
SHORT COMMUNICATION

**PRELIMINARY STUDIES EVALUATING CYTOTOXIC EFFECT
OF COMBINED TREATMENT WITH METHOTREXATE AND SIMVASTATIN
ON GREEN MONKEY KIDNEY CELLS**

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Abstract: Patients, affected by neoplastic disease, take usually other drugs and this may lead to a number of often rather unpredictable interactions. The statins are among the most commonly prescribed drugs in medicine but have also adverse side effects and come into interactions with other drugs. The aim of this study was to investigate the cytotoxic effect of methotrexate (5.5 or 16.5 $\mu\text{mol/L}$), simvastatin (100 or 300 $\mu\text{mol/L}$) and their combination on green monkey kidney (GMK) cells culture using cytotoxicity detection kit LDH. Besides, the effect of above drugs on the cells viability was estimated by MTT test. After 6, 12 or 24 h of simultaneous incubation of GMK cells with methotrexate (5.5 $\mu\text{mol/L}$) and simvastatin (100 $\mu\text{mol/L}$) the cytotoxicity (about 10%) of the drugs was found in LDH test. Cytotoxicity of combination: methotrexate (5.5 $\mu\text{mol/L}$) with simvastatin (300 $\mu\text{mol/L}$) after 6 or 12 h of incubation with GMK cells was similar (about 10%), but after 24 h of incubation, cytotoxicity increased to 21%. The significant increase of the cytotoxicity (about 30%) was found after 24 h incubation of GMK cells with methotrexate (16.5 $\mu\text{mol/L}$) and simvastatin (100 $\mu\text{mol/L}$). In the MTT assay, the decrease in the cells viability was found also after 12 and 24 h of GMK cells incubation with methotrexate (5.5 or 16.5 $\mu\text{mol/L}$) and simvastatin (100 or 300 $\mu\text{mol/L}$). These results suggest the adverse effect of combined application of both drugs on GMK cells especially after 24 h of incubation.

Keywords: methotrexate, simvastatin, LDH, MTT, green monkey kidney cells

A lack of knowledge about interactions of one or more drugs taken by patients can have different results, sometimes dangerous to the health. Therefore, there is a need for research on the interaction of drugs used simultaneously in the treatment of various diseases. Particular attention should be paid to drugs used in the chronic diseases of civilization (cancer, atherosclerosis or hypertension). One group of anticancer drugs are antimetabolites, which include methotrexate (MTX) – the subject of this study. MTX is a folate antagonist used for patients with acute lymphoblastic leukemia, osteosarcoma, lymphoma and other kinds of cancer. In addition, it is used at lower doses for patients with non-malignant diseases such as rheumatoid arthritis or psoriasis (1–4). Unfortunately, MTX is a highly toxic drug. MTX may cause the bone marrow depression (anemia, leucopenia, thrombocytopenia) and gastrointestinal toxicity (vomiting, diarrhea or ulcerative stomatitis and hemorrhagic enteritis). The renal dysfunction after MTX treatment is a clinical-

ly important side effect. High dose of MTX induced renal failure because drug is mainly eliminated by the kidney (5, 6). Currently, a large part of the population suffers from arteriosclerosis and hypertension. Therefore, in cancer patients, there is a risk of interaction between cytostatic and lipid-lowering drugs. Simvastatin, the drug used in this study, belongs to the statins. Statins block the synthesis of cholesterol, preventing the formation of mevalonic acid, and thus lower the level of lipids (7–9). Simvastatin is a widely used cholesterol-lowering drug in the treatment of atherosclerosis, in the prevention of cardiovascular diseases, as well as reduces stroke incidence. However, the effects of statins extend beyond their lipid-lowering actions. Recent *in vivo* studies with experimental animals and *in vitro* studies in numerous cancer cell lines have shown antitumor properties of statins. Simvastatin is attributed to inhibition of cell cycle both *in vitro* and *in vivo* (10–13). The use of simvastatin as well as other statins are associated with the

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risk of side effects. The adverse effects of some statins are mainly on muscle, such as myopathy and rhabdomyolysis and liver (14, 15). The statins are the most commonly prescribed drugs in medicine but unfortunately can come into interactions with other drugs. The lack of the literature data about the simultaneous treatment with MTX and statins was the inspiration to undertake the research of the presented work. The aim of this pilot study was to

investigate the cytotoxic effects of MTX and simvastatin on green monkey kidney (GMK) cells using LDH and MTT tests.

EXPERIMENTAL

Reagents

The following substances were used in the study: MTX (Metotrexat-Ebewe, Ebewe Pharma, Unterach,

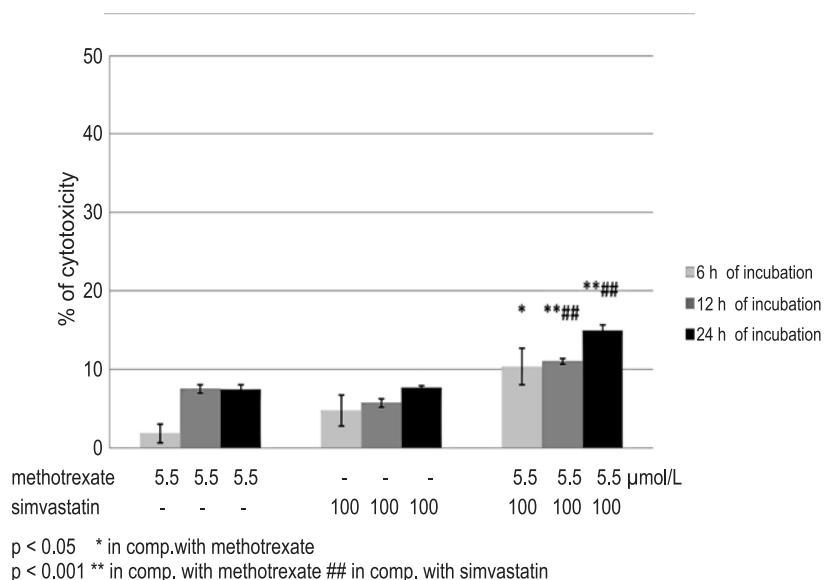


Figure 1. Cytotoxicity of methotrexate (5.5 µmol/L), simvastatin (100 µmol/L) and their combination after incubation with GMK cell culture in LDH test

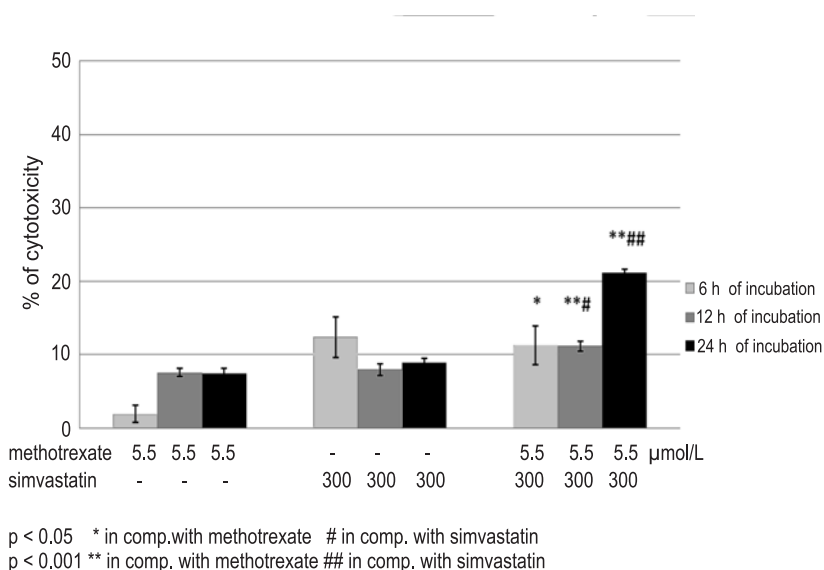
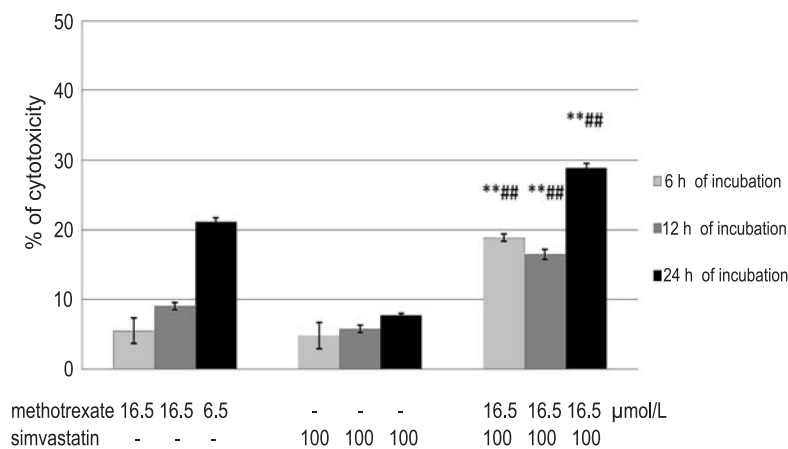


Figure 2. Cytotoxicity of methotrexate (5.5 µmol/L), simvastatin (300 µmol/L) and their combination after incubation with GMK cell culture in LDH test



p < 0.001 ** in comp. with methotrexate ## in comp. with simvastatin

Figure 3. Cytotoxicity of methotrexate (16.5 µmol/L), simvastatin (100 µmol/L) and their combination after incubation with GMK cell culture in LDH test

Austria), simvastatin (Simvacard, Zentiva, Praga, Czech Republic), Cytotoxicity Detection Kit (LDH) (Roche Diagnostic GmbH, Mannheim, Germany), MTT (Thiazolyl blue tetrazolium bromide, Sigma-Aldrich, Steinheim, Germany). The cell culture medium RPMI-1640 (with L-glutamine and phenol red), fetal bovine serum (FBS) and antibiotics solutions: penicillin, streptomycin and amphotericin B (obtained from the PAA – The Cell Culture Company GmbH, Pasching, Austria). MTX and simvastatin were *ex tempore* prepared in RPMI-1640 medium.

Cell culture

The research was performed on green monkey kidney cells (GMK) obtained from the „Biomed” Serum and Vaccine Production Plant Ltd. in Lublin, Poland. GMK cell line was grown in RPMI-1640 medium (with L-glutamine and phenol red) supplemented with 10% fetal bovine serum heat-inactivated, 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B in 25 cm² tissue culture flasks (EasYFlasks™Nunclon™Δ, Nunc GmbH Wiesbaden, Germany). GMK cells were cultured as monolayer in CO₂ cell incubator at 37°C in an atmosphere of 5% CO₂. Afterwards, GMK cells were counted in Neubauer hemocytometer (BlauBrand, BRAND GmbH) by means of the compact inverted microscope Olympus CKX41. The assays of cytotoxicity GMK cells were prepared at density of 2 × 10⁶ cells/cm³.

LDH test

The cytotoxicity detection kit (LDH) is a colorimetric assay for the quantitation of cytotoxicity/cytolysis, based on the measurement of LDH activity released from the damaged cells. To determine the cytotoxic activity of MTX, simvastatin and their combination, drugs were added to GMK cells line and incubated for 6, 12 or 24 h. Both drugs were added together in the same volume 100 µL/well at the following concentrations: (MTX – 5.5 µmol/L and simvastatin – 100 µmol/L); (MTX – 5.5 µmol/L and simvastatin – 300 µmol/L) as well as (MTX – 16.5 µmol/L and simvastatin – 100 µmol/L). Cytotoxicity of MTX, simvastatin and their simultaneous treatment was calculated from equation suggested in the instruction of the manufacturer. Cytotoxicity was expressed in %.

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MTT viability assay

For assay of cell viability, MTT test based on INVITTOX protocol n°17, ECVAM – European Centre for the Validation of Alternative Methods, Database Service on Alternative Methods To Animal Experimentation was used. To determine the effects on cell viability, the combination of MTX and simvastatin were added to GMK cells line in the above concentrations and were incubated for 6, 12 or 24 h. GMK cells viability was expressed in % of control group.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance among groups was determined using analysis of variance (ANOVA) accompanied with *post-hoc* Newman-Keuls test; p-values less than 0.05 were considered significant.

RESULTS

Cytotoxicity of drugs in LDH test

After 6 and 12 h of incubation of GMK kidney cells with the combination of MTX (5.5 $\mu\text{mol/L}$) with simvastatin (100 $\mu\text{mol/L}$), approx. 10% cytotoxic effect was observed, while after 24 h incubation, it rose to about 15% (Fig. 1). It is worth noticing that after the same periods of GMK cell line incubation, with only MTX (5.5 $\mu\text{mol/L}$) or only simvastatin (100 $\mu\text{mol/L}$), cytotoxicity of the drugs was below 10%.

After 6, 12, and 24 h of incubation of GMK cell line with MTX (5.5 $\mu\text{mol/L}$) combined with

simvastatin (300 $\mu\text{mol/L}$), their cytotoxic effect was higher as compared with the groups incubated with each of the drugs separately (Fig. 2). The highest increase (over 20%) was observed after 24 h incubation.

A significant increase in toxicity was also found when using combinations of MTX (16.5 $\mu\text{mol/L}$) with simvastatin (100 $\mu\text{mol/L}$) after the three periods of incubation with GMK cells as compared to the results of groups of GMK cells incubated with each drug separately (Fig. 3). After 24 h of incubation, cytotoxicity of the drugs combined was the highest (about 30%).

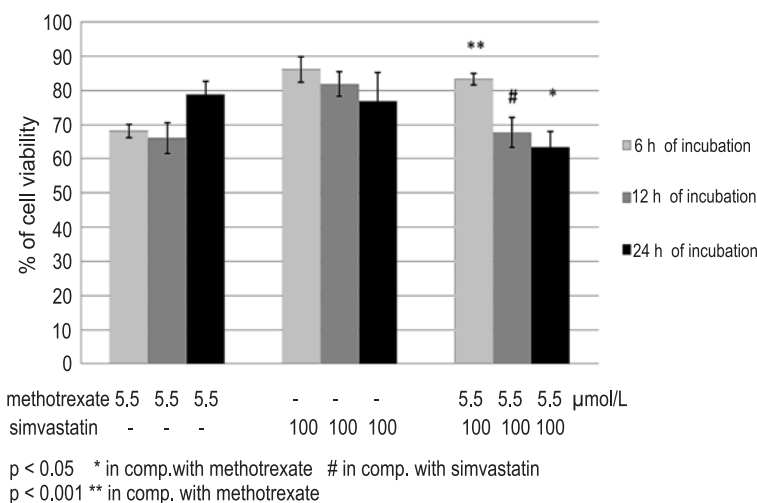


Figure 4. Effect of methotrexate (5.5 $\mu\text{mol/L}$), simvastatin (100 $\mu\text{mol/L}$) and their combination on the GMK cells viability in MTT test

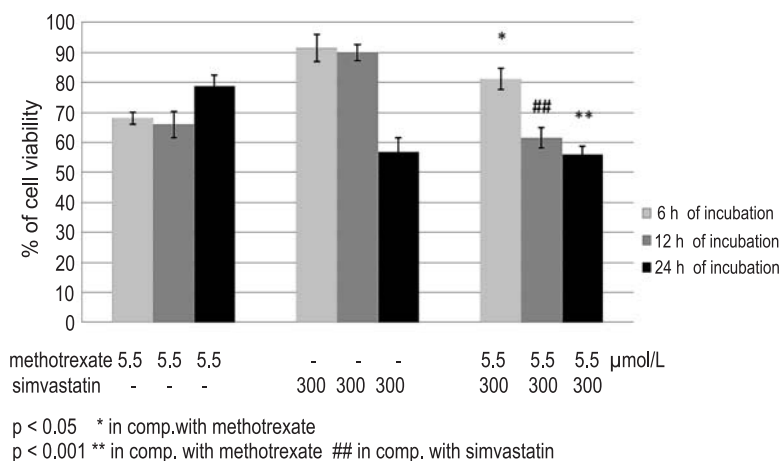


Figure 5. Effect of methotrexate (5.5 $\mu\text{mol/L}$), simvastatin (300 $\mu\text{mol/L}$) and their combination on the GMK cells viability in MTT test

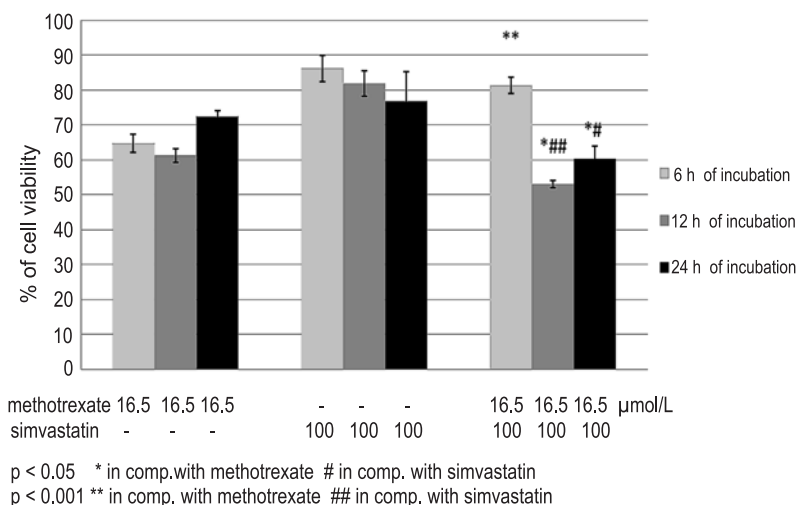


Figure 6. Effect of methotrexate (16.5 µmol/L), simvastatin (100 µmol/L) and their combination on the GMK cells viability in MTT test

The cells viability in MTT test

MTT test was used to measure the effect of administration of the drugs separately and in combination on GMK cell viability after 6, 12 and 24 h incubation. As a result of 6 h incubation of GMK cells with the combination of MTX (5.5 µmol/L) with simvastatin (100 µmol/L), viability was observed to rise when compared with the group of GMK cells incubated with MTX only. Viability of GMK cells after 12 and 24 h incubation was significantly lower as compared with MTX or simvastatin only (67.7 and 63.5%, respectively). It should be noted that after 6, 12 and 24 h incubation of GMK cell line with MTX only (5.5 µmol/L), a significant lowering of cell viability was observed (by about 30% in case of 6 and 12 h incubation and by about 20% after 24 h incubation). After 6, 12 and 24 h incubation of the cell line with simvastatin (100 µmol/L), a decrease was observed in cell viability by 13.7, 18.2 and 23.2%, respectively (Fig. 4).

When both drugs, i.e., MTX (5.5 µmol/L) and simvastatin (300 µmol/L), were applied simultaneously, cells viability was higher after 6 h of incubation, while after 12 and 24 h it decreased by 39 and 45%. It seems, however, that the lowered viability of GMK cells does not result from a point effect of the drug combination but from the effect of the two drugs used separately (Fig. 5).

After 6, 12 and 24 h incubation of GMK cells with MTX (16.5 µmol/L) and simvastatin (100 µmol/L), their viability was about 80, 55 and 60%, respectively. It is worth emphasizing that after 6 h incubation, a significant increase was noted, while

after 12 and 24 h of joint incubation of GMK cells with both the drugs one could see a significant decrease in the cell viability as compared with only MTX or only simvastatin in this case points to the intensification of their cytotoxic effect (Fig. 6).

DISCUSSION AND CONCLUSION

One of important problems in modern pharmacology are drug interactions resulting from a few drugs taken simultaneously by patients. These interactions may lead to a lot of, sometimes dangerous, side effects. Cytostatics used in cancer treatment are definitely drugs with an increased risk of interactions with other drugs and affect also healthy cells (16). Drugs given in combination may produce effects that are greater than or less than the effect predicted from their individual potencies. Administration of high doses of MTX and simultaneous treatment with other drugs increased the risk of damage of kidney, liver, bone marrow, skin or mucous membranes. The kidney are the major route of MTX elimination. Long-term of MTX therapy can cause permanent impairment of kidney function, leading to the delay of drug elimination from the body and the increase of its toxicity. Acute renal failure is the result of intratubular precipitation of the drug crystals in acidic pH of urine (5, 6). There are no data, however, on simultaneous treatment with MTX and a widely used group of drugs – statins (17, 18). Simvastatin, examined in the present study, is one of them. The study evaluated cyto-

toxic effect of MTX, simvastatin and their combination on green monkey kidney cells (GMK) in LDH test, was also evaluated GMK cell viability after incubation with the two drugs in MTT test. The initial MTX concentration (5.5 $\mu\text{mol/L}$) and simvastatin (100 $\mu\text{mol/L}$) were determined in our previous research (19, 20). These concentrations were not toxic (less than the determined IC_{10} inhibitory concentration of 10%) to the GMK cells line after 24 h of incubation. The literature shows that these concentrations of both drugs used in the study are effective for various cells line (21–24). In the case of MTX, the range of cytotoxicity doses for tumor cells used in the works of different authors was quite high and it ranged from $10^9 - 10^6 \text{ mol/L}$ (23–25).

From the research conducted in this study, one can conclude that MTX (5.5 or 16.5 $\mu\text{mol/L}$) in the combination with simvastatin (100 or 300 $\mu\text{mol/L}$) inhibited the growth of GMK cells after 12 or 24 h of incubation. The highest cytotoxicity growth, by about 30% in LDH test, was noted after applying of MTX (16.5 $\mu\text{mol/L}$) into the colony of GMK cells together with simvastatin (100 $\mu\text{mol/L}$). Similarly, the highest decrease of GMK cells viability in the MTT test also was observed after 12 and 24 h of simultaneous incubation of MTX in the combination with simvastatin. The obtained results indicate the negative influence of combined application of both drugs on the cells viability. This observations, in addition to the cognitive aspect, may have practical importance in the treatment of patients with malignant disease, suffering from lipid disorders.

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