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Research Paper

Discovery of Genetic Variants of the Kinases That Activate Tenofovir in a Compartment-specific Manner

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ABSTRACT

Tenofovir (TFV) is used in combination with other antiretroviral drugs for human immunodeficiency virus (HIV) treatment and prevention. TFV requires two phosphorylation steps to become pharmacologically active; however, the kinases that activate TFV in cells and tissues susceptible to HIV infection have yet to be identified. Peripheral blood mononuclear cells (PBMC), vaginal, and colorectal tissues were transfected with siRNA targeting nucleotide kinases, incubated with TFV, and TFV-monophosphate (TFV-MP) and TFV-diphosphate (TFV-DP) were measured using mass spectrometry–liquid chromatography. Adenylate kinase 2 (AK2) performed the first TFV phosphorylation step in PBMC, vaginal, and colorectal tissues. Interestingly, both pyruvate kinase isozymes, muscle (PKM) or liver and red blood cell (PKLR), were able to phosphorylate TFV-MP to TFV-DP in PBMC and vaginal tissue, while creatine kinase, muscle (CKM) catalyzed this conversion in colorectal tissue. In addition, next-generation sequencing of the Microbicide Trials Network MTN-001 clinical samples detected 71 previously unreported genetic variants in the genes encoding these kinases. In conclusion, our results demonstrate that TFV is activated in a compartment-specific manner. Further, genetic variants have been identified that could negatively impact TFV activation, thereby compromising TFV efficacy in HIV treatment and prevention.

1. Introduction

Within the last decade, tenofovir (TFV), prescribed as tenofovir disoproxil fumarate in its prodrug formulation, has emerged as a critical component of antiretroviral combination therapy for the treatment of human immunodeficiency virus (HIV) (Schooley et al., 2002; Robbins et al., 1998). More recently, oral as well as vaginal and rectal microbicide gel preparations of TFV have been investigated for use in pre-exposure prophylaxis (PrEP) as an HIV prevention strategy for individuals at high-risk of viral exposure (Baeten et al., 2012; Mayer et al., 2006; Anton et al., 2012). TFV is a desirable drug candidate for PrEP due to the long half-life of active drug TFV-diphosphate (TFV-DP), reported to be 53 h in vaginal tissue homogenate and up to 139 h in vaginal CD4+ cells following an oral dosing of HIV-uninfected women (Derdelinckx et al., 2006; Louissaint et al., 2013). That being said, there has been discrepancy in the prophylactic effect observed for TFV-based regimens. For example, the Partners in Prevention study demonstrated a 67–75% reduction in HIV acquisition in serodiscordant heterosexual couples, iPrEx demonstrated a 44% reduction in men or transgender women who have sex with men, whereas FEM-PrEP and VOICE trials showed no significant reduction in the rate of infection in heterosexual women (Grant et al., 2010; Van Damme et al., 2012; Marrazzo et al., 2015). This disparity has been primarily due to poor adherence. Adjusting for adherence, the differences among these clinical trials have been attributed to the increased accumulation of active drug in colorectal versus vaginal tissue. As such, these findings suggest that the enzymes responsible for TFV activation may differ between colorectal and vaginal tissue, however, this has not been tested thus far. Further, while yet to be explored, it can be envisioned that genetic variation in the nucleotide kinases that activate TFV could underlie observed inter-individual differences in tissue TFV-DP concentrations that has been noted even when adherence is high (Louissaint et al., 2013; Hendrix et al., 2013; Patterson et al., 2011).

As TFV requires phosphorylation by nucleotide kinases in order to become pharmacologically active, both local mucosal tissue cell

Abbreviations: HIV, human immunodeficiency virus; PrEP, pre-exposure prophylaxis; TFV, tenofovir; PBMC, peripheral blood mononuclear cells; TFV-MP, tenofovir-monophosphate; TFV-DP, tenofovir-diphosphate; MTN-001, Microbicide Trials Network Study MTN-001; AK2, adenylate kinase 2; PKM, pyruvate kinase, muscle; PKLR, pyruvate kinase, liver and red blood cell; CKM, creatine kinase, muscle; NME1, NME/NM23 nucleoside diphosphate kinase 1; GUK1, guanylate kinase 1; SNV, single-nucleotide variant.

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phosphorylation and distant peripheral blood CD4+ cell phosphorylation with secondary migration to mucosal tissue may be critical in achieving effective TFV-DP concentrations. If mucosal tissue phosphorylation is an essential contribution to mucosal tissue cell TFV-DP concentrations, then the kinases responsible for TFV transformation are expressed in cells and tissues associated with HIV infection (Robbins et al., 1998; Kearney et al., 2004). To date, the expression profiles of nucleotide kinases in peripheral blood mononuclear cells (PBMC), vaginal tissue, and colorectal tissue have not been characterized and data demonstrating kinase activity towards TFV and TFV-MP are lacking. In vitro studies using human T-lymphoid cells demonstrated the mitochondrial and cytosolic enzyme adenylate kinase isoform 2 (AK2) could catalyze the phosphorylation of TFV to form TFV-monophosphate (TFV-MP) (Nobumoto et al., 1998; Robbins et al., 1995; Topalis et al., 2008). Experimental evidence is deficient, however, with regard to the nucleotide kinase(s) capable of transforming TFV-MP to TFV-DP. TFV-MP has been reported as a substrate of NME/NM23 nucleoside diphosphate kinase 1 (NME1) as this enzyme demonstrates broad activity towards purine nucleoside diphosphates (Robbins et al., 1998; Bourdais et al., 1996). Yet, in separate studies, NME1 exhibited low to non-detectable rates of catalysis, while creatine kinase, muscle (CKM) efficiently phosphorylated TFV-MP and minor catalytic efficiency towards TFV-MP was demonstrated by rabbit pyruvate kinase, muscle (PKM) (Koch et al., 2009; Varga et al., 2013). Nevertheless, although these kinases exhibit activity towards TFV or TFV-MP using in vitro model systems, whether or not they are expressed in cells and tissues susceptible to HIV infection is unknown.

In the present study, we characterized nucleotide kinase expression in PBMC, vaginal tissue, and colorectal tissue in order to identify those that catalyze the phosphorylation of TFV to TFV-MP and TFV-MP to TFV-DP. Towards this end, we knocked down the protein expression of AK2, CKM, PKM, pyruvate kinase, liver and red blood cell (PKLR), and guanylate kinase 1 (GUK1) using siRNA. Neither PKLR nor GUK1 have been previously reported to activate TFV; however, PKLR exhibits an approximate 70% amino acid sequence identity to the isozyme PKM and GUK1 has been demonstrated to phosphorylate the antiviral drug adefovir which is structurally similar to TFV (Gentry et al., 2011). Taking these studies a step further, we wanted to leverage our identification of the nucleotide kinases that activate TFV and test for the existence of genetic variants in *AK2*, *CKM*, *PKM*, and *PKLR*. To do so, we performed next-generation targeted sequencing of genomic DNA isolated from the plasma of 142 HIV-uninfected female participants of the Microbicide Trials Network study MTN-001 (Hendrix et al., 2013). In this work, we have demonstrated at an enzymatic level that TFV is activated in a tissue-specific manner. We also put forth an innovative concept that variation in the genes that encode the nucleotide kinases that activate TFV may contribute to inter-individual differences observed clinically. Taken together, these findings represent a shift in thinking about the factors that govern variability in TFV efficacy and pharmacokinetics.

2. Materials and Methods

2.1. siRNA Knockdown of Nucleotide Kinases

PBMC were obtained from Bioreclamation (Westbury, NY), and fresh vaginal and colorectal biopsies were obtained from the Johns Hopkins University School of Medicine Tissue Bank (Baltimore, MD). Donor information is as follows: PBMC (n = 3; 38 y.o. male, 24 y.o. female, and 27 y.o. female); vaginal tissue (n = 3; 31 y.o. female, 48 y.o. female, and 37 y.o. female); colorectal tissue (n = 3; 35 y.o. male, 39 y.o. female, 42 y.o. male). All donors were healthy, HIV-uninfected individuals. PBMC, vaginal, and colorectal tissues were transfected with siRNA and incubated with TFV. Homogenized cells and tissues were then immunoblotted for protein expression and intracellular metabolites detected using ultra-high performance liquid chromatography–tandem mass spectrometry (uHPLC–MS/MS). Electroporation, immunoblotting, and uHPLC–MS/MS assay conditions are described in the supplementary methods.

2.2. Clinical Samples

Plasma was obtained from HIV-uninfected females (n = 142) enrolled in the Microbicide Trials Network study MTN-001 across seven study sites: Umkomaas and Botha's Hill, Durban, South Africa; Makerere University–Johns Hopkins University Research Collaboration, Kampala, Uganda; Case Western Reserve University in Cleveland, OH, United States of America (USA); University of Pittsburgh, Pittsburgh, PA, USA; University of Alabama at Birmingham, Birmingham, AL, USA; Bronx-Lebanon Hospital Center, New York City, NY, USA (Hendrix et al., 2013). The current analysis was approved by the Johns Hopkins Medicine IRB (NA_00016287).

2.3. Genomic DNA Isolation

Genomic DNA was isolated from 200 μL of plasma using the GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA). Purified DNA was eluted using 50 μL of elution buffer.

2.4. Next-generation Sequencing Targeted Enrichment Design

Sequencing was performed using the Illumina TruSeq custom amplicon v1.5 kit (San Diego, CA). Custom probes targeting the exonic regions of *AK2*, *CKM*, *PKM*, and *PKLR* were generated in silico using Illumina DesignStudio software. The chromosomal coordinates used were as follows: *AK2* 1:33473531–1:33502522; *CKM* 19:45809661–19:45826243; *PKM* 15:72492805–15:72523694; and *PKLR* 1:155259074–1:155271235. The start and stop coordinates for each target region is detailed in Supplementary Table 1. The final design included 102 amplicons listed in Supplementary Table 2. Sample preparation, sequencing, and data analyses are detailed in the supplementary methods. All genetic variants reported in this study have been submitted to the SNP database under the submitter handle "BUMPUSLAB." The phenotypic consequence of missense variants was assigned using SIFT (sorts intolerant from tolerant substitutions; J. Craig Venter Institute online tool) and PolyPhen (polymorphism phenotyping; Harvard University online tool) in silico prediction tools where amino acid substitutions were scored (Ng and Henikoff, 2001; Ramensky et al., 2002).

2.5. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (San Diego, CA). Two-tailed unpaired *t* tests were performed and significance was denoted as follows: *, $p \le 0.05$; **, $p \le 0.01$; ***, $p \le 0.001$.

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