Original Article

Comparison of the Radiosensitizing Effect of ATR, ATM and DNA-PK Kinase Inhibitors on Cervical Carcinoma Cells

(γ radiation / HeLa cells / ATM / ATR and DNA PK inhibitors / VE-821)

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Abstract. Here, we compared the effects of inhibitors of three phosphatidylinositol-3-kinase-related kinases, ATM, ATR a DNA-PK, on radiosensitization of cervical carcinoma cells. We demonstrated that DNA-PK inhibitor NU7441 enhanced phosphorylation of Chk1 and Chk2 kinases 2 h after irradiation of HeLa cells at a dose of 8 Gy in contrast to ATM kinase inhibitor KU55933, which completely blocked the Chk2 kinase phosphorylation on threonine 68, and ATR kinase inhibitor VE-821, which blocked the Chk1 kinase phosphorylation on serine 345. Most HeLa cells were accumulated in G2 phase of the cell cycle 24 h after irradiation at a high dose of 15 Gy, which was even potentiated after adding the inhibitors NU7441 and KU55933. Compared to all other irradiated groups, inhibitor VE-821 increased the number of cells in S phase and reduced the number of cells in G2 phase 24 h after irradiation at the high dose of 15 Gy. HeLa cells entered the mitotic cycle with unrepaired DNA, which resulted in cell death and the radiosensitizing effect of VE-821. Short-term application of the inhibitors (2 h before and 30 min after the irradiation by the dose of 8 Gy) significantly decreased the colony-forming ability of HeLa cells. Using real-time monitoring of cell proliferation by the xCELLigence system we demonstrated that while the radiosensitizing effect of VE-821 (ATR inhibitor) is manifested early after the irradiation, the radiosensitizing effect of KU55933 (ATM inhibitor) and NU7441 (DNA-PK inhibitor) is only observed as late as 72 h after the irradiation.

Abbreviations: ATM – ataxia-telangiectasia mutated kinase, ATR – ATM and Rad3-related kinase, Cdc25 A and C – cell division cycle 25 phosphatases A and C, Chk1, 2 – checkpoint kinase 1, 2, DMSO – dimethyl sulphoxide, DNA-PK – DNA-dependent protein kinase, DNA-PKcs – DNA-dependent protein kinase catalytic subunit, DSB – double-strand break(s), HeLa – cervical carcinoma cells, HL-60 – human promyelocytic leukaemia cells, HR – homologous recombination, IRIF – ionizing radiation inducing foci, KU55933 – a specific ATM inhibitor (2-morpholine-4-yl-6-thianthrene-1-yl-pyran-4-one), NHEJ – non-homogenous end joining, PBS – phosphate-buffered saline, NU7441 – a specific inhibitor of DNA-PK (2-N-morpholine-8-dibenzothiophenyl-chromen-4-one), SSB – single-strand break(s), VE-821 – a specific ATR inhibitor (amino-6-[4-(methylsulphonyl)phenyl]-N-phenyl-2-pyrazinecarboxamide).

Introduction

The aim of radiotherapy is to destroy tumour cells, while the damage to normal cells should be as low as possible. The purpose of the present work was to sensitize cervical carcinoma cells to ionizing radiation using inhibitors of DNA-PK, ATM and ATR kinases.

Ionizing radiation induces double-strand breaks (DSB) of DNA in the cells, which represent potentially lethal damage. Their repair is difficult and frequently leads to production of modified cells. Phosphatidylinositol-3-kinase-related kinases ATM, ATR and DNA-PK are activated very rapidly after the production of a double-strand break. ATM and ATR have a number of common substrates, but also substrates specific for one kinase only. When comparing the role of these kinases in the cell survival, ATM kinase (unlike ATR kinase) is not essential (Wagner and Kaufmann, 2010). ATM and ATR respond to different types of damage. The activation of ATM is associated with the effects of ionizing radiation and DSB. ATR is activated after UV irradiation and also after oxidative stress and application of chemotherapeutic substances, i.e. after agents particularly causing sin-

gle-strand breaks (SSB) and damage in the replication fork (Dai and Grant, 2010).

The main substrates of ATM kinase are protein p53 (associated with a cell cycle arrest in G1 phase) and Chk2. In contrast to ATM kinase, ATR kinase particularly activates Chk1, which is associated with a cell cycle arrest in S and G2 phases. Phosphorylated Chk1 subsequently phosphorylates and thus inactivates Cdc25A and Cdc25C phosphatases, similarly as ATM kinase. Phosphorylated Cdc25A phosphatase becomes ubiquitinated and degraded, the result being the cell cycle arrest in S and G2 phases. Phosphorylated Cdc25C phosphatase is exported from the nucleus, which also results in the cell cycle arrest in the G2 phase. Signalling through the ATR kinase is of principal importance for the cell division and replication (Dai and Grant, 2010). In spite of the fact that ATR is particularly activated by SSB, larger amounts of SSB in a close vicinity can lead to production of DSB with the ATM kinase activation, establishing a cross-talk between the two kinases.

Cells have developed two basic mechanisms of DSB repair – homologous recombination (HR) and non-homologous joining of ends (NHEJ). The NHEJ pathway requires the activity of DNA-PK, a serine/threonine kinase consisting of two components, a 460 kDa polypeptide catalytic subunit (DNA-PKcs) and autoimmune antigen Ku, which is composed of polypeptides of about 70 (Ku70) and 80 kDa (Ku80) (Novotna et al., 2013). NHEJ is the main mechanism of the DSB repair in eukaryotes, which prevalently occurs in the G0/G1 phase. We demonstrated that the resistance of leukemic HL-60/MX2 cells to mitoxantrone is associated with DNA-PK overexpression. Specific DNA-PK inhibitor NU7026 reduced the amount of DNA-PK and abolished the resistance of these cells to mitoxantrone (Mikusova et al., 2011).

In contrast, HR operates in late S/G2 phase in the presence of a sister chromatid, which is necessary for the appropriate joining of the free ends of DNA chains (Jeggo and Lavin, 2009). In our work on haematopoietic leukemic lines (Vavrova et al., 2001), we demonstrated accumulation of cells in the G2 phase after irradiation, particularly in the cells lacking functional protein p53. For example, HL-60 cells (human promyelocytic leukaemia) are relatively radioresistant, and the radioresistance was enhanced when the cells were accumulated in the G2 phase in the course of irradiation. In the cases when cells entered the cell cycle in the course of irradiation, their sensitivity to radiation was increased (Vavrova et al., 2003).

Effective repair of the radiation-induced damage enhances the cellular resistance to ionizing radiation effects. Specific inhibitors of phosphatidylinositol-3-kinase-related kinases DNA-PK, ATM and ATR are searched for to increase the tumour cell radiosensitivity, which could prevent the radiation damage repair. In the present work, we compared the effect of DNA-PK inhibitor NU7441 (2-N-morpholine-8-dibenzothiophenyl-chromen-4-one), ATM inhibitor KU55933 (2-morpholine-4-yl-6-thianthrene-1-yl-pyran-4-one) and ATR

inhibitor VE-821 (amino-6-[4-(methylsulfonyl)phenyl]-N-phenyl-2-pyrazinecarboxamide) on HeLa cell radiosensitization.

Material and Methods

Cell culture and culture conditions

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in Dulbecco's Modified Earle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% foetal calf serum (PAA Laboratories GmbH, Pasching, AU) at 37 °C, under controlled 5% CO_2 and humidified atmosphere. The cultures were divided every four days. Cells in a maximum range of 20 passages were used for this study.

The selective inhibitor of ATR kinase 3-amino-6-(4-(methylsulphonyl)phenyl)-N-phenylpyrazine-2-carboxamide (VE-821, APIs Chemical Co., Ltd., Shanghai, China), selective inhibitor of ATM kinase 2-morpholine-4-yl-6-thianthrene-1-yl-pyran-4-one (KU55933, Merck KGaA, Darmstadt, Germany) and selective inhibitor of DNA-PK 2-N-morpholine-8-dibenzothiophenyl-chromen-4-one (NU7441, Tocris Bioscience, Minneapolis, MN) were reconstituted in dimethyl sulphoxide (DMSO, Sigma-Aldrich) to 10 mM and diluted to final concentration in culture medium. The final concentrations were 10 μM for VE821, 10 μM for KU55933 and 1 μM for NU7441. The inhibitors were added into the culture 2 h before irradiation at the dose of 1–15 Gy. The final concentration of DMSO in the culture was lower than 0.1%. The cells were incubated in the presence of the inhibitors and/or 0.1% DMSO for the entire period of the experiment.

γ-Ray irradiation

Exponentially growing HeLa cells were suspended in flasks at a concentration of 10^5 cells/ml in the complete medium and irradiated using a ^{60}Co γ-ray source (Chisotron Chirana, Prague, Czech Republic) at a dose-rate of 1 Gy/min. After irradiation, the flasks were placed in an incubator and aliquots of the cells were sampled for analysis at various times after irradiation.

Clonogenic survival assay

VE-821 and KU55933 (10 μM), NU7441 (1 μM) and DMSO were added to the culture 2 h before irradiation and washed away 30 min after irradiation, and the cells were then cultivated in the medium free of inhibitors. Cell aggregates with more than 20 cells were considered to be colonies and were scored with light microscopy at 10–12 days after incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air. Four measurements were performed with cells irradiated by doses of 2, 4 and 8 Gy.

WST assay

The viability of cells treated with irradiation and inhibitors was assessed by means of Cell Proliferation

Reagent WST-1 (Roche, Basel, Switzerland) according to the manufacturer's procedure after 120 h of incubation with inhibitors. Each treatment was performed in five wells of a 96-well plate. The absorbance was detected at 450 nm in a multifunctional microplate reader with Multi Detector Magic XBC (Beckman-Coulter, Fullerton, CA). The results represent an average from five measurements and were expressed with SD (standard deviation of the mean).

xCELLigence – real-time cell analyser

HeLa cells were harvested by trypsinization and the cell number was determined using a haemocytometer. HeLa cells were treated with various doses of radiation in the presence of an inhibitor in tubes. VE-821 and KU55933 (10 μM), NU7441(1 μM) and DMSO were added to the culture 2 h before irradiation and the cells were then cultivated for 5 days in the medium with inhibitors. The cell proliferation and viability were continuously monitored in real time using the xCELLigence System (Roche). The system uses a specially designed 96-well plate (E-plate 96) with an array of gold microelectrodes on the bottom of each well, and the electric impedance signal is recorded. The E-plate 96 was filled with 50 μl of DMEM per well to measure the background signal. Then 100 μl of cell suspension in a density of 10^3/well was added and the E-plate was left for 20 min at room temperature for homogeneous attachment of cells. The E-plate was returned to the incubator and connected to the monitoring device unit. Cell proliferation was monitored for 5 days. The electrical impedance signal expressed as a cell index was automatically recorded every hour for the next 120 h to produce a time-dependent cell-response curve. Each measurement was carried out in four replicates.

Cell cycle analysis

The untreated control and irradiated cells were harvested by trypsinization, washed with cold phosphate-buffered saline (PBS), fixed with 70% ethanol and stained with propidium iodide (PI) in the Vindelov's solution consisting of 100 ml Tris-buffered saline (pH = 7.6), 1 mg ribonuclease A, 7.5 mg PI and 0.1 ml Nonidet p-40 (all components form Sigma-Aldrich) for 60 min at 37 °C in the dark.

Flow-cytometric analysis was performed in FACS analyser CyAnDakoCytomation (Beckman Coulter). At least 50,000 cells were analysed per each sample. List mode data were analysed using the Summit v 4.3 software (Beckman Coulter). At least three measurements were performed.

Western blotting

HeLa cells were harvested by trypsinization, washed with PBS and lysed. Whole-cell extracts were prepared by lysis in 500 μl of lysis buffer (137 mM NaCl; 10% glycerol; 1% n-octyl-β-glucopyranoside; 50 mM NaF; 20 mM Tris, pH = 8; 1 mM Na_3VO_4; 1 tablet of protease inhibitors Complete TM Mini, Roche, Grenzach-Wyh-len, Germany). The lysates containing equal amounts of protein (30 μg) were loaded onto 10% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane and hybridized with an appropriate antibody: anti-Chk1 (1 : 500), anti-Chk2 (1 : 500), anti phospho-Chk2-Thr68 (1 : 250), anti phospho-Chk1-serine-345 (1 : 500) (Cell Signaling Technology, Inc, Danvers, MA) and β-actin (1 : 10,000) (Sigma-Aldrich). After washing, the blots were incubated with secondary peroxidase-conjugated antibody (Dako, High Wycombe, UK) and the signal was developed with a chemoluminescence detection kit (Boehringer, Mannheim, Germany) and exposed to film (Foma, Hradec Králové, Czech Republic).

Statistical analysis

The values represent mean ± SD (standard deviation of the mean) of at least three independent measurements. The Mann-Whitney test (SigmaStat 3.1, Systat Software Inc., Erkhart, Germany) was used for the statistical analysis. The differences were considered significant when $P \leq 0.05$.

Results

The Western blot detection of Chk1, Chk2, phosphorylated Chk1 (serine 345) and phosphorylated Chk2 (threonine 68) 2 h after irradiation was used for evaluation of the specificity of inhibitors. VE-821 (10 μM) reduced Chk1 phosphorylation of serine 345 2 h after the irradiation by 8 Gy, while KU55933 reduced phosphorylation of Chk2 on threonine 68 (Fig. 1). After incubation with DNA-PK inhibitor NU7441, the phosphorylation of both kinases was increased compared to the group treated with irradiation only.
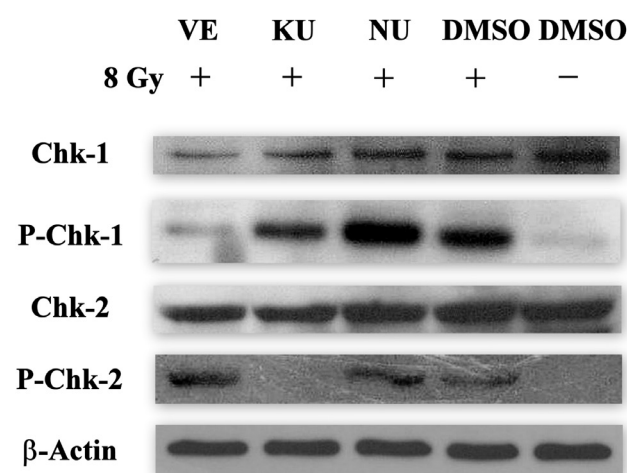
Fig. 1. Effects of inhibitors of DNA-PK (NU7441), ATM (KU55933) and ATR (VE-821) on the phosphorylation of Chk1 on serine 345 and Chk2 on threonine 68, 2 h after irradiation at a dose of 8 Gy. Inhibitors of ATM (KU 55933), ATR (VE-821) and DNA PK (NU7441) were added 2 h before irradiation and left in the culture for the entire period monitored.

The cell cycle was evaluated 24 h after single irradiation by 4 or 15 Gy. Figure 2A shows that neither the dose of 4 Gy nor the inhibitors KU55933 and NU7441 affected the percentage of HeLa cells in the S phase of the cell cycle compared to the control, non-irradiated group. The incubation with VE-821 resulted in a significant increase in the number of cells in the S phase compared to other irradiated groups as well as to the non-irradiated group. In all irradiated groups, there was a significant increase in the number of cells in the G2 phase compared to the non-irradiated group, the increase being more considerable after the incubation with NU7441 (60 %) compared to the solely irradiated group (24 %). A significant increase in the number of cells in the G2 phase compared to the irradiated group was also observed after incubation with the ATM kinase inhibitor KU55933. Twenty four hours after irradiation by 15 Gy, essentially no cells were present in the S phase, and the majority (60 %) of cells were accumulated in the G2 phase. Inhibitors NU7441 and KU55933 increased accumulation of HeLa cells in the G2 phase to 70 % and 90 %, respectively. In contrast to all the other groups, the inhibitor VE-821 significantly reduced (by 30 %) the accumulation of HeLa cells in the G2 phase of the cell cycle (Fig. 2B).

Figure 3 shows the proliferative activity (WST assay) of HeLa cells after 120-h long cultivation with inhibitors after irradiation by doses of 1–8 Gy. The proliferation of HeLa cells decreased dose-dependently within the range of 2 to 8 Gy. Neither the inhibitor KU55933 nor the inhibitor NU7441 significantly affected proliferation of non-irradiated or irradiated cells. The five-day incubation of irradiated cells with the ATR inhibitor VE-821 caused a significant drop in the cell proliferation of HeLa cells. This significant decrease was even more pronounced in irradiated groups pre-incubated with inhibitor compared to the group solely irradiated with the same radiation doses.

The clonogenic survival assay was used to evaluate the sensitivity of HeLa cells to ionizing radiation (2, 4, and 8 Gy). VE-821, KU55933, and NU7441 were added 2 h before irradiation and washed out 30 min after irradiation. We demonstrate a significant radiosensitizing
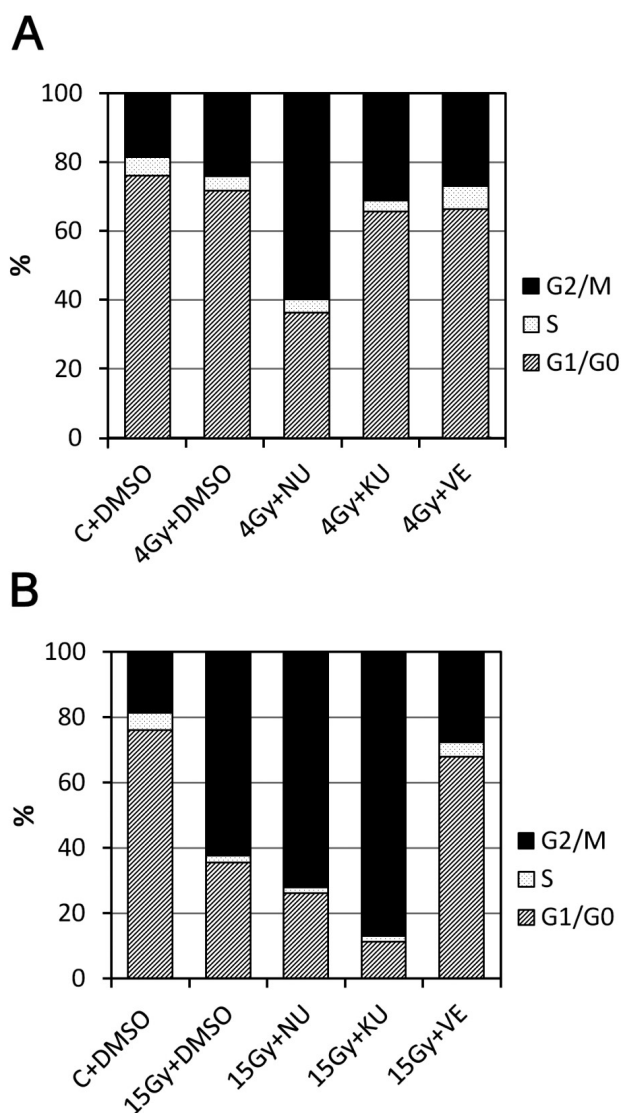

Fig. 2. Effects of inhibitors of ATM, ATR and DNA PK on the percentage of HeLa cells in particular phases of the cell cycle 24 h after irradiation at doses of 4 Gy (A) and 15 Gy (B). Inhibitors of ATM (KU 55933), ATR (VE-821) and DNA PK (NU7441) were added 2 h before irradiation and left in the culture for the entire period monitored.
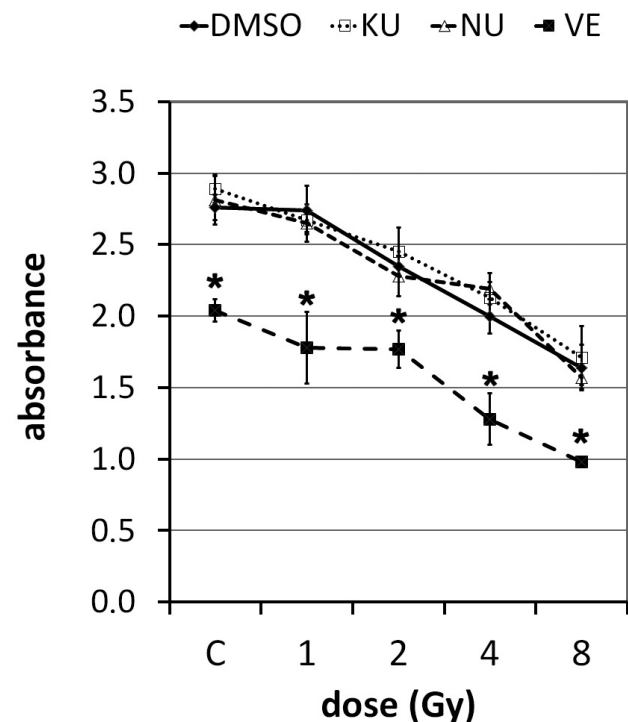

Fig. 3. Effects of inhibitors of ATM, ATR and DNA PK on the proliferation of HeLa cells 120 h after irradiation by doses up to 8 Gy (WST assay). Inhibitors of ATM (KU 55933), ATR (VE-821) and DNA PK (NU7441) were added 2 h before irradiation and left in the culture for the entire period monitored.
*Statistically significant difference from groups with DMSO at $P < 0.05$

effect of VE-821 after all doses of ionizing radiation studied (Fig. 4). A statistically significant radiosensitizing effect of KU55933 and NU7441 was observed after the irradiation by the dose of 8 Gy only (Fig. 4).

Using the xCELLigence System, we followed the proliferative activity of HeLa cells treated for a period of 120 h by inhibitors and ionizing radiation at doses of 1–8 Gy. Figure 5A shows clearly that after the dose of 1 Gy, the proliferative activity of cells was affected relatively moderately and within a long period after irradiation (a decrease from the 60th h after irradiation), whereas after the dose of 4 Gy (Fig. 5B), the proliferation was decelerated from the 36th h after irradiation, and from the 55th h, the proliferation moderately decreased over the entire period monitored (120 h). After the dose of 8 Gy, the cells proliferated again for periods up to 36 h, and in the next period, the proliferation decreased compared to the non-irradiated group, as well as to the group irradiated by 1 Gy, and it was the lowest at 120 h after irradiation, i.e. at the end of the experiment (data not shown). Inhibitors of ATR or ATM kinases (both VE-821 and KU55933) inhibited proliferation of non-irradiated cells. The effect of VE-821 was, however, considerably stronger. The inhibitor NU7441 did not considerably affect proliferation of the non-irradiated cells. All three inhibitors decelerated the proliferative activity of cells irradiated by the doses of 1 and 4 Gy (Fig. 5). The radiosensitizing effect of NU7441 and KU55933 was mani-

fested in a later period only, with a maximum effect at the end of the experiment. The cell proliferation arrest caused by the inhibitor VE-821 was accented after irradiation by doses of 1 and 4 Gy, which was observed as soon as starting from the first hours of the incubation, when using the xCELLigence System.

Discussion

In the present work, we studied the effects of ionizing radiation on human cervical carcinoma cells (HeLa cells). HeLa cells are known to have a poor function of protein p53 due to infection with human papilloma virus
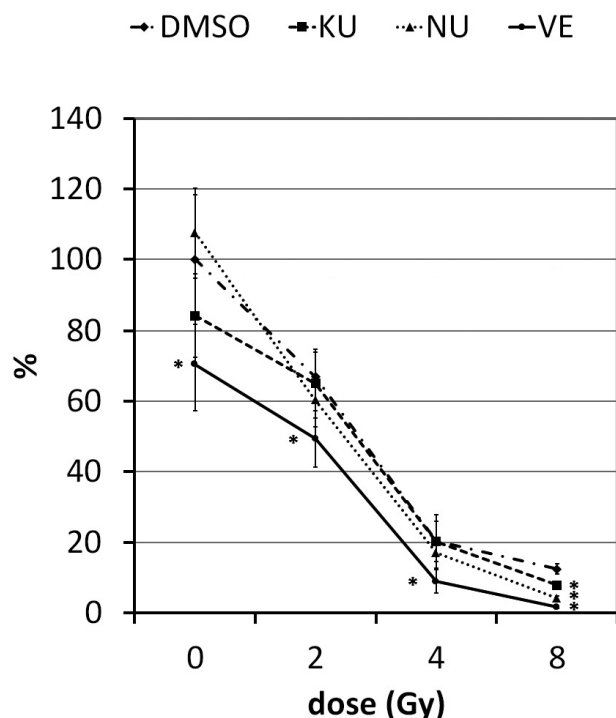

Fig. 4. Clonogenic survival curves of cells treated with inhibitors of ATM, ATR and DNA PK and irradiation. Inhibitors of ATM (KU 55933), ATR (VE-821) and DNA PK (NU7441) were added 2 h before irradiation and washed out 30 min after irradiation.
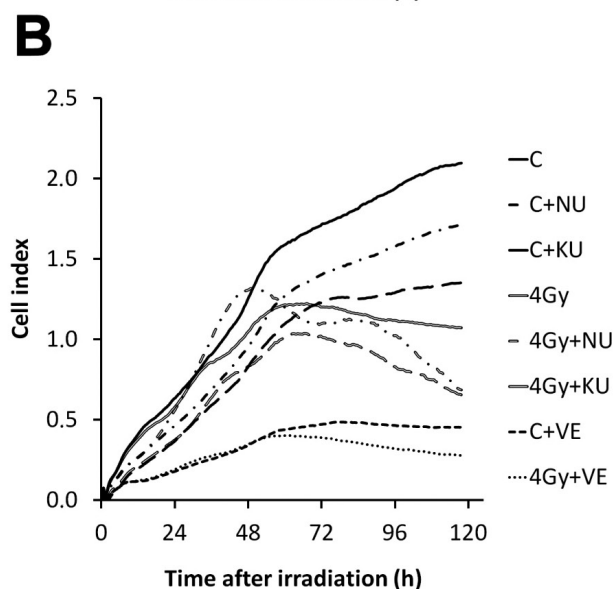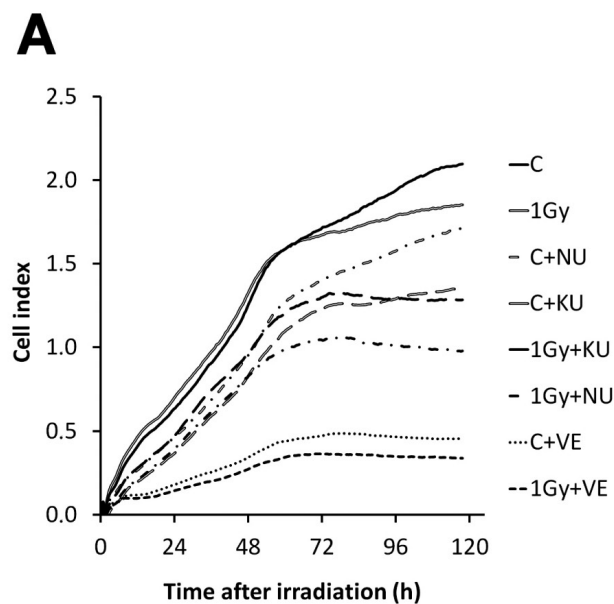*Statistically significant difference from groups with DMSO at P < 0.05

Fig. 5. Monitoring of the proliferative activity of HeLa cells 120 h after irradiation with the help of the xCELLigence System. HeLa cells were irradiated at doses of 1 Gy (A) and 4 Gy (B). Inhibitors of ATM (KU 55933), ATR (VE-821) and DNA PK (NU7441) were added 2 h before irradiation and left in the culture for the entire period monitored.

18. In cervical cancer, inactivation of p53 is attributed to the E6 oncoprotein, which binds to E3 ubiquitin ligase E6-AP and facilitates proteasomal degradation of p53 (Scheffner et al., 1993; Jin et al., 2005). The cells subsequently die by a non-apoptotic type of cell death; in HeLa cells, it is particularly the case of a mitotic catastrophe (Huang et al., 2005). Mitosis in HeLa cells persists for 45 min and in non-irradiated cells, mitosis (82 %) occurs within 12 h (On et al., 2011). On et al. (2011) have reported that after irradiation by a high dose of 15 Gy, no mitosis was revealed using time-lapse microscopy between 16 and 28 h after the irradiation (all the cells were arrested in G2 phase). Treatment with the combination of Chk1 inhibitor UCN-01 and a dose of 15 Gy led to the mitosis of 57 % cells, and these furthermore continued entering the G1 phase. Twenty-four percent of the cells exerted an extremely long mitosis even after 12-h monitoring and were considered as cells in the so-called mitotic catastrophe. In the case of a radiation dose of 40 Gy, the addition of UCN-01 resulted in prolongation of the time of entrance of the cells into mitosis compared to a dose of 15 Gy, but 84 % of the cells were in the condition of the mitosis even after 12 h of monitoring, and this was considered by the authors as a condition of the mitotic catastrophe.

When the cells lack p53, the G1 cell cycle arrest is not fully functional and relies on the activation of Chk1. Activating phosphorylation of Chk1 on serine 345 was observed during our experiments 2 h after the γ-irradiation by the dose of 8 Gy. Further, we proved that in HeLa cells, this process is ATR dependent, as the inhibitor VE-821 prevented this phosphorylation. Therefore, the crucial period of the ATR inhibitor application seems to be 2 h before until 2 h after the irradiation. Inhibition of ATM by KU55933 prevented phosphorylation of Chk2, but not the phosphorylation of Chk1.

We demonstrated that 24 h after γ-irradiation of HeLa cells by the dose of 4 Gy, there was a moderate increase in the number of cells in the G2 phase, which intensified significantly after irradiation by 15 Gy. X-ray irradiation by the dose of 6 Gy and irradiation by carbon ions in the dose of 3 Gy also induce accumulation of HeLa cells in the G2 phase of the cell cycle (Fujisawa et al., 2015). The block in the G2 phase after the dose of 4 Gy observed in our experiments was considerably increased and extended in the case of incubation of the cells with the DNA-PK inhibitor NU7441. DNA-PK is responsible for the NHEJ repair of the radiation damage. While after irradiation of HeLa cells by the dose of 4 Gy maximal accumulation of the cells in the G2 phase was achieved 12 h after irradiation, after inhibition of DNA-PK with siRNA or small molecule inhibitor NU7026, the block in the G2 phase was prolonged and more intensive (8–24 h after irradiation) (Shang et al., 2010). The DNA-PK inactivation results in an increase in polyploid, multinuclear cells and acceleration of the mitotic catastrophe process (Shang et al., 2010). Fuhrman et al. (2008) studied the effects of non-specific DNA-PK inhibitor LY294002 (which also inhibits Akt kinase) on HeLa cells, and based on the clonogenity test, they demonstrated a radiosensitizing effect, which was time-dependent, and the effect of the inhibitor was potentiated by extending the incubation period. They also demonstrated an increase in ionizing radiation inducing foci (IRIF) after the incubation with LY294002 and irradiation by the dose of 2 Gy, particularly 48–96 h after irradiation, which shows a long-term presence of cells with unrepaired DNA. In our experiments (unpublished data) we demonstrated that NU7441 increases the persistence of radiation-induced IRIF (co-location of γH2AX and MDC1) 72 h after irradiation by the doses of 2–8 Gy.

Monitoring of the HeLa cell proliferative activity for 120 h after irradiation with the help of the xCELLigence System revealed the radiosensitizing effects of all three inhibitors. While the effects of NU7441 and KU55933 after the doses of 1 and 4 Gy were manifested after longer time periods only (essentially from the third day after irradiation), VE-821 reduced the proliferative activity in all of the cells (both irradiated and non-irradiated) as soon as from the first hours after the beginning of cell monitoring (inhibitors were added 2 h prior to irradiation). Moreover, the clonogenic survival assay also proved the radiosensitizing effect of short application of ATR inhibitor VE821 (2 h before and 30 min after irradiation), which was significant after all studied irradiation doses (2, 4 and 8 Gy). Fujisawa et al. (2015) studied the effect of VE-821 on the clonogenic survival of HeLa cells irradiated by X-rays or carbon ions and compared the application of the inhibitor from 1 h before until 8 or 24 h after the irradiation. Their work shows that irradiation by the carbon ions is significantly more effective in elimination of tumour HeLa cells compared to X-rays. They also proved that a prolonged period of incubation with the ATR inhibitor after the irradiation was more effective. Based on our results, however, we conclude that the application of VE-821 before irradiation is crucial for enhancement of the radiation-induced cell death in tumour cells. Not only the clonogenic survival evaluated already 30 min after the irradiation decreased significantly, but also the xCELLigence System detected a decrease in the cell index already during the first hours of incubation with VE-821 (unlike the inhibitors of ATM and DNA-PK, where the effect was seen after three days of incubation only).

It is thus obvious that the presence of ATR kinase is required for the cell proliferation. Signalling through the ATR kinase is of principal importance for cell division and replication (Dai and Grant, 2010). Hypermorphic mutations in humans cause a disease known as Seckel syndrome, in which the mutation considerably reduces but does not fully abolish the ATR function (O'Driscoll and Jeggo, 2003). As can be seen with the xCELLigence System, the HeLa cells proliferate for a relatively long period of time (three days) after irradiation and the radiosensitizing effect is manifested later. The effect of DNA-PK inhibitor is not associated with the p53 status of the cells. Similarly as Zhao et al. (2006), we demonstrated that the G2 block induced by ionizing radiation

was prolonged in the group treated with NU7441, and the cells with unrepaired DNA persisted longer in the G2 block. In our experiments, the KU55933 radiosensitizing effect was demonstrated using the xCELLigence System after lower doses of γ-radiation – 1 and 4 Gy.

We and others (Fujisawa et al., 2015) demonstrated that the ATR kinase inhibitor VE-821 reduced the time of the presence of irradiated HeLa cells in the G2 phase, the cells entered mitosis with damaged DNA, and the capability of proliferation was considerably decreased in the cells treated in this way. By WST and xCELLigence System test and by clonogenic survival assay we demonstrated the proliferation capability of HeLa cells to be reduced with increasing radiation dose (1–8 Gy), and VE-821 potentiated this effect. We demonstrated that after incubation of HeLa cells with VE-821, no phosphorylation of Chk1 kinase occurred after the irradiation, and thus ATR kinase inhibition led to inhibition of the Chk1 kinase activity and subsequently to the cell death through the mitotic catastrophe. This means that the ATR kinase inhibitor exerted a similar effect as the Chk1 kinase inhibitor (On et al., 2011). VE-821 also inhibited the proliferative activity in non-irradiated HeLa cells.

Taken together, the ATR kinase inhibitor VE-821 was shown to be an important radiosensitizing agent in HeLa cells. The VE-821 radiosensitizing effect has also been demonstrated in other p53-negative cells (HL-60, prostate carcinoma cells and mammary carcinoma cells) (Reaper et al. 2011; Prevo et al. 2012; Vavrova et al., 2013). VE-821 demonstrated its excellent selectivity for ATR kinase and minimum cross-reactivity with ATM kinase. Reaper et al. (2011) demonstrated that the inhibitor VE-821 sensitized tumour (ATM-negative) cells to the effects of cisplatin. This molecule inhibits Chk1 phosphorylation induced by cisplatin. VE-821 also considerably enhances the sensitivity of pancreatic carcinoma cells to radiation and gemcitabine. Upon the effect of this inhibitor on tumour cells, increased DNA damage and decreased extent of homologous recombination were observed, which was suggested by persisting foci of co-localized 53BP1 and γH2AX (Prevo et al. 2012). The effect of VE-821 seems to be different in normal fibroblasts. When combined with cisplatin, it potentiated inhibition of proliferation but did not induce cell death of normal fibroblasts (Reaper et al., 2011).

We demonstrated an increase in Chk2 phosphorylation of threonine 68 after the dose of 8 Gy in HeLa cells similarly as Shang et al. (2010). The ATM kinase inhibitor KU55933 blocked Chk2 kinase phosphorylation and exerted a relatively weak effect on the HeLa cell proliferative activity. The incubation of HeLa cells with KU55933 before irradiation by the dose of 4 Gy resulted in a decrease in the cell number in S phase and increase in the cell number in G2 phase. Shaheen et al. (2011) studied the effects of KU55933 on prostate carcinoma cells (PC3) lacking p53. The dose of 2 Gy resulted in an increase in the cell number in G2 phase and a decrease in the cell number in S phase 48 h after irradiation. After incubation with KU55933, the decrease in the number of cells in the S phase was even potentiated and the incubation exerted radiosensitizing effects.

To conclude, in our work we demonstrated the antiproliferative effect of ATR, ATM a DNA-PK kinase inhibitors VE-821, KU55933 a NU7441 on HeLa cells, using the xCELLigence System and clonogenic survival assay. Transient application of any of the inhibitors 2 h before until 30 min after the irradiation by 8 Gy resulted in a significant decrease of HeLa cell clonogenicity. However, when combined with lower doses (1 or 4 Gy), only ATR inhibitor VE-821 was effective in decreasing the clonogenicity. Using real-time monitoring of the cell growth by xCELLigence System we demonstrated that while the radiosensitizing effect of VE-821 manifests early after irradiation, the effect of KU55933 and NU7441 is observed as late as 72 h after the irradiation.

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