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LCH-7749944, a novel and potent p21-activated kinase 4 inhibitor, suppresses proliferation and invasion in human gastric cancer cells

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ABSTRACT

P21-activated kinase 4 (PAK4), a serine/threonine protein kinase, has involved in the regulation of cytoskeletal reorganization, cell proliferation, gene transcription, oncogenic transformation and cell invasion. Moreover, PAK4 overexpression, genetic amplification and mutations were detected in a variety of human tumors, which make it potential therapeutic target. In this paper we found that LCH-7749944, a novel and potent PAK4 inhibitor, effectively suppressed the proliferation of human gastric cancer cells through downregulation of PAK4/c-Src/EGFR/cyclin D1 pathway. In addition, LCH-7749944 significantly inhibited the migration and invasion of human gastric cancer cells in conjunction with concomitant blockage of PAK4/LIMK1/cofilin and PAK4/MEK-1/ERK1/2/MMP2 pathways. Interestingly, LCH-7749944 also inhibited the formation of filopodia and induced cell elongation in SGC7901 cells. Importantly, LCH-7749944 caused successful inhibition of EGFR activity due to its inhibitory effect on PAK4. Taken together, these results provided novel insights into the development of PAK4 inhibitor and potential therapeutic strategies for gastric cancer.

1. Introduction

Gastric cancer is the first leading cause of mortality in Asia and the second one in the world [1]. Although the surgical techniques are improving and some chemotherapic regimens are available, the outcomes of patients with advanced disease are usually poor [1]. So it is very important to investigate proteins controlling metastasis and to identify novel therapeutic targets to improve the survival of patients with gastric cancer.

The p21-activated kinases (PAKs) are a family of serine/threonine protein kinases which act as effectors for Rac and Cdc42 [2]. PAKs play important roles in cytoskeletal reorganization, cell survival, hormone signaling, gene transcription and tumorigenesis [2,3]. There are six mammalian isoforms of PAKs which can be classified into group I PAKs (PAK1–3) and group II PAKs (PAK4–6) [4]. PAK4 is the most extensively and profoundly studied member among the group II PAKs. Overexpression of PAK4 has been found in a variety of cancer cell lines, including breast, prostate, gall bladder and stomach [5–7] and also in several primary tumors [8]. Subsequent studies demonstrated that PAK4 could promote cell survival via interacting with TNFα receptor complex [9] and regulate cell proliferation involving c-Src/EGFR/cyclin D1 pathway [10]. On the other hand, PAK4 could mediate the induction of filopodia in response to activated Cdc42 [11], inhibit cell adhesion [12] and promote anchorage-independent growth [5,8,12]. Importantly, it could also enhance cell migration and invasion through HGF/LIMK1/cofilin and MEK-1/ERK1/2/MMP2 pathways [10,13]. Many of these functions rely on PAK4 kinase activity.

The important functions of PAK4 provide a rationale for the discovery of PAK4 inhibitors as anti-cancer therapeutics. Nowadays several small molecular compounds identified just act as ATP-competitive inhibitors which typically target the highly conserved ATP-binding pocket of kinase domain. Staurosporine and its related molecule K252a were reported to successfully inhibit the kinase activity of PAK4 [14]. SU11652 and three compounds derived from the inhibitor of CDK (cyclin-dependent kinases) were also identified as the inhibitors of group II PAKs [15]. Recently, Pfizer identified PF-3758309 as a potent, ATP-competitive inhibitor of PAK4

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[16]. Although all of these compounds are not particularly specific for PAK4, they may provide helpful scaffolds from which more selective and potent inhibitors could be developed.

Here we report the identification and characterization of LCH-7749944 as a novel and potent inhibitor targeting PAK4 in human gastric cancer cells with the expected cellular functions of a PAK4 inhibitor. This study expands the scope of the exploitation and application of PAK4 inhibitors, and provides a new therapeutic strategy targeting gastric cancer cells by blockade of PAK4 signaling.

2. Materials and methods

2.1. Cell culture

Human gastric cancer cell lines SGC7901, BGC823, MGC803, MKN-1 and MKN-45 were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) at 37 °C in incubator with humidified atmosphere of 5% CO_2 and 95% air.

2.2. Reagents

LCH-7749944, N2-(3-methoxyphenyl)-N4-((tetrahydrofuran-2-yl)methyl) quinazoline-2,4-diamine was synthesized according to the procedure provided in Supplementary data and its structure was shown in Fig. 1A. The purity of LCH-7749944 was determined by mass spec and ^{1}H NMR, indicating that LCH-7749944 synthesized by us is pure enough for biological activity test (Supplementary Fig. S1B and C). WYA-3, N^2,N^4-dimethyl-N^2,N^4-diphenylquinazoline-2,4-diamine was also synthesized according to the procedure provided in Supplementary data and its structure was shown in Fig. 1B. LCH-7749944 and all of its structurally related compounds were dissolved in 100% dimethyl sulfoxide (DMSO) at the concentration of 20 mM and stored as small aliquots at −20 °C. Then thawed and diluted as needed in cell culture medium.

2.3. Immunoprecipitation and kinase assays

Cells transfected with myc-PAK4 wt were pre-incubated with the indicated concentrations of LCH-7749944 before further incubation in the absence or presence of HGF (R&D). Then cells were washed with ice-cold PBS and suspended in cold lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.5 and 150 mM NaCl) supplemented with protease and phosphatase inhibitors as described. Supernatants were preadsorbed with protein A-Sepharose 4B beads (GE Healthcare Bio-Science Inc., Sweden) for 1 h at 4 °C before incubation with myc-tagged antibody (Santa Cruz). Then the supernatants with equal amounts of protein were subjected to immunoprecipitation using myc-tagged antibody and the protein A-Sepharose 4B beads. PAK4 kinase assays were performed using the exogenous Histone H3 as substrate to assess activity. Protein A-Sepharose 4B beads containing immunoprecipitated myc-PAK4 were washed twice with lysis buffer and three times with kinase buffer (50 mM HEPES, pH7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$ and 0.2 mM DTT). Kinase activity was measured in 40 μL of kinase buffer containing 10 μCi of [γ-^{32}P] ATP (5000 Ci/mmol) for 30 min at 30 °C. Reactions were stopped by addition of 6× SDS buffer and loading on a 12% SDS–PAGE. Proteins were transferred onto PVDF membranes and ^{32}P-labelled proteins were visualized by autoradiography with Molecular Imager RX (BIO-RAD). To assure equal loading amount, PAK4 were detected by immunoblotting analysis and Histone H3 (Roche) was detected by ponceau stain. For chemicals direct effect on

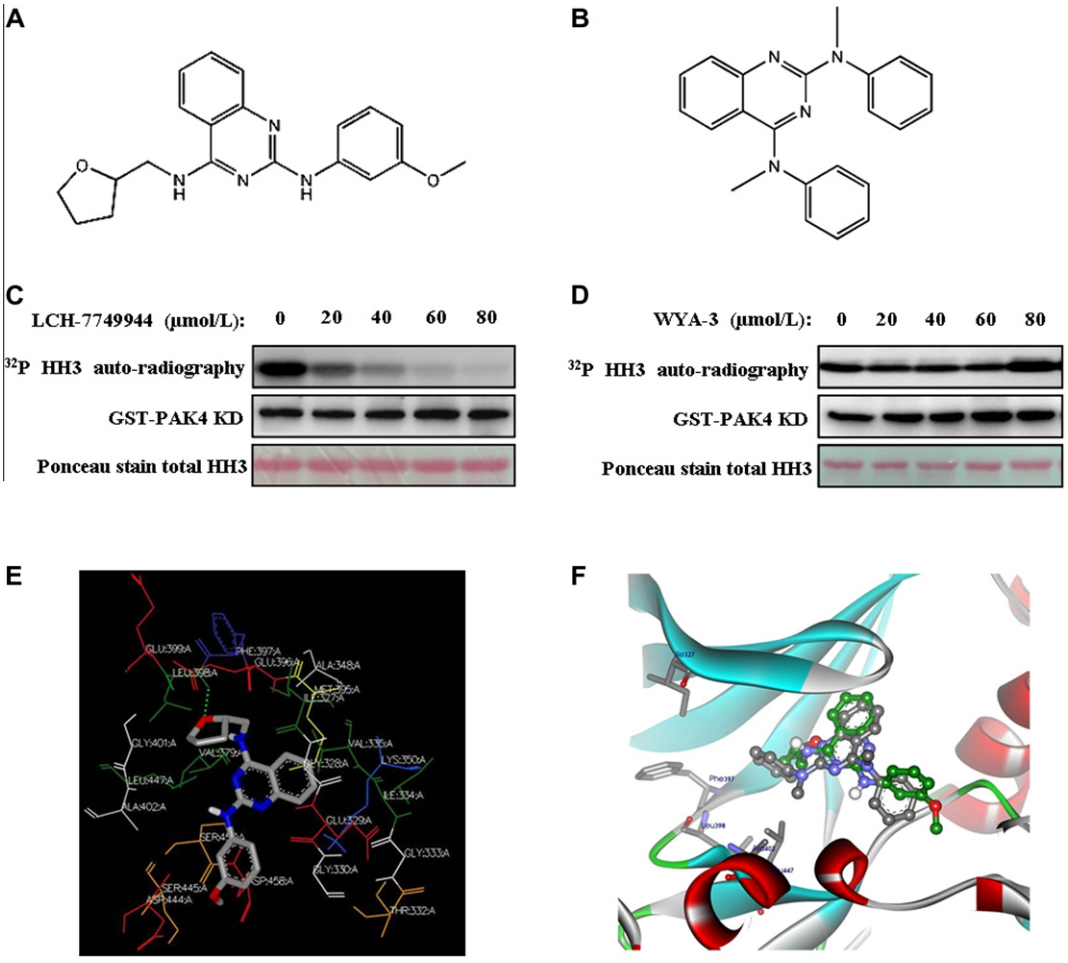

Fig. 1. LCH-7749944 is a novel inhibitor of PAK4. (A) Chemical structure of LCH-7749944 ($C_{20}H_{22}O_2N_4$). (B) Chemical structure of an inactive LCH-7749944 structural relative, WYA-3 ($C_{22}H_{20}N_4$). (C) LCH-7749944 inhibits PAK4 kinase activity in vitro. PAK4 was pre-incubated with the indicated concentrations of LCH-7749944 for 30 min, then the kinase assay were performed. (D) WYA-3 has no inhibitory effect on PAK4 kinase activity in vitro. PAK4 was pre-incubated with the indicated concentrations of WYA-3 for 30 min, then the kinase assay were performed. (E) Binding mode of LCH-7749944 (stick style, gray color) within the PAK4 binding site, which forms hydrogen bond with Leu398. (F) Comparison of compound LCH-7749944 (green color) with WYA-3 (gray color). PAK4 structure was shown in ribbon form and small molecules presented as ball and stick structure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)