ONCOLOGY

Azacitidine enhances sensitivity of platinum-resistant ovarian cancer cells to carboplatin through induction of apoptosis

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OBJECTIVE: The objective of the study was to investigate whether azacitidine sensitizes platinum-resistant ovarian cancer cells to carboplatin and the possible mechanisms involved.

STUDY DESIGN: We tested the in vitro antitumor activity of azacitidine both alone and combined with carboplatin in the ovarian cancer cell line 2008/C13 and Hey by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays and investigated the potential mechanisms by flow cytometry, terminal transferase deoxyuridine 5-triphosphate nick-end labeling assay, Western blot, reverse transcriptase–polymerase chain reaction (PCR), and promoter methylation–specific PCR.

RESULTS: Sequential treatment (ie, 24-hour azacitidine pretreatment followed by 48-hour cotreatment with azacitidine and carboplatin) significantly inhibited growth in 2008/C13 and Hey cells. More apoptotic cells were induced in 2008/C13 cells by sequential treatment than by a single drug. Increased cleaved caspase-3 and -8 were seen in 2008/C13 cells after sequential treatment with azacitidine and carboplatin. DR4 was demethylated, and DR4 messenger ribonucleic acid expression was increased in 2008/C13 cells after the 24-hour azacitidine treatment.

CONCLUSION: Azacitidine enhanced the sensitivity of platinum-resistant ovarian cancer cells to carboplatin associated with caspase-3– and -8–dependent apoptosis pathway and reexpression of DR4.

Key words: apoptosis, azacitidine, carboplatin, DR4, ovarian cancer

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Disease recurrence continues to be a major problem for patients with advanced ovarian cancer; only 10-20% of such patients can be cured.[1] The main reason for disease recurrence is the acquisition of cellular resistance to the platinum-based treatment regimen. New treatment strategies to overcome this drug resistance are urgently needed.

DNA methylation is an epigenetic modification that leads to an alteration of gene expression.[2] Aberrant DNA methylation is involved in the initiation and development of many cancers,[3] including ovarian cancer.[4] Hypermethylation of CpG islands (CG-rich regions, usually associated with transcriptionally active genes) is frequently found in tumor suppressor genes, such as p16[5] and the mismatch repair gene human MutL homologue-1,[6-8] or in proapoptotic genes, such as tumor necrosis factor–related apoptosis-inducing ligand receptor 1 (TRAIL-R1, also known as death receptor 4 [DR4], Apo-2, and TNFRSF10A).[9] Loss or decreased expression of these tumor suppressor or proapoptotic genes is thought to be one of the factors that confer resistance to chemotherapeutic agents.[6,9]

One strategy used to overcome drug resistance is to reverse the methylation-induced silencing of the tumor suppressor or proapoptotic genes. Azacitidine (Pharmion Corp, Boulder, CO), a cytidine analog, is a methylation inhibitor.[10,11] Although different mechanisms are also involved in the antitumor action of azacitidine, including inhibition of ribonucleic acid (RNA) metabolism and DNA replication and induction of apoptosis,[12] methylation inhibition via inhibiting DNA methyltransferase has been proposed as the main mechanism responsible for the antineoplastic action of azacitidine, when azacitine is administered at the Food and Drug Administration–recommended dosage (75 mg/m^2 per day × 7).[10,13,14]

Little information is available in the literature about the in vitro antiproliferative effect of azacitidine in combination with carboplatin on ovarian cancer cells, especially in platinum-resistant ovarian cancer.[15] As a demethylating agent, azacitidine might be effective at overcoming drug resistance in ovarian cancer. In this study, we sought to investigate whether azacitidine sensitizes platinum-resistant ovarian cancer cells to carboplatin in vitro and which possible mechanisms are involved in this sensitization.

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MATERIALS AND METHODS

Cell lines and drug treatment

Azacitidine was dissolved in distilled water at a stock concentration of 10^{-2} M. Carboplatin (Bristol-Myers Squibb Co, Princeton, NJ) was obtained from the pharmacy of our institute. Experiments were conducted on 6 epithelial ovarian cancer cell lines, including 5 platinum-resistant (2008/C13, Hey,[16] NMP-1,[17] OVCAR3, and SKOV3) and 1 platinum-sensitive cell line (A2780). All cell lines were obtained from Dr Ralph S. Freedman (Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center).[18]

Cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum and incubated at 37°C in 5% CO_2 and 95% humidity. For cotreatment, cells were treated with azacitidine and carboplatin simultaneously for 48 hours. For sequential treatment, cells were pretreated with azacitidine alone for 24 hours followed by the concurrent treatment with azacitidine and carboplatin for 48 hours. Cells treated with vehicle or 72 hours of azacitidine or 48 hours of carboplatin at the same concentration were used to compare with sequential treatment. All experiments were repeated 3 times.

Cell proliferation and drug cytotoxicity assays

Cell growth and viability were determined by using a nonradioactive cell proliferation and cytotoxicity assay via the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method with an EZ4U kit (catalog #04-BI-5000; American Laboratory Products Co, Windham, NH). Cells were seeded in quadruplicate in 96-well plates at 4000 cells/well and allowed to adhere to the plate overnight. The next day, cells were treated with azacitidine and carboplatin alone or in combination. A series of carboplatin concentrations (4-250 μg/mL) and 3 levels of azacitidine concentrations (0.1, 1, and 10 μM) were used for cotreatment and sequential treatment with the 2 drugs.

After the treatment periods, MTT assays were performed according to the manufacturer's instructions. The percentage of cell survival was determined by the ratio of absorbance of the sample vs that of the control. The combination index, calculated as follows, was used to determine whether there was a synergistic or additive effect between azacitidine and carboplatin:

$$\text{Combination index} = \frac{\begin{array}{c}\text{cell survival (\%) after}\\ \text{azacitidine treatment} \times\\ \text{cell survival (\%) after}\\ \text{carboplatin treatment}\end{array}}{\begin{array}{c}\text{Cell survival (\%) after combined}\\ \text{treatment with the two agents}\end{array}}$$

A combination index of 1 indicated an additive effect, and a value > 1 indicated a synergistic effect.[19,20]

Flow cytometry for detection of the sub-G_1 fraction

A total of 1.8×10^6 cells were seeded in a T25 flask and were allowed to adhere to the flask overnight. The next day, they were treated with azacitidine and carboplatin either alone or in combination for indicated time. After treatment, the cells were harvested using 0.5% EDTA-trypsin, washed 3 times with ice-cold 1× phosphate-buffered saline (PBS), and fixed with 70% ethanol at room temperature for 15 minutes. Cell pellets were then stained with propidium iodide solution (50 μg/mL of propidium iodide [Calbiochem, San Diego, CA] and 20 μg/mL of ribonuclease A [Sigma-Aldrich Corp, St Louis, MO] at room temperature for 15 minutes. The fraction of cells that were in the sub-G_1 phase was determined using a Coulter XL-MCL flow cytometer (Coulter, Miami, FL).

Terminal transferase deoxyuridine 5-triphosphate nick-end labeling (TUNEL) apoptosis assay

For TUNEL assay, 2008/C13 cells were treated with azacitidine (10 μM, 72 hours) and carboplatin (63 μg/mL, 48 hours) alone or with sequential treatment of the 2 drugs as described above. After treatment, cells were harvested and washed. Cytospin preparations were fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS, air dried, and then stored at -20°C until use. The TUNEL assay was done using an APO-BRDU-IHC kit (catalog #AH1001; Phoenix Flow System, Inc, San Diego, CA) according to the manufacturer's instructions. We counted a total of 300 cells for each treatment to determine the percentage of apoptotic cells.

Western blot analysis

To examine the effect of single azacitidine on protein expression, 2008/C13 cells were treated with 0-50 μM azacitidine for 24 hours. Sequential treatment was performed as described in previous text with 10 μM of azacitidine and 63 μg/mL of carboplatin. After being harvested and washed, the cells were lysed with a modified radioimmunoprecipitation lysis buffer (10 mM Tris HCl [pH, 8.0], 10 mM EDTA [pH, 8.0], 0.15 M NaCl, 1% Nonidet P-40, and 0.5% sodium dodecyl sulfate [SDS]) containing a freshly added protease inhibitor. After 30 minutes' incubation on ice, lysates were cleared by centrifugation at 12,000 rpm for 30 minutes at 4°C.

Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Thirty-five micrograms of protein was separated on 8-15% SDS-polyacrylamide gradient gel, transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK), and probed with the following diluted antibodies: poly-[adenosine diphosphate-ribose] polymerase (PARP) (1:800; Promega Corp, Madison, WI), caspase-3 (1:200; Cell Signaling Technology, Danvers, MA), cleaved caspase-8 (18C8; Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2500; Santa Cruz Biotechnology, Santa Cruz, CA).

Signals were visualized on reaction with enhanced chemiluminescence detection reagent (Amersham Biosiences). GAPDH was used as the internal control. Image analysis (Scion Image for Windows; Scion Corp, Frederick, MD) was used for semiquantitative measurement of protein expression.