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# Defining And Characterizing The Step In The Hiv-1 Viral Life Cycle Affected By Fullerene Derivatives

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DEFINING AND CHARACTERIZING THE STEP IN THE HIV-1 VIRAL LIFE CYCLE  
AFFECTED BY FULLERENE DERIVATIVES

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2016

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by

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DISSERTATION

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## ABSTRACT

Fullerene derivatives, both C<sub>60</sub> and C<sub>70</sub>, were synthesized and characterized to test their activity against HIV-1 infection. The derivatives tested have previously been reported to inhibit HIV-1 replication and/or *in vitro* activity of reverse transcriptase. It was found that viruses produced in the presence of low micromolar concentrations of most of these fullerene derivatives exhibit a reduction in viral infectivity greater than 99%. Quantification of virion-associated viral RNA and p24 indicates that RNA packaging and viral production were unremarkable in these viruses. These compounds did not affect infectivity of mature virions, indicating no effect on the early steps of the viral life cycle, thus suggesting an effect on viral maturation. Analysis of Gag processing confirmed this mechanism of action, which is independent of the activity of protease as demonstrated by an *in vitro* enzymatic assay. As a result, fullerene derivatives potentially impair viral infectivity of viruses harboring mutant proteases that developed resistance to multiple protease inhibitors in patients. Pull-down experiments using magnetic bead-immobilized compounds demonstrated that some C<sub>70</sub> fullerene derivatives strongly bind to the HIV capsid-spacer peptide 1 Gag proteolytic fragment, showing that this viral protein is the target. Comparison of the antiviral activity of fullerene derivatives to that of other small molecules and peptides that block maturation by binding to the HIV-1 capsid protein indicates that fullerene derivatives exhibit a new mechanism of action.

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# CHAPTER 1: INTRODUCTION

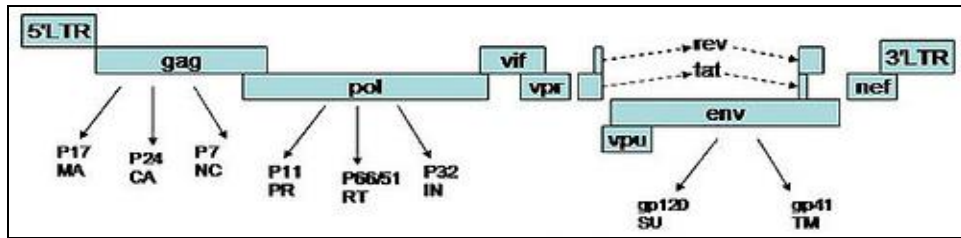
## 1.1 HIV Overview

Since the early 1980's, the Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immune Deficiency Syndrome (AIDS), has been a major global epidemic. While much has been learned about how the virus is transmitted and how it attacks the immune system, the HIV/AIDS epidemic has expanded on an unprecedented scale over the last three decades since its identification (1). HIV cases have been reported in all regions of the world, with the largest part existing in low and middle income countries, particularly in sub-Saharan Africa (1). As a leading cause of deaths worldwide and the number one cause of deaths in Africa, HIV prevention, care, and treatment are not easily accessible to most people living with HIV or at risk for HIV infection (1).

HIV is a lentivirus from the *Retroviridae* family that infects mainly CD4<sup>+</sup> T lymphocytes and has also been shown to target macrophages and dendritic cells (2). Many infected individuals will undergo a progressive depletion of CD4<sup>+</sup> T-cells, rendering them susceptible to opportunistic infections that may be fatal (3, 4). HIV exists as two different species, HIV-1 or HIV-2, with HIV-1 being the dominant species in the HIV/AIDS epidemic (2, 3, 5). HIV-2 causes similar illnesses as HIV-1, however progression of immunodeficiency is slower and less transmissible for HIV-2 (3). There are four groups of HIV-1, group M is the cause of the global HIV epidemic and consist of nine different subgroups, and groups N, O, and P are restricted to West Africa (2, 3, 5).

The marked genetic diversity of HIV-1 is a consequence of the error-prone function of the viral enzyme reverse transcriptase, resulting in a high mutation rate (3).

HIV is a spherical enveloped particle consisting of a single-stranded positive sense RNA genome (4). There are two strands of RNA surrounded by a cone-shaped capsid consisting of p24 viral proteins, with each strand containing a copy of the virus's nine genes (2, 5). A viral envelope composed of a lipid bilayer membrane that is formed during budding from the cellular membrane of the host cell surrounds the capsid (5). The HIV genome is approximately 9.7 kilobases in length and contains three genes that are common to all retroviruses, the *gag*, *pol*, and *env* genes (refer to figure 1) (2, 4-6). The *gag* gene encodes for the core proteins that package the viral genomic RNA such as the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins (2, 4, 6). The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase that are essential for HIV replication (2, 4, 6). The *env* gene encodes for the envelope protein that consists of two membrane proteins, the outer envelope glycoprotein (gp) 120 and the transmembrane protein gp41 (2, 4, 6).



**Figure 1.** Genetic organization of the human immunodeficiency virus (HIV-1).

The genome is made of three large open-reading frames that express structural (Gag), enzymatic (Pol) and envelope (Env) proteins, surrounded by terminal repeated sequences LTR (Long Terminal Repeat). The genome also encodes regulatory (Tat and Rev) and auxiliary (Nef, Vif, Vpr, and Vpu) proteins (Google images).

## 1.2 HIV-1 Life Cycle

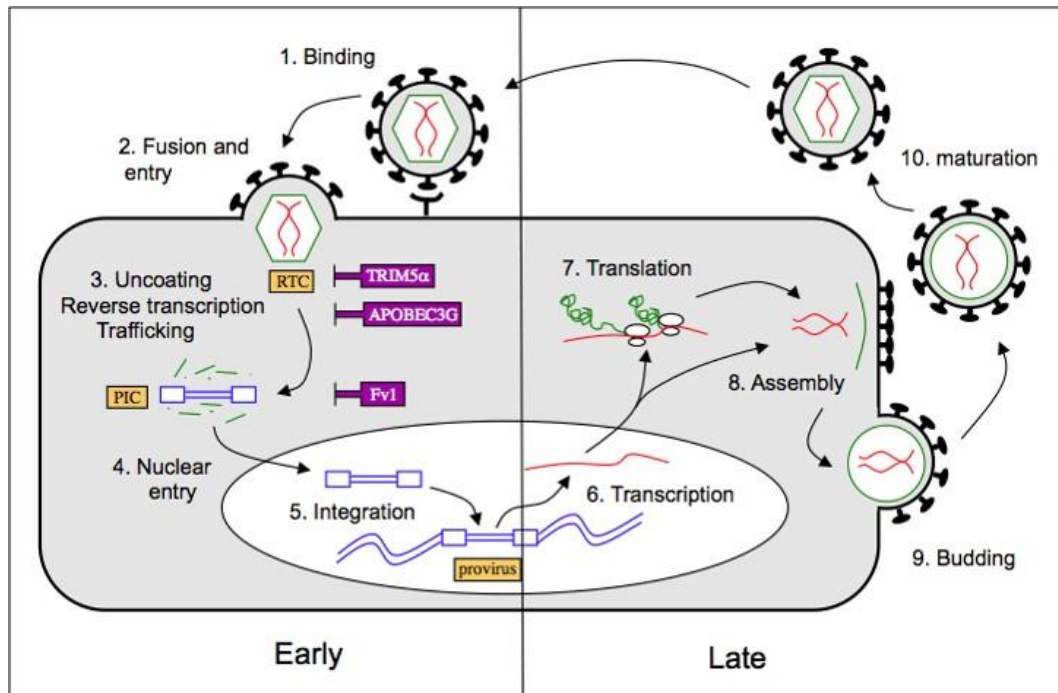
The HIV life cycle has two stages throughout the replication process, the early stage and the late stage (depicted in figure 2). In the early phase, HIV first binds to the CD4 receptor on T-cells mediated by the gp120 subunit on the viral envelope protein (7, 8). This is followed by conformational changes in both gp120 and CD4 leading to coreceptor binding to chemokine receptors CCR5 or CXCR4, depending on the HIV strain (7, 8). After coreceptor binding, conformational shifts in the envelope glycoprotein leads to the dissociation of gp120 from gp41, allowing the fusogenic conformation of gp41 (8). Moreover, movement of the virus particle to the site of productive membrane fusion can now occur (7). HIV virions enter the cell by insertion of the gp41 fusion peptide into the target membrane, resulting in the fusion of viral and cellular membranes and the release of the viral core in the cytoplasm (8). The entry of viral content is followed by uncoating of the viral core and reverse transcription (8). Single-stranded viral RNA is reverse transcribed by viral reverse transcriptase, generating a linear double-stranded DNA molecule (7, 9). Reverse transcriptase contains two enzymatic activities needed for efficient reverse transcription which include a DNA polymerase that copies either a RNA or a DNA template and an RNase H that degrades RNA in a RNA-DNA duplex (9).

Once viral DNA is synthesized in the cytoplasm, it stably associates with HIV integrase, reverse transcriptase, protease, and Vpr to form the pre-integration complex (PIC) (8). Also predicted to be associated with the PIC are structural and cellular proteins, however there is much controversy as to whether these proteins are present in the complex (8). The PIC is transported to the nucleus by mechanisms still not fully

understood, however several known components of the PIC exhibit nuclear localization properties when fused to a polypeptide that does not normally localize to the nucleus (10). Once inside the nucleus, viral integrase facilitates the integration of viral DNA into the host chromosomal DNA (10). Viral RNA synthesis is directed by the HIV long terminal repeat (LTR) and requires the host RNA polymerase II (2, 11).

The late stage of the viral life cycle begins with transcription of RNA followed by translation of the viral proteins in the cytoplasm, a process that is dependent on the host machinery (12). Contents required for HIV infection, such as two copies of viral RNA, cellular tRNA molecules, the viral envelope (Env) protein, the Gag polyprotein, and the Gag-Pol polyprotein are assembled at the plasma membrane (13). The Gag and Gag-Pol polyproteins mediate virion assembly events as well as binding to the plasma membrane (13). The virion acquires its lipid envelope from the plasma membrane of the cell and the Env protein spikes this envelope as the immature virion buds from the cell (13). During the budding process HIV protease is simultaneously activated and proteolytic cleavage of the Gag and Gag-Pol polyproteins occur, leading to a change in virion morphology allowing for maturation (2, 13-15). Virion maturation is the final step in the HIV life cycle and is essential for the conversion of the immature virion into its mature infectious form (2, 13).





**Figure 2.** *The life cycle of HIV-1 replication.*

The early and late stages of the HIV-1 life cycle are depicted above. Early stages of the life cycle include virion binding to target cells, entry, reverse transcription, nuclear entry, and integration. The late stages include transcription, translation, assembly of viral contents, budding from the cell, and finally maturation (Google images).

### 1.3 HIV Maturation

HIV maturation is a dynamic, multistep process that involves a series of conformation adjustments and subunit rearrangements (13). Viral maturation begins concurrently with or immediately following viral budding and is driven by viral protease cleavage of the Gag and Gag-Pol polyproteins (2, 13-15). This cleavage process occurs at 9 different sites, producing fully processed matrix, capsid, nucleocapsid, spacer peptide 1 (SP1), spacer peptide 2 (SP2), p6, protease, reverse transcriptase, and integrase proteins (2, 13-15). During maturation, individual structural components should be released in a defined sequence to allow for dramatic viral protein rearrangement to establish the mature infectious virion (13, 15, 16). Many things about viral maturation are still not fully understood, such as the nature of the triggering event and its timing with respect to virus formation (16). Also, the kinetics of polyprotein processing in the context of the assembled virus is unknown, as well as the dynamics and pathway of structural rearrangements (16).

HIV immature virions are incomplete spherical particles harboring radially arranged Gag molecules (17). The nucleocapsid (NC) domains point toward the center of the particle where they cluster Gag molecules together via interactions with the RNA genome and possibly between the NC domains themselves (17). The capsid surrounds the NC, the structure required for specific encapsidation of the HIV genome and viral proteins (13). During the maturation process the Gag shell is disassembled resulting in the reassembly of approximately 1200 – 1500 copies of CA forming the conical fullerene-shaped core (13, 17). The matrix (MA) protein remains associated with the inner leaflet of the viral membrane, forming a discontinuous matrix shell throughout

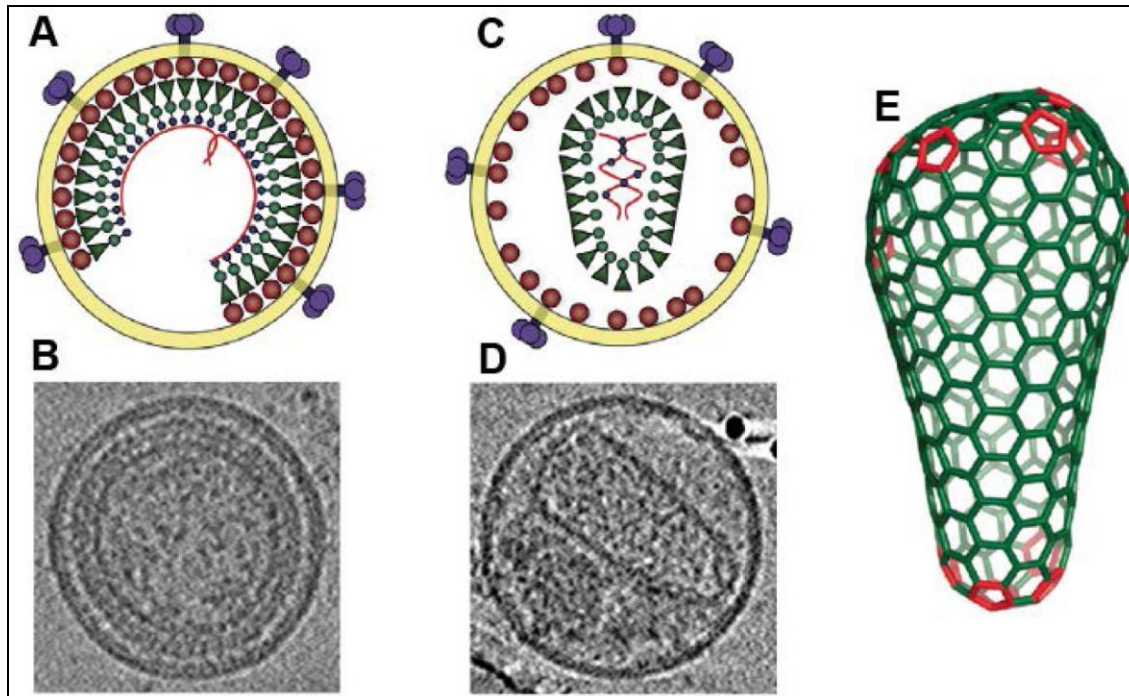
maturation (13, 17). These structural rearrangements required for HIV maturation are depicted in figure 3 (17). Other important changes, aside from capsid assembly, that occur during HIV-1 maturation include activation of the fusogenic activity of the viral Env protein, stabilization of the genomic RNA dimer, and rearrangement of the tRNA primer-genome complex (13).

Temporal control of viral maturation is provided in part by the extremely different rates of processing at the five Gag processing sites, whose cleavage rates vary by up to 400-fold (13). These cleavage sites are separated into three different categories: rapid (SP1/NC), intermediate (SP2/p6, MA/CA), and slow (NC/SP2, CA/SP1) (13, 15). The SP1/NC cleavage activates Env and promotes condensation of the ribonucleoprotein (RNP) complex formed between the viral RNA and NC (13, 15). SP2 processing frees NC, MA/CA cleavage disassembles the immature lattice and releases CA-SP1, and CA-SP1 cleavage which is thought to be the final cleavage step frees CA to form the conical capsid core (13, 15). This highly ordered processing of Gag is mainly regulated by those amino acids in the substrate that are in direct contact with the viral protease (15). Many mutational studies have shown that complete cleavage of Gag at multiple sites is essential for successful maturation (16). Blocking protease-mediated Gag processing by mutagenesis at a single site severely reduces or even abolishes HIV infectivity (16). It has been demonstrated that partially processed intermediates, in particular the slowly cleaved CA-SP1 intermediate, can exert a strong dominant-negative effect on viral infectivity (16).

The HIV-1 capsid conical fullerene-shaped core comprises of conical hexagonal rings that close at both ends by the introduction of exactly 12 pentagonal rings with five

of these pentamers at the narrow end of the cone and seven at the broad end, as shown in figure 3E (13, 17). Positioning of these pentamers at alternate sites accounts for the different capsid shapes seen in other retroviruses (13). The “tubular” or “conical” capsids are created as described above, whereas the “spherical” capsids are created when the pentamers are distributed more evenly throughout the hexagonal rings (13). These rings are stabilized by interactions between the first three helices of the N-terminal domain of CA (CA<sub>NTD</sub>) (13). The capsid rings are supported by an exterior restraint formed by the C-terminal domain of CA (CA<sub>CTD</sub>), with each CA<sub>CTD</sub> contacting the CA<sub>NTD</sub> of a neighboring subunit in the ring (13). CA<sub>CTD</sub> also makes important inter-ring contacts across the local two- and three-fold axes stabilizing the extended lattice (13). Modifications in lattice curvature across different regions of the cone surface are accommodated by flexibility in the NTD-CTD linker and in the NTD-CTD interface, allowing the two CA domains to move relative to one another (13).

Aside from key roles the capsid plays in maturation of the virion, this structure also performs essential functions during the early stages of HIV-1 replication (13, 16). It has been reported that CA mutations that block capsid assembly or destabilize the capsid inhibit the process of reverse transcription, suggesting that the capsid helps with the organization of the replicating genome (13, 16). Nuclear localization has been shown to be inhibited by CA mutations as well, implicating the capsid in roles of nuclear targeting or import of the pre-integration complex, or both (13, 16).



**Figure 3.** *HIV Maturation.*

Cartoon depiction of the immature (A) and mature (C) HIV-1 virion architecture. Central slices through cryo-electron tomograms of the immature (B) and mature (D) HIV-1 virions. The different components of the virions are color-coded: envelope glycoprotein (purple), MA domain (dark red), CA N- (dark green) and C-terminal (light green) domains, NC domain (dark blue) and viral RNA (red trace). (E) The conical fullerene HIV capsid. The CA protein forms both hexamers (green) and pentamers (red) (17).

## 1.4 HIV Gag as a Therapeutic Target

HIV-1 Gag represents a novel therapeutic target as it plays a critical role in infectious virion production, however it is not targeted by any of the currently approved antiretroviral drugs (18-21). Disrupting cleavage at individual sites or altering the order of cleavage results in aberrant virus particles that have significantly reduced infectivity (18). This disruption of Gag processing therefore represents an appealing therapeutic target for inhibiting HIV-1 (18-22). Inhibitors that act by specifically targeting individual Gag cleavage sites have been classified as maturation inhibitors and represent a new class of potential anti-HIV-1 drugs (18, 20-22).

The first drug candidate, as well as the best characterized, of maturation inhibitors was 3-O-(3', 3'-dimethylsuccinyl)betulinic acid, better known as bevirimat (18, 22-29). Bevirimat, also known as PA-457 or DSB, has been shown to potently inhibit wild-type HIV-1 as well as viral isolates that are resistant to currently approved reverse transcriptase, protease, and fusion inhibitors (18, 27, 30-32). Bevirimat retains activity against drug-resistant HIV-1 isolates due to its novel mechanism of action involving specifically blocking the cleavage of SP1 from the C-terminus of CA (18, 22, 25-31). Disrupting the cleavage of CA-SP1 with either bevirimat treatment or by mutation of the CA-SP1 cleavage site results in the formation of virions that exhibit an aberrant morphology characterized by an acentric core and an electron-dense layer of Gag inside the viral membrane (18, 22, 25-29). The precise mechanism by which bevirimat prevents cleavage of CA-SP1 is still unknown, however experimental data suggests that the CA-SP1 junction of an oligomeric form of Gag within immature HIV particles is the molecular target (22). Single amino-acid substitutions that confer resistance to

bevirimat all map to the CA-SP1 junction and not elsewhere in Gag (18, 25, 33-35). Three substitutions are located at the first and third residues of SP1 (A1V, A3V, and A3T) while the other three are located at the C-terminus of CA (H226Y, L231M, and L231F), with mutations A3V and A3T occurring in SP1 having the most severe impairment of HIV replication (18, 22, 33-35). Despite promising data in a phase IIa clinical trial, further development of bevirimat was suspended in 2010, nearly 10 years after its identification, due to bevirimat-resistance resulting in Gag SP1 polymorphisms present in nearly 50% of HIV-1 infected patients (22, 25, 27, 29). The discovery and characterization of bevirimat represented a novel class of anti-HIV drugs termed maturation inhibitors that exploit a previously unidentified viral target (22).

Interrupting the CA-CA interactions required for core formation is another potential therapeutic strategy for inhibiting HIV-1 replication (18). There are three major CA-CA interfaces required for core formation that facilitate intrahexamer CA<sub>NTD</sub>-CA<sub>NTD</sub>, intrahexamer CA<sub>NTD</sub>-CA<sub>CTD</sub> and interhexamer CA<sub>CTD</sub>-CA<sub>CTD</sub> interactions (18, 36, 37). Previously reported, two independent inhibitor screens have identified molecules that appear to target pockets within or adjacent to the CA<sub>NTD</sub>-CA<sub>CTD</sub> interface in the mature CA lattice (18). The first compound identified was CAP-1 (N-(3-chloro-4-methylphenyl)-N'-{2-[(5-[dimethylamino)-methyl]-2-furyl)-methyl]-sulfonyl}ethyl}urea) (18, 25, 26, 29, 38, 39). It has been shown that HIV-1 particles produced from cells treated with CAP-1 did not contain conical capsid core structures (18, 25, 26, 29, 38, 39). The primary mechanism of action of this compound appears to involve inhibition of core formation during maturation as binding studies showed that CAP-1 interacts with the C-terminal end of CA<sub>NTD</sub> in both processed CA and precursor Gag (18, 25, 26, 29, 38, 39).

The second inhibitor screen revealed the 12-mer peptide CAI (capsid assembly inhibitor), which inhibits HIV-1 immature and mature assembly *in vitro* (18, 25, 26, 29, 39, 40). CAI acts by first blocking the CA<sub>NTD</sub>-CA<sub>CTD</sub> interaction in mature core assembly by competing for the natural binding region in the CA<sub>NTD</sub> (18, 25, 26, 41, 42). The second effect of CAI is to modify the CA<sub>CTD</sub>-CA<sub>CTD</sub> dimeric interaction that is important for connecting the hexameric lattice (18, 26, 41, 42). The identification of these two compounds and their mechanism of action is interesting, however, they are not viable drug candidates due to their weak binding affinity for CA, and also in the case of CAI, poor cell permeability (18, 25, 26, 39). Despite these limitations, the discovery of their molecular targets can be exploited for the rational design of future effective maturation inhibitors (18).

## **1.5 HIV Protease**

The first cleavage events catalyzed by HIV protease serve to liberate protease itself from the Gag-Pol precursor (2). The mechanism by which protease is activated during Gag assembly and budding is still not fully understood (13-15). It is hypothesized that Gag trafficking probably helps to regulate protease activation by preventing premature protease dimerization until the Gag molecules coalesce at the plasma membrane (13). Like other retroviral proteases, the active HIV protease enzyme is a homodimer, with each monomer consisting of 99 amino acids (13, 15, 16). The two monomers are held together in part by a four-stranded, antiparallel  $\beta$ -sheet derived from both N- and C-terminal ends of each monomer (2). Protease uses two aspartic acid side chains within a characteristic Asp-Thr-Gly motif to activate the



nucleophilic water molecule that catalyzes the hydrolysis of the peptide bond of substrates (2, 13). Two extended flexible loops lie on the other side of the active site and act as flaps that open or close to allow substrates access to the active site (13). These flaps consist of two  $\beta$ -hairpins and undergo significant conformational changes between the open, semi-open and closed conformations depending on substrate or inhibitor binding (16). Numerous experimental and computational studies have revealed that hydrogen bonding interactions between substrate or inhibitors and the active site residues of protease, the hydrophobic nature of the peptide, the orientation it acquires once inside the active site and the presence of a water molecule close to the active site and flap residues are necessary for effective protease binding (43).

The efficiency with which protease cleaves the individual target sites in Gag and Gag-Pol polyproteins varies widely and is influenced by two major factors: the amino acid sequence at the site of cleavage and the degree of exposure and accessibility of the cleavage site (2). Studies have shown that the binding cleft of protease can accommodate a peptide of approximately seven residues in length (2). Due to the diverse target sequences in the Gag and Gag-Pol polyproteins, protease cleavage takes place as an ordered step-wise cascade (2). Mutations present in these polyproteins that disturb the ordered nature of protease processing severely disrupt virus assembly and maturation (2). It has also been found that HIV-1 mutants engineered to overexpress protease exhibit rapid premature processing of Gag and Gag-Pol and subsequently block maturation (2). The activation of protease must therefore be tightly regulated to prevent premature processing prior to the completion of assembly (2).

## 1.6 Protease Inhibitors

Advances in anti-HIV retroviral drugs have led to a significant reduction in AIDS-related deaths, delayed disease progression, and diminished the rates of HIV transmission (16, 44). Current therapeutic treatments for effective repression of HIV replication are administered in a cocktail regimen known as highly active antiretroviral therapy (HAART) (16). The antiretroviral activity of these drugs is mainly due to their inhibition of reverse transcriptase and protease (2, 3, 15). These therapies efficiently suppress the spread of HIV in patients (2, 16). Protease inhibitors do not prevent uninfected cells from becoming infected, as reverse transcriptase inhibitors do, but rather result in the release of noninfectious virions (2). Drugs targeting protease prevent the cleavage of the Gag and Gag-Pol polyproteins, leading to immature virions that are non-infectious (3, 45). There are currently nine different HIV protease inhibitors approved by the US Food and Drug Administration (FDA), these include saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), fosamprenavir (FPV), lopinavir (LPV), atazanavir (ATV), tipranavir (TPV), and darunavir (DRV), and a number of them are employed as part of HAART (15, 16, 45). All these FDA-approved protease inhibitors are competitive inhibitors that bind to the active site of protease (15, 16). Most protease inhibitors are peptidic or peptidomimetic compounds designed as analogs of the cleavage sites within the Gag and Gag-Pol polyproteins (2, 15, 16).

While the current treatments are effective at inhibiting HIV replication, the emergence of drug-resistant viruses is a continuous challenge to the effectiveness of this intervention. The extreme variability and the high evolution rate of HIV favor the development of antiviral resistance (46). Since the introduction of protease inhibitors,

drug-resistant mutations in protease have become prevalent (15, 47). Mutations in at least 34 of the 99 HIV protease residues have shown to have clinical relevance (47). HIV strains found in the most highly protease inhibitor-experienced patients show between 5 and 15 mutations in protease which are often combinations of mutations both inside and outside the active site (47). Cross-resistance between protease inhibitors is another common characteristic of protease mutations (47). Even though there are chemical differences between all the currently available protease inhibitors, when they are bound to the active site of protease they occupy a similar space and have analogous functional groups at similar positions within protease (47). This causes mutations in protease to produce multi-drug resistant viruses (47). Aside from mutations in protease that allow for drug-resistance, it has been shown that Gag substrate mutations can confer protease inhibitor resistance in the absence of protease mutations (15, 45). In addition, the need for lifelong treatment, a high pill burden, and the frequently associated side effects of protease inhibitors due to toxicity (refer to table 1) are major obstacles for patient adherence to these drug regimens (48-52). Therefore the development of new and safer anti-HIV compounds is in critical need (47-52).

**Table 1.** *Summary of toxic effects of HIV protease inhibitors.*

Drug	GI toxicity	Dyslipidemia	Other toxicity
Saquinavir (SQV)	Yes	Yes	–
Lopinavir (ABT)	Yes	Yes	Risk for MI
Darunavir (DRV)	Yes	–	Rash
Indinavir (IDV)	Yes	Yes	Unconjugated hyperbilirubinemia, nephrolithiasis
Tipranavir (TPV)	Yes	Yes	Intracranial hemorrhage, rash
Atazanavir (ATV)	Yes	–	Unconjugated hyperbilirubinemia
Nelfinavir (NFV)	Yes	–	–
Fosamprenavir (FPV)	Yes	–	Rash, risk for MI
Ritonavir (RTV)	Yes	Yes	Risk for MI

Gastrointestinal (GI) toxicity: abdominal pain, nausea, emesis, diarrhea; Dyslipidemia: abnormal amounts of lipids in the blood, including hypercholesterolemia and hypertriglyceridemia. Myocardial infarction (MI) (52).

## 1.7 Fullerene Compounds and Their Biological Activities

Fullerene compounds have been reported to possess many different biological functions and have become increasingly popular as novel medicinal treatments (53-55). These compounds are highly symmetrical and have the potential to be extensively derivatized to possess different chemical and physical properties (53-55). Fullerenes consist mainly of 60 carbon atoms in the shape of a hollow sphere containing 20 hexagon and 12 pentagon rings, resembling that of a soccer ball (53, 54). Due to its hydrophobic core, derivatized fullerene can efficiently cross the cell membrane while water solubility can be engineered by attaching hydrophilic moieties (53-55). As a result of  $C_{60}$ 's chemical nature, fullerenes can react with free radical species acting as a free scavenger in diseases where reactive oxygen species (ROS) are relevant in pathogenesis (54, 55, 57). This antioxidant property in turn allows fullerene to possess antiapoptotic activity as it is able to decrease neuronal cell death, reduce the toxicity of free radical damage on neuronal tissue, and stabilize the redox status of the cell and preserve mitochondrial membrane potential integrity (54, 55, 57).

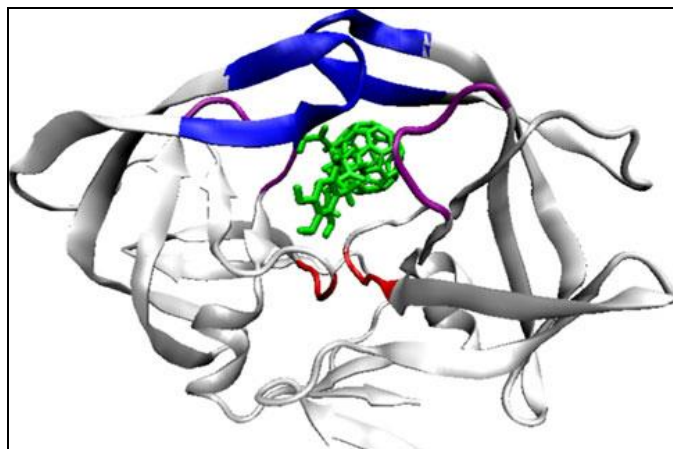
Aside from reacting with ROS, these compounds can generate singlet oxygen after irradiation and can therefore be used to cleave nucleic acids and oxidize lipids (55). This property of fullerenes renders them potential photosensitizers for photodynamic therapy and many fullerene derivatives containing functional groups with affinity to nucleic acids are being investigated for anticancer activity (54). Antimicrobial studies have been performed with fullerene derivatives and Gram positive and Gram negative bacteria (55). Results showed that various Gram positive bacteria were inhibited by fullerenes with a minimum inhibitory concentration of 5  $\mu\text{g/ml}$  in contrast to

Gram negative bacteria in which these same fullerene derivatives had no effect (55). This bactericidal action was later found to be based on the insertion of the compounds into the microbial cell wall (55). It is also possible to entrap metal atoms into the fullerene cage to obtain endohedral metallofullerenes that can be used as radiotracers in magnetic resonance and X-ray imaging (MRI and XRI) (55). Fullerene compounds also have the capability of carrying drugs and genes for cellular delivery when hydrophilic groups are attached to its core (53-55).

### **1.8 Anti-HIV Activity of Fullerene Compounds**

Aside from the aforementioned biological applications that fullerenes possess, antiviral activity, more specifically anti-HIV activity, is extremely notable as the potential of these compounds to suppress HIV replication is invaluable. It has been reported that fullerene derivatives are able to inhibit HIV-1 replication in human cells with concentrations as low as 0.22  $\mu\text{M}$  and display no cytotoxic effects at concentrations as high as 100  $\mu\text{M}$  (55, 58-63). However, the step of the viral life cycle targeted by these compounds is unknown. Molecular docking simulations predict fullerene derivatives to bind to HIV protease due to the size and conformation of the enzyme active site and the structure of certain fullerene derivatives as depicted in figure 4 (54, 58, 59, 64-67). In addition, *in vitro* analyses indicate that fullerene derivatives possess anti-reverse transcriptase activity (61). The positioning of substituent groups on the fullerene core has been shown to have an influence on anti-HIV activity (54). This inhibition of enzymatic activity is very promising for the use of fullerene derivatives as anti-HIV compounds; however, much of this work has been carried out in a non-viral context

using only recombinant viral enzymes and/or molecular docking simulations. Further assessment of these compounds is needed, particularly in a viral and cellular setting, to enhance the characterization of the anti-HIV activity they exhibit.



**Figure 4.** Representation of a fullerene derivative (green) inside the HIV-1 protease active site. Active site residues (Asp25-Thr26-Gly27, Asp25'-Thr26'-Gly27', red), flap residues (44-55/44'-55', blue), and the solvent-accessible region involving residues 79-83/79'-83' (purple) are also displayed (67).



## 1.9 Project Significance and Hypothesis

Since the start of the HIV epidemic, over 71,000,000 people have become infected with the virus, with nearly 35,000,000 people living with HIV in 2013 (68). The current therapeutic treatments have significantly impacted the number of AIDS-related deaths and have reduced the rates of HIV transmission (16, 44). However, the emergence of drug-resistant HIV viruses continues to threaten the effectiveness of clinically used drugs. Drug toxicity leading to adverse side effects has also been a major concern for patients on HAART, alongside with a high pill burden and the conditions established for taking medication. These problematic challenges have led to vast efforts to search for novel therapeutic alternatives that can inhibit HIV replication when the virus becomes resistant to the current treatments and that are safer for patient health.

Fullerene compounds have been reported to exhibit anti-HIV activity, however the mechanism of action by which they inhibit HIV replication has yet to be understood (55, 58-63). The lack of characterization of these compounds and their anti-HIV activity has hindered their development as future therapeutic treatments. Characterization of the mechanism of action of fullerene derivatives on HIV replication must be achieved to unlock their potential as anti-HIV agents. Once the step of the viral life cycle that is affected by these compounds is defined, optimization of compound structure can lead to enhanced inhibitory effects on HIV replication. Therefore the anti-HIV activity of fullerene compounds must be better understood and well defined.

Molecular docking experiments have predicted fullerene derivatives to bind to the active site of HIV protease (54, 58, 59, 64-67). Based on the size and chemical nature

of these derivatives, it is plausible that the anti-HIV activity they possess is due to inhibition of protease by occupying the active site. This competitive binding of fullerenes would prevent the Gag and Gag-Pol polyproteins from being processed and lead to the formation of immature virions unable to infect target cells. We hypothesize that fullerene derivatives are able to inhibit HIV-1 replication due to competitive binding to the protease active site, not allowing for Gag and Gag-Pol polyproteins to be fully processed, leading to immature virus particles.

## **CHAPTER 2: DEFINING THE STEP IN THE HIV-1 VIRAL LIFE CYCLE AFFECTED BY C<sub>60</sub> FULLERENE DERIVATIVES**

### **2.1 Introduction**

Advances in anti-Human Immunodeficiency Virus (anti-HIV) retroviral drugs have led to a significant reduction in acquired immune deficiency syndrome (AIDS) related deaths, delayed disease progression, and diminished the rates of HIV transmission (44). Current therapeutic treatments for effective repression of HIV replication are administered in a cocktail regimen known as highly active antiretroviral therapy. The antiretroviral activity of these drugs is mainly due to their inhibition of HIV reverse transcriptase and protease, essential enzymes for HIV replication. Drugs targeting protease prevent the cleavage of the Gag and Gag-Pol polyprotein, leading to immature virions. These therapies efficiently suppress the spread of HIV in patients; however, the emergence of drug-resistant viruses is a continuous challenge to the effectiveness of these interventions. In addition, these anti-retroviral drugs have important side effects that limit their use (46, 72, 73). Therefore the development of new and safer anti-HIV compounds is a critical need (44, 49, 50).

Fullerenes consist of carbon atom cages, some, like C<sub>60</sub>, have the shape of a hollow sphere, similar to a soccer ball (72). Due to their ability to be extensively derivatized, functionalized fullerenes have shown several biological applications (53, 54). It has been shown that fullerene derivatives are capable of efficiently crossing the cell membrane due to their hydrophobic core while water solubility can be achieved by attaching hydrophilic moieties (53, 54, 56). The first fullerene derivatives that exhibited

anti-HIV activity were reported in 1993 (59, 62). However, the lack of comprehensive characterization of the antiviral mechanisms of fullerene derivatives has hindered their further development into therapeutic drugs (54, 58, 59, 60-63, 75, 76). Since the original report, it has been assumed that the anti-HIV activity of fullerene derivatives is mediated mainly, if not exclusively, by inhibition of the viral protease. Evidences supporting this mechanism are mostly based molecular docking simulations that predict the binding of these compounds to the active site of HIV-1 protease due to the size and conformational complementarity (58, 59, 64-66, 75). However, this model lacks support from empirical evidences. In addition, *in vitro* assays indicate that some fullerene derivatives possess anti-reverse transcriptase activity (63).

Therefore, to better understand the mechanism of action of fullerene derivatives in HIV replication, we investigated the effect of these compounds on the different steps of the HIV-1 life cycle in human CD4<sup>+</sup> T-cells.

## **2.2 Materials and Methods**

**2.2.1 Synthesis of compounds 1 and 2.** Compounds 1, 2, and 3 have been previously reported (61). Slight modifications for the synthesis of compounds 1, 2, 3 and the synthesis of compound 4 were performed by Edison Castro from the Department of Chemistry at the University of Texas at El Paso. Solvents and reagents were obtained from commercial sources (Rieke Metals Inc., Nano-C Inc., Sigma-Aldrich and Fisher Scientific) and were used as received. A mixture of C<sub>60</sub> (72.00 mg, 0.10 mmol), *N*-methyl glycine (35.40 mg, 0.40 mmol), and paraformaldehyde (18.00 mg, 0.60 mmol) was refluxed in toluene (100 mL) for 2 h. The solution was evaporated under reduced

pressure, and the mixture of bis-adducts was isolated by column chromatography on silica gel using toluene:ethyl acetate (10:1) as eluent. The first fraction after the pristine fullerene was eluted corresponded to the monoadduct and the next 5 fractions were collected in one flask and corresponded to the regioisomeric mixture of bis-adducts. Bis-adduct *trans*-3 isomer was purified from the regioisomeric mixture of bis-adducts by column chromatography on silica gel using toluene:ethyl acetate (20:1.5) as eluent. The third fraction corresponded to the *trans*-3 isomer, which was confirmed by  $^1\text{H-NMR}$ , matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and UV-Vis. The *trans*-3 isomer or the regioisomeric mixture of bis-adducts (10.00 mg, 0.01 mmol) were dissolved in methyl iodide (10 mL) and stirred at room temperature for 48 h to give a brown precipitate. The precipitate was collected by filtration, and then washed with  $\text{CS}_2$ ,  $\text{CHCl}_3$ , toluene, MeOH and acetone in this order; to afford a brown powder corresponding to compounds 1 or 2.

**2.2.2 Synthesis of compound 3.** Diethyl iminodiacetate (28.35 mg, 0.150 mmol) and glyoxylic acid ethyl ester (0.03 mL,  $\sim 0.150$  mmol) were added to a solution of  $\text{C}_{60}$  (72.00 mg, 0.100 mmol) in toluene (100 mL) (64). The mixture was refluxed for 30 min, and then the solvent was evaporated under reduced pressure. The tricarboxylic ethyl ester  $\text{C}_{60}$  derivative was purified by column chromatography on silica gel using toluene:ethyl acetate (15:1) as the eluent. The first fraction after pristine fullerene was eluted corresponded to the tricarboxylic ester  $\text{C}_{60}$  derivative. The tricarboxylic ethyl ester  $\text{C}_{60}$  derivative (15.00 mg, 0.015 mmol) was dissolved in toluene (20 mL), and NaH (120.00 mg,  $\sim 0.120$  mmol) 50-60% in oil was slowly added and the reaction was stirred at room temperature for 1 h, then ethanol (3 mL) was added followed by the addition of HCl (3M,

2 mL), then the reaction was stirred at room temperature overnight (64). The solvent was evaporated under reduced pressure, the precipitate was washed with chloroform, methanol and cold water, and finally compound 3 was recrystallized from methanol/acetone. The structures of the compounds were confirmed by <sup>1</sup>H-NMR and MALDI-TOF.

**2.2.3 Synthesis of compound 4.** The *cis*-2 isomer (15.00 mg, 0.016 mmol) was dissolved in *ortho*-dichlorobenzene (*o*-DCB, 4 mL), and methyl iodide (4 mL) and stirred at 40°C for 2 weeks to give a brown precipitate. The precipitate was collected by filtration and washed with CS<sub>2</sub>, CHCl<sub>3</sub> and MeOH in this order; to afford a brown powder corresponding to compound 4.

**2.2.4 Plasmids.** The plasmids used to generate retroviral vectors were described previously (20). HIV-1-derived vectors were produced using pHIV Luc and pMD.G. pHIV Luc was derived from pNL4-3.Luc.R<sup>-</sup>E<sup>-</sup> (70) by introducing a deletion in the env open reading frame. pHIV Luc containing multi-protease inhibitors resistant to protease mutants were constructed by swapping a 4.3 Kb Sal I/Spe I fragment in pHIV Luc with this fragment from pNL4-3 containing the mutant proteases. pMD.G encodes the Vesicular Stomatitis Virus glycoprotein G (VSV-G). HIV-1NL4-3 was produced from the corresponding expression plasmid whereas multi-protease inhibitor resistant viruses were produced with plasmids obtained from the NIH AIDS Reagent Program (77).

**2.2.5 Cell lines.** SupT1 and HEK293T cells were grown in RPMI 1640 and in DMEM, respectively. All culture media were supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

**2.2.6 Generation of retroviruses.** Procedures previously described were followed (71).  $3 \times 10^6$  HEK293T cells were plated in a T75 cm<sup>2</sup> tissue culture flask and co-transfected the next day with the corresponding plasmids by the calcium-phosphate precipitation method. 18 h later the transfection medium was replaced with fresh medium containing no drug, fullerene derivatives, Indinavir, or dimethyl sulfoxide (DMSO, vehicle control). The cells were cultured for 48 h until the viral supernatant was harvested and filtered. Single-round infection viral vectors were further concentrated by ultracentrifugation at 124,750 g for 2 h on a 20% sucrose cushion. Viral preparations were stored at -80°C until used. VSV-G-pseudotyped HIV-derived reporter virus expressing firefly luciferase (HIVluc) and harboring wild type or multi-protease inhibitor resistant protease mutants were prepared by co-transfection of 15 µg of the corresponding pHIV luc and 5 µg of pMD.G. HIV-1 wild type viruses were produced by transfection of 15 µg of the corresponding expression plasmids.

**2.2.7 Single-round infectivity assay.** SupT1 cells were plated at  $1 \times 10^5$  cells in 500 µL of RPMI 1640 culture medium in 24-well plates and infected with HIVluc. 4 days post-infection, cells were collected by centrifugation at 1000 g for 6 min and the pellet resuspended in 200 µL of phosphate-buffered saline (PBS). Half of the sample was mixed with 100 µL of luciferase substrate (Bright-Glow™ Luciferase Assay System, Promega) and the other half with 100 µL of cell viability substrate (CellTiter-Glo® Assay, Promega). Cell lysates were incubated for 10 min at room temperature in the dark and then luminescence was measured in triplicate in 50 µL-samples using a microplate luminometer reader (Thermo Scientific, Luminoskan Ascent).

**2.2.8 HIV-1 replication assays.** SupT1 cells ( $0.25 \times 10^6$  cells in 3 mL RPMI 1640) were infected with HIV-1NL4-3 (2.1 ng of HIV-1 p24) wild type or multi-protease inhibitor resistant viruses in the presence of fullerene derivatives or DMSO. 24 h after infection, the cells were washed 3 times by centrifugation in 10 mL (total 30 mL) of culture medium to remove the input virus and compounds. Cell supernatant was then collected at different days post-infection and used for HIV-1 p24 quantification by ELISA.

**2.2.9 HIV-1 p24 ELISA.** HIV-1 p24 levels were determined by a sandwich ELISA following manufacturer instructions. Briefly, 200  $\mu$ L of the viral samples were diluted appropriately and incubated on the ELISA wells overnight at 37°C. Unbound proteins were removed by washing the wells 6 times with 200  $\mu$ L of washing buffer, and bound HIV-1 p24 was detected by incubating each well with 100  $\mu$ L of the anti-HIV-1 p24 secondary antibody for 1 h. Unbound antibodies were removed by washing as described above and bound antibodies were detected by incubating each well with 100  $\mu$ L of substrate buffer for 30 min at room temperature until the reaction was stopped by adding 100  $\mu$ L of stop solution into each well. The absorbance of each well was determined at 450 nm using a microplate reader (Molecular Devices, Versa max microplate reader).

**2.2.10 Cellular viability assay.**  $1 \times 10^4$  SupT1 cells were plated in a 96-well plate in 100  $\mu$ L RPMI1640 culture media and left untreated or treated with fullerene derivatives, DMSO (control), or 2 mM hydrogen peroxide (positive control). Fullerene derivatives were evaluated at a concentration that ranged from 3  $\mu$ M to 32  $\mu$ M. The cells were cultured in the presence of the indicated compounds for 24 h and then 20  $\mu$ L of the MTS were added to each well of cells. Incubation with the reagent for an additional 3 h was



allowed. The colored formazan product was measured by absorbance at 490 nm with a reference wavelength of 650 nm using a microplate reader (SpectraMax 190, Molecular Devices). Control wells containing the same volumes of culture medium and MTS reagent were used to subtract background absorbance.

**2.2.11 Exogenous Reverse Transcriptase assay.** Reverse transcriptase levels in HIV-1 reporter viruses produced in the presence of DMSO, fullerene derivatives 1 and 2, and Indinavir were measured using the EnzChek® Reverse Transcriptase Assay following the manufacturer instructions. HIV-1 p24-normalized amounts (4.34 µg) were analyzed for each compound treated virus.

**2.2.12 HIV-1 protease in vitro activity assay.** The effect of DMSO, compounds 1 and 2 (3 and 10 µM), and Indinavir (0.1 µM) on HIV-1 protease activity was measured using the ProAssay™ HIV-1 Protease Assay Kit. This assay uses purified recombinant HIV-1 protease and a fluorescence resonance energy transfer (FRET) peptide derived from the native p17/p24 cleavage site of HIV-1 protease on Gag. Briefly, HIV-1 protease (0.2 µL) and FRET peptide (final concentration 0.5 µM) were mixed in HIV-1 protease buffer supplemented with 1 mM DTT (final concentration) on ice and protected from light, and immediately transferred into a black 96-wells plate that contain the compounds being evaluated. The reaction was measured by determining the Relative Fluorescing Intensity (RFI) with a fluorometer at excitation/emission wavelengths of 490 nm / 530 nm every 5 min during 90 min.

**2.2.13 Quantification of virion associated ribonucleic acid (RNA).** Real-time quantitative reverse transcription-PCR was used to quantify virion associated RNA. RNA was purified from HIV-1 p24-normalized compound-treated virions (5 ng) after their

treatment with 1 µg of RNase and 2 units of DNase (Qiagen® RNAEasy™ Miniprep kit). cDNAs were generated from all the viral RNA extracted with random oligonucleotides using the High Capacity RNA-to-cDNA Kit from Applied Biosystems®. cDNA (1 µg) was then analyzed using qPCR using Gag-hybridizing primers (iQTM SYBR® Green Supermix BIO-RAD®).

**2.2.14 Immunoblotting.** Proteins of HIV-1 p24-normalized amounts of virions (0.38 µg) were resolved by 13% SDS-PAGE and transferred overnight to PVDF membranes at 100 mA at 4°C. Membranes were blocked in TBS containing 10% milk for 1 h and then incubated in the corresponding primary antibody diluted in TBS containing 5% milk and 0.05% Tween 20 (antibody dilution buffer) overnight at 4°C. HIV-1 p24 was detected with anti-p24 obtained from the NIH AIDS Reagent Program (Catalog # 1513). HIV-1 integrase was detected with anti-integrase (Catalog # sc-69721, Santa Cruz Biotechnology). Primary antibody-bound membranes were washed in TBS containing 0.1% Tween 20 and all bound antibodies were detected with goat anti-mouse IgG-HRP (1/2000, KPL, 074-1806) followed by chemo-luminescence detection.

**2.2.15 Real time PCR analysis of early steps of the HIV-1 life cycle.** Procedures previously described were followed (77). Briefly,  $1 \times 10^6$  SupT1 cells were challenged with DNase-treated single-round infection HIVluc virus produced in the presence of fullerenes or DMSO and 24 h later 90% of the cells were used for DNA extraction (High pure PCR template preparation kit, Roche) whereas 10% were cultured for four days to evaluate infectivity. Extracted DNA (20 ng) was used for the detection of total HIV-1 cDNA, mitochondrial DNA, and 2LTR circles while 0.2 ng of DNA were used for the Alu-LTR junctions PCR. Total HIV-1 cDNA, Alu-LTR junctions, and 2LTR circle products

were normalized to mitochondrial DNA. Real time PCRs were performed in a MiniOpticon system (Bio-rad) with primers and conditions previously described (77). Fold change was calculated using the  $\Delta C_t$  method as recommended in the thermocycler manual.

## **2.3 Results**

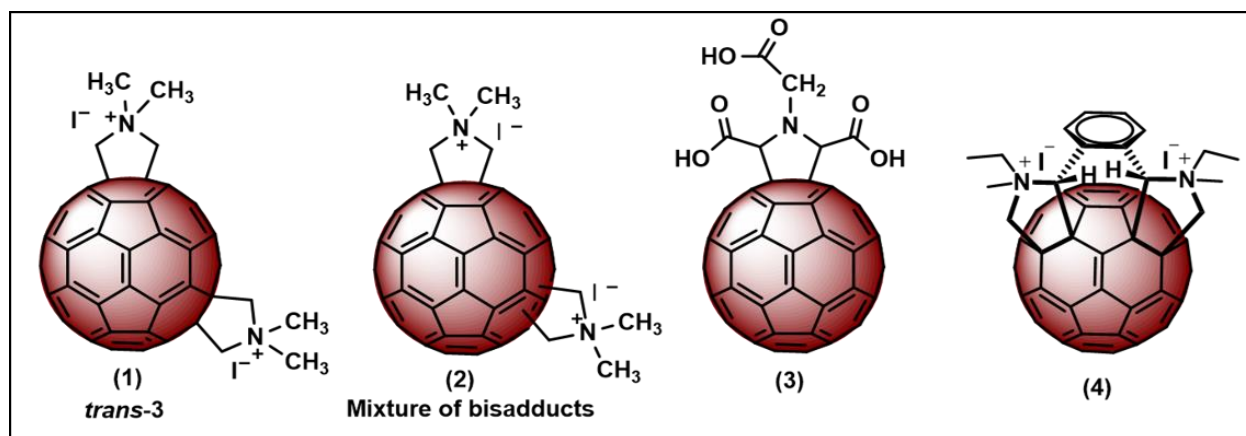
### **2.3.1 Effect of fullerene derivatives on HIV-1 replication in human CD4<sup>+</sup> T-cells.**

Compound 1 (Figure 5) was previously shown to inhibit HIV-1 replication in CEM cells and primary lymphocytes, but its effect on the different steps of the viral life cycle was not evaluated (61). In addition, compounds 1, 2, and 3 (Figure 5) have been shown to inhibit reverse transcriptase in vitro (63). Therefore, to fully characterize the anti-HIV-1 activity of fullerene derivatives in biologically relevant environments, compounds 1, 2, and 3 were evaluated for their ability to affect HIV-1 replication in human CD4<sup>+</sup> T-cells (Figure 6). SupT1 cells were infected with HIV-1NL4-3 in the presence of compounds 1, 2, and 3 at 1, 3, and 10  $\mu$ M concentrations, and compounds and input viruses were removed 24 h later. Infected cells were cultured for approximately 2 weeks and HIV-1 p24 levels in the cell supernatant were measured by ELISA.

Data in figure 6 indicate that compounds 1 and 2 potently inhibited viral replication at 3  $\mu$ M. Similarly, compound 1 was active at 10  $\mu$ M (Figure 6a). The effect of compound 1 on HIV-1 replication at 3 and 10  $\mu$ M were indistinguishable and showed a 300-fold inhibition of HIV-1 viral replication. The regioisomeric compound mixture 2 at 3  $\mu$ M inhibited HIV-1 replication to a similar extent as compound 1 (Figure 6b). However, the inhibitory activity of compounds 1 and 2 dropped at 1  $\mu$ M to 3-fold and 5-

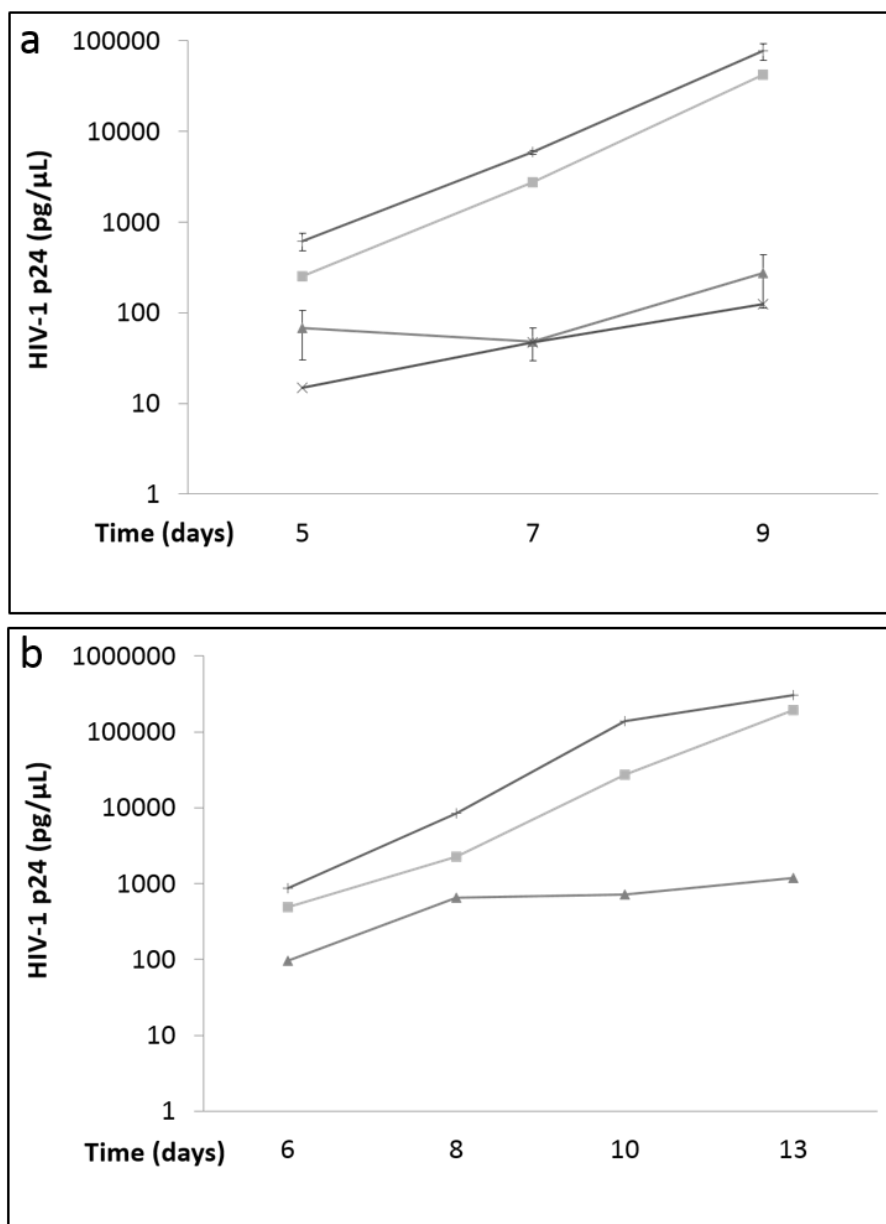
fold, respectively (Figure 6). Contrary to the effect of compounds 1 and 2 on HIV-1 replication, no effect was observed when cells were treated for 24 h at the time of infection with compound 3 at 1, 3 and 10  $\mu$ M (data not shown). Therefore, our data support the anti-HIV-1 activity reported for compounds 1 and 2 (61) but failed to validate in human cells the effect reported for compound 3 using in vitro assays (63).

Function/structure analysis of the fullerene derivatives characterized (Figure 5) indicate that similar chemical addends present in the regioisomeric mixture 2 and the pure regioisomer (trans-3) compound 1 most likely explain their comparable inhibitory effect on HIV-1 replication. However, when the quaternized nitrogen in 1 and 2 was eliminated and the pyrrolidine ring was modified with carboxylic acids, compound 3, the anti-HIV activity was completely lost, indicating the relevance of the addends in the activity of the fullerene derivatives.



**Figure 5.** Fullerene derivatives 1-4.

Fullerene derivatives 1 and 2 (C<sub>60</sub>-bis(N,N-dimethylpyrrolidinium iodide), 3 (fullereno-C<sub>60</sub>-pyrrole-2,5-dicarboxylic acid-1-(carboxymethyl)-1,5-dihydro and 4 (cis-2-C<sub>60</sub>-bis(N,N-ethylmethylpyrrolidinium iodide)).

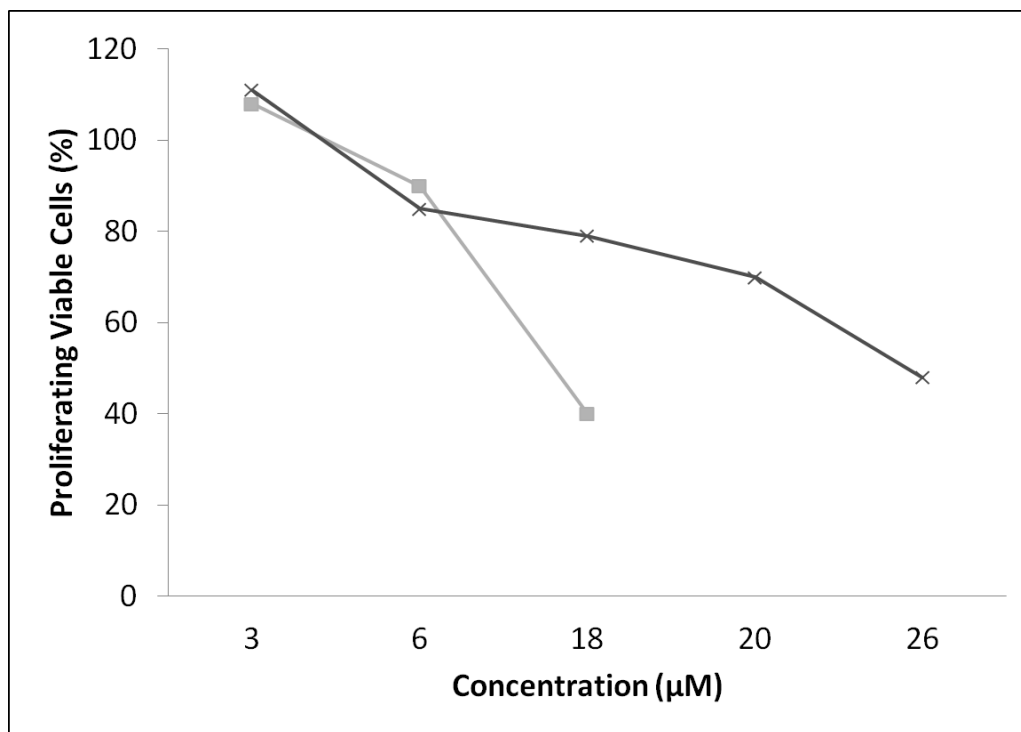


**Figure 6.** *Effect of fullerene derivatives on HIV-1 replication.*

SupT1 cells were treated with DMSO (+) or compounds 1 a) and the regioisomeric mixture 2 b) at 1 μM (□), 3 μM (Δ), or 10 μM (x) at the time of infection with HIV-1 NL4-3. 24 h later the compounds and virus were removed and infected cells were cultured for up to 2 weeks. The amount of HIV-1 p24 antigen was determined in cell supernatant by ELISA. Results from one experiment are shown, standard deviations are from three independent experiments.

**2.3.2 Effect of fullerenes on cellular viability.** We have observed that compounds 1 and 2 potently inhibit HIV-1 infection (Figure 6). A possibility is that these compounds compromise cellular viability affecting viral infection nonspecifically. To rule out this possibility, the effect of compounds 1 and 2 on cell viability was evaluated by the tetrazolium dye reduction assay. In these experiments, SupT1 cells were treated with compounds 1 and 2 at concentrations of 3 and 6  $\mu$ M, or similar amounts of DMSO, for 24 h and then cell viability was measured. At these concentrations none of these compounds showed cytotoxic activity (Figure 7). Therefore, the effects on HIV-1 infection observed with compounds 1 and 2 at 3  $\mu$ M most likely are not due to cell toxicity.

To further define the cytotoxic activity of these compounds the drug concentration that kills approximately 50% of SupT1 cells (LC50) was determined for compounds 1 and 2 after 24 h of treatment. In these experiments, compound 1 at 18  $\mu$ M and the regioisomeric mixture 2 at 26  $\mu$ M killed 60% and 52% of the treated cells, respectively, as compared to DMSO-treated cells (Figure 7). These results also indicate that the doses evaluated in the HIV-1 infection experiments unlikely impaired HIV-1 replication by affecting cellular viability. In summary, experiments in figure 7 indicate that compounds 1 and 2 inhibit HIV-1 infection at doses that are not toxic to the target cells.



**Figure 7.** *Assessing the cytotoxicity of compounds 1 and 2.*

SupT1 cells were treated with DMSO, compound 1 (□), or regioisomeric mixture 2 (x) at varying concentrations for 24 h and the amounts of viable cells were determined by the tetrazolium dye reduction assay. Cell viability values were normalized to DMSO-treated cells. Results shown are of one experiment.

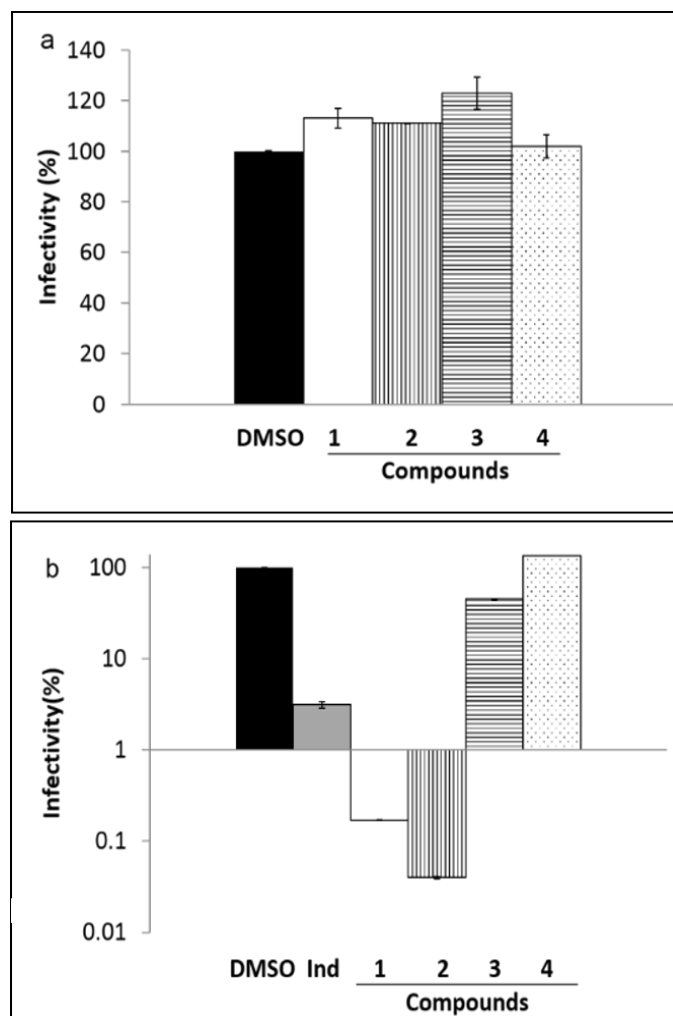


**2.3.3 Effect of fullerene derivatives on single-round infection.** We have provided evidence that fullerene derivatives, specifically compounds 1 and 2, inhibit HIV-1 replication; however the viral step implicated in this effect is unknown. In order to determine the step in the viral life cycle that is affected, the early phase of HIV-1 infection was first analyzed. The effect of compounds 1, 2 and 3 on the infection of VSV-G pseudotyped HIV-1 single-round infection viruses expressing luciferase was evaluated in SupT1 cells. Although compound 3 showed no signs of inhibition of HIV-1 replication, it was evaluated as a control. In addition, we analyzed the activity of compound 4 (Figure 5), an isomerically pure regioisomer (cis-2, endo-endo) with two pyrrolidinium rings connected by a benzene bridge (78). This compound was incorporated in the analysis to provide additional information about the involvement of different addends attached to fullerene and their anti-HIV-1 activity. Cells were exposed to 1, 3, and 10  $\mu$ M of each compound and infected with the reporter virus. 24 h later, drugs and input virus were removed and four days post-infection luciferase and ATP levels were measured. Luciferase was normalized to ATP to standardize for cell viability and number. Data in figure 8a clearly indicate that none of the fullerene derivatives affect the early steps of the viral life cycle at the highest concentration of compound (10  $\mu$ M). These results also demonstrated that LTR-driven transcription and translation of viral proteins, or cellular viability, was not affected by fullerene derivatives. Combined analysis of data in figures 6 and 8a suggest that the late phase of the viral life cycle is targeted by compounds 1 and 2.

#### **2.3.4 Effect of fullerene derivatives on the late phase of the HIV-1 life cycle.**

Compounds 1, 2, 3 and 4 were further evaluated to determine their effect on the late phase of the HIV-1 life cycle. VSV-G pseudotyped, HIV-1 single-round infection viruses expressing luciferase were produced in the presence of DMSO, compounds 1, 2, 3, and 4 (3  $\mu$ M), or Indinavir (0.1  $\mu$ M) in HEK293T cells cotransfected with plasmids expressing these retroviruses and plasmids encoding eGFP. Then, produced viruses were concentrated by ultracentrifugation on a sucrose cushion and HIV-1 p24 levels were measured by ELISA.

In order to analyze the effect of fullerene derivatives on HIV-1 infectivity, SupT1 cells were infected with p24-normalized single round infection viruses and luciferase and ATP levels were determined four days later. Data in figure 8b indicate that infectivity of viruses produced in the presence of compounds 1 and 2 was dramatically reduced by more than 99%. This inhibitory effect was similar to the impairment caused by Indinavir (95%). In contrast, compounds 3 and 4 did not affect the infectivity of the viruses produced, again highlighting the functional relevance of the addends modifying the fullerene cage.

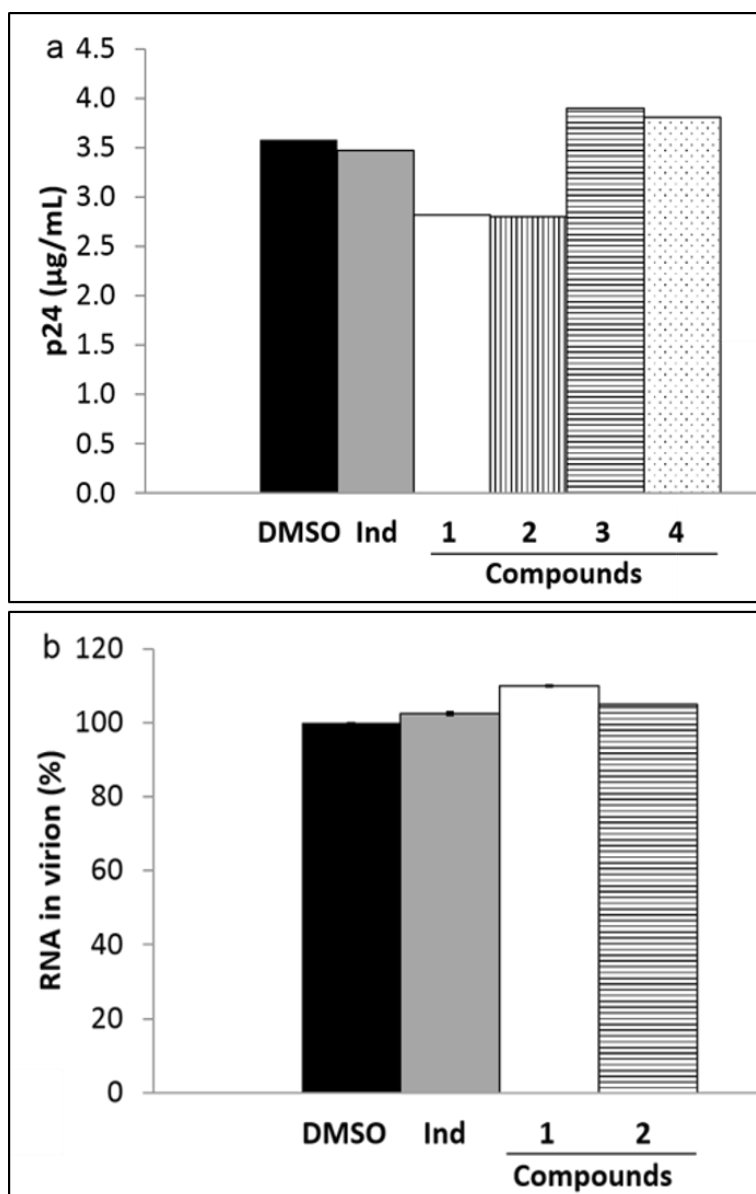


**Figure 8.** Analysis of the viral life cycle step affected by compounds 1, 2, 3 and 4.

a) Effects on the early stages of the HIV-1 viral life cycle. SupT1 cells were infected with single-round infection HIV-1 viruses in the presence of DMSO or fullerene derivatives (10  $\mu$ M), and analyzed for luciferase expression and cellular viability (ATP content) three days later. Luciferase was normalized to cellular viability. b) Effects on the late phase of HIV-1 infection. Single-round infection HIV-1 virus were produced in the presence of DMSO, Indinavir (0.1  $\mu$ M), or fullerene derivatives (3  $\mu$ M) and their infectivity analyzed in single-round infection assays using HIV-1 p24-normalized viruses. Results shown are the average and standard deviation of triplicate readings of one experiment representative of three independent experiments.

HIV-1 p24 levels were similar among viruses produced in the presence or absence of fullerene derivatives or Indinavir, indicating that these compounds did not affect LTR transcription, translation, and viral budding, nor cellular viability (Figure 9a). These observations also correlate with the lack of an effect for compounds 1, 2, 3 and 4 on the expression of luciferase in the single-round infection assays (Figure 8a). The similar levels of HIV-1 p24 also correlated with equivalent eGFP expression in the producer cells (Data not shown), indicating comparable transfection efficiency.

To further investigate the effect of fullerene derivatives on viral assembly, we determined whether compounds 1 and 2 as well as Indinavir affected the amount of virion-associated viral RNA. HIV-1 RNA was extracted from p24-normalized amounts of compound-treated virions and then converted into cDNA that was quantified by real time PCR with primers hybridizing to Gag. Using this method, similar RNA levels were found in virions produced in the presence of DMSO, compounds 1, 2, and Indinavir, indicating that these compounds do not affect RNA packaging (Figure 9b). Altogether, the data shown in figures 4b and 5a-b show that fullerene derivatives target the viral maturation process.



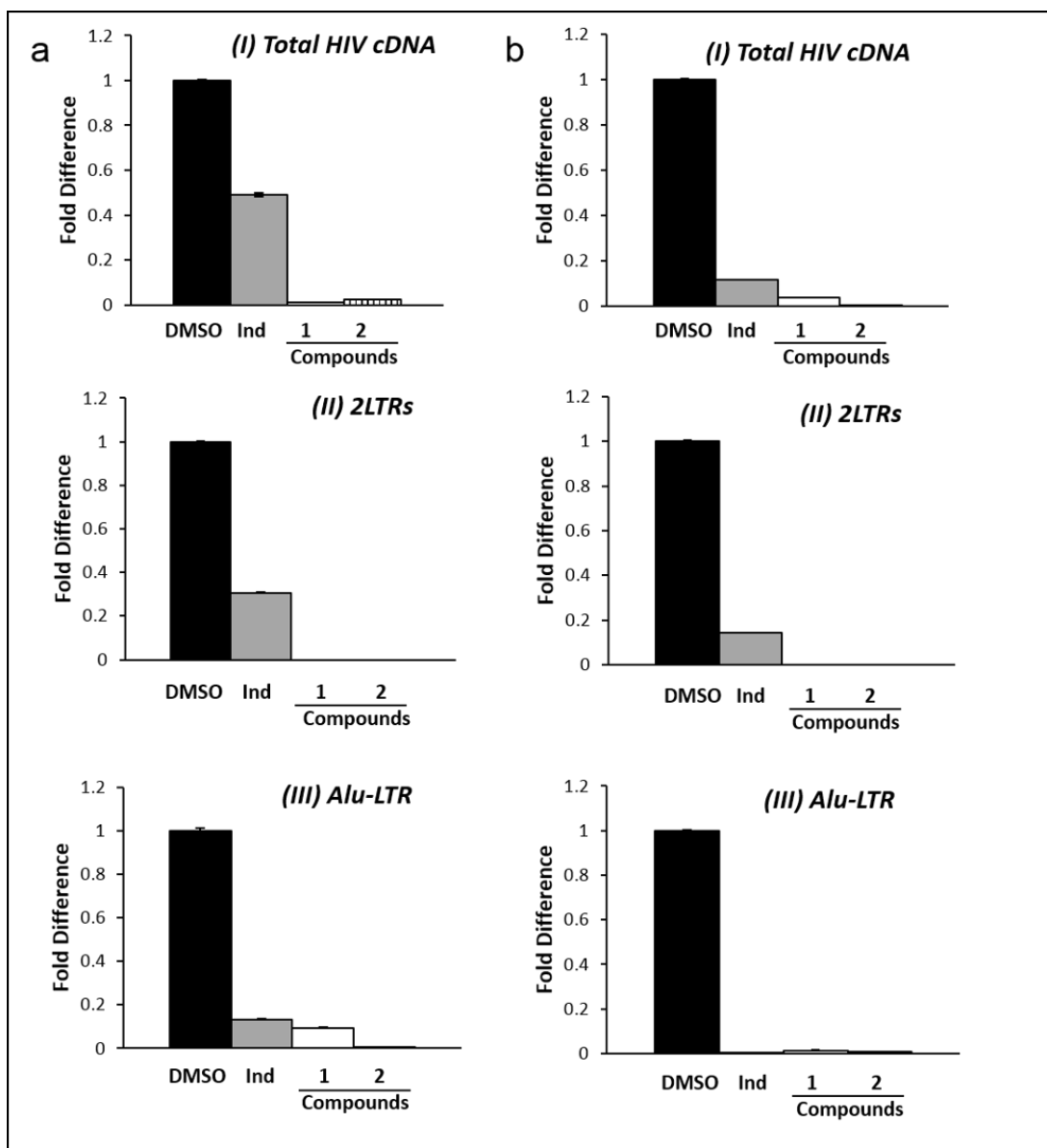
**Figure 9.** Effects of compounds 1, 2, 3 and 4 on virion production.

VSV-G pseudotyped, single-round HIV-1 expressing luciferase were produced in the presence of DMSO or fullerene derivatives (3 µM) and then concentrated by ultracentrifugation and analyzed. Indinavir (0.1 µM) was used as a control. a) Virion production as determined by HIV-1 p24 levels quantified by ELISA. b) Virion-associated RNA as quantified by real time PCR analysis of reverse transcribed cDNA using primers that hybridize to Gag. Results shown in (a) are of one experiment. Results in (b) are representative of two experiments, standard deviations indicate the variability of multiple readings.

### **2.3.5 Characterization of the early steps of the viral life cycle of virions produced in fullerene derivative-treated cells.**

We have shown that compounds 1 and 2 allude to impairing the maturation step of HIV-1, drastically reducing virion infectivity. Using real time PCR analysis we determined the competence of fullerene derivative-treated viruses to complete the different steps of the early phase of the HIV-1 viral life cycle. SupT1 cells were infected with DNase-treated HIV-1 p24-normalized amounts of concentrated HIV-1 produced in the presence of DMSO, Indinavir, or compounds 1 and 2. DNA was extracted from these cells 24 h and 4 days after infection and total HIV-1 cDNA that is formed only after efficient reverse transcription, 2LTR circles that is synthesized upon nuclear import of the linear HIV-1 cDNA, and Alu-LTR junctions, products indicative of HIV-1 DNA integration, were quantified by real-time PCR.

In these analyses, we found that total HIV-1 cDNA was dramatically reduced in viruses produced in the presence of compounds 1 and 2, and this effect was of a higher magnitude than in virions produced in Indinavir-treated cells (Figure 10a I). As a consequence, 2LTR circles and Alu-LTR junctions were also markedly diminished in cells infected with fullerene-treated than DMSO-treated viruses (Figure 10a II and III, respectively). As expected, this inhibitory effect of fullerene derivatives was also observed in DNA extracted four days after infection (Figure 10b). Importantly, findings in figure 10 correlated with the lack of infectivity reported in figure 8b, indicating a severe defect in the infectivity of viruses produced in the presence of compounds 1 and 2.



**Figure 10.** Evaluation of the early steps of the HIV-1 life cycle of virions produced in cells treated with compounds 1 or 2.

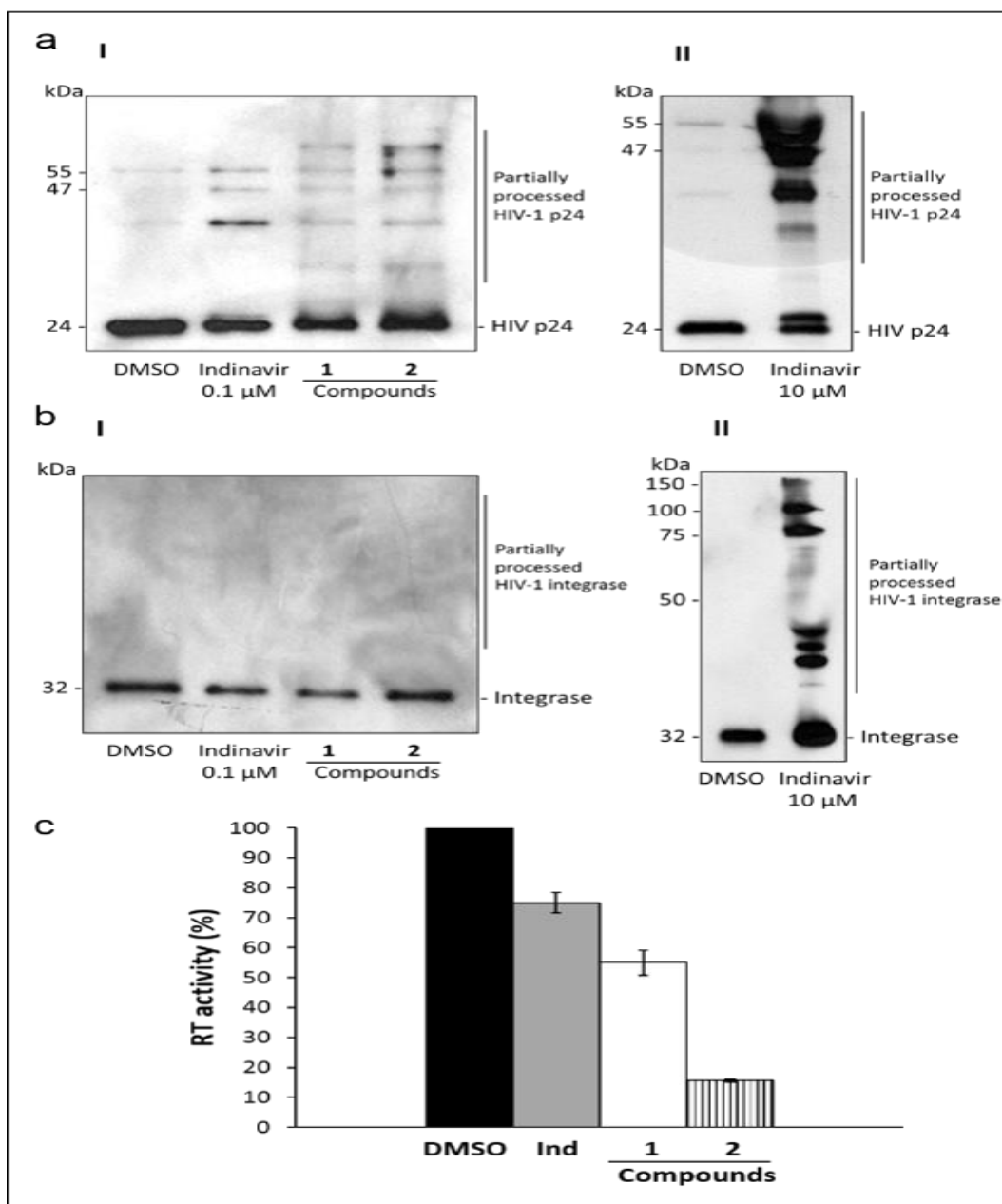
SupT1 cells were infected with HIV-1 p24-normalized, DNase-treated, single-round HIV-1 viruses produced in the presence of DMSO, Indinavir (0.1  $\mu$ M), or fullerene derivatives (3  $\mu$ M). DNA was extracted from infected cells 24 h (a) and 4 days post-infection (b) and used to detect total HIV cDNA (I), 2LTR junctions (II), and proviruses (III). Results shown are the average of triplicate readings of one experiment.

**2.3.6 HIV-1 Gag processing in fullerene-treated virions.** The fact that fullerene severely blocks infectivity of viruses produced in their presence (Figure 8b and 10) but not mature viruses (Figure 8a) suggests that these compounds affect virion maturation. This step of the viral life cycle is initiated by the required proteolytic processing of Gag and Gag-Pol polyproteins by HIV-1 protease and the further assembly of the viral components. To formally evaluate the effect of fullerenes on maturation we determined Gag-Pol processing in virions treated or not with fullerene derivatives. HEK293T cells were transfected with pHIV luc and pMD.G and the next day the transfection medium was replaced with culture medium containing DMSO, compounds 1 and 2 (3  $\mu$ M), or Indinavir (0.1  $\mu$ M or 10  $\mu$ M). Produced viruses were concentrated by ultracentrifugation on a sucrose cushion and used for quantification of HIV-1 p24 by ELISA. Then, HIV-1 p24-normalized amounts of viruses were used for Gag and Pol processing analysis. As shown in figure 11a I, Gag processing was affected by compounds 1 or 2 at 3  $\mu$ M to a similar extent of Indinavir at 0.1  $\mu$ M. However, integrase processing was not altered by fullerene derivatives at 3  $\mu$ M or Indinavir at 0.1  $\mu$ M (Figure 11b I) although at these doses these drugs significantly affected HIV-1 infection (Figure 6 and 8b). Nevertheless, Indinavir at higher doses (10  $\mu$ M) severely impair Gag and integrase processing (Figure 11a II and b II). Therefore, data in figures 11a and b indicate that fullerene derivatives impair Gag processing.

To further evaluate the effects of fullerene derivatives (3  $\mu$ M) and Indinavir (0.1  $\mu$ M) on HIV-1 maturation, we also determined the virion-associated reverse transcriptase activity of p24-normalized viruses using an exogenous ssDNA template (exogenous reverse transcription assay). Although Indinavir impaired reverse



transcriptase activity, the effect of fullerenes, and in particular of the regioisomeric mixture 2, were more pronounced (Figure 11c), indicating a defect in HIV-1 maturation in these viruses. The fact that the inhibitory effect on reverse transcriptase activity of the regioisomeric mixture 2 was more potent than compound 1 (Figure 11c) highlights the relevance of the fullerene addend groups in their activity.



**Figure 11.** Effects of compounds 1 and 2 on Gag and Gag-Pol processing.

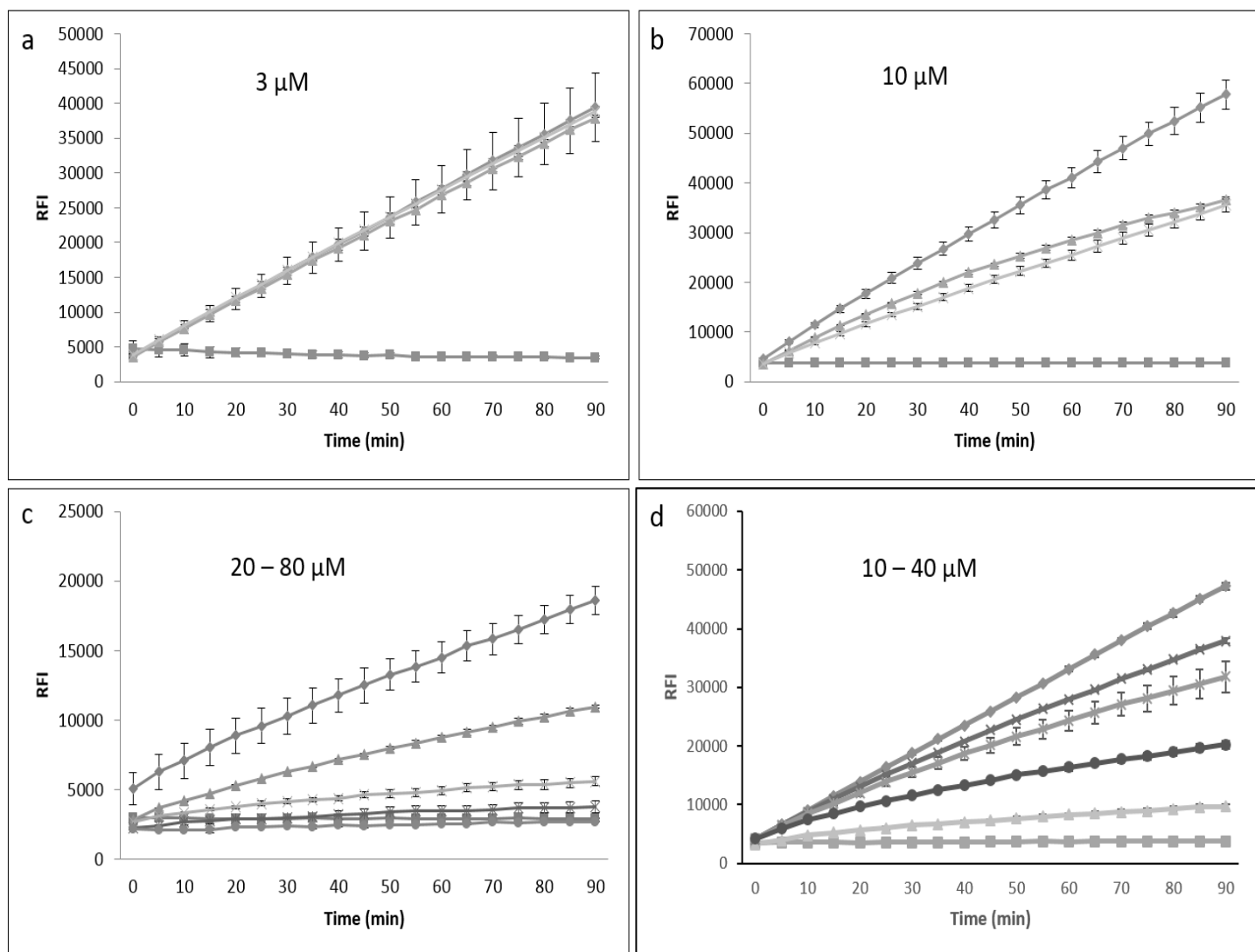
Protease-mediated processing of capsid (p24) (a) and integrase (b) was evaluated in virions by immunoblot. c) Reverse transcriptase activity of virions was measured by the exogenous reverse transcription assay. Results are representative of one (a and b) or three (c) independent experiments. Standard deviations are of multiple readings.

**2.3.7 Effect of fullerene derivatives on HIV-1 protease activity.** In silico analysis suggest that fullerene derivatives 1 and 2 could bind to the active site of HIV-1 protease (58, 59, 64-66, 75). This enzyme is essential for viral maturation, the step of the viral life cycle that we have identified to be affected by fullerenes. However, fullerenes 1 and 2 did not affect integrase processing (Figure 11b) although this event also depends on the HIV-1 protease activity. Therefore, to further characterize the specific mechanism of action of these compounds, we determined their effect on the in vitro activity of HIV-1 protease using a FRET peptide-based assay. Recombinant protease was incubated with the substrate, an HIV-1-derived FRET peptide containing the native p17/p24 cleavage site of HIV-1 protease on Gag, in the presence of DMSO, fullerene derivatives, or Indinavir, and fluorescence emission was tracked for 90 mins (Figure 12).

As expected, Indinavir completely blocked the activity of protease but surprisingly, compounds 1 and 2 were inactive at 3  $\mu$ M (Figure 12a), a concentration that severely impairs HIV-1 infection (Figures 6 and 8b). However, fullerene derivatives 1 and 2 at 10  $\mu$ M inhibit 40% of protease activity (Figure 12b). Only at toxic concentrations (Figure 7), above 40  $\mu$ M, fullerene 1 inhibits protease activity (Figure 12c). In order to determine if this inhibition of protease activity was specific, we also tested fullerene 3, which is inactive against HIV replication, at concentrations ranging from 10 – 40  $\mu$ M, using fullerene 1 at 40  $\mu$ M as a control for comparison (Figure 12d). Fullerene 3 clearly shows a concentration-dependent inhibition of protease activity as did fullerene 1 in figure 12c, with 40  $\mu$ M of this compound inhibiting 50% of activity. While this concentration of fullerene 3 is not as inhibitory of protease activity as fullerene 1, the same concentration-dependent inhibition is seen, suggesting that the

higher concentrations of fullerene 1 (40  $\mu$ M and above) are non-specifically inhibiting protease activity.

Therefore, our data definitively demonstrate that fullerene derivatives fail to inhibit HIV-1 protease at doses that potently block HIV-1 maturation. These results conclusively show that fullerenes 1 and 2 are strongly anti-HIV active, but most likely not via HIV-1 protease, contradicting the multiple *in silico* studies previously reported (58, 59, 64-66, 75). Therefore, our results clearly show that fullerene derivatives are potent anti-HIV agents but their mechanistic activity unlikely involves HIV-1 protease binding.



**Figure 12.** Effect of compounds 1 and 2 on the *in vitro* activity of HIV-1 protease.

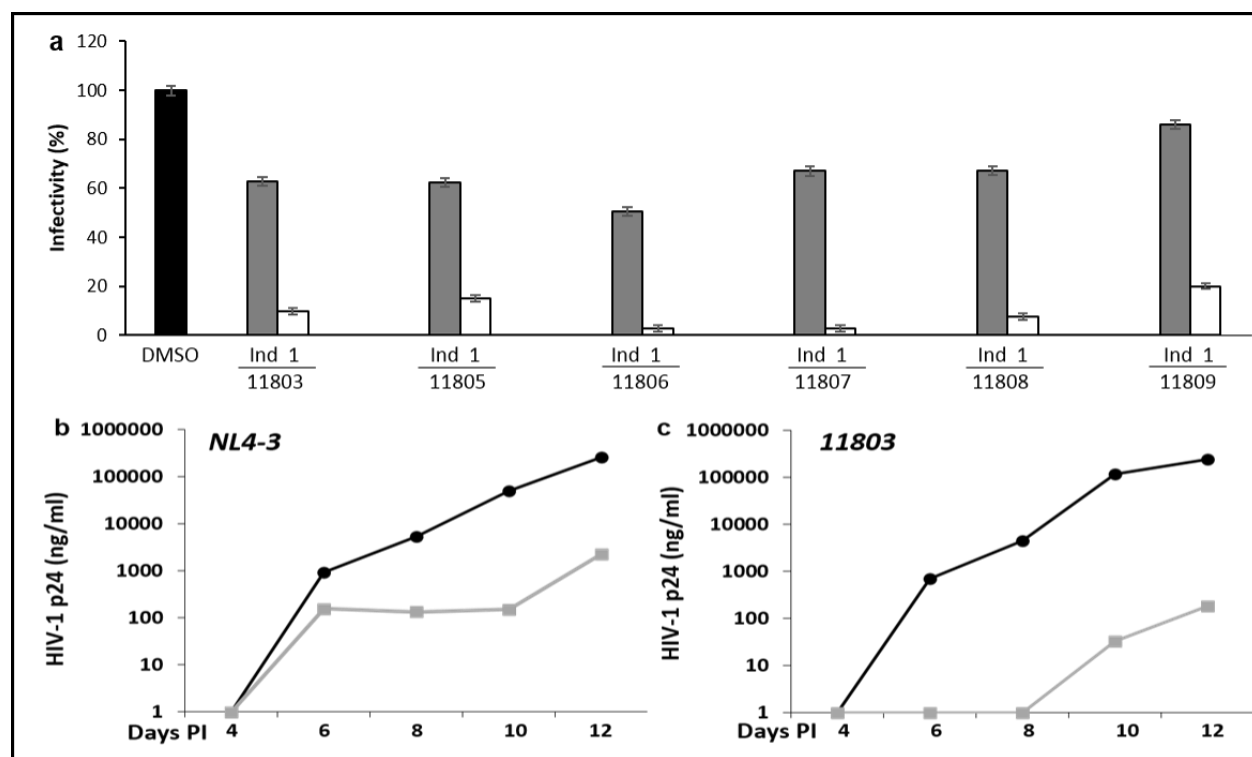
The cleavage of an HIV-derived FRET peptide by recombinant HIV-1 protease in the presence of fullerenes 1 ( $\Delta$ ) and 2 (x) at 3  $\mu$ M a) or 10  $\mu$ M b) was determined by fluorescence measurements. c) Fullerene 1 was used at 20  $\mu$ M ( $\Delta$ ), 40  $\mu$ M (x), 60  $\mu$ M ( $\times$ ), or 80  $\mu$ M (o). d) Fullerene 1 was used at 40  $\mu$ M ( $\Delta$ ), and fullerene 2 was used at 10  $\mu$ M (x), 20  $\mu$ M ( $\times$ ), or 40  $\mu$ M (o). DMSO ( $\diamond$ ) and Indinavir ( $\square$ ) were used as negative and positive controls, respectively. Experiments were performed in duplicates and standard deviations indicate the variability of duplicate readings.

**2.3.8 Inhibitory activity of fullerene derivatives on multi-protease inhibitor resistant HIV-1 viruses.** Our data indicate that fullerene derivatives do not affect HIV-1 protease; thus it is possible that these compounds could inhibit the infectivity of HIV-1 viruses that are resistant to the clinically used protease inhibitors. To test this hypothesis, we determined the sensitivity to fullerene derivatives of multi-protease inhibitor-resistant HIV-1 recombinant infectious molecular clones previously described (77). The mutant viruses analyzed included 11803, 11806, 11807, 11808, and 11809 that are resistant to nelfinavir, Fosamprenavir, Saquinavir, Indinavir, Atazanavir, Lopinavir, Tipranavir, and Darunavir; and 11805 that is also resistant to these drugs except for Tipranavir and Darunavir. The protease (99 amino acids) in these viruses contains between 10 and 24 point mutations (77).

Single-round infection HIV-1 expressing luciferase that harbor the multi-protease inhibitor-resistant protease mutants were produced in HEK293T cells as described above in the presence of DMSO, compound 1 (3  $\mu$ M), or Indinavir (0.1  $\mu$ M). The viruses were concentrated by ultracentrifugation on a sucrose cushion, normalized for p24 content, and used to infect SupT1 cells. In these experiments we observed that compound 1, but not Indinavir, potently blocked the infectivity of all the mutant viruses with a similar efficiency (Figure 13a).

To further verify these data, the effect of fullerenes on the replication of HIV-1 11803 was evaluated. This mutant was selected for analysis because, although it contains 18 point mutations in protease that renders the virus resistant to eight different protease inhibitors, it has a replication capacity of 63% of protease wild type HIV-1 (77). SupT1 cells were infected with NL4-3 or 11803 in the presence of DMSO or compound

1 (3  $\mu$ M) and 24 h later the cells were washed to remove the input drugs and viruses, and viral replication was evaluated by quantification of HIV-1 p24 in the cell supernatant at different times post-infection. Viruses were used at p24 levels that result in robust viral replication one week after infection to compensate for differences in viral fitness. Data in figure 13c confirmed that compound 1 strongly inhibits viral replication of the multi-protease inhibitor-resistant virus 11803 to a similar extent of the protease wild type NL4-3 virus (Figure 13b). Therefore, data in figure 13 highlight the potential clinical relevance of fullerene derivatives to block replication of multi-protease inhibitor resistant viruses.



**Figure 13.** Activity of compound 1 on the infectivity of HIV-1 harboring multi-protease inhibitor-resistant protease mutants.

a) HIV-1 viruses harboring protease mutants were produced in the presence of DMSO, compound 1 (3  $\mu$ M), or Indinavir (0.1  $\mu$ M), and their infectivity evaluated in single-round infection assays. b-c) SupT1 cells were infected with HIV-1 NL4-3 harboring a wild type (b) or a multi-protease inhibitor-resistant protease mutant (virus 11803) (c) in the presence of DMSO (○) or compound 1 (3  $\mu$ M) (□). Results are representative of one experiment, standard deviations in 13a are of triplicate readings.



## 2.4 Discussion

Our data has corroborated the reported anti-HIV-1 activity of compounds 1 and 2 but do not support findings or predictions indicating reverse transcriptase (63) or protease (58, 59, 65, 66, 75, 76) as their targets. Instead, we have demonstrated for the first time that these compounds strongly inhibit viral maturation by a protease-independent mechanism. As a consequence, multi-protease inhibitor resistant viruses are susceptible to the inhibitory effects of fullerene derivatives highlighting the potential clinical relevance of fullerene derivatives to block resistant viruses.

HIV-1 produced in the presence of fullerenes showed a severe defect in infectivity that correlated with a defect in virion-associated Gag processing. Gag is produced as a polyprotein that is cleaved by protease at specific sites to generate several mature proteins including matrix, capsid, nucleocapsid, p6, and two spacer peptides SP1 and SP2. Gag processing occurs in a strictly organized manner and interfering with this highly ordered process results in immature virions (29, 13). As a consequence of their mechanism of action, virions produced in the presence of fullerenes exhibited a defect in virion-associated reverse transcription activity and a blockage in post-entry reverse transcription and viral integration. On the contrary, fullerenes do not affect the infectivity of mature virions, indicating that they target the maturation step. Similar to our findings, the maturation inhibitor 3-O-(3',3'-dimethylsuccinyl)betulinic acid (bevirimat) impairs Gag processing affecting the infectivity of immature, but not mature, viruses (30, 34, 79).

We have also demonstrated that the addends on the C60 fullerenes have pronounced effects on their anti-HIV-1 activity, beyond solubility effects. The difference

in anti-HIV-1 activity between compounds 1, 2, 3 and 4 is completely reliant on the chemical nature of their side chains. Previously it has been reported that different regioisomers of 2 exhibit similar HIV-1 inhibitory activity and it was concluded that the trans-3 compound 1 is more potent than the corresponding cis-3 isomer (60, 61).

In summary, our data indicate that fullerene derivatives affect virion maturation of HIV-1 wild type and multi-protease inhibitor resistant viruses. We also observed that the chemical nature of the addends and their regiochemistry importantly affect the HIV-1 inhibitory activity of these compounds. Furthermore, the action mechanism is likely to not target the HIV-1 protease, a paradigm-shifting finding.

## **CHAPTER 3: DEFINING THE STEP IN THE HIV-1 VIRAL LIFE CYCLE AFFECTED BY C<sub>70</sub> FULLERENE DERIVATIVES**

### **3.1 Introduction**

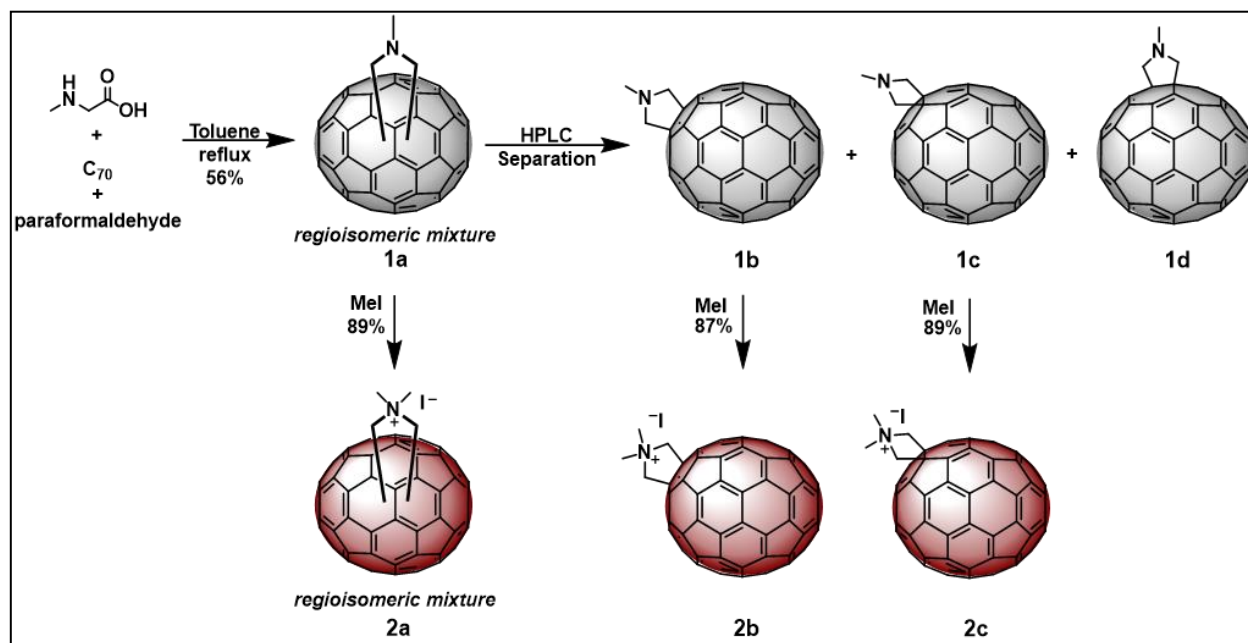
Maturation is a fine tuned mechanism orchestrated by HIV protease that begins following virion release from infected cells (13, 29). Gag and Gag-Pol polyproteins are sequentially cleaved by protease to produce viral enzymes and structural proteins required in the early steps of viral replication (13, 80, 81). Maturation can be impaired by altering the activity of protease, the structure of the substrate, or molecular interactions between the different viral structural proteins. Inhibitors targeting protease are widely used in the clinic (82, 83). Small molecules or peptides that act as maturation inhibitors by binding to the HIV capsid protein have been described (84-86). Among them, the best characterized maturation inhibitor is Bevirimat that inhibits the protease cleavage of capsid-spacer protein 1 (CA-SP1) (28, 30, 31, 84, 86, 87). Despite its potency against HIV-1 strains resistant to clinically approved drugs and its good pharmacological and safety profiles, further clinical development of Bevirimat was stopped due to the emergence of resistance caused by naturally occurring SP1 polymorphisms (28, 35, 88, 89). New Bevirimat-derived compounds have been recently developed and reported to be effective against HIV-1 strains harboring some of the SP1 polymorphs reported to block Bevirimat activity (22, 29). However, no maturation inhibitors are currently used clinically (29).

Since the discovery of fullerene C<sub>60</sub>, efficient synthetic methods for fullerene functionalization have been developed (74, 90). Functionalization with highly polar or

ionic groups is the most commonly used approach to obtain water soluble fullerene derivatives for biomedical applications (91). C<sub>60</sub> and C<sub>70</sub> fullerene derivatives have been shown to affect HIV-1 replication by unknown mechanisms of action (59, 62, 75). Regioselective functionalization of the C<sub>70</sub> cage is challenging partly due to its lower symmetry compared to C<sub>60</sub>. This may be one reason why only a few examples of water soluble C<sub>70</sub> fullerene derivatives have been reported (75, 92). To better understand the mechanism of action of C<sub>70</sub> fullerene derivatives in HIV replication, we investigated the effect of these compounds on the different steps of the HIV-1 life cycle in human CD4<sup>+</sup> T-cells.

### 3.2 Materials and Methods

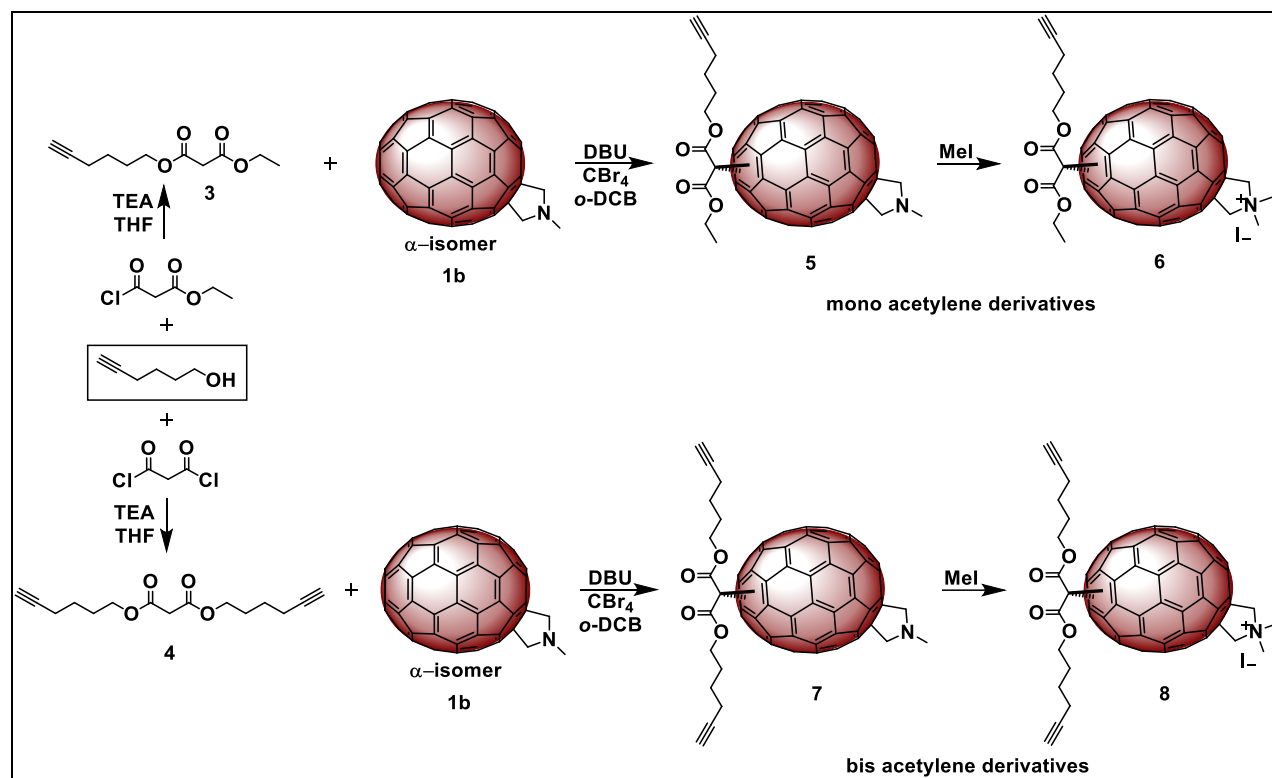
**3.2.1 Syntheses of C<sub>70</sub>-(N,N-dimethylpyrrolidinium iodide) 2a-c.** Synthesis of compounds 2a-c were performed by Edison Castro from the Department of Chemistry at the University of Texas at El Paso. The pure isomers 1b-d and the regioisomeric mixture 1a were each dissolved in methyl iodide (6 mL) and stirred for 48 h at room temperature to give a black precipitate. The precipitate was collected by filtration, and washed with CS<sub>2</sub>, CHCl<sub>3</sub>, toluene, MeOH and acetone, in this order, to give the corresponding C<sub>70</sub>-(N,N-dimethylpyrrolidinium iodide) 2b, c and those of the regioisomeric mixture of 2a (Figure 14). The ratio of the monoadducts in the mixture was 25:64:11 (γ:β:α) as determined by <sup>1</sup>H NMR, and their molecular masses were determined by matrix assisted laser desorption ionization-time of flight (MALDI-TOF).



**Figure 14.** Syntheses of  $C_{70}$ -(*N,N*-dimethylpyrrolidinium iodide) derivatives 2a-c.  $\alpha$  isomers (1b and 2b),  $\beta$  isomers (1c and 2c) and 1d  $\gamma$  isomer.

**3.2.2 Syntheses of C<sub>70</sub> mono and bis acetylene derivatives 5-8.** Synthesis of compounds 5-8 were performed by Edison Castro from the Department of Chemistry at the University of Texas at El Paso. Compounds 3 and 4 were synthesized according to procedures previously reported (93). DBU (0.025 mL, 0.172 mmol) was added to a stirred solution of 1b (30 mg, 0.033 mmol), CBr<sub>4</sub> (0.331 mg, 0.100 mmol), and 3 or 4 (0.066 mmol) in toluene (20 mL) at room temperature. The solution was stirred overnight at this temperature, then it was evaporated under reduced pressure, and the mixture of bis-adducts was isolated by column chromatography on silica gel using toluene:ethyl acetate (10:1) as eluent. The first fraction after the starting material corresponded to the mixture of bis-adducts 5 or 7, respectively. Compounds 5 and 7 were separately dissolved in methyl iodide (6 mL) and stirred for 48 h at room temperature to give a brown precipitate. The precipitate was collected by filtration, and washed with CS<sub>2</sub>, CHCl<sub>3</sub>, toluene and acetone, in this order, to give the corresponding salts 6 and 8 (Figure 15).

**3.2.3 Magnetic beads-immobilized fullerene (MBIF) derivatives.** Immobilized fullerene derivatives were prepared by click chemistry using a freshly prepared copper catalyst solution CuSO<sub>4</sub> (10 mmol/L) and ascorbic acid (20 mmol/L) in H<sub>2</sub>O:THF (1:1) ratio. Then, one equivalent of azide with one equivalent of alkyne or half equivalent of bis alkyne were added and the resulting mixture was shaken for 6 h at room temperature. Finally, the immobilized fullerene derivatives were washed with water and stored in DMSO at 4°C until used. Fullerene-coupled beads were produced by Edison Castro from the Department of Chemistry at the University of Texas at El Paso



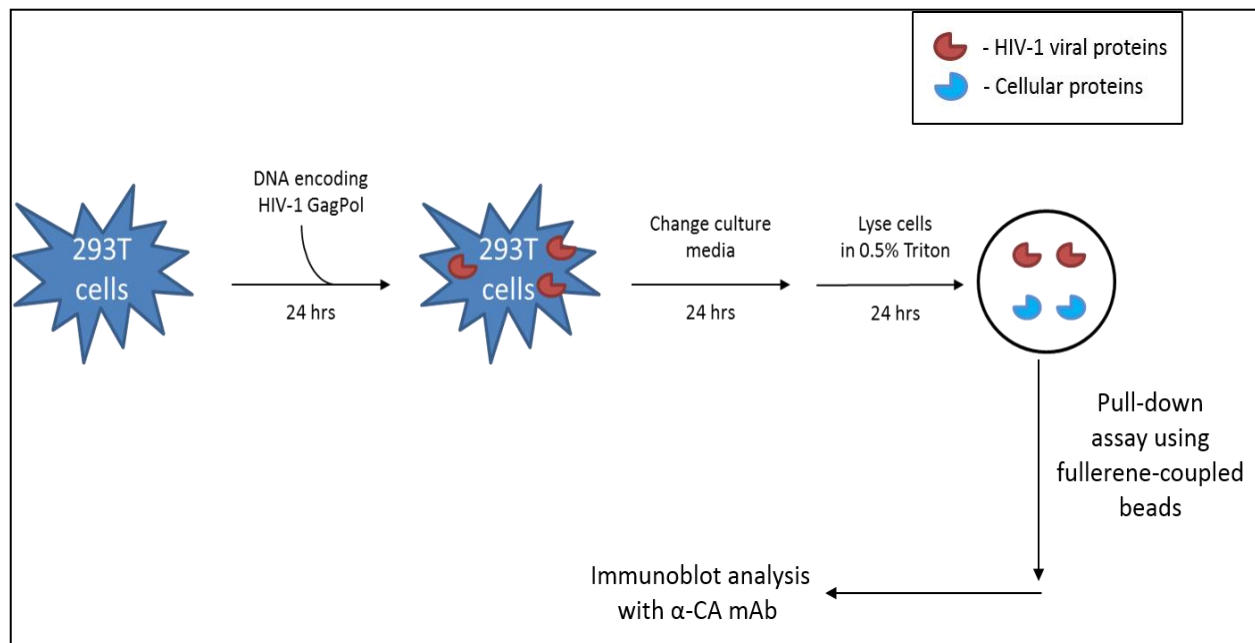
**Figure 15.** Syntheses of  $C_{70}$  mono and bis acetylene derivatives 5-8.

**3.2.4 Pulldown assay.**  $3 \times 10^6$  HEK293T cells were plated in a T75 cm<sup>2</sup> tissue culture flask and transfected the next day with 20 µg of the Gag-Pol expression plasmid ΔR8.9 by the calcium-phosphate precipitation method. After 18 h, the transfection medium was replaced with fresh medium, and 24 h later the cells were washed in 1x PBS and lysed in 500 µL of 0.5% Triton in 1x PBS. The cell lysate was incubated on ice for 15 min, centrifuged at 22000 g for 10 min, and the supernatant was incubated with 50 µL of magnetic beads for 1 h at 4°C to remove proteins binding non-specifically to the beads. 50 µL of the precleared lysate were saved as input sample, and 150 µL were mixed with 100 µL of non-functionalized magnetic beads or functionalized with fullerene derivative 6 or fullerene derivative 8. Beads and cell lysates were then rotated overnight at 4°C, followed by one 5 min wash using 0.5% Triton in 1x PBS. Washed beads were boiled 10 min at 100°C in 50 µL of 2x Laemmli buffer, and the eluted proteins were analyzed by immunoblotting with an anti HIV-1 p24 monoclonal antibody as described below. A schematic of this assay is depicted in figure 16.

**3.2.5 Plasmids.** The plasmids used to generate retroviral vectors were described previously (69). HIV-1-derived vectors were produced using pHIV luc and pMD.G. pHIV Luc was derived from pNL4-3.Luc.R-E-(70) by introducing a deletion in the env open reading frame. pMD.G encodes the Vesicular Stomatitis Virus glycoprotein G (VSV-G).

**3.2.6 Cell lines.** SupT1 and HEK293T cells were grown in RPMI 1640 and in DMEM, respectively. All culture media were supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin.





**Figure 16.** Schematic of pull-down assay with fullerene-coupled magnetic beads.

**3.2.7 Generation of retroviruses.** Procedures previously described were followed (71). Briefly, HEK293T cells were co-transfected with the corresponding plasmids by the calcium-phosphate precipitation method. After 18 h the transfection medium was replaced with fresh medium containing no drug, fullerene derivatives 2a-c, derivatives 6 and 8, Indinavir or DMSO (vehicle control). The cells were cultured for 48 h until the viral supernatant was harvested and filtered. Single-round infection viral vectors were further concentrated by ultracentrifugation. VSV-G-pseudotyped HIV-derived reporter virus expressing firefly luciferase (HIVluc) was prepared by co-transfection of 15 µg of pHIV luc and 5 µg of pMD.G. HIV-1 wild type viruses were produced by transfection of 15 µg of the corresponding expression plasmids.

**3.2.8 Single-round infectivity assay.** SupT1 cells were plated at  $1 \times 10^5$  cells in 500 µL of RPMI 1640 culture medium in 24-well plates and infected with HIVluc. 4 days post-infection, cells were collected by centrifugation at 1000 g for 6 min and the pellet resuspended in 200 µL of phosphate-buffered saline (PBS). Half of the sample was mixed with 100 µL of luciferase substrate (Bright-Glow™ Luciferase Assay System, Promega) and the other half with 100 µL of cell viability substrate (CellTiter-Glo® Assay, Promega). Cell lysates were incubated for 10 min at room temperature in the dark and then luminescence was measured in triplicate in 50 µL-samples using a microplate luminometer reader (Thermo Scientific, Luminoskan Ascent).

**3.2.9 HIV-1 replication assays.** SupT1 cells were infected with HIV-1<sub>NL4-3</sub> (2.1 ng of HIV-1 p24) in the presence of the fullerene derivatives 2a-c or DMSO. 24 h after infection, the cells were extensively washed and viral replication was monitored by HIV-1 p24 quantification by ELISA.

**3.2.10 Cellular viability assay.**  $1 \times 10^4$  SupT1 cells were plated in a 96-well plate in 100  $\mu$ L RPMI1640 culture media and left untreated or treated with fullerene derivatives 2a-c, DMSO (control), or 2 mM hydrogen peroxide (positive control). Fullerene derivatives 2a-c were evaluated at a concentration that ranged from 3  $\mu$ M to 32  $\mu$ M. The cells were cultured in the presence of the indicated compounds for 24 h and then 20  $\mu$ L of the MTS were added to each well of cells. Incubation with the reagent for an additional 3 h was allowed. The colored formazan product was measured by absorbance at 490 nm with a reference wavelength of 650 nm using a microplate reader (SpectraMax 190, Molecular Devices). Control wells containing the same volumes of culture medium and MTS reagent were used to subtract background absorbance.

**3.2.11 Immunoblotting.** Proteins of HIV-1 p24-normalized amounts of virions (0.38  $\mu$ g) were resolved by 13% SDS-PAGE and transferred overnight to PVDF membranes at 100 mA at 4°C. Membranes were blocked in TBS containing 10% milk for 1 h and then incubated in the corresponding primary antibody diluted in TBS-5% milk-0.05% Tween 20 (antibody dilution buffer) overnight at 4°C. HIV-1 p24 was detected with anti-p24 obtained from the NIH AIDS Reagent Program (Catalog # 1513). Primary antibody-bound membranes were washed in TBS containing 0.1% Tween 20 and all bound antibodies were detected with goat anti-mouse IgG-HRP (1/2000, KPL, 074-1806) followed by chemo-luminescence detection.

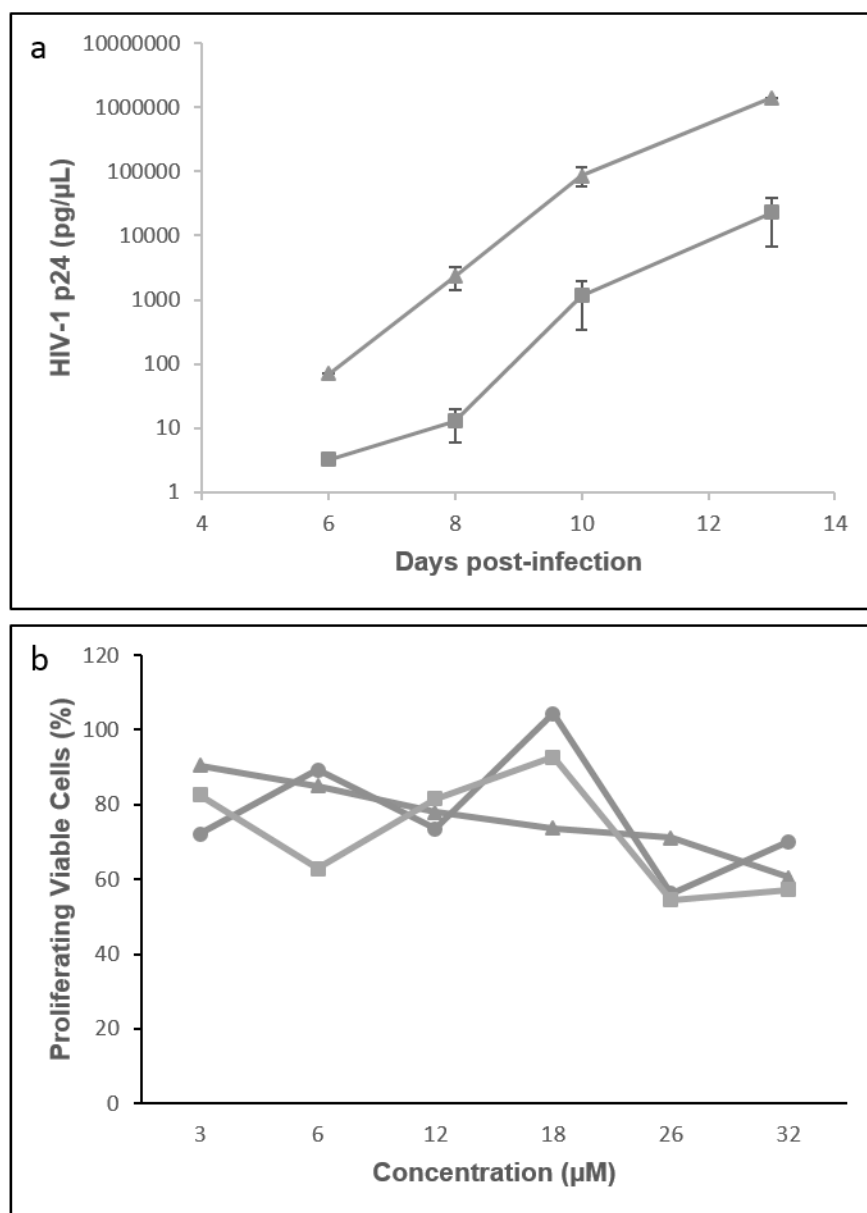
**3.2.12 HIV-1 protease in vitro activity assay.** The ProAssay™ HIV-1 Protease Assay Kit was used to determine the effect of derivatives 2a and 2b on HIV-1 protease activity *in vitro*. Briefly, HIV-1 protease and FRET peptide were treated or not with DMSO, fullerene derivatives 2a, b, and Indinavir. The reaction was measured by determining

the Relative Fluorescing Intensity (RFI) with a fluorometer using excitation/emission wavelengths of 490 nm / 530 nm.

### 3.3 Results

**3.3.1 Effect of fullerene derivatives on HIV-1 replication in human CD4<sup>+</sup> T-cells and cellular viability.** We determined the effect of C<sub>70</sub> fullerene derivatives on HIV-1 NL4-3 replication. SupT1 cells were infected with 2.1 ng of HIV-1 p24 in the presence of the regioisomeric mixture 2a (3  $\mu$ M) or DMSO. 24 h later the input virus and compounds were extensively washed and viral replication was followed by quantification of HIV-1 p24 in the cell culture supernatant by ELISA. Results in figure 16a show a potent inhibitory effect of the regioisomeric mixture 2a on HIV-1 replication. Fullerene derivative 2a reduced viral replication between 31 and 160-fold during the course of the experiment.

In order to confirm that fullerene derivative 2a was non-toxic at the concentration demonstrated to block HIV-1 infection, we treated SupT1 cells with different concentrations of fullerene 2a, as well as 2b and c, and DMSO for 24 h and measured cell viability using the tetrazolium dye reduction assay (MTS assay). As figure 16b shows, the concentration that kills approximately 50% of SupT1 cells (LC<sub>50</sub>) was higher than 32  $\mu$ M for each of these fullerene derivatives. This confirms that derivative 2a used at a 3  $\mu$ M concentration does not affect cellular viability, indicating the HIV-1 inhibitory effect witnessed in figure 16a was not due to cellular toxicity.



**Figure 17.** *Effect of fullerene derivative 2a on HIV-1 infection and derivatives 2a-c on cell viability.*

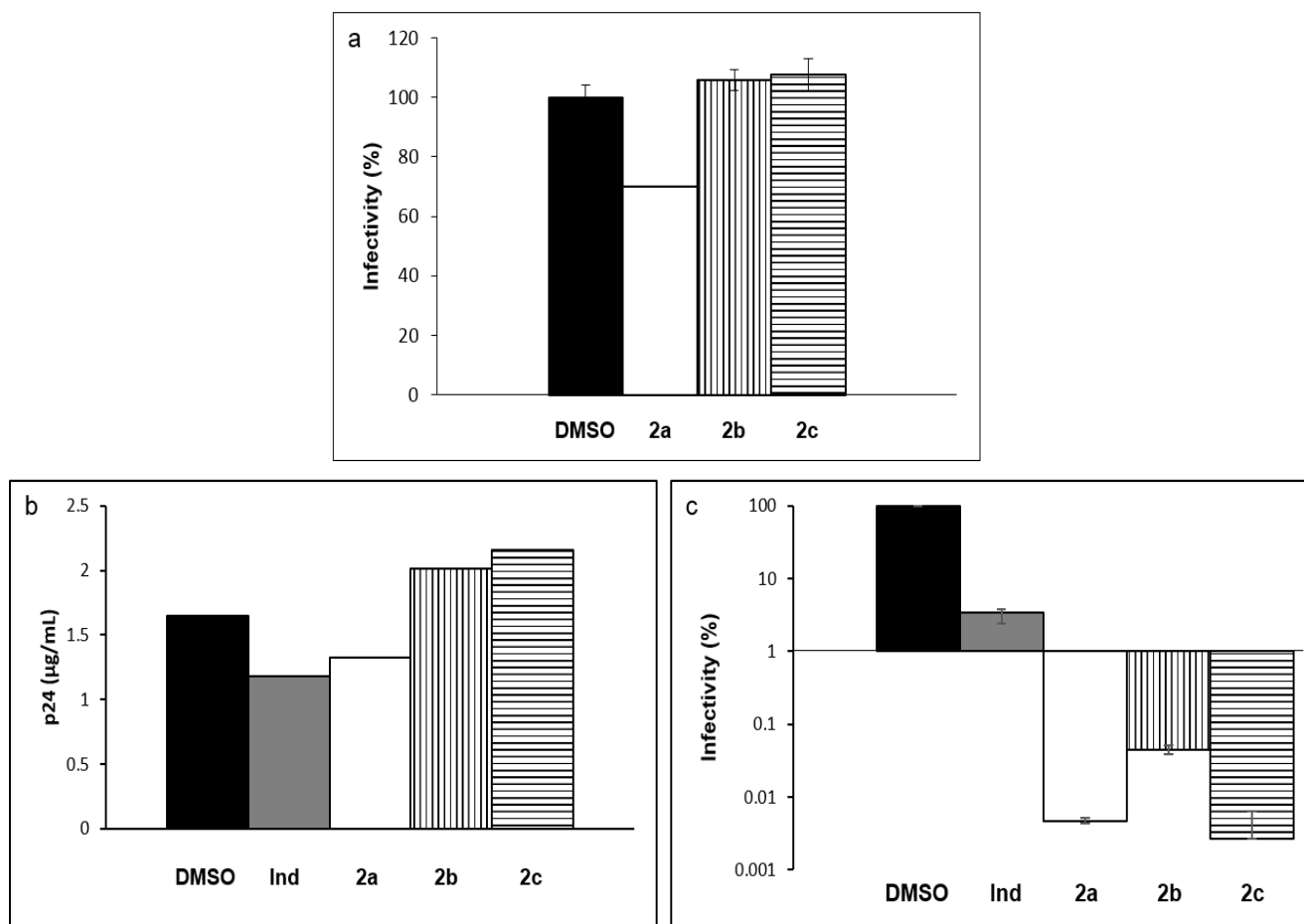
(a) SupT1 cells were treated with DMSO (Δ) or compound 2a at 3 μM (□) at the time of infection with HIV NL4-3 (2.1 ng of p24). (b) SupT1 cells were treated with different concentrations of fullerenes 2a-c (Δ, ○, and □, respectively) and DMSO for 24 h and then cell viability was measured using the tetrazolium dye reduction assay. Results and standard deviations shown for (a) are of two independent experiments. Results shown for (b) are of one experiment.

### **3.3.2 Effect of fullerene derivatives 2a-c on the early stages of the HIV-1 life cycle.**

We next investigated the viral life cycle step affected by the cationic fullerene derivatives 2a-c. The effect of 2a-c on the infection of VSV-G pseudotyped HIV-1 single-round infection viruses expressing luciferase (HIVluc) was evaluated in the human CD4<sup>+</sup> T cell line SupT1. The cells were exposed to fullerene derivatives 2a-c (10  $\mu$ M), or DMSO as a control, and infected with the reporter virus and 24 h later the compounds and the input virus were removed and three days later cellular luciferase and ATP levels were measured. Luciferase was normalized to ATP to adjust for cell viability and number. As expected, fullerene derivatives 2a-c did not affect the early stages of the viral life cycle (Figure 17a).

### **3.3.3 Effect of fullerene derivatives 2a-c on the late stages of the HIV-1 life cycle.**

The effect of fullerene derivatives 2a-c on the late phase of the HIV-1 life cycle was further evaluated. HIVluc was produced in HEK293T cells in the presence of DMSO, fullerene derivatives 2a-c (3  $\mu$ M), or Indinavir (0.1  $\mu$ M) and their infectivity was evaluated in single-round infection assays using SupT1 cells. Similar levels of HIV-1 p24 were found among these viruses (Figure 17b) indicating that fullerene derivatives did not affect LTR transcription, translation of viral proteins, immature particle assembly, viral budding, or cellular viability. The infectivity of viruses produced in the presence of C<sub>70</sub> fullerene derivatives 2a-c and Indinavir was dramatically reduced. Compounds 2a-c, caused more than 99% reduction of infectivity at 3  $\mu$ M (Figure 17c).



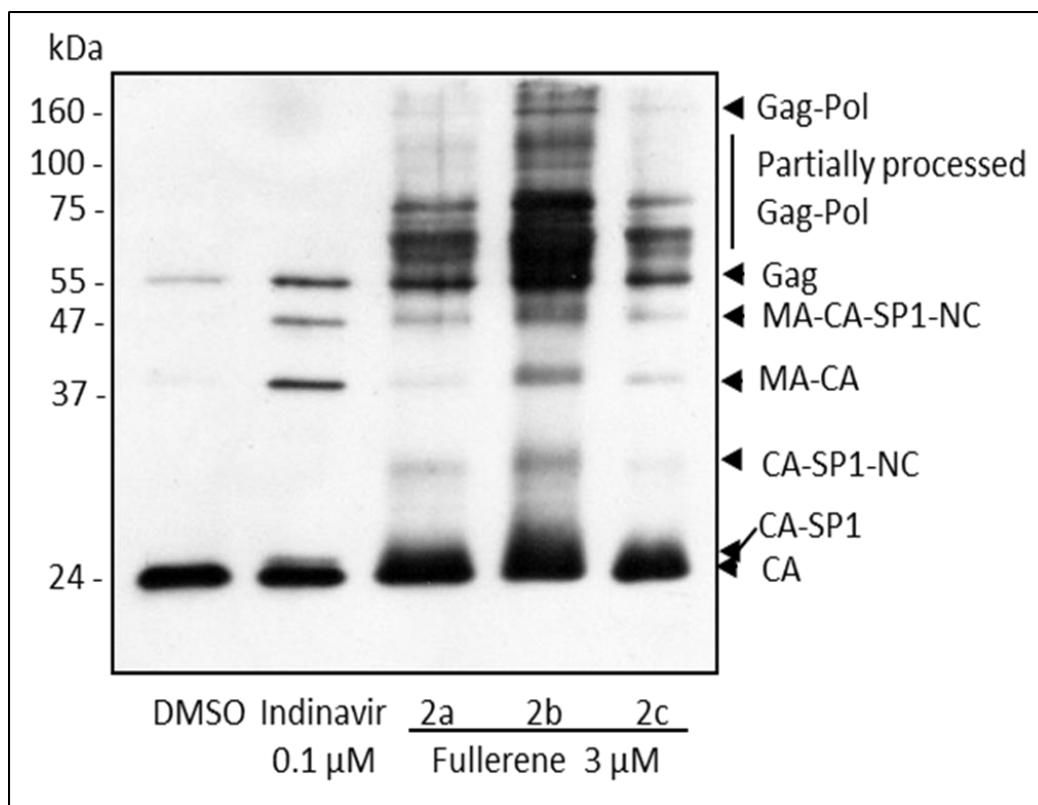
**Figure 18.** Analysis of the viral life cycle step affected by derivatives 2a-c.

a) Effects on the early stages of the HIV-1 viral life cycle. SupT1 cells were infected with single-round infection HIV-1 viruses in the presence of DMSO or fullerene derivatives 2a-c (10  $\mu$ M), and analyzed for luciferase expression and cellular viability (ATP content) three days later. Luciferase was normalized to cellular viability. Single-round infection HIV-1 virus were produced in the presence of DMSO, Indinavir (0.1  $\mu$ M), or fullerene derivatives 2a-c (3  $\mu$ M). b) Virion production as determined by HIV-1 p24 levels quantified by ELISA. c) Effects on the late phase of HIV-1 infectivity analyzed in single-round infection assays using HIV-1 p24-normalized viruses. Results shown for a) and c) are the average and standard deviation of triplicate readings of one experiment representative of two independent experiments. Results shown for (b) are of one experiment.

**3.3.4 Effect of fullerene derivatives 2a-c on HIV-1 Gag processing.** Data in figure 17 suggest that fullerenes 2a-c, block HIV-1 maturation. In order to define whether C<sub>70</sub> derivatives inhibit HIV-1 infection by similar mechanisms, we determined the efficiency of Gag processing in virions produced in the presence of DMSO, fullerenes 2a-c (3  $\mu$ M), and Indinavir (0.1  $\mu$ M) by immunoblot analysis with an anti-capsid (p24) monoclonal antibody. Capsid is a domain in Gag, therefore, non- or partially processed Gag proteins containing CA are detected in this assay. Data in figure 18 indicate a pronounced decrease in Gag processing in the virions produced in the presence of C<sub>70</sub> fullerene derivatives that correlate with the decrease in infectivity observed in these same viruses (Figure 17c).

C<sub>70</sub> fullerenes impair Gag processing at a higher magnitude than Indinavir. As well, fullerene-treated virions contain a larger proportion of Gag processing intermediates in contrast with virions produced in the presence of Indinavir. HIV-1 proteins in Gag are ordered from N- to the C-terminus as matrix (p17, MA, 17 kDa), capsid (p24, CA, 24 kDa), spacer peptide 1 (SP1, 2 kDa), nucleocapsid (NC, 7kDa), spacer peptide 2 (SP2, 1 kDa), and late domain-containing protein P6 (P6, 6 kDa) (13, 95). Protease-mediated Gag processing occurs in a fixed order, resulting in several processing intermediates, some of which are indicated in figure 18. Several CA-containing Gag fragments, presumably identified as CA-SP1-NC (~33 kDa) and CA-SP1 (~25 kDa), were enriched in the fullerene- but not in the Indinavir-treated virions (13). Therefore, these data suggest that C<sub>70</sub> fullerene derivatives 2a-c and Indinavir affect Gag processing by different mechanisms.

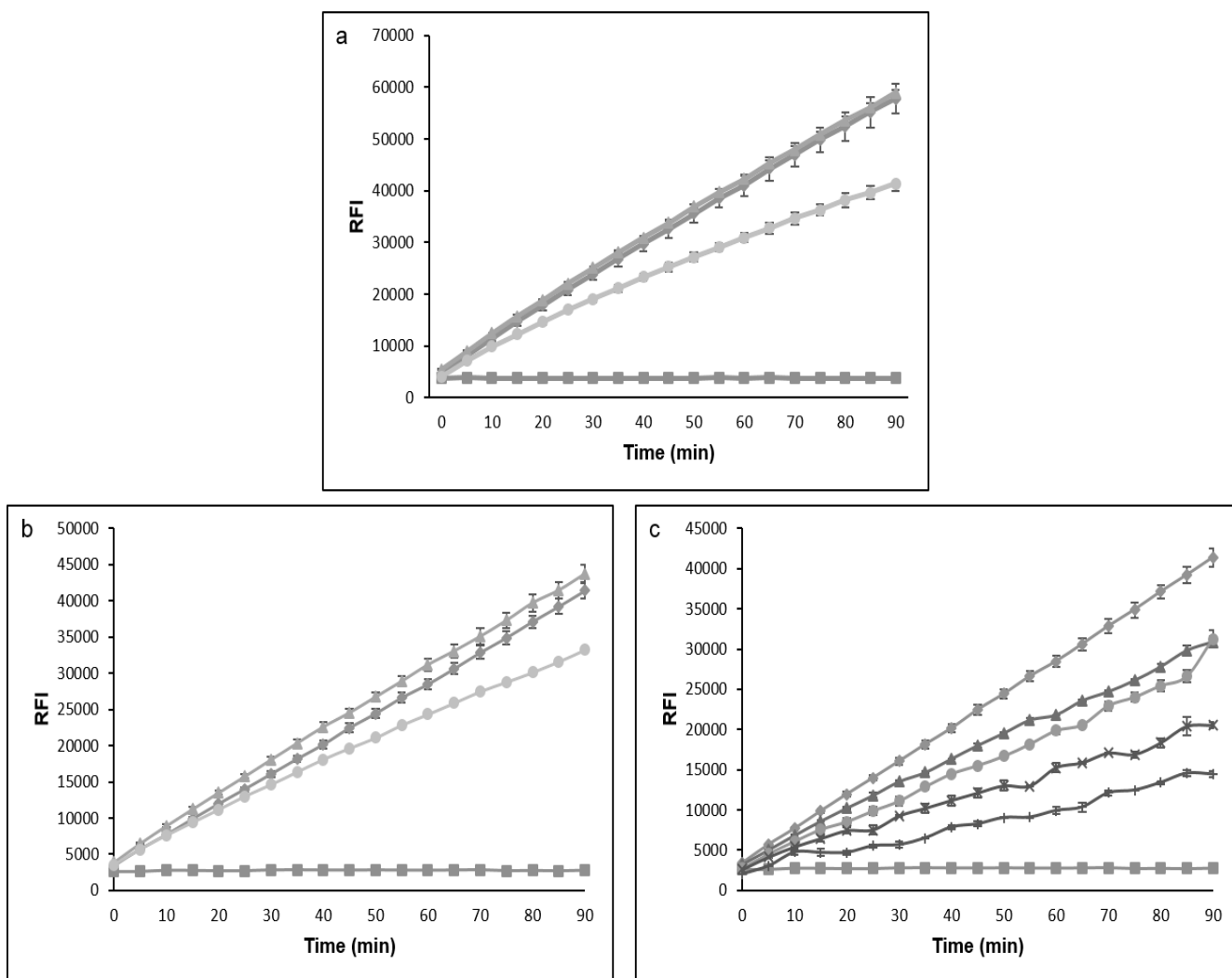




**Figure 19.** Effects of  $C_{70}$  fullerene derivatives 2a-c on HIV-1 Gag processing.

Immunoblot analysis of the effect of derivatives 2a-c on Gag processing. Results are representative of two independent experiments. The identity of the Gag proteolytic fragments have been previously described (13).

**3.3.5 Effect of fullerene derivatives on HIV-1 protease activity.** Gag processing can be affected by protease inhibition or by altering the conformation of the protease substrate (13, 29). Therefore, we determined the effect of the C<sub>70</sub> fullerene derivatives 2a and 2b on the ability of recombinant HIV-1 protease to cleave a peptide derived from the native p17/p24 (MA-CA) cleavage site on Gag. Figures 19a and b show that derivatives 2a and 2b, respectively, did not significantly affect HIV-1 protease activity at doses that strongly block HIV-1 infection (3 and 10  $\mu$ M). Even at 40  $\mu$ M, a concentration that exceeds the LD<sub>50</sub> (16b), fullerene 2b only inhibited 24% of protease activity and reached 65% inhibition at 80  $\mu$ M. Again, as with the C<sub>60</sub> fullerene derivative 1 reported in chapter 2.3.7, derivative 2b exhibits a concentration-dependent inhibition of protease activity. This can be regarded as non-specific inhibition of protease activity due to the C<sub>60</sub> derivative 3 having a similar concentration-dependent inhibition while having no effect on HIV replication. Therefore, these results indicate that C<sub>70</sub> fullerene derivatives 2a, b are not protease inhibitors but rather HIV-1 maturation inhibitors.



**Figure 20.** Effect of derivatives 2a and 2b on the *in vitro* activity of HIV-1 protease.

The cleavage of an HIV-derived FRET peptide by recombinant HIV-1 protease in the presence of a) fullerene 2a at 3  $\mu\text{M}$  ( $\Delta$ ) or 10  $\mu\text{M}$  (o) or b) fullerene 2b at 3  $\mu\text{M}$  ( $\Delta$ ) or 10  $\mu\text{M}$  (o) was determined by fluorescence measurements. c) Fullerene 2b was used at 20  $\mu\text{M}$  ( $\Delta$ ), 40  $\mu\text{M}$  (o), 60  $\mu\text{M}$  (x), or 80  $\mu\text{M}$  (+). DMSO ( $\diamond$ ) and Indinavir ( $\square$ ) were used as negative and positive controls, respectively. Experiments were performed in duplicates and standard deviations indicate the variability of duplicate readings. Results for a-c are representative of one experiment.

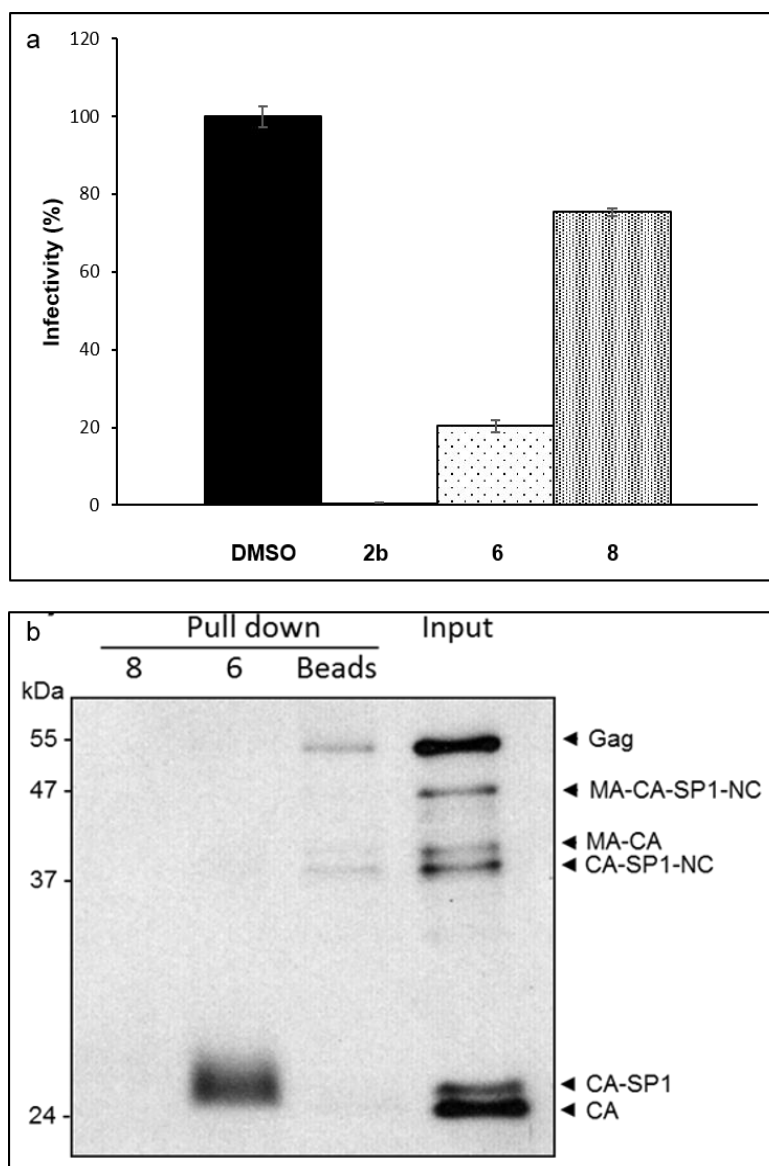
### 3.3.6 Analysis of the mechanism of action of C<sub>70</sub> fullerene derivatives 2a-c in HIV-1

**maturation.** The fact that fullerene derivatives 2a-c generated more CA-containing Gag processing intermediates than Indinavir (Figure 18) suggests that compounds 2a-c could bind to CA. Similarly, Bevirimat and related compounds that bind to the region flanking the CA-SP1 junction selectively produce the accumulation of this processing intermediate in the virion (38, 30, 31, 84, 86, 87). Therefore, we explored the interaction of 2a-c with HIV-1 CA in the context of Gag polyproteins using a pull-down assay with derivative 2b as bait, followed by anti-CA immunoblot analysis. C<sub>70</sub> fullerene derivative 2b was functionalized with one or two acetylene groups, compounds 6 and 8 respectively (Figure 15), and then coupled to the azide magnetic beads via click chemistry. Infrared (IR) analysis showed that the C<sub>70</sub> derivatives were successfully coupled covalently to the beads.

To evaluate if the functionalization of 2b with malonates 3 and 4 (compounds 6 and 8) disrupted its anti-HIV-1 activity, HIVluc was produced by transfection in HEK 293T in the presence of fullerene derivatives 2b, 6, 8 or DMSO. The infectivity of the produced viruses was evaluated in single-round infection assays in SupT1 cells. Data in figure 20a indicate that functionalization of fullerene 2b using malonate 4 resulted in the total loss of its anti-HIV-1 activity. Fortunately, the inhibition activity was preserved for compound 6 containing only one acetylene group.

Lysates from HEK 293T cells transiently expressing HIV-1 Gag polyproteins were incubated overnight with magnetic beads with no fullerenes attached or beads containing active or inactive fullerene derivatives 6 and 8, respectively. Then, the beads were washed and the bound proteins were analyzed by immunoblot with an anti-p24

(HIV-1 CA) monoclonal antibody. Data in figure 20b demonstrate that a CA-containing Gag product of ~25 kDa, suggesting the CA-SP1 fragment, strongly binds to beads containing active fullerene derivative 6, but not to those with the inactive derivative 8 or no fullerene attached. Therefore, these results demonstrate the specific interaction of active fullerene derivative 6 with HIV-1 CA-SP1 protein. Importantly, no other CA products were pulled-down suggesting that stable binding of fullerene derivative 6 to CA-SP1 requires Gag processing.

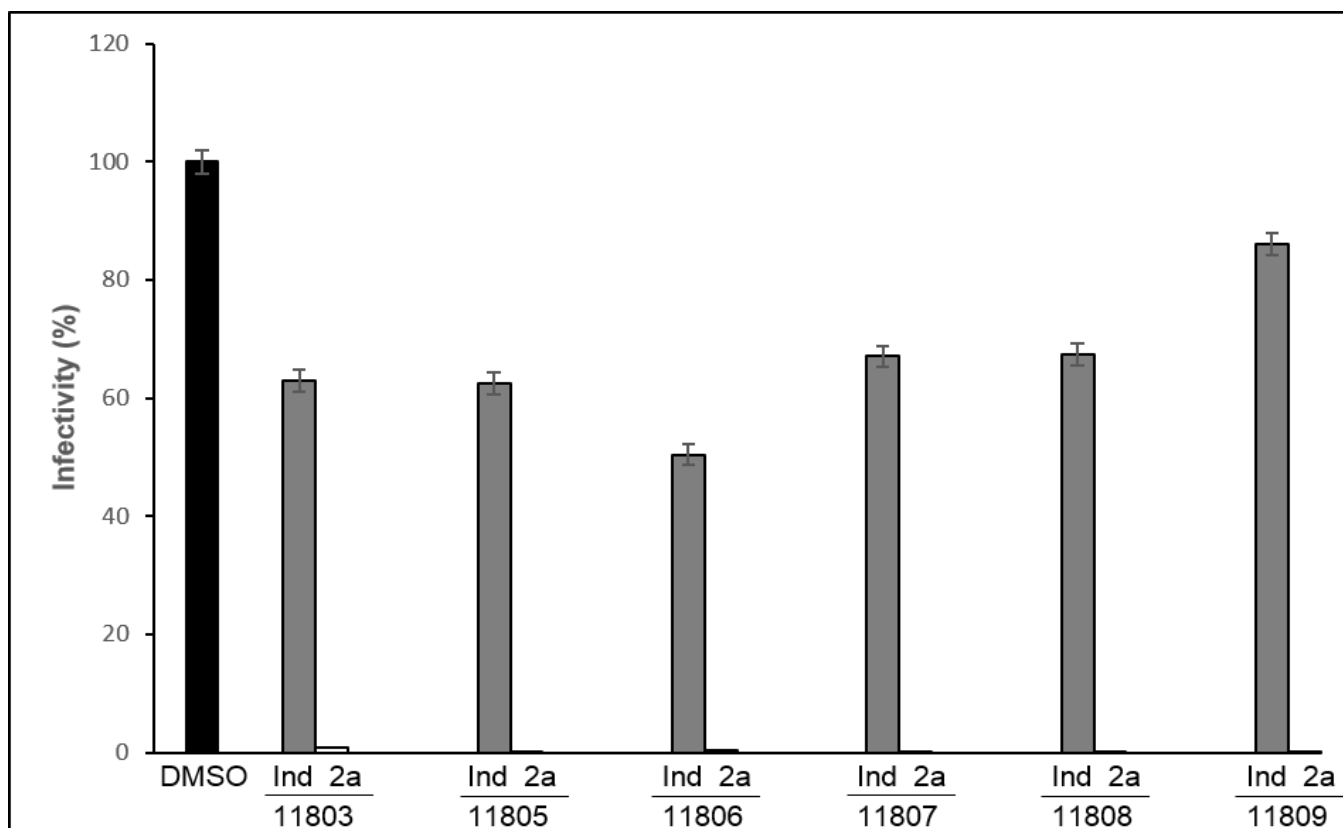


**Figure 21.** Analysis of the mechanism of action of  $C_{70}$  fullerene derivative 2b.

(a) Effect on HIV-1 infection of fullerene 2b, and compounds 6 and 8 that were coupled to the beads used in (b). (b) Analysis of the interaction of HIV-1 Gag with beads coated with active or inactive fullerene derivatives 6 and 8, respectively. Results shown for a) and b) are the average and standard deviation of triplicate readings of one experiment representative of two independent experiments.

**3.3.7** Anti-viral activity of C<sub>70</sub> fullerene derivative 2a on HIV-1 molecular clones harboring mutant proteases resistant to protease inhibitors. Typically, HIV-1 maturation inhibitors block infection by HIV-1 strains resistant to protease inhibitors, indicating that these type of drugs would be excellent additions to current anti-HIV-1 therapy (13, 29). In order to evaluate the effect of C<sub>70</sub> fullerene derivative 2a on the infectivity of HIV-1 strains resistant to multiple protease inhibitors, HIVluc-derived viruses harboring a panel of protease inhibitor-resistant mutant proteases were produced by plasmid transfection in HEK 293T cells in the presence of DMSO, Indinavir (0.1  $\mu$ M), or derivative 2a (3  $\mu$ M). The mutant viruses analyzed included 11803, 11806, 11807, 11808, and 11809 that are resistant to Nelfinavir, Fosamprenavir, Saquinavir, Indinavir, Atazanavir, Lopinavir, Tipranavir, and Darunavir; and 11805 that is also resistant to these drugs except for Tipranavir and Darunavir. The protease (99 amino acids) in these viruses contains between 10 and 24 point mutations (77).

Produced viruses were normalized for p24 and used to infect SupT1 cells. Four days after infection, cellular ATP and luciferase levels were measured and luciferase was normalized to ATP. We found that the regioisomeric mixture 2a potently inhibits all the multi protease inhibitor resistant viruses (Figure 21) to a similar extent as reporter viruses harboring wild type protease (Figure 17c). As expected, Indinavir failed to significantly affect the infection of these viruses. These findings highlight the therapeutic potential of fullerene derivatives further supporting their protease-independent mechanism of action.



**Figure 22.** *Effect of the regioisomeric mixture 2a and Indinavir on the infectivity of HIVluc-derived viruses carrying protease mutants resistant to multiple clinically approved protease inhibitors.*

HIV-1 viruses harboring protease mutants were produced in the presence of DMSO, derivative 2a (3  $\mu$ M), or Indinavir (0.1  $\mu$ M), and their infectivity was evaluated in single-round infection assays. Results are representative of one experiment, standard deviations are of triplicate readings.



### 3.4 Discussion

C<sub>70</sub> fullerene derivatives, similar to some C<sub>60</sub> compounds, have been demonstrated to impair HIV-1 replication in human cells, however a lack of virological characterization of their activity has limited their development as anti-HIV-1 therapeutic agents (58-63, 75, 76). We have conducted an extensive analysis of the anti-HIV-1 activity of these compounds and demonstrated for the first time that C<sub>70</sub> fullerene derivatives 2a-c inhibit viral maturation most likely by interacting with HIV-1 CA-SP1 protein instead of HIV-1 protease, as previously predicted by *in silico* analyses of C<sub>60</sub> fullerene derivatives (58, 62, 63, 65, 66, 96). In further support of this mechanism of action, C<sub>70</sub> fullerene derivatives 2a-c potently block the infection of viruses carrying protease mutants resistant to multiple protease inhibitors, highlighting their potential therapeutic value. Furthermore, these compounds exhibit significant potency at low micromolar concentrations.

Our data indicate that C<sub>70</sub> fullerene derivatives strongly bind to CA-SP1 and alter Gag processing. A similar specificity has been described for the maturation inhibitors Bevirimat and PF-46396. However, in contrast with these compounds that only affect Gag processing at the CA-SP1 cleavage site, fullerene derivatives 2a-c broadly affect the processing of Gag polyproteins, indicating a different mechanism of action (30, 31, 66, 84). The inhibitory effect of C<sub>70</sub> fullerenes on Gag processing suggest that these compounds also bind CA-SP1 in the context of unprocessed or partially processed polyproteins, altering their protease-mediated cleavage. However, the interaction of C<sub>70</sub> fullerenes with CA-SP1 in Gag is weaker than with the released protein, as indicated by the pull-down experiments. Similarly to C<sub>70</sub> fullerenes, peptides and small molecules

binding to a hydrophobic groove in the C-terminal domain of CA impair Gag processing (85). These results suggest that the potential distortion of the CA conformation caused by the binding of these interacting molecules could impair the ability of HIV-1 protease to reach multiple cleavage sites within Gag. Importantly, these peptides and small molecules act through a different mechanism than that of fullerene derivatives, since they impair both early and late phases of the viral life cycle in addition to viral production (85, 97-99).

Other peptides and small molecules binding to CA have been described to affect its multimerization state and the formation of mature viral capsids, but not Gag processing, hence differing with fullerene derivatives in their mechanism of action (38, 39, 99, 100). Therefore, fullerene derivatives seem to act through a novel anti-HIV-1 mechanism not reported yet for other CA-interacting compounds. The lack of effect on viral production or infectivity of mature virions suggests that fullerene derivatives do not affect immature viral particle assembly or higher-order structures of HIV CA in the mature virion. In summary, our data indicate that C<sub>70</sub> fullerene derivatives are promising HIV-1 maturation inhibitor compounds with a unique mechanism of action.

## CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

### 4.1 Overview and Conclusion

C<sub>60</sub> and C<sub>70</sub> fullerene derivatives have previously been demonstrated to inhibit HIV-1 replication in human cells, predicted to bind to HIV-1 protease via its catalytically active site, and in the case of C<sub>60</sub> derivatives, inhibit recombinant HIV-1 reverse transcriptase in vitro activity. The anti-HIV-1 mechanism of action of these compounds have yet to be elucidated, hindering their potential as therapeutic anti-HIV-1 agents. No previous studies have shed light on the molecular target of fullerene derivatives other than computational predictions and molecular docking simulations, suggesting the viral protease as the target. While the enzymatic site of protease is nearly identical to the size of C<sub>60</sub> derivatives (1 nm), the concept of these compounds binding to protease and inhibiting protease activity has been the dominate theory for the anti-HIV-1 properties of fullerene derivatives for over 20 years. This widely accepted belief has lacked detailed virological characterization and experimental proof displaying or disproving protease as the target.

The work presented in this dissertation has determined that fullerene derivatives, both C<sub>60</sub> and C<sub>70</sub>, have no effect on the early stages of HIV-1 replication including entry, reverse transcription, nuclear import, and integration. Transcription and translation of viral proteins was also seen to not be affected by fullerenes. Production of VSV-G pseudotyped HIV-1 single-round infection viruses expressing luciferase in the presence of C<sub>60</sub> and C<sub>70</sub> derivatives exhibited a severe defect in HIV infectivity, indicating viral maturation as the step in the viral life cycle affected by these compounds. Immunoblot

analysis revealed that protease-mediated Gag and Gag-Pol processing were defective in single-round infection viruses produced in the presence of fullerenes, verifying maturation as the viral step in the HIV-1 life cycle affected.

Protease was evaluated as the molecular target by use of an *in vitro* FRET-based protease assay. Results displayed in this dissertation conclude, for the first time, that fullerene derivative concentrations that inhibit HIV-1 replication have no inhibitory effect on viral protease. While inhibition of protease activity is seen at concentrations that cause cytotoxic effects in human cells, this concentration-dependent inhibitory effect is also seen with fullerene derivatives that exhibit no anti-HIV-1 activity, implying this is a non-specific inhibitory effect. This protease-independent mechanism of action displayed by fullerenes was further evidenced as multi-protease inhibitor resistant HIV-1 viruses were shown to be as susceptible to fullerene derivatives C<sub>60</sub> and C<sub>70</sub> as wild-type HIV-1. This finding emphasizes the potential clinical relevance of fullerene derivatives to inhibit resistant viruses.

Data presented here implicates binding of C<sub>70</sub> derivatives to the CA-SP1 fragment in unprocessed and partially processed Gag, disrupting Gag and Gag-Pol processing. It is known that complete cleavage of Gag and Gag-Pol at multiple sites is essential for successful viral maturation (16). Prevention of protease-mediated Gag and Gag-Pol processing at any of the 9 cleavage sites severely reduces, and may even abolish HIV infectivity (16). In particular, the CA-SP1 partially processed intermediate has been demonstrated to exert a strong dominant-negative effect on viral infectivity (16). The defective Gag and Gag-Pol processing witnessed by immunoblot analysis combined with the binding of C<sub>70</sub> derivatives to the CA-SP1 fragment strongly suggests

that fullerene derivatives exhibit their anti-HIV-1 activity by binding to the unprocessed and partially processed CA-SP1 fragment in Gag, disrupting the highly ordered maturation process, thereby severely reducing HIV infectivity. Therefore, fullerene derivatives can be classified as maturation inhibitors, possibly with a novel mechanism of inhibitory action.

It has also been demonstrated throughout this work that the addends on the C<sub>60</sub> derivatives have prominent effects on their anti-HIV-1 activity. Differences in inhibition of viral replication of these compounds is entirely dependent on the chemical nature of the substituent groups attached to the C<sub>60</sub> core. Also, the regiochemistry of fullerene derivatives influences their anti-HIV-1 activity, an important finding to better understand their mechanism of action and improve the design of new fullerene derivatives.

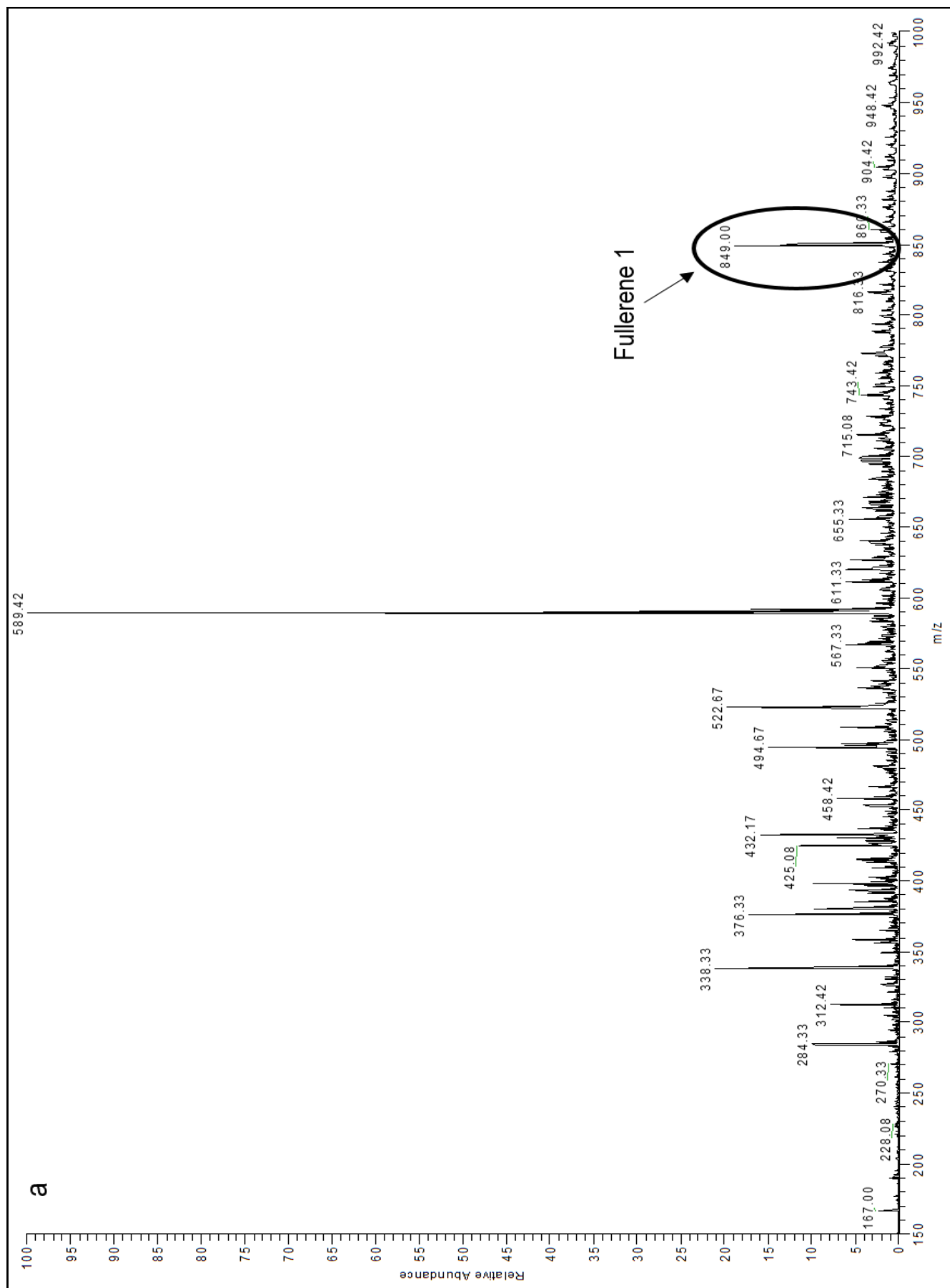
## **4.2 Future Directions**

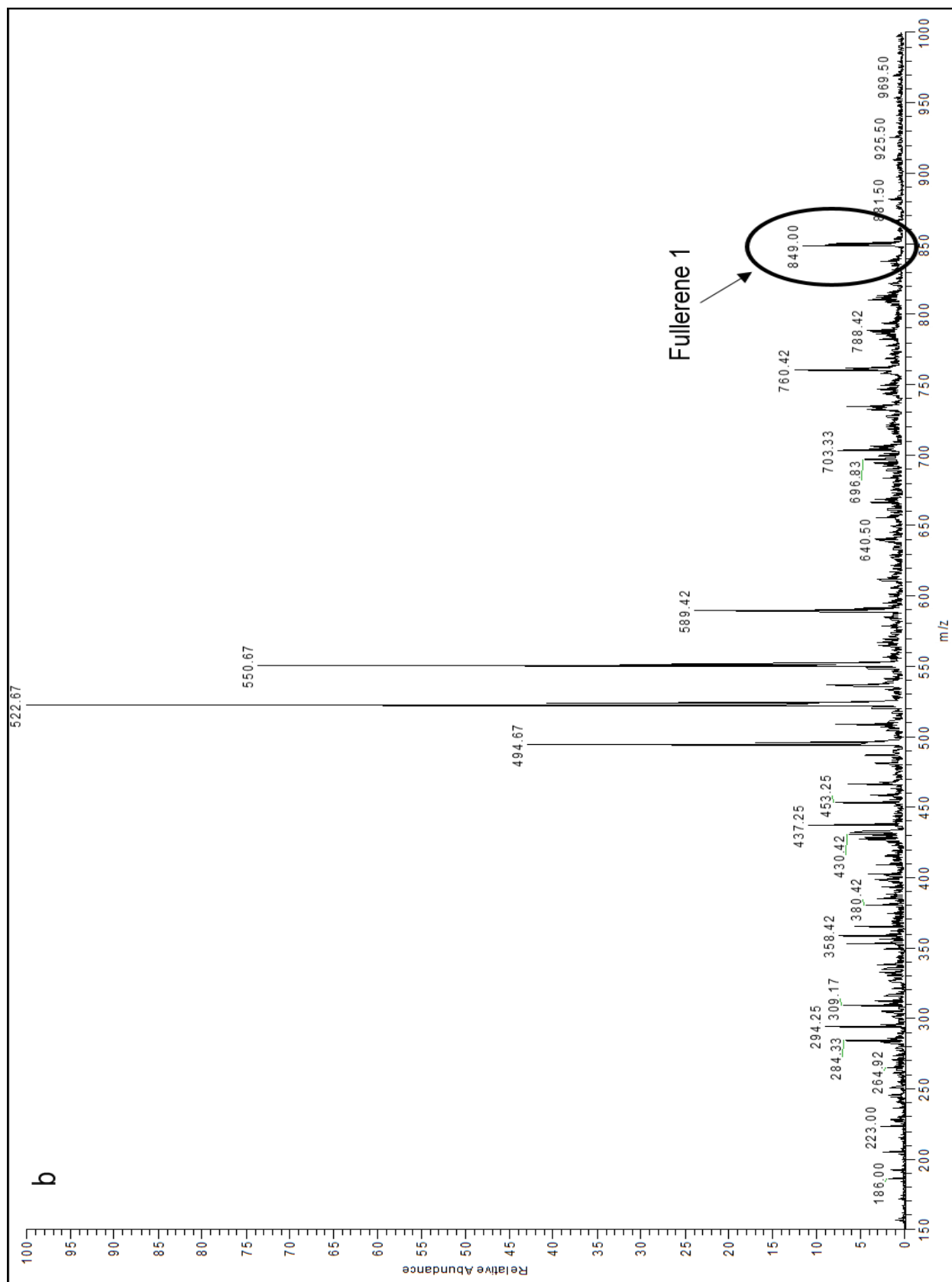
To better define the mechanism of action of fullerene derivatives, it is important to determine if the fullerene compounds are being incorporated inside the virion. While we have demonstrated that fullerene derivatives can bind to the CA-SP1 fragment of Gag, this may occur as the immature virus is being assembled at the cellular membrane, while the virus is budding from the cell, or when the immature virion is released from the cell and undergoing maturation. To determine whether fullerene derivatives are being incorporated inside the virion, single-round infectious HIV viruses will be produced in the presence of fullerene derivatives, either C<sub>60</sub> or C<sub>70</sub> derivatives, or DMSO. These viruses will then be concentrated by ultracentrifugation on a sucrose cushion and resuspended in urea to lyse the virion. Next, these viral samples will be

analyzed for the presence of fullerene, or lack of fullerene in viruses produced in the presence of DMSO, on an electrospray ion-trap mass spectrometer.

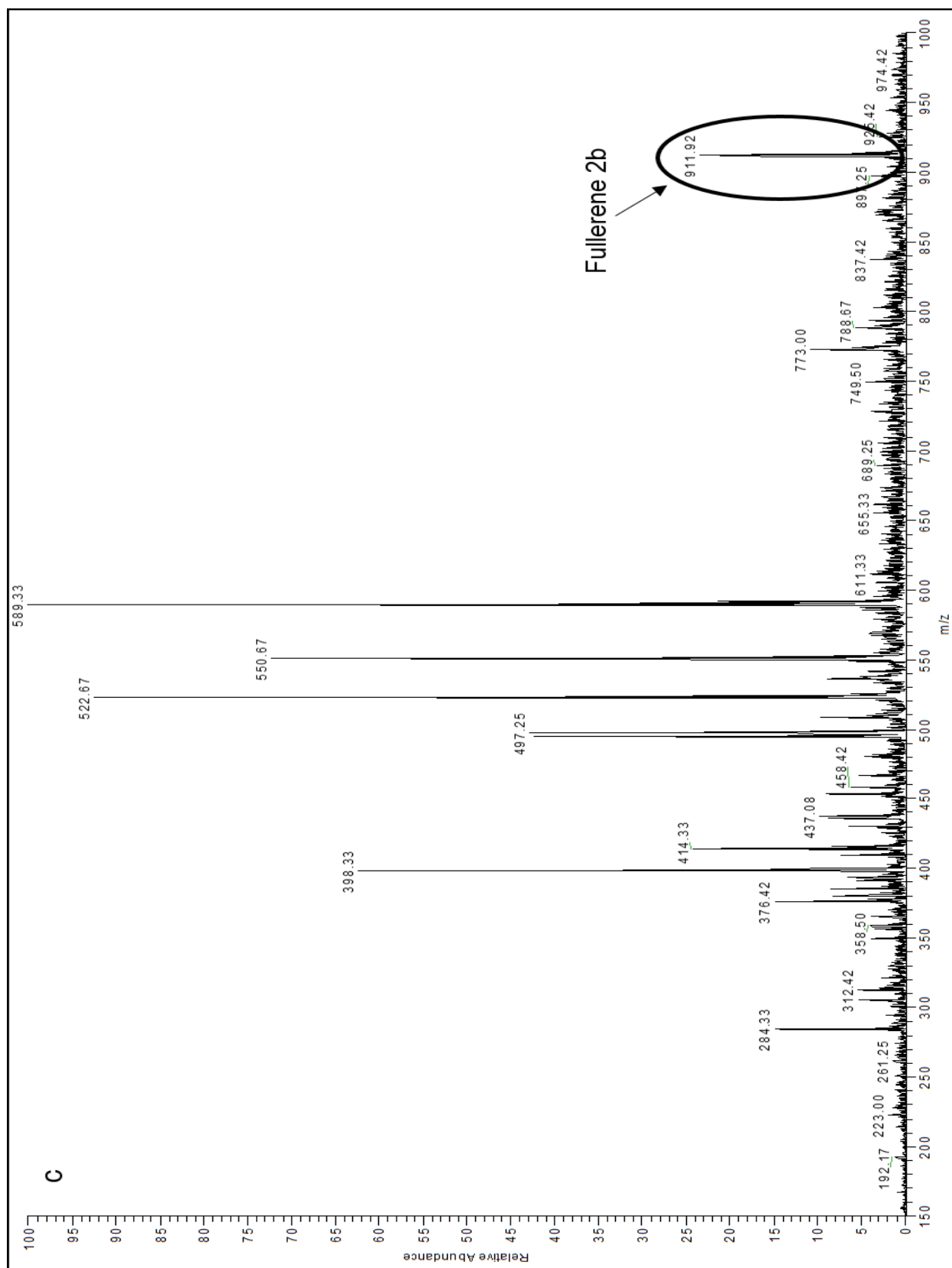
Preliminary results show that fullerene derivatives 1 and 2b, when dissolved in DMSO, can be detected by mass spectrometry with a mass-to-charge ratio of 849.00 and 911.92, respectively (Figure 22a and c, respectively). Viruses produced in the presence of these two compounds were analyzed next to determine if fullerene derivatives are being incorporated inside the virion. Figures 22b and c reveal that derivatives 1 and 2b are able to be detected in the viral samples giving identical mass-to-charge peaks. DMSO treated viruses contained neither of the peaks exhibited by derivatives 1 and 2b (Figure 22e), indicating that the mass-to-charge peaks seen in figures 22b and d are specific to fullerenes. These data suggest that fullerene derivatives are accumulating inside the virion.

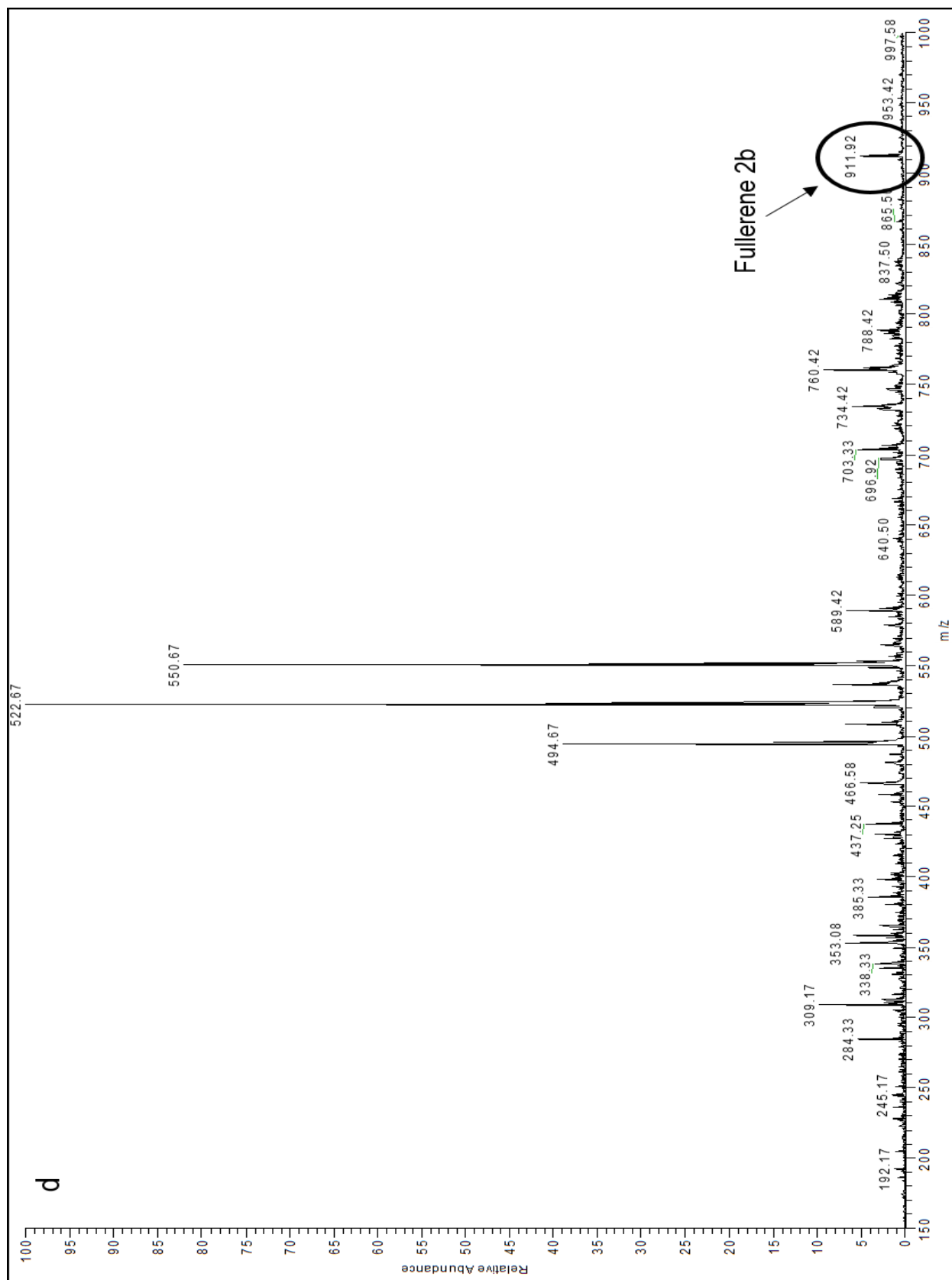
While these data are promising, this experimental procedure needs to be optimized to determine if the mass-to-charge peaks we observe for derivatives 1 and 2b are due to fullerenes accumulating inside the virion. It may be possible for these derivatives to be trapped inside VSV-G forming vesicles that are allowed to pass through the sucrose cushion upon ultracentrifugation. Should this happen, we may be detecting fullerene specific mass-to-charge peaks due to these derivatives being entrapped in VSV-G forming vesicles and not virions. Also, the presence of contaminants in these samples is another problem that must be addressed to obtain cleaner spectra and higher quality data. Currently, optimization of experimental protocols are being established to overcome these challenges.

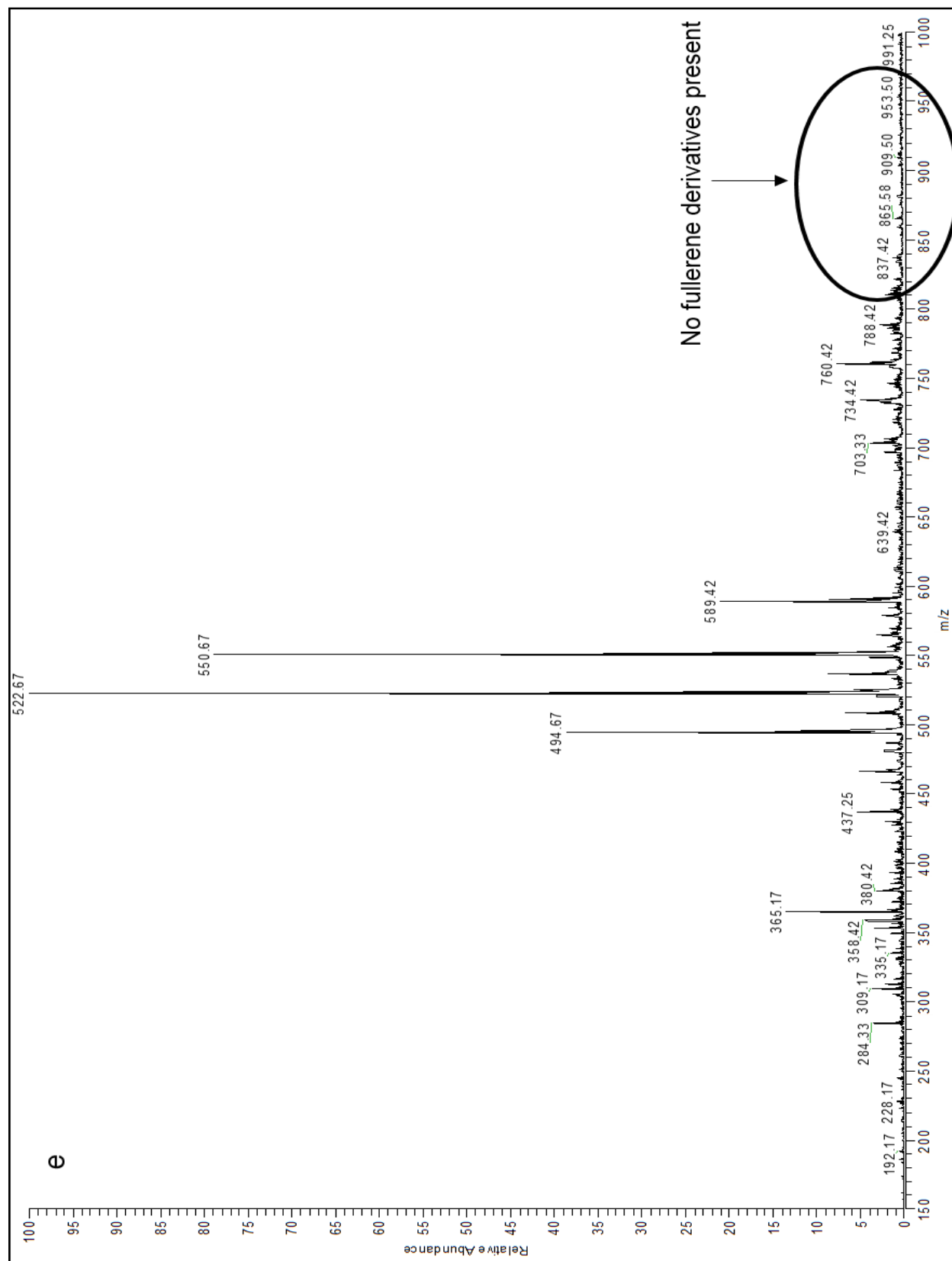












**Figure 23.** *Analysis of fullerene accumulation inside single-round infectious HIV virions.*

Single-round infectious HIV viruses were produced in the presence of fullerene derivative (b) 1, (d) 2b, or (e) DMSO (negative control), and analyzed by mass spectrometry. (a) Derivatives 1 and (c) 2b dissolved in DMSO were also analyzed as positive controls. Results from one experiment are shown.

Aside from determining if fullerene derivatives are being incorporated inside the virion, experiments are underway to investigate the amino acids in the CA-SP1 fragment of Gag involved in fullerene binding. Importantly, identification of these amino acid residues will give prominent insight into fullerenes mechanism of action. These mutations will also be mapped in comparison with those in CA-SP1 that confer resistance against the maturation inhibitor Bevirimat. In the event that the residues required for fullerene binding differ from those necessary for binding to Bevirimat, the anti-HIV-1 mechanism of action of fullerene derivatives will be completely novel, enhancing their potential therapeutic use.

To determine the amino acid residues required for fullerene binding to CA-SP1, viral evolution experiments are currently in progress. Human CD4<sup>+</sup> T-cells were infected with HIV-1NL4-3 and washed 24 h after infection. The infected cells were then treated with fullerene derivative 2b at varying concentrations and the compound was allowed to remain present until viral passages were performed. This will force the virus to undergo mutations, most likely in CA-SP1 or other regions of Gag, to overcome the anti-HIV-1 activity of fullerene 2b, thus conferring resistance to fullerene. DNA from

fullerene-resistant HIV-1 infected cells will then be isolated and sequenced. Analysis of these sequences, specifically in the CA-SP1 and Gag regions, will reveal the specific amino acid residues that have been mutated when compared to DMSO treated HIV-1NL4-3 infected cells that will also undergo viral passages. Results from these sequencing analyses will identify the residues necessary for fullerene binding, which will then be verified by introducing these mutations into single-round infectious HIV-1 viruses, producing these fullerene-resistant viruses in the presence of fullerenes, and evaluating their infectivity.

Finally, we are attempting to obtain a crystal structure of CA-SP1 and derivative 2a by the use of cryo-electron microscopy (cryo-EM). Currently, there are no crystal structures of fullerene derivatives binding to CA-SP1 or HIV-1 protease. This endeavor will definitively demonstrate that fullerene derivatives bind to CA-SP1, verify the amino acid residues required for fullerene binding, and help to better optimize fullerene derivative compounds to have greater potency against HIV-1 replication. Prokaryotic expression vectors encoding CA, CA-SP1, and protease have been generated and are awaiting protein expression for cryo-EM analysis.

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## ABBREVIATIONS

AIDS – Acquired Immune Deficiency Syndrome	FPV – Fosamprenavir
ATP – Adenosine Triphosphate	FRET – Fluorescence Resonance Energy Transfer
ATV – Atazanavir	gp – HIV-1 Glycoprotein
CA – HIV-1 Capsid protein	h - Hour
CAI – Capsid Assembly Inhibitor	<sup>1</sup> H-NMR – Proton Nuclear Magnetic Resonance
CAP-1 - N-(3-chloro-4-methylphenyl)-N'-{2-[(5-[dimethylamino)-methyl]-2-furyl)-methyl)-sul-fanyl]ethyl}urea	HAART – Highly Active Antiretroviral Therapy
CCR5 – C-C Chemokine Receptor type 5	HEK293T – Human Embryonic Kidney cells 293T
cDNA – Complementary DNA	HIV – Human Immunodeficiency Virus
CD4 <sup>+</sup> - Cluster of Differentiation, member 4 activated	HIV Luc – Single-round infectious HIV expressing the firefly luciferase gene
CEM – Human T lymphoblast cell line	IDV – Indinavir
CTD – C-terminal domain	IgG-HRP – Immunoglobulin G-Horseradish peroxidase
CXCR4 – C-X-C Chemokine Receptor type 4	IR – Infrared
DMEM – Dulbecco's Modified Eagle Medium	Kb – Kilobase
DMSO – Dimethyl Sulfoxide	LPV – Lopinavir
DNA – Deoxyribonucleic Acid	LTR – HIV-1 Long Terminal Repeat
DRV – Darunavir	MA – HIV-1 Matrix protein
DSB – Bevirimat	MALDI-TOF – Matrix Assisted Laser Desorption Ionization-Time of Flight
DTT – Dithiothreitol	MBIF – Magnetic Beads-Immobilized Fullerene
eGFP – Enhanced Green Fluorescent Protein	MRI – Magnetic Resonance Imaging
ELISA – Enzyme-Linked Immunosorbent Assay	MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
FDA – Food and Drug Administration	



NC – HIV-1 Nucleocapsid protein  
NFV – Nelfinavir  
NIH – National Institute of Health  
NTD – N-terminal domain  
PBS – Phosphate-Buffered Saline  
PCR – Polymerase Chain Reaction  
PIC – Pre-Integration Complex  
PVDF – Polyvinylidene Fluoride  
RFI – Relative Fluorescing Intensity  
RNA – Ribonucleic Acid  
RNP – Ribonucleoprotein complex  
ROS – Reactive Oxygen Species  
RPMI – Roswell Park Memorial Institute  
RTV – Ritonavir

SDS-PAGE – Sodium Dodecyl Sulfate –  
Polyacrylamide Gel Electrophoresis  
SP1 – HIV-1 Spacer Peptide 1  
SP2 – HIV-1 Spacer Peptide 2  
SQV – Saquinavir  
SupT1 – Human T-cell lymphoblast cell  
line  
TBS – Tris-Buffered Saline  
TPV – Tipranavir  
tRNA – Transfer RNA  
VSV-G – Vesicular Stomatitis Virus  
Glycoprotein  
XRI – X-ray Imaging

## VITA

Zachary Sean Martinez earned his Bachelor of Science degree in Chemistry as well as Microbiology from The University of Texas at El Paso in 2011. Later that year he joined the doctoral program in Pathobiology at The University of Texas at El Paso. While pursuing his degree, he worked as a teaching assistant for the Department of Biological Sciences, teaching Anatomy and Physiology labs. Dr. Martinez presented his research at the 2015 Border Biomedical Research Center Symposium. Before graduating, Zachary submitted two manuscripts for publication, one to Antimicrobial Agents and Chemotherapy entitled, "Fullerene Derivatives Strongly Inhibit HIV-1 Replication by Affecting Virus Maturation without Impairing Protease Activity", and the other to the Journal of the American Chemical Society entitled, "New Cationic [70] Fullerene Derivatives Inhibit HIV-1 Maturation in a Protease-Independent Manner, and Specifically Interact with the HIV-1 Capsid Protein. His dissertation entitled, "Defining and Characterizing the Step in the HIV-1 Life Cycle Affected by Fullerene Derivatives," was supervised by Dr. Manuel Llano.

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