Journal of Controlled Release 161 (2012) 949–958

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

NANOMEDICINE

Carbosilane dendrimer nanotechnology outlines of the broad HIV blocker profile

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ARTICLE INFO

Article history:
Received 31 March 2012
Accepted 26 April 2012
Available online 28 May 2012

Keywords:
Dendrimer
Microbicide
Anti-HIV activity
Biocompatibility
Molecular modeling

ABSTRACT

Researchers have been working hard for more than 20 years to develop safe and effective microbicides to empower women to better control their own sexual life and to protect themselves against HIV and other sexually transmitted infections (STIs). Microbicide classes include moderately specific macromolecular anionic polymers that block HIV and other STIs, and HIV specific drugs that inhibit viral entry and reverse transcription. Based on innovative nanotechnology design, we showed a novel water-soluble anionic carbosilane dendrimer (2G-S16) as a propitious molecule against HIV-infection. A state-of-the-art research was accomplished that focused on biomedical cutting-edge techniques such as in vitro and in vivo cytotoxicity assays performed on female rabbit genital tracts, simulate in vitro model of vaginal epithelium in order to evaluate HIV transmission blockade through the monolayer, complete gene expression profiling experiment to study deregulated genes after 2G-S16 exposition, molecular dynamics simulation of 2G-S16 molecule against principal proteins of HIV particles and pro- and anti-inflammatory cytokine profile study. Therefore, a high-throughput study and detailed analysis of the results were achieved in this article. We provided promising outcomes to encourage 2G-S16 as a hopeful microbicide.
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1. Introduction

About 33.3 million adults were living with HIV/AIDS at the end of 2009, the majority of them in developing countries. More than 50% adults living with HIV/AIDS are women and more than 90% of all adolescent and adult HIV infections have resulted from heterosexual intercourse (UNAIDS, http://www.unaids.org/data/epi2010/). Women and girls are especially vulnerable and account for slightly more than half of all people living with HIV worldwide, notably in Sub-Saharan Africa. Effective HIV prevention options for women are clearly needed in this setting. While vaccines hold promise [1] and that the use of highly active antiretroviral therapy in HIV-infected patient could have a preventive effect on HIV transmission [2], approaches to completely avoid HIV transmission remain elusive. Pre-exposure prophylaxis with orally administered antiretroviral drugs may prove to be effective, but there are substantial concerns for toxicities associated with long-term exposure, the risk for selecting resistant viral variants and the side effects of treatment could lead to a low adherence to this type of treatment [3]. Microbicides could be an alternative way to build preventative approaches to battle HIV transmission [4–7].

Topical drugs at different stage of development emerge as potential efficient prophylaxis candidates, notably the CAPRISA 004 study (http://www.caprisa.org) that is based on 1% tenofovir vaginal gel albeit recent announcements of CAPRISA group of work mentioned that efficiency needs to be put in perspective regarding the last results related to tenofovir gel [8–10]. Inside the field, several nanoparticles have been tested as topical microbicide by themselves due to their chemical characteristics [7,10,11]. Candidate microbicides derived from nano-device until now have showed no significant protection against HIV, with higher rates of infection in the treatment compared to the placebo arms in clinical trials [12–14]. Two clinical trials with the naphthalene sulfonate polymer PRO 2000 gel were published. No protection in HIV or HSV-2 acquisition was observed among women randomized to coitally dependent intravaginal application of PRO 2000 [5,15]. A phase I randomized placebo controlled clinical trial of the safety of SPL7013 dendrimer-based polyanions (VivaGel®) in healthy women showed no toxicity [16,17] and potent antiviral activity was observed against HIV-1 and HSV-2 immediately following vaginal administration [18]. But some limitations were still detected such as lack of large spectrum microbicide against HIV-1 strains using CCR5 as co-receptor or against other viral types. Because of this high amount of new synthesized or natural molecules, a complete

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doi:10.1016/j.jconrel.2012.04.050

state-of-the-art screening is required before facing up clinical trials [5,19].

We have previously shown that cationic dendrimers could be used as internalizing agents for gene therapy because they present low toxicity, retain and internalize genetic material as oligonucleotide or siRNA [20–24]. However, no application concerning the use of anionic carbosilane dendrimers has been reported so far. In this research we present a new water soluble second generation anionic sulfonate-terminated carbosilane dendrimer, 2G-S16 (Supplemental Fig. 1) with the aim to employ it as antiviral microbicide ready for clinical evaluation (manuscript submitted for publication).

The aim of this study was to evaluate 2G-S16 as potential microbicide candidate and whether 2G-S16 may inhibit HIV replication in different target cells and if it could affect the vaginal environment in the purpose to develop a new and harmless microbicides against HIV transmission.

2. Materials and methods

2.1. Dendrimer synthesis

2G-S16 was prepared according to reported methods (manuscript submitted for publication), see Supplemental Fig. 1. 2G-S16 was dissolved in distillated water in a final volume of 1 mM (37 mg/mL). Dilutions to μM range were generated in PBS (BioWhittaker, Walkersville, MD) from this stock. Control represents the volume of PBS added in every assay without dendrimer.

2.2. Cell culture

Blood samples were obtained from healthy anonymous donors from the transfusion centers of Albacete and Madrid following national guidelines. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll–Hypaque density gradient (Rafer, Spain) following the current procedures of Spanish HIV HGM BioBank [25]. PBMC and MT2 were cultured in RPMI 1640 medium (Gibco, UK) supplemented with 10% heat-inactivated FCS, 1% L-glutamine and antibiotic cocktail (125 mg/mL ampicilin, 125 mg/mL cloxacillin and 40 mg/mL gentamicin; Sigma, St-Louis, MO, USA). PBMC were cultured with 60 IU/mL of interleukine-2 (IL-2, Bachem, Switzerland) and stimulated with phytohemagglutinin (PHA, 2 μg/mL, Remel, Santa Fe, USA) for 48 h. HEC-1A (ATCC) is a epithelial cell line derived from a human endometrial carcinoma (uterus mucosa carcinoma) and was grown in McCoy's 5A Medium Modified (Biochrom AG, Germany) supplemented with 10% FCS and antibiotic cocktail. VK2/E6E7 (ATCC) is an epithelial cell line derived from normal vaginal mucosa tissue and was grown in serum-free keratinocyte medium (Gibco) supplemented with recombinant human epidermal growth factor (rEGF, 0.2 ng/mL, Immunotools, Friesoythe, Germany), bovine pituitary extract (BPE, 30 μg/mL, Sigma-Aldrich), 1% L-glutamine and antibiotic cocktail. HeLa P4.R5 MAGI (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: P4.R5 MAGI from Dr. Nathaniel Landau [26] stably expressed co-receptors CXCR4/CCR5 and containing ß-galactosidase gene (β-gal) under the control of HIV long terminal repeat were grown in DMEM medium supplemented with 5% FCS, 1% glutamine and antibiotic cocktail. Cells were seeded in several well plates, optimized for each performed assay (10^5 cells in p96, 2×10^5 in p48 and 3×10^5 in p24 well-plates).

2.3. Virus production

Virus stocks were prepared by amplification of R5-HIV-1_{BaL}, X4-HIV-1_{NL4-3} and HIV-2_{CBL23} in MT-2 cell line (ATCC) or PHA + IL-2 activated PBMC. Virus stock HIV-$1_{NL(AD8)}$ was produced by transient transfection of pNL(AD8) in 293T cell line (ATCC). Physical titers of all HIV viral stocks were evaluated by quantification of HIV $p24^{gag}$ by ELISA kit (Innogenetics, Belgium). To obtain SIVmac239 virus stocks [27], pSIVmac239 was transiently transfected in 293T cells and secondly, the virus produced by single-cycle replication in 293T cells was used to infect human CEMx174 (ATCC), HUT78 (ATCC) and HeLa P4.R5 MAGI. Viral stock SIVmac239 was then clarified by centrifugation prior to determination of viral infectivity in HeLa P4.R5 MAGI. SIVmac239 viral stock was determined as HeLa infectious unit per mL (IU/mL).

2.4. Reagents

Reagents used as controls for inhibition of viral replication were: AZT (Retrovir, GSK, Spain) nucleoside reverse transcriptase inhibitor; T-20 (Genentech, South San Francisco, Ca, USA) inhibitor of HIV fusion with cells; dextran (Dx, 4.85×10^5 g/mol, Sigma-Aldrich) harmless molecule that is used as negative control of toxicity; Dextran Sulfate (DextranS, 5×10^5 g/mol, Sigma-Aldrich) to demonstrate the inhibition of viral transmission in the presence of sulfate groups [28]; Suramin (Sigma-Aldrich) as a positive control of inhibition of adhesion [29]; and Sodium Vinyl Sulfonate (SVSF, Sigma-Aldrich) carbosilane dendrimer's monomer with sulfonate groups at the periphery that was used as negative control of antiviral effect.

2.5. Cell viability assays

Cell viability was determined by MTS (Promega, Madison, WI, USA), MTT (Sigma-Aldrich) or LDH (CytoTox 96®, Promega) assays following manufacturer's instructions. We included dextran to evaluate its innocuous effect in cell cultures, DMSO 20% (Sigma-Aldrich) as positive control of cell death and PBS as negative control. Each experiment was performed in triplicate.

2.6. Inhibition of HIV replication

Activated PBMC were treated with serial dilution of 2G-S16. Cells were then infected with 200 ng of $p24^{gag}/10^6$ cells of HIV-1_{NL4-3} for 2 h with frequent agitation. After 24 h, 48 h or 72 h, supernatant of infected cells was collected and HIV concentration was measured using the $p24^{gag}$ ELISA kit. HEC-1A, VK2/E6E7 epithelial cells were treated with PBS, dextran, DextranS or suramin as negative or positive controls of HIV-inhibition or with serial dilutions of 2G-S16. Cells were then infected for 3 h with 200 ng of $p24^{gag}$ of HIV-1_{NL4-3}, HIV-1_{BaL} or HIV-$1_{NL(AD8)}$ and further extensively washed. After 24 h, 48 h or 72 h, supernatant of infected epithelial cells was collected and HIV was measured using the $p24^{gag}$ ELISA kit.

2.7. Inhibition of HIV internalization

HEC-1A or VK2/E6E7 was plated overnight and was treated with PBS, suramin or 2G-S16 for 1 h. Cells were infected 3 h with HIV-1_{NL4-3}, HIV-1_{BaL} or HIV-2_{CBL23} (50 or 200 ng/10^6 cells). Cells were washed with PBS and lysed by adding 1% Triton X-100 for 45 min at 37 °C. HIV was measured on cellular lysate using the $p24^{gag}$ ELISA kit.

2.8. Direct HIV viral inactivation by dendrimer

Flat-bottom 96 well plate was treated with poly-L-lysine for 1 h at 37 °C. After 3 washes, 4 ng of HIV-1_{NL4-3}, HIV-1_{BaL} or HIV-2_{CBL23} or 35 IU of SIVmac239 was incubated overnight at 4 °C, to ensure that the viral particles adhered to the bottom of the well. Each well was washed 3 times with PBS to remove unbound HIV or SIV. Wells were then treated with PBS, suramin, 1% Tx, SVSF or serial dilution of 2G-S16 for 1 h. 2×10^5 activated PBMC (or 2×10^5 M8166 in the case of SIV) were then added in the wells. Cell culture supernatants were collected after 4 days and $p24^{gag}$ was quantified by ELISA. In the case of SIV, infectious particles were quantified in HeLa P4.R5

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