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CYTOTOXICITY OF ETOPOSIDE IN CANCER CELL LINES *IN VITRO* AFTER *BCL-2* AND *C-RAF* GENE SILENCING WITH ANTISENSE OLIGONUCLEOTIDES

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Abstract: *BCL-2* and *C-RAF* genes are overexpressed in most types of cancers. Although these genes are mediators in different molecular pathways their main characteristic is the antiapoptotic activity, thus cells that overexpress either *BCL-2* or *C-RAF* lose their ability to undergo apoptotic death being resistant to chemotherapeutic agents and/or physiologic mediators of cell death (e.g., TNF-α). Both anti-*C-RAF*, and anti-*BCL-2* oligonucleotides were tested as chemosensitizers in cancer therapy. The aim of the study was to investigate the effects of the combined use of antisense oligonucleotides (ASOs) targeting *BCL-2* and *C-RAF* transcripts on the *in vitro* cancer cell cultures exposed to etoposide. Cells were transfected with phosphorothioate *BCL-2* and *C-RAF* ASOs. To sustain high intracellular level of ASOs, 3-day transfection was used, and it was followed by a single treatment with 20 μM etoposide for 5 h. The following cancer cell lines were tested: A549, HeLa, and T24. Sequence-specific decrease in *BCL-2,* and *C-RAF* mRNA levels were confirmed by real-time RT-PCR: after 1-day treatment mRNA levels decreased by 9–42% of the normal expression in cells treated with 50–1200 nM ASOs. Also, the induction of cell death in all transfected cultures in a concentration-dependent manner was confirmed by MTT assay, microscopic analysis of cell morphology, and the measurement of histone H3 expression. Results also showed that both ASOs effectively potentiated etoposide-induced cytotoxicity; the strongest effects were obtained in A549 (lung cancer). This observation suggests that lower concentrations of both antisense oligonucleotides may be used, at least for this type of cancer, to obtain high efficiency of etoposide-induced cell death enhancement. Simultaneous use of two ASOs in 3-day treatment allows us to lower concentrations needed to obtain significant treatment results thus enabling to diminish sequence-unspecific toxicity.

Keywords: antisense oligonucleotides, apoptosis, *BCL-2*, cancer, *C-RAF*, etoposide, PTGS, real-time RT-PCR

Abbreviations: Apaf-1 – apoptotic protease activating factor 1, ASO – antisense oligonucleotide, BAX – BCL2-associated X protein, BCL-2 – B-cell lymphoma 2, DMSO – dimethyl sulfoxide, ERK – extracellular signal-regulated kinase, FBS – fetal bovine serum, PBS – phosphate-buffered saline, MEK – mitogen-activated protein/extracellular signal-regulated kinase, MDR1 – multidrug resistance gene 1 (P-glycoprotein), PTGS – post-transcriptional gene silencing, RNAi – RNA interference

C-RAF-1 is a protooncogene encoding a serine/threonine kinase that is implicated in mediation of the signal transduction from mitogenic growth factor receptors inside cells. The main effector of *C-RAF* is MEK kinase which regulates cell growth, survival, and proliferation. There are three *RAF* isoforms (isozymes), *A-, B-*, and *C-RAF* (1). *RAF* isoforms exert their effects through the RAF/MEK/ERK cascade when it is initiated by interaction of certain receptor with its ligand. *RAF* kinases are critical mediators of signaling cascades induced by growth factor receptors (2). Moreover, activated *RAF* also increases expression of c-Myc, and P-glycoprotein (MDR1) which is responsible for extrusion of many anticancer drugs (including etoposide and other topoisomerase II inhibitors); it has been suggested that this mechanism is involved in the occurrence of multidrug resistance in tumor cells. Additionally, the RAF/MEK/ERK pathway has also been shown to synergize with *BCL-2* overexpression and to stimulate proliferation rate of hematopoietic cells. The main cause of *C-RAF* over-

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expression in many cancers is the activation of oncogenes, mainly RAS, that promote cell proliferation through *C-RAF* (3, 4).

The second often found phenomenon in cancer cells is the overexpression of *BCL-2*. *BCL-2* belongs to a family of proteins exerting both pro- and antiapoptotic activities. Most extensively studied members of this family are antiapoptotic*BCL-2* and proapoptotic *BAX* (5). The *BCL* family proteins form homodimers as well as heterodimers thus influencing apoptosis. Pro-survival members of *BCL-2* family directly inhibit the ability of Apaf-1 to activate caspases. They are also required for the maintenance of mitochondrial integrity and disruption of the mitochondrial transmembrane potential and leakage of apoptosis inducing factors precedes the onset of apoptosis in many cell types (6). *BCL-2* gene encodes a protein which is located in the outer mitochondrial membrane, endoplasmic reticulum, and the nuclear membrane (5). It has been found to be overexpressed in more than 70% of all breast cancers, and in many other cancer types including prostate, colon, ovary and lung.

BCL-2 and *C-RAF* proteins play crucial role in the growth and apoptosis of cancer cells of different origin. Additionally, they play a role in cancer resistance to chemotherapeutic agents, which is a major obstacle in the successful therapy. Tumor cells have either primary drug resistance or the phenomenon develops during the course of therapy. One of the mechanisms of tumor chemoresistance is based on the failure to initiate apoptosis (7). Elimination of drug resistance may become the main goal for the design of suitable adjuvant therapy i.e., based on apoptosis initiation and stimulation. Both anti-*C-RAF*, and anti-*BCL-2* molecular interventions, including antisense oligonucleotides and siRNA, were extensively tested for the cancer therapies. Our purpose was to investigate whether simultaneous silencing of *BCL-2* and *C-RAF* expressions in cancer cells lead to improvement of the antitumor potential of etoposide. To achieve the aim we analyzed effects of the combined use of antisense oligonucleotides (ASOs) targeting *BCL-2* and *C-RAF* transcripts in the *in vitro* cancer cell cultures exposed to etoposide.

EXPERIMENTAL

Chemicals

Agarose, DMSO, etoposide, Hoechst 33258, magnesium chloride, MTT, potassium ferricyanide, potassium ferrocyanide, 0,4% trypan blue solution, and X-gal were purchased from Sigma-Aldrich. All media and other chemicals used for *in vitro* cell cultivation (RPMI-1640, FBS, PBS, gentamicin) were purchased from PAA; UltraMEM was purchased from Lonza.

Cell cultures conditions and etoposide treatment

Experiments were carried out on the *in vitro* cultures of cell lines representing different cancers: HeLa (ATCC: CCL-2; cervical adenocarcinoma), A549 (ATCC: CCL-185; lung cancer), and T24 (ATCC: HTB-4; urinary bladder transitional cell carcinoma). Cell cultures were routinely propagated in a humidified incubator Hera-Cell (Heraeus), at 37°C, in 5% CO_2/95% air atmosphere; cells were grown in RPMI-1640 medium supplemented with 10% FBS and gentamicin (20 μg/mL). Cell number and dead/alive ratio were estimated microscopically (Axiovert 40CFL, Zeiss) by trypan blue staining in Neubauer hemacytometric chamber. All experiments were carried out either in 96-well (transfection, MTT tests) or 24-well (Hoechst staining) plates (PAA). For apoptosis stimulation, cells were exposed to etoposide (20 μM) for 1 h in RPMI-1640 without FBS or antibiotic.

Antisense oligonucleotides

The following sequences of antisense oligonucleotides were used in the study: *BCL-2*: 5'-AAT CCT CCC CCA GTT CAC CC-3'; cRAF: 5'-TCC CGC CTG TGA CAT GCA TT-3'; *SCR* (*scrambled*): 5'-TCC CGC GCA CTT GAT GCA TT-3'. BCL-2 ASO is complementary to *BCL-2* mRNA within 915–934 nt (accession No. NM_000633) binding both α-, and β–isoforms (141–147 codon). This ASO was selected from the group of four other sequences complementary to different localizations within *BCL-2* mRNA that were tested earlier at our department (8). *C-RAF* ASO is complementary to mRNA of *C-RAF-1* within 2771–2790 nt (accession No. NM_002880.3). The control antisense oligonucleotide is not complementary to any human mRNA sequence in the NCBI database. All ASOs had modified phosphorothioate backbones; they were synthetized and purified at the Institute of Biochemistry and Biophysics (Warszawa, Poland).

Transfections

Antisense oligonucleotides were transferred into cells by lipofection with the use of Oligofectamine Reagent (Invitrogen) according to manufacturer's protocol. All wells were washed twice with RPMI-1640 without FBS or antibiotic directly before transfections. Oligonucleotides were mixed with suitable amounts of oligofectamine (1

mg/mL) and UltraMEM and incubated for 30 min. Then, they were transferred to each well on the 96-well plates. Time of lipofection was 5 h. The following concentrations of oligonucleotides were used: 50, 100, 200, 400, 800 nM, and 1.2 μM. Lipofections were carried out either once or for thrice: for three days in a row. Twenty four hours after the last transfection, cell cultures were further analyzed (MTT, RNA extraction or Hoechst staining). To assess transfection efficiency two plasmids containing extragenomic sequences were introduced in the same conditions: pEF/*myc*/cyto/GFP (Invitrogen) encoding *green fluorescence protein* (GFP) and pcDNA 3.1/His/*lacZ* (Invitrogen) encoding β-galactosidase. GFP expression was detected under fluorescence inverted microscope (Nikon Eclipse). β-Galactosidase activity was visualized *in situ* (cells fixed for 5 min) on inverted microscope (Axiovert 40CFL, Zeiss) after overnight incubation with substrate solution containing: 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg/mL substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in PBS. Additionaly, suitable controls were included: untreated cultures (no ASO, oligofectamine or etoposide), cultures exposed to oligofectamine (liposomes toxicity), cultures transfected with scrambled sequence ASO (unspecific toxicity of ASO), and cultures treated with ASO without oligofectamine (DNA toxicity).

Cytotoxicity assay

Total numbers of alive cells in the studied cultures were estimated by cytotoxicity-proliferation MTT test based on reduction of a substrate dye 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltetrazolium bromide (MTT) in alive cells to produce formazan crystals which are then solubilized by ethanol-DMSO mixture (1 : 1, v/v). MTT reduction rate is proportional to alive cell number. To carry out the test, 100 μL of RPMI-1640 without phenol red containing the MTT substrate (0.5 mg/mL) was inserted into each well. After 2-h incubation at 37°C, an equal volume of 96% ethanol-DMSO mixture (1 : 1, v/v) was added and plates were incubated for another 2 h. Then, the intensity of the colorimetric reaction was measured by Triad LT (Dynex) microplate reader at λ = 570 nm.

Cell proliferation

To test cell proliferation molecular test was carried out based on histone H3 expression. Quantitative RT-PCR with specific primer set was carried out in the same conditions as described below for other genes mRNA detection. The following primer set was used: H3 F: 5' TGC CAT TTC AGC GCC TGG TG 3'; H3 R: 5' CAC GGA TAC GAC GCG CAA GC 3'(amplification product: 200 bp) (9).

Cell morphology analysis

To directly detect apoptotic cells, microscopic analysis of fluorescent staining was performed. After medium removal, cells were washed with PBS and fixed directly on the 24-well culture plates by 4% paraformaldehyde (10 min, RT), and then stained with Hoechst 33258 (1 mg/mL). Stained cultures were analyzed for apoptosis signs (e.g., chromatin condensation, apoptotic bodies) under fluorescence inverted microscope (Nikon Eclipse). In each well at least 100 cells were analyzed.

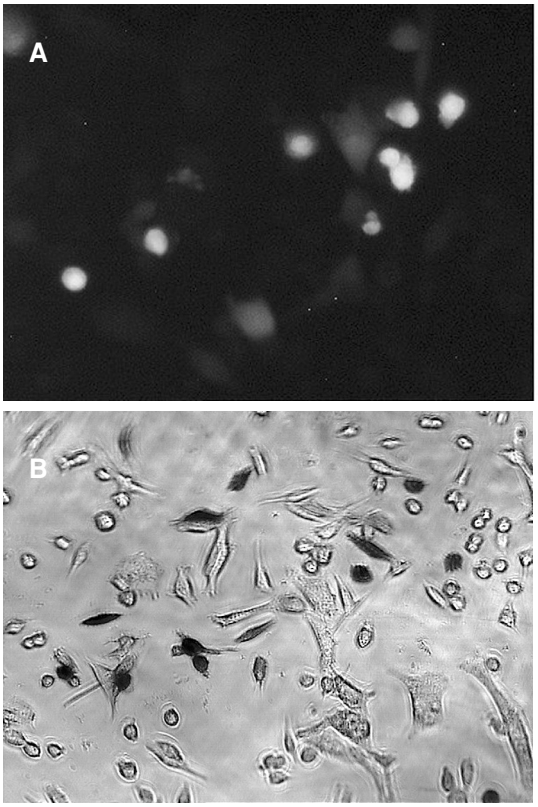
RNA extraction

Total RNA was extracted by phenol-chloroform method using TRI-Reagent (Sigma-Aldrich) according to manufacturer's protocol. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm (BioPhotometer, Eppendorf). RNA quality was estimated by 2% agarose gel electrophoresis with GelRed staining (Invitrogen).

Real-time RT-PCR assay

Levels of mRNA of *BCL-2* and *C-RAF* were determined by SYBR Green Real-Time RT-PCR assay. RT-PCR reaction tubes contained 12.5 μL of 2x QuantiTect SYBR Green RT-PCR Master Mix (containing HotStartTaq DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer with fluorescent dyes SYBR Green I and ROX, and dNTP mix), 0.5 μL of reverse transcriptase mix (Omniscript and Sensiscript), 0.3 μM of each sense and antisense primer, 0.4 μg of unknown RNA template, and water to a total volume of 25 μL. All reagents were purchased from Qiagen. One-step real-time RT-PCR assay was carried out using Mx3000P thermal cycler (Stratagene). The thermal profile was 50°C for 30 min (reverse transcription), and 95°C for 15 min, 40 two-step cycles of 94°C for 15 s and 55°C for 30 s, and 72°C for 10 min (real-time PCR), followed by a dissociation protocol (60–95°C, 30 min).

Sequence-specific PCR primers for mRNA of *BCL-2* and *C-RAF* were designed using computer software Primer Express v.1.0 ABI PRISM (Applera). The following oligonucleotide 5' and 3' primer sequences were used: β-actin – sense: 5'TCA CCC ACA CTG TGC CCA TCT ACG A3'; anti-



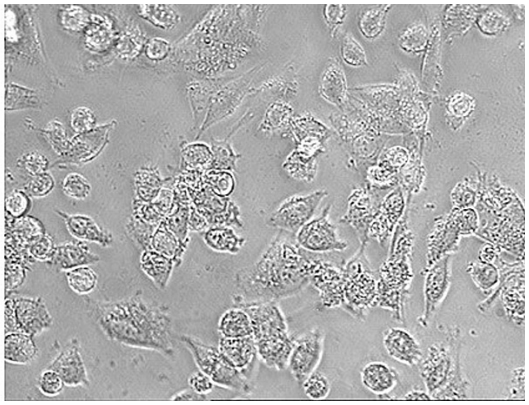


Figure 1. Expression of green fluorescent protein (GFP) (A) and β-galactosidase (B) in HeLa cultures 24 h after transfection with plasmid vectors pEF/*myc*/cyto/GFP and pcDNA 3.1/His/*lacZ*, respectively. For GFP detection cells were visualized in UV and in visible light for comparison (magnif. 100×)

sense: 5' CAG CGG AAC CGC TCA TTG CCA ATG G3'; (amplification product: 295 bp); *BCL-2*– sense: 5' TTG TGG CCT TCT TTG AGT TCG GTG 3'; antisense: 5' GGT GCC GGT TCA GGT ACT CAG TCA 3' (amplification product: 113 bp); *C-RAF* – sense: 5'-GCC TTC AGG AAT GAG GTG GC-3'; antisense: 5'-CCGTTAACACTGGGT-CACC-3' (amplification product: 104 bp).

The amplification products were separated in 2% agarose gels and visualized using ethidium bromide staining. The specificity of PCR products was additionally confirmed by determination of dissociation curves assay run after every real-time RT-PCR amplification by measuring fluorescence intensity during temperature elevation between 60 and 95°C. mRNA levels of the studied genes (*BCL-2* and *C-RAF*) were normalized to endogenous control (β-actin mRNA) by ΔΔCt method, and the calibrators were the control (untreated) cultures. Presented values were the results of mathematical equation $2^{-\Delta\Delta Ct}$ which indicated the increase or decrease in mRNA expression of certain gene compared with the control sample (untreated culture) after normalization to the expression of the housekeeping gene (β-actin). All the results are presented as the means ± range of three independent experiments, each performed in triplicate.

Statistical analysis

The quantitative data were compared by Student *t*-test; $p < 0.05$ was considered significant; calculations were performed using Statistica Version 6.0 software.

RESULTS

Effects of *BCL-2* and *C-RAF* silencing in cancer cell cultures

The first step of the study was to standardize transfection method and to assess whether the selected antisense oligonucleotides were efficient enough to perform significant down-regulation of *BCL-2* and *C-RAF* expression. For this purpose we used HeLa cultures. The mean efficiency of lipofection with oligofectamine was about 21% for GFP detection and 35% for LacZ activity. The efficiency was estimated by calculation of positive cells (expressing transgenic GFP or β-galactosidase) in the whole cell pool (Fig. 1). Sequence-specific down-regulation of *C-RAF* and *BCL-2* mRNA levels was confirmed by real-time RT-PCR after single treatment with antisense oligonucleotides. Relative decrease (compared to untreated controls) for *BCL-2* mRNA varied from 8.6% (50 nM anti-*BCL-2*) to 41.8% for the highest concentration (1.2 μM) of *BCL-2* ASO used; *C-RAF* mRNA decreased by

61–83.5%. All calculations were normalized to β-actin mRNA (Fig. 2). Treatment with anti-*C-RAF* and anti-*BCL-2* ASOs also lead in all transfected cultures to decrease of histone H3 mRNA levels – molecular marker of proliferation rate (9). Relative decrease ranged from 34.1% to 66.8% of the levels measured in the control cultures (Fig. 3). We also tested the cytotoxic effects of the studied ASOs using morphological analysis to detect apoptosis symptoms e.g., apoptotic bodies formation and/or nuclear DNA condensation. *In situ* staining with Hoechst 33258 revealed occurrence of many cells undergoing apoptosis when 50–400 nM of ASOs were used while almost no apoptosis signs were observed in untreated controls or cultures transfected with the control (*scrambled*) ASO. The number of cells undergoing apoptosis increased when higher concentrations of ASOs were used: for cultures transfected with 100, 200, and 400 nM of anti-*BCL-2* or anti-*C-RAF* the apoptotic cells percentage was

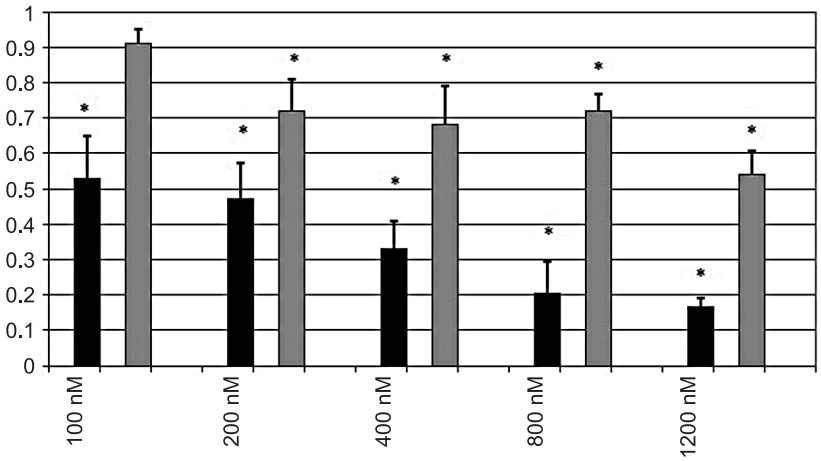


Figure 2. Changes in mRNA levels of BCL-2 (gray bars) and C-RAF (black bars) in HeLa cultures after antisense oligonucleotides transfection. Relative units of ΔΔCt method are shown. Results were normalized to β-actin expression; the calibrators were cultures transfected with *scrambled* (nonsense) ASO. * indicates a statistically significant difference in comparison to control ($p < 0.05$)

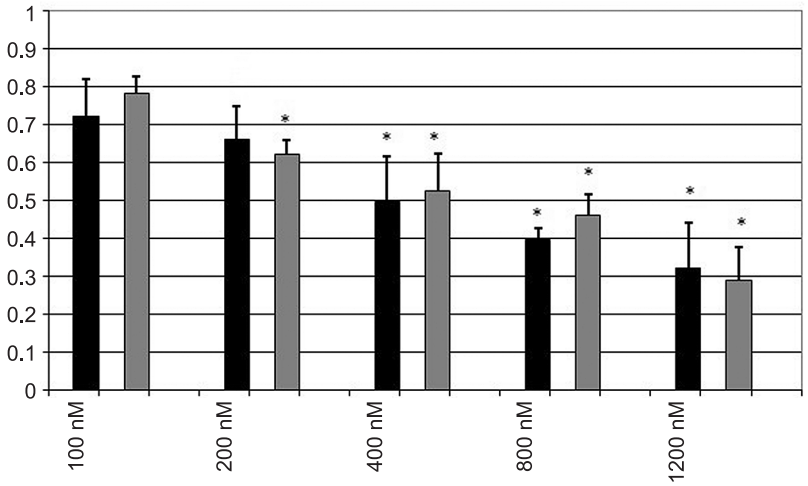


Figure 3. Changes in mRNA levels of histone H3 in HeLa cultures after transfection with anti-BCL-2 (gray bars) or anti-C-RAF (black bars) oligonucleotides. Relative units of ΔΔCt method are shown. Results were normalized to β-actin expression; the calibrators were cultures transfected with *scrambled* (nonsense) ASO. * indicates a statistically significant difference in comparison to control ($p < 0.05$)

15, 22, and 30%, respectively. Even when the lowest (50 nM) concentrations were used, apoptotic cells number was increased compared with the untreated controls (Fig. 4). In the following step we tested *in vitro* cytotoxicity of the studied ASOs towards different cancer cell lines.To obtain stable suppression of *BCL-2/C-RAF* expression we performed repeated, 3-day period experiments instead of single implementation of oligonucleotides. Three cell lines corresponding to different cancer types

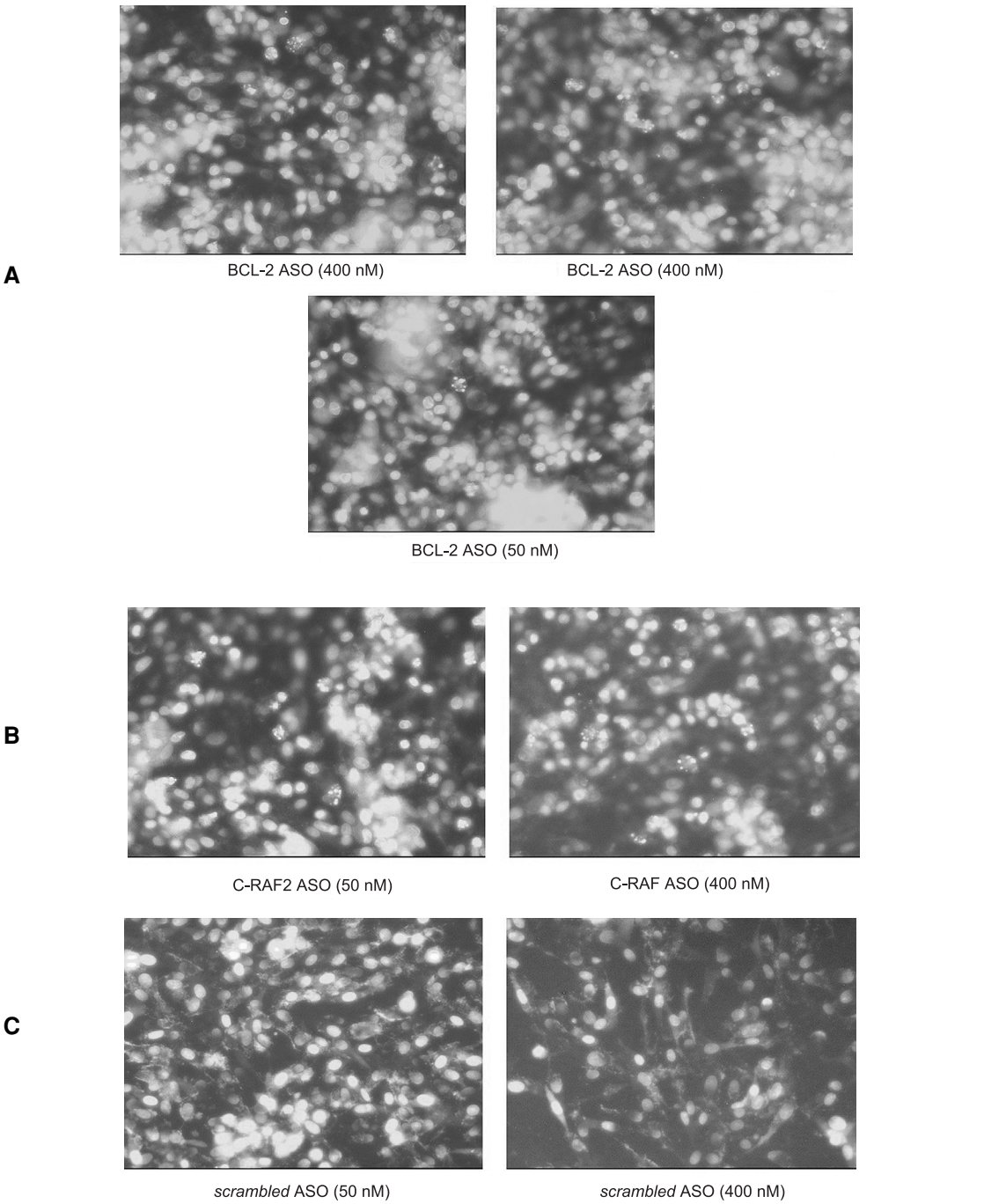


Figure 4. Apoptosis detection using morphological analysis of HeLa cultures treated with anti-BCL-2 (**A**), anti-RAF (**B**) or scrambled sequence (control) (**C**) oligonucleotides. Cells were stained with Hoechst 33258 and visualized in fluorescent microscope (Nikon Eclipse, magnif. 200×).

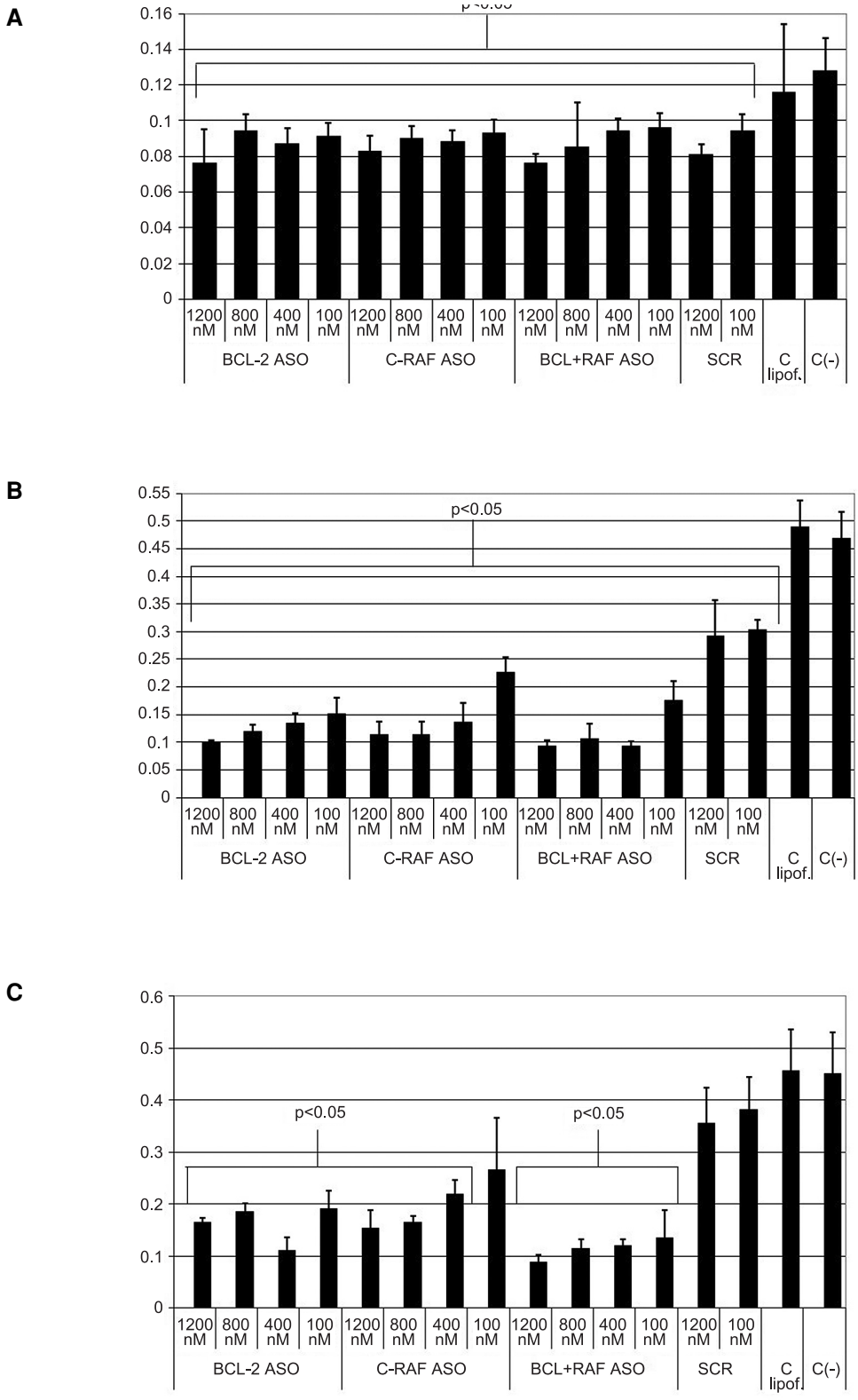


Figure 5. MTT results obtained for HeLa (**A**), T24 (**B**), and A549 (**C**) cultures after 3-day transfections with anti-BCL-2 and/or anti-RAF oligonucleotides. X axis presents values of absorption at λ = 550 nm of *in vitro* cultures transfected with antisense oligonucleotides within range 100–1200 nM. Bars indicate the means +/– standard deviations. The following controls were included: cultures not stimulated with etoposide, SCR – cultures stimulated with scrambled (nonsense) ASO (the reference cultures), C lipof. – cultures treated with empty liposomes, and C (-) – cultures not treated with any agent.

were submitted to transfections. Significant differences were evidenced in the response of certain cancer models analyzed in that study (Fig. 5). Most significant results were obtained for A549 (Fig. 5 C) and T24 (Fig. 5 B) cultures, while in HeLa cultures cytotoxic effects of *BCL-2* and *C-RAF* ASOs were not as strong (Fig. 5 A). The biggest differences among different conditions used in the experiments were obtained for A549 cultures. The strongest resistance to ASOs treatment was observed in HeLa cultures while the most promising results were obtained in A549 (lung cancer) cells. Interestingly, in A549 cultures there was a significant decrease in the number of alive cell when both ASOs were used (by about 10%) compared to cultures transfected with anti-*BCL-2* or anti-*C-RAF* only. This suggests that A549 cells were more sensitive to both oligonucleotides action.

Etoposide-induced cytotoxicity in cancer cell cultures after *BCL-2* and *C-RAF* silencing

The last step of the study was to investigate whether the anti-*BCL-2* and *C-RAF* oligonucleotides may enhance chemosensitivity of cancer cells to etoposide treatment. We used A549 cultures basing on the previous results indicating the strongest cytotoxic effects of both ASOs in the cultures (Fig. 6). The maximal growth inhibition was at the level of about 20% as compared with untreated control cultures. For etoposide-treated cultures that were previously transfected with 800 nM or 1.2 μM concentrations of ASOs there were not significant differences between cultures transfected with both ASOs or with either anti-*BCL-2* or anti-*C-RAF*. The same evaluation for cultures transfected with 400 nM concentration showed a slight decrease in the number of alive cells in cultures transfected with both ASOs compared with cultures transfected with anti-*C-RAF* only. That observation was even more evident when 100 nM concentrations were used. The same observations were made for anti-*BCL-2* oligonucleotide: at 100 nM concentrations cytotoxicity of etoposide increased when both ASOs were used compared with cultures transfected with anti-*BCL-2* only (however, statistically significant differences were not observed). Simultaneous implementation of *BCL-2* and *C-RAF* ASOs generally does not perform stronger cytotoxicity, which suggests that the highest effects attainable for the studied cancer cell cultures are already obtained when the studied ASOs are used separately; however, it allows us to use lower concentrations because even then the cytotoxic effects are as strong as with the higher concentrations in the simultaneous transfections. It is noteworthy that generally neither anti-*BCL-2* nor anti-*C-RAF* oligonucleotides significantly sensitized the transfected cultures to etoposide treatment; cell death extent after etoposide treatment was similar when etoposide was the only factor or when additional incorporation of anti-*BCL-2*, anti-*C-RAF* or both ASOs was implemented.

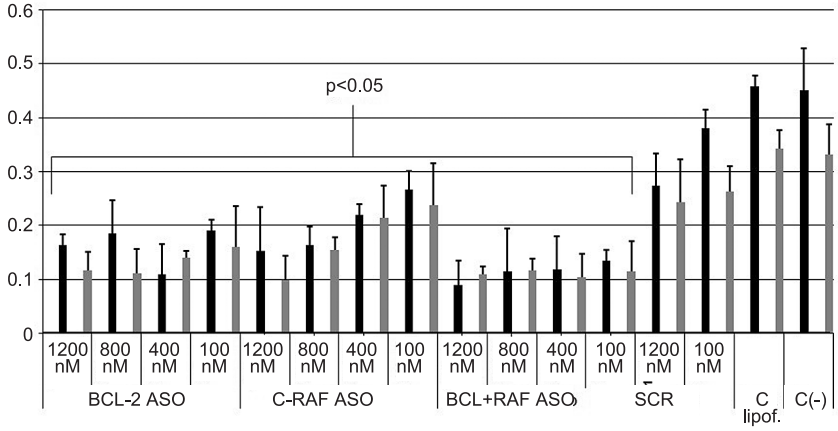


Figure 6. MTT results obtained for the A549 *in vitro* cultures after 3-day transfections with anti-BCL-2 and/or anti-RAF oligonucleotides (black bars) followed by 1-h treatment with 20 μM etoposide (gray bars). X axis presents values of absorption at λ = 550 nm for cultures transfected with antisense oligonucleotides within the range 100–1200 nM. Bars indicate the means ± standard deviations. The following controls were included: cultures not stimulated with etoposide, SCR – cultures stimulated with scrambled (nonsense) ASO (the reference cultures), C lipof. – cultures treated with empty liposomes, and C (-) – cultures not treated with any agent.

DISCUSSION AND CONCLUSION

Antisense oligonucleotides (ASO) are short single-stranded DNA molecules complementary to a chosen mRNA sequence. They are used in post-transcriptional gene silencing (PTGS) and act according to two separate mechanisms: direct inhibition of mRNA translation, and through activation of endogenous RNase H responsible for DNA-RNA hybrids degradation. The overall result of ASO activity is the down-regulation of both transcript, and protein (10). That is unlike in the RNAi (RNA interference) mechanism where mRNA pool degradation is virtually the only way of PTGS. Recently it was demonstrated for *C-RAF*, which was one of the target genes in our study, that the degree of RNase H activation or the balance between RNA and protein degradation depends on antisense ODN sequence (11). When antisense ODN with sequence resembling natural miRN125b was used, no reduction in *RAF* mRNA was demonstrated although the oligonucleotide down-regulated *RAF* protein by 50%; on the other hand, anti-*RAF* siRNA resulted in reduction of both *C-RAF-1* transcripts and protein levels by about 70% (11). In this study we present results of the use of antisense oligonucleotides to down-regulate the expression of two genes: *BCL-2* and *C-RAF*. Both genes encode proteins that are crucial for survival and proliferation of cancer cells (12); they are also involved in multidrug resistance phenomenon, however, only *C-RAF* can stimulate expression of P-glycoprotein mRNA (13). Significant differences were evidenced in the response of certain cancer types analyzed in our study. The strongest resistance to antisense ODNs was observed in HeLa cultures while the most promising results were obtained in A549 (lung cancer) cells. This suggests that anti-*BCL-2* or anti-*C-RAF* ASO stimulation is difficult to implement as a 'golden standard' in tumor treatment even at the *in vitro* level. In A549 cell cultures simultaneous *BCL-2* and *C-RAF* antisense oligonucleotides treatment resulted in the greatest decrease in viable cells level at all ASOs concentrations used in the study. This observation suggests that lower concentrations may be used for this type of cancer to obtain high efficiency of etoposide-induced apoptosis. The main effect observed was the demonstration that optimal concentrations of antisense oligonucleotides could be decreased to as little as 100 nM. At that concentrations of both anti-*BCL-2*, and anti-*C-RAF* antisense oligonucleotides, the number of alive cells decreased to the same levels that were observed for much higher concentrations (400 nM – 1,2 μM) of each oligonucleotide separately. This effect was evident both in the etoposide-treated cultures (induced cell death), and in the untreated cultures (spontaneous cell death).

One of the factors that significantly limit the PTGS efficiency is the rate of transfection. *In vitro* efficiency of antisense oligonucleotides action may be enhanced either by elongation of treatment time or through repeated treatment prolonged to 3–4 days in a row (14). Nevertheless, the overall effects are often not satisfactory, and higher ASO concentrations do not necessarily increase their efficiency. What is more, even lipofection itself may induce apoptosis in the sequence-independent manner (15). DNA or RNA delivery inside the cells therefore remains the main obstacle in cancer molecular therapies. In our study, even single treatment with antisense oligonucleotides induced apoptotic cell death in HeLa cultures, as was revealed by morphology analysis, and impaired proliferation as was measured indirectly by histone H3 expression (in our study not only were the H3 mRNA levels decreased after *C-RAF* and *BCL-2* genes silencing, but also it seems that proliferation rate measured in that way preceded other symptoms of cell death). According to these results, and the literature data cell death increase is followed by higher ASO concentrations but this increase may be a result of unspecific toxicity of oligonucleotide DNA and liposome carrier as it was demonstrated by comparison with the effects of the control (*scrambled*) ASO. Comparison of the scrambled sequence-ASO with sequence-specific anti-*BCL-2* or anti-*C-RAF* ASOs indicates that even though very short (20 nucleotides) DNA sequences may perform cytotoxic effect towards cancer cells. Similar observations were made by Zangemeister-Wittke et al., who indicated that sequence-specific cytotoxic effect of anti-*BCL-2* ASO was gained under 600 nM concentrations while higher concentrations did not lead to stronger results (16). Additionally, cancer cells may perform both primary or treatment-induced compensation mechanisms of *BCL-2* and/or other pro-survival genes overexpression when only one target gene is submitted to silencing. Such phenomenon was demonstrated in lung, prostate, ovary or cervical cancers (17). Liu et al. reported that four days of myeloma cell cultures treatment with *BCL-2* ASO alone (5 μg/mL) lead to 50% decrease in *BCL-2* protein level, however, only 13–14% of cells underwent apoptosis (18). The authors conclude that high *BCL-2*-expressing myeloma cells are practically resistant to *BCL-2* ASO treatment, and that *BCL-2* ASO enhances

spontaneous apoptosis mainly in low *BCL-2* expressing cells.

Alternative approach include combined treatment with antisense oligonucleotide and traditionally used chemo- or radiotherapy agents. Antisense oligonucleotides or siRNA can be used for therapeutic purposes both as single agents, and as modulators of traditional anti-cancer chemotherapeutics or radiation therapy. For *BCL-2* or *RAF* such combination strategy was used for etoposide, dexamethasone, paclitaxel, vinorelbine, vincristine, carboplatin, cyclophosphamide and many others (4, 18–20). Those papers indicate that cells overexpressing *BCL-2* are basically resistant to antisense oligonucleotides; however, when ASOs were combined traditional chemotherapeutic drugs significant synergy could be obtained. Our results confirm that pretreatment of the *in vitro* cultures with antisense oligonucleotides lead to enhanced cytotoxicity and apoptosis rate induced by etoposide in comparison to ASO treatment only. Etoposide exerts its cytotoxic and antitumor action primarily by inhibiting DNA topoisomerase II activity thus decreasing cellular replication and provoking DNA damage. Similar combinations of antisense oligonucleotides and chemo- or radiotherapeutic agents were proved to be efficient in irradiated C666-1 nasolaryngeal cancer cells (21) or in mice treated with cisplatin (22) or cyclophosphamide (23); anti-*BCL-2* oligonucleotides also potentiated sensitivity of prostate cancer cells to paclitaxel (24).

Another way of antisense oligonucleotides potentiation achievement is the use of a mixture of ASOs down-regulating genes acting on different cellular processes. Such strategy was used for *BCL-2*, epidermal growth factor receptor, and protein kinase A providing a strong overall anticancer effect both *in vitro*, and in mouse model *in vivo* (25). Similar strategy of *dual targeting* was used by Lang and co-workers, who decreased growth and metastasis of pancreatic cancer cells by NVP-AAL881: small inhibitor of *RAF* and VEGF receptor (26). Those papers prompted us to use a strategy based on simultaneous implementation of two antisense oligonucleotides designed to silence different target genes expression. In summary, the most important finding of our results is the demonstration that our strategy indicate the possibility to use lower concentrations of two antisense oligonucleotides when they are used simultaneously. Elimination of high dose-treatment may lead to better sequence-specific results. This finding was most relevant in repeated transfections providing better results instead of the use of high ASOs doses (leading to increased unspecific toxicity). This strategy performs different efficiencies in certain types of cancer cell lines: the strongest results were obtained in A549 cells representing a model of lung cancer. We also confirmed earlier papers reporting that the optimal application of *BCL-2* and *C-RAF* silencing is the potentiation of apoptosis induced by etoposide or other anticancer agents.

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REFERENCES

1. Niault T.S., Baccarini M.: Carcinogenesis 31, 1165 (2010).
2. Madhunapantula S.V., Robertson G.P.: Cancer Res. 68, 5 (2008).
3. Haferlach C., Dicker F., Kohlmann A. et al.: Leukemia 24, 1065 (2010).
4. Sun H., He H., Zhang S., Liu T., Ren K., He Q., Shao R.: Cancer Gene Ther. 16, 693 (2009).
5. Sasi N., Hwang M., Jaboin J., Csiki I., Lu B.: Mol. Cancer Ther. 8, 1421 (2009).
6. Brunelle J.K., Letai A.: J. Cell Sci. 122, 437 (2009).
7. Tredan O., Galmarini C.M., Patel K., Tannock I.F.: J. Natl. Cancer Inst. 99, 1441 (2007).
8. Bednarek I., Sypniewski D., Solarz J., Machnik G., Gałka S., Loch T.: Wiad. Lek. 61, 4 (2008).
9. Orchel J., Słowiński J., Mazurek U., Wilczok T.: Biochim. Biophys. Acta 1689, 42 (2004).
10. Wu H., Lima W.F., Zhang H., Fan A., Sun H., Crooke S.T.: J. Biol. Chem. 279, 17181 (2004).
11. Hofmann M.H., Heinrich J., Radziwil G., Moelling K.: Mol. Cancer Ther. 7, 1635 (2009).
12. Benvenuti S., Sartore-Bianchi A., Di Nicolantonio F. et al.: Cancer Res. 67, 2643 (2007).
13. Davis J.M., Navolanic P.M., Weinstein-Oppenheimer C.R. et al.: Clin. Cancer Res. 9, 1161 (2003).
14. Guinness M.E., Kenney J.L., Reiss M., Lacy J.: Cancer Res. 60, 5354 (2000).
15. Bednarek I., Czajka M., Wilczok T.: Folia Histochem. Cytobiol. 40, 133 (2002).
16. Zangemeister-Wittke U., Leech S.H., Olie R.A. et al.: Clin. Cancer Res. 6, 2547 (2000).
17. Nahta R., Esteva F.J.: Semin. Oncol. 30, 143 (2003).
18. Liu Q., Gazitt Y.: Blood 101, 4105 (2003).

19. Hu Y.P., Bebb G., Tan S. et al.: Clin. Cancer Res. 10, 7662 (2004).
20. Rudin C.M., Kozloff M., Hoffman P.C. et al.: J. Clin. Oncol. 22, 1110 (2004).
21. Yip K.W., Mocanu J.D., Billie Au P.Y. et al.: Clin. Cancer Res. 11, 8131 (2005).
22. Zupi G., Scarsella M., Semple S.C., Mottolese M., Natali P.G., Leonetti C.: Clin. Cancer Res. 11, 1990 (2005).
23. Klasa R.J., Bally M.B., Ng R., Goldie J.H., Gascoyne R.D., Wong F.M.: Clin. Cancer Res. 6, 2492 (2000).
24. Yamanaka K., Rocchi P., Miyake H., Fazli L., Vessella B., Zangemeister-Wittke U., Gleave M.E.: Mol. Cancer Ther. 4, 1689 (2005).
25. Tortora G., Caputo R., Damiano V. et al.: Clin. Cancer Res. 9, 866 (2003).
26. Lang S.A., Schachtschneider P., Moser C. et al.: Mol. Cancer Ther. 7, 3509 (2008).

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