Electronic Supplementary Material (ESI) for ChemComm.
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Supplementary information

ProTide Generated Long-Acting Abacavir Nanoformulations

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1. Fig S1-S6.

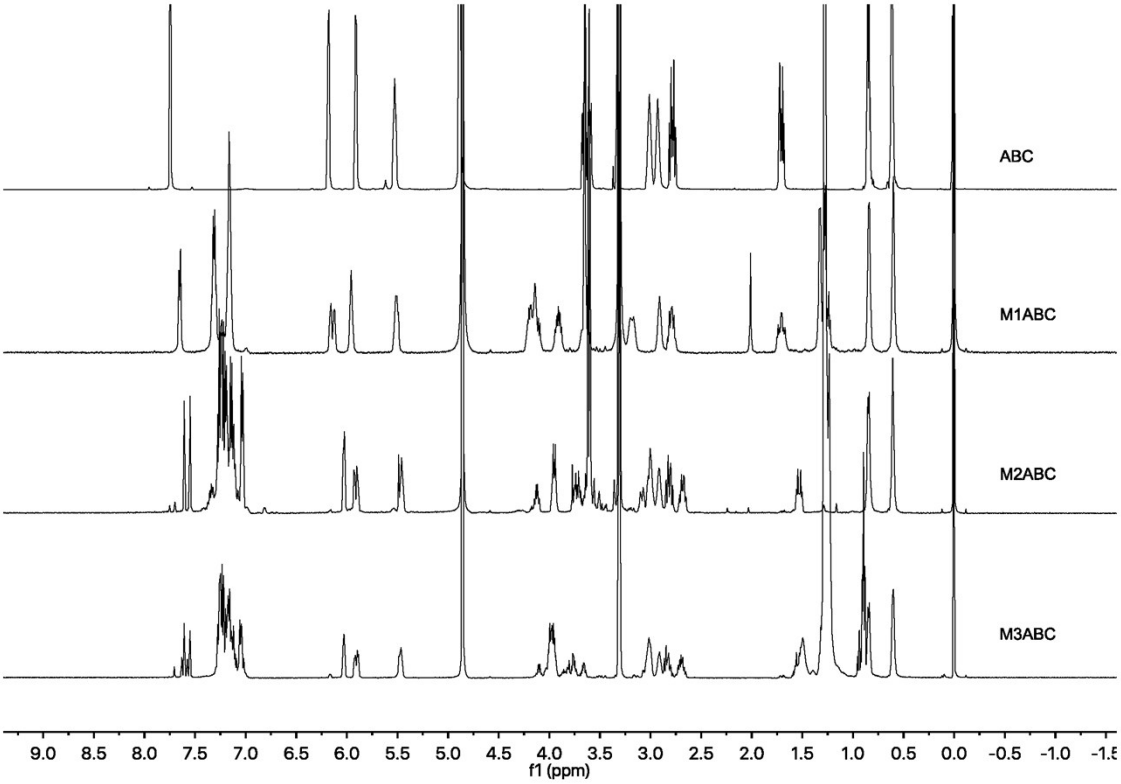


Fig S1: ^{1}H-NMR spectra for ABC, **M1ABC**, **M2ABC** and **M3ABC**.

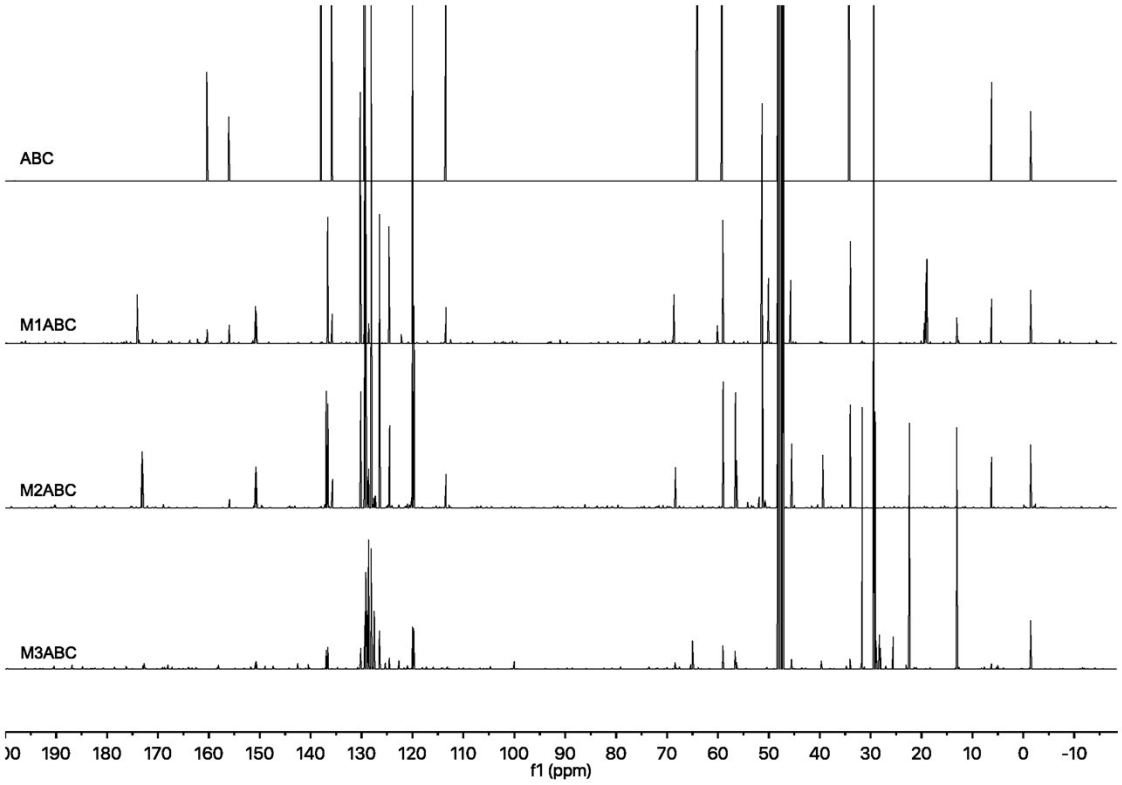


Fig S2: ^{13}C-NMR spectra for ABC, **M1ABC**, **M2ABC** and **M3ABC**.

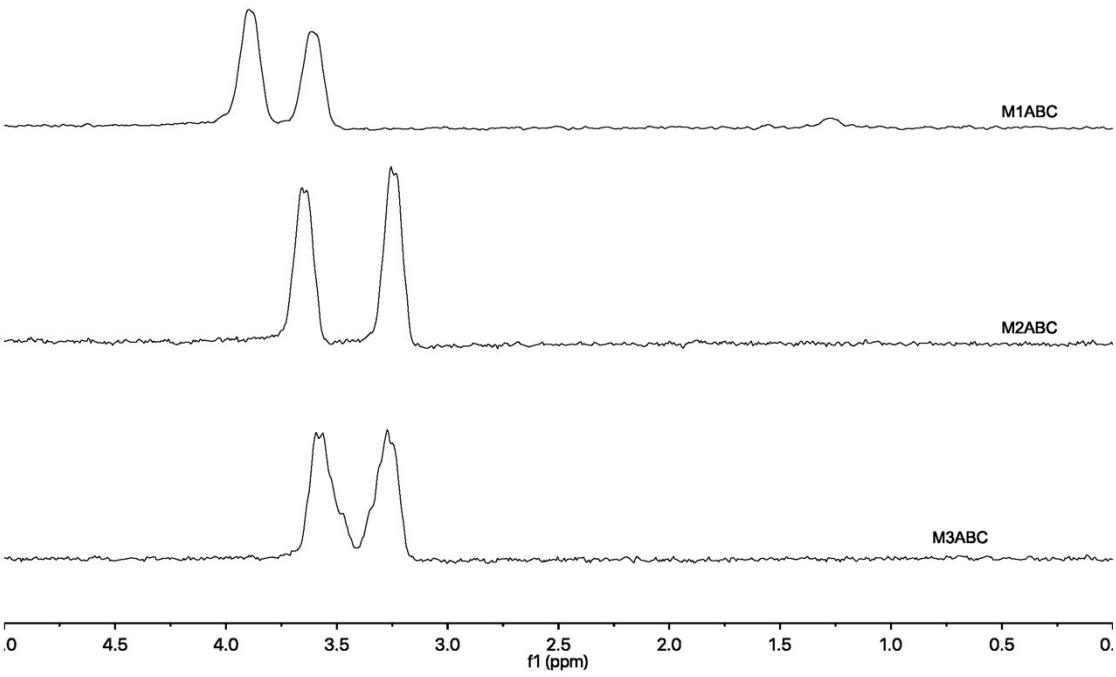


Fig S3 ^{31}P-NMR spectra for **M1ABC**, **M2ABC** and **M3ABC**.

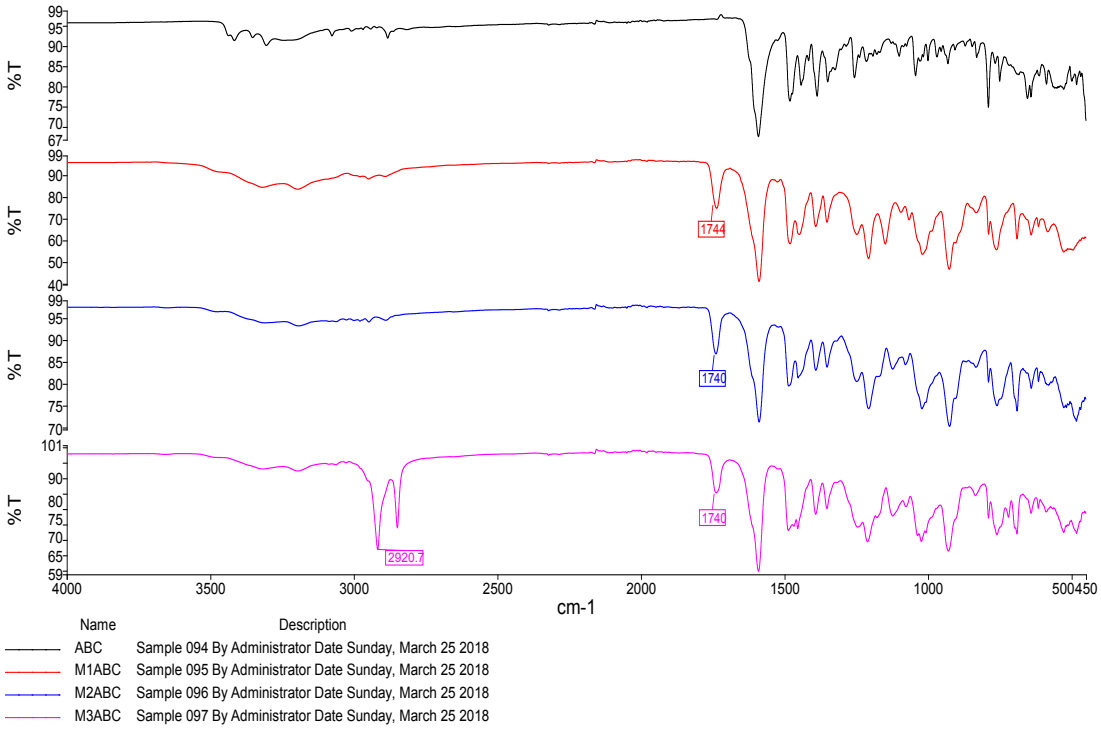
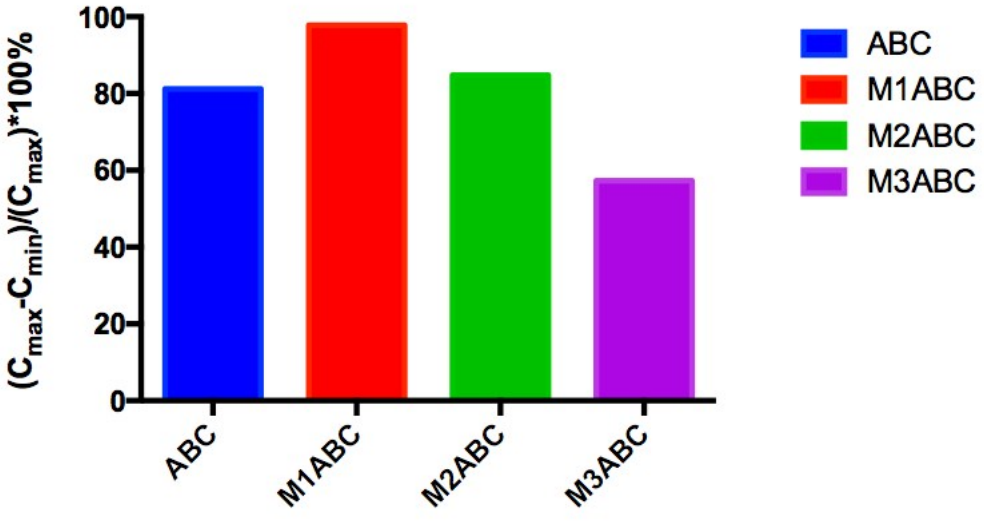


Fig S4: FTIR spectra for ABC, **M1ABC**, **M2ABC** and **M3ABC**.

Fig S5: Intracellular levels of CBV-TP were detected and declined at variant rates. The percent change was calculated as $(C_{max}-C_{min})/(C_{max})*100$. For ABC, **M1ABC**, **M2ABC** and **M3ABC**, the percentage decreases were 81.2%, 97.8%, 84.8% and 57.3% from C_{max}, at 48 h, respectively.



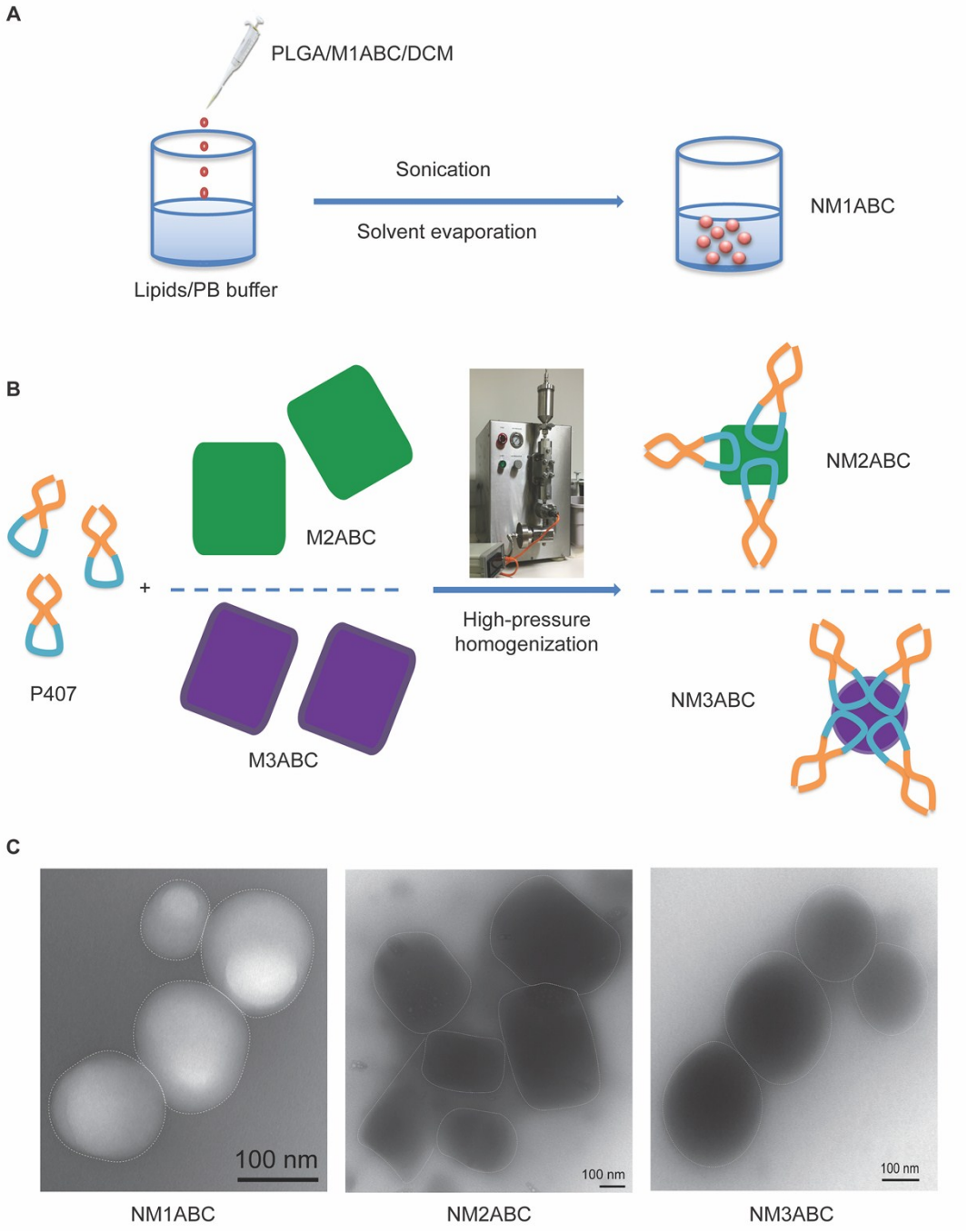


Fig S6. Preparation and characterization of nanoformulations. (A) N**M1ABC** was prepared by a modified single emulsion solvent evaporation technique. **(B)** N**M2ABC** and N**M3ABC** were produced by high pressure homogeinization. **(C)** TEM images of N**M1ABC**, N**M2ABC** and N**M3ABC**.

2. Table S1-S2.

Table S1 Characterization of Nanoformulations[a]

Name	D_{eff} (nm)	PdI	ζ- potential (mV)	Drug loading (%)
N**M1ABC**	237 ± 2	0.15 ± 0.03	- 43.4 ± 0.7	3.4 ± 0.2
N**M2ABC**	339 ± 17	0.35 ± 0.01	- 14.3 ± 0.3	43.4 ± 0.5
N**M3ABC**	329 ± 3	0.28 ± 0.01	- 45.0 ± 0.4	47.4 ± 2.0

Effective diameter (D_{eff}), polydispersity index (PdI) and ζ- potential of nanoformulation samples diluted in water were determined by dynamic light scattering (DLS). Drug loadings of lyophilized nanoformulations were determined by HPLC. Drug loading (%) = [entrapped drug/nanoparticles weight] x 100. Data are expressed as mean ± SD.

Table S2 CBV-TP level in PBMC for pilot *in vivo* study

Time (Hours)	CBV-TP level (fmol/million cells)	
	Rat 1	Rat 2
2	1.07	7.84
24	4.23	5.36
168	4.51	9.37

Rats were injected Intramuscularly with 45mg/kg ABC equivalents using NM3ABC. PBMC were extracted from whole blood samples collected 2, 24 and 168 hours after injection. CBV-TP level were analyzed as reported[1].

3. General methods.

Chemicals and reagents: All chemical synthesis reactions were performed under a dry argon atmosphere unless otherwise noted. Reagents were obtained from commercial sources and used directly; exceptions are noted. Abacavir was purchased from BOC Sciences Inc. (Shirley, NY). Phenyl dichlorophosphate, *L*-alanine methyl ester hydrochloride salt, *L*-phenylalanine methyl ester hydrochloride salt, *N*-(carbobenzyloxy)-*L*-phenylaline, docosanol, dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), *N,N*-dimethylformamide (DMF), triethylamine (Et_3N), diethyl ether, tetrahydrofuran (THF), *tert*-Butylmagnesium chloride solution (*tert*-BuMgCl, 1.0M in THF), triethylsilane (Et_3SiH), methanol and poly (D, L-lactide-*co*-glycolide; lactide:glycolide (75:25), mol wt 66,000-107,000) (PLGA) were purchased from Sigma-Aldrich (St. Louis, MO). Distearoyl-phosphatidylethanolamine-methyl-polyethylene glycol conjugate-2000 (DSPE-mPEG_{2k}), 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1, 2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) were purchased from Corden Pharma (Cambridge, MA). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*] pyridinium 3-oxid hexafluorophosphate (HATU) was obtained from Bachem Inc. (Torrance, CA) while palladium, 10% on activated carbon, was purchased from STREM Inc. (Newburyport, MA). Flash chromatography was performed using flash silica gel (32-63 µ) from SiliCycle Inc. (Quebec, Canada). Chemical reactions were analyzed by thin layer chromatography (T.L.C) on precoated silica plates (200 µm, F-254) from Sorbtech technologies Inc. (Norcross, GA). The compounds were visualized by UV fluorescence or by staining with ninhydrin or $KMnO_4$ reagents.

High Performance Liquid Chromatography (HPLC): Prodrug quantitation was performed on a Waters Breeze HPLC system (Waters, Milford, MA, USA) using a Kinetex 5 µ C18 100A Phenomenex column. HPLC grade acetonitrile and methanol were purchased from Fisher Chemical. For all three ProTide samples, 10 mM potassium phosphate monobasic buffer solution (10 mM KH_2PO_4) was used at a flow rate of 1 mL/min. Specifically, **M1ABC** was eluted in 10 mM KH_2PO_4/acetonitrile (65/35, v/v) mobile phase with a retention time of 3.8 min; **M2ABC** had a retention time of 4.2 min in 10 mM KH_2PO_4/acetonitrile (55/45, v/v), while **M3ABC** had a retention time of 7.0 min in 10 mM KH_2PO_4/methanol (2/98, v/v).

Nuclear Magnetic Resonance (NMR): NMR spectra were recorded on a Bruker Avance-III HD (Billerica, MA) operating at 500 MHz, a magnetic field strength of 11.7T. Proton NMR data is reported in ppm downfield from TMS as an internal standard.

Mass Spectrometry (MS): Mass spectra were obtained on a Waters Xevo TQ-S micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA).

Fourier-transform infrared spectroscopy (FTIR): FTIR spectra were recorded on a Perkin-Elmer-spectrum attenuated total reflectance (ATR)-FTIR equipped with a UATR-accessary (Perkin-Elmer, Inc., Waltham, MA, USA).

4. Synthesis and characterization

Z-Phe-Odoc (2): Triethylamine (1.35 g, 1.86 mL, 13.36 mmol, 2.0 equiv.), imidazole (454 mg, 6.68 mmol, 1.0 equiv.) and HATU (3.81 g, 10.02 mmol, 1.5 equiv.) were added to a solution of Z-Phe-OH (2 g, 6.68 mmol, 1.0 equiv.) and docosanol (2.4 g, 7.35 mmol, 1.1 equiv.) in a mixture of $CHCl_3$ (25 mL) and DMF (25 mL) at 0 ºC under an argon atmosphere.

The mixture was then heated at 45 °C for 48 h and concentrated. The crude product was diluted with CH_2Cl_2 (100 mL), washed successively with 1 M HCl, saturated $NaHCO_3$ and brine (80 mL each). The organic extract was dried over Na_2SO_4 and concentrated. The residue was purified by flash chromatography eluting with 4:1 Hex/EtOAc to give **Z-Phe-Odoc** (3.46 g, 85 %) as a colorless solid. ^{1}H NMR (500 MHz, CD_3OD): 7.24-7.42 (m, 9H), 7.13 (d, *J* = 6.7 Hz, 2H), 5.25 (d, *J* = 4.0 Hz, 1H), 5.07-5.17 (m, 1H), 4.68 (dd, *J* = 13.6, 6.0 Hz 1H), 4.12 (td, *J* = 13.6, 6.7 Hz 2H), 3.14 (m, 2H), 1.60 (br, 2H), 1.29 (br, 38H), 0.92 (t, *J* = 6.9 Hz 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.6, 155.6, 136.2, 135.7, 129.2, 128.5, 128.4, 29.2, 28.4, 25.8, 22.7, 14.1. MS-ES+ (m/z): calcd. for $C_{39}H_{61}NO_4$, 607.46 (100%), 608.46 (42.2%), 609.47 (8.7%); found, 608.46 [$M+H^+$].

H-Phe-Odoc (3c): To a solution of **2** (3.46 g, 5.695 mmol, 1.0 equiv.) in mixture of anhydrous MeOH (20 mL) and $CHCl_3$ (10 mL) was added Pd/C (1.4g, 40% wt). The reaction mixture was cooled to 0°C followed by drop wise addition of triethylsilane[2] (6.6 g, 9.1 mL, 59.95 mmol, 10.0 equiv.). The reaction mixture was then stirred under an atmosphere of argon at room temperature for 16 h, filtered through Celite TM ® and then concentrated to give **H-Phe-Odoc** (quantitative yield), that was precipitated from ether and used in the next step without further purification.

General Procedure A: Aryl aminoacyl phosphorochloridates[3]

The amino acid ester (1 mol equiv) and phenyl dichlorophosphate (1 mol equiv) were suspended in anhydrous CH_2Cl_2 (15 mL) and cooled to -78°C in a dry ice/ acetone bath. To

this mixture, a pre-cooled (-78°C) solution of anhydrous trimethylamine (2 mol equiv) in CH_2Cl_2 was added drop-wise, and the resultant solution was stirred and gradually warmed to room temperature over 32 h under an inert argon atmosphere. The reaction mixture was concentrated on a rotary evaporator to yield a white solid that was suspended into diethyl ether and filtered. The filtrate was concentrated to give aryl aminoacyl phosphochloridates that were used in the next coupling step without further purification.

General Procedure B: Abacavir ProTides[3]

ABC (1 mol equiv) was dried by azeotroping from anhydrous pyridine, then suspended in anhydrous THF and cooled to -78 °C under an argon atmosphere. *tert*-Butylmagnesium chloride (2 mol equiv, 1.0 M solution in THF) was added drop wise to the ABC suspension and the reaction mixture allowed to stir for an additional 10 min with cooling. A solution of aminoacyl phosphorochloridate (1.5 mol equiv) in THF was then added drop-wise and the resulting mixture was gradually warmed to room temperature and stirred for 48-90 h. The reaction mixture was then cooled to 0 °C, quenched with aqueous saturated ammonium chloride solution or methanol, concentrated, and the desired ABC ProTides isolated by flash column chromatography on silica gel eluting with 95%-90% CH_2Cl_2/CH_3OH mobile phase.

Preparation of M1ABC (5a). Synthesized according to general procedure B. On a scale of 1.0 g (3.49 mmol) of ABC, 1.4 g (2.66mmol, 76 % yield) of **M1ABC** product was formed: ^{1}H NMR (500 MHz, CD_3OD): 7.66 (d, *J* = 6.6 Hz 1H), 7.10-7.40 (m, 6H), 6.15 (dd, *J* = 13.7, 5.7

Hz 1H), 5.97 (br, 1H), 5.53 (br, 1H), 4.08-4.30 (m, 2H), 3.85-4.0 (m, 1H), 3.23 (s, 3H), 3.21 (br, 1H), 2.93 (br, 1H), 2.81 (td, *J* = 16.9, 8.3 Hz 1H), 1.65-1.80 (m, 1H), 1.20-1.40 (m, 4H), 0.85 (app. d, *J* = 5.8 Hz 2H), 0.62 (br, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.4, 161.7, 157.4, 152.2, 138.1, 137.2, 131.7, 130.6, 126.1, 123.7, 121.4, 114.8, 70.1, 60.5, 52.7, 51.5, 47.1, 35.4, 24.3, 20.8, 20.5, 14.5, 7.6. ^{31}P NMR (202 MHz, CD_3OD); 3.87, 3.58. MS-ES+ (m/z): calcd. for $C_{24}H_{30}N_7O_5P$, 527.20 (100%), 528.21 (26.0%), 529.21 (2.7%); found, 528.2 [M+H$^+$].

Preparation of M2ABC (5b). Synthesized according to general procedure B. On a scale of 1.6 g (5.59 mmol) of ABC, 1.9 g (3.15mmol, 55 % yield) of **M2ABC** was formed**:** ^{1}H NMR (500 MHz, CD_3OD): 7.59 (d, *J* = 28 Hz 1H), 7.09-7.40 (m, 10H), 7.06 (d, *J* = 7.6 Hz 1H), 6.01-6.08 (m, 1H), 5.87-5.95 (m, 1H), 5.43-5.53 (m, 1H), 4.15 (dd, *J* = 14.8, 9.1 Hz 1H), 3.92-4.04 (m, 1H), 3.45-3.80 (m, 5H), 2.88-3.14 (m, 3H), 2.78-2.87 (m, 1H), 2.65-2.74 (m, 1H), 1.50-1.58 (m, 1H), 0.82-0.89 (m, 2H), 0.57-0.65 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.5, 157.4, 152.2, 152.1, 151.6,138.3, 138.1, 137.2, 131.5, 130.7, 130.6, 129.5, 127.9, 126.0, 125.9, 121.6, 121.1,114.8, 69.8, 69.7, 60.4, 57.9, 57.7, 52.6, 46.8, 40.8, 35.4, 24.4, 7.6. ^{31}P NMR (202 MHz, CD_3OD); 3.62, 3.22. MS-ES+ (m/z): calcd. for $C_{30}H_{34}N_7O_5P$, 603.24 (100%), 604.24 (32.4%), 605.24 (2.4%); found, 604.24 [M+H$^+$].

Preparation of M3ABC (5c). Synthesized according to general procedure B. On a scale of 1.1 g (3.84 mmol) of ABC, 2.4 g (2.64mmol, 68% yield) of **M3ABC** product was formed: ^{1}H NMR (500 MHz, CD_3OD): 7.59 (d, *J* = 28 Hz 1H), 6.95-7.44 (m, 11 H), 6.04 (br, 1H), 5.88-5.95

(m, 1H), 5.48 (br, 1H), 4.13 (d, *J* = 15.2, 8.7 Hz 1H), 3.89-4.09 (m, 3H), 3.47-3.59 (m, 2H), 2.64-3.15 (m, 5H), 1.45-1.64 (m, 3H), 1.07-1.39 (br, 42H), 0.91 (t, *J* = 6.6 Hz 3H), 0.84-0.87 (m, 2H), 0.61 (br, 2H). ^{13}C NMR (125 MHz, CD_3OD): δ 174.2, 157.4, 154.3, 152.1, 144.9, 138.3, 138.2, 138.0, 137.9, 137.1, 131.5, 130.6, 130.5, 130.3, 130.1, 129.6, 128.9, 127.9, 126.7, 125.9, 124.1, 121.9, 121.4, 121.3, 121.1, 114.8, 69.9, 66.7, 66.4, 63.2, 60.4, 58.1, 57.8, 46.8, 41.1, 35.4, 33.1, 31.2, 30.7, 30.5, 29.6, 29.5, 26.9, 23.8, 14.5, 7.6; ^{31}P NMR (202 MHz, CD_3OD); 3.25, 3.54. MS-ES+ (m/z): calcd. for $C_{51}H_{76}N_7O_5P$, 897.56 (100%), 898.57 (55.2%), 899.57 (14.9%); found, 898.56 [$M+H^+$].

5. Cell model for *in vitro* studies.

Human peripheral blood monocytes were obtained and cultured as previously described[4]. Briefly, human peripheral blood monocytes were obtained by leukapheresis from HIV-1/2 and hepatitis B seronegative donors, followed by purification via countercurrent centrifugal elutriation. Elutriated monocytes were cultured as adherent cells in Dulbecco's mimimum essential medium (DMEM) supplemented with 10% heat-inactivated pooled human serum, 10 µg/mL ciprofloxacin, 50 µg/mL gentamicin, and 1000 U/mL recombinant macrophage colony stimulating factor. Cells were maintained at 37 °C in a 5% CO_2 incubator. Seven days later, differentiated macrophages (MDM) were used for the experiments.

6. Antiretroviral activity of ABC and its ProTides.

The antiviral activities of native ABC and ABC ProTides against HIV-1_{ADA} were determined in MDM as previously described[5]. MDM were incubated with various concentrations of ABC or ABC ProTides for 60 min followed by infection with HIV-1_{ADA} at a multiplicity of infection (MOI) of 0.01 for 4 h. The MDM were washed extensively with phosphate buffered saline (PBS) to remove excess virus particles. The cells were incubated an additional 10 days in the presence of the same concentration of drug used before infection. Cell culture medium was changed every other day with replacement of equivalent drug containing media. At day 10 post infection, supernatants were collected and analyzed for HIV-1 reverse transcriptase (RT) activity [6].

7. Intracellular level of CBV-TP of ABC and its ProTides.

Intracellular levels of CBV-TP were measured as previously described[7]. MDM were treated with 10 µM ABC and ABC ProTides. At 3, 6, 12, 24 and 48 h after treatment, MDM were washed with PBS to remove excess free drug. The cells were then collected in 70% methanol. CBV-TP from MDM was extracted and quantitated by LC-MS/MS as previously described[1]. Sep-Pak QMA cartridges (360 mg, 37-55 µm; Waters) were used to separate CBV-TP from their mono- and di-phosphates counterparts. The QMA cartridges were conditioned with 10 ml of 500 mM KCl followed by 10 ml of 5mM KCl. Samples were loaded onto the cartridges and washed with 15 ml of 75mM KCl. The triphosphate fraction was eluted with 3 ml of 500 mM KCl and collected for de-phosphorylation. The pH of the TP fraction was lowered to 4.25 by adding 15 µl ammonium acetate buffer (pH 4,10 mM) per ml eluate, and dephosphorylated by adding one unit of type XA sweet

potato acid phosphatase per ml eluate and incubating at 37 °C for 30 min. The $^{15}N_2{}^{13}C$-3TC and d_4-ABC internal standard was added at this point. Samples were then loaded onto Waters OASIS HLB cartridges (60 mg, 30 µm; Waters) pre-conditioned with 3 ml MeOH and 3 ml H_2O, and washed with 3.5 ml H_2O to remove salts. The nucleosides of interest were then eluted with 1.5 ml of MeOH and evaporated under vacuum. Once dry, the residue was reconstituted with a 100 µl of 25% MeOH and stored in the -20 °C freezer until the time of LC–MS/MS analyses. The LC-MS/MS system comprised of a 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). For the indirect quantification of TPs, chromatographic separation was performed with an ACQUITY UPLC using CSH analytical column (2.1×100 mm, 1.7µm; Waters) equipped with a guard column (Waters, Milford, MA). Mobile phase A consisted of ammonium bicarbonate (pH 7, 7.5 mM) and mobile phase B was methanol. The flow rate was 0.25 ml/min. The initial mobile phase composition was 12 % B for the first 2.5 min, gradually increased to 30 % B over 4 min, gradually increased again to 95 % B over 3.5 min, and then held constant for one min. Mobile phase B was then reset to 12 % over 0.25 min and the column was equilibrated for 2.75 min before the next injection. The total run time was 13 min. The mass spectrometer was operated in the positive ion mode using multiple reaction monitoring (MRM). The following transitions were monitored: m/z 230→112 for 3TC, m/z 248→152 for CBV, m/z 287→191 for ABC, m/z 233→115 for the internal standard (IS) $^{15}N_2{}^{13}C$-3TC, and m/z 291→195 for IS d_4-ABC. 3TC,

CBV, ABC, $^{15}N_2{}^{13}C$-3TC, and d_4-ABC were detected at a cone voltage of 22, 2, 4, 12, and 2 V, respectively, and a collision energy of 12, 12, 20, 10, and 20 V, respectively.

8. Cell viability of ABC and its ProTides.

A CCK-8 Kit was purchased from Dojindo and used to test cell viability after treatment with ABC and its ProTides. Briefly, MDM plated in a 96-well plate were treated with 100 µl of 0-400 µM ABC and its ProTides for 24 h. Drug containing medium was removed and replaced with fresh medium. Ten µl of CCK-8 solution was added to each well followed by incubation of the plate for 2 h at 37˚C. The absorbance (at 450 nm) of each well was measured using a microplate reader. The cell viability was calculated according to manufacturer's instructions.

9. Stability Assay in Serum.

The experiment was carried out by adding each ProTide into 100 µl human serum to reach a final concentration of 15ug/ml in glass vial, and duplicate samples were used for each ProTide. After incubating the samples at 37 °C with shaking for 24 h, 900 µl of MeOH was added to stop the reaction. Samples were vortexed for 3min, then centrifuged down at 16,000 g for 10 min. The supernatants were transfer to new 2 ml tubes and evaporated to dryness under vacuum. The samples were reconstituted using 50 µl of MeOH, vortexed for 3 min and centrifuged down at 16,000 g for 10 min. The supernatants were analyzed by HPLC for drug concentrations. For control sample (0 min), MeOH were added first to stop the enzyme before adding the ProTides.

10. Preparation and characterization of ABC phosphoramidate prodrug nanoformulations.

Lipid-PLGA hybrid nanoparticles loaded with **M1ABC** (N**M1ABC**) were prepared using a modified single emulsion solvent evaporation technique as described previously [8]. Briefly, a mixture of lipids consisting of DSPC, DSPE-PEG_{2k} and DSPG in 10:5:1 weight ratio was coated over drug loaded PLGA nanoparticles to enable sustained release of the loaded cargo. A 2:1 weight ratio of PLGA core to lipid shell was used. N**M2ABC** and N**M3ABC** were prepared by high-pressure homogenization (Avestin EmulsiFlex-C3; Avestin Inc., Ottawa, ON, Canada) as previously reported[9]. Briefly, **M2ABC** or **M3ABC** (1% w/v) and poloxamer 407 (P407, 0.2% w/v) were premixed at room temperature overnight followed by homogenization at 20,000 psi until the desired particle size and polydispersity index (PdI) were achieved. Effective diameter (D_{eff}), PdI, and ζ-potential were measured by dynamic light scattering (DLS) (Malvern Zetasizer Nano Series Nano-ZS, Malvern Instruments, Westborough, MA, USA).

11. Cell uptake and retention of ABC phosphoramidate prodrug nanoformulations.

Uptake and retention of nanoformulations were determined in MDM as previously reported[5, 9-12]. Briefly, MDM were treated with different nanoformulations at a concentration of 100 µM for 1, 2, 4 and 8 h. At select time points, adherent MDM were collected in 1 mL PBS after washing with 1 mL PBS twice. MDM were pelleted by centrifugation at 3,000 rpm for 8 min. The cell pellet was reconstituted in 200 µl methanol and probe sonication (10 sec) was used to break the cells and extract drugs. After probe

sonication, cell debris was pelleted by centrifugation at 14,000 rpm for 10 min and 4˚C. The supernatant was analyzed for prodrug level using HPLC-UV/Vis. For retention, MDM were treated with 100 µM different nanoformulations for 8 h. The nanoformulation-containing media were replaced with fresh media after washing MDM with PBS twice. At days 1, 5, 10, 15, 20, 25 and 30, MDM were collected in 1mL PBS after washing with 1 mL PBS twice. The samples were processed as described above for analysis using HPLC.

Intracellular CBV-TP levels were also measured for as an indicator of cell uptake and retention. Briefly, cells were scraped into PBS, pelleted by centrifugation at 3,000 rpm for 8 min, and reconstituted in 200 µl 70% methanol. CBV-TP was extracted, and the samples were analyzed by LC-MS/MS as previously reported[1].

12. Antiretroviral efficacy of nanoformulations.

Antiviral efficacies of ABC and the ProTide nanoformulations in MDM were assessed as previously described5. MDM were treated with 100 µM different nanoformulations for 8 h. Then nanoformulation-containing medium was replaced with fresh medium after washing MDM with PBS twice. Half media changes were conducted every other day. At predetermined time points, days 1, 5 ,10, 15, 20, 25 and 30, MDM were infected with HIV-1ADA for 4 h at a MOI of 0.1 and cultured with every-other-day half media changes for an additional 10 days. At day 10, supernatant was collected for RT assay and MDM were fixed with paraformaldehyde and immunostained for expression of HIVp24. Untreated, uninfected MDM served as negative controls (control), while MDM exposed to HIV-1ADA but not treated with nanoformulations, served as positive controls (HIV-1).

For N**M3ABC**, a concentration response study was also conducted. MDM were treated with 1, 10, 25, 50 or 100 µM N**M3ABC** for 8 h. The treated MDM were then washed twice with PBS. MDM were cultured for an additional 30 days in fresh media with half media changes every other day. At day 30, MDM were challenged with HIV-1_{ADA} at a MOI of 0.1 for 4 h. Excess virus was washed out with PBS and MDM were cultured for another 10 days. At day 10 post-exposure, media were collected for RT assay and MDM were fixed and immunostained for expression of HIVp24 antigen.

13. *In vivo* study with rats.

Animal PK studies were conducted in accordance with the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Male Sprague Dawley rat (250g; Jackson Labs, Bar Harbor, ME) were injected with NM3ABC (45 mg/kg ABC-eq.) intramuscularly (IM; caudal thigh muscle). Whole blood samples were collected at 2, 24 and 168 hours after injection. PBMC were separated from whole blood samples using HISTOPAQUE 1083 (Sigma# 1083-1) following manufacturer's instruction. CBV-TP were extracted and quantified as mentioned above[1].

14. Acknowledgement.

We wish to thank the University of Nebraska Medical Center Cores for Electron Microscopy (Tom Bargar & Nicholas Conoan), NMR Spectroscopy (Ed Ezell), Elutriation and Cell Separation (Myhanh Che and Na Ly). We also thank Bhavesh Kevadiya, Dhruvkumar Soni, Chang Liu and Nathan Smith for technical assistance. The research was supported by the University of Nebraska Foundation, which includes donations from the Carol Swarts, M.D. Emerging Neuroscience Research Laboratory, the Margaret R. Larson Professorship, and the Frances, and Louie Blumkin, and Harriet Singer Endowment, the Vice Chancellor's Office of the University of Nebraska Medical Center for Core Facility Developments, and National Institutes of Health grants R01 MH104147, P01 DA028555, R01 NS36126, P01 NS31492, 2R01 NS034239, P01 MH64570, P30 MH062261, P30 AI078498, and R01 AG043540. The authors have no affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

15. References.

1. N. Gautam, Z. Lin, M. G. Banoub, N. A. Smith, A. Maayah, J. McMillan, H. E. Gendelman and Y. Alnouti, *J Pharm Biomed Anal*, 2018, **153**, 248-259.
2. P. K. Mandal and J. S. McMurray, *The Journal of Organic Chemistry*, 2007, **72**, 6599-6601.
3. C. McGuigan, S. A. Harris, S. M. Daluge, K. S. Gudmundsson, E. W. McLean, T. C. Burnette, H. Marr, R. Hazen, L. D. Condreay, L. Johnson, E. De Clercq and J. Balzarini, *J Med Chem*, 2005, **48**, 3504-3515.
4. H. E. Gendelman, J. M. Orenstein, M. A. Martin, C. Ferrua, R. Mitra, T. Phipps, L. A. Wahl, H. C. Lane, A. S. Fauci, D. S. Burke and et al., *J Exp Med*, 1988, **167**, 1428-1441.
5. D. Singh, J. McMillan, J. Hilaire, N. Gautam, D. Palandri, Y. Alnouti, H. E. Gendelman and B. Edagwa, *Nanomedicine (Lond)*, 2016, **11**, 1913-1927.
6. S. S. Kalter, R. L. Heberling, J. D. Barry, I. K. Kuramoto, P. V. Holland and K. Sazama, *Journal of clinical microbiology*, 1992, **30**, 993-995.
7. J. Balzarini, S. Aquaro, A. Hassan-Abdallah, S. M. Daluge, C. F. Perno and C. McGuigan, *FEBS Lett*, 2004, **573**, 38-44.
8. Y. Liu, K. Li, J. Pan, B. Liu and S. S. Feng, *Biomaterials*, 2010, **31**, 330-338.
9. D. Guo, T. Zhou, M. Araínga, D. Palandri, N. Gautam, T. Bronich, Y. Alnouti, J. McMillan, B. Edagwa and H. E. Gendelman, *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 2017.
10. B. Sillman, A. N. Bade, P. K. Dash, B. Bhargavan, T. Kocher, S. Mathews, H. Su, G. D. Kanmogne, L. Y. Poluektova and S. Gorantla, *Nature communications*, 2018, **9**, 443.
11. T. Zhou, H. Su, P. Dash, Z. Lin, B. L. D. Shetty, T. Kocher, A. Szlachetka, B. Lamberty, H. S. Fox and L. Poluektova, *Biomaterials*, 2018, **151**, 53-65.
12. T. Zhou, Z. Lin, P. Puligujja, D. Palandri, J. Hilaire, M. Arainga, N. Smith, N. Gautam, J. McMillan, Y. Alnouti, X. Liu, B. Edagwa and H. E. Gendelman, *Nanomedicine (Lond)*, 2018, DOI: 10.2217/nnm-2017-0381.