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

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**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 antiviral activity on the replication of HIV-1 in human CD4$^+$ T cells. However, these compounds did not inhibit infection by single-round infection vesicular stomatitis virus glycoprotein G (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 compound 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 by HIV-1 resistant to protease and maturation inhibitors.**

Advances in anti-HIV retroviral drugs have led to a significant reduction in AIDS-related deaths, delayed disease progression, and diminished rates of HIV transmission (1). 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 due mainly 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 antiretroviral drugs have important side effects that limit their use (2–4). Therefore, the development of new and safer anti-HIV compounds is a critical need (1, 5, 6).

Fullerenes consist of carbon atom cages, some, like $C_{60}$ fullerenes, having the shape of a hollow sphere, similar to a soccer ball (7). Due to their ability to be extensively derivatized, functionalized fullerenes have shown several biological applications (8, 9). 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 (8–10). The first fullerene derivatives that exhibited anti-HIV activity were reported in 1993 (11, 12). However, the lack of comprehensive characterization of the antiviral mechanisms of fullerene derivatives has hindered their further development into therapeutic drugs (9, 11–18). 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. Evidence supporting this mechanism is based mostly on molecular docking simulations that predict the binding of these compounds to the active site of HIV-1 protease due to their size and conformational complementarity (11, 13, 19–21). However, this model lacks support from empirical data. In addition, *in vitro* assays indicate that some fullerene derivatives possess anti-reverse transcriptase activity (16).

Therefore, to better understand the mechanism of action of fullerene derivatives in HIV replication, we investigated the effects 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 and 3 and the regioisomeric mixture 2 have been previously reported (16, 22–24); here we report slight modifications for the synthesis of compounds 1, 2, and 3 and the synthesis of compound 4 (25) (Fig. 1). Please refer to the supplemental

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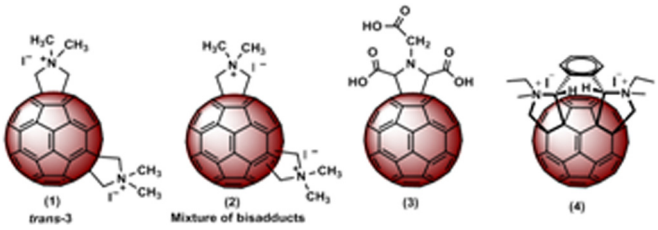


**FIG 1** Structures of fullerene derivatives 1 and 2, $C_{60}$-bis(N,N-dimethylpyrrolidinium iodide), derivative 3, fullereno-$C_{60}$-pyrrole-2,5-dicarboxylic acid-1-(carboxymethyl)-1,5-dihydro, and derivative 4, cis-2-$C_{60}$-bis(N,N-ethylmethylpyrrolidinium iodide).

material for details on the synthesis and physicochemical characterization of these compounds.

**Plasmids.** The plasmids used to generate retroviral vectors were described previously (26). HIV-1-derived vectors were produced using pHIV Luc and pMD.G. pHIV Luc was derived from pNL4-3.Luc.R$^-$E$^-$ (27) by introducing a deletion in the env open reading frame (see Fig. 4). pHIV Luc, containing multiple protease inhibitors resistant to protease mutants was constructed by swapping a 4.3-kb SalI/SpeI 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 (28).

**Cell lines.** SupT1 and HEK293T cells were grown in RPMI 1640 and in Dulbecco's modified Eagle medium (DMEM), respectively. All culture media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin.

**Generation of retroviruses.** Procedures previously described were followed (29). Briefly, $3 \times 10^6$ HEK293T cells were plated in a T 75-cm$^2$ tissue culture flask and cotransfected the next day with the corresponding plasmids by the calcium-phosphate precipitation method. Eighteen hours later, the transfection medium was replaced with fresh medium containing no drug or containing 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 $\times$ *g* for 2 h on a 20% sucrose cushion. Viral preparations were stored at −80°C until used.

VSV-G-pseudotyped HIV-derived reporter viruses expressing firefly luciferase (HIVluc) and harboring wild-type or multi-protease-inhibitor-resistant protease mutants were prepared by cotransfection 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 $1 \times 10^5$ cells in 500 μl of RPMI 1640 culture medium in 24-well plates and infected with HIVluc. Four days postinfection, cells were collected by centrifugation at 1,000 $\times$ *g* for 6 min, and the pellet was resuspended in 200 μl of phosphate-buffered saline (PBS). One 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 (Luminoskan Ascent; Thermo Scientific).

**HIV-1 replication assays.** SupT1 cells ($0.25 \times 10^6$ cells in 3 ml RPMI 1640) were infected with HIV-1NL4-3 harboring wild-type protease or mutant protease resistant to multiple protease inhibitors (2.1 ng of HIV-1 p24) in the presence of fullerene derivatives or DMSO or were infected in the absence of any derivative or vector with HIV-1NL4-3 containing CA mutations L363F and V362L/L363M (VL/LM) that were produced in the presence of DMSO or fullerene derivatives. Twenty-four hours 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 postinfection and used for HIV-1 p24 quantification by ELISA.

TZM-bl cells were also used for infection assays with replication-competent HIV-1. This HeLa-derived indicator cell line expresses CD4, CXCR4, and CCR5 and has luciferase and β-galactosidase expression cassettes driven by the HIV-1 promoter stably inserted in the genome (30–34). TZM-bl cells ($1 \times 10^5$ cells/well) were plated in a p24 well plate and the next day infected with p24-normalized HIV-1, respectively. Seventy-two hours postinfection, cells were lysed in PBS–1% Triton X-100, and luciferase activity was measured as described above.

**HIV-1 p24 ELISA.** HIV-1 p24 levels were determined by a sandwich ELISA according to the manufacturer's instructions. Briefly, 200 μl of the viral samples was 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 min at room temperature until the reaction was stopped by adding 100 μl of stop solution into each well. The absorbance of each well was determined at 450 nm using a microplate reader (Versa max microplate reader; Molecular Devices).

**Cellular viability assay.** SupT1 cells ($1 \times 10^4$) were plated in a 96-well plate in 100 μl RMPI 1640 culture medium 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 μM to 32 μM. The cells were cultured in the presence of the indicated compounds for 24 h, and then 20 μl of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium (MTS) reagent was 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.

**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 according to the manufacturer's instructions. HIV-1 p24-normalized amounts (4.34 μg) were analyzed for each compound-treated virus.

**HIV-1 protease *in vitro* activity assay.** The effects of DMSO, compounds 1 and 2 (3 and 10 μM), and indinavir (0.1 μM) on HIV-1 protease activity were measured using the ProAssayTM 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 into HIV-1 protease buffer supplemented with 1 mM (final concentration) dithiothreitol (DTT) on ice and protected from light, and the mixture was immediately transferred onto a black 96-well plate containing the compounds being evaluated. The relative fluorescence intensity (RFI) was measured with a fluorometer at excitation/emission wavelengths of 490 nm/530 nm every 5 min during 90 min.

**Quantification of virion-associated 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 an-

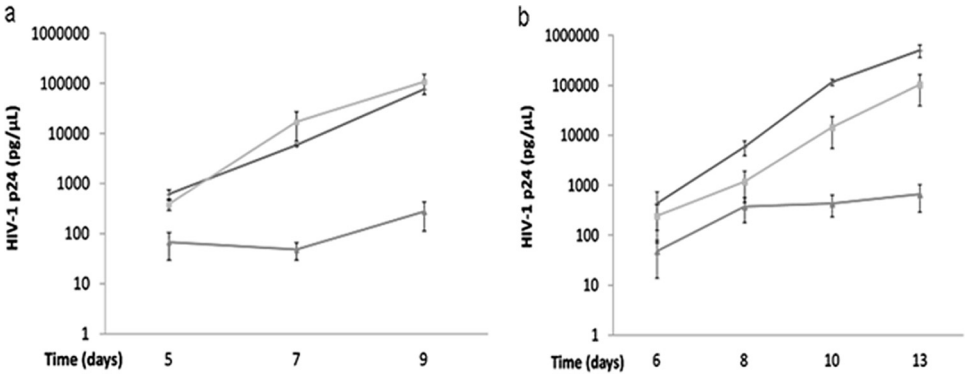


**FIG 2** Effects of fullerene derivatives on HIV-1 replication. SupT1 cells were treated with DMSO (+) or compound 1 (a) and the regioisomeric mixture 2 (b) at 1 μM (■) or 3 μM (Δ) at the time of infection with HIV-1 NL4-3. Twenty-four hours later, the compounds and virus were removed, and infected 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.

alyzed using a quantitative real-time PCR (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 polyvinylidene difluoride (PVDF) membranes at 100 mA at 4°C. Membranes were blocked in Tris-buffered saline (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 antibody obtained from the NIH AIDS Reagent Program (catalog number 1513). HIV-1 integrase was detected with anti-integrase antibody (catalog number sc-69721; Santa Cruz Biotechnology). Primary-antibody-bound membranes were washed in TBS–0.1% Tween 20, and all bound antibodies were detected with goat anti-mouse IgG-horseradish peroxidase (IgG-HRP; 1/2,000; catalog number 074-1806; KPL) followed by chemoluminescence detection.

**Real-time PCR analysis of early steps of the HIV-1 life cycle.** Procedures previously described were followed (28). 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 4 days to evaluate infectivity. Extracted DNA (20 ng) was used for the detection of total HIV-1 cDNA, mitochondrial DNA, and the circular forms containing two copies of the viral long terminal repeat (2LTR circles), while 0.2 ng of DNA was used for the Alu-LTR junction PCR. Total HIV-1 cDNA, Alu-LTR junctions, and 2-LTR circle products were normalized to mitochondrial DNA. Real-time PCRs were performed in a MiniOpticon system (Bio-Rad) with primers and under conditions previously described (28). Fold change was calculated using the $\Delta C_T$ method (where $C_T$ is threshold cycle) as recommended in the thermocycler manual.

## 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 their effects on the different steps of the viral life cycle were not evaluated (14, 15, 17, 35). In particular, compounds 1, 2, and 3 (Fig. 1) have been shown to inhibit HIV-1 reverse transcriptase or protease in *in vitro* assays (11, 16, 18, 35). 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 (Fig. 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 shown in Fig. 2 indicate that compounds 1 and 2 potently inhibited viral replication at 3 μM. The effects 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 an extent similar to the effect of compound 1 and was still active at 1 μM (Fig. 2b); however, compound 1 was inactive at 1 μM (Fig. 2a). In contrast 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 (14, 15, 17, 35) but failed to validate in human cells the effect reported for compound 3 using *in vitro* assays (16).

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

**Effects of fullerenes on cellular viability.** We have observed that compounds 1 and 2 potently inhibit HIV-1 infection (Fig. 2). A possibility is that these compounds compromise cellular viability affecting viral infection nonspecifically. To rule out this possibility, the effects of compounds 1 and 2 on cell viability were 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 with similar amounts of DMSO for 24 h, and then cell viability was measured. At these concentrations, none of these compounds showed cytotoxic activity (Fig. 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 (50% lethal concentration [$LC_{50}$]) was determined for com-

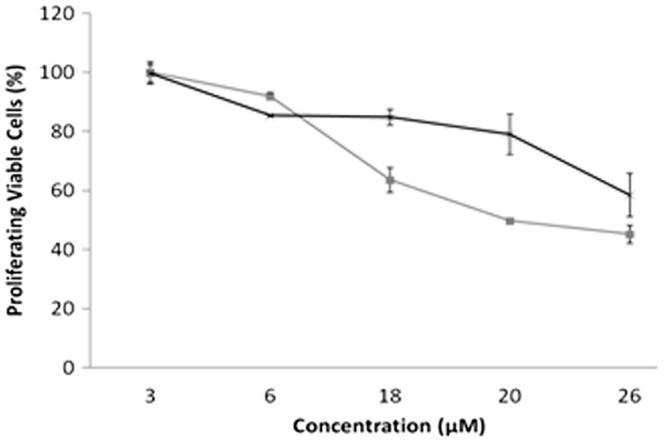


**FIG 3** Assessing the cytotoxicity of compounds 1 and 2. SupT1 cells were treated with DMSO, compound 1 (■), or regioisomeric mixture 2 (×) at various concentrations for 24 h, and the amounts of viable cells were determined by the tetrazolium dye reduction assay. Cell viability values were normalized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

pounds 1 and 2 after 24 h of treatment. In these experiments, the $LC_{50}$s for compounds 1 and 2 were 22.9 μM and 38.3 μM, respectively, as calculated by regression analysis of data shown in Fig. 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 effects of compounds 1, 2, and 3 on the infection of VSV-G-pseudotyped HIV-1 single-round infection viruses expressing luciferase were 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 (Fig. 1), an isomerically pure regioisomer (cis-2, endo-endo) (25) 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 each compound and infected with the reporter virus. Twenty-four hours later, drugs and input virus were removed, and 4 days postinfection luciferase and ATP levels were measured. Luciferase was normalized to ATP to standardize for cell viability and number. The data shown in Fig. 4a clearly indicate that none of the fullerene derivatives affect the early steps of the viral life cycle. These results also demonstrated that neither LTR-driven transcription and translation of viral proteins nor cellular viability was affected by fullerene derivatives. Combined analyses of data shown in Fig. 2 and 4a suggest that the late phase of the viral life cycle is targeted by compounds 1 and 2.

**Effects 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, and 3 (3 μM), and/or indinavir (0.1 μM) in HEK293T cells cotransfected with plasmids expressing these retroviruses and plasmids encoding enhanced green fluorescent protein (eGFP). Then, the produced viruses were concentrated by ultracentrifugation on a sucrose cushion and HIV-1 p24 levels were measured by ELISA. Transfection efficiency was quantified by fluorescence-activated cell sorter (FACS) analysis of eGFP.

In order to analyze the effects 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 4 days later. The data shown in Fig. 4b indicate that infectivity of viruses produced in the presence of compounds 1 and 2 was dramatically reduced, by more than 99%. This inhibitory ef-

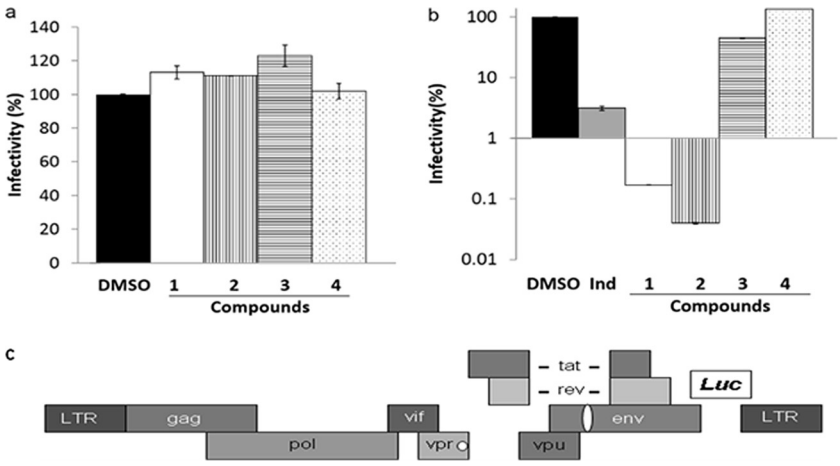


**FIG 4** Analysis of the viral life cycle step affected by compounds 1, 2, 3, and 4. (a) Effects on the early stages of the HIV-1 viral life cycle. SupT1 cells were infected with single-round infection HIV-1 viruses (c) in the presence of DMSO or fullerene derivatives (10 μM) and analyzed for luciferase expression and cellular viability (ATP content) 3 days later. Luciferase was normalized to cellular viability. (b) Effects on the late phase of the HIV-1 infection. Single-round infection HIV-1 was produced in the presence of DMSO, indinavir (Ind; 0.1 μM), or fullerene derivatives (3 μM), and their infectivity was analyzed in single-round infection assays using HIV-1 p24-normalized viruses. Results shown are the averages and standard deviations of triplicate readings of one experiment representative of three independent experiments. (c) The HIV-1 reporter virus used was previously described (26, 27, 51) and includes a frameshift mutation in vpr (circle) and a deletion of 430 nucleotides in env (oval); the luciferase open reading frame is replacing nef.

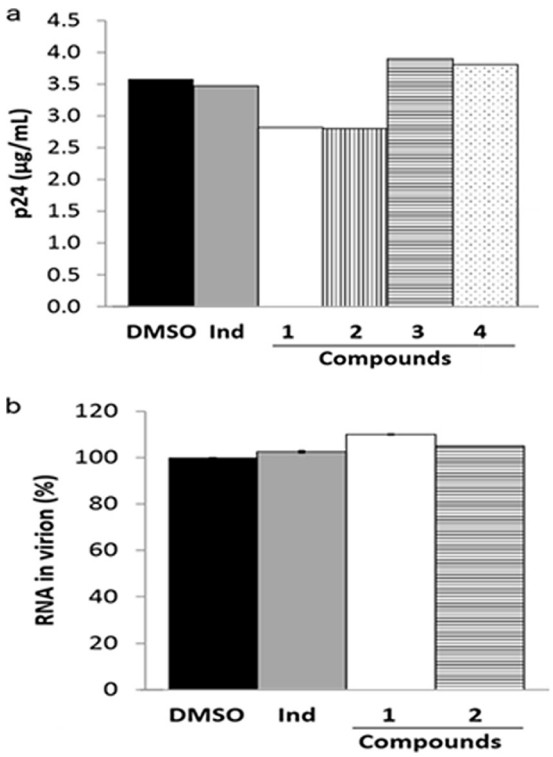


**FIG 5** Effects of compounds 1, 2, 3, and 4 on virion production. VSV-G-pseudotyped, single-round HIV-1 expressing luciferase was produced in the presence of DMSO or fullerene derivatives (3 μM) and then concentrated by ultracentrifugation and analyzed. Indinavir (Ind; 0.1 μM) was used as a control.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

fect 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 or cellular viability (Fig. 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 (Fig. 4a). The similar levels of HIV-1 p24 also correlated with equivalent eGFP expression in the producer cells (data not shown), indicating comparable levels of transfection efficiency.

To further investigate the effects 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 and 2, and indinavir, indicating that these compounds do not affect RNA packaging (Fig. 5b). Altogether, the data shown in Fig. 4b and 5a and 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 (which is formed only after efficient reverse transcription), 2LTR circles (which are 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 (Fig. 6aI). As a consequence, 2LTR circles and Alu-LTR junctions were also markedly diminished in cells infected with fullerene-treated viruses compared with those infected with DMSO-treated viruses (Fig. 6aII and aIII, respectively). As expected, this inhibitory effect of fullerene derivatives was also observed in DNA extracted 4 days after infection (Fig. 6b). Importantly, findings shown in Fig. 6 correlated with the lack of infectivity reported by data shown in Fig. 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 the infectivity of viruses produced in their presence (Fig. 4b and 6) but not mature viruses (Fig. 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 effects 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 Fig. 7a, Gag processing was affected by compound 1 or 2 at 3 μM to an extent similar to that of indinavir at 0.1 μM. These three inhibitors blocked Gag processing at different cleavages sites, including MA-CA-SP1-NC (~50 kDa), MA-CA (~41 kDa), and CA-SP1-NC (~33 kDa) (Fig. 7b). However, integrase processing was not altered by fullerene derivatives at 3 μM or indinavir at 0.1 μM (Fig. 7b), although at these doses these drugs significantly affected HIV-1 infection (Fig. 2 and 4b). Nevertheless, indinavir at higher doses (10 μM) severely impaired Gag and integrase processing (data not shown).

Therefore, the data in Fig. 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 single-stranded DNA (ssDNA) template (ex-

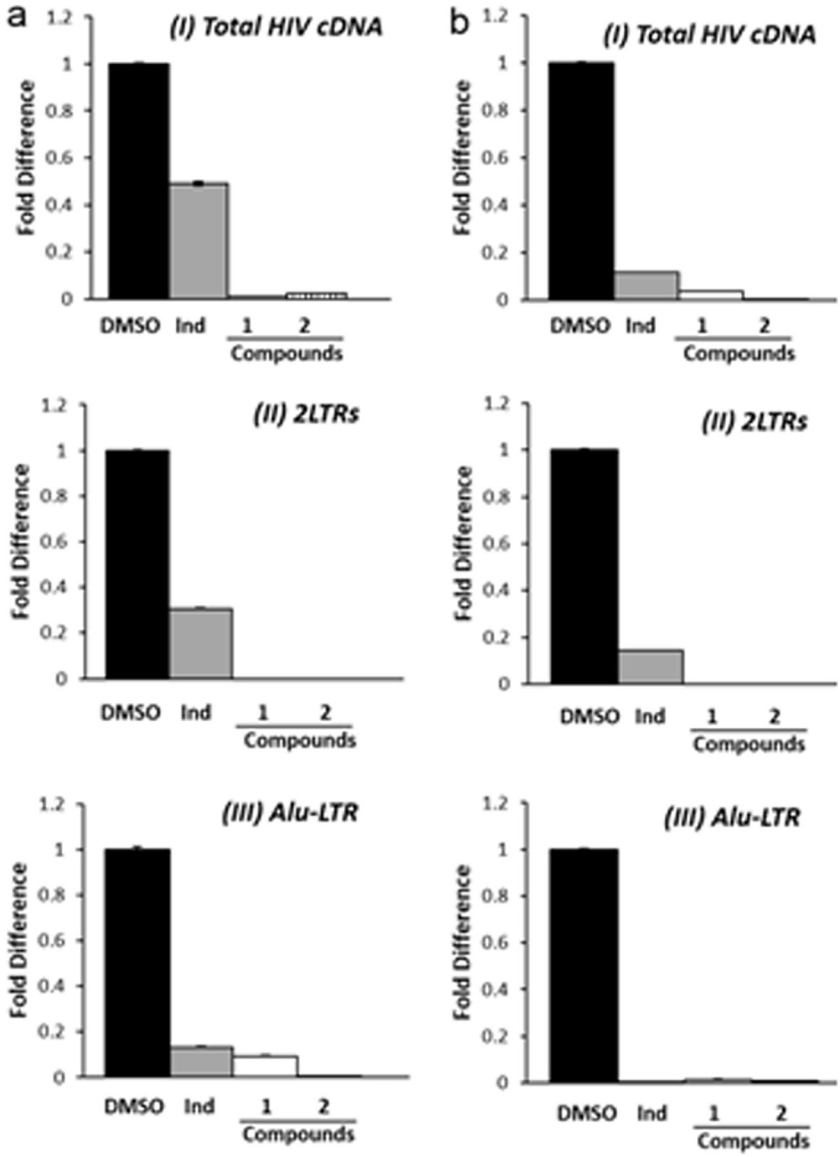


**FIG 6** Evaluation of the early steps of the HIV-1 life cycle of virions produced in cells treated with compound 1 or 2. SupT1 cells were infected with HIV-1 p24-normalized, DNase-treated, single-round HIV-1 viruses produced in the presence of DMSO, indinavir (0.1 μM), or fullerene derivatives (3 μM). DNA was extracted from infected cells 24 h (a) and 4 days (b) postinfection and used to detect total HIV cDNA (I), 2LTR junctions (II), and proviruses (III). Results shown are the averages of triplicate readings of one experiment.

ogenous reverse transcription assay). Although indinavir impaired reverse transcriptase activity, the effects of fullerenes and, in particular, of the regioisomeric mixture 2 were more pronounced (Fig. 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 that of compound 1 (Fig. 7c) highlights the relevance of the fullerene addend groups in their activity. Future work will address the effects of specific regioisomers in compound 2.

**Effects of fullerene derivatives on HIV-1 protease activity.** *In silico* analysis suggests that fullerene derivatives 1 and 2 might be able to bind to the active site of HIV-1 protease (11, 13, 14, 19–21). 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 (Fig. 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 effects on the *in vitro* activity of HIV-1 protease using a FRET peptide-based assay. The data shown in Fig. 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 effects 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 min (Fig. 8).

As expected, indinavir completely blocked the activity of protease, but surprisingly, compounds 1 and 2 were inactive at 3 μM

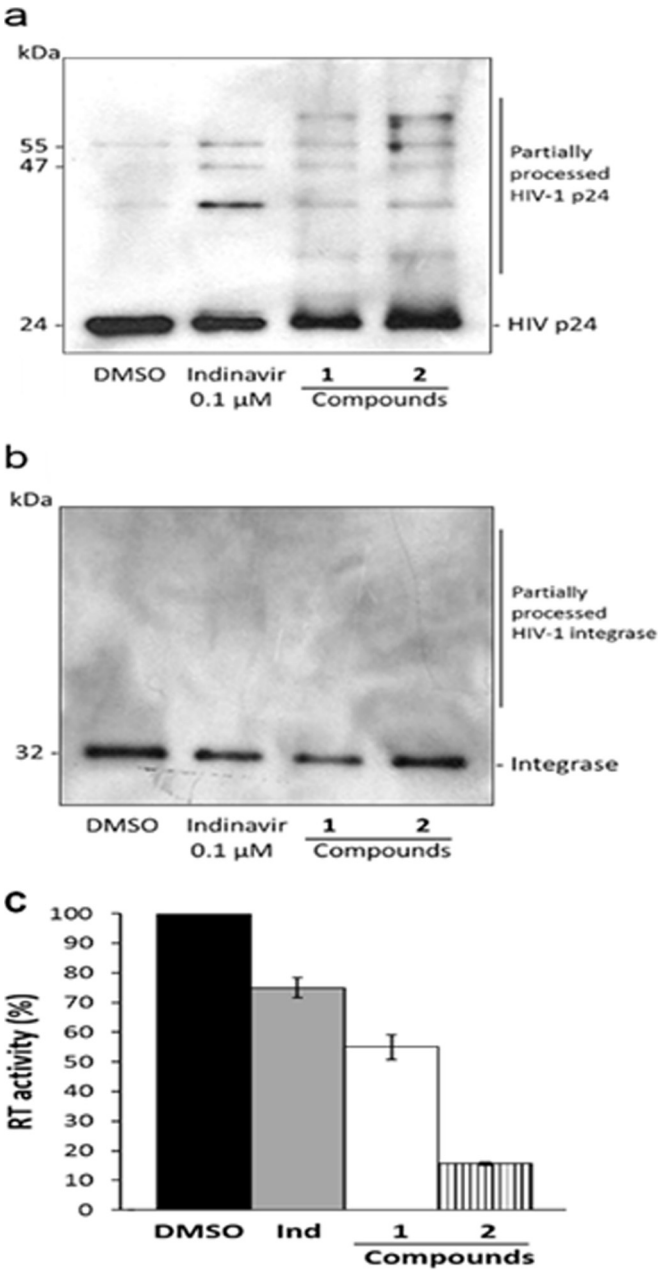


**FIG 7** Effects of compounds 1 and 2 on Gag and Gag-Pol processing. Protease-mediated processing of capsid (p24) (a) and integrase (b) was evaluated in virions by immunoblotting. (c) Reverse transcriptase (RT) activity of virions was measured by the exogenous reverse transcription assay. Results are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

(Fig. 8a), a concentration that severely impairs HIV-1 infection (Fig. 2 and 4b). In further support, even at 10 μM, fullerene derivatives only minimally affect protease activity (Fig. 8b). Only at toxic concentrations (Fig. 3), above 40 μM, did fullerenes 1 and 2 inhibit 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 (11, 13, 18–21, 35).

Given that our results challenge the existing fullerene-protease paradigm, for completeness we decided to study the HIV-1 protease activity of the compound originally reported by Friedman et al. in 1993 (see scheme 4S, compound 5, in the supplemental material) (11). As expected, compound 5 inhibited HIV-1 viral replication (see Fig. A12 in the supplemental material) 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 (see Fig. A13 in the supplemental material). Therefore, our results clearly show that fullerene derivatives are potent anti-HIV agents but their mechanistic activity does not involve HIV-1 protease binding.

**Effects 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 (28). 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 HIV-1 strains 11803, 11806, 11807, 11808, and 11809, which are resistant to nelfinavir, fosamprenavir, saquinavir, indinavir, atazanavir, lopinavir, tipranavir, and darunavir, and strain 11805, which 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 (28).

Single-round infection HIV-1 expressing luciferase and harboring the multi-protease-inhibitor-resistant protease mutants was 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 similar levels of efficiency (Fig. 9a).

To further verify these data, the effects of fullerenes on the replication of HIV-1 11803 were evaluated. This mutant was selected for analysis because, although it contains 18 point mutations in protease that render the virus resistant to eight different protease inhibitors (28), it has a replication capacity of 63% of wild-type protease 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 postinfection. Viruses were used at p24 levels that result in robust viral replication 1 week after infection to compensate for differences in viral fitness. Data shown in Fig. 9b confirmed that compound 1 strongly inhibited viral replication of the multi-protease-inhibitor-resistant virus 11803 to an extent similar to that of the wild-type protease NL4-3 virus. Therefore, data in Fig. 9 highlight the potential clinical relevance of fullerene derivatives to block replication of multi-protease-inhibitor-resistant viruses.

In addition, we evaluated the effects of compound 1 on the infectivity of HIV-1 NL4-3 harboring mutations L363F and V362L/L363M at the end of CA. These viruses have been demonstrated to be resistant to the maturation inhibitor 3-O-(3′,3′-di-

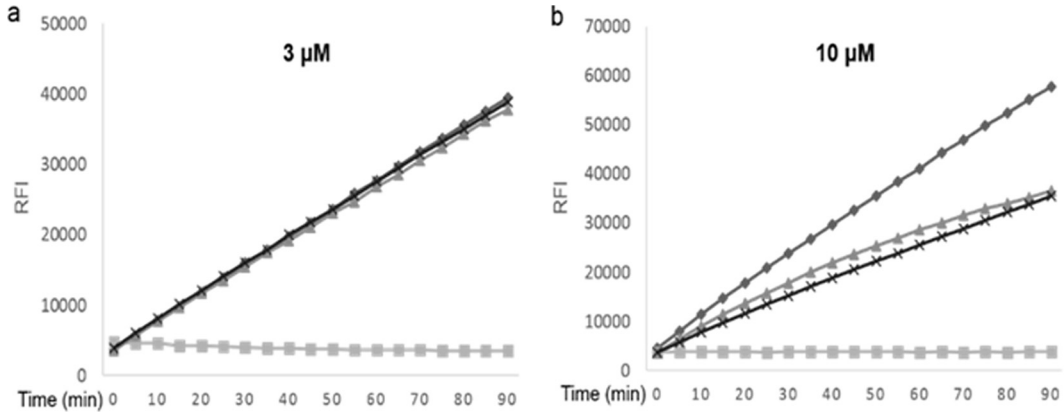


**FIG 8** Effect of compounds 1 and 2 on the *in vitro* activity of HIV-1 protease. The cleavage of an HIV-derived FRET peptide by recombinant HIV-1 protease in the presence of compounds 1 (Δ) and 2 (×) at 3 μM (a) or 10 μM (b) was determined by fluorescence measurements. DMSO (◆) and indinavir (■) were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

methylsuccinyl) betulinic acid (DSB) (36, 37) and fail to interact with DSB (38, 39). Therefore, compounds blocking these mutant viruses have the potential to be clinically relevant (40). 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 affects by only 50% the infectivity of HIV-1 wild-type NL4-3, 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 (Fig. 9d) indicated that compound 1 was as active against HIV-1 NL4-3 V362L/L363M (Fig. 9d, lane 1) and L363F (Fig. 9d, lane 2) mutants as the wild-type viruses (Fig. 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.

## DISCUSSION

Fullerene derivatives have been demonstrated to impair HIV-1 replication in human cells (11, 14, 15, 17, 35); however, detailed

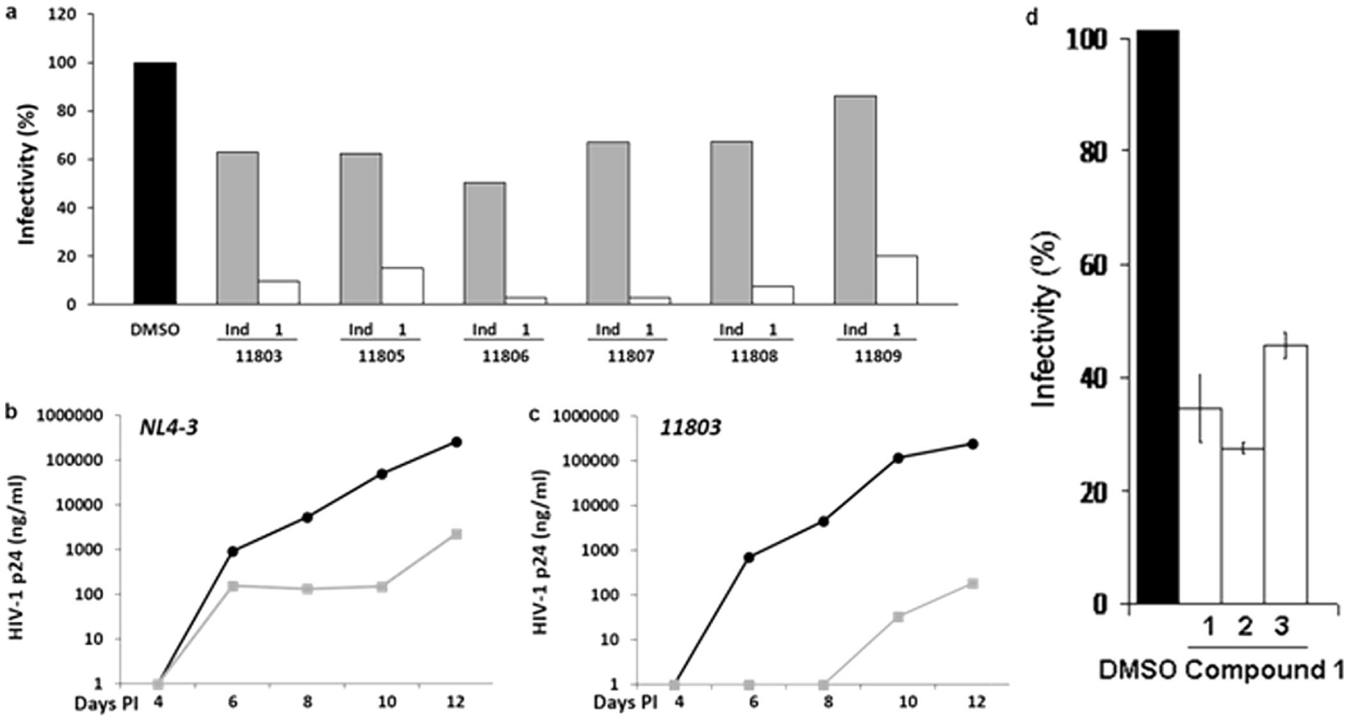


**FIG 9** Activity of compound 1 on the infectivity of HIV-1 harboring multi-protease-inhibitor-resistant protease mutants. (a) HIV-1 viruses harboring wild-type or mutant protease were produced in the presence of DMSO, compound 1 (3 μM), or indinavir (0.1 μM), and their infectivity was evaluated in single-round infection assays. (b and 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 μM) (■). (d) Infectivity in TZM-bl cells of HIV-1-harboring CA mutants resistant to maturation inhibitors (lanes 1 and 2) or wild-type HIV-1 (lane 3) produced in the presence of DMSO or fullerene 1. Results are representative of one (a), two (b), more than four (c), or three (d) independent experiments.

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 (11, 13, 19–21). Other groups have proposed that certain derivatives inhibit recombinant HIV-1 reverse transcriptase *in vitro* activity (16).

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 have corroborated the reported anti-HIV-1 activity of compounds 1 and 2 but do not support findings or predictions indicating reverse transcriptase (16) or protease (11, 13, 18–21, 35) 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, noninfectious 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 that assemble into the mature virion (41, 42). 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 provides an opportunity to profoundly decrease HIV-1 infectivity by protease and maturation inhibitors (41, 42).

We have provided evidence that fullerene derivatives block HIV-1 maturation by inhibiting Gag processing through a protease-independent mechanism. We speculate that interaction of fullerene derivatives with unprocessed Gag could modify the conformation or assembly of these substrates, altering the specificity of protease, and thereby causing defective processing. Similarly, binding of maturation inhibitors to CA result in structural changes at remote regions on this protein (43, 44). Protease-mediated processing of Gag and Gag-Pol occurs in a strictly organized manner, and interfering with this highly ordered process results in immature virions (41, 42); hence, local changes in the protein conformation could globally impact its processing. Importantly, the physical parameters that regulate the interaction of protease with its substrates are unclear (45, 46); perhaps gaining an understanding of fullerene activity will shed light on this phenomenon.

Our findings also highlight the potential therapeutic relevance of fullerene derivatives to block clinically relevant resistant viruses. HIV-1 resistant to multiple clinically used protease inhibitors or to the maturation inhibitors DSB and PF-46396 (36, 37, 39, 47–50) were also potently blocked by compound 1.

We have also demonstrated that the addends on the $C_{60}$ fullerenes and their regiochemistry have pronounced effects on their anti-HIV-1 activity, beyond simple water 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 (15, 16). The fact that the regiochemistry influences the anti-HIV-1 activity of these fullerene derivatives is important and is under investigation in our laboratories.

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

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