some reports of the clinical evaluation of such a drug combination, namely irinotecan (CPT-11) and etoposide (VP-16) [12,24,25]. Although Masuda et al. [12] concluded that the combination regimen of CPT-11 and VP-16 was effective against refractory or relapsed SCLC, no further studies have been reported. In this study, we investigated the feasibility and effectiveness of the combination chemotherapeutic regimen of AMR (DNA topoisomerase II inhibitor) and TOP (DNA topoisomerase I inhibitor) in patients with relapsed or ED-SCLC.

The rationale for combining DNA topoisomerase I and II inhibitors is that such a combination of drugs would yield greater inhibition of the DNA topoisomerase activity resulting in more potent cytotoxicity, because each topoisomerase enzyme has some compensatory activity in the event of deficiency of the other. It has been reported that the cytotoxicity of such a drug combination increases when the drugs are administered sequentially [22,23]. Kim stressed the importance of the administration sequence in a preclinical study, and showed that administration of CPT-11 (topoisomerase I inhibitor) before doxorubicin (topoisomerase II inhibitor) resulted in a synergistic effect against human tumour xenografts in nude mice [23]. However, Masuda reported that administration of VP-16 (topoisomerase II inhibitor) before CPT-11 was also effective in a clinical study [12,25]. In this study, we administered TOP before AMR, and obtained favourable results. Therefore, clinically, the sequence of administration of the two drugs may not be very important.

The present study demonstrated that treatment with the drug combination of TOP and AMR is feasible in patients with relapsed or ED-SCLC. Negoro, et al. [14] reported the results of a phase I study of AMR monotherapy, with daily administration of the drug for three consecutive days. The MTD was 50 mg/m^2/day (150 mg/m^2/course), and the DLTs were leukopenia, neutropenia, thrombocytopenia and gastrointestinal toxicities. On the other hand, the MTD of TOP during 5 days' administration was estimated to be 1.5–2.0 mg/m^2/day, and the DLTs were reversible leukopenia and neutropenia [15,16]. Subsequently, the clinical effectiveness of a combination of DNA topoisomerase I and II inhibitors, that is, CPT-11 and VP-16, was reported by Karato [24] and Masuda [25]. In Karato's study [24], both drugs were administered on Days 1–3 with G-CSF support. The MTDs of VP-16/CPT-11 were 60/80 or 80/60 mg/m^2, and the DLTs were weight loss and diarrhoea. In Masuda's study [25], CPT-11 was administered on Days 1, 8 and 15, and VP-16 was given on Days 1–3 with G-SCF support. The MTD of CPT-11 was 90 mg/m^2 and that of VP-16 was 80 mg/m^2. The DLTs were diarrhoea and leukopenia. During treatment with the chemotherapeutic combination of TOP and AMR in our study, we determined the MTD of TOP and AMR to be 0.75 mg/m^2 and 50 mg/m^2, respectively. The DLT was almost limited to haematological toxicities and seemed severe, however, all these toxicities were reversible, and we finally considered the phase II dose to be the level 2 dose according to the initial definition for the recommended dose, although further investigation is needed to confirm its safety profiles in the following studies using larger cohorts.

In this study, the C_{max} and AUC of AMR increased in a dose-dependent manner, and statistical significance was not reached. However, the corresponding values of 13-OH-AMR varied markedly among the patients, perhaps attributable partly to our small patient population. However, Ohe et al. also demonstrated similar results with respect to 13-OH-AMR in red blood cells in a phase I/II trial of AMR and CDDP in 45 chemo-naïve patients with ED-SCLC [26]. Negoro, et al. [14] also documented that the plasma concentrations of 13-OH-AMR were very low as compared to those of AMR. Thus, it may be difficult to construct a limited sampling model for estimating the AUC of 13-OH-AMR in either single-agent therapy or combination therapy. The C_{max} and AUC of TOP were not significantly different among the first three dose levels, or between Days 2 and 3, which indicates that AMR did not influence the pharmacokinetics of TOP.

In the pharmacodynamic analysis, we demonstrated that the Cmax and AUC of AMR were correlated with the duration of grade 4 neutropenia. In addition, the mean C_{max} of TOP on Day 2 in seven responders was significantly higher than that in two non-responders. Concerning the relationship between the antitumour effect and pharmacokinetics of AMR, Noguchi et al. reported that the AUC of intracellular 13-OH-AMR was related to the anti-tumour effect of the drug [27]. However, these relationships were not observed in our study. It remains unknown why the Cmax of TOP on the previous day used together with AMR was associated with an objective response. Further investigation is warranted to confirm the role of pharmacokinetic and pharmacodynamic monitoring during treatment with the combination regimen of AMR and TOP.

Using CPT-11 and VP-16, a combination of DNA topoisomerase I and II inhibitors, Masuda et al. [12] reported favourable outcomes in cases of refractory or relapsed SCLC. Among the 24 assessable patients, complete response was observed in three (13%), while 14 (58%) patients showed a PR, with an overall response rate of 71%. The response rate was particularly high (80%) in patients with relapsed SCLC. In this study also, the PR rate in relapsed cancer patients was extremely high (80%). Kubota et al. [28] reported a high response rate of 88% to the CODE regimen in 17 relapsed SCLC patients, which was associated with an encouraging survival rate (MST: 245 days). Therefore, we may expect survival benefit with the use of this combination, and this should be confirmed in future studies.

5. Conclusion

In conclusion, this phase I study showed both the feasibility and effectiveness of the two-drug combination of TOP and AMR in patients with relapsed or ED-SCLC. Since this combination seems to be particularly effective for relapsed SCLC, a phase II trial of this drug regimen in this subset of patients (relapsed SCLC) is warranted.

References

[1] Noda K, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med 2002;346:85–91.

[2] Livingston RB, Stephens RL, Bonnet JD, Grozea PN, Lehane DE. Long-term survival and toxicity in small cell lung cancer. Southwest Oncology Group study. Am J Med 1984;77:415–7.

[3] Johnson BE, Ihde DC, Bunn PA, Becker B, Walsh T, Weinstein ZR, et al. Patients with small-cell lung cancer treated with combination chemotherapy with or without irradiation. Data on potential cures, chronic toxicities, and late relapses after a five- to eleven-year follow-up. Ann Intern Med 1985;103:430–8.
[4] Ihde DC, Pass HI, Glatstein E. Small cell lung cancer. In: De Vita Jr VT, Hellman S, Rosenberg SA, editors. Cancer: principles and practice of oncology. 5th ed. Philadelphia: Lippincott-Raven; 1997. p. 911–49.
[5] Albain KS, Crowley JJ, Hutchins L, Gandara D, O'Bryan RM, Von Hoff DD, et al. Predictors of survival following relapse or progression of small cell lung cancer. Southwest Oncology Group Study 8605 report and analysis of recurrent disease data base. Cancer 1993;72:1184–91.
[6] Ettinger DS. New drugs for chemotherapy-naive patients with extensive-disease small cell lung cancer. Semin Oncol 2001;28:27–9.
[7] Yana T, Negoro S, Takada Y, Yokota S, Fukuoka M, The West Japan Lung Cancer Group. Phase II study of amrubicin (SM-5887), a 9-amino-anthracycline, in previously untreated patients with extensive stage small-cell lung cancer (ES-SCLC): A West Japan Lung Cancer Group Trial. Proc Am Soc Clin Oncol 1998;17:450c (abstr 1734).
[8] Schiller JH, Kim K, Hutson P, DeVore R, Glick J, Stewart J, et al. Phase II study of topotecan in patients with exensive-stage small-cell carcinoma of the lung: an Eastern Cooperative Oncology Group Trial. J Clin Oncol 1996;14:2345–52.
[9] Gupta RS, Gupta R, Eng B, Lock RB, Ross WE, Hertzberg RP, et al. Camptothecin-resistant mutants of Chinese hamster ovary cells containing a resistant form of topoisomerase I. Cancer Res 1998;48:6404–10.
[10] Eng WK, McCabe FL, Tan KB, Mattern MR, Hoffman GA, Woessner RD, et al. Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. Mol Pharmacol 1990;38:471–80.
[11] Sugimoto Y, Tsukahara S, Oh-hara T, Liu LF, Tsuruno T. Elevated expression of DNA topoisomerase II in camptothecin-resistant human tumor cell lines. Cancer Res 1990;50: 7962–5.
[12] Masuda N, Matsui K, Negoro S, Takifuji N, Takeda K, Yana T, et al. Combination of irinotecan and etoposide for treatment of refractory or relapsed small-cell lung cancer. J Clin Oncol 1998;16:3329–34.
[13] Mountain CF. Revisions in the international system for staging lung cancer. Chest 1997;111:1710–7.
[14] Negoro S, Fukuoka M, Nakamura S, Ikegami H, Sugiura T, Ariyoshi Y, et al. Phase I-II study of amrubicin (SM-5887), a novel 9-amino-antracycline, by IV administration for 3 consecutive days in patients with advanced non-small cell lung cancer. Proc Am Soc Clin Oncol 1995;14:361 [Abstract 1105].
[15] Kobayashi K, Hino M, Fukuoka M, Takeuchi K, Furuse K, Yoneda S, et al. Phase I studies of nogitecan hydrochloride for Japanese. Int J Clin Oncol 2002;7:177–86.
[16] Rowinsky EK, Grochow LB, Henricks CB, Ettinger DS, Forastiere AA, Hurowitz LA, et al. Phase I and pharmacologic study of topotecan: a novel topoisomerase I inhibitor. J Clin Oncol 1992;10:647–56.
[17] Shimoyama M. The Japanese edition of the National Cancer Institute-Common Toxicity Criteria (in Japanese). Gan To Kagaku Ryoho 1999;26:1084–109.
[18] Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2002;92: 205–16.
[19] Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, et al. Effects of CPT-11 in combination with other anti-cancer agents in culture. Int J Cancer 1992;50:604–10.
[20] D'Arpa P, Beardmore C, Liu LF. Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Res 1990;50:6919–24.
[21] Kaufmann SH. Antagonism between camptothecin and topoisomerase II-directed chemotherapeutic agents in a human leukemia cell line. Cancer Res 1991;51:1129–36.
[22] Bertrand R, O'Connor PM, Kerrigan D, Pommier Y. Sequential administration of camptothecin and etoposide circumvents the antagonistic cytotoxicity of simultaneous drug administration in slowly glowing human colon carcinoma HT-29 cells. Eur J Cancer 1992;28A:743–8.
[23] Kim R, Hirabayashi N, Nishiyama M, Jinushi K, Toge T, Okada K. Experimental studies on biochemical modulation targeting topoisomerase I and II in human tumor xenografts in nude mice. Int J Cancar 1992;50:760–6.
[24] Karato A, Sasaki Y, Shinkai T, Eguchi K, Tamura T, Ohe Y, et al. Phase I study of CPT-11 and etoposide in patiets with refractory solid tumors. J Clin Oncol 1993;11:2030–5.
[25] Masuda N, Fukuoka M, Kudoh S, Matsui K, Kusunoki Y, Takada M, et al. Phase I and pharmacologic study of irinotecan and etoposide with recombinant human granulocyte colony-stimulating factor support for advanced lung cancer. J Clin Oncol 1994;12:1833–41.
[26] Ohe Y, Negoro S, Matsui K, Nakagawa K, Sugiura T, Takada Y, et al. Phase I–II study of amrubicin and cisplatin in previously untreated patients with extensive-stage small-cell lung cancer. Ann Oncol 2005;16:430–6.
[27] Noguchi T, Ichii S, Morisada S, Yamaoka T, Yanagi Y. In vivo efficacy and tumor-selective metabolism of amrubicin to its active metabolite. Jpn J Cancer Res 1998;89: 1055–60.
[28] Kubota K, Nishiwaki Y, Kakinuma R, Hojo F, Matsumoto T, Ohmatsu H, et al. Dose-intensive weekly chemotherapy for treatment of relapsed small-cell lung cancer. J Clin Oncol 1997;15:292–6.

ONCOLOGY REPORTS 15: 837-842, 2006

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

NAGIO TAKIGAWA[1,2,3], MASAKO TAKEYAMA[2], TAKUO SHIBAYAMA[1,2], ATSUHIKO TADA[1,2], NORIKO KAWATA[1,2], CHIHARU OKADA[1,2], KEISUKE AOE[4], TOSHIYUKI KOZUKI[3], KATSUYUKI HOTTA[3], MASAHIRO TABATA[3], KATSUYUKI KIURA[3], HIROSHI UEOKA[4], MITSUNE TANIMOTO[3] and KIYOSHI TAKAHASHI[1]

[1]Department of Internal Medicine and [2]Clinical Research Institute, National Hospital Organization, Minami-Okayama Medical Center, 4066 Hayashima, Okayama 701-0304; [3]Department of Respiratory Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558; [4]Department of Respiratory Medicine, National Hospital Organization, Sanyo Hospital, 685 Higashikiwa, Ube 755-0241, Japan

Received September 26, 2005; Accepted November 21, 2005

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

Correspondence to: Dr Nagio Takigawa, Department of Respiratory Medicine, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
E-mail: ntakigaw@md.okayama-u.ac.jp

Key words: amrubicin, irinotecan, cisplatin, small-cell lung cancer, median-effect plot analysis

water. SN-38, etoposide, and paclitaxel were dissolved in dimethylsulfoxide. Drug solutions were stored at -20˚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 μl 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 μl 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˚C for 96 h in a highly humidified incubator with 5% CO_2 and 95% air, and then 10 μl of AlamarBlue was added to each well. After incubation at 37˚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: [(mean fluorescence in 4 test wells - fluorescence in background wells)/(mean fluorescence in control wells - fluorescence in background wells)] x100. The drug concentration required to inhibit growth of tumor cells by 50% (IC_{50}) was determined by plotting the logarithm of drug concentration versus the percentage of surviving cells. 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 IC_{50} value of resistant subline cells by the IC_{50} of SBC-3 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).

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

IC_{50}, 50% inhibitory concentration; SD, standard deviation; R.R, relative resistance value (IC_{50} value of resistant cells/IC_{50} value of SBC-3 cells). Data are expressed as mean ± SD.

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.

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

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

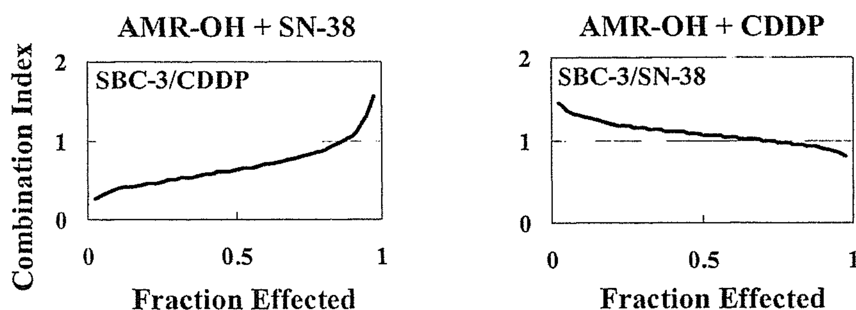

Figure 1. Combination index and surviving fraction of SBC-3/CDDP cells treated with amrubicinol (AMR-OH) in combination with SN-38 simultaneously for 96 h (left). Combination index and surviving fraction of SBC-3/SN-38 cells treated with AMR-OH in combination with cisplatin (CDDP) simultaneously for 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: $fa/fu = (D/Dm)^m$ (equation 1).

In equation 1, D is the dose, Dm 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 (Dm) and shape (*m*) were taken into account as parameters in this method. Equation 2 was rearranged from equation 1 as follows: $D = Dm[fa/(1-fa)]^{1/m}$ (equation 2).

The Dm 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 (Dm) 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: $CI = (D)A/(Dx)A + (D)B/(Dx)B$ (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. (Dx)A and (Dx)B in denominators of equation 3 indicate doses of drug A and drug B alone, respectively, that also inhibit x%. Dx 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 ± 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±46 nM; irinotecan, 195±10.2 nM; etoposide, 270±170 nM; and paclitaxel, 0.55±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

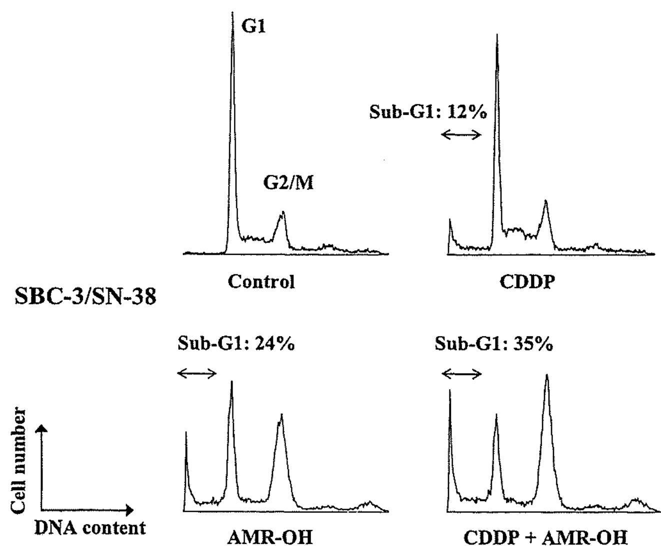

Figure 2. Effect of cisplatin (CDDP), amrubicinol (AMR-OH), or the combination of CDDP and AMR-OH induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3/SN-38 cells.

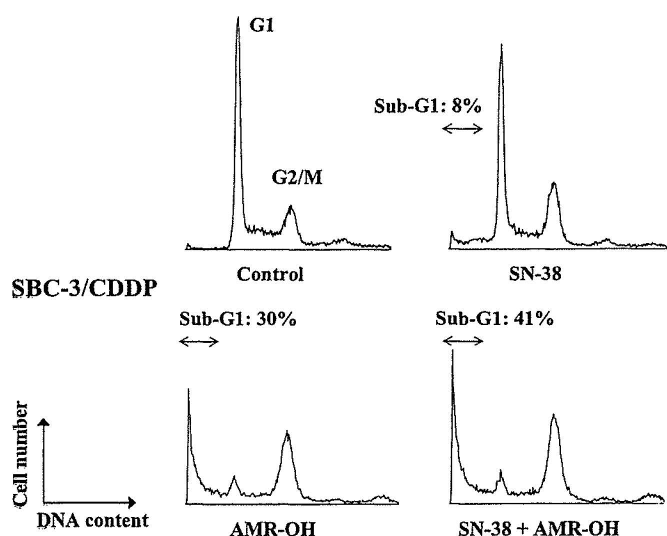

Figure 3. Effect of SN-38, amrubicinol (AMR-OH), or the combination of SN-38 and AMR-OH induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3/CDDP cells.

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±0.21 at IC_{70} and 1.0±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.

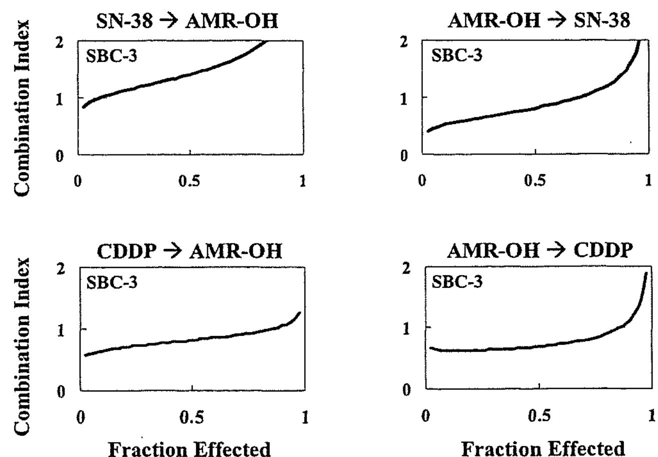

Figure 4. Combination index and surviving fraction of SBC-3 cells treated sequentially with SN-38 or cisplatin (CDDP) for 24 h followed by amrubicinol (AMR-OH) for 24 h and the reverse sequence.

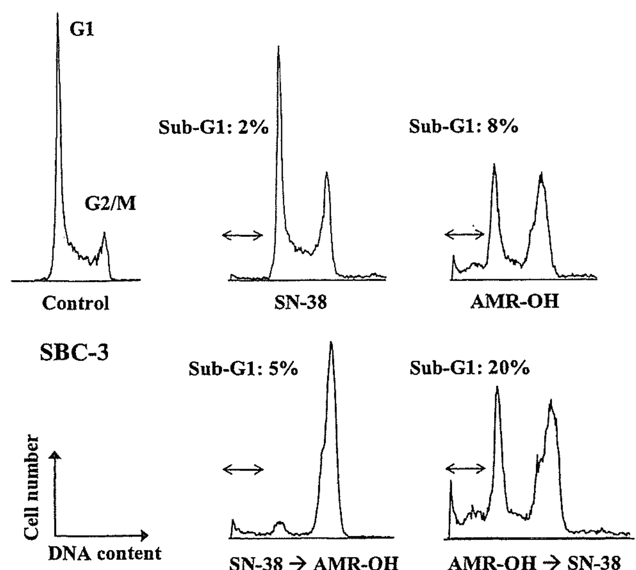

Figure 5. Effect of SN-38, amrubicinol (AMR-OH), SN-38 followed by AMR-OH, or AMR-OH followed by SN-38 induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3 cells.

CI values were 0.99±0.17 at IC_{70} and 0.89±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

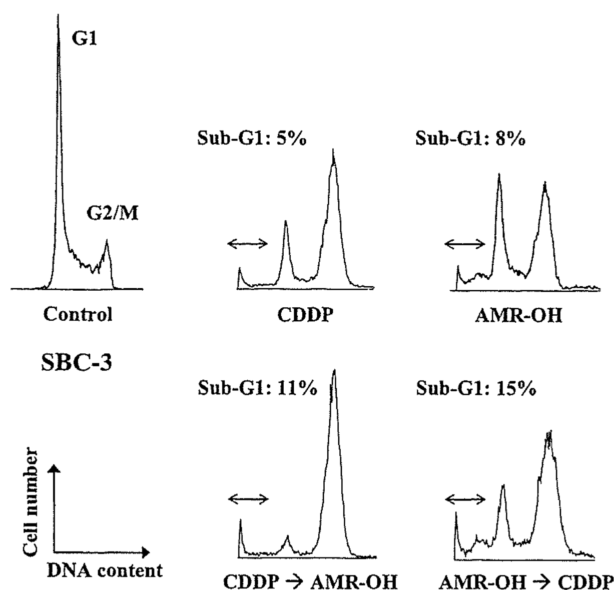

Figure 6. Effect of cisplatin (CDDP), amrubicinol (AMR-OH), CDDP followed by AMR-OH, or AMR-OH followed by CDDP induced cell cycle traverse perturbations and apoptosis (% cells in sub-G1 fraction) in SBC-3 cells.

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±0.12 at IC_{70} and 1.0±0.06 at IC_{90}, and 0.86±0.15 at IC_{70} and 0.93±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 amrubicin 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 amrubicin and cisplatin was reported to be highly effective for untreated ED-SCLC (20). A combination of amrubicin 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 amrubicin and cisplatin or irinotecan is also worth evaluating in relapsed SCLC patients.

Amrubicin 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 amrubicin.

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.

References

1. Doyle LA: Mechanisms of drug resistance in human lung cancer cells. Semin Oncol 20: 326-337, 1993.
2. Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S, Saijo N; Japan Clinical Oncology Group: Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. N Engl J Med 346: 85-91, 2002.
3. Ishizumi K, Ohashi N and Tanno N: Streospecific total synthesis of 9-aminoanthracyclines: (+)-9-amino-9-deoxydaunomycin and related compounds. J Org Chem 50: 4477-4485, 1987.
4. Yamaoka T, Hanada M, Ichii S, Morisada S, Noguchi T and Yanagi Y: Cytotoxicity of amrubicin, a novel 9-aminoanthracycline, and active metabolite amrubicinol on human tumor cells. Jpn J Cancer Res 89: 1061-1067, 1998.
5. Hanada M, Mizuno S, Fukushima A, Saito Y, Noguchi T and Yamaoka T: A new antitumor agent amrubicin induces cell growth inhibition by stabilizing topoisomerase II-DNA complex. Jpn J Cancer Res 89: 1229-1238, 1998.
6. Morisada S, Yanagi Y, Noguchi T, Kashiwazaki Y and Fukui M: Antitumor activities of a novel 9-aminoanthracycline (SM5887) against mouse experimental tumors and human tumor xenografts. Jpn J Cancer Res 80: 69-76, 1989.
7. Suzuki T, Minamide S, Iwasaki T, Yamamoto H and Kanda H: Cardiotoxicity of a new anthracycline derivative (SM-5887) following intravenous administration to rabbits: comparative study with doxorubicin. Invest New Drugs 15: 219-225, 1997.
8. Noda T, Watanabe T, Kohda A, Hosokawa S and Suzuki T: Chronic effects of a novel synthetic anthracycline derivative (SM-5887) on normal heart and doxorubicin-induced cardiomyopathy in beagle dogs. Invest New Drugs 16: 121-128, 1998.
9. Yana T, Negoro Y, Takada S, Yokota M and Fukuoka M: Phase II study of amrubicin (SM-5887), a 9-amino-anthracycline, in previously untreated patients with extensive stage small-cell lung cancer (ES-SCLC): a West Japan Lung Cancer Group Trial. Proc Am Soc Clin Oncol 17: 450, 1998.
10. Miyamoto H: Establishment and characterization of an adriamycin-resistant subline of human small cell lung cancer cells. Acta Med Okayama 40: 65-73, 1986.
11. Chikamori M, Takigawa N, Kiura K, Tabata M, Shibayama T, Segawa Y, Ueoka H, Ohnoshi T and Tanimoto M: Establishment of a 7-ethyl-10-hydroxy-camptothecin-resistant human small-cell lung cancer cell line. Anticancer Res 24: 3911-3916, 2004.
12. Moritaka T, Kiura K, Ueoka H, Tabata M, Segawa Y, Shibayama T, Takigawa N, Ohnoshi T and Harada M: Cisplatin-resistant human small cell lung cancer cell line shows collateral sensitivity to vinca alkaloids. Anticancer Res 18: 927-933, 1998.
13. Ahmed SA, Gogal RM Jr and Walsh JE: A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Methods 170: 211-224, 1994.
14. Chang TT and Chou TC: Rational approach to the clinical protocol design for drug combinations: a review. Acta Paediatr Taiwan 41: 294-302, 2000.
15. Chou TC and Talaly P: A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. J Biol Chem 252: 6438-6442, 1977.
16. Aoe K, Kiura K, Ueoka H, Tabata M, Matsumura T, Chikamori M, Matsushita A, Kohara H and Harada M: Effect of docetaxel with cisplatin or vinorelbine on lung cancer cell lines. Anticancer Res 19: 291-299, 1999.
17. Kiura K, Ohnoshi T, Tabata M, Shibayama T and Kimura I: Establishment of an adriamycin-resistant subline of human small cell lung cancer showing multifactorial mechanisms of resistance. Acta Med Okayama 47: 191-197, 1993.
18. Takigawa N, Ohnoshi T, Ueoka H, Kiura K and Kimura I: Establishment and characterization of an etoposide-resistant human small cell lung cancer cell line. Acta Med Okayama 46: 203-212, 1992.
19. Takigawa N, Ohnoshi T, Ueoka H, Kiura K and Kimura I: Comparison of antitumor activity of new anthracycline analogues, ME2303, KRN8602, and SM5887 using human lung cancer cell lines. Acta Med Okayama 46: 249-256, 1992.
20. Ohe Y, Negoro S, Matsui K, Nakagawa K, Sugiura T, Takada Y, Nishiwaki Y, Yokota S, Kawahara M, Saijo N, Fukuoka M and Ariyoshi Y: Phase I-II study of amrubicin and cisplatin in previously untreated patients with extensive stage small cell lung cancer. Ann Oncol 16: 430-436, 2005.
21. Hotta K, Takigawa N, Kiura K, Tabata M, Umemura S, Ogino A, Uchida A, Bessho A, Segawa Y, Shinkai T, Nogami N, Harita S, Okimoto N, Ueoka H and Tanimoto M: Phase I study of irinotecan and amrubicin in patients with advanced non-small-cell lung cancer. Anticancer Res 25: 2429-2434, 2005.
22. Takagi T, Yazawa Y, Suzuki K, Yamauchi Y and Kano Y: Effects of 13-hydroxy SM5887 in combination with other anticancer agents on human tumor cell lines. Invest New Drugs 14: 357-363, 1996.
23. Yamauchi S, Kudoh S, Kimura T, Hirata K and Yoshikawa J: Additive effects of amrubicin with cisplatin on human lung cancer cell lines. Osaka City Med J 48: 69-76, 2002.
24. Rubin EH, Li TK, Duann P and Liu LF: Cellular resistance to topoisomerase poisons. Cancer Treat Res 87: 243-260, 1996.
25. Xu Y and Villalona-Calero MA: Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. Ann Oncol 13: 1841-1851, 2002.
26. Mitsuoka S, Kudoh S, Takada Y, Matsui K, Eguchi K, Ichinose Y, Nakagawa K, Takada M, Saito H and Ariyoshi Y: Phase II study of cisplatin, etoposide and concurrent thoracic radiotherapy (TRT) followed by irinotecan and cisplatin in patients with limited stage small-cell lung cancer (SCLC); updated results of WJTOG9902. Proc Am Soc Clin Oncol 22: 7044, 2004.