METAPHASE-ARRESTING ACTION OF CARCINOSTATIC TENUAZONIC ACID*1

(Plate XI)

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Synopsis

Carcinostatic 5-benzyl-3-(1'-anilinoethylidene)pyrolidine-2, 4-dione (TN-16) and 5-(p-hydroxybenzyl)-3-(1'-anilinoethylidene)pyrolidine-2,4-dione (TN-17), structural analogs of tenuazonic acid, showed a remarkable metaphase-arresting effect on Yoshida sarcoma cells in vitro. Minimum effective concentration of TN-16 to arrest tumor cells at metaphase was 0.1 μM, which was one-tenth as active as colchicine.

In contrast to colchicine, metaphase-arresting effect of TN-16 and TN-17 was expressed only when the compound was present in the culture medium. Thus Yoshida sarcoma cells arrested at metaphase by TN-16 or TN-17 divided within 1 hr after release from the compound.

The fact that the metaphase-arresting action of TN-16 did not compete with colchicine, and colchicine action overcame that of TN-16 suggests that the receptor sites of Yoshida sarcoma cells for the two compounds may be different, or the affinity of TN-16 to the receptor site is weaker than that of colchicine.

Activities of tumor cells to synthesize nucleic acids and protein were not specifically inhibited by TN-16.

INTRODUCTION

Recently we have reported that several synthetic tenuazonic acid analogs showed carcinostatic activities against Yoshida sarcoma cells and Ehrlich carcinoma cells in vitro and in vivo.7,8) In these studies it was found that the cytocidal effect of these analogs was mainly expressed by their metaphase-arresting action on tumor cells. The present paper reports details of the cytological effect of these analogs on Yoshida sarcoma cells in vitro.

MATERIALS AND METHODS

Tumor Cells  Yoshida ascites sarcoma cells were obtained from a Donryu rat which had been transplanted with the tumor cells 6 days previously. The cells were washed with Eagle’s medium and suspended in Eagle’s minimum essential medium containing 1mM sodium pyruvate, 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 15% heat-inactivated calf serum.

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Compounds Tenuazonic acid, 5-benzyl-3-(1'-anilinoethylidene)pyrolidine-2,4-dione (TN-16), and 5-(p-hydroxybenzyl)-3-(1'-anilinoethylidene)pyrolidine-2,4-dione (TN-17) were previously synthesized by Yuki et al. Colchicine was purchased from the Tokyo Kasei Kogyo Co.

Thymidine[methyl-3H], uridine[5-3H], and DL-leucine[4,5-3H] were obtained from the New England Nuclear, U.S.A.

Cytological Test A test compound dissolved in ethanol at 1mM was serially diluted with physiological saline, and 1 volume of the solution was added to 9 volumes of a tumor cell suspension containing 50 x 10^3 cells/ml. One ml of the cell suspension was placed in a test tube with a rubber stopper and incubated at 37°C. At specified intervals, 0.5 ml of the suspension was centrifuged on a slide glass by a cytocentrifuge (Tommy Co., Tokyo). Cells fixed on the slide glass were stained with Wright-Giemsa stain, and 1000 tumor cells in interphase and in mitotic phases were differentially counted. At the same time, the number of viable tumor cells in the suspension was counted under a phase-contrast microscope with a Bürker hemocytometer.

Radioactivity of isotope-labeled tumor cells was measured by the method described previously.

RESULTS

Cytological Effect of Tenuazonic Acid Analogs As presented in Table I, mitosis of Yoshida sarcoma cells was blocked at metaphase when the cells were incubated with 1μM of TN-16 or TN-17. The number of living tumor cells decreased after 24 hr and many polynuclear cells were observed by the treatment. Tenuazonic acid itself showed neither cytotoxicity nor metaphase-arresting activity on tumor cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incubation period (hr)</th>
<th>Mitotic and polynuclear cells (%)</th>
<th>Living tumor cell (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prophase</td>
<td>Metaphase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenuazonic acid</td>
<td>0</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>1μM</td>
<td>4</td>
<td>1.3</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.9</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.6</td>
<td>4.7</td>
</tr>
<tr>
<td>TN-16 1μM</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1μM</td>
<td>4</td>
<td>1.9</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.6</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.9</td>
<td>4.5</td>
</tr>
<tr>
<td>TN-17 1μM</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1μM</td>
<td>4</td>
<td>1.0</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.8</td>
<td>42.6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td>32.6</td>
</tr>
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<td></td>
<td>0</td>
<td>1.2</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.5</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td>32.6</td>
</tr>
</tbody>
</table>

Values are average of 3 samples. ND=not determined

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Comparative Studies with Colchicine  

Metaphase-arresting effect and cytotoxicity of TN-16 on Yoshida sarcoma cells were compared with those of colchicine in respect to the following points: (1) Metaphase-arresting activity of TN-16 and colchicine, (2) fate of metaphase-arrested tumor cells after release from the chemical, and (3) competitive action of the two compounds.

(1) As illustrated in Fig. 1, TN-16 was approximately one-tenth active as colchicine, both in its cytotoxicity and metaphase-arresting activity in terms of molar concentration.

(2) Tumor cells were incubated with TN-16, TN-17, or colchicine for 4 hr, washed twice with the medium, and then incubated in a fresh medium without the chemical. As shown in Fig. 2 and in Photos la to lc, metaphase-blocked chromosomes, seen in about 30% of the tumor cells at the end of the treatment with 1 μM of TN-16, were arranged on the metaphase plates 10 min after release of the cells from TN-16. About 50% of the mitotic cells entered into anaphase and telophase after 30 min, and most of the mitotic cells divided within 1 hr. TN-17 showed similar effect on tumor cells as TN-16. The tumor cells treated with 0.01 to 1 μM of colchicine for 4 hr did not recover from the metaphase for 1 hr after release from the chemical, even though 0.1 μM of colchicine was equivalent to 1 μM of TN-16 in the metaphase-arresting activity. Growth rate of tumor cells pretreated with 1 μM of TN-16 for 4 or 8 hr was equal to that of untreated tumor cells, but the tumor cells pretreated with 0.1 μM of colchicine decreased to 50% of the initial count 24 hr after the end of the treatment.
These findings suggest that a major difference of TN-16 and TN-17 from colchicine is the recovery of the metaphase-arrested cells by the chemical to continuation of mitotic cycle when the cells are released from the chemical.

(3) Tumor cells were incubated with a mixture of various ratios of TN-16 and colchicine. After the first incubation with the chemicals for 4 hr, the cells were washed and then incubated in a fresh medium without the chemicals for 1 hr. Percentages of metaphase arrested cells after the first and the second incubation were examined. As
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illustrated in Fig. 3, percentage of metaphase-arrested cells decreased after the second incubation if the tumor cells had been incubated with TN-16 alone. In the presence of colchicine with TN-16, percentage of metaphase-arrested cells at the end of the first incubation did not change after the second incubation.

Tumor cells were incubated for 2 hr with 1 \( \mu M \) of TN-16, colchicine was added later to the cell suspension to the concentration of 0.1 \( \mu M \), and then the cells were incubated for further 1 hr. The tumor cells were washed and incubated in a fresh medium for 4 hr. As shown in Fig. 4, percentage of metaphase-arrested cells observed at the end of the treatment with TN-16 and colchicine increased after the following 4-hr incubation in the medium without the chemicals.

These findings indicate that the affinity of TN-16 to Yoshida sarcoma cells is weaker than that of colchicine, and the effect of colchicine overcomes that of TN-16.

Effect of TN-16 on Syntheses of Nucleic Acids and Protein in Yoshida Sarcoma Cells In order to know whether TN-16 specifically affect any of DNA, RNA, or protein synthesis of tumor cells, Yoshida sarcoma cells were incubated with 1 \( \mu M \) of TN-16 and 0.2 \( \mu Ci/ml \) of \(^{3}H\)-thymidine, \(^{3}H\)-uridine, or \(^{3}H\)-leucine, and uptake of a precursor into tumor cells was determined periodically. As shown in Table II, neither DNA, RNA, nor protein synthesis was inhibited until 8 hr. The extent of inhibition observed at the time later than 8 hr was proportional to the percentage of damaged tumor cells.
Table II. Effect of TN-16 on DNA, RNA, and Protein Syntheses of Yoshida Sarcoma Cells

<table>
<thead>
<tr>
<th>Incubation period (hr)</th>
<th>Thymidine</th>
<th>Incorporation of $^3$H-labeled precursor into tumor cells (cpm±S.D.)</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TN-16</td>
<td>% to control</td>
</tr>
<tr>
<td>4</td>
<td>1629±62</td>
<td>1467±45</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>2929±15</td>
<td>2226±49</td>
<td>76</td>
</tr>
<tr>
<td>24</td>
<td>7305±148</td>
<td>2524±86</td>
<td>35</td>
</tr>
</tbody>
</table>

A Yoshida sarcoma cell suspension containing $5 \times 10^3$ cells/ml, 1 $\mu$M TN-16, and 0.2 $\mu$Ci of a precursor was incubated at 37°. After the specified incubation time, 0.8 ml of the suspension was filtered through a Millipore membrane filter (0.45 $\mu$m pore size) and washed with 20 ml of cold 5% trichloroacetic acid solution. Radioactivity was measured in 10 ml of toluene-base scintillator. Each value represents an average of 3 samples.
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DISCUSSION

The present results indicate that tenuazonic acid analogs, TN-16 and TN-17, specifically affect mitotic cells and arrest them at metaphase. However, when the chemical is removed from the culture medium, the metaphase-arrested cells recover immediately from the arrest and readily divide; TN-16 and TN-17 are weakly bound to the cells and they are removed from the cells by washing.

It has been known that colchicine binds to the protein of mitotic apparatus of the cells, a subunit of microtubules.\(^1,2\)\) Comparative study of metaphase-arresting effects of TN-16 and colchicine showed that the effect of TN-16 does not compete with colchicine and the effect of colchicine overcomes that of TN-16, suggesting that the receptor site on the cells for these two compounds may be different. Since TN-16 did not inhibit specifically any of the activities of tumor cells to synthesize nucleic acids and protein, the mechanism of metaphase-arresting action of the compound may be related to its effect on mitotic apparatus of the cells and the effect is expressed only when the compound is present in the culture medium.

TN-16 and TN-17 may be applicable to synchronization of tissue cultured cells since the recovery of cells from metaphase to further mitotic cycle takes place readily if the compound is removed from the culture medium, a character which is similar to colcemid.\(^3,5\)\)

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REFERENCES

2) Idem, ibid., 34, 535 (1967).

EXPLANATION OF PLATE XI

Photo 1. Yoshida sarcoma cells arrested at metaphase by treatment with 1\(\mu\)M of TN-16, and mitotic change of the cells after release from the treatment.

a: Tumor cells treated with TN-16 for 4 hr.
b: Tumor cells incubated for 10 min in fresh medium after the above treatment. Chromosomes are arranged on metaphase plates.
c: Tumor cells incubated for 30 min in fresh medium after the treatment in a. Most of the mitotic cells enter into ana- and telo-phases. Wright-Giemsa stain. 40\(\times\)10.

Photo 2. Yoshida sarcoma cells treated with 1 \(\mu\)M of TN-16 for 24 hr. Many ploynuclear cells are observed. Wright-Giemsa stain. 40\(\times\)10.