Bimodal Effects of 1R,2R-Diaminocyclohexane(trans-diacetato)(dichloro)platinum(IV) on Cell Cycle Checkpoints

Jian Kuang, Guangan He, Zaifeng Huang, Abdul R. Khokhar, and Zahid H. Siddik
Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030-4009

ABSTRACT
1R,2R-Diaminocyclohexane(trans-diacetato)(dichloro)platinum(IV) (DACH-acetato-Pt) is a novel platinum-based agent that is highly effective against cisplatin-resistant ovarian tumors. To probe its cellular mechanism, the effects of DACH-acetato-Pt (0–6.4 μM) on cell cycle checkpoints were examined using the ovarian cancer A2780 cell line as the model system. We found that DACH-acetato-Pt at ≥0.2 μM dramatically inhibited cell growth and induced cell death. At concentrations ≤0.6 μM (low effective concentrations), DACH-acetato-Pt specifically induced G1 phase arrest by selectively inhibiting cyclin-dependent kinase 4 (Cdk4) and Cdk2 activities. The Cdc2 activity, which regulates G2–M phase progression, was unaffected by the drug at these concentrations. At concentrations >0.6 μM (high effective concentrations), DACH-acetato-Pt first transiently inhibited S-phase progression and then blocked cell cycle progression at both G1 and G2 phases. These cell cycle effects were associated with sequential inhibitions of Cdk2/cyclin A activity, Cdk4 and Cdk2 activities, and Cdc2 kinase activity. Following the cell cycle effects, both the low and high effective concentrations of DACH-acetato-Pt induced cell death through apoptosis. These results indicate that DACH-acetato-Pt activates multiple cell cycle checkpoints in a bimodal manner and suggest that the cell cycle effects demonstrated in these studies may be linked to its ability to induce apoptosis.

INTRODUCTION
Cisplatin is a highly potent DNA-damaging agent that is widely used in cancer chemotherapy against several types of cancers, including those of the ovary, testes, and head and neck (1). Although the initial response rates can be high with cisplatin-based regimen, the clinical utility of the drug is often limited by the onset of drug resistance (2, 3). To circumvent this limitation, a number of cisplatin analogues with non-cross-resistant properties have been developed and introduced into clinical trials. These include oxaliplatin, neodecanoato-trans-1R,2R-diaminocyclohexane-platinum(IV) (NDDP), JM216, and tetraplatin (4). In addition, we have identified DACH-acetato-Pt1 as a promising analogue that is effective against both cisplatin-sensitive and -resistant ovarian cancer cells (5, 6). Moreover, the drug has shown effectiveness against tumor cells that are also resistant to other platinum complexes, such as oxaliplatin and tetraplatin (7). The substantial clinical potential of DACH-acetato-Pt necessitates studies to uncover the mechanism of action of this novel platinum drug.

It is well recognized that antitumor effects of cisplatin are mediated through induction of intranastand DNA cross-links (8). Although the exact DNA lesions induced by DACH-acetato-Pt remain to be established, we provided evidence previously that this analogue also interacts with DNA to form adducts (5). DNA damage and other cellular lesions arising from exposure to cytotoxic agents usually activate cell cycle checkpoints, which inhibit cell cycle progression by decreasing the activities of Cdns. The inhibition of Cdk4/cyclin D and/or Cdk2/cyclin E activity leads to cell cycle arrest at the G1 phase, the inhibition of Cdk2/cyclin A activity delays or prevents S-phase progression, and the inhibition of Cdc2/cyclin A and Cdc2/cyclin B activities induces G2 arrest (9). The cell cycle arrest at these phases allows DNA repair and prevents DNA replication or mitosis in the presence of damaged chromosomes (9). On the other hand, there is evidence to suggest that perturbation of cell cycle progression itself may induce apoptosis under certain circumstances (9, 10).

We and others have reported previously that cisplatin and its cross-resistant and non-cross-resistant analogues arrest the cell cycle at S and G2 phases (11–14). This is consistent with the demonstration that cisplatin inhibits the activity of Cdc2 kinase (15). However, we have obtained contrasting results on the effects of DACH-acetato-Pt on cell cycle progression (14). In cisplatin-resistant ovarian OVCA-429 and OVCA-433 tumor models, the analogue arrested cells in G1. On the other hand, the new agent induced S and G2 arrest in the OVCA-432 model. These observations indicate that DACH-acetato-Pt has multiple

Received 4/26/01; revised 7/17/01; accepted 7/17/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 Supported by NIH Grants CA77332 and CA82361 (to Z. H. S.).
2 To whom requests for reprints should be addressed, at Department of Experimental Therapeutics-104, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: (713) 792-7746; Fax: (713) 745-1176; E-mail: zsiddik@mdanderson.org.

3 The abbreviations used are: DACH-aceto-Pt, 1R,2R-diaminocyclohexane(trans-diacetato)(dichloro)platinum(IV); Cdk, cyclin-dependent kinase; FACS, fluorescence activated cell sorter; IC50, concentration of drug to reduce final cell numbers to 50% of control; PI, propidium iodide; RB, retinoblastoma; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
effects on cell cycle checkpoints and suggest that they differ from that of cisplatin. Understanding the specific mechanisms producing the cell cycle effects is important for defining the mechanism of action of DACH-acetato-Pt. Therefore, the present study was undertaken to systematically characterize the effect of this agent on various cell cycle checkpoints. The ovarian A2780 cancer cell line was selected as the experimental system because this cell line was established from a cancer patient before chemotherapy was administered (16), thus ensuring that its genetic background was not influenced by prior drug exposure. Indeed, previous studies indicate that A2780 cells have wild-type p53 and possess intact G1 and G2 checkpoints (17–19). We report here that DACH-acetato-Pt has a bimodal effect on cell cycle checkpoints; the drug activates the G1 checkpoint at low effective drug concentrations (<0.6 μM) and S and G2 checkpoints at high effective drug concentrations (>0.6 μM). We also demonstrate that the cell cycle effects of this drug are followed by induction of apoptosis.

MATERIALS AND METHODS

Cell Culture, Drug Treatment, and Determination of Growth Rate. A2780 cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% fetal bovine serum and an antibiotic mixture (100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml neomycin). The doubling time of this cell line under our culture condition of 37°C and 5% CO2 was ~20 h. The saturation density of this cell line in 100-mm Petri dishes was ~4 × 106 cells. DACH-acetato-Pt was synthesized and purified as described previously (20). The drug was dissolved in deionized water and sterile filtered, and the concentration of the solution was determined by flameless atomic absorption spectrophotometry (5). For maximum drug stability, the solution was stored at −70°C and used within 1 month. To measure the effect of the drug on growth of A2780 cells, 1.3 × 106 cells were plated in 100-mm Petri dishes and cultured for at least 24 h before DACH-acetato-Pt was added to the dish immediately after its dilution in the culture medium. The final volume of medium in the Petri dish was 10 ml. At the indicated times after drug addition, duplicate dishes were trypsinized, and cells collected from each were washed, resuspended, and counted twice using a Coulter Counter. The number of cells in each dish was averaged, and the mean value was calculated for duplicate dishes.

Trypan Blue Staining and FACS Analysis. Trypsinized cells were stained with 0.4% of trypan blue at room temperature for 5 min before being examined under the microscope. Cells that stained blue were scored positive and counted against the total number of cells. Cell cycle distribution of A2780 cells was analyzed by standard FACS (21). Briefly, trypsinized cells were first washed and suspended as single cells at 106 cells/ml in PBS. Next, they were brought to 70% ethanol by slowly dropping cold ethanol into the cell suspensions during vortexing. The samples were then incubated overnight on ice at 4°C or stored at −20°C. For FACS analysis, the cells were spun down, washed twice in PBS, resuspended in PBS containing 50 μg/ml PI and 20 μg/ml RNase A, and incubated at room temperature for 15 min. The FACS analysis was performed on a flow cytometer (Coulter Profile, Miami, FL) in the Flow Cytometry Core Laboratory at M. D. Anderson Cancer Center. The data were analyzed using the Multicycle software (Phoenix Flow System, San Diego, CA).

Immunoblotting, Immunoprecipitation, and Kinase Assay. To prepare protein lysates for immunoblotting, cells were scraped from the dishes using a rubber policeman and then pelleted by centrifugation at 13,000 × g at 4°C for 5 min. The cell pellets were resuspended in four volumes of ice-cold extraction buffer containing 80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 200 mM NaCl, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 0.1 mg/ml each of leupeptin, pepstatin, and chymostatin (Boehringer Mannheim, Indianapolis, IN), and 1 mg/ml benzamidine, aprotinin, soybean trypsin inhibitor, and antipain (Sigma Chemical Co.). After sonication, the resultant cell lysates were centrifuged at 13,000 × g at 4°C for 10 min, and the supernatants were collected. For immunoblotting, 50–100 μg of protein from each sample were separated by 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Immunoblotting was performed by using horseradish peroxidase-conjugated goat antirabbit or mouse IgG as the secondary antibody and visualized by ECL fluorescence detection (Amersham Corp., Arlington Heights, IL). For quantitation, the X-ray films were scanned, and the signals were analyzed by the NIH Image 1.62f software.

Free antibodies for immunoblotting or conjugated antibodies for immunoprecipitating Cdk4, Cdk2, Cdc2, and cyclin E were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). For isolation of Cdk4, Cdk2, Cdc2, or cyclin E complex, 10 μl of the diluted immunoaffinity beads containing 5–10 μg of the antibodies were incubated with ~200 μg of total protein lysates while rotating at 4°C for at least 4 h. After the beads were pelleted, they were washed three times with the immunocomplex wash buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM sodium β-glycerophosphate, 10 mM NaF, 5 mM EGTA, 2 mM EDTA, 200 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 0.1% Tween 20 and rinsed twice with the kinase reaction buffer containing 50 mM Tris-HCl (pH 7.5), 15 mM MgCl2, and 1 mM DTT.

For measurement of the Cdk2–, Cdc2–, or cyclin E-associated kinase activity, 10 μl of the immobilized Cdk2, Cdc2, or cyclin E immunocomplex mixture were mixed with 4 μl of a histone H1 phosphorylation mixture containing 0.5 mg/ml histone H1 (Boehringer Mannheim, Indianapolis, IN), 100 μM ATP, and 0.5 μCi/μl [γ-32P]ATP also in the kinase buffer. For measurement of the Cdk4 kinase activity, the immobilized Cdk4 immunocomplex was mixed with 4 μl of a RB protein phosphorylation mixture containing 0.75 mg/ml recombinant RB fusion protein (Santa Cruz Biotechnology), 100 μM ATP, and 0.5 μCi/μl [γ-32P]ATP in the kinase buffer. The kinase reaction mixtures were incubated at room temperature for 30 min, and the reactions were stopped by addition of SDS-PAGE sample buffer. The phosphorylated reaction products were resolved by 12.5% SDS-PAGE, and the incorporation of 32P was visualized by autoradiography and quantitated by scintillation counting.

TUNEL Assay and Gel Electrophoresis of Fragmented DNA and Annexin V Staining. TUNEL assay, which involves in situ labeling of free DNA ends (22), was performed on cytoperparations using the Promega Apoptosis Detection Sys-
system according to the manufacturer’s instruction (Promega Corp., Madison, WI). Briefly, the cells on slides were fixed in 4% paraformaldehyde for 25 min at 4°C. After the fixed cells were washed twice with PBS, they were permeabilized with 0.2% Triton X-100 for 5 min and washed with PBS two more times. Each slide was then covered with the equilibration buffer provided by the manufacturer for 15 min. The buffer was then aspirated, and the slides were incubated with the solution containing fluorescein-labeled dUTP and terminal deoxynucleotide transferase at 37°C for 1 h. After the reaction was stopped with 2× SSC, the slides were examined under the fluorescence microscope to count fluorescence-positive and -negative nuclei. For each sample, a total of 500 nuclei were counted, and the percentage of TUNEL-positive cells was calculated.

Agarose gel electrophoresis of fragmented DNA was performed essentially as described previously (23). After exposure to DACH-acetato-Pt (0–6.4 μM) in 10-cm dishes, attached and floating cells were collected, washed in PBS, and lysed with 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100 for 20 min on ice. The lysates were centrifuged at 27,000 × g for 20 min, and the supernatants were collected and subjected to RNase (20 mg/ml) and proteinase K (20 mg/ml) treatment for 1 h at 37°C. DNA was precipitated in 50% isopropanol, washed with 70% ethanol, and redissolved in 100 μl of TE buffer 10 mM Tris-1 mM EDTA, pH 8.0. Approximately 10 μl of each sample were electrophoresed on a 1.5% agarose gel containing ethidium bromide and photographed.

Annexin V staining of cells was conducted essentially as reported previously (24). Cells from each culture were trypsinized and washed with PBS, and 5 × 10^5 cells were pelleted by centrifugation. The cell pellet was then resuspended in 500 μl of annexin V binding buffer supplemented with 1.25 μl of 200 μg/ml FITC-annexin V (Oncogene Research Products, Boston, MA), and the cell suspension was incubated at room temperature for 15 min in the dark. The cells were pelleted again by centrifugation and resuspended in 500 μl of fresh annexin V binding buffer supplemented with 10 μl of 30 μg/ml of PI. The samples were analyzed within 1 h by two-parameter FACS on a Coulter XL-MCL flow cytometer. The percentage of cells that stained either with annexin V alone or with annexin V plus PI were calculated by the System II software (Coulter Corp., Miami, FL).

RESULTS

Effects on Cell Growth and Cell Death. Before investigating the effect of DACH-acetato-Pt on various cell cycle checkpoints, it was first necessary to establish a range of drug concentrations that inhibit cell growth and to ascertain a relationship between its inhibition of cell growth and induction of cell death. For this objective, A2780 cells were treated continuously with 0–6.4 μM DACH-acetato-Pt for 4 days, and cell growth and death were monitored daily by counting the total number of cells/dish and determining the percentage of dead cells by trypan blue staining. As seen in Fig. 1A, DACH-acetato-Pt inhibited cell growth to a small extent at 0.1 μM but inhibited cell growth substantially at ≥0.2 μM. At >0.8 μM, the inhibition of cell growth was almost complete within the first 24 h, whereas the growth-inhibitory effect at <0.8 μM became prominent on day 2. On day 4, reductions in total cell numbers at DACH-acetato-Pt concentrations ≥0.2 μM ranged from 85 to 98%. Fig. 1B demonstrates that, consistent with the dose-dependent effect on cell growth, DACH-acetato-Pt induced cell death to a small extent at 0.1 μM and dramatically at ≥0.2 μM. The increase in cell death was most striking on day 2 after drug treatment at all concentrations. Thereafter, the percentage of dead cells increased progressively with time and reached 45–80% on day 4. From this data, we estimate that the concentration (IC50) of DACH-acetato-Pt inducing cell death in 50% of the population of A2780 cells on day 4 is about 0.2 μM. Together, these results demonstrate that DACH-acetato-Pt inhibited cell growth and induced cell death to similar extents in A2780 cells at similar drug concentrations and suggest that the induction of cell death by DACH-acetato-Pt closely correlates with its inhibitory effect on cell growth.

Effects on Cell Cycle Progression. To examine the effect of DACH-acetato-Pt specifically on cell cycle progression, A2780 cells were treated with 0–6.4 μM DACH-acetato-Pt for 24 h and subjected to FACS analysis. Fig. 2A illustrates the DNA content histograms of cells treated with DACH-acetato-Pt.
The estimated percentages of cells in G1, S, and G2-M phases are summarized in Fig. 2B. At concentrations up to 0.4 μM, DACH-acetato-Pt caused a concentration-dependent increase in the relative number of cells in G1 from 44 to 76% and a decrease in the relative number of cells in S-phase from 37 to 10% but had little or no effect on the cell population in G2-M phase. Subsequent studies have identified 0.6 μM as also having no effect on cell numbers in the G2-M phase (Fig. 3). At 0.8 μM or greater, the drug again increased cells in G1 and decreased those in S-phase relative to controls, but the changes were progressively less as the concentration was increased (Fig. 2). Moreover, a concentration-dependent increase from 22 to 34% in G2-M cells and a small increase in the percentage of cells in S-phase were observed. These results show that over the range of drug concentrations examined, DACH-acetato-Pt at ≤0.6 μM (designated low effective concentrations) primarily inhibits G1 progression, whereas at >0.6 μM (designated high effective concentrations), it additionally inhibits S and G2-M phase progression.

Kinetics of Inhibition of G1 Progression. To define the kinetics of inhibition of cell cycle progression at G1, we treated A2780 cells with 0.6 μM DACH-acetato-Pt for 36 h and monitored cell cycle distribution, cell growth, and mitotic index at 6-h intervals. As shown in Fig. 3, A and B, there was no change in cell cycle distribution during the first 12 h by this low effective drug concentration. Thereafter, the percentage of cells in G1 increased from 40 to 80%, and cells in S-phase decreased from 40 to 10%. In contrast, and consistent with Fig. 2, there was no change in the percentage of cells in G2-M during the first 24 h. After this time, the percentage of G1 cells fell from 23 to 9% as expected, because this followed a substantial decrease in the abundance of S-phase cells. The decrease coincided with the sharp drop in the mitotic index and a cessation of cell growth soon after 24 h (Fig. 3C). These results clearly demonstrate that the low effective concentration of DACH-acetato-Pt specifically arrests A2780 cells at G1 after a 12-h delay.

Selective Inhibition of G1 Cdk Activities by Low Effective Drug Concentrations. It is likely that the inhibition of cell cycle progression at G1 by low effective concentrations of
DACH-acetato-Pt was attributable to activation of the G₁ cell cycle checkpoint. To confirm this, we measured the activities of the two G₁-phase Cdks (Cdk4 and Cdk2) and the mitotic Cdk (Cdc2) over the 36-h period of treatment of A2780 cells with 0.6 μM DACH-acetato-Pt. Total protein was extracted from tumor cells collected at different time points after initiating drug exposure, and Cdk4, Cdk2, and Cdc2 complexes were isolated by immunoprecipitation and measured for kinase activity. As shown in Fig. 4, all three Cdk activities increased during the first 6 h. Because control cells also showed this phenomenon (data not shown), the increase was probably attributable to the effect of changing cells to fresh medium to initiate drug exposure. More importantly, Cdk4 and Cdk2 activities decreased dramatically between 6 and 24 h and remained at low levels thereafter. The decrease in these two G₁-phase Cdk activities preceded the increase in the percentage of cells in G₁. These observations indicate that the G₁ checkpoint was activated. In contrast with the inhibition of G₁-phase Cdks, the mitotic Cdc2 kinase activity remained at high levels for the first 24 h. After this time, the decrease in the Cdc2 kinase activity closely paralleled the decrease in the percentage of G₂-M cells, as would be expected. Thus, the G₂ checkpoint was not activated. Taken together, these results demonstrate that the selective inhibition of G₁-S-phase progression by the low effective concentrations of DACH-acetato-Pt is attributable to the specific inhibition of G₁-phase Cdks that is a consequence of activation of the G₁ checkpoint.

Sequential Inhibition of S, G₁, and G₂ Phase Progression by High Effective Drug Concentrations. To characterize the effects of the high effective concentrations of DACH-acetato-Pt on cell cycle progression, we exposed A2780 cells to 3.2 μM DACH-acetato-Pt and monitored cell cycle kinetics for up to 48 h by FACS analysis. Both the DNA content histograms and plots of the percentage of cells in G₁, S, or G₂-M phase demonstrated that at this concentration, the drug induced a triphasic response in A2780 cells (Fig. 5, A and B). During the first 9 h after drug treatment, S-phase cells increased from 33 to 51%. In contrast, the percentage of cells in other phases decreased slightly (G₁) or dramatically (G₂-M). This indicated that progression through S-phase was acutely inhibited, whereas progression through other phases of the cell cycle was not. In the second phase, i.e., between 12 and 24 h, the percentage of S-phase cells decreased to ~10%, whereas the percentage of G₁ and G₂-M phase cells increased to 60 and 33%, respectively. From this, we can deduce that S-phase progression resumed during this period. In the third phase, from 24 to 48 h, the percentage of S-phase cells decreased to ~10%, whereas the percentage of G₁ and G₂-M phase cells increased to 60 and 33%, respectively.
Effects of DACH-acetato-Pt on Cell Cycle Checkpoints

The initial transient inhibition of S-phase progression by high effective concentrations of DACH-acetato-Pt suggests that the S-phase checkpoint may be activated. To examine this possibility, we measured the activity of Cdk2/cyclin A complex, which is required for normal S-phase progression and is a frequent target of S-phase checkpoint response. To achieve this, lysates were prepared from A2780 cells collected at different time points after treatment of cells with 2.4 μM DACH-acetato-Pt. The lysates were then incubated first with the conjugated anti-cyclin E antibody to remove Cdk2/cyclin E complex and then with the anti-Cdk2 antibody to specifically isolate the Cdk2/cyclin A complex. The results in Fig. 6A demonstrate that although the amount of cyclin A protein in the Cdk2 immunocomplex did not decrease for the first 15 h, the Cdk2/cyclin A activity decreased to ~3% of the initial level during this period and then remained at negligible levels throughout the rest of the time course. These results indicate that the S-phase checkpoint was lastingly activated by high effective concentrations of DACH-acetato-Pt although the inhibition of S-phase progression was only transient. It is noteworthy that cyclin A protein became undetectable in the Cdk2 complex at 24 h, raising the possibility that high effective concentrations of DACH-acetato-Pt may not only inhibit the activity of Cdk2/cyclin A complex but also induce cyclin A degradation or its dissociation from Cdk2.

To evaluate the basis for the subsequent inhibition of G1 and G2-M phase progression by DACH-acetato-Pt at the high effective concentrations, we measured the Cdk4-, Cdk2-, and Cdc2-associated kinase activities after treating cells with 2.4 μM DACH-acetato-Pt. Although similar amounts of Cdk4 and Cdk2 proteins were present at all time points, the corresponding kinase activities decreased abruptly between 9 and 12 h after initiation of drug exposure, coinciding with the start of an increase in the percentage of cells in G1, and then remained at negligible levels throughout the ensuing 12 h (Fig. 6, B and C). These results demonstrate that after the activation of the S-phase checkpoint, high effective concentrations of DACH-acetato-Pt activated the G1 checkpoint abruptly. In contrast to the sudden decrease in the Cdk4 and Cdk2 activities, the level of Cdc2-associated kinase activity declined gradually to negligible levels from 9 to 24 h, and this preceded the increase in the percentage of G2-M cells (Fig. 6D). These results indicate that activation of the G1 checkpoint coincided closely with activation of the G1 checkpoint, although the kinetics between the two was different. It is noteworthy that the level of Cdc2 protein was similar for the first 15 h and then decreased at 24 h, raising the possibility that, as with cyclin A, high effective concentrations of DACH-acetato-Pt may induce some Cdc2 protein degradation.

Induction of Cell Death by Apoptosis. The different effects of DACH-acetato-Pt on cell cycle checkpoints raised the question of whether the mode of cell death was by apoptosis at all effective drug concentrations. For this reason, A2780 cells were treated continuously with 0–6.4 μM DACH-acetato-Pt for up to 3 days, and the cells collected daily were analyzed for DNA and nuclear fragmentation and the presence of phosphatidylserine on the cell surface as major hallmarks of apoptosis. Fig. 7 summarizes the results from assays that measure the level of DNA fragmentation. In Fig. 7A, the results from TUNEL assay show that DACH-acetato-Pt at all concentrations examined induced TUNEL-positive cells in a time- and dose-dependent manner, starting as early as the first day and increasing to relatively high levels on days 2 and 3. On day 3, 0.1–6.4 μM DACH-acetato-Pt induced 10–100% TUNEL-positive cells, respectively. It is noteworthy that although higher concentrations

readily from changes in cells in S-phase (Fig. 5C). Together, these results support the conclusion that high effective concentrations of DACH-acetato-Pt initially inhibit S-phase progression in an acute and transient manner and subsequently inhibit cell cycle progression at both G1 and G2-M phases over a protracted period.

Sequential Inhibition of Different Cdk Activities by High Effective Drug Concentrations. The initial transient inhibition of S-phase progression by high effective concentrations of DACH-acetato-Pt suggests that the S-phase checkpoint may be activated. To examine this possibility, we measured the activity of Cdk2/cyclin A complex, which is required for normal
of the drug induced greater percentages of TUNEL-positive cells, the induction kinetics were similar at all drug concentrations, i.e., ~10% of the final TUNEL-positive value on day 3 was reached in general within the first day. In Fig. 7B, the result from gel electrophoresis of fragmented DNA at 48 h after the initiation of drug treatment shows that all drug concentrations of DACH-acetato-Pt induced DNA ladder formation, indicative of internucleosomal cleavage. Again, the concentration dependency was evident, with the higher concentration of the drug correlating with more fragmented DNA. No obvious DNA ladder formation was observed at day 1 (data not shown). These results confirmed the results of the TUNEL assay.

Fig. 8A shows the results of staining unfixed cells at days 1, 2, and 3 with annexin V, which binds specifically to membrane phosphatidylserine that becomes externalized during apoptotic cell death. The externalization of phosphatidylserine is an early apoptotic event occurring around the time of DNA fragmentation (25). Moreover, the annexin V staining quantified by two-parameter FACS analysis is a relatively sensitive method for the detection of apoptosis. The results from this assay showed that DACH-acetato-Pt induced annexin V-positive cells at all drug concentrations starting on day 2. Again, the effect was concentration dependent. On day 2, 0.1–6.4 μM DACH-acetato-Pt induced 13–68% annexin V-positive cells. On day 3, this range increased to 20–75%. In general, the kinetics of appearance of annexin V-staining cells was similar to that of the occurrence of DNA fragmentation, consistent with the expected temporal relationship between DNA fragmentation and phospholipid externalization.

The results of FACS analysis of the cells on days 2 and 3 are shown in Fig. 8B. The sub-G₁ population from FACS analysis indicates nuclear fragmentation and formation of apoptotic bodies, which are relatively late apoptotic events. The sub-G₁ population was not detectable on day 1 at any drug concentration (data not shown), consistent with the results of FACS analysis in Fig. 2. On day 2, DACH-acetato-Pt induced a dose-dependent increase in the sub-G₁ population. This event was further increased on day 3 at all drug concentrations.
Interestingly, the appearance of sub-G1 population lagged behind the appearance of annexin V-positive cells, compatible with the expected timing of nuclear fragmentation. Collectively, these results demonstrate that DACH-acetato-Pt at all effective concentrations induces cell death through apoptosis.

**DISCUSSION**

Activation of cell cycle checkpoints is a general cellular response after exposure to cytotoxic agents. These checkpoints arrest cells in G1, S, or G2-M phase of the cell cycle to enable critical cellular functions, such as DNA repair, to be performed (10). Previous flow cytometric studies have indicated that cisplatin and other platinum agents predominantly inhibit cell cycle progression at S and/or G2-M phase, independent of drug concentration in the range 1–5 × IC50 (11). In contrast, the results from the present study demonstrate clearly that the effect of the novel cisplatin analogue DACH-acetato-Pt on cell cycle checkpoints is concentration dependent. At low effective concentrations (0.1–0.6 μM), the analogue selectively activates the G1 checkpoint. Thus, the primary cell cycle effect of DACH-acetato-Pt is distinct from that of cisplatin and other analogues. At the higher effective concentrations (≥0.8 μM), DACH-acetato-Pt not only activates the G1 checkpoint but also activates S and G2 checkpoints in sequence. The S-phase arrest, however, was found to be transient, which is a typical response to a number of cytotoxic agents, including cisplatin (11, 26). Because the sequential S and G2 phase arrest is characteristic of cisplatin and other platinum-containing agents in a number of cell lines, including A2780 cells (11, 13), this endorses the possibility that the secondary effect produced by the high effective concentrations of DACH-acetato-Pt is similar to that caused by cisplatin. One plausible explanation for this bimodal effect of DACH-acetato-Pt on cell cycle checkpoints is that the drug activates two signal transduction pathways, possibly because of distinct cellular lesions: (a) which is exclusive to this agent and occurs...
at a low threshold drug concentration, activates the G₁ checkpoint; and (b) which occurs at a high threshold drug concentration, may be common to both cisplatin and the analogue and activates S and G₂ checkpoints. Further studies will examine the intriguing possibility that the unique effect of DACH-acetato-Pt on the G₁ checkpoint may be linked to the ability of DACH-acetato-Pt in overcoming cisplatin resistance.

On the basis of our present understanding of cell cycle control, activation of cell cycle checkpoints in most cases culminates in the inhibition of Cdk activities (9, 10, 27, 28). In the present study, we have shown for the first time that the selective inhibition of G₁-S-phase progression by the low effective concentration of DACH-acetato-Pt is associated with the inhibition of both Cdk4 and Cdk2 activities. Thus, the checkpoint control pathway(s) activated by the cellular lesion induced by the low effective concentrations of DACH-acetato-Pt impinges specifically on Cdk regulation of G₁-S-phase progression. Several mechanisms can be proposed to explain the inhibition of these kinases. They include down-regulation of the cyclin components, induction of tyrosine phosphorylation on the Cdk component, and binding of the Cdk inhibitors p16, p21, or p27 to the Cdk complexes (10, 29). We have reported previously that treatment of human ovarian OVCA-429 tumor cells with DACH-acetato-Pt increased the level of p21 protein 3–4-fold as a result of induction of wild-type p53 (14). Furthermore, OVCA-432 tumor cells harboring mutant p53 failed to arrest in G₁ but instead arrested in G₂-M (14). These earlier observations suggest that the inhibition of Cdk4 and Cdk2 activities by DACH-acetato-Pt demonstrated in the present study is mediated via the induction of p53 and p21. Because p21 induction involves de novo RNA transcription and protein synthesis, this may explain the long lag period before G₁ arrest is observed with both low and high effective concentrations of DACH-acetato-Pt. In this context, it is of interest to note that inhibition of G₁ CdkS in p53-proficient Chinese hamster ovary cells and mouse lung fibroblasts by cells undergoing radiation has been demonstrated to occur within 1–2 h (30, 31). This prompts us to speculate that DACH-acetato-Pt and ionizing radiation activate G₁ checkpoints through different mechanisms.

At the higher effective concentrations, DACH-acetato-Pt caused inhibition of S-phase Cdk, which occurred much earlier than the inhibition of G₁ and G₂ Cdns. This indicates that the first checkpoint control pathway activated by the high effective concentrations of DACH-acetato-Pt impinges selectively on the regulators of Cdk2/cyclin A activity and suggests that the process may not involve de novo transcription and translation of Cdk inhibitors, such as p21. In this regard, the S-phase checkpoint response induced by ionizing radiation has been shown to also involve rapid inhibition of the S-phase Cdk2/cyclin A activity that was independent of p53 and p21 but dependent on the ATM gene product (30). This raises the speculation that DACH-acetato-Pt may activate the S-phase checkpoint via this mechanism.

After the rapid inhibition of S-phase Cdk by the high effective concentration of DACH-acetato-Pt, both G₁ and G₂ Cdns were inhibited but with different kinetics. Although the inhibition of G₁ Cdk activities was expected based on results from the study with the low effective concentration, it is important to note that the higher concentration of the drug correlated with a faster and more abrupt inhibition of G₁ Cdns, indicating that the severity of inhibition of these Cdns by DACH-acetato-Pt is dose dependent. In contrast to the kinetics of inhibition of G₁ Cdk activities by the high effective concentration of DACH-acetato-Pt, the inhibition of G₂ Cdk activity was observed as a slow and gradual process. This suggests that the inhibition of G₂ Cdk activity results from activation of a different checkpoint control pathway from that leading to the inhibition of G₁ Cdk activities. Similar temporal relationships for the activation of the G₂ checkpoint have also been reported with cisplatin and nitrogen mustard (15, 32, 33). This was in contrast to a more immediate activation of G₂ checkpoint response by ionizing radiation, with only a 1-h lag period (34). It is possible, therefore, that DACH-acetato-Pt and cisplatin may activate G₂ phase checkpoint through similar mechanisms. The mechanism for cisplatin may involve drug-induced modulation of Cdc2 because of decreased Cdc25 phosphatase activity (27), which is consistent with the reported findings that cisplatin inhibits G₂ Cdk by maintaining tyrosine-15 of Cdc2 in an inactive phosphorylated state (15). In addition, because it is reported that the S-phase Cdk activity is required for subsequent activation of G₂ Cdk activity (35), it is possible that the inhibition of Cdc2 is a direct result of inhibiting the activity of S-phase Cdk. This would offer a plausible explanation for the sequential activation of S and G₂ checkpoints in both cisplatin- and DACH-acetato-Pt treated cells.

Apart from the activation of cell cycle checkpoints, another important cellular event activated in response to cytotoxic agents is programmed cell death (apoptosis). Apoptosis is characterized by cytoskeleton disruption and decreased cell adhesion, DNA fragmentation, the appearance of phosphatidylserine on the external surface of the plasma membrane, and nuclear fragmentation (25, 36). We have used four separate assays to demonstrate that DACH-acetato-Pt induced all of these characteristics of apoptosis in a concentration- and time-dependent manner, thereby establishing that apoptosis was the fundamental mechanism of cell death. In addition, even at the lowest concentration of the drug examined, 0.1 μM (0.5 × IC₅₀), which only slightly inhibited cell growth, induction of these events was observed. Thus, the inhibition of cell growth and induction of apoptosis went hand in hand within the range of concentrations examined. These results indicate that DACH-acetato-Pt is both an effective inhibitor of cell growth and a potent inducer of apoptosis.

Apoptosis has also been demonstrated with cisplatin in a number of cell lines. Using gel electrophoresis as an indicator of DNA fragmentation, cisplatin was shown to induce apoptosis beginning on day 2 in Chinese hamster ovary cells at concentrations ranging from 0.4 to 40 μM (37). Similar results have been reported in L1210 leukemic cells (38). In both these tumor models, cell death occurred only after cells had arrested in the G₂ phase of the cell cycle. In the human ovarian CH1 cells, marked morphological features of apoptosis were noted at concentrations of 3–25 μM of cisplatin (39). In another study, Ormerod et al. (12) have reported that L1210 cells underwent apoptosis at ≥50 μM (≥25 × IC₅₀) but only growth arrest at lower concentrations. This result, however, contrasts with that obtained by Sorenson et al. (38) in the same L1210 cell line, which underwent apoptosis at low and high concentrations. The
Inconsistency between the two studies has been attributed to different tissue culture conditions (12). Our results from the present study, however, are in agreement with the general findings with cisplatin, in that growth arrest and apoptosis were observed at all concentrations in the range 0.1 to 6.4 μM (0.5 to 32 × IC50). In contrast to the observation that progression to the G2 was associated with cisplatin-mediated apoptosis (37, 38), our data with DACH-acetato-Pt provide evidence that apoptosis also occurs after cell cycle arrest at G1. This comparison between cisplatin and the analogue again highlights that cell cycle effects for the two agents are different.

Induction of apoptosis by cytotoxic agents can occur in either an acute or delayed manner (40). The acute induction of apoptosis does not involve prior induction of cell cycle arrest. In contrast, delayed apoptosis occurs after the initial induction of cell cycle arrest, which may last for 2–3 days. We showed that at all drug concentrations examined, DACH-acetato-Pt first induced cell cycle arrest and then induced apoptosis, although the interval was only ~1 day. Because this lag time was observed even at the highest drug concentration that was 32-fold greater than the IC50, the results suggest that DACH-acetato-Pt is not an inducer of acute apoptosis, but that the induction of apoptosis is linked to its ability to elicit a checkpoint response.

A checkpoint response induced by cytotoxic agents can have both positive and negative effects on the induction of apoptosis, depending on the cell cycle stage of the checkpoint response, the cellular context, and the cytotoxic agent examined (10, 40, 41). Because abrogation of the G2 checkpoint response elicted by cisplatin and other cytotoxic agents often increases the cellular sensitivity to these drugs (9, 40), it is generally believed that the G2 checkpoint response antagonizes the apoptotic response of the cell. On the other hand, the relationship between the checkpoint response in other phases of the cell cycle and the induction of apoptosis is less unified (9, 40). We speculate that a normal p53-mediated checkpoint response may facilitate DACH-acetato-Pt-induced apoptosis. In this context, it is of interest to note that evidence exists to indicate that inhibition of Cdk activity alone can induce apoptosis under certain circumstances (40).

In summary, we can advance a tentative model to describe the bimodal effect of DACH-acetato-Pt on cell cycle checkpoints in A2780 tumor cells (Fig. 9). In this model, the antitumor agent activates two distinct signal transduction pathways. One pathway, which is activated at a low threshold drug concentration, is unique to the novel analogue and leads to the activation of the G1 checkpoint. This pathway may contribute to the effectiveness of DACH-acetato-Pt against cisplatin-resistant tumor cells. The second pathway, which is activated at a high threshold drug concentration, is similar to that stimulated by cisplatin and results in sequential activation of S and G2–M checkpoints. It is likely that these cell cycle effects of DACH-acetato-Pt may facilitate the induction of apoptosis.

ACKNOWLEDGMENTS

We thank Dr. Richard B. Clark for critically reviewing the manuscript, Kay Biessbar for providing editorial assistance, and Dr. Junichi Nakamura and Patrick Hennessey for preparing and analyzing solutions of DACH-acetato-Pt.

REFERENCES

13. Eiliopoulos, A. G., Kerr, D. J., Herod, J., Hodgkins, L., Krajewski, S., Reed, J. C., and Young, L. S. The control of apoptosis and drug


Bimodal Effects of 1R,2R-Diaminocyclohexane\textit{trans}-diacetato(dichloro)platinum(IV) on Cell Cycle Checkpoints

Jian Kuang, Guangan He, Zaifeng Huang, et al.


Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/7/11/3629

This article cites 40 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/7/11/3629.full.html#ref-list-1

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.