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The Influence of Maltotriose-Modified Poly(propylene imine) Dendrimers on the Chronic Lymphocytic Leukemia Cells *in Vitro*: Dense Shell G4 PPI

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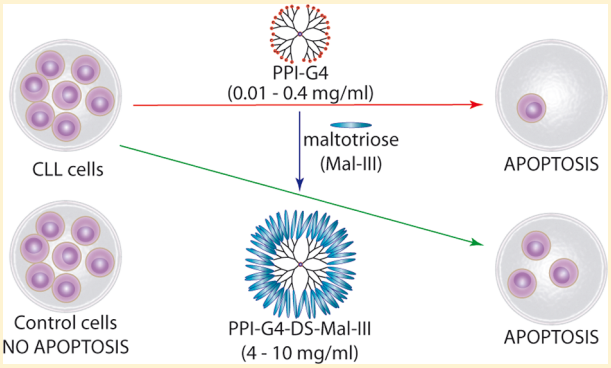
Supporting Information

ABSTRACT: Chronic lymphocytic leukemia (CLL) is the most common leukemia in Europe and North America. For many years scientists and doctors have been working on introducing the most effective therapy into CLL as prognosis of survival time and the course of the disease differ among patients, which might pose a problem in treating. Nanotechnology is providing new insights into diagnosis and, compared with conventional treatments, more efficient treatments, which might improve patients' comfort by decreasing side effects. Among the various nanoparticles that are available, dendrimers are one of the most promising. The aim of this study was a preliminary assessment of the clinical value of treating CLL patients with fourth generation poly(propylene imine) (PPI) dendrimers—either unmodified (PPI-G4) or approximately 90% maltotriose-modified (PPI-G4-DS-Mal-III). PPI-G4-DS-Mal-III dendrimers have, in contrast to the cationic PPI-G4, a neutral surface charge and are characterized by low cyto-, geno-, and hematotoxicity *in vitro* and *in vivo*. For the *in vitro* study we used blood mononuclear cells collected from both untreated CLL patients and from healthy donors. Apoptosis was measured by an annexin-V (Ann-V)/propidium iodide (IP) assay, and mitochondrial membrane potential was estimated with use of Mito Tracker Red CMXRos. Presented results confirm the influence of dendrimers PPI-G4 and PPI-G4-DS-Mal-III on apoptosis and CLL lymphocytes viability in *in vitro* cultures. Both tested dendrimers demonstrated higher cytotoxicity to CLL cells than to healthy donors cells, whereas unmodified dendrimers were more hematotoxic. The surface modification clearly makes glycodendrimers much more suitable for biomedical applications than unmodified PPI-G4; therefore further biological evaluations of these nanoparticles are conducted in our laboratories.



KEYWORDS: *apoptosis, chronic lymphocytic leukemia (CLL), dendrimers, modified dendrimers, poly(propylene imine) (PPI)*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in Europe and North America. It usually affects elderly people (81% of patients are over 60), but recently it has been observed more frequently in younger people. Prognosis of survival time and the course of the disease differs among patients, and this can pose a problem in treating this usually benign disease. In about one-third of patients the course of the disease is stable, and early decisions about chemotherapy do not seriously affect survival times.[1,2] However, in most patients, early intensification of symptoms and rapid progression are observed, and survival time is consequently considerably shorter despite prompt treatment.[3,4] For many years, scientists and doctors have been working on introducing the most effective therapy into CLL.

Nanotechnology is a new and promising scientific tool in medicine and the pharmaceutical industry where nanoparticles can be used to deliver drugs (hydrophilic and hydrophobic), proteins, vaccines, and different biological macromolecules to the body. This may open a window of therapeutic opportunity for some known drugs. Nanoparticles are better suited for intravenous delivery than larger microparticles. Most narrow capillaries in the body are 5−6 μm in diameter. Therefore, preventing particle-induced embolism, particles distributed in the bloodstream, must be significantly smaller than 5 μm. It can

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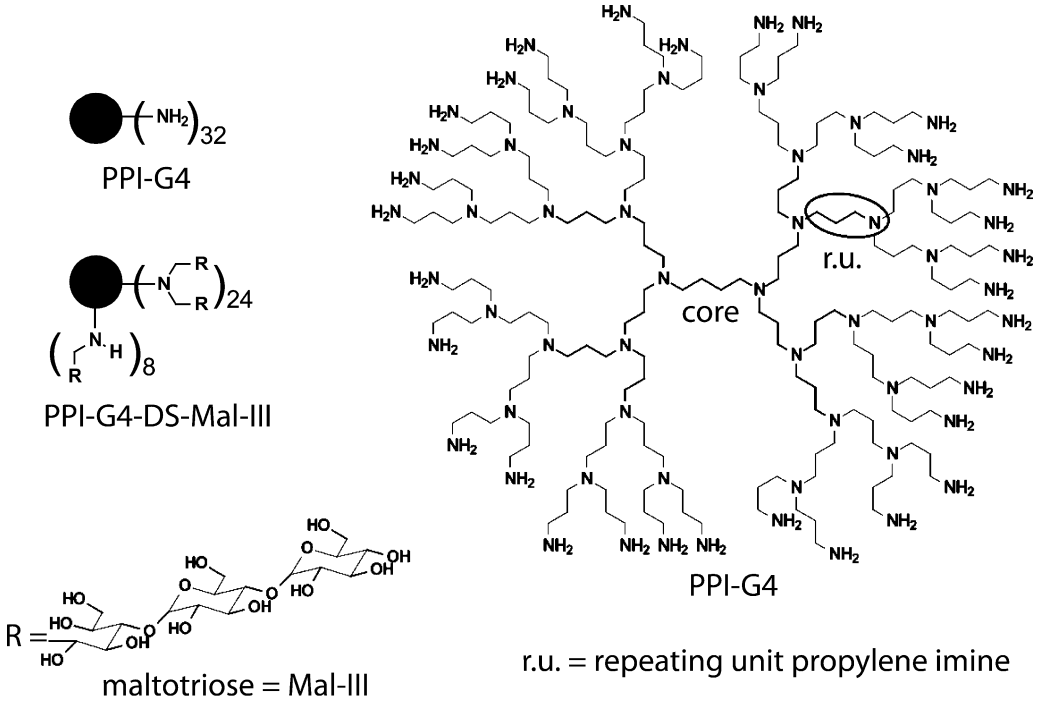

Figure 1. Simplified structure of PPI-G4 and PPI-G4-DS-Mal-III.

be assumed that molecular medicine technologies at the nanolevel are providing new insights into diagnosis, more efficient treatments, which might improve patients' comfort decreasing side effects compared with conventional treatments.[5,6]

Among the various nanoparticles that are available, macromolecules of dendritic architecture, called dendrimers, deserve attention. For the first time they were synthetized in 1985 by two independent scientists' teams, Tomalia et al.[7] and Newkome et al.,[8] and since then the interest in this dendritic architecture has been escalating. They are perfectly branched, monodisperse macromolecules with the precisely controlled chemical structure and a high density of surface functionalities. This also exhibits a three-dimensional structure and different density of molecular structure,[9,10] in which all bonds emerge radially from a central core with repeating and branching units. Furthermore, each additional layer of repeating and branching units creates the next generation of the dendrimer. Depending on the generation, dendrimers size can be up to 10 nm. Their structure provides two strategies of carrying drugs—complexation and conjugation. It has already been reported in oncological research papers that chemotherapeutics such as paclitaxel, 5-fluorouracyl, doxorubicin, and methotrexate were encapsulated inside dendrimers when applying unimolecular micelles[11−14] or attached to their surfaces.[15−18] However, they can also interact with nucleic acids, where cationic dendrimers are involved as vectors for gene transfection.[19] In addition to being molecular carriers and therapeutic agents, they were also used as contrast agents in magnetic resonance imaging (MRI).[20] Furthermore, the application of poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) dendrimers to the encapsulation of photosensitizers for use in photodynamic therapy has been reported.[21]

The aim of this study was a preliminary assessment of the clinical value of treating chronic lymphocytic leukemia (CLL) patients with PPI dendrimers. The assessment was based on the *in vitro* induction of cytotoxicity and apoptosis by fourth generation PPI dendrimers—either unmodified (PPI-G4) or with approximately 90% of the surface amino groups substituted with maltotriose [Mal-III] residues [dense shell (PPI-G4-DS-Mal-III)][22] (Figure 1). PPI-G4-DS-Mal-III dendrimers have, in contrast to the parental cationic PPI-G4, a neutral surface charge as known from PPI dendrimers with dense maltose shell.[23] Because of this neutral surface charge, they are preferentially involved in H-bond driven interactions.[24] Based on others and own expertise dendrimer's toxicity with peripheral amino groups is generation-dependent where the higher generations are more toxic as the number of surface groups increases with each generation. Thus, we have chosen PPI dendrimers of the fourth generation (G4), as they appear to have relatively low toxicity as well as satisfactory biodistribution.[25,26] The reduced cytotoxicity of PPI dendrimers was obtained by the surface modification with galactose[27] or lactose and mannose.[28] Moreover, as we previously described, a dense maltose shell on the PPI dendrimers surface significantly reduced the hemolysis level.[24] The choice of dendrimers for this study was based on our previous data with PPI glycodendrimers (maltose or maltotriose modified) and their low cyto- and hematotoxicity *in vitro*[24,25,29] and *in vivo*.[26]

EXPERIMENTAL SECTION

Patients. Fresh peripheral blood samples were collected from 20 untreated CLL patients (10 men, 10 women) who were diagnosed and followed at the Hematology Department, Medical University, Łódź, Poland and 5 healthy volunteers. The mean age of CLL patients was 64.70 years (range 49−85). The CLL diagnosis was based on IWCLL criteria.[30] The Ethics Committee of the Medical University of Lodz, Poland approved the study (RNN/75/10/KE). Informed consent was obtained from all patients involved in the study.

Dendrimers. Uncoated fourth generation PPI dendrimers (PPI-G4) were purchased from Symo-Chem (Eindhoven, The Netherlands). Dendrimers in which approximately 90% of peripheral amino groups were coated with maltotriose have been defined as PPI-G4-DS-Mal-III. The abbreviation DS describes the dense shell for the structure of carbohydrate modified dendrimers. The synthesis and characterization of maltotriose-modified fourth generation PPI dendrimers were carried out as described previously.[22] The structures of dendrimers used are shown in Figure 1. The characterization of the dendrimers is shown in Table 1.

Table 1. Molar Mass (MM[a]) for Commercially Available PPI-G4 and Synthesized PPI-G4-DS-Mal-III and the Number and the Percentage of Surface Maltotriose Groups

dendrimer	$MM_{theoretical}$, g/mol	$MM_{observed}$, g/mol	number (percentage) of surface Mal-III $groups_{theoretical}$	number (percentage) of surface Mal-III $groups_{observed}$
PPI-G4	3514	3514		
PPI-G4-DS-Mal-III	34774	31000	64 (100%)	56 (87%)

[a]MM of PPI-G4-DS-Mal-III determined by LILBID MS method described in ref 22.

Isolation of Mononuclear Cells and CLL Cell Cultures *in Vitro*. Peripheral blood mononuclear cells (MNCs) were separated from EDTA blood by layering on the Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuging on a density gradient. An enriched buffy coat of MNCs was isolated and washed twice in RPMI-1640 medium (Invitrogen, Scotland). Afterward MNCs at a concentration of 1×10^6 cells/mL were suspended in the above-mentioned medium supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen, Scotland) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Invitrogen, Scotland) and cultured for up to 48 h in an atmosphere of 5% CO_2, at a temperature of 37 °C, and in full humidity (98%). MNCs were incubated with dendrimers at the following concentrations:

1. PPI-G4: 0.01 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.2 mg/mL, 0.4 mg/mL;
2. PPI-G4-DS-Mal-III: 4 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/mL.

The cultures without dendrimers were considered as control.

Cytotoxicity and Apoptosis Assays. Apoptosis was measured by annexin-V (Ann-V)/propidium iodide (IP) assay. IP is a cationic compound with autofluorescence feature, which is actively eliminated from normal cells keeping membrane cell integrity (IP^- cells). Ann-V changes the integrity of the cell membrane. It releases phosphatidylserine (PS) into the outer monolayer of the bilayer. Cytotoxicity of the compound is visible in the IP release to the environment (IP^+ cells). One of early apoptosis features is phosphatidylserine externalization in phospholipid cell membrane. Ann-V—a protein dependent on Ca^{2+} ions—binds in a close proximity to PS, which gives a possibility to quantify the level of apoptosis observed in the examined cell population. IP dye is used to distinguish between apoptotic and necrotic cells. Due to its electric charge IP does not penetrate the healthy cell membranes. Necrotic and apoptotic cells, characterized by the disturbed membrane integrity, allow IP in. IP binds to nucleic acids, giving a red fluorescent signal. The results were given as a percentage of cells positively stained with Ann-V-FITC (not IP) and cells stained with IP (not Ann-V). Positive reaction with Ann-V, which locates closely to PS, indicates the presence of cells in an early apoptosis stage. PS relocation from the inner monolayer of the membrane to the membrane surface layer is an initial phase of commencing apoptosis. A positive reaction to IP indicates the cells in the late phase of apoptosis. The examined probes were washed twice in 5 mL of PBS (Lonza Ltd.) and then centrifuged 5 min at 1100 rotations/minute (218*g*). Then, cells were suspended in the mixture of 50 μL of PBS and 10 μL of IP (Sigma Aldrich, Germany). After 10-min incubation in the darkness at room temperature the viability of cells was estimated in relation to the appropriate controls. All fluorescence measurements were performed by flow cytometry FACSCalibur (Becton Dickinson, USA) equipped with argon laser 488 nm and computer program CellQuestPro (Becton Dickinson, USA). Low fluorescence debris and necrotic cells, permeable to IP, were gated out before the analysis, and 10 000 events were evaluated. The fluorescence was measured using standard emission filters: green − FL1 (wavelength λ = 490 ± 20 nm) and red − FL3 (λ = 530−630 nm). This assay was also used to measure spontaneous apoptosis in freshly isolated MNCs.

Estimation of Mitochondrial Membrane Potential. Mitochondrial membrane potential changes are one of the first markers of apoptosis. *Chloromethyl-X-rosamine* (Mito Tracker Red CMXRos, Molecular Probes, USA) and a monoclonal antibody to glycophorin A conjugated with FITC (Anti-Glycophorin A FITC, Dako, Denmark) were used for CMXRos evaluation. CMXRos is a lipophilic cationic dye that accumulates in the mitochondrial matrix of viable cells when there is an electronegative load on the inner surface of the mitochondrial membrane. This asymmetric location of protons along the inner mitochondrial membrane leads to a high mitochondrial membrane potential ($\Delta\Psi$m) in living cells. Decreased $\Delta\Psi$m is one of the first consequences of apoptosis, lowering the mitochondrial uptake of CMXRos.

In the executive apoptosis phase, consisting of many distinct pathways typical of excitation phase, irreversible triggering of the death program takes place. The early signal of apoptosis opens the mitochondrial mega-channels, which leads to an increase in membrane potential ($\Delta\Psi$m), limitation of ATP synthesis, and the increase of Ca^{2+} concentration in mitochondrial matrix. As a consequence there is a leak of cytochrome c (also called APAF2) and other proapoptotic proteins from mitochondria to cytosol.

The stock CMXRos solution was prepared by dilution with 94 μL of DMSO (dimethyl sulfoxide) and stored at −20 °C. The working solution was prepared with DMSO (1:10) and stored at 4 °C. MNCs (1×10^6 /ml) was incubated with CMXRos (2.5 μL of working solution in 500 μL f RPMI 1640 medium) at 37 °C for 15 min before the addition of 5 μL of monoclonal antibody (MoAb) against glycophorin A (conjugated with FITC) and then incubated for a further 15 min. This MoAb eliminates any contaminating erythrocytes in the sample, which may influence the results owing to their low mitochondrial potential. The samples were measured using flow cytometry. The percentage of cells not bound to antiglycophorin A-FITC ($\Delta\Psi m^{low}$/Gly-A cells), with low mitochondrial potential, was calculated.

Statistical Analysis. Descriptive statistics—mean (X), standard deviation (SD), median (Me), lower quartile ($Q_{0.25}$), and upper quartile ($Q_{0.75}$)—were calculated for all data. The

Table 2. Influence of PPI-G4 Dendrimers on the Apoptosis and Viability of CLL Cells in *in Vitro* Cultures[a]

	PPI-G4																		
	control			0.01 mg/mL			0.025 mg/mL			0.05 mg/mL			0.2 mg/mL			0.4 mg/mL			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	statistical analysis
	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	(P value)
										24 h									
n	20	20	20	10	10	10	5	5	5	10	10	10	6	6	6	6	6	6	1 vs 4 = 0.02; 2 vs 5 = 0.001; 2 vs 8 = 0.001; 2 vs 11 = 0.001; 2 vs 14 = 0.001; 2 vs 17 = 0.001; 3 vs 6 = 0.05; 3 vs 18 = 0.05
X	5.01	6.45	4.04	13.70	22.16	2.28	15.44	31.09	3.97	9.08	41.02	4.39	8.15	75.44	3.41	11.41	82.63	2.08	
SD	3.90	5.55	2.72	10.75	14.28	2.34	17.00	13.81	2.72	5.58	27.02	3.43	4.39	22.47	5.76	8.95	8.53	3.91	
Me	4.40	3.82	3.19	13.79	15.84	0.78	4.48	25.31	3.27	9.10	38.30	4.2	6.77	87.49	0.15	11.08	85.18	0.35	
$Q_{0.25}$	2.19	2.54	1.70	4.35	11.23	0.41	2.46	21.42	1.55	4.00	15.03	0.64	5.22	51.28	0.00	3.69	73.13	0.00	
$Q_{0.75}$	6.75	11.40	6.77	23.20	40.90	3.34	33.91	43.64	6.75	13.37	70.58	7.19	11.29	91.52	8.05	15.92	88.61	3.85	
CAI (%)					24.4			35.07			38.64			72.13			82.58		
IC_{50}											0.13 mg/mL								
										48 h									
n	20	20	20	10	10	10	4	4	4	10	10	10	6	6	6	6	6	6	1 vs 16 = 0.05; 2 vs 5 = 0.004; 2 vs 8 = 0.04; 2 vs 11 = 0.008; 2 vs 14 = 0.001; 2 vs 17 = 0.001; 3 vs 6 = 0.002; 3 vs 15 = 0.04
X	10.19	11.50	9.12	16.64	30.95	1.62	9.87	23.21	15.97	20.35	37.01	10.52	15.01	77.54	3.03	19.94	70.16	8.41	
SD	7.85	10.17	7.89	14.30	20.20	1.37	3.85	7.97	23.55	21.07	30.37	21.14	10.96	14.65	4.60	15.05	9.37	12.95	
Me	7.60	8.04	10.08	12.85	23.78	1.40	12.85	23.78	1.40	12.85	23.78	1.40	18.15	80.65	0.25	23.35	74.80	0.25	
$Q_{0.25}$	3.92	3.92	2.30	4.90	14.50	0.30	6.31	13.88	2.01	5.30	11.31	1.01	1.98	62.33	0.00	2.34	60.26	0.00	
$Q_{0.75}$	14.40	15.75	12.14	20.64	47.23	2.96	14.54	31.31	43.27	22.45	52.16	11.39	24.17	90.26	7.03	29.55	76.74	20.2	
CAI (%)					25.9			11.39			35.67			70.86			68.41		
IC_{50}											0.12 mg/mL								

[a] n − number of samples; X − mean percentage of apoptotic or necrotic cells; SD − standard deviation; Me − median percentage of apoptotic or necrotic cells; $Q_{0.25}$ − lower quartile; $Q_{0.75}$ − upper quartile; IC_{50} − the concentration of the dendrimer that caused 50% cytotoxicity of MNCs; early apoptosis − Ann^+IP^-; late apoptosis − Ann^+IP^+; necrosis − Ann^-IP^+; Ann − annexin-V; IP − propidium iodide; CAI − compensating apoptotic index.

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Table 3. Influence of PPI-G4-DS-Mal-III Dendrimers on the Apoptosis and Viability of CLL Cells in *in Vitro* Cultures[a]

	PPI-g4-DS-Mal-III															
	control			4 mg/mL			6 mg/mL			8 mg/mL			10 mg/mL			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	statistical analysis
	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	(*P* value)
									24 h							
n	20	20	20	20	20	20	20	20	20	20	20	20	15	15	15	1 vs 4 = 0.002;
X	5.01	6.45	4.04	15.35	13.62	3.11	19.90	15.51	2.79	22.01	15.74	4.15	27.97	18.90	6.73	1 vs 7 = 0.002;
SD	3.90	5.55	2.72	12.74	13.34	3.89	15.43	13.48	4.40	16.43	13.34	7.53	19.04	15.45	7.16	1 vs 10 = 0.002;
Me	4.40	3.82	3.19	10.40	8.95	1.40	16.20	11.15	0.85	18.75	13.15	0.86	23.70	14.68	3.68	1 vs 13 = 0.001;
$Q_{0.25}$	2.19	2.54	1.70	7.35	3.17	0.72	8.62	4.91	0.28	7.97	4.15	0.40	13.72	6.22	1.10	4 vs 10 = 0.008;
$Q_{0.75}$	6.75	11.40	6.77	21.95	19.42	4.67	27.26	18.85	4.04	30.53	19.02	3.69	35.22	27.58	14.70	2 vs 5 = 0.007;
CAI (%)					17.51			23.95			26.02			35.41		2 vs 8 = 0.002;
																2 vs 11 = 0.002;
																2 vs 14 = 0.002;
																5 vs 11 = 0.05
									48 h							
n	20	20	20	20	20	20	20	20	20	20	20	20	15	15	15	1 vs 4 = 0.007;
X	10.19	11.50	9.12	16.03	19.80	8.36	19.09	22.69	10.28	19.87	25.70	7.79	22.91	40.71	9.77	1 vs 7 = 0.002;
SD	7.85	10.17	7.89	13.60	17.32	8.05	17.47	18.94	10.23	16.69	19.84	9.17	15.37	18.58	5.60	1 vs 10 = 0.003;
Me	7.60	8.04	10.08	11.20	13.6	5.20	12.00	20.32	4.10	14.40	25.81	2.79	31.50	46.60	5.28	1 vs 13 = 0.002;
$Q_{0.25}$	3.92	3.92	2.30	3.50	3.90	1.80	4.70	5.00	2.09	5.40	6.07	1.92	11.20	13.74	3.21	2 vs 5 = 0.009;
$Q_{0.75}$	14.40	15.75	12.14	28.70	37.58	13.09	31.20	40.70	21.60	34.90	41.44	16.39	40.76	59.87	16.21	2 vs 8 = 0.0009;
CAI (%)					14.14			20.09			23.88			41.93		2 vs 11 = 0.002;
IC_{50}														10 mg/mL		2 vs 14 = 0.001;
																5 vs 8 = 0.03;
																5 vs 11 = 0.02

[a] *n* − number of samples; *X* − mean percentage of apoptotic or necrotic cells; SD − standard deviation; Me − median percentage of apoptotic or necrotic cells; $Q_{0.25}$ − lower quartile; $Q_{0.75}$ − upper quartile; IC_{50} −the concentration of the dendrimer that caused 50% cytotoxicity of MNCs; early apoptosis − Ann^+IP^-; late apoptosis − Ann^+IP^+; necrosis − Ann^-IP^+; Ann − annexin-V; IP − propidium iodide; CAI − compensating apoptotic index.

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Table 4. Influence of PPI-G4-DS-Mal-III Dendrimers on CLL Cells' Mitochondrial Potential in Cultures *in Vitro*; The Comparison with Purine Analogue (Fludarabine; FA) and Monoclonal Antibody (Rituximab; Rit)[a]

	control	PPI-G4-DS-Mal-III 4 mg/mL	PPI-G4-DS-Mal-III 6 mg/mL	PPI-G4-DS-Mal-III 8 mg/mL	FA 1.6 μM	Rit 10 μg/mL	statistical analysis
	1	2	3	4	5	6	(P value)
				24 h			
n	16	16	16	16	16	16	1 vs 2 = 0.015; 1 vs 3 = 0.003; 1 vs 4 = 0.002; 1 vs 5 = 0.001; 1 vs 6 = 0.018; 5 vs 6 = 0.004; 2 vs 6 = 0.01; 3 vs 6 = 0.01; 4 vs 6 = 0.001; 2 vs 3 = 0.026; 3 vs 4 = 0.001
X	34.23	44.79	48.44	56.21	50.09	41.79	
SD	16.02	13.41	12.91	13.26	16.66	16.60	
Me	31.30	44.55	46.90	55.00	47.90	35.65	
$Q_{0.5}$	21.45	36.30	38.12	44.92	39.30	28.22	
$Q_{0.75}$	50.57	53.20	58.27	70.02	61.80	53.65	
				48 h			
n	16	16	16	16	16	16	1 vs 2 = 0.013; 1 vs 3 = 0.005; 1 vs 4 = 0.005; 1 vs 5 = 0.005; 1 vs 6 = 0.013; 5 vs 6 = 0.002; 3 vs 6 = 0.006; 2 vs 5 = 0.009; 4 vs 5 = 0.046; 2 vs 3 = 0.002; 2 vs 4 = 0.002; 3 vs 4 = 0.002
X	38.44	58.21	65.76	75.40	69.94	53.34	
SD	10.81	15.85	16.88	15.58	17.49	16.67	
Me	33.65	51.70	56.30	71.50	64.80	47.70	
$Q_{0.25}$	29.36	41.85	46.10	56.71	50.60	35.20	
$Q_{0.75}$	45.15	65.70	78.20	85.75	83.45	59.45	

[a] n − number of samples; X − mean percentage of cells with lower mitochondrial potential ($\Delta\Psi m^{low}$/Gly-A$^-$ [%]); SD − standard deviation; Me − median percentage of cells with lower mitochondrial potential ($\Delta\Psi m^{low}$/Gly-A$^-$ [%]); $Q_{0.25}$ − lower quartile; $Q_{0.75}$ − upper quartile.

Shapiro-Wilk test was used to evaluate the normality of distribution, and th eF-Snedecor test was used as an equal variance test. Comparisons of values between the experimental and control cultures were made with the independent t test. When the normality test failed, the Mann−Whitney Rank Sum test was used. A P value of < 0.05 was considered to be statistically significant.

IC_{50} value was defined as the concentration of dendrimers that caused 50% cytotoxicity of MNCs. The apoptotic index (AI) was expressed as the percentage of Ann-V positive cells. The compensating apoptotic index (CAI) was calculated as the difference between AI in the presence of dendrimer and the percentage of cells undergoing the spontaneous apoptosis in control cultures. For significant CAI the values of 10% or greater were chosen.

RESULTS

CLL Cells. The percentage of apoptotic MNCs induced by dendrimers PPI-G4 (c = 0.01−0.4 mg/mL) and PPI-G4-DS-Mal-III (c = 4−10 mg/mL) after 24 and 48 h incubations was significantly higher than the percentage of spontaneous apoptotic leukemic cells (Tables 2 and 3, Supporting Information). The incubation of leukemic cells with PPI-G4 dendrimers resulted in the increasing CAI values along with the higher concentration of the dendrimer (Table 2). However, a longer incubation period did not increase significantly the cytotoxic effect of the dendrimer. High CAI values (range 24.4−82.58%) indicate the high cytotoxicity of the PPI-G4 dendrimer. Lower CAI values were quantified for PPI-G4-DS-Mal-III (range 14.14−41.93%) which indicates the smaller toxicity of this nanoparticle (Table 3). However, a longer incubation time of the dendrimer at the concentration of 10 mg/mL significantly increased CAI to 41.93%. PPI-G4-DS-Mal-III glycodendrimers have induced MNCs' apoptosis better after 48 h than 24 h. The greatest differences were observed for Ann-V$^+$IP$^+$ cells at the concentration 8 mg/mL (p = 0.002) and 10 mg/mL (p = 0.001) (Table 3). Similar results were observed for PPI-G4 at the concentration of 0.01 mg/mL (p = 0.001) (Table 2). PPI-G4-DS-Mal-III markedly induced early apoptosis (Ann-V$^+$IP$^-$) of MNCs after 24 h incubation at concentrations of 4 mg/mL, 6 mg/mL, 8 mg/mL (p = 0.002), and 10 mg/mL (p = 0.001) as well as after 48 h (Ann-V$^+$IP$^-$) at the above-mentioned concentrations (p = 0.007; p = 0.002; p = 0.003; p = 0.001; respectively). PPI-G4 dendrimers at concentrations 0.01 mg/mL and 0.05 mg/mL induced late apoptosis more after 24 h (p = 0.001 at both concentrations) than after 48 h (p = 0.004 and p = 0.04, respectively), whereas at concentrations of 0.2 mg/mL and 0.4 mg/mL the mechanism of late apoptosis was equally induced after 24 and 48 h (p = 0.001) (Table 2). The late apoptosis (Ann-V$^+$IP$^+$) was more efficiently induced by PPI-G4-DS-Mal-III at the above-mentioned concentrations after 48 h incubation (p = 0.009; p = 0.0009; p = 0.002; p = 0.001); however the late apoptosis was statistically higher also after 24 h (p = 0.007; p = 0.002; p = 0.002; p = 0.002). PPI-G4-DS-Mal-III at the concentration of 8 mg/mL efficiently initiated the late apoptosis after 48 h incubation in comparison with control cells (p = 0.002), as opposed to 24 h (p = 0.002). The difference in percentage of Ann-V$^+$IP$^+$ cells between 24 and 48 h incubation was also statistically significant (p = 0.022) (Table 3). The differences between the control cells and necrotic cells (Ann-V$^-$IP$^+$) at any concentration of PPI-G4-DS-Mal-III after 24 and 48 h were not statistically significant, whereas the difference in percentage of necrotic cells between 24 and 48 h was significant at the concentration of 6 mg/mL (p = 0.037). The greatest percentage of necrotic cells was observed after the influence of PPI-G4 dendrimers at the concentration of 0.01 mg/mL and 0.4 mg/mL for 24 h incubation (p = 0.05) as well

Table 5. Influence of PPI-G4 Dendrimers on the Apoptosis and Viability of Healthy MNCs in *in Vitro* Cultures[a]

	control			PPI-G4															
				0.01 mg/mL			0.025 mg/mL			0.05 mg/mL			0.2 mg/mL			0.4 mg/mL			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	statistical analysis
	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	(P value)
										24 h									
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	2 vs 8 = 0.04; 2 vs 11 = 0.04; 2 vs 14 = 0.04; 2 vs 17 = 0.04; 3 vs 6 = 0.04
X	7.56	5.65	6.79	7.21	6.83	4.76	6.13	10.95	6.07	6.74	14.36	4.84	18.55	35.08	5.99	23.64	48.65	5.11	
SD	1.90	1.78	1.58	1.25	2.83	1.46	2.66	4.17	2.68	2.32	4.21	2.23	12.27	11.54	5.02	12.39	13.83	4.87	
Me	7.33	5.90	6.95	7.26	5.53	4.53	6.70	10.48	4.69	8.11	12.91	3.75	10.14	36.36	4.02	22.58	43.33	2.23	
$Q_{0.25}$	6.01	4.06	5.37	6.12	4.78	3.04	2.80	7.13	3.41	4.03	9.83	2.59	7.42	20.66	0.70	8.68	34.03	0.47	
$Q_{0.75}$	9.23	7.10	8.11	8.26	9.54	6.59	9.16	14.99	9.41	8.75	19.62	7.63	33.89	48.87	12.25	39.13	65.94	11.19	
CAI (%)					0			1.97			5.99			38.52			57.18		
IC_{50}															0.37 mg/mL				
										48 h									
n	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	2 vs 11 = 0.03; 2 vs 14 = 0.03; 2 vs 17 = 0.03
X	18.05	13.02	10.56	10.66	11.07	7.29	8.74	17.87	8.34	9.94	30.50	10.22	20.50	56.70	11.55	26.45	58.00	10.08	
SD	5.88	1.22	4.78	5.55	3.26	1.49	5.27	5.41	2.07	7.25	4.39	3.34	12.41	6.26	7.80	16.35	13.60	9.80	
Me	18.76	13.86	10.81	9.67	9.58	7.44	6.04	18.29	8.19	8.48	29.02	10.83	15.13	56.47	14.33	24.53	65.21	4.06	
$Q_{0.25}$	12.16	11.50	6.29	5.70	7.74	5.43	4.45	11.11	5.83	3.72	25.75	6.05	7.67	48.99	1.80	6.97	41.00	0.84	
$Q_{0.75}$	23.59	14.12	14.69	16.10	15.14	9.08	14.37	24.41	10.93	16.88	35.98	14.09	36.00	64.52	19.90	46.88	71.38	22.32	
CAI (%)					0			0			9.37			46.13			53.38		
IC_{50}												0.15 mg/mL							

[a] n − number of samples; X − mean percentage of apoptotic or necrotic cells; SD − standard deviation; Me − median percentage of apoptotic or necrotic cells; $Q_{0.25}$ − lower quartile; $Q_{0.75}$ − upper quartile; IC_{50} − the concentration of the dendrimer that caused 50% cytotoxicity of MNCs; early apoptosis − Ann^+IP^-; late apoptosis − Ann^+IP^+; necrosis − Ann^-IP^+; Ann − annexin-V; IP − propidium iodide; CAI − compensating apoptotic index.

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Table 6. Influence of PPI-G4-DS-Mal-III Dendrimers on the Apoptosis and Viability of Healthy Cells in *in Vitro* Cultures[a]

	PPI-g4-DS-Mal-III												
	control			4 mg/mL			6 mg/mL			8 mg/mL			
	1	2	3	4	5	6	7	8	9	10	11	12	statistical analysis
	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	Ann^+IP^-	Ann^+IP^+	Ann^-IP^+	(*P* value)
							24 h						
n	5	5	5	5	5	5	5	5	5	5	5	5	1 vs 7 = 0.04; 1 vs 10 = 0.04; 2 vs 5 = 0.04; 2 vs 8 = 0.04; 2 vs 11 = 0.04
X	11.00	4.11	8.27	15.42	5.99	6.80	16.66	6.28	5.41	17.43	6.56	6.34	
SD	1.97	0.92	1.57	1.61	0.65	1.48	1.05	0.88	1.72	1.72	1.38	3.32	
Me	10.26	3.90	8.74	14.59	6.07	6.75	16.08	6.02	4.58	17.07	5.50	4.18	
$Q_{0.25}$	8.93	3.27	6.85	13.82	5.18	5.45	15.63	5.30	3.88	15.45	5.36	3.27	
$Q_{0.75}$	13.44	5.06	9.45	17.43	6.76	8.16	17.97	7.38	7.35	19.58	8.27	10.48	
CAI (%)					6.3			7.83			8.88		
							48 h						
n	5	5	5	5	5	5	5	5	5	5	5	5	1 vs 7 = 0.03; 1 vs 10 = 0.03
X	18.05	13.02	10.56	18.16	14.09	10.20	22.88	13.59	10.18	26.40	17.26	7.93	
SD	5.88	1.22	3.41	6.07	1.66	2.15	7.34	2.56	3.59	7.62	4.29	2.17	
Me	18.76	13.86	10.81	19.20	14.48	11.13	25.44	13.44	8.73	25.36	15.59	7.47	
$Q_{0.25}$	12.17	11.51	6.30	12.25	12.02	7.51	15.22	10.47	6.42	19.50	12.73	5.44	
$Q_{0.75}$	23.59	14.13	14.69	23.57	15.97	12.42	29.28	16.80	14.66	33.82	22.62	10.64	
CAI (%)					1.18			5.4			12.59		

[a] *n* − number of samples; *X* − mean percentage of apoptotic or necrotic cells; SD − standard deviation; Me − median percentage of apoptotic or necrotic cells; $Q_{0.25}$ − lower quartile; $Q_{0.75}$ − upper quartile; IC_{50} −the concentration of the dendrimer that caused 50% cytotoxicity of MNCs; early apoptosis − Ann^+IP^-; late apoptosis − Ann^+IP^+; necrosis − Ann^-IP^+; Ann − annexin-V; IP − propidium iodide; CAI − compensating apoptotic index.

as for 48 h incubation at the concentration of 0.01 mg/mL (p = 0.002) and of 0.2 mg/mL (p = 0.04). Although PPI-G4 dendrimers at the concentration of 0.4 mg/mL caused the death of 83% cells after 24 h incubation and 70% cells after 48 h incubation, the mechanism of apoptosis prevailed upon necrosis (Table 2).

The results of this study show that the analyzed dendrimers exert significant inhibitory effects on the viability of leukemic cells, with IC_{50} value of 0.13 mg/mL for PPI-G4 dendrimer after 24 h incubation. After 48 h incubation IC_{50} value for the PPI-G4 dendrimers was 0.12 mg/mL (Table 2). For PPI-G4-DS-Mal-III dendrimer IC_{50} value was calculated of 10 mg/mL concentration after 48 h (Table 3). Moreover, CMXRos technique revealed apoptosis induction by PPI-G4-DS-Mal-III at each examined concentration in comparison with control cultures (Table 4). Its potency to trigger apoptosis is even better than fludarabine (FA) and monoclonal antibodies (Rit) widely used in CLL therapy.

Healthy Donors' Cells. The influence of PPI-G4 and PPI-G4-DS-Mal-III dendrimers on MNCs retrieved from healthy donors was also examined (Supporting Information). PPI-G4 at the concentrations of 0.01 mg/mL and 0.025 mg/mL indicated a very low toxicity (IP^+ cells) after 24 h incubation (11.59% and 17.02%, respectively) as well as after 48 h incubation (18.36% and 26.21%, respectively) (Table 5). For each concentration of PPI-G4 late apoptosis predominated for shorter as well as for longer incubation time. An IC_{50} value of 0.37 mg/mL after 24 h incubation was detected, whereas it was 0.15 mg/mL after 48 h incubation. A statistically significant difference in percentage of necrotic cells between control cells and those CLL cells exposed to the concentration of 0.01 mg/mL (p = 0.04) was observed only after 24 h incubation (Table 5).

PPI-G4-DS-Mal-III dendrimers at concentrations of 6 mg/mL and 8 mg/mL exerted a significant cytotoxic effect on healthy MNCs after 24 h incubation mostly via the early and late apoptosis (Table 6). After 48 h incubation with the above-mentioned dendrimer, statistically significant differences were determined only for early apoptotic cells at dendrimer's concentrations of 6 mg/mL (p = 0.03) and 8 mg/mL (p = 0.03). However, the cell viability was considerably greater after 24 h as well as 48 h in healthy lymphocytes as opposed to CLL cells (Table 6).

CAI values were much lower for healthy cells incubated with glycodendrimer than for uncoated PPI-G4. The highest CAI percentage (12.59%) was observed PPI-G4-DS-Mal-III after 48 h incubation at the concentration of 8 mg/mL (Table 6), whereas for PPI-G4 the highest CAI was 57.18% at the concentration of 0.4 mg/mL after 24 h incubation (Table 5).

CLL Cells vs Healthy Donors' Cells. Significant differences concerning the influence of PPI-G4 dendrimers on CLL MNCs and healthy donors' MNCs were found for late apoptotic cells at all examined concentrations except for the highest one (0.4 mg/mL) (p = 0.04). In healthy samples a significant percentage of necrotic cells was induced by the concentration of 0.1 mg/mL (p = 0.04), similarly as in CLL cells. After 48 h of incubation substantial differences were noticed at the concentration of 0.2 mg/mL for $Ann\text{-}V^+IP^+$ cells (p = 0.04) and for $Ann\text{-}V^-IP^+$ cells (p = 0.04).

Although there are visible differences between the groups in mean percentage of cells' viability after the influence of PPI-G4-DS-Mal-III dendrimers, they are not statistically significant. A further study on a bigger group of patients is to be conducted.

DISCUSSION

The development of highly selective drugs triggering specific molecular target is a desirable and ambitious objective in CLL treatment. The main reason for malignant disease formation, including CLL, is an imbalance between cells proliferation and their programmed death, called apoptosis.[31] Therefore, the majority of therapeutic strategies are based on the modulation

of apoptosis and cell cycle of malignant cells. The cytotoxic effect, which is caused by a majority of antitumor drugs used in oncohematology, including cytostatics or radiotherapy, is based on the induction of apoptosis, in spite of other effects, such as DNA damage. For instance, antracyclins or purine analogues commonly used in CLL, such as fludarabine or cladribine, not only act in the mechanism of apoptosis. 2-CdA induces mitochondrial pathway of caspases activation, like fludarabine, but it additionally causes the direct cytotoxic effect on mitochondria. Cladribine also inhibits DNA synthesis in leukemic cells, exhibiting strong synergistic cytotoxicity in addition to UV radiation.[32,33] In the case of CLL, its heterogeneous nature and the resistance to conventional treatments developed in the majority of patients hinder the progress in therapy. The scarcely published results of the research on dendrimers mechanisms revealed their influence on both the changes in apoptotic gene expression[34] as well as on mitochondrial functions.[35,35] Moreover, the cationic dendrimers manifested relatively high effectiveness in destroying malignant cells simultaneously acting on low hematotoxicity level.[24] These findings prompted us to determine whether dendrimers will destroy CLL cells by means of apoptosis. Our study opens new applicative perspectives supported by the observations that dendrimers can act through the mechanism of apoptosis that constitutes a paradigm of CLL cell existence.

The cytotoxicity studies of nanoparticles are essential for providing safe therapeutic applications. Various nanoparticles showed limited cytotoxicity, but size-dependency was not observed.[37] Although the cytotoxicity in our study was concentration-dependent; however in the other study[38] for both dendrimers (unmodified and modified with sugar), it was more pronounced in longer times of incubation. As it is known from literature, positively charged moieties on dendrimer scaffold can interact with negatively charged phospholipids in the plasma membrane.[39,40] The high density of surface groups combined with the small size of dendrimers results in a high area/volume ratio. This confers on dendrimers an unusual capacity to establish surface interactions with the cell membrane.[41,42] Interactions with membrane proteins and phospholipids seem to disturb membrane structure and function.

Unmodified PPI-G4, which possesses cationic amino surface groups, demonstrated higher cytotoxicity than PPI-G4-DS-Mal-III. It is clearly visible in concentration of dendrimers used. PPI-G4 dendrimers were used in 10 times lower concentrations as opposed to maltotriose decorated PPI dendrimers. Similar results were obtained for PPI-G4 and maltotriose conjugated PPI-G4 dendrimers *in vivo*[26] and *in vitro*, where relatively high dosage of PPI-G4-DS revealed minimal or no toxicity against human astroglioma (U-87) and human T4-lymphoblastoid (CEM-SS) cell lines, while PPI-G4 used in 10 times lower concentrations caused cells mortality over 70%.[43]

Cell death can occur by a spectrum of morphologically and biochemically distinct pathways, including apoptosis, necrosis, and autophagy.[44] Although apoptosis refers to a purely morphological change, biochemical events accompany these morphological changes. These events include three stages: decision, commitment, and execution phases with intrinsic and extrinsic pathways. In these stages there are initiator and effector caspase activation, release of cytochrome c from mitochondria, externalization of PS on the plasma membrane, and internucleosomal DNA fragmentation.[45] During the early process of apoptosis, cell shrinkage and pyknosis occur, and they are visible by light microscopy.[46] The late apoptotic events take place after activation of the effector caspases and include exposure of PS on the external surface of the plasma membrane.

To determine the nature of leukemic cell death induced by PPI-G4 and PPI-G4-DS-Mal-III, fluorescence was measured by flow cytometric analysis after Ann-V-FITC and IP staining. Early stages of apoptosis are characterized by translocation of phosphatidylserine from the interior to the outer leaflet of the bilayer as it becomes exposed at the cell surface.[47,48] Ann-V binds with high affinity to phosphatidylserine and thus can be an identifier of cells in all stages of apoptosis. IP is a cationic autofluorescent compound, which stains cells with a disrupted cell membrane, and can be used to identify late apoptosis and necrosis. The incubation of MNCs, retrieved from CLL patients, with PPI-G4 and PPI-G4-DS-Mal-III dendrimers induced visible phosphatidylserine exposure after 24 and 48 h. It was observed that maltotriose modified PPI dendrimers (PPI-G4-DS-Mal-III) evoked strongly early apoptosis after 24 h as well as after 48 h at every examined concentration (Table 3), whereas unmodified dendrimers PPI-G4 strongly induced late apoptosis (Table 2). The results show that the analyzed PPI-G4 nanoparticles act on leukemic cells in mechanism of apoptosis even if the concentration is high (0.4 mg/mL) and the cytotoxicity is about 80%, and the late apoptosis predominated (82.63% and 70.16%, after 24 and 48 h, respectively) over the necrosis (2.08% after 24 h and 8.41% after 48 h). The necrosis was significant only for PPI-G4 at the concentration of 0.01 mg/mL as compared with the control cells (Table 2). Both dendrimers induced the late apoptosis but only a very slight necrosis using the same mechanism. PPI-G4 is a small cationic particle. Cationic dendrimers, due to their positively charged surface interact with negatively charged cell membrane. Dendrimers have a great number of surface function groups, which by means of covalent bonds react with receptors on the cells. Dendrimers join membrane proteins and change their conformation. They probably bind to proteins involved in extrinsic apoptosis path and trigger apoptosis. The above-mentioned path induces the intrinsic one in mitochondria. Uncoated PPI-G4 dendrimer permeates the cell via endocytosis or through nanopores in the cell membrane. It can also reach mitochondria,[49] disrupting electron transport in the respiratory chain and stimulating the overproduction of reactive oxygen species (ROS), thus triggering oxidative stress leading to DNA damage and cell apoptosis.

There is an absence of data concerning the influence of nanoparticles on CLL cells. This work represents, to the best of our knowledge, the first evidence concerning apoptotic changes in CLL cells under the influence of dendrimers. A common paradigm for cationic nanoparticles cytotoxicity is one of endocytosis, encapsulation in endosomes and then lysosomes, which leads to the increase in lysosomal activity. As it has been described for cationic particles there is a mechanism based on a localization in mitochondria, thus triggering a cytotoxic response via the mitochondrial injury pathway[50,51] and generating reactive oxygen species (ROS) as a result.[49,50] The apoptotic effect of dendrimers by means of Ann-V/IP method (PAMAM and PPI dendrimers) on breast cancer cells,[38,52] prostate cancer cells,[53] and human lung cells[49] has already been reported. PPI dendrimers, which were used as carriers for nucleic acid delivery, were shown to induce gene expression changes and apoptosis.[34] Kuo et al.[35] observed in human macrophages the PPI dendrimers influence on mitochondrial

membrane potential and apoptosis. Our study depicts the great PPI-G4-DS-Mal-III dendrimers influence on inhibiting the mitochondrial potential and thus activating the apoptosis in leukemic lymphocytes. A CMXRos assay has not been conducted with PPI-G4 dendrimer due to its high toxicity to healthy cells. Regardless of CMXRos test results, this dendrimer will not be used in further research on the application of dendrimers as a potential cure for leukemia. A mitochondrial membrane potential assay revealed apoptosis induction by PPI-G4-DS-Mal-III at each examined concentration in comparison with control cultures. Its potency to trigger apoptosis is even better than fludarabine (FA) and monoclonal antibodies (Rit) widely used in CLL therapy. The concentration of FA was calculated on the basis of our previous studies with *in vitro* cultures,[54] and it is closely linked with the concentration of this drug given to patients *in vivo* during the standard treatment. As far as Rit is concerned, the required concentration of this drug for *in vitro* culture is higher. This is a monoclonal antibody, and its effectiveness in natural environment depends on known cytotoxic mechanism, caspase-dependent apoptotic pathway, and possible influence of other extracellular factors present in the patient's serum. If the autologous serum had been used, better results would have been obtained with a lower Rit concentration.[55]

The initial cause of CLL remains an open question, but it is currently well-accepted that the accumulation of leukemic cells is partially explained by a defect in apoptosis. However, recent studies suggest that CLL is not solely an accumulation disease but also a proliferative one.[56] As it was described in another publication,[25] evaluated dendrimers did not reveal an antigenic stimulus to the cells. However PPI-G4 strongly inhibited cell proliferation starting with a very low concentration. One of the explanations to this phenomenon is that the lack of proliferation is a consequence of dendrimer genotoxicity.[34,36,57] Due to their ability to interact with nucleic acids,[19] dendrimers might bind to DNA after entering the nucleus and prevent cell division. Therefore there is a danger that a prolonged dosage of PPI-G4 dendrimers may result in immunodeficiency caused by defects in cell-mediated response.[58,59]

Before testing dendrimers in a further study in a particular disease, it is essential to evaluate their safety at the cellular level. Therefore, we have checked in our earlier experiments the influence of examined dendrimers on red blood cells hemolysis and platelets (PLT) aggregation. PPI-G4 was the most hemolytic dendrimer, even at the lowest concentration (1 μM) after only 2 h of incubation. The surface modification of the dendrimer by Mal-III significantly reduced hemolysis. After 24 h incubation, the glycodendrimers at the highest concentration (300 μM) were significantly less damaging than the unmodified PPI-G4 at 30 μM.[25] As far as PLT aggregation is concerned, results obtained from PPI-G4 are comparable with those of trypsin even at low concentrations. The Mal-III modification of PPI dendrimers intensely reduced their ability to induce PLT aggregation. No PLT aggregation was seen in the presence of PPI-G4-DS-Mal-III at any of the studied concentrations.[25]

PPI-G4 and PPI-G4-DS-Mal-III dendrimers demonstrated higher cytotoxicity to CLL cells than to healthy donor cells, whereas unmodified dendrimers were more hematotoxic. Their potency in the triggering of apoptosis is similar to many PNAs and monoclonal antibodies widely used in CLL treatment. There are still no curable approaches for CLL. For this reason, it is necessary to search for new and effective therapeutic compounds. Dendrimers are a promising therapeutic approach for CLL. Further biological evaluations of these nanoparticles are currently conducted in our departments. The results from apoptotic mechanism, gene expression profiling, and studies comparing the influence of dendrimers and drugs on the apoptosis in CLL cells will be described in due course.

■ CONCLUSIONS

The most required method of cell death is apoptosis. It is a genetically caused process of active cell destruction. Cells undergo apoptosis without losing integrity, lysis, inflammation, and damage of neighboring cells. The full clinical potential of dendrimers is still unknown, but the presented results confirm the influence of dendrimers PPI-G4 and PPI-G4-DS-Mal-III on apoptosis and CLL lymphocyte viability in *in vitro* cultures. Flow cytometry confirms that, with the increasing dendrimer dose, the percentage of early and late leukemic apoptotic cell population increases. Since the positive charge of dendrimers is responsible for their high toxicity, surface modification makes glycodendrimers much more suitable for biomedical applications than unmodified PPI-G4. This enables significant reduction of dendrimer toxicity while maintaining their functionality. The cause of such a significant limitation of adverse effects of positive charges by a modification of dendrimer surface lies in the sugar particle itself. Maltotriose covers positive charges present on dendrimer surface, limiting its influence on vulnerable structures, that is, lipids and membrane proteins. However, the modified dendrimers, despite the total surface modification, retain the activity as apoptosis inductors mostly in cancer cells. The results obtained earlier by our group suggest that the actual influence of dendrimers on blood components *in vivo* is lower than that seen *in vitro*.[25,26,60] These findings justify the need for further research on the nature and the mechanism of interactions between these dendrimers and CLL cells.

■ ASSOCIATED CONTENT

Supporting Information

Supporting figures—flow cytometric plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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DS-Mal-III was claimed to the Polish Patent Office in December 2012.

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