Alkylating Agents and Immunotoxins Exert Synergistic Cytotoxic Activity against Ovarian Cancer Cells

Mechanism of Action

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Abstract

Alkylating agents can be administered in high dosage to patients with ovarian cancer using autologous bone marrow support, but drug-resistant tumor cells can still persist. Immunotoxins provide reagents that might eliminate drug resistant cells. In the present study, concurrent treatment with alkylators and immunotoxins proved superior to treatment with each agent alone. Toxin immunoconjugates prepared from different monoclonal antibodies and recombinant ricin A chain (rRTA) inhibited clonogenic growth of ovarian cancer cell lines in limiting dilution assays. When alkylating agents and toxin conjugates were used in combination, the addition of the immunotoxins to cisplatin, or to cisplatin and thiotepa, produced synergistic cytotoxic activity against the OVCA 432 and OVCAR III cell lines.

Studies performed to clarify the mechanism of action showed that cisplatin and thiotepa had no influence on internalization and binding of the 317G5-rRTA immunotoxin. Intracellular uptake of $^{[195m]}$Pt-cisplatin was not affected by the immunoconjugate and thiotepa. The combination of the 317G5-rRTA and thiotepa, as well as 317G5-rRTA alone, increased $^{[195m]}$Pt cisplatin-DNA adduct levels. The immunotoxin alone and in combination with the alkylators decreased intracellular glutathione levels and reduced glutathione-S-transferase activity. Repair of DNA damage induced by the combination of alkylators and 317G5-rRTA was significantly reduced when compared to repair after damage with alkylators alone. These findings suggest that immunotoxins affect levels and activity of enzymes required for the prevention and repair of alkylator damage. (*J. Clin. Invest.* 1993. 92:2440–2447.) Key words: cisplatin • ovarian neoplasms • immunoconjugates • drug synergy

Introduction

Most ovarian cancer is diagnosed in advanced stage and, therefore, cannot be cured by surgery. Thus, after excision of as much tumor as possible, ovarian cancer patients are treated aggressively with platinum-based combination chemotherapy. Most patients respond to cytotoxic chemotherapy and many appear to be cured on clinical evaluation. Unfortunately, a majority of these patients will develop recurrent disease refractory to other available therapy. This is felt to result from the regrowth of drug resistant tumor cells. Thus, there is a major need to find approaches to prevent or reverse drug resistance when it occurs.

Immunotoxins are anticancer agents produced by conjugation of an antibody and a toxin. The antibody permits selective targeting of the toxin to tumor cells (1). Since the mechanisms by which immunotoxins exert their cytotoxic effects differ from those of alkylating agents including platinum drugs, toxin immunoconjugates could potentially eliminate alkylator resistant cells. Moreover, the concurrent use of alkylators and immunotoxins in combination might prove superior to treatment with the individual agents alone.

In a previous study, serial dilution clonogenic assays were used to evaluate the impact of alkylating agents on growth of established ovarian cancer cell lines (2). Isobolographic analysis showed that the combinations of cisplatin together with thiotepa, melphalan, or 4-hydroxyperoxy-cyclophosphamide exerted synergistic cytotoxic activity against the OVCA 432, 420, 429, and 433 cell lines. Other studies have shown that immunotoxins exert cytotoxic and antitumor effects against ovarian cancer cell lines and heterografts (3, 4). The effects of concurrent treatment of ovarian cancer cells with immunotoxins and alkylators has not, however, been previously described.

In the present study, we have shown that immunotoxins prepared from two distinct monoclonal antibodies and recombinant ricin A chain (rRTA)[1] could synergistically enhance the cytotoxic activity of a combination of alkylating agents against the OVCA 432 and OVCAR III cell lines. Possible mechanisms that may explain, in part, the observed synergistic interaction have been evaluated.

Methods

Cell lines

OVCA 432 was established from a human ovarian cancer of epithelial origin (5). OVCAR III was purchased from American Type Culture Collection (Rockville, MD) (6). Cells were maintained in Eagle's minimum essential medium with Earle's salts (OVCA 432), or RPMI 1640

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1. *Abbreviations used in this paper:* BSO, buthionine sulfoximine; cisplatin, *cis*-diamminedichloro-platinum (II); GSH, glutathione; GST, glutathione-S-transferase; SSA, S-sulfosalicylic acid; TCM, tissue culture media; thiotepa, trimethyleneiminethiophosphoramide.

(OVCAR III), supplemented with 10% heat inactivated FBS, 1 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids (0.1 mM each), 100 μg/ml streptomycin, and 100 U/ml penicillin (tissue culture media [TCM]). Unless otherwise indicated, cells were trypsinized before the experiments and aliquoted in 5-ml polypropylene tubes. Trypsinized cells were treated with drugs or immunotoxin at 37°C in an atmosphere of 5% CO_2 and 95% humidified air for 3 h on a rocking platform.

Drugs and immunoconjugates

Cisplatin (*cis*-diamminedichloro-platinum [II]) (Bristol-Myers Co., New York) was stored at room temperature, and thiotepa (tri-methyleneiminethiophosphoramide) (Lederle Laboratories, Pearl River, NY) was stored at 4°C. Alkylating agents were dissolved in H_2O immediately before use and diluted to appropriate concentrations in TCM. rRTA was generously provided by Cetus Corp., Emeryville, CA. The 317G5 murine monoclonal antibodies were obtained as described (7). The BT11A9.13 antibody was prepared in our laboratory after immunization of mice with BT20 breast cancer cells. Both 317G5 and BT11A9.13 bind to a 42–43-kD glycoprotein on the cell surface of human breast and ovarian cancer cells (8). The preparation of the rRTA conjugates was previously described (9).

Serial dilution clonogenic assay and isobolographic analysis

Drug cytotoxicity was evaluated using a limiting dilution technique as described previously (10). Briefly, after trypsinization, 10^6 tumor cells were incubated with drugs and/or immunotoxins in a total volume of 1 ml. Cells were then washed twice with TCM. A series of nine fivefold dilutions was prepared. Six aliquots (100 μl) of each dilution were plated in 96-well flat-bottomed microtiter plates that had been preloaded with 100 μl TCM. Plates were incubated for 14 d at 37°C in 5% CO_2 and 95% humidified air. Growth of colonies (> 50 cells) was evaluated by visual scoring (2). Each value was calculated from a mean of duplicate plates. Limiting-dilution analysis was performed as described (10).

Isobolographic analysis, a geometric method to explore drug interactions, was performed as described by Berenbaum (11, 12) and Steel and Peckham (13). Isoboles for different levels of cytotoxicity were drawn from dose-response curves, in which the log effect by dose of one agent was plotted for each constant dose of the other agents in the combination. The calculation of an "envelope of additivity" between modes I and II, which indicated the theoretical limits of the additive effects obtained from an interaction of two agents, is described elsewhere (2, 13).

An interaction between three agents was considered to be synergistic when the combined cytotoxic effects exerted by the three different agents created a concave isobolar surface (the surface joining all the dose combinations producing a given quantitative effect). According to Berenbaum (11), the interaction between three agents could be generalized using the iso-effective dose fraction formula:

$$\frac{\text{dose of A}}{A_e} + \frac{\text{dose of B}}{B_e} + \frac{\text{dose of C}}{C_e} \begin{cases} < 1 \text{ for synergy} \\ = 1 \text{ for additivity} \\ > 1 \text{ for antagonism,} \end{cases}$$

where A, B, and C are doses of three drugs used in combination to produce a certain effect, whereas A_e, B_e, and C_e are the doses of those drugs used alone to produce the same effect. When doses of drugs A, B, and C are represented by three coordinate axes, one can analyze their interaction geometrically. Thus, if the doses of A, B, and C are represented by points on three axes, then the points representing all doses and dose combinations with the same effect as A_e, B_e, and C_e will lie on the plan connecting these three points. The isobolar surface is flat when all the combinations of A, B, and C producing a certain effect are additive (and the sum of the iso-effective dose fractions constituting any combination of this surface equals 1). The isobolar surface for synergy is concave and the sum of these fractions is < 1.

Internalization of 317G5-rRTA into drug-treated cells

The 317G5-rRTA conjugate was labeled with Na ^{125}I using the iodogen method (14). Internalization was measured as previously described (15). Briefly, radioiodinated 317G5-rRTA (1 μg/ml) was incubated with drug-treated OVCA 432 cells (5×10^5) at 4°C for 1 h. After removal of excess conjugate with cold culture medium containing 1% BSA, the cells were incubated either at 4°C or 37°C for a period of 4 h, and then treated with proteinase K (100 μl of 2.5 mg/ml solution) for 1 h at 37°C with gentle shaking. Cells were then washed three times with cold medium containing 1% BSA and 0.1% NaN_3. Radioactivity was measured in a gamma counter (Packard Instruments (Meriden, CT).

Uptake of $^{[195m]}$Pt-cisplatin

$^{[195m]}$Pt-cisplatin (84 μCi/mmol) was obtained from Oak Ridge National Laboratories (Oak Ridge, TN). Cell size was determined with a Coulter electronic particle counter equipped with a channel analyzer (models ZB and C1000; Coulter Electronics, Hialeah, FL).

The uptake of $^{[195m]}$Pt-cisplatin was determined as described before (16) with minor variations. Briefly, 2×10^6 OVCA 432 cells were incubated in 5-ml polypropylene tubes with different concentrations of $^{[195m]}$Pt-cisplatin (10–100 μmol) for 0.5–30 min, in a volume of 1 ml TCM at 37°C. A 600-μl aliquot of each sample was then diluted 10-fold with ice-cold 0.9% NaCl, and cells were centrifuged at 500 *g* for 5 min at 4°C. The pellet was washed twice with ice-cold 0.9% NaCl and solubilized overnight at 37°C in Aquasol-2 (New England Nuclear Research Products, Boston, MA). Radioactivity was measured in a scintillation counter (Tri-carb 4640). Uptake velocity was calculated from the linear portion of a curve after correction for the initial rapid binding of the drug. The reciprocal velocities were plotted against the reciprocal $^{[195m]}$Pt-cisplatin concentrations. The resulting Lineweaver-Burk plots were analyzed by linear regression and the K_m and V_{max} of each treatment modality were determined from the slopes and intercepts of these plots. Data were also analyzed for two-component transport without correction for the initial rapid binding of the drug according to the formula of Neal (17).

Intracellular levels of glutathione (GSH)

Cells ($2–5 \times 10^6$; ~ 60% confluent) were lysed by sonication in 1 ml of PBS at 4°C. The supernatant was obtained for assay after centrifugation (10,000 *g*, 10 min, 4°C). The protein was precipitated by adding 12% 5-sulfosalicylic acid (SSA) (1 vol SSA to 3 vol of sample). After standing on ice for 1–4 h, the samples were centrifuged (10,000 *g*, 10 min). The SSA extract was assayed as described by Tietze (18) and modified by Griffith (19, 20), using 100 μl of sample and 0.5 U of glutathione reductase per assay. Protein in the PBS lysate was determined by the Lowry assay (21) (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as standard.

Intracellular activity of glutathione-S-transferase (GST)

Activity was measured with 1-chloro-2,3-dinitrobenzene (22). The PBS extract (above) was used after centrifugation. The reaction mixture contained glutathione (1 mM), 1-chloro-2,3-dinitrobenzene (1 mM), and sample (100 μM) in a final volume of 1 ml. The rate of increase in absorbance at 340 nm was measured at 25°C. The results are expressed as nmol/min per 10^6 cells.

DNA repair

$^{[195m]}$Pt-cisplatin DNA adducts. Drug-treated cells (2×10^7) were treated with 50 μm of $^{[195m]}$Pt-cisplatin (123 μCi/mmol) on a rocking platform for 1 h at 37°C. Cells were washed twice in PBS, once in 0.144 M NH_4Cl with 0.01 M $NaHCO_3$ (10:1 vol/vol), and lysed with 0.5% SDS in TNE buffer (10 mM Tris HCl, pH 8.2, 400 mM NaCl, and 2 mM EDTA). Cell lysate was then mixed with proteinase K (250 μg/ml) and incubated at 37°C for 3 h. After phenol/chloroform extraction and ethanol precipitation (23), the DNA was dissolved in 200 μl of TE buffer, pH 8.0, and quantitated in a mini fluorometer (model TKO 100; Hoefer Scientific Instruments, San Francisco, CA). Radioactivity was quantitated as described above. Radiolabeled cisplatin-DNA ad-

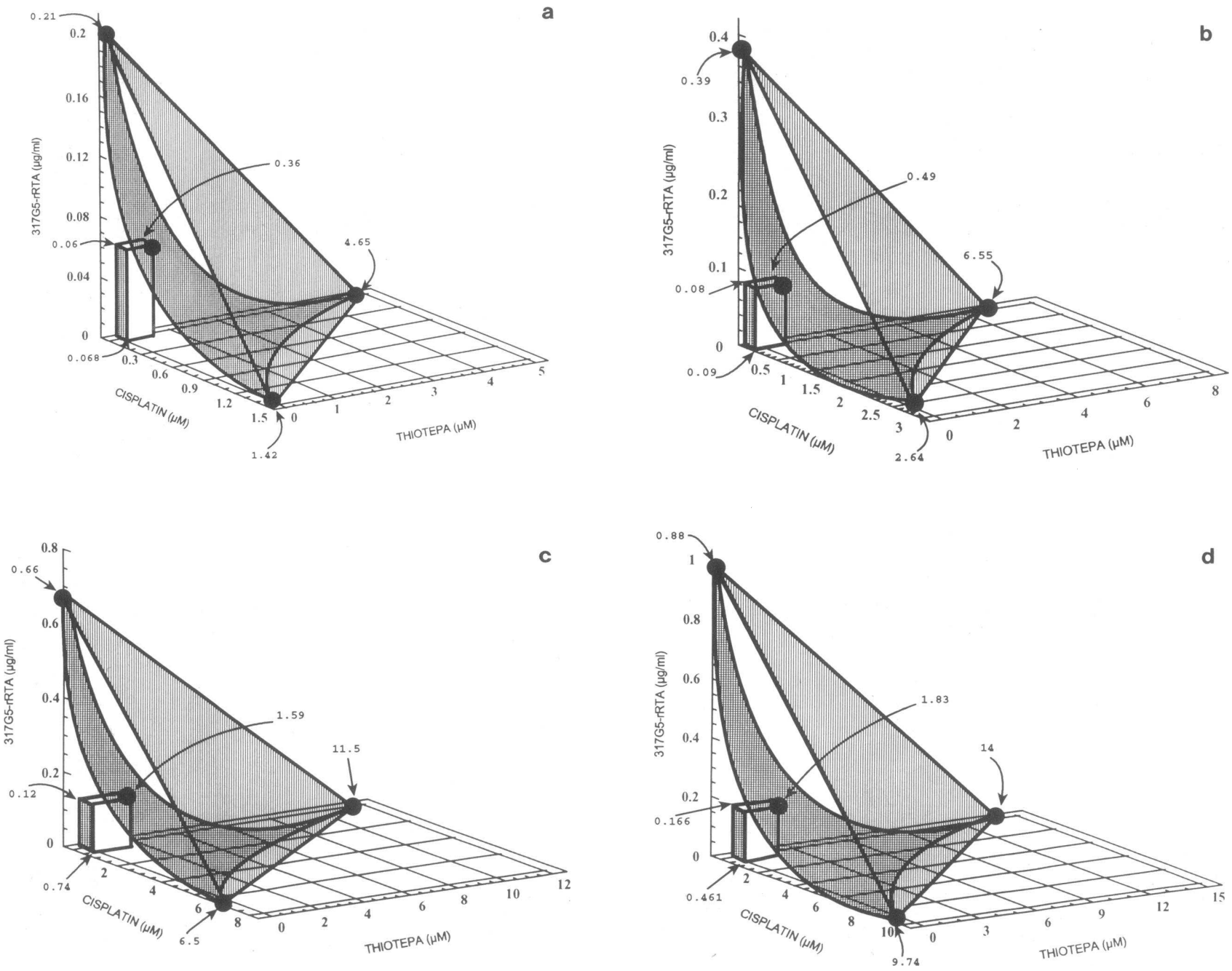

Figure 1. Isobolograms for 1.5 (*a*), 2 (*b*), 3 (*c*), and 4 (*d*) log reduction of clonogenic cell growth. The flat isobolar surfaces represent an additive effect. The concave surfaces indicate that the three agents interact synergistically. Each point is the average of data obtained from three different experiments.

duct levels were expressed in picograms of cisplatin per nanograms of DNA.

Unscheduled DNA synthesis. Unscheduled DNA synthesis after drug damage was taken as a measure of total DNA repair activity. The assay was performed according to a modification (24, 25) of a standard procedure (26, 27). In summary, exponentially growing OVCA 432 cells in TCM were prelabeled with 0.004 μCi/ml [^{14}C]deoxythymidine (54 mCi/mmole; New England Nuclear) for 24 h. 48 h later, the cells were harvested, pooled, and seeded in culture dishes (100 × 20 cm). At full confluency, TCM was replaced with arginine-deficient medium containing 2.5% dialyzed FBS, and medium was replaced every 24 h for a total of 72 h to decrease replicative DNA synthesis. To further suppress and to density label any residual replicative DNA synthesis, hydroxyurea (2 μM) and bromodeoxyuridine (10 μM) were then added to the cultures. Treatment with alkylating agents and/or immunotoxins began 2 h thereafter. Cells were treated with drug and immunotoxin combinations for 3 h and washed three times with PBS. Cells were then incubated with 5 μCi [^{3}H]deoxythymidine/ml (80 Ci/mmol; New England Nuclear) in arginine-deficient medium containing 2.5% dialyzed FBS, hydroxyurea, and bromodeoxyuridine for 4 h, to radiolabel and density label the postdamage unscheduled DNA synthesis. Dishes were then washed three times with cold PBS and the cells were solubilized with 1 ml 10 mM Tris-HCl, 1 mM EDTA (pH 8), 0.5% sodium dodecyl sulfate, and 200 μl 5 N NaOH, to which 0.15 M NaCl and 0.015 M sodium citrate was added to a total lysate weight of 4.8 g. Each lysate was added to 6-g portions of CsCl in polyallomer centrifuge tubes to obtain a density of ~ 1.72 g/ml and centrifuged to equilibrium at 100,000 *g* for 36 h. Multiple fractions of 0.5 ml were obtained from each gradient and DNA was collected on cellulose filters (17 Chr; Whatman Inc., Clifton, NJ). Filters were then washed with 5% cold trichloroacetic acid and 100% ethanol and acetone for 5 min each and allowed to dry. Radioactivity was determined as described above with correction for ^{14}C and ^{3}H channel overlap. Fractions comprising the ^{14}C peak were pooled and dialyzed against water for ≤ 24 h. The DNA content of the normal density DNA was determined spectrophotometrically. The ^{14}C-specific activity of the DNA was calculated and repair activity was expressed as ^{3}H counts per minute per microgram of DNA. The difference of ^{3}H counts per minute per microgram of DNA between treated and control was used as an index of repair activity.

Results

Synergistic interaction between immunotoxin and cisplatin. Using serial dilution clonogenic assays of OVCA432 growth, dose response curves were generated for the cytotoxic effects of cisplatin, thiotepa, and 317G5-rRTA, alone and in combination. Three-dimensional isobolographic analysis was then performed. Isoboles for 1.5, 2, 3, and 4 log-reduction of clonogenic cell growth are plotted in Fig. 1, *a–d,* respectively. Three identical experiments were performed at different times and showed similar results. The resultant concave isobolar surfaces indicate that the three agents interacted synergistically over several orders of magnitude. Using a combination of agents, 99.99% of the cells could be killed.

In a previous study, synergistic cytotoxic activity was obtained with a combination of cisplatin and several other alkylating agents against ovarian cancer cells that were sensitive to the individual alkylators. Synergy could not be demonstrated against the OVCAR III cell line, known to be resistant to cisplatin (2, 28). In the present study, using a combination of cisplatin and 317G5-rRTA, we obtained synergistic cytotoxic activity against OVCAR III (Fig. 2). Thus, supra-additive cytotoxicity could be obtained against cisplatin resistant or cisplatin sensitive ovarian cancer cells when an immunotoxin was

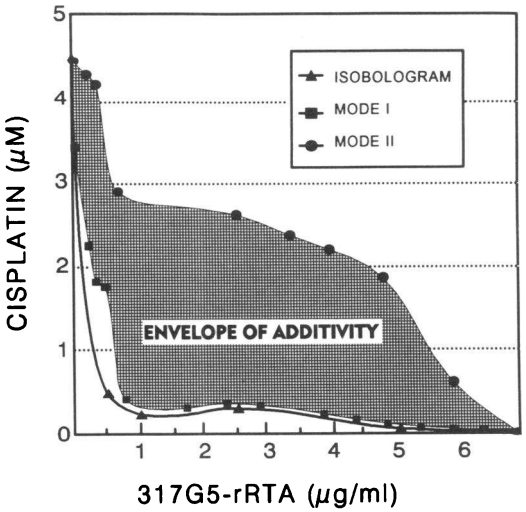

Figure 2. Synergistic cytotoxic interaction of cisplatin (*CPPD*) and 317G5-rRTA against the cisplatin-resistant OVCAR III cell line. Modes I and II enclose an envelope of additivity for 1.5 log reduction of clonogenic cell growth. The isobole of the combined effect is mostly supra-additive, since it falls below the envelope of additivity.

added to cisplatin alone or to a combination of cisplatin and thiotepa.

The synergistic effect against OVCAR III was maintained when 317G5-rRTA was replaced with BT11A9.13-rRTA (Fig. 3). 317G5 and BT11A9.13 recognize different epitopes on the same 42-kD cell surface glycoprotein expressed by human breast and ovarian cancer cells.

Internalization of 317G5-rRTA into drug-treated cells. One important determinant of the cytotoxic activity exerted by ricin A chain on malignant cells is the rate at which bound immunotoxin is internalized into the cytoplasmic compartment. The conjugate is transported into receptosomes through clathrin-coated pits in a process that might be influenced by treatment with alkylators, since alkylating agents have been shown to inhibit uptake of amino acids through the ASC carrier system (16), as well as to damage cell membranes (29).

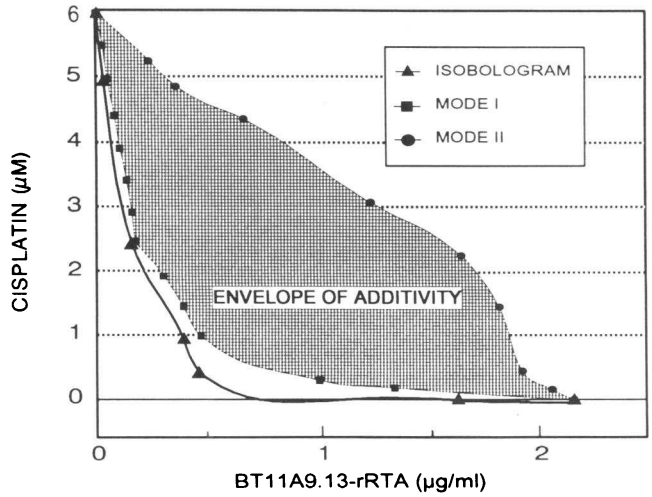

Figure 3. Synergistic cytotoxic interaction of cisplatin and BT11A9.13-rRTA against the OVCAR III cell line.

Experiments were carried out to measure the amount of 317G5-rRTA immunotoxin internalized into OVCA 432 cells during 4 h incubation at 37°C, after treatment of the cells with cisplatin and thiotepa. Internalization was not affected by drug treatment (Table I). The small reduction noted in the amount of conjugate internalized into cells treated with both cisplatin and thiotepa was not statistically significant. In general, 2–3 ng of immunotoxin were internalized into 5×10^5 cells in the presence or absence of alkylating agents.

Kinetic analysis of $^{[195m]}$Pt-cisplatin uptake. Indirect data suggest that uptake of platinum compounds involve an active carrier mechanism and that depletion of this carrier may affect drug transport into the cells (27, 30). Since immunotoxins may affect the carrier system, we compared the uptake of $^{[195m]}$Pt-cisplatin into tumor cells that had been treated with diluent or 317G5-rRTA in the presence or absence of thiotepa. The initial uptake of 100 μm cisplatin by OVCA 432 cells during 10 min is illustrated in Fig. 4. Similar plots were obtained with lower concentrations of cisplatin (10, 25, and 50 μm). The initial slope may reflect rapid binding of the drug to the plasma membrane before internalization actually occurs. Consequently, the V_{max} and K_m for each treatment modality were calculated according to Neal (for two-component transport) and Lineweaver-Burke (with correction for the initial rapid binding) and were found to be similar (data not shown). These findings indicate that the 3-h exposure to 317G5-rRTA and thiotepa did not alter the regular uptake of cisplatin by the OVCA 432 cells.

Intracellular levels of GSH. Glutathione is a nonprotein tripeptide thiol that participates in many important cellular functions, including protection from free radical damage and conversion of cytotoxic agents to less active compounds (31). Depletion of intracellular GSH renders cells more radiosensitive (28), and elevated GSH levels result in radioprotection and decreased sensitivity to many anticancer drugs (32). To study the role played by glutathione in the interaction between 317G5-rRTA and alkylating agents, we measured the intracellular concentration of GSH after treatment of OVCA 432 cells with diluent, alkylating agents, or immunotoxins. Glutathione levels in cells treated with a combination of cisplatin, thiotepa, and 317G5-rRTA were lower than the levels found in the diluent treated cells or in cells treated with cisplatin alone (Table II). The observed GSH depletion was statistically significant and reproducible (Student's t test and ANOVA $P < 0.003$).

Table I. Binding and Internalization of Immunotoxin by OVCA 432 Cells after Treatment with Cisplatin and Thiotepa

Treatment	Immunotoxin bound	
	4°C incubation followed by protease stripping	37°C incubation followed by protease stripping
	ng/5 × 10⁵ cells	
Diluent	12.3±2.1	15.2±1.5
Cisplatin	11.7±0.7	14.5±0.5
Thiotepa	12.9±0.3	15.4±0.5
Cisplatin/thiotepa	12.9±1.9	14.7±0.5

Internalization of 1 μg/ml 317G5-rRTA into 5×10^6 drug-treated OVCA 432 cells. Each value represents the mean±SD for quintuplicate samples of 5×10^6 cells.

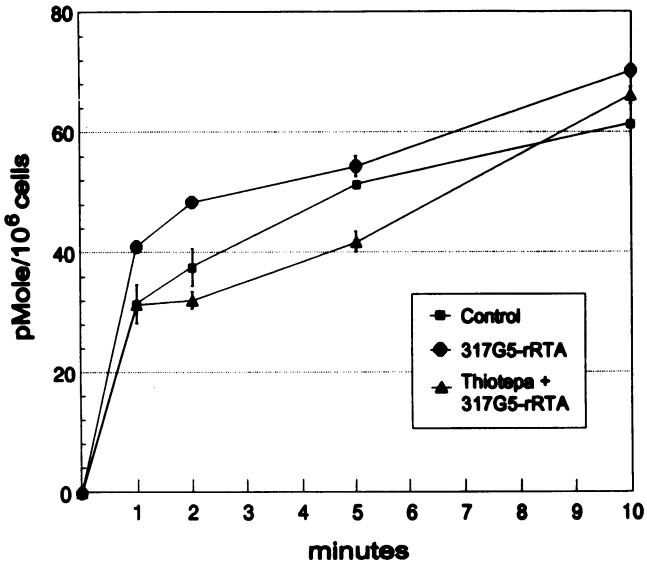

Figure 4. Uptake of 100 μM $^{[195m]}$Pt-cisplatin by 2×10^6 untreated OVCA 432 cells and cells exposed to 317G5-rRTA, with and without thiotepa, during the first 10 min of exposure.

Intracellular levels of GSH also decreased somewhat after treatment with the immunotoxin and either single alkylating agent, but the decrease was not statistically significant.

Intracellular activity of GST. Glutathione S-transferase catalyzes reactions in which GSH, as a nucleophile, can bind to compounds bearing electrophilic atoms (33). As a result, the GST group of enzymes takes part in the detoxification of alkylating agents, converting them to less active compounds (22, 34). The intracellular activity of GST was measured in OVCA 432 cells treated with alkylating agents and 317G5-rRTA. Glutathione S-transferase activity in cells treated with the combinations of cisplatin, thiotepa, and 317G5-rRTA was lower than the activity found in untreated cells and in those treated with the alkylators alone. A reduction of 22% was observed in cells treated with the three agents together as compared to the activity of GST found in the cells treated with cisplatin alone (Table

Table II. Effect of Treatment with Alkylating Agents and Immunotoxins on GSH Levels in OVCA 432 Cells

Treatment	GSH levels
	nM/10⁶ cells
Diluent	41.5±1.6‡
Cisplatin	43.2±0.7‡
Thiotepa	44.0±0.8‡
Cisplatin/thiotepa	43.1±0.6‡
317G5-rRTA	40.6±0.2
Cisplatin/317G5-rRTA	41.0±0.2
Thiotepa/317G5-rRTA	40.4±0.6
Cisplatin/thiotepa/317G5-rRTA	36.1±0.2*

* $P < 0.05$ vs ‡ Student's t test. (The variance of the group was statistically significant.) Intracellular glutathione levels in 6×10^6 treated and untreated OVCA 432 cells. Cisplatin concentration, 1 μM; thiotepa, 2 μM; 317G5-rRTA, 0.4 μg/ml. The experiment was repeated three times with triplicate samples of 6×10^6 cells.

Table III. Effect of Treatment with Alkylating Agents and Immunotoxins on Glutathione-S-transferase Levels in OVCA 432 Cells

Treatment	GST activity
	nM conjugate/min per ng protein
Diluent	374.9±2.6‡
Cisplatin	393.0±5.3‡
Thiotepa	375.9±3.7‡
Cisplatin/thiotepa	375.9±5.9‡
317G5-rRTA	362.3±4.8‡
Cisplatin/317G5-rRTA	354.6±3.2‡
Thiotepa/317G5-rRTA	332.1±8.7‡
Cisplatin/thiotepa/317G5-rRTA	308.3±3.8*

* $P < 0.05$ vs ‡ Student's t test. (The variance of the group was statistically significant.) GST activity in 5×10^6 OVCA 432 cells treated with the combinations of 1 μM cisplatin, 2 μM thiotepa, and 0.4 μg/ml 317G5-rRTA. GST activity in the supernatant was determined spectrophotometrically at 340 nm by measuring the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene and was expressed as nanomoles of conjugate formed per minute per nanogram protein. Quadruplicates of 5×10^6 OVCA 432 cells were treated with each agent as described above.

III). This reduction was highly significant (Student's t test $P < 0.006$ and ANOVA $P < 0.008$).

Adduct formation. Intrastrand, interstrand, and DNA-Pt-protein adducts are formed when cisplatin binds covalently to DNA. Intrastrand adducts are observed most frequently and exhibit sequence-specific binding of cisplatin to DNA forming bidentate N^7-deoxy (G-Pt-G) linkages between adjacent bases on the same strand. Interstrand DNA-Pt adducts are composed of cisplatin bound to two guanines on opposite DNA strands, perturbing G-C base pairing, leading to unwinding and shortening of DNA (35–37). CDDP-DNA adduct formation in OVCA 432 cells was studied after incubation with $^{[195m]}$Pt-cisplatin. OVCA 432 cells were treated for 1 h with thiotepa, 317G5-rRTA, neither, or both agents. Cells were then incubated for 1 h with or without $^{[195m]}$Pt-cisplatin. The concentration of cisplatin-DNA adducts in cells treated with a combination of cisplatin, thiotepa, and 317G5-rRTA was about twice that found in the cells treated with the alkylators (Student's t test $P < 0.017$ and ANOVA $P < 0.008$) (Fig. 5). A significant increase in adduct formation was also observed in cells treated with a combination of cisplatin and 317G5-rRTA, when compared to adducts formed in cells treated with alkylators alone ($P < 0.03$).

DNA repair. DNA repair after alkylator damage can play a major role in acquired resistance to cisplatin (38). The most general repair mechanism known is nucleotide excision that removes ultraviolet-induced lesions and chemical adducts from damaged DNA (39). Excision repair of total cellular DNA was evaluated in OVCA 432 cells by measuring unscheduled DNA synthesis following alkylator damage. When compared to diluent treated cells, bulk repair activity was substantially increased in cells treated with cisplatin (Fig. 6). When compared to levels of repair observed after treatment with cisplatin alone or in combination with thiotepa, a significant reduction of bulk repair activity was observed in cells treated

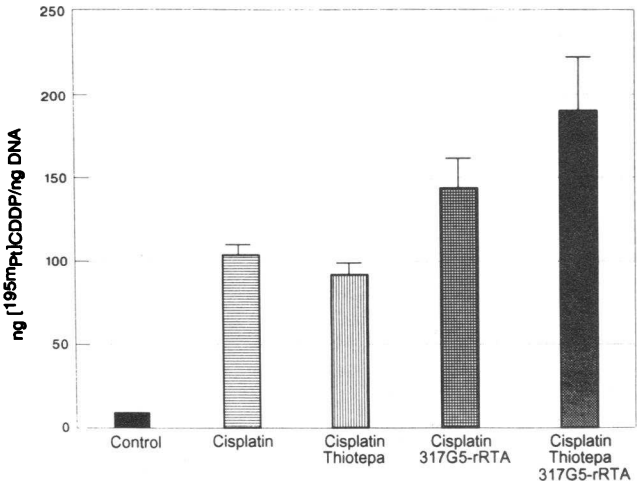

Figure 5. Cisplatin-DNA adduct formation in 1.5×10^7 OVCA 432 cells after incubation with 50 μmol $^{[195m]}$Pt-cisplatin. Cells were treated for 1 h with 2 μM thiopeta and 0.4 μg/ml 317G5-rRTA, neither, or both.

with 317G5-rRTA in addition to the alkylators ($P < 0.004$, ANOVA $P < 0.001$).

Discussion

Approximately 60–75% of ovarian cancer patients are diagnosed initially with advanced stage disease (40). Alkylating agents have proven beneficial in the treatment of these patients (41, 42), but most often only temporary remissions are achieved with platinum-based combination chemotherapy. Thus, the long term survival of ovarian cancer patients with bulky disease has only slightly improved during the last two decades (43–45). It is generally accepted that the emergence of drug resistance is the cause for treatment failure (46, 47). If

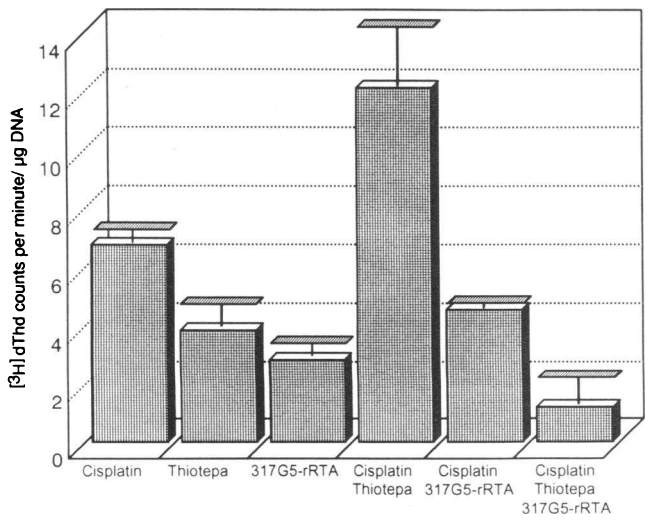

Figure 6. Postdamage unscheduled DNA synthesis as a measure of total DNA repair activity in OVCA 432 cells. Cells were exposed for 3 h to 1 μM CDDP, 2 μM thiopeta, and 0.4 μg/ml 317G5-rRTA. The difference of ^{3}H cpm per micrograms of DNA between treated and control was taken as the measurement of repair activity.

such is the case, modulation of resistant cells should markedly improve the efficacy of chemotherapy in ovarian cancer.

In several preclinical studies, resistance to cisplatin in ovarian cancer has been associated with increased cellular levels of GSH, increased activity of GST, and increased DNA repair of drug induced damage (48). Several compounds can partially restore the sensitivity of resistant ovarian cancer cells to alkylating agents: buthionine sulfoximine (BSO), a specific inhibitor of gamma-glutamyl cysteine synthetase, is capable of lowering cellular GSH; aphidicolin, a specific inhibitor of DNA polymerase α and γ, can reduce DNA repair activity; and ethacrynic acid is capable of inhibiting GST. While, in theory, chemical modulators of drug resistance do not discriminate between normal and malignant cells, in practice, their action appears to be more pronounced in malignant tissues. For example, BSO reduced GSH concentration in most normal tissues studied, but the degree of reduction was frequently not as great as in tumor tissues, and toxicity to normal tissues was generally not augmented at BSO doses that enhanced chemotherapy against human xenograft tumors (49). Consequently, both BSO and aphidicolin are currently undergoing early clinical trials (48, 50).

Effects on normal cells produced by most potential chemical modulators of anticancer drug resistance may limit the utility of those compounds. Extended exposure to BSO, sometimes critical for therapeutic success (51), may potentiate toxicity of alkylators to marrow progenitor cells (52). GSH depletion may also inhibit normal and activated lymphocytic proliferative response (53, 54). Aphidicolin may induce breaks at fragile sites on human chromosomes and telomere association in normal cells (55, 56).

In the present study, we have demonstrated that immunotoxins modulate sensitivity of ovarian cancer cells to alkylators. In contrast to chemosensitizers such as BSO and aphidicolin, immunotoxins have strong cytotoxic activity and this activity is produced by mechanisms distinct from alkylating agents. Thus, these agents may produce efficacy in two ways. First, by direct action, and second, like BSO and aphidicolin, immunotoxins may inhibit enzymes required for the prevention and repair of alkylator damage. In a previous study, nontoxic doses of BSO decreased GSH levels in ovarian cancer cells to 20% of control, potentiating cytotoxicity of alkylators in OVCA cells, and reversing completely resistance to melphalan in the 1847^{ME} OVCA cell line (57). In the present study, GSH depletion of up to 16.4% of controls was obtained with combinations of 317G5-rRTA and cisplatin, and synergistic modulation of cisplatin resistance in the OVCAR III cell line was observed.

Several enzymes take part in drug detoxification and in the removal of drug-induced free radicals by GSH (including gamma-glutamyl cysteine synthetase, glutathione reductase, and glutathione peroxidase). The effect of immunotoxins on enzymes other than GST remain to be explored, but based on the mechanism of action of ricin, indirect effects might be anticipated. Ricin toxin inactivates protein synthesis at the translational stage, by depurinatation of a single adenine base at the 28S rRNA of the 60S ribosomal subunit, near the elongation factor 2 binding site (58, 59). Unlike BSO or aphidicolin, specific inhibition of enzymes by ricin toxin has not been previously demonstrated. Thus, reduction of level or activity of enzymes participating in intracellular thiol and DNA repair pathways is most likely secondary to the general arrest of protein synthesis induced by the immunotoxin. Moreover, depletion of intracellular glutathione could account, at least in part, for the modulation of DNA repair observed in the present study (25).

Selective sensitization of tumor cells to alkylator damage could provide an additional therapeutic advantage, particularly in the setting of high dose therapy with autologous bone marrow support, where appropriate concentration of alkylating agents can be achieved in vivo. In this setting, immunotoxins might be given on a single occasion obviating the effects of human antiovarian antibodies that arise after repeated injections of murine immunoglobulins. The specificity of the immunotoxin will also be important. The 317G5 antibody binds to a 42–43-kD protein that can be found in a majority of ovarian and breast cancers, as well as the normal epithelium of the intestine and renal collecting tubules. Whether this specificity will permit effective use of 317G5 in clinical trials remains to be determined, but the principle of synergistic interactions may apply to reagents with many different specificities.

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