Cotylenin A, a Differentiation-inducing Agent, and IFN-α Cooperatively Induce Apoptosis and Have an Antitumor Effect on Human Non-Small Cell Lung Carcinoma Cells in Nude Mice[1]

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ABSTRACT

Cotylenin A, a novel inducer of the differentiation of leukemia cells, and IFN-α synergistically inhibited the growth of and induced apoptosis in several human non-small cell lung carcinoma cell lines. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor DR5 were the early genes induced by the combination of cotylenin A and IFNα in lung carcinoma cells. Neutralizing antibody to TRAIL inhibited apoptosis, suggesting that cotylenin A and IFNα cooperatively induced apoptosis through the TRAIL signaling system. This combined treatment preferentially induced apoptosis in human lung cancer cells while sparing normal lung epithelial cells and significantly inhibited the growth of human lung cancer cells as xenografts without apparent adverse effects, suggesting that this combination may have therapeutic value in treating lung cancer.

INTRODUCTION

NSCLC[3] is one of the most common malignant diseases in the world. Surgical resection is the only treatment modality with a reasonable chance of offering cure when applied to appropriately selected patients. Only ~40% of patients have resectable disease, and only one-quarter of these (10–12% overall) are still alive at 5 years and apparently cured of their disease. Chemotherapy is reserved solely for patients with advanced stages of NSCLC and until now has only brought marginal benefits (1). Efforts are underway to optimize chemotherapeutic strategies and discover new agents. Attempts to translate recent findings regarding the biology of lung cancer into therapeutic strategies, such as the use of biological response modifiers, monoclonal antibodies, and inhibitors of signal transduction of oncogenes, may some day lead to significant progress.

IFNs are pleiotropic cytokines that block viral infection, inhibit cell proliferation, induce apoptosis, and modulate cell differentiation (2). IFNα has therapeutic activity as a single agent in some types of hematological malignancies but is less effective in the therapy of solid tumors, including NSCLC (2–4). To overcome this resistance, various therapeutic approaches have been developed. The combination of IFNα with conventional chemotherapeutic agents has been reported to be effective at inducing tumor regression in some tumors, including NSCLC (5–7). However, the precise mechanisms of action and optimal dosing and sequencing in combination with chemotherapy are unclear. Additional studies are needed to more clearly define the role of IFNα as a modulator of cytotoxic chemotherapeutic agents.

Differentiation-inducing agents can alter the phenotype of cancer cells, including their drug sensitivity (8, 9). Retinoids in combination with IFNα are highly effective against several malignancies (10–12) but do not affect the sensitivity of NSCLC cells to IFNα (13). Furthermore, although some differentiation-inducing agents effectively enhance the sensitivity of lung cancer cells to IFNα with regard to the inhibition of cell proliferation, retinoids do not (14). Although IFNα alone only slightly inhibited the growth of lung cancer cells at high concentrations, combined treatment with IFNα and suboptimal concentrations of some differentiation-inducing agents greatly reduced the growth of a variety of human lung cancer cell lines both *in vitro* and *in vivo* (14). Although this is a promising approach to lung cancer therapy, DMSO and sodium butyrate are not suitable for use in the treatment of patients with NSCLC. In the present investigation, we examined the synergistic effects of various differentiation-inducing agents and IFNα on the growth of lung cancer cells to identify the most potent and clinically applicable drugs. The most effective agent was cotylenin A, a novel inducer of differentiation of myeloid leukemia (15, 16). Cotylenin A, which has a novel fusicoccane-diterpene glycoside with the complex sugar moiety, was isolated as a plant growth regulator and has been shown to affect several physiological processes in higher plants (17, 18). Cotylenin A also affected the differentiation of leukemic cells that were freshly isolated from acute myelogenous leukemia patients in primary culture (19). It significantly stimulated both the functional and morphological differentiation of leukemia cells in 9 of 12 cases. This differentiation-inducing activity was more potent than those of all-*trans* retinoic acid and 1α,25-dihydroxyvitamin D₃ (19). Because cotylenin A is potent at stimulating differentiation *in vitro,* it may have therapeutic effects in experimental models of leukemia and acute myelogenous leukemia patients. Injection of the human promyelocytic leukemia cell line NB4 into mice with severe combined immunodeficiency resulted in the death of all mice caused by leukemia. Administration of cotylenin A significantly prolonged the survival of mice inoculated with retinoid-sensitive and -resistant NB4 cells, and no appreciable adverse effects were observed in the experiment (20). These results suggest that cotylenin A may be useful in therapy for leukemia and some other malignancies. Therefore, in the present study, we sought to clarify the synergistic effect of cotylenin A and IFNα on human lung carcinoma cells and to examine the therapeutic effects on xenografts of human lung carcinoma cells.

MATERIALS AND METHODS

Chemicals. Cotylenin A was purified from the culture filtrate of *Cladosporium* fungus sp. 501–7W by flash chromatography on silica gel with >99% purity (17, 18). A stock solution of cotylenin A was prepared in absolute ethanol at 20 mg/ml. Human natural IFNα (Sumiferon) was a kind gift from Sumitomo Pharmaceuticals (Tokyo, Japan). MTT, Fas ligand, and anticancer drugs were obtained from Sigma Chemical (St. Louis, MO). DMSO, recombinant human TRAIL, recombinant human TNFα, and Na-butyrate were obtained from Wako Pure Chemicals (Osaka, Japan). TGFβ, caspase inhibitors, and antihuman TRAIL antibody were obtained from R&D Systems (Minneapolis, MN).

Cells and Cell Culture. Human lung carcinoma cell lines were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air (14). A549 was established from a human lung carcinoma with properties of type II alveolar epithelial cells. PC9 and PC14 were well- and poorly differentiated adenocarcinoma cell lines, respectively. Human normal human bronchial epithelial and SAE cells, which were derived from human lung bronchus and the small airway of healthy donors, respectively, were purchased from Clonetics (San Diego, CA) and grown in serum-free Bronchial Epithelial Cell and Small Airway Epithelial Cell Growth Medium Bullet kits (Clonetics), respectively.

Assay of Cell Growth and Apoptosis. The cells were seeded at 1×10^4/ml in a 24-well multidish. After culture with or without the test compounds for the indicated times, viable cells were examined by the modified MTT assay. Briefly, 100 μl of MTT solution (1 mg/ml in PBS) were added to each well. After incubation with MTT for 4 h, the cells were centrifuged at $1000 \times g$ for 10 min. The precipitates were dissolved in 1 ml of DMSO, and their absorption at 560 nm was determined. Assay of the cumulative cell number was determined as described elsewhere (21). The cellular DNA content was analyzed using propidium iodide-stained nuclei (21). Caspase activity in intact cells was measured using PhiPhiLux by flow cytometric analysis according to the manufacturer's instructions (OncoImmunin, Inc., Gaithersburg, MD).

Analysis of TRAIL-, DR4-, and DR5-positive Cells. We detected the expression of TRAIL and its receptors by flow cytometry. Cells were suspended in 100 μl of cold PBS with 2.5% FBS and incubated with antihuman TRAIL, DR4, or DR5 IgG (Cayman Chemical, Ann Arbor, MI) for 30 min on ice. Cells were washed once with cold PBS with 2.5% FBS and incubated with 100 μl of fluorescein-isothiocyanate-conjugated goat antirabbit IgG antibody for 30 min at 4°C. The cells were then washed with PBS with 2.5% FBS and analyzed on an Epics XL flow cytometer (Beckman-Coulter Electronics, Miami, FL). The percentage of fluorescence-positive cells was determined by setting gates to exclude $\geq$99% positive cells (fluorescent) in the isotype control.

Gene Expression Analysis by RT-PCR. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μg) from lung cancer cells was converted to first-strand cDNA primed with random hexamer in a 20-μl reaction using an RNA PCR kit (Takara Shuzo Co., Ltd., Tokyo, Japan), and 4 μl of this reaction was used as a template in the PCR. The oligonucleotides used in PCR amplification were as described elsewhere (22, 23), and a quantitative RT-PCR reaction was performed as described in the literature (24).

Transplantation of Lung Cancer Cells into Nude Mice and Treatment. Female athymic nude mice with a BALB/c genetic background were supplied by CLEA Japan (Tokyo, Japan). They were housed under specific pathogen-free conditions. The *in vivo* experiments were performed in accordance with the guidelines of our institute (Guide for Animal Experimentation, Saitama Cancer Center). Mice were inoculated s.c. with 6×10^6 PC14 cells. A stock solution of cotylenin A for administration was prepared in DMSO at 100 mg/ml. Mice were given a daily s.c. injection of 0.1 ml of PBS, including 3×10^4 IU of IFNα, and/or s.c. injections every other day of 0.2 ml of PBS, including 100 μg of cotylenin A (6.7 mg/kg body weight) at a site distant to the tumors, with the first injection given 7 days after the inoculation of tumor cells. Tumor size was measured with vernier calipers every other day. Statistical analysis was performed using Student's *t* test.

RESULTS

Combined Effects of IFNα and Various Drugs on the Growth of NSCLC Cells. To measure the effects of various drugs on the growth of lung carcinoma A549 cells, the number of viable cells was determined by the MTT assay after 6 days of exposure to various concentrations of drugs with or without 300 IU/ml IFNα. The growth-inhibiting effects of the drugs were examined by determining the concentrations of drugs required to reduce the cell number to one-half of that in untreated cells (IC_{50}). The sensitivity to anticancer agents, such as 5-fluorouracil, *cis*-platin, or doxorubicin, was not affected by IFNα, whereas the sensitivity to hydroxyurea was significantly enhanced (Table 1). Most of the differentiation-inducing agents for

Table 1 *Potentiation of the growth-inhibitory activities of various agents in human lung carcinoma A549 cells by IFNα[a]*

Compound	Growth inhibition (IC_{50})		Ratio ($-/+$)
	$-$ IFNα	$+$ IFNα	
5-Fluorouracil (μg/ml)	0.81 $\pm$ 0.06	0.59 $\pm$ 0.04	1.4
Hydroxyurea (μM)	407 $\pm$ 38	149 $\pm$ 11	2.7
Doxorubicin (ng/ml)	31.4 $\pm$ 2.8	22.8 $\pm$ 1.9	1.4
Cis-platin (ng/ml)	312 $\pm$ 3.0	259 $\pm$ 22	1.2
Etoposide (μg/ml)	5.13 $\pm$ 0.4	3.03 $\pm$ 0.2	1.7
Camptotecin (ng/ml)	124 $\pm$ 8.1	72.2 $\pm$ 4.8	1.7
Actinomycin D (ng/ml)	0.31 $\pm$ 0.02	0.24 $\pm$ 0.02	1.3
All-*trans* retinoic acid (μM)	>36	>36	
13-*Cis* retinoic acid (μM)	>36	>36	
1α,25-Dihydroxyvitamin D_3 (ng/ml)	>90	>90	
Cotylenin A (μg/ml)	15.4 $\pm$ 1.1	1.8 $\pm$ 0.1	8.6
Sodium butyrate (μM)	521 $\pm$ 43	164 $\pm$ 11	3.2
Dimethyl sulfoxide (mM)	257 $\pm$ 21	78.2 $\pm$ 7.3	3.3
Hexamethylene bisacetamide (mM)	12.7 $\pm$ 1.1	4.4 $\pm$ 0.4	2.9
Isopentenyladenine (μg/ml)	86.1 $\pm$ 8.2	69.3 $\pm$ 5.9	1.3
8-Cl-cAMP (μM)	24.7 $\pm$ 1.9	18.8 $\pm$ 1.6	1.3
Bufalin (nM)	1.6 $\pm$ 0.1	1.8 $\pm$ 0.1	0.9
Trichostatin A (ng/ml)	71.5 $\pm$ 7.6	48.2 $\pm$ 4.1	1.5
Genistein (μg/ml)	8.2 $\pm$ 0.7	6.3 $\pm$ 0.6	1.3
Wortmannin (μM)	4.9 $\pm$ 0.5	3.2 $\pm$ 0.3	1.5

[a] Cells were incubated with various concentrations of the test compounds in the presence or absence of 600 IU/ml IFNα for 6 days. Ratio ($-/+$), IC_{50} without IFNα:IC_{50} with IFNα. The values are the mean $\pm$ SD of four determinations.

myeloid leukemia cells were not toxic toward the lung carcinoma cell line when used within a range of concentrations that were effective at inducing the differentiation of human myeloid leukemia cells, suggesting that the lung cancer cells were less sensitive to agents that inhibited cell growth. Retinoids and vitamin D_3 did not affect the growth of A549 cells even at high concentrations (Table 1). The growth-inhibitory effect of DMSO was significantly enhanced by IFNα, although IFNα alone hardly inhibited cell growth (Table 1). We next examined the effects of >50 compounds that induce the differentiation of myeloid leukemia cells (25) and found that although the effects of some agents were strongly affected by IFNα, the effects of other agents were not. Among the differentiation-inducing agents tested, the sensitivity of lung cells to cotylenin A was most strongly affected by IFNα (Table 1). The synergistic effects of cotylenin A and IFNα were also observed in other lung carcinoma cell lines, although the sensitivity of lung carcinoma cell lines to IFNα varied among the cell lines (Fig. 1 and Table 2).

Next, we examined the combined effects of cotylenin A and various cytokines on the growth of A549 cells. Unlike IFNα, IFNγ did not affect cotylenin A-induced growth inhibition (Fig. 2b). TNFα and TGFβ also showed cooperative effects with cotylenin A in the inhibition of cell proliferation (Fig. 2, *c* and *d*), whereas other cytokines, such as interleukin 1, interleukin 4, leukemia-inhibitory factor, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and keratinocyte growth factor, did not show such cooperation with cotylenin A (data not shown). IFNα was the most potent cytokine at inhibiting cell growth in the presence of cotylenin A. These results suggest that the combination of cotylenin A and IFNα is the most potent for inhibiting the growth of human lung cancer cells.

Induction of Apoptosis in A549 Cells Treated with IFNα Plus Cotylenin A. When exposed to cotylenin A in the presence of 600 IU/ml IFNα for 4 days, the number of viable A549 cells decreased in a dose-dependent manner. After exposure for 4 days, a morphological analysis showed shriveled cells, chromatin condensation, nuclear fragmentation, and cytoplasmic blebbing (data not shown). Induction of apoptosis (cells in sub-G_1 phase) in treated A549 cells was confirmed by an analysis of DNA histograms (Fig. 3A) and caspase-3 activation (Fig. 3B). Treatment with cotylenin A alone also inhibited
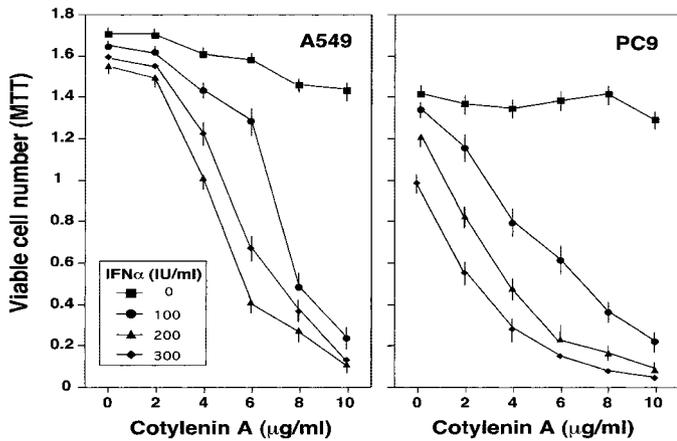
Fig. 1. Synergistic effects of IFNα and cotylenin A on the growth of A549 and PC9 cells. Cells were cultured with various concentrations of cotylenin A in the presence of 0–300 IU/ml IFNα for 6 days. The values are mean ± SD of four determinations.

Table 2 *Synergistic effects of IFNα and cotylenin A on the growth of human lung carcinoma cell lines[a]*

Cell line[b]	IC$_{50}$ for cotylenin A (μg/ml)		Ratio (−/+)
	− IFNα	+ IFNα	
Adenocarcinoma			
A549	15.4 ± 1.1	1.8 ± 0.1	8.6
PC7	37.6 ± 3.1	3.6 ± 0.3	10.4
PC9	34.8 ± 2.9	7.9 ± 0.5	4.4
PC14	28.6 ± 2.2	2.1 ± 3.2	13.6
ABC-1	16.5 ± 1.8	2.2 ± 0.2	7.5
Squamous cell carcinoma			
EBC-1	6.3 ± 4.2	0.8 ± 0.1	7.9
LK2	26.8 ± 2.4	6.1 ± 0.8	4.4
Large cell carcinoma			
Lu65	16.3 ± 1.4	3.1 ± 0.3	5.3
Lu99	7.5 ± 0.7	0.8 ± 0.1	9.4
Small cell carcinoma			
Lu135	4.8 ± 0.4	1.5 ± 0.1	3.2
H69	14.8 ± 1.2	3.4 ± 0.2	4.4

[a] Cells were cultured with various concentrations of cotylenin A in the presence or absence of 600 IU/ml IFNα for 6 days. IC$_{50}$ of Lu65 cells for IFNα alone was 3402 ± 194 IU/ml, and the IC$_{50}$s of the other cells were >6000 IU/ml. The values are the mean ± SD of four determinations.

[b] p53 status is wild type in A549 and Lu99 cell lines and mutated in the other cell lines.

cell growth in a concentration-dependent manner. Cotylenin A alone induced G$_1$ arrest, but not apoptosis, even at a high concentration (Fig. 3A). Combined treatment with 300 IU/ml IFNα plus 4 μg/ml cotylenin A caused significant growth inhibition, and this effect was similar to that with 24 μg/ml cotylenin A alone (Fig. 4). When the cells were treated with cotylenin A and IFNα for 7 days, and then washed and cultured without the drugs, cell growth was greatly inhibited at day 16, indicating that the growth-inhibitory effect of cotylenin A plus IFNα was irreversible in long-term culture. On the other hand, cotylenin A-treated cells began to grow within a few days after removal of the drug (Fig. 4). These results were consistent with the finding that the combination of cotylenin A and IFNα induced apoptosis, whereas a high concentration of cotylenin A induced G$_1$ arrest in A549 cells (Fig. 3), and suggest that this combined treatment may have therapeutic value in the chemotherapy of some lung cancers.

Fas and/or TRAIL signaling pathways are involved in IFN-induced apoptosis in some malignant cells (26–29). Therefore, we examined the effects of Fas ligand and TRAIL on the growth of A549 cells in the presence of IFNα or cotylenin A. The combination of IFNα and Fas ligand or TRAIL scarcely inhibited the proliferation of A549 cells (data not shown), whereas cotylenin A cooperated with TRAIL at inhibiting cell growth (Fig. 5a). However, Fas ligand did not affect the growth inhibition induced by cotylenin A (Fig. 5b). Similar results

were obtained when the cells were treated with anti-Fas monoclonal antibody CH-11, which was highly effective at inducing apoptosis in lymphoid cells. Although TRAIL significantly enhanced the growth inhibition induced by DMSO, the enhancing effects were less than those of cotylenin A (Fig. 5c). Similar results were observed when
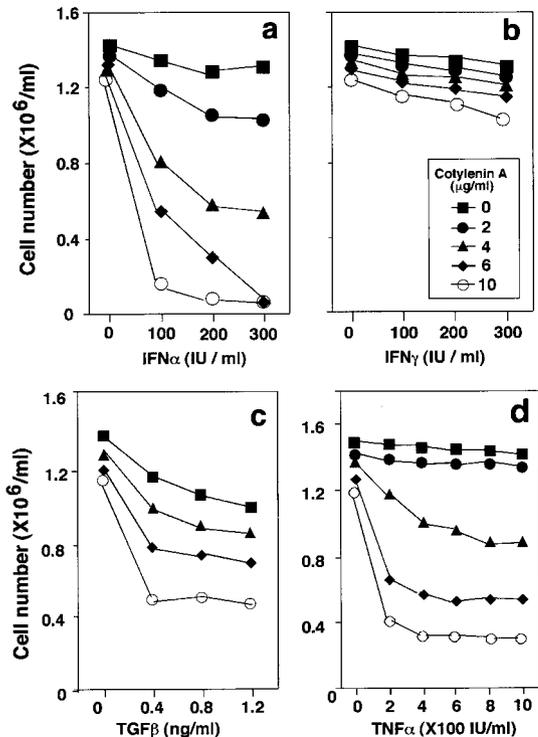

Fig. 2. Effect of cytokines on growth of A549 cells in the presence of cotylenin A. Cells were cultured with various concentrations of IFNα (a), IFNγ (b), TGFβ (c), or TNFα (d) in the presence of cotylenin A for 6 days. The values are mean of four determinations.
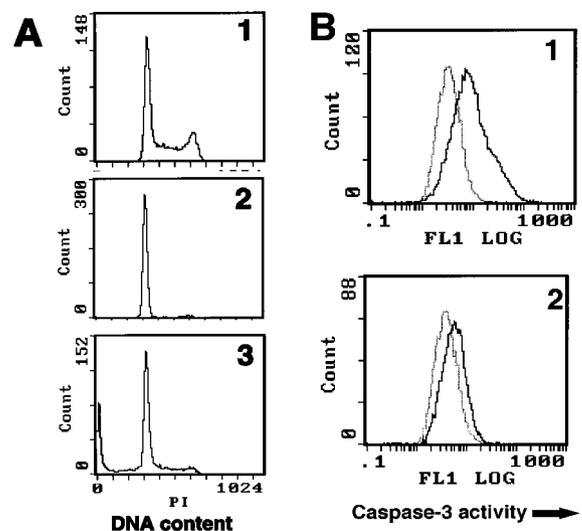

Fig. 3. A, induction of G$_1$ arrest and apoptosis in A549 cells treated with a high concentration of cotylenin A and a combination of cotylenin A and IFNα, respectively. Cells were cultured without (1) or with 24 μg/ml cotylenin A (2) or a combination of 300 IU/ml IFNα and 4 μg/ml cotylenin A (3) for 4 days, and DNA histograms were then analyzed. The apoptotic cell population is shown according to the sub-G$_1$ fraction. B, increase in cells with caspase-3 activity by cotylenin A plus IFNα but not by cotylenin A. Cells were treated with 24 μg/ml cotylenin A (1) or 300 IU/ml IFNα plus 4 μg/ml cotylenin A (2) for 4 days. Cells were then incubated with a fluorogenic caspase substrate PhiPhiLux-G1D2 for 60 min and then analyzed by flow cytometry. Faint line, untreated controls.
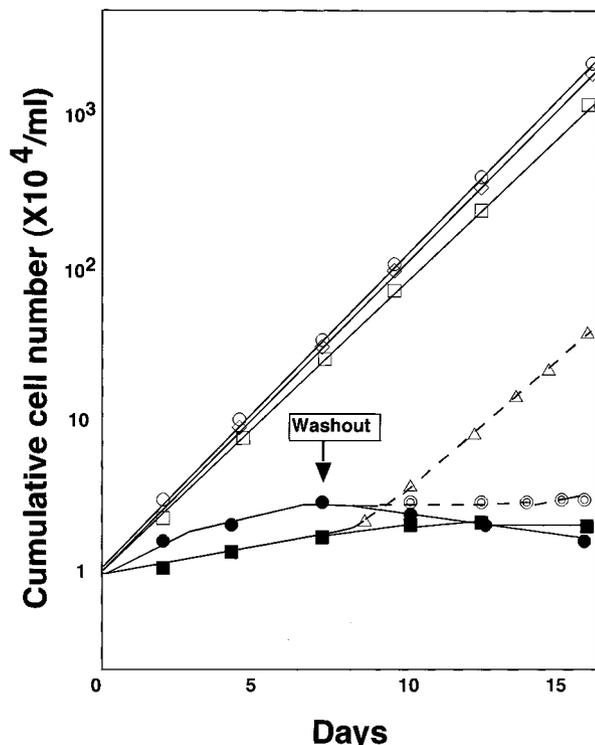
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Fig. 4. Proliferation of A549 cells in long-term culture with IFNα and cotylenin A. Cells were cultured without (○) or with 300 IU/ml IFNα (□), 4 μg/ml cotylenin A (◇), 24 μg/ml cotylenin A (■), or 300 IU/ml IFNα plus 4 μg/ml cotylenin A (●). ◎ and ▽ represent cells that were cultured with 300 IU/ml IFNα plus 4 μg/ml cotylenin A and 24 μg/ml cotylenin A for 7 days and then washed and cultured further without drugs, respectively. The culture medium was replaced by fresh medium at least once every 3 days. The cell density was kept at $1–8 \times 10^4$/ml. The values are means of four separate experiments.

cells were treated with hydroxyurea or doxorubicin (Fig. 5c). Treatment with anti-TRAIL antibody partially blocked the apoptosis mediated by cotylenin A plus IFNα (Fig. 5d). These results suggest that a TRAIL signaling pathway plays a role in the apoptosis induced by cotylenin A plus IFNα.

The expression of TRAIL and its receptors in A549 cells was examined by flow cytometric analysis. Untreated cells expressed very low levels of TRAIL and its receptors DR4 and DR5 (Fig. 6). The cell surface expression of DR5 significantly increased after treatment with cotylenin A plus IFNα but not after treatment with cotylenin A or IFNα alone. Although the expression of TRAIL was dose dependently increased by high concentrations of IFNα, no significant increase in TRAIL expression was observed on treatment with 300 IU/ml IFNα. However, this concentration of IFNα significantly enhanced the cotylenin A-induced expression of TRAIL (Fig. 6). The changes in DR4 expression were minimal in lung carcinoma cells treated with cotylenin A and/or IFNα.

TRAIL-induced apoptosis is known to be mediated by the activation of caspase-8 (30, 31). Therefore, we examined the effect of a caspase-8 inhibitor on the growth inhibition induced by cotylenin A plus IFNα (Fig. 5e). Caspase-8 inhibitor significantly blocked growth inhibition, whereas caspase-4 inhibitor had only modest effects, and other caspase inhibitors, such as inhibitors of caspase-1 and -9, did not essentially affect growth inhibition (data not shown), suggesting that the activation of caspase-8 plays a role in the induction of apoptosis.

The Combined Effects of IFNα and Cotylenin A on the Growth of Normal Lung Epithelial Cells. TRAIL selectively induces apoptosis in some cancer cells while sparing normal human epithelial cells (32–34). Therefore, we compared the combined effects of IFNα

and cotylenin A in two types of normal human lung epithelial cells, normal human bronchial epithelial and SAE cells, which were derived from human lung bronchus and the small airway of healthy donors, respectively, with those in lung cancer cell lines (Fig. 7). The optimal culture conditions for the normal cells were serum-free medium, whereas lung cancer cells grow in the presence of 10% FBS. In serum-free culture conditions, cells were more sensitive to the combined treatment with IFNα and cotylenin A. Therefore, we examined the effects on the growth of normal and cancer cells in both the presence and absence of FBS. Most of the cancer cells also grew in the serum-free medium for normal epithelial cells. In this condition, cancer cells were more sensitive to treatment, whereas weak growth inhibition was seen in normal lung epithelial cells (Fig. 7). Normal lung epithelial cells were still less sensitive to this treatment in the presence of FBS (data not shown). When cultured with 4 μg/ml cotylenin A and 300 IU/ml IFNα for 6 days, A549 cells underwent morphological changes characteristic of apoptosis, such as rounding, detachment, and floating, whereas the morphology of normal lung epithelial cells did not change, although they did show a slight decrease in cell number. Basal mRNA levels of DcR1 and DcR2, two decoy receptors for the death ligand TRAIL (32–34), which antagonize its action, were higher in both normal lung epithelial cells than in cancer cells. The expression of DcR1 mRNA in A549 cells was about one-tenth that in SAE cells, whereas the DcR2 mRNA level was one-half that in SAE cells (Fig. 8). mRNA levels of death receptors DR4 and DR5 in normal lung epithelial cells were also higher than those in cancer cells (Fig. 8).

Up-Regulation of TRAIL and DR5 Receptor mRNA Expression by Cotylenin A and IFNα in Cancer Cells. To determine whether cotylenin A and IFNα affected mRNA levels, quantitative RT-PCR analysis was carried out on normal lung epithelial and lung carcinoma A549 cells. The amounts of DcR1 and DcR2 mRNA were not essentially affected by IFNα, cotylenin A, or IFNα plus cotylenin A, whereas the osteoprotegerin mRNA level was significantly reduced by these treatments in A549 cells (Fig. 8c). The mRNA expression in normal lung epithelial cells was not affected by these treatments (data not shown). The expression of DR5 mRNA was significantly induced by the combination of IFNα and cotylenin A and was comparable with that in normal lung epithelial cells (Fig. 8a), whereas the up-regulation of the expression of DR4 mRNA was moderate (Fig. 8c). IFNα alone significantly up-regulated TRAIL mRNA expression, although combined treatment with IFNα and cotylenin A was more effective (Fig. 8b). Similar results were seen in PC9 lung carcinoma cells (data not shown). These results are compatible with those from flow cytometric analysis of the cell surface expression of TRAIL and its receptors (Fig. 6).

Effects of Cotylenin A and IFNα on the *in Vivo* Growth of PC14 Cells as Xenografts. The *in vitro* studies described above suggested that combined treatment with cotylenin A and IFNα should be more effective therapeutically than treatment with cotylenin A or IFNα alone. At day 7 after the inoculation of human lung adenocarcinoma PC14 cells, the mean tumor volume was 33.4 ± 12.8 mm^3 ($\pm$SD), and treatments were then started. Because of the low solubility of cotylenin A, a dose-escalating effect was not observed in the therapeutic experiments (20). Therefore, we administered 100 μg/mouse cotylenin A, which had no appreciable adverse effects on mice, even in the presence of IFNα. The mice were injected with 3×10^4 IU of IFNα every day. This dose is equivalent to $\sim 3 \times 10^6$ IU/m^2 daily in humans using the calculations of Freireich *et al.* (35). The combined treatment significantly inhibited the growth of PC14 cells as xenografts (Fig. 9). At day 12 after treatment, the mean tumor volumes of untreated, cotylenin A-, IFNα-, and cotylenin A plus IFNα-treated nude mice were 520.4 ± 82.7, 318.5 ± 65.4, 391.6 ± 61.5, and
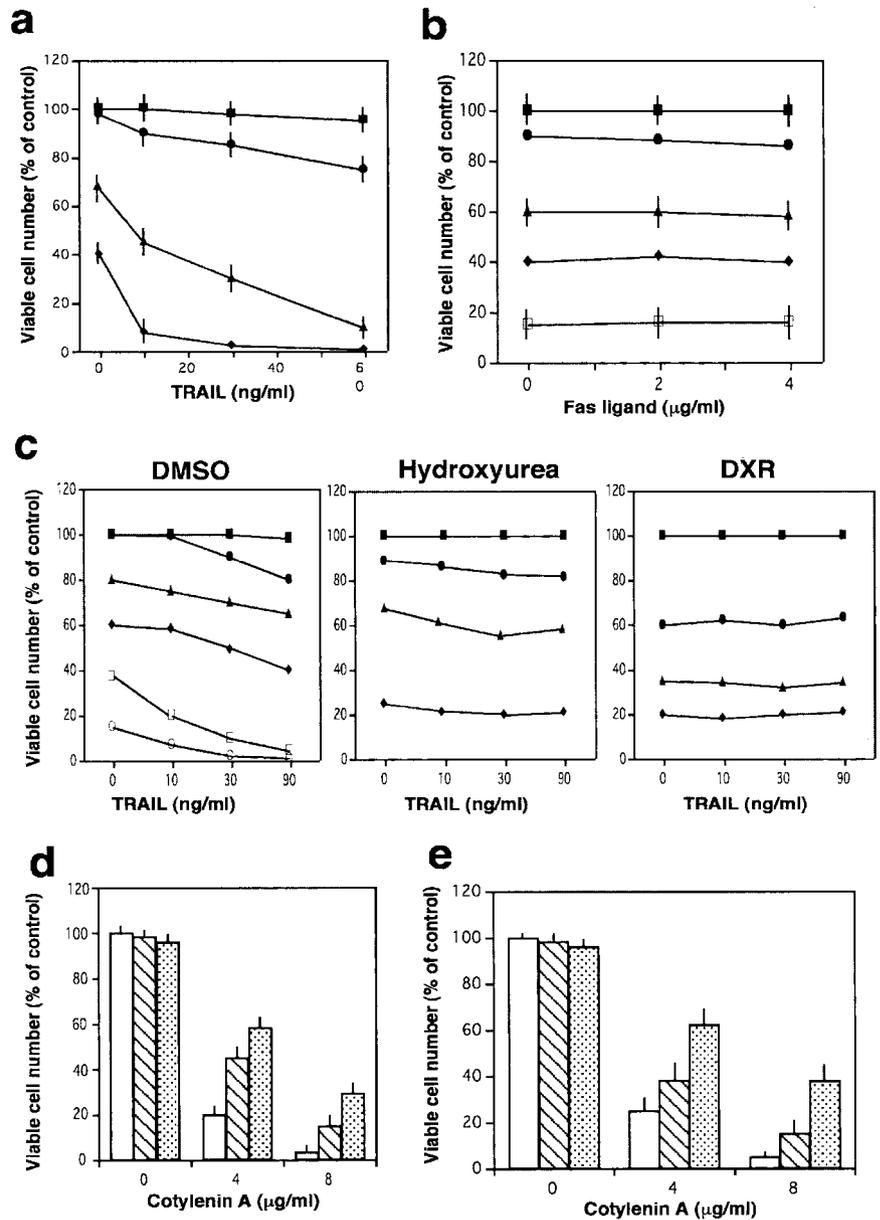
Fig. 5. Combined effects of cotylenin A and TRAIL (*a*) or cotylenin A and Fas ligand (*b*) on the growth of A549 cells. Cells were cultured with TRAIL or Fas ligand in the presence of 0 (■), 4 (●), 8 (▲), 12 (◆), or 16 (□) μg/ml cotylenin A for 6 days. Combined effects of drugs and TRAIL on the growth of A549 cells (*c*). Cells were cultured with various concentrations of TRAIL in the presence of 0 (■), 70 (●), 140 (▲), 210 (◆), or 280 (□) mM DMSO (*left*); 0 (■), 0.1 (●), 0.3 (▲), or 0.9 (◆) mM hydroxyurea (*middle*); and 0 (■), 5 (●), 10 (▲), or 20 (◆) ng/ml daunorubicin (*DXR*) for 6 days (*right*). Effect of anti-TRAIL antibody (*d*) or caspase-8 inhibitor (*e*) on the growth inhibition induced by cotylenin A plus IFNα. Cells were simultaneously treated without (*open bar*) or with 0.3 (*striped b*ar) or 1 (*dotted bar*) μg/ml anti-TRAIL antibody or 20 (*striped bar*) or 60 (*dotted bar*) μM caspase-8 inhibitor in the presence of 300 IU/ml IFNα plus cotylenin A for 6 days. Viable cell number was determined by the MTT assay. The values are mean ± SD of four determinations.

21.8 ± 20.6, respectively. Although cotylenin A and IFNα each significantly retarded tumor growth (*P* < 0.05), combined treatment induced tumor regression. The treatment was continued for 12 days and then stopped, with a follow-up on day 26. All of the untreated mice had a large tumor burden at day 26. On the other hand, >50% of the treated mice escaped from the disease (13 of 20 mice), and the rest had only a small tumor burden, suggesting that the therapeutic effects were still maintained after treatment was terminated. These results indicate that the combination of cotylenin A and IFNα is more effective therapeutically than treatment with cotylenin A or IFNα alone, and the combined treatment had a significant antitumor effect (*P* < 0.001).

Fig. 6. Combined treatment with cotylenin A and IFNα up-regulates TRAIL and DR5 expression in A549 cells. Cells were treated with various concentrations of cotylenin A in the presence of 0 (■), 150 (●), 300 (◆), or 450 (▲) IU/ml IFNα for 4 days. Expression was assayed by flow cytometry. The values are mean ± SD of four determinations.
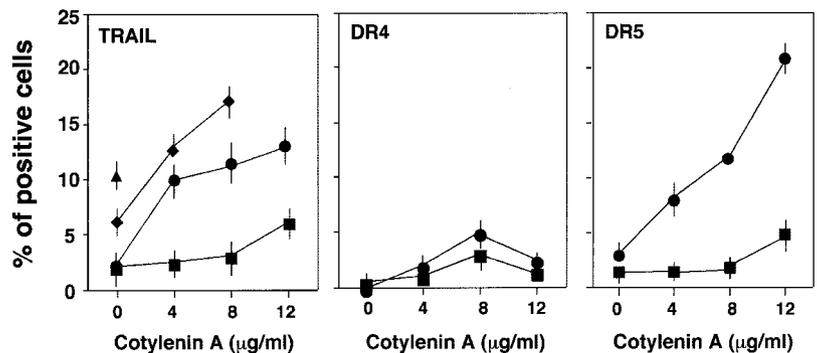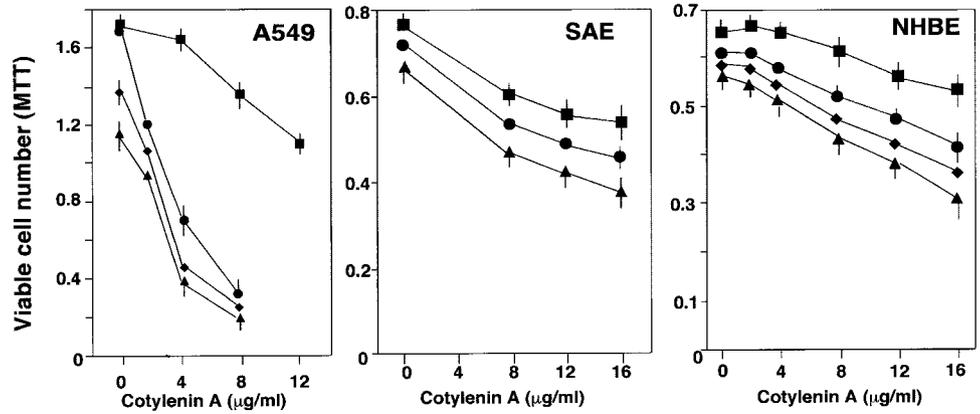

Fig. 7. Differential effects of cotylenin A plus IFNα on the growth of A549 and normal lung epithelial cells. Cells were cultured with various concentrations of cotylenin A in the presence of 0 (■), 150 (●), 300 (◆), or 450 (▲) IU/ml IFNα for 6 days.

DISCUSSION

IFNα alone had no significant effects on the growth or viability of human lung cancer cell lines, even at 2,000 IU/ml. However, we found previously that several human lung cancer cell lines similarly expressed IFNα receptors (3,000–10,000 receptors/cell) with high affinity, and activation of the transcription factor ISGF-3, which has been shown to be required for the transcriptional activation of IFN-induced genes, was induced by IFNα alone in lung carcinoma PC9 cells (14). Moreover, IFNα rapidly induced the expression of some IFNα-inducible genes, such as *IRF-1* and *PML,* in PC9 cells (data not shown). The present study also showed that the expression of TRAIL mRNA was induced by IFNα alone (Fig. 8), suggesting that resistance and its restoration by differentiation-inducing agents, such as cotylenin A and DMSO, in some lung carcinoma cells act downstream of the activation of ISGF-3. Although IFNα alone up-regulated the expression of some genes associated with apoptosis and growth inhibition, it might be inadequate for activating some important genes to exert significant effects on apoptosis. Expression of the death receptor DR5

was not essentially induced by IFNα or cotylenin A alone, but combined treatment greatly up-regulated its mRNA and cell surface expression in lung cancer cells. Wild-type p53 has been shown to up-regulate DR5 gene expression (36). However, expression of DR5 protein expression in A549 cells was not up-regulated by some anticancer drugs that up-regulate p53 protein expression, and the combined effects of cotylenin A and IFNα were observed in both cancer cells with wild-type and mutant/deleted p53. A receptor of fusicoccin, closely related to cotylenin A, has been reported to be a member of the family of 14-3-3 proteins that are commonly found in a huge array of signaling and regulatory pathways (37). A special subfamily of 14-3-3 proteins may bind cotylenin A and affect the interaction with some signaling molecules, including ISGF-3. This modification may lead to up-regulation of DR5 gene expression. However, further investigation is required to explain how up-regulation of DR5 is related to its effects on the 14-3-3 signaling pathway.

To investigate the role of IFNα in the induction of apoptosis in human lung carcinoma cells, we examined the effects of IFNα and/or
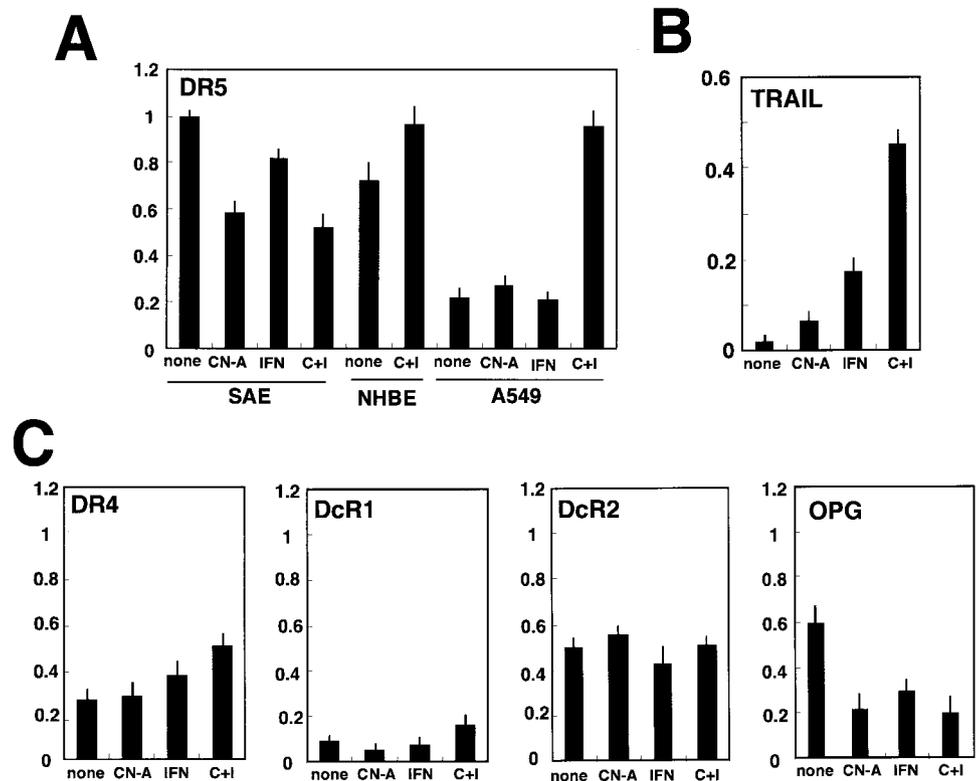

Fig. 8. Comparison of the effects of cotylenin A and IFNα on the gene expression of TRAIL and its receptors in normal lung epithelial and lung cancer cells. Cells were treated with 4 μg/ml cotylenin A (*CN-A*), 300 IU/ml IFNα (*IFN*), or 4 μg/ml cotylenin A plus 300 IU/ml IFNα (*C+I*) for 2 days. mRNA levels were determined by quantitative RT-PCR and then normalized to the amount of glyceraldehyde-3-dehydrogenase mRNA. The value in untreated normal lung epithelial SAE cells is defined as 1. *OPG,* osteoprotegerin. The values are mean ± SD of four determinations.
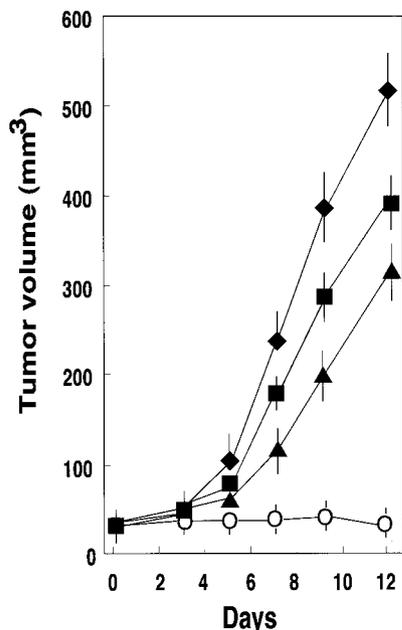
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Fig. 9. Effects of cotylenin A and IFNα on the growth of PC14 cells as xenografts. Mice received daily s.c. administration of 3 × 10⁴ IU of IFNα (■, ○) and s.c. injection of 100 μg of cotylenin A (▲, ○). ◆, untreated mice. Values are mean (±SD) of 10–20 mice.

cotylenin A on the expression of caspases, Apaf-1, and bcl-2 family genes by RT-PCR. The expression of caspase-4 mRNA was induced by treatment with IFNα alone but not by treatment with cotylenin A. The combination of IFNα and cotylenin A did not cause an additional increase in the expression of caspase-4 mRNA. However, treatment with an inhibitor of caspase-4 did not affect the apoptosis induced by IFNα and cotylenin A (data not shown). The expression of other caspase, Apaf-1 and bcl-2 family genes was unchanged by IFNα and/or cotylenin A (data not shown). IFNα alone significantly induced the expression of TRAIL mRNA, and the combination of cotylenin A and IFNα caused an additional increase in expression. The gene expression of the TRAIL receptor DR5 was greatly induced by the combination of cotylenin A and IFNα (Fig. 8). TRAIL is one of the early genes induced by IFN in apoptosis-sensitive melanoma and lymphoma cells, and apoptosis is mediated by the autocrine and/or paracrine loop involving TRAIL and its receptors (26, 27). These results suggest that the activation of TRAIL and DR5 genes is an important process in the induction of apoptosis in NSCLC cells by cotylenin A plus IFNα.

Although natural retinoids did not affect the growth of NSCLC cells, even in the presence of IFNα, some synthetic retinoids might be effective in synergism with IFNα. CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) induces gene expression of DR4 and DR5 and then the apoptosis of NSCLC cells (38). However, normal lung epithelial cells are less sensitive to the retinoid. CD437 and IFNα might cooperatively induce apoptosis in lung cancer cells. Some triterpenoids induce apoptosis of several tumor cell lines, including NSCLC (39, 40). Because cotylenin A is a diterpenoid and resembling in structure to some extent, the antitumor triterpenoids may also cooperate with IFNα in inducing apoptosis of cancer cells.

Recent published results indicate that TRAIL and chemotherapeutic drugs act synergistically to kill cancer cells (22, 41). In the present study, the cells were more sensitive to the combination of cotylenin A and IFNα than to chemotherapeutic drugs and TRAIL. We did not investigate the maximal tolerable dose or dose-limiting toxicity of cotylenin A in the absence or presence of IFNα, because the maximal

concentration of cotylenin A was 100 μg/0.2 ml saline. This treatment has no apparent effects on mice (body weight and behavior). Potent derivatives of cotylenin A that are readily soluble in saline will be required to further develop this therapeutic strategy.

REFERENCES

1. Hoffman, P. C., Bitan, I. D., and Golomb, H. M. Chemotherapy of metastatic non small cell bronchogenic carcinoma. Semin. Oncol. 10 (Suppl. 4): 111–122, 1983.
2. Pestka, S., Langer, J. A., Zoon, K. C., and Samuel, C. E. Interferons and their actions. Ann. Rev. Biochem., 56: 727–777, 1987.
3. Agarwala, S. S., and Kirkwood, J. M. Interferons in the therapy of solid tumors. Oncology, 51: 129–136, 1994.
4. Gutterman, J. U. Cytokine therapeutics: lessons from interferon α. Proc. Natl. Acad. Sci. USA, 91: 1198–1205, 1994.
5. Olsen, K., Ernst, P., Nissen, M. H., and Hansen, H. H. Recombinant interferon A (IFL-rA) therapy of small cell and squamous cell carcinoma of the lung. A phase II study. Eur. J. Cancer Clin. Oncol., 23: 987–989, 1987.
6. Wadler, S., Schwartz, E. L., Goldman, M., Lyver, A., Rader, M., Zimmerman, M., Itri, L., Weinberg, V., and Wiernik, P. H. Fluorouracil and recombinant alfa-2a-interferon: an active regimen against advanced colorectal carcinoma. J. Clin. Oncol., 7: 1769–1775, 1989.
7. Wadler, S., and Schwartz, E. L. Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. Cancer Res., 50: 3473–3486, 1990.
8. Okabe-Kado, J., Hayashi, M., Honma, Y., and Hozumi, M. Enhancement by hemin on the sensitivity of K562 human leukemic cells to 1-β-D-arabinofuranosylcytosine. Cancer Res., 46: 1239–1243, 1986.
9. Okabe-Kado, J., Hayashi, M., Honma, Y., Hozumi, M., and Tsuruo, T. Inhibition by erythroid differentiation factor (activin A) of p-glycoprotein expression in multidrug-resistant human K562 erythroleukemia cells. Cancer Res., 51: 2582–2586, 1991.
10. Moore, D. M., Kalvakolanu, D. V., Lippman, S. M., Kavanagh, J. J., Hong, W. K., Borden, E. C., Paredes-Espinoza, M., and Krakoff, I. H. Retinoic acid and interferon in human cancer: mechanistic and clinical studies. Semin. Hematol., 31: 31–37, 1994.
11. Adamson, P. C., Reaman, G., Finklestein, J. Z., Feusner, J., Berg, S. L., Blaney, S. M., O'Brien, M., Murphy, R. F., and Balis, F. M. Phase I trial and pharmacokinetic study of all-trans-retinoic acid administered on an intermittent schedule in combination with interferon-α2a in pediatric patients with refractory cancer. J. Clin. Oncol., 15: 3330–3337, 1997.
12. Stadler, W. M., Kuzel, T., Dumas, M., and Vogelzang, N. J. Multicenter phase II trial of interleukin-2, interferon-α, and 13-cis-retinoic acid in patients with metastatic renal-cell carcinoma. J. Clin. Oncol., 16: 1820–1825, 1998.
13. Arnold, A., Ayoub, J., Douglas, L., Hoogendoorn, P., Skigley, L., Gelmon, K., Hirsh, V., and Eisenhauer, E. Phase II trial of 13-cis-retinoic acid plus interferon α in non-small-cell lung cancer. J. Natl. Cancer Inst. (Bethesda), 86: 306–309, 1994.
14. Goto, I., Yamamoto-Yamaguchi, Y., and Honma, Y. Enhancement of sensitivity of human lung adenocarcinoma cells to growth-inhibitory activity of interferon-α by differentiation-inducing agents. Br. J. Cancer, 74: 546–554, 1996.
15. Asahi, K., Honma, Y., Hazeki, K., Sassa, T., Kubohara, Y., Sakurai, A., and Takahashi, N. Cotylenin A, a plant-growth regulator, induces the differentiation in murine and human myeloid leukemia cells. Biochem. Biophys. Res. Commun., 238: 758–763, 1997.
16. Yamamoto-Yamaguchi, Y., Yamada, K., Ishii, Y., Asahi, K., Tomoyasu, S., and Honma, Y. Induction of the monocytic differentiation of myeloid leukaemia cells by cotylenin A, a plant growth regulator. Br. J. Haematol., 112: 697–705, 2001.
17. Sassa, T., Tojyo, T., and Munakata, K. Isolation of a new plant growth substance with cytokinin-like activity. Nature (Lond.), 227: 379, 1970.
18. Sassa, T., Ooi, T., Nukina, M., Ikeda, M., and Kato, N. Structural confirmation of cotylenin A, a novel fusicoccane-diterpene glycoside with potent plant growth-regulating activity from Cladosporium fungus sp. 501–7W. Biosci. Biotechnol. Biochem., 62: 1815–1818, 1998.
19. Yamada, K., Honma, Y., Asahi, K., Sassa, T., Hino, K., and Tomoyasu, S. Differentiation of human acute myeloid leukaemia cells in primary culture in response to cotylenin A, a plant growth regulator. Br. J. Haematol., 114: 814–821, 2001.
20. Honma, Y., Ishii, Y., Sassa, T., and Asahi, K. Treatment of human promyelocytic leukemia in the SCID mouse model with cotylenin A, an inducer of myelomonocytic differentiation of leukemia cells. Leuk. Res., in press.
21. Honma, Y., Yamamoto-Yamaguchi, Y., and Kanatani, Y. Vesnarinone and glucocorticoids cooperatively induce G1 arrest and have an anti-tumour effect on human non-small cell lung carcinoma cells grown in nude mice. Br. J. Cancer, 80: 96–103, 1999.
22. Evdokiou, A., Bouralexis, S., Atkins, G. J., Chai, F., Hay, S., Clayer, M., and Findy, D. M. Chemotherapeutic agents sensitize osteogenic sarcoma cells, but not normal human bone cells, to Apo2L/TRAIL-induced apoptosis. Int. J. Cancer, 99: 491–504, 2002.
23. He, Q., Lee, D. I., Rong, R., Yu, M., Luo, X., Klein, M., El-Deiry, W. S., Huang, Y., Hussain, A., and Sheikh, M. S. Endoplasmic reticulum calcium pool depletion-induced apoptosis is coupled with activation of the death receptor 5 pathway. Oncogene, 21: 2623–2633, 2002.
24. Yokoyama, A., Okabe-Kado, J., Sakashita, A., Maseki, N., Kaneko, Y., Hino, K., Tomoyasu, S., Tsuruoka, N., Kasukabe, T., and Honma, Y. Differentiation inhibitory factor nm23 as a new prognostic factor in acute monocytic leukemia. Blood, 88: 3555–3561, 1996.

25. Honma, Y. Cotylenin A-A plant growth regulator as a differentiation-inducing agent against myeloid leukemia. Leuk. Lymph., *43*: 1169–1178, 2002.
26. Oshima, K., Yanase, N., Ibukiyama, C., Yamashina, A., Kayagaki, N., Yagita, H., and Mizuguchi, J. Involvement of TRAIL/TRAIL-R interaction in IFN-α-induced apoptosis of Daudi B lymphoma cells. Cytokine, *14*: 193–201, 2001.
27. Chawla-Sarkar, M., Leaman, D. W., and Borden, E. C. Preferential induction of apoptosis by IFN-β compared with IFN-α2: correlation with TRAIL/Apo2L induction in melanoma cell lines. Clin. Cancer Res., *7*: 1821–1831, 2001.
28. Kumar-Sinha, C., Varambally, S., Sreekumar, A., and Chinnaiyan, A. M. Molecular cross-talk between the TRAIL and interferon signaling pathways. J. Biol. Chem., *277*: 575–585, 2002.
29. Martin, C. A., and Panja, A. Cytokine regulation of human intestinal primary epithelial cell susceptibility to Fas-mediated apoptosis. Am. J. Physiol. Gastrointest. Liver Physiol., *282*: G92–G104, 2002.
30. Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., and Ashkenazi, A. Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity, *12*: 611–620, 2000.
31. Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H., and Walczak, H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity, *12*: 599–609, 2000.
32. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science (Wash. DC), *277*: 815–818, 1997.
33. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science (Wash. DC), *277*: 818–821, 1997.
34. Ashkenazi, A., and Dixit, V. M. Death receptors: signaling and modulation. Science (Wash. DC), *281*: 1305–1308, 1998.
35. Freireich, E. J., Gehan, R. A., Rall, D. A., Schmidt, L. H., and Skipper, H. E. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. Cancer Chemother. Rep., *50*: 219–244, 1966.
36. Fei, P., Bernhard, E. J., and El-Deiry, W. S. Tissue-specific induction of p53 targets *in vivo*. Cancer Res., *62*: 7316–7327, 2002.
37. Oecking, C., Eckerskorn, C., and Weiler, E. W. The fusicoccin receptor of plants is a member of the 14–3–3 superfamily of eukaryotic regulatory proteins. FEBS Lett., *352*: 163–166, 1994.
38. Sun, S.-Y., Yue, P., Chen, X., Hong, W. K., and Lotan, R. The synthetic retinoid CD437 selectively induces apoptosis in human lung cancer cells while sparing normal human lung epithelial cells. Cancer Res., *62*: 2430–2436, 2002.
39. Mujoo, K., Haridas, V., Hoffmann, J. J., Wachter, G. A., Hutter, L. K., Lu, Y., Blake, M. E., Jayatilake, G. S., Bailey, D., Mills, G. B., and Gutterman, J. U. Triterpenoid saponin from *Acacia victoriae* (Bentham) decrease tumor cell proliferation and induce apoptosis. Cancer Res., *61*: 5486–5490, 2001.
40. Kim, K. B., Lotan, R., Yue, P., Sporn, M. B., Suh, N., Gribble, G. W., Honda, T., Wu, G. S., Hong, W. K., and Sun, S-Y. Identification of a novel synthetic triterpenoid, methyl-2-cyano-3, 12-dioxooleana-1, 9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells. Mol. Cancer Ther., *1*: 177–184, 2002.
41. Nakata, T., Sugamura, K., Hylander, B. L., Widmer, M. B., Rustum, Y. M., and Repasky, E. A. Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice. Cancer Res., *62*: 5800–5806, 2002.