Haloperidol inhibits Memapsin 2: Innovation by docking simulation and *in vitro* assay

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Abstract: A number of drugs exhibit unexpected pharmacological effects related to their ability to bind more than one receptor in humans. Haloperidol a typical antipsychotic drug appeared in several reports to be used in schizophrenia patients in which the significant of Alzheimer's disease has been reduced. The etiology of the disease is characterized by aggregates of amyloid plaques, largely composed of amyloid-β peptide formed from the amyloid precursor protein cleaved by Memapsin 2. To investigate if haloperidol can bind to Memapsin 2 active site, an initial molecular docking was performed as a preliminary *in-silico* screening test followed by *in vitro* enzyme inhibition assay. Haloperidol was found to fit readily in Memapsin binding site with IC_{50}value 250mM. Haloperidol can be considered as important lead or important target can be modified for more inhibitory activity, with the intention of protection or treatment for Alzheimer's disease.

Keywords: Haloperidol, Memapsin 2 inhibitors, Alzheimer's, Docking, In-silico.

INTRODUCTION

A number of drugs exhibit unexpected pharmacological effects related to their ability to bind more than one receptor in humans. Determining the molecular mechanism of these effects might lead to new targets or new therapies for the treatment of different disorders. As well, such research may expose ways to design new drugs with fewer side effects or have multiple targets. Well-known examples of drug effects are seen in atypical antipsychotics. Antipsychotic medications are a backbone in the treatment of schizophrenia and are widely used in other psychiatric conditions (Tauscher and Kapur, 2001; Cohen, 2002; Bhugra, 2010). Moreover these drugs can be used to treat related schizophrenia symptoms in other disease state related to age, like Alzheimer's disease (AD) (Turner *et al*. 2008).

Alzheimer's disease (AD) is the most common neurodegenerative disorder that affects elderly. The etiology of the disease is characterized by aggregates of amyloid plaques, largely composed of amyloid-β peptide (Aβ) (Waldemar *et al*, 2007; Bulbarelli *et al*, 2012). The accumulation of Aβ is thought to be the central feature in the progression of AD and, as such, many accepted therapies for the treatment of AD are currently targeting inhibition of Aβ production (Hardy and Selkoe, 2002; Tiraboschi *et al*., 2004). Aβ is derived from proteolytic cleavage of the membrane bound amyloid precursor protein (APP). APP is processed by two routes, firstly, nonamyloidgenic where APP is cleaved by α-secretase to yield soluble APPα. The cleavage site is within the Aβ sequence, thereby precluding its formation. Secondly, amyloidgenic where APP is cleaved by memapsin 2 (β-secretase) to yield soluble APPβ and also Aβ. Cleavage of APP by γ-secretase is common to both pathways. Based on the pathophysiology, various pharmacologic approaches are developed for the treatment of AD. The approved treatment strategies provide symptomatic improvement in AD. The therapies under evaluation for the treatment of AD have disease modifying and neuro-protective approaches (Xiong, 2005; Zec and Burkett, 2008; Christopher *et al*, 2008; Prerna *et al*., 2010). Pharmacological agents used for treatment of Neuropsychiatric illnesses include antipsychotics, antidepressants and mood stabilizers (Zec *et al*., 2008). Early interest in developing therapies for AD focused on the cholinergic system as disease progression is known to be accompanied by loss of cholinergic neurons (Muir, 1997; Christopher *et al*, 2008). In reality, cholinergic deficit and Aβ levels correlate well in the disease state (Beach *et al*., 1997; Shinoe *et al*., 2005). Memantine (an NMDA receptor antagonist) apart, all current FDA-approved therapies for the symptomatic treatment of AD are acetylcholine esterase inhibitors (AChEIs) (Ibach & Haen, 2004; Alvin *et al*. 2011). However, most of the clinical efficacy observed in AD is largely restricted to the first two years from inception of treatment. Moreover, AChEIs have a number of undesirable side effects, including nausea, sweating, salivation and gastrointestinal disturbances (Ibach & Haen, 2004; Christopher *et al*, 2008; Alvin *et al*. 2011). Therefore, there remains a significant need for treatments with less side effect potential, or, preferably, treatments targeting AD progression.

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During the course of the disorder, late-stage AD is characterized by loss of cognitive abilities, delusions, paranoia, and personality changes that range from passivity to aggression (Patterson *et al*. 1990, Turner *et al*. 2008, Terry at al. 2011), these psychiatric symptoms require antipsychotic medication. Recent reports demonstrate that AD pathology is infrequent in patients with schizophrenia (Suna *et al*, 2002; Palotás *et al*, 2003), as well as it has been proposed that antipsychotic medications used to treat schizophrenia might in fact have a protective effect against developing AD neuropathology. Traditional antipsychotic haloperidol (fig. 1) is dopamine-2 (D2) and sigma-1 (σ1) receptor antagonist with apoptotic activity (Walker *et al*., 1990; Behl *et al*., 1996; Kapur and Mamo 2003). Haloperidol is involved in neurotoxicity, causing clinically troublesome adverse events such as extra-pyramidal and cardiac side-effects (Behi *et al*. 1996; Yen *et al*., 2004; Giegling *et al*., 2011). Haloperidol was also demonstrated to efficiently inhibit βAP formation from APP *in vitro* (Higaki *et al*., 1997). The mechanism by which it might exert such an effect is not known even though a patent (US patent 2011) in 2011 had represent haloperidol as memapsin inhibitor but no study so far show the inhibition profile or even the important features of this drug that interact with memapsin 2 binding site. Additionally, no studies were conducted to investigate the direct effect of haloperidol on β-secretase. Accordingly we were encouraged to further evaluate the effect of haloperidol on β-secretase. To investigate if haloperidol can bind to memapsin active site, an initial molecular docking was performed as a preliminary in-silico screening test. Exploring the effect of haloperidol on such pivotal enzyme and its effect on Ab levels could be useful for designing new analogues that could be used to manage AD whether for treatment or as protective method in high risk patient as well as it can be a starting point for research to modify such drug to be more potent inhibitor.

MATERIALS AND METHODS

Molecular Modeling

Software and Hardware

The following software packages were utilized in the present research

- CS ChemDraw Ultra (Version 11.0), Cambridge Soft Corp., USA.
- DiscoveryStudio (DS 2.5), Accelrys, Inc., USA
- Ligandfit within CERIUS2 (Version 4.10), Accelrys, Inc., USA.
- Libdock within Discovery Studio (Version 2.0), Accelrys, Inc., USA.
- Catalyst (version 5.11), Accelrys, Inc., USA

Preparation of Crystal Structures

The 3-D coordinate of memapsin 2 was retrieved from the Protein Data Bank (PDB code: 2IQG, resolution: 1.7 Å (Maillard *et al*, 2007)). Hydrogen atoms were added to the protein utilizing DS 2.0 templates for protein residues. Gasteiger-Marsili charges were assigned to the protein atoms as implemented within DS 2.5 (Gasteiger and Marsili 1980; Discovery Studio manual 2009). The protein structures were utilized in subsequent docking experiments without energy minimization. Explicit water molecules were kept, i.e., docking in the presence of explicit water molecules.

Docking Configurations

(1) Ligand Fit Docking and Scoring.

Ligand Fit considers the flexibility of the ligand and treats the receptor as rigid. There are two steps implemented in the Ligand Fit process:

(A) Defining the location(s) of potential binding site(s) (Venkatachalam *et al*, 2003; Gehlhaar *et al*, 1995). In the current docking experiments, the binding site was generated from the co-crystallized ligand F2I (fig. 2)**(**N'-{(1S, 2R)-1-(3, 5-Difluorobenzyl)-2-Hydroxy-3-[(3-Iodobenzyl) Amino] Propyl}-5-Methyl-N, N-Dipropylisophthalamide) within the targeted protein.

(B) Docking the ligands in the binding site (Venkatachalam *et al*, 2003; Gehlhaar *et al*, 1995).

In LigandFit,docking is composed of few substeps: (i) Conformational search of flexible ligands employing Monte Carlo randomized process. (ii) Pose and conformation selection based on shape similarity with the binding site. (iii) Candidate conformers and poses exhibiting low shape discrepancy are further enrolled in calculation of the dock energies. (iv) Each docked conformation and pose is further fitted into the binding pocket through a number of rigid-body minimization iterations. (v) Docked conformers and poses that have docking energies below certain user-defined threshold are subsequently clustered according to their *rms* similarities. Representative conformers and poses are then selected, further energy-minimized within the binding site, and saved for subsequent scoring.

In the current docking experiments, haloperidol in its un-ionized form was docked into the binding site with a non bonded cutoff distance of 10.0 Å and distance dependent dielectric. The interaction energy was estimated by a trilinear interpolation value using soft potential energy approximations (Venkatachalam *et al*, 2003).

- Rigid body ligand minimization parameters: 30 steepest descend followed by 60 BFGS minimization iterations were applied to every orientation of the docked ligand. The best10 poses were further energy minimized within the binding site for a maximum of 300 rigid body iterations.
- High-ranking docked conformers and poses were scored using seven scoring functions: Jain (Jain 1996), LigScore1, LigScore2 (Krammer *et al*, 2005; Venkatachalam 2003), PLP1 (Gehlhaar *et al*, 2995),

PLP2 (Gehlhaar *et al*, 1999), PMF, and PMF04 (Muegge 2000; Muegge 2001; Muegge 2006).

LigScore1 and LigScore2 scores were calculated employing CFF force field (version 1.02) and using grid-based energies with a grid extension of 7.5 Å across the binding site. PMF scores were calculated employing cutoff distances of 12.0 Å for carbon-carbon interactions and other atomic interactions, while PMF04scores were calculated employing cutoff values of 6.0 and 9.0 carbon-carbon interactions and other atomic interactions, respectively.

LibDock Docking: LibDock docks ligands (after removing hydrogen atoms) into a binding site guided by binding hotspots. It aligns docked ligand conformations to polar and a polar receptor interactions sites, i.e., hotspots. Conformations can be either pre-calculated or generated on the fly. Because some of the output poses may have hydrogen atoms in close proximity to the receptor, a CHARMm minimization step can be optionally enabled to further optimize the docked poses (Diller and Merz 2001; Rao *et al*, 2007). LibDock performs the following steps using a set of pre-generated ligand conformations and a receptor with a specified binding site: (Diller and Merz 2001; Rao *et al*, 2007). (i) Remove hydrogen atoms. (ii) Rank ligand conformations and prune by solvent accessible surface area (SASA). (iii) Find hotspots using a grid placed into the binding site and using polar and apolar probes. The numbers of hot spots are pruned by clustering to a user-defined value. (iv) Dock ligand poses by aligning to the hotspots. This is performed by using triplets (i.e., three ligand atoms are aligned to three receptor hotspots). Poses, which result in protein clashes, are removed. (v) A final BFGS pose optimization stage is performed using a simple pair wise score. The top scoring ligand poses are retained.(vi) Hydrogen atoms are then added back to the docked ligands. (vii) Optionally, CHARMm minimization can be carried out to reduce steric clashes caused by added hydrogen atoms.

In the current docking experiments we employed the following parameters.

- Binding site sphere radius of 11.8 Å surrounding the center of the co-crystallized ligand (**F2I**) (fig. 2).
- Ligand-to-hotspots matching rmsd tolerance value was set to 0.25 Å.
- Maximum number of saved poses for each ligand = 100.
- Maximum number of poses saved for each ligand during hotspots matching before entering the final pose minimization= 100.
- Minimum LibDock score (poses below this score are not reported) = 100.
- Fraction of reported top scoring poses = 0.5.
- Maximum number of rigid body minimization steps during final pose optimization phase (using BFGS method) = 50.
- Maximum number of evaluated poses for each conformation= 30.
- Maximum number of steric clashes allowed before the pose-hotspot alignment is terminated (specified as a fraction of the heavy atom count) = 0.10.
- Cluster similarity cutoff value = 0.5 Å (docked poses are rigid-body minimized and clustered using this cutoff value).
- Maximum value for non-polar solvent accessible surface area for a particular pose to be reported as successful= 15.0 $Å^2$.
- Maximum value for polar solvent accessible solvent area for a particular pose to be reported as successful = 5.0 $Å^2$.
- Number of grid points used for calculating solvent accessible surface area = 18.
- Conformation generation method: The CATALYST module CATCONFIRM implemented in DS 2.0 was implemented employing the BEST conformation generation option to ensure the best coverage of the compound's conformational space. Maximum number of conformations to be generated per ligand = 255 not exceeding an energy threshold of 20 kcal/mol from the most stable conformer. No final ligand minimization was implemented (i.e., in the binding pocket).
- The docked poses were scored employing the same seven scoring functions that were implemented in Ligand Fit docking experiment and employing identical parameters.

In vitro Memapsin 2 Enzyme Inhibition Assay.

Materials

Materials were purchased from corresponding companies (Sigma-Aldrich and BDH Laboratory Supplies) and were used in the experimentation without further purification. Memapsin2 assay kit (Sigma-Aldrich CS0010) Dimethylsulfoxide (DMSO, BDH Laboratory Supplies, England). Haloperidol (Sigma-Aldrich)

Preparation of Hit Compounds for In vitro Assay

The tested compounds were provided as dry powders. They were initially dissolved in DMSO to provide 0.02 mM stock solutions and subsequently diluted to the required concentrations using 50mM sodium acetate, pH 4.5. The inhibition of memapsin activity by the hit compounds was measured using the fluorometric assay. The final concentration of DMSO was adjusted to be less than 0.1%.

Quantification of memapsin 2 activity in a fluorometric assay

The memapsin 2 fluorescence resonance energy transfer (FRET) assay was performed as described by the manufacturer (Sigma, CS0010) (Sigma-Aldrich). Principle of the Assay: The substrate is linked to a fluorescent dye on one end and to a quenching group on its other end. The fluorescence of the substrate is

significantly reduced due to intra-molecular resonance energy transfer to the quenching group. Upon substrate cleavage by the enzyme, there is a disturbance of the energy transfer resulting in the enhancement of the fluorescent signal. The assay procedure can be described briefly as follows The memapsin substrate is prepared in the buffer to a concentration of 50μM. Memapsin enzyme is prepared in the same buffer to a concentration of approximately 0.3units/μl. Stock solutions of test samples are prepared in DMSO, and then serially diluted in the buffer to give the desired working concentrations. Triton X-100 was added to each well to a final concentration of 160μM. memapsin enzyme, substrate, standard, test samples and buffer are then added to the wells for a total volume of 100μL, with the memapsin 2 enzyme being added last, just prior to reading. Baseline fluorescence is recorded immediately after the addition of the memapsin2enzyme on a fluorometer set at excitation 320 nm, emission 405 nm. The reaction rate was monitored for 2h at 37°C using FLX800TBI Microplate Fluorimeter (Biotek Instruments, Winooski, USA) and the linear time-relative fluorescence units (RFU) sections were taken for rate calculation (Al-Nadaf *et al*., 2010, Al-Nadaf and Taha 2012).

Memapsin 2 Inhibition by Hit Compounds

The inhibition of memapsin 2activity by the hit compounds was measured using the fluorometric assay described above. The percentage of residual activity of memapsin 2was determined for each compound by comparing the activity of memapsin 2in the presence and absence of the tested compound. Blank and standard inhibitor (Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe) (Sigma-Aldrich, product (A1847)) was used as negative and positive controls, respectively. Measurements were conducted at least in duplicates.

RESULTS

Molecular modeling

Our efforts to investigate the effect of haloperidol on memapsin 2commenced by evaluating the possibility of binding via computer-aided molecular modeling techniques. Accordingly, wedocked haloperidol into the binding pocket of memapsin 2 (PDBcode: 2IQG).The molecular interactions of the highest ranking binding mode can be summarized in fig. 3. Clearly from the fig. the (OH) group of haloperidol interacts with the carbonyl moiety of PHE-108 by hydrogen bond. And the carbonyl side chain of GLY-230 interacts with benzene ring holding chlorine atom via π-π interaction. On the other hand, the nitrogen of piperidin moiety can forms electrostatic interaction with ASP-228 and hydrophobic interaction with the CH2-CH2 part of GLN -73.Moreover the carbonyl moiety forms hydrogen bond with TYR-198, while the benzene ring holding the florin atom interacts with TYR-198 via p-stacking. Interestingly, these interaction signals are among the hot spots provided by the co-crystallized ligand (fig. 3). Where the terminal amidic carbonyl oxygen atom adjacent aliphatic terminal chain is hydrogen bonded to THR-232 and the central amidic NH group is hydrogen bonded to carbonyl moiety of GLY-230. Moreover, the carbonyl part for the central amide is situated in position that is suitable to interact via hydrogen bond with NH group of GLN-73 or THR-72. In addition the aromatic fluorine atom can do hydrogen bond with GLY-74 where the benzene ring itself involved with PHE-108 in aromatic interaction and with aliphatic side chain (CH_2-CH_2) of GLN-73 via hydrophobic interaction. The terminal NH group adjacent to benzene ring holding Iodine atom situated in position suitable to interrelate with ASP-228 through electrostatic interaction (fig. 3).

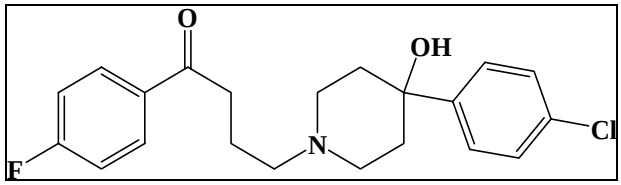

Fig. 1: Molecular structure of haloperidol

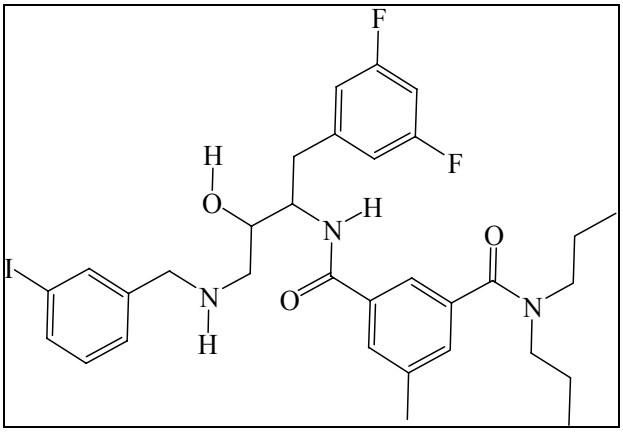

Fig. 2: Molecular structure of co-crystallized structure

Effect of haloperidol on Memapsin 2 Activity

To evaluate the inhibitory effect of haloperidol against memapsin 2, an *in vitro* memapsin 2 inhibitory assay was conducted. In this inhibitory assay; the concentration of haloperidol that inhibits 50% of the enzyme, IC_{50}, was measured. Fig. 4 shows the effect of different concentrations of haloperidol on the relative activity of memapsin 2. The drug produced intermediate potent inhibition with IC_{50} value of 250μM. The validity of the test was established by testing the inhibitory action of the standard inhibitor A1847 (Sinha *et al*, 1999) on memapsin 2, which showed an IC_{50} value of 40nM that is comparable to the published value (Sinha *et al*, 1999).

DISCUSSION

Based on previous results about the ability of haloperidol to decrease levels of APP *in vivo* (Higaki *et al*, 1997;

Palotás *et al*, 2003; US patent 2011) this work intended to investigate the direct effect of this drug on memapsin 2. This is the first published report on the effect of haloperidol on memapsin 2.

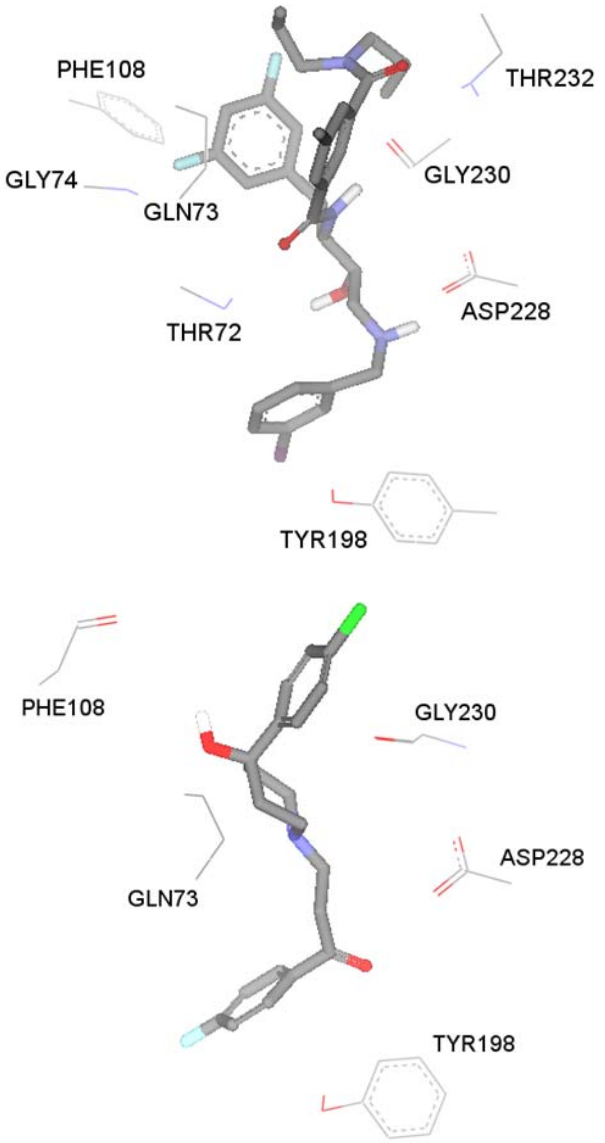

Fig. 3: (A) Detailed view of the co-crystallized structure F21 and the corresponding interacting amino acids within the binding site of memapsin 2. (B) Detailed view of the docked Haloperidol structure and the corresponding interacting amino-acid moieties within the binding site of memapsin 2.

As we discussed earlier haloperidol has been reported to block the amyloidogenic processing of APP, which, in turn, prevent βAP production. Our findings support these reports with the addition of the definite proof for the direct interaction with memapsin 2 binding site that have been conducted by docking study either ligandfit or libdock or by *in vitro* assay.

The conducted preliminary molecular modeling study has shown that haloperidol can be successfully docked within the binding pocket of memapsin 2 making several significant interactions with key hot spots within binding pocket. The validation for our docking-scoring procedure was performed through employing the same conditions to dock a well-known memapsin 2 inhibitor **F2I** (Maillard *et al*, 2007) (fig. 5) into the binding pocket of this enzyme. The docking simulation resulted in a close model to the crystallographic structure, which supports our conclusions regarding haloperidol memapsin 2 binding (fig. 5). Furthermore, five of the important interactions are shared between the co-crystallized ligand and haloperidol (fig. 3). Where one of the most important interactions is ionic with ASP-228 can be found, beside the other aromatic; hydrogen bond and hydrophobic interaction which increase the confidence in the docking configuration and results. Moreover the other interactions can be targeted with possible modifications in haloperidol structure in order to synthesize more potent analogues keeping in mind all attractive feature in our drug as it is already can cross blood brain barrier and its toxicity profile is well known.

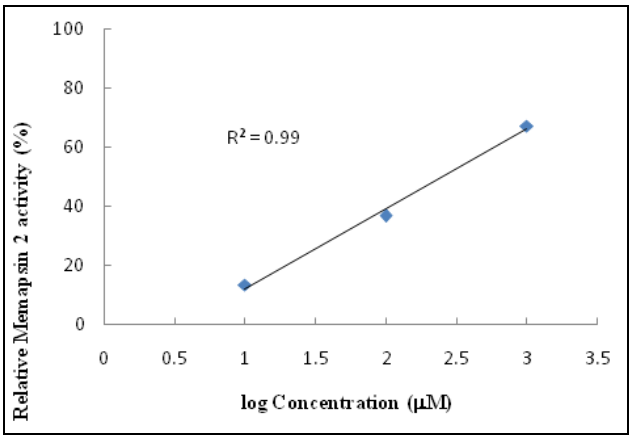

Fig. 4: Effect of Haloperidol concentrations on the relative activity of Memapsin 2.

The preliminary docking study has supported our hypothesis and literature data that haloperidol has an inhibitory activity against memapsin 2. The data presented here demonstrate that haloperidol with IC_{50} of 250.5 μM, explain and justify why several investigators have found a low frequency of AD neuropathology in schizophrenia, as many patients are likely to have been treated chronically with antipsychotics, including haloperidol, a commonly prescribed drug for this disorder. Chronic treatment with haloperidol results in stable APP levels, which leads to decreased deposition of βAP in the brain over time by inhibiting βAP formation and alleviating βAP-induced toxicity.

Pathophysiology of AD has different features and abnormal signal transduction systems have been implicated. But their precise role has been difficult to

establish. We theorize that the action of haloperidol on AD processing and is an improvement mechanism involving various second messengers. Haloperidol acts as D2-antagonists;σ1-antagonist and we can add new mechanism of action that can explain different reports about effect of this drug on AD patients or schizophrenia patients.

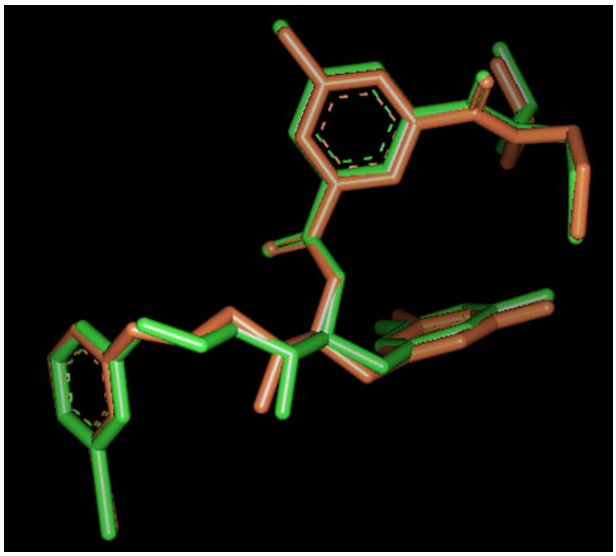

Fig. 5: Comparison between the docked conformer/pose of inhibitor (Orange) as produced by the docking simulation and the crystallographic structure of this inhibitor within Memapsin 2 (green, PDB code: 2IQG)

This may explain why several investigators have found allow frequency of AD neuropathology in schizophrenia, as many patients are likely to have been treated chronically with antipsychotics, including haloperidol, a commonly prescribed drug for this disorder.

Questions that must be answered are: if we can give haloperidol for peoples with high risk potential for AD in very low doses , as we need to decrease the activity of memapsin 2 not to have 100% inhibition?, or can we modify this drug to be more potent inhibitor for memapsin 2 and decrease its side effect if possible? . We believe that this dilemma is important to be considered as this drug is now in clinical use for such patients even though for different cause.

CONCLUSION

These data suggest that haloperidol-induced memapsin 2 inhibition may represent an important new suggestion for the AD treatment. With IC_{50} of 250.5 μM, haloperidol cannot be considered as potent inhibitor but it is important lead or important target that can be modified for more inhibitory activity, which can be used as protective agent.

To address this issue synthetic modification on haloperidol structure depending on this in-silico docking study can be considered to increase possible interactions with β-secretase binding site. Moreover clinical study can be made to investigate sub-therapeutic doses of haloperidol to individuals with high risk to be patients with AD, to investigate the use of haloperidol can be used as protective agent against this disease as we all hope to find such agents.

Table 1: Ligandfit scoring values for the 10 poses of haloperidol

POSE_NUMBER	LigScore1	LigScore2	PLP1	PLP2	Jain	PMF	PMF04	Consensus
1	4.08	5.98	98.45	92.02	3.63	116.59	59.88	4
2	3.9	5.69	97.47	93.83	3.44	76.93	48.2	1
3	3.77	5.62	99.59	95.03	**3.71**	79.92	48	2
4	4.03	5.67	98.41	95.17	3.62	78.07	47.71	2
5	**4.19**	5.92	**102.2**	98.15	3.53	85.69	44.79	5
6	4.11	5.89	99.7	96.02	3.21	81.74	43.27	3
7	4.1	5.81	100.18	95.46	3.27	83.57	44.31	4
8	3.8	**6.13**	91.51	85.43	3.6	**125.08**	**65.41**	5
9	3.95	5.75	98.91	93.2	2.91	79.15	45.24	2
10	3.31	5.45	100.77	**99.18**	2.98	73.46	40.26	4

*Bolded values corresponds to highest value for each scoring function

Table 2: Lib dock scoring out put

POSE_NUMBER	LigScore1	LigScore2	PLP1	PLP2	Jain	PMF	PMF04	Consensus
47	2.36	3.74	**88.03**	84.62	4.5	69.52	**45.69**	3
48	**3.6**	**4.08**	83.91	**87.4**	**6.17**	55.74	23.51	3
53	2.59	4.01	81.11	81.86	4.24	**90.71**	32.27	3

*Bolded values corresponds to highest value for each scoring function

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