RESEARCH ARTICLE

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Cite this: *Med. Chem. Commun.*,
2017, 8, 1914

Received 21st June 2017,
Accepted 24th August 2017

DOI: 10.1039/c7md00305f

rsc.li/medchemcomm

Identification of a potent kinase inhibitor targeting EML4-ALK fusion protein in non-small cell lung cancer

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ALK-fusion proteins play a fundamental role in the development of about 5% of non-small cell lung cancers. Herein, we identified the compound 5067-0952 as a potent ALK inhibitor, which inhibited cell growth, induced apoptosis, and suppressed the phosphorylation of ALK, subsequently blocking its downstream signaling pathway.

Introduction

Receptor tyrosine kinases (RTKs) are vital mediators of extracellular signals that control the key cell survival, growth, and motility pathways.¹ Conversely, abnormal RTK activation through point mutations, chromosomal rearrangements, and gene amplification is responsible for the initiation and progression of many cancers, including non-small cell lung cancer (NSCLC), which is the leading cause of cancer-related mortality in the world.² NSCLC can be classified into distinct molecular subsets based on specific genomic alterations that drive tumorigenesis.³ Anaplastic lymphoma kinase (ALK) oncogenic fusions define a unique molecular subset of NSCLC.⁴ Approximately 3–7% of NSCLCs harbor the ALK chromosomal rearrangement,⁵ most commonly with echinoderm microtubule-associated protein-like 4 (EML4). The constitutive kinase activity associated with ALK fusions seems to play an essential role in cell growth, survival, and motility pathways.⁶ ALK serves as a potent oncogenic “driver”, and cancers with ALK rearrangements are highly sensitive to ALK tyrosine kinase inhibition.⁷ The first generation ATP-competitive ALK/ROS1 (ROS Proto-Oncogene1)/Met (mesenchymal-epithelial transition) inhibitor crizotinib has demonstrated promising clinical benefits in ALK-fusion positive NSCLC and was approved by the Food and Drug Administration (FDA) for the treatment of ALK-fusion positive NSCLC in 2011. Although many patients with ALK-positive NSCLC derive substantial

clinical benefits from crizotinib,^{8,9} unfortunately, as seen with most kinase inhibitors, durable (usually within half a year) responses to crizotinib therapy have been hampered because of acquired resistance.^{10,11} The emergence of drug resistance during crizotinib treatment promoted the discovery of second generation ALK inhibitors.¹² Now alectinib,^{13,14} ceritinib,¹⁵ brigatinib (AP26113),¹⁶ and lorlatinib¹⁷ for NSCLC are used in clinical or preclinical assignment. Resistance to crizotinib in ALK-positive cancers can be overcome by novel inhibitors,¹⁸ so it is urgent to discover and develop new agents targeting ALK.

In this study, to identify effective ALK inhibitors targeting the ALK fusion protein, we performed a molecular docking-based virtual screening from a small molecule database. We then used enzymatic and cell-based assays to evaluate the activity of the selected compounds. The activity of the selected compounds was evaluated by inhibition of the growth of H2228 cells, a NSCLC cell line harboring ALK fusion. Finally, we identified 5067-0952 as a potent inhibitor of ALK, which markedly inhibited the cell viability, reduced the colony formation capacity and induced cell apoptosis in H2228 cells. The antitumor efficacy of 5067-0952 was dose dependent and strongly suppressed the phosphorylation of ALK as well as its downstream signaling molecules ERK1/2, STAT3 and AKT. These data suggested that 5067-0952 might represent a new therapeutic opportunity for patients bearing ALK-dependent tumors as a new ALK inhibitor.

Results and discussion

To discover small molecule compounds that can bind to the active pocket of ALK with potent binding affinity, molecular docking based on virtual screening was employed on the ChemDiv chemical database consisting of about 1.3 million

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compounds. The most promising compound 5067-0952 was selected for further investigation. The structure of compound 5067-0952 is shown in Fig. 1A and the corresponding activities of 5067-0952 are shown in Table 1. In order to gain insight into the binding mode between ALK and 5067-0952, 5067-0952 was docked into the active site of ALK. The docking score of 5067-0952 binding to ALK is -8.70 kcal mol $^{-1}$, indicating a good binding affinity with ALK (see Table 1). The S atom of the thiophene ring moiety of 5067-0952 forms a crucial hydrogen bond with the M1199 residue in the hinge region. The NH group and tertiary amine group of 5067-0952 also form hydrogen bonds with the side chain of residues L1122 and E1210, respectively (see Fig. 1B). Another remarkable feature found in the 5067-0952-ALK complex is the hydrophobic interactions between the side chain of residues A1148, L1196, L1122, V1180, M1199, L1256, G1202, F1207 and E1210. In addition, the polar group of 5067-0952 has polar interaction with the side chain of S1206, L1122 and E1210.

We performed an enzymatic activity assay to evaluate the inhibition activity of 5067-0952 to ALK. The half maximal effective concentration (EC_{50}) of 5067-0952 for the enzymatic inhibition activity of ALK is 19.63 nM (Fig. 2A and Table 1), while the EC_{50} of crizotinib is 0.47 nM.

The antiproliferative effect of 5067-0952 was tested by the MTT assay. Treatment with 5067-0952 significantly decreased the cell viability in a concentration-dependent fashion, with an IC_{50} value of 4.11 ± 0.96 μ M against H2228 cells harboring EML4-ALK fusion (Fig. 2B and Table 1).

A significant reduction in colony formation was observed in the presence of 5067-0952, as compared with untreated cells. Notably, H2228 cells formed no visible colonies when the 5067-0952 concentration reached 10 μ M (Fig. 3A). To investigate whether the induction of apoptosis also contributed to the 5067-0952-mediated growth inhibition of ALK-driven cells, we used Annexin V-FITC/PI flow cytometry to analyze the population of apoptotic cells. Flow cytometry analysis showed that 5067-0952 decreased the cell viability, partly due to the induction of apoptosis in H2228 cells in a concentration-dependent manner. Compared with the control group, treatment of H2228 cells with 5067-0952 significantly induced the level of cell apoptosis (Fig. 3B).

Fig. 1 (A) The structure of 5067-0952. (B) The binding mode of 5067-0952 with ALK at the ATP-binding site. ALK is depicted as the green cartoon. 5067-0952 is depicted as the yellow sticks. Red dotted lines represent hydrogen bonds.

Table 1 The activities of 5067-0952

Compound	IC_{50} (μ M)	EC_{50} (nM)	Docking score
5067-1952	4.11 ± 0.96	19.63	-8.70

To gain insights into the anti-cancer mechanism of 5067-0952, we further examined the downstream pathways of ALK, including MAPK/ERK and PI3K/AKT/mTOR signaling cascades by using western blotting. 5067-0952 and crizotinib could prevent the autophosphorylation of ALK in H2228 NSCLC cells harboring EML4-ALK fusion, which resulted in substantial suppression of the phosphorylation of downstream signaling molecules ERK1/2, STAT3 and AKT in a dose-dependent manner (Fig. 4). Thus, our results indicated that 5067-0952 is a novel ALK inhibitor that can suppress ALK activity and block ALK downstream signaling pathways, resulting in the induction of apoptosis and cancer suppression.

Conclusions

The growing recognition of patients harboring EML4-ALK fusions as a distinct subgroup of NSCLC provides impetus for the development of ALK-directed therapeutic strategies. 5067-0952 was identified as a new inhibitor of ALK in H2228 cells harboring EML4-ALK by *in silico* molecular docking and cell-based screening approaches. 5067-0952 was shown to have a potent binding affinity with ALK and it blocked the growth of the human NSCLC cell line H2228. In addition, 5067-0952 reduced the phosphorylation of ALK and its downstream signaling molecules AKT, ERK and STAT3, leading to significant apoptosis in H2228 cells. 5067-0952 is a highly effective and potent ALK inhibitor and has potential therapeutic benefits for patients with ALK-driven malignancies.

Fig. 2 5067-0952 as a potent ALK inhibitor. (A) Enzymatic assay for an ALK domain was conducted using the LanthaScreen® Eu kinase binding assay. (B) Cell lines were seeded in 96-well plates and treated with various concentrations of crizotinib and 5067-0952 for 72 h. Cell survival was analyzed using the MTT assay.

Fig. 3 5067-0952 inhibits proliferation and induces apoptosis in H2228 cells. (A) Colony formation of H2228 cells after 5067-0952 (2.5, 5, and 10 μM) treatment for 10 days; colony numbers are plotted and photographs of violet-stained colonies are shown. (B) H2228 cells were treated with the indicated drug concentrations (2.5, 5, and 10 μM) for 48 h; the apoptotic cells were measured by the Annexin V/PI staining method. $**P < 0.01$ vs. vehicle; $***P < 0.001$ vs. vehicle.

Experimental

Cell culture and reagents

CCD19-Lu and H2228 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). H2228 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS). CCD19-Lu cells were grown in MEM (Gibco) supplemented with 10% FBS. To all cells were added 100 units mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin under a humidified atmosphere of 5% CO_2 at 37 $^{\circ}\text{C}$. Compound 5067-0952 was purchased from ChemDiv. Crizotinib was purchased from Selleck, and dissolved in DMSO.

Molecular docking calculations

The binding mode of ALK with the compound 5067-0952 was analyzed using a molecular docking method. The 3D structure of the compound 5067-0952 was obtained from the ChemDiv database. Then, 5067-0952 was preprocessed and optimized using the *LigPrep* module with the OPLS-2005 force field. The ionized state was assigned by using *Epik* at a target pH value of 7.0 ± 2.0 . The 3D crystal structure of Anaplastic Lymphoma Kinase (ALK) for molecular docking was retrieved from the Protein Data Bank (PDB ID code 2XP2). *Protein Preparation Wizard* was used to remove crystallographic water molecules, add hydrogen atoms, and assign partial charges. Crizotinib and 5067-0952 were docked into

Fig. 4 Effects of 5067-0952 on the expression and phosphorylation of ALK as well as its downstream signaling pathway in H2228. H2228 cells were treated with 5067-0952 at the indicated concentrations for 48 h, and the levels of p-ALK, ALK, p-STAT3, STAT3, p-AKT, AKT, p-ERK, and ERK were evaluated by western blot analysis of cell lysates using specific antibodies. GAPDH was used as a loading control.

the binding site of ALK using the *Glide* docking program with the standard precision (SP) scoring mode. The docking grid box was defined by centering on the crizotinib in the ALK–crizotinib complex. In molecular docking, 500 poses were generated during the initial phase of the docking calculation, out of which the best 100 poses were chosen for energy minimization by 1000 steps of conjugate gradient minimization.

ALK enzymatic inhibition assay

Enzymatic inhibition assay for the recombinant ALK domain was conducted using the LanthaScreen® Eu Kinase Binding Assay. Briefly, the assay was performed at room temperature for 1 hour in a total volume of 15 μL , including 5 μL of the test compound, 5 μL of the kinase/antibody mixture and 5 μL of the tracer. After 1 hour of incubation, the fluorescence was measured. The emission ratio was calculated *via* dividing the acceptor/tracer emission (665 nm) by the antibody/donor emission (615 nm).

MTT cell viability assay

Cells were seeded at a density of 3×10^3 cells per well in 96-well plates. Following incubation with 5067-0952 of different

concentrations for 72 h, 10 μL of MTT solution (5 mg mL^{-1} in PBS) were added to each well and incubated for 4 h. Then, the reaction was stopped by adding 100 μL of the resolved solution (10% SDS and 0.01 M HCL) followed by incubation overnight. The optical density was measured using a BioRad microplate reader (FluoDIA T70, Photon Technology International, Lawrenceville, NJ) at a wavelength of 570 nm. The cell viability was calculated as the percentage change of the absorbance of treated cells divided by the absorbance of untreated cells. 50% inhibition of cell growth (IC_{50}) value for the compound was determined using the GraphPad Prism 5.0 software.

Colony formation assay

Cells were plated in 6-well plates at 500 cells per well. Twenty-four hours later, 5067-0952 with increasing concentration was added. The growth medium with the drug was replaced every 3 days. The cells were washed with cold PBS, then fixed in 4% paraformaldehyde (PFA) for 15 min, and stained with a 0.5% crystal violet staining solution (1% paraformaldehyde, 0.5% crystal violet, and 20% methanol in ddH₂O) for 15 min. The colonies were photographed.

Annexin V/PI assay

Cells were grown in 6-well plates to ~60% confluency and incubated with 5067-0952 for 48 h. The cells were then stained with 2.5 $\mu\text{g mL}^{-1}$ Annexin V-FITC and 50 $\mu\text{g mL}^{-1}$ propidium iodide in 100 μL of 1 $\times$ Annexin binding buffer for 15 min at room temperature. 400 μL of 1 $\times$ binding buffer was added and flow cytometry was performed (FACSCalibur; BD Biosciences, San Jose, CA, USA). At least 10 000 events were used for the analysis, and data were analyzed with the Flow J software.

Western blotting analysis

The cells were lysed with RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease/phosphatase inhibitor cocktail (Roche), incubated on ice for 10 min, and centrifuged at 14 000 $\times g$ for 10 min at 4 $^{\circ}\text{C}$; equal amounts (20–40 μg) of total proteins for each sample were mixed with a 5 $\times$ NuPAGE LDS loading buffer. The mixture was heated at 100 $^{\circ}\text{C}$ for 5 min and separated using a 10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane (Millipore). The membranes were blocked with 5% skim milk in Tris buffered saline with Tween 20 (TBST) buffer for 1 h at RT, followed by overnight incubation at 4 $^{\circ}\text{C}$ with primary anti-bodies. After washing with TBST 3 times, the membranes were incubated with secondary rabbit or mouse fluorescent antibodies for 1 h in the dark, and then the signal intensity of the membranes was scanned on a LI-COR Odyssey imaging system (Belfast, ME, USA).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5.0. One-way analysis of variance (ANOVA) or student's *t* test was used to assess significant differences between datasets. Values less than 0.05 were considered as significant.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the Macao Science and Technology Development Fund (Project No.: 082/2013/A3, 086/2015/A3, 005/2014/AMJ & 046/2016/A2).

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