# A novel synthetic derivative of biaryl guanidine as a potential BACE1 inhibitor, to treat Alzheimer's disease: *In-silico*, *in-vitro* and *in-vivo* evaluation

Sayyad Ali<sup>1,7</sup>, Muhammad Hassham Hassan Bin Asad<sup>1</sup>\*, Muhammad Arslan Javed<sup>2</sup>, Tariq Javed<sup>3</sup>, Yasser MSA Al-Kharaman<sup>1</sup>, Muhammad Latif<sup>4\*</sup>, Sabeeh Mohsin<sup>1</sup>, Taufiq Nawaz<sup>5</sup>, Syed Muhammad Farid Hasan<sup>6</sup>, Jamshed Iqbal<sup>1</sup>, Borhan Babak<sup>7</sup> and Izhar Hussain<sup>1</sup>

<sup>1</sup>Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Pakistan

<sup>2</sup>Department of Medicine, Services Institute of Medical Sciences, Services Hospital Lahore, Pakistan

<sup>6</sup>Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Karachi, Pakistan

<sup>7</sup>Department of Chemistry, Michigan State University, East Lansing, Michigan, U.S.A

**Abstract**: BACE1 enzyme has been known a potential target involved in Alzheimer's disease (AD). Present research was focused on the principles of virtually screening, chemical synthesis and protease inhibitory effect of BACE1 enzyme via biaryl guanidine derivatives. *In-silico* based paradigm (ligand binding interaction within active domain of BACE 1 enzyme i.e., aspartate Asp32 and Asp228) a novel compound was synthesized and subsequently subjected to *in-vitro* and *in-vivo* evaluation. 1,3-di(isoquinolin-6-yl) guanidine was synthesized and found potent (IC<sub>50</sub> 6±0.56 µM) and active to arrest (99 %) β-secretase enzyme (FRET assay). Furthermore, it was found to improve novel object recognition test (RTI =56.55%) and Morris water maze test (32.26±3.45s) significantly (p<0.05). Enhanced pharmacokinetics and related properties (high iLOGP and Log *S* =-3.98) along with improved permeation to the blood brain barrier (BBB) (zero Lipinski violation) made it feasible to inhibit BACE1 as a novel therapeutic source to treat AD in future.

Keywords: Biaryl guanidine, Alzheimer's disease, BACE1, inhibition.

#### **INTRODUCTION**

One of the known causative pathogenesis of Alzheimer's disease (AD) is amyloidogenesis (Castro and Martinez, 2006). The occurrence of extra cellular "amyloid plaques" in AD have been of great research interest (Tariot and Federoff, 2003; Gilbert, 2013). The amyloid- $\beta$  (A $\beta$ ) peptides are the chief constituent of these amyloid plaques and have a very great tendency self-agglutination which ultimately forms the fibril (Conway et al., 2003). These plaques are formed from a portion of a prominent type-I transmembrane proteins known as amyloid precursor protein (APP) (Bignante et al., 2013). During AD, the APP is cut down consecutively by enzymes called  $\beta$ -site amyloid precursor protein cleaving enzyme 1 (BACE1) which are also known as  $\beta$ -secretase.  $\gamma$ -secretase is responsible for A $\beta$  from 39 to 43 amino acids. A $\beta$  of 42 residues in length (A $\beta_{1-42}$ ) represents the most toxic species, have the capability of the formation of fibrils (LeVine, 2004). A $\beta$  oligomers generates peroxides directly from molecular oxygen in AD patient brain (Tougu et al., 2011). These peroxides then producing the hydroxyl radical and creating a lasting condition of aerophilic stress contributing to cytotoxicity and ultimately cell death (Zhu et al., 2007). It is believed that

the AD has a complex pathogenesis, with various features like deficit cholinergic activity,  $A\beta$  aggregation, and multifactorial oxidative stress (Selkoe and Podlisny, 2002).

To the best of our knowledge guanidine derivatives have been documented previously in literature to antagonize various nervous disorders (Selkoe and Podlisny, 2002). Eli Lilly discovered LY2811376 a biaryl amino thiazine having IC50 =0.27µM (represented as structure 1), a small molecule to inhibit BACE1 enzyme in 2009. It showed significant decrease in  $A\beta$  in healthy volunteer's brain post peroral administration. Later on, clinical utilization of (LY2811376) was stopped owing to the targeted toxicity for the retina (Portelius et al., 2014; Wu et al., 2016). On these basis, it was suggested to enhance inhibitory potential of such molecules via different approaches including incorporation of fluorines or -CF3 moieties along with heterocyclic rings to the parent nucleus (Ali et al., 2019). Therefore, present study was focused to synthesize biaryl guanidine derivative after virtually screening to inhibit effectively BACE1 protein (PDB ID=1FKN) in ameliorate of AD (structure 2 represented general scaffold for biaryl guanidine derivative).

<sup>&</sup>lt;sup>3</sup>Department of Pharmacy, LMDC, University of Health Sciences, Lahore, Pakistan

<sup>&</sup>lt;sup>4</sup>Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan

<sup>&</sup>lt;sup>5</sup>Department of Food Sciences and Technology, The University of Agriculture, Peshawar, Pakistan

<sup>\*</sup>Corresponding authors: e-mail:hasshamasad@yahoo.com; muhammad.lateef@ue.edu.pk



### MATERIALS AND METHODS

#### Virtual screening with molecular docking

The derivative of the biaryl guanidine fragment has been synthesized after a thoroughly in silico authentication. The chemical synthetic compound has been evaluated through molecular docking by means of MGL tools v1.5.6 and Auto Dock v4.2. Chem Draw ultra 16.0 was used to draw the structures, subsequently converted to the 3D via chem-3D pro 16.0. Furthermore, the binding energies of the ligand were minimized with MM2. The  $\beta$ -secretase (PDB ID: 1FKN) has been retrieved from RCSB protein data bank in less than 2.0 Å resolution. Addition of water, gastigeir's charges, hydrogen atom along with the removal of co-crystallized ligand, protein structures were prepared for molecular docking. All the active sites were specified via grids box around the co-crystallized ligands before the removal of ligands. For the sake of docking, nine poses of the compound via Lamarkian Genetic Algorithm were generated. The possible binding sites and docked poses were selected visualizing and binding free energies. PyMol tool v2.1 and similarly visualizer discovery studio v17.2 was employed to obtain the potential binding pose (Santos-Martins et al., 2014). The ligand-enzyme interactions were rerun in new docking procedure and validate via Molecular Operating Environment (MOE) drug discovery software by Chemical Computing group and similarly the pharmacokinetic properties of the ligand i.e. ClogP, (Lipinski), BBB permeation were calculated through MOE while online database (ww.swissadme.ch) was used to calculate pharmacokinetics and drug likeness properties (Vilar et al., 2008).

#### Synthesis of biaryl guanidine derivatives

The general procedure for the chemical synthesis of N, N'- Biaryl guanidines; Compound 4a was CuI catalyzed arylation of guanidine moiety. A Schlenk-line flask set up been filled with guanidine nitrate (1.0 mmol), aryl halides (1.0 mmol), a ligand 'N-methyl glycine' 17.80 mg, 0.20 mmol (aryl bromides), recrystallized CuI 19.00 mg, 0.10 mmol and  $K_3PO_4$  (1.270g, 6.00 mmol). The Schlenk flask was completely evacuated and backfilled with nitrogen before acetonitrile (5 ml) was added. The Stirring of the

reaction mixture was kept continued at a temperature of  $115^{\circ}$ C till the complete consumption of aryl halide which was 20 h as checked by the periodic TLCs. The final reaction mixture was removed through ethyl acetate. The upper organic layer was collected and washed with distilled water, brine solution was added and dried using Na<sub>2</sub>SO<sub>4</sub>. The solvent has been removed in vacuum accompanied by purification on column chromatography upon silica gel or separating TLCS (25:1-40:1 methanol as eluent /methylene chloride) gave us the anticipated final product (Xing *et al.*, 2012).

#### Spectral analysis

The <sup>1</sup>H-NMR and <sup>13</sup>CNMR were recorded by Agilent (DDR2 500 MHz NMR spectrometers) equipped with 7600AS 96 sample auto samplers running Vnmr *J* 3.2A. The instruments have TMS as internal standards. The values of the chemical shift were described in ppm ( $\delta$ ) units and the coupling constants (*J*) were recorded in MHz.

# In-vitro studies (Fluorescence resonance energy transfer (FRET) assay

BACE1 inhibiting activity for the newly synthesized guanidine derivatives was performed via Sigma –Aldrich FRET-based screening assay activity kit (product # CS0010).

Briefly, this assay was performed in a total 100µl volume composed of BACE1 substrate (20µl, 50µM, Catalog # A1472), fluorescent assay buffer (78µl /78-Xµl, pH 4.5, catalog # F8303), test samples (Xµl, 100µM) and BACE1 enzyme (2µl,~0.3 unit/µl, Catalog # B9059) in 96 well plate reaction mixture incubated at 37°C for 1:15 min at optimized assay condition (usually 5%-20% fluorescent product produced from substrate within 1-2h). The baseline fluorescence interpretation (time zero reading) was noticed instantly following the adding of BACE1 enzyme (fluorometer sets at excitation of 320 nm) while the emission at 405nm at room temperature. Both enzyme and BACE1 substrate were formulated in the buffer, whereas the verified samples were dissolved in DMSO (5%) ab initio, this mixture was then diluted sequentially with the provided buffer in the desired volume (2µl, 3µl

& 5µl) and strength (200 pmol, 300 pmol & 500 pmol) respectively. A standard curve was produced among the fluorescent unit (FU) against the standard (100µM, 1-µl) solution concentration (100-500 pmol) to find out 50 % BACE1 cleavage. FU of the blank was subtracted from all signal readings of the reaction mixtures. Sample blank (buffer & substrate) was treated as a negative control, however, positive control was a sample (buffer & substrate) with enzyme mixture (www.sigmaaldrich.com). IC<sub>50</sub> of the tested compound was compared and calculated using GraphPad Prizm v5 software.



**Fig. 1**: Shows the ligand (green) within the active pocket of BACE1 (blue), within the active domain (red).



**Fig. 2**: Represents the <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra of the compound 1,3-di(isoquinolin-6-yl) guanidine.

#### In vivo study: Experimental animals

Male BALB/c mice were utilized for this experiment arranged from (National Health Institute Islamabad, Pakistan). The experimental procedures were worked in exact accordance with Helsinki declaration and Animal Scientific procedure act 1986 (UK) as guided by the research ethics committee (REC) on campus. The experiments employed in this research work were accepted by REC for the appropriate methodological evaluation (Experiments No. PHM. Eth/CS-M01/18-001 dated 05/2018 for a one-year duration). The mice housing was in standard Type III cages of Makrolon TM in a group of 4 or 5 mice with sawdust bedding. Food and water were supplied spontaneously, the water for drinking was supplied on everyday base to avoid any the infectious agent', similarly the sawdust bed was periodically examined and substituted to keep away all the probable infectious organisms happens from mice' feces. All the rodents were separated into nine groups, each group (n = 10) were between 3.5 to 5.5 months of age. Aluminum chloride induced neurotoxicity and used as control for Morris water maze test (Bromley-Brits *et al.*, 2011; Mahboob *et al.*, 2016).



**Fig. 3**: Morris water maze test: Escape latency time period of the mice to arrive at the platform on various trial days of the AlCl<sub>3</sub> triggered neurotoxicity, control and the compound treated group (a); Signify the 5<sup>th</sup>-day trials evaluation studies among the three groups (b). Per hundred total times of the mice spent in the investigation of the target quadrants in days (c); Manner in which time each mouse crosses the targeted quadrant (d).



**Fig. 4**: Improved three-dimensional spatial learning/ memory of  $AlCl_3$  induced model in mice via newly synthesized compound (a & b).

#### Morris water maze test

The test is used know the remembrance of the animal about the spatial remembrance and surrounding. The device utilized for this method has a round pool with 120-centimeter diameter and a depth of 60 cm. This pond is separated into 4 imaginary quarter-circles South, West, East, and North. The first trial commenced on the 27<sup>th</sup> day of dosing and 5 tests were performed for 5 days. Each mouse is allowed for only 60 s to discover the concealed stand in each test, a pause of ten min was given within the 2 consecutive trials. The typical period necessary for a

mouse to reach at the stand was videotaped and an average of 5 trials was supposed as the escape-latency of the mice for that day. The probe test was carried out on the  $32^{nd}$  day of intervention with no hidden stand in water although the release location has not been changed as North-East (NE). The spatial memory was checked from the time spent in the quadrant with previously located stand in it, moreover the number of crossing in the said quadrant were also noted (Bromley-Brits *et al.*, 2011; Mahboob *et al.*, 2016)

#### Novel object recognition test

This experiment is carried out a box of  $(25 \text{cm} \times 25 \text{cm} \times$ 25cm) at day time with maximum light. The methodology is comprised by the (i) Pre-habituation (ii) habituation (iii) Training and (iv) Testing. The mice were adopted with the testing room a day before the initiation of the experiment to get familiarized with the surrounding. On the first day they were allowed to freely explore the empty box for 5 min followed by 20 min for the 2<sup>nd</sup> and 3<sup>rd</sup> days (Akkerman *et al.*, 2012). On the fourth day, every mouse was exposed to a trial of training followed by a testing trial. In the training trial, 2 objects were located oppositely to each other inside the box at a similar space from the adjacent corner (Hughes, 2007). The mice were allowed to explore the objects for ten min and after that were relocated to the home cage. After an hour the mice were put back in the experimental box for ten min, but this time one of the familiarized objects were substituted by a novel one. The behaviors of the mouse were recorded via video-came. The recognition index can be calculated by the following equation

Recognition Test = [Time spent with novel object/Time spent with novel object + familiar object]  $\times 100$ 

# STATISTICS ANALYSIS

All numerical values were given as mean  $\pm$  SEM via using Microsoft Office version 2010. Column statistic (students *t* test) was used to compare the results and level of significance was set at p<0.05.

# RESULTS

Docking study manifested quite impressive binding poses with lower affinities and rankings (rank 1 to upper level) for a proposed newly synthesized compound (table 1). Fig. 1 represents the proposed ligand within the active pocket of the BACE1 enzyme. On the account of spectral and virtually obtained parameters compound was concluded 1,3-di(isoquinolin-6-yl) guanidine having IC<sub>50</sub> 6±0.56µM as summarized in table 2 and fig. 2. Morris water maze test showed elevated time of escape-latency for aluminum chloride induced neurotoxicity (45.10±2.40s) in comparison with control group of mice (26.60±2.12s). 1,3-di(isoquinolin-6-yl) guanidine advocated quite better graphical record with better

memory recalling behaviors (32.26±3.45 s; p<0.05) when compared with AlCl<sub>3</sub>-induced affected mice (fig. 3). Treated group of mice experienced more time in exploration/examination in quadrant with previously lying stand in it (31.13±1.50 s) and AlCl<sub>3</sub>-treated mice (25.12±1.10 s). Alzheimer's disease (AD) models crossed the respective quadrant  $(5.88 \pm 1.01 \text{ s})$  along with control group (12.44±1.01 s), however, treated group value was found in between  $(9.33\pm1.07 \text{ s})$  the two extremities (fig. 4). The average exploration time and the recognition index was significant (p<0.0002) in one-way ANOVA while f-ratio was found 12.94. The results were found significant at p<0.05. In novel object recognition test normal mice explored the new object for an extended time period with a recognition test index (RTI) (67.8%), however, AD model mice displayed (44.55%) and correspondingly the AD-mice treated with developed compound revealed a substantial smaller RTI value (56.55%) as depicted in fig. 4. The normal explore time and the RTI was showed significant at p<0.0002 and the synthesized compound was noticed the most effective to improve the 3-D spatial learning/memory of AlCl<sub>3</sub> inducted model at p<0.05.

# DISCUSSION

One of the identified causative pathogenesis of Alzheimer's disease (AD) is amyloidogenesis (Castro and Martinez, 2006) owing to the  $\beta$ -secretase led to amyloid- $\beta$ (Aβ) peptide (amyloid plaques) self-agglutination resulted in fibril formation (Conway et al., 2003). To the best of our knowledge guanidine derivatives have been recognized earlier to mask barin disorders as proved by Gerritz et al. for acyl-guanidine derivatives (Conway et al., 2003). On these grounds present study was designed to synthesize a bi-aryl guanidine derivative (1,3di(isoquinolin-6-yl) guanidine) with particular interest to cure AD. Interestingly, molecular docking supported derivatization of synthesized compound with BACE1 protein (PDB ID= 1FKN) molecule. In silico study pointed out non-covalent bonding interaction of amide (-NH) group with active site of  $\beta$ -secretase (Asp228 and Asp32) enzyme, could be approachable for Tyr71, Tyr 198 and Thr231 active sites. All these results triggered to synthesize and evaluate newly proposed compound to hit BACE1 activity. Newly synthesized compound 1,3di(isoquinolin-6-yl) guanidine was found potent (IC<sub>50</sub> 6  $\pm$ 0.56µM) and effective to mask (99%) BACE1 activity. Moreover, it was found to increase novel object recognition test inde and Morris water maze test significantly (p<0.05) as compared to the control AlCl<sub>3</sub> induced toxicity in mice. Improved pharmacokinetics and associated parameters (Log S = -3.98; high iLOGP values; zero Lipinski violation resulted in BACE1 inhibition drastically and to declare this compound as a novel source to treat AD in future. Newly synthesized compound 1,3di(isoquinolin-6-yl) guanidine was found the best in trials.

S. No.	Ligand (active site)	3D structure of ligand and receptor complex with the surrounding charges	The 2D structure of complex of ligand with the active domain
1		tomplex with the same same set of the set of	
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**Table 1**: Represents initial four ranks of a proposed newly synthesized compound with different poses having dual active aspartate Asp32 and 228 pockets as per *in-silico* evaluation.

**Table 2**: Describes different spectral detail (<sup>1</sup>H-NMR, <sup>13</sup>C-NMR and HREI MS) of a newly synthesized compound along with virtually obtained parameters.

S. No.	Various parameters	Description
1		(500 MHz, CD3OD): δ 9.2 (2H, s), 8.6 (1NH, s), 8.1 (2H, d, <i>J</i> = 8Hz),
1	II-INIVIK	8.0 (4H, d, J=8.5Hz), 7.9 (4H, t, J=7.5Hz), 7.7 (2H, d, J= 8.5Hz)
2	13C ND 40	δ (175 MHz, CD3OD) 161.9, 152.1(2C), 148.2 (2C), 143.9 (2C),
2	<sup>13</sup> C-NMR	137.6 (2C), 128.3 (2C), 124.6 (2C), 118.0 (2C), 117.9 (2C), 104.1 (2C)
3	HREI MS	m/z 313.13265; [(M+1) <sup>+</sup> Calculated for C <sub>19</sub> H <sub>15</sub> N <sub>5</sub> 313.13275].
4	% yield	93%
5	Color/state	White solid
6	Melting point	136-138 °C
	Molecular weight	313.13275 g/mol
7	Hydrogen Donor	4
8	Hydrogen Acceptor	5
9	(iLOGP) (Platts et al., 2006)	High
10	Log S (Ali <i>et al.</i> , 2012)	-3.98
11	$IC_{50} \pm SD$	$6\pm0.56~\mu M$
12	Lipinski (Lipinski et al., 2001)	Yes; 0; violation.
13	Ghose (Ghose, 1987)	Yes
14	Veber (Veber et al., 2002)	Yes
15	Egan (Kahneman, 2013)	Yes
16	Muegge (Bromley-Brits et al., 2011)	0.55
17	GI-absorption	High
18	GP-Substrate	Yes

**Note**: BA is the Bioavailability, GI signifies Gastrointestinal, GP represents glycoprotein, HB denote the hydrogen bonding, iLOGP is the *n*-octanol/water partition coefficient, the Log *S* represents the experimental aqueous solubility and  $\delta$  represents the delta scale.

# CONCLUSIONS

1,3-di(isoquinolin-6-yl) guanidine was synthesized for the first time and found potent (IC<sub>50</sub> 6±0.56 µM) and active to arrest (99 %)  $\beta$ -secretase enzymatic activity which might have pivotal role in amelioration of Alzheimer's disease in future.

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