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Identification of Potential Lead Molecule against Sphingosine kinase-1: An *in vitro* Study

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Abstract

Sphingosine kinase 1 (sphk1) and lipid mediator sphingosine-1 phosphate plays a key role in the survival and proliferation of breast cancer cells which makes it an attractive target in the field of drug discovery and therapeutic strategies. Previously it has been identified by *in silico* analysis that lead compound 1, viz. ZINC6823429 and compound 2, viz. ZINC95421070 extracted from ZINC database were potent and novel inhibitor of sphk1. This research is based on our *in vitro* study by targeting sphk1 in MCF-7 breast cancer cell line. The enzyme inhibition assay was performed to check the S1P activity in cell lysate. The results reveal the successful inhibition of sphk1 by compound 1 at 1 µM while compound 2 at 20 µM. In the proposed work, we elucidated and validated inhibitory potential of newly deciphered lead compounds 1 and 2 using enzyme inhibition and MTT assay which comfirm the anticancer potential of compound 1. These findings might be useful to hone the computer-aided drug design against breast carcinoma, albeit required more wet-lab based validation.

Keywords: Sphingosine kinase-1, *in silico*, Breast cancer, PF-543, MTT assay.

INTRODUCTION

Breast carcinoma is the most commonly diagnosed aggressive malignancy and second leading cause of cancer death among females after lung cancer globally (Sukocheva, 2018). It is more likely to occur as women get older but it can also occur in younger women too (Campbell *et al*., 2015). It is highly curable, when detected early (Leong *et al*., 2007) but majority of patients are diagnosed at an advanced stage. The awareness of the symptoms, risk factors and the need for screening are the important ways of reducing the risk and chance of successful treatment of breast cancer resulting in improvement in survival rate and quality of life (Gupta *et al*., 2015). Today the major healthcare burden of breast cancer is due to prolong exposure to risk factors. Obesity, certain inherited genes including BRCA1 and BRCA2 mutation, family history, race and ethnicity, not having children, not breast feeding, drinking alcohol, starting menstruation early, going through menopause after age 55, having radiation to your chest, night shift work, exposure to diethylstilbestrol (DES) drug during pregnancy to lower the chances of miscarriage are the major risk factors which have been previously suggested (Becker., 2015). Different forms of treatments are involved for breast cancer patients such as surgery, chemotherapy, radiation therapy, and hormone therapy (Goel *et al*., 2009) but as a consequences, breast cancers are resistant to current therapies due to lack of targeted therapies. Therefore, there is an urgent need to identify more targeted therapies for breast cancer treatment.

From the last 20 years it has been well documented that sphingosine 1 phosphate, (a bioactive compound of sphingolipid) that regulates cell growth, survival, migration, angiogenesis, inflammation and cancer (Shida *et al*., 2008, Hait *et al*., 2017) which has been produced by the phosphorylation of sphingosine catalyzed by lipid kinase sphk1 & sphk2 (Nakajima *et al*., 2017, Wallington *et al*., 2013, Ogretmen., 2018). Sphk1 is highly expressed in various carcinomas especially in breast carcinoma (Spiegel *et al*., 2005). The S1P mediated signalling pathway (Geffken *et al*., 2017) towards the progression of breast cancer is depicted in Figure 1.

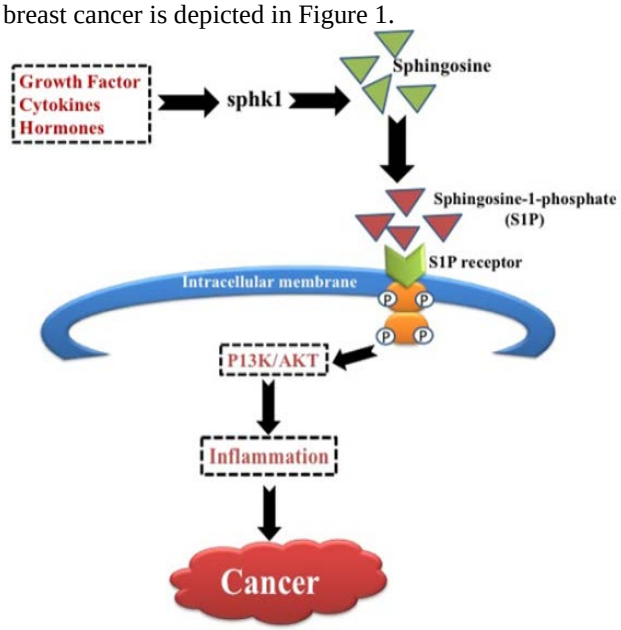

Figure 1: An overview of S1P mediated signalling pathway towards the initiation of cancer.

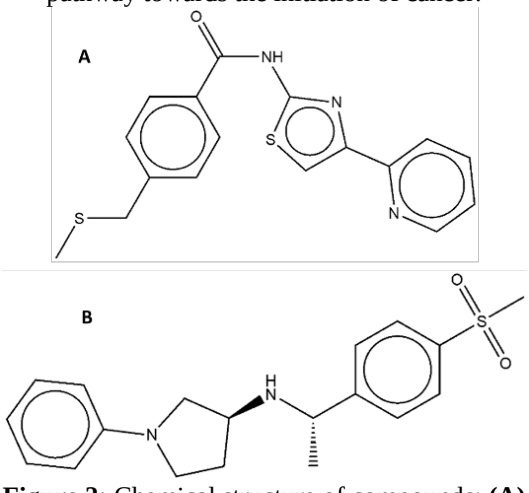

Figure 2: Chemical structure of compounds: **(A)** ZINC6823429, **(B)** ZINC95421070.

In our previous work, the potential hits (Khan *et al*., 2019) (Figure 2) selected *via in silico* study (Arif et al., 2018; Khan *et al*., 2018) including ligand-based virtual screening (Rehman *et al*., 2016; Rehman *et al*., 2019) strategy, molecular docking (Khan *et al*., 2013; Arif *et al*., 2013; Sharma *et al*., 2019) and molecular dynamics simulation (Khan *et al*., 2017). This work is based on our *in vitro* work which has been carried out by targeting sphk1 in MCF7 breast cancer cell line. The two leads have been validated in this section by using enzyme inhibition assay and antiproliferative assay. In particular, enzyme inhibition assay demonstrated that compound 1 exhibited potent inhibitory potential, as well as the antiproliferative assay, suggested that compound 1 exhibited potent anticancer activities against breast cancer cell line MCF-7.

MATERIALS AND METHODS

Chemicals and reagents

The chemical compounds i.e., compound 1, compound 2 and standard PF-543 (Lynch., 2012) were purchased from Sigma-Aldrich (St. Louis, USA). S1P ELISA kit (CD creative diagnostic, USA), MCF-7 breast cancer cell line (NCCS PUNE), phosphate-buffered saline (PBS), antibiotic antimycotic solution, trypsin and MTT dye (Thiazolyl Blue Tetrazolium Bromide) were purchased from HiMedia Laboratories (Mumbai, India). EMEM media; fetal bovine serum (FBS; Gibco, MA); 0.2µm syringe filters (Sartorius AG, Gottingen, German); DMSO (Sigma-Aldrich); Isopropanol (Sigma-Aldrich).

Cell culture

The MCF-7 breast cancer cells were maintained in EMEM media, supplemented with 10% FBS, 1% antibiotic, containing 10 000 U of penicillin, 10 mg of streptomycin, and 25 µg/mL of amphotericin band 0.9% normal saline, in a humidified atmosphere in an incubator at 37°C with 5% CO2.

Enzyme inhibition assay

S1P activity was evaluated using an S1P ELISA kit in MCF-7 cell lysate. It is an enzyme linked immunoassay designed for *in vitro* measurement of S1P in human or animal biological fluids. The S1P ELISA is a competitive ELISA in which the colorimetric signal is inversely proportional to the amount of S1P present in the sample. The samples are premixed with the anti- S1P antibody while the microtiter plate is blocked. Once the block step is complete, the sample/Anti-SIP antibody mixtures are added to the blocked microtiter plate for competitive binding. Streptavidin-HRP and calorimetric detection is used to detect the Anti-S1P bound to the microtiter plate. The concentration of S1P in the sample is determined using a standard curve of known amounts of S1P. This assay should be read at 450 nm and requires 3.5 hours to run.

Procedure for drug treatment

All the molecules were dissolved in DMSO. Different concentrations of drugs were treated into 90 mm culture (MCF-7 cell line) dish and allowed to incubate for 4 hrs at 37°C (5% CO_2 incubator). After incubation, cells (0.5 X 10^5) were harvested and subjected for homogenization and sonication (2 strokes for 30 seconds) in PI cocktail buffer. ELISA assay was performed according to instruction mentioned in the kit manual (CD creative diagnostic, USA, Cat. No: DEIA-XYZ5).

In vitro cell proliferation assay

Cell proliferation was measured by an MTT assay as described by (Mossman *et al*., 1983). The reaction involves the conversion of tetrazolium salt (3-[4, 5-dimethyl thiozol-2yl]-2yl]-2,5-diphenyl tetrazolium bromide), a pale yellow substrate to formazon which was purple in colour by the enzymes present in the living cells. This was dissolved in isopropanol and absorbance was measured at 550nm or 660nm by micro plate reader. The MCF-7 cells were treated with different concentrations of drug leads (9.4-300 µg/ml) for 24hours. After 24 hours of treatment MTT was added to cell culture at 0.5mg/ml concentration and further incubated for 4hours. At the end of the experiment, supernatant was removed and cell lysate was dissolved in DMSO and read in a micro plate reader (BioTek Instruments Inc, Vermont, USA) at 550 nm and 660 nm.

RESULTS AND DISCUSSION

Enzyme inhibition assay

The inhibitory activities of the two final virtual hits against sphk1 in MCF7 cell lysate were determined by using an ELISA based activity assay. The compound 1 showed specific inhibition of sphk1 and decreases the S1P level at 1µM. Similarly, compound 2 showed a significant decrease of sphk1 and reduce the S1P level at 20µM, while the known inhibitor of sphk1 PF-543 decreases the S1P level at 2.5µM. The results of enzyme inhibition assay demonstrated that compound 1 showed the greatest inhibitory potential as compared to compound 2 and PF-543 (Figure 3).

Anti-proliferative assay against MCF7

The lead compounds 1 and 2 identified *via in silico* strategies from the ZINC databases were tested for their *in vitro* antiproliferative activity against human breast cancer cell line MCF-7 at different concentrations (0-300µg/ml) by MTT assay. The cell inhibition percentages of tested compounds were determined after taking the measurement at 24 hours of exposure. The compound 1 showed 50% cytotoxic effect on MCF-7 cell line at 136.69 µg/ml and compound 2 did not show any cytotoxicity in MCF-7 cell line, whereas PF-543 showed 50% cytotoxic effect on MCF-7 cell line at 179.11 µg/ml. Results of MTT assay suggested that compound 1 was found to be the most potent against MCF-7 cell line (Figure 4) and could be used as a potential anti-cancer agents.

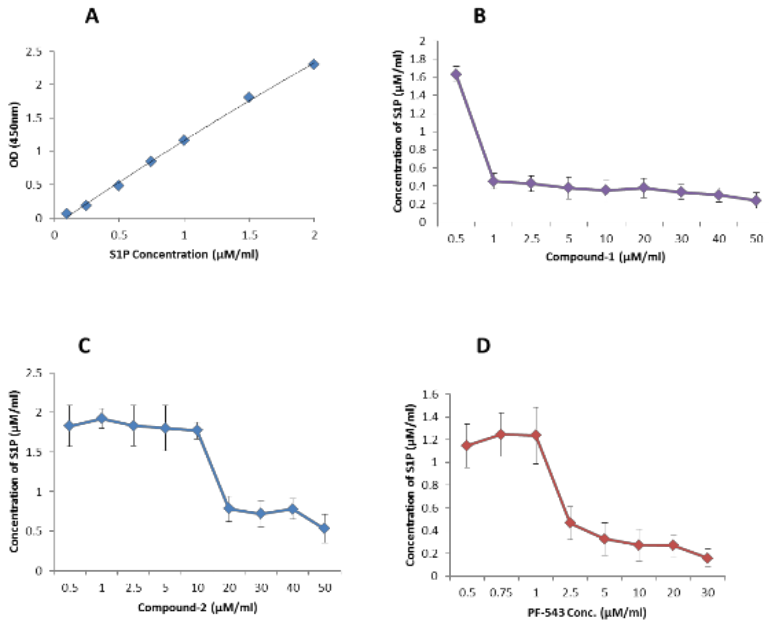

Figure 3: Enzyme inhibition assay: **(A)** Standard Curve for the concentration of S1P**, (B)** Inhibition of S1P by ZINC6823429, **(C)** Inhibition of S1P by ZINC95421070, **(D**) Inhibition of S1P by PF-543.Chemical structure of compounds: (A) ZINC6823429, (B) ZINC95421070.

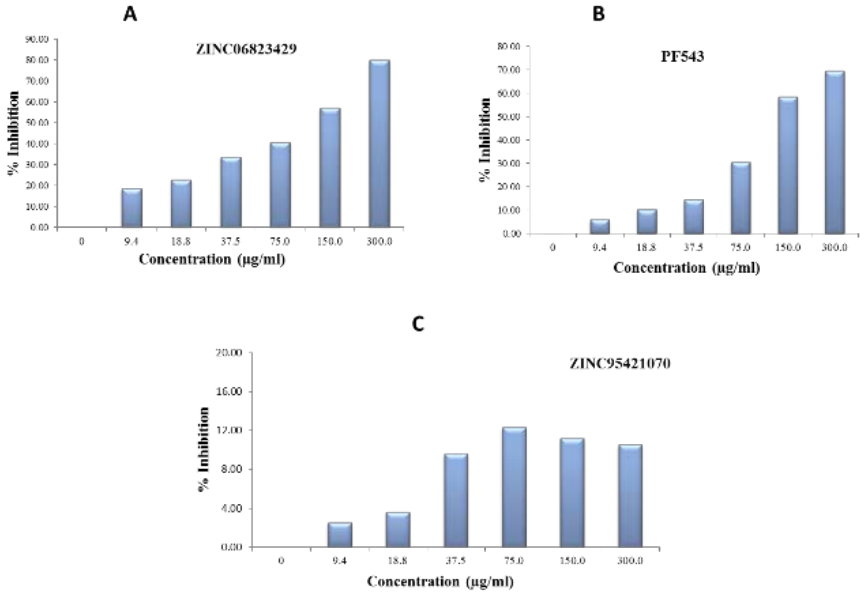

Figure 4: Anti-proliferative activity of: **(A)** ZINC6823429, **(B)** PF-543, and **(C)** ZINC95421070 against MCF-7 breast cancer cell line**.**

CONCLUSION

Our result showed a plausible inhibition of S1P in the cell lysate of MCF-7 breast cancer cell line. The compound 1 showed more potent inhibition against S1P in comparison to compound 2, and standard PF-543. Likewise, compound 1 showed high antiproliferative activity against MCF-7cell line in comparison to PF-543 and compound 2. Moreover, compound 2 did not show any toxicity towards the proliferation of MCF-7 cell line. The compound 1 impairs the proliferation of MCF-7, thereby inhibition of sphK1 observed, while cells remain intact while treating through compound 2. It means compound 1, viz. ZINC6823429 has substantial anticancer potential that could be useful as a pro-drug molecule against breast carcinoma via inhibition of sphk1 activity. Despite preliminary *in vitro* findings, more rigours experimentations are required to check consistency.

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Conflict Of Interest

The authors declare no conflict of interest and disclosures associated with the manuscript

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