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ORIGINAL ARTICLE

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In vitro interactions of a new derivative of spicamycin, KRN5500, and other anticancer drugs using a three-dimensional model

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Abstract *Purpose*: KRN5500 is a new derivative of spicamycin produced by *Streptomyces alanosinicus* and is known to have a wide range of antitumor activities against human cancer cell lines. Because of its unique structure, this compound seems to have a different mode of action from other antitumor drugs and nonoverlapping toxicities. Therefore, KRN5500 is expected to be a suitable candidate for combination chemotherapy. *Methods*: We investigated the effects of combinations of KRN5500 and other anticancer drugs on the growth of a human non-small-cell lung cancer cell line, PC14, using a revised three-dimensional model. *Results*: Synergism was observed when KRN5500 and cisplatin were combined at concentrations in the ranges 0.005 to 0.25 μg/ml and 0.025 to 0.25 μg/ml, respectively. In combination with carboplatin, an analog of cisplatin, and etoposide, a marked synergistic interaction was also found. *Conclusion*: These results suggest the usefulness of combinations of KRN5500 with cisplatin, carboplatin or etoposide for chemotherapy for non-small-cell lung cancer.

Key words Spicamycin analog · KRN5500 · Combination effect · Synergism · 3-D model analysis

Introduction

The antibiotic spicamycin, isolated from the culture broth of a streptomycete, *Streptomyces alanosinicus* 879-MT3, is a potent inducer of differentiation of human promyelocytic leukemia cells (HL60) and M1 myeloid leukemia cells [8, 9]. The compound, which is composed of a long-chain fatty acid, glycine, aminoheptose, and adenine, is probably the 2′ epimer of septacidin, an antitumor and antifungal antibiotic [1]. During studies with purified semisynthetic spicamycin derivatives, KRN5500 (6-[4-deoxy-4-(2E, 4E)-tetradecadienoylglycyl]amino-L-glycero-β-L-mannoheptopyranosyl] amino-9H-purine, Fig. 1), differing in the chain length of the fatty acid moiety has been found to exhibit marked antitumor activity in human tumor xenograft models of various human stomach, breast and lung cancer cell lines [10, 15]. Kamishohara et al. have reported that KRN5500 exerts its cytotoxicity by inhibiting protein synthesis in P388 cells [11]. Further, Burger et al. have observed that KRN5500 alters glycoprotein processing [2]. Therefore, the mechanism of the toxicity of KRN5500 might be different from that of other antitumor drugs. Based on empirically demonstrated antitumor activity and favorable toxicological and pharmaceutic properties, a clinical study of this compound is now in progress in Japan and the United States.

In cancer chemotherapy the therapeutic effect of almost all anticancer drugs with few exceptions is in general limited when they are used as single agents, and it can be difficult to continue chemotherapy because of cumulative toxicity of the drug or drug resistance. Combination chemotherapy seems to conquer those problems. In patients with advanced non-small-cell lung cancer (NSCLC), currently available single agents including cisplatin, ifosfamide, vinblastine, vindesine, mitomycin C, irinotecan, paclitaxel, docetaxel, gemcitabine, navelbine and etoposide have demonstrated a maximum response rate of 15–30%, whereas combination chemotherapy can yield responses of more than 40% in selected patients [6, 17].

KRN5500 seems to be a suitable candidate for combination chemotherapy for the reasons mentioned above, such as different modes of action, responses as a single agent, and nonoverlapping toxicities.

We performed this study to determine the interactions between KRN5500 and other anticancer drugs and

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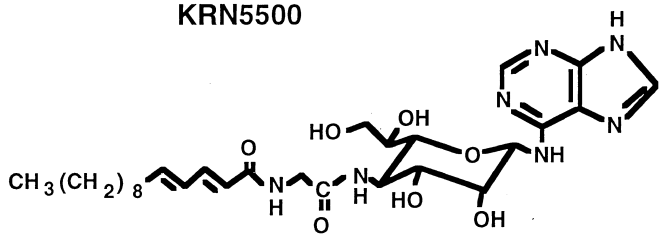


Fig. 1 Structural formula of KRN5500

to select the most favorable drug among them for combination with KRN5500 using a three-dimensional (3-D) model and a statistical method to evaluate the combination effects.

Materials and methods

Materials

KRN5500 was provided by Kirin Brewery Co. (Tokyo, Japan). Cisplatin, carboplatin and etoposide were gifts from Bristol Myers Squibb Japan (Tokyo). Adriamycin was purchased from Kyowa Hakko Kogyo Co. (Tokyo). Irinotecan, vinorelbine and vindesine were obtained from Daiichi Pharmaceutical Co. (Tokyo), Kyowa Hakko Kogyo Co. (Tokyo), and Shionogi Pharmaceutical Co. (Osaka, Japan), respectively. RPMI-1640 medium (Gibco-BRL) and fetal bovine serum were purchased from Nissui (Tokyo).

Cell line and culture

The cell line used in this study was the human NSCLC cell line, PC-14, which was kindly provided by Professor Y. Hayata (Tokyo Medical College, Tokyo). The cells were propagated in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum, and 100 μg streptomycin and 100 units penicillin per ml in an incubator under a humidified atmosphere of 5% CO_2 and air, as described previously [13].

Antiproliferative activity

We measured the inhibition of cell proliferation as the antitumor activity after continuous drug exposure, using a previously described regrowth assay [13]. Briefly, 10-ml culture flasks, initially containing 1.5×10^4 cells/ml of medium and various concentrations of the drugs, were incubated for 6 days, after which the cells were counted with a TOA Microcell counter CC-108 (TOA Medical Electronics Co., Kobe, Japan), and the cell proliferation ratio ($f_u = 1-f_a$) of treated to control cultures was calculated as

$$\mathrm{T/C}\ (\%) = \frac{\text{number of treated cells on day 6/number of treated cells on day 0}}{\text{number of control cells on day 6/number of control cells on day 0}} \times 100$$

The IC_{50} was defined as the drug concentration required for a 50% reduction in the number of cells. Four or five independent experiments were carried out for each dose.

Combination effect analysis

Combination index method

Using the median-effect method described by Chou and Talalay [3], the dose-effect curve was plotted for each agent and for multiple dilutions of a fixed-ratio combination using the equation

$$f_a/f_u = (D/D_m)^m \quad (1)$$

In this equation, D is the dose administered, D_m is the dose required for 50% inhibition of growth, f_a is the fraction affected by dose D, f_u is the unaffected fraction, and m is a coefficient denoting the sigmoidicity of the dose-effect curve. A combination index (CI) equation was used for mutually nonexclusive inhibitors with (f_a) and (f_u) subscripts designating the drug or combination used:

$$\frac{(f_a)_{A,B}}{(f_u)_{A,B}} = \frac{(f_a)_A}{(f_u)_A} + \frac{(f_a)_B}{(f_u)_B} + \alpha\frac{(f_a)_A(f_a)_B}{(f_u)_A(f_u)_B} \quad (2)$$

In this equation, $(f_a)_A$, $(f_a)_B$ and $(f_a)_{A,B}$ are the fractions affected by drug A and drug B, and their combination, respectively, and $\alpha = 0$ for mutually exclusive drugs and $\alpha = 1$ for mutually nonexclusive drugs.

Since $(f_u)_{A,B} = 1 - (f_a)_{A,B}$, $(f_u)_A = 1 - (f_a)_A$, and $(f_u)_B = 1 - (f_a)_B$, the following relationship can be obtained from Eq. 1:

$$\mathrm{CI} = \frac{(D)_A}{(D_x)_A} + \frac{(D)_B}{(D_x)_B} + \alpha\frac{(D)_A(D)_B}{(D_x)_A(D_x)_B} \quad (3)$$

where $(D_x)_A$ is the dose of drug A required to produce x% effect alone and $(D)_A$ is the dose of drug A required to produce the same x% effect in combination with drug B; similarly, $(D_x)_B$ is the dose of drug B required to produce x% effect alone and $(D)_B$ is the dose of drug B required to produce the same x% effect in combination with drug A. Theoretically, CI is the ratio of the combination dose to the sum of the single-agent doses at an isoeffective level. Consequently, CI values of <1 indicate synergism, values of >1 show antgonism, and values of 1 indicate an additive effect [3].

Isobologram method

The effects of KRN5500 in combination with other agents at the IC_{50} point were analyzed by the isobologram method described by Drewinko et al. [5]. The isobologram for representing the isoeffective graph of the combination of two drugs at various doses was previously proposed by Loewe [16] for analyzing the synergism, additivity or antagonism of effects. It may be seen from Eqs. 2 and 3 that, for the special case of $(f_a)_{A,B} = 0.5$, the above relationships are all equal to 1 and hence the magnitude of the values of m (i.e. the sigmoidicity of the dose-effect curve) for the drugs is irrelevant. For instance, Eq. 2 or 3 (for mutually exclusive drugs) become the equation which describes Loewe's isobologram:

$$\mathrm{CI} = \frac{(D)_A}{(D_x)_A} + \frac{(D)_B}{(D_x)_B} \quad (4)$$

Data were also evaluated at the biological effect levels of IC_{20} and IC_{80}. Isoeffect curves were also produced at levels of 20% and 80% growth inhibition.

Three-dimentional model analysis

We have established a 3-D analytical method, which compensates for the many deficiencies of 2-D models, as a practical new tool for clinical investigators to use for determining favorable drugs to use in combination with a new drug [12, 14]. Previous 3-D models have a tendency to under- or overestimate combination effects by reason of a fixed variance as a standard of evaluation.

The theoretical basis of the 3-D model was the median-effect method described by Chou and Talalay described previously [3]. In the case of the combination of KRN5500 and other drugs, $\alpha = 1$ was adopted, because the other drugs are known to act by different mechanisms. The following equation is obtained from eq. 2.

$$\frac{(f_a)_{A,B}}{1-(f_a)_{A,B}} = \frac{(f_a)_A}{1-(f_a)_A} + \frac{(f_a)_B}{1-(f_a)_B} + \frac{(f_a)_A(f_a)_B}{\{1-(f_a)_A\}\{1-(f_a)_B\}} \quad (5)$$

Fig. 2 Three-dimensional analysis of the antitumor interaction between KRN5500 and cisplatin (CDDP), including the stages in the data transformation required to produce the synergy plots. Cells were treated with KRN5500 and cisplatin alone or in combination at a fixed concentration ratio. The experimental dose-response surface is represented in **D.** This surface is not convex as the drug concentrations are plotted on a log scale. Two matrices representing the cytotoxic effects of KRN5500 (**B**), and cisplatin (**A**) alone were used to calculate the theoretical additive effect using the dissimilar site assumption. The resulting calculated additive surface (**C**) was then subtracted from the observed surface (**D**), yielding the difference between the calculated and observed effect plot in surface form (**E**) and contour plot form (**F**). Statistically significant synergy and antagonism are represented by the blue and red regions in **G**, respectively. No statistically significant synergy or antagonism, i.e. more or less than the additive effect, was seen as shown by the diagonal lines

finding a common denominator and simplifying yields

$$(f_a)_{A,B} = (f_a)_A + (f_a)_B - (f_a)_A(f_a)_B \quad (6)$$

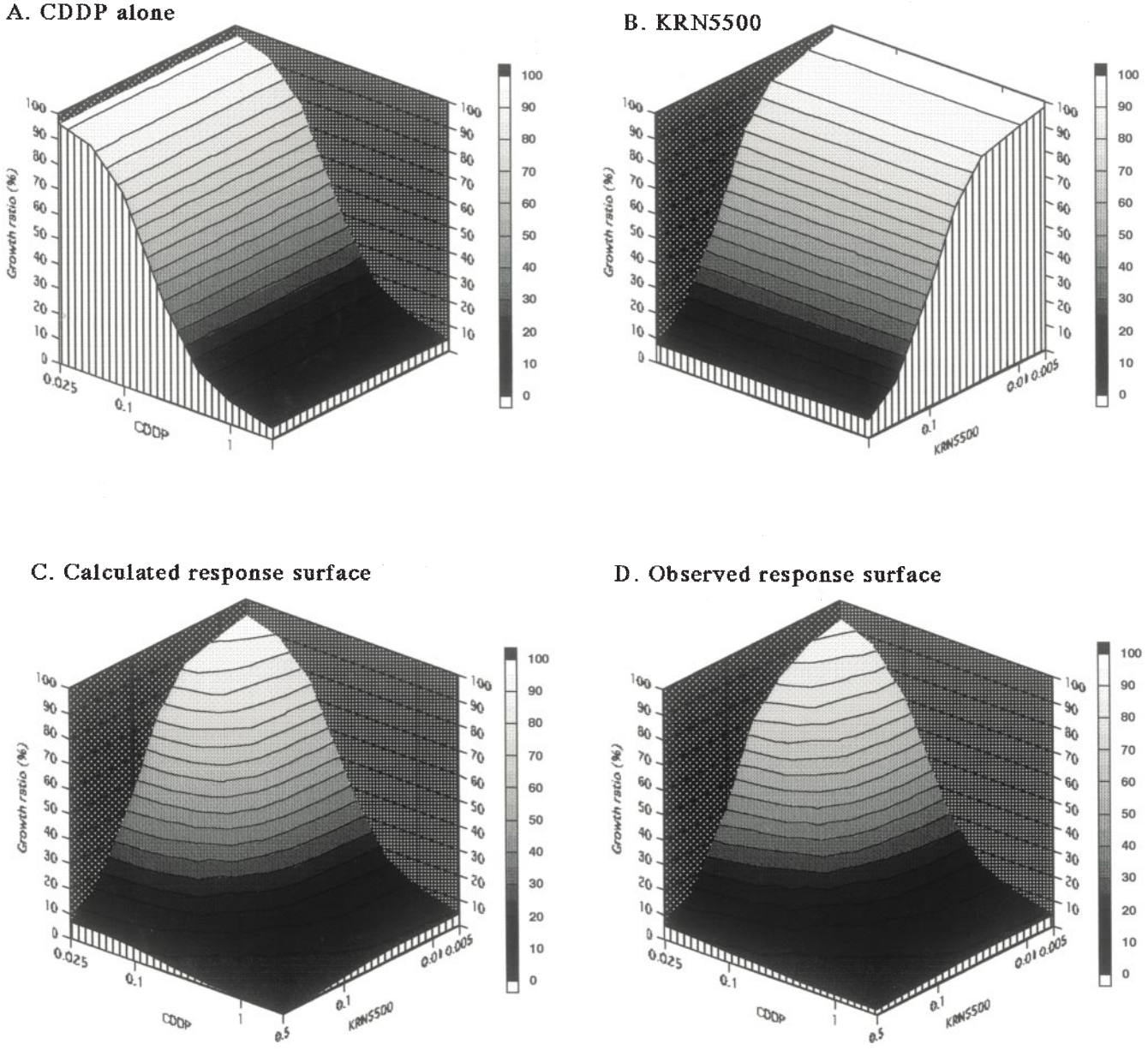
Here $(f_a)_{A,B}$ is defined as theoretical additivity (TA), since Eq. 2 was designated from the TA:

$$TA = (f_a)_A + (f_a)_B - (f_a)_A(f_a)_B \quad (7)$$

Integrating with respect to concentrations of $(f_a)_A$ and $(f_a)_B$, the resulting calculated response surface $(S_{A,B})_{cal}$ can be simulated as shown in Fig. 2C.

$$(S_{A,B})_{cal} = \int\int TA \quad (8)$$

In practice, cytotoxicity data obtained from the experiments were directly entered into a Microsoft Excel spreadsheet (Microsoft Corporation, Redmond, Wash.) with the linearized data matrix of



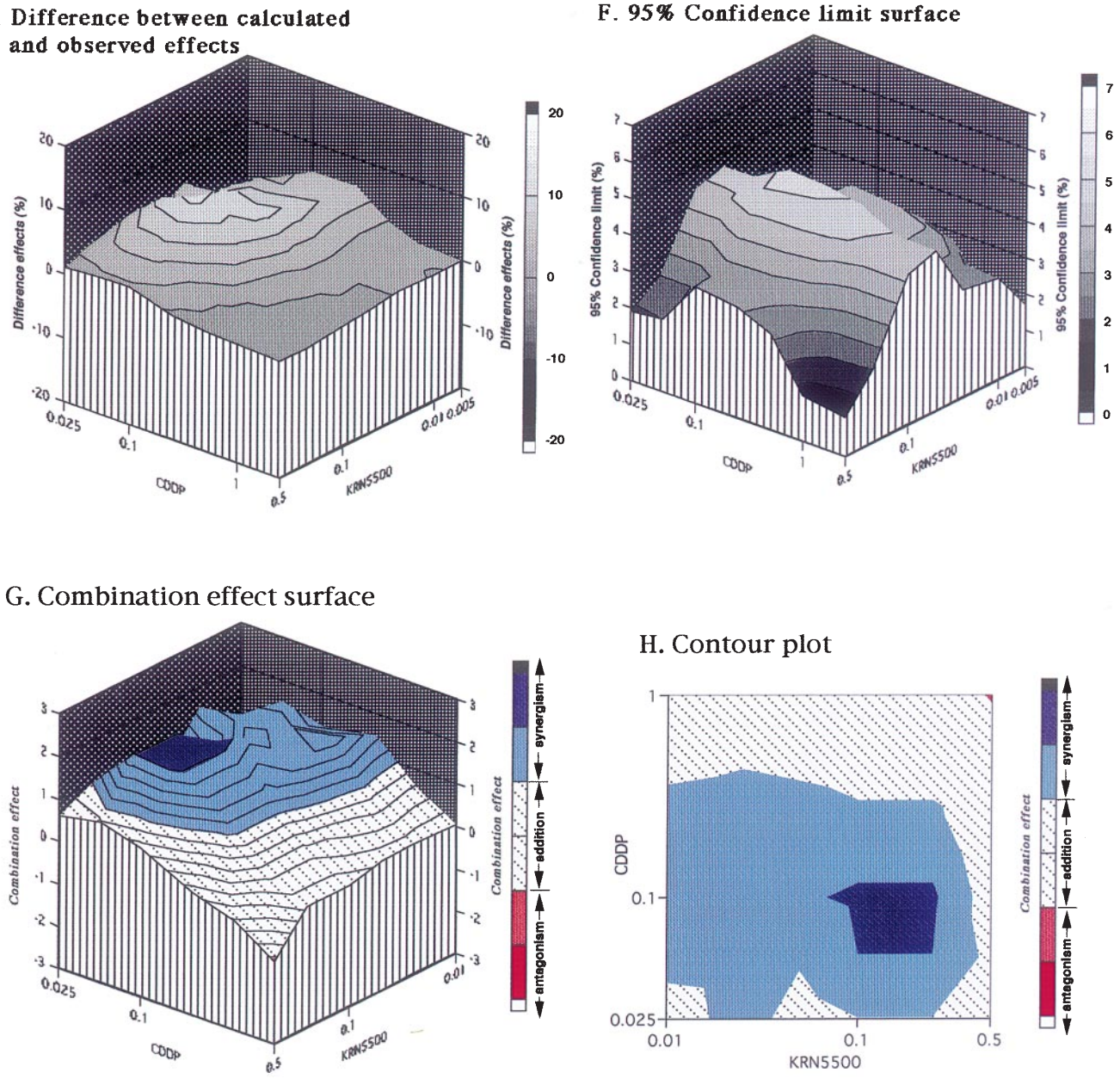


Fig. 2 (*Contd.*)

Delta Graph Pro (Systat-soft, Deltapoint, Monterey, Calif.), on a Macintosh computer (Apple Computers, Cupertino, Calif.), thus generating a dose-response surface, that is, the observed response surface, $(S_{A,B})_{obs}$, as shown in Fig. 2D.

The $(S_{A,B})_{obs}$, is subsequently subtracted from the above $(S_{A,B})_{cal}$ to reveal the surface of difference between two effects, $(S_{A,B})_{DE}$.

$$(S_{A,B})_{DE} = (S_{A,B})_{obs} - (S_{A,B})_{cal} \tag{9}$$

The observed combination effect can be defined as synergy or antagonism when it is greater or less than the calculated combination effect with a statistically significant difference, respectively. To determine the significance, the 95% confidence limits, $(CL_{A,B})_{95\%}$, of each data point were estimated from the variance, S_A^2 and S_B^2 of drug A and B used alone, respectively, by the following equation:

$$(CL_{A,B})_{95\%} = t_\alpha^{df} \sqrt{\frac{S_A^2(n_A - 1) + S_B^2(n_B - 1)}{(n_A + n_B)(n_A + n_B - 2)}} \tag{10}$$

Where t_α^{df} is the t-value (df $= n-1$, n is number of tests, $\alpha = 0.05$). The 95% confidence limit surface, $(S_{A,B})_{CL}$, is obtained by integrating with respect to concentrations of drug A and B, from the following equation:

$$(S_{A,B})_{CL} = \int\int (CL_{A,B})_{95\%} \tag{11}$$

The surface of difference between observed and calculated effects, $(S_{A,B})_{DE}$, is divided by the 95% confidence limits surface, $(S_{A,B})_{CL}$, to reveal the combination effect surface, $(S_{A,B})_{CE}$, for the reason that the true combination effect is evaluated when $(S_{A,B})_{DE}$ exceeds $(S_{A,B})_{CL}$.

$$(S_{A,B})_{CE} = \frac{(S_{A,B})_{DE}}{(S_{A,B})_{CL}} \tag{12}$$

Table 1 Inhibitory concentrations at the 50% level (IC_{50} values) of anticancer drugs tested and the concentration ranges tested in the combination study

Drug	IC_{50} (μg/ml)	Concentration of ranges (μg/ml)
KRN5500	0.091	0.005–0.25
Cisplatin	0.18	0.025–2.5
Etoposide	0.38	0.025–2.5
Adriamycin	0.017	0.001–0.025
Vindesine	0.0022	0.00025–0.005
Carboplatin	21	0.25–10
Irinotecan	0.65	0.1–2.5
Vinorelbine	0.0033	0.0001–0.005
Paclitaxel	0.0015	0.0001–0.0025

Table 2 Combination effect (expressed as percent of control) of KRN5500 and cisplatin on PC-14 cells. Numbers in the rectangle are the data for combination effects. Each data shows the means of four or five experiments

Concentration of CDDP (μg/ml)	Concentration of KRN5500 (μg/ml)							
	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5
0	100.0	97.5	94.7	90.0	75.0	46.2	16.7	7.0
0.025	96.2	93.1	90.1	81.6[b]	69.8	40.1	13.2	5.6
0.05	91.8	86.7[a]	82.2	77.0	63.5	33.6	10.8	4.9
0.1	77.6	70.2	68.7[a]	62.8	48.5	25.2[b]	8.3	3.8
0.25	38.1	31.4	30.5	26.7[a]	19.8	11.3	5.2[b]	3.1
0.5	16.9	14.4	14.0	12.5	10.1[a]	6.7	3.7	2.3[b]
1	9.1	8.7	8.5	8.0	6.6	4.8[a]	2.8	2.0
2.5	4.6	4.4	4.4	4.0	4.0	3.3	2.6[a]	2.0

[a] Data at a KRN5500: cisplatin dose ratio of 1:10 used in the combination index analysis
[b] Data at a KRN5500: cisplatin dose ratio of 1:1 used in the combination index analysis

If it is more than 1 or less than −1, the effect is defined as synergism or antagonism, respectively. On the other hand, $(S_{A,B})_{CE}$ between −1 and 1, not being significant, is defined as additivity.

Results

Antitumor effects of KRN5500 and other drugs on the growth of NSCLC PC-14 cells

KRN5500 and the other anticancer drugs tested produced significant growth inhibition against the human NSCLC cell line, PC-14. As shown in Fig. 3, KRN5500 inhibited the growth ratio to 97.5%, 94.7%, 90.0%, 75.0%, 46.2%, 16.7% and 7.0% at 0.005, 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5 μg/ml, respectively. The IC_{50} against PC-14 cells was 0.091 μg/ml.

The cytotoxicities of vindesine and vinorelbine were also high; their IC_{50} values were 0.0022 and 0.0033 μg/ml, respectively. The IC_{50} values of cisplatin, etoposide and irinotecan were 0.18, 0.38 and 0.65 μg/ml respectively. Carboplatin was moderately cytotoxic against PC-14 cells.

We studied the combination effect in the dose ranges around the IC_{50} values as shown in Table 1. Each combination experiment was repeated at least four times. The average raw data on the combination of KRN5500 and cisplatin shown in Table 2 were used for each of the different analytical methods (3-D model, CI and isobologram analysis).

Reformulation of the 3-D model

First, we tried to improve the previous 3-D model which had been established in our laboratory [14] by adopting the 95% confidence limits calculated from the variance in the presence of each drug, because the variance varies with the presence of the drug and its concentrations.

Figure 2 shows the 3-D model analysis of the cytotoxic interaction between KRN5500 and cisplatin, including the stages in the data transformation required to produce the synergy plots. Figure 2A shows a matrix consisting of the dose-response curve for cisplatin. The resulting surface represents the predicted inhibitory effect of the drug in all wells of the experimental plate and is a replica of the first dose-response curve plotted in three dimensions. Figure 2B shows the dose-response curve for KRN5500. The resulting surface represents the inhibitory effect of KRN5500 in all wells of the experimental plate. The *x*-axes of the two dose-response surfaces are perpendicular to each other, as in the later theoretical plot. These two matrices, when transformed by Eq. 7, yielded further matrices which represent the theoretical effects of an additive combination. The resulting surface (Fig. 2C) was then subtracted from the observed response surface (Fig. 2D) and plotted as a 3-D graph of the difference effect surface (Fig. 2E).

In order to assess statistically the difference between the observed and calculated effects, the 95% confidence limits surface, $(CL_{A,B})_{95\%}$, (Fig. 2F) was produced by integration with respect to the concentrations of each drug. The difference effect surface (Fig. 2E) was divided by the 95% confidence limit surface (Fig. 2F) to produce the combination effect surface 3-D graph (Fig. 2G) and its contour plot (Fig. 2H). This clearly reveals a drug relationship depending on the concentration of cisplatin but not of KRN5500. The section of the surface above 1, where the difference between the observed and calculated effects was higher than the 95% confidence limit, indicating statistically significant synergy, is depicted in blue. In combinations of KRN5500 with relatively high concentrations of cisplatin almost all were the sections between 1 and −1 as indicated by the diagonal line and were identified as additive effects. There was no section below −1 with this combination.

Evaluation by the customary 2-D models

In order to confirm the reliabilities and advantages of the 3-D model analysis, the customarily used 2-D models, i.e. the CI and isobologram analysis, were applied to the data in Table 2.

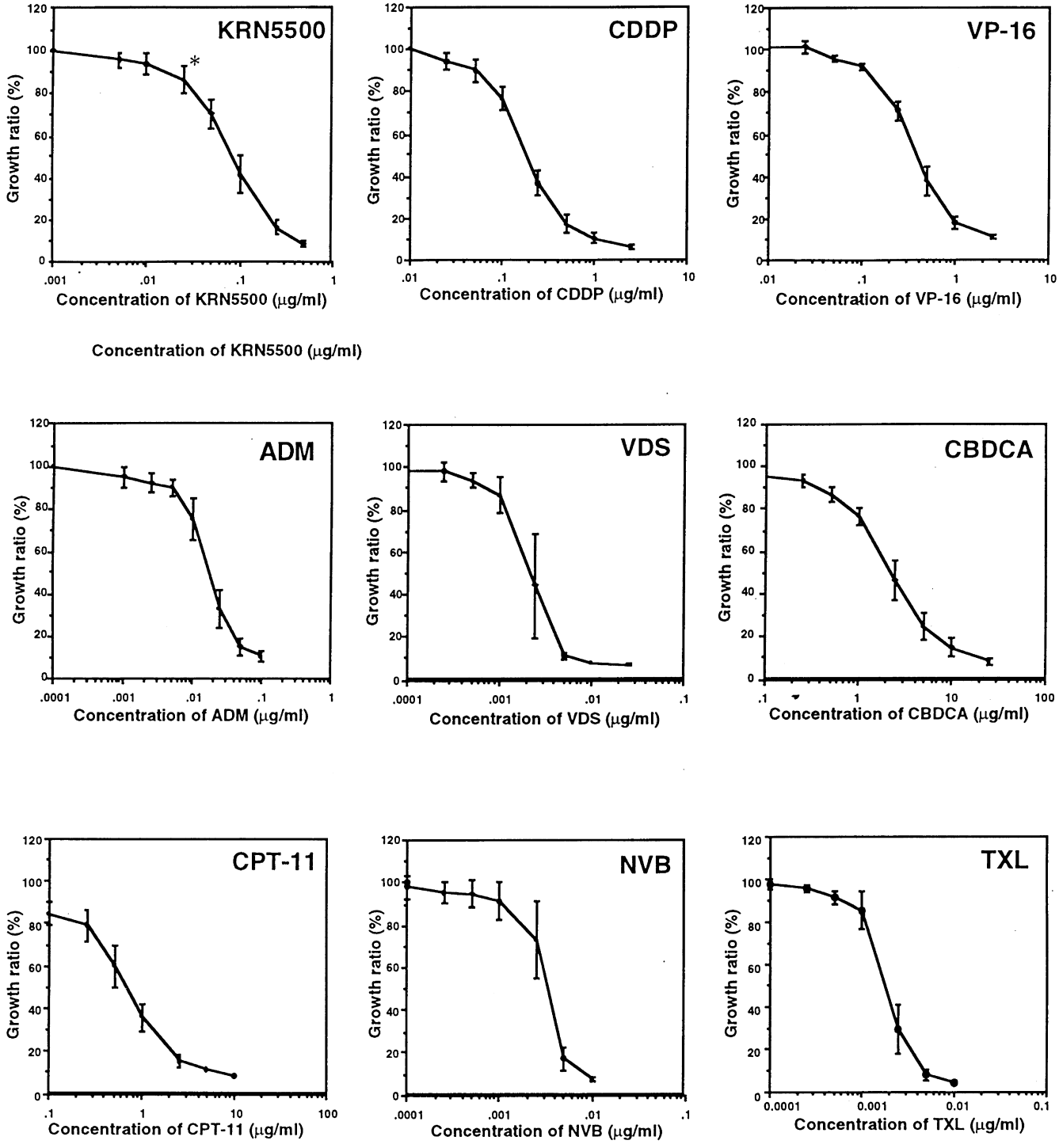


Fig. 3 Dose-response curves of the effect of KRN5500 and other drugs on the growth of the NSCLC cell line PC-14. Cells were exposed to drugs at various concetrations for 6 days, after which the cell proliferation ratio of treated to control cultures was calculated. The anticancer drugs tested were KRN5500, cisplatin (CDDP), etoposide (VP-16), adriamycin (ADM), vindesine (VDS), carboplatin (CBDCA), irinotecan (CPT-11), vinorelbine (NVB) and paclitaxel (TXL). Each plot represents the results of four or five independent experiments, and shows the mean values and the standard deviations

First, we analyzed the cytotoxic interaction between KRN5500 and cisplatin by the CI method described by Chou and Talalay [3]. Figure 4 was constructed by computer from the raw data marked with symbols of a and b in Table 2. For the mutually nonexclusive case (thin line), CI plots below 1 are shown for a wide range of inhibition levels at the KRN5500:cisplatin dose ratio of 1:10, and at the level indicating more than 60% inhibition at a dose ratio of 1:1 as shown in Fig. 4A and

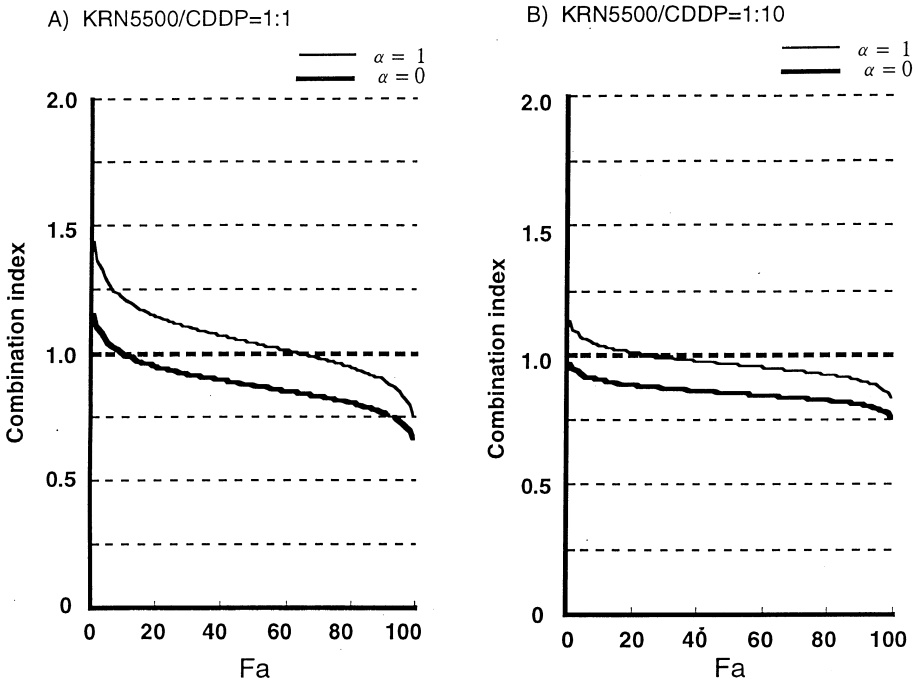


Fig. 4A,B Combination index (CI) plots for the interaction between KRN5500 and cisplatin in PC-14 cells. Cells were treated with KRN5500 and cisplatin alone or in combination at the fixed KRN5500 to cisplatin concentration ratios of 1:10 (**A**) and 1:1 (**B**). Using the conservative isobologram equation, affected fraction (Fa) CI plots for PC-14 cells were constructed by computer analysis of the data generated from the median effect analysis. CI values of < 1 occurred over a wide range of inhibition levels, indicating synergy

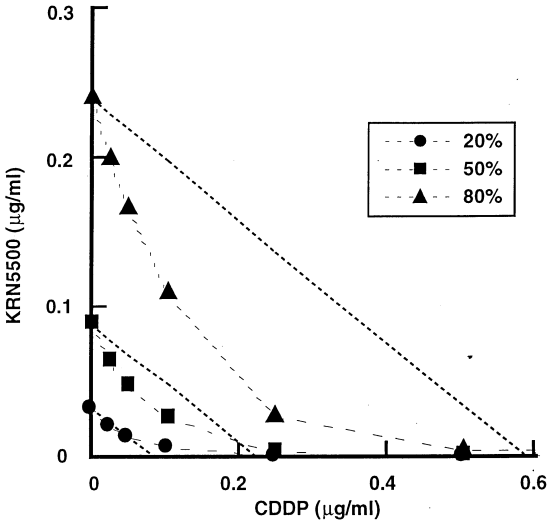


Fig. 5 Multiple isobologram of the interactions between KRN5500 and cisplatin in PC-14 cells. Cells were treated with KRN5500 and cisplatin as single drugs or in combination. The combination effects were analyzed at the 20%, 50%, and 80% growth inhibition ratio endpoints. The isobolograms were based upon the dose response curves in Fig. 2. The *x*-axis and *y*-axis indicate the KRN5500 and cisplatin concentration (μg/ml), respectively. With this system, synergism, additivity and antagonism can be identified from the shape of the curve, based on a plot of the concentrations causing the stated effects. The isobologram plotted at the 80% endpoint is curved downwards, suggesting synergism. The isobolograms plotted at the 50% and 20% endpoints indicate additive and antagonistic interactions, respectively

Fig. 4B, respectively. The results suggest that the combination effects are synergistic.

Next, the combination effect was analyzed by the isobologram method at isoeffect levels of 20%, 50% and 80% reduction in the cell count after drug treatment (IC_{20}, IC_{50} and IC_{80}, respectively; Fig. 5). With this system, synergism, additive effects, and antagonism can be identified from the positions of the data points in relation to straight lines connecting the isoeffect concentrations of the single drugs. The isobologram plotted at the 80% isoeffect level is located below the straight line, suggesting a synergistic interaction. The isobologram plotted at the 50% isoeffect level indicates an additive cytotoxic interaction since it is almost on the line. On the other hand, the isobologram plotted at the 20% isoeffect level is located above the straight line suggesting an antagonistic interaction. Thus, in the isobologram analysis, it should be noted that the combination effects differed according to the isoeffect level.

Combination effects of KRN5500 with other anticancer drugs

The 3-D contour forms of Fig. 6 show the drug-drug interactions which occured when KRN5500 was combined with cisplatin, etoposide, adriamycin, vindesine, carboplatin, irinotecan, vinorelbine and paclitaxel at the various concentrations indicated in Table 1.

A marked synergistic interaction was observed when KRN5500 was combined with carboplatin, an analog of cisplatin, at concentrations in the range 0.03–5.0 μg/ml as shown in Fig. 6E. With the combination of KRN5500 and etoposide, a synergistic interaction was observed when they were combined at concentrations in the range 0.025–0.03 μg/ml and less than 0.5 μg/ml, respectively, but antagonistic interactions also occurred at higher concentrations of both drugs. Thus the combination of KRN5500 and etoposide showed biphasic effects

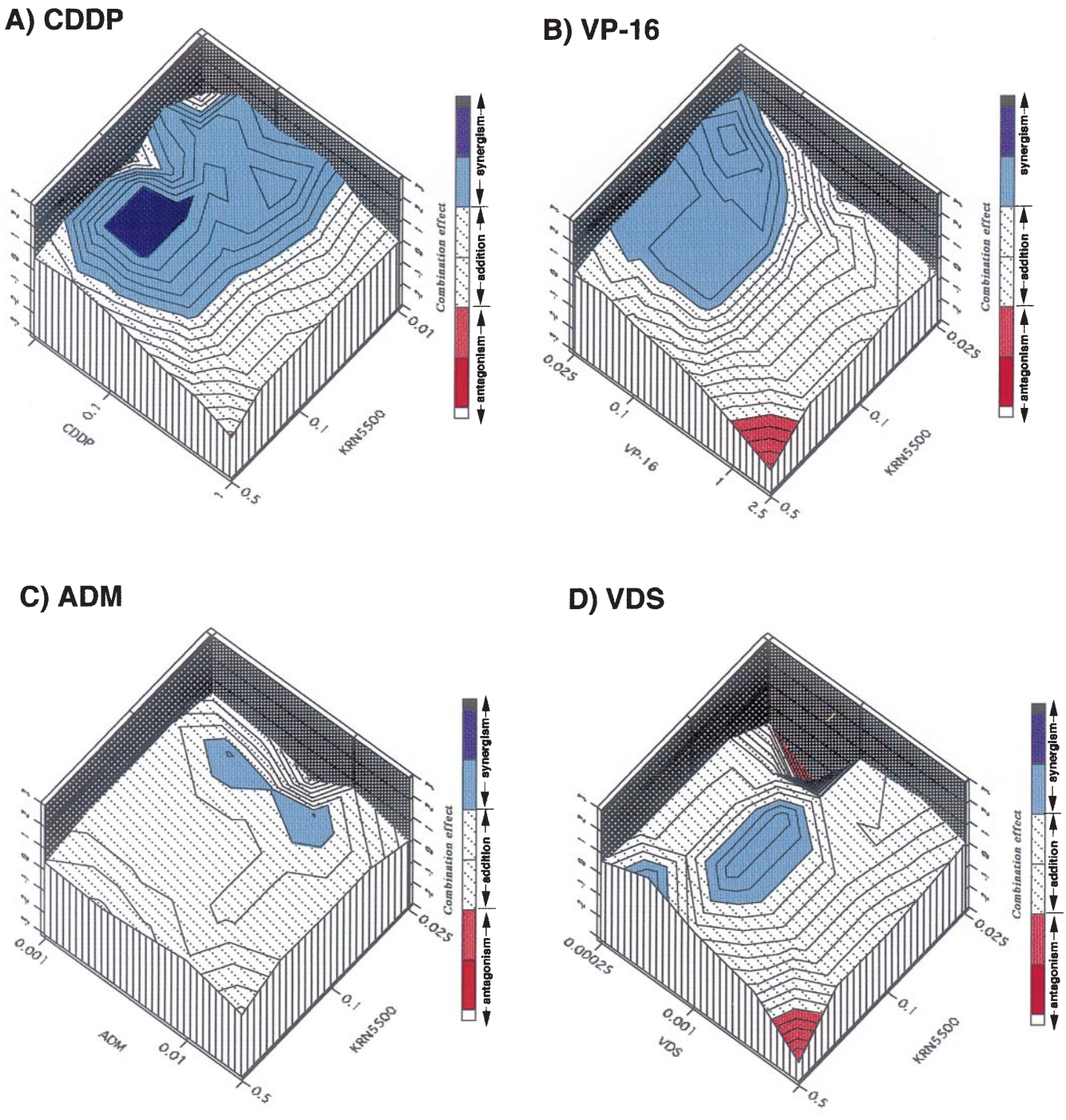


Fig. 6A–H Contour plots representing interaction between KRN5500 and other anticancer drugs in the 3-D model analyzing the combination effect. The anticancer drugs tested are cisplatin (CDDP, **A**), etoposide (VP-16, **B**), adriamycin (ADM, **C**), vindesine (VDS, **D**), carboplatin (CBDCA, **E**), irinotecan (CPT-11, **F**), vinorelbine (NVB, **G**) and paclitaxel (TXL, **H**) in the range of concentrations listed in Table 1

depending on the concentrations. The interaction of KRN5500 with vindesine was also biphasic and was very complicated as shown in Fig. 6D. Only weak synergism was found when KRN5500 was combined with vinorelbine, an analog of vindesine. The interactions of KRN5500 with adriamycin, irinotecan and paclitaxel were almost additive.

Discussion

In the mature field of anticancer chemotherapy, combination chemotherapy is required to cure drug-sensitive cancers [4] and is of great research interest because of its great clinical potential [21]. Over 5000 articles in

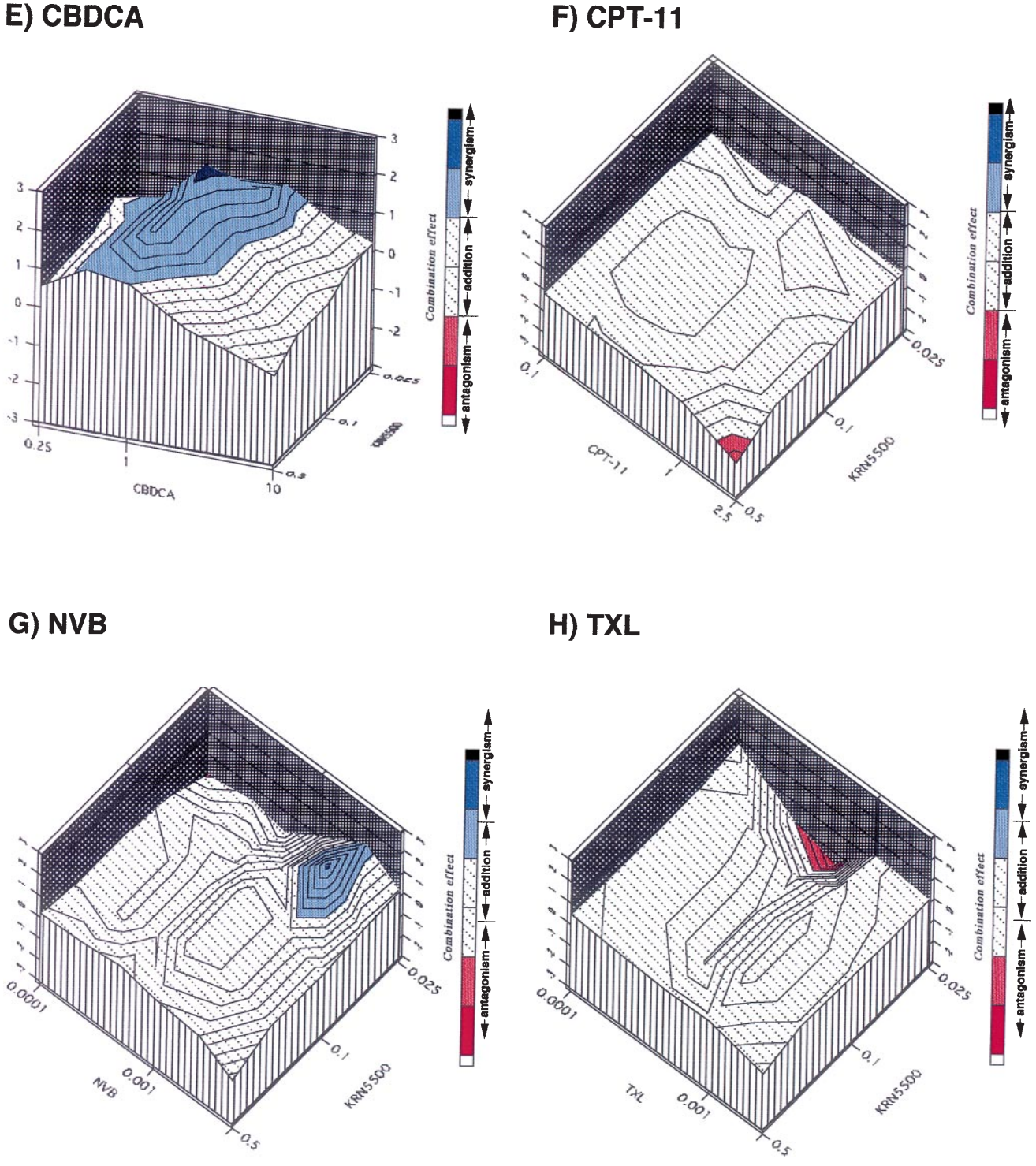


Fig. 6 (*Contd.*)

MEDLINE from 1966 to 1998 include "synergism" as a keyword. The search for synergy has followed many difficult paths during the past 30 years. There is no uniform agreement on the results of combination effects. For example, a high clinical response rate has been reported for small-cell lung cancer after combination therapy with cisplatin and etoposide, but conflicting results have sometimes been obtained in in vitro experimental studies. The discrepancies in the results of the many studies of combination effects seem to be a result of the analytical methods employed in the experiments. When two drugs are combined, there are generally three variables: the concentrations of the two drugs and the resulting biological effect. When 2-D models are used to analyze interactions, one variable must be held constant, thus making the analysis incomplete. Important

synergistic or antagonistic interactions therefore can be underestimated or missed entirely by standard 2-D models [12]. Thus, 2-D models are often unable to detect complex interactions, if discrete isoeffect levels or molar ratios are not chosen. Analyses need to be performed for many different dose ratios and many isoeffect levels, so that interactions between two drugs are fully revealed. However, even if multiple dose ratios and isoeffect levels are used, it is difficult to determine any coherent relationship.

Since combination therapy is defined by three variables, a 3-D equation is necessary to describe it completely. A theoretical 3-D model has been developed and shown to be the most accurate and complete way to describe complicated interactions [16]. However, technological barriers in producing 3-D graphs limited the practical application of this model. The advent of computer-assisted 3-D graph drawing techniques now allows most investigators to use this type of model. Suhnel succeeded in determining synergism or antagonism in the combined action of antiviral agents using such a 3-D model [22]. Prichard and Shipman [18] and Greco et al. [7] reviewed its application.

We have made an effort to simplify the 3-D model, based on the median-effect principle and Michaelis-Menton equations. The resultant 3-D model has many advantages over the 2-D approximations currently used. The 3-D graph can be visualized directly, and plainly shows synergistic and/or antagonistic drug interactions occurring when two drugs are combined. And its shape is characteristic of the particular drug combination used.

In the previous study, combination effects were evaluated to compare the difference between observed and calculated effects with the fixed figure of 5.5%, which was a 95% confidence limit obtained from the variation in cells cultured in drug-free medium. When cells are treated with drugs at concentrations higher than the IC_{95} of each drug, their growth fractions are below 5%. If the drug interaction is more than additive, the calculated growth fraction and then the observed growth fraction would also be below 5%. Consequently, the combination effect obtained by subtracting the calculated growth fraction from the observed growth fraction would also become less than the 95% confidence limit, that is, its effect would be evaluated as less than additive. Therefore, in short, the interaction would be underestimated and it is necessary to avoid underestimation or missed reading in a 3-D graph. As shown in Fig. 3, the variation differed with the drug concentrations. Therefore, in this study, 95% confidence limits in all drug combinations were calculated from the variance of the growth fraction of cells treated with each drug and used for evaluation of combination effects.

Synergism, with statistical significance at the level of the 95% confidence limit, was observed when KRN5500 was combined with cisplatin at relatively low concentrations. Such concentrations are available under clinical conditions. The peak plasma concentration has been observed to be more than 3 μg/ml in patients given 80 mg/m^2 cisplatin by intravenous drip infusion over 30 min [20]. Reece et al. have reported similar results [19].

Synergistic drug interaction was also observed when KRN5500 was combined with carboplatin, an analog of cisplatin. These results seem to indicate that the combination effect is related to the cytotoxic mechanisms of these drugs.

As seen in Fig. 6, each of 3-D graph plainly shows complicated drug interactions peculiar to each combination over the entire range of concentrations tested. The shape of each 3-D graph was characteristic of the particular drug combination used, and offers important information concerning the proper molar ratios or correct drug doses that should be used in further experiments such as in vivo or clinical combination studies.

In this study, KRN5500 was confirmed to be a good candidate for a combination regimen with cisplatin, carboplatin or etoposide for NSCLC.

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References

1. Aszabs A, Lemanski P, Berk B, Dutcher JO (1966) Septacidin analogues. Antimicrob Agents Chemother 1965: 845
2. Burger AM, Kaur G, Hollingshead M, Fischer RT, Nagashima K, Malspeis L, Duncan KLM, Sausville EA (1997) Antiproliferative activity in vitro and in vivo of the spicamycin analogue KRN5500 with altered glycoprotein expression. Clin Cancer Res 3: 455
3. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27
4. DeVita VT Jr (1989) Principles of chemotherapy. In: DeVita VT Jr, Hellman S, Rosenberg SA: (ed) Cancer principles and practice of oncology. JB lippincott, Philadelphia, p 276
5. Drewinko B, Loo TL, Brown B, Gottlieb JA, Freireich EJ (1976) Combination chemotherapy in vitro with adriamycin. Observations of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells. Cancer Biochem Biophys 1: 187
6. Gralla RJ, Casper ES, Kelsen DP, Braun DW Jr, Dukeman ME, Martini N, Young CW, Gplbey RB (1981) Cisplatin and vindesine combination chemotherapy for advanced carcinoma of the lung: a randomized trial investigating two dosage schedules. Ann Intern Med 95: 414
7. Greco WR, Bravo G, Parsons JC (1995) The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47: 331
8. Hayakawa Y, Nakayama M, Kawai H, Tanabe K, Nakayama H, Shimizu A, Seto H, Otake N (1983) Studies on the differentiation inducers of myeloid leukemia cells III. Spicamycin, a new inducer of differentiation of HL-60 human promyelocytic cells. J Antibiot (Tokyo) 36: 934
9. Hayakawa Y, Nakayama M, Kawai H, Tanabe K, Nakayama H, Shimizu A, Seto H, Otake N (1985) Spicamycin, a new differentiation inducer of mouse myeloid leukemia cells (M1) and human promyelocytic leukemia cells (HL-60). Agric Biol Chem 49: 2685

10. Kamishohara M, Kawai H, Odagawa A, Isoe T, Mochizuki J, Uchida T, Hayakawa Y, Seto H, Tsuruo T, Otake N (1993) Structure-antitumor activity relationship of semi-synthetic spicamycin analogues. J Antibiot (Tokyo) 46: 1439
11. Kamishohara M, Kawai H, Sakai T, Isoe T, Hasegawa K, Mochizuki J, Uchida T, Kataoka S, Yamaki H, Tsuruo T, Otake N (1994) Antitumor activity of a spicamycin derivative, KRN5500, and its active metabolite in tumor cells. Oncol Res 6: 383
12. Kanzawa F, Saijo N (1997) In vitro interaction between Gemcitabine and other anticancer drugs using a novel three-dimensional model. Semin Oncol 24: s7–8
13. Kanzawa F, Nishio K, Kubota N, Saijo N (1995) Antitumor activities of a new indolocarbazole substance, NB-506, and establishment of cell lines, SBC-3/NB. Cancer Res 55: 2806
14. Kanzawa F, Nishio K, Fukuoka K, Fukuda M, Kunimoto T, Saijo N (1997) Evaluation of synergism by a novel three-dimensional model for the combined action of cisplatin and etoposide on the growth of a human small-cell lung-cancer cell line, SBC-3. Int J Cancer 71: 311
15. Lee Y-S, Nishio K, Ogasawara H, Funayama Y, Ohira T, Saijo N (1995) In vitro cytotoxicity of a novel antitumor antibiotic, Spicamycin derivative, in human lung cancer cell lines. Cancer Res 55: 1075
16. Loewe S (1953) The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3: 285
17. Miller TP (1990) Rationale for the use of chemotherapy in non-small cell lung cancer. Semin Oncol 17: 11
18. Prichard MN, Shipman C Jr (1990) A three-dimensional model to analyze drug-drug interactions. Antiviral Res 14: 181
19. Reece PA, Stafford I, Russell J, Khan M, Gill PG (1987) Creatinine clearance as a predictor of ultrafilterable platinum disposition in cancer patients treated with cisplatin: relationship between peak ultrafilterable platinum plasma levels and nephrotoxicity. J Clin Oncol 5: 304
20. Sasaki Y (1992) Pharmacological approach to the platinum compounds. Jap J Cancer Chemother 19: 456
21. Schinazi RF (1991) Combined chemotherapeutic modalities for viral infections: rationale and clinical potential. In: Chou TC, Rideout DC (eds): Synergism and antagonism in chemotherapy. Academic Press, New York p 109
22. Suhnel J (1990) Evaluation of synergism or antagonism for the combined action of antiviral agents. Antiviral Res 13: 23