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Schedule-dependent synergism of edatrexate and cisplatin in combination in the A549 lung-cancer cell line as assessed by median-effect analysis

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Abstract. The methotrexate analog edatrexate has been shown to have greater antitumor activity and an improved therapeutic index as compared with its parent compound in preclinical systems. These studies suggest that edatrexate may have a broad role in the treatment of solid tumors. Information regarding edatrexate in combination with other chemotherapeutic agents is limited. We evaluated the interaction of edatrexate with cisplatin in vitro as assessed by median-effect analysis in the A549 human lung-cancer cell line. The effects of dose, exposure time, and schedule dependence were assessed. Cytotoxicity was evaluated using the tetrazolium-based colorimetric (MTT) assay. The inhibitory concentration producing 50% absorbance (IC_{50} for edatrexate with 1 h exposure was 1.4 μM. For all combination experiments, the edatrexate dose was fixed at 0.2 μM (IC_{10}) whereas cisplatin (CDDP) concentrations were varied for 1-, 3-, and 24-h exposures either before or after edatrexate treatment. Drug interactions were assessed using the combination-index method as defined by median-effect analysis. A synergistic interaction was documented in experiments when edatrexate was applied prior to CDDP (combination index, <1). The combination studies in which edatrexate was used prior to CDDP resulted in significant reduction of all three CDDP IC_{50} values: 1-h IC_{50}, from 30.0 to 3.9 μM; 3-h IC_{50}, from 21.3 to 1.4 μM; and 24-h IC_{50}, from 1.7 to 0.03 μM. In contrast, synergism was not observed in experiments in which edatrexate treatment occurred after cisplatin exposure. Median-effect analysis is a useful method of determining drug interactions. In the present study, the combination of edatrexate and CDDP demonstrated schedule-dependent synergism, with the synergism being observed only in the setting of edatrexate treatment before CDDP exposure. Due to the potential broad spectrum of activity of edatrexate plus CDDP, further studies are warranted to determine the mechanism responsible for the synergism and to investigate this combination in a variety of tumor models.

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Introduction

An established principle of chemotherapy as treatment for cancer is that multidrug regimens are frequently superior to single-agent treatment. The rationale for combination chemotherapy is based in part on diverse mechanisms of action that may enhance the anticancer efficacy. Optimally, combination therapy results in greater than additive cytotoxicity, defined as synergism [3].

Edatrexate (10-ethyl-10-deaza-aminopterin) is a methotrexate analog specifically designed to take advantage of the active transport and polyglutamation properties associated with the N^{10} position of folate [22]. Edatrexate has several potential advantages over methotrexate, including increased specific transport and enhanced polyglutamation within tumor cells. In transplanted murine tumor systems it is therapeutically superior [11, 15, 23]. Although in a variety of tumor types the results of preclinical [15] and initial clinical studies of edatrexate have been encouraging [11, 12], little data are available regarding the drug's interaction with other chemotherapeutic agents.

Studies by Schmid et al. [16] and Sirotnak et al. [23a, 24] have evaluated the activity of edatrexate, methotrexate, and cisplatin in an established mouse ovarian teratoma tumor model and against E0771 mammary adenocarcinoma, T241 fibrosarcoma, and L2310 leukemia. They demonstrated a substantial improvement in the antitumor efficacy of edatrexate in comparison with methotrexate. In addition, the therapeutic activity and duration of survival were significantly greater when the chemotherapeutic drugs were given in combination than when each agent was given alone. Furthermore, tumor-free long-term survivors were seen only following treatment with the edatrexate plus cisplatin combination. These authors concluded that

the observed improved therapeutic index of edatrexate appeared to be related to increased cell entry and polyglutamation and to its relative exclusion and rapid elimination from host tissues as compared with methotrexate [11].

Non small-cell lung cancer is characterized by a poor response to standard chemotherapeutic agents. Cisplatin has been identified as one of the most active drugs for the treatment of this malignancy. In view of the activity of cisplatin in human non-small-cell cancer and the encouraging preliminary activity of edatrexate in this tumor type [10, 12, 19], we evaluated the in vitro activity of cisplatin and edatrexate applied alone and in combination in an established human non-small-cell lung cancer line previously characterized with regard to cisplatin cytotoxicity [14]. We present observations on the schedule-dependent synergism of edatrexate in combination with cisplatin in the A549 cell line.

Materials and methods

Cell lines. The human adenocarcinoma cell line A549 was obtained from ATCC (Rockville, Md.). The cells were maintained in RPMI medium supplemented with 10% fetal calf serum and were grown as monolayers in a humidified atmosphere containing 5% CO_2 at 37° C. Cell lines were used within a defined range of 20 passages.

Drugs. Cisplatin was obtained from Bristol Laboratories (Evansville, Ind.). Edatrexate was provided by Ciba-Geigy Laboratories (Summit, N. J.). Both agents were prepared following the manufacturers' instructions and were stored frozen at –20° C for up to 30 days before their use.

Cytotoxicity assays. Cytotoxicity was assessed with the in vitro semi-automated colorimetric tetrazolium-based (MTT) assay [1]. In brief, cells were harvested from exponential-phase maintenance cultures (T-75-cm^2 flasks) and counted by trypan blue exclusion, and 125-µl volumes were dispensed in replicate 96-well culture plates. Following a 24-h incubation as attachment period (37° C, 5% CO_2, 100% relative humidity), 125 µl of culture medium, which was used alone or with drug (vehicle control group, $n = 6$; each drug-treatment group, $n = 3$), was dispensed into each well. Peripheral wells of each plate that lacked cells were utilized as controls for drug blank ($n = 2$) and medium/tetrazolium-reagent blank ($n = 6$) determinations. After appropriate exposure periods, the drug was removed, the wells were washed twice with phosphate-buffered saline (PBS), and 175 µl of fresh media was added. Each drug treatment was sequential, not concurrent, for the combination experiments. Culture plates were then incubated for 4 days (with the control wells being >80% confluent and the viability being 90% as determined by trypan blue dye exclusion) prior to the addition of tetrazolium reagent. MTT stock (5 mg MTT/ml PBS) was sterilized by filtration and stored at 4° C for a maximum of 1 month.

MTT working solution was prepared just prior to culture application by diluting MTT stock solution to 2.0 mg/ml (v/v) in prewarmed culture medium containing 10% fetal bovine serum (FBS). Then, 25 µl of MTT working solution was added to each culture well (resulting in 100 µg MTT/200 µl total medium volume) and cultures were incubated at 37° C for 4 h. All of the culture medium supernatant was removed from wells by slow aspiration and replaced with 175 µl of dimethylsulfoxide (DMSO) using a multichannel pipet. Within 30 min of DMSO application and following thorough formazan solubilization, the absorbance of each well was measured at 540 nm using a Titertek scanning microculture plate reader (single wavelength; calibration factor, 1.00). Cell line growth and growth inhibition were expressed in terms of mean ±SD absorbance units and/or the percentage of control absorbance ±SD following the subtraction of mean "background" absorbance.

Seven different drug concentrations were used for each experiment. The results were displayed by computer-generated fits using these variables. IC_{10}, IC_{50}, and IC_{90} were defined as the inhibitory concentrations of drug that produced 10%, 50%, and 90% of absorbance, respectively, and were determined using computer software [4, 5] on the basis of the dose-effect relationships. IC_{10}, IC_{50}, and IC_{90} values were determined for variable periods of exposure to cisplatin (1, 3, and 24 h) and for a 1-h exposure to edatrexate. We then evaluated the effect of exposure to edatrexate at its IC_{10} level (0.2 µ*M*) on cisplatin cytotoxicity. Two treatment schedules were studied, involving either edatrexate pretreatment or edatrexate treatment following cisplatin exposure. Experiments were performed with quadruplicate determinations and each experiment was repeated three times.

Analysis of drug interaction. The combined effects of the two drugs in terms of synergism, additivity, or antagonism were quantitatively analyzed by the median-effect plot and multiple-drug equation derived by Chou and Talalay [5, 7] for calculating the combination index (CI), whereby CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism, respectively. This method involves the plotting of dose-effect curves by using the median-effect equation:

$$fa/fu = (D/Dm)^m,$$

where D is the dose (concentration), Dm is the dose required for a 50% effect (e.g., 50% inhibition of cell growth), fa is the fraction affected by dose D (e.g., 0.9 if cell growth is inhibited by 90%), fu is the unaffected fraction (1–fa), and m is a coefficient of the sigmoidicity of the dose-effect curve. A plot of $x = \log (D)$ vs $y = \log [fa/(1–fa)]$ determines m (slope) and Dm (log Dm as x-intercept) values, respectively. The dose for any effect level (Dx) can be determined by $Dx = Dm [fa/(1–fa)]^{1/m}$.

The CI for mutually exclusive drugs (similar modes of actions) that follow the classic isobologram equation can be calculated by:

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2},$$

where $(Dx)_1$ is the dose of agent 1 required to produce x percentage of effect alone; $(D)_2$ is the dose of agent 2 required to produce the same x percentage of effect in combination with agent 1, $(D)_1$, and $(Dx)_2$ is the dose of agent 2 required to produce x percentage of effect alone. For mutually nonexclusive drugs (totally independent actions), it has been proposed that a third term be added to the above CI equation that is the product of the first two terms [5, 7]. In most cases, exclusivity is unknown; therefore, the computer software [4, 5] automatically calculates the CI values for both mutually exclusive and nonexclusive assumptions.

The median-effect curves are generated from dose-effect data by using the median-effect plot to obtain the values of D_m (IC_{50}), m (sigmoidicity), and r (linear correlation coefficient), which indicates conformity. D_m is obtained from the x-intercept and m is obtained from the slope of the median-effect plot, respectively, by using the computer software. The computer software automatically simulates the dose-effect curves and fa-CI plots on the basis of the available data.

Statistical analysis. A one-factor analysis of variance (ANOVA) was used to compare the differences between the means for each set of experiments using STATVIEW 512+ on a Macintosh computer. A P value of <0.05 was used as a criteria of statistical significance. The concentration of edatrexate or cisplatin needed to achieve the respective IC of the cell line was expressed as the mean value ±SE.

Results

The results displayed in figure form represent computer-generated fits obtained using the variable data points described in Materials and methods. The error bars used in these figures are a reflection of this statistical analysis, as the surviving fraction is the dependent variable. Figure 1 demonstrates the dose-response curve for edatrexate in the A549 human lung-cancer cell line. From this dose-effect relationship, the IC_{10} was determined to be 0.2 µ*M*. This

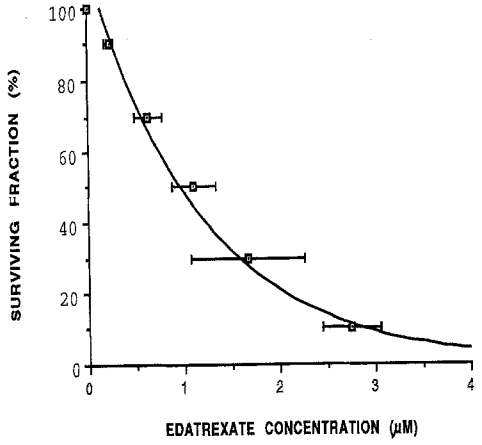

Fig. 1. Dose-effect curve generated for edatrexate with 1 h exposure as described in Materials and methods

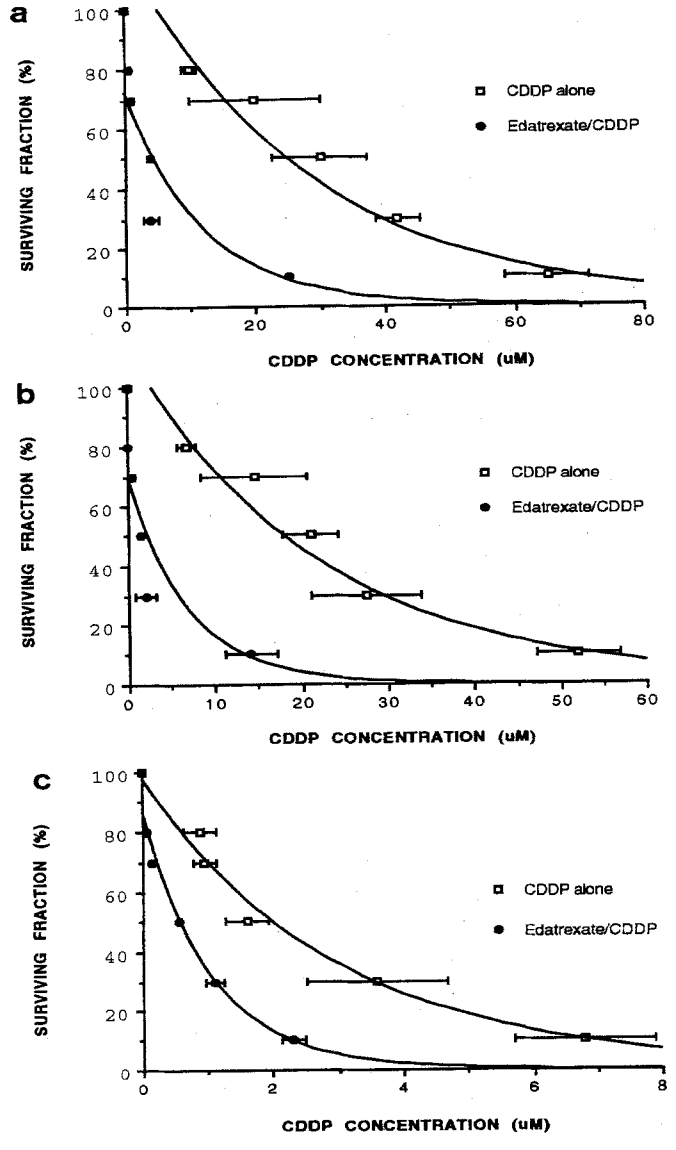

Fig. 2 a–c. Cytotoxicity of cisplatin (*CDDP*) with or without edatrexate pretreatment. CDDP alone (○) and CDDP and edatrexate in combination (■) were applied on the following schedules. **a** Edatrexate followed by CDDP, 1 h. **b** Edatrexate followed by CDDP, 3 h. **c** Edatrexate followed by CDDP, 24 h

Table 1. Dose-modification factor of cisplatin with edatrexate pretreatment or posttreatment

	IC (CDDP)	DMF	*P* value
CDDP + edatrexate pretreatment:			
1 h exposure	IC_{50}	7.7	0.01
3 h exposure	IC_{50}	14.6	0.0002
24 h exposure	IC_{50}	3.0	0.01
CDDP + edatrexate posttreatment:			
3 h exposure	IC_{50}	1.0	0.91
24 h exposure	IC_{50}	1.2	0.24

CDDP, Cisplatin; DMF, dose-modification factor

Table 2. Dose-effect relationship parameters for 1-h edatrexate pre-exposure and cisplatin exposure for 1, 3, and 24 h

Compound	Exposure period	Parameters[a]		
		Dm (μ*M*)	m	*r*
Edatrexate	1 h	1.47	1.171	0.994
Cisplatin	1 h	23.3	1.871	0.974
	3 h	16.7	1.740	0.982
	24 h	1.81	1.709	0.994

[a] The median-effect concentration (Dm) signifies the potency and the m value signifies the shape of the dose-effect curve, whereby m values of 1, >1, and <1 indicate a hyperbolic, sigmoidal, and negative sigmoidal shape, respectively The Dm and m values are obtained from the slope and the x-intercept of the median-effect plot, respectively. The linear correlation coefficient (*r*) of the median-effect plot represents the conformity of the data to the method employed for analysis

1-h IC_{10} of edatrexate was used for combination experiments with cisplatin.

Figure 2 demonstrates the effect of single-agent cisplatin versus the combination experiments in which edatrexate was applied prior to cisplatin (1-, 3-, and 24-h) exposure. These combination studies resulted in significant modification of all three cisplatin IC_{50} values (1-hr IC_{50}, from 30.0 ± 7.4 to 3.9 ± 0.6 μ*M*, $P < 0.02$; 3-h IC_{50}, from 21.3 ± 3.2 to 1.4 ± 0.2 μ*M*, $P < 0.0003$; and 24-h IC_{50}, from 1.7 ± 0.3 to $0.6 \pm .03$ μ*M*, $P < 0.02$).

Table 1 shows drug interaction analysis by dose-modifying factor (DMF), defined as the ratio of the cell inhibitory concentration (IC) of cisplatin alone divided by the cell IC of the cisplatin after the combination experiments. For the experiments in which there was edatrexate pretreatment, 3.0–14.6 times less cisplatin was required to achieve the IC_{50} as compared with experiments in which the cells were exposed to cisplatin alone. With 0.2-μ*M* edatrexate pretreatment (a clinically achievable dose), the cisplatin IC_{50} for all the exposure periods evaluated diminished to levels of cisplatin that are also clinically achievable (≤ 4 μ*M*). In contrast, a favorable DMF was not observed when CDDP was applied prior to edatrexate. When CDDP (3 and 24 h) was applied prior to edatrexate (1 h), there was no significant modification of the cisplatin IC_{50} values (3-h

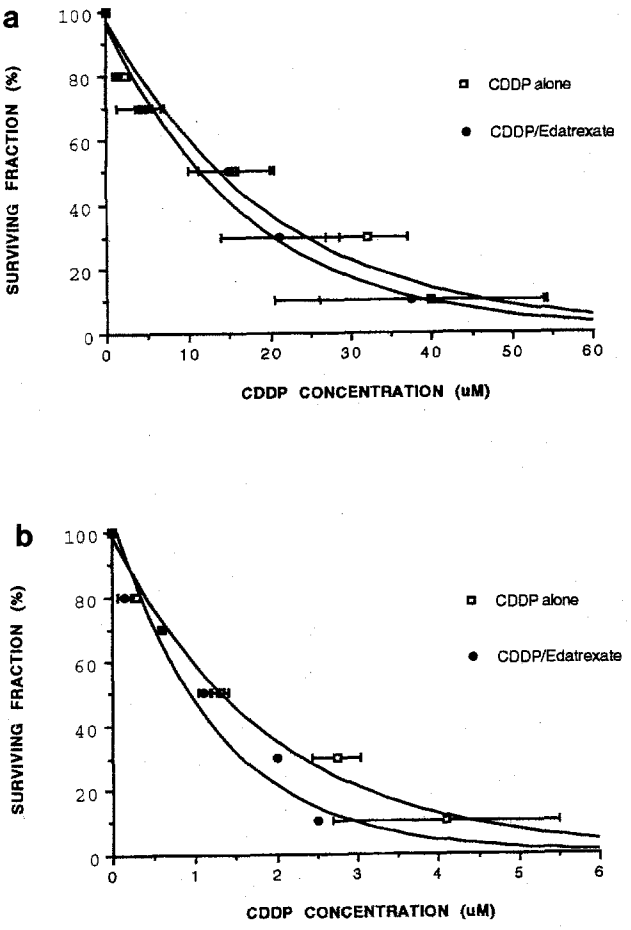

Fig. 3 a, b. Cytotoxicity of cisplatin (*CDDP*) with or without edatrexate posttreatment. CDDP alone (○) and CDDP and edatrexate (■) in combination were applied on the following schedules. **a** CDDP, 3 h, followed by edatrexate. **b** CDDP, 24 h, followed by edatrexate

IC_{50}, from 15.8 ± 4.6 to 15.0 ± 5.0 μM, $P > 0.9$; 24-h IC_{50}, from 1.3 ± 0.1 to 1.1 ± 0.07 μM, $P > 0.2$; Fig. 3).

The dose-effect relationship parameters for cisplatin and edatrexate pretreatment are described in Table 2. The combination-index determinations for both cisplatin and edatrexate pretreatment (Table 3) or posttreatment (Table 4) demonstrate the schedule-dependent synergism between these two compounds. Analysis of the combination index for the cisplatin and edatrexate pretreatment experiments disclosed synergistic activity, whereas this synergism was not observed for the edatrexate posttreatment experiments (whereby the combination indices were close to or more than 1). Figure 4 is a computer-generated plot demonstrating these drug interactions.

Discussion

Although methotrexate is well established as an active agent in the chemotherapeutic armamentarium, its usefulness is limited. Newly developed antifolates with enhanced intracellular uptake and polyglutamation demonstrate improved cytotoxicity and an increased spectrum of anti-

Table 3. The combined concentrations of edatrexate and cisplatin required for the specified levels of effect and their interaction at different schedules of exposure: pretreatment with edatrexate followed by exposure to cisplatin

Effect level (fa)	Exposure schedule and required concentrations (μM)		Combination (CI)[a]	Dose-reduction index[b] index x (DRI) for	
	Edatrexate	CDDP		Edatrexate	CDDP
	1 h:	1 h:			
0.2	0.2	+ 0.55	0.493 (0.515)	2.25	20.18
0.5	0.2	+ 3.9	0.303 (0.326)	7.37[b]	5.97[b]
0.9	0.2	+55	0.352 (0.359)	48.13	3.02
	1 h:	3 h:			
0.2	0.2	+ 0.05	0.450 (0.453)	2.25	150.6
0.5	0.2	+ 1.4	0.220 (0.231)	7.37	11.93
0.9	0.2	+14.7	0.261 (0.266)	48.13	4.17
	1 h:	24 h:			
0.2	0.2	+ 0.05	0.506 (0.533)	2.25	16.11
0.5	0.2	+ 0.55	0.439 (0.480)	7.37	3.30
0.9	0.2	+ 2.3	0.372	48.13	2.85

CDDP, Cisplatin

[a] Combination indices (CI) were calculated on the basis of the multiple-drug-effect equations based on the classic mutually exclusive drugs (no parenthesis) and based on the mutually nonexclusive assumption (in parentheses) as described in Materials and methods (also see [12, 13]). Computer software [9] was used for automated analysis. CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism, respectively

[b] The dose-reduction index denotes how many orders of magnitude of dose reduction are allowed for a given effect level as a result of synergism. Computer software [11] was used for automated analysis. For example, given edatrexate (EDX, 1 h) + CDDP (1 h) for 50% inhibition, the EDX dose can be reduced 7.37-fold and the CDDP dose can be reduced 5.97-fold as compared with the respective IC_{50} values for single-agent EDX and CDDP

tumor activity as compared with the parent compound [20, 21, 23]. One of the most promising of the new analogs is edatrexate.

Initial clinical studies with edatrexate have been encouraging, suggesting a much broader role for this antifolate in the treatment of solid tumors. However, information regarding edatrexate in combination with other antineoplastic agents is limited [8, 11], and observations on combinations have generally been empiric. In this report we present data demonstrating that edatrexate enhances the in vitro cytotoxicity of cisplatin in a synergistic and schedule-dependent manner in a human lung-cancer cell line. In view of the wide use of cisplatin in a variety of tumor types, including lung cancer, these data may have significant clinical implications worthy of further study.

The methodology used in the present studies quantitates the assessment of these types of drug interaction. The

Table 4. The combined concentrations of edatrexate and cisplatin required for the specified levels of effect and their interaction at different schedules of exposure: pretreatment with cisplatin followed by exposure to edatrexate

Effect level (fa)	Exposure schedule and required concentrations (μM)		Combination index x (CI)[a]	Dose-reduction index[b] (DRI) for	
	CDDP	Edatrexate		Edatrexate	CDDP
0.5	3 h: 15	1 h: +0.2	1.095 (1.225)	1.25	7.37
	24 h: 1.13	1 h: +0.2	0.796	1.53 (0.886)	7.37

CDDP, Cisplatin

[a] Combination indices (CI) were calculated on the basis of the multiple-drug-effect equations based on the classic mutually exclusive drugs (no parenthesis) and based on the mutually nonexclusive assumption (in parentheses) as described in Materials and methods (also see [12, 13]). Computer software [9] was used for automated analysis. CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism, respectively

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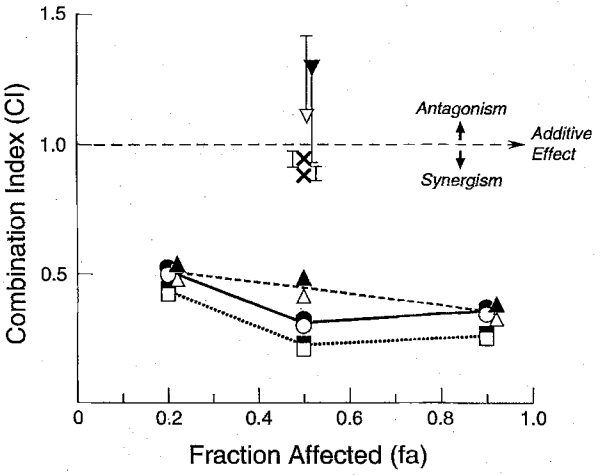

Fig. 4. Combination index (*CI*) as a function of effect levels (*fa*) in various edatrexate and cisplatin (*CDDP*) combination schedules. *Open and filled symbols* represent values calculated on the basis of mutually exclusive and mutually nonexclusive CI equations, respectively. The exposure schedules were: edatrexate (1 h) → CDDP (1 h), (○, ●); edatrexate (1 h) → CDDP (3 h), (□, ■); edatrexate (1 h) → CDDP (24 h), (△, ▲); CDDP (3 h) → edatrexate (1 h), (▽, ▼), mean ± SE; and CDDP (24 h) → edatrexate (1 h), (x, x), mean ± SE. CI values of <1, 1, and >1 indicate synergism, additivity, and antagonism, respectively

combination-index method offers a quantitative assessment of the synergism or antagonism between edatrexate and cisplatin at different doses and different effect levels. Using the median-effect principle and a computer software package [4, 5], data can be efficiently analyzed. The combination index has not only been used in enzyme and receptor systems but also widely used in cellular or clinical systems as shown in recent reviews [6, 7]. Distinct from other methods for dose-effect analysis, the present method takes into account not only the potency but also the shapes of dose-effect curves generated for each drug alone and their combinations.

Recent studies by Chou and co-workers [9] also demonstrate synergistic effects for edatrexate plus cisplatin. In the HL-60 promyelocytic leukemia cell line, a 48-h continuous exposure to both agents at constant combination ratios showed synergistic effects. Delivering one drug for 4 h preceding application of the other during the 48-h incubation period did not enhance the degree of synergism as compared with simultaneous exposure to both agents. These investigators also reported that edatrexate plus cisplatin produced more synergism than methotrexate plus cisplatin [9]. However, the mechanism of synergism between cisplatin and antifolates has remained obscure.

Folate antagonists such as methotrexate and edatrexate act during the S phase of the cell cycle, competitively inhibiting the enzyme dihydrofolate reductase and thus indirectly blocking the synthesis of nucleotides required for DNA synthesis. In view of this mechanism of action, the complimentary effects of antifolates with cisplatin may be explained by the interaction between DNA damage and repair. Li and Kaminskas [13] found a progressive formation of strand breaks in mature DNA of Ehrlich ascites tumor cells that were treated with methotrexate. These findings suggested that cell death was due to a lethal accumulation of DNA strand breaks, probably due to inefficient DNA repair, resulting from inhibition of the synthesis of thymidylate as well as purine nucleotides. It is possible that both the misincorporation of deoxyuridine triphosphate (dUTP) into DNA, due to the blockage of thymidylate synthase by antifolates such as edatrexate, and a general depletion of deoxynucleotides necessary for ongoing repair may contribute to the accumulation of DNA damage [2, 17, 18]. Thus, the synergism between edatrexate and cisplatin may result from an edatrexate-mediated reduction in the deoxynucleotide pool, inhibiting the repair of cisplatin-induced DNA damage.

Our observations suggest that pretreatment with edatrexate is important in enhancing the cytotoxicity of cisplatin. Specifically, we demonstrate schedule-dependent synergism, observed only in the setting of edatrexate treatment before cisplatin exposure. These findings demonstrate methodology in assessing these types of drug interaction. This strategy may have clinical relevance and lead to improved antitumor effects. Even though the mechanism of this synergistic interaction remains unclear, further studies are warranted to optimize synergistic clinical strategies.

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