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Human immunodeficiency virus (HIV) establishes a latent infection in cells to ensure a persistent infection throughout an infected individual's life. HIV can establish this latent infection in a variety of cells. Highly Active Anti-Retroviral Treatment (HAART) is a selection of drugs used to inhibit the production of new HIV and new infections and can effectively diminish virus population in blood. However, due to the pathological mechanism of the virus, it is not possible yet to completely eradicate virus as it remains immunologically invisible in latent cellular reservoirs. The cellular reservoirs where HIV evades the immune system are not known completely. Current research efforts are focused on identifying the cellular populations where HIV remains latent and determine how those latent reservoirs are established. By identifying latent cellular reservoirs where HIV resides strategies can be developed to target and kill infected cells or prevent emergence of virus. We hypothesized that primary, skin, human mast cells may represent a previously unknown latent reservoir for HIV. Because mast cells can be activated through IgE-and non-IgE-dependent stimulation, we further hypothesized activated mast cells may be more vulnerable to infection. Our experimentations suggest that skin-derived mast cells are not susceptible to HIV infection and are not an inducible reservoir for HIV.

One strategy for inhibiting viral replication has been with fullerenes. Fullerenes are carbon spheres that can be functionalized for use in various biological systems. Fullerenes functionalized with large dendrimeric moieties have been shown previously to

inhibit viral replication in vitro, but the majority of investigations that have explored fullerenes as an inhibitor of HIV were assessed computationally. Based on these previous studies we hypothesized that certain functionalized fullerenes will suppress HIV infectivity and/or replication. We hypothesized that these fullerenes may interact with HIV protease and performed molecular modeled docking studies to investigate this idea. We also performed *in vitro* dose response assays on certain fullerene derivatives and our findings suggest they were effective at suppressing the virus.

STUDIES EXAMINING THE INFECTIVITY OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) ON HUMAN IMMUNE CELLS

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Since its discovery in 1983, HIV has infected over 70 million people and killed 35 million people [1]. The virus targets the immune system of infected individuals and if left untreated leads to a condition known as Acquired Immune Deficiency Syndrome (AIDS). An individual is classified as having AIDS when their CD4+ cell count drops below 200 cells per mm^3 of blood [2]. At this point, opportunistic infections have much larger effects, including death in some cases. Many methods have been discovered and employed to help infected people survive the infection, but there still is no cure or effective vaccine to stop the virus. Of those methods available to effectively decrease the probability of progression to AIDS for infected individuals, Highly Active Antiretroviral Therapy (HAART) has been used with great success and is the most often used option for infected individuals. HAART works by inhibiting viral functions and/or by bolstering the host's immune system [3].

I.1 Human Immunodeficiency Virus as a Medical Problem

HIV primarily infects CD4+ cells *in vivo* and a select population of all cells infected becomes long-lived latent reservoirs from which new virions can be produced long after levels of viral markers are no longer detectable in blood samples [4,5]. Latent reservoirs are effectively invisible to the immune system and nearly all treatments [6].

Latency is maintained through a variety of mechanisms and those mechanisms are often reinforced through HAART medications [7]. After the suspension of HAART medication, patients experience explosions of virus production [8]. Consequently, CD4+ cell counts drop and AIDS can be developed.

Current research efforts have focused on identifying the latent reservoirs *in vivo* and one possible latent reservoir may be established in the mast cell. Several studies have examined the possibility of mast cells from various tissue sites for the susceptibility to HIV with varied results [9-20]. Their examination is due to the fact that they are ubiquitously expressed and are often one of the first immune cells to come into contact with the virus. As demonstrated below, mast cells derived from human, excised skin are assessed for their susceptibility to HIV both with and without degranulating activation.

The HAART therapy is often comprised of several components that target many viral processes and interrupt them. One enzyme process that is often targeted for interruption is the HIV protease. Disruption of the proteases activity disables the newly-budded, immature virus from effectively infecting new cells by stopping the cleaving process necessary for matrix proteins to assemble [21,22]. Because HIV protease is a desirable target for drug therapies, protease inhibitors were developed as a class of anti-retroviral drugs that are effective at limiting the virus' potential to infect new cells [23,24]. Fullerenes and their derivatives were identified as potential analogs to these protease inhibitors by Friedman et al because of the hydrophobicity of both the fullerene cage and the active site of HIV protease [25]. Following that initial realization and computational calculation, researchers have identified several fullerene derivatives that

likely or experimentally have inhibitory effects on the HIV protease or suppressive effects on the virus' proliferation [26-28]. This thesis included both preliminary computational evaluations of a panel of previously untested fullerene derivatives and their potential interaction to HIV protease as well as a dose response assay assessing their effectiveness as a viral suppressor.

CHAPTER II

REVIEW OF THE LITERATURE

II.1 HIV Infection

HIV is well known for infecting human helper T-cells. There are several different variations of T-cells, some being classified as being a part of the adaptive immune system and others being classified as being part of the innate immune system [29,30]. Helper T-cells are susceptible to infection because HIV primarily targets the surface receptor CD4 [31]. When HIV infects a cell, it follows a multi-step process. The first step is the attachment of CD4 to viral gp120. This binding event causes conformational changes in gp120 and CD4 allowing for a second binding event with between gp120 and either CCR5 or CXCR4, depending on the tropism of the virus [32-34]. After this binding event, viral gp41 is able to pierce the cell's membrane. Then, conformational changes in gp41 allow the membrane of the virus to fuse with the membrane of the cell and the viral capsid is injected into the cell. The viral capsid dissociates and reverse transcriptase binds to and transports the viral RNA toward the nucleus [21,35]. On the way, viral RNA is reverse transcribed into DNA by viral reverse transcriptase. The viral DNA is then transported into the nuclease by viral integrase [36]. There, viral integrase inserts the viral DNA into the host cell's DNA. From here, the virus can continue to spread the infection by entering into an active replication phase or can enter into a latency phase and

is termed the “point of no return” because of the irreversibility of the integration process [37].

In the former, the virus uses nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and other host transcription factors and later viral transcription factors to transcribe various viral transcripts [38]. These transcripts are then translated into the viral proteins necessary for new virion production. As new viral material is produced it gathers at the cell membrane and is packaged into budding, immature virions [21]. After budding away from the cell, these immature virions require the proteolytic activity of HIV protease to allow for the construction of the viral capsid and therefore mature. Without maturation, the virions have high failure rates trying to infect new cells [22].

II.2 HIV Latency

The other phase that the cell may enter is latency. The latently infected cell harbors viral DNA and is effectively protected from immunogenicity. Latently infected cells can remain dormant indefinitely and may be activated at any time allowing the virus to begin producing new virions and infecting new cells [8]. Therefore, continued administration of HAART drugs is necessary to disallow progression to AIDS. Several latent reservoirs have been identified in memory T-cells, tissue macrophages, as well as possibly hematopoietic stem cells, though this is disputed [5,9,39-43]. Mechanisms that cause latency vary from cell to cell [7]. Also, there may be more than one mechanism that is working in the cell that maintains the latency. Several mechanisms may inhibit viral gene expression, including non-functional mutations in the viral DNA, transcriptional

interference, epigenetic silencing, changes in chromatin structure, negative transcription factor interference, absence of positive transcription factors, and problems with RNA transport and translation [44-59]. An important factor in the integration process of infection is that the virus preferentially inserts its DNA into genes that are actively being expressed [60]. This means that for cells that are latently infected, they can be activated to produce native proteins while at the same time upregulating the genes necessary for viral replication [43]. While not all CD4+ cells are prone to activation, many are and their activation may have an effect on the pathology of the virus.

II.3 Medical Response to HIV Infection

HAART is a treatment regimen composed of drugs that either boost the infected individual's immune system or target viral processes to stop them from progressing. Viral inhibitors used range in specific viral process targeting, but generally work in one of six ways. CCR5 antagonists, also known as entry inhibitors, bind to the active site of the CCR5 surface receptor on host cells [61]. By binding to CCR5, these drugs disallow the secondary binding event of HIV to the cell, thereby stopping the virus from entering the cell. A second drug class used in the inhibition of HIV processes is fusion inhibitors. Fusion inhibitors bind to envelope proteins on the virus and stop the conformational changes necessary to actually allow fusion of the viral and host cell membranes [62,63]. There are two types of reverse transcriptase inhibitors used in HIV medication. The first are termed non-nucleoside reverse transcriptase inhibitors and they work by directly binding to the reverse transcriptase enzyme and stop it from working [64-66]. The second type is nucleoside reverse transcriptase inhibitors. These were the first drugs available to

treat HIV infected individuals and work by terminating newly reverse transcribed DNA [67]. It does this by mimicking naturally available deoxyribonucleotides that are used to synthesize new DNA. These mimics lack a necessary 3'-hydroxyl group and cannot bind additional nucleotides effectively terminating the reverse transcription. This process is called chain termination and can cause adverse side effects. Integrase inhibitors are another class of drugs used in anti-retroviral therapy. As their name suggests, they stop integrase from functioning properly and stop the virus from infecting the cell [68,69]. The final type of drug used in HAART therapies are protease inhibitors. HIV protease (HIVP) is used by the virus to mature newly formed virions [22,70]. After budding away from the host cell, HIVP chops proteins packaged in the virion and allows for their organization into the viral capsid leading to a mature, infectious virion. Protease inhibitors block this process and make these new virions forever immature and unable to infect new cells [71]. There are specific guidelines from the FDA for the correct and effective combination of these drugs to treat infected individuals. Research continues in this field to develop more drugs that may interact less with the host biology and less with other drugs allowing for more effective and comprehensive treatment. Without continued administration of HAART medication, HIV can restart viral replication and poses a risk to develop AIDS [8].

II.4 Mast Cell Biology

In order to fully eradicate HIV from an infected individual, latent reservoirs need to be identified. One focus of research today is identifying latent reservoirs present in the body and many have been identified [5,9,39-43]. Because of their similarity to T-cells

and their ubiquitousness in the body, mast cells have been studied for their susceptibility to HIV infection. Mast cells are important immune effector cells that are most often characterized for their role in allergic response [72-74]. Hematopoietic progenitor cells called myeloid progenitors differentiate into immature mast cells [72-76]. These immature mast cells migrate to fibroblast-rich regions in tissue where they then undergo limited replication producing daughter mast cells that are fully differentiated [72]. Depending on the eventual resting place that the immature cells find, mast cells can differentiate into one of two phenotypes in humans. These separate phenotypes are identified by their neutral protease composition: either having tryptase, chymase, carboxypeptidase A3, and cathepsin G-like enzyme or only tryptase (designated MC_{TC} or MC_T respectively) [77]. MC_{TC} generally are localized to connective tissue but also have populations in some mucosal regions. MC_T are more localized to mucosal regions and are generally not seen in connective tissue [72].

II.5 Mast Cell Activation and Degranulation

Mast cells are granulocytes having pre-stored inflammatory compounds that are rapidly released after introduction to activators [78]. FcεRI is a high-affinity immunoglobulin E (IgE) receptor that is typically involved in the allergic response and is present on mast cell membrane surfaces [79]. IgE is produced by B-cells as a part of the immune system response to allergens [80,81]. FcεRI binds to the heavy chain on the Fc region of IgE. IgE molecules are antibodies that are integral parts of the adaptive immune system and typically cover the surface of mast cells having bound to FcεRI. When the Fab regions of IgE bind to an antigen and the Fc regions bind to FcεRI receptors, a cross-

linking event occurs that causes a downstream pathway to activate leading to mast cell degranulation [82-84]. The first event in this downstream pathway can be the cross-linking of two or more FcεRI receptors. Following a complex downstream activation, mast cells will release their granules filled with various bioactive compounds. Alternative methods for activation can circumvent the FcεRI receptor. Toll-like receptors (TLR) are pattern recognizing receptors that can recognize various bacterial compounds that are structurally conserved [85]. Upon activation, TLR begin a downstream pathway that activates mitogen-activated protein kinase (MAPK) leading to degranulation [85]. Another method for activating mast cells is by causing release of calcium stores and translocating calcium from outside of the cell to the interior [86]. This method can circumvent both TLR and FcεRI receptors by interacting with the downstream components of the activation pathway like Protein Kinase C (PKC).

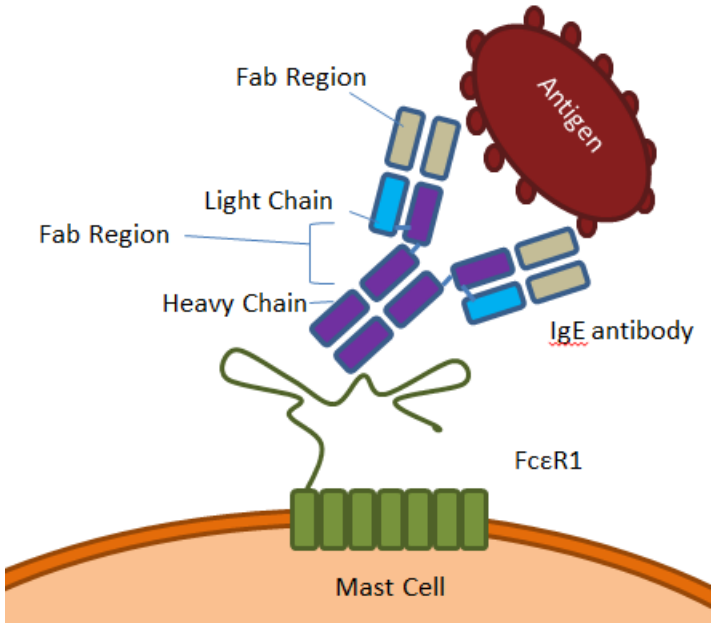

Figure 2.1 The Binding of an Antigen to the FcεRI-IgE Complex

Mast cells are known to have altered gene expression in the hours following either their sensitization or their activation [87,88]. As its terminology suggests, sensitizing a mast cell primes the cell to release granules, but little to no granules are actually released [89]. After following the activation pathway and releasing granules, mast cells undergo many gene upregulations and downregulations. These upregulations help the mast cell to continue to help in the immune response even after releasing granules that can recruit other immune cells to the site of the antigen [87].

II.6 Mast Cell Sources

There are a few ways to procure mast cells. Firstly, there are immortalized mast cell lines that have been isolated. HMC-1 was derived from a patient with mast cell leukemia [90]. It is noted as being similar to normal mast cells but lacks surface FcεRI

receptors which are vital for degranulation. LAD2 cells have minor differences in their FcεRI receptors and can effectively degranulate, but there are major differences discovered in both LAD2 tryptase and chymase [90]. In an effort to circumvent these differences, methods have been developed to procure primary and primary-derived mast cells from human donors and have been described previously [91-93]. Mast cells can be procured from various tissues including skin, umbilical cords, lung, and most recently fat tissue, each with subtle changes in protocol from procurement. Skin mast cells are investigated in this dissertation for their susceptibility to HIV infection following activation and degranulation. Briefly, their isolation involves the mechanical separation of tissue coupled with enzymatic digestion of tissue and followed by separation techniques. Culture of mast cells is accomplished generally by the addition of specialized X-Vivo media supplemented with human stem-cell factor to encourage multiplication.

II.7 Fullerenes

Fullerenes have been the subject of investigations in biological applications for some time. Their use has been included in demonstrations as antioxidants, drug delivery vehicles, therapeutics, diagnostic tools, photo-sensitizers, anti-cancer, and as anti-viral treatments [94-106]. The native fullerene is not soluble in water and can be derivatized for various functional moieties. This ability to bind moieties to the carbon cage can lead to various functionalizations. Fullerenes are unique because of their electrochemical characteristics and are capable of undergoing various chemical reactions that can alter their reactivity, permeability, solubility, or targeting ability. The fullerene derivative (FD) has new physical and chemical properties.

Many fullerenes are known to be effective antioxidants. They act as "free radical sponges," and can readily accept electrons from reactive oxygen species (ROS) found commonly in cellular systems [107]. ROS many times have unpaired electrons that readily react with native molecules inside the cell causing oxidative stress. This oxidative stress can lead to problems including cellular malfunction or dysfunction and can lead to cell death. Accordingly, oxidative stress has been implicated as a primary method for aging [108]. The cell has systems in place to either use these free radicals as signaling molecules or as cellular defense mechanisms, but more often uses proteins like Superoxide Dismutase (SOD) to convert them into either harmless compounds or into more manageable compounds [109]. There are several known ROS known to cause cell damage. They include hydroxyl radicals (HO•), superoxide anions (O_2^-), and peroxynitrites ($ONOO^-$).

Because of the commonality of these damaging compounds, much research has pointed to the benefits of antioxidants against more systemic diseases. It has become widely accepted that antioxidants provide benefits to general health. Following the discovery of fullerene's ability to counteract ROS, various FD have been developed and used in experiments with promising results. N. Gharbi et al showed that micro-dispersed C60 fullerenes had no adverse effects on liver toxicity and worked to stem the effects of an oxidant challenge (CCl_4) in rats [94]. This group went on to show that lifespans of rats is almost doubled with the administration of oral olive oil dosed with C60 fullerene [110]. Another study showed their comparative effectiveness of a FD to the native SOD-1 enzyme and found it to mimic its activity [111]. The researchers did this by dosing SOD2

(-/-) mice with a Tris-malonic acid C60 fullerene (C3) and increased their lifespan by 300%. Another study by some of these authors showed that FD had neuroprotective capabilities in neuronal cell cultures [112]. Fullerenes and their derivatives show potential as antioxidants in treatments for diseases caused by ROS.

Many researchers today are investigating alternative methods for drug therapy [96]. Fullerenes have been investigated as drug delivery vehicles because of their potential for derivation and their small size. Water soluble FD can easily cross cell membranes or, depending on the functionalization of the carbon cage, can bind specifically to a target biomolecules [113]. Beyond water solubility and biomolecular targeting, fullerenes can also be functionalized to become carriers of drugs or genes. Some examples of this possible use are their use as allergic response mediation, their ability to suppress the asthma reaction, their ability to dampen inflammation caused by arthritis, their potential as a therapy for multiple sclerosis, and their ability to inhibit viral process, including in HIV infections [26,98-102,104,114].

Fullerenes may also be shown to be effective diagnostic tools. Researchers at Virginia Tech were able to successfully produce fullerenes that encapsulated 3 gadolinium atoms bound by a central nitrogen atom contained inside the carbon cage. These “Trimetaspheres” (TMS) were demonstrated to be strong MRI contrast agents [98,115]. Researchers hope to overcome limitations caused by more conventional MRI contrast agents like Magnevist^{TM} that can release its toxic, chelated gadolinium ion upon host degradation of the bound carrier. TMSs may also outperform Magnevist^{TM} as far as body retention over time and body targeting ability because of the functionalization of the

carbon cage. This leaves a possibility for fullerenes to be used as both a therapeutic and diagnostic tool simultaneously. By selectively functionalizing moieties to the surface of a fullerene cage, while all the while leaving the gadolinium triad unchanged within the cage, a FD can be made to perform targeting, therapy, and diagnostics. The term "theranostic" has been used for this functionality and very well could be the future of medical practices [100]. Nanoparticles of various compositions are being investigated as diagnostic tools already and some fullerene platforms are attractive targets for the theranostic technology. One particular investigation by Dellinger et al used functionalized TMSs in liposomes to target atherosclerotic plaque lesions in ApoE knock out mice [98]. These TMSs were further functionalized with CD36 ligands which were instrumental in the cellular uptake of the fullerenes into the cells in the atherosclerotic plaque. Mice were then imaged using MRI showing the gadolinium present in the atherosclerotic plaques. This technology can be adapted to be used in various other treatments by changing the functionalization of the TMS.

There are also variations of fullerenes. The classic and most well-known fullerene is the C60 "buckyball", comprising of 60 carbons arranged in a "soccer ball" configuration. Other fullerene configurations include C20, C26, C70, C72, C76, C80, C84, and C100, each being empty carbon cages with varying amounts of 5 and 6 membered rings. As mentioned above, fullerenes have been designed that carry a triad of metal ions, usually gadolinium, and can be used as an effective MRI contrast agent.

II.8 Toxicity of Fullerenes

The basis for determining toxicity and consequently determining the possibility for use in humans is regulated by the FDA. Any new chemical compound or treatment is required to be evaluated separately, despite the similarity to other chemicals in the same class. Beyond the purely chemical variations between fullerene derivatives, there are variations between different fullerenes. Differences in isomeric configurations and differences in cage size allow for even more variations making the toxicologist's job even more intricate. According to FDA guidelines, each isomer and each cage can have different interactions with biological molecules and should be investigated accordingly.

One of the common and primary functionalizations of fullerenes is water solubilization, but not all water soluble fullerenes act similarly. Investigating each fullerene separately should be a priority of each toxicity study. It is clearly evident that certain FDs behave differently than others in biological systems and that fundamental understanding helps to ensure limited negative effects caused by small changes from one to another fullerene derivative.

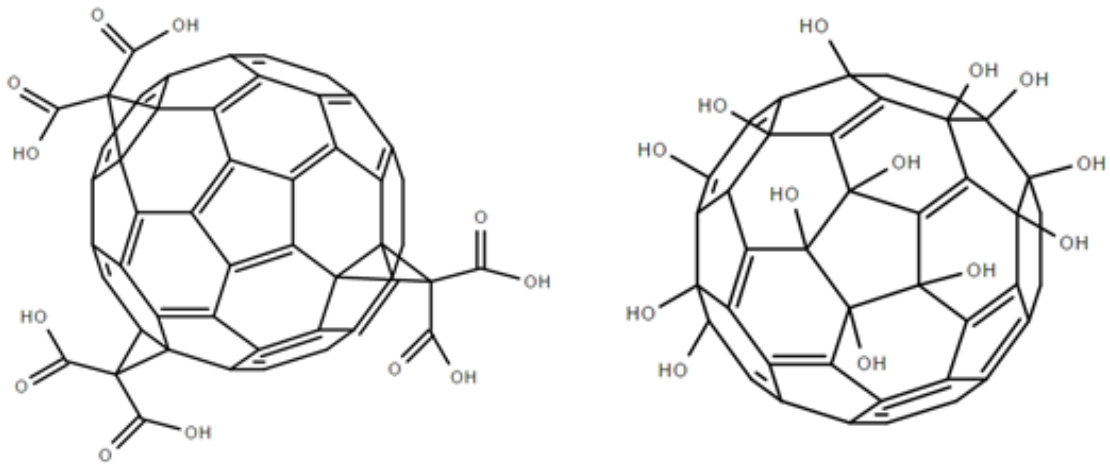

Figure 2.2 Representative C60 Fullerene Derivatives.

Because of the wellspring of opportunity presented in fullerenes and their derivatives, much attention has been given to their potential toxicity. Many of these studies have conflicted with each other and have led to a debate over the nature of their toxicity. The most notable and notorious study concerning the toxicity of fullerenes was performed by Oberdorster [116]. In this study, fullerenes were given to juvenile largemouth bass following an incomplete characterization and lacked a proper control which led to conclusions that could not determine if the toxicity was due to the size of the aggregates, the composition or chemical nature of the aggregates, or the tetrahydrofuran contamination. Unfortunately, media outlets discovered this study and publicized its unfounded conclusions, which led to an atmosphere of wariness of the fullerene's future in the field of biology or medicine. Because of backlash over the failures of the original study, researchers followed with a more expansive examination that concluded that the impurities in the sample were the cause for any toxicity seen in the first study [117].

Other investigations into the subject of fullerene toxicity have demonstrated increases in the lifespan of mice [118] and rats [110]. Intending to explore the over dosing of fullerenes to organisms, other researchers treated mice with excessive dosages but still showed no overt toxicity [94,119]. More recently fullerenes suspended in olive oil were fed to rats and led to a 90% longer lifespan compared to control mice [110].

Conflicts, especially between highly publicized studies, make it difficult for researchers to effectively develop novel treatments based on a fullerene platform because of the perceived potential harm that they could cause. It is therefore necessary to ensure that further investigations into the toxicity of fullerenes (and any nanomaterial for that matter) are carefully constructed to account for the many variables that could contribute to the perceived toxicity.

II.9 Mast Cells and HIV

Mast cells are often found in mucosa and other host-environment interfaces. Because of their positioning and because they are important immune effector cells that help in pathogen defense, MC's role in HIV infection is unclear [11]. The genital mucosae of HIV infected women showed increased MC density. Other examples of noted mast cell population increases were found in men with AIDS associated diarrhea [12]. These increased MC populations suggest that MCs are involved in the body's response to the infection.

II.10 Mast Cells' Susceptibility to HIV Infection

Previous investigations into the hypothesis that mast cells are susceptible to HIV infection have shown varying results. It has been shown that progenitor mast cells are

susceptible to infection by HIV, suggesting that the low levels of CD4 that were detected on these cells were enough to establish an infection [9,10,13-16]. Importantly, these progenitor mast cells appear to lose their susceptibility to HIV after a certain point in their ontogeny. These infected progenitor cells then produce daughter cells that terminally differentiated into mast cells that then reside in tissue and may establish a latent reservoir [13,14]. This evidence that mast cell progenitors and other hematopoietic progenitors can be infected allows for possible HIV reservoirs even after maturation.

Other primary mast cells have been examined. Fetal liver blood-derived mast cells were examined for their susceptibility to X4 tropic HIV and were productively infected [17]. It was unclear in this study if the primary cells in the experiments were progenitors or terminally differentiated. Another study demonstrated that p24 does not co-localize to skin mast cells obtained from various tissue sites [18]. Multiple tissue sites were examined including the lymph nodes, cervix, GI tract, and parotid glands but no mast cells were found to be actively producing p24, a HIV specific protein by immunohistochemistry.

Most recently, mast cells taken from gastrointestinal mucosa of healthy patients undergoing surgery and were introduced to HIV. The researchers found that mast cells are able to present virus to other CD4+ cells. This is facilitated by some HIV-1 attachment factor; some of these factors include DC-SIGN, HSPG, and α4 β7 integrin [19]. This means that mast cells can support HIV infections by capturing HIV and presenting it to susceptible cells. The researchers also investigated the susceptibility of these mast cells to infection and found that they could be infected.

Further studies have examined the immortalized mast cell line, HMC-1, and found that infection was possible [17,20]. The cell line was infected in both cases. It should be noted that HMC-1 is a poor substitute for primary human mast cells because it lacks the expression of FcεRI [120,121].

To our knowledge, primary, skin, human mast cells have not been studied for their susceptibility to HIV in vitro. Further, no studies have examined the effect of activators on primary mast cells and their potentially altered susceptibility to HIV as a result of activation. Mast cells are one of the first immune cells that HIV encounters in vivo and may become a target for HIV under certain inflammatory conditions. We hypothesized that terminally differentiated human mast cells (possibly through IgE-dependent and/or independent activation) can be infected by HIV and represent a new viral reservoir. We therefore tested this hypothesis and the results are discussed in later portions of this dissertation. By identifying various cellular reservoirs of HIV, a greater understanding of the life cycle of the virus is revealed and this understanding may present new opportunities for medical professionals to more efficiently treat individuals infected with the virus.

II.11 Fullerenes and HIV

There have been many studies investigating the possibility of use of fullerene derivatives as HIV inhibitors. The first study following this hypothesis was done by Friedman et al who identified the similarity in size and hydrophobicity of the carbon cage of fullerenes and the viral enzyme protease (HIVP) [25]. They suggested that FDs may act as powerful HIVP inhibitors by acting as antagonists to the HIVP active site. They

identified two compounds using molecular modeling that seem to affect the enzyme's ability to work effectively and tested both computationally and experimentally. Researchers from this original work then continued their work and generated 6 new FD as possible HIVP inhibitors [26]. The next study investigated a highly water-soluble dendro[60]fullerene originally synthesized by Brettreich and Hirsch [122]. This investigation showed this fullerene to be highly effective at inhibiting HIV [27]. More studies have looked at the inhibitory effects of fullerene derivatives on HIVP because of the fullerene derivatives' interaction with the active site of HIVP [28,123-130]. From all of these, many different FDs have been identified as HIVP inhibitors. Most recently, fullerene derivatives that used the carbon cage as the platform for an amino acid inhibitor were shown to be effective HIVP inhibitors [130]. Modeling studies performed in this study indicated that the amino acid chain was more likely to interact with the active site of the protease and overestimated their inhibitory effect. Subsequent experiments utilized a FRET-based assay that revealed strong inhibitory effects on the protease.

However, HIVP may not be the only target for fullerene derivatives. Some fullerene derivatives may target other essential components of the viral reproduction cycle. Fullerenes have been shown to interact with NF-κB functionality [131], G-protein coupled receptors (GPCR) [132], amyloids [133], coagulation [134], and, most interestingly for this discussion, HIV Reverse Transcriptase [135]. Amyloids are important transmission vehicles for HIV [136]. HIV uses amyloids as attachment and fusion facilitators and fullerenes can be used as anti-amyloid compounds [133]. The relationship between HIV and coagulation is still unclear. It has been shown that

thrombocytopenia is a common symptom of infected individuals [137] and the addition of certain fullerene derivatives may help with that condition. HIV hijacks the NF-κB transcription factor to produce transcripts of viral genes initially and the interruption of this process can decrease virion production [38,138]. CCR5 and CXCR4 are GPCRs that are used during the attachment and fusion steps of HIV infection. It has been shown that certain fullerene derivatives can interact with certain GPCRs and this interaction may also be produced with either CCR5 or CXCR4 [132]. This may lead to the fullerene acting as an attachment or fusion inhibitor disallowing infection.

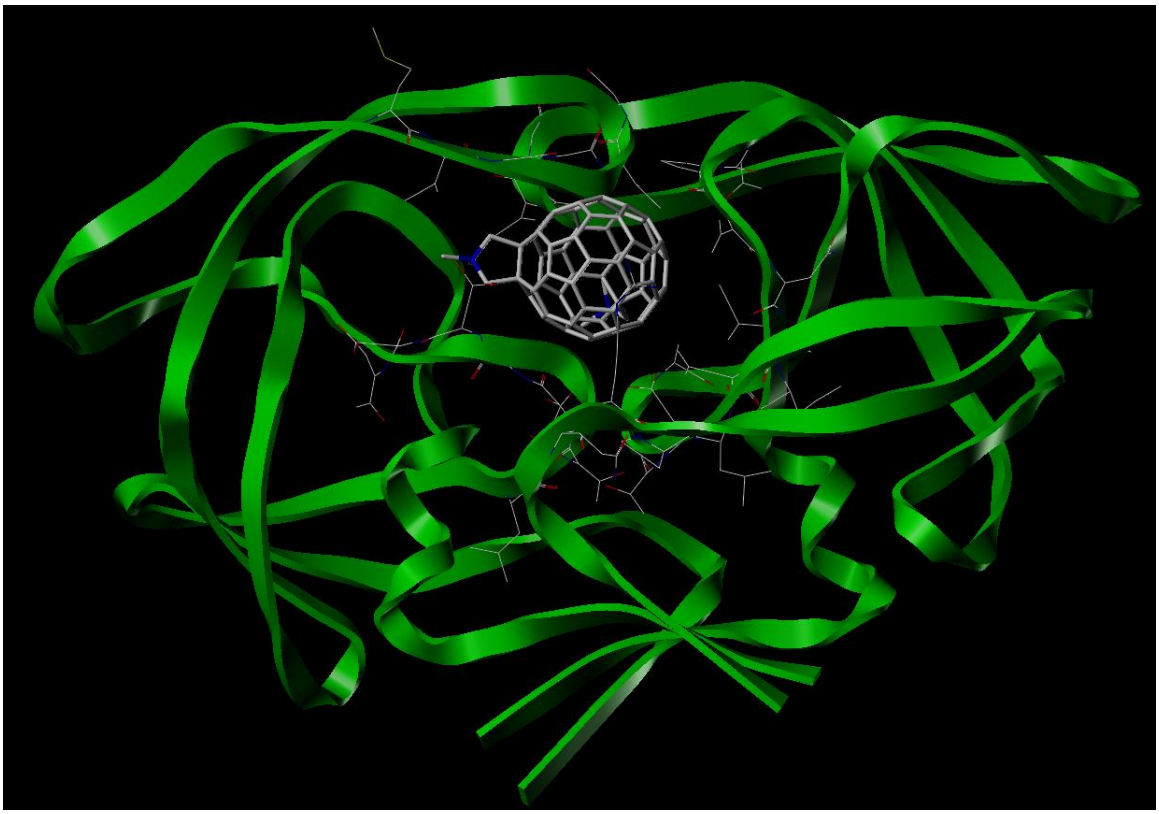

Figure 2.3 Fullerene Derivative Docked into the Active Site of HIV Protease.

Though most studies involving HIV and fullerenes have focused on the HIVP-fullerene interaction and the resulting proliferation inhibition, Mashino et al investigated the role of eight different FDs as HIV Reverse transcriptase inhibitors [135]. Each described had an inhibitory effect on reverse transcriptase's activity. Using a radiometric method, IC_{50} values were obtained and the fullerene derivatives scored moderately well compared to a Nevirapine control. Nevirapine is a commonly used non-nucleoside reverse transcriptase inhibitor in HAART. Interestingly, these same fullerene derivatives were examined as hepatitis C virus RNA polymerase as well in this study. Comparatively, the fullerene derivatives were better inhibitors of the HCV polymerase than the HIVP.

Fullerene derivatives are an exciting new platform for the design of novel anti-HIV compounds. The primary mechanism for HIV inhibition seems to be through HIVP, but there are other relevant mechanisms through which a fullerene derivative can interrupt or retard the processes of HIV. Alternatively, by binding a previously known inhibitor to the carbon cage, fullerene derivatives may be able to serve as effective carriers of inhibitory compounds and their use can be expanded beyond HIVP or reverse transcriptase inhibition. Various fullerene derivatives previously not investigated for their role in HIV inhibitions are investigated in this dissertation as potential anti-HIV compounds and their likelihood for acting as protease inhibitors is explored through computational docking studies.

CHAPTER III

PRIMARY HUMAN SKIN-DERIVED MAST CELLS ARE NOT A RESERVOIR FOR HIV

III.1 Introduction

Human immunodeficiency virus (HIV) is the virus that causes acquired immune deficiency syndrome (AIDS). While Highly Active Anti-Retroviral Treatment (HAART) medications have been efficacious in suppressing viral replication, once it is discontinued, virus emerges from cellular reservoirs [39,43]. However, it is not clear what cell types the virus uses as latent cellular reservoirs. Mast cells are ubiquitously expressed immune effector cells residing in tissue and mast cell progenitors can be infected with HIV, which can lead to a latent reservoir in tissue in humans [9,10,13,17]. Other studies using mast cell-like cell lines or mast cells derived from progenitor cells in the blood can be infected [17]. Most recently, mast cells from gastrointestinal mucosa were shown to be susceptible to HIV infection [19] while other studies found no evidence of active replication in mast cells [18]. Given that mast cells are one of the first immune cells that HIV encounters *in vivo*, we hypothesized that primary human mast cells would be susceptible to infection by HIV. We also recognized that mast cells have altered gene expression following a degranulation event and the method of that degranulation may affect the altered gene expression as described previously [87,88]. We therefore further hypothesized that mast cells could be infected by HIV following FcεRI or non-FcεRI

receptor dependent challenge with various secretagogues and serve as a latent reservoir for HIV.

III.2 Methods

III.2.1 Mast cell culture and degranulation

To test this hypothesis human, skin-derived, mast cells, which proliferate from a yet to be defined stem cell population, were challenged with live HIV [139]. Mast cells were cultured in X-VIVO media (Lonza Inc., Allendale, NJ) supplemented with human Stem Cell Factor (SCF) for 12 to 20 weeks. At this point, cells were collected, counted, and examined for viability with Trypan-Blue exclusion. Mast cells, at <90% viability, were plated in duplicate in a 24 well plate (5×10^5 cells/well) and were activated with FcεRI-dependent (anti-FcεRI-alpha receptor antibody 3B4) or non-FcεRI-dependent stimuli Calcium Ionophore (A23187, 0.1 μg/mL) or Lipopolysaccharide (LPS; 1.5 μg/ml) overnight.

III.2.2 Viral introduction to mast cells

The following day, media was removed and replaced by fresh X-VIVO supplemented with SCF, and then live HIV_{LAI} (NIH AIDS Reagent Program, Germantown, MD) was added to each well at a multiplicity of infection between 0.1 and 0.5 and incubated for 5 days. After 5 days cells were washed three times and left to incubate overnight in new, virus-free media to allow for any non-internalized virus to either infect or be released from non-specific adhesion to cellular plasma membranes. After a final wash, cells were suspended in water for subsequent PCR preparation.

III.2.3 Viral DNA detection by PCR

Mast cell cultures were suspended in water and sonicated in heated water baths (70^{o}C) to lyse cells and disrupt membranes as well as kill any remaining virus present for ease of handling. A phenol extraction was then used to isolate DNA and purity was assessed by a NanoDrop 2000 Spectrophotometer. For detection of viral DNA by PCR, the primers used were: Alu FWD: 5' – GCC TCA ATA AAG CTT GCC TTG A – 3' and gag REV: 5' – CAT CTC TCT CCT TCT AGC CTC – 3' (Integrated DNA Technologies Coraville, Iowa).

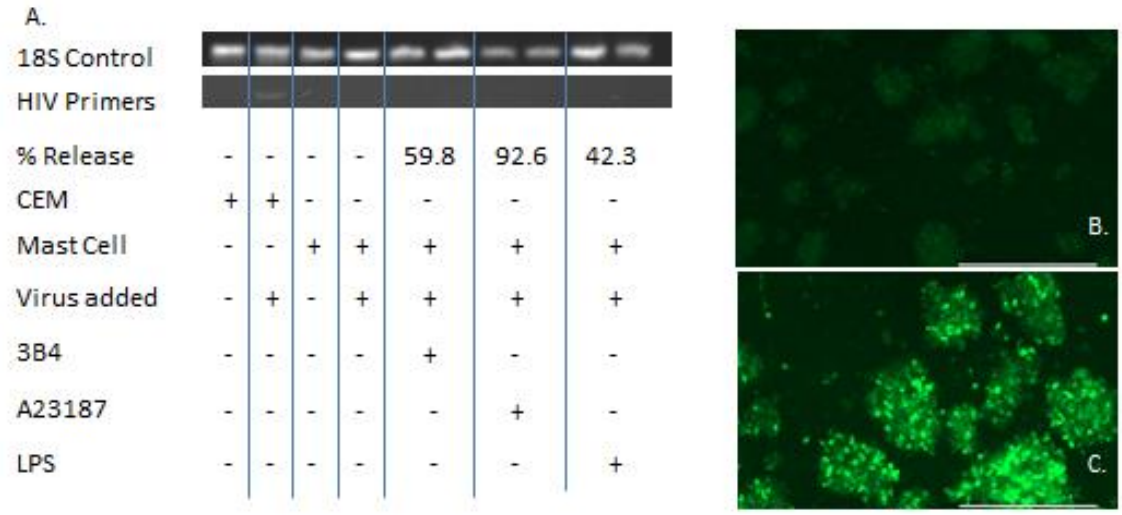

Figure 3.1 HIV Does Not Infect Human Skin Mast Cells

III.3 Results

III.3.1 Human, skin-derived mast cells are not susceptible to HIV

As shown in Figure 1, mast cells did not become infected with HIV under resting or stimulated conditions as assessed by PCR of the HIV-specific marker DNA (Figure 1).

Previous to introduction of HIV to mast cells, mast cells were activated with 3B4, Calcium Ionophore A23187, or LPS and the percent release compared to total and spontaneous release controls. As a control, a t-lymphoblast cell line with a green fluorescent reporter gene called CEM-gfp were actively infected as assessed by p24 expression and gfp upregulation (Figure 1B,C). As expected no viral proteins were detected in the mast cell lysates using Western blotting (not shown).

III.4 Conclusions

Though other mast cell sources have produced mast cells that are susceptible to HIV infection, this study suggests HIV does not infect human skin mast cells or the progenitors that give rise to them and therefore do not serve as a reservoir for HIV. There are some possible explanations for this. There are at least two different phenotypes of mast cells in the body and they are distinguished by the neutral protease composition found in their granules and are designated with the notation MC_T and MC_{TC} (representing Tryptase (T) and Chymase (C)) [72,74,140,141]. Skin mast cells are generally considered as being comprised of primarily MC_{TC} and their composition of these tissues can be a contributing factor to the susceptibility of these skin-derived mast cells. Another possibility is that contrary to previous investigations, our cell populations, which do undergo some limited replication, are devoid of the CD4+ progenitor mast cell populations. This hypothesis could be examined by a more complete characterization of this mast cell population that would separate the fully differentiated mast cells and the progenitors and examine the presence of CD4 on both populations. This characterization would be greatly improved with multiple samplings throughout the culture of the mast

cells from tissue as it could show if and when the CD4 surface receptor as well as any other important or interesting biomolecules.

CHAPTER IV

FULLERENE DERIVATIVES SHOW POTENTIAL ANTI-HIV ACTIVITY

IV.1 Introduction

IV.1.1 HIV

Human immunodeficiency virus (HIV) causes acquired immune deficiency syndrome (AIDS). Current treatments for HIV+ individuals include Highly Active Anti-Retroviral Therapy (HAART) designed to inhibit retroviral proliferation and survival and is commonly used in the treatment of retroviruses [142]. This therapy is responsible for increased longevity for HIV+ individuals by decreasing the probability of progression to AIDS. This treatment, however, is not able to fully eradicate the virus from an infected individual as HIV establishes long term latent reservoirs in cells that harbor the virus indefinitely and protect it from immunogenicity. An individual who is HIV+ and suspends their HAART treatments quickly progresses to AIDS because of these latent reservoirs. One of the focuses of HIV research today is the discovery of new treatments that inhibit viral processes. Researchers have identified many fullerene derivatives (FD) that may serve as a new platform for new anti-viral compounds and this study adds to that body of knowledge.

IV.1.2 Fullerene derivatives viral inhibition

Fullerenes are an allotrope of carbon that features a closed cage of carbon that is hollow inside and this class of compounds has been used extensively for a variety of

biological applications [112,132,143]. Several studies have looked at the modeled dynamics of the interaction between various functionalized fullerenes and the viral protein protease or used fullerene derivatives (FDs) as viral inhibitors *in vitro* [25-28,123-130,144,145]. HIV protease is an especially desirable therapeutic target as it is responsible for cleaving immature viral proteins in the life cycle of HIV [146]. Without protease, newly budded HIV virions do not mature and are less likely to be infectious [22]. Because these FD have some sort of a competitive mode of action by binding to the active site, HIV protease generally is the studied protein in docking simulations. Fullerenes were originally identified as promising protease antagonists because of the hydrophobicity of the active site of protease [25]. Thus, some functionalized fullerenes that are water soluble but still have large portions of their hydrophobic cages accessible can fit directly into the active site of the protease effectively acting as an antagonist. Some fullerenes have been tested in this capacity and were shown to be effective at limiting the virus's proliferation [25,27,126,127,130,135].

Our lab has been interested in fullerene interaction with biological processes and we have previously used fullerenes in other studies as both therapeutics and diagnostics [98,99,101,102,114,147]. We wanted to see if these fullerenes that had many beneficial effects on other systems could be employed in an antiviral capacity following the precedent set by other researchers using FDs as anti-retroviral compounds. We therefore set to test a panel of seven FDs as potential HIV inhibitors with the hypothesis that, similarly to other fullerene-HIV studies, these FDs would also be effective at inhibiting HIV.

IV.2 Methods

IV.2.1 Modeling methods

A panel of FDs (Figure 1) were modeled and minimized in Sybyl modeling package using the Tripos molecular mechanic force field. Another fullerene (named Trans-3)(structure not shown) was also modeled and examined as a positive control in modeling experiments because it had been described previously as being effective at inhibiting HIV [127]. Another positive control used in modeling experiments was Ritnoavir, a commonly used drug in HAART medication for the inhibition of HIV protease [148]. Since the HIV protease structure has not been determined experimentally with a fullerene bound in the active site, the initial structure for HIV protease used in these modeling experiments was derived from a databased structure complexed with a Fluoro-substituted diol based C2-symmetric inhibitor (pdb code 1W5X). Water molecules and the bound inhibitor were removed and analyzed using the Surflex Docking Module within the Sybyl modeling package. Residues present in the protease were amended to be neutrally charged based on previous investigations that suggested that there appears to be no significant differences in docking of FD into HIV protease between charged and neutral protease residues [128]. FDs were then docked into HIV protease using the Surflex Docking module within the Sybyl modeling package for preliminary indications of FD ligand docking. Hydrogens and heavy atoms were allowed to move and minimized before and after ligand fitting. Results were derived from Total Score values produced from the docking program.

IV.2.2 *In Vitro* methods

IV.2.2.1 *CEM-gfp cell line*

In vitro experiments were performed in CEM-gfp cells. CEM-gfp cells are an immortalized T-cell line that has been stably transfected with a gene that codes for green fluorescent protein with a promotor region of the gene that is bound by the HIV transcription factor *tat* (trans-activator of transcription). Therefore, gpf is produced in response to the presence of viral protein *tat*, indicating a particular cell is infected by HIV.

IV.2.2.2 Experimental design

CEM-gfp cells were plated at 100,000 cells per well in a 24-well plate. FDs were then added and cells were allowed to incubate overnight. Seven different fullerenes were surveyed and are shown in Figure 1. HIV populations were expanded in separate CEM-gfp cultures from an original HIV_{LAI} stock (NIH AIDS Reagent Program, Germantown, MD). Expanded viral supernatant was then added the next day equally in appropriate wells at a multiplicity of infection between 0.1 and 0.5. Measurements of gfp fluorescence were taken each day using a BioTek SynergyMx Microplate Photometer. Cells were incubated in the presence of FDs and virus for at least five days. Media was not changed during the duration of the test to allow HIV population growth to be unhindered. Mean fluorescence intensity values were used to determine the proliferation of the virus in comparison to a control population that received no FDs. For the survey, the concentration of each FD was 5μg/mL.

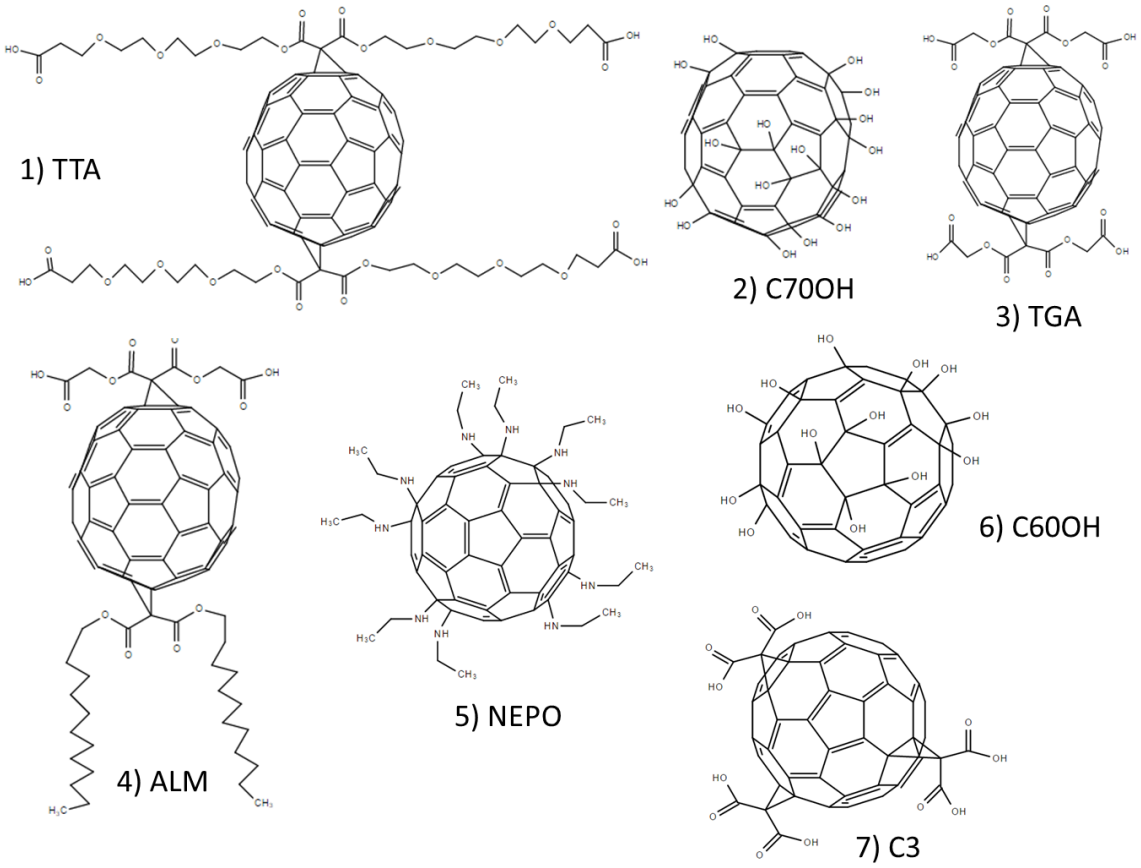

Figure 4.1 Graphical Representations of the FDs Used in this Study.

IV.2.2.3 Fullerene dose response experiments

Following results of the initial survey of seven fullerenes, dose response experiments were performed following a similar protocol used for the initial survey. Cells were plated at 100,000 cells per well in 24 well plates. Varying concentrations (0.01, 0.1, 1, 5, and 20 μg/mL) of three FDs that appeared to work well in the original survey were used. Readings of gfp fluorescence were taken each day and compared to negative and positive samples present in each plate. Dose response experiments using the three selected FD were repeated three times in duplicate.

IV.3 Results

IV.3.1 Modeling results suggest complementarianism

To test the hypothesis that any of these FD had any effect on the activity of HIV Protease, FD (Figure 1) were submitted as ligands to the Surflex docking module in the Sybyl modeling package. The module produced Total Scores that ranked ligands as candidates for fitting into the active site. Total score values are listed in Table 1. Scores are ranked by their positive magnitude meaning that the highest scoring ligands were those that received larger positive values and those that scored low are those close to or below zero. Additionally, these score values suggest the relative likelihood that an interaction was possible in that conformation between the ligand and the viral protease. Table 1 also lists the various residues which participated in hydrogen bonding with the ligand docked in the most highly scored ligand orientation. Interestingly, there appeared to be no correlation between the multiplicity of hydrogen bonding instances and Total Scores. Certain FDs were scored very highly in possible interactions with HIV protease including C3, NEPO, ALM, and C60OH. For both ALM and TTA, the highest scoring conformation did not include the integration of the fullerene cage within the active site of the viral protease. Instead, the moieties decorating the surface of the fullerene cage were occupied the active site more exclusively.

IV.3.2 *In Vitro* experiments show some inhibitory effect of FD

Mean Fluorescence Intensity was measured over the course of five days. The raw values were then normalized and compared to a positive control that had no FDs but did receive virus. This Percent Inhibition of gfp is displayed in Figure 3. After an initial

survey of the seven FDs, three FDs (NEPO, TTA, and C3) with stronger suppressive effects at the surveyed concentration of 5 μg/mL were selected for dose response experiments. Dose response experiments used concentrations of the three FD of 0.01, 0.1, 1, 5, and 20 μg/mL. Raw values were compared to positive controls that were introduced to virus but not FDs to give a percent inhibition compared to control. Values were then averaged together from three dose response experiments and are presented in Figure 4. Dose response experiments were repeated three times in duplicate.

Table 4.1. FDs and Their Total Scores Assigned to Them by the Docking Module. Certain residues are also listed that had hydrogen bonding with the ligand. Positive controls of Ritonavir and a FD that had been shown previously to be a suppressor of HIV Protease are also present [128]. Ligands marked with a (*) had their strongest interaction with HIV protease only with the fullerene cage outside of the active site. These ligands generally interacted with the active site with their bound moieties. Residues marked with a (**) participated in multiple instances of hydrogen bonding; multiple atoms of the residue was able to participate in hydrogen bonding.

Ligand (FD)	Total Scores	Protease Residues involved in H-Bonding
C3	4.8628	ASP30, ASP25, ASP29, LYS45, ARG8
NEPO	12.6611	ASP25, GLY27, GLY48, ILE50, THR80
C60OH	5.2109	ARG8, ASP29, ILE47, GLY48, GLY49, ILE50, THR80, VAL82
ALM*	10.5172	ARG8, ILE50
C70OH	0.2040	ASP25, GLY27, ALA28, ASP29, ILE47, GLY48**, ILE50
TGA	-2.0682	ARG8, ASP29, ASP30, LYS45, GLY48
TTA*	-1.9429	ARG8, ASP29, ASP30
Ritonavir	1.9402	ASP25**
Trans-3	4.7939	none

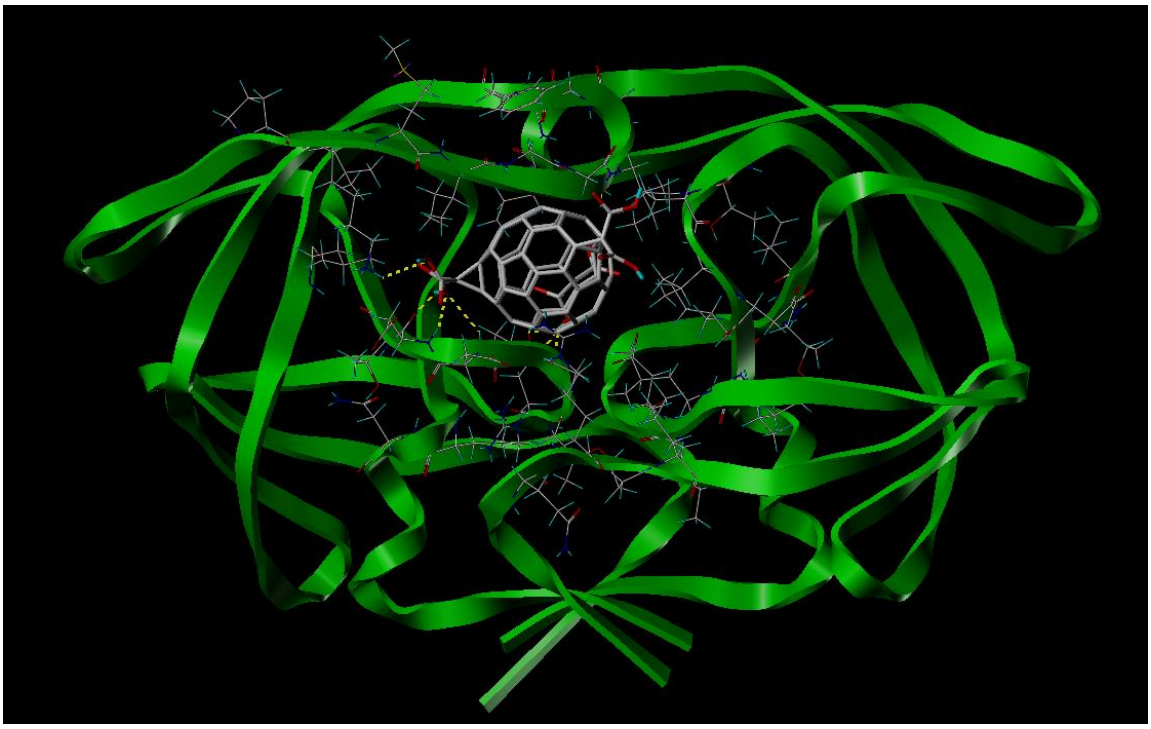

Figure 4.2 Representative Picture of a FD (C3) Docked Into HIV Protease Active Site.

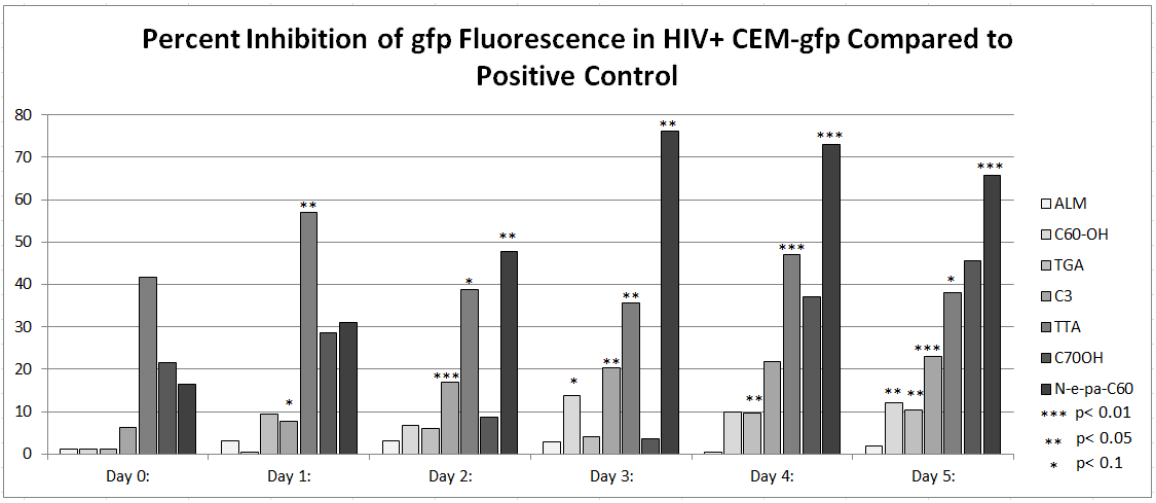

Figure 4.3 Percent Inhibition of gfp Fluorescence in HIV+ CEM-gfp Compared to Positive Control.

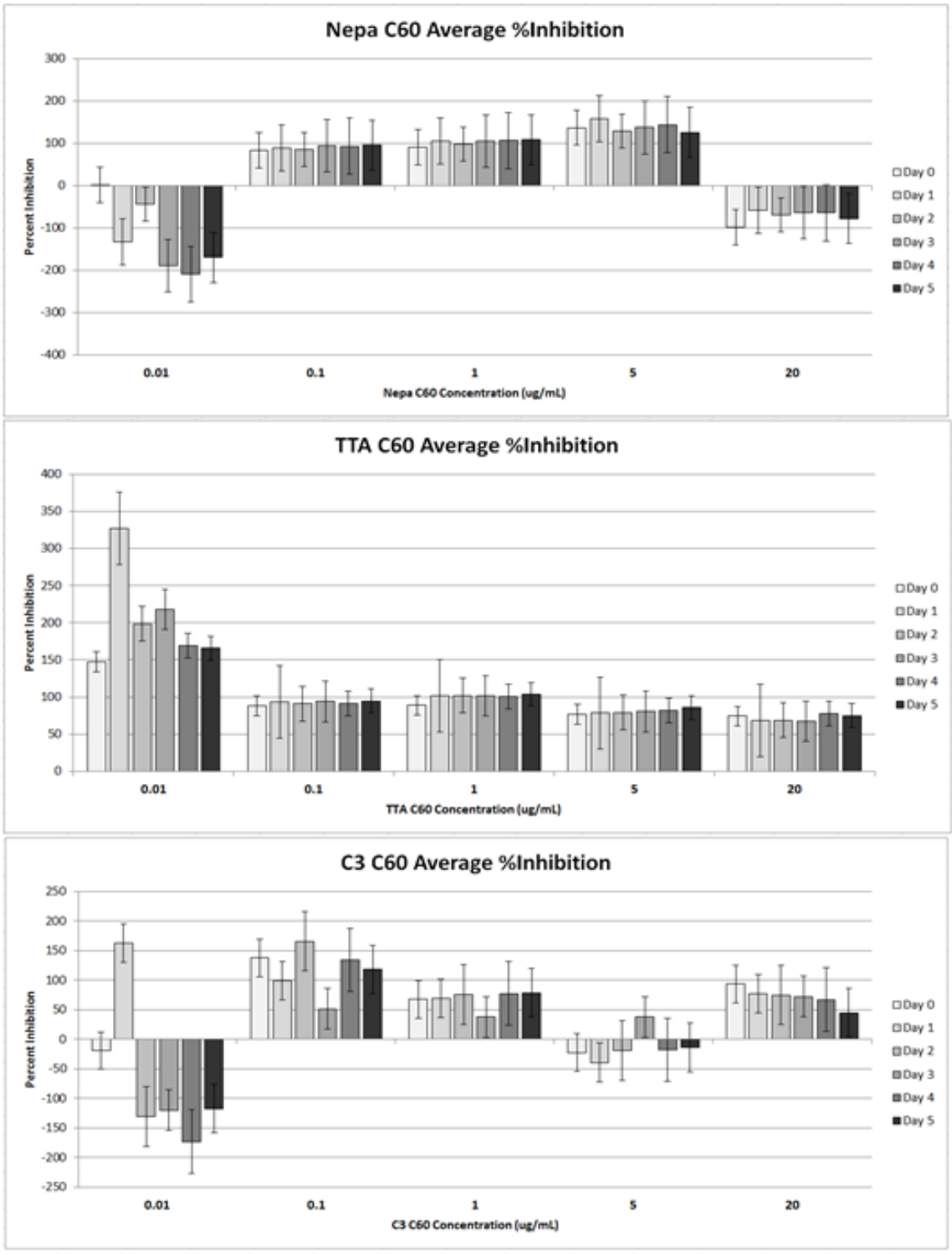

Figure 4.4 Average Percentage of MFI Inhibition.

IV.4 Discussion

Fullerenes and their derivatives represent a promising platform for the design of targeted therapies because of their predisposition to chemical attachment. Chemical moieties decorating the surface of fullerenes allow for the compound to be customized to various applications. This study explored the possibility of various C60 and C70 FDs to be used as HIV inhibitors. Previous investigations have explored FD interactions with both HIV protease [25-28,124,126-128,130] and HIV reverse transcriptase [135] and this study aims to add to that knowledge.

While FDs C3, NEPO, C60OH, and ALM scored well in the docking simulations, not all of these were effective *in vitro*. The reasoning for this is unclear, but there likely are multiple interactions in which these FDs are involved. Further studies are required to more concretely elucidate the possible interactions these FDs have *in vitro*. C3 and NEPO were originally effective in the survey and had some apparent interaction at other concentrations as demonstrated with the dose response experiments. This may mean that these two FDs have some interaction with protease, but the data is unexpected. Generally, dose response assays show a characteristic increase in efficacy over the dosage range until a threshold concentration where toxicity is apparent is reached. Instead, the dose response results suggest that these FDs are likely involved in other interactions. Alternatively, like other FDs that appeared to have no effect, these FDs are not interacting with the viral protease at all, but are instead interacting with other mechanisms that result in inhibition. To examine these possibilities, further experiments are needed. Examinations of the FD's interaction with various viral proteins through

reporter kits would be invaluable for the determination of the method of action. Alternatively, reporter substrates generated from transfected genes for the possible mechanisms of action could be used as a screening tool for the presence of inhibition at various steps of the viral infection pathway. For example, a gene that codes for both the natural substrate for HIVP as well as for a reporter that fluoresces after the natural substrate has been cleaved would help identify interactions of the protease with a FD. Importantly, this example would only be able suggest an interaction and would not show exclusivity of the interaction and would not show other characteristics of the interaction like the length of the interaction.

Interestingly, though TTA did not score well in simulations, it was effective at limiting viral proliferation *in vitro*. There are some possible explanations for this result. While HIVP is the most common target for HIV inhibition using FDs, it is not the only one that has been reported [135]. TTA very likely is having some effect on some other viral process and stopping the virus from replicating. Similar studies suggested earlier could also be translated to determine TTA's mechanism of action by including analytical tests for other viral specific proteins like reverse transcriptase or integrase. Alternatively, TTA may be intimately involved with some host mechanism that HIV requires for replication. TTA may have interactions with the surface chemistry necessary for HIV internalization and disable the virus from entering the cell entirely. To test for these interactions, reporter viral strains that have been engineered to report upon internalization could be employed. Fullerenes have also been shown to interact with various cellular processes; this includes the NF- κB pathway [131]. Because the activation of NF- κB is

regulated by a large downstream pathway consisting of many proteins and protein complexes, the most comprehensive test to examine all interactions would be a phosphoproteomic study of the NF- κB pathway. This examination would allow us to determine the phosphorylation state of each of the proteins in the pathway and look for any blocking caused by a FD dose. While we could examine individual bonding instances with FDs and certain parts of the pathway, this proposed examination would be able to screen multiple FD treatments as well as screen multiple possible instances of interactions.

In the instance of ALM, this FD is known to have interactions with other biological molecules including lipid bilayers. Accordingly, ALM may be present only in areas of the cell that are not involved with viral processes. As such, even though ALM scored highly on the docking simulations, it likely was not effective as a protease inhibitor (or as any other inhibitor for that matter) because it was sequestered away in membranes. In HIV protease interaction experiments examining other FDs, ALM should still be included. ALM itself may not have any *in vitro* effect, but its moieties could still be of use as an inhibitor, just bound to another platform. This study is helpful as screening method development for detecting inhibition of HIV proliferation, but admittedly does only suggest an interaction. Additional computational studies may illuminate interactions between FDs and other viral proteins like reverse transcriptase, but, like the computational studies performed here, they will only be able to suggest interactions. Further experimentation that analyzes specific FD interactions with host and

viral proteins is needed to fully determine the mechanisms of action through which these (and other) FD disrupt the natural viral processes.

CHAPTER V

FUTURE PROSPECTIVES

HIV has been studied extensively. Continued research efforts are likely to focus on identifying new latent reservoirs and drug classes intended to further inhibit the virus's pathology. The hope is that at some point a way to fully eradicate the latent reservoir will be discovered. However, this solution will likely include some measure of either permanently disabling the integrated viral DNA or killing the latently infected cells altogether. Some options for the latter have been explored by firstly activating the latently infected cell population to produce virus and then targeting the virus expressing cell for extermination [6]. There are complications that make this approach difficult and researchers are actively exploring other opportunities.

Another method for the eradication of HIV and the latent reservoir is through bone marrow transplantation. To date, one individual has been functionally cured of HIV infection. The highly publicized study described the case of Timothy Brown, commonly referred to as the "Berlin Patient", as his medical problems were solved [149]. Briefly, Brown was diagnosed with HIV in 1995. He was prescribed anti-retroviral treatment and remained on the treatment until 2007 when he was diagnosed with Acute Myeloid Leukemia. Brown was living in Germany at the time and his doctors decided to give him a stem cell transplant from a donor who expressed a homozygous CCR5 Δ32 mutation and, after enduring many complications, recovered from both his leukemia and HIV

infection. This mutation is present in a small percentage of the population and it causes the CCR5 receptor to no longer have an extracellular region. This then means that the virus struggles to infect cells because it cannot perform the secondary binding event with CCR5. For the "Berlin Patient," this transplantation resulted in a newly acquired natural resistance to the virus. Brown reports that he has since discontinued his HAART medication and remains free of detectable virus to this day. This same method for treatment was lauded as a possible blueprint for the eradication of HIV in infected individuals and thus is being explored to solve complications caused by the treatment and in turn make it more robust.

The option to more fully eradicate the presence of detectable virus in HIV+ individuals has further spurred the creation of more diverse treatment options. Because HAART medication only reduces the probability of progression to AIDS and because the virus easily mutates to circumvent treatment and immune responses, more robust treatment options are necessary for more indefinite control of the virus [150,151]. Furthermore, many of the currently available HAART treatments are accompanied by unwanted side-effects. These are some of the main reasons that fullerene derivatives and other more unconventional drug development strategies have been employed in research studies to find replacements and alternatives [130]. This trend will likely continue until a reliable, functional cure for HIV is produced.

There are complications, however, with the development of FD based treatments. Though fullerenes and their derivatives are generally regarded as non-toxic and have notable beneficial effects [110,112,152-155], the FDA is understandably strict with the

composition and predictability of drug interactions [156]. Accordingly, many FD drug candidates are expected to be comprised of the C70 fullerene cage over the C60 cage because of the increased predictability of moiety binding positions. C70 cages are arranged in a more ellipsoidal shape and that ellipsoidal shape prefers binding on the opposite ends of the major axis [147,157,158]. This characteristic allows for more predictable attachment of surface moieties and is likely to be preferred by the FDA.

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