

# The combination effect of amrubicin with cisplatin or irinotecan for small-cell lung cancer cells

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**Abstract.** The single agent of amrubicin is active in untreated small-cell lung cancer (SCLC). Cytotoxicity of amrubicinol, the active form of amrubicin, was evaluated in a parent SCLC cell line (SBC-3); an active metabolite of irinotecan, 7-ethyl-10-hydroxy-camptothecin (SN-38)-resistant subline (SBC-3/SN-38); and cisplatin-resistant subline (SBC-3/CDDP) using AlamarBlue assay. Interaction of the combined drugs was evaluated by median-effect plot analysis, and the fraction of apoptotic cells was determined using flow cytometry. SBC-3/SN-38 was 34-fold more resistant to SN-38 and SBC-3/CDDP was 7.2-fold more resistant to cisplatin than parental SBC-3. However, these resistant sublines retained sensitivity to amrubicinol (1.8- and 1.7-fold, respectively). Simultaneous exposure of SBC-3/SN-38 cells to amrubicinol and cisplatin showed a synergistic effect. Simultaneous exposure of SBC-3/CDDP cells to amrubicinol and SN-38 displayed synergistic or additive effects. The two-drug combination produced an increase of apoptotic cells compared to each single agent alone in both resistant cells. These findings suggest that amrubicin alone and in combination with cisplatin or irinotecan is effective against SCLC refractory to irinotecan and/or cisplatin.

## Introduction

More than 80% of patients with small-cell lung cancer (SCLC) receiving chemotherapy achieve an objective response;

however, most responders eventually relapse because of drug resistance (1). Since a phase III study in patients with extensive disease (ED)-SCLC demonstrated that a combination regimen of cisplatin and irinotecan yielded a highly significant improvement in survival over a standard regimen consisting of cisplatin and etoposide (2), the combination may be considered the current standard treatment for ED-SCLC. However, the median survival time and 2-year survival rate were only 12.8 months and 19.5%, respectively (2). The development of irinotecan or cisplatin resistance in tumor cells is assumed to play a major role in these unsatisfactory results.

Amrubicin is a totally synthetic 9-aminoanthracyclin (3). Amrubicinol, its converted active form, has 10 to 100 times higher activity than amrubicin in cytotoxicity by inhibiting topoisomerase II (4,5). Antitumor activity of amrubicin was superior to that of the mother compound, adriamycin in human tumor xenografts (6). In addition, amrubicin had less toxicity, including cardiotoxicity, than adriamycin, in experimental animal models (7,8). Amrubicin was highly active (response rate, 78.8%; median survival time, 11.3 months) and well tolerated in a phase II study in untreated patients with ED-SCLC (9). The objectives of this study were to evaluate the antitumor activity of amrubicin for SCLC cells, especially for irinotecan- or cisplatin-resistant cells, and the combination effect of amrubicin with commonly used anticancer drugs against SCLC.

## Materials and methods

**Chemicals and reagents.** Drugs in this study were provided by the following sources: amrubicin (SM5887) and amrubicinol (SM5887-13-OH) from Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan; irinotecan and 7-ethyl-10-hydroxycamptothecin (SN-38) from Yakult Honsha, Tokyo, Japan; etoposide and paclitaxel from Bristol-Myers Squibb, Tokyo, Japan; and cisplatin from Nippon Kayaku Kogyo Co., Ltd., Tokyo, Japan. Amrubicin, irinotecan and cisplatin were dissolved in 0.9% saline, and amrubicinol was dissolved in distilled

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water. SN-38, etoposide, and paclitaxel were dissolved in dimethylsulfoxide. Drug solutions were stored at  $-20^{\circ}\text{C}$ . AlamarBlue (UK Serotec Ltd., Oxford) was purchased from Dainippon Pharmaceutical Co. Ltd, Osaka, Japan.

**Cell culture.** The SBC-3 parent cell line was established from a bone marrow aspirate of a previously untreated patient with SCLC (10). The growth medium (RPMI-FBS) was RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The SN-38-resistant subline (SBC-3/SN-38) (11) and cisplatin-resistant subline (SBC-3/CDDP) (12) were established by continuous exposure of the SBC-3 cells to increasing concentrations of SN-38 and cisplatin, respectively.

**Assay of drug sensitivity.** Drug sensitivity was determined using AlamarBlue assay (13). Briefly, 50  $\mu\text{l}$  of RPMI-FBS containing serial concentrations of each chemotherapeutic agent was prepared in 96-well flat-bottomed microplates (Coster 3596; Corning Inc., Corning, NY, USA). The 50  $\mu\text{l}$  of RPMI-FBS containing 500 cells for SBC-3, 1500 cells for SBC-3/SN-38 and 2000 cells for SBC-3/CDDP was then added to each well. Cells were incubated at  $37^{\circ}\text{C}$  for 96 h in a highly humidified incubator with 5%  $\text{CO}_2$  and 95% air, and then 10  $\mu\text{l}$  of AlamarBlue was added to each well. After incubation at  $37^{\circ}\text{C}$  for 5 h, the fluorescence of each well was measured using Fluoroskan Ascent (LabSystems Inc., Franklin, MA, USA) with 544 nm excitation and 590 nm emission. Fluorescence of a well without chemotherapeutic agents was used as the control, and a well containing only RPMI-FBS and AlamarBlue was used to determine the background. The percentage of surviving cells was calculated using the formula:  $[(\text{mean fluorescence in 4 test wells} - \text{fluorescence in background wells}) / (\text{mean fluorescence in control wells} - \text{fluorescence in background wells})] \times 100$ . The drug concentration required to inhibit growth of tumor cells by 50% ( $\text{IC}_{50}$ ) was determined by plotting the logarithm of drug concentration versus the percentage of surviving cells.

Table I. Drug sensitivity in the SBC-3 parent line, SN-38-resistant subline (SBC-3/SN-38), and cisplatin-resistant subline (SBC-3/CDDP).

	$\text{IC}_{50}$ value (nM)		
	SBC-3	SBC-3/SN-38	SBC-3/CDDP
SN-38	4.1 $\pm$ 1.5	139 $\pm$ 16	13 $\pm$ 4.5
R.R.		34	3.2
Cisplatin	345 $\pm$ 39	120 $\pm$ 15	2480 $\pm$ 120
R.R.		0.35	7.2
Amrubicinol	33 $\pm$ 16	60 $\pm$ 26	57 $\pm$ 20
R.R.		1.8	1.7

$\text{IC}_{50}$ , 50% inhibitory concentration; SD, standard deviation; R.R., relative resistance value ( $\text{IC}_{50}$  value of resistant cells/ $\text{IC}_{50}$  value of SBC-3 cells). Data are expressed as mean  $\pm$  SD.

Determinations were carried out in quadruplicate for each experiment, and results were confirmed by 3 or more separate experiments. Relative resistance was calculated by dividing the  $\text{IC}_{50}$  value of resistant subline cells by the  $\text{IC}_{50}$  of SBC-3 cells.

**Design for drug combination.** The constant-ratio design for the combination assay is highly recommended as it allows the most efficient data analysis (14). After simultaneous exposure of the cells to two drugs for 96 h, growth inhibition was determined using AlamarBlue assay. Sequential exposure of two drugs was performed as follows. After exposure to the first drug for 24 h, cells were twice washed in drug-free medium, and the second drug was then added to the 96-well microplates for 24 h. At the end of exposure, the cells were washed in drug-free medium, re-incubated in drug-free medium for 48 h, and proliferation was measured with AlamarBlue. Experiments were repeated 3 times.

Table II. Combination effect of amrubicinol and other agents.

Cell line	Drugs	Combination index (mean $\pm$ SD)	
		$\text{IC}_{70}$	$\text{IC}_{90}$
SBC-3	AMR-OH + SN-38	1.2 $\pm$ 0.1	1.0 $\pm$ 0.02
	AMR-OH + CDDP	0.82 $\pm$ 0.05	0.35 $\pm$ 0.17
	AMR-OH + PTX	1.3 $\pm$ 0.26	2.4 $\pm$ 0.52
	AMR-OH + ETP	1.1 $\pm$ 0.02	0.85 $\pm$ 0.21
	AMR-OH $\rightarrow$ SN-38	1.0 $\pm$ 0.02	1.1 $\pm$ 0.25
	SN-38 $\rightarrow$ AMR-OH	1.5 $\pm$ 0.32	2.2 $\pm$ 0.17
	AMR-OH $\rightarrow$ CDDP	0.86 $\pm$ 0.15	0.93 $\pm$ 0.32
	CDDP $\rightarrow$ AMR-OH	0.93 $\pm$ 0.12	1.0 $\pm$ 0.06
SBC-3/CDDP	AMR-OH + SN-38	0.76 $\pm$ 0.21	1.0 $\pm$ 0.35
SBC-3/SN-38	AMR-OH + CDDP	0.99 $\pm$ 0.17	0.89 $\pm$ 0.24

AMR-OH, amrubicinol; CDDP, cisplatin; PTX, paclitaxel; ETP, etoposide.

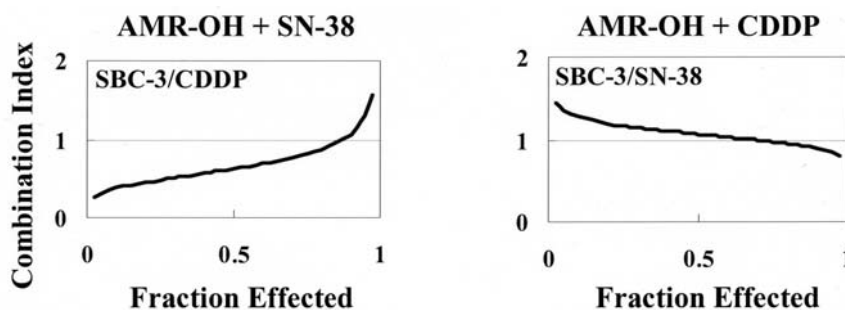


Figure 1. Combination index and surviving fraction of SBC-3/CDDP cells treated with amrubicinol (AMR-OH) in combination with SN-38 simultaneously for 96 h (left). Combination index and surviving fraction of SBC-3/SN-38 cells treated with AMR-OH in combination with cisplatin (CDDP) simultaneously for 96 h (right).

**Median-effect principle for dose-effect analysis.** The multiple drug effect analysis of Chou and Talaly, based on the median-effect principle, was used to calculate the combined drug effect (15). This method involved plotting dose-effect curves for each agent and its combination with other agents by using the median-effect equation:  $fa/fu = (D/D_m)^m$  (equation 1).

In equation 1,  $D$  is the dose,  $D_m$  is the required dose for 50% inhibition of cell growth,  $fa$  is the fraction affected by dose  $D$  (e.g. 0.9 if cell growth is inhibited by 90%),  $fu$  is the unaffected fraction (therefore,  $fa = 1 - fu$ ), and  $m$  is a coefficient of the sigmoidicity of the dose-effect curve;  $m=1$ ,  $m>1$ , and  $m<1$  indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curves, respectively, for an inhibitory drug. Thus, both potency ( $D_m$ ) and shape ( $m$ ) were taken into account as parameters in this method. Equation 2 was rearranged from equation 1 as follows:  $D = D_m[fa/(1-fa)]^{1/m}$  (equation 2).

The  $D_m$  and  $m$  values were easily determined by the median-effect plot;  $x = \log(D)$  versus  $y = \log(fa/fu)$  was based on the logarithmic form of equation 1. In the median-effect plot,  $m$  was slope and  $\log(D_m)$  was the x-intercept. Conformity of data to the median-effect principle could be readily manifested by the linear coefficient ( $r$ ) of the median-effect plot. To obtain a reasonable  $m$  and  $r$ , non-linear points, usually at the lowest or the highest concentrations, were excluded. The 5 to 9 concentrations on a linear line were employed in this analysis. Computer programs based on the median-effect plot parameters and combination index equation have been used for data analysis in the present study (16).

**Combination index for determining synergism and antagonism.** The combination index (CI) isobologram equation was used for data analysis of the two-drug combination:  $CI = (D)A/(D_x)A + (D)B/(D_x)B$  (equation 3).

$CI < 1$ ,  $CI = 1$ , and  $CI > 1$  indicate synergism, additive effect, and antagonism, respectively. Equation 3 dictates that drug A, i.e.  $(D)B$  in the numerators inhibit  $x\%$  when drugs A and B are combined.  $(D_x)A$  and  $(D_x)B$  in denominators of equation 3 indicate doses of drug A and drug B alone, respectively, that also inhibit  $x\%$ .  $D_x$  can be readily calculated from equation 2, where  $D$  is designated for  $x\%$  inhibition. When equation 3 equals 1 (i.e.  $CI=1$ ), it represents the classic isobologram equation. CI at the inhibitory concentration of

70% ( $IC_{70}$ ) and 90% ( $IC_{90}$ ) levels was used for determining synergism, additive effect, or antagonism.

**Flow cytometry.** Flow cytometry for cell cycle traverse perturbations was carried out after staining with propidium iodide using CycleTest Plus DNA Reagent kit (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Drug concentration was based on the  $IC_{50}$  value of a single drug. After 96 h simultaneous exposure to single drug or combined drugs, cells were stained according to the instruction manual. For sequential schedules, after 24 h of exposure to the first drug, cells were twice washed in drug-free medium, and the second drug was then added to cells for 24 h. At the end of exposure, cells were stained with propidium iodide. Flow cytometric analysis was performed on a FACSCalibur (Becton-Dickinson Immunocytometry Systems). Data were analyzed according to ModFit LT software (Verity Software House Inc, Topsham, ME, USA).

## Results

**Cytotoxicity of amrubicinol and other drugs.** Values (mean  $\pm$  standard deviation) for  $IC_{50}$  and relative resistance of SN-38, cisplatin, and amrubicinol for SBC-3, SBC-3/SN-38, and SBC-3/CDDP cells are shown in Table I. Although SBC-3/SN-38 was 34-fold more resistant to SN-38 and SBC-3/CDDP was 7.2-fold more resistant to cisplatin than the parental SBC-3, they retained sensitivity to amrubicinol with relative resistance values of 1.8 and 1.7, respectively.  $IC_{50}$  values of other drugs for SBC-3 cells were: amrubicin,  $862 \pm 46$  nM; irinotecan,  $195 \pm 10.2$  nM; etoposide,  $270 \pm 170$  nM; and paclitaxel,  $0.55 \pm 0.25$  nM.

**Combination effect of amrubicinol with other drugs for SBC-3.** To equalize the contribution of each drug, the ratio of  $IC_{50}$  value for each drug was used as the concentration ratio for the combination (14). Thus, concentration ratios of amrubicinol, SN-38, cisplatin, paclitaxel, and etoposide were designed to be relative ratios of 100: 10: 1000: 1:1000, respectively. CI values for SBC-3 cells treated with amrubicinol after 96 h simultaneous exposure to SN-38, paclitaxel, cisplatin or etoposide are shown in Table II. Amrubicinol and cisplatin showed a synergistic effect, however, amrubicinol and paclitaxel exerted an antagonistic

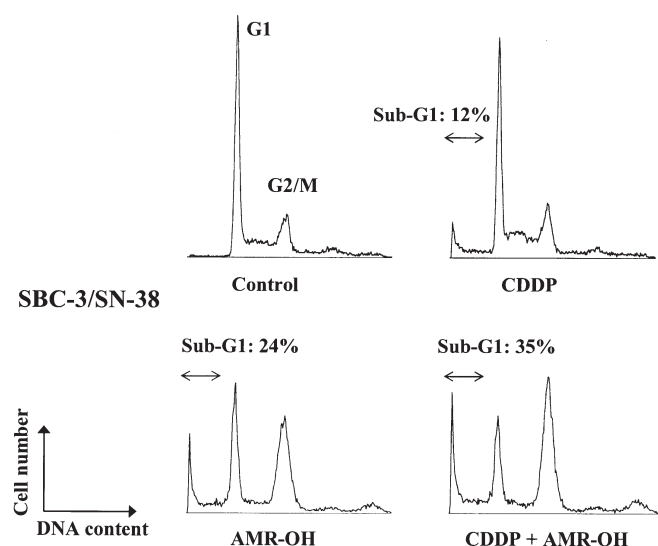


Figure 2. Effect of cisplatin (CDDP), amrubicinol (AMR-OH), or the combination of CDDP and AMR-OH induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3/SN-38 cells.

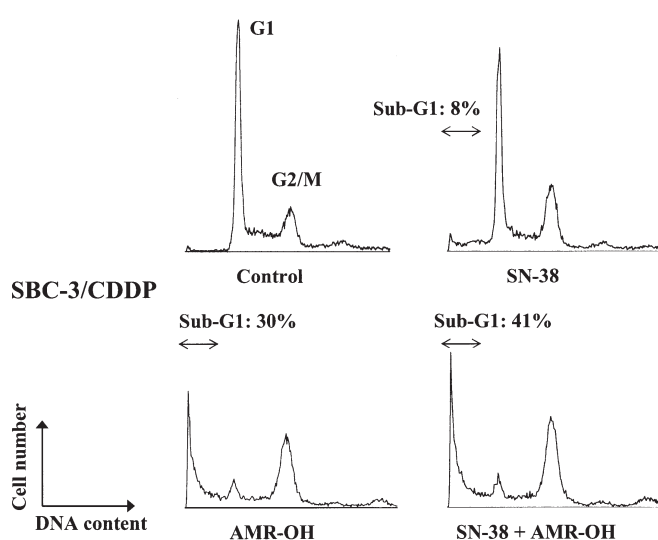


Figure 3. Effect of SN-38, amrubicinol (AMR-OH), or the combination of SN-38 and AMR-OH induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3/CDDP cells.

effect. At  $IC_{90}$ , the combination of amrubicinol and SN-38 showed an additive effect and that of amrubicinol and etoposide displayed a synergistic effect.

**Combination effect of amrubicinol with SN-38 for SBC-3/CDDP and cisplatin for SBC-3/SN-38.** CI values and the surviving fraction of SBC-3/CDDP cells treated by 96 h simultaneous exposure to amrubicinol and SN-38 are drawn in Fig. 1 (left). Based on  $IC_{50}$  values in resistant cells, the concentration ratio of amrubicinol and SN-38 was determined to be 5:1. CI values were  $0.76 \pm 0.21$  at  $IC_{70}$  and  $1.0 \pm 0.35$  at  $IC_{90}$ . Similarly, CI values and the surviving fraction of SBC-3/SN-38 cells treated by 96 h simultaneous exposure to amrubicinol and cisplatin are drawn in Fig. 1 (right). The concentration ratio of amrubicinol to cisplatin was 1:2.

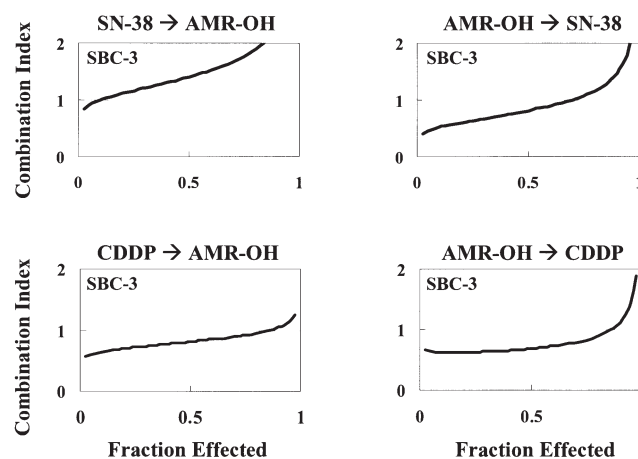


Figure 4. Combination index and surviving fraction of SBC-3 cells treated sequentially with SN-38 or cisplatin (CDDP) for 24 h followed by amrubicinol (AMR-OH) for 24 h and the reverse sequence.

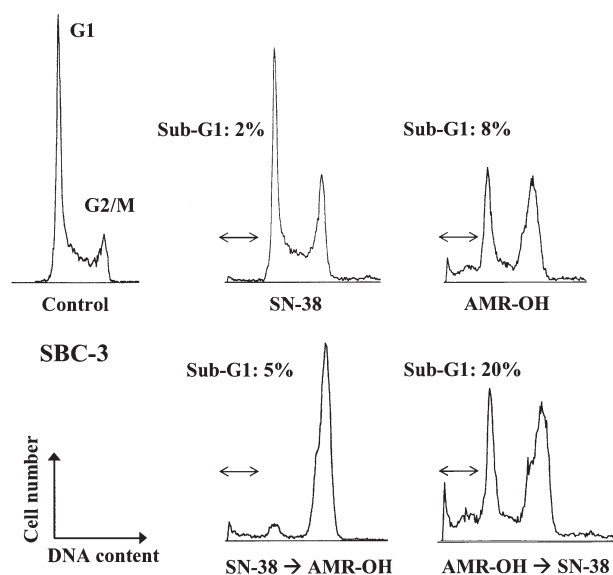


Figure 5. Effect of SN-38, amrubicinol (AMR-OH), SN-38 followed by AMR-OH, or AMR-OH followed by SN-38 induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3 cells.

CI values were  $0.99 \pm 0.17$  at  $IC_{70}$  and  $0.89 \pm 0.24$  at  $IC_{90}$ . Thus, the combination of amrubicinol with SN-38 showed synergistic or additive effects for cisplatin-resistant cells, and amrubicinol with cisplatin displayed a synergistic effect for SN-38-resistant cells. As shown in Fig. 2, an analysis of cell cycle traverse perturbations demonstrated that treating SBC-3/SN-38 cells with amrubicinol (50 nM) alone resulted in an accumulation of cells in the S+G2/M boundary and a measurable increase in the apoptotic cell population (sub-G1, 24%). Cisplatin (100 nM) alone increased apoptotic cells to 12%, however, the combination of these two drugs induced more apoptosis (35%). Similarly, treating SBC-3/CDDP cells with the combination of SN-38 (10 nM) and amrubicinol (50 nM) produced more apoptotic cells (sub-G1, 41%) than SN-38 alone (8%) or amrubicinol alone (30%) (Fig. 3).

**Analysis of combination effect by exposure schedule of amrubicinol and SN-38 or cisplatin for SBC-3.** CI values and



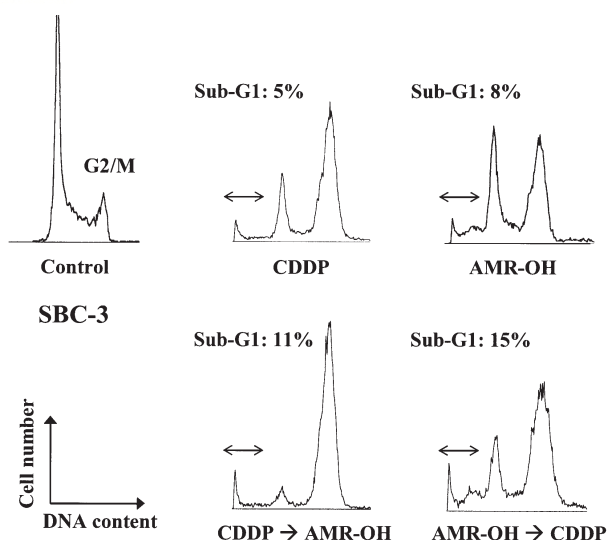


Figure 6. Effect of cisplatin (CDDP), amrubicinol (AMR-OH), CDDP followed by AMR-OH, or AMR-OH followed by CDDP induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3 cells.

the surviving fraction of SBC-3 cells treated sequentially with amrubicinol for 24 h followed by SN-38 or cisplatin for 24 h and those with a reverse sequence are shown in Fig. 4. The sequence of amrubicinol followed by SN-38 was more effective than the reverse sequence. As shown in Fig. 5, analysis of cell cycle traverse perturbations demonstrated that treatment of SBC-3 cells with amrubicinol alone resulted in an accumulation of cells in the S+G2/M boundary and a measurable increase in the apoptotic cell population (sub-G1, 8%). Treating the cells with SN-38 (5 nM) followed by amrubicinol (50 nM) resulted in no marked accumulation of cells at sub-G1 (5%), but the reverse sequence exposure produced a marked increase in apoptotic cells (20%). CI values after exposure to cisplatin followed by amrubicinol were  $0.93 \pm 0.12$  at  $IC_{70}$  and  $1.0 \pm 0.06$  at  $IC_{90}$ , and  $0.86 \pm 0.15$  at  $IC_{70}$  and  $0.93 \pm 0.32$  at  $IC_{90}$  for the reverse sequence. This combination of two drugs appears effective irrespective of sequence. Treatment with amrubicinol (50 nM) followed by cisplatin (500 nM) and the reverse sequence exposure increased the number of apoptotic cells (15% and 11%, respectively) as shown in Fig. 6.

## Discussion

We have established adriamycin-resistant SBC-3/ADM (17), etoposide-resistant SBC-3/ETP (18), cisplatin-resistant SBC-3/CDDP (12), and SN-38-resistant SBC-3/SN-38 cells from SBC-3, which was derived from an untreated SCLC patient (11). Amrubicinol was found to be completely cross-resistant to adriamycin and etoposide in experiments using SBC-3/ADM and SBC-3/ETP cells (19). SBC-3/SN-38 cells had decreased topoisomerase I and II activity and over-expressed breast cancer-resistant protein compared to the SBC-3 cells (11). SBC-3/CDDP cells showed increased intracellular glutathione and glutathione S-transferase content

and decreased intracellular accumulation of cisplatin (12). In the present study, SBC-3/SN-38 and SBC-3/CDDP retained sensitivity to amrubicinol. These results suggest that amrubicinol may be effective for SCLC patients who were previously treated with cisplatin and irinotecan. In addition, the combination of amrubicinol and cisplatin showed a synergistic effect for SBC-3/SN-38 and that of amrubicinol and SN-38 displayed additive or synergistic effects for SBC-3/CDDP. In a phase II study, the combination of amrubicinol and cisplatin was reported to be highly effective for untreated ED-SCLC (20). A combination of amrubicinol and irinotecan was feasible and effective in some patients with relapsed non-small cell lung cancer in our phase I study (21). The present study suggests that combination of amrubicinol and cisplatin or irinotecan is also worth evaluating in relapsed SCLC patients.

Amrubicinol had additive effects in combination with cisplatin for several human tumor cells, including lung cancer cells, by isobologram analysis (22,23). The present study confirmed those results using SBC-3, as both simultaneous and sequential combinations of the two drugs displayed synergistic or additive effects by median-effect plot analysis. In addition, flow cytometric analysis showed that exposure of the two drugs produced an increase of apoptotic cells compared to that for each single agent. It was difficult to draw a conclusion about the effect of the combination of amrubicinol and SN-38. However, sequential exposure of amrubicinol followed by SN-38 may be considered for further studies since: i) CI values after simultaneous exposure of amrubicinol and SN-38 were 1.2 at  $IC_{70}$  (antagonistic) and 1.0 at  $IC_{90}$  (additive); ii) the effect of SN-38 followed by amrubicinol was antagonistic; and iii) CI values after sequential exposure of amrubicinol followed by SN-38 were 1.0 at  $IC_{70}$  (additive) and 1.1 at  $IC_{90}$  (antagonistic), and this sequence produced a marked increase in apoptotic cells. Amrubicinol had an additive effect with etoposide for T-cell leukemia cells and osteosarcoma cells, although the effects were antagonistic at  $IC_{70}$  and synergistic at  $IC_{90}$  for SBC-3 (22). To our knowledge, the combination of amrubicinol with paclitaxel, which had an antagonistic effect in this study, has not been reported. More cell lines should be investigated to further evaluate these combinations.

The mechanisms of drug interaction between amrubicinol and other drugs have not been elucidated. Flow cytometry data in the present study suggested the presence of apoptotic cells based on the sub-G1 peak. Biochemical analysis for apoptotic cell death should be carried out for further investigation. Yamauchi *et al* reported that cisplatin enhanced the topoisomerase II inhibitory effect of amrubicinol and amrubicinol enhanced the formation of cisplatin-induced DNA interstrand cross-links (23). A combination of topoisomerase I inhibitors and topoisomerase II inhibitors is thought reasonable because reciprocal enhancement of one enzyme in the resistant cell lines develops an inhibitory effect on the other enzyme (24). However, the effectiveness of a combination and administration schedule has been a controversial issue in clinical trials to date (25). Thus, additional research will be needed to establish a rationale for the combination of irinotecan and amrubicinol.

The combination of irinotecan and cisplatin is accepted as the standard treatment for ED-SCLC (2). Concurrent

chemoradiotherapy consisting of cisplatin, etoposide and thoracic radiotherapy followed by cisplatin and irinotecan is considered to be very active in limited disease SCLC (26). The present study indicated that further studies are warranted on amrubicin alone and in combination with cisplatin or irinotecan in relapsed SCLC patients.

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