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Synthesis and Characterization of Long Acting Darunavir Prodrugs

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Abstract

Antiretroviral therapy (ART) has improved the quality of life in patients infected with HIV-1. However, complete viral suppression within anatomical compartments remains unattainable. This is complicated by adverse side effects and poor adherence to lifelong therapy leading to the emergence of viral drug resistance. Thus, there is an immediate need for cellular and tissue targeted long acting (LA) ART formulations. Herein, we describe two LA prodrug formulations of darunavir (DRV), a potent antiretroviral protease inhibitor. Two classes of DRV prodrugs; M1DRV and M2DRV were synthesized as lipophilic and hydrophobic prodrugs and stabilized into aqueous suspensions designated NM1DRV and NM2DRV. The formulations demonstrated enhanced intracellular prodrug levels with sustained drug retention and antiretroviral activities for 15 and 30 days compared to native DRV formulation in human monocyte derived macrophages. Pharmacokinetics tests of NM1DRV and NM2DRV administered to mice demonstrated sustained drug levels in blood and tissues for 30 days. These data, taken together, support the idea that LA DRV with sustained antiretroviral responses through prodrug nanoformulations is achievable.

SYNOPSIS/TOC

Modifying DRV into more hydrophobic prodrugs allowed better drug permeation inside cells. Modifications led to longer retention and sustained antiretroviral activities.

Graphical Abstract

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Author Contributions

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Keywords

Darunavir; prodrugs; long acting; LASER ART; protease inhibitors

INTRODUCTION

While antiretroviral therapy (ART) has revolutionized human immunodeficiency virus type one (HIV-1) infection, treatment and preventative drug regimens require strict adherence to daily dosing. With substantive extensions in longevity and quality of life, ART still remains associated with a number of adverse drug reactions, poor regimen compliance and drug resistance(1–3). Comorbid events include stigma of infection, depression and neuropsychiatric disorders associated with or without substance abuse. These also affect regimen adherence(4, 5) and can lead to the emergence of virus drug resistance mutations(6). Moreover, associated acquired opportunistic infections and drug-drug interactions commonly emerge(7, 8). These affect clinical outcomes, such as viral transmission and disease progression, impede the ART virologic suppression goals set by the United Nations Programme on HIV and AIDS (UNAIDS) and the US national HIV/AIDS strategic plan(9–12). Such complications have set the stage for the development of long acting (LA) ART(13–16).

Recent surveys support an immediate need for LA ART for HIV-1 infected patients including those who would prefer the ease of extended release regimens compared to taking daily pills(14). Thus, currently in development are monthly injectables of cabotegravir and rilpivirine (CAB and RPV) for treatment and prevention of viral infection(13, 17). These suffer limitations that include large injection volumes, injection site reactions and restricted access to tissue compartment sites of viral replication(13, 17, 18). Moreover, most antiretroviral drugs (ARVs) used in combination therapy have either poor physicochemical properties or require high doses limiting their potential reformulation into long acting therapies. To overcome such obstacles, we transformed ARVs into lipophilic and hydrophobic prodrugs (18–23). These long acting slow effective release ART (LASER ART) are surfactant stabilized nanocrystals with enhanced drug loading and tissue penetrance

properties. Indeed, each of our LASER ART formulations were designed to facilitate rapid diffusion across physiological barriers allowing sustained therapeutic drug concentrations at sites of viral replication(18–24). With these advances established, we now describe the development of two long acting darunavir (DRV) prodrug formulations with extended apparent drug half-lives and improved antiretroviral activities.

While protease inhibitors are second line agents after integrase inhibitors, they remain an important component of ARV regimens. Emerging data supports the use of dual regimens such as ritonavir or cobicistat boosted darunavir and dolutegravir for maintenance therapy in virally suppressed patients (25, 26). Moreover, combinations of DTG with RPV or boosted DRV with lamivudine (3TC) were found to be non-inferior to standard three-drug regimens (25). DRV is a second-generation unnatural peptidomimetic protease inhibitor (PI) used in combination ART for treatment of HIV-1 infection(27, 28). Compared to other approved PIs, DRV has a higher genetic barrier to resistance with potent antiviral activity against multidrug resistant viral strains(29). However, limitations of DRV use include its short half-life and restricted access to cellular and tissue reservoirs of infection. Moreover, DRV and other PIs require high doses and pharmacokinetic (PK) boosting agents, increasing the potential for secondary toxicities(30). Thus, there is an immediate need to develop improved means for delivery of these medicines. One strategy is utilizing prodrugs to extend the half-life and biodistribution of the agent. While such prodrug approaches were previously explored in attempts to improve absorption, distribution, metabolism and drug excretion (ADME) none have yet to be applied to the generation of reservoir targeted LA DRV(30–33). Prior efforts included, but were not limited to, carrier-mediated drug delivery approaches that incorporate amino acids to minimize p-glycoprotein efflux and mitigate first pass metabolism(30, 34). Notably, extensive prodrug approaches have led to development and approval of fosamprenavir, a phosphorylated water-soluble prodrug of amprenavir(35). Various fatty ester prodrugs of indinavir and saquinavir have been described but are significantly less potent compared to the parent drugs(31). Overall, the reported prodrug and formulation approaches sought to improve aqueous solubility of native drugs and the few lipophilic ester modifications made had limitations(31, 32, 36). Specifically, PI prodrug designs were exclusively focused on modifications of the free hydroxyl group and such attempts have either resulted in a marked loss of antiviral activity with comparable half-lives to that of parent drugs(36–38). Thus, LA delivery systems for PIs have remained elusive. Based on extensive prior works from our laboratory on development of LASER ART platforms we sought to transform DRV into a LA formulation. We reasoned that conversion of DRV into prodrugs was not only feasible and economical but also a faster strategy for overcoming ADME and formulation challenges associated with the parent drug(39). Lipophilic ester prodrugs, M1DRV and M2DRV, were created by covalent linkage of hydrolysable fatty acid promoieties to functional groups present in the parent drug. These chemical modifications improved physicochemical properties of the modified compounds compared to the parent drug, thereby enabling transformation of DRV into LA nanosuspensions with improved intracellular and tissue drug accumulation and sustained antiretroviral activities.

EXPERIMENTAL SECTION

Reagents

Darunavir (DRV) was purchased from Boc Sciences (Shirley, NY). Anhydrous pyridine, dichloromethane (CH_2Cl_2), chloroform (CHCl_3), dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), deuterated chloroform (CDCl_3), triethylamine (Et_3N), diethyl ether, tetrahydrofuran (THF), triethylsilane (Et_3SiH), pluronic F127 (P407), 4-dimethylaminopyridine (DMAP), stearoyl chloride, myristoyl chloride, zinc chloride, paraformaldehyde (PFA), sodium iodide (NaI), sodium hydride (NaH), potassium iodide (KI), hexanes, ethyl acetate, ciprofloxacin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 3,3'-diaminobenzidine (DAB) were purchased from Sigma-Aldrich (St. Louis, MO). Benzyl chloroformate was purchased from MilliporeSigma (Burlington, MA). Live/Dead Fixable Green Dead Cell Stain Kits were purchased from Thermo Fisher Scientific (Waltham, MA). LC-MS grade methanol (MeOH), acetonitrile (ACN), cell culture grade water (endotoxin-free), gentamicin, ammonium formate, bovine serum albumin (BSA) and Triton X-100 were purchased from Fisher Scientific (Hampton, NH). Palladium, 10% on activated carbon, was purchased from STREM Inc. (Newburyport, MA). All chemical synthesis reactions were performed under a dry argon atmosphere. Flash column chromatography was performed on 32–63 μm flash silica gels from SiliCycle Inc. (Quebec, Canada). Reactions were monitored by thin-layered chromatography on precoated silica plates (250 μm , F-254 from SiliCycle Inc.). The compounds were visualized by UV fluorescence or stained with potassium permanganate. Heat-inactivated pooled human serum was purchased from Innovative Biologics (Herndon, VA). Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Corning Life Sciences (Tewksbury, MA). CEM CD4⁺ T cells were obtained from the National Institute of Health. Monoclonal mouse anti-human HIV-1p24 (clone Kal-1), monoclonal mouse anti-human leukocyte antigen (HLA-DP/DQ/DR; clone CR3/43), and the polymer-based HRP-conjugated anti-mouse EnVision⁺ secondary were purchased from Dako (Carpinteria, CA).

Prodrug synthesis and physicochemical characterization

To synthesize M1DRV, DRV (0.5 g, 0.91 mmol, 1 equivalent) was dried from anhydrous toluene (20 mL), dissolved in anhydrous THF (10 mL), cooled to -78°C , followed by slow addition of NaH (0.07 g, 1.83 mmol, 2 equivalents) and allowed to stir for 15 minutes under an argon atmosphere. A solution of iodomethyl tetradecanoate (0.58 g, 1.37 mmol, 1.5 equivalents) was prepared as previously described(40) and added to the precooled DRV solution. The reaction mixture was gradually warmed to room temperature and stirred for 48h after which M1DRV was isolated by silica column chromatography purification using 2:1 ethyl acetate-hexanes to form a colorless powder (84% yield). ^1H NMR (500 MHz, CDCl_3): δ 7.59 (br, 2H), 7.17–7.35 (m, 6H), 6.66–6.78 (m, 1H), 5.67 (dd, J = 20.5, 4.9 Hz, 1H), 5.55 (dd, J = 32.9, 5.8 Hz, 1H), 5.25–5.40 (m, 1H), 4.88–5.22 (m, 2H), 4.73 (br, 1H), 4.12–4.23 (m, 1H), 3.63–4.10 (m, 3H), 3.45–3.59 (m, 1H), 3.36 (app. d, J = 11.4 Hz, 1H), 2.80–3.20 (m, 4H), 2.63–2.75 (m, 1H), 2.60 (t, J = 12.5 Hz, 1H), 2.25–2.45 (m, 2H), 1.85–1.97 (m, 1H), 1.50–1.74 (m, 4H), 1.20–1.40 (m, 20H), 0.80–1.05(m, 9H). ^{13}C NMR (125 MHz, CDCl_3): δ 173.1, 155.1, 137.7, 129.6, 129.2, 128.3, 126.4, 113.9, 112.3, 109.4, 81.7,

73.5, 73.4, 73.3, 71.4, 69.6, 58.9, 53.7, 45.3, 36.3, 34.2, 33.6, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 26.9, 25.9, 24.6, 22.6, 20.3, 20.2, 20.1, 19.9, 14.1. HRMS (ESI-TOF) m/z : $[M + Na]^+$ Calcd for $C_{42}H_{65}N_3NaO_9S^+$, 810.43 (100%), 811.44 (45.4%), 812.44 (10.1%); found, 811.05. M2DRV was synthesized in a three-step process that involved protection of the primary amine in the parent drug using a carbobenzoxy (Cbz) group, coupling to stearoyl chloride and a final Cbz deprotection to yield the desired product as a colorless powder. Specifically, DRV (1.0 g, 1.83 mmol, 1 eq.) was dried from anhydrous pyridine (10 mL) and dissolved in a mixture of anhydrous THF (10 mL) and DMF (10 mL) under an argon atmosphere. The reaction mixture was cooled to 0°C, followed by drop wise addition of Et_3N (254 μ L, 1.83 mmol, 1 eq.) then benzyl chloroformate (388 μ L, 2.74 mmol, 1.5 eq.) and left to stir for 48h under protection from light. The desired protected Cbz-DRV was isolated by silica column chromatography purification eluting with 1:1 ethyl acetate-hexanes. The Cbz-DRV was dissolved in 15 mL of DMF and cooled to 0°C followed by addition of 1.5 equivalents of 4-dimethylaminopyridine and 2 equivalents of Et_3N . The mixture was stirred for 15 minutes after which 1.5 equivalents of stearoyl chloride were added and warmed to 45°C over 24 hours. The formed Cbz-M2DRV was isolated by column chromatography and subjected to catalytic hydrogenolysis using 10% palladium on activated carbon and 20 equivalents of Et_3SiH in a mixture of MeOH and $CHCl_3$ (1:1) solvents. After reaction completion, the mixture was filtered through a pad of Celite to remove carbon followed by further purification by column chromatography eluting with a mixture of 2:1 ethyl acetate-hexanes to isolate M2DRV as a colorless powder. ^1H NMR (500 MHz, $CDCl_3$): δ 7.58 (d, J = 8.3 Hz, 2H), 7.18–7.34 (m, 7H), 6.70 (d, J = 4.8 Hz, 2H), 5.65 (d, J = 4.8 Hz, 1H), 5.17 (br, 1H), 5.10 (d, J = 9.0 Hz, 1H), 5.02 (dd, J = 13.9, 6.9 Hz, 1H), 4.21–4.39 (m, 1H), 3.98 (t, J = 7.8 Hz, 1H), 3.82 (t, J = 7.6 Hz, 1H), 3.76 (app. t, J = 7.7 Hz, 1H), 3.67 (dd, J = 15.8, 8.9 Hz, 1H), 3.53 (dd, 1H J = 14.8, 5.3 Hz, 1H), 3.11 (app. d, J = 10.7 Hz, 1H), 2.93–3.09 (m, 2H), 2.82–2.92 (m, 1H), 2.65–2.79 (m, 2H), 2.22–2.40 (m, 2H), 1.93 (app. t, J = 6.3 Hz, 1H), 1.50–1.70 (m, 6H), 1.20–1.38 (m, 28H), 0.81–0.95 (m, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.3, 155.1, 150.7, 137.4, 129.5, 129.2, 128.4, 126.7, 126.6, 113.9, 109.3, 73.9, 73.3, 70.8, 69.6, 57.7, 53.5, 49.3, 45.4, 36.9, 34.2, 31.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.8, 25.7, 24.6, 22.6, 20.2, 20.2, 19.9, 14.1. HRMS (ESI-TOF) m/z : $[M + Na]^+$ Calcd for $C_{45}H_{71}N_3NaO_8S^+$, 836.49 (100%), 837.49 (48.7%), 838.49 (11.6%); found, 837.14. Nuclear magnetic resonance (^1H -NMR and ^{13}C -NMR) spectra were recorded on a Varian Unity/Inova-500 NB (500 MHz; Varian Medical Systems Inc., Palo Alto, CA, USA). Fourier Transform Infrared Spectroscopy (FTIR) was performed on a Spectrum Two FT-IR spectrometer (PerkinElmer, Waltham, MA, USA). Infusion on a Waters TQD triple quadrupole mass spectrometer (Boston, MA) was performed to confirm the desired molecular ion peaks of M1DRV and M2DRV. Solubilities of DRV, M1DRV, and M2DRV were determined in water. Briefly, homogeneous saturated solutions of each compound were mixed at room temperature for 24 hours and centrifuged at $17,000 \times g$ for 10 minutes to pellet insoluble drug. The amount of drug in the supernatants was quantified by multiple reaction monitoring (MRM) on a liquid chromatography tandem mass spectrometry (LC-MS/MS) system comprised of an Waters ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to a triple quadrupole mass spectrometer with electrospray ionization (ESI) source (Waters Xevo TQ-XS).

Prodrug plasma cleavage

To evaluate plasma stability of M1DRV and M2DRV, 100 μ L plasma samples from mouse or rat were spiked with 1 μ M M1DRV or M2DRV. Triplicate samples were kept on a shaker at 37°C. At various time points (0, 2, 6, 24, and 48 hours), 1 mL of methanol was added to each tube and vortexed for 3 minutes to stop enzymatic cleavage, then stored at –80°C until drug quantitation. Negative controls were used to normalize for non-specific binding. Specifically, 100 μ L ice cold plasma was added to 1 mL MeOH followed by addition of the prodrugs. In addition, negative controls were prepared using heat-inactivated plasma to determine whether drug hydrolysis was due to enzymatic activity or non-enzymatic hydrolysis. At various time points, 1 mL MeOH was added to the samples. Samples were then vortexed for 3 minutes and centrifuged at $15,000 \times g$ for 15 minutes. To quantify prodrugs, 40 μ L of supernatant was added to 10 μ L of internal standard (IS) and 50 μ L of 75% MeOH. For DRV quantitation, 50 μ L of sample was added to 10 μ L of IS and 40 μ L of water. Samples were vortexed and plated for analyses by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

Nanoformulations of DRV, M1DRV and NM2DRV

Poloxamer 407 (P407) coated DRV (NDRV), M1DRV (NM1DRV) and M2DRV (NM2DRV) nanoformulations were produced by high-pressure homogenization (Avestin EmulsiFlex-C3; Avestin Inc, Ottawa, ON, Canada). Briefly, each compound (1% w/v) was premixed in a P407 solution (0.5% w/v in PBS) for 16 hours at room temperature followed by homogenization at 20,000 psi until the desired particle size was achieved (150 – 450 nm). Particle size, polydispersity index (PDI), and zeta potential were determined by dynamic light scattering (DLS) on a Malvern Nano-ZS (Worcestershire, UK). Drug nanoparticles were diluted in water and triplicate measurements were recorded. Nanoparticle morphologies were analyzed by FEI Tecnai G2 Spirit transmission electron microscopy (TEM) (Hillsboro, OR) using negative staining. An aliquot (10 μ L) of the diluted nanoformulation (1 mg/mL) was applied on a formavar/silicone monoxide coated 200 mesh copper grid. The grid was left for 2–5 minutes to absorb the nanoparticles. Excess sample was dried carefully using a clean filter paper and allowed to dry for 2 minutes. A drop of Nanovan negative stain was added on the grid using a pipette and allowed to stain for no longer than a minute while shielding the sample using a filter paper tent to minimize any air contaminants. Excess stain was blotted and the grid was allowed to dry prior to viewing under TEM.

Cytotoxicity and cell viability

Effects of NDRV, NM1DRV, and NM2DRV on cell viability were assessed in human monocyte-derived macrophages (MDM) and CEM-SS CD4+ T cells. Briefly, human monocytes were isolated from one donor at a time then cultured for 7–10 days in 96-well culture plates (80,000 cells/well) with Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% heat-inactivated pooled human serum, 1,000 U/mL of macrophage colony stimulating factor, 1% glutamine, 10 μ g/mL ciprofloxacin, and 50 μ g/mL gentamicin in a 37°C in a 5% CO₂ incubator(41–43). A stock solution of each drug nanoformulation was serially diluted in DMEM to produce drug concentrations ranging from 50–400 μ M.

Cells were treated for 24 hours, washed and incubated with 200 μ L/well 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) for 45 minutes. Upon MTT removal, 200 μ L/well of DMSO was added and absorbance measured at 490 nm on a SpectraMax M3 plate reader with SoftMax Pro 6.2 software (San Jose, CA). Sample absorbance was normalized to that of non-treated controls. For CEM-SS CD4+ T cell studies, the cells were seeded in round bottom 96-well culture plates at a density of 80,000 cells/mL per well in Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin and treated with each nanoformulation as described for MDM. After 24 hours, cells were centrifuged at $650 \times g$, dispersed in PBS, centrifuged again at $650 \times g$, and resuspended in PBS. Excitation dye (0.1 μ L) (Molecular Probes, Live/Dead Fixable Green Dead Cell Stain Kits) was added to each well. Cells were excited at 488nm and the percent dead cells determined by flow cytometry.

Evaluation of drug potency

To evaluate drug potency, MDMs were seeded on round bottom 96-well culture plates (80,000 cells/well) and exposed to DRV, NM1DRV or NM2DRV containing media at concentrations ranging from 0.005 nM-50 μ M in tenfold dilution increments for 60 minutes and replaced with drug-containing HIV-1_{ADA} media at a multiplicity of infection (MOI) of 0.1 infectious particles per cell. After 4 hours, infection medium was removed, and the cells were incubated an additional 10 days in the presence of the same concentration of drug prior to infection. Half media changes were done every other day. After 10 days of infection, culture media was collected for measures of HIV-1 reverse transcriptase (RT) activity(41–43). For CEM-SS CD4+ T cells studies, the cells were cultured in suspension in 96 well plates, pelleted at $650 \times g$ and re-dispersed in 100 μ L drug-containing media. After 60 minutes, cells were challenged with HIV-1_{NL4-3-eGFP} at a MOI of 0.1 by spin-inoculation followed by incubation in drug-containing and FBS-free media for 2 hours. Cells were kept at 37°C in a 5% CO₂ incubator for 12 hours, after which media containing drug and FBS was added. Twenty-four hours post infection, all cells were washed twice with PBS to remove extracellular virus and drug-containing media was replaced with medium containing the same drug concentration prior to infection. Every other day, cells were centrifuged at $650 \times g$ and resuspended in fresh drug-containing medium. Ten days post HIV-1 challenge; supernatants were collected for RT activity measurements. The 50% effective concentration (EC₅₀) was calculated using sigmoidal 4-point logarithmic regression of RT activity curves using GraphPad Prism v7 (San Diego, CA).

Cell drug uptake and retention

For determination of nanoformulation cell uptake and retention, MDM were cultured on 12-well culture plates and treated with media containing 100 μ M NDRV, NM1DRV or NM2DRV (41–43). Treatment concentration was determined based on cytotoxicity studies as well as previous studies in our laboratory(18–21). At 2, 4, and 8 hours post treatment, media was removed and adherent cells were washed twice with PBS then scraped into 1 mL PBS. Cells were counted on an Invitrogen Countess Automated Cell Counter (Carlsbad, CA) then pelleted by centrifugation at $956 \times g$ for 8 minutes. The cell pellets were resuspended in 100 μ L MeOH and sonicated for 5 minutes followed by centrifugation at $20,817 \times g$ for 5 minutes. For CD4+ T cell experiments, the CEM-SS cell line was used(44, 45). For uptake

studies, CEM-SS CD4⁺ T cells were plated on poly-L-lysine pre-coated cell culture plates that allow cell adherence and cell uptake was assessed as described for MDM. For drug retention studies, MDM were treated for 8 hours with 100 μ M NDRV, NM1DRV or NM2DRV, then washed twice with PBS and maintained with half-media changes every other day until collection days. Intracellular and supernatant drug contents were analyzed using a Waters Alliance e2695 HPLC Separations Module or a Waters Acquity H-class UPLC with a Waters Acquity QDa detector (Waters, Milford, MA). For the HPLC analysis, a Kinetex 5 μ m C18 column (150 $\times$ 4.6 mm) (Torrance, CA) was used for separation using mobile phases of 55% 10 mM ammonium formate, pH 3.0/45% ACN, 20% 10 mM ammonium formate pH 3.0/80% ACN, and 10% water/90% MeOH for DRV, M1DRV and M2DRV, respectively, with a flow rate of 1.0 mL/min and UV detection at 265 nm. For UPLC-QDa analysis, an Acquity UPLC® BEH Shield RP18 1.7 μ m analytical column (2.1mm $\times$ 100mm, Waters Corp.) was used for separation using a mobile phase of 60% MeOH/40% 7.5 mM ammonium formate, pH 3.0, at a flow rate of 0.25 mL/min with a mass range of 50 – 600 Da and cone voltage of 12 mV. Transmission electron microscopy (TEM) was used to image the morphology of MDM loaded with the different drug nanoparticles. Cells were loaded with the different nanoparticles for 4 hours then washed twice with PBS. Cells were collected in PBS, centrifuged at 956 $\times$ g for 8 minutes, fixed in 2% paraformaldehyde (PFA) and kept at 4°C until TEM processing.

Antiretroviral activities

To assess long-term antiretroviral efficacy, MDM were treated for 8 hours with 100 μ M NDRV, NM1DRV or NM2DRV as previously described for uptake studies. After treatment, cells were washed twice with PBS and cultured in fresh media without drug followed by half-media changes every other day. At 5-day intervals from days 1 to 30 after treatment, cells were challenged with HIV-1_{ADA} at a MOI of 0.1 for 16 hours. HIV-1_{ADA} media was then replaced with fresh media without drug. Ten days after viral challenge culture media were analyzed for RT activity, while adherent MDM were fixed with 2% PFA and assessed for HIV-1p24 protein expression by immunocytochemistry(41–43)

NDRV, NM1DRV and NM2DRV pharmacokinetics (PK)

Male BALB/cJ mice (25 g) (Jackson Labs, Bar Harbor, ME, USA) were administered a single 100 mg/kg DRV-equivalent dose of NDRV, NM1DRV or NM2DRV intramuscularly (IM) into the caudal thigh muscle to determine PK and biodistribution over 4 weeks. Heparinized tubes were used for blood collection to obtain plasma and blood samples at days 1, 7, 14, 21, and 28 from treated mice. Tissues were collected at the end of the study and stored at –80°C until drug analysis. Plasma, blood and tissue samples were analyzed by UPLC-MS/MS to determine parent and prodrug levels.

Drug quantitation by UPLC-MS/MS

To determine drug concentrations in blood, 25 μ L of blood/plasma from mice were immediately mixed with 1 mL ice-cold ACN, and spiked with internal standard (IS) of (500 ng/mL of a fourteen carbon fatty-acid modified dolutegravir prodrug (MDTG)(20) and d3-dolutegravir (DTG-d₃), final concentration of 50 ng/mL). Samples were then vortexed, and centrifuged at 17,000 $\times$ g for 10 minutes. The resulting supernatants were evaporated to

dryness on a Speedvac, reconstituted in 100 μ L. For DRV analysis, 45 μ L supernatant was mixed with 30 μ L of H₂O and for prodrug analysis 35 μ L supernatant was mixed with 35 μ L of 85% MeOH. Finally 10 μ L of samples were injected for separate drug and prodrug LC/MS/MS analyses. Blood and plasma standards were extracted at final DRV or prodrug concentrations of 0.5–500 ng/mL. For tissue sample preparation, approximately 20–100 mg of spleen, lymph nodes, and liver were weighed and homogenized in 5–20x dilutions of 90% MeOH. A 100 μ L aliquot of homogenate was mixed with 10 μ L IS, vortexed, and centrifuged at $16,000 \times g$ for 15 min. For DRV analysis, 60 μ L of supernatant was mixed with 40 μ L of 50% MeOH and for prodrug analysis, 60 μ L supernatant was mixed with 40 μ L of 85% MeOH before LC/MS/MS analyses. Tissue standards were extracted at final DRV or prodrug concentrations of 0.5–500 ng/mL. DRV and prodrugs were quantified by UPLC/MS/MS consisting of a Waters ACQUITY H class UPLC coupled to a Waters Xevo TQ-Smicro mass spectrometer (Waters Corp., Milford, MA, USA) with an ESI source in positive mode. Analyte separation was achieved on an ACQUITY UPLC® BEH C18 1.7 μ m analytical column (2.1 mm $\times$ 50 mm, Waters Corp.). Mobile phase A was 7.5 mM ammonium formate in water (MS grade, Fisher), pH adjusted to 3.0 with formic acid (MS grade, Fisher). Mobile phase B was 100% MeOH (MS grade, Fisher). For prodrug analyses, mobile phase was held at 20% A for 10 minutes, decreased to 5% A, and held for one minute, then increased to 40% A for and held for 2 minutes using flow rate of 0.28 mL/min. For DRV analysis, mobile phase was held at 40% A for 4.5 minutes, decreased to 5% A, held for another minute and then increased to 40% and held for 2 minutes using a flow rate of 0.25 mL/min. Multiple reaction monitoring (MRM) transitions, cone voltage and collision energy were optimized for DRV, M1DRV, M2DRV and IS. MRM transitions for DRV were $548.00 < 112.81$ m/z for quantification, and $548.00 < 68.89$ m/z and $548.00 < 392.2$ m/z for identification. Internal standard (DTG-d₃) was monitored at $422.84 < 129.94$ m/z. Dwell time was 20 msec for each transition. Transitions for M1DRV were $758.4 < 155.93$, $758.4 < 112.89$ m/z and $758.4 < 602.33$ m/z for quantification and identification respectively. Transitions for M2DRV were $814.52 < 155.93$, $814.52 < 240.97$ m/z and $814.52 < 658.44$ m/z for quantification and identification respectively. The internal standard (MDTG) was monitored at transition $616.27 < 126.95$ m/z. Dwell time was 25 ms for each transition.

Study Approvals

All animal studies were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee in accordance with the standards incorporated in the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011). Human blood cells were isolated by leukapheresis from HIV-1/2 and hepatitis B seronegative donors and were deemed exempt from approval by the Institutional Review Board of UNMC. Monocytes were isolated from one donor at a time and studies were repeated to confirm experimental results using cells from different donors each time.

Statistics

All data sets are presented as mean $\pm$ standard error of the mean (SEM). *In vitro* studies were performed using three biological replicates (n=3). *In vivo* studies included a minimum of 5 animals per group (n=5). For comparisons of two groups, Student's t test (two-tailed)

was used. For comparison between multiple groups, one-way ANOVA with Bonferroni correction were performed. For studies with multiple time points, two-way ANOVA and Bonferroni's post hoc tests for multiple comparisons were performed. Results with $P < 0.05$ were considered significant. All data were graphed and analyzed using GraphPad Prism 7.0 software (La Jolla, CA).

RESULTS

M1DRV and M2DRV synthesis and characterization

M1DRV and M2DRV ester prodrugs were synthesized by either derivatizing the amine (M1DRV) or hydroxyl (M2DRV) group in the parent drug using fatty acids of variable carbon chain length to form the desired lipophilic compounds in high chemical yields after purification by column chromatography (Fig. 1A). The chemical structures were confirmed by ^1H -NMR and ^{13}C -NMR spectroscopy (Fig S1 and S2). Specifically, broad peaks at 0.9 and 1.26 ppm in the ^1H NMR spectra of both prodrugs correspond to the terminal methyl and methylene protons of the fatty esters (Fig S1A and S2A). Additional peaks from the carbonyl groups at 173 ppm and aliphatic carbon atoms from the fatty acids in the ^{13}C -NMR spectra further confirmed M1DRV and M2DRV prodrug synthesis (Fig S1B and S2B). Further prodrug characterization by Fourier transform infrared (FTIR) spectroscopy showed absorption bands at 2917, 2915 and 2848 cm^{-1} representing asymmetric and symmetric C-H stretches in the long chain fatty acids (Fig S3A, B). Mass spectrometric analysis showed the desired molecular ion peaks of 811.05 $[\text{M}+\text{Na}]^+$ and 837.14 $[\text{M}+\text{Na}]^+$, for M1DRV and M2DRV, respectively. The water solubility of the prodrugs was at 212 ng/mL (M1DRV) and 130 ng/mL (M2DRV) compared to 150 $\mu\text{g/mL}$ for the parent drug (Fig 1B). The 700-fold decrease in aqueous solubility for the two prodrugs confirmed their hydrophobicity as conferred by the lipid conjugates. The stability of the prodrugs was determined in plasma of mice and rats. Incubation of the prodrugs in plasma resulted in a decline in prodrug concentrations by more than 45 % over 24 h (Fig 1C, D). In both plasma matrices, M1DRV and M2DRV concentrations declined over time and levels of DRV cleaving from the prodrugs were detected at increasing concentrations. Parallel studies in heat inactivated plasmas showed no decrease in prodrug concentrations or formation of DRV indicating that the compounds are activated enzymatically through ester bond cleavage.

Characterization of NM1DRV and NM2DRV

Poloxamer 407 (P407) stabilized native drug and prodrug nanoparticles (NDRV, NM1DRV and NM2DRV) were produced by high-pressure homogenization with drug encapsulation efficiencies of 57, 74 and 85% for NDRV, NM1DRV and NM2DRV, respectively. The NDRV, NM1DRV and NM2DRV nanoparticles exhibited uniform particle sizes of 384 ± 10 , 322 ± 7 and 149 ± 2 nm, narrow polydispersity indices (PDI) of 0.27 ± 0.01 , 0.26 ± 0.01 and 0.28 ± 0.01 , and zeta potentials of -7.78 ± 0.36 , -7.03 ± 0.02 , and -1.74 ± 0.09 mV, respectively, and remained stable at 4°, 25° and 37°C for 13 weeks (Fig S4). Nanoparticle morphologies were examined by transmission electron microscopy (TEM). NDRV and NM2DRV exhibited uniform rod-shaped morphologies, while NM1DRV showed spherical morphologies (Fig S5), all with diameters in the nanometer range (150 – 450 nm). It is worth noting that DLS and TEM are complementary techniques used to measure size

distribution and actual particle radius, respectively. Physicochemical and particle stability supported further preclinical development of the prodrug formulations.

Cytotoxicity and efficacy of NM1DRV and NM2DRV

Toxicity of the prodrug nanoformulations was assayed by mitochondrial function in human monocyte-derived macrophages (MDM) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Similarly, toxicity of the different drug nanoformulations was determined in CEM-SS CD4⁺ T cells by MTT as well as Live/Dead Fixable Dead Cell Stain Kits. No adverse effects on cell vitality were observed in MDM (Fig. 2A) and CEM-SS CD4⁺ T cells (Fig 2B) after 24 hours of incubation with 400 and 200 μ M of drug nanoformulations, respectively. Given the hydrophobic nature of the two prodrugs, homogenous nanosuspensions of the prodrug with enhanced apparent aqueous solubility were employed for efficacy studies. The amine modified ester prodrug formulation of DRV (NM1DRV) demonstrated comparable antiretroviral activity to native DRV formulation in human MDM and CEM-SS CD4⁺ T cells. Modification of the hydroxyl functional group in DRV to form NM2DRV nanoparticles resulted in a 10-fold loss in activity, nevertheless, the half maximal effective concentration (EC_{50}) of NM2DRV against HIV-1_{ADA} in MDM and CD4⁺ T cells was in the nanomolar range and exhibited long-term efficacy when compared to native drug or NM1DRV formulations. The EC_{50} values for NDRV, NM1DRV and NM2DRV in MDM were 20.2, 19.1 and 260 nM (Fig 2C), respectively. Similarly, NDRV (1.48 nM) and NM1DRV (0.97 nM) exhibited comparable activity in CEM-SS CD4⁺ T cells, while NM2DRV (18.7 nM) showed decreased potency compared to NDRV (Fig 2D). The observed antiviral activity for NM1DRV and NM2DRV formulations is likely linked to improved cellular drug uptake and variable prodrug activation rates in HIV target cells. However, unlike M1DRV that hydrolyses back to DRV inside cells, M2DRV was found to be hydrolytically stable, suggesting that the prodrug could be intrinsically active. Overall, these data sets suggest that both prodrug formulations could facilitate long-lasting antiretroviral efficacy. Development of long acting stable DRV prodrug nanoformulations could also affect drug administration and drug cell penetrance and tissue biodistribution.

Uptake of NM1DRV and NM2DRV in MDM and CD4⁺ T cells

We evaluated NDRV, NM1DRV and NM2DRV cellular activities in both MDM and CD4⁺ T cells. These were assessed in parallel studies after a single exposure to equivalent concentrations of drug or prodrug formulations followed by quantitation of native drug and prodrugs. Both NM1DRV and NM2DRV were readily taken up in MDM, increasing over time to peak levels at 24 hours (Fig 3A). At 24 hours, intracellular prodrug concentrations were 126 nmol/10⁶ cells and 74 nmol/10⁶ cells for NM1DRV and NM2DRV, respectively. Intracellular DRV levels remained less than 0.4 nmol/10⁶ cells over 24 hours after NDRV treatment. The amount of DRV formed from M1DRV was 2.8 nmol/10⁶ cells at 24 hours and an 8-fold enhancement compared to peak drug levels after NDRV treatment (0.31 nmol/10⁶ cells) (Fig 3A). However, DRV formation from M2DRV was consistently at the limit of detection, suggesting that the prodrug was protected against intracellular hydrolysis. Similarly, prodrug uptake in CEM-SS CD4⁺ T cells was rapid for NM1DRV and NM2DRV. Intracellular peak prodrug levels for NM1DRV and NM2DRV treatments were 71 and 49

nmol/10⁶ cells, respectively (Figure 3B). In contrast, NDRV treatment did not produce detectable intracellular drug concentrations at all time points. Taken together, these findings demonstrate formulation stability and rapid prodrug uptake in HIV-1 target cells. Intracellular nanoparticle uptake was confirmed by TEM (Fig 3C). After 4-hours of treatment, both NM1DRV and NM2DRV demonstrated significant nanoparticle accumulation in macrophages compared to NDRV. The electron micrograph images corroborate our prior findings on nanoparticle trafficking and storage in endosomal compartments(22, 46).

Retention and antiretroviral activities of NM1DRV and NM2DRV

To determine the potential for long-term and extended drug release of the DRV prodrug formulations, retention of M1DRV and M2DRV was assessed in MDM after an 8- hour loading. M1DRV and M2DRV were retained within macrophages for 14 and 31 days, respectively, further suggesting their extended release potential (Fig 4A). M1DRV levels in MDM were 0.10 nmol/10⁶ cells at two weeks following NM1DRV treatment, while NM2DRV treatment provided M2DRV concentrations of 25.5 nmol/10⁶ cells at day 31 (Fig 4A). In contrast, drug from NDRV was not detected in MDM within hours of treatment. Drug released from MDM into culture media showed a burst release followed by a rapid decline for M1DRV compared to low but steady release of M2DRV during the 31-day observation period (Fig 4B), presumably due to slow intracellular dissolution of NM2DRV nanoparticles. Overall, these data sets suggest that transformation of DRV into lipophilic prodrugs could facilitate creation of long-acting and slow-release formulations for efficient intracellular drug delivery.

To assess long term intracellular antiretroviral responses, MDM were treated with NDRV, NM1DRV or NM2DRV at equimolar concentrations for 8 hours, followed by drug washout and HIV-1_{ADA} challenge at five-day intervals for a period of 1 to 30 days. In cells treated with NM2DRV no HIV RT activity was detected during the 30-day observation period (Fig 4C). In contrast, viral breakthrough, as determined by RT activity, was observed at days 5 and 20 after treatment with NDRV and NM1DRV, respectively. Cross validation with HIV-1p24 antigen staining mirrored RT results, demonstrating complete viral inhibition for up to 30 days after a single exposure of MDM to NM2DRV (Fig 4D). These findings demonstrate that NM2DRV nanoparticles could potentially sustain therapeutic drug concentrations at cellular sites of infection.

NM1DRV and NM2DRV PK

To determine whether MDM and CD4+ T cell targeted lipophilic prodrug nanoformulations would provide sustained drug levels in plasma and tissues, a PK study was performed in BALB/cJ male mice. Specifically, mice were administered a single intramuscular injection of NDRV, NM1DRV or NM2DRV at 100 mg/kg DRV equivalents. NM1DRV and NM2DRV formulations provided high and sustained prodrug concentrations in plasma. M1DRV concentrations in plasma were 1297.7 ng/mL at day 1 and declined to 5.6 ng/mL at day 14. Plasma M2DRV concentrations at day 28 were 252.4 ng/mL (Fig 5B). Similarly, M1DRV concentrations in blood were 826 ng/mL at day 1 and declined to 5.3 ng/mL at day 28. M2DRV blood concentrations were 2890.2 ng/mL at day 1 and declined to 120.1 ng/mL by

day 28 (Fig 5C). At day 28, levels of DRV generated from hydrolysis of M1DRV and M2DRV in plasma were 2.2 and 8.4 ng/mL, respectively. In contrast, DRV levels after NDRV treatment fell below the limit of quantitation within one day (Fig 5A). Notably, spleen ($8216.5 \text{ ng/g} \pm 1745.2$), liver ($1516.8 \text{ ng/g} \pm 217.2$), lymph nodes ($26848.7 \text{ ng/g} \pm 15689.5$), and brain ($12.2 \text{ ng/g} \pm 3.5$) exhibited high prodrug concentrations at day 28 (Fig 5E) after NM2DRV treatment while NM1DRV provided prodrug concentrations of $55.9 \text{ ng/g} \pm 11.7$, $11.1 \text{ ng/g} \pm 2.3$ and $42.7 \text{ ng/g} \pm 6.7$ in the spleen, liver and lymph nodes, respectively, with detectable DRV levels at day 28. In contrast, NDRV treatment provided drug levels of 3 ng/g or less in lymph nodes and spleen (Fig 5D). High prodrug levels in tissues compared to plasma is suggestive of tissue drug depots for controlled and sustained release into blood and other restricted sites of infection.

DISCUSSION

Antiretroviral therapy has revolutionized HIV-1 treatment and enabled millions of patients to lead near normal lives. However, ART regimens require strict adherence in order to effectively suppress viral replication and achieve optimum therapeutic outcomes. Daily doses of ART cause adverse drug reactions that may yield poor outcomes such as associated anemia, lipodystrophy, skin rashes, bone homeostasis impairment, toxicities to liver, heart, and skeletal muscle among others (1–3). In addition, a plethora of other factors including social stigma, depression, bipolar spectrum disorder, posttraumatic stress disorder and substance abuse, common between HIV-1 patients, can negatively and significantly affect adherence (4, 5). These may lead to avoidance of the prescribed daily regimens giving rise to emergence of drug resistance mutations(6). Moreover, many HIV-1 patients suffer co-infections of associated opportunistic infections further increasing the risk of drug-drug interactions and their associated side effects (7, 8). Collectively, these factors ultimately lead to patterns of non-adherence to the oral regimens as evident in the frequency of missed doses (9). Therefore there has been a paradigm shift towards development of long acting injectable ART formulations and delivery devices to lower organ-specific and systemic toxicities and potentially improve the lifestyle of patients by lessening the burden of pill fatigue and dosing intervals. Hence improving current ART therapy available for HIV-1 patients is a critical area for study (47).

Long-acting formulations could potentially have a significant impact on HIV treatment and prevention strategies (13, 15, 48). In a survey of 400 HIV-1 positive individuals, 73% expressed their interest in long acting injectable therapy (14). Despite ongoing efforts and documented benefits from long acting agents in other chronic disease conditions, only two long acting ARV formulations containing rilpivirine (RPV LA) and cabotegravir (CAB LA) have successfully progressed into clinical trials (49–53). Moreover, these LA drugs were recently submitted to the FDA for approval as the first monthly injectable two-drug regimen for treatment of HIV. It is worth noting that the selection of an agent for a LA injectable formulation requires compounds with high potency and appropriate physicochemical features since there are limitations for the volume administered in a single injection (54). CAB and RPV are hydrophobic and potent compounds making them suitable candidates for extended release aqueous nanosuspensions. On the other hand, protease inhibitors are known for their short half-lives, low plasma concentrations, high dosing regimens, poor cell

penetration, and low oral bioavailability (30, 36). Since darunavir and most protease inhibitors are hydrophobic, attempts have been made to enhance drug targeting and metabolic stability through nanoformulation of parent drugs (55, 56). However, native drug formulations exhibited limited improvement in drug biodistribution and PK parameters (55). Darunavir was also found to be incompatible with lipid-based nanoparticles due to poor formulation stability (57). Others have explored prodrug strategies to improve ADME properties of PIs (30–33). Such attempts have either resulted in a marked loss of antiviral activity or exhibited comparable half-lives to that of parent drugs (36–38). Thus, the realization of long acting delivery systems for PI has remained elusive for decades.

Our laboratory has previously demonstrated extension of apparent half-lives of hydrophilic and hydrophobic ARVs through synthesis and creation of lipophilic antiretroviral prodrug nanocrystals coined as long acting slow effective release antiretroviral therapy (LASER ART) (16, 18–22). LASER ART enabled slow release and activation of prodrugs within cellular and tissue reservoirs of infection after parenteral administration (18, 20). We therefore sought to transform DRV into LASER ART nanocrystals. We reasoned that conversion of DRV into prodrugs was not only feasible and economical but also a reasonable strategy for overcoming ADME and formulation challenges associated with the parent drug. Herein, we describe the development of two long acting DRV prodrug formulations, NM1DRV and NM2DRV, with extended in vitro antiretroviral activities and improved drug half-lives.

Darunavir ester prodrugs were synthesized by conjugating reversible fatty acid lipids to either primary amine (M1DRV) or secondary hydroxyl (M2DRV) groups on the native drug. To our knowledge, amine modification has not previously been explored for protease inhibitor prodrugs. After successful synthesis and characterization of the prodrugs, we then developed stable poloxamer stabilized nanosuspensions of the prodrugs using simplified top down technologies used in the manufacture of LASER ART nanocrystals and other pharmaceutical LA injectable products (18, 50, 52, 58). The amine modified ester prodrug formulation (NM1DRV) demonstrated comparable EC_{50} to native DRV in human MDM and CEM CD4+ T cells. This is significant since previous studies with ester prodrugs of PIs in similar cell lines demonstrated diminished potency when compared to native drugs (37, 38). However, modification of the hydroxyl functional group in NM2DRV resulted in a 10-fold loss in potency. Nevertheless, the EC_{50} of NM2DRV against HIV-1 in MDM and CD4+ T cells was in the nanomolar range and exhibited long-term efficacy when compared to native drug or M1DRV nanoformulations. The observed antiviral activity for NM1DRV and NM2DRV formulations is likely linked to improved cellular prodrug uptake. However, unlike M1DRV that hydrolyses back to DRV, M2DRV was found to be hydrolytically stable inside cells, suggesting that the prodrug is predominantly responsible for efficacy. Prodrugs are generally pharmacologically inactive compounds that require enzymatic or chemical hydrolysis to produce the corresponding active parent drugs (39, 59, 60). The observed efficacy data sets for NM2DRV were surprising in the context of previous studies that demonstrated an almost complete loss in antiviral activity when the secondary hydroxyl group in PIs was irreversibly blocked from interacting with the catalytic site of HIV protease (38). Therefore, future studies will be required to elucidate association of M2DRV with the viral enzyme.

Ideal LA ARV formulations should be potent, safe, scalable, and stable to deliver therapeutic concentrations of active agents at infection sites. Given HIV-1 has been shown to infect and replicate in macrophages and CD4+ T cells (61–63), optimal drug delivery strategies should target ART to these cell subsets and other tissue reservoirs of infection where conventional therapies and parent drug formulations have limited access (63). We have previously shown that macrophages can serve as drug depots (64, 65) and play a pivotal role in nanoparticle trafficking and dissemination to CD4+ T cells and tissues (20, 66–68). We have also shown that macrophage targeting prolongs drug half-life to improve efficacy and PK profiles (18, 20). Uptake studies confirmed that NM1DRV and NM2DRV were rapidly taken up and retained by MDM and CD4+ T cells compared to NDRV. Of significance, the presence of high intracellular prodrug concentrations had no negative effect on cell viability. As a result of enhanced MDM drug uptake and sustained retention, the prodrug formulations exhibited superior antiretroviral activities compared to native DRV formulation. Notably, complete viral inhibition was maintained for up to 30 days after single exposure to NM2DRV compared to 15- and 5-days protection for NM1DRV and NDRV treatments, respectively. The ability of NM2DRV to protect MDM from HIV-1 infection for extended periods of time is a significant step towards transforming DRV into long acting cell targeted formulations that could be used in combination with other long acting ART for HIV-1 treatment.

It has previously been demonstrated that therapeutic drug concentrations in plasma after administration of native ART formulations does not reflect cellular and tissue levels presumably due to limited drug access to restricted sites of viral growth (18, 20). Single intramuscular administration of NM1DRV or NM2DRV to BALB/cJ mice provided sustained prodrug concentrations in blood, plasma and tissues for one month. In contrast, drug concentrations from NDRV treatment declined to undetectable levels within a day. Notably, M2DRV was detected in the brain at day 28, which is significant since protease inhibitors have limited permeability across the blood brain barrier (30, 38). High prodrug levels in blood and tissue reflect the formation of cellular and tissue drug depots for sustained release. Overall, these data sets suggest that transformation of DRV into lipophilic prodrugs could enable creation of cellular and tissue targeted long-acting formulations.

In summary, amine and alcohol ester lipophilic prodrugs of darunavir were successfully synthesized and stabilized into long acting slow effective release aqueous nanosuspensions. NM2DRV was found to exhibit enhanced intracellular prodrug levels with sustained retention and efficacy. PK tests of NM1DRV and NM2DRV administered in mice demonstrated sustained drug levels in blood and tissues with higher concentrations of the prodrugs observed over 28 days. These data, taken together, support the idea that tissue and cell targeted lipophilic prodrugs could facilitate transformation of DRV into long acting sustained release formulations with improved antiretroviral activities. Future studies will investigate antiretroviral efficacy of the prodrug formulations in humanized mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Synthesis and characterization of DRV prodrugs.

(A) Amine (M1DRV) and hydroxyl (M2DRV) modified DRV ester prodrugs were synthesized in high chemical yields. (B) Aqueous solubility demonstrated enhancement in prodrug hydrophobicity (**** $P < 0.0001$ for DRV compared to M1DRV or M2DRV, * $P = 0.0258$ for M1DRV versus M2DRV). (C) and (D) Hydrolysis of M1DRV and M2DRV in mice and rat plasma over 48 hours at 37°C demonstrating enzymatic degradation of the prodrugs over time. Data are expressed as mean $\pm$ SEM for $n = 3$ samples per group.

Figure 2. Biological characterizations of NDRV, NM1DRV and NM2DRV.

Cytotoxicity of drug formulations in MDM (A) and CEM-SS CD4+ T cells (B) was assessed by MTT and Live/Dead Fixable Dead Cell Stain Kits. Antiretroviral activities against HIV-1 were determined in MDM (C) and CEM-SS CD4+ T cells (D). Data are expressed as mean $\pm$ SEM for n=3 samples per group.

Figure 3. NDRV, NM1DRV and NM2DRV uptake in MDM (A) and CEM-SS CD4+ T cells (B). Results demonstrate high intracellular drug levels for NM1DRV and NM2DRV treatments compared to undetectable levels for NDRV (^^^^P<0.0001 for NDRV versus NM1DRV for parent drug quantitation and ****P<0.0001 and ***P=0.0007 for NM1DRV versus NM2DRV for prodrugs quantitation). (C) TEM images of MDM loaded with native drug and prodrug nanoformulations. Data are expressed as mean $\pm$ SEM for n=3 samples per group.

Figure 4. Drug retention and antiretroviral activities in MDM after a single 8-hour 100 μ M drug treatment.

(A) **NM1DRV** and **NM2DRV** were retained in MDM for 14 and 31 days, respectively, while **NDRV** was washed out within hours. **** $P < 0.0001$ for **NM1DRV** versus **NM2DRV**. (B) Consequently, **NM1DRV** and **NM2DRV** exhibited sustained release of drug into culture media. Antiretroviral activity was determined by RT activity (C) and HIV-1p24 antigen staining (D). **NM2DRV** formulation demonstrated long-term viral suppression for a period of 30 days compared to viral breakthrough within 15 days and one day for **NM1DRV** and **NDRV**, respectively. Data are expressed as mean $\pm$ SEM for $n=3$ samples per group.

Figure 5. PK in mice.

(A) Plasma DRV levels demonstrating sustained drug release for NM1DRV and NM2DRV formulations. (B) Plasma M1DRV and M2DRV levels. (C) Blood M1DRV and M2DRV levels. (D) Tissue DRV concentrations at day 28. (E) Prodrug levels in the lymph nodes, spleen, liver, and brain demonstrating high prodrug concentrations for NM2DRV compared to NM1DRV treatment. Data are expressed as mean $\pm$ SEM for n=5 mice per group.