Supplementary Information

A Year-Long Extended Release Nanoformulated Cabotegravir Prodrug

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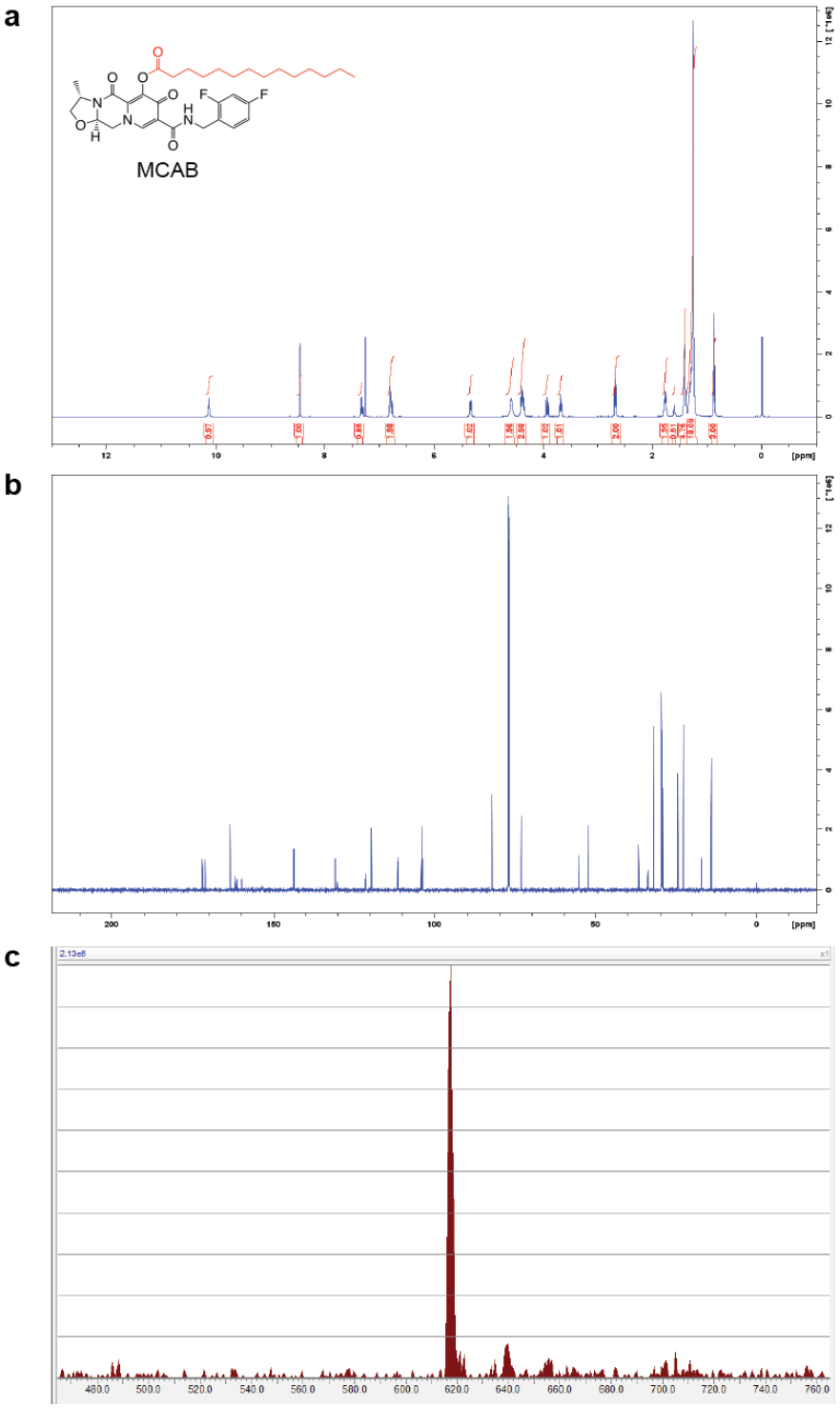
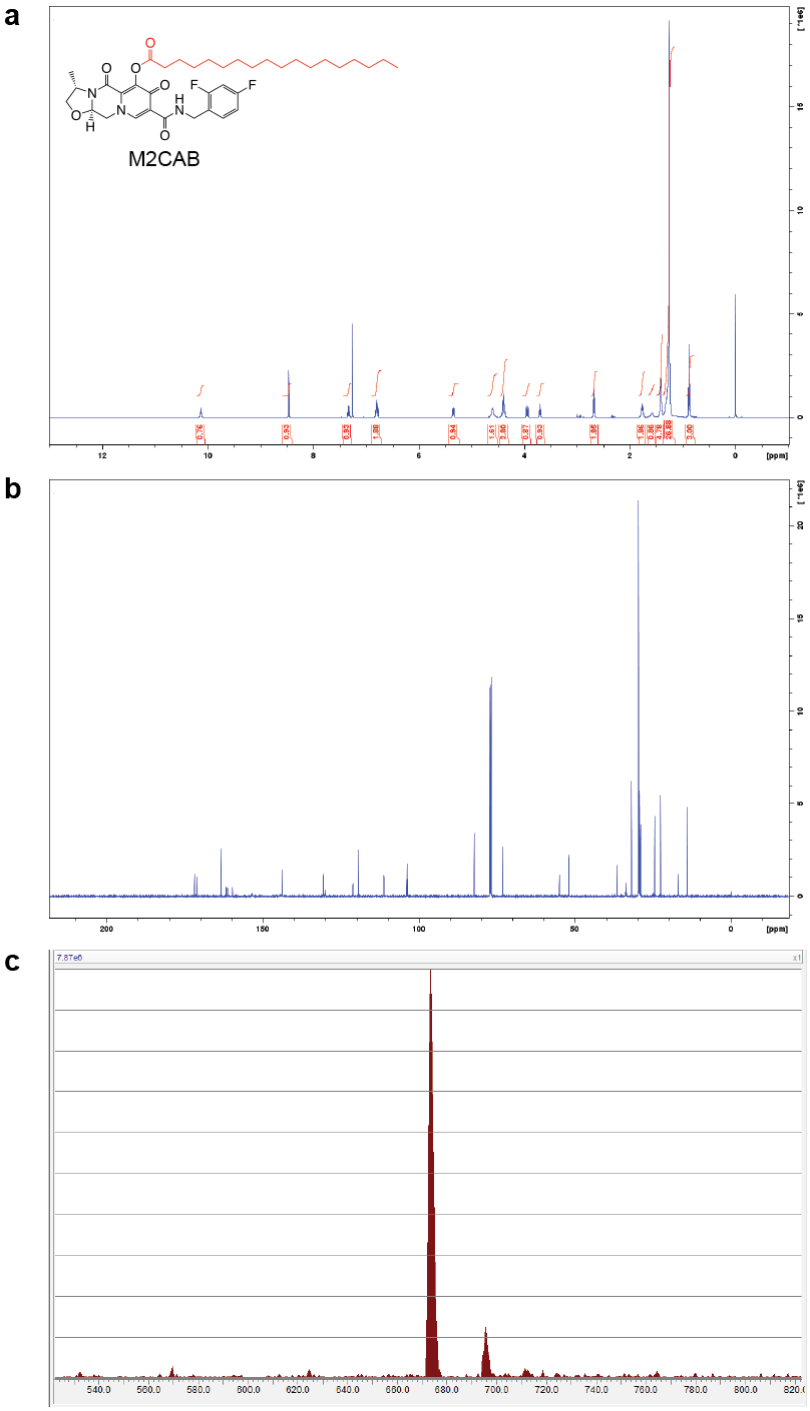
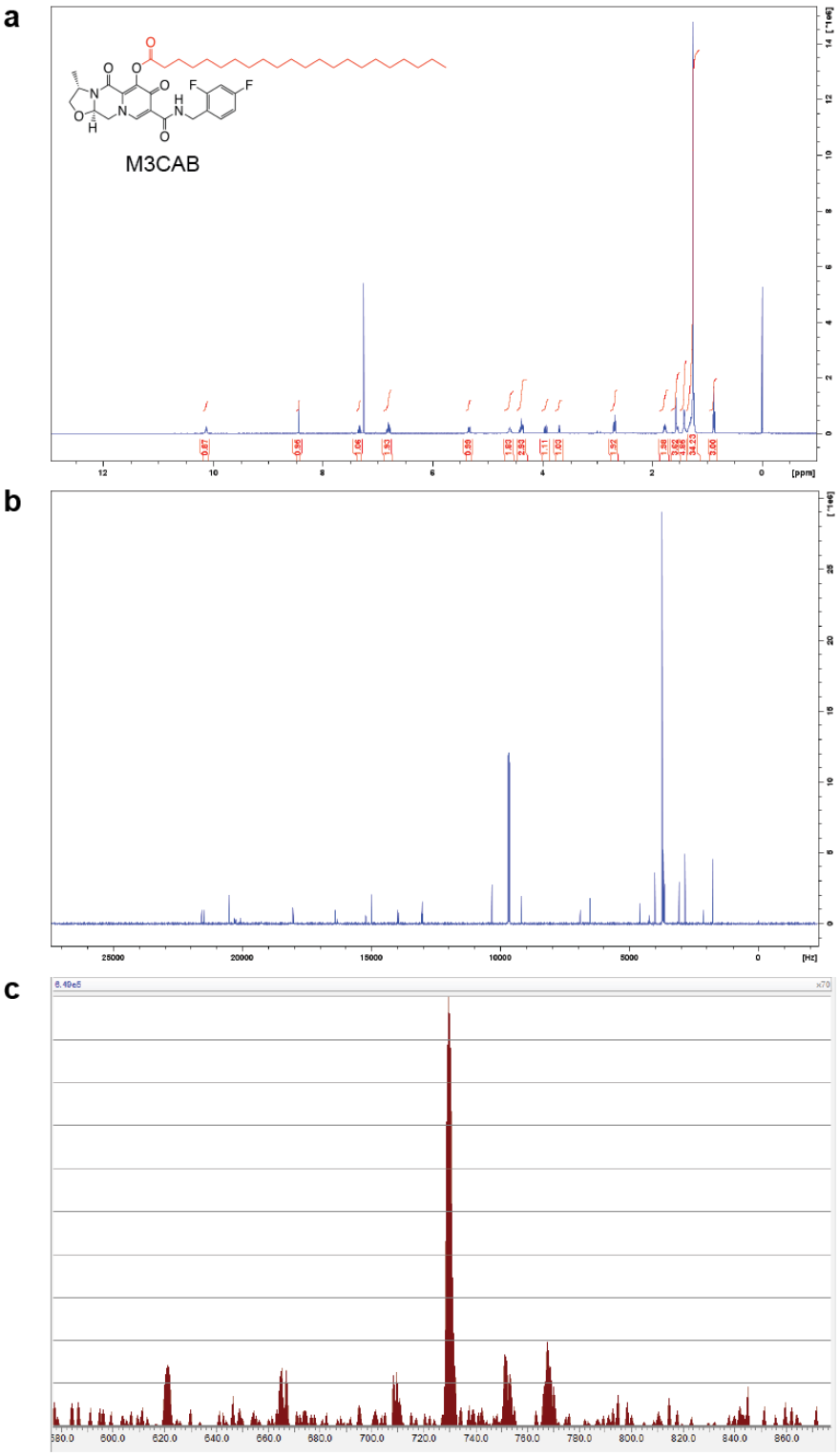
Supplementary Figures



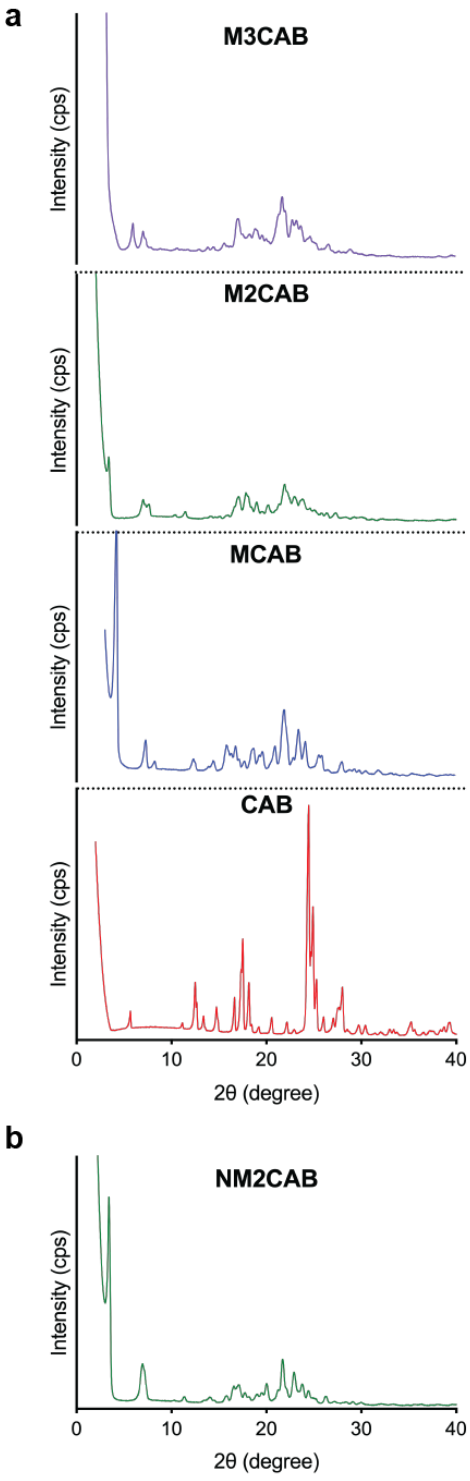
Supplementary figure 1. *(**a**) **^{1}H and** (**b**) **^{13}C NMR of MCAB.** Proton and carbon-13 NMR spectra confirmed the successful synthesis of MCAB. (**c**) ESI-MS infusion of MCAB generated a strong signal at 615.31 m/z. (**a-c**) Experiments were repeated independently five times with equivalent results.*



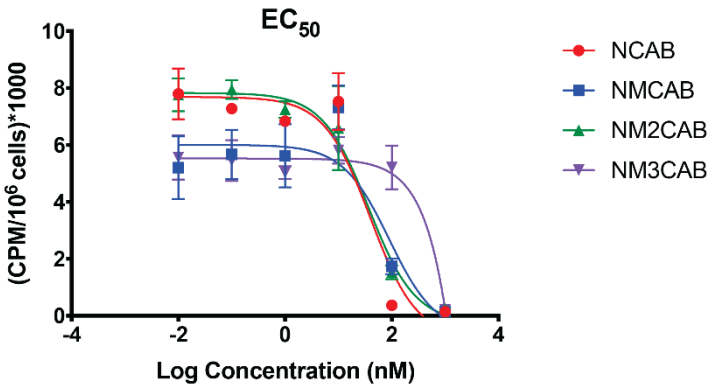
Supplementary figure 2. *(**a**)* ***^{1}H and*** *(**b**)* ***^{13}C NMR of M2CAB.*** *Proton and carbon-13 NMR spectra confirmed the successful synthesis of M2CAB. (**c**) ESI-MS infusion of M2CAB generated a strong signal at* 671.37 *m/z. (**a-c**) Experiments were repeated independently five times with equivalent results.*



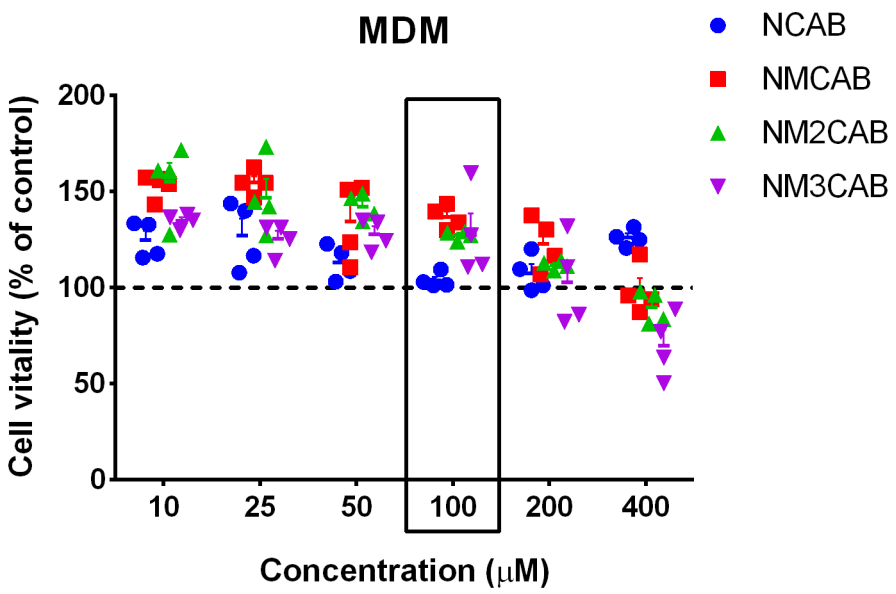
Supplementary figure 3. *(**a**)* ***^{1}H and*** *(**b**)* ***^{13}C NMR of M3CAB.*** *Proton and carbon-13 NMR spectra confirmed the successful synthesis of M3CAB. (**c**) ESI-MS infusion of M3CAB generated a strong signal at 727.44 m/z. (**a-c**) Experiments were repeated independently five times with equivalent results.*



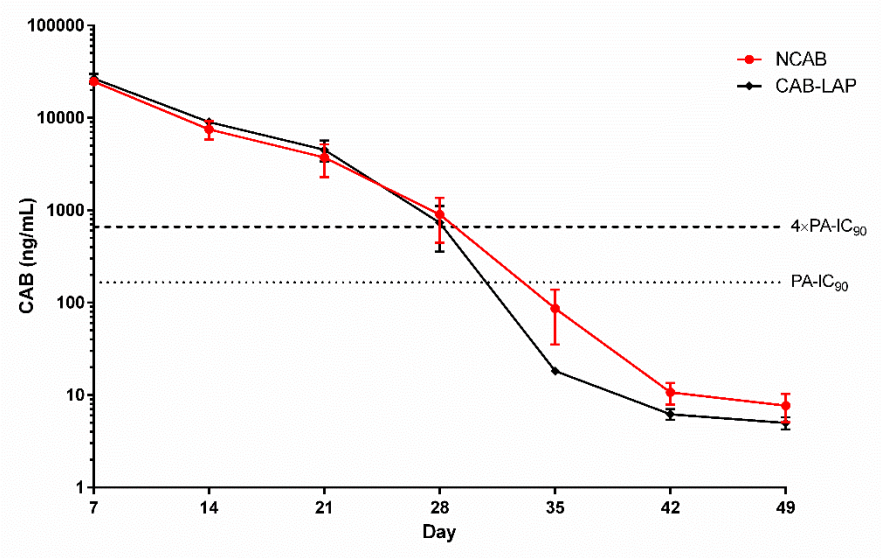
Supplementary figure 4. (a) X- ray diffraction (XRD) of CAB and prodrugs. *XRD analysis of CAB, MCAB, M2CAB, and M3CAB at 2θ = 2-70 at 1 °/min.* ***(b) XRD of NM2CAB.*** *XRD analysis of NM2CAB at 2θ = 2-70 at 1 °/min. (**a-b**) Experiments were repeated independently three times with equivalent results.*



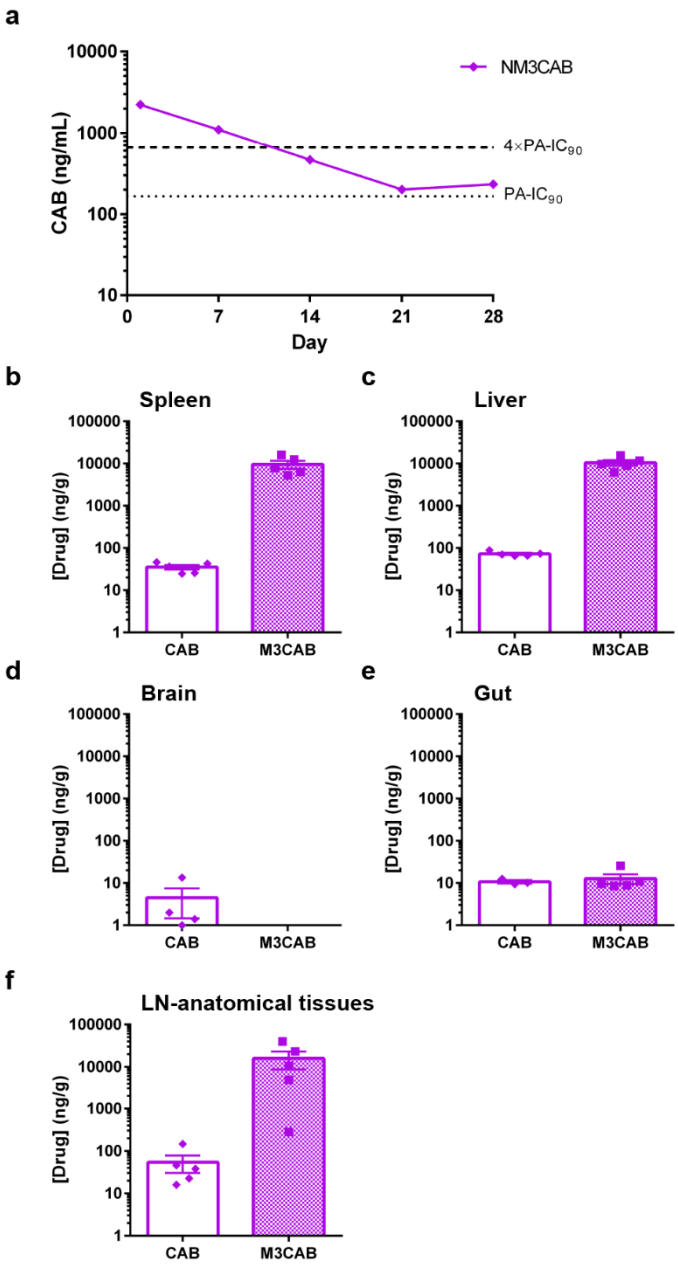
Supplementary figure 5. EC_{50} assessment of nanoformulations. *Antiviral activity of NCAB, NMCAB, NM2CAB, or NM3CAB was determined in MDM over a range of concentrations (0.01-1000 nM) by measuring HIV-1 RT activity after HIV-1_{ADA} challenge with at an MOI of 0.1. Data are expressed as the mean ± SEM for N = 3 biological replicates.*



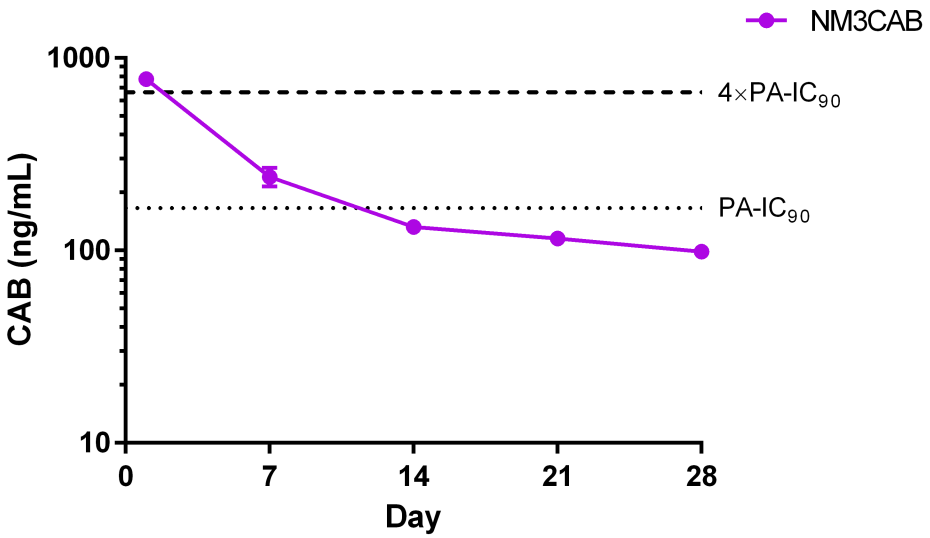
Supplementary figure 6. Cytotoxicity assay*. The cellular vitality in MDM was determined after 24 hours of MTT treatment over a range (10-400 μM) of concentrations. The concentration in the box did not show cytotoxicity. Thus, 100 μM treatment concentration was chosen for further analyses. Data are expressed as the mean ± SEM for N = 4 biological replicates.*



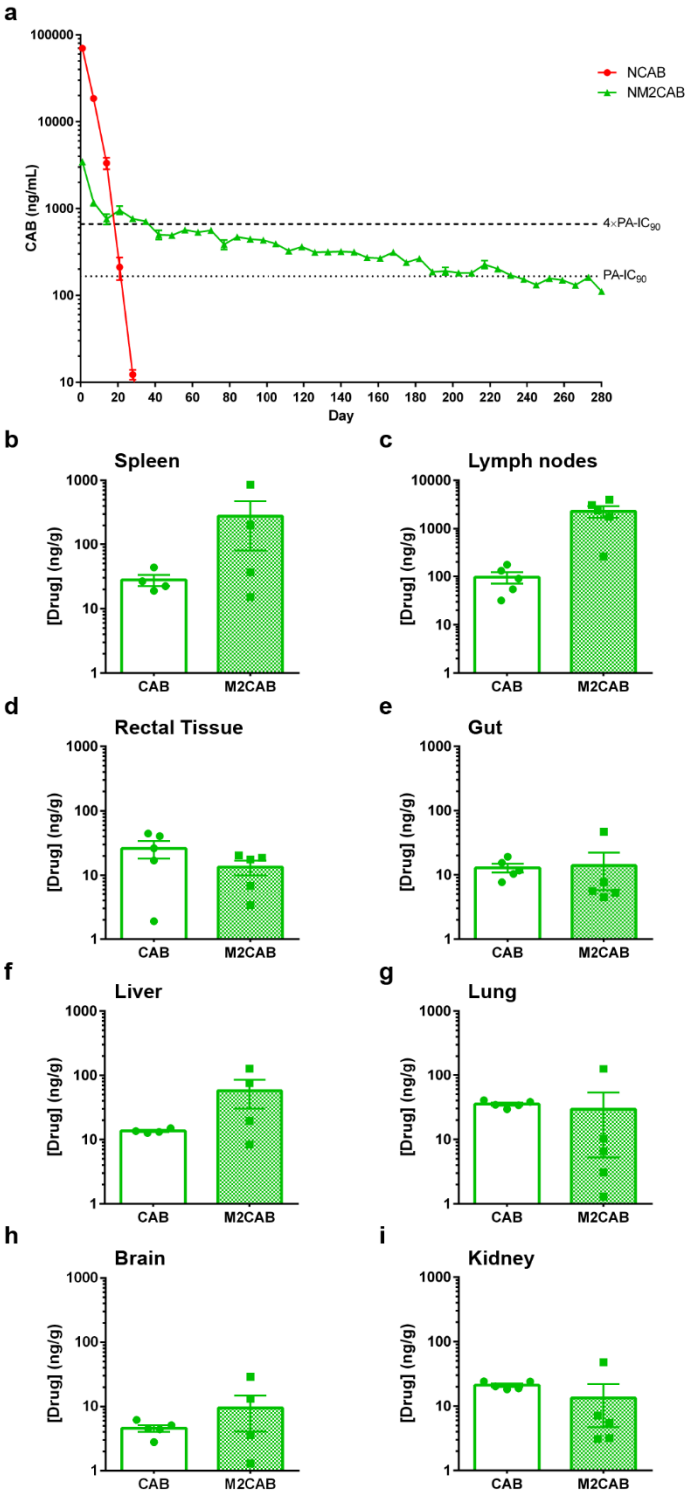
Supplementary figure 7. Comparative PK assessment for NCAB and CAB LA. *Male BALB/cJ mice were administered a single IM 45 mg/kg dose of NCAB or CAB-LAP (currently in clinic). Plasma CAB levels were measured weekly up to day 49. Top bold dashed line indicates plasma CAB 4×PA-IC_{90} of 664 ng/mL, and the bottom stippled line shows the plasma CAB PA-IC_{90} of 166 ng/mL. Plasma CAB levels in both treatments were equivalent. Data are expressed as mean ± SEM. For NCAB, N = 10 animals and for CAB LA, N = 5 animals. Data for CAB-LAP were extracted from[14] and adapted for comparisons.*



Supplementary figure 8. NM3CAB PK and BD testing in NSG mice. *(**a**) Plasma CAB levels were measured 28 days after a single IM injection of NM3CAB (45 mg/kg CAB-equivalent dose) in female NSG mice. Drug levels in plasma were monitored weekly. Top bold dashed line indicates plasma CAB 4×PA-IC_{90} of 664 ng/mL, and the bottom stippled line shows the plasma CAB 1×PA-IC_{90} of 166 ng/mL. (**b - e**) Tissue concentrations of CAB and M3CAB at day 28 following NM3CAB administration were quantitated in (**b**) spleen, (**c**) liver, (**d**) brain, (**e**) gut, and (**f**) lymph nodes-anatomical associated tissues. Data are expressed as mean ± SEM. Animal numbers, N = 5.*

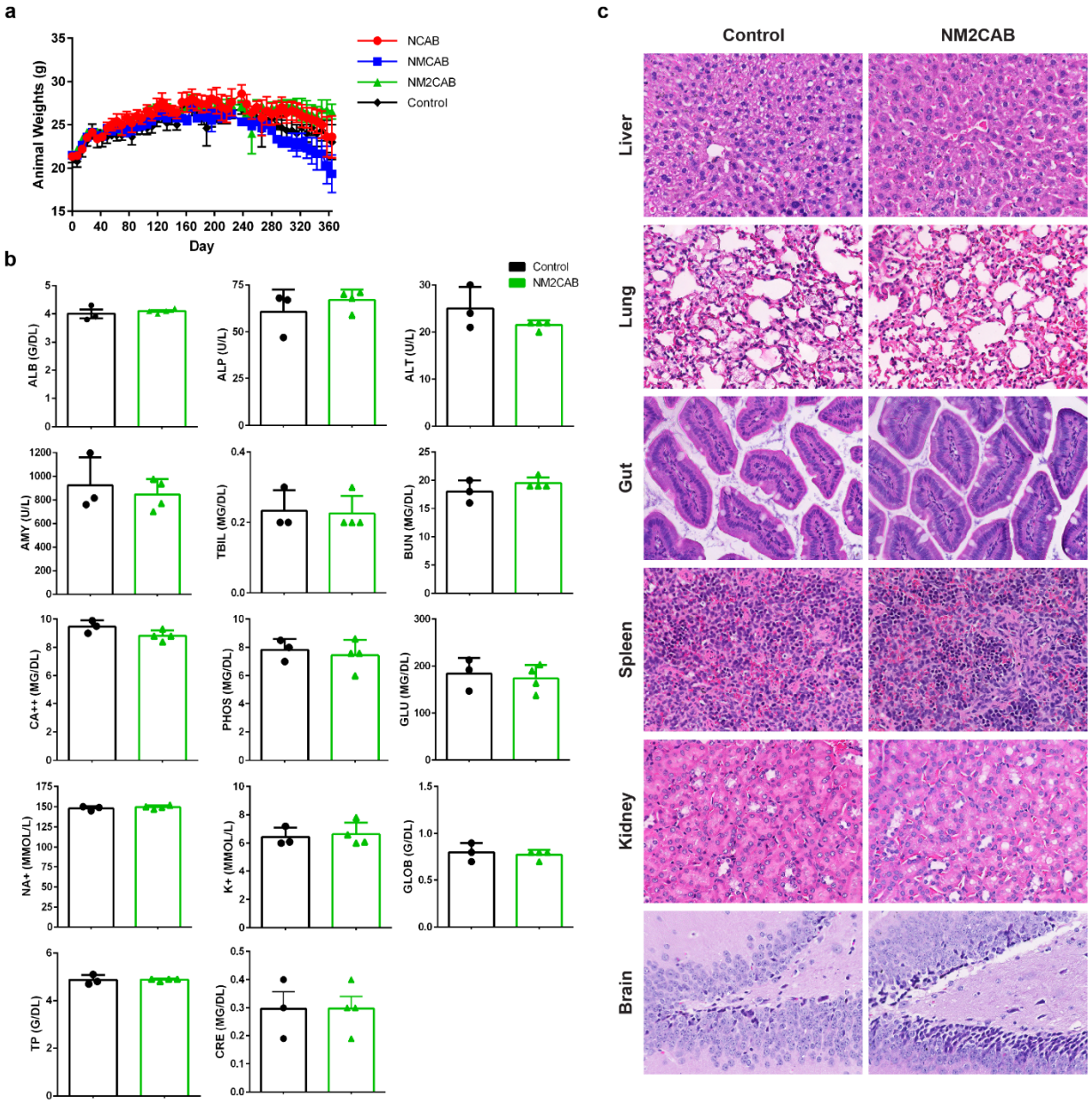


Supplementary figure 9. NM3CAB PK testing in BALB/cJ mice. *Plasma CAB levels were assessed 28 days after a single IM injection of NM3CAB (45 mg/kg CAB-equivalent dose) in male BALB/cJ mice. Drug levels in plasma were monitored weekly. Top bold dashed line indicates plasma CAB 4×PA-IC$_{90}$ of 664 ng/mL, and the bottom stippled line shows the plasma CAB 1×PA-IC$_{90}$ of 166 ng/mL. Data are expressed as mean ± SEM. Animal numbers, N = 4.*



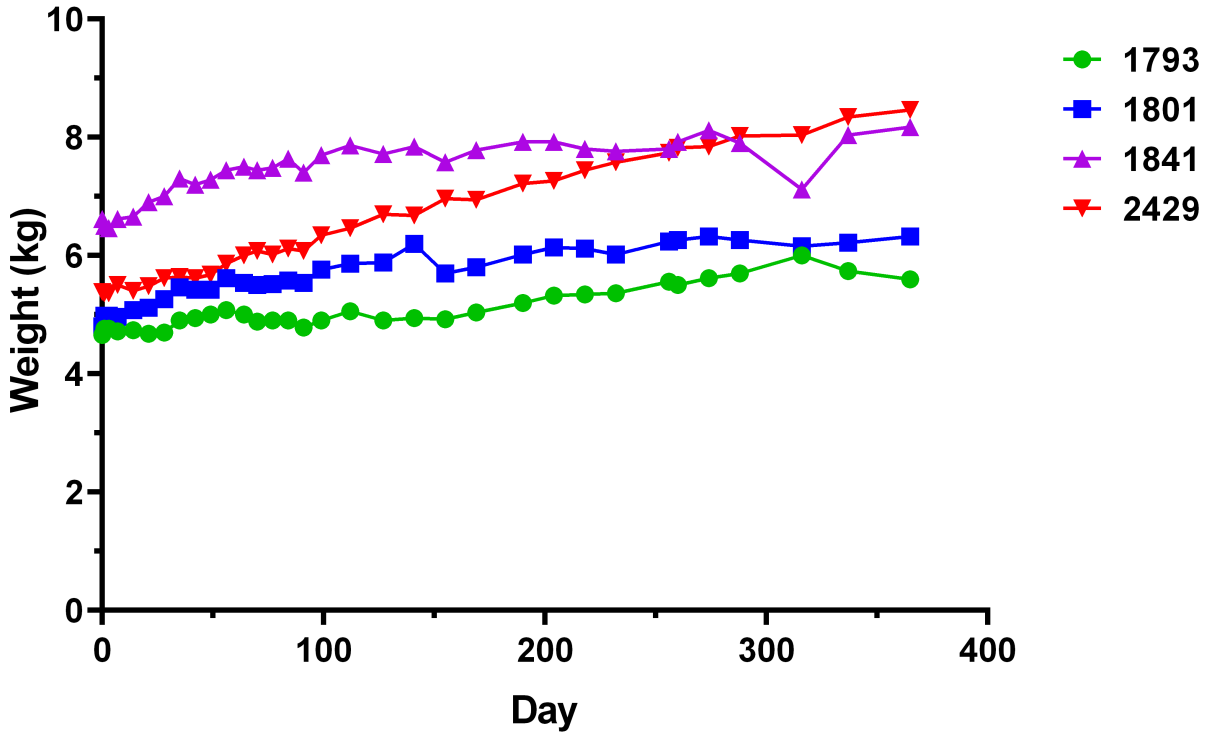
Supplementary figure 10. NCAB or NM2CAB BD in male BALB/cJ mice. *(**a**) CAB and M2CAB levels were assessed 280 days after a single IM injection of NCAB or NM2CAB (45 mg/kg CAB-equivalents) in the plasma. Drug levels in plasma were monitored weekly. Top bold dashed line indicates plasma CAB*

*4×PA-IC_{90} of 664 ng/mL, and the bottom stippled line shows the plasma CAB 1×PA-IC_{90} of 166 ng/mL. (**b - i**)Tissue concentrations of CAB and M2CAB at day 280 following NM2CAB administration were quantitated in (**b**) spleen, (**c**) lymph nodes, (**d**) rectal tissue, (**e**) gut, (**f**) liver, (**g**) lung, (**h**) brain, and (**i**) kidney. Data are expressed as mean ± SEM. Animal numbers/group, N = 5.*

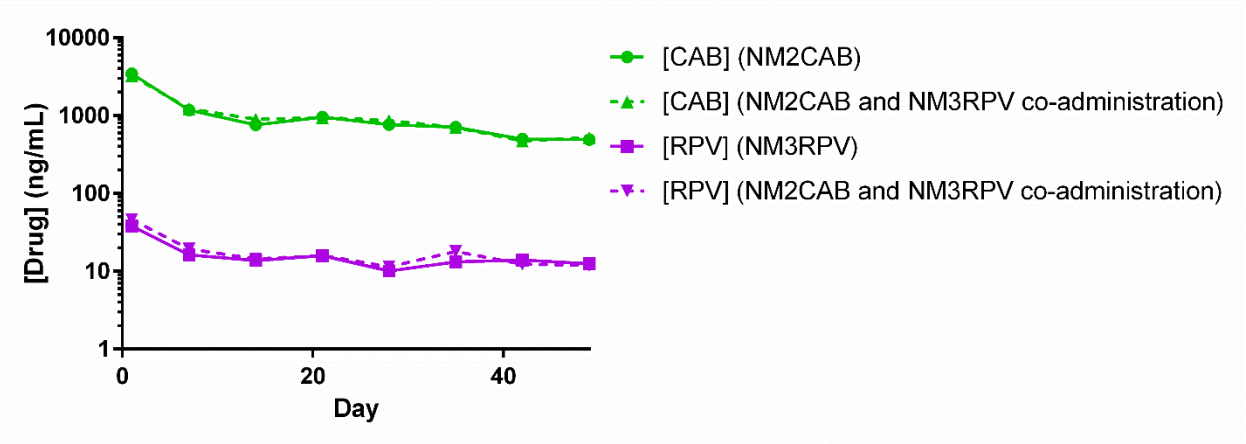


Supplementary figure 11. Toxicity profiles in female NSG mice. *(**a**) Weights of mice treated with NCAB, NMCAB, or NM2CAB, and of controls were recorded. Age matched untreated mice were used as controls. Data are expressed as mean ± SEM, and animal numbers in each group, N = 3 (control), N = 5 (NCAB), N = 3 (NMCAB), and N = 4 (NM2CAB). (**b**) Comprehensive serum chemistry profiles were analyzed in mice post-NM2CAB administration. Examined parameters were albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), blood urea*

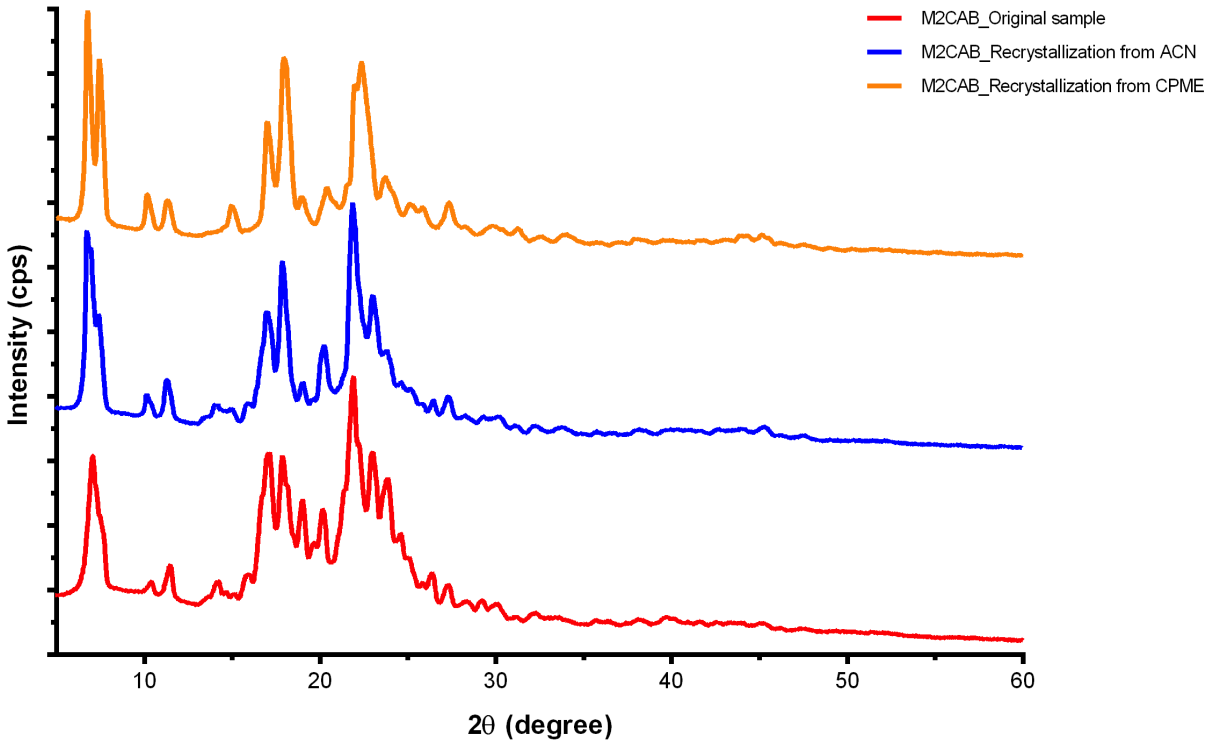
*nitrogen (BUN), total calcium (CA++), phosphorus (PHOS), glucose (GLU), sodium (NA+), potassium (K+), globulin (GLOB), total protein (TP), and creatinine (CRE). Data are expressed as mean ± SEM, and animal numbers/group, N = 3 (for control) and N = 4 (for NM2CAB). (**c**) Histopathology was performed on tissues of mice treated with NM2CAB. Tissue sections (5 µm) of liver, lung, gut, spleen, kidney, and brain were stained with hematoxylin and eosin (H&E) and evaluated by a certified pathologist. Images were captured with a 40× objective. No adverse events or abnormal pathology was detected in any of the tests. Animal numbers in each group, N = 3 (for control) and N = 4 (for NM2CAB). Histopathology evaluation of tissues of mice at 364 days post-treatment was done a single time.*



Supplementary figure 12. Weights of rhesus macaques *were recorded to day 365 for each animal. This was performed to evaluate well-being of the animals following NM2CAB treatments. N = 4 rhesus macaques*



Supplementary figure 13. Potential drug-drug interactions. *Male Balb/cJ mice were administered via IM injection, NM2CAB alone, NM3RPV alone, or both NM2CAB and NM3RPV (all doses as 45 mg/kg CAB or RPV equivalents) to determine drug-drug interactions of prodrug nanoformulations. Plasma levels of CAB or RPV were measured up to day 49. Data are expressed as mean ± SEM. Animal numbers per group, N = 5.*



Supplementary figure 14. X- ray diffraction (XRD) of M2CAB before and after recrystallization using ACN (acetonitrile) or CPME (cyclopentyl methyl ether). *XRD analysis of M2CAB at 2θ = 2-70 at 1 °/min. Experiments were repeated independently two times with equivalent results.*

Supplementary Tables

Supplementary Table 1. Comparative physicochemical characterization of CAB prodrugs.

	CAB	MCAB	M2CAB	M3CAB
Molecular weight (g/mol)	405.36	615.31	671.37	727.44
Aqueous solubility (µg/mL)	34.31	0.13	0.10	0.04
1-Octanol solubility (µg/mL)	49.10	5796.8	8057.2	6159.5
$LogP_{ow}$	0.16	4.56	4.89	5.12

ow = 1-octanol/water

Supplementary Table 2. Characterization of M2CAB nanoformulation.

	NCAB	NM2CAB
Encapsulation efficiency (%)	76.83	75.07
Drug Loading (%)	84.50	87.10
Free drug in nanoformulation (%)	0.81	0.41

Supplementary Table 3. Reproducibility of NM2CAB manufacture. N = 3 biologically independent samples

Manufacture Batch no.	Size ± SEM (nm)	PDI ± SEM
1	320.13 ± 6.81	0.23 ± 0.02
2	322.10 ± 2.99	0.21 ± 0.01
3	328.67 ± 1.60	0.18 ± 0.03
4	311.43 ± 4.53	0.33 ± 0.03
5	287.17 ± 0.94	0.22 ± 0.01
6	317.57 ± 2.83	0.27 ± 0.01
7	378.00 ± 1.90	0.26 ± 0.01
8	244.63 ± 5.85	0.19 ± 0.01
9	268.97 ± 7.66	0.19 ± 0.02
10	259.77 ± 2.09	0.23 ± 0.01
11	243.00 ± 2.48	0.22 ± 0.01

Supplementary Table 4. Correlation between in vitro and in vivo PK assessments.

Dependent Variables	P-Value	R^2
Potency, RT activity CPM/10e6 cells EC50, nM	0.88	0.04
Potency, RT activity CPM/10e6 cells AUC, CPM/10e6 cells*nM	0.26	0.84
Cell Vitality MDM, Percent*uM AUC	0.77	0.13
log MDM uptake mmol CAB/10e6*hr AUC	0.02	0.99
In vitro antiviral activity, Days @ 100 uM CAB eq AUC	0.03	0.99

*Linear regression models were used to examine the association between in vitro parameters and area-under-the-curve (AUC) of in vivo PK data of CAB and CAB prodrugs. In vivo PK AUC: N = 5 animals (NCAB), N = 3 animals (NMCAB), and N = 4 animals (NM2CAB); Potency, RT activity CPM/10e6cells EC50, nM : N = 3 biological replicates; Potency, RT activity CPM/10e6cells AUC, CPM/10e6 cells*nM: N = 3 biological replicates; Cell Vitality MDM, Percent*uM AUC: N = 4 biological replicates; log MDM uptake mmol CAB/10e6*hr AUC: N = 3 biological replicates; In vitro antiviral activity, Days @ 100 uM CAB eq AUC: N = 3 biological replicates.*

Supplementary Table 5. Characterization of M2CAB before and after recrystallization.

Recrystallization solvent	Recovery yield (%)	Melting point range after recrystallization (˚C)	M2CAB purity
Original sample	NA	116-122	98.86%
ACN	**70%**	123-125	99.08%
MeTHF	15%	120-121	-
CPME	**85%**	121-122	98.99%
PhMe	10%	120-121	-

Supplementary Methods

CAB prodrugs chemical characterization

Proton (^{1}H)- and carbon (^{13}C)- NMR spectra for MCAB, M2CAB, and M3CAB were recorded on a Varian Unity/Inova-500 NB (500 MHz; Varian Medical Systems Inc., Palo Alto, CA).

MCAB: ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.13 (t, *J* = 5.7 Hz, 1H), 8.47 (s, 1H), 7.32 (dd, *J* = 15.0, 8.3 Hz, 1H), 6.74-6.84 (m, 2H), 5.34 (dd, *J* = 9.9, 3.7 Hz, 1H) 4.59 (b, 2H), 4.33-4.46 (m, 3H), 3.94 (app t, *J* = 11.1 Hz, 1H), 3.69 (app. t, *J* = 7.3 Hz, 1H), 2.68 (t, *J* = 7.6 Hz, 2H), 1.76 (p, *J* = 7.5 Hz, 2H), 1.40 (app. d, *J* = 5.6 Hz, 5H), 1.20-1.38 (b, 18H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 171.0, 163.3, 161.7, 161.3, 159.7, 153.5, 143.7, 130.5, 130.0, 121.2, 121.1, 119.5, 111.3, 111.1, 104.0, 103.8, 103.6, 82.3, 73.1, 54.9, 52.0, 36.5, 33.7, 31.9, 29.7, 29.5, 29.3, 29.0, 24.5, 22.7, 17.0, 14.1. HRMS (ESI-TOF) m/z: $[M + H]^+$: calculated for $C_{33}H_{44}F_2N_3O_6^+$, 615.31 (100.0%), 616.32 (37.7%), 617.32 (3.5%); found, 616.28.

M2CAB: ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.13 (t, *J* = 5.7 Hz, 1H), 8.47 (s, 1H), 7.33 (dd, *J* = 14.9, 8.4 Hz, 1H), 5.34 (dd, *J* = 9.9, 3.8 Hz, 1H), 4.60 (b, 2H), 4.33-4.47 (m, 3H), 3.94 (t, *J* = 11.1 Hz, 1H), 3.70 (app. t, *J* = 7.4 Hz, 1H), 2.68 (t, *J* = 7.6 Hz, 2H), 1.77 (p, *J* = 7.6 Hz, 2H), 1.41 (app. d, *J* = 5.8 Hz, 4H), 1.20-1.38 (b, 27H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 171.1, 163.3, 163.2, 161.7, 161.3, 159.7, 153.5, 143.7, 130.6, 130.5, 130.0, 121.3, 121.1, 119.5, 111.3, 111.1, 104.0, 103.8, 103.6, 82.3, 73.1, 54.9, 52.0, 36.6, 33.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 24.5, 22.7, 17.1, 14.1. HRMS (ESI-TOF) m/z: $[M + H]^+$: calculated for $C_{37}H_{52}F_2N_3O_6^+$, 671.37 (100%), 672.38 (40.0%), 673.38 (7.8%); found, 672.34.

M3CAB: ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.13 (t, *J* = 5.8 Hz, 1H), 8.46 (s, 1H), 7.33 (dd, *J* = 14.9, 8.4 Hz, 1H), 6.72-6.87 (m, 2H), 5.35 (dd, *J* = 9.9, 3.8 Hz, 1H), 4.60 (b, 2H), 4.34-4.48 (m, 3H), 3.95 (t, *J* = 11.1 Hz, 1H), 3.71 (app. t, *J* = 7.4 Hz, 1H), 2.68 (t, *J* = 7.6 Hz, 2H), 1.98 (p, *J* = 7.5

Hz, 2H), 1.41 (app. d, *J* = 5.8 Hz, 5H), 1.20-1.38 (b, 34H), 0.87 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.9, 171.1, 163.4, 163.3, 163.2, 161.7, 161.3, 159.7, 153.5, 143.7, 130.6, 130.5, 129.9, 121.2, 121.1, 119.5, 111.3, 111.1, 104.0, 103.8, 103.6, 82.3, 73.1, 54.9, 52.0, 36.6, 33.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 24.5, 22.7, 17.1, 14.1. HRMS (ESI-TOF) m/z: $[M + H]^+$: calculated for $C_{41}H_{60}F_2N_3O_6$, 727.44 (100%), 728.44 (44.3%), 729.44 (6.9%); found, 728.47.

Quantitation of CAB, MCAB, M2CAB and M3CAB by UPLC-MS/MS

CAB, MCAB, M2CAB, and M3CAB were quantitated in mouse and rhesus macaque plasma, blood and tissues by UPLC-MS/MS using a Waters ACQUITY H-class UPLC (Waters, Milford, MA, USA) connected to a Xevo TQ-S micro mass spectrometer. All solvents for sample processing and UPLC-MS/MS analysis were LC-MS-grade (Fisher). For plasma and blood samples, 25 µL of sample was added into 1 mL ACN spiked with 10 µL internal standard (IS). d3-Dolutegravir (d3-DTG), myristoylated dolutegravir (MDTG), and stearoylated darunavir (SDRV), at a final concentration of 40, 20 and 20 ng/mL, respectively, were used as ISs for CAB, MCAB, and M2CAB/M3CAB analyses, respectively. Samples were vortexed and centrifuged at 17,000 × g for 10 minutes at 4 °C. The supernatants were collected and dried using a SpeedVac and reconstituted in 100 µL 80% methanol; 10 µL was injected for MCAB, M2CAB, and M3CAB UPLC–MS/MS analyses. For CAB analysis, 20 µl of above reconstitute in 80% methanol was mixed with 80 µl of 50% ACN before LC-MS/MS injections. Standard curves were prepared in blank mouse or rhesus macaque plasma/blood in the range of 0.2-500 ng/mL for CAB, MCAB, M2CAB, and M3CAB. For tissue drug quantitation, 3-200 mg of sample was homogenized in 4-29 volumes of 0.1% v/v formic acid and 2.5 mM ammonium formate containing 90% methanol. To 100 µL of tissue homogenate was added 280 µl methanol containing 0.1% formic acid and 2.5 mM ammonium formate, 80% methanol (10 µL), and IS (10 µL), followed by vortexing for 3 minutes and centrifugation at 17,000 × g for 15 minutes. For

MCAB, M2CAB, and M3CAB analyses, 85 µl of supernatant was mixed with 15 µl water. For CAB analysis, 20 µl of supernatant was mixed with 80 µl of 50% ACN. These aliquots were vortexed, centrifuged at 17,000 x g for 10 minutes, and 10 µl of supernatant was used for UPLC-MS/MS analysis. Standards were prepared similarly using blank tissue homogenates with 10 µL of spiking solution (CAB/MCAB/M2CAB/M3CAB, 5-5,000 ng/mL in 80% MeOH containing 0.1% formic acid and 2.5 mM ammonium formate). For CAB quantitation, chromatographic separation of 10 µL CAB sample was performed on a Waters ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm x 100 mm) using a 7-minutes gradient of mobile phase A (7.5 mM ammonium formate in water, adjusted to pH 3 using formic acid) and mobile phase B (100% ACN) at a flow rate of 0.25 mL/minute. For the first 3.5 minutes, the mobile phase composition was 35% B and was increased to 95% B in 0.5 minute and held constant for 1.5 minutes. Mobile phase B was then reset to 35% in 0.5 minute, and the column was equilibrated for 1 minute before the next injection. For MCAB and M2CAB quantitation chromatographic separation was achieved on a Waters ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 mm × 30 mm) using an 8-minute gradient method at a flow rate of 0.28 mL/minute. For MCAB, the initial mobile phase composition was 80% B for the first 2 minutes, and increased to 95% B in 4 minutes, held constant for 0.75 minute, reset to 80% in 0.25 minute, and the column was equilibrated for 1 minute before the next injection. For M2CAB, the initial mobile phase composition was 85% B for the first 5 minutes, and increased to 95% B in 0.25 minute, held constant for 1 minute, reset to 85% in 0.25 minute and the column was equilibrated for 1 minute before the next injection. For M3CAB quantitation chromatographic separation was achieved with the same column using an 8-minute gradient method at a flow rate of 0.35 mL/minute. The initial mobile phase composition was 88% B for the first 5 minutes, and increased to 95% B in 0.25 minute, held constant for 1.5 minutes, reset to 88% B in 0.25 minute, and the column was equilibrated for 1 minute before the next injection. CAB, MCAB, M2CAB, and M3CAB were detected at a cone voltage of 10 V, 24 V, 2 V, and 20 V, respectively, and a collision energy of

24 V, 18 V, 24 V, and 26 V, respectively, in the positive ionization mode. Multiple reaction monitoring (MRM) transitions used for CAB, MCAB, M2CAB, M3CAB, d3-DTG, MDTG, and SDRV were 406.04 > 126.93, 616.28 > 406.09, 672.34 > 406.07, 728.47 > 406.09, 422.84 > 129.99, 630.20 > 420.07, and 814.52 > 658.44, respectively. Spectra were analyzed and quantified by MassLynx software version 4.1. All quantitation's were determined using analyte peak area to internal standard peak area ratios.