

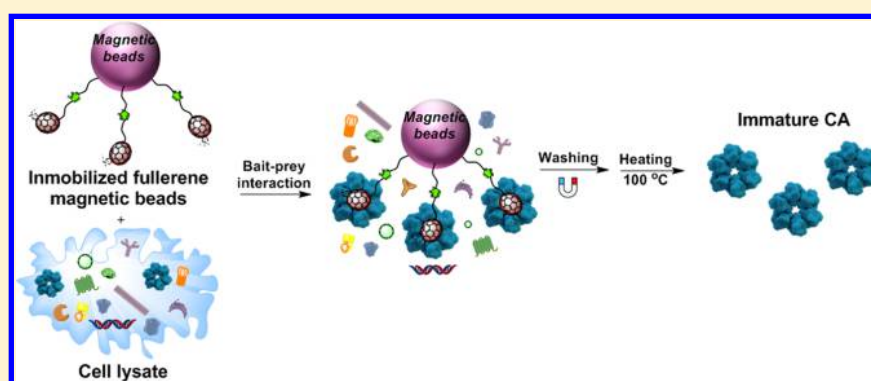
Characterization of New Cationic *N,N*-Dimethyl[70]fulleropyrrolidinium Iodide Derivatives as Potent HIV-1 Maturation Inhibitors

Edison Castro,[†] Zachary S. Martinez,[‡] Chang-Soo Seong,[†] Andrea Cabrera-Espinoza,[†] Mauro Ruiz,[†] Andrea Hernandez Garcia,[†] Federico Valdez,[‡] Manuel Llano,^{*,‡} and Luis Echegoyen^{*,†}

[†]Department of Chemistry, University of Texas at El Paso, 500 West University Avenue, CCSB #3.0302, El Paso, Texas 79968, United States

[‡]Department of Biological Sciences, University of Texas at El Paso, 500 West University Avenue, El Paso, Texas 79968, United States

S Supporting Information



ABSTRACT: HIV-1 maturation can be impaired by altering protease (PR) activity, the structure of the Gag–Pol substrate, or the molecular interactions of viral structural proteins. Here we report the synthesis and characterization of new cationic *N,N*-dimethyl[70]fulleropyrrolidinium iodide derivatives that inhibit more than 99% of HIV-1 infectivity at low micromolar concentrations. Analysis of the HIV-1 life cycle indicated that these compounds inhibit viral maturation by impairing Gag and Gag–Pol processing. Importantly, fullerene derivatives **2a–c** did not inhibit *in vitro* PR activity and strongly interacted with HIV immature capsid protein in pull-down experiments. Furthermore, these compounds potently blocked infectivity of viruses harboring mutant PR that are resistant to multiple PR inhibitors or mutant Gag proteins that confer resistance to the maturation inhibitor Bevirimat. Collectively, our studies indicate fullerene derivatives **2a–c** as potent and novel HIV-1 maturation inhibitors.

INTRODUCTION

The emergence of resistant human immunodeficiency virus (HIV) strains limits the therapeutic efficiency of current antiretroviral therapies.¹ Therefore, discovery of new antiviral agents remains an important goal for HIV-1 infection treatment. These agents may act by impairing viral maturation, a process in which Gag and Gag–Pol polyproteins are sequentially cleaved by PR to produce viral enzymes and structural proteins that are required for viral replication.²

Pharmacological and genetic evidence demonstrates that impairment of viral maturation is a powerful strategy to block HIV-1 replication *in vivo* and *in vitro*. HIV-1 is released from infected cells in the form of immature, noninfectious virions that must undergo maturation before acquiring full infectivity. Viral maturation is triggered by proteolytic processing of Gag and Gag–Pol polyproteins by HIV-1 PR. This processing results in the production of functional viral proteins including capsid (CA) proteins, which assemble into the viral core. HIV-1 maturation can be hindered by drugs that act as PR inhibitors

(PIs) or as maturation inhibitors (MIs). The latter can bind to Gag and affect its processing or to CA, thus impairing core assembly. MIs binding to mature CA do not affect PR-mediated processing, whereas those targeting CA–SP1 selectively block the cleavage between CA and SP1, allowing normal processing of Gag and Gag–Pol at other cleavage sites.³ Currently, there are no maturation inhibitors used clinically.⁴

Since the discovery of fullerene C₆₀,⁵ efficient synthetic methods for fullerene functionalization have been developed.⁶ Functionalization with highly polar or ionic groups is the most commonly used approach to obtain water-soluble fullerene derivatives for biomedical applications.⁷ C₆₀ and C₇₀ fullerene derivatives have been shown to affect HIV-1 replication.⁸ The currently accepted fullerene-induced inhibition mechanism suggests binding to the PR active site⁹ as was determined by analysis of the effect of these compounds on the *in vitro* activity

Received: July 7, 2016

Published: November 17, 2016

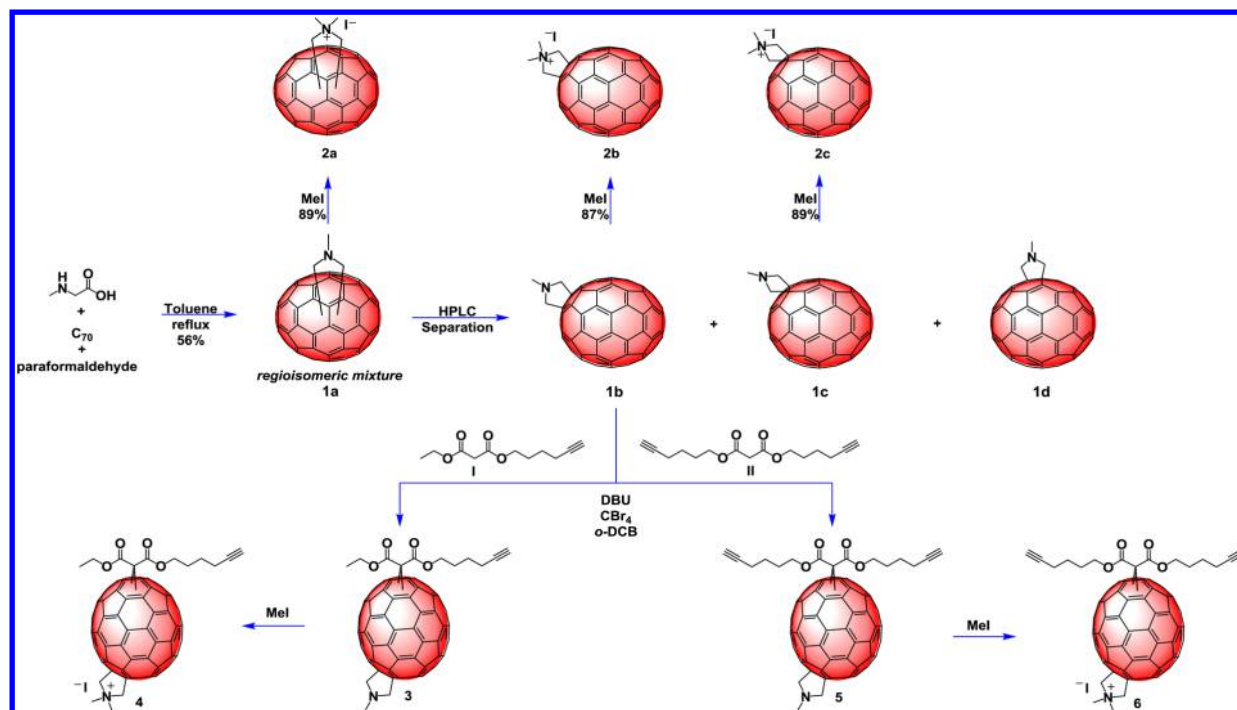


Figure 1. Chemical structures of the C_{70} fullerene derivatives 2a–c, 4, and 6 synthesized in this work.

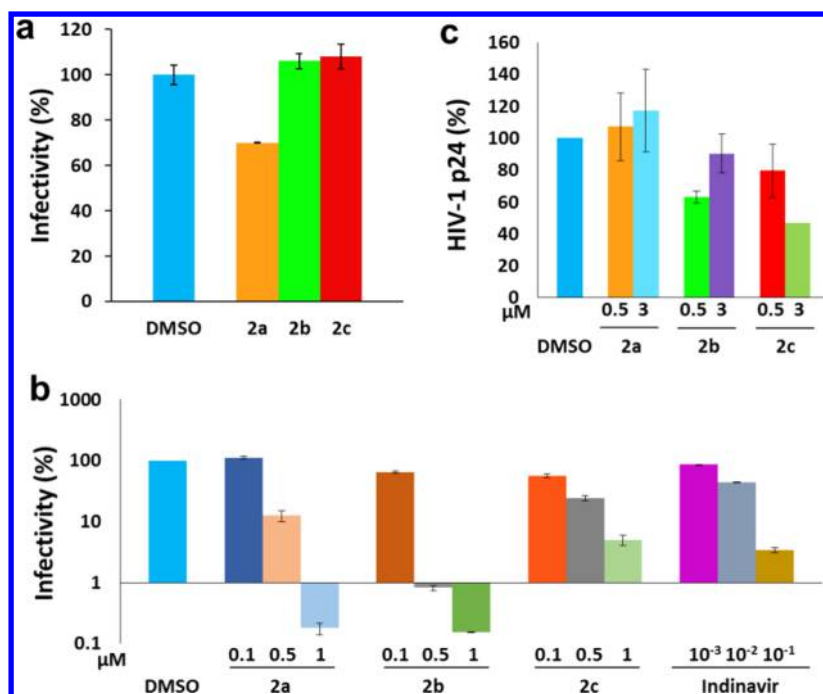


Figure 2. Effect of fullerene derivatives 2a–c on HIV-1 infection. (a) Effect on the early steps of the viral life cycle. (b) Effect on the infectivity of the viruses produced in the presence of fullerene derivatives. (c) Effect on the amount of virions produced in the presence of fullerene derivatives. Means and standard deviations in parts a and b represent luciferase values normalized to ATP from three independent experiments, while data in part c correspond to two independent viral productions (viruses produced in the presence of compound 2c at 3 μ M were generated only once).

of this enzyme combined with *in silico* predictions of the interaction of these compounds with the active site of HIV-1 PR. However, this paradigm has recently been challenged¹⁰ by the fact that C_{60} fullerene derivatives failed to significantly inhibit HIV-1 PR in *in vitro* assays at doses that potentially blocked HIV-1 maturation and infection.

To date, there are only a few examples of water-soluble C_{70} derivatives,^{8b,11} partly due to the challenges involved in

regioselective functionalization. *N*-Quaternization of C_{60} -fullerenopyrrolidines is an excellent and high-yielding reaction to synthesize water-soluble C_{60} -fullerenopyrrolidinium salts.¹² In this study, we report the first synthesis and characterization of C_{70} quaternary ammonium salts and their strong anti-HIV properties. Our data demonstrated that these compounds blocked HIV-1 maturation. Importantly, at doses that potentially blocked Gag and Gag-Pol processing, these compounds did not

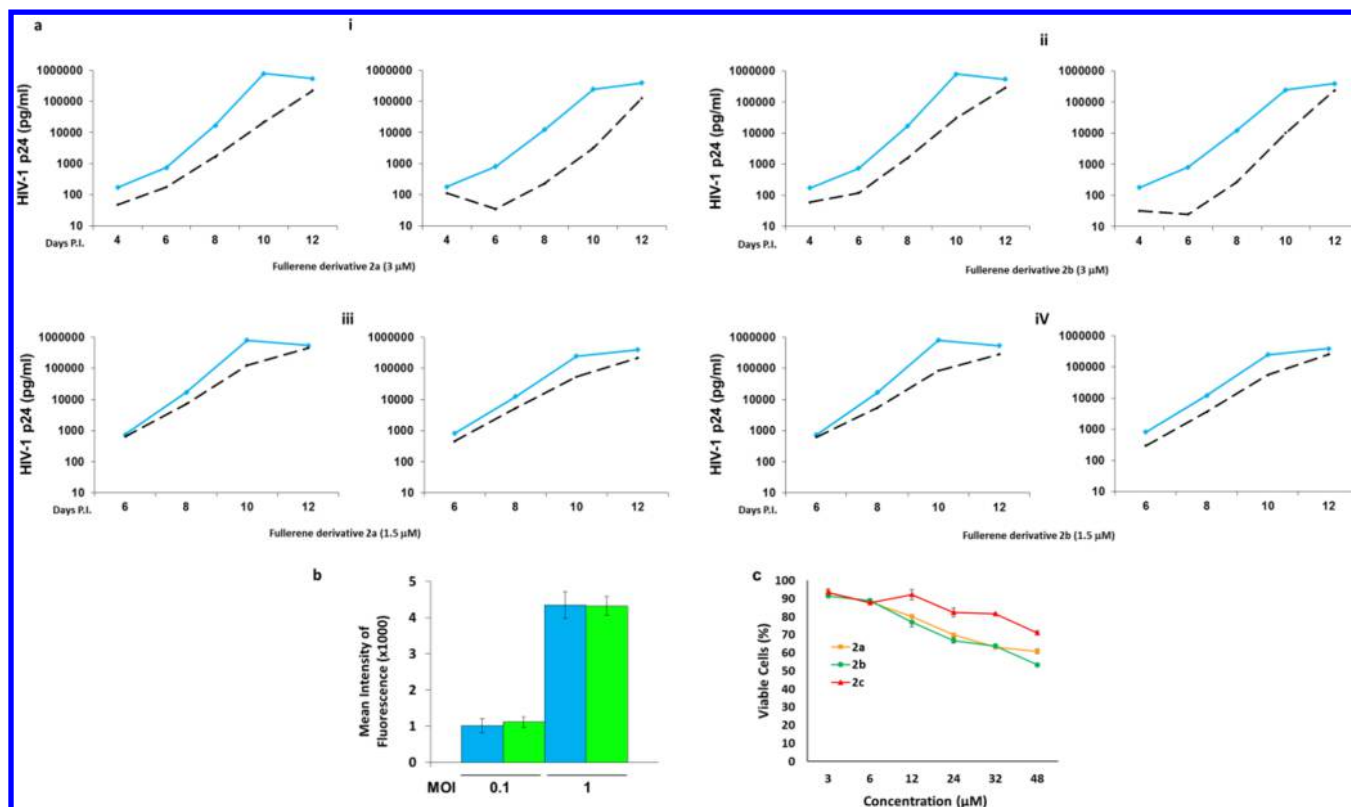


Figure 3. (a, i–iv) Effect of fullerene derivatives **2a** and **2b** on NL4-3 replication. SupT1 cells were infected and then cultured in the presence of DMSO (continuous lines) or fullerene derivatives (discontinuous lines). Concentration and fullerene derivative used are indicated in the graphics. HIV-1 p24 was quantified in the cell supernatant collected at different days postinfection (PI). Two independent experiments are represented for each dose and compound. (b) Effect of fullerene derivative **2b** on the infectivity of a vesicular stomatitis virus engineered expressing eGFP. SupT1 cells were infected at MOIs 0.1 and 1 in the presence of DMSO (blue bars) or **2b** (3 μM, green bars), and eGFP was determined 24 h later by FACS analysis. Average and standard deviations of two experiments are represented. (c) Effect of C₇₀ fullerene derivatives **2a–c** on cell viability. SupT1 cells were treated with different concentrations of fullerenes **2a–c** and DMSO for 24 h before cell viability was measured. Values detected in the cells treated with fullerene derivatives were expressed as a function of those found in DMSO-treated cells. Means and standard deviations in parts a and b are representative of two independent experiments and in part c of three independent experiments.

affect HIV-1 PR *in vitro* activity; therefore, not supporting the current paradigm that fullerene derivatives are HIV-1 PR inhibitors. Furthermore, pull-down experiments using magnetic bead-immobilized fulleropyrrolidinium derivatives demonstrated their strong interaction with immature HIV-1 CA proteins and this specificity correlated with the anti-HIV-1 activity of these compounds. However, further studies are necessary to validate immature CA as the molecular target of the anti-HIV activity of C₇₀ fullerene derivatives.

RESULTS

Design and Synthesis of C₇₀ Fullerene Derivatives.

The synthesis and characterization of C₇₀ pyrrolidinium iodide salts **2a–c**, **3**, and **5**, is schematically represented in Figure 1, as described in the Experimental Section. The structure of the C₇₀ derivatives **2b** and **2c** was confirmed by ¹H-, ¹³C NMR, and by matrix assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) (Supporting Information, Figures 4 and 5). Their synthesis is very reproducible, and their high purity is optimal for virological assays. Functionalization of **1b** with malonate addends **I** and **II** yielded the regioisomeric mixtures **3** and **5**, respectively (detailed synthesis and characterization of malonates **I** and **II** is presented in the Supporting Information, Figures 1, 6, and 7). N-Quaternization of these compounds afforded the corresponding regioisomeric mixtures **4** and **6** (Supporting Information, Figures 6, 10, and

11), which were characterized by MALDI-TOF-MS (Supporting Information, Figures 8 and 9).

Having prepared the cationic C₇₀ derivatives, we first studied their anti-HIV-1 activity and the viral life cycle step affected by the regioisomeric mixture **2a** and the pure compounds **2b** and **2c**.

Effect of C₇₀ Fullerene Derivatives on the Early Stages of the HIV-1 Life Cycle. The effect of compounds **2a–c** on the infectivity of VSV-G pseudotyped HIV-1 single-round infection viruses expressing LTR-driven luciferase (HIV-luc) was evaluated using the human CD4⁺ T cell line SupT1. These cells were infected with HIV-luc in the presence of DMSO (vehicle control) or fullerene derivatives **2a–c** (10 μM), and 24 h later the compounds and the input virus were removed. After 3 days, cellular luciferase and ATP levels were measured and luciferase was normalized to ATP to adjust for cell viability and number. In these experiments, compound **2a** only minimally affected HIV-1 infectivity, whereas compounds **2b** and **2c** were inactive (Figure 2a). These data indicate that fullerene derivatives did not significantly affect the early steps of the HIV-1 life cycle required for HIV-driven luciferase expression including viral entry and uncoating, reverse transcription, integration of viral cDNA, and HIV-1 Tat-mediated gene expression. These results also demonstrated that these compounds were not toxic to SupT1 cells at 10 μM.

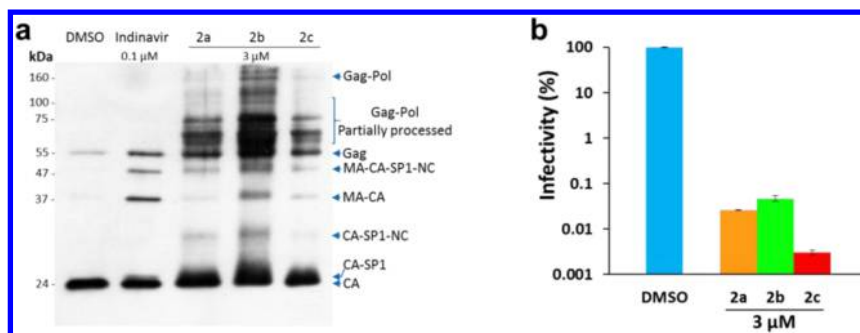


Figure 4. Effect of fullerene derivatives on PR-mediated Gag and Gag-Pol processing and infectivity. (a) Immunoblot analysis of Gag and Gag-pol processing in virions. (b) Infectivity of virions evaluated in part a. Data are representative of two independent experiments.

Effect of C₇₀ Fullerene Derivatives on the Late Stages of the HIV-1 Life Cycle. To evaluate whether the fullerene derivatives affect the late steps of viral development, we produced HIV-luc by plasmid transfection in HEK293T cells in the presence of DMSO, fullerene derivatives 2a–c (0.1, 0.5, and 1 μM), or indinavir^{2b} (0.1, 0.01, and 0.001 μM). The latter is a PR inhibitor known to potentially inhibit HIV-1 maturation. Viruses were concentrated by ultracentrifugation through a sucrose cushion, normalized for p24 levels, and their infectivity evaluated in untreated SupT1 cells as described in Figure 2a. As expected, indinavir produced 96%, 56% and 14% inhibition at 0.1, 0.01, and 0.001 μM, respectively (Figure 2b), as compared to DMSO. Importantly, compounds 2a–c caused more than 99% reduction of infectivity at 1 μM, whereas at 0.5 μM these compounds inhibited 87.4% (2a), 99.2% (2b), and 76% (2c) of viral infectivity (Figure 2b). However, none of the compounds were significantly active at 0.1 μM. Linear regression analysis of these data also indicated that the concentration at which 50% of their maximum response was reached (EC₅₀) was 0.41, 0.33, and 0.54 μM for compounds 2a, 2b, and 2c, respectively. Comparison with indinavir (EC₅₀ 39.23 nM) indicates that compounds 2a–c were between 8- and 13-fold less potent than this therapeutic drug.

To evaluate the late step of the viral life cycle affected by fullerene derivatives, we analyzed their effect on viral particle production by measuring the levels of p24 (Capsid protein, CA) in virions concentrated by ultracentrifugation through a 20% sucrose cushion. Importantly, the ELISA used to quantify p24 detects both HIV-1 PR-fully processed mature CA and partially or nonprocessed immature CA (Supporting Information, Figure 13). Data in Figure 2c indicate that at either 0.5 or 3 μM fullerene compounds minimally affected virion production, although viral infectivity was blocked at these concentrations (Figure 2b). These findings indicate that fullerene derivatives did not affect LTR-driven gene expression, virion production, or cellular viability.

In addition, results in Figure 2 clearly indicate that fullerene derivatives drastically reduce the infectivity of virions produced in their presence.

Effect of C₇₀ Fullerene Derivatives 2a and 2b on HIV-1 Replication. Because fullerene derivatives 2a and 2b were more potent than 2c (Figure 2b), we characterized them in further detail by evaluating their effect on HIV-1 replication in SupT1 cells. Cells were infected with HIV-1 NL4-3 for 24 h. Then the input virus was removed and the infected cells distributed in cultures containing DMSO or fullerene derivatives 2a and 2b at 3 or 1.5 μM. Cells were cultured for 2 weeks by replacing every 48 h half of the culture with fresh

medium containing no drugs. Supernatant was collected every 48 h, before feeding the cells, and used for quantification of HIV-1 p24 by ELISA. In these experiments, we observed a robust inhibitory effect of fullerene derivatives 2a and 2b at 3 μM, but this activity decayed at 1.5 μM (Figure 3a, i–iv). A the replication peak (day 10 postinfection) fullerene derivatives inhibited more than 92% of viral replication and that effect dropped to 71–80% inhibition at 1.5 μM, whereas at 1 μM these compounds did not inhibit viral replication (data not shown).

The inhibitory potency of fullerene derivatives 2a and 2b was markedly different between wild type and single-round infection, VSV-G-pseudotyped HIV-1 (Figure 3a vs 2b). To exclude any potential effect of fullerene derivatives on VSV-G virion incorporation, we determined the effect of fullerene 2b, the most potent of all the compounds evaluated, on the replication of the rhabdovirus vesicular stomatitis virus which requires VSV-G for infection. Findings represented in Figure 3b indicated that fullerene 2b at 3 μM did not interfere with the replication of vesicular stomatitis virus in SupT1 cells, thus excluding any deleterious effect of fullerene derivatives on VSV-G-dependent functions. Furthermore, these data indicate the HIV-1-specificity of these compounds, excluding any toxic effect on the target cells.

Effect of C₇₀ Fullerene Derivatives on Cell Viability. Several experimental results (Figures 2a,c and 3b) demonstrated that at doses that fullerene derivatives block HIV-1 infectivity (Figure 2b and 3a) other viral processes that requires preservation of cell viability are not affected, indirectly indicating that the inhibitory effects observed with these compounds are not due to cellular toxicity. To further evaluate the toxicity of these compounds, we calculated the concentration of fullerene derivatives 2a–c that kills approximately 50% of SupT1 cells (LC₅₀). SupT1 were treated with different doses of the compounds for 24 h, and then cell viability was measured by quantifying the cellular ATP levels. In these experiments, we observed approximately 92% of viability in cells treated with 3 μM of fullerene derivatives 2a–c (Figure 3c), whereas at 48 μM, the viability dropped to 60–70%. Linear regression analysis of these data indicated an LC₅₀ of 60.6, 53.8, and 104.2 μM for compounds 2a, 2b, and 2c, respectively. This indicated more than 148-fold difference between the LC₅₀ and EC₅₀ of these compound, therefore, ratifying that the inhibitory effects observed on HIV-1 infection are not due to decreased viability of the target cells.

Effect of Fullerene Derivatives 2a–c on HIV-1 Gag and Gag-Pol Processing. Our data clearly show that fullerenes 2a–c impair HIV-1 viral infectivity by affecting

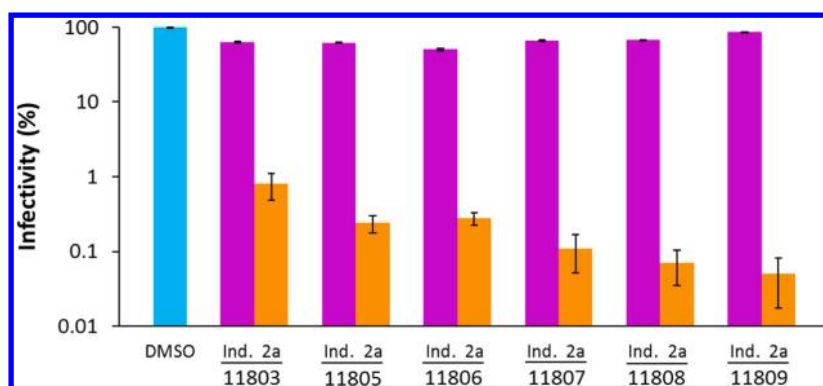


Figure 5. Effect of fullerene derivative **2a** ($3 \mu\text{M}$) and indinavir ($0.1 \mu\text{M}$) on the infectivity of HIV-luc-derived viruses carrying PR mutants resistant to multiple clinically approved PR inhibitors. Mean and standard deviation values represent the variability of triplicate readings of one experiment.

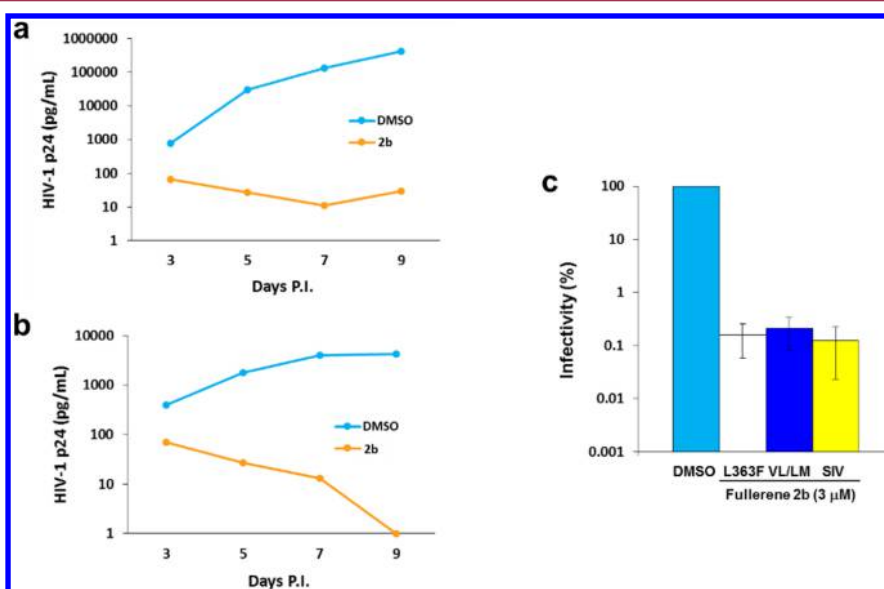


Figure 6. Effect of fullerene **2b** on the infectivity of wild-type SIV and HIV-1 NL4-3 harboring mutations at the C-terminus of CA. Replication of HIV-1 produced in the presence of DMSO or fullerene **2b** ($3 \mu\text{M}$) in SupT1 cells was evaluated by HIV-1 p24 ELISA. (a) NL4-3 VL/LM CA mutation. (b) NL4-3 L363F CA mutation. (c) The infectivity of HIV-1 NL4-3 CA mutants L363F and V362L/L363 M (VL/LM) and wild-type SIV produced in the presence of DMSO or fullerene **2b** ($3 \mu\text{M}$) was analyzed in the reporter cell line TZM-bl. Data in parts a and b correspond to one experiment. Mean and standard deviation values in part c represent the variability of three independent experiments.

virion maturation. To evaluate this inhibition mechanism, we determined the effect of these compounds on PR-mediated Gag and Gag-Pol processing. HEK293T cells were transfected with the HIV-luc expression plasmid and culture for 24 h in the presence of DMSO, fullerenes **2a–c** ($3 \mu\text{M}$), and indinavir ($0.1 \mu\text{M}$). Then cell lysates were analyzed by immunoblot using an anticapsid (CA, p24) monoclonal antibody. Capsid is a domain in Gag, therefore, non- or partially processed Gag and Gag-Pol proteins containing CA are detected in this assay. Data in Figure 4a indicate a pronounced defect in Gag and Gag-Pol processing of virions produced in the presence of C_{70} fullerene derivatives, which directly correlate with the decreased infectivity observed for these viruses (Figure 4b). Fullerene-treated virions presented partially processed Gag-Pol in contrast with virions produced in the presence of indinavir. HIV-1 proteins in Gag are ordered from the N- to the C-terminus as matrix (p17, MA, 17 kDa), capsid (p24, CA, 24 kDa), spacer peptide 1 (SP1, 2 kDa), nucleocapsid (NC, 7 kDa), spacer peptide 2 (SP2, 1 kDa), and late domain containing protein P6 (P6, 6 kDa).^{2c,13} PR-mediated Gag processing occurs in a fixed order that results in several

processing intermediates, some of which are indicated in Figure 4a. Several CA-containing Gag fragments, presumably identified as CA-SP1-NC (~ 33 kDa) and CA-SP1 (~ 25 kDa),^{2c} were enriched in the fullerene- but not in the indinavir-treated virions. Therefore, indinavir and C_{70} fullerene derivatives **2a–c** affect Gag and Gag-Pol processing by different mechanisms.

Antiviral Activity of C_{70} Fullerene Derivative **2a on Drug-Resistant HIV-1.** The effect of fullerene derivatives on HIV-1 molecular clones harboring a panel of PR mutants that are resistant to multiple PR inhibitor or Gag mutants resistant to the maturation inhibitor Bevirimat^{3m} was determined to assess the potential clinical relevance of the molecular target of fullerene inhibitors. HIV-luc-derived viruses harboring the PR mutants were produced by plasmid transfection in HEK293T cells in the presence of DMSO, indinavir ($0.1 \mu\text{M}$), or compound **2a** ($3 \mu\text{M}$). The mutant viruses analyzed included 11803, 11806, 11807, 11808, and 11809 that are resistant to nelfinavir,¹⁴ fosamprenavir,¹⁴ saquinavir,¹⁴ indinavir, atazanavir,¹⁴ lopinavir,¹⁴ tipranavir,¹⁴ and darunavir, respectively,¹⁴ and 11805 that is resistant to these drugs except for tipranavir and darunavir. The PR (99 amino acids) in these viruses contains

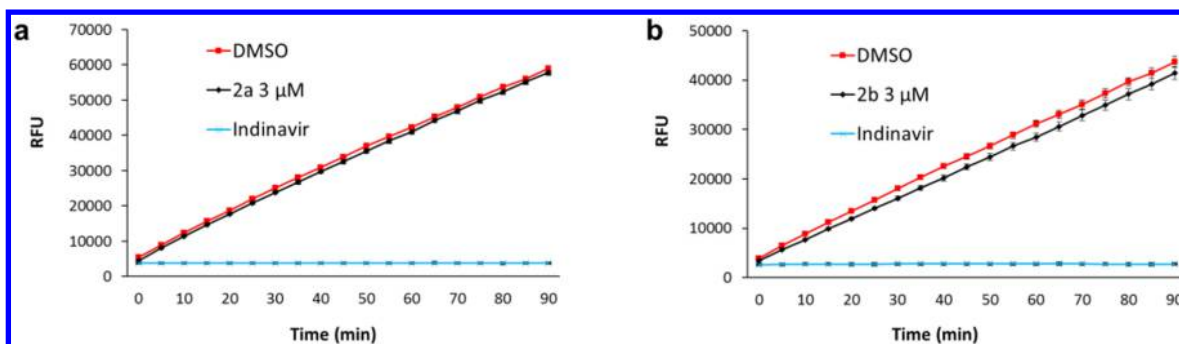


Figure 7. Effect of compounds **2a** and **2b** on the in vitro activity of HIV-1 PR. The cleavage of an HIV-derived FRET peptide by recombinant HIV-1 PR in the presence of compounds **2a** (a) and **2b** (b) at 3 μM determined by fluorescence measurements. Indinavir at 0.1 μM was used as the control. Mean and standard deviation values represent the variability of three independent experiments.

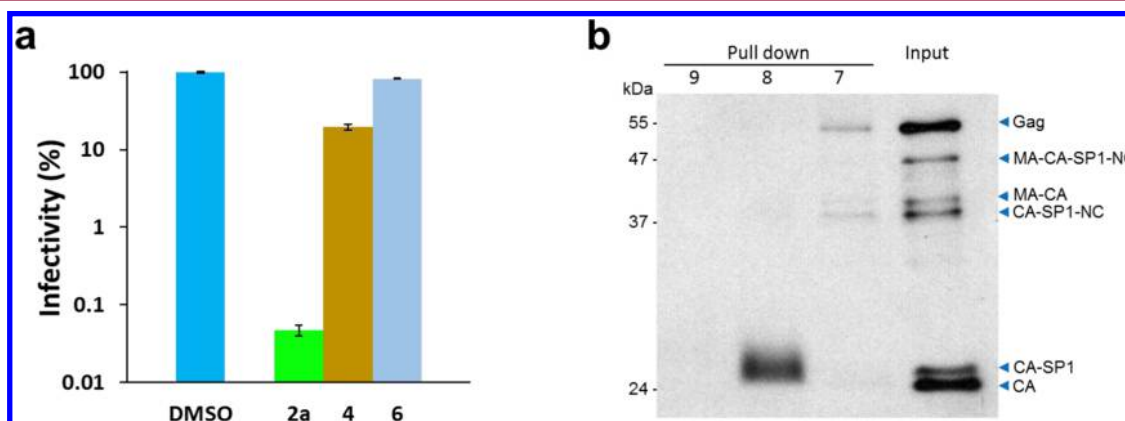


Figure 8. (a) Effect on HIV-1 infectivity of fullerene **2b** and compounds **4** and **6**. (b) Analysis of the pull-down products using beads functionalized with active or inactive fullerene derivatives **4** and **6**, respectively. Mean and standard deviation values in part a represent the variability of two independent experiments, and data in part b is representative of three independent experiments.

between 10 and 24 point mutations that evolved in patients subjected to highly active antiretroviral therapy. The produced viruses were normalized for p24 and used to infect SupT1 cells. Four days after infection, luciferase levels were measured and normalized to ATP. In these experiments, we found that compound **2a** potently inhibited all the multiprotease inhibitor resistant viruses (Figure 5) and the reporter viruses harboring wild-type protease to a similar extent (Figures 2b). As expected, indinavir failed to significantly affect the infection of the resistant viruses.

We also evaluated the effect of fullerene **2b** on the infectivity of HIV-1 NL4-3 harboring Gag mutations at the C-terminus of CA [L363F and V362L/L363 M (VL/LM)], as well as wild-type SIV. These viruses are resistant to the maturation inhibitor Bevirimat.^{3a,n} Viruses were produced by plasmid transfection in HEK293T cells in the presence of DMSO or fullerene **2b** (3 μM), and their infectivity was determined in SupT1 cells or TZM-bl cells using p24- or p27-normalized HIV-1 or SIV, respectively. Comparison of the infectivity of viruses produced in the presence of DMSO or fullerene **2b** demonstrated that this compound potently inhibits the ability of these viruses to replicate in SupT1 cells (Figure 6a,b) or to infect the reporter cell line (Figure 6c), demonstrating no cross-reactivity between fullerene **2b** and Bevirimat. This is expected because Bevirimat only impairs processing of CA-SP1, whereas the effect of fullerene **2b** on Gag processing is much broader.

In addition, these findings highlight the potential clinical relevance of the molecular target of fullerene derivatives.

Effect of Derivatives 2a and 2b on HIV-1 PR in Vitro Activity. Our data indicate that fullerene derivatives impair HIV-1 maturation by affecting Gag and Gag-Pol processing. This effect could be the result of direct inhibition of HIV-1 PR or due to a modification of the substrate or its accessibility to this enzyme. To understand better the mechanism and to test the established paradigm that HIV-1 PR is the target for fullerene inhibition, we determined their effect on the activity of HIV-1 PR in vitro.

Enzymatic assays are the oldest and still the most common approach to test the inhibition of HIV-1 PR.¹⁵ The assay we used is based on the PR cleavage of a fluorogenic substrate derived from the native p17/p24 (MA/CA) cleavage site on Gag. As expected, indinavir at 0.1 μM completely inhibited processing of the substrate (Figure 7). However, C₇₀ derivatives **2a** and **2b** at concentrations that inhibit more than 99% of viral infectivity (~ 7 – 9 -fold IC₅₀) showed no effect on PR activity (Figure 7).

Evaluating the Interaction of Fullerene 2b with Gag-Derived Proteins. Having established that PR is not the direct target of these fullerenes against HIV-1 infection, and considering that these compounds generate more CA-containing Gag processing intermediates than indinavir (Figure 4a), which also suggest that compounds **2a**–**c** likely act through a different mechanism of inhibition, we evaluated the interaction of Gag-derived proteins with compound **2b**.

Azide magnetic beads (**7**) were functionalized using fullerene derivatives **2b** as bait. The synthesis of functionalized magnetic beads starts from the α -isomer **1b**, which after a Bingel

nucleophilic cyclopropanation with acetylenes **I** or **II** yields the regioisomeric mixtures **3** and **5**, respectively (Figure 1). After quaternization of these compounds to yield **4** and **6**, respectively, they were coupled to the azide magnetic beads via “click chemistry” to obtain functionalized magnetic beads with one (**8**) and two (**9**) acetylene groups, respectively, as described in the Experimental Section. Proof that the “click reaction” was successful comes from the disappearance of color when a magnet is placed close to the reaction vessel (Supporting Information, Figure 2). Additional proof comes from infrared (IR) spectroscopy (Supporting Information, Figure 12), which showed the disappearance of the azide and acetylene stretching vibration bands near 2100 cm^{-1} and the appearance of new bands between 900 and 1700 cm^{-1} and 2900 and 3000 cm^{-1} , confirming the presence of the fullerene cage. Magnetic beads coupled to fullerene **6** showed similar IR characteristics.

To evaluate whether the functionalization of **2b** with malonates **I** and **II** disrupted its anti-HIV-1 activity, HIV-luc was produced by plasmid transfection in HEK293T cells in the presence of fullerene derivatives **2b**, **4**, **6** ($3\text{ }\mu\text{M}$), or DMSO. The infectivity of the produced viruses was evaluated in single-round infection assays in SupT1 cells as described in Figure 2b. Data in Figure 8a shows that functionalization of fullerene **2b** using a symmetric dihexynyl malonate **II** (compounds **6**) resulted in the total loss of its anti-HIV-1 activity, whereas substantial inhibitory activity was preserved for compounds **4**, which has only one acetylene group (Figure 1). These findings highlight the relevance of the addends in the anti-HIV activity of fullerene derivatives.

Next, we determined the interaction of fullerene derivatives with HIV-1 Gag. Cell lysates obtained from HEK293T cells that were transfected with a plasmid expressing Gag and Gag-Pol polyproteins (pCMVΔR8.91) were incubated overnight with pristine beads, beads coupled to fullerenes **4** and **6** followed by washing and boiling of the beads. Bound proteins were analyzed by immunoblot with an anti-p24 (HIV-1 CA) monoclonal antibody (183-H12-5C). This antibody recognizes nonprocessed and processed Gag proteins containing the p24 domain. Data in Figure 8b demonstrate that an immature CA-containing Gag product of $\sim 25\text{ kDa}$, potentially corresponding to the CA-SP1 fragment, strongly and specifically binds to beads coupled to fullerenes **4**. This protein was not recovered from the nonfunctionalized beads or beads coupled to fullerenes **6** pull-down experiments, in accordance with the anti-HIV-1 activity of compounds **4** and **6**. Similar results were obtained with an anti-p24 monoclonal antibody that reacts against an epitope located within amino acids 48–62 of CA, highlighting the identity of the pulled-down protein (data not shown).

DISCUSSION AND CONCLUSIONS

C_{70} and C_{60} fullerene derivatives have been reported to impair HIV-1 replication in human cells by ill-defined mechanisms.^{8,9,16} Only recently,¹⁰ our group systematically characterized the anti-HIV-1 activity of C_{60} fullerene derivatives, discovering that these compounds severely impair HIV-1 maturation at doses that do not affect the in vitro activity of HIV-1 PR. Here, we described the synthesis and potent anti-HIV-1 activity of novel C_{70} derivatives and confirmed that C_{70} and C_{60} fullerene derivatives share a similar mechanism of action. We demonstrated, for the first time, that C_{70} fullerene derivatives **2a–c** inhibit HIV-1 maturation by blocking Gag and

Gag-Pol processing. The mechanism seems to be PR independent because these compounds did not inhibit in vitro PR activity at doses that potentially block Gag and Gag-Pol processing. These findings, however, contrast with a previous report^{8a} indicating that fullerene derivatives affect in vitro PR activity. Important differences in the methodology used could explain these contradictory findings. Perhaps the most relevant is the nature of the substrate used in the assays; the previous study used a non-HIV peptide as substrate, whereas we used a peptide representing the natural substrate found at the MA–CA cleavage site. The artificial peptide substrate has a 3-fold higher turnover rate (kCat) than the peptide that we used. According to calculations using the Hopp–Woods hydrophobicity scale, the artificial peptide was significantly more water-soluble than the HIV-derived peptide that we used, potentially explaining its higher kCat. Aqueous solubility is an important property of peptide substrates with improved kCat.^{15b,17} Therefore, the addition of hydrophobic compounds to the in vitro PR assay, such as the C_{60} fullerene derivatives assayed in the reported study, could have compromised the aqueous solubility of the peptide affecting its rate of cleavage by PR. Thus, our findings illustrate the relevance of studying natural vs artificial substrates in the case of HIV-1 PR. Another important difference between our assay and the one previously reported is that we used a continuous assay that allowed analysis of the enzymatic activity at multiple time points, whereas the previous report used a single-point assay that measured the effect of the compounds when $<15\%$ of the product was generated. Then, our findings also point out another advantage of continuous vs single-point assays to evaluate inhibitors of HIV-1 PR. Therefore, our data here, and recently published,¹⁰ alter the long-established and unchallenged paradigm that fullerene derivatives inhibit HIV-1 infectivity via binding to PR.^{8a,9,16c,d,18}

Importantly, the antiviral activity of fullerene derivatives correlated with their interaction with an immature CA protein of $\sim 25\text{ kDa}$, presumably CA-SP1. However, we have not unequivocally demonstrated that the binding to immature CA proteins is responsible for the antiviral activity of fullerene derivatives. Nevertheless, we propose a model indicating that interaction of fullerene derivatives to CA in unprocessed Gag or Gag-Pol modifies the conformation or assembly of these substrates, altering the specificity and/or activity of PR and therefore causing defective processing. Importantly, the parameters that regulate the interaction of PR with its multiple natural, nonhomologous, substrates is unclear; thus clarification of the mechanism of action of the C_{70} fullerene derivatives will potentially increase our understanding of this process.

Similar to C_{70} fullerenes **2a–c**, Bevirimat and 1-[2-(4-*tert*-butylphenyl)-2-(2,3-dihydro-1*H*-inden-2-ylamino)ethyl]-3-(trifluoromethyl)pyridin-2(1*H*)-one (PF-46396)¹⁹ interact with immature CA. However, in contrast with these compounds that affect Gag processing only at the binding site, CA-SP1,^{3a,h,19,20} fullerene derivatives **2a–c** produce a global impairment of processing indicating a distinct mechanism of action. In further support of this, fullerene derivatives block the infectivity of Bevirimat-resistant HIV-1 molecular clones and viruses. Similarly, HIV-1 viruses resistant to multiple PR inhibitors are also blocked by C_{70} fullerenes. These findings highlight the potential clinical relevance of the molecular target of fullerene derivatives.

In summary, our studies show that fullerene derivatives **2a–c** act through a novel anti-HIV-1 mechanism not yet reported for

other CA-interacting compounds. Unraveling the details of this unprecedented mechanism will open new avenues for the discovery of novel anti-HIV-1 inhibitors.

EXPERIMENTAL SECTION

Chemical Synthesis of C₇₀ Fulleropyrrolidinium Derivatives.

We initially synthesized the neutral C₇₀ pyrrolidine derivatives **1a–d**,²¹ and the key intermediates for the synthesis of the quaternary ammonium salts **2a–c**, **4**, and **6**; the purity of these compounds was higher than 99% as determined by HPLC. Subsequent *N*-methylation of compounds **1a–c** yielded the regioisomeric mixture **2a**, and the pure C₇₀-(*N,N*-dimethylpyrrolidinium iodide) isomers **2b** and **2c**. Malonates **I** and **II** were synthesized by the reaction of hexynyl alcohol **S1** with the corresponding acid chlorides **S2** and **S3**, see Supporting Information, Figure 1. These addends were used to strategically introduce the acetylene functionalities (compounds **3** and **5**) starting with **1b**. The C₇₀ fulleropyrrolidinium iodides **4** and **6** were synthesized by following a similar procedure to that used for the synthesis of compounds **2a–c**. Finally, magnetic beads were independently functionalized using compounds **4** and **6** using “click chemistry” (Figures 1 and 7). Detailed descriptions of the synthetic procedures can be found in the Supporting Information.

Plasmids. HIV-1-derived vectors were produced using pHIV-luc and pMD.G. pHIV-luc was derived from pNL4-3.Luc.R-E²² by introducing a deletion in the env open reading frame. pMD.G encodes the vesicular stomatitis virus glycoprotein G (VSV-G). Gag and Gag Pol were expressed with the HIV-1 packaging plasmid pCMVΔR8.91 (a gift of D. Trono) and Gag with the expression plasmid p96ZM651gag-opt (8675, NIH AIDS Reagent Program).²³

Cell Lines. SupT1 cells were grown in RPMI 1640 and HEK293T, TZM-bl, and A-172 in DMEM. Both culture media were supplemented with 10% of heat-inactivated fetal calf serum, 2 mM L-glutamine, and 1% penicillin/streptomycin.

Generation of Retroviruses. HEK293T cells were cotransfected with the corresponding plasmids by the calcium-phosphate precipitation method.²⁴ VSV-G-pseudotyped HIV-derived reporter virus expressing firefly luciferase (HIV-luc) was prepared by cotransfection of 15 μg of pHIV-luc and 5 μg of pMD.G. Replicating competent virus [HIV-INL4-3 wild-type and Gag mutants, and SIV (SIVmac239 SpX)] were produced by transfection of 15 μg of the corresponding expression plasmids. After 18 h, the transfection medium was replaced with fresh medium containing no drug, fullerene derivatives **2a–c**, indinavir, or DMSO. 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 through a 20% sucrose cushion.

Single-Round Infectivity Assay. SupT1 cells were plated at 1 × 10⁵ cells in 500 μL of RPMI 1640 culture medium in 24-well plates and infected with HIV-luc. Four days postinfection, cells were collected by centrifugation at 1000g for 6 min, and the resulting pellet was 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 were infected with HIV-1_{NL4-3} wild-type or mutants in the presence or absence of fullerene derivatives **2a–c** or DMSO. Then 24 h after infection, the cells were extensively washed and viral replication was monitored for several days by quantification of HIV-1 p24 in the cell supernatant by ELISA.

In experiments described in Figure 3a, SupT1 cells were infected with HIV-1_{NL4-3} wild-type (92 pg of p24), and 24 h later, the input virus was extensively washed and then the cells were distributed in cultures containing DMSO or fullerene derivatives (3, 1.5, and 1 μM). During the two-week analysis, half of the culture medium was replaced every 48 h with fresh medium containing no drugs. Viral replication

was monitored by HIV-1 p24 ELISA analysis of the cell culture supernatants that were collected every 48 h before culture feeding.

TZM-bl cells were also used for evaluation of the infectivity of replication competent HIV-1 and SIV. This HeLa-derived indicator cell line expresses CD4, CXCR4, CCR5, and HIV-1 promoter-driven luciferase and β-galactosidase expression cassettes. These cells are permissive to infection by different isolates of HIV-1, HIV-2, and SIV.²⁵ TZM-bl cells (1 × 10⁵/well) were plated in a p24-well plate and the next day infected with p24 or p27-normalized HIV-1 or SIV, respectively. Then 72 h postinfection, cells were lysed in PBS-1% Triton X-100 and luciferase activity was measured.

Vesicular Stomatitis Virus Replication Assay. SupT1 were infected in the presence of DMSO or fullerene derivative **2b** (3 μM) with different multiplicities of infection (MOIs) of the rhabdovirus vesicular stomatitis virus, that was engineered to express eGFP (VSV-eGFP).²⁶ Then 24 h later, the cells were analyzed for eGFP expression by FACS.

HIV p24 and SIV p27 ELISAs. The instructions of commercially available ELISA kits were followed (HIV, zeptomatrix, and SIV, XpressBio). Briefly, 200 μL of the viral samples were diluted and incubated on the ELISA wells overnight (HIV p24) or 1 h (SIV p27) at 37 °C. Unbound proteins were removed by washing the wells six times with 200 μL of washing buffer. Bound HIV-1 p24 or SIV p27 was detected by incubating each well with 100 μL of the respectively anti-CA HRP-labeled secondary antibodies for 1 h. Unbound antibodies were removed by washing as described above. Bound antibodies were detected by incubating each well with 100 μL of substrate buffer for 30 min at room temperature after 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 (Molecular Devices, Versa Max microplate reader).

Cellular Viability Assay. First, 25 × 10⁴ SupT1 cells were plated in a 96-well plate in 50 μL of RPMI 1640 culture media and left untreated or treated with fullerene derivatives **2a–c**, DMSO, or 2 mM hydrogen peroxide (positive control). Then fullerene derivatives **2a–c** were evaluated at concentrations ranging from 3 to 48 μM, with each concentration evaluated in triplicate. The cells were cultured in the presence of the indicated compounds for 24 h, and then 50 μL of cell viability substrate (CellTiter-Glo Assay, Promega) was added to each well of cells. Cells were incubated for 10 min at room temperature in the dark, and then luminescence was measured using a microplate luminometer reader (Thermo Scientific, Luminoskan Ascent). Control wells containing the same volumes of culture medium were used to subtract the background.

Immunoblotting. Proteins of HIV-1 p24-normalized amounts of virions (0.38 μg p24) were resolved by 13% SDS-PAGE and transferred 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 monoclonal antibodies 183-H12-5C, obtained from the NIH AIDS Reagent Program (catalogue no. 1513), and SC-130531 (Santa Cruz Biotechnology). Primary antibody-bound membranes were washed in TBS-0.1% Tween 20; all bound antibodies were detected with goat antimouse IgG-HRP (1/2000, KPL, 074-1806), followed by chemoluminescence detection.

Cells of the glioblastoma cell line A172 were transfected with 1 μg of either pCMVΔR8.91 or p96ZM651gag-opt plasmids using Lipofectamine 3000 (Invitrogen) according to manufacturer's protocol. Then 4 h later, the transfection media was replaced with fresh culture medium. Then 24 h post-transfection, supernatants were collected and cell lysates were prepared and analyzed by immunoblotting with an anti-p24 monoclonal antibody (183-H12-5C). HIV-1 p24 levels were determined by ELISA analysis of the cell supernatants.

HIV-1 PR in Vitro Activity Assay. We measured the effect of fullerene derivatives on the in vitro activity of HIV-1 PR with a commercially available kit (Protein One, catalogue no. P9003). This assay uses purified recombinant HIV-1 PR and a fluorescence resonance energy transfer (FRET) peptide derived from the native p17/p24 cleavage site of HIV-1 PR on Gag. HIV-1 PR (0.2 μL) and

FRET peptide (final concentration 0.5 μM) were mixed in HIV-1 PR buffer supplemented with 1 μM DTT (final concentration) on ice and protected from light and immediately transferred into a black 96-well plate that contain the compounds being evaluated. The reaction was measured by determining the relative fluorescing units (RFU) with a fluorometer at excitation/emission wavelengths of 490 nm/530 nm every 5 min during 90 min.

Pull-Down Assay. First, 3×10^6 HEK293T cells were plated in a T75 cm^2 tissue culture flask and transfected the next day with 20 μg of the Gag-Pol expression plasmid pCMV Δ R8.91 by the calcium-phosphate precipitation method. After 18 h, the transfection medium was then replaced with fresh medium, and 24 h later, the cells were washed in 1 \times PBS and lysed in 500 μL of 0.5% Triton in 1 \times PBS. The cell lysate was incubated on ice for 15 min, centrifuged at 22000g for 10 min, and the supernatant was incubated with 50 μL of nonfunctionalized magnetic beads for 1 h at 4 $^\circ\text{C}$ to remove proteins binding nonspecifically to the beads. Then 50 μL of the precleared lysate were saved as input sample, and 150 μL were mixed with 100 μL of nonfunctionalized magnetic beads or functionalized with fullerene derivatives 4 or fullerene derivatives 6. Beads and cell lysates were then rotated overnight at 4 $^\circ\text{C}$, followed by one 5 min wash using 0.5% Triton in 1 \times PBS. Washed beads were boiled 10 min at 100 $^\circ\text{C}$ in 50 μL of 2 \times Laemmli buffer, and the eluted proteins were analyzed by immunoblotting with anti HIV-1 p24 monoclonal antibodies as described above.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00994.

Detailed synthesis and characterization of fullerene derivatives 2a–c, 3–6, functionalized magnetic beads with fullerenes 4 and 6 (PDF)
Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Authors

*For L.E.: phone, 915-747-7573; E-mail, echegoyen@utep.edu.

*For M.L.: phone, 915-747-7573; E-mail, mlano@utep.edu.

ORCID

Edison Castro: 0000-0003-2954-9462

Chang-Soo Seong: 0000-0001-9426-9534

Luis Echegoyen: 0000-0003-1107-9423

Author Contributions

E.C. and Z.S.M. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

L.E. thanks the U.S. National Science Foundation (NSF), grant CHE-1408865, the NSF-PREM program (DMR 1205302) and the Robert A. Welch Foundation for an endowed chair, grant no. AH-0033, for generous support. M.L. thanks the National Institute of General Medical Sciences (NIGMS) grant no. 5 SC1 AI098238-02 and the National Institutes of Health (NIH). We also thank the College of Science at University of Texas at El Paso for providing seed funds for this work through the grant 5G12MD007592 from the National Institutes on Minority Health and Health Disparities (NIMHD-NIH). The University of Texas at El Paso core facilities are funded by the BBRC grant 5G12RR008124. We also thank Diana Torres (UTEP) for technical help. The following reagents were obtained through the NIH AIDS Reagent Program, Division of

AIDS, NIAID, NIH: (1) from Dr. Robert Shafer, catalogue no. 11803, pF190755_3; catalogue no. 11805, pV38886_2; catalogue no. 11806, pV48334_2; catalogue no. 11807, pM135498_1; catalogue no. 11808, pT450023_2; catalogue no. 11809, pV16970_2. (2) HIV-1 p24 hybridoma (183-H12-5C) from Dr. Bruce Chesebro. (3) SIV expression plasmid SIVmac239 SpX from Dr. Ronald C. Desrosiers. (4) TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. (5) Plasmid p96ZM651gag-opt from Drs. Yingying Li, Feng Gao, and Beatrice H. Hahn. HIV-1 NL4-3 CA mutants L363F and V362L/L363M expression plasmids were a gift of Dr. Christopher R. Aiken (Vanderbilt University School of Medicine).

■ ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; VSV-G, vesicular stomatitis virus G protein; LTR, long terminal repeat; HEK293, human embryonic kidney cells 293; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorting; SIV, simian immunodeficiency virus; FRET, fluorescence resonance energy transfer

■ REFERENCES

- (1) Wheeler, W. H.; Ziebell, R. A.; Zabina, H.; Pieniazek, D.; Prejean, J.; Bodnar, U. R.; Mahle, K. C.; Heneine, W.; Johnson, J. A.; Hall, H. I. Prevalence of Transmitted Drug Resistance Associated Mutations and Hiv-1 Subtypes in New Hiv-1 Diagnoses, U.S.-2006. *AIDS* **2010**, *24* (8), 1203–1212.
- (2) (a) Babine, R. E.; Bender, S. L. Molecular Recognition of Protein-ligand Complexes: Applications to Drug Design. *Chem. Rev.* **1997**, *97* (5), 1359–1472. (b) Leung, D.; Abbenante, G.; Fairlie, D. P. Protease Inhibitors: Current Status and Future Prospects. *J. Med. Chem.* **2000**, *43* (3), 305–341. (c) Sundquist, W. I.; Krausslich, H. G. Hiv-1 Assembly, Budding, and Maturation. *Cold Spring Harbor Perspect. Med.* **2012**, *2* (7), a006924.
- (3) (a) Zhou, J.; Yuan, X.; Dismuke, D.; Forshey, B. M.; Lundquist, C.; Lee, K. H.; Aiken, C.; Chen, C. H. Small-Molecule Inhibition of Human Immunodeficiency Virus Type 1 Replication by Specific Targeting of the Final Step of Virion Maturation. *J. Virol.* **2004**, *78* (2), 922–929. (b) Zhou, J.; Huang, L.; Hachey, D. L.; Chen, C. H.; Aiken, C. Inhibition of Hiv-1 Maturation Via Drug Association with the Viral Gag Protein in Immature Hiv-1 Particles. *J. Biol. Chem.* **2005**, *280* (51), 42149–42155. (c) Zhou, J.; Chen, C. H.; Aiken, C. Human Immunodeficiency Virus Type 1 Resistance to the Small Molecule Maturation Inhibitor 3-O-(3',3'-Dimethylsuccinyl)-Betulinic Acid Is Conferred by a Variety of Single Amino Acid Substitutions at the Ca-Sp1 Cleavage Site in Gag. *J. Virol.* **2006**, *80* (24), 12095–13101. (d) Wang, D.; Lu, W.; Li, F. Pharmacological Intervention of Hiv-1 Maturation. *Acta Pharm. Sin. B* **2015**, *5* (6), 493–499. (e) Urano, E.; Ablan, S. D.; Mandt, R.; Pauly, G. T.; Sigano, D. M.; Schneider, J. P.; Martin, D. E.; Nitz, T. J.; Wild, C. T.; Freed, E. O. Alkyl Amine Bevirimat Derivatives Are Potent and Broadly Active Hiv-1 Maturation Inhibitors. *Antimicrob. Agents Chemother.* **2016**, *60* (1), 190–197. (f) Tang, C.; Loeliger, E.; Kinde, I.; Kyere, S.; Mayo, K.; Barklis, E.; Sun, Y.; Huang, M.; Summers, M. F. Antiviral Inhibition of the Hiv-1 Capsid Protein. *J. Mol. Biol.* **2003**, *327* (5), 1013–1020. (g) Salzwedel, K.; Martin, D. E.; Sakalian, M. Maturation Inhibitors: A New Therapeutic Class Targets the Virus Structure. *AIDS Rev.* **2007**, *9* (3), 162–172. (h) Sakalian, M.; McMurtrey, C. P.; Deeg, F. J.; Maloy, C. W.; Li, F.; Wild, C. T.; Salzwedel, K. 3-O-(3',3'-Dimethylsuccinyl) Betulinic Acid Inhibits Maturation of the Human Immunodeficiency Virus Type 1 Gag Precursor Assembled in Vitro. *J. Virol.* **2006**, *80* (12), 5716–5722. (i) Li, J.; Goto, M.; Yang, X.; Morris-Natschke, S. L.; Huang, L.; Chen, C. H.; Lee, K. H. Fluorinated Betulinic Acid Derivatives and Evaluation of Their Anti-Hiv Activity. *Bioorg. Med. Chem. Lett.* **2016**, *26* (1), 68–71. (j) Li, F.; Zoumplis, D.; Matallana,

- C.; Kilgore, N. R.; Reddick, M.; Yunus, A. S.; Adamson, C. S.; Salzwedel, K.; Martin, D. E.; Allaway, G. P.; Freed, E. O.; Wild, C. T. Determinants of Activity of the Hiv-1 Maturation Inhibitor Pa-457. *Virology* **2006**, *356* (1–2), 217–224. (k) Li, F.; Goila-Gaur, R.; Salzwedel, K.; Kilgore, N. R.; Reddick, M.; Matallana, C.; Castillo, A.; Zoumplis, D.; Martin, D. E.; Orenstein, J. M.; Allaway, G. P.; Freed, E. O.; Wild, C. T. Pa-457: A Potent Hiv Inhibitor That Disrupts Core Condensation by Targeting a Late Step in Gag Processing. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (23), 13555–13560. (l) Adamson, C. S.; Waki, K.; Ablan, S. D.; Salzwedel, K.; Freed, E. O. Impact of Human Immunodeficiency Virus Type 1 Resistance to Protease Inhibitors on Evolution of Resistance to the Maturation Inhibitor Bevirimat (Pa-457). *J. Virol.* **2009**, *83* (10), 4884–4894. (m) Adamson, C. S.; Ablan, S. D.; Boeras, I.; Goila-Gaur, R.; Soheilian, F.; Nagashima, K.; Li, F.; Salzwedel, K.; Sakalian, M.; Wild, C. T.; Freed, E. O. In Vitro Resistance to the Human Immunodeficiency Virus Type 1 Maturation Inhibitor Pa-457 (Bevirimat). *J. Virol.* **2006**, *80* (22), 10957–10971. (n) Zhou, J.; Chen, C. H.; Aiken, C. The Sequence of the Ca-Sp1 Junction Accounts for the Differential Sensitivity of Hiv-1 and Siv to the Small Molecule Maturation Inhibitor 3-O-{3',3'-Dimethylsuccinyl}-Betulinic Acid. *Retrovirology* **2004**, *1*, 15. (o) Adamson, C. S.; Sakalian, M.; Salzwedel, K.; Freed, E. O. Polymorphisms in Gag Spacer Peptide 1 Confer Varying Levels of Resistance to the Hiv-1 Maturation Inhibitor Bevirimat. *Retrovirology* **2010**, *7*, 36–43.
- (4) Freed, E. O. Hiv-1 Assembly, Release and Maturation. *Nat. Rev. Microbiol.* **2015**, *13* (8), 484–496.
- (5) Kroto, H. W.; Heath, J. R.; O'Brien, S. C.; Curl, R. F.; Smalley, R. E. C60: Buckminsterfullerene. *Nature* **1985**, *318* (6042), 162–163.
- (6) (a) Thilgen, C.; Diederich, F. Structural Aspects of Fullerene Chemistry Journey through Fullerene Chirality. *Chem. Rev.* **2006**, *106* (12), 5049–5135. (b) Tzirakis, M. D.; Orfanopoulos, M. Radical Reactions of Fullerenes: From Synthetic Organic Chemistry to Materials Science and Biology. *Chem. Rev.* **2013**, *113* (7), 5262–5321.
- (7) (a) Cantalado, F.; Da Ros, T. *Medicinal Chemistry and Pharmacological Potential of Fullerenes and Carbon Nanotubes*; Springer: Dordrecht, The Netherlands, 2008; Vol. 1, pp 107–121 (b) Yang, X.; Ebrahimi, A.; Li, J.; Cui, Q. Fullerene–Biomolecule Conjugates and Their Biomedical Applications. *Int. J. Nanomed.* **2014**, *9*, 77–92.
- (8) (a) Friedman, S. H.; DeCamp, D. L.; Sijbesma, R. P.; Srdanov, G.; Wudl, F.; Kenyon, G. L. Inhibition of the Hiv-1 Protease by Fullerene Derivatives: Model Building Studies and Experimental Verification. *J. Am. Chem. Soc.* **1993**, *115* (15), 6506–6509. (b) Kornev, A. B.; Peregodov, A. S.; Martynenko, V. M.; Balzarini, J.; Hoorelbeke, B.; Troshin, P. A. Synthesis and Antiviral Activity of Highly Water-Soluble Polycarboxylic Derivatives of [70]Fullerene. *Chem. Commun.* **2011**, *47* (29), 8298–8300.
- (9) (a) Sijbesma, R.; Srdanov, G.; Wudl, F.; Castoro, J. A.; Wilkins, C.; Friedman, S. H.; DeCamp, D. L.; Kenyon, G. L. Synthesis of a Fullerene Derivative for the Inhibition of Hiv Enzymes. *J. Am. Chem. Soc.* **1993**, *115* (15), 6510–6512. (b) Saleh, N. A. The QSAR and Docking Calculations of Fullerene Derivatives as Hiv-1 Protease Inhibitors. *Spectrochim. Acta, Part A* **2015**, *136* (Part C), 1523–1529.
- (10) Martinez, Z. S.; Castro, E.; Seong, C. S.; Ceron, M. R.; Echegoyen, L.; Llano, M. Fullerene Derivatives Strongly Inhibit Hiv-1 Replication by Affecting Virus Maturation without Impairing Protease Activity. *Antimicrob. Agents Chemother.* **2016**, *60* (10), 5731–5741.
- (11) Witte, P.; Beuerle, F.; Hartnagel, U.; Lebovitz, R.; Savouchkina, A.; Sali, S.; Guldi, D.; Chronakis, N.; Hirsch, A. Water Solubility, Antioxidant Activity and Cytochrome C Binding of Four Families of Exohedral Adducts of C60 and C70. *Org. Biomol. Chem.* **2007**, *5* (22), 3599–3613.
- (12) Okuda, K.; Hirota, T.; Hirobe, M.; Nagano, T.; Mochizuki, M.; Mashino, T. Synthesis of Various Water-Soluble G60 Derivatives and Their Superoxide-Quenching Activity. *Fullerene Sci. Technol.* **2000**, *8* (3), 127–142.
- (13) Pettit, S. C.; Moody, M. D.; Wehbie, R. S.; Kaplan, A. H.; Nantermet, P. V.; Klein, C. A.; Swanstrom, R. The P2 Domain of Human Immunodeficiency Virus Type 1 Gag Regulates Sequential Proteolytic Processing and Is Required to Produce Fully Infectious Virions. *J. Virol.* **1994**, *68* (12), 8017–8027.
- (14) Varghese, V.; Mitsuya, Y.; Fessel, W. J.; Liu, T. F.; Melikian, G. L.; Katzenstein, D. A.; Schiffer, C. A.; Holmes, S. P.; Shafer, R. W. Prototypical Recombinant Multi-Protease Inhibitor Resistant Infectious Molecular Clones of Human Immunodeficiency Virus Type-1. *Antimicrob. Agents Chemother.* **2013**, *57* (9), 4290–4299.
- (15) (a) Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.; Erickson, J. Novel Fluorogenic Substrates for Assaying Retroviral Proteases by Resonance Energy Transfer. *Science* **1990**, *247* (4945), 954–958. (b) Windsor, I. W.; Raines, R. T. Fluorogenic Assay for Inhibitors of Hiv-1 Protease with Sub-Picomolar Affinity. *Sci. Rep.* **2015**, *5*, 11286.
- (16) (a) Marchesan, S.; Da Ros, T.; Spalluto, G.; Balzarini, J.; Prato, M. Anti-Hiv Properties of Cationic Fullerene Derivatives. *Bioorg. Med. Chem. Lett.* **2005**, *15* (15), 3615–3618. (b) Mashino, T.; Shimotohno, K.; Ikegami, N.; Nishikawa, D.; Okuda, K.; Takahashi, K.; Nakamura, S.; Mochizuki, M. Human Immunodeficiency Virus-Reverse Transcriptase Inhibition and Hepatitis C Virus Rna-Dependent Rna Polymerase Inhibition Activities of Fullerene Derivatives. *Bioorg. Med. Chem. Lett.* **2005**, *15* (4), 1107–1109. (c) Tanimoto, S.; Sakai, S.; Kudo, E.; Okada, S.; Matsumura, S.; Takahashi, D.; Toshima, K. Target-Selective Photodegradation of Hiv-1 Protease and Inhibition of Hiv-1 Replication in Living Cells by Designed Fullerene-Sugar Hybrids. *Chem. - Asian J.* **2012**, *7* (5), 911–914. (d) Tanimoto, S.; Sakai, S.; Matsumura, S.; Takahashi, D.; Toshima, K. Target-Selective Photo-Degradation of Hiv-1 Protease by a Fullerene-Sugar Hybrid. *Chem. Commun.* **2008**, *44*, 5767–5769.
- (17) Nashed, N. T.; Louis, J. M.; Sayer, J. M.; Wondrak, E. M.; Mora, P. T.; Oroszlan, S.; Jerina, D. M. Continuous Spectrophotometric Assay for Retroviral Proteases of Hiv-1 and Amv. *Biochem. Biophys. Res. Commun.* **1989**, *163* (2), 1079–1085.
- (18) (a) Friedman, S. H.; Ganapathi, P. S.; Rubin, Y.; Kenyon, G. L. Optimizing the Binding of Fullerene Inhibitors of the Hiv-1 Protease through Predicted Increases in Hydrophobic Desolvation. *J. Med. Chem.* **1998**, *41* (13), 2424–2429. (b) Strom, T. A.; Durdagi, S.; Ersoz, S. S.; Salmas, R. E.; Supuran, C. T.; Barron, A. R. Fullerene-Based Inhibitors of Hiv-1 Protease. *J. Pept. Sci.* **2015**, *21* (12), 862–870. (c) Al Garalleh, H.; Thamwattana, N.; Cox, B.; Hill, J. Modeling Interactions between C₆₀ Antiviral Compounds and Hiv Protease. *Bull. Math. Biol.* **2015**, *77* (1), 184–201. (d) Hassanzadeh, Z.; Ghavami, R.; Kompany-Zareh, M. Radial Basis Function Neural Networks Based on the Projection Pursuit and Principal Component Analysis Approaches: Qsar Analysis of Fullerene[C60]-Based Hiv-1 Pr Inhibitors. *Med. Chem. Res.* **2016**, *25*, 19–29. (e) Ahmed, L.; Rasulev, B.; Turabekova, M.; Leszczynska, D.; Leszczynski, J. Receptor- and Ligand-Based Study of Fullerene Analogues: Comprehensive Computational Approach Including Quantum-Chemical, Qsar and Molecular Docking Simulations. *Org. Biomol. Chem.* **2013**, *11* (35), 5798–5808.
- (19) Blair, W. S.; Cao, J.; Fok-Seang, J.; Griffin, P.; Isaacson, J.; Jackson, R. L.; Murray, E.; Patick, A. K.; Peng, Q.; Perros, M.; Pickford, C.; Wu, H.; Butler, S. L. New Small-Molecule Inhibitor Class Targeting Human Immunodeficiency Virus Type 1 Virion Maturation. *Antimicrob. Agents Chemother.* **2009**, *53* (12), 5080–5087.
- (20) Blair, W. S.; Pickford, C.; Irving, S. L.; Brown, D. G.; Anderson, M.; Bazin, R.; Cao, J.; Ciaramella, G.; Isaacson, J.; Jackson, L.; Hunt, R.; Kjerrstrom, A.; Nieman, J. A.; Patick, A. K.; Perros, M.; Scott, A. D.; Whitby, K.; Wu, H.; Butler, S. L. Hiv Capsid Is a Tractable Target for Small Molecule Therapeutic Intervention. *PLoS Pathog.* **2010**, *6* (12), e1001220.
- (21) Wilson, S. R.; Lu, Q. 1,3-Dipolar Cycloaddition of N-Methylazomethine Ylide to C70. *J. Org. Chem.* **1995**, *60* (20), 6496–6498.
- (22) He, J.; Choe, S.; Walker, R.; Di Marzio, P.; Morgan, D. O.; Landau, N. R. Human Immunodeficiency Virus Type 1 Viral Protein R (Vpr) Arrests Cells in the G2 Phase of the Cell Cycle by Inhibiting P34cdc2 Activity. *J. Virol.* **1995**, *69* (11), 6705–6711.
- (23) Gao, F.; Li, Y.; Decker, J. M.; Peyerl, F. W.; Bibollet-Ruche, F.; Rodenburg, C. M.; Chen, Y.; Shaw, D. R.; Allen, S.; Musonda, R.; Shaw, G. M.; Zajac, A. J.; Letvin, N.; Hahn, B. H. Codon Usage

Optimization of Hiv Type 1 Subtype C Gag, Pol, Env, and Nef Genes: In Vitro Expression and Immune Responses in DNA-Vaccinated Mice. *AIDS Res. Hum. Retroviruses* **2003**, *19* (9), 817–823.

(24) Garcia-Rivera, J. A.; Bueno, M. T.; Morales, E.; Kugelman, J. R.; Rodriguez, D. F.; Llano, M. Implication of Serine Residues 271, 273, and 275 in the Human Immunodeficiency Virus Type 1 Cofactor Activity of Lens Epithelium-Derived Growth Factor/P75. *J. Virol.* **2010**, *84* (2), 740–752.

(25) (a) Derdeyn, C. A.; Decker, J. M.; Sfakianos, J. N.; Wu, X.; O'Brien, W. A.; Ratner, L.; Kappes, J. C.; Shaw, G. M.; Hunter, E. Sensitivity of Human Immunodeficiency Virus Type 1 to the Fusion Inhibitor T-20 Is Modulated by Coreceptor Specificity Defined by the V3 Loop of Gp120. *J. Virol.* **2000**, *74* (18), 8358–8367. (b) Platt, E. J.; Bilska, M.; Kozak, S. L.; Kabat, D.; Montefiori, D. C. Evidence That Ecotropic Murine Leukemia Virus Contamination in Tzm-BL Cells Does Not Affect the Outcome of Neutralizing Antibody Assays with Human Immunodeficiency Virus Type 1. *J. Virol.* **2009**, *83* (16), 8289–8292. (c) Platt, E. J.; Wehrly, K.; Kuhmann, S. E.; Chesebro, B.; Kabat, D. Effects of CCR5 and CD4 Cell Surface Concentrations on Infections by Macrophagetropic Isolates of Human Immunodeficiency Virus Type 1. *J. Virol.* **1998**, *72* (4), 2855–2864. (d) Takeuchi, Y.; McClure, M. O.; Pizzato, M. Identification of Gammaretroviruses Constitutively Released from Cell Lines Used for Human Immunodeficiency Virus Research. *J. Virol.* **2008**, *82* (24), 12585–12588. (e) Wei, X.; Decker, J. M.; Liu, H.; Zhang, Z.; Arani, R. B.; Kilby, J. M.; Saag, M. S.; Wu, X.; Shaw, G. M.; Kappes, J. C. Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy. *Antimicrob. Agents Chemother.* **2002**, *46* (6), 1896–1905.

(26) Stojdl, D. F.; Lichty, B. D.; tenOever, B. R.; Paterson, J. M.; Power, A. T.; Knowles, S.; Marius, R.; Reynard, J.; Poliquin, L.; Atkins, H.; Brown, E. G.; Durbin, R. K.; Durbin, J. E.; Hiscott, J.; Bell, J. C. Vsv Strains with Defects in Their Ability to Shutdown Innate Immunity Are Potent Systemic Anti-Cancer Agents. *Cancer Cell* **2003**, *4* (4), 263–275.