

**ANTIRETROVIRAL DRUGS EFAVIRENZ AND TENOFOVIR AND THEIR  
EFFECTS ON ARTERIAL REMODELING AND PROTEASE ACTIVITY**

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LaDeidra Monét Roberts

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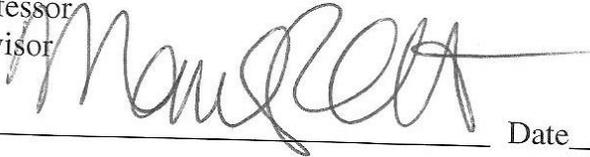
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Manu Platt, Ph.D.  
Assistant Professor  
Research Advisor

Signature



Date

12/21/13

Rudolph Gleason, Ph.D.  
Associate Professor

Signature



Date

11/21/13

Approved by:  
Dr. Manu Platt, Advisor  
School of Biomedical Engineering  
*Georgia Institute of Technology*  
Dr. Rudolph Gleason, Associate Professor  
School of Biomedical Engineering  
*Georgia Institute of Technology*

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## LIST OF SYMBOLS AND ABBREVIATIONS

APOE <sup>-/-</sup>	Apolipoprotein E knockout
AZT	Azidothymidine
CVD	Cardiovascular Disease
DMSO	Dimethyl Sulfoxide
EFV	Efavirenz
FTC	Emtricitabine
ECM	Extracellular Matrix
3TC	Lamivudine
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Disease
NRTI	Nucleoside Reverse Transcriptase Inhibitor
PBMCs	Peripheral Blood Mononuclear Cells
TDF	Tenofovir Disoproxil Fumurate

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## SUMMARY

Highly antiretroviral therapies (HAART) have been implemented to slow the progression of the human immunodeficiency virus (HIV). Although these new advances in the medications for HIV-positive patients have contributed in longer life expectancy, comorbidities, such as cardiovascular disease, still cause higher number of deaths among HIV-positive patients than in the regular population. Because of the intrinsic inflammation caused by the HIV virus, atherogenesis is more likely to occur and is driven by infected macrophages. These macrophages are known to secrete cathepsins, but infection causes the macrophages not to perform their function properly as an immune agent. I hypothesize that antiretroviral drugs play an important role in arterial remodeling by affecting cells within the artery and causing an alteration of cathepsin activity, leading to an increased risk of atherosclerosis in HIV patients. To test this hypothesis, we incubated THP-1 monocytes with antiretroviral drugs efavirenz and tenofovir individually to observe any changes in cathepsin activity. These lysates were analyzed through multiplex cathepsin zymography and quantified through densitometry. We found that our hypothesis held true for efavirenz and tenofovir in THP-1 monocytes, which caused decreased cathepsin K activity compared to vehicle controls. Still, stimulation of peripheral blood mononuclear cells (PBMCs) with efavirenz and tenofovir caused differential effects. Together, our data suggest that the HAART interaction with monocytes that are physiologically relevant to our system possibly contributes to the advancement of atherogenesis in HIV+ patients.

# CHAPTER I

## INTRODUCTION

Currently, over 34 million people are infected with the human immunodeficiency virus (HIV) worldwide<sup>1</sup>. Major advances in treatment with highly active antiretroviral therapy (HAART) have contributed to longer life expectancies, suppressed viral load, and slowed progression of Acquired Immunodeficiency Syndrome (AIDS) in HIV+ patients. Although combination HAART regimens are attributed to these improvements, prevalent cohort studies have shown that long-term exposure to these treatments increase progression of cardiovascular disease (CVD) in infected patients<sup>2</sup>. HAART has been associated with adverse metabolic side effects such as hypertension, diabetes, and lipodystrophy that could be contributing to the cardiovascular complications<sup>2-4</sup>.

Since cardiovascular risk was first suggested to be associated with antiretroviral exposure, few studies have examined the molecular mechanisms of HAART in the increased risk of cardiovascular disease in HIV+ patients. Degradation of the extracellular matrix (ECM) within arteries, which serves as a key factor in atherogenesis and arterial stiffening, is prevalent in cardiovascular disorders. This remodeling has been associated with cysteine proteases called cathepsins, specifically K, L, S, V, potent collagenases and elastases in the body that have been shown to be upregulated in atherosclerotic plaques in CVD<sup>5,6</sup>.

Expounding upon HAART and arterial remodeling through proteolytic activity, protease inhibitor azidothymidine (AZT) has been shown to promote arterial remodeling by increasing the proteolytic activity of cathepsins in mouse arteries<sup>7</sup>. It has also been shown in preliminary studies in our lab that HIV+ patients in Ethiopia and South Africa on HIV regimens including reverse transcriptase inhibitors (RTIs) efavirenz and tenofovir

have decreased cathepsin activity in their peripheral blood mononuclear cells (PBMCs). Studies in both clinical settings as well as animal models support that cathepsin activity should be further studied in association with cardiovascular disease in HIV+ patients.

In light of previous studies, **the purpose of this study is to investigate how HAART drugs alter cathepsin activity in monocytes, which could contribute to cardiovascular disease in HIV+ patients.** Therefore, the central hypothesis is that antiretroviral drugs efavirenz and tenofovir cause a decrease in cathepsin activity in monocytes. Additionally, HAART conditioned monocytes are then hypothesized to contribute to cardiovascular disease by differentiating into macrophages as well as reducing cathepsin activity. I propose to test these hypotheses first using a monocytic cell line and PBMCs, then to verify the results from *in vitro* conditions with *in vivo* samples from humans and mice. This method will also aid in parsing out each specific antiretroviral effect on proteolytic activity within these cell types to provide insight on future therapeutic targets.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Cell Culture and Treatment

Human THP-1 acute monocytic leukemia cells (American Type Culture Collection) THP-1 monocytes were treated with vehicle controls (0.079% dimethyl sulfoxide [DMSO] (Sigma-Aldrich); 0.159% methanol (EMD)) or efavirenz (EFV) or tenofovir (TDF) (100 uM each) (NIH AIDS Reagent Program) in RPMI 1X 1640 medium (Corning Cell Gro) containing 10 % FBS, 0.05 %  $\beta$ -mercaptoethanol, 1 % L-glutamine, and 1 % penicillin/streptomycin for 24 h at 37 degrees Celsius prior to lysing. After consent from patients, human peripheral blood mononuclear cells were isolated from non-infected patients. Whole blood samples were centrifuged for 30 minutes at 400 x g at 4°C after adding Ficoll-Paque (GE Healthcare) to separate layers of PBMCs from red blood cells. Following centrifugation, the cells were collected, resuspended in PBS, and centrifuged for 10 minutes at 4°C. The cells were then washed in RBC lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) and centrifuged for 10 minutes at 4°C and subsequently with PBS to completely remove remaining red blood cells out of sample. To remove platelets from PBMCs, the cells are then centrifuged at room temperature. Cells were counted using the Vi-Cell XR (Beckman Coulter) for cell count and viability.

#### 2.2 *ApoE*<sup>-/-</sup> Mouse Peripheral Blood Mononuclear Cell Lysates

Whole blood samples donated by Gleason Lab from Apolipoprotein E knockout (*ApoE*<sup>-/-</sup>) mice fed with 7.5 mg/kg EFV as a dosage. EFV was diluted to 1 mg/ml and fed to the mouse based on the weight of the mouse. Water was administered in an equivalent dose to the control group. The blood samples were centrifuged for 30 minutes

at 400 x g at 4°C after adding Ficoll-Paque (GE Healthcare) to separate layers of PBMCs from red blood cells. Following centrifugation, the cells were collected, resuspended in PBS, and centrifuged for 10 minutes at 4°C. The cells were then washed in RBC lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) and centrifuged for 10 minutes at 4°C and subsequently with PBS to completely remove remaining red blood cells out of sample. To remove platelets from PBMCs, the cells are then centrifuged at room temperature.

### **2.3 Multiplex Cathepsin Zymography**

Cathepsin zymography was performed on each of the samples<sup>8</sup>. Prior to loading, non-reducing 5X loading buffer was added to all of the samples used for the experiment. Gel electrophoresis of the samples within 12.5% SDS-polyacrylamide gels containing gelatin at 4°C was performed for 2 hours at 100 V. Gels were removed and washed in cathepsin renaturing buffer (65 mM Tris buffer, pH 7.4 with 20 % glycerol) for 3 washes for 10 minutes each. Gels were then assayed in respective activity buffer (0.1 M sodium phosphate buffer, pH 6.0, 1 mM EDTA, and 2 mM DTT freshly added as well as 0.1 acetate buffer, pH 4.0, 1 mM EDTA, and 2 mM DTT) for 30 minutes at room temperature. Then, fresh activity buffer was added to the respective gels and incubated for an overnight incubation at 37°C. After the overnight incubation, each gel was rinsed with distilled water and incubated for one hour in Coomassie Blue stain (10 % acetic acid, 25 % isopropanol, 4.5 % Coomassie Blue) followed by destain (10% isopropanol and 10% acetic acid). Gels were then scanned using GE Healthcare LifeSciences ImageQuant LAS 4010. After imaging, each gel was quantified through densitometry using ImageJ software.

## **2.4 Statistical Analyses**

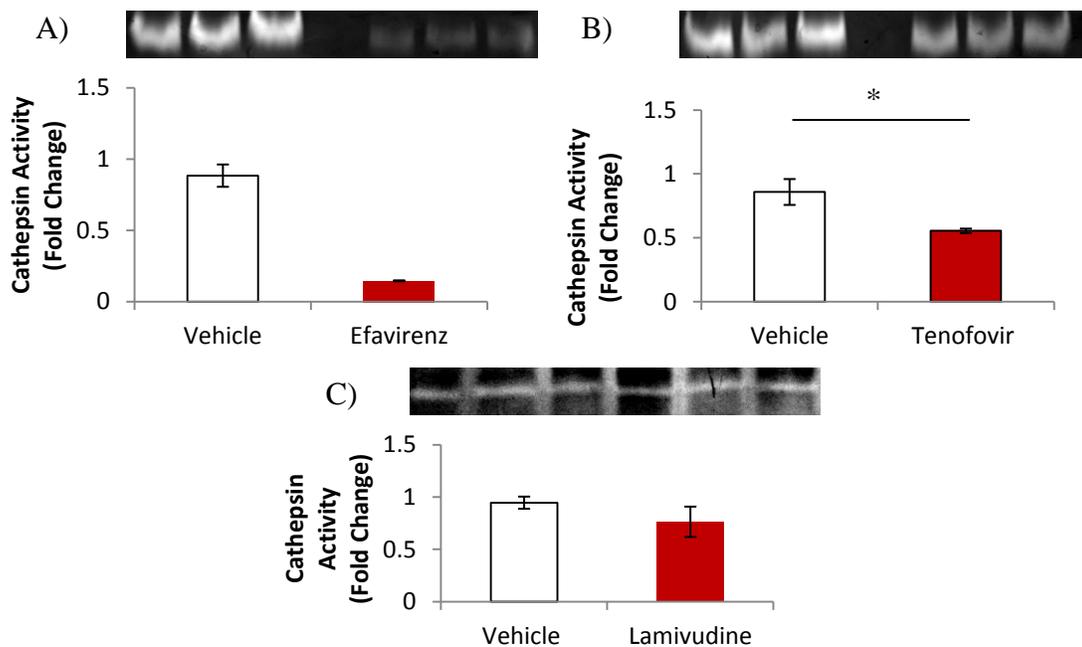
Each experimental condition was repeated with a minimum of three biological replicates and each data point is presented as the mean value and standard deviation of the mean. Representative images are shown. Unpaired Student's t tests were used to determine the statistical significance ( $p < 0.05$ ) between experimental groups.

## CHAPTER 3

### RESULTS

#### 3.1 Efavirenz and tenofovir significantly decreased cathepsin activity in monocytes

To determine how tenofovir and efavirenz regulate cathepsin activity in monocytes during inflammatory conditions such as plaque formation, THP-1 monocytes were incubated with 100  $\mu$ M efavirenz and tenofovir for 24 h. The respective concentration was found in previous literature to observe a response at the highest possible non-cytotoxic concentration that compared both antiretroviral drugs uniformly<sup>9,10</sup>. Both of these antiretroviral drugs decreased mature cathepsin activity, efavirenz by fourfold (Fig. 1A) and tenofovir by twofold (Fig. 1B) ( $n = 3$ ,  $p < 0.05$ ) compared to each respective vehicle. To ensure that efavirenz and tenofovir were the most active in decreasing cathepsin activity, monocytes were also incubated in 100  $\mu$ M lamivudine where there was no significant difference from the respective vehicle (Fig. 1C).

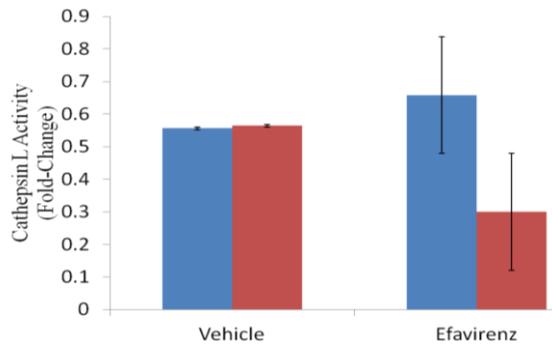


**Figure 1. EFV & TDF decreases cathepsin activity in THP-1 monocytes.** Densitometry showed that both of these antiretroviral drugs decreased mature cathepsin activity, (A) efavirenz by fourfold and (B) tenofovir by twofold ( $n = 3$ ,  $p < 0.05$ ) compared to each respective vehicle.

Results also showed that (C) lamivudine does not significantly alter cathepsin activity (n = 3) compared to its respective vehicle.

### 3.2 Efavirenz induced differential cathepsin activity in human PBMCs

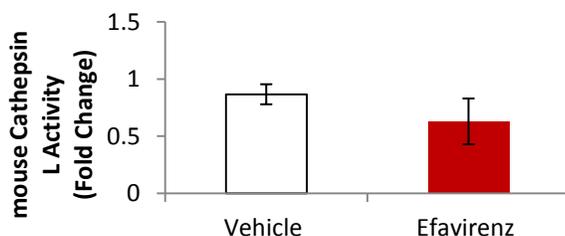
Since efavirenz showed the most significant decrease in cathepsin activity in monocytes, the next objective was to see what kind of effects were seen in human PBMCs, which include monocytes, macrophages, and other types of leukocytes intrinsic to inflammatory response. Cells were isolated from two different non-infected patients and subsequently incubated with 100  $\mu$ M efavirenz for 24 h. In patient 1, efavirenz increased cathepsin L activity while cells from patient 2 showed a decrease in cathepsin activity in comparison to the vehicle (n = 3, p > 0.05) (Fig. 2).



**Figure 2. EFV causes differential effects on cathepsin L activity in human PBMCs.** Efavirenz stimulated variable cathepsin L activity in two different patients. (patient 1: blue, patient 2: red) (n = 3, error bars represent the mean  $\pm$  standard deviation).

### 3.3 Efavirenz showed decreasing trends in cathepsin V activity in *ApoE*<sup>-/-</sup> mice

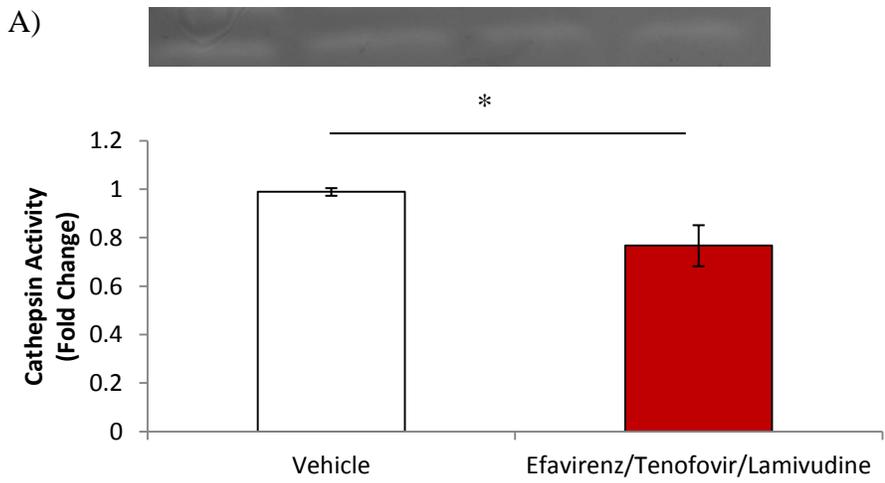
PBMCs from *ApoE*<sup>-/-</sup> mice that were fed 7.5 mg/kg efavirenz and compared to PBMCs from the control group that were fed water as the vehicle. Results showed decreases in cathepsin V activity in the presence of efavirenz compared to the vehicle. Although not significant, the general trend among the mice was comparable to the littermate controls (Fig. 3).

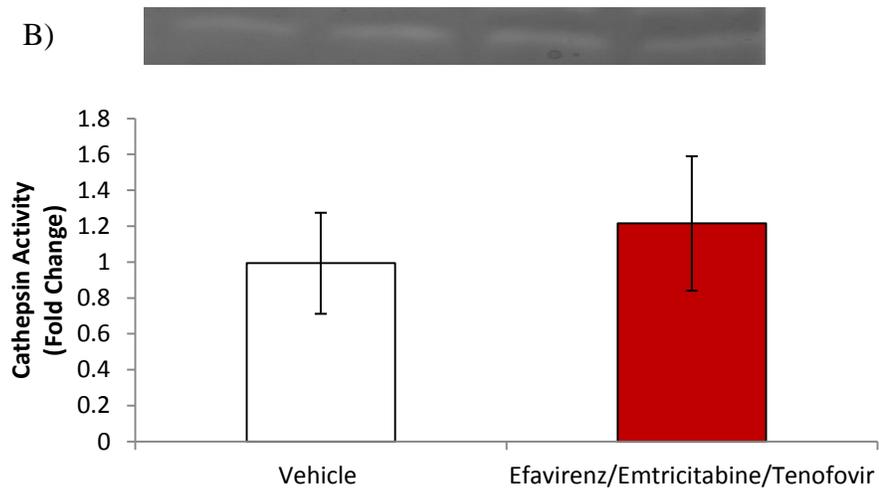


**Figure 3. Efavirenz caused a decrease in cathepsin V activity in *ApoE*<sup>-/-</sup> mice PBMCs.** Compared to the vehicle, efavirenz induced a decrease in cathepsin V activity in PBMCs from *ApoE*<sup>-/-</sup> mice. (n = 3, error bars represent the mean  $\pm$  standard deviation)

### 3.4 Combination HAART stimulated cathepsin activity in monocytes

Since it is physiologically relevant, combination HAART was implemented in this study to observe changes in cathepsin activity in monocytes. Using common drugs in regimens, monocytes were incubated in 5  $\mu$ M efavirenz, tenofovir, and emtricitabine as well as efavirenz, tenofovir, and lamivudine in combination. Lamivudine and emtricitabine are common NRTIs considered to be clinically interchangeable in combination with tenofovir and lamivudine; however, emtricitabine has been suggested to be pharmacologically different from lamivudine through interactions with tenofovir, thereby increasing its half-life. Overall, the two drugs are comparable, so observing each particular drug in combination therapy was relevant<sup>11</sup>. Cathepsin activity was significantly decreased in the presence of efavirenz, tenofovir, and lamivudine (Fig 4A.). Although not significant, cathepsin activity showed an increased in cathepsin activity in the presence of efavirenz, tenofovir, and emtricitabine (Fig. 4B).





**Figure 4: Combination HAART suggests that NRTI backbone stimulated differential cathepsin activity.** (a) Cathepsin S activity decreased in the presence of combination HAART using efavirenz, tenofovir, and lamivudine (n=3, p< 0.05). (b) Increased cathepsin activity was seen in the presence efavirenz, emtricitabine, and tenofovir (n = 3, p < 0.05).

## CHAPTER IV DISCUSSION

Increased cathepsin activity has been associated with arterial remodeling in atherosclerosis, abdominal aortic aneurysms, and arterial stiffening<sup>6</sup>. Plaque biology in atherosclerosis has been elucidated<sup>12</sup> but how HIV antiretroviral drugs interact with the physiology in the arterial wall remains unknown. There was a need to study treating a monocytic cell line and peripheral blood mononuclear cells (PBMCs) of patients in the presence of some common antiretroviral drug regimens to observe the effects that it has on cells that are prevalent in the inflammatory response in HIV as well as CVD. I proposed that stimulation of active cathepsins by efavirenz and tenofovir could provide insight in arterial remodeling in HIV+ patients, which could possibly contribute to the narrowing of the lumen and impeding bloodflow involved in myocardial infarctions. In THP-1 monocytes, there was significant reduction of cathepsin expression in the presence of the two drugs. These results are similar compared to previous studies within our lab in clinical settings involving Ethiopian and South African HIV+ patients on regimens with efavirenz and tenofovir.

In human PBMCs from two different subjects, there was differential stimulation of cathepsin L activity. The difference suggests which suggested variability and possible patient-specific proteolytic activity as a factor in the treatment of HIV<sup>13</sup>. Overall, these results suggest that these antiretroviral drugs affect cathepsin activity and this alteration could affect other cells within the vascular network. Furthermore, PBMCs from *ApoE*<sup>-/-</sup> mice showed that trend of decreased mouse cathepsin L (homologue of human cathepsin V) activity compared to the vehicle. In the future, I expect that a larger sample size will increase the significance of the decrease in mouse cathepsin L activity in the PBMCs. These results were useful to show if efavirenz promotes more proteolytic activity than

what is seen at baseline in induced atherosclerosis in mice. Also, the decreases in cathepsin activity matched what we have seen in monocytes.

Limitations in this study included the cell types used within this study. By using monocytes, interactions with other cell types within the body was not taken into account in this study. Also, by incubating PBMCs from non-infected patients with efavirenz after they were isolated from the blood, this study did not take into account processing of the drug into metabolites, thereby causing a physiological response through metabolite-cell interactions. Lastly, proteolytic activity was only analyzed and was not compared to physical arterial remodeling. However, future studies will include looking at arterial stiffening and other remodeling characteristics seen in the mice to make this study impactful.

Also, inherent inflammation involves endothelial cell-monocyte interaction as well as inflammatory cytokines that activate cell adhesion molecules to promote monocyte transendothelial migration<sup>14</sup>. To further study how this contributes to proteolysis and vascular remodeling in atherosclerosis, future studies will include a co-culture using human PBMCs and human arterial endothelial cells (HAECs) incubated with antiretroviral drugs can be to model an arterial system of a HIV+ patient under HAART regimen.

In a physiologically relevant regimen of a HIV+ patient, combination HAART therapy stimulated cathepsin activity in monocytes. Using lamivudine or emtricitabine as the nucleoside reverse transcriptase inhibitor (NRTI) backbone of the antiretroviral regimen stimulated differential effects in cathepsin expression, which enhances the need for HAART drugs to be examined closely in terms of molecular mechanisms.

In mechanistic terms, Keegan et al. showed that increases in cathepsin K and V activity, which are the most potent collagenase and elastase, respectively, and key phosphorylation of the Jun N-terminal kinase linked intracellular pathways of TNF $\alpha$  and monocyte binding stimulation to cathepsin activity<sup>14</sup>. In coordination with this study,

oxidative stress and an increase in reactive oxygen species causes vascular damage during inflammation, so future studies could include the observation of increasing oxidative stress, thereby increasing recruitment of PBMCs and/or THP-1 monocytes by endothelial cells<sup>15</sup>. Cathepsin activity should then be analyzed by multiplex cathepsin zymography to help explain specific protease activity that could ultimately lead to establishing mechanisms between atherosclerosis and HIV infection<sup>8</sup>.

Overall, this study is a novel way to approach the molecular mechanisms of HAART drugs have on monocytes. HAART interactions with each other in combination therapy affect protease activity, so this proposed study provides insight on focusing on potential new therapeutic targets to consider in HAART regimens as well as CVD treatment.

## CHAPTER V

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