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Tenofovir Induced Nephrotoxicity: A Mechanistic Study

Rachel A. Murphy
murphyrachel63@gmail.com

TENOFOVIR INDUCED NEPHROTOXICITY: A MECHANISTIC STUDY

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by
Rachel A. Murphy
Approved by
Dr. Monica Valentovic, Committee Chairperson
Dr. Gary Rankin
Dr. Richard Egleton
Dr. Travis Salisbury
Dr. Todd Green

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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Rachel Ann Murphy, affirm that the dissertation, *Tenofovir-Induced Nephrotoxicity: A Mechanistic Study*, meets the high academic standards for original scholarship and creative work established by the Biomedical Science Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Monica Valentovic — 6-8-17

Dr. Monica Valentovic, Committee Chairperson Date
Department of Biomedical Sciences

Gary Rankin — 6/8/17

Dr. Gary Rankin, Committee Member Date
Department of Biomedical Sciences

Richard Egleton — 6/8/17

Dr. Richard Egleton Committee Member Date
Department of Biomedical Sciences

Travis Salisbury — 6/8/17

Dr. Travis Salisbury Committee Member Date
Department of Biomedical Sciences

Todd L. Green — 6/8/17

Dr. Todd Green Committee Member Date
Department of Biomedical Sciences

DEDICATION

I dedicate this work to my incredible parents Wendy and Matt, my siblings Joe, Nicole, and Mary, and my best friend, Brandon. There is no way I would have made it through this program without your love and support. Thank you so much for answering all the panicked FaceTime calls, reminding me that I was capable, and for talking and joking with me until I was calm enough to continue with whatever I was trying to complete at the time. There is no way I would have been able to finish my PhD without your help; I love you guys so much!

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ABSTRACT

Tenofovir (TFV) is a reverse transcriptase inhibitor that is approved by the United States Food and Drug Administration (FDA) to treat HIV and chronic Hepatitis B. It has a long half-life, allowing for once a day dosing and is effective in treatment of both naive and experienced patients. It is administered orally as tenofovir disoproxil fumarate (TDF) and is deesterified in plasma to the active drug TFV. However, renal impairment is associated with its use; TFV can induce decreased glomerular filtration rate (GFR) and free calcitriol, renal failure, and Fanconi Syndrome. The exact mechanism of toxicity currently remains unknown, largely due to limited experimental models. The purpose of this study was to investigate the mechanisms of cytotoxicity and oxidative damage observed in HK-2 cells following treatment with TFV and to determine if managing oxidative damage mitigates toxicity. TFV is the active form of TDF and was used for all studies. HK-2 cells were grown to confluency for 48 h and then exposed to 0-28.8 µM TFV for 24, 48, or 72 h. The vehicle used for all studies was phosphate buffered saline (PBS). TFV induces a loss of cell viability compared to the control within 24 h as shown by an MTT assay, Trypan Blue Exclusion cell counts, and lactate dehydrogenase (LDH) leakage. Oxidative stress and mitochondrial damage were assessed in whole cell lysate and different cell fractions using OxyBlot and western blot for 4-hydroxynonenol (4-HNE), tumor necrosis factor alpha (TNFα), caspase 3, 8, and 9, MnSOD, ATP Synthase, and cytochrome c and showed an increase in protein carbonylation and loss of mitochondrial membrane integrity following 72 h exposure to 28.8 µM TFV. TFV induces apoptosis at 72 h exposure as shown by western blot analysis of cytochrome c leakage and activation of caspase 3 and 9. Studies conducted using Seahorse XFp technology determined that TFV alters mitochondrial function. Studies were conducted using a 1 h pretreatment with antioxidants resveratrol, N-acetyl-L-cysteine, or

ascorbic acid, and results showed protection of cell viability following 24 h exposure to 3 and 14.5 μM TFV. These studies suggest that mitochondrial damage and oxidative stress occur in HK-2 cells treated with TFV and that controlling oxidative damage may help prevent toxicity from developing. Additional knowledge of subcellular events associated with tenofovir nephrotoxicity can be used to develop clinical methods to mitigate toxicity.

CHAPTER 1

FACTORS CONTRIBUTING TO THE ANTIVIRAL EFFECTIVENESS OF TENFOVOIR

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Rachel A. Murphy; Monica A. Valentovic

Rachel A. Murphy*, Monica A. Valentovic*[1]

*Department of Biomedical Sciences

[1]Correspondence should be addressed to: Department of Biomedical Sciences; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755. Phone (307-696-7332) Fax (304-696-7391) Email: valentov@marshall.edu

Abstract

Over one million people in the United States are living with Human Immunodeficiency Virus (HIV) which may progress to Acquired Immunodeficiency Syndrome (AIDS). The use of antiviral therapy has successfully controlled the rate of viral growth in patients. Antiviral agents improve the quality of life and reduce the potential for spreading HIV; HIV is currently considered a chronic disease provided patients are compliant with their antiviral medications. Tenofovir is a nucleoside transcriptase inhibitor that prevents viral replication and is approved for treatment of HIV and chronic hepatitis B infection. Tenofovir is an antiretroviral drug used alone and in combination with other nucleoside reverse transcriptase inhibitor agents to lower viral load in HIV patients. Tenofovir is administered as a prodrug in order to increase bioavailability. The prodrug forms of tenofovir are tenofovir disoproxil fumarate approved in 2001 and tenofovir alafenamide approved in 2016. Tenofovir is extensively used in controlling HIV as it is administered once daily allowing for good compliance. This minireview discusses the changes in dosing that are needed in the presence of renal impairment which is a common occurrence with HIV chronic disease progression. The impact of food, age, and drug transporters on tenofovir absorption and clearance will be discussed. The potential special conditions occurring with fixed combination doses containing tenofovir will also be reviewed, including the use of cobicistat, a cytochrome P450 inhibitor. The short review also addresses some newer preparations using niosomes to improve tenofovir absorption and delivery to the target cells.

Introduction

Human Immunodeficiency Virus (HIV) is a virus that decimates the immune system. HIV targets a specific type of white blood cells known as T-cells, T-lymphocytes or CD4 cells. A decline in CD4 cell count mediated by HIV leaves the body open to opportunistic infections.

At one time, HIV rapidly progressed to Acquired Immunodeficiency Syndrome (AIDS) and death, but advancements in highly active antiretroviral therapy (HAART) have made HIV a chronic condition rather than a death sentence. Tenofovir (TFV), formerly known as PMPA [9-(R)-(2-phosphonomethoxypropyl) adenine] is a reverse transcriptase inhibitor that has potent and selective inhibition of HIV and herpes viruses' reverse transcriptase. TFV is an acyclic nucleotide analogue of adenosine 5' monophosphate that is phosphorylated intracellularly by adenylate kinase to its active form, tenofovir diphosphate. TFV is effective in cases of nucleoside resistant HIV infection, making it a first line agent for treatment of this disease (Miller, Margot, Hertogs, Larder, & Miller, 2001; Squires et al., 2003).

Despite its efficacy, poor bioavailability was a limiting factor in development of TFV as a clinical agent. Oral administration of TFV had 18% bioavailability in beagles and 5.3% in monkeys (Cundy et al., 1998; Shaw et al., 1997). Research efforts subsequently focused to improve bioavailability by altering TFV formulation but maintaining antiviral activity. Two methyl carbonate esters were added to form the prodrug tenofovir disoproxil fumarate (TDF), which demonstrated equivalent antiviral activity with bioavailability increased to 30.1% in beagles and 25% in humans (Barditch-Crovo et al., 2001; Shaw et al., 1997). TDF was approved in Fall 2001 by the United States Food and Drug Administration (FDA) to treat chronic Hepatitis B and HIV in conjunction with other agents (Gilead Sciences, 2001). There are several factors that alter TDF bioavailability and pharmacokinetics, including age, sex, and food intake. This article provides a review of tenofovir bioavailability as well as factors that have an impact on TFV pharmacokinetics in human and animal models.

Pharmacokinetics and Bioavailability of Tenofovir Disoproxil Fumarate

TDF is formulated into a tablet for oral administration with a standard dose of 300 mg. TDF has a higher lipophilicity than TFV. The LogP, a measure of the partition coefficient between octanol and water, is 2.1 for TDF compared to -1.6 for TFV. The higher partition coefficient would enable increased intestinal drug absorption. As a result of higher lipophilicity, TDF has an oral bioavailability of 25% in HIV-1 infected patients (Barditch-Crovo et al., 2001). Following absorption, TDF is cleaved in the plasma by esterases (Figure 2) first to a monoester intermediate, and then to TFV (Choi, Bui, & Ho, 2008; Shaw et al., 1997). This hydrolysis is effective enough that TDF is not observed in systemic plasma samples.

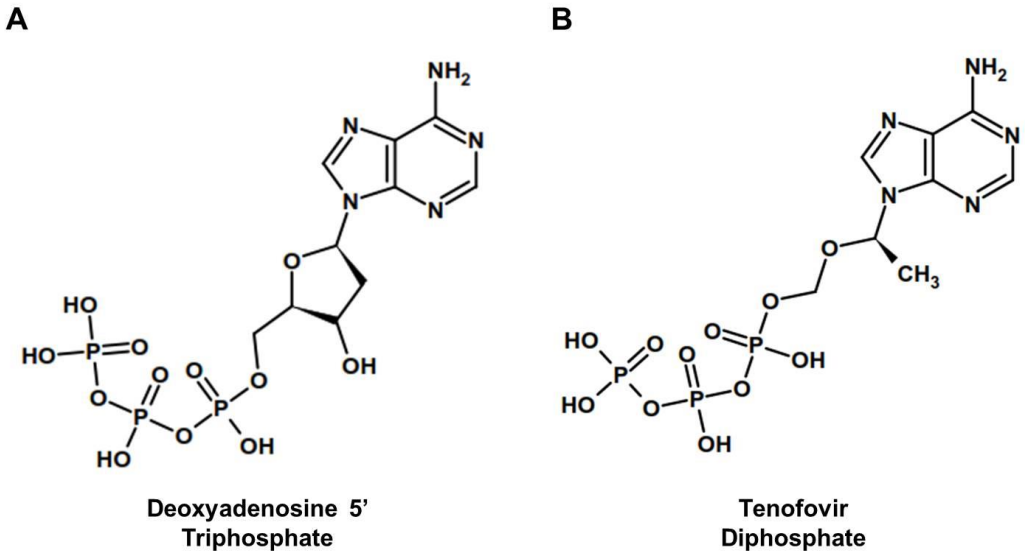

Figure 1. Structure of deoxyadenosine 5' triphosphate (A) and tenofovir diphosphate (B)

After intestinal absorption and plasma hydrolysis, TFV is phosphorylated intracellularly by adenylate kinase to its active form, tenofovir diphosphate (TDP) (Figure 3). As a nucleotide analogue, TFV diphosphate competes with deoxyadenosine 5' triphosphate (Figure 1) for incorporation into the growing DNA strand during HIV transcription and blocks the activity of

reverse transcriptase, leading to elongation termination (Balzarini et al., 1993; Robbins, Srinivas, Kim, Bischofberger, & Fridland, 1998).

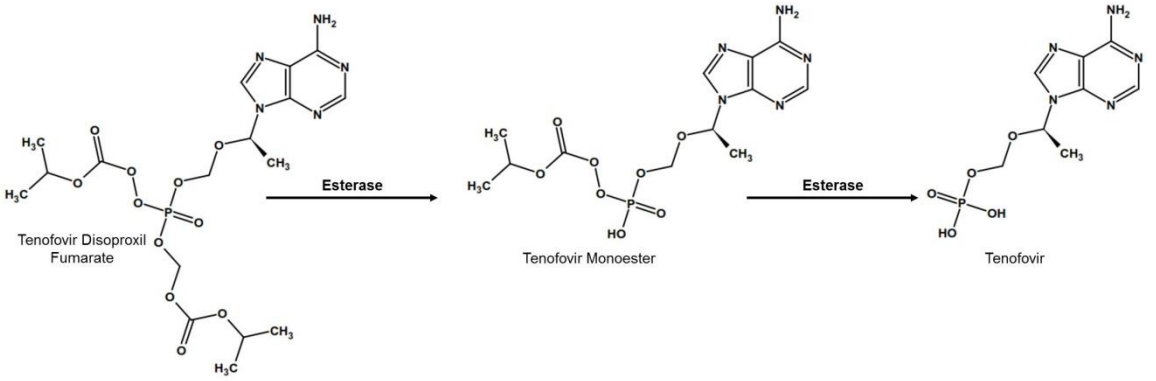

Figure 2. Conversion of tenofovir disoproxil fumarate to tenofovir by intestinal and plasma esterases.

Intravenous administration of 1 mg/kg TDF has a steady state volume of distribution of 813 mL/kg, indicating that tissue distribution is extensive and exceeds total body water. C_{max} of the same intravenous dose is approximately 2.7 μg/ml, and TFV serum concentration decreases in a biphasic manner (Deeks et al., 1998). The measured area under the curve (AUC) was 3.024 μg ·h/mL. The oral bioavailability of TDF was calculated by comparing the AUC for a 300 mg oral dose to the AUC for a 1 mg/kg intravenous dose; oral TDF has 25% bioavailability when compared to an intravenous dose (Barditch-Crovo et al., 2001).

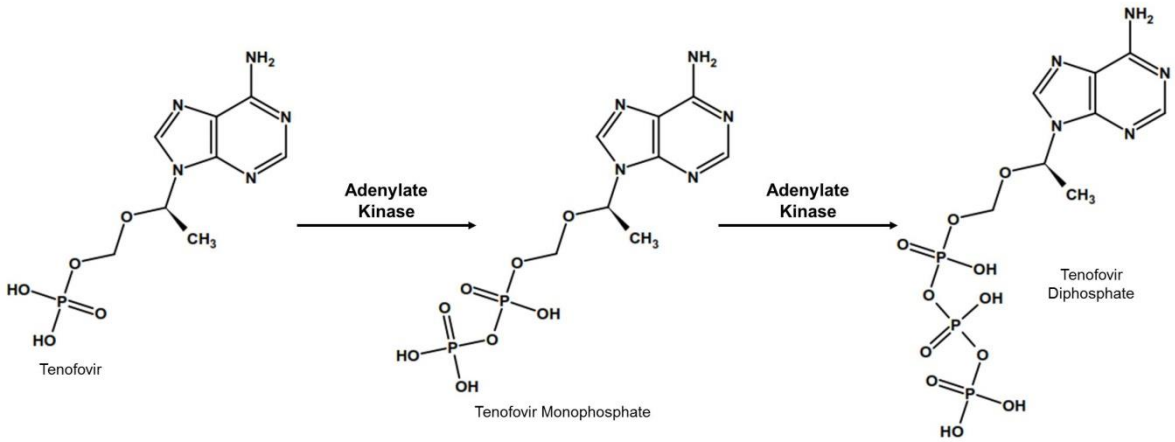

Figure 3. Intracellular activation of tenofovir to tenofovir diphosphate by adenylate kinase

The half-life for a single intravenous TDF injection was 7 h compared to 12 h for a single oral dose of 300 mg TDF (Barditch-Crovo et al., 2001; Deeks et al., 1998; Hawkins et al., 2005). The intracellular half-life of TDF is estimated to be 12-50 h (Robbins et al., 1998).

The pharmacologic antiviral activity of TDF and ultimately TFV is proportional to the intracellular concentration in target cells. A single oral dose of TDF to macaques provided almost 8 fold higher intracellular levels of the active TFV-diphosphate compared to a single subcutaneous injection of TFV (Durand-Gasselin et al., 2009). Additionally, oral TDF shows greater accumulation in peripheral blood mononuclear cells (PBMCs) when given as an oral prodrug rather than a subcutaneous injection of TFV to macaques (Durand-Gasselin et al., 2009). The bioavailability in macaques was 31%, which was similar to humans (Barditch-Crovo et al., 2001). These findings suggest that TDF first enhanced bioavailability, but also provided improved delivery to the target cells. The excellent uptake into cells allows for once daily dosing. Once daily dosing increases compliance, which will promote therapeutic success, which is important in controlling HIV as a chronic disease.

In general, differences in TDF pharmacokinetics are not apparent when comparing oral and intravenous routes of administration. Oral TDF has equivalent pharmacokinetics to intravenous TDF. Once C_{max} is reached, serum TFV concentrations decline in a biphasic manner. TFV is excreted 92% unchanged by the kidney and 4.5% unchanged in feces. Renal TFV clearance exceeds calculated creatinine clearances, indicating that TFV is actively secreted by the kidneys (Barditch-Crovo et al., 2001; Cundy et al., 1998; Durand-Gasselin et al., 2009). In vitro transport studies indicate that TFV is a substrate for the multidrug resistance-associated protein 4 (MRP4), an apical membrane efflux transporter (A. S. Ray et al., 2006). TFV was not a substrate for MRP2. These in vitro studies would suggest that active tubular secretion of TFV

into the nephron lumen is mediated by MRP4. It is worth noting that while TFV is not a substrate of MRP1, MRP2, or MRP 3, at concentrations exceeding 50 μM, TFV does inhibit these transporters in Madin-Darby canine kidney II (MDCKII) cells (Weiss, Theile, Ketabi-Kiyanvash, Lindenmaier, & Haefeli, 2007).

Pharmacokinetics and Bioavailability of TDF in Patients with Renal Insufficiency

TDF pharmacokinetics and bioavailability differ in patients with renal insufficiency, which is an important consideration in individuals with HIV. The frequency of acute kidney injury is more common in HIV infected individuals and can progress to end stage renal disease. In 2005, it was estimated that in the United States, almost 3,000 people had end stage renal disease related to HIV. A decline in renal function is a frequent occurrence in HIV disease progression (Schwartz & Klotman, 1998; Wyatt et al., 2009). HIV-associated nephropathy (HIVAN) primarily affects the glomerulus, and is characterized by proteinuria, elevated serum creatinine and blood urea nitrogen (BUN), focal segmental glomerulosclerosis (FSGS), and microcystic tubular dilation. Renal lesions lead to the collapse of the glomeruli and rapid progression to kidney failure (Laurinavicius, Hurwitz, & Rennke, 1999; P. E. Ray, 2012).

While HIVAN occurs less frequently in patients whose HIV is well controlled with antiretroviral therapy (ART) (Fabian, Naicker, Goetsch, & Venter, 2013), renal insufficiency should be considered when using TDF. In a study conducted in patients with varying degrees of renal insufficiency, patients with moderate and severe renal impairment (noted as creatinine clearance (CL_{CR}) of 30-49ml/min and <30ml/min, respectively) had an AUC 2-6 fold higher than in patients with normal renal function. The increase in AUC was most likely due to a decrease in TFV clearance that was directly proportional to the observed decreases in CL_{CR} (Kearney, Flaherty, & Shah, 2004).

Patients treated with TDF do have an increased incidence of renal impairment, and there is a positive correlation between increased TFV serum concentrations, duration of treatment with TDF, and incidence of renal toxicity (Ezinga, Wetzels, Bosch, van der Ven, and Burger, 2014; Fux et al., 2007; Quesada et al., 2015; Verhelst et al., 2002). TDF is associated with increased serum creatinine and plasma vitamin D binding, decreased free calcitriol levels and glomerular filtration rate (GFR), renal failure, and Fanconi Syndrome (Del Palacio, Romero, & Casado, 2012; Hall, Hendry, Nitsch, & Connolly, 2011; Havens et al., 2013). Fanconi syndrome is a disorder of renal tubular function characterized by excess renal excretion of potassium, phosphate salts, protein, urate, and glucose. Clinical studies indicate that the mitochondria of the proximal tubules are the target of TFV induced toxicity as shown by enlarged and malformed mitochondria in electron microscopy cross sections (Cote et al., 2006; Hall et al., 2011; Herlitz et al., 2010; Woodward et al., 2009). Proximal tubular mitochondrial toxicity has also been described in mice and rats treated with TFV as shown by disruptions in mitochondrial cristae and a reduction in mitochondrial DNA (mtDNA) levels (Kohler et al., 2009; Lebrecht et al., 2009).

Changes in TDF administration are recommended for patients with decreased renal function to reduce plasma concentrations and to prevent worsening of renal damage. In patients with moderate impairment (CL_{CR} 30-49ml/min), 300 mg dose of TDF should be reduced in frequency to once every 48 h instead of once daily. In patients with severe impairment (CL_{CR} 10-29ml/min) or those who are on hemodialysis, the 300 mg dose should be administered every 72-96 h or once every 7 days, respectively (Gilead Sciences, 2001).

Pharmacokinetics and Bioavailability of TDF in Fixed Dose Combination Tablets

To simplify HIV treatment regimens and increase compliance, TDF is commonly paired with other antiretrovirals in single dose combination tablets. The drugs currently combined with TDF in fixed dose combinations are: emtricitabine (FTC, 200 mg), efavirenz (EFV, 600 mg), rilpivirine (RPV, 25 mg), the integrase strand transfers inhibitor elvitegravir (EVG, 150 mg) and the CYP 3A4 inhibitor cobicistat (COBI, 150 mg). The most common formulations are: TDF/FTC, TDF/FTC/EFV, TDF/FTC/RPV and TDF/FTC/EVG/COBI. Each formulation includes TDF as a standard 300 mg dose. Bioequivalence is evaluated in combination tablet formulations by maintenance of pharmacokinetic parameters C_{max}, AUC, and $t_{1/2}$ within a confidence interval (CI) of 80-125% of the reference formulation.

The first fixed dose combination approved by the FDA for treatment of HIV contained TDF/FTC/EFV. FTC, EFV and TDF all have half-lives that are suitable for once a day dosing (FTC, 39h; EFV, 23 h; TDF, 15-60 h) (Barditch-Crovo et al., 2001; DiCenzo et al., 2003; Hawkins et al., 2005; Saag, 2006). When studied as a fixed-dose combination tablet, all pharmacokinetic parameters had geometric mean ratios close to 100% and 90% CI within the bioequivalence bounds of individual formulations, indicating that TDF, EFV, and FTC can be combined into a once daily combination tablet (Mathias et al., 2007). The combination of TDF/FTC/EFV is FDA approved as a complete regimen for HIV treatment and can also be used in combination with other HIV medications (Gilead Sciences, 2006).

TDF/FTC/RPV combination provides two nucleos(t)ide reverse transcriptase inhibitors (TDF/FTC) with a non-nucleoside reverse transcriptase inhibitor, RPV to treat HIV. Initial studies showed that addition of 25mg RPV to a TDF/FTC treatment regimen showed equivalence in HIV suppression when compared to TDF/FTC + EFV regimens and had less

adverse effects than EFV (Cohen et al., 2011; Molina et al., 2011). RPV has a $t_{1/2}$ of approximately 50 h, making it a suitable candidate for inclusion in a once daily combination tablet (Cohen et al., 2013). In a bioequivalence study, all pharmacokinetic parameters of a TDF/FTC/RPV single dose tablet demonstrated a 90% CI within the bioequivalence bounds of individual formulations, indicating that TDF, FTC, and RPV can be combined into a once daily combination tablet. While both TDF and FTC are approved to be taken with or without a meal, RPV bioavailability is reduced 40% in a fasted state alone and in a combination tablet. Therefore, TDF/FTC/RPV should be taken with food (Mathias et al., 2012).

TDF/FTC combination tablets also demonstrate bioequivalence for all tested pharmacokinetic parameters when compared to individual therapy (Blum, Chittick, Begley, & Zong, 2007). TDF/FTC is FDA approved to treat HIV-1 in conjunction with other therapies, but is not considered a complete regimen. TDF/FTC is also FDA-approved for use in pre-exposure prophylaxis (PrEP) therapy to prevent the transmission of HIV-1 between sexual partners (Anderson et al., 2012; Baeten et al., 2012; Grant et al., 2010). The use of TFV-containing regimens for PrEP is discussed in further detail in the "Tenofovir as a Prophylactic Agent" section below.

Recently COBI has been included in HAART to treat HIV. While it has no antiviral activity on its own, COBI is a pharmacoenhancer for drugs that are metabolized by cytochrome P450 3A enzymes (CYP3A). COBI is a potent CYP3A inhibitor, and is commonly used with protease and integrase inhibitors such as EVG in the treatment of HIV. In a fixed dose combination study, administration of COBI/TDF/EVG combination tablets showed bioequivalence; however, TDF C_{max} was increased 30% but AUC was largely unaffected (German, Warren, West, Hui, & Kearney, 2010). COBI may alter the half-life of other drugs

taken concurrently that are biotransformed by CYP3A as inhibition by COBI may slow biotransformation by CYP3A. COBI is a substrate for CYP3A and treatment with agents that induce or inhibit CYP3A has the potential to alter the effects of COBI (Sherman, Worley, Unger, Gauthier, & Schafer, 2015).

This increase in C_{max} may be due to COBI's transient inhibition of P-glycoprotein efflux transporter (Pgp); TFV is a recognized substrate of the Pgp transporter (German et al., 2010; Lepist et al., 2012). It is worth noting that COBI inhibition of P-glycoprotein is weak, much like other protease inhibitors such as ritonavir (RTV); COBI, RTV, and other protease inhibitors do not inhibit efflux transporters at pharmacologically relevant concentrations in systemic circulation (Cihlar et al., 2007; Polli et al., 1999; Ray et al., 2006; van der Sandt et al., 2001; Washington, Duran, Man, Sikic, & Blaschke, 1998) but may reach concentrations high enough to inhibit efflux transporters in the gut (Ray et al., 2006; Tong et al., 2007).

Effect of Food Intake on TDF Bioavailability

Taking TDF with food has shown to improve bioavailability. However, it does not appear that the type of meal matters, as no changes in pharmacokinetic parameters are seen with administration of a standard meal, high fat meal, or protein-rich meal. In a study of healthy Japanese males, participants ingested a standard meal (11.4g protein, 9.6g fat, and 72.2g carbohydrates) or a protein-rich drink (8.8g protein, 8.8g fat, 34.3g carbohydrates) prior to administration of a 300 mg dose of TDF. There was a 28% decrease in both AUC and C_{max} when TDF was administered in a fasted state compared to administration following a standard meal or a protein-rich drink. Although C_{max} was slightly lower in the protein-drink group (554 ng/ml vs. 613 ng/ml), AUC and $t_{1/2}$ were not different between meals, indicating that a standard meal and a

protein-rich drink produce bioequivalent increases in TDF bioavailability over fasted administration (Shiomi et al., 2014).

Bioavailability of TDF was increased in one study using a very high fat meal in HIV uninfected males. Effects of a very high fat meal were evaluated during phase I testing of TDF. Participants ingested a high fat meal (50% fat, 1000kcal) 30 minutes prior to administration of TDF. TDF bioavailability in the fed group was increased 40% over the fasted group, and C_{max} was increased 14%. T_{max} also increased from 0.5-1 h in a fasted state to 1.3-3 h in a fed state, consistent with a slowing of gastric emptying following a meal (Barditch-Crovo et al., 2001; Kearney et al., 2004). This same group noted that a high fat meal was not indicative of normal diet, and studied the effects of a light meal on TDF bioavailability and found no significant effect. According to its prescribing information, TDF can be administered with or without a meal (Gilead Sciences, 2001). It should be noted that certain TDF fixed-dose combination formulations such as TDF/FTC/RPV and TDF/FTC/ EVG/COBI should be taken with food due to significant decreases in RPV (40%) and EVG (50%) bioavailability in a fasted state (German et al., 2010; Mathias et al., 2012; Shiomi et al., 2014).

Though TDF is intended to be cleaved by plasma esterases, it is susceptible to cleavage by intestinal carboxylesterases when administered by the oral route. It is hypothesized that this intestinal hydrolysis and efflux via Pgp is what limits TDF bioavailability in humans (Shaw et al., 1997; van Gelder et al., 2002; Yuan, Dahl, & Oliyai, 2001). Ester rich fruit juices such as grapefruit, orange, cranberry, and concord grape have been shown to increase oral absorption of drugs such as lopinavir (LPV) and saquinavir (SQV) through inhibition of Pgp, MRP-2, and various cytochrome P450s (CYPs) (Bailey, Malcolm, Arnold, & Spence, 1998; Honda et al., 2004; Ravi, Joseph, Avula, & Anthireddy, 2015). As TDF is a substrate of Pgp, studies were

conducted to determine if ester rich fruit juices increase TDF bioavailability and intestinal absorption. Strawberry juice extract increased TDF intestinal absorption in Caco-2 cell lines to 67%, while grapefruit and cranberry juice extracts increased TDF bioavailability by 97 and 24% in an in vivo rat study (Shailender, Ravi, Saha, & Myneni, 2017; Van Gelder et al., 1999). Additional investigation needs to be done to determine if this effect is also seen in humans.

Age Related Differences in Tenofovir Bioavailability and Pharmacokinetics

TDF is FDA approved for use in the US in children age two and older. As HIV is a chronic disease, it is important to determine the developmentally associated changes in pharmacokinetic parameters of antiretroviral therapy. Studies have been conducted to determine the pharmacokinetic profile of TDF administration in neonates, children/adolescents, and adults. TDF pharmacokinetics vary in children comparatively to adults, and these variables need to be considered when determining dosing. Initial pharmacokinetic studies were run following TDF's approval for use in adults. Hazra and colleagues (2004) determined the adult-equivalent dose in children to be ~175 mg/m^2 based on calculations from initial pharmacokinetic studies in adults. In a cohort of children ranging from 4-18 years of age given 175 mg TDF for four weeks, AUC and C_{max} were reported to be 34 and 27% lower than the equivalent dose in adults. The decrease in AUC may be explained by the observed 1.5 times faster clearance in children (Barditch-Crovo et al., 2001; Hazra et al., 2004).

Studies where HIV-positive children were administered a full adult dose of TDF once daily showed similar pharmacokinetic changes observed in the initial pharmacokinetic study. Children and adolescents (defined as <25 years old) have lower plasma concentrations of TFV compared to adult patients and clearance in children was 36% faster, which may contribute to the lower plasma concentration. The increase in clearance may be due to a larger kidney relative to

body size as well as increased renal function (Baheti, King, Acosta, & Fletcher, 2013; King et al., 2011). Additionally, Baheti and colleagues (2013) reported that children have almost 50% higher intracellular concentrations of TDP following oral administration compared to adults. This phenomenon may be due to a lower effective concentration, EC_{50} seen in children rather than adults for TFV (100 ng/mL vs 192 ng/mL respectively).

Though no significant adverse effects were initially observed in studies using an adult dose, studies have also been conducted to determine effective weight-band TDF dosing in children due to concerns of bone density loss at different stages of bone dynamics. Longitudinal assessment of available data determined that overall incidence of bone mineral density (BMD) loss in children taking TDF at 24-32%; this loss occurs within 6-12 months of initiation of treatment and then stabilizes (Aurpibul et al., 2015; Jacobson et al., 2010; O'Brien, Razavi, Henderson, Caballero, & Ellis, 2001; Puthanakit et al., 2012; Schtscherbyna et al., 2012).

A study conducted in children age 9 to 18 years also saw increased TFV clearance relative to adults, although clearance increased with body weight. Because of the differences in clearance, this model recommends prescribing TDF to children based on weight to achieve adult AUC: 150 mg at 20-30 kg body weight, 225 mg at 30-40 kg body weight, and the full adult dose of 300 mg at over 40 kg bodyweight (Bouazza et al., 2011); no mention of adverse effects was made in this study. Another study conducted based on weight-band dosing in children 3-17 years determined similar dosing parameters: 150 mg at 15-21 kg body weight, 225 mg at 22-33 kg body weight, and a full adult dose of 300 mg at >33 kg body weight. In this 96 week study, 28% of patients experienced a decrease in BMD by 24 weeks, at which point BMD remained constant (Aurpibul et al., 2015).

TDF is most commonly administered in children as part of a treatment regimen also containing LPV and RTV, unlike adults who are commonly prescribed single dose combination tablets (see previous section). It is relevant to note that LPV increases TDF AUC by 34% and serum concentration by 30% in adults (Barditch-Crovo et al., 2001; Kearney et al., 2004). This phenomenon is also seen in children, and is attributed to the observed 25% decrease in TDF clearance when co-administered with LPV (Aurpibul et al., 2015; Bouazza et al., 2011). It is recommended that TDF dosing be adjusted in children when co-administered with LPV: 150 mg at 20-40 kg body weight, 225 mg at 40-50 kg body weight, and 300 mg at >55 kg body weight (Bouazza et al., 2011).

TDF is approved to treat HIV in children >2 years of age in combination with other medications. It is recommended that dosage be adjusted according to body weight in children <12 years of age (Gilead Sciences, 2001). Additional study needs to be conducted on the observed interaction of TDF with LPV, as well as to monitor the incidence of bone density loss in children. Additional research is needed to evaluate the effect of age on the renal tubular transporters and potential impact on TFV clearance. TFV is a substrate for the efflux transporter MRP4. A study in rats reported that MRP4 expression developed slower than the basolateral membrane transporters OAT1 and OAT3 (Nomura, Motohashi, Sekine, Katsura, & Inui, 2012). The developmental expression of MRP4 and other transporters is an area of limited information and may explain some of the differences related to TFV clearance with age.

Tenofovir as a Prophylactic Agent: Sex Related Differences in Pharmacokinetics

TDF is commonly used for antiretroviral pre-exposure chemoprophylaxis (PrEP) in HIV-uninfected patients who engage in high risk sexual encounters. Studies evaluating oral TDF/FTC PrEP efficacy show modest protection (76%) when taken twice a week and 75-99% infection

protection when taken every day. Studies using oral TDF 300 mg tablets as PrEP show a 67% reduction in HIV incidence but high adherence was necessary (Anderson et al., 2012; Baeten et al., 2012). It should be noted that while TFV pharmacokinetics are similar, there are documented differences in TDP intracellular concentrations in HIV-infected and uninfected patients; TDP concentrations are higher in HIV-infected patients (120 fmol/million cells) than in healthy patients (42 fmol/million cells) (Anderson et al., 2012; Baheti et al., 2013). Currently, TDF/FTC is the only FDA approved PrEP regimen; it must be taken daily in conjunction with safer sex practices and HIV-status monitoring every three months. HIV-status monitoring is critical as HIV-1 resistance has been documented in cases of undetected infection when using TDF/FTC (Gilead Sciences, 2004).

To evaluate PrEP options for women, studies have been conducted to determine the efficacy of a topical 1% TDF gel formulation in the prevention of HIV infection. The original CAPRISA clinical trial showed modest protection, with an incidence of reduction ranging from 39-54% depending on adherence (Abdool Karim et al., 2010). TDP intracellular concentrations were not measured. A study of PrEP regimens of TDF, TDF/FTC, or 1% TDF vaginal gel in African women determined that there was no effect in the prevention of HIV transmission (Marrazzo et al., 2015); again TDP concentration was not measured. Currently 1% gel TDF formulation is not approved for PrEP, as efficacy has thus far been shown to be moderate at best.

Recently clinical studies were done to determine the differences in TFV-DP distribution in healthy women using the TDF 1% gel, TDF 300 mg oral tablet, or both. TDP vaginal concentration was 130-fold higher in patients using the topical gel than oral dosing, but systemic TDP concentrations were 56-fold lower. Combination regimens of oral and topical TDF resulted in anal tissue concentrations 3-fold higher than in groups that used the oral formulation alone

(Burns, Hendrix, & Chaturvedula, 2015; Hendrix et al., 2013). Efficacy as a PrEP regimen was not directly measured in this study.

The extremely high vaginal concentrations of TDP may be due in part to hormonal regulation. Estradiol increases the amount of TDP present in isolated epithelial cells from the female reproductive tract, progesterone has no effect, and estradiol-progesterone combination treatment negates the stimulatory effect of estradiol. In CD4 cells isolated from the female reproductive tract, progesterone alone and in combination with estradiol greatly reduced TDP concentrations (Shen et al., 2014). These results suggest that hormonal regulation of TFV phosphorylation is dependent on cell type. It has been hypothesized that women experience a 7-10 day window of increased susceptibility to HIV-infection due to immune suppression by sex hormones following ovulation (Wira & Fahey, 2008). The changes in TDF-uptake and activation in the female reproductive tract may be due to the hormonal responses seen in previous studies.

It is worth noting that while TDP concentrations vary in the female reproductive tract, administration of TDF with oral contraceptive pills (OCP) did not change either TFV or OCP systemic levels (Kearney & Mathias, 2009). The observed responsiveness to sex hormones may be limited to tissues within the reproductive tract; additional study needs to be done in this area to fully determine the relationship between TDF pharmacokinetics and sex hormones.

TDF has also been examined in late pregnancy and labor to prevent vertical transmission of HIV from expectant mothers to their unborn children. One study gave 300 mg TDF at the onset of labor and to neonates within 12 h of birth to infected mothers; a 13 mg/kg neonate dose produced intracellular TDP concentrations similar to adult levels (Hirt et al., 2011). However plasma concentration remains low as seen in children taking TDF, and there is a delay in the

presence of TDP in neonatal PBMCs. There was no quantifiable TDP in fetal PBMCs. The delay in TFV phosphorylation could be due to differences in enzyme expression and activity in neonates vs. children. Another study showed similar results: HIV-infected mothers were given a 600 mg dose of TDF and neonates were given a 6 mg/kg daily dose, resulting in plasma concentrations similar to those seen in adults. Infant clearance was also equivalent to that seen in older children (Mirochnick et al., 2014).

It is important to note that TDF pharmacokinetics are slightly altered during pregnancy. During the third trimester TDF AUC is 20% lower compared to postpartum levels. This transient difference may be directly attributable to increased weight and faster clearance during the last trimester of pregnancy (Best et al., 2015). Additional research needs to be done to determine changes in pharmacokinetic parameters during pregnancy, and how these changes affect vertical HIV transmission to children.

Alternative Tenofovir Prodrug Formulations

Recently, tenofovir alafenamide (TAF) has undergone phase I-III testing and was approved to treat chronic Hepatitis B (HBV) in 2016. TAF has greater plasma stability, more effective cellular loading than TDF, and maintains a similar oral bioavailability at 17%. TAF has a 50% effective concentration (EC_{50}) of 0.005 uM rather than 5 uM seen with TDF, leading to significantly less plasma exposure, and, by association, lower systemic side effects (Buti et al., 2016; Lee et al., 2005; Sax et al., 2015). TAF underwent phase I-II testing in HIV patients, but did not make it to phase III due to the risk of HIV-1 resistance (Gilead Sciences, 2017; Markowitz et al., 2014).

TAF has been approved to replace TDF in fixed-dose combination tablets to treat HIV following phase III trials. The combination product of TAF/FTC/COBI/EVG demonstrated 90%

virologic success in both treatment naïve and treatment experienced patients with a lower incidence of adverse renal and bone effects when compared to TDF/FTC/COBI/EVG (Gilead Sciences, 2015b; Mills et al., 2016; Pozniak et al., 2016; Sax et al., 2015; Wohl et al., 2016). The combined preparation TAF/FTC demonstrated 93% virologic success when compared to the combination of TDF/FTC, with no documented renal tubulopathy in either test group. It should be noted that while TDF/FTC is recommended as a PrEP regimen, TDF/FTC is not approved for PrEP regimen by the FDA as of May 2017 (Gallant et al., 2016; Gilead Sciences, 2015a; Mills et al., 2016; Sax et al., 2015). While TDF is currently FDA approved for HIV-1 treatment in patients two years of age and older, TAF fixed dose combinations are only indicated for patients greater than 12 years of age.

The use of liposomes and niosomes has been investigated to improve the bioavailability of TDF. When encapsulated in a liposome made of phospholipids and 7.5% cholesterol, TDF had an increased permeability rate across Caco-2 cells (3.71E-07 cm/s) compared to TDF alone (4.18E-07 cm/s) (Spinks, Zidan, Khan, Habib, & Faustino, 2017). When encapsulated in a niosome created with sorbitan monoesterate in a 1:1 ratio, >99% of the TDF dose was released in vitro within 24 h. In a subsequent in vivo study in rats, TDF AUC was increased two-fold when encapsulated in a niosome compared to TDF alone; C_{max}, $T_{1/2}$, and T_{max} were also increased, possibly due to increased GI absorption and the release retarding effects of niosomes (Azmin et al., 1985; Kamboj, Saini, & Bala, 2014). No studies have yet been conducted to determine if liposomes or niosomes are more efficient in improving TDF bioavailability or if encapsulation is effective in humans for the treatment of HIV using TDF.

Conclusions

TDF is the prodrug for TFV, which is a nucleotide analogue reverse transcriptase inhibitor that is very effective in the treatment of HIV and chronic HBV. TDF has a favorable pharmacokinetic profile, allowing for once a day dosing, and its pharmacokinetics are largely unchanged when given in fixed-dose combination tablets. TDF has been shown to be effective as a prophylactic agent to prevent HIV-1 transmission, leading to site-specific formulations; this area of research remains highly active. TDF does have limited oral bioavailability, and research is ongoing to determine if bioavailability can be increased. More importantly, TDF improves intracellular concentration in PBMCs. Overall, TDF remains one of the first line agents in the treatment of HIV and HBV providing once daily dosing which improves patient compliance.

CHAPTER 2

TENOFOVIR INDUCED NEPHROTOXICITY: THE SCOPE OF THE CURRENT WORK

Tenofovir Induced Nephrotoxicity

TDF is the prodrug for TFV which is a nucleotide analogue reverse transcriptase inhibitor that is approved in the United States for the treatment of HIV and chronic Hepatitis B. TDF has a favorable pharmacokinetic profile, allowing for once a day dosing and has limited incidence of day to day adverse effects; both of these factors are important to increase compliance in chronic disease treatment (Barditch-Crovo et al., 2001). TDF is also effective in both treatment naïve and treatment experienced patients, particularly in patients who have developed HIV-1 resistance (Miller et al., 2001; Squires et al., 2003). These attributes make TDF a first line agent in the treatment of HIV according to the World Health Organization.

Despite its many positive attributes, renal toxicity is an adverse effect associated with TDF use clinically. TFV can induce reductions in GFR and free calcitriol, increases in serum creatinine and plasma vitamin D binding, renal failure, and in extreme cases Fanconi Syndrome. Fanconi syndrome is described as a renal tubular function disorder that results in excess excretion of phosphate salts, protein, potassium, glucose, and urate (Del Palacio et al., 2012; Hall et al., 2011; Havens et al., 2013). Clinical studies indicate that the mitochondria of the proximal tubules are the target of TFV induced toxicity as shown by enlarged and malformed mitochondria in electron microscopy cross sections (Cote et al., 2006; Hall et al., 2011; Herlitz et al., 2010; Woodward et al., 2009). Proximal tubular mitochondrial toxicity has also been described in mice and rats treated with TFV as shown by disruptions in mitochondrial cristae and a reduction in mtDNA levels (Kohler et al., 2009; Lebrecht et al., 2009).

Renal excretion requires both glomerular filtration and tubular secretion. Following glomerular filtration, TFV is transported from blood into proximal tubular epithelial cells by organic anion transporters 1 and 3 (OAT1, OAT3, respectively) (Kohler et al., 2011; Uwai, Ida, Tsuji, Katsura, & Inui, 2007). TFV can then undergo tubular secretion into the lumen via the multidrug resistant proteins 2 (MRP2) and 4 (MRP4) (Izzedine, Launay-Vacher, & Deray, 2005; Kohler et al., 2011). While there is knowledge of the mechanisms contributing to tubular transport, the mechanism of TFV-associated proximal tubule damage is not extensively known at this time. This lack of knowledge is due largely to limited experimental models.

HK-2 Cell Model

Mechanistic studies require a model that is consistent with what occurs in humans. The selection of suitable models to study TFV cytotoxicity has been problematic as most in vivo models require subchronic treatment of animals. TFV toxicity in rodents such as rats has required eight weeks of daily treatment in order to develop nephrotoxicity (Lebrecht et al., 2009). In this study, rats treated for eight weeks exhibited diminished renal function and proximal tubular damage along with enlarged mitochondria. Another study showed that treatment for five weeks with TFV induced oxidative stress in Wistar rats dosed with very high levels of TFV (Abraham, Ramamoorthy, & Isaac, 2013).

An in vitro model was first reported by Wang and Flint (2013) that developed TFV cytotoxicity using primary human kidney cells (Wang & Flint, 2013). Primary human kidney cells required culturing for 19 days with 200 μM TFV to induce cytotoxicity, which was a long duration at concentrations higher than pharmacological levels. The need for higher concentrations of TFV to induce toxicity in primary proximal tubule cell lines may be due to differences in OAT1 and OAT3 expression. Endogenous expression of OAT1 and OAT3 in

primary human proximal tubule cells has been shown to vary greatly from sample to sample (Lash, Putt, & Cai, 2006) and TFV-induced cytotoxicity is dependent on OAT1 and OAT3 expression (Kohler et al., 2011; Nieskens et al., 2016).

Renal toxicity was diminished in OAT1 knockout mice, indicating that OAT1 has a primary contribution to TFV renal uptake into proximal tubular epithelial cells (Kohler et al., 2011). In vitro studies reported that HEK293 cells transfected with OAT1 were much more sensitive to TFV cytotoxicity than cells transfected with OAT3 (Izzedine et al., 2005); further, intracellular TFV concentrations were higher in HEK-OAT1 transfected cells compared to wild type and these findings support the concept that OAT1 is important for TFV uptake.

Human Kidney 2 (HK-2) cells were selected as a model to investigate the in vitro cytotoxicity of TFV. HK-2 cells are an immortalized, noncancerous, human epithelial proximal tubular cell line that maintains activity and biochemical properties similar to in vivo proximal tubule cells (Gunness, Aleksa, Kosuge, Ito, & Koren, 2010; Paolicchi et al., 2003; Ryan et al., 1994). HK-2 cells express OAT1 and OAT 3, which are necessary to transport TFV into proximal tubule cells as well as multidrug resistance protein 4 (MRP4), which is necessary for efflux from the proximal tubule cell (Miyamoto et al., 2012). HK-2 cells are commonly used to study drug toxicity and in mechanistic studies as they eliminate potential species differences that occur in porcine, dog, and mouse cell lines (Andreucci et al., 2015; Gunness et al., 2010; Jia, Ha, Yang, Chang, & Yang, 2011; Kondo, Inamura, Matsumura, & Matsuoka, 2012). This thesis establishes that HK-2 cells are a viable model to study TFV induced cytotoxicity as well as utilize them to gain insight into the mechanism of this toxicity.

Statement of Hypothesis

Current published studies have determined that TFV induces damage to proximal tubule epithelial cells. The purpose of my dissertation was to investigate a mechanistic understanding of TFV induced cytotoxicity and investigate potential methods to mitigate or prevent it. To that end, I tested the following hypotheses in this work. First, TFV is toxic to HK-2 cells at clinically relevant concentrations. Second, the mechanism of this toxicity is due to oxidative stress and mitochondrial dysfunction. Lastly, pretreatment with antioxidants mitigates TFV induced cytotoxicity.

CHAPTER 3

ESTABLISHMENT OF HK-2 CELLS AS A RELEVANT MODEL TO STUDY TENOFOVIR-INDUCED CYTOTOXICITY

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Rachel A. Murphy [1], Reagan M. Stafford [1], Brooke A. Petrasovits [1], Megann A. Boone [2] and Monica A. Valentovic [1,*]

[1]Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755

[2] West Virginia Wesleyan College, Buckhannon, WV, 26201

[1]Correspondence should be addressed to: Department of Biomedical Sciences; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755. Phone (307-696-7332) Fax (304-696-7391) Email: valentov@marshall.edu

Abstract

Tenofovir (TFV) is an antiviral drug approved for treating Human Immunodeficiency Virus (HIV) and Hepatitis B. TFV is administered orally as the prodrug tenofovir disoproxil fumarate (TDF) which then is deesterified to the active drug TFV. TFV induces nephrotoxicity characterized by renal failure and Fanconi Syndrome. The mechanism of this toxicity remains unknown due to limited experimental models. This study investigated the cellular mechanism of cytotoxicity using a human renal proximal tubular epithelial cell line (HK-2). HK-2 cells were grown for 48 h followed by 24 to 72 h exposure to 0–28.8 µM TFV or vehicle, phosphate buffered saline (PBS). MTT (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and Trypan blue indicated that TFV diminished cell viability at 24–72 h. TFV decreased ATP levels at 72 h when compared to vehicle, reflecting mitochondrial dysfunction. TFV increased the oxidative stress biomarkers of protein carbonylation and 4-hydroxynonenol (4-HNE) adduct formation. Tumor necrosis factor alpha (TNFα) was released into the media following exposure to 14.5 and 28.8 µM TFV. Caspase 3 and 9 cleavage was induced by TFV compared to vehicle at 72 h. These studies show that HK-2 cells are a sensitive model for TFV cytotoxicity and suggest that mitochondrial stress and apoptosis occur in HK-2 cells treated with TFV.

Introduction

Tenofovir disoproxil fumarate (TDF) is a nucleotide reverse transcriptase inhibitor that is approved in the United States to treat Human Immunodeficiency Virus (HIV) and chronic Hepatitis B. Tenofovir (TFV) is administered as the prodrug TDF; TDF was designed to improve the bioavailability of TFV by the addition of 2-methyl carbonate esters which are deesterified to the active drug TFV (Shaw et al., 1997). TFV is efficacious to both treatment-naïve and

treatment-experienced patients and has been beneficial in patients that have acquired resistance to other HIV medications (Dore et al., 2004; Miller et al., 2001). TFV is considered a first line agent essential for treatment of HIV by the World Health Organization. TFV is the only antiretroviral that is a nucleotide analogue reverse transcriptase inhibitor. Compliance is higher with TFV than with other antiretroviral agents due to fewer side effects and once daily dosing (Barditch-Crovo et al., 2001).

Despite many positive therapeutic attributes, an adverse effect of TFV is nephrotoxicity. TFV-induced nephrotoxicity is characterized by a decreased glomerular filtration rate (GFR), increased serum creatinine, renal failure, and Fanconi-like Syndrome (Del Palacio et al., 2012; Hall et al., 2011). Fanconi Syndrome is a proximal tubular transport abnormality associated with impaired tubular reabsorption resulting in excess glucose, protein, urate, and phosphate excretion.

Renal excretion requires both glomerular filtration and tubular secretion. TFV is transported from blood into proximal tubular epithelial cells by organic anion transporters 1 and 3 (OAT1, OAT3, respectively) (Kohler et al., 2011; Uwai et al., 2007). Renal toxicity was diminished in OAT1 knockout mice, indicating that OAT1 has a primary contribution to TFV renal uptake into proximal tubular epithelial cells (Kohler et al., 2011). In vitro studies reported that HEK293 cells transfected with OAT1 were much more sensitive to TFV cytotoxicity than cells transfected with OAT3 (Zhang, Piotrowski, Zhang, Leach, 2015); further, intracellular TFV concentrations were higher in HEK-OAT1 transfected cells compared to wild type and these findings support the concept that OAT1 is important for TFV uptake. The drug can then undergo tubular secretion into the lumen via the multidrug resistant proteins 2 (MRP2) and 4 (MRP4) (Izzedine et al., 2005; Kohler et al., 2011). While there is knowledge of the mechanisms

contributing to tubular transport, the mechanism of TFV-associated proximal tubule damage is not extensively known at this time. This knowledge gap is due largely to limited experimental models; in vivo studies have used TFV treatment for three to eight weeks (Lebrecht et al., 2009; Ramamoorthy, Abraham, & Isaac, 2014), or primary renal cells exposed for up to 22 days (Vidal et al., 2006). The long duration of these experiments is not ideal and can hamper mechanistic studies. Additionally, daily treatment of rats for an extended period can cause significant stress, adding other complications to a mechanistic study. Cell lines can circumvent the contribution of stress as well as eliminate extrarenal factors and hemodynamics.

The purpose of this study was to explore the in vitro cytotoxicity of TFV in HK-2 cells. We selected Human Kidney 2 (HK-2) cells as a model as these cells are an adult, noncancerous, immortalized human epithelial cell line. HK-2 cells maintain biochemical properties and activity similar to in vivo proximal tubule cells (Gunness et al., 2010; Paolicchi et al., 2003; Ryan et al., 1994). Additionally, HK-2 cells express OAT1 and OAT3 proteins (Miyamoto et al., 2012), which facilitate TFV transport into proximal tubule cells.

In this manuscript, we demonstrate that the HK-2 cell is a viable model to study TFV-induced cytotoxicity at clinically relevant concentrations. Further, these studies revealed that oxidative stress, induction of apoptosis, and mitochondrial decline in ATP are associated with TFV renal cytotoxicity.

Results

TFV Effects on Cell Viability

Based on the MTT (MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) assay, TFV reduced cell viability within 24 h ($p < 0.05$) at all tested concentrations (Figure 4). MTT values continued to show diminished cell viability at 48 and 72 h ($p < 0.001$)

for all TFV concentrations when compared to vehicle control (Figure 4). TFV final concentrations of 4.75 and 14.5 µM showed additional decline in cell viability at 72 h compared to other treatment groups ($p < 0.05$) (Figure 4). Trypan blue exclusion and cell counts were conducted in order to confirm that the TFV mediated decline in formazan formation could not be attributed to a direct effect of TFV on mitochondrial reductase enzymes converting MTT to formazan. Trypan blue cell counts (Figure 5) confirmed that TFV diminished cell viability beginning at 24 h and continuing for 72 h when compared to vehicle control. There was no difference in cell viability between groups. IC50 values calculated using MTT data were 9.21 and 2.77 µM TFV at 48 and 72 h, respectively. MTT viability studies conducted with the less nephrotoxic antiviral agent, abacavir, indicated that abacavir did not reduce cell viability at 24 h (Figure 4D) suggesting that our model is an appropriate model to examine nephrotoxic agents. Further experiments were initiated with TFV to explore the cellular mechanism of TFV cytotoxicity.

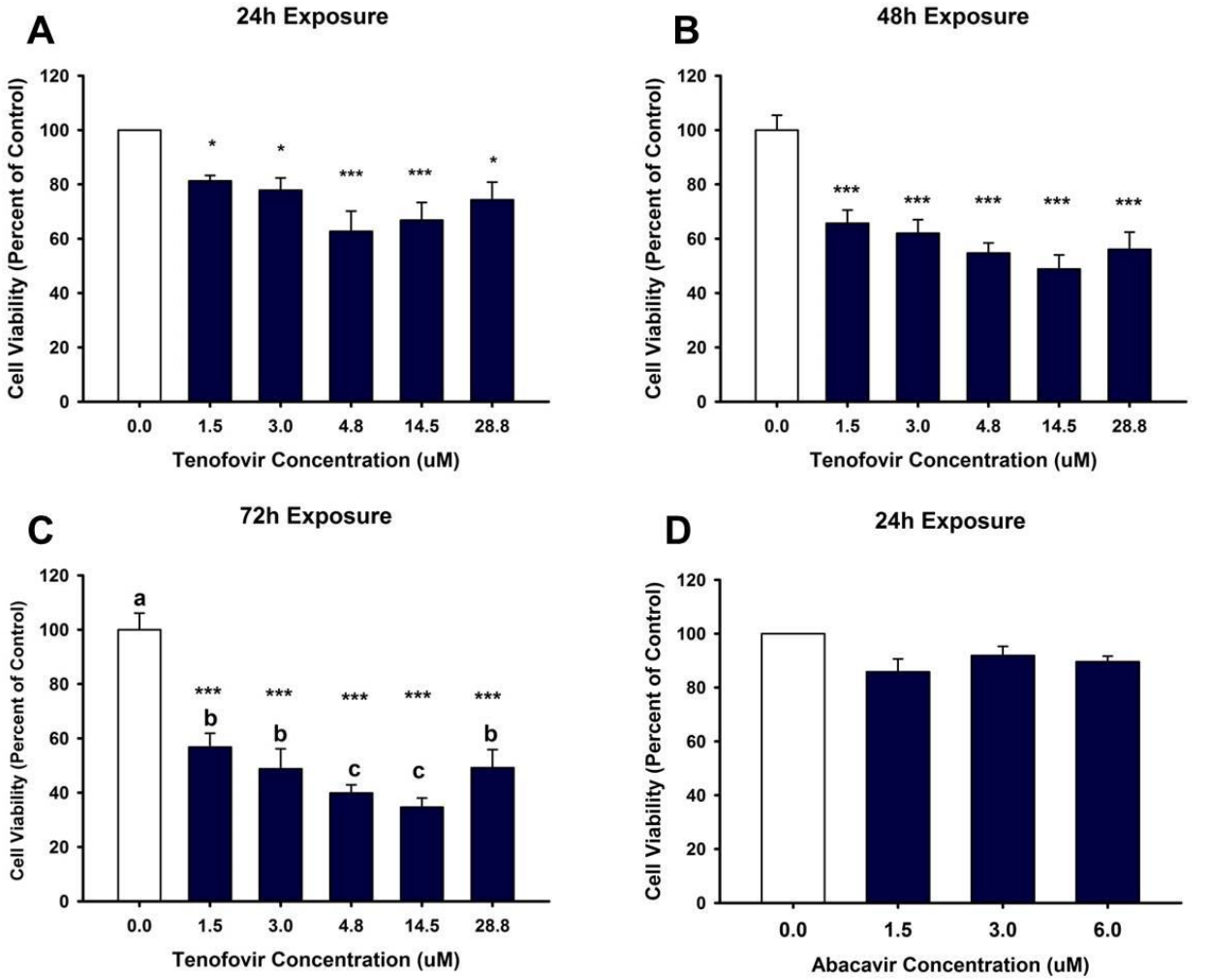

Figure 4. Tenofovir cytotoxic effects on cell viability in HK-2 cells using MTT. Tenofovir diminished cell viability at 24 (A), 48 (B) and 72 h (C). Statistical difference from 0 μM tenofovir depicted in (A and B) by asterisks (* $p < 0.05$, *** $p < 0.001$). Different superscript letters (C) indicate groups that are different from each other ($p < 0.05$); (D) depicts cell viability following exposure for 24 h to another antiviral agent, abacavir. Abacavir did not alter viability at the concentrations tested. Each bar represents Mean ± SEM for three independent experiments run with two biological replicates.

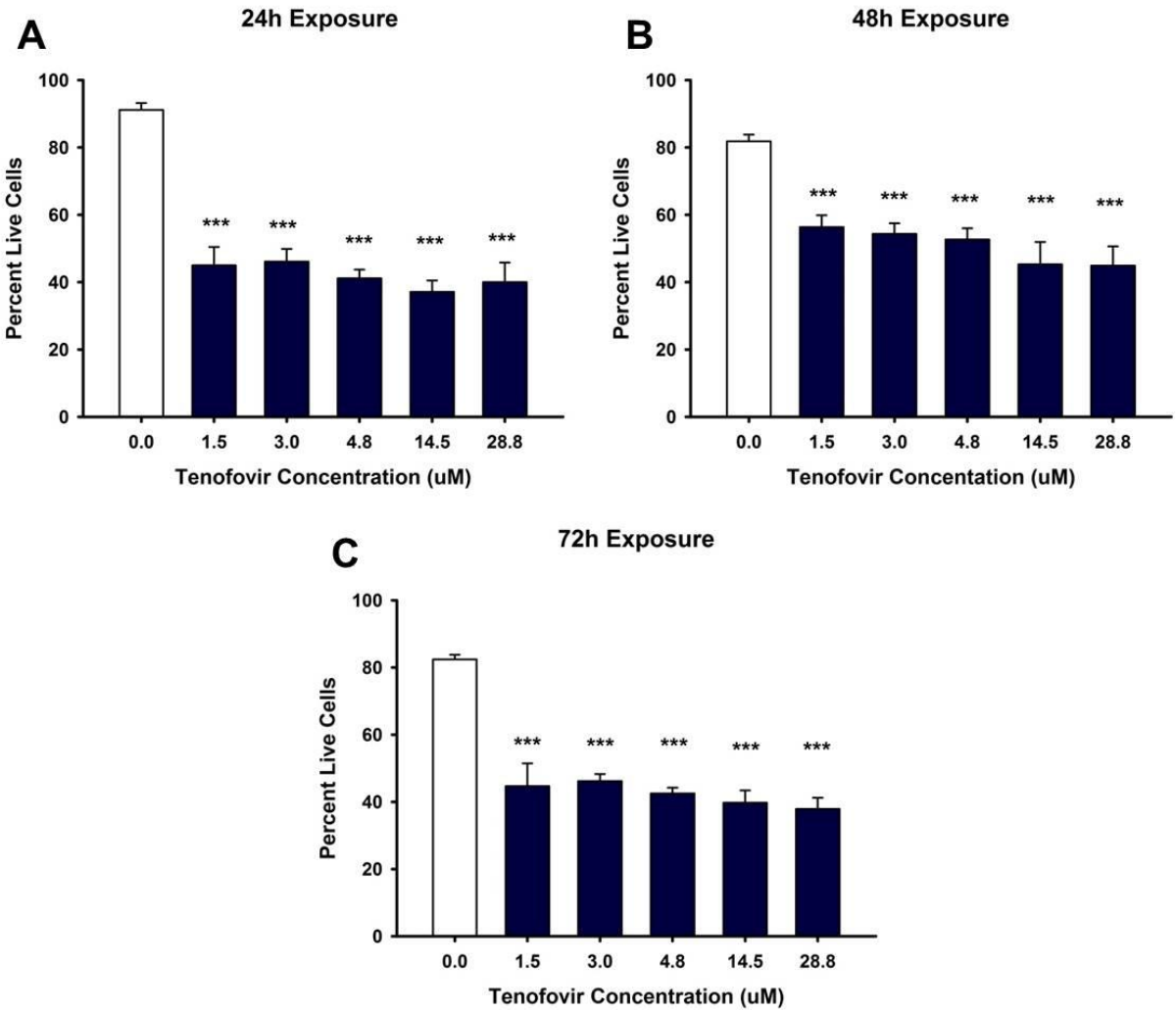

Figure 5. Tenofovir cytotoxic effects on cell viability in HK-2 cells using Trypan Blue Exclusion. Tenofovir diminished cell viability at 24 (A), 48 (B) and 72 h (C). Statistical difference from 0 μM tenofovir is denoted by asterisks (*** $p < 0.001$). Each bar represents Mean ± SEM for three independent experiments run with two biological replicates.

TFV Effects on Mitochondrial Function

Our hypothesis was that TFV impairs mitochondrial function resulting in decreased ATP levels. ATP levels were unchanged compared to vehicle by TFV after 24 or 48 h incubation (Figure 6). ATP levels were diminished by all concentrations of TFV at 72 h when compared to vehicle ($p < 0.001$) (Figure 6). These results suggest that multiple mechanisms of cytotoxicity

may be induced by TFV since the reduction in ATP levels was not apparent prior to a reduction in cell viability.

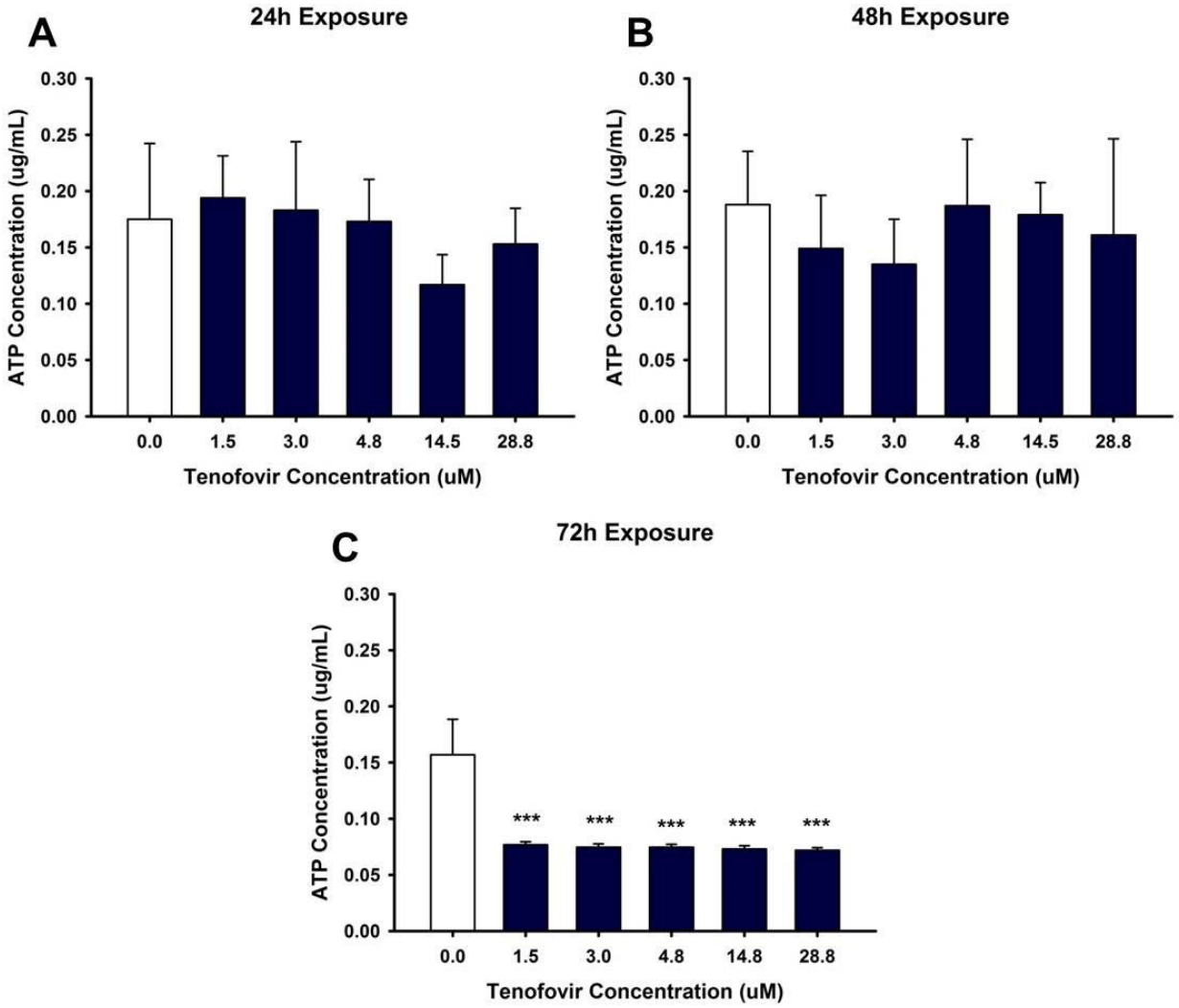

Figure 6. Tenofovir effects on ATP levels in HK-2 cells. ATP levels were measured following 24 (A), 48 (B) and 72 h (C) exposure to tenofovir. ATP was measured using an ATP Assay kit (Biovision) and simultaneously run ATP standards. Statistical difference from 0 μM tenofovir denoted by asterisks (*** $p < 0.001$). ATP levels at 24 and 48 h represent Mean ± SEM for two independent experiments run with three biological replicates. ATP levels at 72 h represent Mean ± SEM for four independent experiments run with three biological replicates.

TFV Effects on Oxidative Stress

TFV increased oxidative stress as shown by Oxyblot analysis at 72 h relative to control in groups treated with 14.5 and 28.8 μM TFV ($p < 0.001$, Figure 7). Protein carbonylation was not

increased relative to control at 48 h or at lower TFV concentrations at 72 h. 4-HNE adduct formation was increased following 72 h exposure for treatment groups of 3.0 to 28.8 μM TFV (Figure 8). TNFα secretion into the media was increased at 72 h exposure to TFV compared to vehicle ($p < 0.001$). TNFα expression in cell lysate was decreased at 48–72 h ($p < 0.05$) as shown by Western blot (Figure 9). These results suggest that TNFα is released into the media as TFV cytotoxicity occurs and that TFV does not increase TNFα protein expression. TNFα expression in the TFV treated cells was similar to vehicle control at 24 h when cell viability was diminished. These results suggest that it is unlikely that TNFα is the primary event in the mechanism of TFV cytotoxicity but rather occurs as a result of initial toxicity. The subcellular generation of oxidative stress may be due to mitochondrial damage, as ATP levels were also diminished by TFV. It is still not clear if TFV directly damages the mitochondria, leading to oxidative stress, or if TFV induces reactive oxygen species that subsequently damage mitochondria; additional studies are needed to further explore the mechanism of cytotoxicity.

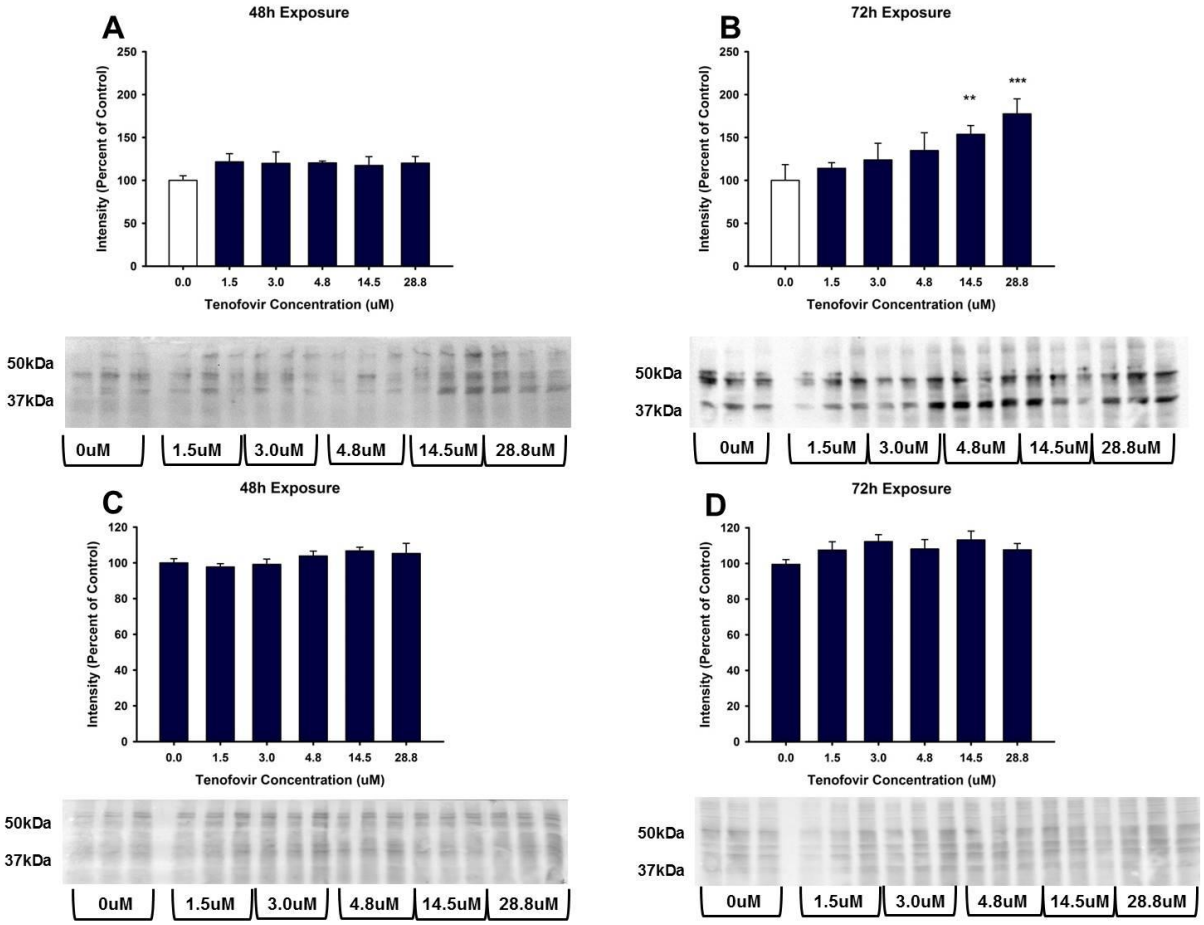

Figure 7. Tenofovir treatment for 72 h increased protein carbonylation in HK-2 cells. Protein carbonylation was measured in HK-2 cell lysate following 48 (A) and 72 h (B) exposure to TFV. Representative gel and cumulative densitometry included in each panel. Representative blot with MemCode Reversible staining for equal 25 µg protein loading and cumulative protein densitometry depicted for 48 (C) and 72 h (D) exposure. Asterisks (** $p < 0.01$, *** $p < 0.001$) indicate statistical difference from the vehicle control group. Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

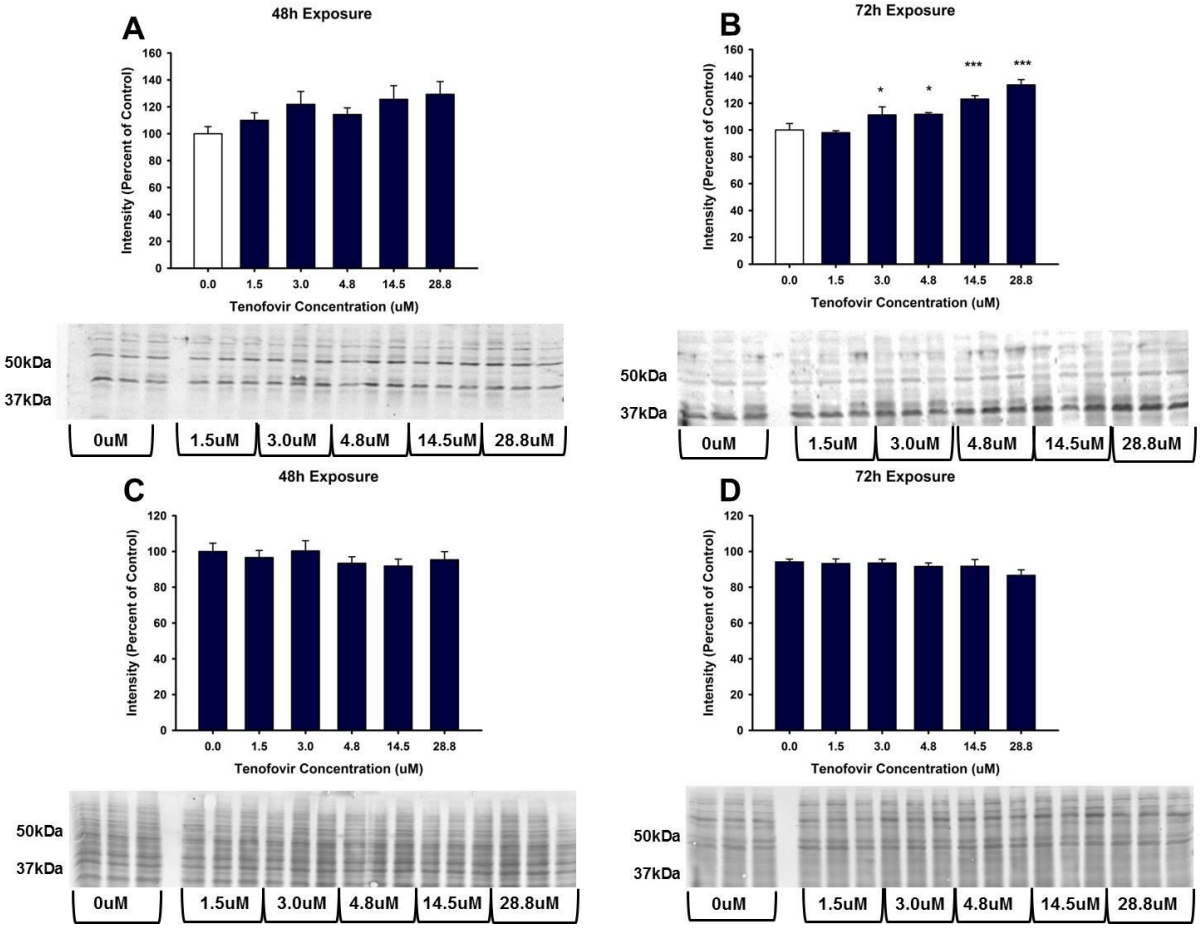

Figure 8. Tenofovir effects on 4-HNE adduct formation in HK-2 cells. 4-HNE adduct formation was measured in HK-2 cell lysate following 48 h (A) and 72 h (B) exposure to TFV. Representative gels and cumulative densitometry included in each panel. Representative blot with MemCode Reversible staining for equal 40 μg protein loading and cumulative protein densitometry depicted for 48 h (C) and 72 h (D) exposure. Asterisks (* $p < 0.05$, *** $p < 0.001$) indicate statistical difference from the vehicle control group. Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

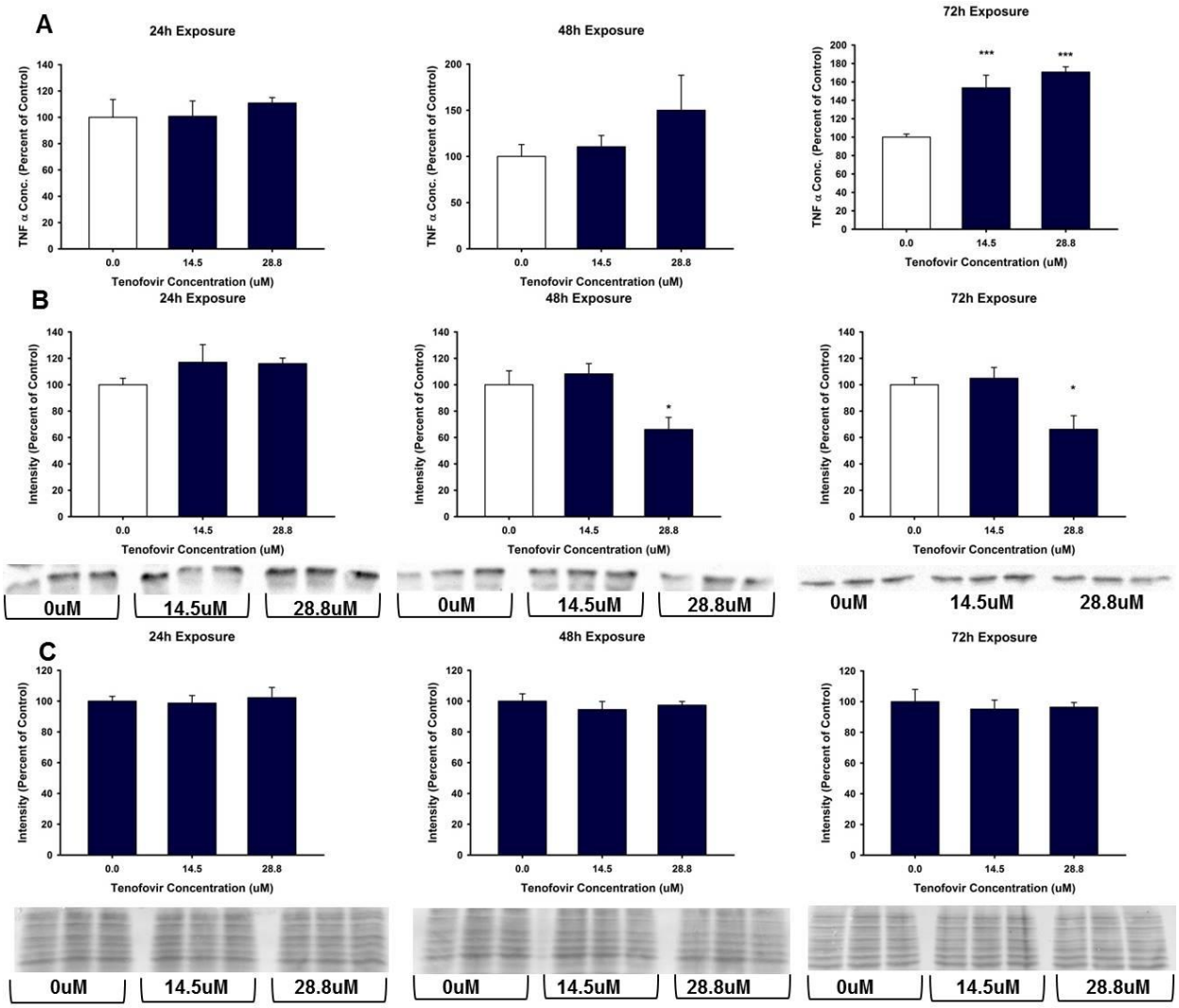

Figure 9. TNFα expression in HK-2 cell lysate and media following tenofovir exposure. (A) TNFα release into media expressed as a percent of control following 24, 48, and 72 h TFV exposure. TNFα in media was increased following a 72 h exposure for all concentrations relative to control (*** $p < 0.001$). (Panel B) provides representative blot and cumulative densitometry for TNFα expression in cell lysate following 24, 48, 72 h TFV exposure. Statistical difference from 0 μM tenofovir depicted by asterisk (* $p < 0.05$); (C) depicts cumulative densitometry and representative blots for 50 μg protein loading of cell lysates visualized with MemCode Reversible Protein staining. Each bar represents Mean ± SEM for two independent experiments run with three biological replicates.

TFV Effects on Apoptosis Initiation

TFV increased cleaved caspase 3 ($p < 0.0.05$) and cleaved caspase 9 relative to control for all treatments at 72 h exposure (Figure 10). Caspase 8 expression was measured after 48 and

72 h exposure to TVF. TFV did not increase caspase 8 cleavage relative to control at either time point. Cleaved caspase 8 was minimal in all groups and was decreased by higher TFV treatment relative to vehicle control. These results suggest that the increase in TNFα in the media at 72 h was not sufficient to stimulate caspase 8 cleavage. These data show that TFV induces apoptosis in HK-2 cells, and that apoptosis is induced via mitochondrial damage.

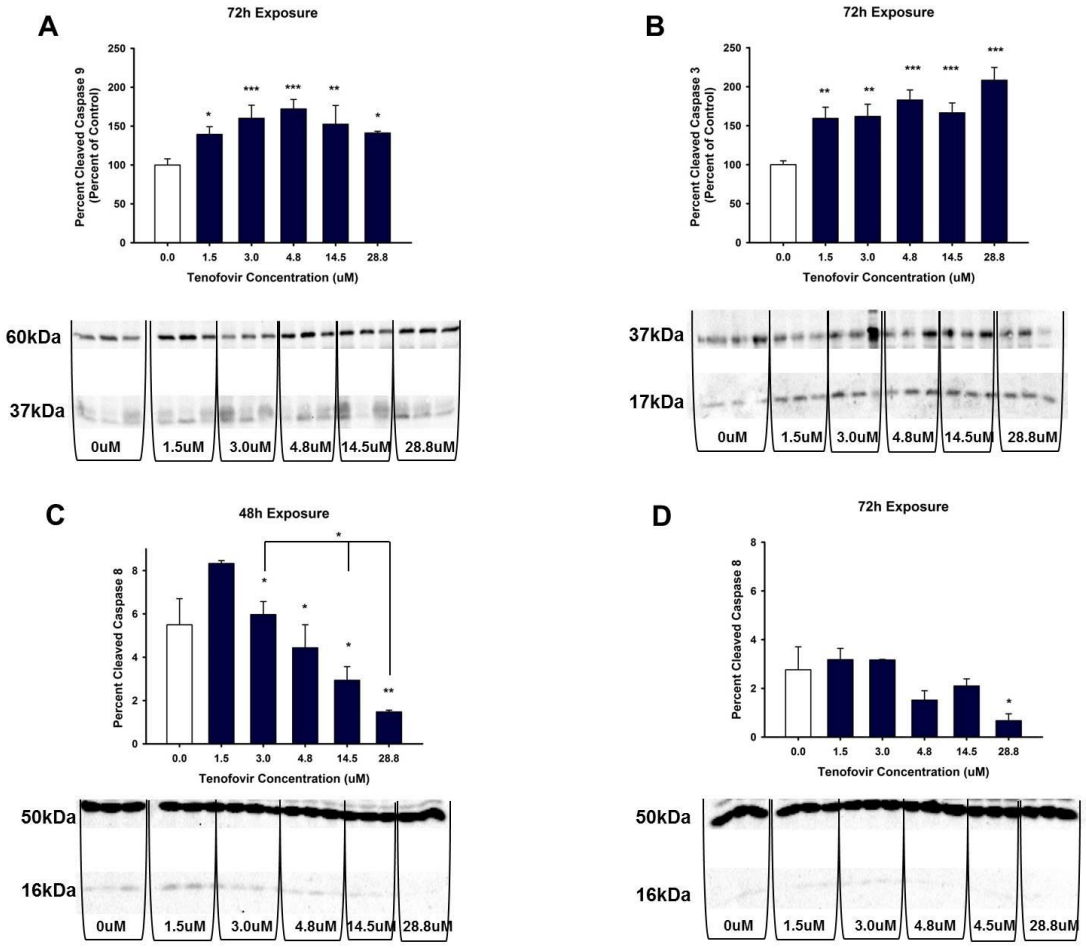

Figure 10. *Cont'd.*

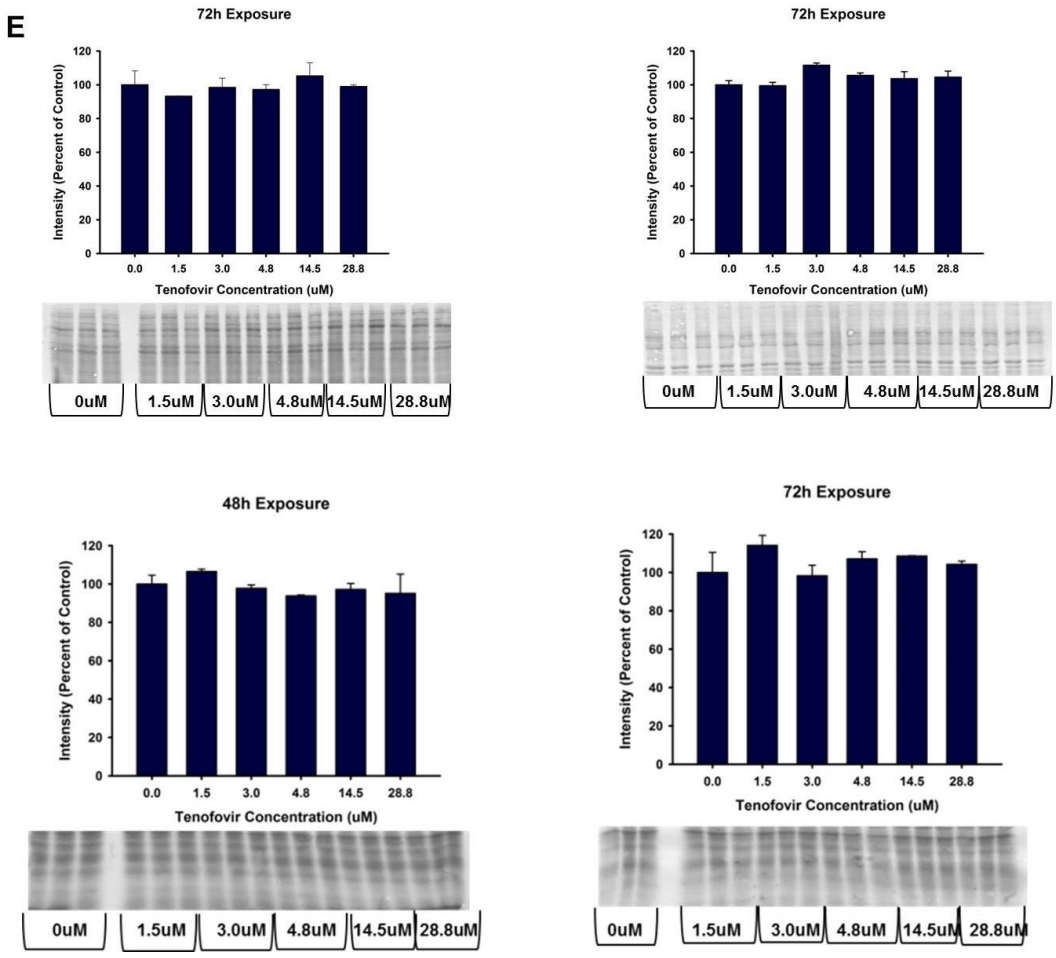

Figure 10. Tenofovir effects on the expression of cleaved caspase 3, 8 and 9 in HK-2 cells. Representative blot and cumulative densitometry for cleaved to total caspase 9 (A), caspase 3 (B) and caspase 8 (D) protein expression following 72 h. Asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) indicate statistical difference from the vehicle control group; (C) Depicts 48 h caspase 8 cleavage. (E) representative blots of MemCode reversible staining for 40 µg protein loading and cumulative densitometry; blots and densitometry depicted (from left to right) are for (A) (72 h Caspase 3), (B) (72 h Caspase 9), (C) (48 h Caspase 8), and (D) (72 h Caspase 8). Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

Ascorbic Acid Protection of TFV Cytotoxicity

Oxidative stress was increased by TFV resulting in increased protein carbonylation (Figure 4) and increased 4-HNE protein adduction (Figure 5) suggesting that a rise in reactive oxygen species occurs with TFV cytotoxicity. Pretreatment with ascorbic acid reduced TFV

toxicity in HK-2 cells (Figure 11) as cell viability was higher in cells exposed to TFV in the presence of ascorbic acid compared to TFV alone. These findings suggest that an antioxidant can reduce TFV cytotoxicity in HK-2 cells.

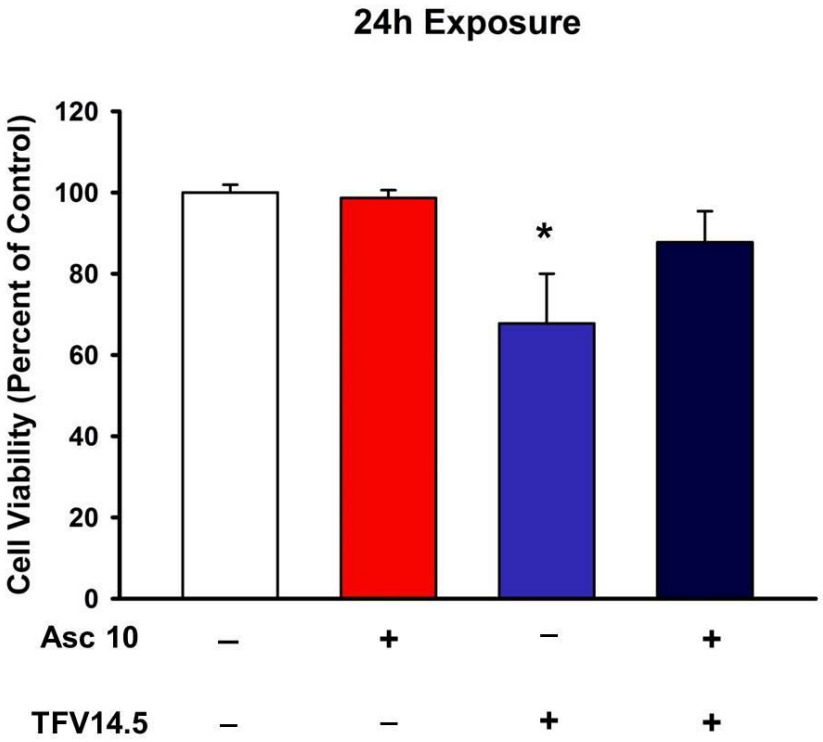

Figure 11. **Effects of ascorbic acid on tenofovir cytotoxicity**. Ascorbic acid did not alter cell viability relative to vehicle control. Ascorbic acid protected HK-2 cells from tenofovir cytotoxicity as assessed by MTT viability. An asterisk (* $p < 0.05$) indicates statistical difference from all other groups. Values represent Mean ± SEM for three independent experiments run with three biological replicates.

Discussion

TFV is a very effective antiviral nucleotide reverse transcriptase inhibitor prescribed worldwide in the treatment of HIV and Hepatitis B. Patients treated with TFV have an increased incidence of renal impairment (Fux et al., 2007; Verhelst et al., 2002). Renal TFV toxicity in humans is characterized by Fanconi Syndrome, and depending on the dose, development of irreversible impaired renal function. There is a positive correlation between increased TFV

plasma concentration, renal toxicity and the duration that patients were treated with TFV Ezinga et al., 2014; Quesada et al., 2015) because treatment with TFV is chronic, examination of the mechanism of renal toxicity is clinically relevant. Understanding the mechanism is essential for the development of methods to mitigate TFV renal impairment.

Mechanistic studies require a model that is consistent with what occurs in humans. The selection of suitable models to study TFV cytotoxicity has been problematic as most in vivo models require subchronic treatment of animals. TFV toxicity in rodents such as rats has required eight weeks of daily treatment in order to develop nephrotoxicity (Lebrecht et al., 2009). In this study, rats treated for eight weeks exhibited diminished renal function and proximal tubular damage along with enlarged mitochondria. Another study showed that treatment for five weeks with TFV induced oxidative stress in Wistar rats dosed with very high levels of TFV (Abraham et al., 2013).

An in vitro model was first reported by Wang and Flint (2013) that developed TFV cytotoxicity using primary human kidney cells (Wang & Flint, 2013). Primary human kidney cells required culturing for 19 days with 200 μM TFV to induce cytotoxicity, which was much longer and at concentrations higher than pharmacological levels. The need for higher concentrations of TFV to induce toxicity in primary proximal tubule cell lines may be due to differences in OAT1 and OAT3 expression. Endogenous expression of OAT1 and OAT3 in primary human proximal tubule cells has been shown to vary greatly from sample to sample (Lash, Putt, & Cai, 2006) and TFV-induced cytotoxicity is dependent on OAT1 and OAT3 expression (Kohler et al., 2011; Nieskens et al., 2016). Our HK-2 cell model is more sensitive than human primary proximal tubule cells as we have shown induction of cytotoxicity in 24 h compared to 22 days and at a concentration almost ten times lower than reported for human

primary proximal tubular epithelial cells (Vidal et al., 2006). In addition to inducing cytotoxicity at 24 h, our HK-2 cell model has an IC_{50} of 9.2 μM TFV at 48 h, and an IC_{50} of 2.77 μM TFV at 72 h. Other cell types such as HEK293 cells have been used to study tenofovir cytotoxicity, but appear less sensitive than our HK-2 model. One study showed that TFV-induced cytotoxicity within 48 h in HEK293 cells transfected with OAT1; however, the IC_{50} was reported to be 316 μM (Zhang et al., 2015).

Our study is the first to show that HK-2 cells can be used to evaluate TFV cytotoxicity. TFV renal cytotoxicity was evident within 24 h at clinically relevant concentrations. Primary human kidney cells required a concentration of 200 μM, which is higher than reported maximal clinical steady state plasma concentration (Wang & Flint, 2013). In comparison, in our study, viability was diminished in HK-2 cells with TFV concentrations ranging from 1.5 to 28.8 μM. The concentrations used in our current study are clinically relevant as plasma TFV concentrations have been reported to be 2.2 μM in HIV-1 infected patients (Barditch-Crovo et al., 2001; Zhang et al., 2015). Additionally, TFV plasma concentrations are higher in HIV-1 patients and non-infected individuals with existing renal impairment; patients with renal impairment also experience a longer duration of TFV exposure, as shown by a 15-fold increase in $AUC_{0-24\,h}$ (Custodio et al. 2016; Ezinga et al. 2014; Sentenac, Fernandez, Thuillier, Lechat, and Aymard, 2003). Therefore, HK-2 cells provide a model that can be used to examine the mechanism of toxicity without the compounding physiological parameters influencing the response of the kidney to a toxicant. Our studies further showed that HK-2 cells can differentiate the toxicity of different antiviral agents as abacavir, an agent that is considered less toxic to the kidney which was not toxic in our system (Figure 4D).

It is probable that TFV renal cytotoxicity involves multiple mechanisms and that these mechanisms are not yet fully understood. TFV treatment of HK-2 cells decreased ATP levels when compared to control at 72 h exposure (Figure 6). Diminished ATP levels can be mediated by numerous pathways, including alterations in mitochondrial DNA stability, impaired mitochondrial function, or increased oxidative stress.

Mitochondrial function relies on many factors, including the stability and integrity of mitochondrial DNA (mtDNA). Decreased mtDNA could result in a decline in cellular ATP levels. There are conflicting reports regarding the effects of TFV on mtDNA. The differences may be due to species variation and differences in experimental models. For example, TFV diminished mtDNA in HIV-1 transgenic mice (Kohler et al., 2009) while a 19 day exposure of human primary renal proximal tubule cells to 2 and 200 μM TFV showed no change in mtDNA compared to control (Paolicchi et al., 2003; Wang & Flint, 2013). However, effects of TFV on mtDNA may vary between renal and nonrenal cells (Wang & Flint, 2013). Additionally, mtDNA was diminished in human primary proximal tubule cells by another nucleoside reverse transcriptase inhibitor, adefovir, within nine days of incubation with TDF (Wang & Flint, 2013), indicating that TFV may be a weaker inhibitor of DNA polymerase gamma.

TFV increased expression at 72 h of cleaved caspase 3 and 9 in HK-2 cells (Figure 10). Apoptosis can be induced via many pathways, including extracellular signals and mitochondrial dysfunction. Each pathway activates different initiator caspase proteins; for example, cleavage of caspase 9 occurs when mitochondrial damage initiates the intrinsic apoptotic pathway. Initiator caspases then interact with other cellular proteins, forming an apoptosome that cleaves executioner caspases such as caspase 3 and activates the induction of cell death. The increased expression of cleaved caspase 3 and 9 in HK-2 cells treated with TFV indicated that apoptosis

was occurring, and that mitochondrial dysfunction may be a major contributing factor to loss of cell viability (Figure 10).

The renal proximal tubular epithelial cell has a high requirement for ATP generation to maintain normal cellular processes such as active transport. The decline in cellular ATP levels caused by TFV would impair normal proximal tubular epithelial cell function. TFV increased oxidative stress, as protein carbonylation was elevated at 72 h exposure to 14.5 and 28.8 μM TFV (Figure 7). The HK-2 cells were more sensitive to TFV and displayed increased oxidative stress at clinically relevant concentrations as compared to previously examined models. A five week treatment of rats with 12 times higher dose than what is clinically used reported a 25% increase in renal protein carbonylation (Abraham et al., 2013); the HK-2 cells demonstrated a much greater increase in protein carbonylation within a shorter time period than reported in vivo treatment of rats (Figure 7), suggesting that HK-2 cells may be a more sensitive model to examine TFV cytotoxicity.

TNFα is a proinflammaotry cytokine which is expressed in proximal tubular epithelial cells. TNFα can be secreted from cells to induce an inflammatory response as a protective mechanism during initial exposure to toxicants (Gu, Xing, Xu, & Lu, 2016). TNFα expression can induce reactive oxygen species generation via mitochondria and through activation of NADPH oxidase (Morgan, 2010). Mitochondrial damage and generation of reactive oxygen species can stimulate the release of various cytokines including TNFα (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014). These changes in TNFα expression and secretion can modulate various parts of the cellular antioxidant system and directly increase the production of mitochondrial reactive oxygen species through damage to the electron transport chain. In experiments involving exposing HK-2 cells to toxicants and/or ischemic injury, TNFα levels rose along with many

other markers of toxicity, including increases in oxidative stress via dysfunction of cellular antioxidant systems, activation of an inflammatory response and associated proteins, and induction of programmed cell death (Gong, Ivanov, Davidson, & Hei, 2015; Zager, Johnson, & Geballe, 2007; Zhang et al., 2013). We examined TNFα as a potential indicator of oxidative stress. Our study showed that TNFα was released into the media at 72 h, while tissue showed a decline in expression at 48 and 72 h (Figure 9). Because TNFα expression was unaltered at 24 h (Figure 9) it is unlikely that activation of inflammatory response is the cause of the oxidative stress observed. The decline in TNFα in the cells is consistent with loss from cells into the media; TNFα released into the media may contribute to greater oxidative stress. Further studies need to explore the cellular mechanism for increased oxidative stress, TNFα secretion in HK-2 cells, specifically, evaluation of mitochondrial alterations that may mediate either a decline in mitochondrial function or increased reactive oxygen species.

Methods

Chemicals and Reagents

TFV was purchased from Cayman Chemicals (Item No. 13874, Ann Arbor, MI, USA) and was used for all studies. The vehicle for cell treatments was phosphate buffered saline (Invitrogen, Carlsbad, CA, USA, Item No. 14175095). MTT and other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburg, PA, USA). Antibodies and kits were purchased as indicated in the sections below.

Cell Lines and Tenofovir (TFV) Treatment

Human immortalized epithelial HK-2 cells were purchased from the American Type Culture Collection (ATCC) and were cultured according to ATCC guidelines. Briefly, cells were grown in a keratinocyte-free media with 50 μg/mL bovine pituitary extract and 5 ng/mL

recombinant epithelial growth factor from Invitrogen (Carlsbad, CA, USA, Item No. 17005-042). Cells were grown in a warm humidified incubator with constant settings of 37 °C and 5% CO_2. HK-2 cells were plated into six-well tissue culture plates (750,000 cells/mL) (Corning, Sigma Aldrich Item No. CLS3516) and allowed to grow for 48 h. Media was replaced and cells were treated with a final concentration of 0, 1.5, 3.0, 4.75, 14.5, or 28.8 μM TFV for 24, 48, or 72 h. The vehicle was an equal volume of phosphate buffered saline (PBS). Abacavir was prepared in sterile water and cells were treated for 24 h with 0, 1.5, 3 or 6 μM of abacavir to evaluate renal sensitivity to an agent recognized to be less nephrotoxic. Following the treatment period, cells were collected with Trypsin-EDTA (0.25%) (Invitrogen, Item No. 25200072) for sample analysis.

Cell Viability

Cells were plated into 48-well tissue culture plates (39,000 cells/mL) (Cyto One, USA Scientific, Ocala, FL, USA, Item No. CC7682-7548) and allowed to grow for 48 h followed by treatment with vehicle or TFV. Following the treatment period, cell viability was assessed using the MTT assay (Humphrey, Cole, Pendergrass, & Kiningham, 2005). The MTT assay relies on the conversion of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, Item No. M5655-5X1G) to formazan by NAD(P)H-dependent oxidoreductases.

To ensure that results of the MTT Assay were not due to mitochondrial damage rather than cell death, a trypan blue exclusion test was run based on a previously published protocol. In brief, an aliquot of collected cells are diluted 1:10 with 40% *w*/*v* Trypan Blue solution (Sigma Aldrich, Item No. T6146). The suspension was mixed via pipetting and allowed to sit for

approximately 2 min. A 10 μL aliquot of suspension was transferred to a hemocytometer and total cells, live cells, and dead cells were manually counted.

ATP Measurement

ATP levels were assessed using the ADP/ATP Ratio Bioluminescence Assay Kit (Biovision Inc., Milpitas, CA, USA, Item No. K255-200). Briefly, 100 μL of reaction mix was plated into a 96-well plate (Corning, Sigma Aldrich, Item No. 356519) and allowed to establish a baseline during a 2 h room temperature incubation. A 10 μL aliquot of the sample was added to each well and bioluminescence was measured using a luminometer. Immediately following the measurement, 10 μL of diluted ADP-converting enzyme was added and bioluminescence was measured again. Lysate concentration of ATP and ADP was determined using a standard curve.

Western Blot

Western blot analysis was conducted to assess the expression of active caspase 3, 8 and 9 and the formation of 4-hydroxy-2-nonenal (4-HNE) protein adducts. Protein concentration in each sample was determined using the Bradford protein assay (Bradford, 1976). A 40 μg aliquot of each sample was denatured by boiling for five min. Proteins were then separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA, Item No. 1620115); successful transfer and protein loading were verified using MemCode Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, IL, USA, Fisher Scientific, Item No. PI-24580). Membranes were blocked using either a 5% *w*/*v* milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) or a 1% Bovine Serum Albumin (BSA)/TBST solution for 1 h. Membranes were next incubated with continual shaking overnight at 4 ◦C with a rabbit polyclonal antibody for caspase 3 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA, Item No. 9662), caspase 8 (1:400 dilution, Biovision Inc., Item

No. 3020-100), caspase 9 (1:1000 dilution, Cell Signaling Technology, Item No. 9502) or 4-HNE (1:1000 dilution, Cell Bio Labs Inc., San Diego, CA, USA, Item No. STA-035) diluted in 5% milk/TBST or 1% BSA/TBST. The membranes were washed four times with TBST and goat anti-rabbit HRP-linked secondary antibodies were diluted to 1:5000 in 5% milk/TBST and added for 1 h. Membranes were washed again with TBST and then developed using Amersham ECL Western Blotting Detection Agent (GE Healthcare Life Sciences, Marlborough, MA, USA, Item No. RPN2232). A BioRad chemic-doc system was used to capture the gel image and used for densitometry analysis.

Oxidative Stress

In addition to 4-HNE adduction of protein, another indicator of oxidative stress is protein carbonylation, which can produce an aldehyde or ketone side chain on amino acids. Protein carbonylation was analyzed using the Oxyblot Protein Oxidation Detection Kit (EMD Millipore, Billerica, MA, USA, Item No. S7150). Following treatment for 24–72 h with vehicle or TFV, cells were pelleted, rinsed with 500 µL PBS, and centrifuged. The pellet was then disrupted with lysis buffer. Protein content was measured, and a 25 µg aliquot was derivatized as previously described (Terneus, Kiningham, Carpenter, Sullivan, & Valentovic, 2007). Protein carbonyl moieties on amino acids generated by oxidative stress are derivatized in the presence of 2,4-dinitrophenylhydrazine to stable 2,4-dinitrophenylhydrazone groups. The primary antibody recognizes 2,4-dinitrophenylhydrazone groups on proteins and was used at a dilution of 1:150. Protein loading was verified using the MemCode Protein Stain and results were analyzed with BioRad Chemidoc densitometry software (version 4.0.1, Catalog No. 170-9690, BioRad, Hercules, CA, USA). A series of studies examined whether ascorbic acid, an antioxidant, could provide protection from TFV. HK-2 cells were allowed to grow for 48 h followed by a one h

pretreatment with 0 or 10 µM ascorbic acid in sterile water followed by a 24 h incubation with 0 or 14.5 µM TFV. Viability of cells was assessed using the MTT assay. All media and MTT was removed from the cells prior to the addition of DMSO to prevent any interaction of ascorbic acid with formazan.

TNFα in Cell Media and Cell Lysate

Proximal tubular epithelial cells express TNFα. TNFα expression was measured in cell culture media using an ELISA assay kit and in cell lysate by Western blot. Release of TNFα into the cell culture media was measured using an ELISA kit (Abcam, Cambridge, MA, USA, Item No. ab181421) per the manufacturer's instructions. Briefly, 50 µL of collected media and a capture/detector antibody cocktail were added to precoated wells and incubated for 1 h shaking at 400 rpm. Following the immunocapture incubation period, the wells were washed and TMB (TMB, 3,3*t*,5,5*t*-Tetramethylbenzidine) substrate was added, producing a color change based on the amount of bound TNFα, which was then read at 450 nm. TNFα concentration was determined using a standard curve. TNFα expression in TFV treated cell lysate was determined using Western blot as described above. Each lane was loaded with 50 µg of protein; membranes were probed using a rabbit-polyclonal HRP-linked antibody for TNFα diluted to 1:1000 in 5% BSA/TBST (Abcam, Item No. ab66579). TNFα was normalized to protein and compared relative to control.

Statistical Analysis

Values represent Mean ± SEM with 2–4 independent experiments conducted with 2–4 biological replicates. Differences between groups were determined with a one-way ANOVA followed by a Holm–Sidak post-hoc test with $p < 0.05$ (SigmaStat, SSPS, Systat Software, Chicago, IL, USA, Item Sigma Plot SPW11).

Conclusions

HK-2 cells are a sensitive mode for examining TFV renal cytotoxicity. The concentrations of TFV used in these studies are clinically relevant. Intracellular concentrations of TFV can be higher than plasma levels as proximal tubule cells utilize active transport. TFV reduced cell viability within 24 h. This is the first report to characterize TFV toxicity using HK-2 cells. Our results show that TFV causes mitochondrial damage resulting in diminished cellular ATP levels and cleavage of caspase 3 and 9. Further studies are needed to explore the specific cellular mechanism of mitochondrial toxicity and oxidative stress. The study also showed that ascorbic acid, an antioxidant, could reduce TFV cytotoxicity.

Acknowledgments

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Author Contributions

Rachel A. Murphy; Monica A. Valentovic designed the studies. Rachel A. Murphy treated all cells. Reagan M. Stafford; Megann A. Boone conducted the MTT and assisted Rachel A. Murphy with Western blot experiments. Brooke A. Petrasovits; Rachel A. Murphy conducted the ATP experiments, measurements and data analysis. Monica A. Valentovic; Rachel A. Murphy wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

CHAPTER 4

ANTIVIRAL AGENT TENOFOVIR CAUSES MITOCHONDRIAL DAMAGE AND OXIDATIVE STRESS IN HK-2 CELLS

A manuscript submitted to *Toxicology.*

Rachel A. Murphy [1],Daniel Rodriguez de Anda[2], Kathleen Brown [1], and Monica A. Valentovic [1,*]

[1]Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755

[2] University of Texas Rio Grande Valley, Brownsville, TX, 78539

[1]Correspondence should be addressed to: Department of Biomedical Sciences; 1 John Marshall Drive, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755. Phone (307-696-7332) Fax (304-696-7391) Email: valentov@marshall.edu

Abstract

Tenofovir (TFV) is an antiviral drug approved in the United States for treating Human Immunodeficiency Virus (HIV) and Hepatitis B. TFV is administered orally as the prodrug tenofovir disoproxil fumarate (TDF) which then is deesterified to the active drug TFV. TFV induces nephrotoxicity characterized by decreased glomerular filtration rate (GFR), renal failure, and Fanconi Syndrome. The mechanism of this toxicity remains largely unknown due to limited experimental models. In a previously published study, we determined that TFV reduces cell viability, causes oxidative stress, and induces apoptosis in the human renal proximal tubular cell line (HK-2). This study further investigated the cellular mechanism of cytotoxicity using HK-2 cells. HK-2 cells were grown for 48 h followed by 24 to 72 h exposure to 0-28.8 μM TFV or vehicle, phosphate buffered saline (PBS). A parallel study was run investigating the effects of antioxidant pretreatment on TFV induced toxicity. HK-2 cells were treated with N-acetyl-L-cysteine (NAC), ascorbic acid, or resveratrol followed by 24 h exposure to 3 or 14.5 μM TFV, or PBS. TFV reduced cell viability in 24-72 h as shown by MTT. Lactate dehydrogenase (LDH) was released into the media following 24 h exposure to 14.5 and 28.8 μM TFV. TFV increased the oxidative stress biomarker of protein carbonylation in the mitochondrial subcellular fraction relative to control following TFV exposure. Manganese superoxide dismutase (MnSOD) and cytochrome c were released into the cytosolic subcellular fraction relative to control following TFV exposure. Mitochondrial function was assessed using Seahorse XFp assays; TFV reduced basal and uncoupled cellular respiration when compared to vehicle at 24-72 h, but did not alter spare respiratory capacity. Pretreatment with NAC, ascorbic acid, or resveratrol reduced TFV toxicity in HK-2 cells. These studies suggest that TFV induces necrotic cell death at 24-48 h, causes oxidative damage to the mitochondria leading to increased membrane permeability, and

alters mitochondrial function. Damage from oxidative stress may be an important factor in the mechanism of TFV induced cytotoxicity, as toxicity was prevented using an antioxidant pretreatment.

Introduction

Tenofovir disoproxil fumarate (TDF) is an antiretroviral agent that is approved in the United States to treat Human Immunodeficiency Virus (HIV) and chronic Hepatitis B (HBV). Tenofovir (TFV) is a nucleotide analogue of adenosine 5'-monophosphate that competes for incorporation into the growing viral strand during chain elongation and blocks reverse transcriptase, leading to chain termination (Balzarini et al., 1993; Robbins et al., 1998). To improve its oral bioavailability, it is administered as the prodrug TDF, which is deesterified intracellularly to active TFV (Choi et al., 2008; Shaw et al., 1997). Its long half-life provides for once a day dosing, which is beneficial for compliance (Barditch-Crovo et al., 2001; Deeks et al., 1998; Durand-Gasselin et al., 2009). TFV is also effective in patients who have resistant HIV-1, making it an essential drug in the treatment of HIV according to the World Health Organization (Miller et al., 2001; Squires et al., 2003).

While TFV has many positive attributes, renal toxicity remains an adverse effect associated with clinical use. TFV can induce increases in serum creatinine levels and higher plasma vitamin D binding, decreases in glomerular filtration rate (GFR) and free calcitriol, Fanconi Syndrome, and renal failure (Del Palacio et al., 2012; Hall et al., 2011; Havens et al., 2013). Fanconi syndrome is a disorder of renal tubular function that results in excess excretion of potassium, phosphate salts, protein, urate, and glucose. Clinical studies indicate that the mitochondria of the proximal tubules are the target of TFV induced toxicity as shown by enlarged and malformed mitochondria in electron microscopy cross sections (Cote et al., 2006;

Hall et al., 2011; Herlitz et al., 2010; Woodward et al., 2009). Proximal tubular mitochondrial toxicity has also been described in mice and rats treated with TFV as shown by disruptions in mitochondrial cristae and a reduction in mtDNA levels (Kohler et al., 2009; Lebrecht et al., 2009).

Though TFV induced renal toxicity has been documented, very little is known about the mechanism; this knowledge gap may be due in part to limited experimental models. In vitro studies have used primary renal proximal tubule cells treated for up to 22 days and in vivo mice and rat models were treated with oral gavage for 5-8 weeks (Kohler et al., 2009; Lebrecht et al., 2009; Vidal et al., 2006; Wang & Flint, 2013). The duration of these experiments can hamper mechanistic studies, and the added stress of daily oral gavage can complicate mechanistic studies using animal models. Cell lines with better responsiveness to TFV could circumvent these issues.

In our previous study, our laboratory reported that Human Kidney-2 cells (HK-2) were a suitable model to study TFV induced renal cytotoxicity. HK-2 cells retain similar activity and biochemical properties to in vivo cells and are commonly used in renal mechanistic studies (Gunness et al., 2010; Miyamoto et al., 2012; Paolicchi et al., 2003; Ryan et al., 1994). Using clinically relevant concentrations, we determined that TFV causes oxidative stress, reduced cellular ATP levels, and induced apoptosis within 72 h (Murphy, Stafford, Petrasovits, Boone, & Valentovic, 2017). The purpose of this study was to further establish the mechanism of TFV induced renal cytotoxicity as well as to begin preliminary tests on toxicity prevention.

In this manuscript, we demonstrate that TFV causes lactate dehydrogenase (LDH) leakage and reduces mitochondrial function via oxidative stress and loss of membrane integrity.

Additionally, our studies showed that TFV cytotoxicity may be prevented or mitigated using antioxidant pretreatment.

Methods

Chemicals and Reagents

TFV was purchased from Cayman Chemicals (Item No. 13874, Ann Arbor, MI) and was used for all studies. The cell treatment vehicle was phosphate buffered saline (Invitrogen, Carlsbad, CA, Item No. 14175095). Resveratrol, N-acetyl-L-cysteine (NAC) and other chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburg, PA). Antibodies and kits were purchased as indicated in the sections below.

Cell Lines and Tenofovir Treatment

Human immortalized epithelial HK-2 cells were purchased from the American Type Culture Collection (ATCC) and were cultured according to ATCC guidelines. In brief, cells were grown in a keratinocyte-free media with 50 µg/ml bovine pituitary extract and 5ng/ml recombinant epithelial growth factor from Invitrogen (Carlsbad, CA, Item No. 17005-04) in a warm humidified incubator with constant settings of 37^{o}C and 5% CO_2. HK-2 cells were plated into six-well tissue culture plates (Corning, Sigma Aldrich Item No. CLS3516), 75 ml tissue culture flasks (USA Scientific, Item No. CC7682-7506) or T75 tissue culture flasks (USA Scientific, Item No. CC7682-4175) at a concentration of 750,000 cells/ml and allowed to grow for 48 h. Media was replaced and cells were treated with a final concentration of 0, 14.5, or 28.8 uM TFV for 24, 48, or 72 h. The vehicle was an equal volume of phosphate buffered saline (PBS). Following each treatment period, cells were collected with Trypsin-EDTA (0.25%) (Invitrogen, Item No. 25200072) for sample analysis.

Cell Viability

Cells were plated into 48-well tissue culture plates (39, 000 cells/ml) and allowed to grow for 48 h followed by treatment with vehicle or TFV. Following the treatment period, cell viability was assessed using the MTT assay (Humphrey et al., 2005). This assay relies on the conversion of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by NAD(P)H-dependent oxidoreductases.

When cells lose membrane integrity as necrosis develops following exposure to a toxicant, LDH is released from the cell into the extracellular space. This leakage can be used as a quantification of cell death via necrosis. Western blot analysis was used to evaluate LDH leakage from HK-2 cells into the media. Following the 24-48 h treatment period with vehicle or TFV, cells were pelleted, rinsed with 500 µl PBS, and centrifuged. The pellet was then disrupted with lysis buffer. Media samples were centrifuged at 2,000xg for ten min to remove any cell debris. Protein content was measured, and each lane was loaded with 10 µg of protein. Membranes were probed using a rabbit polyclonal antibody for LDH (Abcam, Item No. ab47010; 1:500 dilution in 5% BSA/TBST). Results from three independent experiments were analyzed with BioRad Chemidoc densitometry software (version 4.0.1).

Mitochondrial Isolation

Following collection with Trypsin-EDTA, mitochondria were isolated using a Mitochondrial Isolation Kit for Cultured Cells (Thermo Scientific, Item No. 89874) and differential centrifugation. Mitochondria were isolated according to the manufacturer's directions, with the exception of added protease inhibitor. Briefly, cells were centrifuged at 850xg for ten min and resuspended in 800 µL of Reagent A with added protease inhibitor. Samples were vortexed for five sec at maximum speed and then incubated on ice for two min.

Following addition of 10 μL of Reagent B, samples were vortexed for five sec at maximum speed and incubated on ice for ten minutes, vortexing every minute. Next, 800 μL of Reagent C with added protease inhibitor was added and the samples were centrifuged at 750xg for ten min. The supernatant was transferred to new sample tubes and centrifuged at 12,000xg for 15 min at 4^{o}C. The supernatant (cytosolic fraction) was transferred to a new tube and the pellet (mitochondrial fraction) was resuspended in 75 μL cell lysis buffer.

Oxyblot and Western Blot

Western blot analysis was conducted to assess protein expression of manganese superoxide dismutase (MnSOD), ATP Synthase, and cytochrome c in mitochondrial and cytosolic subcellular fractions. Protein concentration in each sample was determined using the Bradford protein assay (Bradford, 1976). A 40 μg aliquot of each sample was boiled for five min to denature it. Proteins were then separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Item No. 1620097); successful transfer and protein loading were verified using Memcode Reversible Protein Stain Kit (Pierce Biotechnology, Fisher Scientific, Item No. PI-24580). Membranes were blocked using either a 5% w/v milk/TBST solution (10mM Tris-HCl, 150mM NaCl, 0.1% Tween-20; pH 8.0) or a 3% Bovine Serum Albumin (BSA)/PBST solution for one h. Membranes were next incubated with continual shaking overnight at 4^{o}C with a rabbit polyclonal antibody for MnSOD (Abcam, Item No. ab13534, 1:500 dilution), ATP Synthase (1:1000 dilution) (Cell Signaling Technology, Item No. 9502) or cytochrome c (Cell Signaling, Item No. 4272, 1:1000 dilution). The membranes were washed four times with TBST and goat anti-rabbit HRP-linked secondary antibodies (Santa Cruz Biotechnology, Item No. sc-2030, 1:5000 dilution in 5% milk/TBST) and added for one h. Membranes were washed again with TBST and then developed using Amersham ECL Western

Blotting Detection Agent (GE Healthcare Life Sciences, Item No. RPN2232). A BioRad chemic-doc system was used to capture the gel image and used for densitometry analysis. All western analyses were conducted as three independent experiments. Western blot was also conducted as described above using two mouse polyclonal antibodies, cytochrome c oxidase IV (COX-IV, Abcam, Item No. ab14744) and cytochrome c oxidase subunit 1 (MtCO-1, Abcam, Item No. 14705). COX-IV was diluted 1:1000 in 5% w/v milk/TBST and MtCO-1 was diluted 1:2000 in 3% w/v milk/TBST. Following washing with TBST, membranes were treated with a goat anti-mouse HRP-linked secondary antibody for one h (Santa Cruz Biotechnology, Item No. sc-2005). Membranes were washed and developed as described previously.

There are several markers of oxidative stress, including protein carbonylation, which can produce an aldehyde or ketone side chain on cellular proteins. Protein carbonylation was analyzed using the Oxyblot Protein Oxidation Detection Kit (EMD Millipore, Item No. S7150). A 25 μg aliquot of sample was derivatized as previously described and protein loading was verified using the MemCode Protein Stain (Terneus et al., 2007). Briefly, protein carbonyl moieties on cellular proteins generated by oxidative stress are derivatized in the presence of 2,4-dinitrophenylhydrazine (DNPH) to stable 2,4-dinitrophenylhydrazone groups; the primary antibody (1:150 dilution in 1% BSA/PBST) recognizes these 2,4-dinitrophenylhydrazone groups on proteins. Results from three independent experiments were analyzed with BioRad Chemidoc densitometry software (version 4.0.1, Catalog No. 170-9690, BioRad, Hercules, CA, USA).

Seahorse XFp Assays

Seahorse XFp Assays allow measurement of basal Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR), as well as following injection of compounds to identify sources of mitochondrial impairment. Mitochondrial function was measured using Agilent Cell

Phenotype, Mitochondrial Stress, and Glycolytic Stress Assays following optimization of cell number per well.

Briefly, cells were cultured in XFp Culture Miniplates (25,000 cells/ml) (Agilent Technologies, Item No. 103025-100) and allowed to grow for 48 h followed by treatment with vehicle or TFV. Prior to the assay, cells were washed with assay media (Agilent Technologies, Item No. 103334-100) supplemented with 1 mM pyruvate, 2 mM glutamine, and 0 mM glucose and equilibrated in 180 μL pre-warmed assay media at 37^{o}C with no CO_2 for one h. In each assay, three basal OCR/ECAR measurements were taken at seven minute intervals using the Seahorse XFp instrument system. Following basal measurements, various probes were injected and additional OCR and ECAR measurements were taken. Maximal respiration was stimulated by the addition of Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) an uncoupler of mitochondrial function. In the Cell Phenotype Assay, stressor mix was injected at approximately minute 15 (50 μM FCCP/50 μM oligomycin) to uncouple the electron transport chain (Agilent Technologies, Item No. 103275-100) and OCR and ECAR were measured in six minute intervals. In the Mitochondrial Stress Test (Agilent Technologies, Item No. 103015-100), injections of oligomycin (0.5 μM), FCCP (0.5 μM), and a mixture of Rotenone/Antimycin A (0.5 μM) were added and OCR and ECAR were measured. Preliminary studies optimized the FCCP concentration to 0.5 μM. In the Glycolysis Stress Test (Agilent Technologies, Item No. 103020-100), glucose (10 mM), oligomycin (1 μM), and 2-DG (50 mM) were injected and OCR and ECAR were measured.

Following each assay, cells were washed with 200 μL PBS and lysed. Protein concentration was assessed using the Bradford Assay as previously described (Bradford, 1976).

Results were normalized to total protein concentration and analyzed using Wave Software (Agilent Technologies, Wave for Desktop, Version 2.3.0.19).

Antioxidant Protection Studies

A separate series of experiments evaluated whether various antioxidants could provide protection from TFV. HK-2 cells were allowed to grow for 48 h followed by a one h pretreatment with resveratrol (5, 7.5, or 10 μM in DMSO), N-acetyl-L-cysteine (NAC, 0.5 or 1 mM in sterile water), or ascorbic acid (10 or 25 μM in sterile water) followed by a 24 h incubation with 0, 3, or 14.5 μM TFV. Viability of cells was assessed using the MTT assay. All media and MTT were removed from the cells prior to the addition of DMSO to prevent any interaction of antioxidants with formazan.

Statistical Analysis

Data are presented as Mean ± SEM with 2–4 independent experiments with 2–4 biological replicates. Differences between groups were determined with a one-way ANOVA followed by a Holm–Sidak post-hoc test with $p < 0.05$ (SigmaStat, SSPS).

Results

TFV Effects on Cell Viability

TFV was cytotoxic to HK-2 cells as cell viability was diminished within 24 h when compared to vehicle based on the MTT assay. TFV reduced cell viability at 24 h ($p<0.001$) at both concentrations tested (Figure 12). Cell Viability continued to be diminished at 48 and 72 h, with 28.8 μM TFV showing additional decline in viability ($p<0.05$).

In our previous study, we determined that TFV initiates apoptosis at 72 h, but not at 24 or 48 h. Western blot was run on tissue and media samples treated with TFV to determine if

multiple mechanisms of toxicity occur. LDH leakage from tissue into media was increased at 24 and 48 h ($p<0.01$) but not at 72 h (Figure 13). This leakage is indicative of necrosis.

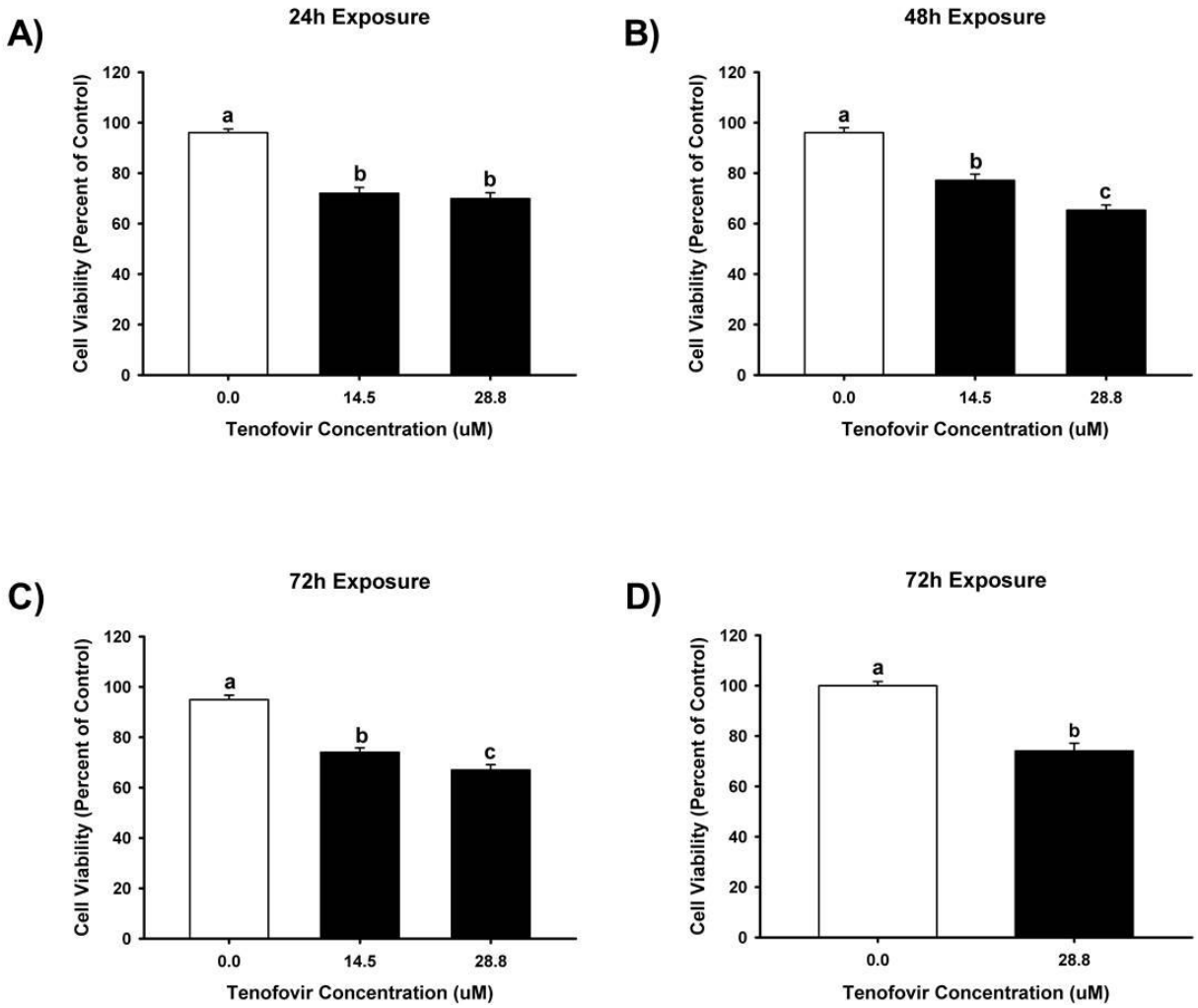

Figure 12. **TFV cytotoxic effects on HK-2 cell viability using MTT.** Tenofovir decreased cell viability at 24 (A), 48 (B), and 72 h (C). Different superscript letters (a-c) indicate difference between treatment groups ($p<0.05$) at the designated time period. D) Depicts cell viability following 72 h exposure to tenofovir in experiments where mitochondria were isolated to ensure treatment success. Each bar represents Mean ± SEM for four independent experiments run with three biological replicates.

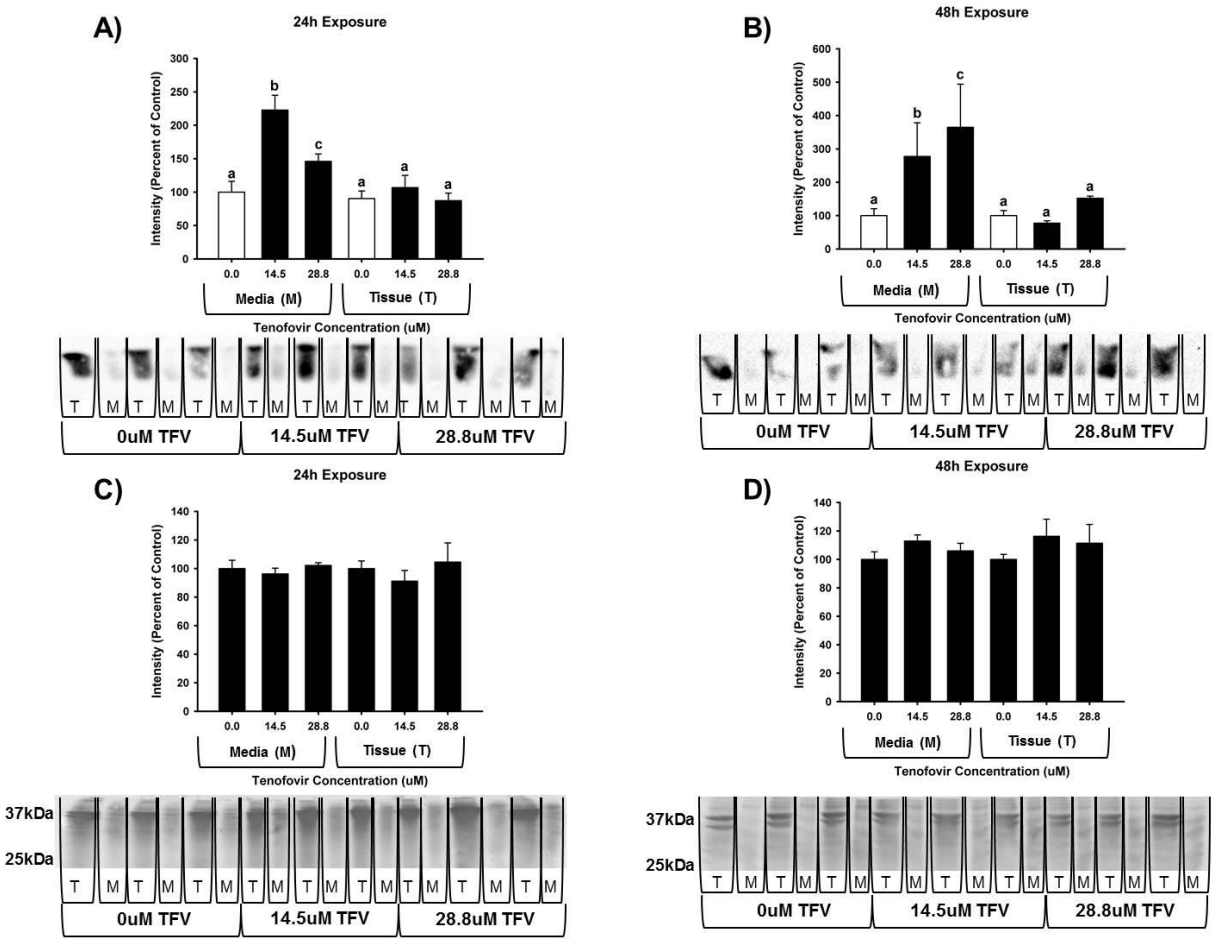

Figure 13. LDH expression in HK-2 cell lysate and media following tenofovir (TFV) exposure. LDH release into media (M) and HK-2 cell lysate (T) expressed as percent of control following 24 (A) and 48 h (B) exposure to TFV. LDH release into the media was increased ($p<0.01$) relative to control at all concentrations and time points. Different superscripts (a-c) indicate differences between treatment groups at designated time periods. C) and D) depict densitometry and representative blots for 10 µg protein loading of cell lysates and media samples for 24 (C) and 48 h (D) TFV exposure visualized with Memcode Reversible Protein Staining. Each bar represents Mean ± SEM for two independent experiments run with three biological replicates.

TFV Effects on Mitochondrial Stress and Membrane Integrity

Mitochondrial oxidative stress was induced by 28.8 µM TFV relative to control at 72 h ($p<0.05$) as shown by Oxyblot analysis (Figure 14). Protein carbonylation was not increased in the cytosolic fraction relative to control. A 72 h exposure to TFV also caused leakage of MnSOD from mitochondria to the cytosol relative to control ($p<0.001$, Figure 14). Loss of this

enzyme within the mitochondrial antioxidant system may further contribute to the observed oxidative damage.

TFV alters mitochondrial membrane integrity as shown by MnSOD leakage (Figure 14). Additional studies on mitochondrial proteins were conducted to assess the extent of mitochondrial membrane damage. TFV caused leakage of cytochrome c from the mitochondria to the cytosol relative to control ($p<0.001$) as shown by western blot (Figure 15). However, TFV did not induce leakage of ATP Synthase into the cytosol (Figure 15). The observed difference may be due to localization of proteins within the mitochondria. ATP synthase is located in the inner mitochondrial membrane while cytochrome c is located in the intermitochondrial space.

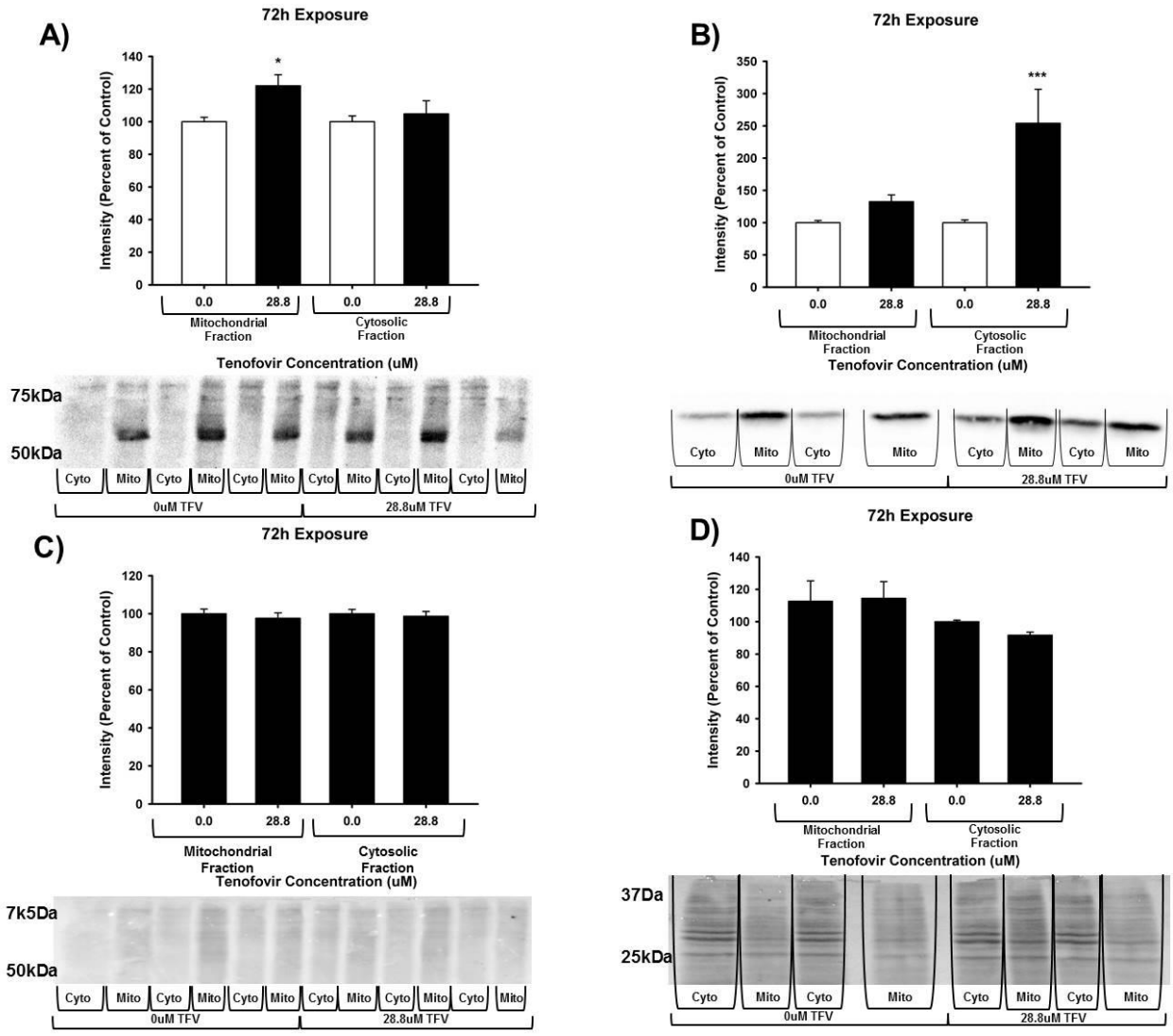

Figure 14. Tenofovir (TFV) treatment of HK-2 cells for 72 h increases protein carbonylation and induces leakage of manganese superoxide dismutase (MnSOD). A) Protein carbonylation in HK-2 mitochondrial and cytosolic fractions following 72 h exposure to 28.8 µM TFV. B) MnSOD expression in mitochondrial and cytosolic fractions following 72 h exposure to 28.8 µM TFV. C) and D) depict densitometry and representative blots for 25 µg (C) and 40 µg (D) protein loading of cytosolic and mitochondrial fractions of cell lysates for 72 h TFV exposure visualized with Memcode Reversible Protein Staining. Asterisks represent differences between groups (*$p<0.05$, ***$p<0.001$). Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

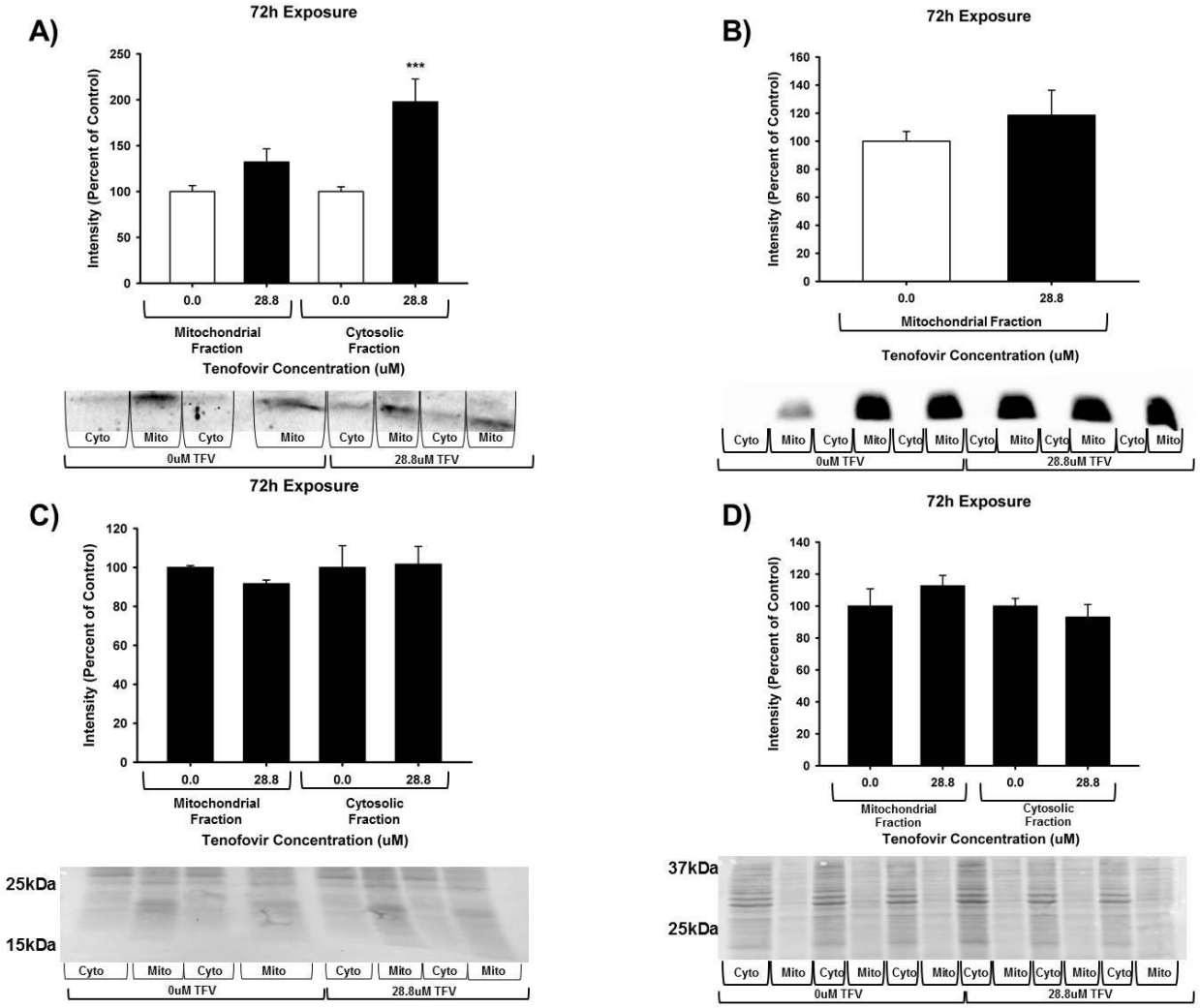

Figure 15. Tenofovir (TFV) alters mitochondrial membrane integrity. A) Expression of cytochrome c in mitochondrial and cytosolic fractions of cell lysate following 72 h exposure to 28.8 µM TFV. B) Expression of ATP Synthase in mitochondrial and cytosolic fractions of cell lysate following 72 h exposure to 28.8 µM TFV. C) and D) depict densitometry and representative blots for 40 µg protein loading of cytosolic and mitochondrial fractions of cell lysates for 72 h TFV exposure visualized with Memcode Reversible Protein Staining. Asterisks represent differences between groups (***$p<0.001$). Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

TFV Effects on Mitochondrial DNA

There are conflicting reports on whether TFV inhibits mitochondrial DNA replication, as some studies have reported TFV is a weak inhibitor of DNA Polymerase γ (Pol γ). We assessed this effect in our cell model via expression of cytochrome c oxidase subunits following 72 h

exposure to TFV. TFV did not alter the expression or localization of the nuclear-encoded subunit, COX-IV, or the mitochondrial-encoded subunit, MtCO-1 (Figure 16). These data indicate that 28.8 μM TFV does not affect mitochondrial DNA replication under the experimental conditions of 72 h exposure.

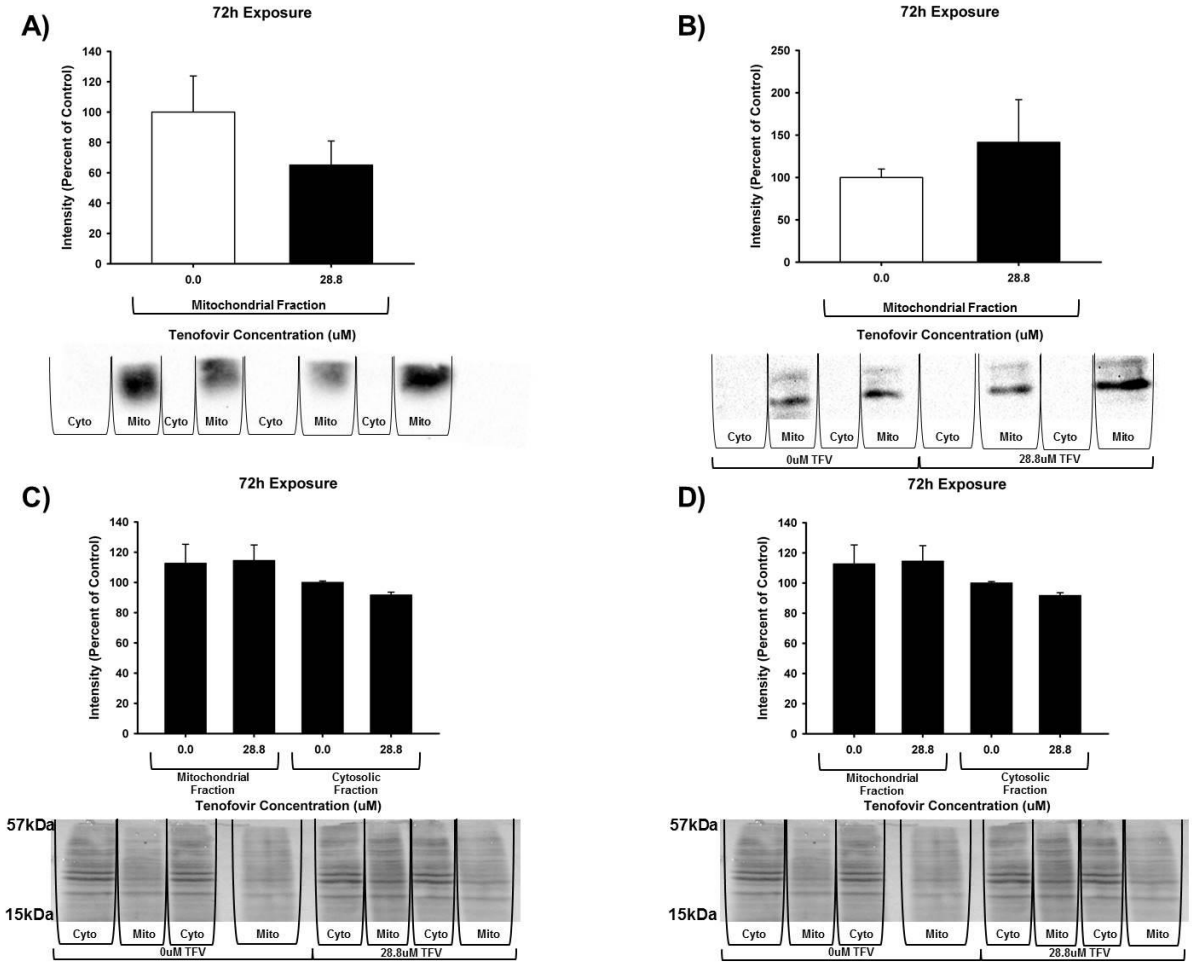

Figure 16. TFV does not alter expression or localization of subunits of cytochrome c oxidase. A) Expression of nuclear-encoded subunit, COX-IV, in mitochondrial and cytosolic fractions of cell lysate following 72 h exposure to 28.8 μM TFV. B) Expression of mitochondrial-encoded subunit, MtCO-1, in mitochondrial and cytosolic fractions of cell lysate following 72 h exposure to 28.8 μM TFV. C) and D) depict densitometry and representative blots for 40 μg protein loading of cytosolic and mitochondrial fractions of cell lysates for 72 h TFV exposure visualized with Memcode Reversible Protein Staining. Each bar represents Mean ± SEM for three independent experiments run with three biological replicates.

TFV Effects on Mitochondrial Function

Mitochondrial function was assessed using a Seahorse XFp instrument. In the cell phenotype assay, basal oxygen consumption rate (OCR) was reduced by 27% and basal extracellular acidification rate (ECAR) was reduced by 16% at 72 h exposure to TFV when compared to vehicle ($p<0.05$) (Figure 19); however, basal OCR and ECAR were similar to control at 24 and 48 h TFV exposure (Figure 17, Figure 18). The addition of FCCP and oligomycin uncouples the electron transport chain and inhibits ATP synthase, respectively, causing a compensatory increase in glycolysis and OCR to try to restore membrane potential and meet energy demands. At 24-72 h exposure to TFV, stressed OCR were reduced relative to control at all concentrations tested ($p<0.05$, Figure 17, Figure 18, Figure 19). Stressed ECAR was reduced at 72 h exposure ($p<0.05$, Figure 19) but not at 24 or 48 h exposure (Figure 17, Figure 18).

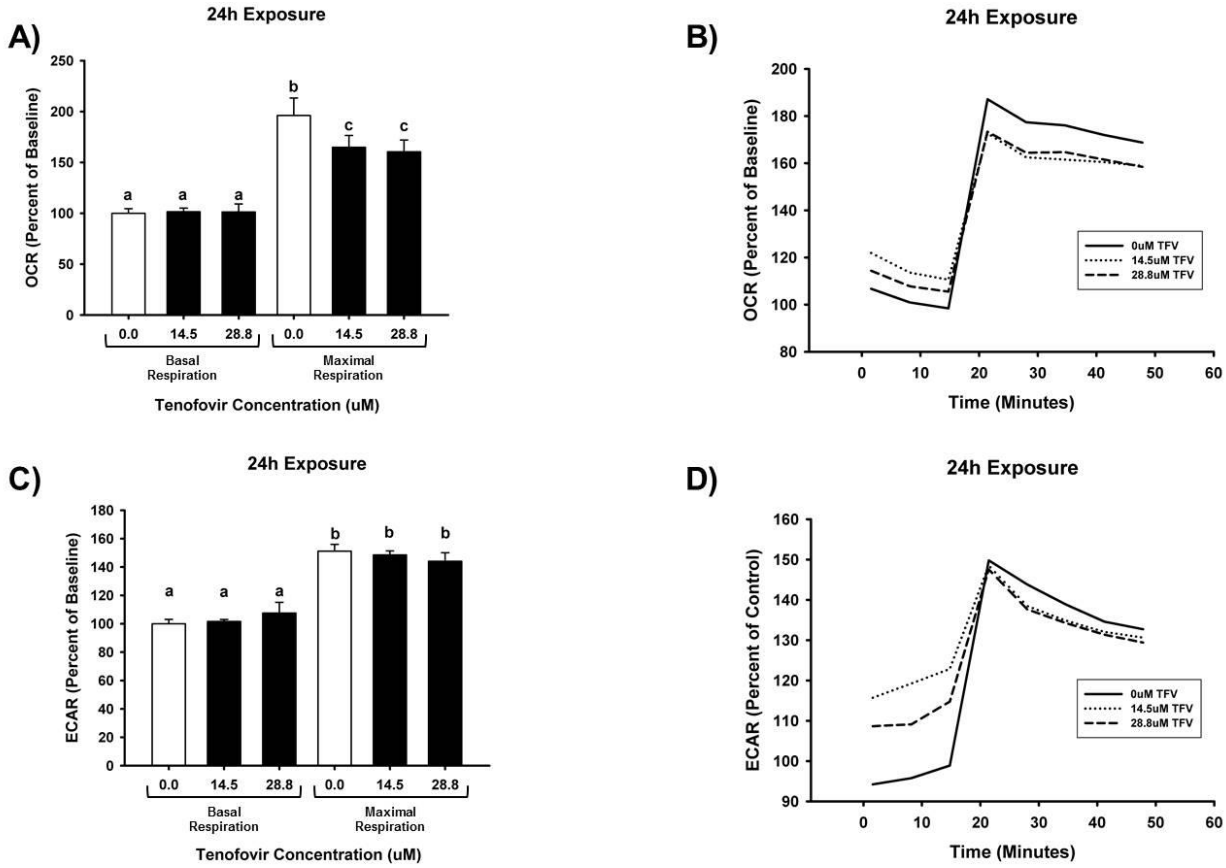

Figure 17. Cell Phenotype Assay, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) following 24 h TFV exposure. A) Basal vs. stressed OCR following 24 h exposure to TFV. B) Time course graph of OCR in the cell phenotype assay following 24 h TFV exposure. C) Basal vs. stressed ECAR following 24 h TFV exposure. Different superscripts (a-d) represent differences between groups ($p<0.05$). D) Time course graph of ECAR in the cell phenotype assay following 24 h TFV exposure. Bar graphs are presented as Mean ± SEM for four independent experiments run with two biological replicates.

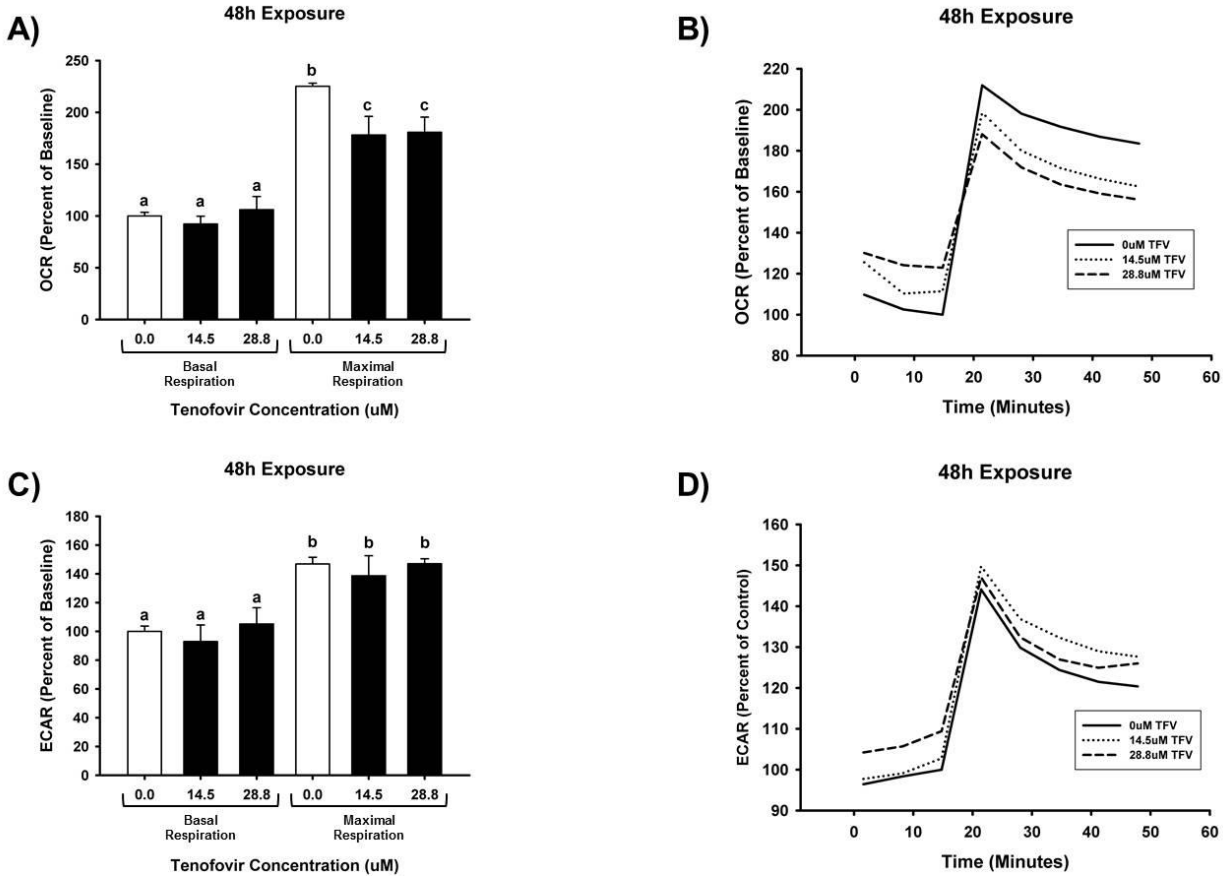

Figure 18. Cell Phenotype Assay, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) following 48 h TFV exposure. A) Basal vs. stressed OCR following 48 h exposure to TFV. B) Time course graph of OCR in the cell phenotype assay following 24 h TFV exposure. C) Basal vs. stressed ECAR following 48 h TFV exposure. Different superscripts (a-d) represent differences between groups ($p<0.05$). D) Time course graph of ECAR in the cell phenotype assay following 48 h TFV exposure. Bar graphs express data as Mean ± SEM for four independent experiments run with two biological replicates.

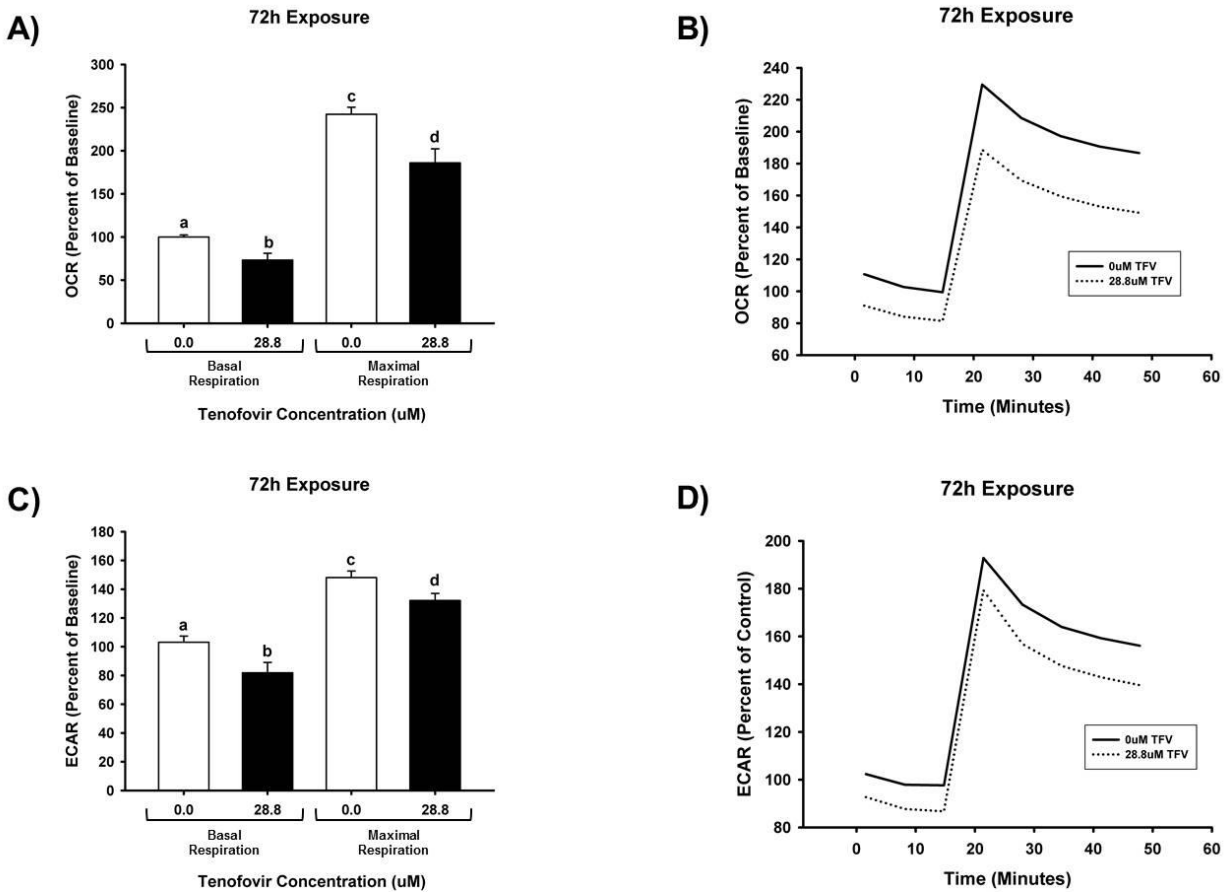

Figure 19. Cell Phenotype Assay, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) following 72 h TFV exposure. A) Basal vs. stressed OCR following 72 h exposure to TFV. B) Time course graph of OCR in the cell phenotype assay following 72 h TFV exposure. C) Basal vs. stressed ECAR following 24 h TFV exposure. Different superscripts (a-d) represent differences between groups ($p<0.05$). D) Time course graph of ECAR in the cell phenotype assay following 72 h TFV exposure. Bar graph values expressed as Mean ± SEM for four independent experiments run with two biological replicates.

A mitochondrial stress test was run following 24-72 h TFV exposure to further explore the observed reduction in stressed OCR in the cell phenotype assay. In this assay, probes are serially injected to reveal key parameters of mitochondrial function. Oligomycin inhibits ATP synthase and the resulting decrease in OCR correlates to mitochondrial respiration linked to ATP production. FCCP is an uncoupling agent that increases oxygen consumption to maximal levels,

which can be used to calculate spare respiratory capacity. The final injection of antimycin A/rotenone inhibits complex I and III, allowing for calculation of non-mitochondrial respiration.

Exposure to 14.5 and 28.8 μM TFV caused a decrease in basal ($p<0.001$) and maximal ($p<0.05$) OCR at 72 h exposure, but not at 24-48 h (Figure 20, Figure 21, Figure 22). ATP production was also decreased at 72 h following exposure to 28.8uM TFV ($p<0.05$, Figure 22) but not at 24-48 h (Figure 20, Figure 21). Spare respiratory capacity was not affected at any time point or concentration tested (Figure 20, Figure 21, Figure 22).

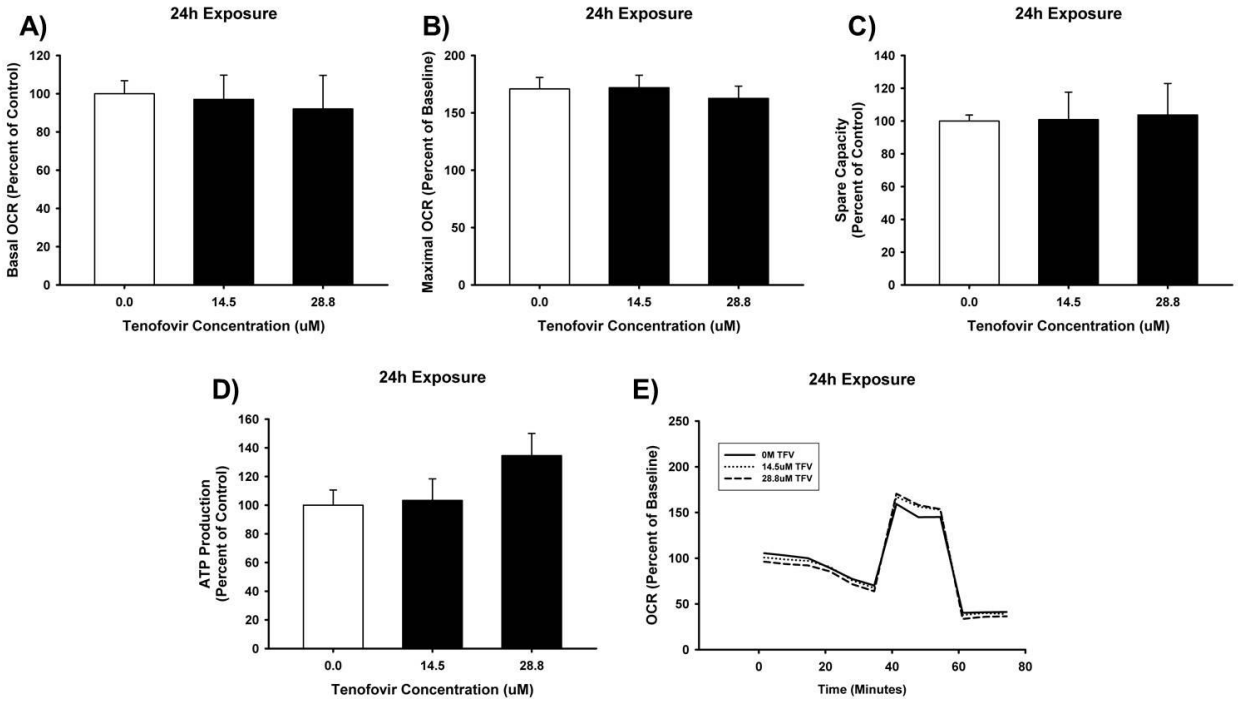

Figure 20. Mitochondrial Stress Test, effects of TFV on ATP production and mitochondrial respiratory capacity. Basal (A) and Maximal (B) OCR following 24 h exposure to TFV. C) Spare respiratory capacity following 24 h TFV exposure. D) Time course graph of OCR in the mitochondrial stress test following 24 h TFV exposure. Bar graph values represent Mean ± SEM for four independent experiments run with two biological replicates.

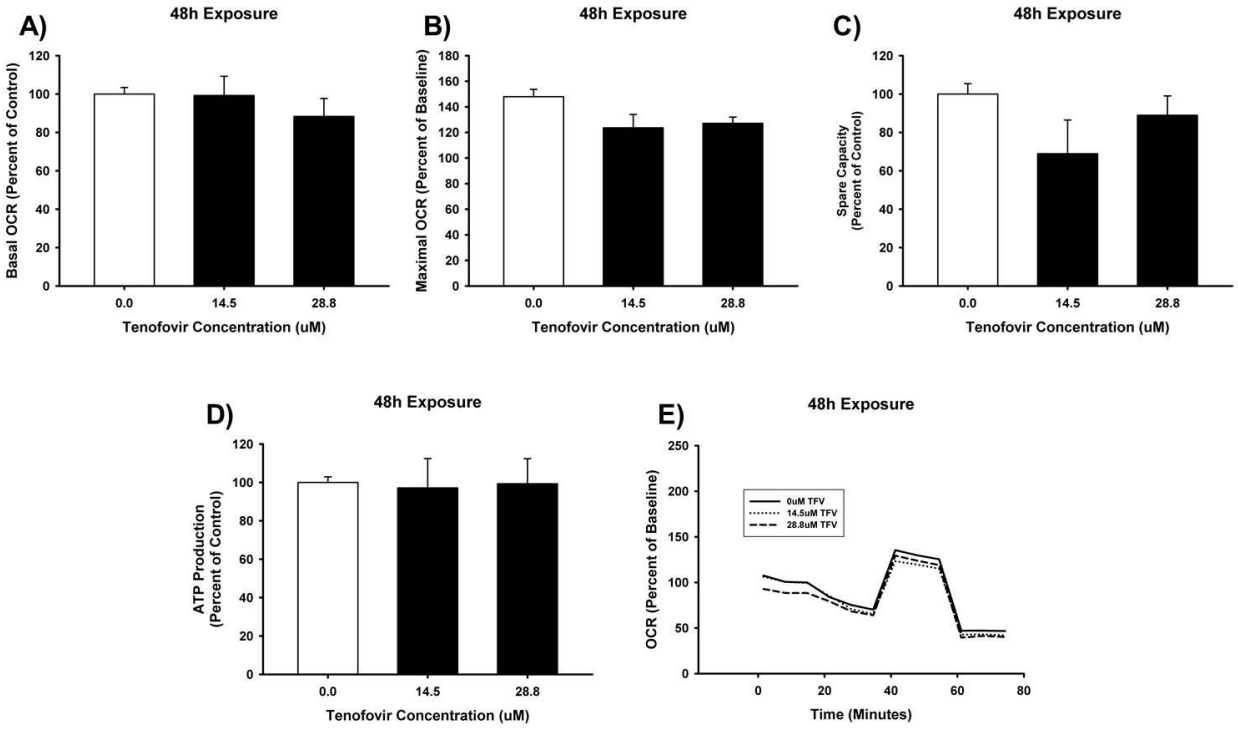

Figure 21. Mitochondrial Stress Test, effects of TFV on ATP production and mitochondrial respiratory capacity. Basal (A) and Maximal (B) OCR following 48 h exposure to TFV. C) Spare respiratory capacity following 48 h TFV exposure. D) Time course graph of OCR in the mitochondrial stress test following 48 h TFV exposure. Bar graph values are presented as Mean ± SEM for four independent experiments run with two biological replicates.

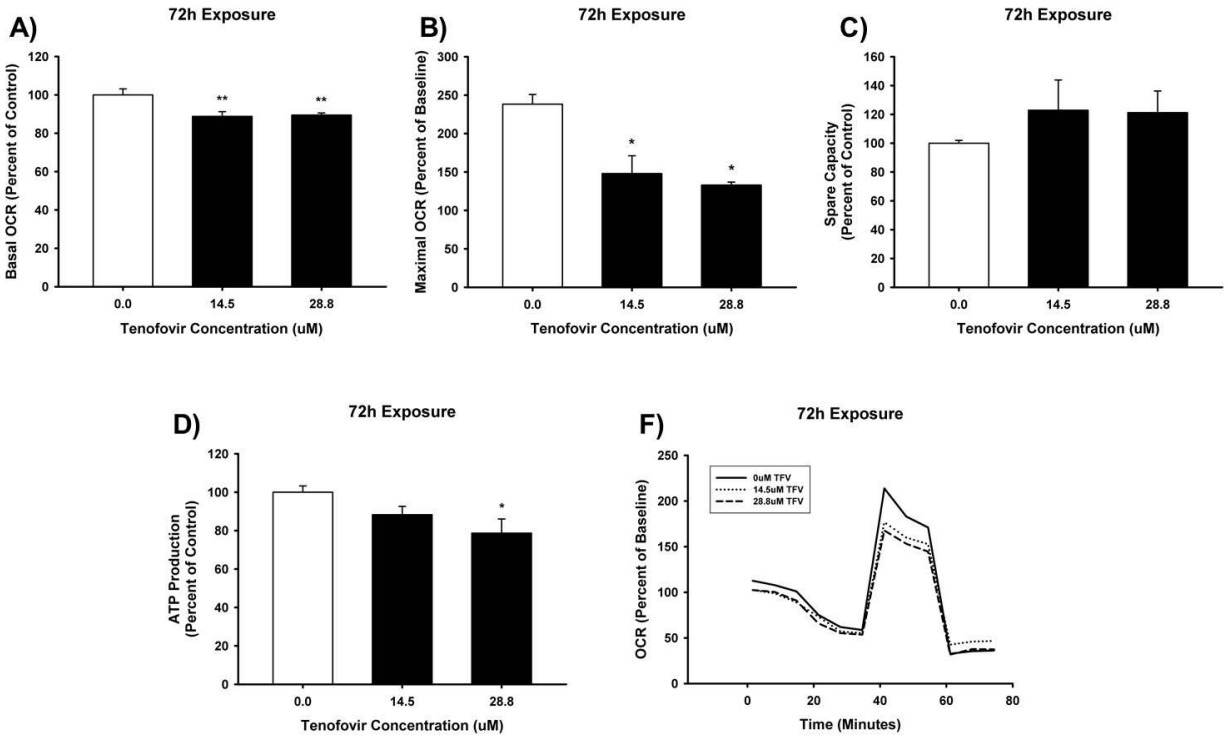

Figure 22. Mitochondrial Stress Test, effects of TFV on ATP production and mitochondrial respiratory capacity. Basal (A) and Maximal (B) OCR following 72 h exposure to TFV. C) Spare respiratory capacity following 72 h TFV exposure. Asterisks indicate differences between groups (*$p<0.05$, **$p<0.01$). D) Time course graph of OCR in the mitochondrial stress test following 72 h TFV exposure. Bar graph values are presented as Mean ± SEM for four independent experiments run with two biological replicates.

Based on the ECAR results in the cell phenotype assay, glycolytic stress tests were run at 72 h TFV exposure to further elucidate the effect of TFV on glycolysis. Cells are saturated with glucose, and basal ECAR was assessed. Oligomycin is then injected to inhibit ATP synthase and drive glycolysis to maximal capacity. Finally, 2-DG, a glucose analog, is injected to inhibit the first enzyme in the glycolytic pathway, hexokinase; the resultant decrease in ECAR ensures that the ECAR produced in the experiment is due to glycolysis. TFV exposure had no effect on any measured glycolytic parameters (Figure 23).

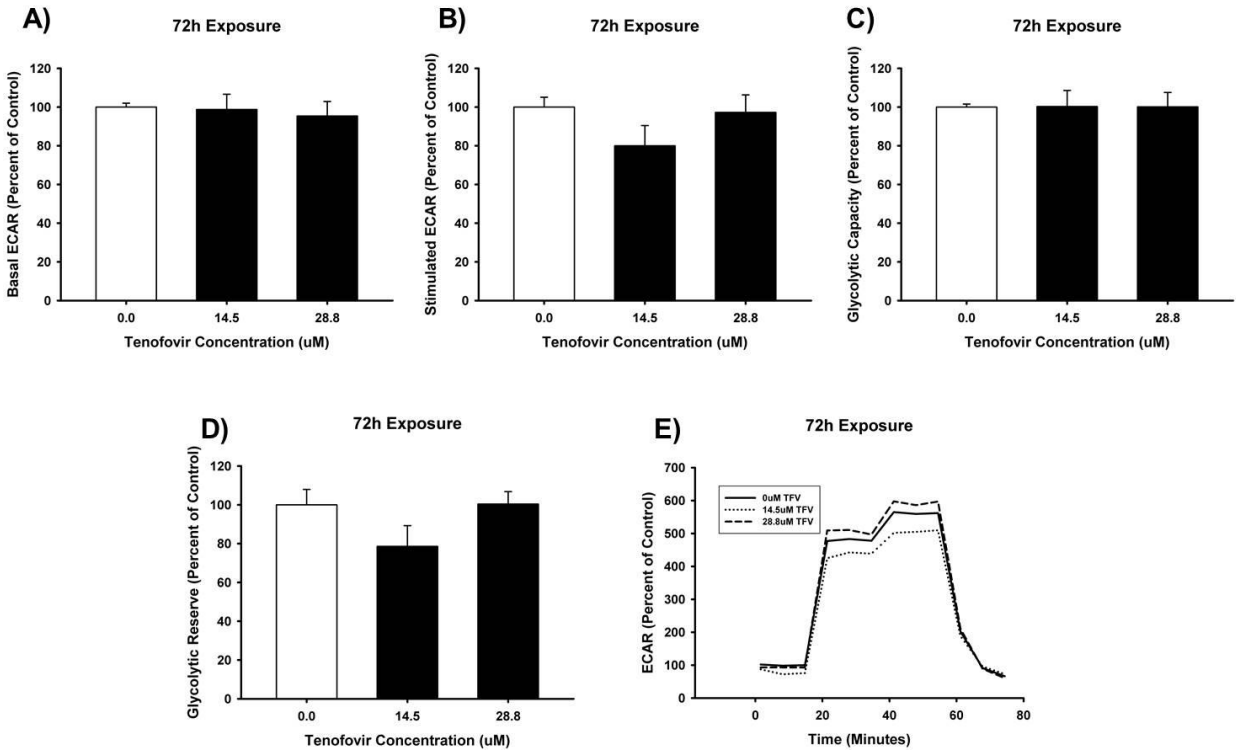

Figure 23. Glycolytic Stress Test, effects of TFV on glycolytic capacity and reserve. Basal (A) and Maximal (B) ECAR following 72 h exposure to TFV. C) Spare glycolytic capacity following 72 h TFV exposure. D) Glycolytic reserve following 72 h exposure to TFV. E) Time course graph of OCR in the glycolytic stress test following 72 h TFV exposure. Bar graph values are presented as Mean ± SEM for four independent experiments run with two biological replicates.

Antioxidant Protection of TFV Cytotoxicity

Our previous study showed that oxidative stress was increased by TFV resulting in increased protein carbonylation and 4-hydroxynonenol (4-HNE) adduct formation, suggesting that a rise in reactive oxygen species occurs with TFV cytotoxicity. Pretreatment with ascorbic acid, resveratrol, and N-acetyl-L-cysteine (NAC) reduced TFV toxicity in HK-2 cells; cell viability was higher in cells exposed to TFV in the presence of antioxidants compared to TFV alone (Figure 24, Figure 25, and Figure 26). While 0.5 mM NAC did protect from TFV cytotoxicity, it did not provide enough protection to return viability to the same level as control (Figure 24). Additionally, 25 μM ascorbic acid did protect from 14.5 μM TFV cytotoxicity, but

it did not provide enough protection to return viability to the same level as control (Figure 25). These findings suggest that use of an antioxidant pretreatment can reduce TFV cytotoxicity in HK-2 cells.

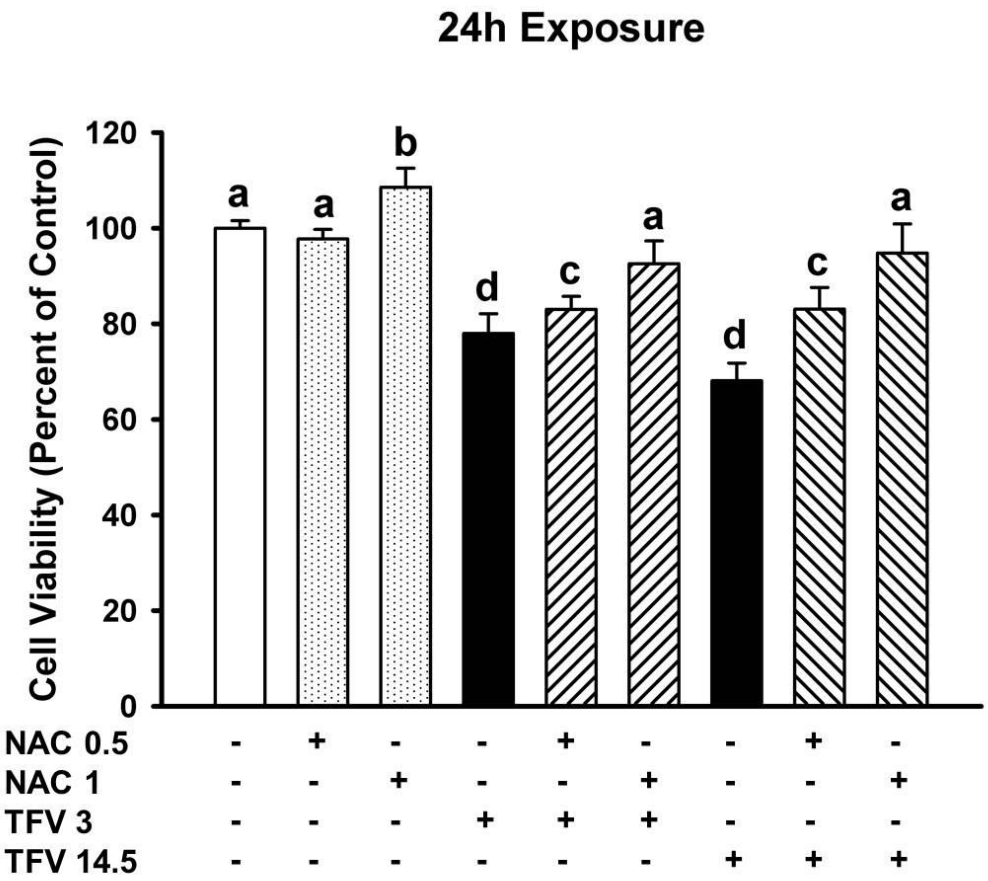

Figure 24. Effects of N-acetyl-L-cysteine (NAC) pretreatment on TFV cytotoxicity. N-acetyl-L-cysteine (NAC) protected HK-2 cells from tenofovir cytotoxicity as assessed by MTT viability. Cells were treated with 0 (−), 0.5 mM (+), or 1 mM (+) NAC followed by a 24 h exposure to 0 (−), 3 μM (+) or 14.5 μM (+) TFV. Different superscript letters (a-d) indicate statistical difference from all other groups ($p<0.05$). Values represent Mean ± SEM for three independent experiments run with three biological replicates.

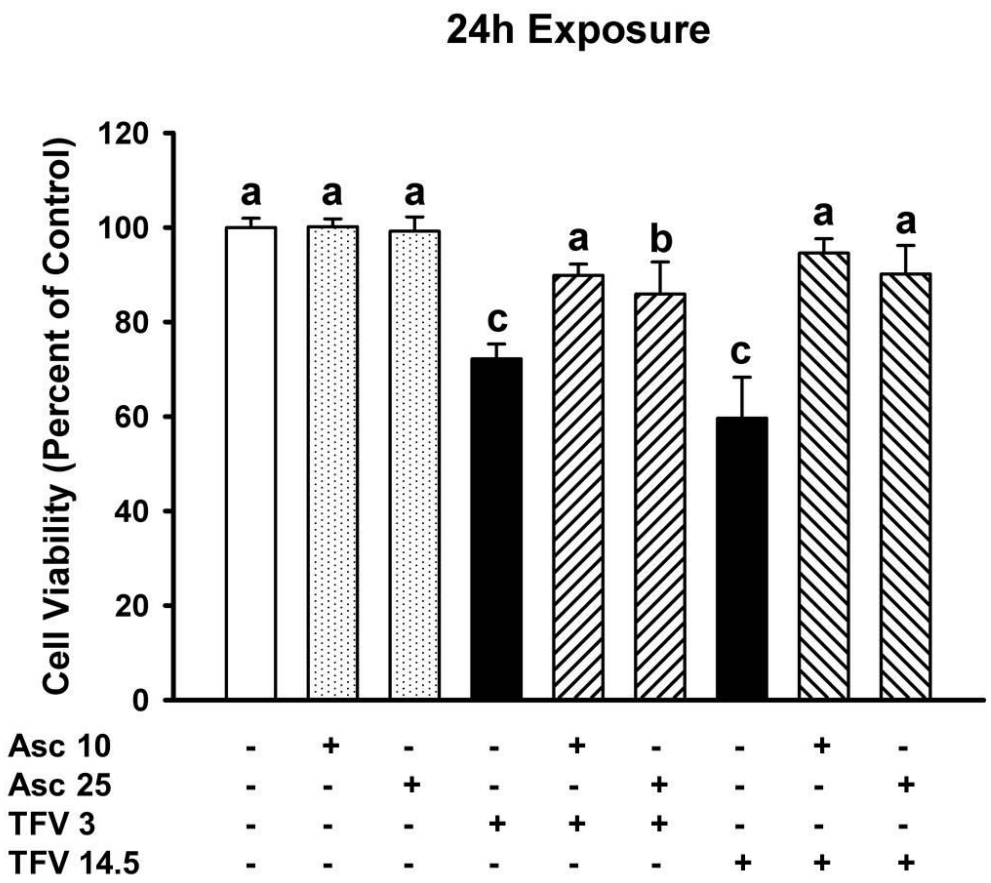

Figure 25. Effects of ascorbic acid (Asc) pretreatment on TFV cytotoxicity. Asc protected HK-2 cells from tenofovir cytotoxicity as assessed by MTT viability. Cells were treated with 0 (−), 10 μM (+), or 25 μM (+) Asc followed by a 24 h exposure to 0 (−), 3 μM (+) or 14.5 μM (+) TFV. Different superscript letters (a-c) indicate statistical difference from all other groups ($p<0.05$). Values represent Mean ± SEM for three independent experiments completed after publication of the previous study run with three biological replicates.

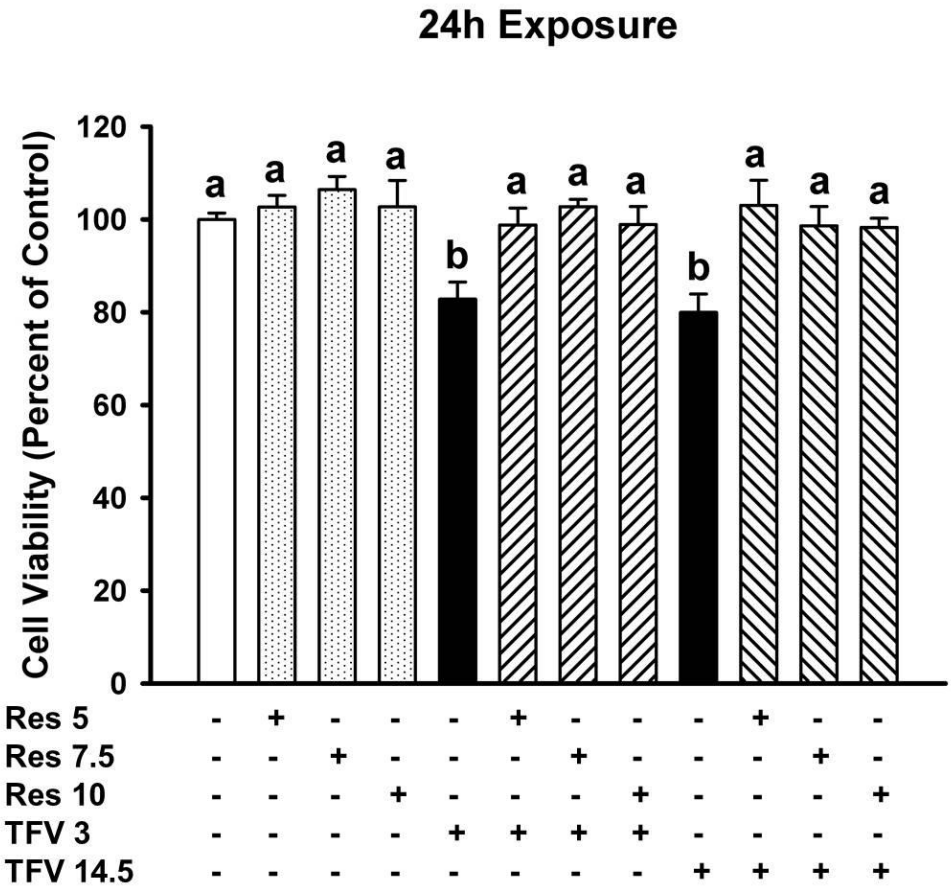

Figure 26. Effects of resveratrol (Res) pretreatment on TFV cytotoxicity. Res protected HK-2 cells from tenofovir cytotoxicity as assessed by MTT viability. Cells were treated with 0 (−), 5 μM (+), 7.5 μM (+), or 10 μM (+) Asc followed by a 24 h exposure to 0 (−), 3 μM (+) or 14.5 μM (+) TFV. Different superscript letters (a-b) indicate statistical difference from all other groups ($p<0.05$). Values represent Mean ± SEM for three independent experiments run with three biological replicates.

Discussion

TFV is a very effective antiviral nucleotide reverse transcriptase inhibitor prescribed worldwide in the treatment of HIV and Hepatitis B. Unfortunately, patients treated with TFV have an increased incidence of renal impairment (Fux et al., 2007; Verhelst et al., 2002). Renal TFV toxicity in humans is characterized by Fanconi Syndrome and development of irreversible impaired renal function depending on the dose. There is a positive correlation between increased TFV plasma concentration, renal toxicity and the duration that patients were treated with TFV

(Ezinga et al., 2014; Quesada et al., 2015). Examination of the mechanism of TFV induced renal toxicity is clinically relevant because HIV treatment is chronic. Understanding the mechanism of TFV nephrotoxicity is essential for the development of methods to mitigate TFV renal impairment.

Our current studies further probed TFV renal cytotoxicity in HK-2 cells, which are an effective model for examining TFV nephrotoxicity. In a previously published paper, we showed that treatment with clinically relevant concentrations of TFV reduced cell viability in HK-2 cells within 24 h. We also showed that TFV increased protein carbonylation and 4-HNE adduct formation in whole cell lysate at 72 h exposure and induced apoptosis via activation of caspase 3 and 9. There is also marked reduction in intracellular ATP at 72 h, but not 24 or 48 h TFV exposure (Murphy et al., 2017). These data provide insight into the mechanism of TFV induced cytotoxicity, but also leave many questions.

Our previous study (Murphy et al., 2017) supported the hypothesis that mitochondrial damage and oxidative stress play a role in TFV induced cytotoxicity; the current study further probed this mechanism. While TFV induced apoptosis at 72 h exposure as shown by caspase 3 and 9 cleavage, activation of these proteins was not observed at lower time points. Additionally, intracellular ATP was not affected until 72 h exposure, indicating that multiple mechanisms of toxicity may be involved. When cells undergo necrosis, organelles swell and break down, leading to disruption of plasma membrane integrity (Challa & Chan, 2010; Moquin & Chan, 2010; Schweichel & Merker, 1973). This loss in membrane integrity leads to the release of intracellular contents. LDH is a stable enzyme that is expressed in every cell type; it is commonly used as a marker of necrosis (Burd & Usategui-Gomez, 1973; Chan, Moriwaki, & De Rosa, 2013). Our study showed an increase in LDH release from HK-2 cells into the media

following 24 and 48 h exposure to TFV (Figure 13), indicating that TFV induces necrosis in HK-2 cells within 24 h exposure. These findings are in line with clinical data of patients with TDF induced renal injury, which show marked tubular necrosis (Herlitz et al., 2010).

The results of our previous study also indicated that the mitochondria are the target of TFV renal cytotoxicity. Clinical studies found that the mitochondria of the proximal tubules are the target of TFV induced toxicity as shown by enlarged and malformed mitochondria in electron microscopy cross sections from patients taking 300 mg TDF for approximately seven years (Cote et al., 2006; Hall et al., 2011; Herlitz et al., 2010; Woodward et al., 2009). Cytochrome c excretion into urine is observed clinically in HIV patients who have developed TFV induced nephropathies (Maggi et al., 2012). Proximal tubular mitochondrial toxicity has also been described in mice and rats treated with TFV as shown by disruptions in mitochondrial cristae and a reduction in mtDNA levels (Kohler et al., 2009; Lebrecht et al., 2009). The results of our current study are in accordance with these findings. TFV increased oxidative damage in the mitochondrial fraction of HK-2 cells, but not the cytosolic subcellular fraction (Figure 14). Additionally, there is a loss in mitochondrial membrane integrity, as indicated by the presence of cytochrome c and MnSOD in the cytosolic subcellular fraction following 72 h TFV exposure (Figure 14 and 15). Release of cytochrome c to the cytosol induces apoptosis via binding to Apoptotic protease activating factor 1 (Apaf-1) to form an apoptotic complex that activates caspase 9 (Jiang & Wang, 2000; Liu, Kim, Yang, Jemmerson, & Wang, 1996).

Cytochrome c is a key component of the electron transport chain; cytochrome c mitochondrial leakage into the cytosol may contribute to the loss of cellular ATP production observed in our previous study. Interestingly, analysis of ATP synthase expression showed no leakage into the cytosolic fraction or alteration in expression (Figure 15); ATP synthase is a

membrane bound protein in the inner mitochondrial membrane and membrane breakdown may not be severe enough to induce release of ATP synthase. It is unlikely that loss of ATP synthase from the mitochondrial membrane contributes to the observed reduction in cellular ATP following TFV exposure.

MnSOD is a nuclearly encoded mitochondrial antioxidant enzyme that is essential in maintaining the balance of generation and detoxification of reactive oxygen species (ROS). MnSOD functions mainly via detoxifying superoxide free radicals that are generated during mitochondrial respiration. Oxidative stress occurs when there is an imbalance between the generation and detoxification of ROS and reactive nitrogen species (RNS)(Fridovich, 1995). The role of MnSOD in toxicity development in HIV patients has not been extensively studied. However, one study has shown that Blood Urea Nitrogen (BUN) is elevated in wild type mice treated with 50 mg/kg TDF but not in transgenic mice that overexpress MnSOD (Glover et al., 2014). Our study shows that following 72 h exposure with 28.8 μM TFV, MnSOD leakage into the cytosol increased 1.5-fold (Figure 14). This data indicates a loss in mitochondrial membrane integrity following TFV exposure and may indicate a decreased ability to respond to oxidative stress. Whether MnSOD leakage contributes to the observed oxidative damage is not yet clear as there was no change in mitochondrial expression following TFV treatment. Additional study needs to be conducted to determine more fully what role MnSOD plays in the development of TFV renal cytotoxicity.

TFV has been shown to be a weak inhibitor of Pol γ, but effects on mitochondrial DNA (mtDNA) show conflicting results, possibly due to variation between cell types. In a clinical study, mtDNA was increased 1.4 fold in peripheral blood mononuclear cells (PBMCs) in HIV-1 infected patients taking 300mg TDF daily for 96 weeks (Curran et al., 2012). In a different study

using an HIV transgenic mouse model, mtDNA was elevated following five weeks of daily TDF treatment. However, further investigation into this study detected diminished mtDNA by TDF compared to the vehicle group when laser capture microscopy was used to microdissect proximal tubules of HIV-1 transgenic mice (Kohler et al., 2009). Conversely, exposing human primary renal proximal tubule cells to 2 and 200 uM TFV for 19 days showed no change in mtDNA compared to control (Wang & Flint, 2013). However, this same study reported TFV decreased mtDNA in primary human adipocytes cultured for 19 days in the presence of 2 and 200 uM TDF. No change in mtDNA was also reported when human renal proximal tubular epithelial cells were incubated for 22 days with 30 uM TFV(Vidal et al., 2006).

The results of our study indicate that TFV does not alter mitochondrial DNA (Figure 5). While we did not measure mtDNA directly, we assessed expression of both nuclear and mitochondrial-encoded subunits of cytochrome c oxidase. TFV did not alter the expression or localization of the nuclear-encoded subunit, COX-IV, or the mitochondrial-encoded subunit, MtCO-1 (Figure 16). While it is possible that mtDNA is affected and there is a compensatory increase in translation or a lower rate of enzyme turnover, our results are similar to another study that assessed both mtDNA and cytochrome c oxidase expression following TFV exposure. Rhesus monkeys, rats, and woodchucks were treated with varying doses of TDF for 56, 28, and 90 days respectively. Following the treatment period, cytochrome c oxidase and mtDNA levels were unaltered in all models and treatment groups (Biesecker et al., 2003).

This is the first study to conduct functional studies to determine the effect of TFV on the mitochondria. Using Seahorse XfP technology, we show that TFV alters mitochondrial function. At 24 and 48 h TFV exposure, maximal OCR is lower relative to control but basal OCR is unaffected (Figure 17, Figure 18). ECAR is not affected at either time point. However, at 72 h

TFV exposure, basal OCR and ECAR, and maximal OCR and ECAR are lower relative to control. Upon further investigation in the mitochondrial stress test, 72 h TFV exposure results in lower ATP production, but does not affect spare capacity. This decline in ATP production is not observed in lower time points, which is consistent with our previous study results.

The results of our study suggest TFV induces mitochondrial changes similar to other mitochondrial toxicants. One study using hepatocellular carcinoma cells to evaluate 27 compounds found that Seahorse technology can be helpful in mitochondrial mechanistic studies. Compounds such as rotenone, a known complex I inhibitor, show a decrease in OCR and spare capacity, but an increase in ECAR. ATP synthase inhibitors such as oligomycin, show a decrease in OCR with no effect on reserve capacity, while uncouplers such as 2,4-dinitrophenol increase OCR and ECAR with no effect on reserve capacity (Eakins et al., 2016). These extracellular flux assays were able to correctly predict the mechanisms of mitochondrial toxicity in 76% percent of known test compounds used. As TFV reduces OCR but does not alter spare capacity, it is possible that TFV inhibits ATP synthase activity; additional study needs to be done to determine the direct inhibitory effects of TFV. Our studies indicated ATP synthase expression was not diminished by TFV.

Eakins and associates (2016) further determined that known glycolytic inhibitors such as clotrimazole induce reduced OCR and reserve capacity but no effect on ECAR levels. The results of our glycolytic stress test show no effect on ECAR at 72 h exposure, but there is reduced OCR (Figure 22); based on results of published glycolytic inhibitors, it is possible that TFV alters glycolysis in some way. It remains unknown if the cause of decreased OCR and ECAR following TFV exposure is due to loss of mitochondrial membrane integrity, direct

enzyme inhibition, fewer functional mitochondria, or a combination of several factors. Further studies need to probe the mechanism of mitochondrial toxicity.

Resveratrol and ascorbic acid are widely known as antioxidants and have been shown to prevent renal toxicity of a number of chemotherapeutic agents that induce oxidative stress in vitro and in vivo rodent models, including cisplatin and cadmium (Do Amaral et al., 2008; Fu, Zhao, Peng, Wu, & Zhang, 2017; Koyuturk, Yanardag, Bolkent, & Tunali, 2007; Maliakel, Kagiya, & Nair, 2008; Valentovic et al., 2014). N-acetyl-L-cysteine (NAC) is the precursor to glutathione (GSH), an important cellular antioxidant. NAC is most notably used to treat acetaminophen overdose by providing cysteine for glutathione synthesis to mitigate hepatic toxicity, but has been shown to protect hepatocytes from oxidative damage induced by cadmium and mercury in rats (Joshi, Mittal, Shukla, Srivastav, & Srivastav, 2014; Wang, Zhu, Liu, & Liu, 2014).

Our study shows that an hour pretreatment with resveratrol, NAC, or ascorbic acid protects HK-2 cell viability when exposed for 24 h to 3 or 14.5 μM TFV (Figure 17). It should be noted that high concentrations of NAC (0.5-1 mM) were required to induce a protective effect; resveratrol may be the best choice in prevention of TFV induced cytotoxicity as it required the lowest concentration to induce a protective effect (5 μM). It is unclear by what mechanism antioxidants protect against TFV toxicity. Our previous studies did not show markers of oxidative damage (protein carbonylation and 4-HNE adduct formation) until 72 h exposure, but protection was observed in our current study within 24 h TFV exposure. Additional study needs to be done to determine the role of reactive oxygen and nitrogen species at lower time points as well as to elucidate the mechanism of antioxidant protection.

Conclusion

This study provides additional insight into the mechanism of TFV induced renal cytotoxicity. TFV induces necrosis within 24 h as shown by LDH release from HK-2 cells. Oxidative stress is increased in mitochondria, and there is a loss of mitochondrial membrane integrity, resulting in the release of MnSOD and cytochrome c into the cytosol. Cytochrome c release further validates that TFV induces apoptosis at 72 h exposure. Mitochondrial function is also altered, as shown by changes in OCR and ECAR in Seahorse XFp Assays. The role of MnSOD in TFV induced cytotoxicity remains unclear. However, antioxidant pretreatment attenuates TFV induced cytotoxicity, but the mechanism by which this occurs is unknown at this time. Additional studies need to be conducted to determine to what degree TFV modulates mitochondrial function and the mitochondrial antioxidant system, as well as investigate the mechanism by which antioxidants protect against TFV induced oxidative damage.

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CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

At the end of my dissertation research, this project remains as exciting and fascinating as it was when I came across it in my first year communications class. TDF is one of the most widely prescribed drugs to treat HIV and it is effective in patients who have resistant HIV-1. As resistance to HAART can be acquired and transmitted, medications with low resistance profiles are key in successful treatment of HIV. This low resistance profile makes the renal toxicity associated with TFV use particularly relevant. The goal of my work was to determine the mechanism of TFV induced nephrotoxicity and to investigate potential methods to mitigate or prevent said toxicity. My work provides insight into this mechanism as well as evidence that antioxidant pretreatment mitigates this toxicity; this evidence warrants further study and has potential clinical implications.

HK-2 Cells are a Suitable Model to Study TFV Cytotoxicity

One of the primary reasons that there is a knowledge gap regarding the mechanism of TFV toxicity is a lack of suitable experimental models; mechanistic studies require a model that is consistent with what occurs in humans. The selection of suitable models to study TFV cytotoxicity has been problematic to date. For example, most in vivo models require subchronic treatment of animals. TFV toxicity in rodents such as rats has required eight weeks of daily treatment in order to develop nephrotoxicity (Lebrecht et al., 2009). In this study, rats treated for eight weeks showed declined renal function and proximal tubular damage along with enlarged mitochondria. Another study showed that treatment for five weeks with TFV induced oxidative stress in Wistar rats. However, they were dosed with very high concentrations of TFV (Abraham et al., 2013).

An in vitro model was first reported by Wang and Flint that developed TFV cytotoxicity using primary human kidney cells (Wang & Flint, 2013). Primary human kidney cells required culturing for 19 days with 200 μM TFV to induce cytotoxicity, which was much longer and at concentrations higher than pharmacological levels. The need for higher concentrations of TFV to induce toxicity in primary proximal tubule cell lines may be due to differences in OAT1 and OAT3 expression. Endogenous expression of OAT1 and OAT3 in primary human proximal tubule cells has been shown to vary greatly from sample to sample (Lash et al., 2006) and TFV-induced cytotoxicity is dependent on OAT1 and OAT3 expression (Kohler et al., 2011; Nieskens et al., 2016). Other cell types such as HEK293 cells have been used to study tenofovir cytotoxicity, but appear less sensitive than our HK-2 model. One study showed that TFV-induced cytotoxicity within 48 h in HEK293 cells transfected with OAT1; however, the IC_{50} was reported to be 316 μM (Zhang et al., 2015).

Our HK-2 cell model is more sensitive than human primary proximal tubule cells and HEK293 cells as we have shown induction of cytotoxicity in 24 h compared to 22 days and at a concentration almost ten times lower than previously reported for human primary proximal tubular epithelial cells and almost 12 times lower than previously reported for HEK293 cells (Vidal et al., 2006; Zhang et al., 2015). In addition to inducing cytotoxicity at 24 h, our HK-2 cell model has an IC_{50} of 9.2 μM TFV at 48 h, and an IC_{50} of 2.77 μM TFV at 72 h compared to an IC_{50} at 48 h of 316uM (Zhang et al., 2015).

In our study, cell viability was diminished in HK-2 cells with TFV concentrations ranging from 1.5 to 28.8 μM. These concentrations are clinically relevant as plasma TFV concentrations have been reported to be 2.2 μM in HIV-1 infected patients (Barditch-Crovo et al., 2001; Zhang et al., 2015). As described in Chapter 1, TFV plasma concentrations are higher

in HIV-1 patients with existing renal insufficiency and patients with renal insufficiency also experience a longer duration of TFV exposure, as shown by a 15-fold increase in $AUC_{0-24\,h}$ (Custodio et al., 2016; Ezinga et al., 2014; Sentenac et al., 2003). Therefore, HK-2 cells provide a model that can be used to examine the mechanism of toxicity without the compounding physiological parameters influencing the response of the kidney to a toxicant. Our studies further showed that HK-2 cells can differentiate the toxicity of different antiviral agents as abacavir, an agent that is considered less toxic to the kidney which was not toxic in our system (Figure 4D, Chapter 2).

TFV Causes Oxidative Stress and Alters Mitochondrial Integrity in HK-2 Cells

In our study, we first probed whole cell lysate for markers of oxidative damage, protein carbonylation, and 4-HNE adduct formation. Oxidative damage occurs following periods of oxidative stress, an imbalance between the generation and detoxification of ROS and reactive nitrogen species (RNS) (Fridovich, 1995). The HK-2 cells were more sensitive to TFV and displayed increased oxidative stress at clinically relevant concentrations as compared to previously examined models. A five week treatment of rats with 12 times higher dose than what is clinically used reported a 25% increase in renal protein carbonylation (Abraham et al., 2013); the HK-2 cells demonstrated a much greater increase in protein carbonylation within a shorter time period than reported in vivo treatment of rats (Figure 7, Chapter 2). Additionally, we saw a large increase in 4-HNE adduct formation at 72 h exposure (Figure 8, Chapter 2), indicating that TFV induced oxidative stress leads to lipid peroxidation.

We further investigated oxidative damage following TFV exposure in subcellular fractions. Clinical studies show that the mitochondria of the proximal tubules are the target of TFV induced toxicity as shown by enlarged and malformed mitochondria in electron microscopy

cross sections from patients taking 300 mg TDF for approximately seven years (Cote et al., 2006; Hall et al., 2011; Herlitz et al., 2010; Woodward et al., 2009). Cytochrome c excretion into urine is observed clinically in HIV patients who have developed TFV induced nephropathies (Maggi et al., 2012). Proximal tubular mitochondrial toxicity has also been described in mice and rats treated with TFV as shown by disruptions in mitochondrial cristae and a reduction in mtDNA levels (Kohler et al., 2009; Lebrecht et al., 2009). The results of our current study are in accordance with these findings. TFV increased oxidative damage in the mitochondrial fraction of HK-2 cells, but not the cytosolic subcellular fraction (Figure 14, Chapter 3).

Additionally, there is a loss in mitochondrial membrane integrity, as indicated by the presence of cytochrome c and MnSOD in the cytosolic subcellular fraction following 72 h TFV exposure (Figure 14 and 15, Chapter 3). This loss in membrane integrity may be due to the lipid peroxidation indicated by 4-HNE adduct formation. Interestingly, analysis of ATP synthase expression showed no leakage into the cytosolic fraction or alteration in expression (Figure 15, Chapter 3); ATP synthase is a membrane bound protein in the inner mitochondrial membrane and membrane breakdown may not be severe enough to induce release of ATP synthase.

MnSOD is a nuclearly encoded mitochondrial antioxidant enzyme that is essential in maintaining the balance of generation and detoxification of reactive oxygen species (ROS). MnSOD functions mainly via detoxifying superoxide free radicals that are generated during mitochondrial respiration. The role of MnSOD in toxicity development in HIV patients has not been extensively studied. However, one study has shown that Blood Urea Nitrogen (BUN) is elevated in wild type mice treated with 50 mg/kg TDF but not in transgenic mice that overexpress MnSOD (Glover et al., 2014). Our study shows that following 72 h exposure with 28.8 μM TFV, MnSOD leakage into the cytosol increased 1.5-fold (Figure 3, Chapter 2). This

data indicates a loss in mitochondrial membrane integrity following TFV exposure and may indicate a decreased ability to respond to oxidative stress. Whether MnSOD leakage contributes to the observed oxidative damage is not yet clear as there was no change in mitochondrial expression following TFV treatment.

TNFα is a proinflammaotry cytokine which is expressed in proximal tubular epithelial cells. TNFα can be secreted from cells to induce an inflammatory response as a protective mechanism during initial exposure to toxicants (Gu et al., 2016). TNFα expression can induce reactive oxygen species generation via mitochondria and through activation of NADPH oxidase (Morgan, 2010). Mitochondrial damage and generation of reactive oxygen species can stimulate the release of various cytokines including TNFα (Mittal et al., 2014). These changes in TNFα expression and secretion can modulate various parts of the cellular antioxidant system and directly increase the production of mitochondrial reactive oxygen species through damage to the electron transport chain (Mittal et al., 2014). In experiments involving exposing HK-2 cells to toxicants and/or ischemic injury, TNFα levels rose along with many other markers of toxicity, including increases in oxidative stress via dysfunction of cellular antioxidant systems, activation of an inflammatory response and associated proteins, and induction of programmed cell death (Gong et al., 2015; Zager et al., 2007; Zhang et al., 2013).

We examined TNFα as a potential indicator of oxidative stress. Our study showed that TNFα was released into the media at 72 h, while tissue showed a decline in expression at 48 and 72 h (Figure 9, Chapter 2). Because TNFα expression was unaltered at 24 h (Figure 9, Chapter 2) it is unlikely that activation of inflammatory response is the cause of the oxidative stress observed. The decline in TNFα in the cells is consistent with loss from cells into the media; TNFα released into the media may contribute to greater oxidative stress.

TFV Alters Mitochondrial Function in HK-2 Cells

The renal proximal tubular epithelial cell has a high requirement for ATP generation to maintain normal cellular processes such as active transport. The decline in cellular ATP levels caused by TFV would impair normal proximal tubular epithelial cell function. In our initial study, we determined that TFV greatly diminished ATP levels following 72 h exposure but not at 24 or 48 h exposure (Figure 6, Chapter 2). Loss in cellular ATP was also seen in our subsequent studies using Seahorse XFp; ATP production was not altered at 24 or 48 h TFV exposure, but was diminished at 72 h (Figure 20, 21, and 22, Chapter 3). While what is causing this loss in ATP production remains unclear, it may be due to several factors. Cytochrome c is a key component of the electron transport chain; cytochrome c mitochondrial leakage into the cytosol may contribute to the observed loss in ATP.

TFV appears to directly alter the electron transport chain, which may also contribute to loss of cellular ATP. At 24 and 48 h TFV exposure, maximal OCR is lower relative to control but basal OCR is unaffected (Figure 17, Figure 18, Chapter 3) when measured using Seahorse XFp technology. ECAR is not affected at either time point. However at 72 h TFV exposure, basal OCR and ECAR, and maximal OCR and ECAR are lower relative to control. Upon further investigation in the mitochondrial stress test, 72 h TFV exposure results in lower ATP production, but does not affect spare capacity. The results of our study suggest that TFV induces mitochondrial changes similar to other mitochondrial toxicants.

One study using hepatocellular carcinoma cells to evaluate 27 compounds found that seahorse technology can be helpful in mitochondrial mechanistic studies. Compounds such as rotenone, a known complex I inhibitor, show a decrease in OCR and spare capacity, but an increase in ECAR. ATP synthase inhibitors such as oligomycin, show a decrease in OCR with

no effect on reserve capacity, while uncouplers such as 2,4-dinitrophenol increase OCR and ECAR with no effect on reserve capacity (Eakins et al., 2016). These extracellular flux assays were able to correctly predict the mechanisms of mitochondrial toxicity in 76% percent of known test compounds used. As TFV reduces OCR but does not alter spare capacity, it is possible that TFV inhibits ATP synthase activity. Our studies indicated that ATP synthase expression was not diminished by TFV. It is unlikely that loss of ATP synthase from the mitochondrial membrane contributes to the observed reduction in cellular ATP following TFV exposure.

TFV Causes Multiple Mechanisms of Cell Death

Our study shows a loss of cell viability following TFV exposure within 24-48 h via necrosis. However, markers of apoptosis are not observed until 72 h exposure. Release of cytochrome c to the cytosol induces apoptosis via binding to Apoptotic protease activating factor 1 (Apaf-1) to form an apoptotic complex that activates caspase 9 and eventually caspase 3 (Jiang & Wang, 2000; Liu et al., 1996). Following 72 h exposure to TFV, there is observable release of cytochrome c into the cytosolic subcellular fraction as well as cleavage of caspase 3 and 9. These results are indicative that TFV causes mitochondrial damage that initiates apoptosis.

When cells undergo necrosis, organelles swell and break down, leading to disruption of plasma membrane integrity (Challa & Chan, 2010; Moquin & Chan, 2010; Schweichel & Merker, 1973). This loss in membrane integrity leads to the release of intracellular contents. LDH is a stable enzyme that is expressed in every cell type; it is commonly used as a marker of necrosis (Burd & Usategui-Gomez, 1973; Chan et al., 2013). Our study showed an increase in LDH release from HK-2 cells into the media following 24 and 48 h exposure (Figure 13, Chapter 3), indicating that TFV induces necrosis in HK-2 cells within 24 h exposure. These findings are

in line with clinical data of patients with TDF induced renal injury, which show marked tubular necrosis (Herlitz et al., 2010).

Antioxidant Pretreatment Prevents TFV Cytotoxicity

Resveratrol and ascorbic acid are widely known as antioxidants and have been shown to prevent renal toxicity of a number of chemotherapeutic agents that induce oxidative stress in vitro and in vivo rodent models, including cisplatin and cadmium (Do Amaral et al., 2008; Fu et al., 2017; Koyuturk et al., 2007; Maliakel et al., 2008; Valentovic et al., 2014). N-acetyl-L-cysteine (NAC) is the precursor to glutathione (GSH), an important cellular antioxidant. NAC is most notably used to treat acetaminophen overdose by providing cysteine for glutathione synthesis to mitigate hepatic toxicity, but has been shown to protect hepatocytes from oxidative damage induced by cadmium and mercury in rats (Joshi et al., 2014; Wang et al., 2014).

Our study shows that a one h pretreatment with resveratrol, NAC, or ascorbic acid protects HK-2 cell viability when exposed for 24 h to 3 or 14.5 μM TFV (Figure 6, Chapter 2). It should be noted that high concentrations of NAC (0.5-1 mM) were required to induce a protective effect; resveratrol may be the best choice in prevention of TFV induced cytotoxicity as it required the lowest concentration to induce a protective effect (5 μM). It is unclear by what mechanism antioxidants protect against TFV toxicity. Our studies did not show markers of oxidative damage (protein carbonylation and 4-HNE adduct formation) until 72 h exposure, but protection was observed within 24 h TFV exposure.

Conclusions

My dissertation research over the last four years has led to a general understanding of the mechanism of TFV induced cytotoxicity. This mechanistic understanding is essential if clinical methods of prevention are to be developed. We have determined that upon entry into the

proximal tubule cell, TFV causes necrotic cell death within 24- 48 h. TFV enters the mitochondria and causes oxidative stress, leading to increased protein carbonylation, a loss of cellular ATP production, lipid peroxidation, and a loss of mitochondrial membrane integrity. This loss of membrane integrity leads to leakage of cytochrome c and MnSOD to the cytosol, where cytochrome c activates caspase 9 and 3, leading to apoptotic cell death within 72 h exposure (Figure 27). Although multiple mechanisms of cell death appear to be involved, pretreatment with NAC, ascorbic acid, or resveratrol protected against TFV cytotoxicity within 24 h. Resveratrol showed the most effective protection, and warrants further study into possible clinical prevention of TFV induced nephrotoxicity.

Future Directions

My study was the first to investigate the mechanism of TFV induced cytotoxicity in depth. While it has answered many questions, there are several left unanswered. It remains unknown what causes the observed loss in cellular respiration and ATP levels; while inhibition of ATP synthase may play a role, more work needs to be done to determine whether TFV directly alters function of electron transport enzymes. Additionally, what triggers necrotic cell death at lower exposure time points is unknown at this time. This alternate mechanism of toxicity may play a role in the observed antioxidant protection at 24 h TFV exposure. Additional research needs to be conducted to determine what mechanism triggers necrotic cell death, how antioxidants prevent this, and if antioxidants are also effective at longer treatment periods.

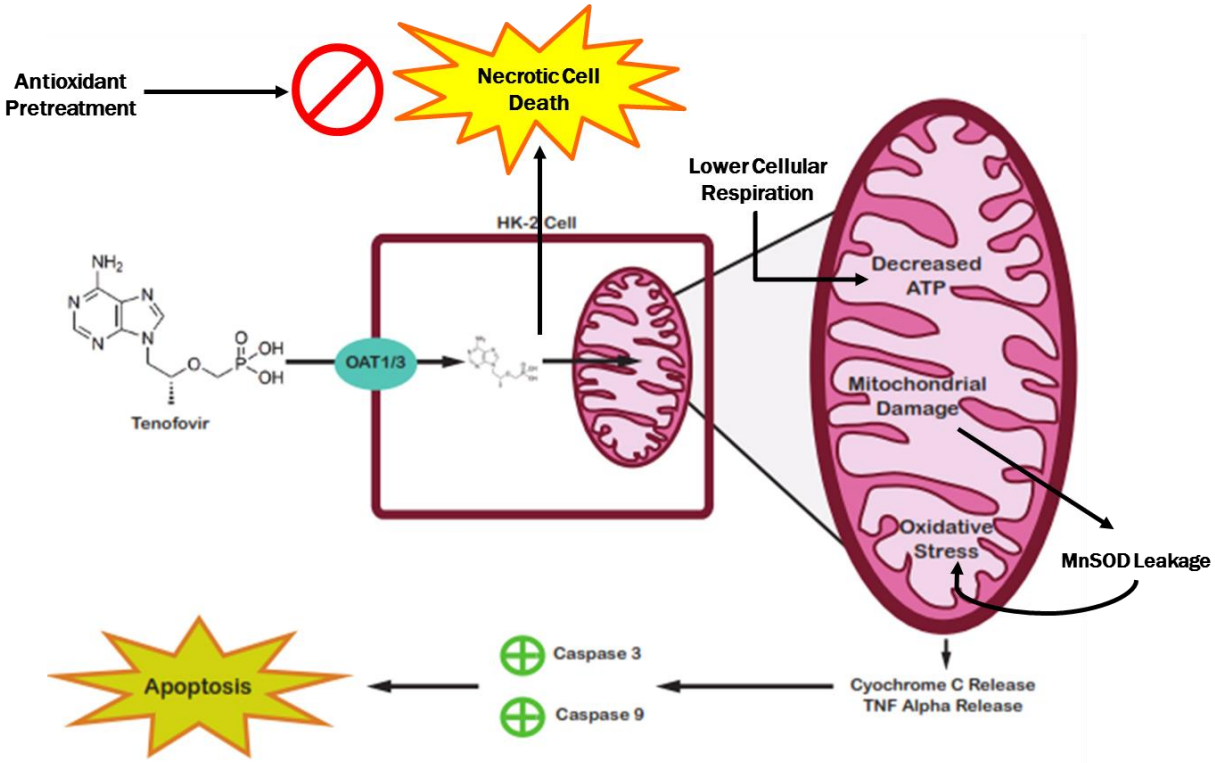

Figure 27. Summary of TFV induced cytotoxicity. TFV enters the proximal tubule cell via OAT 1/3 transporters. TFV induces necrotic cell death by an unknown mechanism. TFV enters the mitochondria and reduces cellular respiration and ATP levels, causes oxidative stress and mitochondrial damage. Mitochondrial damage leads to the release of MnSOD, cytochrome c, and TNFα. Cytochrome c release activates caspase 9 and 3, leading to apoptotic cell death. Necrotic cell death can be prevented with antioxidant pretreatment.

REFERENCES

Abdool Karim, Q., Abdool Karim, S. S., Frohlich, J. A., Grobler, A. C., Baxter, C., Mansoor, L. E., . . . Group, C. T. (2010). Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science, 329*(5996), 1168-1174. doi:10.1126/science.1193748

Abraham, P., Ramamoorthy, H., & Isaac, B. (2013). Depletion of the cellular antioxidant system contributes to tenofovir disoproxil fumarate - induced mitochondrial damage and increased oxido-nitrosative stress in the kidney. *J Biomed Sci, 20*, 61. doi:10.1186/1423-0127-20-61

Anderson, P. L., Glidden, D. V., Liu, A., Buchbinder, S., Lama, J. R., Guanira, J. V., . . . iPrEx Study, T. (2012). Emtricitabine-tenofovir concentrations and pre-exposure prophylaxis efficacy in men who have sex with men. *Sci Transl Med, 4*(151), 151ra125. doi:10.1126/scitranslmed.3004006

Andreucci, M., Faga, T., Pisani, A., Sabbatini, M., Russo, D., Mattivi, F., . . . Michael, A. (2015). Reversal of radiocontrast medium toxicity in human renal proximal tubular cells by white grape juice extract. *Chem Biol Interact, 229*, 17-25. doi:10.1016/j.cbi.2014.12.035

Aurpibul, L., Cressey, T. R., Sricharoenchai, S., Wittawatmongkol, O., Sirisanthana, V., Phongsamart, W., . . . Chokephaibulkit, K. (2015). Efficacy, safety and pharmacokinetics of tenofovir disoproxil fumarate in virologic-suppressed HIV-infected children using weight-band dosing. *Pediatr Infect Dis J, 34*(4), 392-397. doi:10.1097/INF.0000000000000633

Azmin, M. N., Florence, A. T., Handjani-Vila, R. M., Stuart, J. F., Vanlerberghe, G., & Whittaker, J. S. (1985). The effect of non-ionic surfactant vesicle (niosome) entrapment on the absorption and distribution of methotrexate in mice. *J Pharm Pharmacol, 37*(4), 237-242.

Baeten, J. M., Donnell, D., Ndase, P., Mugo, N. R., Campbell, J. D., Wangisi, J., . . . Partners Pr, E. P. S. T. (2012). Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med, 367*(5), 399-410. doi:10.1056/NEJMoa1108524

Baheti, G., King, J. R., Acosta, E. P., & Fletcher, C. V. (2013). Age-related differences in plasma and intracellular tenofovir concentrations in HIV-1-infected children, adolescents and adults. *AIDS, 27*(2), 221-225. doi:10.1097/QAD.0b013e32835a9a2d

Bailey, D. G., Malcolm, J., Arnold, O., & Spence, J. D. (1998). Grapefruit juice-drug interactions. *Br J Clin Pharmacol, 46*(2), 101-110.

Balzarini, J., Holy, A., Jindrich, J., Naesens, L., Snoeck, R., Schols, D., & De Clercq, E. (1993). Differential antiherpesvirus and antiretrovirus effects of the (S) and (R) enantiomers of acyclic nucleoside phosphonates: potent and selective in vitro and in vivo antiretrovirus activities of (R)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. *Antimicrob Agents Chemother, 37*(2), 332-338.

Barditch-Crovo, P., Deeks, S. G., Collier, A., Safrin, S., Coakley, D. F., Miller, M., . . . Lietman, P. S. (2001). Phase i/ii trial of the pharmacokinetics, safety, and antiretroviral activity of tenofovir disoproxil fumarate in human immunodeficiency virus-infected adults. *Antimicrob Agents Chemother, 45*(10), 2733-2739. doi:10.1128/aac.45.10.2733-2739.2001

Best, B. M., Burchett, S., Li, H., Stek, A., Hu, C., Wang, J., . . . Adolescent, A. C. T. P. T. (2015). Pharmacokinetics of tenofovir during pregnancy and postpartum. *HIV Med, 16*(8), 502-511. doi:10.1111/hiv.12252

Biesecker, G., Karimi, S., Desjardins, J., Meyer, D., Abbott, B., Bendele, R., & Richardson, F. (2003). Evaluation of mitochondrial DNA content and enzyme levels in tenofovir DF-treated rats, rhesus monkeys and woodchucks. *Antiviral Res, 58*(3), 217-225.

Blum, M. R., Chittick, G. E., Begley, J. A., & Zong, J. (2007). Steady-state pharmacokinetics of emtricitabine and tenofovir disoproxil fumarate administered alone and in combination in healthy volunteers. *J Clin Pharmacol, 47*(6), 751-759. doi:10.1177/0091270007300951

Bouazza, N., Urien, S., Hirt, D., Frange, P., Rey, E., Benaboud, S., . . . Treluyer, J. M. (2011). Population pharmacokinetics of tenofovir in HIV-1-infected pediatric patients. *J Acquir Immune Defic Syndr, 58*(3), 283-288. doi:10.1097/QAI.0b013e3182302ea8

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248-254.

Burd, J. F., & Usategui-Gomez, M. (1973). A colorimetric assay for serum lactate dehydrogenase. *Clin Chim Acta, 46*(3), 223-227.

Burns, R. N., Hendrix, C. W., & Chaturvedula, A. (2015). Population pharmacokinetics of tenofovir and tenofovir-diphosphate in healthy women. *J Clin Pharmacol, 55*(6), 629-638. doi:10.1002/jcph.461

Buti, M., Gane, E., Seto, W. K., Chan, H. L., Chuang, W. L., Stepanova, T., Investigators, G.-U.-. (2016). Tenofovir alafenamide versus tenofovir disoproxil fumarate for the treatment of patients with HBeAg-negative chronic hepatitis B virus infection: a randomised, double-blind, phase 3, non-inferiority trial. *Lancet Gastroenterol Hepatol, 1*(3), 196-206. doi:10.1016/S2468-1253(16)30107-8

Challa, S., & Chan, F. K. (2010). Going up in flames: necrotic cell injury and inflammatory diseases. *Cell Mol Life Sci, 67*(19), 3241-3253. doi:10.1007/s00018-010-0413-8

Chan, F. K., Moriwaki, K., & De Rosa, M. J. (2013). Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol Biol, 979*, 65-70. doi:10.1007/978-1-62703-290-2_7

Choi, S. U., Bui, T., & Ho, R. J. (2008). pH-dependent interactions of indinavir and lipids in nanoparticles and their ability to entrap a solute. *J Pharm Sci, 97*(2), 931-943. doi:10.1002/jps.21020

Cihlar, T., Ray, A. S., Laflamme, G., Vela, J. E., Tong, L., Fuller, M. D., . . . Rhodes, G. R. (2007). Molecular assessment of the potential for renal drug interactions between tenofovir and HIV protease inhibitors. *Antivir Ther, 12*(2), 267-272.

Cohen, C. J., Andrade-Villanueva, J., Clotet, B., Fourie, J., Johnson, M. A., Ruxrungtham, K., . . . group, T. s. (2011). Rilpivirine versus efavirenz with two background nucleoside or nucleotide reverse transcriptase inhibitors in treatment-naive adults infected with HIV-1 (THRIVE): a phase 3, randomised, non-inferiority trial. *Lancet, 378*(9787), 229-237. doi:10.1016/S0140-6736(11)60983-5

Cohen, C. J., Molina, J. M., Cassetti, I., Chetchotisakd, P., Lazzarin, A., Orkin, C., . . . Echo, T. s. g. (2013). Week 96 efficacy and safety of rilpivirine in treatment-naive, HIV-1 patients in two Phase III randomized trials. *AIDS, 27*(6), 939-950. doi:10.1097/QAD.0b013e32835cee6e

Cote, H. C., Magil, A. B., Harris, M., Scarth, B. J., Gadawski, I., Wang, N., . . . Montaner, J. S. (2006). Exploring mitochondrial nephrotoxicity as a potential mechanism of kidney dysfunction among HIV-infected patients on highly active antiretroviral therapy. *Antivir Ther, 11*(1), 79-86.

Cundy, K. C., Sueoka, C., Lynch, G. R., Griffin, L., Lee, W. A., & Shaw, J. P. (1998). Pharmacokinetics and bioavailability of the anti-human immunodeficiency virus nucleotide analog 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in dogs. *Antimicrob Agents Chemother, 42*(3), 687-690.

Curran, A., Martinez, E., Podzamczer, D., Lonca, M., Barragan, P., Crespo, M., . . . Ribera, E. (2012). Changes in body composition and mitochondrial DNA in HIV-1-infected patients switching to fixed-dose abacavir/lamivudine or tenofovir/emtricitabine: a substudy of the BICOMBO trial. *Antivir Ther, 17*(4), 711-718. doi:10.3851/IMP2081

Custodio, J. M., Fordyce, M., Garner, W., Vimal, M., Ling, K. H., Kearney, B. P., & Ramanathan, S. (2016). Pharmacokinetics and Safety of Tenofovir Alafenamide in HIV-Uninfected Subjects with Severe Renal Impairment. *Antimicrob Agents Chemother, 60*(9), 5135-5140. doi:10.1128/AAC.00005-16

Deeks, S. G., Barditch-Crovo, P., Lietman, P. S., Hwang, F., Cundy, K. C., Rooney, J. F., Kahn, J. O. (1998). Safety, pharmacokinetics, and antiretroviral activity of intravenous 9-[2-(R)-(Phosphonomethoxy)propyl]adenine, a novel anti-human immunodeficiency virus (HIV) therapy, in HIV-infected adults. *Antimicrob Agents Chemother, 42*(9), 2380-2384.

Del Palacio, M., Romero, S., & Casado, J. L. (2012). Proximal tubular renal dysfunction or damage in HIV-infected patients. *AIDS Rev, 14*(3), 179-187.

DiCenzo, R., Forrest, A., Squires, K. E., Hammer, S. M., Fischl, M. A., Wu, H., . . . Adult, A. C. T. G. P. S. T. (2003). Indinavir, efavirenz, and abacavir pharmacokinetics in human immunodeficiency virus-infected subjects. *Antimicrob Agents Chemother, 47*(6), 1929-1935.

Do Amaral, C. L., Francescato, H. D., Coimbra, T. M., Costa, R. S., Darin, J. D., Antunes, L. M., & Bianchi Mde, L. (2008). Resveratrol attenuates cisplatin-induced nephrotoxicity in rats. *Arch Toxicol, 82*(6), 363-370. doi:10.1007/s00204-007-0262-x

Dore, G. J., Cooper, D. A., Pozniak, A. L., DeJesus, E., Zhong, L., Miller, M. D., . . . Cheng, A. K. (2004). Efficacy of tenofovir disoproxil fumarate in antiretroviral therapy-naive and -experienced patients coinfected with HIV-1 and hepatitis B virus. *J Infect Dis, 189*(7), 1185-1192. doi:10.1086/380398

Durand-Gasselin, L., Van Rompay, K. K., Vela, J. E., Henne, I. N., Lee, W. A., Rhodes, G. R., & Ray, A. S. (2009). Nucleotide analogue prodrug tenofovir disoproxil enhances lymphoid cell loading following oral administration in monkeys. *Mol Pharm, 6*(4), 1145-1151. doi:10.1021/mp900036s

Eakins, J., Bauch, C., Woodhouse, H., Park, B., Bevan, S., Dilworth, C., & Walker, P. (2016). A combined in vitro approach to improve the prediction of mitochondrial toxicants. *Toxicol In Vitro, 34*, 161-170. doi:10.1016/j.tiv.2016.03.016

Ezinga, M., Wetzels, J. F., Bosch, M. E., van der Ven, A. J., & Burger, D. M. (2014). Long-term treatment with tenofovir: prevalence of kidney tubular dysfunction and its association with tenofovir plasma concentration. *Antivir Ther, 19*(8), 765-771. doi:10.3851/IMP2761

Fabian, J., Naicker, S., Goetsch, S., & Venter, W. D. (2013). The clinical and histological response of HIV-associated kidney disease to antiretroviral therapy in South Africans. *Nephrol Dial Transplant, 28*(6), 1543-1554. doi:10.1093/ndt/gft010

Fridovich, I. (1995). Superoxide radical and superoxide dismutases. *Annu Rev Biochem, 64*, 97-112. doi:10.1146/annurev.bi.64.070195.000525

Fu, B., Zhao, J., Peng, W., Wu, H., & Zhang, Y. (2017). Resveratrol rescues cadmium-induced mitochondrial injury by enhancing transcriptional regulation of PGC-1alpha and SOD2 via the Sirt3/FoxO3a pathway in TCMK-1 cells. *Biochem Biophys Res Commun, 486*(1), 198-204. doi:10.1016/j.bbrc.2017.03.027

Fux, C. A., Simcock, M., Wolbers, M., Bucher, H. C., Hirschel, B., Opravil, M., . . . Furrer, H. (2007). Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV Cohort Study. *Antivir Ther, 12*(8), 1165-1173.

Gallant, J. E., Daar, E. S., Raffi, F., Brinson, C., Ruane, P., DeJesus, E., . . . Rhee, M. S. (2016). Efficacy and safety of tenofovir alafenamide versus tenofovir disoproxil fumarate given as fixed-dose combinations containing emtricitabine as backbones for treatment of HIV-1 infection in virologically suppressed adults: a randomised, double-blind, active-controlled phase 3 trial. *Lancet HIV, 3*(4), e158-165. doi:10.1016/S2352-3018(16)00024-2

German, P., Warren, D., West, S., Hui, J., & Kearney, B. P. (2010). Pharmacokinetics and bioavailability of an integrase and novel pharmacoenhancer-containing single-tablet fixed-dose combination regimen for the treatment of HIV. *J Acquir Immune Defic Syndr, 55*(3), 323-329. doi:10.1097/QAI.0b013e3181eb376b

Gilead Sciences, L. (2001, April 2017). Full Prescribing Information, Viread.

Gilead Sciences, L. (2004, 2017). Full Prescribing Information, Truvada.

Gilead Sciences, L. (2006, April 2017). Full Prescribing Information, Atripla.

Gilead Sciences, L. (2015a, 2017). Full Prescribing Information, Descovy.

Gilead Sciences, L. (2015b, 2017). Full Prescribing Information, Genvoya.

Gilead Sciences, L. (2017, April 2017). Full Prescribing Information, Vemlidy

Glover, M., Hebert, V. Y., Nichols, K., Xue, S. Y., Thibeaux, T. M., Zavecz, J. A., & Dugas, T. R. (2014). Overexpression of mitochondrial antioxidant manganese superoxide dismutase (MnSOD) provides protection against AZT- or 3TC-induced endothelial dysfunction. *Antiviral Res, 111*, 136-142. doi:10.1016/j.antiviral.2014.09.010

Gong, X., Ivanov, V. N., Davidson, M. M., & Hei, T. K. (2015). Tetramethylpyrazine (TMP) protects against sodium arsenite-induced nephrotoxicity by suppressing ROS production, mitochondrial dysfunction, pro-inflammatory signaling pathways and programed cell death. *Arch Toxicol, 89*(7), 1057-1070. doi:10.1007/s00204-014-1302-y

Grant, R. M., Lama, J. R., Anderson, P. L., McMahan, V., Liu, A. Y., Vargas, L., . . . iPrEx Study, T. (2010). Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med, 363*(27), 2587-2599. doi:10.1056/NEJMoa1011205

Gu, Xin-Yue; Xing, Wen-Min; Xu, Jia-Dong; Lu, Hong. (2016). Andrographolide-induced apoptosis in human renal tubular epithelial cells: Roles of endoplasmic reticulum stress and inflammatory response. *Environmental Toxicology and Pharmacology*(45), 257-264. doi: 10.1016/j.etap.2016.02.004

Gunness, P., Aleksa, K., Kosuge, K., Ito, S., & Koren, G. (2010). Comparison of the novel HK-2 human renal proximal tubular cell line with the standard LLC-PK1 cell line in studying drug-induced nephrotoxicity. *Can J Physiol Pharmacol, 88*(4), 448-455. doi:10.1139/y10-023

Hall, A. M., Hendry, B. M., Nitsch, D., & Connolly, J. O. (2011). Tenofovir-associated kidney toxicity in HIV-infected patients: a review of the evidence. *Am J Kidney Dis, 57*(5), 773-780. doi:10.1053/j.ajkd.2011.01.022

Havens, P. L., Kiser, J. J., Stephensen, C. B., Hazra, R., Flynn, P. M., Wilson, C. M., . . . Adolescent Medicine Trials Network for, H. I. V. A. I. S. T. (2013). Association of higher plasma vitamin D binding protein and lower free calcitriol levels with tenofovir disoproxil fumarate use and plasma and intracellular tenofovir pharmacokinetics: cause of a functional vitamin D deficiency? *Antimicrob Agents Chemother, 57*(11), 5619-5628. doi:10.1128/AAC.01096-13

Hawkins, T., Veikley, W., St Claire, R. L., 3rd, Guyer, B., Clark, N., & Kearney, B. P. (2005). Intracellular pharmacokinetics of tenofovir diphosphate, carbovir triphosphate, and lamivudine triphosphate in patients receiving triple-nucleoside regimens. *J Acquir Immune Defic Syndr, 39*(4), 406-411.

Hazra, R., Balis, F. M., Tullio, A. N., DeCarlo, E., Worrell, C. J., Steinberg, S. M., . . . Zeichner, S. L. (2004). Single-dose and steady-state pharmacokinetics of tenofovir disoproxil fumarate in human immunodeficiency virus-infected children. *Antimicrob Agents Chemother, 48*(1), 124-129.

Hendrix, C. W., Chen, B. A., Guddera, V., Hoesley, C., Justman, J., Nakabiito, C., . . . Bumpus, N. N. (2013). MTN-001: randomized pharmacokinetic cross-over study comparing tenofovir vaginal gel and oral tablets in vaginal tissue and other compartments. *PLoS One, 8*(1), e55013. doi:10.1371/journal.pone.0055013

Herlitz, L. C., Mohan, S., Stokes, M. B., Radhakrishnan, J., D'Agati, V. D., & Markowitz, G. S. (2010). Tenofovir nephrotoxicity: acute tubular necrosis with distinctive clinical, pathological, and mitochondrial abnormalities. *Kidney Int, 78*(11), 1171-1177. doi:10.1038/ki.2010.318

Hirt, D., Ekouevi, D. K., Pruvost, A., Urien, S., Arrive, E., Blanche, S., . . . Treluyer, J. M. (2011). Plasma and intracellular tenofovir pharmacokinetics in the neonate (ANRS 12109 trial, step 2). *Antimicrob Agents Chemother, 55*(6), 2961-2967. doi:10.1128/AAC.01377-10

Honda, Y., Ushigome, F., Koyabu, N., Morimoto, S., Shoyama, Y., Uchiumi, T., . . . Sawada, Y. (2004). Effects of grapefruit juice and orange juice components on P-glycoprotein- and MRP2-mediated drug efflux. *Br J Pharmacol, 143*(7), 856-864. doi:10.1038/sj.bjp.070600

Humphrey, M. L., Cole, M. P., Pendergrass, J. C., & Kiningham, K. K. (2005). Mitochondrial mediated thimerosal-induced apoptosis in a human neuroblastoma cell line (SK-N-SH). *Neurotoxicology, 26*(3), 407-416. doi:10.1016/j.neuro.2005.03.008

Izzedine, H., Launay-Vacher, V., & Deray, G. (2005). Antiviral drug-induced nephrotoxicity. *Am J Kidney Dis, 45*(5), 804-817.

Jacobson, D. L., Lindsey, J. C., Gordon, C. M., Moye, J., Hardin, D. S., Mulligan, K., . . . Pediatric, A. C. T. G. P. t. (2010). Total body and spinal bone mineral density across Tanner stage in perinatally HIV-infected and uninfected children and youth in PACTG 1045. *AIDS, 24*(5), 687-696. doi:10.1097/QAD.0b013e328336095d

Jia, Q. H., Ha, X. Q., Yang, X. P., Chang, Y. W., & Yang, Z. H. (2011). [The toxic effects of lead acetate on the apoptosis and the ultrastructure in human renal tubular epithelial cells (HK-2)]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi, 29*(9), 674-677. doi:10..3760/cma.j.issn.1001-9391.2011.09.010

Jiang, X., & Wang, X. (2000). Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1. *J Biol Chem, 275*(40), 31199-31203. doi:10.1074/jbc.C000405200

Joshi, D., Mittal, D. K., Shukla, S., Srivastav, A. K., & Srivastav, S. K. (2014). N-acetyl cysteine and selenium protects mercuric chloride-induced oxidative stress and antioxidant defense system in liver and kidney of rats: a histopathological approach. *J Trace Elem Med Biol, 28*(2), 218-226. doi:10.1016/j.jtemb.2013.12.006

Kamboj, S., Saini, V., & Bala, S. (2014). Formulation and characterization of drug loaded nonionic surfactant vesicles (niosomes) for oral bioavailability enhancement. *ScientificWorldJournal, 2014*, 959741. doi:10.1155/2014/959741

Kearney, B. P., Flaherty, J. F., & Shah, J. (2004). Tenofovir disoproxil fumarate: clinical pharmacology and pharmacokinetics. *Clin Pharmacokinet, 43*(9), 595-612. doi:10.2165/00003088-200443090-00003

Kearney, B. P., & Mathias, A. (2009). Lack of effect of tenofovir disoproxil fumarate on pharmacokinetics of hormonal contraceptives. *Pharmacotherapy, 29*(8), 924-929. doi:10.1592/phco.29.8.924

King, J. R., Yogev, R., Jean-Philippe, P., Graham, B., Wiznia, A., Britto, P., . . . Team, P. P. (2011). Steady-state pharmacokinetics of tenofovir-based regimens in HIV-infected pediatric patients. *Antimicrob Agents Chemother, 55*(9), 4290-4294. doi:10.1128/AAC.01334-10

Kohler, J. J., Hosseini, S. H., Green, E., Abuin, A., Ludaway, T., Russ, R., . . . Lewis, W. (2011). Tenofovir renal proximal tubular toxicity is regulated by OAT1 and MRP4 transporters. *Lab Invest, 91*(6), 852-858. doi:10.1038/labinvest.2011.48

Kohler, J. J., Hosseini, S. H., Hoying-Brandt, A., Green, E., Johnson, D. M., Russ, R., . . . Lewis, W. (2009). Tenofovir renal toxicity targets mitochondria of renal proximal tubules. *Lab Invest, 89*(5), 513-519. doi:10.1038/labinvest.2009.14

Kondo, M., Inamura, H., Matsumura, K., & Matsuoka, M. (2012). Cadmium activates extracellular signal-regulated kinase 5 in HK-2 human renal proximal tubular cells. *Biochem Biophys Res Commun, 421*(3), 490-493. doi:10.1016/j.bbrc.2012.04.028

Koyuturk, M., Yanardag, R., Bolkent, S., & Tunali, S. (2007). The potential role of combined anti-oxidants against cadmium toxicity on liver of rats. *Toxicol Ind Health, 23*(7), 393-401. doi:10.1177/0748233707081907

Lash, L. H., Putt, D. A., & Cai, H. (2006). Membrane transport function in primary cultures of human proximal tubular cells. *Toxicology, 228*(2-3), 200-218. doi:10.1016/j.tox.2006.08.035

Laurinavicius, A., Hurwitz, S., & Rennke, H. G. (1999). Collapsing glomerulopathy in HIV and non-HIV patients: a clinicopathological and follow-up study. *Kidney Int, 56*(6), 2203-2213. doi:10.1046/j.1523-1755.1999.00769.x

Lebrecht, D., Venhoff, A. C., Kirschner, J., Wiech, T., Venhoff, N., & Walker, U. A. (2009). Mitochondrial tubulopathy in tenofovir disoproxil fumarate-treated rats. *J Acquir Immune Defic Syndr, 51*(3), 258-263.

Lee, W. A., He, G. X., Eisenberg, E., Cihlar, T., Swaminathan, S., Mulato, A., & Cundy, K. C. (2005). Selective intracellular activation of a novel prodrug of the human immunodeficiency virus reverse transcriptase inhibitor tenofovir leads to preferential distribution and accumulation in lymphatic tissue. *Antimicrob Agents Chemother, 49*(5), 1898-1906. doi:10.1128/AAC.49.5.1898-1906.2005

Lepist, E. I., Phan, T. K., Roy, A., Tong, L., Maclennan, K., Murray, B., & Ray, A. S. (2012). Cobicistat boosts the intestinal absorption of transport substrates, including HIV protease inhibitors and GS-7340, in vitro. *Antimicrob Agents Chemother, 56*(10), 5409-5413. doi:10.1128/AAC.01089-12

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., & Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell, 86*(1), 147-157.

Maggi, P., Montinaro, V., Bellacosa, C., Pietanza, S., Volpe, A., Graziano, G., . . . Angarano, G. (2012). Early markers of tubular dysfunction in antiretroviral-experienced HIV-infected patients treated with tenofovir versus abacavir. *AIDS Patient Care STDS, 26*(1), 5-11. doi:10.1089/apc.2011.0185

Maliakel, D. M., Kagiya, T. V., & Nair, C. K. (2008). Prevention of cisplatin-induced nephrotoxicity by glucosides of ascorbic acid and alpha-tocopherol. *Exp Toxicol Pathol, 60*(6), 521-527. doi:10.1016/j.etp.2008.04.015

Markowitz, M., Zolopa, A., Squires, K., Ruane, P., Coakley, D., Kearney, B., . . . Lee, W. A. (2014). Phase I/II study of the pharmacokinetics, safety and antiretroviral activity of tenofovir alafenamide, a new prodrug of the HIV reverse transcriptase inhibitor tenofovir, in HIV-infected adults. *J Antimicrob Chemother, 69*(5), 1362-1369. doi:10.1093/jac/dkt532

Marrazzo, J. M., Ramjee, G., Richardson, B. A., Gomez, K., Mgodi, N., Nair, G., . . . Team, V. S. (2015). Tenofovir-based preexposure prophylaxis for HIV infection among African women. *N Engl J Med, 372*(6), 509-518. doi:10.1056/NEJMoa1402269

Mathias, A. A., Hinkle, J., Menning, M., Hui, J., Kaul, S., Kearney, B. P., & Efavirenz/Emtricitabine/Tenofovir Disoproxil Fumarate Single-Tablet Regimen Development, T. (2007). Bioequivalence of efavirenz/emtricitabine/tenofovir disoproxil fumarate single-tablet regimen. *J Acquir Immune Defic Syndr, 46*(2), 167-173. doi:10.1097/QAI.0b013e3181427835

Mathias, A. M., M.; Wiser, L.; Wei, X; Dave, A.; Chuck, S.; Kearney, BP. (2012). Bioequivalence of the Emtricitabine/Rilpivirine/Tenofovir Disoproxil Fumarate Single Tablet Regimen *J Bioequiv Avilab, 4*, 100-105.

Miller, M. D., Margot, N. A., Hertogs, K., Larder, B., & Miller, V. (2001). Antiviral activity of tenofovir (PMPA) against nucleoside-resistant clinical HIV samples. *Nucleosides Nucleotides Nucleic Acids, 20*(4-7), 1025-1028. doi:10.1081/ncn-100002483

Mills, A., Arribas, J. R., Andrade-Villanueva, J., DiPerri, G., Van Lunzen, J., Koenig, E., . . . team, G.-U.-. (2016). Switching from tenofovir disoproxil fumarate to tenofovir alafenamide in antiretroviral regimens for virologically suppressed adults with HIV-1 infection: a randomised, active-controlled, multicentre, open-label, phase 3, non-inferiority study. *Lancet Infect Dis, 16*(1), 43-52. doi:10.1016/S1473-3099(15)00348-5

Mirochnick, M., Taha, T., Kreitchmann, R., Nielsen-Saines, K., Kumwenda, N., Joao, E., . . . Team, H. P. (2014). Pharmacokinetics and safety of tenofovir in HIV-infected women during labor and their infants during the first week of life. *J Acquir Immune Defic Syndr, 65*(1), 33-41. doi:10.1097/QAI.0b013e3182a921eb

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal, 20*(7), 1126-1167. doi:10.1089/ars.2012.5149

Miyamoto, Y., Iwao, Y., Mera, K., Watanabe, H., Kadowaki, D., Ishima, Y., . . . Maruyama, T. (2012). A uremic toxin, 3-carboxy-4-methyl-5-propyl-2-furanpropionate induces cell damage to proximal tubular cells via the generation of a radical intermediate. *Biochem Pharmacol, 84*(9), 1207-1214. doi:10.1016/j.bcp.2012.07.033

Molina, J. M., Cahn, P., Grinsztejn, B., Lazzarin, A., Mills, A., Saag, M., . . . group, E. s. (2011). Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naive adults infected with HIV-1 (ECHO): a phase 3 randomised double-blind active-controlled trial. *Lancet, 378*(9787), 238-246. doi:10.1016/S0140-6736(11)60936-7

Moquin, D., & Chan, F. K. (2010). The molecular regulation of programmed necrotic cell injury. *Trends Biochem Sci, 35*(8), 434-441. doi:10.1016/j.tibs.2010.03.001

Morgan, M. J. L., Z.G. (2010). Reactive oxygen species in TNF alpha-induced signaling and cell death. *Mol Cells, 30*(1), 1-12.

Murphy, R. A., Stafford, R. M., Petrasovits, B. A., Boone, M. A., & Valentovic, M. A. (2017). Establishment of HK-2 Cells as a Relevant Model to Study Tenofovir-Induced Cytotoxicity. *Int J Mol Sci, 18*(3). doi:10.3390/ijms18030531

Nieskens, T. T., Peters, J. G., Schreurs, M. J., Smits, N., Woestenenk, R., Jansen, K., . . . Masereeuw, R. (2016). A Human Renal Proximal Tubule Cell Line with Stable Organic Anion Transporter 1 and 3 Expression Predictive for Antiviral-Induced Toxicity. *AAPS J, 18*(2), 465-475. doi:10.1208/s12248-016-9871-8

Nomura, M., Motohashi, H., Sekine, H., Katsura, T., & Inui, K. (2012). Developmental expression of renal organic anion transporters in rat kidney and its effect on renal secretion of phenolsulfonphthalein. *Am J Physiol Renal Physiol, 302*(12), F1640-1649. doi:10.1152/ajprenal.00525.2011

O'Brien, K. O., Razavi, M., Henderson, R. A., Caballero, B., & Ellis, K. J. (2001). Bone mineral content in girls perinatally infected with HIV. *Am J Clin Nutr, 73*(4), 821-826.

Paolicchi, A., Sotiropuolou, M., Perego, P., Daubeuf, S., Visvikis, A., Lorenzini, E., . . . Pompella, A. (2003). gamma-Glutamyl transpeptidase catalyses the extracellular detoxification of cisplatin in a human cell line derived from the proximal convoluted tubule of the kidney. *Eur J Cancer, 39*(7), 996-1003.

Polli, J. W., Jarrett, J. L., Studenberg, S. D., Humphreys, J. E., Dennis, S. W., Brouwer, K. R., & Woolley, J. L. (1999). Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharm Res, 16*(8), 1206-1212.

Pozniak, A., Arribas, J. R., Gathe, J., Gupta, S. K., Post, F. A., Bloch, M., . . . Team, G.-U.-S. (2016). Switching to Tenofovir Alafenamide, Coformulated With Elvitegravir, Cobicistat, and Emtricitabine, in HIV-Infected Patients With Renal Impairment: 48-Week Results From a Single-Arm, Multicenter, Open-Label Phase 3 Study. *J Acquir Immune Defic Syndr, 71*(5), 530-537. doi:10.1097/QAI.0000000000000908

Puthanakit, T., Saksawad, R., Bunupuradah, T., Wittawatmongkol, O., Chuanjaroen, T., Ubolyam, S., . . . Chokephaibulkit, K. (2012). Prevalence and risk factors of low bone mineral density among perinatally HIV-infected Thai adolescents receiving antiretroviral therapy. *J Acquir Immune Defic Syndr, 61*(4), 477-483. doi:10.1097/QAI.0b013e31826ea89b

Quesada, P. R., Esteban, L. L., Garcia, J. R., Sanchez, R. V., Garcia, T. M., Alonso-Vega, G. G., & Ferrandez, J. S. (2015). Incidence and risk factors for tenofovir-associated renal toxicity in HIV-infected patients. *Int J Clin Pharm, 37*(5), 865-872. doi:10.1007/s11096-015-0132-1

Ramamoorthy, H., Abraham, P., & Isaac, B. (2014). Mitochondrial dysfunction and electron transport chain complex defect in a rat model of tenofovir disoproxil fumarate nephrotoxicity. *J Biochem Mol Toxicol, 28*(6), 246-255. doi:10.1002/jbt.21560

Ravi, P. R., Joseph, S., Avula, U. S. R., & Anthireddy, S. (2015). A Simple Liquid Chromatographic Method for the Determination of Tenofovir in Rat Plasma and Its Application to Pharmacokinetic Studies. *Acta Chromatographica, 27*(4), 597-612. doi:10.1556/AChrom.27.2015.4.2

Ray, A. S., Cihlar, T., Robinson, K. L., Tong, L., Vela, J. E., Fuller, M. D., . . . Rhodes, G. R. (2006). Mechanism of active renal tubular efflux of tenofovir. *Antimicrob Agents Chemother, 50*(10), 3297-3304. doi:10.1128/AAC.00251-06

Ray, P. E. (2012). HIV-associated nephropathy: a diagnosis in evolution. *Nephrol Dial Transplant, 27*(11), 3969-3972. doi:10.1093/ndt/gfs114

Robbins, B. L., Srinivas, R. V., Kim, C., Bischofberger, N., & Fridland, A. (1998). Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-R-(2-phosphonomethoxypropyl)adenine (PMPA), Bis(isopropyloxymethylcarbonyl)PMPA. *Antimicrob Agents Chemother, 42*(3), 612-617.

Ryan, M. J., Johnson, G., Kirk, J., Fuerstenberg, S. M., Zager, R. A., & Torok-Storb, B. (1994). HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int, 45*(1), 48-57.

Saag, M. S. (2006). Emtricitabine, a new antiretroviral agent with activity against HIV and hepatitis B virus. *Clin Infect Dis, 42*(1), 126-131. doi:10.1086/498348

Sax, P. E., Wohl, D., Yin, M. T., Post, F., DeJesus, E., Saag, M., . . . Team, G.-U.-S. (2015). Tenofovir alafenamide versus tenofovir disoproxil fumarate, coformulated with elvitegravir, cobicistat, and emtricitabine, for initial treatment of HIV-1 infection: two randomised, double-blind, phase 3, non-inferiority trials. *Lancet, 385*(9987), 2606-2615. doi:10.1016/S0140-6736(15)60616-X

Schtscherbyna, A., Pinheiro, M. F., Mendonca, L. M., Gouveia, C., Luiz, R. R., Machado, E. S., & Farias, M. L. (2012). Factors associated with low bone mineral density in a Brazilian cohort of vertically HIV-infected adolescents. *Int J Infect Dis, 16*(12), e872-878. doi:10.1016/j.ijid.2012.07.019

Schwartz, E. J., & Klotman, P. E. (1998). Pathogenesis of human immunodeficiency virus (HIV)-associated nephropathy. *Semin Nephrol, 18*(4), 436-445.

Schweichel, J. U., & Merker, H. J. (1973). The morphology of various types of cell death in prenatal tissues. *Teratology, 7*(3), 253-266. doi:10.1002/tera.1420070306

Sentenac, S., Fernandez, C., Thuillier, A., Lechat, P., & Aymard, G. (2003). Sensitive determination of tenofovir in human plasma samples using reversed-phase liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci, 793*(2), 317-324.

Shailender, J., Ravi, P. R., Saha, P., & Myneni, S. (2017). Oral pharmacokinetic interaction of ester rich fruit juices and pharmaceutical excipients with tenofovir disoproxil fumarate in male Wistar rats. *Xenobiotica*, 1-8. doi:10.1080/00498254.2016.1269375

Shaw, J. P., Sueoko, C. M., Oliyai, R., Lee, W. A., Arimilli, M. N., Kim, C. U., & Cundy, K. C. (1997). Metabolism and pharmacokinetics of novel oral prodrugs of 9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) in dogs. *Pharm Res, 14*(12), 1824-1829.

Shen, Z., Fahey, J. V., Bodwell, J. E., Rodriguez-Garcia, M., Kashuba, A. D., & Wira, C. R. (2014). Sex hormones regulate tenofovir-diphosphate in female reproductive tract cells in culture. *PLoS One, 9*(6), e100863. doi:10.1371/journal.pone.0100863

Sherman, E. M., Worley, M. V., Unger, N. R., Gauthier, T. P., & Schafer, J. J. (2015). Cobicistat: Review of a Pharmacokinetic Enhancer for HIV Infection. *Clin Ther, 37*(9), 1876-1893. doi:10.1016/j.clinthera.2015.07.022

Shiomi, M., Matsuki, S., Ikeda, A., Ishikawa, T., Nishino, N., Kimura, M., & Irie, S. (2014). Effects of a protein-rich drink or a standard meal on the pharmacokinetics of elvitegravir, cobicistat, emtricitabine and tenofovir in healthy Japanese male subjects: a randomized, three-way crossover study. *J Clin Pharmacol, 54*(6), 640-648. doi:10.1002/jcph.283

Spinks, C. B., Zidan, A. S., Khan, M. A., Habib, M. J., & Faustino, P. J. (2017). Pharmaceutical characterization of novel tenofovir liposomal formulations for enhanced oral drug delivery: in vitro pharmaceutics and Caco-2 permeability investigations. *Clin Pharmacol, 9*, 29-38. doi:10.2147/CPAA.S119875

Squires, K., Pozniak, A. L., Pierone, G., Jr., Steinhart, C. R., Berger, D., Bellos, N. C., . . . Study, T. (2003). Tenofovir disoproxil fumarate in nucleoside-resistant HIV-1 infection: a randomized trial. *Ann Intern Med, 139*(5 Pt 1), 313-320.

Terneus, M. V., Kiningham, K. K., Carpenter, A. B., Sullivan, S. B., & Valentovic, M. A. (2007). Comparison of S-Adenosyl-L-methionine and N-acetylcysteine protective effects on acetaminophen hepatic toxicity. *J Pharmacol Exp Ther, 320*(1), 99-107. doi:10.1124/jpet.106.111872

Tong, L., Phan, T. K., Robinson, K. L., Babusis, D., Strab, R., Bhoopathy, S., . . . Ray, A. S. (2007). Effects of human immunodeficiency virus protease inhibitors on the intestinal absorption of tenofovir disoproxil fumarate in vitro. *Antimicrob Agents Chemother, 51*(10), 3498-3504. doi:10.1128/AAC.00671-07

Uwai, Y., Ida, H., Tsuji, Y., Katsura, T., & Inui, K. (2007). Renal transport of adefovir, cidofovir, and tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2). *Pharm Res, 24*(4), 811-815. doi:10.1007/s11095-006-9196-x

Valentovic, M. A., Ball, J. G., Brown, J. M., Terneus, M. V., McQuade, E., Van Meter, S., . . . Williams, T. (2014). Resveratrol attenuates cisplatin renal cortical cytotoxicity by modifying oxidative stress. *Toxicol In Vitro, 28*(2), 248-257. doi:10.1016/j.tiv.2013.11.001

van der Sandt, I. C., Vos, C. M., Nabulsi, L., Blom-Roosemalen, M. C., Voorwinden, H. H., de Boer, A. G., & Breimer, D. D. (2001). Assessment of active transport of HIV protease inhibitors in various cell lines and the in vitro blood--brain barrier. *AIDS, 15*(4), 483-491.

Van Gelder, J., Annaert, P., Naesens, L., De Clercq, E., Van den Mooter, G., Kinget, R., & Augustijns, P. (1999). Inhibition of intestinal metabolism of the antiviral ester prodrug bis(POC)-PMPA by nature-identical fruit extracts as a strategy to enhance its oral absorption: an in vitro study. *Pharm Res, 16*(7), 1035-1040.

van Gelder, J., Deferme, S., Naesens, L., De Clercq, E., van den Mooter, G., Kinget, R., & Augustijns, P. (2002). Intestinal absorption enhancement of the ester prodrug tenofovir disoproxil fumarate through modulation of the biochemical barrier by defined ester mixtures. *Drug Metab Dispos, 30*(8), 924-930.

Verhelst, D., Monge, M., Meynard, J. L., Fouqueray, B., Mougenot, B., Girard, P. M., . . . Rossert, J. (2002). Fanconi syndrome and renal failure induced by tenofovir: a first case report. *Am J Kidney Dis, 40*(6), 1331-1333. doi:10.1053/ajkd.2002.36924

Vidal, F., Domingo, J. C., Guallar, J., Saumoy, M., Cordobilla, B., Sanchez de la Rosa, R., . . . Domingo, P. (2006). In vitro cytotoxicity and mitochondrial toxicity of tenofovir alone and in combination with other antiretrovirals in human renal proximal tubule cells. *Antimicrob Agents Chemother, 50*(11), 3824-3832. doi:10.1128/AAC.00437-06

Wang, F., & Flint, O. P. (2013). BMS-986001, an HIV nucleoside reverse transcriptase inhibitor, does not degrade mitochondrial DNA in long-term primary cultures of cells isolated from human kidney, muscle, and adipose tissue. *Antimicrob Agents Chemother, 57*(12), 6205-6212. doi:10.1128/aac.01206-13

Wang, J., Zhu, H., Liu, X., & Liu, Z. (2014). N-acetylcysteine protects against cadmium-induced oxidative stress in rat hepatocytes. *J Vet Sci, 15*(4), 485-493.

Washington, C. B., Duran, G. E., Man, M. C., Sikic, B. I., & Blaschke, T. F. (1998). Interaction of anti-HIV protease inhibitors with the multidrug transporter P-glycoprotein (P-gp) in human cultured cells. *J Acquir Immune Defic Syndr Hum Retrovirol, 19*(3), 203-209.

Weiss, J., Theile, D., Ketabi-Kiyanvash, N., Lindenmaier, H., & Haefeli, W. E. (2007). Inhibition of MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 by nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors. *Drug Metab Dispos, 35*(3), 340-344. doi:10.1124/dmd.106.012765

Wira, C. R., & Fahey, J. V. (2008). A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *AIDS, 22*(15), 1909-1917. doi:10.1097/QAD.0b013e3283060ea4

Wohl, D., Oka, S., Clumeck, N., Clarke, A., Brinson, C., Stephens, J., . . . Study, T. (2016). Brief Report: A Randomized, Double-Blind Comparison of Tenofovir Alafenamide Versus Tenofovir Disoproxil Fumarate, Each Coformulated With Elvitegravir, Cobicistat, and Emtricitabine for Initial HIV-1 Treatment: Week 96 Results. *J Acquir Immune Defic Syndr, 72*(1), 58-64. doi:10.1097/QAI.0000000000000940

Woodward, C. L., Hall, A. M., Williams, I. G., Madge, S., Copas, A., Nair, D., . . . Connolly, J. O. (2009). Tenofovir-associated renal and bone toxicity. *HIV Med, 10*(8), 482-487. doi:10.1111/j.1468-1293.2009.00716.x

Wyatt, C. M., Morgello, S., Katz-Malamed, R., Wei, C., Klotman, M. E., Klotman, P. E., & D'Agati, V. D. (2009). The spectrum of kidney disease in patients with AIDS in the era of antiretroviral therapy. *Kidney Int, 75*(4), 428-434. doi:10.1038/ki.2008.604

Yuan, L. C., Dahl, T. C., & Oliyai, R. (2001). Degradation kinetics of oxycarbonyloxymethyl prodrugs of phosphonates in solution. *Pharm Res, 18*(2), 234-237.

Zager, R. A., Johnson, A. C., & Geballe, A. (2007). Gentamicin suppresses endotoxin-driven TNF-alpha production in human and mouse proximal tubule cells. *Am J Physiol Renal Physiol, 293*(4), F1373-1380. doi:10.1152/ajprenal.00333.2007

Zhang, Yijian; Liu, Yu; Xiang, Xudong; Dong, Zheng. (2013). Paclitaxel Ameliorates Lipopolysaccharide-Induced Kidney Injury by Binding Myeloid Differentiation Protein-2 to Block Toll-Like Recepor 4-Mediated Nuclear Factor-kB Activation and Cytokine Production. *J Pharmacol Exp Ther, 345*, 69-75.

Zhang, X. W., R; Piotrowski, M; Zhang, H: Leach, K. (2015). Intracellular concentrations determine the cytotoxicity of adefovir, cidofovir, and tenofovir. *Toxicol In Vitro*(29), 251-258.

APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

June 5, 2017

Rachel A. Murphy
Department of Biomedical Sciences
Toxicology Research Cluster
Byrd Biotech Science Building Rm 435
Marshall University School of Medicine

Dear Ms. Murphy:

This letter is in response to the submitted dissertation abstract entitled *"Tenofovir-Induced Nephrotoxicity: A Mechanistic Study."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: LIST OF ABBREVIATIONS

4-HNE….4-hydroxynonenal

ADP….adenosine diphosphate

AIDS….Acquired Immunodeficiency Syndrome

Apaf-1….Apoptotic protease activating factor 1

ART….antiretroviral therapy

Asc….ascorbic acid

ATCC….American Type Culture Collection

ATP….adenosine triphosphate

AUC….area under the curve

BSA….bovine serum albumin

BMD….bone mineral density

BUN….blood urea nitrogen

CI….confidence interval

COBI….cobicistat

COX-IV….cytochrome c oxidase IV

CYP….cytochrome P450

DMSO….dimethylsulfoxide

DNPH….dinitrophenylhydrazine

EC50….effective concentration in 50% of population

ECAR….extracellular acidification rate

EFV….efavirenz

EVG….elvitegravir

FCCP….carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

FDA….Food and Drug Administration

FSGS….focal segmental glomerulosclerosis

FTC….emtricitabine

GFR….glomerular filtration rate

GSH….glutathione

HAART….highly active antiretroviral therapy

HBV….hepatitis B

HIV….human immunodeficiency virus

HIVAN….HIV-associated nephropathy

HK-2….human kidney 2

HRP….horse radish peroxidase

LDH….lactate dehydrogenase

LPV….lopinavir

MDCKII….Madin-Darby canine kidney II

MnSOD….manganese superoxide dismutase

MtCO-1….mitochondrial-encoded cytochrome c oxidase subunit

MRP1….multidrug resistance-associated protein 1

MRP2….multidrug resistance-associated protein 2

MRP3….multidrug resistance-associated protein 3

MRP4….multidrug resistance-associated protein 4

MtDNA….mitochondrial DNA

MTT….3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC….N-acetyl-L-cysteine

NADPH….nicotinamide adenine dinucleotide phosphate

OAT1….organic anion transporter 1

OAT3….organic anion transporter 3

OCP….oral contraceptive pills

OCR….oxygen consumption rate

PBMCs….peripheral blood mononuclear cells

PBS….phosphate buffered saline

Pgp….P glycoprotein

PrEP….pre-exposure chemoprophylaxis

Res….resveratrol

RNS….reactive nitrogen species

ROS….reactive oxygen species

RPV….rilpivirine

RTV….ritonavir

SQV….saquinavir

TAF….tenofovir alafenamide

TBST….tris buffered saline plus Tween 20

TDF….tenofovir disoproxil fumarate

TDP….tenofovir diphosphate

TFV….tenofovir

TMB….3,3*t*,5,5*t*-tetramethylbenzidine

TNFα….tumor necrosis factor alpha

APPENDIX C: VITA

Rachel A. Murphy
2299 Knobhill Drive, Apt. 13
Okemos, MI, 48864
(540) 604-3126
murphyrachel63@gmail.com

August 2017

Education

July 2013 – July 2017	Doctor of Philosophy in Biomedical Sciences, Marshall University School of Medicine, Huntington, WV
August 2010 – June 2013	Bachelor of Science in Biochemistry, Benedictine College, Atchison, KS

Research

2013-Present "Tenofovir Induced Nephrotoxicity: A Mechanistic Study"

- Collaborators: Monica Valentovic, PhD; Kathleen Brown, MS; Reagan M. Stafford, BS; Brooke Petrosavits, MS
- Research conducted at Marshall University School of Medicine as part of the requirement for the degree of Doctor of Philosophy
- Funding: I was awarded a graduate fellowship through the NASA West Virginia Space Grant Consortium which was renewed for a second year. This work was also supplemented from Dr. Valentovic through the West Virginia IDeA Network of Biomedical Research Excellence (NIH Grant 5P02RR016477)

Peer-Reviewed Publications

March 2017 Murphy, R.A.; Stafford, R.M.; Petrasovits, B.A.; Boone, M.A.; Valentovic, M.A. Establishment of HK-2 Cells as a Relevant Model to Study Tenofovir-Induced Cytotoxicity. *Int. J. Mol. Sci.* 2017, Mar 1;18(3). pii: E531.

Manuscripts Submitted

May 2017 Murphy, R.A.; Rodriguez de Anda, D.; Brown, K.; Valentovic, M.A. Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells. Submitted to *Toxicology*, May 2017.

May 2017 Murphy, R.A.; Valentovic, M.A.; Factors Contributing to the Antiviral Effectiveness of Tenofovir. Submitted to the *Journal of Pharmacology and Experimental Therapeutics*, May 2017.

Recent Presentations

Oral

May 2017	"Tenofovir Induced Nephrotoxicity: A Mechanistic Study" Presented to faculty and staff at Michigan State University
December 2016	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" Presented at the British Pharmacology Society Annual Meeting, London, UK
December 2016	"Does Illicit Drug Use Have an Effect on HIV and Hepatitis C Treatment?" Lecture presented to graduate students at Marshall University as part of the 2016 Fall Seminar Series, Huntington, WV
October 2016	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" Presented at the 2016 Appalachian Regional Cell Conference, Charleston, WV
May 2016	"Considerations in Study Design" Lecture presented to graduate students at Marshall University, Huntington, WV
May 2016	"Preparing for your Oral Qualifiers" Lecture presented to students at Marshall University as part of the Spring 2016 Seminar Series, Huntington, WV
March 2016	"Tenofovir-Induced Nephrotoxicity: What is Known and Where do we go Next?" State of the Art Review presented to graduate students at Marshall University as part of the 2016 Spring Seminar Series, Huntington, WV
March 2015	"Establishment of HK-2 Cells as a Relevant Model for Investigating Tenofovir Renal Cytotoxicity" Presented at the 27th Annual Joan C. Edwards Research Day at Marshall University, Huntington, WV
October 2014	"HIV: An Overview" Lecture presented to undergraduate students at Marshall University, Huntington, WV

Poster

March 2017	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" by Murphy, RM; Rodriguez de Anda, D; Brown, K; and Valentovic, MA. Presented at the Society of Toxicology Annual Meeting, Baltimore, MD
April 2016	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" by Murphy, RM; Rodriguez de Anda, D; Brown, K; and Valentovic, MA. Presented at NASA S.P.A.C.E Day, Fairmont, WV
April 2016	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" by Murphy, RM; Rodriguez de Anda, D; Brown, K; and Valentovic, MA. Presented at Experimental Biology 2016, San Diego, CA
November 2015	"Antiviral Agent Tenofovir Causes Mitochondrial Damage and Oxidative Stress in HK-2 Cells" by Murphy, RM; Rodriguez de Anda, D; Brown, K; and Valentovic, MA. Presented at the 2015 Appalachian Regional Cell Conference

Honors and Awards

February 2017	Travel Award to Attend the 2017 Society of Toxicology Meeting
August 2016	Best Overall Performance as a Graduate Student • Award granted for a combination of outstanding research and service to the Marshall University Biomedical Science Program
April 2016	First Place, Graduate Student Poster Competition, NASA S.P.A.C.E Day 2016
April 2016	Third Place, Graduate Student and Postdoctoral Fellow Poster Competition, American Society of Pharmacology and Experimental Therapeutics, Experimental Biology 2016
February 2016	Travel Award to Attend Experimental Biology 2016 Meeting
August 2014	Goran Boskovic Best Academic Performance • Award granted to the graduate student with the highest GPA upon completion of their first year

Professional Activities

May 2016	Selected to present seminar series to graduate students based on personal performance within the Biomedical Science program at Marshall University. • Seminar Series: "Passing Your Qualifiers: Studying, Writing, and Presenting your NIH-Style Qualifying Grant", "Experimental Design: Where Do I Begin?", and "Experimental Design: Active Learning Workshop".
2016 - Present	Women in Medicine and Science, Graduate Student Council Member, Huntington, WV • Coordinated formation of a certificate program for Joan C. Edwards School of Medicine for MD residents, PhD candidates, and pharmacy students • Student leader on fundraising and budgeting committee for philanthropy events to support Branches Domestic Violence Shelter
2016 – Present	Society of Toxicology, Member
2015-2016	Served as Marshall University Chair for the 2016 Appalachian Regional Cell Conference. Responsible for budgeting, planning, and execution of educational regional conference involving several universities with over 100 attendees. Sat on planning committee for the same conference in 2015.
2015 – Present	American Medical Writers Association, Member
2015 – Present	Text and Academic Authors Association, Member
2015 - Present	American Society for Pharmacology and Experimental Therapeutics, Member
2014 – Present	American Association for the Advancement of Science, Member

2014 – Present	Society for Experimental Biology and Medicine, Member
2013 - Present	Marshall University School of Medicine Graduate Student Organization • President: May 2015 – April 2016 • Historian: May 2014 – April 2015 • Member: July 2013 – Present

Community Service

2016 – Present	Volunteer, Huntington City Mission, Huntington, WV
2016 – Present	Volunteer, Veteran Administration Hospital, Huntington, WV
December 2015	Coordinator, Jared Box Toy Drive for In-Patient Children, Huntington WV
October 2013	Proctorville High School Science Fair Judge