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Relationships between various uses of antineoplastic drug-interaction terms

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Summary. In in vitro testing, no pharmacologic synergism has been found for the combination of cisplatin and etoposide in P388 leukemia in contrast to the demonstration of therapeutic synergism in the same model. No pharmacologic synergism has been found for the same combination in the treatment of four small-cell lung-cancer cell lines, although clinical results obtained using this combination in small-cell lung cancer and other cancers suggest a therapeutic advantage. The popular concept of synergy, implying a therapeutic advantage, is different from the pharmacologic meaning, which generally implies that less drug is required in a combination for an equal effect. Therapeutic advantage may be obtained regardless of whether drugs are synergistic in the pharmacologic sense in the treatment of a tumor. To gain a more comprehensive insight into concepts of drug interaction, it is important to recognize that the type of drug interaction seen is dependent on the drug doses used and may vary with the treatment of different cell lines. All of these factors complicate the use of the word synergism, or any associated term, in a categorical manner to describe the effects of combinations of antineoplastic drugs.

Introduction

The terms synergism, additivity, and antagonism are best applied to linear dose-response relationships or to those that can be transformed to linearity by changing the axis by probit, logit, or other scaling. In this case, a change to a steeper slope of the dose-response relationship can be used as a definition of synergy. Changes in slope in the opposite direction have been shown to occur progressively as cells become more resistant to drug treatment [14]. Thus, antagonism corresponds to resistance, whereas synergy implies sensitization of the cells to the treatment. The isobologram method [18] was developed to compensate for the problem of nonlinear response and considers drug effects at a fixed level of response. Nevertheless problems arise; different types of interactions may be seen at different dose levels, and no dose of any one of the drugs will achieve the desired level of response. The latter situation commonly exists in the case of cancer chemotherapy. For this reason, other definitions of synergy have been developed.

The concept of therapeutic synergism was generated during a period of animal testing of cancer chemotherapeutic agents and was considered to be operative when any combination dose was shown to give a greater effect than that achieved by any dose of an individual drug [34]. Originally there was no statistical quantitation of this difference, but the use of response surface methodology enables the determination of statistically significant therapeutic synergism [9]. Clinical synergism has also been loosely defined as the situation in which the response rate of a combination is greater than that obtained by adding the individual response rates of the single drugs in the combination. It may also imply that the survival rate produced by the combination is greater than anticipated [17]. Another term, cellular synergism, has been developed [32] to describe the results obtained in tissue-culture experiments using the isobologram approach together with statistical analysis. A number of definitions of synergism from in vitro studies have slightly different meanings [13, 19, 23, 31], but these tend to follow the pharmacologic principle that synergism requires the use of less drug in combination for an equal effect.

This paper considers the relationship between these definitions of synergy. The combination of cisplatin and etoposide in vitro, given as an example, is therapeutically synergistic in P388 leukemia [27], is clinically synergistic in the treatment of small-cell lung cancer and testicular tumors [17], and is synergistic in the treatment of Chinese hamster V79-171B lung cells [16]. However, in in vitro testing of a number of lung cancer lines, no evidence of cellular synergism could be documented [32]. This was disappointing in view of the hope that in vitro methods

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could be developed that would predict favorable clinical drug interactions. Unless otherwise qualified, further use of the terms synergism and antagonism in this paper refer to the pharmacologic meaning.

Materials and methods

Tumor lines. Small-cell lung cancer (SCLC) lines were obtained through the courtesy of Dr. A. F. Gazdar at the NCI (Navy) and included (1) NIH H209, a classic cell line developed from an untreated patient, exhibits tightly packed floating cellular aggregates, a long doubling time, a low colony-forming efficiency, and a biochemical profile indicating active neurosecretory function; (2) NIH H345, a classic line derived from a patient who had received treatment for SCLC; (3) NIH N417, an untreated variant type that displays discordant expression of biochemical markers, often together with altered (large-cell) morphology and altered (loosely attached floating aggregates and a short doubling time) growth characteristics and cloning efficiencies; and (4) NIH H82, a variant cell line developed from a treated patient. The P388 leukemia line used was obtained from Dr. H. Bear at the Massey Cancer Center, Medical College of Virginia (Richmond, VA.).

Culture methods. Cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). SCLC cell lines growing in the logarithmic phase were dispersed after centrifugation by suspension in a mixture of 0.1% trypsin (10 ml), 0.1% collagenase (10 ml), and 2% bovine serum albumen (10 ml) followed by incubation for 30 min. The cells were washed, exposed to 1% trypsin inhibitor in 5 ml phosphate-buffered saline (PBS), and resuspended in medium at a concentration of 10^5 cells/ml. Cells (1.5×10^4) were then placed in each well in 150–180 μl RPMI 1640 medium, drug or drugs in PBS solution, or PBS alone (20–50 μl) for a total of 0.2 ml/well. Final concentrations of each drug ranged from 0 to 14.3 μg/ml for the SCLC cell lines. Each drug was used at concentrations of 0–5 μg/ml for P388 leukemia cells in replication 1; in the other P388 studies, cisplatin concentrations of 0–12.5 μg/ml and etoposide concentrations of 0–100 μg/ml were applied. Drug concentrations were increased at logarithmic intervals.

After 1 h drug treatment, the cells were washed and resuspended in fresh medium. For sequential drug treatments, extra washing and feeding of the cells was required following the second 1-h drug exposure, after which incubation was continued for a total of 4 days (96 h). At this time, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT dye) was added, which is metabolized by the mitochondria of living cells to a blue formazan product. After 3 h exposure of the SCLC cell lines or 6–24 h exposure of the P388 lines, the formazan product was solubilized in dimethylsulfoxide (DMSO) and read at 540 nm using a scanning multiwell spectrophotometer. In two of the P388 runs using a 6-h period of exposure to the MTT dye, a shaker was used to aid in solubilization of the dye.

Experimental design. Factorial designs were employed. Simple 6×6 designs were used in testing of the four SCLC lines, to which drugs were applied at five logarithmically spaced doses plus a zero dose. The interval between the two drug administrations was varied in additional experiments using the NIH H209 and P388 cell lines. The drugs were tested in both sequences, cisplatin first and etoposide first, using five intervals (0, 2, 4, 24, and 48 h) between drug treatments for each sequence. The 6×6 experiments consisted of 144 wells, 4 at each dose level, and the $6 \times 6 \times 5$ experiment, 720 wells. The term replication refers to the repetition of an entire experiment.

Response surface modeling. The following model was used to describe the dose-response relationship for these experiments:

$$E(Y) = B \frac{1}{[1 + e^{-(\beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3)}]},$$

where $E(Y)$ is the expected absorbance at 540 nm, B is the parameter associated with control absorbance, β_1 is an unknown constant associated with the effect of the first drug treatment, β_2 is an unknown constant associated with the effect of the second drug treatment, β_3 (when used) is an unknown constant associated with the interval between the two injections of the drug (the scheduling parameter), β_{12} is an unknown constant associated with the interaction between the two drugs, β_{13} (when used) is an unknown constant associated with the interaction between the first drug and the scheduling effect, β_{23} (when used) is an unknown constant associated with the interaction between the second drug and the scheduling effect, and β_{123} (when used) is an unknown constant associated with any triple variable interaction (drug 1, drug 2, and the scheduling effect).

Model parameters were estimated from the experimental data using the SAS procedure NLIN [26]. The model used in this project is empirical. Consequently, it is useful to consider the differences between observed and predicted results. A substantive deviation would suggest inadequacy of the model. In the absence of model inadequacy, useful information can be obtained from the results.

The importance of each of the variables can be gauged by examining the test of significance of the appropriate regression coefficient. The relative contributions of each drug can be assessed from the value of the associated parameter estimates when the dose units are scaled to be equally effective. When a parameter estimate representing a drug, the treatment interval, or an interaction is significantly different from zero, this information may be useful in the formulation of hypotheses for the construction of improved treatment regimens or schedules in vivo.

Negative coefficients are favorable in terms of treatment effectiveness, since they would indicate a reduction in tumor cell survival. For example, if the coefficient associated with the drug dose or the treatment interval is negative, the use of higher doses or longer intervals would be expected to lead to lower absorbance (increased cell kill).

Results

In the initial experiments (Table 1) in which the drugs were given together, statistically significant effects ($P < 0.05$) were seen for both drugs when the classic SCLC lines were tested. However, only cisplatin was active in the variant

Table 1. Parameter estimates for the cisplatin-etoposide combination in human SCLC lines

Parameter	Classic lines		Variant lines	
	NIH H209	NIH H345	NIH N417	NIH H82
B	5.23×10^{-1}*	7.97×10^{-1}*	6.53×10^{-1}*	7.12×10^{-1}*
β_1	-9.63×10^{-3}*	-1.05×10^{-2}*	-4.92×10^{-3}*	-3.84×10^{-3}*
β_2	-5.82×10^{-3}*	-5.17×10^{-3}*	4.83×10^{-4}	-2.48×10^{-3}
β_{12}	4.71×10^{-5}	4.80×10^{-5}	-8.88×10^{-5}	-5.51×10^{-5}

Cells were exposed for 1 h to each drug at concentrations of 0–14.3 μg/ml. B, Parameter associated with control absorbance; β_1, parameter associated with the cisplatin dose; β_2, parameter associated with the etoposide dose; β_{12}, parameter associated with the interaction effect

* $P \leq 0.05$

Table 2. Parameter estimates for sequential use of cisplatin and etoposide in human SCLC line NIH H209

Drug sequence	Parameter	Replication 1	Replication 2
Cisplatin→ etoposide	B	4.84×10^{-1}*	1.54×10^{0}*
	β_1 (cisplatin)	-1.29×10^{-2}*	-1.86×10^{-1}*
	β_2 (etoposide)	-7.94×10^{-3}*	-1.21×10^{-2}*
	β_3 (interval)	-5.14×10^{-3}*	-2.19×10^{-2}*
	β_{12} (drug interaction)	1.21×10^{-4}*	1.51×10^{-3}*
	β_{13}	2.18×10^{-5}	1.87×10^{-3}*
	β_{23}	7.91×10^{-5}*	1.38×10^{-4}*
	β_{123}	-7.56×10^{-7}	1.64×10^{-5}*
Etoposide→ cisplatin	B	4.26×10^{-1}*	3.45×10^{-1}*
	β_1 (etoposide)	-2.57×10^{-3}*	-4.58×10^{-3}*
	β_2 (cisplatin)	-7.01×10^{-3}*	-6.98×10^{-3}*
	β_3 (interval)	1.48×10^{-3}*	1.25×10^{-4}
	β_{12} (drug interaction	2.70×10^{-6}	5.48×10^{-5}*
	β_{13}	-7.87×10^{-6}	5.11×10^{-6}
	β_{23}	6.05×10^{-5}*	5.89×10^{-5}*
	β_{123}	3.27×10^{-7}	5.36×10^{-7}

Cells were exposed for 1 h to each drug at concentrations of 0–14.3 μg/ml. β_1, Parameter associated with the first drug used; β_2, parameter associated with the second drug used; β_3, parameter associated with the interval between drug treatments (time); β_{12}, parameter associated with the drug-interaction effect; β_{13}, parameter associated with the interaction of the first drug and time; β_{23}, parameter associated with the interaction of the second drug and time; β_{123}, parameter associated with the interaction of the drugs and with time (triple interaction effect)
* $P \leq 0.05$

Table 3. Parameter estimates for sequential use of cisplatin and etoposide in P388 leukemia

Drug sequence	Parameter	Replication 1	Replication 2
Cisplatin→ etoposide	B	1.01×10^{0}*	3.31×10^{-1}*
	β_1 (cisplatin)	-4.42×10^{-3}*	-2.34×10^{-3}*
	β_2 (etoposide)	-1.17×10^{-4}	-3.74×10^{-5}
	β_3 (interval)	1.41×10^{-2}*	3.72×10^{-3}*
	β_{12} (drug interaction)	2.14×10^{-5}	2.14×10^{-6}
	β_{13}	-4.60×10^{-5}	1.42×10^{-6}
	β_{23}	-9.43×10^{-7}	-4.81×10^{-6}
	β_{123}	-4.68×10^{-7}	-3.27×10^{-8}
Etoposide→ cisplatin	B	3.47×10^{-1}*	
	β_1 (etoposide)	-3.07×10^{-4}	
	β_2 (cisplatin)	-1.58×10^{-3}*	
	β_3 (interval)	6.62×10^{-3}*	
	β_{12} (drug interaction	3.54×10^{-5}*	
	β_{13}	3.40×10^{-5}	
	β_{23}	-3.04×10^{-6}	
	β_{123}	-7.46×10^{-7}	

For replication 1, cells were exposed for 1 h to each drug at concentrations of 0–5 μg/ml. For replication 2, cells were exposed for the same period to drug concentrations of 0–12.5 μg/ml (cisplatin) and 0–100 μg/ml (etoposide). β_1, Parameter associated with the first drug used; β_2, parameter associated with the second drug used; β_3, parameter associated with the interval between drug treatments (time); β_{12}, parameter associated with the drug-interaction effect; β_{13}, parameter associated with the interaction of the first drug and time; β_{23}, parameter associated with the interaction of the second drug and time; β_{123}, parameter associated with the interaction of the drugs and with time (triple interaction effect)
* $P \leq 0.05$

lines. The interaction effects were not statistically significant. In the sequential experiments, cisplatin was used first in two experiments and etoposide was applied first in two additional experiments.

The results obtained in the NIH H209 cell line are shown in Table 2 and Fig. 1. The effect of both drugs against this cell line was confirmed in all experiments as indicated by the statistical significance of parameters β_1 and β_2. In addition, the two parameter estimates associated with the time between treatments when cisplatin was used first were statistically significant and negative, indicating that longer intervals result in greater cell kill. When etoposide was applied first, the interval estimates were positive and one was statistically significant, which suggests that the use of cisplatin immediately after etoposide results in greater cell kill. Three of the four estimates of the drug-interaction parameter β_{12} were statistically significant, but all were positive and, hence, of the antagonistic type.

In P388 leukemia, cisplatin was shown to be active in all experiments as shown in Table 3 by negative parameter estimates that were statistically significant (β_1 values were associated with the effect of the first drug and β_2 values were associated with that of the second drug). No activity was documented for etoposide, although it has been shown to be active in P388-bearing animals [27]. The interaction terms were positive but statistically significant only in the experiment in which etoposide was used before cisplatin.

Discussion

It is important to recognize that a statistically significant drug effect, such as that demonstrated in the present study for cisplatin against all of the cell lines tested and for etoposide against the classic SCLC lines, is not synonymous with drug sensitivity as reported from in vitro studies in which sensitivity has been set at a given level of cell kill to give as great a correlation as possible with clinical activity [24]. It simply indicates a statistically significant cell-killing effect. In each of the variant human SCLC cell lines, we could not show significant activity for etoposide. Variant cell lines are known to be more resistant to treatment than are classic lines [6].

Ruckdeshel et al. [25] have reported activity for etoposide in NIH N417 cells, but these authors used a higher dose of 125 μg/ml [9]. Etoposide is known to be more effective both in animal systems [15] and in man [28] when given in multiple doses. Since we could not show the expected activity of single doses of etoposide in P388 leukemia, we performed additional experiments in which two doses of etoposide were separated by varying intervals. In only one experiment using the highest range of etoposide concentrations, 0–800 μg/ml, was statistically significant activity shown in P388 leukemia. The variant SCLC cell lines were not tested in this manner. Thus, the perception of inactivity may be misleading when doses or schedules of treatment are not optimal. It would seem

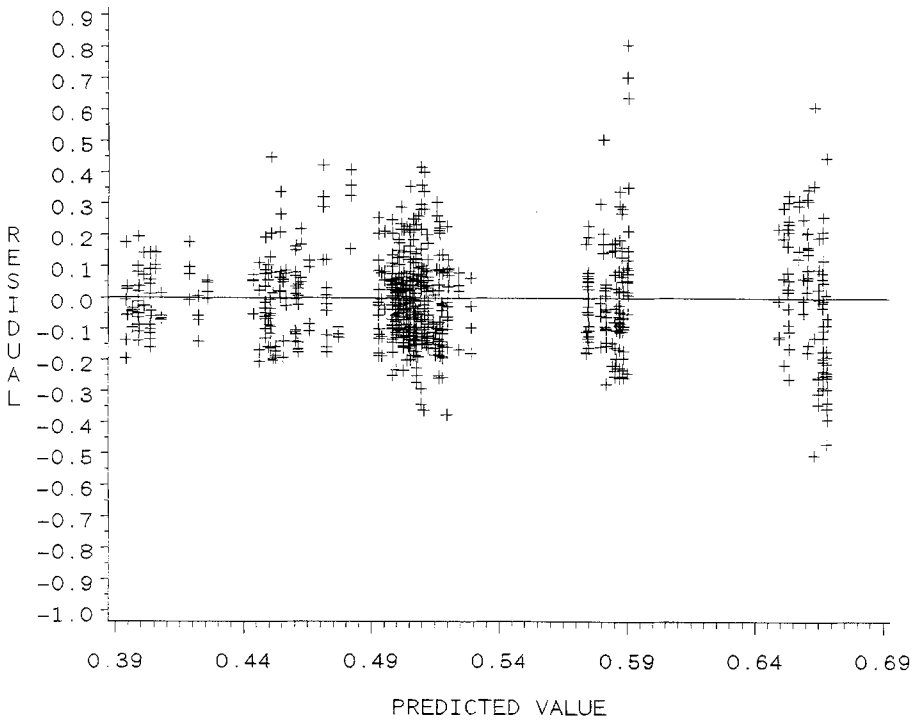

Fig. 1. Plot of the values predicted by the model versus the residual for the experimental results summarized in Table 2 (replication 1). This plot represents what is considered to be an adequate fit of data to the model. For all experiments, similar plots were scrutinized before the results were accepted as valid

reasonable to conclude that classic cell lines are more sensitive to etoposide treatment than are variant lines.

In the experiments summarized in Tables 1–3, no significant interaction occurred that was associated with negative parameter estimates as defined for cellular synergism. These estimates were positive except in the variant SCLC lines, in which the negative parameter estimates were not significant. However, some of the positive estimates of the drug-interaction parameter were statistically significant, indicating an antagonistic interaction. Since therapeutic synergism has been shown to occur in situations in which one drug has no activity [7] and since interactions involving inactive drugs are well-described phenomena [2, 4], the absence of significant interactions in the variant cell lines or P388 leukemia was not due solely to a lack of effectiveness of etoposide. Thus, we conclude that additivity or antagonism occurs at the cellular level in these SCLC lines and in P388 leukemia.

These studies confirm the findings of Tsai et al. [32] that no synergy exists at the cellular level for the combination of cisplatin and etoposide in the treatment of SCLC lines, although there is a large body of clinical evidence indicating a therapeutic advantage for this combination. We extended their results, since our conclusions apply to all possible dose combinations within the dose ranges tested. Tsai et al. [32] used fixed dose-ratio combinations. For the NIH H209 line the present conclusions also apply to both sequences of drug use, cisplatin first and etoposide first, as well as to the intervals between doses within the ranges tested. In addition we found no in vitro synergy for this combination in the treatment of P388 cells, although therapeutic synergism has been reported by Schabel et al. [27] in P388 leukemia.

There are major differences between cell-culture and animal studies that could easily account for the differences seen. For instance, in animal experiments, multiple injections of both etoposide and cisplatin were given (days 5, 9, and 13). This allowed a reduction of the tumor burden to levels that were not measurable in tissue culture. Tissue-culture systems primarily show the effects of treatment on sensitive cells. The use of the animal system offers the possibility of showing combination effects on cells that are resistant to one drug or partially resistant to both agents. In the experiments reported by Schabel et al. [27], survivors were seen only in the combination-treatment groups. It is possible to set conditions in animal experiments such that single drug treatments reduce the tumor-cell burden to low numerical levels. In combination therapy some animals may be cured with only a small additional effect of the second agent. This tends to exaggerate the effects of the experiment such that therapeutic synergism can occur in the absence of cellular synergy. That is, if conditions are such that each individual drug reduces the tumor burden to 10^1 or 10^2 cells, the combination may produce cures when the effect is only additive or even antagonistic. The point is that these two concepts are largely independent of each other, and there is no compelling reason to suggest that one should predict the other.

Drugs that are cellularly synergistic may not show therapeutic synergism or clinical synergism if the synergistic effect is more pronounced in the normal tissues than in the tumor. Conversely, drugs that are antagonistic may be therapeutically synergistic if the antagonistic effect is more pronounced in the normal tissues [3].

Interactions vary at different response levels. A general curve for a drug-dose response tends to be sigmoidal. Combination experiments conducted at the point at which the response just begins to appear are likely to show synergism. On the linear portion of the curve, as previously implied, definitions of synergy or antagonism seem to be most applicable. Experiments using antibiotics or antihypertensives involve this portion of the curve. However, for antineoplastic agents, only the maximal responses are of any real interest. These may be antagonistic.

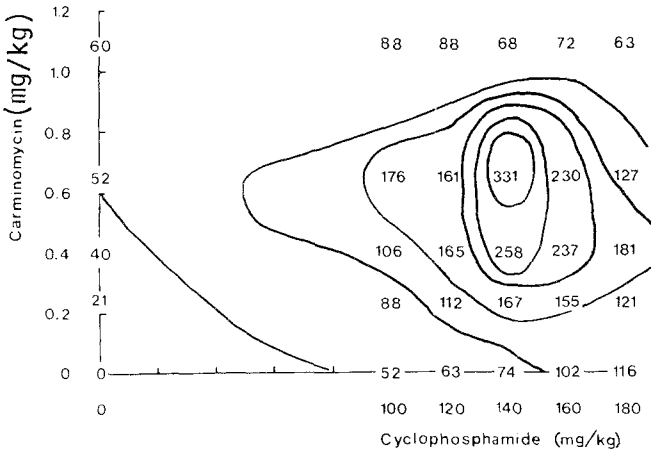

Fig. 2. Isoboles of the percentage of increased life span over controls as determined for mice bearing L1210 leukemia and treated with the combination of carminomycin and cyclophosphamide (reprinted from [2], p. 330, with the permission of Academic Press). The lines represent 50%, 100%, 150%, 200%, 250%, and 300% increases in life span as compared with controls as optimal doses of 140 mg/kg cyclophosphamide and 0.65 mg/kg carminomycin are approached. (The carminomycin dose was corrected from data obtained from the original source [1].) Synergistic isoboles are located in the *bottom left* portion of the graph, whereas antagonistic isoboles are present in the *upper right-hand portion* of the figure

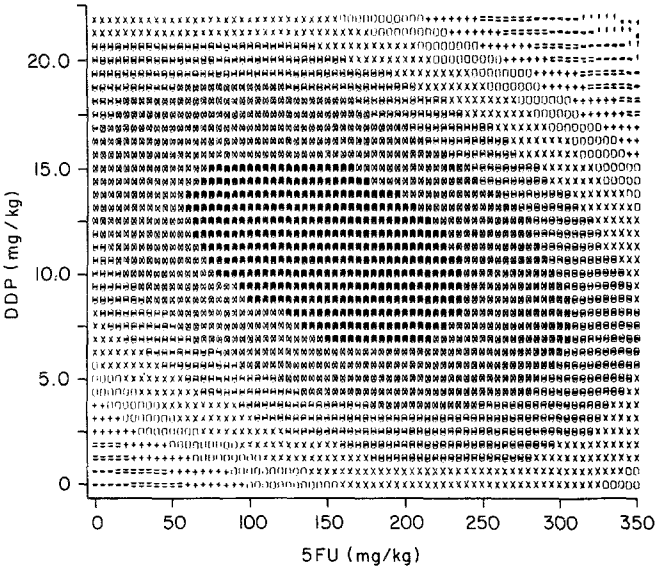

Fig. 3. Computer-generated isoboles of the estimated relative hazard for mice bearing L1210 leukemia and treated with cisplatin (*DDP*) and 5-fluorouracil (*5FU*); reprinted from [10], p. 67, courtesy of Marcel Dekker, Inc.). These plots show the same relationships vis-à-vis synergism and antagonism as those shown in Fig. 2 in which no modeling was involved. Anytime therapeutic synergism with toxic drugs occurs, closed isoboles of necessity are present; this requires that some portions of any such isobole indicate synergism and other portions indicate antagonism

We have published many contour plots of the effects of antineoplastic agents in the treatment of tumor-bearing animals [7–10, 29, 30, 35–37]. The contours on these plots are estimated isoboles, contours of isobiologic effect. A bowing toward the origin indicates synergy, whereas a bowing away from the origin indicates antagonism. In such plots, both antagonistic and synergistic relationships are frequently seen for combinations of drugs, with synergism typically being observed at low dose levels and antagonism, at high levels. Admittedly, model misfit is a factor in these presentations, yet the claim of different interactive effects under differing treatment conditions, dose levels in this case, seems to be secure on the basis of the accumulated evidence as further demonstrated in Fig. 2 and 3. Both antagonism and synergism or additivity and synergism have been reported for the same combination by various authors for different response levels [16], different dose levels [12], or different dose ratios [4, 11].

Etoposide and cisplatin were synergistic in Chinese hamster V79-171B lung cells, albeit not under all conditions or at all response levels [16], but were not synergistic in human lung-cancer cell lines [32]. Thus, like resistance, drug-interaction properties are cell-specific. Moreover, as discussed above, the conditions under which synergy has been demonstrated need to be specified.

One problem associated with the in vitro study of drug interactions in cancer chemotherapy that has never been adequately addressed relates to the prominent role of resistant or partially resistant cells in determinations of the outcome of treatment. Small numbers of resistant cells (2% or less) [14, 20] are lost in the noise of tissue-culture experiments. Even if this were not the case, their effect would be represented at the extreme end of the response curve and would be subject to distortion through the use of parametric modeling. It should be noted that our models as well as those advocated by Chou and Talalay [13] or those revised by Greco [21] and Greco and Lawrence [22]; are intended to apply to a uniform population of cells. The applicability of such models to cancer chemotherapy is prone to error, since certain populations of cancer cells are likely to be resistant to any drug used. For radiation and alkylating agents, the accepted mechanism that results in the initial resistance to low doses (survival curve shoulder) involves the accumulation of damage in the target (DNA) before cell killing has begun [5]; the mechanism suggested to underlie resistance to dose escalation at the high-dose region on the curve is related to the presence of subpopulations of resistant cells [14]. Thus, changes in the shoulder and slope of the curve may occur independently of one another and for different reasons [14]. Therefore, we regard any parametric representation as being empirical.

A number of the recent parametric approaches to this problem have used models that assume Michaelis-Menten kinetics [13, 21–23, 32]. However, radiation and alkylating agents have no receptors and the term nonexclusivity, which is associated with these models, has no meaning in the absence of a receptor. Therefore, the parametric models that have been advanced, which may be useful in some applications, are unlikely to be more advantageous for the study of cancer-chemotherapy interactions than the one we used in the present study. Our model offers an advantage in situations in which it is of interest to examine the effect of the interval between treatments. One problem with many studies of drug interactions in cell culture is that the ana-

lyses are conducted at concentrations producing 50% growth inhibition (IC_{50}). As we have argued, this is not the appropriate part of the dose-response curve for antineoplastic agents. Another problem is that fixed dose ratios of the drugs are often employed [32, 33]. A very limited cross section of the possible dose combinations is tested in such a design.

It is important to recognize that there are different definitions of synergy. One must carefully interpret just what each author means by the term. For example, the universal response surface approach [21–23] requires that the overall effect of a combination be evaluated, thus excluding the possibility of different interactions under different conditions as discussed above. Variations of the definitions have been comprehensively reviewed by Berenbaum [4]. He prefers to use synergy and related terms to refer to cellular interactions. Therapeutic optimization [2] or therapeutic advantage [4] are terms that he would substitute for therapeutic synergism and, possibly, clinical synergy. Although we suspect that the generic or unspecified use of the term synergy is so well entrenched that it cannot be eliminated from popular use, we stress the necessity to define accurately the meaning of any term used and to realize that no relationship has been established between the various types of synergy occurring at the cellular, preclinical (animal), and clinical levels. Only with a clear discernment of the formidable limitations associated with the study of antineoplastic drug interactions can there be a reasonable hope of using the concepts and information obtained from laboratory studies in any meaningful way.

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