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Research paper

Multi-target screening mines hesperidin as a multi-potent inhibitor: Implication in Alzheimer's disease therapeutics

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ABSTRACT

Alzheimer's disease (AD) is the most frequent form of neurodegenerative disorder in elderly people. Involvement of several pathogenic events and their interconnections make this disease a complex disorder. Therefore, designing compounds that can inhibit multiple toxic pathways is the most attractive therapeutic strategy in complex disorders like AD. Here, we have designed a multi-tier screening protocol combining ensemble docking to mine BACE1 inhibitor, as well as 2-D QSAR models for anti-amyloidogenic and antioxidant activities. An *in house* developed phytochemical library of 200 phytochemicals has been screened through this multi-target procedure which mine hesperidin, a flavanone glycoside commonly found in citrus food items, as a multi-potent phytochemical in AD therapeutics. Steady-state and time-resolved fluorescence spectroscopy reveal that binding of hesperidin to the active site of BACE1 induces a conformational transition of the protein from open to closed form. Hesperidin docks close to the catalytic aspartate residues and orients itself in a way that blocks the cavity opening thereby precluding substrate binding. Hesperidin is a high affinity BACE1 inhibitor and only 500 nM of the compound shows complete inhibition of the enzyme activity. Furthermore, ANS and Thioflavin-T binding assay show that hesperidin completely inhibits the amyloid fibril formation which is further supported by atomic force microscopy. Hesperidin exhibits moderate $ABTS^{\bullet+}$ radical scavenging assay but strong hydroxyl radical scavenging ability, as evident from DNA nicking assay. Present study demonstrates the applicability of a novel multi-target screening procedure to mine multi-potent agents from natural origin for AD therapeutics.

1. Introduction

Alzheimer's disease (AD) emerges as the most common form of neurodegenerative disorder in elderly people [1]. Current AD therapies are palliative and only temporarily slow cognitive decline [2]. None of the available medications are able to cure Alzheimer's dementia or even stop the disease progression. Moreover, treatments addressing the underlying pathological mechanisms of AD are completely lacking. The key histopathological feature of AD is the presence of extracellular aggregates of proteinaceous debris, the "amyloid plaques", and intracellular filamentous "neurofibrillary tangles" (NFT) in the region of the brain involved in memory and cognition [3,4]. Plaques are insoluble, dense protein masses mostly composed of aggregates of a peptide called amyloid-β peptide (Aβ) [5,6]. On the other hand, tangles are insoluble twisted fibers inside the nerve cell composed of hyper-phosphorylated tau, a microtubule associated protein [7]. Over the years "amyloid hypothesis" emerges as the principal pathological mechanism in AD and the evidence came from the transgenic mice model which demonstrated that fibrillar Aβ induces neurofibrillary tangle formation [8]. Even, fetal rat hippocampal neurons and human cortical neurons treated with fibrillar Aβ exhibited an increased degree of tau phosphorylation [9] providing indication that amyloid fibril formation might alter the phosphorylation state of tau, which in turn facilitates NFT formation. Aggregates of Aβ peptides have been found to induce neuronal apoptosis by the activation of several

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caspase mediated cell signaling pathways and thereby causing cell death [10,11]. It has also been reported that exposure of synapses to Aβ impairs the function of membrane ion and glutamate transporters and compromises mitochondrial function mediated by oxidative-stress [12].

Aβ is generated by the sequential cleavage of two proteases. β-secretase 1 (BACE1) initially cleaves APP at the N- terminus of the Aβ peptide domain which is followed by the cleavage of γ-secretase in the trans-membrane domain of APP leading to the secretion of Aβ peptide [13,14]. The enzyme BACE1 is considered a prime target to design therapeutics in AD mainly because catalysis by BACE1 is the rate-limiting step in APP proteolysis and also BACE1 knock-out mice lacking Aβ production survives with normal physiology [15]. Recently, there has been a tremendous growth in research interest to design and synthesize new BACE1 inhibitors that include small molecules, peptides to peptidomimetics [16]. Hydroxyethylamine [17] derivatives as well as pyridinium [18], aminoimidazoles [19], aminohydantoin [20] derivatives have been explored as BACE1 inhibitors in several recent QSAR studies. Notably, most of the identified BACE1 inhibitors are of synthetic origin. Studies on BACE1 inhibitors from natural sources are of very recent interest. Chitosan derivatives from crab shell show weak β-secretase inhibitory [21] activity, while catechins from green tea [22], ellagic acid from pomegranate [23] and hispidin from mycelial cultures of *Phellinus linteus* [24] have shown moderate β-secretase inhibitory activity. Recently, Lee et al., have identified a 12 residue BACE1 inhibitory peptide from skate skin hydrolysate with IC_{50} value of 24.26 μM [25]. Phytochemicals have been of particular interest for many years due to their natural availability and wide range of functional diversity. Polyphenols have been demonstrated to inhibit Aβ aggregation [26,27]. Particularly, wine-related polyphenols [27] and several curcumin derivatives [28] have been found to dose-dependently inhibit Aβ fibril formation. In addition, polyphenols are extremely strong antioxidants and have been demonstrated to inhibit reactive oxygen species (ROS) mediated cell death [29–31]. Epidemiological studies have suggested that intake of polyphenols prevent to some extent the decline of cognitive functions with aging and also the development or the course of neurodegenerative diseases [32]. Additionally they possess significant blood–brain barrier penetration ability [33] which enhances the possibility of CNS drugs based on phenolics/polyphenolics structural scaffolds.

Present study focuses to develop a multi-tier screening strategy to identify novel phytochemicals that can act as multi-potent agent and simultaneously block many pathogenic pathways in AD. We have devised a multi-target screening protocol combining ensemble docking to predict BACE1 inhibitory potency as well as developed 2-D QSAR models for the anti-amyloidogenic and anti-oxidant activities. An *in house* developed phytochemical library has been screened using the multi-tier screening protocol to identify phytochemicals that show high BACE1 inhibitory potency as well as strong anti-amyloidogenic activity along with mild to moderate antioxidant activity and thus function as a multi-potent inhibitor in AD therapeutics.

2. Experimental section

2.1. Materials

BACE1 and β-secretase activity assay kit were purchased from Sigma–Aldrich and so was 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and Thioflavin-T. Hesperidin was purchased from EXTRASYNTHESE. Aβ (25-35) was purchased from ANASPEC. ANS was a kind gift from Dr. Rajat Banerjee (Department of Biotechnology, University of Calcutta). All solvents used in the study were of analytical grade (E. Merck) and were checked for absence of absorbing impurities. Deionized water from a Milli-Q system apparatus (Millipore Corp., Billerica, MA) was used throughout the experiments.

2.2. Structure based protocol for BACE1 inhibition

Initially, PDB database was systematically searched to identify BACE1 conformations with widely different active site orientation by superimposing the available structures on each other and subsequently two BACE1 crystal structures (PDB IDs: 3IND & 3TPP) were considered for docking study. 3D structures of the small molecules were generated in SYBYL mol2 format. The active site of the receptor was defined by selecting the bound ligand as the reference ligand and choosing a 10 Å cutoff around the reference ligand. FlexX [34] that uses an incremental docking strategy to dock a ligand in the cavity of a receptor was used to dock compounds into the BACE1 active site. All the docked solutions were ranked using a scoring function which estimates the free energy of binding (ΔG) of the protein-ligand complex. Preparation of the receptor, ligand and all the post docking interactions were analyzed using LeadIT (http://www.biosolveit.de/LeadIT/). Docking procedures were validated by re-docking approach. In the re-docking procedure, the respective ligands from both the 3IND and 3TPP BACE1-ligand complexes were docked back into the respective BACE1 structure.

2.3. Designing a multi-target virtual screening strategy

We employed a multi-tier approach to screen multi-potent inhibitors that can inhibit BACE1 as well as exhibit anti-amyloidogenic and anti-oxidant activity. We therefore applied a structure based screening protocol to identify BACE1 inhibitory phytochemicals, a 2-D QSAR model for anti-amyloidogenic activity as well as another 2-D QSAR model for antioxidant activity and thus identified multi-potent phytochemicals. The two-receptor ensemble docking strategy was tested on a dataset of seven BACE1-inhibitor complexes obtained from PDB. The dataset was selected such that the inhibitory potency of the inhibitor varies from nM to μM range. Complexes were chosen such that the bound inhibitors belonged to diverse structural scaffolds with widely different molecular volume. All the seven inhibitors were docked to their corresponding crystal structure of BACE1 and also to the receptor ensemble of BACE1 comprising of 3IND and 3TPP. We have already designed 2-D QSARs for anti-amyloidogenic and anti-oxidant activity of phytochemicals using the CODESSA package. Details of the QSAR modeling methodology for both anti-amyloidogenic and anti-oxidant activity are provided in Chakraborty et al. [35,36].

2.4. Phytochemical database design and virtual screening

A database of 200 phytochemicals was developed *in house*. Three-dimensional structure of each compound was built from the respective 2D structures using HYPERCHEM 8.0 [37]. Each structure was minimized using MM + molecular mechanical force field with Polak–Ribiere conjugate gradient algorithm to RMS gradient of 0.001 kcal/Å mol. 3D structures of all the phytochemicals were generated in SYBYL mol2 format. The database was screened simultaneously by using the molecular docking algorithm implemented in FlexX [34] with the receptor ensemble of BACE1, two separate 2-D QSARs for anti-amyloidogenic and antioxidant activity to mine novel phytochemicals as potential multi-potent lead in AD therapeutics.

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