Pharmaceutical Research, Vol. 25, No. 10, October 2008 (© 2008)
DOI: 10.1007/s11095-008-9621-4

Research Paper

Diammine Dicarboxylic Acid Platinum Enhances Cytotoxicity in Platinum-Resistant Ovarian Cancer Cells through Induction of Apoptosis and S-Phase Cell Arrest

Hong Zheng,[1,4] Wei Hu,[1,5] Dongfang Yu,[2] De-Yu Shen,[1] Siqing Fu,[3] John J. Kavanagh,[1] I-Chien Wei,[2] and David J. Yang[2,5]

Received January 9, 2008; accepted April 30, 2008; published online May 29, 2008

Purpose. Polysaccharides such as chondroitin play a potent role in tumor growth, tissue repair and angiogenesis. These properties make chondroitin a good candidate for novel drug delivery systems. Diammine dicarboxylic acid platinum (DDAP), a novel polymeric platinum compound, was developed by conjugating the platinum analogue to aspartate–chondroitin for drug delivery to tumor cells. DDAP improves platinum solubility which may reduce systemic toxicity and be more efficacious than cisplatin in killing tumor cells.
Methods. We tested and compared the cytotoxic effects of DDAP and CDDP on the platinum-sensitive 2008 and A2780 ovarian cancer cell lines and their platinum-resistant sublines 2008.C13 and A2780cis; we also investigated DDAP's mechanism of action.
Results. In the platinum-sensitive cell lines, the cytotoxic effects of DDAP and CDDP were comparable. However, in the platinum-resistant sublines, significantly greater cell-growth inhibition was induced by DDAP than by CDDP, especially at lower doses. DDAP also induced more apoptosis than CDDP did in the 2008.C13 subline, which was partially mediated by the caspase 3-dependent pathway. In addition, lower (but not higher) doses of DDAP arrested 90% of S-phase 2008.C13 cells, which might be associated with up-regulation of p21 and maintenance of low cyclin A expression. Furthermore, greater cellular uptake of DDAP was seen in platinum-resistant than in platinum-sensitive ovarian cancer cells.
Conclusions. Low-dose DDAP enhances drug delivery to platinum-resistant ovarian cancer cells and substantially inhibits their growth by inducting apoptosis and arresting cells in the S-phase, suggesting that DDAP may overcome platinum resistance in ovarian cancer.

KEY WORDS: CDDP; CDDP analogue; drug resistance; ovarian cancer.

INTRODUCTION

Among women with ovarian cancer, advanced-stage disease is diagnosed in about 70%. Despite extensive efforts to increase patient survival, the overall survival rates for patients with advanced epithelial ovarian cancer have improved only slightly during the past 20 years (1–3). Combination chemotherapy, consisting of a platinum analogue and paclitaxel, is the current standard of care. Although most patients respond initially, the disease usually relapses because of acquired resistance to the chemotherapeutic agents, including cisplatin (*cis*-diamminedichloroplatinum(II) [CDDP]) and carboplatin (4–10).

CDDP, a clinically important antitumor drug, is particularly effective against ovarian cancer. It is generally accepted that DNA is a crucial target for CDDP cytotoxicity. 1,2-Cross-links account for 90% of the lesions formed by CDDP, with the main lesions made up of intrastrand cross-links between purine bases. Despite the strong antitumor activity of CDDP, dose-related toxicity, such as nephrotoxicity and neurotoxicity, and acquired drug resistance limit its use (11,12). The onset of resistance creates a further therapeutic complication in that tumors failing to respond to cisplatin are cross-resistant to diverse unrelated drugs; thus, the benefits of second-line chemotherapy diminish substantially, and patients eventually succumb to their disease (13). These drawbacks give strong impetus for the development of new platinum-based drugs to circumvent resistance and decrease toxicity to normal cells (11,13–16).

Despite the advances that have been made in the field of targeted cancer therapies, some major limitations remain in

[1] Department of Gynecologic Oncology, Unit 1362, The University of Texas M. D. Anderson Cancer Center, 1155 Pressler Street, Houston, Texas 77030, USA.
[2] Department of Experimental Diagnostic Imaging, Unit 59, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, Texas 77030, USA.
[3] Department of Gynecologic Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA.
[4] Department of Gynecology, Beijing Cancer Hospital, Beijing 100036, China.
[5] To whom correspondence should be addressed. (e-mail: weihu@mdanderson.org; e-mail: dyang@mdanderson.org)

the delivery of cytotoxic drugs *in vivo*. For an anticancer agent to have a high therapeutic index, a carrier is needed that can specifically target tumor cells. A combination of polysaccharides and aspartic acid can be such a carrier. Polysaccharides (17–20) such as heparin, chitosan and chondroitin, play a potent role in tumor growth, tissue repair, and angiogenesis and have been applied to drug delivery (20–22). In order to conjugate platinum to chondroitin, aspartic acid was selected as a spacer. Expression of system L amino acid transporters (LAT) is upregulated in many types of tumor cells (23). Among amino acids, aspartic acid has two acid moieties which could be conjugated with platin derivative. The macromolecule that results from combining platinum with aspartate–chondroitin may have more selectivity and efficacy in cancer treatment.

Diammine dicarboxylic acid platinum (DDAP), a novel, potent chemotherapeutic compound developed by Drs. David Yang and Dongfang Yu at the University of Texas M. D. Anderson Cancer Center, is a sugar-based platinum complex consisting of 21.87% (*w*/*w*) platinum. The novel structure of DDAP was confirmed by ^{1}H nuclear magnetic resonance and elemental analysis. DDAP differs from CDDP in that the dichlorine portion of CDDP is replaced by aspartate acid conjugated to chondroitin in DDAP, and the aspartate–chondroitin is then chelated to platinum. We aimed at enhancing platinum solubility which may reduce systemic toxicity and be more efficacious than cisplatin in killing tumor cells *via* slowly releasing platinum from the platinum–aspartate–chondroitin macromolecule. Compared with the results we have seen with CDDP, we expected DDAP to show increased specificity for tumor cells, to enhance the cytotoxicity of platinum in platinum-resistant ovarian cancer, and to decrease the toxicity of platinum in normal cells.

In this preclinical study, we compared the growth-inhibitory effect of DDAP with that of CDDP on platinum-sensitive and platinum-resistant ovarian cancer cells and investigated the mechanism of action of this new compound. To our knowledge, this is the first study to demonstrate the superior efficacy of low-dose DDAP in inhibiting the growth of platinum-resistant ovarian cancer cells by the enhancement of apoptosis and induction of S-phase arrest, which were associated with up-regulated p21 expression and low cyclin A expression. This study may provide the foundation for clinical applications of DDAP in the treatment of ovarian cancer.

MATERIALS AND METHODS

Chemicals and Reagents

CDDP, obtained from Sigma-Aldrich Corporation (St. Louis, MO), was freshly dissolved in dimethyl sulfoxide (DMSO) at a stock platinum concentration of 3 mg/mL. DDAP (molecular structure shown in Fig. 1) was freshly dissolved in phosphate-buffered saline (PBS) at a stock platinum concentration of 3 mg/mL. Monoclonal antibody against cyclin A (anti-cyclin A [Ab-2] mouse mAb [BF683],

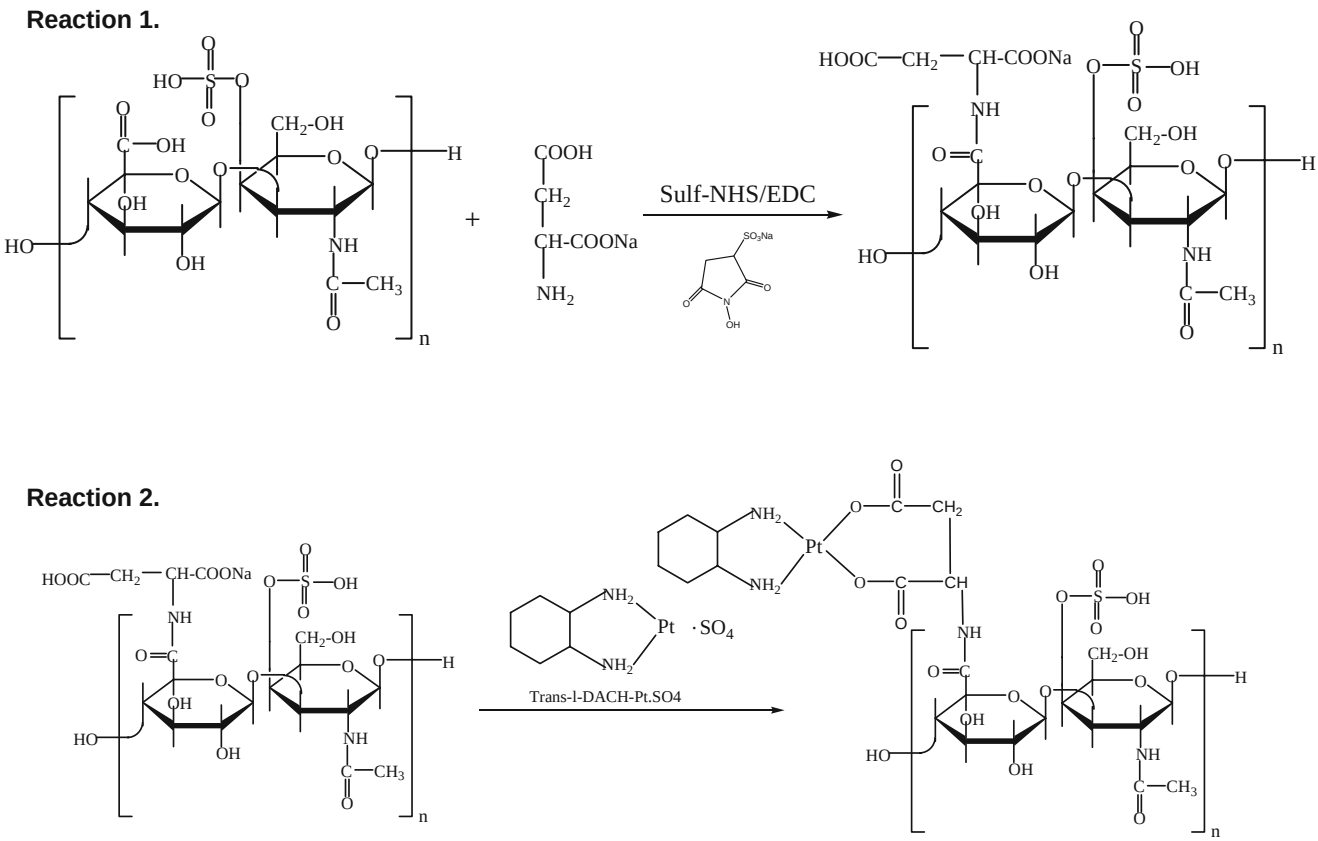

Fig. 1. The synthesis and molecular structure of diammine dicarboxylic acid platinum (DDAP). Sulfo-NHS/EDC, sulfo-*N*-hydroxysuccinimide/1-ethyl-3-[3-dimethylaminopropyl] carbodiimide-hydrochloride.

Calbiochem and Oncogene Research Products product lines) was obtained from EMD Biosciences (San Diego, CA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), polyclonal antibody against p21 (C-19) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody against proliferating cell nuclear antigen (PCNA) (ab18197) was obtained from Abcam (Cambridge, MA). Polyclonal antibody against cleaved caspase 3 was obtained from Cell Signaling Technology (Danvers, MA). Polyclonal antibody against the 85-kDa caspase-cleaved fragment of human poly (ADP-ribose) polymerase (PARP) was obtained from Promega Corporation (Madison, WI).

Cell Cultures

A platinum-sensitive ovarian cancer cell line (2008) and its platinum-resistant subline (2008.C13) were obtained from Dr. Zahid Siddik at M. D. Anderson Cancer Center. A second pair of platinum-sensitive (A2780) and platinum-resistant (A2780cis) ovarian cancer cell lines was obtained from the European Collection of Cell Culture (Wiltshire, SP4 0JG, UK). All cell lines remained in monolayer culture with RPMI 1640 medium supplemented with 10% fetal bovine serum and glutamine (2 mM) in a 5% CO_2 incubator at 37°C.

Synthesis of DDAP

DDAP was synthesized in three steps shown in Fig. 1. In the first step, conjugation of aspartate to chondritin was prepared. Chondroitin sulfate (a sodium salt, 412.4 mg) was dissolved in 2.0 mL of H_2O and 2.5 mL of 1 M $NaHCO_3$. *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) (201.4 mg, 0.927 mmol) was added to the above solution. The mixture was stirred for 6 min at room temperature (25°C). L-aspartic acid (monosodium salt) (514.3 mg, 2.971 mmol) was dissolved in 2.0 mL of water and *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) (195.4 mg, 1.019 mmol) were added to the above reaction solution. In addition, the reaction solution was adjusted a PH of 8.0 by 2.0 mL of 1 M $NaHCO_3$. The reaction mixture was stirred for over night at room temperature (25°C). The reaction solution was dialyzed about 24 h by dialysis membrane (10,000 Da MWCO) and was filtrated by a filter (0.45 μm, 250 mL). The filtrate was frozen at −20°C and was dried under vacuum. A white residue (aspartate–chondroitin) was obtained (373.9 mg, yield=99.7%).

In the second step, *trans*-1,2-DACH-Pt·SO_4 was synthesized. K_2PtCl_4 (5.0 g, 12 mmol) in 120.4 mL of deionized water with potassium iodide (KI) (20 g, 0.12 mol in 12.05 mL of water) and was allowed to stir for 5 min. To this solution one equivalent of the *trans*-l-DACH (1.373 g (1.487 mL), 12 mmol) was added. The reaction mixture was stirred for 30 min at room temperature (25°C). A yellow solid was obtained which was separated by filtration, washed with a small amount of dimethylformamide (DMF), then repeatedly with water, ethanol, and acetone. The final product was dried under vacuum (6.4817 g, yield=95.6%). *Trans*-l-DACH-PtI_2 (6.4817 g, 11.5 mmol) was added as a solid to an aqueous solution of silver sulfate (Ag_2SO_4) (3.44 g, 11 mmol). The reaction mixture was left stirring (protected from light) overnight at room temperature. Silver iodide was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. A yellow residue was obtained which was recrystallized from water. The final product, *trans*-l-DACH-sulfatoplatinum(II)·H_2O (*trans*-1,2-DACH-Pt·SO_4) was dried under vacuum (4.83 g, yield=99.1%), m.p. 230~231°C. Elemental analysis showed that Pt (*w*/*w*) had 44.6% (46.9%, theoretical). The structure was confirmed by proton- and carbon-13 NMR (Figs. 2 and 3).

In the third step, DDAP was synthesized by reacting *trans*-1,2-DACH-Pt·SO_4 to aspartate–chondroitin conjugate. Aspartate–chondroitin (373.9 mg) was dissolved in 4.0 mL of water and *trans*-l-DACH-Pt (II)SO_4·H_2O (299.7 mg, 0.7 mmol) was dissolved in 2.0 mL of water. The reaction mixture was stirred over night at room temperature (25°C). The reaction mixture was dialyzed for 6 h using dialysis membrane (10,000 Da MWCO). After dialysis, the solution was filtered using a 0.45 μm filter. The filtrate was frozen at −20°C and dried under vacuum. A white residue (DDAP) was obtained (508.7 mg, yield=92.0%), elemental analysis showed Pt (*w*/*w*) had 20±2%. The active ingredient of DDAP is *trans*-1,2-DACH-Pt·SO_4. The structure of *trans*-1,2-DACH-Pt·SO_4 is characterized. DDAP is a macromolecule which does not have detectable UV absorbance. Thus, the platin content was determined as a reference standard.

Tetrazolium-based Colorimetric Assay for Growth-inhibition Studies

Cell-growth inhibition mediated by DDAP or CDDP was determined by the MTT cell proliferation assay, a tetrazolium-based colorimetric assay, performed in quadruplicate. Briefly, 2008, 2008.C13, A2780, and A2780cis cells were seeded into 96-well plates ($4–8\times10^3$ cells/well) and maintained in RPMI 1640 medium for 24 h at 37°C. Next, cells were treated with DDAP or CDDP at concentrations of 2.5, 5, 10, 20, 25, and 50 μg/mL for 48 and/or 72 h. Controls were treated with DMSO or PBS. After the cells were treated, we determined their growth and viability by incubating them for 1 to 2 h at 37°C with 20 μL of tetrazolium substrate. Absorbance was measured at 450 nm using a 96-well microplate reader (Synergy HT; BioTek, Winooski, VT). We expressed the rate of cell-growth inhibition as follows: $\% = 100 \times (OD_{controls} - OD_{treated\,cell})/OD_{controls}$. The experiments were repeated separately three times.

In Vitro Cellular Uptake Assay of Technetium 99 m-DDAP

An *in vitro* cellular uptake assay of technetium 99 m (^{99m}Tc)-DDAP was performed to determine the cellular DDAP level in 2008 and 2008.C13 cells. ^{99m}Tc-pertechnetate (0.2 mCi) (Mallincrofdt., Houston, TX) was added to a vial containing the lyophilized residue of DDAP (0.25 mg) and tin chloride (II) ($SnCl_2$, 100 μg, 0.53 μmol) in 1 mL water. The cells were seeded in hexaplicate for each dose and maintained in RPMI 1640 medium for 48 h at 37°C. We added 4 μCi (20 μL) of ^{99m}Tc-DDAP (5 μg/mL) radiotracer to each well and incubated the cells at 37°C for 2 h. We used ^{99m}Tc-1, 4, 8, 11-tetraazacyclotetradecane (^{99m}Tc-N4; Sigma-Aldrich) as the control. After incubation, we washed the cells twice with ice-cold PBS and trypsinized them with 0.25% EDTA-trypsin.

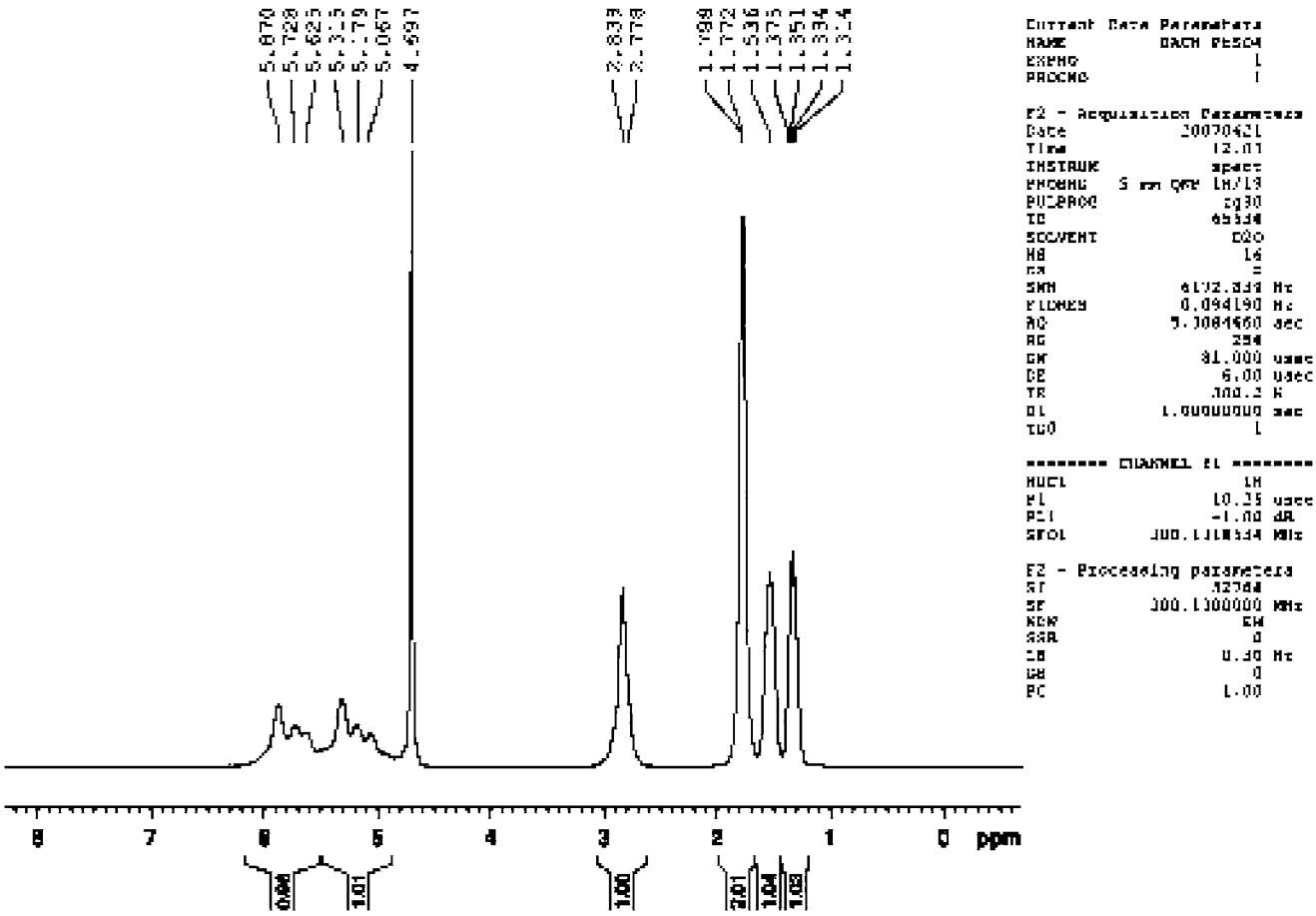

Fig. 2. Proton-NMR of *trans*-1,2-DACH-Pt·SO_4.

We extracted nuclear and cytoplasmic proteins from the cells in triplicate by using the NE-PER and cytoplasmic extraction kit (Pierce Chemical Company); we also collected whole cells in triplicate. The radioactivity of the whole cells and of the cytoplasmic and nuclear extracts was measured with a Packard Cobra gamma counter. We repeated the experiments separately three times and expressed the data as a mean ± SEM percent uptake ratio.

Flow Cytometry for Assessment of Apoptosis and Cell-cycle Analysis

The cell-cycle distribution of cells was analyzed by flow cytometry. The 2008.C13 cells (0.5×10^6) were treated with DDAP or CDDP at concentrations of 2.5, 5, 10, and 20 μg/mL for 48 h. Controls were treated with DMSO or PBS. The cells were trypsinized and subjected to centrifugation at 2,500 rpm for 5 min. After the cells were washed twice with 1× PBS, they were fixed with 70% ethanol overnight. They were then washed twice again with 1× PBS and resuspended in 0.5 mL of propidium iodide solution consisting of 50 μg/mL propidium iodide and 20 μg/mL of ribonuclease in PBS (1×10^6 cells per milliliter of propidium iodide buffer). The samples were incubated at 37°C for 15 min, and propidium iodide fluorescence was analyzed with an EPIS XL analyzer (Beckman Coulter, Miami, FL). We used MultiCycle software from Phoenix Flow Systems (San Diego, CA) for the cell cycle analysis.

TUNEL Assay for Evaluating DNA Strand Breaks in Apoptosis

We treated platinum-resistant (2008.C13) cells with CDDP or DDAP at concentrations of 0, 2.5, 5, 10, and 20 μg/mL for 48 h and then collected the floating and adherent cells. Cytospin preparations were made, and the 2008.C13 cells were fixed with 4% paraformaldehyde at room temperature for 20 min and air-dried. The slides were stored at −20°C until use. Apoptotic cells were evaluated by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling of fragmented DNA) assay, following the manufacturer's protocol (APO-BRDU-IHC kit; Phoenix Flow Systems). We randomly selected five different fields for counting and calculated the percentage of apoptotic cells.

Immunoblot Analysis

Extracts from the 2008.C13 cells treated with DDAP or CDDP at concentrations of 0, 2.5, 5, 10, and 20 μg/mL were prepared from PBS-washed cell pellets for protein analysis. Pellets were lysed in lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and freshly added proteinase inhibitors). Cell lysates were subjected to centrifugation at 14,000 rpm for 10 min, and the supernatants were analyzed. The protein concentrations were estimated by using a protein assay (Pierce Chemical Company). Next, 35 μg of protein was loaded into each lane and fractionated in 10–12%

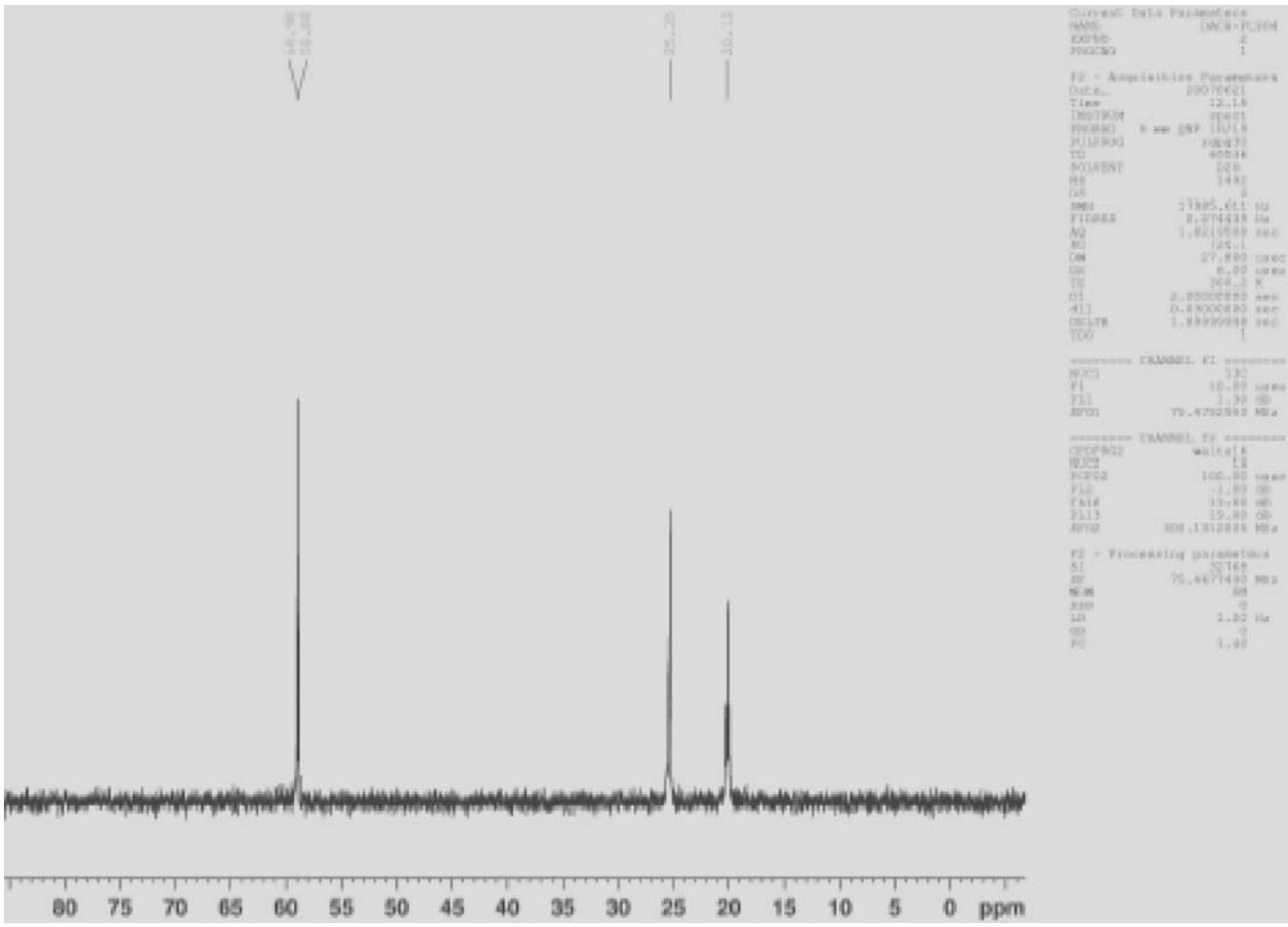

Fig. 3. Carbon 13-NMR of *trans*-1,2-DACH-Pt·SO_4.

sodium dodecyl sulfate-polyacrylamide gels and then transferred to a nitrocellulose membrane. Blots were probed with antibodies against cyclin A, p21, PCNA, 85-kDa caspase-cleaved fragment of PARP, cleaved caspase 3, and GAPDH (dilutions, 1:200–1:500). Anti-rabbit IgG and anti-mouse IgG conjugated with horseradish peroxidase were used as the secondary antibodies. GAPDH was used as a protein loading control. The signal was developed with an enhanced chemiluminescence detection system (Amersham Corporation, Little Chalfont, UK). We used the NIH Image program (Scion Image for Windows) for image analysis. The experiments were repeated separately three times.

Statistical Analyses

The differences between the DDAP and CDDP treatments in terms of percentages of cell-growth inhibition and of cells experiencing apoptosis were assessed using the Student's *t* test. Statistical significance was at α level <0.05.

RESULTS

Growth-inhibitory Effect of DDAP on Ovarian Cancer Cells

As determined by the MTT cell proliferation assay, after 48 and/or 72 h of treatment, cell growth in two pairs of platinum-sensitive (2008, A2780) and platinum-resistant (2008.C13, A2780cis) cell lines was significantly inhibited by DDAP in a dose-dependent manner ($P<0.05$) (Fig. 4). CDDP also significantly inhibited cell growth in all cell lines except in the platinum-resistant 2008.C13 cell line at the lowest dose of 2.5 μg/mL after 48 h of treatment. In the platinum-sensitive 2008 cells, CDDP and DDAP at the same concentrations had similar effects on cell-growth inhibition (Fig. 4A,C).

In the A2780 cell line, one of the most sensitive to CDDP in a panel of ovarian cancer cell lines, greater cell-growth inhibition was observed after CDDP than after DDAP treatment at 5, 10, and 20 μg/mL ($P<0.05$), whereas similar cell-growth inhibition was seen after CDDP and DDAP treatments at 2.5 and 25 μg/mL (Fig. 4E). However, in the platinum-resistant A2780cis cell line, the growth-inhibitory effect was significantly greater for DDAP than for CDDP at each dose ($P<0.05$) (Fig. 4F).

Of note, in the platinum-resistant 2008.C13 cell line after the 48- and 72-h treatments, cell growth was inhibited significantly more in the DDAP than in the CDDP treatment group at each dose ($P<0.05$), except the highest dose after the 72-h treatment ($P>0.05$) (Fig. 4B and D). When 2008.C13 cells were treated with DDAP or CDDP at concentrations of 2.5, 5, 10, and 20 μg/mL for 48 h, DDAP-treated cells showed 7.3-, 6.3-, 3.6-, and 2.4-fold higher growth inhibition rates, respectively, than did CDDP-treated cells; after 72 h of

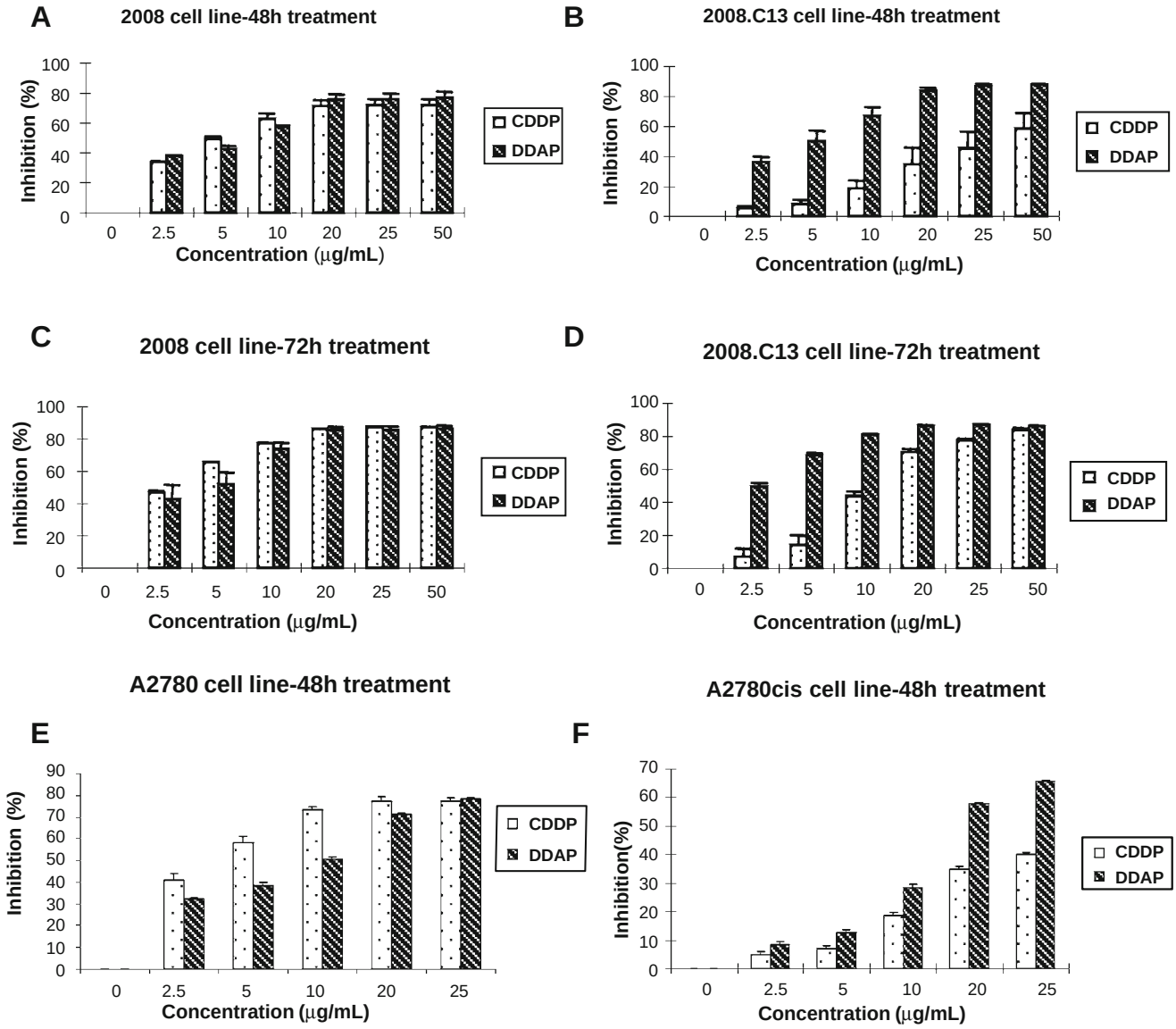

Fig. 4. Cell growth inhibitory effect of *cis*-diamminedichloroplatinum(II) (*CDDP*) and diammine dicarboxylic acid platinum (*DDAP*) on platinum-sensitive (**A**, **C**, **E**) and platinum-resistant (**B**, **D**, **F**) ovarian cancer cells, as detected by the MTT cell proliferation assay. DDAP inhibited cell growth more potently than did CDDP in the platinum-resistant cell lines (2008.C13 and A2780cis). Data are presented as the means of three independent experiments. Error bars ± SEM.

treatment, DDAP-treated cells showed 7.5-, 4.8-, 1.9-, and 1.2-fold higher rates, respectively (Fig. 4B and D).

Our data suggest that DDAP significantly inhibited the cell growth of both platinum-sensitive and platinum-resistant ovarian cancer cells and that it was much more potent than CDDP in inhibiting platinum-resistant ovarian cancer cell growth.

The chemical way used for labeling DDAP with technetium is shown in Fig. 5A. *In vitro* cellular uptake of ^{99m}Tc-DDAP in platinum-sensitive (2008) and platinum-resistant (2008.C13) ovarian cancer cell lines. After a 2-h exposure to DDAP at 5 μg/mL, the whole-cell uptake of ^{99m}Tc-DDAP was 1.62% in the 2008.C13 platinum-resistant cells, two times higher than the 0.82% uptake in the 2008 platinum-sensitive cells (Fig. 5B). Though the uptake was seen in whole cells, the measured uptake of ^{99m}Tc-DDAP in cytoplasmic and nuclear extracts was relatively low. The findings suggest that ^{99m}Tc-DDAP did not enter the cells and the cell death might be due to incorporation of platinum containing cleavage compounds from the DDAP macromolecule.

Apoptosis Induction by DDAP in Platinum-resistant Ovarian Cancer Cells

Apoptosis induced by DDAP or CDDP was tested by flow cytometry in the 2008.C13 cell line after 48 h of drug exposure. Flow cytometric analysis showed a dose-dependent increase in the number of cells in the sub-G_1 fraction after DDAP and CDDP treatments, which represents hypodiploid cells and indicates the induction of apoptosis. At the same

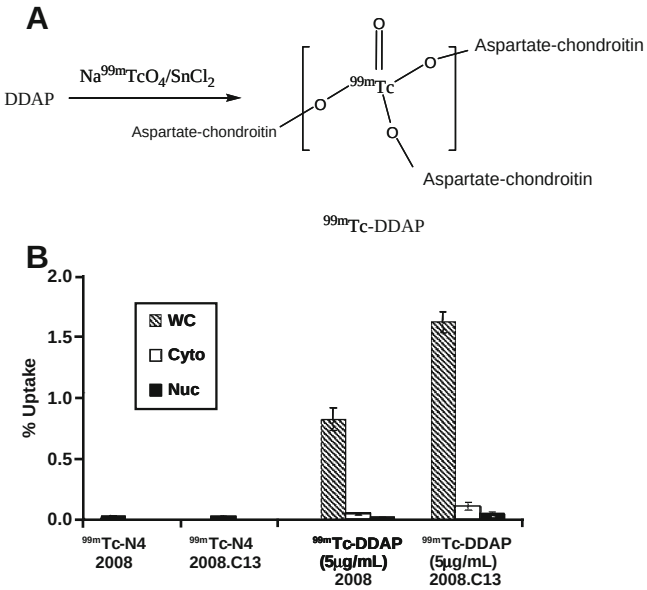

Fig. 5. **A** The chemical way used for labeling DDAP with technetium. **B** *In vitro* cellular uptake assay of technetium 99 m-diammine dicarboxylic acid platinum (*^{99m}Tc-DDAP*) in platinum-sensitive (*2008*) and platinum-resistant (*2008.C13*) ovarian cancer cells after a 2-h exposure to DDAP at 5 μg/mL. ^{99m}Tc-1,4,8,11-tetraazacyclotetradecane (*^{99m}Tc-N4*) was used as the control. Data are presented as the means of three independent experiments. *Cyto* Cytosolic extracts, *Nuc* nuclear extracts, *WC* whole cells. Error bars ± SEM.

doses, however, the use of DDAP, compared with CDDP, resulted in a more pronounced increase in the sub-G_1 fraction (Fig. 6).

DNA fragmentation typical of apoptosis was further determined by the TUNEL assay in three independent experiments. A clear dose-dependent increase in the number of apoptotic cells was detected after exposure to both drugs. However, when compared at each dose, the DDAP-treated cells exhibited much higher levels of apoptosis than the CDDP-treated cells did ($P<0.05$) (Fig. 7A and B).

To determine whether apoptosis is induced through a caspase 3-dependent pathway followed by the cleavage of PARP, levels of cleaved caspase 3 and PARP, which form after caspase 3 activation, were determined by Western blot analysis. PARP is a 113-kDa nuclear protein that has been shown to be specifically cleaved to an 85-kDa fragment during caspase 3-dependent apoptosis. After cells were exposed to CDDP or DDAP for 48 h, cleaved PARP was present at each dose. In the CDDP-treated group, cleaved PARP expression increased from 2.5 to 20 μg/mL, and cleaved caspase 3 was expressed in a pattern similar to that of PARP. In the DDAP-treated group, the expression of cleaved caspase 3 was comparable to that in the CDDP-treated group, except for the lower expression seen at 5 μg/mL of DDAP. Although cleaved PARP expression induced by high-dose (20 μg/mL) DDAP appeared to be lower than that induced by low-dose DDAP, no such difference was detected in its upstream cleaved caspase 3 expression (Fig. 7C).

Cell-cycle Arrest by DDAP in Platinum-resistant Ovarian Cancer Cells

DNA content was analyzed by flow cytometry 48 h after 2008.C13 cells were treated with DDAP or CDDP. Exposure to CDDP induced cell arrest in the S phase and increased the sub-G_1 fraction at the 5 μg/mL dose but not at the lowest dose, 2.5 μg/mL. The numbers of cells arrested in the S phase and the sub-G_1 fraction increased continuously as the CDDP dose increased, with the maximal S-phase arrest (84.8%) occurring at 20 μg/mL. After cells were exposed to DDAP for 48 h, the highest levels of S-phase arrest occurred at the lower doses (2.5 μg/mL [90.3%] and 5 μg/mL [90.1%]). At higher doses (10 and 20 μg/mL), the level of S-phase arrest steadily decreased as the sub-G_1 fraction increased. This can be explained by the fact that under the strong stress of high-dose DDAP, cells underwent apoptosis promptly and directly before they were arrested in the S phase (Fig. 8A).

To elucidate the mechanism underlying S-phase arrest caused by CDDP and DDAP in 2008.C13 cells, we examined the expression of p21 and cyclin A, which are important for cell-cycle regulation in the S phase, in 2008.C13 cells after 48 h of drug exposure. Neither p21 nor cyclin A expression was related to the extent of S-phase arrest after CDDP treatment. After DDAP treatment, however, p21 and cyclin A expression were closely related to the extent of S-phase arrest: p21 was up-regulated with maximal S-phase arrest after low-dose DDAP treatment, but not after high doses;

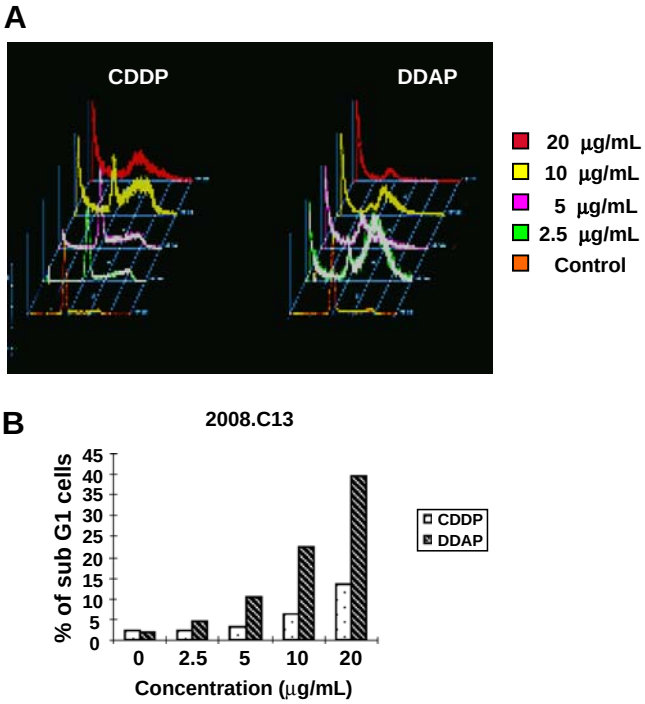

Fig. 6. **A** Flow cytometric analysis of the dose-dependent increase of the sub-G_1 fraction in the platinum-resistant 2008.C13 cell line after 48-h exposure to *cis*-diamminedichloroplatinum(II) (*CDDP*) or diammine dicarboxylic acid platinum (*DDAP*). At the same doses, DDAP induced substantially more sub-G_1 cells than did CDDP. **B** The percentage of the sub-G_1 fraction in 2008.C13 cells after 48-h exposure to CDDP or DDAP.

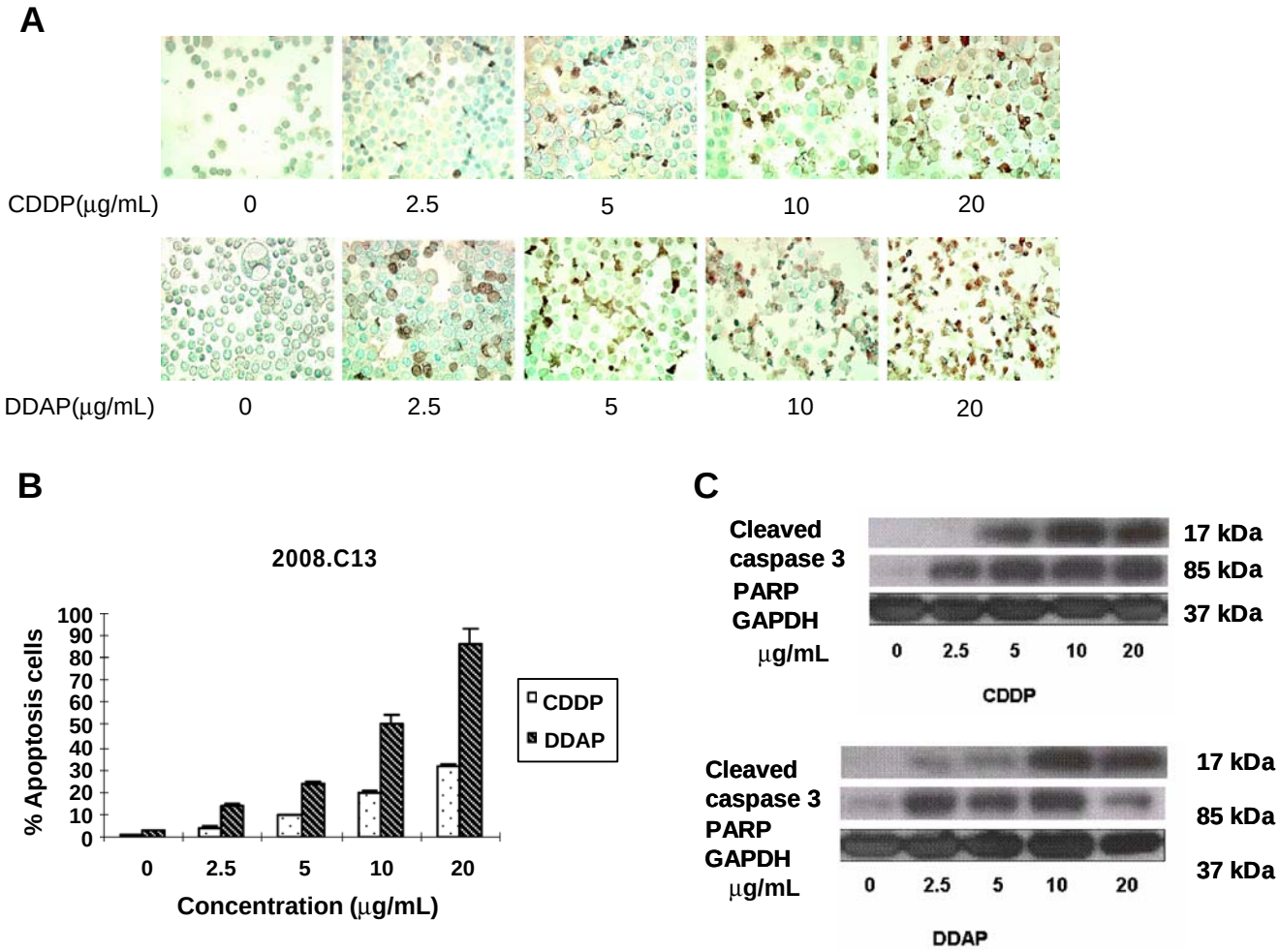

Fig. 7. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling of fragmented DNA) assay of apoptosis induced by *cis*-diamminedichloroplatinum(II) (*CDDP*) and diammine dicarboxylic acid platinum (*DDAP*) after 48 h of drug exposure in platinum-resistant 2008.C13 cell line. **A** The apoptotic morphology is indicated by brown particles. **B** The percentage of cells with apoptotic morphology. Data are presented as the means of three independent experiments. Error bars ± SEM. **C** Western blot analysis of cleaved caspase 3 and poly (ADP-ribose) polymerase (*PARP*) cleavage in 2008.C13 cells treated with CDDP or DDAP. *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase.

cyclin A was up-regulated after high-dose DDAP treatment and was maintained at a low level after low-dose DDAP treatment (Fig. 8B).

DISCUSSION

To our knowledge, this is the first study to demonstrate the superior efficacy of low-dose DDAP in inhibiting the growth of platinum-resistant ovarian cancer cells and to describe its underlying mechanism of action, shown by increased cellular uptake of this compound, enhanced apoptosis, and induced arrest of S-phase cells, accompanied by up-regulated p21 expression and low cyclin A expression. Our results show that DDAP may be of great benefit in the treatment of platinum-resistant ovarian cancer because of its high potency in overcoming platinum resistance at low doses. In our study, we observed greater cellular uptake of DDAP in platinum-resistant ovarian cancer cells than in platinum-sensitive ovarian cancer cells after low-dose DDAP treatment. It suggests that adding aspartate–chondroitin to the dichlorine portion of CDDP specifically enhanced drug delivery to the platinum-resistant ovarian cancer cells. Enhanced permeability and retention effect of the polymer-drug conjugate may also play roles in achieving better cellular uptake (24). The fact that DDAP exerted greater antitumor activity than CDDP in platinum-resistant ovarian cancer cells may be partly due to its ability to achieve better cellular uptake.

CDDP is believed to exert its cytotoxicity on ovarian cancer cells through the formation of different CDDP-DNA adducts that can induce apoptosis. Defective apoptosis has been implicated as an important mechanism affecting the occurrence of platinum resistance (25). In our study, we found that although both CDDP and DDAP induced apoptosis in 2008.C13 cells, the extent of apoptosis induced by DDAP was much greater, indicating that the superior antitumor efficacy of DDAP on CDDP-resistant ovarian cancer cells may be due to its greater ability to induce apoptosis.

CDDP can induce apoptosis through a caspase 3-dependent apoptotic pathway (26,27). Caspase 3 belongs to a family of effector caspases that are activated by upstream initiator caspases, *e.g.*, caspase 8 in the extrinsic pathway and

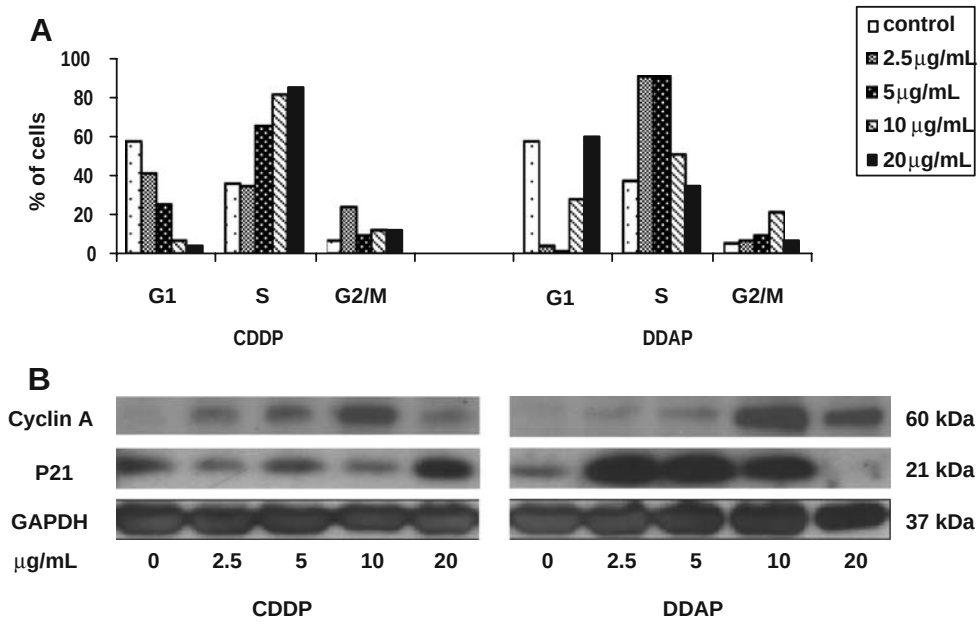

Fig. 8. A Cell-cycle distribution in the 2008.C13 cell line after treatment with *cis*-diamminedichloroplatinum(II) (*CDDP*) or diammine dicarboxylic acid platinum (*DDAP*) for 48 h. G_1, G_2, *M*, and *S* indicate cell phases. **B** Western blot analysis of p21 and cyclin A expression in 2008.C13 cells after exposure to CDDP or DDAP for 48 h.

caspase 9 in the intrinsic pathway of apoptosis. To further elucidate whether DDAP and CDDP induce apoptosis through a caspase 3-dependent pathway, we used Western blot analysis to examine the activated form of caspase 3, cleaved caspase 3. Clear evidence for a caspase 3-dependent apoptotic pathway was obtained from 2008.C13 cells treated with CDDP and DDAP. Since proteolytically active caspase 3 can cleave a number of cellular proteins, such as PARP, we also tested PARP cleavage and found that apoptosis induced by CDDP or DDAP in 2008.C13 cells was accompanied by the cleavage of PARP, suggesting that apoptosis occurs through a caspase 3-dependent pathway and that the cleavage of PARP is involved in DDAP-induced apoptosis in platinum-resistant ovarian cancer cells. We also found that the cleaved PARP expression that occurred after high-dose (20 μg/mL) DDAP treatment was lower than that after low-doses (2.5–10 μg/mL). DDAP treatment and that no such difference was detected in cleaved caspase 3 expression. We therefore speculate that cleavage by caspase 3 on other caspase 3 substrates could be involved in apoptosis induced by high-dose DDAP.

However, despite the fact that greater apoptosis was induced by DDAP than by CDDP, the expression of cleaved caspase 3 in DDAP-treated cells was not greater than that in CDDP-treated cells, prompting us to speculate that the apoptosis induced by DDAP could occur partially through a caspase 3-dependent pathway. Because a lack of caspase 3 activation during CDDP-induced apoptosis has also been demonstrated in other ovarian cancer cell lines (28), other effector caspase pathways, such as caspase 6-mediated apoptotic pathways (29) or caspase-independent cell-death pathways (30,31), could be involved. This needs further investigation.

We also found that in addition to its apoptotic effect on platinum-resistant 2008.C13 cells, DDAP exerted an antiproliferative effect through the arrest of cells in the S phase which was associated with up-regulating p21 expression. As a cyclin-dependent kinase inhibitor (CDKI), p21 plays a key role in controlling cell-cycle progression, including the S phase. In this study, p21 up-regulation was in accordance with the increased S-phase arrest observed in DDAP-treated cells, which is consistent with previous findings that the overexpression of p21 was associated with S-phase arrest (32,33).

DNA-damaging agents such as platinum can induce p21 expression in a p53-dependent or p53-independent manner (34). It has been suggested that p21 can retard the progression of the S phase through its ability to bind to and inhibit the function of the cyclin A-CDK2 complex. Cyclin A-CDK2 is a pivotal regulator of S-phase progression. It has been reported that the ectopic expression of cyclin A can promote the S phase, that the down-regulation of cyclin A can impede the S phase (35), and that the overexpression of cyclin A can reverse the inhibitory action of p21 (32). In our study, after DDAP treatment, S-phase arrest was accompanied by up-regulated p21 expression and low cyclin A expression. It is likely that p21 and cyclin A regulation play an important role in S-phase arrest in platinum-resistant ovarian cancer cells treated with DDAP.

Of note, p21 can also bind to PCNA to block DNA elongation and, consequently, cell progression through the S phase (33). PCNA acts as a recruiting center for DNA replication proteins, and the replication is arrested by the competitive binding of p21 to PCNA (33). We found no difference in PCNA expression after CDDP or DDAP treatment in 2008.C13 cells (data not shown), which can be explained by the fact that p21 preferentially interacts with cyclin A-CDK2 when both cyclin A-CDK2 and PCNA exist (33).

Though there is the possibility of an involvement of chondrotin sulfate or chondroitin aspartate in the effects of DDAP. However, the question whether chondriotin sulfate itself involved in the tumor growth is still debatable. It has

also been reported that chondriotin sulfate can enhance platelet derived growth factor-mediated signaling in fibrosarcoma cells. In our study, we used the platinum-sensitive ovarian cancer cell line 2008 as the control. We did not see greater growth inhibitory effect of DDAP on platinum-sensitive ovarian cancer cells, compared to that of CDDP on platinum-sensitive ovarian cancer cells, suggesting the chondroitin itself might not be involved in the DDAP drug action.

In summary, our study findings indicate that at low doses, DDAP, compared with CDDP, substantially enhances the growth-inhibitory effect on platinum-resistant ovarian cancer cells. The superior efficacy of low-dose DDAP is accomplished through enhanced apoptosis, induced arrest of S-phase cells, associated with up-regulated p21 expression and low cyclin A expression, and increased cellular uptake of DDAP in platinum-resistant ovarian cancer cells.

This preclinical study, the first to our knowledge to demonstrate that DDAP may be a more potent compound than CDDP in the treatment of platinum-resistant ovarian cancer, may provide the foundation for further clinical applications of DDAP in the treatment of platinum-resistant ovarian cancer.

ACKNOWLEDGMENT

We thank Tamara Locke in the Department of Scientific Publications at M. D. Anderson Cancer Center for editing this manuscript, as well as Ling Chen and Richard Mendez for their assistance with the experiments. This work was supported by M. D. Anderson's institutional grant and Core Grant (NCI grant 5P30CA016672-32).

REFERENCES

1. A. Jemal, L. X. Clegg, E. Ward, L. A. Ries, X. Wu, P. M. Jamison, P. A. Wingo, H. L. Howe, R. N. Anderson, and B. K. Edwards. Annual report to the nation on the status of cancer, 1975–2001, with a special feature regarding survival. *Cancer.* **101**:3–27 (2004).
2. J. J. Kavanagh, S. Pecoreli, A. Dipertrillo, and N. Einhorn. *Chemotherapy in advanced ovarian cancer.* Blackwell Scientific, Cambrige, MA, 1998.
3. W. P. McGuire, W. J. Hoskins, M. F. Brady, P. R. Kucera, E. E. Partridge, K. Y. Look, D. L. Clarke-Pearson, and M. Davidson. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer [see comments]. *N Engl J Med.* **334**:1–6 (1996).
4. M. J. Piccart, H. Lamb, and J. B. Vermorken. Current and future potential roles of the platinum drugs in the treatment of ovarian cancer. *Ann Oncol.* **12**:1195–1203 (2001).
5. J. T. Thigpen. Chemotherapy for advanced ovarian cancer: overview of randomized trials. *Semin Oncol.* **27**:11–16 (2000).
6. G. Giaccone. Clinical perspectives on platinum resistance. *Drugs.* **59**(Suppl 4):9–17 (2000)discussion 37–18.
7. S. Manic, L. Gatti, N. Carenini, G. Fumagalli, F. Zunino, and P. Perego. Mechanisms controlling sensitivity to platinum complexes: role of p53 and DNA mismatch repair. *Curr Cancer Drug Targets.* **3**:21–29 (2003).
8. C. Balch, T. H. Huang, R. Brown, and K. P. Nephew. The epigenetics of ovarian cancer drug resistance and resensitization. *Am J Obstet Gynecol.* **191**:1552–1572 (2004).
9. W. P. McGuire and R. F. Ozols. Chemotherapy of advanced ovarian cancer. *Semin Oncol.* **25**:340–348 (1998).
10. W. P. McGuire 3rd, and M. Markman. Primary ovarian cancer chemotherapy: current standards of care. *Br J Cancer.* **89** (Suppl 3):S3–8 (2003).
11. M. A. Fuertes, J. Castilla, C. Alonso, and J. M. Perez. Novel concepts in the development of platinum antitumor drugs. *Curr Med Chem Anti-Canc Agents.* **2**:539–551 (2002).
12. N. J. Moreland, M. Illand, Y. T. Kim, J. Paul, and R. Brown. Modulation of drug resistance mediated by loss of mismatch repair by the DNA polymerase inhibitor aphidicolin. *Cancer Res.* **59**:2102–2106 (1999).
13. Z. H. Siddik. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene.* **22**:7265–7279 (2003).
14. F. M. Muggia and G. Los. Platinum resistance: laboratory findings and clinical implications. *Stem Cells.* **11**:182–193 (1993).
15. A. de Graeff, R. J. Slebos, and S. Rodenhuis. Resistance to cisplatin and analogues: mechanisms and potential clinical implications. *Cancer Chemother Pharmacol.* **22**:325–332 (1988).
16. M. J. McKeage. New-generation platinum drugs in the treatment of cisplatin-resistant cancers. *Expert Opin Investig Drugs.* **14**:1033–1046 (2005).
17. G. Biagini, A. Pugnaloni, A. Damadei, A. Bertani, A. Belligolli, V. Bicchiega, and R. Muzzarelli. Morphological study of the capsular organization around tissue expanders coated with *N*-carboxybutyl chitosan. *Biomaterials.* **12**:287–291 (1991).
18. J. S. Pieper, P. B. van Wachem, M. J. A. van Luyn, L. A. Brouwer, T. Hafmans, J. H. Veerkamp, and T. H. van Kuppevelt. Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats. *Biomaterials.* **21**:1689–1699 (2000).
19. M. Prabaharan and J. F. Mano. Chitosan-based particles as controlled drug delivery systems. *Drug Deliv.* **12**:41–57 (2005).
20. A. Polykratis, P. Katsoris, J. Courty, and E. Papadimitriou. Characterization of heparin affin regulatory peptide signaling in human endothelial cells. *J Biol Chem.* **280**:22454–22461 (2005).
21. M. K. Chourasia and S. K. Jain. Polysaccharides for colon targeted drug delivery. *Drug Deliv.* **11**:129–148 (2004).
22. V. R. Sinha and R. Kumria. Polysaccharides in colon-specific drug delivery. *Int J Pharm.* **224**:19–38 (2001).
23. C. Haase, R. Bergmann, F. Fuechtner, A. Hoepping, and J. Pietzsch. L-type amino acid transporters LAT1 and LAT4 in cancer: uptake of 3-O-methyl-6-18F-fluoro-L-dopa in human adenocarcinoma and squamous cell carcinoma *in vitro* and *in vivo*. *J Nucl Med.* **48**:2063–2071 (2007).
24. C. Li, J. E. Price, L. Milas, N. R. Hunter, S. Ke, D. F. Yu, C. Charnsangavej, and S. Wallace. Antitumor activity of poly(L-glutamic acid)-paclitaxel on syngeneic and xenografted tumors. *Clin Cancer Res.* **5**:891–897 (1999).
25. Y. Sedletska, M. J. Giraud-Panis, and J. M. Malinge. Cisplatin is a DNA-damaging antitumour compound triggering multifactorial biochemical responses in cancer cells: importance of apoptotic pathways. *Curr Med Chem Anticancer Agents.* **5**:251–265 (2005).
26. S. Fulda, M. Los, C. Friesen, and K.M. Debatin. Chemosensitivity of solid tumor cells *in vitro* is related to activation of the CD95 system. *Int J Cancer.* **76**:105–114 (1998).
27. H. Kojima, K. Endo, H. Moriyama, Y. Tanaka, E. S. Alnemri, C. A. Slapak, B. Teicher, D. Kufe, and R. Datta. Abrogation of mitochondrial cytochrome c release and caspase-3 activation in acquired multidrug resistance. *J Biol Chem.* **273**:16647–16650 (1998).
28. K. M. Henkels and J. J. Turchi. Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. *Cancer Res.* **59**:3077–3083 (1999).
29. G. D. Diaz, Q. Li, and R. H. Dashwood. Caspase-8 and apoptosis-inducing factor mediate a cytochrome c-independent pathway of apoptosis in human colon cancer cells induced by the dietary phytochemical chlorophyllin. *Cancer Res.* **63**:1254–1261 (2003).
30. B. S. Cummings, G. R. Kinsey, L. J. Bolchoz, and R. G. Schnellmann. Identification of caspase-independent apoptosis in epithelial and cancer cells. *J Pharmacol Exp Ther.* **310**:126–134 (2004).
31. R. E. Aird, J. Cummings, A. A. Ritchie, M. Muir, R. E. Morris, H. Chen, P. J. Sadler, and D. I. Jodrell. *In vitro* and *in vivo*

activity and cross resistance profiles of novel ruthenium (II) organometallic arene complexes in human ovarian cancer. *Br J Cancer.* **86**:1652–1657 (2002).
32. V. V. Ogryzko, P. Wong, and B. H. Howard. WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Mol Cell Biol.* **17**:4877–4882 (1997).
33. V. Gottifredi, K. McKinney, M. V. Poyurovsky, and C. Prives. Decreased p21 levels are required for efficient restart of DNA synthesis after S phase block. *J Biol Chem.* **279**:5802–5810 (2004).
34. A. L. Gartel and A. L. Tyner. Transcriptional regulation of the p21((WAF1/CIP1)) gene. *Exp Cell Res.* **246**:280–289 (1999).
35. S. Gilfillan, E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol.* **3**:1150–1155 (2002).