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REDUCTION IN VIABILITY OF HUMAN CERVIX CARCINOMA HeLa CELLS UNDER TRANSFER OF *p21* GENE WITH BLOCK POLYMERIC DIMETHYLAMINOETHYL METHACRYLATE-BASED CARRIER

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Background. Various cationic polymers have been proposed for use as carriers of genetic materials in biotechnology and biomedicine. The poly(2-dimethylamino) ethyl-methacrylate (poly(DMAEMA)) demonstrated high transfection efficiency and low cytotoxicity when used as a gene delivery system. The aim of the present study was to evaluate the ability of poly(DMAEMA)-*block*-poly(N-vinylpyrrolidone)-*co*-(butyl-acrylate)-*co*-2-aminoethyl methacrylate carrier, BP83-1, to deliver *p21* gene into human cervix carcinoma HeLa cells and to define its effects on the viability of tumor cells *in vitro*.

Methods. Transfection assay, Western-blot analysis, MTT test, DNA comet analysis in alkaline conditions, diphenylamine assay for DNA fragmentation (Barton's assay), FACS analysis of cell cycling.

Results. The BP83-1 polymer effectively transferred pFlag-P21WT plasmid DNA containing *p21* gene into human cervix carcinoma HeLa cells. The level of BP83-1-facilitated delivery of *p21* into HeLa cells was significantly higher than the level achieved with linear polyethyleneimine (PEI). A significant (26.1 % and 40.1 %) reduction in the viability of HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI polyplexes was detected compared to non-transfected cells. The reverse dependence between the elevated amount of p21 and the reduced amount of Cdk2 was observed in the transfected

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HeLa cells. The number of cells in G1 phase of the cell cycle in HeLa cells increased from 54.9 % to 65.8 % and to 64.9 % after their transfection with pFlag-P21WT/BP83-1 and pFlag-P21WT/PEI polyplexes, correspondingly. Besides, an increased number of single-strand breaks in DNA and content of the fragmented DNA was detected in HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI polyplexes. The DNA damaging effects of the BP83-1 carrier and pDNA/BP83-1 polyplex were less pronounced in treated HeLa cells, compared with such effects of PEI and pDNA/PEI polyplex.

Conclusion. An effective transfer of *p21* gene with BP83-1 carrier into human cervix carcinoma HeLa cells was demonstrated. The overexpression of *p21* gene led to inhibition of viability of HeLa cells, DNA damage, and blocking of cell cycle progression from G1 phase to S phase via a reduction of the amount of cyclin-dependent kinase 2 (Cdk2) and accumulation of cells in G1 phase.

***Keywords*:** poly(2-dimethylamino)ethyl-methacrylate, polymeric carrier, gene transfer, p21, cytotoxic action, cell cycle, DNA damage

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INTRODUCTION

The application of non-viral carriers for effective gene delivery offers ways to overcome the drawbacks of viral vectors. Non-viral vectors possess lower general toxicity and less immunogenicity. They have an improved loading capacity and a high tunability of chemical structure (Salameh *et al.*, 2019). Cationic polymers are widely used as gene delivery vectors in biotechnology and biomedicine (Chen *et al.*, 2020, Richter *et al.*, 2021). Recently, various polycationic gene carries as poly(L-lysine) (PLL), poly(amidoamine) (PAMAM), poly(ethylenimine) (PEI), polymethacrylates (e.g. poly(2-dimethylamino)ethyl methacrylate (poly(DMAEMA)) have been developed (Chen *et al.*, 2020; Santana-Armas, & Tros de Ilarduya, 2021). Cationic polymers that form micelles might enhance the stability of polyplex and gene delivery efficiency (Zheng *et al.*, 2018; Li *et al.*, 2016).

Cervical cancer is one of the top gynecologic cancers worldwide and is the fourth leading reason of death among women. Surgery, radiotherapy, chemotherapy, immunotherapeutic approaches are used for their treatment (Hemminki *et al.*, 2022; Cohenet *et al.*, 2019). It was reported that the activation of tumor-promoting genes and/or the inactivation of tumor suppressor genes affected the progression of cervical cancer (Zhu *et al.*, 2020).

Several gene therapy techniques using both viral and non-viral vectors have been developed for treatment of cancer patients. Transfer of tumor suppressor gene(s) is a promising approach in cancer treatment (Ibnat *et al.*, 2019). p21 (p21 Waf1/Cip1) protein functions as a cell cycle inhibitor and anti-proliferative effector in normal cells, and is dysregulated in different types of cancers (Lin *et al.*, 2019). The modulation of p21 can affect cancer susceptibility to chemotherapy, radiation, and even to targeted therapy. It was reported that the inhibition of p21 transcription in cervical cancer resulted in its progression (Zhu *et al.*, 2020) and modulated resistance to radiotherapy (Pedroza-Torres *et al.*, 2018). Thus, changing p21 expression via gene therapy can be used to suppress the tumorigenesis and reduce drug resistance (Shamloo & Usluer, 2019).

Poly(DMAEMA)-based polymers proved to be efficient for gene transfer into mammalian (Cheng *et al.*, 2018; Paiuk *et al.*, 2019; Guler Gokce *et al.*, 2020), yeast (Filyak *et al.*, 2013), and plant (Finiuk *et al.*, 2017; Finiuk *et al.*, 2021b) cells. We have reported that poly(2-dimethylamino)ethyl-methacrylate-*block*-poly(N-vinylpyrrolidone)-*co*-(butyl acrylate)-co-2-aminoethyl methacrylate carriers are effective in genetic transformation of mammalian cells (Guler Gokce *et al.*, 2020) and plant protoplasts (Finiuk *et al.*, 2017). This poly(DMAEMA)-based block copolymer improved the stability of the plasmid DNA (pDNA) by protecting the pDNA from nuclease degradation (Guler Gokce *et al.*, 2020).

Taking into account an important role of p21 in blocking the development and progression of cancer, the poly(DMAEMA)-based block copolymer BP83-1 facilitated transfer of *p21* gene into cervical cancer cells could provide new perspectives in suppression of tumor growth.

MATERIALS AND METHODS

Cell culture. The human cervix adenocarcinoma HeLa cells were kindly provided by Cell Collection at the Institute of Molecular Biology and Genetics. The National Academy of Sciences of Ukraine (Kyiv, Ukraine) received those cells from the American Type Culture Collection (ATCC). Cells were cultivated in the DMEM medium supplemented with 10 % of fetal bovine serum (all were purchased from Biowest, France) in the CO_2 thermostat at 37 °C in the atmosphere of 95 % air and 5 % CO_2 (Finiuk *et al.*, 2020).

Polymeric carrier used for gene delivery. The synthesis of the block polymer BP83-1 (poly (DMAEMA)-*block*-poly(N-vinylpyrrolidone)-*co*-(butyl acrylate)-*co*-2-aminoethyl methacrylate) was carried out via solution polymerization of the N-vinylpyrrolidone (NVP), butyl acrylate (BA) and aminoethyl methacrylate (AEM) (all abovementioned reagents were purchased from Sigma-Aldrich, USA) initiated by the telechelic poly(dimethylaminoethyl methacrylate) (pDMAEMA-MP) oligoperoxide containing peroxide terminal-group from 1-isopropyl-3(4)-[1-(tert-butyl peroxy)-1-methylethyl]benzene (MP), as described previously (Guler Gokce *et al.*, 2020). The colloidal-chemical characteristics of the BP83-1 polymer were reported by (Guler Gokce *et al.*, 2020). The schematic structure of the polymeric carrier BP83-1 is presented in **Fig. 1**, and its characteristics are shown in **Table 1**.

The stock solution of BP83-1 (10 mg/mL) was prepared by dissolving the polymer in 0.3 % HCl; the solution of NaOH (0.05 M) was added dropwise in order to obtain pH 7.2 in the solution.

Fig. 1. Schematic structure of the polymeric carrier BP83-1 (poly(DMAEMA)-*block*-poly (N-vinylpyrrolidone)-*co*-(butyl acrylate)-*co*-2-aminoethyl methacrylate)

Table 1. **Composition, molecular weight and colloidal-chemical characteristics of BP83-1 polymer**

Composition, %				Molar weight, kDa	Characteristics of micelle-like structures				
Block A	Block B				Critical micelle concentration (CMC) $\times 10^3$, mol/L	Surface tension of water solution σ_{CMC}, mN/m	DLS Z-average hydrodynamic diameter, nm	ξ-potential, mV
DMAEMA(*n*)	NVP(*m*)	BA(*k*)	AEM(*l*)					
40.6	44.2	10.4	4.8	11.0±1.2	0.07	29.5	120	+5.1

Isolation of plasmid DNA. The pFlag-P21WT plasmid vector (Clontech, USA) contained an insert of *p21* (*cip1/waf1*) gene (600 bp) and a tag/fusion protein named Flag (N-terminal on backbone) (Zhou *et al.*, 2001). The Pure Link Hi Pure Plasmid Maxiprep Kit (Invitrogen, Germany) was applied for pDNA isolation after transformation of *Escherichia coli* DH5α using selective growth in Ampicillin (Ibnat *et al.*, 2019). The purity and integrity of pDNA were determined by measuring absorption (A260/A280 ratio) using Nano Drop ND-1000 UV/Vis Spectrometer (USA) and electrophoresis in 1 % agarose gel. pDNA solution was stored at -20 °C.

The transfection of cells. The HeLa cells were seeded at cell density of 50,000 cells/mL in a 24-well plate and incubated overnight. The culture medium was changed before transfection. The polymer/pDNA complexes were prepared by mixing 2.5 μL of the polymer (1 mg/mL) with 1 μg of pFlag-P21WT plasmid DNA and 50 μL of serum free DMEM medium. After 20 min of incubation at room temperature, the complexes were added to the cultured cells. 48 h after transfection, the cells were investigated using Western blotting, MTT test, flow cytometric analysis, comet assay in alkaline conditions, Barton's assay.

Measurement of cytotoxicity of free polymers and polyplexes. The toxicity of BP83-1, the linear polyethyleneimine (PEI, 25 kDa, Polysciences Inc., USA), and their complexes with pFlag-P21WT was studied in HeLa cells by using the MTT assay (Sigma-Aldrich, USA). Cells were incubated for 48 h with pFlag-P21WT plasmid DNA (1 μg/mL), free polymers BP83-1 and PEI (2.5 μg/mL), pDNA (1 μg/mL)/ studied polymer (2.5 μg/mL) complexes. 100 μL of MTT solution (0.5 mg/mL in serum-free DMEM medium) was added to each well and incubated for next 2–4 h at 37 °C for formation of purple formazan crystals by mitochondrial dehydrogenases of viable cells. The media was replaced and dimethylsulphoxide (DMSO) (100 μL) was added to dissolve the crystals. The absorbance was measured by an Absorbance Reader BioTek ELx800 (BioTek Instruments, Inc., USA). The level (%) of the viability inhibition was calculated in comparison with the non-treated controls (Finiuk *et al.*, 2022).

Western-blot analysis. 48 h after HeLa cells transfection, cellular proteins were extracted with RIPA lysis buffer containing 10 μg/mL of protease inhibitors cocktail "Complete" (Basel, Roche, Switzerland). Proteins (30 μg/lane) were separated by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane as previously described (Mahmood & Yang, 2012). The primary antibodies against p21 Waf1/Cip1 (12D1) Rabbit mAb 2947, Cdk2 (78B2)

Rabbit mAb 2546 (Cell Signaling Technology, Austria), β-actin monoclonal mouse AC-15 (Sigma-Aldrich, USA) were used. Secondary peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling Technology, Austria) were applied. The enhanced chemiluminescence (ECL) detection reagents (Sigma-Aldrich, USA) were used for protein visualization. Densitometric analysis of protein level was performed using open access ImageJ software (Finiuk *et al.*, 2022).

Flow cytometric analysis of cell cycling. 48 h after transfection of HeLa cells or their treatment with plasmid DNA pFlag-P21WT (1 μg/mL) and applied polymers (2.5 μg/mL), the cells were harvested and washed with ice-cold phosphate buffered saline (PBS), then the cells were fixed via dropwise adding of 4 mL of cooled absolute ethanol by gentle mixing. The samples were stored at -20 °C until use, but no longer than 1 week. For conducting flow cytometric analysis, cell samples were centrifuged, and the cell pellet was re-suspended in 1 mL of PBS. 50 μL of DNAase-free Ribonuclease A (100 μg/mL, Sigma-Aldrich, USA) were added, and samples were incubated at 37 °C for 30 min. Then, each sample was supplemented with 10 μL of the Propidium iodide (1 mg/mL, Sigma-Aldrich, USA) and incubated at room temperature for next 10 min in the dark. Cells were analyzed using FACScan flow cytometer (BD Biosciences, USA) and Summit v3.1 software (Cytomation, Inc., USA) (Kobylinska *et al.*, 2016).

DNA comet assay in alkaline conditions*.* 48 h after HeLa cells transfection or treatment with pFlag-P21WT plasmid DNA (1 μg/mL), the studied polymers (2.5 μg/mL), doxorubicin (Actavis S.R.L., Romania, 1 μg/mL), 25,000 cells (maximum 10 μL) were mixed with 100 μL of 0.5 % low melting agarose (Promega, USA) and transferred to microscopic glass slides covered with 1 % normal molten agarose (Lachema, Czech Republic). The cells were incubated in lysis buffer for 4–6 h at 4 ºC. Electrophoresis was performed at 25 V for 20 min in the electrophoresis buffer (1 mM Na_2EDTA; 300 mM NaOH, pH 13.0) at 4 ºC. The slides were stained with EtBr (10 μg/mL, Sigma-Aldrich, USA). The slides were examined using Zeiss fluorescent microscope with AxioImager A1 camera (Carl Zeiss, Germany) and AxioVision image analysis software Release 4.6.3.0 for Carl Zeiss microscopy (Imaging Associates Ltd., Cork, Ireland, UK). The Casplab-1.2.3b2 software (CASPLab, Poland) was used for calculation of the percentage of tail DNA and Olive Tail Moment as parameters of DNA damage (Liao *et al.*, 2009; Finiuk *et al.*, 2022).

Diphenylamine (Barton's) assay of DNA fragmentation. 48 h after HeLa cells transfection or treatment with plasmid DNA pFlag-P21WT (1 μg/mL), the studied polymers (2.5 μg/mL), doxorubicin (Actavis S.R.L., Romania, 1 μg/mL), the cells were lysed in 0.5 mL Tris-EDTA buffer, pH 7.4, supplemented with 0.2 % Triton X-100 and centrifuged for 10 min at 12,000 g at 4 °C. The supernatant that contained fragmented DNA (labelled as "B") was transferred into a new tube. The tube with sediment which contained intact chromatin was labelled as "A". 0.5 mL of 25 % trichloroacetic acid (Sfera Sim, Ukraine) was added to tubes "A" and "B", mixed and incubated for 1 h at 56 °C. The samples were centrifuged for 10 min at 14,000 g at 4 °C. 1 mL of freshly prepared 1.5 % diphenylamine reagent (Sigma Aldrich, USA) was added to pellets "A" and "B" and incubated overnight at 37 °C. The optical density (OD) of the solution was measured at 630 nm using Absorbance Reader BioTek ELx800 (BioTek Instruments, USA). The percentage of DNA fragmentation was calculated as {OD tube B / (OD tube A + OD tube B) × 100%} (Arora & Tandon, 2015; Finiuk *et al.*, 2021a).

Statistical analysis. All data are presented as a Mean (M) ± Standard deviation (SD) from three replications. Statistical analysis was performed using one-way ANOVA test at GraphPad Prism 8.0.1 software (RRID:SCR_002798). P value of ≤ 0.05 was considered as statistically significant (Finiuk *et al.*, 2020).

RESULTS AND DISCUSSION

Recently, we reported that cationic block polymer poly(DMAEMA)-*block*-poly(NVP-*co*-BA-*co*-AEM) (BP83-1) formed micelles and complexes with the plasmid DNA (Guler Gokce *et al.*, 2020). Such complexes possessed higher stability against DNAse I degradation. The BP83-1 effectively delivered pEGFP c-1 plasmid DNA that encodes green fluorescent protein (GFP) into cells of HEK293T, MCF-7, and HeLa lines. It possessed low toxicity towards targeted mammalian cells (Guler Gokce *et al.*, 2020).

The transfer of tumor suppressor gene(s) was shown to suppress tumorigenesis (Ibnat *et al.*, 2019). Several cancer cells, including the cervical carcinoma, have an abnormal p21 expression. p21 gene plays an important role in controlling cell cycling and proliferation (Shamloo & Usluer, 2019). It can be speculated that transfection of human cervical carcinoma HeLa cells with *p21* gene may lead to the inhibition of the viability of these cells.

We used poly(DMAEMA) BP83-1 carrier for transferring of *p21* gene present in plasmid DNA pFlag-P21WT into human cervical carcinoma HeLa cells and examined its effect on the viability of these tumor cells (**Fig. 2**). For comparison, we used linear PEI, a commercial cationic carrier. BP83-1 at final 2.5 μg/mL concentration was found to be the most effective in transfecting HeLa cells. 48 h after transfection of HeLa cells with polyplexes, a statistically significant increase in p21 Waf1/Cip1 content was detected in the transfected cells compared to the intact (non-transfected) cells. The level of p21 in HeLa cells transfected with polyplexes pDNA/BP83-1 was significantly higher compared with cells transfected with pDNA/PEI. More than a 2-fold elevation of p21 level was found in HeLa cells transfected with pDNA/BP83-1 polyplexes, and a 1.25-1.38-fold elevation of p21 amount in HeLa cells transfected with pDNA/PEI polyplexes was detected.

Liu *et al*. (2003) reported about efficient application of GE7-poly-l-Lysine/DNA/HA20-poly-l-Lysine polyplex for transferring p21WAF1 and murine cytokine gene of granulocyte–macrophage colony-stimulating factor (GM-CSF) into subcutaneously transplanted hepatoma Hepa cells. Here we demonstrated that poly(DMAEMA)-containing polymer BP83-1 can be effectively used for *p21* gene transferring into HeLa cells.

It was reported that the inhibition of p21 transcription in cervical cancer resulted in its progression (Zhu *et al.*, 2020). We investigated the effect of *p21* overexpression on the viability of HeLa cells. It should be noted that BP83-1 at 2.5 μg/mL did not cause an inhibition of growth of HeLa cells (**Fig. 3**). There were 90.1 % of viable HeLa cells found under BP83-1 treatment. PEI at 2.5 μg/mL significantly (by 26.85 %) inhibited growth of HeLa cells. A reduction of the viability of HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI polyplexes was found (**Fig. 3**). Transfection of HeLa cells with pDNA/BP83-1 reduced cell viability to 73.86 %. HeLa cells transfected with pDNA/PEI exhibited 59.94 % of cell viability, compared to control (non-transfected) cells. 16.24 % reduction in viability of HeLa cells transfected with pDNA/BP83-1 was found compared to the effect of free polymer BP83-1, and 13.2 % reduction of viability of these cells transfected

with pDNA/PEI compared to the effect of free PEI (**Fig. 3**). Thus, overexpression of *p21* gene induced a significant inhibition of viability of HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI.

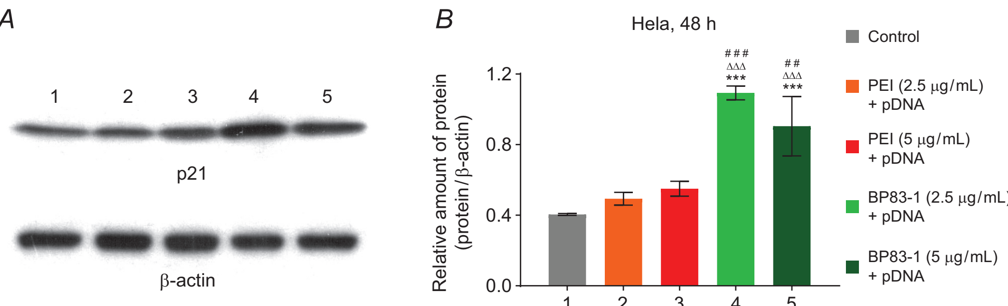

Fig. 2. Results of Western-blot analysis (***A***) of p21 Waf1/Cip1 protein in human cervix adenocarcinoma HeLa cells 48 h after transfection with BP83-1 and linear polyethylenimine (PEI) and pFlag-P21WT (pDNA, 1 μg/mL): 1 – control (non-transfected) cells, 2 – cells transfected with polyplex pDNA pFlag-P21WT/PEI (2.5 μg/mL); 3 – transfected with polyplex pDNA pFlag-P21WT/PEI (5 μg/mL); 4 – transfected with polyplex pDNA pFlag-P21WT / BP83-1 (2.5 μg/mL); 5 – transfected with polyplex pDNA pFlag-P21WT / BP83-1 (5 μg/mL). ***B*** – Densitometric analysis of Western blotting results. *** – $P \le 0.001$ compared to control (non-transfected) cells; $^{\Delta\Delta\Delta}$ – $P \le 0.001$ compared to cells transfected with polyplex pDNA pFlag-P21WT / PEI (2.5 μg/mL); $^{\#\#}$ – $P \le 0.01$ and $^{\#\#\#}$ – $P \le 0.001$ compared to cells transfected with polyplex pDNA pFlag-P21WT / PEI (5 μg/mL)

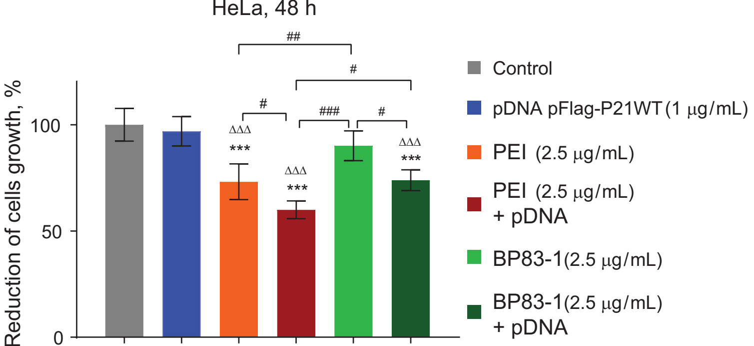

Fig. 3. The viability of human cervix adenocarcinoma HeLa cells after transfection with pFlag-P21WT (pDNA), polymers BP83-1 and PEI, polyplexes pDNA/BP83-1 and pDNA/PEI. The number of cells in the control was equated to 100 %. Results of MTT assay are presented as M±SD. *** – $P \le 0.001$ compared to control (non-transfected) cells; $^{\Delta\Delta\Delta}$ – $P \le 0.001$ compared to cells treated with plasmid DNA pFlag-P21WT; $^{\#}$ – $P \le 0.05$, $^{\#\#}$ – $P \le 0.01$, and $^{\#\#\#}$ – $P \le 0.001$

These results are in agreement with literature data. The transfer of the gene coding for p21-p27 fusion protein into MCF-7 cells resulted in suppression of their viability and induction of apoptosis (García-Fernández *et al*., 2011). A suppression of growth of Hepa cells was found after their transduction with GE7/p21 WAF1 and a combination of GE7/p21WAF1 and GM-CSF using adenovirus vector (Liu *et al*., 2003). Transfer of *p21* gene into MDA-MB-231, MCF-7, and 4T1 cells using inorganic nanoparticles of carbonate apatite was accompanied by a statistically significant decrease in growth of those cells (Ibnat *et al*., 2019).

p21 inhibits cell proliferation using two mechanisms: 1) via binding to cyclin-dependent kinase 2 (Cdk 2) or 2) via binding/inhibition the proliferating cell nuclear antigen, which is a secondary protein in DNA polymerase needed for DNA synthesis and nucleotide excretion and repair (Khanna & Hosenpud, 2000).

To elucidate the mechanisms involved in suppression of viability of HeLa cells after transfer of *p21* gene, the amount of Cdk2 and cell cycle progression were studied. Cdk2 is known to regulate the cell cycle. p21 was shown to inhibit cyclin B/Cdk1, cyclin E/Cdk2, and cyclin D/Cdk4,6 complexes and, thus, it inhibited cell cycle progression during G1 and S phases (Al Bitar & Gali-Muhtasib, 2019). We found that the amount of Cdk2 was decreased in HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI (**Fig. 4**).

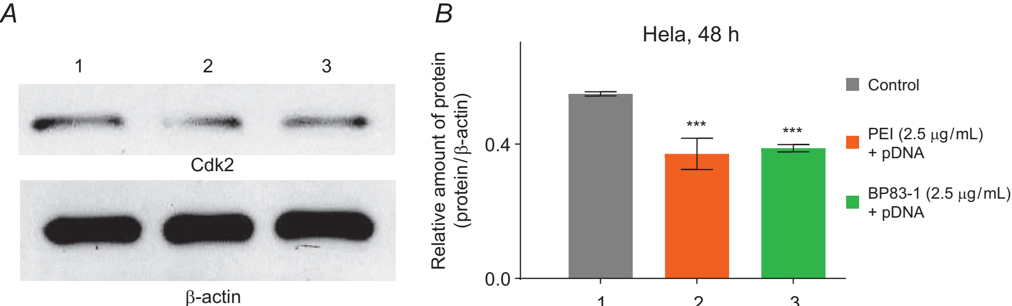

Fig. 4. Results of Western-blot analysis (***A***) of Cdk2 protein in human cervix adenocarcinoma HeLa cells 48 h after transfection with BP83-1 and linear polyethylenimine (PEI) and pDNA pFlag-P21WT: 1 – control (non-transfected) cells; 2 – cells transfected with polyplex pDNA pFlag-P21WT (1 μg/mL)/PEI (2.5 μg/mL); 3 –transfected with polyplex pDNA pFlag-P21WT (1 μg/mL)/BP83-1 (2.5 μg/mL). ***B*** – Densitometric analysis of Western blotting results. *** – P ≤ 0.001 compared to control (non-transfected) cells

The analysis of the cell cycle in the transfected HeLa cells was performed by flow cytometry. A significant increase (from 54.9 ± 3.5 % to 65.8 ± 5.0 %) in the number of cells in G1 phase was found after cell transfection with pFlag-P21WT/BP83-1 polyplex (**Table 2**). There was also an increase (to 64.9 ± 6.4 %) in the number of cells in G1 phase after their transfection with pFlag-P21WT/PEI. These results suggest that a decrease in Cdk2 protein and accumulation of cells in G1 phase in the HeLa cells transfected with pFlag-P21WT/BP83-1 polyplex might cause inhibition of cell cycle progression from G1 to S phase.

Table 2. **The distribution of HeLa cells by phases of the cell cycle (according to flow cytometry of cells in 48 h after their transfection)**

Experimental groups	G1, %	S, %	G2, %
Control (non-transfected) cells	54.9 ± 3.5	25.5 ± 3.9	19.6 ± 1.0
Plasmid DNA pFlag-P21WT (1 μg/mL)	52.3 ± 5.7	27.9 ± 3.4	19.2 ± 0.9
PEI (2.5 μg/mL)	55.6 ± 7.3	25.0 ± 3.2	19.4 ± 2.0
PEI (2.5 μg/mL) + pFlag-P21WT	64.9 ± 6.4*	19.7 ± 2.8	15.4 ± 2.1
BP83-1 (2.5 μg/mL)	53.5 ± 7.0	24.8 ± 3.4	21.7 ± 2.0
BP83-1 (2.5 μg/mL) + pFlag-P21WT	65.8 ± 5.0**	18.6 ± 2.3	15.6 ± 1.3

Comment: Data are presented as M±SD. * – P ≤ 0.05; ** – P ≤ 0.01 compared to control (non-transfected) cells

Jiang *et al.* (2013) reported that overexpression of p21Waf1 and p27Kip1 increased the number of cells in G1 phase and reduced the number of cells in S phase, thus, inhibiting cell cycle progression during the G1/S transition period (Jiang *et al.*, 2013).

It was reasonable to find out if overexpression of *p21* leads to DNA damage in the transfected HeLa cells. Minimum DNA damage was found in non-transfected cells and in cells treated with plasmid DNA: the amount (%) of DNA in the comet tail was 2.24 % and 2.50 %, respectively, and the OTM was 0.41 % and 0.42 %, respectively (**Fig. 5, 6**). BP83-1 (2.5 μg/mL) caused the appearance of 2.57 % of the mean DNA in the comet tail and the OTM = 0.44, whereas the effect of the polyplex pDNA/BP83-1 was 3.91 % of the DNA in the comet tail and OTM = 0.60. At the same concentration (2.5 μg/mL), PEI induced a significantly higher DNA damage of HeLa cells: 9.45 % of the mean DNA in the comet tail and OTM = 1.78. The polyplex pDNA/PEI induced the appearance of 10.78 % of the DNA in the comet tail and OTM = 2.09.

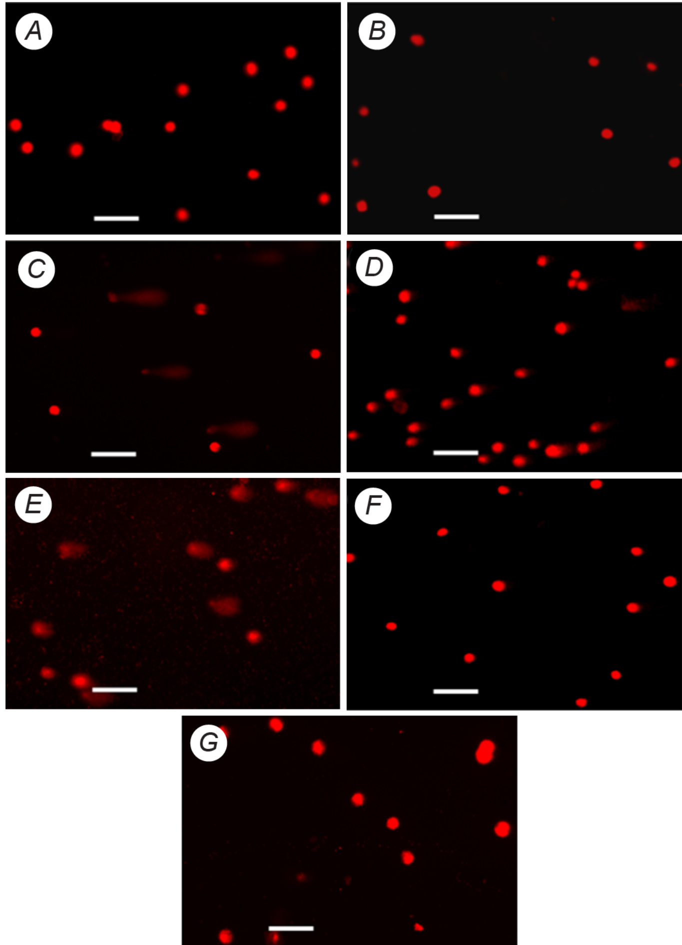

Fig. 5. DNA comets in HeLa cells transfected with pFlag-P21WT, BP83-1, PEI, polyplexes pDNA/BP83-1 and pDNA/PEI, and doxorubicin (48 h): ***A*** – control; ***B*** – pDNA pFlag-P21WT (1 μg/mL); ***C*** – doxorubicin (1 μg/mL); ***D*** – PEI (2.5 μg/mL); ***E*** – PEI (2.5 μg/mL) + pDNA; ***F*** – BP83-1 (2.5 μg/mL); ***G*** – BP83-1 (2.5 μg/mL) + pDNA. Bars are equal 50 μm

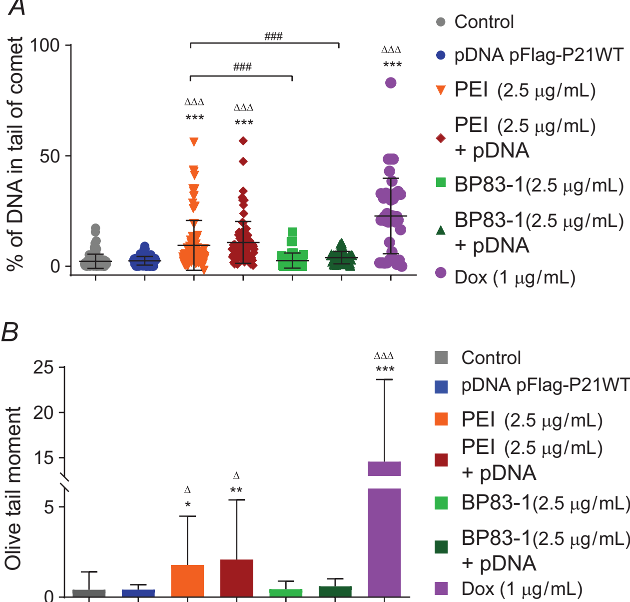

Fig. 6. Quantitative data of DNA damage (DNA comet analysis) in HeLa cells 48 h after transfection with pFlag-P21WT (1 μg/mL), BP83-1 (2.5 μg/mL), PEI (2.5 μg/mL), polyplexes pDNA/BP83-1 and pDNA/PEI, and doxorubicin (Dox): ***A*** – the percentage of DNA in the tail of comet; ***B*** – Olive Tail Moment. * – P ≤ 0.05; ** – P ≤ 0.01; *** – P ≤ 0.001 (significant changes compared with control (non-transfected) cells); $^{\Delta}$ – P ≤ 0.05; $^{\Delta\Delta\Delta}$ – P ≤ 0.001 compared to cells treated with plasmid DNA pFlag-P21WT; $^{\#\#\#}$ – P ≤ 0.001 compared to cells treated with PEI

An increased amount of the fragmented DNA was detected in HeLa cells transfected with polyplexes pDNA/BP83-1 and pDNA/PEI (**Fig. 7**). Free PEI also induced DNA fragmentation in the treated HeLa cells. However, free BP83-1 did not induce DNA fragmentation in the treated HeLa cells. Dox (positive control for DNA damage) caused a significant increase in the fragmented DNA of HeLa cells (**Fig. 7**).

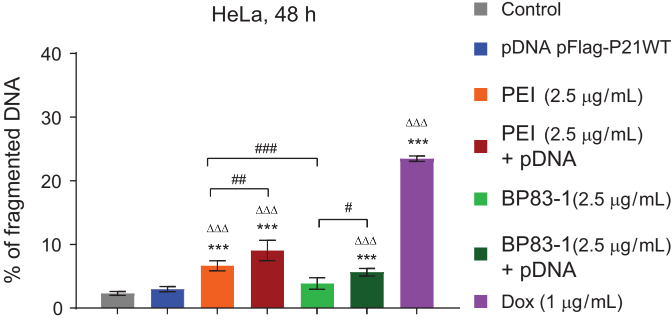

Fig. 7. The amount (%) of fragmented DNA in HeLa cells after their transfection with pFlag-P21WT (1 μg/mL), BP83-1 (2.5 μg/mL), PEI (2.5 μg/mL), polyplexes pDNA/BP83-1 and pDNA/PEI, and doxorubicin (Dox). Barton's method with diphenylamine was used for quantitative identification of DNA fragmentation. *** – P ≤ 0.001 (significant changes compared with control (non-transfected) cells); $^{\Delta\Delta\Delta}$ – P ≤ 0.001 compared to cells treated with plasmid DNA pFlag-P21WT; $^{\#}$ – P ≤ 0.05; $^{\#\#}$ – P ≤ 0.01; $^{\#\#\#}$ – P ≤ 0.001

Thus, we have found that BP83-1 polymer is effective in transfer of *p21* gene into HeLa cells and such transfer significantly inhibited the viability of these cells. This effect might be associated with the accumulation of the treated cells in G1 phase of cell cycle and induction of DNA damage (single strand breaks and DNA fragmentation). DNA damage induced by BP83-1 and polyplex pDNA/BP83-1 in the transfected HeLa cells was less pronounced compared with such effect of PEI and pDNA/PEI polyplex.

CONCLUSION

We synthesized BP83-1 polymer that effectively transferred plasmid DNA pFlag-P21WT into human cervical carcinoma HeLa cells. In the cells transfected with polyplex pDNA/BP83-1, a significantly higher level of p21 protein (inhibitor of cell cycle and regulator of cells proliferation) was detected compared with their transfection with pDNA/PEI. A reduction in the viability of HeLa cells transfected with pDNA/BP83-1 and pDNA/PEI polyplexes was found. The reverse dependence between an elevated amount of p21 protein and a reduced level of Cdk2 protein was detected. A decrease in Cdk2 and accumulation of cells in G1 phase of the cell cycle in HeLa cells transfected with *p21* gene resulted in the inhibition of cell cycle progression from G1 phase to S phase. The overexpression of *p21* in HeLa cells led to DNA damage (single-strand breaks and DNA fragmentation). The DNA damaging effects of BP83-1 and polyplex pDNA/BP83-1 were less pronounced in the treated HeLa cells compared with such effects of PEI and pDNA/PEI polyplex. Thus, the presented data suggest that poly(DMAEMA)-based polymer BP83-1 might be a promising carrier of genes of interest into mammalian cells for suppression of tumor cell growth.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: This article does not contain any studies with human subjects performed by any of the authors.

Animal studies: All international, national and institutional guidelines for the care and use of laboratory animals were followed.

AUTHOR CONTRIBUTIONS

Conceptualization, [R.S.S.; N.S.F.; A.S.Z.]; methodology, [N.S.F.; N.E.M.]; compound synthesis and characterization, [N.E.M.; A.S.Z.]; investigation, [N.S.F.; O.Y.K.; I.Y.K.; N.E.M.]; data analysis, [N.S.F.; N.E.M.; R.S.S]; writing – original draft preparation, [N.F.]; writing – review and editing, [N.S.F.; R.S.S.; O.Y.K.; N.E.M.; A.S.Z.]; visualization, [N.S.F.; O.Y.K.]; supervision, [R.S.S.; A.S.Z.]; funding acquisition, [R.S.S.; N.S.F.].

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ПРИГНІЧЕННЯ ЖИТТЄЗДАТНОСТІ КЛІТИН HeLa КАРЦИНОМИ ШИЙКИ МАТКИ ЛЮДИНИ ЗА ТРАНСФЕКЦІЇ ГЕНА *p21* ЗА ДОПОМОГОЮ ДИМЕТИЛАМІНОЕТИЛМЕТАКРИЛАТ-ВМІСНОГО НОСІЯ З БЛОЧНОЮ СТРУКТУРОЮ

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Вступ. Катіонні полімери широко використовують як носії генів у біотехнології та біомедицині. Продемонстровано високу ефективність трансфекції клітин і низьку цитотоксичність систем доставки генів на основі полі(2-диметиламіно)етилметакрилату (полі(DMAEMA)).

Мета роботи – визначити можливості використання полімеру полі(DMAEMA)-*блок*-полі(N-вінілпіролідон)-*ко*-(бутилакрилат)-*ко*-2-аміноетилметакрилату (BP83-1) блочної будови для доставки гена *p21* в клітини лінії HeLa карциноми шийки матки людини, а також дослідити його вплив на життєздатність пухлинних клітин.

Методи: трансфекція клітин, Вестерн-блот аналіз, МТТ-тест, ДНК-комет аналіз за лужних умов, метод Бартона з використанням барвника дифеніламіну для виявлення фрагментації ДНК, проточна цитофлуориметрія для дослідження клітинного циклу.

Результати. Полімер BP83-1 ефективно переносив ДНК плазміди pFlag-P21WT, що кодує ген *p21*, у клітини HeLa карциноми шийки матки людини. Рівень трансфекції гена *p21* в клітини HeLa за використання BP83-1 був значно вищим, ніж такий рівень, що досягався за допомогою лінійного поліетиленіміну (PEI). Встановлено пригнічення життєздатності клітин лінії HeLa, трансфікованих *p21*-вмісними поліплексами pDNA/BP83-1 і pDNA/PEI на 26,1 % та 40,1 %, відповідно. Виявлено зворотну залежність між підвищеним вмістом білка p21 та зниженим вмістом циклін-залежної кінази 2 (Cdk2) у трансфікованих клітинах лінії HeLa. Встановлено зростання кількості клітин лінії HeLa у фазі G1 клітинного циклу від 54,9 % до 65,8 % та 64,9 % після їх трансфекції поліплексами pFlag-P21WT/BP83-1 та pFlag-P21WT/PEI, відповідно. Виявлено підвищену кількість одноланцюгових розривів ДНК та вміст фрагментованої ДНК у клітинах HeLa, трансфікованих поліплексами pDNA/BP83-1 та pDNA/PEI. Вільний носій BP83-1 і поліплекс pDNA/BP83-1 проявляли менш виражений ДНК-пошкоджувальний ефект щодо клітин лінії HeLa, ніж PEI та поліплекс pDNA/PEI.

Висновок. Продемонстровано ефективний перенос гена *p21* в клітини лінії HeLa карциноми шийки матки людини за допомогою полімерного носія BP83-1.

Надекспресія гена *p21* індукувала пошкодження ДНК та інгібування прогресу клітинного циклу з фази G1 до фази S через зменшення кількості білка Cdk2 і накопичення клітин у фазі G1 клітинного циклу.

Ключові слова: полі(2-диметиламіно)етил-метакрилат, полімерний носій, введення генів, p21, цитотоксична дія, клітинний цикл, пошкодження ДНК

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