NOTE

SR1768A, AN ANTITUMOR ANTIBIOTIC CLOSELY RELATED TO CHROMOMYCIN

TOSHIKAZU OKI, YASUE MATSUZAWA, KENJI NUMATA and AKIRA TAKAMATSU

Central Research Laboratories of
Sanraku-Ocean Co., Ltd.
Fujisawa, 251 Japan

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In the course of our screening program for new antibiotics, seven components of antibiotic SR1768, which exhibit strong growth inhibition against tumor cells and markedly inhibit RNA synthesis of L1210 cells, were isolated from the culture filtrate of *Streptomyces* sp. A468 and were identified as members of the chromomycin A group[1~4].

Streptomyces sp. A468 was isolated from a soil sample collected in Katsunuma-cho, Yamanashi Pref. Preliminary taxonomic studies demonstrated that this organism can be readily differentiated from *Streptomyces aburaviensis*[5] and *Streptomyces griseus* No. 7[1] which produce aburamycin and chromomycin. Vegetative inoculum was grown in the medium containing dry yeast 25 g, Larex (Swift Co., lard oil) 20 g, soluble starch 10 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4 0.5 g, and tap water 1,000 ml and incubated for 48 hours at 28°C~30°C on a reciprocal shaker. The resulting culture was used to provide a 2% inoculum for the fermentation flasks containing the same medium. The antibiotic activity of cultured broth was assayed by the disc plate method using *Bacillus subtilis* PCI 219 and *Xanthomonas citri*. Maximum yields were obtained when the fermentation was carried out at 28°C~30°C for 4~5 days. The fermentation broth was filtered at pH 7, and the filtrate containing the antibiotic activity was extracted with an equal volume of ethyl acetate. The extract was evaporated to dryness. This crude material was dissolved in chloroform and chromatographed on a column of silicic acid (Mallinckrodt Chemical Works, 100 mesh) using the consecutive solvent systems chloroform-ethyl acetate (2:4) and chloroform-ethyl acetate-methanol (2:4:0.1 to 2:4:0.7). The active fractions obtained by this procedure was subjected to further purification. Seven components were isolated using preparative thin-layer chromatography. Silica gel G (Merck) plates were pretreated by soaking in ethyl acetate containing 1% oxalic acid. The solvent was then used for development. The antibiotic-containing portion of the adsorbent was scraped off the glass plates, and the antibiotic absorbed on the silica gel was eluted with acetone. The active yellow eluates were then evaporated *in vacuo* to dryness. Three major components, 35 mg of B, 28 mg of E and 5.2 mg of A, and four minor components were obtained in pure form from 5 liters of cultured broth.

The Rf values on silica gel G plates developed with three solvent systems namely chloroform-ethyl acetate-methanol (2:4:1), benzene-ethyl acetate-dimethylformamide (3:2:1) and ethyl acetate containing 1% oxalic acid, are summarized in Table 1. Rf values of SR1768B and E were the same as those of authentic samples of chromomycin A_2 and A_3, respectively, and identities of the antibiotics were confirmed in cochromatography, mixed melting point determination and physicochemical properties; infrared absorption spectrum: ν_{max}^{KBr} 3425, 1740, 1638, 1240, 1075 cm^{-1}; ultraviolet absorption spectrum: λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$) 230 (296), 281 (618), 320 (92), 335 (65), 416 (135) for SR1768E and 230 (209), 282 (430), 319 (83), 333 (64), 417 (100) for SR1768B. Furthermore, the chromophore and sugars from the seven components, prepared by methanolysis[6] using methanolic hydrogen chloride at room temperature and hydrolysis[7] with 50% acetic acid at 65°C for 30 hours, were compared with those of chromomycin A_2 and A_3. Comparisons were made of infrared, ultraviolet and mass spectra and Rf values on silica gel G plate developed with the solvent *n*-propanol-ethyl acetate-ammonia (6:2:0.45). The very close similarity of these physicochemical properties of chromophores obtained from the seven components and of chromomycinone clearly indicates that SR1768 belongs to the chromomycin A group, and moreover it was demonstrated chromatographically that the water-soluble fraction of the acid hydrolysate of SR1768E con-

Table 1. Rf values of SR1768

Components	Solvent systems		
	(1)	(2)	(3)
A	0.51	0.60	0.26
B	0.47	0.57	0.23
C	0.44	0.43	0.32
D	0.43	0.45	0.18
E	0.28	0.39	0.16
F	0.26	0.28	0.23
G	0.10	0.39	0.07
Chromomycin A_3	0.28	0.39	0.16
Chromomycin A_2	0.47	0.57	0.23

Solvent systems
(1) Chloroform-ethyl acetate-methanol (2: 4: 1)
(2) Benzene-ethyl acetate-dimethylformamide (3: 2: 1)
(3) Ethyl acetate containing 1% oxalic acid
Silica gel (E. Merck) plates were used with solvents (1) and (2), and silica gel plates pretreated with oxalic acid with solvent (3).

tained chromose A, chromose B, chromose C, chromose D, deacetylchromose B and deacetylchromose D. SR1768B afforded chromose B′ (4-O-isobutyryldeacetylchromose B) instead of chromose B[7,8]. Therefore, SR1768B and E were identified with chromomycin A_2 and A_3, respectively.

On the other hand, SR1768A was different from chromomycin A_2 and A_3 in its sugar moiety, thin-layer chromatogram, cytotoxicity, antibacterial spectrum and antitumor activity. Since the sugar (a blue spot was produced with *m*-aminophenol) of SR1768A which corresponds to chromose D migrated a little farther than those of chromomycin A_2 and A_3 on a silica gel G plate in *n*-propanol-ethyl acetate-ammonia (6: 2: 0.45), the chromose D-corresponding portion of the adsorbent was scraped off the glass plates and hydrolyzed with 1 N NaOH at 65°C for 1 hour. Analysis of fatty acids by gas-liquid chromatography using Polypack-2 (F & M Co.) column detected propionic acid and acetic acid from SR1768A and chromomycin A_2, respectively. Chromose B′, chromose A, deacetylchromose B, deacetylchromose D and chromose C were also detected in the hydrolysate of SR1768A. This suggested that SR1768A is an analogue of chromomycin A_2 and has propionyldeacetylchromose D as a substitute for chromose D. The antibiotic melts at 178°C to 181°C and the elementary analysis gave $C_{60}H_{88}O_{26}$ for its molecular formula; calcd. C: 58.81%, H: 7.24%, found C: 59.38%, H: 7.41%. The ultraviolet and infrared spectra exhibit; λ_{max}^{EtOH} nm ($E_{1cm}^{1\%}$) 232 (195), 284 (367), 319 (88), 333 (69), 425 (83); ν_{max}^{KBr} 3425, 1740, 1640, 1200, 1075 cm^{-1}.

Comparative studies of the biological properties of the seven components of SR1768 are shown in Table 2. Activity was examined for inhibition of growth and RNA biosynthesis of L1210 leukemic cells in culture and of EHRLICH ascites carcinoma in mice. When L1210 cells (6×10^4 cells/ml) suspended in RPMI1640 medium containing 20% calf serum were incubated in the presence of 0.01~0.5 μg/ml of SR1768 for 3 days at 37°C, 50% inhibition of cell growth was caused by concentrations of 0.031~0.047 μg/ml for A, B and E, but over 0.2 μg/ml for C, F and G. When L1210 cells were exposed to 0.1~10 μg/ml of SR1768 for 15 minutes prior to a 60-minute pulse of 0.1 μc/ml of uridine-2-^{14}C (New England Nuclear Co.), SR1768A inhibited 50% of the incorporation of ^{14}C-uridine into RNA at a concentration of 0.74 μg/ml, but other components exerted 50% inhibition at higher concentrations, namely 1.25 to 13 μg/ml. Inhibition of EHRLICH ascites carcinoma in mice was observed by intraperitoneal injection of daily doses of 7.8 μg/kg of SR1768A for 6 days, 7.8 μg/kg of B and 15.6 μg/kg of E. The acute toxicity tests in mice by the intraperitoneal route gave a LD_{50} of 0.5~1 mg/kg for A and B, 1~2 mg/kg for E, and 4 mg/kg for C, F and G.

The antimicrobial spectrum obtained by the agar dilution method showed that SR1768 is active against Gram-positive bacteria, and that SR1768A has marked anti-*Xanthomonas* activity with the MIC ranging from 0.75 to 1.56 μg/ml.

From the results, it is concluded that SR1768A is more active than the other components including chromomycin A_2 and A_3 in inhibiting RNA synthesis of L1210, the growth of EHRLICH ascites carcinoma in mice, and in antimicrobial activity.

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Table 2. Biological properties of SR1768

	Components						
	A	B	C	D	E	F	G
Cytotoxicity (ID_{50} of L1210 cell growth) μg/ml	0.031	0.039	0.26	0.15	0.047	0.28	0.24
Inhibition of RNA synthesis (ID_{50} of L1210 cells) μg/ml	0.74	1.25	3.2	2.5	3.1	12.5	13.0
Toxicity in mice (LD_{50}, i.p.) mg/kg	0.5~1	0.5~0.75	4.0	—	1~2	4.0	4.0
Minimum inhibitory concentration μg/ml							
Bacillus subtilis PCI 219	1.56	0.39	1.56	—	0.20	0.20	0.78
Aeromonas punctata ATCC 11163	3.13	50	0.39	—	50	50	0.20
Sarcina lutea PCI 1001	1.56	0.39	1.56	—	0.78	1.56	6.25
Staphylococcus aureus FDA 209P	—	0.39	—	—	0.78	3.13	—
Staphylococcus aureus Smith	1.56	0.39	1.56	—	0.78	3.13	25
Micrococcus flavus	1.56	0.20	3.13	—	1.56	1.56	3.13
Xanthomonas citri	1.56	3.13	1.56	—	6.25	3.13	—
Xanthomonas oryzae	1.56	25	3.13	—	50	3.13	—

Cytotoxicity: L1210 leukemic cells were originally grown in suspension culture with Roswell Park Memorial Institute 1640 medium supplemented with 20% calf serum. Aliquots of the cell suspensions (6×10^4 cells/ml) were placed in replicate tubes, and one tenth volume of diluted SR1768 solution were added to give the desired concentration of 0.01~0.5 μg/ml on day 0. The cell suspensions were incubated at 37°C under a 95% air-5% CO_2 atmosphere, and the cell number was determined daily for 3 days. Concentrations required to produce 50% inhibition of growth on day 2 as compared to untreated controls are expressed as the ID_{50}.

Inhibition of RNA synthesis: One ml of L1210 leukemic cells (8×10^5 cells/ml) suspended in RPMI1640 medium containing 10% calf serum was exposed to 0.1~10 μg/ml of SR1768 for 15 minutes prior to the 60-minute pulse of 0.1 μc/ml of uridine-2-^{14}C. The incorporation of ^{14}C-uridine was stopped by rapid chilling the tubes in ice and the addition of 1.0 ml of cold 10% trichloroacetic acid (TCA). After centrifuging, the acid-insoluble fractions were washed with cold 5 % TCA three times, and then dissolved in 0.2 ml of 88 % formic acid. The radioactivity was measured in 10 ml of BRAY's scinitillator[9] with an Aloka LSC-653 liquid scintillation spectrometer. The dose causing 50% inhibition of ^{14}C-incorporation is expressed as ID_{50} of RNA synthesis.

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