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Identification of novel FAK and S6K1 dual inhibitors from natural compounds via ADMET screening and molecular docking

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ABSTRACT

Focal adhesion kinase (FAK) and human p70 ribosomal S6 kinase (S6K1) are non-receptor protein tyrosine plays a vital role in cell signaling pathways, such as cell proliferation, survival, and migration. In this study, the 3D structure of FAK (PDB ID: 2AL6) and S6K1 (3A60) were chosen for docking 60 natural compounds attempted to identify novel and specific inhibitors from them. The 30 selected molecules with high scores were further analyzed using DSSTox tools and DS 3.5 ADMET software. Based on a high docking score and energy interaction, 3 of the 9 candidate compounds, neferine B, neferine A, and antroquinonol D, were identified and the inhibitory activity of these compounds were subsequently validated in the C6 glioma cell line. All three selected compounds show potential effects on cell viability by MTT assay. Neferine B, neferine A, and antroquinonol D showed an IC_{50} value of 10-, 12-, and 16-μM, respectively. Moreover, these compounds decreased the p-FAk and p-S6k1 proteins in a dose-dependent manner. The results of best docked neferine B, neferine A, and antroquinonol D have the potential for further development as a supplement to treat tumorigenesis and metastasis.

1. Introduction

Focal adhesion kinase (FAK), a 125 KD non-receptor protein tyrosine kinase, regulates cell proliferation, survival, and migration in response to extracellular signals [1,2]. FAK is activated by integrin binding to the extra cellular matrix (ECM) followed by the autophosphorylation of FAK at Tyr-397, which is critical for most cellular functions [3]. The phosphorylation of FAK is important in promoting FAK-dependent signal transduction because its phosphorylation state can regulate the ability of these signaling molecules to interact with Src and phosphatidylinositol 3-kinase, and/or it regulates the catalytic activity of FAK that influences the ability of these molecules to become phosphorylated [4]. The amino-terminal domain of FAK, which contains a region sharing some sequence similarity to FERM domains, may be a clue in regulating the activity and phosphorylation state of FAK in cells [5]. Large truncations of the amino terminus of FAK are hyperphosphorylated, which suggests that amino terminus exert an inhibitory influence on the catalytic domain of FAK [6,7]. Overexpressed FAK has been found in many tumors including those of the head and neck, colon, breast, prostate, liver, brain, and thyroid. Additionally, this overexpression is highly correlated with an invasive phenotype in these tumors [8–10]. The inhibition of FAK signaling by the overexpression of dominant-negative fragments of FAK reduced the invasion of glioblastomas and ovarian cancer cells [11]. Furthermore, FAK is also implicated in cancer cell invasion, metastasis, angiogenesis, and survival [12]. Hence, the exploration of FAK inhibitor provides a new insight into the anti-tumor ability.

The 70-kDa Ribosomal S6 kinase 1 (S6K1) is Ser/Thr protein kinase and belongs to a member of the AGC (protein kinases A, G and C) kinase family [13]. S6K1 is an important downstream effector of the PIK3/mTOR signaling pathway in the nucleus and cytoplasm, which mediates a variety of cellular process, including mRNA process, protein synthesis, cell proliferation, and survival [14]. S6K1 has been shown to control the proliferative aspect of cell division. In addition, it has been reported to drive the G1/S cell cycle progression. S6K1 is activated by the phosphorylation of three important multiple sites. Thr252 is phosphorylated by PDK1 [15] while Thr412 is phosphorylated by mTORC1 [16]. Moreover, Ser394 must also be phosphorylated for the full activation of S6K1. Significantly, the upstream regulators of the mTORC1/S6K1 pathway, such as PIK3CA, PTEN, AKT, PDK1, and TSC1/2, are frequently mutated in cancer and leads to inappropriate hyperactivation of S6K1 [17]. S6K1 is involved in a number of various disease-related aberrations, including diabetes, obesity, and cancer [18]. Recently, S6K1 was found to be over-expressed in brain

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tumors and breast cancer [19–21]. When the *S6K1* gene knockout mouse uncovered a specific role for S6K1 in the regulation of cell growth [21]. Therefore, an investigation of S6K1 inhibitor may clinically afford an alternative cancer therapy.

A molecular docking approach can model the interaction between molecules (ligand) and proteins, which allows for the characterization of the behavior of molecules at the binding site of target proteins [22]. Docking studies include many conformations to predict the binding of free energies (scoring functions) and to search (scoring sampling) for the most representative binding conformations. A recent docking study has shown various properties such as binding energy, electron distribution, geometry complementarity, hydrogen bond donor acceptor properties, hydrophobicity, and polarizability [23]. Computational prediction of pharmacokinetic parameters such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies have become increasingly important in the process of drug selection and also serve as a promising tool for early screening of potential drug candidates [24]. The regulations of FAK and S6K1 could control tumorigenesis and carcinogenesis. For the abovementioned reasons, this study first attempts to discover a new FAK and S6K1 inhibitor using high throughput virtual screening [25]. The 3D structures of FAK (PDB 2AL6) and S6K1 (PDB 3A60) were taken as the targets and virtually screened the library database for tightly binding compounds using the Discovery Studio (DS) molecular modeling tool. The DSSTox and ADMET of tested compounds with higher docking scores were predicted and further evaluated. Next, the compounds with high scores and had good ADMET prediction were selected to measure their effects on the cell growth of C6. Additionally, cisplatin, a chemotherapeutic drug, was employed as a positive control to test the anti-cancer efficacy of the selected compounds.

2. Methods and materials

2.1. Chemicals and antibodies

The antibodies against FAK, p-FAK, S6K1, and p-S6K1 were purchased from Cell Signaling (Danvers, MA, USA). The antibody for β-actin was obtained from Sigma (USA), the anti-mouse and anti-rabbit IgG Horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (UK), and the enhanced chemiluminescence (ECL) Western blotting detection kit were obtained from Perkin Elmer Inc (USA). The structure of neferine B, neferine A, and antroquinonol D [26] was determined by ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).

2.2. Protein structure for docking

The 3D structure PDB 3A60 of human p70 ribosomal S6 kinase-1 (S6K1) [27] and PDB 2AL6 of focal adhesion kinase (FAK) [28] were chosen as protein targets. Proteins 3A60 and 2AL6 were downloaded from the Protein Data Bank (PDB; http://www.rcsb.org/pdb). Selected S6K1 and FAK were fixed with the force field CHARMn (Chemistry at Harvard Macromolecular mechanics) equipped in DS 3.5 (http://accelrys.com/products/discovery-studio) to add up the hydrogen atoms, partial charges, and missing residues so that they can be used properly for the molecular docking processes.

2.3. Docking and score function

S6K1 and FAK downloaded from the PDB database and the potential binding sites were predicted by DS 3.5. A total of 60 compounds available in our laboratory were docked to the binding site of S6K1 and FAK with LigandFit software (DS 3.5), which provides a shape-based method for accurately docking ligands into protein active sites. The docked poses were then analyzed using potential of DOCK scores [29].

2.4. ADMET prediction

The ADMET properties of drug candidates have been considered critical parameters for drug development [30]. Therefore, the ADMETs of the virtually selected compounds were estimated using the web server DSSTox (http://www.epa.gov/ncct/dsstox/) and DS 3.5 ADMET software. These predictions were based on certain animal and cell models with their results serving as good indicators before performing experimental measurements.

2.5. Cell culture

Rat glioma C6 cell lines were used for the experiments. As per our previously described method [31], C6 cell lines were cultured in DMEM that was supplemented with 15% horse serum, 2.5% FBS, and 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO_2. Prior to the experiment, the cells were seeded and cultured for 16–24 h confluence. Human bronchial epithelial cells (BEAS-2B) cells were cultured in DMEM supplemented with 10% FBS and 1%.

2.6. MTT assay

The potential cellular toxicity of the selected compounds on C6 cells was assessed with the MTT method. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells. C6 (1×104 cells per well) and BEAS-2B (5×103 cells per well) were cultured in a 96-well culture plate for 24 h at 37 °C in atmosphere of 5% CO_2. The cultures were treated with different concentrations of the selected compounds for 24 h. The supernatants were removed and MTT (5 mg/ml) was added and incubated for an additional 4 h. The purple formazan crystals that developed from tetrazolium (MTT) within the cells by the action of mitochondrial succinate dehydrogenase were extracted into DMSO. The OD_{570} was measured using an EnSpire Multimode ELISA Plate Reader (PerkinElmer, USA).

2.7. Western blot

After the treatments, the cells were collected and washed twice in cold Krebs-Ringers Henseleit buffer (KRH; 50 mM HEPES, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$) and lysed in ice-cold RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM NaF, 1% NP40, 1 mM sodium orthovanadate, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), Roche protease inhibitors, and phosphatase inhibitors (DE-68305, Mannheim, Germany)) incubated at 4 °C for 1 h. After centrifugation at 12,000*g* at 4 °C for 30 min, the supernatant was kept and quantified for protein content by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). 30 μg of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF (PerkinElmer Life Sciences, Boston, MA, USA) membranes. The blots were blocked with 5% non-fat milk in TBS/T (20 mM Tris-Base, 137 mM NaCl at pH 7.4, and 0.1% Tween-20) at room temperature (RT) for 1 h and then incubated with the appropriate primary antibody at 4 °C overnight. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (General Electric, Little Chalfont, Buckinghamshire, UK) at RT for 1 h. After washing, the bands of blots were developed by Western Lightning™ *Plus*-ECL (PerkinElmer Life Sciences) and the signals were