Anguinomycins C and D, New Antitumor Antibiotics with Selective Cytotoxicity against Transformed Cells

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The retinoblastoma protein (pRB) is inactivated during the development of a wide variety of human cancers. In the course of our screening for antitumor antibiotics by using pRB-inactivated cells, an actinomycete strain was found to produce two active substances, which were elucidated to be new members of the leptomycin-anguinomycin family by NMR spectral analysis and were designated anguinomycins C and D. The anguinomycins induced growth arrest against normal cells and induced cell death against transformed cells, in which pRB was inactivated by viral oncoproteins such as human papillomavirus E7, adenovirus E1A and simian virus 40 large T antigen.

The retinoblastoma tumor suppressor protein (pRB) plays a central role in the mammalian cell cycle control and is inactivated during the development of a wide variety of human cancers[1]. The human papillomaviruses (HPV) are highly associated with human cervical cancers and carry E6 and E7 oncoproteins, which bind and inactivate the tumor suppressors p53 and pRB, respectively[2].

In order to search for antitumor substances with selective cytotoxicity against transformed cells, we established immortalized cell lines with pRB inactivated by HPV16 E7 oncoprotein. Primary rat glia cells were transfected with plasmids containing a neomycin-resistant gene and HPV16 E7 or both E6 and E7 oncogenes[3] by the calcium phosphate method[4]. Drug-resistant colonies were selected by incubation for 3 weeks in the presence of 400 μg/ml of G418, a neomycin analogue. Twelve weeks after transfection, immortalized cells were cloned by limiting dilution and designated RG-E7-6f and RG-E6E7-3d cell lines.

In the course of our screening for antitumor antibiotics by using these transformed cells, a strain belonging to *Streptomyces* was found to produce two active substances, which were elucidated to be new members of the leptomycin-anguinomycin family[5,6] by NMR spectral analysis and were designated anguinomycins C and D. The anguinomycins induced cell death against pRB-inactivated cells and cell-cycle arrest at G1 phase against normal cells. This paper describes the fermentation, isolation, physico-chemical properties, structure elucidation and biological activity of anguinomycins C and D.

Fermentation

The seed medium consisted of soluble starch 1.0%, molasses 1.0%, meat extract 1.0% and Polypepton 1.0% (pH 7.2). Seed tubes containing 15 ml of the medium were inoculated with a stock culture of the producing strain maintained on a BENNET's agar slant and were incubated on a reciprocal shaker at 27°C for 2 days. The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the seed medium. The flasks were incubated on a rotary shaker at 27°C for 2 days. A 600-ml portion of the culture was inoculated into a 50-liter jar fermenter containing 30 liters of a production medium consisting of glycerol 2.0%, molasses 1.0%, casein 0.5%, Polypepton 0.1% and calcium carbonate 0.4% (pH 7.2). The fermentation was carried out at 27°C for 4 days under agitation of 300 rpm and aeration of 30 liters/minute.

Isolation

A mycelial cake obtained from the fermentation broth (60 liters) was extracted with 15 liters of acetone. The extract was concentrated and extracted twice with one liter of ethyl acetate. A chloroform solution (100 ml) of the extract was precipitated by addition of 10 volumes of hexane. The supernatant was applied to a silica gel column (600 ml), which was eluted with hexane-ethyl acetate (2:1). The active fraction was chromatographed on a Sephadex LH-20 column (150 ml) with methanol. The active eluate was subjected to reverse-phase HPLC (YMC-Pack D-ODS-7, Yamamura Chemical Laborato-

ries Co.). Development of the column with 80% methanol gave two active fractions (retention time: 24.4 and 32.9 minutes, 19.8 ml/minute), which were separately evaporated to dryness to yield colorless oils of anguinomycins C (80 mg) and D (56 mg).

Physico-chemical Properties

The physico-chemical properties of the anguinomycins are summarized in Table 1. The ^{1}H NMR spectra of anguinomycins C and D are shown in Figs. 1 and 2, respectively. The high resolution FAB-MS established the molecular formulae of anguinomycins C and D as $C_{31}H_{46}O_4$ and $C_{32}H_{48}O_4$, respectively. Each anguinomycin exhibited IR absorption peaks due to hydroxyls (3460 cm^{-1}) and carbonyls (1710 cm^{-1}).

Structure Elucidation

The ^{13}C NMR spectrum of anguinomycin C confirmed the presence of 31 carbons. A heteronuclear multiple-quantum coherency (HMQC)[7] experiment established all one-bond ^{1}H-^{13}C connectivities as shown in Table 2. A COSY experiment revealed five spin networks to generate partial structures A to E as shown in Fig. 3. The heteronuclear multiple-bond correlation (HMBC)[8] spectrum displayed ^{1}H-^{13}C long-range couplings from 8-CH_3 to C-7, C-8 and C-9, and from 6-H, 7-H and 10-H to C-8, indicating the connection between partial structures A and B *via* C-8 as shown in Fig. 4. ^{1}H-^{13}C long-range correlations from 22-CH_3 to C-21, C-22 and C-23, and from 21-H and 24-H_3 to C-22 established the connection between partial structures D and E *via* C-22 (Fig. 4). Partial structures B, C and D were connected as shown in Fig. 4 by ^{1}H-^{13}C long-range couplings from 14-CH_3 to C-13, C-14 and C-15, from 12-H, 13-H and 16-H to C-14, and from 15-H, 16-H, 18-H, 19-H, 16-CH_3 and 18-CH_3 to a ketone carbonyl carbon (C-17). In addition, a δ-lactone ring was constructed from ^{1}H-^{13}C

Table 1. Physico-chemical properties of anguinomycins C and D.

	Anguinomycin C	Anguinomycin D
Appearance	Colorless oil	Colorless oil
$[\alpha]_D^{19}$	-128° (*c* 0.5, MeOH)	-135° (*c* 0.5, MeOH)
Formula	$C_{31}H_{46}O_4$	$C_{32}H_{48}O_4$
FAB-MS (*m/z*) calcd.	483.3437 $(M+H)^+$ 483.3474	497.3604 $(M+H)^+$ 497.3631
UV λ_{max} nm (ε)	241 (30,400) in MeOH	242 (32,400) in MeOH
IR ν_{max} cm-1	3460, 1710	3460, 1710

Fig. 1. ^{1}H NMR spectrum of anguinomycin C in $CDCl_3$.

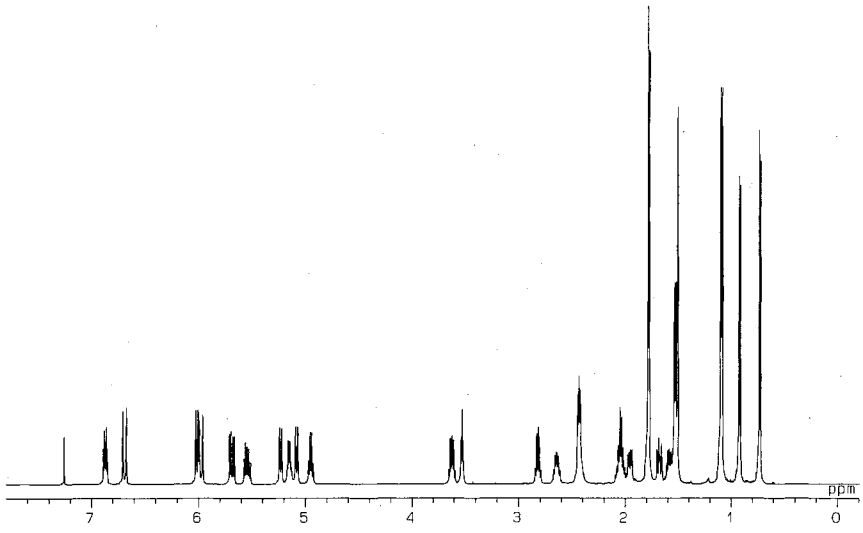


Fig. 2. ^{1}H NMR spectrum of anguinomycin D in $CDCl_3$.

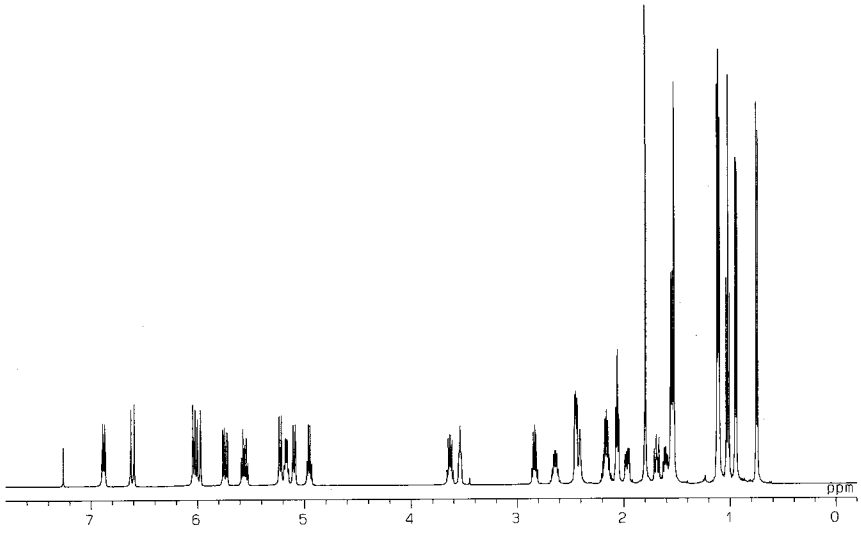


Table 2. ^{13}C and ^{1}H NMR data summary for anguinomycins C and D.

	Anguinomycin C		Anguinomycin D	
No.	δ_C	δ_H (J = Hz)	δ_C	δ_H (J = Hz)
1	163.9 s		164.0 s	
2	121.5 d	6.02 dt (9.7, 1.8)	121.6 d	6.03 dt (9.7, 1.8)
3	144.7 d	6.87 dt (9.7, 4.3)	144.7 d	6.88 dt (9.7, 4.2)
4	29.9 t	2.44 m	30.0 t	2.45 m
5	78.5 d	4.95 dt (6.8, 7.6)	78.8 d	4.95 dt (6.9, 7.5)
6	125.3 d	5.69 dd (15.7, 6.8)	124.7 d	5.74 dd (15.8, 6.9)
7	130.6 d	6.69 d (15.7)	129.9 d	6.61 d (15.8)
8	129.3 s		135.4 s	
9	138.9 d	5.23 d (9.8)	137.1 d	5.22 d (9.7)
10	32.1 d	2.65 m	32.1 d	2.64 m
11	40.6 t	2.05 2H m	40.7 t	2.06 2H dd (7.2, 6.6)
12	127.5 d	5.55 dt (15.5, 7.3)	127.6 d	5.56 dt (15.6, 7.2)
13	135.3 d	5.98 d (15.5)	135.4 d	5.98 d (15.6)
14	136.1 s		136.1 s	
15	128.3 d	5.08 d (10.2)	128.3 d	5.09 d (10.2)
16	45.5 d	3.62 dq (10.2, 6.8)	45.6 d	3.63 dq (10.2, 6.7)
17	215.5 s		215.6 s	
18	46.5 d	2.82 dq (6.0, 7.0)	46.5 d	2.83 dq (5.8, 7.0)
19	74.2 d	3.53 dd (6.0, 5.0)	74.3 d	3.54 dd (5.8, 4.5)
20	33.1 d	1.58 m	33.1 d	1.60 m
21	44.0 t	1.95 dd (13.0, 6.0)	44.1 d	1.97 dd (13.0, 6.0)
		1.68 dd (13.0, 8.6)		1.69 dd (13.0, 8.6)
22	133.9 s		133.9 s	
23	120.3 d	5.15 br q (6.6)	120.4 d	5.17 br q (6.6)
24	13.2 q	1.53 3H d (6.6)	13.4 q	1.55 3H d (6.6)
8-CH_3	20.2 q	1.78 3H d (1.0)		
8-$\mathbf{CH_2}CH_3$			26.3 t	2.16 2H m
8-$CH_2\mathbf{CH_3}$			13.4 q	1.02 3H t (7.5)
10-CH_3	20.6 q	0.93 3H d (6.8)	20.7 q	0.94 3H d (6.6)
14-CH_3	12.9 q	1.79 3H d (1.0)	13.0 q	1.80 3H d (0.9)
16-CH_3	16.1 q	1.09 3H d (6.8)	16.2 q	1.10 3H d (6.7)
18-CH_3	12.2 q	1.10 3H d (7.0)	12.2 q	1.11 3H d (7.0)
20-CH_3	13.9 q	0.73 3H d (6.7)	14.0 q	0.75 3H d (6.8)
22-CH_3	15.2 q	1.51 3H s	15.2 q	1.53 3H s

long-range correlations from 2-H, 3-H and 5-H to an ester carbonyl carbon (C-1) to establish the structure of anguinomycin C except for the stereochemistry.

The geometrical configurations of C-6 and C-12 were determined to be 6*E* and 12*E* by $J_{6\sim7}=15.7$ Hz and $J_{12\sim13}=15.5$ Hz. A lower-field chemical shift for 8-CH_3 (δ_C 20.2) and higher-field chemical shifts for 14-CH_3 (δ_C 12.9) and 22-CH_3 (δ_C 15.2) indicated 8*Z*, 14*E* and 22*E* configurations, which were confirmed by NOEs observed between 9-H and 8-CH_3, between 16-H and 14-CH_3 and between 21-H_2 and 23-H (Fig. 4).

The ^{13}C and ^{1}H NMR spectra of anguinomycin D were very similar to those of anguinomycin C. Anguinomycin D, however, contained an ethyl group in place of 8-CH_3 in anguinomycin C (Table 2) resulting in a downfield shift for C-8 by 6.1 ppm. The substitution of the ethyl group at C-8 in anguinomycin D was further confirmed by COSY, HMQC and HMBC experiments (data not shown).

The planar structures of anguinomycins C and D thus obtained are closely related to those of anguinomycins A and B[6] (Fig. 5), although their geometrical configurations were not reported. Since the ^{13}C chemical shift similarity between the two groups reveals the

Fig. 3. ^{1}H spin networks in anguinomycin C.

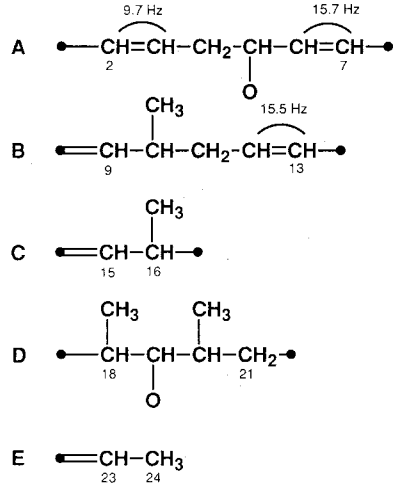
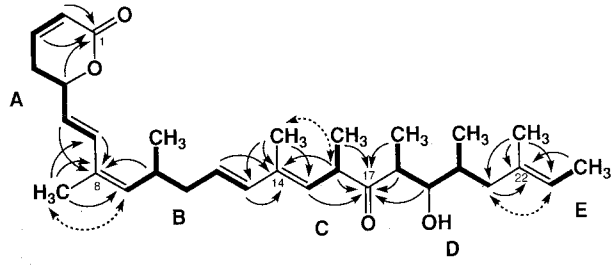
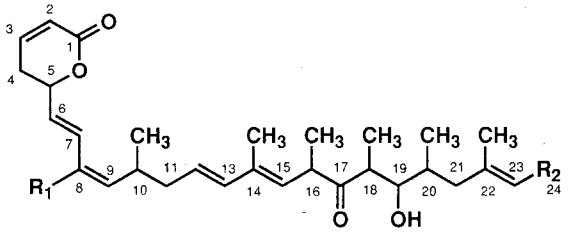


Fig. 4. HMBC and NOESY data summary for anguinomycin C.



Solid arrows show ^{1}H-^{13}C long-range correlations and dashed arrows show NOEs.

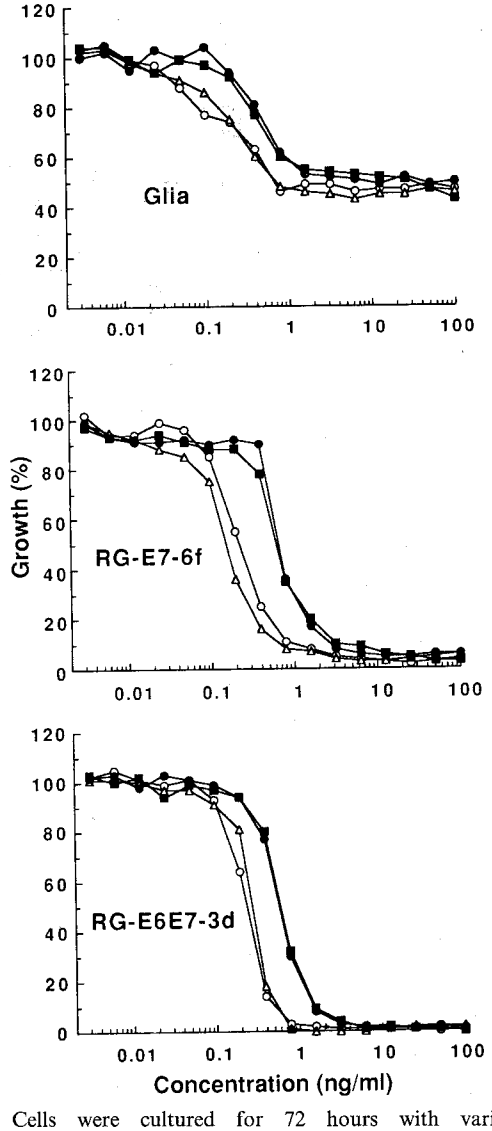
Fig. 5. Structures of anguinomycins A to D.



Anguinomycin A	$R_1 = CH_3$	$R_2 = COOH$
Anguinomycin B	$R_1 = CH_2CH_3$	$R_2 = COOH$
Anguinomycin C	$R_1 = CH_3$	$R_2 = CH_3$
Anguinomycin D	$R_1 = CH_2CH_3$	$R_2 = CH_3$

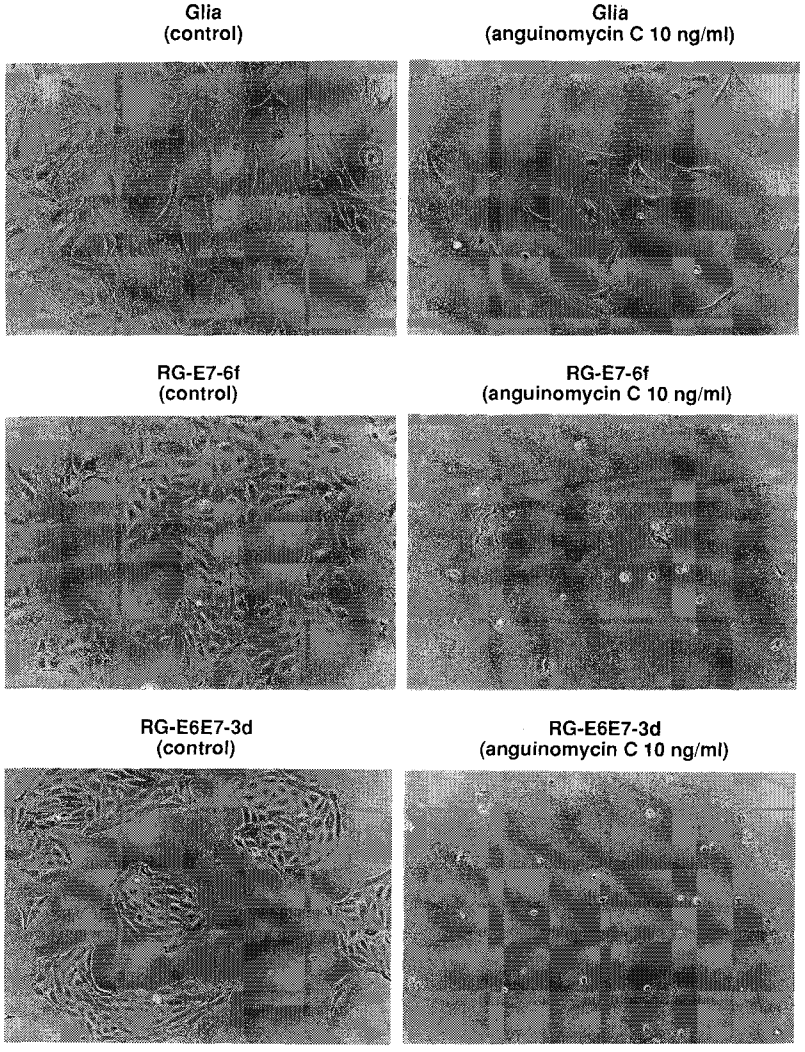
Fig. 6. Effect of anguinomycins A to D on the growth of normal and transformed rat glia cells.

○ Anguinomycin A, △ anguinomycin B, ■ anguinomycin C, ● anguinomycin D.



Cells were cultured for 72 hours with various concentrations of anguinomycins and then the growth was measured by the MTT method.

Fig. 7. Effect of anguinomycin C on the morphology of normal and transformed rat glia cells.



Cells were cultured for 72 hours with or without 10 ng/ml of anguinomycin C.

identical configurations, anguinomycins A and B appear to be C-24 carboxyl derivatives of anguinomycins C and D, respectively.

Biological Activity

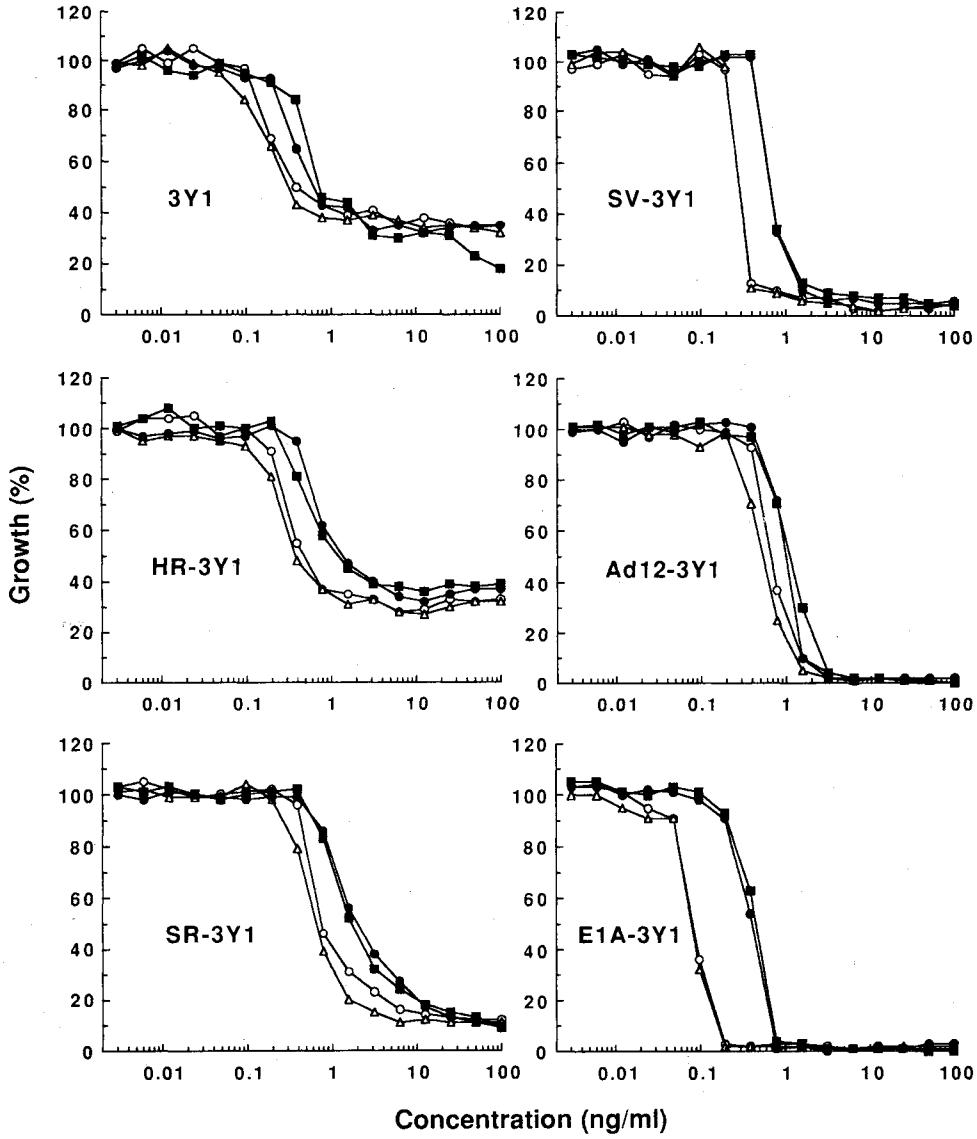
The cytotoxic and cytostatic effects of anguinomycins A to D on normal and transformed cells were examined by using rat glia cells and glia cells transformed with HPV16 E7 or both E6 and E7 genes[3] (RG-E7-6f and RG-E6E7-3d cells). At very low concentrations, anguinomycins induced growth arrest against normal cells and induced cell death against transformed cells as shown in Figs. 6 and 7.

The effects of anguinomycins were further investigated using normal and transformed 3Y1 rat fibroblasts[9~11], since 3Y1 cells show normal characteristics and a variety of transformed cells are available. The results are summarized in Fig. 8. Anguinomycins A to D induced growth arrest against normal 3Y1 cells and v-H-*ras*-transformed cells (HR-3Y1), and caused cell death against 3Y1 cells transformed with v-*src* (SR-3Y1), simian virus 40 (SV-3Y1), adenovirus type 12 (Ad12-3Y1) and its E1A gene (E1A-3Y1). The IC_{50} values of anguinomycins against these cells are summarized in Table 3. Flow cytometric analysis revealed that anguinomycins C and D arrested the cell cycle of 3Y1 cells mainly at G1 phase as shown in Fig. 9.

Except for *src*-transformed cells, cell lines highly sensitive to the killing effect of anguinomycins commonly express viral oncoproteins including HPV E7, adenovirus E1A and simian virus 40 large T antigen, which can bind and inactivate pRB. Elevation of p53 is known to cause G1 arrest in normal cells and apoptotic cell death in pRB-inactivated cells[12~16]. The activities of anguinomy-

Fig. 8. Effect of anguinomycins A to D on the growth of normal and transformed rat 3Y1 fibroblasts.

○ Anguinomycin A, △ anguinomycin B, ■ anguinomycin C, ● anguinomycin D.



Cells were cultured for 72 hours with various concentrations of anguinomycins and then the growth was measured by the MTT method.

cins resemble those of p53, although they induced cell death against cells with p53 inactivated by HPV E6, adenovirus E1B or simian virus 40 large T antigen. It is possible that anguinomycins might activate a signal pathway after p53. Further studies on the biological activity of anguinomycins are in progress.

Experimental

Microorganism

The anguinomycin-producing strain was isolated from a soil sample collected at Takasaki, Gunma Prefecture, Japan. The culture has been deposited with the National Institute of Bioscience and Human-Technology (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. KR2827-2 with the accession number FERM BP-5018.

Spectral Analysis

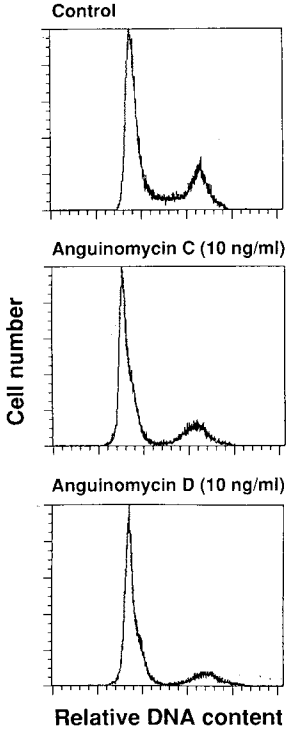
Specific optical rotations were obtained on a Jasco DIP-371 spectropolarimeter at 589.6 nm and 19°C. Mass spectra were measured on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. NMR spectra were obtained on a JEOL JNM-A500 spectrometer with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Table 3. IC_{50} values of anguinomycins A to D against normal and transformed rat cells.

Cell line	A	B	C	D
Glia	0.66	0.66	-*	-
RG-E7-6f	0.22	0.15	0.61	0.66
RG-E6E7-3d	0.24	0.27	0.60	0.58
3Y1	0.39	0.32	0.72	0.62
HR-3Y1	0.47	0.37	1.2	1.3
SR-3Y1	0.73	0.64	1.7	2.0
SV-3Y1	0.29	0.29	0.66	0.66
Ad12-3Y1	0.66	0.55	1.1	1.0
E1A-3Y1	0.082	0.080	0.46	0.42

* Cell growth was arrested around 50% (see Fig. 6).

Fig. 9. Flow cytometric cell cycle analysis of 3Y1 cells.



Cells in G1 phase, G2/M phase and S phase are represented by the first peak, the second peak and the area between the peaks, respectively.

Cells and Cell Culture

All cell lines were maintained in DULBECCO's modified EAGLE's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose, and grown at 37°C in a humidified atmosphere of 5% CO_2. Normal rat glia cells were obtained from primary cultures of Wistar rat (18-day embryo) cerebral cortex cells. All 3Y1 cell lines were obtained from Japanese Cancer Research Resources Bank.

DNA Transfection

Primary rat glia cells were transfected with plasmids containing a neomycin-resistant gene and HPV16 oncogenes (pSVneo-E7P and pSVneo-E6E7)[3] at 2.5 μg DNA per 10^5 cells by the calcium phosphate method[4]. One day after transfection, the transfected cultures were replaced at a split ratio of 1:20 and maintained for 3 weeks with refeeding with a medium containing 400 μg/ml of G418. Twelve weeks after transfection, immortalized cells were cloned by limiting dilution and established as RG-E7-6f and RG-E6E7-3d cell lines.

MTT Assay

Cells at 50% confluence were plated at one tenth lower cell density and incubated for 3 days with various concentrations of samples. The growth was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C.

Flow Cytometry

Rat 3Y1 cells were plated at 1×10^3 cells/cm^2. One day after plating, the cells were incubated with or without 10 ng/ml of anguinomycins C or D for 72 hours. The cells were trypsinized, fixed in 70% ethanol, and stained with 50 μg/ml of propidium iodide. Flow cytometric analysis was performed using a Beckton Dickinson FACScan instrument.

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