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Rilpivirine-associated aggregation-induced emission enables cell-based nanoparticle tracking

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Abstract

Antiretroviral therapy (ART) has improved the quality and duration of life for people living with human immunodeficiency virus (HIV) infection. However, limitations in drug efficacy, emergence of viral mutations and the paucity of cell-tissue targeting remain. We posit that to maximize ART potency and therapeutic outcomes newer drug formulations that reach HIV cellular reservoirs need be created. In a step towards achieving this goal we harnessed the aggregation-induced emission (AIE) property of the non-nucleoside reverse transcriptase inhibitor rilpivirine (RPV) and used it as a platform for drug cell and subcellular tracking. RPV nanocrystals were created with endogenous AIE properties enabling the visualization of intracellular particles in cell and tissue-based assays. The intact drug crystals were easily detected in CD4⁺ T cells and macrophages, the natural viral target cells, by flow cytometry and ultraperformance liquid chromatography tandem mass spectrometry. We conclude that AIE can be harnessed to monitor cell biodistribution of selective antiretroviral drug nanocrystals.

Keywords

Rilpivirine; Antiretroviral therapy; Aggregation-induced emission; Single-crystal x-ray diffraction; CD4⁺ T cell tracking; Monocyte-macrophage; Drug biodistribution

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

1. Introduction

The past three decades have seen the global use of antiretroviral therapy (ART) for those with or at risk for human immunodeficiency virus type one (HIV-1) infection. ART has decreased disease morbidity and mortality, in infected patients, by maintaining viral suppression to undetectable levels [1, 2]. Further progress towards maintaining regimen adherence and slowing viral transmission can now be realized through long-acting (LA) formulations and LA slow effective release (LASER) ART prodrugs [3–9]. Recent advances have shown “proof of concept” that HIV could be eliminated in live animals using LASER ART and CRISPR/Cas9 viral excision [10]. However, HIV-1 elimination has yet to be demonstrated in infected people through medical regimens alone [11–15]. To address this, antiretroviral drugs (ARVs) and viral elimination agents must be precisely delivered to CD4⁺ T cell and monocyte-macrophage, the principal viral cell reservoirs. Assuring that these agents reach viral sites would affect best clinical outcomes. In efforts aimed to develop platforms that could reach viral sites [16, 17] we began to search for better ways to track ARV delivery. Through this search we uncovered the aggregation-induced emission (AIE) property of rilpivirine (RPV), a non-nucleoside reverse transcriptase inhibitor (NNRTI) already approved for treatment of HIV. RPV proved to be an excellent candidate for ARV tracking as it is used as part of LA antiretroviral regimens and its mechanism of action involves interference with the early stage life cycle of HIV replication. Indeed, as an NNRTI, RPV inhibits the activity of reverse transcriptase, preventing synthesis of viral DNA and hence subsequent integration of the viral genome into host DNA [9].

The application of RPV AIE is based on a photophysical phenomenon first discovered in 2001 [18]. This property could easily have been missed as in dilute solution each of the AIEgenic molecules lack luminescence. However, once aggregated as is now demonstrated for RPV they brightly fluoresce [18–22]. This occurs as a result of restricted intramolecular motion (RIM) as the drug retains high structural flexibility. As the restriction of intramolecular rotation (RIR) and vibration (RIV) increases the efficiency of deactivation decreases leading to RPV’s fluorescence properties [18, 21]. Based on such intrinsic AIE properties of RPV we posit that the drug can be used as effective tool to assess nanoparticle tracking in cell and subcellular organelles while, at the same time, also achieving therapeutic outcomes. We observed that the emission of RPV is observed as a blue fluorescence upon excitation by ultraviolet (UV) light. To elucidate the RPV AIE property for use in drug tracking drug nanocrystals (RPV-NC) were synthesized then enabling quantitative analysis of cell targeting by flow cytometry. We were successful in affirming particle biodistribution by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC MS/MS) analysis of the drug in CD4⁺ T cell and monocyte-macrophage populations. We contend that harnessing RPV AIE properties for drug biodistribution could speed the further development LA ARVs and in success for viral elimination strategies.

2. Materials and methods

2.1. Materials

RPV was purchased from Hangzhou Bingo Chemical Co., Ltd. (Hangzhou, ZJ, China). 1, 20-Distearoyl-phosphatidylethanolaminemethyl-polyethyleneglycol conjugate 2000 (DSPE-

PEG₂₀₀₀) was obtained from Corden Pharma International (Plank Stadt, Germany). L- α -phosphatidylcholine (from egg yolk) Chloroform, Dimethyl sulfoxide, N-butanol and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) from Sigma Aldrich (St. Louis, MO, USA). Heat-inactivated, pooled human serum was purchased from Innovative Biologics, Herndon, VA, USA. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Corning Life Sciences (Tewksbury, MA, USA). Monoclonal mouse HIV-1p24 (Clone Kal-1) and Dako EnVision+ detection system HRP labeled polymer anti-mouse antibodies were purchased from Agilent Technologies (Santa Clara, CA, USA). The primary antibodies used in study were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA): Rab5 (D-11) (sc-46692), Rab7 (H-50) (sc-10767), and Rab14 (sc-98610) (H-55). The Alexa FlourTM secondary antibodies were purchased from Thermo Fischer Scientific (Waltham, MA, USA): 594 goat anti-rabbit (A11037), 594 goat anti-mouse (A11005), 488 chicken anti-mouse (A21200), and 488 goat anti-rabbit (A11008). FITC mouse anti-human CD45, BUV805 mouse anti-human CD3, APC mouse anti-human CD4, PE mouse anti-human CD14, BV605 mouse anti-human CD19 and BV421 mouse anti-human CD8 were purchased from BD Biosciences (San Jose, CA, USA). Methanol, hexane and optima-grade solvents were purchased from Thermo Fischer Scientific. Bovine serum albumin (BSA), Gibco RPMI Medium 1640 and HyCloneTM Penicillin Streptomycin 100x solution were purchased from Thermo Fischer Scientific. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA, USA).

2.2. Synthesis of RPV-NC

Formulation of RPV-NC followed a bottom-up top-down approach [27] wherein micron sized crystals of RPV were prepared and then subjected to size reduction to obtain nanocrystals. Approximate 366 mg of RPV was dissolved in 400 ml of methanol and heated for 1 hour at 70°C for complete dissolution of RPV. To obtain a saturated solution, solvent volume was reduced under vacuum in a rotavapor (Heidolph, Hei-VAP Precision, G3B Vertical, Schwabach, Germany). The saturated solution was then added to 300 ml of deionized water at 4°C in a sonication bath under rapid stirring at 2500 rpm (IKA RW 20 Digital overhead stirrer, IKA Works, Inc., Wilmington, NC, USA). The formulation was sonicated and stirred rapidly for 15 min. The white, uniform suspension obtained was purified by centrifugation at 6000 g for 30 min (Thermo ScientificTM SorvallTM LYNX 4000 superspeed centrifuge, Waltham, MA, USA). The solid pellet was lyophilized overnight. For preparation of the lipid film, 200 mg of L- α -phosphatidylcholine and 100 mg of DSPE-PEG₂₀₀₀ were dissolved in a chloroform/hexane mixture (5:1 v/v) contained within a round-bottomed flask and evaporated to form a thin film using a rotavapor. The films were dried overnight to ensure complete removal of organic solvents. Lyophilized RPV crystals were dispersed in 15 ml of endotoxin free water, added to the prepared lipid film and sonicated for 5 min to obtain a uniform surface coating. The mixture was then homogenized at 20,000 psi in an Avestin EmulsiFlex-C3 high-pressure homogenizer (Ottawa, ON, Canada) until the desired particle size was reached (~320 nm DLS). Suspensions were stored at room temperature in amber vials for light protection.

2.3. Particle characterizations

2.3.1. Transmission electron microscopy (TEM), powder X-ray diffraction (XRD), thermal analysis and stability measurements—Nanoparticle morphology were analyzed by TEM. Nanoparticle suspensions were dried on a copper grid at room temperature and bright field images were taken with two second exposure times using the Tecnai G2 Spirit TWIN electron microscope (FEI, Houston, TX, USA) operating at 80 kV. Images were acquired with an AMT digital imaging system [28]. Analyses were carried out with XRD using a PANalytical Empyrean diffractometer (PANalytical, Inc., Westborough, MA, USA). This was performed in the 2θ range of $2\text{--}70^\circ$ with $\text{Cu-K}\alpha$ radiation ($1.5418 \text{ \AA}$) at 40 kV, 45 mA setting. The incident beam path used a mask of 20 mm and divergence slit of $1/32^\circ$. A layer of the powder was placed on a zero-background silicon plate. The sample holder was then spun at the rate of 22.5 deg/sec . During data collection, the solid state PIXcel3D detector (PANalytical, Inc., Westborough, MA, USA) scanned at a rate of 0.053 deg/sec . The PIXcel3D used a beam monochromator used to improve signal to noise ratios. Thermogravimetric analysis was performed under a nitrogen flow of 20 ml/min at a temperature of $30\text{--}600^\circ\text{C}$ and a heating rate of 10°C/min (TGA 209 F1 Libra, Netzsch-Gerätebau GmbH, Selb, Germany). Stability of the RPV-NC nanoparticle suspensions were assessed for 3 weeks at 4°C recording hydrodynamic particle size distribution.

2.3.2. Spectral measurements and drug quantifications—For confocal microscopy, single crystal RPV-NC were imaged using a $20\times$ objective on an LSM 710 confocal microscope (Carl Zeiss Microimaging, Inc., Dublin, CA, USA). Zeiss LSM 710 Image browser AIM software version 4.2 was used to determine pixel numbers and the mean channel intensities. Absorption spectra of dilute RPV suspensions were recorded on Thermo Scientific Evolution 220 spectrophotometer in quartz cuvettes with the optical path length of 1 cm. Fluorescence spectroscopy measurements were performed at 350 nm excitation by SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA), or on the Fluoromax 4 (Horiba Jobin Yvon, Kyoto, Japan) fluorometer ($\lambda_{\text{exc}}=335 \text{ nm}$) in quartz cuvettes with the optical path length of 1 cm. Fluorescence quantum yields were determined using quinine sulfate in $0.5 \text{ M H}_2\text{SO}_4$ as a standard ($\phi=0.546$ [29]). Photostability of RPV-NC was studied under a UV lamp (4 W , $\lambda=365 \text{ nm}$, broadband filter) with constant stirring. Analysis of RPV concentrations were carried out by UPLC using a Waters Acquity UPLC H-Class System with TUV detector and Empower 3 software (Waters, Milford, MA, USA). Sample separation was done on a Phenomenex Kinetex $5 \mu\text{m C18}$ column ($150 \times 4.6 \text{ mm}$, Phenomenex Ltd., Torrance, CA, USA) using an isocratic mobile phase of $65\% 50 \text{ mM KH}_2\text{PO}_4$, $\text{pH } 3.2$ / $35\% \text{ acetonitrile}$ at a flow rate of 1.0 ml/min and detection at 285 nm . Drug content was quantitated by comparison of the peak area to those of known standards ($0.05\text{--}50 \mu\text{g/ml}$ in methanol) [16, 17]. All flow cytometry data was acquired on a BD LSR II flow cytometer and analyzed using FLOWJO analysis software v10.2 (Tree Star, Ashland, OR, USA) and BD FACSDiva™ Software v8 (BD Biosciences, San Jose, CA, USA). Untreated and single stained controls were used to assign gates for nanoparticle positive populations. Light microscopy images of the particles were taken using a Nikon TE300 microscope (Nikon Instrument, Inc., Melville, NY, USA) with a $20\times$ objective. Polarized microscopy images

were taken using an Olympus BX51 (Olympus Scientific Solutions Americas Corp., Waltham, MA, USA) polarizing microscope at 90° polarized light.

2.3.3. Single-crystal x-ray diffraction—Single crystals for x-ray diffraction were prepared from a saturated solution of RPV in a mixture of dimethylsulfoxide (DMSO; 75%) and n-butanol (25%). A suitable crystal was selected and mounted on a Kryolooop using paratone oil on an Xcalibur, Onyx, Ultra Diffractometer (Oxford Diffraction Limited, Oxfordshire, UK). The crystal was kept at 100 K during data collection. Using OLEX2[30], the structure was solved with the SHELXS[31] structure solution program using Direct Methods and refined with the SHELXL[32] refinement package using Least Squares minimization.

2.4. Cell-based studies

Human peripheral blood monocytes were obtained by leukapheresis from HIV-1, 2 and hepatitis B seronegative donors and purified by countercurrent centrifugal elutriation. Monocyte-derived macrophages (MDM) were cultured in DMEM supplemented with 10% heat-inactivated pooled human serum, 10 µg/ml ciprofloxacin, 50 µg/ml gentamicin, and 1,000 U/ml equivalents of macrophage colony stimulating factor for 7 days to promote monocyte-macrophage differentiation (MDM) [23, 24]. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM-SS cells (CD4+ T cells) from Dr. Peter L. Nara [25, 26]. The UPLC-MS/MS parameters for drug concentration determinations were performed as previously reported [24,25] and are briefly described in the supplemental methods.

2.4.1. Cellular uptake in MDM and T cells—Cell vitality studies for MDM were carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) [33]. RPV-NC concentrations from 12 to 200 µM were prepared and added to the cells in triplicate. After treatment for 8 hours, analysis was carried out as described previously [16]. For T cell vitality studies, CD4+ T cells were treated with 3 to 200 µM of RPV-NC diluted in RPMI medium containing 10% FBS and 1% penicillin-streptomycin for 24 hours in a 96-well round-bottom plate. Supernatant was removed by centrifugation at 650 g for 5 min and cells were washed twice with PBS. Cells were then stained using LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to manufacturer's protocol and fixed in 2% paraformaldehyde (PFA).

MDM and CD4⁺ T cell uptake of the particles were assessed by flow cytometry and UPLC-TUV. Adherent MDM were treated in biological triplicates with 100 µM RPV-NC for 8 hours and cells were collected at various time points after washing and scraping into 1 ml PBS. For uptake in lymphocytes, CD4+ T cells were allowed to attach for one hour in poly-L-lysine coated plates at 37°C [34]. Cells were then treated with appropriate concentrations of RPV-NC and collected at aforementioned time points by washing and scraping into 1 ml PBS. Cell counting was done using an Invitrogen Countess Automated Cell Counter (Invitrogen Corp., Carlsbad, CA, USA). Cells were pelleted by centrifugation at 650 g for 8 min at 4°C and lysed in methanol by sonication for 1 min followed by centrifugation to remove cell debris. Drug concentration in methanol extracts were analyzed UPLC-TUV as

previously reported [35, 36]. For analysis by flow cytometry, MDM and CD4⁺ T cells were treated similarly, stained with LIVE/DEAD™ Fixable Green Dead Cell Stain Kit and fixed in 2% PFA. For co-culture experiments, additional staining was performed using mouse anti-human CD4 and CD14 antibodies according to manufacturer's protocol.

2.4.2. Cell-based microscopic assays—For TEM tests, MDM and CD4⁺ T cells were incubated in 12-well plates at 10⁶ cells/well for 8 hours with RPV-NC (100 μM). Cells were washed 2x with PBS and centrifuged at 650 g for 8 min at 4°C after treatment and cell pellets were suspended in a solution of 2% glutaraldehyde and 2% PFA in 0.1 M Sorenson's phosphate buffer (pH 6.2) for a minimum of 24 hours at 4°C. TEM analysis was then carried out as previously described [17, 36] and can be found in the supplemental methods. For AFM analysis, MDM were treated with RPV-NC at 100 μM for 8 hours, washed and fixed in 4% PFA. Coverslips with fixed cells were removed from wells and mounted on a slide for AFM analysis. Images were acquired in air using an MFP-3D™ system (Asylum Research, Santa Barbara, CA, USA) operating in tapping mode. AFM probes MSNL-F with a nominal spring constant ~0.6 N/m were used for imaging. Image processing was performed using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

Subcellular immunocytochemistry localization of RPV-NC treated MDM and T cells and immunocytochemistry staining were performed as previously described with some modifications [37–39]. Briefly, after MDM were treated with RPV-NC (100 μM), cells were washed with PBS and fixed with ice-cold 4% PFA and rinsed three times with PBS. MDM were then treated with 0.5% v/v Triton-X-100 for 15 min to permeabilize the cells and again washed with PBS. A mixture of 5% w/v bovine serum albumin (BSA) in PBS and 0.1% v/v Triton-X-100 was used as a blocking solution for 1 hour; and 50 mM NH₄Cl was used as quenching solution for 15 min and washed once with 0.1% Triton-X-100 in PBS. MDM were next treated with primary antibodies against Rab5 for early endosomes, Rab7 for late endosomes, or Rab14 for recycling endosomes. Primary antibodies were diluted 1:25 (v/v: antibody: blocking solution) and incubated overnight with shaking at 4°C as previously described [17]. MDM were then treated with secondary antibody in blocking solution (1:50) for 2 hours at room temperature. F-actin was stained using Alexa Fluor™ 488 Phalloidin (Thermo-Fischer Scientific, Waltham, MA, USA) according to manufacturer's protocol. Nuclei were stained using NucRed™ Live 647 ReadyProbes™ Reagent (Thermo-Fischer Scientific, Waltham, MA, USA). Cells were imaged using a 63x oil objective on an LSM 710 confocal microscope (Carl Zeiss Microimaging, Inc., Dublin, CA, USA). Zeiss LSM 710 Image browser AIM software version 4.2 was used to determine the number of pixels and the mean intensity of each channel.

2.5. Pharmacokinetic (PK) and biodistribution assays

PK and biodistribution studies for RPV-NC were carried out in rats and mice. For the first study, Sprague-Dawley rats (180–200 g, Jackson Labs, Bar Harbor, ME, USA) were injected IV with 45 mg/kg RPV-NC. At days 1 and 2 post-injection blood was collected. Plasma was subsequently isolated by centrifugation at 2000 g for 5 min for drug quantification tests by UPLC-MS/MS. Five days after injection the animals were sacrificed and tissues (liver, spleen and lymph node) stored. In a replicate study, male 6 to 8 week- old NOD/SCID/

IL2R γ ^{-/-} (NSG) mice (Jackson Labs, Bar Harbor, ME, USA) were injected intraperitoneally with 25×10⁶ human peripheral blood lymphocytes (PBL). These cells were obtained by leukapheresis and centrifugal elutriation and used to reconstitute the NSG mice. Twelve days after reconstitution, mice were injected intravenously with 45 mg/kg RPV-NC. After 10 days, the animals were sacrificed and tissues were collected for drug quantification by LC-MS/MS [35, 36]. For confocal analysis, 5 μ m sections of paraffin-embedded tissue were fixed on glass slides and stained with appropriate antibodies. For rats, spleen tissue sections were probed with rabbit anti-rat polyclonal antibody to ionized calcium binding adaptor molecule-1 (Iba-1) (1:500, Wako Chemicals, Richmond, VA, USA) to detect macrophages [17]. Primary antibody was detected with anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Thermo-Fischer Scientific, Waltham, MA, USA). For PBL mice, spleen and lymph node tissue sections were stained with CD4 Monoclonal Antibody (OKT4 (OKT-4)), Alexa Fluor 488. NucRed™ Live 647 ReadyProbes™ Reagent (Thermo-Fischer Scientific, Waltham, MA, USA) was used as the nuclear stain.

2.6. Nanoparticle tracking in humanized mice

Nanoparticle tracking in CD4⁺ T cells and macrophages was assessed in humanized mice. NSG mice were reconstituted at birth with CD34⁺ HSC isolated from umbilical cord blood [40]. Reconstituted mice, six-to-eight weeks old, were injected IV with 45 mg/kg of RPV-NC. At 24 hours following particle injection, mice were sacrificed, and liver and spleen were collected. Liver and spleen tissue were passed through a 70 μ m cell strainer to obtain single cell suspensions. For flow cytometry analysis, cell suspensions were stained with antibodies to human CD45, CD14, CD4, CD8 and CD3 as per manufacturer's protocol and fixed in 2% PFA. For drug quantification, single cell suspensions from liver and spleen tissue were stained with antibodies to human CD45, CD3, CD4 and CD14 antibodies and subjected to FACS on BD FACSAria II (BD Biosciences, San Jose, CA, USA). Sorted cells were pelleted by centrifugation at 700 g for 5 min at 4°C and analyzed by UPLC-MS/MS as described below.

2.7. Statistical analyses

For all studies, data were analyzed using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA) and are presented as the mean $\pm$ standard error of the mean (SEM). For comparisons of two groups, Student's t-test (two-tailed) was used. Experiments with multiple time points were analyzed using two-way factorial ANOVA and Bonferroni's post-hoc tests for multiple comparisons.

2.8. Study approval

All animal studies were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee in accordance with known standards for the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011).

3. Results

The major target cells for HIV infection are the CD4⁺ T cell and the monocyte-macrophage [38–43]. Thus, detection of viral suppressive agents in these cells could prove valuable towards improving therapeutic and eradication designs. Indeed, the AIE properties of RPV enables drug tracking by fluorescence emission. This ability was affirmed by TEM, UPLC-MS/MS, flow cytometry and confocal microscopy (Table 1).

3.1. RPV-NC preparation

RPV-NC were formulated by Smart nanotechnology [27] (Fig. 1A). Micron-sized particles were prepared by anti-solvent precipitation [44–46]. A consistent molecular RPV crystal structure was achieved. Based on RPV solubility in methanol, a saturated drug solution was prepared by heating. The drug was then rapidly added to ice-cold water under bath sonication and high-speed shearing. The homogenous suspension of particles was purified by high speed ultra-centrifugation and freeze-dried. Lyophilized particles were dispersed in water and coated with a lipid film containing DSPE-PEG₂₀₀₀ and phosphatidyl choline (α -PC) to impart stability and lipophilicity. The suspension was subjected to high pressure homogenization for size reduction and nanocrystal formation [35].

3.2. Physicochemical characterization

The physicochemical properties, fluorescence and structural features of these nanocrystals were studied by various analytical techniques. TEM images taken before homogenization showed micron-sized, ‘flower-shaped’ crystals accumulated in clusters Fig. 1B and C [47]. Fig. 1D demonstrates that the nanocrystals had a uniform shape and smooth surface with a particle size of approximately 200 nm. To ensure that crystal structure was retained, powder x-ray diffraction (XRD) analysis was performed for native RPV and for RPV crystals before and after homogenization. As shown in Fig. 1E, F and G, the characteristic diffraction peaks of native RPV (Fig. 1E) at 12.4°, 17.5°, 20.1°, 21.1° and 27.9° at 2° positions were maintained in the RPV crystals before (Fig. 1F) and after homogenization (Fig. 1G). This was also seen in RPV-NC after storage for 21 days at 25°C (Supplemental Fig. S1). The lack of a shift in the x-ray diffraction patterns demonstrates that the crystalline nature of native RPV in the free drug form was preserved in the micro crystals as well as in the homogenized nanoparticles. Thermal stability of formulated and native drug was shown by thermogravimetric analysis (TGA) analysis. Isothermal heating distribution curve of TGA showed an endothermic peak at 444.22°C and 442.78°C due to complete degradation of native RPV and RPV-NC, respectively. TGA is represented as a derivative thermal analysis (DTA) in Fig. 1H and shows the unchanged thermal properties of RPV-NC with respect to native RPV. The stability of RPV-NC in suspension at room temperature was monitored by dynamic light scattering (DLS). The zeta potential was found to be –24.9mV and as shown in Fig. 1I and J, the particle size, represented by hydrodynamic diameter, and poly dispersity index (PDI) remained constant at 320 nm and 0.2, respectively, for a period of 21 days.

3.3. AIE properties

In its aggregated form, RPV emits blue fluorescence when excited by UV light. This unique property can be used to monitor particle integrity. The AIE properties of RPV were

characterized by various methods [18, 48]. Confocal microscopy images of the RPV crystals overlapped with bright-field microscopic images displayed uniformity of fluorescence emission (Fig. 2A). Since RPV is freely soluble in methanol, the AIE phenomenon of RPV-NC was studied in various ratios of water/methanol. The shift towards longer wavelengths, and the broader width of the peak suggest that it should be attributed to the absorption of the RPV aggregates. The isobestic point at approximately 325 nm supports the conclusion that these two absorption peaks are related. Similar conclusions can be made from analysis of the RPV emission spectra ($\lambda_{\text{exc}}=335$ nm) in the same solvent systems (Fig. 2C). There is no detectable fluorescence from RPV in its solution in methanol. Upon increase of water content to 50%, blue fluorescence rises with the maximum at 385 nm. It reaches its maximum intensity at ~70% water content, and then somewhat decreases, potentially due to partial quenching induced by interactions with water [49]. Measurements of fluorescence quantum yield in solvent systems with varying amount of methanol (%v/v) yielded a sigmoidal curve with high goodness of fit ($R^2 = 0.9981$; Fig. 2D), a trend that was visually discernable (Fig. 2E). Analysis of RPV levels in supernatants of these samples showed an inverse relationship between drug concentration and fluorescence intensity (Fig. 2F). As the particles dissolve rapidly in 50% methanol, the fluorescence intensity drops significantly.

Further characterization of the AIE properties and its fluorescence stability important for prospective theranostic applications were carried out by measuring fluorescence intensities in different conditions. Emission of RPV-NC remained stable over a pH range of 2 to 14, but rapidly declined at pH 1 due to the complete disintegration of RPV aggregates (Fig. 2G). The photostability studies revealed that the fluorescence intensity of RPV-NC only decreases by approximately 30% over the course of 6 hours of constant irradiation (Fig. 2H). The associated absorption and emission spectra (Supplemental Fig. S2) do not show any apparent photooxidation or other degradation processes that affect RPV-NC performance. These observations demonstrate that RPV-NC exhibit AIE.

3.4. Elucidation of crystal structure

Several agents that exhibit the AIE property via RIM have previously been reported [18, 19, 21]. To understand the mechanism of AIE exhibited by RPV, single-crystal x-ray diffraction studies were performed (Fig. 3C). Single crystals of RPV were grown from a mixture of two solvents to obtain clear micron sized crystals (Fig. 3A). By polarized microscopy, the optically anisotropic character of the crystals was observed (Fig. 3B). X-ray crystallographic data revealed that two RPV molecules crystallized in a single cell unit (Fig. 3D). Displacement parameters, structure refinement and crystal data from x-ray studies are described (Supplemental Results SR1). Examination of the packing diagram revealed several close CH- π interactions for adjacent RPV molecules within the crystalline lattice. These contacts ranged from 2.96 – 3.5 Å. Similar intramolecular distances were observed for hexaphenylsilole (HPS) whose crystal structure showed intramolecular distances between C-H bonds and π rings of 2.587 – 3.642 Å [18, 50]. Taken together, these data suggest that restricted rotation of π rings in RPV crystals enable fluorescent emission by an RIM mechanism similar to that observed in other compounds [19].

3.5. Cell-based RPV-NC activities

To assess the cellular kinetics of RPV-NC, cytotoxicity, particle uptake and antiretroviral activities are required in HIV-1 infectible macrophages and CD4⁺ T cells [51–54]. MDM treated with RPV-NC demonstrated no apparent cytotoxicity at concentrations up to 200 μ M over 8 hours using MTT vitality assays (Fig. 4A). To assess cell uptake, MDM were treated with 100 μ M RPV-NC and at 2, 4, 6 and 8 hours, media was removed, and cells lysed for drug analysis. Cellular RPV concentrations increased over time with maximum uptake seen at 6 hours (60 μ g/10⁶ cells) (Fig. 4B). AFM micrographs of replicate treated MDM showed no cell surface distortions upon uptake of RPV-NC (Fig. 4C). The intracellular uptake of RPV-NC was confirmed by TEM demonstrating RPV-NC in the macrophage cytoplasm and in the membrane bound intracellular structures (Fig. 4D). Because AIE relies on particle aggregation, we studied nanoparticle integrity in MDM by flow cytometry. Up to 98.8% of MDM demonstrated nanoparticle uptake at 8 hours (Fig. 4E), further highlighting their ability to phagocytose nanoparticles in large amounts [55, 56].

Uptake of RPV-NC by CD4⁺ T cells was similarly evaluated. No cytotoxicity was observed at concentrations of up to 200 μ M when treated for 8 hours (Fig. 5A). Cell uptake was determined at 2, 4, 6 and 8 hours after 100 μ M RPV-NC treatments. Maximum uptake of 8 μ g/10⁶ cells was seen at 8 hours (Fig. 5B). To assess RPV-NC integrity over time within CD4⁺ T cells, the cells were examined by flow cytometry. Both the percent and median fluorescent intensity (MFI) increased with time, corresponding to efficiency of endocytosis and RPV-NC accumulation within CD4⁺ T cells, respectively. The RPV-NC positive population increased from 11% at 2 hours to 30% at 24 hours along with a 10-fold increase in MFI (Fig. 5C and D).

Pearson correlation between the median fluorescent intensity (MFI) and drug concentration showed an *r* value of 0.8800 when cells were treated with 100 μ M RPV-NC over a time course of 24 hours (Fig. 5E). Correlative studies were also performed by varying the treatment concentration with a fixed harvest after 24 hours. Results revealed that both MFI and intracellular RPV were positively correlated with treatment concentration and showed a Pearson *r* value of 0.7925 (Fig. 5F). TEM images from cells treated for 24 hours with 100 μ M RPV-NC depict discernible presence of nanoparticles in T cell cytoplasm and membrane-bound intracellular structures (Fig. 5G). Thus, RPV-NC uptake kinetics in a model for CD4⁺ T cells can be monitored using AIE.

Monocyte-macrophage and CD4⁺ T cell activities may differ in pure versus combined cell cultures [57], thus, we studied RPV-NC uptake in isolate or co-culture conditions over 24 hours. CD14⁺ MDM demonstrated greater uptake of RPV-NC in co-culture with CD4⁺ T cells (Fig. 6A and B, Supplemental Fig. S5). In contrast, CD4⁺ lymphocytes exhibited less efficient endocytosis of RPV-NC in the presence of macrophages. These differences emphasize the phagocytic potential of macrophages, which readily outcompete T cells to engulf RPV-NC, whereas lymphocyte immune activation of macrophages may enhance their uptake potential [23]. RPV-NC cytoplasmic accumulation in macrophages was further detected using flow cytometry of nanocrystal AIE as exhibited by the rightward shift in histogram peaks with varying treatment concentration (Fig. 6C). Pearson correlation analysis revealed high positive correlation (*r* = 0.9810) between MFI and treatment concentration in

macrophages (Fig. 6D). In contrast, CD8⁺ T cells and CD19⁺ B lymphocytes exhibited no such dose-dependent accumulation of RPV-NC (Supplemental Fig. S6).

These results highlight the preferential sequestration of antiretroviral nanocrystals by HIV-infectable macrophages and to a lesser degree CD4⁺ T cells, rather than by lymphocyte subtypes that are resistant to HIV-infection. To this end, AIE characteristics of RPV-NC facilitate quantitative assessment of drug uptake in primary cells under conditions more similar to those encountered *in vivo*.

3.6. Cellular and sub-cellular colocalizations

To track their fate once inside cells, RPV-NC sub-cellular localization in monocyte-macrophages and CD4⁺ T cells was studied by confocal microscopy. MDM were treated for 8 hours with 100 μ M RPV-NC then immunostained for endosomal markers that included Rab5 (early endosomes), Rab7 (late endosomes) and Rab14 (recycling endosomes). Co-localization of RPV-NC was readily seen in stained endosomal compartments (Fig. 7A) with the Rab14 endosomes indicating that RPV-NC can be recycled for release by macrophages [24, 58]. Another potential means of RPV-NC dissemination during HIV-1 infection is through cytoplasmic bridges, also called tunneling nanotubes (TNTs), that facilitate cell-to-cell communication and intracellular material exchange [38, 59–61]. Accordingly, TNTs of RPV-NC treated, HIV-1 ADA-infected MDM were visualized by f-actin staining. Fig. 7B shows the formation of multinucleated giant cells (i) and cytoplasmic bridges (ii) with RPV-NC observed in both the infected cells and the cytoplasmic bridges (iii and iv). Because uptake in CD4⁺ T cells occurs primarily by endocytosis [62], the extent of uptake is lower than in macrophages. To visualize RPV-NC taken up by T cells, CD4⁺ T cells treated with RPV-NC were analyzed by confocal microscopy. RPV-NC was readily seen in the T cell cytoplasm (Fig. 7C). Once RPV-NC entry and intercellular dissemination had been studied, antiretroviral activities of RPV-NC were also determined in MDM. MDM were treated with 100 μ M RPV-NC for 8 hours followed by replacement with fresh media. At days 1, 5, 7 and 10 following treatment, cells were infected with HIV-1 ADA at a MOI of 0.1. 10 days after infection, cell supernatants were analyzed for reverse transcriptase (RT) activity. Cells were stained for HIV-1 p24 in parallel. Viral suppression was observed up to day 10 (Supplemental Fig. S7). These results indicate that the antiretroviral efficacy of RPV-NC parallels that of native RPV.

3.7. Pharmacokinetics (PK) and biodistribution

To correlate RPV-NC *in vitro* cellular uptake with tissue and cellular uptake *in vivo*, biodistribution and PK profiles of RPV-NC were assessed in rats and humanized mice. Fig. 8A shows a schematic outlining the time course of the studies in the indicated animal models. A short-term biodistribution and PK analysis was carried out in Sprague-Dawley rats injected intravenously (IV) with 45 mg/kg RPV equivalent of RPV-NC. On days 1, 2 and 5 post-injection, blood samples were collected, and plasma was analyzed by UPLC-MS/MS for drug quantification. Animal tissues were collected on day 5 post-injection and drug was quantified by UPLC-MS/MS. Fig. 8B shows plasma drug levels over time as well as day 5 liver, spleen and lymph node drug levels. As shown in Fig. 8B (i), plasma drug levels decreased from 134.5 ± 15.3 ng/ml on day 1 to 1.5 ± 0.2 ng/ml on day 5. In tissues,

drug levels were highest in the liver (3540 ng/g of tissue), followed by the spleen (94.5 ng/g of tissue) and the lymph nodes (9.5 ng/g of tissue) in Fig. 8B (ii).

Toxicological assessments of liver and spleen tissues from rats treated with RPV-NC showed no histological abnormalities when compared to tissues from untreated controls. Also, no biochemical or hematological effects of RPV-NC were seen in treated animals (Supplemental Fig. S8). *To track RPV-NC biodistribution in human immune cells, we* adoptively transferred human peripheral blood lymphocytes into NSG mice (PBL mice) and dosed animals with 45 mg/kg drug. Tissues were collected on day 10 post-injection and RPV concentration was determined. Analysis of RPV drug levels in excised tissue showed highest drug levels in the liver in Fig. 8B(iii). Having observed measurable quantities of RPV in tissues, we searched for intact nanoparticles displaying AIE in HIV-1 infectible cells. Confocal imaging of rat spleens revealed discernable overlap between UV-excitable RPV-NC and Iba-1⁺ rat macrophages (Fig. 8C). This finding extended to human CD4⁺ leukocytes in PBL-reconstituted mice (Fig. 8D, E and F). A high percentage of human CD4⁺ splenocytes exhibited RPV-NC-associated fluorescence (Fig. 8D), whereas this effect was more modest in lymph nodes (Fig. 8E and F). Together, these findings manifest that IV administration of RPV-NC travels via blood to reticuloendothelial organs (i.e. liver, spleen, and lymph nodes), where they are encountered and engulfed by various immunocytes.

3.8. Cellular tracking of RPV-NC in humanized mice

To track nanoparticles in CD4⁺ T cells and monocyte-macrophages, a second murine model was employed to assess uptake of RPV-NC in human immune cells. NSG mice were reconstituted at birth with human CD34⁺ hematopoietic stem cells (HSC). Importantly, the reconstituted mice (humanized mice) show quiescent lymphocyte profiles and as such reflect what would occur in a HIV-1 challenged human host [40]. These humanized mice were injected with 45 mg/kg RPV equivalent of RPV-NC, and after 24 hours, liver and spleen were collected. Single cell suspensions of these tissues were prepared and analyzed *ex vivo* by flow cytometry for nanoparticle distribution in different immune subsets. Fig. 9A shows representative flow cytometry plots of nanoparticle positive CD4⁺ T cells and CD14⁺ macrophages from treated mice. The detailed gating strategy for different immune subsets is provided in Supplemental Fig. S9. Human CD4⁺ and CD14⁺ populations were quantified as percent nanoparticle positive cells for each animal. From the same experimental mice, cellular suspensions from liver and spleen were subjected to fluorescence-activated cell sorting (FACS) to sort CD4⁺ T cells and CD14⁺ monocyte-macrophages. These isolated cell populations were then analyzed by UPLC-MS/MS quantification of RPV. Fig. 9B shows liver CD4⁺ T cell and CD14⁺ macrophage percent populations positive for RPV-NC. RPV levels of about 179 ng/10⁶ cells and 368 ng/10⁶ cells were observed in the CD4⁺ T cells and monocyte-macrophages, respectively, as shown in Fig. 9C. Pearson correlations between RPV levels and percent positive CD4⁺ T cell or CD14⁺ macrophage populations provided *r* values of 0.81 and 0.94, respectively (Fig. 9D & E). Similarly, Fig. 9F shows percent CD4⁺ T cell and monocyte-macrophage populations positive for RPV-NC where a significantly higher particle uptake was observed in macrophages. RPV levels of about 22.6 ng/10⁶ cells and 62.6 ng/10⁶ cells were observed in splenic CD4⁺ T cells and CD14⁺ macrophages, respectively, as shown in Fig. 9G.

Discussion

ART and more recently more long acting versions have positively affected the quality and longevity of life all have failed to eliminate viral infection [63–65]. While viral suppression and restoration of immunity have been achieved elimination of the viral reservoir has not yet been achieved. Nonetheless, a number of research strategies are being developed towards HIV cure [11, 12]. This includes improved ART, viral reversal agents and boosting of adaptive immunity. These have been deployed to modulate viral replication then eliminate the residual infected cells. While stem cell transplantation has been successful it has been seen in only anecdotal cases. Work from our own laboratories have achieved HIV elimination in live infected animals through LASER ART and CRISPR/Cas9 therapies [10] but only in a subset of cases. Concerns for off target toxicities and optimal drug delivery strategies remained. Taken together, a critical obstacle lies in finding then delivering therapeutics to latent viral reservoirs and ultimately eliminated them is of clear immediate need [12, 14, 15, 66]. Since the major leukocyte harboring latent HIV infection is the CD4⁺ T cell [41–43] and to a lesser degree the monocyte-macrophage, tracking of viral cell reservoirs is imperative to seek out sites of low level HIV replication so that viral elimination can be achieved. While such cell tracking could be achieved through gold, magnetic and programmed nanoparticles [67–69] concerns for both sensitivity and specificity of such systems abound. Indeed, these technologies are limited in that an external probe is needed. AIE could overcome such a limitation as it allows for visualization of ARVs or excision-containing nanoparticles endocytosed by these cellular reservoirs as well as simultaneously permitting quantitative drug assessment.

To effectively achieve viral elimination, therapeutically effective amounts of viral suppressive and elimination agents must reach cellular reservoirs of HIV. Thus, ARV tracking to sites of viral infection is required. The development of RPV-NC exhibiting AIE could enable this need to be achieved. Correlations made between amount of therapeutic nanoparticle payloads delivered to cells harboring latent HIV and elimination of infection could be realized. Thus, the technologies developed herein would provide a pathway forward towards such required therapeutic delivery. It is certainly feasible that other antiviral agents would possess AIE properties as a plethora of molecules have been characterized for similar activities and several more have been newly synthesized or discovered. Indeed, molecules with AIE properties have already shown to possess a wide range of applications. These include chemical sensors to biological imaging probes and have been used successfully in a range of applications in diagnosis and therapy [19, 70–73]. One such prototypical molecule is tetraphenylethylene (TPE) [22, 48, 72, 74]. The AIE characteristics of TPE were deployed as an AIE fluorescent probe for real time tracking of cell apoptosis [75]. Other molecules have been derivatized by introducing an AIE-genic fragment to induce strong fluorescence upon aggregation [76, 77]. Clearly, the future use of AIE-active drugs will certainly provide significant added advantages to any presently existing nanoparticle tracking designs.

In the current report, we not simply demonstrate the aggregation-induced emission of RPV, an NNRTI used for HIV therapy but show its utility to find target cell for HIV infection. *First*, we synthesized RPV-NC through a bottom-up top-down approach by antisolvent precipitation and subsequent size-reduction to obtain nanoparticles that can reach viral target

CD4⁺ T cells and monocyte-macrophages. *Second*, we thoroughly characterized the morphological and fluorescent properties of RPV-NC in a wide variety of conditions. These characterizations confirmed the AIE property of RPV as well as the crystallinity of formulated RPV-NC. *Third*, we performed single crystal x-ray crystallography studies and found that RIR was the primary mechanism of AIE expression in RPV-NC ensuring its stability. *Fourth*, we characterized uptake of RPV-NC in CD14⁺ macrophages and CD4⁺ T cells by a broad range of techniques and also ensured cell uptake of RPV-NC in simulated conditions that could reflect what is operative in an HIV infected human host. *Fifth*, we carried out confocal microscopy analyses of macrophages and T cells treated with RPV-NC and confirmed their presence in cellular and sub-cellular compartments where viral replication is known to ensue. *Sixth*, PK and biodistribution studies performed and showed the presence of RPV in liver, spleen and lymph nodes which are major tissue viral reservoirs. *Seventh*, confocal analyses of these tissue sections showed the presence of intact RPV-NC in splenic macrophages and human CD4⁺ T cells of the spleen and lymph node. *Eighth*, nanoparticle tracking was achieved by flow cytometry in human immune cells using a humanized mouse model. CD4⁺ T cells and monocyte-macrophages from these animals were isolated by FACS and subjected to drug quantification. These results, taken together, demonstrate the use of AIE to achieve therapeutic nanoparticle tracking in human cellular immune subsets.

All together, we characterized an AIE drug molecule, RPV, and harnessed its fluorescent property for drug biodistribution assessment. Cellular uptake studies were translated into animal models to observe nanoparticle tracking in CD4⁺ T cells and monocyte-macrophages. When used in combination with other ARVs [78, 79], RPV has the potential to track the biodistribution and cellular uptake of other drugs. In tandem with drug quantification, the AIE phenomena can be used as a platform to optimize drug delivery to cellular viral reservoirs that currently remain elusive. While parallel studies have achieved cellular tracking for other diseases by gold, magnetic and programmed nanoparticles [67–69], the inherent AIE property of RPV enables simultaneous assessment by fluorescence and drug quantification, affirming the presence of intact ARV nanoparticles in target cellular reservoirs. Hence, RPV-NC achieving HIV eradication through improvement in the safety and delivery of existing therapies. Our findings may be readily translated into other nanoformulations and can be used with other AIEs to achieve nanoparticle tracking during the question to uncover and eliminate sites of HIV-1 growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIDS	acquired immune deficiency syndrome
AIE	aggregation-induced emission
AFM	atomic force microscopy
ART	antiretroviral therapy
ARV	antiretroviral drugs
BSA	bovine serum albumin
CD	cluster of differentiation
DMSO	dimethyl sulfoxide
DLS	dynamic light scattering
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]
DTA	derivative thermal analysis
FACS	fluorescence-activated cell sorting
HIV-1	human immunodeficiency virus type 1
HPS	hexaphenylsilole
HSC	hematopoietic stem cells
IP	intraperitoneal
IV	intravenous
LA ARV	long-acting antiretroviral drugs
LA NRPV	Long acting nanoformulated rilpivirine
LASER ART	long acting slow effective release antiretroviral therapy

MDM	human monocyte-derived macrophages
MFI	median fluorescent intensity
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NNRTI	non-nucleoside reverse transcriptase inhibitor
NSG	Nod. <i>Cg-Prkdc^{scid}IL2rg^{tm1Wjl}</i> / SzJ mice
PBS	phosphate-buffered saline
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PC	phosphatidylcholine
PDI	polydispersity index
PFA	paraformaldehyde
PK	pharmacokinetic
RB	round-bottom flask
RIM	restricted intramolecular motion
RIR	restricted intramolecular rotation
RIV	restricted intramolecular vibration
RPV	rilpivirine
RPV-NC	rilpivirine nanocrystals
RT	reverse transcriptase
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TGA	thermogravimetric analysis
TNT	tunneling nanotubes
UPLC-MS/MS	ultraperformance liquid chromatography-tandem mass spectrometry
UPLC-TUV	ultraperformance liquid chromatography-tunable UV detector
UV	ultraviolet

XRD

x-ray diffraction

References

- [1]. Pau AK, George JM, Antiretroviral therapy: current drugs, *Infect Dis Clin North Am* 28(3) (2014) 371–402. [PubMed: 25151562]
- [2]. Vittinghoff E, Scheer S, O'Malley P, Colfax G, Holmberg SD, Buchbinder SP, Combination antiretroviral therapy and recent declines in AIDS incidence and mortality, *J Infect Dis* 179(3) (1999) 717–20. [PubMed: 9952385]
- [3]. Zhou T, Su H, Dash P, Lin Z, Dyavar Shetty BL, Kocher T, Szlachetka A, Lamberty B, Fox HS, Poluektova L, Gorantla S, McMillan J, Gautam N, Mosley RL, Alnouti Y, Edagwa B, Gendelman HE, Creation of a nanoformulated cabotegravir prodrug with improved antiretroviral profiles, *Biomaterials* 151 (2018) 53–65. [PubMed: 29059541]
- [4]. Sillman B, Bade AN, Dash PK, Bhargavan B, Kocher T, Mathews S, Su H, Kanmogne GD, Poluektova LY, Gorantla S, McMillan J, Gautam N, Alnouti Y, Edagwa B, Gendelman HE, Creation of a long-acting nanoformulated dolutegravir, *Nat Commun* 9(1) (2018) 443. [PubMed: 29402886]
- [5]. Lin Z, Gautam N, Alnouti Y, McMillan J, Bade AN, Gendelman HE, Edagwa B, ProTide generated long-acting abacavir nanoformulations, *Chem Commun (Camb)* 54(60) (2018) 8371–8374. [PubMed: 29995046]
- [6]. van 't Klooster G, Hoeben E, Borghys H, Looszova A, Bouche MP, van Velsen F, Baert L, Pharmacokinetics and disposition of rilpivirine (TMC278) nanosuspension as a long-acting injectable antiretroviral formulation, *Antimicrob Agents Chemother* 54(5) (2010) 2042–50. [PubMed: 20160045]
- [7]. Spreen WR, Margolis DA, Pottage JC Jr., Long-acting injectable antiretrovirals for HIV treatment and prevention, *Curr Opin HIV AIDS* 8(6) (2013) 565–71. [PubMed: 24100877]
- [8]. Jackson A, McGowan I, Long-acting rilpivirine for HIV prevention, *Curr Opin HIV AIDS* 10(4) (2015) 253–7. [PubMed: 26049950]
- [9]. Baert L, van 't Klooster G, Dries W, Francois M, Wouters A, Basstanie E, Iterbeke K, Stappers F, Stevens P, Schueller L, Van Remoortere P, Kraus G, Wigerinck P, Rosier J, Development of a long-acting injectable formulation with nanoparticles of rilpivirine (TMC278) for HIV treatment, *Eur J Pharm Biopharm* 72(3) (2009) 502–8. [PubMed: 19328850]
- [10]. Dash PK, Kaminski R, Bella R, Su H, Mathews S, Ahooyi TM, Chen C, Mancuso P, Sariyer R, Ferrante P, Donadoni M, Robinson JA, Sillman B, Lin Z, Hilaire JR, Banoub M, Elango M, Gautam N, Mosley RL, Poluektova LY, McMillan J, Bade AN, Gorantla S, Sariyer IK, Burdo TH, Young WB, Amini S, Gordon J, Jacobson JM, Edagwa B, Khalili K, Gendelman HE, Sequential LASER ART and CRISPR Treatments Eliminate HIV-1 in a Subset of Infected Humanized Mice, *Nat Commun* 10(1) (2019) 2753. [PubMed: 31266936]
- [11]. Palmer S, Josefsson L, Coffin JM, HIV reservoirs and the possibility of a cure for HIV infection, *J Intern Med* 270(6) (2011) 550–60. [PubMed: 21929712]
- [12]. Chun TW, Fauci AS, HIV reservoirs: pathogenesis and obstacles to viral eradication and cure, *AIDS* 26(10) (2012) 1261–8. [PubMed: 22472858]
- [13]. Hocqueloux L, Avettand-Fenoel V, Jacquot S, Prazuck T, Legac E, Melard A, Niang M, Mille C, Le Moal G, Viard JP, Rouzioux C, A.C.o.t.A.N.d.R.s.l.S.e.l.H. Virales, Long-term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and normal T cell counts, *J Antimicrob Chemother* 68(5) (2013) 1169–78. [PubMed: 23335199]
- [14]. Churchill MJ, Deeks SG, Margolis DM, Siliciano RF, Swanstrom R, HIV reservoirs: what, where and how to target them, *Nat Rev Microbiol* 14(1) (2016) 55–60. [PubMed: 26616417]
- [15]. Dahl V, Josefsson L, Palmer S, HIV reservoirs, latency, and reactivation: prospects for eradication, *Antiviral Res* 85(1) (2010) 286–94. [PubMed: 19808057]
- [16]. Ottemann BM, Helmink AJ, Zhang W, Mukadam I, Woldstad C, Hilaire JR, Liu Y, McMillan JM, Edagwa BJ, Mosley RL, Garrison JC, Kevadiya BD, Gendelman HE, Bioimaging predictors of rilpivirine biodistribution and antiretroviral activities, *Biomaterials* 185 (2018) 174–193. [PubMed: 30245386]

- [17]. Kevadiya BD, Woldstad C, Ottemann BM, Dash P, Sajja BR, Lamberty B, Morsey B, Kocher T, Dutta R, Bade AN, Liu Y, Callen SE, Fox HS, Byrareddy SN, McMillan JM, Bronich TK, Edagwa BJ, Boska MD, Gendelman HE, Multimodal Theranostic Nanoformulations Permit Magnetic Resonance Bioimaging of Antiretroviral Drug Particle Tissue-Cell Biodistribution, *Theranostics* 8(1) (2018) 256–276. [PubMed: 29290806]
- [18]. Mei J, Leung NL, Kwok RT, Lam JW, Tang BZ, Aggregation-Induced Emission: Together We Shine, United We Soar!, *Chem Rev* 115(21) (2015) 11718–940. [PubMed: 26492387]
- [19]. Hong Y, Lam JW, Tang BZ, Aggregation-induced emission: phenomenon, mechanism and applications, *Chem Commun (Camb)* (29) (2009) 4332–53. [PubMed: 19597589]
- [20]. Hong Y, Lam JW, Tang BZ, Aggregation-induced emission, *Chem Soc Rev* 40(11) (2011) 5361–88. [PubMed: 21799992]
- [21]. Mei J, Hong Y, Lam JW, Qin A, Tang Y, Tang BZ, Aggregation-induced emission: the whole is more brilliant than the parts, *Adv Mater* 26(31) (2014) 5429–79. [PubMed: 24975272]
- [22]. Kokado* TJTTSK, Control of Aggregation-Induced Emission from a Tetraphenylethene Derivative through the Components in the Co-crystal, *Crystal Growth & Design* (2018).
- [23]. David JR, Macrophage activation induced by lymphocyte mediators, *Acta Endocrinol Suppl (Copenh)* 194 (1975) 245–61. [PubMed: 1092116]
- [24]. Arainga M, Guo D, Wiederin J, Ciborowski P, McMillan J, Gendelman HE, Opposing regulation of endolysosomal pathways by long-acting nanoformulated antiretroviral therapy and HIV-1 in human macrophages, *Retrovirology* 12 (2015) 5. [PubMed: 25608975]
- [25]. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE, Continuous Culture of Human Lymphoblasts from Peripheral Blood of a Child with Acute Leukemia, *Cancer* 18 (1965) 522–9. [PubMed: 14278051]
- [26]. Nara PL, Fischinger PJ, Quantitative infectivity assay for HIV-1 and-2, *Nature* 332(6163) (1988) 469–70. [PubMed: 3281026]
- [27]. Raghava Srivalli KM, Mishra B, Drug nanocrystals: A way toward scale-up, *Saudi Pharm J* 24(4) (2016) 386–404. [PubMed: 27330370]
- [28]. Dou H, Morehead J, Destache CJ, Kingsley JD, Shlyakhtenko L, Zhou Y, Chaubal M, Werling J, Kipp J, Rabinow BE, Gendelman HE, Laboratory investigations for the morphologic, pharmacokinetic, and anti-retroviral properties of indinavir nanoparticles in human monocyte-derived macrophages, *Virology* 358(1) (2007) 148–58. [PubMed: 16997345]
- [29]. Brouwer AM, Standards for photoluminescence quantum yield measurements in solution, *Pure Appl. Chem Vol. 83*(No. 12) (2011) 15.
- [30]. Dolomanov OV, Bourhis LJ, Gildea RJ, Howard JAK, Puschmann H, OLEX2: a complete structure solution, refinement and analysis program, *Journal of Applied Crystallography* 42(2) (2009) 339–341.
- [31]. Sheldrick GM, A short history of SHELX, *Acta Crystallogr A* 64(Pt 1) (2008) 112–22. [PubMed: 18156677]
- [32]. Sheldrick GM, Crystal structure refinement with SHELXL, *Acta Crystallogr C Struct Chem* 71(Pt 1) (2015) 3–8. [PubMed: 25567568]
- [33]. Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Methods* 65(1–2) (1983) 55–63. [PubMed: 6606682]
- [34]. Soni D, Bade AN, Gautam N, Herskovitz J, Ibrahim IM, Smith N, Wojtkiewicz MS, Dyavar Shetty BL, Alnouti Y, McMillan J, Gendelman HE, Edagwa BJ, Synthesis of a long acting nanoformulated emtricitabine ProTide, *Biomaterials* 222 (2019) 119441. [PubMed: 31472458]
- [35]. Hilaire JR, Bade AN, Sillman B, Gautam N, Herskovitz J, Dyavar Shetty BL, Wojtkiewicz MS, Szlachetka A, Lamberty BG, Sravanam S, Fox HS, Alnouti Y, Dash PK, McMillan JM, Edagwa BJ, Gendelman HE, Creation of a long-acting rilpivirine prodrug nanoformulation, *J Control Release* 311–312 (2019) 201–211.
- [36]. Kevadiya BD, Ottemann B, Mukadam IZ, Castellanos L, Sikora K, Hilaire JR, Machhi J, Herskovitz J, Soni D, Hasan M, Zhang W, Anandakumar S, Garrison J, McMillan J, Edagwa B, Mosley RL, Vachet RW, Gendelman HE, Rod-shape theranostic nanoparticles facilitate

antiretroviral drug biodistribution and activity in human immunodeficiency virus susceptible cells and tissues, *Theranostics* 10(2) (2020) 630–656. [PubMed: 31903142]

- [37]. Kadiu I, Gendelman HE, Human immunodeficiency virus type 1 endocytic trafficking through macrophage bridging conduits facilitates spread of infection, *J Neuroimmune Pharmacol* 6(4) (2011) 658–75. [PubMed: 21789505]
- [38]. Kadiu I, Nowacek A, McMillan J, Gendelman HE, Macrophage endocytic trafficking of antiretroviral nanoparticles, *Nanomedicine (Lond)* 6(6) (2011) 975–94. [PubMed: 21417829]
- [39]. Guo D, Zhang G, Wysocki TA, Wysocki BJ, Gelbard HA, Liu XM, McMillan JM, Gendelman HE, Endosomal trafficking of nanoformulated antiretroviral therapy facilitates drug particle carriage and HIV clearance, *J Virol* 88(17) (2014) 9504–13. [PubMed: 24920821]
- [40]. Gorantla S, Sneller H, Walters L, Sharp JG, Pirruccello SJ, West JT, Wood C, Dewhurst S, Gendelman HE, Poluektova L, Human immunodeficiency virus type 1 pathobiology studied in humanized BALB/c-Rag2^{-/-}γ^{-/-} mice, *J Virol* 81(6) (2007) 2700–12. [PubMed: 17182671]
- [41]. Siliciano JD, Kajdas J, Finzi D, Quinn TC, Chadwick K, Margolick JB, Kovacs C, Gange SJ, Siliciano RF, Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4⁺ T cells, *Nat Med* 9(6) (2003) 727–8. [PubMed: 12754504]
- [42]. Schnittman SM, Psallidopoulos MC, Lane HC, Thompson L, Baseler M, Massari F, Fox CH, Salzman NP, Fauci AS, The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4, *Science* 245(4915) (1989) 305–8. [PubMed: 2665081]
- [43]. Agosto LM, Herring MB, Mothes W, Henderson AJ, HIV-1-Infected CD4⁺ T Cells Facilitate Latent Infection of Resting CD4⁺ T Cells through Cell-Cell Contact, *Cell Rep* 24(8) (2018) 2088–2100. [PubMed: 30134170]
- [44]. Zhou Y, Fang Q, Niu B, Wu B, Zhao Y, Quan G, Pan X, Wu C, Comparative studies on amphotericin B nanosuspensions prepared by a high pressure homogenization method and an antisolvent precipitation method, *Colloids Surf B Biointerfaces* 172 (2018) 372–379. [PubMed: 30193196]
- [45]. Thorat AA, Dalvi SV, Liquid antisolvent precipitation and stabilization of nanoparticles of poorly water soluble drugs in aqueous suspensions: Recent developments and future perspective, *Chemical Engineering Journal* 181-182 (2012) 1–34.
- [46]. Reverchon E, Supercritical antisolvent precipitation of micro- and nano-particles, *The Journal of Supercritical Fluids* 15(1) (1999) 1–21.
- [47]. Chang X, Bao J, Shan G, Bao Y, Pan P, Crystallization-Driven Formation of Diversified Assemblies for Supramolecular Poly(lactic acid)s in Solution, *Crystal Growth & Design* 17(5) (2017) 2498–2506.
- [48]. Gao W, Lee D, Meng Z, Li T, Exploring intracellular fate of drug nanocrystals with crystal-integrated and environment-sensitive fluorophores, *J Control Release* 267 (2017) 214–222. [PubMed: 28844755]
- [49]. Dobretsov G, Syrejschikova T, Smolina N, On mechanisms of fluorescence quenching by water, *Biophysics* 59 (2014) 183–188.
- [50]. Zhang T, Jiang Y, Niu Y, Wang D, Peng Q, Shuai Z, Aggregation effects on the optical emission of 1,1,2,3,4,5-hexaphenylsilole (HPS): a QM/MM study, *J Phys Chem A* 118(39) (2014) 9094–104. [PubMed: 24921662]
- [51]. Campbell JH, Hearps AC, Martin GE, Williams KC, Crowe SM, The importance of monocytes and macrophages in HIV pathogenesis, treatment, and cure, *AIDS* 28(15) (2014) 2175–87. [PubMed: 25144219]
- [52]. Lameijer MA, Tang J, Nahrendorf M, Beelen RH, Mulder WJ, Monocytes and macrophages as nanomedicinal targets for improved diagnosis and treatment of disease, *Expert Rev Mol Diagn* 13(6) (2013) 567–80. [PubMed: 23895127]
- [53]. Batrakova EV, Gendelman HE, Kabanov AV, Cell-mediated drug delivery, *Expert Opin Drug Deliv* 8(4) (2011) 415–33. [PubMed: 21348773]
- [54]. Orenstein JM, Fox C, Wahl SM, Macrophages as a source of HIV during opportunistic infections, *Science* 276(5320) (1997) 1857–61. [PubMed: 9188531]

- [55]. MacParland SA, Tsoi KM, Ouyang B, Ma XZ, Manuel J, Fawaz A, Ostrowski MA, Alman BA, Zilman A, Chan WC, McGilvray ID, Phenotype Determines Nanoparticle Uptake by Human Macrophages from Liver and Blood, *ACS Nano* 11(3) (2017) 2428–2443. [PubMed: 28040885]
- [56]. Weissleder R, Nahrendorf M, Pittet MJ, Imaging macrophages with nanoparticles, *Nat Mater* 13(2) (2014) 125–38. [PubMed: 24452356]
- [57]. Cline MJ, Swett VC, The interaction of human monocytes and lymphocytes, *J Exp Med* 128(6) (1968) 1309–25. [PubMed: 5688078]
- [58]. Nowacek A, Kadiu I, McMillan J, Gendelman HE, Methods for isolation and identification of nanoparticle-containing subcellular compartments, *Methods Mol Biol* 991 (2013) 47–55. [PubMed: 23546658]
- [59]. Kadiu I, Ricardo-Dukelow M, Ciborowski P, Gendelman HE, Cytoskeletal protein transformation in HIV-1-infected macrophage giant cells, *J Immunol* 178(10) (2007) 6404–15. [PubMed: 17475870]
- [60]. Eugenin EA, Gaskill PJ, Berman JW, Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking, *Cell Immunol* 254(2) (2009) 142–8. [PubMed: 18835599]
- [61]. Dupont M, Souriant S, Lugo-Villarino G, Maridonneau-Parini I, Verollet C, Tunneling Nanotubes: Intimate Communication between Myeloid Cells, *Front Immunol* 9 (2018) 43. [PubMed: 29422895]
- [62]. Compeer EB, Kraus F, Ecker M, Redpath G, Amiezer M, Rother N, Nicovich PR, Kapoor-Kaushik N, Deng Q, Samson GPB, Yang Z, Lou J, Carnell M, Vartoukian H, Gaus K, Rossy J, A mobile endocytic network connects clathrin-independent receptor endocytosis to recycling and promotes T cell activation, *Nat Commun* 9(1) (2018) 1597. [PubMed: 29686427]
- [63]. Rusconi S, Marcotullio S, Cingolani A, Long-acting agents for HIV infection: biological aspects, role in treatment and prevention, and patient's perspective, *New Microbiol* 40(2) (2017) 75–79. [PubMed: 28368071]
- [64]. Kirtane AR, Langer R, Traverso G, Past, Present, and Future Drug Delivery Systems for Antiretrovirals, *J Pharm Sci* 105(12) (2016) 3471–3482. [PubMed: 27771050]
- [65]. Ma A, Chen DM, Chau FM, Saberi P, Improving adherence and clinical outcomes through an HIV pharmacist's interventions, *AIDS Care* 22(10) (2010) 1189–94. [PubMed: 20640958]
- [66]. Chun TW, Moir S, Fauci AS, HIV reservoirs as obstacles and opportunities for an HIV cure, *Nat Immunol* 16(6) (2015) 584–9. [PubMed: 25990814]
- [67]. Heo MB, Lim YT, Programmed nanoparticles for combined immunomodulation, antigen presentation and tracking of immunotherapeutic cells, *Biomaterials* 35(1) (2014) 590–600. [PubMed: 24125775]
- [68]. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, Weissleder R, Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells, *Nat Biotechnol* 18(4) (2000) 410–4. [PubMed: 10748521]
- [69]. Shi H, Kwok RTK, Liu J, Xing B, Tang BZ, Liu B, Real-Time Monitoring of Cell Apoptosis and Drug Screening Using Fluorescent Light-Up Probe with Aggregation-Induced Emission Characteristics, *Journal of the American Chemical Society* 134(43) (2012) 17972–17981. [PubMed: 23043485]
- [70]. La DD, Bhosale SV, Jones LA, Bhosale SV, Tetraphenylethylene-Based AIE-Active Probes for Sensing Applications, *ACS Appl Mater Interfaces* 10(15) (2018) 12189–12216. [PubMed: 29043778]
- [71]. Zhao Z, Lam JWY, Tang BZ, Tetraphenylethene: a versatile AIE building block for the construction of efficient luminescent materials for organic light-emitting diodes, *Journal of Materials Chemistry* 22(45) (2012) 23726–23740.
- [72]. Wang M, Zhang G, Zhang D, Zhu D, Tang BZ, Fluorescent bio/chemosensors based on silole and tetraphenylethene luminogens with aggregation-induced emission feature, *Journal of Materials Chemistry* 20(10) (2010) 1858–1867.
- [73]. Svehkarev D, Mohs AM, Organic Fluorescent Dye-based Nanomaterials: Advances in the Rational Design for Imaging and Sensing Applications, *Curr Med Chem* 26(21) (2019) 4042–4064. [PubMed: 29484973]

- [74]. Liu JL, Zhang JQ, Tang ZL, Zhuo Y, Chai YQ, Yuan R, Near-infrared aggregation-induced enhanced electrochemiluminescence from tetraphenylethylene nanocrystals: a new generation of ECL emitters, *Chem Sci* 10(16) (2019) 4497–4501. [PubMed: 31057778]
- [75]. Shi H, Kwok RT, Liu J, Xing B, Tang BZ, Liu B, Real-time monitoring of cell apoptosis and drug screening using fluorescent light-up probe with aggregation-induced emission characteristics, *J Am Chem Soc* 134(43) (2012) 17972–81. [PubMed: 23043485]
- [76]. Srivastava AK, Singh A, Mishra L, Tuning of Aggregation Enhanced Emission and Solid State Emission from 1,8-Naphthalimide Derivatives: Nanoaggregates, Spectra, and DFT Calculations, *J Phys Chem A* 120(26) (2016) 4490–504. [PubMed: 27294534]
- [77]. Chen J, Xu B, Ouyang X, Tang BZ, Cao Y, Aggregation-Induced Emission of cis,cis-1,2,3,4-Tetraphenylbutadiene from Restricted Intramolecular Rotation, *The Journal of Physical Chemistry A* 108(37) (2004) 7522–7526.
- [78]. Margolis DA, Gonzalez-Garcia J, Stellbrink HJ, Eron JJ, Yazdanpanah Y, Podzamczek D, Lutz T, Angel JB, Richmond GJ, Clotet B, Gutierrez F, Sloan L, Clair MS, Murray M, Ford SL, Mrus J, Patel P, Crauwels H, Griffith SK, Sutton KC, Dorey D, Smith KY, Williams PE, Spreen WR, Long-acting intramuscular cabotegravir and rilpivirine in adults with HIV-1 infection (LATTE-2): 96-week results of a randomised, open-label, phase 2b, non-inferiority trial, *Lancet* 390(10101) (2017) 1499–1510. [PubMed: 28750935]
- [79]. Cohen C, Wohl D, Arribas JR, Henry K, Van Lunzen J, Bloch M, Towner W, Wilkins E, Ebrahimi R, Porter D, White K, Walker I, Chuck S, De-Oertel S, Fralich T, Week 48 results from a randomized clinical trial of rilpivirine/emtricitabine/tenofovir disoproxil fumarate vs. efavirenz/emtricitabine/tenofovir disoproxil fumarate in treatment-naïve HIV-1-infected adults, *AIDS* 28(7) (2014) 989–97. [PubMed: 24508782]

Fig.1. RPV-NC synthesis and physical characterizations.

(A) Schematic representation of the Smart nanotechnology used to formulate RPV-NC by bottom-up and top-down approaches. (B) Low and (C) high magnification TEM images showing the morphology of RPV crystals made by anti-solvent precipitation. (D) TEM image of RPV-NC shows uniform surface morphology after homogenization. (E, F, G) XRD patterns of native RPV, RPV-NC before and after homogenization, respectively. (H) TGA analysis of native RPV and RPV-NC. (I) Hydrodynamic size distribution of RPV-NC is shown by DLS (average size ~ 320 nm). (J) Stability of RPV-NC was determined by size and PDI measurements over a three-week time period.

Fig. 2. Fluorescence characteristics of RPV-NC.

(A) Confocal and bright field images of RPV-NC merged to show fluorescence distribution. (B) Absorption spectra of RPV-NC in water/methanol ratios. (C) Corresponding fluorescence spectra of RPV-NC in water/methanol ratios. (D) Fluorescence quantum yield in water/methanol ratios. (E) RPV-NC in water/methanol ratios upon UV excitation. (F) Fluorescence (FL) intensity and concentration of RPV in various methanol/water ratios. (G) Fluorescence intensity of RPV-NC at different pH. (H) Photostability of RPV-NC.

Fig. 3. Single crystal x-ray RPV-NC crystallography.

(A) Light microscopy images of single crystals of RPV. (B) Polarized microscopy images of single crystals of RPV. (C) Schematic representation of single crystal x-ray crystallography setup. (D) Molecular conformation of RPV in RPV-NC showing the intermolecular CH- π interactions.

Fig. 4. RPV-NC macrophage targeting.

(A) MDM cell vitality was determined by the MTT assay. Data are expressed as percent of control absorbance. (B) Uptake of RPV-NC in MDM upon treatment with 100 μ M RPV-NC as determined by UPLC-MS. (C) AFM images of control and RPV-NC treated MDM. (D) TEM images of control and RPV-NC treated MDM showing internal morphology. Detailed evaluation of membrane-bound intracellular structures at areas of interest bordered in dotted red lines are presented in the magnified panels (ii) and (iii) and illustrate RPV-NC within membrane-bound intracellular structures. (E) Quantitative assessment of RPV-NC uptake by MDM via flow cytometry. MDM treated with RPV-NC (100 μ M) for 8 hours were stained with live-dead fluorophore and fixed. The percent of live MDM was determined by selecting the fluorophore dim (live) cells sub-gated from single-cells of the main forward-scatter and side-scatter density plot. The percent of RPV-NC and live double positive cells (bottom panel) were gated based on untreated, live-stained negative controls (top panel).

Fig. 5. RPV-NC detection in CD4⁺ T cells.

(A) CD4⁺ T cell vitality assessed by flow cytometry. The percent live population is based on gating for GFP_{dim} cells. (B) Uptake of RPV-NC in CD4⁺ T cells upon treatment with 100 μM RPV-NC for 8 hours as determined by UPLC. (C) Flow cytometric quantitation of RPV-NC uptake in T cells. CD4⁺ T cells were treated with RPV-NC (100 μM) for 2, 4, 6, 12 or 24 hours, then stained with live-dead indicating fluorophore and fixed. Gating for double-positive live, RPV-NC containing cells was performed versus untreated control based on gating strategy noted above (Fig. 4E, full gating shown in Supplemental Fig. S3). (D) MFI for RPV-NC positive populations at each time point. (E) Pearson correlation between MFI and drug concentration; $r = 0.8800$. (F) MFI and RPV levels in CD4⁺ T cells treated with increasing concentrations of RPV-NC. (G) TEM images of control and RPV-NC treated CD4⁺ T cells showing internal morphology. Detailed evaluation of membrane-bound intracellular structures at areas of interest bordered in dotted red lines are presented in the

magnified panels **(ii)** and **(iv)** and illustrate nanoparticles (indicated by arrows) within membrane-bound intracellular structures.

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Fig. 6. RPV-NC in macrophage-T cell co-cultures.

(A) Primary MDM and PBL from human blood elutriation were treated in isolation or co-culture with RPV-NC (100 μM) for 24 hours. Treated and untreated (negative control) cells were stained with antibody cocktail against CD45 (pan-leukocyte), CD3 (T cell), CD4, CD8, and CD14 (monocyte-macrophage) epitopes. Sub-gating from parent populations is depicted in Supplemental Fig. S4 and the percent of RPV-NC containing (bottom plots) CD14⁺ monocyte-macrophage and CD4⁺ T cell populations was determined against negative control (top plots). (B) The percent of each population containing RPV-NC is represented in heat map format for both CD14⁺ monocyte-macrophages and CD4⁺ T cells treated with a range of concentrations of RPV-NC (12.5–100 μM), either in isolation or co-culture. (C) Histogram showing the increasing MFI of RPV-NC positive monocyte-macrophage populations treated in isolation at various concentrations (12.5–100 μM). (D) Pearson

correlation analysis comparing MFI and RPV-NC treatment concentration from (C) yielded an r value of 0.9810.

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Fig. 7. Cellular and sub-cellular RPV-NC localizations.

(A) Representative images of macrophages treated with RPV-NC (pseudo-colored red) and Alexa-Fluor 488 secondary antibody against primary Rab5, Rab7 or Rab14 antibodies (green). Yellow areas (merged images) indicate co-localization of RPV-NC with endosomal compartments. Nuclei are stained with NucRed (pseudo-colored blue). (B) Cellular distribution of RPV-NC (blue) in infected macrophages. Virus particles were visualized by staining with Alexa-Fluor 594 secondary antibody against p24 primary (red). Cell cytoskeleton and tunneling nanotubes were visualized by staining for F-actin (green). (i) Control macrophages showing giant cell formation upon infection. (ii) and (iii) Infected macrophages showing the formation of tunneling nanotubes (indicated by the white arrows) and the presence of RPV-NC in the cell cytoplasm. (iv) Presence of RPV-NC in the tunneling nanotubes of infected macrophages (indicated by the arrows). Nuclei are stained

with NucRed (pseudo-colored white). (C) Presence of RPV-NC (pseudo-colored red) in CD4⁺ T cells. Nuclei are stained with NucRed (pseudo-colored blue).

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Fig. 8. PK and RPV tissue-cell biodistribution.

(A) Schematic representation of the in vivo studies of RPV-NC. (B) RPV concentrations from (i) rat plasma at 1, 2- and 5-days post-injection (ii) rat liver, spleen and lymph node tissue on day 5 post-injection (iii) PBL mouse tissue on day 10 post-injection. Statistical differences were determined using two-way ANOVA among groups; Bonferroni's post-hoc test was used for multiple comparisons. *p < 0.05; **p < 0.01; ***p < 0.001. (C) Representative images of rat spleen tissue sections stained with Iba-1 for activated macrophages (green). Yellow areas indicate co-localization of RPV-NC (pseudo-colored red) in rat splenic macrophages. Representative images of PBL mouse (D) spleen and (E) lymph node tissue sections, stained with anti-human CD4⁺ antibody (green). Yellow areas indicate co-localization of RPV-NC (pseudo-colored red) in human CD4⁺ cells. (F) Magnification of the areas of co-localization from (E). Nuclei are stained with NucRed (pseudo-colored blue).

Fig. 9. Nanoparticle tracking and quantification in humanized mice.

(A) Representative flow cytometry plots showing RPV-NC positive populations in spleen CD4⁺ T cells and CD14⁺ macrophages based on gating relative to splenocytes and hepatocytes from untreated control mice (see Supplemental Fig. S9 for full gating description). (B) Percent populations positive for RPV-NC from the liver. (C) RPV concentrations in hepatic T cells and macrophages. Pearson correlation between RPV-NC percent positive population and RPV drug levels in liver (D) T cells; $r = 0.81$ and (E) macrophages; $r = 0.93$ (F) Percent populations positive for RPV-NC from the spleen. (G) RPV concentrations in splenic T cells and macrophages. Pearson correlation between RPV-NC percent positive population and RPV drug levels in splenic (H) T cells; $r = 0.72$ and (I) macrophages; $r = 0.97$. Pearson correlation analysis for RPV concentration and percent cells positive for RPV-NC provided an r value of 0.72 for the CD4⁺ T cells and 0.99 for the monocyte- macrophages as shown in Fig. 9H and I. Hence, presence of RPV-NC in human monocyte-macrophages and T cells was observed by flow cytometry and substantiated with RPV drug levels from UPLC-MS/MS. The correlation values obtained confirm the presence of RPV-NC in the form of intact nanoparticles in immune cell subsets. These data, taken together, confirm that the AIE property of RPV can be used to track the presence and integrity of RPV-NC in immune cell populations.

Table 1.

Methods of analysis of RPV AIE in cells and in rodent animal models. The table names the tests used to affirm RPV AIE. TEM, UPLC-MS/MS, flow cytometry and confocal microscopy tests were used for drug tracking using fluorescence emission tests.

Models		Dose	Methods of Analysis			
			TEM	UPLC-MS/MS	Flow cytometry	Confocal microscopy
<i>In vitro</i>	Macrophages	100µM	+	+	+	+
	T cells		+	+	+	+
<i>In vivo</i>	Sprague-Dawley rats	45mg/kg	-	+	-	+
	PBL mice		-	+	-	+
	Humanized mice		-	+	+	-