Research article

VIOLOGEN-PHOSPHORUS DENDRIMERS EXHIBIT MINOR TOXICITY AGAINST A MURINE NEUROBLASTOMA CELL LINE

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Abstract: Dendrimers containing viologen (derivatives of 4,4'-bipyridyl) units in their structure have been demonstrated to exhibit antiviral activity against human immunodeficiency virus (HIV-1). It has also recently been revealed that novel dendrimers with both viologen units and phosphorus groups in their structure show different antimicrobial, cytotoxic and hemotoxic properties, and have the ability to influence the activity of cholinesterases and to inhibit α-synuclein fibrillation. Since the influence of viologen-phosphorus structures on basic cellular processes had not been investigated, we examined the impact of such macromolecules on the murine neuroblastoma cell line (N2a). We selected three water-soluble viologen-phosphorus (VPD) dendrimers, which differ in their core structure, number of viologen units and number and type of surface groups, and analyzed several aspects of the cellular response. These included cell viability, generation of reactive oxygen species (ROS), alterations in

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mitochondrial activity, morphological modifications, and the induction of apoptosis and necrosis. The MTT assay results suggest that all of the tested dendrimers are only slightly cytotoxic. Although some changes in ROS formation and mitochondrial function were detected, the three compounds did not induce apoptosis or necrosis. In light of these results, we can assume that the tested VPD are relatively safe for mouse neuroblastoma cells. Although more research on their safety is needed, VPD seem to be promising nanoparticles for further biomedical investigation.

Key words: Apoptosis, Cytotoxicity, N2a cell line, ROS, Viologen-phosphorus dendrimers

INTRODUCTION

The highly branched polymers called dendrimers have several unique features, including monodispersity, multivalency, and precisely determined mass, size, shape and architecture [1, 2]. These special characteristics make them an attractive object of research in terms of their interactions with biological systems. Many already are or may soon become useful tools with applications in various fields of biomedical science. Dendrimers could play a role as efficient drug, nucleic acid and contrast agent carriers [3], or they may be used as anti-pathogenic compounds [4-6]. *In vitro* studies have revealed that these nanoparticles possess the ability to inhibit the fibrillation and aggregation of proteins involved in neurodegenerative disorders [7-9]. Importantly, *in vivo* studies of polyamidoamine (PAMAM) dendrimers have been performed to determine their toxicity to the central nervous system [10], check their ability to localize in inflammatory cells in the brain [11], and show their potential in dendrimer-drug conjugates for the treatment of neuroinflammation [12, 13].
Significant progress has been made in research on the interactions between dendrimers and living cells or organisms, resulting in commercially available products such as transfection agents (SuperFect from Qiagen and PrioFect from EMD-Merck), markers for rapid heart attack diagnosis (Stratus CS Acute Care from Siemens Healthcare), and anthrax-detecting agents (Alert Ticket from the US Army Research Laboratory) [3]. However, there are still many unknowns regarding the influence of these nano-objects on cellular processes and organism functions.
There are several main families of dendrimers, but new types of dendritic structures are constantly being synthesized. One novel class of dendritic compounds is viologen-phosphorus dendrimers (VPD). Phosphorus-containing dendrimers [14-16] are considered to be particularly important for biomedical research because phosphorus is essential for all terrestrial forms of life. The biological properties of these compounds have been recently reviewed in detail, including their positive effect on the growth of neuronal cells, monocytes and natural killer cells, their anti-prion properties, their use as delivery platforms for ocular drugs and transfection and imaging agents, and their potential as highly sensitive biosensors [17]. Viologen derivatives (4,4'-bipyridinium salts) were

commonly used as non-selective herbicides (e.g. Paraquat) [18], but they can also cause severe or lethal poisoning in humans [19]. Interestingly, there are growing data indicating the association between chronic Paraquat exposure and Parkinson's disease [20-22]. The mechanism of viologen toxicity involves the induction of the generation of superoxide anions and other ROS, which leads to cell and tissue damage [23, 24].

Recent studies have shown that incorporating viologen units into the dendritic backbone can yield new positive biological properties. Several polycationic viologen-based dendrimers were tested for antiviral activity against human immunodeficiency virus (HIV-1), herpes simplex virus (HSV), vesicular stomatitis, Punta Toro virus, Sindbis virus, reovirus, and respiratory syncytial viruses and were shown to be good inhibitors of HIV-1 [25]. Ciepluch et al. [26] studied eight novel dendrimers with both phosphorus groups and viologen units in their structure. These VPD differed in the core structure (tri- or hexafunctionalized), generation (G0 or G1), number of viologen units, and the number and type of surface groups, which are aldehydes, phosphonates or polyethyleneglycols (PEG).

The cytotoxicities, hemotoxicities, and antimicrobial and antifungal activities of these dendrimers were examined and it was shown that the macromolecules with the highest number of viologen units (positive charges) and higher generations were more hemolytic. Moreover, all of the tested structures exhibited relatively high toxicity to the N2a cell line but were less toxic to the B14 cell line. All of the dendrimers also showed good antimicrobial activity against the Gram-positive bacteria *Staphylococcus aureus*. These compounds also demonstrated potential against neurodegenerative disorders. They influenced the activity of acetylcholinesterase and butyrylcholinesterase, which play a role in Alzheimer's disease [27]. VPD can also interact with α-synuclein, which is involved in Parkinson's disease, and inhibit its fibrillation [28, 29].

Other than the results of simple cytotoxicity assays, there have been no reports on the influence of viologen-phosphorus structures on the cell condition and basic functions. This inclined us to continue studies on VPD cytotoxicity and examine the impact on murine neuroblastoma cells (N2a) of three of the eight studied by Ciepluch et al. [26]. The tested compounds are water-soluble, zero generation dendrimers, which have either a trifunctionalized (VPD3) or hexafunctionalized (VPD1 and VPD2) core, three (VPD3) or six (VPD1 and VPD2) viologen units with Br⁻ as a counteranion, and phosphonates (VPD1 and VPD3) or PEG (VPD2) as surface groups (Fig. 1).

Since dendrimers are extensively used in anticancer research [30, 31] and analyzed for their potential to prevent neurodegenerative disorders [8, 9], we used a cancerous neuronal cell line (N2a) for our studies. We investigated several aspects of the cellular response, including cell viability, generation of reactive oxygen species (ROS), alterations in mitochondrial function, morphological modifications, and the induction of apoptosis and necrosis.

Compound	MW (Da)	Structure
VPD1	3789.30	
VPD2	14421.60	
VPD3	1890.13	
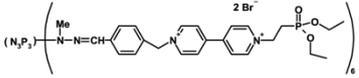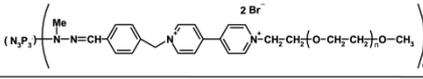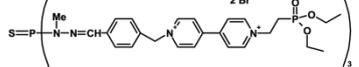
Fig. 1. The structure and molar weight of the studied viologen-phosphorus dendrimers.

MATERIALS AND METHODS

Chemicals

Viologen-phosphorus dendrimers were synthesized by the Laboratoire de Chimie de Coordination du CNRS. 3-[4,5-2-yl]-2-5-diphenyltetrazolium bromide (MTT), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), 2,7-dichlorodihydrofluorescin diacetate (H_2DCFDA), acridine orange (OA), ethidium bromide (EB), camptothecin (CPT), propidium iodide (PI), pentachlorophenol (PCP), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), carbonyl cyanide 3-chlorophenylhydrazone (CCCP), buffered saline (PBS) tablets, fetal bovine serum and trypsin were purchased from Sigma-Aldrich (USA). MitoTracker Orange (CM-H_2TMRos), YO-PRO-1 iodide and actinomycin D were purchased from Molecular Probes (USA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (USA). 4th generation PPI dendrimers were obtained from Symo-Chem. All other reagents and solvents were of analytical grade.

Cell culture

The mouse neuroblastoma cell line (N2a) was purchased from the American Type Culture Collection ATCC (CCL-131). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and maintained at 37ºC in an atmosphere of 5% CO_2. Cells were split for subculture every 2 to 3 days.

Dendrimer treatment

Dendrimer solutions were prepared using 10 mM PBS, pH 7.4. Cells were treated with five concentrations of a given dendrimer chosen based on previous results [26] (1, 2.5, 5, 10 and 20 μM) and incubated for 24 h in growing conditions prior to further experimentation.

Cytotoxicity assay

The cytotoxicity of viologen-phosphorus dendrimers was evaluated using the MTT assay. The assay is based on the reduction of MTT by cellular reductases

of viable cells to a blue formazan product, the absorbance of which can be measured spectrophotometrically after solubilization [32]. The assay was performed as previously described [33]. Cells were seeded in 96-well microplates at a density of 1.5×10^4 cells/well in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment, 0.5 mg/ml MTT was added to each well and incubated for 3 h. After this time, the MTT solution was discarded, DMSO was added to each well to dissolve the formazan crystals and the absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek).

Measurement of reactive oxygen species
Changes in the levels of reactive oxygen species (ROS) were measured using a fluorescent probe H_2DCFDA. H_2DCFDA does not fluoresce until it enters the cell, where the acetate groups are removed by intracellular esterases to form H_2DCF. H_2DCF is then oxidized to fluorescent DCF [34]. Cells were seeded in 12-well plates at a density of 25×10^4 cells/well in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment, the supernatant was discarded, and the cells were washed with PBS and stained with 2.5 μM H_2DCFDA for 15 min in growing conditions. After staining, the dye solution was removed, and the cells were washed with PBS, collected by trypsynization and analyzed using flow cytometry (LSRII, Becton Dickinson) through the FL1 channel. Cells treated with H_2O_2 were used as a positive control. The data were recorded for a total of 10,000 events per sample.

Assessment of oxidative activity of mitochondria
Mitochondrial activity was assessed using the reduced MitoTracker Orange (CM-H_2TMRos) fluorescent probe. CM-H_2TMRos is oxidized inside the cell to the fluorescent CMTMRos and then selectively sequestered in the mitochondria [35]. Cells were seeded in 12-well plates at a density of 25×10^4 cells/well in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment, the supernatant was discarded, and the cells were washed with PBS and stained with 500 nM CM-H_2TMRos for 30 min in growing conditions. After staining, the dye solution was removed, and the cells were washed with PBS, collected by trypsynization and analyzed by flow cytometry (LSRII, Becton Dickinson) through the FL2 channel. Cells treated with G4 PPI were used as a positive control, which was established experimentally. The data were recorded for a total of 10,000 events per sample.

Assessment of mitochondrial membrane potential (ΔΨm)
Mitochondrial membrane potential (ΔΨm) was determined using the fluorescent dye JC-1. JC-1 is a lipophilic cationic dye that accumulates in the mitochondria, where at higher concentrations, it forms J-aggregates, which exhibit red fluorescence (λex = 530 nm, λem = 590 nm). When the mitochondrial membrane is depolarized, the dye does not form J-aggregates and exists in the form of monomers, which exhibit green fluorescence (λex = 485 nm, λem = 538). The loss

of ΔΨm can be indicated by a decrease in the red-to-green fluorescence intensity ratio [36]. Cells were seeded in black 96-well microplates at a density of 1.5×10^4 cells/well in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment the supernatant was removed, and JC-1 at a concentration 5 μM was added to each well and incubated for 20 min in growing conditions. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as a positive control. Fluorescence was measured using a fluorescence microplate reader (Fluoroscan Ascent FL).

Determination of apoptosis and necrosis by acridine orange/ethidium bromide double staining – fluorescence microscopy analyses
Acridine orange/ethidium bromide (AO/EB) double staining was performed according to Ribble *et al.* [37]. AO and EB are DNA-binding fluorescent dyes. The differential uptake of these dyes by cells allows the distinction of viable cells from apoptotic and necrotic ones. AO enters all cells and stains the nucleus green. EB only permeates cells with a damaged cell membrane and stains the nucleus orange or red. Separate fractions of cells were identified as follows:
- Viable cells – morphologically normal, green nucleus;
- Early apoptotic cells – green nucleus with condensed or fragmented chromatin;
- Late apoptotic cells – condensed or fragmented orange/red chromatin;
- Necrotic cells – morphologically normal orange/red nucleus.

Cells were seeded in 96-well microplates at a density of 1.5×10^4 cells/well in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment, OA and EB were added to each well at a concentration of 2 μg/ml and cells were visualized under a fluorescent microscope (Olympus IX 70). Five hundred randomly chosen cells were counted in each experiment. Positive controls were prepared: cells treated with camptothecin (80 μM) for 4 h constituted a control for apoptosis and cells treated for 1 h with pentachlorophenol (600 ppm) constituted a control for necrosis (data not shown).

For fluorescent microscopy images, the cells were seeded in 8-chamber glass slides at a density of 1.5×10^4 cells/chamber in DMEM medium and incubated for 20 h. After 24 h dendrimer treatment, OA and EB were added to each well at a concentration 2 μg/ml and cells were visualized using a fluorescence/phase contrast Optiphot-2 microscope (Nikon) at a magnification of 400x. The microscope is equipped with CCD camera (Nikon, DXM1200). The software used is Nikon ACT-1 version 2.20.

Determination of apoptosis and necrosis by YO-PRO-1 iodide/propidium iodide staining – flow cytometry analyses
YO-PRO-1 is a dye that can enter apoptotic cells, whereas propidium iodide (PI) cannot. Apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence [38]. Cells were seeded in 12-well plates at a density of 25×10^4 cells/well in DMEM medium

and incubated for 20 h. After 24 h dendrimer treatment, the cells were trypsynized and collected, and YO-PRO-1 (0.1 μM) and PI (1.5 μM) were added to each sample and incubated for 20 min on ice. Then the samples were analyzed by flow cytometry (LSRII, Becton Dickinson) with excitation at 488 nm to visualise the YO-PRO-1 green fluorescence (530/30 bandpass filter) and PI red fluorescence (610/20 bandpass filter). The data were recorded for a total of 10,000 events per sample. Single-stained compensation controls were prepared: cells treated with camptothecin (80 μM) for 4 h for positive green fluorescence and cells treated for 1 h with pentachlorophenol (600 ppm) for positive red fluorescence. Actinomycin D was used as a double-stained positive control for apoptosis (data not shown).

Cell morphology analyses
Cell morphology was assessed using a fluorescence/phase contrast microscope (Nikon) equipped with a CCD camera (Nikon, DXM1200). Images were taken at a magnification of 400x. The software used was Nikon ACT-1 version 2.20. Cells were also analyzed by flow cytometry (LSRII, Becton Dickinson). Cell size and granularity were determined with simultaneous separate detection of low-angle (FSC-A) and right-angle (SSC-A) light scattering. The data were recorded for a total of 10,000 events per sample.

Statistics
All of the experiments were conducted in at least three independent replicates. For the MTT assay, H_2DCFDA, $CM-H_2TMRos$, JC-1 and morphological analyses by flow cytometer the results were calculated in relation to untreated cells (100%). For the YO-PRO-1/PI and OA/EB assays, the results are presented as a percentage of a given fraction of cells (viable, apoptotic, necrotic). Statistical analyses were performed using one-way ANOVA followed by Dunnett's (samples compared to control) or Tukey's (comparison between VPD and concentrations of the same VPD) multiple comparison test. Data are presented as the means ± SD of three to five individual experiments.

RESULTS

Cytotoxicity of VPD
The results of the MTT assay revealed that the toxicity of all three VPD to N2a cells is concentration dependent. Fig. 2 shows that there was a statistically significant, although not drastic, reduction in the percentage of viable cells after 24 h exposure to dendrimers, compared to the control. At the highest tested concentration (20 μM), VPD1 and VPD3 caused a comparable decrease in cell viability, which amounts to 72.7% and 74.8% of the control respectively, while VPD2 reduced the percentage of viable cells to only 80.3% of the control. There is a statistically significant difference between the concentrations of 1 and 20 μM, 2.5 and 20 μM for VPD1, and between 1 and 20 μM for VPD2 and

VPD3. These results indicate that VPD are characterized by low cytotoxicity and VPD2 is the least toxic compound of the three dendrimers.
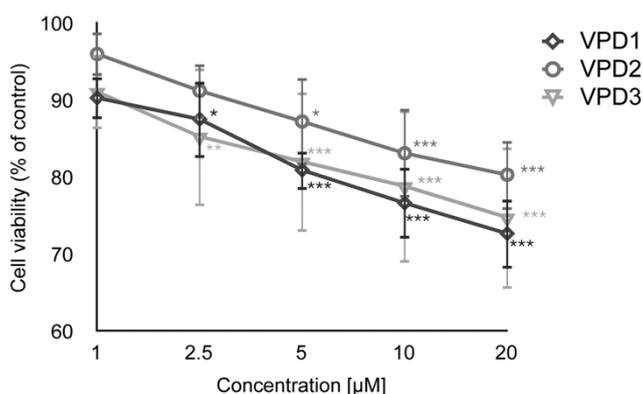

Fig. 2. The viability of N2a cells after 24 h exposure to viologen-phosphorus dendrimers ($n = 5$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistically significant differences occur between 1 and 20 μM of VPD1 (**), 2.5 and 20 μM of VPD1 (*), 1 and 20 μM of VPD2 (*), and 1 and 20 μM of VPD3 (*).

ROS generation

Alterations in the level of ROS were evaluated using a H_2DCFDA fluorescent probe. The intensity of DCF fluorescence was measured using a flow cytometer. The results demonstrate that VPD showed a tendency to decrease the cellular ROS level below the control level, even at the lowest tested concentration (1 μM; Fig. 3A). The strongest effect was caused by VPD1, which led to the reduction in ROS level amounting to 76.4% of the control at the highest tested concentration. VPD3 reduced ROS generation to 80.5% of the control, while VPD2 reduced it to only 86.8% of the control. The effect of cellular ROS reduction by VPD is also presented in the histogram based on the flow cytometry measurements (Fig. 3B). The results are statistically significant compared to the control for VPD1 at the concentrations of 5 to 20 μM and for VPD3 at the concentrations of 2.5, 10 and 20 μM.

Assessment of the oxidative activity of mitochondria

Reduced MitoTracker Orange (CM-H_2TMRos) fluorescent probe was used to stain functional mitochondria. Analysis of CM-TMRos fluorescence showed that VPD slightly decreased the mitochondrial activity, which dropped to 88.9%, 85.1% and 92% of the control for VPD1, VPD2 and VPD3, respectively, at the highest tested concentration (Fig. 4A). Statistically significant effects in comparison to the control were obtained for VPD1 at 10 μM and for VPD2 at 5 to 20 μM. A small reduction in MitoTracker Orange fluorescence is also visible in the histogram from the flow cytometry measurements (Fig. 4B).
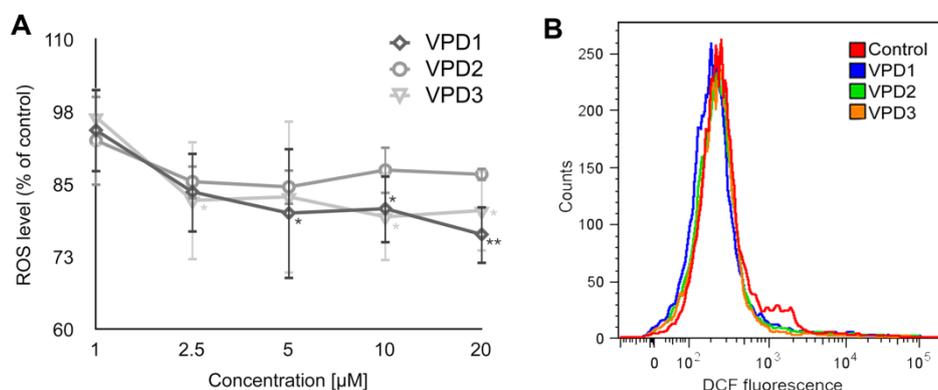
Fig. 3. Changes in the level of reactive oxygen species (ROS) in N2a cells after 24 h exposure to viologen-phosphorus dendrimers measured using a H_2DCFDA probe. A – The intensity of DCF fluorescence was assessed using a flow cytometer (n = 3, * p < 0.05, ** p < 0.01). H_2O_2 was used as a positive control (139.54% of the control ± 19.57 *). B – Representative histogram showing a slight change in the DCF fluorescence intensity between control cells and cells treated with 20 μM of viologen-phosphorus dendrimers.
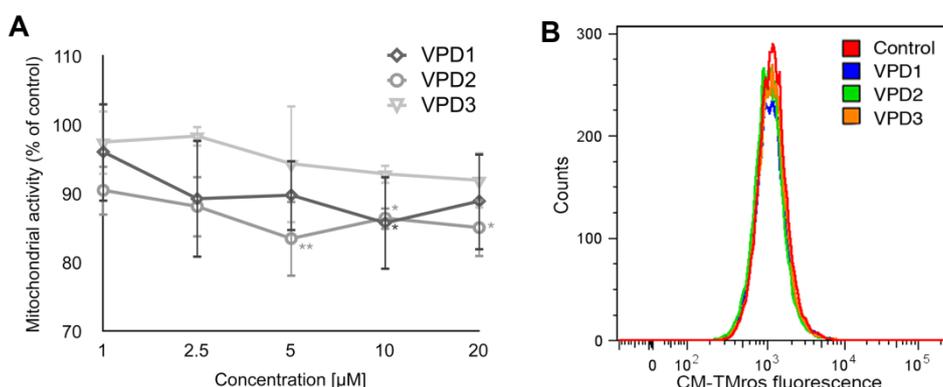

Fig. 4. Mitochondrial activity in N2a cells exposed to viologen-phosphorus dendrimers. A – Flow cytometric analysis of alterations in mitochondrial function in N2a cells after 24 h exposure to viologen-phosphorus dendrimers based on MitoTracker Orange fluorescence intensity (n = 3, * p < 0.05). PPI was used as a positive control (471.72% of the control ± 82.94 ***). B – Representative histogram showing the slight change in MitoTracker Orange fluorescence intensity between control cells and cells treated with 20 μM of viologen-phosphorus dendrimers.

Analysis of mitochondrial membrane potential (ΔΨm)

Alterations in mitochondrial membrane potential (ΔΨm) were determined using JC-1 fluorescent dye. Fig. 5 shows that depolarization of mitochondrial membrane occurred in the case of VPD1 and VPD3, while the PEGylated dendrimer did not exhibit any significant influence on mitochondrial membrane potential. ΔΨm was reduced to 74.9% and 82.4% of the control for VPD1 and VPD3, respectively, at a concentration of 20 μM. The results are statistically

significant compared to the control at 2.5 to 20 μM for VPD1, and 20 μM for VPD3. There are also statistically significant differences between VPD1 and VPD2 at 2.5 to 20 μM, and between VPD2 and VPD3 at 10 μM.
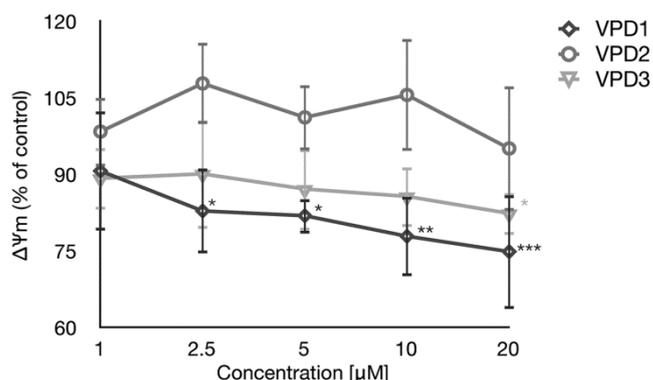

Fig. 5. Alteration in mitochondrial membrane potential (ΔΨm) in N2a cells after 24 h exposure to viologen-phosphorus dendrimers, determined using a JC-1 fluorescent dye (n = 5, * p < 0.05, ** p < 0.01, *** p < 0.001). CCCP was used as a positive control (28.61% of the control ± 10.45**). Statistically significant differences occur between VPD1 and VPD2 at 2.5 μM (**), 5 μM (*), 10 μM (***) and 20 μM (*), and between VPD2 and VPD3 at 10 μM (*).

Table 1. Apoptosis study on N2a cells after 24 h exposure to viologen-phosphorus dendrimers. The analysis was performed via flow cytometry using YO-PRO-1 iodide and propidum iodide stains (n = 3; * p < 0.05).

	Control	VPD1				
c (μM)	0	1	2.5	5	10	20
Healthy	95.62 ± 1.83	93.28 ± 4.23	93.16 ± 4.05	93.48 ± 4.09	93.37 ± 4.17	93.44 ± 4.39
Apoptotic	1.09 ± 0.37	1.76 ± 1.26	1.40 ± 0.85	1.31 ± 0.94	1.41 ± 0.84	1.51 ± 1.07
Necrotic	3.26 ± 1.79	4.97 ± 3.31	5.01 ± 3.42	5.20 ± 3.38	5.20 ± 3.43	5.10 ± 3.48

	Control	VPD2				
c (μM)	0	1	2.5	5	10	20
Healthy	96.87 ± 0.31	96.53 ± 1.10	96.00 ± 0.40	95.13 ± 0.61	96.00 ± 0.72	94.60 ± 1.56
Apoptotic	1.80 ± 0.6	2.87 ± 0.70	3.47 ± 0.64	4.13 ± 0.31	2.93 ± 0.23	4.87 ± 1.63
Necrotic	1.33 ± 0.76	0.73 ± 0.42	0.53 ± 0.42	0.73 ± 0.31	1.07 ± 0.81	0.73 ± 0.12

	Control	VPD3				
c (μM)	0	1	2.5	5	10	20
Healthy	96.87 ± 0.31	96.53 ± 1.21	94.93 ± 1.10	95.20 ± 1.83	95.13 ± 1.60	95.60 ± 1.44
Apoptotic	1.80 ± 0.6	2.87 ± 1.14	4.13 ± 1.10	3.67 ± 2.16	3.33 ± 1.40	3.20 ± 1.71
Necrotic	1.33 ± 0.76	0.60 ± 0.20	0.60 ± 0.20	1.20 ± 0.60	0.93 ± 0.64	1.27 ± 0.46

Apoptosis and necrosis studies

The induction of apoptotic and necrotic processes in VPD-treated cells was studied based on two methods – fluorescence microscopy analysis of AO/EB-stained samples and flow cytometry measurements of YO-PRO-1/PI fluorescence. Results from both methods indicate that none of the tested VPD induced apoptotic or necrotic cell death in N2a cells. Regardless of the tested concentration, only slight alterations in YO-PRO-1 and PI fluorescence were observed and the fraction of healthy cells was comparable to the control samples (Table 1, Fig. 6). Likewise, AO/EB staining revealed only minor, non-significant changes in the fraction of apoptotic and necrotic cells compared to the control (Table 2). Fluorescence microscopy images show that the nuclei of treated cells have normal morphology and fluoresce green similarly to the untreated samples (Fig. 7, upper panel). Overall, the obtained data indicate that VPD-treated cells maintain cell membrane integrity and do not enter the apoptotic pathway or undergo necrotic death.
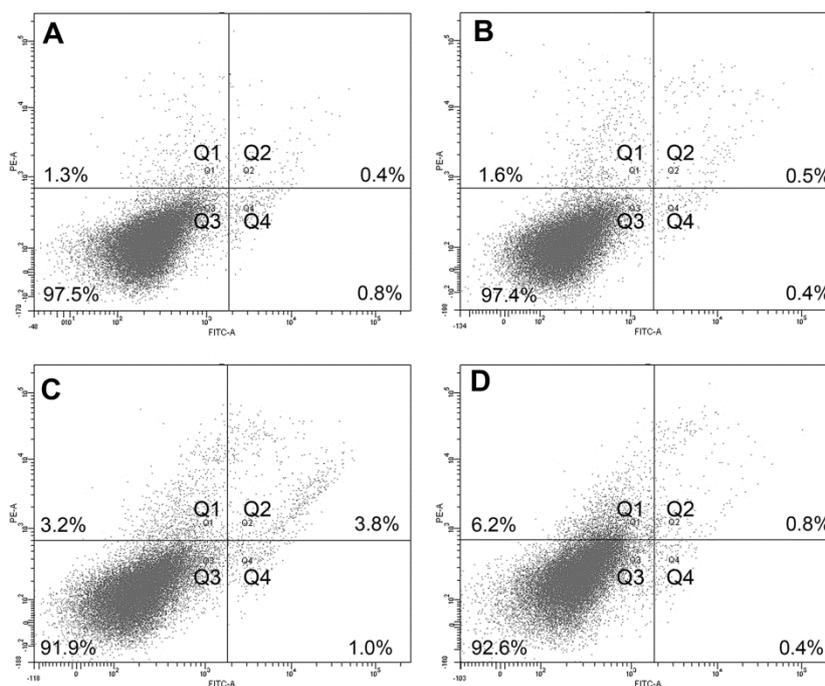

Fig. 6. Representative dot plots showing fractions of healthy (Q3), apoptotic (Q4), and dead (Q1 and Q2) cells after 24 h exposure to 20 μM viologen-phosphorus dendrimers. The analysis was performed by flow cytometry using YO-PRO-1 iodide and propidum iodide stains. A – Control cells. B – Cells treated with VPD1. C – Cells treated with VPD2. D – Cells treated with VPD3.

Table 2. Apoptosis study on N2a cells after 24 h exposure to viologen-phosphorus dendrimers. The cells were analyzed under a fluorescence microscope using acridine orange (AO) and ethidium bromide (EB) stains (n = 3; * p < 0.05).

	Control	VPD1				
c (μM)	0	1	2.5	5	10	20
Healthy	96.87 ± 0.31	97.00 ± 0.87	96.13 ± 0.76	96.20 ± 0.92	95.53 ± 1.50	95.00 ± 0.35
Apoptotic	1.80 ± 0.6	2.33 ± 0.83	2.93 ± 0.42	2.80 ± 0.72	3.53 ± 1.47	3.40 ± 1.06
Necrotic	1.33 ± 0.76	0.67 ± 0.12	1.00 ± 0.53	1.00 ± 0.80	0.87 ± 0.42	1.60 ± 0.72

	Control	VPD2				
c (μM)	0	1	2.5	5	10	20
Healthy	96.87 ± 0.31	96.53 ± 1.10	96.00 ± 0.40	95.13 ± 0.61	96.00 ± 0.72	94.60 ± 1.56
Apoptotic	1.80 ± 0.6	2.87 ± 0.70	3.47 ± 0.64	4.13 ± 0.31	2.93 ± 0.23	4.87 ± 1.63
Necrotic	1.33 ± 0.76	0.73 ± 0.42	0.53 ± 0.42	0.73 ± 0.31	1.07 ± 0.81	0.73 ± 0.12

|	Control	VPD3				
c (μM)	0	1	2.5	5	10	20
Healthy	96.87 ± 0.31	96.53 ± 1.21	94.93 ± 1.10	95.20 ± 1.83	95.13 ± 1.60	95.60 ± 1.44
Apoptotic	1.80 ± 0.6	2.87 ± 1.14	4.13 ± 1.10	3.67 ± 2.16	3.33 ± 1.40	3.20 ± 1.71
Necrotic	1.33 ± 0.76	0.60 ± 0.20	0.60 ± 0.20	1.20 ± 0.60	0.93 ± 0.64	1.27 ± 0.46

Fig. 7. Images of N2a cells after 24 h exposure to 20 μM viologen-phosphorus dendrimers. Cells were stained with acridine orange (AO) and ethidium bromide (EB) and examined by fluorescence (upper panel) and phase contrast (lower panel) microscopy. Images were taken at a magnification of 400x.

Cell morphology analyses
Cell morphology was analyzed using a fluorescence/phase contrast microscope and assessed based on flow cytometry measurement-derived FSC and SSC parameters, which reflect cell size and granularity, respectively. As presented in

Fig. 7 (lower panel), N2a cells exposed to 20 μM VPD maintained normal morphology, with unchanged size, shape and cell adhesion capacity. Moreover, the analysis of the FSC and SSC parameters indicated no alterations in the cell size and granularity after N2a treatment with all concentrations of VPD, except for a small decrease in granularity after the exposure to 20 μM VPD2 (Fig. 8).
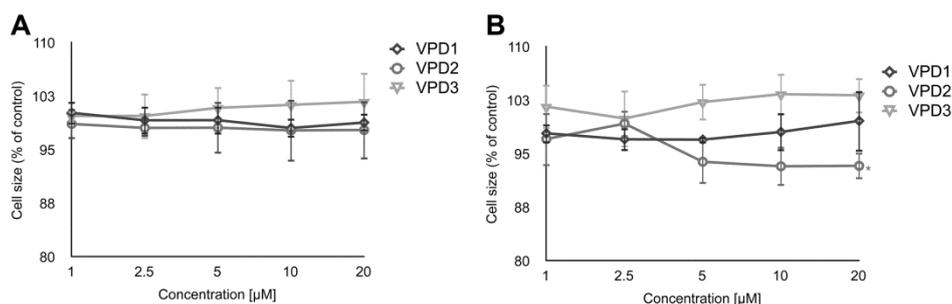

Fig. 8. Analysis of N2a cell morphology, specifically size (A), and granularity (B), after 24 h exposure to viologen-phosphorus dendrimers (n = 3, * $p < 0.05$).

DISCUSSION

The goal of this study was to evaluate the cytotoxicity of three water-soluble viologen-phosphorus dendrimers whose effects on the cell had not been determined before. We focused on monitoring cell responses that reflect the cell condition, such as alterations in mitochondrial functions, ROS generation, and the induction of cell death processes.

The MTT assay after VPD treatment was previously performed on B14 (Chinese hamster) and N2a cell lines [26]. The tested dendrimers were shown to be less toxic to B14 than to N2a cells, the viability of which was reduced to about 40-50% of the control at a concentration of 20 μM. Those data differ from the results presented here. We showed that three tested VPD are characterized by low toxicity. The percentage of living cells did not drop below 70% for VPD1 and VPD3 or below 80% for VPD2 (Fig. 2). The discrepancies in the results presented by Ciepluch *et al.* and ours may stem from different experimental conditions, such as the lower number of cells per well used by Ciepluch *et al.* [26]. Nevertheless, judging by the range of concentrations used (1 to 20 μM), VPD can generally be considered relatively low-toxicity compounds compared to phosphorus dendrimers, which have an IC_{50} of about 1 μM for N2a after 24 h exposure [9] or PAMAM G4 dendrimers, which have an IC_{50} value of 1.7 μM for SH-SY5Y (human neuroblastoma) cells [39].

One of the cell responses to treatment with cationic unmodified dendrimers is the generation of ROS. ROS production was reported after the exposure of human macrophages to polypropyleneimine (PPI) dendrimers [40], mouse macrophages to PAMAM [41] and mouse embryonic fibroblasts to PAMAM [42]. Here, we demonstrated that the ROS level in N2a cells after VPD treatment

falls below the control (Fig. 3). The existence of two mechanisms of ROS level reduction can be assumed. First, the tested dendrimers may directly scavenge ROS. Second, VPD may decrease the ROS level indirectly, by inducing enzymatic or non-enzymatic antioxidant systems. It was shown previously that PAMAM dendrimers cause biphasic production of ROS in a human keratinocyte (HaCaT) cell line and a primary adenocarcinoma cell line of the colon (SW480) and at some time-points after exposure, the level of ROS decreased below the control level. The kinetics of ROS production and levels were also shown to be generation and concentration dependent. For HaCaT cells at concentrations above 1 μM, ROS quenching below the control occurred between 3 and 24 h after PAMAM G6 treatment [43, 44]. It is suggested that such process may be associated with cellular antioxidant levels and the migration of antioxidants to localized subcellular sites. Indeed, the depletion of glutathione level in HaCaT cells after exposure to PAMAM was reported [44]. It is possible that different concentrations of VPD and incubation times would also lead to a transient increase in ROS level. The activity and level of antioxidant systems should also be monitored to understand why VPD treatment led to the reduction in ROS generation.

Measurements of MitoTracker Orange fluorescence revealed a small decrease in the oxidative activity of mitochondria, which respectively fell to 88.9%, 85.1% and 92% below the control for VPD1, VPD2 and VPD3 at the highest tested concentration (Fig. 4). The results of JC-1 assays suggest slightly stronger disturbances in mitochondrial function in comparison to those obtained for CM-H$_2$TMRos staining. Nevertheless, these data also indicate some mitochondrial dysfunction. A decrease in MitoTracker Orange fluorescence was also reported in HaCaT cells after PAMAM treatment [43].

It has been demonstrated that depolarization of the mitochondrial membrane occurred after cell treatment with PAMAM and this process was associated with apoptotic cell death [42, 45], while PPI increased (G2) or caused fluctuations (G3) in ΔΨm [40]. Our studies showed that 24 h exposure of N2a to VPD with phosphonate surface groups led to a small depolarization of the mitochondrial membrane (Fig. 5). ΔΨm fell to 74.9% and 82.4% of the control for VPD1 and VPD3, respectively. The PEGylated dendrimer increased ΔΨm to a small degree at 2.5 to 10 μM, but these alterations were not significant. Although only small dysfunction of mitochondria was found, some apoptotic changes could have been expected. However, neither flow cytometry measurements of YO-PRO-1/PI-stained cells (Table 1, Fig. 6) nor fluorescence microscopy analysis of AO/EB-stained samples (Table 2, Fig. 7, upper panel) revealed any pronounced increase in cell death processes. The differences in healthy, apoptotic, and necrotic cell fractions between control and VPD-treated samples never exceeded 5%, regardless of the method used. These findings indicate that the integrity of the plasma membrane after N2a exposure to VPD is maintained. It is postulated that cationic dendrimers can interact with negatively charged plasma membranes, which may lead to the formation of nanoholes in the membrane and cell dysfunction or

death [46-48]. Therefore, the possible reason for the low cytotoxicity of VPD is the number and location of positive charges. The used VPD possess 6 (VPD3) to 12 (VPD1 and VPD2) positive charges, which are located inside the nanomolecule. By contrast, dendrimers such as PAMAM and cationic phosphorus dendrimers carry a large number of positive charges at the surface of the dendritic structure, which can easily interact with plasma membranes [47, 49]. Furthermore, according to a current theory, dendrimers can enter the cell via clathrin-dependent endocytosis and/or macropinocytosis [50-53] and the process of dendrimer uptake has been shown to reach completion within 4 h [52-54]. Therefore, it is possible that VPD are internalized into the cell within the first hours after treatment, causing minor, reversible changes in the cell membrane integrity, which are repaired during the next hours and undetectable at 24 h. Ciepluch et al. [26] demonstrated that only VPD2 and VPD3 at the highest concentrations caused a small but statistically significant decrease in erythrocyte membrane fluidity, which implies that these VPD can interact with the polar headgroup region of the phospholipid bilayer. However, it should be taken into account that the erythrocytes were incubated with dendrimers for 0.5 h and this time may have been too short to observe a stronger influence of VPD on lipid membranes. By comparison, PAMAM were shown to be bound to the cell membrane at about 1 h after exposure [54].

Morphological alteration, such as cell shrinkage, chromatin condensation, DNA fragmentation and compaction of organelles, are indicative of apoptosis, while necrosis is characterized by cell swelling and final rupture [55]. As an additional confirmation of the condition of cells, we analyzed FCS and SSC parameters from flow cytometry (Fig. 8). No alterations in the size and granularity of cells (apart from a slight decrease in granularity after the exposure to 20 μM VPD2) were found. These results are in accordance with fluorescence/phase microscopy analysis (Fig. 7 lower panel), which did not reveal any changes in cell morphology.

In summary, basic responses of N2a cells to three water-soluble G0 viologen-phosphorus dendrimers were investigated. We found that despite structural differences, all of the studied VPD caused similar effects in the tested cell line. Our results indicate that dendrimer treatment leads to slight mitochondrial dysfunction, which is manifested by a small decrease in mitochondrial membrane potential and MitoTracker Orange fluorescence. Moreover, the dendrimers reduced the level of ROS, which implies that they may have antioxidant properties or influence the intracellular antioxidant level and activity. Importantly, VPD treatment did not induce apoptotic or necrotic cell death at any tested concentration. Therefore, it seems that these nanoparticles are innocuous to N2a cells. As such, they could be good candidates for further studies on their biological properties. Nevertheless, the possibility that VPD undergo degradation or are inactivated in another way, resulting in reduced cell responses, cannot be excluded. Thus, more research is needed to prove the safety of VPD and accept them as low-toxicity compounds.

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