# REPORTS

## Computerized Quantitation of Synergism and Antagonism of Taxol, Topotecan, and Cisplatin Against Human Teratocarcinoma Cell Growth: a Rational Approach to Clinical Protocol Design

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***Background:*** **Cisplatin-based induction chemotherapy may achieve a complete response (i.e., no sign of tumor following treatment) in 70%-80% of patients with germ cell tumors. However, only a minority of patients in whom the first-line regimens fail are cured with the salvage regimens.** ***Purpose:*** **The aim of these studies was to identify new agents or new regimens for the treatment of germ cell tumors by carrying out quantitative assessment in vitro of two promising new antitumor agents (paclitaxel [Taxol] and topotecan) and three more established agents (cisplatin, vincristine, and etoposide). These agents were used singly or in two- and three-drug combinations and were selected because they represent five distinct categories of antineoplastic mechanisms.** ***Methods:*** **The combination index–isobologram method, which is based on the median-effect principle developed by Chou and Talalay, was used for computerized data analysis. This method was selected because it takes into account both the potencies of each drug and combinations of these drugs and the shapes of their dose–effect curves.** ***Results:*** **Synergism against the growth of teratocarcinoma cells resistant to cisplatin (833K/64CP10 cells) was greater than against the growth of parent 833K cells. The degrees of synergism were in the following order: cisplatin + topotecan ≥ paclitaxel + cisplatin + topotecan > paclitaxel + topotecan ≥ paclitaxel + etoposide > paclitaxel + cisplatin + etoposide > paclitaxel + cisplatin. All other combinations showed nearly additive effects or moderate antagonism. The degrees of antagonism were as follows: cisplatin + etoposide ≥ paclitaxel + vincristine > paclitaxel + cisplatin + vincristine > cisplatin + vincristine. The combination of paclitaxel and cisplatin was synergistic against 833K/64CP10 cells and moderately antagonistic against 833K cells. Since the combination of paclitaxel, cisplatin, and topotecan and the two-component combinations of these drugs (cisplatin + topotecan and paclitaxel + topotecan) showed synergism stronger than that of other combinations, these three drugs were selected for illustrating detailed data analysis, using a computer software program developed in this institute.** ***Conclusions and Implications:*** **Our findings suggest that, as a result of synergy, the doses of these agents needed to achieve an antitumor effect may be reduced by twofold to eightfold when these agents are given in combination. The present quantitative data analyses for synergism or antagonism provide a basis for a rational design of clinical protocols for combination chemotherapy in patients with advanced germ cell tumors. [J Natl Cancer Inst 86:1517-1524, 1994]**

Germ cell cancer serves as a model for the chemosensitive tumor. With cisplatin and etoposide therapy, a complete response (i.e., no sign of tumor following treatment) may be achieved in 70%-80% of patients with germ cell tumors (*1,2*). In patients who are resistant to the combination therapy with cisplatin and etoposide, salvage therapies exist that are based on cisplatin and ifosfamide or high-dose carboplatin, but treatment efficacy is limited with these salvage therapies (*3-5*). The identification of new agents or regimens with antitumor activity remains a priority of investigation for the 20%-30% of patients with cisplatin-resistant germ cell cancer.

The aim of our studies was to assess quantitatively in vitro two promising new antitumor agents (paclitaxel [Taxol] and topotecan) and three more established agents (cisplatin, vincristine, and etoposide). These agents were used singly or in two- and three-drug combinations and represent five distinct categories of antineoplastic mechanisms.

The human teratocarcinoma cell line 833K and its subline, 833K/64CP10, which is relatively resistant to cisplatin, were used for the sulforhodamine B assays (*6*). Synergism or antagonism in chemotherapy was quantitated with the median-effect principle (*7,8*) and the combination index–isobologram method (*9-12*) using a computer software program (*13,14*) for automated analysis and simulation. This method takes into account both the potency of each drug and combinations of these drugs and the shapes of their dose–effect curves. Furthermore, it quantitates synergism or antagonism at different concentrations and at different effect levels (*8,15*).

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*See* "Notes" section following "References."

The prerequisite for determining synergism or antagonism is the clear definition of the "additive effect," since synergism is a more than additive effect and antagonism is a less than additive effect. The data analyses can be greatly facilitated by defining the additive effect with a derived general equation (*8,11*). An additive effect is not the simple arithmetic sum of the effects of two drugs. For example, if drug 1 and drug 2 each inhibit the cell growth by 70% (fraction affected by dose [*fa*] = 0.7), the combined effect (if additive) cannot be 140% inhibition (i.e., exceeding 100% inhibition). The calculation of additive effect by an alternative method, the fractional product method of Webb (*16*), can also lead to erroneous conclusions, since this method uses only a single data point for each drug and does not take into account the shapes of the dose–effect curves (*8-11*).

Contrary to common belief, if two drugs in combination produce an effect greater than that of each drug alone, this result does not necessarily indicate synergism, since each drug plus itself (i.e., an increase in the dose) may also produce a greater effect. When applied to therapy studies, this situation is frequently termed "therapeutic synergy." The superior response rate of remission of cancer by drug combination, for example, simultaneously takes into account the therapeutic end results and the maximum tolerated toxicity. Because of intrinsic complexities, such an evaluation of a drug combination frequently lacks dose–effect parameters and is nonquantitative and subjective. Thus, in therapeutic synergy, "synergism" is, in fact, not determined (*8,17*). As indicated earlier, therapeutic synergy may be a result of real synergy, an additive effect, or even a moderate antagonistic effect when two drugs produce nonoverlapping toxicity (*8*).

In the present studies, we have used a quantitative method to determine synergism or antagonism with up to three drugs in a simple system in vitro. For any synergistic interactions, there must be a dose reduction for the component drug(s) for a given degree of effect, as can be determined by the dose-reduction index (DRI) (*8,14,17*). As a general rule, dose reduction (due to efficacy synergy) always leads to reduction in toxicity and, therefore, may improve overall therapeutic results.

Paclitaxel enhances the polymerization of tubulin to stable microtubules and also interacts stoichiometrically with microtubules in the absence of any cofactors (*18-20*). It blocks cells in the mitotic phase of their cycle, and such cells are unable to replicate normally. Paclitaxel and its derivatives are the only antitumor drugs that are known to stabilize microtubules and are the prototype for a new class of cancer therapeutic agents. Paclitaxel has been shown to be effective against ovarian tumors, breast tumors, and some refractory solid tumors (*21-23*). The dose-limiting toxic effects for paclitaxel are hypersensitivity, neutropenia, and peripheral neuropathy (*24*).

Topotecan is another promising antitumor agent currently under extensive clinical investigation (*25-27*). It is a water-soluble derivative of camptothecin that potently and specifically inhibits DNA topoisomerase type I (*28*). It forms a stable, cleavable ternary complex with a single strand of DNA and possibly acts by aborting the advancing DNA replication fork (*29*). The toxic effects of topotecan in phase I trials were neutropenia, thrombocytopenia, and mucositis.

Of the five drugs we studied, cisplatin is the most active drug for treating germ cell tumors (*30-33*). It acts by interstrand or intrastrand cross-linking of DNA (*34,35*). Its dose-limiting toxicity is nausea, vomiting, and renal toxicity (*30,32*).

Etoposide is a podophyllotoxin derivative (*36*) that inhibits DNA topoisomerase II (*37,38*). It has been shown previously to be effective against germ cell cancer (*32,39*) and against many other solid tumors (*39*). The dose-limiting toxic effects of etoposide are leukopenia, nausea, and vomiting.

Vincristine or its derivative, vinblastine, is a vinca alkaloid that is distinct from paclitaxel and blocks the polymerization of the tubulin-form microtubule. It, along with vinblastine, has been among the mainstays in cancer chemotherapy (*32,40-42*). The dose-limiting toxic effects of vincristine are neurotoxicity and nephropathy (*39*).

The toxic effects of paclitaxel, topotecan, cisplatin, etoposide, and vincristine are, therefore, only partially overlapping.

## Materials and Methods

### Compounds

Paclitaxel (Taxol), topotecan (SK&FS-104864-4), and cisplatin (Platinol) were from the Chemotherapeutic Agents Repository, National Cancer Institute, obtained through ERC BioService Corp., Rockville, Md. Etoposide (Vepesid) and vincristine (Oncovin) were obtained from Sigma Chemical Company, St. Louis, Mo.

### Cells and Cell Culture

Human teratocarcinoma cell lines with relative sensitivity (833K cells) and resistance (833K/64CP10 cells) to cisplatin were supplied by Dr. Bruce J. Roth, Indianapolis, Ind., obtained through Dr. Ethan Dmitrosky of the Memorial Sloan-Kettering Cancer Center (*43*). The cell lines were cultured in an initial density of $4 \times 10^5$ cells/mL. The cells were maintained in a 5% $CO_2$-humidified atmosphere at 37 °C in RPMI-1640 medium (GIBCO BRL, Grand Island, N.Y.) containing penicillin (100 U/mL) and streptomycin (100 µg/mL) (GIBCO BRL) and 10% heat-inactivated fetal bovine serum.

### Cytotoxicity Assay

Teratocarcinoma cells were incubated at 37 °C with five to eight drug concentrations for 4 days. The compounds were evaluated alone and in two-drug or three-drug combinations for their cytotoxic effects. The cytotoxicity of the compounds was determined in 96-well microplates by a method described by Skehan et al. (*6*) for measuring the cellular protein content. Cultures were fixed with trichloroacetic acid and then stained for 30 minutes with 0.4% sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by acetic acid washes, and the protein-bound dye was extracted with an unbuffered Tris base [tris(hydroxymethyl)aminomethane] for determination of optical density at 570 nm in a 96-well microplate reader (model EL340; Bio-Tek Instruments, Inc., Winooski, Vt.).

### Median-Effect Principle for Dose–Effect Analysis

The multiple drug effect analysis of Chou and Talalay (*9-12*), which is based on the median-effect principle (*7,8*), was used to calculate combined drug effects. This method involves plotting of dose–effect curves for each agent and their combinations in multiply diluted concentrations by using the median-effect equation (*8,11*)

$$fa/fu = (D/Dm)^m. \quad [1]$$

In equation 1, $D$ is the dose, $Dm$ is the dose required for 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 (therefore, $fa = 1 - fu$), and $m$ is a coefficient of the sigmoidicity of the dose–effect curve; $m = 1$, $m>1$, and $m<1$ indicate hyperbolic, sigmoidal, and negative sigmoidal dose–effect curves, respectively, for an inhibitory drug. Thus, the method takes into account both the potency ($Dm$)

and shape ($m$) parameters. A rearrangement of equation 1 gives the following:

$$D = Dm[fa/(1-fa)]^{1/m}. \quad [2]$$

The $Dm$ and $m$ values are easily determined by the median-effect plot: $x$ = log ($D$) versus $y$ = log ($fa/fu$), which is based on the logarithmic form of equation 1. In the median-effect plot, $m$ is the slope and log ($Dm$) is the $x$-intercept. The conformity of the data to the median-effect principle can be readily manifested by the linear correlation coefficient ($r$) of the median-effect plot. Computer programs (*13,14*) based on the median-effect plot parameters ($Dm$ and $m$) and the combination index equation (*see below*) have been used for data analysis.

## Combination Index for Determining Synergism and Antagonism

The combination index (CI)–isobologram equation (*8,11*)

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad [3]$$

has been used for data analysis of two-drug combinations. For three-drug combinations, a third term, $(D)_3/(D_x)_3$, is added (*8,14*). CI<1, CI = 1, and CI>1 indicate synergism, additive effect, and antagonism, respectively (*8,11*). Equation 3 dictates that drug 1, i.e., $(D)_1$, and drug 2, i.e., $(D)_2$, in the numerators in combination inhibit $x$%. $(Dx)_1$ and $(Dx)_2$ in the denominators of equation 3 are the doses of drug 1 and drug 2 alone, respectively, that also inhibit $x$%. $Dx$ can be readily calculated from equation 2, where $D$ is designated for $x$% inhibition. When equation 3 equals 1 (i.e., CI = 1), it represents the classic isobologram equation

The DRI represents the fold of dose reduction allowed in a combination (for a given degree of effect) as compared with the dose of each drug alone (*8,14*).

Data were also evaluated by the isobologram technique (*8,11*), a dose-oriented geometric method of assessing drug interactions. This method yields conclusions quantitatively identical to those of the effect-oriented CI method described above. Computer programs (*13,14*) for automated construction of mutually exclusive (conventional) isobolograms and mutually nonexclusive (conservative) isobolograms were used with an IBM-PC computer for the present data analysis. For simplicity, a mutually exclusive assumption has been widely used, since it conforms with the classical isobologram equation (*8,17*).

In the present studies, a three-drug combination with paclitaxel + cisplatin + topotecan and their two-drug component combinations showed stronger synergism than other combinations. We have used this dataset as an example to illustrate data analysis of these combinations.

# Results

## Single-Drug Parameters

The dose–effect relationships of paclitaxel, cisplatin, and topotecan were subjected to the median-effect plot to determine their potency ($Dm$), shape ($m$), and conformity ($r$) in both 833K and 833K/64CP10 teratocarcinoma cells. The pooled results are given in Table 1. The $r$ values were .95 or greater. All five compounds were potent cytotoxic agents with $IC_{50}$ values (i.e., concentrations that cause 50% inhibition of growth) ranging from nanomolar to submicromolar levels. The relative potency against 833K cells was in this order (highest to lowest): paclitaxel, vincristine, topotecan, etoposide, and cisplatin. The relative potency against 833K/64CP10 cells followed the same order. 833K/64CP10 cells were 5.5-fold more resistant to cisplatin than were 833K cells, whereas paclitaxel, topotecan, and etoposide showed a partial cross-resistance in 833K and 833K/64CP10 cells. The $Dm$ and $m$ values for single drugs and for their combination mixtures were used for calculating synergism or antagonism based on the CI equation.

## Two- and Three-Drug Combinations

Among five drugs, seven pairs of two-drug combinations and three-drug combinations were studied. The experiments were repeated two to seven times. The average CI values for both 833K and 833K/64CP10 cells are summarized in Table 2. The combination of cisplatin and topotecan yielded superior synergistic effects in both cell lines at broad concentration ranges (e.g., from $IC_{50}$ to $IC_{95}$). The addition of paclitaxel to this two-drug combination continued to yield desirable synergism. This finding is supported by the fact that the combination of paclitaxel and topotecan also showed a synergistic effect. Other combinations that showed a synergistic effect were the two-drug combination of paclitaxel and etoposide and the three-drug combination of paclitaxel, cisplatin, and etoposide.

The interactions of two- and three-drug combinations are illustrated in Fig. 1. The outcomes with the three-drug combinations could be qualitatively predicted from the outcomes with the two-drug combinations.

As shown in Table 2, the cisplatin-resistant cells (833K/64CP10) consistently showed more synergistic inhibition by various combinations when compared with the parent cells (833K). This greater synergism was particularly prominent at high effect levels (e.g., $IC_{90}$ and $IC_{95}$). The combination of paclitaxel and cisplatin yielded moderate antagonistic effects in 833K cells but synergistic effects in 833K/64CP10 cells.

The following combinations showed nearly additive effects or moderate antagonism: cisplatin + etoposide, paclitaxel + vincristine, cisplatin + vincristine + paclitaxel, and cisplatin + vincristine. The combination of cisplatin and etoposide showed strong antagonism in 833K cells but only moderate antagonism in 833K/64CP10 cells. Some combination experiments were not carried out in 833K/64CP10 cells when the correspond-

**Table 1.** Dose–effect relationship parameters of various agents against the growth of 833K and 833K/64CP10 teratocarcinoma cells in vitro*

| Compound | 833K cells | | | | 833K/64CP10 cells | | | |
|---|---|---|---|---|---|---|---|---|
| | $Dm$, μM | $m$ | $r$ | $n$ | $Dm$, μM | $m$ | $r$ | $n$ |
| Paclitaxel | 0.0018 ± 0.0005 | 1.291 ± 0.165 | .969 ± .009 | 9 | 0.0065 ± 0.0036 | 0.759 ± 0.104 | .969 ± .005 | 4 |
| Cisplatin | 0.387 ± 0.076 | 1.296 ± 0.102 | .986 ± .004 | 8 | 2.120 ± 0.099 | 1.660 ± 0.310 | .984 ± .013 | 4 |
| Topotecan | 0.061 ± 0.016 | 1.807 ± 0.030 | .993 ± .002 | 3 | 0.263 ± 0.010 | 1.581 ± 0.136 | .993 ± .005 | 2 |
| Etoposide | 0.190 ± 0.098 | 1.235 ± 0.379 | .950 ± .028 | 3 | 0.690 ± 0.910 | 0.661 ± 0.036 | .974 ± .009 | 2 |
| Vincristine | 0.0075 ± 0.0008 | 2.154 ± 0.236 | .968 ± .010 | 5 | † | † | † | † |

*The parameters $m$, $Dm$, and $r$ are the slope, antilog of $x$-intercept, and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency ($IC_{50}$), and conformity of the data to the mass-action law, respectively (*7,8,11*); $n$ is the number of sets of dose–effect relationship experiments that were carried out. Values = means ± SEM.

†Not done, since preliminary studies indicated that the combination of vincristine with paclitaxel or of vincristine with cisplatin mainly showed antagonism.

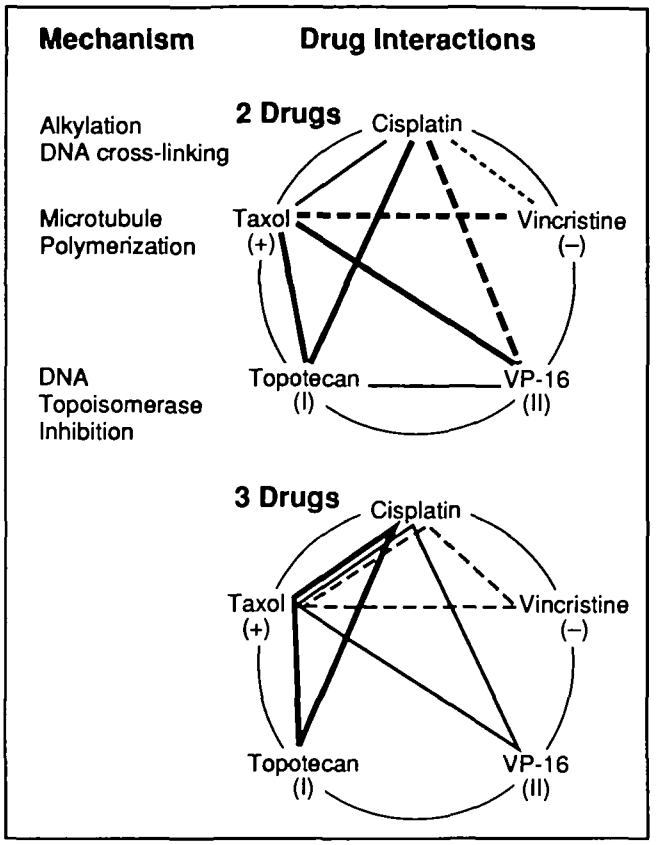


**Fig. 1.** Diagram showing the pharmacologic interactions of the five drugs in two- and three-drug combinations using 833K cells. Two-drug combinations (top) are connected with straight lines, where synergism is represented by solid lines, with heavy line indicating synergism (combination index at 95% inhibition [$CI_{95}$] = 0.1-0.8) and thin line indicating moderate synergism ($CI_{95}$ = 0.8-1.0). Antagonism is represented by broken lines, with heavy line indicating antagonism ($CI_{95}$>2) and thin line indicating moderate antagonism ($CI_{95}$ = 1.1-1.9). Three-drug combinations (bottom) are connected to form triangles, where synergism, antagonism, moderate synergism, and moderate antagonism are designated as above. Note that paclitaxel (Taxol) and vincristine are enhancer and inhibitor, respectively, for microtubule polymerization; topotecan and etoposide (VP-16) are inhibitors of DNA topoisomerases I and II, respectively; and cisplatin is a DNA cross-linking agent.

ing combinations had already shown antagonism in the parent 833K cells.

### Example of Experimental Design and Data Analysis for Two- and Three-Drug Combinations

In the present studies, the combinations of cisplatin and topotecan, of paclitaxel and topotecan, and of paclitaxel, cisplatin, and topotecan showed greater degrees of synergism than all other combinations examined. We have used the data obtained from these two- and three-drug combinations to illustrate the experimental design and the detailed data analysis using computer software (*13,14*) as well as step-by-step manual calculations.

As shown in Table 3, single-drug, dose–effect relationship parameters ($Dm$, $m$, and $r$) were calculated for paclitaxel ($D_1$), cisplatin ($D_2$), and topotecan ($D_3$). For the three-drug combination ($D_1 + D_2 + D_3$), it was desirable also to carry out $D_1 + D_2$, $D_2 + D_3$, and $D_1 + D_3$, since it was not known whether these components were synergistic or antagonistic or both (at different effect levels). The single-drug parameters could be shared for calculating CI values for both two-drug and three-drug combinations and, thus, maximized the efficiency of the study. From the pilot studies of single drugs, the dose ranges were selected to cover the concentrations below and above the $IC_{50}$ values of each drug. The combination ratio was designed to approximate the $IC_{50}$ ratio of the component drugs, so that the contribution of the effect for each drug in the mixture would be about the same (i.e., equipotency ratio), although any other ratios (i.e., nonconstant ratios) could also be used.

Following the entry of data in Table 3 into an IBM-PC using a computer software program (*14*), the dose–effect curves along with the median-effect plots were automatically generated (not shown). The median-effect plots linearized the dose–effect curves with an excellent linear correlation coefficient ($r$). As indicated in the footnote of Table 3, the slope ($m$) of the median-effect plot signifies the shape of the dose–effect curve, whereas

**Table 2.** Combination effects of paclitaxel or cisplatin with other chemotherapeutic agents against 833K and cisplatin-resistant 833K/64CP10 teratocarcinoma cells in two- and three-drug combinations

| Drug combination | CI values* for 833K cells at | | | | CI values* for 833K/64CP10 cells at | | | | Overall ranking† |
|---|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ | $IC_{75}$ | $IC_{90}$ | $IC_{95}$ | $IC_{50}$ | $IC_{75}$ | $IC_{90}$ | $IC_{95}$ | |
| Paclitaxel + cisplatin | 1.22 ± 0.24 | 1.15 ± 0.21 | 1.17 ± 0.23 | 1.26 ± 0.28 | 1.06 ± 0.36 | 0.70 ± 0.18 | 0.61 ± 0.14 | 0.45 ± 0.10 | 6 |
| Paclitaxel + etoposide | 1.10 ± 0.15 | 0.95 ± 0.03 | 0.84 ± 0.05 | 0.77 ± 0.11 | 1.58 | 0.89 | 0.50 | 0.34 | 4 |
| Paclitaxel + vincristine | 1.84 ± 0.62 | 1.77 ± 0.64 | 1.96 ± 0.13 | 2.25 ± 0.96 | — | — | — | — | (9) |
| Paclitaxel + topotecan | 1.17 ± 0.16 | 1.00 ± 0.15 | 0.87 ± 0.13 | 0.79 ± 0.11 | 0.93 ± 0.03 | 0.61 ± 0.08 | 0.43 ± 0.11 | 0.35 ± 0.12 | 3 |
| Cisplatin + etoposide | 1.85 ± 0.33 | 2.12 ± 0.70 | 2.53 ± 1.19 | 2.87 ± 1.58 | 1.10 | 1.05 | 1.12 | 1.26 | (10) |
| Cisplatin + vincristine | 1.14 ± 0.20 | 1.13 ± 0.27 | 1.14 ± 0.32 | 1.16 ± 0.37 | — | — | — | — | (7) |
| Cisplatin + topotecan | 0.66 ± 0.08 | 0.66 ± 0.11 | 0.67 ± 0.14 | 0.67 ± 0.15 | 0.58 ± 0.16 | 0.57 ± 0.22 | 0.58 ± 0.28 | 0.59 ± 0.32 | 1 |
| Paclitaxel + cisplatin + etoposide | 1.28 ± 0.10 | 1.04 ± 0.02 | 0.86 ± 0.03 | 0.77 ± 0.06 | 1.74 ± 0.02 | 1.12 ± 0.11 | 0.82 ± 0.13 | 0.72 ± 0.15 | 5 |
| Paclitaxel + cisplatin + vincristine | 1.18 ± 0.39 | 1.28 ± 0.27 | 1.54 ± 0.03 | 1.84 ± 0.24 | — | — | — | — | (8) |
| Paclitaxel + cisplatin + topotecan | 1.32 ± 0.08 | 0.91 ± 0.13 | 0.63 ± 0.13 | 0.50 ± 0.02 | 0.76 ± 0.10 | 0.67 ± 0.17 | 0.59 ± 0.19 | 0.56 ± 0.21 | 2 |

*CI<1, CI = 1, and CI >1 indicate synergism, additive effect, and antagonism, respectively. Values = means ± SEM; single values without a range are obtained from single drug-combination experiments. In each experiment, four to seven concentrations were used for each drug and each combination drug. Approximated $IC_{50}$ equipotency ratios were used as the drug combination ratios. $IC_{50}$, $IC_{75}$, $IC_{90}$, and $IC_{95}$ = concentration required to inhibit 50%, 75%, 90%, or 95%, respectively.

†Rankings of favorable in vitro drug combination synergism are based on experiments in both 833K and 833K/64CP10 cells. Rankings shown in parentheses are those combinations that showed moderate antagonism.

**Table 3.** Example of experimental design and dose–effect relationships of paclitaxel, cisplatin, and topotecan and their two- and three-drug combinations on growth inhibition of 833K teratocarcinoma cells during 96 hours' exposure*

| Drug, μM | | | | Parameter† | | | |
|---|---|---|---|---|---|---|---|
| Paclitaxel | Cisplatin | Topotecan | Fractional inhibition, *fa* | *m* | *Dm*, μM | *r* | CI‡ |
| $(D)_1$ | | | | | | | |
| 0.002 | | | 0.429 | | | | |
| 0.004 | | | 0.708 | | | | |
| 0.005 | | | 0.761 | | | | |
| 0.01 | | | 0.882 | | | | |
| 0.02 | | | 0.932 | 1.248 | 0.00217 | .990 | |
| | $(D)_2$ | | | | | | |
| | 0.05 | | 0.055 | | | | |
| | 0.1 | | 0.233 | | | | |
| | 0.2 | | 0.301 | | | | |
| | 0.5 | | 0.559 | | | | |
| | 1.0 | | 0.821 | | | | |
| | 2.0 | | 0.953 | 1.458 | 0.320 | .986 | |
| | | $(D)_3$ | | | | | |
| | | 0.01 | 0.069 | | | | |
| | | 0.02 | 0.213 | | | | |
| | | 0.05 | 0.373 | | | | |
| | | 0.1 | 0.785 | | | | |
| | | 0.2 | 0.940 | | | | |
| | | 0.5 | 0.991 | 1.847 | 0.046 | .991 | |
| $(D)_1 + (D)_2$ (1:100)§ | | | | | | | |
| 0.001 | 0.1 | | 0.450 | | | | 0.900 |
| 0.002 | 0.2 | | 0.701 | | | | 0.815 |
| 0.005 | 0.5 | | 0.910 | | 0.000115 | | 0.681‖ |
| | | | | | + | | |
| 0.01 | 1.0 | | 0.968 | 1.572 | 0.1147 | .999 | 0.602 |
| | $(D)_2 + (D)_3$ (100:10) | | | | | | |
| | 0.05 | 0.005 | 0.304 | | | | 0.445 |
| | 0.1 | 0.01 | 0.413 | | | | 0.658 |
| | 0.2 | 0.02 | 0.675 | | | | 0.669 |
| | 0.5 | 0.05 | 0.924 | | 0.1053 | | 0.561 |
| | | | | | + | | |
| | 1.0 | 0.1 | 0.977 | 1.588 | 0.0105 | .989 | 0.522 |
| $(D)_1$ | + | $(D)_3$ (1:10) | | | | | |
| 0.001 | | 0.01 | 0.274 | | | | 1.373 |
| 0.002 | | 0.02 | 0.579 | | | | 1.078 |
| 0.005 | | 0.05 | 0.901 | | 0.0017 | | 0.719 |
| | | | | | + | | |
| 0.01 | | 0.1 | 0.965 | 1.891 | 0.0166 | .998 | 0.681 |
| | $(D)_1 + (D)_2 + (D)_3$ (1:100:10) | | | | | | |
| 0.001 | 0.1 | 0.01 | 0.456 | | | | 1.121 |
| 0.002 | 0.2 | 0.02 | 0.806 | | 0.00116 | | 0.729 |
| | | | | | + | | |
| 0.003 | 0.3 | 0.03 | 0.947 | | 0.11614 | | 0.403 |
| 0.005 | 0.5 | 0.05 | 0.995 | 3.363 | 0.01161 | .984 | 0.136 |

*Incubation conditions are described in the "Materials and Methods" section.

†The parameters *m*, *Dm*, and *r* are the slope, antilog of *x*-intercept, and the linear correlation coefficient of the median-effect plot, which signifies the shape of the dose–effect curve, the potency ($IC_{50}$), and conformity of the data to the mass-action law, respectively (*7,8,11*). *Dm* and *m* values are used for calculating the CI values.

‡CI<1, CI = 1, and CI>1 indicate synergism, additivity, and antagonism, respectively. As based on the classic isobologram equation, CI can be calculated by equation 3: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2]$, where $D_x = Dm[fa/(1-fa)]^{1/m}$.

§Drug mixture was serially diluted and added to incubation mixture at 0 hour. The combination ratio was approximately equal to the *Dm* ratio of the component drugs (i.e., close to their equipotency ratio).

‖Sample calculation for the CI value of 0.005 μM paclitaxel + 5 μM cisplatin that inhibited 833K cell growth by 91.0% (*fa* = 0.910). On the basis of equation 2, for paclitaxel alone to inhibit cell growth by 91% would require $[D_{0.91}]_{paclitaxel} = (D_m)_{paclitaxel}[0.91/(1-0.91)]^{1/1.248} = 0.00217\ \mu M \times 6.385 = 0.01385\ \mu M$ and for cisplatin alone to inhibit cell growth by 91% would require $[D_{0.91}]_{cisplatin} = (D_m)_{cisplatin}[0.91/(1-0.91)]^{1/1.458} = 0.320\ \mu M \times 4.888 = 1.564\ \mu M$. Therefore,

$$CI = \frac{0.005\ \mu M}{0.01385\ \mu M} + \frac{0.5\ \mu M}{1.564\ \mu M} = 0.681$$

at 91% inhibition. Computer software (*13,14*) was used on IBM-PC for automated calculation and simulation.

the $x$-intercept gives the $Dm$ value (i.e., $IC_{50}$). By using the $m$ and $Dm$ values and equation 3, the CI was then calculated (*see* sample calculation in the footnote of Table 3). The computer simulated the CI values at various effect levels to generate the CI plots (not shown) and the CI tables (used in Tables 2-4). CI<1, CI = 1, and CI>1 indicate synergism, additive effect, and antagonism, respectively. The isobolograms for each pair of combination could also be automatically generated (not shown). Both *fa*–CI plot (effect-oriented) and isobologram (dose-oriented) should yield the same conclusion in terms of synergism or antagonism (*8*).

The computer-simulated CI and DRI values at $IC_{50}$, $IC_{75}$, $IC_{90}$, and $IC_{95}$ are given in Table 4. These values could be quite different at different effect levels. Paclitaxel + cisplatin and paclitaxel + topotecan showed the greatest overall synergism, but paclitaxel + cisplatin + topotecan showed the greatest synergism at high effect levels (e.g., $IC_{90}$ and $IC_{95}$). By contrast, cisplatin + topotecan showed moderate synergism.

The concentrations of each drug in a synergistic combination required to achieve a given degree of inhibition (e.g., $IC_{50}$, $IC_{75}$, $IC_{90}$, or $IC_{95}$) could be reduced when compared with the concentration required for each drug alone. The dose reductions for the two-drug combinations as indicated by DRI values ranged from 1.3-fold to 4.4-fold (Table 4). For the three-drug combinations, the DRI tended to be higher, as expected. DRI values greater than 1 may lead to reduced toxicity toward normal tissue while maintaining therapeutic efficacy due to synergism.

## Discussion

Clinical trials of combination therapy against cancer are frequently conducted empirically in the absence of supporting experimental data. However, phase I clinical trials for single drugs are always preceded by extensive pharmacologic, toxicologic, and efficacy studies. In most cases, the results of drug combinations are not predictable; thus, a combination of two drugs, in essence, recreates a third drug with some inherent features. We believe that some supporting data should be provided in directing drug combinations of clinical trials.

The purpose of the present studies was to conduct in vitro drug combination studies prior to their investigation in clinical trials, especially for new drugs. These studies were carried out by quantitative analysis of synergism or antagonism at different drug doses and different effect levels. As the present report illustrates, the prospective studies in vitro were carried out and they provide much of the essential information for rational protocol design.

The median-effect equation and the CI method, derived from the principle of the mass-action law (*7,9,10*) through mathematical induction and deduction in enzyme kinetic models, have been subjected to many extensive reviews (*8,11,12,15*). As registered in the Citation Index (Science Information Service, Philadelphia, Pa., 1987-1993), they have been used not only for anticancer and anti-human immunodeficiency virus agents but also for antimicrobial agents, the purging of leukemia cells for autologous bone marrow transplantation, and the multiple immunosuppressants for organ transplants (*8,11,15*) using the computer software (*13,14*). To date, scientific papers published in more than a hundred biomedical journals and many monographs have used these methods. Therefore, the methods for dose–effect analysis used in the present studies are considered generally applicable in biological systems, which are manifested by the $r$ values.

The scope of the present studies has not included the schedule dependence of synergism. This aspect has been examined in detail in other studies (*8,17*). The determination of synergism or antagonism was based on the principle of the mass action and its related equations; this determination does not require the knowledge of mechanisms of action. In many drug combinations, each drug may have more than one mode of action, and synergism may be due to reasons other than the mechanism of action per se, such as membrane transport, drug resistance reversal, interference of metabolic activa-

**Table 4.** Sample illustration of computer-simulated CI and DRI values for paclitaxel, cisplatin, and topotecan combinations at 50%, 75%, 90%, and 95% inhibition of 833K cell growth*

| Drug combination | Combination ratio | CI values† at inhibition of | | | | DRI values‡ at inhibition of | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 50% | 75% | 90% | 95% | 50% | 75% | 90% | 95% |
| Paclitaxel + cisplatin | 1:100 | 0.888<br>(1.077) | 0.781<br>(0.931) | 0.690<br>(0.808) | 0.635<br>(0.736) | 1.89<br>2.79 | 2.27<br>2.95 | 2.72<br>3.11 | 3.07<br>3.23 |
| Cisplatin + topotecan | 10:1 | 1.125<br>(1.400) | 0.921<br>(1.122) | 0.770<br>(0.916) | 0.689<br>(0.807) | 1.30<br>2.79 | 1.76<br>2.83 | 2.37<br>2.87 | 2.91<br>2.90 |
| Paclitaxel + topotecan | 1:10 | 0.556<br>(0.631) | 0.560<br>(0.637) | 0.567<br>(0.647) | 0.574<br>(0.656) | 3.04<br>4.40 | 3.23<br>4.00 | 3.44<br>3.63 | 3.58<br>3.39 |
| Paclitaxel + cisplatin + topotecan | 1:100:10 | 1.149<br>(1.189) | 0.736<br>(0.750) | 0.478<br>(0.482) | 0.359<br>(0.361) | 1.87<br>2.76<br>3.99 | 3.25<br>4.22<br>5.22 | 5.65<br>6.47<br>6.83 | 8.23<br>8.65<br>8.20 |

*Incubation was carried out for 96 hours under conditions described in the "Materials and Methods" section. Data analysis was carried out for parameters ($Dm$ and $m$) and statistics ($r$) by using a computer software program (*13,14*) as shown in Table 2.

†The first entry of CI values is based on the classic isobologram equation (equation 3). The CI values shown in parentheses are based on a proposed conservative isobologram, which has the third term consisting of the product of the first two terms of equation 3.

‡DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone (*14*). All DRI values are calculated on the basis of the classic isobologram equation and assumptions (*8,11*).

tion, or inactivation (*8*). The present studies emphasize the quantitative end results of drug combinations rather than the mechanism of synergistic or antagonistic interactions. However, the antagonism results obtained for paclitaxel and vincristine with CI values of 1.84, 1.77, 1.96, and 2.25 at $IC_{50}$, $IC_{75}$, $IC_{90}$, or $IC_{95}$, respectively, in 833K cells may be related to the mechanisms per se, since paclitaxel is known to promote tubulin polymerization, whereas vincristine blocks it (Fig. 1).

Our results and conclusions are valid to the experimental system used and the experimental conditions selected. Despite these conditions, we believe that the methods used in the present studies provide a useful approach for the rational clinical protocol design. This report provides the first example that the combination of three anticancer drugs can be analyzed by the quantitative CI method. Among the five drugs selected in the present studies, cisplatin, etoposide, and vincristine have demonstrated utility in the chemotherapy of teratocarcinoma, and two drugs (paclitaxel and topotecan) are potentially new candidates for chemotherapeutic explorations. A phase II trial of paclitaxel in this institution has shown that this drug has antitumor activity in patients with cisplatin-resistant germ cell tumors; however, topotecan as a single agent is currently being studied. The present studies indicate that the study of both paclitaxel and topotecan in clinical trials for teratocarcinoma is highly warranted.

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## Notes

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# Allelic Loss at Chromosomes 3p, 8p, 13q, and 17p Associated With Poor Prognosis in Head and Neck Cancer

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***Background:*** **Little is known about the molecular genetic events that contribute to the pathogenesis of squamous cell carcinoma of the upper aerodigestive tract. Previous molecular genetic studies have been limited to the identification of mutations of the p53 (also known as TP53) tumor suppressor gene, activation of a limited set of oncogenes, allelic loss at 3p and other locations, and occasional association with human papillomavirus infection.** ***Purpose:*** **Our purpose was to screen tumor tissue and blood from patients with squamous cell carcinoma of the upper aerodigestive tract for loss of heterozygosity at polymorphic loci corresponding to each of the autosomal chromosomes and to identify the locations of additional putative tumor suppressor genes, other than RB (also known as RB1) and p53, that may contribute to the pathogenesis of this disease.** ***Methods:*** **Tumor tissue and blood were obtained from 68 consecutive patients with squamous cell carcinoma of the upper aerodigestive tract. In all cases, tumor tissue was obtained from the center of the surgical specimen. The relative absence of non-neoplastic tissue was confirmed by frozen-section histologic examination of immediately adjacent tissue. Initially, 30 paired tissue and blood samples were tested for loss of heterozygosity by polymerase chain reaction (PCR) to amplify 43 different highly polymorphic sequences containing small oligonucleotide repeats. After PCR amplification, with unique oligonucleotides flanking the repeat, visualization and sizing of the alleles on DNA sequencing gels were performed. Specific loss of heterozygosity was distinguished from random genetic loss due to generalized chromosomal instability if it occurred in more than 20% of specimens tested for a particular marker.** ***Results:*** **Significant loss of heterozygosity (>20%) occurred at alleles at chromosome bands 3p21 (32%), 3p25-26 (56%), 8pter-21.1 (31%), 13q14 (27%), and 17p12 (45%). Loss of heterozygosity at more than two loci was significant with a poor prognosis ($P$ = .039).** ***Conclusions:*** **These findings demonstrate that squamous cell carcinoma of the upper aerodigestive tract exhibits genetic alterations at multiple loci and that allelic loss at more than two locations is indicative of a poor prognosis (the likelihood of the patient dying of disease).** ***Implications:*** **While tumor suppressor genes at 3p (VHL), 13q (RB), and 17p (p53) have been identified, altered genes at other loci on 3p and on 8p have not yet been characterized. Furthermore, the genotype at these loci for squamous cell carcinoma of the upper aerodigestive tract has prognostic importance and may identify the patients who should receive the most aggressive treatment. [J Natl Cancer Inst 86:1524-1529, 1994]**

More than 500 000 new cases of squamous cell carcinoma of the upper aerodigestive tract occur worldwide annually (*1*). In 1992, there were 42 800 new cases in the United States and 11 600 people died of this disease (*2*). More than 90% of these tumors occur in individuals who smoke tobacco and/or ingest alcohol (*3*).

While there has been an explosion of new knowledge about genetic alterations of oncogenes and tumor suppressor genes in human cancer, studies of squamous cell carcinoma of the upper aerodigestive tract have been limited (*4,5*). Investigators in our laboratory and in others have identified mutations of the p53 (also known as TP53) tumor suppressor gene in these tumors (*6-10*). In a small number of cases, infection of squamous cell carcinoma of the upper aerodigestive tract cells with human papillomavirus (serotypes 16 and 33) has been reported; the E6 and E7 transforming genes of these viruses interact with, and potentially inactivate, the p53 and RB tumor suppressor genes (*11*). Amplification and overexpression of the MDM2 gene, an alternative mechanism of p53 inactivation, were not detected in these tumors (*12*). Other sporadic reports have identified alterations of EGFR, c-myc, ras, int-2, hst-1, bcl-1, prad-1, and E-cadherin (*4,5*). Cytogenetic studies (*13,14*) of squamous cell carcinoma of the upper aerodigestive tract demonstrated deletions in chromosome regions 1p, 3p, 7q, 9p, 11q, and 17p. Recent allelotyping studies of these tumors identified loss of heterozygosity on chromosome 3 (*15*), chromosome 9 (*16*), and multiple other regions (*17,18*).

Loss of heterozygosity is a frequent mechanism of inactivation of tumor suppressor genes where one allele is already altered. Frequent allelic loss at specific loci in both hereditary and sporadic tumors may therefore indicate the location of putative tumor suppressor genes (*19*). In squamous cell carcinoma of the upper aerodigestive tract, we previously detected increased loss of heterozygosity

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