RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#) Cite this: *Med. Chem. Commun.*,
2017, 8, 621Received 19th November 2016,
Accepted 9th January 2017

DOI: 10.1039/c6md00643d

rsc.li/medchemcomm

Identification of mitoxantrone as a new inhibitor of ROS1 fusion protein in non-small cell lung cancer cells†

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Mitoxantrone, an FDA-approved drug for multiple sclerosis and hormone refractory prostate cancer, is identified as a potent inhibitor of ROS1 fusion protein by *in silico* screening in non-small cell lung cancer cells. Mitoxantrone can suppress the phosphorylation of ROS1 and subsequently inhibit its downstream signaling pathway and thus induce cell apoptosis.

Introduction

Oncogenic c-ros oncogene 1 (ROS1) receptor tyrosine kinase mutations and rearrangements were discovered in a subset of human lung adenocarcinoma.¹ Approximately 1–2% of non-small cell lung cancer (NSCLC) patients harbor rearrangement of gene encoding ROS1.^{2,3} Crizotinib (Xalkori, PF-02341066) as the first generation inhibitor targeting ALK/ROS1 fusion protein demonstrated promising clinical benefits in ALK/ROS1 positive lung adenocarcinoma and was approved by the FDA.^{4,5} Unfortunately, durable responses to crizotinib therapy are problematic due to the occurrence of drug resistance, which is a common feature of tyrosine kinase inhibitor (TKI) drugs.^{6,7} Thus, it is very critical and urgent to develop novel ROS1 inhibitors for NSCLC therapy.

Drug discovery is a lengthy process, translating a novel lead candidate compound into an approved clinical drug takes often more than a decade and the success rate is very low.⁸ The discovery of new ligands for new targets by screening of FDA approved drugs is attracting increasing attention and research interest because it can save time and cost.^{9,10} Antifungal drug itraconazole (ITZ) is identified to be used as a broad-spectrum intestinal virus inhibitor.¹¹ Recently, tolcapone, an FDA-approved drug for Parkinson's disease, has been identified as a potent inhibitor of transthyretin (TTR) amyloidogenesis.¹² Metformin is an approved anti-type 2 diabetes drug, currently being used in cancer treatment.¹³

In order to identify new opportunities for targeting ROS1 fusion proteins, a strategy of molecular docking-based high-throughput drug screening, from anticancer agents used in clinical trials and clinical assessments was implemented in the current study. We used the molecular docking method to search for the drugs with potential binding to ROS1 kinase from the FDA-approved drug library and evaluated their efficiency with HCC78 non-small cell lung cancer cells harboring SCL34A2-ROS1 fusion protein compared to the normal human fibroblast cell line CCD19-Lu. Using this assay, we demonstrated that mitoxantrone is a potent inhibitor of ROS1 kinase, which was originally thought to be an intercalating DNA agent for prevention of DNA synthesis, inhibition of type II topoisomerase, causing DNA aggregation and compaction, and delaying cell cycle progression, particularly in the late S phase.^{14,15} A recent study also identified mitoxantrone as a nanomolar inhibitor of PIM1 (pim-1 oncogene) kinase and Rho GTPase inhibitor, shedding light on the mechanism of mitoxantrone in clinical application.^{14,16} Here we first report mitoxantrone as a new inhibitor of ROS1 fusion protein in non-small cell lung cancer cells.

Fig. 1 Computational binding mode of mitoxantrone in the ROS1 model. (A) The scaffold of mitoxantrone overlapped well with the location of crizotinib. (B) Mitoxantrone was buried in a hydrophobic pocket formed by different amino acids.

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Results and discussion

Molecular docking calculations were performed to screen the inhibitors of ROS1 from the FDA-approved drug library and study the binding mode of mitoxantrone to ROS1. Mitoxantrone had a similar binding mode to ROS1 as crizotinib. The anthracenedione scaffold of mitoxantrone overlapped well with the core structure of crizotinib (Fig. 1A). All of the residues Leu1951, Glu2027, Met2029, Asp2033, Asp2083 and Lys2090 had hydrogen bond interaction with mitoxantrone. Glu2027 and Met2029 in the hinge region formed multiple hydrogen bonds with the polar atoms of the anthracenedione group, while Leu1951, Asp2033, Asp2083 and Lys2090 had hydrogen bonds with the two substituted “tails”. Other residues also had hydrophobic interaction with mitoxantrone, such as Val1959, Leu2012, Leu2026 and Leu2086.

As a matter of fact, it is essential but insufficient to confirm the specificity of an inhibitor only by calculating the compound binding affinity to an enzyme with *in silico* molecular docking because non-specific binding to non-functional sites of the enzyme could be present.¹⁷ Therefore, in order to have better validation, we performed an *in vitro* enzyme-linked immunosorbent assay (ELISA) to determine the inhibition specificity of mitoxantrone to ROS1. The result showed that mitoxantrone inhibited ROS1 enzyme activity in a dose-dependent manner, and the EC_{50} value was 2998.7 ± 608.8 nM (Fig. 2D), indicating that it possessed the ROS1 kinase inhibiting function, despite the fact that its EC_{50} value was larger than crizotinib ($EC_{50} = 7.1 \pm 2.7$ nM). Subsequently, to evaluate the cytotoxicity of mitoxantrone, the MTT assay was performed in NSCLC cell line HCC78 harboring SCL34A2-ROS1 fusion protein, which demonstrated that mitoxantrone had much higher cytotoxicity to HCC78 cells ($IC_{50} = 13.83 \pm 2.49$ nM) compared to normal lung fibroblast cells CCD19-Lu ($IC_{50} > 160$ nM) (Fig. 2A–C).

Next, to assess the anticancer ability of mitoxantrone, analysis of apoptosis was carried out by flow cytometry. The result exhibited that mitoxantrone induced significant apoptosis on HCC78 cells in a concentration-dependent manner, and it even induced higher levels of apoptosis than crizotinib (Fig. 3).

Fig. 2 (A–C) Evaluation of cell growth by mitoxantrone in HCC78 cells and CCD19-Lu cells, as determined using the MTT assay. (D) Enzymatic assay for the ROS1 kinase domain was conducted using the enzyme-linked immunosorbent assay.

Fig. 3 Effect of mitoxantrone on apoptosis of HCC78 cells. HCC78 cells were treated with the indicated drug concentrations (0, 5, 10, 20 nM) 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.

Fig. 4 Mechanism of action of mitoxantrone in HCC78 cells. Immunoblot analysis of p-ROS1, ROS1, p-STAT3, STAT3, p-AKT, AKT, p-ERK, ERK and GAPDH in HCC78 cells, cells were harvested after treatment with mitoxantrone for 48 h.

To explore the anti-cancer mechanism of mitoxantrone, we further examined the downstream pathways of ROS1, including MEK/ERK and PI3K/AKT/mTOR signaling cascades¹⁸ by using western blots. HCC78 cells were treated with mitoxantrone and crizotinib, leading to phosphorylation suppression of ROS1 as well as its downstream signaling molecules ERK1/2, STAT3 and AKT in a dose-dependent manner (Fig. 4). Thus, our results indicated that mitoxantrone is a novel ROS1 inhibitor that can suppress ROS1 kinase activity and block ROS1 downstream signaling pathways, resulting in induction of apoptosis and cancer suppression.

Conclusions

In summary, mitoxantrone was identified as a novel inhibitor of ROS1 in SCL34A2-ROS1-driven human NSCLC cells by *in*

silico molecular docking and the cell-based high throughput screening approach. Mitoxantrone was shown to have a potent binding affinity to ROS1 and it selectively suppressed the growth of human NSCLC cell line HCC78 and spared the normal lung fibroblast cells. In addition, mitoxantrone reduced ROS1 phosphorylation and inhibited PI3K/AKT and MEK/ERK phosphorylation in the downstream signaling pathways, leading to significant apoptosis in HCC78 cells. In summary, mitoxantrone may serve as a useful therapeutic agent against NSCLC harboring ROS1 rearrangements. Moreover, mitoxantrone as a multi-target drug is critical against drug resistance. Identification of new molecular targets of a known drug is a complement to the development of new uses of inhibitors.¹⁹ In addition, computational prediction of promiscuous new targets of compounds would be a great advance. Several computational approaches have been reported to predict the kinase inhibitor selectivity profile.^{20,21}

Experimental

Cell culture and reagents

HCC78 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 units per ml penicillin, and 100 $\mu\text{g ml}^{-1}$ streptomycin under a humidified atmosphere of 5% CO_2 at 37 °C. Mitoxantrone was purchased from ChemDiv and dissolved in DMSO.

Molecular docking calculation

Molecular docking calculation is performed to study the binding mode of mitoxantrone to ROS1 by using the Induced Fit docking module in Schrodinger software (Schrodinger, Inc., New York, NY, 2015). The structure of mitoxantrone is prepared and optimized in the LigPrep module. The 3D structure of ROS1 is derived from the PDB database (PDB ID: 3ZBF) and prepared using the Protein Preparation Wizard. In the molecular docking calculation, crizotinib is used to define the active site and the pose of the ligand is evaluated with the XP docking score. The pose with the highest score is selected for further analysis.

MTT cytotoxicity assay

3000 cells were seeded at 96-well plates, cultured overnight for cell adhesion, and treated with DMSO or various concentrations of mitoxantrone for 72 h. Each well was added with 10 μl of MTT (5 mg ml^{-1} ; Sigma), which was continued for another 4 hours, then the dark blue crystals were dissolved in 100 μl of the resolved solution (10% SDS and 0.1 mM HCL). Finally, the absorbance at 570 nm was measured by a microplate reader (Tecan, Morrisville, NC, USA). The cell viability was calculated relative to untreated controls, with results based on at least 3 independent experiments.

ELISA kinase assay

The effects of mitoxantrone on the activities of ROS1 tyrosine kinases were determined using enzyme-linked immunosorbent assays (ELISAs) with purified recombinant proteins. Briefly, 20 $\mu\text{g ml}^{-1}$ poly (Glu,Tyr)_{4:1} (Sigma, St Louis, MO, USA) was pre-coated in 96-well plates as a substrate. A 49 μl aliquot of 10 $\mu\text{mol l}^{-1}$ ATP solution diluted in kinase reaction buffer (50 mmol l^{-1} HEPES [pH 7.4],¹⁵ 50 mmol l^{-1} MgCl_2 , 0.5 mmol l^{-1} MnCl_2 , 0.2 mmol l^{-1} Na_3VO_4 , and 1 mmol l^{-1} DTT) was added to each well; 1 μl of various concentrations of the compound diluted in 1% DMSO (v/v) (Sigma, St Louis, MO, USA) was then added to each reaction well. DMSO (1%, v/v) was used as the negative control. The kinase reaction was initiated by the addition of purified ROS1 proteins diluted in 49 μl of kinase reaction buffer. After incubation for 60 min at 37 °C, the plate was washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (T-PBS). The anti-phosphotyrosine (PY99) antibody (100 μl ; 1:500, diluted in 5 mg ml^{-1} BSA T-PBS) was then added. After a 30 min incubation at 37 °C, the plate was washed three times, and 100 μl horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, diluted in 5 mg ml^{-1} BSA T-PBS) was added. The plate was then incubated at 37 °C for 30 min and washed 3 times. A 100 μl aliquot of a solution containing 0.03% H_2O_2 and 2 mg ml^{-1} *o*-phenylenediamine in 0.1 mol l^{-1} citrate buffer (pH 5.5) was added. The reaction was terminated by the addition of 50 μl of 2 mol l^{-1} H_2SO_4 as the color changed, and the plate was analyzed using a multi-well spectrophotometer (SpectraMAX 190, from Molecular Devices, Palo Alto, CA, USA) at 490 nm. The inhibition rate (%) was calculated using the following equation: $[1 - (A_{490}/A_{490\text{control}})] \times 100\%$. The IC_{50} values were calculated from the inhibition curves in two separate experiments.

Apoptosis analysis

HCC78 cells were seeded in a 6-well plate, allowed to attach overnight, and treated with a dilution series of mitoxantrone for 48 h, the cells were collected using trypsin and washed twice with ice-cold PBS, and then resuspended in 100 μl 1 $\times$ binding buffer. 2 μl Annexin-V FITC and 4 μl PI (100 $\mu\text{g ml}^{-1}$) were added to the solution and then mixed well. After incubation for 15 min in the dark at room temperature, 400 μl of 1 $\times$ binding buffer was added to each tube, and apoptosis was analyzed using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, California, USA).

Western blot analysis

After the cells were planted on 6-well plates overnight, the indicated concentrations of mitoxantrone were administered for the times indicated. The cells were washed twice with cold PBS then lysed in RIPA lysis buffer containing protease and phosphatase inhibitors, and the protein concentrations of the cell lysates were measured using the Bio-Rad protein Assay kit (Bio-Rad, Philadelphia, PA, USA). After equalizing the protein concentrations of the samples, 5 $\times$ Laemmli buffer

was added and boiled at 100 °C for 5 min. Protein samples were subjected to SDS-PAGE gel, transferred to a Nitrocellulose (NC) membrane, and blocked with 5% non-fat dried milk in TBS containing 0.1% Tween 20 (0.1% TBST) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with primary antibodies following incubation with secondary fluorescent antibodies, finally, the signal intensity of the membranes was detected by anLI-COR Odyssey scanner (Belfast, ME, USA). Antibodies against GAPDH, p-AKT, p-ROS1, ROS1, p-ERK, ERK, p-STAT3, and STAT3 were purchased from Cell Signaling Technology. The anti-AKT antibody was acquired from Santa Cruz Biotechnology.

Statistical analysis

All of the experiments were performed in triplicate. Statistical analysis was conducted using GraphPad Prim5.0 software. Differences between datasets were assessed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Acknowledgements

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

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