Biochemical Pharmacology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Andrographolide reversed 5-FU resistance in human colorectal cancer by elevating BAX expression

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ARTICLE INFO

Article history:
Received 27 July 2016
Accepted 22 September 2016
Available online xxxx

Keywords:
Andrographolide
BAX
5-FU-resistant
Human colorectal cancer

ABSTRACT

5-FU is the first line therapy for colorectal cancer, however, treatment effect is often hampered by the development of drug resistance or toxicity at high doses. Andrographolide is a natural diterpenoid from *Andrographis paniculata* which has anti-bacterial, anti-antiviral and anti-inflammation activities. In the current study, we test the hypothesis that Andrographolide reverses 5-FU resistance in colorectal cancer and examine the underlying mechanism. In vitro and vivo studies indicated that Andrographolide treatment significantly re-sensitizes HCT116/5-FUR cells (HCT116 cells which are 5-FU resistant) to cytotoxicity of 5-FU. Mechanism analysis showed that Andrographolide/5-FU co-treatment elevated apoptosis level of HCT116/5-FUR cells with highly increased level of BAX. By using biotin-Andrographolide pull down and cellular thermal shift assay, we found out that Andrographolide can directly target to BAX. Andrographolide-BAX interaction prevented BAX degradation, enhancing mitochondria-mediated apoptosis thus reversed 5-FU resistance while BAX silence diminished this effect. Further, by analyzing patient samples who received 5-FU involved chemotherapy, we found that expression level of BAX is correlated with PFS. Our results here provide a novel combination treatment strategy, especially for patients with 5-FU-resistant tumors expressing low level of BAX. Meanwhile, we also proposed that BAX expression may be a predicted and prognosis marker of 5-FU involved chemotherapy.

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed solid tumors worldwide. Colorectal cancer is the second leading cause of cancer deaths in the United States and the third most common malignant neoplasm worldwide [1]. Current therapies for the treatment of CRC mainly comprise 5-Fluorouracil (5-FU)-based chemotherapies that are used individually or in combination with oxaliplatin or anti-angiogenic agents, and/or anti-epidermal growth factor agents [2,3]. As a false substrate to thymidylate synthase enzyme that incorporates into DNA and RNA, 5-FU treatment causes the growth restrain and apoptosis of cancer cells [4]. Although CRC incidence rates have declined somewhat, current therapies are associated with significant side effects, high expense and recurrence rates can be as high as 50–60%, primarily due to the development of acquired resistance to 5-FU-based chemotherapeutics, as more than 15% of patients are resistant to 5-FU-based chemotherapies [5,6]. Thus, resistance to 5-FU has been a major obstacle in advanced colorectal cancer chemotherapy and confines its use in clinical practice. Thus novel and safe treatment strategies which can help overcome chemoresistance and enhance CRC response to 5-FU-based chemotherapies are desperately needed.

Andrographolide, a natural diterpenoid from *Andrographis paniculata*, has been used as a herbal medicine in China for thousands of years. Andrographolide has been reported to exert antibacterial, antiviral, anti-inflammation and neuroprotective activities [7–9]. Our previous studies have proved that Andrographolide can alleviate colitis and suppress colitis-associated colon cancer [10,11]. Recent studies showed that Andrographolide alone can induce apoptosis of different cancer cells via regulating pro-apoptotic Bcl-2 family members [12]. Another group has reported that Andrographolide can inhibit tumor growth in mice although at a relatively high dose (about 200 mg/kg) [13]. What's more, Andrographolide can potentiate the cytotoxic effect of various

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http://dx.doi.org/10.1016/j.bcp.2016.09.024
0006-2952/

chemotherapy drugs in different cell lines via enhancing apoptosis pathways or via suppression of pro-survival autophagy [14,15]. However, in colorectal cancer, no investigations about the effects and mechanism of Andrographolide on CRC 5-FU resistance have been reported.

In the present study, we investigated the effect of Andrographolide in combination with 5-FU on various growth regulatory parameters and extensive characterization of underlying mechanisms in a 5-FU-resistant CRC cell line in vivo and vitro. Our data firstly reveal that the bind and up-regulation of BAX expression by Andrographolide is one of the principle mechanisms for resensitizing 5-FU-resistant CRC cells to 5-FU. At the same time, we found that BAX expression level maybe a prognosis marker for 5-FU-based treatment. These findings highlight the potential possibility of using this natural, safe and relatively inexpensive compound as potential adjunctive treatments in improving the overall treatment response of patients with CRC in future.

2. Methods

2.1. Chemicals and reagents

Primary antibodies against BAX, BCL2 and Actin, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against Biotin, PARP, Caspase-3, GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipofectamine 3000 and JC-1 was bought from Life Technology (Carlsbad, CA, USA). TUNEL assay kits were bought from Vazyme Biotech Co., Ltd (Nanjing, China). Aneexin V/PI staining kit were bought from Beyotime Company (Nantong, China). Softlink Soft Release Avidin Resin was purchased from PROMEGA (Madison, WI, USA). GTVisin™ anti-mouse/anti-rabbit immunohistochemical analysis KIT was purchased from Gene Company (Shanghai, China). 5-FU was bought from KingYork Group Co. Ltd. (Tianjin, China). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The HCT116 human colon cancer cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The 5-FU-resistant HCT116 (HCT116/5-FUR) cell line was induced in our lab. Cells were respectively cultured in DMEM culture medium (Gibco) supplemented with 10% FBS (fetal bovine serum, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin, under a humidified 5% CO_2 atmosphere at 37 °C in incubator.

2.3. Induction of 5-FU-resistant HCT116 cell line

5-FU-resistant HCT116 variants (HCT116/5-FU R1, HCT116/5-FU R2 and HCT116/5-FU R) of each cell line were derived from each original parental (PT) cell line (HCT116) by utilizing serial passage in the presence of increasing 5-FU concentrations (continuous exposure). Initially, cells were treated with 5-FU (10 μM) for 72 h. The media and dead cells were removed and cells were allowed to recover for a further 72 h and then treated with higher concentration of 5-FU. This development period was carried out for approximately 6 months and finally we got the HCT116/5-FU R subline while HCT116/5-FU R1, HCT116/5-FU R2 were subline with partly resistant to 5-FU during this process. HCT116/5-FU R subline was then maintained continuously in the presence of 1 mM 5-FU for a further 3 months to make it stable.

2.4. Animals

Nude mice (B6 background), 6–8 weeks of age, were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All experimental protocols were approved by Ethic Committee of Nanjing University. All efforts were made to reduce the number of animals used and to minimize animals' suffering.

2.5. Human tissue samples

Human tissue samples were got from Department of Oncology, The First Affiliated Hospital with Nanjing Medical University. The study was approved by the Ethic Committee of Nanjing University and Nanjing Medical University and the methods and experimental protocols were carried out in accordance with the approved guidelines in Nanjing University. Written informed consent was obtained from all donors involved.

2.6. Xenograft model

HCT116/5-FUR cells (2×10^6) were injected subcutaneously into the right flank of nude mice. Once the tumor reached 200 mm^3, treatments were initiated as follows: group 1, PBS (vehicle); group 2, Andrographolide at 25 mg/kg of body weight once daily, ip; group 3, 5-FU at 25 mg/kg of body weight every 3 days, ip; group 4, combination of Andrographolide and 5-FU. Drugs were administered on days 0–24. The body weight of mice was measured daily. The body weight and tumor volume were measured daily after treatment begin and tumor volumes were calculated using the formula $V = ab^2/2$. After 24 days, mice were sacrificed and solid tumors were separated.

2.7. Cell viability assay

The cells were plated at a density of approximately 4×10^3 viable cells per well in 96-well plates. Various concentrations of compound were used to treat cells in triplicates. After incubation for the indicated time, MTT assay was performed to measure cell viability by a 96-well plate reader (Elx 800, BIOTECH).

2.8. Cell apoptosis assay

Cells were incubated with Annexin V/PI at room temperature for 15 min in dark and then analyzed by FACS Calibur flow cytometry (Becton Dickinson). Annexin V+/PI− and Annexin V+/PI+ cells were considered as apoptotic cells in the early and late phase, respectively.

2.9. Mitochondria membrane potential analysis

Mitochondrial membrane potential was measured by JC-1 stain. Cells were washed with PBS and incubated with 5 μg/ml JC-1 at 37 °C for 30 min. Cells were then washed twice with PBS and immediately assessed FACS. A 488 nm filter was used for the excitation of JC-1. Emission filters of 535 (FL-1) and 595 nm (FL-3) were used to quantify the population of mitochondria with green (JC-1 monomers) and red (JC-1 aggregates) fluorescence.

Please cite this article in press as: W. Wang et al., Andrographolide reversed 5-FU resistance in human colorectal cancer by elevating BAX expression, Biochem. Pharmacol. (2016), http://dx.doi.org/10.1016/j.bcp.2016.09.024