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Reverse phase protein array identifies novel anti-invasion mechanisms of YC-1

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ABSTRACT

YC-1 has recently been demonstrated to have potent anti-invasion and anti-metastatic activity in several cancer models, in addition to its anti-proliferation activity. However, the mechanism underlying its anti-invasion/anti-metastatic activity is largely unknown. Nasopharyngeal carcinoma (NPC) is a highly metastatic head and neck cancer in Southeast Asia. Here, we demonstrated that YC-1 inhibited invasiveness and proliferation of NPC cells, with the latter being accompanied by PARP cleavage, S-phase arrest and activation of Chk1/Chk2. We aimed at identifying novel anti-invasion mechanisms of YC-1 in NPC by a functional proteomic platform, the reverse phase protein array (RPPA). Our study revealed for the first time that multiple invasion-related signaling proteins (β-catenin, caveolin, Src and EGFR), as well as several growth-related proteins (AMPKα, phospho-acetyl-CoA carboxylase (p-ACC), HER-2 and mTOR), which were previously un-described signaling proteins altered by YC-1, were found to be down-modulated by YC-1 in NPC cells. We hypothesized that YC-1-mediated downregulation of these invasion proteins contributed to its anti-invasion activity in NPC cells. Overexpression of EGFR, activated Src or caveolin, but not β-catenin reversed the inhibitory effects of YC-1 on NPC cell invasion, with EGFR and activated Src having additional effects on rescuing NPC cells from YC-1-mediated growth inhibition. In summary, we have identified several novel anti-invasion mechanisms of YC-1 that could impact NPC, and possibly other cancers as well.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic head and neck cancer prevalent in Southeast Asia with a high incidence rate of 15–50/100,000 persons/year (comparable to that of pancreatic cancer in the US) [1–3]. Over 50–70% of NPC patients present with advanced disease (stages IIb–IV) with lymph node invasion or metastasis at the time of diagnosis [4]. Recurrent NPC patients also have a high rate of distant metastasis up to 37% [5]. Although the underlying mechanism for its high metastatic characteristics is not fully understood, the search for anti-invasion or anti-metastatic drugs for advanced NPC is actively being pursued.

YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole] is a synthetic benzylindazole compound originally developed as an activator of guanylyl cyclase to inhibit platelet aggregation and vascular contraction [6]. Recent studies revealed that YC-1 has potent activity against tumor growth, angiogenesis, invasion and metastasis [7–9]. In multiple cancer models, YC-1 induced prominent suppression of tumor growth and prolongation of survival in tumor-bearing mice with established tumors [8–12]. The growth-inhibitory activity of YC-1 has been proposed to be mediated by: HIF-1α [9,12], STAT3 [13], MAPK [14], JNK [15], NF-κB [11], cell cycle-dependent pathway [14,16] and mitochondrial-dependent apoptosis pathway [10]. The recently described inhibitory activity of YC-1 on cellular invasion/metastasis in several *in vitro* and *in vivo* models of hepatoma, neuroblastoma and gastric and lung cancers [7,14] suggest the clinical potential of this agent for anti-invasion/anti-metastatic therapy. Studies are underway to identify the largely unknown anti-invasion or

Abbreviations: NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; RPPA, reverse phase protein array; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MMP-2/-9, Matrix Metalloproteinases 2 and 9; ACC, acetyl-CoA carboxylase; AMPKα, AMP-activated protein kinase α; LMP1, latent membrane protein 1.

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anti-metastatic mechanism(s) of YC-1, in addition to the potential involvement of HIF-1α and Matrix Metalloproteinases 2 and 9 (MMP-2/-9) [7,14]. A more detailed mechanistic understanding of its anti-invasion/anti-metastatic activity may facilitate its clinical application for aggressive cancers.

A quantitative functional proteomic platform, named reverse phase protein array (RPPA), has been recently employed for quantitative profiling of signaling protein expression in cancer [17]. With the capability to detect total, phospho-proteins, and translationally modified proteins in a highly quantitative manner with high sensitivity at femtogram level [18], this high-throughput multiplex functional proteomic approach can be used for signaling profiling, detection of disease marker expression, as well as for mechanistic studies of drug action [19–21]. Recently, we have employed RPPA for the identification of novel signaling changes induced by combined anti-EGFR and anti-GRPR therapies in head and neck cancer, which provide valuable insights for the mechanism of action of combination therapy [22].

In this study, we reported the anti-invasive, as well as anti-proliferative activities of YC-1 in NPC cell lines. We aimed at identifying novel anti-invasion mechanisms of YC-1 by RPPA scanning of signaling changes induced by YC-1 in NPC cells. Our study revealed for the first time that multiple invasion-related signaling proteins (β-catenin, caveolin, Src and EGFR), which were previously un-described signaling proteins altered by YC-1, were found to be down-modulated by YC-1 in NPC cells. We hypothesized that these invasion-related proteins (as identified to be downregulated by YC-1), may contribute to the anti-invasion mechanism of YC-1 in NPC cells. Rescue experiments demonstrated that overexpression of EGFR, activated Src or caveolin in NPC cells all resulted in reversal of YC-1-induced inhibition of cellular invasion, with EGFR and activated Src having additional effects on rescuing NPC cells from YC-1-induced growth inhibition. Using the NPC model, we have identified several novel anti-invasion mechanisms of YC-1.

2. Materials and methods

2.1. Reagents

YC-1 was purchased from AG Scientific, Inc., San Diego, CA, USA and dissolved in DMSO (Sigma–Aldrich, Saint Louis, MO, USA) at a stock concentration of 100 mM. Epidermal growth factor (EGF) was from Invitrogen (BioSource), Camarillo, CA, USA. Fetal bovine serum (FBS) was from Hyclone, Logan, UT, USA. Antibodies against phospho-p42/44 MAPK, phospho-STAT3(Y705), STAT3, cleaved PARP, mTOR, Src, phospho-ACC, caveolin, β-catenin, AMPKα, phospho-Chk1, phospho-Chk2, Chk1 and Chk2 were from Cell Signaling Technology, Danvers, MA, USA. Phospho-Src(Y418) antibody was from Invitrogen, Camarillo, CA, USA. Phospho-EGFR(Y1086) antibody was from Invitrogen (Zymed), Camarillo, CA, USA. EGFR monoclonal antibody was from BD Transduction Laboratories, San Jose, CA, USA. Actin antibody (JLA20) was from Calbiochem, EMD Biosciences, San Diego, CA, USA. Expression plasmids for β-catenin (pCl-neo β-catenin WT), activated Src (pLNCX chick srcY527F) and caveolin (Cav1-GFP) were from Addgene Inc., Cambridge, MA, USA. EGFR expression plasmid (pcDNA-HA-EGFR) was provided by Dr. Lan Ma (Fudan University, Shanghai, China).

2.2. Cell culture

Human nasopharyngeal carcinoma cell lines, HONE-1, HONE-1-LMP1, HK1, CNE-2 and C666-1 were cultured in complete RPMI (Hyclone, Logan, UT, USA) in a humidified incubator at 37 °C in 5% CO_2 as previously described [23]. HONE-1 and CNE-2 were of poorly differentiated NPC origin, while HK1 and C666-1 were derived from well-differentiated and undifferentiated NPC. HONE-1-LMP1 stably expresses the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) (B95.8 variant) and was maintained in puromycin selection medium (400 ng/ml; Merck, Whitehouse Station, NJ, USA).

2.3. Cell viability assay

Cells (1.3×10^4 per well) were treated with various concentrations of YC-1 or vehicle control (DMSO) for 48 h. Cell viability was determined by MTT assay (MTT from Sigma–Aldrich, Saint Louis, MO, USA) as previously described [24]. Percentage of cell growth inhibition was calculated as $(OD_{vehicle} - OD_{drug})/OD_{vehicle} \times 100\%$. The IC_{50} value was the drug concentration at which 50% of maximal growth inhibition was observed.

2.4. Cell cycle analysis

After treatment with DMSO or YC-1 (700 nM) for 12 h, cells were fixed with ice-cold ethanol (70%) and subjected to cell cycle analysis as previously described [25].

2.5. Matrigel invasion assay

Invasiveness of NPC cells was evaluated using Matrigel-coated modified Boyden inserts (Becton Dickinson/Biocoat, BD Biosciences, Bedford, MA, USA) as previously described [23]. Briefly, HONE-1 (7×10^4) or CNE-2 cells (3.5×10^4) were seeded onto the upper chamber in serum-free medium. FBS (10%) or EGF (20 ng/ml) in serum-free medium was added to the bottom chamber in presence of DMSO or YC-1. Cells were incubated for 24 h at 37 °C. The invaded cells were fixed, stained, photographed (100× magnification) and counted (200× magnification) under the microscope. The average number of invaded cells/field of 6–10 fields was presented.

2.6. Transfection

HONE-1 cells (1.5×10^4 or 1.2×10^5) were seeded onto 24- or 6-well plates and incubated for 24 h. Cells were then transfected with 0.5 μg (24-well plate) or 4 μg (6-well plate) plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For Matrigel invasion assay, two days after transfection, 7×10^4 transfectants were replated onto the upper chamber in serum-free medium. DMSO or YC-1 (700 nM) in complete medium was added to the bottom chamber. Cells were incubated for 24 h at 37 °C. For MTT assay, day 2 transfectants were treated with DMSO or YC-1 (1 and 5 μM) for 24 h, followed by MTT assay.

2.7. Western blotting

After drug treatment, protein lysates were collected for Western blot analysis as previously described [26]. Twenty-five micrograms of protein was used for SDS-PAGE. After primary and secondary antibody incubations, the signal was detected by Supersignal West Pico Chemiluminescent detection kit (Thermo Fisher Scientific, Waltham, MA, USA), followed by autoradiography.

2.8. RPPA sample preparation and slide printing

HONE-1 cells (1×10^6) were plated on 10 cm plates. After treatment, cells were collected by washing with ice-cold PBS, scrapping and centrifugation at 1500 rpm at 4 °C for 10 min. Cell pellets were resuspended in RPPA lysis buffer (1% Triton X-100,