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Lipid-like trifunctional block copolymers of ethylene oxide and propylene oxide: Effective and cytocompatible modulators of intracellular drug delivery

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ABSTRACT

A new glycerol-based trifunctional block copolymer (TBC) of propylene oxide and ethylene oxide and its conjugate with succinic acid (TBC–SA) were studied as a drug delivery system and compared with Pluronic L61. TBCs have multiple effects on the plasma membrane of human cells, e.g. increasing its fluidity and ion permeability, inhibiting ATPase activity of efflux transporter P-glycoprotein through reversible membrane destabilization. Such membrane-modulating properties attributed to the unimer form of copolymers increase in the order Pluronic L61 ≪ TBC < TBC–SA and correlate with an ability of TBCs to promote the accumulation of P-glycoprotein substrates in lung cancer A549 cells. Furthermore, TBC, and especially TBC–SA, exhibit substantially lower hemolytic, cytotoxic and proapoptotic activities in comparison with Pluronic L61. Our results demonstrate that TBCs are promising analogs of bifunctional Pluronics in anticancer drug delivery.

1. Introduction

Nonionogenic amphiphilic polymers have a variety of applications in petroleum production, chemical technologies and are also an important component of household products. Depending on their physicochemical properties, e.g. the hydrophilic–lipophilic balance (HLB), such polymers exhibit different effects on living cells as a result of a reversible interaction with amphiphilic cellular components, especially lipid membranes and membrane-associated proteins (Firestone and Seifert, 2005; Batrakova and Kabanov, 2008; Alakhova et al., 2010). The biological properties of these amphiphilic polymers are of particular interest with regard to their potential (cyto-)toxicity, as well as an intriguing possibility of their therapeutic application.

Bifunctional block copolymers of ethylene oxide and propylene oxide (Pluronics™) have been systematically studied as drug carriers (Venne et al., 1996; Kabanov et al., 2002; Batrakova and Kabanov, 2008; Valle et al., 2011; Wei et al., 2013). Pluronic-based micellar formulations allow for the encapsulation of hydrophobic drugs, improving their solubility and increasing blood circulation time (Wang et al., 2007; Yoncheva et al., 2012). Other applications of Pluronics include the development of: hydrogels for topical drug delivery (Jansen et al., 2013), immuno-adjuvants (Newman et al., 1998) and cellular membrane protectors (Serbest et al., 2006; Mina et al., 2009).

Although in above applications Pluronics have been exploited as relatively inert surfactants, a series of recent studies show that Pluronics may directionally affect some cellular functions including: gene expression (Batrakova and Kabanov, 2008), energetic metabolism (Alakhova et al., 2010) and membrane transporter activity (Wei et al., 2013). These effects are more pronounced in Pluronics with low and intermediate HLB, such as Pluronics L61 and P85, which were found to promote drug transport across the blood brain barrier as well as into multidrug resistant cancer cells, making them more sensitive to conventional chemotherapy (Venne et al., 1996; Batrakova and Kabanov, 2008). To date, several compositions of existing anticancer drugs and Pluronics L61/F127 have been developed by Supratek Pharma Inc. to treat solid tumors and leukemia. One formulation SP1049C containing the anthracycline antibiotic doxorubicin is in phase III of clinical trials (Valle et al., 2011).

The principal limitation of hydrophobic Pluronics (e.g. Pluronic L61) as candidates for drug delivery is high cytotoxic/proapoptotic action which restricts their pharmaceutical application. Safer amphiphilic polymers with balanced specific and cytotoxic activities are ones of choice in advanced polymer-based therapy. A promising strategy is the development of polyfunctional analogs of Pluronics on the basis of biochemical structures. Recently, we have demonstrated that the conjugation of hydrophobic Pluronics with succinic acid modulates their interaction with the plasma

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membrane of human cells and dramatically reduces cytotoxicity (Bondar et al., 2013). Here we have studied lipid-like trifunctional block copolymers of ethylene oxide and propylene oxide as a promising polymeric system to promote intracellular drug delivery.

2. Materials and methods

2.1. Reagents

The bifunctional block copolymer of EO and PO (Pluronic™ L61) was purchased from Sigma–Aldrich. Trifunctional block copolymer of EO and PO oxide (Laprol™ 6003), the structural analog of Voranol™ 6008 (Dow Chemical), was produced by JSC Nizhnekamskneftekhim (Russia). Succinic anhydride, inorganic salts and solvents were produced by Acros Organics. Diphenylhexatriene (DPHT), tetramethylrhodamine ethyl ester perchlorate (TMRE) and 3,3-dipentyloxacarbocyanine iodide (DiOC5(3)) were purchased from Sigma–Aldrich. Rhodamine 123 and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich. Verapamil–BODIPY-FL conjugate was purchased from Invitrogen. Annexin V–Cy3 phosphatidylserine probe was purchased from Biovision. Doxorubicin hydrochloride and verapamil hydrochloride were produced by Ferane (Russia). Membrane preparation of recombinant human MDR1 transporter expressed in Sf9 cells was purchased from Sigma–Aldrich. Cell culture reagents were purchased from PAA.

2.2. Modification of Laprol 6003

Terminal hydroxyl groups in Laprol 6003 were modified in a reaction with succinic anhydride. Briefly, 20 g of Laprol 6003 dissolved in 62 mL of toluene were mixed with 1.1 g of succinic anhydride. The mixture was kept initially for 2 h at 90 °C and then for 2 h at 100 °C. After solvent evaporation, the residue was dissolved in water and the pH of the solution was adjusted to pH > 9 with sodium carbonate and then to pH < 2 with diluted sulfuric acid. The product was extracted into *n*-butanol (2 × 180 mL). The extract was exsiccated in a water-free sodium sulfate; additionally extracted into chloroform (3× 100 mL) and carefully dried in a vacuum. Finally, a yellow oily product was obtained with a yield of 87.5%. The structure of modified Laprol was verified by FTIR spectroscopy (Vector 22, Bruker) and acid-base titration.

2.3. Analysis of micelle formation

The critical micelle concentration (CMC) of polymers was determined with the use of a pyrene probe as described earlier (Bondar et al., 2013). 200 μL of serially diluted polymer solutions in PBS (pH 7.4) were pipetted into 96-well plate, pre-covered with 2.5 nmol of pyrene, from methanol solution. The plate was incubated for 1 h at 37 °C under agitation to allow the pyrene to dissolve and redistribute into polymeric micelles. Emission spectra of pyrene were detected at RT using an Infinite 200 PRO multimode microplate analyzer (Tecan) in 365–410 nm wavelength range (λ_{ex} 339 nm). CMC was calculated by the relationship between fluorescence intensity at λ_{max} 373 nm and the logarithm of copolymer concentration.

Polymeric micelles were characterized by the use of a dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments). The hydrodynamic diameter of micelles was analyzed in PBS (pH 7.4) at RT or in DMEM supplemented with a 10% fetal bovine serum (FBS) at 37 °C. Zeta potential of micelles was analyzed in 50 mM HEPES (pH 7.4). A multimodal (mean number) distribution based on nonnegative least squares algorithm was utilized to evaluate DLS data. The measurements were performed in triplicates.

2.4. Cell isolation and culturing

A549 cells (human lung adenocarcinoma epithelial cells) were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin under standard conditions (37 °C, 5% CO_2 atmosphere). Adherent cells were collected from a culture flask by detaching them in a trypsin–EDTA solution. Suspended cells were washed by centrifugation at 200 × *g* in PBS and cell viability was verified by trypan blue staining.

Human skin fibroblasts were isolated from the skin explant according to the conventional protocol (Rittie and Fisher, 2005). Primary fibroblasts were expanded by culturing them under the above conditions, but in α-MEM, and analyzed in the early passages.

2.5. Analysis of plasma membrane fluidity and potential

Aliquots of freshly prepared suspension of A549 cells in PBS (2×10^6 cells/mL) were placed into 96-well plate and equilibrated with 1 μM DPHT. Polymers were added to the cell suspension with a final concentration of 1 mg/mL. After 30-min incubation, the fluorescence polarization of DPHT in the suspension was detected on an Infinite 200 PRO analyzer (Tecan) using λ_{ex} 340 nm.

The transmembrane potential of A549 cells was assessed with the aid of a DiOC5(3) probe on a BD FACSCalibur System flow cytometer (BD Biosciences). The cells were suspended in PBS at a density of 2.5×10^5 cells/mL and incubated with a polymer (0.1 mg/mL) for 10 min at 37°C. Then, the DiOC5(3) solution was added to the suspension to obtain a final concentration of 0.5 μM and an additional incubation was carried out for 20 min at 37°C.

2.6. Hemolysis assay

A hemolysis assay was performed according to ISO 10993-4:2002 recommendations in a microplate format. The human erythrocyte fraction isolated from fresh peripheral blood was diluted 100-fold with PBS (pH 7.4). Polymers were added to the erythrocyte suspension at final concentrations of 0.1 and 1.0 mg/mL. The mixture was kept at 37°C for 4 h with moderate shaking; then intact erythrocytes were removed by centrifugation. The optical absorption of the supernatant, proportional to the concentration of hemoglobin released, was measured at 540 nm on the Infinite 200 PRO analyzer against appropriate blank solutions. The extent of hemolysis in the samples was expressed as a percentage relative to complete hemolysis induced by 0.2% Triton X-100.

2.7. Assessment of cell viability

The cytotoxic concentrations (IC_{50}) of polymers were determined with the use of a MTT assay. A549 cells and skin fibroblasts were pre-seeded in a 96-well plate at a density of 1000 cells per well and cultured with serially-diluted polymers for 3 days under standard conditions. The culture medium in the plate was then replaced by the fresh one supplemented with 0.5 mg/mL MTT and additionally kept for 4 h to reduce the MTT into a colored product (formazan) by metabolically active cells. The optical absorbance of the formazan produced in proportion to viable cell number was registered on the Infinite 200 PRO analyzer at 550 nm.

To detect apoptotic changes, A549 cells were cultured in a 24-well plate in the presence of polymers at a concentration of 0.5 and 1.0 mg/mL for 13 and 24 h. Treated cells were collected by trypsinization, washed by centrifugation and suspended in PBS. The cells were stained with 100 nM TMRE, a marker of the transmembrane potential of mitochondria (Haugland, 2002), or annexin V–Cy3 to reveal the redistribution of phosphatidylserine onto the cell surface (according to manufacturer's protocol). The fluorescence intensity of TMRE and Cy3 in stained cells was analyzed by