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The novel HDAC inhibitor AR-42-induced anti-colon cancer cell activity is associated with ceramide production

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ABSTRACT

In the current study, we investigated the potential activity of AR-42, a novel histone deacetylase (HDAC) inhibitor, against colon cancer cells. Our *in vitro* results showed that AR-42 induced ceramide production, exerted potent anti-proliferative and pro-apoptotic activities in established (SW-620 and HCT-116 lines) and primary human colon cancer cells. Exogenously-added sphingosine 1-phosphate (S1P) suppressed AR-42-induced activity, yet a cell-permeable ceramide (C4) facilitated AR-42-induced cytotoxicity against colon cancer cells. In addition, AR-42-induced ceramide production and anti-colon cancer cell activity were inhibited by the ceramide synthase inhibitor fumonisin B1, but were exacerbated by PDMP, which is a ceramide glucosylation inhibitor. *In vivo*, oral administration of a single dose of AR-42 dramatically inhibited SW-620 xenograft growth in severe combined immunodeficient (SCID) mice, without inducing overt toxicities. Together, these results show that AR-42 dramatically inhibits colon cancer cell proliferation *in vitro* and *in vivo*, and ceramide production might be the key mechanism responsible for its actions.

1. Introduction

Colon cancer and other colorectal carcinomas (CRC) are one leading cause of cancer-related mortalities around the world [1–3]. Surgery, radiotherapy or chemotherapy are the current treatment options of colon cancer patients [1–3]. However, above managements only showed limited value in improving overall survival (OS) of affected patients, especially for those with advanced or metastatic diseases [4,5]. Many different anti-colon cancer drugs are being utilized clinically, yet associated-side effects and drug resistance are two major drawbacks [4,5]. Thus, the development of more effective agents is urgent and necessary for colon cancer treatment [1,2].

Histone deacetylases (HDACs) are a family of enzymes that are capable of removing acetyl group from histone lysine, leading to chromatin condensation and transcriptional repression [6,7]. There are at least four classes of HDACs have been characterized thus far, including class I (HDAC1, 2, 3, and 8); class II (II-a: HDAC4, 5, 7, and 9; II-b: HAC6, and 10); class III HDACs or sirtuins, and class IV (HDAC11) [6,7]. Groups all over the world are focusing on the development of small-molecule HDAC inhibitors, and their use in preclinical and clinic cancer models [6,7]. Several of these HDAC inhibitors have displayed promising results against colon cancer cells [8].

In the past decade, there has been a significant expansion of HDAC inhibitors [6,7]. Recent studies have characterized a phenylbutyrate derivative AR-42 as a novel class I and class II-b HDAC inhibitor [9–12]. Existing evidences have demonstrated that AR-42 induced potent anti-tumor activities, both alone and in combination with other treatments [9–12]. At sub μM concentrations, this novel HDAC inhibitor was shown to exert anti-proliferative and cytotoxic activities against lymphocytic/acute myeloid leukemia cells, and B-cell lymphoma cells [9–12]. Studies have also shown that AR-42 could directly target leukemic stem cells [10]. However, the potential activity of AR-42 in colon cancer cells is not extensively studied, and the underling mechanisms need further characterizations.

Ceramide is a well-known lipid mediator of cell apoptosis [13,14]. In fact, a large amount of anti-cancer cytotoxic drugs were shown to activate ceramide-mediate apoptosis pathways [13,14]. In the current study, we show that AR-42 potently inhibits colon cancer cell proliferation *in vitro* and *in vivo*. Ceramide production might be the key signaling mechanism responsible for its actions.

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2. Materials and methods

2.1. Chemicals and reagents

AR-42 was obtained from Selleck (Shanghai, China). AR-42 was dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiments, and was dissolved in 0.5% methylcellulose/0.2% Tween 80 for *in vivo* experiments. Fumonisin B1 and D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) were obtained from Sigma (St. Louis, MO); The apoptosis inhibitors including z-VAD-fmk, Ac-DEVD-CHO, and NS3694 were also purchased from Sigma (St. Louis, MO). Sphingosine 1-phosphate (S1P) and C4 ceramide were from Avanti Polar Lipids, Inc. (Alabaster, AL). Anti-cleaved caspase-3, cleaved-poly (ADP-ribose) polymerase (PARP) and tubulin antibodies were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

Established human colon cancer SW-620 and HCT-116 lines were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China), cells were maintained in RPMI 1640 medium (Invitrogen, NY, USA) with 10% fetal bovine serum (FBS, Invitrogen) in a CO_2 incubator.

2.3. Primary colon cancer cells preparation and culture

Experiments requiring clinical samples were approved by the Research Ethics Board of authors' institution. The patients were fully written informed. Surgery-isolated colon cancer tissues were minced into approximately 1 mm^3-size pieces, and washed four times in PBS with 100 unit/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Shanghai, China). After removal of the wash solution, tissue fragments were placed in serum-free RPMI 1640 (Invitrogen) with 1 mg/mL Collagenase Type IV (Sigma), and incubated for 1.5 h at 37 °C to obtain enzymatic disaggregation. Every 15 min, the solution was vigorously shaken for 15 s to encourage dissociation. Cells were then sieved through a 40-μm filter and resuspended in RPMI 1640 with 20% FBS and necessary supplements [15]. Primary cells at passage 2–7 were utilized for experiments.

2.4. The MTT cell proliferation assay

Colon cancer cell proliferation was assessed through [3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-2H-tetrazolium bromide] (MTT) assay. Briefly, cells were seeded into 96-well plates at a density of 5×10^3 cells/well. After treatment, 20 μL/well of MTT (5 mg/ml, Sigma) solution was added, the OD value was determined by measuring absorbance using a microplate spectrophotometer at 490 nm (Molecular Devices, Sunnyvale, CA).

2.5. Colony formation assay

SW-620 cells (1×10^5/well) were plated onto a 24-well plate. After treatment, the cells were collected and seeded (1000/well) in a fresh 12-well plate for 10 days. The cells were then stained with hematoxylin solution, and the survival colonies (>50 cells/per colony) were manually counted.

2.6. Apoptosis assay

After treatment, cells (1×10^5/well) were stained with Annexin V (allophycocyanin [APC] conjugated) and 7-aminoactinomycin D (7-AAD) according to the manufacturer's instructions (BD Biosciences, Erembodegem, Belgium). Apoptosis was assessed by flow cytometry (BD FACSCalibur, Shanghai, China). The percentage of Annexin V positive cells was recorded as a quantitative measurement of cell apoptosis.

2.7. LDH detection

Lactate dehydrogenase (LDH) content was analyzed by a two-step enzymatic reaction LDH assay kit (Takara, Tokyo, Japan). Percentage of released LDH was calculated by the following formula: LDH released in conditional medium/(LDH released in conditional medium + LDH in cell lysates) × 100%.

2.8. Caspase-3 activity assay

After treatment, SW-620 cells were lysed by the caspase lysis buffer [2.5 mM HEPES (pH 7.5), 5 mM EDTA, 2 mM DTT, 0.1% CHAPS]. A total of 100 μg protein/100 μL was collected, and 2 μL of the caspase-3 substrate (Ac-DEVD-pNA, Calbiochem) was added to the wells. Cells were further incubated in a shaking incubator at 37 °C for 3 h. The absorbance at 405 nm was then determined using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, CA).

2.9. Western blots

After treatment, cancer cells or tissues were lysed in RIPA lysis buffer [50 mM Tris–HCl (pH 7.4), 1% NP-40, 40 mM NaF, 10 mM NaCl, 10 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM dithiothreitol and EDTA-free protease inhibitor tablets], and the protein concentration was measured by the Bradford DC protein assay (Bio-Rad, Shanghai, China). Afterwards, 40 μg proteins from each sample were separated on 10% Bis-Tris polyacrylamide gel through electrophoresis, and blotted onto polyvinylidene fluoride (PVDF) membranes. Blots were immunostained with applied primary antibody at 4 °C overnight, and secondary antibody at room temperature for 1 h. The blots were then visualized by the ECL Plus Western Blotting Detection Reagents (GE Healthcare, Shanghai, China).

2.10. Intracellular ceramide measurement

After treatment, cells were washed in PBS and lysed. The lysates were then heated at 70 °C for 5 min and centrifuged at 12,000 rpm for 10 min at 4 °C. The reaction was started by adding 10 μL of supernatant to the tube containing 10 ng recombinant human neutral ceramidase enzyme (10 μL) and incubating for 1 h at 37 °C. The reaction was stopped by adding 55 μL of stop buffer (1:9, 0.07 M potassium hydrogen phosphate buffer: methanol). The released sphingosine was derivatized with o-phthaladehyde (OPA) reagent. After the reaction was stopped, 25 μL of freshly prepared OPA reagent was added for 30 min. An aliquot of 25 μL was analyze by HPLC using a Waters 1525 binary pump system [16]. The fluorescence detector (Waters 2475) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm [16].

2.11. Tumor xenograft animal model

Experiments were performed on male severe combined immunodeficient (SCID) mice according to the regulation of the Institutional Animal Care and Use Committee (IACUC). Tumors for implantation were initially grown from subcutaneous injections of SW-620 cells (200 μL of 3×10^6 cells per mouse) into the right flanks. After 21 days of tumor establishment in mice, when the tumor reached a volumes around 100 mm^3, animals were randomly