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RESEARCH ARTICLE

In silico Identification of Novel Indole Analogues as β-Secretase Inhibitor in the Treatment of Alzheimer's Disease

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ABSTRACT

Alzheimer's disease is a serious health issue with serious social and economic consequences. The availability of drugs based on symptoms is not adequate, and treatment of AD remains difficult. The condition is brought on by the body's multidirectional pathology, which necessitates the employment of the multi-target-directed ligand (MTDL) strategy. Based on this, the current work with the indole fused pyrimidine inspired molecule as a lead provides hope for a novel lead for AD. To evaluate the β-Secretase inhibitory activity of designed indole fused pyrimidine analogues using insilico docking studies. Insilico docking studies were carried out using Auto Dock Vina. To synthesis substituted 4-(1H-indol-3-yl)-1,6-diphenyl-5,6- dihydropyrimidin-2(1H)-one and the synthesized lead molecules were characterized by means of FTIR, ESI-Mass spectral analysis. The newly synthesized lead molecule was assessed for anti-oxidant activity by DPPH assay and their neuroprotective activity by using SH-SY5Y cell lines by MTT assay method. The binding energy of indole fused pyrimidine analogues were varied between -7.0 to -7.8kcal/mol β-Secretase. When compared to the standard binding energy, test compound IP-2 was shown to be more potent. Five analogues were selected for wet lab synthesis based on docking score and availability of reagents. Conventional technique was used for the synthesis of selected compounds with satisfactory yield. Purity of the synthesized compounds was ensured by repeated recrystallization. Further the compounds were identified by TLC and Melting point determination. The synthesized lead compounds were confirmed by means of their IR, 1H NMR and ESI MS. The synthesized compound IP2 and IP-3 were found to be having significant hydrogen peroxide scavenging activity and reduces the oxidative stress. The invitro studies showed compound IP-2 possessing good neuroprotection. SH-SY5Y neuroblastoma cell line which was induced with beta amyloid, on treatment with the test

compounds shows increased percentage inhibition 89.33% which is necessary for a neuroprotective effect. The present work conclude that identified a finest molecule of indole-fused-pyrimidine analogue IP-2 as an innovative drug against Alzheimer's, oxidative stress can be considered as a promising lead molecule for further research.

Keywords: Insilico drug design, Auto Dock, Lipinski rule, β-Secretase, Alzheimers disease, Indole

INTRODUCTION

Alzheimer's disease is a brain disorder that gradually impairs memory and ability to think as well as the capacity to do even the most basic duties. 2 According to Alzheimer's disease was officially classified as the sixth-leading cause of death in the United States in 2019, with 121,499 fatalities from the condition documented in official death certificates. Alzheimer's was the seventh most common cause of death in 2020 and 2021 when COVID-19 broke into the top ten causes of mortality. For Americans 65 and older, Alzheimer's disease continues to rank fifth in terms of causes of death. Deaths from heart disease, stroke, and HIV declined between 2000 and 2019, while recorded deaths from AD rose by more than 145%. The COVID-19 pandemic in 2020 most likely made this trajectory of AD mortality worse. An estimated 18 billion hours of care were given to persons with Alzheimer's or other dementias in 2022 by more than 11 million family members and other unpaid caregivers [3]. More than 25% of COVID-19 patients suffer neurological symptoms such headaches, confusion, anosmia, hyposmia, and memory loss. The neurological effects could be due to viral entrance into the central nervous system (CNS) and/or subsequent neuroinflammation, both of which are associated with an increased risk of Alzheimer's disease (AD).

Interrelationship between Antioxidant and Alzheimers [4-5]

Analyzing the etiology and pathogenesis of AD involves comprehending a number of factors, but oxidative stress is one of the main causes. The development of neurological disorders is caused by oxidative stress, which is the impairment of the antioxidant response and the generation of both reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress is primarily caused by the inactivation and shortage of antioxidant enzyme machinery, such as glutathione peroxidase (GSH-Px), lipid peroxidase (LPO), superoxide dismutase (SOD), and catalase (Cat) [4]. The primary indicators of oxidative stress induction during Alzheimer's disease (AD) progression include inflammation, elevated metal accumulation, mitochondrial dysfunction, hyperphosphorylation of tau protein (microtubule-associated protein), and amyloid beta peptide (ABP) aggregation. The two most uncommon histopathological features of AD are neurofibrillary tangents (NFTs), which are made of the filament protein tau.[5] Figure 2

INDOLE[6-7]

The gut microbiota produces indole compounds, which have important biological functions such as antioxidant and neuroprotective qualities. Subsequent investigations have confirmed these compounds' anti-inflammatory, immunoregulatory, and amyloid anti-aggregation properties. Some indoles may be great candidates to reduce neurodegeneration because their actions simultaneously target multiple pathogenic pathways. Our hypothesis is that controlling the microbiota to increase the generation of neuroprotective indoles (such indole propionic acid) will improve brain function as we age. This field of study offers a novel therapeutic approach that may prolong the functional years of life for those who would otherwise experience dementia. The significance of the pyrimidine nucleus in the management of AD has been proven and confirmed by multiple research.In an attempt to identify lead ,multifunctional candidates as part of our multitargeted approach to treat AD, thesubstituted pyrimidine derivatives were generally found with the multi-target approaches like anticholinesterase (AChE and BuChE), anti-Aβ aggregation (AChE- and self-induced), and anti-β-secretase (BACE-

inhibitory activity. [8]

Based on the above facts, the present work focused on designing two different heterocyclic nucleuses Indole fused pyrimidone to inhibit β-Secretase and to assess its activity through *insilico* molecular docking studies.

EXPERIMENTAL WORK

Computational Techniques:

Chem Draw Ultra and Chem Sketch [9]

The computationally designed ligand was drawn by using Chem Draw Ultra 8.0. developed by Cambridge Pvt.Ltd.

Preparation of Ligand

The enzyme-ligand complex with improved binding affinity. For this, the lead molecules must contain following requirements.

- ❖ Ligand prepared in PDB format and it's containing all hydrogen.
- ❖ It must present in a single molecule that has no covalent bonds to the receptor, with no fused fragments such as counter ions and solvents. Must contain realistic bond lengths and bond angles.

Design of Ligand

Lipinskis Rule of Five: [10-11]

Lipinskis rule of five is used to evaluate drug likeness or to determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans.

Pro-Tox II [12-13]

ProTox-II, a virtual lab for the prediction of toxicities of small molecules.

The prediction of compound toxicities is an important part of the drug design development process. Computational toxicity estimations are not only faster than the determination of toxic doses in animals, but can also help to reduce the amount of animal experiments. It is based a total of 33 models for the prediction of various toxicity endpoints such as acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes (Tox21) pathways and toxicity targets.

Protein Selection and Preparation of Protein [14]

The selected protein target which has the specific biological activity was downloaded in the RCSB PDB format using PDB ID 2IRZ from Protein Data Bank (www.rcsb.org). Prior to docking, the protein crystal structures are prepared by removing the ligands and water molecules from the protein and adding hydrogen atoms to optimize hydrogen bonds.

β Secretase [15-17]

The enzyme known as β secretase, or β-site amyloid precursor protein cleaving enzyme 1 (BACE1), is responsible for the initial generation of toxic amyloid β (Aβ), which is a critical precursor in the development of Alzheimer's disease. In Alzheimer's disease, BACE1 is a potential therapeutic target for reducing cerebral Aβ concentrations, and BACE1 inhibitor clinical development is being intensively pursued. Several promising BACE1 inhibitors have recently entered human clinical trials, despite the difficulty of developing BACE1 inhibitor drugs.

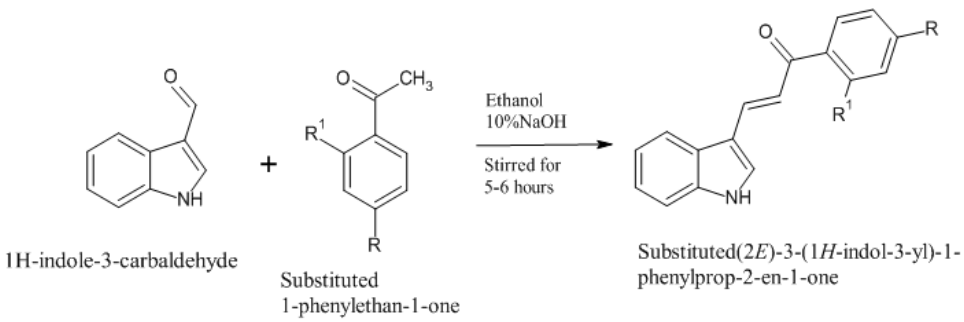
In silico Molecular Docking Studies [18]

To perform the docking studies of the compounds by using a nuclear protein, **β Secretase (Protein ID: 2IRZ)**, was obtained from the RCSB Protein Data Bank. (www.rcsb.org). All the water molecules in the crystal structure were deleted, bond orders were assigned, hydrogens were added and the protein was then further refined for docking studies by using Auto Dock Vina.

SYNTHETIC PROCEDURE [19-20]

Step 1: Synthesis of Chalcone

To a solution of 1H indole - 3-carbaldehydein ethanol substituted ketone was added.To this aqueous potassium hydroxide (40%) was poured. The reaction mixture was stirred at a reaction time of 6hours and acidified with concentrated HCl. The product obtained was filter and recrystallized from ethanol.



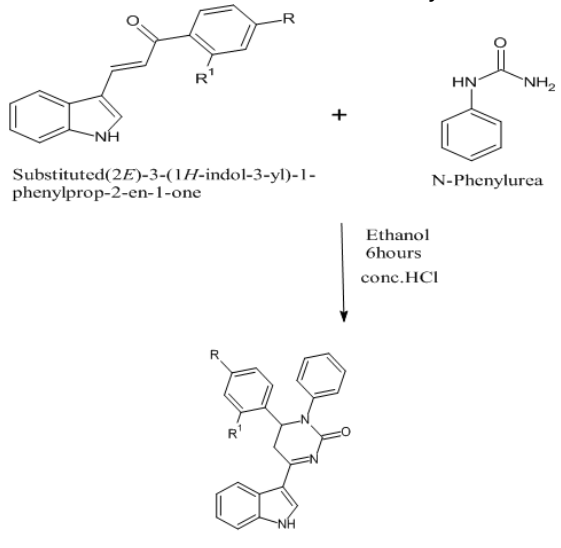
1H-indole-3-carbaldehyde

Substituted
1-phenylethan-1-one

Substituted(2*E*)-3-(1*H*-indol-3-yl)-1-phenylprop-2-en-1-one

Step 2 Synthesis of Pyrimidine

In R.B.F take (0.01 mol) chalcone and add p-Nitro phenylurea or phenylurea was dissolved in 10ml of ethanol and refluxed for about 6hrs and poured intothe ice water then add catalytic amount of conc. HCl. The solid product obtained was filtered and washed with water, recrystallized from absolute alcohol.



Substituted(2*E*)-3-(1*H*-indol-3-yl)-1-phenylprop-2-en-1-one

N-Phenylurea

Substituted 4-(1*H*-indol-3-yl)-1,6-diphenyl-5,6- dihydropyrimidin-2(1*H*)-one

BIOLOGICAL SCREENING

Anti-oxidant activity [23]

Radical scavenging action of synthesized compounds against stable1, 1 – diphenyl-2-picrylhydrazyl radical was measured spectrophotometrically. In order to measurethe DPPH scavenging activity of synthesized compounds, DPPH solution was prepared by adding DPPH (12.5mg) in 50ml of methanol. Absorbance of this stock solution was measured at 517nm. The DPPH solution was diluted with methanol to an absorbance of 0.98 and stored amber colour bottle ready for use. An aliquot of 100µl of synthesized compounds at different concentrations (20, 40, 60, 80 and 100 µl prepared in methanol) was added to 1ml of DPPH solution. The reaction mixture was incubated at 37^{0}C in

darkness for 20-30 min and absorbance was determined at 517nm. As a standard, ascorbic acid was used. The reaction was performed in triplicate. Scavenging activity was calculated by using the following equation.

$$Q\ (\%) = \frac{\text{Absorbance of Control - Sample}}{\text{Absorbance of Control}} \times 100$$

Q =DPPH Scavenging activity

Anti-Alzheimer's Activity [24-25]

Invitro Neuroprotective Effect Determination by MTT Assay

SHSY5Y (Neuroblastoma) cell line was purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium(DMEM) (Sigma Aldrich, USA).

Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize theformazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\%\ \text{of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

RESULT AND DISCUSSION

COMPUTATIONAL TECHNIQUES

Computer aided drug design was successfully carried out with the aid of commercially available free software as well as online tools. A series of derivatives were designed using these softwares.

Ligand Validation

(www.molinspiration.com) Evaluation of physiochemical properties of lead molecule using molinspiration software. Analysis of Lipinski's rule of five revealed that all the selected five lead compounds were showed zero violation that indicates lead molecules were orally active.

Docking results of anti-alzheimers activity

- The molecular docking study was carried out into the active site of β-secretase enzymes (PDB: 2IRZ) using Auto dock vina.
- Molecular modelling of the selected lead molecules showed significant binding to a β-secretase enzymes (-7.3 to -7.8kcal/mol) as compared to standard Donepezil (-7.3 kcal/mol) given in Table.
- Hence the compounds with highest binding energy were selected for synthesis. Docking scores for anti-alzheimer's activity are presented in the Table 5.7and the 2D structure of the selected lead compounds is shown in Figure The PyRx results between the interactions of 2IRZ inhibitor with the designed compounds have given an account result of -7.8 Kcal/mol binding energy for the IP_2.

EXPERIMENTAL WORK

Five analogues were selected for wet lab synthesis based on docking score and availability of reagents. Conventional technique was used for the synthesis of selected compounds with satisfactory.

Characterization of Synthesized Compounds

Physical characterization

Purity of the synthesized compounds was ensured by repeated recrystallization. Further the compounds were identified by TLC and Melting point determination.

Spectral characterization [21-22]

The synthesized lead compounds were confirmed by means of their IR, 1H NMR and ESI MS.

BIOLOGICAL ACTIVITY

Anti-oxidant activity

The antioxidant activity of synthesized lead compounds performed using DPPH method and the results given in table 5.14. The results revealed that synthesized compounds IP_2 and $IP_3$was found to be having significant hydrogen peroxide scavenging activity and their IC_{50} value is32.5 and 31.70 respectively as compared to the standard ascorbic acid with IC_{50} value of 24.32 µM.

Anti-Alzheimer's activity

Neuroprotective Effect Determination by MTT Assay

The newly synthesized lead molecule was assessed for their neuroprotective activity. The activity was carried out by using SH-SY5Y cell lines by MTT assay method. The *invitro* studies showed compound IP-2 possessing good neuroprotection. SH-SY5Y neuroblastoma cell line which was induced with beta amyloid, on treatment with the test compounds shows increased percentage inhibition 89.33% which is necessary for a neuroprotective effect. (Fig.6.) The synthesized lead compound showed significant activity against beta amyloid induced neurotoxicity at 25 µg/ml concentrations and possesses significant percentage cell viability compared to standard.

SUMMARY AND CONCLUSION

The present work conclude that identified a finest molecule of indole-fused-pyrimidine analogue IP-2 (Figure 4) as an innovative drug candidate who was docked against β-Secretase in a deliberate attempt to discover an MTDL, able to interfere with diverse key target points of AD Neurodegeneration. The molecular properties were calculated from suitable computational tools like molinspiration and toxicity by protox-II. These lead molecules were investigated for drug-like properties by calculating Lipinski's rule of five using molinspiration. All of the selected lead molecules showed zero violations of the rule of 5, which indicates good bioavailability. Since **IP-2** was well thought-out as a lead molecule and active against Alzheimer's and oxidative stress in all *in-vitro* assays and also elicited high docking score in docking studies, can be considered as a promising lead molecule for further research.

Future work needs to be focused on targeting tau protein being the prime factors of the pathophysiology of Alzheimer. Thus these novel leads may be promising candidates for the treatment of Alzheimer disease.

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Table. 1 Compound Code and Substitution

COMPOUND CODE	R	R^1
IP_1	H	H
IP_2	Cl	H
IP_3	OH	H
IP_4	OH	OH
IP_5	CH_3	H

Table 2.Substitution of Acetophenones

COMPOUND CODE	R	R^1
IP_1	H	H
IP_2	Cl	H
IP_3	OH	H
IP_4	OH	OH
IP_5	CH_3	H

Table 3. Physiochemical Properties of Lead Molecule

Code	Log P	TPSA	No of atoms	MW	noN	Noh NH	No of violations	nrotb	Volume
IP1	5.30	48.46	28	365.44	4	1	1	3	333.65
IP2	5.98	48.46	29	399.88	4	1	1	3	347.19
IP3	4.82	68.69	29	381.44	5	2	0	3	341.67
IP4	4.74	88.92	30	397.43	6	3	0	3	349.69
IP5	5.75	48.46	29	379.46	4	1	1	3	350.21

Table 4. Docking results of lead molecules towards 2IRZ

Code	Binding Energy (Kcal/mol)	Amino Acid Interactions
IP_1	-7.4	GLN73, ASN233, THR232, ARG307
IP_2	-7.8	PRO308, VAL309, ILE110, THR232
IP_3	-7.4	LYS321, PRO308, ILE110,VAL309
IP_4	-7.7	THR232,ALA323,ILE110, LYS321
IP_5	-7.5	GLU290, PRO192, ARG194, MET288
Donepezil	-7.3	TYR190,ARG331,VAL292,PRO373

Table 5. Physical Characterization of Synthesized Lead Compounds

Compound Code	Molecular formula	Molecular weight (gm)	Melting point	R_f value	Percentage Yield	Colour
IP_1	$C_{24}H_{19}N_3O$	365.42	170°C	0.72	74%	Pale Yellow
IP_2	$C_{24}H_{18}ClN_3O$	399.87	150°C	0.38	65%	Yellow
IP_3	$C_{24}H_{19}N_3O_2$	381.42	160°C	0.47	82%	Cream
IP_4	$C_{24}H_{19}N_3O_3$	397.42	165°C	0.42	78%	Brown
IP_5	$C_{25}H_{21}N_3O$	379.45	170°C	0.64	80%	Cream

Table 6: IR Interpretation of Synthesized Lead Compounds

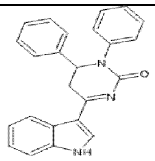
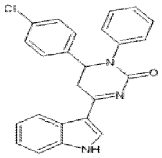
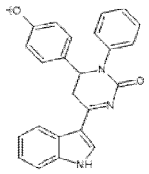
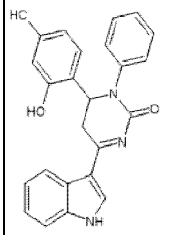
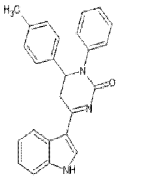
COMPOUND CODE	STRUCTURE	IR INTERPRETATION (Frequency in cm-1)
IP_1		3041.74(CH Aromatic stretching vibration), 1633.71(C=C Stretching vibration), 1496.76 (C=N Stretching vibration), 3579.88(NH), 1458.18(C-N Stretching vibration), 1747.51(C=O Stretching vibration)
IP_2		3051.39(CH Aromatic stretching vibration), 1633.71(C=C Stretching vibration), 1519.91(C=N Stretching vibration), 3545.16(NH), 1444.68(C-N Stretching vibration), 1749.44(C=O Stretching vibration), 759.95(C-Cl Stretching vibration)
IP_3		3145.90(CH Aromatic stretching vibration), 1614.42(C=C Stretching vibration), 1539.20(C=N Stretching vibration), 3562.52(NH), 1446.61(C-N Stretching vibration), 1747.50(C=O Stretching vibration), 1336.67(C-OH Stretching vibration)
IP_4		3113.11(CH Aromatic stretching vibration), 1614.40(C=C Stretching vibration), 1521.84(C=N Stretching vibration), 3566.38(NH), 1458.18(C-N Stretching vibration), 1732.08 (C=O Stretching vibration), 1548.58.(C-OH Stretching vibration) 3041.34 (C-OHStr vibration)
IP_5		3149.76(CH Aromatic stretching vibration), 1633.71(C=C Stretching vibration), 1519.91(C=N Stretching vibration), 3585.67(NH), 1446.61(C-N Stretching vibration), 1732.08(C=O Stretching vibration), 1446.61(C-CH3Str vibration)

Table 7:^{1}H NMR and ESI MS of Synthesized Lead Compounds

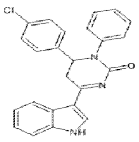
STRUCTURE	^{1}H NMR	^{13}C NMR	MASS (M+1)
IP$_2$ 	^{1}H NMR DMSO - δ10.04 (s 1H) 9.06 (s 1H) 8.00-8.31 (d 3H) 7.98 -7.82 (d 3H) 7.59-7.46 (d 3H) 7.44-7.30 (s 2H) 7.29-7.08(d 2H) 7.06 – 6.97 d 2H) 1.24 (s 2H)	^{13}C NMR: δ 29.38 (1C, s), 76.78 (1C, s), 118.7 (1C, 77.10-77.42 (3C, s), 111.66 (1C, s), 112.08 (1C, s), 1117.30(1C, s),119.73(1C, s),121.04(1C, s),122.02(1C, s),123.14(1C, s), 124.30-124.92, (2C, s), 129.92-129.94, (2C, s), 130.77(1C, s),135.59(1C, s),136.78(1C, s),137.37(1C, s),138.21(1C, s), 139.56(1C, s),185.37(1C, s)	**402**

Table 8. DPPH Radical Scavenging Activity of the Synthesized Compounds

Comp. Code	DPPH radical scavenging activity					IC$_{50}$ Value(µM)
	20µg/ml	40µg/ml	60µg/ml	80µg/ml	10µg/ml	
IP$_2$	25.50	43.21	57.64	74.4	86	32.25
IP$_3$	23.97	39.69	56.53	66.80	78.24	31.70
Ascorbic acid	29.59	46.83	58.97	78.36	87.24	24.32

Table 9: Percentage of Growth Inhibition

Comp Code	% Cell Viability				
	1.5 µg/ml	3.1 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml
IP-2	53.44	64.65	74.23	82.67	89.33
Standard	70.70	85.28	89.20	91.89	98.25
CONTROL	100.00				
βamyloid	49.72				

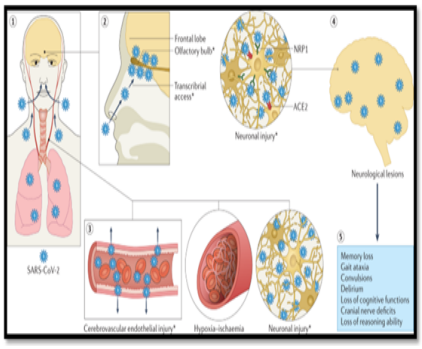


Fig. 1: Routes of the spread of SARS-CoV-2 across the CNS and the possible mechanisms involved in neuronal injury, AD

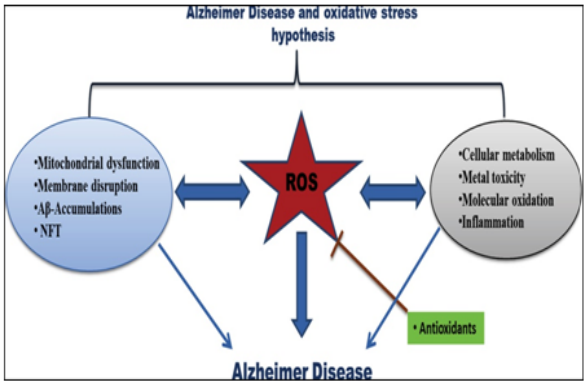


Fig. 2: Relationship between antioxidant and alzeihmers

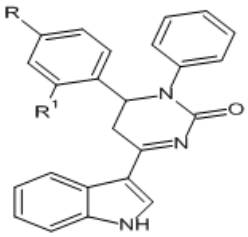


Fig:3 Design of Ligand

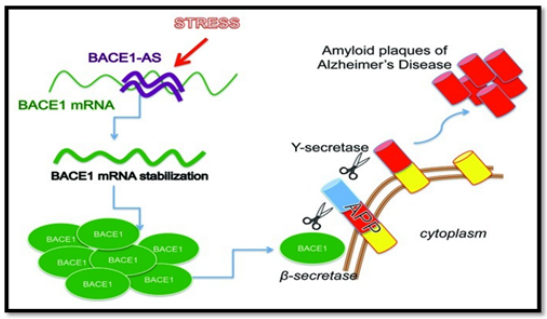
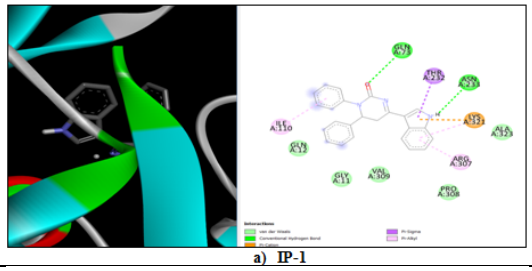
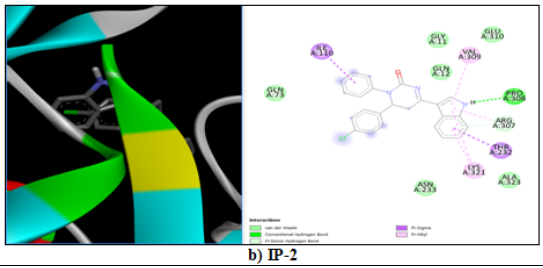


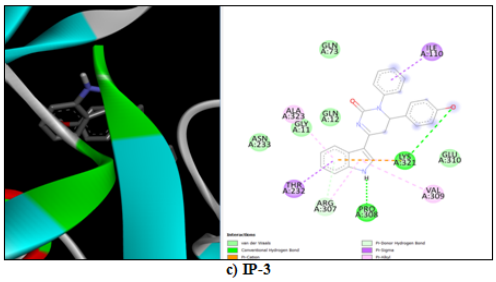
Fig 4 : Action of β Secretase



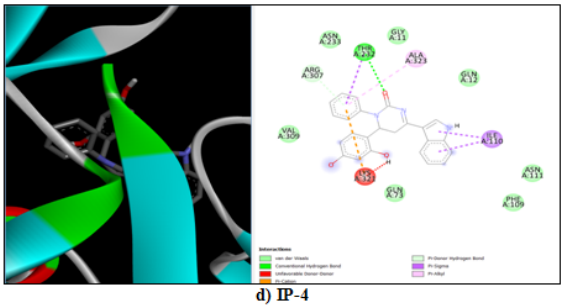
a) IP-1



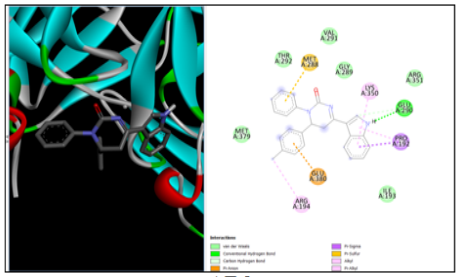
b) IP-2



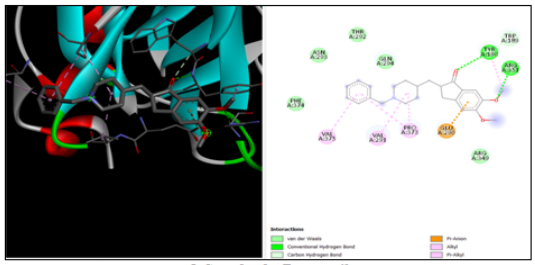
c) IP-3



d) IP-4



e) IP-5



f) Standard - Donepezil

Fig. 5 : (a-f)Ligand Were Docked Against β-Secretase –Target (2IRZ)

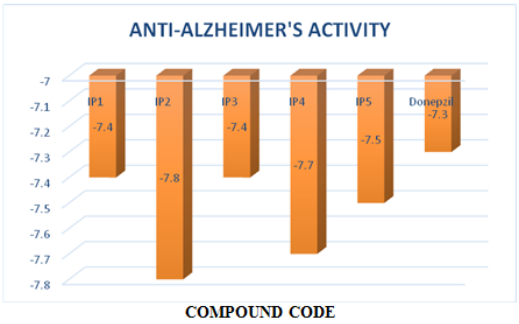


Fig. 6: Graphical representation of anti-Alzheimer's activity Binding Affinity

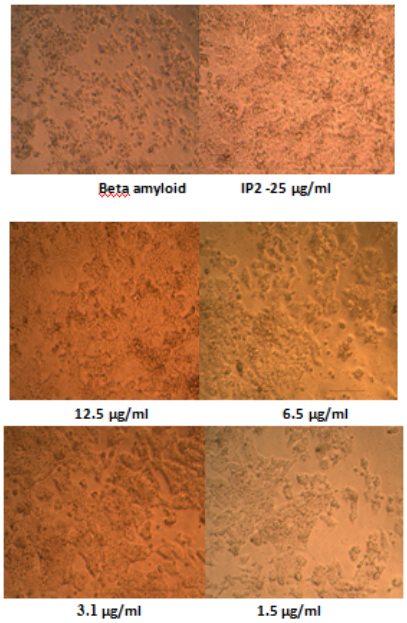


Fig. : Percentage of growth of inhibition

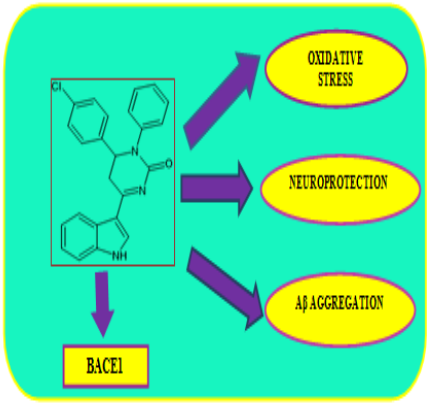


Fig:8