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Kinetic Analysis of Combination Effect of Navelbine (KW-2307) with Cisplatin against Human Lung Adenocarcinoma PC-12 Cells in Culture

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The combination effect of navelbine (NVB, KW-2307), a newly synthesized vinca alkaloid, and cisplatin (CDDP) was compared with that of vindesine (VDS) and CDDP using human lung adenocarcinoma PC-12 cells. The growth-inhibitory activity of NVB or VDS was time-dependent, whereas that of CDDP was AUC (area under the curve)-dependent. When NVB or VDS was used in combination with CDDP simultaneously for 24 h, antagonism was observed in terms of growth-inhibitory activity. However, additive combination effect was observed when NVB or VDS treatment was followed by CDDP treatment. On this treatment schedule, a synergistic combination effect was observed in terms of the cell-killing activity assessed by colony formation assay. The growth-inhibitory activity of NVB or VDS was detected 24 h after the treatment, whereas that of CDDP became significant 72 h after the treatment. NVB and VDS caused cell accumulation in G2M phase at 10 times their IC_{80} values, and cells with less than diploid DNA content were detected after 24 h at IC_{80}. CDDP caused accumulation of cells in S phase, and the effect became detectable 16 h after the treatment. The DNA histogram of cells treated with NVB or VDS in combination with CDDP was a superposition of those of cells treated with each drug alone. Significant differences in the characteristics of anticellular activity were not detected between NVB and VDS, although NVB inhibited cell growth at a slightly lower concentration than VDS at the short exposure time of 1–8 h.

Key words: Navelbine — Vindesine — Cisplatin — Lung cancer — Combination effect

Vinca alkaloids such as vincristine (VCR), vinblastine (VLB) and vindesine (VDS) are mitotic inhibitors and have been used clinically as antitumor chemotherapeutic drugs with a broad spectrum and strong activity.[1] Among them, VDS is more effective against solid tumors such as non-small cell lung carcinoma and esophageal carcinoma, and its efficacy is enhanced by combination chemotherapy with cisplatin (CDDP).[2–6]

Recently a new vinca alkaloid analog, 5′-nor anhydrovinblastine (NVB, KW-2307), was synthesized by Potier *et al.*[7,8] NVB showed antitumor activity equal or superior to that of VCR, VLB or VDS in experimental murine and human tumor models including VCR-resistant murine P388 leukemia.[9,10] NVB was also demonstrated to be a clinically effective antitumor drug against non-small cell lung cancer[11] and advanced breast cancer.[12]

The present study was carried out in order to characterize the combination effect of NVB and CDDP using human lung adenocarcinoma cell line PC-12, and furthermore, to compare it with those of VDS and CDDP.

Abbreviations: AUC, area under the curve; CDDP, cisplatin; CI, combination index; NVB, navelbine; PBS(−), Dulbecco's phosphate-buffered saline; VCR, vincristine; VDS, vindesine; VLB, vinblastine; IC_{50}, concentration required for 50% growth inhibition; IC_{80}, concentration required for 80% growth inhibition.

MATERIALS AND METHODS

Chemicals NVB was provided by Pierre Fabre Médicament, Castre-Cedex, France. VDS was purchased from Shionogi & Co., Osaka. CDDP was purchased from Sigma Chemical Co., St. Louis, Mo. These compounds were dissolved in sterile distilled water and diluted to the designated concentration with culture medium.

Cell line Human lung adenocarcinoma PC-12 cells[13] were passaged in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal bovine serum, 100 units of penicillin and 100 μg/ml of streptomycin (Grand Island Biological Co.).

Growth-inhibitory activity The cells were precultured for 24 h in 24-well multidishes (Nunc, Roskilde, Denmark) containing 0.25 ml of the culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air. Then cells were treated with each drug prepared by two-fold dilution for the indicated time. The drug treatment was terminated by washing the cells twice with PBS(−), and they were placed in drug-free medium. The cell number was counted with a Toa micro-cell counter (Toa Medical Electronics, Ltd., Hyogo) according to the

method previously reported.[14] The combination effect of two drugs was analyzed by means of isobolograms, which were generated from the ratio of IC_{80} values of two combined drugs to that of a single drug.[15-17] Analysis of drug interactions was performed by constructing "an envelope of additivity."[15-17] When the isobolograms are within the envelope of the Model I line and the Model II line, the combination effect of the two drugs is considered to be additive. When the isobolograms fall to the left of the envelope, the two drugs have a synergistic interaction. When the isobolograms are in the area to the right of the envelope, the drugs have a subadditive or protective interaction.

Colony formation-inhibitory activity The cell-killing activity of drugs was determined as follows. PC-12 cells (8×10^2/2 ml/well) were cultured on day 0 in 6-well plates (NUNC), and treated with drugs for the indicated period. The number of colonies was counted on day 8 by staining them with 0.05% (w/v) crystal violet containing 10% (v/v) formaldehyde. The combination effect of 2 drugs was analyzed by the median effect plot method established by Chou *et al.*[18,19] A combination index (CI) was determined by drawing a least-squares regression line on a computer graphic system.[19] When CI is 1, the interaction is considered to be additive; when CI is less than 1, synergism is indicated, and when CI is over 1, antagonism is indicated.

Cell cycle analysis PC-12 cells (3×10^6/30 ml) were cultured in a plastic flask (NUNC), and NVB, VDS or CDDP was added according to the appropriate treatment schedule. At the indicated time, cells were harvested by treatment with 0.02% EDTA, fixed with 70% ethanol solution, hydrolyzed with 25 μg/ml of ribonuclease A (type 1-A, Sigma Chemical Co.) at 37°C for 30 min, and stained with propidium iodide (Sigma Chemical Co.). These cells were analyzed with a Coulter EPICS C cell sorter (Coulter Electronics, Inc., Hialeah, Fla.). Analysis of cell cycle distribution was carried out by using Bagwell's algorithm.[20]

RESULTS

Kinetic analysis of cell growth-inhibitory activity of NVB, VDS and CDDP Recently Inaba *et al.* demonstrated that cancer chemotherapeutic agents could be classified into two groups, i.e., "AUC-dependent drugs" and "time-dependent drugs," and the appropriate group for a given drug can be assessed by analyzing the relationship between drug concentration and exposure time with a log-log scale graph.[21,22] AUC-dependent drugs had previously been called concentration-dependent drugs, and recently AUC-dependence was demonstrated by the above analysis. We applied this method for the analysis of growth-inhibitory activity of NVB, VDS and CDDP against PC-12 cells (Fig. 1). CDDP showed linear plots with a slope of -1 between 1 and 24 h, suggesting that its growth-inhibitory activity is AUC-dependent. The plots between 24 and 72 h might be explained by its instability in the culture medium, since the residual anticellular activity of CDDP was only about 30% after 24 h (data not shown). On the other

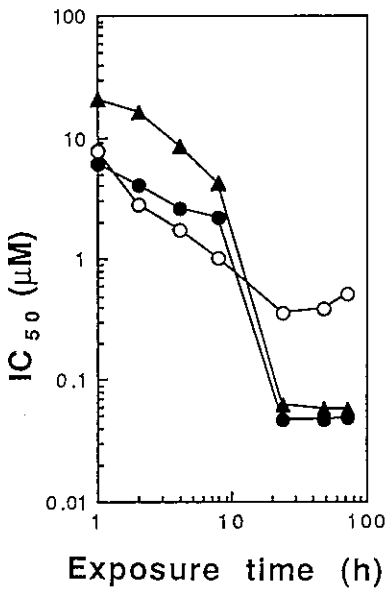

Fig. 1. Exposure time dependency of anticellular activity of NVB (●), VDS (▲) or CDDP (○). PC-12 cells (2.5×10^4/well) were cultured on day 0, and treated with each drug from day 1 for the indicated exposure time. Cell number was counted on day 4.

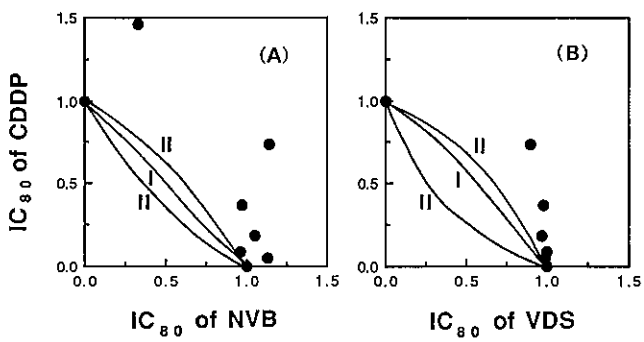

Fig. 2. Isobologram of NVB (A) or VDS (B) in combination with CDDP, in the case of simultaneous exposure for 24 h. PC-12 cells (2.5×10^4/well) were cultured on day 0, and treated with NVB or VDS in combination with CDDP from day 1 for 24 h. Cell number was counted on day 4. The definition of lines I and II is given in "Materials and Methods."

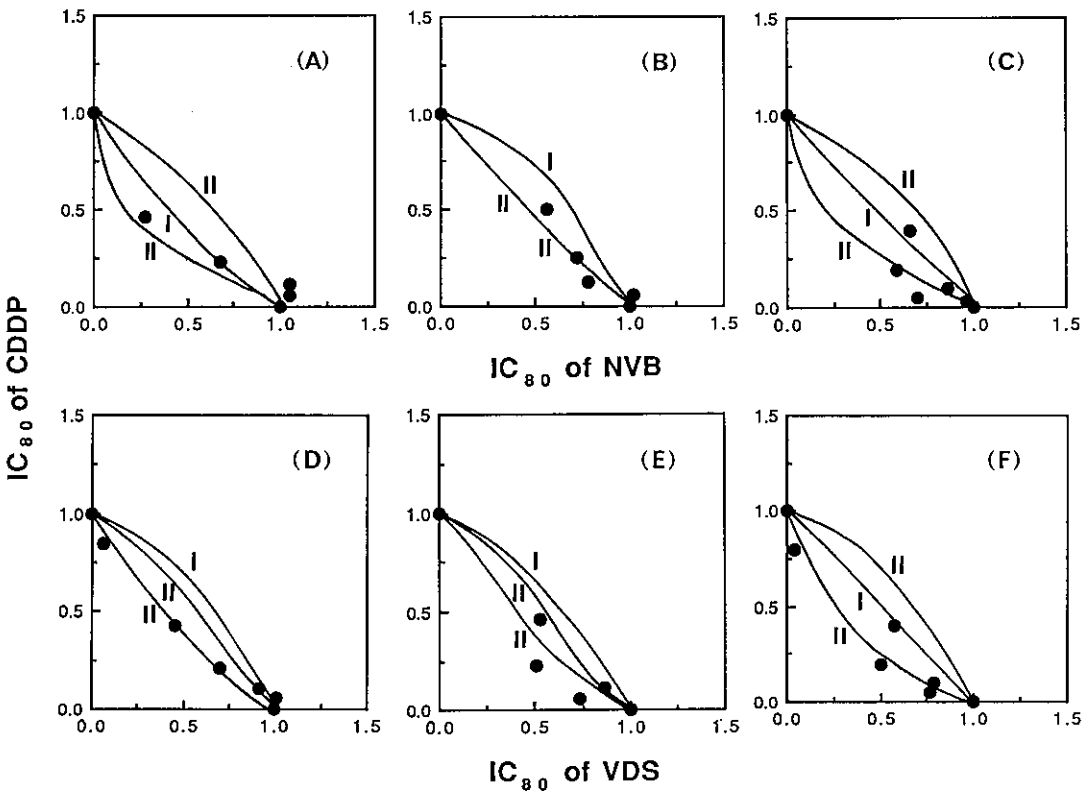

Fig. 3. Isobologram of NVB (A–C) or VDS (D–F) in combination with CDDP, in the case of sequential exposure. PC-12 cells (6.1×10^3/well) were cultured on day 0, and treated with NVB or VDS from day 1 for 24 h. Then the cells were washed and treated with CDDP for 8 h immediately (A, D), or after an interval of 8 h (B, E) or 16 h (C, F). Cell number was counted on day 6. The definition of lines I and II is given in "Materials and Methods."

hand, NVB and VDS showed plots with a steep slope between 8 and 24 h, suggesting that the growth-inhibitory activity of both compounds is time-dependent and cell cycle phase-specific. Since both drugs were stable in the culture medium (data not shown), the plots between 24 and 72 h might be explained by other factors, presumably the threshold of their growth-inhibitory activity. NVB inhibited cell growth at a slightly lower concentration than VDS at the short exposure time of 1–8 h.

Combination effect of NVB or VDS with CDDP The combination effect of NVB and CDDP was compared with that of VDS and CDDP by the isobologram method (Figs. 2 and 3). The IC_{80}, but not IC_{50}, values were used to analyze the combination effect in terms of the cytotoxic action rather than cytostatic action. First the combination effect was examined in the case of simultaneous exposure for 24 h (Fig. 2). Under this condition, both combination regimens of NVB plus CDDP and VDS plus CDDP showed antagonism, since the isobolograms of both combination regimens were outside the envelope. In the next experiment, the combination effect was examined on a sequential treatment schedule, since clinically the administration of VDS is followed by CDDP administration.[3] A 24-h treatment with NVB or VDS was followed immediately by an 8-h treatment with CDDP (Fig. 3 (A, D)), or after an 8-h (B, E) or a 16-h (C, F) interval. Some plots fell to the left of the envelope of the Model II line, suggesting a synergistic interaction of the two drugs. However, as a whole, the effect of all combination regimens was indicated to be additive, since most of the plots were within the envelope. On the basis of this result, further analysis of the combination effect of NVB or VDS with CDDP was performed by sequential treatment as shown in Fig. 3 (A, D).

Analysis of combination effect in terms of colony formation The combination effect of NVB or VDS with CDDP was further examined in terms of the cell-killing activity assessed by colony formation (Fig. 4). All three drugs inhibited the colony formation of PC-12 cells dose-dependently. The cell-killing activity of NVB was nearly equal to that of VDS. For the analysis of combination

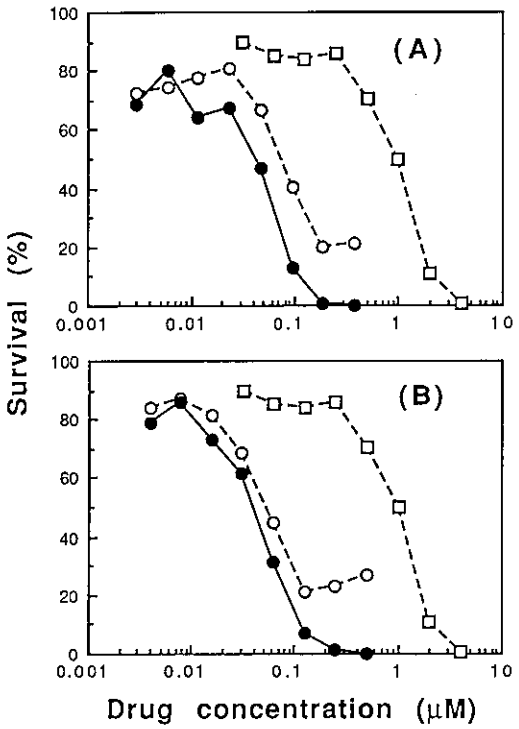

Fig. 4. Inhibition of colony formation by NVB (A) or VDS (B) in combination with CDDP. DC-12 cells (800/well) were cultured on day 0, and 5 h later NVB or VDS was added for 24 h. Then the cells were washed and treated immediately with CDDP for 8 h. Colony number was counted on day 8. The efficiency of colony formation of untreated cells was 20%. ○, NVB or VDS alone; □, CDDP alone; ●, NVB or VDS in combination with CDDP.

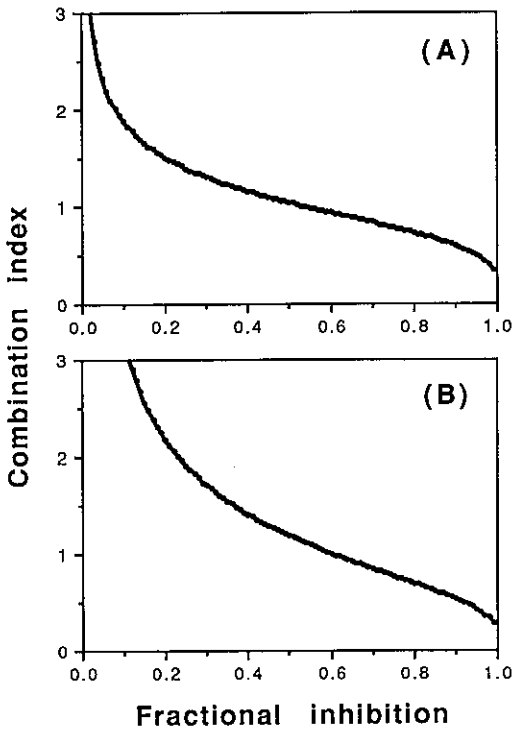

Fig. 5. Combination effect of NVB plus CDDP (A) or VDS plus CDDP (B) was analyzed from the results of Fig. 4 (A) or (B), respectively, by the median effect plot method.

effect, two-fold dilutions of two drugs were prepared, and they were combined with each other from the lowest concentration. For example, 0.00294–0.376 μM NVB and 0.0313–4 μM CDDP were combined, respectively, and 0.00389–0.496 μM VDS and 0.0313–4 μM CDDP. The colony number was counted and the combination effect was analyzed by the median effect plot method (Fig. 5). The CI values of groups treated with NVB plus CDDP or VDS plus CDDP were less than 1 at high concentrations, suggesting that the cell-killing activity in both combination regimens was synergistic. This synergistic activity of NVB and CDDP was almost equal to that of VDS and CDDP. Although antagonism was observed at relatively low concentrations, it was suggested to be irrelevant to the clinical usefulness of both combination regimens, since the cell-killing activity of each drug alone was marginal at these concentrations.

Growth curve of PC-12 cells treated with drugs In advance of the cell cycle analysis, the growth pattern of PC-12 cells was examined on the combination schedule as in Fig. 4 (Fig. 6). The growth-inhibitory effect of NVB or VDS, which was added on day 1, became detectable on day 2 and remained apparent for 2 days. Then the cell number increased gradually. On the other hand, the growth pattern of CDDP-treated cells was different, since the cells grew at almost the same speed as the untreated cells till day 4, and then the cell number decreased, suggesting that the cytotoxicity of CDDP was a delayed type. The combination of NVB or VDS with CDDP decreased the cell number rapidly, indicating that both combination regimens are rational.

Cell cycle analysis A DNA histogram of PC-12 cells treated with NVB or VDS alone is shown in Fig. 7. At 10 times the IC_{80} values, the decrease of cell population in the G1 phase and the accumulation of cells in the G2M phase were observed at 8 h after treatment. After 16 h, most of the cells were arrested in the G2M phase, as expected from the mode of action of the drugs as mitotic inhibitors.

Then the combination effect of NVB or VDS with CDDP was compared by analyzing DNA histograms (Fig. 8). In this experiment, IC_{80} values were used to verify the synergistic cell-killing activity of NVB plus

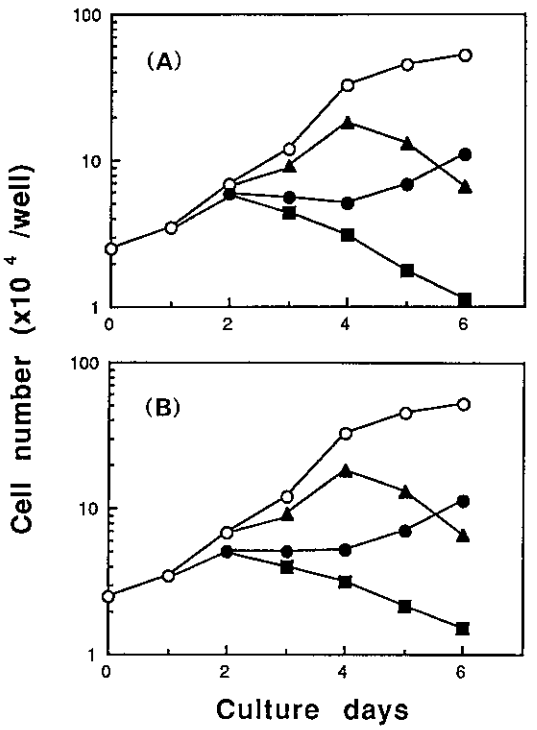

Fig. 6. Growth curve of PC-12 cells treated with NVB plus CDDP (A) or VDS plus CDDP (B). PC-12 cells (2.5×10^4/well) were cultured on day 0, and treated with IC_{80} of NVB (0.073 μM) or VDS (0.11 μM) from day 1 for 24 h. Then the cells were washed and treated immediately with IC_{80} of CDDP (1.45 μM) for 8 h. Cell number was counted on the indicated day. ○, untreated; ●, NVB or VDS alone; ▲, CDDP alone; ■, NVB or VDS in combination with CDDP.

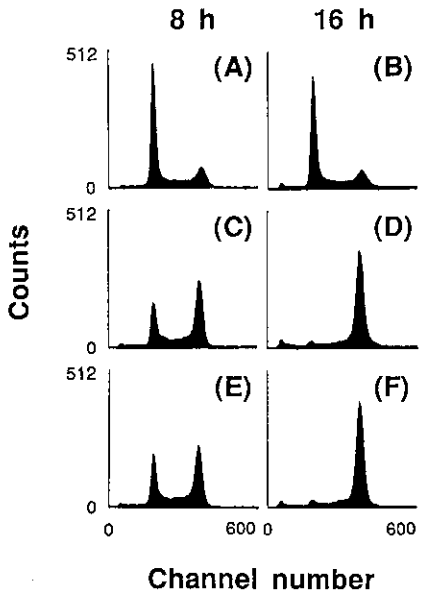

Fig. 7. DNA histogram of PC-12 cells untreated (A, B) or treated with 0.73 μM NVB (C, D) or 1.1 μM VDS (E, F). PC-12 cells (3×10^6/30 ml/flask) were cultured on day 0, and treated with each compound from day 1 for the indicated time. The DNA histogram was obtained by flow cytometry.

CDDP or VDS plus CDDP as shown in Fig. 5. At these concentrations, the effect of NVB or VDS alone was less significant than that shown in Fig. 7, although a slight decrease of cell population in the G1 phase and accumulation of cell population in the G2M phase were observed after 8 h, or rather, cells with DNA content less than that in G1 phase became detectable after 24 h. In the cells treated with CDDP alone, the accumulation of cells in the S phase became detectable after 16 h. The DNA histogram in the case of the combination of NVB plus CDDP or VDS plus CDDP was a superposition of those for each drug alone.

DISCUSSION

To elucidate the feasibility of combination cancer chemotherapy, it is important to understand the characteristics of each drug in terms of kinetics of anticellular activity or cell cycle specificity.[21,22] We observed various differences between NVB or VDS and CDDP as follows: namely, in the kinetics of anticellular activity (Fig. 1), pattern of cell growth (Fig. 6), and DNA histogram (Figs. 7 and 8). These different characteristics and kinetics of anticellular activity might be involved in the synergistic cell killing activity as shown in Figs. 4 and 5.

When NVB or VDS was used in combination with CDDP simultaneously for 24 h, antagonism was observed (Fig. 2). Antagonism between vinca alkaloids and CDDP in the case of simultaneous exposure had already been reported in human lung carcinoma A549 cells.[23,24] The antagonism of vinca alkaloids and CDDP may be explained by their different effects on the cell cycle distribution.[24] Namely, NVB or VDS caused cell accumulation in the G2M phase (Figs. 7 and 8), and as a result, the growth-inhibitory activity of CDDP might be suppressed. In fact, the isobologram plots shown in Fig. 2 seem to indicate that NVB or VDS suppressed the growth-inhibitory activity of CDDP. However, another possibility exists, i.e., that CDDP interferes with the major action site of vinca alkaloids.[24] In any case, sequential treatment with VDS and CDDP was preferable as regards the combination effect.[23] Our results also indicate the superiority of sequential treatment with vinca alkaloids and CDDP (Figs. 3–5). The combination effect of NVB or VDS with CDDP was additive in terms of cell growth-inhibitory activity (Fig. 3), and synergistic

Fig. 8

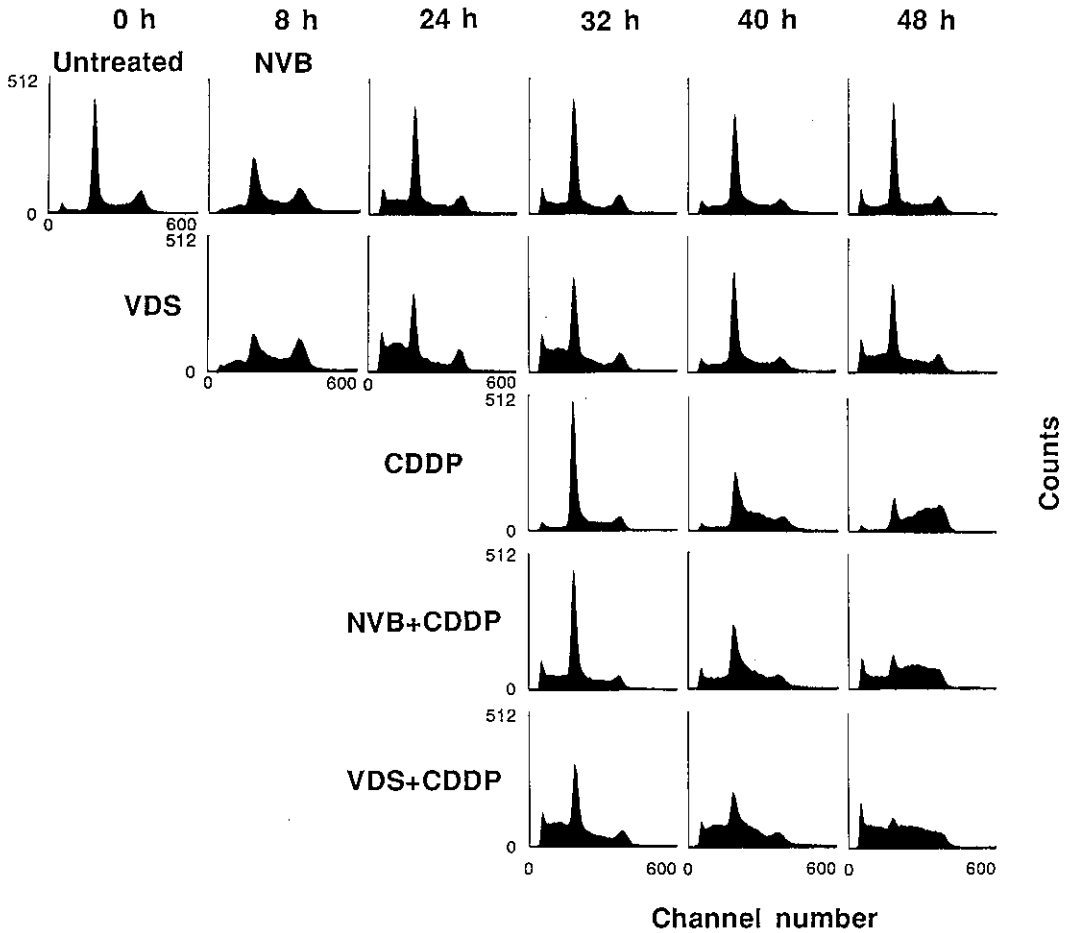

Fig. 8. DNA histogram of PC-12 cells treated with NVB or VDS in combination with CDDP, in the case of sequential exposure. PC-12 cells (3×10^6/30 ml/flask) were cultured on day 0, and treated with 0.073 μM NVB or 0.11 μM VDS from day 1 (designated as 0 h) for 24 h. The cells were then washed and treated with 1.45 μM CDDP for 8 h. DNA histogram was obtained by flow cytometry.

in terms of cell-killing activity (Figs. 4 and 5). In the assay of cell-killing activity, two drugs were combined from low concentration to analyze their combination effect in terms of CI values. The synergism was observed with the combinations of two drugs at relatively high concentration (Fig. 5), suggesting that combination chemotherapy of NVB and CDDP may be rational when they are given at full doses.

NVB and VDS caused cells to accumulate in the G2M phase at 10 times IC_{80} (Fig. 7), and CDDP caused accumulation in the S phase at IC_{80} (Fig. 8). These results on VDS and CDDP are in agreement with the previous reports.[25-28] However, in cells treated with NVB or VDS at IC_{80}, the accumulation of cells in the G2M phase was less significant as compared with that at 10 times their IC_{80}, or rather, the cells with less-than-diploid DNA content became detectable after 24 h (Fig. 8). The accumulation of these cells has already been reported for VDS, VCR and VLB,[27] and camptothecin,[29] although its significance remains undetermined. Since IC_{80} value may be a better measure of physiological significance, the results on NVB or VDS shown in Fig. 8 seem to be meaningful.

The cell cycle-phase specificity of NVB was similar to that of VDS (Figs. 7 and 8). However, the IC_{50} value of NVB against PC-12 cells was a little smaller than that of VDS (Fig. 1). On the other hand, the maximum tolerated dose of NVB was severalfold higher than that of VDS in both animal experiments[9,10] and clinical studies.[11,12] The superior antitumor efficacy of NVB in clinical studies may be due to its reduced toxicity in humans.

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