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

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A B S T R A C T
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.

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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 management 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 IIa (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 lymphomas 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 underlying 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.
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 (SIP) 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 CO2 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 mm3-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 and 10 mM dithiothreitol and EDTA-free protease inhibitor tablets, and 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 (SIP) 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).

2.4. 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 $5 \times 10^3$ 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 $\times 10^5$/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 $\times 10^5$/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]) $\times$ 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 immunosorbsent 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 Na2VO4, 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-phthaldehyde (OPA) reagent. After the reaction was stopped, 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 $\times 10^6$ cells/mouse) into the right flanks. After 21 days of tumor establishment in mice, when the tumor reached a volumes around 100 mm3, animals were randomly...
divided into two groups. Each animal received daily gastric lavage of 20 mg/kg body weight of AR-42 or vehicle control for a total of five weeks. The tumor volume (in mm$^3$) was calculated by the formula: volume = (width)$^2$*length/2, and the tumor growth curve was presented. At the termination of experiments (week-6), xenograft tumors were isolated and weighted.

2.12. Statistical analysis

The data presented were mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. AR-42 exerts potent anti-proliferative and cytotoxic activities against colon cancer cells

First, we tested the effect of AR-42 on colon cancer cell proliferation. MTT assay was performed. As demonstrated in Fig. 1A, AR-42 dose-dependently inhibited SW-620 cell proliferation. The effect of AR-42 was potent, with an IC-50 less than 1 μM. Further, as shown in Fig. 1B, the anti-proliferative activity of AR-42 was also time-dependent. At least 48 h were needed for AR-42 (1 μM) to exert the anti-SW-620 cell activity. Results from the colony formation assay showed that AR-42 treatment at the concentrations between 0.3 and 3 μM remarkably decreased the number of survival SW-620 colonies (Fig. 1C). In addition, LDH content in conditioned medium of AR-42-treated SW-620 cells was significantly increased (Fig. 1D). The anti-proliferative activity of AR-42 was also observed in HCT-116 colon cancer cells (Fig. 1E), and in ex-vivo cultured primary human colon cancer cells (Fig. 1F). These data together demonstrate the anti-proliferative and cytotoxic activities of AR-42 in colon cancer cells.

3.2. AR-42 induces apoptotic death in colon cancer cells

Proliferation inhibition and cytotoxicity could be the result of cell apoptosis. Thus, the potential effect of AR-42 on colon cancer cell apoptosis was examined. Results from Annexin V FACS assay showed that AR-42 treatment induced significant apoptosis activation in SW-620 cells, and the effect of AR-42 was again dose-dependent (Fig. 2A). The caspase-3 activity of AR-42-treated SW-620 cells was also remarkably increased (Fig. 2B). Meanwhile, expression of cleaved-caspase-3 and cleaved-PARP was induced after corresponding AR-42 treatment, further confirming apoptosis activation (Fig. 2C). Notably, three different apoptosis inhibitors, including the pan-caspase inhibitor z-VAD-fmk (FMK), the caspase-3 inhibitor Ac-DEVD-CHO (CHO), and the apoptosome inhibitor NS3694, dramatically ameliorated AR-42-induced anti-proliferative and cytotoxic activities in SW-620 cells (Fig. 2D and E). Similar results were also obtained in HCT-116 cells (Data not shown). Apoptosis activation, evidenced by Annexin V FACS assay, was also observed in AR-42 (1 μM)-treated HCT-116 cells and primary colon cancer cells.
cancer cells (Fig. 2F). Together, these results show that AR-42-induced cytotoxicity against colon cancer cells is associated with significant apoptosis activation.

3.3. Ceramide production mediates AR-42-induced cytotoxicity in colon cancer cells

One aim of the current study is to investigate the potential involvement of ceramide in AR-42-induced cytotoxicity. We first examined cellular ceramide level in AR-42-treated colon cancer cells. As shown in Fig. 3A, AR-42 dose-dependently increased ceramide production in SW-620 cells. Cellular ceramide started to go up 12 h after AR-42 (1 μM) stimulation, and reached peak level after 24 h (Fig. 3B). The ceramide level in AR42-stimulated HCT-116 cells and primary colon cancer cells was also increased after applied AR-42 treatment (Fig. 3 C). Importantly, exogenously-added cell permeable ceramide (C4) mimicked AR-42’s actions, and induced cytotoxic effects in SW-620 cells (Fig. 3D and E). Meanwhile, C4 ceramide could further increased AR-42-mediated activities (Fig. 3D and E). On the other hand, fumonisin B1, the ceramide synthase inhibitor [19], dramatically attenuated AR-42-induced ceramide production (Fig. 3F), and its activities in SW-620 cells (Fig. 3G and H). Similar results were also reproduced in HCT-116 cells and primary cancer cells (Data not shown). Based on these results, we propose that AR-42 induces ceramide production through the de novo synthesis pathway (ceramide synthase-dependent), which mediates AR-42-induced anti-proliferative and pro-apoptotic activities in colon cancer cells.

3.4. AR-42 inhibits SW-620 cell proliferation in SCID mice

To test the in vivo efficacy of AR-42, SCID mice implanted with SW-620 cells were orally administrated with AR-42. The tumor growth curve results showed that daily gastric lavage of a single dose of AR-42 (20 mg/kg) led to significant inhibition of SW-620 xenografts growth in SCID mice (Fig. 4A). For example, at week-6 of treatment, the volumes of SW-620 tumors in AR-42-treated mice were over 60% smaller than those in the vehicle (0.5% methylcellulose/0.2% Tween 80 gastric lavage) control mice (Fig. 4A). Also at week-6, the AR-42-treated tumors were dramatically lighter than vehicle control tumors (Fig. 4B). Notably, the treated mice
demonstrated no overt clinical toxicities. Nor the mice body weights were significantly affected by the AR-42 treatment (Fig. 4C). Western blot results in Fig. 4D showed that cleaved caspase-3 and cleaved-PAR expressions were dramatically upregulated in AR-42-treated tumors, indicating apoptosis activation. Together, these results show that oral administration of a single dose of AR-42 dramatically inhibits proliferation of SW-620 cells in SCID mice.

4. Discussions

Ceramide is a well-known signaling mediator of cell apoptosis [13,14]. A large number of cytotoxic agents, including numerous anti-cancer drugs, were shown to induce ceramide production and subsequent cell apoptosis [13,14]. At the meantime, however, cancer cells have the ability to remove excess ceramide through diverse metabolic pathways, causing chemo-resistance [20,21]. It has been suggested that agents that would facilitate intracellular ceramide accumulation, either through de novo synthesis and/or metabolic clearance inhibition, could favor a pro-apoptotic outcome [22–25]. In this study, we showed that AR-42 induced an early (24 h) but significant ceramide de novo production in colon cancer cells, which was required for subsequent cell apoptosis. Fumonisin B1, a ceramide synthase inhibitor [19], suppressed AR-42-mediated ceramide production and cell apoptosis. Yet, exogenously-added cell permeable ceramide (C4) mimicked AR-42’s phenotypes, and induced cytotoxic effects in SW-620 cells. Meanwhile, this ceramide could further increased AR-42-mediated activities in colon cancer cells. All these findings support an important role of ceramide in AR-42-mediated in vitro anti-colon cancer cell activity.

Based on our results, we propose that AR-42-induced ceramide production was subject to metabolic clearance in colon cancer cells.
Co-administration of sphingolipid glucosylation inhibitor PDMP [25,26] could further increase ceramide accumulation, leading to substantial colon cancer cell apoptosis. The results of this study are consistent with previous findings showing that PDMP could potentiate cancer cell death induced by Taxol [27], vincristine [27] and curcumin [25] through augmenting ceramide production. The detailed underlying mechanisms of AR-42-induced ceramide pathway grantees further characterizations. More studies are also needed to explore the link between HDAC inhibition and ceramide production by AR-42.

Thus, we show that AR-42, a novel pan HDAC inhibitor, induces potent proliferation inhibition and apoptosis in both established and primary human colon cancer cells. In vivo, oral administration of a single dose of AR-42 could dramatically restrain SW-620 xenografts growth in SCID mice. We propose that ceramide production might be the key signaling mediator for AR-42-exerted activities in colon cancer cells. Also much still need to be done, the preclinical results of this study suggest that AR-42 might be further be inspected as a novel anti-colon cancer agent.

Conflict of interests

The authors have declared no conflict of interests.

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References