# Avemar, a nontoxic fermented wheat germ extract, attenuates the growth of sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells through induction of apoptosis

PHILIPP SAIKO[1], MARIA OZSVAR-KOZMA[1], GERALDINE GRASER[1], ANDREAS LACKNER[3], MICHAEL GRUSCH[3], SIBYLLE MADLENER[2], GEORG KRUPITZA[2], WALTER JAEGER[4], MATE HIDVEGI[5], RAM P. AGARWAL[6], MONIKA FRITZER-SZEKERES[1] and THOMAS SZEKERES[1]

[1]Clinical Institute of Medical and Chemical Laboratory Diagnostics, [2]Institute of Clinical Pathology, Medical University of Vienna, General Hospital of Vienna, Waehringer Guertel 18-20; [3]Department of Medicine I, Division of Cancer Research, Medical University of Vienna, Borschkegasse 8a; [4]Department of Clinical Pharmacy and Diagnostics, Faculty of Life Sciences, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria; [5]Jewish University, Berkocsis utca 2, H-1084 Budapest, Hungary; [6]Department of Medicine, Division of Hematology/Oncology, University of Miami, 1600 N.W. 10th Avenue, Miami, FL 33136, USA

Received August 21, 2008; Accepted October 31, 2008

DOI: 10.3892/or_00000285

**Abstract.** Avemar (MSC) is a nontoxic fermented wheat germ extract, which has been shown to significantly improve the survival rate in patients suffering from various malignancies. We investigated its effects in sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells. After 48 and 72 h of incubation, Avemar inhibited the growth of sensitive H9 cells with $IC_{50}$ values of 290 and 200 $\mu$g/ml, whereas the growth of 5-FdUrd/Ara-C cross-resistant H9 cells was attenuated with $IC_{50}$ values of 180 and 145 $\mu$g/ml, respectively. Treatment with 300 $\mu$g/ml MSC for 48 h caused dose-dependent induction of apoptosis in 48% of sensitive H9 cells. In cross-resistant H9 cells, incubation with 200 $\mu$g/ml Avemar for 48 h led to 41% of apoptotic tumor cells. Growth arrest of sensitive H9 cells after exposure to various concentrations of MSC occurred mainly in the S phase of the cell cycle, thereby increasing the cell population from 54 to 73% while depleting cells in the G0-G1 phase from 40 to 19%. Growth arrest in cross-resistant H9 cells occurred also mainly in the S phase, increasing the cell population from 45 to 68% while depleting cells in the G0-G1 phase from 45 to 31%. As MSC treatment likely overcomes 5-FdUrd/Ara-C resistance, further investigations to elucidate the exact mechanisms are warranted. We conclude that Avemar exerts a number of beneficial effects which could support conventional chemotherapy of human malignancies.

*Correspondence to:* Professor Thomas Szekeres, Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, General Hospital of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria
E-mail: thomas.szekeres@meduniwien.ac.at

*Key words:* Avemar (MSC), sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells, growth inhibition, apoptosis, cell cycle

## Introduction

Avemar (MSC) is a fermented wheat germ extract standardized to methoxy-substituted benzoquinones. This extract, invented by Mate Hidvegi, has been demonstrated to exert numerous cancer-fighting effects. Taken orally, MSC can inhibit metastatic tumor dissemination and proliferation (1). Avemar has a marked inhibitory effect on metastasis formation in tumor-bearing animals (2-4), which results in a decreased survival time of skin grafts and reduced cell proliferation while increasing apoptosis. Moreover, MSC 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 (5). Oral co-administration of Avemar inhibits tumor metastasis formation and prolongs survival after chemotherapy and surgery in advanced colorectal cancers (6,7) and high-risk melanoma (8).

MSC was demonstrated to induce apoptosis in pancreatic carcinoma cells, T and B lymphocytic tumor cell lines, leukemia and melanoma cells *in vitro* (5,9-11). In lymphoid tumor cells, apoptosis was selectively induced via tyrosine phosphorylation and $Ca^{2+}$ influx (10). Avemar was also shown to have a selective inhibitory effect on glycolysis and pentose-cycle enzymes and to cause the down-regulation of major histocompatibility complex class I proteins in tumor cells (9-11). Incubation of Jurkat leukemia cells with MSC led to an $IC_{50}$ value of 200 $\mu$g/ml which was preceded by the appearance of a sub-$G_1$ peak in 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.

Previously, we investigated the effects of MSC in HT29 human colon cancer cells as well as in HL-60 human promyelocytic leukemia cells. Avemar induced both apoptosis and necrosis and significantly inhibited the activity of ribonucleotide reductase by depleting dCTP, dTTP and dATP pools (12,13). In HL-60 cells, the cell cycle progression was attenuated only at high doses (13).

In order to underpin these results and to determine whether MSC is also able to overcome both 5-FdUrd and Ara-C resistance, we now examined the effects of Avemar in a recently described 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cell line in comparison to sensitive H9 cells. The activity of 5-fluorodeoxyuridine (5-FdUrd) is similar to that of 5-FU and due to extensive hepatic extraction, 5-FdUrd has a marked pharmacological advantage when administered via the hepatic artery (14,15).

We determined the effects of Avemar on the growth of both cell lines and investigated the induction of apoptosis and necrosis employing a specific double staining method developed by our group. The effect of MSC on the cell cycle phase distribution of sensitive and 5-FdUrd/Ara-C cross-resistant H9 cells was evaluated by FACS.

## Materials and methods

*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 the highest purity.

*Cell culture*. Sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells were provided by Ram P. Agarwal, University of Miami, Florida. 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% $CO_2$. 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.

*Growth inhibition assay*. H9 cells ($0.1x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37˚C under cell culture conditions. Cell counts and $IC_{50}$ values were determined after 48 and 72 h using the microcellcounter CC-108. Viability of cells was determined by trypan blue exclusion. Results were calculated as numbers of viable cells.

*Hoechst dye 33258 and propidium iodide double staining*. The Hoechst staining was performed according to the method described by our group (16). H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ 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) were added directly to the cells to final concentrations of 5 and 2 $\mu$g/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.

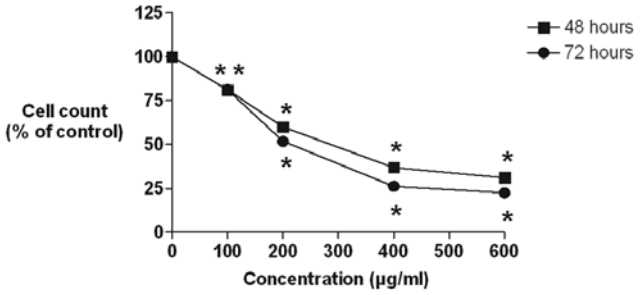


Figure 1. Growth inhibition of sensitive H9 human lymphoma cells after incubation with Avemar for 48 and 72 h. H9 cells ($0.1x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37˚C under cell culture conditions. Cell counts and $IC_{50}$ 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 mean ± standard errors of three determinations out of one representative experiment. Values significantly ($p<0.05$) different from control are marked with an asterisk (*).

*Cell cycle distribution analysis*. H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ 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 $\mu$g/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).

*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.

## Results

*Effect of Avemar on the growth of sensitive H9 cells*. Logarithmically growing cells were seeded at a concentration of $0.1x10^6$ cells per ml. After 48 and 72 h of incubation, Avemar inhibited the growth of sensitive H9 cells with $IC_{50}$ values of 290 and 200 $\mu$g/ml, respectively. Results are depicted in Fig. 1.

*Effect of Avemar on the growth of 5-FdUrd/Ara-C cross-resistant H9 cells*. Logarithmically growing cells were seeded at a concentration of $0.1x10^6$ cells per ml. After 48 and 72 h of incubation, Avemar inhibited the growth of 5-FdUrd/Ara-C

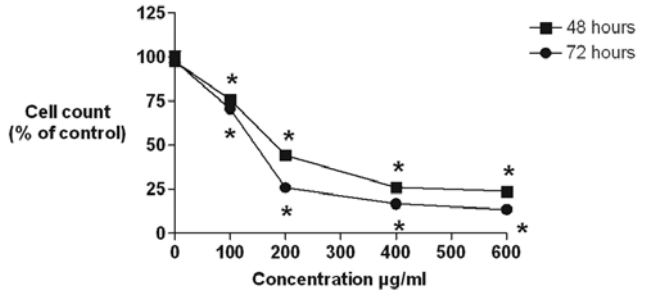


Figure 2. Growth inhibition of 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells after incubation with Avemar for 48 and 72 h. H9 cells ($0.1x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37˚C under cell culture conditions. Cell counts and $IC_{50}$ 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 mean ± standard errors of three determinations out of one representative experiment. Values significantly ($p<0.05$) different from control are marked with an asterisk (*).

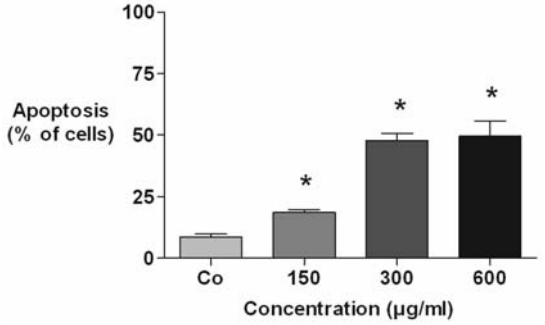


Figure 3. Induction of apoptosis in sensitive H9 human lymphoma cells after incubation with Avemar for 48 h. H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and exposed to increasing concentrations of Avemar for 48 h. Hoechst 33258 (HO, Sigma) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 and 2 $\mu$g/ml, respectively. After 60 min of incubation at 37˚C, cells were counted under a fluorescence microscope and the number of apoptotic cells was given as percentage value. Data are mean ± standard errors of three determinations out of one representative experiment. Values significantly ($p<0.05$) different from control are marked with an asterisk (*).

cross-resistant H9 cells with $IC_{50}$ values of 180 and 145 $\mu$g/ml, respectively. Results are shown in Fig. 2.

*Induction of apoptosis after treatment of sensitive H9 cells with Avemar*. Logarithmically growing cells were incubated with 150, 300 and 600 $\mu$g/ml Avemar for 48 h. Then cells were double stained with Hoechst 33258 and propidium iodide as described in the methods section. 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 with 48% of cells showing hallmarks of apoptosis at 300 $\mu$g/ml. Results are depicted in Fig. 3.

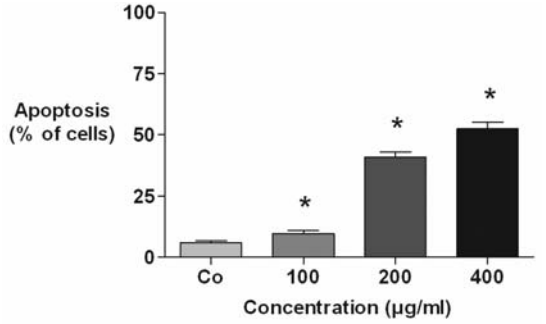


Figure 4. Induction of apoptosis in 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells after incubation with Avemar for 48 h. H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and exposed to increasing concentrations of Avemar for 48 h. Hoechst 33258 (HO, Sigma) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 and 2 $\mu$g/ml, respectively. After 60 min of incubation at 37˚C, cells were counted under a fluorescence microscope and the number of apoptotic cells was given as percentage value. Data are mean ± standard errors of three determinations out of one representative experiment. Values significantly ($p<0.05$) different from control are marked with an asterisk (*).

*Induction of apoptosis after treatment of 5-FdUrd/Ara-C cross-resistant H9 cells with Avemar*. Logarithmically growing cells were incubated with 100, 200 and 400 $\mu$g/ml Avemar for 48 h. Then cells were double stained with Hoechst 33258 and propidium iodide as described in the methods section. 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 with 41% of cells showing hallmarks of apoptosis at 200 $\mu$g/ml. Results are shown in Fig. 4.

*Cell cycle distribution after treatment of sensitive H9 cells with Avemar*. Sensitive H9 cells were prepared as described in the methods section and incubated with increasing concentrations of Avemar for 24 h. Growth arrest after exposure to MSC occurred mainly in the S phase, increasing the cell population from 54 to 73% while depleting cells in the G0-G1 phase from 40 to 19%. Results are summarized in Fig. 5.

*Cell cycle distribution after treatment of 5-FdUrd/Ara-C cross-resistant H9 cells with Avemar*. Cross-resistant H9 cells were prepared as described in the methods section and incubated with increasing concentrations of Avemar for 24 h. Growth arrest after exposure to MSC also occurred mainly in the S phase, increasing the cell population from 45 to 68% while depleting cells in the G0-G1 phase from 45 to 31%. Results are summarized in Fig. 6.

## Discussion

Increased cell proliferation and decreased cell death (by means of apoptosis) are two major processes that contribute to the progression of tumor cell growth. Apoptosis induction has emerged as a significant therapeutic principle for the effective

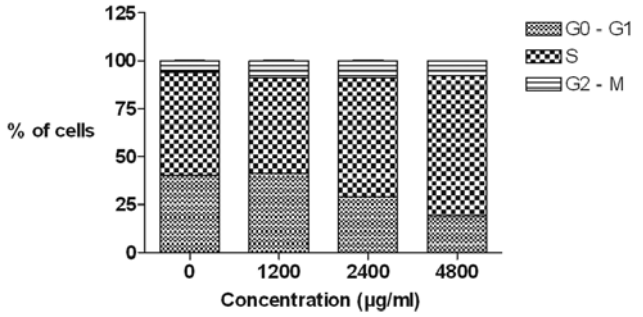


Figure 5. Cell cycle distribution in sensitive H9 human lymphoma cells after incubation with Avemar for 24 h. H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37˚C under cell culture conditions. Cells were analyzed on a FACSCalibur flow cytometer and cell cycle distribution was calculated with ModFit LT software. Data are mean ± standard errors of three determinations out of one representative experiment.

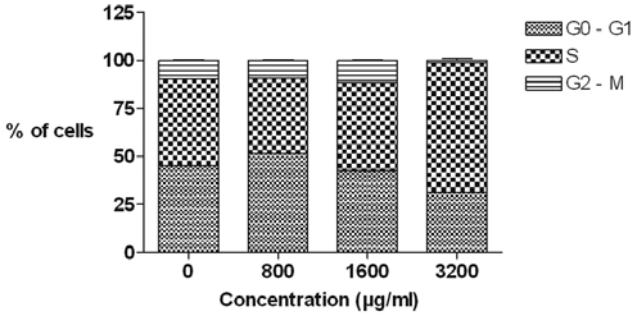


Figure 6. Cell cycle distribution in 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells after incubation with Avemar for 24 h. H9 cells ($0.4x10^6$ per ml) were seeded in 25 $cm^2$ Nunc tissue culture flasks and incubated with increasing concentrations of Avemar at 37˚C under cell culture conditions. Cells were analyzed on a FACSCalibur flow cytometer and cell cycle distribution was calculated with ModFit LT software. Data are mean ± standard errors of three determinations out of one representative experiment.

elimination of cancer cells (17,18). Thus, the intrinsic propensity to undergo apoptosis is a general determinant for chemotherapeutic sensitivity and therefore represents a target for pharmacological modulation (19,20).

In this study, we evaluated the effects of Avemar (MSC), a non-toxic fermented wheat germ extract on sensitive and 5-FdUrd/Ara-C cross-resistant H9 human lymphoma cells.

Avemar is the first fermented and concentrated wheat germ extract given as a nutritional supplement for cancer patients. 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 and showed no disadvantageous interactions with cytostatic drugs being widely used in clinical practice (21). Previously, it has also been revealed that Avemar reduced the incidence of febrile neutropenia in pediatric cancer patients (22).

Avemar induces apoptosis in lymphoid tumor cells but not in healthy resting mononuclear cells (11), which prompted us to examine the apoptosis-inducing effects in sensitive and 5-FdUrd/Ara-C cross-resistant H9 lymphoma cells. The activity of 5-fluorodeoxyuridine (5-FdUrd) is comparable to that of 5-Fluorouracil (5-FU) and due to extensive hepatic extraction, 5-FdUrd has a marked pharmacological advantage when administered via the hepatic artery (14,15). 5-FU is a pyrimidine analogue with the capacity to inhibit the biosynthesis of pyrimidine nucleotides. Following rapid transport into the cell, a significant amount of the drug is converted by ribosylation and phosphorylation reactions into three main metabolites, two of which, fluorouridine triphosphate (5-FUTP) and fluorodeoxyuridine monophosphate (5-FdUMP), are known to be active. 5-FUTP and 5-FdUMP are being further metabolized and cause inhibition of RNA and DNA synthesis. However, the development of drug resistance remains one of the major problems in anticancer chemotherapy. Several mechanisms have been hypothesized for observed resistance to 5-FU in tumor cells. These include loss or decreased activity of the key enzyme required for its activation, increased clearance and overproduction of thymidylate synthase (acquired resistance) through gene amplification, overexpression, or mutation (23). In attempts to circumvent resistance to 5-FU by tumor cells, a number of modulators have been used to increase the antitumor effects of the drug, including leucovorin and eniluracil (24). Accordingly, the overcoming of 5-FU/5-FdUrd resistance is an important goal for the development and clinical establishment of anticancer drugs.

Using growth inhibition assays, a recently described double staining method, and flow cytometry, we were able to study a number of cellular response mechanisms such as cell growth inhibition, apoptosis, necrosis and attenuation of cell cycle progression.

Morphological 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. The percentage values of the apoptotic cell fractions observed correlated to the $IC_{50}$ values yielded in the growth inhibition assays, indicating that apoptosis induction is likely to be the main mechanism by which Avemar exerts its antitumor effect. However, the percentage of apoptotic cells reached a maximum of 48% at a concentration of 300 $\mu$g/ml since higher dosage of the drug (600 $\mu$g/ml) was not able to significantly intensify the observed effect. This might be the reason for the fact that Avemar demonstrated no signs of toxicity in various studies (25), whereas more potent anticancer drugs exert a number of dose-limiting toxic side effects when applied to humans.

Furthermore, Avemar stopped the cell cycle transition of both sensitive and 5-FdUrd/Ara-C cross-resistant H9 cells in the S 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 >3000 $\mu$g/ml, indicating that the drug does not primarily exert its activity by influencing the cell cycle of sensitive or cross-resistant H9 cells. This finding underpins the effects seen in HL-60 human promyelocytic leukemia cells (13).

Regarding our results, we conclude that Avemar is able to overcome 5-FdUrd/Ara-C cross-resistance in H9 human lymphoma cells and induces dose-dependent apoptosis. Additionally, this non-toxic fermented wheat germ extract

exerts its cytotoxicity without causing remarkable cell cycle perturbations in both sensitive and cross-resistant H9 cells. Based on our data, further investigations against hematological malignancies within the framework of animal and clinical studies are warranted.

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