Fullerene Derivatives Strongly Inhibit HIV-1 Replication by Affecting Virus Maturation without Impairing Protease Activity.

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Running Head: Fullerene derivatives block HIV-1 maturation

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ABSTRACT

Three compounds (1, 2, and 3) previously reported to inhibit HIV-1 replication and/or in vitro activity of reverse transcriptase were studied, but only fullerene derivatives 1 and 2 showed strong anti-viral activity on the replication of HIV-1 in human CD4+ T cells. However, these compounds did not inhibit infection by single-round infection VSV-G pseudotyped viruses, indicating no effect on the early steps of the viral life cycle. In contrast, analysis of single-round infection VSV-G pseudotyped HIV-1 produced in the presence of 1 or 2 showed a complete lack of infectivity in human CD4+ T cells, suggesting that the late stages of the HIV-1 life cycle were affected. Quantification of virion-associated viral RNA and p24 indicates that RNA packaging and viral production were unremarkable in these viruses. However, Gag and Gag-Pol processing was affected, as evidenced by immunoblot analysis with an anti-p24 antibody and the measurement of virion-associated reverse transcriptase activity, ratifying the effect of the fullerene derivatives on virion maturation of the HIV-1 life cycle. Surprisingly, fullerenes 1 and 2 did not inhibit HIV-1 protease in an *in vitro* assay at the doses that potently blocked viral infectivity, suggesting a protease-independent mechanism of action. Highlighting the potential therapeutic relevance of fullerene derivatives, these compounds block infection of HIV-1 resistant to protease and maturation inhibitors.

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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 (12). 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 (28, 40, 44). Therefore the development of new and safer anti-HIV compounds is a critical need (11, 12, 23).

Fullerenes consist of carbon atom cages, some, like C60, have the shape of a hollow sphere, similar to a soccer ball (27). Due to their ability to be extensively derivatized, functionalized fullerenes have shown several biological applications (2, 3). It has been hypothesized 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 (2, 3, 13). The first fullerene derivatives that exhibited anti-HIV activity were reported in 1993 (15, 46). However, the lack of comprehensive characterization of the antiviral mechanisms of fullerene derivatives has hindered their fur-

ther development into therapeutic drugs (3, 15, 17, 25, 33, 36, 47, 50, 53). 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 (8, 15, 17, 35, 63). However, this model lacks support from empirical evidences. In addition, *in vitro* assays indicate that some fullerene derivatives possess anti-reverse transcriptase activity (37).

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+ T cells. Our data demonstrate for the first time that the viral maturation process is the step of the HIV-1 life cycle affected. After 23 years of assuming that inhibition of HIV-1 was due to the interaction between the fullerene and the hydrophobic pocket of the protease, unexpectedly we discovered that fullerene derivatives do not inhibit HIV-1 protease at doses that potently inhibit HIV-1 infection. Furthermore, we observed that these compounds are effective in blocking replication of viruses that are resistant to the clinically approved protease inhibitors.

MATERIALS AND METHODS

Synthesis of fullerene derivatives. Compounds 1, 3 and the regioisomeric mixture 2 have been previously reported (10, 32, 36, 39), here we report slight modifications for the synthesis of compounds 1, 2, 3 and the synthesis of compound 4 (24) (Figure 1). Please, refer to the supporting information for details on the synthesis and physico-chemical characterization of these compounds.

Plasmids. The plasmids used to generate retroviral vectors were described previously (31). HIV-1-derived vectors were produced using pHIV Luc and pMD.G. pHIV Luc was derived from pNL4-3.Luc.R^-E^- (22) by introducing a deletion in the env open reading frame (diagrammed in figure 4). 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 (56).

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.

Generation of retroviruses. Procedures previously described were followed (19). Briefly, 3 x 106 HEK293T cells were plated in a T75 cm2 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 124750 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.

Single-round infectivity assay. SupT1 cells were plated at 1 x 105 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).

HIV-1 replication assays. SupT1 cells (0.25 x 10^6 cells in 3 mL RPMI 1640) were infected with HIV-1NL4-3 harboring protease wild type or mutant resistant to multiple protease inhibitors (2.1 ng of HIV-1 p24) in the presence of fullerene derivatives or DMSO, or in the absence of any with HIV-1NL4-3 containing CA mutations L363F and V362L/L363M (VL/LM) that were produced in the presence of DMSO or fullerene derivatives. 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.

TZM-bl cells were also used for infection assays with replicating competent HIV-1. This HeLa-derived indicator cell line expresses CD4, CXCR4, CCR5 and has luciferase and ß-galactosidase expression cassettes driven by the HIV-1 promoter stably inserted in the genome (7, 41, 42, 49, 58). TZM-bl cells (1x10^5 cells/well) were plated in a p24-wells plate and the next day infected with p24-normalized HIV-1, respectively. Seventy-two hours post-infection cells were lysed in PBS-1% Triton X-100 and luciferase activity measured as described above.

HIV-1 p24 ELISA. HIV-1 p24 levels were determined by a sandwich ELISA following manufacturer instructions. Briefly, 200 µL 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 µL of washing buffer, and bound HIV-1 p24 was detected by incubating each well with 100 µL 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 µL of substrate buffer for 30

137 min at room temperature until the reaction was stopped by adding 100 µL of stop solu-
138 tion into each well. The absorbance of each well was determined at 450 nm using a mi-
139 croplate reader (Molecular Devices, Versa max microplate reader).

140 **Cellular viability assay**. 1 x 104 SupT1 cells were plated in a 96-well plate in 100 µL
141 RMPI1640 culture media and left untreated or treated with fullerene derivatives, DMSO
142 (control), or 2 mM hydrogen peroxide (positive control). Fullerene derivatives were eval-
143 uated at a concentration that ranged from 3 µM to 32 µM. The cells were cultured in the
144 presence of the indicated compounds for 24 h and then 20 µL of the MTS were added
145 to each well of cells. Incubation with the reagent for an additional 3 h was allowed. The
146 colored formazan product was measured by absorbance at 490 nm with a reference
147 wavelength of 650 nm using a microplate reader (SpectraMax 190, Molecular Devices).
148 Control wells containing the same volumes of culture medium and MTS reagent were
149 used to subtract background absorbance.

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

155 **HIV-1 protease in vitro activity assay**. The effect of DMSO, compounds 1 and 2 (3
156 and 10 µM), and Indinavir (0.1 µM) on HIV-1 protease activity was measured using the
157 ProAssayTM HIV-1 Protease Assay Kit. This assay uses purified recombinant HIV-1
158 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.

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®). The sequences of the oligonucleotides used in the qPCRs are available upon request.

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 mAmp 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). HIV-1 integrase was

detected with anti-integrase (Catalog # sc-69721, Santa Cruz Biotechnology). Primary antibody-bound membranes were washed in TBS-0.1% Tween 20 and all bound antibodies detected with goat anti-mouse IgG-HRP (1/2000, KPL, 074-1806) followed by chemo-luminescence detection.

Real time PCR analysis of early steps of the HIV-1 life cycle. Procedures previously described were followed (56). Briefly, 1 x 106 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 (56). Fold change was calculated using the ΔCt method as recommended in the thermo-cycler manual.

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RESULTS

Effect of fullerene derivatives on HIV-1 replication in human CD4+ T cells. C_{60} fullerene derivatives were previously shown to inhibit HIV-1 replication, but its effect on the different steps of the viral life cycle was not evaluated (26, 34, 45, 51). In particular, compounds 1, 2, and 3 (Figure 1) have been shown to inhibit HIV-1 reverse transcriptase or protease in *in vitro* assays (18, 37, 45, 52). 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+ T cells (Figure 2). SupT1 cells were infected with HIV-1NL4-3 in the presence of compounds 1, 2, and 3 at 1, 3, and 10 μM 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 2 indicate that compounds 1 and 2 potently inhibited viral replication at 3 μM. The effect of compound 1 on HIV-1 replication at 3 and 10 μM were indistinguishable and showed a 300-fold inhibition of HIV-1 viral replication (Data not shown). The regioisomeric compound mixture 2 at 3 μM inhibited HIV-1 replication to a similar extent as compound 1, and was still active at 1 μM (Figure 2b); however, compound 1 was inactive at 1 μM (Figure 2a). 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 μM (data not shown). Therefore, our data support the anti-HIV-1 activity reported for compounds 1 and 2 (26, 34, 45, 51) but

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failed to validate in human cells the effect reported for compound 3 using *in vitro* assays (37).

Function/structure analysis of the fullerene derivatives characterized (Figure 1) 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.

Effect of fullerenes on cellular viability. We have observed that compounds 1 and 2 potently inhibit HIV-1 infection (Figure 2). 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 μM, 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 3). Therefore, the effects on HIV-1 infection observed with compounds 1 and 2 at 3 μM 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, LC50 for compound 1 and 2 was 22.9 μM and 38.3 μM respectively, as calculated by regression analysis of data in figure

3. These results also indicate that compounds 1 and 2 inhibit HIV-1 infection at doses that are not toxic to the target cells.

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 1), an isomerically pure regioisomer (cis-2, endo-endo) (24) with two pyrrolidinium rings connected by a benzene bridge. 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 μM 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 4a clearly indicate that none of the fullerene derivatives affect the early steps of the viral life cycle. 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 2 and 4a suggest that the late phase of the viral life cycle is targeted by compounds 1 and 2.

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 (3 μM) and/or Indinavir (0.1 μM) 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. Transfection efficiency was quantified by FACS analysis of eGFP.

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 4b 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.

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 5a). 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 4a). 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 5b). Altogether, the data shown in figures 4b and 5a-b show that fullerene derivatives target the viral maturation process.

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 impair 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 6a 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 6a II and III, respectively). As expected, this inhibitory effect of fullerene derivatives was also observed in DNA extracted four days after infection (Figure 6b). Importantly, findings in figure 6 correlated with the lack of infectivity reported in figure 4b, indicating a severe defect in the infectivity of viruses produced in the presence of compounds 1 and 2.

HIV-1 Gag processing in fullerene-treated virions. The fact that fullerene severely blocks infectivity of viruses produced in their presence (Figure 4b and 6) but not mature viruses (Figure 4a) 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 μM), or Indinavir (0.1 μM). 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 7a, Gag processing was affected by compounds 1 or 2 at 3 μM to a similar extent of Indinavir at 0.1 μM. These three inhibitors blocked Gag processing at different cleavag-

es sites including MA-CA-SP1-NC (~50 kDa), MA-CA (~41 kDa), and CA-SP1-NC (~33 kDa) (Figure 7b). However, integrase processing was not altered by fullerene derivatives at 3 μM or Indinavir at 0.1 μM (Figure 7b) although at these doses these drugs significantly affected HIV-1 infection (Figure 2 and 4b). Nevertheless, Indinavir at higher doses (10 μM) severely impaired Gag and integrase processing (Data not shown).

Therefore, data in figures 7a and b indicate that fullerene derivatives, similar to indinavir, impair Gag processing. To further evaluate the effects of fullerene derivatives (3 μM) and Indinavir (0.1 μM) 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 7c), 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 7c) highlights the relevance of the fullerene addend groups in their activity. Future work will address the effects of specific regioisomers in 2.

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 (8, 15, 17, 25, 35, 63). 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 7b) 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. Data in figure 7a indicated that indinavir and compounds 1 and 2 block the processing of the native MA-CA (p17/p24) cleavage site of HIV-1 protease on Gag, therefore a peptide containing this site was selected for evaluation of the effect of these compounds on the activity of protease. Recombinant protease was incubated with the substrate, an HIV-1-derived FRET peptide containing the MA-CA cleavage site, in the presence of DMSO, fullerene derivatives, or Indinavir, and fluorescence emission was tracked for 90 mins (Figure 8).

As expected, Indinavir completely blocked the activity of protease but surprisingly, compounds 1 and 2 were inactive at 3 μM (Figure 8a), a concentration that severely impairs HIV-1 infection (Figures 2 and 4b). In further support, even at 10 μM, fullerene derivatives only minimally affect protease activity (Figure 8b). Only at toxic concentrations (Figure 3), above 40 μM, fullerenes 1 and 2 inhibited protease activity (Data not shown). 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 not via HIV-1 protease, contradicting the multiple studies previously reported (8, 15, 17, 35, 45, 52, 63).

Given that our results challenge the existing fullerene-protease paradigm, for completeness we decided to study the HIV-1 protease activity of the originally reported compound by Friedman in 1993 (scheme 4S in supplementary information, compound 5) (15). As expected, compound 5 inhibited HIV-1 viral replication (Figure A12) as compounds 1 and 2 did, but was inactive in the *in vitro* activity of HIV-1 protease using the

FRET peptide-based assay at 3 and 10 μM (Figure A13). Therefore, our results clearly show that fullerene derivatives are potent anti-HIV agents but their mechanistic activity does not involve HIV-1 protease binding.

Effect of fullerene derivatives on the infectivity of HIV-1 resistant to protease or maturation inhibitors. 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 further, we determined the sensitivity to compound 1 of multi-protease inhibitor-resistant HIV-1 recombinant infectious molecular clones previously described (56). Compound 1 and not 2 was chosen for these experiments because compound 1 is the pure regioisomer (trans-3) contained in compound 2, a regioisomeric mixture. 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 (55).

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 μM), or Indinavir (0.1 μM). 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 9a).

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 (55), it has a replication capacity of 63% of protease wild type HIV-1. SupT1 cells were infected with NL4-3 or 11803 in the presence of DMSO or compound 1 (3 μM) 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 9b confirmed that compound 1 strongly inhibits viral replication of the multi-protease inhibitor-resistant virus 11803 to a similar extent than the protease wild type NL4-3 virus. Therefore, data in figure 9 highlight the potential clinical relevance of fullerene derivatives to block replication of multi-protease inhibitor resistant viruses.

In addition, we evaluated the effect of compound 1 on the infectivity of HIV-1 NL4-3 harboring mutations at the end of CA, L363F and V362L/L363M. These viruses have been demonstrated to be resistant to the maturation inhibitor 3-O-(3',3'-dimethylsuccinyl) betulinic acid (DSB) (60, 62), and to fail to interact with DSB (38, 61). Therefore, compounds blocking these mutant viruses have the potential to be clinically relevant (57). Viruses were produced by plasmid transfection in HEK293T cells in the presence of DMSO or compound 1 and their infectivity was determined in TZM-bl cells

using p24-normalized HIV-1. In these experiments compound 1 was used at a concentration that only affect by 50% the infectivity of HIV-1 NL4-3 wild type, allowing detection of small differences in the sensitivity of mutant and wild type viruses. Comparison of the infectivity of viruses produced in the presence of DMSO or compound 1 (Figure 9d) indicated that compound 1 was as active against HIV-1 NL4-3 V362L/L363M (Figure 9d lane 1) and L363F (Figure 9d lane 2) mutants as the wild type viruses (Figure 9d lane 3). Therefore, these data indicated no cross-resistance between DSB and compound 1, further highlighting the potential anti-HIV clinical relevance of fullerene derivatives.

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DISCUSSION

Fullerene derivatives have been demonstrated to impair HIV-1 replication in human cells; (15, 25, 33, 45, 50) however, detailed virological characterization of the activity of these compounds is absent, limiting their development as anti-HIV therapeutic agents. Defining the step in the viral life cycle affected by fullerene derivatives allows for a better understanding of their anti-HIV mechanism. Molecular docking predictions have proposed that fullerene derivatives could bind to the active site of HIV-1 protease while the enzyme is in its catalytically active conformation as a homodimer (9, 15, 16, 35, 63). Other groups have proposed that certain derivatives inhibit recombinant HIV-1 reverse transcriptase in vitro activity (37).

Encouraged by these initial findings and the need for novel anti-HIV therapeutics, we decided to fully characterize the anti-HIV-1 activity of fullerene derivatives 1 and 2. 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 (37) or protease (9, 15, 16, 35, 45, 53, 63) as their targets. Instead, we have demonstrated for the first time that fullerene inhibitors block HIV-1 maturation. HIV-1 is released from infected cells in the form of immature, non-infectious virions that then proceed through maturation before gaining full infectivity. Viral maturation is triggered by proteolytic processing of Gag and Gag-Pol polyproteins by HIV-1 protease. This processing results in the production of functional viral proteins which assemble into the mature virion (14, 48). HIV-1 maturation can be pharmacologically hindered by drugs that affect the activity of the viral protease (protease inhibitors) or that bind to the polyproteins (maturation inhibitors). The importance of Gag- and Gag-Pol-derived proteins in every step of the viral life cycle

447 provides an opportunity to profoundly decrease HIV-1 infectivity by protease and matu-
448 ration inhibitors (14, 48).

449 We have evidenced that fullerene derivatives block HIV-1 maturation by inhibiting
450 Gag processing through a protease-independent mechanism. We speculate that inter-
451 action of fullerene derivatives with unprocessed Gag could modify the conformation or
452 assembly of these substrates, altering the specificity of protease, and thereby causing
453 defective processing. Similarly, binding of maturation inhibitors to CA result in structural
454 changes at remote regions on this protein (20, 54). Protease-mediated processing of
455 Gag and Gag-Pol occurs in a strictly organized manner and interfering with this highly
456 ordered process results in immature virions (14, 48); hence, local changes in the protein
457 conformation could globally impact its processing. Importantly, the physical parameters
458 that regulate the interaction of protease with its substrates is unclear (1, 29), perhaps
459 gaining an understanding of fullerene activity will shed light on of this phenomenon.

460 Our findings also highlight the potential therapeutic relevance of fullerene deriva-
461 tives to block clinically relevant resistant viruses. HIV-1 resistant to multiple clinically
462 used protease inhibitors, or to the maturation inhibitors DSB and PF-46396 (4, 5, 43,
463 59-62) were also potently blocked by compound 1.

464 We have also demonstrated that the addends on the C_{60} fullerenes and their re-
465 giochemistry have pronounced effects on their anti-HIV-1 activity, beyond simple water
466 solubility effects. The difference in anti-HIV-1 activity between compounds 1, 2, 3 and 4
467 is completely reliant on the chemical nature of their side chains. Previously it has been
468 reported that different regioisomers of 2 exhibit similar HIV-1 inhibitory activity and it

469 was concluded that the trans-3 compound 1 is more potent than the corresponding cis-3
470 isomer (33, 36). The fact that the regiochemistry influences the anti-HIV-1 activity of
471 these fullerene derivatives is important and is currently under investigation in our labora-
472 tories.

473 In summary, our data indicate that fullerene derivatives affect virion maturation of
474 HIV-1 wild type and protease and maturation inhibitors resistant viruses by impairing
475 viral polyprotein processing through a protease-independent mechanism, a paradigm-
476 shifting finding.

477

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704 **FIGURE LEGENDS**

705 **Figure 1**. Fullerene derivatives 1 and 2 (C60-bis(N,N-dimethylpyrrolidinium iodide), 3
706 (fullereno-C60-pyrrole-2,5-dicarboxylic acid-1-(carboxymethyl)-1,5-dihydro and 4 cis-2-
707 C60-bis(N,N-ethylmethylpyrrolidinium iodide).

708 **Figure 2**. Effect of fullerene derivatives on HIV-1 replication. SupT1 cells were treated
709 with DMSO (+) or compounds 1 **a**) and the regioisomeric mixture 2 **b**) at 1 μM (□), 3 μM
710 (Δ), or 10 μM (x) at the time of infection with HIV-1 NL4-3. 24 h later the compounds
711 and virus were removed and infected cells were cultured for up to 2 weeks. The amount
712 of HIV-1 p24 antigen was determined in cell supernatant by ELISA. Results from one
713 experiment are shown.

714 **Figure 3**. Assessing the cytotoxicity of compounds 1 and 2. SupT1 cells were treated
715 with DMSO, compound 1 (□), or regioisomeric mixture 2 (x) at varying concentrations
716 for 24 h and the amounts of viable cells were determined by the tetrazolium dye reduc-
717 tion assay. Cell viability values were normalized to DMSO-treated cells. Results shown
718 are representative of two independent experiments.

719 **Figure 4**. **Analysis of the viral life cycle step affected by compounds 1, 2, 3 and 4.**
720 **a**) Effects on the early stages of the HIV-1 viral life cycle. SupT1 cells were infected with
721 single-round infection HIV-1 viruses (**c**) in the presence of DMSO or fullerene deriva-
722 tives (10 μM), and analyzed for luciferase expression and cellular viability (ATP content)
723 three days later. Luciferase was normalized to cellular viability. **b**) Effects on the late
724 phase of the HIV-1 infection. Single-round infection HIV-1 virus were produced in the

presence of DMSO, Indinavir (0.1 μM), or fullerene derivatives (3 μM) 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. **c**) The HIV-1 reporter virus used was previously described (6, 21, 30) and includes a frameshift mutation in vpr (circle) and a deletion of 430 nts in env (oval), the luciferase open reading frame is replacing nef.

Figure 5. 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 represent two experiments, standard deviation indicate the variability of multiple readings.

Figure 6. 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 μM), or fullerene derivatives (3 μM). 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.

747 **Figure 7**. Effects of compounds 1 and 2 on Gag and Gag-Pol processing. Protease-
748 mediated processing of capsid (p24) (**a**) and integrase (**b**) was evaluated in virions by
749 immunoblot. **c**) Reverse transcriptase activity of virions was measured by the exoge-
750 nous reverse transcription assay. Results are representative of one (**a** and **b**) or three
751 (**c**) independent experiments.

752 **Figure 8**. Effect of compounds 1 and 2 on the *in vitro* activity of HIV-1 protease. The
753 cleavage of an HIV-derived FRET peptide by recombinant HIV-1 protease in the pres-
754 ence of compounds 1 (Δ) and 2 (x) at 3 μM a) or 10 μM b) was determined by fluores-
755 cence measurements. DMSO (◊) and Indinavir (□) were used as negative and positive
756 controls, respectively. Experiments were performed in duplicates.

757 **Figure 9**. Activity of compound 1 on the infectivity of HIV-1 harboring multi-protease
758 inhibitor-resistant protease mutants. **a**) HIV-1 viruses harboring protease wild type or
759 mutants were produced in the presence of DMSO, compound 1 (3 μM), or Indinavir (0.1
760 μM), and their infectivity evaluated in single-round infection assays. **b**-**c**) SupT1 cells
761 were infected with HIV-1 NL4-3 harboring a wild type (**b**) or a multi-protease inhibitor-
762 resistant protease mutant (virus 11803) (**c**) in the presence of DMSO (o) or compound 1
763 (3 μM) (□). Results are representative of one (**a**), two (**b**), or more than four (**c**) inde-
764 pendent experiments.

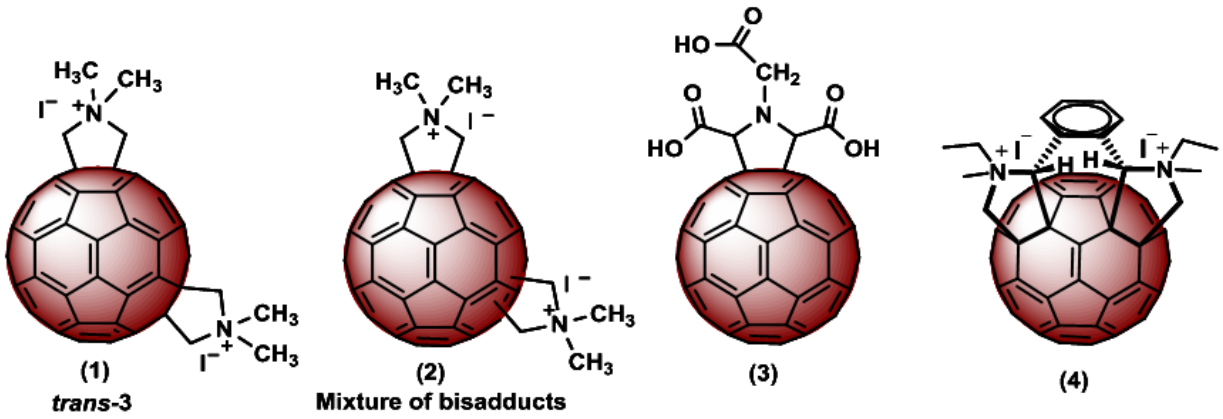

Figure 1. Fullerene derivatives 1 and 2 (C60-bis(N,N-dimethylpyrrolidinium iodide), 3 (fullereno-C60-pyrrole-2,5-dicarboxylic acid-1-(carboxymethyl)-1,5-dihydro and 4 cis-2-C60-bis(N,N-ethylmethylpyrrolidinium iodide).

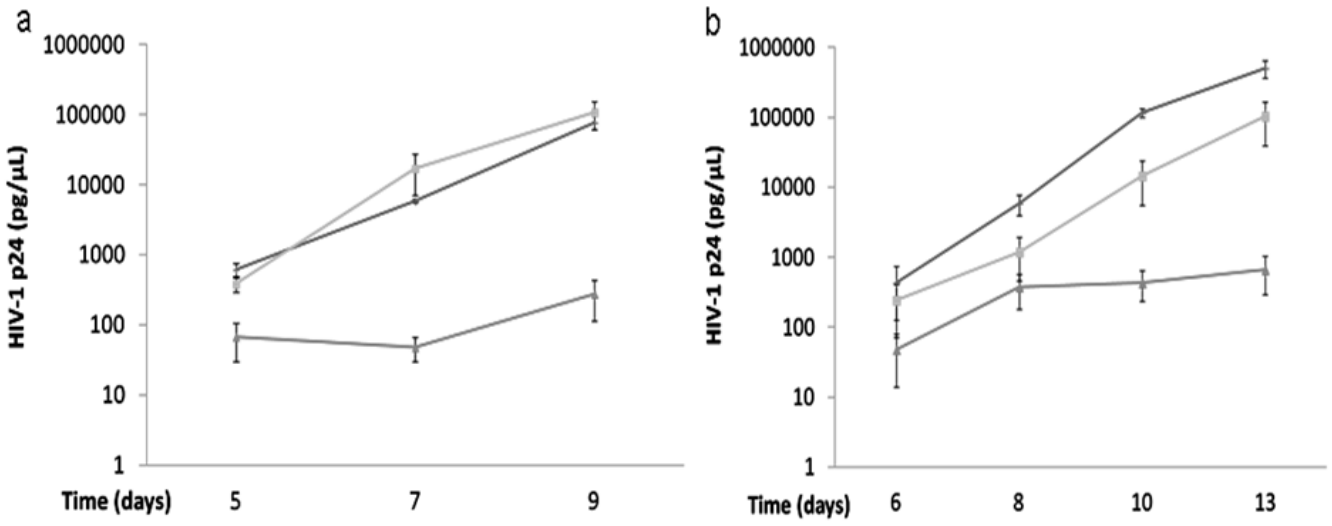

Figure 2. Effect of fullerene derivatives on HIV-1 replication. SupT1 cells were treated with DMSO (+) or compounds 1 **a**) and the regioisomeric mixture 2 **b**) at 1 µM (□) or 3 µM (Δ) at the time of infection with HIV-1 NL4-3. 24 h later the compounds and virus were removed and infected cells were cultured for up to 2 weeks. The amount of HIV-1 p24 antigen was determined in cell supernatant by ELISA. Mean and standard deviation from two experiments are shown.

Martinez et. al. Figure 2

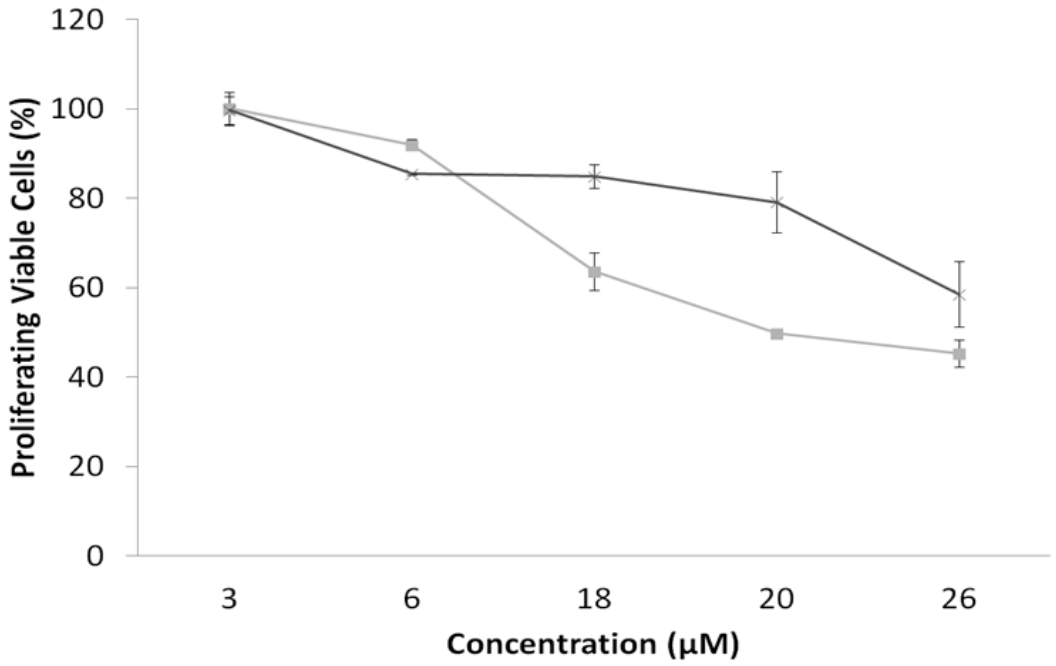

Figure 3. Assessing the cytotoxicity of compounds 1 and 2. SupT1 cells were treated with DMSO, compound 1 (□), or regioisomeric mixture 2 (x) at varying concentrations for 24 h and the amounts of viable cells were determined by the tetrazolium dye reduction assay. Cell viability values were normalized to DMSO-treated cells. Results shown are representative of two independent experiments.

Martinez et. al. Figure 3

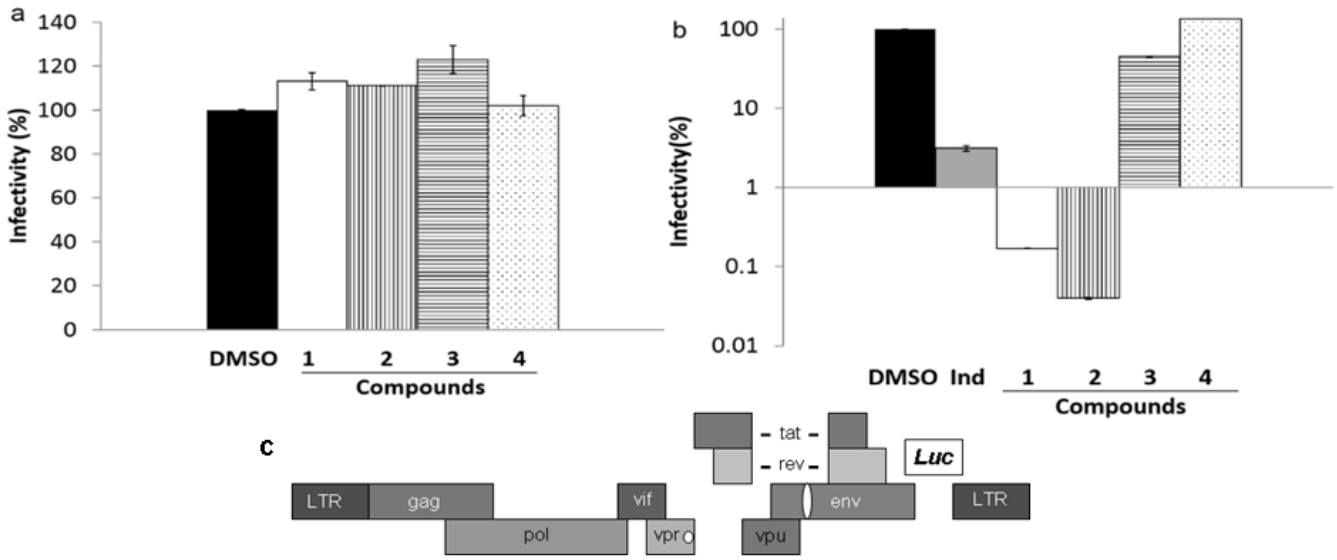

Figure 4. Analysis of the viral life cycle step affected by compounds 1, 2, 3 and 4. **a**) Effects on the early stages of the HIV-1 viral life cycle. SupT1 cells were infected with single-round infection HIV-1 viruses (**c**) in the presence of DMSO or fullerene derivatives (10 μM), and analyzed for luciferase expression and cellular viability (ATP content) three days later. Luciferase was normalized to cellular viability. **b**) Effects on the late phase of the HIV-1 infection. Single-round infection HIV-1 virus were produced in the presence of DMSO, Indinavir (0.1 μM), or fullerene derivatives (3 μM) 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. **c**) The HIV-1 reporter virus used was previously described (6,21,30) and includes a frameshift mutation in vpr (circle) and a deletion of 430 nts in env (oval), the luciferase open reading frame is replacing nef.

Martinez et. al. Figure 4

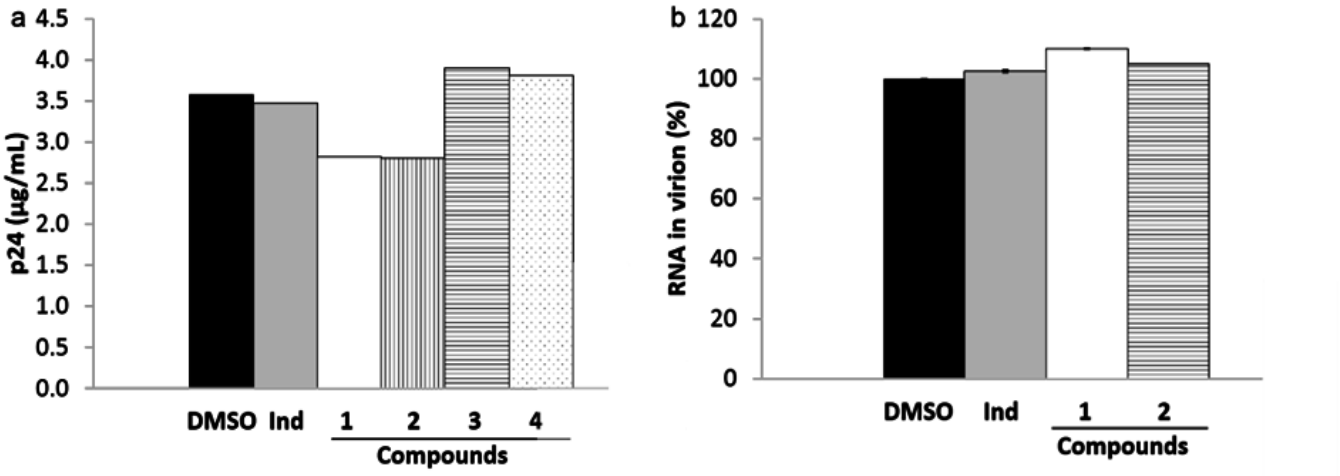

Figure 5. Effects of compounds 1, 2, 3 and 4 on virion production. VSV-G pseudotyped, single-round HIV-1 expressing luciferase were produced in the presence of DMSO or fullerene derivatives (3 µM) and then concentrated by ultracentrifugation and analyzed. Indinavir (0.1 µM) was used as a control. **a**) 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 represent two experiments, standard deviation indicate the variability of multiple readings.

Martinez et. al. Figure 5

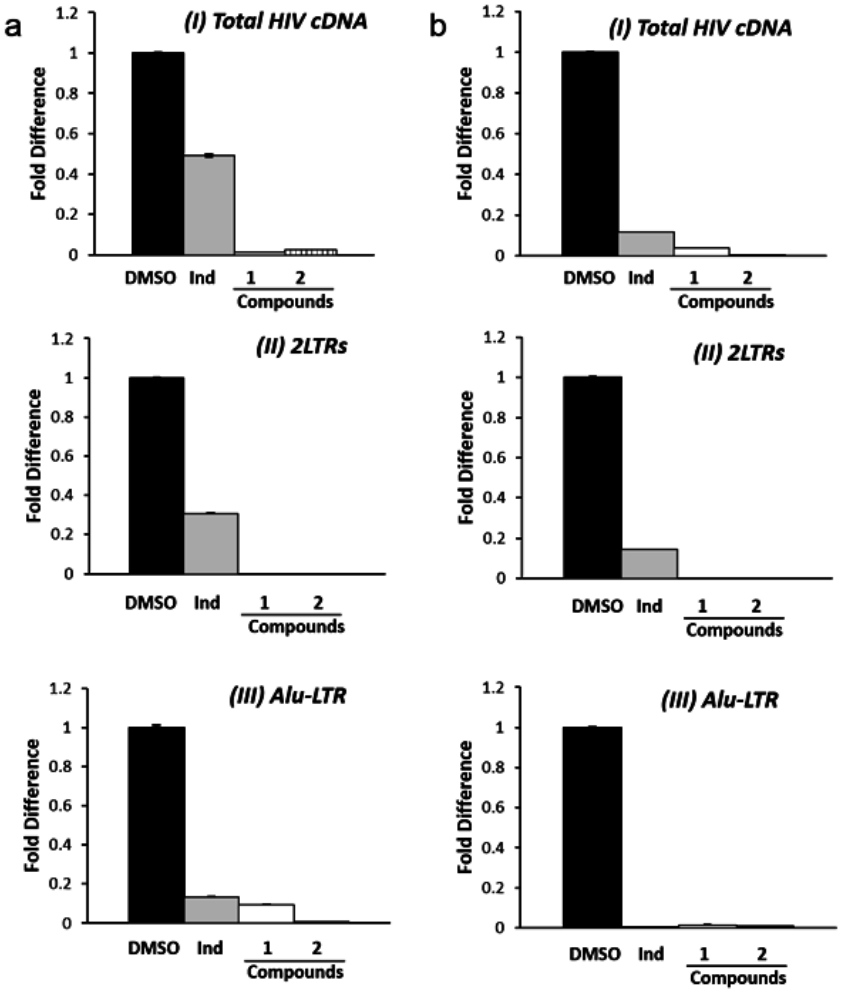

Figure 6. Evaluation of the early steps of the HIV-1 life cycle of virions produced in cells treated with compounds 1 or 2. SupT1 cells were infected with HIV-1 p24-normalized, DNase-treated, single-round HIV-1 viruses produced in the presence of DMSO, Indinavir (0.1 μM), or fullerene derivatives (3 μM). 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.

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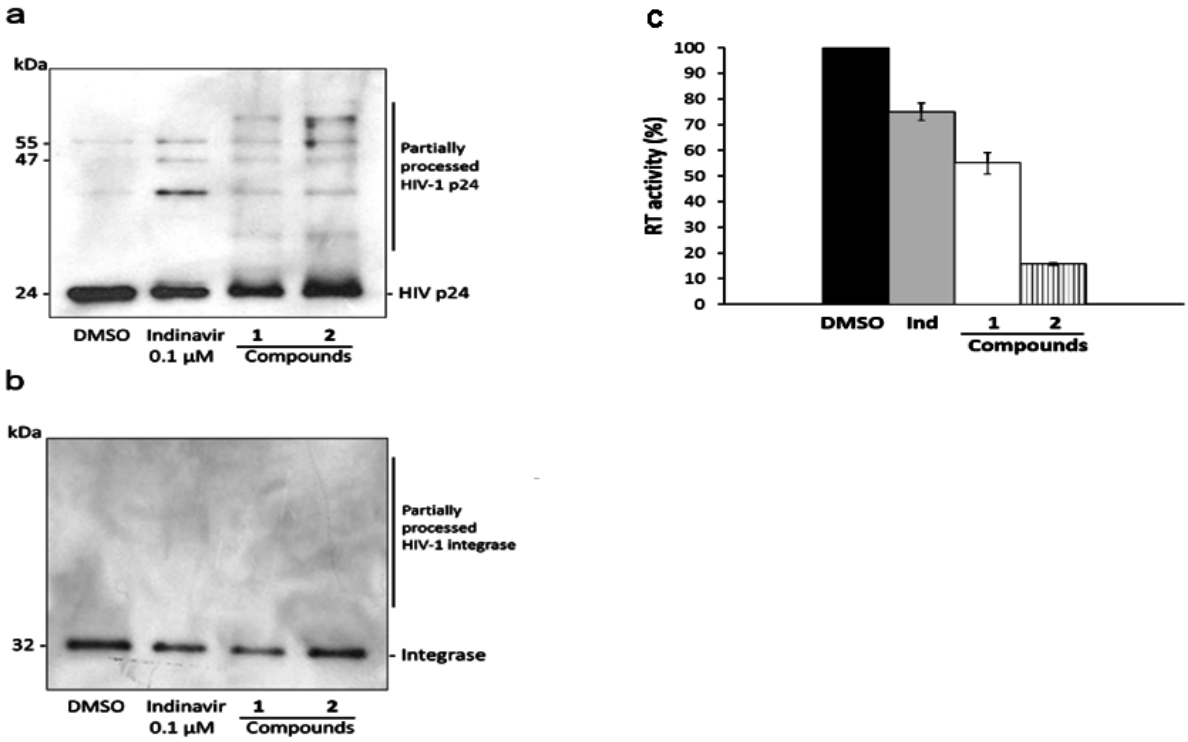

Figure 7. Effects of compounds 1 and 2 on Gag and Gag-Pol processing. Protease-mediated processing of capsid (p24) (**a**) and integrase (**b**) was evaluated in virions by immunoblot. **c**) Reverse transcriptase activity of virions was measured by the exogenous reverse transcription assay. Results are representative of one (**a** and **b**) or three (**c**) independent experiments.

Martinez et. al. Figure 7

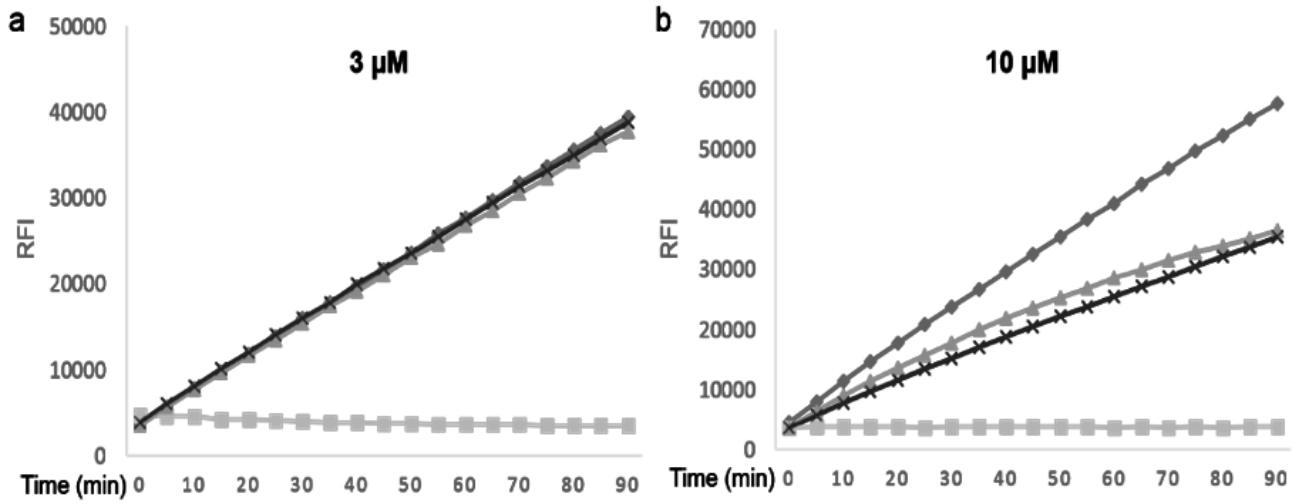

Figure 8. Effect of compounds 1 and 2 on the *in vitro* activity of HIV-1 protease. The cleavage of an HIV-derived FRET peptide by recombinant HIV-1 protease in the presence of compounds 1 (Δ) and 2 (x) at 3 µM a) or 10 µM b) was determined by fluorescence measurements. DMSO (◊) and Indinavir (□) were used as negative and positive controls, respectively. Experiments were performed in duplicates.

Martinez et. al. Figure 8

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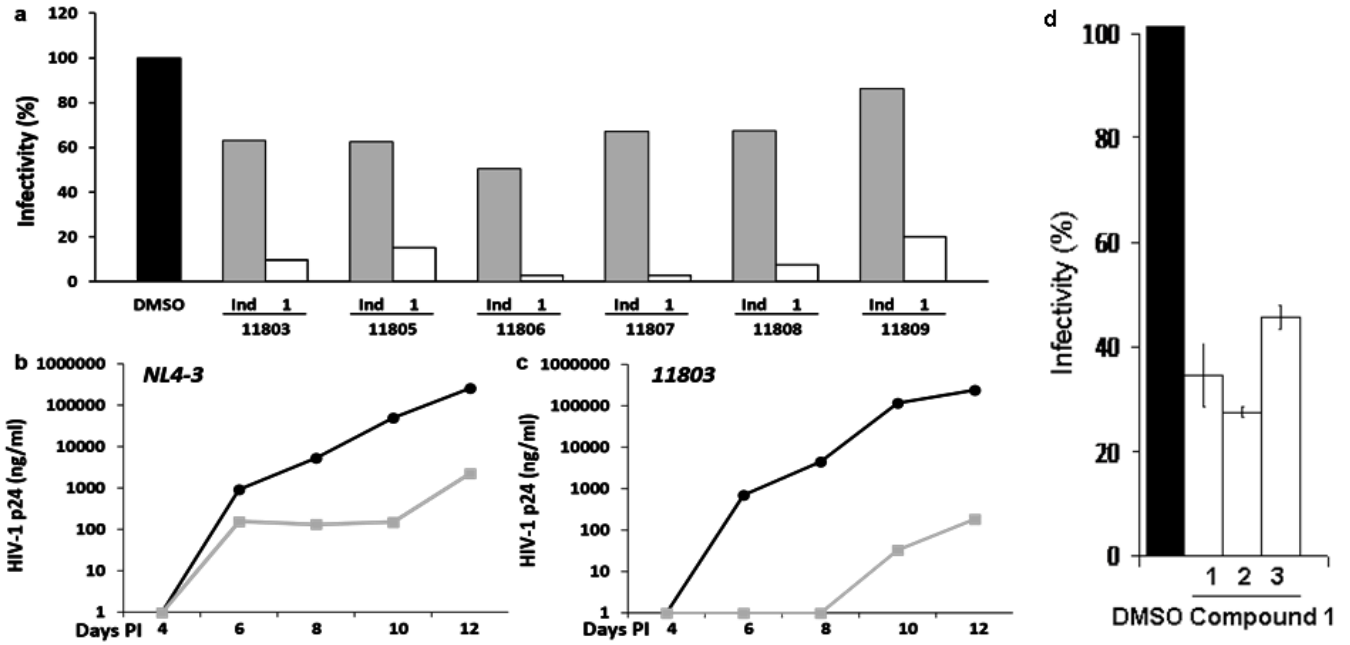

Figure 9. Effect of compound 1 on the infectivity of HIV-1 resistant to protease and maturation inhibitors. (**a**) Single-round infection of HIV-1 harboring protease inhibitor-resistant protease mutants produced in the presence of DMSO, fullerene 1 (3 μM), or Indinavir (0.1 μM) in SupT1 cells. (**b-c**) Replication of HIV-1 wild type or strain 11803 (described in **a**) in SupT1 cells treated with DMSO (o) or compound 1 (3 μM) (□) at the time of infection. (**d**) Infectivity in TZM-bl cells of HIV-1 harboring CA mutants resistant to maturation inhibitors (**1-2**), or wild type (**3**) produced in the presence of DMSO or fullerene 1. Results are representative of one (**a**), two (**b**), five (**c**), and three (**d**) independent experiments.

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