Thermosensitive Vaginal Gel Containing PLGA-NRTI Conjugated Nanoparticles For HIV Prophylaxis

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) have shown efficacy as pre-exposure prophylaxis (PrEP). The research goal is conjugating NRTIs to biodegradable polymer poly (lactic-co-glycolic acid; PLGA) by an amide bond. NRTIs-PLGA conjugates were chemically activated and conjugated to free amine group of emtricitabine (FTC) and lamivudine (3TC). The conjugates were made into nanoparticles (NPs) and incorporated into a thermosensitive gel for vaginal PrEP. PLGA-FTC and PLGA-3TC NPs were < 200nm and zeta potential of -13.7 and -14.1 mV, respectively. The conjugated NPs were not toxic to HeLa cells up to 100 mcg/ml. Thermosensitive gel containing fluorescent NPs fabricated similarly and administered vaginally, were seen in submucosa for up to 5 days. FTC-NPs showed similar EC50 compared to FTC solution. This thermosensitive gel would be a novel vaginal sustained-release PrEP option.

Keywords: HIV, Conjugated NRTIs, Pre-exposure prophylaxis, Sustained vaginal delivery, nanoparticles

1 BACKGROUND

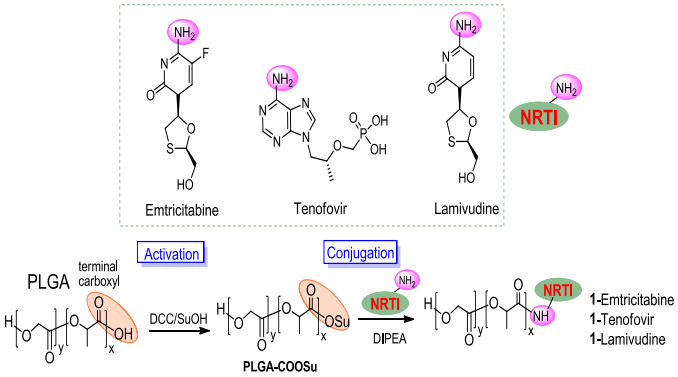
Nucleoside reverse transcriptase inhibitor (NRTIs) have shown efficacy against HIV-1 when used as pre-exposure prophylaxis (PrEP). Indeed, the CAPRISA 004 clinical trial demonstrated 1% tenofovir gel administered close to the time of sexual intercourse reduced the incidence of contracting HIV 39% of the time [1]. However, adherence to the treatment regimen is a major component of success. In the CAPRISA 004 trial, women who used > 80% of the tenofovir vaginal gel applicators demonstrated 54% success. However, a coitus-independent regimen has not shown success and several trials have been stopped for fuility [2,3]. Recently reported at the 2013 CROI meeting, the VOICE trial showed low adherence to study drugs (oral tenofovir, or tenofovir/emtricirabine or vaginal gel tenofovir) and did not reduce HIV acquisition risk. Long-acting products requiring minimal daily adherence may be more suitable in this young, unmarried population [4].

The use of antiretroviral nanoparticles in the prevention of HIV is undergoing pre-clinical development. These particles has been shown to offer sustained release of antiretroviral drugs over an extended time period [5,6]. Our goal of this research is to conjgate NRTI drugs to an FDA-approved polymer (poly (lactic-co-glycolid acid; PLGA) to make NRTI-PLGA conjugates to fabricate into nanoprticles and incorporate into a thermosensitive gel for PrEP.

2 METHODS

Acid terminated PLGA was activated using N,N-dicyclohexylcarbodiimide and di-iso-prophylethyl amine and was conjugated to the free amine group of emtricitabine (FTC) and lamivudine (3TC) (Figure 1). This PLGA conjugated product was used to fabricate FTC-NP and 3TC-NP nanoparticles using nanoprecipitation. PLGA conjugated product was dissolved in 10 ml ethyl acetate along with additional free PLGA. The organic phase was added to an aqueous phase of 30 ml 2% Pluronic F127 on a magnetic stirrer. The combined emulsion was allowed to evaporate the organic solvent over several hours and added to the thermosensitive gel for vaginal delivery. The NRTI-NPs emulsion was characterized for size and zeta potential using dynamic light scattering (DSC).

Figure 1. Chemical synthesis of PLGA to FTC and 3TC. Synthesis of PLGA-NRTI conjugates: DCC = *N,N*'-dicyclohexylcarbodiimide, SuOH = *N*-hydroxysuccinimide, DIPEA = di-*iso*-propylethyl amine.



Nanoparticle conjugated FTC was evaluated in triplicate experiments using HeLa cells for cytotoxicity. HeLa cells (4,000 cells/well) were placed in a 96-well flat-bottomed plate in DMEM with 10% fetal bovine serum (FBS), 4mM L-glutamine, and 1% penicillin/streptomycin at 37C in 5% CO_2. Cells were allowed to adhere to the plate overnight and then drugs in nanoparticle or solution were added. Viability of the HeLa cells was assessed at 48 h after administration of gel, FTC-NPs (100 mcg/mL) or FTC solution (100 mcg/mL) using the CellTiter Glo protocol according to the manufacturer's instructions. Triplicate experiments were performed. Results were averaged and analyzed.

For antiviral activity, TZM-bl indicator cells (2 x 10^5 per well) in 24-well plates were used. The cells were incubated for 24 h and then treated with seven 10-fold dilutions of FTC solution or FTC-NPs. After 24 h, media from all wells was aspirated to remove the particles and solution and replaced with fresh media. Subsequently, the cells were inoculated with HIV-1_{NL4-3} virus (25uL) for 4 h. The cells were washed and incubated for 48 h. Cells were washed with PBS, lysed with 150 uL M-PER solution and clarified by centrifugation. A luciferease substrate was added to the lysate and the resulting luminescence, expressed as relative luminexcence units (RLU) was determine by the luminometer. The results were normalized by comparing RLU of the treatment groups with that of the positive control and data obtained was pltted to obtain an EC_{90} concentration response curve. All experiments were performed in triplicate.

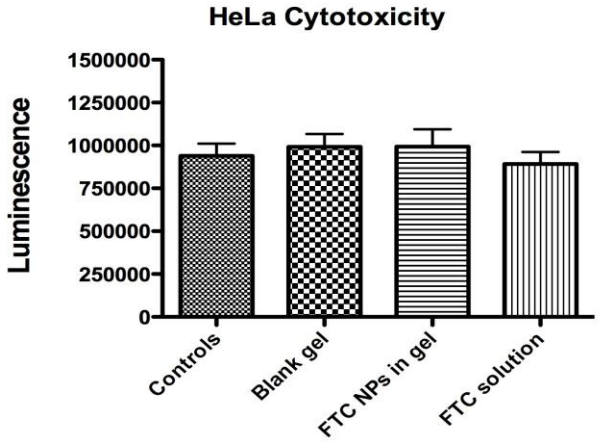
A thermosensitive gel containing 20% Pluronic F127, 1% Pluronic F68 was developed for vaginal delivery [6]. The thermosensitive gel (30uL) containing Rhodamine-6G labeled PLGA-NP was fabricated and instilled vaginally into Balb/c mice. At sepcific time points, the mice were euthanized, vagina and cervix were harvested, fixed, embedded for histological assessment of fluorescent NP using confocal microscopy at 1- and 5-day time period. Fixed slides were counter stained with DAPI (green).

3 RESULTS

NRTI-PLGA conjugates were successfully synthesized and the conjugation was confirmed by NMR. NRTI conjugate to PLGA ratio of 1:4, Pluronic F127 + Solutol HS 15 (2.5%) and organic to aqueous phase ratio of 1.25:1 was found to be optimal to yield PLGA-FTC NP and PLGA-3TC NP with size < 200 nm and zeta potential of -13.7 $\pm$ 2.3 and -14.1 ± 2.7 mV, respectively.

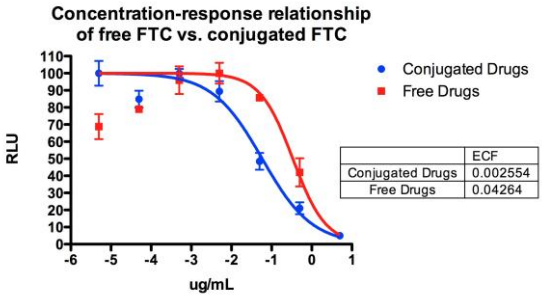
FTC-NPs were not toxic to HeLa cells at a concentration up to 100 mcg/mL (Figure 2). These results leads one to conclude that emtricitabine either in the nanoparticle or in solution would not cause an increase risk of contracting HIV-1 through sexual intercourse. Further toxicity trials are necessary for a longer timeframe to confirm these preliminary results.

Figure 2. Cytotoxicity comparison of HeLa cells (controls), blank gel, gel containing 100 mcg/ml FTC-NPs, and FTC solution (100 mcg/ml).



Antiviral activity was evaluated using the EC_{50} model. The results show that the EC_{50} for the FTC-NPs was similar to the FTC solution. (Figure 3). Therefore, the conjugated FTC is able to cleave the amine bond and inhibit HIV-1 reverse transcriptase. Since these *in vitro* results are encouraging, further studies in a humanized mouse model of HIV-1 are planned. We and others have already demonstrated that nanotechnology formulations show high intracellular drug levels in monocyte-derived macrophages as well as other cell types [7,8]. Since the activity of reverse transcriptase is also intracellular, it stands to reason that high intracellular drug levels would be important from an efficacy standpoint. Further research in this area is necessary to translate intracellular drug levels and effect.

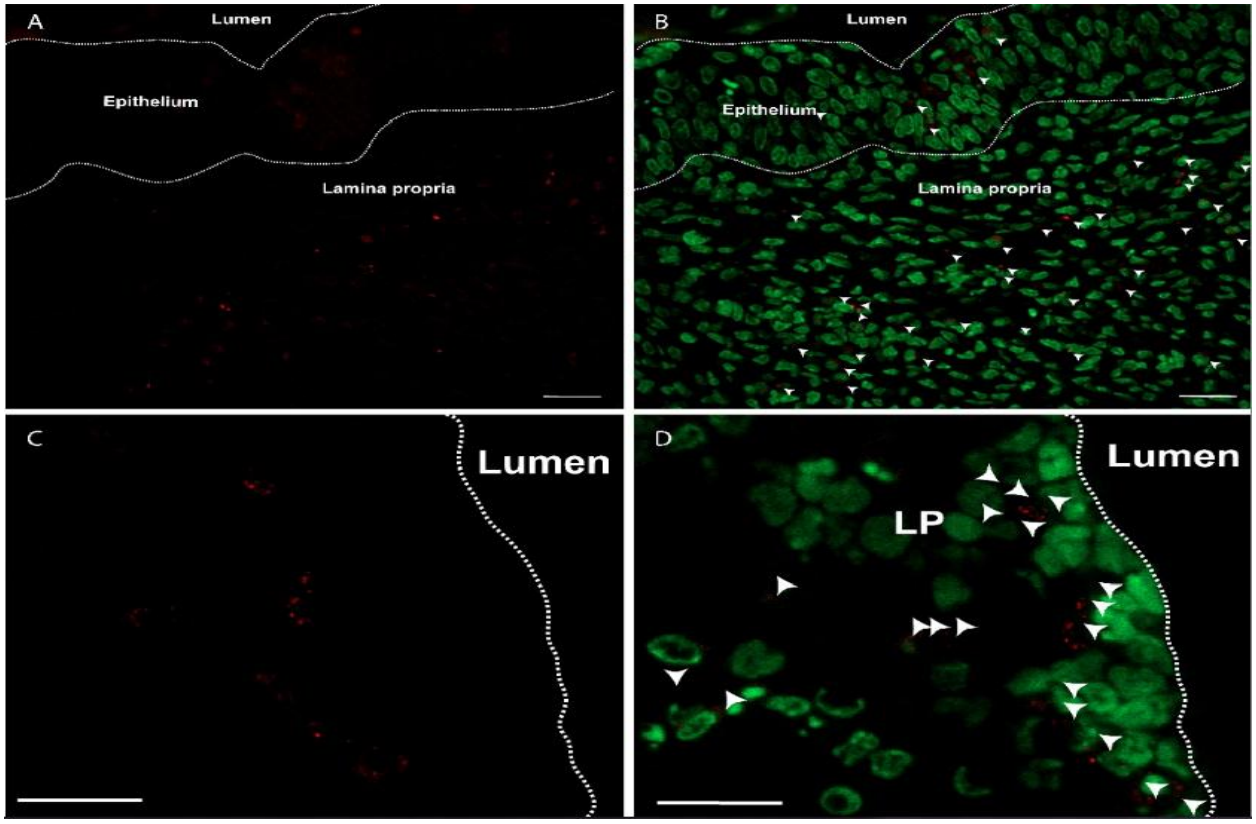
Figure 3. Comparison of EC_{90} for soluble FTC and conjugated-FTC NPs



The thermosensitive gel was fabricated and Rhodamine 6G fluorescent nanoparticles were incorporated into the gel. Thirty microliters were instilled vaginally into BALB/c mice. The thermosenstive gel was allowed to dwell in the vaginal cavity for up to 5 days. As shown in Figure 4, the Rhodamine containing nanoparticles had transferred from the thermosensitive gel into the vaginal mucosal

tissues at 24 h. Additionally, the Rhodamine nanoparticles were still present in the lamina propria of the mouse vaginal tissue at 5 days after intravaginal administration. These results show for the first time that antiretroviral drug therapy can be administered in a nanoparticle formulation that allows for sustained release. This could pave the way for incorporating antiretroviral nanoparticles into a prevention strategy that is coitus-independent. Further studies are needed in either humanized mouse model of HIV or non-human primates to futher characterize the release of antiretroviral drug concentrations over time with HIV-1 infection.

Figure 4. *Rhodamine containing nanoparticles (Rho-NP) was enriched in the epithelial cells and lamina propria of vagina after 5 days intravaginal administration. Adult female Balb/c mice were intravaginally administrated 30 ul of Rho-NP and were euthanized 1, 3, 5 days later. Cervical and vaginal tissues were harvested, fixed with SafeFix II, embedding in paraffin, sectioned, and counter-stained with DAPI (green). Rho-NP (red) in vaginal mucosal tissues at 1 (A, B) and 5 (C, D) days post intravaginal administration, white arrows point to nanoparticles, scale bar 20um*



4 DISCUSSION

The use of nanomedicine for the prevention of HIV-1 infection is undergoing preclinical investigation. The results of these experiments demonstrate that hydrophilic antiretroviral drugs can be chemically conjugated with an FDA-approved polymer. These nucleoside reverse transcriptase inhibitors have been difficult to incorporate into a nanomedicine due to their physical-chemical characteristics. However, chemically conjugating these drugs to PLGA allows for incorporation into the nanoparticle fabrication system that we have designed. This fabrication process allows for easy scale-up.

Successful vaginal delivery of nanoparticles should be optimized for vaginal administration. Nanoparticles should have a small size and the ability to rapidly penetrate through vaginal mucus to deliver the antiretroviral drug to the vaginal epithelium. This report demonstrates the successful development of sub-200 nm nanoparticles composed of PLGA and emtricitabine or lamivudine, two nucleoside reverse transcriptase inhibitors. Others have demonstrated rapid penetration of Pluronic F127-coated polystyrene nanoparticles through cervicovaginal mucus [9]. Incorporating Pluronic F127 for fabricating the nanoparticles may have resulted in the mucus-penetrating ability. Osmolarity is another important criteria for development of successful vaginal formulations. The 1% tenofovir gel used in the CAPRISA 004 trial was hyperosmolar (3111 mOsm/kg) and may result in epithelial stripping of polarized explants [10]. Preferrably, vaginal gels with an osmolarity < 100 mOsm/kg prevents mucosal irritation and damage to epithelial lining of the vagina [11]. Finally, development of a suitable vehicle to enable vaginal delivery of the nanoparticles is an important aspect for translational studies. The use of a thermosensitive gel for vaginal delivery allows for contact between the gel and vaginal epithelial surface for an extended timeframe. This type of gel remains a liquid when the average temperature is < 30ºC. However, at 32ºC, the gel increases its viscosity allowing the messiness associated with liquid gels to be minimized. The nanoparticle incorporation into the gel was without any visible signs of aggregation and could be an advantageous vaginal delivery system for sustained release of antiretroviral drugs.

5 CONCLUSIONS

The results of these experiments show the ability to conjugate nucleoside reverse transcriptase inhibitors to an FDA-approved polymer. The utility of the conjugation to fabricate antiretroviral nanoparticles for HIV-1 prevention in a thermosensitive gel formulation allows for sustained release of the antiretroviral compound within the vaginal epithelial surface into the submucosa. The concentration of the active antiretroviral triphosphate drug at the time of HIV-1 will need to be examined in small animals. Finally, the prevention of HIV-1 using a humanized mouse model needs to be investigated. This nanoparticle delivery system should move forward in pre-clinical development.

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