Acta Biomaterialia 44 (2016) 332–340

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Development and *in vivo* safety assessment of tenofovir-loaded nanoparticles-in-film as a novel vaginal microbicide delivery system

Alexandra Machado [a,b,1], Cassilda Cunha-Reis [c,1], Francisca Araújo [a,b,d], Rute Nunes [a,b,d], Vítor Seabra [c], Domingos Ferreira [e], José das Neves [a,b,c,*], Bruno Sarmento [a,b,c,*]

[a] *i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal*
[b] *INEB – Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal*
[c] *CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde & Instituto Universitário de Ciências da Saúde, Gandra, Portugal*
[d] *ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal*
[e] *Laboratório de Tecnologia Farmacêutica, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal*

ARTICLE INFO

Article history:
Received 9 May 2016
Received in revised form 18 July 2016
Accepted 16 August 2016
Available online 17 August 2016

Keywords:
Antiretroviral
HIV/AIDS
Nanotechnology
Pre-exposure prophylaxis
Safety
Vaginal drug administration

ABSTRACT

Topical pre-exposure prophylaxis (PrEP) with antiretroviral drugs holds promise in preventing vaginal transmission of HIV. However, significant biomedical and social issues found in multiple past clinical trials still need to be addressed in order to optimize protection and users' adherence. One approach may be the development of improved microbicide products. A novel delivery platform comprising drug-loaded nanoparticles (NPs) incorporated into a thin polymeric film base (NPs-in-film) was developed in order to allow the vaginal administration of the microbicide drug candidate tenofovir. The system was optimized for relevant physicochemical features and characterized for biological properties, namely cytotoxicity and safety in a mouse model. Tenofovir-loaded poly(lactic-co-glycolic acid) (PLGA)/stearylamine (SA) composite NPs with mean diameter of 127 nm were obtained with drug association efficiency above 50%, and further incorporated into an approximately 115 μm thick, hydroxypropyl methylcellulose/poly (vinyl alcohol)-based film. The system was shown to possess suitable mechanical properties for vaginal administration and to quickly disintegrate in approximately 9 min upon contact with a simulated vaginal fluid (SVF). The original osmolarity and pH of SVF was not affected by the film. Tenofovir was also released in a biphasic fashion (around 30% of the drug in 15 min, followed by sustained release up to 24 h). The incorporation of NPs further improved the adhesive potential of the film to ex vivo pig vaginal mucosa. Cytotoxicity of NPs and film was significantly increased by the incorporation of SA, but remained at levels considered tolerable for vaginal delivery of tenofovir. Moreover, histological analysis of genital tissues and cytokine/chemokine levels in vaginal lavages upon 14 days of daily vaginal administration to mice confirmed that tenofovir-loaded NPs-in-film was safe and did not induce any apparent histological changes or pro-inflammatory response. Overall, obtained data support that the proposed delivery system combining the use of polymeric NPs and a film base may constitute an exciting alternative for the vaginal administration of microbicide drugs in the context of topical PrEP.

Statement of Significance

The development of nanotechnology-based microbicides is a recent but promising research field seeking for new strategies to circumvent HIV sexual transmission. Different reports detail on the multiple potential advantages of using drug nanocarriers for such purpose. However, one important issue being frequently neglected regards the development of vehicles for the administration of microbicide nanosystems. In this study, we propose and detail on the development of a nanoparticle-in-film system for the vaginal delivery of the microbicide drug candidate tenofovir. This is an innovative approach that, to our best knowledge, had never been tested for tenofovir. Results, including those from *in vivo* testing,

* Corresponding authors at: i3S – Instituto de Investigação e Inovação em Saúde, Room 116.S3, Rua Alfredo Allen 208, 4200-135 Porto, Portugal.
E-mail addresses: j.dasneves@ineb.up.pt (J. das Neves), bruno.sarmento@ineb.up.pt (B. Sarmento).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.actbio.2016.08.018

sustain that the proposed system is safe and holds potential for further development as a vaginal microbicide product.

1. Introduction

Vaginal microbicides hold the potential to play a major role in the fight against HIV infection as well as other important sexually transmitted pathogens. Proof-of-concept has been achieved in recent clinical trials for topical pre-exposure prophylaxis (PrEP) of HIV-1, namely using a tenofovir (TFV) gel [1,2] and a dapivirine ring [3,4]. Still, protection levels reported for such products have been mild or even in some cases not significant [5], which may impair their translation into real-life settings. Poor adherence to vaginal microbicides by women undergoing clinical testing is considered one of the most important issues responsible for limited effectiveness [6], thus highlighting the need for further development of optimal microbicide products. Adherence is highly influenced by differences in women's acceptability and preferences, and a "one-size-fits all" microbicide is unlikely to be widely used in a consistent way [7]. While gel-based products are typically limited due to the need for multiple and coitus-dependent use (i.e., require pericoital administration), the continuous and prolonged presence in the vagina of rings, irrespectively of sexual activity status, may be considered troublesome. Hence, there is a real need for developing new microbicide dosage forms and technologies. Nanotechnology-based systems could serve this purpose. In particular, drug nanocarriers are currently recognized for potentially possessing advantageous features, including (i) increased active payload solubility and stability, (ii) modified drug release, (iii) enhanced intracellular drug delivery, (iv) improved drug activity, (v) optimized intravaginal drug distribution and retention, and (vi) sustained local pharmacokinetics (PK) [8]. Poly(lactic-*co*-glycolic acid) (PLGA)-based systems have been preferential in the development of nano-microbicides [9–13], namely due to the polymer's extensive safety, biocompatibility and biodegradability records [14], in addition to the well described ability to be used for the production of precisely engineered nanoparticles (NPs) that can bear specific colloidal properties [15]. Moreover, the application of PLGA NPs is well described as potentially safe and versatile enough to deliver different active molecules presenting various physicochemical features by the vaginal route [16].

One significant aspect regarding the development of nanotechnology-based microbicides concerns the choice and optimization of delivery platforms for nanosystems. Gels have been mostly explored, including those undergoing sol-gel transition upon administration [17,18]. However, even thermosensitive gels cannot fully minimize disadvantages associated with intravaginal gel products, namely leakage, leading to discomfort and rapid drug clearance from the vagina, as well as the need for an applicator. In contrast, vaginal films may overcome these last issues due to the ability to be administered with the aid of the fingers and lead to only small increases in volume of the fluid present in the vagina upon disintegration/dissolution [19]. Films also favor drug stability and potentially minimize drug release form nanocarriers due to typical low water content, being portable and discrete to use, and providing more accurate dose administration than gels [20]. The combination of drug nanocarriers and films may therefore constitute an interesting approach for the development of new and improved microbicide products.

The present work details on the development of a novel vaginal microbicide formulation based on the incorporation of drug-loaded NPs into polymeric films. TFV, a nucleotide reverse transcriptase inhibitor (NtRTI) and leading microbicide drug candidate, was chosen as model and its association to PLGA-based NPs was optimized. NPs-in-films were characterized for relevant physicochemical and biological properties, both *in vitro* and in a mouse model, with particular focus on safety.

2. Materials and methods

2.1. Materials

Ester-terminated PLGA (Purasorb PDLG 5002, 50:50 D,L-lactide: glycolide ratio, 0.2 $dL.g^{-1}$ inherent viscosity) was kindly provided by Corbion (Gorinchem, The Netherlands), TFV monohydrate by Gilead Sciences (Foster City, CA, USA), and poloxamer 407 (Kolliphor® P 407) by BASF (Ludwigshafen, Germany). Stearylamine (SA; octadecylamine), poly(vinyl alcohol) (PVA; 87–90% hydrolyzed, 30–70 kDa), purified type II mucin, triazolyl blue tetrazolium bromide (MTT reagent), and ethyl acetate were acquired from Sigma-Aldrich (St. Louis, MO, USA). Hydroxypropyl methylcellulose (HPMC; Methocel E4M) was purchased from Colorcon (Dartford, UK) and glycerin from Aliand (Mem Martins, Portugal). All other materials and reagents were of analytical grade or equivalent.

2.2. Cell lines and culture conditions

All cell lines were from ATCC (Manassas, VA, USA). Human HEC-1-A endometrial, CaSki cervical, and HeLa cervical cells were maintained in, respectively, McCoy's 5A modified medium (Invitrogen, Carlsbad, CA, USA), RPMI-1640 medium (Invitrogen), and Dulbecco's Modified Eagle medium with Ultraglutamine 1 (Lonza, Verviers, Belgium). All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (all from Invitrogen). Cells were maintained at 37 °C, 5% CO_2 and 95% humidity, and media renewed every 2–3 days.

2.3. Preparation and characterization of nanoparticles

TFV-loaded, PLGA-based NPs were prepared by a double emulsion/solvent evaporation method. Briefly, TFV (5 mg) dissolved in ultra-pure water (1 mL) was mixed with ethyl acetate (4 mL) containing PLGA (40 mg) by vortexing for 30 s. The resulting *w*/*o* emulsion was added to 0.5% (*w*/*v*) poloxamer 407 (10 mL), and the mixture was sonicated for 60 s at 70% intensity using a Vibra-Cell™ VC 50 (Sonics & Materials, Danbury, CT, USA). The *w*/*o*/*w* emulsion was transferred into an additional 10 mL of 0.5% poloxamer 407, and left overnight under magnetic stirring (300 rpm) to allow organic solvent evaporation. NPs were then concentrated by centrifugation (4000×*g*, 30 min) using Amicon Ultra-15 filter units (MWCO 100 kDa; Merck Millipore, Tullagreen, Ireland) and washed twice with 15 mL of water. Collected filtered fluids were used for TFV quantification and calculation of the percentage of drug association efficiency (AE%) and loading degree (LD%) as previously described [12]. TFV was assayed by a reversed-phase high performance liquid chromatography with UV detection (HPLC-UV) method, using a Merck-Hitachi LaChrom® HPLC system (Merck, NJ, USA). A Zorbax Eclipse XDB-C18 column (3.5 μm, 3.0 × 75 mm; Agilent Technologies, Santa Clara, CA, USA) was used