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In vitro interactions between platinum analogues in human ovarian-carcinoma cell lines

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Summary. In vitro and clinical data suggest that cisplatin and carboplatin resistance may be overcome in some cases by dose escalation, although clinical toxicities limit this approach. Administration of platinum analogues in combination is an alternative dose-intensification strategy that has been little studied. The cytotoxicities of cisplatin (CDDP), carboplatin (CBDCA), and tetraplatin (TP, ormaplatin) alone and in combination were assayed by inhibition of the clonogenic survival of human ovarian-carcinoma cell lines (a) from an untreated patient (A2780), (b) selected for CDDP resistance in vitro (2780-CP70), and (c) from patients presenting with clinically refractory disease (OVCAR3, OVCAR10). The sensitivity patterns of these cell lines to platinum analogues were consistent with the existence of at least two platinum-resistance phenotypes – one being moderately resistant to CDDP and CBDCA but highly resistant to TP and the other being highly resistant to CDDP and CBDCA but only partially cross-resistant with TP. Effects of drug combinations were determined by median-effect analysis. Interactions between platinum analogues were variable in different cell lines. Synergistic cytotoxicity was apparent for the CDDP-CBDCA combination in the A2780 and OVCAR-3 cell lines and for the CDDP-TP combination in 2780-CP70 and OVCAR-3. Strong antagonistic effects were seen for CBDCA-TP in 2780-CP70. Platinum analogues showed additive effects in the remaining cell lines. These data suggest that there may be distinct sensitivity phenotypes for platinum-analogue combinations. The demonstration of in vitro synergy between platinum analogues supports their combined clinical use.

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Introduction

Chemotherapy for ovarian cancer remains suboptimal. Although cisplatin-based chemotherapy has produced improved objective response rates, a minority of patients presenting with advanced ovarian cancer are cured. Despite the high percentage of patients who achieve a complete clinical response (60%–80%), virtually all subjects eventually succumb to recurrent, refractory disease [17]. Resistance to available chemotherapy is therefore a major contributor to the 10%–20% 5-year survival of these patients.

Accumulating evidence suggests that cisplatin resistance may be overcome by dose escalation in some cases. Relatively low-level (i.e., $\leq$5-fold) resistance is commonly observed in vitro assays of cell lines selected for resistance in the laboratory or derived from clinically refractory patients [2, 12, 21]. Similar levels of resistance have been demonstrated in a human ovarian-carcinoma cell line selected by weekly cisplatin treatments in an athymic mouse xenograft model [1]. Finally, approximately 30% of cisplatin- and carboplatin-refractory patients show objective responses after 2- to 3-fold dose escalation [19, 20, 23], although these individuals are rarely, if ever, cured. Thus, dose escalation remains an intriguing strategy for improving the efficacy of chemotherapy for ovarian cancer.

Unfortunately, renal and neurologic toxicities limit the dose of cisplatin that can be safely given to patients. Alternative dose-escalation strategies include combinations of cisplatin with platinum analogues exhibiting different toxicity and/or activity profiles. Carboplatin appears to display a spectrum of activity very similar to that of cisplatin in vitro [2, 12, 21, 24] and in the clinic [7, 10] but produces relatively little nephro- or neurotoxicity. Preliminary clinical investigations of the cisplatin/carboplatin combination have been initiated [9, 16, 22, 25, 26]. The clinical toxicity profile of tetraplatin (ormaplatin) remains to be defined, but its in vitro activity profile appears to be distinct from that of cisplatin [2, 12, 21]. Although there may be some rationale for the administration of platinum analogues in

Table 1. Cytotoxicity of platinum analogues

Cell line	CDDP	CBDCA	Tetraplatin
A2780:			
IC_{50} (μM)	0.08 ± 0.04	0.55 ± 0.31	0.05 ± 0.03
Rel. resist.	1	1	1
2780-CP70:			
IC_{50} (μM)	2.91 ± 1.40	7.24 ± 4.67	0.25 ± 0.25
Rel. resist.	36	13	5
OVCAR3:			
IC_{50} (μM)	0.35 ± 0.25	1.06 ± 0.36	0.51 ± 0.32
Rel. resist.	4	2	10
OVCAR10:			
IC_{50} (μM)	7.37 ± 3.80	42.74 ± 30.08	0.52 ± 0.34
Rel. resist.	70	62	10

IC_{50} data represent mean values ± SD (n = 7–12 separate experiments). Within experiments, triplicate platings were done at each concentration. Rel. resist., resistance relative to A2780; CDDP, cisplatin; CBDCA, carboplatin

combination, in vitro interactions between these drugs have not previously been studied in human ovarian-carcinoma cell lines. We therefore investigated cytotoxic interactions of cisplatin, carboplatin, and tetraplatin combinations in an assay system that required maintenance of the capacity for substrate-independent growth.

Materials and methods

Ovarian-carcinoma cell lines. A2780, an ovarian-cancer cell line from an untreated patient, was originally provided by Dr. S. Aaronson (NCI, Bethesda, Md.) [8]. The 2780-CP70 cell line was produced by intermittent, incremental exposure of the sensitive parental A2780 cell line to cisplatin. The OVCAR3 cell line was isolated from a patient who was clinically refractory to cisplatin, cyclophosphamide, and doxorubicin combination chemotherapy. The OVCAR10 cell line was derived from an unrelated individual who was clinically refractory to high-dose cisplatin and high-dose carboplatin. The characteristics of these cell lines have been reviewed in detail elsewhere [11]. All cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Grand Island, N. Y.), 0.28 IU/ml insulin (Squibb-Novo, Inc., Princeton, N. J.), 100 μg streptomycin/ml, 100 IU penicillin/ml, and 0.3 mg glutamine/ml. Cells were grown at 37° C in a humidified atmosphere comprising 5% CO_2 in air.

Drugs. Cisplatin and carboplatin were furnished by Bristol-Myers Oncology Division, Bristol Laboratories (Evansville, Ind.). Tetraplatin (ormaplatin) was obtained from the Investigational Drug Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). The clinical formulations of all drugs were used. Drugs were reconstituted in sterile water according to the manufacturers' directions and were diluted to their final concentrations immediately before use.

Cytotoxicity assay. Cytotoxicity was assayed in a soft agarose bilayer system as previously described [18, 21]. Top layers consisting of single-cell suspensions in media, in the presence or absence of drugs, plus agarose to 0.3% (w/v) were plated over chilled 0.6% agarose (w/v) feeder layers. Cells were plated at 10,000–30,000 cells/ml. Depending on the cloning efficiency of the individual cell lines, approximately 2,000 colonies/10 cm^2 were obtained in untreated controls. Colonies were counted with an Artek Omnicon FAS IV image-analysis system following incubation of the plated cells under routine culture conditions for 7–21 days. Within experiments, the percentage of clonogenic survival was determined as the mean number of colonies obtained from triplicate platings at each drug concentration relative to untreated control values.

Experimental design and data analysis. Drug concentrations required to inhibit colony formation by 50% (IC_{50} values) were determined from median-effect plots generated for each experiment. Data were expressed as the mean value ± SD for 7–12 individual experiments unless otherwise indicated. Resistance was arbitrarily expressed relative to A2780. In drug-combination experiments, between three and five concentrations of individual drugs and from three to five combinations of drugs at a constant molar ratio were tested.

Data were analyzed on a microcomputer using commercially available software [4, 5]. Correlation coefficients for cytotoxicity curves used to generate median-effect plots were ≥0.9, with few exceptions. Cytotoxicity data were fitted to regression lines, and the concentration of each drug producing a given level of cytotoxicity (fractional effect, Fa) alone or in combination was determined. These values were used to calculate the "combination index" (CI) for a given fractional effect:

$$CI = \frac{d_1}{D_1} + \frac{d_2}{D_2},$$

where D_1 and D_2 represent the doses of drugs 1 and 2 that by themselves produce a given fractional effect (i.e., IC_{50}) and d_1 and d_2 indicate the doses that produce the same fractional effect in combination. Ideally, CI = 1 indicates zero interaction (additive cytotoxicity), CI = <1 indicates synergy, and CI >1 indicates antagonism. Sham treatments (i.e., combinations of a platinum analogue with itself) were included to determine the experimental variation in CI values for combinations that by definition exhibit additive cytotoxicity.

The standardized difference between control and experimental means (Cohen's d) [6], an estimate of effect size, was calculated:

$$d = \frac{\text{Mean CI (controls)} - \text{mean CI (experimental)}}{\text{Pooled standard deviation}}.$$

This test quantitatively describes the difference between the means of two groups and is generally interpreted as follows: d between 0 and 0.2, no effect; d between 0.2 and 0.5, small effect; d between 0.5 and 0.8, moderate effect; and d greater than 0.8, large effect.

Results

Cytotoxicity data for individual platinum analogues are summarized in Table 1. Resistance was arbitrarily expressed relative to A2780 to facilitate comparisons between cell lines. On a molar basis, carboplatin was consistently the least potent analogue, whereas tetraplatin was the most potent in all cell lines except OVCAR3. High levels of resistance to cisplatin and carboplatin were evident in the 2780-CP70 and OVCAR10 cell lines, whereas more modest resistance was seen in OVCAR3. Conversely, the 2780-CP70 and OVCAR10 cell lines were incompletely cross-resistant with tetraplatin (5- and 10-fold resistance, respectively), and the OVCAR3 cell line was more highly resistant to tetraplatin than to the other analogues.

The effects of platinum-analogue combinations were variable in individual cell lines. Combinations were tested at constant molar ratios based on the cytotoxicity of individual analogues. The results are summarized in Table 2. The cisplatin-carboplatin combination apparently produced moderate synergistic cytotoxicity in the A2780 and OVCAR3 cell lines, whereas additive effects were seen in the 2780-CP70 and OVCAR10 cell lines. The cisplatin-tetraplatin combination exhibited synergistic cytotoxicity in the 2780-CP70 and OVCAR3 cell lines. This combination

Table 2. CI values for platinum-analogue pairs

Cell line	Combination	Ratio	CI
A2780	CDDP + CBDCA	1:1	0.791 ± 0.176
	CDDP + tetraplatin	1:1	0.911 ± 0.091
	Tetraplatin + CBDCA	1:10	1.166 ± 0.230
2780-CP70	CDDP + CBDCA	1:1	0.932 ± 0.311
	CDDP + tetraplatin	10:1	0.694 ± 0.184
	Tetraplatin + CBDCA	1:10	1.898 ± 1.09
OVCAR3	CDDP + CBDCA	1:1	0.733 ± 0.195
	CDDP + tetraplatin	1:1	0.535 ± 0.103
	Tetraplatin + CBDCA	1:1	1.013 ± 0.233
OVCAR10	CDDP + CBDCA	1:1	1.206 ± 0.620
	CDDP + tetraplatin	10:1	0.968 ± 0.315
	Tetraplatin + CBDCA	1:100	0.877 ± 0.322

CI data represent mean values ± SD. CDDP, cisplatin; CBDCA, carboplatin

Table 3. CI values for sham combinations

Drug combination	Ratio	*n*	CI at Fa = 0.5
CDDP + CDDP	1:1	12	0.953 ± 0.289
CBDCA + CBDCA	1:1	12	1.126 ± 0.708[a]
Tetraplatin + tetraplatin	1:1	7	1.008 ± 0.309
Overall mean ± SD			1.032 ± 0.490[b]

[a] 0.935 ± 0.271 excluding outlier
[b] 0.959 ± 0.279 excluding outlier
Fa, Fractional effect; CDDP, cisplatin; CBDCA, carboplatin

Table 4. Magnitude of effect for platinum-analogue pairs

Cell line	Combination	Cohen's *d*	Magnitude of effect
A2780	CDDP + CBDCA	0.55	Moderate
	CDDP + tetraplatin	0.28	Small
	Tetraplatin + CBDCA	–0.31	Small
2780-CP70	CDDP + CBDCA	0.23	Small
	CDDP + tetraplatin	0.77	Moderate
	Tetraplatin + CBDCA	–1.97	Large
OVCAR3	CDDP + CBDCA	0.68	Moderate
	CDDP + tetraplatin	1.13	Large
	Tetraplatin + CBDCA	0.35	Small
OVCAR10	CDDP + CBDCA	–0.40	Small
	CDDP + tetraplatin	0.15	None
	Tetraplatin + CBDCA	0.35	Small

CDDP, cisplatin; CBDCA, carboplatin

may have been synergistic in the A2780 cell line as well, and it exhibited additive effects in the OVCAR10 cell line. Effects of the tetraplatin-carboplatin combination were more difficult to interpret due to the greater experimental variability. However, this combination appeared to produce additive cytotoxicity in all cell lines except 2780-CP70, in which strong antagonism was apparent.

Interpretation of these data is complicated due to the lack of formal significance tests. Application of standard significance tests is difficult because the distribution of CI data is not known. As an initial approach to this problem, we determined the CI values for sham combinations of platinum analogues, which produce additive cytotoxicity by definition (Table 3). The pooled mean CI value for sham combinations was 1.032. However, there was substantial variability in the data as indicated by the relatively large standard deviation (0.490). On close inspection, there was much greater variability for the carboplatin sham combination than for the other two sham combinations. Moreover, the higher variability found for this combination resulted from a single aberrant sham CI value (3.220); exclusion of this aberrant finding yielded a CI value of 0.935 ± 0.259 for the carboplatin sham combination and a mean overall value of 0.961 ± 0.270. The cause of the extreme variation observed for the single carboplatin sham-combination experiment was not apparent.

The standardized difference (*d*) between sham and experimental CI values was calculated to provide an estimate of effect size. It is apparent from the effect-size equation than mean experimental CI values lower than the mean control (sham) value will be positive. The results are shown in Table 4. Based on this criterion, moderate to strong synergy was apparent for the carboplatin-cisplatin combination in the A2780 and OVCAR3 cell lines and for the cisplatin-tetraplatin combination in the 2780-CP70 and OVCAR3 cell lines. In addition, the tetraplatin-carboplatin combination was strongly antagonistic in the 2780-CP70 cell line. Small effects produced by platinum-analogue combinations in these cell lines were more difficult to interpret due to the variability in the data.

The magnitude of effect was recalculated following exclusion of the aberrant carboplatin sham CI value (data not shown). Moderate antagonism was found for cisplatin-carboplatin in OVCAR10 (revised $d = -0.78$) and for tetraplatin-carboplatin in A2780 (revised $d = -0.65$). The remaining results were not substantially affected by recalculation.

Discussion

The cellular mechanisms responsible for resistance to platinum analogues are incompletely characterized. However, comparative cytotoxicity studies may identify characteristics of resistant cells that have potential laboratory and/or clinical significance. Specifically, resistance phenotypes in different cell lines may be described in terms of the patterns and magnitudes of cross-resistance between platinum analogues. The present data confirm previous observations in this laboratory [21] and others [12], which have demonstrated nearly complete cross-resistance between cisplatin and carboplatin. These findings are not surprising, given that the same active species is formed by the aquation of either platinum analogue. In addition, at least two platinum-resistance phenotypes were apparent – one in which

cells were highly resistant to cisplatin/carboplatin but only moderately resistant to tetraplatin (OVCAR10, 2780-CP70) and a second in which cells were somewhat more resistant to tetraplatin than to either cisplatin or carboplatin (OVCAR3). The recognition of distinct resistance phenotypes suggests that the determinants of platinum-analogue sensitivity/resistance may vary in different cell lines [21].

These observations are consistent with clinical data demonstrating significant cross-resistance between cisplatin and carboplatin [7, 10]. However, clinical trials have also shown that 2- to 3-fold dose escalations may produce objective responses in approximately one-third of patients who have been refractory to conventional-dose cisplatin or carboplatin treatment [19, 20, 23]. These individuals must have shown relatively low-level resistance to have achieved a response following dose escalations of this magnitude. These observations are consistent with in vitro data demonstrating that modest levels of resistance to cisplatin and carboplatin are not infrequently seen [2, 12, 21, 24]. Moreover, the failure of many patients to respond to dose escalation suggests that more highly cisplatin-resistant phenotypes, such as that seen in the OVCAR10 cell line, are also clinically relevant. Whether platinum analogues exhibiting distinct activity profiles, such as tetraplatin, will benefit individuals who are refractory to high-dose cisplatin/carboplatin remains to be determined.

Overall, these observations provided a rational basis for the present investigations. Classically, chemotherapy combinations have included agents that produce different clinical toxicities, enabling drugs to be used at or near their maximal tolerated single-agent doses. Thus, the administration of cisplatin and carboplatin in combination may result in increased "dose intensity" along with tolerable toxicity. Combinations of cisplatin with tetraplatin are potentially more interesting because the activity profiles of these analogues are distinct and because tetraplatin is frequently more potent than either cisplatin or carboplatin in vitro [2, 12, 21].

In the present experiments, interactions between platinum analogues were variable in different cell lines. The tetraplatin-cisplatin combination was synergistic in the 2780-CP70 and OVCAR3 cell lines but showed additive effects in the remaining two cell lines. The tetraplatin-carboplatin combination was antagonistic in the 2780-CP70 cell line but produced additive effects in the other cell lines. These results are particularly interesting because the effects of the latter combination appear to be distinct from those of the former, although cisplatin and carboplatin are known to form identical active species and to exhibit quite similar activity profiles. Equally interesting results were obtained for the cisplatin-carboplatin combination, which produced additive cytotoxicity in the 2780-CP70 cell line, additive or antagonistic cytotoxicity in OVCAR10, and synergistic cytotoxicity in the A2780 and OVCAR3 cell lines. These results were unexpected, given that the active component of both of these compounds is the same. Few data have been published on platinum-analogue combinations. In the preliminary report of one study [13], the cisplatin-carboplatin combination produced additive cytotoxicity in vitro in a human squamous lung-carcinoma cell line; however, the information provided in that report was insufficient to enable a comparison of these data with the present observations.

The present investigations were not designed to determine the mechanisms of interactions between platinum analogues. However, it is known that platinum analogues may interact with cellular macromolecules at different rates [14, 15]. It is therefore possible that platinum compounds might interact with specific molecular determinants of cytotoxicity (such as DNA) in ways that enhance or oppose the actions of other platinum analogues. Detailed mechanistic investigations of these interactions should prove to be interesting. It is not surprising that platinum-analogue combinations produced different effects in individual cell lines. Prior findings in our laboratory [21] and by Hills et al. [12] have clearly demonstrated the existence of distinct platinum-analogue-resistance phenotypes in different cell lines. Our data suggest that there may be distinct sensitivity/resistance phenotypes for platinum-analogue combinations as well.

Analysis of the interactions between drugs used in combination is difficult. Terminology and methodologies are not standardized, results are often reported in ambiguous terms, and criteria for statistical validity are lacking [3]. We approached these problems in several ways. First, straightforward definitions of interactions were used, with additivity being defined as cytotoxicity that was no greater than the effects of individual drugs and synergy and antagonism describing effects that were greater and smaller, respectively, than those of additive combinations. Sham combinations necessarily produce additive cytotoxicity according to this definition. Second, the combination-index equation used is equivalent to the general isobologram equation and is broadly applicable [3]. Third, experimental variability for additive cytotoxicity as based on sham combinations was described. Finally, a descriptive statistic was used to estimate the magnitude of effects with respect to experimental control values. The latter two approaches offer a conceptually appealing alternative to traditional analysis (i.e., comparison of experimental results with an "ideal" combination index of 1 for additive cytotoxicity). The observed variability in sham combination indices provides direct experimental support for this contention. However, it should be emphasized that our approach does not obviate the need for the development of valid, formal significance tests for these data.

Three important caveats should be considered in the interpretation of these data. First, our observations were descriptive rather than mechanistic. Information on specific mechanisms of cytotoxicity was not considered in the experimental design and cannot be inferred from the results. Second, the experimental design did not address the schedule dependence of effects, which is a potentially important consideration whenever one drug modulates the effects of another. Finally, the clinical significance of in vitro synergy has yet to be established. The clinical utility and toxicities of platinum-analogue combinations remain to be defined in clinical trials.

Resistance to platinum analogues used alone or in combination appears to be a complex problem. Our investigations were conducted in a limited number of cell lines that were chosen as representatives of particular resistance

phenotypes [21]. Clearly, more cell lines need to be screened to establish whether the effects of platinum-analogue combinations are consistent and predictable and to identify cellular mechanisms responsible for these phenomena. However, the demonstration of in vitro synergy and antagonism between platinum analogues suggests that combinations of these agents may potentially benefit some but not all ovarian cancer patients. This contention awaits clinical validation.

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