Acta Biochimica Polonica

Vol. 54 No. 4/2007, 873–876

on-line at: www.actabp.pl

Communication

New cationic polyprenyl derivative proposed as a lipofecting agent★

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Received: 24 August, 2007; revised: 19 September, 2007; accepted: 22 September, 2007
available on-line: 08 December, 2007

Cationic linear poly-*cis*-isoprenoid prepared from natural plant polyprenol in a mixture with dioleyl phosphatidylethanolamine was found to be an effective lipofection agent for eukaryotic cells. The transfecting activity is related to the poly-*cis* structure of the polyprenyl chain.

Keywords: cationic polyprenyl, lipofection, transfection, GFP

INTRODUCTION

The linear polymers of isoprene residues were found to be lipid components of the systems of glycosylation in prokaryotes and eukaryotes (Robbins *et al*, 1967; Chojnacki & Dallner, 1988), thus establishing this new class of lipids as specific, crucial elements of cellular metabolism. The discovery of this class of lipids documented in the nineteen sixties (Burgos *et al*., 1963; Lindgren, 1965; Stone *et al*., 1967) was followed by testing their effect on membrane structure (Valterson *et al*., 1985; Janas *et al*., 1986) and thus approaching the mechanism of their coenzymatic functions. There were also trials to find the possible effect of these substances on eukaryotic cells (Korohoda *et al*., 1980) and the motility of cells was affected even by low amounts of dolichol (α-saturated poly-*cis*-prenol). The linear isoprenoids known so far occur in nature as alcohols, esters with acetic acid or fatty acids, phosphates or pyrophosphates and also as diesters with sugars. The cationic form of polyprenols or dolichols which are most probably absent from living organisms and have never been the subject of interest in bioorganic chemistry may be interesting substances as components of typical lipofecting cocktails allowing the transfer of genetic material into the cells of interest. So far, only non-isoprenoid methylene based cationic lipids have been used in the lipofecting cocktails of Lipofectamine 2000 (cf. leaflet of Lipofectamine Reagent, 2004).

The aim of this report is to present the case a lipofecting activity of heptaprenyltrimethylammonium iodide in a model system of human prostate cancer cells.

MATERIALS AND METHODS

Heptaprenyltrimethylammonium iodide is one of the set of compounds of this type prepared by the method of Sen & Roach (1995) from naturally occuring poly-*cis*-prenols. The biological results

★This paper is dedicated to Professor Tadeusz Chojnacki from the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw on the occasion of the 50th anniversary of his scientific activity and 75th birthday.

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Abbreviations: DOPE, dioleyl phosphatidylethanolamine; FCS, fetal calf serum; GFP, green fluorescent protein; PBS, phosphate-buffered saline.

(negative) obtained on using all-*trans*-nonaprenyltrimethylammonium iodide prepared by the same method are also stated in this report.

The preparation of a range of these compounds from naturally occurring polyprenols and dolichols is described elsewhere (Masnyk *et al.*, in preparation) and these substances are the subject of a patent application (No. PL 382448).

The heptaprenyltrimethylammonium iodide (Fig. 1A) was prepared from poly-*cis*-heptaprenol isolated from wood of *Betula verrucosa* (Lindgren, 1965) and nonaprenyltrimethylammonium iodide (Fig. 1B) from all-*trans*-nonaprenol (solanesol) isolated from commercially available tobacco leaves (Rowland *et al.*, 1956). Both products were found to be stable when checked by thin-layer chromatography on silica gel plates (Merck) (single spots in chloroform/methanol/water (65 : 25 : 4, by vol.) $R_F = 0.66$ in n-butanol/acetic acid/water (66 : 17 : 17, by vol.) $R_F = 0.23$; in n-propanol/ammonia/water (8 : 1 : 1, by vol.) $R_F = 0.10$ and in ethyl acetate/methanol/acetic acid (75 : 20 : 5, by vol.); $R_F = 0.03$; R_F the same for both compounds after one year storage in a dry state under argon atmosphere). Dioleyl phosphatidylethanolamine (DOPE) was from Avanti Polar Lipids (USA).

Lipofecting mixtures. The Lipofectamine 2000 Reagent was from Invitrogen Corporation (USA). The lipofecting mixture containing our new cationic isoprenoids (Fig. 1) was made by making a suspension with dioleyl phosphatidylethanolamine. In the control experiments with Lipofectamine Reagent the polycationic lipid 2,3-dioleyl-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleyl phosphatidylethanolamine (Invitrogen Corporation, Carlsbad, CA, USA) in a 3 : 1 (w/w) were used. Other proportions of the ingredients were also used with the aim of finding optimal conditions for transfection in the liposome formulation.

Cell culture. DU145 cells (human prostate cancer cells) were cultured in DMEM-F12 medium (Sigma, St. Louis, MO, 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.

Transfection procedure. DU145 cells were seeded one day before transfection at density 0.08×10^6 per well of 24-well plate in DMEM-F12 with 10% FCS but without antibiotics and grown to 70–80% confluency. Heptaprenyltrimethylammonium salt and helper lipid dioleylphosphatidylethanolamine (DOPE) were diluted in 99% ethanol to achieve final concentrations of 10 mg/ml and 3.2 mg/ml, respectively. Next, they were diluted and vortexed to suspend the lipid in DMEM-F12 medium without serum, at the indicated heptaprenyltrimethylammonium iodide/DOPE ratio (w/w). The plasmid pEGFP-C1 (kindly supplied by Professor A. F. Sikorski, University of Wrocław, Poland) was dissolved in DMEM-F12 without serum to a final concentration of 0.01 μg /μl. Equal volumes of cationic liposome suspension (the final lipid concentration indicated in the text) and plasmid DNA were gently mixed and incubated for 30 min at room temperature and then diluted 4 times with DMEM-F12 without serum. Medium from cells was aspirated and replaced with 200 μl of DMEM-F12 without serum and 200 μl of diluted complexes (transfection mixture). Cells were incubated at 37°C in 5% CO_2 for 5 h. Then 400 μl DMEM-F12 containing 2 × the normal concentration of serum and antibiotics was added without removing the transfection mixture. The transfection efficiency was calculated after 24 h. Cells were washed with PBS and incubated in the presence of Hoechst 33342 (Sigma, St. Louis, MO, USA) (1 μg/ml in PBS) for 10 min. After this time cells were again washed with PBS and growing medium was added. The proportion of transfected cells was calculated under fluorescence Leica DM IRE2 microscope equipped with Leica DC350 FX digital camera as follows:

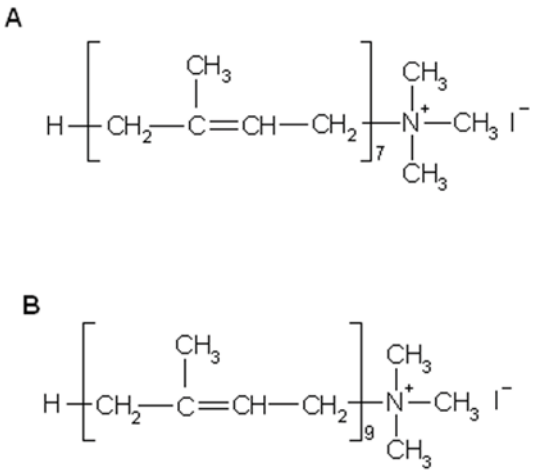

Figure 1. Structures of heptaprenyltrimethylammonium (A) and nonaprenyltrimethylammonium (B) iodides.

(Number of cells expressing GFP/Number of cells stained with Hoechst 33342) × 100%.

Lipofectamine 2000 reagent was used as a positive control. Diluted plasmid was combined with diluted lipofectamine 2000 (1 μl of lipofectamine : 0.25 μg of plasmid) total volume (50 μl) and incubated for 30 min at room temp. Then, the diluted complexes were used for the transfection procedure as described above.

RESULTS

Transfection efficiency on using heptaprenyltrimethylammonium salt

Transfection efficiency of this polyprenyl derivative was estimated by means of GFP-expression assay (Fig. 2). Cationic liposomes were prepared by mixing heptaprenyltrimethylammonium iodide and dioleyl phosphatidylethanolamine in ratio 3 : 1, followed by mixing with a plasmid DNA, pEGFP-C1, which encodes green fluorescent protein (GFP). DU145 cells were transfected with the liposome-DNA complex and the transfection efficiency was evaluated by means of fluorescence microscopy. As shown in Fig. 2, heptaprenyltrimethylammonium salt containing liposomes induced efficient transfection of DU145 cells. The highest transfection efficiency (32% of transfected cells) was observed at 1 μg of lipids per well (Fig. 3).

Interestingly, another tested compound – nonaprenyltrimethylammonium iodide under the same experimental procedure induced transfection with low efficiency, not exceeding 5% (not shown). This result seems to be consistent with previous observations that shorter-chain lipids are more fusogenic than long-chain ones (Tarahovsky *et al.*, 2004). Cationic lipids are usually formulated as liposomes using co-lipids such as DOPE (Rao & Gopal, 2006). In our preliminary research we prepared cationic liposomes by mixing heptaprenyltrimethylammonium salt with DOPE in ratio 3 : 1 (Fig. 3). However, transfection efficiency of such liposomes was lower than with commercially available lipofectamine 2000, used in our experiments as a positive control (Fig. 3). To improve efficiency of transfection we investigated the effect of liposomes prepared in various heptaprenyltrimethylammonium salt and DOPE ratios. The results presented in Fig. 4 show that optimal efficiency (exceeding 40%) was obtained for the lipids in a ratio of 1 : 1. This is comparable to other highly efficient lipofectamines, e.g., lipofectamine 2000 (Fig. 4).

DISCUSSION

The data presented in this report extend the list of cationic lipids that may be used as components of lipofecting mixtures. The idea of constructing cationic lipids built from linear isoprene polymers came from the already known information on the effect of polyprenols and dolichols on artificial lipid membranes

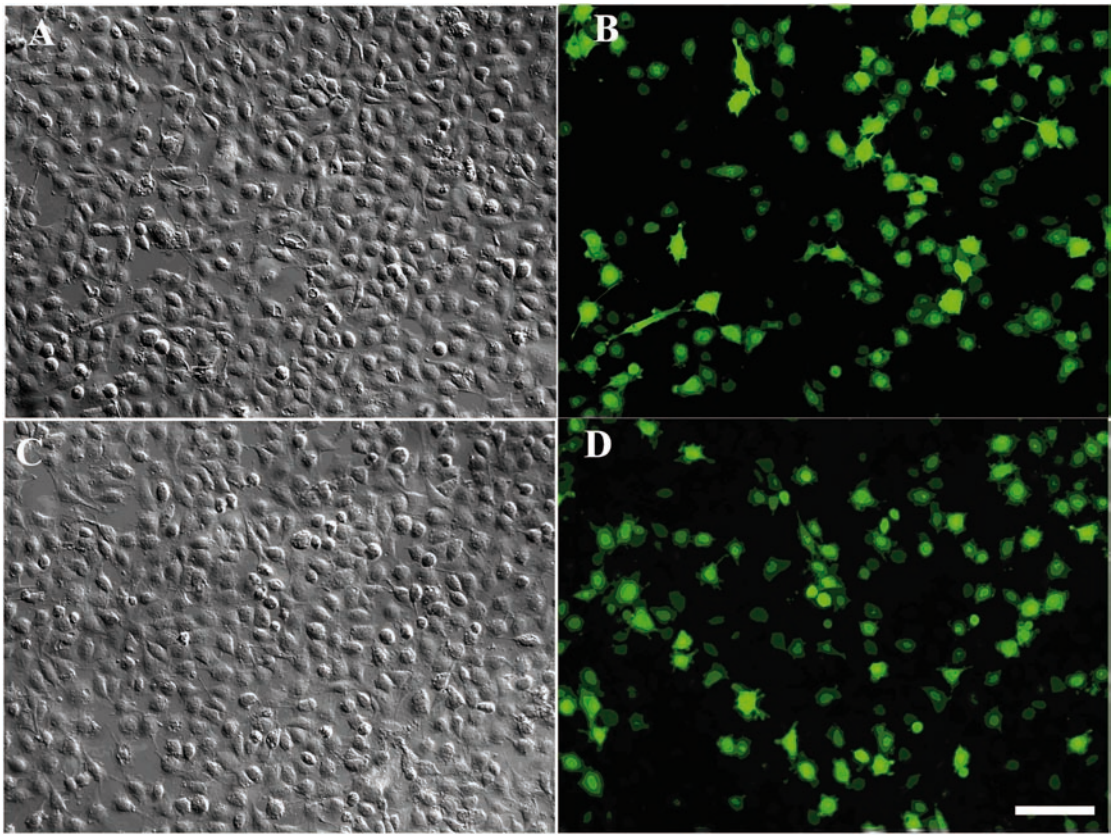

Figure 2. Transfection of DU145 cells *in vitro* with heptaprenyltrimethylammonium salt pEGFP–C1 complexes and lipofectamine 2000-pEGFP-C1 complexes.
(A,B) DU145 cells treated with heptaprenyltrimethylammnium salt contaning pEGFP-C1 complexes or Lipofectamine 2000–pEGFP-C1 complexes (C, D). (A, C) Nomarski Interference contrast. (B, D) Epifluorescence microscopy with FITC filter. Scale bar 100 μm.

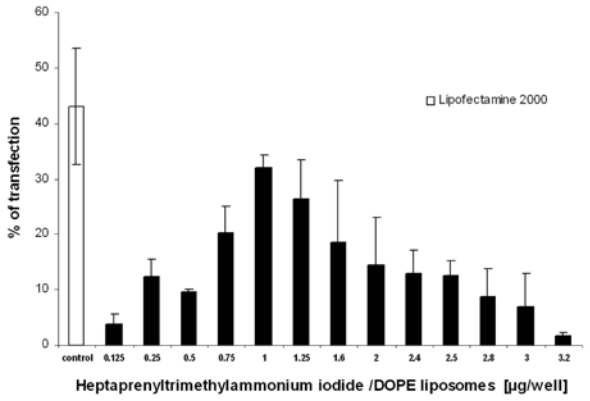

Figure 3. Transfection efficiency of various concentration of heptaprenyltrimethylammonium salt in lipofecting mixture.
Transfection activities of cationic liposomes prepared with heptaprenyltrimethylammonium iodide and DOPE (ratio 3 : 1) were expressed as percentage of cells emitting green fluorescence. Each value represents the mean ± S.D. (n = 4).

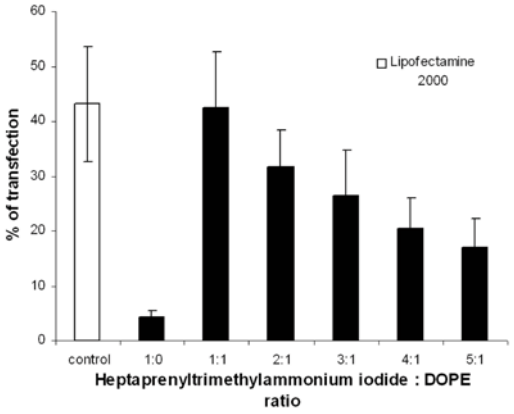

Figure 4. The effect of heptaprenyltrimethylammonium iodide : DOPE ratio on transfection efficiency.
Transfection activities of cationic liposomes prepared with heptaprenyltrimethylammonium iodide and DOPE (final lipid concentration 1 µg/well) at various ratios were expressed as percentage of cells emitting green fluorescence. Each value represents the mean ± S.D. (n = 4).

(Valtersson *et al.*, 1985; Janas *et al.*, 1986). It was also known that the phosphate derivatives of these compounds function as lipid coenzymes of transglycosylation in the cell and may play an important role in natural cellular membranes (Chojnacki & Dallner, 1988).

The cationic forms of linear polymers of isoprene units do not occur in living organisms. Data are reported concerning the synthesis of such substances from naturally occurring plant isoprenoid alcohols, heptaprenol of the wood of *Betula verrucosa* (Lindgren, 1965) and other members of *Betulaceae* (Chojnacki, unpublished) and nonaprenol from leaves of *Nicotiana tabacum* (Rowland, 1955) and from several other plants. Several linear poly-*cis*-prenols occur in many plants as secondary metabolites (e.g.: Stone *et al.*, 1967; Swiezewska *et al.*, 1994).

The lack of the lipofecting activity of nonaprenyltrimethylammonium salt observed in the present study may be due not only to the length of the isoprenoid chain of nonaprenyl radical as postulated by Tarahovsky *et al.* (2004) but also to the stiff, all-*trans* structure of this unit. The current studies of our laboratories on derivatizing several linear cationic polyprenoids available in the Collection of Polyprenols of the Institute of Biochemistry and Biophysics, PAS, (Masnyk *et al.*, in preparation) should solve this question.

Acknowledgements

We acknowledge the cooperation of the Larodan Fine Chemicals AB (Limhamngardens alle 9, SE 216 16 Malmo, Sweden).

The Larodan company will be ready to supply free of charge a limited number of 0.5 mg samples of heptaprenyltrimethylammonium iodide to the laboratories wishing to perform similar trials of transfection. This substance is protected in the patenting system (Appl. No PL 382448).

The support within the State Committee of Scientific Research (KBN, Poland) grants PB 2PO4C 008 28 and PBZ-KBN-110/P04/19 and by the European Community grant (CT-2004-005151) and the cooperation of Professor W. Krzyżosiak of the Institute of Bioorganic Chemistry of PAS in Poznan, Poland, in performing preliminary biological experiments are greatly acknowledged.

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