Communications to the Editor

CYTOGENIN, A NOVEL ANTITUMOR SUBSTANCE

Sir:

In the course of a screening for antitumor substances against a solid tumor, we found a novel antitumor substance named cytogenin in culture filtrate of the strain, MI43-37F11 which was isolated from the soil sample collected in Yokohama city, Kanagawa Prefecture, Japan. The strain was identified as *Streptoverticillium eurocidicum* on the basis of its cultural properties. Cytogenin showed low cytotoxicity and no antimicrobial activity and inhibited the growth of Ehrlich carcinoma. In this communication, the production, isolation, physico-chemical properties, structure and biological properties are reported.

A slant culture of MI43-37F11 was inoculated into 110 ml of the medium consisting of galactose 2.0%, dextrin 2.0%, soy peptone 1.0%, corn steep liquor 0.5% $(NH_4)_2SO_4$, 0.2%, $CaCO_3$ 0.2%, silicon oil 0.003% (adjusted to pH 7.4 before sterilization) and incubated at 30°C for 2 days on rotary shaker (180 rpm). For production of cytogenin, 2.5 ml of the culture was transferred to 125 ml of the production medium consisting of glycerol 2.0%, soy bean meal (Ajinomoto Co., Inc.) 1.5%, KH_2PO_4 0.1%, $CoCl_2 \cdot 6H_2O$ 0.0005%, silicon oil 0.003%, (pH 6.2 adjusted with 1 N K_2HPO_4 before sterilization) in Sakaguchi flask (500 ml) and cultured at 27°C for 4 days on a reciprocating shaker at 120 times per minute.

The culture filtrate (9 liters) was adjusted at pH 6.0 and extracted with EtOAc. The EtOAc extract was concentrated under a reduced pressure to give an oily residue (2.0 g) and it was applied to silica gel column. After washing the column with hexane-EtOAc (7:3), the active substance was eluted with the solvent ratio at 1:1. The active eluate was concentrated under a reduced pressure to give a crude powder (0.2 g) and subjected to a reverse phase HPLC (Senshu pak ODS330IN, 20× 250 mm). It was eluted with linear gradient from 20 to 50% acetonitrile in H_2O. After concentration of the active fraction, the resultant crude powder (50 mg) was dissolved in chloroform containing methanol and purified by silica gel column chromatography again. After washing the column with chloroform, cytogenin was eluted with chloroform-methanol (100:1). The active eluate was evaporated to dryness *in vacuo* (20 mg). Cytogenin was crystallized from chloroform-hexane. The crystal (10 mg) was obtained as colorless needles.

Physico-chemical properties of cytogenin are summarized in Table 1. The molecular formula was established at $C_{11}H_{10}O_5$ (MW 222) by elemental analysis and EI-MS. The UV spectrum in MeOH was very similar to that of isocoumarin. The IR spectrum (KBr) showed an intense carbonyl bond at 1680 cm^{-1} suggesting 8-hydroxyisocoumarin nucleus. The ^1H NMR spectrum of cytogenin in CD_3CN showed the signals of one hydroxymethyl, one methoxy, three aromatic protons, one phenol group (11.2 ppm) forming a hydrogen bonding. The ^{13}C NMR spectrum (CD_3CN, 100 MHz) indicated eleven carbon signals as shown in Table 2. The positions of the substituents (CH_2OH, OCH_3 and OH) on the isocoumarin nucleus were determined by the analysis of the spectrum of heteronuclear multiple-bond connectivity. On the basis of these

Table 1. Physico-chemical properties of cytogenin.

Appearance	Colorless needle
EI-MS (*m/z*)	222 (M^+)
Anal Calcd for $C_{11}H_{10}O_5$:	C 59.46, H 4.54, O 36.00
Found:	C 59.40, H 4.54, O 35.79
MP	148.5~149.5°C
$[\alpha]_D^{25}$ (*c* 0.2, CH_3CN)	0°
UV λ_{max}^{MeOH} nm (log ε)	238 (4.63), 244 (4.65), 256 (sh, 4.05), 274 (sh, 3.82), 286 (sh, 3.67), 330 (3.78)
IR (KBr) cm^{-1}	3480, 1680, 1630, 1570, 1510, 1230, 1190, 1170, 1110, 1090, 980, 860, 840, 710, 690
Solubility	
Soluble in:	Acetonitrile, acetone, DMSO
Slightly soluble in:	Chroloform, methanol, ethyl acetate
Insoluble in:	Hexane, H_2O

spectroscopic analysis, the structure of cytogenin was determined to be 8-hydroxy-3-hydroxymethyl-6-methoxyisocoumarin (Fig. 1). Although various isocoumarin analogues have been found in natural and synthetic products[1], among them, three analogues which have hydroxymethyl group at 3-position have been reported[2]: these are 3-hydroxymethyl-6,7-dimethoxy-3-hydroxyisocoumarin, 3-hydroxymethyl-6,8-dihydroxy-7-methoxyisocoumarin and 3-hydroxymethyl-6,7,8-trimethoxyisocoumarin.

Antitumor activity of cytogenin was tested against Ehrlich carcinoma. Ehrlich ascites tumor cells (2×10^6) were inoculated sc to mice (female ICR mice, 6 weeks old) at inguinal region and 1 day thereafter, mice were given cytogenin orally, daily for 9 days. On day 14, tumors were removed and weighed. As shown in Table 3, cytogenin retarded growth of the solid tumor by 45~74% at 6.3 to 100 mg/kg/day. Cytogenin showed a weak cytotoxicity against L1210 cells, P388 cells, EL-4 cells, IMC carcinoma cells, human lung cancer cells, LX-1 at 50 to 100 μg/ml. It had no antimicrobial activity at 100 μg/ml against bacteria and fungi. Single ip or po administration of 200 mg/kg of cytogenin into ICR mice (female, 6 weeks old) was not toxic. These results suggest that the antitumor activity of cytogenin may be due to the activation of host mediated events rather than its direct cytotoxicity to tumor cells.

Since most of antitumor substances which act through host mediated events are known as a cytokine inducer, the effect of cytogenin on cytokine production was investigated. It was found that cytogenin stimulated macrophages in peritoneal exudate cells (PEC) and spleen cells to produce interleukin 1 (IL-1). CDF1 mice (female, 10 weeks old) were given 25 mg/kg of cytogenin po and at days 7, 5, 3 and 1 after the administration, adherent cells in PEC and adherent cells in spleen cells were prepared by incubation in a plastic dish for 2 hours. These cells (2×10^6 and 2.5×10^6 cells/ml, respec-

Table 2. ^{1}H and ^{13}C NMR data of cytogenin in CD_3CN.

Position	δ_C (100 MHz)	δ_H (400 MHz)
1	166.93	—
3	157.86	—
4	104.14	6.55 (d, 2.0)[a]
4a	140.37	—
5	103.59	6.53 (d, 2.0)
6	167.99	—
7	101.50	6.48 (br t, −1)
8	164.34	—
8a	101.02	—
9	61.04	4.33 (dd, 6.0, −1)
6-OCH_3	56.66	3.86 (s)
9-OH	—	11.20 (t, 6.0)

δ values relative to TMS = 0 ppm.
[a] Proton signal multiplicity and coupling constant (J = Hz).

Fig. 1. Structure of cytogenin.

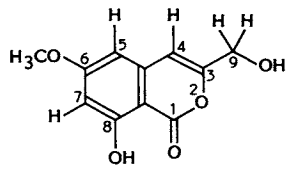

Table 3. Effect of cytogenin on Ehrlich carcinoma.

Dose (mg/kg/day)	Tumor weight (mean ± SD)	Inhibition (%)
0	1,084 ± 256	—
6.3	586 ± 282*	45
12.5	391 ± 266**	63
25.0	280 ± 152***	74
50.0	485 ± 123**	55
100.0	566 ± 421**	47

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Table 4. Effect of cytogenin on IL-1 production.

Days after administration (25 mg/kg, po)	Uptake of [^{3}H]TdR			
	Adherent cells in PEC		Adherent cells in spleen cells	
	cpm ± SD	T/C (%)	cpm ± SD	T/C (%)
Control	10,561 ± 1,241	100	2,091 ± 481	100
1 day	17,510 ± 810**	165.8	1,741 ± 352	83.3
3 days	13,912 ± 1,367*	131.7	4,868 ± 497**	232.8
5 days	26,759 ± 3,601**	253.4	2,140 ± 121	102.3
7 days	17,639 ± 2,699**	167.0	2,385 ± 124	114.1

* $P<0.05$, ** $P<0.01$.

tively) were cultured for 24 hours and IL-1 activity in the cultured supernatant was examined. D10.G4.1 cells (1×10^5 cells/ml) were cultured with 100 μl of the cultured supernatant and 2.5 μg/ml of concanavalin A for 48 hours and pulsed with [^{3}H]-thymidine([^{3}H]TdR) for 16 hours. IL-1 activity was determined by measuring the incorporation of [^{3}H]TdR into D10.G4.1 cells[3]. As shown in Table 4, at day 3 or 5 after administration of cytogenin, IL-1 production by adherent cells in PEC or adherent cells in spleen cells was induced. From these results, it can be considered that the antitumor activity of cytogenin may be due to activation of host mediated mechanisms. The mechanism of action are now under study.

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References

1) BARRY, R. D.: Isocoumarins. Developments since 1950. Chem. Rev. 64: 229~260, 1964
2) FURUTANI, Y.; H. NAGANAWA, T. TAKEUCHI & H. UMEZAWA: Isolation and structure of new isocoumarins. Agric. Biol. Chem. 41: 1179~1183, 1977
3) KAYE, J. & C. A. JANEWAY, Jr.: Induction of receptors for interleukin 2 requires T cell Ag: Ia receptor crosslinking and interleukin 1. Lymphokine Res. 3: 175~182, 1984