Pathways for Dolutegravir Transformation from a Daily Oral to a Once-a-Year Parenteral Medicine

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Pathways for Dolutegravir Transformation

from a Daily Oral to a Once-a-Year Parenteral Medicine

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Abstract

An “ultra” long-acting (LA) integrase strand transfer inhibitor was created by screening a library of monomeric and dimeric dolutegravir (DTG) prodrug nanoformulations. This led, to the best of our knowledge, a “first in class” 18-carbon chain modified prodrug nanocrystal (coined as NM2DTG). Ideal physiochemical and pharmacokinetic (PK) properties facilitated slow drug release from tissue macrophage prodrug depot stores for up to one year after a single intramuscular injection. Both the muscle injection site and secondary lymphoid tissues were depots of prodrug hydrolysis; dependent on nanocrystal dissolution and prodrug release rates, drug-depot volume, perfusion and cell-tissue pH. Each affected an extended NM2DTG apparent half-life by recorded PK parameters. The NM2DTG LA product can impact therapeutic adherence, tolerability, and access of a widely used integrase inhibitor in resource limited and rich settings to reduce HIV-1 transmission and achieve optimal treatment outcomes.

One-Sentence Summary (125 characters)

A single parenteral dose of lipophilic dolutegravir prodrug nanocrystal sustained plasma drug levels above PA-IC₉₀ for up to a year.

Introduction

Vaccination remains the principal means to prevent viral infections enabling elimination of smallpox, measles, polio, and rubella [1-4]. However, such success has not been achieved for the human immunodeficiency virus type one (HIV-1). Despite four decades of research, complete prevention of HIV-1 transmission has not been achieved. Viral suppression and pre-exposure prophylaxis (PrEP) was realized only by antiretroviral therapy (ART) [5]. While ART treated HIV-1-infected or susceptible persons has reduced morbidity and mortality signs and symptoms of infection continue coordinate with low-level viral replication [6, 7]. ART remains the “gold standard” of treatment for people living with HIV-1 (PLWH). Nonetheless regimen limitations in drug compliance, toxicities, and tolerability affect viral drug resistance [8, 9]. Treatment cessation leads to viral rebound coincident with co-morbid cancers and opportunistic infections [10-12]. This includes hepatitis B and C infections where chronic antiviral therapy is mandated [13]. Thus, while ART profoundly improves PLWH life quality and longevity, therapeutic limitations remain [14].

The major deterrent to ART efficacy is regimen adherence linked to the social stigma of storing and taking daily medicines along with depression and substance abuse disorders. These also affect HIV-1 transmission rates [15-17]. These concerns have ushered in an era of long-acting (LA) parenteral ART. LA ART drugs include cabotegravir (CAB), rilpivirine, and lenacapavir amongst others [18-22]. Each are designed for therapeutic and or PrEP applications [22-24].

Injection site reactions, administration volume, drug-drug interactions, resistance, and the required monthly to bimonthly parenteral drug administration limit LA ART use presenting therapeutic

challenges [25-27]. One remedy rest in achieving extended plasma drug levels above the protein-adjusted (PA) IC₉₀ for up to one year. With this need in mind, we have now achieved such a drug dosing interval by creating a DTG prodrug encased in a surfactant-coated nanocrystal (coined as NM2DTG). NM2DTG significantly extends the DTG apparent half-life for up to one year. Microscopy and spectroscopy studies affirm the stable and unique prodrug-nanoparticle composition. Prodrug release from the nanocrystal is linked to its novel physiochemical properties and tissue protein and lipid compositions. Drug concentrations, injection volume and content, tissue perfusion and composition, pH, chemical and enzymatic hydrolysis rates, and prodrug dissolution are linked to an optimal monomeric 18-carbon fatty-acid carbon chain length that affects an extended apparent DTG half-life. The interplay between prodrug hydrophobicity and lipophilicity, particle composition, and tissue environment determine the rate at which the prodrug nanocrystals release the active medicine. The prodrug presence at the injection site allows a convenient drug removal if and when warranted for secondary adverse reactions. These biochemical, pharmacological, and biological events underly DTG's transformation pathway from a daily oral to a year-long parenteral medicine. The impact of the reported NM2DTG formulation rests in its utility to prevent HIV-1 transmission and adherence to drug regimens where extended dosing can significantly affect disease prevention and treatment outcomes [28].

Materials and methods

Reagents

DTG was purchased from BOC Sciences (Shirley, NY, USA). Pyridine, dimethylformamide (DMF), N,N-diisopropylethylamine (DIEA), myristoyl chloride, stearoyl chloride, behenic acid, octadecanedioic acid, Pluronic F127 (poloxamer 407; P407), polyethylene glycol₃₃₅₀ (PEG₃₃₅₀),

ciprofloxacin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 1-octanol, paraformaldehyde (PFA), and 3,3'-diaminobenzidine (DAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl ether, ethyl acetate, hexanes, dichloromethylene (DCM), acetonitrile (ACN), methanol, optima-grade water, Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), gentamicin, L-glutamine, potassium phosphate monobasic (KH₂PO₄), bovine serum albumin (BSA), and Triton X-100 were purchased from Thermo Fisher Scientific/Gibco (Waltham, MA, USA). Cell culture grade water (endotoxin-free) was purchased from Cytiva (Logan, UT, USA). Monoclonal mouse anti-human HIV-1p24 (IgG_{2a}; clone 05-001) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Polymer-based HRP-conjugated anti-mouse EnVision+ secondary was purchased from Dako (Carpinteria, CA, USA). Heat-inactivated pooled human serum was purchased from Innovative Biologics (Herndon, VA, USA).

Synthesis and characterization of DTG prodrugs

Three monoester prodrugs were synthesized by esterifying the DTG hydroxyl group, yielding lipophilic prodrugs with 14-, 18-, or 22-carbon chains. They are named MDTG [29], M2DTG, and M3DTG, respectively. Additionally, a fourth prodrug M4DTG was synthesized by parallel esterification with two DTG molecules on either end of a single 18-carbon chain. For synthesis, DTG was dried from anhydrous pyridine then suspended in anhydrous DMF. The mixture was cooled to 0°C under argon. DIEA (2 equivalents) deprotonated the hydroxyl group of DTG, which was then reacted with 2 equivalents myristoyl- or stearyl-chloride for 18 hours to create MDTG or M2DTG. M3DTG and M4DTG were prepared by first activating behenic or octadecanedioic acid to their acylchloride forms using thionyl chloride. The formed chlorides were then reacted

with deprotonated DTG enabling the creation of the final prodrugs. These were purified by silica gel column chromatography employing an eluent of 4:1 then a 9:1 mixture of ethyl acetate and hexanes. The desired compound fractions were acquired from the columns, then dried on a rotary evaporator, precipitated from diethyl ether, and recrystallized from DCM. Finally, the prodrug powders were further dried under a high vacuum providing average chemical yields of 85-95%. Prodrug synthesis was confirmed by proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) spectroscopy using Bruker Avance-III HD (Billerica, MA, USA) operating at 500 MHz, a magnetic field strength of 11.7 T.

Solubility

Solubility was determined by adding an excess of the drug to water or 1-octanol at room temperature and mixing for 24 h. Samples were centrifuged at $20,000 \times g$ for 10 min to pellet insoluble drug. Aqueous supernatants were frozen, lyophilized then resuspended in methanol. 1-octanol products were prepared for analysis by dilution in methanol, and samples were analyzed for drug content by UPLC-TUV.

Nanoparticle preparation and characterization

Nanoformulations of DTG (NDTG) and all prodrugs (NMDTG [\[29\]](#), NM2DTG, NM3DTG, and NM4DTG) were manufactured by high-pressure homogenization using P407 as the surfactant. Each solid drug or prodrug was dispersed in a P407 solution in endotoxin-free water to form a presuspension. The drug or prodrug to surfactant ratio was maintained at 10:1 (w/w), and a suspension concentration was in the range of 1%-30% (w/v) of drug/prodrug and 0.1-3% (w/v) of

P407. The presuspension was homogenized on an Avestin EmulsiFlex-C3 high-pressure homogenizer (Ottawa, ON, Canada) at $20,000 \pm 1,000$ PSI to form the desired particle size. Nanoparticles were characterized for hydrodynamic particle diameter (size), polydispersity indices (PDI), and zeta potential as measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS (Worcestershire, UK). The physical and chemical stabilities of the nanoformulations were monitored at 4, 22 and 37°C. Drug and prodrug concentrations were determined by dissolving the nanoformulations in methanol (1,000 - 100,000-fold dilutions). These were then analyzed by UPLC-TUV and processed for sterile use.

Isolation and cultivation of human monocyte-derived macrophages (MDM)

Human monocytes were obtained by leukapheresis from HIV-1/2 and hepatitis B seronegative donors and purified by counter-current centrifugal elutriation. Monocytes were cultured in conditions detailed in our previous works [[29](#), [30](#)]. After differentiation, MDM were used for the drug-particle uptake, retention, and antiretroviral assays.

Drug-nanoparticle uptake and retention

The *in vitro* assessment of nanoformulation uptake and retention in MDM were performed as detailed in our previous works [[29](#), [30](#)]. For drug nanoparticle cellular uptake studies, MDM were treated with 5 or 25 μ M NDTG, NMDTG, or NM2DTG and collected at 2, 4, 8, and 24 h following treatment. For retention studies, MDM was treated with 5 or 25 μ M NDTG, NMDTG, or NM2DTG for 8 h, and washed cells were collected at days 1, 10, 20, and 30, to be analyzed for drug and prodrug content by UPLC-TUV.

Morphological evaluation of intracellular nanoparticles

MDM were treated with 25 μ M NM2DTG for 8 h, and collected at days 0, 10, 20, and 30 after treatment and analyzed by transmission electron microscopy (TEM) to visualize intracellular nanoparticles as detailed in our previous works[[29](#), [30](#)]. Images were acquired digitally with an AMT digital imaging system (Woburn, MA, USA).

Measurements of antiretroviral activities in HIV-1 challenged MDM

The study comprised of MDM treatments with 1 or 10 μ M NDTG or NM2DTG for 8 h. At 5, 10, 15, 20, 25, and 30 days after treatment, the cells were challenged with HIV-1_{ADA} at a multiplicity of infection (MOI) of 0.1 infectious particles/cell for 4 h according to previous established protocols [[29-31](#)]. Cells were fixed in 4% PFA at each time point, and expression of HIV-1p24 antigen was determined by immunocytochemistry.

Measures of the half maximal inhibitory concentration (IC₅₀) of DTG formulations

The IC₅₀ determination in MDM was conducted as per previously established protocols [[29](#), [30](#)], wherein the cells were treated with a range of drug concentrations, 0.01-1,000 nM of DTG, MDTG, or M2DTG dissolved in 0.1% (v/v) DMSO for 1 h prior to challenge with HIV-1_{ADA} (MOI of 0.1) for 4 h. Cell supernatants were collected on day 10 and assayed for HIV-1 RT activity.

PK studies in rodents and rhesus macaques (RM)

Male Balb/cJ mice (6-8 weeks, Jackson Labs, Bar Harbor, ME, USA) were administered a single intramuscular (IM; caudal thigh muscle) of 45 mg DTG-equivalents (eq.)/kg of NDTG, NMDTG, NM2DTG, NM3DTG, or NM4DTG in a maximum volume of 40 μ L/25 g mouse [[29](#)]. Following

injection, blood samples were collected into heparinized tubes on day 1 post-drug administration and then weekly until 1 year by cheek puncture (submandibular vein) using a 5 mm lancet (MEDIpoint, Mineola, NY, USA). Blood samples were centrifuged at $2,000\times g$ for 8 min for plasma collection and drug content quantitation. On day 367, after drug administration, animals were humanely euthanized using isoflurane followed by cervical dislocation.

Male Sprague-Dawley (SD) rats (186-225 g, SASCO, Wilmington, MA, USA) were administered a single intramuscular (IM) dose in the caudal thigh muscle of 45 mg DTG-eq./kg of NDTG, NM2DTG, or sterile saline in a maximum volume of 200 μ L/200 g rat. Following injection, blood samples were collected into heparinized tubes at 4 h, day 1, 4, and 7 after administration, then weekly for 6 months and monthly from time points of 6 months to 1 year. Blood draws were made through retro-orbital plexus bleeds. Blood samples were centrifuged at $2,000 \times g$ for 8 minutes for plasma collection and quantitation of plasma drug contents. At days 57, 175, and 364 following drug administrations, animals were humanely euthanized and tissues (spleen, liver, lymph nodes, muscle [site of injection], kidney, lung, gut, brain, heart, and rectal tissue) were collected for quantitation of DTG and prodrug levels and/or histology. Part of each tissue was placed in an Eppendorf tube on dry ice and stored at -80°C for later drug analysis. Each tissue was placed in 10% neutral buffered formalin for immunohistochemistry, pathology, and toxicity studies. On day 3, following drug administration, animals were humanely euthanized, and muscle tissue from the injection site and contralateral control muscle were collected for drug analysis, immunohistochemistry, pathologic and electron microscopy studies. The muscle tissue containing the site of injection was collected, divided in half, and fixed appropriately for either immunohistochemistry/pathology or electron microscopy. For histological examination, 5 μ m

sections of paraffin-embedded tissues were stained with hematoxylin and eosin (H&E). Images were captured using a Nuance EX multispectral imaging system affixed to a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY, USA). A board-certified pathologist conducted histopathological assessment according to the Society of Toxicologic Pathology [32]. Toxicity in SD rats was assessed by evaluating complete blood counts, serum chemistry profiles, and histological examination [33]. At sacrifice time points, blood was collected into potassium-EDTA coated tubes for hematology analysis using a VetScan HM5 veterinary hematology blood analyzer (Abaxis Veterinary Diagnostics, Union City, CA, USA). Serum chemistry profiles were determined using a VetScan comprehensive diagnostic profile disc and a VetScan VS-2 instrument (Abaxis). Results for treated animals were compared to those from age-matched untreated control rats.

Female rhesus macaques (RM) were administered an intramuscular (IM) dose in the quadriceps muscle of 45 mg DTG-eq./kg NM2DTG in a maximum volume of 0.5 mL/kg (not to exceed 1.5 mL/injection sites). The NM2DTG nanoformulations were prepared in the Nebraska Nanomedicine Production Plant by established good laboratory practice (GLP) protocols [34]. Following injection, blood samples were collected into EDTA tubes at days 1, 3, 7, and 14 after administration, then biweekly until 3.5 months and monthly from 3.5 months to 6.5 months. Blood draws were made following ketamine anesthesia. Blood samples were centrifuged at $1,000 \times g$ for 20 minutes to collect and quantify plasma drug contents. General animal well-being and recorded movement and skin reactions were recorded. A second booster dose was given on day 217 in the same manner. Blood samples were collected on days 1, 3, 7, 14, and 21 after a boost, then biweekly

until 11.25 months (total) and monthly until day 345. Complete blood count and comprehensive metabolic panel were performed all the time of blood collections.

DTG and M2DTG were quantitated in mouse, rat, and rhesus plasma and tissues by UPLC-tandem mass spectroscopy (MS/MS) using a Waters ACQUITY H-class UPLC connected to a Xevo TQ-S micro mass spectrometer and described in the supplementary methods. All solvents for sample processing and UPLC-MS/MS analysis were Optima-grade (Fisher). Non-compartmental PK for plasma DTG in mice and rats was performed with Phoenix WinNonlin-8.0 software (Certara, Princeton, NJ, USA).

Transmission electron microscopy (TEM)

TEM imaging tissue and cell samples were processed according to previously optimized protocols [30, 35] and examined on a Tecnai G² Spirit *TWIN* (Thermo Fisher Scientific) operating at 80kV.

Prodrug hydrolysis kinetics in tissue and plasma

Male SD rats (SASCO) were humanely euthanized, perfused, and tissues collected as described previously. Samples were stored at -80 °C until further processed as described previously. M2DTG prodrug solution, dissolved in 1% (v/v) methanol and aqueous prodrug nanoformulation (NM2DTG) were used as substrates for cleavage in various rat tissue homogenates. After preincubation of 100 µL of tissue homogenate at 37 °C for 5 min, the reactions were initiated by the addition of the substrates and stopped by the addition of 900 µL of acidified methanol (0.1% formic acid and 2.5 mM ammonium formate in Optima-grade methanol) at 30 min, 2, 6, 12, 24, and 48 h time points. Control samples were incubated using the same method but with substrates

added after adding acidified methanol. The mixtures were centrifuged at 16,000 x g for 10 min to remove precipitated protein. The supernatants were aspirated and stored at -80 °C until analysis. The supernatant was diluted 1:1 with internal standard (IS; DTG-d3 20 ng/mL, 40 ng/mL SDRV, 40 ng MDRV), vortexed for 30 seconds and transferred to a 96-well plate to be injected onto the UPLC-MS/MS system for drug quantitation and described in the supplementary methods.

pH affects for prodrug cleavage

The contribution of pH to prodrug cleavage was studied in pH buffers including 7.5 mM ammonium acetate (pH 6.0, adjusted with acetic acid), 7.5 mM ammonium bicarbonate (pH 8.0, adjusted with acetic acid) and pH adjusted solutions including 0.1% formic acid (pH 2.0), and 0.1% ammonium hydroxide (pH 10.3). M2DTG prodrug solution and NM2DTG suspension were incubated in these matrices as per the protocol for rat tissue homogenates and analyzed as such.

Statistical analyses

For all studies, data were analyzed using GraphPad Prism 7.0 software (La Jolla, CA, USA) and presented as the mean $\pm$ the standard error of the mean (SEM). Experiments were performed using a minimum of three biologically distinct replicates. Samples sizes were not based on power analyses. Animal studies included a minimum of three animals per group, with the number of replicates (N) listed. Extreme outliers beyond the 99% confidence interval of the mean and 3-fold greater than the SEM were excluded. Significant differences were determined at $P < 0.05$.

Study approvals

All experimental protocols involving the use of laboratory animals were approved by the UNMC 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) ensuring the ethical care and use of laboratory animals in experimental research with protocol 20-075-09-FC. All animal studies were performed according to University of Nebraska Medical Center (UNMC) institutional and National Institutes of Health guidelines for laboratory animal housing and care. Human blood cells were isolated by leukapheresis from HIV-1,2 and hepatitis seronegative donors and were deemed exempt from approval by the UNMC Institutional Review Board.

Results

Prodrug synthesis and physicochemical characterizations

We now demonstrate that optimal ester carbon lengths can yield substantive changes to the pharmacological properties of LA antiretroviral drugs (ARVs) [30, 36]. The current report demonstrates that attachment of DTG on one side of an 18-carbon fatty acid chain promoiety through an ester linkage markedly extends the drug's apparent half-life when compared to other prodrug monomers of varied chain lengths or an 18 carbon DTG dimer. Notably, twenty-two carbon fatty acid chain prodrug modifications adversely influenced PK tests (Fig. 1a). The DTG and its prodrugs were characterized by Fourier-transformation infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR), and electrospray ionization mass spectrometry (ESI-MS) (Fig. 1b, S1-5). Altogether the results showed that attachment of variable carbon chains to the parent drug affect both the PK parameters and aqueous solubility (Fig. 1c). Octanol solubility for

MDTG, M2DTG, and M3DTG was dependent on the hydrocarbon chain length (Fig. 1d). However, M4DTG, with an 18-carbon fatty acid with DTG molecules attached on both ends, exhibited higher aqueous solubility than the 18-carbon lipid bearing a single DTG attachment. This also resulted in the octanol solubility reflective of the parent drug. To determine whether the chemical modifications influenced antiviral activity, the half-maximal inhibitory concentration (IC_{50}) of the prodrugs were tested in human MDM challenged with HIV-1_{ADA}. HIV-1 reverse transcriptase (RT) activities from each of the treatment groups demonstrated comparable IC_{50} values for DTG, MDTG, and M2DTG (Fig. 1f; 2.4, 3.2, and 3.1 nM, respectively). Such modifications also elicited stable drug-to-polymer interactions through increased hydrophobicity and lipophilicity, limiting degradation of the nanoparticles and drug dissolution from the solid nanocrystal matrix. Nanoformulations of DTG (NDTG), MDTG (NMDTG), and M2DTG (NM2DTG) were generated by high-pressure homogenization. XRD confirmed the crystalline form of the drug nanoformulations, with NMDTG and NM2DTG having similar diffraction patterns divergent from that of NDTG (Supplementary figure 6). The thermal properties and physical states of the prodrugs and their respective nanoformulations were studied by differential scanning calorimetry (DSC) (Fig. 1e, S7) and thermogravimetric analysis (TGA) (Supplementary figure 8). The DSC thermograms affirmed content uniformity and lack of thermally distinct polymorphs. The TGA thermograms showed that the drugs (Supplementary figure 8a) and nanoformulations (Supplementary figure 8b) lacked solvent residues and were stable across temperatures of 30-300°C. Physical stability of NDTG and NM2DTG was monitored by measuring particle hydrodynamic diameter (size), polydispersity (PDI), and zeta potential during storage by dynamic light scattering (DLS; Fig. 1g-h, S9). Particle size and PDI was unchanged over 265 days. The stability of the nanoformulations were investigated by parent drug and or

prodrug quantification over 301 days during room temperature storage (Fig. 1i). The nanoformulations showed consistent drug levels with limited evaporation of the NM2DTG formulation.

Macrophage uptake, retention, and antiretroviral activities

NM2DTG was readily taken up by MDM at both 5 and 25 μM treatment concentrations during a 24 h evaluation (Supplementary figure 11a, 2a, respectively). Dose-dependent differences in uptake were observed, with 13.9 and 75.5 nmol M2DTG/ 10^6 cells following 5 or 25 μM treatment at 24 h. Higher drug uptake was seen for NM2DTG when compared against NMDTG. For each drug concentration, free drug and nanoformulation showed no cytotoxicity by MTT tests (Supplementary figure 10). Following 8 h drug loading, MDM retained significantly higher amounts of NM2DTG at both 5 and 25 μM concentrations over a 30-day test period (Supplementary figure 11b, 2b, respectively). NMDTG was near baseline at day 10 for both doses, while M2DTG levels were consistent over 30 days. Dose-dependent differences in retention were also observed, with 5.7 and 21.07 nmol M2DTG/ 10^6 cells present at day 30 following 5 or 25 μM treatment. NDTG yielded little to no uptake or retention of drug in MDM at any recorded time point. TEM visualized intracellular particles after treatment with 25 μM NM2DTG for 8 h in MDM (Supplementary figure 12). NM2DTG was observed in intracellular MDM vesicles immediately after treatment and extending to day 30. These data sets affirmed the recorded retention drug levels. The antiretroviral activities of the DTG prodrug nanocrystals were evaluated after a single 8 h administration of 1 or 10 μM to MDM followed by a challenge with HIV-1_{ADA} at five-day intervals to day 30. Viral infection was assessed by HIV-1p24 antigen expression in cells by immunocytochemical staining (Fig 2c). NDTG showed evident viral replication beginning at day

5 at both 1 and 10 μ M treatments, while NM2DTG protected MDM against HIV-1 challenge for 30 days at both drug concentrations.

Histopathology of NM2DTG intramuscular injection

The intramuscular delivery of NM2DTG demonstrated that the muscle was the primary drug depot site. The histiocytic infiltration and the generation of a local injection site granulomatous reaction was induced by intramuscular delivery of NM2DTG at 45 mg DTG-eq./kg, as visualized by H&E staining at day 3 after drug administration (Fig. 2f-h). Amorphous material believed to be part of the formulation depot, was also observed in the muscle surrounded by histiocytic cells (Fig. 2f, g). The macrophages present at the NM2DTG injection site readily phagocytose the nanoformulation with intracellular storage. The low pH, in the cellular and tissue microenvironments, resulted in slow prodrug cleavage rates. Uninjected tissue showed normal muscle histology (Fig. 2d). Sham, saline-injected, controls showed limited macrophage responses (Fig. 2e). H&E staining at day 57 after drug administration showed a return to normal muscle histology, like that seen in the uninjected controls (Fig. 2i). Electron microscopic images of uninjected or sham controls affirm regular or minimal changes in muscle histology (Fig. 2j-m). In contrast, the NM2DTG-injected group showed extensive histiocytic infiltration with clusters of fused nanocrystals and endocytic drug particle contents (Fig. 2n-o).

Pharmacokinetics (PK)

PK tests were performed in male Balb/cJ mice, male SD rats, and female rhesus macaques. Male Balb/cJ mice were administered a single 45 mg DTG-eq./kg (equimolar DTG) dose of NDTG, NM2DTG, NM3DTG, or NM4DTG intramuscularly (IM) into the caudal thigh muscle

to determine PK profiles during a one-year observation period. Plasma samples were analyzed by UPLC-MS/MS to assess parent drug levels (Fig 3a). NM2DTG displayed a significantly reduced DTG decay curve compared to any of the other formulations, with plasma drug levels dropping below the protein-adjusted IC_{90} ($PA-IC_{90} = 64$ ng/mL) at > 367 days. Plasma levels were at or below the limit of quantitation ($LOQ = \sim 1$ ng/mL) at day 35 for NDTG, day 287 for NM2DTG, and day 210 for NM4DTG, while they remained above the $PA-IC_{90}$ for the entire test period for NM2DTG (69.6 ng/mL at day 367). DTG apparent half-life increased from 3.48 days for NDTG and 39.1 days for NM2DTG, to 167.9 days for NM2DTG in plasma of mice after receiving single drug formulation injections (Supplementary Table 2).

Male SD rats were administered a single 45 mg DTG-eq./kg dose of NDTG or NM2DTG intramuscularly (IM) into the caudal thigh muscle to determine PK over 1 year. Plasma samples were analyzed by UPLC-MS/MS to assess parent drug levels (Fig 3b). NM2DTG displayed a significantly reduced DTG decay curve compared to NDTG in mice and rats. Plasma levels were at or near the $LOQ = \sim 1$ ng/mL at day 42 for NDTG (1.1 ng/mL at day 42), while they remained above the $PA-IC_{90}$ until day 308 for NM2DTG (77.4 ng/mL at day 308). Peak plasma M2DTG levels were at 4 hours (100.8 ng/mL), but only detectable at day 7 (1.4 ng/mL) where they fell below the LOQ (Fig. 3b insert). Tissue biodistribution was assessed at days 57, 175, and 364 for both prodrug (M2DTG) and parent drug (DTG) in muscle injection site, spleen, lymph node, and liver (Fig. 3d-g). Parallel drug measurements were done in the lung, gut, kidney, rectum, and brain (Supplementary figure 14a-e). Tissue drug levels following NDTG treatment were limited, with a maximum of 66.7 ng/g in lymph nodes at day 57. Tissue drug levels following NM2DTG were notably higher, with measurable parent and prodrug at all time points in all tissues apart for the

brain. Liver and spleen exhibited higher levels of prodrug than parent drug, while lymph nodes had similar levels at each time point. Kidney showed prodrug at or slightly above baseline measurement levels. Significant active drug levels were observed. This likely reflects native drug excretion. Lymph nodes, lungs and rectum showed the highest drug levels. The highest prodrug levels were detected at the site of injection and maintained throughout the year-long study. Taken together, the data sets indicate that the muscle represented the primary drug depot. There the nanocrystals were either slowly absorbed or dissociated releasing the prodrug which was then converted into the active native drug. Neither NDTG nor NM2DTG treatments had any adverse effect on animal weight or metabolic profiles, with no lasting differences between treatments and controls (Supplementary figure 13 and supplementary table 1). Modest metabolic differences were observed briefly after NM2DTG administration at day 3 but were quickly resolved without lasting effects. No erythema or swelling were observed at the injection site. DTG apparent half-life increased from 4.53 days for NDTG to 108.76 days for NM2DTG in rat plasma after receiving single drug formulation injections.

Female rhesus macaques (RM) were administered a 45 mg DTG-eq./kg dose of NM2DTG intramuscularly (IM) into the quadriceps muscle to determine PK. A first dose was given at the start of the study and a second booster dose was administered on day 217. Plasma samples were analyzed by UPLC-MS/MS to determine parent drug levels (Fig 3c). As observed in rodents, NM2DTG displayed a greatly reduced DTG decay curve. Following the first dose, plasma levels were at or above the PA-IC₉₀ until day 154 (65.3 ng/mL). Before the second booster dose on day 217 plasma DTG levels fell to 45.3 ng/mL. After boosting, plasma DTG levels rose to match those seen after the first dose (974.7 vs 1,102 ng/mL, respectively), however, plasma prodrug levels

were 1.6 times higher following the boost than the first dose (Fig. 3c). The PK parameters have been provided in supplementary table 3. This greatly outperformed previous studies in rhesus macaques given a single IM injection of NM2DTG at 25.5 mg DTG-eq./kg [34]. Plasma DTG levels of only 86 and 28 ng/mL were observed on days 35 and 91, respectively, with concentrations above the PA-IC₉₀ for only 35 days. Even given the dose differences, NM2DTG was able to maintain stable plasma drug levels for significantly longer than the first-generation prodrug formulation.

Prodrug hydrolysis

Given that the activation of the ester prodrugs is mediated, in largest measure, by enzymatic processes, we assessed the mechanisms underlying the conversion by measurements of two isoforms of carboxylesterases [(CES), CES1, and CES2]. These tests were completed in rat tissues and plasma (Supplementary figure 15). Quantification of the CES isoforms demonstrated that CES1 was the most dominant. Notably, no relationships were found between CES levels and prodrug cleavage rates, indicating the process of native prodrug to drug release was independent from a specific CES. Next, we investigated the stability of solubilized and nanoformulated M2DTG solid drug suspensions, in rat tissue homogenates, to assess time-dependent prodrug tissue cleavage. The prodrug formulation remained stable in all tissue matrices tested (Fig. 4a-h). The findings were cross validated by quantifying parent drug levels from the same samples. The prodrug solution showed complete cleavage in spleen and kidney homogenates at 48 h (Fig. 4c-d). For liver and muscle tissues, only half of the prodrug solution was depleted at 48 h (Fig. 4a, e). However, the prodrug solution was rapidly cleaved in plasma, while heat-inactivation led to a significant reduction in the rates of plasma cleavage (Fig. 4g-h). Prodrug to parent drug conversion were calculated (Supplementary figure 17a-f). Lymph node showed the most rapid cleavage rates

of the nanoformulation. Notably, spleen and kidney showed the highest rate of prodrug cleavage when exposed to the prodrug solution. The decreasing concentration of total prodrug levels in the tissue homogenates followed second order kinetics for all nanoformulation, and most solution sample sets; with the rates obtained from the incubation of the solution in spleen and kidney following mixed order kinetics.

The influence of pH on the intracellular fate of the nanoformulation in macrophage endosomal microenvironments was evaluated. Tissue homogenates for the study were prepared and used for pH prodrug activation studies over 48 h (Supplementary figure 19). pH-dependent differences in prodrug release and hydrolysis were found linked to prodrug form, with the nanoformulation being more stable in acidic conditions than alkaline (Fig. 5a-d). The findings were cross validated by the corresponding increase in the parent drug levels from replicate samples. The prodrug solution also displays a pH-dependent hydrolysis within 48 h and found to be more stable under acidic conditions (Fig. 5a, d). The pH-dependent prodrug activation of the nanoformulation and prodrug solution at pH 2.0, 6.0, 8.0 and 10.3 shows the profile differences linked to reaction rate kinetics (Supplementary figure 18a-d).

While the prodrug nanoformulation showed only 48% total prodrug left at pH 6.0 (Fig. 5b), the same formulation showed about 85% prodrug left in the spleen tissue homogenate corroborated with parent drug levels (Fig. 4c). The findings show that the nanoformulation remained stable in tissues of the same pH as the tested buffers. The reduced dissolution of the nanocrystals can explain this in the tissue matrices due to protein binding to the nanoformulation. It can thus be concluded

that the dissolution of the nanocrystals to release the prodrug is the critical factor that governs the PK outcomes.

Discussion

Herein, an optimal novel M2DTG prodrug and its formulation were created to transform the drug's apparent half-life. The highest prodrug and drug levels were at the muscle injection site which were sustained at a million ng/g of DTG for a year. Based on the stability of the nanoformulations, the sustained nanocrystal dissolution, the slow tissue prodrug hydrolysis and release from the injection site provide the platform necessary for continuous drug seeding to blood. Rapid plasma prodrug hydrolysis concordant with sustained tissue prodrug levels provides a vehicle for the observed novel PK profiles. The lipophilic C18 moiety on the monomeric DTG prodrug enabled its optimal physicochemical and extended PK properties. Reflective of our prior works, any of the “ultra” LA antiretroviral prodrugs [\[30\]](#) require water insoluble compounds for compatibility with scalable top down nanocrystal formulation technologies. The resultant formulation need be crafted into an optimal shape, size, and solubility, allowing for final lipophilicity, stability, and safety. These parameters also aid in optimizing timed tissue parent drug conversion with slow plasma drug release. The reported improvements in the drug's PK also permit parallel pathways for broader prodrug transformation in drug classes targeting different parts of the viral life cycle [\[20\]](#). The formulation lipophilicity facilitates drug targeting to CD4⁺ T cells and monocyte-macrophages, the natural HIV-1 cell reservoirs, for maximal viral suppression. LA slow effective release (LASER) ART also facilitates the required chemical stability during

extended *in vivo* drug-release and attenuated cytotoxicity [28]. The tissue pH and microenvironment, and prodrug hydrolysis were shown to be critical PK predictive parameters and may also account for the seen species differences. Equivalence in prodrug nanoparticle release rates and hydrolysis equity from plasma from different species suggest that PK differences may be based on creating optimal formulations [30, 33]. Therapeutic efficacy may also facilitate viral elimination through co-administration of clustered regularly interspaced short palindromic repeats (CRISPR) gene editing [37].

Notably, other groups have also modified the structure and or delivery of DTG to develop the means to extend the drug's half-life [29, 38-41]. In one LA biodegradable polymeric solid implants were developed by phase inversion producing removable implants [41]. However, large scale implants have not yet been realized. In another, palmitic acid (PA) conjugated prodrug of DTG was produced by esterification [38]. Biodegradable microparticles produced extended the drug half-life and enabled slow drug release but formulation safety, scale-up and long-term stability remain as limitations. A third removable ultra-LA system was reported for DTG delivery for up to 9 months but with PK variability [39].

Interestingly, we found that at the injection site within the muscle served as a primary stable depot for the nanoformulated prodrug. This provides an avenue for potential removal. This study reports that following NM2DTG parenteral injection, sustained plasma drug levels can be achieved above the PA-IC₉₀ in Balb/cJ mice and SD rats. Modest increases in prodrug cleavage obtained in rodents showed modest differences in RMs where an injection boost was required to sustain plasma drug levels. We offer that the identified prodrug profiles are based, in whole or part, on the chemical

properties of the nanoformulation and its tissue penetration, perfusion, cell infiltration, and nanoparticle uptake, tissue prodrug biodistribution, and drug release kinetics [42-45]. Accurate predictions for human doses will use allometric scaling and while empiric can be derived through dose normalization of our acquired mouse, rat and monkey data sets linked to unique human anatomical, physiological, and biochemical process.

The LASER ART NM2DTG formulation provide a critical link between the hydrophobic-lipophilic character of the prodrug and the apparent half-life. In contrast, NDTG, with the highest aqueous solubility, showed rapid native drug clearance. Moreover, the C18 lipophilic promoiety grafted onto a native DTG facilitates transport across cell and tissue barriers and slows the drug's excretion rates. Thus, we posit that aqueous solubility is one of several predictive markers for this LASER ART. Interestingly, while M2DTG and M4DTG both had 18-carbon fatty acid modification, the *in vivo* fates differed. Such differences underscore that the hydrophobicity and lipophilicity of the DTG prodrugs provide one, but not all, of the predictive parameters for the unique LASER ART PK profiles. For example, poor dissolution of the prodrug, as demonstrated by NM3DTG, affected plasma DTG levels which fell below the PA-IC₉₀ at one week. Taken together, the current study establishes an ideal range of prodrug hydrophobicity and lipophilicity required for optimal clinical PK parameters. To this end, NM2DTG was identified as our lead candidate for further study and product development.

The PK boost seen during the second injection of NM2DTG in RM likely reflects an extension of the established depot present in the muscle at the site of injection, as well as within the reticuloendothelial system. The established depot in muscle represents a long-lived reservoir. Indeed, for the macrophage, the formation of granulomas at the injection site occurs consequent

to cellular infiltration and uptake of the DTG prodrug nanoformulations. This notion is supported by the fact that macrophages serve both as reservoirs for persistent infection and for the drug nanoparticles [44]. In this manner, the macrophage represents a cell depot from which the prodrug is released then hydrolyzed. In support of this idea is that at the site of injection, extensive histocyte infiltration is observed. Morphologically, rearrangement of the muscle with nanoformulation present in intracellular endosomes characterizes how the macrophage handles LASER ART formulations [29-31, 33, 35].

Interestingly, the nanoformulation and the prodrug are more stable at lower pH conditions, which characterizes the macrophage subcellular microenvironments that the nanoparticles are exposed to as part of the phagocytic cascade. The retention studies of the nanoformulation in macrophages showed persistent prodrug levels with limited concentrations of the native drug across 30 days. These observations provide insight into the slow rate of release and hydrolysis of the prodrug from the nanoformulation. Thus, intracellular accumulation of drug nanocrystals is stabilized and retained in these compartments, allowing them to release the drug into blood slowly and as such extend the ARV's apparent half-life. The nanoformulations are stored, in measure, in lymphoid organs using these organs as secondary tissue depots. Therefore, the release of the prodrug into the extracellular matrix and plasma, resulting in its subsequent hydrolysis, underlie the unique PK profiles of NM2DTG, as seen previously for NM2CAB [30, 33].

We suggest that a contributing factor responsible for the release of the prodrug from the nanoformulation and the subsequent hydrolysis could be tissue enzymatic hydrolysis. Testing of different CES tissue isoforms shows the prominence of CES1. While liver and kidney have the

highest concentrations of CES1, those of CES2 are significantly less. However, in contrast, plasma shows no discernible levels of either of the CES species. Thus, in attempts to better appreciate the influence of hydrolysis and the prodrug physiochemical properties, we conducted computational modeling. This was completed to simulate the enzymatic prodrug hydrolysis of MDTG and M2DTG by CES. CES1 was chosen as it is known to hydrolyze ester-bond-containing drugs and is the most abundant CES enzyme [46]. MDTG, a prodrug with a shorter alkyl side chain (14-carbon units), had superior docking with the highest level of hydrolysis (Supplementary figure 16). The carbonyl group in MDTG faces the Ser221 residue in CES1, and the hydrophobic tail is next to a hydrophobic patch identified on the enzyme surface. Other prodrugs showed reduced hydrolysis rates that paralleled the increases in apparent half-life. Indeed, the molecular docking experiments showed that the increased length of the fatty acid chain led to decreased favorable enzyme binding of the prodrug and was linked to the PK profiles. Failure to produce immediate binding to the enzyme allows these long-side chain prodrugs to persist in biological matrices and results in slower cleavage rates. Prodrug cleavage studies from the nanoformulation and free prodrug in solution while of interest, its role in predicting PK parameters and the effects of different levels of tissue esterases while providing evidence of species and tissue dependence the actual mechanisms that occur in variant tissue environments await further study. This is highlighted by the failures to bridge CES levels with rates of prodrug hydrolysis. Indeed, 85% of the prodrug remained in the spleen by *ex vivo* tests. However, 48% of the total prodrug was seen at pH 6.0 in solution. These studies show that the nanoformulation is stable in tissues and explained through the reduced dissolution of the nanocrystals, likely through protein-nanoformulation binding. A summative understanding points towards a cluster of variable conditions contributing towards release and hydrolysis from the prodrug formulation. A clear delineation of prodrug

stability, formulation, and solution is required in future studies to decipher the contribution of nanocrystal dissolution and release of free prodrug under divergent tissue and cell environments and in blood. In conclusion, the dissolution of the prodrug is a major component governing the extended PK profiles of the nanoformulated prodrug. Comprehensive dose-escalating studies will be required in future studies to determine human dosing. NM2DTG elicited plasma drug levels just above the PA-IC₉₀ requiring a drug boost in RMs after six months, while the murine models showed persistent plasma drug levels just above the PA-IC₉₀ for a year. This is in line with the previously reported terminal phase half-life of cabotegravir in rhesus macaques of 3 to 12 days compared to 21 to 50 days for humans [47, 48]. However, rats have shown concentration vs. time profiles for paliperidone with similarity to human deltoid/gluteal injection data [49]. The human equivalent dose of a rat 45 mg/kg translates to a human equivalent of 438 mg of DTG in a 60 kg person. Dose extrapolation achieving drug concentrations of 330 mg/mL prodrug or 201 mg/mL of DTG equivalents an annual human injection volume will be 2.2 mL; about equal to the intramuscular monthly injection volume of CAB-LA [50]. Future dose-escalating studies must also take into account other contributing variables that include protein binding, of the nanoformulations and or prodrug, hepatic microsomal metabolic stability.

Arguably, the most important utility of the current NM2DTG formulation rests in PrEP by providing greater access in resource-limited settings. Recent reports demonstrated that CAB-LA is superior to daily oral tenofovir disoproxil fumarate–emtricitabine combinations in preventing HIV-1 infection in studies populations of men who have sex with men and transgender women. NM2DTG is a particularly attractive candidate as a LA ARV based on its safety profile

and resistance patterns. Moreover, the extended NM2DTG's PK profile now characterized as a multi-step slow prodrug release-hydrolysis for a year or longer can now lead to clinical translation.

References

1. Centers for Disease, C. and Prevention, *Progress toward global poliomyelitis eradication, 2000*. MMWR Morb Mortal Wkly Rep, 2001. **50**(16): p. 320-2, 331.
2. Fenner, F., *Smallpox and its eradication*. History of international public health. 1988, Geneva: World Health Organization. xvi, 1460 p.
3. Goldenthal, K.L., K. Midthun, and K.C. Zoon, *Control of Viral Infections and Diseases*, in *Medical Microbiology*, th and S. Baron, Editors. 1996: Galveston (TX).
4. Goodson, J.L., et al., *Measles and rubella elimination: learning from polio eradication and moving forward with a diagonal approach*. Expert Rev Vaccines, 2017. **16**(12): p. 1203-1216.
5. Baril, J.G., et al., *Dual Therapy Treatment Strategies for the Management of Patients Infected with HIV: A Systematic Review of Current Evidence in ARV-Naive or ARV-Experienced, Virologically Suppressed Patients*. PLoS One, 2016. **11**(2): p. e0148231.
6. Jain, R., et al., *Limitations of current antiretroviral agents and opportunities for development*. Curr Pharm Des, 2006. **12**(9): p. 1065-74.
7. Mtambo, A., et al., *Treatment limitations imposed by antiretroviral drug resistance mutations: implication for choices of first line regimens in resource-limited settings*. HIV Med, 2012. **13**(3): p. 141-7.
8. Bartlett, J.A. and J.F. Shao, *Successes, challenges, and limitations of current antiretroviral therapy in low-income and middle-income countries*. Lancet Infect Dis, 2009. **9**(10): p. 637-49.
9. Montessori, V., et al., *Adverse effects of antiretroviral therapy for HIV infection*. CMAJ, 2004. **170**(2): p. 229-38.
10. *Panel on Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV. Guidelines for the Prevention and Treatment of Opportunistic Infections in HIV-infected Adults and Adolescents: Recommendations from the Centers for Disease Control and Prevention, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America*. p. Available at https://clinicalinfo.hiv.gov/sites/default/files/inline-files/adult_oi.pdf. Accessed October 2021.
11. Mofenson, L.M., et al., *Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-exposed and HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases*

- Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. MMWR Recomm Rep, 2009. 58(RR-11): p. 1-166.*
12. Sneller, M.C., et al., *Kinetics of Plasma HIV Rebound in the Era of Modern Antiretroviral Therapy*. J Infect Dis, 2020. **222**(10): p. 1655-1659.
 13. Lai, C.L., et al., *Rebound of HBV DNA after cessation of nucleos(tide) analogues in chronic hepatitis B patients with undetectable covalently closed*. JHEP Rep, 2020. **2**(3): p. 100112.
 14. Marcus, J.L., et al., *Comparison of Overall and Comorbidity-Free Life Expectancy Between Insured Adults With and Without HIV Infection, 2000-2016*. JAMA Netw Open, 2020. **3**(6): p. e207954.
 15. Centers for Disease Control and Prevention. HIV Surveillance Report, 2018 (Updated). **31**: p. <http://www.cdc.gov/hiv/library/reports/hiv-surveillance.html>. Published May 2020. Accessed October 2021.
 16. Chen, Y., K. Chen, and S.C. Kalichman, *Barriers to HIV Medication Adherence as a Function of Regimen Simplification*. Ann Behav Med, 2017. **51**(1): p. 67-78.
 17. Cohen, J., et al., *Real-world adherence and persistence for newly-prescribed HIV treatment: single versus multiple tablet regimen comparison among US medicaid beneficiaries*. AIDS Res Ther, 2020. **17**(1): p. 12.
 18. *HIV Glasgow - Virtual, 5-8 October 2020*. J Int AIDS Soc, 2020. **23 Suppl 7**: p. e25616.
 19. Abbasi, J., *Promising Early Results for Potent, Long-Acting HIV Injection*. JAMA, 2020. **324**(6): p. 539.
 20. Labh, R. and R. Gupta, *Emerging Trends in the Long-Acting Antiretroviral Therapy: Current Status and Therapeutic Challenges*. Curr HIV Res, 2021. **19**(1): p. 4-13.
 21. Rizzardini, G., et al., *Long-Acting Injectable Cabotegravir + Rilpivirine for HIV Maintenance Therapy: Week 48 Pooled Analysis of Phase 3 ATLAS and FLAIR Trials*. J Acquir Immune Defic Syndr, 2020. **85**(4): p. 498-506.
 22. Scarsi, K.K., *Chasing the cabotegravir tail: implications for prevention*. Lancet HIV, 2020. **7**(7): p. e451-e453.
 23. Currier, J.S., *Monthly Injectable Antiretroviral Therapy - Version 1.0 of a New Treatment Approach*. N Engl J Med, 2020. **382**(12): p. 1164-1165.
 24. Margolis, D.A., et al., *Long-acting intramuscular cabotegravir and rilpivirine in adults with HIV-1 infection (LATTE-2): 96-week results of a randomised, open-label, phase 2b, non-inferiority trial*. Lancet, 2017. **390**(10101): p. 1499-1510.
 25. Fernandez, C. and C.L. van Halsema, *Evaluating cabotegravir/rilpivirine long-acting, injectable in the treatment of HIV infection: emerging data and therapeutic potential*. HIV AIDS (Auckl), 2019. **11**: p. 179-192.
 26. Havlir, D. and M. Gandhi, *Implementation challenges for long-acting antivirals as treatment*. Curr Opin HIV AIDS, 2015. **10**(4): p. 282-9.
 27. Soriano, V., P. Barreiro, and C. de Mendoza, *Long-acting antiretroviral therapy*. Nat Mater, 2020. **19**(8): p. 826-827.
 28. Edagwa, B., et al., *Long-acting slow effective release antiretroviral therapy*. Expert Opin Drug Deliv, 2017. **14**(11): p. 1281-1291.
 29. Sillman, B., et al., *Creation of a long-acting nanoformulated dolutegravir*. Nat Commun, 2018. **9**(1): p. 443.
 30. Kulkarni, T.A., et al., *A year-long extended release nanoformulated cabotegravir prodrug*. Nat Mater, 2020. **19**(8): p. 910-920.

31. Zhou, T., et al., *Creation of a nanoformulated cabotegravir prodrug with improved antiretroviral profiles*. Biomaterials, 2018. **151**: p. 53-65.
32. Crissman, J.W., et al., *Best practices guideline: toxicologic histopathology*. Toxicol Pathol, 2004. **32**(1): p. 126-31.
33. Gautam, N., et al., *Lipophilic nanocrystal prodrug-release defines the extended pharmacokinetic profiles of a year-long cabotegravir*. Nat Commun, 2021. **12**(1): p. 3453.
34. McMillan, J., et al., *Pharmacokinetics of a Long-Acting Nanoformulated Dolutegravir Prodrug in Rhesus Macaques*. Antimicrob Agents Chemother, 2018. **62**(1).
35. Cobb, D.A., et al., *Transformation of tenofovir into stable ProTide nanocrystals with long-acting pharmacokinetic profiles*. Nat Commun, 2021. **12**(1): p. 5458.
36. Hilaire, J.R., et al., *Creation of a long-acting rilpivirine prodrug nanoformulation*. J Control Release, 2019. **311-312**: p. 201-211.
37. Dash, P.K., et al., *Sequential LASER ART and CRISPR Treatments Eliminate HIV-1 in a Subset of Infected Humanized Mice*. Nat Commun, 2019. **10**(1): p. 2753.
38. Khuroo, T., et al., *Ultra-long acting prodrug of dolutegravir and delivery system - Physicochemical, pharmacokinetic and formulation characterizations*. Int J Pharm, 2021. **607**: p. 120889.
39. Kovarova, M., et al., *Ultra-long-acting removable drug delivery system for HIV treatment and prevention*. Nat Commun, 2018. **9**(1): p. 4156.
40. Maturavongsadit, P., et al., *A new engineering process of biodegradable polymeric solid implants for ultra-long-acting drug delivery*. Int J Pharm X, 2021. **3**: p. 100068.
41. Maturavongsadit, P., et al., *Biodegradable polymeric solid implants for ultra-long-acting delivery of single or multiple antiretroviral drugs*. Int J Pharm, 2021. **605**: p. 120844.
42. Cao, Z.T., et al., *Protein Binding Affinity of Polymeric Nanoparticles as a Direct Indicator of Their Pharmacokinetics*. ACS Nano, 2020. **14**(3): p. 3563-3575.
43. Darville, N., et al., *Modeling the Time Course of the Tissue Responses to Intramuscular Long-acting Paliperidone Palmitate Nano-/Microcrystals and Polystyrene Microspheres in the Rat*. Toxicol Pathol, 2016. **44**(2): p. 189-210.
44. Herskovitz, J. and H.E. Gendelman, *HIV and the Macrophage: From Cell Reservoirs to Drug Delivery to Viral Eradication*. J Neuroimmune Pharmacol, 2019. **14**(1): p. 52-67.
45. Nara, E., et al., *Pharmacokinetic analysis of drug absorption from muscle based on a physiological diffusion model: effect of molecular size on absorption*. Pharm Res, 1992. **9**(2): p. 161-8.
46. Laizure, S.C., et al., *The role of human carboxylesterases in drug metabolism: have we overlooked their importance?* Pharmacotherapy, 2013. **33**(2): p. 210-22.
47. Andrews, C.D., et al., *Cabotegravir long acting injection protects macaques against intravenous challenge with SIVmac251*. AIDS, 2017. **31**(4): p. 461-467.
48. Andrews, C.D. and W. Heneine, *Cabotegravir long-acting for HIV-1 prevention*. Curr Opin HIV AIDS, 2015. **10**(4): p. 258-63.
49. Patel, H., et al., *Rats and rabbits as pharmacokinetic screening tools for long acting intramuscular depots: case study with paliperidone palmitate suspension*. Xenobiotica, 2019. **49**(4): p. 415-421.
50. Clement, M.E., R. Kofron, and R.J. Landovitz, *Long-acting injectable cabotegravir for the prevention of HIV infection*. Curr Opin HIV AIDS, 2020. **15**(1): p. 19-26.

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Competing interests

BE and HEG are Co-Founders of Exavir Therapeutics, Inc. a biotechnology company that is developing extended-release long-acting antiretroviral drugs.

Data and materials availability

All data and materials used in these analyses are included in the main text and supplemental materials will be made available on request to reproduce or extend the works. Patented additions are listed as references.

Supplementary Materials:

Materials and Methods

Figs. S1 to S19

Tables S1 to S4

Fig. 1. Synthesis and characterization of DTG prodrugs. (A) DTG was synthesized by esterification of the DTG hydroxyl group yielding lipophilic prodrugs with 14, 18, or 22-carbon chains. They are named MDTG, M2DTG, and M3DTG. A DTG dimer with an 18-carbon chain ester linkage was named M4DTG. (B) FT-IR overlays of DTG, MDTG, and M2DTG identified the presence of specific molecular groups in the prodrugs affirmed by spectroscopy. (C) Aqueous and (D) octanol solubility of DTG and prodrugs are illustrated. Results are expressed as the mean $\pm$ SEM for N = 3. (E) Differential scanning calorimetry (DSC) thermogram overlays of NDTG, NMDTG, and NM2DTG determined changes in physical state. (F) The antiretroviral half maximal inhibitory concentration (IC₅₀) in macrophages were assessed at 0.1-1,000 nM concentrations by measurements of HIV-1 reverse transcriptase (RT) activity in culture supernatants. Results are expressed as the mean $\pm$ SEM for N = 3. Nanoformulations that enhance particle properties include (G-H) the hydrodynamic diameter (size) of NM2DTG formulations were tested at 22°C over 120 days by dynamic light scattering (DLS). (G) NM2DTG (268 mg/mL of M2DTG) formulated with Tween 20 and PEG₃₃₅₀ in PBS (pH 7.0). (H) NM2DTG (330 mg/mL of M2DTG) formulated with Tween 20 and PEG₃₃₅₀ in PBS (pH 7.0).

Fig. 2. Biological, histological and electron microscopic characterization of NM2DTG. (A) drug uptake and (B) retention in MDM measured over 24 h and 30 days, respectively, after treatment with prodrug nanoformulations at concentration of 25 μ M. Results are expressed as the mean $\pm$ SEM for N = 3. (C) Antiretroviral responses were recorded after HIV-1_{ADA} challenge at a multiplicity of infection (MOI) of 0.1 infectious virions/cell at recorded times following treatment with either NDTG or NM2DTG at 1 or 10 μ M concentrations for 8 h. HIV-1p24 antigen levels were assessed in fixed MDM by immunohistochemical staining. (D-H) Hematoxylin and eosin (H&E) staining of caudal thigh muscle in histological sections after dissection from rats 3 days following IM injections. (D) Control (uninjected), (E) sham (saline-injected) control and (F-H) NM2DTG (45 mg DTG-eq./kg) treated muscle sections. (I) H&E staining of caudal thigh muscle in histological sections after dissection from rats 57 days following IM injection of NM2DTG at 45 mg DTG-eq./kg. Representative images for panel (F) at 10X magnification have been provided at 40X in panel (G). (J-O) Replicate muscle samples were examined by transmission electron microscopy (TEM) from rats three days post-treatment. (J-K) uninjected and (L-M) Sham (saline-injected) controls show normal muscle histology. (N-O) Rats which were injected with NM2DTG show cell infiltration with ingestion of the nanoformulation into endosomal vesicles. Representative images for panel (L) at 10X magnification have been provided at 40X in panel (M). Scale bars – 500 nm (J-M), 10 μ m (N), 2 μ m (O).

Figure 3. DTG PK studies in Balb/cJ mice, Sprague Dawley rats, and rhesus macaques. (A)

Plasma DTG levels in male Balb/cJ mice administered a single IM dose of NDTG, NM2DTG, NM3DTG, or NM4DTG. Drug formulations were injected at concentrations of 45 mg DTG-eq./kg IM in the caudal thigh and native drug levels monitored to day 367. The dotted line indicates the DTG protein-adjusted IC_{90} ($PA-IC_{90} = 64 \text{ ng/mL}$) and dashed line indicates four-times the $PA-IC_{90}$ ($4 \times PA-IC_{90} = 256 \text{ ng/mL}$). **(B)** Plasma DTG levels in male SD rats administered a single 45 mg DTG-eq./kg IM dose of NDTG or NM2DTG in the caudal thigh were recorded. Plasma prodrug (M2DTG) levels are shown in the insert to day 7. **(C)** Plasma DTG (solid line) and M2DTG (dashed line) levels in female rhesus macaques given a 45 mg DTG-eq./kg IM dose of NM2DTG in the quadriceps muscles, followed by an equivalent booster dose on day 217 (arrow). Plasma samples were collected, and drug levels determined up to day 428. **(D-G)** Tissue biodistribution of NDTG and NM2DTG in SD rats was assessed on days 57, 175, and 364. Parent drug (DTG) and prodrug (M2DTG) levels were determined in **(D)** muscle (at the site of injection), **(E)** spleen, **(F)** lymph nodes, and **(G)** liver. All drug levels were quantified by UPLC-MS/MS. Results are expressed as mean $\pm$ SEM.

Figure 4. NM2DTG prodrug cleavage studies in tissue drug depots. Cleavage of M2DTG solution (dissolved in 1% (v/v) methanol) or NM2DTG nanoformulation in SD rat tissue homogenates (**A-F**) and plasma (**G-H**). Prodrug (M2DTG) and parent drug (DTG) were quantified following M2DTG or NM2DTG incubation over 48 h in (**A**) liver, (**B**) heat-inactivated liver, (**C**) spleen, (**D**) kidney, (**E**) muscle, (**F**) lymph node, (**G**) plasma, and (**H**) heat inactivated plasma. All drug levels were quantified by UPLC-MS/MS. Results are expressed as mean $\pm$ SEM for N = 3.

Figure 5. pH-dependent prodrug hydrolysis. Hydrolysis of M2DTG solution (dissolved in 1% (v/v) methanol) or NM2DTG nanoformulation in buffers of various pH. Prodrug (M2DTG) and parent drug (DTG) were quantified following M2DTG or NM2DTG incubation over 48 h in buffers at **(A)** pH 2.0, **(B)** pH 6.0, **(C)** pH 7.0, and **(D)** pH 10.3. All drug levels were quantified by UPLC-MS/MS. Results are expressed as mean $\pm$ SEM for N = 3.

Figure 6. Characterization of the NM2DTG extended PK profile. The illustration shows the sequential steps determining the *in vivo* fate of the NM2DTG nanoformulation. **(a)** DTG was first esterified with an 18 C fatty acid to produce M2DTG which was then nanoformulated to yield NM2DTG. **(b)** Intramuscular injection of NM2DTG illustrates the formation of the primary drug depot from which the prodrug dissolves from the nanoformulation. **(c, d)** Histiocytic infiltration ensues at the injection site leading to NM2DTG uptake by macrophages. **(e)** Biodistribution of drug to HIV-1 target organs amongst other tissue sites leads to sustained drug levels in liver, spleen, lymph node, and lung end organs. **(f)** M2DTG is slowly dissolved from the nanoformulation in the low pH microenvironment in macrophages then hydrolyzed to release DTG. **(g)** The slow rate of dissolution of the DTG nanocrystals from tissues and rapid hydrolysis of M2DTG prodrug in plasma allow active DTG to enter the blood stream and subsequent antiretroviral activities. The two-stage process of dissolution and hydrolysis leads to the plasma DTG concentrations, at or above protein adjusted IC₉₀, for up to one year. **(h)** DTG shows potent integrase strand inhibition to the integration of viral DNA into the host genome.

Supplementary Files

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