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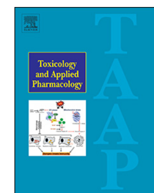

Affecting NF-κB cell signaling pathway in chronic lymphocytic leukemia by dendrimers-based nanoparticles

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ABSTRACT

The complex genetic diversity of chronic lymphocytic leukemia (CLL) makes it difficult to determine the effective and durable therapy beneficial to patients. During the several past years' significant insights in the biology of the disease and its treatment have been made, allowing for the identification of promising novel therapeutic agents. The investigation of signaling pathways to understand the biological character of CLL together with the development of molecular profiling is key in personalized approach in therapy for this disease. As it was already proven, maltotriose (M3) modified fourth generation poly(propylene imine) dendrimers (PPI-G4) modulate *BCR, TRAIL* and *WNT* signaling pathway gene expression in CLL cells and strongly influence their survival by inducing apoptosis and inhibiting proliferation. The aim of this study was to evaluate the influence of PPI-G4-M3 dendrimers on NFκB pathway gene expression in CLL (MEC-1) cells with 60 K microarray, as it is one of the major factors in the pathogenesis of B-cell neoplasms. The findings were compared with those obtained with Fludarabine (FA) and the results indicate that PPI-G4-M3 dendrimers affect the expression of the examined genes and exert comparable effect on the CLL cells to FA. Dendrimers are one of the most potent groups of nanometer-sized macromolecules for closing the gap between the present ineffective treatment and the future effective personalized therapy due to their potential versatile biological properties.

1. Introduction

Despite the enhanced molecular understanding and the clinical diagnosis improvement, chronic lymphocytic leukemia (CLL) still remains a disease without a well-defined genetic alteration responsible for its onset (Doménech et al., 2012). As B-CLL cells are characterized by a low dividing rate, they are less susceptible to chemo- and radiation therapy commonly used in cancer treatment, which make CLL difficult to cure. Standard therapy for CLL includes a combination of chemotherapeutics such as fludarabine (FA), cyclophosphamide and rituximab (FCR) but it is not suitable for all the patients because of the drug resistance or the side effects of strong chemotherapy (Smolej, 2016; Tam et al., 2008). Although the past several years have brought huge and significant changes in therapeutic paradigms of CLL while novel drugs like ibrutinib or idelalisib have been approved (Byrd et al., 2015; Ramanathan et al., 2016), alternative therapeutics approaches are still necessary if longer survival rates and an ultimately cure in CLL patients are be achieved.

One of the proposed alternative therapeutic approaches in CLL treatment is a substitution of a standard chemotherapy by polymeric therapeutics, which possesses another molecular biological pathway for inducing apoptosis of CLL cells. For substituting anticancer drug fludarabine, open shell (OS) and dense shell (DS) maltotriose (M3)-modified fourth-generation poly(propylene imine) (PPI-G4) dendrimers (PPI-G4-OS-M3 or PPI-G4-DS-M3; Fig. 1) have been found and further validated as possible alternative drug for fludarabine(Franiak-Pietryga

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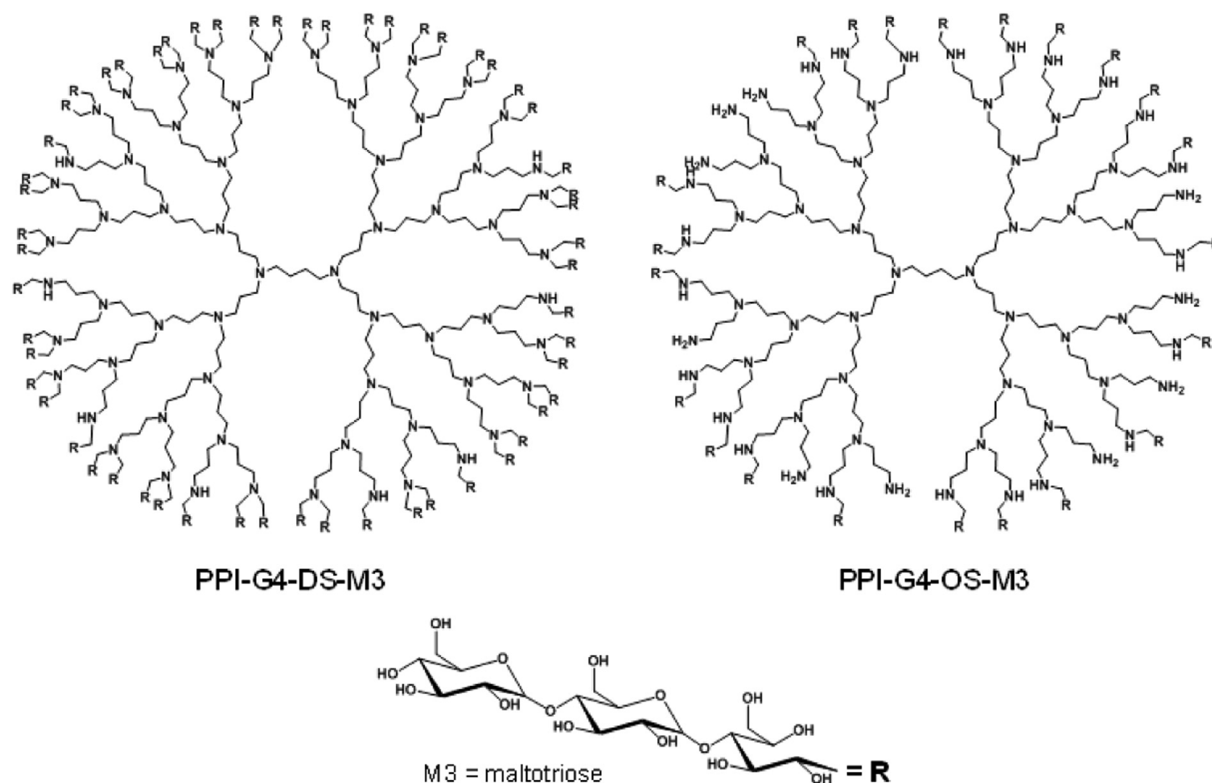

Fig. 1. Simplified structure of PPI-G4-DS-M3 and PPI-G4-OS-M3 dendrimers.

et al., 2017a; Franiak-Pietryga et al., 2013; Franiak-Pietryga et al., 2017b). Dendrimers are currently one of the most popular nanoparticles and they have been well described in many previous publications, e.g. (de Brabander-Van Den Berg and Meijer, 1993; Jain et al., 2010; Tomalia and Fréchet, 2002; Ziemba et al., 2012a). In short, they are well-defined, monodisperse, three-dimensional structures with a central core and functional terminal groups (Tomalia et al., 1985). Because of their unique structure and properties, dendrimers have attracted great interest in biomedical applications e.g. as polymeric agents in cancer diagnosis and therapy (Baker, 2009; Kesharwani et al., 2014; Menjoge et al., 2010; Svenson and Tomalia, 2012).

In previous works it was proved that PPI-G4-OS/DS-M3 dendrimers reveal low cyto- (Ziemba et al., 2014), geno- (Ziemba et al., 2012b), hemo-(Ziemba et al., 2012c) and *in vivo*[21] toxicity (Ziemba et al., 2011). However, it was also indicated that PPI glycodendrimers induce apoptosis in cancer cells, including CLL, and inhibit their proliferation without significant influence on normal lymphocytes viability (Franiak-Pietryga et al., 2013; Ziemba et al., 2014). Moreover, results in one of our recent study show that PPI-G4-OS-M3 dendrimer significantly influences pro- and antiapoptotic gene and protein expression in CLL cells (Franiak-Pietryga et al., 2017b).

In this paper, we focused on the influence of PPI-G4-OS/DS-M3 dendrimers on nuclear factor-κB (NFκB) signaling pathway genes expression as it is one of the potential targeting patterns for CLL therapy. NF-κB transcription factors family consists of p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) proteins. These proteins play a meaningful role in inflammation and immunity, as well as in cells proliferation, differentiation, and survival (Oeckinghaus and Ghosh, 2009). The major role of NF-κB in the pathogenesis of B-cell neoplasms is well established and a broad array of, mostly extracellular, stimuli has been reported to directly activate NF-κB in CLL cells (Liu et al., 2011). The stimuli include: induction of GSK-3 β (Ougolkov et al., 2007), activation of the Tumor Necrosis Factor receptor (TNFR) family members, leukemia/lymphoma-1 (TCL1) oncogene deregulation of the T cell (Liu et al., 2011), activation of Notch signaling (Rosati et al., 2009), activation of the cell surface receptor CD40 (Polager and Ginsberg, 2008) or modulation of epigenetic regulators (Chen et al., 2009). Recent data pointed out that NF-κB is activated by the binding of unphosphorylated signal transducer and activator of transcription (STAT3) to the NF-κB dimers p65/p50 in competition with the inhibitor of NF-κB proteins (IκB) (Liu et al., 2011; Hazan-Halevy et al., 2010).

The present work is part of a series of publications describing the effect of PPI glycodendrimers on signaling pathway gene expression in CLL cells. The results of microarray analysis for *BCR, TRAIL* and *WNT* have already been published (Franiak-Pietryga et al., 2017a; Franiak-Pietryga et al., 2017c; Franiak-Pietryga et al., 2016) and they indicate that glycodendrimers significantly modulate expression of the examined genes thereby reducing the CLL cells survival. Findings of these preliminary results might be of an importance considering the usage of PPI dendrimers as active compounds in the future.

2. Material and methods

2.1. Fludarabine

Commercially available Fludarabine (FA, Genzyme) was used in the study. Chosen concentration of 1.6 μM is the IC_{50} of FA in 24-h culture and was calculated on the basis of previous studies (Ziółkowska et al., 2012; Zaborowska et al., 2009). It is close to the concentration of the drug given to patients during the standard treatment protocol (Korycka and Robak, 2003).

2.2. Dendrimers

Two sets of 4th generation poly(propylene imine) dendrimers (PPI-G4) with peripheral amino groups modified with maltotriose units (M3) in approximately 90% (labeled as the dense shell (DS); PPI-G4-DS-M3) and 35% (labeled as the open shell (OS); PPI-G4-OS-M3) were used. The concentration of 8 mg/mL was chosen based on the results of our previous study (Franiak-Pietryga et al., 2015) since it is the IC_{50} value for

Table 1
Molar mass (MM[a]) for synthesized dendrimers, with the number and the percentage of surface maltotriose groups.

Dendrimer	MM [g/mol]	Number (percentage) of surface M3 groups
PPI-G4-OS-M3	14,260	22 (35%)
PPI-G4-DS-M3	31,000	56 (87%)

[a] MM of glycodendrimer determined by ^{1}H NMR approach [(500 MHz, D_2O, δ) for the molar ratio between x-times of 2 protons for anomeric H of maltotriose and 124 protons for internal CH_2 groups, used as reference, of dendritic poly(propylene imine) scaffold: 4.9–5.3 (s, x-time 2H, anomeric H of maltotriose) and 1.4–2.3 ppm (124H for internal CH_2-groups).] approach described in (Mkandawire et al., 2009).

PPI-G4-OS-M3 in 48-h culture. Synthesis of the glycodendrimers (GD) was carried out as described elsewhere (Appelhans et al., 2010; Mkandawire et al., 2009). The dendrimer structures are given in Fig. 1 and the general characteristics is presented in Table 1.

2.3. Cell culture and treatment

The MEC-1 homogenous cell line with del(17p)(11q) mutation (DSMZ no. ACC 497) was used. Cells were treated as described in previous papers (Franiak-Pietryga et al., 2017b; Franiak-Pietryga et al., 2017c; Franiak-Pietryga et al., 2016). For the treatment cells were incubated with either FA (1.6 μM) or GD (8 mg/mL), up to 4 h. Cultures without GD or FA were used as reference (Ref).

2.4. Microarray gene expression analysis

Microarray gene expression assay (Agilent SurePrint Technologies, USA) was performed as it was described previously (Franiak-Pietryga et al., 2017b). Samples were hybridized to an 8 × 60 K whole human genome microarray and the arrays were scanned on an Agilent DNA Microarray Scanner. Two replicate hybridizations and dye-swap were carried out for each experiment. All microarray data were deposited at the Gene Expression Omnibus (GEO) with the accession number GSE68094.

Microarray data analysis was performed as it was described previously (Franiak-Pietryga et al., 2017b).

3. Results

3.1. Microarray gene expression profiling

We investigated the influence of PPI-G4-OS/DS-M3 dendrimers on NFκB pathway genes expression in MEC-1 cells as on the one of the major factors in the pathogenesis of B-cell neoplasms. We compared the findings with results obtained for FA, a drug commonly used in CLL treatment (Fig. 2).

The gene descriptions and the details concerning their expression with the *p*-value indicating differential expression between the sample of interest (FA, PPI-G4-OS-M3, PPI-G4-DS-M3) and the Ref are collected in Table 2. Comparison of gene expression between GD and FA treated cells is presented in Table 3.

A comparison between PPI-G4-OS-M3 and PPI-G4-DS-M3 dendrimers mode of action on the cell culture revealed that both groups of dendrimers induced the same sets of genes (*REL, RELB* and *NFKBIB*) (Table 3). Analogous comparison between FA and dendrimers showed differences in the gene expression in the NF-κB signaling pathway. We have noted that FA in the 4-h culture caused overexpression of *REL, RELB* and *NFKBIB* (Table 2). The heatmap reflects significant genes expression differences in NF-κB signaling pathway under the influence of both FA and GD in comparison with reference (FA/Ref and Dendrimer/Ref), shown in Fig. 3.

4. Discussion

For decades, treatment of CLL has been established on chemotherapy. Despite the improvement of survival by the use of chemoimmunotherapy, most patients carrying CLL burden will relapse eventually. Currently the treatment of patients with CLL is undergoing fundamental changes (Burger et al., 2017). One promising approach to achieve more durable responses might be the development of glycodendrimers coated with sugar layer which presents unique and functional characteristics (Kakkar and Balakrishnan, 2015; Eichhorst et al., 2016).

Following many novel approaches in CLL examination and the molecular methodology our study is based on the gene expression profiling (GEP) by microarrays to identify the specific target in CLL treatment to apply maltotriose-modified PPI glycodendrimers. In the current study we found a direct link between different expression profile and the examined compounds. By using the network analysis a unique gene expression signature composed of three genes: *NFKBIA, BCL3* and *CHUK* differentiated between cultures treated with PPI dendrimers and FA.

Conserved helix-loop-helix ubiquitous kinase (CHUK) encoded by the *CHUK* gene is also known as the inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK-α or IKK1) and it is a part of the IκB kinase (IKK) complex that plays an important role in regulating the NF-κB transcription factor.

NF-κB mediates cross-talk between BCR and TNFR signaling (Rickert et al., 2011). As discussed earlier, activation of the proximal BCR signaling complex ultimately results in activation of IKK1/IKK2, phosphorylation and proteosomal degradation of IκBα and induction of the canonical NF-κB signaling. NF-κB transcriptional activity leads to upregulation of a number of chemokine molecules (CCL17, CCL22, CXCR5); cell cycle regulators (Cyclin D) and the anti-apoptotic proteins mentioned above. In a positive feedback mechanism, canonical NF-κB signaling enhances transcription of the non-canonical NF-κB pathway components (such as p100), as well as c-Rel and BAFF-R which are critical for BAFF-R signaling. Hence the NF-κB pathway represents an attractive target in B-cell malignancies. Moreover, CD40L- or BAFF-mediated NF-κB activation has strong clinical implications in that it may render malignant B cells less dependent on BCR signaling targeting agents ultimately leading to resistance to BCR-targeting agents (Rickert et al., 2011). However, our data shows that FA more upregulate the expression of *CHUK* gene rather than both glycodendrimers, the differences are not very significant as well as it is still very short time of observation (4 h) and we expect different results after extended incubation time.

The gene B-cell CLL/lymphoma 3 (*BCL3*) belongs to IκB family and was initially identified as a candidate for a proto-oncogene located adjacent to the breakpoint of t(14;19)(q32;q13) in some patients with CLL (Nishikori et al., 2003; McKeithan et al., 1994; Ohno et al., 1990; Chang and Vancurova, 2014; Mckeithan et al., 1997; Mitchell et al., 2001). Unlike other IκBs, BCL3 is a predominantly nuclear protein, containing a transactivation domain, and can be recruited to NFκB-responsive promoters. This results in a transcriptional activation or repression, depending on the composition of NFκB complexes (Ge et al., 2003). Overexpression of BCL3 is also directed to an increased survival, cell proliferation and malignant potential.

The hallmark of NFKBI protein family is the presence of multiple ankyrin repeats and their ability to associate with the NF-κB protein. The NFKBIA (IκBα) is characterized by 3 regions: an N-terminal region with phosphorylation sites, a C-terminal PEST region regulating basal degradation and an ankyrin repeat domain (Jacobs and Harrison, 1998; Zhao et al., 2014). The NFKBIA gene is found on chromosome 14 and contains 6 exons. Polymorphisms and haplotypes of NFKBIA and mutations have been found in many diseases such as melanoma, Hodgkin's lymphoma, colorectal cancer, hepatocellular carcinoma, multiple myeloma or breast cancer (He et al., 2009; Liu et al., 2010; Bu et al.,

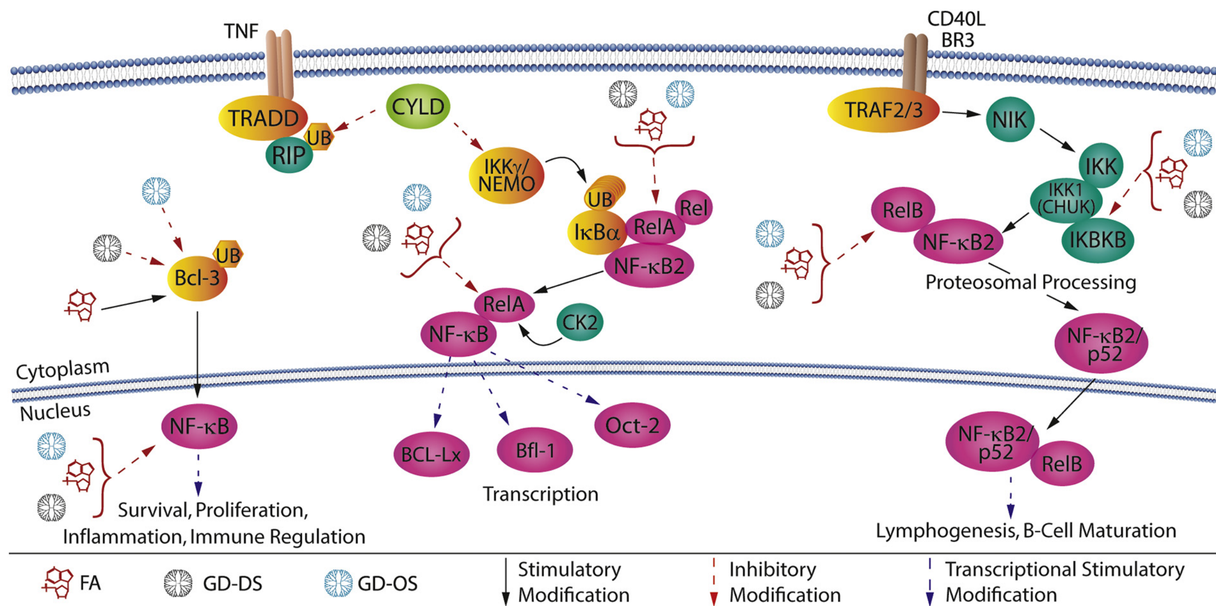

Fig. 2. A comparison between glycodendrimers (GD) and fludarabine (FA) mode of action on NF-κB cell signaling pathway in chronic lymphocytic leukemia cells.

Table 2
NF-κB gene expression in MEC-1 cells under the influence of PPI-G4-OS-M3, PPI-G4-DS-M3 and FA in 4-h-cultures; comparison with Ref.

	Probe set	Gene symbol	Gene full name	LogFC	Statistical analysis *P* value
PPI-G4-OS-M3/ Ref	P330895	*NFKBIB*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, beta	1.2943	0.0002
	P56938	*REL*	V-rel avian reticuloendotheliosis viral oncogene homolog	0.7639	0.0005
	P106002	*NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alfa (IκBα)	−0.6581	0.001
	P55706	*RELB*	V-rel avian reticuloendotheliosis viral oncogene homolog B	0.6076	0.002
	P30655	*NFKBIE*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, epsilon	−0.8388	0.01
	P3209433	*RELA*	V-rel avian reticuloendotheliosis viral oncogene homolog A	0.2533	0.05
PPI-G4-DS-M3/Ref	P56938	*REL*	V-rel avian reticuloendotheliosis viral oncogene homolog	0.9975	0.0001
	P330895	*NFKBIB*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, beta	1.2535	0.0002
	P55706	*RELB*	V-rel avian reticuloendotheliosis viral oncogene homolog B	0.6551	0.001
FA/Ref	P56938	*REL*	V-rel avian reticuloendotheliosis viral oncogene homolog	0.6598	0.001
	P55706	*RELB*	V-rel avian reticuloendotheliosis viral oncogene homolog B	0.6953	0.001
	P330895	*NFKBIB*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	0.8904	0.001

Table 3
Apoptotic gene expression in MEC-1 cells under the influence of glycodendrimers in 4-h-cultures; comparison with FA.

	Probe set	Gene symbol	Gene full name	LogFC	Statistical analysis P value
PPI-G4-OS-M3/FA	P106002	*NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alfa (IκBα)	−0.4580	0.04
	P4662	*BCL3*	B-cell CLL/lymphoma 3	−0.6212	0.04
	P3258141	*CHUK*	Conserved helix-loop-helix ubiquitous kinase	−0.3954	0.05
PPI-G4-DS-M3/FA	P4662	*BCL3*	B-cell CLL/lymphoma 3	−0.6488	0.04
	P56938	*REL*	V-rel avian reticuloendotheliosis viral oncogene homolog	0.3376	0.06
	P3258141	*CHUK*	Conserved helix-loop-helix ubiquitous kinase (*IKK1*)	−0.3363	0.08
	P106002	*NFKBIA*	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alfa	0.3208	0.09

2007; Spink et al., 2007). Alterations render NFKBIA proteins important from a clinical point of view mainly because they are incapable of interacting with NF-κB, NFKBIA lose their activity, and thus resulting in the loss of protection of tumor cells from apoptosis. *RELB* silencing may be involved in the development of resistant subtypes of CLL in males (Marteau et al., 2010). Our results show that glycodendrimers increase the expression of this gene.

Lymphoid microenvironment promotes survival, proliferation and finally progression of CLL cells through chronic activation of NF-κB while suppressing the immune response (Mittal et al., 2014). In the current study, we show that the addition of PPI-G4-OS-M3 and PPI-G4-DS-M3 dendrimers to B-CLL cells significantly induces apoptosis phenomenon within this lymphocytes by significant reduction of their survival ratio.

Since 2012 our team has investigated the PPI-G4-M3 dendrimers as potential drug in CLL therapy (Franiak-Pietryga et al., 2013) evidently proving that PPI glycodendrimers strongly influence the survival of cancer cells by inducing apoptosis and inhibiting their proliferation but do not exert significantly harmful effect on normal lymphocytes (Franiak-Pietryga et al., 2013; Ziemba et al., 2014). Moreover, PPI-G4 glycodendrimers mainly modulate pro- and antiapoptotic gene and protein expression in CLL cells (Franiak-Pietryga et al., 2017b).

5. Conclusions

According to our previous results, we can indicate many genetic

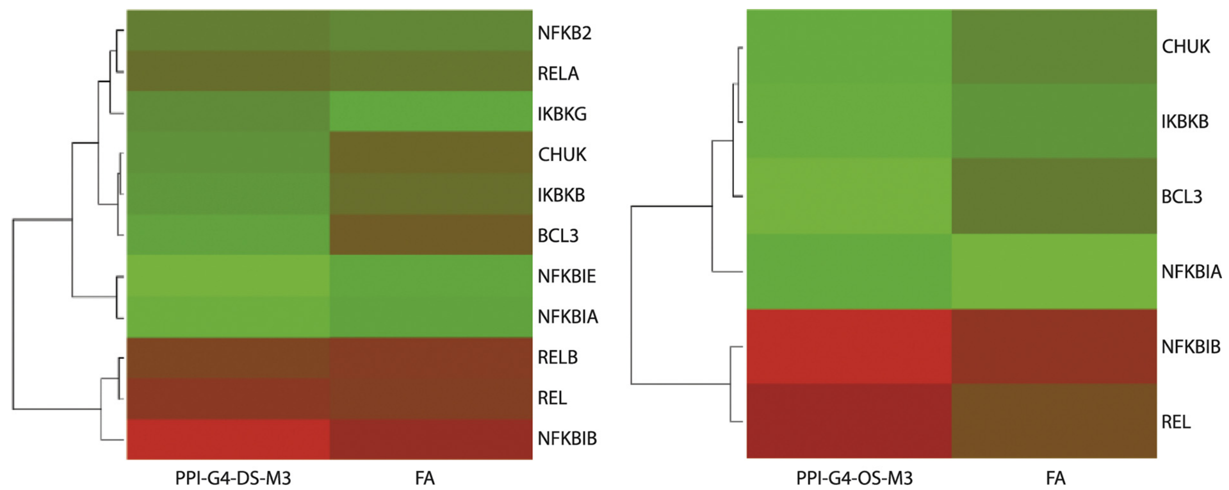

Fig. 3. Heatmaps illustrating the results of hierarchical clustering of rows (genes) and columns (samples) in four-hour cultures (Glycodendrimers vs. FA). Green represents the lowest and red the highest signal in FA or dendrimer samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

targets in CLL that glycodendrimers (GD) may work with. This is totally different from the drug currently available in the market. The effect of GD is based on triggering different genes simultaneously, starting a domino effect with the rest of the pathways. The effect of apoptosis begins in the inner part of the cell as soon as the dendrimer is endocytozed. The apoptosis comes directly from the mitochondria and secondly triggers external pathway. Since the mechanism starts in the middle of cell, it is much easier to turn on different genes simultaneously. This is the 'trojan horse' effect – the most efficient sort of treatment. We already know a lot about GD but there are still uncharted waters remaining where we can find tremendous potential of GD in biomedical applications. Thus, further research is fully justified for validating the potential of glycodendrimers as polymeric drug under in vivo conditions.

Conflicts of interest

There are no conflicts to declare.

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