Original article

The Effect of ATM and ERK1/2 Inhibition on Mitoxantrone-Induced Cell Death of Leukaemic Cells

(mitoxantrone / ATM / ERK1/2 / p53 / leukaemia)

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Abstract: The relationship between signal pathways MEK1/2-ERK1/2 and ATM-p53 in the response to DNA damage is not well understood. The aim of our study was to investigate the effect of mitoxantrone and two protein kinase inhibitors – caffeine (inhibitor of ATM kinase) and U0126 (inhibitor of MEK1/2 kinase) – on MOLT-4 and Jurkat leukaemic cell lines. In this work we show that the inhibition of MEK1/2 is associated with an increased mortality of cells after mitoxantrone treatment. Inhibition of ATM by caffeine delayed mitoxantrone-induced cell death in MOLT-4 cells. Mitoxantrone itself induced cell-cycle arrest and accumulation of the cells in late S and G2/M phase. Inhibition of ATM, but not of MEK1/2, abrogated mitoxantrone-induced cell-cycle arrest. Inhibition of MEK1/2 did not change mitoxantrone-induced up-regulation of p53 and p21, but inhibition of ATM markedly decreased up-regulation of p53 and p21, and p53 phosphorylation on serine 15 and serine 392. It can be concluded that: 1) mitoxantrone-induced phosphorylation of p53 on serine 15 and serine 392 is ATM dependent and MEK1/2-ERK1/2 independent. 2) ATM inhibition by caffeine prevents G2 cell arrest and in p53-positive cells MOLT-4 delays the onset of mitoxantrone-induced cell death. 3) Inhibition of MEK1/2-ERK1/2 cascade potentiates the cytostatic effect of mitoxantrone regardless of the p53 status.

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Abbreviations: Ann – annexin V, ATM – ataxia-teleangiectasia mutated, ATR – ataxia-teleangiectasia and Rad3-related, Chk2 – checkpoint kinase 2, DSB – double-strand breaks of DNA, ERK – extracellular signal-regulated kinase, JNK – Jun N-terminal kinase, MAPK – mitogen-activated kinases, MTX – mitoxantrone, PI – propidium iodide, SAPK – stress-activated protein kinase.

Introduction

Mitoxantrone (MTX), 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino] 9,10-anthracendione dichloride, is a synthetic derivative of anthracendione with anticancer effect. It is used in treatment of both, haematological and solid tumours. Its mechanism of action involves intercalation and topoisomerase II inhibition. Unlike anthracyclines MTX does not increase reactive oxygen species formation. It forms a covalent complex with topoisomerase II, which prevents rejoining of DNA strands during replication and induces double-strand breaks (DSB) of DNA.

Early response of human cells to DSB is by activation of kinases – casein kinase 1, DNA protein kinase (DNA-PK), mitogen-activated kinases (MAPK), ataxia-teleangiectasia and Rad3-related (ATR), and predominantly ataxia-teleangiectasia mutated (ATM) kinase. ATM is a 350 kDa protein from the family of PI3 kinases. Its main role is to transmit the signal by phosphorylation of other target proteins (Khanna et al., 2001) such as Chk2 or p53.

Nuclear phosphoprotein p53 is a tumour suppressor that is important for cells as a transcription factor (Harris, 1996). The DNA damage results in the activation of p53 phosphorylation. Two protein kinases play an important role in this activation in G1 phase. ATM-kinase phosphorylates serine 15 and after this activation checkpoint kinase 2 (Chk2) phosphorylates serine 20. The phosphorylation continues on serine 37 and serine 392 in the phase G2/M (Buschmann et al., 2000). Shieh et al. (1997) reported that phosphorylation of serine 15 prolonged the life span of protein p53 due to the inhibition of Mdm2 activity. The binding ability of p53 to specific DNA sequences increases in case of serine 392 phosphorylation (Thompson et al., 2004).

Chk2 is a protein kinase which is activated in response to DNA damage and is involved in the cell cycle checkpoint control, DNA damage repair and DNA damage-

induced apoptosis. Chk2 is phosphorylated by ATM kinase on Thr68 and the activated Chk2 phosphorylates various downstream targets including Cdc25C, p53, and others. The main effect of Chk2 activation is that phosphorylated Chk2 inhibits the function of Cdc25C phosphatase, which in turn leads to G2/M cell cycle arrest. Chk2 was also found to phosphorylate p53 on Ser20 and thus contributes to increased stability of p53 (Matsuoka et al., 2000).

DNA damage simultaneously provokes activation of at least two types of signals – signals resulting in cell death and signals contributing to the cell survival and proliferation. The activation of a wide spectrum of MAPK is an important component of the processes leading to cell survival. Five MAPK systems have been classified in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2, JNK3), p38 kinases, ERK3/ERK4 and ERK5 (Qi and Elion, 2005). Three groups of MAPK have been characterized in greater detail: ERK1 and ERK2, JNK (also known as stress-activated protein kinases – SAPK), and p38 kinases.

According to our current knowledge, double-strand breaks of DNA result in the compensatory activation of many MAPK signal pathways. This is accompanied with simultaneous activation of the ATM-Chk1/2-p53 cascade. It includes not only the activation of mitogen-activated MEK1/2-ERK1/2 cascade, but also the activation of JNK and p38 MAPK. The latter kinases are known for their pro-apoptotic effects. On the other hand, the signals mediated by the ERK cascade are associated with cell proliferation and anti-apoptotic effects (Golding et al., 2007). The response to the double-strand breaks of DNA can lead to the modulation of expression and the release of autocrine factors (e.g. TGF-α, TNF-α) and death receptors that are also involved in the activation of MAPK.

The aim of our study was to investigate the effect of mitoxantrone and two protein kinase inhibitors – caffeine (inhibitor of ATM kinase) and U0126 (inhibitor of MEK1/2 kinase) – on MOLT-4 (p53-positive) and Jurkat (p53-negative) leukaemic cell lines.

Material and Methods

Cell cultures and culture conditions

The experiments were carried out with the MOLT-4 cell line (human leukaemic T lymphocytes) and Jurkat leukaemia T cells from the American Type Culture Collection (ATCC, Manassas, VA). MOLT-4 cells were cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 20% foetal calf serum in a humidified incubator at 37 °C and a controlled 5% CO_2 atmosphere. Jurkat leukaemic cell line was maintained in 90% RPMI medium supplemented with 10% foetal bovine serum, L-glutamine, geneticin, penicillin and streptomycin in a humidified incubator in the presence of 5% CO_2. The cultures were

divided every 2nd day by dilution to a concentration of 2×10^5 cells/ml. The cell counts were performed with a haemocytometer; the cell membrane integrity was determined using the Trypan blue exclusion technique. The cell lines in the maximal range of up to 20 passages were used for this study.

Mitoxantrone and inhibitors

Stock solution of mitoxantrone (Sigma-Aldrich) was prepared by dissolving 1.3 mg of the substance in 2.5 ml of distilled water (final concentration c = 1 mmol/l). For the experiments the stock solution was diluted to final concentration 5 nmol/l. Caffeine (Sigma-Aldrich) was dissolved in culture medium to reach the final concentration of 2 mmol/l and applied 45 min before MTX application. U0126 (Merck, Wilmington, DE) was dissolved in DMSO to stock solution with concentration 10 mmol/l. The stock solution was mixed with culture medium to reach the final concentration of U0126 10 μmol/l and applied 30 minutes before MTX application.

Flow cytometry

For apoptosis detection we used the flow cytometric Apoptest-FITC kit (DakoCytomation, Copenhagen, Denmark) according to manufacturer's instructions. The kit is based on Annexin V (Ann) binding to phosphatidylserine at the cell surface of apoptotic cells. Propidium iodide (PI) is used as a marker of cell membrane permeability. For cell cycle analysis the cells were washed with cold PBS and fixed with 70% ethanol. For detection of low-molecular-weight fragments of DNA, the cells were incubated for 5 min at room temperature in a buffer (192 ml 0.2 mol/l Na_2HPO_4 + 8 ml 0.1 mol/l citric acid, pH 7.8) and then stained with PI in Vindelov's solution for 60 min at 37 °C. The measurements were performed in a Cell Lab Quanta flow cytometer (Beckman Coulter, Brea, CA); the data were analysed using WinMDI 2.9 software.
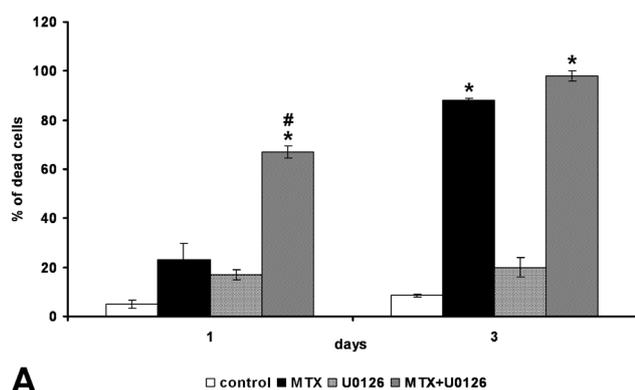
Western blotting

The cells were harvested for preparation of whole-cell lysates (Cell Lysis Buffer, Cell Signaling Technology, Inc, Danvers, MA). The protein content was quantified using BCA assay (Sigma-Aldrich). The lysates containing an equal amount of protein (20 μg) were loaded into each lane of a polyacrylamide gel. After electrophoretic separation, the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA). The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk and then incubated with primary antibody (p53, p53_serine392 – Exbio, Prague, Czech Republic; β-actin, p21, – Sigma-Aldrich; p53_serine15 – Calbiochem, Merck; Chk2_threonin68 – Cell Signalling) at 4 °C overnight. After washing, the membranes were incubated with appropriate secondary antibody (DakoCytomation) for one hour at room temperature. The signal was developed with a chemiluminescence detection kit (Roche, Basel, Switzerland). To

confirm equal protein loading each membrane was re-probed and re-incubated with β-actin.
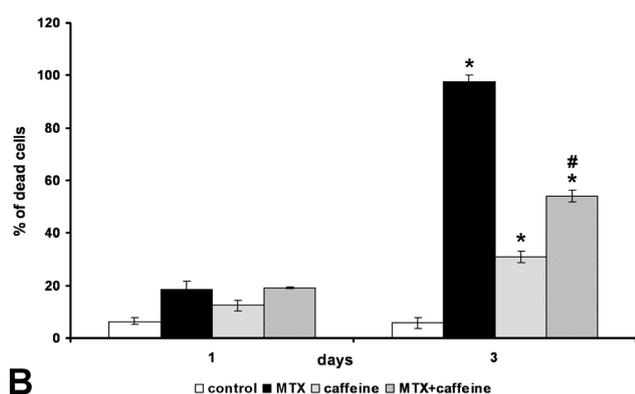
Results

Viability

The effect of mitoxantrone and two protein kinase inhibitors – caffeine (inhibitor of ATM kinase) and U0126 (inhibitor of MEK1/2 kinase) – on cell viability was studied first in the p53-positive MOLT-4 leukaemic cell line (Fig. 1). The exposure to a single application of 5 nmol/l mitoxantrone caused a significant decrease in cell viability (as determined by the Trypan blue exclusion technique) 72 h after incubation in comparison with the control group, reaching almost 100 % of dead cells. The combination of U0126 (MEK1/2 inhibitor) with MTX was significantly more effective already 24 h after application (67 % of dead cells); complete eradication was reached after three days (Fig. 1A). Preincubation with caffeine (ATM-kinase inhibitor) prior to MTX administration did not influence the viability of cells in comparison with caffeine or MTX alone after 24 h.
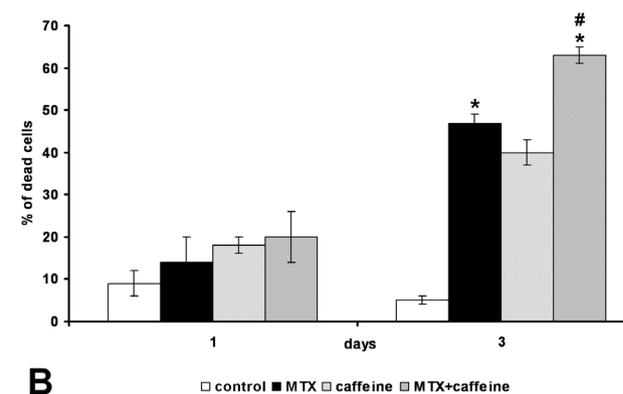
Surprisingly, the effect of caffeine in combination with MTX was temporarily protective – 54 % of dead cells in comparison with MTX alone after three days (Fig. 1B), and the eradication was delayed to day 6 (not shown).

Next we studied the effect of mitoxantrone and the inhibitors on the viability of p53-negative Jurkat leukaemic cell line (Fig. 2). The effect of a single application of 5 nmol/l mitoxantrone caused a significant decrease in cell viability (as determined by the Trypan blue exclusion technique) 72 h after incubation in comparison with the control group, reaching 50 % of dead cells. The combination of U0126 (MEK1/2 inhibitor) with MTX was significantly more effective 24 h after application (32 % of dead cells); application of U0126 with MTX reached 56 % of dead cells after three days (Fig. 2A). Preincubation with caffeine (ATM-kinase inhibitor) prior to MTX administration did not significantly influence the viability of cells in comparison to caffeine or MTX alone after 24 h. The effect of caffeine in combination with MTX was not significantly different in comparison to MTX alone after one day. Caffeine with MTX caused a significant increase in the percentage of dead cells (63 %) in comparison to MTX alone (47 %) after three days (Fig. 2B).

Fig. 1. Effect of 5 nmol/l mitoxantrone in combination with **(A)** U0126 (inhibitor of MEK1/2 kinase) and **(B)** in combination with caffeine (inhibitor of ATM kinase) on the viability of the MOLT-4 cell line. The viability was determined using the Trypan blue exclusion technique. The results are shown as mean ± SD from three experiments.
* - significantly different to control ($P < 0.001$), # - significantly different from MTX alone ($P < 0.05$).
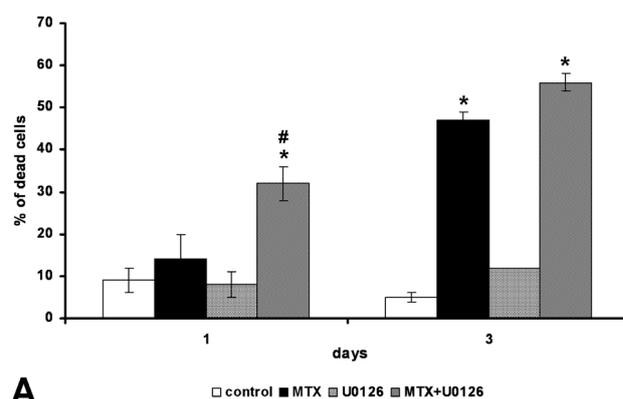
Fig. 2. Effect of 5 nmol/l mitoxantrone in combination with **(A)** U0126 (inhibitor of MEK1/2 kinase) and **(B)** in combination with caffeine (inhibitor of ATM kinase) on the viability of the Jurkat cell line. The viability was determined using the Trypan blue exclusion technique. Results are shown as mean ± SD from three experiments.
* - significantly different to control ($P < 0.001$), # - significantly different from MTX alone ($P < 0.05$).

Annexin/PI assay

Apoptosis induction was analysed in MOLT-4 cells 24 h after the application of the agents (Fig. 3). The treatment with single doses of caffeine or U0126 influenced the apoptosis in a very similar manner. The total amount of apoptotic cells was increased in case of MTX (57 %) in comparison with the control group (19 %). The combination of caffeine with MTX had a protective effect; we observed 21 % of dead cells in comparison to MTX alone (57 %). On the other hand, the combination of U0126 with MTX was effective and the inhibitor sensitized the cells – 26 % of them were in the early phase and 49 % in the late phase of apoptosis.

Cell cycle

The treatment of MOLT-4 cells with both inhibitors resulted in a small decrease in the percentage of cycling cells in S+G2/M phase – caffeine 21 %, U0126 32 % – in comparison with the control – 45 %. MTX alone induced cell-cycle arrest and accumulation of the MOLT-4 cells in late S and G2/M phase (71 %). The application of ATM kinase inhibitor caffeine completely abrogated MTX-induced cell-cycle arrest (25 %). On the other hand, application of U0126 (inhibitor of MEK1/2) did not abrogate MTX-induced cell-cycle arrest – 62 % of cells occurred in S+G2/M phase (Fig. 4A, B). MTX induced DNA fragmentation during apoptotic cell death
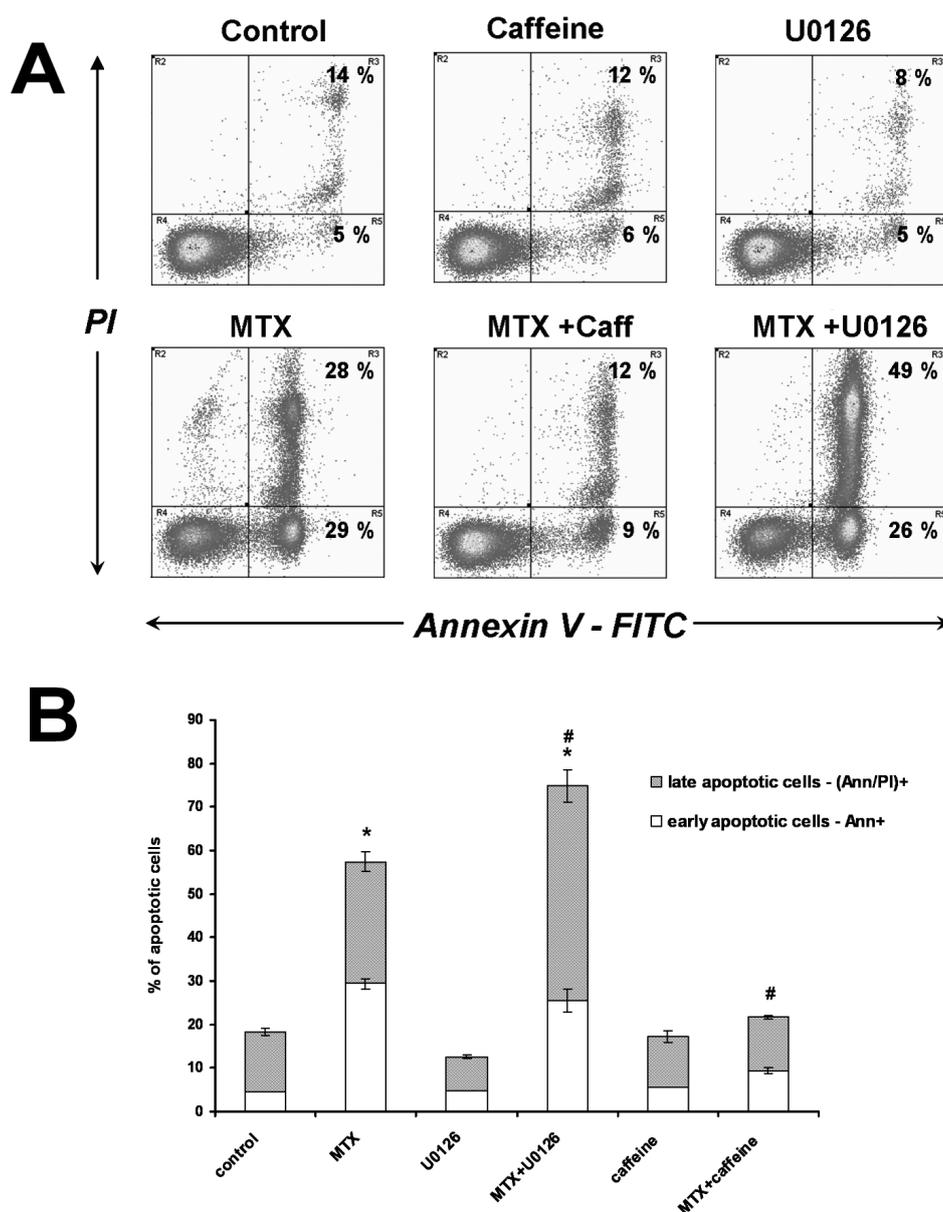

Fig. 3. Apoptosis detection by Annexin V/PI staining 24 h after the application of 5 nmol/l mitoxantrone in combination with caffeine (inhibitor of ATM kinase) or U0126 (inhibitor of MEK1/2 kinase) in MOLT-4 cells. **(A)** Representative results of dotplot analysis. **(B)** Percentage of early and late apoptotic MOLT-4 cells detected by flow cytometry. Results are shown as mean ± SD from three experiments.
* - significantly different to control ($P < 0.001$), # - significantly different from MTX alone ($P < 0.05$).

(detected as sub G1 peak), which was potentiated by U0126 (Fig. 4C).

In the case of p53-negative Jurkat cells MTX alone induced stronger cell-cycle arrest and accumulation of the cells in late S and G2/M phase (92 %) when compared to p53-positive MOLT-4 cells (71 %). The application of ATM kinase inhibitor caffeine completely abrogated MTX-induced cell-cycle arrest, while inhibition of MEK1/2 by U0126 did not significantly affect MTX-induced cell-cycle arrest (Fig. 5A, B). MTX induced DNA fragmentation during apoptotic cell death (detected as sub G1 peak), which also occurred when combined with U0126 (Fig. 5C).

ATM-Chk2 pathway

To confirm the effect of caffeine as an inhibitor of ATM kinase we determined the Chk2 phosphorylation of threonine 68 in both MOLT-4 and Jurkat cells. The level of Chk2_Thr68 increased markedly after the exposure to MTX alone in both cell lines. As we expected, pre-treat-

ment with caffeine in combination with MTX decreased markedly the expression of Chk2_Thr68 (Fig. 6).

Pathway p53-p21

The effect of MTX and caffeine (inhibitor of ATM kinase) and U0126 (inhibitor of MEK1/2 kinase) on activation of the p53-p21 pathway was studied on p53-positive MOLT-4 cells (Fig. 7). As we expected, the expression of protein p53 was low in the control group. The amount of protein p53 increased markedly as a response to DNA damage caused by MTX. Pre-treatment with U0126 prior to MTX did not change the cell response – the amount of p53 was comparable with the effect of MTX alone. However, pre-treatment with caffeine – an inhibitor of ATM kinase – decreased markedly the up-regulation of p53.

We were also interested in the effect of the tested substances on posttranslational modification of p53, namely phosphorylation of Ser15 and Ser392. No phosphorylation of the serines in question was detected in the case of
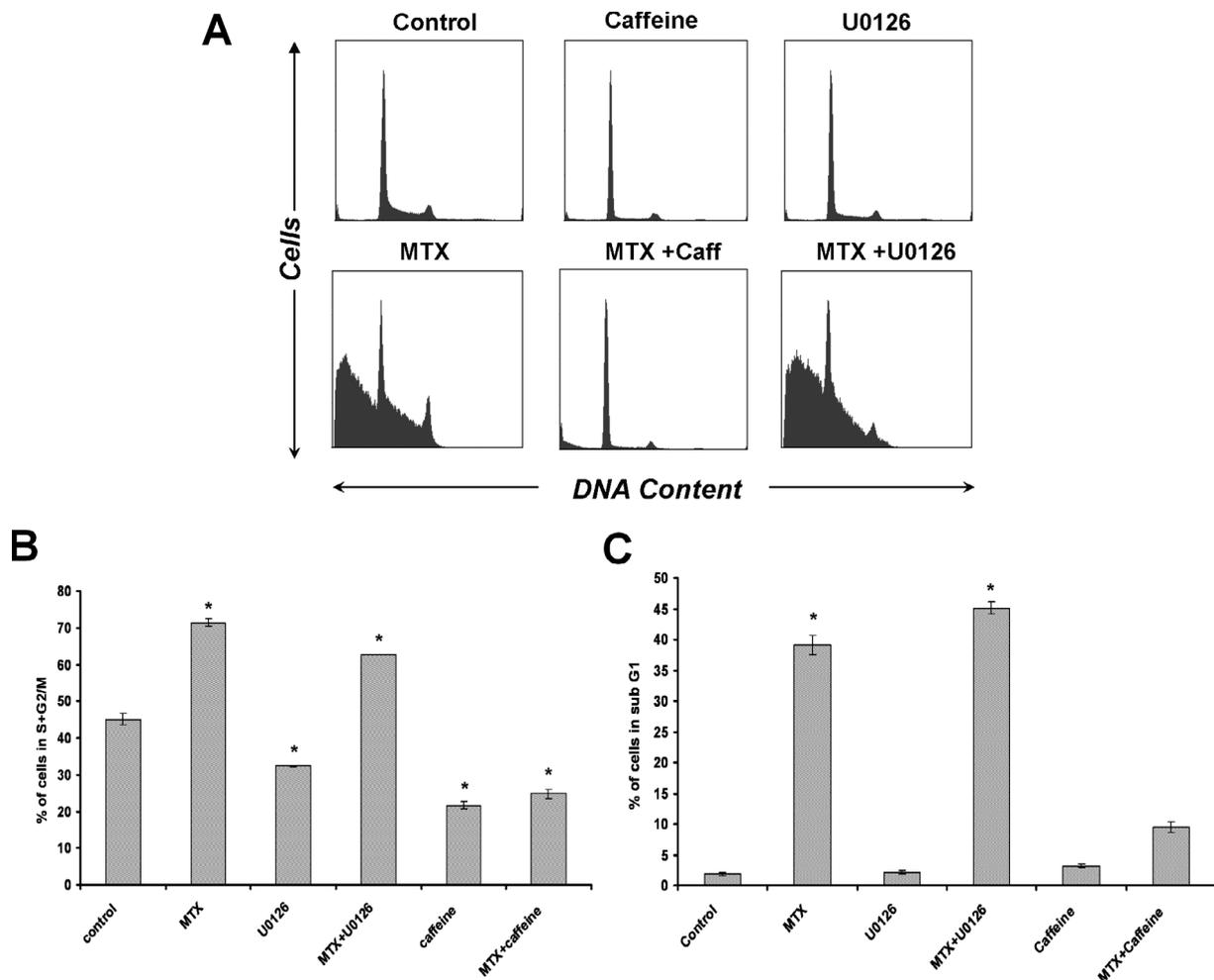

Fig. 4. Analysis of cell cycle of MOLT-4 cells 24 h after the application of 5 nmol/l mitoxantrone in combination with caffeine (inhibitor of ATM kinase) or U0126 (inhibitor of MEK1/2 kinase). **(A)** Representative results of cell cycle analysis, **(B)** percentage of cells in S+G2/M, and **(C)** percentage of early apoptotic cells represented by sub G1 peak after the application of 5 nmol/l mitoxantrone in combination with caffeine or U0126. Results are shown as mean ± SD from three experiments.
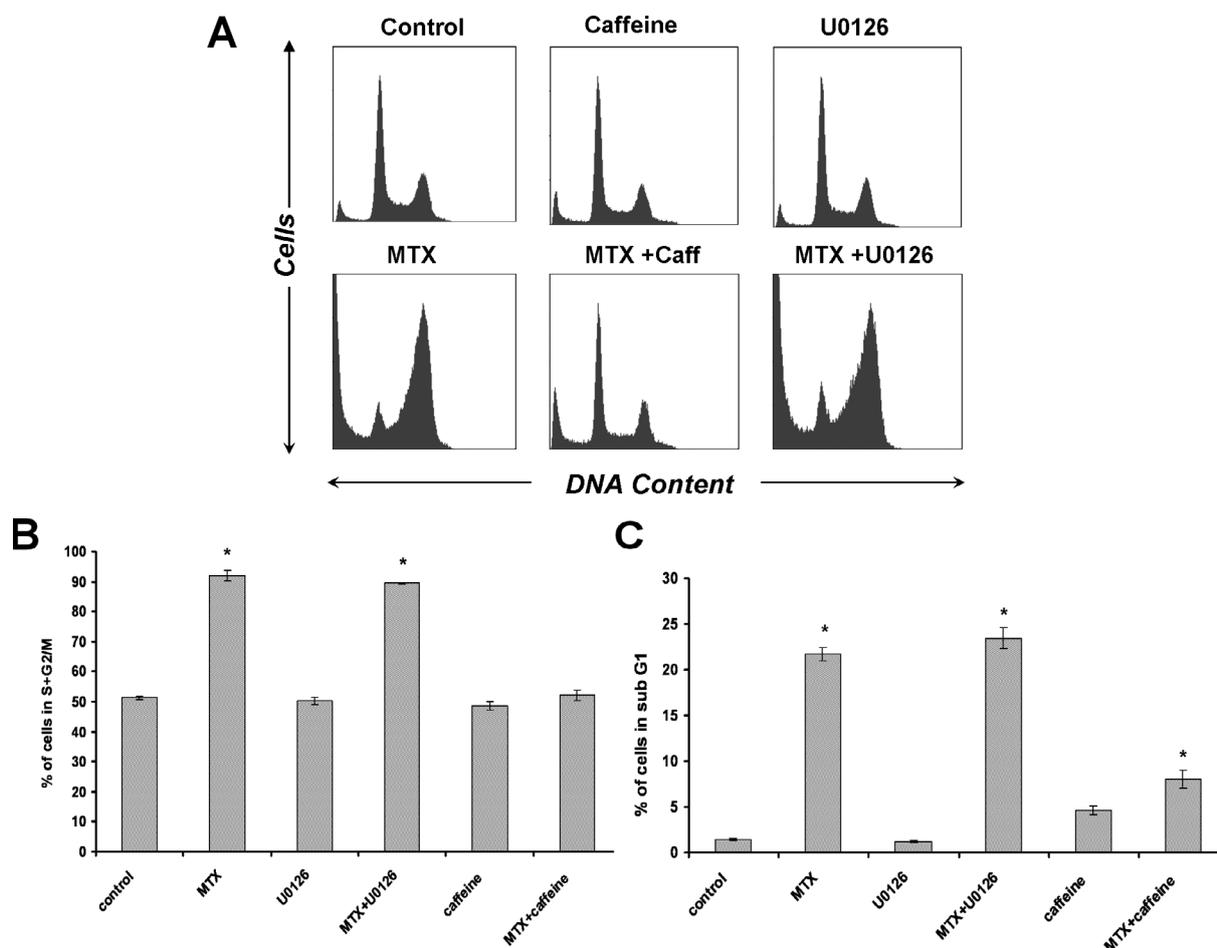* - significantly different from control (P < 0.001)

Fig. 5. Analysis of cell cycle of Jurkat cells 24 h after the application of 5 nmol/l mitoxantrone in combination with caffeine (inhibitor of ATM kinase) or U0126 (inhibitor of MEK1/2 kinase). **(A)** Representative results of cell cycle analysis, **(B)** percentage of cells in S+G2/M, and **(C)** percentage of early apoptotic cells represented by sub G1 peak after the application of 5 nmol/l mitoxantrone in combination with caffeine or U0126. Results are shown as mean ± SD from three experiments.
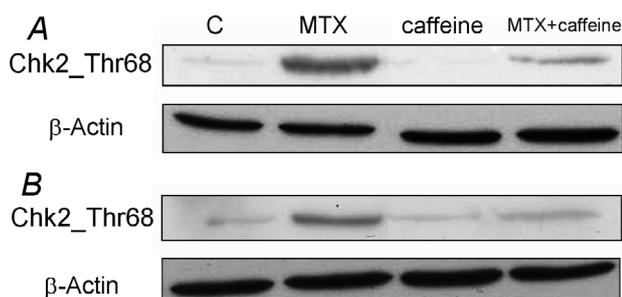* - significantly different from control (P < 0.001)

Fig. 6. Activation of Chk2_Thr68 in MOLT-4 cells **(A)** and Jurkat cells **(B)** exposed to 5 nmol/l mitoxantrone in combination with inhibitor of ATM kinase caffeine 24 h after the application. To confirm equal protein loading, membranes were re-incubated with β-actin. Representative results of one of three experiments.

the control group and after the treatment with either caffeine or U0126. The application of MTX supported phosphorylation of both serines 15 and 392 and the same response was detected after the combination U0126 + MTX. Caffeine in combination with MTX protected both serines from phosphorylation.

The expression of protein p21 occurred after the treatment with MTX alone and its combination with U0126. Caffeine in combination with MTX inhibited the expression of p21 to some extent in comparison with the effect of MTX. No expression of p21 was detected in the control group and after treatment with single doses of both inhibitors.

Discussion

Transcription factor protein p53 is known as a tumour suppressor that defends cells against tumour transformation. This protein is said to play a key role in the regulation of the cell response to various forms of stress. DNA damage leads to phosphorylation of p53 by ATM/ATR kinases. ATM kinase is believed to play a crucial role in the response to DNA double-strand breaks. Phosphorylation of protein p53 on Ser15 is taken as a
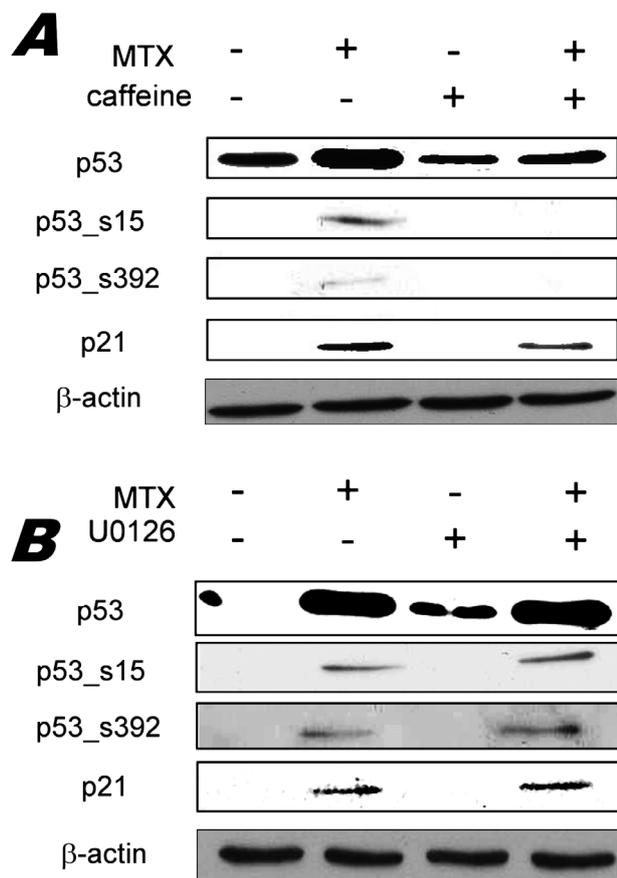
Fig. 7. Induction and activation of p53 in MOLT-4 cells exposed to 5 nmol/l mitoxantrone in combination with inhibitor of ATM kinase caffeine **(A)** or inhibitor of MEK1/2 kinase U0126 **(B)** 24 h after the application. To confirm equal protein loading, membranes were re-incubated with β-actin. Representative results of one of three experiments.

decisive point of posttranslational modification associated with DSB. It is known that phosphorylation of Ser 15 inhibits the interaction with Mdm2 and protects protein p53 from ligation to ubiquitin, stabilizes it (Toledo and Wahl, 2007) and prolongs its longevity (Shieh et al., 1997).

We tested caffeine as ATM kinase inhibitor. At the first glance, the results seem to be slightly controversial. Despite the toxic effect of caffeine alone on the MOLT-4 cell line, caffeine in combination with MTX protected these cells from the damage caused by MTX on day 3 after application. However, this protection was temporary; the complete eradication occurred on day 6 after the treatment (data not shown). Protein p53-negative Jurkat cells were much less sensitive to MTX treatment, the percentage of dead cells 72 h after MTX treatment was 98 % for MOLT-4 (Fig. 1) and 47 % for Jurkat cells (Fig. 2). As expected, due to p53 absence the MTX-induced cell-cycle arrest in G2/M was much stronger in Jurkat cells. In Jurkat cells, inhibition of ATM kinase caused almost complete abrogation of this cell-cycle arrest 24 h after MTX treatment and caused a significant increase in the number of dead cells 72 h after MTX

treatment. This probably resulted from shorter time for DNA repair. Our data are in good correlation with a similar response described by Vávrová et al. (2003). These authors were focused on the effect of ATM kinase inhibition on apoptosis following ionizing radiation that provoked DNA double-strand breaks, which is also the principle of cytostatic effect of MTX. In their experiments the radiation-induced G2/M cell-cycle arrest was abrogated, the onset of apoptosis occurred in later intervals, and an overall effect of ATM kinase inhibition was radiosensitizing. Also, the cells originating from patients suffering from ataxia-teleangiectasia, a disease in which the gene encoding ATM is mutated and ATM protein absent, are markedly radiosensitive (Derheimer and Kastan, 2010). Thus, in the conditions of normal active ATM the p53 absence increases the resistance of cells to MTX, as this allows complete G2/M cell-cycle arrest mediated by kinase Chk2 and temporarily protects the cells from apoptosis induction. When ATM is inhibited in p53-positive cells, p53 cannot be efficiently activated, delaying the onset of cell death, but also affecting DNA repair. However, even in p53-negative cells the inhibition of ATM does not allow G2/M cell-cycle arrest mediated by Chk2, which shortens the time for DNA repair, causes DNA damage accumulation and results in increased sensitivity to MTX.

The phosphorylation of Ser392 mediates stabilization of protein p53 through its ability to interact with specific sequences of DNA (Thompson et al., 2004) and some reports state that this phosphorylation is catalysed by casein kinase II (Sakaguchi et al., 1997, Szkanderová et al., 2003). The treatment with caffeine in our experiments diminished this phosphorylation, which allows speculating that this process may not be directly catalysed by ATM but is ATM dependent.

U0126 eliminates one of the pathways leading to the support of cell growth due to the inhibition of MEK1/2 (Roux and Blenis, 2004). In our work we show that this inhibition is associated with an increased mortality of cells after MTX treatment. As the treatment with inhibitor U0126 strengthened the MTX effect, we can conclude from our results that the MEK1/2-ERK1/2 pathway is involved in the pro-survival signalling following the DNA damage caused by MTX. The viability of cells was decreased in comparison with the effect of MTX alone due to the inhibition of MEK1/2 that is necessary for ERK1/2 activation.

Based on our results, the MEK1/2-ERK1/2 pathway seems to be involved to some extent in the cascade regulating the level of protein p53 in the MOLT-4 cells. Malmlöf et al. (2007) described the phosphorylation of a negative regulator of protein p53 – Mdm2 – on Ser 166 mediated by MEK1/2-ERK1/2 kinases in several cell types. An increased synthesis of Mdm-2 after activation of the MEK1/2-ERK1/2 pathway was reported by Phelps et al. (2005). These findings could explain the accumulation of protein p53 caused by U0126 alone. The absence of p53 phosphorylation after inhibition of MEK1/2 supports the hypothesis that the accumulation

of p53 in the MOLT-4 cell line is not linked to p53-modification after treatment with U0126 but is associated with the regulation of Mdm-2 activity.

One of the mechanisms of the cell-cycle arrest is inhibition of cyclin-dependent kinases due to transcription of the protein 21 gene that is triggered by protein p53 following DNA damage (Agarwal et al., 1998). We also found a correlation between increased p21 and the amounts of p53 and its phosphorylated forms. We assume that in consequence of processes occurring in MTX-damaged MOLT-4 cells the activated protein p53 initiates transcription of the *p21* gene. Caffeine – an inhibitor of ATM kinase – decreases both the phosphorylation of p53 and as a consequence the amount of p21.

The relationship between signal pathways MEK1/2-ERK1/2 and ATM-p53 in the response to DNA damage has been poorly understood. Golding et al. (2007) reported in cells of malignant glioma that the inhibition of MEK1/2-ERK1/2 decreased significantly the activation of ATM kinase following DBS and also that ATM inhibition decreased the activation of ERK1/2 by ionizing radiation. On the other hand, Yan et al. (2007) did not find decreased ATM activity after MEK1/2-ERK1/2 inhibition in cells of breast carcinoma after radiation; however, the ATR activity was decreased. In their experiments, ATM and ATR inhibition did not influence the activation of ERK1/2 by radiation. Our results allow us to conclude that the inhibition of the signal cascade MEK1/2-ERK1/2 potentiates the sensitivity of MOLT-4 and Jurkat leukaemic cells to MTX-induced apoptosis, but does not affect activation and accumulation of p53 in MOLT-4 cells. The activation and accumulation of p53 in MOLT-4 cells treated by mitoxantrone is ATM dependent.

Conclusions

– MTX-induced phosphorylation of p53 (Ser15 and Ser 392) is ATM dependent and MEK1/2-ERK1/2 independent

– Inhibition of the MEK1/2-ERK1/2 cascade potentiates the cytostatic effect of MTX regardless of the p53 status

– ATM inhibition conditioned by caffeine prevents G2 cell arrest and delays the onset of MTX-induced cell death in p53-positive MOLT-4 cell line

– Inhibition of ATM by caffeine causes abrogation of G2 cell-cycle arrest and increased MTX-induced cell death in p53-negative Jurkat cell line

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