Antiviral Research 171 (2019) 104614

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Octadecyloxyethyl benzyl tenofovir: A novel tenofovir diester provides sustained intracellular levels of tenofovir diphosphate

James R. Beadle, Kathy A. Aldern, Xing-Quan Zhang, Nadejda Valiaeva, Karl Y. Hostetler*, Robert T. Schooley

Department of Medicine, Division of Infectious Disease and Global Public Health, University of California San Diego, La Jolla, CA, 92093, USA

ARTICLE INFO

Keywords:
Tenofovir
HIV-1 prophylaxis
Long-acting antiretroviral
Adherence

ABSTRACT

Pre-exposure prophylaxis (PrEP) with topically or systemically administered antiretroviral agents can prevent acquisition of human immunodeficiency virus type 1 (HIV-1) infection. However, in clinical trials using tenofovir-containing agents, HIV-1 acquisition is reduced but not eliminated. Incomplete adherence remains the major contributor to failure. Sustained release or long-acting antiretroviral agents may provide better HIV-1 protection by reducing the clinical impact of incomplete adherence. To reduce dosing frequency, we synthesized a novel tenofovir prodrug, octadecyloxyethyl benzyl tenofovir (ODE-Bn-TFV), that is designed to release TFV slowly in tissues, and showed potent anti-HIV activity *in vitro* (EC_{50} = 1.7 nM). In cells exposed to ^{14}C labeled TFV, ODE-Bn-TFV or the quickly activated monoester ODE-TFV, rapid cellular uptake for both lipophilic analogs was noted, achieving 50-fold higher levels than unmodified TFV after 48 h. Following exposure to ODE-[8–^{14}C]TFV, the intracellular diphosphate levels were approximately four-fold higher than with ODE-Bn-TFV. However, intracellular TFVpp drug levels fell rapidly yielding a half-life of about two days. TFVpp levels in ODE-Bn-TFV treated cells decreased much more slowly and reached half-maximal levels in about seven days. These results suggest early accumulation of ODE-Bn-TFV followed by sustained intracellular release following cleavage of the ester bonds linking the ODE and benzyl moieties to the active molecular precursor, thereby potentially allowing for less frequent administration than with more rapidly activated forms of tenofovir.

1. Introduction

The recent call for elimination of AIDS in the United States by 2030 relies heavily on the use of antiretroviral drugs to prevent transmission of HIV to uninfected persons (Fauci et al., 2019). The elimination plan requires substantial reductions of HIV-1 RNA among infected persons at the population level, as well as a major increase in the availability and effectiveness of topical and systemically administered antiviral agents to uninfected persons at elevated risk of HIV infection. Studies of antiretroviral drugs for both treatment and prevention indicate that failure in either setting is most often attributable to incomplete levels of adherence (Amico, 2012). In an effort to alleviate barriers to adherence, especially in vulnerable populations, intensive efforts are underway to develop an array of more potent, longer acting antiretroviral compounds that can be administered topically or systemically. Some of the most promising candidates are antiretrovirals that feature prolonged intracellular half-lives (Gulick and Flexner, 2019). Anti-HIV acyclic nucleoside phosphonates (ANPs) such as tenofovir (TFV) are among the more promising compounds in this regard. Data from pre-exposure prophylaxis (PrEP) trials suggest that the presence of therapeutically relevant tenofovir concentrations in vaginal tissue is effective in reducing sexual transmission of HIV-1 (Abdool Karim et al., 2010). However, there are two major problems reducing the efficacy of vaginal mucosal PrEP: 1) suboptimal drug concentrations in the target mucosal tissues due to the cellular permeability limitations of unmodified ANPs; and 2) lack of adherence to the frequent dosing required for optimal efficacy. To address both challenges, we designed a "controlled release" prodrug strategy for anti-HIV ANPs.

Abbreviations: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; PrEP, preexposure prophylaxis; HAART, highly active antiretroviral therapy; ANP, acyclic nucleoside phosphonate; TFV, tenofovir; ODE-TFV, octadecyloxyethyl tenofovir; ODE-Bn-TFV, octadecyloxyethyl benzyl tenofovir; TFVp, tenofovir monophosphate; TFVpp, tenofovir diphosphate; HFF, human foreskin fibroblast; PBMC, peripheral blood mononuclear cell; EC_{50}, 50% effective concentration; CC_{50}, 50% cytotoxic concentration; NMR, nuclear magnetic resonance spectroscopy; ESI-MS, electrospray ionization mass spectroscopy; HPLC, high performance liquid chromatography; TLC, thin layer chromatography

* Corresponding author. University of California, San Diego, 9500 Gilman Drive, mail code 0711, La Jolla, CA, 92093, USA.
E-mail address: khostetler@ucsd.edu (K.Y. Hostetler).

https://doi.org/10.1016/j.antiviral.2019.104614
Received 7 June 2019; Received in revised form 19 September 2019; Accepted 21 September 2019
Available online 21 September 2019
0166-3542/

2. Materials and methods

2.1. Compounds and radiochemicals

Tenofovir was purchased from Matrix Scientific (Columbia, SC USA). All other reagents were of commercial quality and used without further purification. Chromatographic purification was done using the flash method with silica gel 60 (EMD Chemicals, Inc., 230–400 mesh). ^{1}H, ^{13}C,and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on either a Varian VX-500 or a Varian HG-400 spectrometer and are reported in units of ppm relative to internal tetramethylsilane at 0.00 ppm. Electrospray ionization mass spectra (ESI-MS) and high resolution mass spectra (HRMS) were recorded on a Thermo LCQDECA mass spectrometer at the small molecule facility in the Department of Chemistry at University of California, San Diego. Purity of the target compounds was characterized by high performance liquid chromatography (HPLC) using a Beckman Coulter System Gold chromatography system. The analytical column was Phenomenex Synergi™ Polar-RP (4.6 × 150 mm). Mobile phase A was 95% water/5% methanol and mobile phase B was 95% methanol/5% water. At a flow rate of 0.8 mL/min, gradient elution was as follows: 10% B (0–3 min); 10–95% B (3–20 min); 95% B (20–25 min); 95%–10% B (25–34 min). Compounds were detected by ultraviolet light (UV) absorption at 274 nm. Purity of compounds was also assessed by thin layer chromatography (TLC) using Analtech silica gel-GF (250 μm) plates and the solvent system: $CHCl_3$/MeOH/conc NH_4OH/H_2O (70:30:3:3 v/v). TLC results were visualized with UV light, phospray (Supelco, Bellefonte, PA, USA) and charring at 400 °C. Octadecyloxyethyl benzyl [8–^{14}C]-tenofovir (specific activity = 55 mCi/mmol), ODE-[8–^{14}C]-tenofovir (specific activity = 51.1 mCi/mmol) and [8–^{14}C]-tenofovir (specific activity = 51.6 mCi/mm were purchased from Moravek Biochemicals (Brea, CA).

2.2. Synthesis of octadecyloxyethyl tenofovir (ODE-TFV)

Tributylamine (388 mg, 2.1 mmol) was added to a stirred solution of anhydrous tenofovir (500 mg, 1.74 mmol) and octadecyloxyethan-1-ol (659 mg, 2.1 mmol) in dry DMF (35 mL) and the mixture was heated to 50 °C. To the mixture was added (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP, 1.56 g, 3 mmol). After stirring 5h, water (5 mL) was added and DMF was evaporated under reduced pressure. The residue was purified by silica gel chromatography (gradient 0–30% MeOH/CH_2Cl_2) and gave ODE-TFV as a white powder (385 mg, 38% yield). ^{1}H NMR (CD_3OD) δ 8.38(s, 1H), 8.21 (s, 1H), 4.40 (dd, 1H), 4.22 (dd, 1H), 4.02 (m, 3H), 3.75 (dd, 2H), 3.59 (t, 2H), 3.45 (t, 2H), 1.53 (t, 2H), 1.25–1.30 (br s, 30H), 1.15 (d, 3H), 0.89 (t, 3H). ^{13}C NMR (CD_3OD) δ 157.27, 153. 55, 151.03, 144.28, 119.70, 77.25, 77.08, 72.42, 71.86, 71.76, 66.77, 65.29, 65.21, 64.65, 33.22, 30.95, 30.92, 30.80, 30.63, 27.36, 23.89, 17.04, 14.66. ^{31}P NMR (CD_3OD) δ 17.94, ESI MS: 584.48 $(M + H)^+$, 606.44 $(M + Na)^+$. HRMS calcd for $[C_{29}H_{55}N_5O_5P]^+$ 584.3935, found 584.3927. HPLC analysis: R_t = 11.68 min, purity = 97.2%.

2.3. Synthesis of octadecyloxyethyl benzyl tenofovir (ODE-Bn-TFV)

Diisopropylethylamine (DIEA, 155 mg, 1.2 mmol) was added to a stirred solution of octadecyloxyethyl tenofovir (583 mg, 1.0 mmol), benzyl alcohol (130 mg, 1.2 mmol) and PyBOP (780 mg, 1.5 mmol) in dry DMF (25 mL) at room temperature. After stirring overnight, DMF was evaporated under reduced pressure, and the residue was dissolved in ethyl acetate (40 mL) and extracted with water (3 × 10 mL). The organic layer was concentrated, and the residue was purified by silica gel chromatography (gradient 0–20% EtOH/CH_2Cl_2) to give ODE-Bn-TFV as a mixture of diastereomers (waxy solid, 256 mg, 38% yield). ^{1}H NMR (DMSO-d_6) δ 8.12(s, 1H), 8.04 (s, 1H), 7.35 (s, 3H), 7.34 (d, 2H), 7.18 (s, 2H), 4.99–4.93 (m, 2H), 4.28–4.12 (m, 2H), 4.00-3-80 (m, 6H), 3.47–3.42 (m, 1H), 3.36–3.29 (m, 2H), 1.43 (m, 2H), 1.22–1.12 (m, 30H), 1.07 (d, 3H), 0.85 (t, 3H). ^{13}C NMR (CD_3OD, 75 MHz,) δ 157.40, 153.82, 151.00, 143.73, 137.65, 137.59, 137.52, 129.76, 129.71, 129.18, 119.82, 77.77, 77.61, 72.42, 72.39, 70.88, 70.82, 70.74, 69.29, 69.24, 69.21, 67.35, 67.31, 67.26, 67.21, 64.53, 62.30, 33.22, 30.95, 30.90, 30.75, 30.63, 27.37, 23.89, 16.90, 14.66. ^{31}P NMR (DMSO-d_6) δ 22.55, 22.51, ESI MS: 674.61 $(M + H)^+$, 696.54 $(M + Na)^+$. HRMS: calcd for $[C_{36}H_{61}N_5O_5P]^+$ 674.4405, found 674.4400. HPLC analysis: Rt = 16.37 min, purity = 97.14%.

2.4. Antiviral assays

Anti-HIV assays in human peripheral blood mononuclear cells (PBMC) were performed as described previously (Zhang et al., 2006).

2.5. Cell uptake studies

1.0 μM [8–^{14}C]-TFV, ODE-[8–^{14}C]-TFV or ODE-Bn-[8–^{14}C]-TFV was added to 24-well plates containing subconfluent monolayers of human foreskin fibroblast (HFF) cells and incubated at 37 °C . At the indicated time points the supernatant was removed, cells were washed with cold phosphate buffered saline (PBS), trypsinized and transferred to scintillation vials. Radioactivity in the cell lysate was determined by liquid scintillation counting.

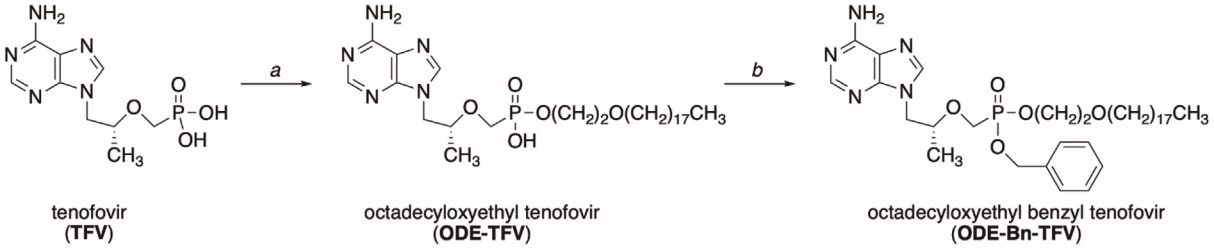
2.6. Intracellular metabolism studies

^{14}C-labeled ODE-TFV and ODE-Bn-TFV at 1.0 μM were added to 25 cm^2 flasks of subconfluent HFF cells and incubated at 37 °C for 48 h. The media was removed, cells were washed with PBS and drug-free media was added. The flasks were further incubated with cell harvesting at intervals up to 14 days. Then 0.6 ml of distilled water was added, and the flasks were frozen and thawed twice, followed by sonication in a cold bath sonicator for 5 min. The cells were removed by scraping and transferred to a glass tube. Cold trichloroacetic acid was added to a final concentration of 8%, and the contents were vortexed and centrifuged for 10 min at 4 °C. The supernatant was removed, counted, and immediately analyzed by HPLC. HPLC was done by applying the sample to a 4.6 × 15cm Partisil 10 SAX column with a SAX guard column. The column was eluted at a flow rate of 1 ml/min using a potassium phosphate buffer gradient of 20–700 mM, pH 5.8, beginning at 9 min for a duration of 20 min and a terminal hold of 5 min. Fractions (1 min/ml) were collected, FloScint IV scintillation fluid was added and the samples were analyzed by liquid scintillation counting (Aldern et al., 2003).

3. Results and discussion

3.1. Compounds

Esterification of various ANPs with long-chain alkoxyalkyl promoieties has been shown to promote rapid cellular uptake, enhanced antiviral activity, and oral bioavailability (Beadle and Hostetler, 2011). To prepare longer-acting ANP prodrugs suitable for injection or topical administration, we added a benzyl (or phenyl) promoiety aiming to create analogs that are metabolized more slowly than conventional alkoxyalkyl ANPs. This strategy was recently employed to develop an anti-human papillomavirus topical treatment (Beadle et al., 2016; Banerjee et al., 2018). To assess application of this strategy to sustained release tenofovir analogs, we synthesized ODE-Bn-TFV and ODE-TFV from commercially available tenofovir by sequential esterification using the condensation reagent (1H-benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (PyBOP) (Campagne et al., 1995). As shown in Scheme 1, monoester ODE-TFV was prepared, and then additional esterification with benzyl alcohol afforded the desired phosphonodiester. ODE-Bn-TFV was isolated as a pair of diastereomers



Scheme 1. Synthesis of octadecyloxyethyl benzyl tenofovir. *Reagents*: a) octadecyloxyethan-1-ol, tributylamine, PyBOP, DMF; b) benzyl alcohol, diisopropylamine, PyBOP, DMF.

(1:1 ratio by ^{31}P NMR) due to the creation of a stereogenic phosphorous center. Both compounds were analyzed by ^{1}H and ^{31}P NMR as well as electrospray ionization mass spectroscopy to establish chemical identity and purity was confirmed to be greater than 95% using HPLC analysis.

3.2. Antiviral activity

The antiretroviral activity of ODE-Bn-TFV against HIV-1 was assessed in PBMCs and is compared to unmodified tenofovir and monoester ODE-TFV (Table 1). ODE-Bn-TFV exhibited potent inhibition of HIV-1 replication (EC_{50} = 1.7 nM) and excellent selectivity (5130). ODE-Bn TFV was approximately 700-fold more active than TFV against HIV and displayed a 2.8-fold improvement in selectivity versus tenofovir. Compared to monoester ODE-TFV, ODE-Bn-TFV was slightly less active, suggesting that the lipophilic diester might be more slowly activated to the active metabolite, tenofovir diphosphate (TFVpp) in PBMCs. We hypothesize that cleavage of the benzyl ester is rate limiting (Serafinowska et al., 1995) and required prior to rapid phospholipase-mediated cleavage of the octadecyloxyethyl promoiety which liberates TFV.

3.3. In vitro uptake

Uptake of ODE-Bn-TFV was evaluated in HFF cells and compared to unmodified tenofovir and ODE-TFV using a previously described method (Aldern et al., 2003). Cells were exposed to 1 μM [8–^{14}C]-TFV, ODE-[8–^{14}C]-TFV or ODE-Bn-[8–^{14}C]-TFV and then processed at the indicated time points for ^{14}C content (Fig. 1). Tenofovir uptake was relatively slow, reaching maximum loading of 1.6 pmol/well after 24 h exposure. In contrast, both ODE-TFV and ODE-Bn-TFV were taken up rapidly and reached maximal levels of 81 and 102 pmol/well at 24 h, respectively, representing a 62- and 81-fold increase in cell uptake versus unmodified TFV. These results confirm that ODE-Bn-TFV is similar to previously studied phospholipid-like ANP analogs (Hostetler, 2009) as it readily permeates cell membranes, and might be retained for extended periods of time while slowly releasing TFV.

3.4. Intracellular pharmacokinetics

To compare sustainability of the antiviral metabolite in cells, intracellular levels of TFV, TFV monophosphate (TFVp) and TFV diphosphate (TFVpp) in HFF cells were measured following 48 h of exposure to 1 μM [^{14}C]-ODE-TFV or ODE-Bn-TFV. After exposure, drug was removed and replaced with drug free media and cellular levels of TFV, TFVp and TFVpp were measured at intervals for 14 days. As expected,

Table 1
Anti-HIV activity in human PBMCs.

Compound	EC_{50} (nM)	CC_{50} (nM)	Selectivity
TFV*	950 ± 50	$2.63 \times 10^6 \pm 7.6 \times 10^5$	2770
ODE-TFV	1.1 ± 1.0	1284	1170
ODE-Bn-TFV	1.7 ± 1.5	8720	5130

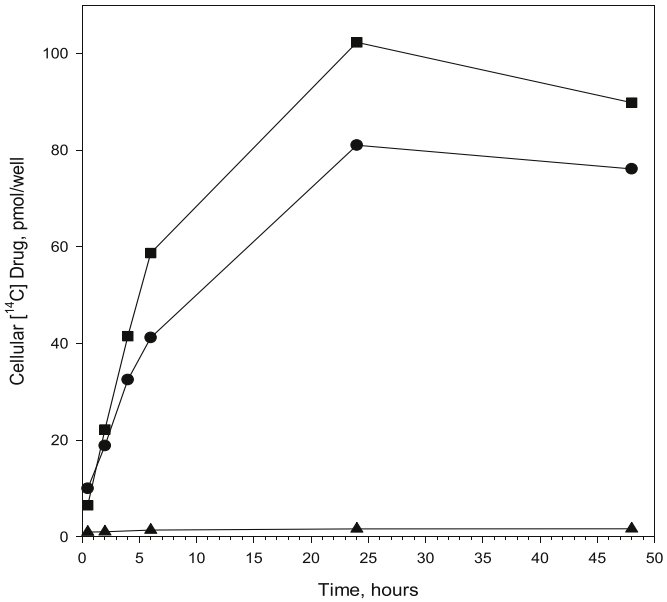


Fig. 1. Cellular uptake in HFF cells exposed to 1 μM ^{14}C-labeled tenofovir, ODE-tenofovir or ODE-Bn-tenofovir. Data are the average of two independent replicates per time point. Symbols: triangle, tenofovir; circle, ODE-tenofovir; square, ODE-Bn-tenofovir.

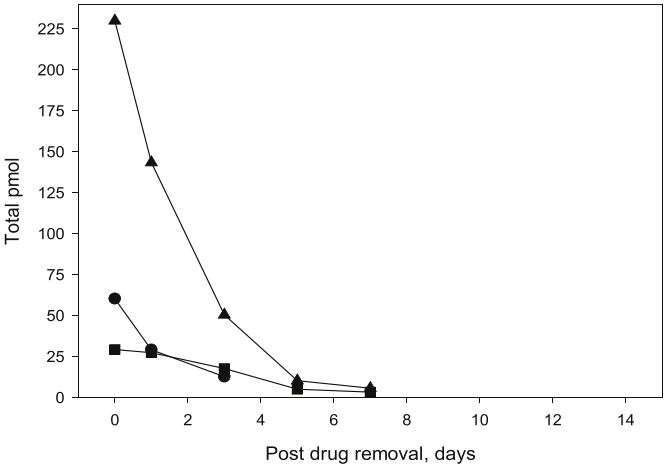


Fig. 2. Cellular metabolite levels following a 48-hour exposure to 1 μM ODE-[8–^{14}C]-tenofovir followed by replacement with drug-free medium. Data are the average of two independent replicates per time point. Symbols: circle, tenofovir; square, tenofovir monophosphate; triangle, tenofovir diphosphate.

ODE-TFV generated high intracellular levels of TFVpp at day zero following drug removal (230 pmol/well) which declined by 50% at day 2 and 90% at day 4 (Fig. 2). For comparison, results following ODE-Bn-TFV treatment are shown in Fig. 3. Although lower levels of TFVpp

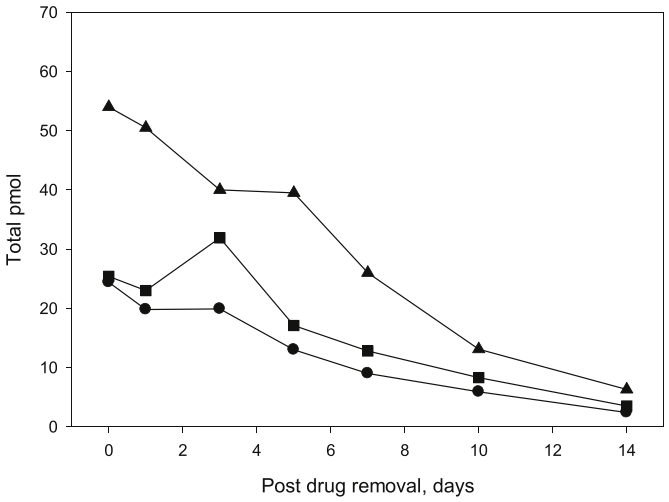


Fig. 3. Cellular metabolite levels following a 48-hour exposure to 1 μM ODE-Bn-[8–^{14}C]-tenofovir followed by replacement with drug-free medium. Data are the average of two independent replicates per time point. Symbols: circle, tenofovir; square, tenofovir monophosphate; triangle, tenofovir diphosphate.

were found immediately after the 48-hour exposure (54 pmol/well), the levels of TFVpp declined more slowly, reaching 50% at day 7 and 90% at day 14. The slower release of TFV from ODE-Bn-TFV prolongs the intracellular persistence of TFVpp by a factor of 3.5.

4. Conclusion

We synthesized ODE-Bn-TFV and have demonstrated that it provides potent *in vitro* anti-HIV activity and 3.5-fold longer duration of TFVpp in cells compared to the rapidly activated monoester ODE-TFV. The sustained release properties observed are presumably due to hindrance by the benzyl moiety of phospholipase activity required for release of TFV (Hostetler, 2009). Because cleavage of the benzyl ester appears to be rate-limiting for intracellular activation to TFVpp, the sustained release of tenofovir potentially could be improved using alternate substituted benzyl, aryl or aliphatic esters. Availabilty of long-acting analogs of TFV such as ODE-Bn-TFV could enable less frequent dosing and improved adherence by patients receiving preexposure prophylaxis or HAART. We plan to assess the therapeutic potential of ODE-Bn-TFV in animal models of HIV infection.

Author disclosure statement

Dr. Hostetler is the founder, a consultant and has an equity interest in Antiva Biosciences. Although this NIH grant has been identified for conflict of interest management based on the overall scope of the project, the research findings included in this publication do not relate to the interests of Antiva Biosciences. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. J.R.B., K.A. A., N. V. and R. T. S. are equity holders in Antiva Biosciences, Inc. The other authors have no competing interests to declare.

Acknowledgements

This work was supported by NIAID grant RO1 AI131424, the San Diego Center for AIDS Research and Antiva Biosciences. Portions of this work were presented in abstract form at the 2016 and 2018 International Conferences on Antiviral Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104614.

References

Abdool Karim, Q., Abdool Karim, S.S., Frohlich, J.A., Grobler, A.C., Baxter, C., Mansoor, L.E., Kharsany, A.B., Sibeko, S., Mlisana, K.P., Omar, Z., Gengiah, T.N., Maarschalk, S., Arulappan, N., Mlotshwa, M., Morris, L., Taylor, D., 2010. CAPRISA 004 Trial Group. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 329, 1168–1174.

Aldern, K.A., Ciesla, S.L., Winegarden, K.L., Hostetler, K.Y., 2003. Increased antiviral activity of 1-O-Hexadecyloxypropyl-[2-14C]cidofovir in MRC-5 human lung fibroblasts is explained by unique cellular uptake and metabolism. Mol. Pharmacol. 63, 678–681.

Amico, K.R., 2012. Adherence to preexposure chemoprophylaxis: the behavioral bridge from efficacy to effectiveness. Curr. Opin. HIV AIDS 7, 542–548.

Banerjee, N.S., Wang, H., Beadle, J.R., Hostetler, K.Y., Chow, L.T., 2018. Evaluation of ODE-Bn-PMEG, an acyclic nucleoside phosphonate prodrug, as an antiviral against productive HPV infection in 3D organotypic epithelial cultures. Antivir. Res. 150, 164–173.

Beadle, J.R., Hostetler, K.Y., 2011. Alkoxyalkyl ester prodrugs of nucleoside phosphates and phosphonates. In: De Clerq, E. (Ed.), Antiviral Drug Strategies. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 181–208.

Beadle, J.R., Valiaeva, N., Yang, G., Yu, J.-H., Broker, T.R., Aldern, K.A., Harden, E.A., Kathy Keith, K.A., Prichard, M.N., Hartman, T., Buckheit Jr., R.W., Chow, L.T., Hostetler, K.Y., 2016. Synthesis and antiviral evaluation of octadecyloxyethyl benzyl 9-[(2-Phosphonomethoxy)ethyl]guanine (ODE-Bn-PMEG), a potent inhibitor of transient HPV DNA amplification. J. Med. Chem. 59, 10470–10478.

Campagne, J., Coste, J., Jouin, P., 1995. (1H-Benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate- and (1H-benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate-mediated activation of monophosphonate esters: Synthesis of mixed phosphonate diesters, the reactivity of the benzotriazolyl phosphonic esters vs the reactivity of the benzotriazolyl carboxylic esters. J. Org. Chem. 60, 5214–5223.

Fauci, A.S., Redfield, R.R., Sigounas, G., Weahkee, M.D., Giroir, B.P., 2019. Ending the HIV epidemic: a plan for the United States. J. Am. Med. Assoc. 321, 844–845.

Gulick, R.M., Flexner, C., 2019. Long-acting HIV drugs for treatment and prevention. Annu. Rev. Med. 70, 137–150.

Hostetler, K.Y., 2009. Alkoxyalkyl prodrugs of acyclic nucleoside phosphonates enhance oral antiviral activity and reduce toxicity: current state of the art. Antivir. Res. 82, A84 – 98.

Serafinowska, H.T., Ashton, R.J., Bailey, S., Harnden, M.R., Jackson, S.M., Sutton, D., 1995. Synthesis and in vivo evaluation of prodrugs of 9-[2-(Phosphonomethoxy) ethoxy]adenine. J. Med. Chem. 38, 1372–1379.

Zhang, X.-Q., Sorensen, M., Fung, M., Schooley, R.T., 2006. Synergistic *in vitro* antiretroviral activity of a humanized monoclonal anti-CD4 antibody (TNX355) and enfuvirtide. Antimicrob. Agents Chemother. 50, 2231–2233.