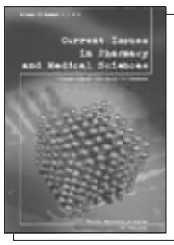

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Interaction between drug Ukrain and Simvastatin *in vitro* studies

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ABSTRACTS

The aim of this study was to investigate the interaction between the drugs Ukrain and Simvastatin *in vitro* studies. The research was conducted on green monkey kidney cells (GMK). The experiment used the drug Ukrain (at concentrations of 50 μmol/l and 150 μmol/l), the drug Simvastatin (at concentrations of 100 μmol/l and 300 μmol/l), and the combination of these two drugs in the ratio of 1:1, 1:3 and 3:1. The cytotoxicity of both drugs were evaluated using a Cytotoxicity Detection Kit (LDH), while cell viability was determined using the MTT assay (based on the MTT assay protocol INVITTOX n° 17, ECVAM), after 6, 12 and 24 h incubation with the tested drugs. Cytotoxicity of drug Ukrain (50 μmol/l) and Simvastatin (100 μmol/l) alone was observed after 6 h incubation of GMK cells with both drugs., while, Ukrain in combination with Simvastatin (300 μmol/l) showed cytotoxicity only after 12 h of incubation. However, cytotoxicity of the drug Ukrain (150 μmol/l), together with Simvastatin (100 μmol/l) was observed after 6 and 12 h incubation with GMK cells. The greatest inhibition growth of green monkey kidney cells was noted after 24 h incubation with all concentrations of these drugs.

Keywords: Ukrain, Simvastatin, LDH, MTT, *in vitro* studies

INTRODUCTION

The semisynthetic drug Ukrain (NSC-631570), a thiophosphoric-acid derivative of alkaloids from *Chelidonium majus* L., exhibits cytotoxic and/or cytostatic activity in cancer cells [5,15,18]. Ukrain also influences the human immune system: increasing the amount of lymphocyte Th, but decreasing the amount of lymphocyte Ts, while increasing NK cells and macrophage activity [5,7,14]. In addition, Ukrain inhibits DNA, RNA and protein synthesis in cancer cells [5,8,13]. This drug, moreover, induces apoptosis [4,5,9,15,18]. Ukrain selectively accumulates in cancer cells [5,18] and leads to a reduction of tumor mass and partial/total remission [5,7,8]. Besides the previous, Ukrain reduces undesirable and toxic effects while it is used with chemotherapy and/or with radiotherapy [5,18].

Among the cancer patients of mature age, treatment often involves frequently combining anticancer drugs with HMG-CoA inhibitors (statins). What is more, statins are widely used for the treatment of hypercholesterolemia [11,12]. In addition, statins demonstrate multi (pleiotropic) action. They improve endothelial function in people with cardiovascular disease. Also, they exhibit antithrombotic and profibrinolytic activity [1,11,12]. Moreover, statins have beneficial effects on the kidney. These drugs inhibit the release of pro-inflammatory cytokines and prevent the accumulation of monocytes in kidney cells [6]. Statins have as well, a cytostatic and cytotoxic effect on tumor cells. They inhibit cell growth and decreased their viability [3,16,17]. In addition, statins inhibit DNA synthesis in cancer cells [17]. It should be noted too, that statins selectively induce apoptosis in cancer cells. This discovery is important because the majority of cytostatic drugs equally destroy cancer cells and normal dividing cells [2,10,17]. The significant role of statins in inhibiting cancer metastasis is explained by researchers as preventing the formation of new blood vessels within the tumor. This is because statins decrease the production of many cytokines, consequently, they inhibit capillary tube formation [17].

The lack of literature of data about the effects of the combined treatment with statins and Ukrain was the inspiration to undertake the studies that form this present paper. The aim of this study was to investigate the interaction between Ukrain and Simvastatin *in vitro* studies.

MATERIAL AND METHODS

***Drugs utilized*:** Ukrain (aqueous high-purity concentrate 1:30, Ukrainian Anti-Cancer Institute, Vienna, Austria), Simvastatin (Simvacard, Zentiva, Prague, Czech Republic).

***Cell culture*:** The research was conducted on green monkey kidney cells (GMK) that were obtained from the "Biomed" Serum and Vaccine Production Plant Ltd. in Lublin, Poland. GMK cells were grown in RPMI-1640 medium (with L-glutamine and phenol red), supplemented with 10% Foetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2,5 μg/ml amphotericin B (all from the PAA – The Cell Culture Company GmbH, Pasching, Austria), in 25 cm^2 tissue culture flasks (EasYFlasks™, Nunclon™Δ, Nunc GmbH, Wiesbaden, Germany). The GMK cells were

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cultured as monolayer in a humidified atmosphere of 5% CO_2, at 37 C, in a cell incubator.

Cytotoxicity assay: In this study, a Cytotoxicity Detection Kit (LDH) (from Roche Diagnostic GmbH, Mannheim, Germany) was used. It is a colorimetric assay that can quantitate cytotoxicity/cytolysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant. The culture supernatant is collected cell-free and incubated with the reaction mixture from the kit. The LDH activity is determined in the enzymatic test. In the first step: NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step: the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is then reduced to formazan: the amount of formazan being proportional to the number of lysed cells. Formazan, being characterized by a maximum absorption at 490 nm, can then be measured.

Three controls were prepared for each marking in order to calculate the percent of cytotoxicity of Ukrain, Simvastatin and their combination on GMK cells: A background control provides information about the LDH activity contained in the assay medium; A low control provides information about the LDH activity released from the untreated normal cells; and a high control provides information about the maximum releasable LDH activity in the cells.

Green monkey kidney cells (GMK) were suspended in growth medium RPMI-1640 and prepared in a concentration of 2×10^6 cells/ml. These cells were then pipetted (100 µl/well) into a sterile 96-well culture plate (from Nunc 96 Microwell™ Plater, Nunc GmbH, Wiesbaden, Germany). Following this, these GMK cells were incubated for 24h in an incubator (37°C, 5% CO_2, 90% humidity) for cell adhesion. Afterwards, medium from cells adherent was removed (as LDH may be released from GMK cells during night-time incubation) and fresh growth medium was added at 100 µl/well.

To determine the cytotoxic activity of Ukrain, Simvastatin and their combination, these drugs were administrated to the GMK cell culture and incubated for 6, 12 and 24 h. Ukrain, Simvastatin and their combination were added in a constant volume, 100 µl/well, in the following concentrations: Ukrain 50 µmol/l or 150 µmol/l, Simvastatin 100 µmol/l or 300 µmol/l and combinations of both drugs: Ukrain 50 µmol/l and Simvastatin 100 µmol/l (in ratio 1:1), Ukrain 50 µmol/l and Simvastatin 300 µmol/l (in ratio 1:3), Ukrain 150 µmol/l and Simvastatin 100 µmol/l (in ratio 3:1). To determine the LDH activity, 100 µl cell-free growth medium was taken from each well and transferred to the appropriate wells of a new clear 96-well microplate, after the incubation.

The next step was to add at 100 ěl/well, the reaction mixture (diaphorase/NAD^+ and iodotetrazolium chloride INT and sodium lactate) prepared *ex tempore* and incubated for 30 min at 15–25°C, without light. The absorbance of each well was measured immediately after incubation by using an automated absorbance microplate reader EL_x808_{IU} (Bio-Tek Instruments Inc.) at a wavelength of 490 nm. The cytotoxicity of Ukrain, Simvastatin and their combination was expressed in %.

MTT viability assay: The study was performed by using the MTT test based on INVITTOX protocol n°17, ECVAM (European Centre for the Validation of Alternative Methods), in accordance with the Database Service on Alternative Methods To Animal Experimentation. This test is a simple colorimetric method for evaluating the viability/number of cells in culture. The principle of the test is based on the determination of the coloured product formazan, produced only in living cells incorporating the tetrazolium salt MTT. The amount of formazan is proportional to the number of living cells. The ability of cells to reduce MTT provides information on the integrity and activity of mitochondria, hence, this data can be interpreted as a measure of the viability and/or the number of cells. The purple formazan crystals are then washed out of the cells by using DMSO. Formazan is released and determined by colorimetry. Two controls were then prepared for each marking to determine GMK cell viability after administration of the drugs Ukrain, Simvastatin and their combination, to cell cultures. These were a background control (involving only the growth medium RPMI-1640) and a low control, which provided information on the amount of formazan formed in normal (not tested) cells. Ukrain and Simvastatin were added to the GMK cells line at similar concentrations as in the LDH test, and were incubated for 6, 12 and 24 h. After the incubation, 20 µl MTT (Thiazolyl blue tetrazolium bromide, Sigma-Aldrich, Steinheim, Germany**)** solution (5 mg/ml) was added to each well microplate and incubation continued for an additional 3 h at 37°C. Afterwards, the culture medium was removed from each well, and 100 µl DMSO was added to rinse the formazan from the cells. The absorbance of each well was then measured at 550 nm, by using an EL_x808_{IU} (Bio-Tek Instruments Inc.) automated absorbance microplate reader. GMK cell viability after incubation with Ukrain, Simvastatin and their combination was expressed in %.

***Statistical analysis*:** The results were calculated as mean ($\bar{x}$) ± SEM. The statistical analysis of the results was performed using Student's t-test. *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

***LDH test results*:** Results reveal that the cytotoxic effects was less then 10% after 6, 12 and 24 h incubation of GMK cells with the drug Ukrain (50 µmol/l) or with Simvastatin (100 µmol/l). It should be noted that Ukrain (50 µmol/l) clearly evidenced cytotoxic activity against tumor cells [5]. After only 12 h incubation of green monkey kidney cells with Simvastatin (300 µmol/l), minor cytotoxicity (14,6%) was also already notable. In the GMK cell group which received the drug Ukrain (150 µmol/l), minor cytotoxic effect was, as well, evident after 6, 12 and 24 h incubation. Similar cytotoxicity of Ukrain (50 µmol/l) and Simvastatin (100 µmol/l) was observed after 6 and 12 h incubation of GMK

cells with both drugs (Fig. 1, 2), but only 24 h incubation shown a significant increase in their cytotoxicity (56,73 %) (Fig. 3). No adverse effects on cell growth with Ukrain (50 μmol/l) and Simvastatin (300 μmol/l) in combination was seen after 6 h incubation (Fig. 1). Increasing concentrations of both drugs had, however, negatively effects on the GMK cells. This was observed after 12 h incubation with these drugs (Fig. 2). Furthermore, incubation of cells for a period more than 24 h with a combination of Ukrain (50 or 150 μmol/l) and Simvastatin (100 or 300 μmol/l) significantly inhibited cell growth (Fig. 3).

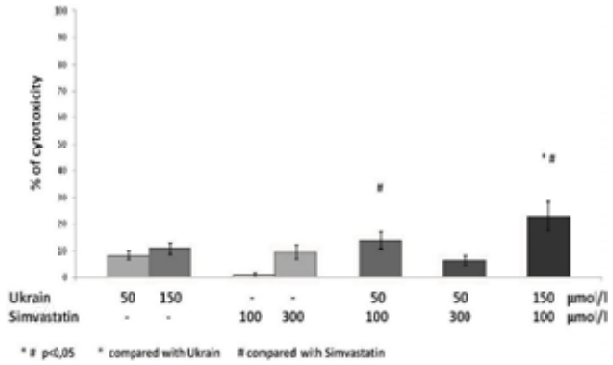

Fig. 1. Cytotoxicity of Ukrain, Simvastatin and their combination after 6 hours incubation with GMK cell culture in the LDH test

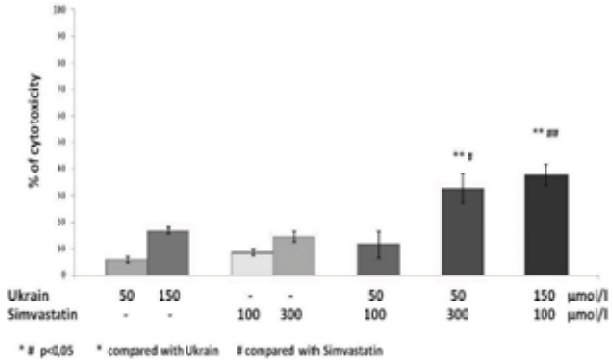

Fig. 2. Cytotoxicity of Ukrain, Simvastatin and their combination after 12 hours incubation with GMK cell culture in the LDH test

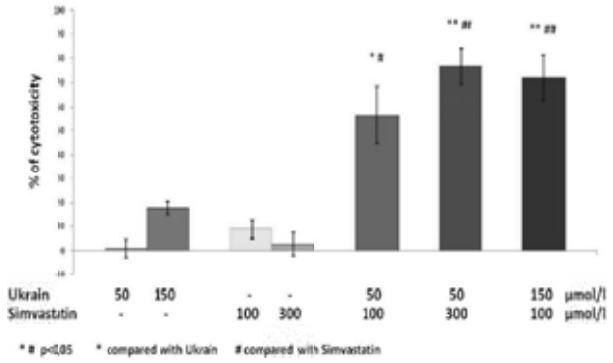

Fig. 3. Cytotoxicity of Ukrain, Simvastatin and their combination after 24 hours incubation with GMK cell culture in the LDH test

***MTT test results*:** Ukrain (50 μmol/l) decreased by about 20 % the GMK cell viability after 6, 12 and 24 h incubation. This drug, at a concentration of 150 μmol/l, caused a gradual decrease in GMK cell viability in proportion to the time of incubation (Fig. 4–6), while Simvastatin (100 μmol/l) practically did not affect GMK cell viability after the same incubation time. However, Simvastatin (300 μmol/l) eventually did considerably decrease the GMK cell viability, but only after 24 h (Fig. 6). When the green monkey kidney cells were incubated 6 h to the cumulative action of Ukrain (50 or 150 μmol/l) and Simvastatin (100 or 300 μmol/l), there was a significant decrease in cell viability compared to the group receiving only Simvastatin or Ukrain alone (only 150 μmol/l) (Fig. 4). Furthermore, GMK cell viability was significantly inhibited after 12 h incubation with a combination of these drugs (Fig. 5). Similar results (but more cytotoxicity effect) were seen after 24 h incubation of the GMK cell line with the drugs discussed above (Fig. 6).

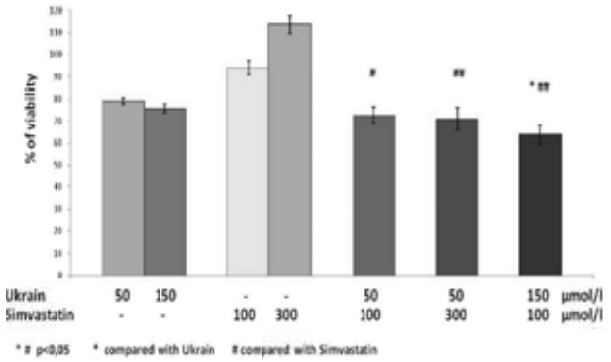

Fig. 4. Effect of Ukrain, Simvastatin and their combination on the GMK cells viability after 6 hours incubation in the MTT test

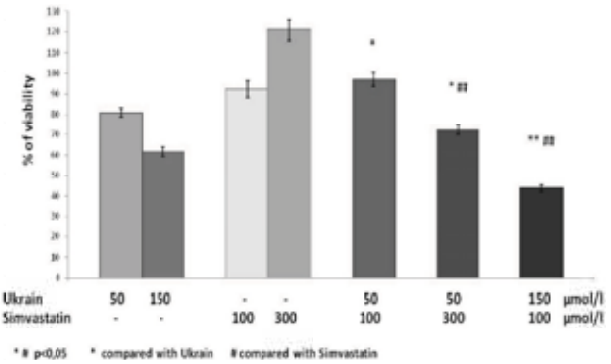

Fig. 5. Effect of Ukrain, Simvastatin and their combination on the GMK cells viability after 12 hours incubation in the MTT test

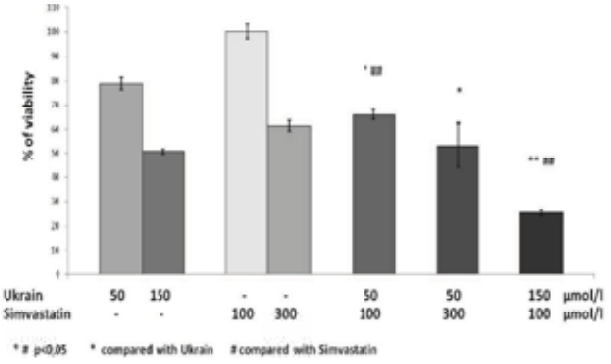

Fig. 6. Effect of Ukrain, Simvastatin and their combination on the GMK cells viability after 24 hours incubation in the MTT test

A combination of Ukrain and Simvastatin significant decreased the GMK cell viability. However, both drugs given individually to the cultures do not show a very negative impact upon the GMK cells. This effect is not expected in regard to studies on normal cell line like the green monkey kidney cells GMK.

REFERENCES

1. Adam O. and Laufs U.: Antioxidative effects of statins. *Arch. Toxicol.*, 82, 885, 2008.
2. Cheng G. et al.: Apoptosis induced by simvastatin in rat vascular smooth muscle cell through Ca^{2+}-calpain and caspase-3 dependent pathways. *Pharmacol. Res.*, 48, 571, 2003.
3. Graaf M.R. et al.: Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer. *Cancer Treat. Rev.*, 30, 609, 2004.
4. Habermehl D. et al.: Pro-apoptotic activity of Ukrain is based on *Chelidonium majus* L. alkaloids and mediated via a mitochondrial death pathway. *BMC Cancer*, 6, 14, 2006.
5. Jagiełło-Wojtowicz E, Kleinrok Z. and Urbanska E.M.: Ukrain (NSC-631570) in experimental and clinical studies: a review. *Drugs Exptl. Clin. Res.*, 24, 213, 1998.
6. Kassimatis T.I. and Konstantinopoulos P.A.: The role of statins in chronic kidney disease (CKD): Friend or foe?. *Pharmacol. Ther.*, 122, 312, 2009.
7. Korolenko T.A. et al.: Macrophage stimulation and anti- tumour effect of Ukrain. *Drugs Exptl. Clin. Res.*, 24, 253, 1998.
8. Koshelnick Y. et al.: Ukrain (NSC-631570) inhibits angiogenic differentiation of human endothelial cells in vitro. *17th Internat. Cancer Congress*, Rio de Janeiro, 24-28 August, 91, 1998.
9. Liepins A. et al.: Induction of bimodal programmed cell death in malignant cells by the derivative Ukrain (NSC-631570). *Drugs Exptl. Clin. Res.*, 22, 73, 1996.
10. Liu H. et al.: Statins induce apoptosis in ovarian cancer cells through activation of JNK and enhancement of Bim expression. *Cancer Chemother. Pharmacol*, 63, 997, 2009.
11. Mach T.: Statyny a ryzyko uszkodzenia wątroby. *Przegl. Gastroenterol.*, 2, 111, 2007.
12. Merx M.W. and Weber C.: Benefits of statins beyond lipid lowering. *Drug Discov. Today Dis. Mech.*, 5, 325, 2008.
13. Nowicky J.W., Hiesmayr W.and Liepins A.: Influence of Ukrain on DNA, RNA and protein synthesis in malignant cells. *Drugs Exptl. Clin. Res.*, 22, 81, 1996.
14. Nowicky J.W., Hiesmayr W. and Liepins A.: Influence of Ukrain on immunological blood parameters in vitro and in vivo. *Drugs Exptl. Clin. Res.*, 22, 163, 1996.
15. Roublevskaia I.N. et al.: Induced apoptosis in human prostate cancer cell line LNCaP by Ukrain. *Drugs Exptl. Clin. Res.*, 26, 141, 2000.
16. Seeger H. Wallwiener D. and Mueck A.O.: Statins can inhibit proliferation of human breast cancer cells in vitro. *Exp. Clin. Endocrinol. Diabetes*, 111, 47, 2003.
17. Sławińska A. and Kandefer-Szerszeń M.: Właściwości przeciwnowotworowe statyn. *Postepy Hig. Med. Dosw.*, 62, 393, 2008.
18. Uglyanitsa K.N. et al.: Ukrain: A novel anti-tumour drug. *Drugs Exptl. Clin. Res.*, 26, 341, 2000.