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Dihydroartemisinin suppresses STAT3 signaling and Mcl-1 and Survivin expression to potentiate ABT-263-induced apoptosis in Non-small Cell Lung Cancer cells harboring EGFR or RAS mutation

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ABSTRACT

Non-small cell lung cancer (NSCLC) is the most common malignancy worldwide. A significant fraction of NSCLC carries activating mutations in epidermal growth factor receptor (EGFR) or RAS oncogene. Dihydroartemisinin (DHA) is a semisynthetic derivative of the herbal antimalarial drug artemisinin that has been recently reported to exhibit anti-cancer activity. To develop new therapeutic strategies for NSCLC, we investigated the interactions between DHA and ABT-263 in NSCLC cells harboring EGFR or RAS mutation. Our data indicated that DHA synergized with ABT-263 to trigger Bax-dependent apoptosis in NSCLC cells in culture. DHA treatment antagonized ABT-263-induced Mcl-1 upregulation and sensitized NSCLC cells to ABT-263-triggered apoptosis. Additionally, DHA treatment caused downregulation of Survivin and upregulation of Bim, which also contribute to cotreatment-induced cytotoxicity. Moreover, DHA effectively suppressed STAT3 phosphorylation, and STAT3 inactivation resulted in the downregulation of Mcl-1 and Survivin, functioning to enhance ABT-263-induced cytotoxicity. Finally, cotreatment of DHA and ABT-263 significantly inhibited xenograft growth in nude mice. Together, DHA effectively inhibits STAT3 activity and modulates expression of Mcl-1, Survivin and Bim, thereby synergizing with ABT-263 to trigger apoptosis in NSCLC cells harboring EGFR or RAS mutation. Our data provide a novel therapeutic strategy for EGFR or RAS mutant NSCLC treatment.

1. Introduction

Despite the advances in both medical and surgical management in the last decades, non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related death worldwide [1]. One of the key reasons is that NSCLC cells usually harbor specific mutated oncogenes that are thought to be the primary genetic "driver" to trigger cancer. Epidermal growth factor receptor (EGFR) and KRAS are two of the most commonly mutated oncogenes found in NSCLC.

EGFR is a receptor tyrosine kinase that activates cellular signaling pathways such as the PI3K/AKT, STAT, and MAPK pathways and leads to enhanced cell proliferation and migration. EGFR mutation has been found in more than 20% of NSCLC patients and was established as a rational therapeutic target. A group of small-molecule tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, have been developed and showed significant clinical benefit in patients with activating EGFR mutations. However, most patients develop resistance to those TKI treatments quickly because of the appearance of T790M point mutation in EGFR [2]. Thus, a third generation of EGFR inhibitor was developed and approved as osimertinib [3]. However, laboratory studies have indicated that the efficacy of this agent can be compromised by Cys797 mutation [4].

The oncogenes KRAS, HRAS and NRAS are founding members of the RAS superfamily of GTPases. KRAS is most commonly mutated in NSCLC. Lying downstream of EGFR and other receptor tyrosine kinases, KRAS mutation is linked to constitutive and hyperactive activation of the RAS-MAPK cascade and other growth and survival pathways, contributing to the initiation and maintenance of the malignant phenotype. KRAS itself has been proven difficult to inhibit. The strategies to target RAS effectors, including RAF, MEK and ERK, have been thwarted by adaptive resistance mechanisms [5,6]. In addition, KRAS mutation could be an additional mechanism of escape from EGFR-TKI inhibition [7]. Therefore, it is critically important to develop new therapies for NSCLC patients with EGFR and RAS mutation.

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Currently, chemotherapy remains the primary options to treat NSCLC. Given the side effects and acquired resistance to synthesized chemotherapy drugs, natural plant-derived compounds with low toxicity and non-mutagenic properties have received increasing attention as alternative therapies against various cancers.

Dihydroartemisinin (DHA) is a semisynthetic derivative of the herbal antimalarial drug artemisinin. It has much better water solubility and stronger antimalarial activity than other artemisinin derivatives [8,9]. Current studies have shown that DHA exhibits antitumor effects via modulating the cell cycle process, triggering cell death and inhibiting tumor angiogenesis [10]. During those processes, multiple signaling pathways are modulated by DHA, including the MAPK/ERK, Wnt/β-catenin, JAK/STAT and translationally controlled tumor protein (TCTP) pathways. In most of those studies, however, DHA only moderately inhibited tumor cells when administered as a single chemotherapy agent. Combinatorial therapies with other anti-cancer drugs seem to be a promising application of DHA to enhance its anti-cancer effects [11].

In this study, we investigated the interaction between DHA and a BH3-mimetic ABT-263 in NSCLC cell lines carrying oncogenic EGFR or RAS mutation. We demonstrated that cotreatment of these cells with DHA and ABT-263 synergistically triggered apoptosis *in vitro* and reduced their tumorigenicity *in vivo*. Mechanistically, DHA inhibited the JAK-STAT3 pathway to control expression of the STAT3-regulated Mcl-1 and Survivin. These molecular targets of DHA are frequently activated by EGFR or RAS mutation in NSCLC [12,13].

2. Materials and methods

2.1. Reagents

Dihydroartemisinin, ABT-263, Stattic and AZD1480 were purchased from Selleck (Selleck Chemicals, Houston, USA). FBS was obtained from BI (Biological Industries, Shanghai, China). Antibodies of cleaved PARP (#5625), cleaved caspase-3 (#9661), Mcl-1 (#39224), Bak (#6947), Bcl-xL (#2764), Survivin (#2808), Bim (#2933), STAT3 (#12640), p-STAT3 (Y705) (#9145), p-JAK2 (T1007/T1008) (#3776), p-EGFR (Y1068) (#2234), p-Hsp27 (S82) (#9709), p-Hsp27 (S78) (#2405), p-STAT3 (S727) (#9134), p-p38/MAPK (T180/T182) (#9211), p-ERK1/2 (T202/Y204) (#4370), p-AKT(S473) (#4060) were obtained from Cell Signaling Technologies (Beverly, MA, USA). The antibodies p-Bmx (Y566) (#AB12321) and Bcl-2 (#AB40639) were from Aboci (Aboci, MD, USA). Bax (#23931-1-AP) and β-actin (#60008-1-lg) was obtained from Proteintech (Wuhan, Hubei, China).

2.2. Cell culture

EGFR-mutant (H1975, HCC827, H1650, H3255), KRAS-mutant (A549, H727), NRAS mutant (H1299) and BRAF mutant (MV522) cell lines were obtained from the American Type Culture Collection (ATCC). IRM-90 cells were kindly provided by Dr. Mingxiong Guo, Wuhan University, China. All cancer cell lines were cultured in RPMI-1640 media supplemented with 10% FBS (Biological Industries, Shanghai, China) and 1% penicillin-streptomycin (Solarbio, Beijing, China) and were incubated in a humidified incubator at 37 °C containing 5% CO_2.

2.3. Flow cytometry analysis of apoptosis

Following drug treatment, floating and adherent cells were harvested and stained with FITC-conjugated annexin V and propidium iodide (PI) according to the manufacturer's instruction (#FXP018, 4A Biotech, Beijing, China). The stained cells were analyzed for annexin V-positive apoptotic cells by flow cytometry (Guava easyCyte 8HT, Millipore, MA, USA). The extent of apoptosis was quantified as a percentage of annexin V FITC-positive cells. Each experiment was performed in triplicate for at least three times.

2.4. Colony formation assays

Cells were seeded into 24-well plates at approximately 200 cells per well. After overnight incubation, the cells were treated with the indicated agents and were incubated at 37 °C in 5% humidified CO_2 for 14 days. The media were changed every 3–4 days. The colonies were washed with PBS twice and were fixed with methanol for 15 min before staining with 0.1% crystal violet.

2.5. Western blotting analysis

Whole-cell lysates were prepared with cell lysis buffer (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, and 1 × proteinase inhibitor (Roche, Basel, Switzerland)). The protein concentration was determined using the BCA protein assay kit (Solarbio, Beijing, China). Equal amounts of protein (50 μg) were separated by SDS-PAGE and were transferred to polyvinylidene difluoride (PVDF) membranes (IPVH 00010, Millipore, MA, USA). The membranes were blocked and then were probed with primary antibodies overnight at 4 °C. Beta-actin was used as an endogenous loading control. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000, Cell Signaling, Beverly, MA, USA) and were visualised by ECL chemiluminescence (Millipore, MA, USA). For western blotting quantification, the densitometry measurements of cleaved PARP and cleaved caspase 3 are normalized to β-actin, respectively. For others, the densitometry measurements, normalized to β-actin and then compared to vehicle-treated control, are indicated below the corresponding blot. The data are represented as the means of at least three independent experiments.

2.6. Plasmids

The structure of pCDNA3.1-Survivin was described previously [14]. The lentiviral vector expressing constitutively active STAT3 was obtained from Addgene (MA, USA). The pGreenPuro shRNA plasmid was purchased from System Biosciences (SBI, CA, USA). The shRNA lentivirus vectors were generated by cloning target-specific oligonucleotides into the lentiviral vector according to the manufacturer's protocol. STAT3 shRNA (sh-STAT3) oligonucleotide: 5′-CTTCAGACCCGTCAAC AAA-3′; and Bim shRNA (sh-Bim) oligonucleotide: 5′-GACCGAGAAGG TAGACAATTG-3′. The details of sh-ctrl, sh-Bax and sh-Mcl-1 were described previously [14,15].

2.7. Lentivirus packaging

The viruses were produced by co-transfection of 293TN cells with a lentiviral vector, and packaging and envelope plasmids using lipofectamine 2000 (Life Technology, Thermo Fisher Scientific, MA, USA) as previously described [15]. The virus-containing supernatants were harvested at 48 and 72 h post transfection and were used for infection or were stored in aliquots at −80 °C.

2.8. Real-time PCR

Total RNA was extracted from H1975 cells with a total RNA Simple Kit (TIANGEN, Beijing, China). The first-strand cDNA was synthesized with Max DNA (TaKaRa, Dalian, China) according to the manufacturer's instructions. Fluorogenic probes were purchased from TaKaRa (#RR820A, Shiga, Japan). The relative mRNA levels of Mcl-1 and Survivin were quantified using a real-time PCR reaction platform (CFX96 Touch, BioRad, MA, USA). The following paired oligos were used for Mcl-1 (5′-GGGCAGGATTGTGACTCTCATT-3′ (forward) and 5′-GATGCAGCTTTCTTGGTTTATGG-3′ (reverse)) and Survivin (5′-CTGCCTGGCAGCCCTTT-3′ (forward) and 5′-CCTCCAAGAAGGGCCA GTTC-3′ (reverse)). GAPDH (5′-GGGAAGGTGAAGGTCGGA-3′ (forward) and 5′-GCAGCCCTGGTGACCAG-3′ (reverse)) were detected as