Effect of Simultaneous Administration of Avemar® and Cytostatic Drugs on Viability of Cell Cultures, Growth of Experimental Tumors, and Survival of Tumor-Bearing Mice

Béla Szende, Zoltán Marcsek, Zsuzsanna Kocsis, and Anna Tompa
National Institute of Chemical Safety, “József Fodor” National Center for Public Health, Budapest, Hungary

ABSTRACT
Avemar® (Biromedicina Co., Budapest, Hungary), a wheat germ preparation with immunomodulant and antimetastatic activity, was applied simultaneously with cytostatic drugs of different modes of action, in vitro and in vivo, in order to find out whether this simultaneous administration exerts an antagonistic or a synergistic effect on the viability of cell cultures, tumor growth, and survival of animals, inoculated with a transplantable mouse tumor (3LL-HH). In vitro, Avemar did not influence the effect on the viability of MCF-7, HepG2, or Vero cells, exerted by Dacarbazine, 5-fluorouracyl, or Adriblastina. In vivo, Avemar, combined with Endoxan, Navelbine, and doxorubicin, did not prevent the tumor growth inhibitory effect of the cytostatic drugs. The combination of Avemar with the cytostatic drugs did not increase the toxicity of the cytostatic compounds, and did not exert any toxic effect. Avemar may be administered together with cytostatic drugs, without the risk of increasing toxicity or decreasing antiproliferative activity.

Key words: Avemar®, Navelbine, 5-fluorouracil, Endoxan, Adriblastina, Dacarbazine

INTRODUCTION
An orally applicable fermentation product of wheat germ containing 0.4% of substituted benzozquinone (MSC, Avemar®) has been invented by Hungarian chemists under the trade name of Avemar.1,2 Oral administration (3 g/kg body weight) of MSC enhances the blastic transformation of splenic lymphocytes in mice. The same treatment shortens the survival time of skin grafts in a co-isogenic mouse skin transplantation model, pointing to the immune-reconstructive effect of MSC.2 A highly significant antimetastatic effect of MSC has been observed in three metastasis models (3LL-HH, B16, HRC25).3

The simultaneous administration of MSC and 5-fluorouracil (5FU), as well as dacarbazine (DTIC), resulted in a significant, more than additive, increase of antimetastatic activity of MSC, 5FU, or DTIC. Although the therapeutic effects of both of the combinations were considerable, the usual side-effects of cytostatics, e.g., decrease of body mass, were not observed.1,4

The antimetastatic effect of MSC—besides the immune-reconstruction—may also be the result of its cell-adhesion inhibitory, cell-proliferation inhibitory, apoptosis-enhancing, and antioxidant characteristics, also observed in in vitro experiments.2
The aim of our study was to investigate whether the simultaneous administration of Avemar and various cytostatic drugs exerts an antagonistic or a synergistic effect on the viability of cell cultures as well as on tumor growth and the survival of animals, inoculated with a transplantable mouse tumor.

Recently, the antimetastatic effect of Avemar has been confirmed on human colon cancer.6,7

MATERIALS AND METHODS

Chemicals

MTT reagent was purchased from Sigma (St. Louis, MO), fetal calf serum from GIBCO (Grand Island, NY), streptomycin (Sigma, St. Louis, MO). Cells growing as a monolayer were kept in an isolated 37°C, 5% CO2 tissue incubator compartment.

Treatment

Cytotoxicity testing of Avemar (at 24 hours) was performed in the concentration range of 156 μg/mL and 5 mg/mL using two estrogen receptor (ER) positive (MCF-7 and HepG2) and an ER negative (Vero) cell lines. The noncytotoxic concentration (500 μg/mL) of Avemar was used in the experiments.

Avemar and the cytostatics were administered to MCF-7 cell cultures 24 hours after plating. Control cultures were maintained in DMEM similar to the treated cultures. Four samples of cells were cultured and treated in a volume of 100 μl in 96-well tissue culture plates for a further 24 hours for the MTT assay. 5 FU was applied in a dose range between 5 and 2000 μg/mL; DTIC (Dacarbazine) between 5 and 1200 μg/mL; Adriablastina between 1 and 400 μg/mL.

MTT Assay

Cytotoxic effects on the viability of cells were determined using tetrazolium dye (MTT; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as described by Horiuchi et al. 3 × 10⁴ cells/well were plated in 96-microwell plates. An MTT solution was prepared at 5 mg/mL in PBS, filter sterilized and stored in the dark at 4°C for a maximum of 1 month. 20 μl of MTT reagent was added to each 100 μL of culture. After incubation for 3 hours at 37°C, the formazan crystals were dissolved by the addition of 100 μl of propanol to the culture wells. The plates were further incubated for 20 minutes at room temperature, and optical density (O.D.) of the wells was determined using an Anthos 2020 (Salzburg, Austria) ELISA microplate reader at a test wavelength of 570 nM and a reference wavelength of 690 nM. Each plate contained “blank” background control wells holding an appropriate volume of media, but no cells. All experiments were performed three times, with 4 wells for each concentration of the tested agents. The control cells were grown under the same conditions, without the addition of the test compounds. Cell survival (% of control) was calculated relative to untreated control cells.

In Vivo Studies

Experimental Animals

In all experiments, C57BL inbred mice were used. The animals were 8–10 weeks old and weighed 20–22g. They were kept in plastic cages (5 animals per cage) and were fed with rodent pellet (Charles River Hungary Ltd., Gödöllő, Hungary) and tap water ad libitum.

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water *ad libitum*. The room temperature was 20°C–22°C, with a relative humidity of 55% ± 5%.

**Tumor Model**

The transplantable, highly metastatic variant of Lewis lung carcinoma (3LL-HH) tumor line grown on mice was used in the experiments. 3LL-HH cells were inoculated intramuscularly into the muscles of the left hind leg of the mice. The inoculated tumor cell number was $2 \times 10^5$ per animal.

**Treatment**

Avemar, and also cytostatic treatment, was started 24 hours after tumor implantation. Avemar was dissolved in water and administered by means of a gastric tube. The daily dose was 3g/kg body weight per os administered in 0.1 mL of water. Control animals received tap water daily (0.1 mL), also by means of a gastric tube.

The dose of Endoxan was 250 mg/kg body weight, intraperitoneally, as a single injection, 24 hours after tumor inoculation.

The dose of doxorubicin was 0.2 mg per mouse, as a single injection, 24 hours after tumor inoculation.

The dose of Navelbine was 4–6–16 mg/kg body weight, respectively, as intraperitoneal injections, 24 hours and 8 days after tumor inoculation.

The following groups were formed:

1. Solvent-treated control (inoculated with 3LL-HH tumor)—10 animals for each experiment, except in the case of doxorubicin, where 5 animals were used.
2. Endoxan treatment—10 animals
3. Endoxan treatment—10 animals
4. Adriblastina treatment—5 animals
5. Adriblastina + Avemar treatment—5 animals
6. Navelbine treatment—10 animals
7. Navelbine + Avemar treatment—10 animals

A group of 10 mice was established in each experiment, treated only with Avemar.

Overall clinical status of the experimental mice, tumor growth, and spontaneous death of the tumor-bearing animals was followed. The usual tumorous cachexia and the toxic signs caused by cytostatics (such as anemia, spotted hemorrhages of the mucous membranes in a few cases) were observed in the case of single treatment and also in the case of combination therapy. No dif-

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**Figure 1.** The effect of 5FU and Avemar® (Biromediana Co., Budapest, Hungary) (500 µg/mL) administration on the viability of cultured cells at 24 hours of treatment (MTT assay). (A) MCF-7 cells, 24 h, (B) HepG2 cells, 24 h, (C) Vero (European Collection of Cell Cultures, Salisbury, UK) cells.
Figure 2. Effect of DTIC (Dacarbazine™); Pliva-Lachema, Brno, Czech Republic on the viability of different cell lines with Avemar® (500 µg/mL) in 24 hours of treatment (MTT assay). (A) MCF-7 cells; (B) HepG2 cells; (C) Vero (European Collection of Cell Cultures, Salisbury, UK) cells.

Figure 3. Effect of Adriablastina™ (Farmitalia, Milan, Italy) and Avemar® (Biomedicina Co., Budapest Hungary) (500 µg/mL) on the viability of different cell lines determined by MTT assay in 24 hours of treatment. (A) MCF-7 cells; (B) HepG2 cells; (C) Vero (European Collection of Cell Cultures, UK) cells.
ference could be observed between these signs shown by the groups which received Endoxan, Adriblastina, or Navelbine treatment or a combination of one of the drugs and Avenmar. The death of the animals was the result of tumor growth in each case. Mice treated with Endoxan and Adriblastina were followed up until the spontaneous death of the animals. Mice treated with Navelbine were sacrificed on day 13 after tumor inoculation. Tumor weight was measured in the case of Navelbine-treated animals.

Statistical Analysis

Statistical analyses were performed with the Student’s paired t test and p values <0.05 were considered to be significant.

RESULTS

In Vitro Studies

DITC, Adriblastina, and 5FU treatment resulted in a dose-dependent decrease in viability of the cell cultures, depending also on the estrogen receptor positivity or negativity of the cell lines. The 500 µg/mL dose of Avenmar, which proved to be noncytotoxic in our previous experiments, did not increase or decrease the viability of any of the cell cultures treated also with DITC, Adriblastina, or 5FU. This effect was not influenced by the dose of the cytostatics. The results are documented in Figs. 1, 2, and 3.

In Vivo Studies

All tumor-bearing control and treated mice of the experimental groups 2, 3, 4, 5 and the Avenmar-treated group died because of the growth and propagation of the tumor. The mice treated with Endoxan and those which received combination therapy (Endoxan and Avenmar) showed significant survival compared to the control group (Table 1). Adriblastina and Adriblastina plus Avenmar treatment did not cause any increase or decrease in survival (Table 2). Avenmar treatment did not influence the effect of Navelbine on the weight of 3LL-HH tumors (Table 3).

Table 1. Effect of Endoxan (1 × 200 mg/kg i.p.) and Avenmar (3 g/kg per os, daily) on the Survival of C57Bl6 Mice Inoculated with 3LL-HH Tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of death after tumor inoculation</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13, 20, 20, 21, 22, 22, 22, 22</td>
<td>20.5 ± 2.62</td>
</tr>
<tr>
<td>Avenmar</td>
<td>18, 18, 18, 19, 20, 22, 22, 22</td>
<td>20.1 ± 1.70</td>
</tr>
<tr>
<td>Endoxan</td>
<td>31, 38, 39, 47, 48, 49, 54, &gt;60, &gt;60</td>
<td>43.2 ± 9.33</td>
</tr>
<tr>
<td>Endoxan + Avenmar</td>
<td>33, 35, 39, 43, 43, 49, 58, &gt;60, &gt;60</td>
<td>42.1 ± 9.91</td>
</tr>
</tbody>
</table>

Table 2. Effect of Adriblastina (1 × 0.2 mg/mouse i.p.) and Avenmar (3 g/kg per os, daily) on the Survival of C57Bl6 Mice Inoculated with 3LL-HH Tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of death after tumor inoculation</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11, 12, 20, 24, 24</td>
<td>18.2 ± 6.34</td>
</tr>
<tr>
<td>Avenmar</td>
<td>12, 18, 18, 20, 21</td>
<td>17.8 ± 3.49</td>
</tr>
<tr>
<td>Doxorubicin*</td>
<td>16, 16, 18, 20, 20</td>
<td>18.0 ± 2.00</td>
</tr>
<tr>
<td>Doxorubicin + Avenmar</td>
<td>16, 16, 18, 20, 21</td>
<td>18.2 ± 2.28</td>
</tr>
</tbody>
</table>

(*Doxorubicin was ineffective in this dose, and against this tumor. Higher dose (2 mg/mouse) proved to be lethal; thus, the dose-spectrum is narrow.)

SD, standard deviation.
DISCUSSION
The immunomodulatory and antimetastatic effect of Avemar has been established earlier. 1,3,4,6,8 Our present studies were aimed to investigate whether the combined administration of Avemar® and cytostatics of various modes of action may or may not increase the toxicity or adversely influence the cell proliferation and tumor-growth inhibitory effect of the cytostatics.

The mode of action of Adriblastina (doxorubicin), an anthracycline family antibiotic from Streptomyces spp., is to intercalate with DNA and partially uncoil the double-stranded helix. The binding of anthracyclines to DNA inhibits DNA polymerase and nucleic acid synthesis, which stabilizes the complex between DNA and the topoisomerase II enzyme, resulting in DNA double-strand breaks.9 In tumor cells, these processes may initiate endonucleolytic DNA fragmentation, known as apoptosis.

SFU is a nucleoside analogue, which inhibits the S phase of the cell cycle as well as the RNA polymerase system. DTIC is an alkylating agent, which inhibits DNA polymerase activation in Jurkat T-cell leukemia tumor cells. 

SFU, an alkylating agent, results observed in the case of the three other cytostatic drugs (i.e., the effect of these compounds on primary tumor growth and death of animals caused by tumor growth was not influenced by simultaneous Avemar treatment). Our results suggest that Avemar, a new immunomodulant with antimetastatic activity, may be administered together with cytostatic drugs, without increasing toxicity or decreasing the antiproliferative effect of the cytostatics.

ACKNOWLEDGMENTS
This work was supported from grant NKFP 1/016/2001. Z.L.M. is a recipient of the grant OTKA T030799. We thank Katalin Molnar for her excellent technical help.

REFERENCES

Table 3. Effect of Navelbine (2 × 4–8–16 mg/kg i.p.) and Avemar (3 g/kg p.o. daily) on the Weight of 3LL-HHTumors on Day 13 after Tumor Inoculation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor weight (average ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.0 ± 0.46</td>
</tr>
<tr>
<td>Avemar</td>
<td>4.1 ± 0.61</td>
</tr>
<tr>
<td>Navelbine 4 mg/kg</td>
<td>3.3 ± 0.39</td>
</tr>
<tr>
<td>Navelbine 8 mg/kg</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Navelbine 16 mg/kg</td>
<td>2.8 ± 0.47</td>
</tr>
<tr>
<td>Navelbine 4 mg/kg + Avemar</td>
<td>3.0 ± 0.46</td>
</tr>
<tr>
<td>Navelbine 8 mg/kg + Avemar</td>
<td>3.1 ± 0.35</td>
</tr>
<tr>
<td>Navelbine 16 mg/kg + Avemar</td>
<td>2.8 ± 0.48</td>
</tr>
</tbody>
</table>

SD, standard deviation.


