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ORIGINAL ARTICLE

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Combination effects of cisplatin, vinorelbine and irinotecan in non-small-cell lung cancer cell lines in vitro

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Abstract *Purpose*: Isobologram analysis has been widely used for evaluating the combined effect of two antitumor drugs in vitro as a pre-clinical screening test. In this study, we tried to extend two-dimensional isobologram analysis to three dimensions for evaluating the effects of a three-drug combination. *Methods*: We selected three anticancer agents, cisplatin, vinorelbine and irinotecan. Each of them has been classified as having good single-agent activity against non-small-cell lung cancer (NSCLC). Human NSCLC cell lines (EBC-1, PC-3, RERF-LC-MS) were incubated for 4 days in the presence of the three drugs and cytotoxic activities were determined by a tetrazolium-based colorimetric assay (MTT assay). The data were analyzed by three dimensional isobologram analysis. *Results*: The effects of the three drugs were additive against EBC-1 (a squamous cell carcinoma cell line), subadditive against PC-3 (an adenocarcinoma cell line) and from subadditive to supraadditive against RERF-LC-MS (an adenocarcinoma cell line). *Conclusions*: Our findings suggest that the effects of cisplatin, vinorelbine and irinotecan incombination are additive against NSCLC in vitro. These results encourage clinical trials of the three agents in combination chemotherapy for the treatment of NSCLC.

Key words Cisplatin · Vinorelbine · Irinotecan · Lung cancer · Isobologram

Introduction

Numerous systemic combination chemotherapy programs have been tested in patients with advanced non-small-cell lung cancer (NSCLC) during the past two decades. No regimen has produced a significant survival improvement or changed the natural history of patients with advanced NSCLC [10]. However, the combination of cisplatin (CDDP) and vindesine or vinblastine for advanced NSCLC has been widely studied, and a survival benefit has been observed [20–22]. Recently, newer anticancer agents have demonstrated some activity. A new derivative of camptothecin, 7-ethyl-10-[4-(1-pi-peridino)-1-piperidino] carbonyloxycamptothecin (irinotecan), has been reported to have a high antitumor activity in patients with advanced NSCLC [9, 13]. A new semisynthetic vinca alkaloid, vinorelbine (NVB), has also been added to the group of active antitumor agents [6, 7]. Further, in clinical trials, the combination of CDDP and irinotecan or CDDP and NVB for advanced NSCLC has been reported to be beneficial [3, 16, 18]. It was therefore considered worthwhile to evaluate the combination of CDDP, NVB and irinotecan for advanced NSCLC. We report an in vitro study of this combination.

Isobologram analysis has been widely used for evaluating the combined effect of two antitumor drugs in vitro [14, 23, 24]. We extended two-dimensional isobologram analysis to three dimensions for evaluating the combined effect of the three drugs.

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Materials and methods

Cell lines

The human NSCLC cell lines EBC-1 (squamous cell carcinoma), PC-3 (adenocarcinoma) and RERF-LC-MS (adenocarcinoma) were obtained from the Japanese Cancer Resources Bank (JCRB). These cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin and 10% fetal calf serum (FCS).

Chemicals

CDDP was provided by Bristol-Myers-Squibb Co., Tokyo, Japan. NVB was provided by Kyowa Hakko Kogyo Co. Ltd., Shizuoka, Japan. SN-38 (7-ethyl-10-hydroxycamptothecin) was provided by Yakult Honshya Co. Ltd., Tokyo, Japan. SN-38 is an active metabolite of irinotecan [13]. CDDP and NVB were dissolved in physiological saline at a concentration of 2 m*M*, respectively, and SN-38 was dissolved in physiological saline at a concentration of 1 mg/ml. Drugs were diluted to the designated concentrations with culture medium.

Cell growth inhibition and MTT assay

Growing cells in the logarithmic phase were harvested and resuspended to a final concentration of 1.6×10^5 cells/ml (EBC-1, PC-3) or 4×10^4 cells/ml (RERF-LC-MS) in fresh medium with 10% FCS. Cell suspensions (50 µl) were dispensed into 96-well tissue culture plates. Each plate had one 12-well control row containing medium alone and one 12-well control row containing cells without chemotherapeutic agents. After incubation at 37 °C for 24 h, solutions of the three drugs at various concentrations were added into 96-well tissue culture plates. The layout of the three-drug combinations on the 96-well plates is shown in Fig. 1. After 4 days exposure to the chemotherapeutic agents, viable cell growth was determined using a tetrazolium-based colorimetric assay (MTT assay) [19]. The assay is dependent on the cellular reduction of a tetrazolium salt, 3-(4, 5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of viable cells to blue formazan which can be measured spectrophotometrically. A final concentration of 0.5 mg/ml MTT was added to each well. After 3–4 h incubation at 37 °C with MTT, the unreacted MTT and medium were removed and 150 µl dimethyl sulfoxide (DMSO) was added to solubilize the MTT formazan.

After shaking the plates, the optical density (OD) of each well was measured with a microplate spectrophotometer (EAR 400 AT, SLT-Labinstruments, Salzburg, Austria) equipped with a 540-nm filter. The spectrophotometer was calibrated at 0 absorbance using wells that contained only medium and MTT. The OD of wells containing cells and MTT (maximum dye reduction) was used as control. The percent cytotoxicity was calculated from the following equation: % cytotoxicity = $[1 - (\text{OD drug-treated})/(\text{OD control})] \times 100$. All tests were performed on six samples and mean values were calculated. Each experiment was repeated at least four times. Drug concentrations producing 50% inhibition of cell growth (ID_{50}) were estimated from a graph drawn by the curve-fitting program NGRAPH (NEC Fundamental Research Laboratories, Tsukuba, Japan).

Isobologram analysis

The effect of combining two agents on the ID_{50} was analyzed using an improved isobologram method described previously [14, 23, 24]. Based on the dose-response curves of two agents, three isoeffect lines, mode I, mode II_a and mode II_b, were drawn (Fig. 2). Two isoeffect lines which surrounded the maximum area (mode I and mode II_a in Fig. 2) were selected. Three sets of isoeffect lines for two agents (drug A vs drug B, drug A vs drug C, and drug B vs drug C) were then constructed with three-dimensional coordinates (Fig. 3). The three-dimensional coordinates of the isoeffect lines and surfaces (inner surface and outer surface) were drawn with a three-dimensional computer aided design (CAD) graphics system. The space surrounded by six isoeffect lines and five surfaces was defined as the envelope of additivity corresponding to the envelope of additivity in two-dimensional isobologram analysis (Figs. 2, 3). Each point from the three-drug combination experiment was then plotted on the three-dimensional isobologram. When each point of a drug combination fell within the space of the envelope of additivity (Ad), the combination was regarded as additive. When each point fell in the space surrounded by the inner surface of the envelope of additivity and the three axes of the coordinates (Su), the combination was-regarded was supraadditive. When each point fell in the space between the outer surface of the envelope and the cube produced from the 1.0 position on the three axes (Sb), the combination was regarded as subadditive. When each point was outside the cube (Pr), the combination was considered to be mutually protective.

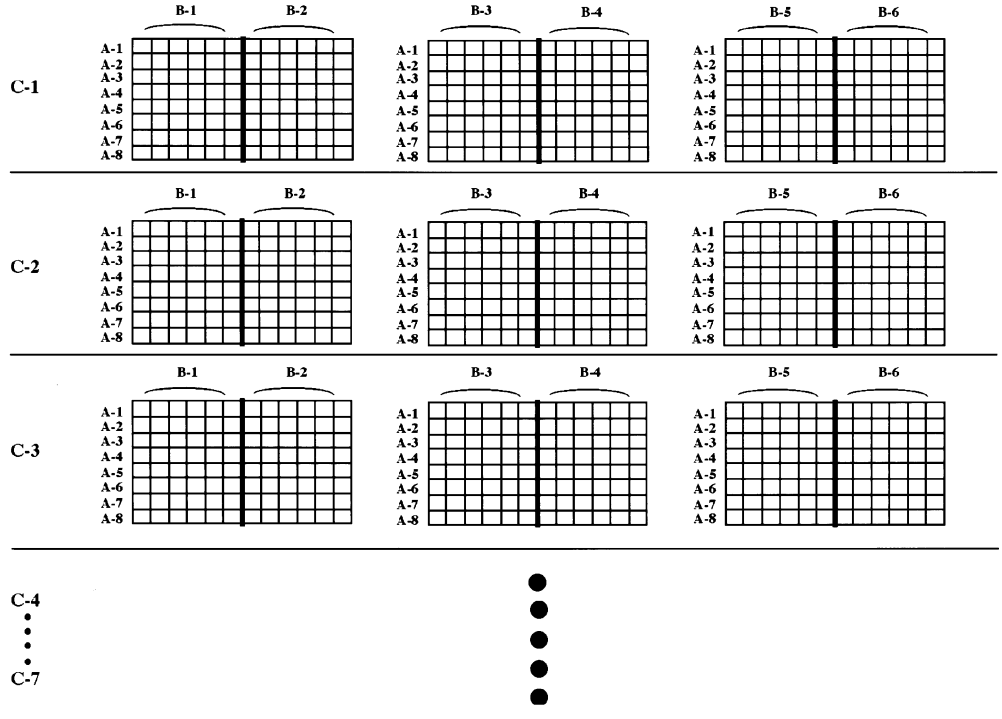

Fig. 1 Layout of the three-drug combinations in the 96-well plates. Serial dilution of drug A is shown from A-1 to A-8 including both negative and positive controls. Serial dilution of drug B is shown from B-1 to B-6. Serial dilution of drug C is shown from C-1 to C-7

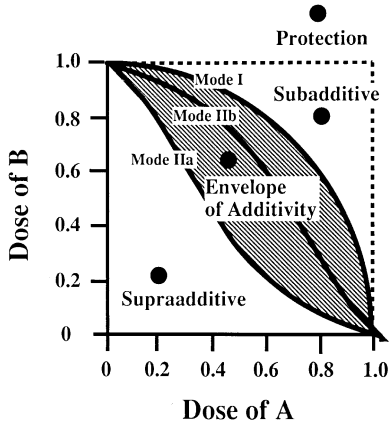

Fig. 2 An isobologram of the combination of drug A and drug B. Mode I, Mode IIa and Mode IIb lines are isoeffect lines drawn from the data from the dose response curves of drug A and drug B

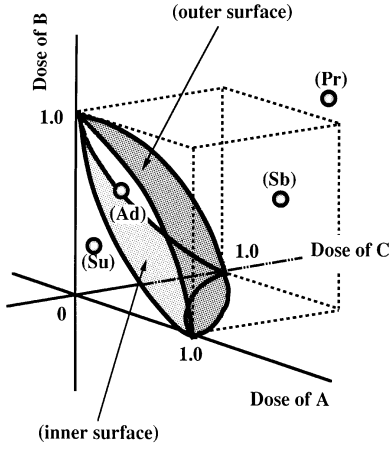

Fig. 3 Three-dimensional isobologram. Three sets of isoeffect lines for two agents (drug A vs drug B, drug A vs drug C, and drug B vs drug C) were constructed in three-dimensional coordinate system

Results

Cytotoxicity of CDDP, NVB and SN-38 in vitro

The cytotoxicity of CDDP, NVB and SN-38 in vitro was assayed against NSCLC cell lines. Single-agent ID_{50} values of these drugs against each of the cell line are shown in Table 1. RERF-LC-MS was much more sensitive to CDDP than EBC-1 and PC-3. In contrast, EBC-1 was much more sensitive to SN-38 than PC-3 and RERF-LC-MS. As for NVB, the values of ID_{50} among these cell lines were not so different.

Table 1 ID_{50} values of CDDP, NVB and SN-38 in non-small cell-lung cancer cell lines. Each value is the mean ± SEM of at least four independent experiments

	ID_{50}		
Cell line	CDDP (μ*M*)	NVB (n*M*)	SN-38 (n*M*)
EBC-1	13.4 ± 1.8	11.7 ± 1.0	2.2 ± 0.2
PC-3	4.6 ± 0.3	13.6 ± 1.6	12.4 ± 0.3
RERF-LC-MS	1.2 ± 0.2	7.6 ± 0.9	81.4 ± 6.1

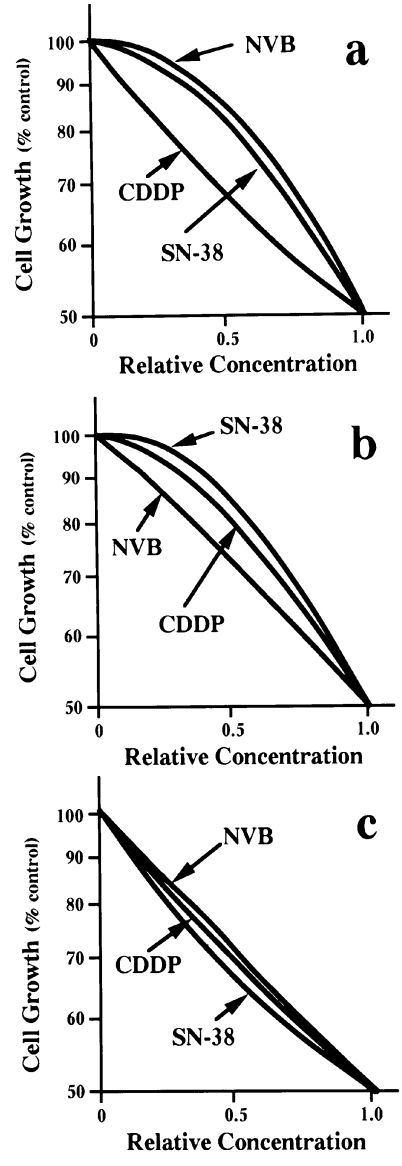

Fig. 4 a–c Dose response curves of three drugs (CDDP, NVB and SN-38) against three human NSCLC cell lines. The growth of cells as a percentage of the growth of control cells were plotted on a log scale versus drug concentrations relative to the ID_{50} on a linear scale (**a** EBC-1, **b** PC-3, **c** RERF-LC-MS)

Isobologram analysis for the three-drug combinations

Figure 4 shows the dose response curves of CDDP, NBV and SN-38 against NSCLC cell lines. Cell survival was plotted on a log scale versus relative concentration of each drug on a linear scale. The relative concentration was defined as the drug concentration divided by the ID_{50} concentration for the particular

cell line. Based on these dose response curves, isoeffect lines were drawn. Figure 5 shows the isoeffect lines for EBC-1 based on the representative results from Fig. 4a. After three-dimensional coordinates of these isoeffect lines were constructed, the data points from the three-drug combination experiments were plotted. Figure 6a shows the three-dimensional isobologram for the cell line EBC-1. The data points fell in the envelope of additivity. This suggests that simultaneous and continuous exposure to CDDP, NVB and SN-38 for EBC-1 produced an additive effect. Figure 6b shows the effect of the three-drug combinations for PC-3. The data points fell in the subadditive area. As for RERF-LC-MS, the envelope of additivity was so narrow that the data points were scattered from the supraadditive to the subadditive area (Fig. 6c). These isobologram

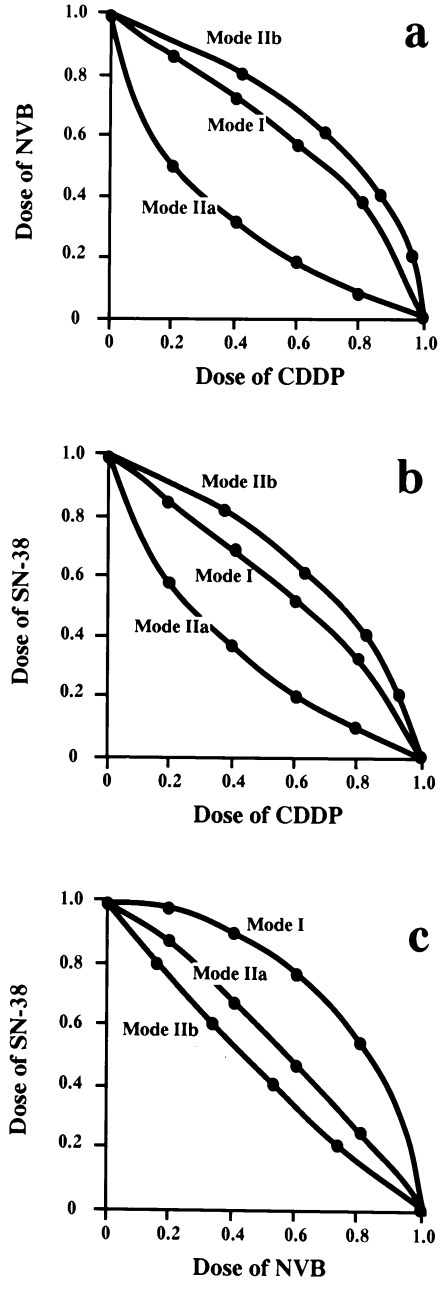

Fig. 5 a–c Isoeffect lines (ID_{50}) against the EBC-1 cell line for two agents out of CDDP, NVB and SN-38. These isoeffect lines were drawn from the data shown in Fig. 4a (**a** CDDP vs NVB, **b** CDDP vs SN-38, **c** NVB vs SN-38)

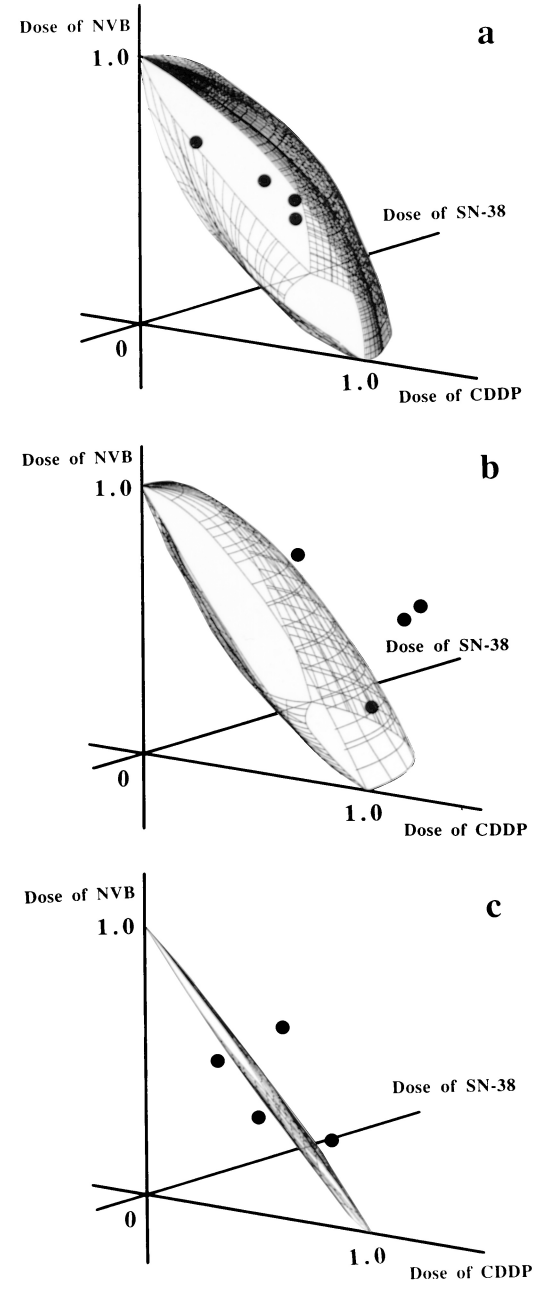

Fig. 6 a–c Three-dimensional isobolograms (ID_{50}) of combinations of CDDP, NVB and SN-38. **a** Combined effect against EBC-1 cells (the data points fell in the envelope of additivity); **b** combined effect against PC-3 cells (the data points fell in the area of subadditivity); **c** combined effect against RERF-LC-MS cells (the data points were scattered from the supraadditive to the subadditive area)

patterns of the drug combinations were reproduced at least three times from independent experiments on each cell line.

Discussion

In most clinical settings, two or more antineoplastic agents are used for chemotherapy of NSCLC because no single agent is likely to be curative [10]. Isobologram analysis has been widely used for evaluating the combined effect of two drugs in vitro. However, evaluation of three-drug combinations in vitro as a preclinical screening test has rarely been reported. We evaluated the effects of a three-drug combination against human NSCLC cell lines in vitro. We modified the isobologram analysis from two agents to three agents. The concept of this isobologram analysis for three drugs was to extend the two-dimensional analysis to three dimensions. Although the method designed for this study was more complex than two-dimensional isobologram analysis, it could be a useful and important method in the selection of chemotherapeutic agents for the treatment of NSCLC patients.

Recently, various approaches to the assessment of drug interactions in in vitro systems have been initially reviewed by Greco et al. [12]. In the review, they mentioned the similarities and differences between the improved isobologram analysis of Steel and Peckham [23] and various other approaches used in in vitro assessment of drug interactions. They suggested that no-interaction null-reference models for the Steel-Peckham approach are based on the Bliss independence reference model [4] (mode I), and another model (mode II) which is not the same as the Loewe additivity model [17] used by many other approaches such as the classical isobologram approaches by Loewe and Muischnek [17] and Elion et al. [8], the interaction index and the mutually exclusive model method of Berenbaum [1, 2], and the median-effect method of Chou and Talalay [5]. Greco et al. [12] also pointed out that the envelope of additivity in the improved isobologram in the method of Steel and Peckham is not a statistical interval, and the method lacks the variability resulting from experimental data. We thus repeated the three-dimensional isobologram experiment independently at least three-times, confirming that the isobologram patterns of the three-drug combination were reproduced.

We selected three anticancer agents, CDDP, NVB and irinotecan. Each of them has been shown to have good single-agent activity against NSCLC. The effect of the combination of SN-38 and CDDP against the hematopoietic cell line MOLT-3 has been assessed by isobologram analysis. It was shown that simultaneous and continuous exposure to SN-38 and CDDP for 3 days has a supraadditive effect (synergy) [15]. In contrast, isobologram analysis has shown that simultaneous exposure of the NSCLC cell line PC-12 to NVB and CDDP for 24 h has a protective effect (antagonism) [11]. However, the results obtained from phase II clinical trials indicate that combination chemotherapy with CDDP and NVB or CDDP and irinotecan is very promising for the treatment of NSCLC [3, 16, 18]. The present study showed that the effects of the combination of CDDP, NVB and SN-38 were additive against EBC-1, subadditive against PC-3 and from subadditive to supraadditive against RERF-LC-MS. Our findings suggest that continuous and simultaneous exposure to CDDP, NVB and irinotecan has an additive effect against NSCLC. Although experimental results obtained from in vitro systems do not always correlate with clinical results with drug combinations, the effect of this three-drug combination showed more than a subadditive effect, and did not show an antagonistic effect in vitro. These results encourage clinical trials using these three agents in combination chemotherapy for the treatment of NSCLC.

References

1. Berenbaum MC (1977) Synergy, additivism and antagonism in immunosuppression. Clin Exp Immunol 28:1
2. Berenbaum MC (1985) The expected effect of a combination of agents: the general solution. J Theor Biol 114:413
3. Berthaud P, Le Chevalier T, Ruffie P, Baldeyrou P, Arriagada R, Besson F, Tursz T (1992) Phase I-II study of vinorelbine (Navelbine) plus cisplatin in advanced non-small cell lung cancer. Eur J Cancer 28A:1863
4. Bliss CI (1939) The toxicity of poisons applied jointly. Ann Appl Biol 26:585
5. 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
6. Depierre A, Lemarie E, Dabouis G, Garnier G, Jacoulet P, Dalphin JC (1989) Efficacy of Navelbine (NVB) in non-small cell lung cancer (NSCLC). Semin Oncol 16:26
7. Depierre A, Lemarie E, Dabouis G, Garnier G, Jacoulet P, Dalphin JC (1991) A phase II study of Navelbine (vinorelbine) in the treatment of non-small-cell lung cancer. Am J Clin Oncol 14:115
8. Elion GB, Singer S, Hitchings GH (1954) Antagonists of nucleic acid derivatives: part VIII. Synergism in combinations of biochemically related antimetabolites. J Biol Chem 208:477
9. Fukuoka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, Kuriyama T, Ariyoshi Y, Negoro S, Masuda N, Nakajima S, Taguchi T (1992) A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer . J Clin Oncol 10:16
10. Ginsberg RJ, Kris MG, Armstrong JG (1993) Cancer of the lung. In: DeVita VT, Hellman S, Rosenberg SA (eds) Cancer: principles and practice of oncology, 4th edn. JB Lippincott Company, Philadelphia, p 673
11. Gomi K, Ohno H, Nomura K, Okabe M, Kobayashi K, Niitani H (1992) Kinetic analysis of combination effect of navelbine (KW-2307) with cisplatin against human lung adenocarcinoma PC-12 cells in culture. Jpn J Cancer Res 83:532
12. Greco WR, Bravo G, Parsons AJ (1995) The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 47:331

13. Kaneda N, Nagata H, Furuta T, Yokokura T (1990) Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. Cancer Res 50:1715
14. Kano Y, Ohnuma T, Okano T, Holland JF (1988) Effects of vincristine in combination with methotrexate and other antitumor agents in human acute lymphoblastic leukemia cells in culture. Cancer Res 48:351
15. Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S, Miura Y (1992) Effects of CPT-11 in combination with other anti-cancer agents in culture. Int J Cancer 50:604
16. Le Chevalier T, Brisgand D, Douillard JY, Pujol JL, Alberola V, Monnier A, Riviere A, Lianes P, Chomy P, Cigolari S, Gottfried M, Ruffie P, Panizo A, Gaspard MH, Ravaioli A, Besenval M, Besson F, Martinez A, Berthaud P, Tursz T (1994) Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small-cell lung cancer: results of a European multicenter trial including 612 patients. J Clin Oncol 12:360
17. Loewe S, Muischnek H (1926) Effect of combinations: mathematical basis of problem. Arch Exp Pathol Parmakol 114:313
18. Masuda N, Fukuoka M, Takada M, Kusunoki Y, Negoro S, Matsui K, Kudoh S, Takifuji N, Nakagawa K, Kishimoto S (1992) CPT-11 in combination with cisplatin for advanced non-small-cell lung cancer. J Clin Oncol 10:1775
19. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55
20. Pignon JP, Stewart LA, Souhami RL, Arriagada R. (1994) A meta-analysis using individual patient data from randomized clinical trials of chemotherapy in non-small cell lung cancer: (2) survival in the locally advanced setting. Proc Am Soc Clin Oncol 13:334
21. Rapp E, Pater JL, Willan A, Cormier Y, Murray N, Evans WK, Hodson DI, Clark DA, Feld R, Arnold AM, Ayoub JI, Wilson KS, Latreille J, Wierzbicki RF, Hill DP (1988) Chemotherapy can prolong survival in patients with advanced non-small-cell lung cancer – report of a Canadian multicenter randomized trial. J Clin Oncol 6:633
22. Souquet PJ, Chauvin F, Boissel JP, Cellerino R, Cormier Y, Ganz PA, Kaasa S, Pater JL, Quoix E, Rapp E, Tumarello D, Williams J, Woods BL, Bernard JP (1993) Polychemotherapy in advanced non small cell lung cancer: a meta-analysis. Lancet 342:19
23. Steel GG, Peckham MJ (1979) Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. Int J Radiat Oncol Biol Phys 5:85
24. Tsai CM, Gazdar AF, Venzon DJ, Steinberg SM, Dedrick RL, Mulshine JL, Kramer BS (1989) Lack of in vitro synergy between etoposide and cis-diamminedichloroplatinum(II). Cancer Res 49:2390