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Antiretroviral hydrophobic core graft-copolymer nanoparticles: the effectiveness against mutant HIV-1 strains and *in vivo* distribution after topical application.

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Abstract

Purpose.—Developing and testing of microbicides for pre-exposure prophylaxis and post-exposure protection from HIV are on the list of major HIV/AIDS research priorities. To improve solubility and bioavailability of highly potent anti-retroviral drugs, we explored the use of a nanoparticle (NP) for formulating a combination of two water-insoluble HIV inhibitors.

Methods.—The combination of a non-nucleoside HIV reverse transcriptase inhibitor (NNRTI), Efavirenz (EFV), and an inhibitor of HIV integrase, Elvitegravir (ELV) was stabilized with a graft copolymer of methoxypolyethylene glycol-polylysine with a hydrophobic core (HC) composed of fatty acids (HC-PGC). Formulations were tested in TZM-bl cells infected either with wild-type HIV-1_{IIIB}, or drug-resistant HIV-1 strains. *In vivo* testing of double-labeled NP formulations was performed in female rats after a topical intravaginal administration using SPECT/CT imaging and fluorescence microscopy.

Results.—We observed a formation of stable 23–30 nm NP with very low cytotoxicity when EFV and ELV were combined with HC-PGC at a 1:10 weight ratio. For NP containing ELV and EFV (at 1:1 by weight) we observed a remarkable improvement of EC₅₀ of EFV by 20 times in the case of A17 strain. *In vivo* imaging and biodistribution showed *in vivo* presence of NP components at 24 and 48h after administration, respectively.

Conclusions.—Insoluble orthogonal inhibitors of HIV-1 life cycle may be formulated into the non-aggregating ultrasmall NP which are highly efficient against NNRTI-resistant HIV-1 variant.

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Introduction.

Both HIV-1 reverse transcriptase and integrase are initially present in retroviral particles and play essential roles in the initiation of retroviral life cycle via reverse transcription of viral RNA and integration of double stranded DNA into the host genome (1, 2). Therefore, both reverse transcriptase and integrase represent the most immediate and feasible drug targets for pre-and post-exposure interventions (3–6). Unfortunately, many first- and second-generation non-nucleoside reverse transcriptase (NNRTI) inhibitors of HIV replication machinery as well as inhibitors of HIV integration are insoluble in water. Improving bioavailability and efficacy of these insoluble anti-retroviral drugs presents important technical challenges. In particular, Efavirenz (EFV), a World Health Organization essential medicine and a highly efficient NNRTI, is practically insoluble in water (solubility in water - 10 mg/L) (7). Elvitegravir (ELV), an FDA approved integrase strand transfer inhibitor (INSTI), is water insoluble (solubility <0.3 mg/L) (8). One of potential strategies of improving the bioavailability of these hydrophobic drugs is a co-delivery in NP. Nanoparticles (e.g. polymeric mixed micelles) dramatically increase the surface area of insoluble drug cargo and promote potential uptake of the contents of the NP by the cells (9–11). Recent evidence suggests that after a HIV-1 challenge tenofovir/ELV NP combinations were 100% efficient in protecting humanized animals for 4 days post subcutaneous administration of the NP (12). The design of the above pre-exposure strategy is based on poly(lactic-co-glycolic acid), PLGA-mediated entrapment of NNRTI mixtures into 200 nm nanoparticles. Another recently reported approach for delivering antiretroviral drugs to mucosal surfaces also included PLGA-based NP, which were stearylamine-stabilized and then incorporated into polymer films for local application onto the mucosal surfaces (13). Poly(lactic-co-glycolic acid) copolymer has a definite advantage over other NP components since it has gained FDA acceptance and nanoformulations containing PLGA are commonly used (14, 15). These NP benefit from PLGA matrix biodegradability, however, hydrolytic biodegradation with the breakdown of ester bonds eventually results in development of negative charge in these particles (16), which may require additional polymer coatings, incorporation of PEG blocks and other polymer blends or some other alternative techniques resulting in masking of negative charge (13, 17–21). It has been demonstrated that strongly charged NP, which are mucoadhesive (22), have limited distribution and are retained mainly at the epithelial surfaces without penetration through the mucus. In contrast, PEGylated NP (i.e. mucus-penetrating particles), which do not exhibit mucoadhesiveness were found to interact with the entire epithelial lining of intestine and rectum (23). Similar improved penetration through mucosal barriers was observed when PEGylated NP were tested for intravaginal delivery (23–25). It should be noted that to obtain PLGA NP loaded with a combination of several antiretroviral drugs one has to use multiple time-consuming procedures, such as sonication for generating water-in-oil emulsions required for interfacial polymer deposition, sequential evaporations and extractions, dialysis and/or centrifugation as well as the use of additional stabilizing di-block surfactants such as Pluronics® which may introduce additional variables (26). Meanwhile a complete elimination of PLGA from the composition of NNRTI-loaded nanoparticles by using either pure Pluronic® –127, or mixed Pluronics®/Tetronic® micelles loaded with EFV showed a drastic increase of EFV stabilization in water solutions and improvement of oral bioavailability (27, 28). In our

current research we used previously tested technology based on NP formed in the presence of hydrophobic core PEGylated graft copolymers (HC-PGC (29, 30), which were loaded with insoluble NNRTIs (31). This approach is founded on a premise that large amphiphilic copolymers instantly form ultrasmall NP after the solubilization of co-lyophilized water-insoluble drugs along with the stabilizing HC-PGC layer (31). The advantage of HC-PGC stabilized NP is not only in the simplicity of preparation but also the modular design of NP stabilizing copolymer (30, 32). This report is focused on investigating the anti-viral activity of these graft-copolymer stabilized NP developed to block the initiation of wild-type and drug-resistant HIV-1 life cycle by formulating EFV/ELV into NP. The overall goal of our study was to develop a single composition of HC-PGC based molecules which would 1) stabilize NP composed of insoluble anti-HIV-1 drugs and 2) prevent infection with both NNRTI-sensitive and NNRTI resistant HIV-1 strains. We also performed initial testing of NP biodistribution *in vivo* by using non-invasive imaging and microscopy after a local application onto mucosal surface.

Materials and methods.

Simulations.

Molecular dynamics simulations were performed by using molecular operation environment (MOE, MOE2016 software (Chemical Computing Group, Montreal CA) (33). We applied the assisted model building with energy refinement (Amber14:EHT), an all-atom force field combining EHT and Amber14, parameterized for proteins and nucleic acids as well as for small molecules according to 2D-extended Hückel's theory conventions (34). Force field was applied to model systems containing various numbers of EFV and ELV molecules as well as a combination of EFV/ELV in the presence of four shortened fragments of hydrophobic core graft copolymer carrying two MPEG residues ($n=50$ ethylene oxide units) linked to N- ϵ - amino groups of poly-L-lysine and eight stearic acid residues (PLL, DP=20). The mixtures were included into NaCl/water cell of simulated solvent with $n=8$ molecular margin followed by partial charges calculations and potential energy minimization. Dynamics trajectories were calculated by using Nosé-Poincaré-Andersen (NPA) integration algorithm for constant pressure and temperature ensembles with a 0.001 ps time step.

Synthesis of MPEG-gPLL.

The synthesis of NP-stabilizing hydrophobic core pegylated co-polymers (HC-PGC) was performed by covalent modification of a graft copolymer of MPEG carboxylate and PLL with hydrophobic residues according to a procedure previously described in (35) by acylating $22\pm2\%$ of free amino groups of poly-L-lysine (PLKB₅₀, average m.w. (NMR) 11,500, DP_n (NMR) =55, (dispersity) =1.04; (Alamanda Polymers Inc.) with N-hydroxysuccinimide ester of MPEG5 methoxypoly(ethylene glycol) carboxylate (M-SCM-5000, m.w. 5000 ± 500 Da, JenKem Technology USA, Plano TX) followed by blocking remaining free amino groups with N-hydroxysuccinimide esters of stearic acid or oleic acid, respectively (Millipore-Sigma). The obtained products, M5PL10OL and M5PL10ST (gram-scale) were purified by hollow-filter ultrafiltration on UFP-100 cartridges (GE Healthcare Bio-Sciences Corp., Piscataway NJ) using 30% isopropanol (20x volume changes) followed by water (40x volume changes). The obtained products were analyzed on 1×30 cm Superose

6 Increase column (GE Healthcare Bio-Sciences), eluted 0.1 ammonium acetate, pH 7.0 (0.5 ml/min). The degree of modification of PLL with MPEG carboxylate and fatty acids was determined by 2,4,6-trinitrobenzenesulfonic acid titration and ^1H NMR (DMSO- d_6 , 500 MHz Bruker Avance NMR Spectrometer, see Supplementary Material, Figure S1).

Formulation of EFV and ELV in the presence of HC-PGC.

Stock solutions of EFV (NIH AIDS Reagent Program, Division of AIDS, NIAID) and ELV (Santa Cruz Biotechnology) were prepared at 70°C in dry *tert*-butyl alcohol (1 mM each). Solutions of HC-PGC (8 mg/ml) in *tert*-butyl alcohol were mixed with the required volumes of EFV, or a combination of both EFV and ELV (10% inhibitor by weight of HC-PGC) and kept at 60°C for 10 min. The solutions were snap-frozen and lyophilized overnight and the obtained powders were kept under argon. To achieve spontaneous formation of NP the lyophilized powders were reconstituted by adding pre-heated sterile HBS (10 mM Hepes, 0.15M NaCl, pH 7.4, 60°C) to obtain a solution containing 8 mg/ml of either M5PL10OL, M5PL10ST, or a combination of both at 1:1 by weight. The resultant NP contained approximately 10% by weight of EFV, or a combination of EFV and ELV (each 5% by weight). Empty NP were prepared as described above with no inhibitors added. After 15 min equilibration at 60°C the NP solutions were cooled at room temperature and an aliquot diluted 1:10 in 0.22 μm -filtered HBS (Millex-GV, Merck) for analysis using dynamic light scattering after filtration (Zetasizer Nano ZS, Malvern Instruments, Westborough MA) followed by transmission electron microscopy and cell culture testing (see below). To determine concentrations of both anti-retroviral drugs in the final filtered NP preparations the aliquots were diluted 50–100 times in methanol in glass vials, heated at 50°C for 30 min and analyzed using LC-MS (6120 LC/MS single quadrupole mass spectrometer system, 1260 series HPLC; Agilent Technologies, Santa Clara CA) on a reversed phase HPLC column (100 $\times$ 2 mm, 3 μm , C18 Hydrosphere, YMC group) using a gradient of 0.1% formic acid in 0–100% acetonitrile (0.5 ml/min). Calibration curves (concentration ranges: 5–100 $\mu\text{g/ml}$ for EFV and 1–80 $\mu\text{g/ml}$ for ELV) were obtained using the standard solutions of EFV and ELV with signal detection using two MS m/z selective ion monitoring (SIM) channels for EFV and ELV detection, respectively. The detection was performed in positive mode (Figure S2) by summation of integrated signals of the following ions : EFV- m/z =316, 297.2 and 271.9; ELV - m/z =449.8 and 431.8. For detection of each ion the optimized fragmentor voltages were individually specified.

HIV-1 infection inhibition assays in reporter cells.

TZM-bl cells stably expressing CD4/CCR5 and integrated copies of *Ppyralis* luciferase cDNA under control of the HIV-1 long terminal repeats (LTRs) were provided by (NIH AIDS Reagent Program, Division of AIDS, NIAID). Cell culture was maintained in 10% fetal bovine serum/ DMEM (Thermo Fisher Scientific); HIV-1_{IIIB} virus and HIV-1_{IIIB} A17 Variant (NIH AIDS Reagent Program, Division of AIDS, NIAID) were propagated in H9 cells cultured in 10% fetal bovine serum, RPMI1640 with l-glutamine (Gibco, ThermoFisher Scientific). HIV-1_{RTMDR1}/MT-2 variant NIH AIDS Reagent Program, Division of AIDS, NIAID) was propagated in MT-2 cells. Cell culture media were analyzed for p24 using ELISA assay (QuickTiter, Lentivirus Titer Kit, Clontech) and HIV-1 particles were harvested when 24–34 ng p24/ml was achieved corresponding to the viral content of

3·10⁶–3·10⁷ infectious units. Virus stocks were obtained after removing cell debris by centrifugation and stored frozen at –80°C. Adherent TMZ-bl cells (10⁵/well 96-well plates) were washed with fresh medium and infected by using a co-addition of 4 ng HIV-1 p24/well and serially diluted test compounds (EFV and ELV from stock solutions in DMSO and NP from stock solutions in HBS in complete cell culture medium). Three days post infection, cells were lysed and luciferase activity was determined in the lysates by using luciferin/ATP reagent (Promega) by using SpectraMAX M5 plate reader (Molecular Devices). The obtained results were analyzed and fitted using response (luminescence) vs. log(inhibitor) equation $y=b + (a-b)/(1+10^{(x-\lg EC_{50})})$, Prism 7 (Graph Pad Software Inc.). Toxicity to cells was determined by using WST-1 reagent (Roche).

NP radioiodination.

For SPECT imaging and fluorescence detection concentrated NP were formulated with HC-PGC that carried covalently linked residues of both Alexa Fluor488 (AF488) and hydroxyphenylpropionate (at approximately 1% of total available amino groups of PLL). Hydroxyphenylpropionate residues were labeled with a gamma-emitting I-123 isotope (half-Life: 13.2 h, Na[¹²³I], Nordion) in the presence of iodogen-coated tubes (Pierce™ Iodination Tubes, Thermo Fisher) according to the manufacturer's instructions. Briefly, 0.1 mg of a mixture of AF488 and hydroxyphenylpropionate- labeled M5P10OL and M5P10ST (1:1 by weight) containing a mixture of EFV and ELV were combined with 2 mCi of Na¹²³I dissolved in 25mM Tris hydrochloride, 0.4M NaCl pH 7.5, the reaction was quenched by removing labeled NP from the tube and adding 0.01 mM L-tyrosine followed by 0.1 mM sodium iodide to displace free [¹²³I]iodide. The resultant dual-labeled HC-PGC NP were purified by using Biogel P-30 column blocked with 0.01% Tween-20 and equilibrated with sterile HBS. Radioiodination labeling efficiency was approximately 50% as determined by using iTLC-SG paper plates (Agilent).

In vivo application of NP and imaging.

Animal protocols were approved by the Institutional Animal Care and Use Committee at UMMS. To study biodistribution after a local administration we introduced double labeled NP into the cervicovaginal tract of rats. The estrous cycles of these rats were timed, and the animals were in confirmed estrus and metestrus stages (36) as determined by cytology of vaginal smears (37) (see Supplementary Figure S3). Initially n=3 female Fisher rats were briefly anesthetized using 1.8% isoflurane/oxygen and 30 µl of concentrated dually labeled HC-PGC NP (80 mg/ml, 230±25 µCi/animal) were administered by gentle pipetting into the vaginal tract using sterile 0.2 ml pipette tips. For SPECT/CT imaging, the animals were anesthetized with 1.8% isoflurane/oxygen, and imaged at 24h after the administration using NanoSPECT/CT (Bioscan). Acquisition time was approximately 30 min. The CT and SPECT reconstruction was performed using InVivoScope 1.37 software (Bioscan) and images were quantified using a volume-of-interest (VOI) approach. For biodistribution the animals were euthanized at 48 h, the blood samples were weighed and radioactivity in blood was counted using a gamma-counter and normalized for weight and radioactivity decay.

Microscopy.

Transmission electron microscopy (TEM) characterization of NP was performed by incubating NP at a dilution with 1:10 phosphate-buffered saline (Ambion) on carbonfilm/formvar-coated grids with negative staining with 1% uranyl acetate and further examining the grids under EM (FEI Tecnai 12 Spirit, at 16–128K magnification). Fluorescence microscopy of frozen sections was performed by using an inverted microscope TE2000-S (Nikon, Melville, NY) and processed using IPLab Spectrum software (BD Bioimaging, Rockville, MD, USA). Consecutive (serial) sections were stained with Hematoxylin/Eosin.

Results.

Simulations.

To assess the feasibility of incorporating and retaining the insoluble anti-retroviral drug molecules in the presence of HC-PGC graft copolymers we performed molecular dynamics simulations by using molecular operation environment (MOE). Initially we performed simulations to test whether EFV and ELV molecules interact with each other. Molecular dynamics simulations in 0.1 M NaCl water environment showed that an equimolar mixture (1:1 mol/mol) resulted in a formation of heterodimeric associates of EFV/ELV (Figure 1A). Furthermore, the simulations pointed to a high probability of the formation of periodic associates composed of EFV/ELV dimers (Figure 1 B). Second, simulations by using simplified approximations of HC-PGC assuming PLL DP=20 and MPEG length of n=50 ethylene oxide units (approximately 50% of MPEG5000 molecule, Figure 1C) showed that fatty acid interior environments in HC-PGC strongly favored stable association of multiple EFV/ELV associates (n=8) with the hydrophobic components of HC-PGC (n=8) within the internal core (hydrophobic-red, hydrophilic- blue, Figure 1D).

Characterization and testing of nanoparticle formulations in cell assays.

We tested several types of hydrophobic-core graft copolymers (HC-PGC) as potential stabilizers of insoluble NNRTI and INSTI. HC-PGC carrying either saturated stearic (M5PL10ST) or unsaturated oleic acids (M5PL10OL) were synthesized in two steps. The first step modification was verified by both TNBS titration and ^1H NMR spectroscopy analyses which were consistent with a covalent modification of $22\pm 2\%$ amino groups with MPEG5 resulting in methoxy polyethylene glycol (5000)-poly-L-lysine graft copolymers (38). The second step included acylation of residual primary amino groups with fatty acids. According to ^1H NMR results and assuming degree of polymerization DP=55, HC-PGC molecules carried 12 mol MPEG and 42 mol. fatty acid per mol. of HC-PGC. In the absence of any drug cargo M5PL10OL (modified with oleic acid residues) formed elongated (“worm-like”) and branched nanoparticles after the reconstitution of lyophilized HC-PGC in water (Figure 2A). Unlike oleic acid-containing HC-PGC copolymer, M5PL10ST formed spherical NP under identical conditions (Figure 2B). Next, NP were prepared by using hydration of HC-PGC co-lyophilized in the presence of EFV only or a drug combination of EFV/ELV at 1:1 w/w ratio. In the case of M5PL10OL the observed elongated structures (Figure 2A) underwent fragmentation into smaller, non-spherical segments (nanoparticles, NP, Figure 2C). HC-PGC acylated with stearic acid residues (M5PL10ST) showed the formation of predominantly spherical NP with non-crystalline core with amorphous

morphology in the presence of EFV/ELV (Figure 2D). Electron microscopy of a combination of both M5PL10ST and M5PL10OL after “loading” with an EFV/ELV mixture and resulted in homogenous non-spherical NP preparation (Figure 2E). Dynamic light scattering (DLS) analysis of NP solutions (Table 1) confirmed the results of electron microscopy and showed that in all cases the combination of EFV and ELV with HC-PGC resulted in ultra-small nanoparticles with average hydrodynamic diameters (Z_{av}) less than 50 nm (Table 1). A typical preparation containing 10% by weight of either EFV, ELV, or their 1:1 (w/w) mixture resulted in stable, weakly negatively charged NP (zeta potential range of -4 to -2 mV) with number average hydrodynamic diameters of less than 30 nm at pH 7.4. According to LC-MS data, after formulating EFV/ELV drug combination into NP the final concentration of EFV was 1.44 ± 0.01 mM and ELV concentration was 1.01 ± 0.39 mM. The actual concentrations of antiretroviral drugs in NP preparations were used in calculations of drug concentrations that were used in further experiments.

We performed the assessment of antiretroviral activity of NP formulations by using test assays involving TZM-bl reporter cells to which HIV-1 was added together with the NP, i.e. a cell culture co-addition assay. Initially EFV was assayed as a single drug cargo component of NP in cells infected with the wild-type HIV-1_{IIIB} (Table 1). The anti-retroviral activity of EFV-loaded NP (EC_{50}) was in the low nM range ($EC_{50} = 2.3\text{--}4.1$ nM) and did not differ significantly between M5PL10ST and M5PL10OL-stabilized NP in a co-infection *in vitro* assay ($p > 0.05$), Table 1, Figure 3A. A comparison of EC_{50} values corresponding to EFV dissolved in DMSO vs. EFV NP stabilized with HC-PGC also revealed similar anti-retroviral activity in the case of a multiple drug resistant HIV-1_{RTMDR1/MT-2} variant (39), as well as in the case of a A17 HIV-1 NNRTI-resistant mutant (Table 2, Figure 3A). In our experiments NP stabilizers with short PLL backbones exhibited very low cytotoxicity (50% cytotoxic concentrations (CC_{50}) were determined by using WST-1 assay, Table 1). EFV-loaded NP, which were stabilized with M5P10OL (MPEG-gPLL acylated with oleic acid, M5P10OL) showed lower cytotoxicity ($CC_{50} > 75 \mu\text{M}$ of EFV formulated in NP) than M5P10ST-stabilized NP. By comparing EC_{50} values for M5PL10ST and M5P10OL NP we noticed almost 2-times higher anti-HIV efficacy of EFV in M5PL10ST NP. Therefore, we used a combination of both HC-PGC types for formulating a combination of EFV and ELV (Tables 1 and 2). The combination NP containing both EFV and ELV were 19.5 ± 5.3 nm in diameter (with $Z_{av} = 37$ nm) after the reconstitution in water and showed high efficacy in inhibiting HIV-1_{IIIB} infection combined with low cytotoxicity, Table 1. If NP-stabilizing HC-PGC was omitted from formulation, we consistently observed large and unstable EFV/ELV particles with hydrodynamic diameters exceeding 1 μm formed after reconstitution of co-lyophilized inhibitors in water. Those large particles showed very low anti-HIV-1 effect even in the case of wild-type HIV-1_{IIIB} infection (Table 2). However, in the case of the M5PL10ST/M5P10OL stabilized NP the resultant combination NP containing both EFV and ELV showed a considerably improved antiretroviral activity with a 5-times lower EC_{50} (mean EC_{50} of 0.9 nM EFV) compared to pure EFV-containing NP, Table 2 and Figure. 3B. As expected, in contrast to the wild type HIV-1_{IIIB}, the NNRTI-resistant A17 HIV-1 variant, which carries double mutations K103N and Y181C in the viral RT domain (40)) showed resistance to EFV and EFV NP with a resultant EC_{50} that was more than 10 times higher than in the case of a wild-type HIV-1_{IIIB} (36.8 vs 2.4 nM), Table 2, Figure 3A,

B. However, nanoparticles containing EFV/ELV combination instead of EFV alone caused a drastic improvement as evident from the decrease of EC₅₀ to 2.9 nM (expressed in concentration units of EFV to enable quantitative comparison), Figure 3C. In the case of HIV-1_{RTMDR1}/MT-2 variant, which did not show strong resistance to EFV the use of a combination of EFV with ELV in NP also resulted in a complete inhibition of HIV-1 LTR-driven reporter construct expression and also caused appreciable but statistically insignificant decrease of EC₅₀ values (overlapping of 95% confidence intervals, $p > 0.05$, Table 2). It should be noted that the presence of pure ELV or ELV NP in the incubation medium was insufficient to achieve complete inhibition of the expression of HIV-1 genome products. The level of luciferase marker gene expression under the control of the HIV-1 LTRs could not be decreased below 10% of the initial levels of expression (i.e. in the absence of ELV) even in the presence of micromolar concentrations of ELV and regardless of the type of HIV-1 isolate used in infection assays (Figure 3B, green trace).

***In vivo* experiments.**

To investigate whether radioactive labeling of NP could be useful in tracking the distribution of NP *in vivo* we performed a pilot experiment in female rats which were confirmed in estrus/metestrus stages of estrous cycle by cytology of vaginal smears (see Supplemental Figure S2) with cornified epithelial cells and nucleated epithelial cells mainly present. The imaging performed at 24 h after local cervicovaginal administration of concentrated double-labeled (i.e. AF488 and ¹²³I) NP showed radioactivity distribution mainly along the whole reproductive system of rats (Figure 4A). Since some of the administered dose could have also been ingested by the animals there was also activity noted in the stomach (near body center, indicated with crosshairs on the images). By performing SPECT and CT imaging with images registration (fusion of SPECT image with CT map) we could track the distribution of NP components over time in both uterine horns, which showed variability of dose distribution (see Supplemental movies 1 and 2) 24 h after the administration (Figure 4A). Despite the fact that we used no barrier for preventing the loss of administered NPs from the site of administration we could track the distribution by imaging over time. As expected, the highest NP-associated radioactivity was contained in the vagina and the lower part of cervicovaginal tract. At 48 h endpoint post administration the biodistribution revealed variable residual fractional content of the delivered dose of labeled NP in the reproductive system and blood, with much smaller amounts in other organs (e.g. kidneys and liver), Figure 4B. Histology of frozen vaginal cross-sections stained with H&E did not show signs of infiltration of inflammatory cells (Figure 5A). Fluorescence microscopy showed that green AF488 fluorescence was associated mainly with the epithelial lining (Figure 5B). We also observed variable number of highly fluorescent cells ($n=10-30$ / section) within the epithelial lining and detected fluorescence within cells that were distally located and had high amounts of nanoparticles in the cytoplasm as judged by total green (AF488) fluorescence intensity of cells (Figure 5B).

Discussion.

There are four major approaches that can be pursued for blocking transmission of HIV-1 via mucosal barriers as pre-exposure prophylaxis: 1) inactivation of viral particles by

compounds interfering with the retroviral particle integrity; 2) the use HIV-1 fusion inhibitors; 3) co-receptor blocking agents (antagonists); 4) the use of HIV-1 reverse transcriptase and/or integrase inhibitors. The latter approach may involve oral dosing of highly effective combination of reverse transcriptase inhibitors, which is currently getting wider acceptance as a pre-exposure prophylaxis regimen (i.e. tenofovir/emtricitabine combination, a.k.a. PrEP (41–43). However, PrEP regimen availability world-wide is limited primarily to high-risk populations because it requires high daily doses with enteral (systemic) administration associated with the overall high costs of medication (43, 44). These factors necessitate the need for novel combinations of highly efficient antiretroviral formulations for topical delivery of much smaller amounts of HIV-1 inhibitors. In a local administration scenario all four approaches listed above ideally should target the events preceding HIV-1 life cycle or early events in the life cycle in the context of complex mucosal epithelial microenvironments. These microenvironments are affected by migrating surveying cells and are populated by macrophages, dendritic (Langerhans cells), CD4+T cells and other subpopulations of immune cells (45), many of which are susceptible to secondary HIV-1 infection (46). We investigated the approach that we initially developed for anti-HIV-1 protection (31) based on formulations stabilized with novel hydrophobic core HC-PGC graft copolymers. We report the results obtained by using a potentially more efficient combination nanoparticles that were designed to simultaneously deliver sufficient concentrations of insoluble inhibitors simultaneously targeting two enzymes of the HIV-1 life cycle, i.e. reverse transcriptase and integrase. We investigated this combination NP as a potential strategy for improving the response to treatment of NNRTI-resistant HIV-1 infection. Synergistic effects of a combination of two HIV reverse transcriptase inhibitors (tenofovir/emtricitabine) with INSTI were previously observed *in vitro* (47), while the response to a combination of highly efficient insoluble NNRTI and INSTI has not been yet studied under the conditions of drug resistant HIV-1 infection. In attempt to improve bioavailability we used non-toxic hydrophobic core graft copolymers (30, 32) which allowed us to select optimized combinations of stabilizers for formulating either EFV, or the EFV/ELV combination into nanoparticles (NP) with average hydrodynamic diameters below 50 nm. As expected, EFV and NP-formulated EFV showed more than 12-times lower anti-HIV-1 efficacy in the case of A17 variant when we compared it to the wild-type isolate because common mutations K103N and Y181C confer drug resistance to NNRTI inhibitors such as to nevirapine and efavirenz (48). In contrast, NP containing both orthogonal inhibitors, i.e. EFV and ELV showed a potent synergistic effect due to independently blocking both reverse transcription of HIV-1 genome and integration of HIV-1 DNA into the genome of reporter cells. While a combination of EFV with ELV improved EC50 of EFV in the case of all viral isolates we tested, in the case of NNRTI-resistant A17 HIV-1 mutant the decrease of mean EC50 was especially drastic and exceeded 12 times. The observed persistence of luciferase marker expression in reporter cells after a co-addition of HIV-1 and ELV in the absence of EFV indicates that transcription of viral genes was still ongoing in TZM-bl reporter cells even in the absence of integration of viral cDNA into the cell genome. As the next step we conducted a pilot *in vivo* study to estimate the local concentration of NP components that would be required to protect against HIV infection. Our *in vivo* experiment in a rat model showed that we were able to deliver NP-formulated combination of inhibitors locally and observe its distribution along the cervicovaginal tract using SPECT/CT imaging

at 24 h post administration. Furthermore, *ex vivo* biodistribution revealed that the formulation was still present in the cervicovaginal space at 48 h after the application. If we assume that NP stabilizer was associated with anti-HIV-1 drug cargo at the same ratio as in the initial NP formulation, the radioactivity tracer concentration determined in reproductive organs at 48h corresponded to approximately 50 nM. This concentration is approximately 2-times higher than the concentration of EFV/ELV combination required to achieve 90% protection from HIV-1 A17 variant infection as determined in cell culture assays (i.e. EC90=26.5 nM). This assumption implies that even though our study was limited because we did not perform NP topical delivery optimization, the hypothetical concentration of anti-HIV-1 drugs in the surrounding tissue was higher or on par with the *in vitro* EC90 value. This result is in agreement with the conclusion of another study, in which the actual concentrations of NNRTI were determined after mucosal biopsies (49).

Conclusions.

In conclusion, our results indicate that 1) by varying the type of HC-PGC components, i.e. hydrophobic modifier linked to PLL, the length of PLL backbone and the density of PEGylation it is potentially feasible to change NP diameter and their shapes depending on HC-PGC type and the content of the water-insoluble drug or insoluble drug combination; 2) a facile approach was developed for formulating a combination of insoluble inhibitors of HIV-1 reverse transcriptase and integrase into the ultrasmall NP in the presence of hydrophobic core copolymers carrying fatty acid residues; 3) these non-toxic NP exhibit strong anti-retroviral activity against drug-resistant HIV-1 isolates with more than 12-fold improvement of EC50 values; 4) dual labeling of NP enabled imaging of their biodistribution *in vivo* with microscopic corroboration *ex vivo*; 4) in 48 h after the topical intravaginal application a fraction of the NP components was found to be distributed along the reproductive system and in the blood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations.

DP	degree of polymerization
EFV	efavirenz
ELV	elvitagavir

INSTI	strand transfer inhibitor of HIV integrase
NNRTI	non-nucleoside reverse transcriptase inhibitor
MPEG-gPLL	methoxypoly(ethylene glycol)- graft-N-ε-poly-l-lysine
HC-PGC	hydrophobic core protected graft copolymer
M5P100L	methoxypoly(ethylene glycol)5000- graft-N-ε-poly-l-lysine (DP 55) acylated with oleic acid
M5P10ST	methoxypoly(ethylene glycol)5000-graft-N- ε-poly-l-lysine (DP55) acylated with stearic acid
AF488	Alexa Fluor 488

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Figure 1.

Molecular dynamics simulations. A - the formation of a dimer of EFV and ELV (the dimer is shown at 0° and -90° rotation); B- the formation of periodic associate of EFV/ELV dimers in simulated water solution, $n=8$ dimers; C- hydrophobic core of HC-PGC complex formation: shown are the steps applying molecular dynamics simulations to a system of EFV and ELV in the presence of a simplified fragments of hydrophobic core graft copolymer in simulated water environment. For simulations four shortened fragments of hydrophobic core graft copolymer carrying two MPEG residues ($n=50$ ethylene oxide units) linked to N-e-amino groups of poly-l-lysine with $DP=20$ and eight stearic acid residues; D- a molecular hydrophobicity surface map shown with the solvent removed (red- hydrophobic, blue- hydrophilic). Atoms of EFV and ELV in A, C and D are rendered as spheres.

Figure 2.

Transmission electron microscopy of nanoparticles. A - empty NP composed of M5P10OL (0.8 mg/ml); B- empty NP composed of M5P10ST (0.8 mg/ml); C- EFV- loaded (10%, w/w) M5P10OL NP (0.8 mg M5P10OL/ml); D- EFV-loaded (10% w/w) M5P10ST NP (0.8 mg M5P10ST/ml); E- EFV, ELV combination (loaded at 5% w/w each) NP composed of 1:1 mixture of M5P10OL and M5P10ST. The samples on carbon-coated TEM grids were stained with uranyl acetate for negative contrast.

Figure 3.

TZM-bl cell infectivity assays showing concentration-dependent inhibition of luciferase marker gene expression inhibition by added EFV and ELV. A- a comparison of titration curves in the infectivity assay using HIV-1_{RTMDR-1/MT-2} (blue traces) and A17 HIV-1 (red traces) after a co-addition of either DMSO-solubilized EFV (open symbols) or NP-formulated EFV (closed symbols) and the virus; B- antiretroviral activity of EFV-NP (red) and combination EFV/ELV NP (blue) compared to EFV (black) and ELV (green). EFV and ELV dilutions were performed by using stock solutions of these drugs in DMSO; C- the 20-fold decrease of effective EC50 value after combining EFV and ELV in NP (red) compared to single NNRTI NP (EFV-NP, black) in the case of A17 HIV-1 mutant.

Figure 4.

In vivo imaging and biodistribution. A- SPECT-CT imaging of the persistence of radioiodinated (^{123}I) and AF488 –labeled HC-PGC NP 24 h after intravaginal local application in rats. SPECT images are shown in pseudocolor, as an overlay of CT images (in grayscale). MIP (maximum intensity pixel projection), SAG- sagittal, and COR- coronal projections. B- 48h biodistribution (n=3) based on ^{123}I -radioactivity counting of major organs, blood and reproductive system organs shows the prevalence of NP components in the reproductive system and in the blood.

Figure 5. Histology and fluorescence microscopy of representative thin cross-sections of rat vaginal tissue performed after the double-labeled combination NP biodistribution study. A- hematoxylin and eosin staining of frozen section B- fluorescence microscopy of a parallel section. Blue- DAPI (nuclei), green – AF488 (nanoparticles).

Table 1.

Properties of HC-PGC nanoparticles.

	EFV-NP (M5P10ST) ¹⁾	EFV-NP (M5P10OL) ¹⁾	EFV/ELV-NP (M5P10ST/M5P10OL) ²⁾
Zav, nm ³⁾	33.1	29.8	37.1
Hydrodynamic diameter, by number mean±SD)	24.1±3.2	18.9±4.7	19.5±5.3
Zeta potential (1:10 HBS), mV	−4.1	−2.0	−3.0
logEC50 ⁴⁾ (mean±SD)	−8.7±0.3	−8.4±0.2	−9.0±0.1
EC50 ⁴⁾ , nM	2.3	4.1	1.0
CC50 ⁴⁾ , μM	>50	>75	>75

¹⁾ NP composition: 8 mg HC-PGC/ml, 0.8 mg EFV/ml (10% drug by weight).

²⁾ NP composition: 4 mg M5P10OL/ml, 4 mg/M5P10ST/ml, 0.4 mg EFV/ml, 0.4 mg ELV/ml (10% drug by weight)

³⁾ NP were diluted 10 times before DLS analysis. Polydispersity index (from DLS) measured for every one of NP preparations (n=4) was in the range of 0.25–0.35.

⁴⁾ EC50, logEC50 and CC50 were determined in TZM-bl cell culture infected with the wild-type HIV-1_{IIIB} isolate (3.5–4 ng HIV-1 p24/well) in the presence of serially diluted NP formulations. 50% cytotoxic concentrations (CC50) were determined by using WST-1 test. No toxicity was recorded below indicated concentrations. The experiments were performed in triplicate.

Table 2.

Testing of antiretroviral properties of HC-PGC stabilized nanoparticles (NP) containing Efavirenz (EFV) or a combination of Efavirenz and Elvitegravir (ELV) compared to non-formulated EFV, ELV and EFV/ELV combination.

Test compound/ HIV strain	EFV ¹⁾			EFV NP ²⁾			ELV ¹⁾			EFV/ELV NP ³⁾			EFV/ ELV ⁴⁾
	HIV-1IIB	A17 ⁵⁾	MT2 ⁶⁾	HIV-1IIB	A17	MT2	HIV-1IIB	A17	MT2	HIV-1IIB	A17	MT2	
logEC50±SD (n=5)	-8.4±0.1	-7.3±0.1	-8.5±0.1	-8.6±0.1	-7.4±0.1	-8.7±0.1	-8.3±0.1	No Fit ⁷⁾	-7.7±0.1	-9.0±0.1	-8.5±0.1	-8.6±0.1	-7.3±0.1
Log EC50 (95% CI), r ²	-8.5 to -8.2, 0.95	-7.4 to -7.2 0.97	-8.6 to -8.3 0.84	-8.7 to -8.5 0.97	-7.6 to -7.3 0.95	-8.7 to -8.5 0.89	-8.5 to -8.1 0.89		-7.9 to -7.4 0.90	-9.1 to -8.9 0.89	-8.6 to -8.4 0.96	-8.7 to 8.4 0.97	-7.4 to -7.2 0.96
EC50, nM	4.2	45.8	3.3	2.4	36.8	3.6	4.9	No Fit	19.2	0.9	2.9	2.6	45.2
EC90, nM	37.8	412.4	29.7	21.3	331.1	32.4	81.3		173.1	8.1	26.5	23.1	407.0

1) EFV and ELV were diluted in cell culture medium from stock solutions in DMSO.

2) Nanoparticle composition: 4 mg/ml M5P10ST, 4 mg/ml M5P10OL (100 nM HC-PGC), 0.9 mg/ml EFV (2.8 µM).

3) Nanoparticle composition: 4 mg/ml M5P10ST, 4 mg/ml M5P10OL (100 nM HC-PGC), 0.45 mg/ml EFV (1.44 µM); 0.44 mg/ml Elvitegravir (1.0 µM).

4) EFV/ELV mixture was lyophilized, dispersed in PBS (at 0.4 mg/ml EFV; 0.4 mg/ml Elvitegravir); and diluted in cell culture medium before adding to the cells.

5) HIV-1IIB A17 variant, highly resistant to inhibition by RT nonnucleoside inhibitors, mutations K103N and Y181C in the viral RT domain, uses CXCR4 and CCR5 as co-receptors.

6) HIV-1RTMDR1/MT-2 multi-drug resistant variant containing mutations of RT amino acid residues 74V, 41L, 106A, and 215Y, uses CXCR4 as a co-receptor.

7) HIV-1IIB A17 mutant data obtained by using ELV alone could not be fitted by using log[EFV] vs. normalized response model (no concentration-dependent inhibition).