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Development of peptide inhibitor as a therapeutic agent against head and neck squamous cell carcinoma (HNSCC) targeting p38α MAP kinase

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ABSTRACT

Background: The p38α MAP kinase pathway is involved in inflammation, cell differentiation, growth, apoptosis and production of pro-inflammatory cytokines TNF-α and IL-1β. The overproduction of these cytokines plays an important role in cancer. The aim of this work was to design a peptide inhibitor on the basis of structural information of the active site of p38α.
Methods: A tetrapeptide, VWCS as p38α inhibitor was designed on the basis of structural information of the ATP binding site by molecular modeling. The inhibition study of peptide with p38α was performed by ELISA, binding study by Surface Plasmon Resonance and anti-proliferative assays by MTT and flow cytometry.
Results: The percentage inhibition of designed VWCS against pure p38α protein and serum of HNSCC patients was 70.30 and 71.5%, respectively. The biochemical assay demonstrated the K_D and IC_{50} of the selective peptide as 7.22×10^{-9} M and 20.08 nM, respectively. The VWCS as inhibitor significantly reduced viability of oral cancer KB cell line with an IC_{50} value of 10 μM and induced apoptosis by activating Caspase 3 and 7.
Conclusions: VWCS efficiently interacted at the ATP binding pocket of p38α with high potency and can be used as a potent inhibitor in case of HNSCC.
General significance: VWCS can act as an anticancer agent as it potentially inhibits the cell growth and induces apoptosis in oral cancer cell-line in a dose as well as time dependent manner. Hence, p38α MAP kinase inhibitor can be a potential therapeutic agent for human oral cancer.

1. Introduction

In India cancer registries have confirmed an incidence of head and neck squamous cell carcinoma (HNSCC). The case control and cohort studies have established that the high incidence is due to widespread habits of tobacco chewing and smoking [1]. Tobacco is a major determinant in the etiology of a number of cancers. Despite advances in surgery, chemotherapy and radiation, the five year survival rate for HNSCC has not improved significantly over the past several decades and remains about 50 to 55% [2]. Prevention and early detection is important in the management of these cases. Unfortunately, there has been little improvement in the early detection of HNSCC as most patients present for diagnosis and treatment in stage III or stage IV of the disease. This work aims to develop peptide inhibitor for HNSCC targeting p38α as a therapeutic agent. The p38α MAP kinase pathway is involved in inflammation, cell differentiation, growth, apoptosis and production of pro-inflammatory cytokines-TNF-α and IL-1β. The overproduction of these cytokines plays an important role in supporting the pro-inflammatory microenvironment of the tumor. There are four isoforms of p38MAPK: α, β, γ and δ. All mitogen-activated protein kinases (MAPK) pathways operate through sequential phosphorylation events phosphorylating transcription factors and regulate gene expression. These MAP kinases are activated by dual phosphorylation of Thr and Tyr residues in "TXY", respectively (where X is Gly in case of p38α) and further activate transcription factors, by phosphorylation using ATP as a substrate. They can also phosphorylate cytosolic targets to regulate intracellular events. MAPKs are phosphorylated and activated by MAPK kinases (MKKs), which in turn are phosphorylated and activated by MKK kinases (Raf and MKKK) [3]. The final goal of these cascades is the regulation of

Abbreviations: MAPK, mitogen activated protein kinase; HNSCC, head and neck squamous cell carcinoma; MKK, MAPK kinase; MKKK, MKK kinase; LB, Luria-Bertani; IPTG, isopropyl-ß-D-galactosidase; DLS, dynamic light scattering; ATF, activated transcription factor; NPP, 4-nitrophenyl phosphate; HTVS, high throughput virtual screening; SP, standard precision; XP, extra precision; DMF, dimethyl formamide; HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TFA, trifluoro acetic acid; RPC, reverse phase column; PBS, phosphate buffer saline; MHC, minimal hemolytic concentration; PI, propidium iodide; HRP, horse radish peroxidase; SPR, surface plasmon resonance; RU, resonance unit

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cellular proliferation, differentiation, development, regulation of cell cycle, induction of G2/M checkpoint due to double stranded breaks in DNA during V(D)J recombination in B cells [4], and transmission of oncogenic signals through gene transcription. Significantly high levels of p38α MAPK have been associated with non-small cell lung cancer [5] and HNSCC [6].

p38α can be one of the biomarker for the detection of HNSCC in early stage [6]. This has implications in examining a variety of natural and synthetic compounds that selectively inhibit this enzyme and thus provides suitable drug target for the control of carcinogenic events in oral mucosa. The development of p38α MAP kinase inhibitors is considered the main therapeutic strategy for preventing the production of pro-inflammatory cytokines involved in inflammatory and cancer diseases. Nowadays the pharmaceutical companies tend to focus on the discovery and development of peptide as a drug. The peptides are advantageous as they are easy to synthesize, less immunogenic due to small size, easily absorbed in the body and they mimic the peptide–protein interaction providing the powerful means of signal regulation and potential drug with therapeutic implications. This study develops peptide inhibitor against p38α on the basis of structural information of the active site which showed an anticancer activity by using oral cancer cell-line.

2. Materials and methods

2.1. Expression and purification of p38α

Human p38α cDNA in pET14b expression vector was transformed in bacterial *Eschericha coli* BL21 (DE3) competent cells (Novagen). The cells were grown in Luria-Bertani (LB) broth at 37 °C containing 100 μg/ml ampicillin and the expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactosidase (IPTG). After 16 h growth, the cell pellet was resuspended in 10 ml of lysis buffer (25 mM Tris pH 7.9, 300 mM NaCl, 0.8 mM PMSF and 10 mM imidazole). The cell lysate was centrifuged at 12,000 *g* for 20 min and was loaded onto the Ni^{2+}-NTA-Agarose column (1 ml, QIAGEN) pre-equilibrated with lysis buffer. It was washed with the lysis buffer and the protein was eluted with the same buffer containing 100 mM imidazole.

The p38α was further purified by anion exchange chromatography on DEAE-Sephadex A50 column at 4 °C pre-equiliberated with buffer (25 mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 5% glycerol and 1 mM DTT) and was eluted with a concentration gradient of 0.05–0.2 M NaCl. The protein elutes at 0.1 M NaCl.

2.2. Characterization of p38α

The p38α (10.9 mg/ml) was assessed by SDS-PAGE using Laemmli buffer system [7]. Protein spots were excised from the gel and subjected to ingel reduction, alkylation and tryptic digestion [8]. The MS/MS (Bruker Daltonics, USA) was used to determine the mass and sequence of the protein. Each of the peptide obtained was used to BLAST search the protein and identified by Mascot search program (matrixscience) [9]. The hydrodynamic radius of p38α (10 mg/mL) was determined by dynamic light scattering assay (DLS) using N-octyl-β-D-glucopyranoside and NaCl as additives in a RiNA Spectroscatter 201. The data were analyzed using PMgr v3.01p17 software.

2.3. In vitro p38α kinase assay using ELISA

The activity of p38α was measured by the Forrer et al. method [10] using ATF-2 as a substrate. Microtiter plates were coated with 10 μl ATF-2 (bioPLUS Fine Research Chemicals) solution (10 μg/ml) at 4 °C. The plates were washed and purified p38α (12 μg) protein in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 100 μg/ml BSA, 1 mM DTT, 0.1 mM Na_3VO_4, 100 μM ATP) was added to the wells and incubated for 1 h at 37 °C. The kinase mixture without the p38α protein was used as a blank. The plates were incubated with anti-phospho ATF-2 antibody (1:400) (Biovision) for 1 h at 37 °C which interacts with the phosphorylated form of ATF-2 and subsequently incubated with alkaline phosphates conjugated goat anti-rabbit IgG (1:4000) (Chemicon) for 1 h at 37 °C. Finally, the chromogenic substrate solution 4-nitrophenyl phosphate (4-NPP) in 0.1 M Tris–HCl, pH 8.1, 0.01%$MgCl_2$) (Cayman Chemical Company, USA) was added for 1.5 h at 37 °C. The reaction was stopped by adding 100 μl of 3 N NaOH. The formation of nitrophenolate was measured at 405 nm using ELISA reader (Quanta Biotech, UK).

2.4. Design of peptide inhibitor using computational studies

The structure of complex of p38α protein (PDB id 1A9U) with known inhibitor SB203580 was obtained from the PDB database (www.rcsb.org). It was prepared using GLIDE v 9.1 [11] docking tool involving removal of heteroatoms including SB203580, water molecules, and addition of hydrogen atoms. Finally, the protein structure was minimized using impref minimization with default settings of OPLS_2005 molecular mechanics force field.

Using a python script and build module of PYMOL peptide libraries of 1.6 lakhs possible combinations of tetrapeptides were generated and prepared using Ligprep wizard of GLIDE tool generating a maximum of 32 tautomers per peptide within a pH range of 7.0 ± 2.0. Similarly, the known inhibitor SB203580 was prepared using Ligprep wizard.

A grid of 17A° size was generated with GLIDE grid preparation wizard using the amino acid residues of active site of p38α viz. Val(30), Ser(32), Gly(33), Arg(34), Tyr(35), Val(38), Arg(51), Val(52), Lys(53), Glu(71), Leu(74), Leu(75), Ile(84), Leu(86), Leu(104), Val(105), Thr(106), His(107), Leu(108), Met(109), Asp(112), Leu(167), Asp(168), Phe(169) and Leu(171). The docking experiments were performed keeping this receptor grid rigid. The entire ligand library was screened against p38α using three docking modes of GLIDE. High throughput virtual screening (HTVS) and standard precision (SP) docking modes were initially used and the resultant 16000 structures exhibiting highest GLIDE score were further processed under extra precision (XP) mode. Finally, five tetrapeptides with lowest energy conformations were considered.

Post docking analysis for these top five hits was performed using the Getneares program. It calculates the protein atoms that are involved in making interactions with the ligand atoms within a 5 Å radius. Finally, the best docked molecules for MAP kinase p38α were visually analyzed by the visualizing tool PYMOL [12].

2.5. Synthesis of peptides

The peptides were synthesized by solid phase peptide synthesis in PS3 synthesizer (Protein Technology, USA) using Fmoc and Wang resin chemistry [13]. The solvent used for the synthesis was dimethylformamide (DMF). 2-(1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-methylmorpholine (NMM) were used as activators of the Fmoc amino acids (Chem Impex, USA). Fmoc was deprotected by 20% piperidine and Wang resin was cleaved by trifluoroacetic acid (TFA). The peptides were precipitated from dry ether.

2.6. Analytical RP-HPLC of peptides

The purity of peptides was verified by analytical RP-HPLC, C18 reversed phase column (RPC) (1.6 × 10 cm, Amersham Bioscience). The 1 mg/ml of peptide was loaded to the RPC. The linear gradients were formed by passing two different solvents, where solvent A was 0.05% aqueous TFA, pH 2 and solvent B was 0.05% TFA in acetonitrile. The flow rate was 0.25 ml/min at room temperature.