Application of new lysine-based peptide dendrimers D3K2 and D3G2 for gene delivery: Specific cytotoxicity to cancer cells and transfection in vitro


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ABSTRACT

In order to enhance intracellular uptake and accumulation of therapeutic nucleic acids for improved gene therapy methods, numerous delivery vectors have been elaborated. Based on their origin, gene carriers are generally classified as viral or non-viral vectors. Due to their significantly reduced immunogenicity and highly optimized methods of synthesis, nanoparticles (especially those imitating natural biomolecules) constitute a promising alternative for virus-based delivery devices. Thus, we set out to develop innovative peptide dendrimers for clinical application as transfection agents and gene carriers. In the present work we describe the synthesis of two novel lysine-based dendritic macromolecules (D3K2 and D3G2) and their initial characterization for cytotoxicity/genotoxicity and transfection potential in two human cell line models: cervix adenocarcinoma (HeLa) and microvascular endothelial (HMEC-1). This approach allowed us to identify more cationic D3K2 as potent delivery agent, being able to increase intracellular accumulation of large nucleic acid molecules such as plasmids. Moreover, the dendrimers exhibited specific cytotoxicity towards cancer cell line without showing significant toxic effects on normal cells. These observations are promising prognosis for future clinical application of this type of nanoparticles.

1. Introduction

Despite the numerous research and great interest in the development of an effective delivery system for gene therapy, success in this field has not been achieved so far. Carriers based on viral systems are the most popular, due to their natural capacity to transport genetic material and introduce it into the cells [1]. However, the applicability restricted mostly to dividing cells, difficult large-scale production, as well as immunogenicity and carcinogenicity raised serious concerns about their use in gene therapy [2]. Moreover, several deaths during clinical trials have been reported, further delaying the possibility of medical application of viral vectors. As a promising alternative, numerous non-viral carriers have been studied, exhibiting lower toxicity, non-immunogenicity and high loading capacity. Compounds such as cationic lipids, cyclodextrins or dendritic polymers can prove effective in overcoming limitations associated with viral vectors [3–6]. Cationic dendrimers are of particular interest for gene delivery, primarily due to the ability of forming stable complexes with negatively-charged nucleic acids and efficient cellular uptake. Highly-branched macromolecules such as poly(amideamine) (PAMAM) or poly(propyleneimine) (PPI) dendrimers have been widely studied for DNA delivery, enhancing the transfection efficiency by endocytosis and direct transport into the cell nucleus [7–11]. Recent reports indicate that dendritic poly(lysine) polymers could be used as gene carriers for both in vitro and in vivo transfection. In contrast to classical cationic dendrimers such as PAMAM and PPI with symmetric architecture, composed of multiple branches radially attached to the central core, the structure of dendritic poly(lysine) compounds is more asymmetric. Moreover, peptide dendrimers usually exhibit reduced cytotoxicity [12,13] and ability to mimic cell-penetrating peptides for enhanced cellular uptake [14]. Finally, biodegradable nanocarriers composed of naturally occurring monomers such as amino acid residues can be subjected to facile degradation inside the organism, generating non-toxic metabolites [12,15]. All these features indicate the high potential of poly(lysine) dendrimers for clinical application as drug or gene delivery devices.

Our research focused on the development and in vitro evaluation of
new derivatives of poly(lysine) dendrimers as potential drug/gene nanocarriers. Two lysine-based dendrimers (D3K2 and D3G2, Fig. 1) were synthesized and tested for cytotoxicity, DNA damage and transfection capacity in two cell line models: human cervix adenocarcinoma (HeLa) and human microvascular endothelial (HMEC-1). The present work was meant to verify the hypothesis that poly(lysine) dendrimers are suitable for the transport of genetic material and to select a better carrier from the two proposed nanoparticles for further studies.

2. Results

2.1. Cytotoxicity of D3G2 and D3K2 dendrimers

First, the cytotoxic effect of analyzed dendrimers was assessed by the MTT method. The MTT approach is the most common assay and has been extensively applied to determine the cytotoxic effect of a variety of nanoparticles [16,17]. This method takes advantage of the reduction of...
MTT salt to a colored, water-insoluble formazan by NADH or NADPH-dependent oxidoreductases in viable cells. Moreover, this assay can be applied for both cytotoxicity and cell proliferation studies. However, due to the specific mechanism, this method can display varied sensitivity. The utilization of complementary assays in the same study, based on the different mechanism for toxicity detection, is therefore strongly recommended [18]. In the course of our experiments, the crystal violet assay was used additionally. The method is based on binding of triarylmethane dye to ribose-containing molecules such as DNA in the nuclei. Dead adherent cells, detached from cell culture plates are removed from viable cell population during washing steps. Crystal violet assay measures total DNA quantity of the remaining living population and therefore determines cell viability.

Fig. 2 and Fig. 3 show the influence of the analyzed dendrimers on the viability of human cervix adenocarcinoma cells (HeLa) and non-cancer human microvascular endothelial cells (HMEC-1) estimated by MTT and crystal violet assays. Data presented as percentage of viability of control (untreated) cells, mean ± SD of 3 independent experiments.

2.2. Measurements of DNA damage - comet assay

An important stage of our investigation was to explain the intracellular mechanisms associated with the toxic action of lysine-based dendrimers. Thus, we evaluated the impact of investigated compounds on nucleic acid degradation. In our study, the alkaline version of DNA comet assay (pH > 13) was performed to assess the degree of DNA damage in cancer HeLa and non-cancer HMEC-1 cells. This method enables the detection of various types of DNA damage, such as single- and double-stranded DNA breaks, DNA fragmentation induced by free radicals, cross-type DNA-DNA bonds or DNA-protein interactions [19].

For the purpose of establishing the dendrimer concentration sufficient to induce detectable DNA cleavage, we exposed the cells to D3K2 and D3G2 dendrimers in the subtoxic and toxic concentration range (5–50 μM) for 24 and 72 h. This period of time was chosen on the basis of cytotoxicity assays described above. The results presented in Fig. 4 and Fig. 5 indicate that HMEC-1 cell line was less susceptible to induction of DNA damage by both D3K2 and D3G2 dendrimers, which is consistent with the results of cytotoxicity assays. Both cell lines were significantly more sensitive to D3K2 than to D3G2 dendrimer. The increased level of DNA damage was dependent on the dendrimer type and its concentration as well as on the length of the incubation time. In HMEC-1 cells, 72 h incubation with 50 μM D3K2 or D3G2 led to a similar (approx. 10–15%) increase in percentage of the DNA in the...
comet tail. On the other hand, in HeLa cells, these concentrations of dendrimers caused different levels of damage - to approx. 30% (D3K2) and 18% (D3G2).

2.3. TUNEL assay

DNA comet assay did not give an unambiguous answer to the question whether DNA breaks are the result of the mobilization of the apoptotic cell death machinery or result from the direct genotoxic activity of dendrimers.

Therefore, we decided to use TUNEL assay as an alternative method for the evaluation of DNA damage associated with apoptotic changes. Since the activation of endonucleases that cleave chromosomal DNA preferentially at internucleosomal sections is a hallmark of apoptosis, DNA fragmentation revealed by the presence of multitude strand breaks is considered the gold standard for the identification of apoptotic cells [20].

Using the TUNEL method, which enabled quantitative (Fig. 6) and qualitative (Fig. 7) assessment of molecular events associated with apoptosis, we investigated the average effects of D3G2 and D3K2 dendrimers on HeLa cells population, as well as morphological changes at the single cell level. Again, D3K2 induced higher level of DNA damage than D3G2. The maximal increase in the percentage of apoptotic cells (TUNEL positive population) was noted for 24 h (57%) and 72 h (45%) after treatment with 50 µM of D3K2 dendrimer. The effect observed after incubation with the same concentration of D3G2 (24 h − 38% and 72 h − 35%) was considerably lower.

Fig. 4. Influence of D3K2 and D3G2 dendrimers on the DNA content in comet tail [%] of HeLa human cervix adenocarcinoma cells (upper panel) and HMEC-1 human microvascular endothelial cells (lower panel), estimated by comet assay. The number of analyzed cells in each treatment was equal to 150. DAPI stained cells were analyzed under fluorescence microscope Olympus CX 40, magnification 200 × . Data presented as mean ± SD of 3 independent experiments. *p < 0.05 relative to the control (untreated cells).

Fig. 5. Fluorescence images of DNA content in comet tail of HeLa human cervix adenocarcinoma cells (upper panel) and HMEC-1 human microvascular endothelial cells (lower panel) after 72 h incubation with D3G2 and D3K2 dendrimers (50 µM concentration). DAPI stained cells were analyzed under fluorescence microscope Olympus CX 40, magnification 200 × .

Fig. 6. Influence of D3K2 and D3G2 dendrimers on the induction of apoptosis in HeLa human cervix adenocarcinoma cells, estimated with the use of TUNEL assay. Quantitative results of the dendrimer effect on the level of TUNEL positive cells presented as mean ± SD of 3 independent experiments. *p < 0.05 relative to the control (untreated cells).
2.4. Transfection

pcDNA3-EGFP - enhanced green fluorescent protein plasmid assay was used to investigate the transfection efficiency of D3K2 and D3G2 dendrimers in cancer HeLa and non-cancer HMEC-1 cells. The results of our experiments showed that D3K2 dendrimer possesses significant transfection efficiency, comparable with commercially available lipofectamine.

As expected, the control group without treatment and the control group without the use of vectors did not show any EGFP fluorescence (data not shown). Results presented in Fig. 8 demonstrated high D3K2-EGFP transfection potential comparable to lipofectamine. After 72 h incubation with 50 µM D3K2 dendrimer the transfection efficiency was slightly below 60%, while for the commercial lipofectamine it was about 70% for the HeLa cell line. In HMEC-1 cells, after 72 h incubation with D3K2-EGFP dendriplex it was approx. 60%, while in the case of lipofectamine ~ 55%. Importantly, commercial transfection reagent revealed high toxicity (especially upon longer incubation times), while the D3K2 dendrimer complexed with plasmid showed a very low toxicity profile towards non-cancer HMEC-1 cells and moderate towards HeLa cells. The observed transfection efficiency of less toxic D3G2 dendrimer was significantly lower and incomparable with D3K2 dendrimer.

3. Discussion

Modern diagnostic methods and progress in biomedical science allowed to prove that the source of many diseases lies in the defects or lack of single genes. For the treatment of genetic disorders, several techniques of so-called gene therapy have been elaborated, involving the intracellular delivery of foreign nucleic acid. These are meant either to introduce the correct version of a gene or to change the activity of the defective one [3,5,21]. The concept of gene therapy was launched in 1962, when the scientists from the University of Wisconsin introduced a piece of DNA into human bone marrow cells [22]. In 1990, the first clinical trial of gene therapy was reported, when 4-year-old Ashanthi De Silva, suffering from severe combined immunodeficiency (SCID) syndrome, has been treated with modified lymphocytes containing the correct copies of previously mutated gene [23]. Since then, several approaches to the successful clinical application of gene therapy have been developed.

Among the most frequently used nucleic acid molecules, next to RNA, genomic DNA or mitochondrial DNA, plasmid DNA [24] has been applied in gene therapy. This type of DNA is found mainly in the cells of Prokaryotes in three forms: the CCC-DNA (so-called supercoiled form, which is a circular DNA molecule), the CC-DNA (circular but open molecule), and the least frequent linear type. In general, plasmids exist as autonomous molecules, physically separated from chromosomal DNA, with the ability of independent replication. Plasmid DNA can carry a number of genes responsible for cell survival, providing e.g. resistance to antibiotics or decomposition of toxic chemical agents [25–27]. The use of plasmids in gene therapy gives the opportunity to carry the correct genes to the selected chromosomal target sites. Additionally, it can be designed as an expression vector that allows direct production of a functional protein [27]. This make them particularly fine candidates for the application in gene therapy.

The nucleic acid introduced externally should be effectively transported into the cell to enable the expression of transported gene and production of properly functioning protein. This process is extremely difficult, being a major obstacle for the development of effective gene therapy methods. All nucleic acids possess negative charge, hampering their transport through the equally charged cellular membranes. Further, these molecules are extremely sensitive to enzymatic degradation, both in the bloodstream and in the cytoplasm. Therefore, to enable efficient cellular uptake of therapeutic nucleic acids and to protect them from degradation, it is necessary to use appropriate carrier systems.

In the 1990s, scientists focused on the idea of using viruses as carriers of genetic material, due to their innate mechanisms of introduction of DNA and RNA into the cells [28]. Retroviruses seemed to be the most promising since they possess the ability to convert RNA into double-stranded DNA molecule that integrates with the genome in an efficient and stable way [1,29]. Further, the use of adenoviral vectors enabled the introduction of large nucleic acid fragments (up to 8 kbp) into various types of cells, both dividing and non-dividing [30,31]. However, these types of carriers may cause various side effects, of which the most severe involve strong immune responses lowering the treatment efficacy [32,33]. The dangers associated with clinical use of viral vectors forced scientists to seek other, non-viral nucleic acid delivery agents for gene therapy. For this purpose, the systems based on the use of liposomes, gold nanoparticles, carbon nanotubes or dendrimers have been proposed, of which the latter attract particular attention. Dendrimers constitute a class of core-shell monodisperse globular nanostructures with precisely designed, highly-branched architecture and defined molecular size [34]. For the application in gene therapy, cationic dendrimers seem to be the most legitimate choice, providing the formation of stable complexes (called dendriplex) [4,7] due to the strong electrostatic interactions with negatively charged nucleic acids. Thanks to its characteristic structure and high loading capacity, dendrimer macromolecule is able to bind and transport relatively large amounts of RNA or DNA, at the same time protecting them from...
Moreover, positive charge enables efficient transport of dendriplexes through the cell membrane [36,37]. In particular, poly(lysine) dendrimers are attractive candidates for medical applications [38]. These polymers, containing lysine amino acids as branching units, were synthesized for the first time in the 1980’s [39,40]. Since then, their properties have been thoroughly studied both experimentally [41–43] and in computer simulations [44–47]. Branched peptide macromolecules are usually called “asymmetric dendrimers” as they contain “long” and “short” branches that significantly affect the structure density. Short branches cause the densification of the dendrimer interior, altering overall size of macromolecule and facilitating its cellular uptake. In case of poly(lysine) dendrimers, such architecture additionally influences the availability of amino groups with the affinity for negatively charged nucleic acids [48–50]. Lysine-based dendrimers have been used as drug delivery systems, antiviral and antimicrobial agents, amyloid aggregation inhibitors and DNA carriers, enhancing the transfection efficacy and transporting nucleic acids into the cell nucleus [9,51–57]. In 2012, Starpharma (Melbourne, Australia) started two pivotal phase III clinical research for the treatment of bacterial and viral vaginosis with Vivagel® (www.starpharma.com/vivagel/vivagel_clinical_trials). This product, containing anionic, lysine-based dendrimer of the 4th generation with thirty-two naphthalene disulfonate groups on the surface, showed potent topical vaginal microbicide activity [58]. What is important, the integration of properties of dendrimers and peptides might provide relevant synergistic effects. These include the increase of peptide biological activity due to the polyvalent structure of dendrimers, multiplication of peptide functions by allowing simultaneous interactions with multiple receptors, improvement of biocompatibility and biodegradability, as well as enhanced resistance to proteolysis and delayed renal clearance [59,60]. Thus, understanding of interactions between peptide dendrimers and cell cultures in vitro is particularly important in order to fully exploit their potential and define the mechanisms of action. Several recent studies combined dendrimers or dendritic structures with amino acids and peptides to improve their delivery potential or gene transfection properties without degradation [35]. Figure 8. Transfection efficiency of D3K2 and D3G2 dendrimers in HeLa and HMEC-1 cells (left panel), estimated with the use of pcDNA3-EGFP assay. Comparison of cytotoxic activity of dendriplexes with plasmid DNA and lipofectamine:plasmid DNA complex towards HeLa and HMEC-1 cells (right panel), estimated with the use of MTT assay. Results are presented as mean ± SD of 3 independent experiments. *p < 0.05 relative to the lipofectamine. **p < 0.05 relative to the cells after 72 h incubation.
significantly increasing their cytotoxicity [61]. However, these studies did not provide full insight into the essential cellular mechanisms underlying the toxic activity of these dendrimers.

The aim of our work was to elaborate and characterize novel delivery system based on poly(lysine) dendrimers. For this purpose, we synthesized two types of macromolecules: D3K2 (containing two additional lysine residues (Lys-Lys) with charged NH$_3^+$ groups between each pair of neighboring branching points of standard poly(lysine) dendrimer of 3rd generation (D3)) and D3G2 (containing two additional glycine residues (Gly-Gly) at the same points). Recent studies demonstrated that structural flexibility, size and charge density of the dendrimers may significantly influence their gene transfection activity [62]. Thus, introduced modifications were meant to provide additional properties that could improve the interactions with genetic material, making the dendrimer more positively charged (D3K2) or more flexible (D3G2) [63].

An overall positive charge of the dendrimer:nucleic acid complex is required for effective cellular uptake. On the other hand, highly cationic systems are usually featured with high cytotoxicity [64,65]. Thus, we decided to determine the cytotoxicity of D3K2 and D3G2 macromolecules and to evaluate their gene delivery potential, in order to select the best concentration range ensuring proper balance between cytotoxicity and transfection efficiency for further, more detailed investigations. For this purpose, we focused on the application of well-known methods used as screening tests on the stage of preliminary evaluation.

Since the difference in the effect of dendrimers on cancerous and normal cells has important medical implications, for our studies we chose cervix adenocarcinoma (HeLa) and microvascular endothelial (HMEC-1) human cell lines. HeLa has been the first immortalized human cell line cultured and studied for many years, which makes it well characterized model for cancer research, providing clinical relevance of the results [66]. On the other hand, HMEC-1 is immortalized human microvascular endothelial cell line that retains the morphologic, phenotypic and functional characteristics of normal human microvascular endothelial cells [67]. The choice of this cell line was dictated by the fact that most of currently used therapeutics are intended for intravenous administration, where the endothelial cells are the first barrier.

Our research started with the evaluation of cytotoxic activity of lysine-based dendrimers in selected cell line models using MTT and crystal violet assays. Both D3K2 and D3G2 dendrimers revealed almost no toxicity against HMEC-1 cells. However, HeLa cell line was more sensitive to these macromolecules, with D3K2 exhibiting higher, time- and concentration-dependent toxicity compared to D3G2. This is not unexpected, since cationic dendrimers are usually characterized by high cytotoxicity activity due to non-specific interactions with cellular membranes, mitochondrial damage and triggering of reactive oxygen species (ROS) production [68,69]. Thus, enhanced toxicity of D3K2 may be explained by its higher positive charge. Notably, the observed selective cytotoxic action of lysine-based dendrimers towards HeLa cell line is of great importance, considering their application as anticancer drug carriers or drugs per se. In these cases, our polymers may provide synergistic therapeutic effect and reduce detrimental side effects of classical chemotherapy.

Further examination of the impact of investigated compounds on nucleic acid degradation using DNA comet assay corroborated the results of cytotoxicity evaluation. D3K2 dendrimer treatment resulted in significantly higher level of DNA breaks in cancer HeLa cell line in comparison to HMEC-1 cells, especially after longer incubation times. These observations may indicate, on the one hand, the significant involvement of apoptotic processes in the death of cells treated with poly (lysine) dendrimers [70] or their direct genotoxic activity [71], since comet assay does not allow to distinguish between these two phenomena [72].

Thus, we decided to apply TUNEL assay to conclude whether the DNA degradation determined with comet assay is associated with the cell death via apoptosis. This test was conducted on HeLa cell line owing to its higher sensitivity to dendrimer treatment. Once again, D3K2 was able to induce higher level of DNA breaks than D3G2. Notably, the TUNEL showed higher percentage of cells with DNA damage compared to the results obtained with comet assay. This outcome is not unexpected, since the cells in further stages of apoptosis cannot be efficiently detected with comet assay [73]. However, the time-dependent increase in DNA damage observed during comet assay did not occurred in case of the TUNEL, suggesting that during longer incubation periods poly(lysine) dendrimers may exhibit direct genotoxic activity. Nevertheless, taking these results into consideration, we postulate that the observed DNA fragmentation triggered by poly(lysine) dendrimers is primarily associated with the induction of apoptosis (e.g. by increased ROS production) and not by their genotoxicity [71].

Knowledge and understanding of peptide dendrimer cytotoxicity is also important for the efficient transfection. The possibility of using plasmid DNA or siRNA for enhanced treatment and prevention of diseases that are currently incurable is very attractive. However, in contrast to siRNA (20–25 bp) plasmid DNA is very large (from 1 to even 2000 kbp), which makes it particularly susceptible to enzymatic degradation and hampers its efficient cellular uptake. To overcome these obstacles, linear poly(lysine) peptides have been investigated as a plasmid DNA delivery vectors owing to their cationic nature and bio-compatibility. However, these carriers showed low transfection efficiency and high cytotoxicity which ruled out their further clinical application [62,74]. The shaping of poly(lysine) into a branched or star-shaped forms might overcome these problems. Dendritic poly(lysine) polymers turned out to be promising candidates for the plasmid DNA transfection agents, although such compounds are synthetically challenging and usually do not show high transfection capacity [75,76]. By contrast, we were able to synthesize monodisperse poly(lysine) dendrimers that could be used as gene carrier for in vitro gene delivery.

Our experiments with pcDNA3-EGFP showed that D3K2 dendrimer was able to introduce a large plasmid inside both cell types with efficiency comparable to commercially available lipofectamine, in some cases even outweighing its effect. For HeLa cells, a higher transfection rate was associated with a change in D3K2 concentration to more toxic. This dependency has been observed previously for different nanoparticles and cell line models [77]. However, in case of HMEC-1 cells, the limited toxicity of D3K2 did not decrease the transfection efficacy, pointing to the lack of correlation between these two phenomena. Moreover, the dendrplexes were found to be less toxic to both cell lines than lipofectamine, especially after longer incubation times. This ultimately points to high transfection potential of D3K2 dendrimer towards various cell types. Considering that D3G2 dendrimer showed almost no transfection capacity, in can be concluded that higher positive charge of the macromolecule is in this case more important than its flexibility. This is also indicated by the fact that the high transfection efficiency was achieved for higher D3K2 concentration, which may be associated with concentration-dependent cellular uptake of cationic dendrimers [78,79], possibly due to the various degree of cell membrane permeability.

4. Conclusions

This manuscript presents the results of in vitro cytotoxicity and gene delivery tests with the use of newly synthesized lysine-based polymeric nanoparticles. The applied assays showed significant differences in cytotoxic activity of both D3K2 and D3G2 dendrimers, which were concentration-, time- and charge-dependent. Interestingly, both dendrimers exhibited higher toxicity towards cancer cells, which indicates that this macromolecules may serve as promising nanocarriers for anticancer therapeutics, providing a synergistic effect and reducing
interactions of the drugs with healthy tissues. Further, we proved the great transfection potential of more cationic D3K2 dendrimer, which was able to deliver plasmid DNA into both cancer and normal cells to a degree comparable with commercial lipofectamine, at the same time causing less cell damage. These observations bring hope for elaboration of efficient gene delivery system based on poly(lysine) dendrimers, which require further research.

5. Experimental section

5.1. Materials

Amino acids (l-lysine, L-glycine, L-alanine) were purchased from Iris Biotech GMBH (Germany); trifluoromethanesulfonic acid (TFSA), d,l-sipropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt), thioniane, ethanedithiol and other reagents were purchased from Sigma-Aldrich (Germany) and used as received. Triethylamine (Et3N), di- and chloromethane were purchased from Vexton Ltd. (Russia) and distilled before use. Dimethylformamide (DMF), also purchased from Vexton Ltd. (Russia), was dried under molecular sieves (4 Å) and distilled under vacuum. Trifluoroacetic acid (TFA) purchased from Panreac (Spain) was distilled before use. All solvents were purified and distilled using standard procedures. Flasks and multiwell plates were obtained from Nunc (Germany). Cell culture media and supplements were obtained from Gibco (Thermo Fisher Scientific, USA).

5.2. Synthesis and characterization of a trispherical lysine-based dendrimers

Lysine-based dendrimers were prepared using standard phase peptide synthesis (SPPS) performed on p-methylbenzhydrylamine resin (BachemBiochemica GmbH) using the BOC-strategy, DIC/HOBt as a condensing mixture, and trifluoroacetic acid for deblocking at the acylation stage. To protect functional groups of amino acids, the tert-butyloxycarbonyl (Boc), and 2-chlorobenzylhydroxycarbonyl groups were used. At the C-terminus of the dendrimers of this series, an alanine (Ala) residue was introduced so that the amino acid analysis of the synthesized dendrimers enabled the control of their structure. Nε,Nα-di-[(tert-butyloxycarbonyl)lysine was introduced into the branching point and, subsequently, double amounts of derivative amino acids were added. The excess of the reagent introduced into the reaction was generally 3–4 equivalents per amino group based on the initial capacity of the polymeric carrier. The efficiency of the acylation reaction was controlled by the determination of free amino groups using the Kaiser test (ninhydrin test). The protective BOC-group was removed during the synthesis by the addition of trifluoroacetic acid (TFA). The final stage of the synthesis involved the cleavage of the target dendrimer molecule from the polymeric carrier with the simultaneous complete deprotection of the trifluoromethanesulfonic acid/trifluoroacetic acid (TFSA/TFA) system in the presence of scavengers. Isolation and purification of the target molecules were carried out by chromatographic methods.

The general protocol, involving the use of 0.2 g of p-methylbenzhydrylamine resin (capacity 0.85 mmol/g) was as follows: (1) deprotection, 50% TFA/CH2Cl2 (5 ml), 20 min; (2) deprotection, 10% Et3N/DMF (5 ml × 2), 15 min; (3) coupling, 1.5 mM Boc-amino acid, 15 mM DIC, 1.5 mM HOBt/DMF (5 ml), 2 h; (4) ninhydrin test. For stage (4), the resin was washed with dimethylformamide and dichloromethane. In case of incomplete coupling (indicated by positive ninhydrin reaction) the protocol was repeated from stage (2).

Note that the problem of exhaustive acylation of branched oligo-lysyl polymer appeared during the transition from dispherical to trispherical construct in the process of D3G2 and D3K2 dendrimers synthesis: two acylation reactions with the activated derivative of Nε,Nα-di-[(tert-butyloxycarbonyl)lysine and an increase in the acylation time were necessary to complete the reaction. Further, the reaction times were prolonged after one (180 min), two (240 min) and three branching units (360 min). The amounts of amino acids, DIC and HOBt were increased respectively. During the last stage of the growth of the dendrimer, it was necessary to add 4-N,N-dimethylaminopyridine (DMAP) as a catalyst to the reaction mixture for complete conversion.

The complete deprotection and detachment of the dendrimer from polymer was carried out using TFSA (1 ml) in TFA (10 ml) in the presence of thioniane (1 ml) and ethanedithiol (0.5 ml) for 1 h at 0 °C and then 1.5 h at room temperature. The mixture was diluted with ethyl ether (30 ml) and filtered. The dendrimer was dissolved in TFA (10 ml), filtered to remove the resin, and precipitated by dry ether (100 ml).

The crude D3G2 and D3K2 dendrimers were isolated by gel filtration on Sephadex G-50 column (2.5 × 50 cm) using 10% acetic acid as eluent and purified by RP-HPLC in water-acetonitrile-0.1% trifluoroacetic acid system with the use of linear gradient of acetonitrile. The isolated fractions had 95% purity by counting from the baseline (UV detection performed at 230 nm). After lyophilization of the corresponding fractions, the purification degree of product was analyzed by RP-HPLC on the Shimadzu LC-20 Prominence system (Japan) chromatograph. Luna C18(2), 4.6 × 150 mm column, 5 μm for analytical chromatography and Discovery C18 21.6 × 250 mm column, 5 μm for preparative chromatography were used.

Characterization of D3K2 and D3G2 dendrimers [63] was performed by an established NMR approach [80,81].

5.3. Cell culture

HeLa human cervix adenocarcinoma (ATCC® CCL-2™) and HMEC-1 human microvascular endothelial (ATCC® CRL-3243™) cell lines were purchased from American Type Culture Collection (ATCC, Rockville, USA). The newly received cells were expanded and aliquots of less than 10 passages were stored in liquid nitrogen. Cells were kept at low passages, returning to original frozen stocks every 3 months. During the course of the study, the cells were thawed and passaged within 3 months in each experiment. HeLa cells were grown as a monolayer in DMEM growth medium supplemented with 10% fetal bovine serum, penicillin (10 U/ml) and streptomycin (50 μg/ml), while HMEC-1 cells were grown as a monolayer in MCDB131 growth medium supplemented with 10 ng/ml Epidermal Growth Factor (EGF), 1 μg/ml hydrocortisone, 10 mM glutamine, 10% fetal bovine serum, penicillin (10 U/ml) and streptomycin (50 μg/ml), at 37 °C in an atmosphere of 5% CO2. HeLa and HMEC-1 cells were subcultured 24 h prior the treatment to ensure they were in the exponential growth phase. The cells were trypsinized, suspended in fresh complete medium, centrifuged at 200 × g for 5 min and seeded into appropriate plates at the optimal density. After 24 h the dendrimers at selected concentrations were added to the plates for 24 or 72 h, depending on the experiment.

5.4. Cytotoxicity assays

5.4.1. MTT assay

The cytotoxic effect of analyzed dendrimers was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazol (bromide) method. Briefly, different concentrations of D3K2 and D3G2 dendrimers were added to the 96-well plates containing cells at the density of 2.0 × 104 cells/well. Cells were incubated with the dendrimers for 24 and 72 h in a 37 °C humidified atmosphere containing 5% CO2. After the incubation period, cells were washed with phosphate buffered saline (PBS). Next, 50 μL of a 0.5 mg/mL solution of MTT in PBS was added to each well and cells were further incubated under standard conditions for 3 h. The residue MTT solution was subsequently removed and the obtained formazan precipitate was dissolved in DMSO (100 μL/well). The conversion of the tetrazolium salt (MTT) to a colored formazan by mitochondrial and cytosolic dehydrogenases is a marker of cell viability. Plates were shaken for 1 min and absorbance at 570 nm
was measured using the PowerWave HT Microplate Spectrophotometer (BioTek, USA).

5.4.2. Crystal violet assay
Cytotoxic effect of the analyzed dendrimers was additionally assessed by the crystal violet method. Briefly, different concentrations of D3K2 and D3G2 dendrimers were added to the 96-well plates containing cells at the density of 2.0 × 10^5 cells/well. Cells were incubated with the dendrimers for 24 and 72 h in a 37 °C humidified atmosphere containing 5% CO₂. After the incubation period, cells were fixed with 70% ethanol for 30 min in room temperature. Then crystal violet staining solution (0.2% crystal violet in 20% methanol) was added for 10 min. After incubation, the staining solution was removed and the obtained precipitate was dissolved in 0.1 M sodium acetate ethanol solution (50 μL/well). Crystal violet cannot penetrate the cellular membrane of living cells, and stains only the DNA of dead cells, thus being a marker of cell viability. The plates were shaken for 1 min and the absorbance at 570 nm was measured using the PowerWave HT Microplate Spectrophotometer (BioTek, USA). Crystal violet penetrates the cellular membrane of living cells, and stains only the DNA of dead cells, thus being a marker of cell viability. The plates were shaken for 1 min and the absorbance at 570 nm was measured using the PowerWave HT Microplate Spectrophotometer (BioTek, USA).

5.5. Measurement of DNA damage - comet assay
DNA comet assay was performed under alkaline conditions. Briefly, different concentrations of D3K2 or D3G2 dendrimers were added to the 12-well plates containing cells at the density of 2.0 × 10^5 cells/well. Cells were incubated with the dendrimers for 24 and 72 h in a 37 °C humidified atmosphere containing 5% CO₂. After the incubation period, cells were washed with PBS, trypsinized and suspended in 0.75% low melting point agarose in PBS, pH 7.4. Next, 50 μL of this suspension was spread on frozen microscope slides coated with 1% normal melting agarose. After gelling, the slides were treated with lysis buffer containing 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10% DMSO and 10 mM Tris, pH 10 at 4 °C for 1 h. The slides were subsequently placed in the electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13) for 40 min to allow DNA unwinding. Electrophoresis was performed at 0.73 V/cm and 30 mA for 30 min. Then the slides were neutralized with 0.4 M Tris, pH 7.5 and stained with 2 μg/ml DAPI. All these steps were performed in the dark to prevent additional DNA damage. 150 randomly selected cells from each slide were analyzed using fluorescence microscope (Olympus CX 40), equipped with a video camera and CaspLab freeware image analysis software. The percentage of DNA in the comet tail was chosen as an indicator of DNA damage.

5.6. Measurement of DNA damage during apoptosis - TUNEL assay
The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used in order to examine DNA damage associated with apoptosis. This method enables the detection of early stages of apoptosis by labeling 3'-OH ends of single- and double-stranded DNA fragments with Br-dUTP (bromlated deoxyuridine triphosphate nucleotides). Br-dUTP is detected by the fluorescein-labeled anti-BrdU monoclonal antibody. Both dendrimer-treated and control cells were processed according to the Apo-BrdU In Situ DNA Fragmentation Assay Kit protocol supplied by the manufacturer (BioVision). Briefly, different concentrations of D3K2 and D3G2 dendrimers were added to the 12-well plates containing HeLa cells at the density of 2.0 × 10^5 cells/well. Cells were incubated with the dendrimers for 24 and 72 h in a 37 °C humidified atmosphere containing 5% CO₂. After the incubation period, cells were washed with PBS, trypsinized and fixed with 4% paraformaldehyde freshly prepared in PBS and incubated for 1 h at 37 °C in DNA Labeling Solution containing TdT Reaction Buffer, terminal deoxynucleotidyl transferase (TdT) and Br-dUTP. Next, the cells were suspended in solution containing anti-BrdU-FITC antibody (in total darkness for 30 min in room temperature) and incubated with the propidium iodide/RNase A solution. The cell fluorescence was measured using Becton Dickinson LSR II flow cytometer (BD Biosciences, USA). The green fluorescence of FITC at 520 nm and the red fluorescence of propidium iodide at 623 nm were detected. The number of TUNEL-positive cells was expressed as a percentage of the total number of cells in the sample.

In addition, fluorescent images were taken for each variant of the experiment. Confocal microscopy images were obtained under 6300 × magnification with SP-8, Leica (USA). For this purpose, cells were grown on 96-well glass-bottom plates and incubated with both dendrimers for 24 and 72 h in 37 °C humidified atmosphere containing 5% CO₂. After the incubation, cells were treated as before and imaged in green channel (excitation 490 nm, emission 510–575 nm) and far-red channel (excitation 595 nm, emission 600–740 nm).

5.7. Transfection
5.7.1. Plasmid preparation
Plasmid pcDNA3-EGFP with enhanced green fluorescent protein (a gift from Doug Golenbock (Addgene plasmid # 13031)) was amplified in E. coli. Then the transfection-grade plasmid DNA was isolated from E. coli with PureLink™ HiPure Plasmid MaxiPrep Kit (Thermo Fisher Scientific, USA). DNA purity assessment was performed on NanoDrop (Thermo Fisher Scientific, USA).

5.7.2. Transfection procedure
Briefly, different concentrations of plasmid transfection complexes were added to 12-well plates containing cells at the density of 2.0 × 10^5 cells/well. Plasmid transfection complexes were prepared by mixing D3K2 and D3G2 dendrimers at concentrations of 5 or 50 µM with an enhanced green fluorescent protein plasmid (pcDNA3-EGFP; 3, 4 or 5 µg) in OptiMEM medium (Gibco) and incubating for 15 min in room temperature. Then, the cells were incubated with plasmid transfection complexes for 24 and 72 h in a 37 °C humidified atmosphere containing 5% CO₂. Transfection control complexes with lipofectamine (Lipofectamine 2000, Thermo Fisher Scientific, USA) in OptiMEM medium were also prepared by mixing with an enhanced green fluorescent protein plasmid (pcDNA3-EGFP; 3, 4 or 5 µg) at appropriate concentrations according to the manufacturer’s protocol. Before the treatment, OptiMEM medium was added to dilute the complexes so that each complex contained 0.25 µg of DNA in a total volume of 100 µL in one well of a 96-well plate. After removing the complete medium from the cells, complexes were added. Upon the incubation period, the cells were washed with PBS, trypsinized, suspended in full growth medium and transferred to flow cytometry tubes. The fluorescence was measured using Becton Dickinson LSR II flow cytometer (BD Biosciences, USA). The green fluorescence at 520 nm was measured, and the number of EGFP-positive cells was expressed as a percentage of total number of cells in the sample. Moreover, cytotoxicity of dendriplexes with plasmid DNA (dendrimer:pcDNA3-EGFP; 50 µM:3 µg) and lipofectamine with plasmid DNA complex (lipofectamine:pcDNA3-EGFP = 3 µL:3 µg) towards HeLa and HMEC-1 cells was assessed with the use of MTT assay.

5.8. Statistics
For statistical analysis we used one-way ANOVA for concentration series followed by post-hoc Tukey's test for pairwise difference testing. For single pairwise comparisons, Student’s t test was applied. In all tests, p values < 0.05 were considered to be statistically significant. Data were presented as arithmetic mean ± S.D.

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