Cancer Chemother Pharmacol (1989) 25: 89–94

Cancer Chemotherapy and Pharmacology

In vitro evaluation of cisplatin interaction with doxorubicin or 4-hydroperoxycyclophosphamide against human gynecologic cancer cell lines*

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Summary. Doxorubicin, cisplatin, and cyclophosphamide are the three drugs most commonly used in the treatment of ovarian cancer, but no effect greater than additivity was observed for any combination of these drugs in the present study. Only a few studies have been reported concerning the degree of their additivity or their best order of sequencing. In our in vitro studies, cisplatin in combination with doxorubicin or 4-hydroperoxycyclophosphamide (4HC) was tested against seven human gynecologic tumor-cell lines in different sequences, using a double-agar layer tissue-culture system. Drug interactions with respect to inhibition of tumor clonogenicity were evaluated by isobologram and fractional survival methods. Doxorubicin and 4HC were sequenced simultaneously and at 1, 6 and 24 h after cisplatin, and cisplatin was sequenced at 1, 6 and 24 h after 4HC. The isobolograms constructed for doxorubicin or 4HC plus cisplatin revealed strict additivity between these agents against ovarian cancer clonogenicity. Both doxorubicin and 4HC showed the greatest additivity when used simultaneously and at 1 h vs 6 or 24 h after cisplatin. Although the mechanisms by which these sequencing effects occur are unknown, these studies provide new leads for the design of clinical trials with combinations of these three agents.

Introduction

Doxorubicin, cyclophosphamide, and cisplatin are the three drugs most commonly used in the treatment of advanced ovarian cancer [5–7, 12, 39, 40]. Virtually all patients receive two or three of these agents in combination as initial therapy after exploratory laparotomy. Decker et al. [13] proved extreme additivity between cisplatin and cyclophosphamide in a randomized trial comparing these two agents with cyclophosphamide alone in patients with stage III and IV disease. Both the progression-free interval and overall survival were significantly prolonged by the addition of cisplatin to cyclophosphamide in these patients. Bruckner et al. [8, 9] have also shown additivity between doxorubicin and cisplatin in this patient population. More recent results [10] suggest that doxorubicin plus cisplatin at optimal doses may be as active as the three-drug combination including cyclophosphamide. Finally, Omura et al. [26] and Alberts et al. [2] have shown in phase III trials that the addition of cisplatin to regimens containing doxorubicin and cyclophosphamide results in significantly higher objective response rates and longer response and survival durations.

In most clinical trials using these three agents the drugs are given simultaneously. Few data exist concerning the effect of their sequencing on the optimization of their additive anticancer activity. Thus, we used in vitro human tumor-cloning assays to quantitate the additive effects of these drug combinations and then determine the optimal interval for drug administration with respect to their additive antitumor effects.

Materials and methods

Human tumor-cell lines. Seven human gynecologic tumor-cell lines were studied in the logarithmic phase of growth. Table 1 gives the name, passage number, source, tissue type, medium for culture, and method of harvesting used for each of the cell lines. Cell lines were cultured in a room air incubator at 37° C and 95% humidity in an atmosphere containing 5% CO_2. During exponential growth (i.e., 5–7 days after growth initiation in tissue-culture flasks), the cells were harvested using a hypoosmolar medium that had an osmolarity of about 200 mosmol and included bovine serum albumin and methocel as well as PVP-40 to help retain cell viability [20]. Approximately 5 ml hypoosmolar medium was added to cover cells adhering to the bottom of the flask. These cells were then placed in an incubator for 5–10 min and shaken off the bottom of the flask.

Preparation of single-cell suspension. After the harvested cells were washed twice in McCoy's 5A (or RPMI 1640) medium with 10% fetal calf serum, the suspension was aspirated into pipettes of decreasing diameter to break up cell clumps. If clumps still remained, the suspension was passed through a 30-μm nylon mesh (Tekto, Elmsfore, NY) to make a single-cell suspension. To 20 μl cell suspension was added 180 μl of 0.4% trypan blue: phosphate-buffered solution (PBS) (1:5). Cell counts and viability were then determined.

* This work was supported in part by grants CA-17094, CA-21839, and CA-23074 from the National Institutes of Health, Bethesda, Maryland, and the Phi Beta Psi National Sorority

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Table 1. Cell sources and numbers of inoculation

Cell line (Passage number)	Source	Tissue	Medium[a]	Harvesting	Plating efficiency
OVCA-433 (P72)	[3, 23]	Ovarian	RPMI 1640	Hypoosmolar [20]	3.2%
OVCA-420 (P20)	[3, 23]	Ovarian	RPMI 1640	Hypoosmolar	4.0%
UACC-326 (P18–21)	UACC	Ovarian	RPMI 1640	Hypoosmolar	3.8%
UACC-66 (P34–36)	UACC	Ovarian	McCoy's 5A	Hypoosmolar	6.8%
UACC-166 (P15)	UACC	Uterine	McCoy's 5A	Hypoosmolar	5.0%
UACC-38 (P30)	UACC	Fallopian	McCoy's 5A	Hypoosmolar	2.6%
UACC-169 (P23–28)	UACC	Ovarian	McCoy's 5A	Hypoosmolar	8.2%

[a] Add 10% heat-inactivated fetal calf serum (Flow Laboratories. Inglewood, Calif), 1% penicillin (100 IU/ml) – streptomycin (1 mg/ml) (Gibco, Grand Island, NY), and 1% heparin sodium (1,000 USP units/ml) (O'Neal, Jones & Feldman, St. Louis, Mo)
UACC, University of Arizona Cancer Center

Plating of cells. A double-layer soft-agar system (0.3%) on a 0.5% base layer was used as described by Hamburger and Salmon [16, 17, 29]. The plating media consisted of 80 ml enriched McCoy's 5A media, 20 ml tryptic soy broth (3% in DDH_2O), and 1.2 ml asparagine (6.6 mg/ml). The top layer was made of 3% agar diluted to 0.3% with enriched CMRL 1066 media. Single-tumor-cell suspensions were diluted to the desired cell concentrations, mixed quickly with the agar at 50° C, and plated on culture dishes at final concentrations of 10,000–25,000 cells/agar plate, depending on the plating efficiencies of the respective cell lines. All plates were examined by inverted microscopy on the day of plating (to assure that a good single-cell suspension had been obtained) and placed in a 5% CO_2 incubator at 37° C after drug addition.

Preparation and handling of drugs. Doxorubicin was obtained from Adria Laboratories (Columbus, Ohio), cisplatin was obtained from Bristol Laboratories (Syracuse, NY), and 4HC was supplied by Dr. Peter Hilgard (Asta-Werke Degussa Pharma Gruppe, Bielefeld, FRG). Drug stock solutions were prepared at the intermediate concentrations of 1.5 mg/ml and 150 μg/ml in 0.85% NaCl solution and stored in cryotubes as 0.5- to 1.0-ml aliquots in a dark environment at −80° C until used. The time of storage was <10 weeks [1, 14]. Just prior to use, each of the above drug preparations was allowed to warm to room temperature before being diluted with 0.85% NaCl solution and culture media to those concentrations to be used against the human tumor-cell lines.

The desired concentrations for use were generally 22 times the final drug concentrations when two drugs were combined but amounted to 11 times the final concentration if only one drug was tested. Drug exposure was continuous by adding 0.2 ml diluted drug onto the upper agar layer. Thus, there was a 2.2-ml mixture of agar, media, tumor cells, and drug in each dish. All platings were carried out at least in triplicate.

Tumor-colony counting. The tissue-culture plates were scanned every 2–3 days by inverted microscopy to evaluate growth. Viable counting was done as follows: for background subtraction, 1 ml 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution [32] was added to the surface of a control plate on the day of plating. Colony counting was carried out after incubation for 24 h. Also, INT solution was added to each plate on day 13–19 after plating to terminate colony growth. Final colony counting was done 24 h later using a Bausch and Lomb Omnicon FAS II image analysis system [30]. The median number of tumor colonies per control plate was approximately 400.

Results

Dose-response curves

The activity of doxorubicin, cisplatin, and 4HC against seven different human gynecologic tumor-cell lines was evaluated. The final concentrations tested ranged from 0.0001 to 1.0 μg/ml for doxorubicin and from 0.001 to 10.0 μg/ml for both cisplatin and 4HC. Each tumor-cell line was exposed to at least five different concentrations of each drug in an attempt to construct 5-log dose-response curves.

Figures 1 and 2 show the dose-response curves for doxorubicin and cisplatin, respectively, in seven different tumor-cell lines. Figure 3 shows the dose-response curves for 4HC in five of the tumor-cell lines (i.e., excluding

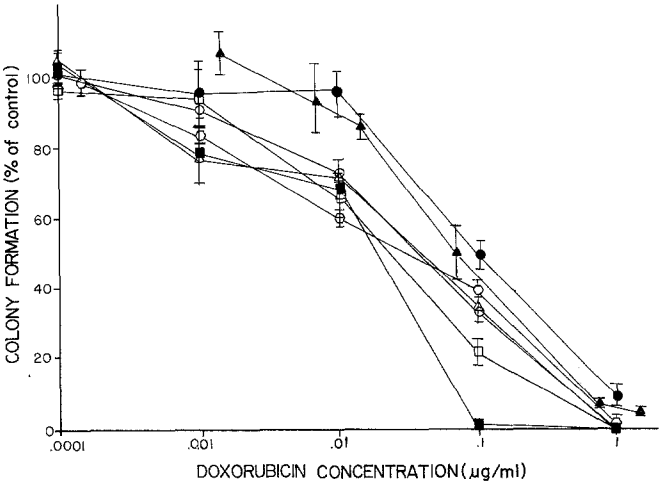

Fig. 1. Doxorubicin dose-response curves during continuous exposure of seven human gynecologic tumor-cell lines. *Open circles* (○) represent data points for the OVCA-433 cell line; *closed circles* (●), OVCA-420; *triangles* (△), UACC-326; *closed triangles* (▲, UACC-66; *squares* (□), UACC-166; *closed squares* (■), UACC-38; *hexagons* (⬡) UACC-169. Each symbol represents the mean percentage of survival for tumor colony-forming units (±SE) obtained from three separate experiments, each of which was carried out in triplicate

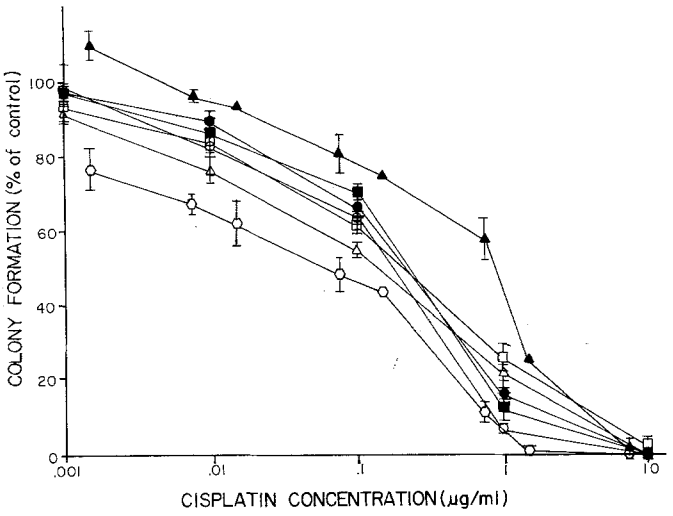

Fig. 2. Cisplatin dose-response curves during continuous exposure of seven human gynecologic tumor-cell lines. Symbols are as detailed in Fig. 1

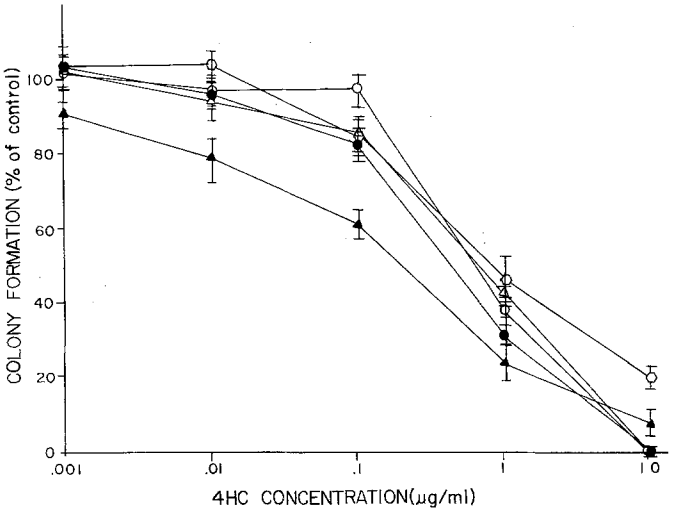

Fig. 3. 4HC dose-response curves during continuous exposure of five human gynecologic tumor-cell lines. Symbols are as detailed in Fig. 1

UACC-38 and UACC-166). To assure reproducibility, dose-response curves were carried out again at the dose range that resulted in between 20% and 80% survival of tumor-colony-forming units (TCFUs) using the same cell passage number. Listed in Table 2 are the ID_{40} values (i.e., drug concentration associated with 60% survival of TCFUs for each of the three anticancer drugs) calculated from the dose-survival curves for each tumor cell line.

Isobolograms

Isobolograms [15, 22, 28, 31] were constructed for each drug combination to determine whether the growth-inhibitory interaction between cisplatin and doxorubicin or cisplatin and 4HC was additive, synergistic, or antagonistic. The ID_{40} concentration of each drug alone was arbitrarily set to equal a fractional inhibitory concentration (FIC) of 1.0 on the vertical and horizontal axes. Each drug was diluted to six different concentrations corresponding with FICs of 0.2, 0.4, 0.6, 0.8, 0.9, and 1.0 of the ID_{40} concentration. Isobolograms were completed by combining the drugs in a variety of proportions as described above. Figures 4 and 5 show the isobolograms for combinations of cisplatin plus doxorubicin and cisplatin plus 4HC, respectively, in three ovarian cancer-cell lines (i.e., OVCA-420, OVCA-433, and UACC-326). Each point on the isobologram curves represents the relative concentration of each drug used in combinations that caused 40% inhibition of tumor-colony growth. Almost all of the concentration points fall to the right of the "line of additivity" but lie mostly within the envelope of additivity [27, 33].

Table 2. ID_{40} Concentrations of doxorubicin, cisplatin, and 4HC against seven human gynecologic tumor-cell lines

Cell line	ID_{40} (μg/ml):		
	Doxorubicin	Cisplatin	4HC
OVCA-433	0.04	0.15	0.50
OVCA-420	0.05	0.15	0.40
UACC-326	0.03	0.13	0.50
UACC-66	0.075	0.75	0.25
UACC-166	0.015	0.15	–
UACC-38	0.015	0.16	–
UACC-169	0.015	0.10	0.75

Drug-sequencing experiments

After the additive effects of cisplatin and doxorubicin or 4HC were verified in three ovarian cancer-cell lines by the isobologram technique, different sequences of administration for cisplatin and doxorubicin or cisplatin and 4HC were explored using fractional survival techniques to evaluate additive effects of the drug combinations [35, 37].

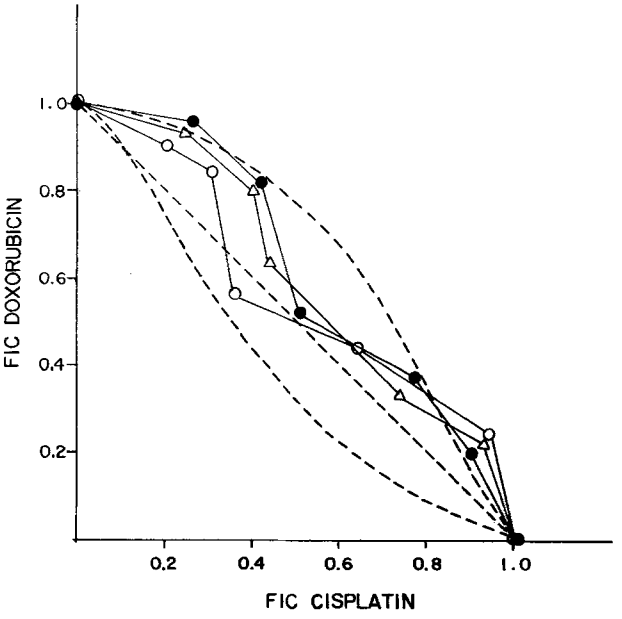

Fig. 4. Isobolograms of cisplatin in combination with doxorubicin against three human ovarian cancer-cell lines in cloning culture. *Open circles* (○) represent data points for the OVCA-433 cell line; *closed circles* (●), OVCA-420; *triangles* (△), UACC-326. Each symbol represents the mean of two or three separate experiments, each of which was carried out in triplicate

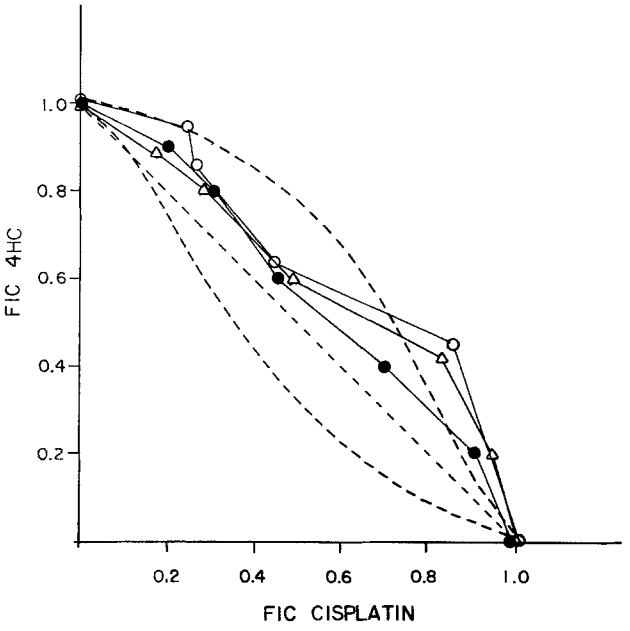

Fig. 5. Isobolograms of cisplatin in combination with 4HC against three human ovarian cancer-cell lines in cloning culture. Symbols are as detailed in Fig. 4. Each symbol represents the mean of two or three separate experiments, each of which was carried out in triplicate

Table 3. Evaluation of the interaction of cisplatin plus doxorubicin with respect to antitumor activity following different sequences of combination against seven human gynecologic tumor-cell lines

Cell line tests (n)	$SF_{DOX} \times SF_{CDDP} - SF_{DOX+CDDP}$:			
	Simultaneous DOX + CDDP	DOX after CDDP		
		1 h	6 h	24 h
OVCA-433 (3)	−0.13	−0.06	−0.19	−0.17
OVCA-420 (3)	−0.09	−0.03	−0.21	−0.23
UACC-326 (3)	−0.07	−0.03	−0.13	−0.18
UACC-66 (7)	−0.15	−0.09	−0.22	−0.21
UACC-166 (3)	−0.05	0	−0.11	−0.13
UACC-38 (3)	−0.01	+0.03	−0.07	−0.10
UACC-169 (7)	−0.14	−0.04	−0.17	−0.16
$\bar{X}$ (29)	−0.09	−0.03	−0.16	−0.17

SF, the surviving fraction of tumor colony formation; DOX, doxorubicin; CDDP, cisplatin; DOX + CDDP, these two drugs used in combination

In all experiments, the ID_{40} concentrations (Table 2) of each of the drugs were used against all seven gynecologic tumor-cell lines. Experiments were repeated three to seven times with each cell line as shown in Tables 3 and 4, using the same cell-line passage number. Tables 3 and 4 and Fig. 6 show the results obtained from these drug-sequencing experiments. Note that in every experiment the surviving fraction (SF) of tumor-colony-forming units (TCFUs) associated with a simultaneous or asynchronous combination of drugs was lower than the SF associated with each of the agents alone. With respect to the effect of the sequence of drug addition on the SF of TCFUs, it is noteworthy that the 1-h interval between drugs was consistently associated with the lowest SFs ($P < 0.01$ in comparison with the simultaneous, 6-h, and 24-h sequences), regardless of whether cisplatin was used before or after 4HC.

To determine the effects of drug sequencing on cytotoxicity, we arbitrarily defined synergism, additivity, and antagonism of the two drug combinations based on differences between calculated and experimental SFs (i.e., $SF_1 \times SF_2 - SF_{1+2}$) as follows: (1) synergism, $SF_1 \times SF_2 - SF_{1+2} \geq 0.15$; (2) additivity, $SF_1 \times SF_2 - SF_{1+2}$, $-0.15 - 0.15$; and (3) antagonism, $SF_1 \times SF_2 - SF_{1+2}$, ≤ -0.15. For the combinations of cisplatin plus 4HC and cisplatin plus doxorubicin, simultaneous and 1-h asynchronous drug administration resulted in additivity for all tumor-cell lines studied. In contrast, for both drug combinations, asynchronous drug administration at 6- and 24-h intervals resulted in antagonism for all tumor-cell lines studied.

Discussion

Although cisplatin has proved to be the most active single agent in the treatment of advanced ovarian cancer, there is ample evidence that cyclophosphamide or doxorubicin can potentiate its cytotoxicity in such patients [4, 8, 9, 13, 18, 25, 34, 36, 38, 40]. Omura et al. [26] have recently shown that the addition of cisplatin to a doxorubicin/cyclophosphamide combination significantly increases the objective response rate and response and survival durations in ovarian cancer patients with clinically measurable disease; however, the combination of cisplatin/doxorubicin/cyclophosphamide may be more effective than cisplatin/cyclophosphamide without doxorubicin in such patients [15a].

We used two different methods of analysis to evaluate the additivity of the cisplatin/doxorubicin and cisplatin/

Table 4. Evaluation of the interaction of cisplatin plus 4HC with respect to antitumor activity following different sequences of combination against five human ovarian tumor-cell lines

Cell line tests (n)	$SF_{CDDP} \times SF_{4HC} - SF_{DDP+4HC}$:						
	Simultaneous CDDP + 4HC	CDDP after 4HC			4HC after CDDP		
		1 h	6 h	24 h	1 h	6 h	24 h
OVCA-433 (3)	−0.09	+0.04	−0.14	−0.18	−0.02	−0.16	−0.19
OVCA-420 (3)	−0.12	−0.06	−0.16	−0.20	−0.06	−0.16	−0.22
UACC-326 (3)	−0.08	−0.02	−0.14	−0.20	−0.12	−0.15	−0.21
UACC-66 (7)	−0.13	−0.11	−0.18	−0.27	−0.07	−0.14	−0.22
UACC-169 (7)	−0.20	−0.15	−0.21	−0.27	−0.12	−0.17	−0.23
$\bar{X}$ (23)	−0.13	−0.06	−0.17	−0.23	−0.08	−0.16	−0.22

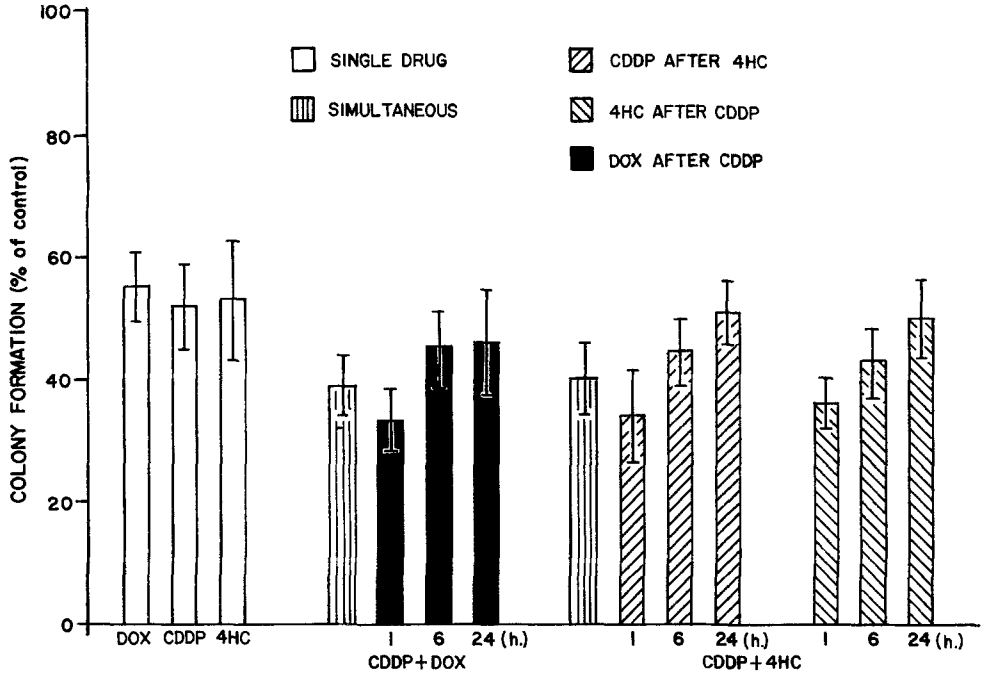

Fig. 6. Comparison of the effects of different drug-combining sequences in the clonogenic assay of seven human gynecologic tumor-cell lines. The ID_{40} concentrations listed in Table 2 were used in all drug-sequencing experiments. *DOX*, doxorubicin; *CDDP*, cisplatin; *CCDP + DOX, CDDP + 4HC*, two drugs used in combination

cyclophosphamide combinations, including Loewe's isobologram technique [22] and the fractional survival method formalized by Webb [37]. Despite the many factors that can affect the in vitro testing of drug combinations using tumor-cloning assays, both isobologram and fractional survival methodologies demonstrated the strict additivity of cisplatin added to either doxorubicin or cyclophosphamide. Of considerable interest is the fact that the greatest degree of cytotoxic additivity was demonstrated in vitro when a 1-h period separated the addition of either cyclophosphamide or doxorubicin to cisplatin. The cytotoxic additivity was lost when the interval increased to 6 or 24 h. Since the distribution half-lives for both cyclophosphamide and doxorubicin are in the range of 1 h, our data provide a good rationale for the present clinical approach of simultaneously giving cisplatin with cyclophosphamide and/or doxorubicin.

The explanation for the cytotoxic additivity of cisplatin with cyclophosphamide or doxorubicin has not been completely explained but may relate to the way in which each of these drugs interacts with tumor-cell DNA. Cyclophosphamide, a bifunctional alkylating agent, is metabolized to compounds that exert their cytotoxicity through interstrand DNA cross-linking and subsequent inactivation of the DNA template with the cessation of DNA synthesis. In contrast, the formation of intrastrand DNA cross-links appears to play a major role in the cytotoxicity of cisplatin against human tumors. Doxorubicin appears to have an entirely different mechanism of DNA interaction, with intercalation between DNA base pairs and inhibition of DNA-dependent DNA and DNA-dependent RNA syntheses; however, doxorubicin's biologic effects are complex. Besides DNA binding, free radical formation, membrane binding, and metal ion chelation are all likely to occur in vitro [24]. It is also known that doxorubicin can decrease the ability of tumor cells to repair damaged DNA by interfering with the activity of a DNA gyrase, thus increasing the killing effect of various agents that exert their cytotoxicity by causing DNA degradation [11, 19, 21]. Of course, it is possible that either doxorubicin or cyclophosphamide might interact with the tumor-cell DNA repair system to increase the cytotoxicity of cisplatin, and cisplatin or doxorubicin could interact with tumor-cell membrane proteins to increase the cellular accumulation of each of these compounds. Obviously, the exact mechanism by which doxorubicin or cyclophosphamide potentiates cisplatin cytotoxicity is unknown.

Our in vitro data clearly indicate the in vitro additivity of both cyclophosphamide and doxorubicin with cisplatin against gynecologic cancer cells and provide a pharmacologic rationale for the efficacy of cisplatin/doxorubicin and cisplatin/cyclophosphamide combination therapies against ovarian cancer.

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Received 26 June 1988/Accepted 12 May 1989