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Research paper

Design, synthesis of allosteric peptide activator for human SIRT1 and its biological evaluation in cellular model of Alzheimer's disease

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Sirtuin 1 (SIRT1) is one of the member of the mammalian proteins of the Sirtuin family of NAD^+ dependent deacetylases, has recently been shown to attenuate amyloidogenic processing of amyloid protein precursor (APP) in in-vitro cell culture studies and transgenic mouse models of Alzheimer's disease (AD). SIRT1 has been shown to have a protective role against (AD). It has been reported earlier that increasing SIRT1 activity can prevent AD in mice model. Tripeptide as an activator of SIRT1 were screened on the basis of structural information by molecular docking and synthesized by solid phase method. The enhancement of biochemical activity of pure recombinant SIRT1 as well as SIRT1 in serum of AD patients in presence of tripeptide was done by Fluorescent Activity Assay. The activity of SIRT1 by peptide was assessed in IMR-32 cell line by measuring acetylated p53 level. Further the protective effect of SIRT1 activator in cellular model of AD was analyzed by MTT assay. We find CWR tripeptide as a SIRT1 activator by molecular docking, enhanced the activity of SIRT1 protein by lowering the Michaelis constant, Km by allosteric mechanism. The activity of serum SIRT1 of AD was also increases by CWR. It also decreased the acetylation of p53 in IMR32 neuroblastoma cells and protected the cell death caused by Aβ amyloid fragments in cell line model of AD. Thus, it can be concluded that CWR may serve as platform to elucidate further small molecule activator as a therapeutic agent for AD targeting SIRT1.

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1. Introduction

Cognitive decline is a feature of ageing process; ranging from age associated memory impairment and Mild Cognitive Impairment (MCI) to Alzheimer's disease (AD). All these conditions require substantial care and are emerging as a burden on society as well as on healthcare system. As the pathogenesis is yet unclear, treatment is mostly symptomatic and the search for better understanding continues. Sirtuin are a group of proteins whose role in ageing and protection from various age related pathologies are currently under scrutiny [1]. Sirtuin 1 (SIRT1) is one of the members of the mammalian proteins of the Sirtuin family of NAD+-dependent deacetylases. It has recently been shown to attenuate amyloidogenic processing of β-amyloid precursor protein (APP) in cell culture studies in vitro and in transgenic mouse models of AD [2]. In our earlier studies it has been shown that the levels of serum SIRT1 declines in case of AD patients as compared to healthy controls [3]. Therapeutic potential of SIRT1 against AD in mice model have also been reported [4]. SIRT1 constitutes a unique molecular link between aging and neurodegenerative disorders and provides a promising avenue for therapeutic intervention. This has implications in examining a variety of natural and synthetic compounds that selectively modulates SIRT1 and thus offer suitable drug target for the AD. Many small molecule sirtuin-activating compounds (STAC) have been developed, which increases the catalytic deacetylation of Lys382 residue of p53 by SIRT1. A single point mutation of Glu-230 decreases the activity of the enzyme [5]. The crystal structure of SIRT1 complex with resveratrol (Res) explain the allosteric activation of SIRT1 which showed Glu-230 is critical residue for the catalytic activation. Res, the SIRT1 activator, mediate

Abbreviations: MCI, (Mild Cognitive Impairment); AD, (Alzheimer's disease); SIRT1, Sirtuin1; SPR, surface Plasmon resonance; RU, resonance unit; PVDF, Polyvinylidene fluoride; NTD, N domain; AFU, (arbitrary fluorescence units); Aβ, (Amyloid beta).

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the interaction between the p53-AMC peptide and N terminal domain (NTD) of SIRT1 thus elevated the SIRT1 activity [6]. However, mechanism of action of small molecule as activator is still elusive. In the present study, novel peptide molecule as therapeutic agent against AD by activating SIRT1 based on the available structural information of SIRT1 complex with STAC were designed and synthesized [6]. Peptides were chosen as drug candidate as they had gained interest during recent years for being potent, specific, and having safe mode of action. We have analyzed the effect of designed peptide on activity of recombinant SIRT1 protein as well as in IMR32 neuroblastoma cell line treated with amyloid fragment $A\beta_{25-35}$ peptide induced cytotoxicity associated amyloid plaque of AD [7]. $A\beta_{25-35}$ peptide is the crucial fragment of Aβ amyloid peptide containing neurotoxicity [8–10] and IMR32 neuroblastoma cell line is the well established cell line for AD research [11].

2. Materials and methods

2.1. Molecular docking of tripeptide that binds to the allosteric site of SIRT1

A tripeptide library with all possible combinations of amino acids was generated using python script in molecular visualisation tool PYMOL [12]. The resultant library was further subjected to Ligprep wizard of Glide module in *Schrödinger* molecular docking software [13] to generate maximum of 32 tautomers for each tri peptide within the pH range of 7.0 ± 2.0.

Crystal structure of SIRT1 in open state (PDB id: 5BTR) was downloaded from PDB database (www.rcsb.org) [6]. Using prime module of Glide, missing residues were built and then the final structure was submitted to protein preparation wizard of Glide for assignment of bond orders and addition of hydrogen atoms. Finally using OPLS_2005 force field as default settings the structure was minimized using impref minimization. To define the allosteric active site residues which involved Leu-220, Ile-223, Ile-227, Asn-226, Glu-230, Pro-447 and Leu-450 were selected. A grid of 20 Å was prepared around the active site residues by grid preparation wizard of Glide. *In silico* screening and docking experiments were conducted keeping grid as rigid and all the tri-peptides ligands flexible. Screening was performed using High Throughput Virtual Screening (HTVS) and the top 25% tri-peptide ligands were subjected to Standard Precision (SP) docking mode. The top 500 ligands were then submitted for the Extra Precision (XP) mode. Finally top ten tri-peptide ligands were selected for further analysis of their predicted affinity. To cross validate docking results post-docking analysis was done using X-score v1.2.1 which computes -log of dissociation constant and binding energy of ligands with the target protein. Ligplot [14] was used for determination of hydrophobic interactions and hydrogen bond formation between the protein and ligand complexes.

2.2. Screening of the synthesized peptides as activators of SIRT1 by fluorescent activity assay and determination of $EC_{1.5}$ value

The peptides were synthesized by solid phase peptide synthesizer PS3 (Protein technology, USA) using Fmoc and Wang resin chemistry [15]. For the synthesis of CWR peptide, Fmoc of Fmoc-Arg-Wang resin was deprotected by 20% piperidine and Fmoc-Trp-OH was activated with uronium salt 2-(1Hbenzotrizole-1yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) in the presence of base N-methylmorpholine (NMM) and coupled for 1 h to obtained Fmoc-Trp-Arg-Wang resin. The above procedure was repeated and coupled with Fmoc-Cys-OH to get the final sequence Fmoc-Cys-Trp-Arg-Wang resin. Finally, Fmoc was removed and the resin was cleaved from the peptide with trifluoroacetic acid. The peptide was purified by reverse phase chromatography on C18 Pep-RPC column (1.6 × 10 cm, Amersham Bioscience) and was characterized by MS/MS (Applied Biosystems, Foster City CA, USA) (Fig S1)

To perform the activity assay, SIRT1 was expressed and purified using bacterial system as mentioned earlier [16]. The top 10 peptide selected by molecular docking as SIRT1 activators were biochemically screened using fluorescence based deacetylase assay [17] to find out whether synthesized peptides have the ability to enhance the activity of SIRT1. Each well consisted of 35 ng of SIRT1 Enzyme, 500 μM of NAD^+ (Enzo Life Sciences), 200 μM of SIRT1 peptide substrate (Enzo Life Sciences) and SIRT1 assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA) along with the test compound i.e. peptides at a concentration of 100 μM. The plate was incubated at 37 °C for 30 min and the reaction was stopped using 1×Fluor de Lys developer solution (Enzo Life Sciences) containing 2 mM nicotinamide. The plate was further incubated at 37 °C for another 30 min and the samples were read by a fluorimeter with an excitation wavelength of 360 nm and emission wavelength of 460 nm. All assays were performed in triplicates and results represent the mean of these triplicates. The maximum percentage activation achieved with all peptides was also determined.

Potency of the best peptide molecule as an activator was tracked by determining the concentration which was sufficient to increase the enzyme activity by 50% ($EC_{1.5}$) using fluorescent activity assay. To calculate the $EC_{1.5}$, a graph was plotted between AFU (arbitrary fluorescence units)/min and different concentrations (0 μM, 40 μM, 80 μM, 160 μM and 200 μM) of the peptide.

2.3. Mechanism of activation of SIRT1 by peptide

The effect of screened peptide on the V_{max} (Maximal velocity infinite concentration of substrate) and the K_m (Michaelis constant) of SIRT1 for its substrate was determined to identify the mechanism through which the screened peptide activate SIRT1 protein. K_m and V_{max} values were determined by performing the assay with different concentrations of SIRT1 Fluor de Lys substrate (100 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM, 900 μM and 1000 μM). In a similar way, same set of reaction were performed in the presence of different concentrations of the screened peptide (0 μM, 40 μM, 80 μM, 160 μM and 200 μM).

2.4. Assessment of SIRT1 activity in serum of AD patients by CWR

The effect of CWR on SIRT1 in serum of AD patients was determined by the above mentioned method. Endogenous SIRT1 (1 μl) was treated with different concentrations of CWR (1.5 mM, 2 mM, 3 mM) for 1 h, then incubated with SIRT1 substrate peptide (Enzo Life Sciences) for 1 h at 37 °C. The activity of SIRT1 was determined by fluorescence spectroscope (Synergy2, BioTek Instruments, VT USA) measuring the formation of deacetylated peptide substrate after adding the developer solution. SIRT1 inhibitor; nicotinamide (5 mM) was used as negative control.

To rule out the activity of CWR on SIRT2, the activity assay of serum was also done by using SIRT2 substrate.

2.5. Assessment of SIRT1 activity by CWR in IMR-32 cell line by measuring acetylated p53

IMR-32 cells were purchased from National Centre for Cell Science (Pune, India). The cells were routinely grown in DMEM supplemented with 10% Fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h of seeding, cells were given treatment as follows: 1) vehicle control group, which was cultured with the addition of equal volume of DMSO (less than

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