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The mixed lineage kinase-3 inhibitor URM-099 improves therapeutic outcomes for long-acting antiretroviral therapy.

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The mixed lineage kinase-3 inhibitor URM-099 improves therapeutic outcomes for long-acting antiretroviral therapy

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Abstract

During studies to extend the half-life of crystalline nanoformulated antiretroviral therapy (nanoART) the mixed lineage kinase-3 inhibitor URM-099, developed as an adjunctive neuroprotective agent was shown to facilitate antiviral responses. Long-acting ritonavir-boosted atazanavir (nanoATV/r) nanoformulations co-administered with URM-099 reduced viral load and the numbers of HIV-1 infected CD4+ T-cells in lymphoid tissues more than either drug alone in infected humanized NOD/SCID/IL2R γ –/– mice. The drug effects were associated with sustained ART depots. Proteomics analyses demonstrated that the antiretroviral responses were linked to affected phagolysosomal storage pathways leading to sequestration of nanoATV/r in Rab-associated recycling and late endosomes; sites associated with viral maturation. URM-099 administered with nanoATV induced a dose-dependent reduction in HIV-1p24 and reverse transcriptase activity. This drug combination offers a unique chemical marriage for cell-based viral clearance.

From the Clinical Editor: Although successful in combating HIV-1 infection, the next improvement in antiretroviral therapy (nanoART) would be to devise long acting therapy, such as intra-cellular depots. In this report, the authors described the use of nanoformulated antiretroviral therapy given together with the mixed lineage kinase-3 inhibitor URM-099, and showed that this combination not only prolonged drug half-life, but also had better efficacy. The findings are hoped to be translated into the clinical setting in the future.

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Key words: HIV-1; Long-acting nanoformulations; URM-099; Humanized mice; Phagolysosome; Rab proteins

Long-acting nanoformulated antiretroviral therapy (nanoART) offers advantages for the management of human immunodeficiency virus type one (HIV-1) infection.¹ Long-lived intracellular drug

depots, at or adjacent to the viral life cycle, can improve both regimen adherence and antiretroviral responses.² NanoART could affect chemical viral eradication.^{3,4} The accumulation of nanoART

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Competing interests: H.A.G. and H.E.G. are members of the Scientific Advisory Board of WavoDyne Therapeutics, Inc. that has exclusive rights to URM-099 development.

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particles in late and recycling mononuclear phagocyte (MP: monocytes, macrophages and dendritic cells) endosomes^{1,5–7} can limit hepatic ART metabolism and renal secretion.^{8–10} Metabolism of HIV-1 protease inhibitors by hepatic cytochrome P450 3A (CYP3A)^{11,12} may also be slowed by drug intracellular carriage. However, the limitations of nanoART reside in the effectiveness of the cells' depot storage capacity. We posit that the longer the storage the longer the drug half-life. As preventative vaccines for HIV have thus far proven ineffective, extending the time intervals for ART administration could serve as effective pre-exposure prophylaxis measures and as surrogate vaccination measures. Moreover, drug patient adherence to complex regimens may also be improved as the effectiveness of ART has been plagued by limitations in taking the drugs at needed time intervals. Novel platforms designed to achieve such goals represent “cutting edge” bench to bedside research pursuits.

To such ends we now report a novel means to perpetuate the ART depot in subcellular organelles. The discovery was made during the evaluation of the mixed lineage kinase-3 (MLK3) inhibitor URM-099 as an adjunctive neuroprotective HIV/AIDS therapy.^{13,14} Here we have shown potentiation of nanoART effected antiretroviral responses. HIV-1 infected, nanoART- and URM-099-treated humanized NOD/SCID/IL2R γ ^{-/-} (NSG) mice showed reductions in viral load (VL) and decreased numbers of infected lymphocytes in spleen and lymph nodes with restoration of CD4+ T-cells in peripheral blood. In HIV-1 infected human monocyte-derived macrophages (MDM), URM-099 increased nanoART retention in recycling and late Rab protein-associated endosomes. URM-099 facilitated nanoART actions by reducing residual HIV-1 in infected NSG mice. Reduction of virus to undetectable levels beyond what either drug can achieve alone was observed. Notably, virus-infected lymphocytes were not observed in such treated and infected animals. The establishment of an extended ART depot in monocyte-macrophages is a significant step forward in development of long-acting ART strategies for HIV/AIDS. Moreover, combination of URM-099 and nanoART can extend ART half-life and improve treatment outcomes for those infected while also serving to protect those at risk for infection.

Methods

Ethics statement

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (UNMC). Animal studies were performed in compliance with UNMC institutional policies and the National Institutes of Health (NIH) guidelines for housing and care of laboratory animals. Human tissues were obtained from the University of Washington, supported by NIH award 5R24HD000836. These studies were approved by the UNMC Institutional Review Board (IRB), in accordance with Health and Human Services Regulations, for the protection of human subjects. All fetal liver tissues were anonymized without patient information or identifiers when obtained. Human monocytes were isolated by leukopheresis from HIV-1/2 and hepatitis seronegative donors according to an approved UNMC IRB exempt protocol.

Study design

The MLK3 inhibitor URM-099 was tested for its abilities to affect nanoART antiretroviral activities. *First*, URM-099 was administered to an HIV-1-infected humanized mouse model treated with folic acid decorated-nanoART (FA-nanoART) and antiretroviral drug concentrations measured in plasma and tissues. *Second*, putative mechanisms of URM-099 action against HIV-1 infection were examined by SWATH proteomics and Western blot. *Third*, HIV-1 integrated viral DNA, RNA, HIV-1p24 protein and HIV-reverse transcriptase (RT) activities were measured. *Fourth*, URM-099-induced nanoART retention in cells and cellular compartments was determined by endosomal immunoaffinity isolation, HPLC drug analysis, mathematical simulation and confocal microscopy.

Preparation of nanoformulated ATV/r

Folic acid(FA)-conjugated-P407 was synthesized as previously described.^{15,16} FA-targeted and non-targeted nanoformulations of atazanavir (ATV) and ritonavir (r, RTV) were prepared as described in the [Supplementary materials](#). Fluorescently-labeled nanoART was made with CF568-modified P407 or CF633-modified P188.¹⁵

Viral infection and treatment of CD34+ NSG mice

NSG mice were reconstituted with human fetal hematopoietic CD34+ stem cells as previously described.⁶ After 22–26 weeks, mice were infected intraperitoneally (IP) with HIV-1_{ADA} at 10⁴ TCID₅₀/mouse. Ten weeks after infection, mice were given URM-099 (10 mg/kg, IP) daily for three weeks with weekly intramuscular (IM) injections of 100 mg/kg FA-modified nanoATV/r (FA-nanoATV/r) (1:1). One week after the third FA-nanoATV/r injection spleen, brain, liver and plasma were collected for drug levels and antiviral activity determination.¹⁷

Immunohistochemistry

Immunohistochemistry on spleen and lymph nodes was performed.⁶ Tissues were stained for HLA-DR, CD45 and HIV-1p24. Spleen sections were double stained with antibodies to human CD3, CD68, Rab7 and HIV-1p24. Immunofluorescence staining was captured using an LSM 510 confocal microscope (Carl Zeiss Microimaging Inc., Dublin, CA) or by Nuance microscopy.⁶ Replicate tissues were collected from BALB/c mice 12 hours-post IP injection with 100 mg/kg CF633-labeled nanoATV and flash-frozen in Tissue-Tek O.C.T. Cryosections were stained with antibodies to F4/80 (Abcam, Cambridge, MA) followed by a Alexa Fluor 488 conjugated secondary antibody.

Monocyte-macrophage cultures

Human MDM cultures were established as previously described.¹⁸

SWATH-MS proteomics and data analyses

Human MDM were infected with HIV-1_{ADA} for 4 hours, treated with/without 100 μ M nanoATV and 10 ng/mL URM-099 for 16 hours. Cells were harvested and proteins digested.¹⁹ Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic resource 6.7 was used for high-stringency functional

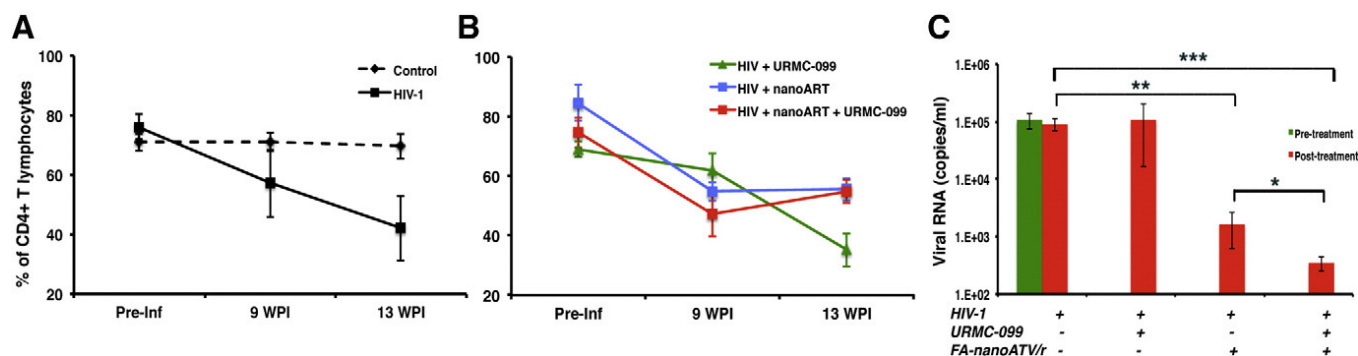


Figure 1. URM-099 potentiates FA-nanoATV/r antiretroviral activities in humanized NSG mice. Humanized NSG mice infected with HIV-1_{ADA} for 10 weeks were administered weekly intramuscular injections of 100 mg/kg FA-nanoATV/r with or without daily IP injections of 10 mg/kg URM-099. All nanoparticles used were coated with FA to facilitate macrophage depot formation. After three weeks, antiviral activity was determined. (A) Human CD4+ T cells in peripheral blood during infection in untreated (n = 5) and HIV-1 infected (n = 5) mice. (B) CD4+ T cells in mice treated with FA-nanoATV/r with (n = 7)/without URM-099 (n = 5) or URM-099 only (n = 5) are presented before and after treatment. Preserved CD4+ T cell counts in nanoART alone and dual treatment groups were found. The study was repeated 2 times. (C) Plasma viral load measures are illustrated as average \pm SEM. *, **, *** $P < 0.05$.

annotation clustering for protein identification.²⁰ The Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper Search & Color Pathway tool was used to visualize the endocytosis pathway with proteomics data overlay.^{21,22}

NanoATV and URM-099 treatment

MDM were treated with 100 μ M native ATV or nanoATV with or without 0.1–100 ng/ml of URM-099. After 16 hours, MDM were infected with HIV-1_{ADA} at an MOI of 0.1 for 4 hours on day 0, 5 or 10 after drug loading. Cells were cultured for an additional 2 weeks in the absence or presence of URM-099.

Western blots

Western blots on MDM were performed as described²³ and membranes were probed with Rab5, Rab7, Rab11, LAMP1 or STAT1 antibodies.

HIV-1 measurements

MDM were treated with nanoATV and URM-099, infected on day 0 with HIV-1_{ADA} and cultured for an additional 14 days. Genomic DNA from MDM was extracted using the Puregene Core Kit. Integrated HIV-1 DNA was assessed by Alu-gag real time PCR using a modified protocol.²⁴ The HIV standards were generated DNA H3B, ACH-2 and 8E5 cell lines.²⁵ In replicate wells, RNA from MDM was isolated using RNeasy mini kit (Qiagen). HIV-1 gag RNA was assessed by real-time RT-PCR.²⁶ Culture media were collected until 14 days from HIV-1-infected MDM for RT activity measures as previously described.⁹ HIV-1 p24 staining was performed as described.

MDM nanoATV uptake and retention and endocytic trafficking

Uptake and retention of nanoATV in MDM were studied.²⁷ MDM cultured on LabTek CC₂ chamber slides were treated with 100 μ M CF-568-labeled nanoATV with or without 10 ng/mL URM-099 for 16 hours. Following HIV-1_{ADA} infection at an MOI of 0.1 for 4 hours, the infected MDM were cultured for an additional 14 days with 0 or 10 ng/ml URM-099. MDM not infected with HIV-1_{ADA} were cultured for 12, 24, 48 or 144 hours after nanoART

loading with 0 or 10 ng/ml URM-099. All cells (infected and non-infected) were fixed with ice-cold 4% PFA for 30 min, treated with permeabilizing/blocking solution (0.1% Triton, 5% BSA in PBS) then incubated with rabbit anti-human Rab7 or 11 or mouse anti-human HIV-1p24. Alexa Fluor 488 goat anti-rabbit secondary antibody detected Rab7 and 11 antibodies and Alexa Fluor 633 goat anti-mouse secondary antibody was used for HIV-1p24. Slides were covered with ProLong Gold anti-fade reagent with DAPI and imaged on an LSM 510 confocal microscope (Carl Zeiss Microimaging Inc.). Double or triple co-localization images were analyzed using Zeiss LSM 510 Image browser AIM software version 4.2 and Image J (NIH, Bethesda, MD) software with BlobProb plug-in for percent overlap.⁸

Statistical analyses

The sample size was determined according to published guidelines with a minimum of 5 animals per group (n = 5). All experiments were replicated three times and results were blinded until statistical analyses. No outliers from animal or cell experiments were excluded. Data comparisons were analyzed using Prism (GraphPad Software Inc, La Jolla, CA). One-way ANOVA was used to compare three or more samples followed by unpaired, two-tailed Student's *t* test. The Student's *t* test was also used to compare two samples. All significant differences were determined at $P < 0.05$.

Results

URM-099 potentiates nanoART activities in humanized CD34+ NSG mice

Chronic immune activation potentiates HIV-1 infection and CD4+ T-cell loss.^{28–30} Notably, immune deactivation reduces pro-inflammatory activities and end-organ tissue injury.^{31–33} Recently, URM-099, a MLK3 inhibitor with excellent end-organ pharmacokinetic profiles became available to our laboratory based on its effectiveness as an anti-inflammatory neuroprotective agent.^{14,34} We reasoned that it could potentiate nanoART as inflammation regulates Rab endosomal networks and extend drug depot sites.⁸ Thus, we reasoned that URM-099

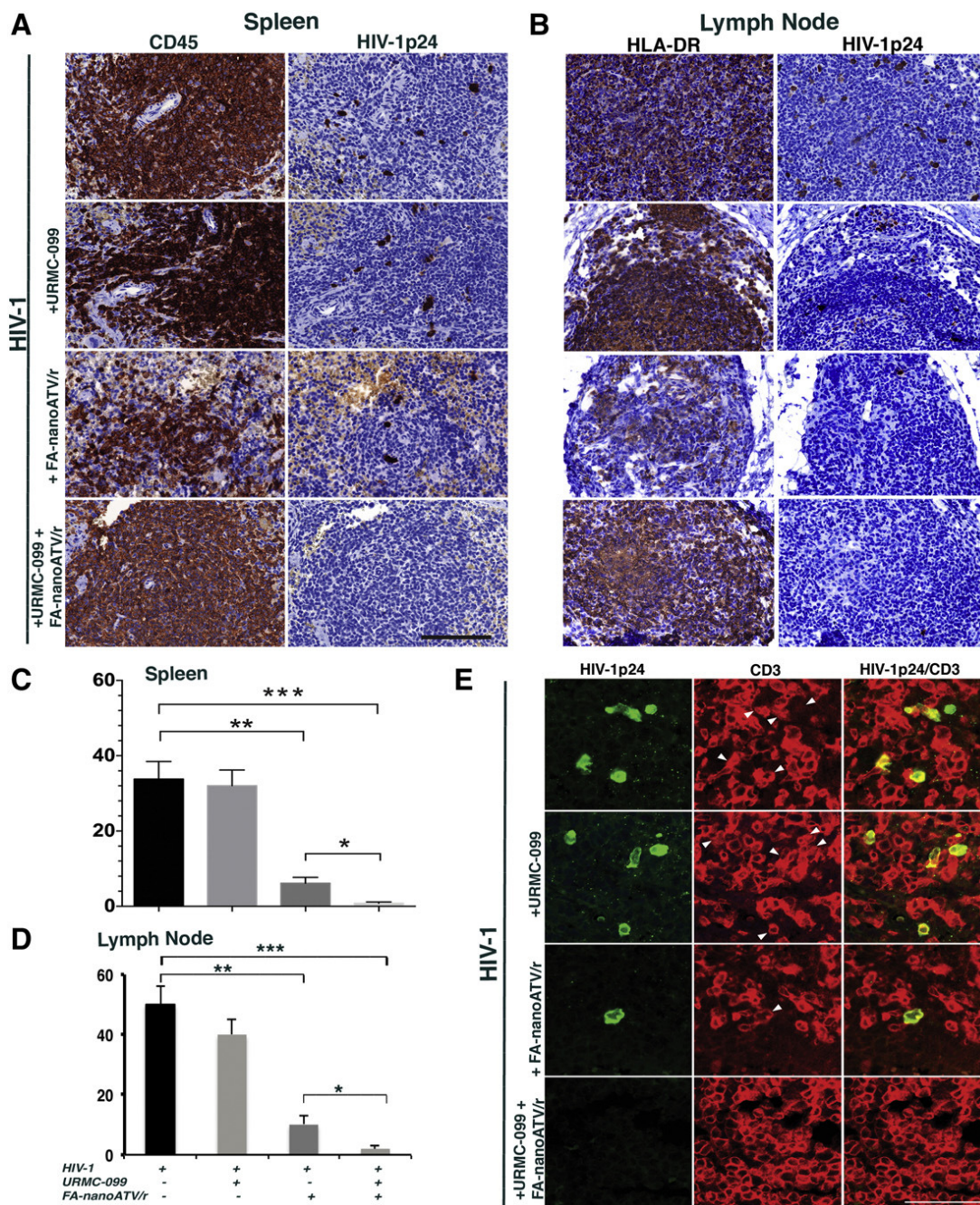


Figure 2. Histopathological evaluation of spleen and lymph nodes from humanized mice. (A) Rare HIV-1p24+ cells are seen with FA-nanoATV/r. These were eliminated by the addition of URMC-099. Representative images of human CD45+ and HIV-1p24+ staining (brown) are shown, 40 \times magnification. Scale bar = 100 μ m. (B) Lymph nodes showed rare HIV-1p24+ cells in FA-nanoATV/r treated mice, which were eliminated in with URMC-099 co-treatment. (C) Number of HIV-1p24+ cells/1000 human CD45+ cells in spleen and (D) total number of HIV-1p24+ cells in the lymph nodes are shown from five to seven animals/group (average \pm SEM). *, **, *** $P < 0.05$. (E) Immunofluorescence double staining for human CD3+ (red) and HIV-1p24+ (green) cells in spleen demonstrated that HIV-1p24 infected cells are T lymphocytes. URMC-099 co-treatment with FA-nanoATV/r reduces residual lymphocyte infection in spleen. White arrows point to T lymphocytes that are HIV-1p24+. 100 \times magnification. Scale bar equals 50 μ m.

would extend drug nanoparticle activities.^{35,36} To test this idea human CD34+ hematopoietic-reconstituted NSG mice were infected with HIV-1_{ADA} at a tissue culture infective dose of 0.1 infectious virions/ml for nine weeks. Infected mice were divided into untreated controls, URMC-099 treated, FA-nanoATV/r treated or both. To facilitate particle entry into cells, FA was used as the targeting ligand for nanoATV/r.^{15,16} FA-nanoATV/r

was administered once a week with daily URMC-099 for three weeks. FACS tests of peripheral blood over 13-weeks demonstrated significant gradual depletion of CD4+ T-cells in HIV-1 infected mice when compared to uninfected controls (Figure 1, A). In HIV-1 infected mice, CD4+ T-lymphocytes were restored in the FA-nanoATV/r and URMC-099 co-administered group when compared to URMC-099 only treated mice (Figure 1, B). Before

Table 1
Endolysosomal proteins in HIV-1 or nanoATV + 099 treated MDM.

	Protein	HIV-1		HIV+nanoATV+099	
		Ztest	Regulation	Z test	Regulation
Early endosomes	Rab5	4.22	↑	--	↔
	TAP	2.39	↑	2.11	↑
	TfR	3.91	↑	--	↔
	vATPase	4.48	↑	--	↔
	Stx13	--	↔	--	↔
	EEA1	--	↔	-3.27	↓
	VPS34	--	↔	--	↔
	CALR	--	↔	--	↔
	Calnexin	--	↔	--	↔
Late endosomes	Rab7	2.12	↑	2.89	↑
	vATPase	4.48	↑	--	↔
	Dynein	4.49	↑	5.27	↑
	TUBB	--	↔	-3.38	↓
	TUBA	--	↔	--	↔
	RILP	--	↔	--	↔
	Stx7	--	↔	--	↔
Lysosomes	LAMP	--	↔	--	↔
	vATPase	4.48	↑	--	↔
	TAP	2.39	↑	2.11	↑
	M6PR	2.00	↓	--	↔
	Cathepsin	-2.52	↓	3.17	↑

↓ Downregulated ↔ Unchanged ↑ Upregulated

starting treatment regimens, the median plasma VL in infected mice was 10^5 copies/ml. Mice treated with FA-nanoATV/r had a mean plasma VL of 1.6×10^3 copies/ml. Mice treated with URMC-099 and FA-nanoATV/r had mean VL of 284 copies/ml, a 5.6-fold reduction over FA-nanoATV/r treatment alone ($P < 0.01$, Figure 1, C). Treatment with URMC-099 alone did not affect the VL compared to infected controls (NS, $P = 0.2$). Immunohistochemical staining for HIV-1p24 in spleen confirmed the VL tests (Figure 2, A). Untreated, infected animals had 40 HIV-1p24+ cells/1000 CD45+ cells in spleen. After three weeks of FA-nanoATV/r treatment, HIV-1p24+ cells were reduced to 4 HIV-1p24+ cells/1000 CD45+ cells ($P < 0.01$; Figure 2, A and C). Treatment with URMC-099 and FA-nanoATV/r further reduced this number to < 0.1 HIV-1p24/1000 CD45+ cells ($P < 0.001$; Figure 2, A and C). Double immunofluorescence staining for human CD3 and HIV-1p24 showed that the decreases were in numbers of infected T-cells (Figure 2, E). Pharmacokinetic analyses showed increases in RTV from 411 to 1053 ng/g and 1471 to 3238 ng/g in spleen and liver ($P < 0.05$) co-administered with URMC-099 (Supplementary Figure 1, A and B). ATV levels were also increased from 215 to 326 ng/g in spleen, but the differences did not reach statistical significance ($P = 0.065$) (Supplementary Figure 1, C). No differences in liver ATV levels were seen (Supplementary Figure 1, D).

URMC-099 facilitates nanoATV/r antiretroviral activities in lymph nodes

Elimination of HIV-1p24+ cells in lymph nodes, by non-targeted nanoATV/r, was not achieved in humanized NSG mice.⁶ In this study, HIV-1p24+ HLA-DR+ cells in lymph nodes were reduced in URMC-099 and FA-nanoATV/r treated mice. Untreated, infected animals had 50.3 HIV-1p24 cells/1000 CD45+ cells in lymph nodes.

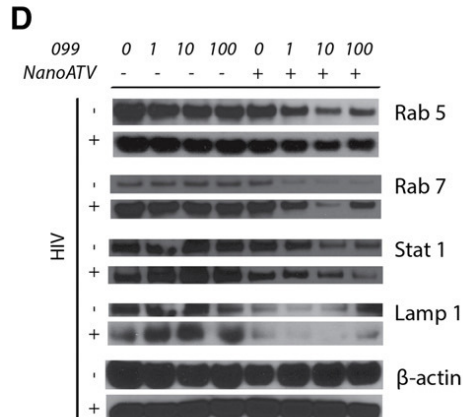
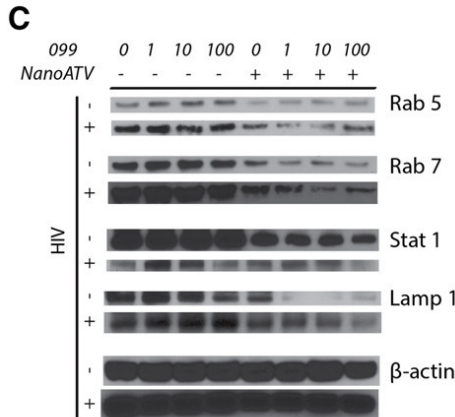
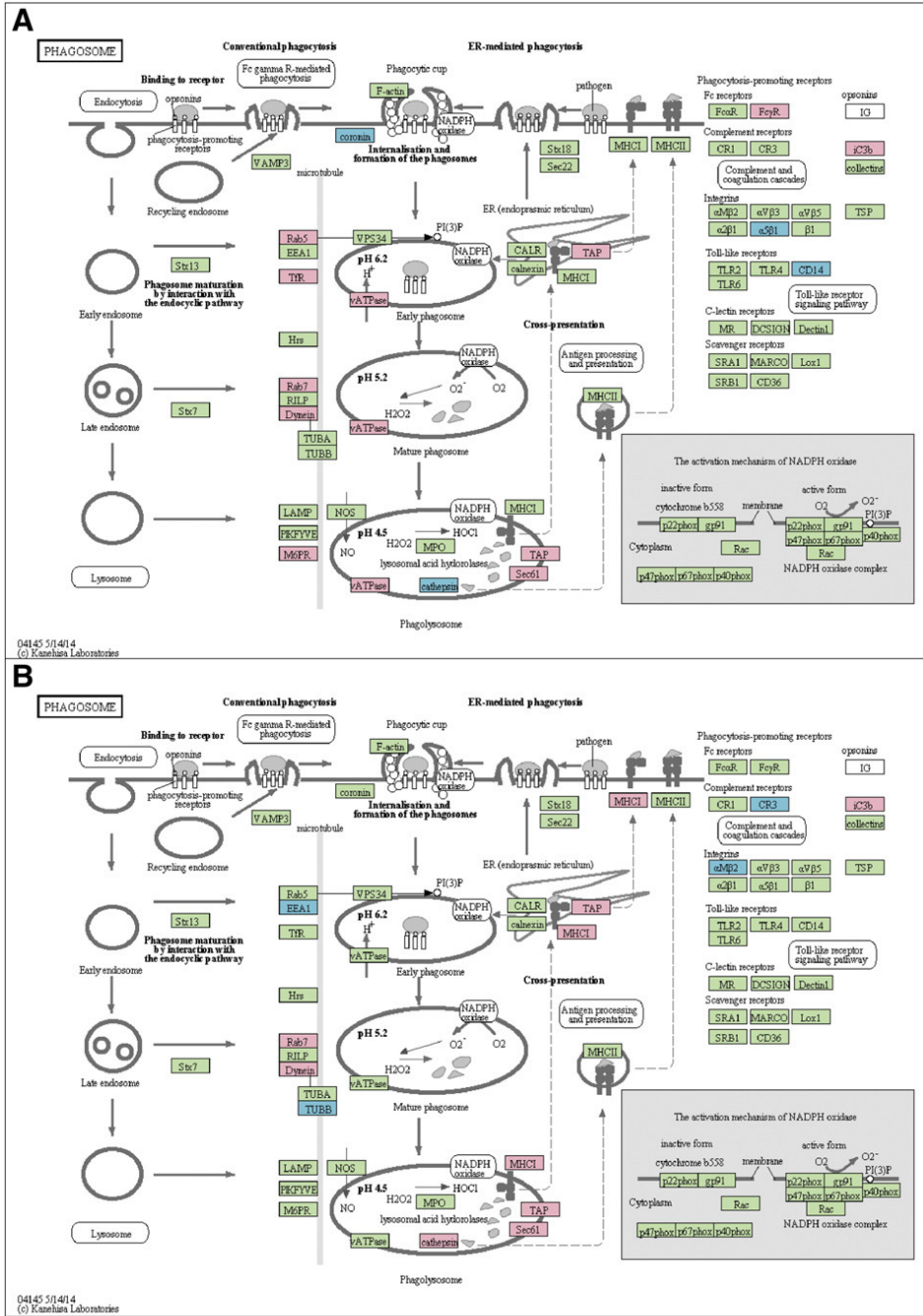
Three weeks of FA-nanoATV/r treatment significantly reduced the number of HIV-1p24+ cells to 10.8 HIV-1p24 cells/1000 HLA-DR+ cells ($P < 0.005$; Figure 2, B and D), but treatment with URMC-099 and FA-nanoATV/r further reduced this number to < 2.3 HIV-1p24/1000 HLA-DR+ cells ($P < 0.001$; Figure 2, B and D).

Proteomic analyses of virus-infected MDM

We theorized that the locus of URMC-099's interactions with nanoATV could be the site of virion assembly based on ATV's action. Thus, we focused our investigation on the effects of HIV-1 infection on the host cell proteome,^{37–41} particularly in cellular organelles where the virus is assembled in macrophages.^{10,42} As nanoATV/r accumulates in macrophages the gradual elimination of the viral T cell reservoir is linked to drug release from its intracellular depots. To assess the effects of nanoATV/r and URMC-099 treatments on viral replication in macrophages, proteomic tests of whole cell lysates were performed using Sequential Windowed data independent Acquisition of the Total High-resolution Mass Spectra (SWATH-MS). Quantitative profiling of HIV-1 infected human MDM demonstrated 181 up-regulated and 162 down-regulated protein species ($P < 0.05$) in four replicate samples evaluated by the paired-samples Z-test as compared to uninfected cells (Supplementary Figure 3, A and B). DAVID and KEGG bioinformatics tools enabled functional enrichment and pathway analyses on a dataset of 438 proteins. The effect of HIV-1 infection, URMC-099 and nanoATV is summarized in Table 1 and Figure 3, A and B. Few numbers of proteins were altered as a consequence of URMC-099 or nanoATV treatment in uninfected MDM. Rab5 and Rab7, specifically, were upregulated by viral infection. This result was in agreement with our prior findings.^{8,43–45} Proteins deregulated by URMC-099 and nanoATV included Rab5, TfR, vATPase and FcγR. Opposing regulation of phagolysosomal and endosomal proteins between HIV-1 infection and those virus-infected treated with nanoATV with and without URMC-099 were found. To determine if URMC-099 could alter the expression of Rab proteins beyond what was seen by nanoATV, we examined the effects of URMC-099 and nanoATV in HIV-1-infected or uninfected MDM. Quantitative Western blot analyses of cell lysates showed increases in Rab5, Rab7 and LAMP1 expressions in infected macrophages treated with increasing concentrations of URMC-099 alone compared with uninfected macrophages (Figure 3, C and D). Importantly, co-treatment of uninfected and virus-infected MDM with nanoATV and URMC-099 led to reductions in Rab5, Rab7, STAT1 and LAMP1 expressions. These reductions were more significant in HIV-1 infected cells for Rab7, STAT1 and LAMP1. In the combined treatment groups, with the increasing URMC-099 concentrations, the greatest decreases were in Rab7, STAT1 and LAMP1.

Localization and regulation of Rab proteins in macrophages containing nanoATV/r

To confirm these proteomic results for nanoART-macrophage interactions, we determined the cellular distribution and subcellular localization of nanoATV/r in vivo by injecting CF633-labeled nanoATV/r into mice. Splenic and hepatic tissues were recovered after animal sacrifice then stained with F4/80 antibodies to identify macrophages. CF633 dye co-localized in macrophages (Figure 4, A)



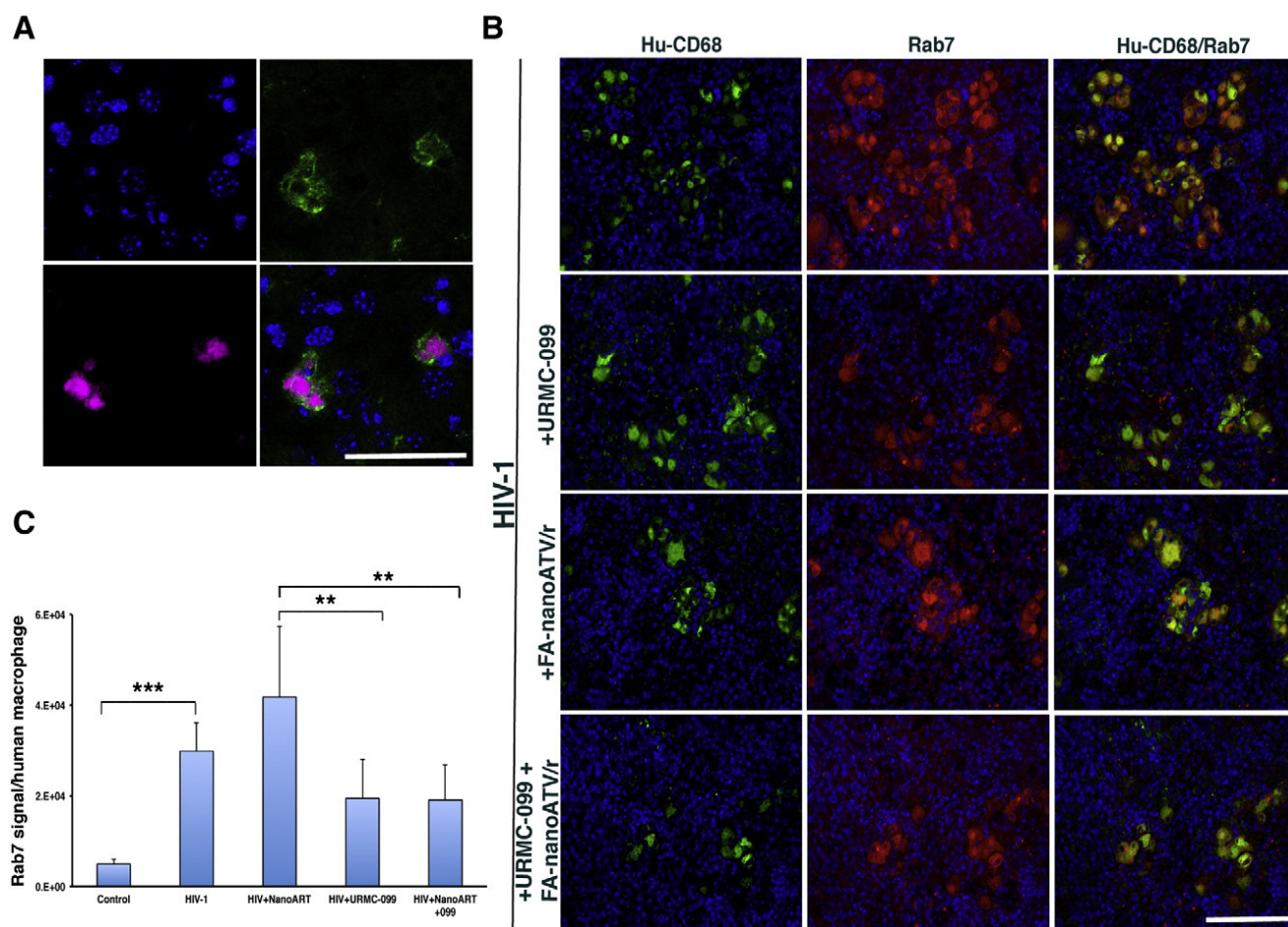


Figure 4. Nanoparticle uptake and characterization of Rab7 expression in human macrophages with HIV-1 infection. (A) Liver showed the presence of CF633-labeled nanoATV (red) in mouse macrophages (F4/80-green), demonstrating clear uptake and co-localization of nanoATV in tissue resident macrophages. Results were confirmed using 5 mice. (B) Immunofluorescence double staining of human macrophages (CD68-green) and human Rab7 (red) in spleens of humanized mice showed an increase in the expression of co-localized Rab7 in HIV-1 infected or FA-nanoATV/r ($n = 5$) treated groups compared to uninfected controls, infected mice treated with URMC-099 alone ($n = 5$) or double treatment ($n = 7$) and (C) significant increase in expression in HIV-1 infected group as compared to controls. *, **, *** $P < 0.05$. Scale bar equals 100 μm (Figure 4, A), 50 μm (Figure 4, B).

but not CD3 positive T cells in the reticuloendothelial system. In HIV-1 infected humanized NSG mice, Rab7 expression was increased when compared to uninfected controls ($P = 0.0003$; Figure 4, B and C). A decrease of Rab7 expression, by 50%, was observed within human macrophages in infected NSG mice treated with URMC-099 or with nanoATV/r and URMC-099 compared to mice treated with nanoART/r alone ($P < 0.01$ and 0.001, respectively, Figure 4, B). Immunofluorescence quantitation showed that Rab7 levels were similar in murine macrophages likely as a result of macrophage viral infection in human cells (Supplementary Figure 2, A and B).

URMC-099 potentiates nanoATV antiretroviral activities

Human MDM endosomes harbor both progeny virus and ART nanoparticles.⁸ To assess how these drugs could influence viral replication we treated MDM with nanoATV and/or URMC-099 prior to viral exposure. After 14 days of HIV-1_{ADA} infection administered at a multiplicity of infection (MOI) of 0.1, integrated HIV-1 DNA was measured by Alu-long terminal repeat (LTR)-based real-time nested PCR. URMC-099 had no effect on HIV-1 DNA in infected MDM. Administration of nanoATV reduced HIV-1 integrated DNA (74 copies per 1000 cells with nanoATV compared to 11,471 copies/1000

Figure 3. Schematic representation of the MDM phagosome network. MDM phagosome network identified in (A) HIV-1-infected and (B) HIV-1-infected with URMC-099 and nanoATV treated cells. Proteins identified were compared against uninfected MDM cultures ($P < 0.05$). The acquired profiles were analyzed through the bioinformatics tools DAVID and KEGG pathway. Statistical significance was determined using a P -value < 0.05 . Proteins in red and blue, display up- and down-regulation, respectively. Proteins in green belong to the phagosome network and not deregulated by ATV treatment. URMC-099 deregulates phagosomal protein expressions in human MDM. The proteomics data were based on experiments conducted from 4 donor MDMs. MDM were treated with 100 μM nanoATV for 16 hours with 0, 1, 10 or 100 ng/mL URMC-099. HIV-1_{ADA} infection was performed at (C) day 0 or (D) day 5 after drug treatment. Cell lysates were collected on day 7 following infection. Expression of Rab5, Rab7, STAT1, and LAMP1 were determined by Western blot in URMC-099/nanoATV treated MDM. Results are from a representative experiment. Western blots were performed in duplicates from 2 donor MDMs.

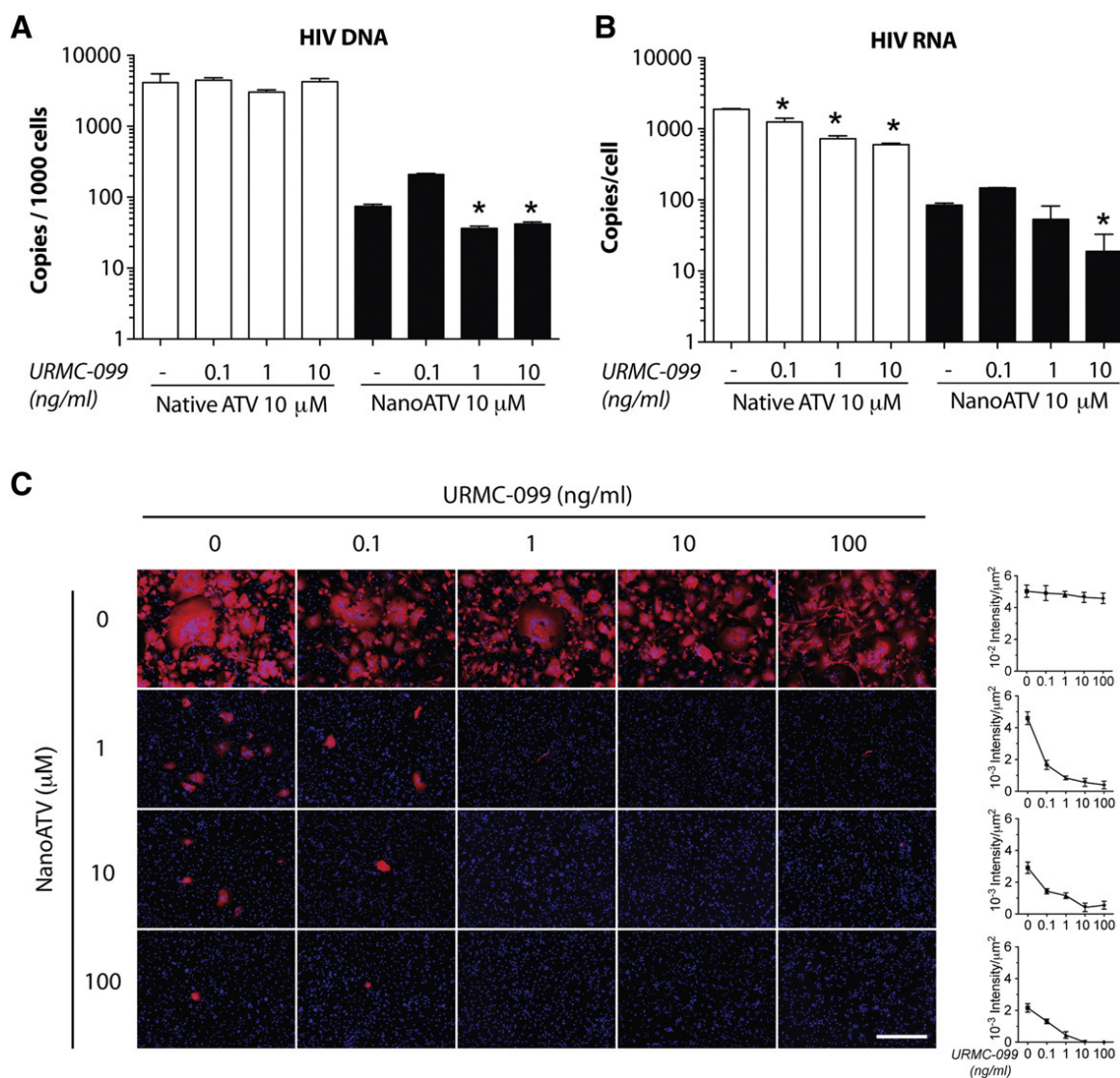


Figure 5. URM-099 potentiates nanoATV antiretroviral activities in HIV-1 infected MDM. URM-099 and native ATV or nanoATV treated human MDM were infected with HIV-1_{ADA} and cultured for 14 days. (A) HIV-1 LTR DNA was measured by Alu-gag PCR amplification (n = 4). (B) HIV-1 RNA was measured by HIV-1 gag amplification (n = 4); * $P < 0.05$ compared to ATV treatment without URM-099. (C) Immunocytochemical staining of HIV-1p24 expression (red). Nuclei are stained with DAPI (blue). Immunofluorescence was quantified and is expressed as mean \pm SEM. 100 \times Magnification. Scale bar – 100 μ m.

cells without nanoATV). Further, suppression to 33 copies per 1000 cells was observed when URM-099 and nanoATV were co-administered (Figure 5, A). Reductions in RNA levels to 19 copies of HIV-1p24 gag RNA per MDM were seen, 3-fold less than with nanoATV alone ($P < 0.05$; Figure 5, B). URM-099 also significantly boosted antiretroviral effects of native ATV, although to a markedly lesser extent. HIV-1p24 viral RNA was reduced to 1885 copies/cell following native ATV treatment and reduced in a dose-dependent manner to 1250, 725 and 663 copies after co-treatment with 0.1, 1 and 10 ng/ml URM-099. Treatment with URM-099 alone did not affect HIV-1 integrated DNA and viral RNA levels. Immunofluorescence staining of HIV-1p24 antigen in MDM showed parallel reductions after URM-099 and nanoATV treatments (Figure 5, C). NanoATV treatment alone resulted in a decrease in HIV-1p24 expression of 90.8, 94.2 and 95.7% with 1, 10 and 100 μ M nanoATV, respectively. Co-treatment with URM-099 (0.1, 1, 10 and 100 ng/ml) enhanced nanoATV

HIV-1p24 suppression by 63.8, 81.7, 87.8 and 91.3%, respectively ($P < 0.05$) (Figure 5, C). These results signaled the abilities of nanoATV and URM-099 to work in tandem to reduce viral production but the pathway for such effects remain unclear.

To cross validate the HIV-1p24 assays and to substantiate the antiretroviral efficacy of nanoATV and URM-099 we next determined levels of HIV-1 RT activity in cell culture supernatants. Human MDM were treated with increasing concentrations of native or nanoATV (0.1, 1, 10 and 100 μ M) with or without 0.1 or 1 ng/ml URM-099. The cells were then infected with HIV-1_{ADA} at an MOI of 0.1 for 4 hours at day 0, 5 or 10 (Figure 6, A and B, Supplementary Figure 4). RT activity was measured over 14 days following viral infection. The rationale for waiting up to 10 days to infect the MDM rested in the idea that URM-099 would affect the ATV depot and as consequence extend the drug half-life. In cells infected on day 0, nanoATV at 0.1 μ M suppressed RT activity by approximately 10-fold while concentrations of 1–100 μ M

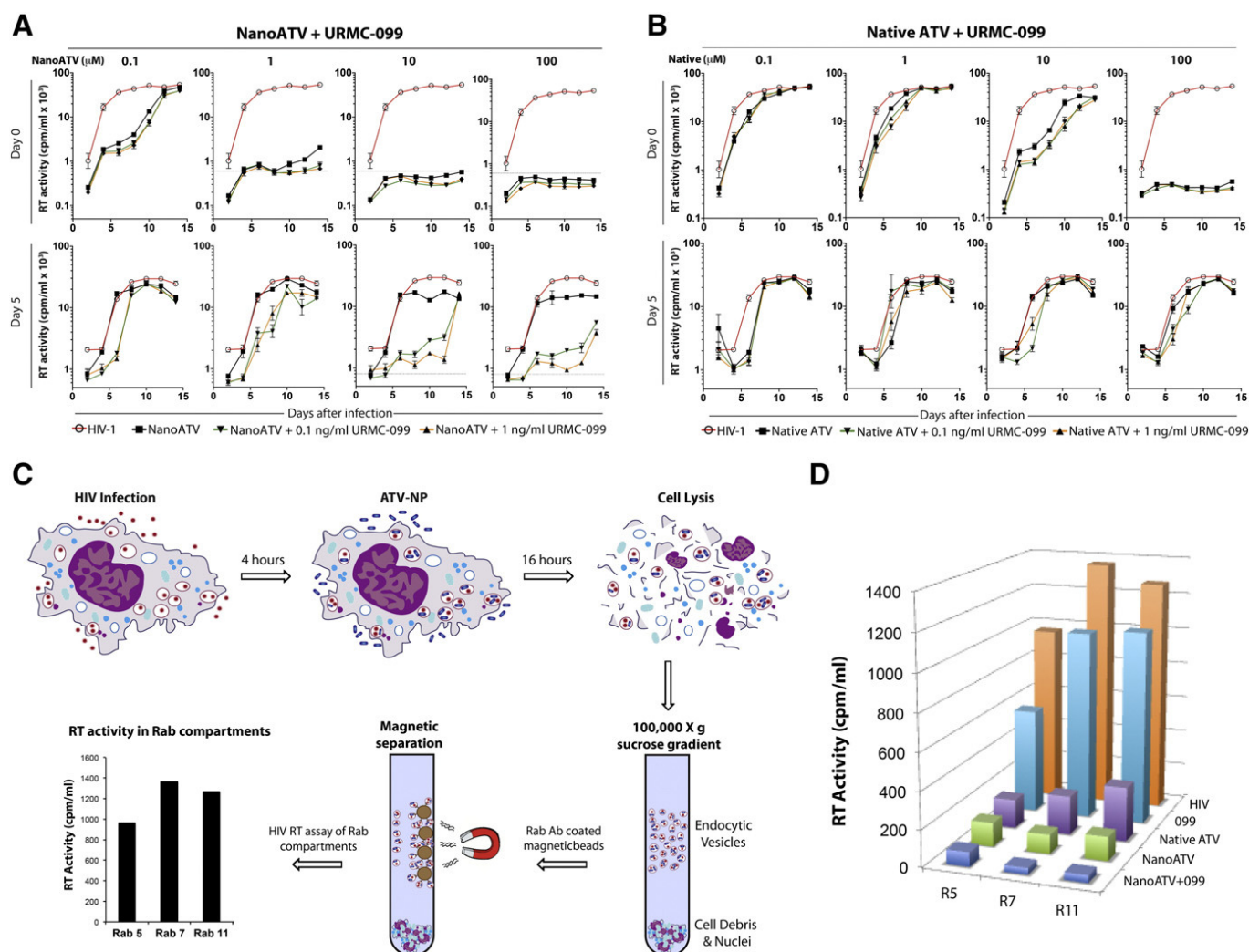


Figure 6. URMC-099 prolongs antiretroviral activities of nanoATV in HIV-1-infected human MDM. MDM were treated with URMC-099 (0.1 or 1 ng/ml) and native ATV or nanoATV (100 μ M) for 8 hours, and infected with HIV-1_{ADA} at day 0 or day 5 after drug treatment. HIV-1 RT activity was measured in culture supernatants 14 days after infection. RT activity in MDM treated with (A) nanoATV and URMC-099 or (B) native ATV and URMC-099 are shown. Gray line = limit of detection for assay. Data are expressed as average \pm SEM (n = 4). (C) Endosomal compartments isolated from URMC-099 (10 ng/ml), native ATV or nanoATV (100 μ M) treated MDM 14 days after HIV-1 infection were evaluated for RT activity. (D) HIV-1 RT activity was determined in Rab (R)-5, -7 and -11 positive compartments. Average activities of n = 5 replicates are shown.

suppressed RT activity to detectable limits of assay. Co-treatment with URMC-099 (0.1 or 1 ng/ml) reduced RT activity at all nanoATV concentrations. When the time of HIV-1 infection was delayed to days 5 or 10 after drug treatments, co-administration of URMC-099 at either concentration augmented 10 or 100 μ M nanoATV-induced inhibition of RT activity by an order of magnitude (Figure 6, A, Supplementary Figure 4, C). Furthermore, URMC-099 (1 ng/ml) and nanoATV (10 or 100 μ M) inhibited RT activity to its limit of assay detection. Treatment with native ATV and URMC-099 did not affect RT activity levels (Figure 6, B, Supplementary Figure 4, B).

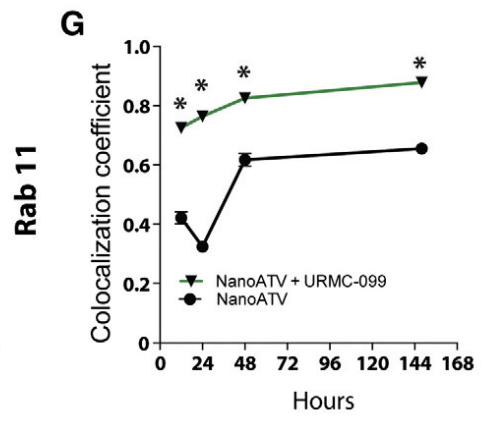
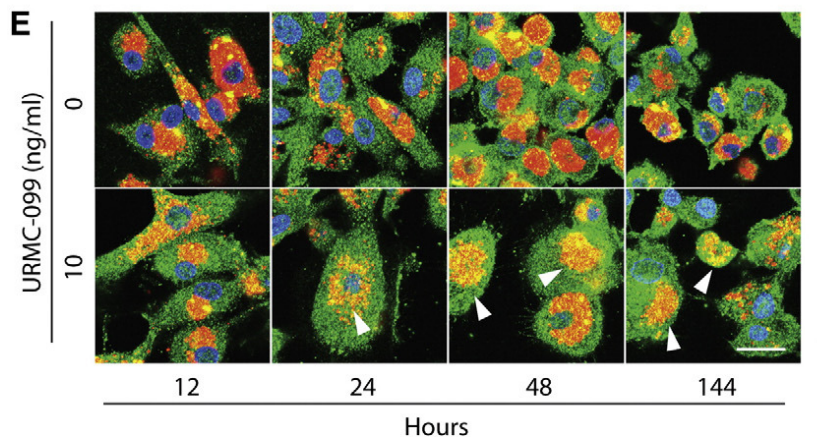
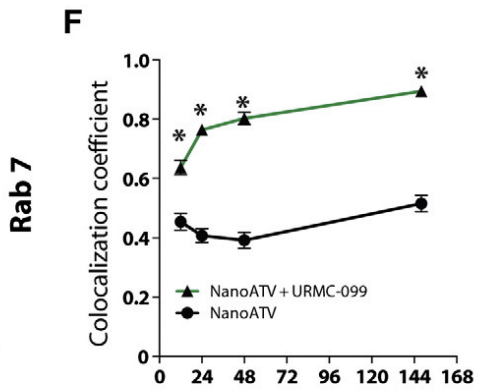
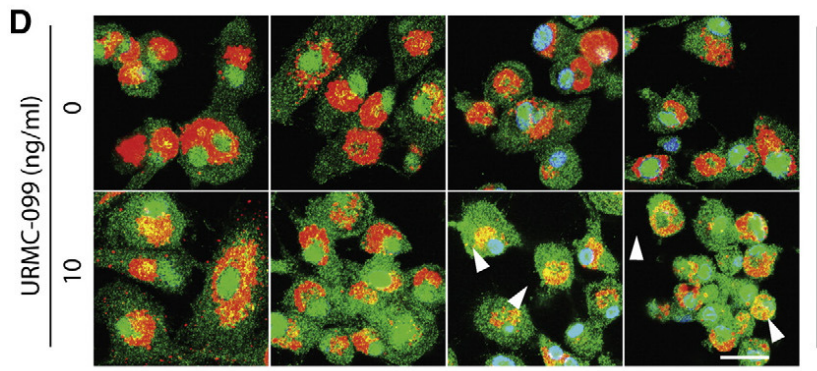
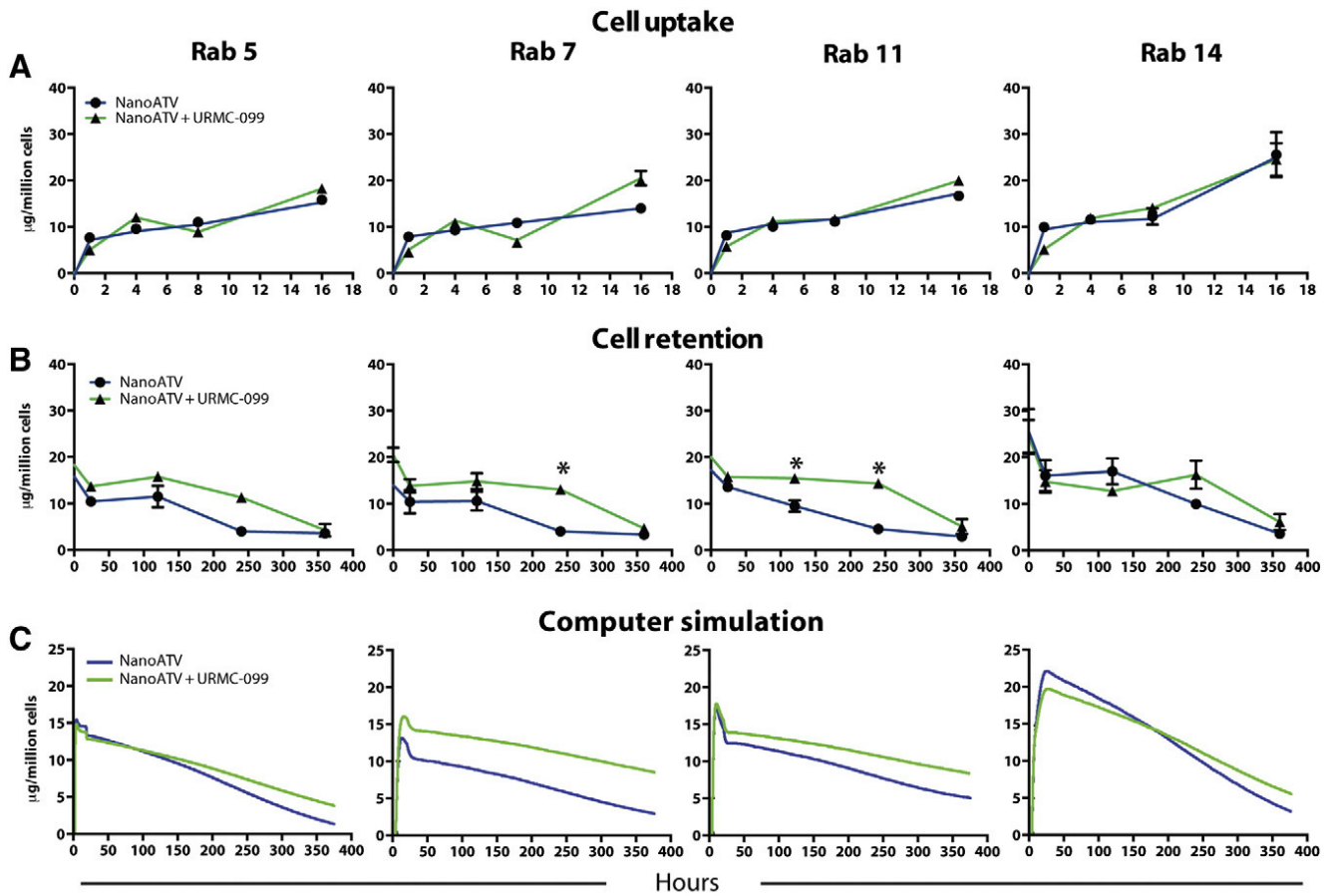
URMC-099 affects nanoATV antiretroviral activities in endosomal compartments

To determine the effect of URMC-099 on subcellular trafficking of nanoATV and virus assembly, we posited that decreased Rab7 and Rab11 expressions might also reflect a decreased number of progeny

virions in endosomal compartments. Thus, we measured RT activity in the early, late and recycling endosomes. We isolated Rab compartments from HIV-1 infected cells treated with nanoATV and URMC-099, using specific Rab antibody-coated magnetic beads and measured HIV-1 RT activity in each of these compartments (Figure 6, C). At 14 days after viral infection, 100 μ M nanoATV alone significantly reduced RT activity in all Rab-labeled endosomal compartments, by 86, 92 and 89% for Rab5, 7- and 11-labeled endosomes, respectively ($P < 0.01$, Figure 6, D). Combination of 10 ng/ml URMC-099 and 100 μ M nanoATV further decreased RT activity by 38% in Rab5-, 65% in Rab7- and 69% in Rab11-labeled compartments compared to nanoATV alone (Figure 6, D).

URMC-099 enhances nanoATV retention in Rab7 and Rab11 endosomes

We reasoned based on deregulation of Rab endosomal proteins by URMC-099 and nanoATV and its relationship to



antiretroviral responses that the result was linked to retention of the antiretroviral drug at or adjacent to sites of the viral replication cycle. To this end the role of URM-099 in potentiation of nanoATV antiviral activity was measured by assessment of nanoATV uptake and retention in Rab-labeled endosomes. MDM were treated with 100 μ M nanoATV and 10 ng/ml URM-099; and endosomal compartments were immunoisolated. URM-099 had no effect on nanoATV uptake into either whole cells or subcellular compartments (Figure 7, A, Supplementary Figure 5). However, 10 ng/ml URM-099 significantly enhanced cellular retention of nanoATV from 35.9 to 52.3 μ g ATV/10⁶ cells at 3 days ($P < 0.05$; Supplementary Figure 5), and increased nanoATV retention in Rab5, Rab7, and Rab11 endosomal compartments compared to MDM treated with nanoATV alone ($P < 0.05$; Figure 7, B). A simulated flow of nanoparticles and mathematical analyses is presented in Figure 7, C and Supplementary Figure 6, which demonstrates the enhanced retention of nanoATV in Rab7 and Rab11 compartments.

URM-099 enhances nanoATV at sites of virion assembly

We next evaluated the ability of URM-099 to enhance nanoATV accumulation in Rab-labeled endosomal compartments. The co-localization of fluorescently labeled nanoATV with endosomes labeled by fluorescently tagged Rab antibodies was determined in human MDM. URM-099 increased the level of nanoATV in Rab7 and Rab11 endosomes (Figure 7, D–G). Ten nanograms per milliliter of URM-099 increased nanoATV levels in both Rab7 and Rab11 endosomes (Figure 7, D–G). ATV concentrations persisted to 6 days. As Rab7 and Rab11 endosomes contain HIV-1 virions, we determined whether HIV-1p24 and nanoATV were co-localized in these organelles (Supplementary Figure 7, A and B). Confocal microscopy demonstrated that Rab7- or 11-labeled endosomes (expressed as a coefficient of overlap in a Z-stack of XY images) contained HIV-1p24 and nanoATV. When treated with nanoATV alone, these coefficients of overlap were 0.41 and 0.48, respectively (Supplementary Figure 7, C and D). Importantly, they were significantly increased to 0.55 and 0.66 ($P < 0.05$), when URM-099 was co-administered with nanoATV. To explore the relationship by which URM-099 could affect such viral and endosomal drug trafficking we are currently studying how Rab proteins are regulated in HIV-1 infected and nanoATV-treated macrophages.

Discussion

URM-099 boosts the antiretroviral activities of long-acting nanoART. As progeny virion trafficking occurs largely within these endosomal compartments, the nanoparticle–cell interactions of

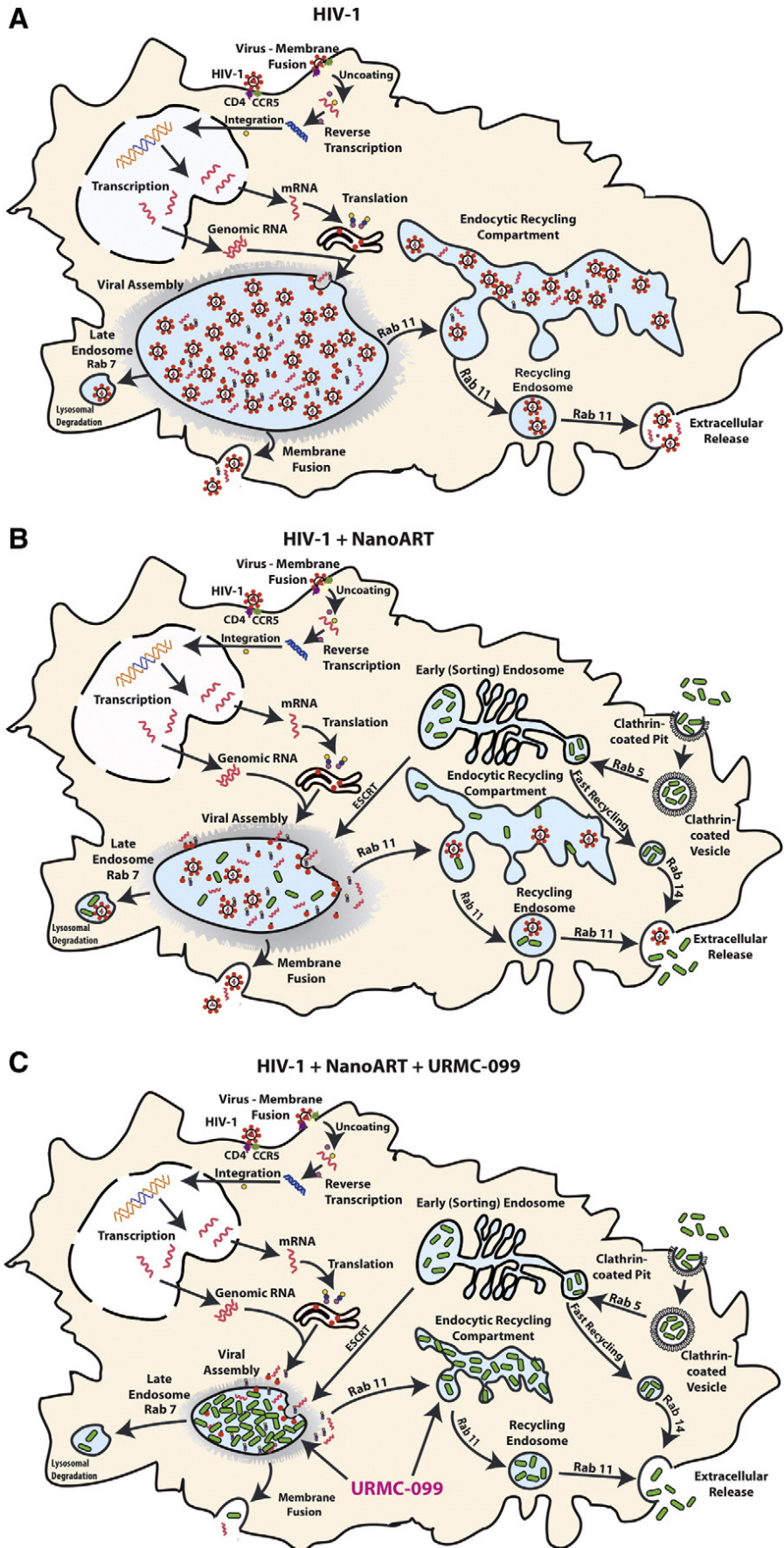
URM-099 led to improved antiretroviral outcomes. The findings buttress an URM-099-ART marriage by a surprising ability of the drugs to increase ART retention in virus-containing subcellular compartments. This occurred in lymph node viral reservoir compartments. The extension of the half-life of ART by URM-099 provides novel therapeutic opportunities for HIV-1 prevention and buttresses what is now known for nanoART (Figure 8).

The convergence of the drug–drug actions revolves around the therapeutic potential of long-acting nanoART. This is based on the nanoparticles' ability to maintain effective drug concentration in cellular and tissue depots and thus improve drug pharmacokinetics. Indeed, poorly water-soluble crystalline nanoformulations affect drug stability. Drug particles can also remain within sites of injection in subcutaneous tissue or muscle.^{6,15} Utilization of peripheral blood mononuclear cells as drug carriers thus presents a novel treatment scheme.^{46,47} Cell based drug delivery can now be harnessed to improve disease outcomes while overcoming physiologic barriers such as improved drug entry into viral reservoirs.^{48,49} Such therapeutic enhancements may be further improved by modification of the coating surface of nanoparticles.⁵⁰ Targeting ligands, such as FA, mannose, hyaluronic acid or HIV-1gp120, are being developed to facilitate uptake and retention of nanoART.^{15,51}

The endocytic pathway constitutes the uptake to cells, the out-going route being the biosynthetic pathway.⁵² In addition the endosome is responsible for mediating the uptake of nutrients, the propagation of signaling and regulation of receptors.⁵³ The endosomal system also plays an indispensable role in retroviral assembly. The envelope glycoprotein, known as Env, is one of the vital components of a retrovirus, which traffics through the endocytic pathway.⁵⁴ Env expressed in infected macrophages is located in intracellular membranes of the endocytic pathway or in trans-Golgi network.⁵⁵ HIV-1 assembly in macrophages may occur, in part, from the limiting membrane of a late endosomal compartment linked to in multi-vesicular bodies.²⁵ Viruses might be able to hide in these late endosomes, sequestered away from the immune system. What's more, late endosomes and lysosomes are part of the major histocompatibility complex class II compartment, a lysosome-related organelle with properties similar to secretory lysosomes.⁵⁶

Viral particle accumulation can occur inside late endosomes.⁵⁷ During native drug administration, such intracellular virus-containing compartments are resistant to antiretroviral drug penetration and retention since less endolysosomal proteins are related to native drug treatment.⁵⁸ The modification of the endosomal trafficking route by drug-loaded nanoparticles could significantly improve antiretroviral drug efficacy as our data demonstrate that HIV-1 can hijack Rab7 and Rab11 endosomes for virus maturation; this could be circumvented through URM-099. Indeed, URM-099's dual effects on Rab protein

Figure 7. URM-099 facilitates retention of nanoATV in endosomal compartments. Human MDM were treated with 100 μ M nanoATV with or without 10 ng/ml URM-099 for 16 hours then maintained for an additional 15 days. Subcellular compartments were isolated by immunoaffinity chromatography using antibody coated magnetic beads. (A) MDM nanoATV drug levels into individual endosomal compartments over 16 hours were measured. (B) Retention of nanoATV over 15 days (360 hours) in endosomes after the 16-hour loading (average \pm SEM, $n = 5$). All cell retention and uptake studies are repeated 3 times. (C) Mathematical simulation of nanoATV retention in individual endosomal compartments over the same time frame. (D) URM-099 potentiates nanoATV accumulation in Rab7 endosomal compartments. (E) URM-099 increases nanoATV accumulation in Rab11 endosomal compartments. (D and E) Arrowheads point to dual labeled Rab compartments and nanoparticles. CF568-labeled nanoATV shows as red, while AlexaFluor 488-labeled endosomal compartment shows as green. DAPI (blue) indicates cell nuclei, 1260 \times magnification. Percent overlap of nanoATV with (F) Rab7 and (G) -11 was quantitated (average \pm SEM, $n = 30$, unpaired 2-tailed t test); * $P < 0.05$ compared to respective time point. Scale bar = 20 μ m.



regulation and nanoparticle trafficking could lead to improvements in drug action by improving ART delivery to subcellular compartments by several divergent means. *First*, URM-099 can affect redistribution of nanoparticles in the late and recycling endosomes. *Second*, URM-099 cannot, by itself, inhibit HIV-1 replication. *Third*, deactivation of macrophages through suppression of the MAP kinase signaling pathway can restrict transcription of pro-inflammatory cytokine-induced immune responses.^{59,60} *Fourth*, virus-immune control of Rab protein expression and function is operative.⁶¹ Modulation of macrophage activation can affect Rab protein expression and regulate intracellular endocytosis. *Fifth*, progeny virions can be assembled in identical or adjacent compartments affected by immune responses. *Sixth*, this is the first combinatorial formulation known to date to reach the lymph nodes of infected animals and reduce, by more than 90%, the numbers of infectious viral particles. Thus, nanoART and URM-099 would perform synergistic functions by affecting intrinsic macrophage activation. Both would result in reduction of Rab protein expression. Perhaps more importantly, the pharmacologic and pharmacodynamic characteristics of URM-099 bode well for inclusion in the next generation of nanoformulated antiretroviral therapy to combat persistent HIV-1 infection. While targeting viral monocyte-macrophage viral reservoirs is one component that supports the abilities of this cell carriage system to reduce residual HIV-1, nanoparticles encased in cells can affect drug transfer to CD4+ T lymphocytes and improve antiretroviral activities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2015.09.009>.

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Figure 8. Schematic diagrams reveal URM-099 affects nanoART endosomal trafficking and HIV-1 progeny virion production. (A) HIV-1 budding, assembly and maturation in macrophage Rab7 and Rab11 endosomal compartments. (B) NanoATV targets endosomal compartment for storage and inhibits viral maturation at the site of viral assembly. (C) URM-099 boosts nanoATV antiviral activity through increased nanoATV accumulation in macrophage Rab7 and Rab11 endosomal compartments.

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