ORIGINAL ARTICLE

DZNep inhibits the proliferation of colon cancer HCT116 cells by inducing senescence and apoptosis

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Abstract EZH2 is over-expressed in human colon cancer and is closely associated with tumor proliferation, metastasis and poor prognosis. Targeting and inhibiting EZH2 may be an effective therapeutic strategy for colon cancer. 3-Deazaneplanocin A (DZNep), as an EZH2 inhibitor, can suppress cancer cell growth. However, the anti-cancer role of DZNep in colon cancer cells has been rarely studied. In this study, we demonstrate that DZNep can inhibit the growth and survival of colon cancer HCT116 cells by inducing cellular senescence and apoptosis. The study provides a novel view of anti-cancer mechanisms of DZNep in human colon cancer cells.

1. Introduction

Colon cancer has become the third leading cause of tumor-related deaths in the world[1], and its incidence is rapidly rising in Chinese cities along with economic development[2]. Colon cancer normally develops through a multistep process that results from the progressive accumulation of mutations and epigenetic alterations in tumor suppressor genes and oncogenes, which include both DNA hypermethylation and chromatin modifications such as histone methylation and deacetylation[3]. Recently, the study of epigenetic gene silencing by histone methylation has gained attention in the development of colon cancer.

Enhancer of zeste homolog-2 (EZH2), a histone methyltransferase, is the catalytic subunit of polycom repressive complex 2 (PRC2) that methylates H3K27me3 and results in epigenetic control of cancer genes expression[4,5]. Up-regulation of EZH2 has been found in many malignant human tumors, including colorectal carcinomas, and a high level of EZH2 positively correlates with colon cancer metastasis[6]. Thus, EZH2 has become a promising therapeutic target for colon cancer treatment.

3-Deazaneplanocin A (DZNep), a S-adenosyl-L-homocysteine (AdoHcy) hydrolase, has been shown to be capable of reducing the expression of EZH2[7]. Although DZNep was shown to be able to inhibit the proliferation as well as the metastasis and invasion of a variety of cancer cells, such as lung cancer and breast cancer[8,9], little is known with regard to the effects and mechanisms of DZNep on colon cancer cells.

Cellular senescence has become a vital anti-cancer mechanism due to irreversible cell cycle arrest and permanent inhibition of cancer cell growth[10,11]. Inhibition of EZH2 was shown to promote senescence and apoptosis induced by doxorubicin in gastric cancer cells harboring mutant $p53$[12]. However, direct evidence linking EZH2 inhibition by pharmacological intervention with cell senescence in colon cancer cells is still lacking. Here, we investigated the role of DZNep in colon cancer HCT116 cells, and found that both senescence and apoptosis contribute to the anti-cancer effects of the compound.

2. Materials and methods

2.1. Reagents and materials

3-Deazaneplanocin A hydrochloride (DZNep) and propidium iodide (PI) were purchased from Sigma-Aldrich. MTT was purchased from Amresco (Solon, OH). AnnexinV-PI was purchased from Beijing 4A Biotech Co., Ltd. Senescence associated β-galactosidase (SA-β-gal) staining kit was purchased from Beyotime Institute of Biotechnology. Antibody for β-actin and EED were purchased from Sigma. EZH2, SUZ12 and other antibodies were purchased from Cell Signaling Technology.

2.2. Cell culture

HCT116 was cultured and maintained in DMEM (Hyclone, Logan, UT) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technology), 100 U/mL penicillin G (Amresco) and 100 µg/mL streptomycin (Amresco). The cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Cellular viability measurement

Cellular viability was performed with the MTT assay as described previously[7]. The cells were seeded at 5000 cells/well in 96-well plates for 24 h and treated with different concentrations of DZNep for another 48 h, then incubated in MTT solution for 4 h at 37 °C. MTT solution was then carefully removed and replaced by 100 µL DMSO to dissolve formazan crystals. Absorbance was measured using a microplate reader (Bio-Rad) at a wavelength of 570 nm. The control group was set as 100%, and IC_{50} values were calculated and plotted with Graphpad Prism 5. This experiment was conducted in triplicates three times.

2.4. Colony formation and cell growth assay

Cellular sensitivity to DZNep was determined using a previously described protocol[13]. In brief, HCT116 cells were seeded at a density of 500 cells/well into 35 mm dishes and treated as indicated. After incubation for 2 weeks, the colonies obtained were fixed in 3% paraformaldehyde for 15 min and then washed with PBS followed by staining with hematoxylin. HCT116 cells were seeded into 96-well plate with 10^4 cells/well in triplicate for cell counting at indicated time points with a Countess Cell Counter (Life Technology).

2.5. SA-β-gal staining

SA-β-gal activity staining was performed using SA-β-gal staining kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The cells were seeded at 5×10^4 cells/well in 6-well plates, and treated with DZNep at 5 µmol/L for the indicated time. The cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells then were incubated overnight at 37 °C with the working solution containing 0.05 mg/mL X-gal using SA-β-gal staining kit (Beyotime Biotechnology).

2.6. Apoptosis assay

Apoptosis assay was performed using the Annexin V Alexa Fluor 488/PI apoptosis detection Kit (Beijing 4A Biotech Co., Ltd.) according to the manufacturer's instructions. Briefly, after treated as indicated, both floating and non-floating cells were collected after trypsinization, and then washed twice in PBS, followed by re-suspension in Annexin-V binding buffer at a concentration of 10^6 cells/mL. An aliquot of 100 µL of this suspension was stained with 5 µL of Annexin-V Alexa Fluor 488 and 5 µL PI, and incubated at room temperature in the darkness for 15 min. Subsequently, the cells were added to 400 µL PBS and subjected to FACS analysis in a FACS Calibur (Becton Dickinson instruction, BD Bioscience).

2.7. Flow cytometry and cell cycle analysis

The cells were harvested and fixed in 70% ethanol at -20 °C overnight. The fixed cells were stained with PI for 1 h after treatment with RNase at 37 °C for 30 min in the darkness. The stained cells were analyzed for DNA content by FACS. The cell cycle fractions were quantified using the Cell Quest Acquisition and Analysis (BD Bioscience).

2.8. Immunoblotting analysis

Immunoblotting analysis was determined using a previously described protocol[14]. The cells were washed with icy PBS and

lysed with lysis buffer containing protease inhibitors cocktail (CST) plus 1 mmol/L PMSF. Protein concentration was determined by Bradford assay. Equal amounts of protein were separated by 10%–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Whatman). The membranes were blocked with 5% nonfat milk PBS-T buffer at room temperature for 1 h, and then incubated with primary antibodies at 4 °C overnight. The membranes were then incubated for 1 h with an appropriate horseradish peroxidase linked secondary antibody, and electrochemiluminescence was performed with a ChemiImager 5500 imaging system (Alpha Innotech Corporation).

2.9. Statistical analysis

SPSS 19.0 statistical software was used for statistical analysis. Values are shown as the mean ± SD. Statistical analysis was carried out using Student's t-test. Differences between groups were identified as statistically significant at $P < 0.05$.

3. Results

3.1. DZNep inhibits the growth of HCT116 cells

MTT assay was used to screen the effective concentration of DZNep on human colon cancer HCT116 cells. The cells were treated with DZNep for 48 h at the concentration of 0, 0.08, 0.4, 2, 10 and 50 µmol/L. DZNep induced potent growth inhibition in a dose-dependent manner (Fig. 1A). We used 5 µmol/L DZNep at IC_{50} range for further study, and found that DZNep significantly inhibited the cellular proliferation in a time-dependent way, as indicated by the growth curve (Fig. 1B). Longer treatment of DZNep also displayed significant growth inhibition with colony formation assay (Fig. 1C).

3.2. DZNep induces G1 cell cycle arrest in HCT116 cells

We next used flow cytometry to determine whether the suppression of proliferation was due to cell cycle arrest in HCT116 cells. After treatment with DZNep for 24 h, the cellular ratio in G1 phase increased from 41.09% to 55.14%, and the ratio in S phase decreased from 38.24% to 30.73% (Fig. 2). Higher ratio of G1 phase was seen upon DZNep treatment for 48 h and 72 h (Fig. 2). This suggests that DZNep induces G1 cell cycle arrest in HCT116 cells.

3.3. DZNep induces cellular senescence in HCT116 cells

Inhibition of EZH2 is suggested to induce cancer cell senescence[15]. We thus checked whether DZNep was able to induce cell senescence in HCT116 cells as well. After the cells were treated with DZNep for 48 h, 72 h and 96 h, we used SA-β-gal staining to observe the senescence-like phenotype, and found that many cells became positively stained upon DZNep exposure (Fig. 3A). This suggests that DZNep induces cellular senescence simultaneously under these conditions. Similarly, senescence was observed upon EZH2 siRNA treatment for 72 h (Fig. 3B).
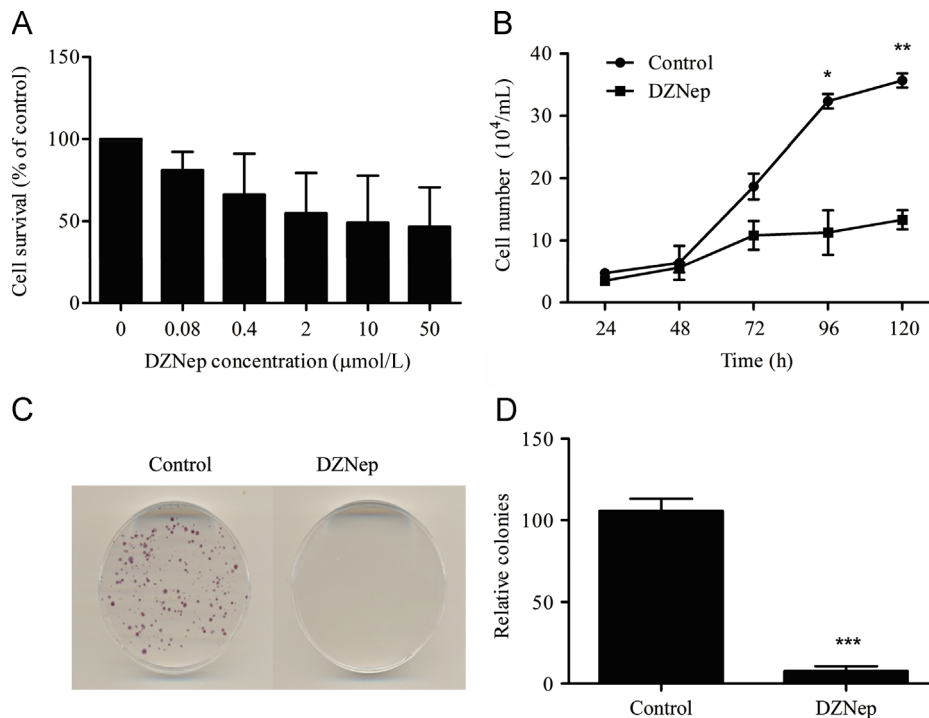

Figure 1 DZNep inhibited the proliferation of HCT116 cells. (A) Effect of DZNep on the proliferation of HCT116 determined by MTT assay. The cells were treated in 96-well plates for 48 h with DZNep at the indicated concentration. (B) Effect of DZNep on the growth curve. The cells were seeded into 24-well plates with 1×10^4 cells/well for 24 h, and then treated with or without 5 µmol/L DZNep and counted at indicated points ($^*P < 0.05$, $^{**}P < 0.01$ vs. control, $n = 3$). (C) Colony formation upon DZNep treatment. The cells were seeded in 35 mm dishes with 500 cells per dish, and then treated with 5 µmol/L DZNep for 2 weeks. The percentage of the colonies was normalized relative to the control ($^{***}P < 0.001$ vs. control, $n = 3$).

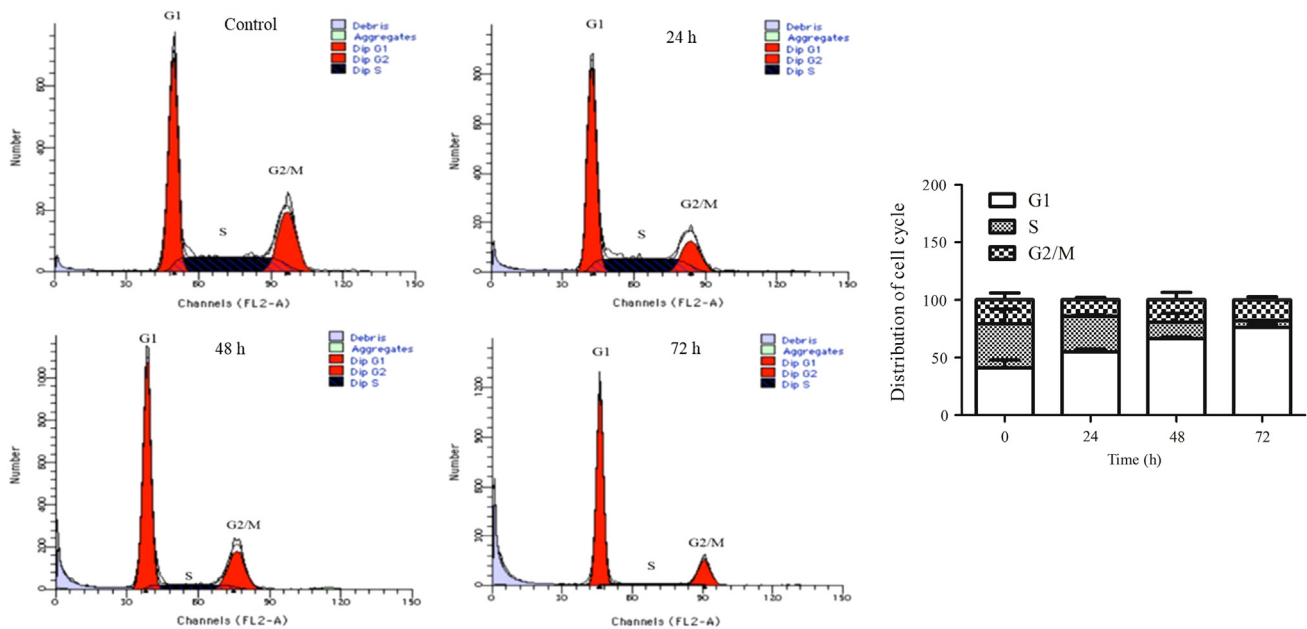**Figure 2** DZNep induced cell cycle arrest in HCT116 cells. The cells were treated with 5 µmol/L DZNep for the indicated time before being subjected to PI staining and FACS analysis as described in Section 2.

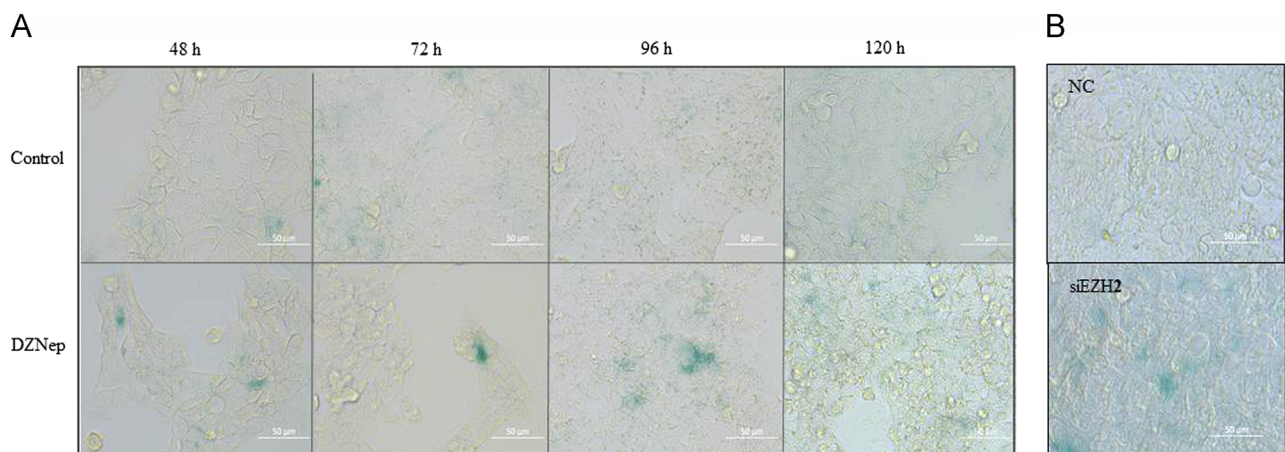**Figure 3** Inhibition the expression of EZH2 induced cellular senescence in HCT116 cells. The cells were treated with 5 µmol/L DZNep (A) or with siRNA (B) for the indicated time, and senescence was analyzed with SA-β-gal staining and photographed (40 ×). Scale bar: 50 µm.

3.4. DZNep regulates cell cycle and senescence related proteins expression in HCT116 cells

We further investigated the possible molecular mechanisms involved. The expression levels of PRC2 proteins (EZH2, EED and SUZ12) were confirmed to be suppressed after treatment with DZNep for 48 h, 72 h, 96 h and 120 h, as shown by immunoblot analysis in HCT116 cells (Fig. 4). Cell cycle and senescence regulatory proteins were investigated to clarify the underlying mechanism[16], and a significant increase of p21 and decrease of p-RB protein expression were found upon exposure to the agent. In addition, we examined the expression of p27 and p-CDC2, which are cell cycle regulators as well[17], and found that DZNep

increased p27 and decreased p-CDC2 expression in a time-dependent manner (Fig. 4).

3.5. DZNep induces apoptosis in HCT116 cells

We also asked whether apoptosis is involved. Flow cytometry analysis using Annexin V/PI demonstrated that the apoptotic fraction of HCT116 cells was increased in a time-dependent manner following treatment with DZNep (Fig. 5A). We also analyzed PARP cleavage, a well-known molecular event indicating apoptosis induction[18], and found a significant increase of PARP cleavage upon treatment with 5 µmol/L DZNep for 24 h,

48 h and 72 h (Fig. 5B). This indicates that DZNep induces apoptosis as well.

4. Discussion

EZH2-targeted therapy has become an effective strategy for cancer treatment. For example, blocking the expression of EZH2 by siRNA leads to G1/S cell cycle arrest and growth inhibition in colon cancer[19]. Moreover, decreased EZH2 expression is found to be able to increase the sensitivity to TRAIL-mediated apoptosis in

Figure 4 Relative protein expression levels affected by DZNep in HCT116 cells. The cells were treated with 5 μmol/L DZNep for 72 h before being subjected to immunoblot assay as described in Section 2.

human colon cancer HT29 cells[20]. In the present work, we provide first evidence to show that inhibition of EZH2 by DZNep can directly induce cell senescence, along with apoptosis, in human colon cancer HCT116 cells.

Cellular senescence of HCT116 cells induced by DZNep is demonstrated by G1 phase arrest and an increased number of cells with positive SA-β-gal staining, which is one of most commonly used senescence markers. We found that the cell cycle arrest caused by DZNep can be addressed by the increase of p21 expression and decrease of p-RB expression. Moreover, p27 and p-CDC2, which blocks cell cycle progression at the G1 transition and promotes G1 and G2/M transition[17,21], respectively, are found to be separately up-regulated and down-regulated as well. In line with our data, silencing of EZH2 is reported to be linked to the increase of p27 expression in pancreatic cancer cells and HCT116 cells[11,22]. We also checked the expression of p16, another molecular marker associated with senescence and cell cycle, but did not find any significant change in the protein level (data not shown).

In our work, we also found that DZNep can dose- and time-dependently induce colon cancer cell apoptosis, as confirmed by FACS analysis and PARP cleavage (Fig. 5). Accordingly, DZNep-induced apoptosis has been described in osteosarcoma U2OS and breast cancer MCF7 cells[23,24]. More importantly, DZNep at a similar dose range was found to induce apoptosis in human colon cancer stem cells as well[25]. Therefore, combined mechanisms are involved in the anti-cancer effects of DZNep on colon cancer. A study by Wu et al.[23] has shown that DZNep induces apoptosis and G1 cell cycle arrest in response to DNA damage in both *p53*-proficient and *p53*-deleted HCT116 cells, resulting in induction of FBXO32, a decrease of p21 and an increase of PARP cleavage. This seems to be inconsistent with our data which suggests an increase of p21 upon DZNep exposure. The discrepancy in p21 regulation may rely on the different stresses imposed on the cells.

Taken together, our results demonstrate that DZNep inhibits the growth and survival of colon cancer HCT116 cells by inducing
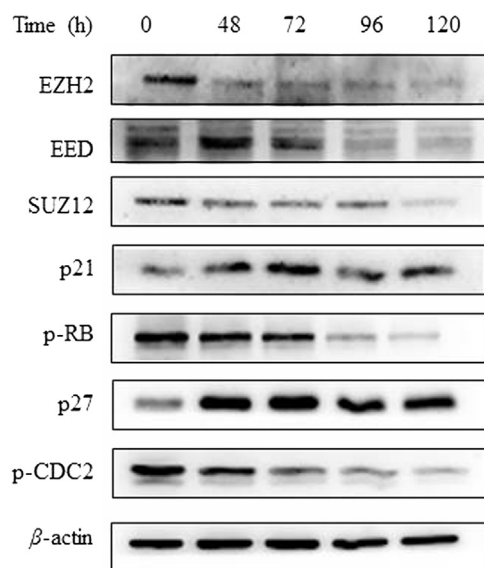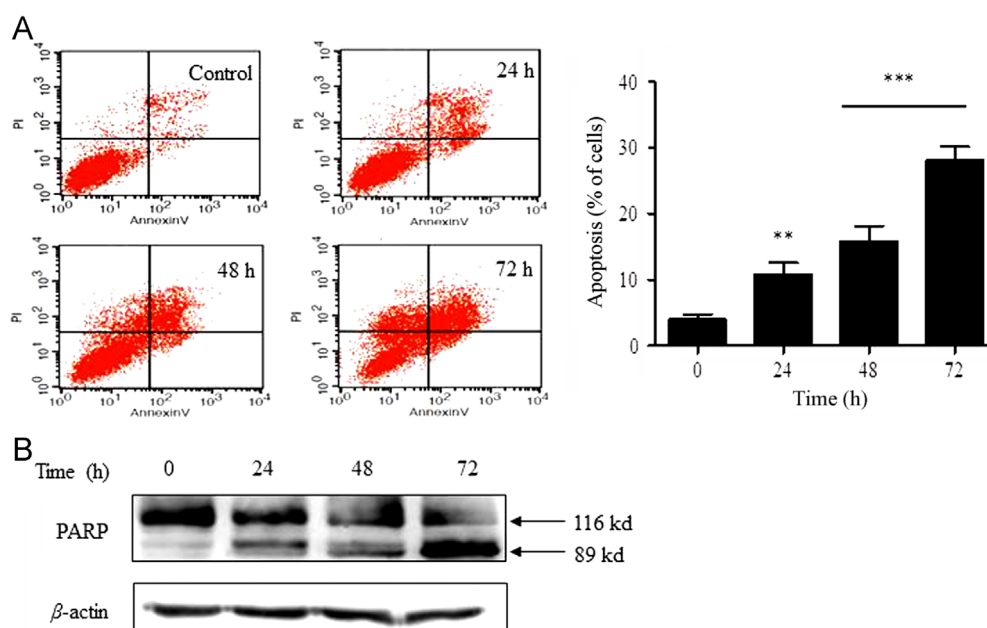
Figure 5 DZNep induced apoptosis in HCT116 cells. (A) Apoptosis induced by DZNep was detected by AnnexinV/PI staining. The cells were seeded in 6-well plates and treated with 5 μmol/L DZNep for the indicated time, and then subjected to FACS analysis to determine the percentage of early apoptosis cells (lower right quadrant) and late apoptosis cells (upper right quadrant) ([**]$P < 0.01$, [***]$P < 0.001$ *vs.* control). (B) PARP cleavage analysis was performed in HCT116 cells following treatment with DZNep (5 μmol/L) as indicated. β-actin was used as loading control.

cellular senescence and apoptosis. The study also provides first evidence showing the direct correlation between inhibition of EZH2 and cellular senescence in colon cancer cells.

Acknowledgments

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