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The radiosensitising effect of olomoucine derived synthetic cyclin-dependent kinase inhibitors

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Bohemine and roscovitine are the most important representatives of the group of compounds structurally derived from olomoucine. Biologically they function as inhibitors of cyclin-dependent kinases (CDKs), the key regulators of cell cycle, which is often disrupted in cancer cells resulting in uncontrollable proliferation. Bohemine and roscovitine have demonstrated their cytostatic and cytotoxic *in vitro* and also *in vivo* effects. Currently the phase II clinical trials for roscovitine are underway. The aim of the study was to evaluate the potential *in vitro* radiosensitising effect of bohemine (BOH) and roscovitine (ROS).

Clonogenic survival assay and human lung adenocarcinoma cell line A549 were used. Tested schedules were: A-pretreatment, B-concomitant application and C-posttreatment. Concentrations corresponded to IC10, IC25 and IC50 for BOH/ROS (0.1-30 μM). The radiation doses were 1, 2 and 3 Gy. Flow cytometry and western blot analysis were used to characterize cell cycle distribution, BrdU incorporation and DNA repair processes.

The highest *in vitro* radiosensitising effect of BOH/ROS was observed for Schedule A in all tested concentrations (SER(37%) 1.46-3.20). Cell cycle analysis showed an inclination towards G0/G1 delay 48 hours posttreatment and unaltered level of apoptosis. Changes in the DNA repair processes were observed - inhibition of DNA-PK kinase, inhibition of BrdU incorporation, strong and enduring induction of p21 protein and long-lasting phosphorylation of γH2AX(Ser139). Certain low concentration activities of BOH/ROS in monotherapy were detected, mainly the activation of DNA-PK kinase.

The results demonstrated strong *in vitro* radiosensitising effect of BOH/ROS that is concentration and especially schedule dependent. The strong cytostatic effect of the pretreatment schedule is mediated through the inhibition/rearrangements of DNA repair processes.

Keywords: Bohemine, roscovitine, radiosensitisation, CDK inhibitor, DNA repair.

Bohemine and roscovitine, compounds structurally derived from olomoucine, are inhibitors of cyclin-dependent kinases (CDKs) [1, 2]. They inhibit kinase CDK2, CDK7 and CDK9. CDK1 and CDK5 are affected as well [2–4]. These compounds have demonstrated their potential to block proliferation and induce apoptosis under both *in vitro* and *in vivo* conditions [2–5].

Recently the role of CDK kinases in the regulation of RNA polymerase II (RNAP II) transcription has been highlighted. Kinases CDK 1, 7, 8 and 9 activate RNA II polymerase and thus facilitate efficient initiation and elongation of transcription [6]. Moreover RNAP II polymerase blocked at sites of DNA lesions triggers transcription coupled repair (TCR) and stabilises p53 protein [7, 8]. The stabilization of p53 protein [5, 6, 9], which is accompanied by nucleolar fragmentation [5], down-regulation of antiapoptotic factor Mcl-1 [10], depletion of cyclins [4] and induction of apoptosis [10], occur due to the inhibition of RNA polymerase II by roscovitine (above 20 μM) and bohemine (above 50 μM) [4, 6, 10].

Treatment of cell lines with BOH/ROS at micromolar concentration leads to the arrest of the cell cycle on G1/S and G2/M boundaries and deceleration of S phase. The synchronised cells, especially those blocked on the G2/M boundary, could sensitively respond to consequent irradiation. The po-

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tential radiosensitising effect has already been proposed based on possible hyperstimulation of p53 activity using roscovitine in combination with irradiation [9, 11].

Repair of radiation incurred DNA double strand breaks (DSBs) is essential for cell survival. In eukaryotes, DSBs are either repaired by accurate homologous recombination (HR) or by potentially error-prone non-homologous end joining (NHEJ) [12]. The central role in HR is played by ATM and ATR kinases, which phosphorylate diverse components of the repair network/apoptosis pathway either directly or through the transducing kinases CHK2 and CHK1 [13, 14]. These kinases can rapidly and transiently delay cell–cycle progression through the CDC25s phosphatase pathway or they can also impose a delayed and enduring cell-cycle arrest through the p53/MDM2-p21 pathway [13]. The key role in NHEJ is played by the kinase DNA-PK [12]. ATM, ATR and also DNA-PK kinases directly activate and stabilize p53 protein by phosphorylation of its Serine-15 site. CHK1 and CHK2 kinases phosphorylate p53 on Ser20 and Thr18 [13].

The aim of this study was to evaluate the *in vitro* radiosensitising effects of BOH/ROS using the p53 wild-type human lung adenocarcinoma cell line A549 in the three treatment schedules. Flow cytometry methods and western blot analysis were used to study final effects.

Materials and methods

Cell culture and cell line. The human lung adenocarcinoma cell line A549 was purchased from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI medium supplemented with 10% fetal calf serum and containing 100 U/ml penicilin, 100 μg/ml streptomycin at 37°C and 5% CO2. All experiments were performed in exponentially and asynchronously growing cultures.

Tested compounds and irradiation conditions. Tested compounds were bohemine and roscovitine (Laboratory of Growth Regulators, Palacky University, Olomouc, Czech Republic). 10 mM stock solutions were prepared in 10% DMSO in saline and stored at -20°C. γ-irradiation was delivered at room temperature using ^{60}Co source (Chisostat, Chirana, Czech Republic).

Clonogenic survival assay. Cells were seeded in triplicates into 6-well plates (250 cells per well) and were left to stabilize for 24 hours. The incubation with drugs always lasted for 24 hours. Three different schedules were used: *A–pretreatment* - preincubation of cells before irradiation; *B–concomitant* - concomitant application of radiation and drugs and *C–post-treatment* – incubation of cells 1 day after irradiation. Treated cultures were incubated for additional 7 days in drug-free medium. Finally, the cultures were fixed, stained with crystal violet and colonies containing >50 cells were counted.

Flow cytometry methods. For BrdU (bromodeoxyuridine) incorporation assay, cell populations were exposed to 10 μM BrdU for 30 minutes before trypsinization and fixed in ice-cold ethanol. DNA content and cell cycle analysis was performed using hypotonic citrate buffer and propidium iodide staining method [15]. BrdU positive cells were visualized using anti-BrdU-FITC antibody (Becton Dickinson, San Jose, CA) after chemical DNA denaturation according to the manufacturer's instructions.

Cell suspensions were measured on Becton Dickinson FACS Calibur flow cytometr (Becton Dickinson, San Jose, CA) at excitation wavelength of 488 nm. Cell cycle histograms were analyzed using ModFit software (Verity Software House, Inc., Topsham, ME). Cell Quest software (Becton Dickinson, San Jose, CA) was used for quantification of both apoptotic subG1 and BrdU positive cells. The ratio of G2/M BrdU positive versus G2/M BrdU negative cells was calculated (ratio = G2/M BrdU positive/ G2/M BrdU negative x 10).

Immunoblotting. Cell lysates were analysed by Cell Signaling immunoblotting protocol (Cell Signaling, Danvers, MA) in combination with the enhanced chemiluminescent detection ECL system (Amersham, Little Chalfont, UK). Cells were resuspended in sodium dodecyl sulphate sample buffer supplemented with protease inhibitor coctail (Sigma, St Louis, MO). The used antibodies were: polyclonal antibody anti-phospho H2AX(Ser139) (Upstate, Lake Placid, NY); polyclonal antibodies p53, CHK1, CHK2 and phospho p53(Ser15), p53(Ser20), p53(Ser46), p53(Ser392), CHK1(Ser345), CHK2(Thr68) (Cell Signaling, Danvers, MA); monoclonal antibody p21CIP1/WAF1 (Exbio, Prague, Czech Republic); monoclonal antibodies PARP, MSH6, MSH2 and DNA-PKcs/p350 (BD Biosciences Pharmingen, Philadelphia, PA); secondary peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Pierce- SuperSignal West Dura Extended Duration kit, Rockford, IL). Protein loading was controlled by monoclonal antibody against β-tubulin (Sigma, St Louis, MO).

Statistical methods. Every data point of the survival curves was calculated from two or three independent experiments with three replicates each. The values of the sensitising enhancement ratio (SER) for 37% survival level were calculated. The radiation dose that reduced the surviving colonies to 37% of the non-treated controls was divided by the radiation dose that reduced survival to 37% in combination with BOH/ROS. Statistical comparison of the survival curves was performed using the one-tailed t-test for each data point. Data analysis and graphics were calculcated using PRISM 4.01 software (Graph-Pad, San Diego).

Results

Cytotoxic/cytostatic activities of monotherapy. Monotherapy survival curves were analyzed for proper dosage of drugs and radiation in combinations (Fig. 1). The concentrations inhibiting colony formation for 10, 25 and 50% (IC10, IC25 and IC50) were calculated (0.1 μM, 12.5 μM and 30 μM for bohemine; 0.1 μM, 9 μM and 13 μM for roscovitine). The A549 cells were sensitive to radiation, they significantly responded to irradiation in low, clinically relevant doses (1-3 Gy).

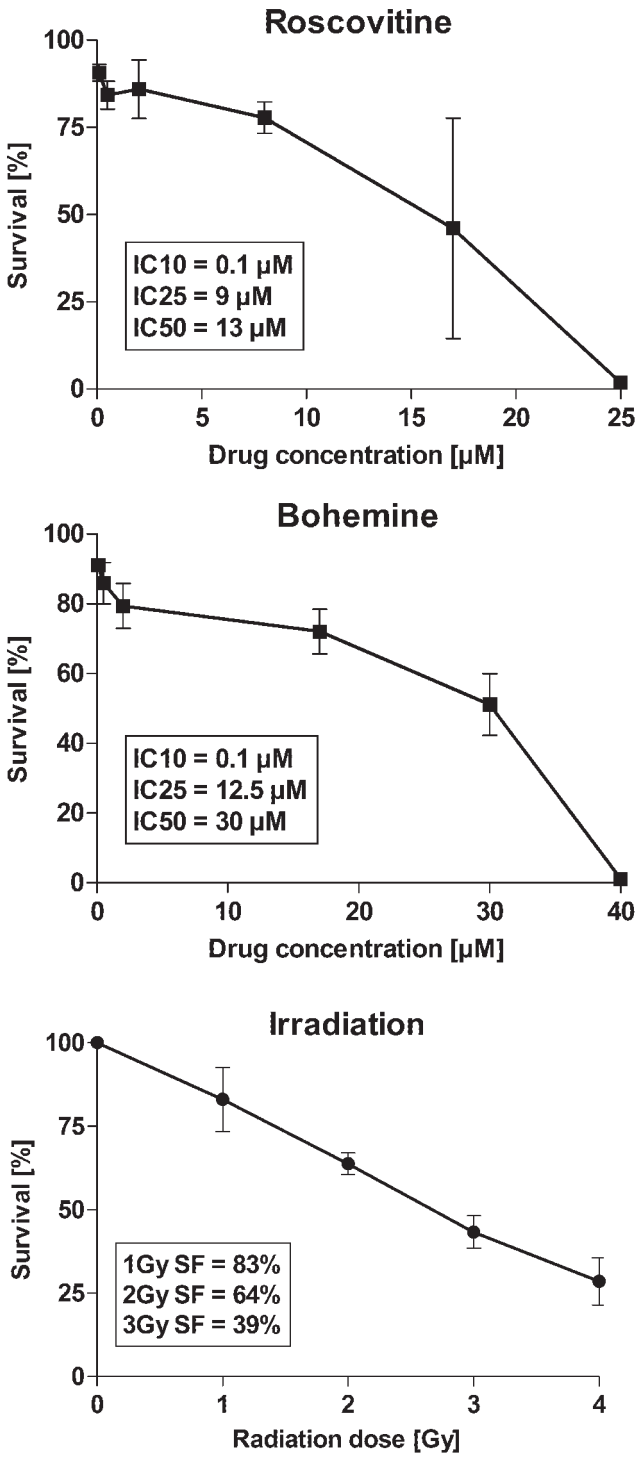

Figure 1. Cytotoxic/cytostatic activity of monotherapy. Each data point represents the mean ± S.D. from at least three separate experiments.

Cytotoxic/cytostatic activities of combined treatment

Schedule A-pretreatment. Compounds clearly demonstrated radiosensitising effects (Fig. 2) even in the lowest tested concentrations. The SER(37%) values for IC10 concentrations were 1.55/1.47 for BOH/ROS respectively. However, the higher radiosensitising effect of CDKIs (IC50) was more apparent at higher concentrations. The highest SER(37%) values achieved were 3.20 for BOH and 2.53 for ROS at level of IC50. Decrease of survival fraction was significant for all tested concentrations of BOH/ROS ($p<0.0001$).

Schedule B–concomitant application. The only significant radiosensitising effect was registered for the highest concentrations (IC50 levels) of both BOH ($p<0.0001$) and ROS ($p<0.0204$) (Fig. 2). The relevant SER(37%) values were 1.77/1.76 for BOH/ROS respectively. At lower concentrations the effects were merely additive.

Schedule C–posttreatment. Similarly to Schedule B, the radiosensitising effect was found in the highest concentrations of CDKIs (IC50) with SER(37%) 1.56($p<0.0058$)/1.61($p<0.0001$) for BOH/ROS, respectively (Fig. 2). A weak radiosensitising effect was also demonstrated for ROS at level of IC25 with SER(37%) 1.18 ($p<0.0464$).

Cell cycle and subG1 peak analysis. Figure 3 summarizes the effects of radiation (2 Gy) and drugs (cBOH/ROS = 12.5/9 μM) on cell cycle 24 and 48 hours after the wash-out of the drugs. Unfortunately the results for 48 hours in Schedule C could not be included because the cell populations were overgrown (the experiment is 24 hours longer than Schedule A & B and the cell viability is the highest of all the schedules). Our data (24 hours interval) demonstrate that the combined therapy leads to substantially decreased count of the S phase cells and increase in the G2/M population. The most noticeable redistribution of cell cycle phases occurred in Schedule A (pretreatment). Bohemine and roscovitine alone reduced the S phase of the cell cycle (29.5% for BOH and 29.7% for ROS; control cells 40%). In Schedule A, the level of S phase was decreased to 22.6%/19.8% for BOH/ROS. The decreased level of S phase remained on the same level 48 hours after the wash-out and the percentage of cells in G0/G1 phase was increased (67.5%/65.5% for BOH/ROS respectively) at the expense of G2/M phase. In the same time interval the irradiated cells and the cells treated with BOH/ROS demonstrate the common profile of exponentially growing A549 cells (S phase 30-37%, G2/M phase 13-14% and G0/G1 48-58%). Similar although inferior trend to develop the G1 arrest was also shown in Schedule B.

The analysis of the subG1 peak (Fig. 3) showed that the level of apoptosis for the irradiated cells was slightly increased 24 h after irradiation (8%). Bohemine or roscovitine only delayed the apoptotic process in irradiated cells to 48 hours (BOH, Schedule A; 9.6% and ROS, Schedule B; 8.3%).

BrdU incorporation assay and DNA repair. Next we have implemented the BrdU incorporation assay for time interval 24 hours after wash-out of BOH/ROS. In this analysis, the BrdU positive cells were either the S phase cells (S-phase DNA

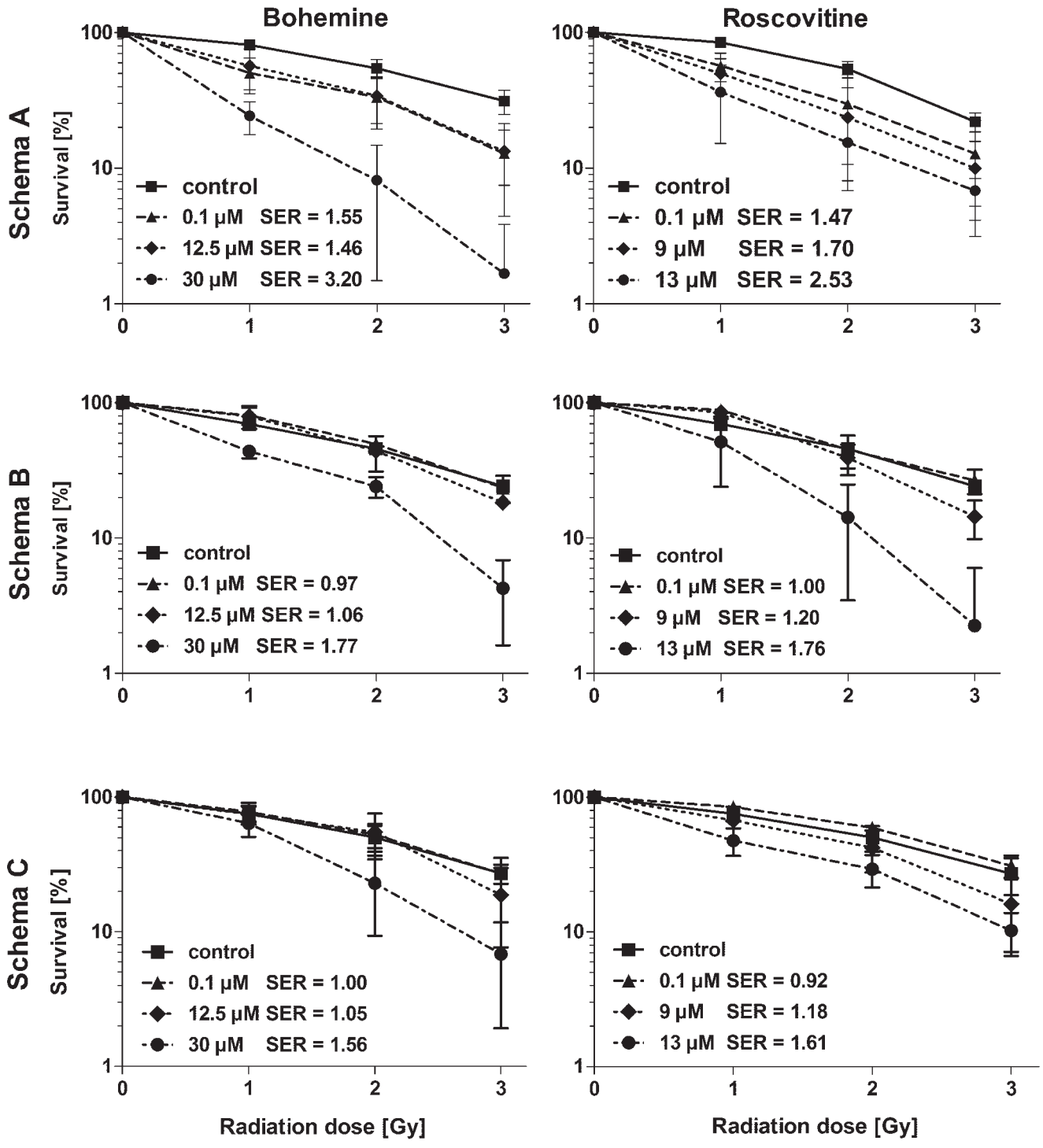

Figure 2. Cytotoxic/cytostatic activities in Schedule A, B and C. Each data point represents the mean ± S.D. from three separate experiments.

content) or the cells undergoing DNA repair (G2/M DNA content). The ratio of BrdU positive/negative cells in the G2/M phase reflects the percentage of DNA repairing cells. Figure 4 shows a decrease of DNA repair in Schedule A (0.7/0.6-fold for BOH/ROS). Mild inhibition was also registered in Schedule B (0.85/0.85-fold for BOH/ROS).

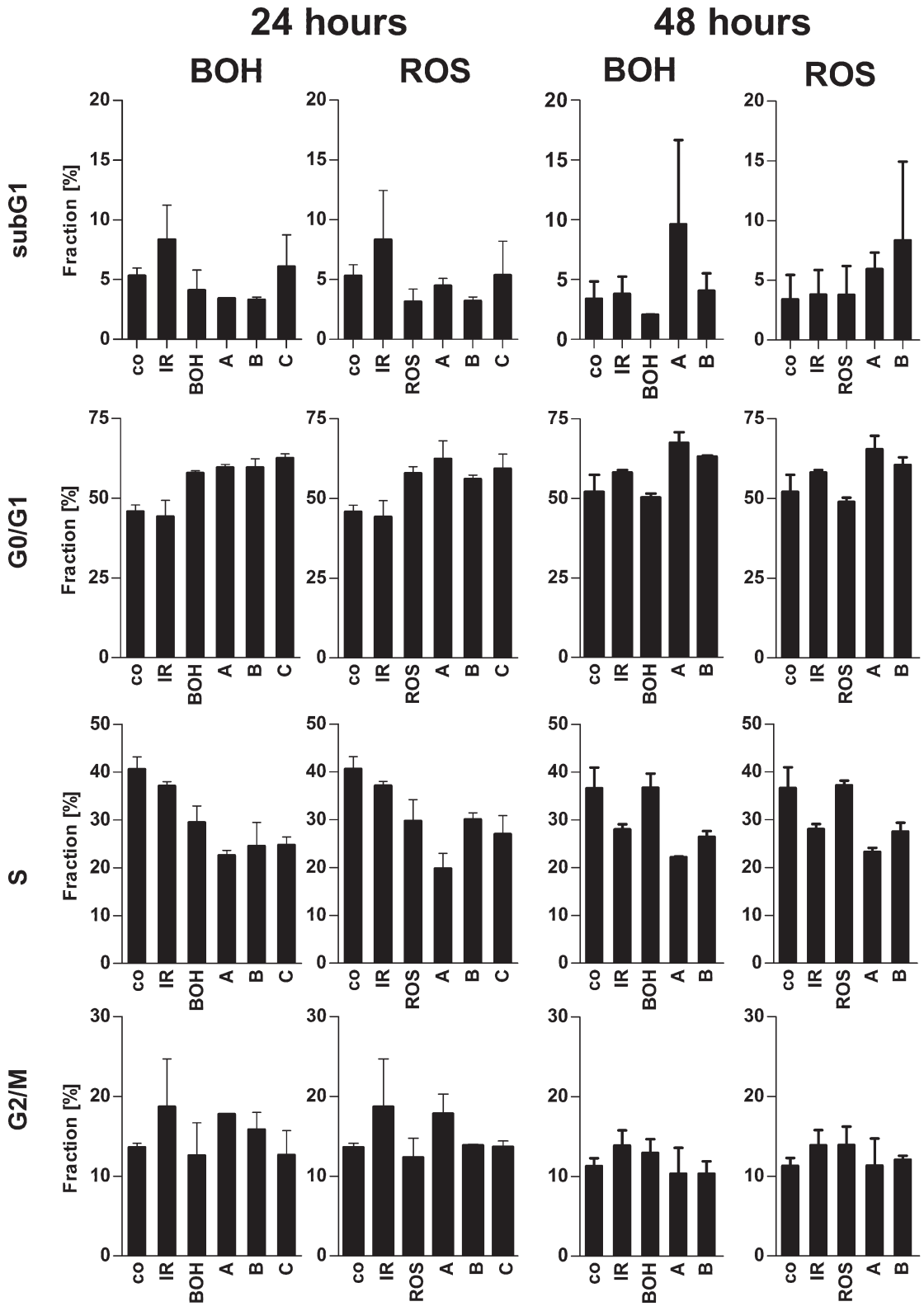

Figure 3. Cell cycle and subG1 peak analysis. Data correspond to 24 and 48 hours after the wash-out of the drugs (rad. dose 2 Gy, cBOH/ROS = 12.5/9 μM). Each data point represents the mean ± S.D. from two separate experiments.

Immunoblotting relating to Schedule A-pretreatment . Cell lysates corresponding to the intervals 1, 3, 6 and 12 hours after the wash-out of the drugs were analysed. The irradiation dose was increased to 10 Gy in order to enhance the chances of capturing some of the DNA DSBs repairs (cBOH/ROS = 12.5/9 μM). Resulting western blot pictures are shown in Figure 5. Initially we used antibody against phosphorylated form of histone H2AX (γH2AX Ser139), because this phosphorylation could be an indicator of ongoing DNA DSBs repair. Our data demonstrated more noticeable phosphorylation of histone H2AX at the time interval 6 and 12 hours after preincubation with BOH/ROS, indicating enduring DNA repair.

Unambiguous inhibition of DNA-PKcs (catalytic subunit of DNA-PK kinase) by BOH/ROS gives evidence of their influence on NHEJ repair (time interval 3 hours). On the contrary BOH/ROS alone slightly activate DNA-PK kinase (time interval 1 and 3 hours). ATM/ATR kinases (HR) phosphorylate mainly Thr68 site of CHK2 kinase and Ser345 or Ser317 sites of CHK1 kinase. In our experiments inhibition of CHK1 and CHK2 kinases in the case of combined therapy was not detected. Minor induction of CHK1 kinase with different dynamics for bohemine and roscovitine was visible.

Up-regulation of MSH6 protein by BOH/ROS indicates activation of methyl directed mismatch repair system (MMR). The MSH2-MSH6 complex probably also participates in correcting base mispairs that can arise from the strand reactions of HR [12]. The induction of MSH6 protein, observed after irradiation, points to the involvement of MSH2-MSH6 complex in DSBs repairs. In the combined therapy the signal of MSH6 protein was completely absent at the 3 hour interval and the overall duration of the protein induction was longer. The levels for MSH2 protein were not affected.

BOH/ROS alone did not influence the stability of p53 protein. Our results in the combined therapy demonstrated only weak upregulation of p53(Ser15) protein compared to irradiation control at the time interval of 12 hours. Strong upregulation of p21 protein at 3 hours in response to irradiation was transient; however, strong induction of p21 protein caused by combined therapy was delayed: starting at 3 hours and clearly visible at 6 and 12 hours. Induction of p21 was also observed after incubation with roscovitine and, to lesser extent, after incubation with bohemine. Phosphorylation of p53 on Ser20 showed no remarkable differences between irradiation and combined therapy. No signal was observed using p53(Ser46) and p53(Ser392) antibodies. Our data showed no cleavage of PARP protein.

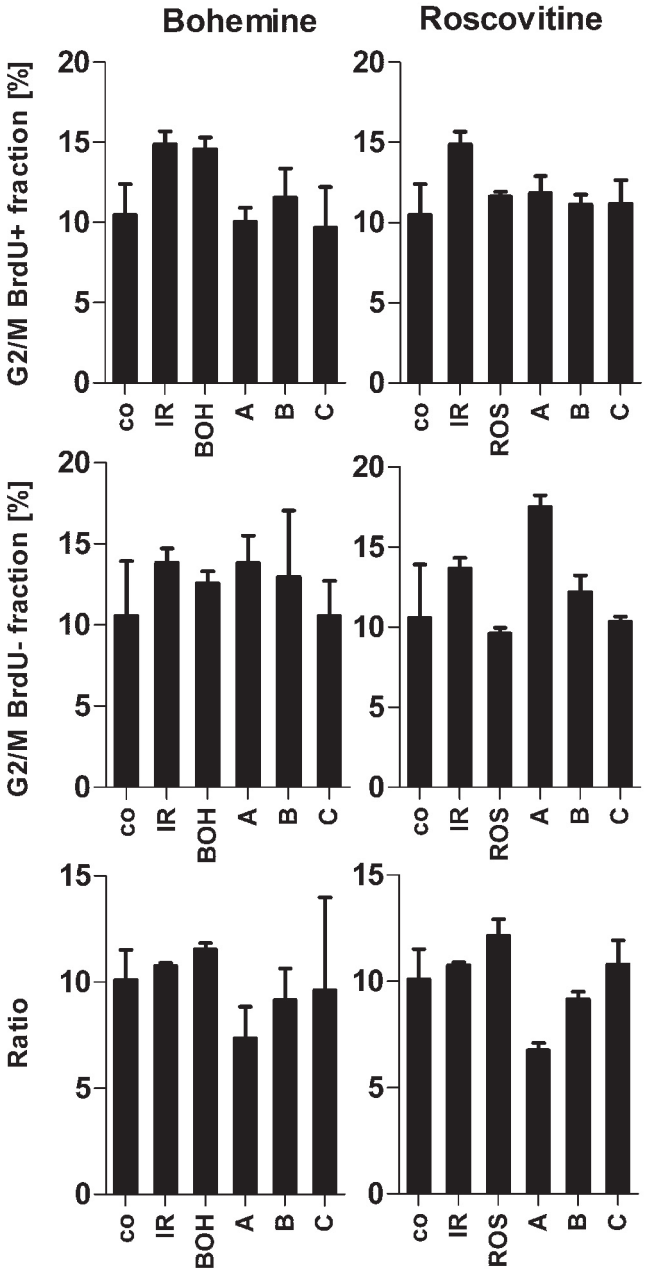

Figure 4. BrdU incorporation assay. Data correspond to 24 hours after the wash-out of the drugs (rad. dose 2 Gy, cBOH/ROS = 12.5/9 μM). The ratio of G2/M BrdU positive versus negative cells was calculated. Each data point represents the mean ± S.D. from two separate experiments.

Discussion

We observed strong *in vitro* radiosensitising effect of BOH/ROS for Schedule A (pretreatment) in all tested concentrations. Redistribution of the cell cycle related to the radiosensitising effect (Schedule A) was only moderate, cells displayed tendency to block cell cycle in the G0/G1 phase 48 hours posttreatment.

During the experiments there occured certain changes of DNA DSBs repair processes related to radiosensitisation; mainly strong inhibition of DNA-PK kinase (NHEJ), although BOH/ROS in monotherapy caused its activation (Fig. 5). The hypothesis of significant change in HR repair mechanism is supported only by the different dynamics of MSH6 protein

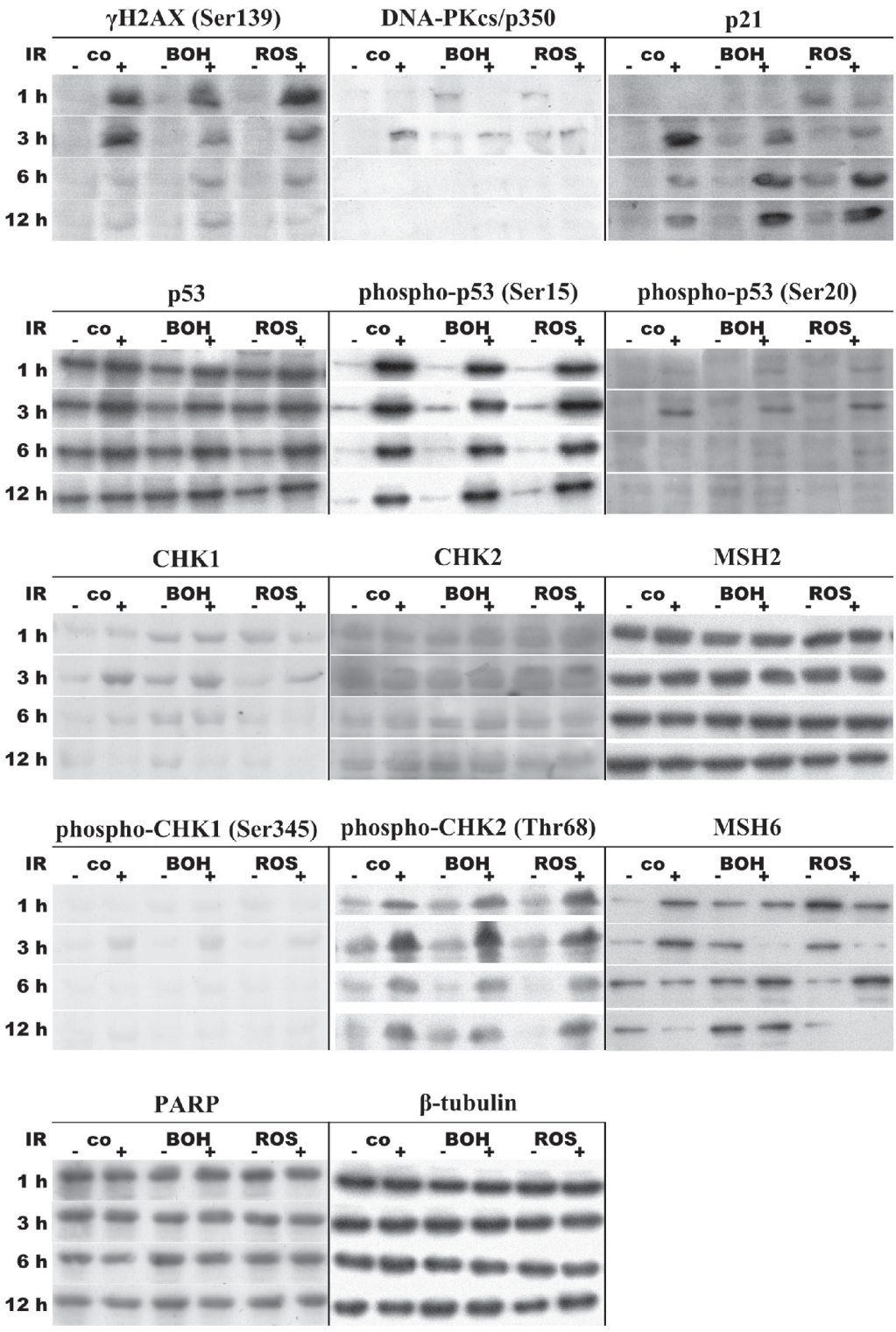

Figure 5. Immunoblotting of selected proteins related to Schedule A. Cell lysates corresponding to the intervals 1, 3, 6 and 12 hours after the wash-out of the drugs were tested (rad. dose 10 Gy, cBOH/ROS = 12.5/9 μM). Loaded protein volumes were usually 20 μg or 50 μg (CHK1, CHK1(Ser345), CHK2(Thr68), CHK2) or 100 μg (DNA-PKcs/p350, γH2AX(Ser139), p21).

induction (Fig. 5). Immunodetection of the CHK1 and CHK2 transducing kinases did not show any changes in the time interval of 1-12 hours. Unfortunately the direct activity of ATM and ATR kinase was not tested. The exact coordination of HR and NHEJ mechanisms is yet unknown [13, 14]. It seems that the DNA DSBs repairs are mediated primarily by the NHEJ mechanism, while the HR mechanism is activated in more persistent lesions. Also HR repair process functions preferentially in the late S/G2 phase, while NHEJ predominates in the G0/G1 phase [13, 14]. BrdU incorporation assay (Fig. 4), immunodetection of histon γH2AX(Ser139), protein MSH6 and protein p21 (Fig.5) imply inhibited and longlasting DNA repair process. We suppose that the DSBs caused by combined therapy are repaired mainly by the HR mechanism. Possible explanation is that combined therapy leads to higher proportion of cells in the G2/M phase immediately after irradiation, where the HR predominates, or that the combined treatment induces more persistent lesions.

Our findings correlate with the results published by Maggiorella, describing the radiosensitising effect of concomitantly administered roscovitine using p53 mutated breast cancer MDA-MB231 cell line under *in vitro* and also *in vivo* conditions [16]. Using pulse-field gel electrophoresis he showed the inhibition of DNA DSBs repair 24 hours after treatment (cROS = 5 μM, rad. dose 4 Gy) and suggested that the inhibition of DNA-PK activity, which was tested measuring the DNA-PK activity on nuclear extracts, is caused by the direct interaction of roscovitine with DNA. Nevertheless he did not study the possible effect on HR. There are more concordant data in our and Maggiorella's results – e.g. that in the treatment Schedules B and C the synergistic effect was achieved in the highest concentrations only (IC50). Maggiorella described this effect of concomitantly administered ROS only for the higher concentration he used (5 μM and not for 2.5 μM). Next, he used p53 mutated cell line and therefore this effect did not depend on the p53 protein. Our results did not show hyperstimulation of p53 protein activity either, although we did not test in detail its nuclear accumulation. The malfunction of the ATM/ATR-CHK1/CHK2-p53/MDM2-p21 cascade can be compensated by the ATM/ATR-CHK1/CHK2-CDC25s and ATM-NBS1-SMC1 signal cascades. Finally, radiosensitising effect of BOH/ROS is not based on hyperstimulation of p53 activity.

Upregulation of p53 protein by the low concentration of BOH/ROS (12.5/9 μM) used in monotherapy was not demonstrated. However, induction of p21 protein was detected for roscovitine and weakly also for bohemine (Fig. 5). Upregulation of MSH6 protein and mild induction of CHK1 kinase may suggest evidence of HR activation that responds to an arrest of replication fork or to occurrence of DNA DSBs (Fig. 5). Ljungman proposes that ATR-CHK1 repair pathway could be activated primarily not only by the arrest of the DNA replication fork, but also in reaction to blocked transcription [7, 8]. Maude and Savio described the replication inhibition caused by roscovitine (20 μM) accompanied by the activation of the DNA damage response system [17, 18]. According to the first study the histon γ-H2AX phosphorylation corresponded to roscovitine caused DNA DSBs [17]. According to the second study the phosphorylation of γ-H2AX was limited only to the arrested replication fork site [18]. The activation of DNA-PK (NHEJ) we discovered might rather point towards the DNA DSBs caused by BOH/ROS even at low concentrations (cBOH/ROS = 12.5/9 μM) (Fig. 5, time interval 1 and 3 hours). Using micronuclei assay and by measurement of DNA-PK activity Maggiorella also demonstrated induction of DSBs and activation of DNA-PK by roscovitine (5 μM), though he did not comment on the activation of DNA-PK [16].

The detailed mechanism by which BOH/ROS might cause DNA DSBs is yet unclear. First, DSBs may arise in response to an aberrant replication process either due to the inhibition of CDK2 kinase or due to the possible collision of the replication machinery with stalled or collapsed RNA polymerase complex. Second, under *in vitro* conditions during mitosis CDK1 kinase may form molecular complexes with topoisomerase IIα, and its inhibition can lead to possible occurrence of DSBs [19]. Inhibition of CDK2 or CDK1 kinase is important, because inhibitor of RNAII polymerase 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB; inhibitor of CDK7 and CDK9) does not induce DNA damage [8]. Finally, bohemine and roscovitine at very low concentrations (app. 10 μM) alter transcription and also replication process. These events lead to the occurrence of DNA DSBs. This could possibly suggest that in the combined therapy the drug induced damages and radiation induced damages accumulate, leading to more persistent lesions.

Strong and longlasting induction of p21 protein, no cleavage of PARP protein (Fig. 5) and no significant increase of subG1 peak in cell cycle analysis (Fig. 3) then point to possible enduring or permanent G1 arrest (senescence), and therefore strong cytostatic effect. The fact that the combined therapy does not lead to increase of apoptosis was also supported by other studies [16, 20].

Considering our findings, it seems obvious, that inhibition of transcription and replication processes by the CDK inhibitors bohemine and roscovitine is an important phenomenon not only for their radiosensitising effect. Importantly, the transformed cells seem to be more sensitive to disruption of RNA and DNA synthesis than corresponding normal cells. We can presume that the cyclin-dependent kinase inhibitors may have a promising therapeutic potential as radiosensitisers. Currently the phase II clinical trials are in progress for roscovitine.

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References

[1] VESELÝ J, HAVLÍČEK L, STRNAD M, BLOW JJ, DONELLA-DEANA A, et al. Inhibition of cyclin-dependent kinases by purine analoques. Eur J Biochem 1994; 224: 771–786. doi:10.1111/j.1432-1033.1994.00771.x

[2] MEIJER L, BORGNE A, MULNER O, CHONG JP, BLOW JJ, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 1997; 243: 527–536. doi:10.1111/j.1432-1033.1997.t01-2-00527.x

[3] HAJDÚCH M, HAVLÍČEK L, VESELÝ J, NOVOTNÝ R, MIHÁL V, et al. Synthetic cyclin dependent kinase inhibitors. New generation of potent anti-cancer drugs. Adv Exp Med Biol 1999; 457: 341–353.

[4] RAYNAUD FI, WHITTAKER SR, FISCHER PM, MAC CLUE S, WALTON MI, et al. In vitro and in vivo pharmacokinetic-pharmacodynamic relationships for the trisubstituted aminopurine cyclin-dependent kinase inhibitors olomoucine, bohemine and CYC202. Clin Cancer Res 2005; 11: 4875–4888. doi:10.1158/1078-0432.CCR-04-2264

[5] WOJCIECHOVSKI J, HORKY M, GUEORGUIEVA M, WESIERSKA-GADEK J. Rapid onset of nucleolar disintegration preceding cell cycle arrest in roscovitine-induced apoptosis of human MCF-7 breast cancer cells. Int J Cancer 2003; 106: 486–495. doi:10.1002/ijc.11290

[6] LJUNGMAN M, PAULSEN MT. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at ser15 and lys383. Mol Pharmacol 2001; 60: 785–789.

[7] LJUNGMAN M. The transcription stress response. Cell cycle 2007; 6: 2252–2257.

[8] DERHEIMER FA, CHANG C, LJUNGMAN M. Transcription inhibition: A potential strategy for cancer therapeutics. Eur J Cancer 2005; 41: 2569–2576. doi:10.1016/j.ejca.2005.08.012

[9] BLAYDES JP, CRAIG AL, WALLACE M, BALL HM, TRAYNOR NJ, et al. Synergistic activation of p53-dependent transcription by two cooperating damage recognition pathways. Oncogene 2000; 19: 3829–3839. doi:10.1038/sj.onc.1203773

[10] MAC CALLUM DE, MELVILLE J, FRAME S, WATT K, ANDERSON S, et al. Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. Cancer Res 2005; 65: 5399–5407. doi:10.1158/0008-5472.CAN-05-0233

[11] LU W, CHEN L, PENG Y, CHEN J. Activation of p53 by roscovitine–mediated suppresion of MDM2 expression. Oncogene 2001; 20: 3206–3216. doi:10.1038/sj.onc.1204412

[12] BERNSTEIN C, BERNSTEIN H, PAYNE CM, GAREWAL H. DNA repair/pro-apoptotic proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat Res 2002; 511: 145–178. doi:10.1016/S1383-5742(02)00009-1

[13] KASTAN MB, BARTEK J. Cell-cycle checkpoints and cancer. Nature 2004; 432: 316–323. doi:10.1038/nature03097

[14] SONODA E, HOCHEGGER H, SABERI A, TANIGUCHI Y, TAKEDA S. Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. DNA Repair 2006; 5: 1021–1029. doi:10.1016/j.dnarep.2006.05.022

[15] DIAMOND RA, DE MAGGIO S. In living color: Protocols in flow cytometry and cell sorting. 1st Edition, Berlin: Springer Verlag, 2000.

[16] MAGGIORELLA L, DEUTSCH E, FRASCOGNA V, CHAVAUDRA N, JEANSON L, et al. Enhancement of radiation response by roscovitine in human brest carcinoma in vitro and in vivo. Cancer Res 2003; 63: 2513–2517.

[17] MAUDE SL, ENDERS GH. Cdk inhibition in human cells compromises Chk1 function and activates a DNA damage response. Cancer Res 2005; 65: 780–786.

[18] SAVIO M, CERRI M, CAZZALINI O, PERUCCA P, STIVALA LA, et al. Replication-dependent DNA damage response triggered by roscovitine induces an uncoupling of DNA replication proteins. Cell cycle 2006; 18: 2153–2159.

[19] ESCARQUIEL AE, PLISOV SY, SKLADANOWSKI A, BORGNE A, MEIJER L, et al. Recruitment of cdc2 kinase by DNA topoisomerase II is coupled to chromatin remodeling. FASEB J 2001; 15: 2288–2290.

[20] BOUTIS A, PAPAZISIS K, PISTEVOU-GOMPAKI K, LAMBROPOULOS A, SOFRONIADIS I, et al. Cyclin-dependent kinase (CDK) inhibitor olomoucine enhances γ-irradiation-induced apoptosis and cell cycle arrest in Raji cells. Anticancer Res 2006; 26: 3493–3498.