Reprinted from:

2nd Congress of the World Society for Breast Health

Budapest (Hungary), June 24-28, 2003

MONDUZZI EDITORE

INTERNATIONAL PROCEEDINGS DIVISION

Chemoprevention with Tamoxifen and Avemar® by Inducing Apoptosis on MCF-7 (ER+) Breast Cancer Cells

A. Tompa, Zs. Kocsis, Z. Marcsek, M. Jakab, B. Szende and M. Hidvégi*

*National Institute of Chemical Safety, Budapest, Hungary. *Biromedicina First Hungarian Corporation for Cancer Research and Oncology, Budapest, Hungary*

Summary

In the present study the combined effect of in vitro tamoxifen and Avemar® treatment was studied on MCF-7 (ER+) breast cells as a model of a breast cancer situation. Cells were treated for 24, 48 and 72 hours, cytotoxicity was measured by MTT assay, the percentage of apoptosis and cell proliferation was determined by flow cytometry, hematoxilin/eosin staining and by immunochemistry using the ApopTag® reaction. Estrogen receptor activation was studied by semi-quantitative determination of the estrogen-responsive pS2 gene mRNA production. Tamoxifen had no effect on apoptosis but significantly reduced the percentage of the S-phase. Avemar® significantly increased apoptosis after 48 hours of the treatment. Apoptosis was increased when Avemar® was added to tamoxifen treatment, but tamoxifen induced reduction in cell proliferation remained decreased. Estrogen receptor activity of MCF-7 cells was enhanced by Avemar®, decreased by tamoxifen and combined tamoxifen+Avemar® treatments. Results suggest that the use of the supplementary therapy with Avemar® in the case of ER+ breast cancer tumors is not contra-indicated and it may enhance the effect of tamoxifen on ER+ breast tumors.

Introduction

Tamoxifen, an anti-estrogen drug is commonly used in the therapy of ER+ breast cancer. Tamoxifen is decreasing cell proliferation of ER+ cells. Avemar® (a fermented extract of wheat germ, a complex mixture containing biologically active molecules) is used to supplement different therapies

of tumors, as it is reported to decrease the incidence of metastases - especially in combination with cytostatics - and Avemar® was reported to influence tumor cell proliferation by inducing rogrammed cell death.

In the present study the effect of the combination of in vitro tamoxifen and Avemar® treatment was investigated on ER+ and ER- cell lines in order to detect possible agonistic or antagonistic reactions on the cytotoxicity of in vitro Avemar® treatment, on the effects of Avemar® on apoptosis, on cell cycle and estrogen receptor activation. 17β estradiol was used as positive control in the experiments

Materials and Methods

Cell lines

MCF-7 (European Collection of Cell Cultures, UK ECACC 86012803) ER+ and MDA-MB-231 (ECACC 92020424) ER- human breast adenocarcinoma cells, MRC-5 (ECACC 84101801) ER- human fetal lung cells, HepG2 (American Type Culture Collection, USA, ATCC HB-8065) ER+ cells.

Cells were cultured in DMEM with 10%(v/v) heat-inactivated fetal calf serum, 2mM L-glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin.

Treatments

Cytotoxicity testing of Avemar® (at 24 hours) was performed in the concentration range of 156 μg/ml and 5000 mg/ml. Experiments to determine apoptosis, cell proliferation and ER activation were made with an Avemar® concentration of 625 μg/ml (highest non-cytotoxic dose), the concentrations of tamoxifen and 17β estradiol were: 1 nM. Tamoxifen and 17b-estradiol were administered to cell cultures 24 h after plating.

MTT assay

Cytotoxic effects were determined using tetrazolium dye (MTT; 3[4,5 dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) and optical density (O.D.)was determined using an Anthos 2020 (Salzburg, Austria) ELISA microplate reader at wavelength of 570nm and a reference wavelength of 690nm.

Flow cytometry

MCF-7 cells were cultured for 48h. The cells were treated at hour 24. Pulse labeling of MCF-7 cells with 5mg/ml 5-bromo-2'-deoxyuridine was made 2 hours prior to the termination of the cultures.

After trypsinisation cells were washed twice with PBS, fixed in 1 ml of ice-cold 70% ethanol and stored at –20 oC until further processing.

DNA denaturation prior to PI and FITC-anti-BrdU staining was performed at room temperature with 2M HCl containing 0.2mg/ml pepsin. DNA was stained with propidium iodide (PI) and the incorporated BrdU was detected immunocytochemically with FITC-labeled monoclonal antibody.

Flow cytometric analysis was performed in a FACSCalibur (Beckton-Dickinson) flow cytometer, CellQuest software (Beckton-Dickinson) was used for the analysis of the obtained data.

Morphological detection of apoptosis and mitosis

MCF-7 human breast cancer cells were plated on glass coverslips, fixed in methanol: acetic acid. Cells were stained with haematoxylin and eosin. Apoptosis was detected immunocytochemically by the ApopTag® reaction (Intergen Company) using terminal deoxynucleotidyl transferase to bind digoxigenin- nucleotides to the apoptotically fragmented DNA. Apoptotic cells were visualized with the peroxidase reaction of peroxidase conjugated anti-digoxigenin (Intergen Company) and diamino-benzidine as chromogene (Vector Laboratories).

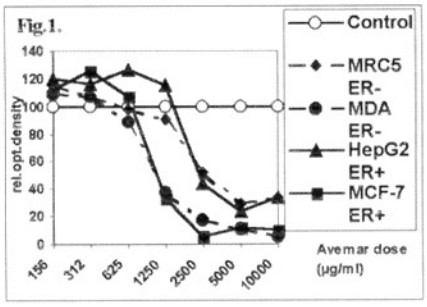
Apoptotic and mitotic figures among 200 cells were counted and the values (apoptotic and mitotic index) were expressed as a percentage.

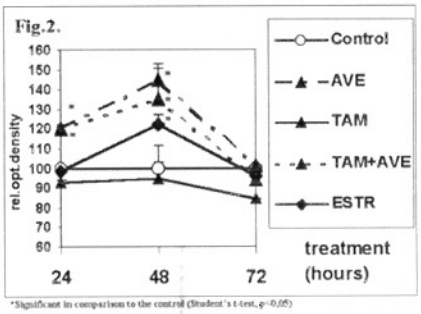
Estrogen receptor activation (semi-quantitative PCR protocol determination of the estrogen-responsive pS2 gene mRNA production)

RNA was isolated from cell cultures using GenElute total RNA isolation kit. Reverse transcriptase reaction was performed in 20ml reaction volumes using 1ml of isolated total RNA, 0.1mg oligo-dT12-18 primers, 15mM deoxyribonucleoside triphosphates each dATP, dCTP, dGTP and TTP, 200U of M MLV reverse transcriptase. In the PCR reactions parallel amplification of 18S ribosomal RNA (rRNA) was performed as an internal control of the constantly expressed RNA. pS2-specific primers were 5'CATGGAGAACAAGGTGATCTG and 5'CAGAAGCGTGTC TGAGGTGTC amplifying 336 bp DNA, 18S-rRNA primers were 5'GTAACCCGTTGAACCCCATT 3' and 5'CCATCCAATCGG TAGTAGCG 3' producing 151 bp PCR fragment. PCR reactions were performed in a Techne Progene thermocycler (Cambridge, U.K.) PCR products were analyzed on 2% agarose TBE gels containing ethidium bromide. Gels were analyzed by the Kodak EDAS 290 image analysis system Ethidium bromide-stained band intensities were evaluated as relative intensity values to the 18S rRNA PCR product and expressed as percent of control.

Results

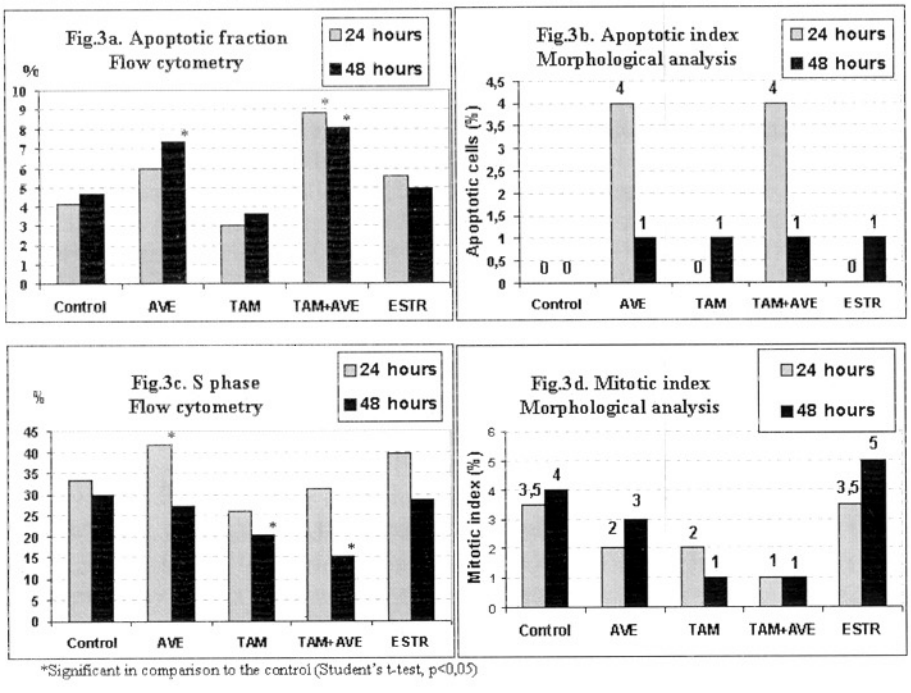
The cytotoxicity of Avemar® on ER- and ER+ cell lines is shown in Fig. 1.

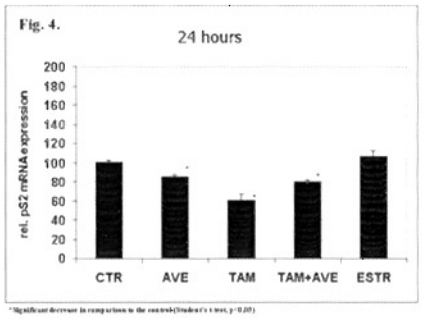


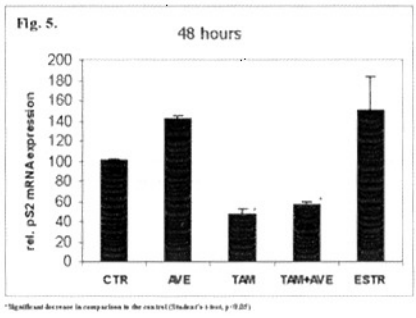


and the effect of 24, 48 and 72 hour Avemar® (AVE, 625 µg/ml), tamoxifen (TAM, 1 nM), 17β-estradiol (ESTR, 1 nM), TAM+AVE treatment of MCF-7 ER+ cells on values obtained by MTT assay is summarized in Fig. 2.

The percentage of APOPTOTIC CELLS measured by flow cytometry and changes in APOPTOTIC INDEX, assessed by morphological analysis are shown in Fig.3a. and Fig.3b., the percentage of cells in S PHASE measured by flow cytometry and the changes in MITOTIC INDEX assessed by morphological analysis are shown in Fig.3c.and Fig.3d., respectively in MCF-7 ER+ cultures after 24 and 48 hour Avemar® (AVE, 625 µg/ml), tamoxifen







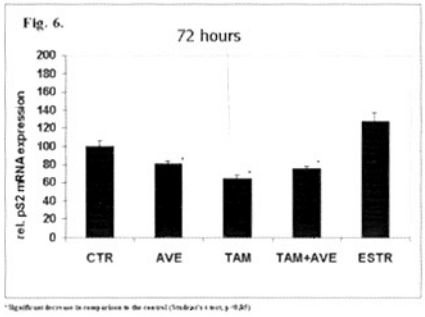
(TAM, 1 nM), 17β-estradiol (ESTR, 1 nM), and TAM+AVE treatment.

Results of estrogen receptor activity measurements in MCF-7 cells after 24 (Fig.4.), 48 (Fig.5.) and 72 (Fig.6.) hours Avemar® (AVE, 625 μg/ml), tamoxifen (TAM, 1 nM), 17β-estradiol (ESTR, 1 nM), and TAM+AVE treatment show that receptor activity was inhibited by tamoxifen treatment although Avemar® alone increased the receptor expression after 48 hours. The effect caused by continued treatment was similar to the effect caused by tamoxifen alone after 48 and 72 hours (Figs. 5. and 6.).

Conclusions

In vitro Avemar® treatment caused a slight increase in viability of ER+ cell lines (MCF-7 and HepG2). Avemar® treatment on ER+ cells shows a marked apoptosis-inducing effect, which is significantly potentiated by simultaneous tamoxifen administration. Studies on the proportion of cell population in S phase revealed an initial overshoot (at 24 hours) by the cells treated with Avemar®. Tamoxifen decreased the S phase index at any time investigated what was not modified by Avemar®.

Tamoxifen downregulates estrogen receptor activity in MCF 7 cells after 24 and 48 hours of treatment. This downregulation can be prevented by simultaneous Avemar® administration at 24 hours.



In conclusion it appears that supplementary therapy with Avemar® in estrogen receptor positive breast cancer may enhance the effect of tamoxifen.

Acknowledgements

This study was supported by OMFB-02440/2000 and by NKFP-1/016/2001

References

FAJKA-BOJA, R., HIDVÉGI, M., SHOENFELD, Y., ION, G., DEMYDENKO, D., TÖMÖSKÖZI-FARKAS, R., VIZLER,C., TELEKES, A., RESETAR, A., MONOSTORI, E.: 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: 563-70, 2002.

HIDVÉGI, M, RÁSÓ, E., TÖMÖSKÖZI-FARKAS, R., SZENDE, B., PAKU, S., PRÓNAI, L., BOCSI, J., LAPIS, K.: MSC, a new benzoquinone-containing natural product with antimetastatic effect. Cancer Biother Radiopharm., 14: 277-89, 1999.

HIDVÉGI, M., RÁSÓ E., TÖMÖSKÖZI-FARKAS R., PAKU, S., LAPIS, K., SZENDE, B.: Effect of Avemar and Avemar + vitamin C on tumor growth and metastasis in experimental animals. Anticancer Res., 18: 2353-2358, 1998.

ZALATNAI, A., LAPIS, K., SZENDE, B., RÁSÓ, E., TELEKES, A., RESETAR, A., HIDVÉGI, M.: Wheat germ extract inhibits experimental colon carcinogenesis in F-344 rats. Carcinogenesis, 22: 1649-1652, 2001

Volume ISBN 88-323-3165-9
CD ISBN 88-323-3166-7

MONDUZZI EDITORE
Via Ferrarese, 119/2 – 40128 Bologna, Italy
Tel ++ 39-051 4151123 – Fax ++ 39-051 4151125