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Combination effects of TAS-103, a novel dual topoisomerase I and II inhibitor, with other anticancer agents on human small cell lung cancer cells

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Abstract *Purpose*: TAS-103 [6-((2-(dimethylamino) ethyl)amino)-3-hydroxy-7H-indeno(2,1-c)quinolin-7-one dihydrochloride] is a newly synthesized dual inhibitor of topoisomerase I and II. Since anticancer drugs are used in combination with other drugs for effective chemotherapy, we investigated the cytotoxic effect of TAS-103 in combination with other conventional anticancer agents, such as cisplatin, vindesine, doxorubicin, 5-fluorouracil, and the antitopoisomerase inhibitors SN-38 and etoposide in vitro. *Methods*: Inhibition of the growth of the human small-cell lung cancer cell line SBC-3 was evaluated using the tetrazolium dye (MTT) assay. Drug interactions were evaluated by isobologram analysis and the determination of combination indices supplemented by a three-dimensional model. *Results*: Simultaneous use of TAS-103 and cisplatin had a supradditive effect, but combinations of TAS-103 with other drugs had an additive or marginally subadditive effect. Three-dimensional model analysis added more information about the synergistic concentration ranges of two drugs (cisplatin 200–400 n*M* and TAS-103 7–10 n*M*). Sequential use of TAS-103 and cisplatin had only an additive effect. *Conclusion*: These results suggest that the concomitant use of TAS-103 and cisplatin has a greater cytotoxic effect on cancer cells than single drug use, and may provide a beneficial effect in the treatment of small-cell lung cancer.

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Key words Novel anticancer agent · Three-dimensional method · Synergism · Combination effect · Topoisomerase inhibitor

Abbreviations *MTT* tetrazolium dye, IC_{50} drug concentration that inhibits cell growth by 50%, *CDDP cis*-diamminedichloroplatinum(II), *VDS* vindesine, *DOX* doxorubicin, *VP-16* etoposide, *5-FU* 5-fluorouracil, *CPT-11* 7-ethyl-10-[4-(1-piperidyl)-1-piperidyl]carbonyloxy-camptothecin, *SN-38* 7-ethyl-10-hydroxy-camptothecin, *FBS* fetal bovine serum *CI* combination index, *ICL* interstrand crosslink, *TA* theoretical activity

Introduction

DNA topoisomerases are enzymes that alter the topology of DNA by transiently breaking one or both of its strands, passing a single- or double-strand-DNA through the break, and finally rejoining the breaks. These enzymes are involved in a number of critical cellular processes, including replication, transcription, and recombination [10]. They are viewed as important targets for cancer chemotherapy, especially since it has been demonstrated that the topoisomerase I inhibitor, irinotecan chloride (CPT-11), a semisynthetic derivative of camptothecin, shows strong antitumor activity against leukemia, lymphoma [7], small-cell lung cancer [9], non-small-cell lung cancer [24], and colorectal [1], ovarian and cervical (2) cancers. The topoisomerase II inhibitor etoposide (VP-16) is also a potent anticancer agent, and combined use of VP-16 and cisplatin (CDDP) now constitutes one of the major regimens in small-cell lung cancer chemotherapy [4, 15]. It is therefore probable that topoisomerase inhibitors are promising anticancer agents.

Such an inhibitor is the novel anticancer agent 6-[[2-(dimethylamino)ethyl]amino]-3-hydroxy-*7H*-indeno[*2,1-c*] quinolin-7-one dihydrochloride (TAS-103) [26]. Its primary targets are thought to be topoisomerase I and II,

and its mechanism of action is believed to be stabilization of a covalent complex with DNA topoisomerase I and II. The IC_{50} of TAS-103 for topoisomerase I is said to be 2 μM, and the IC_{50} for topoisomerase II has been determined to be 6.5 μM in vitro [11, 20]. TAS-103 has been found to exhibit powerful and broad antitumor activity against 12 of 13 subutaneously implanted human solid tumor xenografts including cancer of the lung, colon, stomach, breast, pancreas, and kidney [20]. However, TAS-103 has topoisomerase I inhibitor activity similar to that of SN-38, and stronger topoisomerase II inhibitory activity than VP-16 [27], and does not show any cross-resistance in several resistant phenotypes such as CDDP resistance, multidrug resistance, or topoisomerase inhibitor resistance [11]. Since anticancer drugs are used in combination with other drugs for effective chemotherapy, we investigated the cytotoxic effect of TAS-103 in combination with other established anticancer agents in vitro.

Materials and methods

Cell line and culture

The human small-cell lung cancer cell line SBC-3, originally established at the Okayama University School of Medicine, was donated by the Japanese Cancer Research Resources Cell Bank. The SBC-3 cells were grown as attached cultures in RPMI-1640 medium (GIBCO Co., Grand Island, N.Y.) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Mo.), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a highly humidified atmosphere of 5% v/v CO_2 in air at 37 °C, as described previously. The cells were harvested routinely by mechanical disaggregation and diluted with medium to the appropriate concentrations. Cell size and numbers were determined with a Coulter Cannalyzer C-256 system (Coulter Electronics, Hialeah, Fl.).

Drugs and chemicals

TAS-103 was provided by Taiho Chemicals Co. (Tokyo, Japan), and CDDP was purchased from Nippon Kayaku Co. (Tokyo, Japan). Paclitaxel, formulated in Cremophor EL (polyethylene glycol 35 castor oil; Parsippany, N.J.) was obtained from Bristol Myers Squibb Co. (Tokyo, Japan), vindesine (VDS) from Shionogi Co. (Osaka, Japan), VP-16 from Sigma-Aldrich Japan Co. (Tokyo Japan) and doxorubicin (DOX) from Kyowa-Hakko-Kogyo Co. (Tokyo, Japan). SN-38 was provided by Daiichi Co. (Tokyo Japan) and a stock solution of SN-38 was prepared in dimethylsulfoxide. Finally, 5-fluorouracil (5-FU) was provided by Mitsui Pharmaceuticals. These stock solutions and chemicals were stored at −20 °C.

Growth inhibition assay

We used the tetrazolium dye (MTT) assay to evaluate the growth inhibitory effects of the drugs, as described previously [8, 18], and designed a two-way exposure schedule: concomitant exposure to two drugs for 96 h, and sequential exposure to the same drugs for 48 h each. For the concurrent schedule, 100 μl of an exponentially growing cell suspension (2.5×10^4 cells/ml) was seeded into each well of a 96-well microtiter plate, then 50 μl of TAS-103, 50 μl of the other drug solution, and nine more steps of 1.5-fold dilution with medium were added to each well. Following exposure to the drugs for 96 h, 20 μl of the MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and the plates were incubated at 37 °C for another 4 h. After centrifuging the plates at 200 *g* for 5 min, the medium was aspirated from each well as completely as possible. Next, 200 μl of dimethylsulfoxide was added to each well to dissolve the formazan, and the optical density (absorbance) was measured at 562 and 630 nm using a Delta Soft ELISA analysis program run on a Macintosh computer interfaced with a Bio-Tek Microplate Reader (EL-340, Bio-Metallics, Princeton, N.J.).

For the sequential schedule, 100 μl of an exponentially growing cell suspension (2.5×10^4 cells/ml) was seeded into each well of a 96-well microtiter plate, then 100 μl of the first drug solution was added to each well at nine concentrations. After incubation for 48 h, the solution containing the first drug was aspirated from each well as completely as possible, 100 μl of the second drug solution was added, and the plates were incubated at 37 °C. Following exposure to the second drug for 48 h, 20 μl of the MTT solution was added to each well, and absorbance was measured at 562 and 630 nm, as described above. The absorbance of wells containing only RPMI-FBS and MTT were also read as an assay control. Each experiment was performed using six replicate wells for each drug concentration. At least three independent experiments were carried out, and the IC_{50} was defined as the concentration needed for a 50% reduction of the absorbance in each test, and the surviving fraction was calculated as: (mean absorbance of six replicate wells containing drugs − mean absorbance of six replicate assay control wells)/(mean absorbance of six replicate drug-free wells − mean absorbance of six replicate assay control wells).

Analysis of combined effects

We analyzed the effects of drug combinations using two-dimensional methods the isobologram method of Steel and Peckham [22] and the combination index (CI) method of Chou and Talalay [3]. If the combination was found to be supraadditive, it was further evaluated by a three-dimensional method.

Combination index

This method is based on the principle that the growth inhibition curve can be represented by the median-effect equation as follows [3]:

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

where D is the dose administered, Dm is the dose required for 50% growth inhibition, 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. Theoretically, the CI is the ratio of the combined dose to the sum of the two single-agent doses at the isoeffective level. Consequently, CI values smaller than, equal to, or greater than 1 represent synergism, additivity, and antagonism, respectively.

Isobologram

The isobologram was proposed by Loewe and Muischnek in 1926 [17], and the "envelope of additivity" was developed and introduced into isobologram analysis by Steel and Peckham [21, 22]. This method is well known and has often been used in recent studies to evaluate synergism or antagonism. If two drugs do not interact, the equation is:

$$(D)_A/(D_X)_A + (D)_B/(D_X)_B = 1 \quad (2)$$

In practice, the concentrations $(D_X)_A$, $(D_X)_B$, $(D_X)_{A,B}$, for each of two drugs and the two drugs combined, respectively, required to produce the same percentage growth inhibition, are obtained from their dose-response curves. The concentrations of drugs A and B are placed on the X and Y coordinates of the isobologram,

respectively. Three isoeffect curves (mode I, mode IIA, and mode IIB) are drawn [19]. The total area enclosed by these three lines represents the "envelope of additivity". When the experimentally determined IC_{50} of the combination is plotted on the left side of the envelope, the interaction between the drugs in combination is considered supraadditive (synergistic). When the experimental data point is plotted within the envelope, the combination is considered additive, and when it falls on the right side of the envelope, but within the square produced by 0–1 IC_{50} units, the combination is considered subadditive. When the point lies outside the square, both drugs are considered protective of each other.

Three-dimensional model

The theoretical basis of this three-dimensional model has been described previously [14]. The CI Eq. 1 for mutually nonexclusive inhibitors f_a and f_u, with subscripts designating the drug or the combination used is,

$$(f_a)_{A,B}/(f_u)_{A,B} = (f_a)_A/(f_u)_A + (f_a)_B/(f_u)_B + \alpha(f_a)_A/(f_a)_B/(f_u)_A(f_u)_B \quad (3)$$

where $(f_a)_A$, $(f_a)_B$, and $(f_a)_{A,B}$ are the fractions affected by drug A, drug B, and their combination, respectively. And $(f_u)_{A,B} = 1 - (f_a)_{A,B}$, $(f_u)_A = 1 - (f_a)_A$, $(f_u)_B = 1 - (f_u)_B$. For mutually exclusive drugs, $\alpha = 0$; for mutually nonexclusive drugs, $\alpha = 1$.

In the case of the combination of TAS-103 and CDDP, $\alpha = 1$ was assumed, since TAS-103 is known to act by different mechanisms from CDDP.

For mutually nonexclusive drugs, where $\alpha = 1$,

$$(f_a)_{A,B}/\{1 - (f_a)_{A,B}\} = (f_a)_A/\{1 - (f_a)_A\} + (f_a)_B/\{1 - (f_a)_B\} + (f_a)_A(f_a)_B/\{1 - (f_a)\}\{1 - (f_a)_B\} \quad (4)$$

Finding a common denominator and simplifying,

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

Here, $(f_a)_{A,B}$ is defined as theoretical additivity (TA), since Eq. 3 was designated from the TA,

$$TA = (f_a)_A + (f_a)_B - (f_a)_A(f_a)_B \quad (6)$$

Integrating with respect to concentration of $(f_a)_A$ and $(f_a)_B$, the resulting calculated response surface $\{(S_a)_{A,B}\}$ is simulated as shown in Fig. 1C:

$$\{(S_a)_{A,B}\}_{cal} = \int_{a=0}^{n}\int_{b=0}^{m} TA \quad (7)$$

Cytotoxicity data obtained from the experiments directly generated a dose-response surface, that is, an observed response surface as shown in Fig. 1A. This observed response surface, $\{(S_a)_{A,B}\}_{obs}$, was subsequently subtracted from the above theoretical calculated response surface, $\{(S_a)_{A,B}\}_{cal}$, as shown in Fig. 1B, to reveal the "combination effect surface", $\{(S_a)_{A,B}\}_{CE}$, calculated from the combination of the two drugs (Fig. 1C):

$$\{(S_a)_{A,B}\}_{CE} = \{(S_a)_{A,B}\}_{cal} - \{(S_a)_{A,B}\}_{obs} \quad (8)$$

The combination effect surface reveals synergy or antagonism for the growth of cells cultured in a drug-free medium. To confirm whether synergism occurred or not, the upper and lower 95% confidence limits of the experimental data were compared with the calculated additivity. As shown in Fig. 4E, the "95% confidence limit surface", $\{(S_a)_{A,B}\}_{95\%C}$, was calculated from standard deviations of the data $\{(S_a)_{A,B}\}_{SD}$ and *t*-values $\{(S_a)_{A,B}\}_t$:

$$\{(S_a)_{A,B}\}_{95\%C} = \{(S_a)_{A,B}\}_{SD}\{(S_a)_{A,B}\}_t \quad (9)$$

The combination effect surface, $\{(S_a)_{A,B}\}_{CE}$, and the 95% confidence limit surface $\{(S_a)_{A,B}\}_{95\%C}$, then yielded "the modified confidence plot surface", $\{(S_a)_{A,B}\}_{MC}$, (Fig. 4G):

$$\{(S_a)_{A,B}\}_{MC} = \{(S_a)_{A,B}\}_{CE}/\{(S_a)_{A,B}\}_{95\%C} \quad (10)$$

If the upper confidence limit is lower than the calculated additivity, the observed synergy would be considered significant. Similarly, if the lower confidence limit is greater than the calculated additivity, the observed antagonism would be considered significant.

Fig. 1A–H Three-dimensional analysis of the antitumor interaction of TAS-103 and CDDP, including the stages in the data transformation required for the synergy plots. **A** shows the empirical dose-response curve. Two matrices representing the cytotoxic effects of TAS-103 and CDDP alone were used to calculate the theoretical additive effect using the assumption of dissimilarity. The resulting calculated additive surface (**B**) was then subtracted from the empirical surface, yielding the synergy plot in surface (combination effect surface **C**) and contour form (**D**). The combination effect surface was then divided according to the upper and lower 95% confidence limits of the experimental data (**E**, and contour form **F**), yielding the modified confidence plot in surface (**G**) and contour form (**H**). The combination effect (%) in **C** and **D** represents there 'quantities' of the additive effects, and confidence grades in **G** and **H** can represent the 'qualities' of the effects. At TAS-103 and CDDP concentrations of approximately 2–10 n*M* and 100–600 n*M*, 6–8% synergism was observed with a statistical confidence of more than 95%

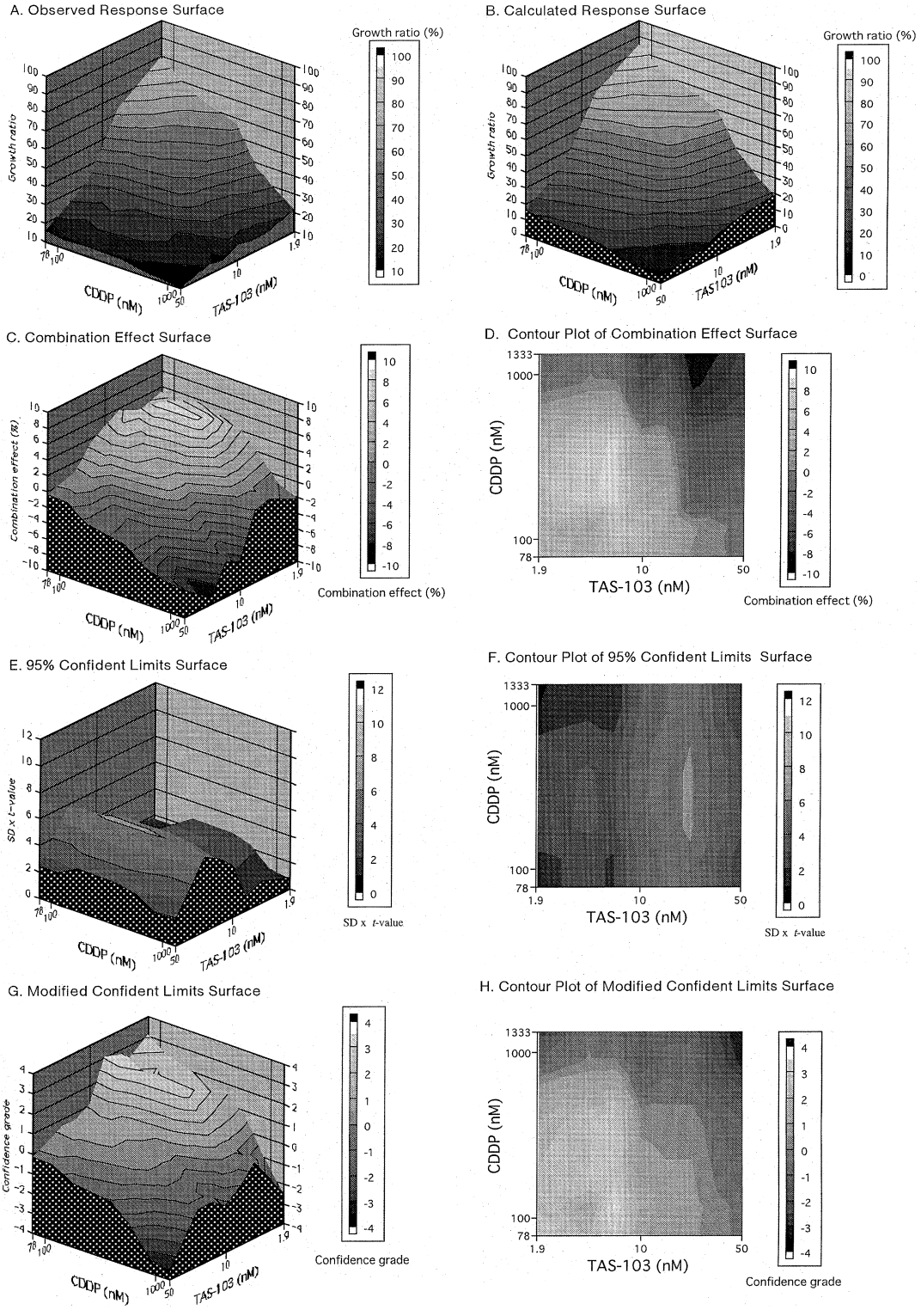
Results

Effects of TAS-103 and other drugs, alone and in combination, on the growth of SBC-3 cells

We repeated each experiment independently at least three times. The data from the experiments were used for the different methods of analysis: the CI, isobologram analysis, and the three-dimensional model analysis. Growth inhibition curves of TAS-103 alone and in combination with other drugs against SBC-3 cells were obtained. The growth inhibition curves of TAS-103 in combination with various concentrations of CDDP (0, 78.0, 117.1, 175.6, 263.4, 395.1, 592.6, 888.9, 1333.3, and 2000 n*M*), and of CDDP alone and in combination with various concentrations of TAS-103 (0, 2.0, 2.9, 4.4, 6.6, 14.8, 22.2, 33.3, 50 n*M*), are shown in Fig. 1A and 1B, respectively. Similar dose-response curves were obtained for paclitaxel, VDS, DOX, 5-FU, SN-38, and VP-16 (data not shown). The shape of these dose-response curves is indicative of a mutually nonexclusive interaction between them. An additive effect is suggested by the difference in slope of the dose-response curves for TAS-103 at various concentrations of CDDP [3].

Evaluation by combination index

We analyzed the cytotoxic interaction between TAS-103 and other drugs by the median-effect method described by Chou and Talalay [3], and evaluated the combinations in terms of synergism or antagonism. The analysis using the CI was performed for many different dose ratios, because CI values depend on drug concentrations and their molar ratios. We constructed CI plots by computer analysis, and the optimal CI values at the IC_{50} level are shown in Table 1.



A potent synergistic effect was observed only with concomitant exposure to TAS-103 and CDDP combined.

Evaluation by isobologram analysis

We analyzed the drug combination effects by the isobologram method of Steel and Peckham, based on the growth inhibition curves of each drug. With this system, supraadditive, additive, and subadditive effects can be identified from the location of data points on the area of the "envelope of additivity". IC_{50} values were calculated on the basis of growth inhibition curves of each drug, and three isoeffect curves, modes I, IIa, and IIb, were drawn. The IC_{50} drug concentrations determined from

growth inhibition curve of each drug in combination with other drugs at various concentrations were plotted on a linear scale in the isobologram.

Simultaneous and continuous exposure of SBC-3 cells to TAS-103 and CDDP (96 h) resulted in a supra-additive effect (Fig. 3A). Exposure to TAS-103 plus paclitaxel was marginally subadditive (Fig. 3B), exposure to TAS-103 plus VDS was marginally subadditive (Fig. 3C), exposure to TAS-103 plus DOX was marginally subadditive (Fig. 3D), exposure to TAS-103 plus 5-FU was additive (Fig. 3E), exposure to TAS-103 plus SN-38 was additive (Fig. 3F), and exposure to TAS-103 plus VP-16 was additive (Fig. 3G). Sequential exposure of SBC-3 cells to TAS-103 (48 h) followed by CDDP (48 h; Fig. 4A), and to CDDP followed by TAS-103 (48 h: Fig. 4B) was additive, not supraadditive. The single agent IC_{50} values for 96 h continuous treatment with CDDP, paclitaxel, VDS, DOX, 5-FU, SN-38, and VP-16 were 330 ± 50, 650 ± 200, 2.3 ± 2, 1.7 ± 1, 650 ± 450, 2200 ± 1020, 1.3 ± 0.77, 780 ± 620 n*M* (average ± SD), respectively.

Evaluation by the three-dimensional model

The evaluations using the CI and isobologram methods revealed a supraadditive effect of the concomitant use of TAS-103 and CDDP, and we used the three dimensional model analysis [14] to evaluate the effects of TAS-103 and CDDP in greater detail than is possible with the conventional two-dimensional model. Figure 1 shows the three-dimensional model of the cytotoxic interaction between TAS-103 and CDDP, including the stages in the data transformation required to produce the synergy plots. The experimental dose-response curve was constructed in three dimensions (observed response surface; Fig. 1A) by using the two dose-response curves of TAS-103 and CDDP. These two curves were transformed to matrices that represented the theoretical effect of an additive combination (calculated response surface; Fig. 1B). The calculated response surface was then subtracted from the observed response surface and plotted as a three-dimensional graph and as contour forms to reveal regions of synergy or antagonism (Fig. 1C,D).

A significant synergistic region was seen in the contour plot (Fig. 1D), at TAS-103 and CDDP concentrations of approximately 2–10 n*M* and 100–600 n*M*, respectively (in the white region, the defference is over 6%). The three-dimensional graph demonstrates the concentration dependence of the drug-drug interaction and shows the complexity of the interaction between the drugs.

►

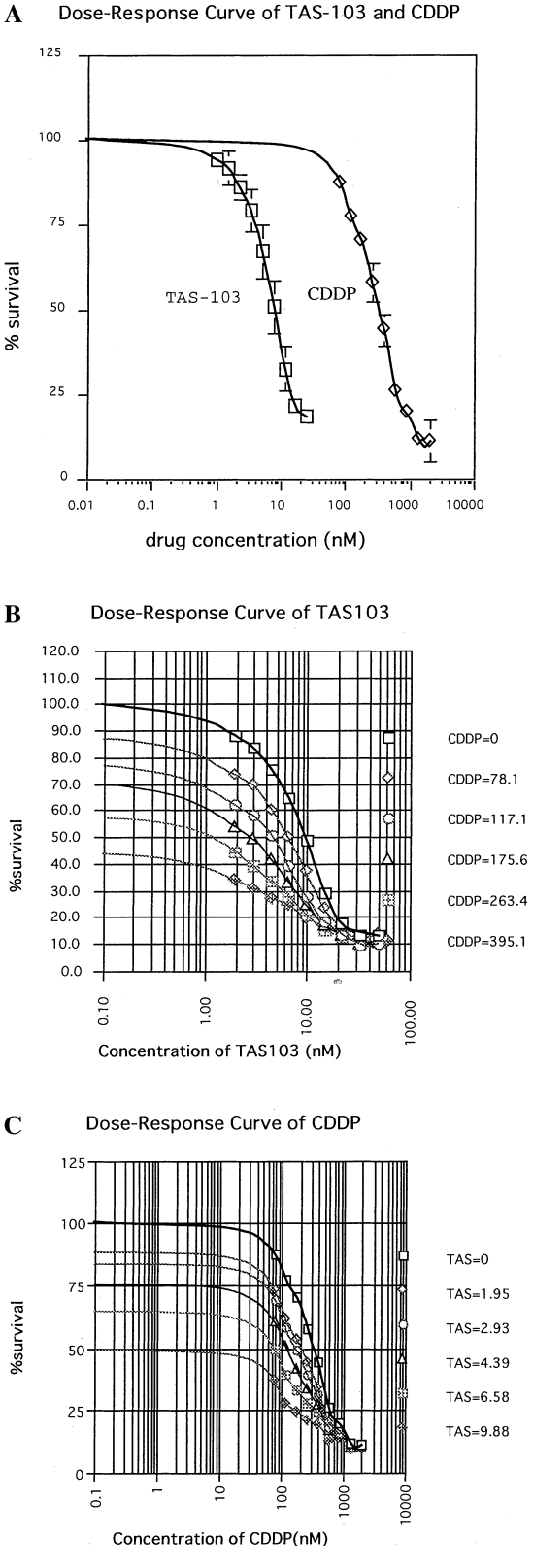


Fig. 2A–C Growth inhibition curves after 96 h continuous exposure to TAS-103 and CDDP of SBC-3 cells (**A**). Surviving fractions were evaluated using the MTT assay. The values shown are the means ± SD from at least six independent experiments, using six replicate wells for each drug concentration. Growth inhibition curves are shown for TAS-103 in combination with various doses of CDDP (**B**), and for CDDP in combination with various doses of TAS-103 (**C**)

Table 1 Optimal combination index (CI) values at 50% growth inhibition of SBC-3 cells by TAS-103 plus other drugs. Cells were treated with TAS-103 in combination with the other drugs at a fixed ratio. CI values <1 indicate synergism. Strong synergy is observed for CDDP

	CDDP	Paclitaxel	VDS	DOX	5-FU	SN-38	VP-16
CI at 50% fraction affected	0.501	1.237	1.341	0.970	0.869	0.934	1.091
Optimal concentration ratio of drug/TAS-103	40	1.67	4	40	40	0.4	40

To determine the significance of the differences between the observed and calculated effects, the upper and lower 95% confidence limits of the experimental data (Fig. 1E, and contour form Fig. 1F) were compared with the calculated additive effect. The modified surface and its contour form were constructed by dividing the calculated surface and plotting as a three-dimensional graph from the 95% confidence surface (Fig. 1G,H).

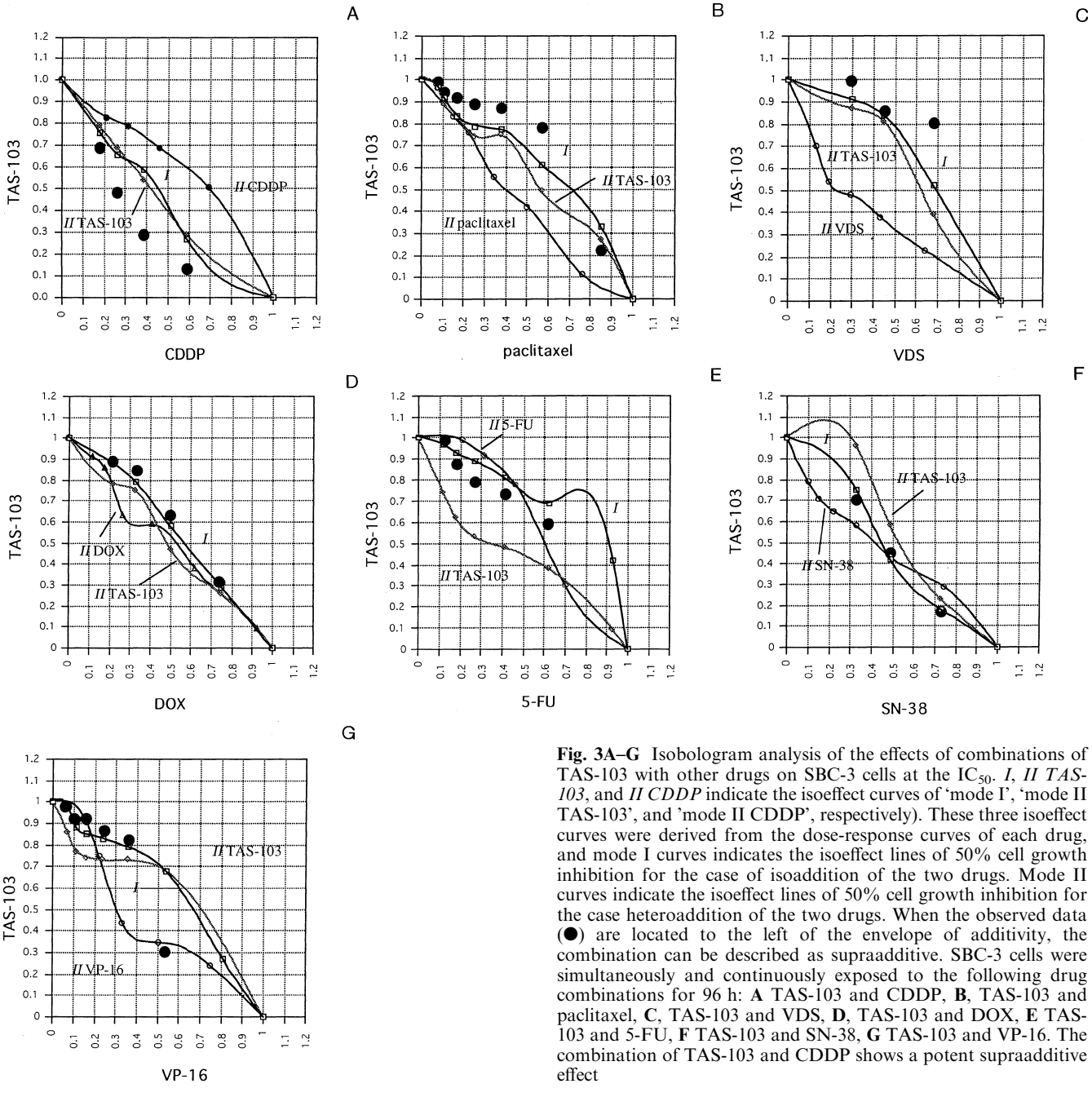


Fig. 3A–G Isobologram analysis of the effects of combinations of TAS-103 with other drugs on SBC-3 cells at the IC_{50}. *I*, *II TAS-103*, and *II CDDP* indicate the isoeffect curves of 'mode I', 'mode II TAS-103', and 'mode II CDDP', respectively). These three isoeffect curves were derived from the dose-response curves of each drug, and mode I curves indicates the isoeffect lines of 50% cell growth inhibition for the case of isoaddition of the two drugs. Mode II curves indicate the isoeffect lines of 50% cell growth inhibition for the case heteroaddition of the two drugs. When the observed data (●) are located to the left of the envelope of additivity, the combination can be described as supraadditive. SBC-3 cells were simultaneously and continuously exposed to the following drug combinations for 96 h: **A** TAS-103 and CDDP, **B**, TAS-103 and paclitaxel, **C**, TAS-103 and VDS, **D**, TAS-103 and DOX, **E** TAS-103 and 5-FU, **F** TAS-103 and SN-38, **G** TAS-103 and VP-16. The combination of TAS-103 and CDDP shows a potent supraadditive effect

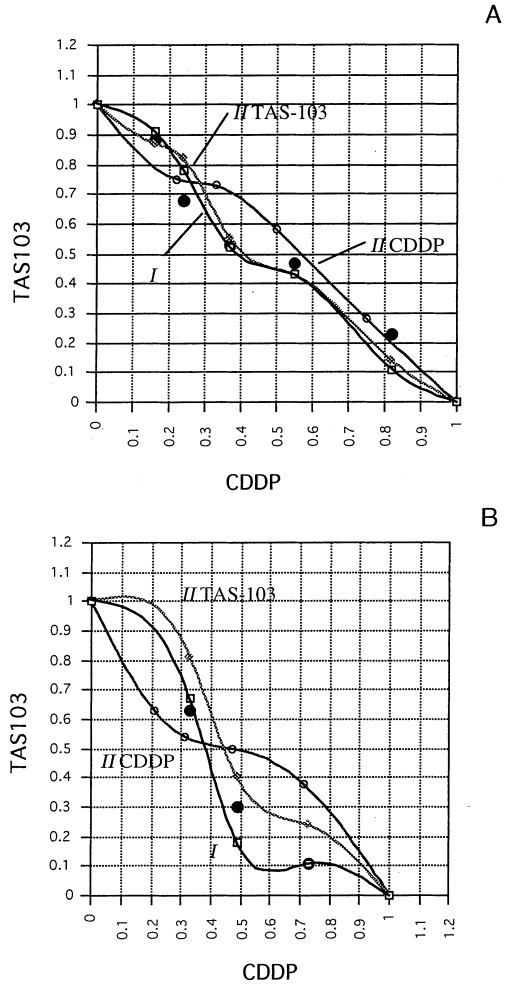


Fig. 4A,B Effects on SBC-3 cells of TAS-103 combined with CDDP using a sequential schedule. The drug concentrations were plotted on a linear scale relative to the IC_{50}. The SBC-3 cells were exposed to TAS-103 for 48 h followed by CDDP for 48 h (**A**), or exposed to CDDP for 48 h followed by TAS-103 for 48 h (**B**)

Since the modified surface is considered to represent statistical significance or real additive effects, the statistically reliable region of the contour plot (Fig. 1H) is at TAS-103 and CDDP concentrations of approximately 2–10 n*M* and 100–600 n*M*, respectively (within the halftone region with confidence grades 1–4).

Thus we conclude that at TAS-103 and CDDP concentrations of approximately 2–10 n*M* and 100–600 n*M*, significant synergism occurred with enough statistical confidence.

Discussion

TAS-103 is a newly synthesized potent inhibitor of topoisomerase I and II that acts by forming a stable covalent complex with DNA-topoisomerase I or II, as described above. DNA topoisomerase is currently one of the most important targets of antitumor agents, and indeed CPT-11 (for topoisomerase I), topotecan (for topoisomerase I), VP-16 (for topoisomerlase II) and other topoisomerase inhibitors have already been demonstrated to have strong antitumor activity against various human malignancies in clinical studies [23]. The combined use of anticancer agents is a common strategy in clinical chemotherapy, and thus the possibility of synergism and the optimal combinations of the anticancer agents should be examined. The results of our study suggest that concomitant continuous exposure to TAS-103 and CDDP has a synergistic effect on the SBC-3 cell line at concentrations of approximately 7–8 n*M* and 80–200 n*M* respectively, but that sequential use of these drugs does not result in synergism.

The combined use of CDDP and a topoisomerase inhibitor is an interesting strategy for cancer chemotherapy. A high clinical response rate has been reported for CDDP plus CPT-11 in lung cancer [5]. On the other hand, CDDP and VP-16 are active against a variety of human tumors, and this combination is considered synergistic based on the fact that therapeutic synergy has been reported in the mouse P388 in vivo model [20] and in human clinical trials.

At the cellular level, some studies have demonstrated a synergistic effect of CDDP combined with CPT-11 and SN-38, an active metabolite of CPT-11. Itoh et al. have observed synergistic effects of SN-38 combined with CDDP on five out of six human lung cancer cell lines [12], and Kano et al. have reported synergistic effects of CPT-11 and SN-38 combined with CDDP on acute lymphoblastic leukemia (MOLT-3) cells [13].

The interaction and biochemical mechanisms of synergy between CDDP and NB-506, a topoisomerase I inhibitor, have been examined [6]. The formation of DNA interstrand crosslinks (ICLs) in the cells was analyzed, and an increased in ICLs was observed after simultaneous exposure to CDDP and NB-506 compared with exposure to CDDP alone. DNA repair after ICL formation induced by a 3 h exposure to CDDP is also reduced by NB-506 exposure [6]. On the other hand, it has previously been reported that the biochemical mechanism responsible for the synergistic effects of CDDP and VP-16 on SBC-3 cells is enhancement of the DNA topoisomerase II inhibitory activity of VP-16 by CDDP, and the analysis in vitro is reflected in vivo [16]. The details of the biochemical mechanisms of synergy between TAS-103 and CDDP are unknown, but similar mechanisms may be applicable to topoisomerase I and II.

Isobologram analysis has shown that the effect of combined CDDP and VP-16 on human SCLC and four human NSCLC cell lines is no more than additive [19]. However, we have previously shown by three-dimensional model analysis that the combination of CDDP and VP-16 has a synergistic effect on human SCLC cell lines at concentrations of approximately 0.025–0.075 μg/ml and 0.01–0.075 μg/ml, respectively [14]. In the present study we were able to demonstrate a synergistic effect of TAS-103 and CDDP by three different methods of analysis, and showed that the synergistic effects depend

not only on the combination but also on the concentrations of the drugs. The three-dimensional model, as a method for evaluating the combined effect of these drugs, provided information about the optimal concentration ranges. It is very important to obtain such information before proceeding to in vivo studies.

Further examination of these drug interactions in preclinical studies, both in vitro and in vivo, and investigation of their mechanisms of action, would provide useful information for future combination chemotherapy regimens.

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