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# Potentiation of antitumor and antimetastatic activities of adriamycin by a novel N-alkylated dihydropyridine, AC394, and its enantiomers in colon cancer-bearing mice

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**Abstract** *Purpose*: We have previously shown that a series of N-alkylated 1,4-dihydropyridines potentiate the therapeutic efficacy of vincristine in vincristine-resistant P388 leukemia. The purpose of this study was to investigate the ability of one of the compounds, AC394, and its enantiomers to potentiate the antitumor activity of adriamycin against colon cancer cells in vitro and in vivo. *Methods*: The effects of AC394 on potentiation of adriamycin cytotoxicity and enhancement of its accumulation were evaluated using colon 26, HCT-15 and MCF-7 cells. Furthermore, the activities of AC394 and its enantiomers were compared. We also studied the combined effects of (+)-AC394 and adriamycin on subcutaneously (s.c.)-implanted and liver metastasis tumor models. *Results*: AC394 potentiated the cytotoxicity of adriamycin and enhanced its accumulation in colon cancer cells (colon 26 and HCT-15), which are known to express P-GP (P-glycoprotein) intrinsically. Enhancement of adriamycin accumulation by AC394 was found in s.c.-implanted colon 26 cells in vivo. Although both enantiomers of AC394 showed equal activity in vitro, (+)-AC394 was more effective than (−)-AC394 given orally. (−)-AC394 was found to be cleared more rapidly from the plasma than (+)-AC394. Thus, (+)-AC394 was evaluated for further study. Administration of (+)-AC394 significantly potentiated the antitumor activities of adriamycin in human colon cancer HCT-15 cells implanted s.c. Furthermore, in the liver metastasis model using colon 26 cells, a model completely resistant to adriamycin, the combination therapy of adriamycin with (+)-AC394 produced superior antitumor effects over adriamycin alone. *Conclusions*: A newly synthesized N-alkylated 1,4-dihydropyridine derivative, (+)-AC394, showed superior effects on the potentiation of adriamycin antitumor and antimetastatic activities in vivo. These results suggest that this combination may have therapeutic efficacy not only against primary colon cancers but also against metastatic liver cancer.

**Key words** AC394 · Multidrug resistance · Colon cancer

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## Introduction

Development of drug resistance during treatment is a major obstacle to cancer chemotherapy. It is well known that myeloma and lymphoma frequently develop drug resistance, despite initial response to chemotherapy. In the past decade, it has become clear that such acquired resistance can be partially explained by a cell membrane 170 000 Da molecular mass glycoprotein, called P-GP (P-glycoprotein), encoded by the *mdr*1 gene [6, 7]. Several classes of compounds have been found to reverse multidrug resistance (MDR) in a mouse P388 leukemia cell line resistant to vincristine or adriamycin in vitro and in vivo [3, 12, 17, 19, 24, 25].

However, a number of solid tumors, such as colon cancer, are resistant to primary chemotherapy, although the mechanisms responsible for the intrinsic drug resistance have yet to be identified. As the *mdr*1 gene has been shown to be present in several untreated human solid tumors, it might be possible that P-GP is partly responsible for intrinsic resistance [5, 14]. In fact, it has been reported that verapamil, an MDR-reversal agent, increases drug sensitivity in some wild-type colon cancer cell lines in vitro [1, 10, 21]. In our

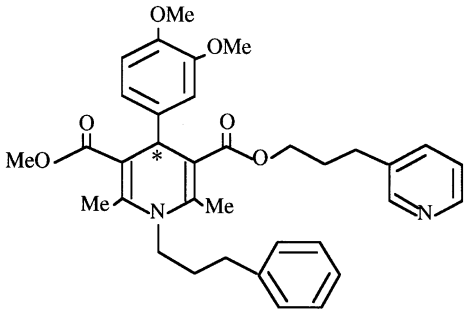


**Fig. 1** Chemical structure of AC394. The asterisk indicates an asymmetric carbon

previous study [15], we showed that a series of N-alkylated 1,4-dihydropyridines potentiate the therapeutic efficacy of vincristine in vincristine-resistant P388 leukemia in vivo and in vitro.

In the present study, we describe how the N-alkylated 1,4-dihydropyridines, AC394, (Fig. 1) and both its enantiomers potentiate the antitumor effect of adriamycin against intrinsically resistant murine and human colon cancer cells in vitro and in vivo. The compound also showed antimetastatic activity in a liver metastasis model.

## Material and methods

### Drugs

AC394, 1,4-dihydro-4-(3,4-dimethoxyphenyl)-2,6-dimethyl-1-(3-phenylpropyl)-3,5-pyridinedicarboxylic acid 3-methyl-5-[3-(3-pyridyl) propyl] ester (Fig. 1) was synthesized as described previously [15]. Its enantiomers were synthesized by esterifying with 3-(3-pyridyl) propanol, the optically pure 1,4-dihydro-4-(3,4-dimethoxyphenyl)-2,6-dimethyl-1-(3-phenylpropyl)-3,5-pyridinedicarboxylic acid, which is optically resolved by forming a salt with (−)-cinchonidine. Optical purity was established by a chiral HPLC (SUMIPAK OA 2000A; Sumika Chemical Analysis Service Ltd., Osaka, Japan) using 0.5 m*M* ammonium acetate in methanol as the mobile phase. Elution was monitored by absorbance at 254 nm. The hydrochloride salts of AC394 and its enantiomers were used in all experiments. All other chemicals used were of analytical grade.

### Animals and tumors

Female $CDF_1$ and ICR nu/nu mice were purchased from Charles River Japan, (Atsugi, Japan). Human colon cancer HCT-15 and human breast cancer MCF-7 cell lines were purchased from the American Type Culture Collection (Rockville, Md.). Murine colon cancer colon 26 cell line was supplied by Simonsen Laboratories (Gilroy, Calif.) under the auspices of the National Cancer Institute, NIH (Bethesda, Md.), and was maintained by the Japanese Foundation for Cancer Research (Tokyo, Japan). RPMI-1640 medium supplemented with 10% fetal calf serum was used as the culture medium.

### In vitro cytotoxicity

Cytotoxicity was evaluated using a tetrazolium-based assay (MTT assay) [13]. First, cells were plated into 96-well microtiter plates at $1 \times 10^4$ cells/well (50 µl). At 24 h after plating, 25 µl adriamycin (Kyowa Hakkou Co., Tokyo, Japan) solution and 25 µl AC394 solution diluted with medium were added. After 2 or 3 days, 10 µl MTT (5 mg/ml) was added to each well, and the cells were incubated for 4 h. Formazan crystals were dissolved by adding 100 µl 10% sodium dodecylsulfate (SDS) in 0.01 *N* hydrochloric acid, and the absorbance of each well was measured on a microplate reader (Vmax Kinetic Microplate Reader; Molecular Devices Co., Menlo Park, Calif.).

### Adriamycin accumulation

To estimate adriamycin accumulation in vitro [27], cells ($1 \times 10^5$ cells/well) were plated into 24-well plates and incubated overnight at 37° C. The cells were then incubated in the presence of adriamycin (10 µg/ml, 17.2 µ*M*) with or without AC394 for 1 or 2 h and washed twice with cold medium. Adriamycin was extracted from the cells with 10% SDS in 0.01 *N* HCl, and quantitated by spectrofluorometry (excitation wavelength, 485 nm; emission wavelength, 590 nm; Cyto Fluor 2300; Millipore Intertech, Bedford, Mass.). For in vivo studies, colon 26 cells ($1 \times 10^6$) were inoculated subcutaneously (s.c.) into $CDF_1$ mice on day 0. On day 7, 10 mg/kg adriamycin was injected intravenously (i.v.), and 800 mg/kg of AC394 was administered orally (p.o.). After 0.5, 1, 4, and 24 h, the mice were sacrificed, and adriamycin was extracted from the tumors [8]. Adriamycin was quantitated by HPLC (YMC-Pack ODS-A; YMC Co., Kyoto, Japan). Acetonitrile with 0.1% trifluoroacetic acid (A) and water with 0.1% trifluoroacetic acid (B) were used as the mobile phases and a linear gradient (A = 20% to 50% in 15 min) was used for elution. Elution was monitored by fluorescence (excitation wavelength, 470 nm; emission wavelength, 550 nm). Each group comprised three mice.

### Plasma concentrations of AC394 enantiomers

(+)-AC394 or (−)-AC394 (800 mg/kg) was administered p.o. to healthy $CDF_1$ mice. After 0.5, 1, 4 and 8 h, blood was drawn, and to 0.2 ml plasma was added 0.8 ml dichloroethanemethanol (2 : 1). After centrifugation at 10 000 *g* for 5 min, the supernatant was evaporated and the residue was dissolved in methanol. The concentrations of AC394 enantiomers were measured by HPLC (YMC-Pack ODS-A). Acetonitrile with 0.1% trifluoroacetic acid (A) and water with 0.1% trifluoroacetic acid (B) were used as the mobile phases and a linear gradient (A = 45% to 60% in 15 min) was used for elution. Elution was monitored by absorbance at 350 nm. Each group comprised five mice.

### In vivo antitumor activities

In transplanted solid tumor models, colon 26 cells ($1 \times 10^6$) were inoculated s.c. into $CDF_1$ mice, and HCT-15 cells ($1 \times 10^6$) were inoculated s.c. into ICR nu/nu mice on day 0. Adriamycin was given i.v. on days 7 and 14 (colon 26 cells) or on days 14 and 21 (HCT-15 cells). AC394 enantiomer was administered p.o. twice a day on the day of and the day after adriamycin administration. On day 21 (colon 26 cells) or day 28 (HCT-15 cells), two diameters of each tumor were measured using a caliper. The tumor weights were calculated using the formula: tumor weight (mg) = [length (mm) × (width (mm))$^2$]/2. The inhibition ratio was evaluated as $(1 - T/C) \times 100$ (%) (where T is the mean tumor weight of the treated group, and C is the mean tumor weight of the control group). To produce the liver metastasis system, colon 26 cells ($1 \times 10^5$) were inoculated into the spleen on day 0. Adriamycin was injected i.v. on days 7 and 14, and (+)-AC394 was administered p.o. as above. On day 19, the mice were sacrificed, and the organs with tumors

were weighed. The inhibition ratio was evaluated as $(1 - (T - N)/(C - N)) \times 100$ (%) (where N is the mean organ weight of the normal group). Five mice were used for each group in these experiments.

## Results

### In vitro effects of AC394 on potentiation of adriamycin cytotoxicity

The in vitro potentiation of adriamycin cytotoxicity by AC394 in colon 26 and HCT-15 colon cancer cell lines and the MCF-7 breast cancer cell line are shown in Fig. 2. MCF-7 cells were used because the tumor showed higher sensitivity to adriamycin than colon tumors as described below. In colon 26 cells, AC394 (0.3 or 1.0 μg/ml) alone did not show cytotoxicity, but the combination of adriamycin with 1.0 μg/ml of AC394 resulted in a fivefold enhancement of adriamycin cytotoxicity as determined by the 50% growth-inhibitory concentration ($IC_{50}$) (Fig. 2A). As shown in Fig. 2B, adriamycin itself was approximately 30 times more cytotoxic to MCF-7 cells than to HCT-15 cells. Although AC394 (1.0 μg/ml) did not potentiate the activity of adriamycin in MCF-7 cells, AC394 significantly potentiated the effect of adriamycin against HCT-15 cells (up to 20 times). Colon 26 and HCT-15 cells are known to express P-GP, but MCF-7 cells do not [11, 18, 21]. These results suggest that AC394 is effective against P-GP-positive tumors.

### Effects of AC394 on adriamycin accumulation in colon cancer cells

Next, the effects of AC394 on adriamycin accumulation were studied in vitro. AC394 increased the accumulation of adriamycin in colon 26 and HCT-15 cells in a dose-dependent manner (Fig. 3A, B). In HCT-15 cells, 3.0 μg/ml AC394 increased adriamycin accumulation about threefold. In contrast, AC394 did not potentiate the accumulation of adriamycin in P-GP-negative MCF-7 cells, even at high concentrations (10 μg/ml), although, without AC394, adriamycin accumulated at higher levels in MCF-7 cells than in colon 26 and HCT-15 cells (Fig. 3C). The enhanced cytotoxicity of adriamycin by AC394 seems to be explained by the enhanced accumulation of adriamycin in the resistant cells.

To investigate whether this holds true in vivo, colon 26 cells were inoculated s.c., and the concentration of adriamycin in solid tumors was measured with or without oral administration of AC394. The concentration of adriamycin in colon 26 tumors was significantly higher at 4 h after adriamycin administration with AC394 than with adriamycin alone (Fig. 4).

### Comparison of the effects of AC394 and its enantiomers in vitro and in vivo

As AC394 is a racemic mixture, we investigated the activities of its enantiomers (+)-AC394 and (−)-AC394 for potentiation of cytotoxicity of adriamycin. No significant difference was observed between these enantiomers in colon 26 cells in vitro (Fig. 5). However, against colon 26 solid tumors in vivo, (+)-AC394 was more effective than (−)-AC394 at the same dose (Fig. 6A–C). On day 21, adriamycin alone showed a low inhibition ratio (49%), and (+)-AC394 alone did not show any antitumor activity at 800 mg/kg (Fig. 6D). The combined administration of adriamycin and 800 mg/kg of (+)-AC394 produced a 79% inhibition ratio. In this experiment, twice the dose of (−)-AC394 showed the same activity as (+)-AC394 in combination with adriamycin on tumor volume suppression. To clarify the reason for the different activities, the concentration in plasma after oral

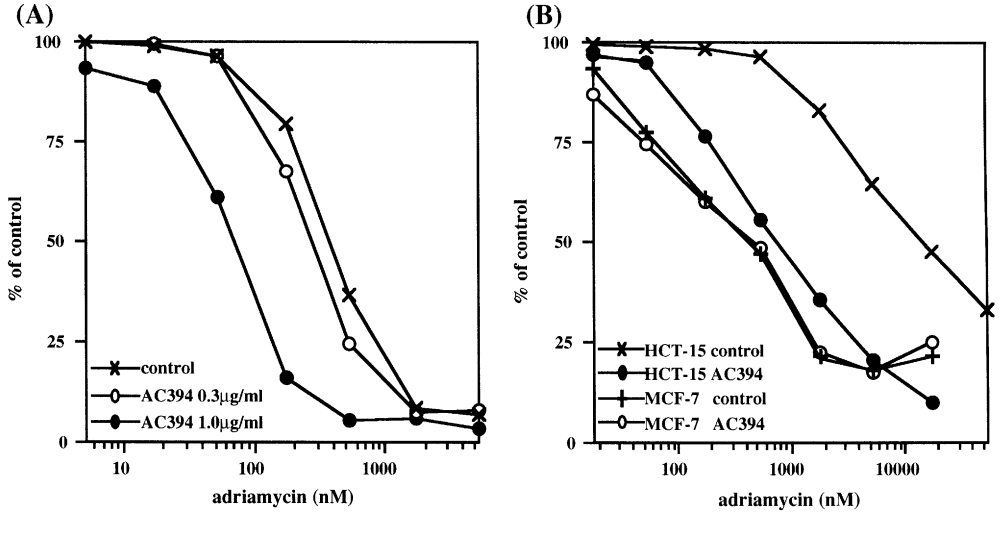


**Fig. 2A, B** Effects of AC394 on the potentiation of adriamycin cytotoxicity in murine colon cancer cell line colon 26 (**A**) and human cancer cell lines HCT-15 and MCF-7 (**B**). Cells were cultured with various concentrations of adriamycin in the absence or presence of 0.3 (○) and 1.0 (●) μg/ml AC394 (**A**), 1.0 μg/ml AC394 (○, ●) (**B**). Each point represents the mean value for three determinations whose coefficient variation was < 5%

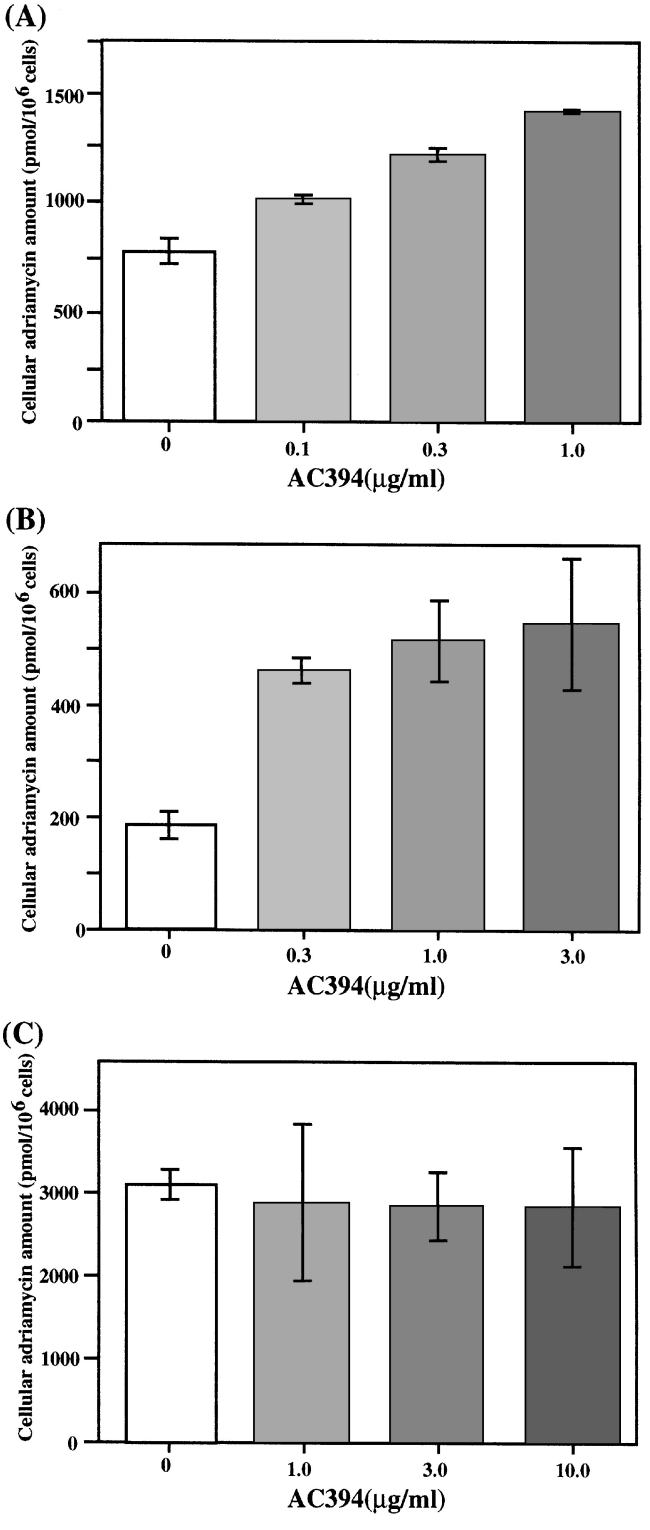


**Fig. 3A–C** Effects of AC394 on the accumulation of adriamycin in colon 26 (**A**), HCT-15 (**B**), and MCF-7 (**C**) cells. Cells were incubated with 10 μg/ml (17.2 μ*M*) of adriamycin in the absence or presence of various concentrations of AC394. The results shown are means ± SD of triplicate determinations

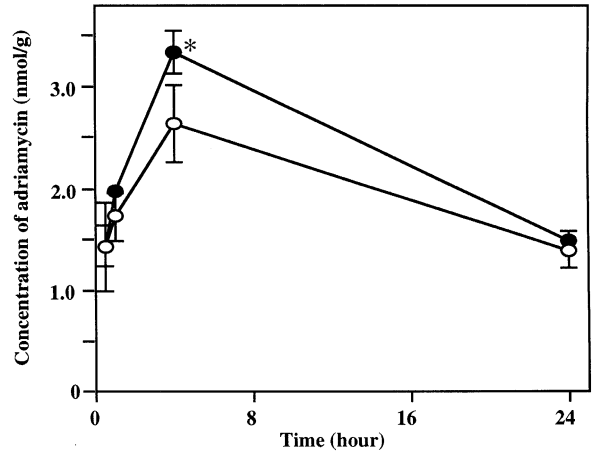


**Fig. 4** Effects of AC394 on the accumulation of adriamycin in colon 26 solid tumor in vivo. Colon 26 cells were inoculated s.c. on day 0. On day 7, adriamycin (10 mg/kg) was injected i.v. with (●) or without (○) AC394 (800 mg/kg p.o.). At the indicated times, mice were sacrificed, and adriamycin was extracted from the tumors. Each point represents the mean ± SD from three mice. $*P < 0.05$, versus adriamycin alone at the same time, Student's *t*-test

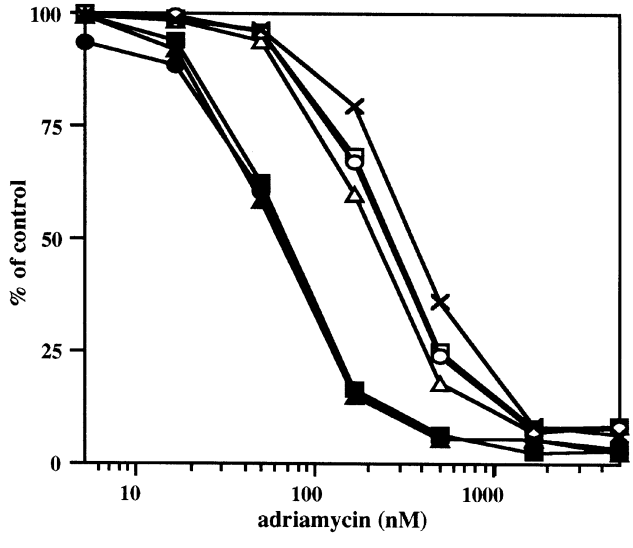


**Fig. 5** Effects of (+)-AC394 and (−)-AC394 on the potentiation of adriamycin cytotoxicity. Colon 26 cells were cultured with various concentrations of adriamycin in the absence (×) or presence of 0.3 (*open symbols*) and 1.0 (*closed symbols*) μg/ml of AC394 (○, ●), (+)-AC394 (□, ■) or (−)-AC394 (△, ▲). Each point is the mean value of three determinations whose coefficient variation was < 5%

administration was measured. As shown in Fig. 7, (−)-AC394 was more rapidly cleared from plasma than (+)-AC394, suggesting that the superior effect of (+)-AC394 depends on its stability in vivo.

## In vivo activity of (+)-AC394 against human colon cancer

Adriamycin (10 mg/kg) alone on days 7 and 14 did not show any antitumor activity against human colon

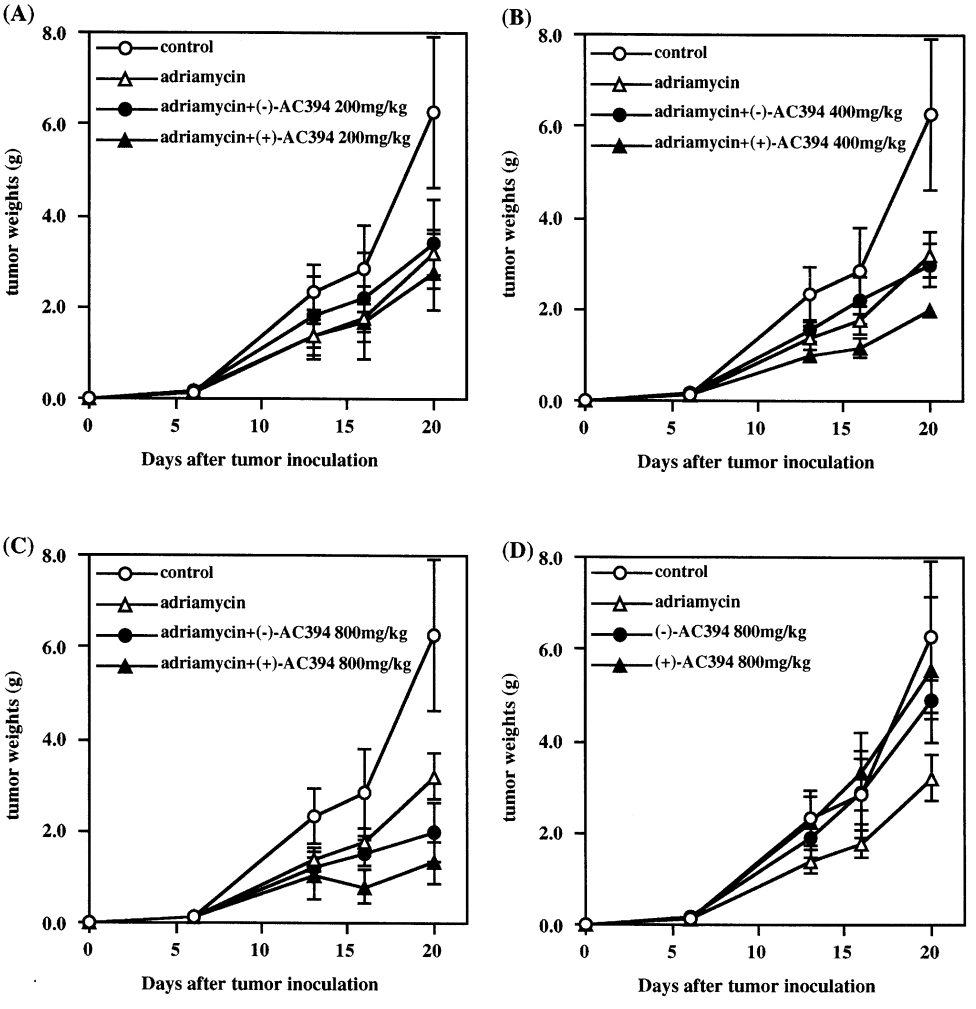


**Fig. 6A–D** Effects of (−)-AC394 and (+)-AC394 on the antitumor activity of adriamycin in colon 26-bearing mice. Colon 26 cells were inoculated s.c. on day 0. On days 7 and 14, adriamycin (10 mg/kg) was injected i.v. and (−)-AC394 or (+)-AC394 was administered p.o. as described in Material and methods, after administration of adriamycin. The results are means ± SD from five mice

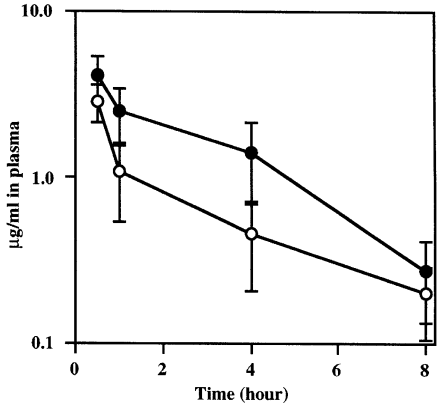


**Fig. 7** Plasma concentrations of (+)-AC394 and (−)-AC394. (−)-AC394 (○) or (+)-AC394 (●) (800 mg/kg) was administered p.o.. At the indicated times, mice were sacrificed, and plasma concentrations were measured. Each point is the mean ± SD from five mice

cancer HCT-15 cells in nude mice. As shown in Fig. 8, (+)-AC394 in combination with adriamycin suppressed tumor growth in a dose-dependent manner, and at a dose of 800 mg/kg p.o. (four times for 2 days after i.v. administration of adriamycin) (+)-AC394 significantly enhanced the antitumor effect of adriamycin.

## The combined effect of (+)-AC394 and adriamycin on liver metastasis

Finally, we investigated the effect of the combination of adriamycin and (+)-AC394 on liver metastasis, since it has been recently reported that tumors in liver are highly resistant to adriamycin compared with those in the subcutis [4, 26]. To produce liver metastasis, colon 26 cells were implanted into the spleen, and the tumor metastasis to the liver was estimated by measuring the weight of the liver after 19 days. When adriamycin was administered alone, the primary tumor in the spleen was sensitive (inhibition ratio 89%) without any antitumor effects on the liver metastasis (Fig. 9). On the other hand, the combination of adriamycin and (+)-AC394 showed superior antitumor effects on both spleen-implanted tumor (inhibition ratio 98%) and liver metastasis (inhibition ratio 97%).

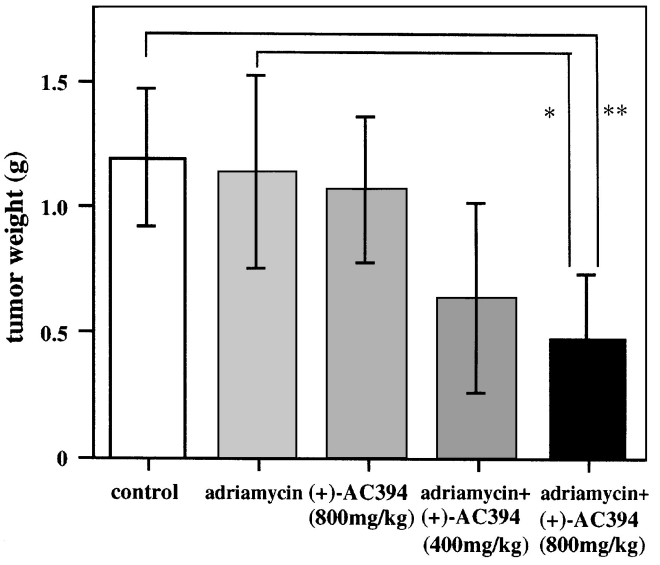


**Fig. 8** Effect of (+)-AC394 on the antitumor activity of adriamycin in HCT-15-bearing mice. HCT-15 cells were inoculated s.c. on day 0. Adriamycin (10 mg/kg) was injected i.v. on days 14 and 21. (+)-AC394 (800 mg/kg) was administered p.o., as described in Material and methods, after administration of adriamycin. On day 28, the sizes of the tumors were determined. The results shown are means ± SD from five mice. $*P < 0.05$, $**P < 0.01$, Student's *t*-test

## Discussion

Although colon cancer is one of the cancers most resistant to chemotherapy, little is known about its mechanisms of resistance. Recent studies have shown that one of the mechanisms is concerned with multidrug resistance [5, 14]. Verapamil, a well-known MDR modifier, enhances adriamycin cytotoxicity in colon cancer cell lines in vitro [1, 10, 21]. Many kinds of compounds, including verapamil analogues, have been used to try to potentiate antitumor activity in MDR-associated drugs not only in in vivo models but also in clinical settings. However, the majority of these compounds have unacceptable side effects or are therapeutically unsatisfactory. We have recently reported that some N-alkylated 1,4-dihydropyridines potentiate the therapeutic efficacy of vincristine in a leukemia model and have no calcium antagonistic activity [15]. In this study, we tested the therapeutic efficacy of a new MDR-modulating agent, N-alkylated 1,4-dihydropyridine (AC394) combined with adriamycin against colon tumors. AC394 potentiates the cytotoxicity of adriamycin in murine colon cancer colon 26 [2] and human colon cancer HCT-15 [23] cells in vitro, but not in human breast cancer MCF-7 cells. Colon 26 and HCT-15 cells express P-GP, but MCF-7 cells do not [11, 18, 21]. Accordingly, it has been suggested that these effects of AC394 correlate with enhanced accumulation of adriamycin within the cells. These results show that AC394 is an MDR modifier affecting P-GP.

AC394 also enhances adriamycin accumulation in colon 26 cells in vivo. So, we tested the efficacy of combination chemotherapy in vivo. AC394 was found to potentiate the antitumor activities of adriamycin in murine and human colon tumors. The effect of orally administered AC394 might be another advantage, and the more metabolically stable isomer (+)-AC394 should be further investigated as a novel MDR modifier in combination chemotherapy.

Recently, it has been shown that the sensitivity of tumor cells to chemotherapeutic agents depends on the tumor site [9, 16, 20, 22], and adriamycin is less effective in liver metastatic tumors than in s.c.-implanted tumors [4, 26]. In the present metastasis model system, the combination of (+)-AC394 and adriamycin is effective both against the primary tumor and at the metastasis site, although the metastatic tumor is completely resistant to adriamycin alone. Fidler reported that the difference in response to adriamycin between s.c.-implanted tumors and liver metastatic tumors is not due to the difference in the cell populations. Elevated levels of P-GP and *mdr*1 mRNA are found in the liver metastasis of colon 26 cells under the influence of the organ

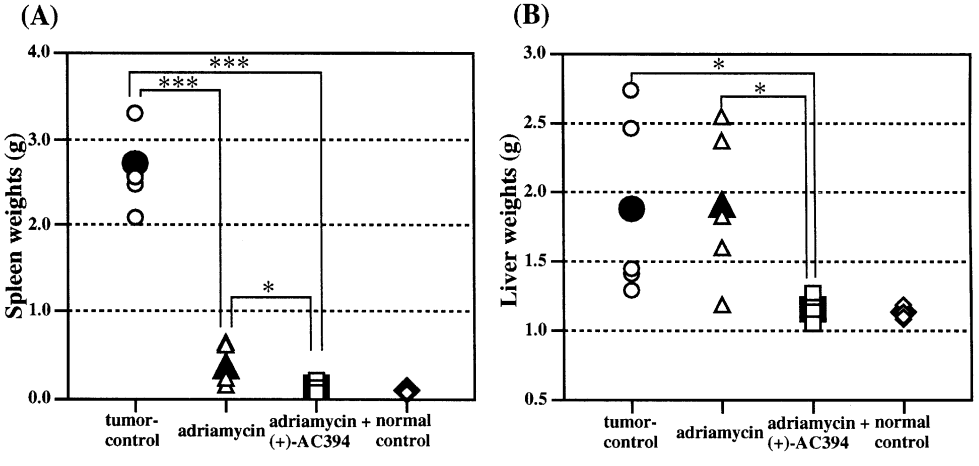


**Fig. 9** Combined effects of (+)-AC394 and adriamycin on liver metastasis. Colon 26 cells were inoculated into the spleen on day 0. Adriamycin was injected i.v. on days 7 and 14. (+)-AC394 (800 mg/kg) was administered p.o., as described in Material and methods, after administration of adriamycin (10 mg/kg). Mice were sacrificed on day 19, and spleens (**A**) and livers (**B**) with tumors were weighed (*normal control* weight of organs from normal mice, *open symbols* individual weights, *closed symbols* mean values). $*P < 0.05$, $***P < 0.001$, Student's *t*-test

environment [4]. The remarkable effect of (+)-AC394 on liver metastasis might be explained by the higher expression of P-GP in the metastatic colon 26 cells in the liver. We submit that the combination therapy presented here may have a therapeutic advantage not only against primary cancers but also against liver metastasis.

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