both symptomatic and disease-modifying effect against AD [11]. Beside this receptor subtypes, other serotonin receptors have been studied in the field of neurodegenerative diseases [12], such as the 5-HT₆R [13,14]. Specifically, 5-HT₆R antagonists increase extracellular levels of acetylcholine, glutamate, and norepinephrine in forebrain regions, whereas activation of 5-HT₆R inhibits corticostriatal glutamatergic transmission. In the end, these various elements (notably, distribution in limbic areas, modulation of different systems of neurotransmission) are in favor of the implication of 5-HT₆R in the modulation of the cognitive processes as well as mood regulation and of numerous behaviors (eating behavior, addictive behavior, etc.). Indeed, they appear as a valuable target to treat cognitive impairments since their blockade confers to 5-HT₆R antagonists’, such as idalopirdine (Figure 1), procognitive effects. [15] It is important to note that during the clinical trials the potential synergistic effect to act simultaneously on 5-HT₆R and AChE has been evaluated since idalopirdine has been tested along with donepezil [16].

![Figure 1. Structures of reference ligands and MTDL (Donecopride, MR33372, benzisoxazole) and targeted structure.](image)

In this context, we recently described initial modulations of donecopride by introducing a series of substituent on the piperidine ring, which yielded the description of a first 5-HT₄R agonist and 5-HT₆R antagonist, [17] and MR33372, the first compound able to modulate simultaneously AChE, 5-HT₄R and 5-HT₆R [18]. In parallel with these first works, we also decided to assess the impact of conformational restriction on the aromatic ring, inspired by both bicyclic donepezil and idalopirdine (Figure 1). Indeed, such restriction are classical in drug discovery [19], and we recently described the preparation and biological evaluation of benzisoxazole analog possessing an oxygen or a methylene linker [20]. We would like to describe in this article, a novel rigidification strategy with the construction of a novel heterocycle between the ketone and its alpha-position. The preparation of novel phenylpyrazole derivatives possessing either a methylene or an oxygen linker (Figure 1), as well as their biological evaluation against AChE and both 5-HT₄R and 5-HT₆R will be described and compared with other members of this series.
2. Results

2.1. Chemistry

The access to targeted compounds was achieved starting from Boc-protected benzophenone 1 that we previously described (Scheme 1) [5]. The latter was treated by DMF–DMA [21] to generate an intermediate dimethyl enamines, which was not isolated, but reacted in boiling ethanol with methylhydrazine to generate the expected pyrazole 2 with 38% yield [22,23]. In order to obtain the final derivatives, the Boc protecting group was removed with TFA, and the resulted piperidine was alkylated with the appropriate bromide to generate the final cyclohexyl and m-tolyl substituted analogs 3a–b. These particular substituents were chosen for a better comparison with either donecopride and MR33372. In order to verify the impact of the aniline both on the reactivity and the biological activities, the corresponding acetamides 4–5 were obtained in acetic anhydride with good yields. Following the same procedure, the cyclohexyl substituted analogs 6 and 7 were prepared after deprotection and alkylation (Scheme 1).

As already described in the benzisoxazole [20] and donecopride series [6], the influence of the linker could be of great importance on the ability of the ligand to bind to AChE. We then investigated the possibility to modulate both the linker and its position on the pyrazole ring. Indeed, the β-ketoester 8 is a crucial intermediate in the preparation of the benzophenone 1 and was then already available [6]. Afterwards, 8 was treated with methylhydrazine in refluxing ethanol to generate the expected pyrazoline 9a with 57% yield (Scheme 2). Unfortunately, the O-alkylation procedure tested was ineffective to generate the expected piperidine 10a, due to the reactivity of the aniline moiety. The pyrazoline 9a was then treated with acetic anhydride to obtain the ester 11 with 94% yield, before being hydrolyzed with LiOH to generate the acetamide 9b. The latter was then alkylated with N-Boc 4-iodomethylpiperidine in DMF to generate this time the expected ether 10b with good conversion. Finally, the protecting group was removed with trifluoroacetic acid, and the m-tolyl analog 12b was obtained after an N-alkylation reaction with the corresponding

![Scheme 1. Synthetic pathways for access to compounds 2–7. Reagents and conditions: (a) i. DMF.DMA, Rflx, ii. CH₃NHNH₂, EtOH, Rflx, mw, 38%; (b) i. TFA, DCM, TA, ii. RCH₂Br, K₂CO₃, DMF, or acetone, 110 °C, 31–46%; (c) Ac₂O, rt, 24 h, 78–83%.](image-url)
bromide. Unfortunately, all attempts aiming at deprotecting the N-acetamide group of 12b failed. In order to verify this time the impact of the substitution of the aromatic ring, commercial pyrazolone 9c–d were engaged in the same procedure. The 3-4-dimethoxyphenyl group of 9d was chosen by analogy with donepezil, a reference AChE inhibitor (AChEI). The expected m-tolyl analogs 12c–d were obtained in a two-step synthesis.

Scheme 2. Synthesis pathways for access to compounds 9–12. Reagents and conditions: (a) CH3NHNH2, EtOH, 160 °C, mw, 57%; (b) N-Boc 4-iodomethylpiperidine, K2CO3, DME, 60 °C, 21–27%; (c) Ac2O, 94%, (d) LiOH, EtOH/H2O, Rfx, 76%, (e) i. TFA, DCM, TA, ii. m-TolylCH2Br, K2CO3, acetone, 110 °C, 32–67%.

2.2. In Vitro Results

The inhibitory activity of novel analogs towards hAChE was evaluated according to the Ellman test [24]. Their affinity for human 5-HT4R and 5-HT6R was assessed using a radioligand displacement assay (Table 1). In these tests, donepezil (DPZ) was used as a reference AChEI, RS67333 as a 5-HT4R ligand, and idalopirdine as a 5-HT6R ligand, respectively.

As already introduced, we demonstrated that pharmacomodulation of the preclinical candidate donecopride could greatly affect the ability of its analog to interact with ChE and 5-HT receptors. Indeed, in our initial study [6], we showed that its ester or amide analogs lose their ability to inhibit AChE. The same tendency has also been observed in the benzisoxazole family with a clear impact of the modulation of the benzenic position on both AChE and 5-HT4R binding. [20] In this context, we decided to explore the modulation of the α-position of the ketone and the possibility to generate conformationally constrained derivatives to increase the elucidation of SAR around donecopride. The preparation of first series of phenylpyrazoles 5a–b and 6, was then achieved from the advance intermediate 1. In this synthesis, the protection of the basic nitrogen with a Boc group is mandatory to obtain the pyrazole cycle, since all attempts to modulate the final donecopride analog in a late stage process failed. For this reason, we also explored in parallel the impact of the
protection of the aniline moiety with an acetamide group, with no clear benefit in terms of synthesis of compound 6. The preparation of the Boc protected 2 was, however, of interest since it allowed us to introduce in a convergent way both the cyclohexyl and the m-tolyl moieties. Indeed if the cyclohexyl present in donecopride yielded low nanomolar affinities for both AChE and 5-HT\(_4\)R, [6] its replacement by a m-tolyl generated triple activity this time towards 5-HT\(_4\)R [18]. If MR33372 possesses affinities in the hundred nM range for the three targets, this compound demonstrated an in vivo anti-amnesic effect in a model of scopolamine induced deficit.

Table 1. hAChE inhibitory activity and affinity for 5-HT\(_4\)R and 5-HT\(_6\)R.

| Compound       | % Inhibition at 10\(^{-6}\) M | IC\(_{50}\) (nM) \((n = 3)\) | % Inhibition at 10\(^{-6}\) M | K\(_i\) (nM) \((n = 3)\) | % Inhibition at 10\(^{-6}\) M | K\(_i\) (nM) \((n = 3)\) |
|----------------|------------------------------|-----------------------------|------------------------------|-----------------------------|------------------------------|-----------------------------|
| Donepezil      | 98%                          | 7.7 ± 1.3                   | –                            | –                           | –                            | –                           |
| RS67333        | –                            | –                           | 100                          | 5.1 ± 0.51                  | –                            | –                           |
| Idalopirdine   | –                            | –                           | –                            | 106                         | 7.05 ± 0.61                  | –                           |
| Donecopride    | 96%                          | 16 ± 5                      | 100                          | 7.05 ± 0.61                 | 30%                         | ND                          |
| MR33372        | 89%                          | (16 ± 4) × 10               | 98%                          | (17 ± 1) × 10               | 80%                         | (23 ± 5) × 10               |
| 7              | 98%                          | 34 ± 5                      | 94%                          | (3 ± 1) × 10\(^2\)         | –                           | ND                          |
| 3a             | 34%                          | ND                          | 34%                          | ND                          | 2%                          | ND                          |
| 3b             | 76%                          | 110 ± 4                     | 20%                          | ND                          | 68%                         | (47 ± 9) × 10               |
| 6              | 31%                          | ND                          | 16%                          | ND                          | 0%                          | ND                          |
| 12b            | 15%                          | ND                          | 9%                           | ND                          | 0%                          | ND                          |
| 12c            | 15%                          | ND                          | 10%                          | ND                          | 6%                          | ND                          |
| 12d            | 6%                           | ND                          | 9%                           | ND                          | 9%                          | ND                          |

IC\(_{50}\) and K\(_i\) values are expressed as mean ± standard error of the mean (SEM) of three experiments; ND: not determined.

Compared with donecopride, the introduction of the acetamide group on 7 had little impact on AChE inhibition (IC\(_{50}\) = 16 vs. 34 nM respectively), but greatly affected the affinity for 5-HT\(_4\)R (Table 1). The introduction of the methylpyrazole ring on 3a led to a complete loss of activities for all targets. Interestingly, however, the introduction of the m-tolyl moiety is again of interest for 3b. Compared to MR33372 this time, 3b is a slightly better AChEI (IC\(_{50}\) = 161 vs. 111 nM respectively) and possesses a decreased affinity for 5-HT\(_4\)R. Contrary, the conformational restriction greatly affects the ability of the compound to bind to 5-HT\(_4\)R. Due to its synthetic accessibility from intermediate 8, a second series of ether substituted analog 12b–d was generated bearing the m-tolyl substituent previously identified. Unfortunately, this modulation led to a complete loss of activities for the three targets.

2.3. In Silico Results

In order to better understand the impact of these SAR on AChE binding, a docking study was performed with 3b in comparison with donecopride (Figure 2). The docking study was carried out with the aim to predict the AChE inhibition of synthesized compounds compared to donecopride, the initial scaffold of the carried out modulations. The docking study into the hAChE active site (PDB code: 4EY7) [25] was performed using Gold 5.7.2 software and the ligand 3D models were built from donecopride X-ray structure solved previously. Firstly, the correctness of employed docking procedure was checked by redocking the co-crystallized ligand, donepezil. Gold program regained the crystallographic donepezil position with a RMSD smaller to 1 Å and with a score fit value of 110.02 [6]. Next, the docking procedure was applied to donecopride and to the newly synthesized compounds (3b, 7, and 12b). The docking generated positions closed to donepezil one for donecopride (score fit ~105.14, Figure 2) and for compound 7 (score fit ~ 117.63, Figure 2) and 12b (score fit ~ 86.79, Figure 2). Indeed, donecopride and compound 7 under the docking results reproduces well the donepezil key binding interactions: (i) the
charged nitrogen of the piperidine ring is oriented in a position suitable for an Hbond with the water molecule in the proximity of Tyr\textsubscript{337} and Tyr\textsubscript{341}, (ii) the carbonyl group of both ligands forms an Hbond with NH of the Phe\textsubscript{295} backbone, (iii) the benzene ring is positioned in a parallel way to the Trp\textsubscript{286} indole ring to favor the π-stacking interaction and (iv) the cyclohexane ring occupied the donepezil benzyl ring place in neighboring of Trp\textsubscript{86}. Even if compound 12b was positioned systematically with the substituted phenyl ring towards the binding cavity’s bottom, its position was higher comparing to previous three compounds. Consequently, 12b lost, more to the loss of Hbond due to carbonyl group absence in its scaffold, also the interaction through the piperidine’s protonated nitrogen as well as π-stacking with Trp\textsubscript{86} (Figure 2).
However, the first docking does not produce conclusive results for compound 3b. Compound 3b was systematically placed with aniline moiety at the bottom of the binding site cavity. Therefore, a second docking with a greater number of docking cycles was tested for compound 3b. This docking generated even some more logical poses of compound 3b with aniline moiety placed at the binding cavity entrance. The generated solution with good orientation of aniline moiety corresponded to the score fit 91.78 is present in Figure 2.

The compound 3b docked in the hAChE active site higher compared to the donecopride in the same way as compound 12b. Like 12b, 3b compound lost the interaction through the piperidine’s protonated nitrogen, π-stacking with Trp86, and the Hbond interaction due to carbonyl group absence in its scaffold (Figure 2). However, on the other hand with compound 12b, 3b establishes new interactions through hydrogen bonds with the hydroxy group of Tyr124 and Ser293.

Based on this study we could state that the introduction of the methylpyrazole ring led to the loss of one of the crucial hydrogen bonds with the backbone NH of Phe295. In this series, the classical cation-π interaction with Tyr337 is not enough to maintain the affinity of the cyclohexyl 3a. The replacement of the cyclohexyl by the aromatic m-tolyl led to clear impact on AChE inhibition surely by increasing the stacking of the aromatic ring of 3b with Phe86 (Figure 2). Both interactions as well as hydrophobic interactions in the aromatic gorge could then justify the interesting properties of the novel phenyl pyrazole 3b.

In order to better understand their worst profile on serotonergic receptors, docking studies were performed in a homology model of the 5-HT4 and 5-HT6 receptors. The docking results on 5-HT4R for compounds 7, 3b, and 12b were compared to donecopride one [6]. From donecopride docking, the selected pose was that where donecopride is placed horizontally in the cavity interacting on one side through an H-bond with Asp100 on the transmembrane helix 3 (TM3, consistent with the constraint used during docking) and on other side through another H-bond with Ser197 on TM5 (in yellow in Figure 3). The donecopride’s group assuring this second H-bond is NH2 substituent on the benzene ring. Therefore, the addition of the substituent on NH2 group as well as the lengthening of the connector between the benzene cycle and the piperidine increase the compounds' length and the compounds can no longer be placed horizontally as donecopride. They take a curve position (Figure 3) and lost the interaction with TM5 in binding site. For compound 7, the position of its carbonyl group allowed it to maintain some electrostatic interactions with Ser197 and Asn279, which is impossible for compound 12b and 3b through the replacement of this carbonyl group by the methylpyrazole ring. Hence, the hypothesis of their loss of affinity.

The comparison of the binding site of 5-HT4R and 5-HT6R showed that the various amino acids change and so modify the binding site topology. For example, in the 5-HT6R, two tryptophan residues are in proximity of TM3 helix, Trp102 on TM3, and Trp92 in the ECL1 loop (Figure 4), and these two residues with a bulky lateral chains obstruct the space between the helices TM2 and TM3, which leads to the ligands no longer accessing this pocket, unlike the 5-HT4R. The binding cavity in 5-HT6R is smaller than that of 5-HT4R and therefore the ligands can take curved conformations to be able to access Asp106. A supplementary interaction through π stacking with Trp102 was only observed for ligand 3b (Figure 4), which could explain the micromolar binding affinity of this ligand to 5-HT6R.
Figure 3. Donecopride (green) position compared to the compound 7 (orange), 3b (pink), and 12b (purple) positioned in h5-HT4R binding sites using the docking studies. The compound and the selected 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, CA, USA) and the protein is presented in the same orientation in all four images.

The comparison of the binding site of 5-HT6R and 5-HT4R showed that the various amino acids change and so modify the binding site topology. For example, in the 5-HT6R, two tryptophan residues are in proximity of TM3 helix, Trp 102 on TM3, and Trp 92 in the ECL1 loop (Figure 4), and these two residues with a bulky lateral chains obstruct the space between the helices TM2 and TM3, which leads to the ligands no longer accessing this pocket, unlike the 5-HT 4R. The binding cavity in 5-HT6R is smaller than that of 5-HT4R and therefore the ligands must take curved conformations to be able to access Asp 106. A supplementary interaction through $\pi$ stacking with Trp 102 was only observed for ligand 3b (Figure 4), which could explain the micromolar binding affinity of this ligand to 5-HT6R.
3. Experimental
3.1. Chemistry
3.1.1. General Methods

All chemical reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a STUART SMP50 melting point apparatus (Cole-Parmer, Vernon Hills, IL, USA). $^1$H, $^{13}$C, and $^{19}$F NMR spectra were recorded on a BRUKER AVANCE III 400 MHz (Bruker, Billerica, MA, USA) apparatus with chemical shifts expressed in parts per million downfield from TMS as an internal standard and coupling in Hertz. IR spectra were recorded on a Perkin-Elmer BX FT-IR apparatus.
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(Perkin Elmer, Wellesley, MA, USA) using KBr pellets. High-resolution mass spectra (HRMS) were obtained by electrospray on a BrukermaXis (Bruker, Billerica, MA, USA). 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 (Waters corporation, Milford, MA, USA) as separating module (column XBridge C18 2.5 µM/4.6 × 50 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 = H2O, B = CH3CN; each containing HCOOH: 0.1%). MS were obtained on a SQ detector by positive ESI.

3.1.2. Synthesis of Compounds (2−7, 9−12)

tert-butyl 4-((3-(4-amino-5-chloro-2-methoxyphenyl)-1-methyl-1H-pyrazol-4-yl)methyl)piperidine-1-carboxylate 2. An amount of 375 mg of compound 1 (0.947 mmol) were refluxed in 5 mL of DMF.DMA overnight. After evaporation under reduced pressure, the residue was dissolved in 10 mL of EtOH and 26 mL of methylhydrazine (3.788 mmol, 4 eq) were added. The mixture was refluxed for 4 h. After concentration, the new residue was dissolved in 20 mL of EtOH and 645 mg of ZnCl2 (4.735 mmol, 5 eq) were added. The new reactional mixture was refluxed for 2 h and then concentrated. The residue was dissolved in DCM and washed 3 times with water. The organic phase was dried on MgSO4, concentrated and purified on neutral Al2O3 to afford 155 mg of product 2 as a brown solid. Yield = 38%; m.p. = 67−69 °C; 1H-NMR (CDCl3, 400 MHz): 7.17 (s, 1H), 7.15 (s, 1H), 6.34 (s, 1H), 4.10 (br s, 2H), 3.99 (br s, 2H), 3.87 (s, 3H), 3.71 (s, 3H), 2.57 (t, J = 13.0 Hz, 2H), 2.29 (d, J = 7.0 Hz, 2H), 1.54 (d, J = 13.1 Hz, 2H), 1.45−1.38 (m, 10H), 0.97 (qd, J = 12.2 Hz, J′ = 4.2 Hz, 2H); 13C-NMR (CDCl3, 100 MHz): 156.8, 154.9, 147.4, 143.5, 131.7, 129.3, 118.7, 114.4, 110.7, 98.7, 79.2, 55.6, 44.0 (2C), 38.9, 37.0, 32.0 (2C), 31.1, 28.5 (3C); MS [M + H]+ = 434.86; IR (KBr, cm−1): 3468, 3351, 2931, 1686, 1624, 1461, 1444, 1426, 1365, 1245, 1211, 1173, 1211.

General Procedure for Acetylation

The aniline was stirred in anhydric acetic (6 mL mmol−1) at room temperature overnight. The mixture was concentrated under reduced pressure. The residue was then dissolved in EtOAc and washed twice with aq. NaHCO3 until the pH was than 95%. Analyses were performed using a Waters Alliance 2695 (Waters corporation, Milford, MA, USA) as separating module (column XBridge C18 2.5 µM/4.6 × 50 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 = H2O, B = CH3CN; each containing HCOOH: 0.1%).
3 mL of EtOH were stirred under microwaves irradiation at 160 °C for 45 min. The solvent was eliminated under reduced pressure and the residue was purified on silica gel (gradient of elution: DCM to EtOAc) to afford 143 mg of compound 9a as a white solid. Yield = 57%; m.p. = 203–205°C; 13C-NMR (CDCl3, 400 MHz): 7.81 (s, 1H), 6.27 (s, 1H), 4.31 (br s, 2H), 3.78 (s, 3H), 3.68 (s, 2H), 3.36 (s, 3H); 13C-NMR (CDCl3, 100 MHz): 172.5, 157.5, 152.4, 145.6, 128.0, 111.7, 111.4, 98.0, 55.6, 42.1, 31.3; MS [M + H]+ = 253.92; IR (KBr, cm⁻¹): 3448, 3331, 2958, 2229, 1677, 1631, 1603, 1572, 1458, 1348, 1214, 1049; HRMS [M + H]+ calcd for C11H13ClN3O2, 254.0696, exp 254.0693.

3-(4-acetamido-5-chloro-2-methoxyphenyl)-1-methyl-1H-pyrazol-5-yl acetate 11. A total of 200 mg of compound 9a (0.80 mmol) were stirred in 2 mL of anhydric acetic acid at room temperature overnight. Water was added to obtain a white suspension. This latter was filtered and washed with water. A total of 250 mg of product 11 were then obtained as a white solid. Yield = 94%. m.p. = 191–193°C; 1H-NMR (CDCl3, 400 MHz): 8.21 (s, 1H), 7.92 (s, 1H), 7.68 (s, 1H), 6.61 (s, 1H), 3.89 (s, 3H), 3.73 (s, 3H), 2.35 (s, 3H), 2.25 (s, 3H); 13C-NMR (CDCl3, 100 MHz): 168.4, 166.1, 155.8, 145.1, 144.8, 134.6, 127.3, 118.8, 113.6, 104.3, 95.5, 55.9, 34.7, 25.1, 20.7; MS [M + H]+ = 337.92; IR (KBr, cm⁻¹): 3422, 2945, 1747, 1722, 1699, 1585, 1511, 1483, 1450, 1352, 1243, 1191, 1011. HRMS [M + H]+ calcd for C15H17ClN3O4, 338.0908, exp 338.0914.

N-(2-chloro-5-methoxy-4-(1-methyl-5-oxo-4,5-dihydro-1H-pyrazol-3-yl)phenyl)acetamide 9b. A total of 250 mg of compound 11 (0.742 mmol) and 21 mg of LiOH (0.890 mmol, 1.2 eq) were dissolved in 14 mL of EtOH/water (5/2). The reactional mixture was stirred at room temperature for 1 h 45 min. After evaporation in vacuo, water was added, adjusted at pH 4–5 and extracted 3 times with CH3Cl. The organic phases were combined, washed once with water, dried on MgSO4, and concentrated to obtain 176 mg of monoprotected compound 9b and used without further purification. Yield = 76%; m.p. = 219–221°C; 1H-NMR (CDCl3, 400 MHz): 8.26 (s, 1H), 7.94 (s, 1H), 7.67 (bs s, 1H), 3.88 (s, 3H), 3.74 (s, 2H), 3.39 (s, 3H), 2.27 (s, 3H); 13C-NMR (CDCl3, 100 MHz) 172.6, 168.6, 156.8, 151.6, 136.9, 126.9, 116.2, 114.0, 104.1, 56.0, 42.0, 31.3, 25.2; MS [M + H]+ = 295.85; IR (KBr, cm⁻¹): 3316, 2340, 1681, 1606, 1586, 1547, 1506, 1450, 1392, 1209, 1015. HRMS [M + H]+ calcd for C13H13ClN3O3, 296.0802, exp 296.0803.

General Procedure for the O-Alkylation

The pyrazolone was dissolved in DMF (3 mL·mmol⁻¹) and K2CO3 (2 eq) and N-Boc-4-iodomethylpiperidine (1.2 eq) were added. The mixture was stirred for 3 h at 60 °C. After cooling, the brown solution was diluted in water and extracted 3 times with EtOAc. The organic phases were combined, washed 3 times with water, dried on MgSO4, and concentrated under reduced pressure. The crude product was then purified on silica gel (eluant DCM to DCM/EtOAc 8/2).

Tert-butyl 4-(((3-(4-acetamido-5-chloro-2-methoxyphenyl)-1-methyl-1H-pyrazol-5-yl)oxy)methyl)piperidine-1-carboxylate 10b. From 167 mg of compound 9b (0.379 mmol), 60 mg of compound 10b were obtained as a white solid. Yield = 22%; m.p. = 182–184 °C; 1H-NMR (CDCl3, 400 MHz): 8.19 (s, 1H), 7.89 (s, 1H), 7.69 (bs s, 1H), 6.00 (s, 1H), 4.15 (br s, 2H), 3.92 (d, J = 6.3 Hz, 2H), 3.89 (s, 3H), 3.66 (s, 3H), 2.73 (t, J = 13.0 Hz, 2H), 2.23 (s, 3H), 1.98 (m, 1H), 1.78 (d, J = 12.6 Hz, 2H), 1.45 (s, 9H), 1.27 (qd, J = 12.1 Hz, J' = 4.2 Hz, 2H); 13C-NMR (CDCl3, 100 MHz): 168.4, 155.7, 154.8, 154.4, 144.7, 134.4, 127.4, 119.3, 113.8, 104.4, 84.0, 79.5, 75.7, 55.9, 43.5 (2C), 36.1, 33.7, 28.6 (2C), 28.5 (3C), 25.1; MS [M + H]+ = 492.76; IR (KBr, cm⁻¹): 3420, 2936, 2853, 1690, 1585, 1557, 1528, 1450, 1436, 1365, 1247, 1173, 1147, 1018.

Tert-butyl 4-(((1-methyl-3-phenyl-1H-pyrazol-5-yl)oxy)methyl)piperidine-1-carboxylate 10c. From 218 mg of compound 9c (0.647 mmol), 129 mg of compound 10c were obtained as a yellow solid. Yield = 27%; m.p. = 133–135 °C; 1H-NMR (CDCl3, 400 MHz): 7.73 (m, 2H), 7.37 (m, 2H), 7.27 (m, 1H), 5.80 (s, 1H), 4.16 (br s, 2H), 3.93 (d, J = 6.4 Hz, 2H), 3.68 (s, 3H), 2.75 (t, J = 12.8 Hz, 2H), 2.00 (m, 1H), 1.80 (d, J = 13.0 Hz, 2H), 1.47 (s, 9H), 1.29 (qd, J = 11.8 Hz,
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2-chloro-4-(4-((1-(cyclohexylmethyl)piperidin-4-yl)methyl)-1-methyl-1H-pyrazol-3-yl)-5-methoxyaniline 3a. From 59 mg of compound 2 (0.136 mmol), 18 mg of compound 3a were obtained as a pale brown gum. Yield = 31%; 1H-NMR (CDCl3, 400 MHz): 7.17 (s, 1H), 7.15 (s, 1H), 6.34 (s, 1H), 4.09 (br s, 2H), 3.88 (br s, 2H), 3.72 (s, 3H), 2.83 (m, 2H), 2.29 (d, J = 6.6 Hz, 2H), 2.10 (m, 2H), 1.78–1.10 (m, 17 H), 0.89–0.80 (m, 2H); 13C-NMR (CDCl3, 100 MHz): 156.8, 147.4, 143.4, 131.8, 129.4, 119.1, 114.6, 110.7, 98.7, 66.0, 55.6, 54.4 (2C), 38.9, 36.9, 35.1, 32.0 (2C), 31.9 (2C), 31.1, 26.7, 26.1 (2C); MS [M + H]+ = 431.02; IR (KBr, cm⁻¹): 3438, 2921, 2849, 1622, 1525, 1461, 1445, 1402, 1211. HRMS [M + H]+ calcd for C24H36ClN4O, 431.2578, exp 431.2576.

2-chloro-5-methoxy-4-(1-methyl-4-((1-(3-methylbenzyl)piperidin-4-yl)methyl)-1H-pyrazol-3-yl)aniline 3b. From 41 mg of compound 2 (0.095 mmol), 19 mg of compound 3b were obtained as a pale yellow gum. Yield = 46%; 1H-NMR (CDCl3, 400 MHz): 7.18 (t, J = 7.6 Hz, 1H), 7.17 (s, 1H), 7.14 (s, 1H), 7.10 (s, 1H), 7.05 (m, 2H), 6.33 (s, 1H), 4.09 (br s, 2H), 3.86 (s, 3H), 3.71 (s, 3H), 3.40 (s, 2H), 2.81 (m, 2H), 2.33 (s, 3H), 2.28 (d, J = 6.8 Hz, 2H), 1.82 (td, J = 11.8 Hz, J' = 2.4 Hz, 2H), 1.55 (d, J = 12.7 Hz, 2H), 1.28 (m, 1H), 1.15 (qd, J = 12.3 Hz, J' = 3.6 Hz, 2H). 13C-NMR (CDCl3, 100 MHz): 156.8, 147.4, 143.4, 134.8, 137.7, 131.8, 130.0, 129.3, 128.0, 127.6, 126.4, 119.2, 114.6, 110.7, 98.7, 63.6, 55.6, 53.9 (2C), 38.9, 36.9, 32.2 (2C), 31.1, 21.4; MS [M + H]+ = 439.74; IR (KBr, cm⁻¹): 3434, 2925, 2623, 1525, 1460, 1445, 1402, 1211. HRMS [M + H]+ calcd for C25H32ClN4O, 439.2265, exp 439.2268.

N-(2-chloro-4-(4-((1-(cyclohexylmethyl)piperidin-4-yl)methyl)-1-methyl-1H-pyrazol-3-yl)-5-methoxyphenyl)acetamide 6. From 60 mg of compound 5 (0.126 mmol), 32 mg of compound 6 were obtained as a beige solid. Yield = 46%; m.p. = 148–150 °C; 1H-NMR (CDCl3, 400 MHz): 8.20 (s, 1H), 7.70 (br s, 1H), 7.31 (s, 1H), 7.16 (s, 1H), 3.87 (s, 3H), 3.80 (s, 3H), 2.77 (d, J = 11.8 Hz, 2H), 2.29 (d, J = 6.8 Hz, 2H), 2.26 (s, 3H), 2.03 (d, J = 7.0 Hz, 2H), 1.74–1.61 (m, 6H), 1.52 (d, J = 12.6 Hz, 2H), 1.43 (m, 1H), 1.28–1.08 (m, 7H), 0.87–0.77 (m, 2H); 13C-NMR (CDCl3, 100 MHz): 168.5, 156.4, 146.8, 135.1, 130.9, 129.6, 119.8, 119.4, 112.9, 103.8, 66.2, 55.8, 54.5 (2C), 38.9, 37.0, 35.3, 32.3 (2C), 32.1 (2C), 31.2, 26.8, 26.2 (2C), 25.2; MS [M + H]+ = 472.92; IR (KBr, cm⁻¹): 3420, 2921, 2849, 1694, 1625, 1584, 1527, 1449, 1388, 1243; HRMS [M + H]+ calcd for C26H34N2O3, 473.2683, exp 473.2682.

N-(2-chloro-4-(3-((1-cyclohexylmethyl)piperidin-4-yl)propanoyl)-5-methoxyphenyl)acetamide 7. From 81 mg of compound 4 (0.185 mmol), 34 mg of compound 7 were obtained as a beige gum. Yield = 42%; 1H-NMR (CDCl3, 400 MHz): 8.26 (s, 1H), 7.77 (s, 1H), 7.75 (br s, 1H), 3.90 (s, 3H), 2.93 (t, J = 7.6 Hz, 2H), 2.83 (d, J = 11.0 Hz, 2H), 2.25 (s, 3H), 2.05 (d, J = 7.1 Hz, 2H),
1.79–1.45 (m, 10H), 1.23–1.05 (m, 8H), 0.83 (m, 2H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): 200.1, 168.6, 158.4, 138.6, 130.6, 123.5, 113.5, 103.9, 66.2, 55.9, 53.3 (2C), 41.2, 35.7, 35.2, 32.2 (2C), 32.1 (2C), 31.0, 26.8, 26.2 (2C), 25.2; MS [M + H]$^+$ = 434.82; IR (KBr, cm$^{-1}$): 3435, 2921, 2849, 1704, 1672, 1599, 1578, 1512, 1450, 1401, 1263, 1241, 1176, 1014; HRMS [M + H]$^+$ calcd for C$_{24}$H$_{33}$ClN$_2$O$_3$, 435.2414, exp 435.2415.

N-(2-chloro-5-methoxy-4-(1-methyl-5-((1-(3-methylbenzyl)piperidin-4-yl)methoxy)-1H-pyrazol-3-yl)phenyl)acetamide 12b. From 70 mg of compound 10b (0.126 mmol), 45 mg of compound 12b were obtained as a beige solid. Yield = 64%; m.p. = 174–176 °C; $^{1}$H-NMR (CDCl$_3$, 400 MHz): 8.22 (s, 1H), 7.90 (s, 1H), 7.67 (brs, 1H), 7.21 (t, $J = 7.4$ Hz, 1H), 7.14–7.06 (m, 3H), 6.00 (s, 1H), 3.93 (d, $J = 6.3$ Hz, 2H), 3.91 (s, 3H), 3.67 (s, 3H), 3.48 (s, 2H), 2.94 (d, $J = 11.2$ Hz, 2H), 2.35 (s, 3H), 2.25 (s, 3H), 2.00 (td, $J = 11.9$ Hz, $J' = 2.5$ Hz, 2H), 1.85–1.77 (m, 3H), 1.44 (qd, $J = 11.9$ Hz, $J' = 3.7$ Hz, 2H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): 168.4, 155.7, 154.7, 144.7, 138.2, 137.8, 134.4, 130.0, 128.1, 127.8, 127.4, 126.3, 119.4, 113.7, 104.3, 86.0, 76.2, 63.5, 56.0, 53.3 (2C), 35.8, 33.7, 28.9 (2C), 25.1, 21.4; MS [M + H]$^+$ = 496.82; IR (KBr, cm$^{-1}$): 3415, 2937, 1694, 1680, 1585, 1557, 1527, 1449, 1361, 1243; HRMS [M + H]$^+$ calcd for C$_{27}$H$_{33}$ClN$_4$O$_3$, 497.2319, exp 497.2319.

4-(((1-methyl-3-phenyl-1H-pyrazol-5-yl)oxy)methyl)-1-(3-methylbenzyl)piperidine 12c. From 62 mg of compound 10c (0.126 mmol), 20 mg of compound 12c were obtained as an oil. Yield = 32%; $^{1}$H-NMR (CDCl$_3$, 400 MHz): 7.73 (m, 2H), 7.37 (m, 2H), 7.27 (t, $J = 7.5$ Hz, 1H), 7.15 (s, 1H), 7.13 (d, $J = 7.5$ Hz, 1H), 7.08 (d, $J = 7.5$ Hz, 1H), 5.80 (s, 1H), 3.94 (d, $J = 6.4$ Hz, 2H), 3.68 (s, 3H), 3.52 (s, 2H), 2.98 (d, $J = 10.7$ Hz, 2H), 2.35 (s, 3H), 2.04 (t, $J = 1.6$ Hz, 2H), 1.86 (m, 1H), 1.80 (d, $J = 13.5$ Hz, 2H), 1.48 (m, 2H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): 155.3, 149.3, 137.9, 137.6, 133.9, 130.1, 128.5 (2C), 128.1, 127.9, 127.5, 126.5, 125.2 (2C), 82.0, 76.2, 63.4, 53.2 (2C), 35.7, 33.8, 28.7 (2C), 21.4; MS [M + H]$^+$ = 376.00; HRMS [M + H]$^+$ calcd for C$_{24}$H$_{32}$N$_2$O$_3$, 376.2389, exp 376.2392.

4-(((3-(3,4-dimethoxyphenyl)-1 methyl-1H-pyrazol-5-yl)oxy)methyl)-1-(3-methylbenzyl)piperidine 12d. From 70 mg of compound 10d (0.126 mmol), 35 mg of compound 12d were obtained as a pale brown oil. Yield = 67%; $^{1}$H-NMR (CDCl$_3$, 400 MHz): 7.33 (d, $J = 2.0$ Hz, 1H), 7.24–7.19 (m, 2H), 7.14–7.06 (m, 3H), 6.87 (d, $J = 8.4$ Hz, 1H), 5.73 (s, 1H), 3.95 (s, 3H), 3.93 (d, $J = 6.4$ Hz, 2H), 3.90 (s, 3H), 3.67 (s, 3H), 3.49 (s, 2H), 2.95 (d, $J = 11.7$ Hz, 2H), 2.35 (s, 3H), 2.00 (td, $J = 11.7$ Hz, $J' = 2.4$ Hz, 2H), 1.85 (m, 1H), 1.81 (d, $J = 13.0$ Hz, 2H), 1.44 (qd, $J = 12.3$ Hz, $J' = 3.7$ Hz, 2H); $^{13}$C-NMR (CDCl$_3$, 100 MHz): 155.3, 149.2, 149.0, 148.7, 138.1, 137.8, 130.0, 128.1, 127.8, 127.1, 126.4, 117.8, 111.0, 108.1, 81.7, 76.3, 63.5, 55.9, 55.9, 53.2 (2C), 35.8, 33.7, 28.8 (2C), 21.4; MS [M + H]$^+$ = 435.94; IR (KBr, cm$^{-1}$): 2935, 1609, 1589, 1558, 1525, 1466, 1435, 1258, 1234, 1029; HRMS [M + H]$^+$ calcd for C$_{26}$H$_{34}$N$_3$O$_3$, 436.2600, exp 436.2596.

3.2. Biological Evaluation

3.2.1. In Vitro Tests of AChE Biological Activity

Inhibitory capacity of compounds on AChE biological activity was evaluated through the use of the spectrometric method of Ellman [24]. Acetylthiocholine iodide and 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) were purchased from Sigma Aldrich. AChE from human erythrocytes (buffered aqueous solution, $\geq 500$ 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 1 mM acetylthiocholine iodide solution. 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 using a 96-well microplate plate reader (Synergy 2, Biotek, Colmar, 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 acetylthiocholine, 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: \(100 - \left(\frac{v_i}{v_0} \times 100\right)\) where \(v_i\) is the rate calculated in the presence of inhibitor and \(v_0\) is the enzyme activity.

First screening of AChE activity was carried out at a \(10^{-6}\) 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 GraphPad Prism 6 software.

3.2.2. Pharmacological Characterization of Drugs on Human 5-HT₄R

For competition studies, 2.5 µg of proteins (5-HT₄(b) membrane preparations, HTS110M, Eurofins. Eurofins’ 5-HT₄(b) 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 \(10^{-8}\) M of each drug (RS67333 was used as a reference standard) and 0.5 nM [³H]-GR113808 (NET 1152, Perkin Elmer) 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 1 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.

3.2.3. Pharmacological Characterization of Drugs on Human 5-HT₆R

Drugs were evaluated through their possibility to compete for the binding of [³H]-LSD on membranes of HEK-293 cells transiently expressing the human 5-HT₆ receptors (ref. RBHS6M, Perkin Elmer). In brief, 4 µg of proteins were incubated at 37 °C for 60 min in duplicate in the absence or the presence of \(10^{-6}\) or \(10^{-8}\) M of each drug (serotonin was used as a reference standard) and 2.5 nM [³H]-LSD (ref. NET638250UC, Perkin Elmer), in 25 mM Tris-HCl buffer (pH 7.4) supplemented with 0.5 mM EDTA. At the end of the incubation, the homogenates were then filtered through Whatman GF/C filters and washed five times with ice-cold 25 mM Tris-HCl buffer. Non-specific binding was evaluated in the presence of 100 µM serotonin. Radioactivity associated to proteins was then quantified and expressed as the percentage of inhibition of the drugs under study.

For some of these compounds, affinity constants were calculated from five-point inhibition curves using the GraphPad Prism 6 software and expressed as Ki ± SD.

3.3. In Silico Study

The X-ray structure of donecopride was used as a 3D model during the docking and four conformers present in the crystal cell were used. The initial model of compounds 7, 3b, and 12b were built from donecopride X-ray structure [6]. The compound’s protonation state at pH 7.4 was predicted using standard tools of the ChemAxon Package (http://www.chemaxon.com/, accessed on 23 February 2021). The majority microspecie protonated on piperidine nitrogen at this pH was used for docking studies of each compounds.

The crystallographic coordinates of human acetylcholinesterase used in this study were obtained from X-ray structure of the donepezil/AChE complex (PDB ID 4EY7, a structure refined to 2.35 Å with an R factor of 17.7%) [25]. The AChE amino acid protonation state was checked before the docking study using the ProPKA software and the proposed protonation for Glu202 was applied.

The docking of the donepezil, donecopride, and compounds 3b, 7, and 12b into the AChE was carried out with the GOLD program (v2020.2.0) using the default parameters [26,27]. 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 used. The binding site in the AChE model was defined as a 7 Å sphere from the co-crystallized donepezil ligand and a water molecule interacting with protonated piperidine ring of donepezil was conserved during the docking (residue number 931). The second docking with the higher number of docking cycles (20 cycles) was carried out for compound 3b.

For the docking studies into 5-HT$_4$ receptor, the 3D model of the human 5-HT$_4$R built previously by homology sequence approach [28] was used. This model was generated using the crystal structure (PDB: 2RH1) of the human $\beta_2$ adrenergic receptor-T4 lysozyme fusion protein complexed with the carazolol [29] as a template. The docking of the donecoppride and 7, 3b, 12b compounds into the generated model was carried out with the GOLD program (v2020.2.0) using the default parameters [26,27] and the proposed ligand positions were evaluated by the ChemPLP fitness function. The binding site in the 5-HT$_4$R model was defined as a 10 Å sphere centered on the aspartic acid residue Asp$_{100}$. As the mutagenesis studies have shown that the interaction between the positively ionizable amine of ligands and Asp$_{100}$ of 5-HT$_4$R is crucial for ligand binding, a hydrogen bond constraint between positively ionizable amine ligand and OD atom of Asp$_{100}$ was used during the docking [30]. Furthermore, special attention was paid during the docking procedure to the following amino acids in the binding site, which were kept flexible: Arg$_{96}$, Asp$_{100}$, Thr$_{104}$, Tyr$_{192}$, Ser$_{197}$, and Trp$_{294}$.

For the docking studies on the 5-HT$_6$ receptor first a homology model was built. The sequence of the human 5-HT$_6$R was retrieved from the UniProt Knowledgebase (UniProtKB) [31] (ID: P50406_HUMAN). Using screening methods like FUGUE [32], SP3 [33], PSIBLAST, [34,35] HHSEARCH [36], and the @tome-2 server [37], the $\beta_2$ adrenergic receptor was identified as the best 3D experimental template for the homology modeling also for the 5-HT$_6$R (Sequence identity = 32%). The high-resolution (2.4 A) crystal structure of the human $\beta_2$ adrenergic receptor ($\beta_2$AR)-T4 lysozyme fusion protein bound to the carazolol (PDB: 2RH1) [29] was used as the 3D template. The alignment between the two sequences was manually optimized to avoid insertions and deletions in secondary structure elements. Disulfide bond: Cys$_{99}$-Cys$_{180}$ between the transmembrane helix 3 (TM3) and the extracellular loop (ECL2) was conserved. This alignment (Figure S2) was used as the basis for the homology modeling with the Modeller software [38]. The resulting model was then evaluated by methods like verify3D [39] and Eval23D [40].

The docking of the donecoppride and 7, 3b, 12b compounds into the generated model was carried out with the GOLD program (v2020.2.0) [26,27] using the same procedure as for 5-HT$_4$R. The binding site in the 5-HT$_6$R model was defined as a 10 Å sphere centered on the cysteine residue Cys$_{110}$. A hydrogen bond constraint between positively ionizable amine ligand and OD atom of Asp$_{100}$ was also applied during the docking and the following amino acids were kept flexible: Trp$_{102}$, Asp$_{106}$, Ser$_{193}$, Thr$_{196}$, and Trp$_{307}$.

4. Conclusions

In conclusion, we prepared two novel series of conformationally constrained donecoppride analogs using a late stage functionalization process. The introduction of the novel pyrazole ring instead of the ketone linked reduces the ability of novel ligands to interact both with AChE and 5-HT$_4$R. These results were confirmed thanks to our docking study. The replacement of the cyclohexyl moiety of donecoppride with a $m$-tolyl group led, however, to a novel phenyl pyrazole 3b as a dual active compound toward AChE and 5-HT$_4$R. According to our docking studies, the introduction of a substituted benzyl moiety on the piperidine ring enhanced the ability of 3b to interact with AChE and 5-HT$_4$R active site thanks to stronger hydrophobic interactions. This preliminary result is of clear interest in the journey toward the identification of valuable pleiotropic compounds for the treatment of neurodegenerative diseases. Indeed, the simultaneous modulation of 5-HT$_6$R and AChE could lead to an interesting synergistic effect in the treatment of AD, as demonstrated with the use of idalopirdine and donepezil in the clinical trials.
Based on these promising preliminary results, a future pharmacomodulation of 3b will be envisaged in order to increase its interaction with 5-HT6R and with AChE.

**Supplementary Materials:** The following data are available online. Including analytical spectrum of the compounds, superimposition of 7, 12b and 3b with donecopride in AChE binding site and amino-acid sequences alignment of 5-HT6R and human β2-adrenergic receptor. Reference [41] is cited in the Supplementary Materials.

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**Sample Availability:** Samples of the compounds are available from the authors.

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