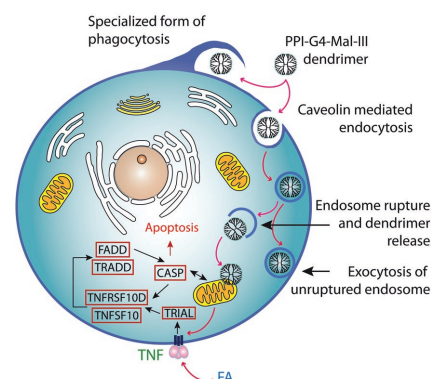


PPI-G4 Glycodendrimers Upregulate TRAIL-Induced Apoptosis in Chronic Lymphocytic Leukemia Cells

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Although chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western world, it remains incurable with conventional chemotherapeutic agents. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is an antitumor candidate in cancer therapy. This study examines the proapoptotic effects of poly(propylene imine) (PPI) glycodendrimers modified with the maltotriose residues (PPI-G4-OS-Mal-III and PPI-G4-DS-Mal-III) on the TNF family in CLL cells. The combination of an understanding of the signaling pathways associated with CLL and the development of a molecular profiling is a key issue for the design of personalized approaches to therapy. Gene expression is determined with two-color microarray $8 \times 60K$. The findings indicate that PPI-G4-OS/DS-Mal-III affect gene expression from the TRAIL apoptotic pathway and exert a strong effect on CLL cells comparable with fludarabine. Dendrimer-targeted technology may well prove to bridge the gap between the ineffective treatment of today and the effective personalized therapy of the future.



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1. Introduction

Disorders in mature B-lymphocytes involving changes in apoptosis dynamics is a hallmark of chronic lymphocytic leukemia (CLL), one of the most common forms of adult leukemia in the North America and the Western world.^[1] In this type of leukemia, cell apoptosis is impaired, leading to the affected cells living longer. Current approaches to treating CLL are based on a combination of both immuno- and chemotherapy in the absence of any effective monotherapy. Forms of CLL with del(17p) or del(11q) have the worst prognoses and remain incurable, with the expected lifespan following diagnosis being very short. The highly heterogeneous disease profile characterized by a variety of signaling pathways contributes to the high level of defective apoptotic response and survival observed in CLL.^[1,2]

Over the last decade increasing attention has been drawn to the role of tumor necrosis factor (TNF) family members (i.e., *APRIL* and *BAFF*), which play an important role in the interaction between malignant B-cells and their microenvironment.^[3] CLL cells express a diverse range of TNF receptor family members including *TNFRSF13B* (*BAFF*), *TNFRSF17*, and B-cell-activating factor receptor (BR-3), these being receptors for *APRIL*, a proliferation-inducing ligand also termed *TALL-2*, *TRAD-1*, and *BAFF*.^[2,4] *APRIL* and *BAFF* are potent regulators of B-cell development and survival. *BAFF* plays a significant role in promoting both neoplastic and normal B-cell survival. *APRIL* potentially stimulates tumor cell growth and can induce primary B-lymphocyte proliferation.^[5] TNF-related apoptosis-inducing ligand (TRAIL) is a candidate for cancer therapy that may cause apoptosis in a variety of human tumors as a death ligand, without affecting normal cells. This ligand is a part of the immune system which helps to prevent the formation and spread of tumors.^[6]

The recalcitrance of CLL cells to treatment may be based on drug-induced resistance and can be enhanced by therapies based on DNA-damaging agents such as fludarabine (FA). Alternatively, this resistance to treatment may occur in response to the use of drugs that activate TNF signaling, which counteract the effect of the therapy. Therefore, while the development of novel treatment approaches is justified, its implementation remains a considerable clinical challenge. Over the past years, great progress has been made in developing nanoparticles in the form of dendrimers, a group of nanomaterials which may prove valuable in combating multiple types of diseases^[7–11] including CLL.^[12] Especially, the treatment of CLL and healthy red blood cells with poly(propylene imine) (PPI) dendrimers decorated with maltotriose units exhibited an overall promising biologically active structure–activity relationship of those nanometer-sized PPI dendrimers toward both cell types. Control experiments with healthy donor cells outlined no apoptotic properties of glycodendrimers used, while

dose-dependent apoptotic properties of glycodendrimers against CLL were observed in cell cultures experiments.^[13] These results pave the way to use such PPI glycodendrimers as potential polymeric therapeutics^[14,15] in clinical trials for the treatment of CLL cells. Moreover, PPI glycodendrimers may further unify two key characteristics that they can be used as polymeric therapeutics as well as delivery system of anticancer drugs for treating CLL. Before such biologically active glycodendrimers are usable for clinical use, our general concern is directed at aiming the evaluation of biological pathways of those dendritic glycoarchitectures in CLL cells, but also exhibiting the indirect influence of those nanoparticles on biologically driven pathway(s) of apoptosis toward CLL cells. Overall, there is a general strong interest in understanding the biological actions of various dendritic (glyco)architectures^[8,9,11] before those nanomaterials can be implemented in (pre)clinical trials.

Thus, the aim of this study was to identify the unique gene expression signature associated with the TNF signaling pathway, which appears during the treatment of CLL with the open shell glycodendrimer PPI-G4-OS-Mal-III and dense shell glycodendrimer PPI-G4-DS-Mal-III (Scheme 1).

2. Experimental Section

2.1. Materials

The synthesis together with specification of PPI-G4 dendrimers modified with maltotriose residues was carried out as described previously.^[13,16] Fourth generation poly(propylene imine) (PPI) dendrimers with peripheral amino groups modified with maltotriose residues in $\approx 90\%$ (PPI-G4-DS-Mal-III) and 35% (PPI-G4-OS-Mal-III) have been used (Table 1). The abbreviation DS and OS describes the dense shell and open shell for the structure of carbohydrate modified glycodendrimers respectively (Scheme 2).

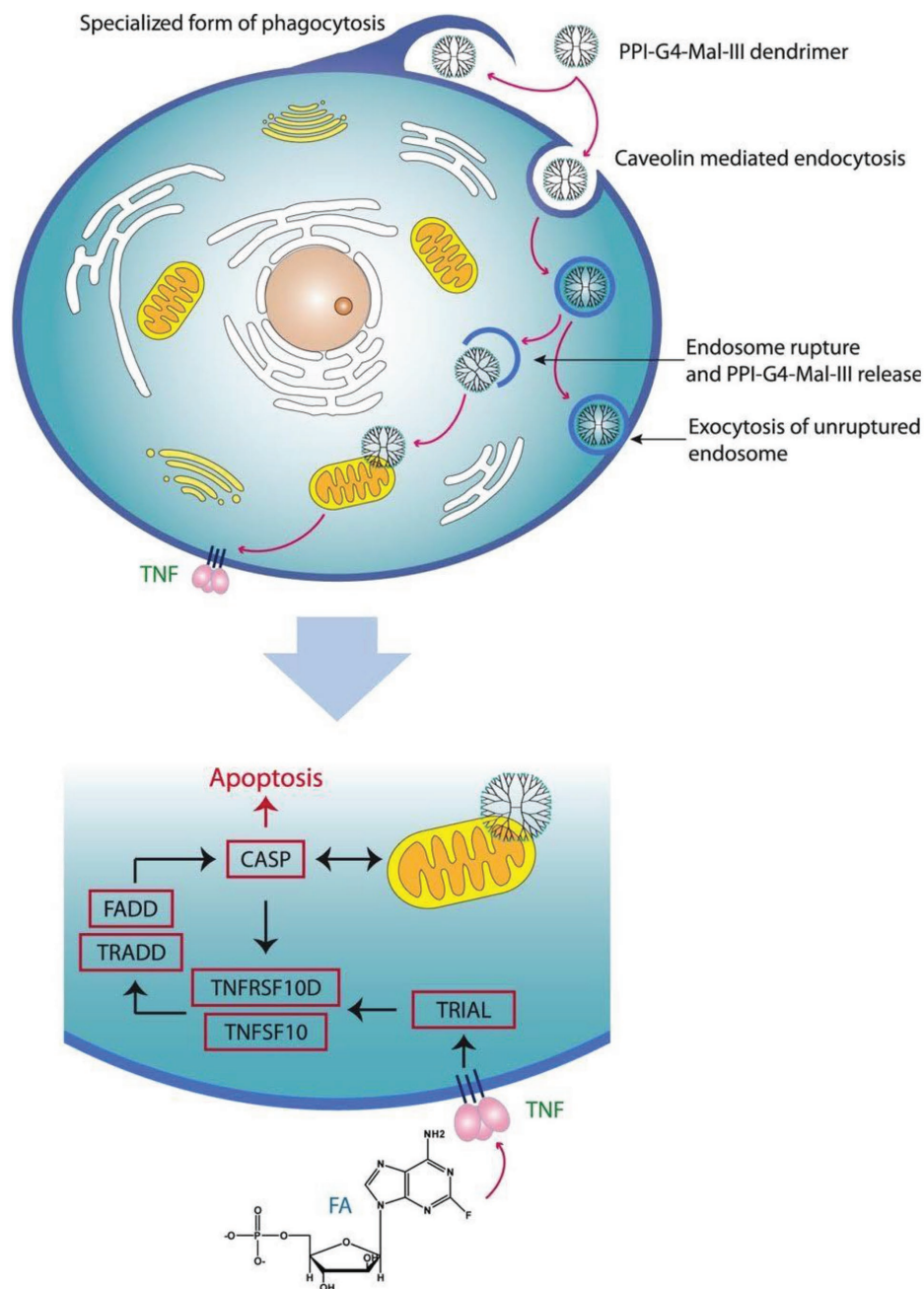
The study used 8 mg mL^{-1} (IC_{50} concentration measured on CD19+, CD5+ B-lymphocytes retrieved from patients as well as on MEC-1 cells) of PPI-G4-OS-Mal-III or PPI-G4-DS-Mal-III dendrimers. Commercially available drug FA (Genzyme) at a concentration of 584.3 mg mL^{-1} ($1.6 \times 10^{-6} \text{ M}$) was chosen to allow comparison with our previous studies.^[18]

2.2. Cell Culture and Treatment

The MEC-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) No. ACC 497) (www.dsmz.de/catalogues/details/culture/ACC-497.html) homogenous cell line with the mutation del(17p)(11q) was used for the in vitro culture. Cells were incubated in culture at a concentration of $1 \times 10^6 \text{ cells mL}^{-1}$ with the addition of either dendrimers or FA. Cultures without active compounds were used as the control.

2.3. Apoptosis and Cell Viability Assays

Cells were harvested after 4 and 24 h to measure apoptosis on a FACSCalibur flow cytometer (Becton Dickinson, USA) by Annexin-V/propidium iodide (Ann-V/PI) assay as described previously.^[13]

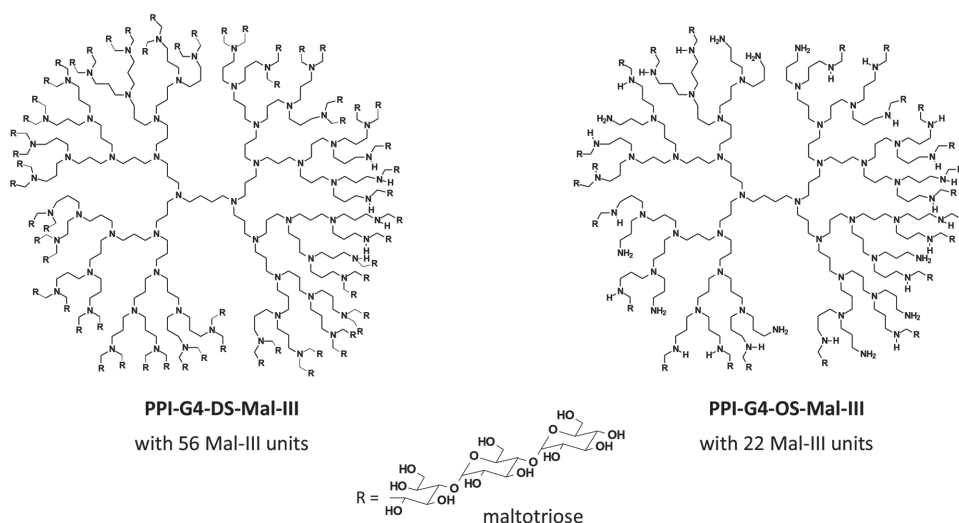


Scheme 1. Schematic presentation of the endocytosis of PPI-G4-OS/DS-Mal-III dendrimers and their targeting mitochondria and TRAIL pathway.

Table 1. Parameters of synthesized PPI-G4 dendrimers modified with maltotriose, including number and the percentage of surface maltotriose groups together with molar mass (MM).

Dendrimer	MM _{theoretical} ^{a)} [g mol ⁻¹]	MM _{observed} ^{a)} [g mol ⁻¹]	Number (percentage) of surface Mal-III groups _{theoretical}	Number (percentage) of surface Mal-III groups _{observed}
PPI-G4-OS-Mal-III	19 144	14 260	32 (50%)	22 (35%)
PPI-G4-DS-Mal-III	34 774	31 000	64 (100%)	56 (87%)

^{a)}MM of PPI-G4-OS-Mal-III determined by H NMR approach described in ref.^[17]



■ Scheme 2. Simplified structure of PPI-G4-OS/DS-Mal-III dendrimers.

The apoptotic index (AI) was expressed as the percentage of annexin V positive cells and the compensating apoptotic index (CAI) was calculated as the difference between the AI in the presence of the dendrimer and the percentage of cells undergoing spontaneous apoptosis in control cultures. CAI values of 10% or greater were regarded as significant.

2.4. Microarray Gene Expression Analysis

An RNeasy total RNA Mini Kit (Qiagen, France) was used for all RNA isolation from the cells cultured for 4 h. To amplify and label the RNA, a two-color microarray-based gene expression $8 \times 60K$ kit (Agilent Technologies) was used. Two replicate hybridizations and dye-swaps (four analyses) were carried out for each experiment. Arrays were scanned on microarray scanner (Agilent Technologies) and all microarray data were deposited at Gene Expression Omnibus (GEO) with the accession number GSE68094.

Microarray analysis was conducted to identify differentially expressed genes between the reference group and samples labeled as FA, PPI-G4-OS-Mal-III, and PPI-G4-DS-Mal-III after 4 h of incubation with MEC-1 cells. The log₂ ratio of the sample channels to the control channels was analyzed, and the signal was median centered. Limma R/Bioconductor software was used with the Benjamin–Hochberg multiple testing corrections for differential gene expression with the stricter Holm correction.^[19]

3. Results and Discussion

3.1. Apoptosis in CLL Cells

The percentage of apoptotic MEC-1 cells induced by 8 mg mL^{−1} PPI-G4-OS-Mal-III and PPI-G4-DS-Mal-III dendrimers was significantly higher than the percentage of spontaneous apoptotic leukemic cells. In the case of the 4 h cultures, no statistically significant differences were observed in apoptotic cells. The CAI value was 22%

($n = 8$) for the 24 h cultures exposed to PPI-G4-DS-Mal-III and 35% ($n = 8$) for those exposed to the PPI-G4-OS-Mal-III dendrimer (Table 2).

3.2. Microarray Gene Expression Profiling (GEP)

The expression of apoptotic gene clusters was compared between samples treated with the DS glycodendrimer and the OS glycodendrimer, FA, and reference (not treated MEC-1 cells) (FA/Ref and Dendrimer/Ref) (Figure 1). The GEP was measured after 4 h, and the expression profiles of the TNF genes demonstrating significant differences in expression were examined.

After 4 h, the comparison of the effects of the PPI-G4-DS-Mal-III and PPI-G4-OS-Mal-III dendrimers on MEC-1 cells revealed that both dendrimers greatly induced the same sets of genes: *TNFRSF17*, *TNFRSF10D*, and *CDT1*. Additionally PPI-G4-OS-Mal-III induced also *TNFAIP3* (Table 2). Moreover, comparative analyses of the action of FA and dendrimers of interest revealed significant changes in the expression of genes from the TNF signaling pathway. FA induced the expression of *TNFRSF17*, *TNFAIP2*, *CDT1*, *TNFAIP8L2*, *TNFSF14*, *TNFSF13B*, and *TNFRSF8* and overexpressed *TNFSF10* compared with either OS or DS glycodendrimers. In opposite OS and DS induced overexpression of *TNFRSF10D*. Although the experiment included a full panel of genes from the TNF pathway, only those that showed statistically significant variation are given in Table 3.

Genetic alterations present in CLL, such as deletions at 17p13 or 11q22–q23, 13q14 or trisomy of chromosome 12, are significantly associated with poor clinical outcome.^[1] Over the last decade, increasing attention has been focused on the role of the TNF family.^[20] This controls the expression of genes that act as crucial growth regulators,

Table 2. The influence of PPI-G4-OS/DS-Mal-III dendrimers and purine analogue (fludarabine, FA) on CLL cells (MEC-) in vitro.

	Control ^{a)}		PPI-G4-DS-Mal-III (8 mg)				PPI-G4-OS-Mal-III (8 mg)				FA 1.6×10^{-6} M				Statistical analysis (P-value)
	24 h														
1	2	3	4	5	6	7	8	9	10	11	12	13			
Ann+ PI- Ann+ PI+ Ann+ PI- Ann+ PI+ Ann+ PI- Ann+ PI+ Ann+ PI- Ann+ PI+ Ann+ PI- Ann+ PI+ Ann+ PI+ Ann+ PI- Ann+ PI+	8	8	8	8	8	8	8	8	8	8	8	8	1 versus 4 < 0.007		
N	5.28	4.10	2.48	12.19	19.25	2.80	15.40	29.08	1.33	12.89	23.34	4.24	1 versus 7 < 0.003		
X	1.31	0.87	0.92	1.88	1.87	1.68	1.77	3.26	0.72	2.48	2.38	1.70	1 versus 10 < 0.001		
SD													2 versus 5 < 0.001		
CAI [%]				22.06				35.10			26.85		versus 8 < 0.001		
													2 versus 11 < 0.001		
48 h															
N	8	8	8	8	8	8	8	8	8	8	8	8			
X	4.41	19.78	6.66	3.46	38.73	8.78	9.72	49.18	3.43	8.17	43.06	6.99	2 versus 5 < 0.006		
SD	0.80	3.72	2.29	1.52	5.32	2.10	2.50	6.78	0.85	1.69	10.26	3.43	2 versus 8 < 0.001		
CAI [%]					18.00			34.71			27.04		2 versus 11 < 0.001		

n: number of samples; X: mean percentage of cells; SD: standard deviation; CAI: compensating apoptotic index.

including many which are important in leukemia.^[21] The recalcitrance of CLL cells to treatment may be based on drug-induced resistance which can be enhanced by therapies based on DNA-damaging agents^[22] such as FA.

Following on from many novel approaches in CLL examination and the microarray methodology, our study uses gene expression profiling (GEP) by microarrays to discover and identify the specific caption in CLL treatment. To achieve this aim, dense and open shell maltotriose-modified PPI dendrimers were employed as the main tools.

Network analysis was used to identify potential TNF pathways, which may be involved in CLL-specific-targeted therapy. A unique gene expression signature composed of four genes, *TNFSF13B* (*APRIL*),^[5,23] *TNFAIP8L2* (*TIPE2*),^[24] *TNFAIP2*,^[25] and *TNFRSF8* (*CD30*),^[26] was found to successfully differentiate between cultures treated with maltotriose-modified PPI dendrimers and FA. *TNFAIP2* encodes for tumor necrosis factor α -induced protein and is greatly expressed in hematopoietic cells. *TNFAIP2* appears to be a direct target for transcriptional repression by the *PML-RAR α* and *PZLF-RAR α* oncogenes.^[26] Although the precise function of *TNFAIP2* is currently not fully known, its transcriptional repression by PML-RAR α and the potential translational repression by the generation of a new miRNA-binding site are consistent with its possible role in leukemic transformation.^[27]

Tumor necrosis factor- α -induced protein 8 (*TNFAIP8L2*, *TIPE2*) is a novel antiinflammatory protein of the *TNFAIP8* family, which is crucial for maintaining immune homeostasis.^[24] The immune cells found in lymphoid tissues are most likely to express *TIPE2* preferentially. *TNFAIP8L2* is significantly downregulated in patients with autoimmune disorders, where its expression inversely correlates with progression of the disease. *TNFAIP8L2* overexpression induces cell death and significantly inhibits Ras-induced tumorigenesis.^[24]

APRIL plays a crucial role in the survival, differentiation, and apoptosis of normal B-cells^[28] and has been identified as a crucial player in the onset of a number of autoimmune diseases. It also might play a prominent and important role in the protective effect provided by nurse-like cells to B-CLL cells, indicating that a crucial role may be played by *APRIL* in B-CLL leukemogenesis.^[29] These genes are capable of protecting the CLL cells against both spontaneous and drug-induced apoptosis. As they have a potent effect on the survival of normal B-lymphocytes, it is certainly worth to investigate their role in the pathogenesis of B-cell malignancies. A desirable effect of the decrease in expression of the above-mentioned TNF signatures (*APRIL*, *TIPE2*, *TNFAIP2*, and *TNFRSF8*) induced by dendrimers might be the triggering of apoptosis in CLL-lymphocytes.

Our results appear promising. Used glycodendrimers indicate the presence of differences in the mode of

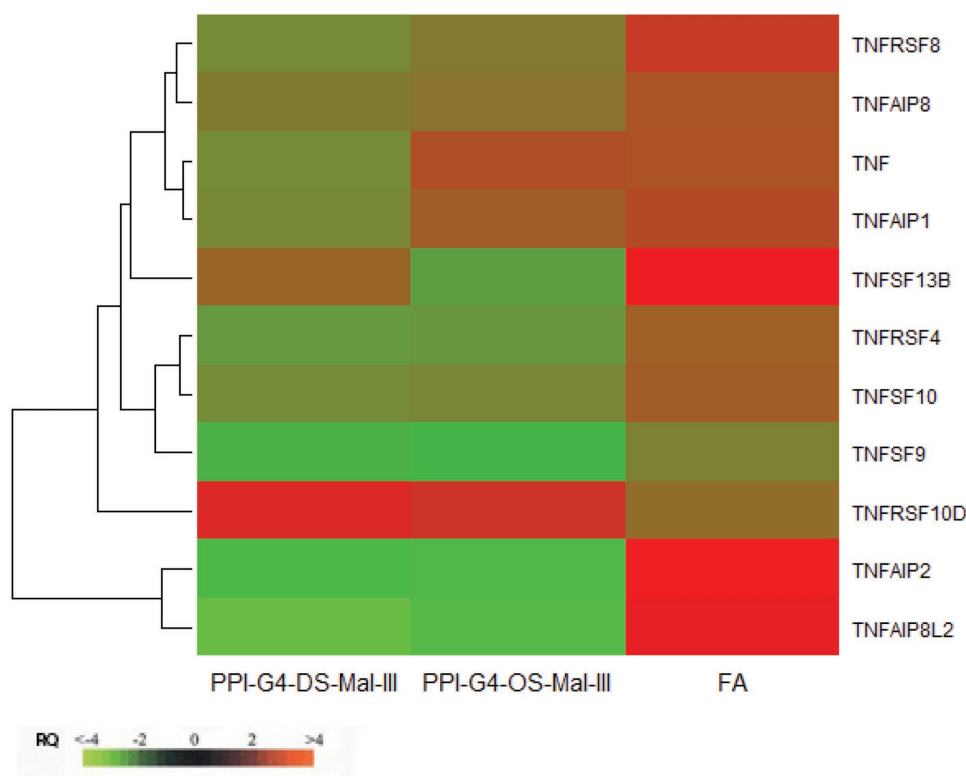


Figure 1. The heat map analysis of significant genes expression differences in TNF signaling pathway. The results are expressed as fold ratio of gene expression between FA and PPI-G4 dendrimers compared to reference. The red marks present high gene expression profile whereas the green ones indicate low gene activity.

biological action against FA. In comparison with other agents, FA is characterized by a significant response rates and progression-free survival. However, although FA prevents smooth uptake by CLL cells due its negative charge, this prodrug form is converted by dephosphorylation, allowing it to enter. This may result in a greater number of overexpressed genes observed after 4 h of stimulation compared to the glycodendrimers used. In contrast, due to their unique dynamic and mode of action (Figure 1), both glycodendrimers are able to boost the expression of genes from the TRAIL apoptotic pathway. This preferentially mediates proteasome inhibitor-induced apoptosis in primary CLL cells.^[30]

The TRAIL may be used as an antitumor factor on the tumor cells and their metastases,^[31] oral cancer,^[32] glioma,^[33] and cervical carcinoma,^[34] because it causes death only in the tumor cells, not in the normal cells.^[35] Our present findings suggest that our glycodendrimers increase TRAIL expression. This might be a new target for personalized therapy of CLL triggered by biocompatible glycodendrimers.^[8] From this one can assume following indirect biologically macromolecular interactions and trafficking of OS and DS glycodendrimers within CLL cells (Scheme 1): As indicated from previous study,

glycodendrimers can be taken up by endocytosis^[36] and other biological uptake processes (e.g., phagocytosis and undefined uptake processes^[36] (Scheme 1)). Preferred endosomal rupture leads to the potential interaction of glycodendrimers with mitochondria surface and simultaneous stimulation of CASP (caspase) pathway. This has an indirect influence on the TRAIL pathway by especially stimulating *TNFRSF10D* gene.

4. Conclusion

Together, our study demonstrates that the lymphoid microenvironment promotes the survival, proliferation, and finally progression of CLL cells through chronic activation of TNF family genes while suppressing the immune response. The addition of PPI-G4-OS-Mal-III and PPI-G4-DS-Mal-III dendrimers to B-CLL cells significantly induces the phenomenon of apoptosis within these lymphocytes by significant reduction of their survival ratio. Moreover our results show that good therapeutic results can be expected in the case of CLL, all the more because of the lack of negative effects demonstrated by the tested glycodendrimers on cells such as erythrocytes or platelets.

Table 3. Comparison of changes in apoptotic gene expression between FA, PPI-G4-DS-Mal-III, and PPI-G4-OS-Mal-III dendrimers versus reference.

Probe set	Gene symbol	PPI-G4-DS-Mal-III		PPI-G4-OS-Mal-III		FA	
		LogFC	P-value	LogFC	P-value	LogFC	P-value
P37736	<i>TNFRSF17</i> (<i>BCM</i>)	1.3719	4.69e-06	1.5347	1.94e-06	1.1973	1.34e-05
P421423	<i>TNFAIP2</i>	−1.2760	2.42e-05	−1.3879	1.28e-05	0.9833	0.0001
P3397763	<i>TNFSF9</i>	−0.9945	3.06e-05	−1.0561	1.94e-05	−0.4601	0.005
P46356	<i>TNFAIP8L2</i>	−1.8788	3.60e-05	−1.5225	0.0001	0.7232	0.01
P3386262	<i>CDT1</i>	0.9043	9.63e-05	0.9683	5.85e-05	0.7507	0.0003
P91764	<i>TNFRSF13C</i> (<i>APRIL</i>)	−0.8297	0.0006	−0.8925	0.0003	−1.1006	8.50e-05
P500614	<i>TNFRSF8</i> (<i>Ki-1</i>)	−0.5621	0.002	−0.3849	0.01	NS	NS
P3326588	<i>TNFRSF10D</i> (<i>TRAILR4</i>)	0.5755	0.002	0.4252	0.01	NS	NS
P376488	<i>TNF</i>	−0.5638	0.002	NS	NS	NS	NS
P49338	<i>TNFRSF12A</i>	−0.7311	0.002	NS	NS	−0.5741	0.01
P3286157	<i>TNFRSF4</i>	−0.7326	0.004	−0.6852	0.005	NS	NS
P121253	<i>TNFSF10</i> (<i>TRAIL</i>)	−0.5556	0.006	−0.7741	0.0005	NS	NS
P245298	<i>TNFSF12</i> (<i>APO3L</i>)	−0.5302	0.007	−0.7267	0.001	−0.8015	0.0005
P38446	<i>TNFAIP1</i>	−0.5278	0.007	NS	NS	NS	NS
P3220437	<i>TNFAIP8L1</i>	NS	NS	−0.4217	0.01	NS	NS
P219520	<i>TNFAIP8</i>	−0.3976	0.01	−0.3043	0.04	NS	NS
P157926	<i>TNFAIP3</i>	NS	NS	0.3732	0.02	NS	NS
P390518	<i>TNFRSF11A</i>	NS	NS	−0.2856	0.05	−0.5479	0.002
P14174	<i>TNFRSF13B</i>	NS	NS	−0.7798	0.03	0.8633	0.02

NS: not statistically significant.

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