Efficacy of Combination Treatment and Influence of Schedule with Irinotecan and Amrubicin in Human Lung Carcinoma Cells *in Vivo* and *in Vitro*

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The aim of this study was to elucidate the efficacy of combination therapy with irinotecan and amrubicin for lung cancer and the influence of administration schedule in a xenograft mouse model and human cancer cell culture. We investigated the antitumor activity of irinotecan and amrubicin on human small cell lung cancer cell line LX-1 inoculated in mice *in vivo* and the cytotoxic effect of SN-38 and amrubicinol on human lung cancer cell lines A549 and PC-6 *in vitro*. Combined administration of irinotecan and amrubicin in divided doses inhibited tumor growth by approximately 90%, with complete recovery observed in one case. Furthermore, combined administration in divided doses induced little loss of body weight. Combination index analysis revealed that the cell growth inhibitory effect of SN-38 combined with amrubicinol was additive, regardless of schedule or cell line. The effect of combination treatment with SN-38 and amrubicinol on cell cycle was investigated. Cell cycle showed arrest at both the S and G2/M phases. The results indicate that combination therapy with irinotecan and amrubicin can be expected to yield improved outcomes, including less toxicity, especially with divided administration.

Key words irinotecan; amrubicin; antitumor effect; combination index; additive effect

Lung cancer is one of the leading causes of cancer death worldwide,[1] including in Japan.[2] Although much work has been done to enable earlier detection, most cases are already advanced on initial presentation, and further improvement is still required in both diagnosis and treatment. Irinotecan is recognized as one of the most important drugs in the treatment of solid tumors, and chemotherapy combining irinotecan with cisplatin has shown great promise in the treatment of small-cell lung cancer (SCLC).[3,4] However, platinum-based regimens are often associated with severe, dose-limiting, systemic toxicities, a factor which hampers their efficacy. The fact is that lung cancer still remains notoriously difficult to treat. Therefore, there is an urgent need to develop more effective combination chemotherapies with novel agents active against lung cancer.

Amrubicin, a completely synthetic 9-aminoanthracycline derivative and anthracyclin analog, is the latest anticancer agent in the treatment of SCLC and non-small cell lung cancer (NSCLC) in Japan, and has demonstrated excellent single-agent activity against extensive-stage SCLC (ES-SCLC).[5] It has also shown promising antitumor activity against advanced NSCLC, with acceptable toxicity.[6] The combination of amrubicin and cisplatin demonstrated an impressive response rate and median survival time in patients with previously untreated ES-SCLC,[7] indicating the potential of this regimen.

Irinotecan is a DNA topoisomerase I (topo I) inhibitor. On the other hand, amrubicin, and especially its 13-hydroxy metabolite, amrubicinol, exhibit antitumor activity through interacting with DNA by intercalation, thus inhibiting DNA topoisomerase II (topo II) by stabilizing the cleavable complex.[8] The combination of topo I and topo II inhibitors such as irinotecan and etoposide has been reported to be effective against SCLC.[9] Therefore, combination treatment with irinotecan and amrubicin offers a potentially promising chemotherapy regimen for refractory or relapsed lung cancer.[10—13] According to the results of another clinical study, combination chemotherapy with irinotecan and amrubicin was well tolerated but produced only a modest antitumor effect in advanced NSCLC.[14]

In this study, we investigated the efficacy of combination therapy with irinotecan and amrubicin for human lung cancer *in vivo* and *in vitro*. The *in vivo* anticancer effect was evaluated using an LX-1 xenograft model in mice. The *in vitro* combination effect of SN-38, the active metabolite of irinotecan,[15] and amrubicinol on proliferation of A549 and PC-6 was analyzed by the combination index method of Chou and Talalay[16] to determine whether synergy was obtained with simultaneous and sequential combination treatment. We also characterized the cell cycle events associated with simultaneous and sequential treatment with the two drugs.

MATERIALS AND METHODS

Reagents Irinotecan hydrochloride, (+)-(4*S*)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione hydrochloride trihydrate (irinotecan) as Campto® Inj. and its active metabolite, SN-38, were prepared by Yakult Honsha Co., Ltd. (Tokyo, Japan). Amrubicin hydrochloride, (+)-(7*S*,9*S*)-9-acetyl-9-amino-7-[(2-deoxy-β-D-*erythro*-pentopyranosyl)oxy]-7,8,9,10-tetrahydro-6,11-dihydroxy-5,12-naphthacenedione hydrochloride (amrubicin) and amrubicinol hydrochloride (amrubicinol) were kindly provided by Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Doxorubicin was purchased as Adriacin® Inj. from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Amrubicin, amrubicinol, and doxorubicin were dissolved in distilled water for injection. SN-38 was dissolved in dimethylsulfoxide.

Animals and Tumor Cell Lines Male BALB/c nude mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and maintained under standard laboratory conditions.

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Human SCLC cell line LX-1 was obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Human NSCLC cell line A549 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Human SCLC cell line PC-6 was kindly provided by Dr. S. Inoue. A549 and PC-6 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.).

Xenograft Studies in Athymic Mice Six-week-old, male, athymic BALB/c nude mice were used for LX-1 xenografts. Fragments of LX-1 tumor (2 to 3 mm square) were implanted subcutaneously into each inguen. When tumors reached a size of 0.1—0.3 cm^3 (width^2×length/2), mice were allocated by tumor volume into 13 groups of 5 mice each to be treated with vehicle, irinotecan (50 mg/kg, total), or amrubicin alone (20 mg/kg, total), or irinotecan + amrubicin (50, 20 mg/kg total, respectively) according to 13 different administration schedules (see Table 1). The administration schedules were designed based on our previous study.[17] Administration *via* the tail vein commenced from day 1. Tumor size was measured on days 1, 5, 9, 14 and 22. Tumors were resected and weighed on day 22, and tumor growth inhibitory ratio (I.R., %) assessed by the equation given below. The total doses of irinotecan and amrubicin, 50 and 20 mg/kg, respectively, were set to allow the effect of combined administration to be detected clearly. On the basis of our preliminary study, we expected a 50% I.R. with single administration at these doses. In addition to tumor volume, body weight was also measured to determine the influence of the drugs used on overall physical condition. All animal studies were conducted in accordance with the Guidelines of the Yakult Central Institute for Microbiological Research and the protocol approved by the study review committee of this institute.

Table 1. Antitumor Activity of Irinotecan and Amrubicin on LX-1 with Either Single or Combined Administration

Schedule (d)		Tumor weight (g) Mean±S.D.	I.R. (%)	Complete recovery
Irinotecan	Amrubicin			
Control		1.81±0.47	—	—
Single administration				
1st	—	1.21±0.25*	32.9	
2nd	—	1.32±0.31	26.8	
1st, 5th, 9th	—	0.72±0.18***	60.3	
—	1st	1.08±0.25**	40.3	
—	2nd	1.72±0.42	4.9	
—	1st, 5th, 9th	0.76±0.36***	58.2	
Combined administration				
1st	1st	0.98±0.24***	45.8	
1st	2nd	1.36±0.13	24.8	
2nd	1st	0.85±0.24***	52.9	
1st	1st, 5th, 9th	0.19±0.20**,#,¶¶¶	89.7	
1st, 5th, 9th	1st, 5th, 9th	0.01±0.01***,##,¶	99.2	1/5
1st, 5th, 9th	1st	0.14±0.06***,###,¶	92.0	

There were 5 mice in each group. $*p<0.05$, $**p<0.01$, $***p<0.001$ *vs.* control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* amrubicin in corresponding schedule of administration; $^{¶}p<0.05$, $^{¶¶}p<0.01$, $^{¶¶¶}p<0.001$ *vs.* irinotecan in corresponding schedule of administration. Total 50 mg/kg irinotecan and 20 mg/kg amrubicin were administered, respectively. Where administered on days 1, 5 and 9, drug given in one-third total doses.

$$\text{I.R. (\%)} = 1 - \frac{\text{mean tumor weight in anti-cancer agent group}}{\text{mean tumor weight in control group}} \times 100$$

Cell Growth Inhibition Assay Cells were plated in triplicate wells (1250 cells/well) in 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and incubated at 37 °C in 5% CO_2 for 24 h. Cells were then exposed to drugs at different concentrations for 72 h until cell growth inhibition assay. Cell growth inhibitory effect of drugs was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay (Promega Co., Madison, WI, U.S.A.). After 80 min incubation with the MTS reagent, absorbance was read at 490 nm using the 96-well microtiter plate reader Spectra Max250® (Molecular Devices Co., Sunnyvale, CA, U.S.A.). Wells containing cells but no drugs and wells containing media but no cells were used as positive and negative controls, respectively.

Single or Concurrent Exposure to SN-38 and/or Amrubicinol Cells were seeded into 96-well microtiter plates as described above and treated with a series of 6 dilutions of each drug individually or both drugs simultaneously at a fixed ratio of concentration that typically corresponded to 0.125, 0.25, 0.5, 1, 2 or 4 times the individual GI_{50}s, the drug concentration required to inhibit cell growth by 50%. After 72 h exposure, growth inhibition was measured by MTS assay. Combination ratios were set at 1 : 3, 2 : 2 and 3 : 1.

Sequential Exposure to SN-38 and Amrubicinol The same *in vitro* experimental setup described above was used. After 4, 8 and 24 h of exposure to the first drug (either SN-38 or amrubicinol), the second drug was added, and cells further incubated. After 72 h exposure to the first drug, growth inhibition was determined by MTS assay.

Analysis of Combined Effect by Construction of Combination Index The combined effect of SN-38 and amrubicinol was analyzed by the combination index (CI) method. The CI was calculated by the Chou–Talalay equation,[16] which takes into account both the potency and shape of the dose–effect curve. A CI of <1 indicates synergism; CI$=1$ indicates an additive effect, and CI>1 indicates antagonism. All experiments were repeated at least 3 times. Data represent the mean of triplicate determinations with the standard deviation (S.D.).

Flow Cytometry Analysis Cells (1×10^6) were trypsinized in a culture dish, fixed with paraformaldehyde and stained with propidium iodide and fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine (BrdU) antibody (BD Biosciences, San Jose, CA, U.S.A.) using the Cycle-TEST™ PLUS DNA Reagent Kit (BD Biosciences). Samples were analyzed for DNA content and DNA synthesis using the Altra® flow cytometer (Beckman Coulter Inc., Fullerton, CA, U.S.A.).

Statistical Analysis Differences in tumor weight at day 22 between the control group and the treated group and between the single administration group and combined administration group were statistically analyzed with the Bonferroni multiple comparison test. Differences in body weight at day 22 between the control group and the treated group were statistically analyzed with the Dunnet multiple comparison test.

RESULTS

Antitumor Activity *in Vivo* Tumor weight in each group on day 22 is shown in Table 1. With single administration of irinotecan or amrubicin, the I.R. in the groups in which the total dose of the drug was administered on day 1 was 30—40% ($p<0.05$ *vs.* control). However, the I.R. in the groups in which the total dose of the drug was administered on day 2 was less than 30%. On the other hand, the I.R. in the groups in which the drug was administered in divided doses of one-third total dose on days 1, 5 and 9 was approximately 60% ($p<0.001$ *vs.* control).

With combined administration of irinotecan and amrubicin, the I.R. was 25—53% with completion of administration on day 2. Combined administration of irinotecan and amrubicin on days 1 and 2 resulted in more potent inhibition of tumor growth compared to irinotecan or amrubicin administered alone on day 1 or 2. However, when irinotecan and amrubicin were administered a) on day 1 (total dosage), and on days 1, 5, and 9 (one-third, divided doses), respectively, b) on days 1, 5, and 9 (one-third, divided doses), and day 1 (total dosage), respectively, or c) on days 1, 5, and 9 (one-third, divided doses) simultaneously, the I.R. was greater than 89% ($p<0.05$ *vs.* irinotecan or amrubicin alone). With schedule c), in particular, a 99.2% I.R. was observed, and the tumor completely disappeared. Tumor volume was measured on days 1, 5, 9, 14 and 22 (Fig. 1). Although tumor volume in each treatment group showed no change until the final day of the administration schedule (day 9), an increase was observed thereafter in the single administration group. In the combined administration groups, however, tumor size showed a reduction or retained its initial size even after completion of the administration schedule. Changes in body weight are shown in Fig. 2. No clear difference in body weight was observed on day 1 or the final day of administration where the drug was given in divided doses (day 9). Recovery of body weight was observed in the group receiving combined administration on day 22; and recovery of body weight was particularly marked in the group receiving simultaneous divided-dose administration on days 1, 5 and 9.

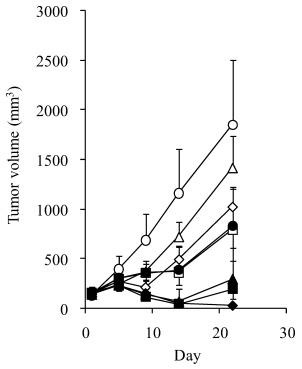

Fig. 1. Effects of Irinotecan, Amrubicin and Their Combination on Tumor Growth in Human Small Cell Lung Cancer LX-1 Xenograft Model

Male mice bearing tumors were randomly allocated into groups of 5 animals each. Irinotecan was administered intravenously (i.v.) on day 1, or in divided doses on days 1, 5 and 9 to a total dose of 50 mg/kg, with or without combined administration with amrubicin. Amrubicin was also administered i.v. on day 1, or in divided doses on days 1, 5 and 9 to a total dose of 20 mg/kg. All results are given as mean±S.D. ○, control; △, irinotecan on day 1; □, irinotecan on days 1, 5 and 9; ◇, amrubicin on day 1; ●, amrubicin on days 1, 5 and 9; ▲, irinotecan on day 1+amrubicin on days 1, 5 and 9; ■, irinotecan on days 1, 5 and 9+amrubicin on day 1; ◆, irinotecan + amrubicin on days 1, 5 and 9.

Cell Growth Inhibition The cell growth inhibitory activities of SN-38, amrubicin, amrubicinol and doxorubicin were investigated by means of a 72-h continuous drug exposure test on 2 human lung cancer cell lines (A549 and PC-6). As shown in Table 2, SN-38 showed the most potent and specific cell growth inhibition activity among the drugs tested. The GI_{50} of amrubicin ranged from 1 to 3 μg/ml, which was 3—19 times less potent than that of doxorubicin. The GI_{50} of amrubicinol was as potent as that of doxorubicin.

Concurrent Exposure (72 h) to SN-38 and Amrubicinol The cell growth inhibitory effect with concurrent exposure to SN-38 and amrubicinol was analyzed by the CI method. In A549, with an SN-38:amrubicinol ratio of 2 : 2, the CI value at the fraction affected (fa) of 0.5 was 0.86±0.32 (actual drug concentrations required to inhibit cell growth by 50% were estimated to be 0.01 μg/ml SN-38, 0.11 μg/ml amrubicinol and 0.01 μg/ml SN-38+0.02 μg/ml amrubicinol). In PC-6, with an SN-38 : amrubicinol ratio of 2 : 2, the CI value at the fa of 0.5 was 0.79±0.33 (actual drug concentrations required to inhibit cell growth by 50% were estimated to be 0.002 μg/ml SN-38, 0.31 μg/ml amrubicinol and 0.001 μg/ml SN-38+0.024 μg/ml amrubicinol). As shown in Fig. 3A, the CI values were approximately 1 for every fa in all combination (SN-38 : amrubicinol) ratios—3 : 1, 2 : 2 and 1 : 3—in both A549 and PC-6.

Sequential Exposure to Second Drug at 4, 8, or 24 h after First Drug The cell growth inhibitory effects of sequential exposure to the 2 drugs at a combination ratio of 2 : 2 were analyzed as shown in Fig. 3B. When SN-38 was administered as the first drug, a CI of >1 was partially observed, but the CI value was generally 1. When amrubicinol was administered as the first drug, the CI value was also gen-

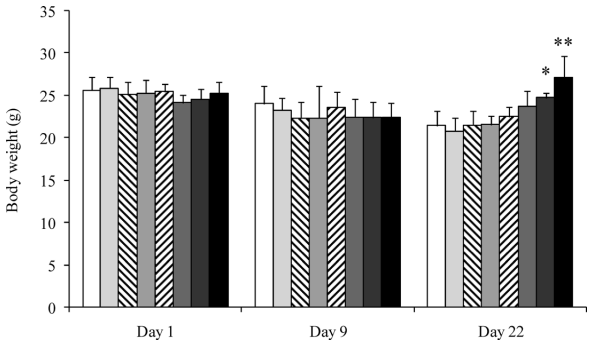

Fig. 2. Body Weight Changes in Mice after Administration of Irinotecan and Amrubicin

□, Control; ▨, irinotecan on day 1; ▧, irinotecan on days 1, 5 and 9; ▩, amrubicin on day 1; ▨, amrubicin on days 1, 5 and 9; ▩, irinotecan on day 1+amrubicin on days 1, 5 and 9; ■, irinotecan on days 1, 5 and 9+amrubicin on day 1; ■, irinotecan+amrubicin on days 1, 5 and 9. Value represents mean±S.D. $*p<0.05$, $**p<0.01$ *vs.* control.

Table 2. GI_{50} of SN-38, Amrubicin, Amrubicinol, and Doxorubicin on Cell Growth of A549 and PC-6 (72 h)

	A549	PC-6
SN-38 (μg/ml)	0.09	0.004
Amrubicin (μg/ml)	1.2	3<
Amrubicinol (μg/ml)	0.15	0.10
Doxorubicin (μg/ml)	0.36	0.16

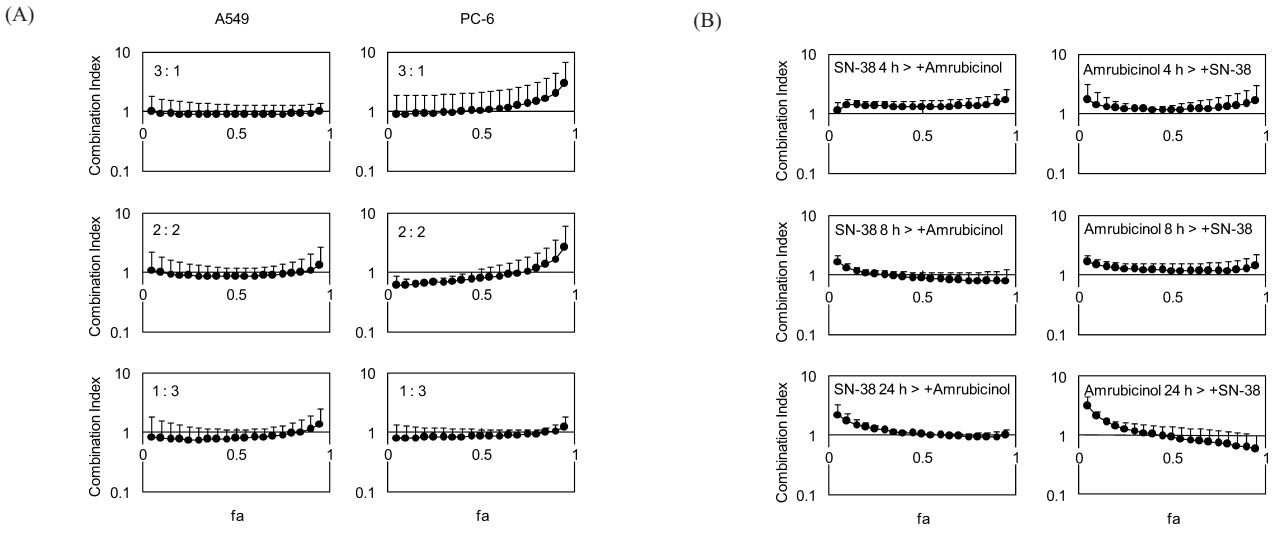

Fig. 3. Combination Index (CI) Values for SN-38 with Amrubicinol

Results are given as means of 3 individual experiments and bars indicate standard deviations. CI<1, CI=1, and CI>1 indicate synergism, additive effect, and antagonism, respectively. (A) Human non-small cell lung cancer cells A549 and human small cell lung cancer cells PC-6 were simultaneously exposed to 2 agents at ratio of 3 : 1, 2 : 2, or 1 : 3 (SN-38 : amrubicinol) for 72 h at 37 °C in 5% CO_2. (B) Human non-small cell lung cancer cells A549 were sequentially exposed to 2 drugs at ratio of 2 : 2 at 37 °C in 5% CO_2. After 4, 8, or 24 h exposure to first drug, treatment with second drug was started.

erally 1, regardless of time or cell line (data for PC-6 not shown).

Cell Cycle and DNA Synthesis with Single or Concurrent Exposure to SN-38 and Amrubicinol Changes in cell cycle and DNA synthesis in A549 with single or concurrent exposure to SN-38 and/or amrubicinol were evaluated at 4, 8, 24 and 48 h (Figs. 4A, B). With single exposure to SN-38 (0.03 μg/ml), the cells in the G0/G1 phase disappeared, and apparent late S to G2/M arrest was observed at 24 and 48 h. The number of BrdU-positive cells, defined as those synthesizing DNA in the *S* phase, showed a reduction by 48 h. With single exposure to amrubicinol (0.1 μg/ml), signs of S-G2/M arrest were observed, even at 4 h. At 24 and 48 h, although a more marked increase in cells in the G2/M phase was observed, a G0/G1 peak remained. BrdU incorporation was also inhibited at 48 h by exposure to amrubicinol. With concurrent exposure to SN-38 and amrubicinol (0.015, 0.05 μg/ml, respectively), change in cell cycle and BrdU uptake appeared to be average that of with single exposure to each drug. Signs of S-G2M arrest were observed, even at 4 h. Although an increase in cells in the G2/M phase was observed by 48 h, a G0/G1 peak remained.

Cell Cycle and DNA Synthesis with Sequential Exposure to Second Drug at 4, 8, or 24 h after First Drug The effects of sequential exposure to the two drugs for 48 h on cell cycle and BrdU incorporation were determined. When SN-38 (0.01 μg/ml) was the first drug (Fig. 5), G2/M arrest was observed at 48 h, regardless of when amrubicinol (0.03 μg/ml) was added (after 4, 8, or 24 h incubation with SN-38), showing no difference to with single exposure to SN-38. When amrubicinol was the first drug (Fig. 6), G2/M arrest was observed at 48 h, regardless of when SN-38 was added (after 4, 8, or 24 h incubation with amrubicinol). However, cells in the G0/G1 phase remained with single exposure to amrubicinol and when SN-38 was added after 24 h incubation with amrubicinol. BrdU-positive cells completely disappeared by 48 h in all cases (Figs. 5B, 6B).

DISCUSSION

Topo I and topo II are primary cellular targets for a number of potent antitumor agents such as doxorubicin, etoposide and irinotecan.[18] The topo I-active agent irinotecan increases expression of topo II mRNA and decreases that of topo I mRNA. In contrast, etoposide and doxorubicin, which are topo-II active, increase expression of topo I mRNA and decrease that of topo II mRNA.[19] Therefore, the combination of topo I and topo II inhibitors offers an attractive proposition in terms of their reciprocal and complementary functions.

With single administration of each drug, the highest I.R., which reached approximately 60%, was obtained with divided administration of the drugs in one-third doses on days 1, 5 and 9. This suggests that divided-dose administration is more effective than administration of the full drug in one dose. An earlier study noted that the effect of amrubicin was enhanced by divided administration.[20,21] This study also found that divided administration promoted a time-dependent accumulation of amrubicinol in tumor tissue, and that there was a good correlation between the total area under the blood concentration–time curve of amrubicinol in tumor tissue and its antitumor effect.

With combined administration, the I.R. in the groups in which the full drugs were administered in one dose did not reach as high a level as that obtained with single administration of irinotecan (60.3%) or amrubicinol (58.2%). Sequential administration of the two drugs showed little benefit in terms of efficacy.

On the other hand, the I.R. in the group in which irinotecan and amrubicin were simultaneously administered on days 1, 5 and 9 reached 99%. Divided administration also yielded a benefit in terms of overall physical condition, with induction of only a little loss in body weight.

According to our preliminary studies, administration of 100 mg/kg irinotecan in one dose or a total of 270 mg/kg irinotecan administered in 3 divided doses resulted in a loss

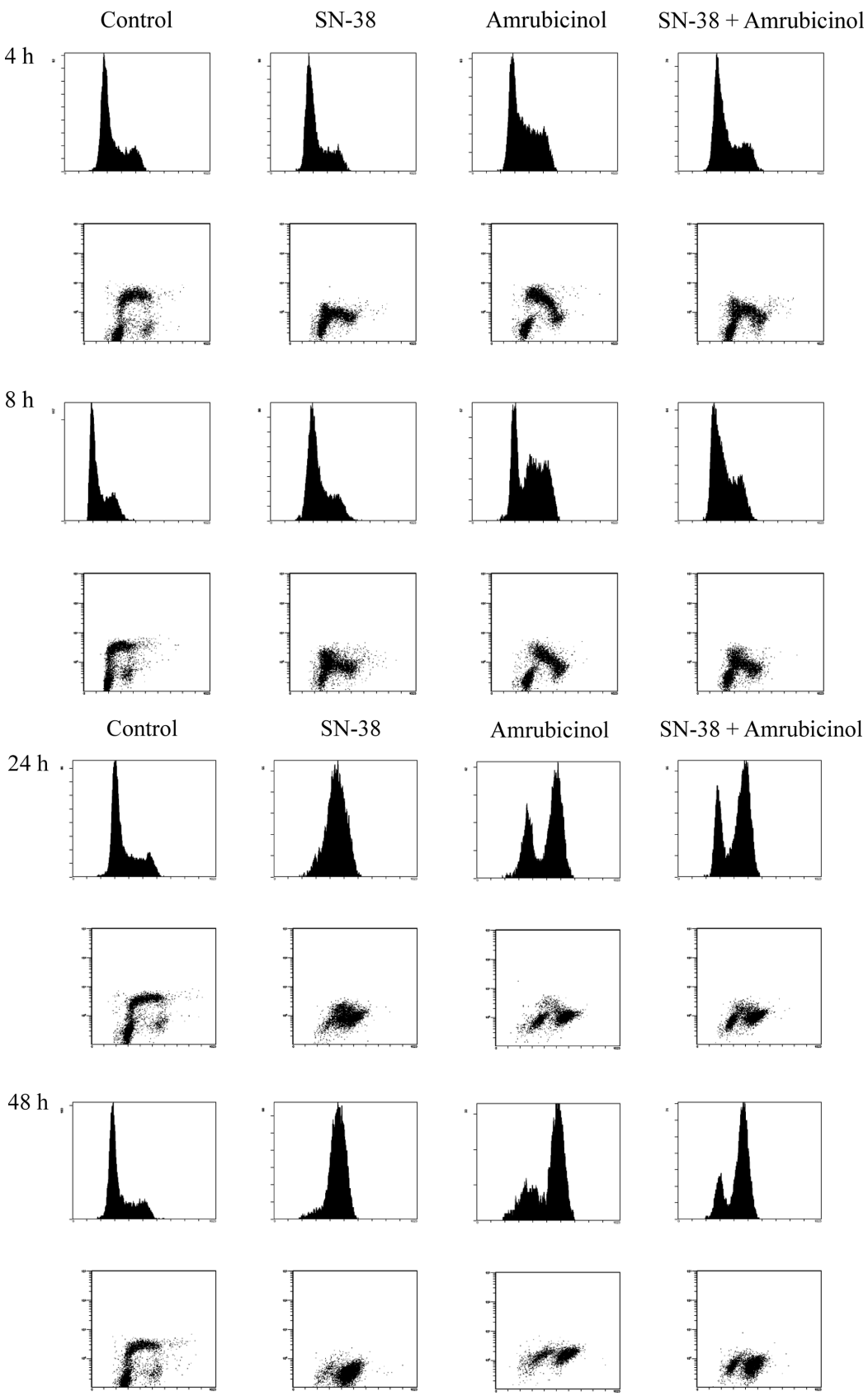

Fig. 4. Effects of SN-38, Amrubicinol and Their Simultaneous Treatment on Cell Cycle and DNA Synthesis in A549 Cells

Cells were treated with SN-38 (0.03 μg/ml), amrubicinol (0.1 μg/ml), or SN-38 (0.015 μg/ml) and amrubicinol (0.05 μg/ml) at 37 °C in 5% CO_2 for (A) 4—8 h, and (B) 24—48 h.

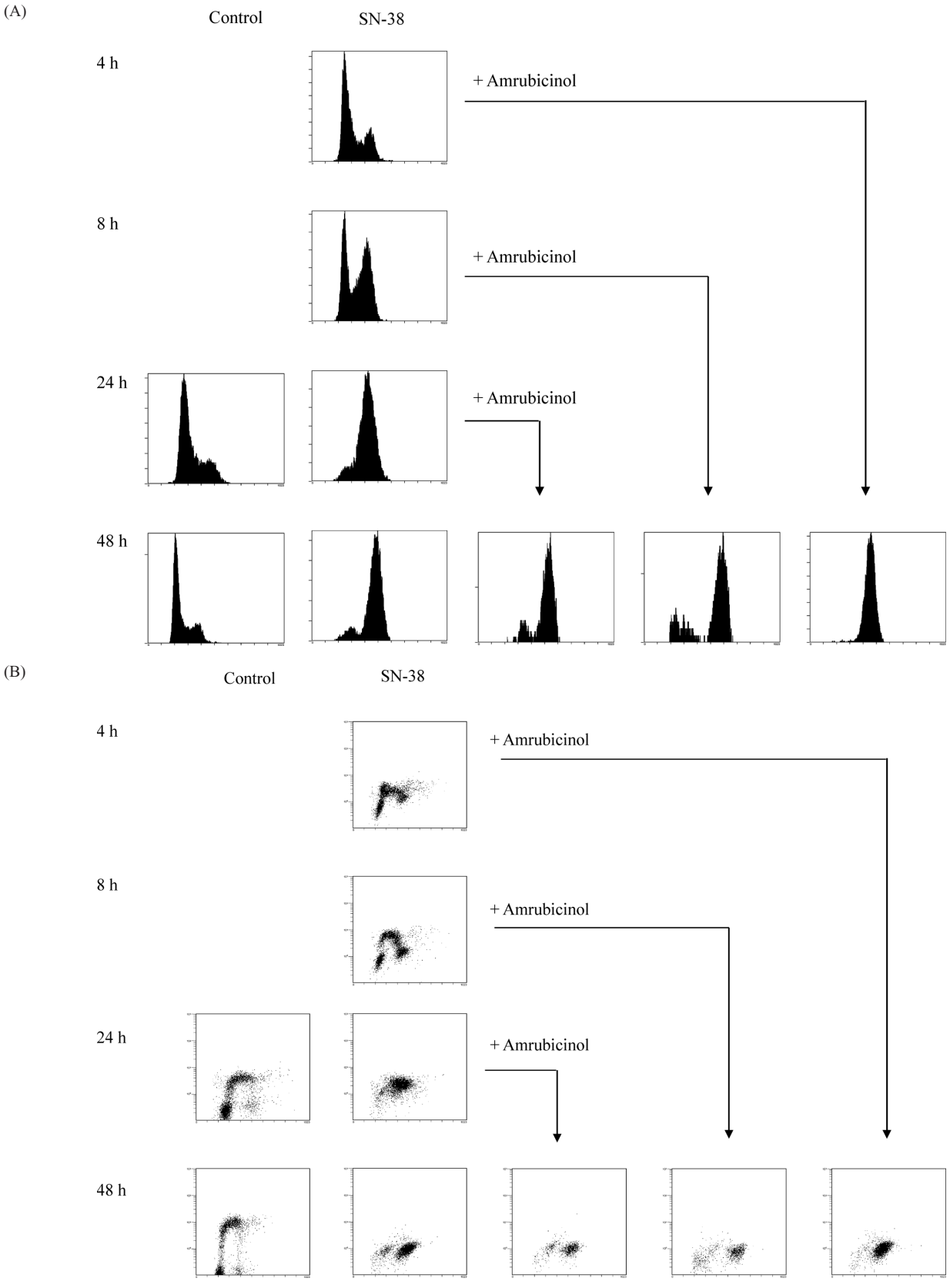

Fig. 5. Effects of SN-38, Amrubicinol and Their Sequential Treatment on (A) Cell Cycle and (B) DNA Synthesis in A549 Cells

Cells were treated with SN-38 (0.01 μg/ml) as first drug and amrubicinol (0.03 μg/ml) as second drug 4, 8, or 24 h after SN-38.

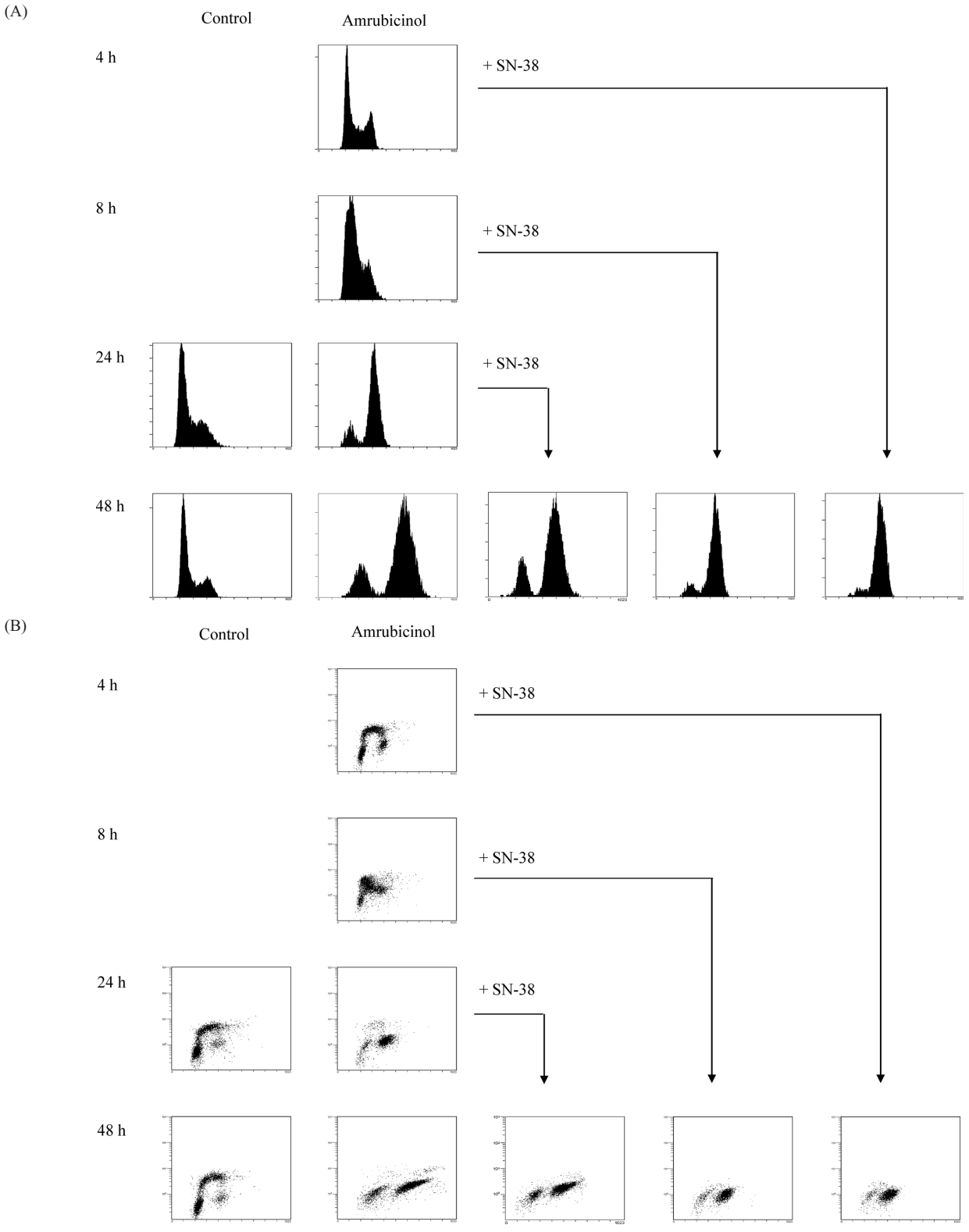

Fig. 6. Effects of Amrubicinol, SN-38 and Their Sequential Treatment on (A) Cell Cycle and (B) DNA Synthesis in A549 Cells

Cells were treated with amrubicinol (0.03 μg/ml) as first drug and SN-38 (0.01 μg/ml) as second drug 4, 8, or 24 h after amrubicinol.

in body weight of up to 20%. On the other hand, the maximum tolerated doses of amrubicinol were 25 mg/kg and 37.5 mg/kg total in one-dose administration and administration in 5 divided doses, respectively, and recoverable myelosuppression was observed at these doses.[21,22] In the present study, irinotecan and amrubicin were administered at lower total doses of 270 and 37.5 mg/kg, respectively. Although it may be possible to raise these doses of irinotecan and/or amrubicin to obtain a schedule which might yield a higher I.R., the resulting adverse effects on the animal's physical condition would be more severe. We did not evaluate the adverse effects of the drugs on bone marrow or hepatic function. However, the dose levels of irinotecan and amrubicin used in the present study were lower than the maximum doses, and the resulting loss in body weight was only up to 10%. This would indicate that there is no cause for concern with regard to severe myelosuppression or hepatic toxicity at these dose levels. These results indicate the importance of the administration schedule, and particularly the importance of divided administration.

In the cell growth inhibition study, amrubicinol showed an 8—30-times higher level of activity than amrubicin in the 72-h continuous drug exposure test on 2 human lung cancer cell lines, A549 and PC-6, and was as potent as doxorubicin. In an earlier study, amrubicinol was found to be an important contributor to the antitumor activity of amrubicin *in vivo* as its active metabolite,[8] which may explain this finding. At this laboratory, we have observed plasma concentrations of SN-38 to reach 0.09 μg/ml (GI_{50} of SN-38 on A549 at 72 h) after intravenous administration of irinotecan at a dose of 40 mg/kg in normal and tumor-bearing mice. Furthermore, the plasma level of amrubicinol has been reported to reach 0.15 μg/ml (GI_{50} of amrubicinol on A549 at 72 h) after intravenous administration of amrubicin at a dose of 25 mg/kg.[23]

The availability of several new active drugs has improved the efficacy of combination regimens and substantially increased the response rate of refractory tumors. Several preclinical studies have shown schedule-dependent drug interaction in combination therapy.[24,25] We, therefore, investigated the relationship between administration schedule and cell growth inhibitory effect.

When A549 and PC-6 were exposed to SN-38 and amrubicinol concurrently, a CI of 1 was observed at each combination ratio. This shows that the inhibitory effect of SN-38 and amrubicinol on cell proliferation in simultaneous treatment is additive. Furthermore, with sequential administration, the outcome was CI=1, with the exception of a few measurement points, regardless of which drug was used to commence incubation. Thus, the results suggest that SN-38 and amrubicinol act additively, with timing of addition and cell line having no effect on the action of the two drugs. Although a drastic enhancement of *in vivo* antitumor effect was observed in combination treatment with divided administration of irinotecan and amrubicin, the difference between single treatment and combination treatment in cell growth inhibition was not as evident as expected. Even when combination treatment was sequential, no difference was observed.

To investigate the mechanism underlying combination therapy, the effects of combined treatment with SN-38 and amrubicinol on cell cycle and DNA synthesis were analyzed. Although a small G0/G1 peak remained even after G2/M arrest in amrubicinol treatment only by 48 h, SN-38 and amrubicinol basically showed similar action: late S to G2/M arrest and inhibition of DNA synthesis were observed with single treatment with each drug. This was probably due to the main mechanisms of action of the two drugs: suppressing DNA synthesis by topo I or II inhibition. With simultaneous treatment with the two drugs, the observed cell cycle arrest was average that obtainable with each drug alone. The present results indicate that the two drugs acted on the cell cycle independently.

In the present study, combined administration of irinotecan and amrubicin, especially when given in divided doses, demonstrated a potent anti-tumor effect with little physical damage in this xenograft model in mice. This seems to correlate with earlier clinical observations.[12] The combination effect of SN-38 and amrubicinol was additive in the cell growth inhibition study, and this was supported by the results of the cell cycle and DNA synthesis analysis, which revealed no evidence of reciprocal interaction in enhancement of cell growth inhibitory action. Since irinotecan and amrubicin are prodrugs, it has been suggested that the tumor-selective metabolism of amrubicin to amrubicinol,[26] and the time-dependent accumulation of amrubicinol in tumor tissue by divided administration,[20] also play an important role in their superior effect *in vivo*. It may be necessary to develop an *in vitro* model which would accurately simulate the pharmacokinetics involved (*i.e.*, conversion, wash-out, *etc.*) to elucidate the mechanism underlying the combination effect of irinotecan and amrubicin in detail. However, we believe that results of this *in vivo* study may be considered representative based on the additive effect observed in the *in vitro* study.

The results of this study suggest that irinotecan and amrubicin play a role in this combination effect independently of each other. Furthermore, the results indicate that combination therapy with irinotecan and amrubicin can be expected to yield improved outcomes, including less toxicity, especially with divided administration.

REFERENCES

1) Perkin D. M., Bray F. I., Devesa S. S., *Eur. J. Cancer*, **37**, S4—S66 (2001).
2) Summary of Vital Statistics 2008 Ministry of Health, Labour and Welfare, 2008.
3) Noda K., Nishiwaki Y., Kawahara M., Negoro S., Sugiura T., Yokoyama A., Fukuoka M., Mori K., Watanabe K., Tamura T., Yamamoto S., Saijo N., *N. Engl. J. Med.*, **346**, 85—91 (2002).
4) Horiike A., Saijo N., *Oncology*, **19**, 54—58 (2005).
5) Yana T., Negoro S., Takada M., Yokota S., Fukuoka M., *Proc. Am. Soc. Clin. Oncol.*, **17**, 450a (1988).
6) Sugiura T., Ariyoshi Y., Negoro S., Nakamura S., Ikegami H., Takada M., Yana T., Fukuoka M., *Invest. New Drugs*, **23**, 331—337 (2005).
7) Ohe Y., Negoro S., Matsui K., Nakagawa K., Sugiura T., Takada Y., Nishiwaki Y., Yokota S., Kawahara M., Saijo N., Fukuoka M., Ariyoshi Y., *Ann. Oncol.*, **16**, 430—436 (2005).
8) Hanada M., Mizuno S., Fukushima A., Saito Y., Noguchi T., Yamaoka T., *Jpn. J. Cancer Res.*, **89**, 1229—1238 (1998).
9) Masuda N., Matsui K., Negoro S., Takifuji N., Takeda K., Yana T., Kobayashi M., Hirashima T., Kusunoki Y., Ushijima S., Kawase I., Tada T., Sawaguchi H., Fukuoka M., *J. Clin. Oncol.*, **16**, 3329—3334 (1998).
10) Hanada M., Noguchi T., Yamaoka T., *Cancer Sci.*, **98**, 447—454 (2007).
11) Oshita F., Saito H., Yamada K., *Oncology*, **74**, 7—11 (2008).
12) Takigawa N., Takeyama M., Shibayama T., Tada A., Kawata N., Okada

C., Aoe K., Kozuki T., Hotta K., Tabata M., Kiura K., Ueoka H., Tanimoto M., Takahashi K., *Oncol. Rep.*, **15**, 837—842 (2006).
13) Yanaihara T., Yokoba M., Onoda S., Yamamoto M., Ryuge S., Hagiri S., Katagiri M., Wada M., Mitsufuji H., Kubota M., Arai S., Kobayashi H., Yanase N., Abe T., Masuda N., *Cancer Chemother. Pharmacol.*, **59**, 419—427 (2007).
14) 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., Tanimoto M., *Anticancer Res.*, **25**, 2429—2434 (2005).
15) Kaneda N., Nagata H., Furuta T., Yokokura T., *Cancer Res.*, **50**, 1715—1720 (1990).
16) Chou T. C., Talalay P., *Adv. Enzyme Regul.*, **22**, 27—55 (1984).
17) Furuta T., Yokokura T., *Jpn. J. Cancer Chemother.*, **17**, 121—130 (1990).
18) D'Arpa P., Liu L. F., *Biochim. Biophys. Acta*, **989**, 163—177 (1989).
19) Eder J. P., Chan V., Wong J., Wong Y. W., Ara G., Northey D., Rizvi N., Teicher B. A., *Cancer Chemother. Pharmacol.*, **42**, 327—335 (1998).
20) Ichii S., Morisada S., Murayama T., Yanagi Y., "48th Annual Meeting of the Japanese Cancer Association," 2165 [Abstract], 1989.
21) Noguchi T., Ichii S., Morisada S., Yamaoka T., Yanagi Y., *Jpn. J. Cancer Chemother.*, **26**, 1305—1312 (1999).
22) Morisada S., Yanagi Y., Kashiwazaki Y., Fukui M., *Jpn. J. Cancer Res.*, **80**, 77—82 (1989).
23) Noguchi T., Ichii S., Morisada S., Yamaoka T., Yanagi Y., *Jpn. J. Cancer Res.*, **89**, 1061—1066 (1998).
24) Zoli W., Ricotti L., Tesei A., Ulivi P., Gasperi Campani A., Fabbri F., Gunelli R., Frassineti G. L., Amadori D., *Clin. Cancer Res.*, **10**, 1500—1507 (2004).
25) Takahashi N., Li W., Banerjee D., Guan Y., Wada-Takahashi Y., Brennan M. F., Chou T. C., Scotto K. W., Bertino J. R., *Cancer Res.*, **62**, 6909—6915 (2002).
26) Noguchi T., Ichii S., Morisada S., Yamaoka T., Yanagi Y., *Jpn. J. Cancer Res.*, **89**, 1055—1060 (1998).