Novel Sunifiram-carbamate hybrids as potential dual acetylcholinesterase inhibitor and NMDAR co-agonist: simulation-guided analogue design and pharmacological screening

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ABSTRACT
An efficient method for synthesising NMDAR co-agonist Sunifiram (DM235), in addition to Sunifram-carbamate and anthranilamide hybrids, has been developed in high yields via protecting group-free stepwise unsymmetric diacylation of piperazine using N-acylbenzotiazole. Compounds 3f, 3d, and 3i exhibited promising nootropic activity by enhancing acetylcholine (ACh) release in A549 cell line. Moreover, the carbamate hybrid 3f was found to exhibit higher in vitro potency than donepezil with IC₅₀ = 18 ± 0.2 nM, 29.9 ± 0.15 nM for 3f and donepezil, respectively. 3f was also found to effectively inhibit AChE activity in rat brain (AChE = 1.266 ng/mL) compared to tacrine (AChE = 1.137 ng/mL). An assessment of the ADMET properties revealed that compounds 3f, 3d, and 3i are drug-like and can penetrate blood–brain barrier. Findings presented here showcase highly potential cholinergic agents, with expected partial agonist activity towards glycine binding pocket of NMDAR which could lead to development and optimisation of novel nootropic drugs.

Introduction
Nowadays, growing numbers of people complain of cognition impairment (CI) arises through degenerative brain disease like Alzheimer (AD) and Parkinsonism. Individuals with CI are usually in need for expensive nursing, safekeeping, and institutional care. Various neurotransmitters are known by their ability to modulate cognitive function; thus they represent potential targets for enhancing cognition. Among these neurotransmitters, acetylcholine (ACh) is well known for its central role in critical physiological processes, such as attention, learning, memory, stress response, wakefulness, sleep, and sensory information. Cholinergic deficit is a reliable early marker in Alzheimer’s disease (AD). So the activation of cholinergic receptors is an attractive therapeutic option for Alzheimer patients, this can be achieved by inhibiting degradation of ACh using acetylcholinesterase inhibitor (AChEI) like rivastigmine, tacrine, and donepezil, which were approved for treatment of AD (Figure 1). However, due to reports hepatotoxicity associated with tacrine, it is no longer in use.

N-methyl-D-aspartate receptor (NMDAR) is another approach in enhancing cognition, it is a glutamate receptor and ion channel protein found in nerve cells as tetrameric complex and is a...
promising target for cognitive enhancement since it is centrally involved in cognitive processes. It was shown that transient activation of NMDAR is the trigger for the induction of long-term potentiation (LTP) at synapses of neurons in the hippocampus which are likely to explain their importance for learning and memory. Also, it has the ability to increase acetylcholine release and its inhibition result in decrease in acetylcholine secretion. Biochemical and molecular studies of NMDA receptor showed that both mRNA and protein levels of NMDARs are reduced in AD brain and AD model, suggesting hypofunction of NMDAR with increasing AD pathologic severity. These observations supported by findings that blocking NMDAR by ketamine and phencyclidine can induce schizophrenic like symptoms including cognitive decline in healthy individuals and exacerbate cognitive deficit in schizophrenic individuals.

Full activation of NMDARs requires agonist binding at two glycine and two glutamates on the tetrameric complex. Several experimental studies showed that the glycine site was likely to be fully occupied in vivo either by glycine itself or by D-serine. On the other hand, it was found that at some locations in the central nervous system, the glycine site is not fully saturated by glycine due to the activity of high-affinity glycine transporters (GlyT-1). The requirement for occupation of the glycine site has been derived from a number of observations that blocking glycine site in NMDAR exacerbates psychotic symptoms in schizophrenic individuals and impairs cognitive performance in healthy individuals.

As a result of this, glycine binding site has attracted attention of many scientists as a potential target for safely elevating the activity of NMDARs. A number of potential strategies for enhancing NMDAR function and hence improving cognition via the glycine site had developed like administration of glycine but this strategy is limited by the high activity of GlyT-1, so effort is moved to develop GlyT-1 inhibitors like Pfizer sarcosine analogue CP-802079. Limitation of this approach is activation of inhibitory glycine receptors. Another promising approach involves monoacylation of piperazine and two glutamates on the tetrameric complex. Several experimental studies showed that the glycine site was likely to be fully occupied in vivo either by glycine itself or by D-serine. On the other hand, it was found that at some locations in the central nervous system, the glycine site is not fully saturated by glycine due to the activity of high-affinity glycine transporters (GlyT-1). The requirement for occupation of the glycine site has been derived from a number of observations that blocking glycine site in NMDAR exacerbates psychotic symptoms in schizophrenic individuals and impairs cognitive performance in healthy individuals.

Results and discussion

Chemistry

We have recently succeeded in monoacylation of aromatic and aliphatic symmetrical diamines using N-acylbenzotriazoles in high yields. Expanding the utility of the earlier method, we first prepared N-acylbenzotriazoles (1a-k) by reacting carboxylic acids, namely, benzoic, propionic, nicotinic, anthranilic, p-aminobenzoic acid, and N-Boc aminoacids with 1 equivalent of 1H-benzotriazole and 1.4 equivalents. N,N'-Dicyclohexylcarbodiimide (DCC) in CH₂Cl₂ at 25 °C for 12 h (Scheme 2). Sunifiram and the intended analogues are prepared via monoacylation of piperazine which is inexpensive and commercially available by simply stirring 1.4 equivalents of it with 1-benzoylbenzotriazole (2a) in n-butanol for 3 h to produce N-benzylopinpiperazine (2a) in 76% yield. Heating of compound (2a) with N-acylbenzotriazoles (1b–k) in n-butanol for 1 h affords Sunifiram and various analogues in high yields in 80–90% yield (Scheme 3). The advantages of this method are (i) short reaction times, (ii) simple work up, (iii) cheap starting materials and reagents, and (iv) benzotriazole can be recycled.
Pharmacological evaluation

The prevention of dementia is a main goal in patients with neurodegenerative diseases like Alzheimer. Basic research efforts have focussed on drugs that restore acetylcholine concentration and by activating long-term potentiation (LTP) at synapses of neurons. Both effects can be achieved by activating NMDAR and also by using classical AChEI to restore acetylcholine levels. Depending on this finding, in the present study, we estimated the efficacy of the designed compounds on the release of acetylcholine and on preventing its degradation.

Cholinergic activity assay

The cholinergic activity of the synthesized compounds 3c–j, was evaluated in comparison with Sunifiram 3a based on the ability of human bronchioalveolar carcinoma cells to produce acetylcholine. The protocol developed by Song et al., and modified by Dasgupta et al., was followed, using adenocarcinomic human alveolar basal epithelial cells A549 that express NMDAR on their surface to measure the amount of acetylcholine released in response to synthesized compounds.

It is possible that using large dose of the tested compounds being toxic to the cells and causes cell death so the levels of acetylcholine become lower. To avoid the possible cytotoxicity, we started by measuring IC50 of the synthesized compounds using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to select safe dose for evaluation of acetylcholine release. The A549 lung adenocarcinoma cells were treated with various concentrations (100, 25, 6.25, 1.56, 0.39 μM) of the target compounds 3c–j and 3a (Sunifiram) and subjected to MTT assay (Table 1, Figure 4). To eliminate the possibility of misleading results due to cytotoxicity, dose of 1/2 IC50 was used in the acetylcholine release assay.

The relative amount of ACh released in response to the target compounds 3c–j was then determined in A549 lung adenocarcinoma cells by Song et al. protocol. Results showed that treatment with the targets 3c-j promotes ACh release in A549 cells (Table 2, Figure 5). Compounds 3d and 3f that contain the carbamate moiety showed a great enhancing activity for ACh release more than two-fold the amount released normally in A549 cells (2.3-fold and 2.9-fold, respectively) with compound 3f (ACh = 176.1 pg/ml) being the most active even higher than the well-known cognitive enhancer Sunifiram (ACh = 144.3 pg/ml). This may be attributed to the ability of these compounds to directly enhance acetylcholine release from A549 cells and/or by inhibiting AChE, thereby resulting in increasing acetylcholine concentration as may be concluded from the lower level of free choline in case of 3f and 3d (221.5 pg/ml and 235.6 pg/ml) compared to control group or Sunifram-treated group with free choline levels of 255.7 pg/ml and 258.2 pg/ml, respectively. Also, compound 3i caused a 1.8-fold increase in acetylcholine concentration compared to the control. These results suggest that targets 3d, 3f, and 3i can be good lead compounds to develop novel cognitive enhancers. Other compounds 3c, 3e, 3g, 3h, and 3j expressed enhanced ACh...
Scheme 2. Synthesis of N-acylbenzotriazoles 1a-k. Reagent condition: DCC (1.4 eq) in CH₂Cl₂, rt, 12 h.

| 1 | R                  |
|---|--------------------|
| a | C₆H₅                |
| b | CH₃CH₂              |
| c | CH₃                 |
| d | Boc-NHCH₂⁺          |
| e | Boc-NHCH(CH₃)⁺      |
| f | Boc-NHCH(CH₂)₂⁻     |
| g | Boc-NHCH(CH₂CH₂SCH₃)⁻ |
| h | Boc-NHCH(CH₂Ph)⁻    |
| i | 4-NH₂-C₆H₄⁺         |
| j | 2-NH₂-C₆H₄⁺         |
| k | 3-pyridinyl         |

Scheme 3. Synthesis of Sunifiram (3a) and Sunifiram analogues (3b-j). Reagent condition: a) n-butanol, rt, 3 h, b) n-butanol, 60 °C, 12 h. For R, see Table 1 and “Experimental” section.

Table 1. Yield % and cytotoxicity IC₅₀ of compounds 3c-j and Sunifiram 3a on A549 cell line.

| Compound no. | Structure | Yield % | Cytotoxicity IC₅₀ μM A549 cells  |
|--------------|-----------|---------|----------------------------------|
| 3a (Sunifiram) (DM235) | ![Structure](image) | 93 | 85.93 ± 4.43 |
| 3b | ![Structure](image) | 86 | Not applied |
| 3c | ![Structure](image) | 91 | 101.5 ± 5.24 |
| 3d | ![Structure](image) | 91 | 49.26 ± 2.54 |
| 3e | ![Structure](image) | 92 | 52.77 ± 2.72 |

(continued)
release with variable increments (1.3- to 1.5-fold) of normal ACh value. The high level of acetylcholine measured with carbamate compounds 3f and 3d may be attributed to a dual effect of these compounds as both AChE inhibitor and NMDAR co-agonist in contrast to the carbamate devoid compounds. So estimating the activity of the most potent compounds on AChE was also performed.

**Table 1.** Cytotoxicity IC₅₀ of compounds 3c-j and 3a (Sunifiram) on A549 cell line.

| Compound no. | Structure | Yield % | Cytotoxicity IC₅₀ µM |
|--------------|-----------|---------|---------------------|
| 3f           | ![Structure](image) | 93      | 32.17 ± 1.66        |
| 3g           | ![Structure](image) | 89      | 37.98 ± 1.96        |
| 3h           | ![Structure](image) | 84      | 43.49 ± 2.24        |
| 3i           | ![Structure](image) | 87      | 96.63 ± 4.98        |
| 3j           | ![Structure](image) | 81      | 57.51 ± 2.97        |

**Figure 4.** Cytotoxicity IC₅₀ of compounds 3c-j and 3a (Sunifiram) on A549 cell line.

**Table 2.** Acetylcholine released in response to compounds 3c-j and 3a from A549 cells.

| Compound | 1/2 IC₅₀ µM | Total ACh (pg/ml) | Free Choline (pg/ml) | Acetyl choline release (pg/ml) |
|----------|-------------|-------------------|----------------------|-----------------------------|
| 3c       | 50.8        | 400.7 ± 4.56      | 320.6 ± 1.16         | 80.1 ± 4.7                  |
| 3d       | 24.6        | 377.4 ± 8.44      | 235.6 ± 1.3          | 141.8 ± 5.01                |
| 3e       | 26.4        | 387.4 ± 8.17      | 307.7 ± 3.44         | 79.7 ± 8.86                 |
| 3f       | 16.1        | 397.6 ± 8.62      | 221.5 ± 2            | 176.1 ± 8.85                |
| 3g       | 19          | 374.5 ± 6.94      | 291 ± 2.83           | 83.5 ± 7.49                 |
| 3h       | 21.7        | 362.9 ± 22        | 275.1 ± 3.1          | 87.8 ± 22.21                |
| 3i       | 48.3        | 351.6 ± 4.24      | 240.8 ± 1.53         | 110.8 ± 4.51                |
| 3j       | 28.8        | 330.7 ± 4.89      | 240 ± 2.59           | 90.7 ± 5.53                 |
| Sunifram(3a) | 43        | 402.5 ± 2.58      | 258.2 ± 1.52         | 144.3 ± 2.99                |
| Control  | –           | 316.7 ± 1.9       | 255.7 ± 9.78         | 61 ± 9.69                   |

**AChE inhibition assay**

In vitro AChE inhibition assay

Our synthesised compounds were evaluated for their in vitro inhibitory activities against AChE based on Ellman’s method in comparison to donepezil.⁴⁶ The best anti-AChE activity was obtained by compound 3f possessing methionine side chain (IC₅₀=0.018 ± 0.0002 µM), a value lower than that of the reference drug donepezil (IC₅₀= 0.0299 ± 0.00015 µM) and close to that of
rivastigmine (IC_{50} = 0.0163 ± 0.00017 μM). Absence of 3f side chain (2-methylthioethyl group resulted in decreasing the potency of the targets to micromolar range (10.36–22.37 μM) (Table 3). It was noticed that (i) 2-methylthioethyl group at the α-carbon of 3f is important for potency and (ii) increasing the size of side chain at the α-carbon (H, CH₃, CH(CH₃)₂, ph) resulted in decreasing anti-AChE activity (Figure 6).

**AChE inhibition assay**

The most active compound 3f was then subjected to ex-vivo experiment to assess its efficacy as AChE inhibitor. The rats were randomly divided into three groups of five animals each: normal control group, 3f-(10 μg/kg) treated group and tacrine- (10 μg/kg) treated groups. AChE activity was determined according to modified Ellman assay method. The percentage of inhibition was calculated by comparison with AChE activity of rats treated with vehicle (Table 4, Figure 7).

The results indicate that compound 3f can inhibit effectively AChE activity in rat brain with efficacy (AChE = 1.266 ng/mL) close to that of tacrine (AChE = 1.137 ng/mL). It could be concluded that:

1. Target 3f can cross BBB
2. Target 3f can inhibit AChE effectively in vivo and could be considered for further investigation as a nootropic agent capable of enhancing cognition in various diseases involving cognitive deficit such as Alzheimer’s disease and Parkinson’s disease.

**In vitro hepatotoxicity screening**

Hepatotoxicity of the active compounds 3f and 3i was evaluated using Transformed Human Liver Epithelial-2 (THLE-2). Compounds 3f and 3i have been incubated with THLE-2 cells for 24 h and MTT assay was used to determine cell viability. Compound 3f was less toxic than compound 3i.

| Group              | Dose ug/kg | AChE (ng/mL) |
|--------------------|------------|--------------|
| Control            | –          | 2.498 ± 0.303|
| 3f                 | 10         | 1.266 ± 0.114|
| Tacrine            | 10         | 1.137 ± 0.064|

**Table 3.** Anticholinesterase activity of synthesised Sunifiram analogues.

| Compound | AChE inhibition (IC_{50} [μM]) | Compound | AChE inhibition (IC_{50} [μM]) |
|----------|---------------------------------|----------|---------------------------------|
| 3c       | 13.9 ± 0.17                     | 3g       | 22.57 ± 0.29                   |
| 3d       | 19 ± 0.00                       | 3i       | 10.36 ± 0.23                   |
| 3e       | 20.1 ± 0.00                     | Donepezil| 0.0299 ± 0.00015               |
|          | (29.9 ± 0.15 mM)                |          | (29.9 ± 0.15 mM)               |
| 3f       | 0.018 ± 0.0002                  | Rivastigmine| 0.0163 ± 0.00017 μM (16.3 nM ± 0.17) |

**Figure 5.** Acetylcholine released in response to compounds 3c-j and Sunifiram (3a) from A549 cells.

**Figure 6.** SAR of novel AChEI.

**Figure 7.** AChE level on rat brain after i.p. administration of 3f and tacrine.

**Table 4.** AChE level on rat brain after i.p. administration of 3f and tacrine.

| Group | Dose ug/kg | AChE (ng/mL) |
|-------|------------|--------------|
| Control | –          | 2.498 ± 0.303|
| 3f     | 10         | 1.266 ± 0.114|
| Tacrine| 10         | 1.137 ± 0.064|

**Table 5.** Molecular docking scores of Sunifiram and synthesised analogues.

| Compound | Docking score (kcal/mol) |
|----------|--------------------------|
| Sunifiram (3a) | –4.5        |
| 3b       | –4.4                    |
| 3c       | –3.8                    |
| 3d       | –3.8                    |
| 3e       | –3.5                    |
| 3f       | –1.7                    |
| 3g       | –0.5                    |
| 3h       | –0.4                    |
| 3i       | –4.5                    |
| 3j       | –2.5                    |
cytotoxic with IC_{50} equal 40.58±1.95 μM while compound 3i cause 50% decrease in cell viability at 26.979±1.29 μM.

**Computational evaluation of the targets ability to bind to the glycine binding pocket of NMDA receptors**

**Molecular modelling insights**

Targets 3a–j were docked into the glycine binding pocket of the NMDA receptor ligand binding domain (LBD) to ascertain their chemical and physical feasibility towards the glycine binding pocket. The scoring functions incorporated in the molecular docking tools allow the evaluation of the binding affinity of each compound\textsuperscript{43} from which we selected the compound with the strongest binding affinity. Compound 3i which exhibited the highest (−4.5 kcal/mol) binding affinities was selected for further in silico investigations. This top compound exhibited relatively similar binding affinity with Sunifiram as seen in Table 5. These docking score hint that this compound favourably bind to the glycine binding pocket. After evaluating the differential docking affinities of the compounds, we proceeded to determine the pharmacokinetics and physicochemical properties of the compounds.

**Assessing the pharmacokinetic and physicochemical properties of analogues**

Upon administration of a drug, the pharmacokinetics and the physicochemical properties of the drug influence their rate of absorption, distribution, metabolism, and excretion in human system.\textsuperscript{44–46} The Lipinski’s rule of five is generally used to predict the drug-likeness of a chemical compound by measuring the biological activity, good oral bioavailability together with the compound’s tendency to cross various aqueous and lipophilic barriers by adhering to certain conditions.\textsuperscript{47,48} SwissADME was used to predict the pharmacokinetic and physicochemical properties of the compounds. As shown in Table 6, all the compounds had molecular weight less than 500 Da, octanol-water partition coefficient of less than 5, H-bond donors less than 5, and H-bond acceptors of less than 10 together with high gastro-intestinal absorption. However, one of the principal conditions that need to be met by all potential nootropic drugs is the ability to traverse the blood–brain barrier (BBB). All targets 3c–j have LogP in the range 1.5–2.36, this value is optimal for BBB penetration as postulated by Hansch and Leo that found that BBB penetration is optimal when the LogP values are in the range of 1.5–2.7, while compound 3b did not met this value (LogP = 0.94).\textsuperscript{49} We then selected the best compound according to its docking score 3i (−4.5 kcal/mol) for molecular dynamics simulation relative to the native ligand glycine.

Conformational and structural dynamics of the LBD of NMDA upon binding of 3i

When chemical compounds bind to biological targets, they usually instigate changes in the primary structure of the biological target which resultanty disrupts the basal functionalities. We therefore investigated the structural changes that occur upon the binding of 3i to the glycine binding pocket using a time-scale analyses of the trajectories generated by the molecular dynamics technique employed. The stability of the systems, mobility, compactness, and the fluctuations of the residues were estimated by computing the C\_\textsubscript{\text{-}} root-mean square deviation (RMSD), C\_\text{\gamma} radius of gyration (RoG), and the C\_\text{\gamma} root-mean-square fluctuation (RMSF). The C\_\text{\gamma} RMSD measures the atomistic deviations as well as reflecting the stability and convergence of the systems. As observed in Figure 8, all the systems converged and were comparatively stable from the beginning to about 175 ns of the simulation. The deviations were then observed to vary with time. The lead compound, Sunifiram, showed the most stable system with an average RMSD value of 1.798 Å. The unbound system (apo) and the 3i system showed average RMSD values of 2.063 Å and 2.213 Å, respectively. Generally, the stability of the systems gives credence to the assumptions derived from the models. We further calculated the RMSF, which is predictive of the flexibility of the systems. As observed from the RMSF graph plots, 3i presents the less flexible domain with an average RMSF value of 13.081 Å, a value lesser that the unbound model and Sunifiram bound model which presented 15.595 Å and 14.127 Å, respectively. This suggests that the binding of 3i to the ligand binding domain induces a less flexible domain compared to Sunifiram. This could indicate that the binding of 3i further decreased structural flexibility reflective of distortions of backbone atoms and corroborated with the findings from RoG where in high RoG values indicate less compactness and high mobility of the C\_\text{\gamma} atoms. RoG average figures of 19.315 Å, 19.448 Å, and 19.452 Å were presented for 3i, Apo, and Sunifiram, respectively. These as well indicate that the 3i induced high compactness and less residual mobility. Taken together, the ability of the compounds to induce a reduction in residual flexibility, compactness, and mobility could induce a signal to the membrane domain of the receptor that resultanty reliefs the TMD of magnesium thereby opening the channel for passage. It could therefore be inferred further that 3i with the most disruptive effect on the Ca atoms could be more potent and of therapeutic use making it a candidate for experimental validation.

**Comparative analysis of the conformational and structural dynamics of the LBD of NMDA upon binding of 3i and Sunifiram relative to the native co-agonist (glycine)**

Finally, the induced conformational and structural dynamics upon the binding of 3i were compared to the native co-agonist glycine
to evaluate their differential effects on the ligand binding domain. As observed from Figure 9, 3i presented similar results as that of the native ligand. Both systems showed similar C-α atoms deviation pattern during the period of simulation via their RMSD calculation with average RMDS values of 2.213 Å and 2.006 Å for 3i and glycine respectively. Relative to the unliganded LBD, both 3i and glycine are shown to decrease the stability of LBD. Also, RMSF and RoG estimations of both 3i and glycine reflect similarity of action on the ligand binding domain. For the residual fluctuations of the domain, both 3i and glycine presented lower figures compared to the unbound system, informative of the reductive effects of both ligands on the flexibility and mobility of the domain. Average RMSF figures of 13.081 Å and 14.398 Å were presented by 3i and glycine, respectively. The compactness of the domain as observed in the RoG figures of 19.315 Å and 19.305 Å for 3i and glycine, respectively, corroborates the reductive effect of both compounds on the mobility which ultimately increase compactness and rigidity of the domain. Presenting a further lower figure compared to the native ligand as observed in the case of 3i could imply more potency though this needs experimental validation.

**Free binding energies and per-residue energy contributions associated with the binding of Sunifiram, and 3i**

Probing further, we sought to ascertain the structural insights into the mechanistic binding and stability of the 3i-receptor complex and Sunifiram-receptor complex during the 250 ns simulation period and the evaluation of their binding energies (∆G) involved in their complex formations. As shown in Table 7, compound 3i and Sunifiram exhibited favourable free binding energies of −30.13 kcal/mol and −6.43 kcal/mol, respectively. Van der Waals and electrostatic energies contributed immensely to their total binding energies which ultimately led to the stability of the compounds within glycine binding pocket. These desirable binding energies of the compounds could explain their modulatory effects on the entire NMDA receptor. Intermolecular interactions between the compounds and the residues at the binding site facilitate the binding and stability of the compounds to the domain. As seen in Figure 10, the residues that contributed the most to the binding of Sunifiram include Ile125 (−0.134 kcal/mol), Pro138 (−0.104 kcal/mol), Tyr141 (−0.310 kcal/mol), Pro165 (−0.166 kcal/mol), Thr191 (−0.151 kcal/mol), Arg194 (−0.173 kcal/mol), Lys198 (−0.143 kcal/mol), and Arg245 (−0.136 kcal/mol). Residues Ile125
Asn126 (1.626 kcal/mol), Asn127 (1.139 kcal/mol), Tyr162 (1.369 kcal/mol), Phe244 (1.061 kcal/mol) and residues Tyr124 (1.211 kcal/mol), Ile125 (1.284 kcal/mol), Asn126 (3.188 kcal/mol), Tyr182 (1.717 kcal/mol), Phe244 (1.187 kcal/mol), and Ser246 (1.767 kcal/mol) contributed the highest energies to the 3i complex. These results present higher residue energy contributions from the residues to the compound 3i relative to Sunifiram.

Table 7. MMGBSA-based binding free energy profile of Sunifiram and compound 3i.

| System  | ΔE_{el} | ΔE_{vdw} | ΔG_{gas} | ΔG_{sol} | ΔG_{bind} |
|---------|---------|----------|----------|----------|-----------|
| Sunifiram | −9.50 ± 7.19 | −4.25 ± 6.29 | −13.75 ± 11.59 | 7.32 ± 6.78 | −6.43 ± 5.68 |
| 3i      | −38.21 ± 2.95 | −17.58 ± 5.64 | −55.80 ± 6.30 | 25.67 ± 4.38 | −30.13 ± 3.09 |

ΔE_{el}: electrostatic energy; ΔE_{vdw}: van der Waals energy; ΔG_{gas}: total binding free energy; ΔG_{sol}: solvation free energy; ΔG: gas phase free energy.

(−1.626 kcal/mol), Asn126 (−1.662 kcal/mol), Asn127 (−1.139 kcal/mol), Tyr162 (−1.369 kcal/mol), Phe244 (−1.061 kcal/mol) and residues Tyr124 (−1.211 kcal/mol), Ile125 (−1.284 kcal/mol), Asn126 (−3.188 kcal/mol), Tyr182 (−1.717 kcal/mol), Phe244 (−1.187 kcal/mol), and Ser246 (−1.767 kcal/mol) contributed the highest energies to the 3i complex. These results present higher residue energy contributions from the residues to the compound 3i relative to Sunifiram.

**Molecular Binding mechanism of novel Sunifiram–Carbamate hybrid towards AChE**

However, there are several crystal structures available for AChE, we have selected structures that contain carbamate derivatives as inhibitors as the synthesised analogues were based on carbamate for activity. Acetylcholinesterase(AChE) complexed with Ganstigmine was retrieved from the protein data bank (PDB) with ID: 2BAG. According to the *in vitro* results as can be seen from Table 3, compound 3f is found to be the most potent in inhibiting AChE depending on that we focussed on it during simulation. The non-covalent binding mechanisms of new compounds were predicted using molecular docking simulation using rivastigmine and donepezil as reference. Molecular docking allowed for the prediction of the most suitable binding conformation of compound 3f which could favour its pocket stability, affinity, and inhibitory potential towards AChE. Molecular docking results as presented in Table 8 and Figure 11 showed that compound 3f exhibited a docking of −8.8 kcal/mol. Relative to other synthesised analogues including the known AChE inhibitor donepezil (−11.1 kcal/mol); 3f and rivastigmine (−7.9 kcal/mol) exhibited the lowest docking score towards AChE as shown in Table 8 although they exhibited the highest AChE inhibitory activity from our experimental investigation. This may attribute to the observation that the crystal structure of carbamate-based AChEi showed catalytic residue Ser200 carbamylated and the inhibitors were hydrolysed as ACh molecule does at the active site. So regardless of its relatively lower docking score, the most favourable binding pose of

**Figure 9.** Comparative RMSD plots of C-a atoms of 3i (red), glycine (magenta), and the unbound Apo (green). B) Comparative RMSF plots of individual residues of 3i (red), glycine (magenta), and the unbound Apo (green). C) Comparative RoG plots of C-a atoms of 3i (red), glycine (magenta) and the unbound Apo (green). D) 3D structural superposition of 3i (red), glycine (magenta) and the unbound Apo (green) to show structural flexibility.
compound 3f (−8.8 kcal/mol) allowed for the formation of strong binding pocket interactions that contributes towards its inhibitory potency.

A comparative analysis of 3f interactions revealed that at a peripheral anionic site (PAS) the −OC(CH₃)₃ moiety is pointed towards TRP 279 forming π-σ interaction also the −SCH₃ moiety participate in π-alkyl interaction with TRP 279 and PHE 290. TYR 70 and TYR 121 are close to show Van der Waals interaction with 3f (Figure 11). Interactions in catalytic anionic site (CAS) was also evident as seen in π-π stacked interaction between the phenyl ring of 3f and TRP 84 and π-alkyl interaction between its piperazine part and PHE 330 and PHE 331. These interactions in addition to π-sulphur interactions with PHE 288 and PHE 331 and several van der Waals interactions collectively anchor compound 3f within the AChE inhibitor binding pocket. Interestingly TRP 279 and TRP 84 were also participate in π-π stacked interaction with donepezil in addition to π-alkyl interaction was also observed with PHE 330 and PHE 331 in similar manner to 3f. Also, PHE 331 involved in π-π interactions with rivastigmine like 3f (Figure 12). All in all, this similarity in binding interactions of 3f, donepezil, and rivastigmine further suggested a similarity in binding mechanism and its potential as an AChE inhibitor.

Conclusions

In conclusion, novel Sunifiram-carbamate and Sunifram-anthranilamide hybrids were designed, synthesised, and evaluated for cholinergic activity. Introducing carbamate to the skeleton of synthesised targets enabled a good AChE inhibitory activity. Amongst all targets compound the Sunifiram-carbamate hybrid 3f showed the most potent AChE inhibitory activity with an IC₅₀ value of 18 ± 0.2 nM. Such ability of 3f together with its good logP value (2.36), its ability to induce ACh release from A549 cells, its in vivo ability to lower AChE activity in rat brain makes it worthy of further investigation as a promising nootropic agent. It also showed molecular docking
score $= -1.7 \text{kcal/mol}$ when docked to glycine binding pocket of NMDA receptor compared to Sunifiram ($-4.5 \text{kcal/mol}$) and the anthranilamide hybrid $3i$ ($-4.5 \text{kcal/mol}$). Compounds $3i$ further bound preferentially to NMDA domain with high binding affinity interaction which enhanced its binding pocket stability and are promising leads as potent co-agonists that binds to the glycine binding pocket of NMDA receptor and expressed good AChE inhibitory activity with IC$_{50}$ value of $10.36 \pm 0.23 \text{mM}$.

Full experimental detail, $^1$H and $^{13}$C NMR spectra, computational methodology, and pharmacological screening can be found via the “Supplementary Content” section of this article’s webpage

**Experimental**

**General information**

Starting materials and solvents were purchased from common commercial sources and used without further purification. Melting points were determined on Fisher melting apparatus and are uncorrected. $^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded on JEOL a 500 MHz NMR Spectrometer and using DMSO-$d_6$ and CDCl$_3$ as solvents, at Faculty of Science, Mansoura University. Also Bruker 400 MHz NMR Spectrometer at Faculty of Pharmacy, Mansoura University was used. The chemical shift ($\delta$) is reported in ppm, and coupling constants ($J$) are given in Hz. The HRMS was recorded on Q-TOF, 6530 (Agilent Technologies) at Faculty of pharmacy, Fayoum University. All reactions were monitored by TLC with visualisation by UV irradiation.

**General procedure for the synthesis of $N$-acylbenzotriazoles**

To the corresponding carboxylic acid (10 mmol), dissolved in dichloromethane (50 ml), benzotriazole (1.19 g, 10 mmol), and dicyclohexylcarbodiimide (2.89 g, 14 mmol) were added. The reaction mixture was left with stirring at 25°C, overnight. Dicyclohexylurea was filtered, and dichloromethane was evaporated. The residue was crystallised from dichloromethane (20 ml) and hexane (30 ml). The product was filtered and dried under vacuum to give the desired compounds.

$^{1H}$benzo[d][1,2,3]triazol-1-yl](phenyl)methanone ($1a$). White microcrystal, yield 2.03 g (91%), mp 111–112°C (lit. 110–112°C).$^{21}$ $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.33–8.28 (m, 2H, Ar–H), 8.13–8.10 (m, 2H, Ar–H), 7.85–7.76 (m, 2H, Ar–H), 7.68–7.63 (m, 3H, Ar–H), $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 166.5 (C=O), 145.2 (C=N = N), 133.5 (Ar–C), 131.7 (Ar–C), 131.5 (Ar–C), 131.3 (Ar–C), 130.7 (Ar–C), 128.3 (Ar–C), 126.6 (Ar–C), 120.0 (Ar–C), 114.4 (Ar–C).

1-(1H-benzo[d][1,2,3]triazol-1-yl)propan-1-one ($1b$). White microcrystal, yield 1.57 g (90%), mp 78–80°C (lit. 80–82°C).$^{22}$ $^1$H
NMR (500 MHz, CDCl₃) δ 8.28 (d, J = 8.5 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 7.64 (t, J = 8.0 Hz, 1H), 7.49 (t, J = 8.2 Hz, 1H), 3.45 (q, J = 7.5 Hz, 2H), 1.40 (t, J = 7.5 Hz, 3H).

1-(1H-benzo[d][1,2,3]triazol-1-yl)ethan-1-one (1c). White microcrystal, yield 1.42 g (88%), mp 49–51°C (lit. 49–51°C).

1H NMR (400 MHz, DMSO-d₆) δ 8.22 (t, J = 8.0 Hz, 2H, Ar–H), 7.78–7.74 (m, 1H, Ar–H), 7.61–7.57 (m, 1H, Ar–H), 2.94 (s, 3H, –CH₃).

13C NMR (100 MHz, DMSO-d₆) δ 169.6 (C=O), 145.4 (C–N=), 130.5 (C=N), 130.4 (Ar–C), 126.1 (Ar–C), 119.8 (Ar–C), 113.8 (Ar–C), 23.0 (CH₃).

tert-Butyl (2-(1H-benzo[d][1,2,3]triazol-1-yl)-2-oxoethyl)carbamate (1d). White microcrystal, yield 2.26 g (82%) mp 139–141°C (lit. 140°C).

tert-Butyl (S)-(1-(1H-benzo[d][1,2,3]triazol-1-yl)-1-oxopropan-2-yl)carbamate (1e). White microcrystal, yield 2.44 g (84%), mp 68–70°C (lit. 68–69°C).

1H NMR (400 MHz, DMSO-d₆) δ 8.28 (d, J = 8.0 Hz, 1H, Ar–H), 8.23 (d, J = 8.0 Hz, 1H, Ar–H), 7.80 (t, J = 7.6 Hz, 1H, Ar–H), 7.63 (t, J = 7.6 Hz, 1H, Ar–H), 7.45 (d, J = 3.2 Hz, 1H, NH), 5.48–5.41 (m, 1H, –CH–), 1.51 (d, J = 7.2 Hz, 3H, –CH–CH₃), 1.38 (s, 9H, –C(CH₃)₃).

13C NMR (100 MHz, DMSO-d₆) δ 173.3 (–CO–N), 156.0 (–OCO–), 145.8 (–N=C=), 131.5 (Ar–H), 131.1 (Ar–H), 127.1 (Ar–H), 120.6 (Ar–H), 114.4 (Ar–H), 79.1 (–C(CH₃)₃), 50.2 (–CH–), 28.6 (–C(CH₃)₂), 17.1 (–CH–CH₃).

tert-Butyl (S)-(1-(1H-benzo[d][1,2,3]triazol-1-yl)-3-methyl-1-oxobutan-2-yl)carbamate (1f). White microcrystal, yield 2.58 g (81%), mp 119–120°C (lit. 120–121°C).

tert-Butyl (S)-(1-(1H-benzo[d][1,2,3]triazol-1-yl)-4-(methylthio)-1-oxobutan-2-yl)carbamate (1g). White sticky, yield 2.90 g (83%).

1H NMR (400 MHz, CDCl₃) δ 8.23–8.09 (m, 1H, Ar–H), 7.87 (d, J = 8.8 Hz, 1H, Ar–H), 7.64–7.50 (m, 1H, Ar–H), 7.38 (d, J = 8.8 Hz, 1H, Ar–H), 7.28 (s, 1H, NH), 5.83–5.73 (m, 1H, CH), 2.71–2.56 (m, 2H, CH₂SCH₃), 2.15–2.06 (m, 5H, CH₂SCH₂CH₂), 1.43 (s, 9H, C(CH₃)₃).

13C NMR (100 MHz, CDCl₃) δ 175.4 (CON), 171.8

Figure 12. Molecular visualisation of 3f-AChE, donepezil-AChE, and rivastigmine-AChE binding pocket.
In a round bottom flask, piperazine (1.30 g, 15 mmol) were dissolved in ethyl acetate (20 ml) and was washed with saturated Na2CO3 (5 ml, 3 x), water (5 ml, 2 x) and brine (5 ml, 1 x). The organic layer was dried over anhydrous sodium sulphate. Hexane (20 ml) was added to the filtrate, and the solid obtained was dried under vacuum to give the target compounds 3a–j.

1-(4-Benzylpiperazin-1-yl)propan-2-ylcarbamate (3a). White microcrystal, yield 0.35g (62%), mp 75–77 °C. 1H NMR (400 MHz, DMSO-d6) δ 7.21 (d, J = 6.8 Hz, 2H, O = C–(o–Ar-H)), 6.66 (t, J = 8.8 Hz, 2H, Ar–H), 6.61 (d, J = 8.4 Hz, 1H, NH), 6.27 (s, br, 1H, Ar–H), 3.95 (t, J = 7.6 Hz, 1H, –NH–CH–), 3.45–3.26 (m, 4H, –C–CO– N(CH2)2–), 3.12–3.03 (m, 4H, –CH(CH3)2–N–CO–CH3–), 1.72–1.67 (m, 1H, –CH–CH3–), 1.13 (s, 9H, –C(CH3)3–), 0.60 (s, br, 6H, –C(CH3)2–). 13C NMR (100 MHz, DMSO-d6) δ 170.8 (~C–CO–), 170.2 (~C–CO–), 155.0 (OOC), 136.1 (~C–CO–), 130.1 (Ar–C), 128.9 (Ar–C), 127.5 (Ar–C), 78.5 (~C–CH3–), 45.0 (~C–CO– N(CH2)2–), 42.3 (~C–CH2–N–CO–CH3–), 30.2 (~CH2CH3–), 28.6 (~CH2CH3–), 19.9 (~CH3–). HRMS (ESI): m/z cald for C19H21N3O4 [M + H]+ 320.1633, found 320.1635.

1-(4-Benzylpiperazin-1-yl)-1-ethan-1-one (3b). White microcrystal, yield 0.31 g (60%), mp 171–173 °C. 1H NMR (400 MHz, DMSO-d6) δ 7.48–7.44 (m, 2H, Ar–H), 6.51 (s, br, 3H, Ar–H), 4.35–3.84 (m, 8H, –N(CH2)2CH2–N–), 2.07 (s, 3H, –CH3–). 13C NMR (100 MHz, DMSO-d6) δ 169.7 (~C–CO–), 161.8 (~C–CO–), 156.2 (~OOC–), 136.1 (~C–CO–), 130.2 (Ar–C), 128.9 (Ar–C), 127.5 (Ar–C), 78.4 ((~C–CO–N(CH2)2–), 44.2 (~C–CO–N(CH2)2–), 42.2 (~CH2–CO–), 41.8 (~CH2–CO–N(CH2)2–), 28.7 ((CH3)3–C–), 21.7 (CH3–). HRMS (ESI): m/z cald for C19H21N3O4 [M + H]+ 320.1633, found 320.1635.

tert-Butyl (2-(4-benzylpiperazin-1-yl)-1-oxo-2-propan-2-yl)carbamate (3d). White microcrystal, yield 0.33 g (91%) mp 70–72 °C. 1H NMR (400 MHz, DMSO-d6) δ 7.50 (d, J = 8.0 Hz, 2H, Ar–H), 7.08–7.0 (m, 3H, Ar–H), 6.69 (s, 1H, NH), 4.51–4.48 (m, 1H, –CH–), 3.53 (s, br, 4H, (~C–CO–N(CH2)2–)), 3.41 (s, br, 4H, (~C–CO–N(CH2)2–)), 1.43 (s, 9H, (~CH3)3C–), 1.19 (d, J = 6.8 Hz, 3H, –CH3), 1.04 (d, J = 6.8 Hz, 3H, –CH(CH2)3–). 13C NMR (100 MHz, DMSO-d6) δ 171.4 (~CH2–CO–), 170.8 (~C–CO–), 155.4 (OOC), 136.1 (~C–CO–), 130.1 (Ar–C), 128.9 (Ar–C), 78.5 (~C–CH3–), 46.3 (~CH–), 45.0 (~C–CO– N(CH2)2–), 41.9 (~C–CH2–N–CO–CH3–), 28.7 (~CH2CH3–), 18.1 (~CH3–). HRMS (ESI): m/z cald for C19H21N3O4 [M + H]+ 320.1633, found 320.1635.
(Ar–C), 127.5 (Ar–C), 126.8 (Ar–C), 78.6 (t (–CH2)3, 51.7 (–CH–), 45.0 (–CO–(CH2)2–N–), 41.9 (–CH–CO–(CH2)3–)), 37.8 (–CH2), 28.6 ((CH2)2–C–). HRMS (ESI): m/z calcd for C23H13N5O4 [M + H]+ 438.2387, found 438.2394.

(4-(4-Aminobenzoyl)piperazin-1-yl)(phenyl)methanone (3h). Buff microcrystal, yield 0.26 g (84%) mp 195–197 °C. 1H NMR (400 MHz, DMSO-d6) δ 7.47–7.43 (m, 5H, Ar–H), 7.17 (d, J = 8.0 Hz, 2H, (H2N–(m–Ar–H)), 6.57 (d, J = 8.0 Hz, 2H, (H2N–(o–Ar–H)), 5.58 (s, 2H, NH2), 5.60–3.54 (m, 8H, (CH2)2–(CH2)2–N–)). 13C NMR (100 MHz, DMSO-d6) δ 170.6 (CO), 169.7 (CO), 151.2 (–C–NH2), 136.2 (Ar–C), 130.1 (Ar–C), 129.9 (Ar–C), 128.9 (Ar–C), 125.0 (Ar–C), 121.9 (Ar–C), 113.8 (Ar–C), 45.1 (Aliph–C), 42.2 (Aliph–C). HRMS (ESI): m/z calcd for C19H15N3O2 [M + H]+ 310.1577, found 310.1591.

(4-(2-Aminobenzoyl)piperazin-1-yl)(phenyl)methanone (3i). Pale yellow microcrystal, yield 0.27 g (87%) mp 170–172 °C. 1H NMR (400 MHz, DMSO-d6) δ 7.45–7.41 (m, 3H, Ar–H), 7.09 (t, J = 2.4 Hz, 2H, Ar–H), 7.01–6.99 (m, 2H, Ar–H), 6.71 (d, J = 8.0 Hz, 1H, H2N–(o–Ar–H)), 6.56 (t, J = 7.2 Hz, 1H, H2N–(p–Ar–H)), 5.21 (s, 2H, NH2), 3.66–3.45 (m, 8H, Aliph–C). 13C NMR (100 MHz, DMSO-d6) δ 169.7 (CO), 169.4 (CO), 146.3 (–C–NH2), 136.1 (Ar–C), 130.6 (Ar–C), 130.1 (Ar–C), 128.9 (Ar–C), 127.5 (Ar–C), 119.5 (Ar–C), 119.4 (Ar–C), 116.0 (Ar–C), 47.4 (Aliph–C), 42.0 (Aliph–C). HRMS (ESI): m/z calcd for C19H15N3O2 [M + H]+ 310.1577, found 310.1590.

(4-benzoylpiperazin-1-yl)(pyridin-3-yl)methanone (3j). Buff microcrystal, yield 0.24 g (81%) mp 145–147 °C. 1H NMR (400 MHz, DMSO-d6) δ 8.66 (s, br, 2H, Ar–H), 7.88 (s, br, 1H, Ar–H), 7.47 (s, br, 6H, Ar–H), 3.69 (s, 4H, –(CH2)2–N–CO–ph), 3.51 (s, 4H, –(CH2)2–N–nitrocinotinyl). 13C NMR (100 MHz, DMSO-d6) δ 167.5 (CO), 151.1 (Ar–C), 148.2 (Ar–C), 136.0 (Ar–C), 135.4 (Ar–C), 131.9 (Ar–C), 130.2 (Ar–C), 128.9 (Ar–C), 127.5 (Ar–C), 124.0 (Ar–C), 47.4 (–(CH2)2–N–CO–ph), 42.2 (–(CH2)2–N–nitrocinotinyl). HRMS (ESI): m/z calcd for C17H14N2O2 ([M + H]+) 296.1421, found 296.1438.

Ex-vivo AChE inhibition assay (for 3f)

Animals

Male Sprague-Dawley rats (180–250 g, 8–10 weeks old) were used in the present study. At our laboratory, the animals were provided from Department of Pharmacology, the Faculty of Veterinary Medicine, Zagazig University, Egypt. Animals were housed under standard conditions (12 h dark/light cycles, temperature 22–26 °C, air humidity 40–60%) in a group of five rats with free access to food and water. The number of animals used and their suffering were minimised as possible as we could. The experimenters and data-processing persons were blind to the treatment of rats. All animal experiments were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committees at Zagazig University (ZU-IACUS), Egypt.

Ex-vivo AChE inhibition assay

The rats were randomly divided into three groups of five animals each: normal control group, 3f (10 μg/kg) treated group and tacrine (10 μg/kg) treated groups. The rats in the normal control group received an equivalent volume of 5% DMSO in saline (vehicle). All the rats were treated with a single intraperitoneal injection of the respective drugs and sacrificed 30 min later. The brains were quickly removed on an ice-cold plate. These tissues were homogenised in a 10-fold volume of cold 10 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 3500 rpm for 10 min, and the supernatants were employed as sources of enzyme in AChE assay. All the above steps were carried out at 4 °C. AChE activity was determined according to a modified Ellman assay method.40–42

Computational methodology

Molecular binding mechanism of novel Sunifiram-Carbamate hybrid towards AChE

Acetylcholinesterase (AChE) complexed with Ganstigmine was retrieved from the protein data bank (PDB) with ID: 2BAG.51 In preparation for molecular docking using AutoDock VINA implicated in the PyRx 0.8 tool,55,56 all non-standard residues including water were removed, and hydrogen ions were subsequently added. The dimensional structures of the synthesised analogues including donepezil and rivastigmine were drawn using Marvin Sketch software. Universal Force Field incorporated into Avogadro 1.2.0 software.57 was then employed to optimise the energy on the 2D structures and to build their 3D structures. The molecular geometries of the compounds were optimised using the steepest descent algorithm and saved for molecular docking. Having established the inhibitory potency of compound 3f via experimental methods, it was then directed into the Ganstigmine binding pocket of AChE. The grid box coordinates for molecular docking included: centre: X = −3.90, Y = 65.61 and Z = 63.99 and dimensions: X = 22.33, Y = 23.89 and Z = 22.28. To view the molecular interactions of the docked complex, we employed Discovery Studio Visualiser.58

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