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Title: Synthesis, biological evaluation and molecular modeling studies of phthalazin-1(2H)-one derivatives as novel cholinesterase inhibitors

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ABSTRACT

A new series of donepezil analogues based on phthalazin-1(2H)-one scaffold was designed and synthesized with the aim of exploring its potential as human ChEIs. The biological results revealed that the structural modifications proposed significantly affected ChE inhibitory potency as well as selectivity AChE/BuChE. Compound **1d** showed promising *in vitro* inhibition of both enzymes, in the µM range. However, most of target compounds were significantly more active against AChE than BuChE, being compounds **1f**, **1h** and **1j**, with IC_{50} values in the low micromolar or submicromolar range, the most active compounds in this series.

Docking simulations suggested that the most active compounds can recognize the binding site of donepezil using a similar interactions network. These results allowed us to rationalize the observed structure-activity relationships. Moreover, the predicted physicochemical and ADME properties were also comparable with those of donepezil.

Keywords: Phthalazinone derivatives, Acetylcholinesterase, Butyrylcholinesterase, Molecular modeling, ADME, Alzheimer's disease

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1. Introduction

Alzheimer's disease (AD) is a complex and progressive neurodegenerative disorder of the central nervous system that constitutes the most common type of dementia in the world. The prevalence of AD, which increases with age, ranges from 1-2% at age 65 years to 35% or higher at age 85 years [1]. Currently, it is estimated that nearly 40 million people worldwide are affected from AD, and the number of patients would be probably increased over the upcoming years because of the rise of life expectancy [2].

This disorder is characterized by a decreased number of brain cholinergic neurons in the hippocampus and frontal cortex, which is clinically reflected in specific symptoms, such as the progressive impairment in memory and intellectual ability to perform basic activities of daily living [1]. Although, the etiology of AD is not yet known, extracellular deposits of aberrant proteins, namely β-amyloid (Aβ) and τ-protein, oxidative stress and neurotoxicity, both related to a dysfunction of the glutamate neurotransmission system, dyshomeostasis of biometals and low levels of acetylcholine (ACh) seems to play significant roles in the pathophysiology of the disease [1,3]. The lack of this pivotal information explains that the pharmacological approaches, in current use, improve symptoms but do not have profound disease-modifying effects [1,4]. They are based on targeting neurotransmitter dysfunctions including acetylcholinesterase inhibitors (AChEIs), and N-methyl-D-aspartic acid (NMDA) glutamate receptor antagonists. Five drugs have been approved by US Food and Drug Administration (FDA) for AD, four AChEIs, tacrine, donepezil, rivastigmine and galantamine, and one NMDA antagonist, memantine.

However, studies developed in the past decades indicate that the acetylcholinesterase (AChE) in AD brains, besides catalysing the hydrolysis of acetylcholine, plays also an important role in Aβ plaques deposition because of its interaction with the Aβ peptide through a set of amino acids located close to the peripheral anionic site (PAS) of the enzyme [5], being proved that AChEIs which bind to PAS could inhibit such processes [6]. Furthermore, the new findings about the roles of the neuronal and nonneuronal cholinergic systems on modulation of regional brain blood flow may also contribute to get better potential for cholinergic therapies in AD [7].

In addition, the levels of ACh are also regulated by butyrylcholinesterase (BuChE), another cholinesterase enzyme with a synaptic ACh hydrolysis role less important than AChE in healthy brains. Recent studies indicate that in AD the AChE activity remains unchanged or even declines while activity of BuChE progressively increases, suggesting

that BuChE inhibition may also be considered a valid approach for AD therapy [8]. The BuChE role in regulation of cholinergic transmission is not yet fully understood and AChE currently remains as the main target. Nevertheless, this new scenario has encouraged the search for drugs inhibiting both cholinesterase enzymes (ChE), AChE and BuChE [9].

Donepezil, a selective inhibitor of AChE acting as dual binding site ligand [10], is the only one of four FDA approved AChEIs which binds simultaneously to CAS (catalytic active site) and PAS sites providing moderate inhibition of the Aβ aggregation [11]. In this respect, recent clinical trials have shown that a continued treatment with donepezil was associated with significant cognitive and functional benefits in patients suffering moderate to severe AD [12].

X-ray crystallographic data of AChE/donepezil complexes together with structure-activity relationships (SAR) results for donepezil-like compounds, indicate that the N-benzylpiperidine group and the dimethoxy benzoyl fragment are key structural features for both dual binding interaction (CAS and PAS sites) and AChE inhibition [13].

Taking into account the growing interest in cholinesterase inhibitors [12,14,15], it is desirable to study new pharmacophoric scaffolds. In this regard, pyridazine could be considered a privileged structure because its derivatives present a large therapeutic potential [16]. Although, several pyridazine derivatives were reported as potential dual binding site AChEIs, most of them show 3,6-disubstitutions on the pyridazine ring [17] and only a few include in their structure the pyridazin-3-(2H)-one framework [18]. However, these last pyridazinone analogues do not share any structural similarity with donepezil.

Considering the above mentioned and as a continuation to our work focused on developing enzyme inhibitors potentially effective in aging-related disorders [19], we have designed a series of donepezil analogues with structure **1** (Figure 1). In these novel compounds the indanone portion was replaced by a phthalazin-1(2H)-one moiety, preserving or not the methoxy groups, and the linking chain between the new bicyclic fragment and N-benzylpiperidine group was ranged from one to three carbon atoms. In addition, the C4 position of this new scaffold could be substituted with groups of different size and electronic properties. These structural modifications were performed with the idea of analyzing the ability of the phthalazinone nucleus to modulate the AChE and BuChE activities.

Figure 1 comes about here

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Thus, in this work we report the synthesis, the AChE and BuChE inhibitory activities and the molecular modeling of representative molecules of a new family of phthalazin-1(2H)-ones (compounds **1**, Figure 1). The molecular modeling studies were performed in order to compare the binding mode and ADME properties of novel compounds with donepezil.

2. Results and discussion

2.1. Chemistry

The proposed strategy to synthesize the designed compounds **1a-j** involves the N-alkylation of corresponding phthalazinone (compounds **10a-f**) with the adequate bromoalkane [20]. Phthalazinones **10a**,**b**, precursors of analogues **1a-d** and **1i**, were commercially available, while phthalazinones **10c-f**, required for preparing the derivatives **1e-h** and **1j**, were synthesized as displayed in Scheme 1. The 4-methylphthalazinone **10c** was obtained in good yield by direct cyclization of 2-acethyl benzoic acid **8** with hydrazine hydrate in ethanol, while, the synthesis of phthalazinones **10d-f**, showing one or two methoxy groups on the phenyl ring, was accomplished adapting several procedures previously described and by using the phthalides **5** as key intermediates, which via benzylic bromination provided the 3-bromo phthalides **9** suitable for cyclization with hydrazine [21].

Scheme 1 comes about here

The 5,6-dimethoxyphthalide **5a** and 6-methoxyphthalide **5b** were synthesized in good to moderate yield by chloroformylation of 3,4-dimethoxybenzoic and 3-methoxybenzoic acids **7a** and **7b,** respectively [22]. However, a multi-step strategy was required to obtain the 5-methoxyphthalide **5c**. The synthetic sequence used involved the alkaline hydrolysis of dimethyl 4-methoxy phthalate **2** to give the dicarboxylic acid **3** and subsequent dehydration of **3** with an excess of acetic anhydride, followed by reduction with $NaBH_4$ of the phthalic anhydride **4** thus obtained [23].

The reaction of 4-substituted phthalic anhydrides with alkaline borohydrides usually provided a mixture of phthalide regioisomers of varying proportions which is consequence of substituent electronic effects. Thus, when the substituent at C4 is an electron-donating group, was detected a preference for reduction at *m*-carbonyl group [24]. In contrast with literature, the treatment of 4-methoxyphthalic anhydride **4** with an equimolecular amount of $NaBH_4$ gave a mixture of compounds, lactone **5c** and hydroxymethyl benzoic acid **6** (ratio**5c**/**6** 1:1.5, very good yield), both resulting from the selective nucleophilic attack by hydride on the *m*-carbonyl carbon. Although compounds **5c** and **6** were initially separated by column chromatography and characterized (method A), the mixture of both compounds was refluxed with hydrochloric acid in order to complete the lactonization of hydroxymethyl benzoic acid **6** (method B). The subsequent radical bromination of phthalides **5a-c** with N-bromosuccinimide (NBS) in carbon tetrachloride produced 3-bromophthalides **9**, which were directly converted into phthalazinones **10d**,**f** by condensation with hydrazine hydrate in ethanol.

Then, the target compounds **1a-h** were prepared in four steps using as starting material the phthalazinones **10** and the commercially available *N*-Boc-4-hydroxyalkylpiperidines **11** (Scheme 2). Thus, firstly, the *N*-Boc-4-hydroxyalkylpiperidines **11a**,**b** were transformed into the corresponding 4-bromoalkylpiperidines **12a**,**b** by treatment with carbon tetrabromide and triphenylphosphine in dichloromethane at reflux. Then, phthalazinones **10a-f** were *N*-alkylated with the appropriate bromoalkyl derivative **12a**,**b** and sodium hydride in dimethylformamide. Finally, the removal of *N*-Boc protecting group from the corresponding 2-(*N*-Boc-4-piperidinylalkyl)phthalazin-1-ones **13a-h** by acid hydrolysis, with 6M HCl in ethyl acetate, was followed by treatment with benzyl bromide in the presence of sodium hydride to give desired compounds **1a-h** in good to moderate yield.

Scheme 2 comes about here

Finally, in scheme 3 was detailed the strategy adopted for the synthesis of target compounds **1i**,**j** bearing an alkyl chain of three carbons between phthalazinone and N-benzylpiperidine fragments.

Scheme 3 comes about here

In this case, the bromoalkane **18** was the reagent involved in N-alkylation of phthalazinones **10a** and **10d**. The compound **18** was synthesized in good yield starting from the commercially available 3-(pyridin-4-yl)propanol **15**. The pyridine ring was reduced via catalytic hydrogenation at atmospheric pressure, under acidic conditions and using platinum (IV) oxide as catalyst, to give **16** as a hydrochloride [25]. The resulting piperidine derivative was N-benzylated by treatment with benzyl bromide in ethanol, in the presence of K_2CO_3, to give **17**, which was converted into bromoalkane **18** by reaction with carbon tetrachloride and triphenylphosphine as described for bromo derivatives **12a-b**.

2.2. Pharmacology

The *in vitro* activity of compounds **1a-j** against AChE and BuChE was determined by Ellman´s method [26] evaluating the hydrolysis of acetylthiocholine and butyrylthiocholine, respectively. The activity was measured by the increased in absorbance at 412 nm due to the yellow color of 5-mercapto-2-nitrobenzoic acid produced by reaction of thiocholine with dithiobisnitrobenzoic acid (DNTB). Thiocholine was generated from acetylthiocholine using AChE isolated from human erythrocytes or from butyrylthiocholine using BuChE from human serum.

The assays were performed with a blank containing all components except AChE or BuChE in order to account for nonenzymatic reaction. The reaction rates were compared and the percent of inhibition due to the presence of test compounds was calculated. The ChE inhibitory activity of target compounds **1a-j** and reference drugs (tacrine and donepezil) was expressed as IC_{50} and these data are summarized in Table 1.

Enzymatic assays revealed that most of the designed compounds showed moderate to good activity with IC_{50} values for AChE and BuChE inhibition ranged from 10.29 to 0.55 μM and from 64.69 to 5.50 μM, respectively. At the same time the tested compounds, with the exception of compounds **1a**, **1c** and **1d**, were remarkably more active against AChE than BuChE. The methoxy substituted compounds **1f**, **1h** and **1j**, with IC_{50} values from submicromolar to low micromolar range, were the most potent AChEIs of this series, resulting almost equipotent with tacrine but slightly less active than donepezil.

The three kind of structural modifications proposed, length variation of alkyl chain linking phthalazinone and benzylpiperine scaffolds, substitution at C4 and inclusion of

methoxy groups in C6 or C7, significantly affected ChE inhibitory potency as well as selectivity AChE/BuChE.

Thus, compounds **1a** and **1c** (both with n = 1) are inactive against AChE at 100 μM, the highest concentration tested, and moderately active against BuChE, with IC_{50} values of 46.14 and 13.26 μM, respectively. By contrast, their homologues **1b**, **1d** and also the analogue **1e** (all them with n = 2) exhibited inhibitory activity against the AChE at low micromolar concentrations, being compound **1b** the most active with an IC_{50} value of 2.58 μM. Compound **1e** (IC_{50} = 2.79 μM), showing a methyl group at C4, was equipotent to **1b** and more potent than compound **1d** (C4 = *p*-Tol, IC_{50} = 3.45 μM). In addition, compound **1d** was the only inhibitor that was equipotent for AChE and BuChE enzymes, behaving as a dual cholinesterase inhibitor (AChE IC_{50} = 3.45 μM and BuChE IC_{50} = 5.50 μM). However, a further elongation of the alkyl linker, such as in compound **1i** (n =3) resulted disadvantageous for AChE inhibition (IC_{50} = 10.29 μM).

With regard to the role of methoxy substituents at C6 and C7 in this new series, it can be noticed that when it is placed at C6, the methoxy group provides a good and selective inhibitory effect against AChE, being compounds **1f** (IC_{50} = 0.67), **1h** (IC_{50} = 0.55) and **1j** (IC_{50} = 1.07) the most potent and selective AChEIs of this series, while when it was moved to C7 position the AChE inhibitory activity seems unaffected (compound **1g**, IC_{50} = 2.40 μM).

Table 1 comes about here

2.3. Molecular Modeling

Dockings studies were carried out in order to analyze the AChE binding mode of the novel inhibitors with the aim to interpret the experimental affinity data.

To identify which docking protocol and which protein-ligand complex were more suitable for our analogues, we performed a benchmark study based on the self-docking procedure. We selected two protein-ligand complexes from the protein data bank (PDB): the complex of human AChE (hAChE) with donepezil (PDB ID: 4EY7), and the complex of human BuChE (hBuChE) with tacrine (PDB ID: 4BDS). The complex including donepezil was particularly interesting due to the molecular similarity with our analogues. The benchmark study revealed that several protocols were able to reproduce

the crystallographic geometries (see the Experimental Section for details). Among them, GOLD software when using ChemPLP as scoring function, gave the best performance (SI Fig.1, panels A and B). In particular, this protocol was able to reproduce both donepezil and tacrine with a RMSD values of 0.34 Å and 0.41 Å, respectively (SI Fig.1, panels C and D).

The selected protocol was used to dock all the analogues into hAChE and hBuChE as described in detail in the Experimental Section.

All analogues showed a common binding mode similarly to the donepezil one (Fig. 2). The N-benzylpiperidine group adopts the same orientation in the active site gorge mainly interacting with Trp86, Tyr337, Phe338 and Ile451. The phthalazin-1(2H)-one moiety mimics the indanone group of donepezil that is placed in the binding site entrance formed by Tyr72, Tyr124, Trp286 and Tyr341 sidechains and the backbone of Phe295 and Arg296.

Based on our docking simulation, all the compounds preserve this network of interactions (Figure 3), as corroborated by the interaction energy values reported in Table 2, with small but significant dissimilarities. While the two important π-π interactions between the indole ring of Trp286 and the phthalazin-1(2H)-one moiety and between the indole ring of Trp86 and N-benzylpiperidine moiety are maintained over the series, one hydrogen bond between the carbonyl oxygen of phthalazin-1(2H)-one and the amide nitrogen of Phe295 is preserved only in compounds with an IC_{50} minor than 10 μM. This hydrogen bond is also present in the crystal structure of donepezil bound to hAChE, mediated by the indanone core.

This observation is more evident focusing on the per-residue analysis (Figure 3), in which contribution to the interaction is computed for each singular residue. In our case, we measured the electrostatic interaction energy and a score taking into account hydrophobic interactions. In Figure 3, is reported the per-residue analysis (panel B) for a subset of relevant residues taking into account their interaction and their conformation respect to the most potent analogue (compound **1h**, Panel A). The minor activity of compounds **1a** and **1c** could be attributed to the lacks of this hydrogen bond. The bulky substituent at the 4-position of the phthalazinone moiety is not well tolerated into the binding pocket, in terms of shape complementary and lead to a slightly different orientation of the phtalazinone core (Fig. 4; **1c** and **1d**). However, in the case the of compound **1d** the longer alkyl linker allows to stabilize the ligand through a similar hydrogen bond with the Arg296 instead of Phe295, and at the same time to maintain the

benzyl group stacked to Trp86, similarly to the other analogues without the *p*-tolyl moiety. The effect of the different linker length between compound **1c** and **1d** is evident observing the difference in the per-residue profile (residues Trp86 and Arg296) and is in good agreement with the experimental IC_{50} values. On the other hand, the *p*-tolyl moiety provides an additional hydrophobic interaction with Trp286 and Leu289 (Fig. 3). The per-residue analysis also highlights the role of methoxy substituent at C6 (compounds **1f**, **1h** and **1j**) and methyl group at C4 (compound **1e**). These compounds neither establish hydrophobic interaction by the methyl moiety nor are involved in hydrogen bond through the ether oxygen atom of methoxy substituent. However, their steric effect improves the orientation of the carbonyl group involved in the hydrogen bond with Phe295, leading to a stronger electrostatic interaction (Fig. 3). Only for compound **1i**, the molecular docking studies have not shown a clear relationship between the complex conformation and the IC_{50} values; the ligand orientation is extremely similar to those of the most active analogues but it lacks in the formation of the H-bond with the Phe295.

The docking studies performed on the hBuChE revealed an overall decrease in scores (Table 2). In this case, while the N-benzylpiperidine portion still occupies the same site as in hAChE, which is also the binding site of tacrine, the phthalazinone core adopts a different conformation, with the plane of the fused rings having an orthogonal angulation respect to the conformation assumed when complex with hAChE (Fig. 5, panel A). The reason of this orientation can be ascribed to the few differences between the two binding sites. Despite the notable similarity between both enzymes (Cα-RMSD 1.36 Å, SI Fig. 1 panel E), they show differences in some fundamental residue for the binding of the phthalazinone group. Thus, the Trp286 that establish the strong stacking interaction is replaced by an alanine. In addition, the amide involved in the hydrogen bond in hAChE (Phe295) is located in a loop that presents a different conformation in hBuChE (Leu286) not compatible with the hydrogen bond formation due to a distortion induced by Pro285, which is not present in hAChE (SI Fig.1, panel E and F).

Interestingly, the analogues showing the *p*-tolyl moiety at C4 present best IC_{50} values for hBuChE. The docking studies revealed that the *p*-tolyl moiety occupy an additional hydrophobic cavity, delimited by Trp231, Phe398, Leu286 and Ala199 (Fig. 5, panel B).

In addition, theoretical calculations were also performed using the StarDrop software to predict physicochemical and ADME (absorption, distribution, metabolism and

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excretion) properties of the novel AChE inhibitors, such as, aqueous solubility (logS), lipophilicity (clogP), cytochrome P450 metabolism (2D6 and 2C9), human Ether-à-go-go Related Gene (hERG) channel inhibition, blood brain barrier (BBB) penetration, human intestinal absorption (HIA), P-glycoprotein binding (P-gp) and plasma protein binding (PPB90). Interestingly, most of the physicochemical and ADME properties of the synthetized compounds were comparable with those of donezepil in particular for compound **1b** and its methoxy derivatives **1f**, **1g**, **1h**, all them potent and selective AChEIs (Table 3).

Table 3 comes about here

3. Conclusions

A series of donepezil analogues based on phthalazin-1(2H)-one scaffold was designed and synthesized with the aim of exploring its potential as ChEIs. In the target compounds the indanone was replaced by the phthalazinone nucleus, preserving or not the methoxy groups, and showing the N-benzylpiperidine fragment linked at N2 by an alkyl chain ranged from one to three carbons. Additionally, The C4 position of the phthalazinone scaffold could be substituted with groups of varying size and electronic properties.

The biological data indicated that most of the designed compounds showed moderate to good activity as AChE and BuChE inhibitors. In addition, the target compounds, with the exception of compounds **1a**, **1c**, were remarkably more active against AChE than BuChE. Thus, compounds **1a** and **1c** (both with n = 1) were inactive against AChE and moderately active against BuChE, while compound **1d** (n =2, C4 = *p*-Tol) resulted equipotent toward AChE and BuChE enzymes, behaving as a dual cholinesterase inhibitor (AChE IC_{50} = 3.45 µM and BuChE IC_{50} = 5.50 µM).

As it can be seen, the AChE inhibition was severely affected by the length of the linking chain between the phthalazinone moiety and the N-benzylpiperidine fragment, since the compounds **1b** and **1d**, homologues of **1a** and **1c**, respectively, as well as the analogue **1e** (all them with n =2) preferentially inhibited AChE, with IC_{50} values ranged from 2.58 to 3.45 µM, while a decreased potency was observed for the homologue **1i** (IC_{50} =

10.29, n = 3). Finally, it is noteworthy the role of methoxy substituents in the activity of these new donepezil analogues. Therefore, when the methoxy group is located at C6 provides a good and selective inhibitory effect against AChE, being compounds **1f** (IC_{50} = 0.67), **1h** (IC_{50} = 0.55) and **1j** (IC_{50} = 1.07), the most potent and selective AChEIs of this series. They are almost equipotent with tacrine but less potent than donepezil. However, when the methoxy group was placed at C7 position the AChE inhibitory activity seems unaffected (compound **1g**, IC_{50} = 2.40 μM).

Docking simulations suggested that the most active compounds can recognize the binding site of donepezil using a similar interactions network. These results allowed us to rationalize the observed structure-activity relationships. In addition, docking analysis also indicated a plausible binding mode of dual inhibitor **1d** along the active site cavity of AChE and BuChE. Therefore, these novel phthalazinone analogues can be considered potential drug candidates to develop new ChEIs, also considering the predicted physicochemical and ADME properties.

4. Experimental section

4.1. Chemistry

All starting materials and common laboratory chemicals were purchased from commercial sources and used without further purification. All solvents were distilled and dried according to standard procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX400 instrument, using TMS as internal standard [chemical shifts (δ) in ppm, *J* in Hz]. The assignment of the signals was performed by COSY, DEPT, HSQC experiments. High resolution mass spectra were recorded using a Bruker microTOF focus spectrometer. Silica gel (Merck 60, 230–400 mesh) was used for flash chromatography (FC). Analytical TLC was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm).

*4.2. 4-Methylphthalazin-1(2H)-one (**10c**)*

To a solution of 2-acetylbenzoic acid (**8**) (1 g, 6.09 mmol) in EtOH (10 mL) was added hydrazine hydrate (0.39 mL, 7.84 mmol). The reaction mixture was refluxed overnight. After cooling, the resulting precipitate was filtered and washed with ethanol to give **10c**

(875 mg, 91%) as a white solid. R_f = 0.5 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$): δ = 8.49–8.45 (m, 1H, H8), 7.89–7.83 (m, 1H, Ar), 7.82-7.77 (m, 2H, Ar), 2.60 (s, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ = 160.8 (C1), 144.8 (C4), 133.6, 131.6, 130.5, 127.9, 127.0 (C8), 125.2, 18.9 (CH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9N_2O$: 161.07094, found: 161.07100.

*4.3. 4-Methoxyphthalic acid (**3**)*

A solution of dimethyl 4-methoxyphthalate (**2**) (1.1 g, 4.76 mmol) in MeOH (5 mL) was treated with aqueous 5M NaOH (3 mL, 15 mmol). The reaction mixture was refluxed overnight. After cooling at r.t., 3M HCl (5 mL) was carefully added until pH = 1 and the mixture was extracted with CH_2Cl_2 (3 x 20 mL), the organic layers were collected, dried over Na_2SO_4 and evaporated to dryness to give **3** (766 mg, 96%). ^{1}H NMR (CD_3OD): δ = 7.91 (d, 1H, *J* = 8.7 Hz, H6), 7.25 (d, 1H, *J* = 2.4 Hz, H3), 7.05 (dd, 1H, *J* = 8.7, 2.4 Hz, H5), 3.85 (s, 3H, OCH_3). ^{13}C NMR (CD_3OD) δ = 172.2 (CO), 169.8 (CO), 163.6 (C4), 138.0 (C2), 132.9 (C6), 123.7 (C1), 116.0 (C5), 114.8 (C3), 56.2 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9O_5$: 197.04445, found: 197.04512.

*4.4. 4-Methoxyphthalic anhydride (**4**)*

A solution of compound **3** (300 mg, 1.53 mmol) in acetic anhydride (5 mL) was refluxed for 1 h. After cooling at r.t., the solvent was removed under reduce pressure to furnish anhydride **4** (266 mg, 98%). ^{1}H NMR ($CDCl_3$): δ = 7.90 (d, 1H, *J* = 8.4 Hz, H6), 7.41 (d, 1H, *J* = 2.2 Hz, H3), 7.35 (dd, 1H, *J* = 8.4, 2.2 Hz, H5), 3.98 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ = 166.2 (C4), 163.0 (CO), 162.5 (CO), 134.0, 127.3 (C6), 123.1 (C5), 122.8, 109.1 (C3), 56.5 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_7O_4$: 179.03389, found: 179.03308.

*4.5. 5-Methoxyisobenzofuran-1(3H)-one (**5c**)*

Method A: to a solution of $NaBH_4$ (11 mg, 0.28 mmol) in THF (2 mL) cooled at 0 ºC was added dropwise a solution of compound **4** (50 mg, 0.28 mmol) in THF (3 mL). The reaction mixture was stirred for 4 h at r.t., treated with 6M HCl until pH = 1 and then

extracted with Et_2O (3 x 10 mL). The combined organic layers were collected, dried over Na_2SO_4 and concentrated under reduced pressure. The solid obtained was purified by column chromatography on silica gel (gradient elution: hexane/EtOAc 4:1 to hexane/EtOAc 1:1) to afford **5c** (15 mg, 33%) and **6** (26 mg, 52%). Compound **5c**: R_f = 0.5 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$): δ = 7.72 (d, 1H, *J* = 8.5 Hz, H7), 6.97 (dd, 1H, *J* = 8.5, 2.2 Hz, H6), 6.09-6.88 (m, 1H, H4), 5.19 (s, 2H, H3), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$, δ): 170.9 (C1), 164.7 (C5), 149.42 , 127.1 (C7), 118.0, 116.5 (C6), 106.0 (C4), 69.1 (C3), 55.9 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9O_3$: 165.05462, found: 165.05451. Compound **6**: R_f = 0.2 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$) δ = 8.00 (d, 1H, *J* = 8.7 Hz, H6), 7.23 (d, 1H, *J* = 2.6 Hz, H3), 6.87 (dd, 1H, *J* = 8.7, 2.6 Hz, H5), 4.92 (s, 2H, CH_2), 3.87 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ = 169.0 (CO), 163.2 (C4), 146.5, 133.3 (C6), 120.2, 112.6 (C3), 111.3 (C5), 62.5 (CH_2), 54.5 (OCH_3).

Method B: following the procedure above described and starting from **4** (190 mg, 1.07 mmol) a crude product containing compounds **5c** and **6** (160 mg) was obtained, which was dissolved in 6M HCl (7 mL) and refluxed for 2 h. After cooling, the reaction mixture was extracted with CH_2Cl_2 (6x12 mL). The combined organic layers were collected, dried over Na_2SO_4 and concentrated under reduced pressure. The solid residue was purified by column chromatography on silica gel (hexane/EtOAc, 4:1) to give **5c** (93 mg, 53%).

*4.6. 5,6-Dimethoxyisobenzofuran-1(3H)-one (**5a**)*

To a solution of 3,4-dimethoxybenzoic acid (**7a**) (50 mg, 0.27 mmol) in 37% HCl (0.75 mL) was added 30% formaldehyde (0.13 mL, 1.25 mmol). The reaction mixture was stirred at 90 ºC for 10 h. After cooling, was quenched with H_2O (5 mL) and extracted with EtOAc (4 x 5 mL). The combined organic layers were washed with 2.5 M NaOH (5 mL) and brine (5 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to afford **5a** (40 mg, 75%). R_f = 0.4 (hexane/EtOAc, 1:2). ^{1}H NMR ($CDCl_3$): δ = 7.10 (s, 1H, H7), 6.83 (s, 1H, H4), 5.08 (s, 2H, H3), 3.85 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$): δ = 171.2 (C1), 154.7, 150.2, 141.0, 117.2, 105.7 (C7), 103.5 (C4),

69.0 (C3), 56.2 (OCH_3), 56.0 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{10}H_{11}O_4$: 195.06519, found: 195.06570.

*4.7. 6-Methoxyisobenzofuran-1(3H)-one (**5b**)*

To a solution of 3-methoxybenzoic acid (**7b**) (1 g, 6.44 mmol) in glacial acetic acid (3.3 mL) was added 37% HCl (4.8 mL, 57.9 mmol) and 30% formaldehyde (1.92 mL, 25.8 mmol) and the reaction mixture was stirred at 100 ºC for 1 h. After cooling, a saturated solution of $NaHCO_3$ was added until pH = 7. The resulting mixture was extracted with CH_2Cl_2 (3 x 20 mL), the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc, 7:1) to afford **5b** (462 mg, 43%). R_f = 0.4 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$) δ = 7.36 (d, 1H, *J* = 8.4 Hz, H4), 7.29 (d, 1H, *J* = 2.2 Hz, H7), 7.22 (dd, 1H, *J* = 8.4, 2.2 Hz, H5), 5.24 (s, 2H, H3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ = 171.3 (C1), 160.6 (C6), 138.9, 127.0, 123.1 (C4), 123.0 (C5), 107.5 (C7), 69.6 (C3), 55.8 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9O_3$: 165.05462, found: 165.05455.

*4.8. General procedure for the preparation of phthalazin-1(2H)-ones (**10d-f**)*

To a solution of the required isobenzofuranone (**5a-c**) (0.51 mmol) in CCl_4 (15 mL) was added NBS (0.51 mmol) and a catalytic amount of benzoyl peroxide. The reaction mixture was stirred at refux for 3 h. After cooling, the mixture was filtered and evaporated to dryness to give a residue containing the corresponding 3-bromoisobenzofuranone (**9a-c**), which were solved in EtOH (5 mL), treated with hydrazine hydrate (1.95 mmol) and refluxed for 14 h. After cooling, the resulting solution was diluted with H_2O (3 mL) and the obtained solid material was filtered and dried to afford the desired phthalazin-1(2*H*)-one (**10d-f**).

*4.8.1. 6,7-dimethoxyphthalazin-1-(2H)-one (**10d**)*

White solid. Yield: 58%. R_f = 0.4 (EtOAc). ^{1}H NMR ($CDCl_3$) δ = 8.08 (s, 1H, H4), 7.78 (s, 1H, H8), 7.04 (s, 1H, H5), 4.05 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3). ^{13}C NMR

($CDCl_3$) δ: 160.3 (C1), 154.4, 153.1, 138.4 (C4), 125.8, 123.0, 106.4 (C5), 106.2 (C8), 56.7 (OCH_3), 56.5 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{10}H_{11}N_2O_3$: 207.07642, found: 207.07693.

*4.8.2. 7-methoxyphthalazin-1-(2H)-one (**10e**)*

White solid. Yield 51%. R_f = 0.4 (hexane/EtOAc, 1:1). ^1H NMR ($CDCl_3$) δ = 10.25 (s, 1H, NH), 8.11 (s, 1H, H4), 7.80 (d, 1H, *J* = 2.6 Hz, H8), 7.66 (d, 1H, *J* = 8.7 Hz, H5), 7.40 (dd, 1H, *J* = 8.7, 2.6 Hz, H6), 3.98 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ = 162.6 (C7), 160.6 (C1), 138.9 (C4), 130.1, 128.4 (C5), 124.5, 124.2 (C6), 106.5 (C8), 56.1 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9N_2O_2$: 177.06585, found: 177.06581.

*4.8.3. 6-methoxyphthalazin-1-(2H)-one (**10f**)*

White solid. Yield 65%. R_f = 0.4 (hexane/EtOAc, 1:1): 0.4. ^1H NMR ($CDCl_3$) δ = 10.29 (s, 1H, NH), 8.34 (d, 1H, *J* = 8.8 Hz, H8), 8.10 (s, 1H, H4), 7.33 (dd, 1H, *J* = 8.8, 2.4 Hz, H7), 7.05 (d, 1H, *J* = 2.4 Hz, H5), 3.96 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ = 163.9 (C6), 160.4 (C1), 138.9 (C4), 132.4, 128.7 (C8), 121.8, 121.1 (C7), 107.4 (C5), 55.9 (OCH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_9H_9N_2O_3$: 177.06585, found: 177.06502.

*4.9. General procedure for the preparation of 4-bromoalkyl-N-Boc-piperidines (**12a**,**b**)*

To a solution of compound **11a** or **11b** (1 mmol) in CH_2Cl_2 (5 mL), CBr_4 (2 mmol) and PPh_3 (2 mmol) were added and the reaction mixture was refluxed for 2 h. After solvent removal, the residue was purified by column chromatography on silica gel (hexane/EtOAc, 9:1) to afford the desired compound.

*4.9.1. 4-Bromomethyl-1-tert-butoxycarbonylpiperidine (**12a**)*

Colorless oil. Yield: 94%. R_f = 0.7 (hexane/EtOAc, 2:1). ^1H NMR ($CDCl_3$): δ = 4.22-4.02 (m, 2H, H2), 3.28 (d, 2H, *J* = 6.2 Hz, H1´), 2.75-2.61 (m, 2H, H2), 1.85-1.72 (m, 3H, H3, H4), 1.44 (s, 9H, 3xCH_3), 1.24-1.10 (m, 2H, H3). ^{13}C NMR ($CDCl_3$): δ = 154.9

(CO), 79.7 ($C(CH_3)_3$), 43.8 (C2), 39.0 (C1´), 38.8 (C4), 31.0 (C3), 28.6 ($(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{11}H_{21}BrNO_2$: 278.07557, found: 278.07502.

*4.9.2. 4-(2-Bromoethyl)-1-tert-butoxycarbonylpiperidine (**12b**)*

Colorless oil. Yield: 89%. R_f = 0.5 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$): δ = 4.18-4.01 (m, 2H, H2), 3.44 (t, 2H, *J* = 6.8 Hz, H2´), 2.74-2.64 (m, 2H, H2), 1.83-1.77 (m, 2H, H1´), 1.70-1.62 (m, 3H, H3, H4), 1.44 (s, 9H, 3xCH_3), 1.16-1.04 (m, 2H, H3). ^{13}C NMR ($CDCl_3$): δ = 155.0 (CO), 79.5 ($C(CH_3)_3$), 44.0 (C2), 39.3 (C1´), 34.5 (C4), 31.6 (C3), 31.2 (C2´), 28.6 ($(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{23}BrNO_2$: 292.09122, found: 292.09021.

*4.10. General procedure for the preparation of 2-(N-Boc-piperidin-4-ylalkyl)phthalazin-1(2H)-ones (**13a-h**)*

A solution of the corresponding phthalazinone (**10a-f**) (0.34 mmol) in DMF (0.8 mL) was added to a suspension of NaH (60% dispersion in mineral oil, 0.53 mmol) in DMF (0.8 mL). After stirring at r.t. for 1 h, a solution of **12a** or **12b** (0.38 mmol) in DMF (0.8 mL) was added. The reaction mixture was stirred at r.t. overnight, followed by quenching with H_2O (15 mL) at 0 ºC. The product was extracted with EtOAc (2x10 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc, 3:1) to obtain the desired compound.

*4.10.1. 2-(1-tert-butoxycarbonypiperidin-4-ylmethyl)phthalazin-1(2H)-one (**13a**)*

Colorless oil. Yield: 78%. R_f = 0.4 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$): δ = 8.42 (d, 1H, *J* = 7.7 Hz, H8), 8.15 (s, 1H, H4), 7.83-7.73 (m, 2H, Ar), 7.71-7.67 (m, 1H, Ar), 4.20-4.00 (m, 4H, H1´, H2´´), 2.73-2.60 (m, 2H, H2´´), 2.22-2.10 (m, 1H, H4´´), 1.68-1.58 (m, 2H, H3´´), 1.43 (s, 9H, 3xCH_3), 1.36-1.22 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.8 (C1), 154.9 ($COC(CH_3)_3$), 137.8 (C4), 133.3, 131.8, 129.7, 128.0, 126.9, 126.1,

79.4 ($C(CH_3)_3$), 56.3 (C1´), 43.8 (C2´´), 35.8 (C4´´), 29.8 (C3´´), 28.6 ($(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{19}H_{26}N_3O_3$: 344.19687, found: 344.19653.

*4.10.2. 2-(2-(1-tert-butoxycarbonylpiperidin-4-yl)ethyl)phthalazin-1(2H)-one (**13b**)*

Colorless oil. Yield: 99%. R_f = 0.4 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$): δ = 8.37 (d, 1H, *J* = 7.6 Hz, H8), 8.12 (s, 1H, H4), 7.79-7.68 (m, 2H, Ar), 7.67-7.63 (m, 1H, Ar), 4.24 (t, 2H, *J* = 7.4 Hz, H1´), 4.14-3.96 (m, 2H, H2´´), 2.71-2.56 (m, 2H, H2´´), 1.81-1.68 (m, 4H, H2´, H3´´), 1.51-1.41 (m, 1H, H4´´), 1.40 (s, 9H, 3xCH_3), 1.19-1.07 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.4 (C1), 155.0 ($COC(CH_3)_3$), 137.9 (C4), 133.1, 131.8, 129.7, 128.0, 126.8, 126.1, 79.3 ($C(CH_3)_3$), 48.9 (C1´), 44.1 (C2´´), 35.2 (C2´), 33.7 (C4´´), 32.1 (C3´´), 28.6 ($(CH_3)_3$). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{20}H_{28}N_3O_3$: 358.21252, found: 358.21164.

*4.10.3. 2-(1-tert-butoxycarbonylpiperidin-4-ylmethyl)-4-p-tolylphthalazin-1(2H)-one (**13c**)*

Colorless oil. Yield: 99%. R_f = 0.4 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$): δ = 8.52-8.47 (m, 1H, H8), 7.78-7.69 (m, 3H, Ar), 7.46 (d, 2H, *J* = 7.9 Hz, Ar), 7.32 (d, 2H, *J* = 7.9 Hz, Ar), 4.28-3.98 (m, 4H, H1´, H2´´), 2.75-2.60 (m, 2H, H2´´), 2.43 (s, 3H, CH_3), 2.28-2.15 (m, 1H, H4´´), 1.71-1.62 (m, 2H, H3´´), 1.42 (s, 9H, 3xCH_3), 1.38-1.25 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.4 (C1), 154.8 ($COC(CH_3)_3$), 146.9, 139.2, 132.8, 132.3, 131.4, 129.4, 129.1, 128.2, 127.2, 126.8, 79.2 ($C(CH_3)_3$), 56.3 (C1´), 43.3 (C2´´), 35.8 (C4´´), 29.8 (C3´´), 28.5 ($(CH_3)_3$), 21.4 (CH_3). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{26}H_{31}N_3O_3$: 433.2365, found: 433.2379.

*4.10.4. 2-(2-(1-tert-butoxycarbonylpiperidin-4-yl)ethyl)-4-p-tolylphthalazin-1(2H)-one (**13d**)*

Colorless oil. Yield: 99%. R_f = 0.4 (hexane/EtOAc, 2:1). ^{1}H NMR ($CDCl_3$): δ = 8.52-8.48 (m, 1H, H8), 7.78-7.70 (m, 3H, Ar), 7.45 (d, 2H, *J* = 7.9 Hz, Ar), 7.32 (d, 2H, *J* = 7.9 Hz, Ar), 4.33 (t, 2H, *J* = 7.5 Hz, H1´), 4.14-3.99 (m, 2H, H2´´), 2.73-2.61 (m, 2H, H2´´), 2.44 (s, 3H, CH_3), 1.87-1.73 (m, 4H, H2´, H3´´), 1.57-1.48 (m, 1H, H4´´), 1.43

(s, 9H, 3xCH_3), 1.20-1.13 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.1 (C1), 155.0 (*C*OC$(CH_3)_3$), 147.1, 139.3, 132.8, 132.5, 131.4, 129.5, 129.4, 128.4, 127.3, 126.8, 79.4 (*C*$(CH_3)_3$), 49.1 (C1'), 43.9 (C2´´), 35.2 (C2´), 33.9 (C4´´), 32.1 (C3´´), 28.6 ($(CH_3)_3$), 21.5 (CH_3). HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{27}H_{34}N_3O_3$: 448.25947, found: 448.26072.

4.10.5. 2-(2-(1-tert-butoxycarbonylpiperidin-4-yl)ethyl)-4-methylphthalazin-1(2H)-one ***(13e)***

Colorless oil. Yield: 99%. R_f = 0.5 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$): δ = 8.42 (d, 1H, *J* = 8.0 Hz, H8), 7.80-7.69 (m, 3H, Ar), 4.21 (t, 2H, *J* = 6.9 Hz, H1´), 4.14-3.95 (m, 2H, H2´´), 2.73-2.60 (m, 2H, H2´´), 2.56 (s, 3H, CH_3), 1.80–1.71 (m, 4H, H2´, H3´´), 1.48-1.40 (m, 10H, H4´´, 3xCH_3), 1.21–1.11 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.3 (C1), 154.9 (*C*OC$(CH_3)_3$), 143.5 (C4), 132.8, 131.3, 129.8, 127.8, 127.1, 124.8, 79.2 (*C*$(CH_3)_3$), 48.6 (C1´), 43,9 (C2´´) 35.1 (C2´), 33.7 (C4´´), 32.0 (C3´´), 28.5 ($(CH_3)_3$), 18.9 (CH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{21}H_{30}N_3O_3$: 372.22817, found: 372.22792.

4.10.6. 2-(2-(1-tert-butoxycarbonylpiperidin4-yl)ethyl)-6,7-dimethoxyphthalazin-1(2H)-one ***(13f)***

Colorless oil. Yield: 79%. R_f = 0.5 (EtOAc). ^{1}H NMR ($CDCl_3$) δ = 8.04 (s, 1H, H4), 7.75 (s, 1H, H8), 6.99 (s, 1H, H5), 4.28–4.24 (m, 2H, H1´), 4.03-4.00 (m, 8H, H2´´, 2xOCH_3), 2.70-2.62 (m, 2H, H2´´), 1.81-1.75 (m, 4H, H2´, H3´´), 1.44-1.42 (m, 10H, H4´´, 3xCH_3), 1.25 – 1.20 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$) δ = 159.0 (C1), 154.9 (*C*OC$(CH_3)_3$), 153.8, 152.9, 137.0 (C4), 124.9, 122.8, 106.3 (C8), 105.6 (C5), 79.3 (*C*$(CH_3)_3$), 56.5 (OCH_3), 56.3 (OCH_3), 48.8 (C1´), 43.6 (C2´´), 35.1(C2´), 33.5 (C4´´), 31.9 (C3´´), 28.4 ($(CH_3)_3$). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{22}H_{32}N_3O_5$: 418.23365, found: 418.23189.

4.10.7. 2-(2-(1-tert-butoxycarbonylpiperidin-4-yl)ethyl)-7-methoxyphthalazin-1(2H)-one ***(13g)***

White solid. Yield: 45%. R_f = 0.5 (EtOAc). ^{1}H NMR ($CDCl_3$) δ = 8.08 (s, 1H, H4), 7.77 (d, 1H, *J* = 2.6 Hz, H8), 7.60 (d, 1H, *J* = 8.7 Hz, H5), 7.34 (dd, 1H, *J* = 8.7, 2.6 Hz, H6), 4.27 (t,

2H, J=7.4 Hz, H1´), 4.13-4.00 (m, 2H, H2´´), 3.95 (s, 3H, OCH_3), 2.72-2.61 (m, 2H, H2´´), 1.84 – 1.75 (m, 4H, H2´, H3´´), 1.44-1.42 (m, 10H, H4´´, 3xCH_3), 1.21-1.11 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$) δ = 162.5 (C7), 159.3 (C1), 155.0 (COC$(CH_3)_3$), 137.6 (C4), 129.8, 128.0 (C5), 123.9, 123.6 (C6), 106.5 (C8), 79.3 ($C(CH_3)_3$), 56.0 (OCH_3), 48.9 (C1´), 44.3 (C2´´), 35.1 (C2´), 33.68 (C4´´), 32.1 (C3´´), 28.6 ($(CH_3)_3$). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{21}H_{30}N_3O_4$: 388.22308, found: 388.22414.

*4.10.8. 2-(2-(1-tert-butoxycarbonylpiperidin-4-yl)ethyl)-6-methoxyphthalazin-1(2H)-one (**13h**)*

Colorless oil. Yield: 86%. R_f = 0.5 (hexane/EtOAc, 1:1). ^{1}H NMR ($CDCl_3$) δ = 8.30 (d, 1H, *J* = 8.9 Hz, H8), 8.06 (s, 1H, H4), 7.28 (dd, 1H, *J* = 8.9, 2.4 Hz, H7), 6.99 (d, 1H, *J* = 2.4 Hz, H5), 4.23 (t, 2H, *J* = 7.4 Hz, H1´), 4.11–3.99 (m, 2H, H2´´), 3.92 (s, 3H, OCH_3), 2.71-2.60 (m, 2H, H2´´), 1.80–1.72 (m, 4H, H2´, H3´´), 1.45-1.40 (m, 10H, H4´´, 3xCH_3), 1.20–1.13 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$) δ) = 163.2 (C6), 159.2 (C1), 154.9 (COC$(CH_3)_3$), 137.4 (C4), 131.7, 128.8 (C8), 121.70, 121.1 (C7), 106.5 (C5), 79.2 ($C(CH_3)_3$), 55.8 (OCH_3), 48.6 (C1´), 44.3 (C2´´), 35.1 (C2´), 33.6 (C4´´), 31.9 (C3´´), 28.5 ($(CH_3)_3$). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{21}H_{30}N_3O_4$: 388.22308, found: 388.22255.

*4.11. General procedure for the preparation of 2-(piperidin-4-ylalkyl)phthalazin-1(2H)-ones (**14a-h**)*

To a solution of compound **13a-h** (0.8 mmol) in EtOAc (0.9 mL), 6 M HCl (0.3 mL) was added. The reaction mixture was stirred at r.t. overnight, followed by addition of saturated aq. $NaHCO_3$ until pH = 12. The solvent was evaporated, MeOH (15 mL) was added and the insoluble material was removed by filtration. The solvent was concentrated to dryness and after a column chromatography on silica gel (EtOAc/MeOH/NH_3, 90/9.5/0.5) a residue that contains the desired compound was obtained.

*4.11.1. 2-(Piperidin-4-ylmethyl)phthalazin-1(2H)-one (**14a**)*

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R_f = 0.3 (CH_2Cl_2/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR (CD_3OD): δ = 8.39 (s, 1H, H4), 8.38-8.32 (m, 1H, H8), 7.96-7.86 (m, 3H, Ar), 4.23-4.19 (m, 2H, H1´), 3.44-3.38 (m, 2H, H2´´), 2.99-2.91 (m, 2H, H2´´), 2.39-2.29 (m, 1H, H4´´), 1.96-1.88 (m, 2H, H3´´), 1.68-1.56 (m, 2H, H3´´). ^{13}C NMR (CD_3OD): δ = 161.5 (C1), 140.2 (C4), 135.0, 133.4, 131.2, 128.6, 128.0, 127.2, 56.4 (C1´), 44.6 (C2´´), 34.9 (C4´´), 27.7 (C3´´). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{14}H_{17}N_3O$: 243.1372, found: 243.1373.

*4.11.2. 2-(2-(Piperidin-4-yl)ethyl)phthalazin-1(2H)-one (**14b**)*

R_f = 0.3 (CH_2Cl_2/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR (CD_3OD): δ =8.33 (s, 1H, H4), 8.28 (d, 1H, *J* = 7.8 Hz, H8), 7.90-7.79 (m, 3H, Ar), 4.26 (t, 2H, *J* = 7.1 Hz, H1´), 3.36-3.29 (m, 2H, H2´´), 2.93-2.83 (m, 2H, H2´´), 2.04-1.96 (m, 2H, H3´´), 1.83-1.77 (m, 2H, H2´), 1.65-1.53 (m, 1H, H4´´), 1.49-1.37 (m, 2H, H3´´). ^{13}C NMR (CD_3OD): δ = 161.3 (C1), 140.2 (C4), 134.8, 133.3, 131.2, 128.5, 127.9, 127.0, 49.3 (C1´), 45.0 (C2´´), 35.7 (C2´), 32.4 (C4´´), 30.0 (C3´´). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{15}H_{19}N_3O$: 257.1528, found: 257.1538.

*4.11.3. 2-(Piperidin-4-ylmethyl)-4-p-tolylphthalazin-1(2H)-one (**14c**)*

R_f = 0.3 (CH_2Cl_2/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR (CD_3OD): δ = 8.46-8.39 (m, 1H, H8), 7.90-7.76 (m, 3H, Ar), 7.51-7.44 (m, 2H, Ar), 7.40-7.31 (m, 2H, Ar), 4.24-4.16 (m, 2H, H1´), 3.23-3.13 (m, 2H, H2´´), 2.78-2.66 (m, 2H, H2´´), 2.44 (s, 3H, CH_3), 2.31-2.19 (m, 1H, H4´´), 1.84-1.74 (m, 2H, H3´´), 1.50-1.40 (m, 2H, H3´´). ^{13}C NMR (CD_3OD): δ = 161.1 (C1), 149.4, 140.7, 134.7, 133.3, 133.1, 130.5, 130.4, 130.3, 129.0, 128.2, 127.8, 56.4 (C1´), 44.7 (C2´´), 34.9 (C4´´), 27.7 (C3´´), 21.4 (CH_3). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{21}H_{23}N_3O$: 333.1841, found: 333.1842.

*4.11.4. 2-(2-(Piperidin-4-yl)ethyl)-4-p-tolylphthalazin-1(2H)-one (**14d**)*

R_f = 0.3 (CH_2Cl_2/MeOH/NH_3, 90/9.5/0.5). ^{1}H NMR (CD_3OD): δ = 8.45-8.40 (m, 1H, H8), 7.88-7.83 (m, 2H, Ar), 7.80-7.76 (m, 1H, Ar), 7.47 (d, 2H, *J* = 8.1 Hz, Ar), 7.36 (d, 2H, *J* = 8.1 Hz, Ar), 4.34 (t, 2H, *J* = 7.1 Hz, H1´), 3.31-3.24 (m, 2H, H2´´), 2.88-2.78 (m, 2H, H2´´), 2.45 (s, 3H, CH_3), 2.04-1.96 (m, 2H, H3´´), 1.90-1.83 (m, 2H, H2´),

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1.69-1.57 (m, 1H, H4´´), 1.43-1.33 (m, 2H, H3´´). ^{13}C NMR (CD_3OD): δ = 160.8 (C1), 149.2, 140.7, 134.7, 133.6, 133.1, 130.9, 130.7, 130.5, 129.4, 128.5, 128.0, 45.4 (C1´) 44.8 (C2´´), 36.1 (C2´), 33.1 (C4´´), 30.7 (C3´´), 21.5 (CH_3). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{22}H_{25}N_3O$: 347.1998, found: 347.2004.

*4.11.5. 2-(2-(Piperidin-4-yl)ethyl)-4-methylphthalazin-1(2H)-one (**14e**)*

R_f = 0.2 (EtOAc/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR (CD_3OD): δ = 8.32-8.19 (m, 1H, H8), 7.94–7.71 (m, 3H, Ar), 4.25-4.11 (m, 2H, H1´), 3.26-3.20 (m, 2H, H2´´), 2.85-2.71 (m, 2H, H2´´), 2.54 (s, 3H, CH_3), 1.98-1.88 (m, 2H, H3´´), 1.80-1.70 (m, 2H, H2´), 1.59-1.46 (m, 1H, H4´´), 1.41–1.27 (m, 2H, H3´´). ^{13}C NMR (CD_3OD): δ = 160.9 (C1), 146.2 (C4), 134.6, 132.9, 130.9, 128.3, 127.4, 126.6, 49.2 (C1´), 45.4 (C2´´), 35.9 (C2´), 32.99 (C4´´), 30.8 (C3´´), 18.9(CH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{16}H_{22}N_3O$: 272.17574, found: 272.17602.

*4.11.6. 2-(2-(Piperidin-4-yl)ethyl)-6,7-dimethoxyphthalazin-1(2H)-one (**14f**)*

R_f = 0.3 (EtOAc/MeOH/NH_3, 90/9.5/0.5). ^{1}H NMR (CD_3OD) δ = 7.79 (s, 1H, H4), 7.17 (s, 1H, H8), 6.85 (s, 1H, H5), 3.89-3.82 (m, 2H, H1´), 3.56 (s, 6H, 2x OCH_3), 2.98-2.93 (m, 2H, H2´´), 2.56-2.46 (m, 2H, H2´´), 1.65-1.59 (m, 2H, H3´´), 1.44-1.37 (m, 2H, H2´), 1.27-1.15 (m, 1H, H4´´), 1.13-1.00 (m, 2H, H3´´). ^{13}C NMR (CD_3OD) δ = 159.3 (C1), 154.3, 153.2, 138.0 (C4), 125.3, 121.7, 106.2 (C5), 105.2 (C8), 55.5 (OCH_3), 55.3 (OCH_3), 47.9 (C1´), 43.6 (C2´´), 34.4 (C2´), 31.1 (C4´´), 28.5 (C3´´). HRMS (EI): *m/z* $[M]^+$ calcd for $C_{17}H_{23}N_3O_3$: 317.1739, found: 317.1745.

*4.11.7. 2-(2-(Piperidin-4-yl)ethyl)-7-methoxyphthalazin-1(2H)-one (**14g**)*

Rf = 0.3 (EtOAc/MeOH/NH_3, 90/9.5/0.5). ^{1}H NMR (CD_3OD) δ = 8.28 (s, 1H, H4), 7.82 (d, 1H, *J* = 8.7, H5), 7.72-7.68 (m, 1H, H8), 7.48–7.43 (m, 1H, H6), 4.30 (t, 2H, *J* = 6.9 Hz, H1´), 3.97 (s, 3H, OCH_3), 3.41-3.34 (m, 2H, H2´´), 2.98-2.88 (m, 2H, H2´´), 2.08-2.02 (m, 2H, H3´´), 1.85 (c, 2H, *J* = 6.9 Hz, H2´), 1.68-1.58 (m, 1H, H4´´), 1.52-1.45 (m, 2H, H3´´). ^{13}C NMR (CD_3OD) δ = 164.3 (C7), 161.0 (C1), 139.9 (C4), 130.5, 130.0 (C5), 125.3, 124.5 (C6), 107.4 (C8), 56.4 (OCH_3), 49.3 (C1´), 45.1 (C2´´), 35.7 (C2´),

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32.4 (C4´´), 29.9 (C3´´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{16}H_{22}N_3O_2$: 288.17065, found: 288.17131.

*4.11.8. 2-(2-(Piperidin-4-yl)ethyl)-6-methoxyphthalazin-1(2H)-one (**14h**)*

R_f = 0.2 (EtOAc/MeOH/NH_3, 90/9.5/0.5). ^{1}H NMR ($CDCl_3$) δ = 8.32 (s, 1H, H4), 8.23 (d, 1H, *J* = 8.7 Hz, H8), 7.40 (dd, 1H, *J* = 8.7, 1.9 Hz, H7), 7.33 (d, 1H, *J* = 1.9 Hz, H5), 4.28 (t, 2H, *J* = 6.9 Hz, H1´), 3.97 (s, 3H, OCH_3), 3.39-3.34 (m, 2H, H2´´), 2.95-2.88 (m, 2H, H2´´), 2.06–2.01 (m, 2H, H3´´), 1.84 (c, 2H, *J* = 6.9 Hz, H2´), 1.65-1.58 (m, 1H, H4´´), 1.52-1.45 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$) δ = 165.3 (C6), 161.0 (C1), 140.0 (C4), 133.4, 129.2 (C8), 122.8 (C7), 122.1, 108.3 (C5), 56.6 (OCH_3), 49.1 (C1´), 44.9 (C2´´), 35.7 (C2´), 32.4 (C4´´), 29.8 (C3´´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{16}H_{22}N_3O_2$: 288.17065, found: 288.17074.

*4.12. General procedure for the preparation of 2-(N-benzylpiperidin-4-ylalkyl)phthalazin-1(2H)-ones (**1a-h**)*

A suspension of NaH (60% dispersion in mineral oil, 0.75 mmol) in DMF (3 mL) was added to a suspension of the residue containing **14a-h** (0.25 mmol) in DMF (3 mL). After stirring at room temperature for 1 h, BnBr (0.38 mmol) was added. The reaction mixture was stirred at room temperature overnight, followed by quenching with H_2O (15 mL) at 0 ºC. The product was extracted with EtOAc (3x10 mL) and dried over Na_2SO_4. After the solvent removal, the residue was purified by column chromatography on silica gel (EtOAc→EtOAc/MeOH 9:1, compounds **1a**-**1d**, hexane/EtOAc 1:1→EtOAc/MeOH 99:1, compound **1f** and hexane/EtOAc 1:1→1:2→1:3, compounds **1e**, **1g** and **1h**) to obtain the desired compound.

*4.12.1. 2-(N-Benzylpiperidin-4-ylmethyl)phthalazin-1(2H)-one (**1a**)*

Colorless oil. Yield: 34% (from **13a**). R_f = 0.4 (EtOAc/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ = 8.42 (d, 1H, *J* = 7.5 Hz, H8), 8.15 (s, 1H, H4), 7.83-7.73 (m, 2H, Ar), 7.71-7.67 (m, 1H, Ar), 7.33-7.28 (m, 3H, Ar), 7.28-7.21 (m, 2H, Ar), 4.14 (d, 2H, *J* = 7.2 Hz, H1´), 3.51 (s, 2H, CH_2Ph), 2.94-2.86 (m, 2H, H2´´), 2.23-2.11 (m, 1H, H4´´), 2.09-1.93 (m, 2H, H2´´), 1.71-1.62 (m, 2H, H3´´), 1.55-1.43 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ =

159.8 (C1), 137.6 (C4), 133.2, 131.8, 129.7, 129.4, 128.3, 127.2, 126.9, 126.1, 63.3 (CH_2Ph), 56.6 (C1´), 53.3 (C2´´), 35.5 (C4´´), 30.0 (C3´´). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{21}H_{23}N_3O$: 333.1841, found: 333.1845.

*4.12.2. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)phthalazin-1(2H)-one (**1b**)*

Colorless oil. Yield: 93% (from **13b**). R_f = 0.3 (EtOAc/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ = 8.42 (d, 1H, *J* = 7.6 Hz, H8), 8.16 (s, 1H, H4), 7.82-7.73 (m, 2H, Ar), 7.71-7.66 (m, 1H, Ar), 7.33-7.29 (m, 3H, Ar), 7.28-7.21 (m, 2H, Ar), 4.27 (t, 2H, *J*= 7.5 Hz, H1´), 3.51 (s, 2H, CH_2Ph), 2.94-2.86 (m, 2H, H2´´), 2.02-1.92 (m, 2H, H2´´), 1.84-1.73 (m, 4H, H2´, H3´´), 1.42-1.32 (m, 3H, H3´´, H4´´). ^{13}C NMR ($CDCl_3$): δ = 159.4 (C1), 137.8 (C4), 133.1, 131.7, 129.7, 129.5, 128.3, 128.1, 127.1, 126.8, 126.1, 63.5 (CH_2Ph), 53.8 (C2´´), 49.1 (C1´), 35.2 (C2´), 33.5 (C4´´), 32.1 (C3´´). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{22}H_{25}N_3O$: 347.1998, found: 347.1995.

*4.12.3. 2-(N-Benzylpiperidin-4-ylmethyl)-4-p-tolylphthalazin-1(2H)-one (**1c**)*

Colorless oil. Yield: 30% (from **13c**). R_f = 0.4 (EtOAc/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ = 8.54-8.50 (m, 1H, H8), 7.79-7.71 (m, 3H, Ar), 7.46 (d, 2H, *J* = 8.0 Hz, Ar), 7.33 (d, 2H, *J* = 8.0 Hz, Ar), 7.31-7.29 (m, 3H, Ar), 7.28-7.21 (m, 2H, Ar), 4.21 (d, 2H, *J* = 7.1 Hz, H1´), 3.50 (s, 2H, CH_2Ph), 2.93-2.86 (m, 2H, H2´´), 2.45 (m, 3H, CH_3), 2.11-1.91 (m, 3H, H2´´, H4´´), 1.73-1.65 (m, 2H, H3´´), 1.57-1.44 (m, 2H, H3´´). ^{13}C NMR ($CDCl_3$): δ = 159.5 (C1), 147.0, 139.3, 132.8, 132.4, 131.4, 129.5, 129.2, 128.4, 128.3, 127.4, 126.8, 63.1 (CH_2Ph), 56.5 (C1´), 53.2 (C2´´), 35.4 (C4´´), 29.7 (C3´´), 21.5 (CH_3). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{28}H_{29}N_3O$: 423.2311, found: 423.2307.

*4.12.4. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)-4-p-tolylphthalazin-1(2H)-one (**1d**)*

Colorless oil. Yield: 63% (from **13d**). R_f = 0.3 (EtOAc/MeOH, 9:1). ^{1}H NMR ($CDCl_3$): δ = 8.54-8.49 (m, 1H, H8), 7.80-7.71 (m, 3H, Ar), 7.47 (d, 2H, *J* = 8.2 Hz, Ar), 7.33 (d, 2H, *J* = 8.2 Hz, Ar), 7.32-7.29 (m, 3H, Ar), 7.28-7.21 (m, 2H, Ar), 4.33 (t, 2H, *J* = 7.5 Hz, H1´), 3.51 (s, 2H, CH_2-Ph), 2.94-2.86 (m, 2H, H2´´), 2.46 (s, 3H, CH_3), 2.02-1.92 (m, 2H, H2´´), 1.88-1.74 (m, 5H, H2´, H3´´, H4´´), 1.41-1.33 (m, 2H, H3´´). ^{13}C NMR

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($CDCl_3$): δ = 159.1 (C1), 147.1, 139.25, 132.7, 132.5, 131.4, 129.5, 129.3, 128.4, 128.3, 127.3, 127.1, 126.8, 63.5 (CH_2-Ph), 53.8 (C2´´), 49.3 (C1´), 35.2 (C2´), 33.6 (C4´´), 32.2 (C3´´), 21,5 (CH_3). HRMS-EI: *m/z* $[M]^+$ calcd for $C_{29}H_{31}N_3O$: 437.2467, found: 437.2463.

*4.12.5. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)-4-methylphthalazin-1(2H)-one (**1e**)*

Colorless oil. Yield: 34% (from **13e**) R_f = 0.6 (EtOAc/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR ($CDCl_3$): δ = 8.44 (d, 1H, *J* = 8.5 Hz, H8), 7.80–7.71 (m, 3H, Ar), 7.31–7.21 (m, 5H, Ar), 4.25–4.19 (m, 2H, H1´), 3.48 (s, 2H, CH_2Ph), 2.91-2.83 (m, 2H, H2´´), 2.57 (s, 3H, CH_3), 1.98-1.90 (m, 2H, H2´´), 1.81-1.74 (m, 4H, H2´, H3´´), 1.39-1.32 (m, 3H, H3´´, H4´´). ^{13}C NMR ($CDCl_3$): δ = 159.2 (C1), 143.4 (C4), 138.4, 132.8, 131.2, 129.7, 129.34, 128.2, 127.8, 127.1, 127.0, 124.7, 63.5 (CH_2Ph), 53.8 (C2´´), 48.9 (C1´), 35.2 (C2´), 33.6 (C4´´), 32.2 (C3´´), 18.9 (CH_3). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}N_3O$: 362.22269, found: 362.22152.

*4.12.6. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)-6,7-dimethoxyphthalazin-1(2H)-one (**1f**)*

White solid. Yield: 66% (from **13f**). R_f = 0.5 (EtOAc/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR ($CDCl_3$) δ = 8.03 (s, 1H, H4), 7.75 (s, 1H, H8), 7.29–7.21 (m, 5H, Ar), 6.97 (s, 1H, H5), 4.24 (t, 2H, *J*=7.5 Hz, H1´), 4.02 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 3.46 (s, 2H, CH_2Ph), 2.88-2.82 (m, 2H, H2´´), 1.96-1.88 (m, 2H, H2´´), 1.80 – 1.73 (m, 4H, H2´, H3´´), 1.35-1.29 (m, 3H, H3´´, H4´´). ^{13}C NMR ($CDCl_3$) δ = 159.0 (C1), 153.8, 152.9, 138.5, 136.8 (C4), 129.3, 128.2, 126.9, 125.0, 123.0, 106.5 (C5), 105.6 (C8), 63.6 (CH_2Ph), 56.6 (OCH_3), 56.4 (OCH_3), 53.8 (C2´´), 49.1 (C1´), 35.2 (C2´), 33.5 (C4´´), 32.3 (C3´´). HRMS (ESI): m/z $[M+H]^+$ calculated for $C_{24}H_{30}N_3O_3$: 408.22817, found: 408.22756.

*4.12.7. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)-7-methoxyphthalazin-1(2H)-one (**1g**)*

Colorless oil. Yield: 59% (from **13g**). R_f = 0.5 (EtOAc/MeOH/NH_3, 90:9.5:0.5). ^{1}H NMR ($CDCl_3$) δ = 8.05 (s, 1H, H4), 7.75 (d, 1H, *J* = 2.4 Hz, H8), 7.57 (d, 1H, *J* = 8.7 Hz, H5), 7.32–7.26 (m, 6H, H6, Ar), 4.23 (t, 2H, *J* = 7.3 Hz, H1´), 3.92 (s, 3H, OCH_3), 3.45 (s, 2H, CH_2Ph), 2.87-2.82 (m, 2H, H2´´), 1.94-1.88 (m, 2H, H2´´), 1.78-1.72 (m,

4H, H2´, H3´´), 1.34-1.29 (m, 3H, H3´´, H4´´). ^{13}C NMR ($CDCl_3$) δ = 162.4 (C7), 159.3 (C1), 138.3, 137.4 (C4), 130.0, 129.4 (C5), 128.2, 127.9, 127.0, 123.8, 123.5 (C6), 106.5 (C8), 63.5 (CH_2Ph), 56.0 (OCH_3), 53.8 (C2´´), 49.2 (C1´), 35.2 (C2´), 33.5 (C4´´), 32.2 (C3´´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}N_3O_2$: 378.21760, found: 378.21775.

*4.12.8. 2-(2-(N-Benzylpiperidin-4-yl)ethyl)-6-methoxyphthalazin-1(2H)-one (**1h**)*

White solid. Yield: 57% (from **13h**). R_f = 0.6 (EtOAc/MeOH/NH_3, 90/9.5/0.5). ^{1}H NMR ($CDCl_3$) δ = 8.31 (d, 1H, *J* = 8.9 Hz, H8), 8.06 (s, 1H, H4), 7.31–7.22 (m, 6H, H7, Ar), 6.98 (d, 1H *J* = 2.4 Hz, H5), 4.23 (t, 2H, J = 7.5 Hz, H2´´), 3.92 (s, 3H, OCH_3), 3.47 (s, 2H, CH_2Ph), 2.89-2.83 (m, 2H, H2´´), 1.97-1.89 (m, 2H, H2´´), 1.80-1.72 (m, 4H, H2´, H3´´), 1.37-1.29 (m, 3H, H3´´, H4´´). ^{13}C NMR ($CDCl_3$) δ = 163.2 (C6), 159.2 (C1), 138.4, 137.3 (C4), 131.7, 129.4, 128.6, 128.2 (C8), 127.0, 121.8, 121.0 (C7), 106.5 (C5), 63.5 (CH_2Ph), 55.8 (OCH_3), 53.9 (C2´´), 48.9 (C1´), 35.2 (C2´), 33.5 (C4´´), 32.2 (C3´´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}N_3O_2$: 378.21760, found: 378.21828.

*4.13. 3-(piperidin-4-yl)propanol hydrochloride (**16**)*

To a solution of 3-(pyridin-4-yl)propanol (**15**) (50 mg, 0.36 mmol) in EtOH (2 mL) was added 4M HCl in dioxane (90 μL, 0.36 mmol) and PtO_2 (3 mg). Then was purged with H_2 for 20 min. The reaction mixture was stirred at 45 ºC under hydrogen atmosphere for 5 h. The catalyst was filtered off and the resulting filtrate was concentrated and dried under vacuum to afford **16** (65 mg, 100%) as a white solid. ^{1}H NMR (CD_3OD) δ = 3.48 (t, 2H, *J* =6.4 Hz, H1), 3.34-3.28 (m, 2H, H2´), 2.96-2.86 (m, 2H, H2´), 1.91-1.83 (m, 2H, H3´), 1.57-1.45 (m, 3H, H4´, H2), 1.42-1.26 (m, 4H, H3´, H3). ^{13}C NMR (CD_3OD) δ = 62.8 (C1), 45.2 (C2´), 34.5 (C4´), 33.2 (C3), 30.3 (C2), 29.8 (C3´). HRMS (ESI): *m/z* $[(M-Cl)+H]^+$ calcd for $C_8H_{18}NO$: 144.13829, found: 144.13843.

*4.14. 3-(1-Benzylpiperidin-4-yl)propanol (**17**)*

To a solution of **16** (41 mg, 0.23 mmol) in EtOH was added BnBr (35μL, 0.29 mmol) and K_2CO_3 (178 mg, 1.28 mmol). The reaction mixture was stirred at r.t. for 1 h and refluxed for 2 h. After cooling, was filtered, concentrated under vacuum and the residue

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was treated with a saturated solution of $NaHCO_3$ (5 mL). The resulting solution was extracted with CH_2Cl_2 (3x5 mL) and the combined layers dried over Na_2SO_4 and concentrated under reduced pressure to afford **17** (40 mg, 76%) as a colorless oil. R_f = 0.2 (EtOAc/MeOH, 97:3). ^{1}H NMR ($CDCl_3$) δ = 7.34–7.22 (m, 5H, Ar), 3.56 (t, 2H, *J* = 6.7 Hz, H1), 3.50 (s, 2H, CH_2Ph), 2.92-2.85 (m, 2H, H2´), 1.99-1.90 (m, 2H, H2´), 1.70-1.63 (m, 2H, H3´), 1.60–1.50 (m, 2H, H2), 1.33–1.21 (m, 5H, H3´, H4´, H3). ^{13}C NMR ($CDCl_3$) δ = 137.8 (C, Ar), 129.4, 128.1, 126.9, 63.4 (CH_2Ph), 62.5 (C1), 53.7 (C2´), 35.6 (C4´), 32.6 (C3), 32.7 (C3´), 29.9 (C2). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{15}H_{24}NO$: 234.18524, found: 234.18520.

*4.15. 1-Benzyl-4-(3-bromopropyl)piperidine (**18**)*

It was prepared as described for compounds **12** by reaction of alcohol **17** (40 mg, 0.17 mmol), CBr_4 (115 mg, 0.34 mmol) and PPh_3 (91 mg, 0.34 mmol) in CH_2Cl_2 (2 mL). The residue was purified by column chromatography on silica gel (EtOAc/MeOH, 95:5) to give **18** (33 mg, 65%) as a white solid. R_f = 0.4 (EtOAc). ^{1}H NMR ($CDCl_3$) δ = 7.36-7.26 (m, 5H, Ar), 3.51 (s, 2H, CH_2Ph), 3.41 (t, 2H, *J* = 6.9 Hz, H3´), 2.93-2.87 (m, 2H, H2), 1.99-1.85 (m, 4H, H2, H2´), 1.70-1.63 (m, 2H, H3), 1.42-1.36 (m, 2H, H1'), 1.32-1.24 (m, 3H, H3, H4). ^{13}C NMR ($CDCl_3$) δ = 138.60 (C, Ar), 129.3, 128.2, 127.0, 63.6 (CH_2Ph), 53.9 (C2), 35.3 (C4), 35.2 (C1'), 34.3 (C3'), 32.4 (C3), 30.3 (C2'). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C15H_{23}BrN$: 296.10084, found: 296.10079.

4.16. *3-(N-benzypiperidin-4-yl)propylphthalazin-1(2H)-one (**1i**)*

It was prepared as described for phthalazinones **1a-h** by reaction of **3a** (11 mg, 0.07 mmol), NaH (60% dispersion in mineral oil, 5 mg, 0.11 mmol) and **18** (25 mg, 0.08 mmol) in DMF (2 mL). The residue was purified by column chromatography on silica gel (hexane/EtOAc 1:1→EtOAc/MeOH 99:1) to afford **1i** (19 mg, 70%) as a colorless oil. R_f = 0.6 (EtOAc/MeOH 99:1). ^{1}H NMR ($CDCl_3$) δ = 8.42 (d, 1H, *J* = 7.4 Hz, H8), 8.15 (s, 1H, H4), 7.83-7.73 (m, 2H, Ar), 7.71–7.66 (m, 1H, Ar), 7.33–7.24 (m, 5H, Ar), 4.21 (t, 2H, *J* = 7.4 Hz, H1´), 3.51 (s, 2H, CH_2Ph), 2.94-2.84 (m, 2H,H2´´), 2.00-1.91 (m, 2H, H2´´), 1.90-1.80 (m, 2H, H2´), 1.70-1.63 (m, 2H, H3´´), 1.36-1.23 (m, 5H, H3´ H3´´, H4´´). ^{13}C NMR ($CDCl_3$) δ = 159.4 (C1), 137.8 (C4), 133.1, 131.7, 129.7, 129.6,

128.3, 128.0, 127.2, 126.8, 126.0, 63.3 (CH_2Ph), 53.7 (C2´´), 51.4 (C1´), 35.5 (C4´´), 33.5 (C3´), 32.1 (C3´´), 26.0 (C2´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}N_3O$: 362.22269, found: 362.22359.

*4.17. 3-(N-benzypiperidin-4-yl)propyl-6,7-dimethoxyphthalazin-1(2H)-one (**1j**)*

It was prepared as described for phthalazinones **1a-h** by reaction of **3d** (19 mg, 0.09 mmol), NaH (60% dispersion in mineral oil, 6 mg, 0.14 mmol) and **18** (30 mg, 0.1 mmol) in DMF (3 mL). The residue was purified by column chromatography on silica gel (EtOAc→EtOAc/MeOH 97:3) to afford **1j** (30 mg, 77%) as a white solid. R_f = 0.3 (EtOAc). ^{1}H NMR ($CDCl_3$) δ = 8.03 (s, 1H, H4), 7.75 (s, 1H, H8), 7.29–7.21 (m, 5H, Ar), 6.98 (s, 1H, H5), 4.19 (t, *J* = 7.3 Hz, 2H, H1´), 4.02 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 3.46 (s, 2H, CH_2Ph), 2.87-2.81 (m, 2H, H2´´), 1.94-1.80 (m, 4H, H2´, H2´´), 1.67-1.60 (m, 2H, H3´´), 1.33-1.22 (m, 5H, H3´, H3´´, H4´´). ^{13}C NMR ($CDCl_3$) δ = 159.1 (C1), 153.8, 152.9, 138.4, 136.9 (C4), 129.4, 128.2, 127.0, 125.0, 122.9, 106.5 (C8), 105.6 (C5), 63.5 (CH_2Ph), 56.6 (OCH_3), 56.4 (OCH_3), 53.9 (C2´´), 51.5 (C1`), 35.6 (C4´´), 33.5 (C3´), 32.3 (C3´´), 26.0 (C2´). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{25}H_{32}N_3O_3$: 422.24382, found: 422.24313.

4.18. Determination of AChE and BuChE activities

Ellman's method was used to determine the *in vitro* ChE activity [26]. The activity was measured by the increase in absorbance at 412 nm due to the yellow colour of 5-mercapto-2-nitrobenzoic acid produced by the reaction of thiocholine with dithiobisnitrobenzoic acid (DTNB). The assay solution consisted of a 50 mM phosphate buffer pH 7.2, with the addition of 0.25 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DNTB), 0.01 U/mL AChE from human erytrocytes or 0.005 U/mL BuChE from human serum (Sigma), and 5 mM substrate (acetylthiocholine or butyrylthiocholine iodide). Test compounds were added to the buffer and preincubated at 37 ^{o}C with the enzyme for 5 min followed by the addition of cromogene and substrate. The activity was determined by measuring the increase in absorbance at 412 nm at 1 min intervals for 10 min at 37 ^{o}C (Fluo-Star Optima^{TM}, BMG LABTECH, Offenburg, Germany). Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. The

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specific absorbance (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from wells containing all components except the AChE or BuChE, which was replaced by a sodium phosphate buffer solution. ChE activity of the test compounds and reference inhibitors is expressed as IC_{50}, ie the concentration of each drug required to produce a 50% decreased on control value AChE or BuChE activity.

4.19. Molecular modeling studies

All AChE inhibitors were built and their partial charges calculated after semi-empirical (PM6) energy minimization [27] using the MOE2014 [28]. Two crystallographic structures were selected to perform docking studies: the hAChE in complex with donepezil (PDB ID: 4EY7) [29] and BuChE in complex with tacrine (PDB ID: 4BDS) [30]. Water molecules and all ligands present in the pdb file were removed and the proteins were subjected to the structure preparation tool of MOE 2014 [31]. Finally protonate 3D tool was used to assign the protomeric state. To identify the more appropriate protocol for the selected complexes we performed a self-docking benchmark using DockBench 1.01a software [32] which compared the performance of 17 different posing/scoring protocols. The active site was defined using a radius of 12 Å from the centre of mass of the co-crystallized ligand. Each ligand was docked 10 times. All synthesized analogues were docked using GOLD [33] using ChemPLP [34] and Goldscore [35] as scoring functions for 4EY7 and 4BDS, respectively, using the virtual screening tool of DockBench adopting the parameters used in the banchmarck study. Finally, the obtained conformations with 4bds from the docking were rescored with ChemPLP.

The predicted ADME and physicochemical properties were calculated using StarDrop program [36].

Acknowledgements

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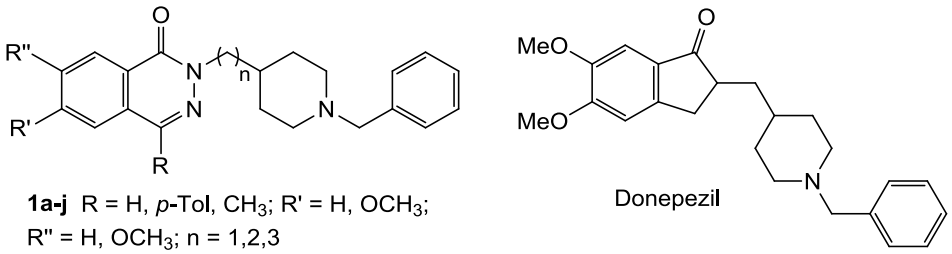

1a-j R = H, *p*-Tol, CH_3; R' = H, OCH_3; R'' = H, OCH_3; n = 1,2,3

Figure 1. General structure of novel ChE inhibitors and donepezil.

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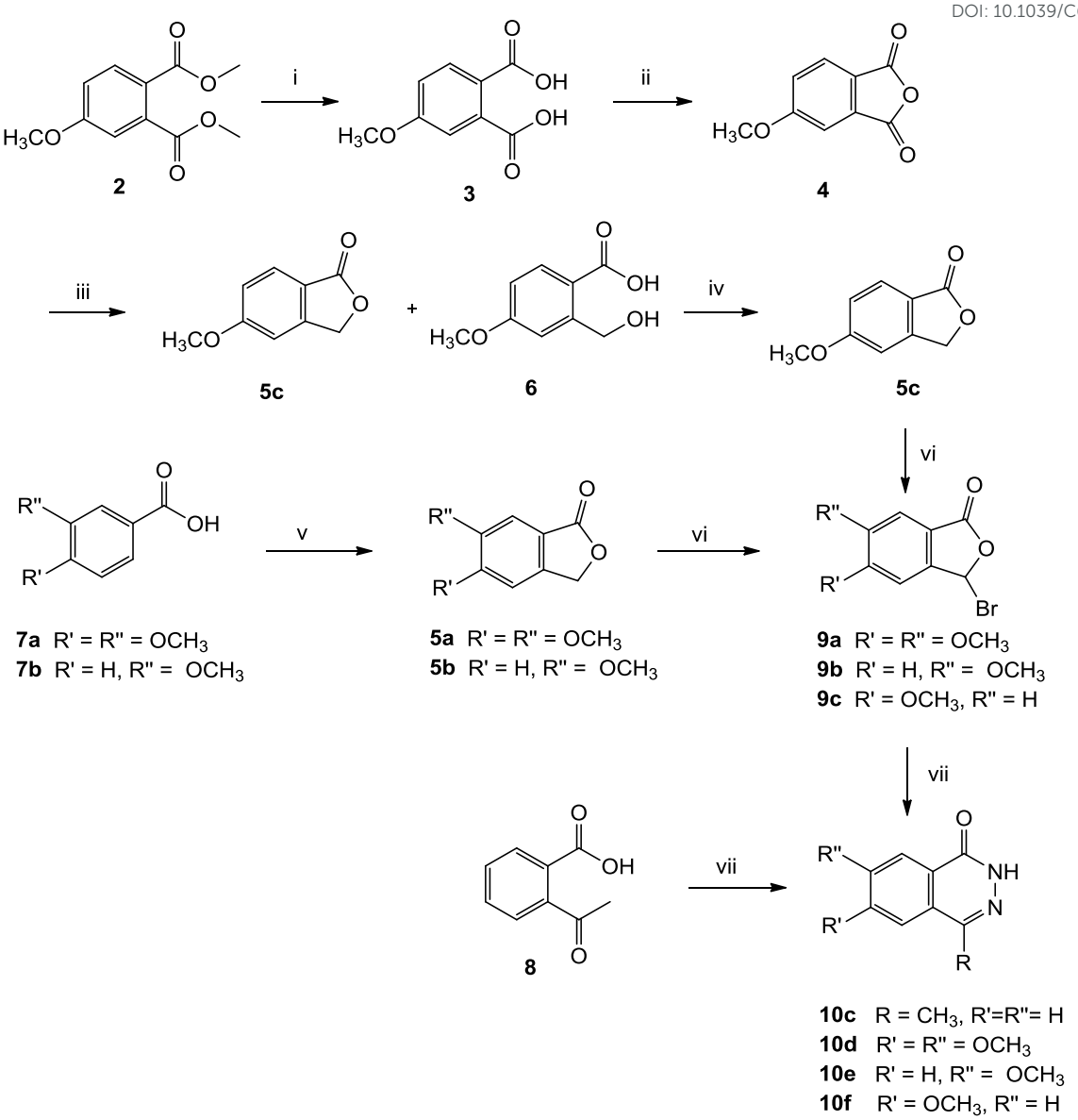

Scheme 1. Reagents and conditions: (i) 5M NaOH, MeOH reflux, 96%; (ii) Ac_2O reflux, 98%; (iii) $NaBH_4$, THF, r.t.; 6M HCl r.t. 33% (**5c**) and 52% (**6**); (iv) 6M HCl reflux, 53 % (two steps); (v) 30% HCHO, 37% HCl, 90 ºC, 75% (**5a**) or 30% HCHO, 37% HCl, acetic acid 100 ºC, 43% (**5b**); (vi) NBS, benzoyl peroxide, CCl_4, reflux; (vii) $NH_2NH_2.H_2O$, EtOH, reflux, 91% (**10c**), 58% (**10d**, two steps), 51% (**10e**, two steps), 65% (**10f**, two steps).

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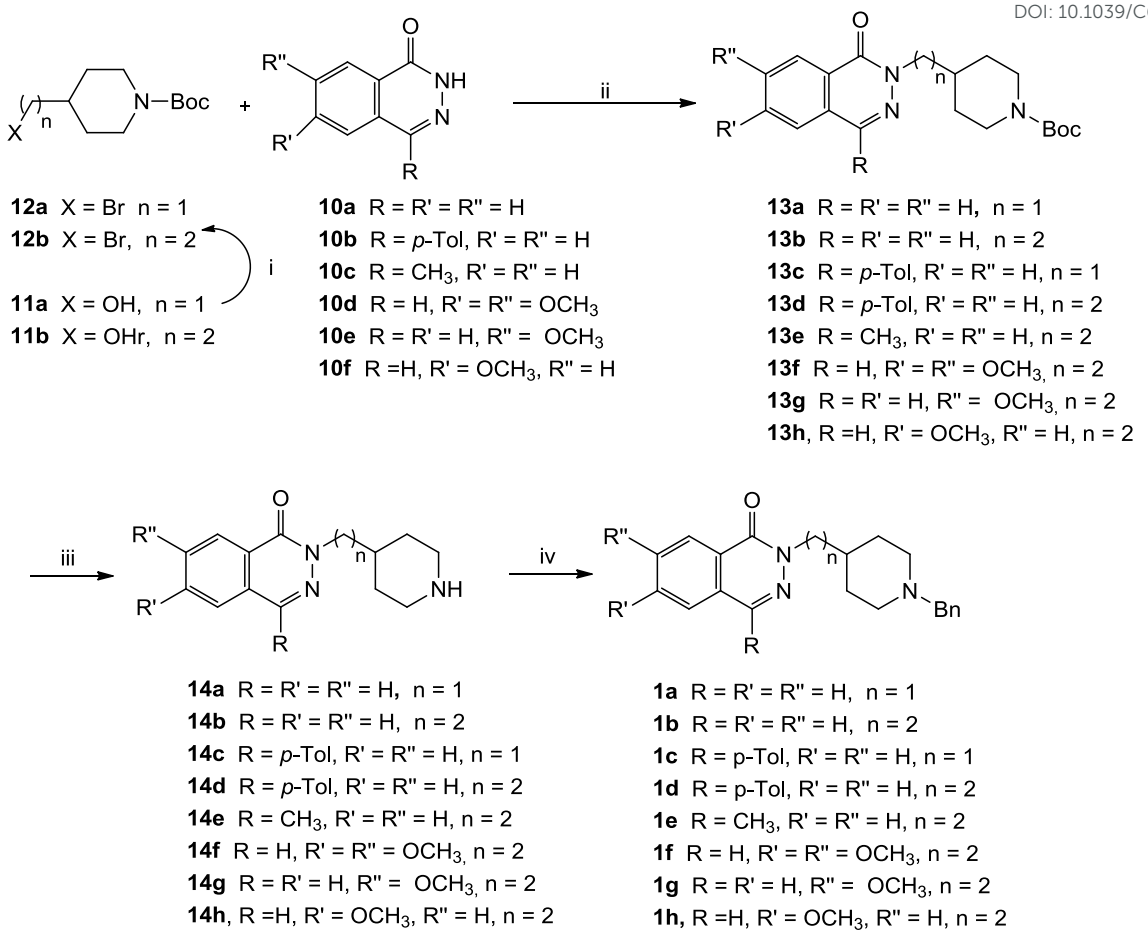

Scheme 2. Reagents and conditions: (i) CBr_4, PPh_3, CH_2Cl_2, reflux, 94% (**12a**), 89% (**12b**); (ii) NaH, **12a** or **12b**, DMF, r.t. 78% (**13a**), 99% (**13b**), 99% (**13c**), 99% (**13d**), 99% (**13e**), 79% (**13f**), 45% (**13g**), 86% (**13h**); (iii) 6 M HCl, EtOAc, r.t; (iv) NaH, BnBr, DMF, r.t. 34% (**1a**, two steps), 93% (**1b**, two steps), 30% (**1c**, two steps), 63% (**1d**, two steps), 34 % (**1e**, two steps), 66% (**1f**, two steps), 59% (**1g**, two steps), 57% (**1h**, two steps).

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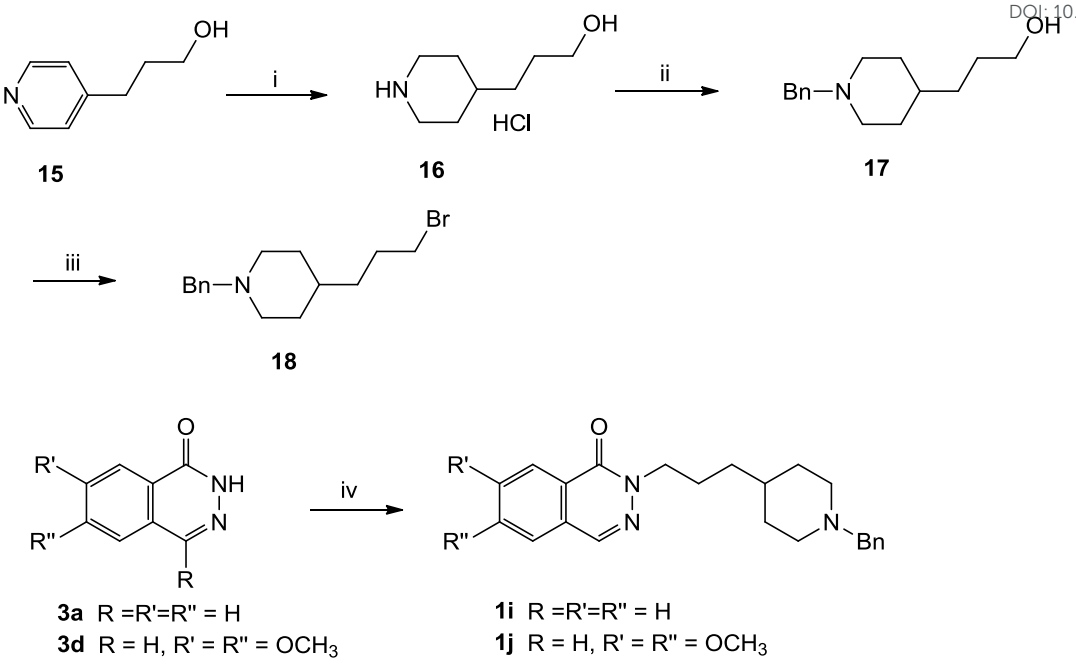

Scheme 3. Reagents and conditions: (i) H_2, PtO_2, 4M HCl dioxane, EtOH, 45 ºC, 100%; (ii) BnBr, K_2CO_3, EtOH, r.t. to reflux 76%; (iii) CBr_4, PPh_3, CH_2Cl_2, reflux, 65%; (iv) NaH, **18**, DMF, r.t. 70% (**1i**), 77% (**1j**).

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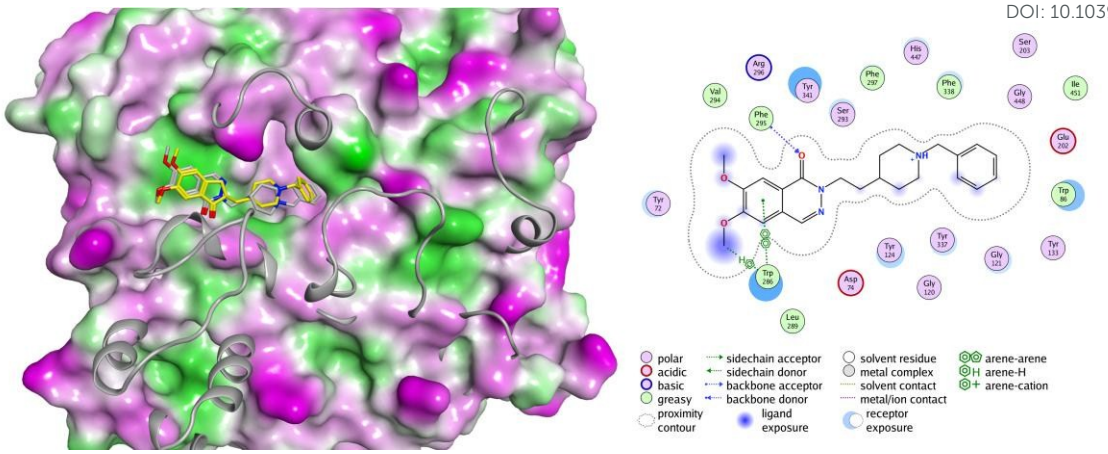

Figure 2. On the left: superposition of the crystallographic complex donezepil (yellow) bound to AChE and compound **1f** (grey) as derived by docking calculation. The molecular surface of the protein (PDB ID: 4EY7) is coloured according its lipophilicity, using the following color scheme: green (lipophilic region) to violet (hydrophilic region). Part of the protein is shown with the ribbon representation (grey) to permit a clear visualization of the ligands**.** On the right, the ligand interaction diagram of **1f** bound to AChE is reported.

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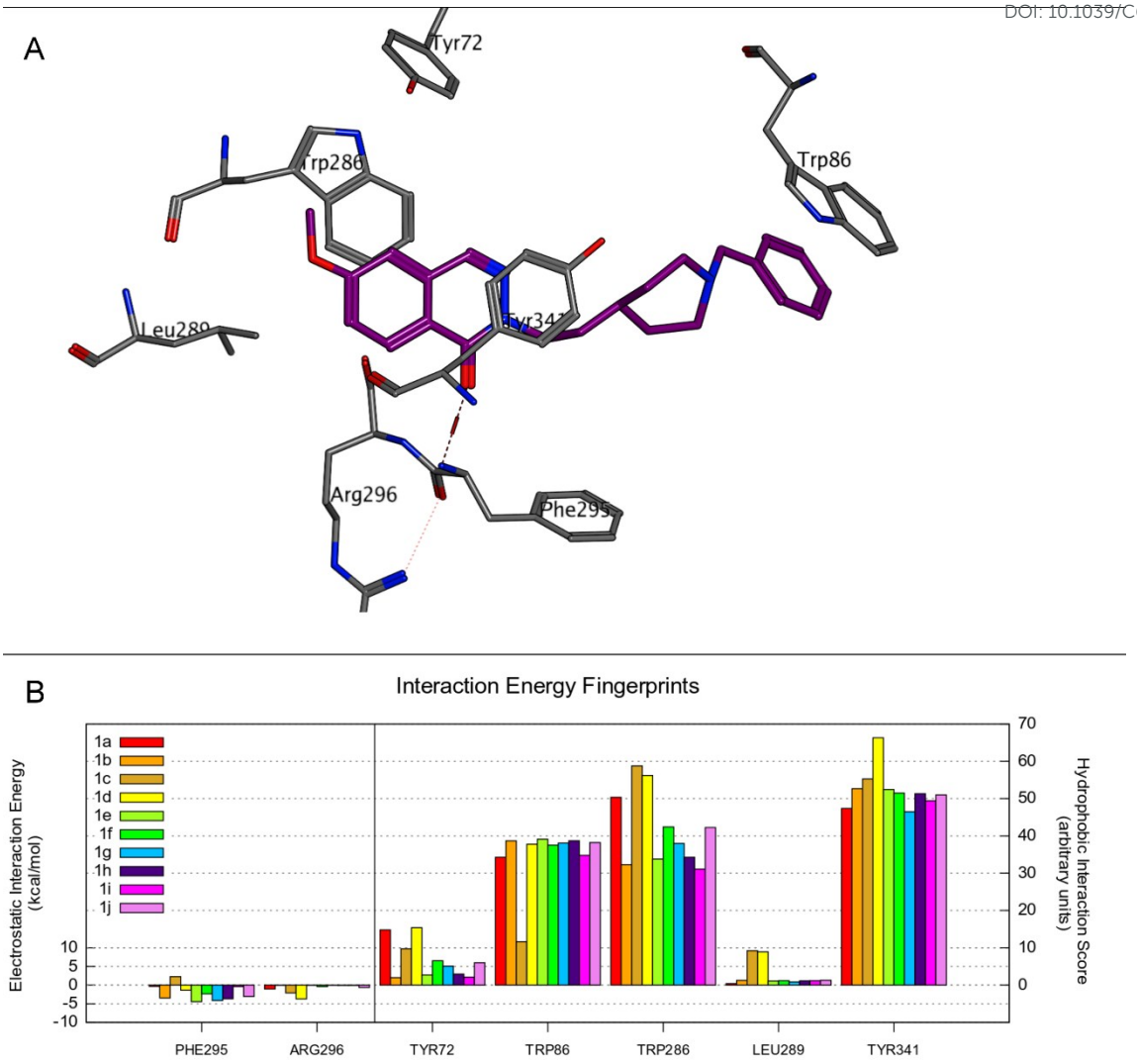

Figure 3. Per-residue analysis of the protein-ligand interaction for each compound. In panel A, the residues considered in the analysis are represented as stick and the carbon atoms are colored in grey, while the ligand carbon atoms are shown using the same representation, but with violet color for carbon atoms.

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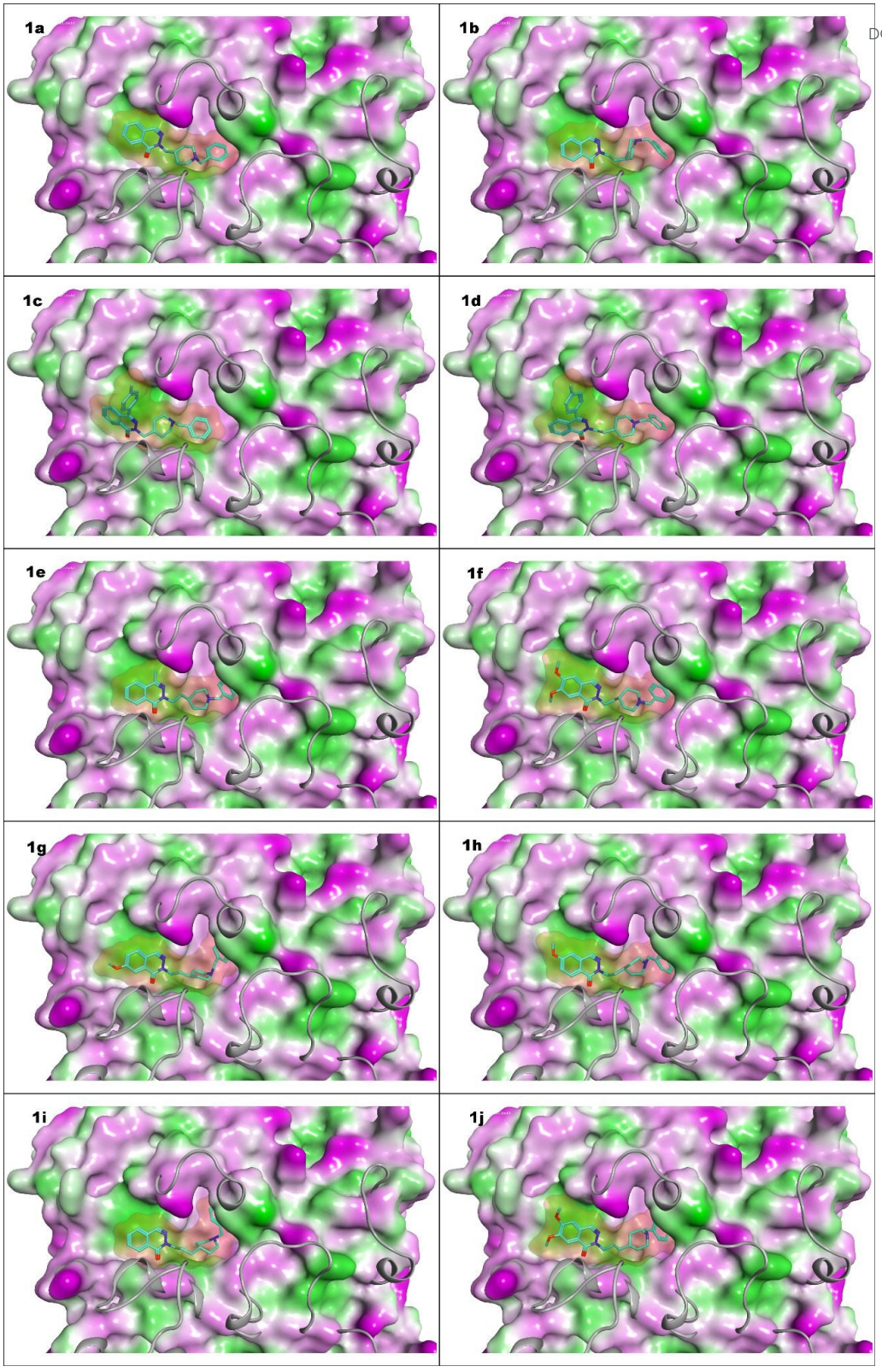

Figure 4. Binding mode of the synthetized compounds. The code of each compound is reported on the upper left of each panel. The molecular surface of the protein (PDB ID: 4EY7) is colored according its lipophilicity, using the following color scheme: green (lipophilic region) to violet (hydrophilic region). Part of the protein is shown with the ribbon representation (grey) to permit a clear visualization of the ligand (azure stick representation). The molecular surface of the ligand (transparent orange) is shown to underline its complementary with the binding site.

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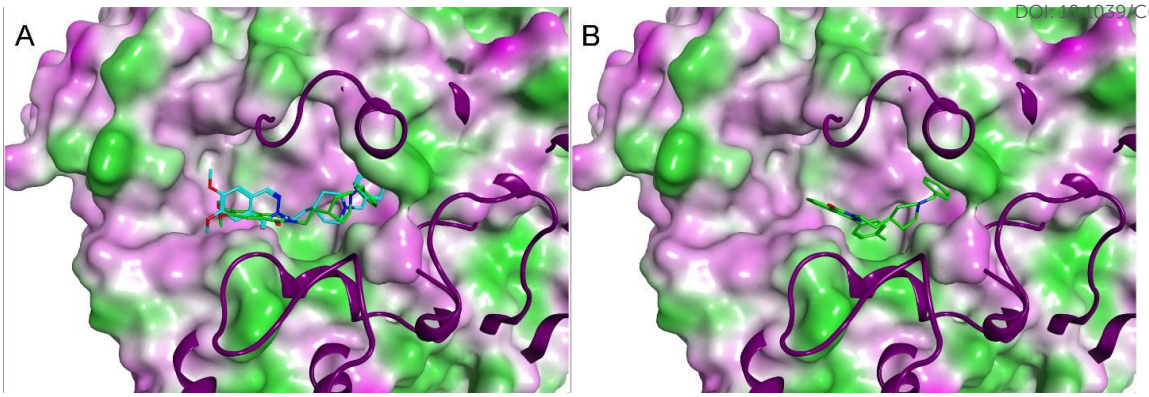
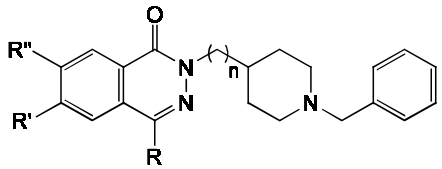


Figure 5. Binding mode of compound **1f** and **1d** to hBuChE. The molecular surface of the protein (PDB ID: 4BDS) is coloured according its lipophilicity using the following colour scheme: green (lipophilic region) to violet (hydrophilic region). In panel A, the conformation of compound **1f** (green) is reported as resulted by molecular docking study. In order to compare the binding mode of compound **1f** in the hAChE, its conformation when docked to hAChE is reported in cyan. In panel B, the binding mode of the most active compound of the series for hBuChE, compound **1d**, in shown in green.

Table 1. IC_{50} values for compounds **1** and reference inhibitors on the enzymatic activity of human AChE and BuChE[a]



Compound	n	R	R'	R''	IC_{50} AChE (μM)	IC_{50} BuChE (μM)
1a	1	H	H	H	>100	46.14±3.08
1b	2	H	H	H	2.58±0.39	64.69±4.13
1c	1	p-Tol	H	H	>100	13.26±0.88
1d	2	p-Tol	H	H	3.45±0.23	5.50±0.37
1e	2	CH_3	H	H	2.79±0.19	>100
1f	2	H	OCH_3	OCH_3	0.67±0.04	39.24±2.62
1g	2	H	H	OCH_3	2.40±0.16	27.47±1.83
1h	2	H	OCH_3	H	0.55±0.04	53.14±3.54
1i	3	H	H	H	10.29±0.69	62.02±4.13
1j	3	H	OCH_3	OCH_3	1.07±0.07	>100
Donepezil	---	---	---	---	0.016±0.003	12.01±0.80
Tacrine	---	---	---	---	0.29±0.06	0.15±0.04

[a]Values are expressed as the mean ± standard error of the mean from three experiments (n = 3).

Table 2. Calculated docking score values for all the synthetized analogues in complex with human AChE and BuChE.

Compound	GOLDPLP SCORE hAChE	GOLDPLP SCORE hBuChE
1a	98.2	68.0
1b	101	74.4
1c	98	70.2
1d	115.6	77.7
1e	111.6	68.3
1f	115.9	70.2
1g	103.3	65.7
1h	104.5	62.6
1j	111.1	67.7
1i	98.3	61.7

Table 3. Calculated physicochemical and ADME properties for all the synthetized analogues, donepezil and tacrine.

compound	Intravenous CNS Scoring Profile_Score[a1]	Oral CNS Scoring Profile_Score[a2]	logS[b]	logP[c]	2C9 pKi[d]	hERG pIC50[e]	BBB category[f]	HIA category[g]	P-gp category[h]	2D6 affinity category[i]	PPB90 category[l]
1a	0.25	0.17	1.71	3.47	4.91	6.59	+	+	yes	medium	low
1b	0.22	0.14	1.77	3.58	4.79	6.69	+	+	yes	medium	low
1c	0.03	0.01	0.16	5.28	5.57	7.15	+	+	yes	medium	high
1d	0.05	0.02	0.36	5.40	5.45	7.24	+	+	yes	medium	high
1e	0.14	0.09	1.61	3.98	4.90	6.68	+	+	yes	medium	low
1f	0.15	0.09	2.10	3.34	5.13	6.69	-	+	yes	medium	high
1g	0.14	0.09	1.91	3.47	4.83	6.69	-	+	yes	medium	low
1h	0.14	0.09	1.91	3.47	4.85	6.70	-	+	yes	medium	low
1i	0.15	0.09	1.64	4.00	4.73	6.81	+	+	yes	medium	low
1j	0.10	0.06	1.98	3.74	4.99	6.81	-	+	yes	medium	high
Donepezil	0.25	0.16	1.87	3.54	4.97	6.41	+	+	yes	medium	low
Tacrine	0.32	0.28	2.77	2.57	4.43	4.84	+	+	no	medium	low

a1, intravenous CNS score: ideal score is 1; a2, Oral CNS score: ideal score is 1; b. aqueous solubility (logS, μM), preferably >1; c. logarithm of partition coefficient between *n*-octanol and water (*c*logP), preferably 0<clogP<3.6; d. CYP2C9 cytochrome metabolism (CYP2C9 affinity, μM), preferably ≤ 6; e. hERG channel inhibition (pIC50), preferably ≤ 5; f. blood brain barrier (BBB) penetration, (+) indicates a ratio ≥ 0.5 and (-) indicates a ratio < 0.5; g. human intestinal absorption (HIA), (+) indicates absorption ≥ 30% and (-) indicates absorption < 30%; h. P-glycoprotein binding (P-gp), "yes" indicates a substrate and "no" indicates a non-substrate; i. CYP2D6 cytochrome metabolism (CYP2D6 affinity, μM), "low" indicates pKi < 5, "medium" indicates 5 < pKi < 6, "high" indicates 6 < pKi < 7, "very high" indicates pKi > 7; l. plasma protein binding (PPB90), "low" indicates < 90% of compound bound to plasma proteins, "high" indicates ≥ 90% of compound bound to plasma proteins. All properties were calculated using StarDrop™, version 6.2 (Optibrium Ltd: 7221 Cambridge Research Park, Beach Drive, Cambridge CB25 9TL, UK).

Graphical abstract

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Synthesis, biological evaluation and molecular modeling studies of phthalazin-1(2H)-one derivatives as novel cholinesterase inhibitors

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A series of donepezil analogues based on phthalazin-1(2H)-one scaffold was studied as hChEIs. The biological results revealed that the structural modifications proposed significantly affected ChE inhibitory potency as well as selectivity AChE/BuChE.