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Cationic phosphorus dendrimers and therapy for Alzheimer's disease

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ABSTRACT: Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by aggregation of extracellular β-amyloid and the intracellular microtubule-associated protein Tau. Neurodegeneration is associated inter alia with activation of microglial cells, neuroinflammation, oxidative stress, and diminished transduction of impulses in cholinergic neurons. Current pharmacotherapy for AD is based mainly on modulation of acetylcholine hydrolysis, administration of non-steroidal anti-inflammatory drugs and antioxidants. Novel drugs with antiamyloidic properties are currently being sought. Cationic phosphorus dendrimers have been proven to modulate amyloidogenesis and stop the aggregation of Tau protein. An ideal drug for AD should demonstrate anti-inflammatory properties, inhibit acetylcholine hydrolysis, and have antioxidant capacity. Cationic phosphorus dendrimers (generation 3 and generation 4) show the foregoing properties. They inhibit acetylcholinesterase activity, can decrease the secretion of TNF-α, and have weak antioxidant effects. The results presented suggest that phosphorus dendrimers may be considered in the future as agents in AD therapy.

Introduction

Alzheimer's disease (AD) has been estimated to be responsible for 50-60% of dementia cases in persons over 65. AD is rapidly becoming an urgent public health challenge. Currently, 35 million individuals worldwide suffer from it and if an effective medicine is not developed before 2050, approximately 115 million people will be suffering from this illness[1]. AD is characterized by the presence of amyloid plaques and neurofibrilar tangles (NFT) in the brain tissue[2]. Studies indicate that forms of β-amyloid peptides (Aβ) that occur during the aggregation process are neurotoxic both *in vitro* and *in vivo*[3]. The mechanisms of Aβ neurotoxicity are unclear, but some evidence points to the involvement of decreased acetylcholinesterase activity[4], oxidative stress[5] and neuro-inflammation[6] in AD pathogenesis.

Acetylcholinesterase (AChE) is a major enzyme involved in acetylcholine-mediated neurotransmission. It occurs in most tissues and can be found in erythrocyte membranes, brain and spinal cord, motor plates of skeletal muscles, bronchial smooth muscles and the bladder[7]. Aβ injures cholinergic neurons leading to the disruption of cholinergic neurotransmission. Peptide fragments

released from the β-amyloid plaques may inhibit the synthesis and reuptake of acetylcholine (ACh). Moreover, Aβ and NFT accumulate butyrylcholinesterase, an enzyme that destroys ACh, resulting in reduced cholinergic transmission[8,9], which deepens the deficit caused by the loss of neurons.

Oxidative stress in an AD brain has two causes. First, reactive oxidative species (ROS) are generated by Aβ-activated microglial cells[10]. Second, amyloids disrupt the mitochondrial respiratory chain, leading to the release of ROS[11]. Micromolar concentrations of Aβ peptide increase the intracellular level of H_2O_2 in culture. Catalase converts H_2O_2 to O_2 and decreases Aβ toxicity[12]. High concentrations of non-aggregated Aβ cause endothelial cell damage, which is prevented by superoxide dismutase (SOD), suggesting that $O_2^{-\bullet}$ may play a role in amyloid toxicity[13]. Zinc, iron and copper are significantly elevated in AD[14]. Epidemiological studies suggest an association between a greater incidence of AD and exposure to environmental zinc, iron and aluminum[15]. Amyloid fibrils reduce copper, suggesting that ROS can be generated both at the start and during subsequent stages of fibril formation[15].

Microglia are glial cells that function as macrophages of the central nervous system and are major agents in the active immune system in the brain[17]. They are involved in neuroprotection as well as neurodegeneration. They show neurotoxic activity when abnormal amounts of pro-inflammatory and pro-apoptotic mediators (IL-1β, IL-6, IL-12, TNF-α) are secreted[18]. In particular, microglial activation has been described in several neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[19]. Plaque-associated microglia display dilated smooth endoplasmic reticulum channels containing amyloid fibrils, suggesting they have a role in the phagocytosis of Aβ in healthy brains[20,21]. In AD, microglia that surround amyloid deposits become dysfunctional and are incapable of removing the amyloids [22].

Nanomedicine is a new, rapidly developing field of science exploiting a variety of nanoscale compounds such as liposomes, metal colloids and dendrimers[23-29]. Dendrimers are macromolecules with defined mass, size, shape, topology and surface chemistry. They are characterized by a neuron-like branching architecture that starts from a core around which the branches are attached[30,31]. It is known that dendrimers have high affinity for proteins[32], nucleic acids[33] and lipids[34]. Polyamidoamine (PAMAM) dendrimers have been found to affect amyloidogenesis[35] and purge the scrapie forms of prion protein from homogenates of infected brains[36].

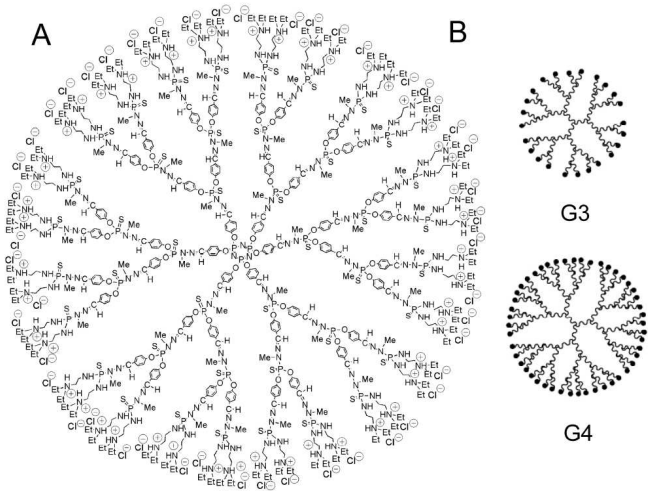

Fig. 1 Structure of cationic phosphorus dendrimers generation 3 (A) and schemes of generations 3 and 4 (B)[35].

This aroused interest in studying dendrimers as promising antineurodegenerative agents[37,38]. Currently there is no effective therapy for most neurodegenerative disorders, including AD. Cationic phosphorus dendrimers (CPDs) have been found either to accelerate or to inhibit fibril formation from $A\beta_{1-28}$, depending on the concentrations used, and to inhibit the aggregation of Tau protein. The most striking effect observed upon addition of CPDs was their ability to diminish the toxicity associated with prefibrillar $A\beta_{1-28}$ forms[39]. Considering these interesting properties, it seems worth checking whether CPDs will act synergistically or antagonistically towards conventional AD therapy.

Pharmacological procedures in AD are directed towards palliative treatment. AChE inhibitors are the only drugs approved by the Food and Drug Administration as therapeutics for AD[40]. Therefore, potential drugs for this condition emerge from inhibitors and modulators of AChE, inhibitors of the synthesis of pro-inflammatory agents, and antioxidants[41]. To date, whether cationic phosphorus dendrimers (Fig. 1) have (1) any influence on AChE activity, (2) antioxidant activity or (3) anti-inflammatory properties have not been investigated. Such information would either exclude CPDs from further studies, or – on the contrary – intensify the effort to study them in the context of AD.

Results and discussion

In this study we focused on cationic phosphorus-containing dendrimers that were characterized in the experimental section.

Acetylcholinesterase is the main enzyme that hydrolyzes acetylcholine. The ability of dendrimers to inhibit AChE activity *in vitro* was checked and the results are presented in Fig. 2 and Fig. 3. The data obtained show that both generations of CPDs at all concentrations used affected the K_M value but the maximum rate of reaction (V_{max}) was changed only at the highest concentration (10 μM) (Fig. 2).

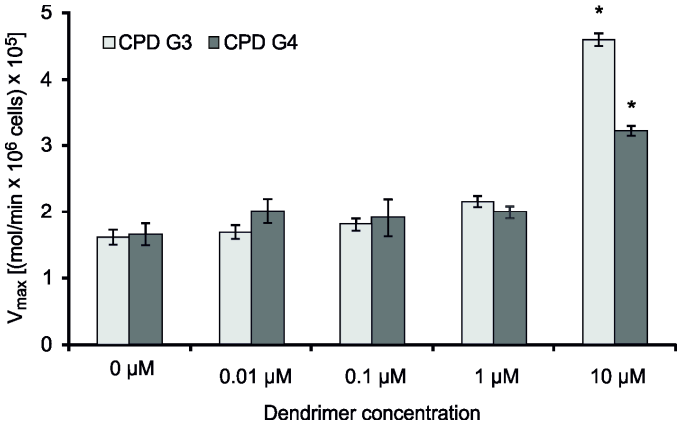

Fig. 2 The effect of cationic phosphorus dendrimers generation 3 and generation 4 on the maximum rate of enzymatic reaction. Results are expressed as means ± SD, n = 15, *P < 0.0005.

The Michaelis-Menten constant (K_M) is correlated with the affinity of the enzyme for the substrate. After addition of dendrimers at the highest concentration (10 μM) the K_M increased 4.4 times for CPD G3 and 3.8 times for CPD G4 (Fig. 3).

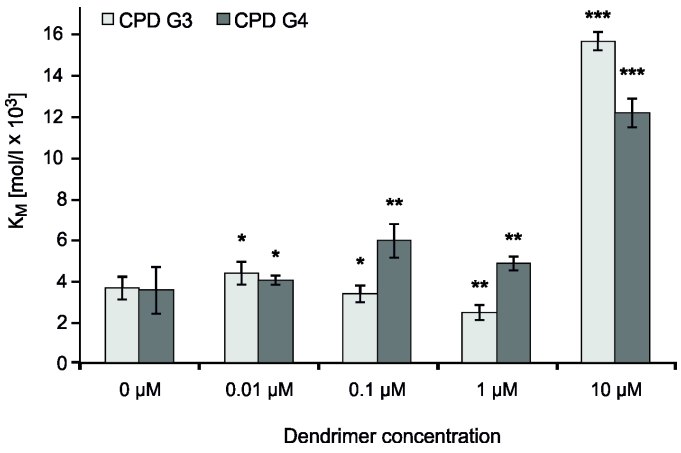

Fig. 3 The effect of cationic phosphorus dendrimers generation 3 and generation 4 on Michaels-Menten constant. Results are expressed as means ± SD, n = 15, *P < 0.05, **P < 0.005, ***P < 0.0005.

Hydrolysis of acetylcholine plays an important role in the pathogenesis of AD. It was observed only at the highest CPD concentration (10 μM) a statistically significant increase in both the maximum enzyme reaction rate (V_{max}) and the Michaelis-Menten constant (K_M), which is a measure of enzyme-substrate affinity. The effect of dendrimers on AChE activity may be due to changes in the cell membrane such as membrane fluidity. There is a relationship between membrane fluidity and the Michaelis-Menten constant. The lower the fluidity, the smaller the K_M value[42], and it is known that CPDs affect the fluidity of the lipid bilayer [34]. Similar effects on changes in V_{max} and K_M were observed for cationic PAMAM G4 dendrimers[43], which are known to affect cell morphology and cell membrane parameters[44,45]. PAMAM dendrimers can also interact with proteins, altering their conformation[32]. Inhibition of AChE activity by the highest concentration of dendrimers may indicate direct interactions between the enzyme and the CPDs. The type of inhibition was determined using Lineweaver-Burk plots as partially non-competitive. In the case of non-competitive inhibition, an inhibitor (I) attaches to an enzyme-substrate complex (ES + I → ESI) blocking the catalytic activity of the enzyme. However, for partially non-competitive inhibition, the complex ESI is still catalytically active. The activity of this complex may be unchanged in comparison to the ES complex, or it can be increased or decreased[31]. Cationic phosphorus dendrimers cannot be considered as traditional inhibitors of AChE activity. They have no structural similarity to ACh, the substrate for the enzyme. The size of the AChE catalytic pocket is 1.8 nm[46], but the diameter of a dendrimer molecule ranges from 4.2 nm to 5 nm for generations 3 and 4, respectively[66]. Therefore, it seems likely that cationic phosphorus dendrimers do not interact directly with the catalytic pocket of the enzyme but change the conformation of the protein, modify the parameters of the plasma membrane or interact with other protein components of the membrane[47].

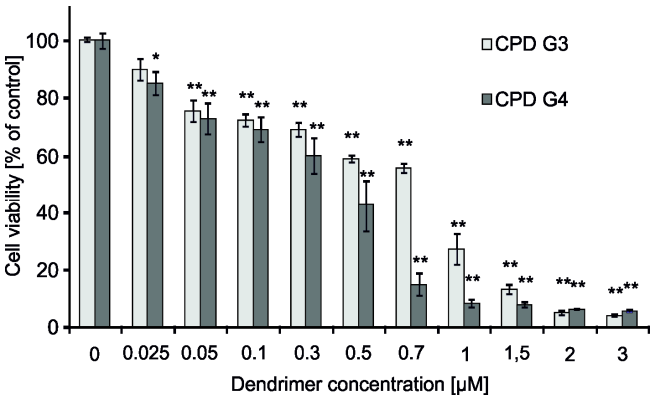

Fig. 4 The effect of increasing concentration of cationic phosphorus dendrimers generation 3 and generation 4 on mouse microglial cell line (BV-2) viability. Results are expressed as means ± SD, n = 3, *P < 0.005, **P < 0.0001.

The cytotoxicity of the CPDs was evaluated on the basis of the IC_{50}, determined from the cell survival curve. The IC_{50} is the concentration of dendrimer that causes a 50% decrease in cell viability (the viability of untreated cells was assumed to be 100%). In addition, the IC_{10} and IC_{25} were calculated for BV-2 cells. These parameters describe the concentrations of dendrimers causing 90% and 75% decreases in cell viability, respectively. These values were needed to establish the concentration used when the dendrimers' influence on the secretion of tumor necrosis factor (TNF-α) was studied. The calculated values are shown in Table 1.

Table 1. Values of IC parameters

IC parametr	CPD G3 [μM]	CPD G4 [μM]
IC10 ± SD	0.025 ± 0.002	0.026 ± 0.002
IC25 ± SD	0.05 ± 0.003	0.052 ± 0.008
IC50 ± SD	0.632 ± 0.032	0.421 ± 0.116

CPD G4 was more cytotoxic for BV-2 cells than CPD G3 (Fig. 4)[38].

Cationic dendrimers exhibit high toxicity both *in vitro* and *in vivo*[48,49] because they interact with cell components. They cause the formation of nanopores in the cell membrane and change the geometry of the membrane by interacting with negatively-charged phospholipids[45,50,51]. In the case of phosphorus dendrimers, the toxicity is limited due to the fact that in physiological pH majority of surface groups are not ionized. Nevertheless, it was necessary to check the toxicity of CPDs on BV-2 cells before studying their influence on the tumor necrosis factor alpha (TNF-α) level, and the IC_{10} and IC_{25} concentrations were used for further investigations.

The influence of CPDs on the secretion of TNF-α was examined in the mouse microglia cell line BV-2. BV-2 cells were incubated for 24 h with bacterial lipopolysaccharide (LPS, 100 ng/ml in PBS) to induce an immune response. LPS is a strong immunogen inducing an immune defense response. N-acetylcysteine (NAC) lowers the secretion of TNF-α. Neither LPS nor NAC in the concentrations used had cytotoxic effects on the BV-2 cell line (Fig. 5).

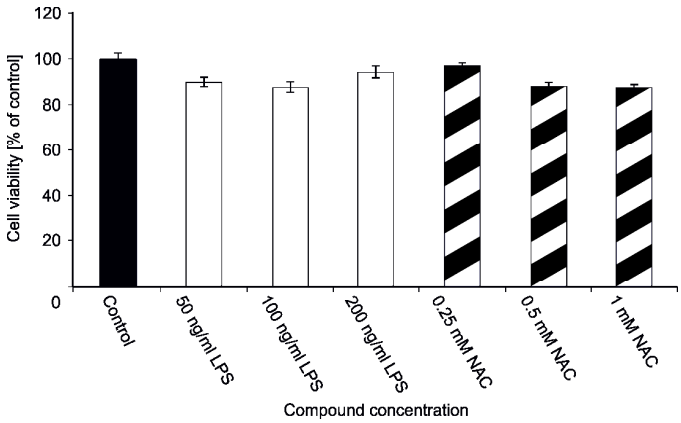

Fig. 5 The effect of increasing concentration of N-acetylcystein and lipopolisaccharide on mouse microglial cell line (BV-2) viability. Results are expressed as means ± SD, n = 3, *P < 0.005, **P < 0.00005.

LPS-activated BV-2 cells secrete proinflammatory cytokines to the extracellular environment and mobilize the immune system. The level of TNF-α rose to double that in the control after pre-incubation of the BV-2 cells with LPS (Fig. 6).

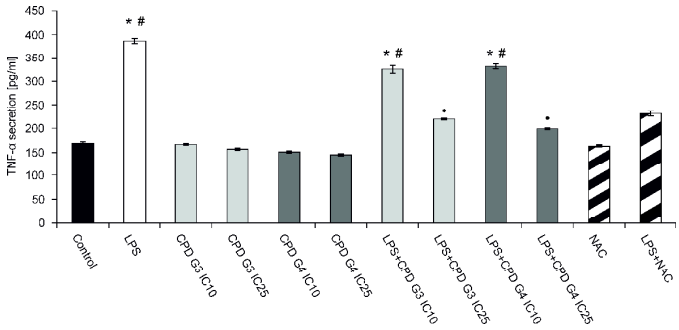

Fig. 6 The effect of cationic phosphorus dendrimers generation 3 and generation 4 on secretion TNF-α. BV-2 cells were activated by 100 ng/ml of LPS for 24h, after preincubation with LPS, CPD G3 and CPD G4 were added in their IC10 and IC25 concentrations. TNF-α in culture medium was measured using mouse TNF-α ELISA Kit, n = 6, *P < 0.0005 (for control), #P < 0.0001 (for LPS + NAC), •P < 0.0001 (for LPS).

A four hour incubation of these cells with CPDs at their IC_{10} and IC_{25} concentrations (Table 1), or with 0.5 mM NAC, caused no statistically significant change in the level of TNF-α; however, incubation of microglia previously activated with LPS for 24 h with CPDs at the IC_{25} though not the IC_{10} concentration caused a statistically significant decrease in the secretion of TNF-α.

Microglial cells are responsible for the inflammatory reaction in a brain affected by AD[52]. The appearance of amyloid plaques is accompanied by chronic inflammation caused by microglia. These cells are activated by amyloid deposits and release pro-inflammatory mediators such as IL-1, IL-6 and TNF-α[53]. Numerous literature reports indicate that an increased TNF-α level plays a major role in the pathogenesis of AD[54-57]. Secretion of TNF-α by microglia induces a cascade of pro-inflammatory activation factors: INF-γ, IL-1, IL-6, GM-CSF, G-CSF, M-CSF, EGF, NGF[51].

To verify whether they affected the TNF-α level, the mouse microglial cell line BV-2 was used because they exhibit the same phenotypic and functional properties as reactive microglial cells[58]. BV-2 cells were activated by bacterial lipopolysaccharide (LPS). In cells of the immune system, LPS activates protein kinase C, leading to the activation of NF-κB and the transcription of protein tyrosine kinases, which in turn are involved in the release of pro-inflammatory cytokines and cytotoxic compounds (reactive oxygen and nitrogen species)[59,60]. As expected, a 24 h incubation of BV-2 cells with LPS resulted in an increased level of TNF-α. It was important to ascertain that CPDs at the IC_{10} and IC_{25} concentrations did not themselves increase the TNF-α level. After addition of CPDs at the IC_{25} concentration to LPS-activated BV-2 cells, the TNF-α level did not differ significantly from controls; the same for the system in which N-acetylcysteine (NAC) was added to the LPS-activated cells (LPS+NAC). NAC suppresses inflammation in the central nervous system[61]. These results indicate that cationic phosphorus dendrimers can modulate the immune response. Similar results were obtained with human monocytes treated with low generations of phosphorus dendrimers, where the surface groups were modified by phosphonic acid[62]; the monocytes were activated[63].

The antioxidant properties of cationic phosphorus dendrimers were examined using two methods: scavenging of the stable radical DPPH and ability to reduce ferric to ferrous ions (FRAP).

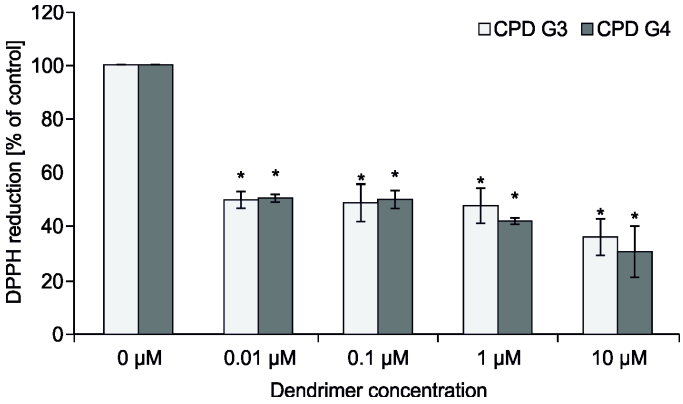

Fig. 7 The effect of cationic phosphorus dendrimers generation 3 and generation 4 on scavenging of DPPH free radical. Results are expressed as means ± SD, n = 4, *P < 0.0005.

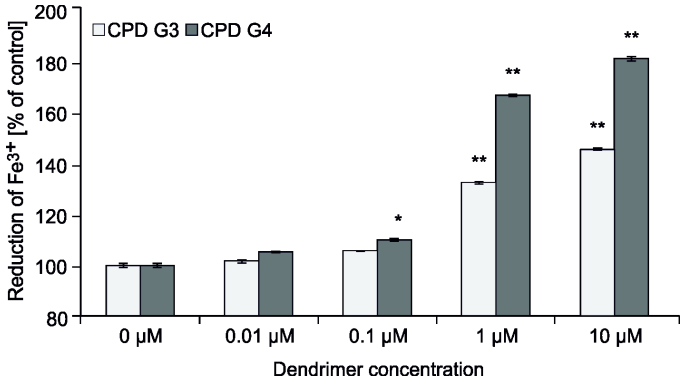

Fig. 8 The effect of cationic phosphorus dendrimers generation 3 and generation 4 on reduction of ferric ions. Results are expressed as means ± SD, n = 4, *P < 0.05, **P < 0.0005.

Data in Fig. 7 and Fig. 8 are presented as percentages of control and those in Table 2 are expressed as the Trolox equivalent antioxidant capacity (TEAC).

Table 2. Trolox equivalent antioxidant capacity (TEAC) values.

Dendrimer concentration	DPPH [mM]		FRAP [mM]	
	CPD G3	CPD G4	CPD G3	CPD G4
Control	0.107	0.167	0.147	0.167
0.01 µM	0.161	0.249	0.152	0.179
0.1 µM	0.161	0.25	0.155	0.187
1 µM	0.163	0.263	0.195	0.282
10 µM	0.173	0.282	0.215	0.309

CPDs after 30 min of incubation reduced the DPPH radicals, as shown by the decrease in solution absorbance. The CPD concentration did not correlate with the extent of DPPH reduction. For both generations of CPDs tested, there was an exponential increase in the reduction of Fe^{3+} after 30 min incubation.

Oxidative stress in the AD brain has two sources. First, reactive oxygen and nitrogen species are generated by activated microglial cells by β-amyloid decomposition[10]. Second, β-amyloid uncouples the mitochondrial respiratory chain leading to the release of reactive oxygen species[11]. In clinical practice, AD patients are treated with a wide range of compounds with antioxidant properties, including vitamin E, flavonoids and NAC[64]. The trolox equivalent antioxidant capacity (TEAC) of CPDs was determined by two methods (reduction of DPPH and a FRAP method). Comparing the TEAC results for the highest CPDs concentration tested (10 µM) with those for various exogenous antioxidants such as vitamin C (1.2 mM), vitamin E (0.9 mM) and orange juice (6.63 mM)[65], it can by concluded that CPDs exhibit weak antioxidant activity.

Conclusions

In this study we have checked whether cationic phosphorus dendrimers act synergistically with the classical pharmacological treatment for Alzheimer's disease (Fig. 9). In the presence of the dendrimers, the level of TNF-α was reduced to values similar to those observed for inactive microglial cells. This effect was observed for the concentration of approx. 0.05 µM. An antioxidant activity, although weaker, was even detected at lower concentration of the dendrimers The third studied aspect - inhibition of AChE – was, however, observed for a much higher concentration (10 µM). It is almost 20 times higher than IC_{50} for BV-2 cells and 10 times more than it is needed to block amyloid fibril formation[39].

To sum up, the cationic phosporus dendrimers, when applied in reasonable concentrations, may act synergistically with antiinflammatory and antioxidant agents and are not antagonistic to AChE inhibitors. In order to translate the tested dendrimers to the clinical evaluation stage, further extensive studies are needed on modifying the dendrimer structure, especially the surface, to reduce the toxic effect and achieve the transport through blood-brain barrier.

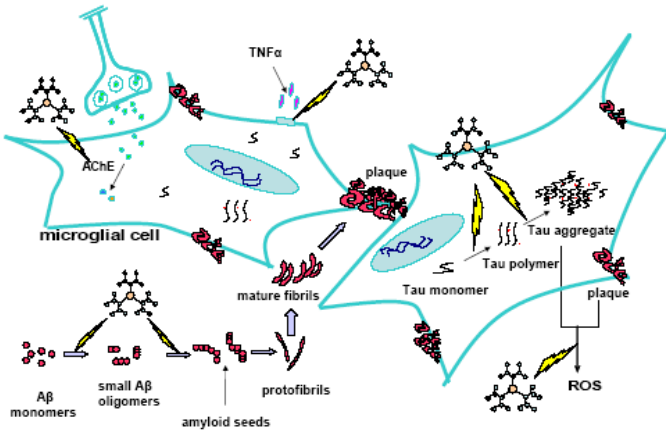

Fig. 9. Influence of cationic phosphorus dendrimers (CPDs) on the: modulation of amyloidogenesis, inhibition of Tau protein aggregation, decreasing of TNF-α secretion, inhibition of the acetylcholinesterase (AChE) activity and antioxidant effect.

Experimental

Materials. Cationic phosphorus dendrimers (CPDs) were synthesized in the Laboratoire de Chimie de Coordination du CNRS[66]. They are characterized by the presence of aminothiophosphates in their backbone at each branch point, which could enhance their biocompatibility. CPD G3, $C_{624}H_{1104}N_{183}Cl_{48}O_{42}P_{45}S_{42}$ (generation 3, 48 cationic surface groups, MW: 16280 Da; diameter: 4.1 nm), and CPD G4, $C_{1296}H_{2256}N_{375}Cl_{96}O_{90}P_{93}S_{90}$ (generation 4, 96 cationic surface

groups, MW: 33702 Da; diameter: 5 nm)[67], were studied. Stock (1 mM) solutions of dendrimers were prepared in PBS (150 mM NaCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4).

Ellman's reagent (DTNB; 5,5'-dithiobis-(2-nitrobenzoic acid)), AChI (acetylthiocholine iodide), DPPH (2,2-dipheny-1-picrylhydrazyl), LPS (lipopolysaccharide) from *E. coli*, TPTZ (2,3,5-triphenytetrazol-2-ium chloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich Chemical Company (Germany). All reagents used were of analytical grade. Water used to prepare solutions was MiliQ.

Cell culture. Mouse microglial cells (BV-2, ICLC No. ATL03001) and mouse neuroblastoma cells (N2a, ATCC No. CCL-131) were used. BV-2 cells were cultured in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Sigma) and 2 mM L-glutamine. N2a cells were cultured in DMEM-GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (Sigma). Cells were grown on a monolayer at 37°C in a 5% CO_2 atmosphere. The medium was replaced every 3 to 4 days and cells were trypsinized and split for subcultures in the ratio 1:6.

Acetylcholinesterase activity. AChE activity was measured by the method of Ellman et al.[68]. AChI was used as a substrate for AChE and was hydrolyzed to thiocholine and acetic acid. Thiocholine reacts with Ellman's reagent and forms a yellow anion, 5-thio-2-nitrobenzoic acid. The formation of this product is an indicator of AChE activity. Cultured N2a cells were trypsynized and resuspended in fresh FBS-free medium (2.5×10^5 cell/ml) in polypropylene tubes. Dendrimers were added to final concentrations of 0.01, 0.1, 1 and 10 μM and incubated for 2 h. After the incubation the cells were centrifuged (600×g, 5 min, 5°C), the medium containing dendrimers was removed, and then the cells were washed twice with cold PBS and centrifuged (600×g, 5 min, 5°C) and 10 mM phosphate buffer (pH 8.0) was added. Ellman's reagent was added to a final concentration of 100 μM in a 1 ml sample. The time course of AChI hydrolysis was recorded spectophotometrically using a JASCO V-650 spectrophotometer at room temperature and the rate of the reaction was calculated from the equation:

$$V = \frac{O.D.^{412} \cdot F}{13600 \cdot 1000}\left[\frac{mol\ \text{acetylocholine}}{\min \cdot 10^6 cells}\right]$$

where O.D.^{412} is the increase of absorbance at 412 nm observed during 1 min, and F is a coefficient taking cell density into account.

Two parameters were calculated from a Lineweaver–Burke graph: V_{max}, the maximum rate of the enzymatic reaction, and the Michaelis–Menten constant (K_M), which corresponds to the concentration of the substrate for which the reaction rate is half maximal.

Cytotoxicity – MTT assay. The MTT reduction assay to assess cell survival, a sensitive marker of mitochondrial activity[69,70], was used to check the cytotoxicity of cationic phosphorus dendrimers on the BV-2 cell line. Cells were seeded in 96-well microplates (Nunc) at 6×10^3 cells/well in 100 μl of RPMI 1640. They were incubated at 37°C in a 5% CO_2 humidified incubator. After 24 h the medium was removed, the cells were washed with PBS and dendrimers were added in the concentration range 0.025 to 3 μM in FBS-free growth medium. After 24 h, the medium containing dendrimers was removed, the cells were washed with PBS, and 100 μl of freshly prepared MTT (0.5 mg/ml of MTT in PBS) was added to each well. After 4 h incubation, the medium was discarded, 100 μl of MTT fixative solution (in DMSO) was added to each well, and the plates were shaken for 60 s. The absorbance was measured at 540 nm (reference 720 nm) using a Cary 50 BIO UV-Visible microplate reader. Cell viability was calculated as follows:

$$viability[\%] = (A/A_C) \times 100\%$$

where A is the absorbance of the investigated sample and A_C is the absorbance of a control (untreated) sample. The parameters IC_{10}, IC_{25} and IC_{50} (the concentrations of dendrimers that reduce cell viability to 90%, 75% and 50%, respectively) were obtained graphically from a semi-logarithmic plot.

Detection of TNF-α. BV-2 cells were seeded in 24-well plates (Nunc) at 10^5 cells/well and incubated for 24 h. After the incubation the medium was removed by aspiration and fresh FBS-free medium containing 100 ng/ml of LPS was added. After a further 24 h incubation, dendrimers were added to their IC_{10} and IC_{25} concentrations. A sample containing 100 ng/ml of LPS was a positive control, while medium with CPDs was used as a negative control. Pure growth medium served as a blank. NAC at 0.5 mM was used as an additional control. Conditioned cell culture medium was collected after 4 h and centrifuged (1000×g, 10 min); the supernatant was used for further analysis. TNF-α secretion was measured using

an ELISA kit according to the producer's protocol (Diaclone, Cat. No. 860.040.096). Supernatant (100 µl) from each sample was added to a 96-well ELISA plate, kept for 2 h and washed three times with PBS-Tween 20. Biotinylated detection antibody was added to each well and the plate was incubated for 60 min at room temperature. After the incubation the plate was washed three times with PBS-Tween 20, Streptavidin-HRP reagent was added, and the plate was incubated for 30 min at room temperature. After the plate was washed again (3 × PBS-Tween 20), the TMB substrate solution was added. The plate was kept in the dark for 30 min, the reaction was stopped by adding H_2SO_4, and absorption at 450 nm (reference 620 nm) was measured immediately using a Cary 50 BIO UV-Visible microplate reader. The concentration of TNF-α was calculated using a mouse TNF-α standard.

DPPH radical scavenging activity. The antioxidant activity of cationic phosphorus dendrimers generations 3 and 4 was checked by investigating their ability to scavenge DPPH free radicals[71]. DPPH has an intense violet color with an absorption maximum at 520 nm but turns colorless when its unpaired electrons are scavenged by an antioxidant. A reaction mixture containing 0.15 mM DPPH (prepared in methanol) and dendrimers in the final concentrations 0.01, 0.1, 1 and 10 µM was incubated for 30 min. After the incubation, the absorbance was measured at 520 nm relative to the reagent blank on 96-well titration plates (Nunc) using a Cary 50 BIO UV-Visible microplate reader. The antioxidant activity of the dendrimer was assessed by comparing the activities with the Trolox equivalent[72,73].

Ferric reduction antioxidant power assay (FRAP). The total antioxidant capacity of cationic phosphorus dendrimers generations 3 and 4 was also analyzed using a modification of the FRAP assay[74]. The method was based on reduction of ferric to ferrous ions at acidic pH causing the formation of a colored complex, ferrous-tripyridyltriazine. FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 20 mM TPTZ and 20 mM $FeCl_3$ in the proportions 10:1:1. To 200 µl of the reaction mixture, dendrimers were added to the final concentrations 0.01, 0.1, 1 and 10 µM. After 30 min incubation, the absorbance was measured at 595 nm relative to the reagent blank. The antioxidant activity of the dendrimer was assessed by comparing the activities with the Trolox equivalent. All measurements were made using a Cary 50 BIO UV-Visible microplate reader on 96-well titration plates (Nunc).

Statistical analysis. Normality of distribution of each variable was examined by the Shapiro-Wilk test. Equality of variance was examined using Bartlett's test. The statistical significance of differences was assessed using a univariate ANOVA Tukey's post hoc t-test for multiple comparisons. For a set of regression curves obtained from the Linewear-Burk equation, the t-test verified the importance of regression coefficients. Differences between results were considered statistically significant when $P < 0.05$. Calculations were performed using Statistica 5.5 (Stat Soft Poland) software. Results were expressed as means ± standard deviation (SD).

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Notes and references

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