International Journal of Antimicrobial Agents 49 (2017) 703–708

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Efficient inhibition of influenza A viral replication in cells by deoxyribozymes delivered by nanocomposites

Marina Repkova [a], Asya Levina [a], Boris Chelobanov [a,b], Zinfer Ismagilov [c], Natalia Shatskaya [d], Sergei Baiborodin [d], Ekaterina Filippova [e], Natalia Mazurkova [e], Valentina Zarytova [a,*]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia
[b] Novosibirsk State University, Novosibirsk, Russia
[c] Boreskov Institute of Catalysis, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia
[d] Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia
[e] FBRI State Research Centre of Virology and Biotechnology 'Vector', Koltsovo, Novosibirsk, Russia

ARTICLE INFO

Article history:
Received 6 July 2016
Accepted 22 January 2017

Keywords:
DNAzymes
TiO_2-based nanocomposites
Immobilization
Cell delivery
Gene silencing
Influenza A virus

ABSTRACT

Nucleic-acid-based drugs are a promising class of novel therapeutics; however, their use in medicine is widely limited because of insufficient delivery into cells. This article proposes a new delivery strategy of nucleic acid fragments into cells as components of TiO_2-based nanocomposites. For the first time, unmodified Dz molecules were non-covalently immobilized on TiO_2 nanoparticles precovered with polylysine (TiO_2•PL) with the formation of (TiO_2•PL)•Dz nanocomposites. DNAzymes in the proposed nanocomposites were shown to retain their ability to cleave the RNA target in a cell-free system with the same selectivity as unbound Dz molecules. It was shown by confocal laser microscopy that the fluorescein-labelled (TiO_2•PL)•Dz^{Flu} nanocomposites penetrate into eukaryotic cells, where Dz^{Flu} is internalized in the cytoplasm and predominantly in nuclei. Delivery of deoxyribozymes into cells in the proposed nanocomposites permits very efficient interactions with RNA targets inside cells. This was demonstrated by an example of inhibition of H5N1 influenza A virus replication (inhibition by a factor of ca. 3000). This effect was one order of magnitude higher than with using lipofectamine as the transfection agent. The proposed (TiO_2•PL)•Dz nanocomposites demonstrated high antiviral activity and are thus potent as nucleic-acid-based drugs.

1. Introduction

Nucleic acid fragments [antisense oligonucleotides (ON), ribozymes, deoxyribozymes (Dz), siRNA, etc.] have attracted increasing attention as promising therapeutic agents because they are involved in complementary interactions with nucleic acid targets, thus affecting the expression of certain genes [1]. They can potentially be used as drugs for the treatment of infectious diseases, inherited diseases, cancer and other pathologies associated with injured nucleic acids in cells.

The most interesting of these compounds are siRNA, catalytically active ribozymes and Dz, which are capable of cleaving complementary RNA targets irreversibly as gene-specific molecular scissors. siRNA is widely used as a powerful tool for target-specific gene silencing [2]. However, Dz have several advantages compared with siRNA, including greater stability, reduced cost and ease of synthesis [3].

The widespread use of nucleic acid fragments in medical practice is hampered by their poor penetration through the cell membrane. Different approaches to delivering these compounds into cells are described in the literature, including the use of liposomes, transporting peptides, polymers of various natures, nanoparticles and physical impact on cells [4,5].

TiO_2 nanoparticles (ca. 5 nm) are known to penetrate through cell membranes [6–8]. Numerous references indicate that TiO_2 nanoparticles at low doses have low toxicity in mammalian cells [9], bacteria (<200 μg/mL) [10] and animals (<150 mg/kg) [11–13]. The cytotoxicity of TiO_2 nanoparticles used in this work did not exceed the level of natural death of Madin-Darby canine kidney (MDCK) cells [14]. Previous work found that the concentration of samples resulting in 50% cell death (toxic concentration, TC_{50}) for TiO_2 nanoparticles was 1.2 mg/mL [15].

* Corresponding author. Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of RAS, pr. Lavrent'eva 8, 630090 Novosibirsk, Russia. Fax: +7 383-363-51-53.
E-mail address: zarytova@niboch.nsc.ru (V. Zarytova).

http://dx.doi.org/10.1016/j.ijantimicag.2017.01.026
0924-8579/

In an earlier publication, the authors proposed a new strategy for ON delivery into cells that involved the use of TiO_2-based nanocomposites, consisting of preparation of TiO_2•(PL-ON) and (TiO_2•PL)•ON nanocomposites by either non-covalent immobilization of ON-polylysine conjugates (PL-ON) on TiO_2 nanoparticles [16] or unmodified ON on preformed TiO_2•PL conjugates [17]. It was demonstrated [15,18,19] that DNA fragments delivered into cells in the proposed system inhibit the reproduction of influenza A virus (IAV) in cells more effectively compared with other systems described in the literature [20–22].

In this work, the (TiO_2•PL)•Dz nanocomposites were obtained for the first time by electrostatic binding of unmodified Dz on preformed TiO_2•PL conjugates. TiO_2 nanoparticles were used in amorphous (Amf) and crystal [anatase (Ans) and brookite (Brt)] forms. This study investigated the ability of the created (TiO_2•PL)•Dz nanocomposites to cleave RNA targets in the cell-free system, penetrate into eukaryotic cells, and affect nucleic acid targets inside cells using the example of H5N1 IAV inhibition.

2. Materials and methods

2.1. Chemicals and materials

Chemicals and materials were obtained from commercial suppliers. Poly-L-lysine hydrobromide (PL, molecular weight 15,000–30,000), trypsin and penicillin–streptomycin were obtained from Sigma-Aldrich St Louis, MO, USA, and DAPI, Phalloidin-TRITC membrane stain, DMEM and Lipofectamine 2000 were obtained from Invitrogen, Carlsbad, CA, USA. MDCK cells, chicken erythrocytes and IAV strain A/chicken/Kurgan/05/2005(H5N1) were obtained from State Research Centre of Virology and Biotechnology 'Vector', Russia, and HeLa cells were kindly provided by Dr. Lactionov (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of RAS, Russia). Microscopic analysis was performed at the Multiple-access Centre for Microscopy of Biological Subjects (Institute of Cytology and Genetics, Siberian Branch of RAS, Russia) on an LSM 710 microscope (Carl Zeiss, Jena, Germany). The optical absorption was measured on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The significance of differences between the log 50% tissue culture infective dose ($TCID_{50}$)/mL values of the studied samples was evaluated by Student's *t*-test using Statistica 6.0 (Statistica, Tulsa, OK, USA).

Dz and their fluorescein-containing derivatives, deoxyribo- and ribooligonucleotides, were synthesized by the phosphoramidite method and kindly provided by the Laboratories of Medicinal Chemistry and Chemistry of Ribonucleic Acids (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of RAS, Russia).

The sequences of the ON used in this study are as follows (5′→3′):

d(GAAATAAGA*GGCTAGCTACAACGA*CCTTCATTA)p Dz
d(TCAACTCCA*GGCTAGCTACAACGA*ATGCCATGT)p $Dz_{n/c}$
d(GAAATAAGAGGC**A**AGCTACA**TC**CACCTTCATTA)p Dz_{Mt}
d(GAAATAAGA*T*CCTTCATTA)p ON
d(GATCAACTCCATATGCCATGT)p $ON_{n/c}$
r(GAAUAAUGAAGGAUCUUAUUUCUUC) synthetic RNA target

The central catalytic fragment in Dz is shown in italic. Dz was targeted to the coding region of (+)RNA of IAV segment 5. $Dz_{n/c}$ had the flanking sequences, which are partially non-complementary to the chosen region; it was used as a non-complementary control. Dz_{Mt} contained arms as in Dz and the central fragment (underlined) containing changed nucleotides (bold) and was used as a mutant control. ON was complementary to the same region as Dz. The ^{32}[P]-labelled synthetic RNA prepared using γ-[^{32}P]ATP and T4 polynucleotide kinase (Biosan, Novosibirsk, Russia) was used as a target in the cell-free cleavage experiments.

2.2. Synthesis of nanocomposites

TiO_2 nanoparticles (ca. 5 nm) in Amf, Ans and Brt forms and TiO_2•PL conjugates (ca. 1 μmol/mg for the amino groups) were prepared as described elsewhere [16,17]. In brief, an aqueous solution of PL (molecular weight 15,000–30,000) was added to TiO_2 nanoparticles in the Amf, Ans or Brt form. After 30 min of intensive stirring, the suspension was washed and resuspended in 0.1 M NaCl. The TiO_2•PL conjugates were obtained with a concentration of 1 mg/mL for the particles and a capacity of 1 μmol/mg for the amino groups of polylysine that corresponded to a PL/TiO_2 molar ratio of ca. 1:1 (taking into account the fact that one polylysine molecule contains, on average, 100 monomer units, 1 mg of TiO_2 nanoparticles contains 8.3 nmol of nanoparticles, and one particle (ca. 5 nm) contains 1500 TiO_2 molecules [17]).

The mixture of Dz (20 μL 10^{-4} M, 2 nmol) and TiO_2•PL (0.1 mg for nanoparticles in Amf, Brt and Ans forms) was stirred for 30 min. The obtained suspensions of (Amf•PL)•Dz, (Brt•PL)•Dz and (Ans•PL)•Dz nanocomposites were centrifuged, washed with 0.1 M NaCl (2 × 100 μL) and resuspended in 1 mL of the same solution. The washing of nanocomposites with 1 M NaCl solution, hot water (90 °C) and saline buffer led to the loss of less than 10–15% of the immobilized Dz.

The efficiency of Dz immobilization was calculated by the ratio of $(A_0 - A_s)/A_0$, where A_0 and A_s are optical absorption values of the added and unbound (in supernatant) Dz, respectively. The (TiO_2•PL)•Dz nanocomposites were prepared in an almost quantitative yield (90–100%), meaning the capacity of the nanocomposites for Dz was 18–20 nmol/mg in all cases.

The (Amf•PL)•Dz^{Flu}, (Brt•PL)•Dz^{Flu} and (Ans•PL)•Dz^{Flu} nanocomposites bearing the fluorescein residue were prepared in the same way using Dz^{Flu}.

All manipulations with nanoparticles and nanocomposites were performed using sonication for 30 s in an ultrasonic bath (Sapphire, Moscow, Russia).

2.3. RNA target cleavage in cell-free system with Dz in (TiO_2•PL)•Dz nanocomposites

(Amf•PL)•Dz, (Brt•PL)•Dz and (Ans•PL)•Dz containing Dz were used in these experiments. The synthetic [^{32}P]RNA (25-mer), a fragment of (+)RNA of H5N1 IAV (nucleoprotein-encoding segment 5), was used as a target. The reaction of [^{32}P]RNA (1 μM) with Dz (10 μM) or (TiO_2•PL)•Dz nanocomposites (0.5 mg/mL for TiO_2 and 10 μM for Dz) was performed at 37 °C in a buffer containing 25 mM $MgCl_2$ and 50 mM Tris–HCl at pH 7.5. The reaction was stopped by the addition of 0.1 M EDTA (1 μL), and the reaction mixtures were analysed by gel electrophoresis under denaturing conditions in 20% PAAG. Gels containing [^{32}P]RNA and products of their cleavage were dried on an FBGD 45 Gel Dryer (Fisher Biotec, Wembley, Australia) and radioautographed on CP-BU NIF 100 films (Agfa, Mortsel, Belgium). The results are presented in Fig. 1b.

2.4. Penetration of nanocomposites into cells

MDCK and HeLa cells were seeded into eight-well Nunc Lab-Tek chamber slides (Thermo Fisher Scientific Inc., Fremont, CA, USA) and cultured for 2–3 days in DMEM media supplemented with 10% foetal calf serum and antibiotics (penicillin and streptomycin, 100 U/mL) at 37 °C and 5% CO_2. At ca. 70% confluence, cells were treated with (Amf•PL)•Dz^{Flu}, (Brt•PL)•Dz^{Flu} and (Ans•PL)•Dz^{Flu} nanocomposites (0.01 mg/mL for TiO_2•PL particles, i.e. 0.2 nmol/mL for Dz) and Dz^{Flu} (0.2 nmol/mL) in DMEM without serum for 4 h, then washed with saline, fixed with formaldehyde (3.7%) for 10 min, and washed again with saline. Cellular actin filaments and nuclei were stained with Phalloidin-TRITC and Fluoroshield with DAPI, respectively. Cells were