



Efficacy of the Antiproliferative Effect of L-Asparaginase from *Streptomyces enissocaesilis* on Different Tumor Cell Lines

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Abstract

The antiproliferative effect of the purified L-Asparaginase enzyme on different tumor human cell lines have been investigated using human leukemic cells. These included MCF 7 (breast cancer cell line), MDA-MB435S (Breast Metastatic cancer), HeLa cell line, Human small cell lung cancer cell line H69PR (ATCC® CRL-11350™) and COLO 205 (ATCC® CCL-222™). The dose- and time-dependent antitumor and cytotoxic effects of L-asparaginase from *Streptomyces enissocaesilis* have been studied. We investigated the sensitivity of tumor cells towards L-asparaginase, as well the effect of L-asparaginase on cell growth rate, protein and DNA synthesis in the presence of various cytostatics. Cell cycle analysis by flow cytofluorimetry and detection of apoptotic cells before and after treatment with L-asparaginase were studied. Experiments have been conducted to investigate whether administration of L-asparaginase, which results in hydrolysis of extracellular asparagine, arrests asparagine dependent protein synthesis and causes subsequent inhibition of cell growth followed by decreased proliferation of leukemic cells.

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L-asparaginase, Tumor cells, Cytotoxicity, DNA and protein synthesis, Apoptosis.

Introduction

L-asparaginase is the first studied enzyme possessing anticancer activity (Pasut *et al.*, 2007). Tumour cells, more specifically lymphatic cells and certain other tumour cells require huge amount of L-asparagine to keep up with their rapid malignant growth but they lack or have a very low level of asparagine synthetase hence do not synthesis L-asparagine denovo. These cells use L-asparagine from the diet (blood stream) to satisfy their large L-asparagine demand (Narta *et al.*, 2007). L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia thereby selectively kills

tumour cells that rely on asparagine supplied by the serum for survival (Uyttebroeck *et al.*, 2008).

Asselin *et al.*, (1989) have studied the cytotoxic action of L-asparaginase both in vitro and in vivo in patients with ALL undergoing treatment with L-asparaginase as a single agent. This study explored the L-asparaginase induced cell cycle arrest in G2/S phase and apoptotic cell death of leukemic cells. It has been reported that there is a requirement of a functional p53 protein for L-asparaginase to induce apoptosis as observed in studies on JURKAT and HL 60 cell lines (Fu *et al.*, 1989, Nandy *et al.*, 1989). Subsequent studies revealed the action of

L-asparaginase on the various leukemic cells. Human acute lymphoblastic leukemia cell line is markedly inhibited by L-asparaginase, in the G2/S phase (Shimizu *et al.*, 1992). The recent research conventional showed out for the production of L-asparaginase from *Penicillium sp.* had shown cytotoxic activity (Patro *et al.*, 2012) and the cytotoxic activity of L-asparaginase from the marine actinomycete *Streptomyces acrimycini* NGP (Selvam and Vishnupriya, 2013).

Materials and Methods

Cancer Cell Lines

Human cancer cell lines - MCF-7 Cell line (Breast Cancer), MDA-MB435S Cell line (Breast Metastatic cancer), Human cervical cancer cell line (HeLa), Human small cell lung cancer cell line H69PR (ATCC® CRL-11350™) and COLO 205 (ATCC® CCL-222™) used in this study were procured from National Centre for Cell Science, Pune.

Culturing of cells

Tumor cells at the logarithmic growth phase with a cell density of $5 \times 10^8/L$ were inoculated into 25 ml culture media containing the purified L-asparaginase at a concentration of 200 mg/L. Cells were grown in 5 % CO₂ incubator at 37°C for 24 h. The morphology of cells was monitored with an inverse microscope. Cells were then isolated by centrifugation at 200×g for 10 min. After discarding the supernatant, cells were fixed with 4 % pentadialdehyde for 24 h followed by a thorough wash with double-distilled water. After dehydration with gradient alcohol washing, cells were embedded with EPON-812 resin. Dissection was accomplished using an LKB-V model microtome. Cell sections were stained with sodium acetate and lead citrate, and subjected to morphology investigation with an H-300 transmission electron microscope.

Cells were cultured in a humid atmosphere (5% CO₂, 37°C) using DMEM and RPMI 1640 media, heat inactivated Embryonic Calf Serum (ECS) and Horse Serum (HS) obtained from Gibco (USA). MCF-7 and MDA-MB435S were cultivated in the DMEM medium, HeLa were cultured in the RPMI 1640, Lung cancer cell lines and Colon cancer cell lines were cultivated in the mixture of DMEM and RPMI 1640 (1:1). All media contained 10% fetal bovine serum (FBS). The media contained 2 mM glutamine, antibiotics penicillin and streptomycin (10 U/mL and 10 µg/mL, respectively).

Determination of cytotoxic activity of asparaginases

For detection of cytotoxic antitumor activity of L-asparaginase, the cells were seeded into 96 well plates in 100µl of respective medium containing 10% FBS at plating density of 10,000 cells/well and incubated at 37°C, 5% CO₂, 95% air and 100% relative humidity for 24 hours prior to addition of extract. The extract was solubilised in Dimethyl Sulfoxide and diluted in respective serum free medium. After 24 hours, 100ml of the medium containing the extract at various concentration (6.25, 12.5, 25, 50, 100 & 200 µg/ml) was added and incubated at 37°C, 5% CO₂, 95% air and 100% relative humidity for 48 hours. Triplicate was maintained and medium containing without extract were served as control. After 48 hours, 15µl of MTT (5mg/ml) in PBS was added to each well and incubated at 37°C for 4 hours. The medium with MTT was flicked off and the formed formazan crystals were solubilised in 100µl of DMSO and then measured the absorbance at 570nm using micro plate reader. The % cell inhibition was determined using following formula.

$$\% \text{ Cell Inhibition} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100$$

Effect of L-asparaginase on protein and cell growth in cancer cells

The combined effect of asparaginase with cytostatics on protein and cell growth was analyzed using the cytostatic methotrexate (5µg/mL). *Streptomyces enissocaesilis* AUBT1404 L-asparaginase concentration was 5.0 IU/ml. The experiments were performed using human leukemia cell lines MCF-7 Cell line (Breast Cancer) and MDA-MB435S Cell line (Breast Metastatic cancer). The cell growth and protein synthesis was evaluated by adding 1 µ Ci/well of [14C] amino acids and [14C] thymidine, respectively 5 h before the end of experiments performed in 96 well plates. After this incubation the growth medium was carefully removed and the cell sediment or monolayer was treated overnight at -10°C with 0.1 ml of the ice cold fixing solution ethanol: acetic acid (9: 1) to remove radioactivity of the acid soluble pool (Hashimoto *et al.*, 2009) were then washed with ice cold Hanks solution and after addition of 50 µL 0.3 M KOH were placed into a thermostat at 37°C for 10–12 h. The resultant cell hydrolyzate was neutralized with 1.0 M HClO₄ up to pH 7.0 and its radioactivity was counted using a scintillation counter Tri Carb 2800 TR (Perkin Elmer) in Bray liquid. After blank deduction results were calculated as cpm per 106 cells and as % of control. The

number of cells was evaluated in the MTT test using data obtained during cell counting in the Goryaev chamber and the extraction value in the MTT test. Statistical treatment of results (including calculation of mean and SD values) and their graphic presentation were performed by the Excel program. Cell cycle analysis by flow cytometry and detection of apoptotic cells before and after treatment of tumor cells with L-asparaginase were performed as described by Walker *et al.*, (1993). Tumor cells (0.5×10^6) were cultivated in 25 cm² flasks containing 5 mL of the growth medium under standard conditions. After cultivation for 24 h asparaginase preparation (5 IU/mL) was added and cultivation continued for 72 h. After this cultivation cell sediment was collected, treated with propidium iodide (Sigma, USA) for analysis in a cytometer.

Results and Discussion

The antiproliferative effect of the purified L-Asparaginase enzyme on different tumor human cell lines; MCF 7 (breast cancer cell line), MDA-MB435S (Breast Metastatic cancer), HeLa cell line, Human small cell lung cancer cell line H69PR (ATCC® CRL-11350™) and COLO 205 (ATCC® CCL-222™) was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan (Mosmann, 1983). The *in vitro* cytotoxic effect of *S. enissocaesilis*

L-Asparaginase enzyme on the growth of five human tumor cell lines showed that the enzyme had significant inhibitory effects in different cell lines (Tables 1 and 2). However, the highest antitumor activity was recorded towards MCF-7(82.3%), MDA-MB435S (71.7%), HeLa and H69PR (ATCC® CRL-11350™) were less sensitive (%CI of 64.2 and 60 respectively), while the least activity was obtained towards COLO 205 (52.1%) when compared with the growth of untreated control cells. The incubation of MCF 7 with gradual doses of *S. enissocaesilis* L-asparaginase lead to a gradual inhibition in the cell growth as depicted by the % CI values. The inhibitory efficiency to all tumor cells was directly proportional to the dosage applied.

Determination of dose response studies using standard drugs

Figures 1 and 2 show results of comparative study of the cytotoxic antitumor activities of L-asparaginase and Tamoxifen on MCF 7 cell line and L-asparaginase and Cisplatin on MDA-MB435S, respectively. The results demonstrate that the incubation of MCF 7 (breast cancer cell line) and MDA-MB435S (Breast Metastatic cancer) with increasing amounts of L-asparaginases resulted in a significant dose-dependent decrease in the number of viable (metabolically active) cells as compared with standard drugs.

Table.1 Antitumor activity of purified L-asparaginase on human carcinoma cell lines

CELL LINES	%CI	%CS
MC-7	82.3	17.7
MDA-MB435	71.7	28.3
HeLa	64.2	35.8
H69PR (ATCC® CRL-11350™)	60	40
COLO 205 (ATCC® CCL-222™)	52.1	47.9

Table.2 The effect of L-asparaginase on the number of tumor cells as shown by results of the MTT test performed at 24, 48, and 72 h and shown as absorption at 540 nm

Leukemia cell line	24 h	48 h	72h
MCF-7, Control	0.230	0.595	1.110
MCF-7, Experiment	0.133	0.468	0.470
MDA-MB435S, Control	0.160	0.196	0.312
MDA-MB435S, Experiment	0.125	0.156	0.166

Table.3 Cell cycle analysis by flow cytometry and detection of apoptotic tumor cells after cultivation with L-asparaginases

Groups	MCF-7	MDA-MB435S
Control		
Apoptosis	21.4	24.7
G0/G1	40.5	37.8
S	17.2	26.1
G2/M	22.5	31.2
<i>S.enissocaesilis</i> L-Asparaginase		
Apoptosis	90.0	87.1
G0/G1	49.5	40.5
S	26.7	15.2
G2/M	13.6	10.5

Fig.1 Dose-response curve of L-asparaginase on MCF-7 Cell line

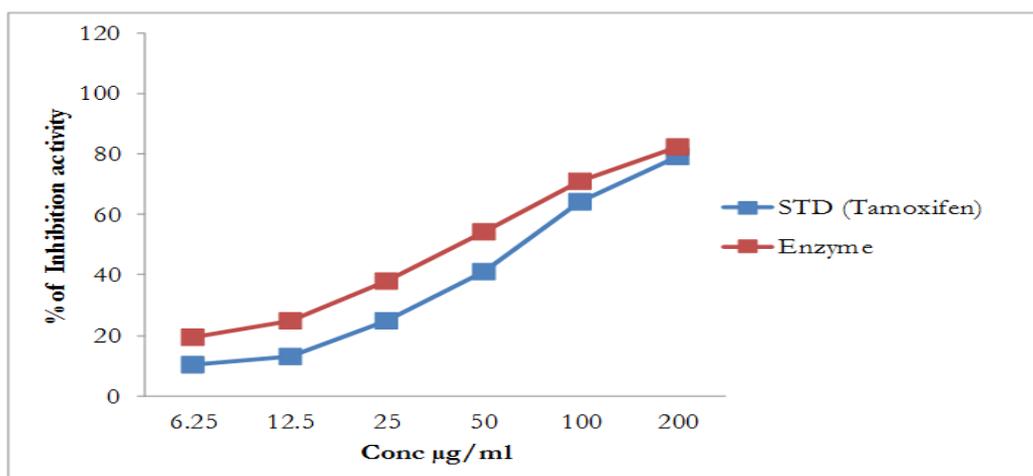


Fig.2 Dose-response curve of L-asparaginase on MDA-MB435S cell line

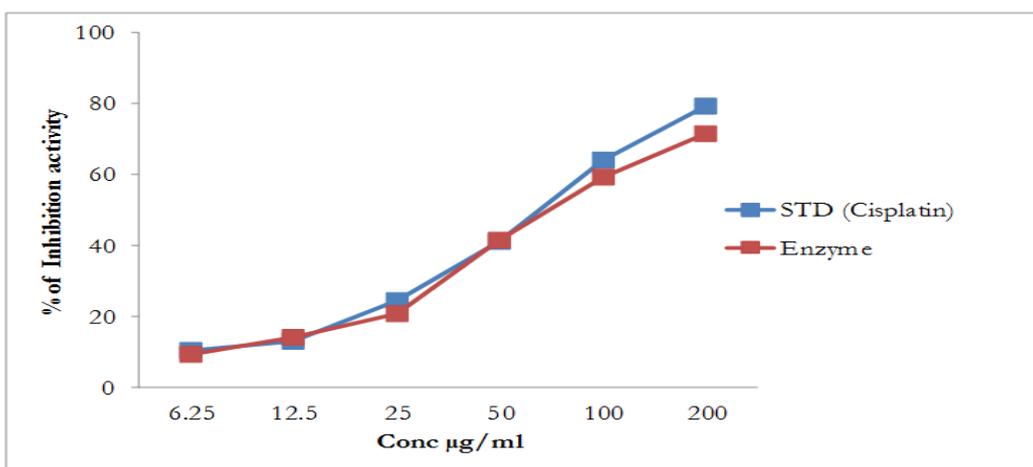


Fig.3 Effect of L-asparaginase on the morphology of the MCF-7cells

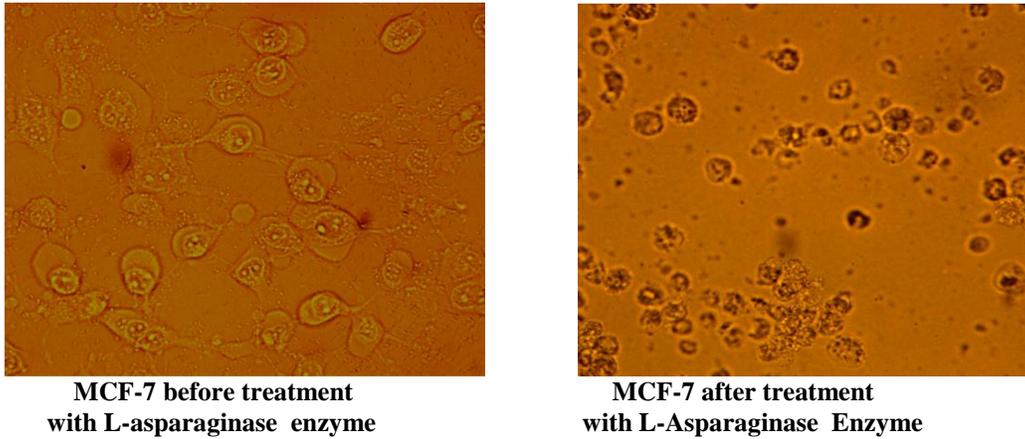


Fig.4 Effect of L-asparaginase on the morphology of MDA-MB435S cell

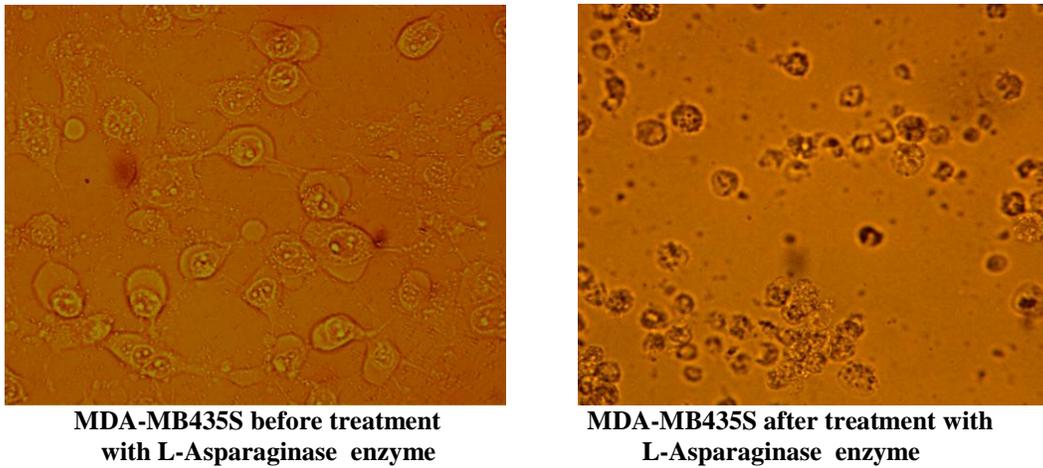


Fig.5 The effect of the combination of L-Asparaginase with cytostatic methotrexate on protein synthesis of MCF-7

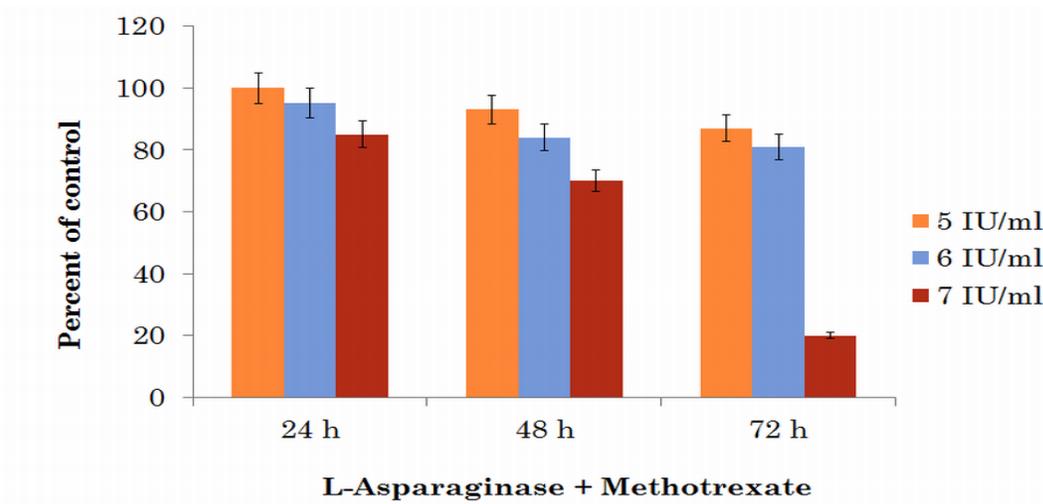


Fig.6 The effect of the combination of L-asparaginase with cytostatic methotrexate on cell growth of MCF-7

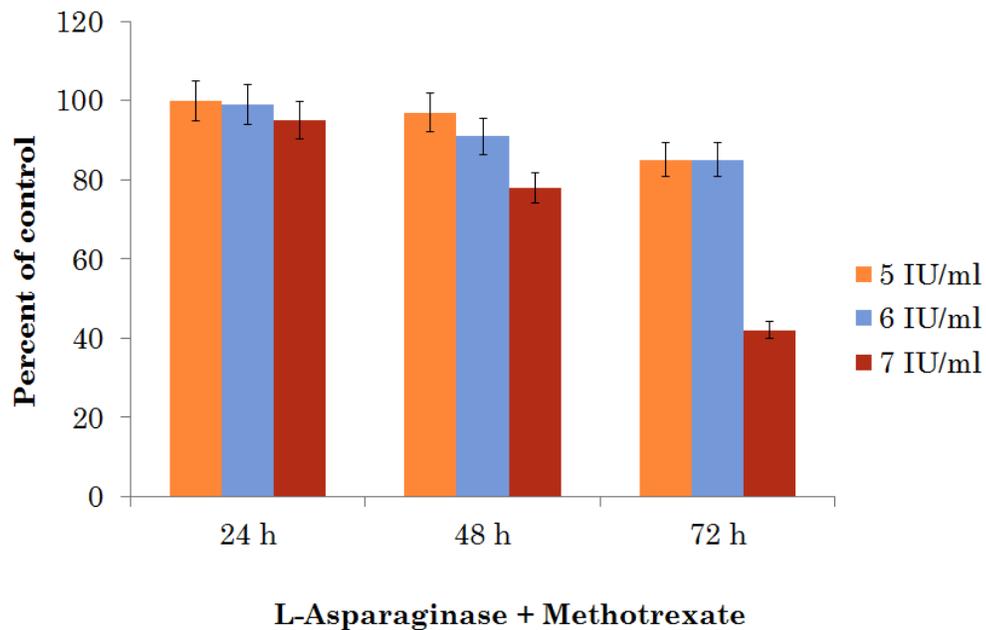
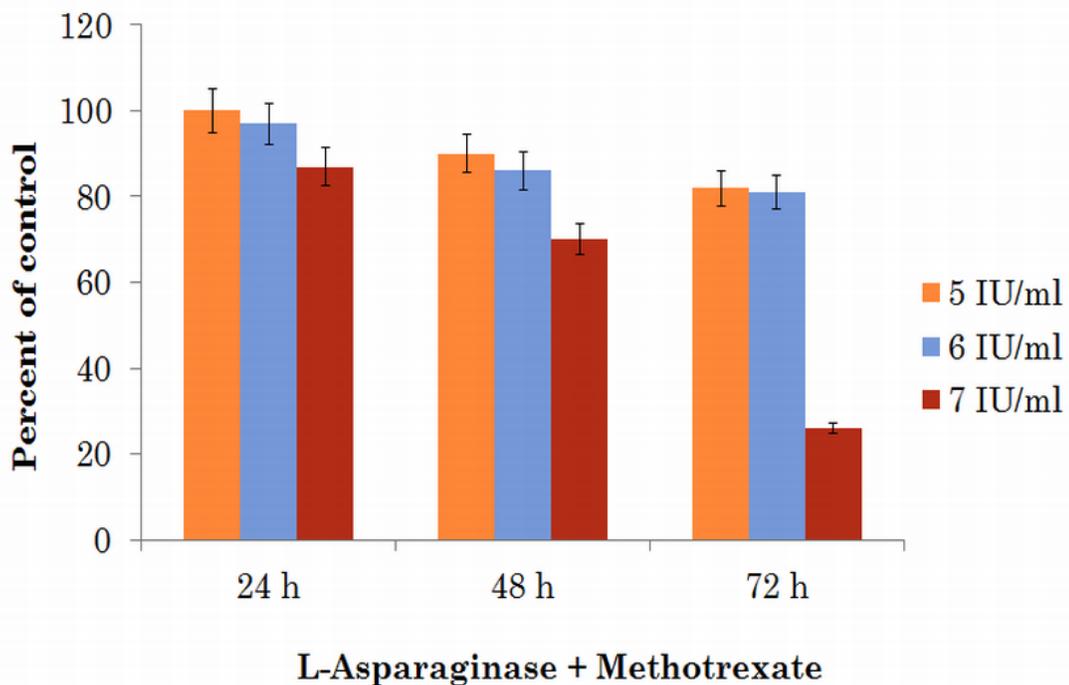


Fig.7 The effect of the combination of L-asparaginase with cytostatic methotrexate on protein synthesis of MDA-MB4352



These results suggest that the isolated L-Asparaginase suppressed cell growth of both the tumor cell lines at certain doses starting from 6.25 µg/ml. Our results on the antitumor cytotoxic toxicity of *S. enissocaesilis* L-asparaginase *in vitro* are rather optimistic. Asselin *et al.*,

(1989) demonstrated linear correlation between death of leukemia cells treated with asparaginases *in vitro* and *in vivo*. Thus, results of *in vitro* tests on asparaginase cytotoxic activity may be used for prediction of the enzyme activity or *in vivo* sensitivity to chemotherapy of

acute lymphoblast leukemia and possibly solid tumors in humans.

Flowcytometry

It was demonstrated that treatments of MCF-7 and MDA-MB435S cell lines with *S. enissocaesilis* L-asparaginase had profound effect on the morphology of the cells (Figures 3 AND 4). At the same time cell cycle analysis by flow cytofluorimetry of MCF-7 and MDA-MB435S cells treated with asparaginase and determination of the number of leukemic cells 24, 48, and 72 h after onset of asparaginase treatment suggest that under asparagine deficit these cells stop normal division (compared with corresponding control) and die. The number of these cells decreased within the time interval of this experiment (24–72 h). This is consistent with observation by Asselin *et al.*, (1989).

Table 3 shows that treatment with L-asparaginases was effective in increasing the number of MCF-7 and MDA-MB435S apoptotic cells. MCF-7 and MDA-MB435S cells containing almost 21.4% and 24.7.7% of apoptotic cells in control demonstrated sensitivity: the number of apoptotic cells significantly increased after L-asparaginase treatment. The increase in the number of apoptotic cells up to 90% and 87% was observed in MCF-7 and MDA-MB435S cell lines, respectively after their combined treatment with L-asparaginase and methotrexate (5 µg/mL).

Effect of L-asparaginase on cell growth and protien synthesis

Experiments have been conducted to investigate whether administration of L-asparaginase, which results in hydrolysis of extracellular asparagine, arrests asparagine dependent protein synthesis and causes subsequent inhibition of cell growth followed by decreased proliferation of leukemic cells.

Figure 5 & 6 show the effect of combined treatment of L-asparaginase and cytostatic methotrexate (5 µg/mL) on the protein synthesis by MCF-7 and Figures 7 and 8 show the effect of combined treatment of L-asparaginase and cytostatic methotrexate (5 µg/mL) on MDA-MB435S cells. Treatment of MCF-7 cells with L-asparaginase and methotrexate for 24–48 h insignificantly decreased protein synthesis, while the protein synthesis significantly decreased (20% of control) after 72 h only at L-asparaginase of 7 IU/mL; at asparaginase concentrations 2 and 5 IU/mL it remained

elevated. The level of secretion demonstrated dependence to the asparaginase concentrations. Cell growth slightly differed from control cell growth at 24 and 48h. Treatment of cells for 72 h was accompanied by a sharp decrease of cell growth (42% of control).

Protein synthesis in MDA-MB435S cells treated with of L-asparaginase and cytostatic methotrexate did not decrease below control values 24 and 48 h after asparaginase addition, but decreased at 72 h at asparaginase concentration of 7 IU/mL (26% of control). The combined treatment for 72 h the cell growth represented 50% of control.

In conclusion, this study implies that *S. enissocaesilis* L-asparaginase may be a potent drug for treatment of MCF-7 and MDA-MB435S leukemia. The Cell cycle analysis by flow cytofluorimetry demonstrated that the L-asparaginase had profound effect on the morphology of the MCF-7 and MDA-MB435S cells and resulted in increased number of apoptotic cells. A linear correlation was observed between the number of the tumor cells and the dose- and time-dependent antitumor and cytotoxic effects of L-asparaginase from *Streptomyces enissocaesilis*. The administration of L-asparaginase resulted in hydrolysis of extracellular asparagines along with arrested asparagine dependent protein synthesis and caused subsequent inhibition of cell growth followed by decreased proliferation of leukemic cells. From the results, it is concluded that this L-asparaginase can be used for the development of new preparations for the therapy of tumors.

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