

RADIO-SENSITIZATION OF HUMAN LEUKAEMIC MOLT-4 CELLS BY DNA-DEPENDENT PROTEIN KINASE INHIBITOR, NU7026

Aleš Tichý^{1,3}, Eva Novotná¹, Kamila Ďurišová¹, Barbora Šalovská^{1,3}, Radka Sedlaříková¹, Jaroslav Pejchal², Lenka Zárybnická¹, Jiřina Vávrová¹, Zuzana Šinkorová¹, Martina Řezáčová³

University of Defence in Brno, Faculty of Health Sciences in Hradec Králové, Czech Republic: Department of Radiobiology¹, Centrum of Advanced Studies²; Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic: Institute of Medical Biochemistry³

Summary: In this paper we describe the influence of NU7026, a specific inhibitor of DNA-dependent protein kinase, phosphoinositide 3-kinase, and ATM-kinase on molecular and cellular mechanisms triggered by ionising irradiation in human T-lymphocyte leukaemic MOLT-4 cells. We studied the effect of this inhibitor (10 μ M) combined with gamma-radiation (1 Gy) leading to DNA damage response and induction of apoptosis. We used methods for apoptosis assessment (cell viability count and flow-cytometric analysis) and cell cycle analysis (DNA content measurement) and we detected expression and post-translational modifications (Western blotting) of proteins involved in DNA repair signalling pathways. Pre-treatment with NU7026 resulted into decreased activation of checkpoint kinase-2 (Thr⁶⁸), p53 (Ser¹⁵ and Ser³⁹²), and histone H2A.X (Ser¹³⁹) 2 hours after irradiation. Subsequently, combination of radiation and inhibitor led to decreased amount of cells in G2-phase arrest and into increased apoptosis after 72 hours. Our results indicate that in leukaemic cells the pre-incubation with inhibitor NU7026 followed by low doses of ionising radiation results in radio-sensitising of MOLT-4 cells via diminished DNA repair and delayed but pronounced apoptosis. This novel approach might offer new strategies in combined treatment of leukaemia diseases.

Key words: Inhibitor NU7026; DNA-PK; ATM, PI3-K; Ionizing radiation; Leukaemia; Apoptosis; Cell cycle

Introduction

Genotoxic agents such as ionising radiation (IR), UV-light or chemical pollutants trigger activation of a complex network of signal transduction pathways collectively referred to as the DNA damage response (31). Typically, double strand breaks (DSB) as one of the most severe forms of DNA damage arise and the signal is sensed and mediated to the effectors, which cause block of cell proliferation followed by DNA repair, senescence, or elimination of unwanted cells by engaging apoptosis.

The chief co-ordinators of the DSB signal are the nuclear protein kinases DNA-dependent protein kinase (DNA-PK) (2) and ataxia-telangiectasia mutated kinase (ATM) (20). DNA-PK is a member of the phosphoinositide 3-kinase-like enzyme family (33). This nuclear serine/threonine protein kinase is required for repairing DSB and for V(D)J recombination. In multi-cellular eukaryotes, the predominant pathway for DSB repair involves a distinct end-joining pathway that requires little or no sequence homology (10). Four gene products required for this activity have been identified, and three of them correspond to the three subunits of DNA-PK, i.e. DNA-PK catalytic subunit (DNA-PK_{CS}), Ku70, and Ku80 (reviewed in (2)). The fourth component, XRCC4, is a 55–60 kDa nuclear

phosphoprotein that is ubiquitously expressed and forms tight complexes with DNA ligase IV (22).

ATM plays no doubt a crucial role in control of cellular responses to DSB, because it regulates all three cell cycle checkpoints, DNA repair, and apoptosis (19). After induction of DSB, it is rapidly activated by intermolecular autophosphorylation (3). ATM is also a member of the phosphoinositide 3-kinase-like enzyme family and it integrates the cellular response to DSB by phosphorylating key proteins involved in these processes, such as p53, murine-double minute protein (Mdm2), and checkpoint kinase-2 (chk-2) managing the G1 checkpoint and many others for the transient S-phase arrest and the G2/M checkpoint (24, 25).

Our understanding of how the irradiated cells sense DNA damage is still unclear but it is more than likely that DNA-PK and ATM are major signal transducers. Since the crucial DNA repair enzymes play a pivotal role in the recent cancer research, the discovery of their inhibitors is of a great interest too.

In this work we used a novel specific DNA-PK inhibitor, NU7026, which has been reported as a radio-sensitiser *in vitro* (13, 39, 40). NU7026 (2-(morpholin-4-yl)-benzo(H) chromen-4-one) inhibits both DNA-PK and ATM but predominantly affects the former one (IC₅₀: 0.23 μ M for

DNA-PK, 13 μ M for phosphoinositide 3-kinase, and more than 100 μ M for ATM) (26).

IR is commonly used in cancer treatment in order to eliminate tumour cells by apoptosis. A wide range of kinase inhibitors is used intentionally to increase the cytotoxic effect of radiation. In our previous work, we showed that ATM/chk-2/p53 pathway is activated upon irradiation in human T-lymphocyte leukaemia cell line MOLT-4 (36). In this paper we report about the effect of NU7026 inhibitor combined with ionising radiation on activation of several components of DNA damage response signalling pathway (p53, chk-2, and H2A.X) and its influence on induction of apoptosis and cell cycle progress.

Methods

Cell cultures and culture conditions

MOLT-4 cells were obtained from the American Type Culture Collections (Manassas, VA, USA). The cells were cultured in Iscove's modified Dulbecco medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum, 0.05% L-glutamine, 150 UI/ml penicillin, 50 μ g/ml streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C. The cultures were split every second day by dilution to a concentration of 2×10^5 cells/ml. The cell counts were performed with a hemocytometer; the cell membrane integrity was determined by using the Trypan blue exclusion technique. Cell lines in the maximal range of up to 20 passages were used for this study.

Gamma irradiation

Exponentially growing MOLT-4 cells were suspended at a concentration of 2×10^5 /ml and divided into four groups: control (intact cells), only NU7026 exposed cells, only irradiated cells and combined exposure. Aliquots of 10 ml of cell suspension were plated into 25 cm² flasks (Nunc, Wiesbaden, GER) and irradiated at room temperature using ⁶⁰Co gamma-ray source with a dose-rate of 0.4 Gy/min, at a distance of 1 m from the source. After the irradiation by the dose of 1 Gy the flasks were placed in a 37 °C incubator with 5% CO₂ and aliquots of the cells were taken at 2 or 24 hours after irradiation for analysis. The cells were counted and cell viability was determined with the Trypan blue exclusion assay.

NU7026 inhibitor exposure

We added 10 μ M NU7026 (Millipore, Billerica, MA USA) to the cells half an hour prior irradiation.

Flow-cytometric analysis

The cells were collected and washed with cold PBS (phosphate-buffered saline) and fixed in 70% ethanol. For

the fixation of low molecular fragments of DNA the cells were incubated for 5 min at room temperature in a phosphate buffer and then stained with propidium iodide in the Vindelov's solution for 30 min at 37 °C. The fluorescence (DNA content) was measured with a Coulter Electronic (Hialeah, FL, USA) apparatus. A minimum of 10,000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle, using the Multicycle AV software. Three independent experiments were performed.

Electrophoresis and Western blotting

At various times after irradiation, the MOLT-4 cells were washed with PBS and lysed. Whole cell extracts were prepared by lysis in 500 μ l of lysis buffer (137 mM NaCl; 10% glycerol; 1% n-octyl- β -glucopyranoside; 50 mM NaF; 20 mM Tris, pH=8; 1 mM Na₃VO₄; 1 tablet of protein inhibitors Complete™ Mini, Roche). The lysates containing equal amount of protein (30 μ g) were loaded onto a 12% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA, USA), and hybridized with an appropriate antibody: anti- γ H2A.X (Ser¹³⁹) and anti-Chk-2 (Thr⁶⁸) from Cell Signaling, Chicago, IL, USA; anti-p53 and anti-p53 (Ser³⁹²) from Exbio, Prague, CZE; anti-p53 (Ser¹⁵) from Calbiochem, San Diego, CA, USA. After washing, the blots were incubated with secondary peroxidase-conjugated antibody (Dako, High Wycombe, UK) and the signal was developed with ECL detection kit (BM Chemiluminescence – POD, Roche, Mannheim, GER) by exposure to a film. The films were scanned and integrated optical density (IOD) was measured by ImagePro 1.0 software (Microsoft, Seattle, WA, USA).

Statistical analysis

The data were statistically analyzed by t-test using SigmaStat software (Aspire Software International, Ashburn, VA, USA). The significance level alpha was 0.025.

Results

Determination of proliferative activity

Initially, we performed the cell count (cell membrane integrity was determined by using the Trypan blue exclusion technique). The number of living intact cells was analyzed 20, 70, and 140 hours after irradiation. The cell were pre-treated with 5 or 10 μ M NU7026 solely or followed by exposure to 0.5 or 1 Gy. After 140 hours a partial growth inhibition was observed in the groups exposed to 1 Gy and those combining 5 μ M NU7026 with 1 Gy dose and 10 μ M NU7026 with 0.5 Gy. The complete growth inhibition (with statistical significance $p \leq 0.001$) was observed after combination of 10 μ M NU7026 and the dose of 1 Gy (Fig. 1). This combination was chosen for further experiments.

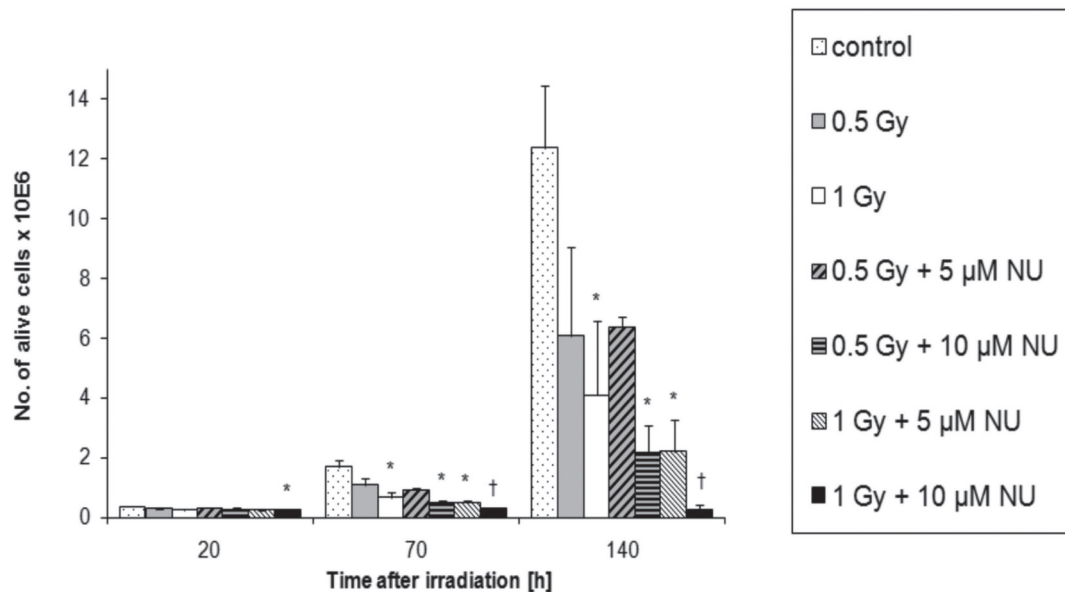


Fig. 1: Proliferation of MOLT-4 cells. Proliferation was measured by trypan blue exclusion technique 20, 70, and 140 hours after irradiation. The maximal growth-inhibition effect was observed after combination of pre-treatment with 10µM NU7026 and the dose of 1 Gy. Results are the mean of three independent experiments (* $p \leq 0.025$; † $p \leq 0.001$).

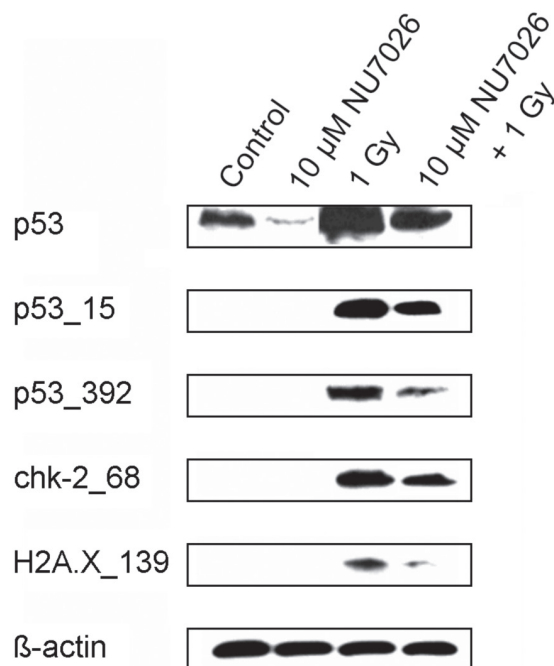


Fig. 2: Western blotting of proteins involved in DNA damage response signalling. MOLT-4 cells were pre-incubated with DNA-PK inhibitor (10 µM NU7026) and exposed to 1 Gy. Whole-cell lysates were analysed 2 h post-irradiation. Combination of inhibitor and gamma-radiation caused substantial decrease of activated forms of all studied proteins. β-actin was used as a gel-loading control. A representative blot from at least three independent experiments is shown.

DNA-repair signalling

We evaluated expression of several proteins involved in DNA repair pathways 2 hours after irradiation. The first one was protein p53. Its basal amount was detected in the control cells. Amount of p53 decreased in the NU7026-treated cells and it increased after irradiation by the dose of 1 Gy. Pre-treatment of irradiated cells with NU7026 caused partial decrease in p53, though its level was higher compared to the control group (Fig. 2).

We also studied two post-translational modifications of p53 on Ser¹⁵ and Ser³⁹². These modifications were not detected in control and NU7026-treated cells, but both serines were phosphorylated after irradiation by 1 Gy and combination of IR with NU7026 caused their decrease.

We observed the same pattern in phosphorylation of chk-2 on Thr⁶⁸, which was also detected only in the cells exposed to 1 Gy. Pre-treatment with NU7026 caused slight decrease in phosphorylated chk-2.

Similar results were obtained when we checked for phosphorylated histone H2A.X (on Ser¹³⁹). It was detected only in 1 Gy irradiated cells and pre-treatment with NU7026 caused its decrease.

Cell cycle analysis

We determined the percentage of cells in G1, S, and G2 phase using flow-cytometric analysis of DNA content. We measured cell cycle distribution 24 and 72 hours after irradiation. Cells in the early phase of apoptosis were detected as subG1 peak (i.e. cells with lower amount of DNA than

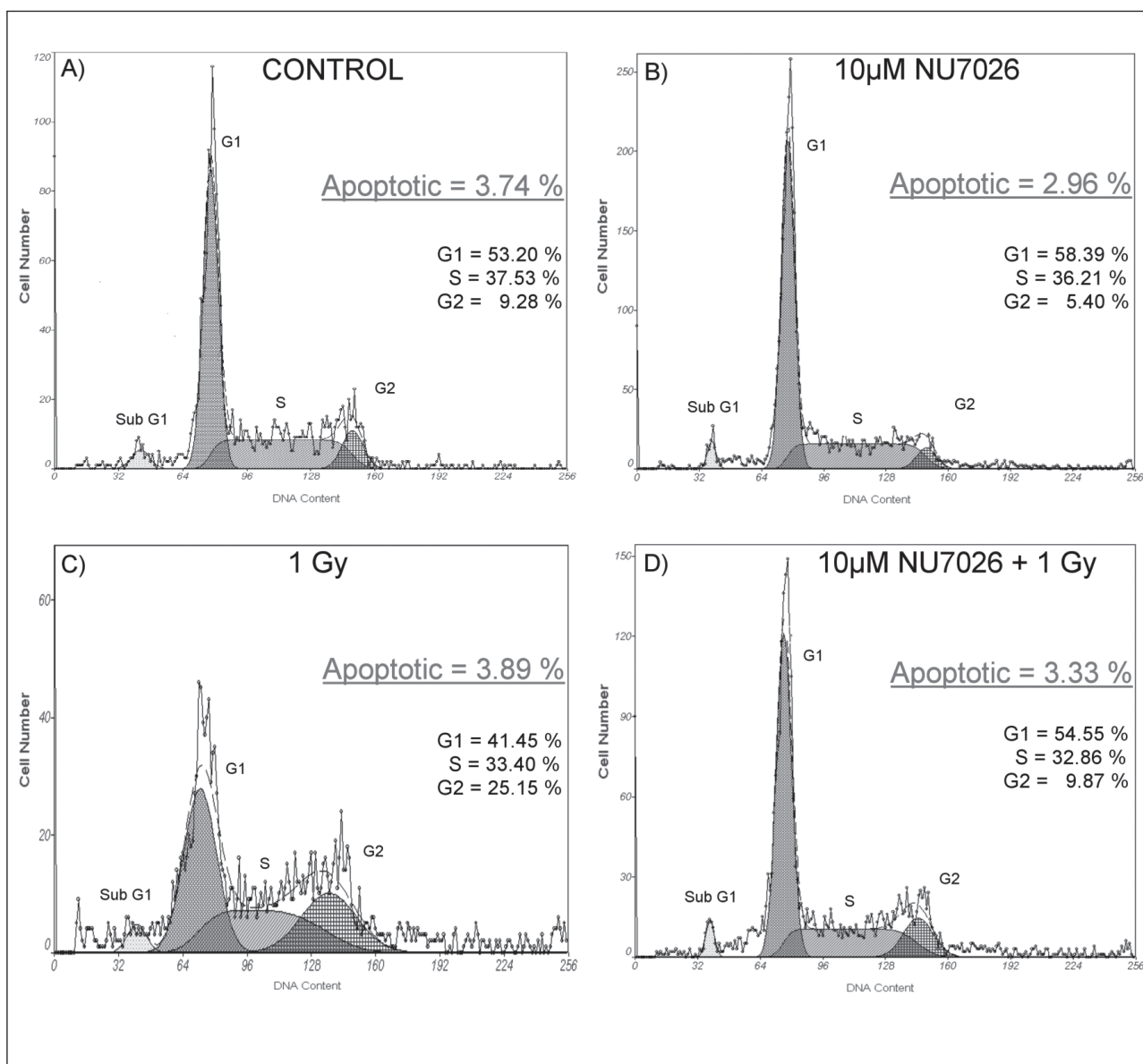


Fig. 3: Flow-cytometric determination of ratio of apoptotic cells and analysis of cell-cycle distribution 24 h after irradiation. Cells in early phase of apoptosis are detected as subG1 peak (i.e. cells with lower amount of DNA than cells in G1 phase of cell cycle) due to fragmentation and loss of DNA during apoptosis. The percentage of subG1 cells is calculated from the total cell count, distribution of the cells in cell cycle (the percentage of cells in G1, S, G2) is calculated only from the cells in the cycle (excluding subG1). Representative results of one out of three independent experiments are shown.

cells in G1 phase of cell cycle) due to fragmentation and loss of DNA during apoptosis. The percentage of apoptotic cells was unchanged in all experimental groups after 24 hours (3.74% in control, 2.96% in NU7026-treated, 3.89% in irradiated, and 3.33% in the group with combination). We observed only modest change in the cell cycle distribution in control and NU7026 treated cells (Fig. 3) but the amount of cells in G2-phase arrest increased when

they were exposed solely to the dose of 1 Gy. On the other hand, the combination of inhibitor and IR returned the amount of the cells in G2-phase arrest to the control level. While the cell cycle distribution remained more or less unchanged 72 hours after irradiation, the amount of sub-G1 (i.e. apoptotic) cells increased dramatically in the group with combination of ionising radiation and inhibitor (Fig. 4).

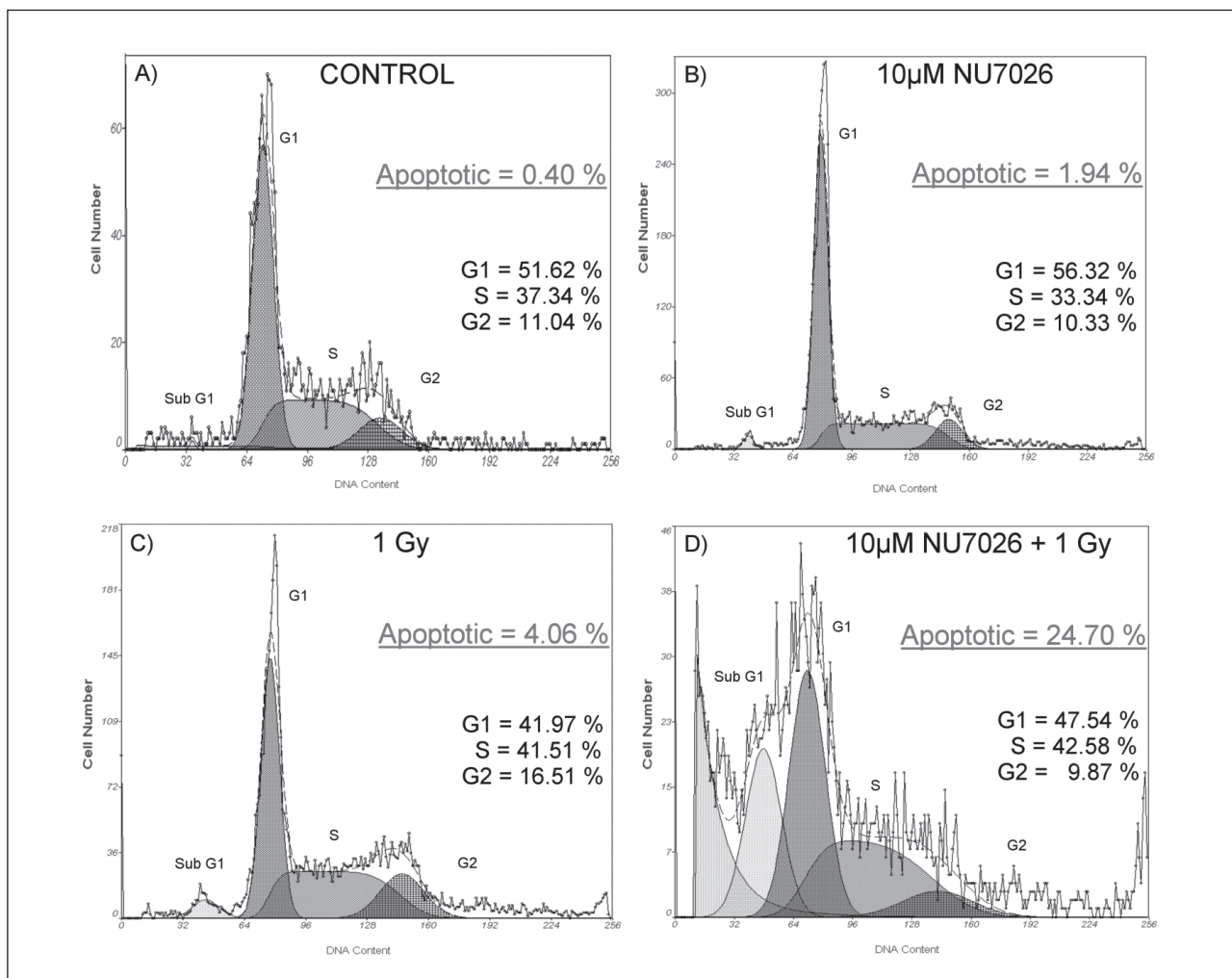


Fig. 4: Flow-cytometric determination of ratio of apoptotic cells and analysis of cell-cycle distribution 72 h after irradiation. Cells in early phase of apoptosis are detected as subG1 peak (i.e. cells with lower amount of DNA than cells in G1 phase of cell cycle) due to fragmentation and loss of DNA during apoptosis. The percentage of subG1 cells is calculated from the total cell count, distribution of the cells in cell cycle (the percentage of cells in G1, S, G2) is calculated only among the cells in the cycle (excluding subG1). Representative results of one out of three independent experiments are shown.

Discussion

Signalling pathways monitoring DNA damage play an important role in maintaining genomic integrity and their malfunctions often result in neoplasia. Nowadays, many anti-tumour drugs are developed to cause deactivation or inhibition of components of these pathways in the cancer cells. Although the biochemical mechanisms for repairing DSB in mammalian cells are still not completely understood, substantial progress has been made in the last few years. Undoubtedly, two members the phosphoinositide 3-kinase-like enzyme family, DNA-PK and ATM, play an irreplaceable role in DNA damage repair.

Bakkenist and Kastan (3) in their work on fibroblasts suggested that raise of DSB causes a rapid change of

higher-order chromatin structure resulting in ATM activation. Bekker-Jensen et al. (4) reported that ATM is spatially redistributed to DSB-flanking chromatin. In our previous work on human T-lymphocyte leukaemic MOLT-4 cells we have shown ATM/chk-2/p53 signalling pathway to be fully functional upon gamma-irradiation (36).

Active ATM subsequently affects plenty of different targets via phosphorylation and some participate in the cell cycle arrest and apoptosis induction. One of them is chk-2, which is activated by ATM on Thr⁶⁸. This relatively stable protein responds to ionising radiation throughout the cell cycle and according to Lukas et al. (23) it appears to be inactive in the absence of DNA damage. We also did not find activated chk-2 in NU7026-treated cells and its phosphorylated form was presented only in irradiated cells. On the other hand,

NU7026 pre-incubation markedly prevented chk-2 phosphorylation after irradiation. It is likely that in spite of the fact that NU7026 affects predominantly DNA-PK than ATM – 430 times more (26), it is still capable of chk-2 inhibition, or another (DNA-PK-dependent) mechanism of chk-2 activation exists, however, it has not been reported yet.

Both ATM and chk-2 contribute to an appropriate control of the central mediator of the DNA damage response, protein p53, which in turn induces cell cycle arrest by up-regulation of p21^{CIP/WAF} and 14-3-3 protein, activates DNA damage repair pathways, and induces apoptosis (8) (11). Commonly, IR induces apoptosis by activating p53, members of Bcl-2 protein family, and caspases, although p53-independent apoptosis has also been reported (38). We have previously described activation of caspases and pro-apoptotic Bid after gamma-irradiation of MOLT-4 cells (37). Also in the recent study, p53 was activated since we detected two of its phosphorylations (Ser¹⁵ and Ser³⁹²) at the N- and C- terminus of p53, respectively, which enable allosteric regulation.

Phosphorylation on Ser¹⁵ is crucial for managing DNA repair post-irradiation. Protein p53 is typically a short-lived protein and its degradation is tightly regulated by Mdm2, p53 negative regulator, which is an E3 ubiquitin ligase (7). Phosphorylation on Ser¹⁵ abrogates the ability of Mdm2 to inhibit p53-dependent transactivation (30). Many studies indicate that ATM is indispensable for prolonged half-life of p53 since it is responsible for this phosphorylation (15, 21, 27). In contrast, we observed pronounced decrease in Ser¹⁵ phosphorylation after irradiation via inhibition of DNA-PK by NU7026 in this study. It is supported by the findings of Boehme et al. (6), who reported signalling cascade for the regulation of p53 on Ser¹⁵ in response to IR that involves activation of DNA-PK and subsequently Akt/PKB and inactivation of Mdm2 and GSK-3. Additionally, Broehme reported that down-regulation of DNA-PK prevented phosphorylation of Akt/PKB and GSK-3 after exposure to IR and strongly reduced the accumulation of p53, which was apparently ATM-independent since this work has been done on lymphoblasts from patients with the absence of functional ATM.

Phosphorylation on Ser³⁹² localized within carboxyl terminus of p53 is no less important for radiation response, because it enhances its sequence-specific DNA binding (12). This site on wild-type p53 is phosphorylated by complexes containing casein kinase 2 (17, 18). Keller and colleagues (17) stated that Ser³⁹² phosphorylation occurs after UV- but not gamma-irradiation. Our results proved that gamma-radiation induces Ser³⁹² phosphorylation, since we detected this phosphorylation in irradiated cells and pre-treatment with NU7026 caused its decrease. Taken together, NU7026 inhibited accumulation and activation of p53 via decreased phosphorylation on Ser¹⁵ and Ser³⁹², which possibly leads to abrogation of cell cycle arrest, and subsequent propagation of unrepaired DNA might be one of reasons of increased apoptosis observed.

Very early step in DNA damage response is phosphorylation of histone subtype H2A, class H2A.X (28). Phosphorylated H2A.X on Ser¹³⁹ (γ H2A.X) can be visualized by suitable antibody via immunofluorescence as a discrete focus and it is localized in the range of 2×10^6 bases from DSB (29). We recently reported flow-cytometric determination of γ H2A.X as a perspective biodosimetric indicator in peripheral lymphocytes of whole-body-irradiated rats by the dose 1–10 Gy (14). DSB induce histone H2A.X phosphorylation, which is associated with the recruitment of repair factors and γ H2A.X is critical for facilitating the assembly of specific DNA-repair complexes on damaged DNA (9). Stiff et al. (34) concluded that under most normal growth conditions, IR-induced H2AX phosphorylation can be carried out by ATM and DNA-PK in a redundant, overlapping manner. In contrast, DNA-PK cannot phosphorylate other proteins involved in the checkpoint response. However, by phosphorylating H2A.X, DNA-PK can contribute to the presence of the damage response proteins MDC1 and 53BP1 at the site of the DSB. Our results are in consistence with these findings since NU7026 inhibited DNA-PK and we observed decreased radiation-induced H2A.X phosphorylation when compared to solely irradiated cells.

DNA-PK is a crucial component of non-homologous end-joining and key determinant of radio- and chemoresistance (40). Lack of DNA-PK causes defective DSB repair and radio-sensitisation. In addition to its role in DNA repair, DNA-PK is also involved in apoptosis. Once gamma-radiation induces DSB, the DNA-PK complex senses and repairs them; on the other hand, when DNA damage is excessive, DNA-PK induces apoptosis (5).

When apoptotic stimuli are released, the key regulators of cell death process are members of Bcl-2 protein family (35). Myeloid cell leukemia 1 protein, Mcl-1, functions among the proteins, which maintain mitochondrial integrity via interaction with pro-apoptotic partners after gamma-irradiation (32). Interestingly, Mcl-1 could act as an adaptor protein essential in ATR-mediated checkpoint kinase-1 phosphorylation (16). In our previous work on MOLT-4 cells (37) we found the amount of Mcl-1 initially increased after irradiation by sublethal but not by lethal dose and later (when apoptosis occurred) its dose-dependent decrease was associated with apoptosis induction. Surprisingly, in the recent experiments, 24 hours after irradiation we expected decrease in Mcl-1 caused by the combination of IR and NU7026, but we did not observe any substantial decrease (data not shown), suggesting that Mcl-1 regulation occurs in DNA-PK-independent manner or that the effect is delayed. Therefore we evaluated apoptosis by flow-cytometric analysis of DNA content. Twenty four hours after irradiation the rate of apoptotic cells was similar (around 3%) in all of the groups but 72 hours after irradiation there was a pronounced and significant effect of combination of inhibitor and radiation and increased apoptosis was measured

(24%). Willmore and colleagues (41) reported that 10 μ M NU7026 potentiated the growth inhibition of topoisomerase II poisons and that NU7026 alone had no effect on cell cycle distribution, but etoposide-induced accumulation in G2/M was increased by NU7026. By contrast, in our experiments the radiation-induced accumulation in G2/M was decreased by NU7026. On the other hand, Zhu and Gooderham (42) observed in human lung adenocarcinoma A549 cells using cryptolepine, a DNA-damaging agent, that 10 μ M NU7026 showed neither induction of cell cycle arrest and apoptosis nor the expression of p53 and p21, while Amrein et al. (1) reported that NU7026 increases chlorambucil-induced G2/M arrest and number of DSBs in chronic lymphocytic leukaemia. Apparently, it is expected that the effect of NU7026 depends on the type DNA-damaging agent and the type of cells used in a particular study.

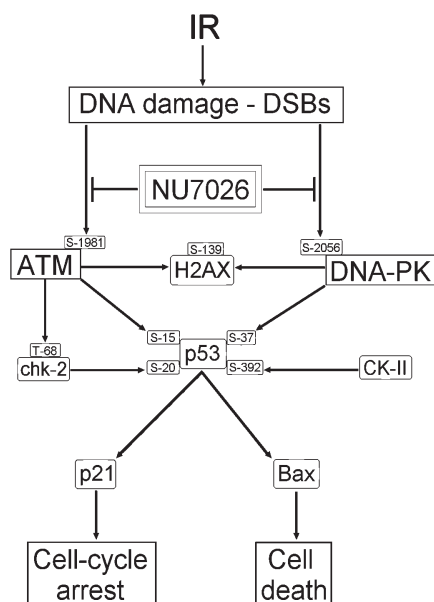


Fig. 5: DNA-damage response pathways targeted by NU7026. Presence of IR-induced DSBs activates two main kinases ATM and DNA-PK by autophosphorylation. ATM and DNA-PK are inhibited by NU7026. Both of them phosphorylate histone H2A.X on Ser 139 in overlapping and independent manner. Another core target is genome guardian – p53. It is phosphorylated directly by ATM on Ser 15 or indirectly via chk-2 on Ser 20. DNA-PK phosphorylates p53 on Ser 37 and casein-kinase II phosphorylates it on Ser 392. All these post-translational modifications contribute to stabilization of p53 and facilitate its transcriptional function (see text). p53 either induces cell cycle arrest via up-regulation of p21 in order to provide sufficient time-window for DNA-repair processes or (in the case of excessive DNA-damage) up-regulates pro-apoptotic factors, which subsequently results into induction of cell death.

Conclusion

Radiation and chemotherapy are two important strategies for cancer treatment. We used DNA repair inhibitor NU7026 in the concentration targeting preferably DNA-PK, to prevent ionizing radiation-induced DSB repair. Post-translational modifications of some DNA-PK and/or ATM downstream effectors (p53, chk-2, and H2A.X) showed substantial decrease, i.e. they were less activated. This led to subdued DNA repair and consequently to increased apoptosis induction.

Taken together, our results indicate that DNA-PK/ATM inhibition by NU7026 concurrent with ionising radiation may be an effective therapeutic modality for radio-sensitisation of leukaemic cells.

Abbreviations

ATM, ataxia-telangiectasia mutated kinase; DNA-PK, DNA-dependent protein kinase; IR, ionising radiation; p53, TP53 tumour suppressor

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Corresponding author:

Aleš Tichý, Department of Radiobiology, Faculty of Health Sciences, University of Defence, Třebešská 1575, 500 01 Hradec Králové, Czech Republic; e-mail: tichy@pmfhk.cz