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 src51-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 reported1. 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 described2. Mitogenic activity can be measured by the incorporation of [3H]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 src51-NRK Cells

Rat kidney cells infected with ts25, a T-class mutant of Rous sarcoma virus Prague strain (src51-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 × 10⁴ cells/200 µl/well) and cultured for 4 hours at 32°C in a CO₂ incubator. Solutions of various concentrations of reveromycins (10 µl each) were added and morphological reversion of src51-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. The cells (4 x 10^3 cells/200 μl/well) were seeded on a 96-well microtiter plate and incubated for 24 hours at 37°C in a CO₂ incubator. Five μl of different concentrations of inhibitors were put into each well and incubated for 72 hours. MTT (dimethylthiazolidinediphenyltetrazolium 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 IC₅₀ 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) 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 10⁴/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 pKa 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⁺-NRK cells (1.5 x 10⁵ 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 [³H]thymidine, [³H]uridine, or [¹⁴C]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. Poly U (Yamasa) was used as mRNA. The in vitro protein synthesis was carried out in the presence of [³⁵S]methionine/cystein or [¹⁴C]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 [³H]thymidine uptake into EGF-stimulated Balb/MK cells by reveromycins were shown in Fig. 1. Reveromycins A, C and D exhibited similar effects (IC₅₀ = 0.7 ~ 1.1 μg/ml), whereas higher
concentrations of reveromycin B (IC₅₀ = 6.0 μg/ml) were needed to inhibit mitogen response induced by EGF in Balb/MK cells.

Reveromycin A did not inhibit EGF-receptor kinase (IC₅₀ > 50 μg/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 erbstatin⁸ or lavendustin A⁹.

Morphological Reversion of src⁶⁶-NRK Cells

When src⁶⁶-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 src⁶⁶-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 μg/ml. Reveromycins A, C and D exhibited EC₅₀s of about 1.58 μg/ml and the activity was observed up to the concentration of 50 μg/ml without cytotoxicity (Table 1). Flow cytometric analysis revealed that reveromycin A inhibited the cell cycle progression of src⁶⁶-NRK cells at G₁ phase (data not shown). It was reported that p60⁰⁴src which possesses tyrosine kinase activity is involved in the G₁/S transition in the cell cycle¹⁰,¹¹. It was possible that both growth factors and p60⁰⁴src kinase act

Table 1. Morphological reversion of src⁶⁶-NRK cells by reveromycins.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>50.0</th>
<th>15.8</th>
<th>5.0</th>
<th>1.58</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reveromycin A</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Reveromycin B</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reveromycin C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reveromycin D</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

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

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

Fig. 2. Morphology of src⁶⁶-NRK cells.

(A) Cells were cultured at 39°C (nonpermissive temperature) (A), at 32°C (permissive temperature) without (B), or with 5 μg/ml of reveromycin A (C).
Table 2. Antiproliferative activity of reveromycins against human tumor cell lines.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (µg/ml)</th>
<th>KB</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reveromycin A</td>
<td>1.9</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Reveromycin B</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>Reveromycin C</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Reveromycin D</td>
<td>1.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>&gt;10</td>
<td>4.6</td>
<td></td>
</tr>
</tbody>
</table>

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-NRK cells (EC100 = 0.5 µg/ml)12. In addition, oxanosome13 as a guanosine analogue, azatyrosine14 as a tyrosine analogue, acetoxycycloheximide, cycloheximide15, epiderstatin16 as glutarimide antibiotics, 2-demethylsteffimycin D17 as an anthracyline antibiotic, and trapoxins A and B18 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 activity10. 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 pKa 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 IC50 for protein synthesis of src-NRK cell was about 1 µM.
Differential 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 (IC₅₀ = 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 src¹⁸-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 p60⁵⁺ and inhibition of protein synthesis in eukaryotic cells by reveromycin A is now under investigation.

Acknowledgment

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