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In vitro antitumor activity of mitomycin C derivative (RM-49) and new anticancer antibiotics (FK973) against lung cancer cell lines determined by tetrazolium dye (MTT) assay

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Summary. Seven small- (SCLC) and four non-small-cell (NSCLC) lung cancer cell lines were used to examine the in vitro cytotoxicity of cytotoxic drugs such as (1aS-(1aα,8β,8aα,8bα))-8-((aminocarbonyl)oxy)methyl)-4,8a-dimethoxy-1,1a,2,8,8a,8b-hexahydro-7-hydroxy-5-methyl-6-nitrosoazirino(2',3':3,4)-pyrrolo-(1,2-a)indole (RM-49) and 11-acetyl-8-carboxymethyl-4-formyl-14oxa-1,11-diaze-tetracyclo(7.4.1.$0^{2,7}$,$0^{10,12}$)tetradeca-2-4-6-trien-6,9-diyl-di-acetate (FK973). In vitro cytotoxicities of RM-49 and FK973 were compared with those of mitomycin C (MMC), cisplatin (CDDP), carboplatin (CBDCA), etoposide (VP16), adriamycin (ADM) and vindesin (VDS). Drug sensitivity was determined using a tetrazolium (MTT)-based assay. Average IC_{50} values of these two drugs were not statistically different compared with that of MMC, although FK973 showed strong antitumor activity against SCLC cell lines such as LT3, N857, and H69 at the same concentration. The predicted peak plasma concentration (predicted PPC) calculated by the formula proposed by Scheithauer, log (predicted PPC) $= -0.788 + (0.755 \times \log(LD_{50}))$, and relative antitumor activity, RAA (PPC/IC_{50}), of RM-49 were higher than those of other drugs such as MMC, CDDP, CBDCA, and ADM against SCLC cell lines ($P \leqq 0.05$), and those of FK973 were also higher than those of other drugs such as MMC, CDDP, CBDCA, and ADM against SCLC cell lines ($P \leqq 0.05$). Based on these promising in vitro studies, the clinical trials of RM-49 and FK973 were warranted.

Introduction

In spite of the progress in our understanding of lung cancer, major problems still remain in the area of treatment. Only 10%–15% of limited disease (LD) SCLC cases experience 3-year disease-free survival, although the response rate to standard combination chemotherapy is as high as 90%. In addition, less than 5% of NSCLC patients experience complete remission. It is essential to develop a new method to identify active antitumor agents against lung cancer. At the National Cancer Institute (NCI; USA), changes have been proposed for the screening of new anticancer drugs, due to the failure to develop any active new agents against common solid tumors by screening a panel of mouse tumors. The first change involves the use of human tumor cell lines in a disease-oriented drug screening program. In this study we used 11 established human lung cancer cell lines, and the in vitro antitumor activities of new anticancer agents such as (1aS-(1aα, 8β,8aα, 8bα))-8-(((aminocarbonyl)oxy)methyl)-4,8a-dimethoxy-1,1a,2,8,8a,8b-hexahydro-7-hydroxy-5-methyl-6-nitro-soazirino(2',3':3,4)-pyrrolo-(1,2-a)indole (RM-49) and 11-acetyl-8-carboxymethyl-4-formyl-14oxa-1,11-diaze-tetracyclo(7.4.1.$0^{2,7}$,$0^{10,12}$)tetradeca-2-4-6-trien-6,9-diyl-di-acetate (FK973) were evaluated by a tetrazolium dye (MTT) assay and compared with those of mitomycin C (MMC), cisplatin (CDDP) carboplatin (CBDCA), etoposide (VP16), adriamycin (ADM), and vindesin (VDS). The results suggested that RM-49 and FK973 could be used against non-small- and small-cell carcinoma of the lung, respectively.

Materials and methods

Tumor cell lines. Seven SCLC cell lines and four NSCLC cell lines were used in the MTT assay. SCLC cell lines such as LT3, LT9, LU130, LU134, and LU135 were established at the National Cancer Center Research Institute (Japan), whereas while N857 and H69 were established at the NCI and were obtained from Prof. Y. Shimosato. The NSCLC cells kindly provided by Prof. Y. Hayata, Tokyo Medical College, were PC7, PC9 (adenocarcinoma), PC13 (large-cell carcinoma), and PC14 (squamous-cell carcinoma). The characteristics of these cell lines are shown in Table 1. The cells were propagated in RPMI-1640 medium (Gibco)

Table 1. Lung cancer cell lines used in this study

Cell line	Tumor type	Doubling time (h)	Cells/well	Duration of drug exposure (days)
LT3	SCLC	92	2×10^4	7
LT9	SCLC	88	2×10^4	7
LU130	SCLC	62	2×10^4	7
LU134	SCLC	67	2×10^4	7
LU135	SCLC	88	2×10^4	7
N857	SCLC	72	2×10^4	7
H69	SCLC	84	2×10^4	7
PC7	NSCLC	52	2×10^3	4
PC9	NSCLC	31	2×10^3	4
PC13	NSCLC	46	2×10^3	4
PC14	NSCLC	35	2×10^3	4

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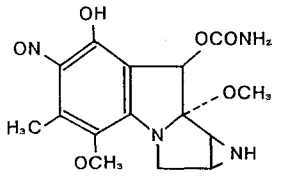
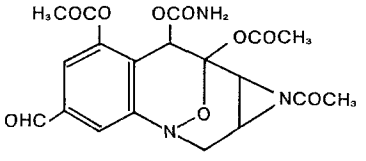
RM-49



[1aS-(1aα, 8β, 8aα, 8bα)]-8-[[(aminocarbonyl)oxy]-methyl]-4, 8a-dimethoxy-1, 1a, 2, 8, 8a, 8b-hexahydro-7-hydroxy-5-methyl-6-nitrosoazirino(2',3':3, 4)-pyrrolo-(1, 2-a)indole

FK973



11-acetyl-8-carboxymethyl-4-formyl-14oxa-1, 11-diazetetracyclo(7, 4, 1, $0^{2,7}$, $0^{10,12}$)tetradeca-2-4-6-trien-6, 9-diyl-diacetate

Fig. 1. Structural formulas of RM-49 and FK973

supplemented with 10% fetal calf serum (Gibco), penicillin (100 μg/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2 in air in a highly humidified incubator at 37° C.

Anticancer drugs. The drugs were obtained from the sources indicated: RM-49, MMC, and ADM, Kyowa Hakko Co. Ltd., Tokyo; FK973, Fujisawa Co. Ltd., Osaka; CDDP and VP16, Nippon Kayaku, Tokyo; CBDCA, Bristol Myers Co. Ltd., New York; VDS, Shionogi Co. Ltd., Osaka.

RM-49 is a derivative of MMC and has been demonstrated to be more active against mouse tumors and human tumor xenografts than MMC. FK973 was developed from *Streptomyces sandaens*. While this compound is not a derivative of MMC, its chemical structure is similar to that of the latter, containing the novel radicals aziridine and carbamoyl (Fig. 1). It has been shown to possess a more potent antitumor activity than that of MMC against B-16 melanoma and P388 leukemia. For the MTT assay, all drugs were dissolved in RPMI medium and stored at −70° C. The drugs were diluted with RPMI medium just prior to each experiment.

MTT assay. This assay is dependent on the reduction of MTT (Shigma) by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically.

Cultures in 96-well microculture plates (Falcon) were established in 135-μl volumes (at the cell concentration shown in Table 1). Serial dilutions of cytotoxic compounds were prepared in separate microculture plates. A solution of drugs at tenfold the required concentration were added to the cultures in 15-μl volumes, the control culture receiving 15 μl RPMI [5].

NSCLC cell lines were incubated for 4 days and SCLC cell lines for 7 days (Table 1). To determine cell growth, 15 μl MTT (5 mg/ml) in phosphate-buffered saline was added to each culture well and incubated at 37° C for 4 hours. The medium was then aspirated from the wells as completely as possible without disturbing the formazan crystal and cells on the plastic surface. DMSO (200 μl) was next added to each well. The plates were then agitated on a plate shaker for 5 min and the optical density (OD) was recorded at 540–690 nm on the plate reader (Titertek Multiscan plate reader) [2, 3, 5, 7, 8]. In all these experiments, three replicate wells were used to determine each point.

Chemosensitivity assays. A direct comparison was made between the effects of RM-49 (0.005–0.5 μg/ml), FK973 (0.01 – 1.0 μg/ml), MMC (0.01 – 1.0 μg/ml), CDDP

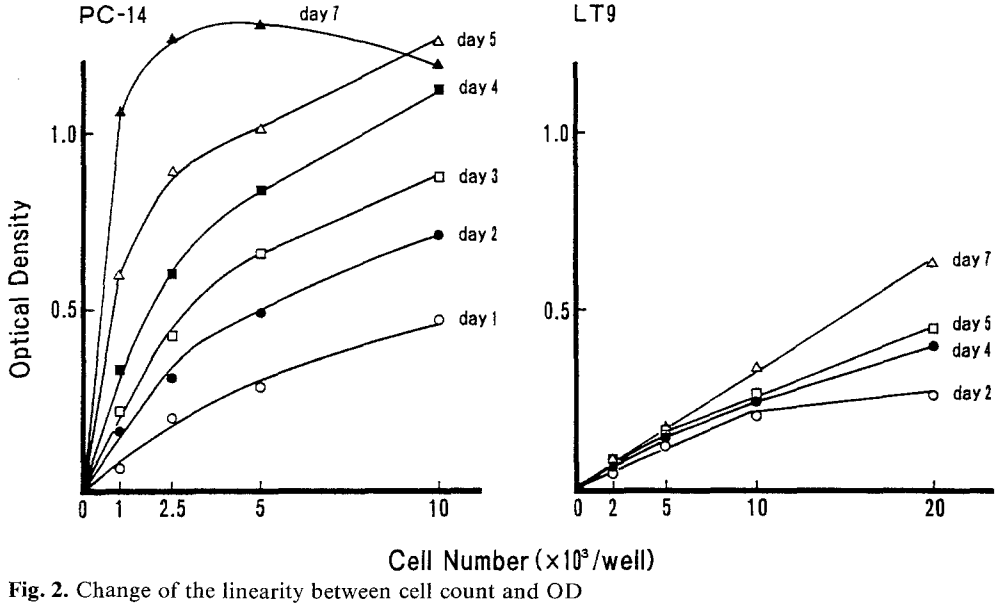


Fig. 2. Change of the linearity between cell count and OD

Table 2. IC_{50} values (μg/ml) for eight cytotoxic drugs in SCLC cell lines

	LT3	LT9	LU130	LU134	LU135	N857	H69	Mean ± SD
RM-49	0.075	0.055	0.070	0.016	0.075	0.12	0.018	0.06 ±0.04
FK973	<0.01	0.033	0.033	0.046	0.06	<0.01	<0.01	0.04 ±0.01
MMC	0.021	0.021	0.039	0.008	0.09	0.32	0.095	0.09 ±0.11
CDDP	0.42	0.34	0.44	0.26	0.64	0.60	0.32	0.43 ±0.14[a, b]
CBDCA	4.4	2.1	1.5	2.4	6.5	5.1	8.0	4.3 ±2.4[a, b]
VP16	1.50	0.75	0.275	<0.25	0.90	<0.25	1.0	0.88 ±0.44[a, b]
ADM	0.20	0.11	0.044	0.088	0.20	0.10	0.11	0.12 ±0.06[a, b]
VDS	0.0075	0.0085	0.013	0.015	0.025	0.0065	0.0076	0.012±0.007[a, b]

[a] Significantly different from RM-49 ($P \leqq 0.05$)
[b] Significantly different from FK973 ($P \leqq 0.05$)

Table 3. IC_{50} values (μg/ml) for eight cytotoxic drugs in NSCLC cell lines

	PC7	PC9	PC13	PC14	Mean ± SD
RM-49	0.11	0.08	0.14	0.50	0.21±0.20
FK973	0.13	0.48	>1.00	1.00	0.52±0.45
MMC	0.24	0.32	0.07	0.40	0.17±0.13
CDDP	2.60	0.82	1.80	0.95	1.54±0.83
CBDCA	32.0	11.5	22.0	17.0	20.6 ±8.7[a, b]
VP16	0.48	10.5	4.6	20.0	8.9 ±8.5
ADM	0.12	0.36	0.064	0.22	0.19±0.13
VDS	0.013	0.037	0.04	0.10	0.05±0.04

[a] Significantly different from RM-49 ($P \leqq 0.05$)
[b] Significantly different from FK973 ($P \leqq 0.05$)

(0.2 – 20 μg/ml), CBDCA (1.0 – 100 μg/ml), VP16 (0.2 – 20 μg/ml), ADM (0.04 – 4.0 μg/ml) and VDS (0.005–0.5 μg/ml) on the growth of 11 cell lines as evaluated by the MTT assay. Wells were also set up in triplicate, with each well containing $2 \times 10^3 - 2 \times 10^4$ cells (Table 1) in 135 μl medium. For continuous drug exposure experiments, drugs were added immediately after the dishes/wells were set up and left in throughout the entire incubation period. All results represent the average of three wells. The surviving fraction or fractional absorbance was calculated by the mean of the test sample of three wells/mean of the nontreated sample of three wells. Each experiment was carried out three times. An additional control consisted of media alone with no cells.

Comparison of drug sensitivity. IC_{50} values were determined graphically a dose response curve was obtained for each tumor cell line. The predicted antitumor activity of each drug was evaluated by the ratio of peak plasma concentration (PPC) to the IC_{50}. Based on preliminary pharmacological studies, the PPCs in this study were considered to be: MMC, 0.52 μg/ml; CDDP, 2.49 μg/ml; CBDCA, 35.0 μg/ml; VP16, 30.0 μg/ml; ADM, 0.60 μg/ml; and VDS, 0.883 μg/ml [1]. Pharmacokinetic data for RM-49 and FK973 in humans are not available. The predicted PPC

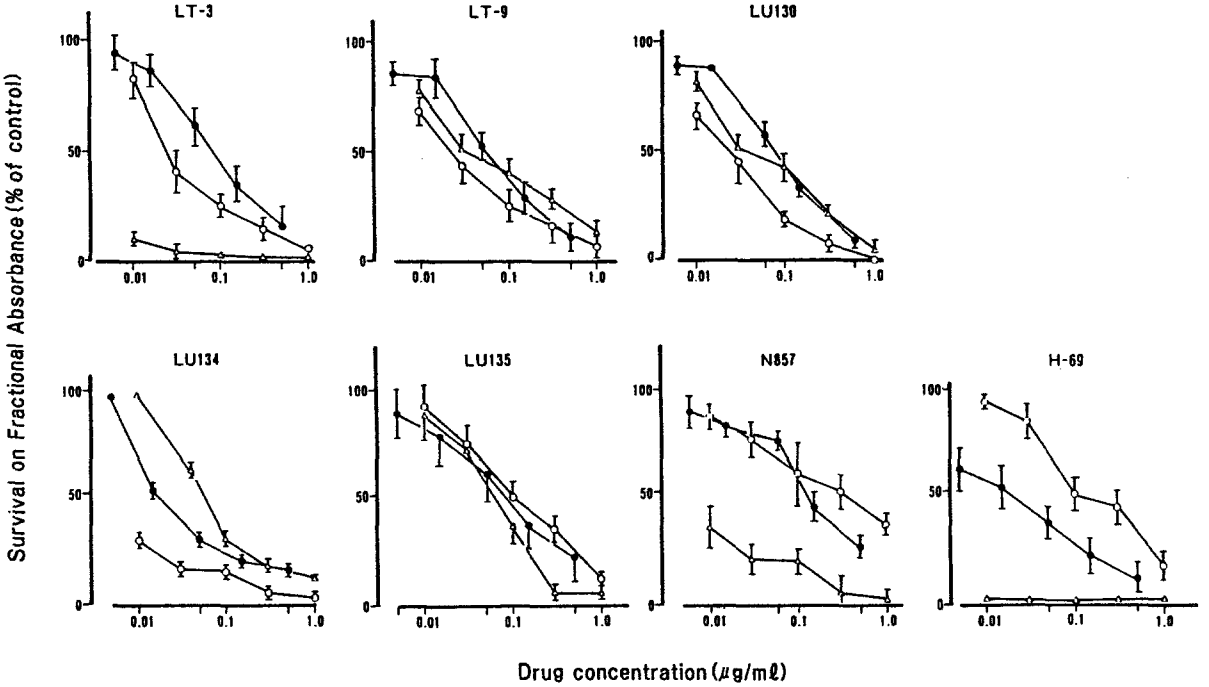


Fig. 3. Dose-response curve of RM-49 (●), FK973 (△), and MMC (○) on human SCLC cell lines. Each point represents the mean ± SD of three plates

was calculated by log (predicted PPC) = $0.755 \times \log$ (mice LD_{50}) $- 0.788$ [9].

Results

Effect of cell count and duration of incubation on MTT formazan production

The growth rate of each cell line in microtiter wells was examined by the changes in OD as well as by cell count. The representative data on LT9 and PC14 are shown in Fig. 2. The doubling time of both cell lines determined by the changes in OD were similar to those determined by direct cell count. For each cell line, the optimal seeding concentration was divided to give maximal absorbance where possible while ensuring that cells remained in a state of exponential growth. The cell counts and incubation times chosen for LT9 and PC14 were 2×10^4 and 2×10^3 cells/well and 7 and 4 days, respectively. The appropriate conditions for cell counts and incubation times for other SCLC and NSCLC were the same as those for LT9 and PC14 cells, respectively (data not presented).

Drug sensitivity test

In vitro chemosensitivity tests for eight drugs (RM-49, FK973, MMC, CDDP, CBDCA, VP16, ADM, ans VDS) were carried out with seven SCLC and four NSCLC cell lines. The IC_{50} values of each drug in seven small-cell lung cancer cell lines are shown in Table 2. The mean IC_{50} values (μg/ml) were 0.06, 0.04, 0.09, 0.43, 4.3, 0.88, 0.12, and 0.012 for RM-49, FK973, MMC, CDDP, CBDCA, VP16, ADM, and VDS, respectively. There are variations of IC_{50} values for each of the SCLC cell lines. In NSCLC cell lines, mean IC_{50} values (μg/ml) were 0.21, 0.52, 0.17, 1.54, 20.6, 8.9, 0.19, and 0.05 for RM-49, FK973, MMC, CDDP, CBDCA, VP16, ADM, and VDS, respectively (Table 3).

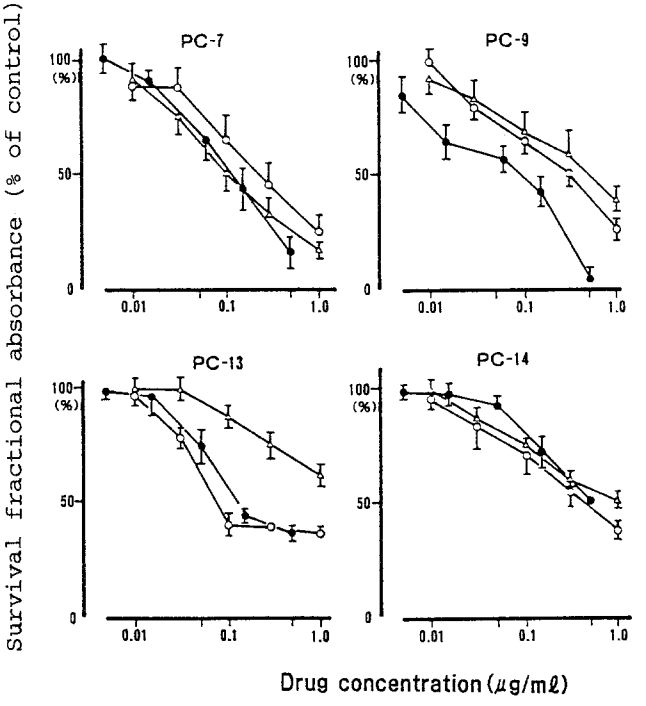


Fig. 4. Dose-response curve of RM-49 (●), FK973 (△), and MMC (○) on human NSCLC cell lines. The points represent the mean ± SD of three plates

The IC_{50} values of all the NSCLC cell lines were significantly higher than those of SCLC cell lines for anticancer drugs such as CDDP, CBDCA, VP16, and VDS ($P \leqq 0.05$). The sensitivity to three drugs (RM-49, FK973, and MMC) was different in each of the cell lines. In SCLC cell lines, the IC_{50} for RM-49 was lower than that for MMC in H69 cells; however, it was higher in LT3, LU130, and LU134 cells than that for MMC. FK973 showed remarkably strong cytotoxicity against LT3, N857, and H69 cells. In LU134 cells, the IC_{50} value was lower for MMC than for the other two drugs. On the other hand, the IC_{50} value for FK973 was higher in PC13 cells (Figs. 3, 4).

Comparison of relative antitumor activity (RAA)

The relative antitumor activity (RAA) was obtained by dividing the peak plasma concentrations (PPC) in humans with the IC_{50} value. For RM-49 and FK973, predicted peak plasma concentrations (predicted PPC) calculated by log (predicted PPC) $= 0.755 \times \log$(mice LD50) $- 0.788$ were used instead of PPC [9]. RAAs for RM-49 and FK973 were significantly higher than for MMC ($P \leqq 0.05$) and were equivalent to those for VP16 and VDS in SCLC cell lines (Table 4). RAAs for RM-49 were also higher than those for MMC in NSCLC ($P \leqq 0.05$) (Table 5), although those for FK973 were higher than those for MMC in three cell lines.

Discussion

In the last 10–15 years, progress in cancer chemotherapy has led to an improvement in the 3-year survival for limited disease cases in SCLC. However, <5% of the patients with extensive disease in SCLC and advanced NSCLC have experienced a 2-year survival, and phase III studies comparing chemotherapeutic effects on NSCLC have failed to show any increase in long-term survival. There is currently strong emphasis on the great need for new drug development for lung cancer treatment. Screening for new anticancer drugs has primarily been conducted using a variety of in vitro tumor systems. One of the important observations involving in vivo mouse-tumor panel screening is that the majority of disease-oriented phase II studies based on the tumor panel are negative.

In this report, we employed the MTT assay, which has great potential as a rapid method of screening for the drug responsiveness of cell lines. It should be stressed that the optimal condition of the MTT assay should be elucidated for each cell line in terms of both cell count and incubation time. It is essential that sufficient time is allowed for cell death and loss of dehydrogenase activity and that control cells are in a state of exponential growth at the time of the assay. Because the IC_{50} is defined as a 50% reduction in absorbance compared with control values, the IC_{50} for a drug in cells growing logarithmically might be lower than in cells that are allowed to plateau. Similarly, if sufficient time is not allowed for cell death and loss of dehydrogenase activity in treated cells, overestimation of the IC_{50} may occur [3]. Our data showed that the increase in OD of PC14 in NSCLC cell lines with short doubling times of 35 h achieved a plateau in 5 days; therefore we used 2×10^3 cells/well and incubated PC14 cells for 4 days. On the other hand, the growth of LT9 cell with long doubling times of 88 h did not achieve the plateau in 7 days; thus, we used 2×10^4 cells/well and incubated LT9 cells for 7 days.

Table 4. Relative antitumor activity (PPC/IC_{50}) for eight cytotoxic drugs in SCLC cell lines

	PPC	LT3	LT9	LU130	LU134	LU135	N857	H69	Mean ± SD
RM-49	5.35*	71.3	97.2	76.4	334.3	71.3	44.6	297.2	111.8 ± 111.4
FK973	1.74*	> 174.0	52.7	52.7	37.8	29.0	> 174.0	> 174.0	99.2 ± 65.3
MMC	0.52	24.8	24.8	13.3	> 65.0	5.8	1.6	5.5	20.1 ± 20.2[a, b]
CDDP	2.49	5.9	7.3	5.6	9.5	3.9	4.2	7.8	6.3 ± 1.9[a, b]
CBDCA	35.0	8.0	16.7	29.3	14.1	5.3	6.8	4.4	12.1 ± 8.2[a, b]
VP16	30.0	20.0	40.0	109.0	> 120.0	33.3	> 120.0	30.0	67.5 ± 42.8
ADM	0.60	3.0	6.0	13.6	6.8	3.0	6.0	6.0	6.3 ± 3.3[a, b]
VDS	0.883	117.7	103.8	67.9	58.9	35.3	135.8	116.2	90.8 ± 34.2

* Predicted peak plasma concentration (Peak plasma concentration, PPC; μg/ml)
[a] Significantly different from RM-49 ($P \leqq 0.05$)
[b] Significantly different from FK973 ($P \leqq 0.05$)

Table 5. Relative antitumor activity (PPC/IC_{50}) for eight drugs in NSCLC cell lines

	PPC	PC7	PC9	PC13	PC14	Mean ± SD
RM-49	5.35*	48.6	66.9	38.2	10.7	41.1 ± 20.3
FK973	1.74*	13.4	3.6	< 1.7	1.7	5.1 ± 4.9[a]
MMC	0.52	2.2	1.6	7.4	1.3	3.1 ± 2.5[a]
CDDP	2.49	1.0	3.0	1.4	2.6	2.0 ± 0.9[a]
CBDCA	35.0	1.1	3.0	1.6	2.1	2.0 ± 0.7[a]
VP16	30.0	62.5	2.9	6.5	1.5	18.4 ± 25.6
ADM	0.60	5.0	1.7	9.4	2.7	4.7 ± 3.0[a]
VDS	0.883	67.9	23.9	22.1	8.8	30.7 ± 22.3

* Predicted peak plasma concentration (Peak plasma concentration, PPC; μg/ml)
[a] Significantly different from RM-49 ($P \leqq 0.05$)

The IC_{50} and RAA values for RM-49 and FK973 were compared with those of six other drugs, including MMC. Although the IC_{50} value for RM-49 and MMC were almost identical, the RAAs for RM-49 were significantly higher than those for MMC in all cell lines, because the predicted PPC for RM-49 (5.35 μg/ml) was higher than that of MMC (0.52 μg/ml). The question remained as to whether such a high PPC would be achievable in the clinical phase I study. FK973 strongly suppressed the growth of three SCLC cell lines: LT3, N857, and H69 (Fig. 3). The RAAs for FK973 against all the SCLC lines were higher than those for MMC ($P \leqq 0.05$) (Table 4). According to Scheithauer, the PPC for anticancer drugs can be predicted by using the formula, log (predicted PPC) = $0.755 \times$ log (mice LD_{50}) $- 0.788$, before the clinical phase I study. The PPC and predicted PPC are 0.52 and 0.82 μg/ml for MMC and 2.49 and 1.95 μg/ml for CDDP, respectively, indicated the validity of this formula. The RAA calculated by using the formula PPC/IC_{50} varied widely in each cell line. Although some discrepancies may exist between RAAs and clinical observations due to the difference in the pharmacokinetics of drugs in vitro and in vivo, the comparison of the cytotoxic activities of CDDP and CBDCA showed that the RAA for CBDCA was higher than that for CDDP in SCLC cell lines and that those of both drugs were the same in NSCLC cell lines. These data corresponded well with the previously reported data, which was determined by a human tumor clonogenic assay (HTCA) [6].

The MTT assay is an excellent method for the rapid, valid, and simple assessment of chemosensitivity in cell lines [4]. However, it should be stressed that results differ depending on the plating cell count and incubation time. With careful use of the MTT assay, it is anticipated that many drugs with in vitro activity against SCLC and NSCLC cell lines will be discovered and subsequently incorporated into clinical trials.

Our experiments with RM-49 and FK973 suggested that they could be used against lung cancer. FK973 is currently undergoing Phase I investigation in Japan. However, it is more urgent to find drugs effective against NSCLC. From this viewpoint, as shown in Table 3, MMC, ADM, and VDS are at least as effective as the two new drugs. Therefore, the development of more effective drugs against NSCLC is essential for the improvement of chemotherapy results in lung cancer.

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