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Preclinical paper

Effect of vesnarinone in combination with anti-cancer drugs on lung cancer cell lines

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Vesnarinone, a quinolinone derivative which is used as an oral inotropic agent in the clinic, has recently also been shown to have anti-cancer activity. We have studied the anti-cancer effect of vesnarinone in combination with cisplatin, VP-16 (etoposide) and gemcitabine, against human lung cancer cell lines (PC-9 and Lu 134A) using the MTT assay and isobologram analysis. Simultaneously, by establishing two cisplatin-resistant sublines, i.e. PC-9/CDDP and Lu 134A/CDDP, we analyzed the cross-resistance between vesnarinone and cisplatin and the resistance-reversing effect of vesnarinone. Nuclear fragmentation, as the presumed mechanism of tumor cell growth inhibition, was further studied quantitatively using flow cytometric analysis. Combination of vesnarinone with the studied anti-cancer drugs had a synergic or additive inhibitory effect on both PC-9 and Lu 134A tumor cell growth. Neither decrease of the sensitivity to vesnarinone nor cross-resistance between vesnarinone and anti-cancer drugs was observed. On the contrary, vesnarinone showed a resistance-reversing effect. Both vesnarinone and the studied anti-cancer drugs could induce tumor cell apoptosis, but a definite correlation between nuclear fragmentation and the growth inhibitory effect was not established. [© 1999 Lippincott Williams & Wilkins.]

Key words: Anti-cancer drugs, apoptosis, lung cancer cell lines, vesnarinone.

Introduction

Vesnarinone (OPC-8212; 3,4-dihydro-6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-2-(1H)-quinolinone), a quinolinone derivative, is widely used to augment myocardial contractility with less influence over heart rate and myocardial oxygen consumption.[1–4] It is known that vesnarinone affects myocardial contractility by decreasing potassium currents, inhibiting phosphodiesterase activity and increasing the inward calcium current.[5–7] Recently some new biological activities of vesnarinone have been reported such as the inhibition of cytokine production,[8] HIV replication[9] and tumor cell proliferation.[10–12] To evaluate the efficacy of the combined use of vesnarinone with some established anti-cancer drugs, we investigated its *in vitro* effect in combination with cisplatin, VP-16 (etoposide) and gemcitabine on human lung cancer cell lines. The lines we used were PC-9, an adenocarcinoma cell line, and Lu 134A, a small cell carcinoma line.The effects of the drugs were studied by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] assay, isobologram and flow cytometric analysis.

Materials and methods

Lung cancer cell lines

Two human lung cancer cell lines, i.e. PC-9 (an adenocarcinoma cell line) and Lu 134A (a small cell carcinoma cell line), were used in the study. PC-9 was established by Y Hayata, (Tokyo Medical College, Japan). Lu134A was established by T Terasaki (National Cancer Reserch Institute, Tokyo, Japan) and deposited by RIKEN Cell Bank (Tsukuba , Japan). Both were maintained as a suspension in complete medium which contained RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Flow, Irvine, UK).

Agents

Vesnarinone was kindly provided by Otsuka Pharmaceutical (Tokushima, Japan), cisplatin and VP-16 by Nihon Kayaku (Tokyo, Japan), and gemcitabine by

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Nihon Eli Lilly (Kobe, Japan). Vesnarinone was first dissolved in 1 ml of 1N HCl, then diluted in 9 ml of fetal bovine serum and added to the culture medium at a final concentration of 30 or 60 μg/ml. The same volume of vehicle solution instead of vesnarinone was used as a control in each experiment. Cisplatin was prepared by dissolving in normal saline solution at final concentrations of 1.0, 0.5, 0.25, 0.125 and 0.0625 μg/ml. VP-16 was dissolved in dimethyl sulfoxide (Sigma, St Louis, MO) at final concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 μg/ml. Gemcitabine was dissolved in sterilized water at 0.05, 0.025, 0.01 and 0.005 μg/ml separately. Aliquots of 25 μl of these agents were added to the complete medium before cell culture.

Evaluation of cell growth inhibition

The inhibitory effect on the tumor cell growth was evaluated by the MTT assay. The cultured cells of PC-9 and Lu 134A in the exponential phase were harvested from the media and resuspended using complete medium to a final concentration of 1×10^4 and 1×10^5 cells/ml, respectively. Then 100 μl of the suspension was seeded into a 96-well tissue culture plate (Iwaki Glass, Tokyo, Japan). In each plate, 12 wells containing medium alone and 12 wells containing cultured cells without drugs were used as controls. They were incubated under a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 4 h, and then 25 μl of the prepared drug solutions at different concentrations as described above was added to each of the wells, followed by additional incubation for 4–7 days. The cell number was determined by the MTT assay.[13] Briefly, MTT (Sigma) was dissolved in calcium-free and magnesium-free PBS buffer (Nissui Pharmaceutical) at a concentration of 5 mg/ml. Then 25 μl of the MTT solution was added to each wells. Following 4 h incubation at 37°C, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added and a further overnight incubation at room temperature was continued to allow the MTT-formazan product to be dissolved. Absorbance at 570 nm was measured using a microplate reader (Model NJ-2300; Nippon BioRad, Osaka, Japan). Dose-response curves were plotted as percentages of the control cell number that were processed simultaneously, but without drug exposure. Each experiment was repeated at least 3 times.

Isobologram analysis

The combination effect of vesnarinone and anticancer drugs at the ID_{60} point was analyzed according to the method of Kano *et al.* by constructing an envelope of additivity on an isobologram.[14,15] The ID_{60} value was defined as the drug concentration inhibiting cell growth by 60% compared to the control cells using MTT assay. Based on the dose-response curves, the combination effect of every two drugs at the ID_{60} point was determined. Three isoeffect curves were drawn as follows.

Mode I line. When the dose of drug A was chosen, there remained an increment in the effect due to drug B. If two drugs acted independently, addition was performed by taking the increment in doses starting from zero, that produced log survivals that added up to the ID_{60}.

Mode II(A) line. When the dose of drug A was chosen, an isoeffect curve could also be calculated by taking the dose increment of drug B that gave the required contribution to the total effect up to the limit, in this case the ID_{60}.

Mode II(B) line. When the dose of drug B was chosen, an isoeffect curve could be calculated by taking the dose increment of drug A that gave the required contribution to the ID_{60}. When the dose-response curve of drug A followed first-order kinetics, the Mode II(B) line would be identical to the Mode I line and vice versa. When both drugs followed first-order kinetics, all three isoeffect lines would converge to make a straight line connecting 1.0 of the ordinate and abscissa. The total area enclosed by these three lines would be considered to represent an additive response or/and an envelope of additivity.

With combinations of graded doses of drug A and a chosen dose of drug B, a single dose-response curve could be drawn. When the experimental ID_{60} concentration in this combination fell into the left of the envelope, it would mean that the two drugs had a synergic interaction. When the experimental data point was within the envelope, this combination was considered to be non-interactive (additive). If this point was in the right area of the envelope, but within the square produced by 1.0 of the ordinate and abscissa, the drugs were considered to have a subadditive interaction. By contrast, if the point lay outside of the square, both drugs were mutually protective. We found that the isoeffect curves had a reasonable variation as the experiment was repeated, but similar tendencies were observed. In this case the typical isobologram was chosen after reproducible data were obtained by repeated experiments.

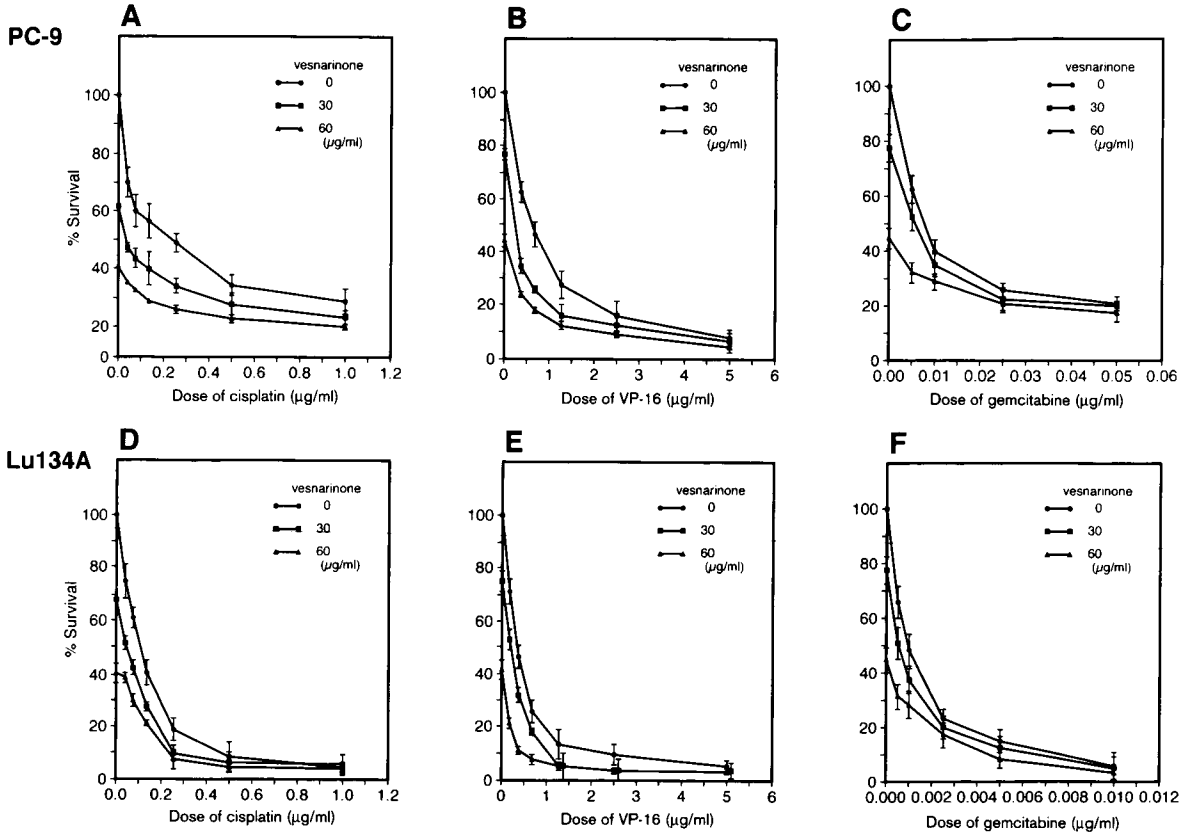

Figure 1. Dose–responses of PC-9 (A–C) and Lu 134A (D–F) against vesnarinone in combination with anti-cancer drugs showing an enhancing inhibitory effect as vesnarinone was combined with cisplatin (A and D), VP-16 (B and E) or gemcitabine (C and F) and as their doses increased.

Cross-resistance and resistance-reversing effect

Cisplatin (CDDP)-resistant sublines (PC-9/CDDP and Lu 134A/CDDP) were established by culturing their parental cell lines with gradually added and increased doses of cisplatin. The PC-9 and Lu 134A cells were cultured first with 0.05 μg/ml of cisplatin for 4–7 days. The live cultured cells were adjusted to a concentration of 1.0×10^4 for PC-9 and 1.0×10^5 for Lu 134A, and further cultured with 0.07 μg/ml of cisplatin. With repeated culture for 2–3 months and gradually increased doses of cisplatin to a final added concentration of 1.8–2.0 μg/ml, the live cells or the sublines (PC-9/CDDP and Lu 134A/CDDP) had acquired resistance to cisplatin. The degree of resistance of each line was expressed as the ID_{50} value, defined as the drug concentration inhibiting cell growth by 50% in comparison with the control cells using the MTT assay. The cross-resistance between vesnarinone and cisplatin was determined by comparing the ID_{50} of parental cell lines to vesnarinone with that of the sublines. The resistant-reversing effect of vesnarinone was analyzed by exposing to vesnarinone (30 μg/ml) and cisplatin simultaneously, and comparing the ID_{50} of parental cell lines to cisplatin with that of the sublines.

Flow cytometric analysis of nuclear fragmentation

Nuclear fragmentation was assayed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using propidium iodide (PI; Sigma).[16] The cultured cells were collected through centrifugation, resuspended gently in 1.0 ml of hypotonic fluorochrome solution (50 μg/ml of PI in 0.1% sodium citrate plus 0.1% Triton X-100) and then placed in the dark at 4°C for overnight before FACScan analysis. The PI fluorescence of individual nuclei was measured and the data were analyzed using specific FACScan research software (Becton Dickinson, San Jose, CA).

Statistical analysis

All data were statistically analyzed by unpaired Student's *t*-test; p values of less than 0.05 were considered to be significant.

Results

Dose–response of the tumor cells against vesnarinone and anti-cancer drugs

Figure 1 showed the dose-response of the tumor cells against vesnarinone in combination with either cisplatin, VP-16 or gemcitabine. As the doses of vesnarinone and anti-cancer drugs increased, the survival rate of the tumor cells reduced markedly ($p<0.01$). A dose-dependent enhancing effect of inhibition was observed on both lung cancer cell lines. Based on the dose-response curves in Figure 1, the isobologram at ID_{60} was produced and is shown in Figure 2.

Inhibitory effect on PC-9 and Lu 134A tumor cell growth

As the cultured PC-9 tumor cells were exposed simultaneously and continuously to vesnarinone and

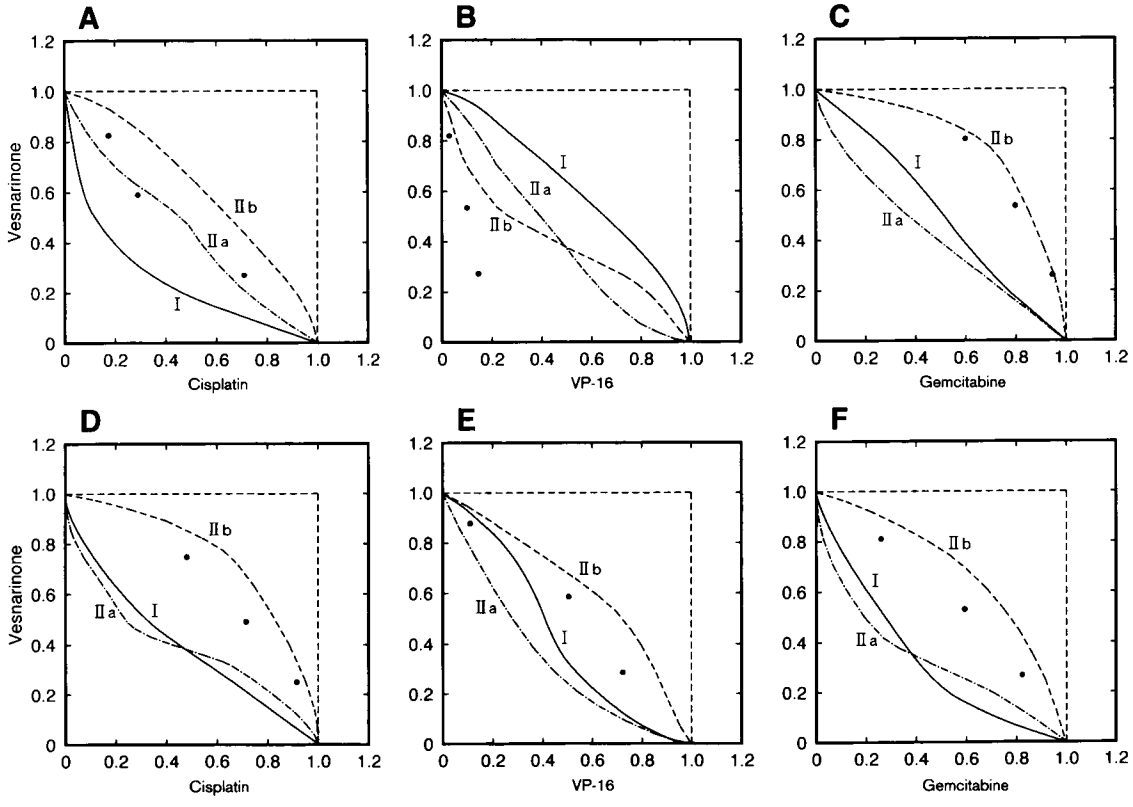

Figure 2. Isobologram analysis indicating a synergic or additive inhibitory effect on PC-9 (A–C) and Lu 134A (D–F) as vesnarinone combined with anti-cancer drugs.

Table 1. Cytotoxicity of vesnarinone on human lung cancer cell lines and their sublines

ID_{50} (mg/ml)[a]		Degree of resistance[b]	ID_{50} (mg/ml)		Degree of resistance
Lu 134A	Lu 134A/CDDP		PC-9	PC-9/CDDP	
46.7±6.4	47.1±5.9	1.0	42.2±3.7	45.7±6.2	1.1

[a]ID_{50} was determined using the MTT assay.
[b]ID_{50} for cisplatin-resistant sublines/ID_{50} for their parental cell lines.

VP-16, the combined data points fell on the left side of the envelope (Figure 2B), indicating a supra-additive (synergic) effect on the PC-9 adenocarcinoma cells. When vesnarinone was combined with cisplatin or gemcitabine, the data points in both combinations fell within the envelope, suggesting an additive effect on the adenocarcinoma cell growth (Figures 2A and C). With the combination of vesnarinone with any one of cisplatin, VP-16 and gemcitabine on Lu 134A cells, all of the data points fell within the envelope as shown in Figure 2(D-F), indicating an additive effect on the small cell carcinoma growth.

Table 2. Cytotoxicity of cisplatin on human lung cancer cell lines and their sublines

	Lu 134A	Lu 134A/CDDP	Lu134A/CDDP with 30 μg/ml vesnarinone	PC-9	PC-9/CDDP	PC-9/CDDP with 30 μg/ml vesnarinone
ID_{50} (μg/ml)	0.14±0.09	0.96±0.21	0.27±0.11	0.28±0.14	2.5±0.9	0.21±0.12
Degree of resistance		6.9	1.9		8.9	0.75

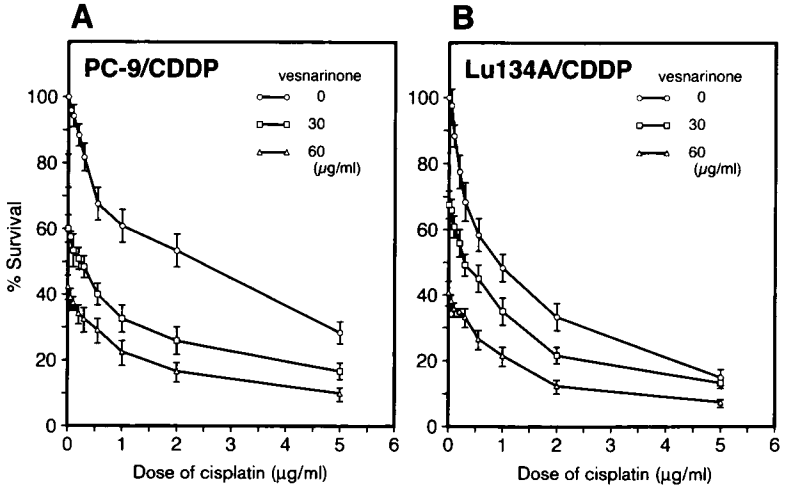

Figure 3. Dose–responses of PC-9/CDDP (A) and Lu 134A/CDDP (B) to vesnarinone and cisplatin demonstrating an enhancing inhibitory effect.

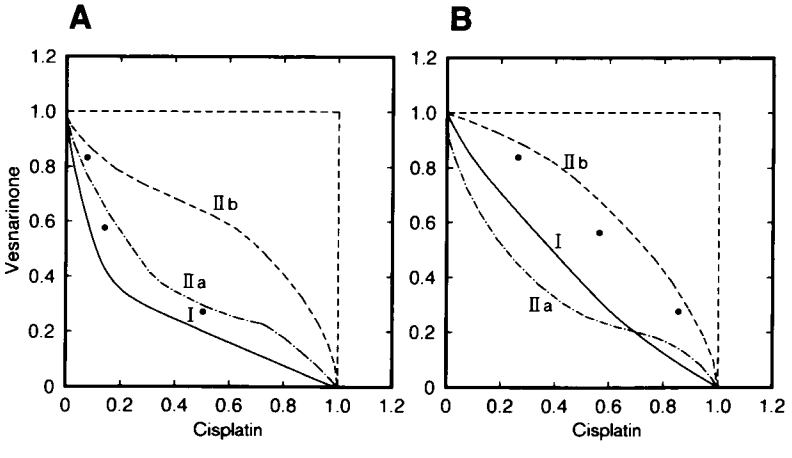

Figure 4. Isobologram analysis indicating an additive effect on PC-9/CDDP (A) and Lu 134A/CDDP (B) by combination of vesnarinone with cisplatin.

Cross-resistance and resistant-reversing effect

The ID_{50} values of cisplatin in PC-9 and PC-9/CDDP cells were 0.28 ± 0.14 and 2.5 ± 0.9, and in Lu 134A and Lu 134A/CDDP cells were 0.14 ± 0.09 and 0.96 ± 0.21, respectively. PC-9/CDDP and Lu 134A/CDDP cells were 8.9 and 6.9 times, respectively, more resistant to cisplatin than their parental cell lines. As the resistance to vesnarinone was analyzed, no significant change was observed between the cisplatin-resistant sublines and their parental cell lines (Table 1) ($p < 0.01$), that suggested that cross-resistance was difficult to induce between cisplatin and vesnarinone. When vesnarinone was used simultaneously with cisplatin, the resistance of PC-9/CDDP and Lu 134A/

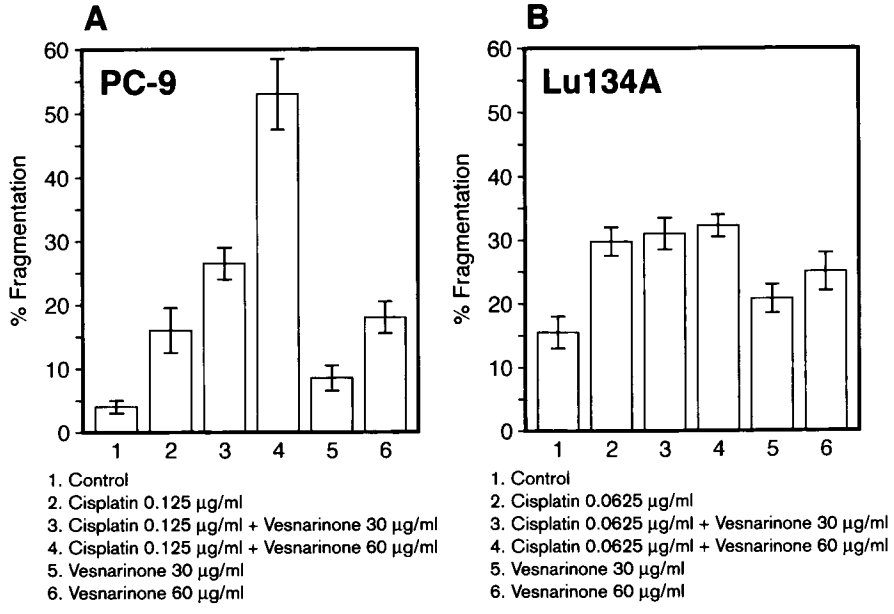

Figure 5. Nuclear fragmentation at the fourth day of exposure to vesnarinone or/and cisplatin. Significant fragmentation of PC-9 was observed (A), but not that of Lu 134A (B).

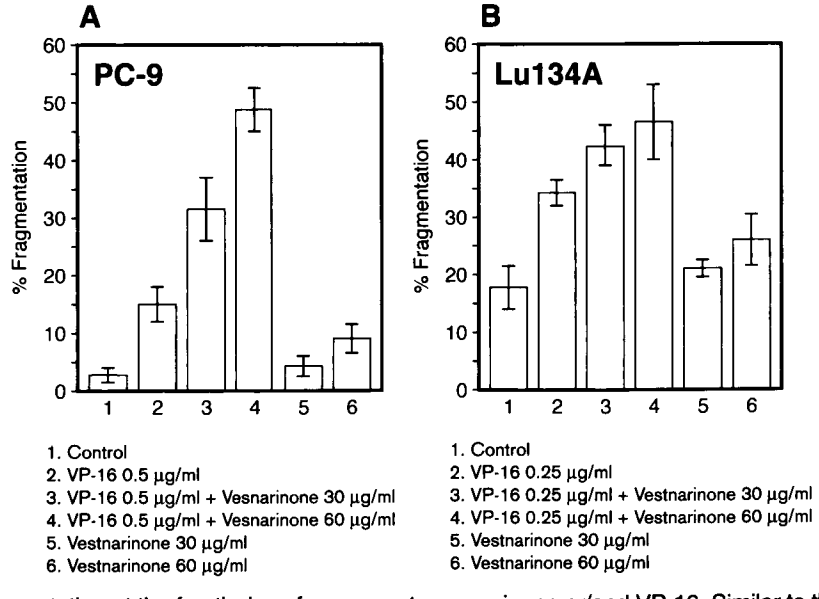

Figure 6. Nuclear fragmentation at the fourth day of exposure to vesnarinone or/and VP-16. Similar to that shown in Figure 5, only PC-9 showed a significant fragmentation (A, PC-9; B, Lu 134A).

CDDP to cisplatin was 0.75 and 1.9 times that of their parental cell lines (Table 2), respectively ($p < 0.01$ for both cell lines), showing a reversing effect of vesnarinone on the resistance of tumor cells against cisplatin. As shown in Figures 3 and 4, the combination of vesnarinone with cisplatin showed an additive inhibitory effect on the growth of either PC-9/CDDP or Lu 134A/CDDP tumor cells.

Nuclear fragmentation of the tumor cells

Nuclear fragmentation was detected quantitatively using flow cytometric analysis. The nuclear fragmentation levels of the PC-9 adenocarcinoma cells significantly increased when vesnarinone was used in combination with either cisplatin, VP-16 or gemcitabine (Figures 5A-7A) ($p < 0.01$ for all combinations), but not the nuclear fragmentation levels of Lu134A small cell carcinoma cells (Figures 5B-7B) ($p < 0.05$).

Discussion

As demonstrated by isobologram analysis, combining vesnarinone with VP-16 showed a synergic inhibitory effect on PC-9 tumor cell growth, whereas combining vesnarinone with each of the other anti-cancer drugs caused additive effects. These results suggest that combination of vesnarinone and VP-16 may be more effective against lung adenocarcinoma than other combinations. Combinations of vesnarinone with any one of the anti-cancer agents demonstrated additive effects on Lu 134A small cell carcinoma cells.

The main side effect of vesnarinone is known to be leukocytopenia with a frequency of 2.5% in the treatment of heart failure.[4] However, this side effect is much lighter than that caused by anti-cancer drugs in general. The usual dose used in the clinic for treating heart failure is sufficient to inhibit tumor cell proliferation. Furthermore, combination of vesnarinone with anti-cancer drugs made it possible to reduce the doses of both vesnarinone and anti-cancer drugs, thus the side effects could be diminished even further. Some chemosensitizing agents, such as caffeine and pentoxyifylline, have only mild anti-cancer effects, but they can improve the cytostatic and cytotoxic effects of anti-cancer drugs.[17-19] Similarly, vesnarinone can possibly also be used as a chemosensitizing drug, as revealed by us.

A gradually acquired resistance to anti-cancer drugs can be acquired by intracellular accumulation of P-glycoprotein.[20-22] This resistance can be circumvented by agents such as verapamil or cyclosporin A which bind with P-glycoprotein and so improve the intracellular accumulation of anti-cancer agents.[23-28] However, severe side effects or too high doses of these agents make them difficult to be applied in the clinic. Using cisplatin-resistant sublines, we have found that vesnarinone had a similar effect to reverse the

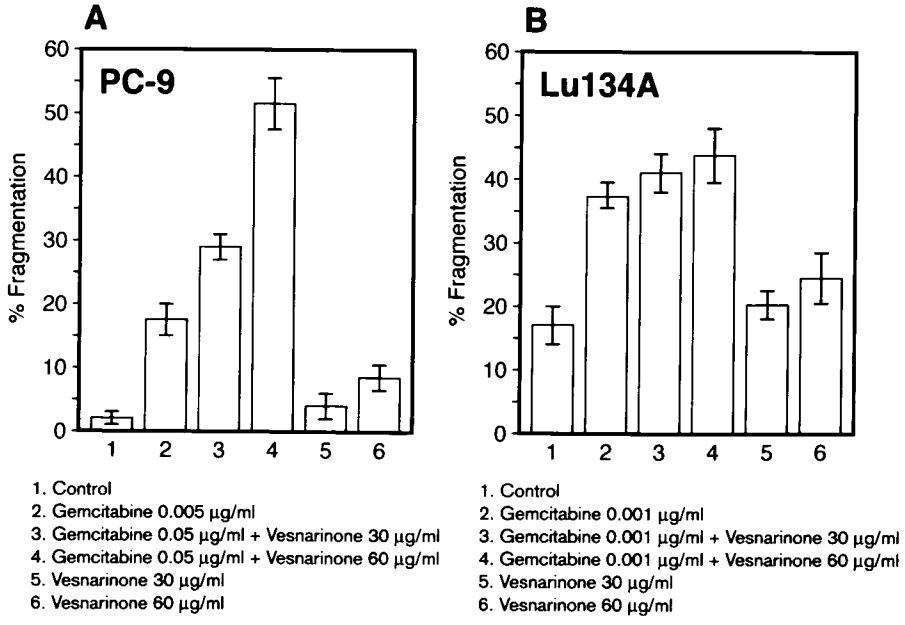

Figure 7. Similar to the results shown in Figures 5 and 6. Combination of vesnarinone with gemcitabine induced an increased level of fragmentation of PC-9 (A), but not of Lu 134A (B).

resistance to cisplatin and there was no evident cross-resistance between them. These results suggest that vesnarinone can also be used as a multidrug-resistant reversing drug. Sato *et al.*[29] have reported the efficacy of vesnarinone in the treatment of a patient with recurrent oral squamous cell carcinoma. Our data suggest that combination of vesnarinone with anti-cancer drugs may be a novel and effective protocol for lung cancer therapy.

The mechanism by which vesnarinone inhibits tumor cell proliferation is still poorly understood. In our previous study, vesnarinone as well as cisplatin, VP-16 and gemcitabine induced the apoptosis of both PC-9 and Lu 134A tumor cells (data not shown), as indicated by nuclear fragmentation. In the present study, although we have confirmed the effects of both tumor cell growth inhibition and apoptosis induction, we did not obtain a correlation between those two effects. This may indicate that the inhibitory effect on the tumor cells is not limited by the apoptosis induction. Some other mechanisms may be also present, e.g. Kumakura *et al.*[30] reported that vesnarinone could prevent the intracellular transport of nucleoside and nucleobase. On the other hand, the reversing effect of vesnarinone on the drug resistance may be similar to other multi-drug-resistant reversing drugs that bind with P-glycoprotein.[31]

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