International Journal of Biological Macromolecules 96 (2017) 200–213

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Identification of natural allosteric inhibitor for Akt1 protein through computational approaches and *in vitro* evaluation

T. Pragna Lakshmi [a], Amit Kumar [a], Veena Vijaykumar [b], Sakthivel Natarajan [b], Ramadas Krishna [a,*]

[a] Centre for Bioinformatics, Pondicherry University, Puducherry 605014, India
[b] Department of Biotechnology, Pondicherry University, Puducherry 605014, India

ARTICLE INFO

Article history:
Received 24 August 2016
Received in revised form 2 December 2016
Accepted 4 December 2016
Available online 9 December 2016

Keywords:
Breast cancer cell line
Cloning
Protein expression and purification
Isothermal titration calorimetry

ABSTRACT

Akt, a serine/threonine protein kinase, is often hyper activated in breast and prostate cancers, but with poor prognosis. Allosteric inhibitors regulate aberrant kinase activity by stabilizing the protein in inactive conformation. Several natural compounds have been reported as inhibitors for kinases. In this study, to identify potential natural allosteric inhibitor for Akt1, we generated a seven-point pharmacophore model and screened it through natural compound library. Quercetin-7-*O*-β-D-glucopyranoside or Q7G was found to be the best among selected molecules based on its hydrogen bond occupancy with key allosteric residues, persistent polar contacts and salt bridges that stabilize Akt1 in inactive conformation and minimum binding free energy during molecular dynamics simulation. Q7G induced dose-dependent inhibition of breast cancer cells (MDA MB-231) and arrested them in G1 and sub-G phase. This was associated with down-regulation of anti-apoptotic protein Bcl-2, up-regulation of cleaved caspase-3 and PARP. Expression of p-Akt (Ser473) was also down-regulated which might be due to Akt1 inhibition in inactive conformation. We further confirmed the Akt1 and Q7G interaction which was observed to have a dissociation constant (K_d) of 0.246 μM. With these computational, biological and thermodynamic studies, we suggest Q7G as a lead molecule and propose for its further optimization.

1. Introduction

Akt or PKB is a key effector of PI3K/Akt/mTOR pathway that plays pivotal role in cellular metabolism, growth, proliferation, differentiation and survival [1]. Currently, there are three known isoforms of Akt: Akt1, 2 and 3 or PKB-α, β and γ [2,3]. Activation of Akt is dependent on stimulation of receptor tyrosine kinase (RTK) or G-protein coupled receptor (GPCR) which triggers phosphatidylinositol 3-kinase (PI3K) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) in the cell membrane. This drives the recruitment of Akt and other pleckstrin homology (PH) domain containing proteins such as phosphoinositide-dependent kinase-1 (PDK1) to the inner leaflet of plasma membrane, where Akt comes in close proximity to PDK1 and gets phosphorylated at Thr308 of catalytic domain. Akt is fully activated when it is phosphorylated at Ser473 either by mammalian target of rapamycin complex2 (mTORC2) or by DNA-dependent protein kinase (DNA-PK). Activated Akt stimulates several downstream substrates which finally lead to inhibition of apoptosis, promotion of cellular growth, survival, glucose metabolism and angiogenesis [4–9]. In various cancers such as breast and prostate, Akt is constantly active or over-expressed due to its own mutations or aberrant activity of upstream proteins of the pathway [10]. Inhibition of Akt could reverse its anti-apoptotic processes and thereby regulate cellular growth, proliferation and survival. Besides, hyper activation of Akt often increases resistance to chemotherapy or radiotherapy. Akt inhibitors have been shown to attenuate this chemotherapeutic resistance when they were administrated along with standard chemotherapy [11–13].

Akt structure comprises of an amino (N)-terminal regulatory PH domain, 42-residue length connecting linker, catalytic

Abbreviations: PKB, protein kinase B; CPH, common pharmacophore hypothesis; RMSE, root mean-square error; PLS, partial least-squares; QSAR, quantitative structure-activity relationship; TCM, traditional Chinese medicine; HTVS, high-throughput virtual screening; SP, standard precision; XP, extra precision; DFT, density functional theory; HOMO, higher occupied frontier orbitals; LUMO, lower unoccupied frontier orbitals; MDS, molecular dynamic simulations.

* Corresponding author.
E-mail addresses: pragnapcu@gmail.com (T. Pragna Lakshmi), amit.kumar10nov@gmail.com (A. Kumar), btveenavijaykumar@gmail.com (V. Vijaykumar), puns2005@gmail.com (S. Natarajan), krishstrucbio@gmail.com (R. Krishna).

http://dx.doi.org/10.1016/j.ijbiomac.2016.12.025
0141-8130/

kinase domain and carboxy (C)-terminal hydrophobic motif. It anchors to plasma membrane *via* pH domain interaction with PI3K products, phosphatidylinositol (4,5)-trisphosphate (PIP_2) and PIP3. Akt exists in both cytoplasmic "PH-in" or inactive and membrane associated "PH-out" or active conformation. In inactive conformation, the PH domain occupies part of the space required for positioning of αC-helix and activation loop to attain active conformation [14]. A large number of inhibitors were developed against PH and catalytic kinase domain of Akt [15,16]. However, due to high degree of similarity of these domains with other serine/threonine kinases, the allosteric pocket of Akt has become an attractive site for drug development. An orally active allosteric inhibitor *i.e.*, MK-2206 (8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2H-[1,2,4]triazolo[3,4-f][1,6]naphthyridin -3(2H)-one, dihydrochloride) has been reported with an IC_{50} of 5, 12 and 65 nM for Akt1/2/3, respectively, [17–23]. However, crystal structure of Akt1 is available with Inhibitor VIII, a compound similar to MK-2206, which depicts the binding of inhibitor in "PH-in" conformation. Inhibitor VIII binds to Akt1 through several hydrophobic contacts and few polar interactions. Its tricyclic imidazo quinoxaline ring forms ring-stacking interaction with Trp80 and H-bond with Ser205 while the other end of tricyclic core interacts with variable loop3 (VL3) residues Glu267, Lys268, and Asn269 [24,13]. The allosterically inhibited Akt1 cannot bind to PIP2 or PIP3 due to blockage by C-lobe of kinase domain and rearrangement of IP4 binding site residues especially, Asn53 and Arg86 which move away from IP4 binding site by about 10 Å and 8 Å, respectively. Pyridopyrimidine biphenyl core analogues for Akt1/2 allosteric site were observed to be more potent and cell-based active inhibitors. Hence in the current study, we utilized the knowledge of these analogues to identify natural allosteric inhibitor.

Virtual screening is a computational alternative for conventional high-throughput screening (HTS). Pharmacophore based virtual screening harnesses the knowledge of key molecular properties such as hydrogen-bond acceptor, donor and aromatic system from active set of ligands in the form of "common pharmacophore" and utilizes it to prioritize library compounds [25]. In the current study, we implemented this screening approach to select compounds from traditional Chinese medicine (TCM) library (http://tcm.cmu.edu.tw/) that could inhibit Akt1. During virtual screening process, the protein structure remains static; however, in nature, the protein exists in different minimum free energy conformations among which, the ligand interacts with only certain conformations. Hence, MDS was performed to evaluate the conformational orientation of protein-ligand complex and to calculate their binding free energy [26]. Integration of *in silico* approaches with *in vitro* studies would help us in understanding the efficiency of compound. Thus, inhibitory potential of the best compound from *in silico* studies was evaluated on MDA MB-231 cancer cell line and its binding affinity with purified human Akt1 was estimated through isothermal titration calorimetry (ITC) experiment.

2. Methodology

2.1. Data set

Pharmacophore modeling and 3D-QSAR (quantitative structure-activity relationship) generation were performed by PHarmacophore Alignment and Scoring Engine (PHASE) module of Schrödinger suite, Maestro [27,28]. Pyridopyrimidine biphenyl core analogues of MK-2206 targeting the allosteric site of Akt1 were considered for generating common pharmacophore [29]. The IC_{50} value of each compound in micromolar range was converted to pIC_{50} (-logIC_{50}). During compound preparation, stereoisomers were generated in all combinations and ionized at a target pH of 7.0. Number of conformations was produced by ConfGen method to which pre and post minimizations were performed for maximum of 100 and 50 iterations respectively, using OPLS2005 force field with a distance-dependent electric solvation treatment [30,31]. Redundancy of conformers was eliminated with a cut-off RMSD of 1.0 Å.

2.2. Common pharmacophore hypothesis generation

The data set was divided into active, moderately active and inactive with threshold value of 7.770 for active and 7.215 for inactive compounds. Among these analogues, PHASE initially searched for common chemical features such as H-bond Acceptor (A), H-bond Donor (D), Hydrophobic group (H), Negatively charged group (N), Positively charged group (P) and aRomatic ring (R). Among the active set compounds, seven variant common pharmacophore hypotheses were generated using tree-based partitioning algorithm, which grouped the similar pharmacophores with a minimum inter site distance of 2.0 Å, maximum tree depth of 5, initial box size (roughly the binding pocket size) of 32.0 Å and final box size (tolerance on matching pharmacophore) of 1.0 Å. Each hypothesis was then scored to identify the one that yields best alignment with active set of compounds. Quality of alignment was assessed with alignment score, vector score and volume score based on which, the survival score of each hypothesis was calculated, using the following formulae:

$$S = W_{site}S_{site} + W_{vec}S_{vec} + W_{vol}S_{vol} + W_{sel}S_{sel} + W^{m}{}_{rew} - W_{E}\Delta E + W_{act}A \quad (1)$$

Where '*S*' and '*W*' represent score and weights respectively. The user definable weights, W_{site}, W_{vec}, W_{vol} and W_{rew} have a default value of 1.0 whereas W_{sel} has a default value of 0.0. $W^{m}{}_{rew}$ is for reward weights, while '*m*' is the number of actives that match hypothesis minus one, $W_E\Delta E$ represents relative energy, W_{act} is for reference ligand activity and *A* is the activity. In this way, S_{site} represents an alignment score, S_{vec} represents vector score, S_{vol} represents volume score and S_{sel} represents selectivity score.

Once the scoring actives are calculated, each ligand was assigned with a fitness score that represents the fitness match of each feature in hypothesis with conformer and is defined by:

$$S = W_{site}(1 - S_{align}/C_{align}) + W_{vec}S_{vec} + W_{vol}S_{vol} \quad (2)$$

where, W_{site}, W_{vec} and W_{vol} represent weights for site, vector and volume score; S_{align}, S_{vec} and S_{vol} represent alignment score, vector score and volume score, respectively whereas C_{align} is for alignment cut-off. The ligand which perfectly matches with hypothesis possessed a fitness score of 3.

2.3. 3D QSAR development

3D QSAR analysis was performed to establish the correlation between biological activity (dependent variable) and structural properties of molecules (independent variable) through chemometric linear method *i.e.*, partial least-squares (PLS). The generated common pharmacophore hypotheses were considered to build atom-based 3D QSAR model by correlating the actual and predicted activity for a set of 16 training molecules using PLS regression analysis. In PHASE, a maximum of N/5 PLS factors (N = number of ligands in training set) with a grid spacing of 1.0 Å is used. An optimal of three PLS factors were used in order to avoid the overfitting of data. All models were validated by predicting the activity of test set molecules.