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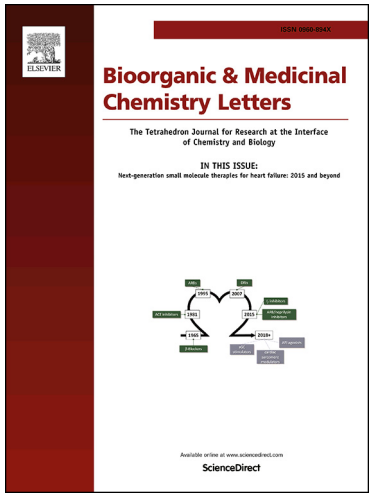

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Synthesis, biological evaluation and molecular dynamic simulations of novelBenzofuran-tetrazole derivatives as potential agents against Alzheimer's disease

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Abstract

A series of novel Benzofuran-tetrazole derivatives were successfully synthesised by integrating multicomponent Ugi-azide reaction with the molecular hybridization approach. Interestingly, a number of synthesized derivatives (**5c**, **5d**, **5i**, **5l**, **5q** and **5s**) exhibited significant reduction of aggregation of "human" amyloid beta peptide, expressing on transgenic *Caenorhabditis elegans* (*C. elegans*) strain CL4176. Further, *in silico* docking results have evidenced the exquisite interaction of active compounds with the help of TcAChE–E2020 complex. These findings underscore the potential of these hybrids as lead molecules against Alzheimers's disease.

Keywords: Benzofuran-tetrazole derivatives, molecular hybridization,Amyloid-beta aggregation, TcAChE–E2020 complex.

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According to data provided by the World health organization, an estimated 524 million people were aged 65 or older i.e. 8% of the world's total population and these data are expected to be nearly triple by the year 2050 due to a combination of declining fertility and increasing life expectancy.[1] The hapless result of this trend is the concomitant rise in the number of people agonized by age-related neurodegenerative disorders. Today, neurodegenerative diseases are the most frequent cause of death and disability worldwide.[2] Alzheimer's disease (AD), is a chronic, fatal, and most common neurodegenerative disease, which is clinically characterized by progressive memory loss, a decline in language skills, and progressive deficits in different cognitive domains. As per a report of the Alzheimer's Association (U.S.), morbidity and mortality rates of patients suffering with AD are high, especially for elderly population over the age of 60.[3] The consistent neuropathologic indicator of the disorder is deposition of aggregated protein breakdown products, amyloid-β(Aβ) plaques and neurofibrillary tangles, generally noted on post-mortem brain examination. Although the primary cause of AD is still speculative due to complex etiology, Aβ plaques are thought to be predominantly responsible for the devastating clinical effects of the disorder.[4] Some other hypotheses also been proposed to understand the pathophysiology of AD and facilitate drug discovery programs, including low levels of acetylcholine (ACh), aggregation of Tau protein, oxidative stress, and the accumulation of biometals.[5]Acetylcholinesterase also known as AChE or acetylhydrolase, is the primary cholinesterase in the body which catalyzes the hydrolysis of acetylcholine to acetate ion and choline.Thus, the principal approach to cure AD has focused on acetylcholinesterase inhibitors (AChEI) that increase the brain ACh levels. However, to date, the therapeutic options for AD treatment are limited to four approved acetylcholinesterase (AChE) inhibitors, tacrine (TC), donepezil, rivastigmine, and galantamine.[6] However, these commercial drugs for AD treatment are unable to meet the market demand. They ameliorate only cognition and the degree of dementia in AD patients and do not reverse the succession of AD.[7] Moreover, high incidence of side effects including hepatotoxicity has been reported after the administration of these drugs.[8] Thus, it is necessary to develop novel anti-AD drugs that are able to act as far upstream as possible, in the neurodegenerative cascade.

As the part of our continuous efforts in drug discovery and development, we describe herein the synthesis and anti-Alzheimer activity of the pyrazole containing benzofuran-tetrazole hybrids (Fig. 1). Benzofurans represent an important class of compounds found in versatile building blocks of biologically important compounds.[9-12] Further, a literature survey based on the framework reveals that the benzofuran bearing moieties display potential anti-Alzheimer

activity by inhibiting AChE.[13-15] On the other hand, tetrazole pharmacophore has also been identified as a novel structural motif that inhibited cholinesterases.[16-17] In addition, pyrazole derivatives have the unique ability to reduce the tau and amyloid β dual aggregation.[18-19] Hybrid molecules which contain multiple structural units of different nature are usually endowed with enhanced biological activities.[20-23] Inspired by the concept, we have designed and synthesized a novel series of compounds that have benzofuran, tetrazole and pyrazole entities in one frame and evaluated them for their anti-Alzheimer activity. This report constitute the first combination of these three pharmacophores into a novel scaffold and their potential anti-Alzheimer's activity.

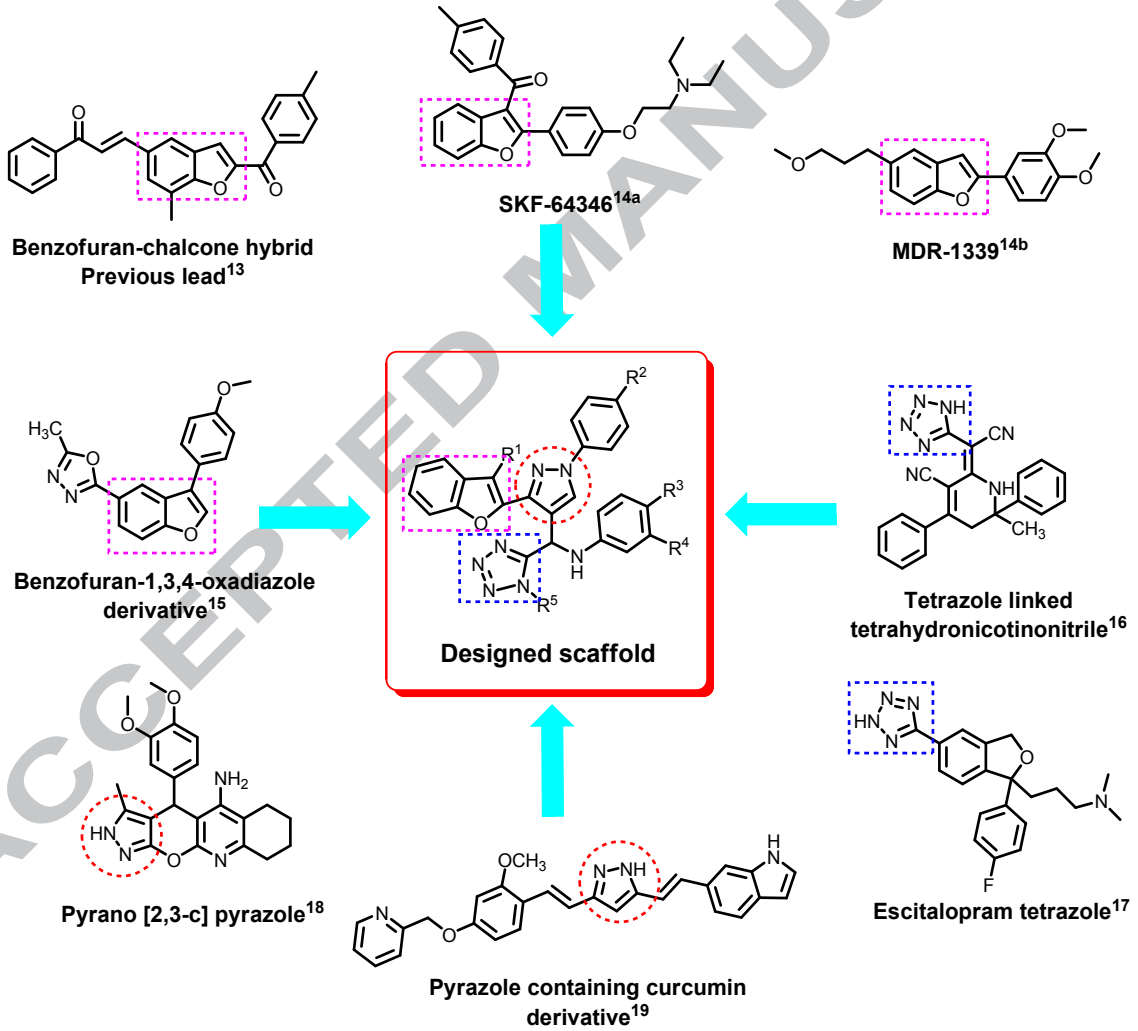
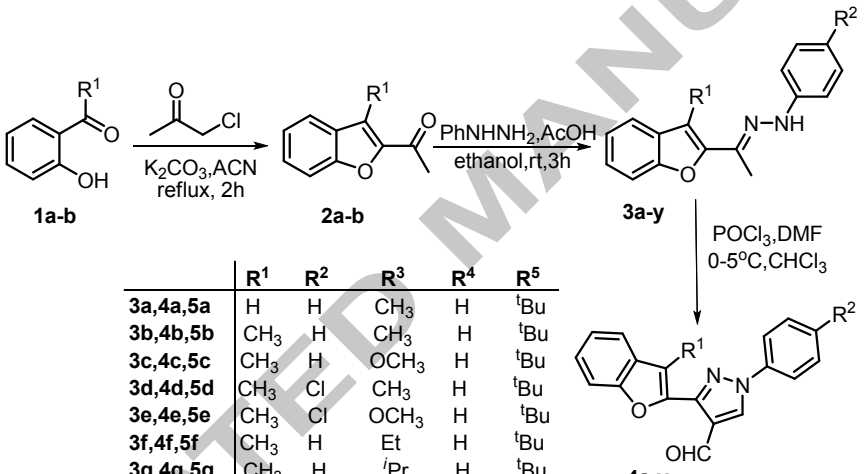


Figure 1. Designing of Benzofuran-tetrazole hybrids.

The detailed synthetic route for the preparation of target and intermediate compounds is outlined in Scheme 1. Initially, *o*-alkynation of salicyladehyde and 2-hydroxy acetophenone

with chloroacetone in the presence of K_2CO_3 furnished 2-acetyl benzofuran **(2a-b)** *via* intramolecular cyclocondensation reaction. The compounds **3a-y** were synthesized by the reaction of acetyl benzofuran with different phenyl hydrazines in ethanol and catalytic amount of acetic acid.[24] These hydrazone intermediates **(3a-y)** were then engaged in Vilsmeier-Haack reaction resulting in the formation of the intermediate aldehydic compounds **4a-y**. Finally, these benzofuran-pyrazole aldehydes **(4a-y)** were subjected to Ugi azide reaction to give the desired hybrids **5a-y**.[25] The atom economical synthesis of target compounds was achieved by operationally simple, metal free, multicomponent reaction. All the synthesized compounds were characterized using ^{1}H NMR, ^{13}C NMR and ESI-MS (Supporting Information).



	R1	R2	R3	R4	R5
3a,4a,5a	H	H	CH_3	H	^{t}Bu
3b,4b,5b	CH_3	H	CH_3	H	^{t}Bu
3c,4c,5c	CH_3	H	OCH_3	H	^{t}Bu
3d,4d,5d	CH_3	Cl	CH_3	H	^{t}Bu
3e,4e,5e	CH_3	Cl	OCH_3	H	^{t}Bu
3f,4f,5f	CH_3	H	Et	H	^{t}Bu
3g,4g,5g	CH_3	H	^{i}Pr	H	^{t}Bu
3h,4h,5h	H	H	OCH_3	H	^{t}Bu
3i,4i,5i	H	H	F	H	^{t}Bu
3j,4j,5j	H	H	Cl	H	^{t}Bu
3k,4k,5k	H	H	Br	H	^{t}Bu
3l,4l,5l	H	F	CH_3	H	^{t}Bu
3m,4m,5m	H	CH_3	CH_3	H	^{t}Bu
3n,4n,5n	H	F	^{i}Pr	H	^{t}Bu
3o,4o,5o	H	Me	Me	H	^{t}Bu
3p,4p,5p	H	H	^{t}Bu	H	^{t}Bu
3q,4q,5q	H	H	CH_3	F	^{t}Bu
3r,4r,5r	H	CH_3	CH_3	F	^{t}Bu
3s,4s,5s	H	H	CF_3	Br	^{t}Bu
3t,4t,5t	H	CH_3	CF_3	Br	^{t}Bu
3u,4u,5u	H	H	CH_3	H	cyclohexyl
3v,4v,5v	H	H	OCH_3	H	cyclohexyl
3w,4w,5w	H	Cl	CH_3	H	^{t}Bu
3x,4x,5x	H	Cl	OCH_3	H	^{t}Bu
3y,4y,5y	CH_3	F	CH_3	H	^{t}Bu

Scheme 1. Synthesis of Benzofuran-tetrazole derivatives.

AD is characterized by the aggregation of *β*-amyloid peptide (A*β*). The transgenic *C. elegans*strain CL4176 exhibits temperature-inducible expression of human*β*-amyloid.This

strain has been developed to express *β*-amyloid specifically in the muscles, thus, elevated *β*-amyloid in the worm which results in early paralysis makes a clear end point to screen the effect of compound on *β*-amyloid aggregation.Thus, strain CL4176 has been chosen for the experiment. In our preliminary screening, we were interested to search for those compounds that were effectively delaying the paralysis rate in worms. To check the effect, these compounds were mixed in the bacterial food diet OP50 (the uracil auxotroph of *E. coli*) and were fed to worms in the working concentration of 1mM, and the percent inhibition of the worms was plotted, by taking into account 50 worms of each group , in duplicate. Among the screened compounds, compound **5c**,**5d**, **5i**, **5l**, **5q** and **5s** were effectively regulating the paralysis rate of worms, with figures of 67.1%, 46.7%, 56.5%, 42.1%, 45.39% and 63.81% respectively, suggesting effective inhibition of *β*-amyloid toxicity (Fig. 2).Therefore we went further with these six compounds and tested their efficacy.

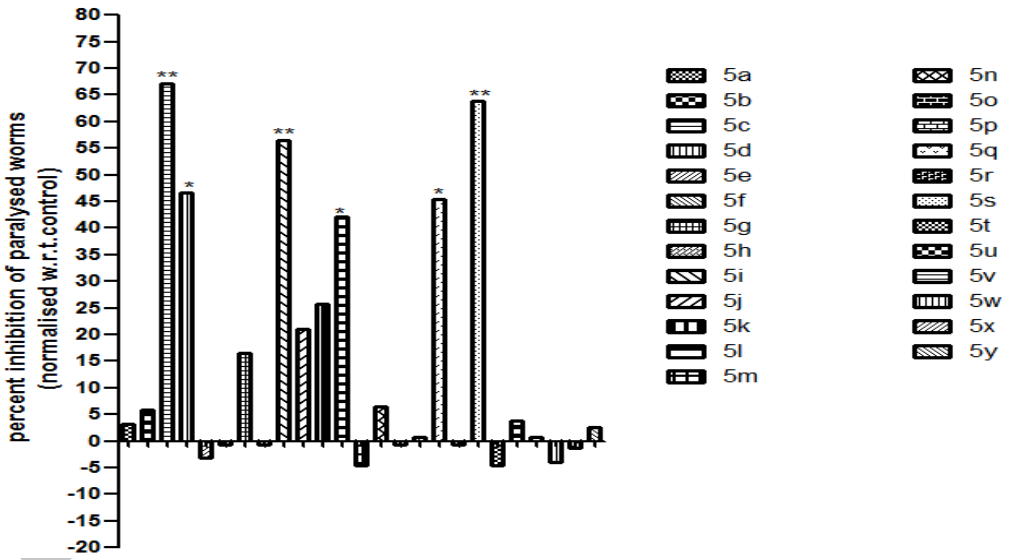


Figure 2. Amyloid-beta induced paralysis assay in the CL4176 strain of *C. elegans* treated with the indicated test compounds. Data are normalized with respect to control; *p<0.05, **p<0.01.

To further strengthen our findings for inhibitory activity of these compounds, the microscopic technique for visualizing amyloid-beta aggregation employing Thioflavin-S has been done.[26] The worms that were exposed to **5c, 5d, 5i, 5l, 5q** and **5s** showed significant decrease in the fluorescent intensity as compared to control OP50 which has been shown in Fig. 3. The

figure is representative of amyloid-beta aggregation for the worms fed with the compounds along with the control OP50 and the positive control EGCG.

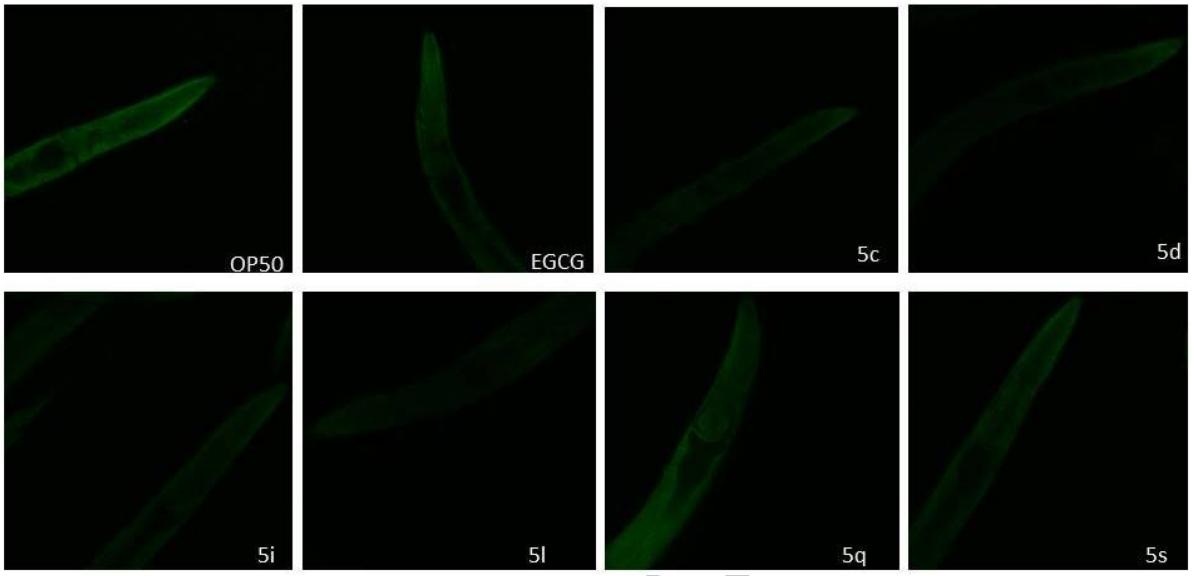


Figure 3. Thioflavin-S imaging for Amyloid-beta aggregation in transgenic strain CL4176 strain of *C. elegans*. OP50 was used as a control and EGCG ((-)-Epigallocatechin-3-gallate) was used as positive control . The fluorescent intensity is compared with respect to control. All images were obtained at 63x magnification.

As increasing cholinergic neurotransmission by inhibiting the enzyme acetylcholinesterase (AChE) still represents one of the mainstream treatment option for AD, these compounds were investigated for their probable role as acetylcholine esterase inhibitors and studied in the worms employing Amplex-Red®. The worms that were exposed to **5c**, **5d**, **5i**, **5l**, **5q** and **5s** in the concentration of 1mM were assayed for AChE activity on their worm extract. Findings exhibited significant inhibition of AChE levels with *p*-values of 0.0461, 0.0360, 0.0175 and 0.0085 particularly with compounds **5c**, **5d**, **5i** and **5q** respectively (Fig. 4). Our results suggested that **5q** has the maximum potential against acetylcholine esterase. *C.elegans* has four acetyl cholinesterase genes viz. *ace-1, ace-2, ace-3* and *ace-4*.[27]Amongst them, *ace-3* and *ace-4* have minor role and a majority of around 95% of AChE activity is encoded by *ace-1* and *ace-2*.[28] We speculate that these compounds interact with *ace-1* and *ace-2*, thereby inhibiting the AChE activity in the model organism *C.elegans*. As we know, AChEIs are currently approved treatment for Alzheimer's disease (AD) type dementia. A huge success is witnessed in this regard as these agents describes a layout for the development of other symptomatic cognitive enhancing agents.[29] In line with this finding, we regard that our compounds could play role in enhancing the memory too, beside lowering the *β*-amyloid

induced toxicity and inhibiting the AChE activity as evident in our results. The activity of these compounds to inhibit the AChE activity would result in delayed breakdown of ACh and prolong its endogenous activity as a neurotransmitter.[30]

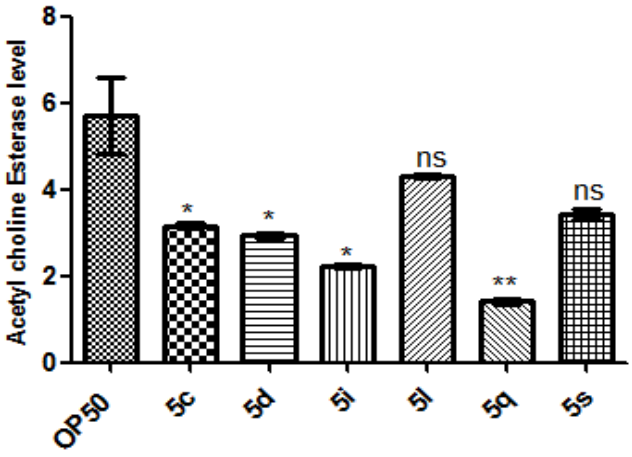


Figure 4. Effect of test compounds on acetylcholinesterase activities, as measured by the Amplex ® Red assay in transgenic strain CL2006 strain of *C. elegans*. OP50 was used as a control. Data are normalized with respect to control. All experiments were performed in triplicate; ns-non-significant, *$p<0.05$, **$p<0.01$.

To support the above experimental results, molecular docking studies were carried out. The structure of *Torpedo californica* AChE (TcAChE) with E2020 (donepezil) (PDB id- 1EVE) has been used extensively for docking studies of AChE inhibitors,[31-32] which suggests that it can be an useful model for evaluating new chemical entities in the drug discovery program against AD. Thus, in the present study, we have used the TcAChE–E2020 complex for the analysis of binding mode of compounds in the active site of TcAChE. Re-docking with E2020 resulted in a conformation close to the co-crystal structure (Fig.5), with an RMSD of 0.88 Å, thus validating our docking method adopted in this study. Residues Trp84, Trp279, Trp290, and Phe330 play main roles in the binding of E2020.[31] Trp84 and Trp279 are predominantly significant, as both can undergo a π- π stacking interaction with E2020.[13] It has been confirmed that E2020 is an active site gorge spanning AChE inhibitor, interfering with both the catalytic active site (CAS) and peripheral active site (PAS) simultaneously.[33] Interestingly, we observed strong non-covalent interactions (H-bond) between these inhibitor compounds and target site of AChE, which were not present in E2020 inhibitor. We also observed π- π stacking interactions between aromatic ring of compounds and residues of the target site. The tetrazole and pyrazole rings increase the binding strength by contributing

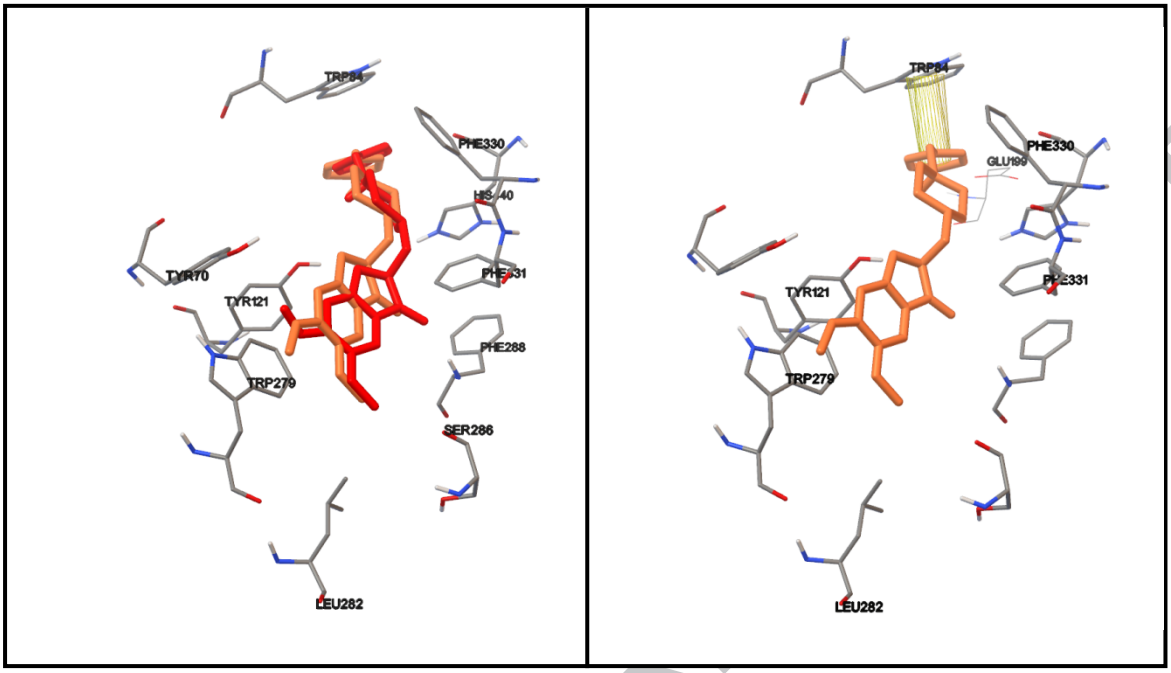


Figure 5.A) Superimpose co-crystallized (orange) and docked conformation (red) of E2020 in the active site TcAChE. B) Interactions between co-crystallized E2020 and TcAChE active site residues (yellow cylindrical pattern shows π-π stacking interactions)

through H-bonds and π- π stacking interactions to the protein-ligand complex respectively. Indole ring of Trp279 appeared to undergo π- π stacking interactions with the pyrazole rings of all the compounds at the entrance of the active site. In the compound **5c**, we observed two H-bonds, one between –OH group of Tyr121 and N-4 of tetrazole ring, another between –NH group of Arg289 and OCH_3group. We also observed π- π stacking interactions between six-membered & five-membered rings of Trp279 and pyrazole ring. Due to strong H-bonds and π- π stacking interactions, this compound shows less binding energy and high binding affinity with the target site of AChE. In all the other five compounds π- π stacking interactions and H-bond interactions stabilizes the binding poses and affinity. We have not observed any π- π stacking interactions with Trp84 at the bottom site of the binding pocket due to the structural variation from the E2020. However, we observed π-π stacking interactions and hydrogen bonding to make these inhibitor-target complexes more stable. Residues Tyr121 and Trp279 belong to the PAS, and in the docking studies, we observed that the pyrazole moieties of all these compounds are present in close proximity to these residues. The role of PAS residues in the formation of amyloid fibrils has been reported.[34] Fig. 6 shows the interactions between the compounds and TcAChE active site residues. Hydrogen bonds are highlighted in green dotted lines and π-π stacking interactions are shown as yellow lined cylindrical pattern. We

hypothesize that the presence of a tetrazole moiety increases the hydrogen bond interactions and pyrazole moiety increases the stacking interactions, allowing a better positioning into the active site of TcAChE.

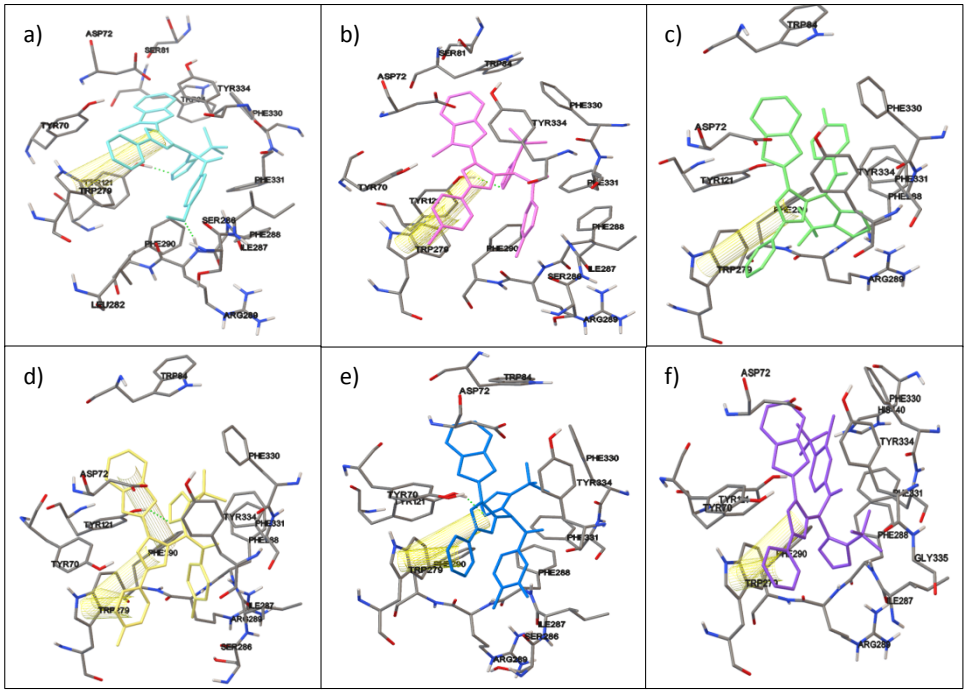


Figure 6. Interactions between compounds and TcAChE active site residues a) **5c**, b) **5d**, c) **5i**, d) **5l**, e) **5q** and f) **5s** (Green dotted lines shows H-bonding, yellow cylindrical pattern shows π-π stacking interactions)

The preceding molecular docking studies revealed that both tetrazole and pyrazole cores are required to show the bonding and nonbonding interactions to fit into the active site of TcAChE, which stabilizes E2020. Initially, all the hybrids were evaluated for Amyloid-*β* toxicityand the result confirmed that **5c**, **5d**, **5i**, **5l**, **5q** and **5s** were the most potent compounds in the series. On the basis of A*β* aggregation results and the diverse chemical structures of synthesized compounds, a preliminary structure activity relationship (SAR) was established. A summary of the SAR is depicted in a pictorial representation (Fig. 7).It was found that the compounds showing >40% inhibition of the paralysis essentially contained *tert*-butyl group (R^5) attached with tetrazole moiety. Further, substitution in the benzofuran ring (R^1) did not affect the activity. The presence of H or Halogen atom at R^2 was favoured over other substitutions. On the other hand, presence of the smaller atom or group (F/CH_3) was preferred over bulkier groups at R^3. Additionally, H-atom at R^4 was found optimal.

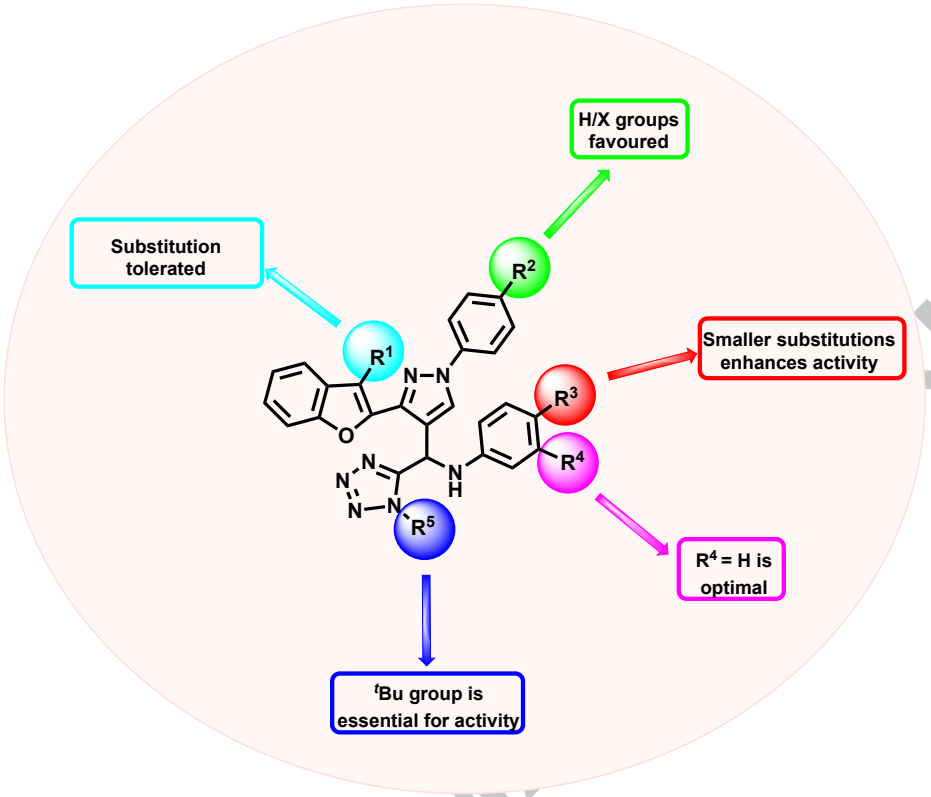


Figure 7. Pictorial representation of SAR studies.

In conclusion, we have synthesized and characterized a novel series of hybrids by using pharmacophore hybridization approach. Among the series compounds **5c**, **5d**, **5i**, **5l**, **5q** and **5s** emerged as promising agents to decrease the disease effects in transgenic *C. elegans* model of AD. Studies such as effects on reducing Aβ aggregation and AChE activity confirm and reinforce the efficacy of these compounds as a new class of anti-Alzheimer agents. Further studies to unravel the mechanism of action and structural optimization of these hybrids are currently underway in our laboratory. It is intended that this communication will help to design and develop a novel class of next generation anti-Alzheimer agents.

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Supplementary data

Details for synthesis and characterization of all compounds together with protocols for biological materials and methods can be found in the supplementary data.

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Graphical abstract

Synthesis, biological evaluation and molecular dynamic simulations of novel Benzofuran-tetrazole derivatives as potential agents against Alzheimer's disease

Pragati Kushwaha[a], Soobiya Fatima[b,#], Akanksha Upadhyay[a], Sampa Gupta[a], Sudha Bhagwati[c], Tanvi Baghel[b], M.I.Siddiqi[c], Aamir Nazir[b,#], Koneni V. Sashidhara[a, # *]

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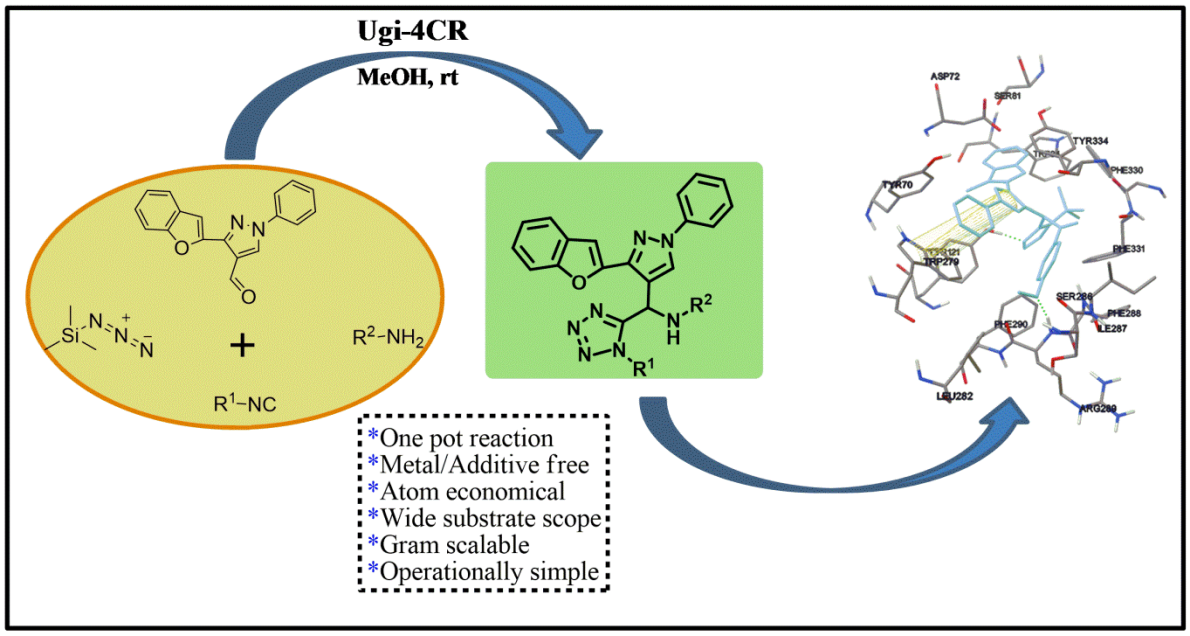

Research Highlights

Synthesis, biological evaluation and molecular dynamic simulations of novel Benzofuran-tetrazole derivatives as potential agents against Alzheimer's disease

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- A series of benzofuran-tetrazole derivatives were synthesized as potent AChE inhibitors.
- Several compounds exhibited significant neuroprotective and Aβ aggregation inhibitory activities.
- Docking studies corroborated well with the anticholinesterase assay results.