



## Avemar, a nontoxic fermented wheat germ extract, induces apoptosis and inhibits ribonucleotide reductase in human HL-60 promyelocytic leukemia cells

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### Abstract

Avemar (MSC) is a nontoxic fermented wheat germ extract demonstrated to significantly improve the survival rate in patients suffering from various malignancies. We investigated its effects in human HL-60 promyelocytic leukemia cells. After 24, 48, and 72 h of incubation, Avemar inhibited the growth of HL-60 cells with IC<sub>50</sub> values of 400, 190, and 160 µg/ml, respectively. Incubation with MSC caused dose-dependent induction of apoptosis in up to 85% of tumor cells. In addition, Avemar attenuated the progression from G<sub>2</sub>-M to G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle and was also found to significantly reduce the in situ activity of ribonucleotide reductase, the key enzyme of de novo DNA synthesis. We conclude that Avemar exerts a number of beneficial effects which could support conventional chemotherapy of human malignancies. © 2006 Elsevier Ireland Ltd. All rights reserved.

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### 1. Introduction

Avemar (MSC), a fermented wheat germ extract standardized to methoxy-substituted benzoquinones,

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possesses cancer-fighting characteristics. Taken orally, MSC can inhibit metastatic tumor dissemination and proliferation [1]. Avemar is capable of synergistically enhancing the effect of 5-fluorouracil (5-FU) and dacarbazine (DTIC) under experimental conditions when applied in combination with these agents [2]. Moreover, MSC has a marked inhibitory effect on metastasis formation in tumor-bearing

animals [3–5], resulting in a decreased survival time of skin grafts and reduced cell proliferation while enhancing apoptosis. Oral co-administration of Avemar inhibits tumor metastasis formation after chemotherapy and surgery in advanced colorectal cancers [6,7].

MSC was demonstrated to induce apoptosis in pancreatic carcinoma cells, T and B lymphocytic tumor cell lines, leukemia, melanoma, breast cancer and gastric carcinoma cells in vitro [2,8–13]. In lymphoid tumor cells, apoptosis was selectively induced via tyrosine phosphorylation and  $\text{Ca}^{2+}$  influx [10]. Avemar was also shown to have a selective inhibitory effect on glycolysis and pentose-cycle enzymes, and to cause down-regulation of major histocompatibility complex class I proteins in tumor cells [8–10,14]. Incubation of Jurkat leukemia cells with MSC led to an  $\text{IC}_{50}$  value of 200  $\mu\text{g}/\text{ml}$  which was preceded by the appearance of a sub-G1 peak on flow histograms. Laser scanning cytometry of propidium iodide- and annexin V-stained cells indicated that the growth-inhibiting effect of Avemar was consistent with a strong induction of apoptosis.

Recently, we investigated the effects of MSC in human HT29 colon cancer cells. Avemar induced both apoptosis and necrosis, attenuated the cell cycle progression, and significantly inhibited the activity of ribonucleotide reductase by depleting dCTP, dTTP, and dATP pools [15].

These results prompted us to investigate the biochemical effects involved in the antitumor activity of Avemar in human HL-60 promyelocytic leukemia cells. We determined its effects on cell growth and investigated the induction of apoptosis and necrosis employing a specific double staining method developed by our group. The effect of Avemar on the cell cycle phase distribution of HL-60 cells was evaluated by FACS. In addition, we examined whether MSC is an inhibitor of ribonucleotide reductase (RR; EC 1.17.4.1), a key enzyme of cancer chemotherapy, which is highly upregulated in rapidly proliferating malignant cells.

## 2. Materials and methods

### 2.1. Chemicals and supplies

Avemar was a gift from Fresenius-Kabi Inc., (Graz, Austria). All other chemicals and reagents used were commercially available (Sigma–Aldrich, Vienna, Austria) and of highest purity.

### 2.2. Cell culture

The human HL-60 promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin–streptomycin in a humidified atmosphere containing 5%  $\text{CO}_2$ . All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). Cell counts were determined using a microcellcounter CC-108 (Sysmex, Kobe, Japan). Cells growing in the logarithmic phase of growth were used for all studies described below.

### 2.3. Growth inhibition assay

HL-60 cells ( $0.1 \times 10^6$  per ml) were seeded in 25  $\text{cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37 °C under cell culture conditions. Cell counts and  $\text{IC}_{50}$  values were determined after 24, 48, and 72 h using the microcellcounter CC-108. Viability of cells was determined by trypan blue staining. Results were calculated as numbers of viable cells.

### 2.4. Hoechst dye 33258 and propidium iodide double staining

The Hoechst staining was performed according to the method described by our group [16]. HL-60 cells ( $0.4 \times 10^6$  per ml) were seeded in 25  $\text{cm}^2$  Nunc tissue culture flasks and exposed to increasing concentrations of Avemar for 48 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added directly to the cells to final concentrations of 5 and 2  $\mu\text{g}/\text{ml}$ , respectively. After 60 min of incubation at 37 °C, cells were examined on a Leica DMR XA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters for Hoechst 33258 and PI. This method allows to distinguish between early apoptosis, late apoptosis, and necrosis. Cells were judged according to their morphology and the integrity of their cell membranes, which can easily be seen after propidium iodide staining. Cells were counted under the microscope and the number of apoptotic cells was given as percentage value.

### 2.5. Cell cycle distribution analysis

HL-60 cells ( $0.4 \times 10^6$  per ml) were seeded in 25  $\text{cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37 °C under cell culture conditions. After 24 h, cells were harvested and suspended in 5 ml cold PBS, centrifuged, resuspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4 °C. After two washing steps in cold PBS, RNase A and propidium iodide were added to a final concentration of 50  $\mu\text{g}/\text{ml}$  each and incubated at 4 °C for 60 min before measurement. Cells were

analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

### 2.6. Determination of deoxyribonucleoside triphosphates (dNTPs)

Logarithmically growing HL-60 cells were incubated with 300, 600, and 1200  $\mu\text{g/ml}$  Avemar for 24 h. Afterwards,  $5 \times 10^7$  cells were separated for the extraction of dNTPs according to a method established earlier [17]. The extracted dNTPs were measured using a Merck “La Chrom” HPLC system equipped with L-7200 autosampler, L-7100 pump, L-7400 UV detector, and D-7000 interface. Samples were eluted with a 3.2 M ammonium phosphate buffer, pH 3.4 (pH adjusted by addition of 3.2 mol/l  $\text{H}_3\text{PO}_4$ ), containing 20 mM acetonitrile using a  $4.6 \times 250$  mm Partisil 10 SAX analytical column (Whatman, Kent, UK). Separation was performed at constant ambient temperature with a flow rate of 2 ml/min. The concentration of dNTPs was calculated as percent of total area under the curve for each sample. Intracellular concentrations of dNTPs in untreated control cells were 1.95, 7.62, 6.65, and 1.32  $\mu\text{M}$  for dCTP, dTTP, dATP, and dGTP, respectively.

### 2.7. Incorporation of $^{14}\text{C}$ -labelled cytidine into DNA

To analyze the effect of Avemar incubation on the activity of DNA synthesis, an assay was performed as described previously [18]. Logarithmically growing HL-60 cells ( $0.3 \times 10^6$  cells per ml) were incubated with various concentrations of Avemar for 24 h. After the incubation period, cells were counted and pulsed with  $^{14}\text{C}$ -cytidine (0.3125  $\mu\text{Ci}$ , 5 nM) for 30 min at 37 °C. Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from  $5 \times 10^6$  cells and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (Perkin-Elmer, Boston, MA).

### 2.8. Statistical calculations

Dose–response curves were calculated using the Prism 4.03 software package (GraphPad, San Diego, CA, USA) and statistical significance was determined by unpaired *t*-test.

## 3. Results

### 3.1. Effect of Avemar on the growth of tumor cells

Logarithmically growing HL-60 cells were seeded at a concentration of  $0.1 \times 10^6$  cells per ml and then incubated with increasing concentrations of Avemar. After 24, 48, and 72 h of incubation, Avemar inhibited the growth of HL-60 cells with  $\text{IC}_{50}$  values of 400, 190, and 160  $\mu\text{g/ml}$ , respectively. Results are depicted in Fig. 1.

### 3.2. Induction of apoptosis after treatment with Avemar

Logarithmically growing HL-60 cells were incubated with 150, 300, and 600  $\mu\text{g/ml}$  Avemar for 48 h. Then cells were double stained with Hoechst 33258 and propidium iodide. After the incubation period, cells were judged according to their morphology and the integrity of their cell membranes. We could observe nuclear condensation and fragmentation (early apoptosis) as well as signs of late apoptosis with membrane damage and incorporation of propidium iodide. Treatment with Avemar led to a dose-dependent increase of apoptotic cells. After treatment with 600  $\mu\text{g/ml}$  of the drug, up to 85% of HL-60 cells showed hallmarks of apoptosis. Results are shown in Fig. 2.

### 3.3. Cell cycle distribution after treatment with Avemar

HL-60 cells were prepared as described in the methods section and incubated with increasing concentrations of Avemar for 24 h. Growth arrest after exposure to 600  $\mu\text{g/ml}$  Avemar occurred mainly in the G2–M phase, increasing the cell population from 10% to 20% while depleting cells in the G0–G1 phase from 44% to 31%. Results are summarized in Fig. 3.

### 3.4. Effect of Avemar on deoxyribonucleoside triphosphates (dNTPs)

HL-60 cells were incubated with 300, 600, and 1200  $\mu\text{g/ml}$  Avemar for 24 h. Then dNTP pool sizes were determined employing the HPLC method described earlier. Avemar treatment caused a remarkable imbalance of dNTPs in HL-60 cells. Treatment with 600  $\mu\text{g/ml}$

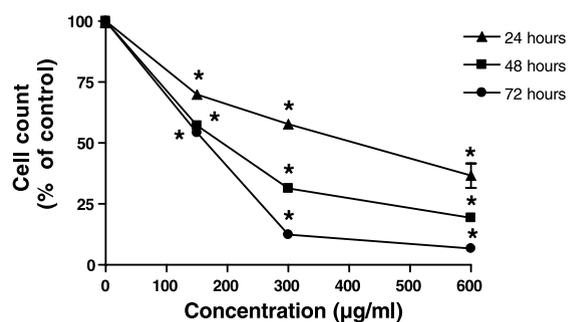


Fig. 1. Growth inhibition of human HL-60 promyelocytic leukemia cells after incubation with Avemar for 24, 48, and 72 h. HL-60 cells ( $0.1 \times 10^6$  per ml) were seeded in 25  $\text{cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37 °C under cell culture conditions. Cell counts and  $\text{IC}_{50}$  values were determined using the microcellcounter CC-108. Viability of cells was determined by trypan blue staining. Results were calculated as number of viable cells. Data are means  $\pm$  standard errors of three determinations out of one representative experiment. Values significantly ( $p < 0.05$ ) different from control are marked with an asterisk (\*).

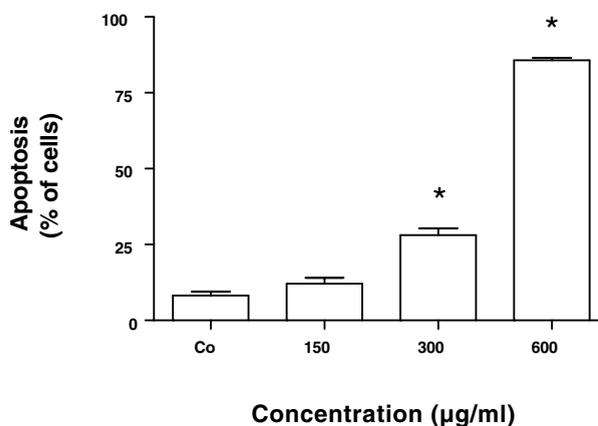


Fig. 2. Induction of apoptosis in human HL-60 promyelocytic leukemia cells after incubation with Avemar. HL-60 cells ( $0.4 \times 10^6$  per ml) were seeded in  $25 \text{ cm}^2$  Nunc tissue culture flasks and exposed to increasing concentrations of Avemar for 48 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added directly to the cells to final concentrations of  $5 \mu\text{g/ml}$  and  $2 \mu\text{g/ml}$ , respectively. After 60 min of incubation at  $37^\circ\text{C}$ , cells were counted under a fluorescence microscope and the number of apoptotic cells was given as percentage value. Data are means  $\pm$  standard errors of three determinations out of one representative experiment. Values significantly ( $p < 0.05$ ) different from control are marked with an asterisk (\*).

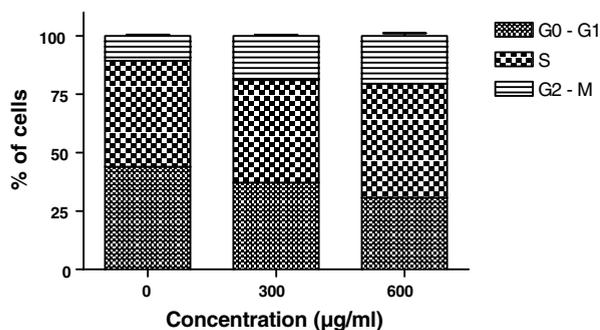


Fig. 3. Cell cycle distribution in human HL-60 promyelocytic leukemia cells after incubation with Avemar for 24 h. HL-60 cells ( $0.4 \times 10^6$  per ml) were seeded in  $25 \text{ cm}^2$  Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at  $37^\circ\text{C}$  under cell culture conditions. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA). Data are means  $\pm$  standard errors of three determinations out of one representative experiment.

Avemar significantly decreased dCTP, dTTP, and dGTP pools to 31%, 27%, and 0% of control values, respectively. In contrast,  $300 \mu\text{g/ml}$  Avemar led to an increase of dATP to 126% of control values, while diminishing dCTP, dTTP, and dGTP pools. After incubation with  $1200 \mu\text{g/ml}$

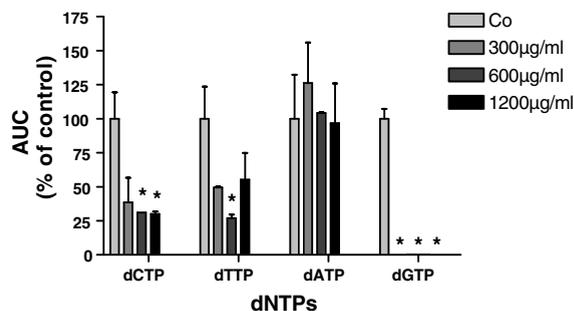


Fig. 4. Concentration of dNTP pools in human HL-60 promyelocytic leukemia cells after treatment with Avemar for 24 h. Logarithmically growing HL-60 cells were incubated with 300, 600, and  $1200 \mu\text{g/ml}$  Avemar for 24 h. Afterwards,  $5 \times 10^7$  cells were separated for the extraction of dNTPs. The concentration of dNTPs was calculated as percent of total area under the curve for each sample. Intracellular concentrations of dNTPs in untreated control cells were  $1.95$ ,  $7.62$ ,  $6.65$ , and  $1.32 \mu\text{M}$  for dCTP, dTTP, dATP, and dGTP, respectively. Data are means  $\pm$  standard errors of two determinations out of one representative experiment. Values significantly ( $p < 0.05$ ) different from control are marked with an asterisk (\*).

$1200 \mu\text{g/ml}$  Avemar for 24 h, all four dNTPs were decreased when compared with untreated controls. Results are shown in Fig. 4.

### 3.5. Inhibition of incorporation of $^{14}\text{C}$ -cytidine into DNA

Incorporation of  $^{14}\text{C}$ -cytidine into the DNA of HL-60 cells was determined after incubation with Avemar. After treatment of HL-60 cells with 150, 300, and  $1200 \mu\text{g/ml}$  Avemar for 24 h,  $^{14}\text{C}$ -cytidine incorporation into DNA was significantly decreased to 75%, 49%, and 47% of control values, respectively. Results are depicted in Fig. 5.

## 4. Discussion

Avemar is the first fermented and concentrated wheat germ extract given as a nutritional supplement for cancer patients. MSC induced apoptosis in lymphoid tumor cells but not in healthy resting mononuclear cells [10], and showed no disadvantageous interactions with cytostatic drugs being widely used in clinical practice [19,20]. Recently, it has also been revealed that Avemar reduced the incidence of febrile neutropenia in pediatric cancer patients [21].

Using a recently described staining method, flow cytometry, and direct enzyme activity measurement, we were able to study a number of cellular response mechanisms such as cell cycle progression, apoptosis, necrosis, cell growth inhibition, depletion of

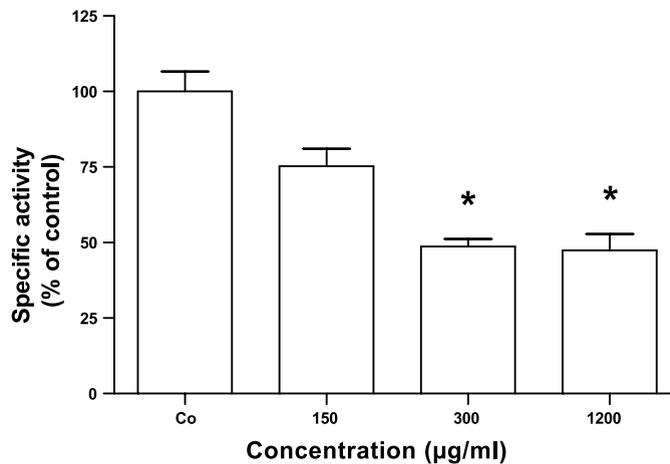


Fig. 5. In situ measurement of ribonucleotide reductase (RR) activity in human HL-60 promyelocytic leukemia cells after incubation with Avemar. Logarithmically growing HL-60 cells ( $0.3 \times 10^6$  cells per ml) were treated with various concentrations of Avemar for 24 h. After the incubation period, cells were counted and pulsed with  $^{14}\text{C}$ -cytidine ( $0.3125 \mu\text{Ci}$ ,  $5 \text{ nM}$ ) for 30 min at  $37^\circ\text{C}$ . Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from  $5 \times 10^6$  cells and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (Perkin-Elmer, Boston, MA). Data are means  $\pm$  standard errors of two determinations out of one representative experiment. Values significantly ( $p < 0.05$ ) different from control are marked with an asterisk (\*).

enzymatic substrates (dNTPs), and direct enzyme attenuation (ribonucleotide reductase; RR).

It has previously been shown that Avemar treatment leads to an increase of apoptosis formation in various tumor cells, such as breast, colon, gastric and pancreatic cancer cells which prompted us to examine the apoptosis-inducing effects of MSC in the human HL-60 promyelocytic leukemia cell line. Morphologic analysis after double staining with Hoechst 33258 and propidium iodide revealed that lower Avemar concentrations mainly resulted in early apoptosis, whereas additional late apoptotic as well as necrotic changes could be observed after incubation with higher doses.

Furthermore, MSC stopped the cell cycle transition of HL-60 cells in the G2–M phase of the cell cycle, resulting in a depletion of G0–G1 phase cells. However, remarkable cell cycle perturbations could only be seen after treatment with  $600 \mu\text{g/ml}$  (corresponding to the 1.5-fold 24 h  $\text{IC}_{50}$ -value yielded in the growth inhibition assay), indicating that the drug does not primarily exert its activity by influencing the cell cycle of HL-60 cells.

Among several other mechanisms made responsible for the effect of Avemar, Comin-Anduix and coworkers speculated that decreased oxidative ribose synthesis might limit the metabolic needs of Jurkat leukemia cells for the conversion of ribonucleotides to deoxyribonucleoside triphosphates (dNTPs) [9], which are the precursors of DNA synthesis. The responsible enzyme, ribonucleotide

reductase (RR) was demonstrated to be significantly up-regulated in tumor cells to meet the increased need for dNTPs of these rapidly proliferating cells [22,23]. The enzyme was therefore considered to be an excellent target for cancer chemotherapy, and various inhibitors of RR have entered clinical practice or are under preclinical or clinical development. To determine whether Avemar is capable of such an inhibition of RR in HL-60 cells, we first employed an in situ enzyme assay. Radiolabeled cytidine has to be reduced by RR to be incorporated into DNA. We were able to demonstrate that the RR in situ activity of HL-60 cells can be inhibited by Avemar in a concentration-dependent manner. However, decrease of RR in situ activity reached a maximum of 49% at a concentration of  $300 \mu\text{g/ml}$  since higher dosage of the drug ( $1200 \mu\text{g/ml}$ ) was not able to intensify the observed effect. This might be the reason for the fact that Avemar demonstrated no signs of additional toxicity in various studies [6,7,9], whereas more potent inhibitors of RR such as gemcitabine exert dose-limiting toxic side effects when applied to humans.

Regarding the incorporation of radiolabeled cytidine into DNA, the obtained results were confirmed by the determination of dNTP pool sizes after incubation of HL-60 cells with the compound. The four dNTPs are being affected differently in different cell lines – such variability is known from the literature for other compounds and cell lines but cannot be explained in detail. Both in situ inhibition of RR

and depletion of dNTP pools could be observed at a concentration of 300 µg/ml, whereas meaningful cell cycle attenuation only occurred after treatment of cells with 600 µg/ml of the drug. Thus, inhibition of RR as caused by Avemar is likely to be one reason for its cytotoxic and apoptotic effects on human HL-60 promyelocytic leukemia cells.

We conclude that Avemar might be a promising additional option for combination treatment of human leukemia. Based on our data, further investigations against hematological malignancies within the framework of animal and clinical studies are warranted.

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