CYTOTOXIC EFFECT OF COMBINED TREATMENT WITH UKRAIN AND METHOTREXATE ON KIDNEY CELLS OF GREEN MONKEY

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Abstract

In in vitro study on green monkey kidney (GMK) cell culture, the cytotoxicity of Ukrain, methotrexate (MTX), and their combination was investigated. The effect of these drugs and their combination on viability (MTT test) and apoptosis of the cells was assessed. The IC_{10} and IC_{50} values for Ukrain and MTX were also indicated. After 24 h of incubation of GMK cells with Ukrain, IC_{10} amounted to 84.6 µmol/L and IC_{50} was 256.3 µmol/L, while MTX IC_{10} amounted to 7.18 µmol/L and IC_{50} was 154.8 µmol/L. After 24 h of simultaneous incubation of GMK cells with 50 µmol/L of Ukrain and 5.5 µmol/L of MTX, 15.33 % of cytotoxicity of the drugs in LDH test was found. The most significant increase in the cytotoxicity (42.10 %) was noted after 24 h incubation of GMK cells with 150 µmol/L of Ukrain and 5.5 µmol/L of MTX. Likewise, in the MTT assay the greatest decrease in the cells viability was found after incubation with 150 µmol/L of Ukrain and 5.5 µmol/L of MTX. The evaluation of apoptosis also indicated the adverse effect of combined application of both drugs on GMK cells.

Key words: cell culture, Ukrain, methotrexate, cytotoxicity, apoptosis.

The anticancer drug Ukrain (NSC–631570), a thiophosphoric acid derivative of alkaloids from Chelidonium majus L., is characterised by cytotoxic and/or cytostatic activity and by immunocorrective properties (11, 18). The clinical investigations suggest beneficial effects of Ukrain in the treatment of patients suffering e.g. from pancreas, breast, ovarian, bladder, and rectal cancer, or Eawing’s sarcoma, when exposed to the medicine as a single drug or in combination with chemotherapeutics or ionizing radiation (1, 16, 26). Pre-clinical in vitro investigations showed selective cytotoxic effects on tumour cells with slight and dose-dependent adverse effects on normal cells and tissues (10, 15, 20). The malignotoxic properties of Ukrain were evaluated by the National Cancer Institute (NCI), Development Therapeutics Programme (USA). Studies in frame of this programme were performed on more than 60 human cell lines representing the most common cancers including brain, ovary, lung, colon and kidney carcinomas, melanoma, leukaemia, and lymphoma (11, 19, 26).

Ukrain is absorbed by the nuclei of the cancer cells within a short time after administration. It causes the inhibition of DNA, RNA, and protein synthesis in cancer cells and induces the programmed cell death (apoptosis) (8, 17, 21). Low doses of Ukrain induce the first classical apoptosis programme, which is based on cell membrane destruction. This process is probably mediated by quinidine sensitive Ca^{2+} – dependent K^{+} channels. High doses of the drug induce the second programme of programmed cell death, in which an intensive DNA polyploidy and their fragmentation were observed (15). In vitro studies also showed that Ukrain has inhibiting effect on tubulin polymerisation and induces antimitotic action to arrest cells in metaphase (20). The lack of literature data about the simultaneous treatment with Ukrain and methotrexate (MTX) was an inspiration to undertake the presented research. MTX in combination with other cytostatics can lead to increased toxicity or can decrease the effects of these anticancer drugs. MTX as an antimitabolite drug is a folate antagonist used in patients with acute lymphoblastic leukaemia, osteosarcoma, lymphoma, breast cancer, bladder cancer, and head and neck cancer. In addition, it is used at lower doses in patients with non-malignant diseases such as rheumatoid arthritis or psoriasis (2, 23, 24). MTX is an inhibitor of dihydrofolate reductase (DHFR), a key enzyme for intracellular folate metabolism and DNA synthesis. DHFR catalyses the conversion of folic acid to tetrahydrofolate. As a consequence of DHFR inhibition, intracellular level of tetrahydrofolate coenzymes is decreased, resulting in inhibition of thymidylate, purine, and consequently DNA biosynthesis (6, 24). Unfortunately, MTX is a highly toxic drug. Patients need to be monitored throughout the treatment, especially in terms of the renal and liver functioning. MTX may cause the bone marrow depression (anaemia, leukopenia, thrombocytopenia)
and gastrointestinal toxicity (vomiting, diarrhoea, or ulcerative stomatitis and haemorrhagic enteritis). The renal dysfunction after MTX treatment is a clinically important side effect (4, 7, 22). High-dose of methotrexate induces renal failure because MTX is mainly eliminated by the kidneys. The aim of this study was to investigate the cytotoxic effects of Ukrain and MTX on green monkey kidney (GMK) cells.

**Material and Methods**

**Preparations and cell culture.** The following preparations were used in the study: Ukrain (aqueous high-purity concentrate 1:30, Ukrainian Anti-Cancer Institute, Vienna, Austria), MTX (Metotreksat-Ebewe, 10mg/mL, Ebewe Pharma, Austria), and MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich, Germany). Cell culture medium RPMI-1640 (with L-glutamine and phenol red), foetal bovine serum (FBS), and antibiotics: penicillin, streptomycin, and amphotericin B were obtained from the PAA - The Cell Culture Company, Austria. Ukrain and MTX were obtained from the Production Plant, Poland. GMK cell line was grown in RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B. GMK cells were propagated in the PAA - The Cell Culture Company, Austria. Ukrain and MTX were ex tempore prepared in RPMI-1640 medium. The following ready-made diagnostic kits were used: Cytotoxicity Detection Kit (LDH) and Cell Death Detection ELISAPLUS, both from Roche Diagnostic, Germany. The experiment was performed on green monkey kidney cells (GMK) obtained from the „Biomed” Serum and Vaccine Production Plant, Poland. GMK cell line was grown in RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B on 25 cm² tissue culture flasks (EasYflasks™Nuncon™, Nunc, Germany). GMK cells were cultured as monolayer in CO₂ cell incubator at 37°C in an atmosphere of 5% CO₂. Afterwards, the cells were counted in Neubauer haemocytometer (BlauBrand, BRAND) by means of the compact inverted microscope Olympus CKX41. For the assays of cytotoxicity, GMK cells were prepared at density of 2×10⁶ cells/cm².

**LDH test.** For the evaluation of IC₁₀ and IC₅₀ values, GMK cells were incubated for 24 h with Ukrain (100 µL/well) at concentrations of 50, 150, 200, 300, 400, and 600 µmol/L, or with MTX (100 µL/well) at concentrations of 5.5, 11.0, 16.5, 22.0, 55.0, 110, and 220 µmol/L in an incubator (37°C, 5% CO₂, 90% humidity). To determine the cytotoxic activity of the combination of Ukrain and MTX, the drugs were added to GMK cell line and incubated for 24 h. Both drugs were added together in the same volume of 100 µL/well at the following concentrations: 1:1 (Ukrain - 50 µmol/L and MTX - 5.5 µmol/L); 1:3 (Ukrain - 50 µmol/L and MTX - 16.5 µmol/L), and 3:1 (Ukrain - 150 µmol/L and MTX - 5.5 µmol/L). After incubation, 100 µL of 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, 100 µL of reaction mixture (diaphorase/NAD⁺, iodotetrazolium chloride INT, and sodium lactate) was added 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 EL₈08urtle (Bio-Tek Instruments Inc.). Cytotoxicity was calculated from equation placed in the manufacturer’s instruction and expressed as a percentage.

**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 (25). To determine the effects on cell viability, the combination of Ukrain and MTX was added to GMK cell line and incubated for 24 h. Both drugs were added together in the same volume of 100 µL/well at the following concentrations: Ukrain - 50 µmol/L and MTX - 5.5 µmol/L, Ukrain - 50 µmol/L and MTX - 16.5 µmol/L, Ukrain - 150 µmol/L and MTX - 5.5 µmol/L. After the incubation, 20 µl MTT solution (5 mg/mL) was added to each well microplate and the mixture was incubated for 3 h at 37°C. During the incubation, formazan crystals developed in living cells. At the end of the incubation, the culture medium was removed carefully from each well and 100 µl DMSO (at room temperature) was added. The absorbance of each well was measured at 550 nm using an automated absorbance microplate reader EL₈08urtle (Bio-Tek Instruments Inc.). GMK cell viability was expressed as a percentage.

**Assessment of apoptosis.** For the evaluation of apoptosis in the GMK cells, Ukrain and MTX were added to GMK cell line and incubated for 24 h. At the end of the incubation period, the cells were lysed and the level of apoptosis was determined using ELISA, which measured the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). The absorbance was measured at 405 nm using an automated absorbance microplate reader EL₈08urtle (Bio-Tek Instruments Inc.). Results were expressed at enrichment factor (the specific enrichment of mono- and oligonucleosomes released into the cytoplasm).

**Statistical analysis.** Results were expressed as mean (X) ± S.E.M. Statistical significance among groups was determined by Newman-Keuls test. P-values less than 0.05 were considered significant.

**Results**

After 24 h incubation of GMK cells with Ukrain in concentrations from 50, 150, 200, 300, 400 to 600 µmol/L designated IC₁₀ (10% inhibitory concentration) and IC₅₀ (50% inhibitory concentration) values were 84.6 and 256.3 µmol/L, respectively (Fig. 1).
**Fig. 1.** Cytotoxicity of Ukrain after 24 h incubation of GMK cell culture.

**Fig. 2.** Cytotoxicity of MTX after 24 h incubation of GMK cell culture.

**Fig. 3.** Cytotoxicity of Ukrain, MTX, and their combination after 24 h incubation of GMK cell culture. LDH test.

Ukrain | 50 | 150 | —  | —  | 50 | 50 | 150  
MTX   | —  | —  | 5.5| 16.5| 5.5| 16.5| 5.5  

* # P<0.05   * comp. with Ukrain   # comp. with MTX
** ## P<0.001
After 24 h of incubation, GMK cells with MTX in concentrations from 5.5, 11.0, 16.5, 22.0, 55.0, 110 to 220 µmol/L, the IC$_{10}$ was 7.18 µmol/L and IC$_{50}$ was 154.8 µmol/L (Fig. 2). The increase in cytotoxicity was dose-related.

After 24 h of incubation of GMK cells with 50 µmol/L of Ukrain, the cytotoxic effect was not detected but at 3 times higher concentration of the drug (150 µmol/L), 17.95% cytotoxic effect was obtained (Fig. 3). In the case of MTX, the initial used concentration (5.5 µmol/L) showed a minimal (2.49%) cytotoxicity in GMK cells, but after applying a 3 times higher concentration (16.5 µmol/L) the cytotoxicity significantly increased to 27.8%. After 24 h of simultaneous incubation of GMK cells with Ukrain (50 µmol/L) and MTX (5.5 or 16.5 µmol/L), the significant increase in cytotoxic effect of both drugs was found by about 15% and 22%, respectively, compared with cells incubated only with Ukrain or only with MTX (5.5 µmol/L). The highest increase in the cytotoxicity (about 42%) was noted after 24 h of simultaneous incubation of GMK cells with Ukrain (150 µmol/L) and MTX (5.5 µmol/L) (Fig. 3).
A decrease in GMK cell viability (by 22% and 49%, respectively) was found after 24 h of incubation with Ukrain (50 or 150 µmol/L). About 25% decrease in cell viability was also demonstrated after 24 h of incubation with MTX (5.5 or 16.5 µmol/L). After 24 h of simultaneous incubation of GMK cells with Ukrain (50 µmol/L) and MTX (5.5 or 16.5 µmol/L), the cell viability decreased by about 40% in comparison with cells incubated only with Ukrain or only with MTX. The most significant decrease (about 50%) in the cell viability was found after incubation with 150 µmol/L of Ukrain and 5.5 µmol/L of MTX (Fig. 4).

Evaluation of apoptosis demonstrated a significant increase in fragmentation of GMK cells' DNA after the 24 h incubation with Ukrain, but only at concentration of 150 µmol/L. The combined application of Ukrain (50 µmol/L) and MTX (5.5 or 16.5 µmol/L) also increased the level of mono- and oligonucleosomes in the cytoplasm of the tested cells in comparison to the results obtained in cells incubated only with Ukrain or only with MTX at appropriate concentrations. The most significant increase in DNA fragmentation was found in the GMK cells incubated with Ukrain (150 µmol/L) together with MTX (5.5 µmol/L). However, it should be noted that these results were statistically significant only in comparison to the group of GMK cells exposed to MTX only (5.5 µmol/L) (Fig. 5).

Discussion

The problems of combined chemotherapy result from dangerous interactions of cytostatics drugs and side effects caused by the lack of them in selective actions on tumour cells (3). MTX belongs to the group of drugs of high-risk side effects. Administration of high doses of MTX and simultaneous treatment with other cytostatics increase the risk of damage of the kidneys, liver, bone marrow, skin, or mucous membranes. The kidneys are the major route of MTX elimination. Long-term MTX therapy can cause permanent impairment of kidney function, leading to the delay of the drug elimination from the body and an increase in its toxicity (4, 7, 22). Ukrain shows selective cytotoxic and/or cytostatic effect on tumour cells, and selectively accumulates in tumour tissue. This interesting phenomenon may be due to greater drug affinity to cancer than to normal cells as confirmed by fluorescent examination at UV light (λ=220-240 nm) (11, 19).

In the presented study, the cytotoxic effect of simultaneous treatment of GMK cells with Ukrain and MTX was evaluated. The cytotoxicity of the drugs was determined using ready-made kit. The GMK cell viability was estimated using the MTT test. In this study the evaluation of the pro-apoptotic action of both drugs was conducted, examining the level of mono- and oligonucleosomes produced in cells as a result of the process of DNA fragmentation that occurs during the programmed cell death – apoptosis.

For the evaluation of cytotoxic activity of the drugs, Ukrain was used at an initial concentration of 50 µmol/L and MTX at a concentration of 5.5 µmol/L. These concentrations were not toxic to the GMK cell line after 24 h of incubation. The literature data show that these concentrations of the drugs used in the presented study are effective and cytotoxic for various tumour cell line (5, 9, 12, 14). The studies with Ukrain were conducted on both physiological and cancer cell lines. This drug has been tested in the National Cancer Institute, Bethesda, USA, where its effect was evaluated on 60 different human tumour lines. The applied drug doses, which inhibited by 50% the cell line vitality, ranged from 0.8 to 13.2 µmol/L. The medium concentration of the drug, which inhibited the growth of tumour cell lines by 50% (GI 50 - 50% growth inhibition) was on the level 10^{-5} mol (11, 19). In the case of MTX, the range of cytotoxicity doses for tumour cells used in the experiments of different authors was quite high (from 10^{-9} to 10^{-6} mol/L) (5, 9, 13). From the results of this study, it can be concluded that 50 µmol/L of Ukrain in the combination with 5.5 or 16.5 µmol/L of MTX inhibited the growth of cells by 15.33% and 23.35%, respectively. The highest growth of cytotoxicity (by about 42%) was noted after applying 150 µmol/L of Ukrain into GMK cell culture together with 5.5 µmol/L of MTX. Similarly, the highest decrease in GMK cell viability, detected with the use of the MTT test, was observed after simultaneous incubation with Ukrain and MTX at the same concentrations. Ukrain (50 µmol/L) in the combination with MTX (5.5 or 16.5 µmol/L) decreased the GMK cell viability by about 40%, compared to the viability of the control groups. This study also showed a statistically significant increase in DNA fragmentation, which resulted in higher apoptosis of GMK cells treated for 24 h with Ukrain (50 or 150 µmol/L) and MTX (5.5 or 16.5 µmol/L). The obtained results can suggest the negative influence of combined application of both studied drugs on the GMK cell viability. These results, in addition to the cognitive aspect, may be helpful in the use of both drugs in the treatment of cancers.

References