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# HIV Tat Protein Activates Plasma Kallikrein-Kinin System in HIV-1 Tat Transgenic Mice

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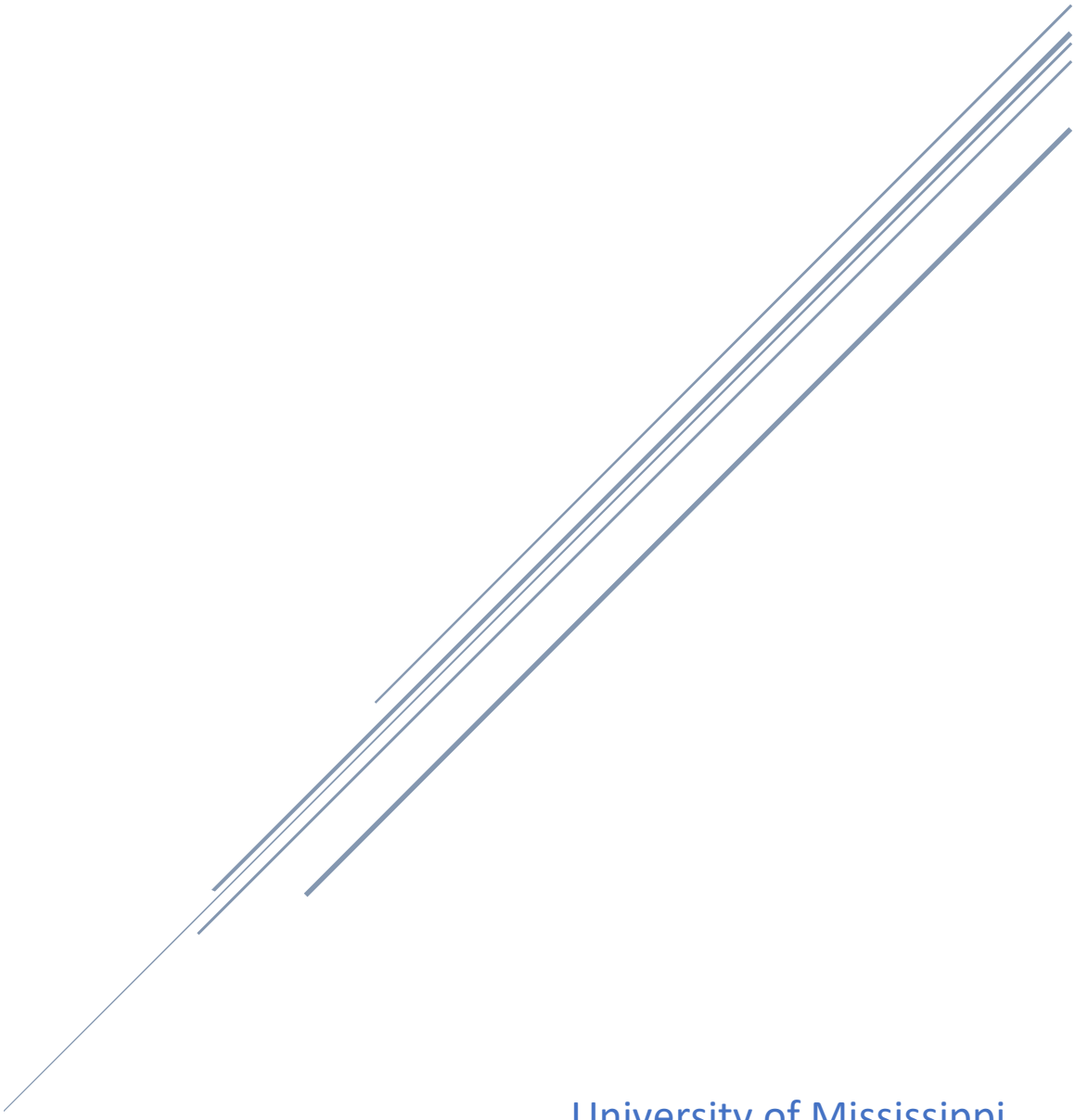

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Approved by

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# Abstract

Human Immunodeficiency Viruses are a group of lentiviruses that have seen a lot of study ever since their discovery. HIV-1, the most common and virulent form of the virus, has proven to be quite deadly when untreated, so any research into the mechanisms of HIV pathogenesis and replication could have major medical applications all over the world. HIV-1 is a sexually transmitted virus that compromises the hosts immune system and eventually leads to the patient developing AIDS, a life-threatening condition that nearly half of people infected with HIV will develop within ten years.

Our study sets out to determine exactly how HIV-1 is able to facilitate its own spread through the use of its viral proteins and genome. The protein we focus on in this study is the Tat protein. Tat is short for Trans-Activator of Transcription. It gets this name from the role that the protein plays in the facilitation of the transcription of the viral genome once it invades a host cell. It performs this role by binding to the RNA stem-loop structure known as TAR located at the 5' end of HIV-1 transcripts. Once bound, Tat assists in recruiting additional transcriptional elements, increasing the transcription of viral RNA. We seek to determine whether the presence of tat also influences the mechanisms of blood coagulation in the body such as the contact activation pathway and the kallikrein-kinin system, due to the high amount of cardiovascular ailments that are often associated with HIV.

` We decided to conduct a time course using the plasma of HIV-1 transgenic mice to see if the presence of Tat protein in the plasma would affect the production of kallikrein and its other downstream products in the kallikrein kinin system. One of these byproducts, bradykinin, has also been implicated in altering endothelial structure and the compromise of vascular selectivity

and permeability. This alteration in selectivity associated with bradykinin may play a role in allowing HIV virus particles to more easily spread to other cells and also to cross the blood brain barrier, compromising the brain to HIV infection.

The results of the study showed that the presence of Tat is indeed associated with higher levels of kallikrein activity in the plasma compared to when Tat is absent. We also found that Tat+ plasma contains a higher level of kallikrein itself, indicating that the byproduct, bradykinin, is also being produced at a higher level. These higher levels of kallikrein activity and increase in kallikrein production, signal that the presence of Tat protein is leading to higher rates of reaction in the kallikrein-kinin system, indirectly resulting in the production of excess bradykinin and also the compromise of endothelial cells.

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## List of Diagrams and Figures

# Introduction

## Human Immunodeficiency Virus

Human immunodeficiency viruses are a group of viruses belonging to the species *Lentivirus* (a subgroup of retrovirus) that infect humans. HIV is mainly spread through sexual contact or during an exchange of bodily fluids such as a blood transfusion. Mothers infected with HIV are also capable of spreading the virus non-sexually to their infants during pregnancy, childbirth, and through breastfeeding. This is due to the fact that HIV stays present as free virus particles within these bodily fluids and also as virus within infected immune cells[8]. Therefore, the exchange of these fluids can result in a new infection. If HIV infection is not treated properly and is able to progress, the patient will eventually develop acquired immunodeficiency syndrome (AIDS). Patients suffering from AIDS will be at a significantly larger risk of developing life-threatening infections and will be much more susceptible to the development of cancer. There are two different species of HIV: HIV-1 and HIV-2, each of which have their own unique characteristics. HIV-1 is the far more prevalent species, responsible for the vast majority of cases of the virus and is believed to be significantly more virulent and has a much higher infectivity[7]. HIV-2, on the other hand, has a lower virulence and infectivity, and because of this, is rarely seen outside of West Africa. The transgenic mice used in this experiment have been infected with HIV-1 so we will be focusing on this specific species of the virus in the rest of the paper.

While the relation between HIV and bleeding disorders has not been researched thoroughly, studies have shown that HIV infection is associated with serious cardiovascular abnormalities. Post-mortem examinations of children and young-patients who had died from AIDS reveal a litany of pathologies including, vascular wall infiltration by mononuclear cells

and lymphocytes, advanced atherosclerotic lesions, and medium to large-artery aneurysms[21]. Another study also showed that HIV-1 infected children, when compared to healthy children, show increased stiffness of arterial walls along with impaired endothelial function measured by fibromuscular dysplasia and reduced distensibility of the carotids[21].

HIV shows both similar and different characteristics in relation to other lentiviruses. Like other lentiviruses, HIV is transmitted as a single-stranded, positive sense, RNA virus. This RNA is responsible for coding the genes necessary for viral function, and thus is protected by a conical capsid consisting of 2,000 copies of the viral protein p24[9]. The RNA is tightly bound to nucleocapsid proteins, p7, and other enzymes, such as reverse transcriptase, proteases, ribonuclease, and integrase, each of which is vital for the development of the virion[9]. This is, in turn, surrounded by a matrix composed of the viral protein p17, which covers the capsid and provides further protection for the virion particle. On top of this, the matrix is also surrounded by a viral envelope characteristic of other lentiviruses. The envelope is created by taking the lipid bilayer from the membrane of a human host cell and integrating it with the newly formed virus particle as it buds from the cell. As a result, the envelope contains relatively few copies of HIV envelope protein, which is made of a cap of three molecules known as glycoprotein (gp) 120 and a stem of three gp41 molecules that provide structural support and anchor the cap into the viral envelope[9]. This envelope protein is encoded by the HIV *env* gene, which gives the virus the ability to attach to host cells and allows for the fusion of the viral envelope to the host's cell membrane, consequently resulting in the release of the viral contents of the infected cell[10].

The RNA genome of HIV-1 consists of seven structural landmarks and nine genes. The landmarks are various sequences of nucleotides that are responsible for different functions such as binding and transcription. These landmarks are as follows: LTR, TAR, RRE, PE, SLIP, CRS,

and INS. The genes present in HIV (See figure 1) are *gag*, *pol*, *env*, *tat*, rev, *nef*, *vif*, *vpr*, *vpu*, and sometimes *tev* (a combination of *tat*, *env*, and *rev*). Gag, pol, and env are the genes containing the necessary information for the production of the structural proteins to be used in new virus particles[9]. The remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*, are all responsible for regulatory function and code for proteins that control the ability of HIV to infect host cells, undergo replication, or cause disease[9]. The *tat* gene in particular is vital for replication as it codes for Tat proteins that drastically enhance viral transcription.

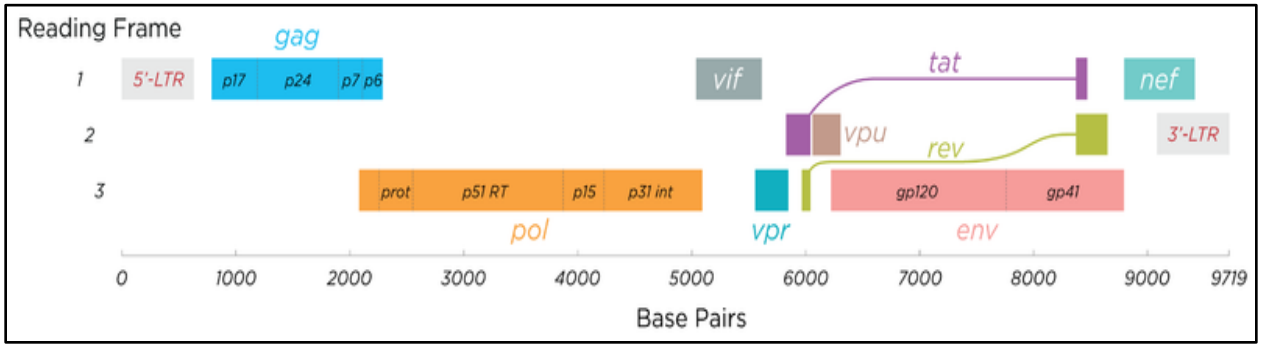



**Figure 1:** Structure of the HIV-1 genome. It is roughly 10,000 base pairs long and consists of nine genes. The *tat* gene can be seen alongside the other regulatory genes responsible for replication and infection.

Credit: Thomas Splettstoesser (www.scistyle.com)

## Tat Protein

A Tat protein is a regulatory protein that has been encoded for by the tat gene in HIV-1. Tat is an acronym that stands for "trans-activator of transcription". This name is derived from its function which is to provide a means for efficient transcription of the viral genome[12]. Tat does this by binding to the RNA stem-loop structure, TAR (trans-activating response element), which

is located on the 5' ends of HIV-1 transcripts. Once bound to TAR, Tat is able to alter the properties of the transcription complex and is able to recruit the positive transcription elongation complex (P-T EFb) of CDK9 and cyclin T1, resulting in the increased production of full-length viral RNA[11].

Tat also contains a transduction domain, also known as a cell-penetrating peptide. Similar to a Trojan horse, this domain gives Tat the ability to enter host cells by way of the cell membrane. This is significant as a major obstacle to neural therapies has been figuring out a way to deliver medication or certain molecules across cellular membranes and the blood/brain barrier[13]. For many potential pharmaceuticals, such as neurotrophins, the endothelial wall of the BBB is not entirely permeable, therefore any mechanism that demonstrates an ability to cross this barrier shows great potential for medical use. It is hypothesized that some HIV virions may gain access to the CNS through this blood/brain barrier paracellularly, aided by breaches in BBB integrity. This is supported by early post-mortem analyses of brains infected with HIV that show high levels of serum proteins, suggesting the degradation of the BBB has taken place.

## Contact Activation Pathway

The contact activation pathway is a group of proteins in the plasma that play a role in surface-activated blood coagulation tests. These proteins are Factor XII, prekallikrein (PK), and High-Molecular Weight Kininogen (HK). Factor XII and PK are proteases while HK is a non-enzymatic co-factor. The contact activation pathway is initiated by the binding of Factor XII to a negatively charged surface and the reciprocal activation of Factor XII and PK into Factor XIIa and PKa respectively[14].

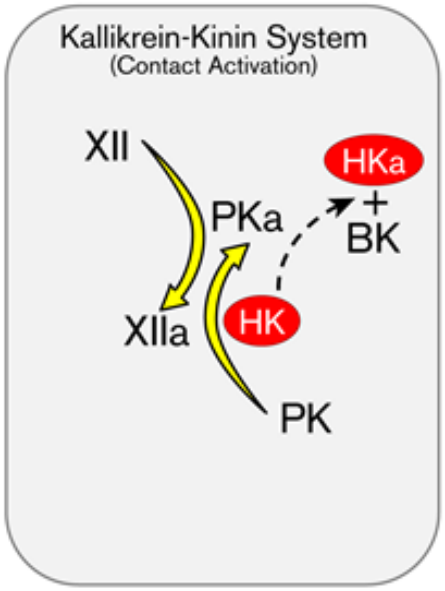



**Figure 2: Kallikrein-Kinin System.**

Michał B. Ponczek, Aleksandr Shamanaev, Alec LaPlace, S. Kent Dickeson, Priyanka Srivastava, Mao-fu Sun, Andras Gruber, Christian Kastrup, Jonas Emsley, David Gailani; The evolution of factor XI and the kallikrein-kinin system. *Blood Adv* 2020; 4 (24): 6135–6147. doi: https://doi.org/10.1182/bloodadvances.2020002456

- Factor XII and PK undergo reciprocal activation to produce Factor XIIa and PKa. PKa then goes on to cleave HK, releasing Bradykinin (BK) and HKa.

Factor XIIa can then go on to initiate the coagulation cascade by activating Factor XI into Factor XIa, leading to the formation of blood clots[2]. The contact activation pathway can also activate the kallikrein-kinin system in which PKa can cleave HK to form HKa, releasing a peptide known as bradykinin (BK). Bradykinin is a molecule that plays an important role in the mediation of inflammation and vascular permeability[3].

## ***Factor XII***

Also known as Hageman Factor, human coagulation factor XII is the zymogen form of a protein found in the plasma that plays a role in the coagulation cascade responsible for blood clotting and hemostasis[15]. It was first discovered in 1955 when the blood sample of 37-year-old John Hageman was found by doctors to have large delays to clotting times in test tubes. This was novel, as the patient was experiencing no hemorrhagic symptoms and led the doctors to determine that this novelty was due to the lack of an unidentified blood clotting factor. This blood clotting factor turned out to be factor XII. The main role of factor XII in the coagulation cascade is to be the starting point of the intrinsic pathway and to activate other coagulation

enzymes such as factor XI and prekallikrein in vitro. Factor XII is thought to play a smaller role in coagulation in vivo, as patients with factor XII deficiencies do not typically experience excessive bleeding symptoms.

As stated earlier, Factor XII is a zymogen and must be activated in order for it to fulfill its enzymatic function. In vitro, Factor XII is activated by negatively charged surfaces such as glass. This is makes it a very good molecule for the initiation of coagulation cascades in laboratory studies[2]. In vivo, Factor XII can be activated by a number of different negatively charged macromolecules including collagen, fibronectin, and certain proteins found in the cell wall or membrane of certain microorganisms. Contact with these polymers activates Factor XII, initiating the intrinsic pathway and contributing to the formation of fibrin. Once activated, Factor XII is converted into its active protease form of Factor XIIa, which in turn goes on to activate Factor XI into Factor XIa and prekallikrein into plasma kallikrein by selectively cleaving the Arg369-Ile370 found in both Factor XI and prekallikrein.

## ***Factor XI***

The next step in the intrinsic coagulation pathway, following the activation of factor XII into factor XIIa is the activation of factor XI into factor XIa. Like factor XII, factor XI is a zymogen that must be activated in order to perform its enzymatic functions[16]. In its activated form, it is a serine protease that starts the next step of the intrinsic pathway by selectively cleaving the arg-ala and arg-val peptide bonds found in factor IX[18]. Factor IX will, in turn, be activated and be able to continue the coagulation cascade.

Factor XI, in its inactive form, is produced by the liver and circulates in the blood as a homo-dimer[17]. The structure of the homo-dimer, formed by the combination of two polypeptide

chains, consists of four apple domains and a fifth serine protease domain. The serine protease domain is responsible for the catalytic activity of factor XI, once activated. This activation is a result of inactive Factor XI coming into contact with activated factor XIIa or thrombin, both of which cleave the Arg369-Ile370 peptide bonds on both subunits of the dimer. It is thought that this cleavage results in the partial detachment of the catalytic protease domain, giving it enough space to properly bind to factor IX and fulfill its protease function.

There is another domain in the factor XI homo-dimer that fulfills an important function as well. Of the four apple domains, there is one that is responsible for the binding site of a molecule known as high molecular weight kininogen (HK). HK is the molecule responsible for carrying factor XI and prekallikrein in the blood. HK forms a complex with inactivated factor XI by binding to its apple domain. HK itself is not enzymatically active but is a necessary component in the complex as factor XIIa would be unable to activate factor XI without it[5].

Even though the contact activation pathway can activate Factor XI and the clotting cascade and is often observed to be activating coagulation in lab settings[3], the actual physiological role of the contact system in vivo is hotly debated. This is due to the fact that people who have deficiencies in the proteins that make up the system (FXII, PK, and HK) rarely experience bleeding disorders as a result, leading many to suggest that the contact activation pathway is a redundant system[3].

## Prekallikrein

Prekallikrein, also known as Fletcher factor, is a serine protease that plays a vital role in the contact activation pathway. In this system, prekallikrein complexes with the protein chaperone high-molecular-weight kininogen to form the HK/PK complex. This complex can then interact with Factor XII which cleaves the prekallikrein from HK to produce kallikrein (PKa)[4].

Prekallikrein is homologous to factor XI in the coagulation cascade as it also consists of four apple domains and a fifth, catalytic serine protease domain[19]. However, unlike Factor XI, prekallikrein does not form dimers. In addition, the bond cleaved by Factor XII in the activation of prekallikrein to kallikrein (Arg371-Ile372) is homologous to the corresponding bond cleaved when Factor XI is activated by Factor XII[20].

As was mentioned in the Factor XI section, prekallikrein and Factor XI are very closely related with 58% identity at the amino acid level[4]. The Factor XI and and prekallikrein N-terminal heavy chains both consist of four apple domains. They are also both activated by the same molecule, Factor XIIa, through cleavage of Arg-Ile bonds (Arg371-Ile372 and Arg369-Ile370, respectively)[4]. They both also circulate as cofactors of High Molecular Weight Kininogen, a protein chaperone vital for the proper functioning of the contact activation pathway.

## High Molecular Weight Kininogen

A key component of the contact activation pathway is the plasma protein high-molecular-weight kininogen (otherwise known as HMWK or HK). High-molecular-weight kininogen acts as a chaperone for prekallikrein (PK). HK is not enzymatically active and acts only as a cofactor in the activation of prekallikrein[5]. In the contact activation pathway, HK forms a complex with

PK, which can be cleaved by Factor XII to produce plasma kallikrein (PKa)[5]. PKa, in turn, may go on to cleave HK to produce HKa and Bradykinin.

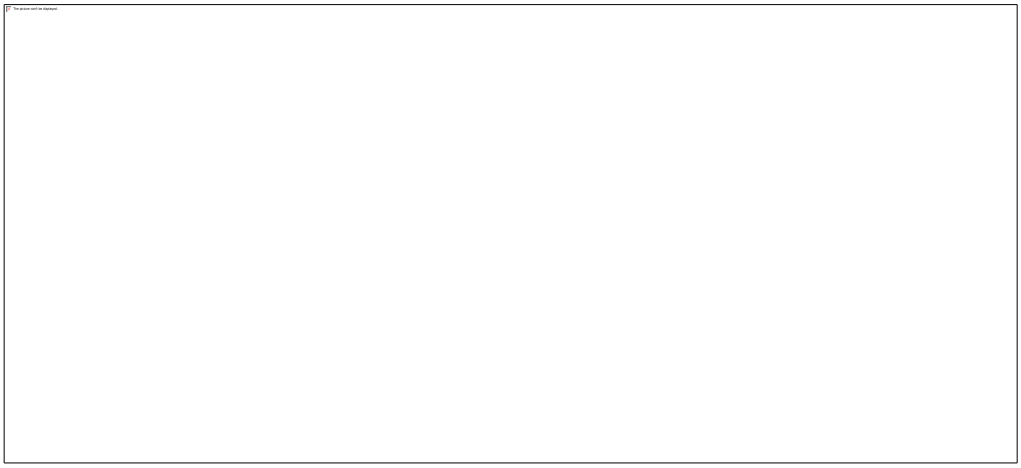


**Figure 3:** The cleavage of HK by Kallikrein (PKa) to produce Bradykinin (BK) and HKa.

High molecular is also necessary for the activation of Factor XI by Factor XIIa. In the same way that HK acts as a chaperone for the protease prekallikrein, it also acts a chaperone for the protease Factor XI. Both Factor XI and prekallikrein circulate in the plasma in the form of noncovalent complexes with HK. These complexes are formed from the binding of HK to the heavy chains located in the two proteases[4].

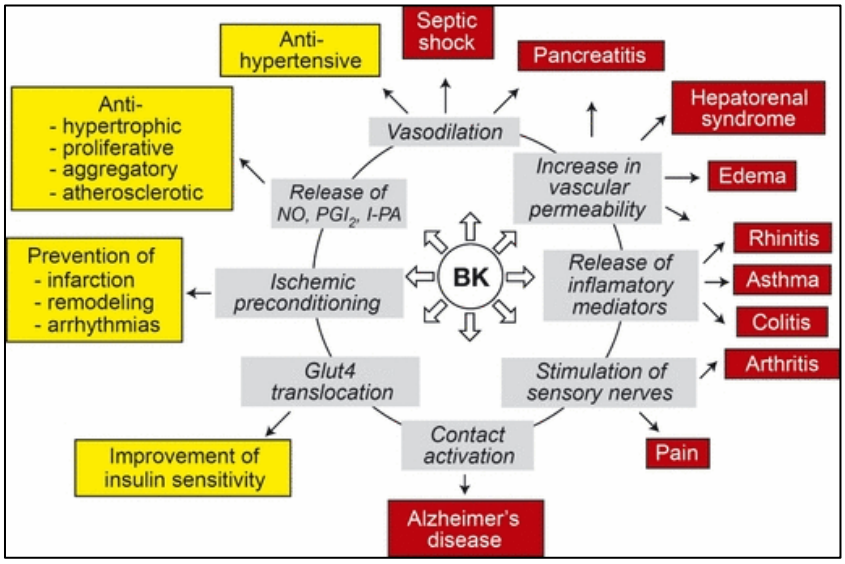
## Bradykinin

Bradykinin, the product of the proteolytic cleavage of HK by PKa, is a vasoactive peptide that plays a major role in inflammation and vascular permeability[6]. It is a low molecular weight nonapeptide, which can be rapidly metabolized by certain metalloproteases such as angiotensin-converting-enzyme (ACE), neutral endopeptidase (NEP), carboxypeptidase N (CPN), and

aminopeptidase P[6]. Bradykinin can influence inflammatory processes through the activation of endothelial cells, resulting in the promotion of vasodilation and increased vascular permeability. It does this by binding to endothelial $B_1$ and $B_2$ receptors and exerting its physiological effects (see Figure 4) These effects are what leads to the classic inflammation symptoms of redness, heat, swelling, and pain[6]. We hypothesize that the vasodilation and vascular permeability increase caused by bradykinin production, may also play a role in facilitating the spread of HIV Tat protein.

**Figure 4:** Effects of bradykinin in different pathophysiological states. This figure demonstrates the various diseases and ailments that can be caused by the physiological effects of BK.

Maurer M, Bader M, Bas M, Bossi F, Cicardi M, Cugno M, Howarth P, Kaplan A, Kojda G, Leeb-Lundberg F, Lötvall J, Magerl M. New topics in bradykinin research. *Allergy* 2011; **66**: 1397–1406.



## Objectives

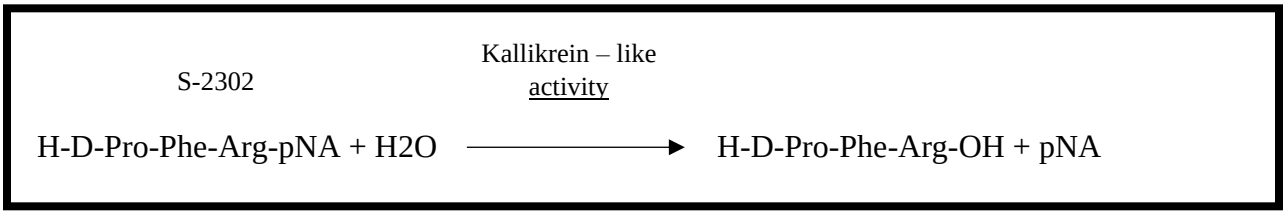
The main objectives of this study is to measure the kallikrein and factor XII activity in the transgenic mice to determine whether HIV trans-activator of transcription, (Tat) protein, alters the structure and function of the endothelium, inducing a prothrombotic and proinflammatory state contributing to the activation of the plasma kallikrein-kinin system (KKS) and favoring the passage of HIV. These changes to the endothelium, possibly due to the presence of HIV tat, facilitate leakage of infected plasma into the brain, compromising the integrity of the blood brain barrier. It is hoped that through isolation and inhibition of tat protein, we may be able to limit the effects of HIV spread.

## Methods and Materials

### Equipment:

- BioTek® Synergy HTX Multi Mode Reader
- 96-well microplate
- Pipette
- Microcentrifuge tubes for sample transfer

### Reagents:




- Chromogenic Substrate S-2302 (2mM)
- Tris Buffer, pH–7.8 (25°C)
    - Tris
    - NaCl
    - Distilled water

### S-2302

In order to conduct our study on the effects of HIV Tat protein on the kallikrein-kinin system. We decided to conduct a time course using Chromogenic Substrate S-2302 to measure the kallikrein-like activity in the plasma. The plasma kallikrein-like activity catalyzes the splitting of p-nitroaniline (pNA) from the substrate H-D-Pro-Phe-Arg-pNA (S-2302). This allows us to use S-2302 to detect the activation of prekallikrein into kallikrein by Factor XII.

The rate at which the pNA is released is measured photometrically at 405 nm. To do this, we prepared a solution of test plasma, Tris Buffer (pH-7.8), and Substrate S-2302.

**Time Course**

As this experiment required the analysis of many samples simultaneously, it was necessary to use a 96 well microplate, where plasma solution samples were loaded into rows based on the identity of the transgenic mouse that the plasma was harvested from. The specific plate reader used in our experiments was the BioTek® Synergy HTX Multi Mode Reader. As the plasma samples being used in the experiment all have different protein concentrations, it was necessary to dilute them to the same concentration before they undergo the 1:10 dilution with Tris Buffer. Each individual well contained 20 µL of 1:10 dilution of the sample plasma, along with 60 µL of Tris Buffer and finally 20 µL of the selected substrate (S-2302/S-2366). This brought the total volume of each well to 100 µL with a final substrate concentration of 400 µM. Before the substrate can be added, it is necessary to incubate the samples in the well along with the Tris Buffer at 37°C for 5 minutes to ensure that reagents can react in conditions resembling the environmental conditions in the human body. After incubation, the substrate can then be added to each well. Each sample solution was arranged into 4 rows of 4 plasma samples harvested from different mice, each row containing three samples from the same mouse (See Figure 5).

| | | | |
|---|---|---|---|
| A | Sample 1 + | Sample 1 + | Sample 1 + |
| B | Sample 2 + | Sample 2 + | Sample 2 + |
| C | Sample 3 - | Sample 3 - | Sample 3 - |
| D | Sample 4 - | Sample 4 - | Sample 4 - |

**Figure 5**: An example of the well setup used in this experiment. Each cell on the table represents one well on the 96-well plate. Samples are arranged in rows, with each row containing 3 identical sample solutions.

It is necessary to be close by to the plate reader when adding the substrate to each well as the reaction takes place extremely quickly. If one takes too long to load the plate into the reader after addition of the substrate, the reader may only catch the tail end of the reaction and not be able to give an accurate result on the rate of reaction. Once the plate was loaded into the reader, the time course was allowed to run for a period of approximately 1 hour, after which the plate was removed and the OD of the solution in the wells had been recorded. OD measurements were taken every 5 minutes throughout the hour bring the total number of readings for each sample to 12 over the course of the hour. The rate of change in OD was used as an indication of the rate of kallikrein substrate cleavage, as the proceeding reaction will change the color of the solution shortly after addition of the substrate. After the data for each sample had been completely recorded, it was compiled together and then the average rate of reaction was calculated for both Tat + samples and Tat- samples.

**Plasma Samples (In order of Use)**

| Transgenic Mouse ID# | Tat | Stock protein concentration (mg/mL) |
|---|---|---|
| 15/675 | + | 2.5 |
| 20/728 | - | 3.2 |
| 7/672 | + | 3.5 |
| 6/714 | - | 2.5 |
| 12/739 | - | 3.0 |
| 16/676 | + | 1.7 |
| 8/673 | + | 1.7 |
| 14/471 | - | 2.5 |
| 5/713 | - | 4.1 |
| 17/677 | + | 3.3 |
| 4/712 | - | 3.15 |
| 18/678 | + | 3.16 |
| 10/737 | - | 4.83 |
| 3/711 | - | 7.04 |
| 19/727 | + | 3.44 |
| 2/690 | + | 5.19 |
| 1/689 | + | 4.7 |
| 19/727 | + | 3.44 |
| 11/738 | - | 4.1 |
| 10/737 | - | 3.44 |

## Inhibition Study

Following completion of the S-2302 time course, we ran an additional similar experiment. The only difference in this experiment relative to the last time course was that after preparing the solution in the microplate wells, 1 µL of 500 µM Soybean Trypsin Inhibitor or Kallistop was added to measure its effect on the rate of kallikrein substrate cleavage. 1 µL of the Tris Buffer was removed to bring the volume of buffer in each well to 59 µL so that the total volume could remain at 100 µL to not alter the concentrations of each reagent. Other than the addition of SBTI, nothing was changed in the methodology of this experiment, relative to the previous one.

## Data and Results

After the experiment was conducted using each plasma sample available, the relative rates of substrate cleavage were analyzed to determine whether there was a noticeable trend in the rate of reaction based on the presence or absence of tat protein. Upon immediate inspection of the wells after the addition of substrate, I found that many of the wells containing Tat protein demonstrated a very rapid color change from clear to yellow. I inferred from this that this color change is most likely indicative of the reaction taking place. This trend did not apply to every round of experiments, but it was observed with enough frequency to assume that the presence of Tat was already playing a role in the rate of reaction.

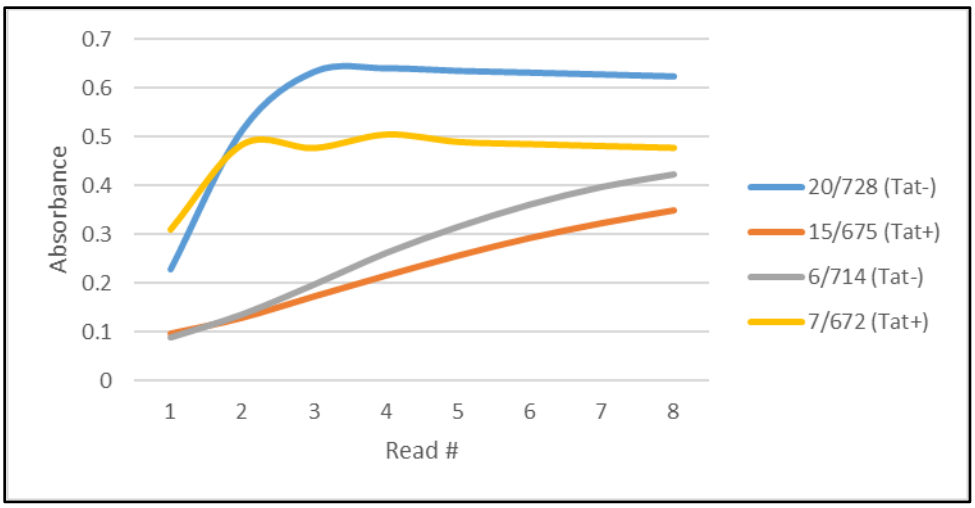



**Figure 6: Experiment 1-** The plasma samples were all diluted to a matching protein concentration of 2.5 mg/mL before their 1:10 dilution. The first experiment was fairly inconclusive as both Tat+ and Tat- samples exhibited similar rates of reaction. Samples 728 (Tat-) and 672 (Tat+) both reacted much quicker than their counterparts 675 (Tat+) and 714 (Tat-).

*Note read numbers are in increments of five minutes, therefore read # 5 = 25 minutes in the spectrophotometer*

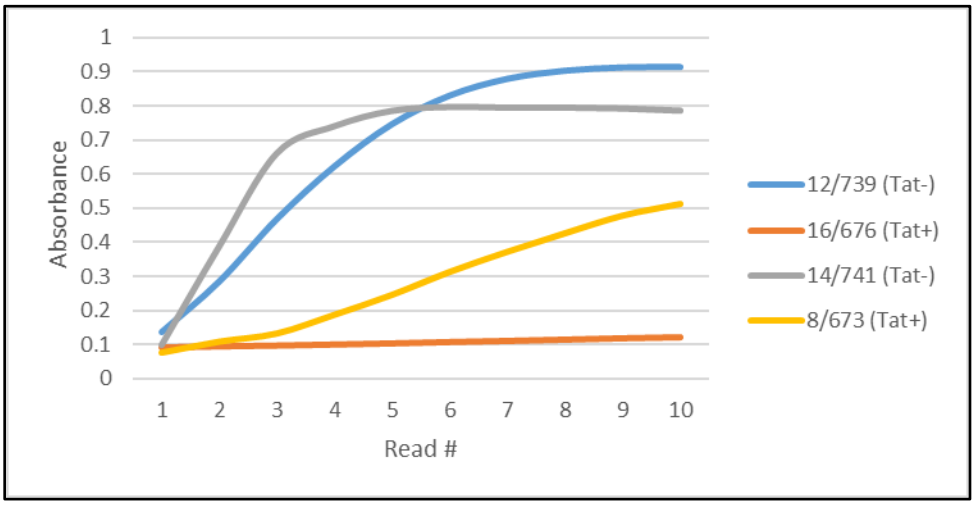



**Figure 7: Experiment 2-** This time the protein concentrations of the plasma samples, pre- 1:10 dilution, were brought to 1.75 mg/mL. The results of the second experiment went against what we were expecting as both of the Tat- samples reacted very quickly while the Tat+ samples lagged behind with sample 676 (Tat+) barely reacting at all. This could mean that there was some sort of error involved in the transfer of reagents into the microplate or that a faulty reagent may have been used.

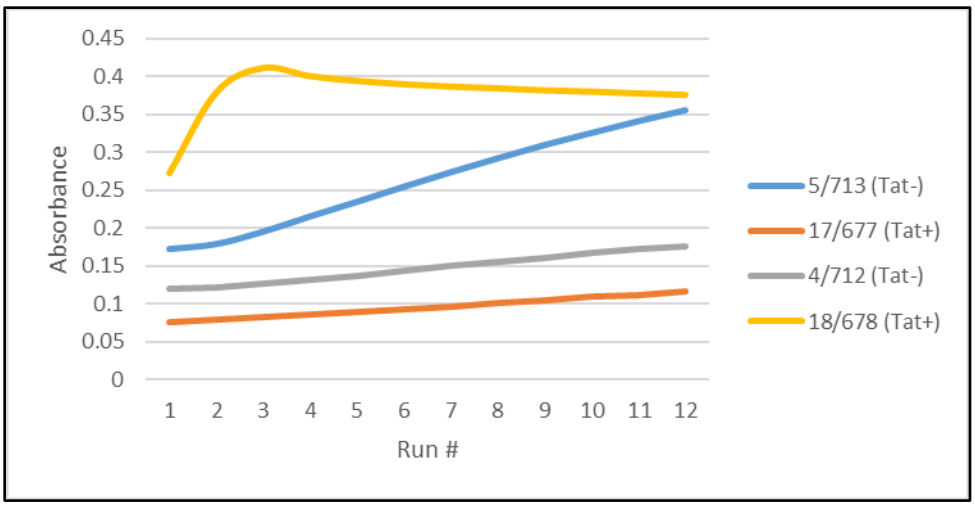



**Figure 8: Experiment 3-** This time, protein concentrations were at 3.15 mg/mL. Sample 678 (Tat+) rapidly reacted compared to the other samples and was able to reach equilibrium at around 15 minutes. It was followed by sample 713 (Tat-), however this sample was never able to reach equilibrium.

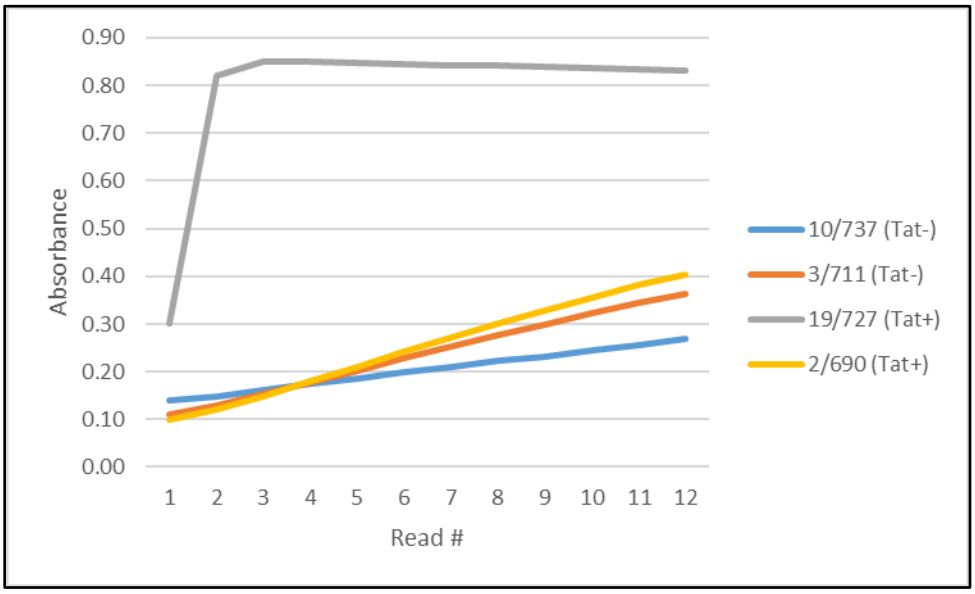



**Figure 9: Experiment 4-** In this experiment, protein concentrations were brought to 3.44 mg/mL. Sample 727 (Tat+) showed by far the most rapid reaction rate up until this point, vastly exceeding the rates of the other samples in the experiment. I am not entirely sure as to what this rapid rate can be attributed to, as all samples were at the same protein concentration and were exposed to the same volume of substrate.

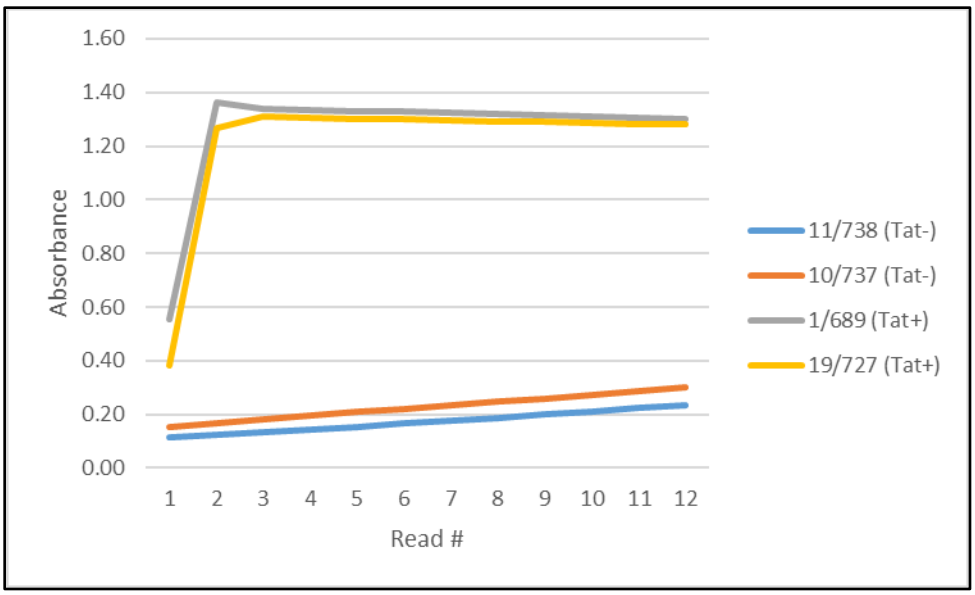



**Figure 10: Experiment 5-** Protein concentrations in this experiment were the same as the previous experiment at 3.44 mg/mL. The extremely rapid reaction rate was observed again, however, this time it was exhibited by both Tat + samples (samples 689 and 727). These samples reached equilibrium before even 10 minutes had passed while the Tat – samples barely reacted at all relative to the others.

Once the data had been compiled, it supported the inference that Tat protein was influencing kallikrein activity in the plasma. On average, Tat+ plasma samples showed a relative rate of substrate cleavage nearly twice as high as the Tat- samples. The graph below is a box and whisker showing the relative reaction rates of all Tat+ samples and all Tat- samples through all 5 experiments. The median reaction rate of the Tat+ samples came in at around a 0.7 while the Tat- samples showed a median relative rate of around 0.25. It is also very interesting to note that the upper quartile range of the Tat- samples fell just below the lower quartile range of the Tat+ samples.

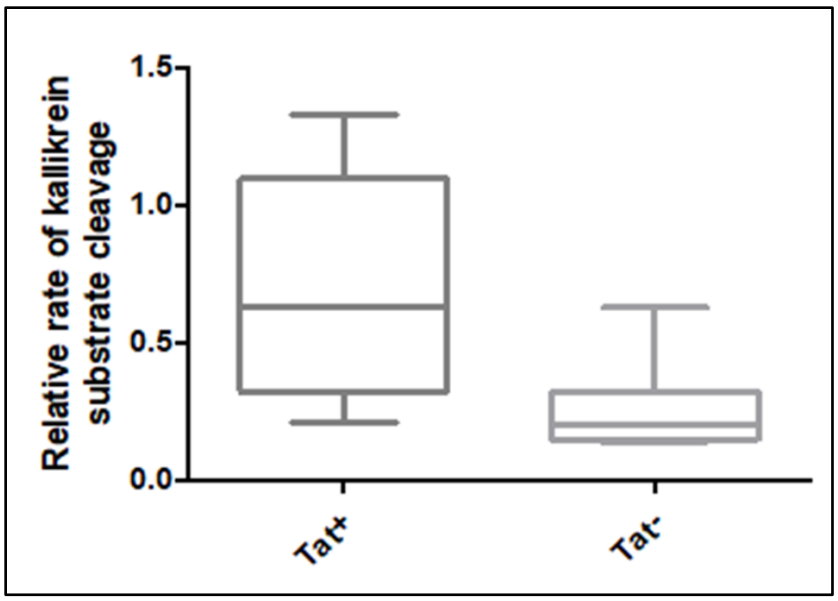



**Figure 11:** Comparing the relative rates of substrate cleavage between Tat+ and Tat- samples shows that Tat+ samples reacted much more quickly than Tat- samples. A higher rate of substrate cleavage indicates higher levels of kallikrein activity.

It is important to note that rates of kallikrein substrate cleavage are indicative of the amount of kallikrein activity and not necessarily the amount of kallikrein itself. While higher kallikrein activity may be a sign of higher kallikrein levels, further investigation was required before we could determine whether Tat protein was leading to increased production of kallikrein or simply just facilitating the cleavage of substrate.

After investigating further, it was in fact found that the HIV-1 Tat transgenic mice demonstrated higher levels of kallikrein present in the plasma (Figure 12). This leads us to believe that Tat is facilitating the production of kallikrein, therefore indirectly facilitating the production of Bradykinin as well.

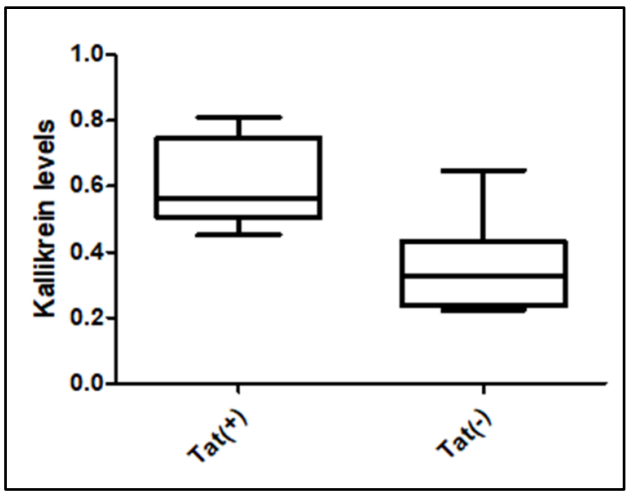



**Figure 12:** HIV-1 Tat transgenic mice demonstrate higher levels of kallikrein in the plasma compared to the plasma of unaffected mice.

Now that we have observed elevated kallikrein levels in the HIV transgenic mice, we can look for ways to inhibit that activity in order to limit the production of the byproduct, bradykinin. As bradykinin is the molecule implicated in the compromise of endothelial selectivity associated with HIV spread, inhibition of kallikrein production may have important medical applications for limiting the spread of HIV throughout the body. We found that plasma kallikrein activity can in fact be inhibited through the use of Kallistop and Soybean Trypsin Inhibitor. Plasma kallikrein activity was reduced by Kallistop and SBTI with $IC_{50}$= 1.1µM and 1.3µM. Introduction of these inhibitors greatly reduced the measured kallikrein activity in the plasma (Figure 13).

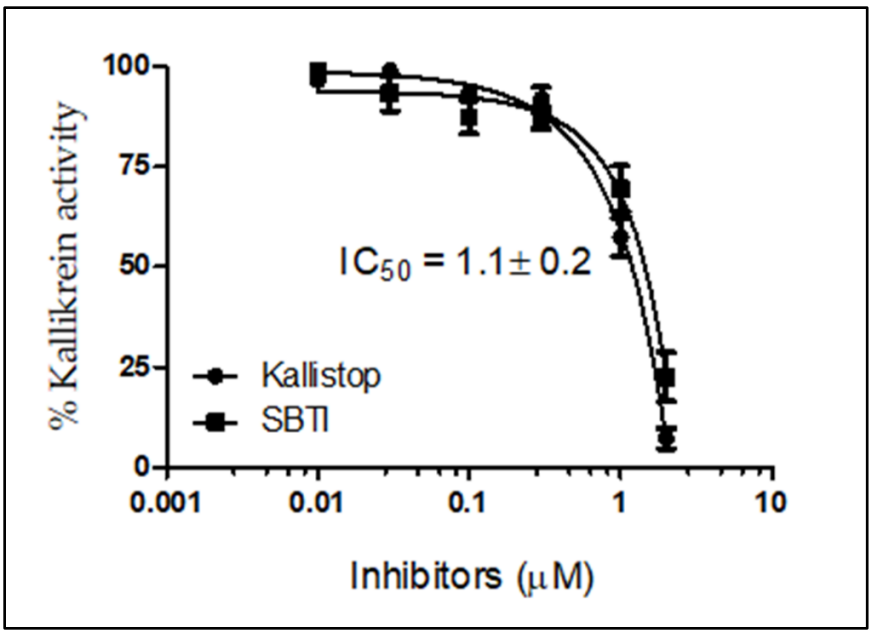



**Figure 13:** Graph demonstrating the inhibition of kallikrein activity by SBTI and Kallistop. As concentrations of inhibitor increase, kallikrein activity, exponentially decreases. An inhibitor concentration of just 1 μM was able to decrease kallikrein activity by approximately 90%.

## Conclusion

Overall, the results of the experiment were pretty supportive of our hypothesis that HIV Tat expression was associated with higher kallikrein activity. This correlation was found to have a p-value of less than 0.05 signaling that this correlation is most likely not due to random chance. We believe that this difference has to do with what was discussed earlier in this paper, that HIV Tat protein is helping to activate the plasma kallikrein system in the plasma of transgenic mice. By utilizing the selectivity of Chromogenic Substrate S-2302, we were able to get a visualization of this increased kallikrein system activity. This was even observable by just looking at the wells of many of the Tat+ samples which showed a higher tendency to quickly change from a clear color to a yellow color within a very short time frame. As was discussed in the data section, this

was not true for every sample as some Tat- samples showed an ability to react quickly as well, however, not at the same frequency as Tat+ samples.

Based on the evidence compiled, I believe that it is safe to say that Tat protein plays a major role in the facilitation of the kallikrein-kinin system. High levels of kallikrein activation in Tat+ samples, has the derivative effect of also increasing levels of Bradykinin. Bradykinin, a known product of kallikrein activation, is a major influence in inflammation and vascular permeability, both of which are conducive to HIV transmission. Therefore, if one were able to inhibit the kallikrein-kinin system, which we were able to show is possible through the use of Kallistop and Soy Bean Trypsin Inhibitor, it could also be assumed that production of Bradykinin would be inhibited as well, leading to a decrease in the inflammation and vascular permeability that allows HIV virus particles to spread so efficiently. This inhibition could be a major benefit to the integrity and structure of the endothelium in the neurovascular unit of the blood brain barrier. As was discussed earlier, the integrity of the neurovascular unit is extremely important in preventing the leakage of plasma into the brain parenchyma, and it is this leaky plasma that HIV and HIV particles use to penetrate the blood brain barrier, compromising the brain to infection in the process. That is why we believe this area deserves further research into exactly how Bradykinin influences the endothelium and its selectivity and also that further research should be done on possible medical applications of kallikrein-kinin inhibition and how it could be a possible treatment for those suffering from HIV infection.

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