Efficient and non-toxic gene delivery by anionic lipoplexes based on polyprenyl ammonium salts and their effects on cell physiology

Short title: Efficient polyprenyl-based gene delivery

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Abstract

Background

One of the major challenges limiting development of gene therapy is absence of efficient and safe gene carriers. Among the non-viral gene delivery methods, lipofection is considered as one of the most promising. In this study a set of cationic polyprenyl derivatives (trimethylpolyprenylammonium iodides – PTAI) with different lengths of polyprenyl chains (from 7, 8 and 11 to 15 isoprene units) was suggested as a component of efficient DNA vehicles.

Methods

Optimization studies were conducted for PTAI in combination with co-lipid DOPE (dioleoylphosphatidylethanolamine) on DU145 human prostate cancer cells using: size and zeta potential measurements, confocal microscopy, fluorescein diacetate/ethidium bromide test, cell counting, time-lapse monitoring of cell movement, gap junctional intracellular coupling analysis, antimicrobial activity assay, red blood cell hemolysis test.

Results

The results show that the lipofecting activity of PTAI allows effective transfection of plasmid DNA complexes in negatively charged lipoplexes of 200 - 500 nm size into cells without significant side effects on cell physiology (viability, proliferation, morphology, migration and gap junctional intercellular coupling – GJIC). Moreover, PTAI-based vehicles exhibit a potent bactericidal activity against *Staphylococcus aureus* and *Escherichia coli*. The developed anionic lipoplexes are safe towards human red blood cell membranes which are not disrupted in their presence.

Conclusions

The developed carriers constitute a group of promising lipofecting agents of a new type and can be utilized as effective lipofecting agents *in vitro* and are a promising basis for *in vivo* applications.

Keywords

Gene – delivery, gene therapy, gene – transfer, plasmid – gene delivery, plasmid – transfection, vector – non-viral, transfection, enhanced green fluorescent protein (eGFP).

Introduction

Progress in understanding the pathogenesis of many disorders and enormous methodological development that has been made in the field of nucleic acid transfer technology has opened perspectives for new applications of DNA and RNA delivery techniques and their introduction into laboratory and clinical practice. In fact, nucleic acids are nowadays regarded as drugs against a constantly increasing number of genetic and acquired diseases. These include hemophilia, autosomal dominant disorders, immune deficiency, chronic inflammation and intractable pain, as well as HIV, cystic fibrosis, artherosclerosis, rheumatoid arthritis and cancer [1]. The promising and continuously growing potential of gene therapy for the treatment of diseases currently considered as incurable, prompts research for effective vectors that could be safely applied *in vivo*.

Whereas the most effective gene delivery strategies are based on viral vectors, their application in clinics meets a number of substantial obstacles, i.e. a limited payload capacity, mutagenic, oncogenic and auto-immunogenic side effects, and expensive large-scale production [2]. Non-viral vehicles represent a promising alternative because they are safe and

relatively cheap in large-scale production. Among the gene transfer strategies employing non-viral vectors, lipofection is considered as one of the most promising options. Lipid-based carriers are appreciated for their versatility, low immunogenicity, simplicity of application and easy modification to meet case-specific requirements [3]. The lipofection strategy is mostly based on cationic lipids which can spontaneously interact with negatively charged nucleic acids to form lipoplexes (complexes of lipids and DNA). Their non-specific interaction with cell surface proteins and sugars on the plasma membrane stimulates the endocytic pathway leading to formation of endosomal vesicles. Mixtures of cationic and neutral lipids (also designated as helper or co-lipids), for instance DOPE (dioleoylphosphatidylethanolamine), change the properties of lipoplexes facilitating endosomal escape of nucleic acids [4],[5]. DOPE promotes formation of more fusogenic inverted hexagonal structures, compared to other helper lipids that form more stable laminar structures e.g. DOPC [6].

Due to their potential as vectors for genetic drug delivery, intensive research has been recently carried out to elaborate an optimal lipid carrier. Despite the fact that numerous cationic and helper lipids have been synthesized, many of them being the subjects of patent applications [7], none of the so far developed lipofecting formulas appear ideal. This may result from the fact that the lipofection efficiency depends not only on the composition of lipids and co-lipids, but also on the properties of target cells and the conditions of lipofection (e.g. *in vitro* vs. *in vivo*). Furthermore, the multitude of pathways and mechanisms implicated in the process of lipofection accounts for the complexity of the structure-safety-efficiency relationships [8]–[10]. Conflicting reports on the effect of vectors and lipoplex properties on transfection efficiency additionally hinder the application of new lipids and co-lipids in gene delivery [7],[11]–[16]. Therefore, trials on the improvement of the lipofection strategy are

still largely based on empirical approaches towards the formulation of optimal lipids, especially in the case of new and distinct classes of lipofecting compounds.

Polyisoprenoids are lipid components of the cellular glycosylation machinery in prokaryotes (polyprenol) and eukaryotes (dolichol) and are biosynthetically interconnected with other crucial elements of cellular metabolism (e.g. ubiquinone, cholesterol, prenylated proteins). Moreover, polyprenols and dolichols are known to increase the fluidity and permeability of lipid membranes [5,6 and references therein], and these physico-chemical properties stimulated us to synthesize the cationic derivatives of polyprenyl alcohols. Such forms of polyprenols or dolichols are most probably absent in living organisms, but can be synthesized chemically from isoprenoid alcohols accumulated in the cells. Previously, we have described preliminary yet encouraging data on the lipofecting activity of polyprenyl derivatives [19], which prompted further chemical approaches to synthesize analogues of a similar structure [20].

The aim of the present study was to estimate the lipofecting potential and cellular effects of the set of cationic polyprenyl derivatives (trimethylpolyprenylammonium iodides – PTAI) with different lengths of polyprenyl chains (from 7, 8 and 11 to 15 isoprene units). Comprehensive analyses of their properties also enabled the optimization of the lipofecting mixture and to define the factors crucial for lipofecting efficiency, cytotoxic and cytostatic effect of PTAI.

Materials and methods

PTAI chemical synthesis and purification

Trimethylpolyprenylammonium iodides (PTAI, Fig. 1) differing in the numbers of isoprenoid residues (n = 7, 8, 11, 15) were prepared from naturally occurring polyprenols according to Madeja *et al.* 2007 [19], with modifications. All polyprenols for the synthesis of cationic derivatives were from the Collection of Polyprenols, Institute of Biochemistry and Biophysics PAS, Warsaw. The relevant preparation procedures were described in a patent application (PL 2012, B1 211824). All products were stable as demonstrated by thin-layer chromatography on silica gel plates (Merck), which showed single spots in chloroform/methanol/water (65 : 25 : 4, ; R_f = 0.66); in n-butanol/acetic acid/water (66 : 17 : 17; R_f = 0.23); in n-propanol/ammonia/water (8 : 1 : 1; R_f = 0.10) and in ethyl acetate/methanol/acetic acid (75 : 20 : 5; R_f = 0.03). No symptoms of decomposition were noticed after the TLC test for any of the compounds after one year of storage in a dry state in the argon atmosphere at -80ºC.

Cell cultures

Human prostate cancer DU145 cells were cultured in DMEM F12 HAM medium (Sigma-Aldrich, St. Louis, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco Lab., New York, USA), 100 IU/ml penicillin, 10/µg/ml neomycin and 10 µg/ml streptomycin (Polfa, Tarchomin, Poland) in a humidified atmosphere with 5% CO_2 at 37°C. *Escherichia coli* and *Staphylococcus aureus* were cultured on tryptic soy agar (TSA; Sigma-Aldrich, St. Louis, USA) – *E. coli* and LB (Bioshop, Burlington, Canada) agar plates – *S. aureus*, respectively.

Preparation of lipofecting mixtures and transfection procedure

PTAIs (PTAI-7, -8, -11, or -15) and helper lipid L-α-phosphatidylethanolamine dioleyl (DOPE, Sigma-Aldrich, St. Louis, USA) were dissolved in 99% ethanol to achieve final concentrations of 10 mg/ml and 3.2 mg/ml, respectively. Next, they were diluted in serum-free DMEM F12 HAM medium at the indicated PTAI/DOPE ratio and vortexed extensively (half of the maximal speed of Grant-bio PV-1 vortex mixer (Grant Instruments, Cambridge, UK) for 3 min to obtain a PTAI+DOPE solution (ethanol content around 47%). The plasmid pEGFP-C1 encoding "enhanced green fluorescent protein GFP" (kindly supplied by Professor A. F. Sikorski, University of Wrocław, Poland) was dissolved in serum-free DMEM F12 HAM to the final concentration of 0.08 µg/µl. Equal volumes of the PTAI+DOPE solution (the final concentration indicated in the text) and plasmid DNA were gently mixed by pipetting (ethanol content 0.2 – 6% depending on the applied PTAI), incubated for 30 min at room temperature to obtain lipoplexes and diluted in serum-free DMEM F12 HAM medium (1:4; ethanol content 0.1 – 1.5 %).

DU145 cells were seeded into the wells of a 24-well plate at a density of 8×10^4 cell/well and cultivated for 24 h in DMEM F12 HAM medium with 10% FCS without antibiotics (to reach 70–80% confluence). Next, the medium was aspirated and replaced with 200 µl of serum-free DMEM F12 HAM medium and 200 µl of the lipoplex suspension. After 5 h of incubation at 37°C in 5% CO_2, 400 µl of DMEM F12 HAM medium supplemented with 20% of FBS and antibiotics was added to the transfection medium. The Lipofectamine 2000 Reagent (Invitrogen–Life Technologies, Carlsbad, USA) was used as a positive control. For cell transfection, diluted pEGFP-C1 plasmid was combined with diluted Lipofectamine 2000 (1 µl of Lipofectamine 2000: 2 µg of plasmid) and incubated for 30 min at RT. Then, the diluted complexes were used for the transfection procedure as described above.

The transfection efficiency was calculated after 24 h of cell incubation with lipoplexes. Cells were washed with PBS, incubated in the presence of Hoechst 33342 (1 µg/ml in PBS; Sigma-Aldrich, St. Louis, USA) for 10 min, washed again with PBS and submerged in DMEM F12 HAM medium with 10% FBS. The percentage of transfected cells was calculated under fluorescent Leica DM IRE2 microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with Leica DC350 FX digital camera according to the following formula: $L_e = (N_{GFP}/N_{Hoe}) \times 100\%$ (where N_{GFP} -Number of cells expressing GFP; N_{Hoe} - Number of cells stained with Hoechst 33342).

Confocal microscopy analysis

The PTAI-7+DOPE-based lipofecting mixture was prepared as described above and used to make a lipoplex mixture with Cy-3-labeled plasmid DNA (2.7 kb, Label IT Plasmid Delivery Control, Mirus Bio LLC, Madison, USA). After transfection in standard conditions, DU145 cells were fixed with 3.7%-formaldehyde, permeabilized with 0.1% Triton X-100 and counterstained with Hoechst 33342. Cells were visualized with Leica TCS SP5 II scanning confocal microscope and data was analysed with Leica Application Suite Advanced Fluorescence (LAS AF) software.

Gel retardation assay

Lipoplexes were prepared as described above and mixed with the loading dye solution to achieve a final concentration of pEGFP-C1 plasmid 0.04 µg/µl, and incubated for 30 min at RT. Then, they were loaded onto 0.8% agarose gel (TAE buffer) stained with SimplySafe dye (EURx, Gdańsk, Poland). Perfect Plus 1 kb DNA Ladder (EURx) was used as an internal control. Electrophoresis was carried out under a constant electric field (65 V, 60 min) at RT.

Size and zeta potential measurements

Vectors and lipoplexes were prepared as described above. Lipoplexes were prepared in DMEM F12 HAM medium. Malvern Nano ZS light-scattering apparatus (Malvern Instrument Ltd., Worcestershire, UK) was used for dynamic light scattering (DLS) and zeta potential measurements. The samples were illuminated with a 633 nm laser, and the intensities of scattered light at the angle of 173° were measured using an avalanche photodiode. A diameter (Intensity PSD mode) and dispersity (PDI) were automatically calculated using the software provided by Malvern. The zeta potential was measured using the technique of laser Doppler velocimetry (LDV).

Cell viability and proliferation assays

To determine the effect of PTAI-7/8/11/15 + DOPE lipoplexes on cell viability, DU145 cells were incubated with optimal concentrations of lipoplexes for transfection. After indicated time intervals, cells were harvested and the number of viable cells was determined by the fluorescein diacetate/ethidium bromide test [21]. At that point the number of cells was also determined for proliferation analysis.

Cell motility assay

Motility of DU145 cells was estimated 24 h after transfection by time-lapse monitoring of their trajectories. Tracks of individual cells were determined from the series of changes in cell centroid positions (8 h with 5 minute intervals), pooled and analysed as previously described [22],[23].

Gap junctional intracellular coupling (GJIC) analyses

GJIC intensity was measured by a fluorescent dye transfer assay as previously described [24] with some modifications. Briefly, donor DU145 cells labelled with calcein and DiI (both from Invitrogen-Life technologies, Carlsbad, USA) were plated (at a ratio of 1:50) onto monolayers of DU145 acceptor cells, 24 h after transfection with PTAI-based lipoplexes. The dynamics of calcein transfer from the donor to acceptor cells was visualized using Leica DMI6000B-AF7000 microscope and the percent of donor cells capable of coupling with at least one acceptor cell within 1 h after seeding was calculated as the coupling index – C_i.

Antimicrobial activity assay

The antimicrobial activity of PTAI-7/8/11/15+DOPE vectors and lipoplexes was analysed by colony reduction assay. *E. coli* (strain ATCC 33694) and *S. aureus* (strain ATCC 29213) were suspended in RPMI-1640 medium (PAA; GE Healthcare Life Sciences, Little Chalfont, UK) with 10% HBSS (PAA; 3.6×10^6 and 3.6×10^5 cells/ml, respectively) and mixed in 1:1 volume ratio with samples of DMEM F12 HAM medium containing vectors or lipoplexes at the final concentrations given in the text and incubated for 2 h at 37°C. Then, the samples were diluted and plated on TSA (Fluka, Sigma-Aldrich, St. Louis, USA; *S. aureus*) or LB agar (Bioshop, Canada) plates (*E. coli*) and incubated for 12 h at 37°C. The CFU (Colony Forming Units; CFU/ml) was calculated and bacteria survival was shown as a percent of control.

Red blood cell hemolysis assay

A red blood cell (RBC) hemolytic activity of PTAI-based vectors and lipoplexes was tested with human RBCs suspended in PBS. RBCs derived from a healthy volunteer donor were purified in 3 centrifugation (1000 × *g* for 10 min at RT)/PBS re-suspension (2 × volume)

cycles and diluted in PBS (50-fold of original blood volume). Vectors and lipoplexes, suspended in F12 HAM medium, were added to RBC suspensions to achieve the concentrations given in the text, and incubated for 1 hr at 37°C. The samples were then centrifuged at 1000g for 10 min and the supernatants were transferred into a 96-well microtiter plate for haemoglobin release analysis. The relative haemoglobin concentration in supernatants was monitored with the Multiscan FC system (Thermo Scientific) by measuring the absorbance of the supernatant at 405 nm. Samples treated with 1% Triton X-100 were used as a positive (100%) control, whereas PBS/F12 HAM medium was used as a negative control.

Statistical analysis

Each parameter was calculated as the mean of three independent measurements +/- standard deviation (SD) or standard error of the mean (SEM) where indicated. In the time-lapse monitoring of movement of individual cells, the statistical significance between the speed of cell movement was determined by the Student's t-test. For the GJIC results the statistical significance was determined by the Dunnett's test. No significant differences were found in both applied tests – Student's t-test ($p>0.01$), Dunnett's test ($p>0.05$).

Results

Efficacy of PTAI-based lipofection is dependent on the length of the PTAI polyprenyl chain

We have previously demonstrated that PTAI-7+DOPE-based lipoplexes show strong lipofecting activity which depends on the cationic lipid helper ratio [19]. Analyses performed in this study with the aid of confocal microscopy revealed the intranuclear localization of Cy-

3-labeled plasmid DNA delivered with PTAI-7-based vectors in DU145 cells (Fig. 2A). These results prompted us to check if the length of the hydrophobic chain affects transfection efficiency of PTAI. GFP expression analyses demonstrated that all tested polyprenyl iodides with different lengths of polyprenyl chains (PTAI-7/8/11/15) were effective components of lipofecting mixtures (Fig. 2B).

Application of 3µg/well AP-7+DOPE mixture at a molar ratio of 1.5:1, along with 2µg/well of pEGFP-C1 plasmid yielded the optimal transfection efficiency of PTAI-7 (60.2 ± 2.2%, Fig. 3A-C). Application of iodides with longer polyprenyl chains (especially PTAI-11 and -15) yielded even higher transfection efficiencies, corresponding to those observed for the commercial lipofectant Lipofectamine 2000. Similar optimization performed for other PTAIs studies showed that the optimal PTAI:DOPE molar ratio for these compounds is 1.5:1 or 1:1 for PTAI-8 or PTAI-11 and -15, respectively (Fig. 3D, Supplementary figures 2, 3, 4). We speculate that the estimated, relatively higher amount of helper lipid in lipoplexes may be necessary to compensate for lower lipid mixing (with cell or endosomal membrane lipids) and fusiogenic capacity of cationic lipids with longer hydrophobic chains as reported for other lipids [25].

Moreover, we observed that PTAI of higher lengths required an increased total lipid amount necessary for the optimal transfection efficiency (Fig. 3D, Supplementary figures 2, 3, 4).

On the other hand, PTAI are monovalent lipids and their charge/mass ratio is strictly dependent on the length of the polyprenyl chain. Therefore, it seems possible that a larger amount of longer-chain PTAI is required to ensure the same pEGFP-C1 plasmid complexation capacity. This hypothesis was verified by carrying out a pEGFP-C1 plasmid gel retardation assay, which demonstrated that 15 µg of PTAI-7+DOPE (1.5:1) was sufficient for the optimal complexation of 2 µg of pEGFP-C1 (Fig. 3E). In contrast, even 20 µg of PTAI-11+DOPE was not enough to achieve such an effect. Furthermore, complete pEGFP-

C1 plasmid complexation seemed unnecessary for optimal efficiency of transfection. In optimal conditions (3 μg of lipids for PTAI-7 and 7.5 μg for PTAI-11) as little as half of the pEGFP-C1 plasmid amount was complexed and protected from DNase degradation (Suppl. Fig. 5). This was illustrated by residual bands corresponding to free pEGFP-C1 plasmid upon electrophoretic analysis (Fig 3E), which on the other hand seemed to have no negative effect on transfection efficiency and was susceptible to DNAse treatment (Suppl. Fig. 5). Importantly, pEGFP-C1 plasmid binding properties of PTAI-based formulations are similar to those estimated for optimal efficiency of transfection of Lipofectamine 2000 (optimal transfection was achieved at 1 μl of Lipofectamine 2000, Fig 3E).

Particle size and ζ potential as determinants of lipofecting activity of PTAI

Our observations indicate that PTAI-based vectors are efficient plasmid DNA carriers and complete DNA complexation is not crucial for the efficiency of PTAI-based gene transfer. Therefore, we further concentrated on the determinants of PTAI-lipoplex efficiency. For instance, the size of lipoplexes is a parameter determining the efficiency of the endocytic pathway of lipoplex internalization. In our experiments, the sizes of the most effective PTAI lipoplexes were 200 – 300 nm, except for PTAI-15+DOPE vehicles which were 310 ± 22 nm and PTAI-15+DOPE +pEGFP-C1 lipoplexes which ranged between 426-485 nm (Fig. 4A). All of them are within the range of 200-500 nm that has been shown to enter cells via caveolae-mediated endocytosis [26]. There was also a small portion of much bigger (approx. 5000 – 5500 nm) particles identified for all vehicles (1.6-3.2%) and PTAI-15+DOPE lipoplexes (4.1-5.1%). All of the lipoplexes maintained their initial size during 5h of experiment (Fig. 4A).

Interestingly, when stored at 4°C, PTAI-7, -8 and 11 - based vectors were effective for at least 18 months of storage (data not shown). In contrast, PTAI-15 - based vectors lost their

lipofecting activity during storage. Their efficiency dropped to 15% and 2% of transfected cells after 1 month and 15 months post preparation, respectively, compared to freshly prepared lipofectant (data not shown). Interestingly, the loss of the lipofecting activity by vehicles during longer storage is characteristic only for PTAI-15+DOPE-based particles creating lipoplexes with a tendency for aggregation (Fig. 4A).

Similarly to the size of lipoplexes, ζ potential has long been considered as a parameter correlated with the lipofecting capacity. Examination of ζ potential is an indirect estimation method of the particle surface charge. The positive charge of vehicles is supposed to enable interactions with negatively charged DNA. ζ potentials estimated for PTAI-based vehicles ranged between +21 and +31mV (Fig. 4B). Interestingly, a small but systematic decrease of zeta potential of vehicles and lipoplexes was observed along with the increasing length of the polyprenyl chain. This is probably connected with the charge/mass ratio of PTAI strictly dependent on the length of the chain. The positive charge of lipoplexes after DNA complexation is generally believed to be necessary to ensure the association of lipoplexes with cell membranes [27],[28]. However, PTAI-based lipoplexes were negatively charged (from -21 to -29 mV; Fig. 4). This somewhat surprising observation confirms that the positive charge of lipoplexes is not an absolute requirement for effective lipofection [29]. Moreover, negative ζ potential values of lipoplexes were reported to be beneficial for *in vivo* applications [30],[31]. All of these examinations consistently characterized PTAI-based DNA delivery vehicles as medium-sized, positively charged particles; their long-lasting DNA complexing capability makes them suitable for storage for at least 18 months (PTAI-7,8,11), while negatively charged lipoplexes work effectively for at least 5 h after preparation.

For comparison, the previously described size of Lipofectamine 2000 liposomes was ~140-500 nm and lipoplexes ~150-600 nm [32],[33]. Lipofectamine 2000-based lipoplexes were reported to have a negative charge from -12 to -25 mV for different Lipofectamine2000/DNA

charge ratios, while Lipofectamine 2000 liposomes proved to be both positive +46.7 mV [33] and negative -4 mV [32]. Our results suggest that PTAI-based lipoplexes have similar characteristics to Lipofectamine 2000. This may be a promising prognostic factor as Lipofectamine2000 is one of the best commercially available lipofecting agents.

Cell type-specific reactions of procaryotic and eukaryotic cells to PTAI-based lipofection

Examination of the effect of PTAI-based vehicles and lipoplexes on procaryotic and eukaryotic cells may provide valuable information about their structure/function relationships. The expected outcome of the lipofection procedure is nucleic acid delivery without affecting target cell survival and physiology. To elucidate the effect of PTAI-lipoplexes on target cells, we used the previously established approach based on the monitoring of basic traits of model prostate DU145 cells. Polyprenyl-based lipoplexes appeared to have no cytotoxic effect on DU145 cells (Fig. 5A). Instead, they exerted a significant effect on their proliferation (Fig. 5B) and morphology (Fig. 5D), which, however, was less pronounced than that of Lipofectamine 2000 (at least for PTAI-7). In order to reduce the unfavourable cytostatic effects of lipoplexes, their concentration was lowered to achieve efficient lipofection without any effect on cell proliferation (Fig. 5C). Thus, the conditions obtained during optimization of lipofection lead to formation of effectively acting lipoplexes which may successfully transfect cells even at lower, non-cytostatic concentration (Fig. 5C,D).

Moreover, it has long been suggested that EGFP expression affects cell physiology [34]. Therefore, experiments with an empty pcDNA3 plasmid were conducted and they revealed (Fig. 5C) that indeed cell proliferation is affected not only by the presence of lipids, but also

by EGFP expression itself. Thus an empty pcDNA3 plasmid was used in all subsequent experiments aimed at testing the impact of lipofection on the cell function.

Non-cytostatic conditions (Fig. 5C,D) of PTAI-based lipofection were used for further analyses of their effects on DU145 cells (Fig. 6). As described above, our results demonstrated that even though lipofection exerted no cytotoxic effect on DU145 cells, it may affect other cellular processes (e.g. proliferation). However, analyses of lipoplex influence cell motility (pEGFP-C1 – Fig. 6A, pcDNA3 – data not shown) and gap junctional intercellular coupling (GJIC) (pcDNA3 – Fig. 6B,C) demonstrated that PTAI-based lipofection remained neutral to both of these traits.

Although, eukaryotic cells were the focus of our attention, simplified cellular systems based on Gram-positive *S. aureus*, Gram-negative *E. coli*, and human red blood cells (RBCs) were also employed to comprehensively estimate PTAI-based carrier interactions with different cell types. Cationic DNA vehicles are believed to act analogously to naturally occurring peptide antibiotics due to their cationicity and amphipathicity, according to data proving their antibacterial activity [35]. Polyisoprenoids, polyprenyl-based vectors and lipoplexes were also tested in this context.

All the vector formulations PTAI-7/8/11/15 + DOPE had strong antimicrobial activity against *E. coli* (Fig. 7A), while only PTAI-7+DOPE and PTAI-8+DOPE were significantly harmful for *S. aureus* (Fig. 7A).

The difference may be due to the fact that Gram-negative *E. coli* has an outer membrane and exposes highly anionic lipids at its surface, which may be the target for positively-charged polyprenyl-based vectors, while Gram-positive *S. aureus* has peptidoglycan on its surface, making lipid interaction more difficult. This activity was maintained for up to 17 months after preparing a ready-to-use reagent (not shown). DOPE addition enhances PTAI activity against *E. coli* and reduces it against *S. aureus* (Fig. 7B). Since DOPE, used in

bacterial membrane-mimicking liposomes [36], strongly influences the membrane-disrupting activity of antibacterial agents [37], we hypothesize that it can facilitate interaction with the membrane of Gram-negative, but not Gram-positive bacterial cell surfaces. Neutralization of the positive charge by plasmid DNA also neutralizes antimicrobial properties (Fig. 7B). In the case of *E. coli* and newly prepared lipoplexes (Fig. 7B), this effect is incomplete. This indicates that not only the surface charge, but also the composition of interacting lipids plays a role in this process [37].

As our carriers are potentially good candidates for *in vivo* applications because of the negative charge of lipoplexes, experiments on the hemolytic activity of PTAI vectors and lipoplexes were conducted. In the case of sole cationic derivatives of polyprenols and the vehicles, the hemolytic activity was correlated with the length of the polyprenyl chain. Derivatives with shorter polyprenyl chains (PTAI-7, 8) ruptured RBC membranes more efficiently than their counterparts with longer ones (PTAI-11,15). A similar tendency was observed for the vehicles, while all the PTAI-based lipoplexes (PTAI-7, 8, 11 and 15) were neutral to RBCs (Tab. 1). Altogether these data confirm that the hemolytic activity (similarly to antimicrobial properties), depends on the positive charge of particles and is no longer present after complexation of plasmid DNA.

Discussion

The idea of constructing cationic vehicles built from linear isoprene polymers was prompted by reports on the effect of polyisoprenoid alcohols and their derivatives (phosphates) on the structure of lipid membranes [17],[38]. Since the pioneering work of Felgner et al. (1987) [39], finding an ideal cationic lipid and optimal formulation for lipofection has been regarded as a great challenge. Previously, we demonstrated that

lipoplexes prepared from a polyprenyl derivative of plant polyprenol (PTAI-7) and DOPE display a transfecting activity corresponding to that of Lipofectamine 2000 [19]. Here we present an optimized lipofecting mixture and show that the entire set of polyprenyl-based derivatives can be considered for creating new, effectively acting and stable lipofection reagents. Moreover, detailed elucidation of lipofection conditions established the determinants of DNA complexing and transfecting capability of PTAI-based lipoplexes.

Whereas all tested PTAI-based compounds have high transfection efficiency, slight differences in optimal transfection conditions hinted towards the identification of features crucial for the transfecting capability. At the level of DNA complexing activity, the ability of vehicles to condense and protect DNA from degradation determines their efficacy. Positively-charged vehicles readily interact with negatively charged nucleic acids and form lipoplexes most probably via electrostatic interaction when dispersed in an aqueous solution. This interaction is strictly connected with the positive charge of the applied lipid. Our observations show that the optimal amount of lipids used for transfection correlates with the increasing length of polyprenyl chain, confirming the relevance of this mechanism for PTAI-based vehicles, because a greater amount of monovalent PTAIs with longer polyprenyl chains is required to ensure the same positive charge and complex DNA as efficiently as short-chain polyprenols. Interestingly, free, non-complexed DNA probably remaining in the form of molecules less firmly bound to the surface of the lipoplex does not seem to have any unfavourable effect on transfection efficiency. Moreover, the hypothesis that it is required for the optimal efficiency and negative charge of lipoplexes can be taken into consideration.

Our data also show that the DNA complexing capability of polyprenyl-based lipoplexes results in formation of middle-sized lipoplexes. The size measurement results show that polyprenols offer a great complexity. This is consistent with the literature. Studies on polyisoprenoids strongly suggest that these long-chain molecules when present in the lipid

bilayer possess a specific coiled conformation and consequently the length of the hydrocarbon chain is much shorter than the predicted sum of respective C-C bonds [40]–[43]. Conflicting data exist on the influence of the lipoplex size on transfection efficiency and the optimal size has not been clearly defined so far [44]. In our experiments, all of the lipoplexes were medium-sized (200-300 nm) and highly effective, despite suggestions that such particles may be limited by diffusion and trigger worse cellular trafficking than larger lipoplexes [45]. Larger particles, up to 400-500 nm, can be efficiently processed by internalization pathways [46] and targeted lipoplexes close to 300 nm can be efficiently used both *in vitro* and *in vivo* [47].

Moreover, positively charged polyprenyl-based vectors meet the crucial requirement for interaction with the negatively charged phosphates on DNA: we demonstrated that efficient transfection was provided by negatively charged polyprenyl-based lipoplexes. This finding is somewhat surprising because positively-charged lipoplexes constitute the overwhelming majority of efficient non-viral vectors described in the literature and are generally considered more efficient [44]. The shift to a negative charge of lipid-based vehicles after DNA condensation was originally thought to ruin DNA protection and inhibit cell association and entry. Our results show that this is not an indispensable condition and negatively-charged lipoplexes still protect a sufficient amount of pDNA and successfully interact with cell membranes. This result finds confirmation in the literature [29],[33],[48]. However, some anionic lipoplexes are accompanied by Ca2+ ions [49]–[51] that are suggested to play a role in the lipoplex-cell membrane interactions. Anionic vehicles for nucleic acids are intensively developed [52]–[54]. Moreover, negatively charged lipoplexes can be useful for *in vivo* applications [30],[55] and are less toxic than positively charged ones [54],[56]. Our observations of the differences between the bacteriolytic and hemolytic activities of the vehicles and lipoplexes may support this notion. The addition of plasmid DNA neutralized

the positive charge and the antimicrobial properties of the lipoplexes. Thus, both the surface charge and the composition of interacting lipids is important for the activity of polyprenyl-based vectors and lipoplexes. Anionic formulations were shown to be useful in lipofection of cells that are hard to transfect e.g. neurons [57], macrophages [58] and to exhibit high efficiency in the presence of serum and antibiotics [49],[57],[59]. Advantages of negatively charged particles in DNA and drug delivery results in different anionic formulations of different design – using anionic lipids [54], anionic polymers [60], anionic lipid bilayers coatings [61]. They are also useful in receptor-mediated transfection [62].

It was suggested that negatively charged lipoplexes enter the cells via caveolae [63] and a temperature-independent mechanism [29], and positively-charged lipoplexes can be the target of negatively charged serum components. This may lead to their opsonization and rapid clearance by the reticulo-endothelial system [31]. Thus, negatively charged lipoplexes may be the best solution for *in vitro* and *in vivo* lipofection. A strategy based on anionic complexes was also used for DNA delivery to protect the cargo from deactivation by polyanions present in tumor ascitic fluid, such as hyaluronic acid (HA) [64]. Amphoteric liposomes (cationic at low pH and anionic at neutral pH) ensure DNA complexation but avoid blood interactions, thus facilitating well-tolerated targeted systemic oligonucleotide delivery to the sites of inflammation [65]. Efficiency of polyprenyl-based lipoplexes as delivery vectors *in vitro* also makes them potentially effective vectors for *in vivo* applications.

Although not cytotoxic, polyprenyl-based lipoplexes, when applied at higher lipid concentrations, exerted an inhibitory effect on DU145 proliferation and morphology. Nevertheless, this unfavourable effect was lower (for PTAI-7+DOPE lipoplexes) than for commercially available Lipofectamine 2000 and could easily be eliminated by lowering their concentration without affecting the efficiency. This cytostatic effect may, however, be the result of GFP expression, since an empty pcDNA3 plasmid did not exert this effect on

DU145. Moreover, PTAI-based lipoplexes had no effect on cell motility, GJIC (Fig. 6) and did not disrupt human RBC membranes (Tab. 1) which is considered as highly beneficial for *in vivo* use. While the lack of cytotoxicity on eukaryotic cells is required to find an ideal DNA vector, its opposite impact on bacteria may be beneficial. Polyprenyl-based vectors meet both of these criteria. All the vector formulations PTAI-7, -8, -11 or -15 + DOPE have antibacterial activity at concentrations not affecting DU145 cell survival. We have also shown that PTAI-7, -8 or -11+DOPE vectors are not only stable and effective, but also maintain their antimicrobial properties (PTAI-7+DOPE) up to 17 months after preparing a ready-to-use reagent. This provides an excellent basis for safe and easy storage.

Conclusions

Although it is generally considered that lipoplexes bearing positive zeta potential are more efficient as lipofectants, our data indicate that this is not always the case. In our experiments, the positive charge is unnecessary for interaction of lipoplexes with the membranes of eukaryotic cells. We demonstrate that the negatively charged polyprenyl-based lipoplexes are efficient plasmid DNA delivery vectors. Moreover, they exhibit many advantages such as effective transfection without cytotoxicity and impact on eukaryotic cell proliferation, motility and GJIC as well as antimicrobial properties and no hemolytic activity against human RBCs. Taken together, the PTAI-based lipoplexes are good candidates for delivery of genetic material to eukaryotic cells.

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Conflicts of Interest Statement

The authors have no competing financial interests to declare.

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TABLES

Table 1. No hemolytic activity of PTAI-based lipoplexes against human RBCs. Each value represents the mean ± S.D. (n = 3), n.a. – not applicable.

	PTAI-7		PTAI-8		PTAI-11		PTAI-15		Lipof. 2000	
PTAI	µg	%	µg	%	µg	%	µg	%	µl	%
	2.5	22.2 ± 0.9	3.0	30.9 ± 0.5	4.0	10.1 ± 0.5	5.0	7.9 ± 0.2	n.a.	n.a.
PTAI	3.0	23.2 ± 0.6	3.5	37.0 ± 0.7	6.0	17.1 ± 0.2	8.5	14.3 ± 0.8		
	4.0	32.7 ± 0.7	4.5	42.4 ± 1.1	7.5	40.1 ± 1.8	10.0	20.5 ± 0.6		
	5.0	40.9 ± 1.6					12.5	25.5 ± 0.2		
	2.5	10.5 ± 0.3	3.0	23.2 ± 0.7	4.0	4.1 ± 0.3	5.0	1.3 ± 0.1	1.0	3.6 ± 0.1
PTAI+	3.0	16.4 ± 0.7	3.5	31.4 ± 1.8	6.0	5.2 ± 0.4	8.5	1.7 ± 0.1	1.5	3.6 ± 0.1
DOPE	4.0	19.4 ± 0.6	4.5	42.7 ± 0.6	7.5	6.8 ± 0.2	10.0	1.6 ± 0.1	3.0	6.9 ± 0.9
	5.0	44.2 ± 1.4					12.5	1.9 ± 0.2	5.0	8.2 ± 0.7
PTAI+	2.5	1.7 ± 0.1	3.0	2.0 ± 0.2	4.0	3.2 ± 0.1	5.0	2.0 ± 0.1	1.0	3.7 ± 0.1
DOPE+	3.0	2.1 ± 0.1	3.5	2.2 ± 0.1	6.0	3.2 ± 0.0	8.5	2.1 ± 0.2	1.5	5.3 ± 0.1
pDNA	4.0	2.5 ± 0.1	4.5	2.4 ± 0.1	7.5	5.7 ± 0.2	10.0	2.3 ± 0.0	3.0	2.3 ± 0.3
	5.0	5.3 ± 0.4					12.5	2.6 ± 0.2	5.0	2.4 ± 0.2

CONTROL	**Ø**	**pEGFP-C1**	**EtOH**	**TRITON X-100**
CONDITIONS	2.8 ± 0.1	3.0 ± 0.0	2.6 ± 0.1	100.0 ± 1.8

% OF HEMOLYSIS	0 – 10 %	10 – 20 %	20 – 30 %	30 – 40 %	40 – 50 %	100 %

FIGURES

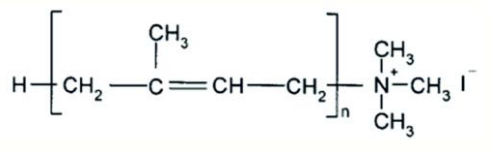


Figure 1. Structure of trimethylpolyprenylammonium iodides (PTAI), n=7/8/11/15 used in the study.

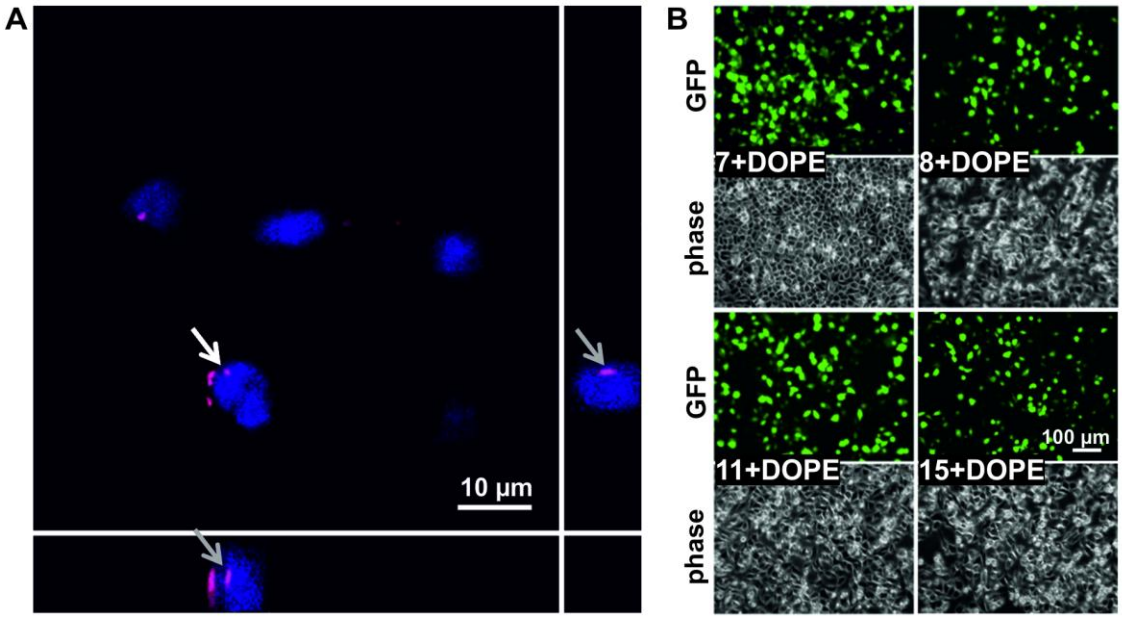
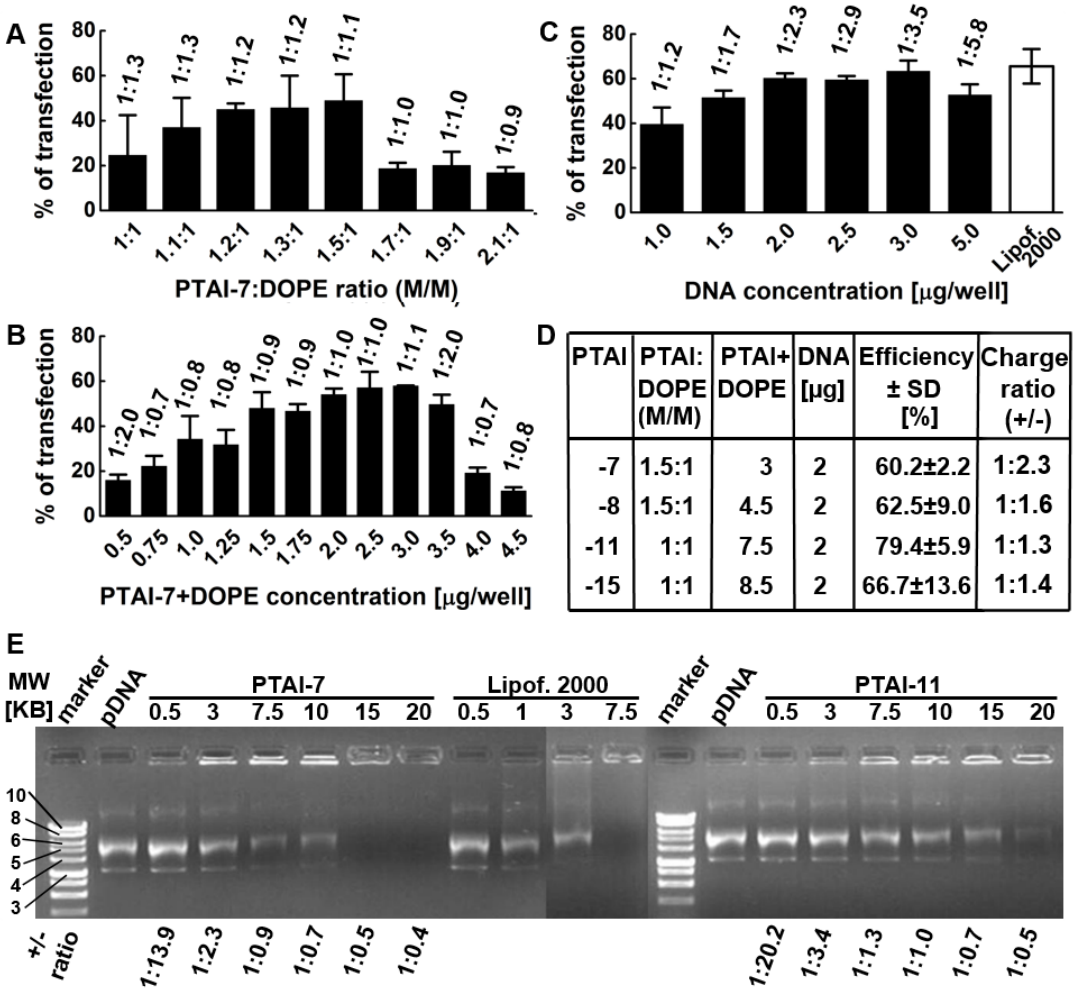


Figure 2. Efficient DNA transfer into DU145 cells. A - Intranuclear localization of Cy-3-labeled pDNA (red) delivered with PTAI-7+DOPE 4 hours after transfection. Hoechst 33342-labeled nuclei - blue. Results illustrated in XY, XZ, and YZ projections of cells. Arrows indicate lipoplexes shown in XY (white), XZ and YZ (grey) projections. PTAI-7:DOPE molar ratio – 1.5:1, 2 µg Cy-3-labeled pDNA/well (24-well plate), 3 µg of PTAI-7+DOPE/well, charge ratio 1:2.3. B - DU145 cells efficiently transfected with PTAI-7+DOPE (1.5:1 M/M, 3 µg/well, charge ratio 1:2.3), PTAI-8+DOPE (1.5:1 M/M, 4.5 µg/well, charge ratio 1:1.6), PTAI-11+DOPE (1:1 M/M, 7.5 µg/well, charge ratio 1:1.3), PTAI-15+DOPE (1:1 M/M, 8.5 µg/well, charge ratio 1:1.4), 2 µg of pEGFP-C1 plasmid/well. Phase contrast (phase) and epifluorescence microscopy with FITC filter (GFP) images.



PTAI	PTAI: DOPE (M/M)	PTAI+ DOPE	DNA [µg]	Efficiency ± SD [%]	Charge ratio (+/-)
-7	1.5:1	3	2	60.2±2.2	1:2.3
-8	1.5:1	4.5	2	62.5±9.0	1:1.6
-11	1:1	7.5	2	79.4±5.9	1:1.3
-15	1:1	8.5	2	66.7±13.6	1:1.4

Figure 3. Optimization of PTAI-based lipofection with pEGFP-C1 plasmid into DU145 cells and DNA complexing ability of PTAI. A – efficiency of transfection for different PTAI-7:DOPE molar ratio; 0.25µg pEGFP-C1/well (24-well plate), 1µg PTAI-7+DOPE/well, B - efficiency of transfection for different PTAI-7+DOPE concentration; PTAI-7:DOPE molar ratio – 1.5:1, 1.5µg pEGFP-C1/well, C – efficiency of transfection for different DNA concentration; 3µg PTAI-7+DOPE/well, PTAI-7:DOPE molar ratio – 1.5:1, control – Lipofectamine 2000 (1µl+2µg pEFP-C1), D – optimal transfection conditions for PTAI+DOPE. E - Gel electrophoresis of PTAI-7+DOPE+pEGFP-C1 lipoplexes (PTAI-7+DOPE [µg] / 2 µg of pEGFP-C1), PTAI-11+DOPE+pEGFP-C1 (PTAI-11+DOPE [µg] / 2 µg of pDNA) and Lipofectamine 2000+pEGFP-C1 lipoplexes (Lipofectamine 2000 [µl] / 2 µg pDNA). Charge ratio ($PTAI^+$:DNA^-) shown above the bars (A, B, C) and below the photo (E). Each value represents the mean ± S.D. (n = 3-7).

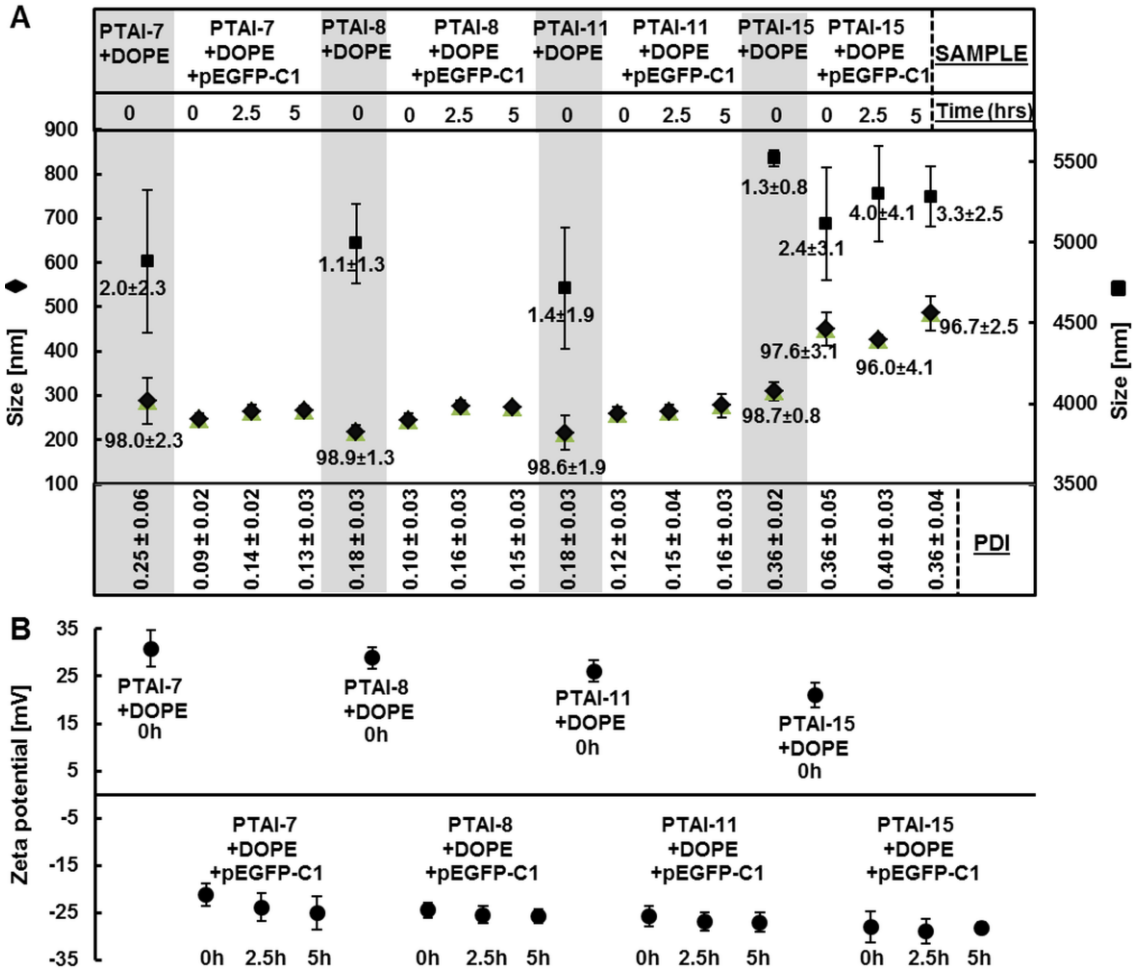


Figure 4. Size and zeta potential of vehicles and lipoplexes. Measurements show the medium size (A) and negative charge (B) of lipoplexes. PDI - polydispersity index. Vehicle and lipoplex characterization shown in fig. 3D. Each value represents the mean ± S.D. (n = 5 [A], n = 16 [B]).

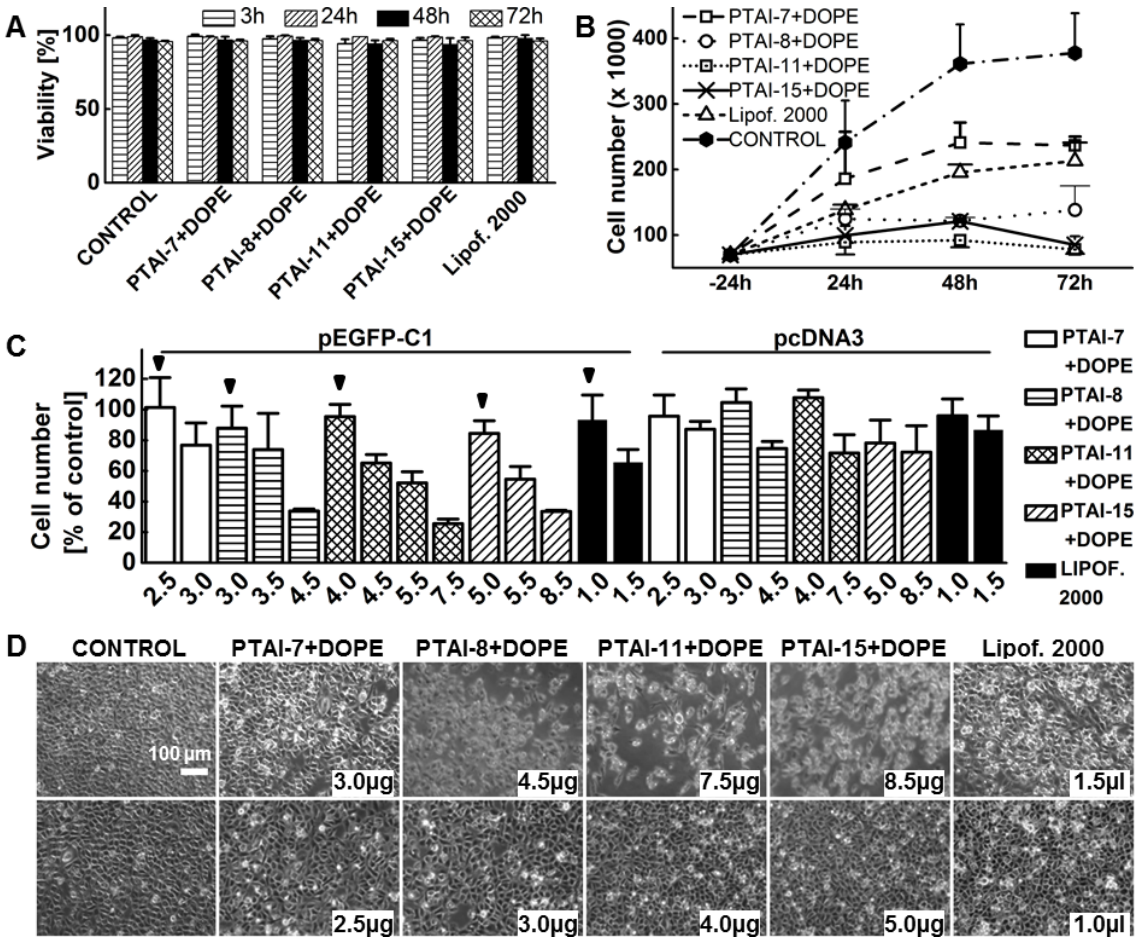


Figure 5. PTAI cytotoxicity and its effects on DU145 cell proliferation and morphology. FDA and EtBr assays revealed no cytotoxicity on DU145 cells (A) and cell counting showed a negative impact on cell proliferation (C) and morphology (D) that was eliminated by lowering the concentration of lipoplexes (C – arrows indicate optimal concentrations of PTAI-based lipoplexes not affecting cell proliferation). Transfection with non-coding pcDNA3 plasmid showed that EGFP expression could be partially the reason of the effect on proliferation (C). A, B - PTAI-7+DOPE (1.5:1 M/M, 3 µg of lipids/well [24-well plate], charge ratio 1:2.3), PTAI-8+DOPE (1.5:1 M/M, 4.5 µg of lipids/well, charge ratio 1:1.6), PTAI-11+DOPE (1:1 M/M, 7.5 µg of lipids/well, charge ratio 1:1.3), PTAI-15+DOPE (1:1 M/M, 8.5 µg of lipids/well, charge ratio 1:1.4), Lipofectamine 2000 (1µl), 2 µg pEGFP-C1/well. C – X-axis – amount of lipids in µg. Decreasing the Lipofectamine 2000+pEGFP-C1 lipoplex amount to 0.75µl/well resulted in a significant loss of transfection efficiency, thus the concentration of Lipofectamine 2000 was increased in the step of lipoplex preparation (1.5µl/2µg pEGFP-C1) and lowered when adding to cells resulting in non-cytostatic, efficient lipofection. Each value represents the mean ± S.D. (n = 3).

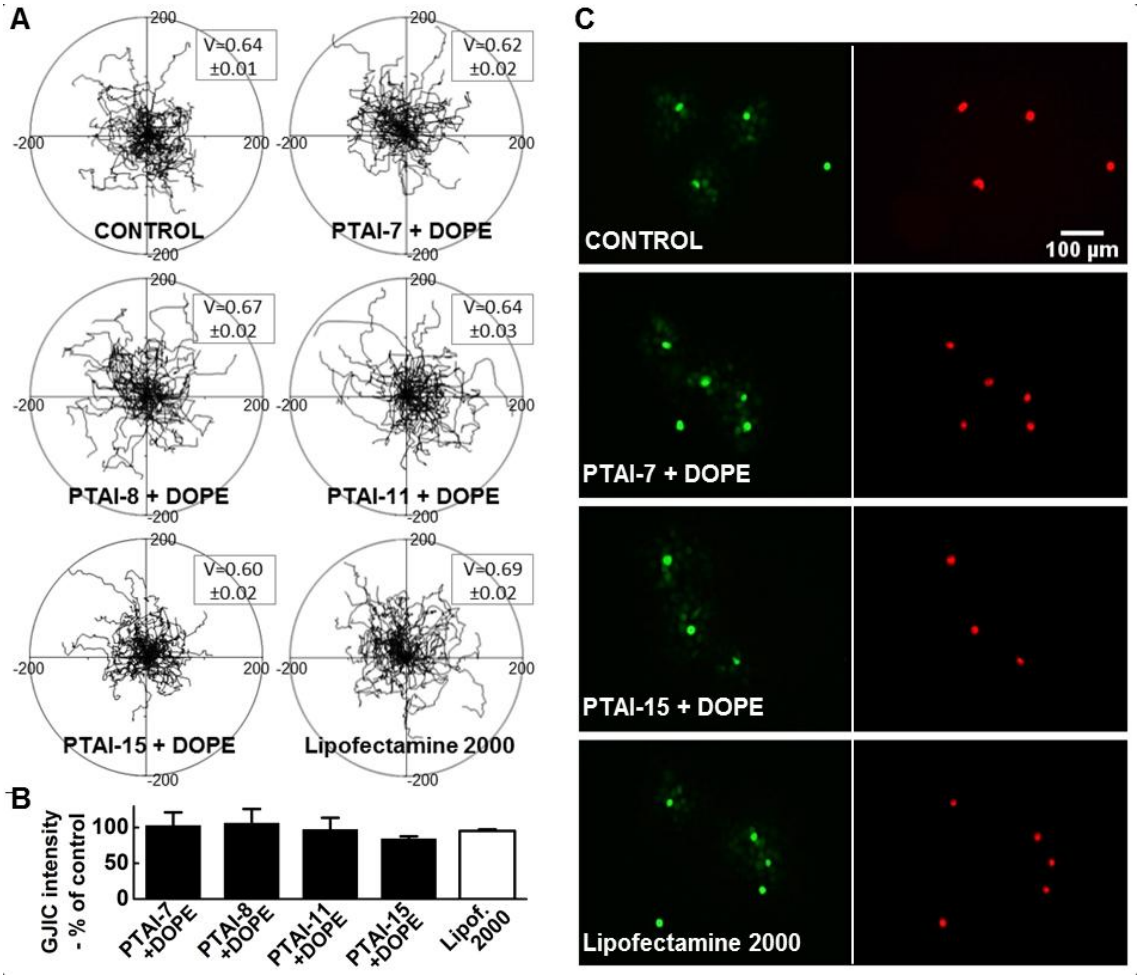


Figure 6. Effects of PTAI on DU145 cell migration and GJIC. Time-lapse monitoring of movement of individual cells 24 h after transfection with pEGFP-C1 plasmid showed no effects of PTAI-based lipofection on DU145 cell migration. Trajectories of cells presented in the form of a circular diagram drawn with the initial point of each trajectory placed at the origin of the plot. V - the speed of cell movement (µm/h). GJIC assay results 24 hours after transfection with PTAI-based lipoplexes with pcDNA3 plasmid revealed no impact on GJIC. Calcein transfer – green, donor cells – red. PTAI-7+DOPE – 2.5 µg/well (24-well plate), charge ratio 1:2.3, PTAI-8+DOPE – 3 µg/well, charge ratio 1:1.6, PTAI-11+DOPE – 4 µg/well, charge ratio 1:1.3, PTAI-15+DOPE – 5 µg/well, charge ratio 1:1.4, pcDNA3 plasmid used in all experiments. A - Each value represents the mean ± SEM (n = 50). The statistical significance between samples was determined by the Student's t-test ($p<0.01$). B - Each value represents the mean ± S.D. (n = 35). The statistical significance was determined by the Dunnett's test ($p<0.05$). No statistically significant differences were found.

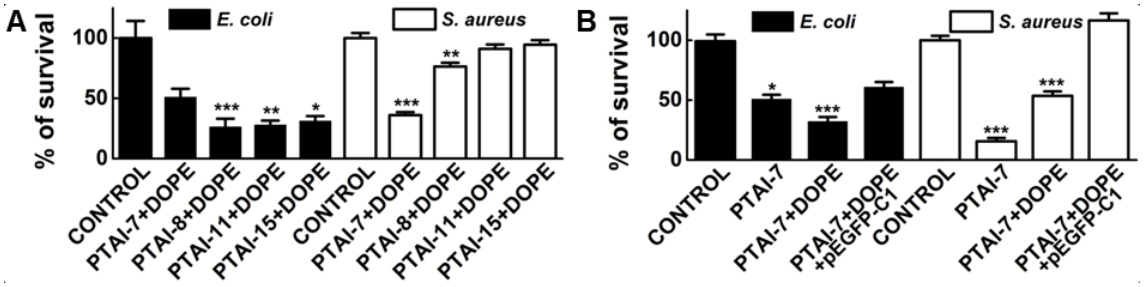


Figure 7. Antimicrobial activity of PTAI. PTAI and PTAI+DOPE showed antimicrobial activity against *E. coli* and *S. aureus* (A, B). Plasmid DNA addition neutralizes antimicrobial properties (B). Lipoplex concentrations represented as the amount of PTAI-7+DOPE – 2.5 µg/well (24-well plate), PTAI-8+DOPE – 3 µg/well, PTAI-11+DOPE – 4 µg/well, PTAI-15+DOPE – 5 µg/well. Each value represents the mean ± SEM (n=12). The statistical significance versus control was determined by Kruskal-Wallis test ($p<0.05$), * - $p < 0.05$, ** < 0.01, *** < 0.001.