Antiviral Research 132 (2016) 50–58

Contents lists available at ScienceDirect

Antiviral Research

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

High resistance barrier to tenofovir alafenamide is driven by higher loading of tenofovir diphosphate into target cells compared to tenofovir disoproxil fumarate

Nicolas A. Margot*, Yang Liu, Michael D. Miller, Christian Callebaut

Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA, 94404, USA

ARTICLE INFO

Article history:
Received 12 March 2016
Received in revised form
13 May 2016
Accepted 16 May 2016
Available online 18 May 2016

Keywords:
Tenofovir alafenamide
Tenofovir diphosphate
Resistance
Tenofovir disoproxil fumarate
HIV-1

ABSTRACT

Tenofovir alafenamide (TAF) is a new oral prodrug of tenofovir (TFV) recently approved for the treatment of HIV-1 as part of the single-tablet regimen containing elvitegravir, cobicistat, emtricitabine, and TAF. Clinical dosing with TAF vs. tenofovir disoproxil fumarate (TDF) has shown improved bone and kidney safety, and has been associated with an increased concentration of the anti-HIV active moiety tenofovir diphosphate (TFV-DP) in the PBMCs of treated patients and a reduction of TFV systemic exposure. We have studied the potential benefit of this increased concentration of TFV-DP observed clinically in an in vitro model system. Using a newly developed virus breakthrough assay with TAF exposure set at physiological concentrations, we show that HIV-1 clinical isolates harboring TFV resistance mutations such as K65R, 3 or 4 thymidine-analog mutations (TAMs), Q151M/K65R, or T69 insertion complex could be inhibited by TAF, but not by TFV when used at clinically relevant concentrations for TDF. These data suggest that the inhibitory quotient (IQ) of TAF is projected to be higher than the IQ of TDF, and that TAF has the potential to inhibit viruses containing TDF resistance in the clinic.

1. Introduction

Tenofovir alafenamide (TAF) is a phosphonamidate prodrug of the nucleotide HIV reverse transcriptase inhibitor (NRTI) tenofovir (TFV). TAF was recently approved for the treatment of HIV-1 infection in the US and EU as part of the single-tablet regimen containing the HIV-1 integrase inhibitor elvitegravir (E), the pharmaco-enhancer cobicistat (C), the NRTI emtricitabine (F, FTC), and TAF (E/C/F/TAF; Genvoya®, GEN). Two large phase 3 clinical studies in over 1700 antiretroviral treatment-naïve HIV-1 infected subjects have demonstrated that treatment efficacy with E/C/F/TAF was non-inferior to treatment with E/C/F/tenofovir disoproxil fumarate (TDF) (E/C/F/TDF; Stribild, STB) (Sax et al., 2015).

Data from several clinical studies of the fixed dose combination E/C/F/TAF vs. E/C/F/TDF have highlighted the better safety profile of TAF compared to TDF with regards to kidney and bone markers (Mills et al., 2016; Sax et al., 2014, 2015). This improved bone and kidney safety profile is attributed to the near 90% reduced TFV plasma exposure upon dosing with TAF vs. TDF, which stems from differences in the metabolic stability of the two prodrugs. TAF has been shown to be stable in the presence of human serum while TDF is rapidly converted to TFV in these conditions (Callebaut et al., 2015; Lee et al., 2005). Indeed, TDF is mostly metabolized to TFV by plasma esterases before entering into target cells, while TAF is metabolized to TFV mostly intracellularly through the action of cathepsin A (Birkus et al., 2007). As a consequence of their different metabolism, TAF in GEN is used at substantially lower dose than TDF in STB, but provides a similar antiviral efficacy in ARV-naïve patients.

In addition, early clinical studies have shown that monotherapy treatment with TAF 25 mg (dose that is equivalent to TAF 10 mg within the context of E/C/F/TAF) (Lepist et al., 2012) led to an enhanced antiviral effect compared to treatment with TDF 300 mg after 10 days of monotherapy (1.46 $\log_{10}$ HIV-1 RNA decline from baseline and 0.97 $\log_{10}$ HIV-1 RNA decline from baseline, respectively) (Ruane et al., 2013). The improved antiviral efficacy was associated with a higher concentration of the anti-HIV active moiety tenofovir diphosphate (TFV-DP) in the PBMCs of subjects receiving TAF vs. TDF. Across Phase 2 and Phase 3 clinical studies overall, there was a 4- to 5-fold increase in intracellular TFV-DP concentration in subjects treated with TAF vs. TDF (Sax et al.,

* Corresponding author.
E-mail address: nicolas.margot@gilead.com (N.A. Margot).

http://dx.doi.org/10.1016/j.antiviral.2016.05.012
0166-3542/

2014, 2015). This selective increase in the concentration of the active drug in vivo upon TAF dosing vs. TDF dosing would be expected to lead to a higher inhibitory quotient (IQ) for TAF compared to TDF, in particular against resistant viruses. However, no information is currently available to assess this potential feature of the new prodrug TAF.

In vitro resistance experiments do not typically take into account quantitative differences in drug concentrations achieved in vivo such as described above for TAF. In dose-escalation resistance selection studies where the drug concentrations used are below therapeutic levels, and usually close to their EC_{50} values, side-by-side comparison of TAF and TFV both led to similar resistant mutants with the RT mutation K65R and reduced phenotypic susceptibility (Margot et al., 2015). These results were expected as both TAF and TFV deliver qualitatively the same anti-HIV active moiety, TFV-DP, inside cells. Phenotypic analyses of a large panel of resistant mutants in the single-cycle PhenoSense assay (Petropoulos et al., 2000) also confirmed the expected result that TAF and TFV have qualitatively the same resistance profile in vitro (Margot et al., 2015).

In the studies described herein, we have adapted some of the standard in vitro methods to address experimentally the potential impact of the higher intracellular concentration of TFV-DP achieved in clinical studies upon dosing with TAF vs. TDF. We have used an in vitro model system to carry out infections with recombinant HIV-1 viruses with genotypic and phenotypic resistance to TFV/TDF to demonstrate that at clinically relevant drug concentration, i.e. concentrations mirroring the differential concentration of TFV-DP observed in PBMCs upon TAF vs. TDF dosing in vivo, TAF could prevent resistant viruses from establishing a spreading infection while TFV/TDF could not.

2. Materials and methods

2.1. Reagents

Tenofovir alafenamide (TAF), tenofovir (TFV), emtricitabine (FTC), elvitegravir (EVG), dolutegravir (DTG), and darunavir (DRV) were synthesized at Gilead Sciences (Foster City, CA, USA). Zidovudine (AZT) was purchased from Sigma-Aldrich (St Louis. MO, USA). HEK293T cells (293T cells) used for virus production were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MT-2 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD). The viral plasmid pXXLAI used to generate the viruses was a gift from John Mellors. The plasmid was derived from the infectious clone pLAI3.2 which was modified to contain an XmaI and an XbaI restriction site within the HIV RT gene (Shi and Mellors, 1997) to facilitate cloning. Polymerase chain reaction (PCR) was conducted using Phusion High-Fidelity DNA polymerase kit (New England Biolabs (NEB), Ipswich, MA). Glutamax® RPMI 1640 and DMEM culture medium, 100 X penicillin/streptomycin, and 100 X HEPES were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from ThermoFisher Scientific (Waltham, MA).

2.2. Patient isolate preparation

Plasma samples from screening visits from treatment-experienced subjects in Study GS-US-183-0145 were used as starting material for the patient isolates. Informed consent was obtained for the use of the plasma samples. Genotypic and phenotypic resistance testing for all these samples had been obtained using the HIV-1 single-cycle PhenoSenseGT™ assay (Monogram Biosciences, South San Francisco, CA) (Petropoulos et al., 2000) as part of the protocol for study GS-US-183-0145. Plasma samples were treated with 4 units of DNase I (NEB) for 45 min at room temperature, and viral RNA was extracted from 400 μL of plasma using the EZ1 Virus Mini Kit v2.0 with the BioRobot EZ1 Workstation (QIAGEN, Valencia, CA), and eluted in 60 μL. Viral RNA was reverse transcribed into cDNA using Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions with the HIV-1 specific primer R4395 (5′–CAGTCTACTTGTCCATGCATGGCTTCTCC–3′; final concentration 0.5 μM). The viral cDNA (10 μL of 40 μL reaction) was amplified through 2 rounds of 30 cycles of PCR to generate DNA fragments containing XmaI and XbaI cloning sites in RT.

2.3. Virus cloning and production

Recombinant mutant HIV-1 viruses were created either by direct PCR of patient isolates as indicated above, or by site directed mutagenesis (SDM) of a wild-type HIV-1 DNA template. One site directed mutant DNA fragment containing the single RT mutation K65R was generated by SDM (direct PCR). PCR products were subcloned (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Carlsbad, CA) and the XmaI/XbaI digestion product was ligated into the pXXLAI vector. Transformed bacteria containing individual clones were amplified, and the DNA sequence of the clones was confirmed by sequencing (ELIM Biopharmaceuticals, Hayward, CA). Plasmid DNA was purified (ELIM Biopharmaceuticals) and transfected into 293T cells using the transfection reagent TransIT-LT1 (Mirus Bio Corporation, Madison, WI) using 7 μg of the viral plasmid DNA in 2 million 293T cells seeded in T-25 cell culture flasks in a 6-mL volume one day before transfection. The cell culture supernatant containing the virus stock was harvested on day 1 and day 2 after transfection and tested in infectivity assays.

2.4. Phenotypic assay

The phenotype of each isolate was determined in a 5-day multicycle antiviral assay in MT-2 cells using a luciferase-based viability readout (CellTiterGlo; Promega, Madison, WI) (Margot et al., 2012). Briefly, MT-2 cells (2.4 million) were incubated with virus for 3 h at 37 °C in 1.5 mL screw-cap tubes with gentle rocking. The amount of virus used was normalized to yield a signal-to-noise (S/N) ratio in the range of 4–7, which was equivalent to an MOI of 0.005 based on the provided titer for the commercially available wild-type isolate HIV-1_{IIIB}. The S/N ratio was calculated from the 100 nM EVG control (50-fold EC_{50}; maximum cell survival) and the no drug control (minimum cell survival). Five-fold dilutions of the drugs of interest were prepared and transferred (50 μL) in triplicate to the inside wells of the 96-well assay plates. After the 3-h incubation, the infected MT-2 cells were diluted 1:14 to a concentration of 0.17 million cells/mL with tissue culture medium, and 50 μL of cell suspension was transferred to all wells in the assay plates. After 5 days of incubation (37 °C, 5% CO_2, 95% humidity), cultures were resuspended and 100 μL of CellTiterGlo reagent was added to each well and luminescence was measured using an Envision plate reader (PerkinElmer, Shelton, CT). Percent inhibition in the drug-containing wells in comparison to the fully protected EVG control, and the associated effective concentration to inhibit 50% of viral replication (EC_{50}) were plotted and calculated using Excel (Microsoft, Redmond, WA) and XL Fit (IDBS, Alameda, CA). Statistical significance ($p < 0.05$) of the fold changes for the mutants compared to the wild-type control was calculated using Excel (two-tailed Student's *t*-test).