REVEROMYCINS, NEW INHIBITORS OF EUKARYOTIC CELL GROWTH

II. BIOLOGICAL ACTIVITIES

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Reveromycins A, B, C and D showed inhibitory activity against EGF-stimulated mitogen response in Balb/MK cells. Furthermore reveromycins A, C and D exhibited morphological reversion of *src*[ts]-NRK cells, antiproliferative activity against human tumor cell lines and antifungal activity. The effects of reveromycins A, C and D on eukaryotic cells were closely similar to each other, but those of reveromycin B were very weak. *In vitro* studies revealed that reveromycin A is a selective inhibitor of protein synthesis in eukaryotic cells.

Reveromycins A, B, C and D were found as inhibitors of mitogenic activity induced by epidermal growth factor (EGF) in Balb/MK cells. In our knowledge, natural products which are structurally related to reveromycins are not reported. We are interested in the biological activities of reveromycins because of their unique chemical structures. Biological activities of reveromycin A on animal cells and fungi were preliminarily reported[1]. In this paper, we report in detail the effects of reveromycin A on eukaryotic cell growth compared with those of reveromycins B, C and D.

Materials and Methods

Inhibition of Mitogen Response Induced by EGF

Quiescent Balb/MK cells for antimitogenic assays were prepared as previously described[2]. Mitogenic activity can be measured by the incorporation of [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA, U.S.A.) into quiescent Balb/MK cells 17 hours after addition of EGF (5 ng/ml, receptor grade, Collaborative Research Inc., Bedford, MA., U.S.A.). Radioactivity of trichloroacetic acid precipitable materials in the presence or absence of reveromycins was quantified.

Morphological Reversion of *src*[ts]-NRK Cells

Rat kidney cells infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (*src*[ts]-NRK)[3] were cultured at permissive temperature (32°C) or at nonpermissive temperature (39°C) in EAGLE's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY., U.S.A.). The cells maintained at 32°C were seeded into a 96-well microtiter plate (2×10^4 cells/200 μl/well) and cultured for 4 hours at 32°C in a CO_2 incubator. Solutions of various concentrations of reveromycins (10 μl each) were added and morphological reversion of *src*[ts]-NRK cells was observed under a microscope after 18 to 20 hours incubation at 32°C. The activity was presented as

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the rate of normal flat cells in total cells.

Antiproliferative Activity Against Human Tumor Cell Lines, KB and K562

DULBECCO's MEM with 5% FBS and RPMI 1640 with 10% FBS were used as culture media for KB and K562 cells, respectively. *In vitro* antiproliferative activity against human tumor cell lines was determined by MTT assay[4,5]. The cells (4×10^3 cells/200 μl/well) were seeded on a 96-well microtiter plate and incubated for 24 hours at 37°C in a CO_2 incubator. Five μl of different concentrations of inhibitors were put into each well and incubated for 72 hours. MTT (dimethylthiazoyldiphenyltetrazolium bromide) solution (50 μg in 10 μl of PBS) was added to each well and the plates were incubated for 4 hours. Then, the medium in each well was changed to equivalent volume of DMSO. After standing for 16 to 18 hours at room temperature, absorbance at 540 nm was measured by InterMed ImmunoReader NJ-2000. The value of IC_{50} were determined by graphic plots. 5-Fluorouracil purchased from Wako Pure Chemical Industries Co., Tokyo was used for comparison.

Antifungal Activity

For evaluation of antifungal activity of reveromycins, synthetic amino-acid medium fungal (SAAMF)[6] was employed as medium. The pH of the medium was adjusted with 1 N HCl between 3.0 and 7.4. *Candida albicans* IFO 1594 was used as test organism. Fluconazole which is a synthetic triazole antifungal agent was purified from Diflucan capsules (Pfizer) and used for comparison.

MICs were determined by broth dilution method using a 96-well microtiter plate. Yeast inocula were prepared in medium to final inocula size of 10^4/ml by hemacytometer counting. Various concentrations of drugs diluted in DMSO were added to the medium. The final concentration of the solvent (DMSO) was 4% (v/v). The plates were incubated at 37°C for 48 hours. The MICs were defined as the lowest concentrations of the drugs at which the growth of yeasts was inhibited.

The *pK*a value of reveromycin A was measured with a Toa AUT-1 automatic titrator in 20% MeOH solution.

Preparation of Trimethyl Ester of Reveromycin A

Reveromycin A (10 mg) was dissolved in MeOH (1 ml) and excess diazomethane-ether solution for 4 hours at room temperature to give quantitative trimethyl ester.

Effect of Reveromycin A on Macromolecular Synthesis

src^{ts}-NRK cells (1.5×10^5 cells/ml) were seeded onto a 48-multiwell plate in MEM supplemented with 10% FBS and cultured at 32°C for 17 hours. The culture medium was changed to 2% FBS containing MEM and [^{3}H]thymidine, [^{3}H]uridine, or [^{14}C]phenylalanine (2 μCi/ml, ICN Radiochemicals) was add to the cells. The serial dilution of reveromycin A was added simultaneously. After 5-hour labeling, the cells were harvested and the acid-insoluble fractions were collected. The radioactivity was counted by a liquid scintillation counter.

Effect of Reveromycin A on *In Vitro* Protein Synthesis

Rabbit reticulocyte lysate and tabacco mosaic virus RNA (Amersham) were used for the assay. *Escherichia coli* S-30 lysate was prepared by NIERENBERG's procedure[7]. Poly U (Yamasa) was used as mRNA. The *in vitro* protein synthesis was carried out in the presence of [^{35}S]methionine/cystein or [^{14}C]phenylalanine (ICN Radiochemicals) under the presence of reveromycin A. The acid-precipitated protein was collected after 30 minutes incubation. The protocol was given in the AMERSHAM's leaflet N-90. The radioactivity was counted by a liquid scintillation counter.

Results and Discussion

Inhibition of Mitogen Response Induced by EGF

Inhibition of [^{3}H]thymidine uptake into EGF-stimulated Balb/MK cells by reveromycins were shown in Fig. 1. Reveromycins A, C and D exhibited similar effects ($IC_{50} = 0.7 \sim 1.1$ μg/ml), whereas higher

concentrations of reveromycin B ($IC_{50}=6.0\,\mu g/ml$) were needed to inhibit mitogen response induced by EGF in Balb/MK cells.

Reveromycin A did not inhibit EGF-receptor kinase ($IC_{50} > 50\,\mu g/ml$). Therefore, it was considered that the inhibitory mechanism of reveromycins against the signal transduction of EGF is different from that of EGF-receptor kinase inhibitors such as erbstatin[8] or lavendustin A[9].

Morphological Reversion of *src*^{ts}-NRK Cells

When *src*^{ts}-NRK cells were cultured at 32°C, spherical transformed cells were observed. On the other hand, when they were cultured at 39°C, flat normal cells appeared taking the place of spherical cells. Reveromycins induced morphological reversion of *src*^{ts}-NRK cells from spherical transformed cells to flat normal cells at 32°C (Fig. 2), except for reveromycin B which showed little morphological reversion even at the concentration of 50 $\mu g/ml$. Reveromycins A, C and D exhibited EC_{50}s of about 1.58 $\mu g/ml$ and the activity was observed up to the concentration of 50 $\mu g/ml$ without cytotoxicity (Table 1). Flow cytometric analysis revealed that reveromycin A inhibited the cell cycle progression of *src*^{ts}-NRK cells at G_1 phase (data not shown). It was reported that p60$^{v\text{-}src}$ which possesses tyrosine kinase activity is involved in the G_1/S transition in the cell cycle[10,11]. It was possible that both growth factors and p60$^{v\text{-}src}$ kinase act

Fig. 1. Inhibition of [^{3}H]thymidine uptake into EGF-stimulated Balb/MK cells by reveromycins.

○ Reveromycin A, ● reveromycin B, ■ reveromycin C, ▲ reveromycin D.

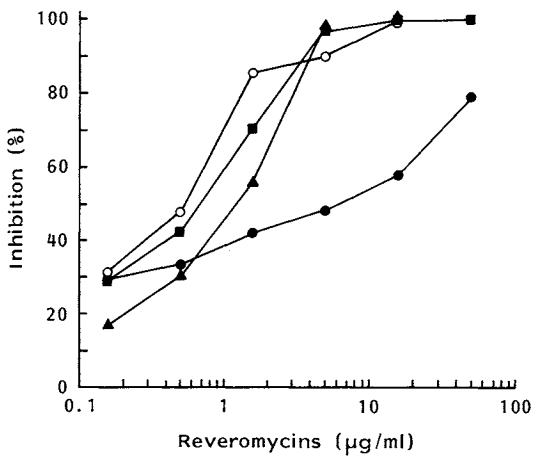
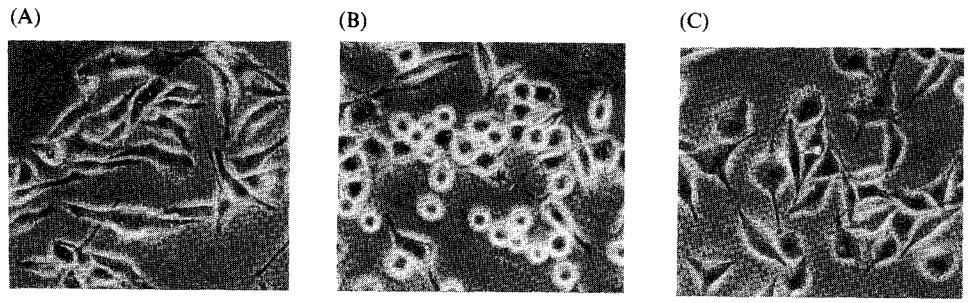


Table 1. Morphological reversion of *src*^{ts}-NRK cells by reveromycins.

Compounds	Concentration ($\mu g/ml$)				
	50.0	15.8	5.0	1.58	0.5
Reveromycin A	+++[a]	+++	+++	++	+
Reveromycin B	+	−	−	−	−
Reveromycin C	+++	+++	+++	++	+
Reveromycin D	+++	+++	+++	++	+

[a] Rate of reversed cells was presented as follows: +++; >80%, ++; 50~80%, +; 20~50%, −; <20%.

Fig. 2. Morphology of *src*^{ts}-NRK cells.



Cells were cultured at 39°C (nonpermissive temperature) (A), at 32°C (permissive temperature) without (B), or with 5 $\mu g/ml$ of reveromycin A (C).

Table 2. Antiproliferative activity of reveromycins against human tumor cell lines.

Compounds	IC_{50} (μg/ml)	
	KB	K562
Reveromycin A	1.9	1.6
Reveromycin B	>50	>50
Reveromycin C	2.0	2.0
Reveromycin D	1.6	1.3
5-Fluorouracil	>10	4.6

Table 3. Antifungal activity of reveromycins against *Candida albicans* IFO 1594.

Compounds	MIC (μg/ml) with an initial pH of:			
	3.0	5.0	6.0	7.4
Reveromycin A	2.0	125	500	>500
Reveromycin B	15.6	NT[a]	NT	>500
Reveromycin C	2.0	NT	NT	>500
Reveromycin D	2.0	NT	NT	>500
Reveromycin A trimethyl ester	>500	NT	NT	>500
Fluconazole	>5.0	>5.0	2.5	0.63

[a] Not tested.

as a mitogen. Reveromycin A might inhibit common factor(s) which is required for the mitogen response.

Several microbial products which induce morphological change of oncogene-expressed cells are known. A benzoquinoid antibiotic, herbimycin A was rediscovered as a compound which induces morphological reversion of *src*^{ts}-NRK cells (EC_{100}=0.5 μg/ml)[12]. In addition, oxanosine[13] as a guanosine analogue, azatyrosine[14] as a tyrosine analogue, acetoxycycloheximide, cycloheximide[15], epiderstatin[16] as glutarimide antibiotics, 2-demethylsteffimycin D[17] as an anthracycline antibiotic, and trapoxins A and B[18] as cyclotetrapeptides have already been reported. The chemical structures of reveromycins distinctly differ from these compounds.

Antiproliferative Activity against Human Tumor Cell Lines, KB and K562

Reveromycins A, C and D exhibited antiproliferative activity against KB and K562 cells *in vitro*, but reveromycin B did not, even at the concentration of 50 μg/ml. Reveromycins A, C and D were more effective than 5-fluorouracil on these cell lines (Table 2). The *in vivo* antitumor activity of reveromycin A will be described elsewhere.

Antifungal Activity

Reveromycin A showed antifungal activity but did not show antibacterial activity[1]. Antifungal activities of reveromycins were examined against *Candida albicans* IFO 1594 at various initial pH of the medium (Table 3). At the physiological pH (7.4), reveromycins had no potency (MIC: >500 μg/ml) in SAAMF. In the case of fluconazole, reducing the pH in SAAMF from 7.4 to 3.0 resulted in a lower of the anticandidal activity. In contrast, anticandidal activity of reveromycins were markedly enhanced when the initial pH of the medium was decreased from 7.4 to 3.0. The drastic change of anticandidal activity was observed in the pH 5 to 3 region. This result was compatible with the data of *pK*a value (4.2) of reveromycin A. However, trimethyl ester derivative of reveromycin A, which was prepared by diazomethane treatment, was not active even at pH 3. These results suggest that the nondissociative form of reveromycin's carboxylic acid are necessary for the anticandidal activity. The MIC value at pH 3 for reveromycins A, C and D was 2.0 μg/ml, but that for reveromycin B was approximately 8-fold higher.

Effect of Reveromycin A on Macromolecular Synthesis and *In Vitro* Protein Synthesis

Reveromycin A inhibited protein synthesis stronger than DNA or RNA synthesis as shown in Fig. 3. The IC_{50} for protein synthesis of *src*^{ts}-NRK cell was about 1 μM.

Fig. 3. Inhibition of macromolecular syntheses by reveromycin A.

○ [^{14}C]Phenylalanine, △ [^{3}H]uridine, □ [^{3}H]thymidine.

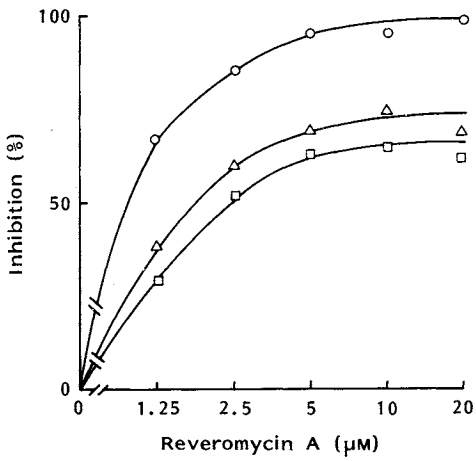
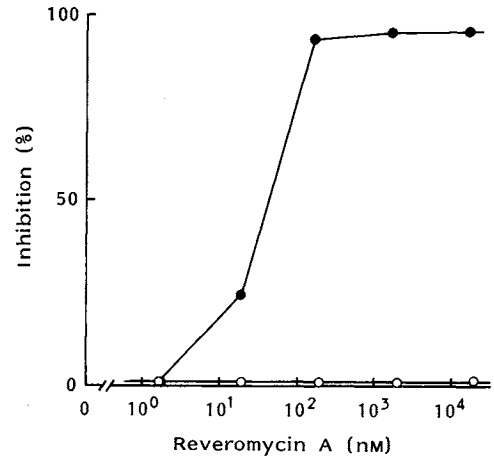


Fig. 4. Inhibition of *in vitro* protein synthesis by reveromycin A.

● Reticulocyte/TMV RNA, ○ *E. coli*/Poly U.



Differencial effect of reveromycin A on *in vitro* protein synthesis of rabbit reticulocyte and *E. coli* was worth noting. No inhibition of *in vitro* protein synthesis of *E. coli* by reveromycin A was found at 10 μM. In contrast, reveromycin A inhibited that of rabbit reticulocyte at the extremely low concentration (IC_{50}=40 nM) (Fig. 4). This observation shows that inhibition of protein synthesis by reveromycin A is selective to eukaryotic cells which was also implied based on the antimicrobial spectrum; reveromycin A inhibited fungal growth but not the bacterial growth. Inhibition of protein synthesis *in vitro* was caused at the lower concentration compared with that in intact *src*^{ts}-NRK cells. This is thought to be due to the low membrane permeability of reveromycin A. The relationship between action on mitogen responses caused by EGF or p60$^{v\text{-}src}$ and inhibition of protein synthesis in eukaryotic cells by reveromycin A is now under investigation.

Acknowledgment

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References

1) OSADA, H.; H. KOSHINO, K. ISONO, H. TAKAHASHI & G. KAWANISHI: Reveromycin A, a new antibiotic which inhibits the mitogenic activity of epidermal growth factor. J. Antibiotics 44: 259~261, 1991
2) OSADA, H.; T. SONODA, H. KUSAKABE & K. ISONO: Epiderstatin, a new inhibitor of the mitogenic activity induced by epidermal growth factor. I. Taxonomy, fermentation, isolation and characterization. J. Antibiotics 42: 1599~1606, 1989
3) CHEN, Y. C.; M. S. HAYMAN & P. K. VOGT: Properties of mammalian cells transformed by temperature-sensitive mutants of avian sarcoma virus. Cell 11: 513~521, 1977
4) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival: Application of proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55~63, 1983
5) ALLEY, M. C.; D. A. SCUDIERO, A. MONKS, M. J. CZERWINSKI, R. H. SHOEMAKER & M. R. BOYD: Validation of

an automated microculture tetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. Proc. Am. Assoc. Cancer Res. 27: 389, 1986

6) HOEPRICH, P. D. & P. D. FINN: Obfuscation of the activity of antifungal antimicrobics by culture media. J. Infect. Dis. 126: 353~361, 1972
7) NIERENBERG, M. W. & J. H. MATTHAE: The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. Proc. Natl. Acad. Sci. U.S.A. 47: 1588~1602, 1961
8) UMEZAWA, H.; M. IMOTO, T. SAWA, K. ISSHIKI, N. MATSUDA, T. UCHIDA, H. IINUMA, M. HAMADA & T. TAKEUCHI: Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. J. Antibiotics 39: 170~173, 1986
9) ONODA, T.; H. IINUMA, Y. SASAKI, M. HAMADA, K. ISSHIKI, H. NAGANAWA & T. TAKEUCHI: Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from Streptomyces griseolavendus. J. Nat. Prod. 52: 1252~1257, 1989
10) DURKIN, J. P.; A. L. BOYNTON & J. F. WHITFIELD: The src gene product ($pp60^{src}$) of avian sarcoma virus rapidly induces DNA synthesis and proliferation of calcium-deprived rat cells. Biochem. Biophys. Res. Commun. 103: 233~239, 1981
11) WELHAM, M. J.; J. A. WYKE, A. LANG & A. W. WYKE: Mitogenesis induced by $pp60^{v\text{-}src}$ is not accompanied by increased expression of immediate early response genes. Oncogene. 5: 161~169, 1990
12) UEHARA, Y.; M. HORI, T. TAKEUCHI & H. UMEZAWA: Screening of agents which convert transformed morphology of Rous sarcoma virus-infected rat kidney cells to 'normal morphology': Identification of an active agent as herbimycin and its inhibition of intracellular *src* kinase. Jpn. J. Cancer Res. (Gann) 76: 672~675, 1985
13) ITOH, O.; S. KUROIWA, S. ATSUMI, K. UMEZAWA, T. TAKEUCHI & H. HORI: Induction by the quanosine analogue oxanosine of reversion toward the normal phenotype of K-ras-transformed rat kidney cells. Cancer Res. 49: 996~1000, 1989
14) SHINDO-OKADA, N.; O. MAKABE, H. NAGAHARA & S. NISHIMURA: Permanent conversion of mouse and human cells transformed by activated ras or raf genes to apparently normal cells by treatment with the antibiotic azatyrosine. Molecular Carcinogenesis 2: 159~167, 1989
15) OGAWARA, H.; Y. HASUMI, K. HIGASHI, Y. ISHII, T. SAITO, S. WATANABE, K. SUZUKI, M. KOBORI, K. TANAKA & T. AKIYAMA: Acetoxycycloheximide and cycloheximide convert transformed morphology of *ras*-transformed cells to normal morphology. J. Antibiotics 42: 1530~1533, 1989
16) OSADA, H.; M. SASAKI, T. SONODA & K. ISONO: Epiderstatin induces the flat reversion of NRK cells transformed by temperature-sensitive Rous sarcoma virus. Biosci. Biotech. Biochem. 56 (11): 1992, in press
17) SUZUKAKE-TSUCHIYA, K.; Y. MORIYA, K. YAMAZAKI, M. HORI, N. HOSOKAWA, T. SAWA, H. IINUMA, H. NAGANAWA, C. IMADA & M. HAMADA: Screening of antibiotics preferentially active against *ras* oncogene-expressed cells. J. Antibiotics 43: 1489~1496, 1990
18) ITAZAKI, H.; K. NAGASHIMA, K. SUGITA, H. YOSHIDA, Y. KAWAMURA, Y. YASUDA, K. MATSUMOTO, K. ISHII, N. UOTANI, H. NAKAI, A. TERUI, S. YOSHIMATSU, Y. IKENISHI & Y. NAKAGAWA: Isolation and structural elucidation of new cyclotetrapeptides, trapoxins A and B, having detransformation activities as antitumor agents. J. Antibiotics 43: 1524~1532, 1990