Potent Antileukemic Activity of the Novel Cytostatic Agent Avarone and Its Analogues in Vitro and in Vivo

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ABSTRACT

Avarone and avarol are novel cytostatic agents which have potent antileukemic activity both in vitro and in vivo (mice). Cell culture experiments revealed that the cytostatic activity of these two compounds on L5178Y mouse lymphoma cells was 13- to 14-fold higher than that determined for HeLa cells and 40- to 43-fold higher than that for human melanoma cells. Nontumor cells (human fibroblasts and human gingival cells) were highly resistant against the two compounds. The inhibitory potency of avarone on L5178Y cells (50% inhibitory concentration, 0.62 μM) was significantly higher than the avarol activity (50% inhibitory concentration, 0.93 μM). Modification of the molecule at the quinone ring or the double bond in the terpenoid skeleton resulted in a significant loss of activity. In vivo studies with L5178Y cells in the ascites of mice confirmed the strong antileukemic effect determined in vitro. At doses of 10 mg/kg given i.p. once daily for 5 days to mice bearing approximately 10^7 leukemia cells, avarone was found to be curative in about 70% of the mice (20% for avarol). The optimal daily i.p. dose of avarone increased life span over controls by 146% when treatment was begun 1 day after tumor implantation and by 87% when treatment was delayed until day 8. Avarol, although active, was less effective. Based on the determined log<sub>10</sub> kill values, avarone can be classified as a highly active and avarol as a markedly active cytostatic agent. The efficacy of the two compounds is also emphasized by the therapeutic index of 11.7 for avarone and of 4.5 for avarol. The two agents were determined not to be either direct mutagens or premutagens in the Ames test.

INTRODUCTION

During the last few years simple biologically active prenylated quinone and hydroquinone derivatives have been discovered which are secondary metabolites from algae (1), tunicates (2), and sponges (3–5). Recently we reported that a main metabolic product of the Mediterranean sponge Dysidea avara, termed avarol, inhibits proliferation of L5178Y mouse lymphoma cells in vitro at low concentrations (5). Avarone has been identified as a sesquerpenoid hydroquinone (5, 6). Its quinone derivatives, avarone, 4′MeA-avarone, and 3′MeA-avarone are present at lower concentrations in the sponge (5, 6); they can easily be synthesized from avarol at high yields (6, 7). Further biological studies revealed that avarol inhibits mitosis of L5178Y cells in vitro, possibly by interfering with polymerization of tubulin (8). Both avarol and avarone have a low antibacterial and antifungal activity in vitro (9).

The work reported herein extends the in vitro testing of avarone and its analogues to include both normal and tumor cell lines in order to determine the breadth of the activity. After knowing that these novel compounds strongly inhibit proliferation of L5178Y cells in vitro, we could demonstrate that they are also highly potent antileukemic compounds in vivo.

MATERIALS AND METHODS

Materials. Benzo(a)pyrene and 3-methylcholanthrene were from Sigma Chemical Co., St. Louis, MO; methyl methanesulfonate was from E. Merck, Darmstadt, West Germany; corn oil was from the “Zvijezda” Oil Refinery, Zagreb, Yugoslavia.

Male outbred NMRI mice (32–35 g; 8–9 months old) were obtained from Zentralinstitut für Versuchstiere, Hannover, West Germany.

Avarone and Its Analogues. Avarone was isolated from the sponge Dysidea avara as described (5); the sponge material was collected in the Bay of Kotor (Yugoslavia) and near Giravaru (Maldives). Avarone was obtained from its corresponding hydroquinone avarol by Ag<sub>2</sub>O oxidation (5). 4′MeA-avarone and 3′MeA-avarone were prepared from avarone (7). Dihydroaavaroal was prepared from avarol hydrogenation in methanol in the presence of palladium-charcoal. For the in vitro tests, the compounds were dissolved in 0.1% dimethyl sulfoxide (final concentration); this solvent had no influence on cell growth. In case of the in vivo i.p. applications, avarone and avarol were suspended in a 0.15% (w/v) solution of methylcellulose (Sigma M-0262).

Cell Culture. L5178Y mouse lymphoma cells (10) were grown in Eagle’s minimum essential medium supplemented with 10% horse serum in roller tube cultures (11, 12). For the dose-response experiments, 5-ml cultures were initiated by inoculation of 5 × 10<sup>4</sup> cells/ml and were incubated at 37°C for 72 h; the controls showed a generation time of 10.5 h. The cell growth was determined by cell count with a Cytocomp counter (128-channel counter; system Michaelis; Mainz, West Germany) (13).

Human gingival cells were obtained from aseptically removed gingiva as previously described (14). Primary cultures were grown as monolayers in Hanks’ medium, containing 10% fetal calf serum (Boehringer Mannheim), in a fully humidified atmosphere of 4% CO<sub>2</sub> and air at 37°C. Gingival epithelial cells (primarily fibroblasts) of the third to fifth passage

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were used for the experiments. The cells were seeded at a density of 2.4 \times 10^5 cells/cm^2; 24 h later the compounds were added to the cultures and incubation was routinely continued for an additional 24 h; a drug incubation time of 72 h revealed almost identical ED_{50} values. Subsequently, the cells were counted optically using a phase contrast microscope; the cell shape was examined in parallel. The generation time of these cells was determined to be 18 h. G-361 human malignant melanoma cells (ATCC CRL 1424) were grown as monolayers on plastic flasks in McCoy Medium 5A supplemented with 10% fetal calf serum. The EDM was estimated by logit regression (17). The data from 10 parallel experiments were evaluated. Student’s t test was used to determine the significance between the ED_{50} values as described (18).

The EDM was estimated by logit regression (17). The data from 10 parallel experiments were evaluated. Student’s t test was used to determine the significance between the ED_{50} values as described (18). The slopes of the ED_{50} values were expressed as the tangent and were obtained by logit regression of the dose-response data.

**Chemotherapy.** The evaluation of the antitumor effect of avarone and avarol in L5178Y leukemia was performed as follows: mice were made leukemic by intraperitoneal injection of 1.8 \times 10^6 L5178Y cells. One or 8 days later, the animals were divided into groups of 10 mice. One group was used as a control (treated with the methylcellulose solution only), whereas the other groups were treated i.p. with the drugs for 5 consecutive days.

The antitumor activity was assessed according to the following criteria:

- **In Vivo Activity**
  - Logarithmic growth plot of the tumor in the control group (19); a value of 2.1 days was obtained. The log_{10} cell kill values were converted to an arbitrary activity rating as described (19).
  - The therapeutic index was calculated according to Skipper and Schmidt (21).

**Mutagenicity Testing.** The mutagenic activity of the avarone derivatives was measured with the Ames microsomal test using Salmonella typhimurium Ta 100 strain (22, 23). The S-9 fraction served as the postmitochondrial fraction. It was isolated from the immature carp liver 48 h after i.p. treatment with 3'-methylcholanthrene (50 mg/kg), as described (23, 24). The number of hist* revertants was counted by a Dynatech Model 980 counter. Benzo(a)pyrene and methyl methanesulfonylamine served as premutagen and direct mutagen standards.

**Mixed Function Oxygenase Induction Testing.** The procedure used was described in earlier papers (24, 25). Briefly, the test samples, dissolved in corn oil (0.1 ml), were i.p. injected into the carp. After 48 h the animals were killed, the livers were removed and immediately processed for enzyme activity determination. The same procedure was used both in positive (receiving 3-methylcholanthrene in corn oil) and negative controls (receiving corn oil alone). The activity of BaPOMO was measured in the postmitochondrial fraction, following the method of Payne and Penrose (26), and expressed in pmol 3-hydroxybenzo(a)pyrene formed per mg protein per min. The protein concentration was determined according to the method of Lowry et al. (27).

**RESULTS**

Initial evidence for the high in vitro cytostatic activity of avarol was obtained from studies with L5178Y mouse lymphoma cells (5). In the present study the corresponding quinone, avarone, was determined to be even more active in this cell system in vitro. Moreover both compounds turned out to be highly potent antileukemic agents in mice. The anticancer activity is coupled with a low toxicity to normal cells.

**In Vitro Activity**

Table 1 summarizes data showing the selective cytotoxicity of avarone and avarol to cultured L5178Y cells relative to the other cells tested. Against L5178Y cells, avarone was the most cytotoxic of the materials tested; avarol and dihydroavarol were moderately cytotoxic, and the methylamino derivatives of avarol were essentially ineffective except at high concentrations.

The slopes of the log-linear dose-response curves of avarone and its analogues at the ED_{50} values were identical within the same cell line. However, they differed from one to another line as follows: L5178Y cells, 1.4; HeLa cells, 3.5; melanoma cells, 5.6; human fibroblasts, 6.1; and human gingival cells, 10.2.

**In Vivo Activity**

**Toxicity.** The i.p. toxicity of both avarone and avarol for male mice is low (Table 2). The LD_{50} values for acute toxicity are 181.2 mg/kg for avarone and 269.1 mg/kg for avarol. The corresponding values for subacute toxicity are 172.1 mg/kg for avarone and 218.4 mg/kg for avarol. The dose-survival curves are steep; the slopes at the LD_{50} values vary between −2.5 and −1.9. In a close approximation, an increase in the dose that was lethal to...
Table 1
Influence of avarone and its analogues on growth of selected cell lines
The ED₉₅s were determined as described under "Materials and Methods." The values represent the means of 10 parallel experiments each; the means ± SD are given.

<table>
<thead>
<tr>
<th>Compound</th>
<th>L5178Y cells</th>
<th>Melanoma cells</th>
<th>HeLa cells</th>
<th>Human fibroblasts</th>
<th>Human gingival cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avarone</td>
<td>0.62 ± 0.11</td>
<td>26.4 ± 3.6</td>
<td>8.7 ± 1.1</td>
<td>11.3 ± 1.7</td>
<td>76.4 ± 15.1</td>
</tr>
<tr>
<td>4'MeA-avarone</td>
<td>4.16 ± 0.52</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3'MeA-avarone</td>
<td>10.02 ± 1.27</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Avarol</td>
<td>0.93 ± 0.13</td>
<td>37.8 ± 5.9</td>
<td>12.4 ± 2.2</td>
<td>16.9 ± 2.9</td>
<td>82.9 ± 16.3</td>
</tr>
<tr>
<td>Dihydroavarol</td>
<td>1.25 ± 0.21</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined.

Table 2
Toxicity of avarone and avarol for male mice
Mice (groups of 5 animals per cytostatic dose) were treated either once (acute toxicity) or daily for 5 days (subacute toxicity) with avarone or avarol. For each compound and each duration of application 5 different doses were chosen. The number of surviving animals was determined after 40 days. The LD⁰ and LD₁₀ values were determined by logit regression (17).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acute LD₁₀</th>
<th>Acute LD₉₀</th>
<th>Subacute LD₁₀</th>
<th>Subacute LD₉₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avarone</td>
<td>181.2</td>
<td>111.1</td>
<td>172.1</td>
<td>109.7</td>
</tr>
<tr>
<td>Avarol</td>
<td>269.1</td>
<td>156.4</td>
<td>218.4</td>
<td>138.6</td>
</tr>
</tbody>
</table>

Only 10% of the mice by 2.5- to 2.8-fold killed 90% of the animals at this increased level.

A daily i.p. injection of 80 mg/kg for 10 days of both avarone and avarol caused no deaths among mice, and the weight of the animals compared to the untreated controls did not change significantly. No toxicity was apparent in the form of loss of hair or nail destruction.

Chemotherapeutic Activity. The median life span of the L5178Y lymphoma-bearing control mice was 14.3 days. Both avarone and avarol were determined to increase the life span of the tumor-bearing mice considerably (Table 3). The animals were given i.p. injections of the compounds for 5 consecutive days, starting at day 1 or day 8; 50 mg/kg/day was chosen as the highest dosage, a dosage which is 2.2- to 2.8-fold lower than the LD₁₀ (see Table 2).

Two schedules for the agent treatment were chosen, days 8–12 and days 1–5 after tumor inoculation (Table 3). In general, the treatment starting at day 1 after tumor inoculation was superior to that beginning the treatment at day 8. The schedule of days 1–5 resulted in a dose-dependent cure of 20–70% of the mice, starting at day 1 or day 8; 50 mg/kg/day was chosen as the highest dosage, a dosage which is 2.2- to 2.8-fold lower than the LD₁₀ (see Table 2).

The influence of avarone and avarol on tumor growth was obtained by plotting the dose-response data reported in Table 4, according to Skipper and Schmidt (21). These values were determined for avarone to be 9.4 mg/kg/day, and for avarol to be 31 mg/kg/day. From these data and the LD₁₀ values (Table 2), the therapeutic ratios were calculated for avarone to be 11.7, and for avarol to be 4.5.

Mutagenicity

Ames Test. Avarone, as well as its analogues, avarol, 3‘MeA-avarone, and 4‘MeA-avarone, were determined not to be mutagenic at concentrations between 50 and 125 μM; the number of revertants per plate varied between 84 and 148, a range which was counted also in the dimethyl sulfoxide controls. By the application of the activating S-9 fraction it could be further elucidated that the compounds displayed neither direct nor activated mitogenic potentials in the Ames microsomal test system. As indirect mutagen benzo(a)pyrene was used (5 μM, 501 ± 45 revertants; and 10 μM, 1591 ± 118 revertants), while methyl methanesulfonate served as direct mutagen (1600 μM caused 1305 ± 117 revertants).

BaPPO Induction Capacity. Neither avarone nor its analogues were inducers of BaPPO activity in experimental fish. At doses of 50 or 100 mg/kg of avarone or its analogues, a BaPPO activity of 30.2 ± 8 pg of 3-hydroxybenzo(a)pyrene formed per mg protein per min was measured; the enzyme in the control animals, given injections of 0.1 ml corn oil, showed an activity of 35 ± 13. Under the identical test conditions 3-methylcholanthrene (40 mg/kg) caused a 15-fold induction of the BaPPO (495 ± 99 pg/mg/min).

DISCUSSION

As demonstrated in this work, the sesquiterpenoids avarone and avarol are cytostatic agents which have an unusually high selectivity for lymphoma cells. The agents have been isolated from the sponge Dysidea avara (5, 6), which is very abundant in...
ANTILEUKEMIC ACTIVITY OF AVARONE

Table 3
Effect of avarone and avarol on L5178Y mouse leukemia in vivo

All mice were given i.p. inoculations of L5178Y cells on day 1 or day 8. Treatment was i.p. as indicated. The assessment of the antitumor activity was performed according to the criteria summarized under "Materials and Methods."

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg/injection)</th>
<th>Schedule</th>
<th>Total dose (mg/kg)</th>
<th>ILS (%)</th>
<th>Weight change (g)</th>
<th>Log kill/dose (Mean)</th>
<th>Total log kill (Mean)</th>
<th>Activity rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avarone</td>
<td>1</td>
<td>Days 8-12</td>
<td>5</td>
<td>43</td>
<td>+0.5</td>
<td>9.7</td>
<td>1.39 ± 0.67 (0/10)</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Days 8-12</td>
<td>50</td>
<td>69</td>
<td>-1.5</td>
<td>12.0</td>
<td>1.72 ± 1.00 (0/10)</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Days 8-12</td>
<td>250</td>
<td>53</td>
<td>-5.3</td>
<td>9.9</td>
<td>1.42 ± 0.70 (0/10)</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>Avarol</td>
<td>1</td>
<td>Days 8-12</td>
<td>5</td>
<td>97</td>
<td>+3.2</td>
<td>14.7</td>
<td>2.11 ± 1.39 (0/10)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Days 8-12</td>
<td>50</td>
<td>148</td>
<td>+0.5</td>
<td>21.2</td>
<td>3.04 ± 2.32 (7/10)</td>
<td>++++++</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Days 8-12</td>
<td>250</td>
<td>93</td>
<td>-6.9</td>
<td>17.2</td>
<td>2.47 ± 1.75 (3/10)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Days 8-12</td>
<td>5</td>
<td>9</td>
<td>+0.4</td>
<td>6.4</td>
<td>0.92 ± 0.20 (0/10)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Days 8-12</td>
<td>50</td>
<td>34</td>
<td>-1.4</td>
<td>8.1</td>
<td>1.31 ± 0.59 (0/10)</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Days 8-12</td>
<td>250</td>
<td>41</td>
<td>-5.5</td>
<td>10.6</td>
<td>1.52 ± 0.80 (0/10)</td>
<td>++ ++ ++ ++ ++</td>
</tr>
</tbody>
</table>

Table 4
Effect of avarone and avarol on mice with L5178Y cells

All mice were given injections of L5178Y cells and were treated with the compounds from day 8 until day 12 as described in Table 3. The tumor cell number was determined at day 13; the results came from five independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg/day)</th>
<th>Mean tumor size (cells/animal × 10^-5)</th>
<th>% of inhibition</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avarone</td>
<td>1</td>
<td>1.5 ± 0.3</td>
<td>78.4</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.0 ± 0.2</td>
<td>91.5</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.9 ± 0.2</td>
<td>92.4</td>
<td>0/10</td>
</tr>
<tr>
<td>Avarol</td>
<td>1</td>
<td>1.6 ± 0.4</td>
<td>83.9</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.6 ± 0.4</td>
<td>86.4</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.1 ± 0.2</td>
<td>90.7</td>
<td>0/10</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>11.8 ± 2.2</td>
<td>0</td>
<td>4/10</td>
</tr>
</tbody>
</table>

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The Mediterranean Sea and in the Indian Ocean.

The in vitro experiments with different cell lines revealed that both avarone and avarol inhibit cell proliferation of L5178Y mouse lymphoma cells with high selectivity. The determined ED50 values are 13- to 90-fold lower compared to those obtained from studies with tumor cell lines (human melanoma cells and HeLa cells) or normal cells (human fibroblasts and human gingival cells). The conclusion from these data, that avarone and avarol are cell type-specific cytostatic agents, was supported by additional findings. The concentration range within which the cytostatic potentials of avarone and avarol are comparably high (ED50, 0.62 and 0.93 μM, respectively). Under otherwise identical conditions the therapeutically used cytostatics bleomycin (13) and 9-β-D-arabinofuranosyladenine (28) reduce cell proliferation by 50% at 1 and 2.9 μM. In all the cell systems studied, avarone was determined to be significantly more active than avarol. In view of these findings and in view of earlier observations (9), demonstrating that avarol is converted at physiological pH into its corresponding oxidized product, more careful investigations will be needed in the future. At present we have no indication that differences in the medium pH account for the differences in drug effects on the various cell lines.

In the preceding study (5) we reported that avarol diacetate has the same cytostatic activity as avarol, suggesting that avarol diacetate has to be enzymatically hydrolyzed before it can interfere with the cell metabolism. We have extended the structural activity relationships and found that a replacement of the hydroquinone ring by a quinone (avarone) unit increased the cytostatic activity of the sesquiterpenoid avarol. A substitution of a hydrogen atom in the quinone ring by a methylamino group significantly diminished the activity. However, hydrogenation of the only double bond in the terpenoid skeleton had no drastic consequence on the biological activity, suggesting that this site is a promising target for further modification, with the aim to increase the water solubility of avarone-avarol.

Of importance for further developments of avarone-avarol as a potential cancer chemotherapeutic agent was the finding that the two compounds exhibit a potent antileukemic activity also in vivo. The reported studies with L5178Y lymphoma cells in mice revealed that both avarone and avarol caused tumor cell kill values which are ranked according to the published arbitrary activity rating scale (19), into the class of markedly active to highly active anticancer agents. The percentage of ILS values achieved by the two compounds are higher than 25% (20), also indicating a marked anticancer activity. From the experiments reported in this study it is clear that avarone has a higher cytostatic and even a more curative activity than does avarol. We have chosen two schedules for the treatment (days 1 to 5 and days 8 to 12), and found that activity of the compounds was higher when the compounds were applied (for 5 days) 1 day after inoculation of the tumor. Studies are under way to clarify
whether an improvement of the schedule is possible.

The acute and subacute toxicities of the two compounds, as well as the 90% inhibitory doses on L5178Y cell growth in mice have been determined. From these data the therapeutic indexes were calculated as follows: avaron, 11.7; and avarol, 4.5. These values are in the range of those determined from cyclophosphamide, daunomycin, and methotrexate (20).

The physiological cause for the potent antileukemic activity of avaron and avarol in vivo is not yet known. At present we are investigating whether differences in the oxidation-reduction potential or pH values between tumor and normal tissue (29) contribute to the tumor-specific effect of the two compounds described. The latter approach seems to be promising in view of recent findings, showing that the biological effects of avaron and avarol are higher at pH 6 than at pH 7 or 8.6 (9), and also that the pH values within the ascites of tumor-bearing mice are lower compared to that of tumor-free animals.4

The potential pharmacological value of avaron and avarol hold further promise, in that these agents possess neither mutagenic nor premutagenic potentials nor induction capacity for benzo(a)pyrene monooxygenase.

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