

The Efficacy of Tamoxifen in Estrogen Receptor-Positive Breast Cancer Cells Is Enhanced by a Medical Nutriment

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ABSTRACT

Avemar, a fermented wheat germ extract, has been applied in the supplementary therapy of human cancers. Because tamoxifen is commonly used in the therapy of ER+ breast cancer, in this study the combined effect of tamoxifen and Avemar treatment was investigated on MCF-7 breast cancer cells, in order to detect a possible agonistic or antagonistic action. Cytotoxicity was measured by MTT assay, the percentage of mitoses and apoptotic cells was determined morphologically, apoptosis and S-phase was measured by flow cytometry, and estrogen-receptor activity was determined by semiquantitative measurement of the estrogen-responsive pS2 gene mRNA production.

Tamoxifen (1 nM) alone had no effect on the percentage of the apoptotic cell fraction and significantly reduced the percentage of the S-phase, compared to untreated cells. Avemar (625 µg/mL) significantly increased apoptosis after 48 hours of treatment. Tamoxifen together with Avemar significantly increased apoptosis already 24 hours after starting treatment but had only a slight (not significant) effect on mitosis and S-phase. Estrogen-receptor activity of MCF-7 cells was enhanced by Avemar, decreased by tamoxifen, and was further decreased by combined tamoxifen and Avemar treatment. As apoptosis increased when Avemar was added to tamoxifen treatment, the use of supplementary therapy with Avemar in the case of ER+ breast tumors may enhance the therapeutic effects of tamoxifen.

Key words: Avemar, tamoxifen, MCF-7, apoptosis, proliferation, estrogen receptor

INTRODUCTION

Tamoxifen is widely used in the chemotherapy of breast cancer and as a preventative agent after its surgery.¹ Tamoxifen has both a pro- and anti-estrogenic effect on the nuclear estrogen recep-

tors (ER), modifies the function of the plasma membrane, the microsomes, the proliferative and antiproliferative factors as TGF or cyclins, et cetera.²⁻⁵ As a "selective ER modulator" (SERM),⁶ tamoxifen significantly influences the activity of ER.

A fermented wheat germ extract (trade name: Avemar, registered in Hungary in 2002 as medical nutriment reg. no. 503), standardized to methoxy-substituted benzoquinones, has been shown to delay disease progression in melanoma,⁷ to prolong progression-free and overall survivals in colorectal cancer,^{8,9} and to improve quality of

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life and to alleviate fatigue in advanced lung cancer¹⁰ patients. As Avemar has been shown to induce apoptosis¹¹ and to inhibit carbon flow to nucleic acid synthesis¹² in tumor cells and tamoxifen has also been reported to negatively influence tumor-cell proliferation by inducing programmed cell death,¹³ the aim of this study was to test the possible interactions of these 2 agents.

The MCF-7 breast cancer cell line is an ideal model to study the mechanism of estrogenic action because these cells express functional wild-type estrogen receptors.¹⁴ In this study, the fermented wheat germ extract in combination with tamoxifen, and 17 β -estradiol as a positive control, were investigated *in vitro* concerning their effects on apoptosis, cell-cycle alterations, and estrogen-receptor activation in the MCF-7 breast cancer cell line.

MATERIALS AND METHODS

Chemicals

MTT reagent, oligo-dT₁₂₋₁₈ primers, deoxyribonucleoside triphosphates, propidium iodide, ethidium bromide, and agarose were purchased from Sigma (St. Louis, MO); fetal calf serum came from GIBCO (Invitrogen Life Technologies, Paisley, Scotland); tamoxifen citrate (Z-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) was the product of TEVA Pharmaceutical Industries, Ltd. (Israel). From Avemar, provided by Biomedicina Co. (Budapest, Hungary), a 10-mg/mL stock solution was dissolved in Dulbecco's modified Eagle's medium (DMEM), filter sterilized, and serial dilutions were made.

Cell Cultures

MCF-7 (ECACC 86012803) and MDA-MB-231 (ECACC 92020424) human breast adenocarcinoma cells, and MRC-5 (ECACC 84101801) human fetal lung cells were purchased from ECACC (European Collection of Cell Cultures, UK), HepG2 (ATCC HB-8065) from ATCC (American Type Culture Collection), and was cultured in DMEM without phenol red (GIBCO; Mecklenheim, Germany), using plastic culture dishes, microwell plates, and thermanox coverslips (Nunc A/S; Roskilde, Denmark). DMEM medium was supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamin, and antibiotics: 100 units/mL penicillin and 100 μ g/mL streptomycin (Sigma, St. Louis,

MO). Cells growing as a monolayer were kept in an isolated 37°C, 5% CO₂ thermostat. For the study of cell death and cell cycle, the cells were seeded into 6-well plates at 5×10^5 cells/well density; for the MTT assay, 96-well microplates were used with 2×10^3 cells/well. 10-cm dishes in triplicate were used to grow cells to isolate RNA.

Treatment

Cytotoxicity testing of Avemar (at 24 hours) was performed in the concentration range of 156 μ g/mL and 5 mg/mL using 2 estrogen receptor (ER)-positive (MCF-7 and HepG2) and two ER-negative (MRC5 and MDA) cell lines. The highest noncytotoxic dose (625 μ g/mL) was used in the experiments.

Avemar, tamoxifen, and 17 β -estradiol were administered to MCF-7 cell cultures 24 hours after plating, while control cultures were maintained in DMEM. Four (4) samples of cells were cultured and treated in a volume of 100 μ L in 96-well tissue culture plates for a further 24 and 48 hours for apoptosis and cell-cycle studies, as well as for the MTT assay and for estrogen-receptor activity measurements. The following treatment schedule was applied. Group 1: Control (DMEM); Group 2: Avemar (625 μ g/mL); Group 3: tamoxifen (1 nM); Group 4: tamoxifen (1 nM) + Avemar (625 μ g/mL).

MTT Assay

Cytotoxic effects on the growth and viability of 2×10^4 cells/mL were determined in 96-microwell plates using tetrazolium dye MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, as described.¹⁵ Optical density (O.D.) of the wells was determined using an Anthos 2020 (Salzburg, Austria) enzyme-linked immunosorbent assay (ELISA) microplate reader at a test wavelength of 570 nm and a reference wavelength of 690 nm. All experiments were performed at least 3 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 controls.

Flow Cytometry

MCF-7 cells were cultured in 10-cm dishes at an initial plating density of 5×10^5 cells/dish in DMEM containing 10% FCS and antibiotics for

24 hours. At hour 24, the cells were treated similarly, as described above for 24 and 48 hours. Pulse labeling of cells with 5 $\mu\text{g}/\text{mL}$ 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO) was performed 2 hours prior to termination of the cultures. DNA was stained with propidium iodide (PI), and the incorporated BrdU was detected immunocytochemically with the FITC-labeled monoclonal antibody (Beckton-Dickinson), as described by others.¹⁶ Flow-cytometry analysis was performed in a FACS-Calibur (Beckton-Dickinson) flow cytometer at a flow rate of approximately 400 cells/sec. CellQuest software (Beckton-Dickinson) was used for the analysis of the obtained data. The percentages of S-phase and apoptotic fractions were determined on the basis of PI and anti-BrdU-FITC/PI fluorescence.

Morphological Detection of Apoptosis and Mitosis

MCF-7 human breast cancer cells were plated on glass coverslips in plastic dishes (diameter, 35 mm) at a density of 1.25×10^4 cells/cm² treated as described above. The coverslip preparates were fixed in methanol: acetic acid (3:1) mixture for 5 minutes and stained with haematoxylin and eosin. The criteria described by Wyllie et al.¹⁷ and Bursch et al.¹⁸ were applied. Apoptotic and mitotic forms among 200 cells were counted and the values (apoptotic and mitotic index) were expressed as a percentage.

Semiquantitative PCR Protocol

RNA was isolated from cell cultures using the GenElute total RNA isolation kit (Sigma, RTN 70) following the manufacturer's instructions. Reverse transcription was performed in 20- μL reaction volumes using 1 μL of isolated total RNA, 0.1 μg oligo-dT₁₂₋₁₈ primers, 15 μM each deoxyribonucleoside triphosphates, buffer (shipped with enzyme) and 200 U of M-MLV reverse transcriptase (GIBCO-BRL, Invitrogen Life Technologies, Paisley, Scotland) at 37°C for 90 minutes. The reaction was stopped in boiling water for 5 minutes, and the synthesized first strand cDNA was stored at -80°C.

Estrogen-receptor activity was monitored by the trefoil factor (pS2) transcript formation. The pS2 gene has an estrogen-responsive element (ERE) in its promoter, so the amount of transcribed pS2 mRNA is proportional to the activity of ER.^{19,20}

In the PCR reactions, parallel amplification of 18S ribosomal RNA (rRNA) was performed as

an internal control.²¹ The pS2-specific primers used were 5'CATGGAGAACAAGGTGATCTG and 5'CAGAAGCGTGTCTGAGGTGTC amplifying 336 bp DNA²⁰ and 18S-rRNA primers were 5'GTAACCCGTTGAACCCATT 3' and 5'CCATCCAATCGGTAGTAGCG 3' producing 151 bp PCR fragment. Primers were synthesized by the Genodia Co. (Budapest, Hungary). The 20- μL PCR reaction volumes contained 1 μL 1st strand cDNA, 15 μM of each primers, 15 μM of each dNTP, 2.5 mM MgCl₂, 1 \times buffer, and 0.5 U RedTaq DNA polymerase (Sigma, St. Louis, MO). PCR reactions were performed in a Techne Progene thermocycler (Cambridge, U.K.). Samples were heated to 94°C for 3 minutes, annealed at 50°C for 1 minute, and DNA was synthesized at 72°C for 10 minutes. 30 cycles of amplification was performed: 94°C for 30 seconds, 50°C for 45 seconds, 72°C for 40 seconds, and last extension at 72°C for 10 minutes. 10 μL of PCR products were analyzed on 2% agarose TBE gels containing ethidium bromide. Gels were analyzed by the Kodak EDAS 290/1Dgel system (Eastman Kodak Co., Rochester, NY) and band intensities were evaluated as relative intensity values to the 18S rRNA PCR product and expressed as a percent of control.

Statistical Analysis

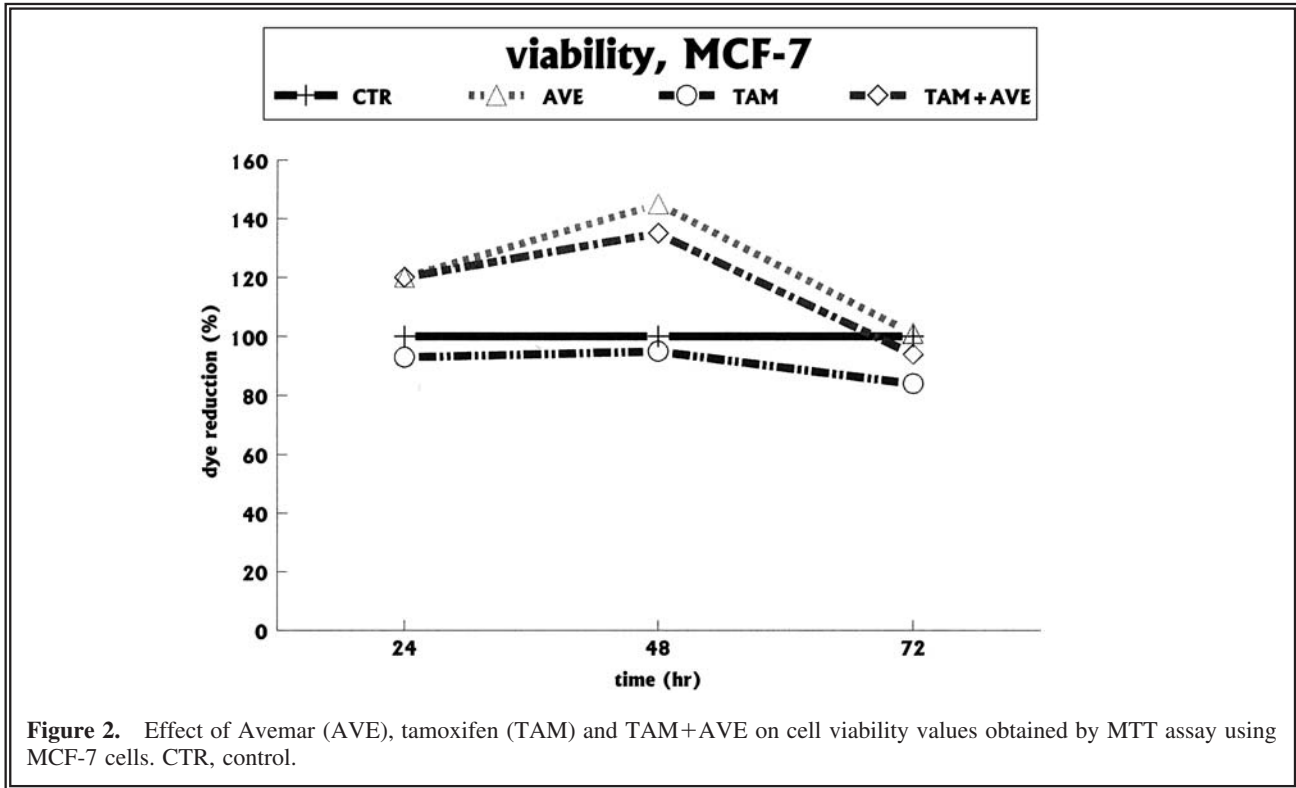
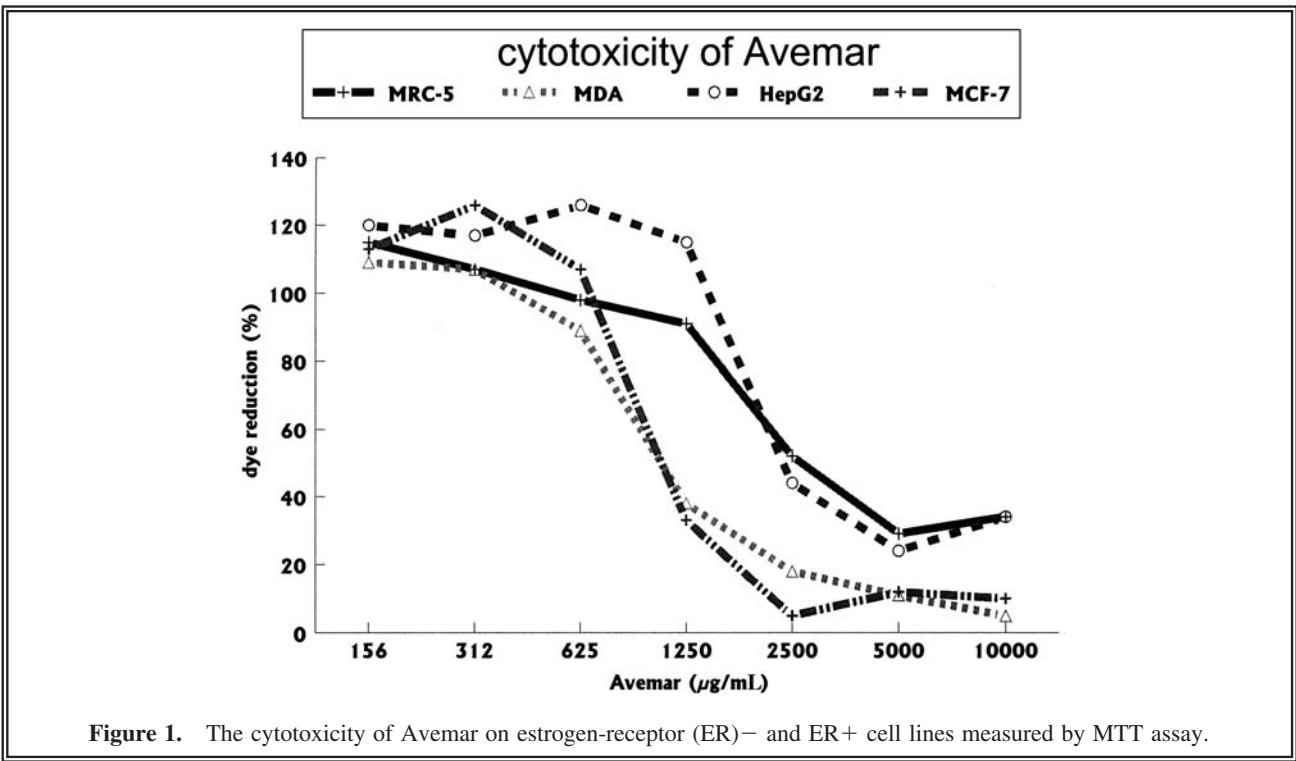
Statistical analyses were performed with a Student's paired *t* test. *P* values of <0.05 were considered to be significant.

RESULTS

Cytotoxicity (MTT Assay)

Avemar exhibited cytotoxicity only in the concentration range over 1.250–2.500 $\mu\text{g}/\text{mL}$ on the cell lines used, as shown in Fig. 1. At lower concentrations both ER+ cell lines (MCF-7 and HepG2) responded with viability increase to Avemar treatment, while ER-cells (MDA-MB231 and MRC5) did not.

The relative optical densities in MTT assays obtained after 24 and 48 hours treatment of MCF-7 cells with the various substances are shown in Fig. 2. Tamoxifen exhibited a slight decrease relative to the control values at both time points. A statistically significant (*p* < 0.02) increase was found at 24 and 48 hours, respectively, after Avemar treatment, and the same phenomenon appeared with Avemar plus tamoxifen.



Flow Cytometry Analysis

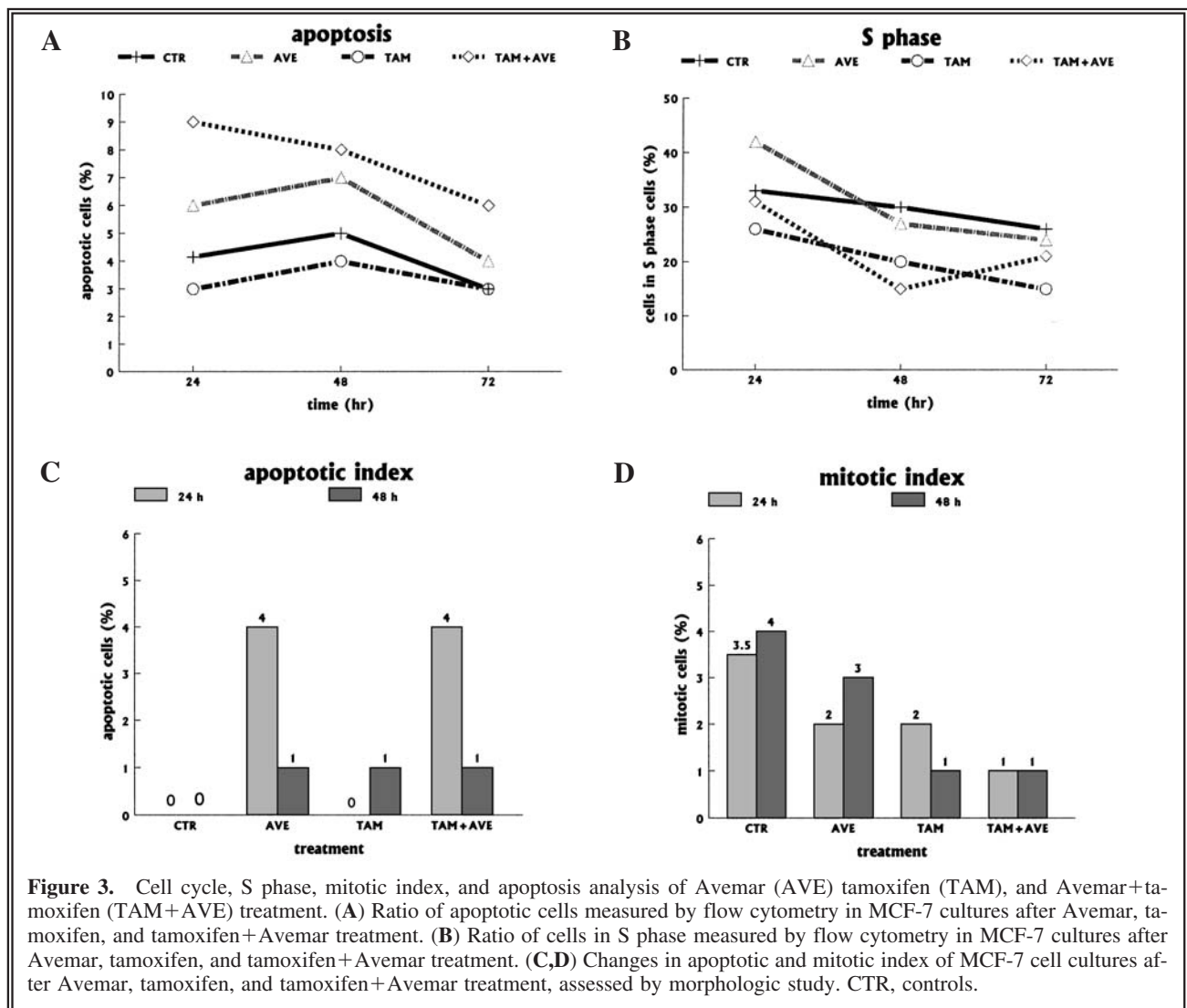
Avemar markedly enhanced apoptosis of MCF-7 cells after 24 hours of the treatment, followed by a further increase at the 48th hour. Tamoxifen alone slightly decreased apoptosis at the 24th hour, and this decrease remained unchanged after 48 hours. On the other hand, the combined treatment of the cell cultures with tamoxifen and Avemar markedly enhanced apoptosis at all time points of the experiments (Fig. 3A).

An elevation of the percentage of cells in the S phase was observed at 24 hours of the Avemar treatment, which was followed by a decrease at the 48th hour, when S-phase ratios became similar to the control values. On the other hand, tamoxifen markedly decreased the ratio of cells in the S phase at the 48th hour of the treatment. Ave-

mar combined with tamoxifen had no influence on the inhibitory effect of tamoxifen alone on the S-phase ratio (Fig. 3B).

Morphological Studies

The ratios of apoptotic and mitotic MCF-7 cells in the control and in the treated cultures are shown in Fig. 3C and Fig. 3D. The results of apoptotic activity correspond—with slight differences—to those obtained by flow cytometry. Regarding apoptosis, both Avemar and Avemar plus tamoxifen treatment induced a higher ratio of apoptosis in comparison to the controls. Mitotic activity was decreased by tamoxifen and, to some extent, by Avemar, compared to the controls. Avemar plus tamoxifen treatment resulted in very low mitotic activity at 24 and 48 hours.



Estrogen-Receptor Activity

Estrogen-receptor activities of MCF-7 cells treated for 24 and 48 hours with various substances are shown in Figs. 4A–B. Both estrogen and Avemar increased the transcriptional activity of ER at 48 hours. The values obtained by tamoxifen treatment at any time point were essentially lower, compared to the controls. The cell culture treated with Avemar plus tamoxifen

showed no significant decrease at 24 hours and significant decrease at hour 48, compared to the controls. No significant difference could be detected between tamoxifen and Avemar plus tamoxifen-treated cells at 24 or 48 hours, respectively.

DISCUSSION

The administration of the medical nutriment, Avemar, is recommended for cancer patients in an average daily dose of 9 g. In our experiments, *in vitro* Avemar treatment caused a slight increase in the viability of ER+ cell lines (MCF-7 and HepG2) when applied in a dose lower than 625 $\mu\text{g}/\text{mL}$. This fact raises the question whether Avemar is able to compete with the antiestrogen effects of tamoxifen.

Our results indicate that tamoxifen downregulates the estrogen-receptor activity in MCF-7 cells after 24 and 48 hours of treatment. The downregulation can be prevented by simultaneous Avemar administration at 24 hours. At 48 hours, the combination of tamoxifen and Avemar combination downregulates the receptor activity to that level seen with tamoxifen alone.

The data show a marked apoptosis-inducing effect of Avemar treatment on ER+ cells, which is significantly potentiated by simultaneous tamoxifen administration. Morphological studies on cells growing on cover-slips gave similar results to those measured by flow cytometry.

Studies on the proportion of cell population in the S phase revealed an initial overshoot (at 24 hours) by the cells treated with Avemar, which was followed by values close to those of the controls. Tamoxifen decreased the S-phase index in all time points investigated, and Avemar did not alter the lowering effect of tamoxifen on the S phase.

Regarding the MTT assay, Avemar exerted an enhancing effect at 24 and 48 hours, respectively, and showed a control value at hour 72. This effect may be explained by the initial increase in S-phase cells (at 24 hours), followed by an increase in apoptosis (at 48 hours). Tamoxifen was ineffective in the first 2 days of treatment and provided a slight decrease in viability at hour 72. The combination of tamoxifen and Avemar resulted in nearly the same effect, as observed after Avemar treatment alone.

The effect of tamoxifen on estrogen receptor-positive breast carcinoma cultures has been

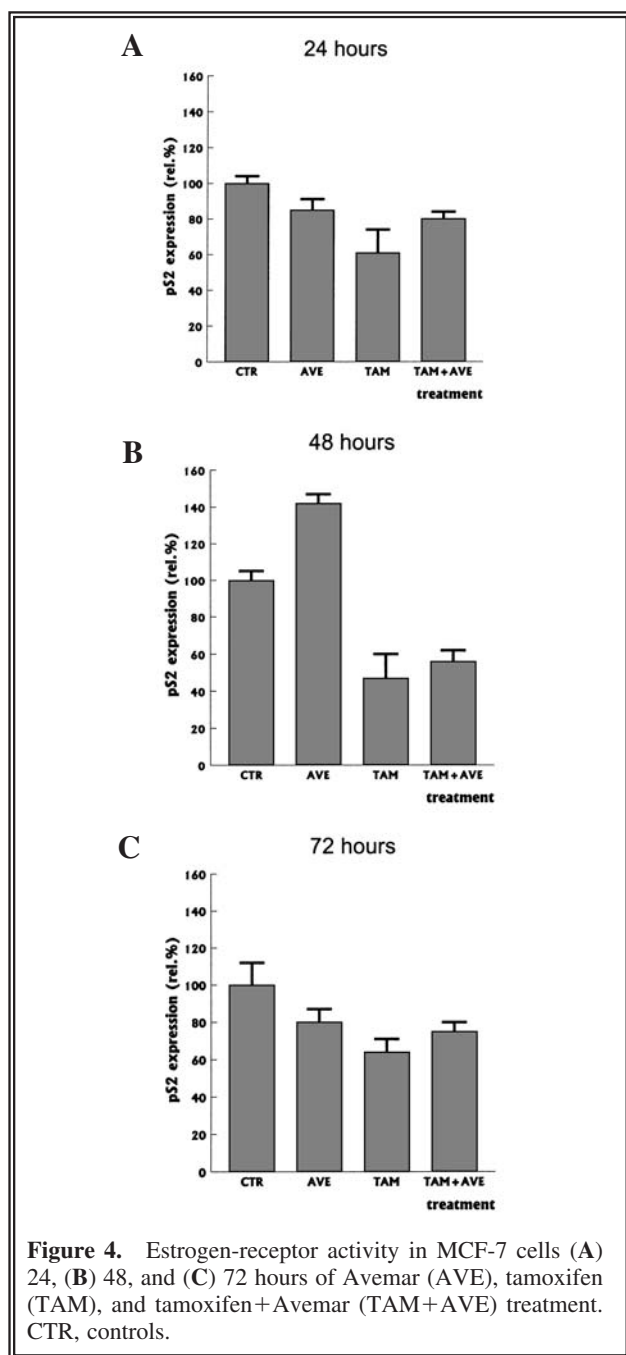


Figure 4. Estrogen-receptor activity in MCF-7 cells (A) 24, (B) 48, and (C) 72 hours of Avemar (AVE), tamoxifen (TAM), and tamoxifen+Avemar (TAM+AVE) treatment. CTR, controls.

widely studied.^{5,22} The type of programmed cell death caused by tamoxifen is predominantly apoptosis, but in cultures cytoplasmic vacuolar cell death has also been shown.^{18,23} In our study, in accordance with the literature,^{24,25} decreased proliferative activity occurred in an earlier period (at 24 hours), and apoptosis could be observed later (at 72 hours).

The mode of action of Avemar regarding *in vivo* retardation of tumor progression is based on its specific metabolic effects in cancer cells and on its apoptotic induction activity. Avemar inhibits glycolysis and pentose cycle enzymes in cancer cells, and induces apoptosis through caspase-3-mediated poly(ADP-ribose)polymerase cleavage.¹¹ In our study, the apoptosis-inducing effect of Avemar became evident in breast cancer cell lines, too. When, in the mentioned cancer cells, the fermented wheat germ extract was applied in combination with tamoxifen, this phenomenon, together with the normalization of the expression of estrogen receptors, was reinforced.

CONCLUSION

In conclusion, the medical nutriment, Avemar, enhances the efficacy of tamoxifen in estrogen receptor-positive breast cancer and, thus, may be recommended as a supportive aid during tamoxifen treatment of such tumors.

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