ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXIV, N 1, 6 SECTIO DDD 2011

Department of Toxicology, Medical University of Lublin, Poland

MAGDALENA IZDEBSKA, IWONA PIĄTKOWSKA-CHMIEL,
EWA JAGIEŁŁO-WÓJTOWICZ

Cytotoxic effects of combined treatment with Ukrain and methotrexate in in vitro studies

Cytotoksyczne efekty łącznego stosowania leku Ukrain i metotreksatu w badaniach *in vitro*

INTRODUCTION

The anticancer drug Ukrain (NSC–631570), a thiophosphoric acid derivative of alkaloids from *Chelidonium majus* L. showed selective cytotoxic effects on tumour cells with slight adverse side effects on normal cells and tissues dependent on doses. Ukrain caused the inhibition of DNA, RNA and protein synthesis in cancer cells and induced the programmed cell death (apoptosis) [7,8,10,12-14]. The clinical investigations suggested beneficial effects of Ukrain in the treatment of patients suffering e.g. from pancreatic, breast, ovarian, bladder, rectal cancer, Eawing's sarcoma when exposed to the medicine as a single drug or in combination with chemotherapeutic drugs or ionizing radiation [1,4,11,16].

The anticancer drug methotrexate (MTX) is used for patients with acute lymphoblastc leukemia, osteosarcoma, lymphoma, breast cancer, bladder cancer and head and neck cancer. MTX belongs to the group of drugs with high-risk side effects. Administration of high doses of MTX and simultaneous treatment with other cytostatics drugs increased the risk of damage of kidney, liver, bone marrow, skin or mucous membranes. The kidneys 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 [2,5,15]. The lack of literature data about the combined treatment with Ukrain and MTX was the inspiration to undertake the research of the presented work. The aim of this study was to investigate the cytotoxic effects of combined treatment with Ukrain and MTX on *green monkey kidney* (*GMK*) *cells* using the Cytotoxicity Detection Kit (LDH). The GMK cell viability was also estimated using the MTT test.

METERIAL AND METHODS

D r u g s : Ukrain (aqueous high-purity concentrate 1:30, Ukrainian Anti-Cancer Institute, Vienna, Austria), metotreksat (Metotreksat-Ebewe, amp. 10mg/1ml, Ebewe Pharma, Unterach, Austria).

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 % foetal bovine serum heat-inactivated, 100 U/ml penicillin, 100 µg/ml streptomycin and 2,5 µg/ml amphotericin B* (from the PAA - The Cell Culture Company GmbH, Pasching, Austria). *GMK cells were cultured as monolayer in CO_2 cell incubator at 37 ^0C in an atmosphere of 5% CO_2.*

Cytotoxicity assay. Cytotoxicity Detection Kit (LDH) from Roche Diagnostic GmbH, Mannheim, Germany was used**.** It is a colorimetric assay for the quantitation of cytotoxicity/cytolysis based on the measurement of LDH activity released from the damaged cells. The cell-free culture medium is collected and incubated with the reaction mixture from the kit. LDH activity is determined in an 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 reduced to formazan. The formazan shows a broad absorption maximum at about 500 nm. GMK cells were suspended in the culture medium RPMI-1640 and prepared in the concentration of 2×10^6 cells/ml. *Afterwards* cells were titrated in sterile 96-well tissue culture plate (Nunc 96 MicroWell™ Plater, Nunc GmbH Wiesbaden, Germany) (100 µl/well cell suspension).

Ukrain and MTX were *ex tempore* prepared in RPMI-1640 medium. To assess cytotoxic effects of both drugs, Ukrain with MTX were added together and incubated with GMK cells for 6 and 12 hrs, at the following concentrations: 1:1 i.e. Ukrain (50 µmol/l) and MTX (5.5 µmol/l); 1:3 i.e. Ukrain (50 µmol/l) and MTX (16.5 µmol/l) as well as 3:1 i.e. Ukrain (150 µmol/l) and MTX (5.5 µmol/l). In our earlier studies [6] these initial concentrations of drug Ukrain (50 µmol/l) and MTX (5.5 µmol/l) were not toxic to the GMK cells line in LDH test after 24 hrs of incubation. The literature shows that these concentrations of both drugs used in the study are effective and cytotoxic for various tumor cells line [7,9,10,16]. After incubation 100 µl cell-free culture medium was removed carefully from each well and was transferred into the corresponding wells of new optically clear 96-well microplate. To determine the LDH activity in cell-free culture medium, we added 100 µl reaction mixture (diaphorase/NAD^+ and iodotetrazolium chloride (INT) and sodium lactate) *ex tempore* prepared to each well and incubated for up to 30 min at 15-25°C. The absorbance of each well was measured immediately after incubation at 490 nm using an automated absorbance microplate reader EL_x808_{IU} (Bio-Tek Instruments Inc.). Cytotoxicity of Ukrain, MTX and their combination was expressed in %.

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 the cells viability the combinations of Ukrain and MTX were added to GMK cells line in the above concentrations and were incubated for 6 and 12 hrs. After the incubation we added to each well microplate 20 µl MTT (Thiazolyl blue tetrazolium bromide, Sigma-Aldrich, Steinheim, Germany) solution (5 mg/ml) and we incubated them for 3 hrs at 37°C. After 3 hrs. of cell incubation with MTT solution formazan crystals develop in living cells. At the end of the incubation the culture medium was removed carefully from each well and added 100 µl DMSO (at room temperature). The microplate was shaken for 5-10 min. The absorbance of each well was measured at 550 nm (formazan solution absorbs light at 550-570 nm) using an automated absorbance microplate reader EL_x808_{IU} (Bio-Tek Instruments Inc.). GMK cells viability was expressed in %.

Statistical analysis. Results are expressed as mean ($\bar{x}$) ± S.E.M. Statistical significance among groups was determined by Student's t-test. *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The major problems of combined chemotherapy are: the dangerous interactions of cytostatics drugs and side effects caused by the lack of them in selective actions on tumor cells [3]. Ukrain selectively accumulates in cancer cells and lead to reduction of tumor mass and partial or total remission. Ukrain reduces undesirable and toxic effects while it is used with another cytostatic drugs or with radiotherapy [7,13,16]. Also, MTX in combination with other cytostatics can lead to the increased toxicity or it can decrease the effects of this anticancer drugs [3].

The results of presented study show that 6 or 12 hrs of simultaneous incubation of GMK cells with drug Ukrain (50 μmol/l) and MTX (5.5 or 16.5 μmol/l) did not affect cytotoxicity in the LDH test (Fig.1). However, after combined application of Ukrain (150 μmol/l) with MTX (5.5 μmol/l) to GMK cells, the significant increase of drugs cytotoxicity were observed (respectively 25% of cytotoxicity after 6 hrs and 33% after 12 hrs).

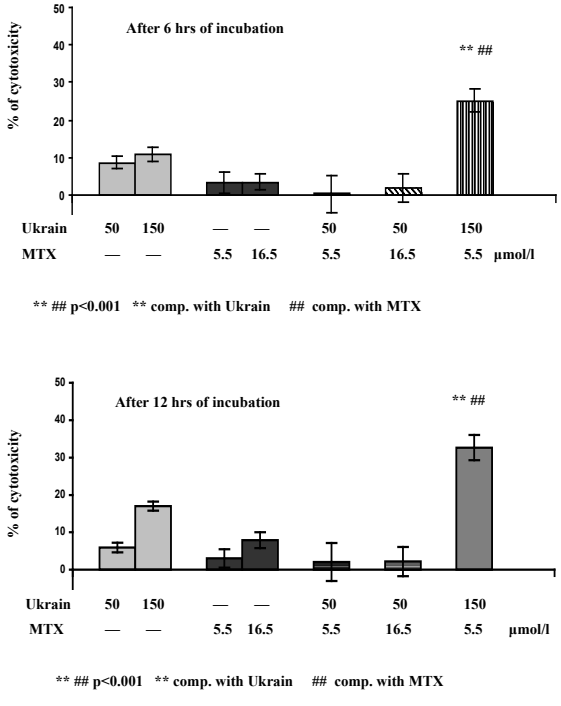

Fig. 1. Cytotoxicity of Ukrain, MTX and their combination after 6 or 12 hrs. incubation with GMK cell culture in the LDH test

After 6 or 12 hrs of incubation of GMK cells only with drug Ukrain (50 µmol/l) the cytotoxic effect was less than 10% but 3 times higher concentrations of drug (150 µmol/l) caused 12% and 17% of cytotoxicity respectively after 6 and 12 hrs incubation with GMK cells.

In the MTT test it was found a significant decrease in the GMK cells viability after 6 and 12 hrs incubation with Ukrain (50 or 150 µmol/l) or MTX (5.5 or 16.5 µmol/l) in comparison with the control GMK cells (Fig.2). Also after 6 or 12 hrs of simultaneous incubation of the GMK cells with Ukrain (50 µmol/l) and MTX (5.5 or 16.5 µmol/l) a decreased GMK cells viability (about 40-45 %) was found in comparison with cell groups incubated only with Ukrain or only with MTX. The most significant decrease (about 55 and 70 %) of the cell viability was found after 6 or 12 hrs incubation GMK cells with Ukrain (150 µmol/l) and MTX (5.5 µmol/l).

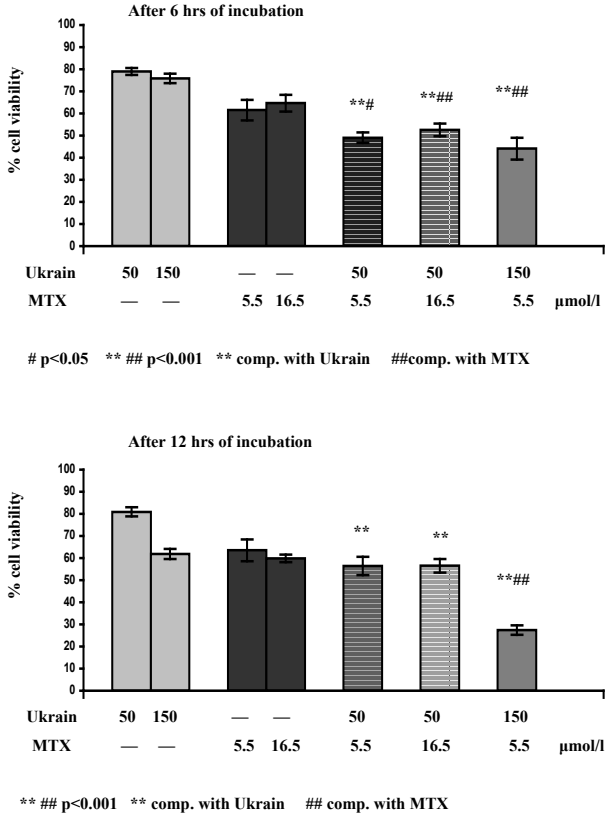

Fig. 2. Effect of Ukrain, MTX and their combination on the GMK cells viability after 6 or 12 hrs. incubation in the MTT test

The initial concentrations combination of both drugs (1:1) were not toxic for GMK cells in the LDH test after 6 or 12 hrs of incubation but in the MTT test we found the significant decrease of GMK cells viability. The obtained results (in LDH and MTT tests) can suggest the toxic influence of the combined application of Ukrain (150 μmol/l) with MTX (5.5 μmol/l) on the GMK cells.

Acknowledgements. The authors wish to express their gratitude to dr J.W. Nowicky (Ukrainian Anti-Cancer Institute, Vienna, Austria) for the generous gift of Ukrain. This research was supported by the grant PW 91/09 from Medical University in Lublin, Poland.

REFERENCES

1. Aschhoff B.: Retrospective study of Ukrain treatment in 203 patients with advanced-stage tumors. Drugs Exp. Clin. Res., 26, 249, 2000.
2. Bangert C.A., Costner M.I.: Methotrexate in dermatology. Dermatol. Ther., 20, 216, 2007.
3. Blower P. et al.: Drug – drug interactions on oncology: Why are they important and can they be minimized?, Crit Rev Oncol. Hematol., 55, 117, 2005.
4. Gansauge F. et al.: NSC-631570 (Ukrain) in the palliative treatment of pancreatic cancer. Results of a phase II trial. Langenbecks Arch. Surg. 386, 570, 2002.
5. Green M.R., Chamberlain M.C.: Renal dysfunction during and after high-dose methotrexate. Cancer Chemother. Pharmacol., 63, 599, 2009.
6. Izdebska M., Piątkowska-Chmiel I., Jagiełło-Wójtowicz E.: Ocena cytotoksycznego działania metotreksatu i leku Ukrain w badaniach *in vitro*. W: II Ogólnopolskie Sympozjum „Nowoczesne techniki badawcze w ocenie jakości produktów leczniczych." Lublin, 24-25 września 2009, P-82, s. 109.
7. Jagiełło-Wójtowicz E. et al. : Ukrain (NSC-631570) in experimental and clinical studies: a review. Drugs Exp. Clin. Res., 24, 213, 1998.
8. Habermehl D. et al.: Proapoptotic activity of Ukrain is based on *Chelidonium majus L*. alkaloids and mediated via a mitochondrial death pathway. BMC Cancer, 6, 14, 2006.
9. Kimura E. et al.: Methotrexate differentially affects growth of suspension and adherent cells. Int. J. Biochem. Cell Biol., 36, 814, 2004.
10. Liepins A. et al.: Induction of bimodal programmed cell death in malignant cells by the derivative Ukrain (NSC-631570). Drugs Exp. Clin. Res., 22, 73, 1996.
11. Lohninger A., Hamler F.: Chelidonium majus L. (Ukrain) in the treatment of cancer patients. Drugs Exp. Clin. Res., 18, 73, 1992.
12. Nowicky J.W. et al.: Influence of Ukrain on DNA, RNA and protein synthesis in malignant cells. Drugs Exp. Clin. Res., 22, 81, 1996.
13. Nowicky J.W. et al.: Ukrain both as an anticancer and immunoregulatory agent. Drugs Exp. Clin. Res., 18, 51, 1992.
14. Roublevskaia I.N. et al.: Induced apoptosis in human prostate cancer cell line LNCaP by Ukrain. Drugs Exp. Clin. Res., 26, 141, 2000.
15. Strang A., Pullar T.: Methotrexate toxicity induced by acute renal failure. J. R. Soc. Med., 97, 536, 2004.
16. Uglyanitsa K.N. et al.: Ukrain: A novel antitumor drug. Drugs Exp. Clin. Res., 26, 341, 2000.

SUMMARY

In *in vitro* studies of this work the cytotoxicity of drug Ukrain, methotrexate and their combination in *green monkey kidney* (*GMK*) *cells culture* was assessed using ready-made kit Cytotoxicity Detection Kit LDH. For assay of cell viability MTT test was used. It was found that 6 or 12 hrs of simultaneous incubation of GMK cells with drug Ukrain (50 μmol/l) with MTX (5.5 or 16.5 μmol/l) did not affect cytotoxicity in the LDH test. The significant increase of the cytotoxicity was found after 12 hrs. incubation GMK cells with Ukrain (150 μmol/l) and MTX (5.5 μmol/l). Likewise, in the MTT assay the greatest decrease in the cells viability was found after GMK cells incubation with Ukrain (150 μmol/l) and MTX (5.5 μmol/l). The results suggest the adverse effect of combined application of both drugs in these concentrations on the GMK cells viability.

Keywords: Ukrain, methotrexate, *in vitro* studies

STRESZCZENIE

W badaniach *in vitro* oceniano cytotoksyczność leku Ukrain, MTX oraz ich kombinacji w hodowli komórek nerki małpy zielonej GMK przy użyciu gotowego zestawu Cytotoxicity Detection Kit LDH. Żywotność komórek GMK po inkubacji z badanymi lekami oceniano stosując test MTT. W pracy wykazano, że 6 lub 12 godz. łączna inkubacja komórek GMK z lekiem Ukrain (50 μmol/l) i MTX (5.5 lub 16.5 μmol/l) nie wpływała na cytotoksyczność w teście LDH. Największy wzrost cytotoksyczności stwierdzono po 12 godz. inkubacji komórek GMK z lekiem Ukrain (150 μmol/l) i MTX (5.5 μmol/l). Podobnie w teście MTT największy spadek żywotności komórek odnotowano w grupie inkubowanej z lekiem Ukrain (150 μmol/l) w połączeniu z MTX (5.5 μmol/l). Uzyskane wyniki sugerują niekorzystny wpływ kombinacji obu stosowanych leków w w/w stężeniach na żywotność komórek GMK.

Słowa kluczowe: Ukrain, metotreksat, badania *in vitro*