ANTIMITOR ACTIVITY OF TRIOXACARCIN C

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The novel antitumor antibiotic, trioxacarcin C, was studied for antitumor activities against murine tumor systems. When mice with i.p.-inoculated B16 melanoma were given intraperitoneal injections of trioxacarcin C, the maximal T/C% was 164 by successive administration of 0.125 mg/kg/day (day 1 – 10). It also gave the prolongation of life span of mice bearing i.p. P388 leukemia (T/C 141%) by i.p. injection for 10 days, and inhibited the growth of sarcoma 180 (T/C 42%) and Lewis lung carcinoma implanted s.c. (T/C 23%) by i.v. administration for 6 or 7 days. It inhibited the growth of P388 leukemia cells in vitro and showed significant inhibition on the colony formation of HeLa S3 cells. DNA and RNA synthesis were more strongly inhibited than protein synthesis by trioxacarcin C. Also, it induced strand scission of PM-2 DNA without reducing agents or metals. It did not effect the number of white blood cells and blood urea nitrogen value of the peripheral blood.

Trioxacarcin, a group of antibiotics with novel polycyclic chromophores1, was isolated from the culture broth of Streptomyces bottropensis DO-45.2 This antibiotic is active against Gram-positive bacteria such as Staphylococcus aureus and Bacillus subtilis, but is weakly active against Gram-negative bacteria such as Escherichia coli and Klebsiella pneumoniae. The preliminary results of antitumor activity of trioxacarcin against sarcoma 180 and P388 leukemia were described in the previous report, but the characterization of trioxacarcin C as an anticancer agent has not been previously elucidated.

This paper describes the antitumor activity against various murine tumors and cell growth inhibition of trioxacarcin C. The mechanism of action of trioxacarcin C was studied on the macromolecular synthesis of P388 leukemia cells in vitro and PM-2 DNA-cleaving activity. Myelosuppression and nephrotoxicity were also studied in mice.

Materials and Methods

Chemicals
Trioxacarcin C was obtained from Dr. F. TOMITA of Tokyo Research Laboratory of Kyowa Hakko. Mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Japan), bleomycin (Nippon Kayaku Co., Ltd., Japan) and neocarzinostatin (Yamanouchi Seiyaku Co., Ltd., Japan) were used reference antitumor agents. [6-3H]Thymidine, [5-3H]Juridine and L-[4,5-3H]leucine were purchased from the Radiochemical Centre Amersham (England). PM-2 phage DNA was purchased from the Boehringer Manheim Yamanouchi Co., Ltd. (Tokyo, Japan)

Animals
Male ddY (18 ~ 20 g weight), CDF1 (5 ~ 8 weeks old) and BDF1 mice (5 ~ 8 weeks old) were purchased from the Shizuoka Agricultural Co-operative Association for Laboratory Animals (Hamamatsu, Japan).

Tumors and Toxicity
Antitumor activity and toxicity were measured and calculated as described previously.3 LD50 was calculated by BEHRETS-KÖRBER method from the number of survivors at 30 days after a single intravenous or intraperitoneal administration of drug into ddY mice.
Peripheral white blood cells and blood urea nitrogen were determined according to the method described previously. Differential counts of leucocytes were made on Giemsa stained smears of pretreatment blood and posttreatment blood on day 4, 7, 10 and 15.

Culture of Leukemia P388 Cells
Leukemia P388 cells were cultured in RPMI 1640 (Gibco Laboratories, New York, U.S.A.) supplemented with 5% fetal bovine serum (Gibco Laboratories), 50 µM of 2-mercaptoethanol, 100 U/ml of benzylpenicillin (Meiji Seika Co., Ltd., Tokyo, Japan) and 100 µg/ml of streptomycin (Kyowa Hakko Co., Ltd., Tokyo, Japan). For testing the growth inhibitory activity of drugs, $2 \times 10^6$ cells precultured for 24 hours at 37°C in a humidified atmosphere of 5% CO₂ in air, were treated with drugs for 1 hour (pulse exposure) or for 72 hours (continuous exposure). The cell count were made at 72 hours after addition of drug.

Incorporation of the Labeled Precursors into Macromolecule
Logarithmically growing P388 cells ($1 \times 10^6$/ml) were cultured with trioxacarcin C. $[^3\text{H}]$Thymidine (103 mCi/mg), $[^3\text{H}]$uridine (114 mCi/mg) or $[^3\text{H}]$leucine (385 mCi/mg) were added at 1, 3 and 5 hours after addition of trioxacarcin C. The cells collected on membrane filter GF/F (Whatman Co., Ltd., England) were washed with cold phosphate buffered saline (pH 7.4), and rinsed with cold 5% trichloroacetic acid several times. The radioactivity on the filter was counted by a liquid scintillation counter (Aloka LSC-653).

Culture of HeLa S₅ Cells
HeLa S₅ cells were cultured in Eagle minimum essential medium (MEM, Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum, 50 µg/ml of kanamycin and 292 µg/ml of L-glutamine.

For testing the lethal effect of drugs, 150 or 750 HeLa S₅ cells were seeded in plastic Petri dishes (60 x 15 mm, Falcon, California, U.S.A.). After incubating for 3 hours at 37°C in a humidified atmosphere of 5% CO₂ in air, the cells were treated with drugs for 1 hour, rinsed with phosphate balanced saline (pH 7.4), and fed the fresh prewarmed medium. The cells were cultured for 10~12 days for colony development, and fixed and stained with 4% Giemsa solution for counting the number of colonies. The plating efficiency of control cells were 70~90% in all experiments.

Analysis of DNA Degradation in Agarose Gel Electrophoresis
The reaction mixtures contained 0.5 µg PM-2 DNA, 40 mM Tris-HCl buffer (pH 7.8) and various concentration of drug. After incubation for 30 minutes at room temperature, the reaction was terminated by addition of 5 µl of 50 mM EDTA. After addition of 0.1% bromophenol blue in 75% glycerol, the resultant mixture was electrophoresed on 0.8% agarose gel according to the procedure described by SUZUKI et al. After electrophoresis, the gels were stained in electrophoresis buffer containing 0.5 µg/ml of ethidium bromide.

Results

Acute Toxicity
The LD₅₀ of trioxacarcin C was about 1.0 mg/kg by a single intraperitoneal (i.p.) or intravenous (i.v.) administration in ddY mice.

Antitumor Activity
Summary of the antitumor activity of trioxacarcin C against murine tumors is shown in Table 1. Trioxacarcin C inhibited the growth of solid sarcoma 180 by successive i.v. injection with minimal T/C% of 42 on day 7. But a single injection gave only 46% inhibition on tumor growth. The growth of Lewis lung carcinoma was inhibited by the successive i.v. treatment of trioxacarcin C with minimal T/C% of 23 on day 13. Maximal T/C% of 121 for survival day was obtained in this schedule, but prolongation of life span was not significant.
Table 1. Effect of trioxacarcin C on murine tumor.

<table>
<thead>
<tr>
<th>System</th>
<th>Site</th>
<th>Drug</th>
<th>Schedule</th>
<th>Dose (mg/kg/day)</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180</td>
<td>s.c.</td>
<td>i.v.</td>
<td>Day 1</td>
<td>0.75</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>i.v.</td>
<td>Days 1-6</td>
<td>0.5</td>
<td>42</td>
</tr>
<tr>
<td>P388</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Day 1</td>
<td>0.25</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>i.p.</td>
<td>Days 1,5,9</td>
<td>0.125</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>i.p.</td>
<td>Days 1-10</td>
<td>0.125</td>
<td>141</td>
</tr>
<tr>
<td>B16</td>
<td>i.p.</td>
<td>i.p.</td>
<td>Day 1</td>
<td>0.25</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>i.p.</td>
<td>Days 1-10</td>
<td>0.125</td>
<td>164</td>
</tr>
<tr>
<td>3LL</td>
<td>s.c.</td>
<td>i.v.</td>
<td>Day 1</td>
<td>0.9</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>i.v.</td>
<td>Days 1,7,13</td>
<td>0.4</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>i.v.</td>
<td>Days 1-7</td>
<td>0.3</td>
<td>23</td>
</tr>
</tbody>
</table>

Trioxacarcin C prolonged the life span of leukemia P388 bearing mice. Almost equal T/C% were observed with three schedules examined (single, intermittent and successive). A significant activity was shown in i.p.-inoculated B16 melanoma. Maximal effect (T/C 164%) was obtained in mice treated with successive i.p. injection of 0.125 mg/kg/day trioxacarcin C.

Effect on the Growth of Leukemia P388 In Vitro

The growth of leukemia P388 cells was markedly inhibited by trioxacarcin C in vitro. As shown in Table 2, the IC% (concentration which gave 50% cell number of control) was $8.24 \times 10^{-6}$ mM at continuous exposure, and $1.39 \times 10^{-4}$ mM at pulse exposure. The IC% value of trioxacarcin C was more than 100 times lower than that of bleomycin by pulse and continuous exposure. Neocarzinostatin was more inhibitory than trioxacarcin C by pulse exposure.

Lethal Effect on HeLa S3 Cells

The dose-survival curve of HeLa S3 cells exposed to trioxacarcin C for 1 hour is presented in Fig. 1. Trioxacarcin C-treated cells showed a dose-dependent exponential survival curve with a slight shoulder at low concentration. The mean lethal dose (D, the dose necessary to give 37% survival) was $1.30 \times 10^{-8}$ mM, and n (extrapolating value of an exponential portion of dose survival curve) was 1.60 for trioxacarcin C. For bleomycin-treated cells, the D and n values were $1.46 \times 10^{-6}$ mM and 0.69, and for neocarzinostatin, $1.32 \times 10^{-6}$ mM and 3.48, respectively.

Effect on Macromolecular Synthesis

Fig. 2 shows the inhibitory effect of trioxacarcin C on the incorporation of 3H-labelled precursors into macromolecules of leukemia P388 cells. At a concentration of $1.39 \times 10^{-4}$ mM (IC% at pulse exposure), trioxacarcin C suppressed DNA and RNA synthesis more strongly than protein synthesis (time

Table 2. Effect of trioxacarcin C on the growth of P388 leukemia cell.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Continuous exposure (72 hours) (mM)</th>
<th>Pulse exposure (1 hour) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trioxacarcin C</td>
<td>$8.24 \times 10^{-6}$</td>
<td>$1.39 \times 10^{-4}$</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>$1.07 \times 10^{-2}$</td>
<td>$2.24 \times 10^{-5}$</td>
</tr>
<tr>
<td>Neocarzinostatin</td>
<td>$4.92 \times 10^{-5}$</td>
<td>$3.44 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

Exponentially growing P388 leukemia cells were treated with each drug for 1 or 72 hours. Count of cells was made at 72 hours after addition of drugs.
Fig. 1. Lethal effect of trioxacarcin C on HeLa S3 cells.

HeLa S3 cells was exposed to each drug for 1 hour. Each point represents mean value of 3 experiments with trioxacarcin C.

Fig. 2. Inhibitory effect of trioxacarcin C on incorporation of labelled precursors into P388 primary culture cells.

(i) The labelled precursors were added at the indicated time after addition of trioxacarcin C and radioactivity was counted after pulse labelling for 1 hour.

(ii) The labelled precursors were added at 3 hours after addition of trioxacarcin C and radioactivity was counted after pulse labelling for 1 hour.

<table>
<thead>
<tr>
<th>Drug</th>
<th>D0 (mm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trioxacarcin C</td>
<td>1.30 x 10^-5</td>
<td>1.60</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.46 x 10^-2</td>
<td>0.69</td>
</tr>
<tr>
<td>Neocarzinostatin</td>
<td>1.32 x 10^-6</td>
<td>3.48</td>
</tr>
</tbody>
</table>

Fig. 3. Agarose gel electrophoresis of PM-2 DNA following incubation with trioxacarcin C.

1 and 8: Control, PM-2 DNA, 2 to 7: trioxacarcin C, 4 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 100 μg/ml, 200 μg/ml.

As shown in Fig. 3, 100 μg/ml of trioxacarcin C caused single strand scission of PM-2 DNA as well as change in the mobility (lane 6). As shown in the lane 7, the amount of the linear DNA was increased, while that of the open circular DNA was decreased. From these results, trioxacarcin C could make double strand scission of PM-2 DNA.

Bone Marrow Toxicity and Renal Toxicity

Fig. 4 shows the time course change of white blood cells and blood urea nitrogen in mice after i.v. treatment of trioxacarcin C and mitomycin C (MMC). Trioxacarcin C exhibited no significant decrease in white blood cells but slight increase at a single dose of 1.2 mg/kg with concomitant increase in neutrophiles (data not shown). Mitomycin C gave significant decrease in white blood cells with concomitant decrease in lymphocytes.

The blood urea nitrogen value of mice treated with trioxacarcin C did not deviate from that of
Fig. 4. Effect of trioxacarcin C on peripheral white blood cells and blood urea nitrogen.

(i) White blood cells

(ii) Blood urea nitrogen

Discussion

Trioxacarcin C gave a prolongation of the life span of mice bearing i.p.-inoculated P388 leukemia and B16 melanoma by a single and successive administrations. For solid tumors, a single administration of trioxacarcin C not inhibit the growth of solid sarcoma 180 and Lewis lung carcinoma. The successive injection (day 1~6) gave T/C% of 42 against sarcoma 180 on day 7 and all animals treated with 0.5 mg/kg/day survived on day 7. But, as mortality of treated animal was not observed thereafter, we could not conclude the effectiveness of trioxacarcin C against sarcoma 180. For solid tumor of Lewis lung carcinoma, 7 successive i.v. administrations of trioxacarcin C inhibited the growth of tumor and gave a slight prolongation of life span with T/C% of 121, showing no sign of toxic death. In the previous report, LD50 was reported to be 2 mg/kg by a single i.p. administration, but further studies with purified sample gave 1 mg/kg for LD50 by a single injection. The discrepancy of the values between previous and this report might result from the purity of sample.

Trioxacarcin C may interact with DNA and give DNA strand scission. But the mechanism of trioxacarcin C might be somewhat different from that of bleomycin, neocarzinostatin and macro-momycin, because reducing agents were required for DNA strand scission by latter compounds. Trioxacarcin C-induced DNA breakage might contribute to its cytotoxicity. Further study on DNA breaking activity of trioxacarcin C will be performed.

Because of DNA strand scission activity of trioxacarcin C, the effect of trioxacarcin C on the in vitro cell growth was compared with those of bleomycin or neocarzinostatin by assays of P388 leukemia cells with suspension culture and HeLa S3 cells with colony formation. Trioxacarcin C was about 100 and 10 times more potent than bleomycin and mitomycin C, respectively, against the growth of P388 leukemia cells in vitro with pulse exposure. It was also more active than bleomycin for the colony form-
ing ability of HeLa S3 cells. A slight shoulder on the dose-survival curve of HeLa S3 cells might suggest that trioxacarcin C induced DNA repair synthesis from sublethal damage.

Trioxacarcin C was reported to inhibit DNA synthesis without significant effect on RNA and protein synthesis in Bacillus subtilis, but, there is no difference on the inhibition % of DNA and RNA synthesis at various periods after addition and the same concentration of drugs in P388 leukemia cells. Trioxacarcin C might inhibit different steps of macromolecular synthesis between mammalian cells and microorganisms.

As the number of peripheral white blood cells did not decrease by a single and successive injection of trioxacarcin C, the bone marrow toxicity of trioxacarcin C might not be significant in mice. But the increase in neutrophiles count might suggest the irritating activities of trioxacarcin C.

While the antitumor activity and spectrum of trioxacarcin C was not superior to mitomycin C or adriamycin, the lower myelosuppressive activity of trioxacarcin C as compared with mitomycin C or adriamycin, might be one of the interesting characteristics for further studies of this compound.

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References