

Pladienolides, New Substances from Culture of *Streptomyces platensis* Mer-11107

III. *In Vitro* and *In Vivo* Antitumor Activities

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We have discovered seven novel 12-membered macrolides, pladienolides A to G, from *Streptomyces platensis* Mer-11107, with pladienolide B the most potently inhibiting hypoxia induced-VEGF expression and proliferation of the U251 cancer cell line. A growth inhibitory study using a 39-cell line drug-screening panel demonstrated that pladienolide B has strong antitumor activities *in vitro*. A COMPARE analysis reveals that it has a unique antitumor spectrum that sets it apart from anticancer drugs currently in clinical use. This result suggests that pladienolide B has a novel mechanism of action. A series of xenograft studies were conducted to evaluate the *in vivo* potency of pladienolides. Pladienolide B extensively inhibited tumor growth in xenograft models. In the most sensitive model, using BSY-1 xenografts, tumors were completely regressed by administration of pladienolide B. For the reason of their novel mechanism of action and excellent *in vivo* efficacy, pladienolides appear to have major potential for use in cancer treatment.

Tumor cells are frequently exposed to severe hypoxia. To survive and grow under hypoxic conditions, tumors adapt to their environment by activating a series of cascading events. This opens the possibility of creating novel antitumor drugs that interfere with the signaling involved in these adaptation processes. One key regulator for hypoxia adaptation is known to be hypoxia-inducible factor-1 (HIF-1)¹. HIF-1 is an essential transcription factor and is composed of two basic helix-loop-helix (HLH) PAS transcription subunits, HIF-1 α and HIF-1 β , which are believed to have potential as targets for antitumor drug development^{2,3}. However, it is difficult for small molecules to inhibit HIF-1 directly, since it is an HLH transcription factor. Accordingly, several groups are searching for compounds that inhibit the HIF-1 pathway, using a reporter gene assay controlled by hypoxia-responsive element (HRE), an HIF-1 binding sequence⁴.

We also have developed a cell-base screening system

using VEGF promoter (2.3 kb) containing endogenous HRE to search for HIF pathway modulators. The system is based on genetically engineered U251 human glioma cells that stably express a recombinant vector in which the placental alkaline phosphatase (PLAP) reporter gene is placed under the control of the human VEGF promoter. We performed a high throughput screening and as a result identified a series of microbial products that inhibit hypoxia-induced PLAP expression⁵. They are structurally novel 12-membered macrolides, which we designate as pladienolides^{5,6}.

In this report, we describe the *in vitro* and *in vivo* antitumor activities of pladienolides. These substances show highly potent antitumor activity both *in vitro* and *in vivo*, and may thus have potential for use in anticancer therapy.

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Materials and Methods

Antitumor Agents

Pladienolides B, C, and D were purified at Eisai Co., Ltd. (Tsukuba Research Laboratories, Ibaraki, Japan); they are shown in Fig. 1. Camptothecin (CPT), vincristine (VCR), etoposide (ETP), 5-fluorouracil (5-FU), and taxol were purchased from Sigma (St. Louis, MO). Cisplatin (CDDP) was purchased from Bristol-Myers Squibb (Tokyo, Japan). All antitumor agents except for CDDP were dissolved in DMSO for *in vitro* use.

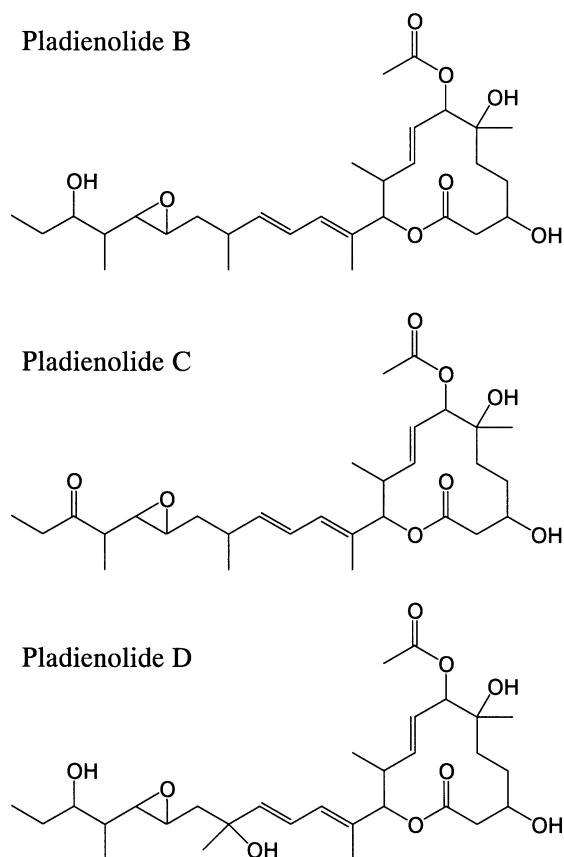
For *in vivo* study, pladienolides B, C, and D were dissolved at appropriate concentrations in 3.5% DMSO and 6.5% Tween 80 in 5% glucose solution.

Human Cancer Cell Line Panel

To evaluate pladienolide B for its cell growth inhibition profile, we used a human cancer cell line panel combined with a database. The system was developed according to the method of the National Cancer Institute^{7,8)}, modified by the Japanese Foundation for Cancer Research

(JFCR)^{9,10)}. The cancer panel experiment for pladienolide B was carried out in JFCR, and the inhibition profile was compared with those of more than 200 standard compounds including various anticancer drugs. The precise methods of experiments and data analysis are described elsewhere⁹⁾. We briefly show the cell lines used and the method for detecting growth inhibition. The following human cancer cell lines were used in cancer panel experiments: breast cancer HBC-4, BSY-1, HBC-5, MCF-7, and MDA-MB-231; brain tumor U251, SF-268, SF-295, SF-539, SNB-75, and SNB-78; colon cancer HCC-2998, KM-12, HT-29, HCT-15, and HCT-116; lung cancer NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273, and DMS114; melanoma LOX-IMVI; ovarian cancer OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; renal cancer RXF-631L and ACHN; stomach cancers St-4, MKN-1, MKN-7, MKN-28, MKN-45, and MKN-74; and prostate cancers DU-145 and PC-3. The cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 $\mu\text{g/ml}$) at 37°C in humidified air containing 5% CO₂. The GI₅₀ values for these cell lines were determined by colorimetric analysis after staining with sulforhodamine B.

Fig. 1. Chemical structures of pladienolides B, C, and D.



Cell Lines

P388 and BSY-1 were supplied by the Cancer Chemotherapy Center, JFCR. HCT-116, PC-3, OVCAR-3, and DU-145 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). WiDr was purchased from Dainippon pharmaceutical Co., Ltd. (Osaka, Japan).

Drug-resistant Cell Lines

P388/CDDP is a cisplatin-resistant cell line provided by the Cancer Chemotherapy Center, JFCR. MES-SA/Dx5, which was purchased from ATCC, is a doxorubicin-resistant cell line known to highly express P-glycoprotein. P388/CPT, P388/ETP, P388/VCR, and HCT116/5-FU cell lines were isolated from P388 or HCT116 by stepwise selection with increasing concentrations of camptothecin, etoposide, vincristine, and 5-fluorouracil, respectively, at our laboratories.

Growth Inhibition Assay

Exponentially growing tumor cells were seeded at 2×10^3 cells/well into 96-well plates. After 24 hours incubation, test compounds with serial dilutions were added to the plates, and the plates were incubated for 72 hours. MTT reagent (Sigma) was used to measure the live adherent cells, and alamarBlue reagent (TREK, Cleveland,

OH) was used for the non-adherent cells according to the manufacturer's manual. Briefly, $180\ \mu\text{l}$ of 2×10^3 cells were seeded into 96-well plates. After overnight incubation, $20\ \mu\text{l}$ of test compounds at serial dilutions were added to the plates and incubated for 72 hours. The cell number was then determined by MTT¹¹⁾ or alamarBlue assay⁵⁾. The growth inhibitory activity (IC_{50}) was determined by calculating the concentration at which cell growth was inhibited to 50% of control growth.

Cell Cycle Analysis

WiDr cells (1.5×10^5 cells/well) were cultured overnight in 6-well plates, and then incubated for 6, 12, 24, and 72 hours in the absence or presence of 10 nM pladienolide B; alternatively, WiDr cells were incubated for 24 hours in several dilutions of pladienolide B, 5-fluorouracil, taxol, or vincristine. After harvesting the cells by trypsinization, the cells were fixed in 70% ethanol at -20°C and stained with propidium iodide ($20\ \mu\text{g}/\text{ml}$) in the presence of RNaseA ($100\ \mu\text{g}/\text{ml}$). DNA content was analyzed by FACS Caliber and quantified using Modifit LT software (Beckton Dickinson, Franklin Lakes, NJ).

In Vivo Antitumor Activities

Female or male BALB/c *nu/nu* mice (7 weeks of age; Clea Japan, Inc., Tokyo, Japan) were housed in barrier facilities on a 12-hour light/dark cycle, with food and water. Cell suspensions of various human cancer cells were implanted subcutaneously (s.c.) into their flank. When the tumor reached $100\sim 250\ \text{mm}^3$ in volume, animals were divided randomly into test groups consisting of five mice per group (Day 1). The administration schedule was daily intravenous injection for five consecutive days (qdx5). The tumor volume (TV) was measured on Days 5, 8, 12, and 15 for PC-3, OVCAR-3, DU-145, WiDr, and HCT-116. In the BSY-1 xenografts, TV was measured twice a week for more than three months. TV was calculated as follows: $\text{TV} (\text{mm}^3) = \text{length} (\text{mm}) \times \text{width}^2 (\text{mm}^2) / 2$. T/C was the ratio of average tumor volume of the treated group to that of the control group. The minimum value for T/C during the experiment was designated as mT/C. Relative body weight (RBW) was calculated as $\text{RBW} = \text{BW} / \text{BW}_1$, where BW_1 is the body weight on Day 1. The minimum value for RBW during the experiment was designated as mRBW.

Results

Cancer Panel Experiment

A cancer panel experiment using 39 cancer cell lines was

carried out at the Cancer Chemotherapy Center. The mean graph of pladienolide B based on the growth inhibition parameter of GI_{50} is shown in Figure 2. Pladienolide B showed differential growth inhibition, and seemed to be most effective against lung and breast cancer cell lines. The BSY-1 MCF-7, NCI-H522, and OVCAR-3 cell lines were more sensitive than other cell lines. The mean log GI_{50} of pladienolide B was -8.65 ($2.2\ \text{nM}$), which was quite a strong activity compared to anticancer drugs in clinical use in the panel. A COMPARE analysis of the mean graph revealed that there is no standard anticancer drug with a high correlation coefficient to pladienolide B. The top five drugs in their rank order of correlation coefficient are listed in Table 1. They are all tubulin binders. However, their correlation coefficients were weak, which indicates that pladienolide B may have a unique mode of action. Taken together, the results of the cancer panel study indicated that pladienolide B is worth further analysis.

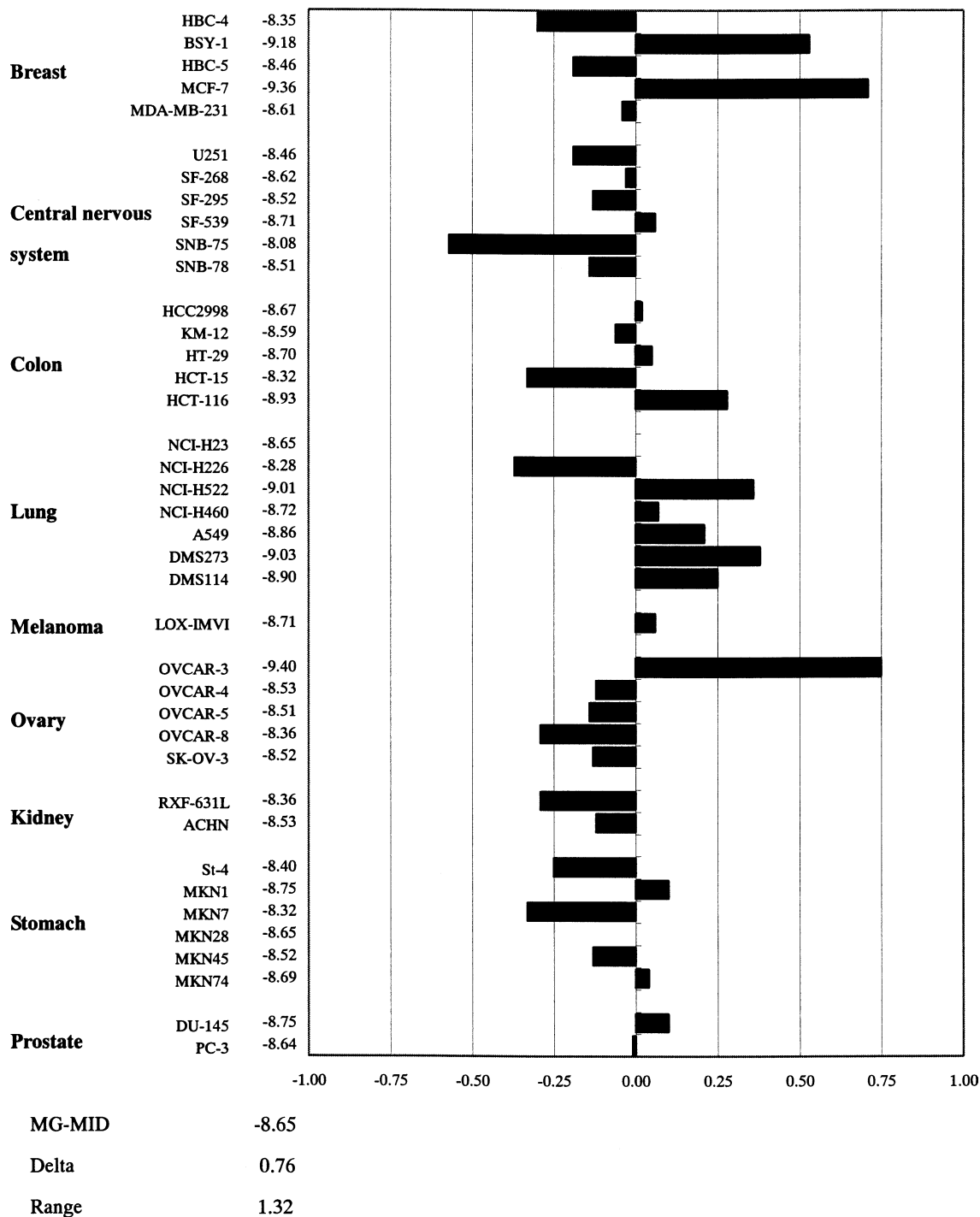
Growth Inhibitory Activities against Drug-resistant Cell Lines

The effect of growth inhibition of pladienolide B on several anticancer drug-resistant cell lines was measured (Table 2). P388/CPT, P388/ETP, P388/CDDP, P388/VCR, HCT-116/5-FU, and MES-SA/Dx5 cell lines were >1596.8 , 29.0, 13.5, 11.3, 12.6, and 498.5-fold resistant to camptothecin, etoposide, cisplatin, vincristine, 5-fluorouracil, and taxol, respectively, for relative resistance calculated by the ratio (IC_{50} of resistant cells/ IC_{50} of parent cells). Pladienolide B displayed unchanged antitumor effects against six drug-resistant cell lines compared with the parental cell lines, since the relative resistance were 2.3, 1.0, 1.0, 0.6, 0.9, and 2.3-fold. This result demonstrates that pladienolide B is also effective against drug-resistant cancer cells.

Effect of Pladienolide B on Cell Cycle Progression

To elucidate the mode of action of the cytotoxic effects of pladienolides, the effects of pladienolide B on cell cycle progression were examined. A human colorectal cancer cell line, WiDr, was treated with pladienolide B and analyzed by flow cytometry. As shown in Fig. 3A, treatment of the cells with pladienolide B caused a cell cycle arrest in both G1 and G2/M-phase in a time-dependent manner. Although several anticancer agents were tested under the same conditions, such as 5-FU, vincristine, and taxol, their cell cycle distribution patterns were different from that of pladienolide B (Fig. 3B).

Fig. 2. GI₅₀ mean graph of pladienolide B against a panel of 39 human cancer cell lines.



The mean graph was produced by computer processing of the GI₅₀s as described in "Materials and Methods." MG-MID: mean of log GI₅₀ values for 39 cell lines. Delta: logarithm of difference between the MG-MID and the log GI₅₀ of the most sensitive cell line. Range: the logarithm of the difference between the log GI₅₀ of the most resistant cell line and the log GI₅₀ of the most sensitive one.

In Vivo Antitumor Activities

Table 1. The COMPARE analysis of pladienolide B.

Drug	Correlation coefficient with the pladienolide B
Navelbine	0.543
Vinblastine	0.522
Vindesine	0.506
Taxol	0.464
Vincristine	0.368

The mean graph of pladienolide B was compared with those of standard compounds using the COMPARE analysis. Drugs were ordered according to the correlation coefficient.

The antitumor activities of pladienolide B, C, and D were evaluated against WiDr human colon tumor xenografts (Table 3). The drugs were intravenously administered once a day for 5 consecutive days (Day 1 to 5) at doses of 2.5, 5, 10, 20, or 40 mg/kg/day to WiDr tumor-bearing nude mice. No fatalities were observed in these dose regimens. At doses of 5 and 10 mg/kg/day, pladienolide B inhibited tumor growth by more than 50%. Pladienolide D also inhibited tumor growth in a dose-dependent manner. Concerning body weight changes, they decreased by day 5 after beginning administration, but recovered during the experiment.

We then examined pladienolide B against six human tumor xenografts at doses of 2.5, 5, and 10 mg/kg/day in nude mice. Pladienolide B showed strong growth inhibitory or regressive activities against these xenografts (Fig. 4). In the BSY-1 xenograft model, the administration of pladienolide B at doses of 2.5, 5, and 10 mg/kg/day completely regressed the BSY-1 tumor at Day 15 after the first administration. To observe tumor recurrence, mice were monitored for more than three months, and only one

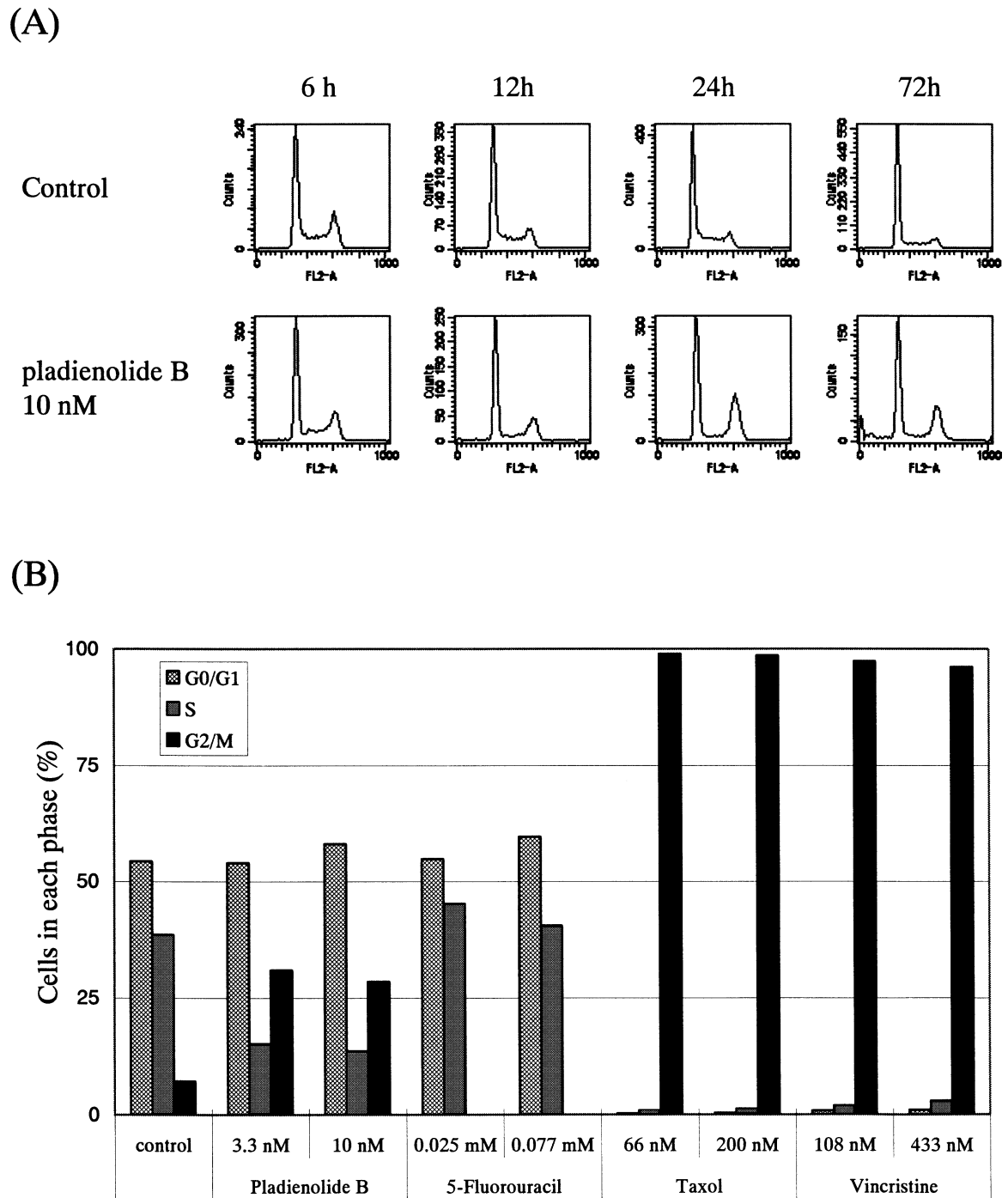
Table 2. Growth inhibitory activities against drug-resistant cell lines.

Compound	Relative resistance (IC ₅₀ value of resistant cell/IC ₅₀ value of parental cell)					
	P388				HCT-116	MES-SA
	^a CPT	^b ETP	^c CDDP	^d VCR	^e 5-FU	^f Dx5
Pladienolide B	2.3	1.0	1.0	0.6	0.9	2.3
Camptothecin	> 1596.8	-	-	-	-	-
Etoposide	-	29.0	-	-	-	-
Cisplatin	-	-	13.5	-	-	-
Vincristine	-	-	-	11.3	-	-
5-Fluorouracil	-	-	-	-	12.6	-
Taxol	-	-	-	-	-	498.5

^a Camptothecin resistant P388, ^b Etoposide resistant P388, ^c Cisplatin resistant P388,

^d Vincristine resistant P388, ^e 5-Fluorouracil resistant HCT-116, ^f Doxorubicin resistant MES-SA

Fig. 3. Flow cytometric cell cycle analysis of WiDr cells treated with pladienolide B and known antitumor drugs.



(A) WiDr cells were incubated for 6, 12, 24, and 72 hours in the presence or absence of pladienolide B.

(B) WiDr cells were treated with pladienolide B, 5-fluorouracil, taxol, and vincristine for 24 hours. The percentage of cells in each phase of the cell cycle is shown graphically.

Table 3. Antitumor effects of pladienolides B, C, and D against WiDr xenografts.

Compounds	Dose ^a (mg/kg/day)	mT/C ^b (on Day)	mRBW ^c (on Day)
Pladienolide B	2.5	0.54 (8)	0.93 (5)
	5.0	0.40 (8)	0.94 (5)
	10.0	0.31 (8)	0.88 (5)
Pladienolide C	2.5	0.76 (5)	1.01 (5)
	10.0	0.60 (5)	0.98 (5)
Pladienolide D	10.0	0.44 (5)	0.93 (5)
	20.0	0.33 (5)	0.90 (5)
	40.0	0.21 (5)	0.84 (5)

^a Compounds were administered i.v., by consecutive injection on Day 1 to Day 5 (qdx5).

^b T/C value was calculated by the following formula; T/C = average tumor volume in treated group / average tumor volume in control group. Minimum T/C (mT/C) is the lowest T/C during the experiment.

^c Relative body weight (RBW) was calculated by the following formula; RBW=average body weight on day N/ average body weight on day 1. Minimum RBW (mRBW) is the lowest T/C during the experiment.

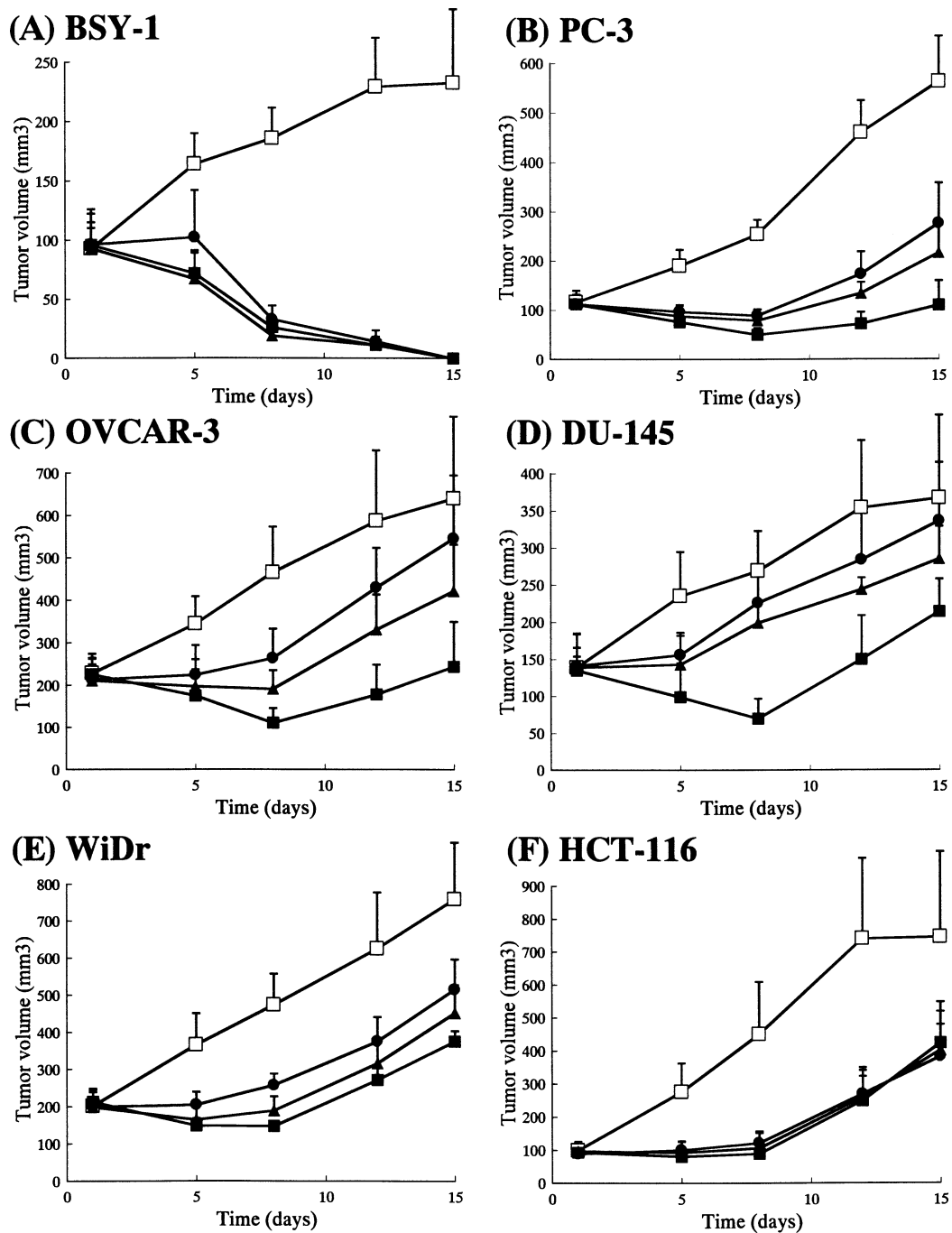
recurrence was seen at Day 37 at a dose of 2.5 mg/kg/day. As a result, fourteen mice out of fifteen were cured by pladienolide B treatment.

Discussion

In the preceding paper, we isolated seven novel macrolide compounds, designated as pladienolides A to G. Six of them inhibited hypoxia-inducible-factor (HIF) dependent transcription⁵⁾. Notably, they inhibited VEGF promoter-dependent placental alkaline phosphatase (PLAP) reporter expression in U251 cells when the cells were exposed to hypoxic conditions⁵⁾. The HIF pathway is the most important physiological regulator of hypoxia signals and is believed to be required for tumor progression *in vivo*, so pladienolides that inhibit the HIF pathway are likely to act as powerful angiogenesis inhibitors. Pladienolide B also

possesses strong cytotoxic effects on a variety of cancer cells *in vitro*, and bases on a novel mechanism of action that is different from the antitumor drugs currently in clinical use. We have not discovered yet whether or not the cytotoxic effects of pladienolides are a result of their inhibition of the HIF-1 pathway. In general, antitumor drugs possessing novel mechanisms of action are in great demand for anticancer therapy. We are thus continuing to examine their antitumor activities and further investigating their mechanism of action on growth inhibition. The other required feature of antitumor drugs is that they cause apoptosis or growth inhibition in cancer cells resistant to currently used drugs, since drug resistance is a major problem with therapy. In our experiment, pladienolide B inhibited the growth of six drug-resistant cell lines similar to that of parental cells. MES-SA/Dx is a typical multi-drug-resistant cell that strongly expresses P-glycoprotein, while P388/CPT, P388/ETP, P388/CDDP, P388/VCR, and

Fig. 4. Antitumor effects of pladienolide B against BSY-1, PC-3, OVCAR-3, DU-145, WiDr, and HCT-116 xenografts.



Tumor inoculation was carried out as described in "Materials and Methods." Pladienolide B was intravenously administered on Day 1 to Day 5 (qd1x5) at doses of 10 (closed squares), 5 (closed triangles), 2.5 (closed circles), and 0 mg/kg/day (open squares, control). Values are the means and standard deviations for five mice.

HCT-116/5-FU are atypical drug-resistant cells that do not show cross-resistance against the others. Pladienolide B may be useful for cancer therapy to combat drug resistance.

To clarify the mechanism of action, effects on cell cycle progression were evaluated using WiDr cells. Pladienolide B clearly arrested the cell cycle at both the G1 and G2/M phases, unlike taxol and vincristine, which arrest the cell cycle at the G2/M phase. This result suggests a novel mechanism of action for pladienolides, although the COMPARE analysis shows a weak correlation with these tubulin binders.

We first demonstrated that pladienolide B and D have antitumor activities against WiDr xenografts. However, pladienolide C showed weak antitumor activities. Since no body weight changes were observed in the experiment, it seems likely that pladienolide C would show antitumor activity if the dosages were raised. Our results indicate that the order of antitumor activities based on mT/C, dosage, and body weight change is as follows: pladienolide B>D>C. This order is the same as those of *in vitro* activities, such as HIF-1 pathway inhibition and growth inhibition⁵⁾. Next, pladienolide B, the most potent compound, was evaluated against a series of tumor xenografts, against which it showed strong tumor growth inhibition (data not shown). It is especially noteworthy that pladienolide B regressed or strongly inhibited tumors in six xenograft models (Fig. 4).

Taken together, due to their strong activities and novel mechanism of action, pladienolides are promising antitumor agents, although further studies need to be conducted to clarify the molecular targets underlying their marked *in vivo* efficacy.

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