5-DI-(2'-TETRAHYDROPYRANYL) SECALONIC ACID D AS A NEW ANTIBIOTIC DERIVATIVE WITH ANTICANCER ACTIVITY*1

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A pyranyl derivative was chemically synthesized from secalonic acid D, an antibiotic obtained from culture filtrates of *Penicillium oxalium*, and its anticancer activity towards a highly antigenic rat bladder cancer, BC-47, implanted into inbred ACI/N rat was studied. The anticancer effect of the drug was similar to that of Adriamycin. Delayed initiation of treatment, starting 5 days after the cancer implantation, was more effective than treatment starting from day 1. In addition, it was less effective in an immunodeficient host subsequently implanted with BC-47. This compound is thought to retard the cancer cell growth until the appearance of tumor immunity in the host.

Key words: Anticancer activity — 5-Di-(2'-tetrahydropyranyl)secalonic acid D — BC-47 tumor — ACI/N rat

Secalonic acid D, an antibiotic obtained from culture filtrates of *Penicillium oxalium*, belongs to the group of ergot pigments (ergochromes) in the sclerotium of the fungus *Claviceps purpurea* grown on rye, and possesses strong antimicrobial activity towards *Bacillus subtilis*. As part of a search for a new chemically modified compound with more potent anticancer activity and lower toxicity than the original antibiotic, 5-di-(2'-tetrahydropyranyl)secalonic acid D (pyranyl secalonic acid) was chemically synthesized by pyranylization of secalonic acid D. This compound has already been reported to possess antitumor activities towards Ehrlich ascites carcinoma, sarcoma-180 and some mice tumors induced with Rous sarcoma virus.

Recently, urinary bladder cancers were induced in ACI/N rats of an inbred strain by oral administration of N-butyl-N-(4-hydroxybutyl)nitrosamine. They were established as several transplantable strains, among which BC-47 was defined as a highly antigenic strain and BC-43 or BC-50 as a poorly antigenic one on the basis of transplantation immunity. Histological observation indicated these strains to be transitional cell carcinomas. Among clinically available chemotherapeutic agents, adriamycin was found to be the most effective in this system. In order to ascertain the effectiveness of our new compound against a rat epithelial neoplasm, its anticancer activity was investigated in this system. The present paper describes the anticancer activity of pyranyl secalonic acid towards rat bladder cancers and compares the activity of this derivative with that of adriamycin.

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Materials and Methods

Animal and Tumors  Inbred ACI/N rats, 8 to 9 weeks old and weighing about 150 g, were purchased from Fuji Animal Farm Co., Tokyo. Transplantable rat bladder cancers, BC-47, BC-43 and BC-50, were maintained by subcutaneous passage. Once every 10 days, four rats bearing the cancer were sacrificed and the cancer mass (about 2 x 4 cm²) was removed from the subcutaneous site under sterile conditions. After careful removal of normal tissues around the cancers, the cancer mass was minced and then 0.5 ml was implanted subcutaneously or intraperitoneally into a normal rat with a trocar. For intravenous implantation, the minced cancer was first trypsinized. The minced cancer was suspended in phosphate-buffered saline (PBS, pH 7.2) containing 0.25% trypsin and 0.01% EDTA and incubated for 30 min at 37°C. The free cells obtained were then filtered through a gauze and washed twice with Eagle's minimum essential medium (MEM). The cells were adjusted to a final concentration of 5 x 10⁵ cells/ml and then implanted intravenously into a rat.

Solubilization of Pyranyl Secalonic Acid

Pyranyl secalonic acid was synthesized according to the method of Ishida.3) This compound was only sparingly soluble in PBS (pH 7.4). Thus, it was emulsified in PBS with 0.1% carboxymethyl cellulose (CMC). Alternatively, PBS-dimethyl sulfoxide (DMSO)-Tween-80 in a ratio of 50:47.5:2.5 was used as a basal solvent to dissolve the compound completely.

Chemicals  Adriamycin was purchased from Kyowa Hakko Co., Tokyo. Other reagents used were of analytical grade.

Anticancer Activity of Pyranyl Secalonic Acid and Adriamycin  The activity was evaluated in terms of the survival of animals at 60 or 90 days after the cancer implantation. Ten rats were used for each experimental group.

Irradiation of the Host  Normal ACI/N rats were exposed to 400 rad (⁶⁷Co) in a Toshiba apparatus (model RCS 16-2) at a dose rate of approximately 68.4 R/min.

Plaque Forming Assay  Sheep red blood cells (SRBC) were purchased from Toyo Kessei Co., Tokyo. Each drug was given intraperitoneally to normal ACI/N rats at a dose of 4 mg/kg for pyranyl secalonic acid and 1 mg/kg for adriamycin, once a day for 7 successive days (three rats per group). SRBC were washed three times with sterilized saline by centrifugation at 700 g for 7 min and the SRBC (2 x 10⁹) were injected intravenously into a rat, 4 days before the plaque forming assay. On appropriate days (Fig. 8), these rats were killed and spleen cells were harvested for the direct plaque forming assay. Spleen cells were suspended in MEM, washed three times with MEM and resuspended at a final concentration of 2 x 10⁷ cells/ml MEM. The spleen cells (0.1 ml), 0.4 ml of guinea pig serum diluted 1/4 and 0.5 ml of 10% SRBC were placed in a parafilm microchamber and kept at 37°C for 30 min. The number of plaque forming cells was counted under a microscope. Details of the assay techniques have been described previously.3)

Induction of Resistance to BC-47 Challenge  One-half ml of the minced BC-47 was implanted subcutaneously or intraperitoneally into a normal rat with a trocar. Drugs were given intraperitoneally with 7 successive daily doses of 4 mg/kg for pyranyl secalonic acid and 1 mg/kg for adriamycin, starting 1 or 5 days after the cancer implantation. Approximately 4 months later, the cured rats received a BC-47 challenge, and the immunoresistance was evaluated from the loss rate of cancer implantations.

Results

The anticancer activity of pyranyl secalonic acid against a highly antigenic bladder cancer, BC-47, was first examined. When the compound was administered intraperitoneally at a dose of 16 mg/kg in phosphate-buffered saline containing 0.1% carboxymethyl cellulose, once a day for 7 successive days, starting 1 day after the cancer implantation, the survival of the animals was significantly increased compared to the untreated group (Fig. 1).

![Fig. 1. Anticancer activity of pyranyl secalonic acid in PBS-DMSO-Tween-80 and PBS-CMC towards BC-47 implanted intraperitoneally.](image-url)

Pyranyl secalonic acid was injected intraperitoneally at a dose of 4 mg/kg (---) in PBS-DMSO-Tween-80 or 16 mg/kg (- - - - - - ) in PBS-CMC, 1 day after the cancer implantation, once a day for 7 successive days. Untreated rats (///). * Statistically significant difference from the untreated group in 60-day survival, P<0.05 (Fisher's test).
tation, the life span of the rats was prolonged as shown in Fig. 1. On the other hand, when it was administered at a dose of 4 mg/kg in PBS-DMSO-Tween-80 solvent, the life span was prolonged to that of the untreated group and some rats appeared to be cured. The difference in activity observed between these two groups is thought to be due to the solubility and permeation properties of pyranyl secalonic acid.

**Dose-Response and Schedule** Anticancer activity was compared by intraperitoneal treatment with 7 successive daily doses of the compound, starting 1 or 5 days after intraperitoneal implantation of the cancer. Contrary to expectation, later initiation of treatment was more effective than earlier initiation, as shown in Fig. 2. A single treatment with a dose of 20 mg/kg 5 days after the cancer implantation was ineffective. With daily therapy for 7 successive days, starting 5 days after the cancer implantation, anticancer activity was not observed at a dose of 2 mg/kg, but was observed at a dose of 4 or 8 mg/kg with similar degrees of effectiveness. At a dose of 8 mg/kg, the body weight of the rats gradually decreased during the treatment and some rats died of the cancer, approximately 80 days after the cancer implantation. As shown in Fig. 3, drug treatment at a dose of 4 mg/kg was found to be the most effective in this schedule.

**Site of Cancer Implantation and Route of Drug Administration** The anticancer activity of pyranyl secalonic acid varied considerably depending upon the site of cancer implantation and the route of drug administration. In the following experiments, cancer cells were implanted subcutaneously or intravenously and then the compound was given intraperitoneally or by painting on the subcutaneous cancer tissues. As shown in Fig. 4, a greater effect was observed in the case of direct contact of this compound with cancer cells. In other words, the anticancer activity depended on the cancer implantation site and drug administration.

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**Fig. 2.** Anticancer activity towards BC-47 implanted intraperitoneally on various dose schedules

Intraperitoneal injection of pyranyl secalonic acid started at a dose of 4 mg/kg 1 day (---) and 5 days (----) after the cancer implantation, once a day for 7 successive days. Untreated rats (//////). *Statistically significant difference from the untreated group in 60-day survival, P<0.05.

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**Fig. 3.** Anticancer activity of various dose of pyranyl secalonic acid towards BC-47 implanted intraperitoneally

Intraperitoneal injection of pyranyl secalonic acid was started at a dose of 2 mg/kg (---), 4 mg/kg (---), or 8 mg/kg (----), 5 days after the cancer implantation, once a day for 7 successive days. Untreated group (//////).
ANTICANCER ACTIVITY OF PYRANYL SECALONIC ACID

Fig. 4. Changes in anticancer activity with implantation site of BC-47
Pyranyl secalonic acid was injected intraperitoneally at a dose of 4 mg/kg or applied onto the cancer tissues at a dose of 3 mg per head 5 days after the cancer implantation, once a day for 7 successive days.
(a) Intraperitoneal implantation of the cancer, (b) subcutaneous implantation of the cancer, (c) intravenous implantation of the cancer; treated (---) and untreated (-) rats. (d) Application of pyranyl secalonic acid onto the cancer implanted subcutaneously into the rats; treated (---) and untreated (//////) rats. * Statistically significant difference from the untreated group in 60-day survival, $P<0.05$.

Effect of Irradiation of the Host on Anticancer Activity
In order to abolish the immune response of the host, the rats received whole body irradiation (400 rad) and 1 day later, BC-47 was implanted intraperitoneally. Intraperitoneal treatment with the drug was initiated 1 day after the cancer implantation at a dose of 4 mg/kg as shown in Fig. 5. A significant loss of anticancer activity of the compound as shown by a shortened life span was observed in the irradiated group. The anticancer activity of pyranyl secalonic acid thus appears to be partly host-mediated.

Anticancer Activity of Pyranyl Secalonic Acid and Adriamycin
Adriamycin has been reported to be most effective in ACI/N rats bearing BC-47. The effectiveness of pyranyl secalonic acid was compared with that of adriamycin. As shown in Fig. 6, the anti-

Fig. 5. Decrease in anticancer activity after irradiation of the host
One day after irradiation, the rat was implanted intraperitoneally with the cancer. Intraperitoneal injection of pyranyl secalonic acid started 1 day after the cancer implantation, once a day for 7 successive days. (a) Non-irradiated group; treated rats (---), (b) irradiated group; irradiated (-----), and treated (-----) rats, untreated rats (//////).
Fig. 6. Anticancer activities of pyranyl secalonic acid and adriamycin towards BC-47 implanted intraperitoneally
Intraperitoneal injection of pyranyl secalonic acid was started at a dose of 4 mg/kg (---), and that of adriamycin at a dose of 1 mg/kg (-----), 5 days after the cancer implantation, once a day for 7 successive days; untreated rats (//////). * Statistically significant difference from the untreated group in 90-day survival, \( P<0.05 \).

Fig. 7. Anticancer activity of pyranyl secalonic acid towards bladder cancer, BC-43 or BC-50, implanted intraperitoneally
Intraperitoneal injection of pyranyl secalonic acid was started at a dose of 4 mg/kg, 5 days after the cancer implantation, once a day for 7 successive days. (a) BC-43 implanted group; treated (---) and untreated (-----) rats. (b) BC-50 implanted group, treated (---) and untreated (-----) rats.

Fig. 8. Comparative effects of pyranyl secalonic acid and adriamycin on direct plaque forming activity
Pyranyl secalonic acid and adriamycin were given intraperitoneally to normal rats, once a day for 7 successive days. Direct plaque forming activity was assayed 5, 8, and 15 days after the first injection of each drug. Columns represent means±standard error.

Anticancer Activity against Other Bladder Cancers, BC-43 and BC-50
Anticancer activity was also investigated against poorly antigenic bladder cancers, BC-43 and BC-50, induced in the same host as BC-47. Rats implanted intraperitoneally with each bladder cancer was given pyranyl secalonic acid intraperitoneally at a dose of 4 mg/kg 5 days after the cancer implantation, once a day for 7 successive days. As shown in Fig. 7, the compound was less effective against both these cancers than against BC-47.
Table I. Induction of Rats Resistant to Challenged BC-47 after Complete Cure by Treatment with Pyranyl Secalonic Acid and Adriamycin

<table>
<thead>
<tr>
<th>Drug treatment day 1 to day 7</th>
<th>Resistant ratio</th>
<th>Drug treatment day 5 to day 12</th>
<th>Resistant ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyranyl secalonic acid-treated</td>
<td>10/19 (53)</td>
<td>10/10 (100)</td>
<td>14/18 (78)</td>
</tr>
<tr>
<td>Adriamycin-treated</td>
<td>5/7 (71)</td>
<td>1/4a (25)</td>
<td>6/10 (60)</td>
</tr>
</tbody>
</table>

Figures indicate number of survivors /total number of rats (%).

a) One rat died as a result of a technical error.

**Effects of Pyranyl Secalonic Acid and Adriamycin on Direct Plaque Forming Activity**

The effects of the drugs on 19S antibody formation in normal ACI/N rats to sheep red blood cells were compared on the same schedule as the drug therapy. As shown in Fig. 8, antibody formation in adriamycin-treated rats decreased 5 days after the first drug administration, while in pyranyl secalonic acid-treated rats, it increased. Although antibody formation in pyranyl secalonic acid-treated rats also declined after drug treatment was concluded, it returned to normal levels two weeks later. Thus, immunosuppression by pyranyl secalonic acid was found to be weaker than that by adriamycin.

**Induction of Rats Resistant to BC-47 Challenge after Complete Cure by Treatment with Pyranyl Secalonic Acid or Adriamycin**

Approximately 4 months after the primary cancer implantation, a BC-47 cancer challenge was applied to rats cured by intraperitoneal administration of pyranyl secalonic acid or adriamycin once a day for 7 successive days, starting 1 or 5 days after the intraperitoneal implantation of the cancer. As shown in Table I, all pyranyl secalonic acid-treated rats had acquired resistance to the implanted cancer graft regardless of any drug administration, while adriamycin-treated rats failed to do so in the case of early drug administration.

**DISCUSSION**

As a model of epithelial neoplasm, a highly antigenic cancer, BC-47, was selected in order to evaluate the anticancer activity of pyranyl secalonic acid. In the case of intraperitoneal implantation of the cancer and intraperitoneal administration of the compound, later administration has been found to be more effective as regards anticancer activity than earlier administration. This seems to be an important phenomenon from an immunological point of view. These data suggest that a certain quantity of cancer cells and a certain period of their residence in the host may be required for functioning of the immunological system in the host and subsequent expression of anticancer activity. In addition, anticancer activity was found to be almost abolished in preirradiated rats. These findings suggest that host-mediated action participates significantly in the anticancer effect of pyranyl secalonic acid, although direct action by the compound on the cancer could not be excluded. On the other hand, pyranyl secalonic acid is less immunosuppressive to the host than adriamycin, as far as 19S antibody formation is concerned. In other words, this compound is not as toxic to immunocompetent cells as other anticancer chemotherapeutic agents. Thus, pyranyl secalonic acid is thought to retard the cell growth of BC-47 without impairment of tumor immunity. The anticancer activity of pyranyl secalonic acid was compared with that of adriamycin by using bladder cancer, BC-47, implanted intraperitoneally into rats. Approximately 4 months after the primary cancer implantation, rats cured by treatment with pyranyl secalonic acid or adriamycin...
were challenged with BC-47 cancer. As shown in Table I, the pyranyl secalonic acid-treated group rejected the cancer graft even in the case of early drug treatment (from 1 to 7 days) after the primary cancer implantation, but the adriamycin-treated group failed to do so after the same schedule. It is probable that adriamycin rapidly reduces the number of cancer cells in the host through a direct cytotoxic action on the cancer before the expression of tumor immunity, while pyranyl secalonic acid may only retard the cancer growth, followed by expression of the host immunity against tumor-specific transplantation antigen. This may be the basis of the difference in the ability of the cured rats to reject the challenge dose of cancer cells. Further investigations are now being carried out.

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REFERENCES