Treating post-HIV infection through molecular target of HIV TAT and PKC regulation with Berberine and Curcumin

Author: Jingyue (Oliver) Zhang

Abstract

Current drugs used for anti-retroviral therapy against HIV have a narrow spectrum of activity and associated adverse events, and possess vulnerability to viral mutation. The HIV TAT protein (TAT), a virus encoded protein required for efficient transcription of the HIV genome, hasn't been intensely researched as a target for developing therapeutics. This study examines Berberine's and Curcumin's effect at very low dosage on diminishing TAT's efficiency in activating Protein Kinase C (PKC), a critical step for HIV transcription.

Berberine is a known PKC inhibitor and Curcumin is previously shown to cause TAT degradation. The extent to which these drugs mitigate the level of TAT functionality is studied with cell migration and viability assays. Matrix Metallopeptidase-9 (MMP-9) levels were measured by Enzyme-linked Immunosorbent Assay (ELISA) as MMP-9 is downstream PKC and plays a role in cell migration and viability. Results showed that cells transfected by TAT had shorter migration bridges (MB), indicating a greater scale of cell migration and reduced viability when compared to the control. Dual-treatment with Berberine and Curcumin significantly restricted TAT induced MB shortening and maintained cell viability, resulting in 45.0% wider MB's (at 48hr) and 73.6% more survivorship in TAT transfected 3T3 cells, significantly exceeding the efficacy of single treatments. ELISA showed 50% greater MMP-9 levels with TAT transfection relative to control, and TAT + dual-treatment reduced that level to 49% of control. Such data shows MMP-9 expression can be explained by PKC activity, resulting in the changes seen with treatments.

This research demonstrates Berberine and Curcumin could work together to mitigate HIV TAT's efficiency in PKC activation.

Introduction

1.1 Background

Human Immunodeficiency Virus (HIV) is able to cause drastic dysfunction in human immune system, ultimately manifesting to a clinical stage that is referred to as Acquired Immunodeficiency Syndrome (AIDS). HIV is capable of infecting the human CD4+ T-cells through direct interaction, sending two RNA strands into the cytosol of the host cell along with several retroviral proteins. Utilizing reverse transcriptases and integrase, HIV is able to transcribe viral RNA's into single stranded DNA's and insert them into the human genome (Volberding & Deeks 2010). The infected CD4+ cells will likely not deteriorate immediately but rather undergo multiple lysogenic cycles until certain virally encoded proteins trigger critical stimulations, which cause CD4+ cells to degrade at a drastic rate; Ultimately, the consequent deterioration of the human immune system is the sole reason for any HIV-related deaths. According to the World Health Organization (WHO), 32 million lives around the globe carried HIV in 2017. In addition, the number of deaths and the average mortality rate associated with AIDS have reached 770,000 annually by 2018 (World Health Organization 2019). To exacerbate the issue, current therapeutic modalities are limited in potency and induce collateral damage to healthy cells.

1.2. Limitations of Current Treatment

According to the FDA, there are currently only five major targets selected by current HIV treatment methods and are accompanied by obvious limitations and adverse events: non-nucleoside reverse transcriptase (NNRT), reverse transcriptase (RT), HIV protease, CCR5, and HIV integrase inhibitors. All of the treatment drugs developed based on these targets are confronted with the issue of causing fatal hepatotoxicity (excluding CCR5 and HIV protease inhibitor drugs). In addition, most current treatments are susceptible to mutations on targeted HIV virally encoded proteins, hence possess insufficient competence in treating post-viral infection. NNRT and Nucleoside Reverse Transcriptase (NRT) inhibitors function differently to prevent HIV utilization of RTs for RNA-dDNA transcription as the prior mainly alter the structure of RTs while the latter competitively bind to RTs. NNRT inhibitor are able to increase CD4 cell count by 1.5 folds after a 48-week treatment duration

(Katlama et al. 2010), but a commonly occurring single point mutation at residue K103N could hinder their performances in addition causing potential teratogenicity (Hughes et al. 2009). Similar to NNRT inhibitors, NRT inhibitors are also decent in increasing CD4 cell counts, but the FDA reported the intake of most NRTIs can manifest to life-threatening expressions of lactic acidosis (U.S. Food and Drug Association 2019). For HIV protease inhibitors, efficacy also comes about with severe adverse events and limitations; these drugs (eg. Darunavir) can remarkably lower the viral load in Stage III HIV patients (those who possess baseline HIV RNA > 100,000 copies/mL or CD4+ cell count < 200 cells/mm^3) but with possibility to stimulate severe skin rash (Oritz et al. 2008). Similarly, HIV integrase inhibitors (eg. Dolutegravir) could uplift CD4+ cell count by 109/mm^3 (1.55 folds) after a 48 week treatment duration in human patients (Cooper et al. 2008), but prospective allergic hypersensitivity degraded its competence. Lastly, the CCR5 antagonists is plainly unproficient; its low rate of efficacy for viral reduction is due to a low bioavailability (approximately 95% of the chemical will be excreted by the feces and urine)(Westby et al. 2007). Concerning the deficiencies associated with current FDA approved post-HIV infection drugs and the lack of diversity in their means of approaching alleviation in HIV pathogenesis, it is paramount to find new, alternative pathways for treating HIV infection.

1.3. TAT Protein in Viral Reproduction

In this study, HIV Trans-Activator Transcription (TAT) protein is examined due to its ability to speed up the rate of viral transcription. By activating the Protein Kinase C Beta II ($PKC\beta_{II}$) (Leghmari et al. 2008), a conventional enzyme in the Protein Kinase C (PKC) class, TAT could lead to the discharge, or freeing, of positive transcription elongation factor (P-TEFb) complex composed of CDK9 and Cyclin T1 (Cary et al. 2016). Precisely, TAT carries out this function by interacting with the 7SK small nuclear ribonucleoprotein (snRNP), which is composed of a La-related protein LARP7, a methyl phosphate capping enzyme MePCE, HEXIM1, and an inactive P-TEFb (Peterlin et al. 2012). HEXIM1 is responsible for occluding the ATP pocket in CDK9 with its Tyr271, thus keeping P-TEFb inactive (Cary et al. 2016). However, PKCs have the capability to phosphorylate both HEXIM1 and P-TEFb, altering the structure of the prior and consequently freeing the ATP pocket of the latter, which, when phosphorylated by PKC, causes the activation and discharge of P-TEFb from the 7SK

snRNP (Planès et al. 2016; Cary et al. 2016). The free and activated P-TEFb can then be recruited by HIV Trans-Activator Receptor (TAR) protein during viral transcription phase to increase the speed of this process: P-TEFb regulated phosphorylation and freeing of both Spt5 and NELF-E, two elements that contributes to the arrest of RNA Polymerase II (Pol II) at its binding site (Yamaguchi et al. 1999), will essentially bypass the promoter proximal pausing site during the beginning of viral transcription and resume Pol II activity (Rice 2018). Therefore, with TAT induced PKC upregulation, HIV viral transcription can still be easily facilitated regardless of the number of P-TEFb suspended by 7SK snRNP.

1.4. TAT Protein and Cellular Response

TAT is responsible for inducing multiple inflammatory responses in various cell types other than lymphocytes due to its genuine capability in activating the PKC pathway. Its ability to agonize PKC, in turn, allows it to upregulate the expression of MAPK and ERK1/2, consequently leading to the overexpression of Matrix Metalloproteinase-9 (MMP-9), a downstream protein in the PKC pathway (Ju et al. 2009; Zhang et al. 2017). Upregulation of MMP-9 expression is critical for excessive cell migration (Newby 2006), and this fundamentally contributes to the development of AIDS Dementia Complex (ADC) as HIV-infected monocytes and macrophages will be able to transpass the blood-brain barrier (BBB) due to loosened tight junction and degraded basal lamina in epithelial cells caused by overexpressed MMP-9 (Xing et al. 2017; Ju et al. 2009). On the other hand, MMP-9 can often induce apoptosis in epithelial, endothelial, and fibroblast cells through the opening of mitochondrial permeability transition pores, subsequently causing mitochondrial dysfunction (depolarization) and leading to mitochondrial emission of Cytochrome c and Apoptosis Inducing-Factor (AIF) into the cytosol (Mishiro et al. 2014). Ultimately, these released factors would cause apoptotic cell death by activating caspase-3.

1.5. Controlling HIV Infection Through P-TEFb Regulation

There are currently two main hypothesized methods in managing HIV pathogenesis via TAT - P-TEFb interaction: “Shock and Kill” and “Block and Lock” (Asamitsu et al. 2018). The “Shock and Kill” theory involves the activation and freeing of P-TEFb from the 7SK snRNP using so-called potent latency reversing agents and then targeting potential viral reservoirs, which would theoretically have a fast viral transcription rate, and killing them

using this specific character (Spivak & Planelles 2018). However, scientists have only successfully carried out the "shock" while not the "kill" part; most importantly, the "Shock and Kill" method is potentially hazardous as it is hard to manage the persistent HIV infection after heightening the viral RNA production with latency reversing agents (Churchill et al. 2016). The "Block and Lock" theory requires the release of inactive P-TEFb from the 7SK snRNP, tricking HIV TAR protein into recruiting an unviable elongation promoting complex during the transcription process as TAR cannot differentiate between the active and inactive P-TEFb; ultimately, chemicals will be introduced to induce the latency of HIV in infected cells, potentially rendering the provirus to enter dormancy (Asamitsu et al. 2018). Currently, the "block" part of the theory can be easily achieved using chimeric proteins, which separate an inactive P-TEFb from the 7SK snRNP (Fujinaga et al. 2002). Unfortunately, only one promising chemical, Didehydro-Cortistatin A, has been identified for its apparent effect in prolonging HIV provirus latency in the nucleus, and its molecular mechanism has not yet being understood (Mousseau & Valente 2017). Although the "Block and Lock" method is potentially safer than "Shock and Kill," it cannot eliminate the latent HIV and provirus. However, the "Block and Lock" strategy is a method of interest as it has the potential in granting patients more time in seeking complete decimation of HIV and better qualities of life.

1.6. Curcumin and Berberine

Berberine is a bioactive alkaloid that can be isolated from several medicinal plants such as Barberry (*Berberis vulgaris*) (Wang et al. 2017). It has been shown that Berberine is a very safe drug when applied at appropriate dosage. It was shown that Berberine is able to mediate improvements in the function of damaged kidneys in diabetic nephropathy through inhibition of several substances including PKCβ (Qiu et al. 2017), a crucial element in the TAT pathogenesis pathway. Berberine is also capable of reducing the overexpression of MMP-9 and MMP-1 caused by TPA, a PKC agonist, in cancer cells (Kims et al. 2012), further consolidating its role in modulating PKC activities. Curcumin, a hydrophobic polyphenol, is an herbal substance extracted from the rhizomes of *Curcuma Longa*, which is typically known as the turmeric plant used for spice (Mirzaei et al. 2016). This chemical has been confirmed to be effective in multiple antiviral and anti-inflammatory diseases (e.g. Parkinsons

[anti-inflammatory] and HPV [antiviral]), and its daily dosage for human adults is estimated to be as high as eight grams per day, revealing a relatively low hazard level upon consuming (Mirzaei et al. 2017). Intriguingly, prior studies found that Curcumin carries very slight inhibitory roles on TAT; its structure provided electrophilic properties that is able to target the Cysteine-rich domain of TAT, altering the structure of protein and obstructing its function (Narayan et al. 2011). Later studies found that Curcumin could degrade the TAT with the activation of ubiquitin independent S20 proteasomal pathway, which proteolyzes the unfolded proteins like TAT but not folded ones such as HIV Gag protein (Ali & Banerjea 2016).

1.7. Hypothesis and Goal

An MTT Assay, Cell Migration Assay, and Enzyme Linked Immunosorbent Assay (ELISA) on MMP-9 overexpression are used to assess the efficacy of Berberine and Curcumin in reducing TAT's efficiency in PKC activation, a critical step in HIV pathogenesis. Since Berberine and Curcumin affect the TAT in different mechanisms, it might be beneficial to combine them during treatment and examine for potential synergistic effects. Ultimately, this study focuses extensively on the degree to which Berberine and Curcumin can deteriorate the function of TAT *in vitro*.

2. Material & Methods

2.1. Cell Incubation and Treatment

The fibroblast NIH-3T3 cell line (ATCC, New York) was grown *in vitro* in MEM media supplemented with 10% fetal bovine serum (Invitrogen, USA). Cells were cultured in a 37 ℃ incubator (NAPCO) supplemented with 95% O_2/5% CO_2. The NIH-3T3 cell line is chosen for its high sensitivity to PKC (Uberall et al. 1997; Bandyopadhyay et al. 1997), a kinase in which TAT intensely utilizes, and its swift speed in uptaking TAT itself *in vitro* (Ziegler et al. 2005). Furthermore, NIH-3T3 cells are also known for its renowned role in tissue repair and wound healing as they can easily stretch over and cover the substratum of site of injury (Men et al. 2019), making it an excellent model for studying PKC overexpression in cell migration. TAT, Curcumin, Berberine, TPA, and Dimethyl Sulfoxide (DMSO) were all purchased from Sigma-Aldrich (St. Louis, MO); TAT was diluted using only distilled water while the rest were all dissolved in DMSO prior to being diluted with 50% H_2O/50% DMSO solution and

ultimate H_2O only for the second dilution. Trypsin (Sigma-Aldrich, St. Louis, MO) was applied during cell seeding to detach the 3T3 cells from the culture flasks prior to entering the centrifuge process, ensuring that all of the 3T3 cells cultured were put in use for the following assays.

2.2 Cell Migration Assay

To determine the potential of cells' ability to migrate, a six well culture plate was prepared. For each well, 2 mL of cell media is introduced. PKC's role in the cell migration process was clarified in an earlier study that confirmed its ability to be agonized for promoting the speed of migration in 3T3 cells (Simpson et al. 2017). In this study, TAT acts as an activator of PKC while two other chemicals, Berberine and Curcumin, were also applied to purposely prevent the TAT induced cell migration. Specifically, each well (excluding the control) was treated with following substances after suspended cells were stable: TAT alone, TAT with Berberine, TAT with Curcumin, TAT with both Berberine and Curcumin, and Berberine plus Curcumin. Finally, three bridges were scratched by the tip of a pipette for each well as these would serve as sites of cell migration. The treated plate was then incubated at 37 ℃ for 24 and 48 hours. After the incubation period, cell media was removed from each well, and 350 μL of Hema 3 fixatives was applied to secure and bound cells to the surface of each well. Afterwards, 350 μL of Hema 3 dye was applied to each well to make the attached cells appear visible and clear for examination under light microscope (40x magnification). The extent of cell migration under each treatment was evaluated using the length (in pixels) of migration bridge.

2.3 MTT Cell Proliferation Assay

100 μL of cell solution is introduced into each well prior to being treated with appropriate chemicals. For each trial, each treatment condition was introduced in eight wells (n=8) of cell media except that the control group without any treatments had a sample size of sixteen (n=16). This 96-well plate is then exposed to an incubation period of 24-hours. Afterwards, 10 μL of MTT is pipetted into each well to react with NADH from mitochondria, and the plate was held for a 90-minute incubation for the reaction to fully take place. Eventually, 50 μL of supernatant is extracted from each well and replaced by the same volume of DMSO, which breaks up the cell membrane and releases the MTT formazan from cytosol. After this step, the

cells are again incubated for 10~20 minutes before the plate is analyzed with MPM6 microplate reader, which was purposely set to read the plate using 595nm wavelength of light to determine the extent of accumulation of MTT formazans that positively correlate to and represent degree of cell viability.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

2mL of 3T3 cells were first applied to each well of a six-well plate; 20 μL of each treatment (TAT alone, Curcumin+TAT, Berberine+TAT, Berberine+Curcumin+TAT, and Berberine+Curcumin) were each introduced to five individual wells. The plate was then incubated for 24 hours and afterwards immediately stored at -20 ℃ for another 24 hours to initiate cold-stress induced cell apoptosis, releasing MMP-9. Then, the cell media was defrosted and brought to room temperature along with all the reagents and materials provided by the PicoKine MMP-9 ELISA kit. In each trial, 100 μL of standard (solution provided for final comparison to determine the concentration of MMP-9; n=16) and sample (n=8) from the cell media was introduced into each of the individual wells. 100 μL sample diluent buffer was added to each of the wells before the plate was incubated for 90 minutes at 37 ℃. Afterwards, any liquid from each of the wells were removed, and 100 μL Biotinylated Anti-Human MMP-9 detection antibody provided by the kit was added to each of the wells. The plate was then incubated for another 90 minutes at 37 ℃ and washed with phosphate-buffered saline for three times before the removal of remaining liquid in each of the wells. 100 μL of the Avidin-Biotin-Peroxidase Complex (ABPC) was then introduced in each well to bound with MMP-9, and the plate was incubated for another 30 minutes at 37 ℃. The unbounded ABPCs were then washed away with wash buffer, and the wells were dried again. At this point, the plate was incubated for 20 minutes at 37 ℃ after 90 μL of Color Developing Reagent was introduced into each of the wells. ultimately, 100 μL of stop solution were introduced to each of the wells, and its color should immediately turn into yellow; microplate readers are then employed to read the absorbance, which positively correlates with the MMP-9 concentration, at 450nm.

2.5 Statistical Analysis

Data from all experiments were statistically analyzed using Microsoft Excel implemented two-way ANOVA followed by a Post-Hoc Tukey test. Statistical significance was defined as $p < 0.05$.

3. Results

To assess the efficacy of single and dual-treatments of Berberine and Curcumin on modulating TAT protein activity in inducing PKC expression in 3T3 fibroblast cells, Cell Migration and Cell Viability assays, along with ELISA to measure MMP-9 expression, were utilized. In the Cell Migration assay, the lengths of Migration Bridges (MB), which are the gaps between cell colonies that would allow space for surface-dependent cell growth, were measured; the values obtained from measurements are negatively correlated to the magnitude of PKC-MMP9 activity. The downstream, overexpressed MMP9 in the TAT protein activated PKC pathway can also lead to cell apoptosis due to its capability of causing mitochondrial dysfunction. Consequently, the Cell Viability assay with MTT was used to evaluate PKC activity that negatively correlates with cell survival rate. Lastly, ELISA was used to directly determine the concentration of MMP9, the resultant values of which positively correlate with the level of PKC animation.

3.1. Cell Migration

At 24 and 48 hours, TAT protein reduced the lengths of MBs by 16.9% and 17.1%, respectively, as compared to its control ($p < 0.01$, ANOVA), whereas the Berberine plus Curcumin dual-treatment (DT) maintained the lengths of MBs, allowing them to be 12.5% and 32.4% longer, respectively, relative to its controls. At the same times, all three TAT protein-plus-treatment groups showed significant differences when compared to the TAT protein alone group; the DT group without TAT possessed significantly greater MB lengths than the respective DT with TAT protein group ($p < 0.001$, ANOVA) in two independent trials for cell migration, demonstrating the maximum efficacy of DT in restricting PKC expression without TAT protein transfection **(Fig. 3)**.

At 24 hours, both of the single treatments (ST) achieved similar MB lengths when compared to control **(Fig. 3 & 4)**. Berberine and Curcumin ST groups resulted in MBs that

are 15.3% and 8.0% longer than those from the TAT protein only group. The DT, however, is only slightly more effective than the STs in alleviating the shortening of MBs at 24 hours with insignificant variance to Berberine-only treatment ($p = 0.32$, ANOVA); it resulted in bridges only 17.8% longer than those from the TAT protein group. At 48 hours, MB lengths determined in all treatment groups were greater than their counterparts from 24 hours except in the cases for TAT protein alone and control groups. At 48 hours, the MBs found in dual-treatment group are 45.0% longer than those from TAT protein only groups, and this percentage of change in MB length exceeds that of ST ($p < 0.001$, ANOVA), which possessed bridges only 26.5% (Berberine) and 24.4% (Curcumin) longer than those in TAT-inclusive group.

TAT protein instigated the shortening of MBs to similar degrees at both 24 and 48 hours and shows only a slight but insignificant ($p = 0.44$, ANOVA) difference in length between the two times. However, when compared with their respective controls at both times, Berberine alone, Curcumin alone, and dual-treatment groups suppressed 7.7%, 13.1%, and 20.8% more MB length recovery, respectively, at 48 hours **(Fig. 4)**, indicating that the DT's impact increases more over time than STs did.

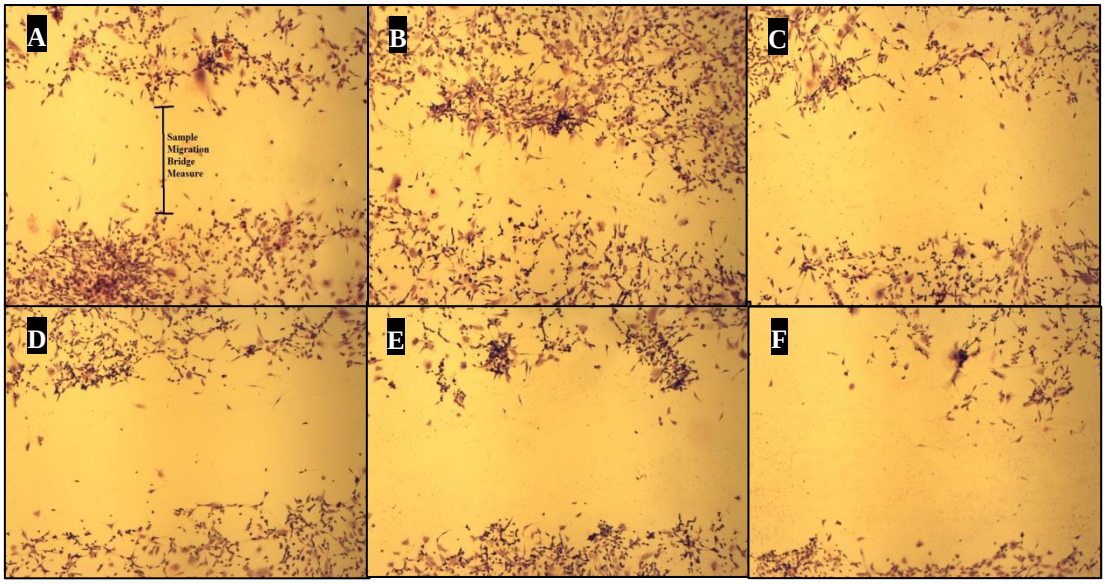

Fig. 1: Samples of microscopic pictures taken for 24hr Cell Migration assay analysis are shown. Picture A shows a sample from control, and one of the MB measurements is indicated for reference (each picture will be assessed ten times for ten measurements of MBs). Pictures B, C, D, E, and F are representative of TAT protein, Berberine+TAT protein, Curcumin+TAT protein, Berberine+Curcumin+ TAT protein, and Berberine+Curcumin samples, respectively. The pictures indicate a magnification of 40x and 3T3 fibroblast cells are assessed.

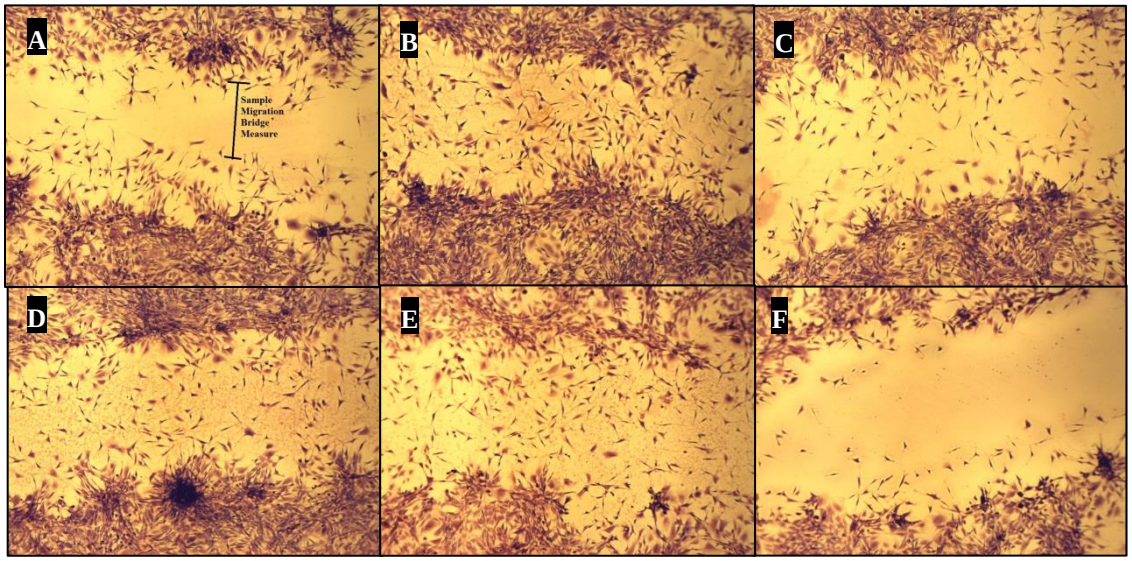

Fig. 2: Samples of microscopic pictures taken for 48hr Cell Migration assay analysis are shown. Picture A shows a sample from control, and one of the MB measurements is indicated for reference (each picture will be assessed ten times for ten measurements of MBs). Pictures B, C, D, E, and F are representative of TAT protein, Berberine+TAT protein, Curcumin+TAT protein, Berberine+Curcumin+ TAT protein, and Berberine+Curcumin samples, respectively. The pictures indicate a magnification of 40x and 3T3 fibroblast cells are assessed.

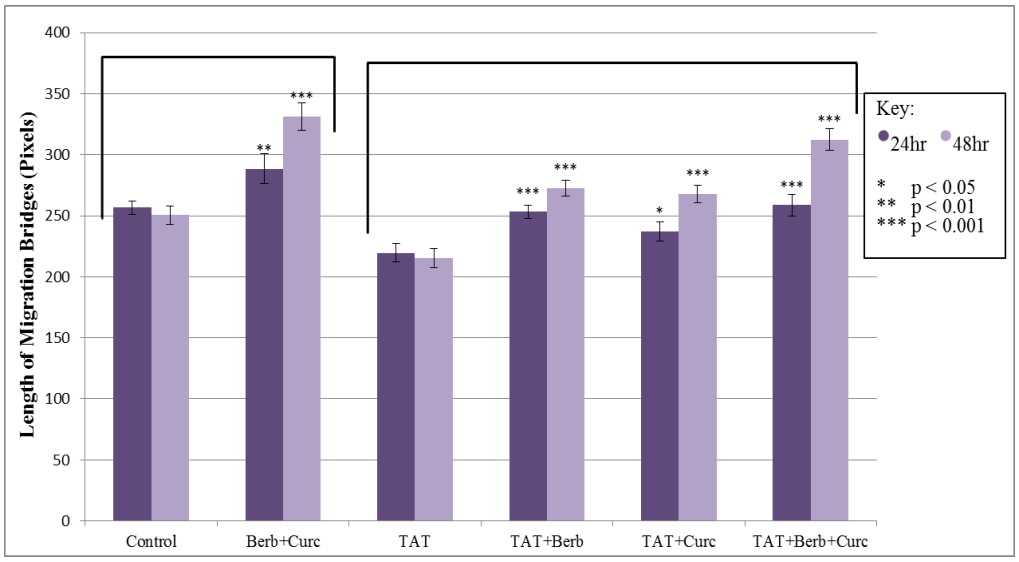

Fig. 3: The length of MBs after 24 and 48 hours from treatments being applied on 3T3 fibroblast cells. Treatment labels TAT, Berb, and Curc represent 3.21*10^-1 uM, 2.98*10^-2 uM, and 2.72*10^-2 uM solution with target chemical, respectively. The values were obtained from measurements with Microsoft Paint using the pixel ruler, and each treatment possesses a representative of 120 measurements of MBs (n=120) in two independent cell migration assay trials with six-well plate (one well per condition in each trial). The error bars represent two ±standard errors within the average mean of bridge lengths.

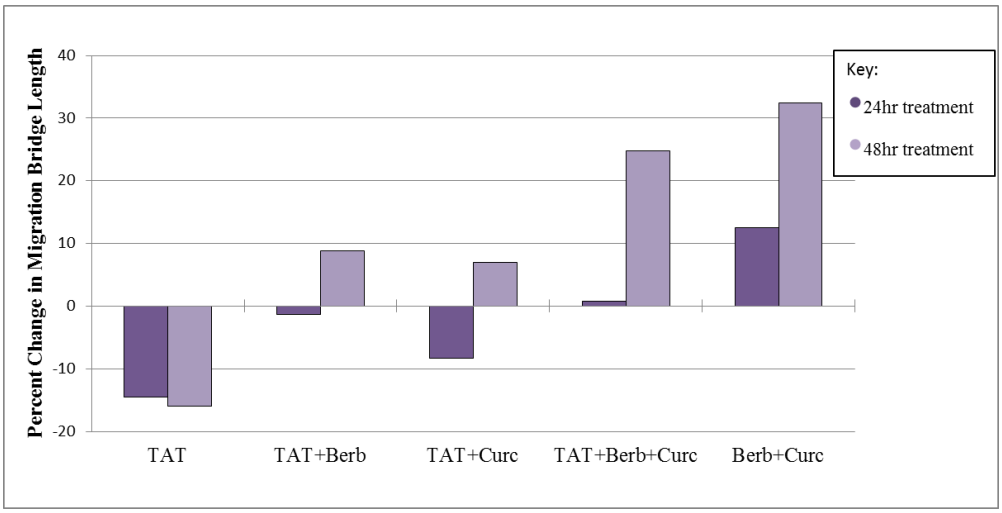

Fig. 4: The variations in the lengths of MBs in percentages after 24 and 48 hours from treatments being applied on 3T3 fibroblast cells. Treatment labels TAT, Berb, and Curc represent 3.21*10^-1 uM, 2.98*10^-2 uM, and 2.72*10^-2 uM solution with target chemical, respectively. The rates of changes at both time points are all calculated based on the bridge lengths of the respective control groups (include cell media only; not shown in this figure). Each treatment possesses a representative of 120 measurements of MBs (n=120) in two independent cell migration assay trials with six-well plate (one well per condition in each trial).

3.2. Cell Viability

The quantity of viable cells remained from PKC-MMP9 induced apoptosis was studied with the MTT Cell Viability Assay. Since TPA is a PKC agonist, resembling the expected function of TAT protein, it is used here to serve as a positive control for TAT protein as they both would instigate apoptotic cell death; in addition, the usage of TPA was of interest in gaining more insight into the mechanism by which TAT protein affects cell viability. In both assays, TAT protein and TPA alone groups reduced cell viability while TAT protein alone led to a 35.1% greater extent of viability decline than TPA alone. Cell viabilities for all treatments' along with TPA and TAT protein groups are significantly different from their respective TPA alone or TAT protein alone control ($p < 0.05$, ANOVA).

TAT protein reduced cell survival by 64.0% relative to control, while Berberine, Curcumin, and their integrated treatment increased cell viability by 33.7%, 46.1%, and 73.6% when compared to the TAT protein group, respectively, while still shows 50% less cell viability on average relative to the control. Furthermore, the percentage cell viability for the DT group is significantly higher from the results for STs by 29.1% and 18.1% to Berberine and Curcumin treatments, respectively ($p < 0.01$, ANOVA).

Curcumin, a substance expected to be unrelated to PKC regulation, treatment with TPA

resulted in cell viability that is not significantly different ($p = 0.81$, ANOVA) from the viability from TPA along treatment **(Fig. 5B)**. On the other hand, Berberine-only and DT groups showed significant differences in percent cell viability when compared with the TPA alone group, promoting viability by 19.0% ($p < 0.05$, ANOVA) and 16.2% ($p < 0.01$, ANOVA), respectively, yet this distinction in resulted cell viability between Berberine single treatment and DT is statistically insignificant ($p = 0.62$, ANOVA) **(Fig. 5B)**.

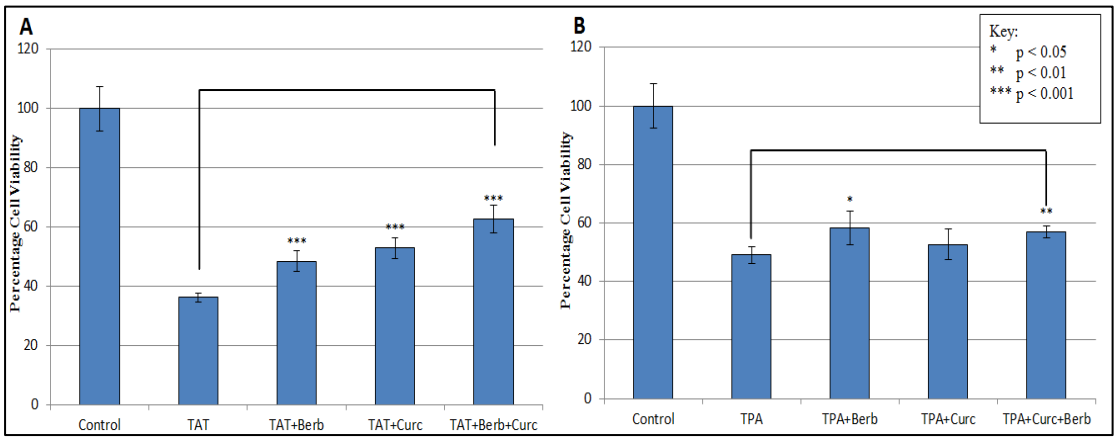

Fig. 5A & 5B: The extent of cell viability in percentages for 3T3 fibroblast cells after the introduction of different treatments; the control group (n=16; n=wells) is the basis for comparison with other groups, and all other columns (n=8) represent the percentage cell viability based on variances from the control; the assay was repeated twice and the sample size "n" represents the wells used for each condition in a single trial. Treatment labels TAT, TPA, Berb, and Curc represent 3.21*10^-1 uM, 8.56 uM, 2.98*10^-2 uM, and 2.72*10^-2 uM solution with target chemical, respectively. The asterisks show the extent of statistical significance between TAT only and TAT + treatment groups for A, and the asterisks show the extent of statistical significance between TPA protein only and TPA + treatment groups for B. The error bars represent two ±standard errors within the average mean of percent cell viability.

3.3. MMP-9 Expression

Quantitative measurements for human MMP9 from treated cells were conducted with ELISA. While TAT protein alone group possessed 50.0% higher MMP9 concentration when compared to the control, all of the treatment groups significantly lowered the MMP9 concentration (for all of them, $p < 0.001$, ANOVA) that is induced by TAT protein transfection. Precisely, Berberine, Curcumin, and their integrated treatment were able to reduce the TAT protein induced MMP9 concentration by 47.2%, 57.6%, and 67.4%, respectively **(Fig. 6)**. These treatments resulted in MMP9 levels lower than that of control while greater than that of the DT of control cells; furthermore, the magnitude of Curcumin in reducing MMP9 count is greater than Berberine by 19.7% ($p < 0.01$, ANOVA). Ultimately, the DT worked most efficaciously on lowering the MMP9 concentration, resulting in 38.2% and 23.0% more reduction in MMP9 count relative to Berberine and Curcumin STs, respectively (ANOVA, $p <$

0.001).

To determine the precision of data in each group for ELISA, two independent trials were conducted; intra-assay analysis for coefficient of variability was applied, and result showed that all groups obtained variances less than 10%, indicating that the same data series obtained from the two trials are similar to each other.

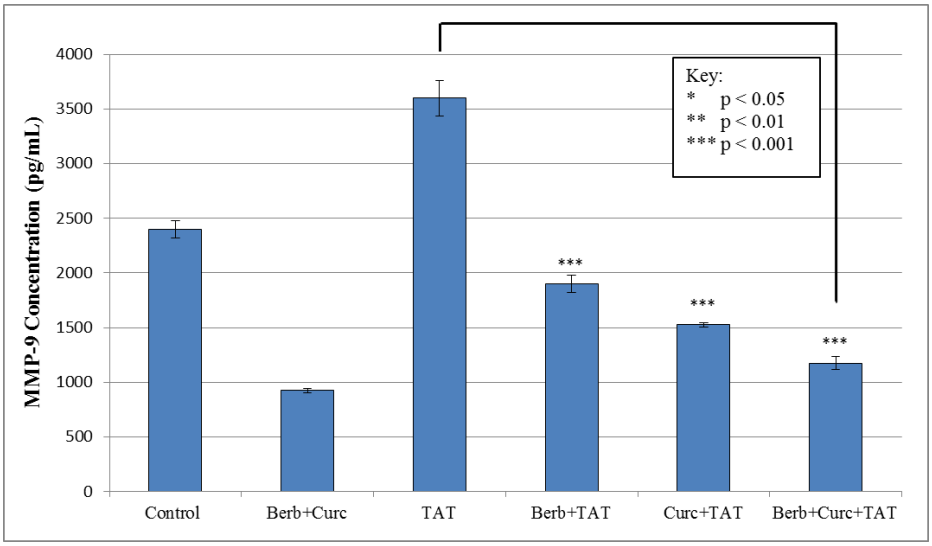

Fig. 6: The accumulation of human MMP9 in 3T3 fibroblast cells after the introduction of different treatments; data is acquired from ELISA with biotinylated capture and detection antibodies specifically made for MMP9, and microplate reader was used to assess the catalysis activity (color developing reagent on antibody-MMP9 interaction) by reading absorbance at 450nm; the resultant values are linearly proportional to the MMP9 concentration. Each column represents a sample size of 24 and an average of measurements from two trials (each trial has 12 wells and thus 12 measurements). Treatment labels TAT, Berb, and Curc represent 3.21*10^-1 uM, 2.98*10^-2 uM, and 2.72*10^-2 uM solution with target chemical, respectively. The TAT protein transfected section is shown under the frame, and the asterisks show the extent of statistical significance among TAT protein and TAT protein + treatment groups. The error bars represent two ±standard errors within the average mean of percent cell viability.

4.Discussion

This study focused on examining the effect of Berberine and Curcumin on reducing the active level of TAT, which is a potent HIV coded protein for viral transcription. Several studies have provided support for multiple mechanisms in which TAT facilitates HIV transcription. TAT could directly interact with HEXIM1 of 7sk snRNP, potentially activating P-TEFb and releasing it from 7sk snRNP (Cary et al. 2016). Consequently, P-TEFb will be made available for recruitment at viral transcription site. On the other hand, TAT is a PKC agonist, and by activating PKC, TAT could cause the phosphorylation of HEXIM1, consequently releasing and activating the initially inhibited P-TEFb from 7sk snRNP (Planès et al. 2016). On the basis of TAT as a PKC agonist, this study determined the efficacy of Berberine and Curcumin in preventing TAT's capability in achieving its normal function. The

degree of PKC activity (an indicator of TAT activity) was determined through the level of MMP-9 expression, cell migration, and cell survival. With the PKC-MAPK-ERK1/2-MMP-9 chain-reaction pathway (Ju et al. 2009; Zhang et al. 2017), change in PKC activity will cause positively correlated variations in MMP-9 protein expression. Overall, Berberine, a plant alkaloid, is expected to inhibit TAT-induced PKC activity (Qiu et al. 2017) (Liu et al. 2019), which would reduce the efficiency of TAT. Curcumin, a turmeric extract, presumably activates the 20s proteasome pathway for degradation of unfolded proteins such as TAT (Ali & Banerjea 2016), lowering the *in vitro* TAT concentration and therefore deducts its efficiency. Altogether, with Berberine and Curcumin treatments, TAT-induced changes in 3T3 fibroblasts can be significantly alleviated.

4.1. Berberine and Curcumin Obstructs TAT Efficiency in PKC Activation through Distinctive Mechanisms

Utilizing a cell migration assay, the activity of PKC can be indicated by examining the length of migration bridges (MB), which can be regulated by MMP-9 (Ramella et al. 2017), a downstream protein to PKC. Cell migration predominantly occurs during tissue injuries, such cuts, resulting in gaps among initially fully grown cell colonies represented by MB's. Ultimately, the MMP-9 expression can be determined from the length of MB's (MMP-9 stimulates cell migration, and thus greater MMP-9 expression results in shorter MB length). A 17% reduction in the length of MB's were observed for TAT transfected 3T3 fibroblasts when compared to the control; This is most plausibly due to a heightened MMP-9 expression, which indicates an increased PKC activity that positively correlates with the efficiency of TAT. The single treatments (ST) on TAT transfected cells with Berberine and Curcumin lead to approximately one-fourth increase in the length of MB when compared to TAT only group at 48 hours. This shows that both treatments are efficacious in hindering TAT's effect on MB's while Berberine shows that blocking of the PKC pathway limits TAT's effect, and Curcumin shows that degradation of TAT limits its efficiency. Although the two treatments appear to have the same effect when measured by MB's, the effect on MMP-9 differs. ELISA demonstrated that Curcumin lowered the MMP-9 concentration to 1525 pg/mL while Berberine to 1900 pg/mL, both under the influence of TAT; theoretically, Curcumin, capable of lowering MMP-9 level to a greater extent, should be more effective in

hindering cell migration as MB's are presumably and predominantly controlled by the level of MMP-9, which can be regulated by TAT-PKC pathway, in this study. However, this could also be indicating implicitly that Berberine may be blockading TAT's function through means other than PKC inhibition or merely restricting cell migration through other pathways such as TNF-JNK, where JNK instigates slight migration (Ma et al. 2014).

The dual-treatment (DT) involving Berberine and Curcumin had greater success (MB were on average ~12.5% longer than those from the ST's at 48 hours) in cell migration assay when compared to the ST's; this signifies that a greater extent of TAT's functionality could be restricted due to both its proteolysis and PKC inhibition with DT: Curcumin presumably reduces a certain amount of TAT but does not lead to the complete decimation of TAT, so having a second mechanism that disrupts TAT efficiency — PKC inhibition by Berberine — is helpful. Meanwhile, ELISA showed a drop of MMP-9 concentration from 3600 pg/mL (TAT alone) to 1175 pg/mL in TAT-transfected cells with DT, which is a greater decline than what was achieved by both ST's. Altogether, there seems to be an additive effect with DT, granting it the ability to significantly exceed the level of suppression on TAT-induced PKC activity approached by ST's.

The efficacy of both ST's and DT was greater at 48 hours than at 24. At 48 hours, Berberine and Curcumin ST's suppressed 7.7% and 13.1% more MB shortening, respectively; Intriguingly, summation of ST's enhanced suppression is equivalent to DT's improvement — DT inhibited 20.8% more MB recovery at 48 hours. These observations suggest that both Berberine and Curcumin may be acting on a time-dependent manner in carrying out their distinct roles to hinder TAT's efficiency in activating PKC.

This study suggests that PKC can be extensively activated with HIV TAT (shown most directly by ELISA on MMP-9), highlighting the probable role of PKC in HIV transcription facilitation process. On this premise, treatment formulation with Berberine and Curcumin is potentially effective for post-HIV infection as this *in vitro* study found TAT efficiency in activating PKC to be significantly down-regulated with these herbal substances.

4.2. Berberine and Curcumin Decrease TAT-Induced Cytotoxicity

MTT cell viability assays were utilized to examine the TAT's role in inducing apoptotic cell death. Since TAT upregulates PKC, a surge in the concentration of MMP-9 is expected

(Zhang et al. 2017) in cell culture. MMP-9, at high concentrations, is able to increase the permeability of mitochondrial transitional pores, causing the mitochondria to depolarize and fragment (Mishiro et al. 2014). When dysfunction and fragmentation of mitochondria occur, potential Apoptosis-inducing Factors such as cytochrome c will be released into the cytoplasm, causing the activation of apoptotic factors such as Caspase-3, hence reducing cell viability (Savitskaya & Onishchenko 2015).

TAT reduced cell viability by 64.0% when compared to the control group while the Berberine and Curcumin ST's recovered the viability by 33.7% and 46.1% in relation to the TAT alone group. In other words, ST's held back the TAT-reduced viability to 50% on average. The DT had even greater efficacy as it induced 73.6% more cell viability when compared to the TAT alone group. Curcumin and Berberine reduced cell death presumably due to their known capability in causing proteolysis of the TAT and inhibiting PKC, respectively, and both of which would lead to reduced downstream MMP-9 expression. An enhanced level of cell viability would mean that more CD4+ cells might survive if *in vivo*, potentially elongating the latency of HIV infection; in turn, this also means that AIDS, which is only dominant after < 200 CD4+ cells/mm^3, will be manifested after a longer duration. Therefore, Berberine and Curcumin are prospectively efficacious in the perspective of postponing HIV to AIDS manifestation.

4.3. Model Novelty

As mentioned earlier in the introduction, only two treatment methods were developed from the known TAT- P-TEFb connection: the "Shock and Kill" and the "Block and Lock" Strategies. The "Shock and Kill" method is no longer under much investigation as the "Kill" part of the strategy — elimination of viral reservoirs after stimulating viral transcription— cannot be easily manifested (Churchill et al. 2016). On the other hand, the more promising "Block and Lock" model possesses a severe limitation as there is only one known-to-date substance capable of prolonging the latency of provirus (Mousseau & Valente 2017), despite the fact that the "Block" part (separation of inactive elongation factor from 7sk snRNP) can be easily achieved.

This study suggests a potentially promising method in confronting TAT during HIV infection. Altogether, there are three major advantages with the Berberine and Curcumin

treatments. Primarily, Berberine and Curcumin are likely to be invulnerable to mutations that might occur in the residues that make up TAT and HIV itself; Instead of directly targeting TAT, Berberine inhibits PKC, directly reducing TAT efficiency in PKC activation. Curcumin, on the other hand, takes advantage of TAT's unfolded structure, and this characteristic of TAT is unlikely to be varied (despite potential point mutations that could occur) as its structure must be retained for PKC activation (Siddharth et al. 2012). Another advantage of Berberine and Curcumin treatment is that, similar to the "Block and Lock" strategy, they could prolong the latency of HIV infection. But unlike the "Block and Lock" method, instead of separating inactive elongation factors to avoid PKC phosphorylation, Berberine and Curucmin directly reduce TAT's potential interactions with PKC by hindering its mechanism of work (through PKC inhibition) and lessening its concentration (through proteasome degradation). Lastly, Berberine and Curcumin are extremely accessible drugs derived from herbal plants, and unlike many other post-HIV infection drugs currently in use, their prices are inexpensive and thus potentially more affordable. Furthermore, these two herbal substances are highly tolerated by human body, as referred earlier in the introduction, and thus may be relatively safer constituents than current HIV drugs.

4.4. Limitations and Future Research

The MB's examined in the cell migration assay were manually created using the 200uL pipette tip that has a <1mm width. Slight variations could occur during the process of carving MB's as the force exerted on tips when pressed on the bottom of the plate may vary; a better method to perform this procedure should involve machine work instead of manual carving. For the cell viability assay, despite that DMSO was used to dissolve Berberine, Curcumin, and TPA, it was disregarded during data analysis as it constitutes only 0.45% of the total volume of cell media used for each well; generally, DMSO can be well tolerated by cells when its concentration is under 0.5% ~ 1.0% by volume (Galvao et al. 2014), but using DMSO alone as a treatment analyzing it in the result might provide better insights of the data.

Furthermore, it is essential to vary the dosage of Berberine and Curcumin to learn the possible differences in the outcomes. By raising the concentration of these treatments, the maximum dosage can be discovered and thus the high limit for efficacy of this

Berberine-Curcumin model on TAT modulation would be clarified. In addition, although a prior study suggested 20s Protease involvement in Curcumin induced TAT degradation, a Western Blotting test can be conducted to confirm whether if the protease is fully activated. Ultimately, this test would further validate and clarify the role of Curcumin. Meanwhile, Curcumin has a relatively low bioavailability and is nearly insoluble in water, and it is also not readily absorbed by the blood upon consumption (Prasad et al. 2014). Therefore, the usage of Curcumin in clinical means may only be pursued after overcoming the issue of its low bioavailability.

Ultimately, the effect of Berberine and Curcumin should be examined *in vivo* with human lymphocyte T-cells to further validate and assess their efficacy in obstructing HIV transcription.

5. Conclusion

Berberine and Curcumin ST's and DT significantly improved the health of the TAT-transfected 3T3 fibroblasts, which is expected to be the result of reduced TAT efficiency in PKC activation, an essential step in promoting the speed of HIV transcription. The two molecules, presumably, worked differently to impact the TAT's regulation on host cellular molecular mechanism: Berberine inhibits PKC, obstructing TAT's mechanism of action, while Curcumin instigates the degradation of unfolded TAT through activation of 20s Proteasome pathway, lowering the concentration of this viral protein. The DT is more efficacious than ST's in every measure from all assays applied. Most evidently, the fact that DT caused a greater extent in the reduction of MMP-9 expression, which is heavily dependent on the activity of PKC, indicates that DT impeded TAT's role in PKC activation at a greater scale. Although PKC activation is not the only route for HIV-TAT pathogenesis, the potential positive correlation between TAT and PKC suggested by study implies that PKC is likely to be involved in HIV infection. Overall, Berberine and Curcumin treatment for HIV infection is promising as these two molecules are able to alleviate the potential impact of TAT *in vitro*, consequently suggesting similar results may be seen *in vivo*.

Ali, A., & Banerjea, A. C. (2016). Curcumin inhibits HIV-1 by promoting Tat protein degradation. *Scientific reports*, *6*, 27539. doi:10.1038/srep27539

Asamitsu, K., Fujinaga, K., & Okamoto, T. (2018). HIV Tat/P-TEFb Interaction: A Potential Target for Novel Anti-HIV Therapies. *Molecules (Basel, Switzerland)*, *23*(4), 933. doi:10.3390/molecules23040933

Bandyopadhyay, G., Standaert, M. L., Zhao, L., Yu, B., Avignon, A., Galloway, L., … & Farese, R. V. (1997) Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. *The Journal of Biological Chemistry*, *272*(4), 2551-2558.

Cary, D. C., Fujinaga, K., & Peterlin, B. M. (2016). Molecular mechanisms of HIV latency. *The Journal of clinical investigation*, *126*(2), 448–454. doi:10.1172/JCI80565

Churchill, M. J., Deeks, S. G., Margolis, D. M., Siliciano, R. F., & Swanstrom, R. (2016). HIV reservoirs: what, where and how to target them. *Natural Reviews Microbiology, 14*(1), 55-60. doi:10.1038/nrmicro.2015.5

Cooper, D. A., Steigbigel, R. T., Gatell, J. M., Rockstroh, J. K., Katlama, C., Yeni, P., Lazzarin, A., … & Nguyen, B. Y. (2008). Subgroup and resistance analyses of raltegravir for resistant HIV-1 infection. *N Engl J Med., 359*(4), 355-365. doi: 10.1056/NEJMoa0708978

Dey, S. S., Xue, Y., Joachimiak, M. P., Friedland, G. D., Burnett, J. C., Zhou, Q., … Schaffer, D. V. (2012). Mutual information analysis reveals coevolving residues in Tat that compensate for two distinct functions in HIV-1 gene expression. *The Journal of biological chemistry*, *287*(11), 7945–7955. doi:10.1074/jbc.M111.302653

Fujinaga, K., Irwin, D., Geyer, M., & Peterlin, B. M. (2002). Optimized chimeras between kinase-inactive mutant Cdk9 and truncated cyclin T1 proteins efficiently inhibit Tat transactivation and human immunodeficiency virus gene expression. *Journal of virology*, *76*(21), 10873–10881. doi:10.1128/jvi.76.21.10873-10881.2002

Hughes, C. A., Robinson, L., Tseng, A., & MacArthur, R. D. (2009) New antiretroviral drugs: a review of the efficacy, safety, pharmacokinetics, and resistance profile of tipranavir, darunavir, etravirine, rilpivirine, maraviroc, and raltegravir. *Expert Opin Pharmacother*, *10*(15), 2445-2466. doi: 10.1517/14656560903176446

Ju, S. M., Song, H. Y., Lee, J. A., Lee, S. J., Choi, S. Y., & Park, J. (2009). Extracellular HIV-1 Tat up-regulates expression of matrix metalloproteinase-9 via a MAPK-NF-kappaB dependent pathway in human astrocytes. *Experimental & molecular medicine*, *41*(2), 86–93. doi:10.3858/emm.2009.41.2.011

Katlama, C., Clotet, B., Mills, A., Trottier. B., Molina, J. M., Grinsztejn, B., Towner, W., … & Witek, J. (2010). Efficacy and safety of etravirine at week 96 in treatment-experienced HIV type-1-infected patients in the DUET-1 and DUET-2 trials. *Antivir Ther., 1*5(7), 1045-1052. doi: 10.3851/IMP1662

Kim, S., Han, J., Lee, S. K., Choi, M. Y., Kim, J., Lee, J., Jung, S. P. … & Nam, S. J. (2012). Berberine suppresses the TPA-induced MMP-1 and MMP-9 expressions through the inhibition of PKC-alpha in breast cancer cells. *The Journal of Surgical Research, 176*(1), e21-9. doi:10.1016/j.jss.2011.11.1041

Leghmari, K., Contreras, X., Moureau, C., & Bahraoui, E. (2008). HIV-1 Tat protein induces TNF-alpha and IL-10 production by human macrophages: differential implication of PKC-betaII and –delta isozymes and MAP kinases ERK1/2 and p38. *Cell Immunology, 254*(1), 46-55.

Liu, P., Li, Y., Qi, X., Xu, J., Liu, D., Ji, X., … Zou, L. (2019). Protein kinase C is involved in the neuroprotective effect of berberine against intrastriatal injection of quinolinic acid-induced biochemical alteration in mice. *Journal of cellular and molecular medicine*, *23*(9), 6343–6354. doi:10.1111/jcmm.14522

Ljubimov, A. V., & Saghizadeh, M. (2015). Progress in corneal wound healing. *Progress in retinal and eye research*, *49*, 17–45. doi:10.1016/j.preteyeres.2015.07.002

Ma, X., Li, W., Yu, H., Yang, Y., Li, M., Xue, L., & Xu, T. (2014). Bendless modulates JNK-mediated cell death and migration in Drosophila. *Cell death and differentiation*, *21*(3), 407–415. doi:10.1038/cdd.2013.154

Men, S. Y., Huo, Q. L., Shi, L., Yan, Y., Yang, C. C., Yu, W., & Liu, B. Q. (2019) Panax notoginseng saponins promotes cutaneous wound healing and suppresses scar formation in mice. *Journal of Cosmetic Dermatology*, doi: 10.1111/jocd.13042.

Mirzaei, H., Naseri, G., Rezaee, R., Mohammadi, M., Banikazemi, Z., Mirzaei, H. R., Salehi, H., … & Sahebkar, A. (2016). Curcumin: A new candidate for melanoma therapy? *Int J Cancer, 139*(8), 1683-1695. doi: 10.1002/ijc.30224

Mirzaei, H., Shakeri, A., Rashidi, B., Jalili, A., Banikazemi, Z., & Sahebkar, A. (2017). Phytosomal curcumin: A review of pharmacokinetic experimental and clinical studies. *Biomed Pharmacother, 85,* 102-112. doi: 10.1016/j.biopha.2016.11.098

Mishiro, K., Imai, T., Sugitani, S., Kitashoji, A., Suzuki, Y., Takagi, T., Chen, H., … & Hara, H. (2014). Diabetes mellitus aggravates hemorrhagic transformation after ischemic stroke via mitochondrial defects leading to endothelial apoptosis. *PloS one, 9,* e103818. Doi: 10.1371/journal.pone.0103818

Moghadamtousi, S. Z., Kadir, H. A., Hassandarvish, P., Tajik, H., Abubakar, S., & Zandi, K. (2014). A review on antibacterial, antiviral, and antifungal activity of curcumin. *BioMed research international, 2014,* 186864. doi:10.1155/2014/186864

Mousseau, G., & Valente, S. T. (2016). Didehydro-Cortistatin A: a new player in HIV-therapy?. *Expert review of anti-infective therapy, 14*(2), 145–148. doi:10.1586/14787210.2016.1122525

Narayan, V., Ravindra, K. C., Chiaro, C., Cary, D., Aggarwal, B. B., Henderson, A. J., & Prabhu, K. S. (2011). Celastrol inhibits Tat-mediated human immunodeficiency virus (HIV) transcription and replication. *Journal of molecular biology*, *410*(5), 972–983. doi:10.1016/j.jmb.2011.04.013

Newby, A. C. (2006). Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. *Cardiovascular Research, 69*(3), 614–624. doi: 10.1016/j.cardiores.2005.08.002

Ortiz, R., Dejesus, E., Khanlou, H., Voronin, E., VanLunzen, J., Andrade-Villanueva, J., Fourie, J., … & Spinosa-Guzman, S. (2008). Efficacy and safety of once-daily darunavir/ritonavir versus lopinavir/ritonavir in treatment-naive HIV-1-infected patients at week 48. *AIDS, 22*(12), 1389-1397. doi: 10.1097/QAD.0b013e32830285fb

Peterlin, B. M., Brogie, J. E., & Price, D. H. (2012). 7SK snRNA: a noncoding RNA that plays a major role in regulating eukaryotic transcription. *Wiley interdisciplinary reviews. RNA, 3*(1), 92–103. doi:10.1002/wrna.106

Planès, R., Ben Haij, N., Leghmari, K., Serrero, M., BenMohamed, L., & Bahraoui, E. (2016). HIV-1 Tat Protein Activates both the MyD88 and TRIF Pathways To Induce Tumor Necrosis Factor Alpha and Interleukin-10 in Human Monocytes. *Journal of virology*, *90*(13), 5886–5898. doi:10.1128/JVI.00262-16

Prasad, S., Tyagi, A. K., & Aggarwal, B. B. (2014). Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. *Cancer research and treatment : official journal of Korean Cancer Association*, *46*(1), 2–18. doi:10.4143/crt.2014.46.1.2

Qiu, Y. Y., Tang, L. Q., & Wei, W. (2017). Berberine exerts renoprotective effects by regulating the AGEs-RAGE signaling pathway in mesangial cells during diabetic nephropathy. *Mol Cell Endocrinol, 5*, 89-105. doi: 10.1016/j.mce.2017.01.009

Ramella, M., Boccafoschi, F., Bellofatto, K., Follenzi, A., Fusaro, L., Boldorini, R., … Cannas, M. (2017). Endothelial MMP-9 drives the inflammatory response in abdominal aortic aneurysm (AAA). *American journal of translational research*, *9*(12), 5485–5495.

Rice, A. P. (2017). The HIV-1 Tat Protein: Mechanism of Action and Target for HIV-1 Cure Strategies. *Current pharmaceutical design*, *23*(28), 4098–4102. doi:10.2174/1381612823666170704130635

Savitskaya, M. A. & Onishchenko, G. E. (2015). Mechanisms of Apoptosis. *Biochemistry, 80*(11), 1393-1405. doi: 10.1134/S0006297915110012

Simpson, M. J., Lo, K. Y., & Sun, Y. S. (2017). Quantifying the roles of random motility and directed motility using advection-diffusion theory for a 3T3 fibroblast cell migration assay stimulated with an electric field. *BMC systems biology*, *11*(1), 39. doi:10.1186/s12918-017-0413-5

Spivak, A. M., & Planelles, V. (2018). Novel Latency Reversal Agents for HIV-1 Cure. *Annual review of medicine*, *69*, 421–436. doi:10.1146/annurev-med-052716-031710

Uberall, F., Giselbrecht, S., Hellbert, K., Fresser, F., Bauer, B., Gschwendt, M. … & Baier, G. (1997). Converntional PKC-alpha, novel PKC-epsilon and PKC-theta, but not atypical PKC-lambda are MARCKS kinases in intact NIH 3T3 fibroblasts. *The Journal of Biological Chemistry, 272*(7), 4072-4078.

Volberding, P. A. & Deeks, S. G. (2010) Antiretroviral therapy and management of HIV infection. The Lancet, 376(9734), 49-62. doi: 10.1016/S0140-6736(10)60676-9

Wang, K., Feng, X., Chai, L., Cao, S., & Qiu, F. (2017). The metabolism of berberine and its contribution to the pharmacological effects. *Drug Metab Rev.*, *49*(2), 139-157. doi: 10.1080/03602532.2017.1306544

Westby, M. (2007). Resistance to CCR5 antagonists. *Curr Opin HIV AIDS.*, 2(2), 137-144. doi: 10.1097/COH.0b013e3280142007

Xing, Y., Shepherd, N., Lan, J., Li, W., Rane, S., Gupta, S. K., … Yu, Q. (2017). MMPs/TIMPs imbalances in the peripheral blood and cerebrospinal fluid are associated with the pathogenesis of HIV-1-associated neurocognitive disorders. *Brain, behavior, and immunity*, *65*, 161–172. doi:10.1016/j.bbi.2017.04.024

Yamaguchi, Y., Takagi, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., & Handa, H. (1999). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. *Cell*, *97*(1), 41-51.

Zhang, J. F., Wang, P., Yan, Y. J., Li, Y., Guan, M. W., Yu, J. J., & Wang, X. D. (2017). IL-33 enhances glioma cell migration and invasion by upregulation of MMP2 and MMP9 via the ST2-NF-κB pathway. *Oncology reports*, *38*(4), 2033–2042. doi:10.3892/or.2017.5926

Ziegler, A., Nervi, P., Durrenberger, M., & Seelig, J. (2005). The cationic cell-penetrating peptide CPP(TAT) derived from the HIV-1 protein TAT is rapidly transported into living fibroblasts: optical, biophysical, and metabolic evidence. *Biochemistry*, *44*(1), 138-148.