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Discovery of new acetylcholinesterase inhibitors for Alzheimer's disease: virtual screening and *in vitro* characterisation

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ABSTRACT

For more than two decades, the development of potent acetylcholinesterase (AChE) inhibitors has been an ongoing task to treat dementia associated with Alzheimer's disease and improve the pharmacokinetic properties of existing drugs. In the present study, we used three docking-based virtual screening approaches to screen both ZINC15 and MolPort databases for synthetic analogs of physostigmine and donepezil, two highly potent AChE inhibitors. We characterised the *in vitro* inhibitory concentration of 11 compounds, ranging from 14 to 985 μM. The most potent of these compounds, S-I 26, showed a fivefold improved inhibitory concentration in comparison to rivastigmine. Moderate inhibitors carrying novel scaffolds were identified and could be improved for the development of new classes of AChE inhibitors.

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Introduction

In 2020, it has been estimated that more than 50 million people have developed dementia, with more than 60% of all cases being associated with Alzheimer's disease (AD)[1,2]. AD is a neurodegenerative disease predominantly characterised by a progressive decline of cognitive abilities and memory impairment[2]. The complete mechanism of the pathogenesis of the multifactorial disease is still unsolved[3]. The "cholinergic hypothisis," which suggests that the progressive degeneration of cholinergic neurons is the main factor contributing to AD, remains one of the major theories to explain the origin of this disease[4]. The decline of the acetylcholine concentration in the brain of AD patients is further amplified by the activity of neuronal acetylcholinesterase (AChE), which regulates the termination of the synaptic signal by hydrolysing the neurotransmitter acetylcholine secreted in the inter-synaptic cleft.

As a result, the inhibition of AChE has become a promising therapeutic strategy for treating the symptoms of AD. The use of cholinesterase inhibitors (ChEIs) reduces these symptoms by increasing the concentration of acetylcholine in the brain, which, in turn, improves patient memory and cognitive function. Many ChEIs have already been developed over the last 20 years. Although ChEIs have been mainly employed to treat AD-induced dementia, they have also proven to be effective for the treatment of glaucoma, myasthenia gravis, and chronic psychiatric diseases such as schizophrenia[5]. Donepezil, galantamine, and rivastigmine are currently the most used commercial ChEIs for the treatment of AD[2]. However, the use of these ChEIs is hampered by severe dose-dependent side-effects. In addition to these respective side-effects, the short half-life of some ChEIs such as rivastigmine and physostigmine also jeopardises their long-term therapeutic use. Synthetic analogs of traditional ChEIs, such as ladostigil, tacrine, and indenyl derivatives, have shown reduced side-effects, but their potency has been strongly limited by their poor ability to cross the blood–brain barrier[2].

Clearly, the development of novel synthetic ChEIs with improved pharmacokinetic properties and potency remains an ongoing priority to overcome the limitations of traditional ChEIs and contribute to the improvement of existing treatments for AD. The use of computational methods for virtual screening of large chemical databases has proven to be a valuable approach for the discovery of new classes of ChEIs[6,7]. Here, we describe the discovery of new ChEIs based on the virtual screening of the ZINC15 and MolPort databases for synthetic analogs of two highly potent ChEIs, physostigmine and donepezil. We report the *in vitro* inhibitory concentration (IC50) of selected hits towards AChE and subsequently explore their respective binding mode with the enzyme by molecular docking.

2. Material and methods

2.1. Reagents

All compounds tested in this study were obtained commercially from MolPort (Riga, Latavia). A detailed list of purchased compounds, including purity and quantity, is available in the supplementary information (Supplementary Table S1). All other chemicals used in this study were purchased by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) if not stated differently.

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2.2. Biological activity

Inhibitory activity of AChE from *Electrophorus electricus* was determined by using the Ellman method[8]. As references, Rivastigmine (TCI Deutschland GmbH, Eschborn, Germany), Physostigmine (EDQM, Strasbourg, France), and Donepezil were used. The determined IC50 values are provided in Table 1. All compounds purchased from Molport were directly dissolved in DMSO to give a 125 mM stock solution and then further diluted with 100 mM potassium phosphate buffer (KPi) pH 8 to give the desired dilution using 96-well-plates (Greiner, Item No.: 655101). 1:4 dilution series were made for each compound to obtain twelve concentrations ranging from 31 mM to 7 nM. Each concentration was tested in six replicates.

A total of 162 µL of 1.5 mM DNTB solution (dissolved in 100 mM KPi pH 8) was first added to each well. Second, 8 µL of each tested compound solution was added to each well except for the positive control, for which 8 µL 25% DMSO was used. Third, 50 µL of AChE enzyme solution (0.5 U/mL dissolved in 100 mM KPi pH 8, activity according to the supplier's instructions) was added to each well, except for the blank row. Following a 10 min preincubation at 23 °C, 30 µL acetylthiocholine iodide solution (15 mM, dissolved in 100 mM KPi pH 8) was added to each well to start the assay. After a 5 s linear shake, the absorbance at 410 nm was measured for 5 min every 30 s using a Tecan Infinite M1000Pro microplate reader. The results are reported as mean ± standard deviation. The inhibition properties are reported as IC50 values, which were determined graphically by using OriginPro 2019 (OriginLab Corporation, Northampton, MA). IC50 values represent the concentration of inhibitor required for the inhibition of 50% of the AChE activity.

2.2. Kinetics

The kinetics of AChE from *Electrophorus electricus* for acetylthiocholine iodide in the presence of inhibitors was evaluated using the method described in the previous section. The velocity was measured as the change of absorption per minute (ΔA/min) at 410 nm over 5 min. Samples were done in triplicates. The tested concentrations of acetylthiocholine iodide ranged from 0.01 mM to 1.8 mM. The EC80, EC50, and EC20 values previously determined for each tested inhibitor were used. Lower concentrations were used for compound S-II 18 due to its low solubility.

Table 1. Compounds successfully tested in this work.

Compound ID	IC_{50}[a]	Docking Score[b]	Ligand Efficiency[c]
S-I 16	220 ± 10	−8.02	−0.038
S-I 18	634 ± 67	−9.68	−0.035
S-I 26	14 ± 1	−9.20	−0.031
S-II 2	481 ± 36	−10.15	−0.029
S-II 6	331 ± 8	−10.03	−0.030
S-II 13[d]	393 ± 27	−10.29	−0.023
S-II 14	985 ± 309	−10.64	−0.032
S-II 16	417 ± 33	−10.74	−0.034
S-II 18	175 ± 16	−10.99	−0.023
S-III 6[d]	120 ± 3	−15.10	−0.052
S-III 12	393 ± 27	−13.48	−0.044
Physostigmine	0.18 ± 0.01	−7.79	−0.028
Donepezil	0.027 ± 0.002	−10.46	−0.025
Rivastigmine	71 ± 3	−7.64	−0.030

[a]Mean ± standard deviation ($n = 6$), concentrations in µM.
[b]In kcal mol^{-1}. The docking scores for both S-III 6 and S-III 12 were predicted by FRED, whereas all other docking scores were predicted by Glide SP.
[c]In kcal mol^{-1} Da^{-1}. The ligand efficiency is defined as the docking score of a given compound divided by the molecular weight.
[d]S-II 13 and S-III 6 were tested as racemic mixtures.

2.3. Virtual screening (S-I) of analogs of physostigmine

The FastROCS program[9] (ROCS 3.3.2.2: OpenEye Scientific Software, Santa Fe, NM) was used to screen a modified version of the ZINC15 database[10] for physostigmine analogs. In this modified version, only the compounds from the original ZINC15 database that passed the "Drug" filter of the FILTER program (OpenEye Scientific Software, Santa Fe, NM) were considered. The 5000 most similar analogs showing the best Tanimoto-Combo scores were considered from the first screening step.

As an alternative protocol, a pharmacophore-based screening was conducted to screen for additional compounds. A pharmacophore model of physostigmine was defined using the ZINCpharmer web-server [11] (Supplementary Figure S1) and subsequently used to screen the ZINC15 database for physostigmine analogs. A total of 4432 unique ligands showing the lowest RMSD to physostigmine were selected from the first screening step. All retained ligands from both screening procedures were subjected to a 3 D conformational sampling using the LigPrep module of the Maestro software (Schrödinger Release 2020–2: Schrödinger, LLC, New York, NY, 2020), and the resulting conformers were subsequently docked to the substrate binding site of the human AChE (PDB ID: 4ey4) *via* the Glide module using the standard precision (SP) flexible docking[12,13] method with the OPLS3 force field[14]. Prior to the docking, the protein structure was prepared using the Protein Preparation Wizard module[15] of the Maestro software. Subsequently, compounds with a docking score larger than physostigmine and exhibiting a greater number of steric clashes with the protein were excluded. The steric clashes (defined as "bad" and "ugly" contacts) were calculated using the script "poseviewer_interactions.py" as part of the Schrödinger suite.

Finally, three independent CNS scores were calculated for each retained compound to evaluate its ability to cross the blood–brain barrier and be active in the central nervous system. The scores were calculated by three distinct algorithms using the QikProp program (QikProp, Schrödinger, LLC, New York, NY, 2020) and a shell script implementation of the Nervous System Multi-Parameter Optimisation (MPO)[16] and the Technically Extended Multi-Parameter Optimisation (TEMPO)[17]. This consensus approach requires that a given compound has to be predicted as CNS positive by all the three methods to be retained for the next filtering step.

The programs QikProp, Ligfilter, Epik[18,19] (Epik, Schrödinger, LLC, New York, NY, 2020), Canvas[20] (Canvas, Schrödinger, LLC, New York, NY, 2020) and calcx (ChemAxon Marvin Suite 20.4.0) were applied to calculate the required descriptors needed for the determination of the CNS scores. A large set of ADME properties were finally computed using the QikProp program in order to estimate the relative likelihood of each retained (CNS positive) compounds to be administered as oral drugs. A compound would not be considered "drug-like" when more than five of its predicted ADME descriptors values fall outside the 95% range of similar values for known drugs. In this respect, all compounds having more than three ADME property violations or which did not satisfy the Lipinski's rule of five[21] were excluded. A total of 155 compounds were retained at that stage and analysed for their toxicity using the Derek program[24–26] (Derek Nexus v2.0, Lhasa Limited). 26 of them were finally ordered for testing based on their availability and their low predicted toxicity.

2.4. Virtual screening (S-II) of analogs of donepezil

A pharmacophore model of donepezil was defined using ZINCpharmer (Supplementary Figure S2) and used to screen the

full ZINC15 database for analogs. A total of 49,113 unique ligands were retained from the first screening step based on their low RMSD value to donepezil. These compounds were then subjected to the same filtering protocol as previously described in the virtual screening S-I. A total of 3250 molecules were finally retained. 22 among those showing the lowest docking scores were ordered and tested.

2.5. Virtual screening (S-III) of analogs of the three best hits from screening I

A search for additional close analogs of compounds S-I 16, S-I 18, and S-I 26 isolated from the first virtual screening was performed using the FastROCS program against the MolPort chemical database. Based on their Tanimoto-Combo score, the 10,000 most similar analogs to each reference compounds (i.e. 30,000 compounds in total) were selected and subsequently docked in the substrate binding pocket of the human AChE.

To speed-up the screening procedure, the docking was performed using the FRED program[22] of the OEDOCKING suite (OEDOCKING 3.4.0.2: OpenEye Scientific Software, Santa Fe, NM) using the high-resolution protocol along with the Chemgauss3 scoring function. Prior to the docking, the 3D conformers of each of the respective screened analogs were sampled using the OMEGA software[23] (OMEGA 3.0.0.1: OpenEye Scientific Software, Santa Fe, NM). Compounds from the MolPort database that did not pass the "BlockBuster" filter of the FILTER program were not considered "drug-like" and were therefore excluded from the screening procedure. In contrast with the "Drug" filter that was initially used to prepare the ZINC15 database employed for the first virtual screening, the "BlockBuster" filter was here chosen as a comparative analysis (data not shown) of both filters has shown that the latter was much less restrictive in the definition of "drug-likeliness."

All docked analogs showing a higher docking score than the respective score of at least one of the reference compounds (S-I 16, S-I 18, and S-I 26) were excluded. Those compounds exhibiting a larger number of steric clashes (see screening S-I) with the protein than the respective reference compounds were also filtered out. The retained compounds were then filtered based on their CNS score as described previously. At this stage, a total of 1063 compounds were retained, and 30 that were directly purchasable were finally analysed for their toxicity as described previously. 13 compounds were finally purchased based on their low predicted toxicity.

3. Results and discussion

3.1. In vitro characterisation

3.1.1. Assay validation and general considerations

The *in vitro* inhibitory concentration (IC50) of compounds top-ranked by virtual screening was evaluated *via* the Ellman's colorimetric assay. Three commercial AChE inhibitors (physostigmine, donepezil, and rivastigmine; Supplementary Figure S3) were used as positive controls, and their respective IC50 values (Table 1) are comparable to those reported in the literature.

When using the Ellman's method, the nature and the concentration of the co-solvent can impact the reproducibility of the results. Therefore, we tested four different solvents, including

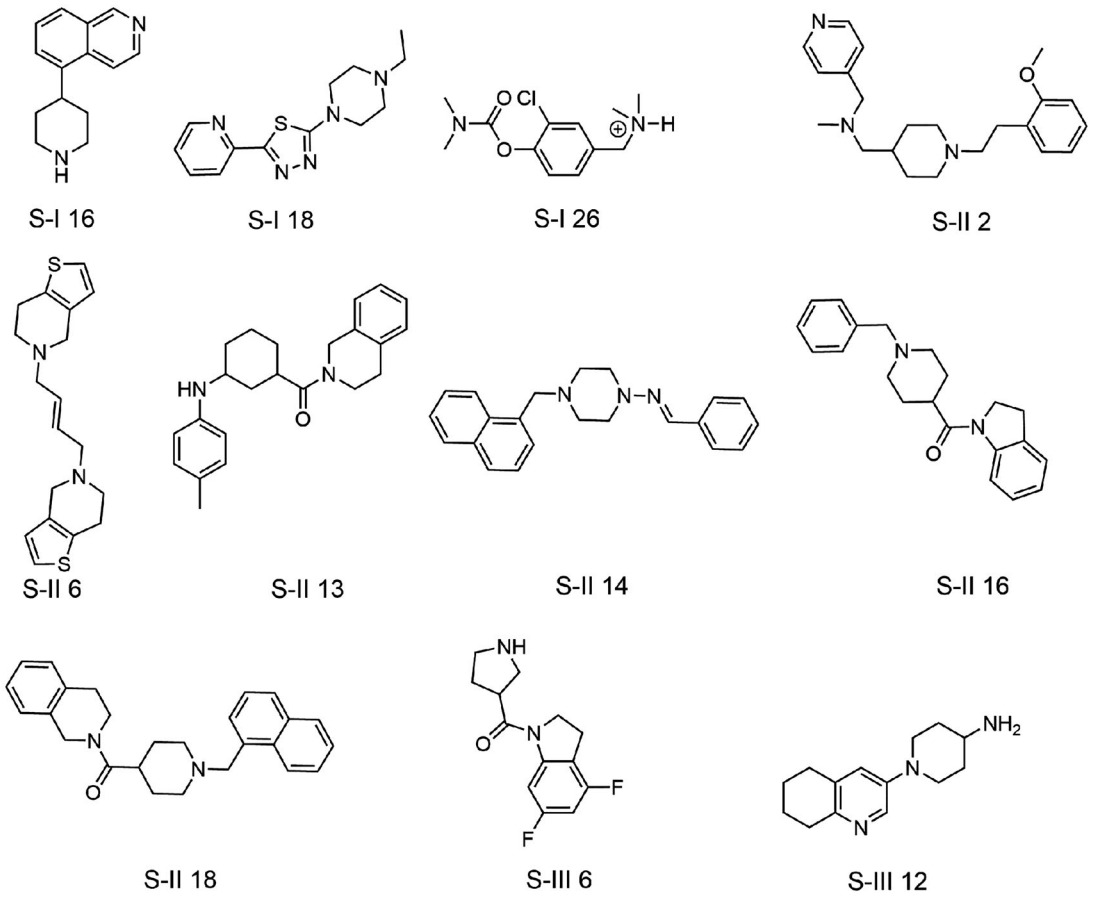

Figure 1. Chemical structures of the new compounds characterised in this work.

DMSO, which is often considered the gold standard for such assays. Interestingly, at common DMSO concentrations (4–8% (V/V)), 80% of the total AChE activity is lost (Supplementary Figure S4) as already published in a similar work[27]. In contrast, the use of EtOH and MeOH as alternative co-solvents did not result in such an activity decrease. For practical reasons, however, these co-solvents were not considered further since their high volatility would have compromised the reproducibility of the dilution series of the tested compounds. Instead, using a concentration of 1.8% (V/V) DMSO led to an acceptable residual AChE activity of ~78%. Based on our findings, we recommend not exceeding DMSO concentrations of between 1 and 2% (V/V) to avoid an excessive use of AChE.

3.1.2. In vitro evaluation of selected compounds

In total, 55 compounds selected from all three virtual screenings (S-I, S-II, and S-III) were tested for their respective IC50. Of these 55 candidates, an IC50 value could be determined for 11 compounds (Table 1), ranging from 14 to 985 μM. No inhibition was observed for the remaining compounds in the tested concentration range. Compound S-I 26 shows the lowest IC50 of all tested compounds (Supplementary Figure S5), which is five times lower than that of its close analogue rivastigmine (71.1 μM). This result suggests that the potency of rivastigmine can be improved with only a few modifications of its scaffold (Figure 1). S-I 26 forms similar interactions with the AChE binding pocket like physostigmine[28], which are in accordance with the mechanism of action of carbamate inhibitors. Likely, S-I 26 shows a pseudo-irreversible inhibition mechanism, similar to all AChE inhibitors with a carbamate moiety. Analyses of the kinetics suggest a mixed inhibition mode of S-I 26, S-II 18, and S-III 6 (Supplementary Figures S6–S11 and Table S5). Note that, apart from a single amino acid difference located more than 5 Å away from the docked S-I 26, all other residues contributing to the binding are identical in both AChE from *Electrophorus electricus* and human AChE (Supplementary Figure S12)[29,30], suggesting that highly similar binding modes can be expected.

In comparison to rivastigmine, the presence of a single chlorine substituent on the phenyl ring of S-I 26 may lead to an increase in potency by favouring van der Waals interactions with the surrounding aromatic residues (Figure 2). Yet, it seems more likely that the absence of the two additional methyl groups in rivastigmine explains the fivefold improvement in IC50 for S-I 26: As suggested by docking, the presence of these methyl groups introduces steric hindrances compromising the formation of key hydrogen bonds between the oxygen atom of the carbamate moiety and the backbone amides of glycine residues (Gly118, Gly119), which form the oxyanion hole (Figure 3). These missing interactions in the predicted rivastigmine docking pose disfavour the formation of the complementary π–π stacking and π–cation interactions, which stabilise the docking poses of both physostigmine and S-I 26.

The second and third best AChE inhibitors are compounds S-II 18 and S-III 6. Despite their structural dissimilarity, these two

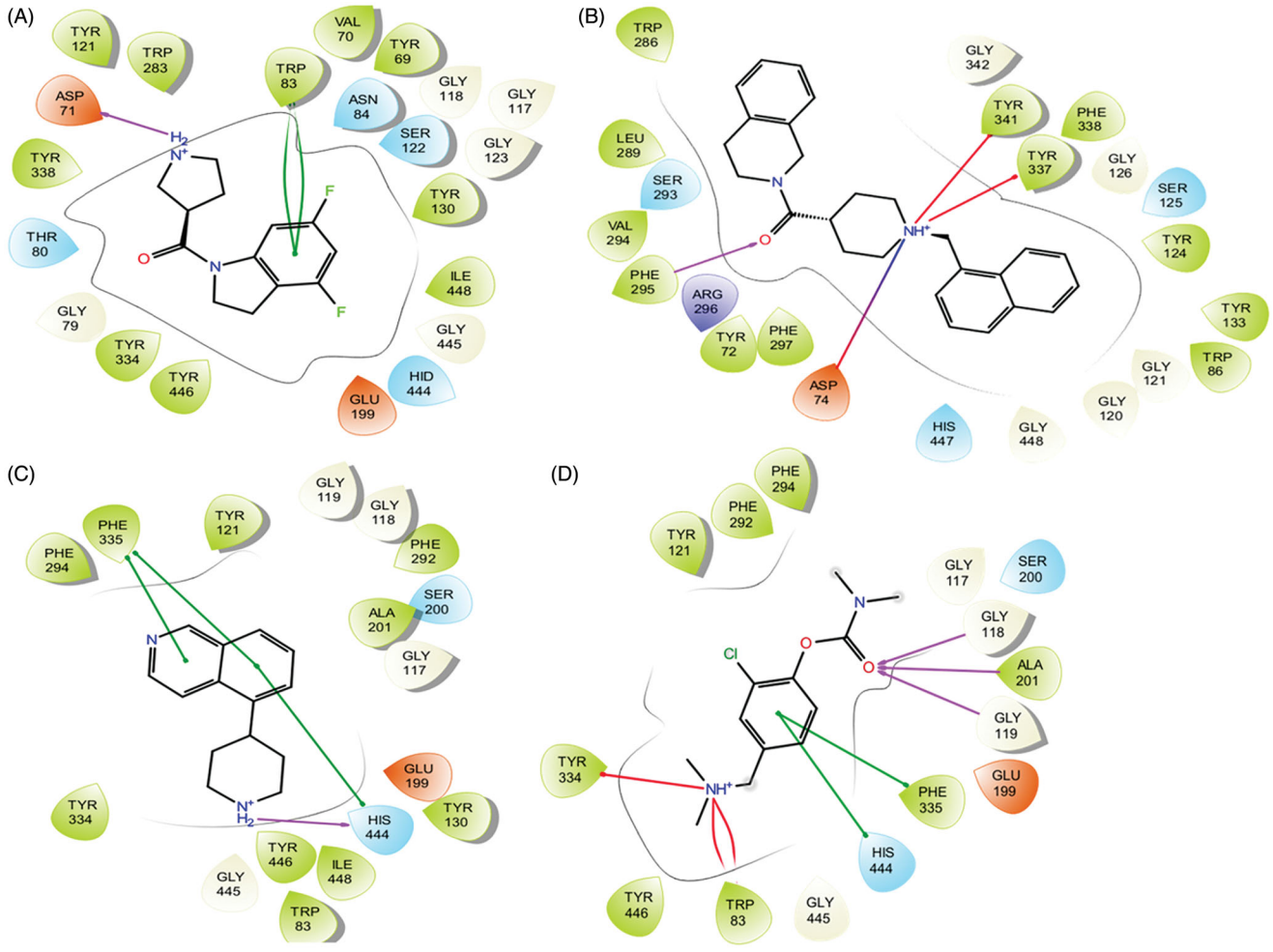

Figure 2. Interaction patterns of the lowest-energy docking poses of the top-four compounds with the surrounding residues in the human AChE binding pocket (PDB ID: 4ey4): S-III 6 (A), S-II 18 (B), S-I 16 (C), and S-I 26 (D). The purple arrows and the blue-red solid lines indicate hydrogen bonds and salt–bridge interactions, respectively. The green and the red solid lines illustrate π–π stacking and π–cation interactions, respectively.

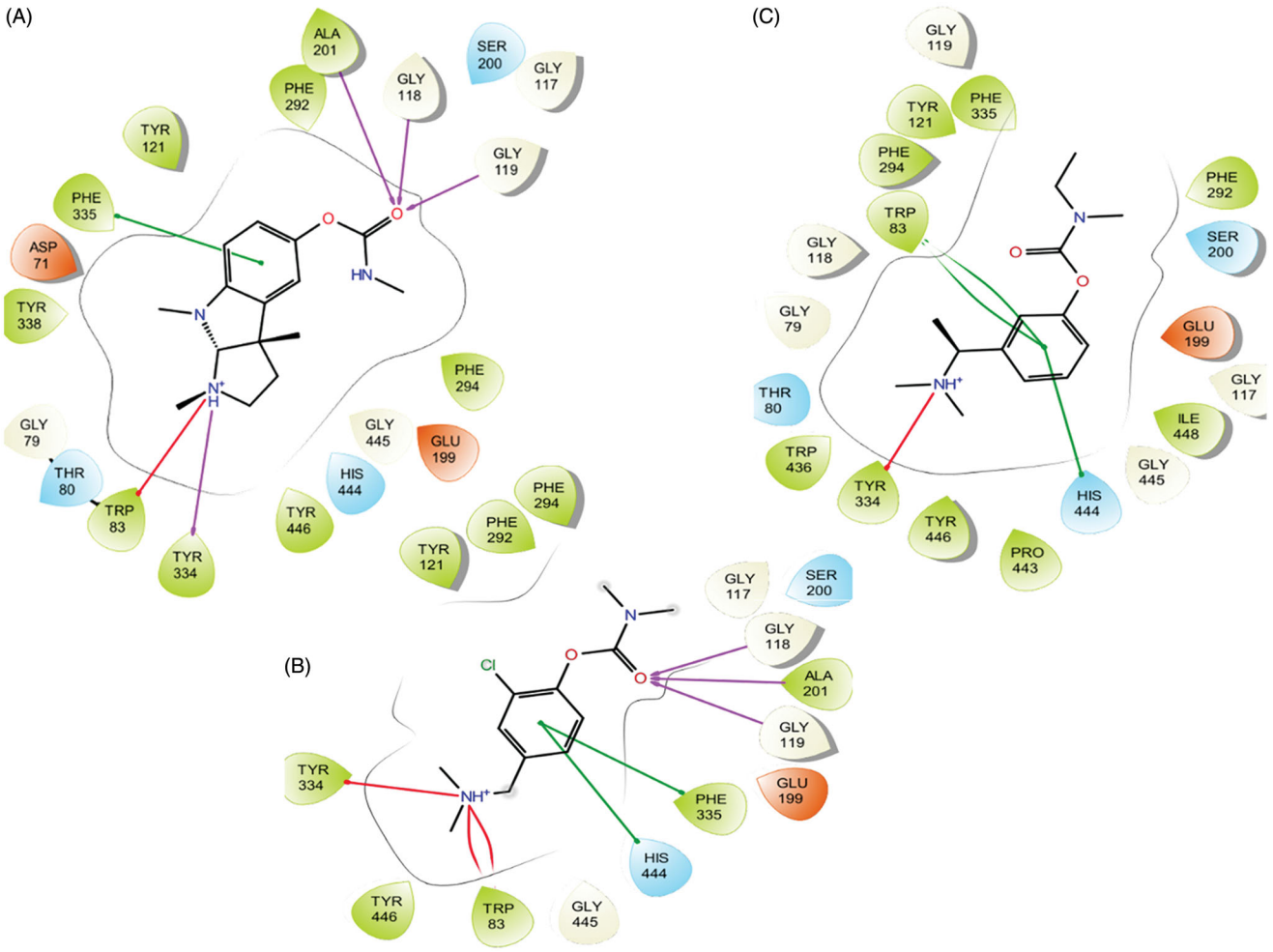

Figure 3. Interaction patterns of the docking poses (predicted by Glide SP) of physostigmine (A), S-I 26 (B), and rivastigmine (C). The legend is identical to Figure 2.

compounds show moderate IC50 of 120 and 175 μM, respectively. This result reflects the ability of the binding pocket to accommodate a broad diversity of compounds for which different binding modes can lead to similar inhibition potencies. Although compound S-III 6 has an IC50 close to rivastigmine, it has a new scaffold which, to our knowledge, has never been described for an AChE inhibitor so far. As S-III 6 was tested as a racemic mixture, the configuration retained by docking (Supplementary Table S3) may show an up to twofold lower IC50 than the racemic mixture.

4. Conclusion

In this study, we describe the discovery of 11 new AChE inhibitors showing IC50 in the μM range. All these compounds satisfy the Lipinski's rule of five and are predicted to pass the blood–brain barrier according to three independent predictors. Compound S-I 26, a close analog of rivastigmine, is the most potent of the 11 AChE inhibitors and shows an IC50 fivefold lower than rivastigmine. Among the most promising hits, both S-I 16 and S-III 6 possess novel scaffolds that have not been previously described for AChE inhibitors. Thus, these new scaffolds could serve as precursors for the design of new classes of cholinesterase inhibitors.

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Disclosure statement

The authors report no conflict of interest.

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References

1. McGill-Carter T. Market analysis Alzheimer's disease 2020. Market Analysis. 2020.

2. Sharma K. Cholinesterase inhibitors as Alzheimer's therapeutics (Review). Mol Med Report 2019;20:1479–87.
3. Fan L, Mao C, Hu X, et al. New insights into the pathogenesis of Alzheimer's disease. Front Neurol 2020;10:1312.
4. Hampel H, Mesulam MM, Cuello AC, et al. Revisiting the cholinergic hypothesis in Alzheimer's disease: emerging evidence from translational and clinical research. J Prev Alzheimers Dis 2019;6:2–15.
5. Colovic MB, Krstic DZ, Lazarevic-Pasti TD, et al. Acetylcholinesterase inhibitors: pharmacology and toxicology. CN 2013;11:315–35.
6. Jang C, Yadav DK, Subedi L, et al. Identification of novel acetylcholinesterase inhibitors designed by pharmacophore-based virtual screening, molecular docking and bioassay. Sci Rep 2018;8:14921.
7. Doytchinova I, Atanasova M, Valkova I, et al. Novel hits for acetylcholinesterase inhibition derived by docking-based screening on ZINC database. J Enzyme Inhib Med Chem 2018;33:768–76.
8. Ellman GL, Courtney KD, Andres V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
9. Hawkins PCD, Skillman AG, Nicholls A. Comparison of shape-matching and docking as virtual screening tools. J Med Chem 2007;50:74–82.
10. Sterling T, Irwin JJ. ZINC 15-ligand discovery for everyone. J Chem Inf Model 2015;55:2324–37.
11. Koes DR, Camacho CJ. ZINCPharmer: pharmacophore search of the ZINC database. Nucleic Acids Res 2012;40:W409–14.
12. Friesner RA, Banks JL, Murphy RB, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J Med Chem 2004;47: 1739–49.
13. Halgren TA, Murphy RB, Friesner RA, et al. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J Med Chem 2004;47:1750–9.
14. Harder E, Damm W, Maple J, Wu C, et al. OPLS3: a force field providing broad coverage of drug-like small molecules and proteins. J Chem Theory Comput 2016;12:281–96.
15. Madhavi SG, Adzhigirey M, Day T, et al. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. J Comput Aided Mol Des 2013;27: 221–34.
16. Wager TT, Hou X, Verhoest PR, Villalobos A. Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties. ACS Chem Neurosci 2010; 1:435–49.
17. Ghose AK, Ott GR, Hudkins RL. Technically extended multiparameter optimization (TEMPO): an advanced robust scoring scheme to calculate central nervous system druggability and monitor lead optimization. ACS Chem Neurosci 2017;8: 147–54.
18. Shelley JC, Cholleti A, Frye LL, et al. Epik: a software program for pK a prediction and protonation state generation for drug-like molecules. J Comput Aided Mol Des 2007;21: 681–91.
19. Greenwood JR, Calkins D, Sullivan AP, Shelley JC. Towards the comprehensive, rapid, and accurate prediction of the favorable tautomeric states of drug-like molecules in aqueous solution. J Comput Aided Mol Des 2010;24:591–604.
20. Duan J, Dixon SL, Lowrie JF, Sherman W. Analysis and comparison of 2D fingerprints: insights into database screening performance using eight fingerprint methods. J Mol Graphics Model 2010;29:157–70.
21. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 2001;46:3–26.
22. McGann M. FRED pose prediction and virtual screening accuracy. J Chem Inf Model 2011;51:578–96.
23. Hawkins PCD, Skillman AG, Warren GL, et al. Conformer generation with OMEGA: algorithm and validation using high quality structures from the protein databank and cambridge structural database. J Chem Inf Model 2010;50:572–84.
24. Marchant CA, Briggs KA, Long A. In silico tools for sharing data and knowledge on toxicity and metabolism: derek for windows, meteor, and vitic. Toxicol Mech Methods 2008;18: 177–87.
25. Barber C, Amberg A, Custer L, et al. Establishing best practise in the application of expert review of mutagenicity under ICH M7. Regul Toxicol Pharmacol 2015;73:367–77.
26. Barber C, Hanser T, Judson P, Williams R. Distinguishing between expert and statistical systems for application under ICH M7. Regul Toxicol Pharmacol 2017;84:124–30.
27. Kumar A, Darreh-Shori T. DMSO: a mixed-competitive inhibitor of human acetylcholinesterase. ACS Chem Neurosci 2017;8:2618–25.
28. Barak D, Ordentlich A, Stein D, et al. Accommodation of physostigmine and its analogues by acetylcholinesterase is dominated by hydrophobic interactions. Biochem J 2009; 417:213–22.
29. Notredame C, Higgins DG, Heringa J. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J Mol Biol 2000; 302: 205–17.
30. Beitz E. TeXshade: shading and labeling of multiple sequence alignments using LATEX2 epsilon. Bioinformatics 2000;16: 135–9.