

Expression of *p53* in Cisplatin-resistant Ovarian Cancer Cell Lines: Modulation with the Novel Platinum Analogue (*1R, 2R*-Diaminocyclohexane)(*trans*-diacetato)(dichloro)-platinum(IV)¹

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ABSTRACT

The compound (*1R,2R*-diaminocyclohexane)(*trans*-diacetato)(dichloro)platinum(IV) (DACH-acetato-Pt) is a novel platinum-based antitumor agent with clinical potential against cisplatin-resistant disease that is under development in our laboratory. In view of the central role of the wild-type *p53* tumor suppressor gene in drug-induced apoptosis, we evaluated the cytotoxicity of cisplatin and DACH-acetato-Pt in a panel of cisplatin-resistant ovarian tumor models with differing *p53* status. Cisplatin was relatively more effective against mutant or null *p53* cell lines (continuous drug exposure IC₅₀, 1.2–3.3 μM) than it was against those harboring wild-type *p53* (IC₅₀, 2.8–9.9 μM). In contrast, DACH-acetato-Pt was considerably more active in wild-type *p53* models (IC₅₀, 0.17–1.5 μM) than it was in mutant or null models (IC₅₀, 2.7–11.3 μM). Inactivation of wild-type *p53* function in OVCA-429 cells by the human papillomavirus type 16 (HPV 16) E6 plasmid increased resistance to DACH-acetato-Pt by 3–5-fold, which confirmed the drug's dependence on wild-type *p53* for its high cytotoxic potency. Differences between the two platinum agents were also evident in cell cycle studies: cisplatin arrested both wild-type and mutant *p53* cells in G₂-M, whereas DACH-acetato-Pt arrested wild-type *p53* cells in G₁ and mutant *p53* cells in G₂-M. The

G₁ arrest by DACH-acetato-Pt was abrogated in HPV 16 E6 transfectant clones of OVCA-429 cells. In agreement with effects on cell cycle progression, a 2-h pulse exposure to low concentrations (≤25 μM) of DACH-acetato-Pt induced marked increases in *p53* and p21^{Waf1/Cip1} expression in OVCA-429 cells. Cisplatin, in direct contrast, had no effect on expression of *p53* or p21^{Waf1/Cip1} until the drug concentration was increased to 125 μM. In HPV 16 E6 transfectants of OVCA-429 cells, induction of *p53* by the two agents was severely attenuated, and corresponding increases in p21^{Waf1/Cip1} were abrogated. This suggests that p21^{Waf1/Cip1} increases were *p53* dependent. Collectively, the results demonstrate that DACH-acetato-Pt is very distinct from cisplatin. In particular, the greater activity of DACH-acetato-Pt in cisplatin-resistant wild-type *p53* ovarian tumor models can be ascribed to its ability to more efficiently induce *p53* protein and activate *p53* functions.

INTRODUCTION

Ovarian cancer is the most lethal gynecological malignancy in the United States; in 1997, it was predicted that 26,800 women would develop the disease, and ~14,200 women would die that year (1). Although a number of factors impact on prognosis, including age, histological type and grade, tumor DNA content, extent of disease at presentation, and volume of residual disease, the most important is International Federation of Gynecology and Obstetrics stage (2). The 5-year survival rates for stages I, II, III, and IV are 74, 58, 30, and 19%, respectively (3). Unfortunately, 75% of women present with advanced disease (stages III and IV; Ref. 4). Current therapy for advanced disease consists of staging laparotomy and cytoreductive surgery (5), followed by chemotherapy with platinum-containing complexes and Taxol (2, 6). Despite initially encouraging response rates of up to 70%, most women develop recurrent disease (7). Thus, given the high response rate and the low 5-year survival rate, cisplatin resistance is a critically important factor that limits the clinical utility of this agent.

In view of the central problem of cisplatin resistance, efforts have focused on the development of alternative platinum-based analogues that can be more effective against resistant disease. The compound DACH-acetato-Pt,³ which bears axial acetate ligands (Fig. 1), represents a novel class of plati-

Received 5/27/98; revised 12/9/98; accepted 12/10/98.

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¹ Supported by American Cancer Society Grant DHP-134 and the Sandra S. Davis Ovarian Cancer Research Fund (to Z. H. S.) and by a grant from the Office of Education and the University Cancer Foundation of the University of Texas M. D. Anderson Cancer Center (to G. S. H.). This work was presented in preliminary form at the 87th Annual Meeting of the American Association for Cancer Research, held April 20–24, 1996, in Washington, DC.

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³ The abbreviations used are: DACH-acetato-Pt, (*1R,2R*-diaminocyclohexane)(*trans*-diacetato)(dichloro)-platinum(IV); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HPV 16, human papillomavirus type 16.

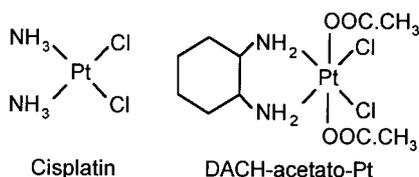


Fig. 1 Structure of platinum complexes.

num-based antitumor agents. The ability of DACH-acetato-Pt to circumvent resistance acquired through exposure to cisplatin is a particularly important observation of our laboratory (8), and mechanistic studies have been in progress as part of its preclinical development for the anticipated clinical trials.

Although the molecular basis of drug sensitivity and resistance is complex, the p53 tumor suppressor protein appears to play a major role in modulating cellular response to therapeutic agents. However, the impact of p53 gene status on chemo- and radiosensitivity varies between different tumor types. This is a function of (a) the integrity of the cellular transduction pathways of the cell activated in response to injury, (b) the specific type of cellular damage produced by the drug, and (c) the inherent cell-specific responses of the particular cell (9). In cells that are programmed for apoptosis (especially hematopoietic and lymphoid cells), loss of p53 function decreases their sensitivity to a wide variety of DNA-damaging agents (10–17). This, however, is not observed in other cell types (18, 19). Presence of wild-type p53, on the other hand, does not necessarily ensure a chemosensitive phenotype, and a recent clinical study has underscored this very convincingly: Righetti *et al.* (20) reported that, among the group of chemoresistant ovarian tumors, 37% had wild-type p53 and 63% had mutant p53. It is also noteworthy that, contrary to expectations, inactivation of wild-type p53 in MCF-7 breast carcinoma cells and in normal human foreskin fibroblasts enhanced sensitivity of these specific cells to cisplatin and other selected chemotherapeutic agents (21, 22).

This study was undertaken to assess the extent of cisplatin resistance in established human ovarian cancer lines with wild-type, mutant, or null p53 status and to examine whether p53 status modulates the ability of DACH-acetato-Pt to circumvent this resistance. We report here that, against cisplatin-resistant tumor cells with wild-type p53, DACH-acetato-Pt was significantly more effective and, unlike cisplatin, caused these cells to arrest in G₁ in a p53-dependent manner.

MATERIALS AND METHODS

Chemicals. Cisplatin was synthesized by us using a standard procedure reported previously (23). We have reported the synthesis and chemical characterization of DACH-acetato-Pt separately (24). Cisplatin and DACH-acetato-Pt were dissolved in normal saline and water, respectively, then sterilized through 0.22- μ m disc filters. The concentration of each drug was confirmed by its platinum metal content, as determined by flameless atomic absorption spectroscopy (25), and then the drugs were diluted to 2 mM stock solutions. MTT was purchased from

Table 1 p53 status of ovarian cancer cell lines

Cell line	p53 gene status	Ref.
OVCA-420	Wild-type	31
OVCA-429	Wild-type	31
OVCA-433	Wild-type	31
OVCAR-10	Wild-type	— ^a
HEY	Wild-type	32
OVCA-432	Mutant	31
OVCAR-3	Mutant	33
OCC1	Mutant	32
SKOV-3	Null	33

^a S. W. Johnson, personal communication.

Sigma Chemical Co. (St. Louis, MO), and FCS was obtained from BioWhittaker, Inc. (Walkersville, MD).

Cell Lines. The nine ovarian carcinoma cell lines used in this study were established from biopsies taken from patients who had failed cisplatin- and/or alkylating agent-based chemotherapy and were classified as cisplatin resistant (26–30). The p53 statuses of these cell lines have been documented (31–33) and are indicated in Table 1. Cells were grown as monolayers in 5% CO₂ and 95% humidified air at 37°C. The OVCA-420, -429, -432, and -433 cell lines were maintained in Eagle's MEM with Earle's salts containing 10% heat-inactivated FCS, 1 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and antibiotics (100 μ g/ml streptomycin and 100 units/ml penicillin). The SKOV-3 cell line was grown in McCoy's 5a medium, 15% heat-inactivated FCS, and 2 mM L-glutamine. The HEY, OCC1, OVCAR-3, and OVCAR-10 cell lines were maintained in RPMI 1640 containing 10% heat-inactivated FCS, and 2 mM L-glutamine. The McCoy's 5a medium and RPMI 1640 were both supplemented with an antibiotic cocktail (100 μ g/ml streptomycin, 100 units/ml penicillin, and 100 μ g/ml neomycin).

HPV 16 E6 Transfection Studies. The HPV 16 E6 gene was cloned into a pCMV plasmid that also contains a neomycin gene (34). This plasmid and the pCMV-neo control vector were obtained from Dr. K. R. Cho (Johns Hopkins University School of Medicine, Baltimore, MD). For transfections, 1×10^6 OVCA-429 cells were seeded in a 100-mm tissue culture dish and incubated until 50–80% confluent. The HPV 16 E6 gene (34) or the pCMV-neo control vector were introduced into cells using Lipofectamine (Life Technologies, Inc.). Approximately 14 days later, G418-resistant colonies were selected and expanded.

The clones were examined for their stable growth characteristics and/or expression of p53. The HPV 16 E6 protein facilitates the degradation of the p53 protein via the ubiquitin-dependent proteolytic pathway (35). Therefore, a reduced level of p53 protein by Western analysis was used as an indication of clones with positive HPV 16 E6-mediated effects. Three such E6 clones were selected, along with two control transfectants, for experimental investigations. These selected clones were maintained in complete Eagle's MEM supplemented with 800 μ g/ml G418. The clones were cultured in G418-free medium for one passage prior to undertaking experiments.

Cytotoxic Evaluations. Cytotoxicity was determined by a modified MTT assay (36), which has been validated against

Table 2 IC₅₀ of cisplatin and DACH-acetato-Pt in wild-type, mutant, and null p53 ovarian cancer cell lines following continuous or 2-h drug exposures

Cell line	Continuous drug exposure			2-h drug exposure		
	Cisplatin	DACH-acetato-Pt	Cisplatin/DACH-acetato-Pt ratio	Cisplatin	DACH-acetato-Pt	Cisplatin/DACH-acetato-Pt ratio
Wild-type p53						
OVCA-420	2.93 ± 0.18 ^a	1.49 ± 0.24	1.97	21.2 ± 1.73	8.39 ± 0.81	2.53
OVCA-429	4.10 ± 0.63	0.79 ± 0.10	5.19	24.8 ± 1.67	5.61 ± 0.75	4.42
OVCA-433	9.90 ± 2.86	1.07 ± 0.33	9.25	103.3 ± 9.70	9.83 ± 1.39	10.5
OVCAR-10	8.90 ± 1.77	0.17 ± 0.01	52.4	109.5 ± 12.2	6.32 ± 0.53	17.3
HEY	2.78 ± 0.23	0.45 ± 0.05	6.18	29.9 ± 2.14	8.08 ± 0.98	3.70
Mutant or null p53						
OVCA-432	2.03 ± 0.25	11.3 ± 2.08	0.18	16.1 ± 1.91	73.2 ± 7.33	0.22
OVCAR-3	1.22 ± 0.33	2.65 ± 0.76	0.46	6.64 ± 0.98	23.5 ± 2.83	0.28
OCC1	3.30 ± 0.92	9.85 ± 3.65	0.34	19.3 ± 2.21	108.1 ± 10.3	0.18
SKOV-3	1.32 ± 0.17	3.18 ± 0.57	0.42	8.87 ± 1.10	44.2 ± 3.81	0.20

^a Mean ± SE; n = 3–5.

the clonogenic assay in the evaluation of new platinum compounds (37). Cells in exponential growth were trypsinized, counted using a hemocytometer, and then diluted to appropriate concentrations. Aliquots (100 µl) of cell suspensions were added to each well of a 96-well microtiter plate. The stock cisplatin or DACH-acetato-Pt solution was serially diluted with complete medium immediately before use. Each diluted solution (100 µl) was added to wells in triplicate, and the cells were incubated at a 37°C in a 5% CO₂ humidified incubator. After 3 or 5 days, when untreated control cells were in logarithmic growth, 50-µl aliquots of an MTT solution (3 mg/ml) were added to the wells, and the microtiter plate was incubated for a further 3 h. The medium was then removed from wells and replaced with 50 µl of 100% DMSO to dissolve MTT formazan crystals. Plates were then agitated on a shaker for 5–10 min, and absorbances were measured at 570 nm with a multiwell scanning spectrophotometer (Dynatech, Chantilly, VA; or Molecular Devices, Sunnyvale, CA).

For cytotoxic evaluations using 2-h drug exposures, 100-µl aliquots of cell suspensions were placed in the 96-well microtiter plate. Following a 2-day attachment period in a 37°C-5% CO₂ humidified incubator, 100-µl aliquots of either complete medium or cisplatin or DACH-acetato-Pt solution in medium were added to wells in triplicate. After 2 h, the cells were washed free of the drug and then incubated in drug-free medium for a further 3 or 5 days. The MTT assay was then performed as described above.

The IC₅₀s, defined as the drug concentration (µM) inhibiting cell growth by 50% compared to control cells, were determined from a plot of log concentration *versus* A₅₇₀ readings (as a percentage of control).

Cell Cycle Studies. Attached cells in an exponential growth phase in 100-mm tissue culture dishes were exposed for 2 h to cisplatin or DACH-acetato-Pt, as described above. The cells were washed and reincubated in drug-free medium. At appropriate time intervals, cells were collected, washed twice with ice-cold PBS, counted, and diluted with PBS to give a concentration of 1 × 10⁶ cells/ml. Paraformaldehyde in PBS was added dropwise to the cell suspension to give a 1% final concentration. The cell suspension was then incubated for 15

min on ice. After this time, the cells were washed with ice-cold PBS and resuspended in ice-cold 70% ethanol at a concentration of 1 × 10⁶ cells/ml. The suspension was stored at –20°C until completion of each set of experiments. Before analysis, the cell suspension was thawed, washed once with ice-cold PBS, resuspended in a solution of propidium iodide (10 µg/ml) in PBS containing 0.5% Tween 20 and 500 units/ml of RNase A (Sigma) and incubated at room temperature for 30 min and then at 4°C overnight. Cell cycle kinetics were determined on a Becton Dickinson flow cytometer.

Western Analysis. Cells were exposed for 2 h to cisplatin or DACH-acetato-Pt as described above, washed, and incubated at 37°C in drug-free medium for 24 h. The cells were then washed with PBS and lysed for 10 min on ice with 1 ml of lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 100 µg/ml phenylmethylsulfonyl fluoride, and 1 µg/ml aprotinin]. The lysates were collected by microcentrifugation at 4°C, and the protein level was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Forty µg of total cell protein were electrophoresed on a 10% SDS-polyacrylamide gel, blotted overnight in TBS-20 buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 3% nonfat milk powder and 0.2% BSA. The membranes were then probed for 2 h with either the DO-1 (for mutant and wild-type p53; Oncogene Science, Cambridge MA) or the sdi1 (for p21^{Waf1/Cip1}; PharMingen, San Diego, CA) antibody. The antibody reaction was visualized by chemiluminescence using a sheep antimouse horseradish peroxidase as a second antibody (Amersham, Arlington Heights, IL) and quantified by laser densitometry. The membranes were then stripped of antibody with a stripping buffer [2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 100 mM 2-mercaptoethanol] at 50°C for 30 min, washed with PBS-Tween 20, and then reprobbed with a β-actin antibody (Sigma) to ensure equal loading of samples.

Statistical Analysis. Differences between groups were evaluated by Student's *t* test and were considered significant for values of *P* < 0.05.

Table 3 IC₅₀ of cisplatin and DACH-acetato-Pt in control clone PVC1 and HPV E6 clone E6B2 from OVCA-429 cells and corresponding resistance factors for E6B2^a

Parameter	Continuous drug exposure		2-h drug exposure	
	Cisplatin	DACH-acetato-Pt	Cisplatin	DACH-acetato-Pt
IC ₅₀ , PVC1	6.41 ± 0.83 ^b	1.04 ± 0.17	22.2 ± 2.06	5.26 ± 0.50
IC ₅₀ , E6B2	6.73 ± 1.54	2.55 ± 0.58 ^c	23.6 ± 2.85	26.3 ± 2.55 ^c
Resistance factor	1.03 ± 0.19	3.25 ± 1.11 ^d	1.06 ± 0.09	4.99 ± 0.12 ^d

^a Resistance factor of E6B2 = (IC₅₀ vs. E6B2)/(IC₅₀ vs. PVC1).

^b Mean ± SE; n = 3–7.

^c Significantly different from PVC1 (P < 0.05).

^d Significantly different from 1.00 (P < 0.05).

RESULTS

Cytotoxicity of Cisplatin and DACH-acetato-Pt. Cytotoxic evaluations were conducted in nine cisplatin-resistant ovarian cancer cell lines of differing p53 status (Table 1), and the results are shown in Table 2. Using the continuous drug exposure protocol, we found the IC₅₀ for cisplatin in the wild-type p53 cell lines to be in the range of 2.78–9.9 μM, with a median value of 4.1 μM. The IC₅₀ for the mutant/null p53 cell lines, on the other hand, ranged from 1.22 to 3.3 μM, with a median of ~1.32–2.03 μM. Thus, cisplatin was more effective against mutant/null p53 cell lines than against the wild-type p53 lines. With DACH-acetato-Pt, the median IC₅₀ in wild-type p53 cell lines was 0.79 μM (range, 0.17–1.49 μM). The median value increased to 3.18–9.85 μM (range, 2.65–11.3 μM) in mutant/null p53 cell lines. It is apparent, therefore, that wild-type p53 cell lines were more sensitive to DACH-acetato-Pt than mutant/null p53 cell lines. This relationship is in sharp contrast to that seen with cisplatin. The differential cytotoxic profile of cisplatin and DACH-acetato-Pt is readily apparent by examining the ratio of IC₅₀ for the two agents (Table 2). This ratio was >1 in wild-type p53 cell lines and <1 in the mutant/null p53 models. The difference between the ratios was statistically significant (P < 0.05), and this further demonstrates the superiority of DACH-acetato-Pt in wild-type p53 cell lines. Similar results were obtained when drug exposure to cells was limited to 2 h (Table 2). However, the IC₅₀s for cisplatin and DACH-acetato-Pt following pulse exposures were 8.1-fold (SE, 0.83) and 13.0-fold (SE, 3.3) greater, respectively, than those following continuous drug exposures. These increases in IC₅₀ for the 2-h exposure were expected and were similar to the 9–10-fold increases reported in human ovarian tumor models for cisplatin (29) and DACH-Pt(IV) analogues (37).

To confirm the role of wild-type p53 in mediating the superior cytotoxic activity of DACH-acetato-Pt, we established stable transfectants of the OVCA-429 cell line with the HPV 16 E6 plasmid to inactivate p53 function. The results with the control PVC1 and the test E6B2 clones indicate that loss of p53 function significantly increased resistance to DACH-acetato-Pt by ~3–5-fold (P < 0.05; Table 3). In contrast, no changes in resistance to cisplatin were noted.

Cell Cycle Kinetics following Exposure to Cisplatin and DACH-acetato-Pt. To gain insights into the mechanism of action of DACH-acetato-Pt, cell cycle studies were performed with two tumor models. With both wild-type p53 OVCA-433 and mutant p53 OVCA-432 cell lines, IC₅₀ concentrations of

cisplatin arrested cells in G₂ (Fig. 2, A and C); by 36–48 h, most of the cells were in G₂-M. In contrast, equitoxic concentrations of DACH-acetato-Pt demonstrated an interesting p53-dependent differential effect on cell cycle kinetics. In the OVCA-433 model with wild-type p53, the analogue arrested cells in G₁, with a concomitant decrease in the S-phase population (Fig. 2B). The kinetic profile returned to normal by 48 h. No accumulation in G₁ was observed in mutant p53 OVCA-432 cells exposed to DACH-acetato-Pt. Instead, the analogue behaved like cisplatin and maximally arrested cells in G₂-M by 48 h (Fig. 2D). In these resistant tumor cell lines, associations between G₂-M arrest and low cytotoxicity of the analogue and between G₁ arrest and high activity are apparent.

Whether the G₁ arrest in wild-type p53 cells by DACH-acetato-Pt was mediated in a p53-dependent manner was examined in wild-type p53 OVCA-429 parental cells and its control and E6 transfectant clones. In parental cells, cisplatin induced the characteristic G₂-M arrest by 24–48 h and a concomitant decrease in the G₁ population (Fig. 3A). This was similar to the effect seen in OVCA-432 and -433 cells (Fig. 2, A and C). DACH-acetato-Pt caused OVCA-429 cells to arrest in G₁ and a decrease in S-phase (Fig. 3B), which is consistent with observations made with OVCA-433 cells (Fig. 2B). The effect of equitoxic concentrations of cisplatin and DACH-acetato-Pt on cell cycle distribution of control transfectant PVA1 and PVC1 clonal cells were similar to those observed in parental cells (Fig. 4). However, the characteristic G₁ arrest produced by DACH-acetato-Pt was abrogated by the HPV 16 E6 plasmid in the three E6 clones (E6B2, E6B4, and E6C6; Fig. 4A). Indeed, there were no gross differences between cisplatin and the analogue with regard to distribution of E6 transfectant cells in G₁, S, or G₂-M (Fig. 4). The results indicate that the presence of wild-type p53 is important for DACH-acetato-Pt to mediate cellular effects with greater potency.

p53 and p21^{Waf1/Cip1} Induction by Cisplatin and DACH-acetato-Pt. To investigate why cisplatin-resistant ovarian cancer cells with wild-type p53 were sensitive to DACH-acetato-Pt, we examined p53 induction by the platinum agents in selected models. Increases in p21^{Waf1/Cip1} were also determined as an indication of the transactivation potential of the p53-dependent *waf1/cip1* gene and to correlate with the G₁ arrest induced by DACH-acetato-Pt. The mutant p53 OVCA-432 cells, which overexpress p53, demonstrated no detectable changes in p53 protein levels 24 h after a 2-h exposure to equitoxic concentrations of cisplatin (up to 80 μM) or DACH-

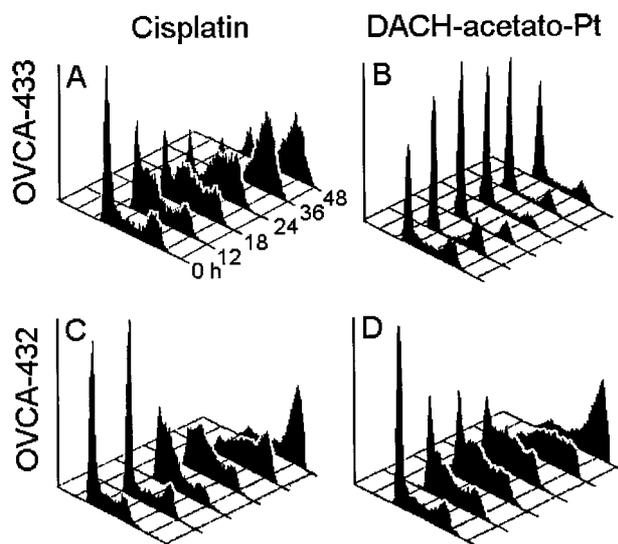


Fig. 2 Cell cycle analysis of the wild-type *p53* OVCA-433 and the mutant *p53* OVCA-432 cell lines exposed to cisplatin or DACH-acetato-Pt. Attached cells in an exponential growth phase were exposed for 2 h to cisplatin or DACH-acetato-Pt. The cells were then washed and reincubated in drug-free medium. Cells were harvested at 12, 18, 24, 36, and 48 h and analyzed by flow cytometry. *A*, OVCA-433, 100 μM cisplatin. *B*, OVCA-433, 10 μM DACH-acetato-Pt. *C*, OVCA-432, 15 μM cisplatin. *D*, OVCA-432, 70 μM DACH-acetato-Pt.

acetato-Pt (up to 400 μM ; data not shown). Levels of $p21^{\text{Waf1/Cip1}}$ were undetectable in these cells before or after drug treatment. The wild-type *p53* OVCA-429 cell line also expressed detectable basal levels of *p53* (Fig. 5), but these were substantially lower than those observed in OVCA-432 cells. With cisplatin, a significant induction ($\geq 20\%$) of *p53*, with coordinate increases in $p21^{\text{Waf1/Cip1}}$, was only observed in OVCA-429 cells at the high concentration of 125 μM ($\sim 5 \times \text{IC}_{50}$). In comparison, DACH-acetato-Pt increased levels of both *p53* and $p21^{\text{Waf1/Cip1}}$ at the lower drug concentration of 5 μM ($\sim \text{IC}_{50}$). Interestingly, the increases in $p21^{\text{Waf1/Cip1}}$ were only observed in parallel with *p53* induction, which suggests that the induced *p53* was functional with regard to its ability to transactivate the *waf1/cip1* gene. Similar differential effects of cisplatin and the analogue on *p53* induction were also seen in wild-type *p53* OVCA-433 tumor cells (data not shown). Thus, the greater cytotoxicity of DACH-acetato-Pt against cisplatin-resistant ovarian tumor cells with wild-type status may relate to its ability to induce *p53* and $p21^{\text{Waf1/Cip1}}$ expression.

To explain the reduction in cytotoxicity of DACH-acetato-Pt by E6, levels of *p53* and $p21^{\text{Waf1/Cip1}}$ were also examined in the control PVC1 and E6-transfectant E6B2 clones of OVCA-429 cells. Compared to parental OVCA-429 cells, untreated PVC1 cells expressed ~ 1.5 -fold greater levels of *p53* and 2.4-fold greater levels of $p21^{\text{Waf1/Cip1}}$ (Fig. 6). As with parental cells, cisplatin increased *p53* by $\sim 30\%$ in PVC1 cells at the high 125 μM drug concentration only. Surprisingly, there was no corresponding increase in $p21^{\text{Waf1/Cip1}}$, which may be due to selective desensitization of transactivation mechanisms by the increased basal expression of *p53* in the PVC1 clone. DACH-acetato-Pt, on the other hand, induced both *p53* and

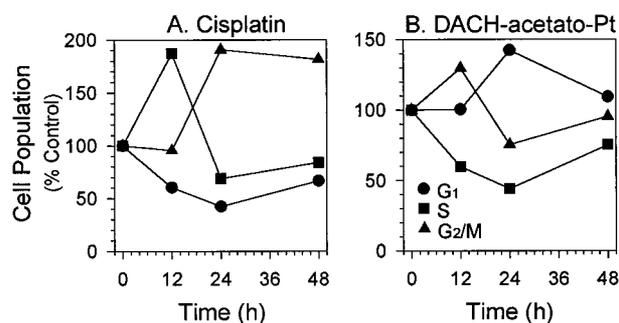


Fig. 3 Cell cycle analysis of the wild-type *p53* OVCA-429 cell line exposed to cisplatin or DACH-acetato-Pt. Attached cells in an exponential growth phase were exposed for 2 h to drug vehicle (control), 25 μM cisplatin, or 5 μM DACH-acetato-Pt. The cells were then washed and reincubated in drug-free medium. Cells were harvested at 12, 24, and 48 h and analyzed by flow cytometry. *A*, cisplatin. *B*, DACH-acetato-Pt.

$p21^{\text{Waf1/Cip1}}$ in PVC1 cells at a lower drug concentration (Fig. 6), and this was consistent with results obtained in parental cells (Fig. 5). However, increases in *p53* and $p21^{\text{Waf1/Cip1}}$ were lower, and this again may be related to higher basal levels of these proteins in the control transfectant. In contrast, the E6 plasmid reduced basal levels of *p53* in E6B2 clone by $\sim 30\%$ compared to parental OVCA-429 cells but had no effect on $p21^{\text{Waf1/Cip1}}$ levels (Fig. 6). Both cisplatin and DACH-acetato-Pt failed to induce *p53* or $p21^{\text{Waf1/Cip1}}$ in this clone. The results indicate that E6 abrogates drug-induced up-regulation of *p53* and $p21^{\text{Waf1/Cip1}}$, and this is consistent with cytotoxicity and cell cycle data obtained with the analogue from the E6B2 transfectant.

DISCUSSION

The initial reports on the activity of cisplatin were made three decades ago by Rosenberg *et al.* (38, 39). Since that time, analogue development has been a major priority and has centered on both improving the toxicity profile of cisplatin and circumventing cisplatin resistance (40). Our efforts have resulted in the identification of DACH-acetato-Pt as an analogue with clinical potential against cisplatin-resistant disease (8). This study reveals that this analogue is selectively active in cisplatin-resistant ovarian tumor cells harboring wild-type *p53*.

The presence of wild-type *p53* in tumor cells generally correlates with a good clinical response to drug therapy (41). The cytotoxic effect is usually preceded by drug-mediated cellular accumulation of wild-type *p53*. This induction can activate *p53*, which can then transactivate a number of genes to regulate cell cycle and apoptosis. The *p53*-dependent transactivation of $p21^{\text{Waf1/Cip1}}$ gene, for instance, appears to be essential in arresting cells in G_1 of the cell cycle. The *p53*-dependent expression of the *bax* gene, on the other hand, triggers apoptosis as a facile cytotoxic response to chemotherapeutic agents. Not surprisingly, loss of apoptotic functions appears to be a major cause of resistance to cytotoxic drugs (42–44). Consistent with this understanding is our observation here that cisplatin-resistant ovarian cells harboring wild-type *p53* were resistant to induction of *p53* by cisplatin, whereas the greater cytotoxicity of DACH-

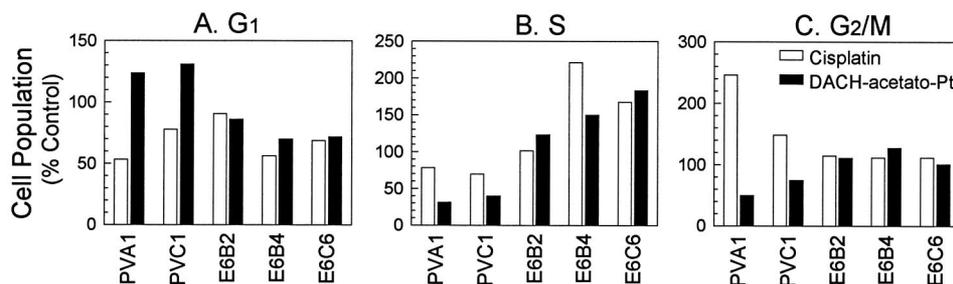


Fig. 4 The effects of cisplatin or DACH-acetato-Pt on cell cycle kinetics of three HPV 16 E6 clones (E6B2, E6B4, and E6C6) and two control vector clones (PVA1 and PVC1) derived from the wild-type p53 OVCA-429 cell line. Attached cells in an exponential-growth phase were exposed for 2 h to drug vehicle (control), 25 μM cisplatin, or 5 (control clones) or 25 (E6 clones) μM DACH-acetato-Pt. The cells were then washed and reincubated in drug-free medium. Cells were harvested at 24 h and analyzed by flow cytometry. A, G₁. B, S phase. C, G₂-M.

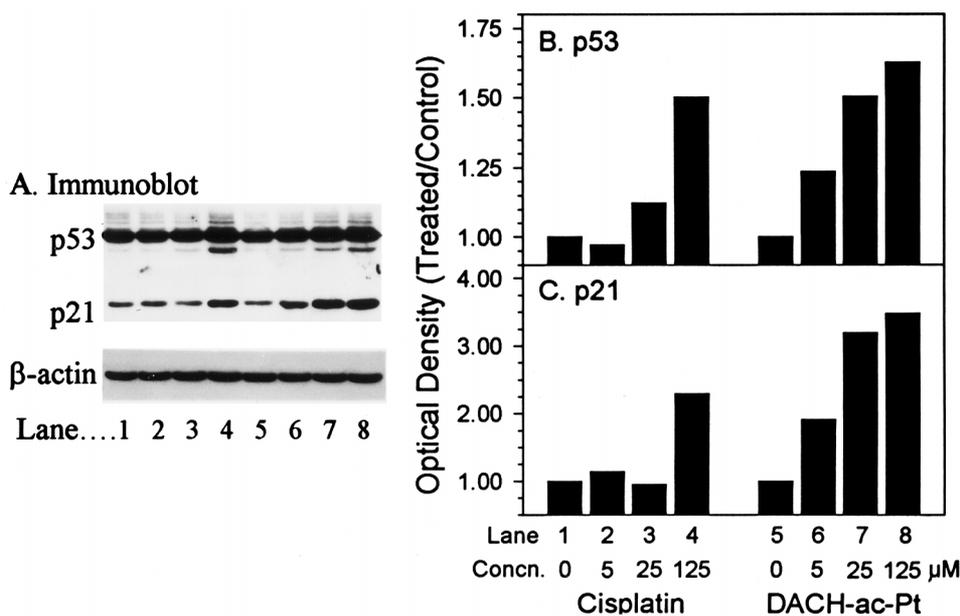


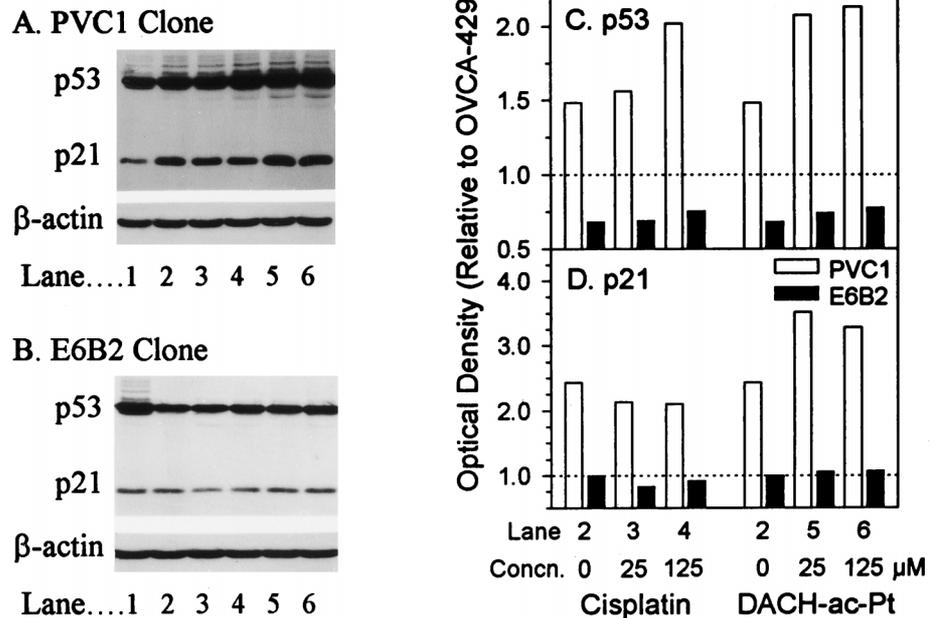
Fig. 5 Induction of p53 and p21^{Waf1/Cip1} in the wild-type p53 OVCA-429 cell line exposed to cisplatin or DACH-acetato-Pt. Cells in exponential growth phase were exposed to 0–125 μM of cisplatin or DACH-acetato-Pt (DACH-ac-Pt) for 2 h, washed and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and examined for p53 and p21^{Waf1/Cip1} levels by Western blotting. A, immunoblot analysis of p53 and p21^{Waf1/Cip1} levels by laser densitometry. B, levels of p53 (relative to control in Lane 1 or 5) estimated from immunoblots by laser densitometry. C, levels of p21^{Waf1/Cip1} (relative to control in Lane 1 or 5) estimated from immunoblots by laser densitometry.

acetato-Pt in these cells was associated with significant induction of p53 and p21^{Waf1/Cip1}. Furthermore, the analogue was considerably less cytotoxic against cisplatin-resistant cells with mutant p53. The demonstration of a loss in the activity of DACH-acetato-Pt in the wild-type p53 OVCA-429 cell line expressing HPV 16 E6, which inactivates the p53 protein via a ubiquitin-dependent pathway (34, 35, 45), confirms the significant role of wild-type p53 and its induction in the cytotoxicity of the analogue. In contrast, the lack of change in the cytotoxicity of cisplatin in the E6 transfectant clone provides credence to the idea that p53 does not participate in mediating the cytotoxic effect of cisplatin in the OVCA-429 model.

The differential molecular and associated cytotoxic effects of cisplatin and DACH-acetato-Pt in resistant wild-type p53 ovarian tumor cell lines are novel observations among platinum antitumor agents. The explanation for the results is not currently known but may be reconciled by considering that the two platinum drugs activate independent signal transduction pathways in response to DNA damage. It is likely that the normal

pathway used by cisplatin in resistant wild-type p53 ovarian cells is down-regulated, which prevents the characteristic induction of p53 and p21^{Waf1/Cip1} proteins in response to DNA damage by clinically relevant concentrations of cisplatin. DACH-acetato-Pt, on the other hand, may use an alternative pathway that appears to be intact and fully capable of regulating p53 levels in response to DNA damage. The existence of alternative pathways for p53 induction has been implicated previously from studies with ataxia-telangiectasia cells. Artuso *et al.* (46) and Zhang *et al.* (47), for instance, reported that p53 induction was poor following treatment with ionizing radiation but was comparable to that in normal cells treated with methylmethane sulfonate, cisplatin, or UV light. Similarly, we have found in a separate study that the cisplatin-resistant ovarian 2780CP model lacked the ability to accumulate p53 in response to cisplatin, but induction of p53 and p53-mediated functions was normal when cells were exposed to X-rays (48). On the basis of these results, it may be reasonable to speculate that methylmethane sulfonate, cisplatin, and UV light activate sig-

Fig. 6 Induction of p53 and p21^{Waf1/Cip1} by cisplatin or DACH-acetato-Pt in the HPV 16 E6 clone E6B2 and the control vector PVC1 clone derived from the wild-type p53 OVCA-429 cell line. Cells in exponential growth phase were exposed to 0, 25, or 125 μ M cisplatin or DACH-acetato-Pt (*DACH-ac-Pt*) for 2 h, washed, and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and examined for p53 and p21^{Waf1/Cip1} levels by Western blotting. **A**, immunoblot analysis of p53 and p21^{Waf1/Cip1} for the PVC1 clone. **B**, immunoblot analysis of p53 and p21^{Waf1/Cip1} for the E6B2 clone. **C**, levels of p53 relative to those in parental OVCA-429 cells (relative to *Lanes 1*) estimated from immunoblots by laser densitometry. **D**, levels of p21^{Waf1/Cip1} (relative to *Lanes 1*) estimated from immunoblots by laser densitometry.



naling pathways that are distinct from those activated by DACH-acetato-Pt and ionizing radiation.

Apart from the differential effect of cisplatin and DACH-acetato-Pt on p53 and p21^{Waf1/Cip1} levels, a second observation of interest noted in this study was the relative abilities of the two agents to induce G₁ arrest. DACH-acetato-Pt demonstrated an ability to arrest wild-type p53 cells in G₁, but this was not apparent with cisplatin. It is well acknowledged that arrest in the G₁ phase of the cell cycle after DNA damage requires participation of wild-type p53 protein (9, 43, 49). Furthermore, transcriptional activation of p21^{Waf1/Cip1} is necessary for p53-mediated G₁ arrest by a number of agents, including γ -rays and Adriamycin (50). Thus, the differential effect of the two platinum agents at G₁ of the cell cycle is consistent with their relative abilities to increase intracellular levels of p21^{Waf1/Cip1}. However, it needs to be stressed that the cisplatin-mediated effect on the cell cycle is generally characterized by a predominant G₂-M arrest (51–53), even in cells in which cisplatin induces wild-type p53 and p21^{Waf1/Cip1} (54). Interestingly, the analogue induced G₂ arrest in cells with mutant p53, an observation that is consistent with literature reports on cells that lack functional p53. We confirmed this in this study using transfectant clones, in which p53 inactivation by HPV 16 E6 expression abrogated the prominent G₁ accumulation and produced G₂-M arrest instead. It is not known at this stage if the G₁ arrest mediated by DACH-acetato-Pt is necessary for its cytotoxicity against wild-type p53 cells. However, it is possible that the cytotoxic effects of the analogue may be distinct from its ability to induce G₁ arrest, as has been proposed from studies in other model systems (55, 56). Nevertheless, the ability of DACH-acetato-Pt to arrest cells in G₁ needs further investigation to better define its mechanism of action. It is feasible, by analogy with other G₁-arresting agents, that DACH-acetato-Pt may induce DNA strand breaks as opposed to the predominant formation of cross-links by cisplatin (57, 58).

Current therapy for effective management of ovarian carcinoma consists of cytoreductive surgery (5), followed by combination chemotherapy with platinum complexes and Taxol (6). Nevertheless, the 5-year survival rate of this disease is still dismal and has not changed significantly over the past 10 years (59). These statistics may change, however, as we begin to more fully comprehend the existence of multiple drug-specific signaling pathways and then use chemotherapeutic agents to activate specific pathways for affecting tumor cell kill. In this regard, the possibility that an alternative p53 regulatory pathway participates in the activity of DACH-acetato-Pt may represent a potentially important development for clinical therapy of subset of platinum-resistant ovarian tumors with wild-type p53 gene status. These results provide evidence for the novelty of DACH-acetato-Pt and endorse the development of this analogue for clinical trials.

ACKNOWLEDGMENTS

We thank Drs. Neely Atkinson, Jon Weiner, and Wei Zhang for helpful discussions and Ruth LaPushin, Long Nguyen, and Gerald Thai for expert technical assistance.

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Expression of *p53* in Cisplatin-resistant Ovarian Cancer Cell Lines: Modulation with the Novel Platinum Analogue (*1R*, *2R* -Diaminocyclohexane)(*trans*-diacetato)(dichloro)-platinum(IV)

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Clin Cancer Res 1999;5:655-663.

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