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

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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-fluorouracyl, Endoxan, Adriblastina, Dacarbazine

INTRODUCTION

An orally applicable fermentation product of wheat germ containing 0.4% of substituted benzoquinone (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.[3] A highly significant antimetastatic effect of MSC has been observed in three metastasis models (3LL-HH, B16, HRC25).[1]

The simultaneous administration of MSC and 5-fluorouracyl (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[5]—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]

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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, MS), fetal calf serum from GIBCO (Invitrogen Life Technologies, Paisley, Scotland), 5-fluorouracyl from TEVA Pharmaceutical Industries Ltd., Netanya, Israel, Adriblastina® (doxorubicin) from Farmitalia, Milan, Italy, Endoxan® (cyclophosphamide) from Asta Medica Ltd., Budapest, Hungary, Dacarbazine (DTIC) from Pliva-Lachema, Beno, Czech Republic, Navelbine® (vinorelbine) from Pierre Fabre Medicament, Boulogne, France. Avemar is a wheat germ extract dispersed in maltodextrine, fructose, and siliciumdioxide to prevent adherence. Avemar was provided by Biromedicina Co. (Budapest, Hungary).

A 40 mg/mL stock solution of Avemar was prepared in Dulbecco's modified Eagle's medium (DMEM), filter sterilized, and serial dilutions were made.

Cell cultures

MCF-7 (ECACC 86012803) and Vero (ECACC 84113001) cell cultures were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK), HepG2 (ATCC HB-8065) from ATCC (American Type Culture Collection, Manassas, VA), and cultured in DMEM without phenol red (GIBCO, Meckenheim, Germany), using plastic culture dishes and microwell plates (Nunc A/S, Roskilde, Denmark). DMEM medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics: 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MS). Cells growing as a monolayer were kept in an isolated 37°C, 5% CO_2 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; Adriblastina 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. 3×10^3 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., Gödöllö, Hungary) and tap

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 2×10^5 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-

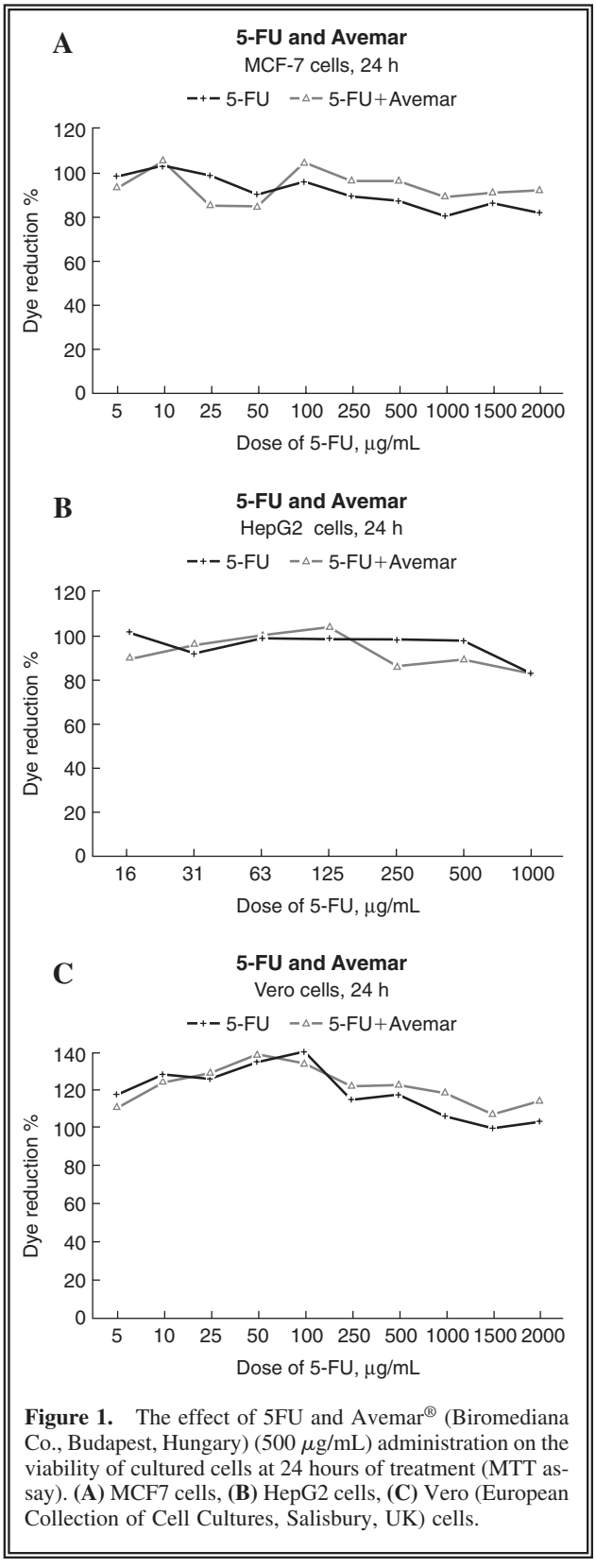

Figure 1. The effect of 5FU and Avemar® (Biromediana Co., Budapest, Hungary) (500 μg/mL) administration on the viability of cultured cells at 24 hours of treatment (MTT assay). **(A)** MCF7 cells, **(B)** HepG2 cells, **(C)** Vero (European Collection of Cell Cultures, Salisbury, UK) cells.

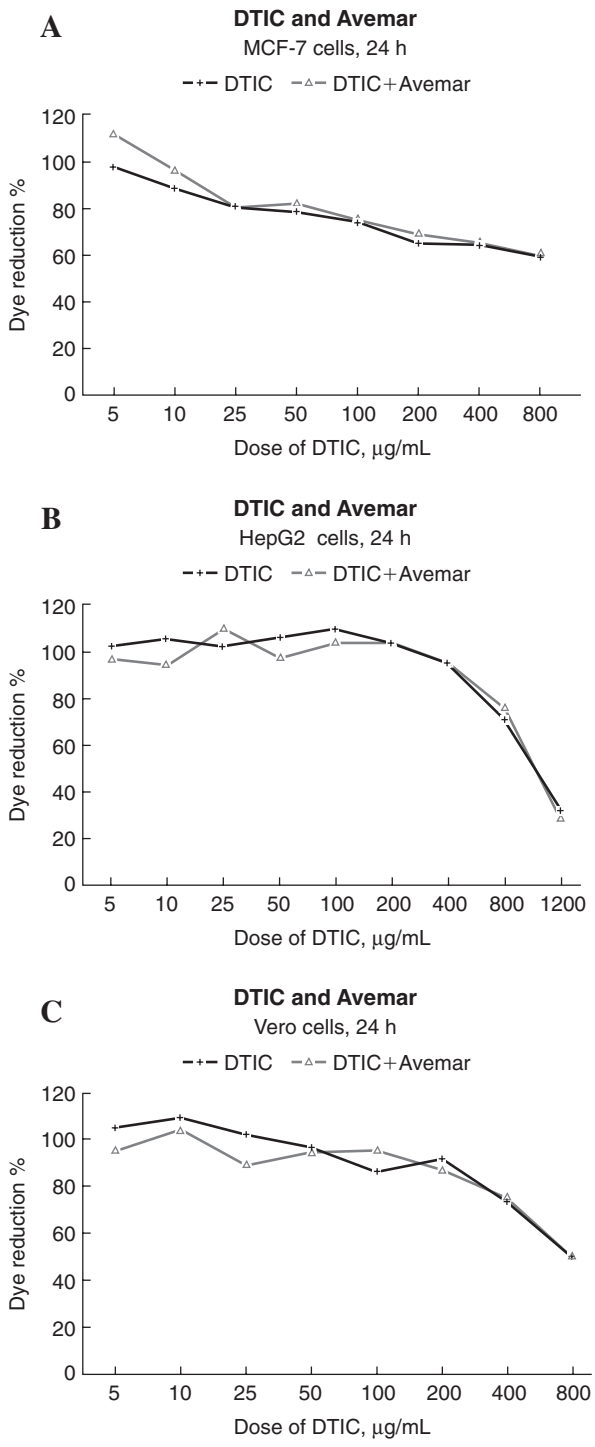

Figure 2. Effect of DTIC (Dacarbazine™); Pliva-Lachema, Brno, Czech Republic on the viability of different cell lines with Avemar® (500 μg/mL) in 24 hours of treatment (MTT assay). **(A)** MCF-7 cells, **(B)** HepG2 cells, **(C)** Vero (European Collection of Cell Cultures, Salisbury, UK) cells.

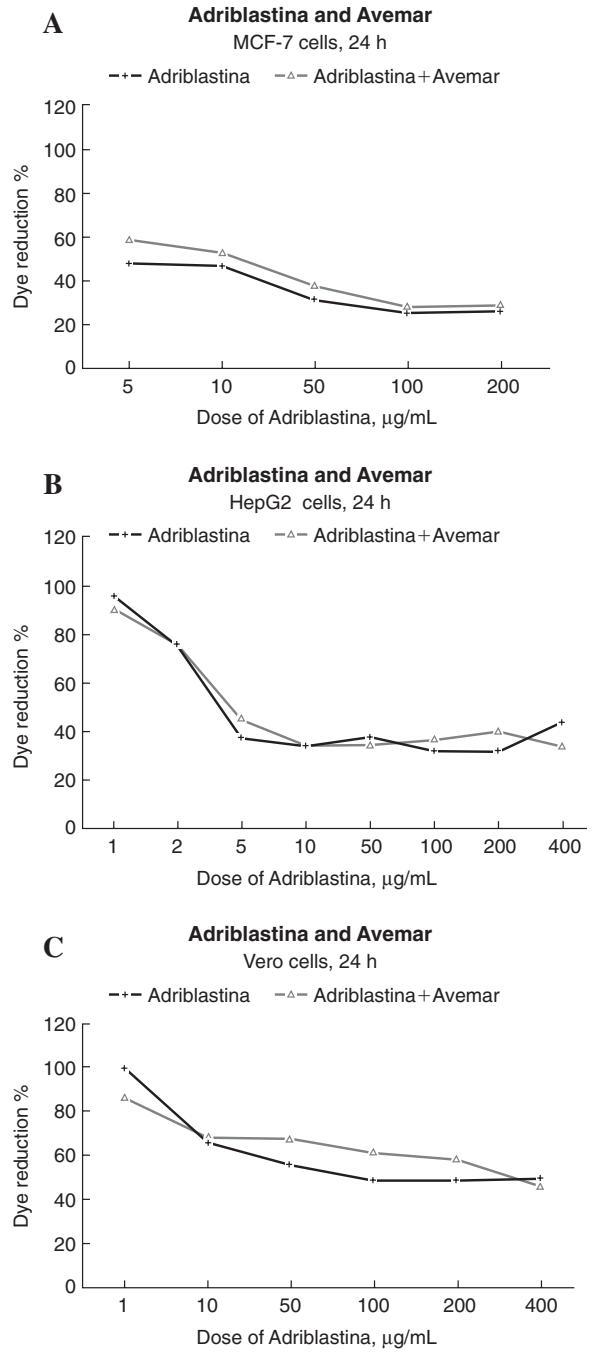

Figure 3. Effect of Adriblastina™ (Farmitalia, Milan, Italy) and Avemar® (Biomedicina Co., Budapest Hungary) (500 μg/mL) on the viability of different cell lines determined by MTT assay in 24 hours of treatment. **(A)** MCF-7 cells; **(B)** HepG2 cells; **(C)** Vero (European Collection of Cell Cultures, UK) cells.

Table 1. Effect of Endoxan (1 × 200 mg/kg i.p.) and Avemar (3 g/kg per os, daily) on the Survival of $C_{57}Bl_6$ Mice Inoculated with 3LL-HH Tumor

Treatment	*Day of death after tumor inoculation*	*Average ± SD*
Control	13, 20, 20, 21, 21, 22, 22, 22, 22, 22	20,5 ± 2.62
Avemar	18, 18, 18, 19, 20, 20, 22, 22, 22, 22	20,1 ± 1.70
Endoxan	31, 38, 38, 39, 47, 48, 49, 54, >60, >60	43,2 ± 9.33
Endoxan + Avemar	33, 35, 37, 39, 43, 43, 49, 58, >60, >60	42,1 ± 9.91

SD, standard deviation.

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 Avemar. 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 Avemar, 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 Avemar-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 Avemar) showed significant survival compared to the control group (Table 1). Adriblastina and Adriblastina plus Avemar treatment did not cause any increase or decrease in survival (Table 2). Avemar treatment did not influence the effect of Navelbine on the weight of 3LL-HH tumors (Table 3).

Table 2. Effect of Adriblastina (1 × 0, 2 mg/mouse i.p.) and Avemar (3 g/kg per os, daily) on the Survival of C57BI6 Mice Inoculated with 3LL-HH Tumor

Treatment	*Day of death after tumor inoculation*	*Average ± SD*
Control	11, 12, 20, 24, 24	18,2 ± 6.34
Avemar	12, 18, 18, 20, 21	17,8 ± 3.49
Doxorubicin*	16, 16, 18, 20, 20,	18.0 ± 2.00
Doxorubicin + Avemar	16, 16, 18, 20, 21	18,2 ± 2.28

(*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.

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-HH Tumors on Day 13 after Tumor Inoculation

Treatment	*Tumor weight (average ± SD)*
Control	4,0 ± 0,46
Avemar	4,1 ± 0,61
Navelbin 4 mg/kg	3,3 ± 0,39
Navelbine 8 mg/kg	3,4 ± 0,5
Navelbine 16 mg/kg	2,8 ± 0,47
Navelbine 4 mg/kg + Avemar	3,9 ± 0,46
Navelbine 8 mg/kg + Avemar	3,1 ± 0,35
Navelbine 16 mg/kg + Avemar	2,8 ± 0,48

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.

5FU is a nucleoside analogue, which inhibits the S phase of the cell cycle as well as the RNA polymerase system. DTIC is an alkylating antitumor drug (5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide) producing O^6-methylguanine, an important DNA adduct that, if not repaired, can lead to mutations and cell death.[10] Endoxan (cyclophosphamide) is a classical alkylating agent that results in DNA double-strand breaks.[11] Navelbine (vinorelbine) is a new member of mitotic spindle poisons.[12]

The viability of various ER positive and ER negative cell lines was not increased or decreased when Avemar was added simultaneously to cytostatic administration. Because, according to our *in vitro* experiments, the effect of the combinations was independent of the type of cell lines, only one tumor line was used in the *in vitro* studies. In addition to the three prototypes of cytostatic drugs (5FU, DTIC, doxorubicin) applied *in vitro*, in the *in vivo* experiments the classical alkylating agent Endoxan and Navelbine, a new member of agents causing mitotic arrest, were also involved. Simultaneous administration of Avemar with these two compounds concluded with the same result 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.

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