

Lack of *in Vitro* Synergy between Etoposide and *cis*-Diamminedichloroplatinum(II)¹

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ABSTRACT

Claims of synergy between etoposide and cisplatin have been based upon preclinical *in vivo* murine P388 models or upon human clinical trials in tumors such as lung cancer. Such *in vivo* studies are useful in exploring therapeutic synergy, *i.e.*, an improved therapeutic strategy. The term "synergy" in this context is sometimes, however, taken to imply greater than additive kill of tumor cells. Unfortunately, it is virtually impossible to document supraadditive tumor cell kill *in vivo*, since *in vivo* curves of therapeutic effect are not linear and drugs are therefore not additive with themselves. Therapeutic synergy may, in fact, occur when two drugs are merely additive (or even antagonistic) with regard to cytotoxicity if the drugs have nonoverlapping host toxicity.

The demonstration of true supraadditive cell kill would imply an interaction of the two agents at a cellular level and would have profound implications for biochemical studies. In order to determine whether the reported therapeutic synergy of etoposide and cisplatin is due, in part, to supraadditive cell kill, we used an *in vitro* tetrazolium-based colorimetric assay for cytotoxicity (MTT assay) and an isobologram analysis to test combinations of the two drugs against four human small cell and four human non-small cell lung carcinoma lines. Using a rigorous test for *in vitro* synergy, we could not establish a greater than additive cytotoxic effect on our cell lines. It thus appears that the clinical synergy between etoposide and cisplatin is not due to a supraadditive effect at the cellular level. Our results have implications for a variety of fields in which claims of "synergy" often appear.

INTRODUCTION

The combination of etoposide and cisplatin is active in a variety of human tumors, including lymphomas, SCLC,⁴ NSCLC, and testicular cancer (1-9). Many of the clinical trials of the combination etoposide-cisplatin were prompted by the report from Schabel *et al.* (10) of therapeutic synergy between the two drugs in the mouse P388 *in vivo* model nearly a decade ago. Although numerous cisplatin-containing regimens exhibited synergy in this system, it was particularly marked with the etoposide-cisplatin combination. In the study, the tumor-related mortality was 100% when a 10% lethal dose of either etoposide or cisplatin was given as a single agent, but there was a 30% cure rate when the two agents were combined. The magnitude of the therapeutic synergy has led some investigators to conclude that there is a strong suggestion of a greater than additive

effect of the two drugs on tumor cells (11). In the *in vivo* setting, however, it is probably impossible to establish true biochemical synergy—supraadditive effects on tumor cells (12-14). One reason is that the dose-response curve *in vivo* depends in some measure upon host tolerance and is, therefore, nonlinear. In other words, the effect of any given increment of drug is not linear along the dose-effect curve; hence, the *in vivo* effects of single cytotoxic agents would not appear to be additive with themselves. A theoretical example shown in Fig. 1 serves to illustrate this. A 1-mg dose of either Drug A or Drug B will, as a single agent, achieve <10% tumor control. An observation that 1 mg of Drug A plus 1 mg of Drug B produce >90% tumor control although impressive to anyone who may anticipate <20% tumor control with the combination, does not necessarily imply greater than additive effect. A 2-mg dose of either drug alone would achieve the same result. Under these circumstances, it is impossible to determine whether or not the effects of two drugs are additive with each other in the clinical setting. An improvement in therapeutic strategy can occur whenever two drugs with nonoverlapping toxicities can be given at close to maximally tolerated doses, producing results not achievable with either of the single drugs. This circumstance has sometimes been declared incorrectly as supraadditivity (13).

The same analogy used in Fig. 1 may be extended to *in vitro* tests of supraadditivity. If the dose-effect curves of the single agents are not linear, one could incorrectly interpret a "greater than anticipated" effect of a drug combination as a greater than additive effect.

We have therefore used an *in vitro* semiautomated colorimetric tetrazolium-based (MTT) assay (15) to determine whether the reported *in vivo* therapeutic synergy evident with etoposide and cisplatin could be, in part, due to true supraadditive anti-tumor effect. If documented, this would suggest synergy at the cellular level and have important implications for biochemical pharmacology. To do this, we generated isoeffect cytotoxicity curves of various ratios of etoposide and cisplatin for a panel of four human small cell and four non-small cell lung cancer lines *in vitro*. Because of the large number of data points which can be obtained in the MTT assay, it is uniquely suited to generate a three-dimensional isobologram when exploring possible supraadditive or infraadditive interactions between two agents. Most other assay methods, such as the clonogenic tumor stem cell assay and dye exclusion assay, are labor intensive and cannot practically generate enough data points to describe the dose-effect surface in nearly the same detail. Our results have implications beyond *in vitro* cytotoxicity assays for anticancer agents, since drug interactions and synergy are frequently discussed in a variety of fields such as infectious diseases and immunosuppressive or immunoaugmentive therapy.

MATERIALS AND METHODS

Cell Lines and Culture Methods. Four SCLC cell lines (NCI-H774, NCI-H1092, NCI-H1284, variant subtype NCI-H841) and four

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⁴The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; MTT, (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (tetrazolyl blue); R10, RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum; PBS, phosphate-buffered saline; IC₅₀, concentration of drug that produced 50% reduction of absorbance; CI, combination index; HITES, Hydrocortisone, Insulin, Transferrin, β -Estradiol, and Selenium.

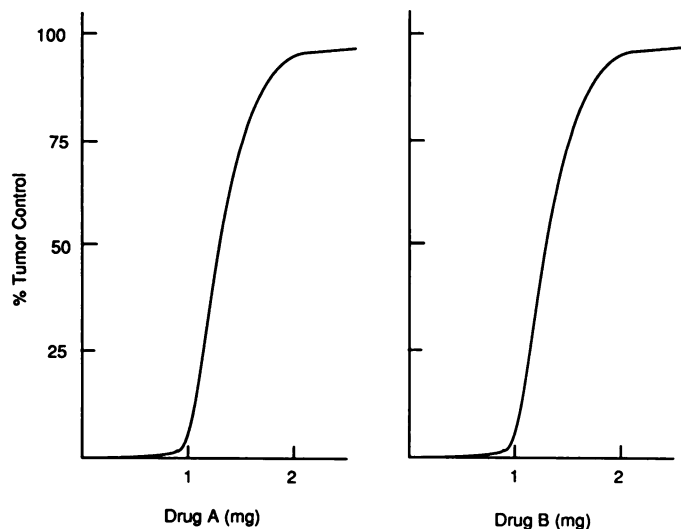


Fig. 1. Dose-effect curves of two theoretical drugs, A and B (percentage of tumor control is plotted versus drug dose).

NSCLC cell lines (adenocarcinoma NCI-H23, squamous cell NCI-H226, bronchioloalveolar cell NCI-H441, large cell NCI-H661) were used. The patient from whom line NCI-H841 was derived had been previously treated with cyclophosphamide, methotrexate, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, vincristine, Adriamycin, and procarbazine. The patient from whom line NCI-H661 was derived had been treated previously with cisplatin and vinblastine. The remaining six cell lines were established from previously untreated patients. All lines were grown in R10 except the three classic SCLC lines NCI-H774, NCI-H1092, and NCI-1284, which were grown in HITES medium (16) supplemented with 5% fetal bovine serum.

Trypsin-EDTA was used to make single cell suspensions after cultures were washed with PBS. Trypsin was neutralized with RPMI 1640 and cells were resuspended in R10 and also in serum-free medium for cytotoxicity assays. The serum-free media were as follows: ACL-4 (17), a modification of ACL-3 originally reported by Brower *et al.* (18) was used for NSCLC lines; and HITES supplemented with 0.2% albumin (HITES-A) was used for SCLC lines. Cells were then plated at the appropriate seeding density, individualized for each line, in 180 μ l of medium into 96-well microtiter plates and incubated for 16 h at 37°C in a 5% CO₂ atmosphere in order to allow recovery from trypsinization before cytotoxicity assays began.

Study Design and MTT Assay. The day after the cells were plated, drugs were added as follows. Twenty μ l of PBS were added to control wells; 10 μ l of PBS and 10 μ l of drug were added to single drug wells; and 10 μ l of each drug were added to drug combination wells. Each plate had 24 control wells with cells but no drug and 8 wells with single agent etoposide. The remaining 8-well columns contained a constant concentration of etoposide plus increasing concentrations of cisplatin in 0.5-log increments to cover a 3-log range. The two drugs were freshly prepared for each experiment with PBS as the diluent. Etoposide (VP-16; Bristol Laboratories, Syracuse, NY) was added 15 min before cisplatin (Ben Venue Laboratories, Bedford, OH). Over a series of 7 plates, the concentration of etoposide was increased in 0.5-log increments to cover a 3-log range. An eighth plate was used for single drug controls and increasing concentrations of single agent cisplatin. With this design, 16 survival curves were generated from every set of 8 microtiter plates, 1 from each of the single agents and 14 from the 49 pairs of drug combinations.

After addition of drug, the microtiter plates were incubated for 96 h. Cell survival was then determined by a semiautomated tetrazolium-based MTT colorimetric assay (15). The assay was performed as published previously by our laboratory (19, 20). This assay is based upon the reduction of light yellow MTT to a blue formazan product in the mitochondria of living cells. The formazan crystals are dissolved in dimethyl sulfoxide, and the absorbance of the blue product is read at 540 nm on a scanning multiwell spectrophotometer. The percentage of control absorbance was considered to be the surviving fraction of cells.

This has been shown to be true by others (15, 21) and confirmed in our laboratory.⁵ Preliminary experiments were performed to determine the appropriate seeding number for each cell line so as to adjust for differences in population-doubling times of the cell lines and to ensure that cell number per microtiter well was proportional to the absorbance of the solubilized formazan (19). The IC₅₀ was defined as the concentration of drug which produced 50% reduction of absorbance.

Data Analysis. The isobologram method of Steel and Peckham (14) and a curve-fitting analysis were used to determine whether etoposide and cisplatin were supraadditive at the cellular level. A brief discussion and a diagram are provided for the isobologram technique (Fig. 2). Fig. 2, *left*, shows the survival curves generated from the MTT assay for etoposide and for cisplatin as single agents against NCI-H774 cells in R10 medium. Cell survival, on a linear scale, is plotted versus relative concentration of each drug, on a log scale. The relative concentration is defined as the drug concentration divided by the IC₅₀ for the particular cell line. An isobologram will thus be generated at a level of 50% cell survival. (The concentrations could also be normalized to another inhibitory level, yielding an isobologram at that level of cell survival.) The data are transformed to the graph in the *middle panel* by plotting the surviving fraction of cells on a log scale versus relative drug concentration on a linear scale (14). This is done because the cytotoxic agents often kill cells in logarithmic fashion. Since even after the transformation the survival curves are not perfectly linear, there is no single isoeffect line, and an "envelope of additivity" which corrects for the nonlinearity of the single agent dose-effect curves must be determined.

The envelope (*shaded area*) is shown in Fig. 2, *right*, and its perimeters are derived by plotting the relative doses of each drug which yield a survival of 0.5 when added together. For example, if a particular concentration of etoposide (C_{ETOP}) results in a reduction in the surviving fraction by an amount y , then an additional amount of cell kill, x , is required to reach the 0.5 level as shown in Fig. 2, *center*. In Mode 1, the addition is performed by taking the relative dose of each drug starting from 100% cell survival, as shown at the *top* of the *panel*. This mode allows for the possibility that the dose of cisplatin that is required to decrease cell survival to 50% is unaffected by the reduction in survival achieved by etoposide (*i.e.*, the drugs act completely independently). The upper perimeter of the envelope is determined by Mode 2, which allows for the possibility that cell kill by cisplatin completely depends upon the effect achieved by etoposide and therefore begins at the level of cell survival which remains after etoposide exposure, as shown at the *bottom* of the *panel*. These two modes span the two extremes of drug interaction in which the mechanisms of action of the two agents are completely independent (Mode 1) or are similar (Mode 2).

Since, as mentioned above, etoposide was always added before cisplatin, we used etoposide as the first drug in generating the envelopes of additivity. The shape of the envelopes would change if cisplatin were to be used as the first drug. The line created by Mode 1, under these circumstances, would remain unchanged. However, the perimeter generated by Mode 2 would often be shifted to the right. In no case, however, would this change the configuration of the region of supraadditivity (the portion of the isobologram to the left of the Mode 1 perimeter). The change in the shape of the envelope of additivity depending on the order in which cells are exposed to two cytotoxic agents is discussed by Steel (22).

Statistical Methods. In order to identify whether the condition of supraadditivity existed, we first considered whether a significantly greater than chance proportion of IC₅₀ values were located below the envelope of additivity in a significantly greater than chance number of cell lines and media conditions. The nonparametric sign test, a binomial test for probability of an event equal to 0.5, was used to formally test the hypothesis of whether or not there was supraadditivity. For a given line and culture medium, if the sign test showed a significantly greater than 50% proportion of IC₅₀ values below the envelope, the condition was scored as a 1 (one). Otherwise, the condition was scored as a 0 (zero). One-sided tests were performed, because we were concerned only about identifying whether proportions were greater than 50%. The 16 tests cannot be considered as independent, since the results from

⁵ Unpublished observations.

Fig. 2. Isobologram method described by Steel and Peckham (14). *Left*, single agent survival curves of line H774 with etoposide and with cisplatin (surviving fraction of cells on a linear scale is plotted versus drug concentration relative to the IC₅₀ plotted on a log scale). *Middle*, transformation of cell survival to log scale and relative concentration to linear scale; sample line lengths (χ) are drawn for Mode 1 and 2 determinations. C_{ETOP} and C_{PLA} are the relative concentrations of etoposide and cisplatin, expressed as a line length (↔), necessary to produce a given amount of cell kill (γ for etoposide and χ for cisplatin). The length of the line representing C_{PLA} depends upon the mechanisms of action for the two drugs (independent for Mode 1; similar for Mode 2). Details are presented under "Data Analysis." *Right*, IC₅₀ isobologram; diagonal dashed line connecting the IC₅₀ of each drug, isoeffect line of additivity for theoretical situations in which the survival curves for both drugs are perfectly linear.

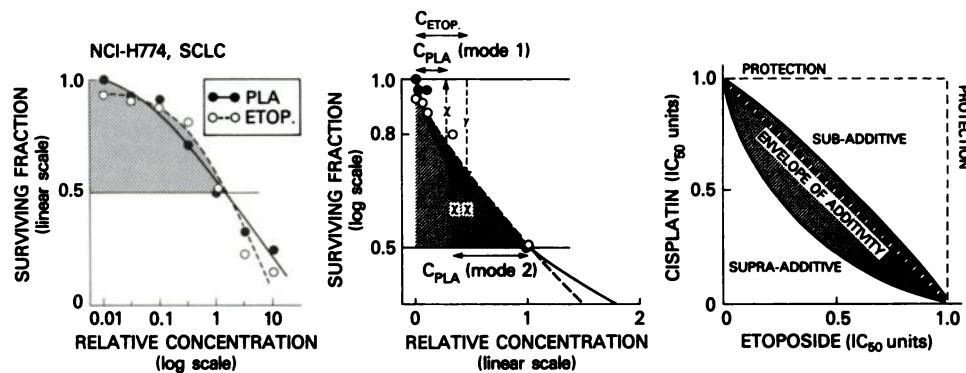


Table 1 IC₅₀ values μM of etoposide and cisplatin as single agents in two experiments

Cell line	Cell type	IC ₅₀ (μM)							
		Etoposide				Cisplatin			
		SCM ^a		SFM		SCM		SFM	
Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2		
H774	SCLC	0.1	0.1	0.1	0.3	2	3	1	3
H1284	SCLC	3	2	1	0.4	3	4	2	3
H841	SCLC ^b	1	0.5	2	2	4	3	6	10
H1092	SCLC	8	8	5	7	15	17	20	19
H226	Squamous	1	0.7	1	0.6	7	3	3	1
H23	Adenocarcinoma	0.6	0.4	3	1	2	1	3	2
H441	Bronchioloalveolar	6	4	5	2	6	6	7	7
H661	Large cell	1	2	80	33	3	5	10	11

^a SCM, serum-containing medium; SFM, serum-free medium (HITES-A for small cell lines and ACL-4 for non-small cell lines).

^b Variant subtype of small cell lung cancer.

Table 2 Combination effect (% control absorbance^a) of etoposide/cisplatin on NCI-H226 in ACL-4 medium

Plate	Control absorbance	Etoposide conc.		Cisplatin conc. μM IC ₅₀ units	Cisplatin IC ₅₀ units									
		μM	IC ₅₀ units		0	0.0054	0.0168	0.054	0.168	0.54	1.68	5.4	16.8	μM
A	0.545	0	0		102	102	97	90	89	66	28	4	2.75	1
B	0.567	0.0043	0.0042	104	101 ^b	100	102	95	88	66	28		2.75	1
C	0.518	0.0136	0.013	102	99	98	101	94	91	68	22		2.65	0.97
D	0.517	0.043	0.042	94	91	91	90	88	83	58	25		2.2	0.8
E	0.6	0.136	0.13	86	84	78	84	79	75	56	21		2.02	0.74
F	0.603	0.43	0.42	66	63	57	62	58	55	46	19		0.98	0.36
G	0.58	1.36	1.3	45	43	42	42	40	39	37	16			
H	0.595	4.3	4.2	24	24	27	22	22	24	26	12			
A	0.545	13.6	13.3	24										
		Etoposide IC ₅₀	μM IC ₅₀ units		1.02	0.87	0.77	0.83	0.70	0.595	0.26			
					1	0.85	0.75	0.81	0.69	0.58	0.25			

^a Mean value of 8 wells.

^b Numbers in the rectangle are the data of combination effects. They are expressed as the percentage of control cell survival.

the same cell line tested in two different media (defined versus serum containing) may be correlated.

In addition to the above nonparametric method, we fitted parametric cell survival curves to the data to test for supraadditivity. The model used for a single agent acting on a cell line is a standard one in biological assays, specifying a logistic response (fraction killed) as a function of the logarithm of drug concentration (23). In Mode 1 of the isobole analysis, the surviving fraction for a two-drug combination is simply the product of the surviving fractions for the two single agents. We also developed a second, more general response function which permits some interaction between the drugs, making the surviving fraction smaller (supraadditive) or larger than in Mode 1. (The explicit para-

metric models that were fitted to the data are described in the "Appendix.") For each set of data, we fitted the Mode 1 formulation by a regression technique and then computed a χ² statistic assessing any evidence of supraadditivity in the second formulation. This was used to test the null hypothesis that Mode 1 is correct (no supraadditivity). When this null hypothesis could be rejected, we proceeded to fit the second model to see whether the improvement in the model was in the direction of supraadditivity. The parametric modeling is an examination of all available data over the dose-effect surface. As such, it examines a three-dimensional isobologram. By comparison, the binomial test described above examines the two-dimensional isobologram in the plane of IC₅₀ only.

**IN VITRO COMBINATION TESTING
OF ETOPOSIDE AND CISPLATIN
(EXPERIMENT 1)**

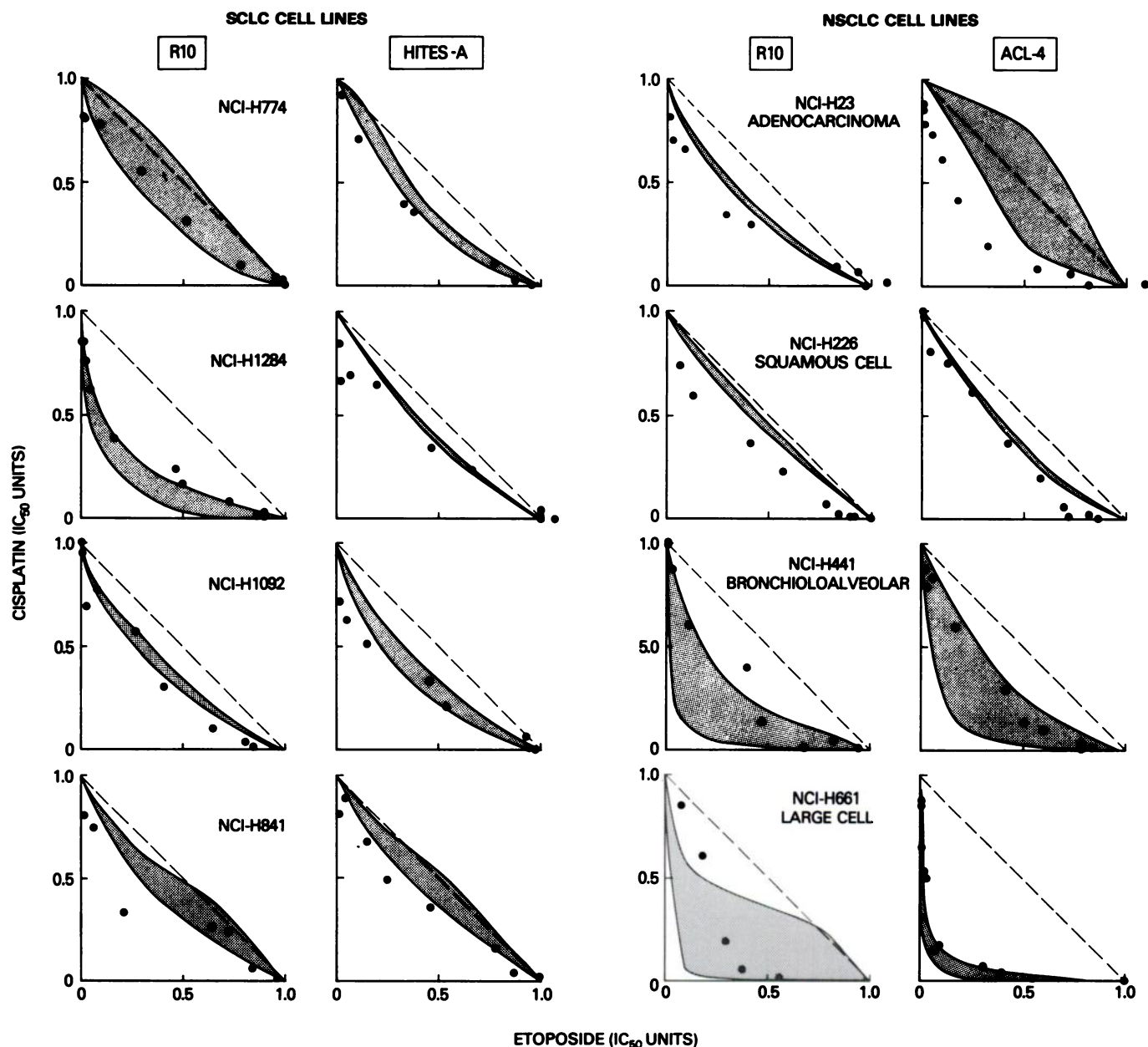


Fig. 3. Isobolograms of IC_{50} for Experiment 1. HITES-A and ACL-4 signify the defined media used for the small cell and non-small cell lines, respectively.

All of the above procedures helped to ensure that we would conclude the condition of supraadditivity only if the evidence clearly supported it. For example, evidence of supraadditivity in only one or two cell lines would be insufficient to generalize a synergistic effect between etoposide and cisplatin to the population of tested lines.

RESULTS

The experiment was performed twice on each of the eight lung cancer cell lines in each of two media (defined serum-free and serum-containing). Hence, there were 32 tests ($2 \times [8 \times 2]$) for supraadditivity. The single-agent IC_{50} values of etoposide and cisplatin for each of the cell lines in the two experiments are compiled in Table 1. The values from the two experiments are similar. In a few instances, the IC_{50} of a drug was consid-

erably higher in serum-free than in serum-containing medium (e.g., NCI-H661 exposed to cisplatin or etoposide). This is presumably because the growth rate of some cell lines is higher in the presence of serum, with resulting increased sensitivity to cytotoxic agents. Table 2 presents typical data used to generate an isobologram (in this case, Experiment 1 on squamous cell line NCI-H226 using ACL-4 growth medium). All of the data from Experiments 1 and 2 are plotted in the isobolograms in Figs. 3 and 4, respectively. It is clear from the figures that the Mode 1 isoeffect line, which presumes independent mechanisms of cell kill by the two agents, is always convex toward the origin of the isobolograms. The isoeffect curve for Mode 1 is always more convex than the curve for Mode 2, which assumes similar mechanisms of action for the two agents. In some cases, such

**IN VITRO COMBINATION TESTING
OF ETOPOSIDE AND CISPLATIN
(EXPERIMENT 2)**

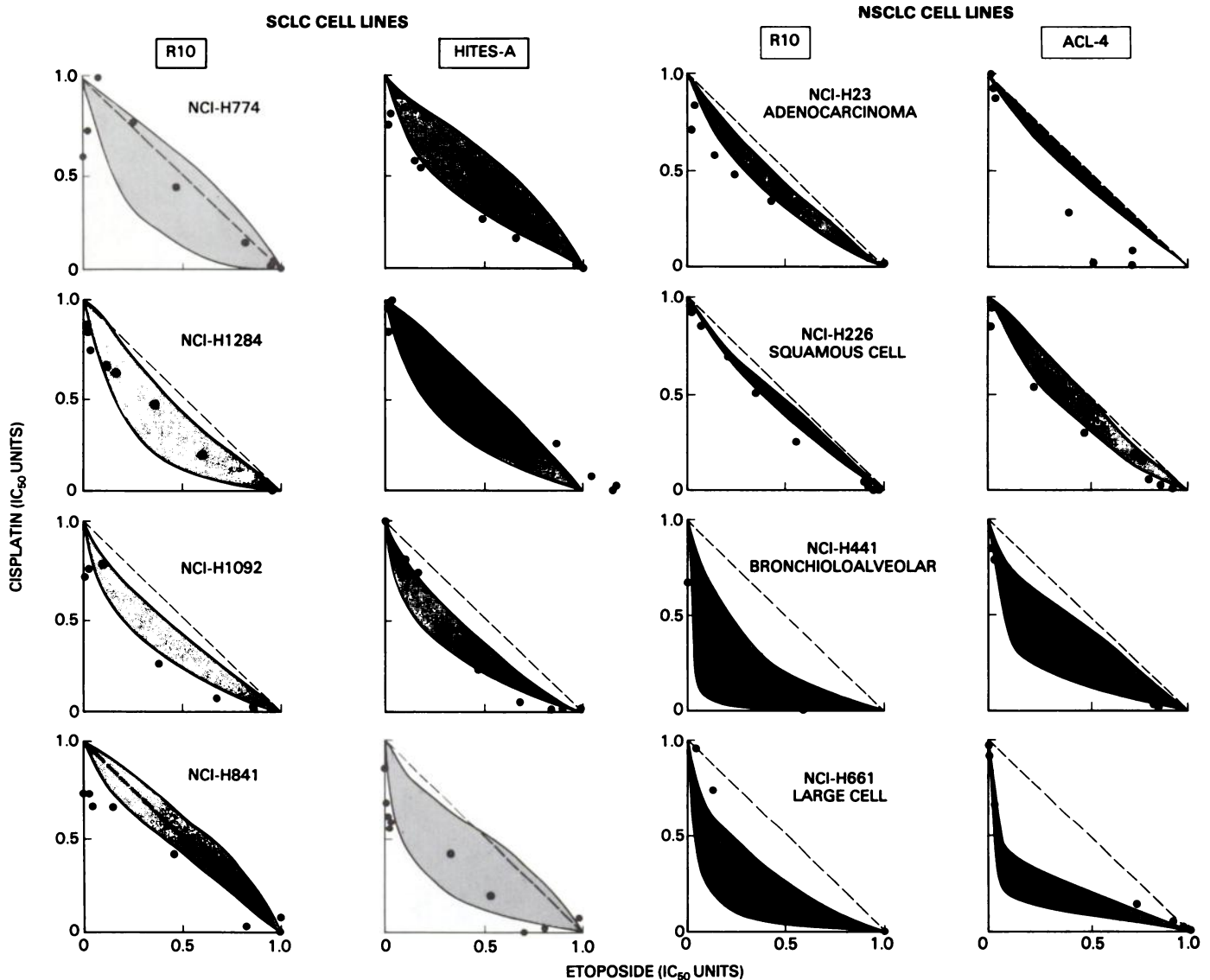


Fig. 4. Isobolograms at IC_{50} for Experiment 2; abbreviations for media are the same as in Fig. 3.

as in lines NCI-H441 and NCI-H661, the convexity of the Mode 1 line is extreme.

Information in Figs. 3 and 4 may be reexpressed according to the numbers of IC_{50} values lying to the left of the Mode 1 isoeffect curve. These results are arranged in Table 3 and are subjected to the sign test as outlined in "Materials and Methods." In Experiment 1, 2 of the 16 cell line/medium combinations were individually associated with a significantly greater than 50% of IC_{50} values below the envelope of additivity: line NCI-H23 in defined medium; and line NCI-H226 in serum-containing medium. In Experiment 2, only line NCI-H226 in serum-containing medium deviated significantly from the null hypothesis. Hence, only one line showed supraadditivity in both experiments in any medium.

It is evident in Figs. 3 and 4 that the shapes of the envelopes of additivity vary between the two experiments. This is because the shapes of the single agent survival curves vary. Nevertheless, the form and position of the Mode 1 lines are quite similar for

the repeated experiments. Since this is the curve which defines the lower edge of the envelope and since the isoeffect points bear similar spatial relationships to this lower perimeter for each cell line on repeat experiment, the sign test yields comparable results between the two experiments.

The results of the parametric model fitting method are also given in Table 3. In Experiment 1, 4 of the 16 cell line/medium conditions showed a significantly better fit with a supraadditive model than with the Mode 1 formulation: NCI-H774 and NCI-H1284 in defined medium; and H841 in both media. The outcome in Experiment 2 was similar, except that results for H841 in defined medium were no longer consistent with greater than additive effect. The inferences for specific lines are clearly different from those of the sign test. There was no overlap in declaration of supraadditivity between the parametric model and the sign test. It should be noted, however, that in the 2 experiments combined, 8 of the 32 conditions had score statistics that would reject the Mode 1 formulation in favor of curves

Table 3 Tests for supraadditivity between etoposide and cisplatin.

Cell line	Media	Experiment 1			Experiment 2		
		Fraction of points below envelope	Sign test	Parametric test	Fraction of points below envelope	Sign test	Parametric test
H774	R10	2/9	0	0	2/11	0	0
	HITES	5/7	0	1	7/10	0	1
H1284	R10	0/11	0	0	4/10	0	0
	HITES	5/9	0	1	1/9	0	1
H1092	R10	5/9	0	0	7/9 ^a	0	0
	HITES	3/8	0	0	3/10	0	0
H841	R10	4/7	0	1	6/9	0	1
	HITES	6/8	0	1	7/12	0	0
H23	R10	9/13	0	0	5/8	0	0
	ACL-4	10/11 ^b	1	0	7/10 ^a	0	0
H226	R10	8/9 ^b	1	0	9/11 ^b	1	0
	ACL-4	8/11 ^a	0	0	7/10 ^a	0	0
H441	R10	0/9	0	0	3/9	0	0
	ACL-4	0/10	0	0	4/9	0	0
H661	R10	0/5	0	0	0/7	0	0
	ACL-4	0/11	0	0	0/11	0	0

^a One-sided $P < 0.25$.^b One-sided $P < 0.05$.

that are supraadditive for some dose combinations but not for others. These include all four of the tests using cell line NCI-H23, for which low doses of etoposide with cisplatin near its IC_{50} had smaller surviving fractions than in Mode 1, whereas low doses of cisplatin with etoposide near its IC_{50} had higher surviving fractions. If one were to try to draw smooth curves through the IC_{50} points in the plots in Figs. 3 and 4 (or generate smooth surfaces in a three-dimensional isobologram), they would therefore in many cases cross the Mode 1 isoeffect line (or surface). Our modeling approach confirmed this impression and gave us an additional assessment of the significance of this effect. Interactions of this type could lead one to different conclusions about supraadditivity depending upon which fraction of cell kill one chose to study and which dose levels one happened to sample.

We emphasize that inferences drawn from any model fitting must be viewed with some caution. The model used here is a simple and reasonable one, but it may not adapt to all the characteristics of every survival curve. Large systematic and random experimental deviations from fitted values appear in a few of the data sets. The score statistic and best fitting model are affected by data at all dose levels, not only at the IC_{50} s. On the other hand, there was remarkable consistency in parameter estimates between the two experiments. These considerations therefore do not affect our conclusion that the results are inconsistent with an overall *in vitro* synergistic interaction between etoposide and cisplatin in the 8 cell lines.

DISCUSSION

This is the first study which explores such a large proportion of the three-dimensional dose-effect surface of two cytotoxic agents. The semiautomated MTT assay is uniquely suited to such a study compared to other existing assays because of the number of data points it can generate. For each isobologram in our study, more than 750 measurements (microtiter wells) were generated. Hence, for the entire study, there were approximately 24,000 data points. This may be why, despite the original description of the envelope of additivity by Steel and Peckham

in 1979 (14), the technique has not been applied to a large set of experimental data using anticancer agents in combination until now.

In only a minority of the 16 cell line/media combinations evaluated in this experiment was there significant evidence of supraadditivity. These particular cell lines and conditions were not predicted in advance to be ones in which supraadditivity was most likely. With multiple tests, a small percentage of such combinations could be associated with "significant" supraadditivity by chance alone. This may be an additional explanation why the sign test and parametric modeling approaches did not identify greater than additive effect in the same lines. Because the number of cell lines in which *in vitro* synergy was identified is a small proportion of all experiments examined, it is clear that supraadditivity is not generally observed and could possibly have occurred by chance where it was noted. The data in these experiments are thus consistent with additivity.

A primary thrust of this report is to emphasize the difference between synergy at the clinical or therapeutic level, and synergy at the cellular or biochemical level of drug interaction (true supraadditivity). Steel has pointed out the difference between these two concepts and has discussed how mechanisms other than supraadditivity may lead to therapeutic synergy (22). As an example, systemic doxorubicin may be combined with local irradiation to achieve improved local tumor control compared with the use of either agent alone when given at the same individual doses. Nevertheless, when combined in an *in vitro* assay, the isoeffect points fall within the isobologram envelope of additivity (22). The clinical synergy, then, is probably due to "spatial cooperation" in Steel's terminology as opposed to supraadditivity.

Likewise, a common strategy for achieving improved response and cure rates in disseminated malignancies has been through the use of drug combinations. Improved therapeutic effect may be achieved when two or more agents with different mechanisms of action and different spectra of toxicities are combined. Schabel *et al.* (10) catalogued 26 such cisplatin-containing regimens which yielded therapeutic synergy in pre-clinical animal tumor models. One of the most impressive combinations was etoposide and cisplatin. These observations led to a number of successful clinical studies. However, enhanced activity achieved with drug combinations, whether in animal tumor models or in the clinical setting, does not prove a greater than additive effect for the component drugs. Clinical success with combination chemotherapy does not require that the antitumor effects of the drugs be supraadditive (or even, for that matter, additive) if the agents are individually active and have nonoverlapping host toxicities (14).

The type of study in which fixed doses of agents are combined and the *in vivo* antitumor effect is compared with that of the single agents does not necessarily imply a greater than additive interaction. This is because the shape of the dose-response curves is not known. The idealized straight line of additivity in an isobologram, represented by the *diagonal dotted line* in Fig. 2, *right*, connecting the IC_{50} of the two drugs, is really a line only for the special case in which the dose-response curve of each of the drugs is a straight line (12-14, 22). Such a case rarely, if ever, exists for cytotoxic agents.

Of importance, if one were to ignore the envelope of uncertainty which occurs in the case of nonlinear dose-response curves, every one of our isobolograms would give the appearance of greater than additive effect (Figs. 3 and 4). However, when the envelopes are plotted, the isoeffect points of even some of the most apparently extreme cases of supraadditivity

fall within the additive region (e.g., lines NCI-H441 and NCI-H661).

Another method for determining *in vitro* synergy has been published recently (24). It involves the calculation of a CI. The theory is based upon equations derived from interactions between two or more reversible enzyme inhibitors which obey Michaelis-Menten or Hill kinetics and has been extended to the use of antineoplastic agents. If the index is 1, the drug combination is thought to be additive. Supraadditivity is indicated by an index <1 , and infraadditivity or antagonism by an index >1 . Over the range of *in vitro* cell survival, the combination index can then be plotted *versus* the fractional cytotoxicity of the drug combination. Frequently, the resulting graph shows synergy at some levels of fractional cytotoxicity, a single point of additivity at one level of cytotoxicity, and antagonism at the remaining levels of cytotoxicity (11, 25). For example, a recent *in vitro* tumor cell spheroid study of the interaction between etoposide and cisplatin showed a $CI < 1$ ("synergy") at low levels of cell survival, a point of additivity, and a $CI > 1$ ("antagonism") at higher levels of cell survival. The precise point of additivity depended upon the ratio of the two drug concentrations and on the depth of the cells within the spheroid. In some circumstances, the slopes of the clinical index curves vary dramatically as the ratios of the drugs are changed. For example, the curves generated by Chang *et al.* (25) indicate that the combination of etoposide and doxorubicin at a ratio of 1:0.06 has synergistic toxicity for bone marrow granulocyte-macrophage-colony-forming units at a low level of fractional cytotoxicity and antagonism at a high level of fractional cytotoxicity. However, when applied at a ratio of 1:0.033, the combination is synergistic at high levels of fractional cytotoxicity and antagonistic at low levels of cytotoxicity. Unlike the method described by Steel and Peckham (14), there is no envelope of uncertainty associated with the combination index of 1. Moreover, translation of the results to the *in vivo* situation may be complicated with drugs of differing half-lives and resulting changes in drug ratios over time.

We cannot transform our data to the method used by Chou and Talalay, for purposes of direct comparison, because it uses fixed ratios of the two drugs over the dose-effect spectrum. As mentioned above, in some instances investigators have identified supraadditivity only at the lowest levels of cell survival. In our assays, however, there was a resistant subpopulation of cells even at the highest drug concentrations used.

The issue of multiple techniques used to identify supraadditivity, with apparently divergent results, is not unique to the field of cancer chemotherapy. Synergy is a prominent concept in the use of antimicrobial agents. Some investigators have pointed out that there are multiple categories of "synergy" and that the clinical relevance of synergy with antibiotics is controversial (26). An historical example of dramatic therapeutic synergy in infectious diseases which was clearly not due to true supraadditivity was the use of "triple sulfa." The administration of three sulfonamides with identical mechanisms of action but independent solubility constants resulted in an increased clinical cure rate due to a reduction in the nephrotoxicity caused by the individual agents given at full dosage. It has even been suggested that the concept of synergy between antimicrobial agents be abandoned altogether (27). The field has been characterized by divergent criteria for *in vitro* greater than additive effects (28). Some have found discordant results when directly comparing techniques. Norden *et al.* (29) compared the checkerboard technique (a modified isobole approach) to the killing-curve method. Using cephalothin and gentamicin against 22

strains of *Klebsiella pneumoniae*, they found very poor agreement in declaring *in vitro* synergy. Calculation of a combination index similar to the one used by Chou and Talalay (24) for mutually exclusive drugs was one of the criteria used to detect synergy. Of note, if the combination index was >1 for some combinations of concentrations but <1 for others, Norden *et al.* considered the result equivocal.

On the basis of our data, we do not find statistical evidence that etoposide and cisplatin interact at the cellular level in a greater than additive manner. We cannot rule out the possibility that there is supraadditivity at certain drug ratios in some regions of the dose-effect spectrum, depending upon the cell line used and the medium. However, the overall pattern does not support *in vitro* synergy, and the minority of instances in which the data suggested it may have been due to random chance. The purpose of the original description by Steel and Peckham (14) of the isobologram approach to drug interactions was not intended to provide a strict definition of additivity, but rather to stress the area of uncertainty which exists whenever dose-response curves are nonlinear (which they nearly always are). They wanted to emphasize that in the case of nonlinear dose-response curves, requirements for establishing synergy are more stringent than for the special case of linear dose-effect relationships.⁶ We chose a drug combination which is widely reported to demonstrate clinical synergy in animal tumor models and in human studies. Nevertheless, we were ultimately unable to prove that any clinical synergy which exists is due to supraadditive killing at the cellular level. When we reviewed the results (which surprised us) we searched the early literature on drug synergy and found the following prophetic statements made more than 35 years ago by Loewe (112):

". . . interpretation of a slight SW-deviation of an isobole of combined lethal effect . . . as "synergism," on the strength of whatsoever definition of the word, may most unfortunately mislead the user of such a combination, who would obtain the same effect more economically with perhaps only half the amount of the "synergistic" mixture if he would employ one of the two components alone.

". . . rarely, if ever, do the dose/effect relations of a combination indicate whether the combined effect is the result of additive or of non-additive behavior. The conclusion is reached that 'synergism' and 'antagonism', which can be recognized only as supra- and infra-additive deviations from a firmly established additive dose/effect relationship, are imaginary magnitudes devoid of a basis of reference and of practical applicability . . . the problem is not solved by confounding the relationship between dose and effect with the relationship between dose and individual tolerance or susceptibility."

While we would not take such an extreme view, it seems clear that the firm establishment of supraadditivity is difficult even using a drug combination with widely accepted clinical synergy.

Finally, the fact that an *in vitro* assay may not identify greater than additive effects with the combined use of etoposide and cisplatin does not detract from the clinical effectiveness of the combination. It is, of course, the latter which is of primary import to the clinician and patient. In the end, what the clinician wants to know is whether a combination of agents has a better therapeutic index than single agents. In fact, supraadditivity achieved in the tumor, even if it were documented, may not be

⁶ G. G. Steel, personal communication.

of any benefit to the patient if it is paralleled by supraadditivity of similar magnitude in critical normal tissues.

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APPENDIX

We give here a brief presentation of the explicit parametric models that were fitted to the data in this paper.

Each observation is the fraction of cells surviving a particular combination of doses of two drugs, say, Drug A and Drug B. For a single drug, the logistic dose-response curve is assumed. For instance, if the dose of Drug A in IC₅₀ units is X_A, the function that gives the surviving fraction for A alone is

$$P_A = (1 + X_A^{\beta_A})^{-1}$$

where β_A is a parameter to be estimated from the data. Since the IC₅₀ level must also be estimated, we use the dose x_A in standard units and let s_A represent the IC₅₀ in these units. Thus

$$P_A = [1 + (x_A/s_A)^{\beta_A}]^{-1}$$

P_B has the same form, with dose x_B and parameters β_B and s_B.

The assumptions for Mode 1 imply that the surviving fraction for the two-drug combination is P = (P_A)(P_B), or

$$P = [1 + (x_A/s_A)^{\beta_A} + (x_B/s_B)^{\beta_B} + (x_A/s_A)^{\beta_A}(x_B/s_B)^{\beta_B}]^{-1}$$

Drug synergy or antagonism would be seen as an increase or decrease in the final term of the above equation. We proposed to model this with an additional exponent for each factor,

$$P = [1 + (x_A/s_A)^{\beta_A} + (s_B/s_B)^{\theta_B} + (x_A/s_A)^{\beta_A}(x_B/s_B)^{\beta_B}]^{-1}$$

If θ_A and θ_B are both less than 1, then the isoeffect curve at 50% cell kill will lie entirely in the supraadditive portion of the graph shown in Fig. 2, compared to the Mode 1 curve using the same βs.

Now, if any observation were truly a binomial proportion with expected value P, its variance would be

$$\frac{P(1-P)}{N}$$

where N is the number of cells, and an approximation using the normal distribution would suffice because N is large. Experimental variation added considerably to the variance of the observations, however. Because of the truncation of the proportions at 0 and 1, it was judged appropriate to model the variances as proportional to the binomial ones, but with a scale factor estimated from the data. The observations are not all independent because, for example, each row of Table 1 is a ratio with the same control as denominator, but the covariance induced should be much smaller than the variance of the independent numerators. Therefore, the models were fitted to the data with weighted nonlinear regression, using independent, normally distributed variables with expected values P and variances

$$\sigma^2 P(1-P)$$

where σ² was estimated from the sum of squared weighted residuals.

REFERENCES

1. Von Heyden, H. W., Scherpe, A., and Nagel, G. A. *cis*-Dichlorodiamineplatinum (II) (*cis*-platinum) and etoposide for patients with refractory lymphomas. *Cancer Treat. Rev.*, 9 (Suppl. A): 45-52, 1987.
2. Cavalli, F. VP-16 in the treatment of malignant lymphomas: a report from the Swiss Group for Clinical Cancer Research (SAKK). *Semin. Oncol.*, 12 (Suppl. 2): 33-36, 1985.
3. Einhorn, L. Initial therapy with cisplatin plus VP-16 in small-cell lung cancer. *Semin. Oncol.*, 13 (Suppl. 3): 5-9, 1986.
4. Evans, W. K., Shepherd, F. A., Feld, R., Osoba, D., and DeBoer, G. VP-16 and cisplatin as first-line therapy for small-cell lung cancer. *J. Clin. Oncol.*, 3: 1471-1477, 1985.
5. Murray, N., Shah, A., Brown, E., Kostashuk, E., Laukkanen, E., Goldie, J., Band, P., Hoek, J. V., Murphy, K., Sparling, T., and Noble, M. Alternating chemotherapy and thoracic radiotherapy with concurrent cisplatin-etoposide for limited-stage small-cell carcinoma of the lung. *Semin. Oncol.*, 13 (Suppl. 3): 24-30, 1986.
6. McCracken, J. D., Lalitha, M. J., Taylor, S. B., Giri, P. G. S., Weiss, G. B., Gordon, W., Jr., Vance, R. B., and Crowley, J. Concurrent chemotherapy and radiotherapy for limited small-cell carcinoma of the lung: a Southwest Oncology Group Study. *Semin. Oncol.*, 13 (Suppl. 3): 31-36, 1986.
7. Splinter, T., Kok, T., Kho, S., Lameris, H., Kate, F., Dalesio, O., Dolman, B., Palmen, F., Bouvy, J., Burghouts, J., Simonis, F., Harper, P., Rankin, E., Reijswoud, I., and Hoogenhuijze, J. A multicenter phase II trial of cisplatin and oral etoposide (VP-16) in inoperable non-small-cell lung cancer. *Semin. Oncol.*, 13 (Suppl. 3): 97-103, 1986.
8. Klastersky, J. Therapy with cisplatin and etoposide for non-small-cell lung cancer. *Semin. Oncol.*, 13 (Suppl. 3): 104-114, 1986.
9. Hainsworth, J. D., Williams, S. D., Einhorn, L. H., Birch, R., and Greco, F. A. Successful treatment of resistant germinal neoplasms with VP-16 and cisplatin: results of a Southeastern Cancer Study Group Trial. *J. Clin. Oncol.*, 3: 666-671, 1985.
10. Schabel, F. M., Jr., Trader, M. W., Laster, W. R., Jr., Corbett, T. H., and Griswold, D. P. Jr. *cis*-Dichlorodiamineplatinum (II): combination chemotherapy and cross-resistance studies with tumors of mice. *Cancer Treat. Rep.*, 63: 1459-1473, 1979.
11. Durand, R. E., and Goldie, J. H. Interaction of etoposide and cisplatin in an *in vitro* tumor model. *Cancer Treat. Rep.*, 72: 673-679, 1987.
12. Loewe, S. The problem of synergism and antagonism of combined drugs. *Arzneim. Forsch.*, 3: 285-290, 1953.
13. Berenbaum, M. C. Synergy, additivism and antagonism in immunosuppression: a critical review. *Clin. Exp. Immunol.*, 28: 1-18, 1977.
14. Steel, G. G., and Peckham, M. J. Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. *Int. J. Radiat. Oncol. Biol. Phys.*, 5: 85-91, 1979.
15. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65: 55-63, 1983.
16. Simms, E., Gazdar, A. F., Abrams, P. G., and Minna, J. D. Growth of human small cell (oat cell) carcinoma of the lung in serum-free growth factor supplemented medium. *Cancer Res.*, 40: 4356-4363, 1980.
17. Gazdar, A. F., and Oie, H. K. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.*, 46: 1092-1096, 1986.
18. Brower, M., Carney, D. N., Oie, H. K., Gazdar, A. F., and Minna, J. D. Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium. *Cancer Res.*, 46: 798-806, 1986.
19. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Gazdar, A. F. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, 47: 936-942, 1987.
20. Park, J-G., Kramer, B. S., Steinberg, S. M., et al. Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. *Cancer Res.*, 47: 5875-5879, 1987.
21. Finlay, G. J., Wilson, W. R., and Baguley, B. C. Comparison of *in vitro* activity of cytotoxic drugs toward human carcinoma and leukemia cell lines. *Eur. J. Cancer Clin. Oncol.*, 22: 655-662, 1986.
22. Steel, G. G. Terminology in the description of drug-radiation interactions. *Int. J. Radiat. Oncol. Biol. Phys.*, 5: 1145-1150, 1979.
23. Finney, D. J. *Statistical Method in Biological Assay*. New York: Macmillan Publishing Co., Inc., 1978.
24. Chou, T-C., and Talalay, P. Quantitative analysis of dose-effects relationships: the combined effect of multiple drugs on enzyme inhibitors. *Adv. Enzyme Regul.*, 22: 27-55, 1985.
25. Chang, T-T., Gulati, S., Chou, T-C., Colvin, M., and Clarkson, B. Comparative cytotoxicity of various drug combinations for human leukemic cells and normal hematopoietic precursors. *Cancer Res.*, 47: 119-122, 1987.
26. Klastersky, J. Antibiotic synergy and antagonism. In: A. M. Ristuccia and B. A. Cunha (eds.), *Antimicrobial Therapy*. New York: Raven Press, 1984.
27. Moellering, R. C., Jr. Antimicrobial synergism—an elusive concept. *J. Infect. Dis.*, 140: 639-641, 1979.
28. Berenbaum, M. C. A method for testing for synergy with any number of agents. *J. Infect. Dis.*, 137: 122-130, 1978.
29. Norden, C. W., Wentzel, H., and Keleti, E. Comparison of techniques for measurement of *in vitro* antibiotic synergism. *J. Infect. Dis.*, 140: 629-633, 1979.