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A novel selective multikinase inhibitor of ROCK and MRCK effectively blocks cancer cell migration and invasion

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ABSTRACT

Two structurally related protein kinase families, the Rho kinases (ROCK) and the myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK) are required for migration and invasion of cancer cells. We hypothesized that simultaneous targeting of these two kinase families might represent a novel therapeutic strategy to block the migration and invasion of metastatic cancers. To this end, we developed DJ4 as a novel small molecule inhibitor of these kinases. DJ4 potently inhibited activities of ROCK and MRCK in an ATP competitive manner. In cellular functional assays, DJ4 treatment significantly blocked stress fiber formation and inhibited migration and invasion of multiple cancer cell lines in a concentration dependent manner. Our results strongly indicate that DJ4 may be further developed as a novel anti-metastatic chemotherapeutic agent for multiple cancers.

Introduction

With the advent of targeted therapeutic strategies, major advancements in the treatment of primary tumors have been achieved. However, treatment of metastatic tumors remains a daunting challenge and successful outcomes are limited. Strategies designed to block primary tumor extravasation and/or secondary site invasion have been developed (i.e. matrix metalloproteinase inhibitors; MMPIs); yet these MMPIs have yielded limited clinical success [1]. Hence, the development of new strategies that specifically target the migratory and invasive properties, which are hallmarks, of metastatic cancer cells could be of enormous therapeutic impact [2].

The importance and regulation of Rho-associated coiled-coil containing protein kinase (ROCK1 and ROCK2; henceforth referred as ROCK) activity in cancer is being discussed extensively [3,4]. Upregulation of ROCK protein/mRNA expression in various tumor types is positively correlated with tumor stage and progression while it is negatively correlated with overall survival and disease prognosis [5–9]. The role of ROCK in cancer cell migration and invasion has been demonstrated by stable overexpression and knockdown studies of ROCK1 and ROCK2 in various cancer cell lines [10–13]. In contrast to ROCK, the expression of myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKα and MRCKβ; henceforth referred as MRCK) in tumor tissues has not been examined.

Individual cancer cells find their way through the extracellular matrix (ECM) by two distinct mechanisms: amoeboid and mesenchymal modes of migration/invasion [14]. Both modes of migration/invasion require contraction of stress fibers mediated by the phosphorylation of myosin light chain (MLC). ROCK and MRCK have been shown to phosphorylate MLC and have been implicated in regulation of cell contractility [15–17]. The amoeboid mode of invasion is primarily dependent on ROCK activity to generate strong contractile forces and is independent of protease activity [18–20], whereas the mesenchymal mode of cell invasion depends on the activity of MRCK to generate contractile forces [21]. Importantly, studies demonstrate that cancer cells often switch between these two mechanisms when an individual mode of migration/invasion is blocked (reviewed in Refs. [14,22]).

The roles of both ROCK and MRCK in cytoskeletal reorganization during cell migration/invasion have been clearly delineated [16,18,23,24]. Indeed, due to their cooperative regulation of cell migration/invasion [21], we believe simultaneous targeting of ROCK and MRCK would be an effective means of inhibiting cancer cell migration/invasion. In support of this, a recent study demonstrated that simultaneous siRNA mediated knockdown of MRCK and ROCK in MDA-MB-231 cells blocked cancer cell invasion more effectively than knockdown of either kinase alone [25]. The strategy of simultaneous inhibition of both ROCK and MRCK for effective inhibition of metastasis is supported widely [3].

Herein, we report the discovery and development of a novel ATP-competitive multikinase inhibitor, (5*Z*)-2–5-(1H-pyrrolo[2,3-*b*]pyridine-3-ylmethylene)-1,3-thiazol-4(5H)-one (DJ4), that

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selectively inhibits the activity of ROCK1 and ROCK2 in addition to MRCKα and MRCKβ. We demonstrate the ability of DJ4 to inhibit the migration and invasion of lung, breast, melanoma and pancreatic cancer cell lines, *in vitro*, and have elucidated the molecular mechanism involved in its inhibition of cancer cell migration/invasion.

Materials and methods

Synthesis of DJ4

DJ4 was synthesized as shown in Fig. 1. Detailed synthesis and structural activity studies of DJ4 will be published elsewhere. Briefly, 2-phenethyl thiourea was reacted with anhydrous sodium acetate and ethyl chloroformate to give 2-phenethylimino-thiazolidin-4-ones (compound 1) in quantitative yield. Compound 1 was reacted with 7-azaindole-3-carboxaldehyde, and catalytic amount of piperidine in absolute ethanol for 12 h at 60 °C to give compound 2 (DJ4) in 64% yield. DJ4 was obtained as a precipitate from the reaction mixture, washed with methanol and diethyl ether. Purity was > 90%. ^{1}H NMR (DMSO-D_6) 8.36–8.33 (m, 2H, aromatic), 7.84 (s, 1H, = CH-NH), 7.68 (s, 1H, CH), 7.35–7.2 (m, 6H, aromatic), 3.75 (t, 2H, CH_2, J = 7.5 Hz), 2.94 (t, 2H, CH_2, J = 7.5 Hz).

Cell lines and cell culture

The following cell lines used in this study were obtained from ATCC: NSCLC (A549, CCL-185; H522, CRL-5810; H23, CRL-5800; H2126, CCL-256; H460, HTB-177), melanoma (A375M, CRL-1619), pancreatic cancer (PANC-1,CRL-1469), breast cancer (MDA-MB-231, HTB-26) and normal human adult fibroblasts (PCS-201-012). The glioblastoma cell line, U251, was kindly provided by Dr. James Connor (Department of Neurosurgery, Penn State Hershey College of Medicine). Cells were maintained in DMEM or RPMI media (Cellgro, Corning) supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin (Gibco) at 37 °C with 5% CO_2.

Western blot analysis

Cells were lysed in 1× lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) containing Mini-EDTA Free protease inhibitor tablets (Roche). The lysates were centrifuged at 20,000×g at 4 °C for 20 min. Total protein was quantified using the bicinchoninic acid (BCA) assay. Equal amounts of total protein were separated on SDS-PAGE gels and expression levels of specific proteins were analyzed by western blot. The following antibodies were employed: pMYPT1 (Thr696, Millipore), MYPT1 (Upstate), pMLC (Ser19, Cell Signaling), ROCK1 (Abcam), ROCK2 (Abcam), β-actin (Cell Signaling), and GAPDH (Cell Signaling).

Protein expression in human lung tumors

To analyze expression of ROCK1/2 and pMYPT1 in lung tumors, tissue samples were obtained from the Penn State Hershey tissue bank with IRB approval. Total protein was isolated and quantified using the Nucleospin RNA/Protein Isolation Kit (Machery Nagel) per manufacturer's instructions. Western blot analysis of ROCK1/2 and pMYPT1 (Thr696) protein expression was performed as stated above. MYPT1 is known to be phosphorylated at Thr853 (myosin-binding regulatory phosphorylation site) [26] by ROCK while at Thr696 (inhibitory phosphorylation site) by both ROCK and MRCK. In this experiment, phosphorylation status of Thr696 was investigated to study total phosphorylation of MYPT1 at inhibitory site.

Kinase activity assays

Cell-free (biochemical) activity assays

Recombinant ROCK1 (9.48 nM) or ROCK2 (8.26 nM; Invitrogen) was incubated in the presence of different concentrations of DJ4 or DMSO in ROCK assay buffer (50 mM Tris pH 7.4, 0.1 mM EGTA, 0.001% β-mercaptoethanol and 10 mM magnesium acetate) at room temperature (RT) for 10 min. MRCKα, MRCKβ, PAK1 and DMPK (2 ng/μL; Invitrogen) assays were performed in assay buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM Na_3VO_4, 5 mM β-glycerophosphate, 2.5 mM DTT and 0.01% Triton X-100. Recombinant MYPT1 (20 ng/μL; Millipore) and ATP (5 μM) were added to initiate the reaction. The reaction was incubated at 30 °C for 20 min. Known ROCK inhibitors Y27632 (Selleck Chemicals LLC) and hydroxyfasudil (Santa Cruz Biotechnology) were used at 1 μM concentration as positive controls. Samples without respective kinases were used as negative controls. Phosphorylation of MYPT1 was determined by western blot analysis using anti-pMYPT1 (Thr696) antibodies. Competitive binding assays for ROCK1 and MRCKβ kinases were performed at 5, 25, 50 μM concentrations of ATP while keeping all other conditions similar.

Activity assays in non-small cell lung cancer (NSCLC) cell lines

A549 cells were treated with different concentrations of DJ4 for 24 h. In an independent experiment, H2126, H23, H460 and H522 cells were treated with 5 μM DJ4 for 24 h. Cell lysates were prepared and protein was quantified per procedure detailed in the 'western blot analysis' section. Equal quantities of total protein were incubated in the presence of ATP (25 μM) with or without recombinant MYPT1 (Millipore) at 30 °C for 25 min. Phosphorylation of MYPT1 was determined by western blot analysis using anti-pMYPT1 (Thr696) antibodies.

DJ4 mediated inhibition of endogenous ROCK/MRCK activity

A549 cells were treated with DMSO or DJ4 for 24 h. Cell lysates were prepared and protein was quantified per procedure detailed in the 'western blot analysis' section. Equal amounts of total protein were separated on SDS-PAGE gels and the levels of pMYPT1 (Thr696, Millipore) and pMLC (Ser19, Cell Signaling) were determined by western blot analysis. To detect phosphorylation of MYPT1 in MDA-MB-231 (breast cancer), cells were treated with the indicated concentration of DJ4 for 24 h and western blot analysis was performed using anti-pMYPT1 (Thr696) antibodies.

Fluorescent microscopy of stress fibers

A549 cells and human adult fibroblasts were plated in DMEM medium containing 10% FBS on glass bottom plates (MatTek Corporation). After treatment with DJ4 or DMSO for 1 h, cells were washed and fixed with 4% paraformaldehyde (alcohol-free) in PBS for 10 min at RT. Fixed cells were washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at RT. Cells were further washed with PBS and stained with 1 μM DAPI (to visualize nucleus; Invitrogen) and 1 U rhodamine-phalloidin (to visualize actin filaments; Invitrogen) for 1 h at RT. Subsequently, cells were washed with PBS three times for 30 min each, and images were captured (600×) using an inverted fluorescent microscope (Nikon Eclipse TE2000-S). Images were merged using Photoshop (Adobe Inc.).

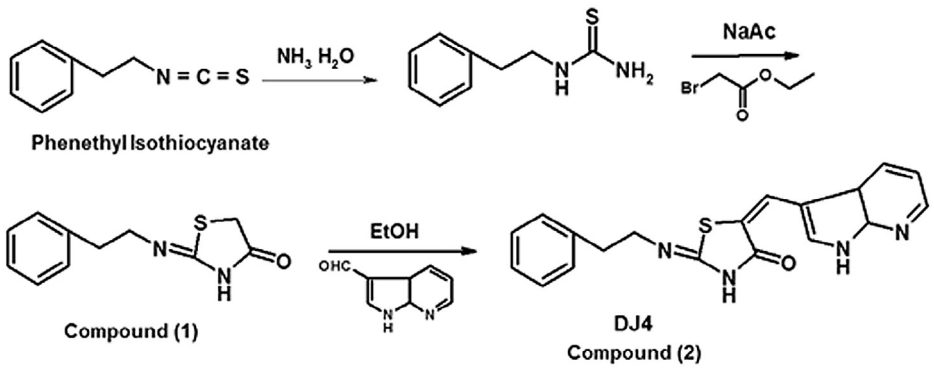

Fig. 1. Chemical synthesis and structure of DJ4.