

Synergistic Effect of Avemar on Proinflammatory Cytokine Production and Ras-Mediated Cell Activation

ANDRÁS TELEKES,^a ENDRE KISS-TÓTH,^b TÜNDE NAGY,^c
EVA E. QWARNSTROM,^b ERZSÉBET KÚSZ,^c TIMEA POLGÁR,^{b,c}
ÁKOS RESETÁR,^d STEVEN K. DOWER,^b AND ERNŐ DUDA^{c,e}

^aNational Institute of Oncology, Budapest, Hungary

^bFunctional Genomics Group, Division of Genomic Medicine, University of Sheffield, Royal Halamshire Hospital, Sheffield, United Kingdom

^cInstitute of Biochemistry, Biological Research Center, Szeged, Hungary

^dBiomedicina First Hungarian Corporation for Cancer Research and Oncology, Budapest, Hungary

^eInstitute of Medical Microbiology and Immunobiology, Szeged University, Szeged, Hungary

ABSTRACT: Macrophages activated by lipopolysaccharide and/or phorbol esters exhibited high sensitivity to Avemar, a fermented wheat germ extract. Avemar synergized with lipopolysaccharide and PMA in the induction of the transcription of cytokine genes and release of inflammatory cytokines. At higher concentrations the preparation had a significant negative effect on the proliferation and survival of activated myeloid cell types. Avemar treatment induced the synthesis of ICAM-1 and synergized with the ICAM-inducing effect of TNF, but had no effect on VCAM-1 expression on microvascular endothelial cells. The effect of Avemar on signaling pathways, which are involved in cell activation was studied on HeLa cells as a model system. Avemar treatment increased the activity of stress kinases in a concentration-dependent way, resulting in the activation of AP-1 transcription factor. NF-kappa B-sensitive reporters were also activated by Avemar; in contrast, no effect of the preparation was observed on PKA-sensitive signaling pathways.

KEYWORDS: cytokine; synergism; Avemar; TNF; ICAM; NF-κB

INTRODUCTION

Avemar, a standardized wheat germ extract, developed by a Hungarian chemist, Máté Hidvégi, was launched as a dietary supplement in 1998. After extensive

Address for correspondence: András Telekes, M.D., Ph.D., Head, Chemotherapy Day-Ward Unit, National Institute of Oncology, Ráth György u. 7-9, 1122 Budapest, Hungary. Voice: +36-1-224-8600; fax: +36-1-224-8620.
telekes@oncol.hu

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research, it was approved in Hungary (Registration No. 503) and in other European countries as a dietary food for cancer patients. The inhibitory effect of Avemar on tumor progression has been proven in several experimental models.¹⁻³ Moreover, Avemar significantly inhibited azoxymethane-induced colon carcinogenesis in F-344 rats determined by the number of animals with tumor as well as the average number of colon tumors per animal.⁴ Avemar induced apoptosis in human cell lines such as MCF-7 breast cancer,⁵ Jurkat acute lymphoid leukemia T cells,^{2,6} A2058 melanoma,¹ and HT-29 colon cancer.³ Avemar induced proteolytic cleavage of poly (ADP-ribose) polymerases (PARPs), which is considered to be a hallmark of activation of caspase-3-like proteases during apoptosis. In fact, incubation of Jurkat cells for 48 h with 0, 0.3, 0.5, and 0.7 mg/ml of Avemar resulted in prominent cleavage of PARP at a concentration of 0.5 mg/ml or greater.⁶ Moreover, Avemar can control tumor propagation by a dose-dependent decrease in cell glucose consumption and regulation of glucose carbon redistribution between cell proliferation-related and cell differentiation-related macromolecules.⁷ Interestingly, glucose deprivation itself can trigger apoptosis through different mechanisms.^{8,9} Avemar showed activity in certain immunologic processes as well. The rejection time of skin graft in thymectomized mice was significantly shortened with Avemar treatment, indicating an immunity-restoring effect.¹⁰ As a result of Avemar treatment, the amount of cell surface MHC class I proteins was downregulated by 90 and 69% in Jurkat and Raji B cells, respectively, compared to nonstimulated controls.² Avemar reduced autoantibody production by inhibiting the Th2 response and it improved clinical manifestations (erythrocyte sedimentation rate [ESR], WBC count, and proteinuria) of the disease in an experimental model of systemic lupus erythematosus.¹¹ Avemar, by its complex mechanism of action, reduced disease progression and the incidence of metastases and consequently significantly improved the survival of patients with colorectal cancer compared to that of patients treated with conventional regimens alone.¹²

Tumor necrosis factor-alpha (TNF- α), first identified as a serum factor from lipopolysaccharide (LPS)-treated mice, induced hemorrhagic necrosis in tumors. In the human body, TNF is mainly produced by activated macrophages and epithelial cells. In contrast, TNF receptor-1 (TNF-R1) is widely expressed in different cells, which could implicate the manifold effect of TNF in physiologic and/or pathophysiologic processes. In fact, the interaction of TNF with TNF-R1 activates several signal transduction pathways. TNF is a major mediator of apoptosis, inflammation, and immunity; therefore, the role of TNF has been implicated in the pathogenesis of a wide range of human diseases including sepsis, diabetes, inflammatory bowel diseases, osteoporosis, multiple sclerosis, rheumatoid arthritis, and cancer.¹³

Because of the importance of TNF in the natural defense against cancer as well as the fact that Avemar induces apoptosis in malignant hematologic and solid tumor cell lines and exhibits immunomodulatory activities, the effect of Avemar treatment on proinflammatory cytokine production and Ras-mediated cell activation was studied, particularly, TNF-mediated cytotoxicity, TNF production of activated leukocytes, and the proliferation, sensitivity, and survival of TNF-producing and target cells. The molecular mechanisms of Avemar were also studied using signaling pathway-specific transcription reporters.

MATERIAL AND METHODS

Cell Line Culture and Reagents

Tumor necrosis factor was measured by bioassay on WEHI 164 cells (ECACC No. 87022501). A genetically modified clone (WC1) of this cell line, produced in our laboratory, was also used.¹⁴ WC1 cells are several hundred-fold more sensitive to TNF and provide us with a reproducible bioassay. WEHI 164 or WC-1 cells were grown in 96-well plates to a density of 3×10^4 cells/well. After 24 h of incubation at 37°C, serial dilutions of TNF were added to the cells containing 5% fetal calf serum (FCS). In WEHI assays, 0.1 µg/ml actinomycin D was also used. After 16-24 h of incubation cell viability was determined using the MTT assay.¹⁵

Several human (THP-1, ECACC No. 88081201, MonoMac6, from Dr. D. Wallach, Rehovot) and mouse (P388D and RAW264.7, ECACC Nos. 85011439 and 91062702, respectively) myeloid leukemia cell lines, an Epstein-Barr virus-immortalized human B-cell lymphoma cell line (Raji, ECACC No. 85011429), Sci1, a human B-cell lymphoma (ECACC No. 960905313), and BCL1, a mouse B-cell lymphoma (ECACC No. 90061904) were used to measure TNF production after *in vitro* activation of the cells by different combinations of Avemar, lipopolysaccharide (LPS), and phorbol-myristyl-acetate (PMA). (The human CDC-HMEC microvascular endothelial cell line was a kind gift of Prof. Hunyadi, Debrecen, Hungary.)

WEHI 164, WC/1, and RAW 264.7 cells were grown on DMEM + 5% FCS, Raji was propagated on RPMI-1640 + 10% FCS, and all other cells (including HeLa and CDC-HMEC) were cultured on DMEM + 10% FCS. Media, sera, and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo). Cell viability, proliferation, and cytotoxicity measurements were done using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.¹⁵

Human microvascular cells were treated *in vitro* with TNF and/or Avemar in six plates (35 mm) overnight. For flow cytometry the cells were dislodged with EDTA, rubber policeman, and repeated pipetting.

PMA and LPS (*Escherichia coli* serotype O55:B5) were purchased from Sigma-Aldrich (Budapest, Hungary). Avemar (Biromedicina, Budapest) was freshly dissolved, cleared by centrifugation, and filtered through a 0.1-µ filter just before the experiments. When cells were treated for extended periods, Avemar was replaced every other day.

Recombinant human TNF and TNF ELISA were purchased from Genzyme (Wiesbaden-Nordenstadt, Germany). Expression of cell adhesion molecules ICAM-1 and VCAM-1 on the surface of CDC-HMEC cells was followed by flow cytometry using monoclonal antibodies from Beckton Dickinson (Soft-Flow, Pécs, Hungary) and a BD FacsCalibur apparatus.

Analysis of RNA

Total cellular RNA was prepared from Avemar-treated macrophages using the guanidium isothiocyanate method according to standard protocols, and the integrity of the RNA samples was checked by running 5 µg RNA on formaldehyde gels.

The ribonuclease protection assay (RPA) was carried out with the ML-11 multiple probe set using 10 μg of total cellular RNA, essentially as described earlier.¹⁶ This set detects transcripts of 10 different murine cytokine genes (lymphotoxin α , TNF, interleukin (IL)-4, IL-5, IL-1 α , interferon (IFN)- γ , IL-2, IL-6, IL-1 β , and IL-3) and the housekeeping L-32 gene used as a control. In each gel, 1/10 part of the total amount of probe and a commonly used mixed spleen sample from LPS-treated mice were always included as a molecular weight marker and a positive control, respectively. TNF mRNA was also detected on Northern blots using 10 μg total RNA for electrophoresis. For these assays a DNA fragment containing the first 885 base-pair of the coding region of the murine TNF gene from the pGMTNF-MJ plasmid (a kind gift from Dr. J.-F. Arrighi) was used as the probe. After stripping, blots were rehybridized with a GAPDH probe, pHcGAP (from ATCC, Rockville, MD) for blotting control. RPA gels and Northern blots were visualized and quantified by using a Phosphorimager 445SI (Molecular Dynamics).

Plasmids and Transformation

All inducible reporter plasmids were purchased from Stratagene; the control, pTK-RL, containing the HSV promoter was from Promega. The reporter plasmid containing the promoter region of the human IL-8 gene (pIL-8-luc) was previously described.¹⁷

Constructs coding for the wild-type and mutant Ras proteins, used for the cotransformation experiments, were derivatives of pCDNA3.

Cells were transformed by liposomes according to standard protocols, as suggested by the manufacturers of Lipofectamine (GIBCO) and Superfect (Qiagen), respectively. For transient expression, $1-2 \times 10^4$ cells were plated into the wells of microtiter plates 1 day before transformation. Next, 500 ng reporter construct and 100 ng control plasmid were mixed with 2.5 μl Superfect or 5 μl Lipofectamine.

Measurement of Reporter Activity

Activity of the individual promoters was measured by luciferase assay in Avemar-treated cells using the Dual Luciferase Assay kit (Promega). Cells were washed in phosphate-buffered saline solution and lysed *in situ* in 25 μl of lysis buffer. Ten microliters of this solution was used for measurement. Because TK promoter was not influenced by Avemar treatment, it was used as an internal control during the experiments. Luciferase activity was expressed, for example, as a ratio of IL8-luc/TK-Rluc. At least two independent transformations were performed in each experiment, and treatments were repeated at least in three parallels.

RESULTS

Effect of Avemar on Proliferation and Survival of Different Cell Lines

Avemar is used by cancer patients as medical nutriment for prolonged periods. Consequently, experiments were planned to be of long duration (6–7 days) so that the proliferation and survival of the investigated cells in the presence of Avemar could be followed. Cells were grown in the presence of Avemar for 72 h before the

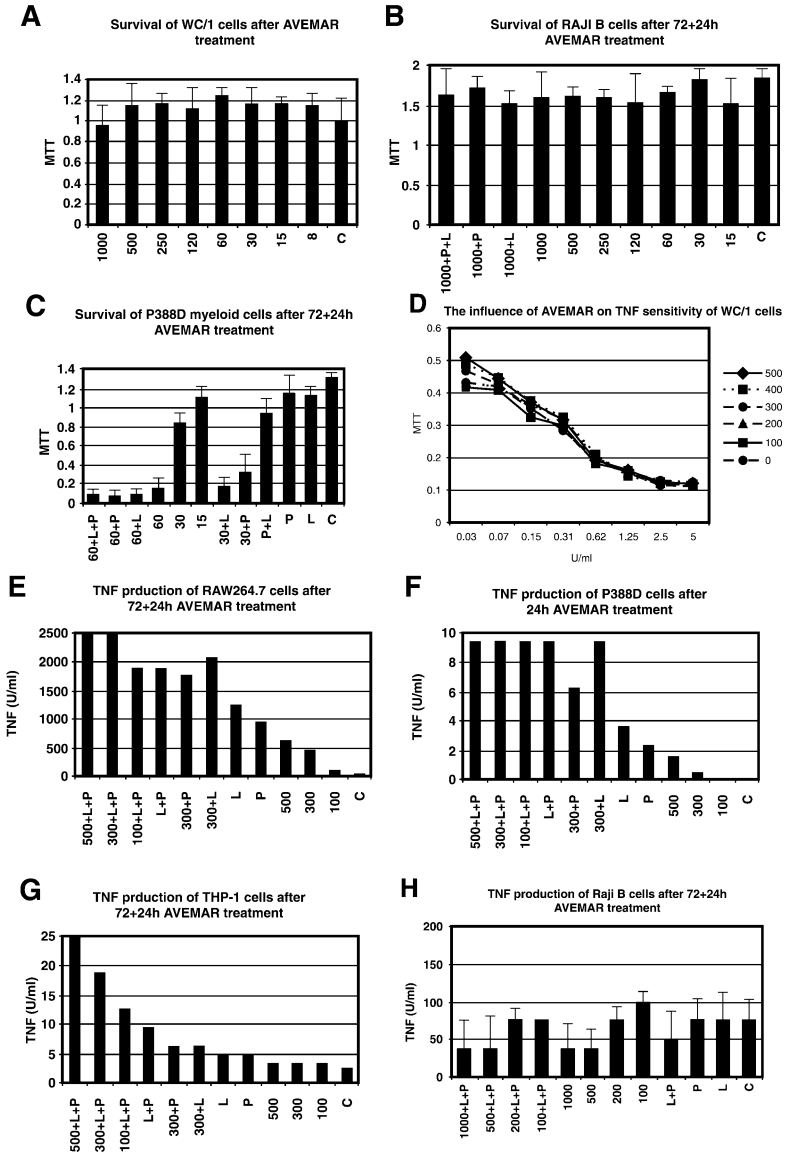


FIGURE 1. Effect of Avemar on proliferation of cells, TNF sensitivity of target cells, and LPS/PMA-induced TNF production of myeloid and lymphoid cells. (A) Presence of Avemar (up to 1,000 µg/ml) did not influence the proliferation rate and survival of (highly TNF-sensitive) WC1 cells (MTT measured at the end of the 24-h experiment; control is 100). (B) In one type of experiment (here EBV-immortalized B cells [Raji cells] are shown) the cultures were exposed to the same concentrations of Avemar for 72 h before the experiment to follow its long-term effects (MTT values were compared to the untreated control,

experiments, followed by another 24 h during the experiment, and the number of living cells was estimated by MTT methods.

Avemar (at a concentration of 8 $\mu\text{g/ml}$ to 10 mg/ml) did not influence the growth rate and survival of any of the investigated, nonmyeloid cell lines (WEHI 164, WC1, Raji, Sci1, BCL1, CDC-HMEC, and HeLa). However, cells of the monocyte-macrophage lineage (MonoMac6, THP-1, P388D, and RAW264.7) were sensitive to the drug, and some lines exhibited highly inhibited growth and limited survival at higher concentrations (0.5 mg/ml and up). This sensitivity was further increased in the presence of macrophage-activating compounds such as LPS or PMA (FIG. 1A, B, and C).

Avemar Did Not Modify the TNF Sensitivity of Target Tumor Cells

WC1 cells, used for the titration of TNF, were grown in the presence and absence of Avemar. In three parallel experiments, Avemar did not modify the TNF sensitivity of target cells significantly. As in FIGURE 1D, slight changes were observed, but in this assay system only a two- to fourfold increase or decrease in TNF sensitivity was judged as significant. Since WC1 is a genetically modified line of WEHI 164, similar experiments were carried out with the parental WEHI 164 line. As expected, WEHI cells, although they were less sensitive to TNF, exhibited similar TNF sensitivities in both the presence and the absence of Avemar (data not shown).

100, at the beginning of the 24-h experiment.) (C) Myeloid cells (here P388D mouse myeloid leukemia cells) showed very strong growth inhibition and decreased survival in the presence of Avemar, especially if TNF-inducing agents were also present. The doses of Avemar applied are as follows: 1,000, 500, 250, 120, 60, 30, 15, and 8 $\mu\text{g/ml}$. L represents 1 $\mu\text{g/ml}$ lipopolysaccharide, and P corresponds to 0.5 μM PMA. (These compounds were used only during the 24 h of the experiment.) C, untreated control cells. Each figure shows the results of at least three parallel experiments. (D) Highly TNF-sensitive WC1 cells were incubated with Avemar for 24 h (pretreatment), and then cells were exposed to a serial dilution of TNF (in the presence of Avemar). TNF sensitivity or survival of WC1 cells was not significantly influenced by Avemar treatment. (Similar results were obtained on WEHI and L929 cells, not shown. 500, 400, 300, 200, 100, and 0 labels Avemar concentrations in $\mu\text{g/ml}$. L corresponds to 1 $\mu\text{g/ml}$ LPS; P, 0.5 μM PMA; C, control cells.) Three experiments were carried out; SD was within 27%. (E-G) Treatment with PMA + LPS induced the production of TNF in different myeloid cell types. They produced very different amounts of TNF in the absence of Avemar; however, all cell types increased TNF production significantly in the presence of Avemar. Cells were exposed to the indicated concentrations of Avemar for 72 h before the experiment. LPS and PMA were added only during the experiment. Avemar concentrations are given in $\mu\text{g/ml}$ (namely, 100, 300, and 500 $\mu\text{g/ml}$.) L, 1 $\mu\text{g/ml}$ lipopolysaccharide; P, 0.5 μM PMA; C, control cells. Each figure shows the results in three independent experiments; SD was within 11%. (H) Raji cells were grown in the absence or presence of the indicated concentrations of Avemar for 72 h, then exposed for 24 h to combinations of Avemar, LPS, and PMA, as labeled. Avemar pretreatment had little influence on the LPS + PMA-induced TNF production of Raji cells, and several other lymphoid lines behaved in a similar way (not shown). (Avemar concentrations are given in $\mu\text{g/ml}$, such as 1,000, 500, 200, and 100 $\mu\text{g/ml}$. L, 1 $\mu\text{g/ml}$ lipopolysaccharide; P, 0.5 μM PMA; C, untreated control).

Effect of Avemar on the Induced Cytokine Production of Myeloid and Lymphoid Cells

Most myeloid cell types produce insignificant amounts of TNF in the absence of activation. LPS and/or PMA induce a high increase in TNF production, usually with slight upregulation of mRNA synthesis, influencing mostly translational activity.

Different myeloid cells responded differently, but all increased LPS/PMA-induced TNF production in the presence of Avemar. In the lower concentration range upregulation of TNF synthesis was dependent on the dose of Avemar (FIG. 1E, F, and G).

High concentrations of Avemar proved to be toxic to certain myeloid cells. Net TNF production of some cultures decreased rapidly in the presence of higher concentrations of Avemar. Activated cells of these lines could not survive the simultaneous presence of Avemar, LPS, and PMA. The molecular mechanisms leading to cell death (overstimulation, production of toxic substances, and apoptosis) remains to be investigated (annexin assays yielded ambiguous results).

This effect of Avemar proved to be specific for myeloid cells. Unlike myeloid cells, lymphoid cells (virus-immortalized and leukemic B cells) were not greatly influenced by the presence of Avemar. LPS and PMA increased TNF synthesis in these cells, but the production rate was much the same in the presence or absence of Avemar. Also, Epstein-Barr virus-immortalized Raji B-cells did not seem to be sensitive to relatively high concentrations of Avemar in either the presence or the absence of LPS and/or PMA (FIG. 1H).

Avemar Upregulated the Synthesis of Cell Adhesion Molecules on Endothelial Cells

TNF has a profound effect on the expression of cell adhesion molecules in endothelial cells, an effect that is mediated by the activation of the NF- κ B/Rel family of transcription factors. The effect of Avemar on ICAM-1 and VCAM-1 expression on cultured human microvascular endothelial cells was investigated. As demonstrated in FIGURE 2A, Avemar induced the production of ICAM-1 and synergized the effect of TNF. VCAM-1 expression or TNF-mediated induction of VCAM-1, however, was not influenced by the presence of Avemar (FIG. 2B).

Avemar Upregulated the Expression of Cytokine Genes on Myeloid Cells

The effect of Avemar on myeloid cells was not restricted to the increased production of TNF. RNase protection experiments (FIG. 3A) showed the upregulated expression of a number of cytokine genes in Avemar and LPS- and/or PMA-treated myeloid cultures. Avemar treatment upregulated the production of mRNAs of IL-1 α , IL-2, IL-5, and IL-6 by 3.7-, 6-, 8.4- and 4.5-fold, respectively, an average of three sets of experiments, with less than 13% SD for the bands of these cytokines. Interestingly, the addition of PMA and/or Avemar to LPS decreased the intensity of IL-1 mRNA synthesis. The pattern of activation was not identical in LPS/PMA- and Avemar-treated cells, suggesting the participation of somewhat different signaling pathways.

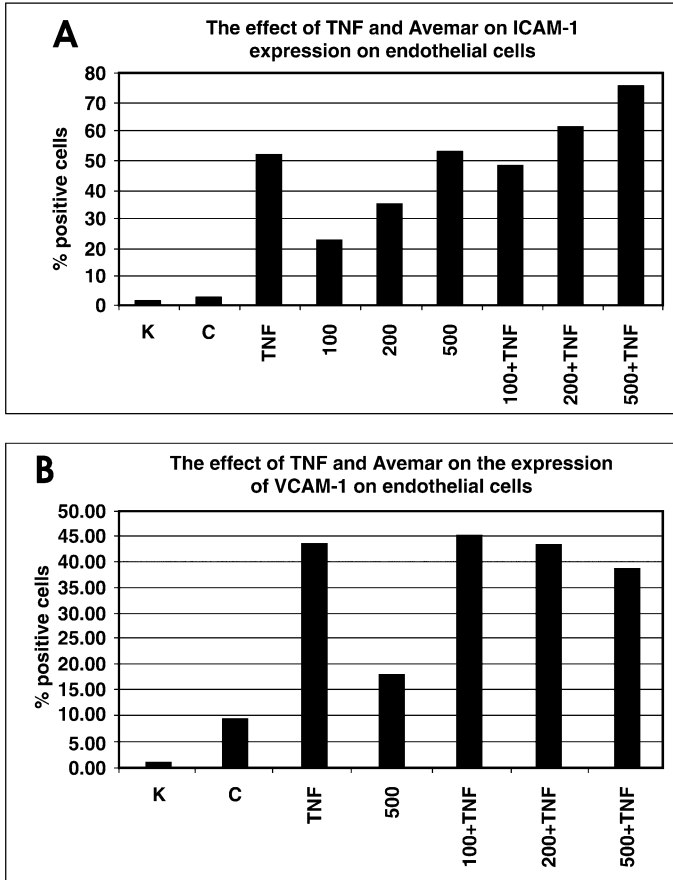


FIGURE 2. Effect of Avemar treatment on the expression of cell adhesion molecules. Human microvascular endothelial cells were exposed to TNF in the presence or absence of Avemar for 24 h, and the expression of cell adhesion molecules was followed by flow cytometry using fluorescent antibodies against CAMs. **(A)** ICAM-1 expression (percentage of fluorescent cells in flow cytometry) after cells were treated with TNF (1,000 U/ml TNF) or Avemar (100, 200, and 500 μ g/ml) or their combinations, as labeled. K, untreated cells labeled with isotype control; C, control cells. Results of three independent experiments yielded values with less than 14% SD. **(B)** VCAM-1 expression on human microvascular cells (percentage of fluorescent cells in flow cytometry). Cells were treated with TNF (1,000 U/ml TNF), Avemar (100, 200, and 500 μ g/ml), or their combination, as labeled; K, untreated cells labeled with isotype control; C, control cells. Results of three independent experiments yielded values with less than 17% SD.

Avemar Upregulated AP-1 and NF- κ B but Not CRE-Sensitive Reporters in Cervix Carcinoma (HeLa) Cells Using a Signaling Pathway, Which Involves P21ras

These results indicate that signaling pathways usually triggered by inflammatory cytokines¹⁸ might be involved in the effects of Avemar. To learn more about the molecular events elicited by Avemar, HeLa cells were transiently transfected with signaling pathway-specific reporters and treated with increasing doses of Avemar. As in FIGURE 3B, Avemar activated the stress kinase-sensitive *AP-1 luc* and *NF- κ B luc* responsive reporters in a dose-dependent manner, whereas it had no effect on the cAMP-responsive *CRE luc* reporter activity. The possible synergism between Avemar and proinflammatory cytokine-mediated reporter activation was investigated by costimulating HeLa cells with nonsaturating and saturating doses of IL-1 or TNF in the presence or the absence of Avemar. A cytokine-sensitive *IL-8 luc* reporter, which contains binding sites for NF- κ B and AP-1 or an *NF- κ B luc* reporter was transiently transfected and used as a readout signal (FIG. 3C, D, E, and F). Except for using IL-1 in a saturating dose on *IL-8 luc*, Avemar was able to synergize with the cytokine-mediated stimulation. The ability to superinduce proinflammatory cytokine stimulation even in a saturating dose suggests that Avemar-mediated responses might be coupled with other second messenger pathways. This hypothesis was tested using *AP-1 luc* as a readout and constitutively active or dominant negative *p21ras* mutants as activators. As in FIG. 3G, Avemar-mediated activation was completely blocked upon transfection with the dominant negative *ras* mutant. These data indicate that Avemar activates the AP-1 reporter via a signaling pathway that is controlled by *p21ras*.

DISCUSSION

Apoptosis is a physiologic process critical for organ development, tissue homeostasis, and elimination of defective or potentially dangerous cells in complex organisms. Apoptosis in mammalian cells can be initiated through two major interrelated pathways, one involving engagement of the TNF family of death receptors and the other involving the release of cytochrome *c* from mitochondria. Apoptosis and proliferation are intimately coupled. Some cell cycle regulators can influence both cell

FIGURE 3. RNase protection assay of cytokine mRNAs produced by Avemar treatment and proinflammatory signaling in HeLa cells. (A) RAW264.7 cells were exposed to different combinations of Avemar, LPS, and PMA, and the cytokine profiles were analyzed using RNase protection assay. T2 and N3 are extracts that induce TNF synthesis. Raw contr, cells without any treatment (negative control); liver + contr, RNA purified from the liver of LPS-treated mice (positive control); Ave, Avemar, 500 μ g/ml, for 24 h. (Protected RNAs run somewhat faster than the probes.) Phosphoimager was used to evaluate the results. Relative band intensities from four experiments differed within 5–22%. (B) HeLa cells were transiently transfected with the appropriate reporter plasmid and treated with none, 30 μ g/ml, 300 μ g/ml, and 3,000 μ g/ml freshly dissolved Avemar for 6 h, respectively. (C–F) Transfected HeLa cells were stimulated with 1 nM (+++) or 10 pM (+) IL-1 or with 1 ng/ml (+++) or 10 pg/ml (+) TNF in the presence or absence of 3,000 μ g/ml Avemar for 6 h. (G) HeLa cells were transfected with AP-1 luc reporter and the stated p21 Ras expression construct and treated with 3 μ g/ml Avemar for 6 h. Results of three parallel experiments.

division and programmed cell death. The linkage of cell cycle and apoptosis has been recognized for c-Myc, p53, pRb, PKA, PKC, Bcl-2, CDK, cyclins, CKI, Ras, and NF- κ B.¹⁹ It is of importance that both NF- κ B and Ras can either induce programmed cell death or protect cells from apoptosis depending on the specific cell type and the type of inducer.^{20, 21} Experimental data clearly indicate that NF- κ B is a major regulator of the inflammatory reaction by controlling the expression of pro-inflammatory molecules in response to cytokines, oxidative stress, and infectious agents.²² Moreover, NF- κ B pathway activation occurs during transformation induced by a number of classic oncogenes, including Bcr/abl, Ras, and Rac, and it is necessary for full transforming potential.²³ TNF plays a critical role in mediating inflammatory responses by upregulation of genes encoding cell-adhesion molecules required for the recruitment of inflammatory cytokines.²⁴ Because the promoter regions of many of the TNF-regulated genes contain DNA binding sites for NF- κ B, activation of NF- κ B by TNF is essential to elicit effective immune and inflammatory responses.¹³ Moreover, the link between NF- κ B and cancer stems, in part, by the fact that this transcription factor can induce gene products that control proliferative responses and that suppress apoptotic cascades, such as those induced by TNF.²⁵

That the regulatory cascades of inflammation, immunity, apoptosis, transformation, and cell cycle seem to entwine, at least partly, the effects of Avemar on TNF-mediated cytotoxicity of sensitive tumor cells is somewhat difficult to interpret. On the one hand, Avemar did not exhibit any significant effect on TNF-mediated killing of tumor cells; it neither protected target cells against TNF nor increased their sensitivity to this cytokine. On the other hand, TNF and IL-6 production of activated macrophages was considerably increased when cells were exposed to Avemar for prolonged periods. IL-6 and TNF are typical examples of multifunctional cytokines involved in the regulation of the immune response, hematopoiesis, and inflammation. Their functions are widely overlapping, but each shows its own characteristic properties.²⁶ It is noteworthy that nonmyeloid cell lines such as WEHI 164, WC11, Raji, Sci1, and BCL1 were not sensitive to Avemar, but monocyte-macrophage lineage (MonoMAc6, THP-1, P388D, and RAW264.7) showed growth inhibition and reduced survival to Avemar treatment at higher concentrations (0.5 mg/ml and up). The oncogenic transformation of myeloid cells in CML results from the expression of a constitutively active tyrosine-kinase signaling protein construct generated by the realignment of the breakpoint cluster region and Abelson leukemia virus protooncogene sequences (Bcr/Abl).²⁷ This construct stimulates glucose transport in multipotent hematopoietic cells.²⁸ Decreased glucose carbon flow towards nucleic acid synthesis has been observed in cancer cells after treatment with Avemar.⁷ It is of interest that Gleevec, one of the most effective agents against CML, has been shown to influence glucose utilization in a similar manner.²⁹ One of the difference between Gleevec and Avemar is that Gleevec decreases glucose intake and carbon flow through the oxidative pentose cycle, whereas Avemar does the same through the nonoxidative pentose cycle.³⁰ The efficacy of Avemar on myeloid cell lines can further be explained by the fact that Avemar not only impedes the enzymes regulating carbon flow in the pentose cycle (transketolase and G6PDH), but also is a potent inhibitor of key enzymes regulating glycolytic flux.⁶

In synergy with LPS and PMA, Avemar induced the activity of a number of cytokine genes. The pattern of activated genes was slightly different in LPS/PMA-treated cells in the absence or presence of Avemar, according to the RNase protec-

tion experiments shown in Figure 3A. These results suggest that the signaling pathways leading to cytokine production were overlapping, but not identical. Further experiments using transcription reporter constructs corroborated this finding and revealed that Avemar activated several intracellular signaling pathways. The MAPK signal transduction cascade plays a pivotal role in cell responses given to extracellular stimuli. Within this system traditionally three subunits are distinguished. The stress kinases activate the AP-1 transcription factor and therefore increase the transcription of several genes including IL-1 and TNF, thus influencing cell growth, survival, differentiation, and apoptosis. The extracellular signal-regulated kinases (ERK) also play an important role in cell growth and differentiation (activation of ERK leads to expression of SRE). Finally, the p38 kinases regulate cytokine production and other processes leading to apoptosis. The activity of p38 kinases can be measured by a reporter construct containing CRE. All inflammatory processes (induced by cytokines, endotoxins, radiation, etc.) activate NF- κ B transcription factor and consequently induce the production of new cytokines, growth factors, adhesion molecules, and immunoreceptors. The signal transduction systems just discussed were measured by reporter constructs containing elements of AP-1, SRE, CRE, and NF- κ B. Avemar increased the activity of constructs containing sequences responsive to AP-1 and NF- κ B in a dose-dependent manner via a mechanism that involves activation of p21 ras, as shown in Figure 3B. SRE showed a dose-dependent increase in activation as well (data not shown).

Endothelial cells in the vasculature of human solid tumors have decreased expression of intercellular adhesion molecule-1 (ICAM-1) compared to normal endothelial cells. Incubation of tumor-derived endothelial cells with TNF resulted in expression levels of only 20%, achieved in similarly treated normal tissue-derived endothelial cells because tumor angiogenesis induces anergy in endothelial cells.¹⁸ This phenomenon may serve as a tumor-protecting mechanism because leukocytes require ICAM-1 to leave the vascular system to infiltrate the tumor tissue. Avemar induced the production of ICAM-1 as well as showing synergy with TNF in this respect. This observation may explain, at least in part, the antitumor activity of Avemar.

In summary, it can be concluded that similar to previous experiments, Avemar showed a wide range of biologic activity. Metabolic pathways triggered by Avemar lead to increased TNF and cytokine production and, if overactivated, to the destruction of the cells. It can be hypothesized that Avemar-treated activated macrophages overproduce proinflammatory cytokines and possibly other mediators (nitric oxide, free oxygen radicals, etc.), so that the cells could not survive ("suicide" mechanism). Alternatively, two signaling pathways, not mutually exclusive, can be envisioned to be set by Avemar in activated myeloid cells, namely, one leading to upregulation of inflammatory mediators and another to apoptosis. Considering the immunologic and anticancer activity of Avemar, further studies are still warranted to elucidate its mechanisms of action.

COMPETING INTEREST: Ákos Resetár is CEO of Biomedicina First Hungarian Corporation for Cancer Research and Oncology, Budapest, Hungary.

REFERENCES

1. HIDVÉGI, M., E. RÁSÓ, R. TÖMÖSKÖZI-FARKAS, *et al.* 1999. MSC, a new benzoquinone-containing natural product with antimetastatic effect. *Cancer Biother. Radiopharm.* **14**: 277–289.
2. FAJKA-BOJA, R., M. HIDVÉGI, Y. SHOENFELD, *et al.* 2002. Fermented wheat germ extract induces apoptosis and downregulation of major histocompatibility complex class I proteins in tumor T and B cell lines. *Int. J. Oncol.* **20**: 563–570.
3. ILLMER, C., S. MADLENER, Z. HORVATH, *et al.* 2005. Immunologic and biochemical effects of the fermented wheat germ extract Avemar. *Exp. Biol. Med.* **230**: 144–149.
4. ZALATNAI, A., K. LAPIS, B. SZENDE, *et al.* 2001. Wheat germ extract inhibits experimental colon carcinogenesis in F-344 rats. *Carcinogenesis* **22**: 1649–1652.
5. MARCSEK, Z., Zs. KOCSIS, M. JAKAB, *et al.* 2004. The efficacy of tamoxifen in estrogen receptor-positive breast cancer cells is enhanced by a medical nutriment. *Cancer Biother. Radiopharm.* **19**: 746–753.
6. COMÍN-ANDUIX, B., L.G. BOROS, S. MARIN, *et al.* 2002. Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly (ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. *J. Biol. Chem.* **277**: 46408–46414.
7. BOROS, L.G., K. LAPIS, B. SZENDE, *et al.* 2001. Wheat germ extract decreases glucose uptake and RNA ribose formation but increases fatty acid synthesis in MIA pancreatic adenocarcinoma cells. *Pancreas* **23**: 141–147.
8. MOLEY, K.H. & M.M. MUECKLER. 2000. Glucose transport and apoptosis. *Apoptosis* **5**: 99–105.
9. KANSARA, M. & M.V. BERRIDGE. 2004. Oncogenes modulate cell sensitivity to apoptosis induced by glucose deprivation. *Anticancer Res.* **24**: 2503–2510.
10. HIDVÉGI, M., E. RÁSÓ, R. TÖMÖSKÖZI-FARKAS, *et al.* 1999. Effect of MSC on immune response of mice. *Immunopharmacology* **41**: 183–186.
11. EHRENFELD, M., M. BLANK, Y. SHOENFELD, *et al.* 2001. AVEMAR (a new benzoquinone-containing natural product) administration interferes with the Th2 response in experimental SLE and promotes amelioration of the disease. *Lupus* **10**: 622–627.
12. JAKAB, F., Y. SHOENFELD, Á. BALOGH, *et al.* 2003. A medical nutriment has supportive value in the treatment of colorectal cancer. *Br. J. Cancer* **89**: 465–469.
13. CHEN, G. & D.V. GOEDDEL. 2002. TNF-R1 signaling: a beautiful pathway. *Science* **296**: 1634–1635.
14. GYÓRFI, Z., S. BENKŐ, E. KÚSZ, *et al.* 1997. Highly increased TNF sensitivity of tumor cells expressing the yeast delta 9-desaturase gene. *Biochem. Biophys. Res. Commun.* **241**: 465–470.
15. DENIYOT, F. & R. LANG. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**: 271–277.
16. HOBBS, M.V., W.O. WEIGLE, D.J. NOONAN, *et al.* 1993. Patterns of cytokine gene expression by CD4+ T cells from young and old mice. *J. Immunol.* **150**: 3602–3614.
17. KISS-TÓTH, E., F.M. GUESDON, D.H. WYLLIE, *et al.* 2000. A novel mammalian expression screen exploiting green fluorescent protein-based transcription detection in single cells. *J. Immunol. Methods* **239**: 125–135.
18. GRIFFIOEN, A.W., C.A. DAMEN, G.H. BLIJHAM, *et al.* 1996. Tumor angiogenesis is accompanied by decreased inflammatory response of tumor-associated endothelium. *Blood* **88**: 667–673.
19. VERMEULEN, K., Z.N. BERNEMAN & D.R. VAN BOCKSTAELE. 2003. Cell cycle and apoptosis. *Cell Proliferat.* **36**: 165–175.
20. SONENSHEIM, G.E. 1997. Rel/NF-kappa B transcription factors and the control of apoptosis. *Semin. Cancer Biol.* **8**: 113–119.
21. DOWNWARD, J. 1998. Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**: 49–54.
22. BOURS, V., G. BONIZZI, M. BENTIREN-ALI, *et al.* 2000. NF-kappa B activation in response to toxic and therapeutical agents: role in inflammation and cancer treatment. *Toxicology* **153**: 27–38.

23. JOYCE, D., C. ALBANESE, J. STEER, *et al.* 2001. NF-kappa B and cell-cycle regulation: the cyclin connection. *Cytokine Growth Factor Rev.* **12**: 73–90.
24. BAZZONI, F. & B. BEUTLER. 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* **334**: 1717–1725.
25. MAYO, M.W. & A.S. BALDWIN. 2000. The transcription factor NF-kappa B: control of oncogenesis and cancer therapy resistance. *Biochim. Biophys. Acta* **1470**: M55–62.
26. AKIRA, S., T. HIRANO, T. TAGA, *et al.* 1990. IL 6 and related molecules (IL 1 and TNF). *FASEB J.* **4**: 2860–2867.
27. GYGER, M., C. PERRAULT, R. BELANGER, *et al.* 1985. Chronic myeloid leukemia with an unusual simple variant translocation: t(22;22)(q13;q11). *Acta Hematol.-Basel* **73**: 193–195.
28. BENTLEY, J., I. WALKER, E. MCINTOSH, *et al.* 2001. Glucose transport regulation by p210 Bcr-Abl in a chronic myeloid leukaemia model. *Br. J. Haematol.* **112**: 212–215.
29. BOREN, J. M. CASCANTE, S. MARIN, *et al.* 2001. Gleevec (ST1571) influences metabolic enzyme activities and glucose carbon flow towards nucleic acid and fatty acid synthesis in myeloid tumor cells. *J. Biol. Chem.* **276**: 27747–37753.
30. BOROS, G.L. M. CASCANTE & W.-N. P. LEE. 2002. Metabolic profiling of cell growth and death in cancer: applications in drug discovery. *Drug Discovery Today* **7**: 18–26.