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In silico and *in vitro* screening of small molecule Inhibitors against SYT-SSX1 fusion protein in synovial sarcoma

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ABSTRACT

Synovial sarcoma (SS) is characterized by a tumour specific chromosomal translocation t(X;18) (p11;q11) which results in the formation of SYT-SSX1 fusion protein. This fusion protein represents a clear therapeutic target and molecules specifically targeting SYT-SSX1 fusion protein are currently not available. In this study, SYT-SSX1 fusion protein sequence was retrieved from Uniprot and 3D structure was generated using I-TASSER modeling program. A structure based computational screening approach has been employed using Glide docking software to identify potential SYT-SSX1 small molecule inhibitors that bind to the junction region of the fusion protein. The obtained inhibitors were further filtered based on the docking score and ADME/T properties. Ten best fit compounds were chosen for *in vitro* studies. The anti-proliferative activities of these 10 compounds were screened in Yamato, ASKA (carries SYT-SSX1 fusion protein) and other sarcoma cell lines such as A673, 143B to understand the specificity of inhibition of the chosen compounds. The *in vitro* activity was compared against HEK293 cell lines. The compound 5-fluoro-3-(1-phenyl-1H-tetraazol-5-yl)-1H-indole (FPTI) was found to be selectively cytotoxic in synovial sarcoma cell lines (Yamato and ASKA) and this compound also showed insignificant anti proliferative activity on other cell lines. Further, target gene expression study confirmed that FPTI treatment down-regulated SYT-SSX1 and modulated its downstream target genes. Cell cycle analysis revealed the involvement of an apoptotic mechanism of cell death. Further experimental validations may elucidate the therapeutic potentials of FPTI against SYT-SSX1 fusion protein.

1. Introduction

Synovial sarcoma (SS) is anaggressive mesenchymal cancer which constitutes 5–10% of all soft tissue sarcomas occurring in children and young adults (Steppan et al., 2017). Despite rapid advancements in treatment modalities, 50% of SS patients eventually show poor recurrence-free survival in the first five years after diagnosis (Lewis et al., 2000; Ferrari et al., 2004). Around 90% of the SS patients exhibit a specific translocation between chromosome X and chromosome 18 t(X; 18) (p11.2; q11.2) that results in the formation of SYT-SSX1, SSX2 and SSX4 fusion transcripts of which SYT-SSX1 being the most common (Clark et al., 1994; Crew et al., 1995; Thway,2009). Prognosis of patients presenting with metastatic disease remains poor, with a median time from 10 to 22 months (Palmerini et al., 2014). Once metastatic, SS invade both locally at the primary site and at distant sites *i.e.*, lungs. Clinical trial enrollment is considered as a standard of care of treatment for those patients who present with a metastatic disease. A Phase II and phase III study from the European Organization for Research and Treatment of Cancer-Soft tissue and Bone Sarcoma group (EORTC) has showed longer progression free survival upon treatment with pazopanib *versus* placebo with a sample size of 37 and 369 patients, respectively (Vlenterie et al., 2015). A phase I study of genetically engineered NY–ESO–1 specific (c259) T cells in HLA–A2+ SS patients was established with the purpose of testing the effects of chemotherapy and the NYESO T cells on patients with metastatic and recurrent SS (Eilber and Dry, 2008; Jungbluth et al., 2001). Several potential enzymatic targets have been identified including, arginine succinate synthetase 1 and histone deacetylases, which are currently in clinical trials (Park et al., 2008; Su et al., 2010; Bolden et al., 2006). The lack of successful treatment regimen for SS highlights the requirement for more effective, less toxic and novel therapies. Though very few studies empirically evaluated the role of SYT-SSX1transcripts as a prognostic marker (Kubo et al., 2015; Mezzelani et al., 2001), several research groups explored the functional relevance of SYT-SSX1 as an oncoprotein in disease pathogenesis and as a molecular therapeutic target against SS (Yang et al., 2002; Xie et al., 2002b; Baird et al., 2005; Pelmus et al., 2002).The

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exact functional mechanism of action of SYT-SSX1 protein in tumorigenesis is unknown. One of the mechanism suggested, SYT-SSX may directly recruit PRC2 and HDAC to ATF2 targets, silencing transcription at those loci (Su et al., 2012; Garcia et al., 2012; Cironi et al., 2009; de Bruijn et al., 2006; Lubieniecka et al., 2008).

Several *in vitro* and *in vivo* studies have demonstrated the requirement of SYT-SSX fusion protein to support tumorigenesis. These studies have also targeted numerous downstream signaling pathways known to play a role towards SS development. SYT-SSX1 was found to be capable of possessing tumour transforming activity along with the involvement of chromatin remodeling factor hBRM/hSNF2 alpha A (Nagai et al., 2001) and cyclinD1 (Xie et al., 2002a). A report suggested the fusion protein to displace wild type SYT and 1NI1 from SWI/SNF complex driving Sox2 mediated proliferation and dedifferentiation (Kadoch and Crabtree, 2013).It is also hypothesized that SYT-SSX could disrupt SWI/SNF complex inhibition of the polycomb complex 2 (PRC2) methyltransferase Enhancer of Zeste Homologue 2 (EZH2), which is a potent histone methyltransferase that targets histone 3 lysine 27 (H3K27), inhibiting proliferation and migration of SS *in vitro*. Another mechanism proposed; SYT-SSX to directly recruit PRC2 to aberrantly silence target genes (Shen et al., 2016). Shen *et al* also showed that knockdown of EZH2 by siRNA and inhibition with a small molecule resulted in suppression of cell growth and migration in SS cell lines. (Shen et al., 2016). By SYT-SSX1 silencing experiments, (Carmody Soni et al., 2014) in 2014 has demonstrated that, SYT-SSX1 is important for cell survival which upon inhibition induces apoptosis and influences cell viability, exhibiting features characteristic of a tumour specific therapeutic target. In a recent study, (Vlenterie et al., 2016) palbociclib effectively inhibited SS cell lines *via* inhibition of cyclinD1-CDK4/6-Rb phosphorylation axis, resulting in G1 arrest. SYT-SSX1 fusion protein promoted tumorigenesis with activation of Wnt/β-catenin signaling. With subsequent administration of Tcf/β-catenin complex inhibitors, tumor growth was significantly reduced, suggesting Wnt/β-catenin signaling pathway to be a potential molecular target (Hartmann, 2014). Another study proposed PI3K-AKT pathway as a molecular target by demonstrating the inhibition of PI3K-AKT pathway with pazopanib that exhibited direct anti-tumour activity in SS cell xenografts (Hosaka et al., 2012).HDAC inhibitors have been shown to reverse SYT-SSX1-mediated polycomb silencing of the tumour suppressor early growth response induced SS tumour growth(Lubieniecka et al., 2008). FOXM1 (Maekawa et al., 2016), ALK,c-MET signaling (Fleuren et al., 2017; Imura et al., 2016; Yamada et al., 2017) and SRC signaling (Michels et al., 2013) were identified as promising SS therapeutic targets.

Although, SYT-SSX1 represents a clear therapeutic target, to our knowledge no studies were available to demonstrate direct inhibition of SS using small molecule inhibitors *via* binding to the SYT-SSX1 fusion protein. In the studies highlighted above, efforts have been taken to target only the downstream and associated pathways involved in the processes of SS tumorigenesis. Indeed, it has been known that several chimeric fusion proteins resulting from chromosomal translocations are necessary for the viability and propagation of human tumors (EWS-FLI, BCR-ABL, EML4-ALK) (Ludwig, 2008; Goldman and Melo, 2008; Sasaki et al., 2010). Functional targeting of these tumour specific targets with small molecules would be ideal and could be considered to be most effective.(Erkizan et al., 2009; Quintás-Cardama and Cortes, 2009; Soda et al., 2007). In this study, a computational approach combined with *in vitro* studies was employed to identify potential small molecules against SYT-SSX1 oncoprotein.

2. Materials and methods

2.1. Protein structure prediction

Physiochemical properties of SYT-SSX1 protein (UniProtKB - A4PIV8) was computed using Expasy ProtParam server (https://web.expasy.org/protparam/) (Gasteiger et al., 2005). Secondary structure prediction of fusion protein was computed using GOR IV(https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page = npsa_gor4.html) (Garnier et al., 1996).The tertiary structure of chimeric protein SYT-SSX1 was predicted using I-TASSER online tool (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) which generates 3D models along with confidence score (C score) (Zhang, 2008).

2.2. Protein preparation

The protein structures were imported into protein preparation wizard of maestro Schrodinger suite 2014-2.The processing, optimization and minimization of fusion protein was carried out. Missing hydrogen atoms were added to the modelled protein to satisfy the valency and bond orders were assigned during the optimization steps. Then the structure was energy minimized to RMSD 0.3A° by applying OPLS-2005 force field (Madhavi Sastry et al., 2013).The structure was validated using Rampage (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) which showed the various residues falling in favored, allowed and in disallowed regions.

2.3. Active site identification

The active sites of the SYT-SSX1 fusion protein were identified using SiteMap tool of Schrodinger 2014-2 and the binding pockets were chosen based on site score as well as the junction region of the fusion protein.

2.4. Receptor grid generation

The position and size of active site in fusion protein was represented by receptor grids. The grids contain the shape and molecular properties of binding site. A grid box size of 20A° was generated using the centroid of selected residues which were identified by Sitemap tool and docking studies were performed (Friesner et al., 2004).

2.5. Ligand preparation

The sdf format of ligands were downloaded from EDULISS (Edinburg University Ligand Selection System) http://eduliss.bch.ed.ac.uk/test/. LigPrep generates accurate, energy minimized 3D molecular structures. It produce a number of structures from each input structure with various ionization states, tautomer, sterochemisteries, ring conformations and applies filters to eliminate compounds and generate ligand library. The resulting structures were saved in maestro format for further docking studies (Chen and Foloppe, 2010).

2.6. Docking

The fusion protein SYT-SSX1 was used as target protein for docking calculations. We employed the Glide script from Schrodinger. LLC. The docking algorithm in Glide performs a complete systematic search of the conformational and positional space of the docked ligand and eliminating unwanted conformations using Glide scoring and after energy optimization. The ligands were docked with active sites using High throughput Virtual Screening (HTVS). First QikProp program predicted pharmacokinetic properties of ligands and pre filtered the ligands based on Lipinski's rule. Ligands which obey the predicted ADME properties were docked with the fusion protein SYT-SSX1 using HTVS, Standard precision (SP) and by Xtra precision mode. Scoring of docked ligands was done based upon the energy minimized poses. Glide Score is based on ChemScore, but includes a steric-clash term and adds buried polar terms devised by Schrödinger to penalize electrostatic mismatches.

2.7. Cell lines

The two synovial sarcoma cell lines Yamato and ASKA were