# Original Article

# Subcellular Localization of Proteins Responding to Mitoxantrone-Induced DNA Damage in Leukaemic Cells

(Chk2 / DNA damage / mitoxantrone / p21 / p53 / subcellular localization)

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**Abstract. The aim of the present study was to investigate the subcellular localization of proteins participating in the double-strand break response pathway – p53, Mdm2, p21 and Chk2. MOLT-4 cells were pre-treated with mitoxantrone in concentrations 1 nmol/l and 5 nmol/l. The trypan blue technique was used to determine cell viability and proliferation. Western blotting was used to evaluate changes in p53, Mdm2 and Chk2 protein expression and sandwich ELISA was used to evaluate changes in the p21 protein amount. After 1 nmol/l mitoxantrone cells did not die, but their ability to proliferate was decreased. The p53 protein was activated and phosphorylated at serines 15 and 392 and accumulated in the nucleus after 24 and 48 h. The Mdm2 protein was present in the cytoplasm with its maximal level after 8 and 16 h. The p21 protein was detected in the nucleus after 24 and 48 h. Increased levels of phosphorylated Chk2 at threonine 68 were observed in the cytoplasmic fraction after 24 and 48 h of mitoxantrone treatment. We used mitoxantrone as an inducer of double-strand breaks to bring new data about the subcellular distribution of proteins responding to DNA damage. In MOLT-4 cells, the p53 protein was activated. p53 was phosphorylated at serines 15 and 392 and accumulated in the nucleus. The Mdm2 protein was activated in advance to p53 and occurred in the cytoplasm. The p21 protein was present in the nucleus. Chk2 kinase was activated by the phosphorylation at threonine 68 and we observed increased levels of this protein in the cytoplasmic fraction.**

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Abbreviation: OD – optical density.

## Introduction

In response to DNA damage caused by genotoxic stress (ionizing radiation, UV radiation, chemical mutagens, and cytostatic drugs), signal cascades resulting in activation of many various processes are activated in cells. The amounts of proteins involved in the response to this damage are changed and their localization in the cell is altered. One of the key regulators is p53 protein. The importance of p53 as a tumour suppressor is emphasized by the fact that the *p53* gene is mutated in more than half of all human cancers (Erikson and Stigrand, 2010).

In response to genotoxic stress, p53 is activated and induces cell cycle arrest, apoptosis, or senescence. It protects the genome from accumulating genetic mutations by giving the chance to repair the DNA damage or by eliminating damaged cells (Zhao and Xu, 2010; Rashi-Elkeles et al., 2011). In normal cells, p53 is a short-lived protein that is present in the cells at a barely detectable level because it is negatively regulated by Mdm2. Mdm2 acts as a specific E3 ligase for p53 by adding ubiquitin chains and targets p53 for degradation through the proteasome (Brooks and Gu, 2003), suppressing the transcriptional activities of p53 (Zhao and Xu, 2010). Under certain circumstances the amount of p53 protein is increased and it triggers off the whole cascade of processes (Rashi-Elkeles et al., 2011; Mirzayans et al., 2012).

The p53 protein is a 393 amino acid transcription regulator consisting of five structural and functional domains. The N-terminal domain is responsible for activation of p53-inducible genes, the central domain enables specific binding to DNA, and the tetramerization domain is essential for the ability of p53 to regulate gene expression. The p53 protein is able to activate a large number of targets through these three domains – including p21 protein and pro-apoptotic proteins (Mirzayans et al., 2012).

The p21 protein is considered to be a universal inhibitor of cell cycle progression. It is able to influence the G1 checkpoint by reaction with cyclin/CDK complexes. It reacts with cyclin-dependent kinases (CDK)

and inhibits their ability to phosphorylate Rb, an event that is required for the release of E2F factor (Mirzayans et al., 2012). However, p21 also plays an important role in the transition of cells from G2 phase of the cell cycle into mitosis through the inhibition of cyclin B1. It is able to bind the PCNA protein to maintain the balance between DNA replication, DNA repair, and cell cycle progression (Cmielová and Rezáčová, 2011; Mirzayans et al., 2012).

Cells can also control the cell cycle by p53-independent mechanisms. One of the main kinase in this pathway is Chk2. Chk2 is a stable protein expressed through the cell cycle, which is activated primarily by ATM in response to DNA double-strand breaks. ATM phosphorylates Chk2 at threonine 68, and the activation involves dimerization and Chk2 auto-phosphorylation. A major target for Chk2 is Cdc25 phosphatase, a positive regulator of cell cycle progression, which is inhibited by Chk2-mediated phosphorylation (Bartek and Lukas, 2003; Reinhardt and Yaffe, 2009; Stracker et al., 2009).

The response of cells to genotoxic stress is widely studied, but the function of the proteins responding to DNA damage depends on their localization in the cell, which is less clear. The aim of the present study was to investigate the subcellular localization of proteins participating in the double-strand break response pathway. Mitoxantrone was used as an inducer of double-strand breaks. It is a synthetic derivative of anthracenedione, which is used in the treatment of tumours. Its mechanism of action involves intercalation and topoisomerase II inhibition.

## Material and Methods

### *Cell isolation and cultivation*

MOLT-4 cells were cultured in 5% $CO_2$ atmosphere under 37 °C in Iscove's modified Dulbecco's medium (Sigma, St Louis, MO) supplemented with 20% FCS (PAA, Dartmouth, MA), 1% glutamine (Gibco, Paisley, UK) and penicillin/streptomycin (Gibco). In all experiments, early passages (< 20 passages) of the cells were used. The cultures were divided every second day by dilution to a concentration of $2 \times 10^5$ cells/ml. Trypan blue staining was used for counting viable and non-viable cells.

### *Treatment of cells*

The stock solution of mitoxantrone (Sigma) was prepared by dissolving 1.3 mg of the substance in 2.5 ml of distilled water to reach the concentration of 1 mmol/l. For the experiments, the stock solution was diluted to final concentrations 1 nmol/l or 5 nmol/l. Cells were irradiated at room temperature by a $^{60}Co$ γ-ray source (Chisotron, Chirana, Prague, Czech Republic) at the photon dose rate of 1 Gy/min in the distance 1 m from the source. Cells were irradiated with the dose of 3 Gy.

### *Viability and proliferation*

Cells were seeded at $1 \times 10^6$ into 5 ml of cultivating media, treated, and after 4, 24 and 48 h counted using the trypan blue exclusion technique.

### *Electrophoresis and Western blotting*

MOLT-4 cells were harvested and whole cell lysates, nuclear and cytoplasmic fractions were prepared for the analysis (Cell Lysis Buffer, Cell Signaling Technology, Boston, MA; NE-PER kit, Thermo Scientific, Rockford, IL). The protein content was determined by bicinchoninic acid assay (Sigma). For the SDS-PAGE electrophoresis, an equal amount of protein (10 μg) was loaded into the gel. The proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories, Prague, Czech Republic), the membrane was blocked in TBS with 5% non-fat dry milk and incubated with primary antibody (β-actin – Sigma; mdm2_s166, Chk2, Chk2_t68 – Cell Signaling Technology; p53, p53_s392 – Exbio, Prague, Czech Republic; p53_s15 – Merck, Prague, Czech Republic) at 4 °C overnight. The membrane was then incubated with polyclonal anti-mouse or anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark). For the signal detection, a chemiluminescence detection kit (Roche, Prague, Czech Republic) was used.

### *ELISA*

For quantitative assay of p21, the PathScan Sandwich ELISA kit by Cell Signaling Technology was used according to manufacturer's instructions. The optical density (OD) was measured at 450 nm. MOLT-4 cells irradiated with the dose of 3 Gy were used as a positive control.

## Results

### *Proliferation and viability*

The MOLT-4 cell line was exposed to a single dose of mitoxantrone in concentrations of 1 or 5 nmol/l for 4, 24 and 48 h. We registered only slight changes in the viability of the cells after the exposure to 1 nmol/l mitoxantrone during the entire time period – 84 % of living cells after 4 h (92 % in control), 94 % of living cells after 24 h (93 % in control) and 84 % of living cells after 48 h (93 % in control) (Fig. 1A). The cells kept their ability to proliferate after 1 nmol/l mitoxantrone, but the number of cells was significantly decreased after 48 h ($4.1 \times 10^6$ cells after 1 nmol/l; $5.8 \times 10^6$ in the control) (Fig. 1B).

The higher concentration of mitoxantrone (5 nmol/l) was toxic for MOLT-4 cells. We detected 82 % of living cells after 4 h (92 % in control), 39 % after 24 h (93 % in control), and only 1 % (93 % in control) of living cells after 48 h of incubation (Fig. 1A). The total number of living cells was decreased as well, and the cells lost their ability to proliferate. After 24 h of incubation with 5 nmol/l mitoxantrone, only $0.7 \times 10^6$ cells were count-

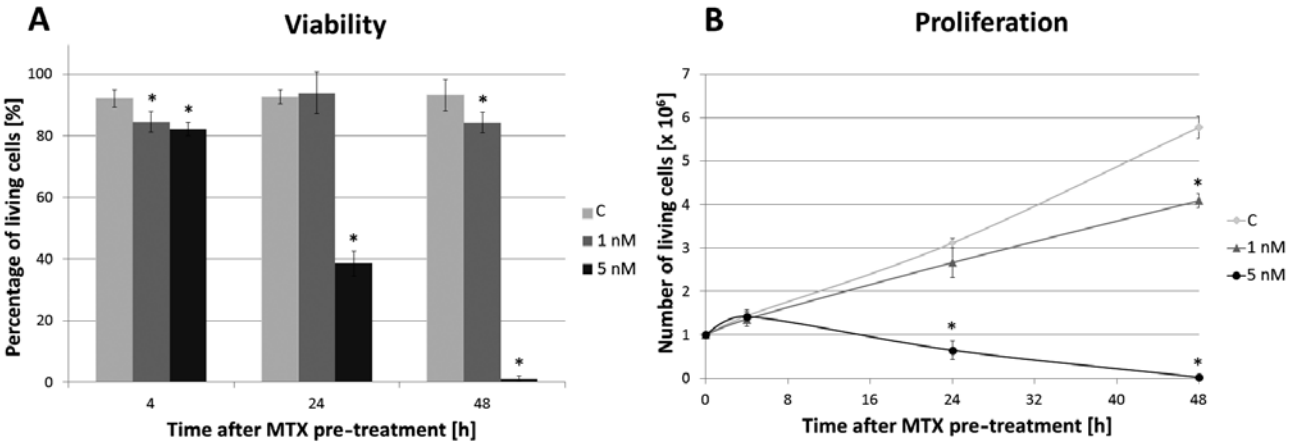


*Fig. 1*. The effect of 1 and 5 nmol/l mitoxantrone on MOLT-4 cells. The values represent means of three independent experiments, P values < 0.001 are considered significant, * indicate values significantly different from the control. **A**. Changes in cell viability during 48 h. **B**. Changes in cell proliferation during 48 h.

ed ($3.1 \times 10^6$ in the control) and $0.03 \times 10^6$ living cells remained after 48 h of incubation ($5.8 \times 10^6$ in the control) (Fig. 1B).

For the detection of proteins responding to DNA damage caused by mitoxantrone we decided to use the concentration 1 nmol/l.

## *Effect of mitoxantrone on p53 and Mdm2 protein activation and subcellular distribution*

We determined the amount of p53 protein and its phosphorylations at serines 15 and 392 in the nuclear, cytoplasmic and whole cell lysates to see the protein changes and the subcellular localization after 1 nmol/l mitoxantrone treatment. As we expected, the level of p53 was low in the control group. The expression of p53 protein increased after 24 and 48 h after the application of 1 nmol/l mitoxantrone. The increase was significantly higher in the nuclear samples. The p53 protein was activated and phosphorylated at serines 15 and 392 and the phosphorylated forms occurred mainly in the nucleus (Fig. 2A).

An increase of Mdm2 phosphorylation at serine 166 was detected after the mitoxantrone exposure. The

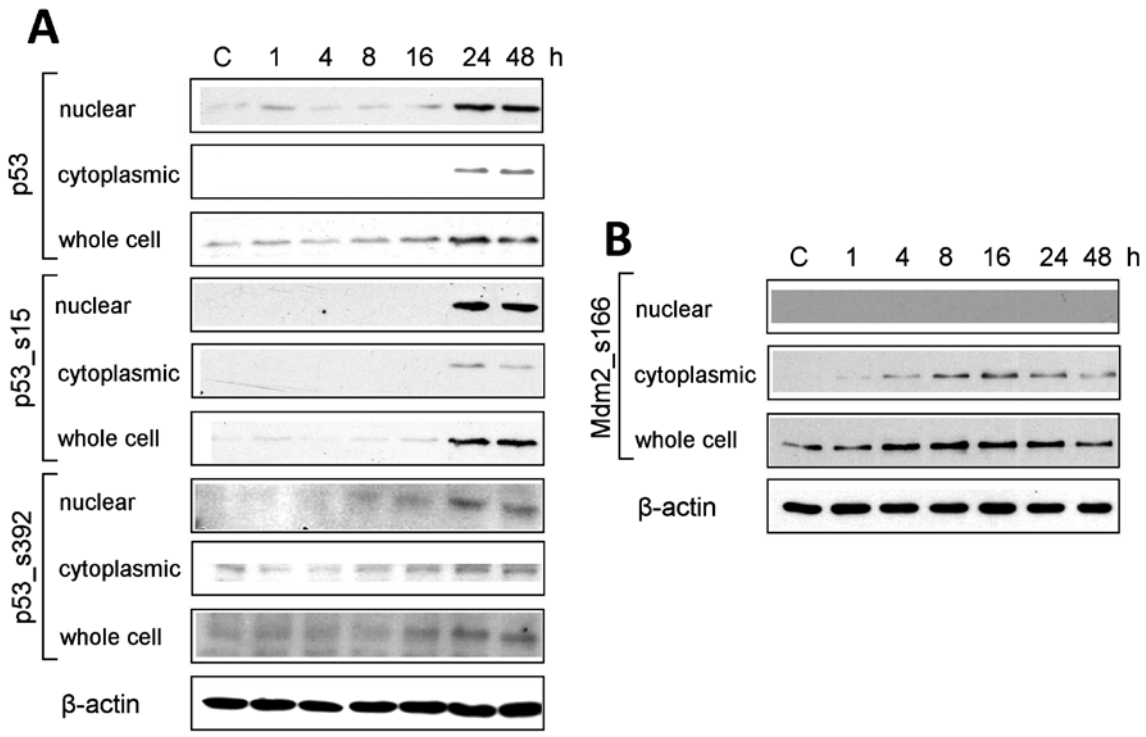


*Fig. 2.* Induction and activation of proteins in MOLT-4 cells in the nuclear, cytoplasmic and whole cell samples after the treatment with 1 nmol/l mitoxantrone in the time intervals of 1, 4, 8, 16, 24 and 48 h. **A**. Changes in p53 protein and phosphorylated p53 at serines 15 and 392. **B**. Changes in phosphorylated Mdm2 protein at serine 166. Representative results of one of three experiments.

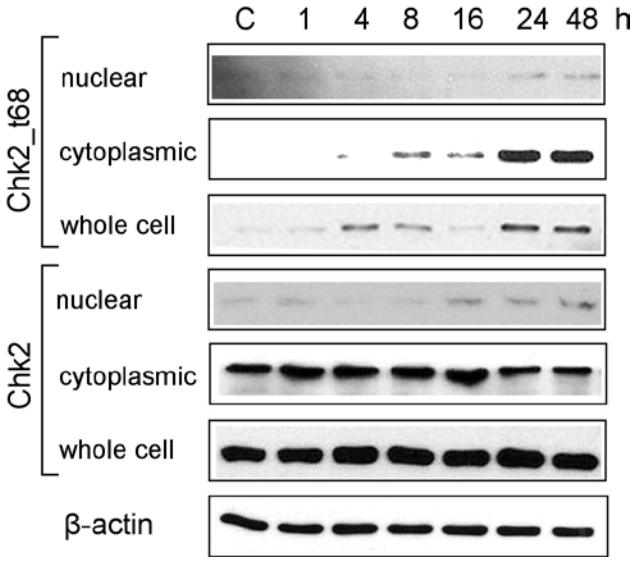


*Fig. 3.* Induction and activation of Chk2 and phosphorylated Chk2 at threonine 68 in MOLT-4 cells in the nuclear, cytoplasmic and whole cell samples after the treatment with 1 nmol/l mitoxantrone in the time intervals of 1, 4, 8, 16, 24 and 48 h. Representative results of one of three experiments.

Mdm2_Ser166 reached the highest levels after 8 and 16 h and then slowly decreased in time in the cytoplasm. The progression was the same in whole cell samples. There was no detectable signal of Mdm2_Ser166 in the nuclear fraction (Fig. 2B).

### *Effect of mitoxantrone on Chk2 activation and subcellular distribution*

We registered an increase of phosphorylated Chk2 at threonine 68 after 24 and 48 h mainly in the cytoplasm after the exposure to mitoxantrone. The level of total Chk2 did not significantly change during the entire time period (Fig. 3).

### *Effect of mitoxantrone on p21 protein activation and subcellular distribution*

The level of cyclin-dependent kinase inhibitor – p21 protein – was changed in response to 1 nmol/l mitoxantrone. The p21 protein was activated and its level was significantly increased in later times of exposure. While we observed no significant increase in the cytoplasm, the p21 level increased in the nucleus after 24 and 48 h. We compared our results with MOLT-4 cells irradiated with the dose of 3 Gy that we used as a positive control. The activation of p21 after irradiation was more pronounced than after 1 nmol/l mitoxantrone exposure. The maximal increase was also detected in the nuclear fraction of the cells (Fig. 4).

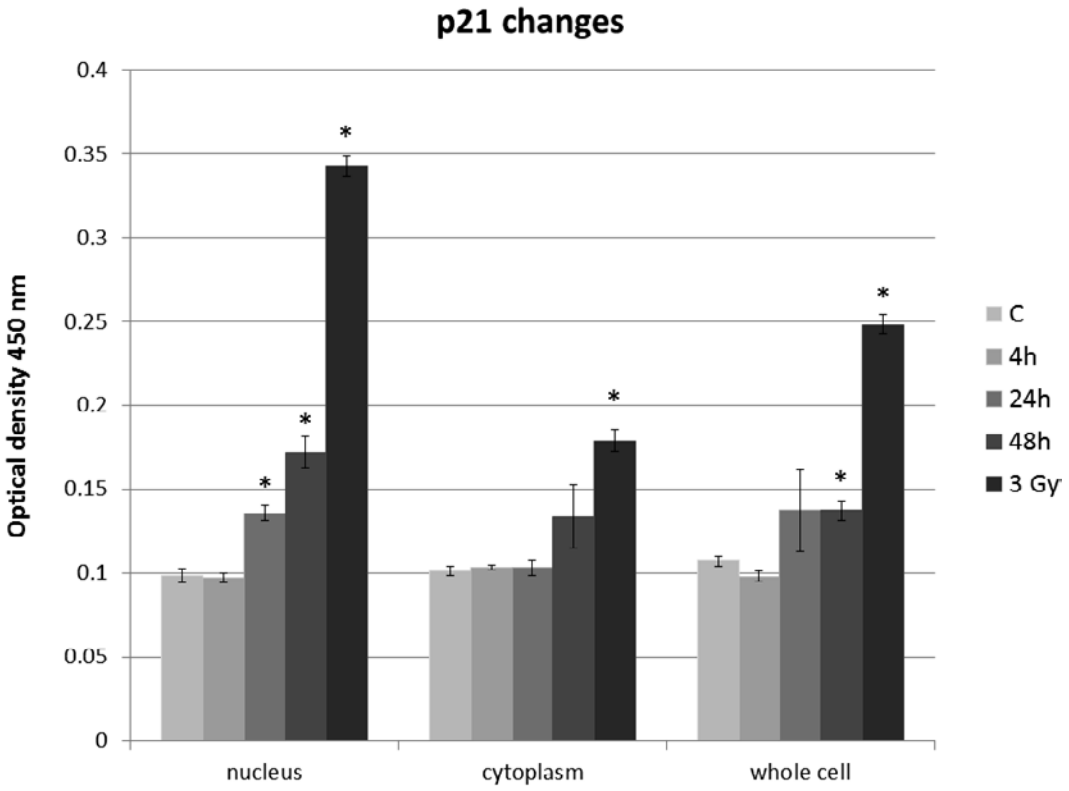


*Fig. 4.* Changes in p21 protein in MOLT-4 cells in the nuclear, cytoplasmic and whole cell samples after the treatment with 1 nmol/l mitoxantrone in the time intervals of 4, 24 and 48 h. Cells irradiated with the dose of 3 Gy lysed after 24 h were used as a positive control. P values < 0.05 are considered significant, * indicate values significantly different from the control.

## Discussion

MOLT-4 cells derived from human T-cell leukaemia are a suitable model for studying the molecular mechanisms of cellular response to genotoxic stress – DNA double-strand breaks. Our previous study showed that MOLT-4 cells respond to mitoxantrone treatment by activation of p53 and p21 proteins (Seifrtová et al., 2011), but it was not clear how these proteins are distributed in the cells. Now we focused on their subcellular localization.

In normal cells under non-stressed conditions, p53 is a short-lived protein, which is maintained in the latent form and shuttles between the nucleus and the cytoplasm according to cell cycle progression. p53 is cytoplasmic in G1 phase, during S phase accumulates in the nucleus, and after DNA synthesis cycles back to the cytoplasm (Maki, 2010). After the DNA damage, p53 rapidly accumulates in the nucleus (Ryan et al., 2001; O'Brate and Giannakakou, 2003; Solozobova et al., 2009; Davis et al., 2013). Our results show that the p53 protein is activated by the phosphorylations at serines 15 and 392, and after 24 and 48 h these phosphorylated forms accumulate in the nucleus. It was found that stress-induced phosphorylation of p53 at serine 15, for example following nitric oxide treatment, can prevent nuclear export of p53 (Maki, 2010). Our findings show that this may apply to mitoxantrone-induced damaged cells as well. The nuclear import and export is a tightly regulated process. Efficient export of p53 to the cytoplasm depends on the ubiquitin ligase activity of Mdm2 protein (Ryan et al., 2001), which is the main protein responsible for the maintenance of p53. Mdm2 and p53 shuttle from the cytoplasm to the nucleus and back, but the ubiquitination of p53 is mostly localized in the nucleus. Mdm2 can efficiently ubiquitinate p53 in the cytoplasm, but it requires p53 to be in tetrameric form (Davis et al., 2013). After mitoxantrone treatment in MOLT-4 cells, the Mdm2 protein was present in the cytoplasm with its maximal level after 8 and 16 h, at which time p53 was not increased. As the amount of Mdm2 in the cytoplasm started to decrease, an increase of p53 in the nucleus was observed.

The p21 protein is a key player in the p53 pathway. It is considered to be a universal inhibitor of cell cycle progression. On the one hand, p53 contributes to transcriptional activation of p21; on the other hand, the p21 protein regulates subcellular localization and transcriptional function of p53. Loss of p21 is associated with increased p53 protein levels without exposure to genotoxic agents. After irradiation, knocking out the *p21* gene resulted in re-localization of p53 protein from the nucleus to the cytoplasm (Mirzayans et al., 2012).

The function of p21 depends on its localization in the cell. The main role in the nucleus is the cell cycle regulation – p21 inhibits cyclin-dependent kinases and induces cell cycle arrest. Cytoplasmic p21 protein influences the cell fate after DNA damage by apoptotic or anti-apoptotic activity depending on the damage range. P21 can also be linked to cytoskeleton regulation and promotes cytoskeleton remodelling and cell motility (Xiong, 2010; Cmielová and Rezáčová, 2011; Qiu et al., 2011). Lee et al. (2009) found that p21 translocates to the nucleus after UV radiation to interact with chromatin-bound PCNA and participate in DNA repair. In MOLT-4 cells after DNA damage caused by mitoxantrone, p21 protein was detected in the nucleus after 24 and 48 h. This result corresponds with the increase of p21 in whole cell lysates. The same cell line irradiated with the dose of 3 Gy was used as a positive control. The level of p21 in irradiated cells was higher than in the cells pre-treated with mitoxantrone, but it had the same tendency and subcellular localization.

Chk2 is a kinase that controls cell cycle progression. Li and Stern (2005) found that before DNA damage, Chk2 is associated with chromatin and irradiation or topoisomerase inhibitors decrease this association. They observed that Chk2 phosphorylated at threonine 68 after DNA damage was released from the chromatin and described concomitant accumulation of the phosphorylated Chk2 in soluble fractions, cytoplasmic and soluble nuclear fraction. Chouinard et al. (2013) focused on cell cycle-dependent localization of Chk2 and they showed that the distribution of Chk2 is different in mitotic cells. Chk2 is localized in the nucleus but not at the centrosomes in interphase cells, whereas a subpopulation of Chk2 associates with centrosomes during mitosis.

Chk2 in human cells exposed to DNA-damaging agents revealed immediate redistribution of the activated Chk2 throughout the nucleus with the need to rapidly spread the checkpoint signal from localized sites of DNA damage to the soluble mobile proteins such as Cdc25 or p53 (Bartek and Lukas, 2003). The reports of the subcellular distribution of Chk2 are inconsistent and the redistribution of Chk2 after DNA damage is very poorly described. Our results bring new insight and show increased levels of phosphorylated Chk2 at threonine 68 in the cytoplasmic fraction after 24 and 48 h of mitoxantrone treatment. We observed only a weak, non-significant signal in the nuclear fraction at the same time points.

In this study we show the changes in subcellular localization of proteins responding to DNA double-strand breaks caused by mitoxantrone in MOLT-4 cells. We found that p53 protein is activated and accumulates mainly in the nucleus. The Mdm2 protein is activated in advance to p53 and occurs in the cytoplasm. The p21 protein is presumably responsible for the cell cycle regulation in these cells in response to DNA damage as it is present in the nucleus. The Chk2 kinase is activated by the phosphorylation at threonine 68 and we observed increased levels of this protein in the cytoplasmic fraction of the cells. Better understanding of the DNA damage response and protein distribution in the cells would help increase the efficiency of treatment of many types of cancer and would enable focusing on individual members of these pathways.

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The authors report no declarations of interest.

## References

Bartek, J., Lukas J. (2003) Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**, 421-429.

Brooks, C. L., Gu, W. (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr. Opin. Cell Biol*. **15**, 164-171.

Chouinard, G., Clément, I., Lafontaine, J., Rodier, F., Schmitt, E. (2013) Cell cycle-dependent localization of CHK2 at centrosomes during mitosis. *Cell Div*. **8**, 7.

Cmielová, J., Rezáčová, M. (2011) p21Cip1/Waf1 protein and its function based on a subcellular localization [corrected]. *J. Cell. Biochem*. **112**, 3502-3506.

Davis, J. R., Mossalam, M., Lim, C. S. (2013) Controlled access of p53 to the nucleus regulates its proteasomal degradation by MDM2. *Mol. Pharm*. **10**, 1340-1349.

Eriksson, D., Stigbrand, T. (2010) Radiation-induced cell death mechanisms. *Tumour Biol*. **31**, 363-372.

Lee, J. Y., Kim, H. S., Kim, J. Y., Sohn, J. (2009) Nuclear translocation of p21(WAF1/CIP1) protein prior to its cytosolic degradation by UV enhances DNA repair and survival. *Biochem. Biophys. Res. Commun*. **390**, 1361-1366.

Li, J., Stern, D. F. (2005) DNA damage regulates Chk2 association with chromatin. *J. Biol. Chem*. **280**, 37948-37956.

Maki, C. G. (2010) P53 localization. *Molecular Biology Intelligence Unit* **1**, 117-126.

Mirzayans, R., Andrais, B., Scott, A., Murray, D. (2012) New insights into p53 signaling and cancer cell response to DNA damage: implications for cancer therapy. *J. Biomed. Biotechnol*. 2012:170325. doi: 10.1155/2012/170325.

O'Brate, A., Giannakakou, P. (2003) The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resist. Updat*. **6,** 313-322.

Qiu, R., Wang, S., Feng, X., Chen, F., Yang, K., He, S. (2011) Effect of subcellular localization of P21 on proliferation and apoptosis of HepG2 cells. *J. Huazhong Univ. Sci. Technolog. Med. Sci*. **31**, 756-761.

Rashi-Elkeles, S., Elkon, R., Shavit, S., Lerenthal, Y., Linhart, C., Kupershtein, A., Amariglio, N., Rechavi, G., Shamir, R., Shiloh, Y. (2011) Transcriptional modulation induced by ionizing radiation: p53 remains a central player. *Mol. Oncol*. **5**, 336-348.

Reinhardt, H. C., Yaffe, M. B. (2009) Kinases that control the cell cycle in response to DNA damage: Chk1, Chk2, and MK2. *Curr. Opin. Cell. Biol*. **21**, 245-255.

Ryan, K. M., Phillips, A. C., Vousden, K. H. (2001) Regulation and function of the p53 tumor suppressor protein. *Curr. Opin. Cell. Biol*. **13**, 332-327.

Seifrtová, M., Havelek, R., Chmelařová, M., Ćmielová, J., Muthná, D., Stoklasová, A., Zemánková, S., Řezáčová, M. (2011) The effect of ATM and ERK1/2 inhibition on mitoxantrone-induced cell death of leukaemic cells. *Folia Biol. (Praha*) **57**, 74-81.

Solozobova, V., Rolletschek, A., Blattner, C. (2009) Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. *BMC Cell. Biol*. **10**, 46.

Stracker, T. H., Usui, T., Petrini, J. H. (2009) Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. *DNA Repair (Amst.*) **8**, 1047-1054.

Xiong, Y. (2010) Targeting p21 degradation locally. *Dev. Cell* **19**, 641-643.

Zhao, T., Xu, Y. (2010) p53 and stem cells: new developments and new concerns. *Trends Cell. Biol*. **20**, 170-175.