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Target-Selective Photodegradation of HIV-1 Protease and Inhibition of HIV-1 Replication in Living Cells by Designed Fullerene–Sugar Hybrids

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Human immunodeficiency virus-1 (HIV-1) infection is considered a pandemic and continues to be a major medical threat to humans.[1] In anti-HIV therapies, HIV-1 protease is still one of the most important targets. HIV-1 protease cleaves high-molecular-weight virus polyproteins to give mature structural proteins,[2] and this function is essential for the replication of infective virus. Hence, HIV-1 protease has been the subject of much attention as a drug target.[3] One effective approach for the inhibition of HIV-1 protease activity is to employ small molecules that have a high affinity for or degrading ability toward HIV-1 protease. In this context, we recently reported that certain fullerene derivatives could degrade HIV-1 protease upon photoirradiation in the absence of any additives and under neutral conditions.[4] Herein, in a significant application of this fundamental result, we report that a newly designed fullerene–sugar hybrid with high water solubility effectively degraded HIV-1 protease and inhibited its enzymatic activity under photoirradiation. Moreover, the fullerene derivative effectively inhibited HIV-1 replication in living cells upon photoirradiation conditions.

In our previous study, the designed and synthesized fullerene–sugar hybrid **1** was found to cause degradation of HIV-1 protease, upon photoirradiation, in the absence of any additives and under neutral conditions.[4] However, **1** could not be applied to further biological studies using cells, mainly due to its low solubility in aqueous media. Spurred by this negative result, in the present study, we designed the novel fullerene–sugar hybrid **2** that consists of a fullerene attached to sugar and carboxylate moieties (Figure 1). The design was based on the expectation that the hydrophobic fullerene moiety of the hybrid would exhibit a high affinity for HIV-1 protease due to hydrophobic interactions, as previously reported by Friedman and coworkers.[5] It was also expected that the hydrophilic hydroxy and/or carboxylate groups of the hybrid would enhance the interaction with HIV-1 protease due to the formation of one or more hydrogen bonds. In addition, these hydrophilic groups were expected to increase the solubility of **2** in aqueous media.

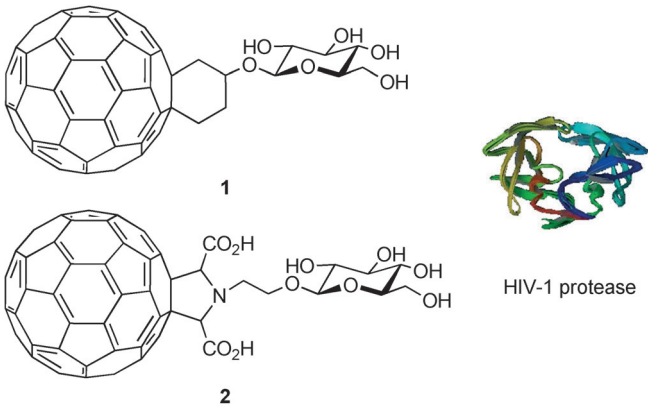

Figure 1. Chemical structure of fullerene–sugar hybrids **1** and **2**, and model structure of HIV-1 protease.

To synthesize **2**, a method involving 1,3-dipolar cycloaddition of an azomethine ylide was employed to modify the fullerene with two carboxylate moieties (Scheme 1).[6] A mixture of *tert*-butyl glyoxylate **4**,[7] ester **5** (prepared from ethanolamine in two steps), and fullerene (**3**) was refluxed in toluene to give the modified fullerene **6**. Deprotection of the *tert*-butyldimethylsilyl (TBS) group of **6** using trifluoroacetic acid (TFA) in dichloromethane gave the primary alcohol **7**, and glycosylation of **7** with **8** using $AgClO_4$ provided protected fullerene–β-glycoside **9** as a 3:2 diastereomeric mixture. This result suggests that **7** consisted of a racemic mixture with *trans* configuration at the C-2 and C-4 positions of the pyrrolidine ring. Cleavage of the acetyl groups of **9** using NaOMe, followed by deprotection of the *tert*-butyl ester groups using BBr_3, gave the designed hybrid **2** as a 3:2 diastereomeric mixture.[8] The isomers of **2** could not be separated by practical methods such as column chromatography using silica gel; thus, the diastereomeric mixture was used in the subsequent biochemical assays.

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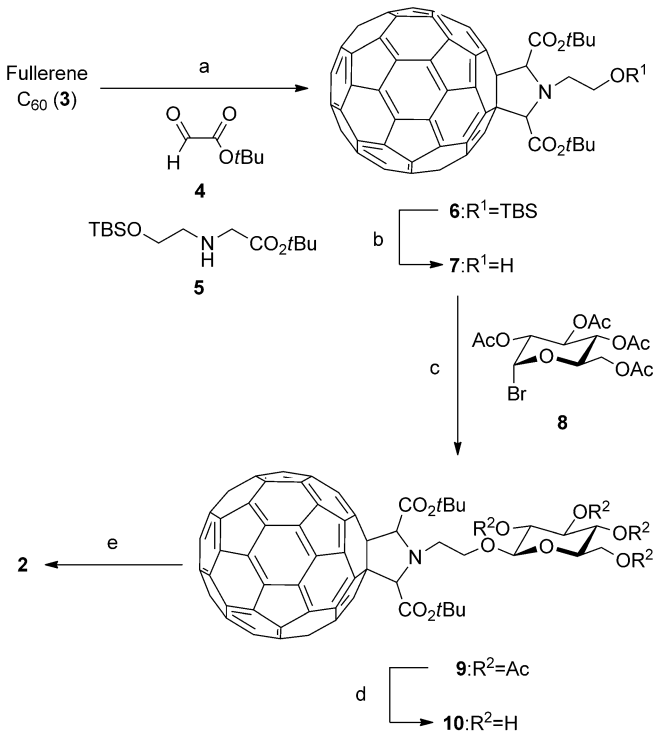

Scheme 1. Synthesis of fullerene-sugar hybrid **2**. a) PhMe, reflux, 18 h, 57%; b) TFA, CH_2Cl_2, 0°C, 10 min, 89%; c) $AgClO_4$, MS 5 Å, CH_2Cl_2, rt, 85 h, 59%, 3:2 dr; d) NaOMe, CH_2Cl_2/MeOH (3/1), 0°C, 1.5 h, 90%; e) BBr_3, $CHCl_3$, rt, 20 h, 45%.

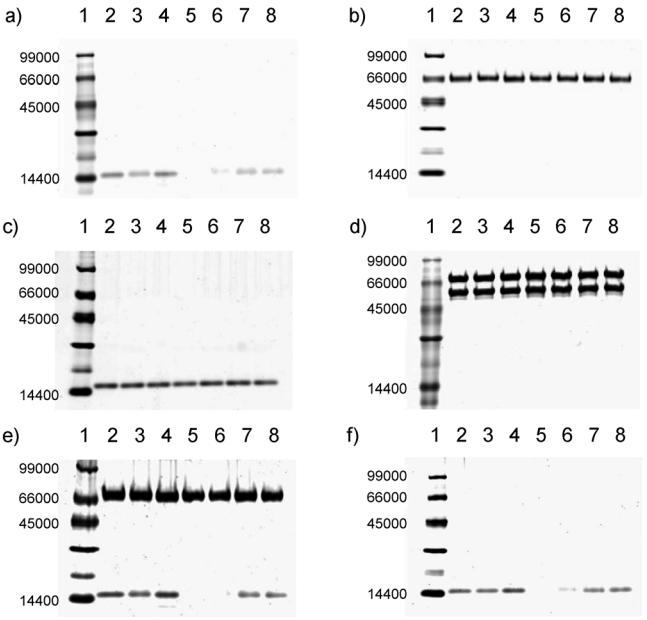

Figure 2. Photodegradation of HIV-1 protease and attempted photodegradation of other proteins using **2** under long-wavelength UV irradiation (panels a–e) or visible-light irradiation (panel f). Each protein (1.5 μM) was incubated with **2** in 50 mM PBS buffer (pH 7.0) containing 10% DMF at 25°C for 2 h under irradiation with a UV lamp (365 nm, 100 W) or visible light (diffuse sunlight, 75 W) placed 10 cm from the sample, and the products were analyzed by tricine-SDS-PAGE. The proteins used are: a, f) HIV-1 protease, b) BSA, c) Lyso, d) HIV reverse transcriptase, e) HIV-1 protease+BSA. Lanes 1, size marker; lanes 2, protein alone; lanes 3, protein upon photoirradiation; lanes 4, protein+**2** (15 nM) without photoirradiation; lanes 5–8, protein+**2** (at concentrations of 15, 5, 1.5, and 0.5 nM, respectively) upon photoirradiation.

After the chemical synthesis of hybrid **2**, photo-induced degradation assays of four proteins—HIV-1 protease, bovine serum albumin (BSA), hen egg lysozyme (Lyso), and HIV reverse transcriptase—were carried out under UV light irradiation (365 nm, 100 W), and the progress of the reactions was monitored by SDS-PAGE (Figure 2). Comparison of lanes 3 and 4 with lane 2 in Figure 2a showed that neither photoirradiation of HIV-1 protease in the absence of **2** nor treatment of HIV-1 protease with **2** without photoirradiation resulted in a change in the SDS-PAGE profile. By contrast, no band corresponding to HIV-1 protease could be detected after exposure to **2** under photoirradiation (lane 5), thus indicating degradation of HIV-1 protease. These results show that **2** is capable of degrading a target protein, HIV-1 protease, upon irradiation with long-wavelength UV light. It was also found that **2** degraded HIV-1 protease in a dose-dependent manner and that a catalytic amount of **2** was sufficient for the degradation. These results were in sharp contrast to those obtained using the other proteins (BSA, Lyso, and HIV reverse transcriptase), which showed no degradation upon photoirradiation (Figure 2b–d). Furthermore, it should be noted that when HIV-1 protease and BSA were both present in the reaction mixture, only HIV-1 protease was degraded by **2**, as shown in Figure 2e. These results clearly indicate that **2** selectively degraded HIV-1 protease upon long-wavelength UV irradiation. Moreover, to examine the possibility of photodegradation of proteins by **2** upon irradiation with visible light, we measured the UV/Vis spectrum of **2**. The results indicated that **2** absorbs not only in the UV region but also in the visible region (see Figure S1 in the Supporting Information). These results prompted us to examine the photodegradation activitity of **2** under visible-light irradiation. As shown in Figure 2f, similar results were obtained when visible light (diffuse sunlight, 75 W xenon lamp) was used instead of UV light, thereby clearly indicating that hybrid **2** degraded HIV-1protease upon irradiation not only with long-wavelength UV light but also with visible light. Moreover, it is noteworthy that the degradation activity of **2** is approximately 100 times higher than that of **1**.[4] The photodegradation of HIV-1 protease using **2** was also confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (see Figure S2 in the Supporting Information).

To investigate the mechanism behind the photodegradation of proteins, electron paramagnetic resonance (EPR) studies were carried out.[9] 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin-trapping agent for the detection of superoxide anions ($O_2^{\bullet-}$) or hydroxyl radicals ($^{\bullet}OH$) (Figure 3a). It was found that photoirradiation of **2** in the presence of DMPO gave products with EPR spectra characteristic of the DMPO–superoxide anion spin adduct DMPO/$^{\bullet}OOH$; no peaks corresponding to DMPO/$^{\bullet}OH$ were detected (Figure 3b). It was also confirmed that no peaks corre-

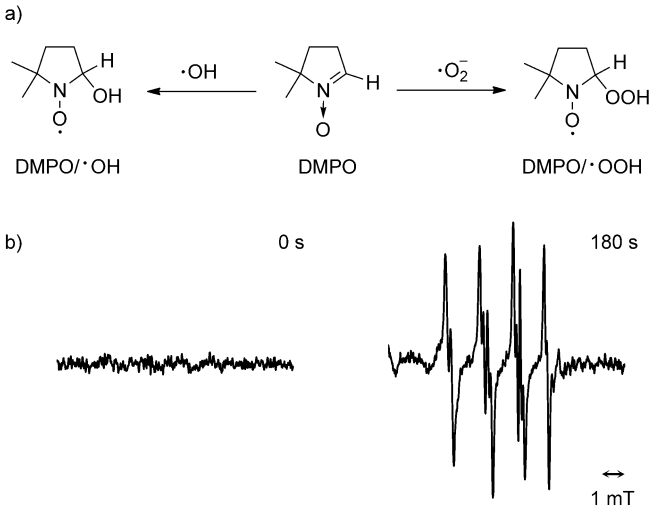

Figure 3. a) Formation of DMPO/·OOH and DMPO/·OH from DMPO. b) EPR spectra obtained during photoirradiation of **2** in the presence of DMPO. **2** (50 μM) was incubated with DMPO (500 mM) in 50 mM phosphate buffer (pH 7.0) containing 20% DMF, 1 mM diethylenetriamine pentaacetic acid, and 10 mM NADH under irradiation with visible light (diffuse sunlight, 75 W) placed 40 cm from a flat cell.

sponding to DMPO/·OOH were detected either upon treatment of DMPO with **2** without photoirradiation or upon photoirradiation of DMPO in the absence of **2**. Although $O_2^{\bullet-}$ is generally regarded as a rather unreactive radical species, it has been reported that $O_2^{\bullet-}$ plays an essential role in DNA cleavage.[10] Thus, we concluded that $O_2^{\bullet-}$, produced by the reaction of the photo-excited fullerene moieties and O_2, contributed mainly to the photodegradation of HIV-1 protease by the fullerene–sugar hybrid.[11]

To confirm that **2** binds to HIV-1 protease, enzyme inhibition assays were carried out using a peptide substrate of HIV-1 protease. As a result, **2** inhibited the enzymatic activity of HIV-1 protease, and the kinetic data fitted well the pattern of competitive inhibition (see Figure S3 in the Supporting Information). This result indicated that **2** bound to the active site of HIV-1 protease. The value of the inhibition constant (K_i) of **2** was determined as 0.33 μM (see Figure S4 in the Supporting Information). The difference in the inhibitory effects of **2** on HIV-1 protease in the presence or absence of photoirradiation was also examined. Half-maximal inhibitory concentrations (IC_{50}) of 2.25 and 15.1 μM with and without photoirradiation, respectively, were observed (Figure 4). The enzyme inhibitory activity of **2** increased 6.7-fold under visible-light irradiation. These results clearly indicate that **2** inhibits the enzymatic activity of HIV-1 protease much more efficiently under photoirradiation.

Next, we examined the inhibitory activity of **2** on the replication of HIV-1 with or without visible-light irradiation using HIV-1-infected Molt-4 T cells. Viral replication was assessed by detection of intracellular expression of p24 using flow cytometry and of p24 in the culture supernatant by ELISA. It was found that **2** significantly inhibited viral replication and decreased the amount of cells expressing

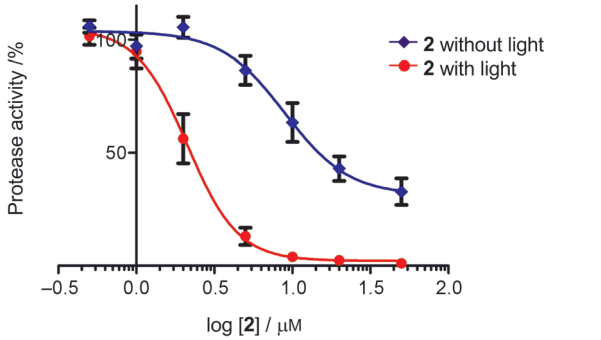

Figure 4. Plot of HIV-1 protease activity as a function of the concentration of **2** without photoirradiation (blue line) and with photoirradiation (red line, diffuse sunlight, 75 W). Assays were performed for 2 h at 25 °C in 50 mM acetate buffer (pH 5.5) containing 1 M NaCl, 2 mM EDTA, 5% glycerol, and 1% DMF. Nonlinear regression analysis with Prism version 5 (Graphpad Software, Inc.) was used for curve fitting of the substrate cleavage reaction. Substrate, H-Lys-Ala-Arg-Val-Nle-*p*-nitro-Phe-Glu-Ala-Nle-NH_2.

p24 upon photoirradiation in a dose-dependent manner (Figure 5b). HIV-1 replication was almost completely suppressed by treatment with 10 μM of **2** under photoirradiation conditions, whereas no significant inhibition was observed in the presence of 10 μM of **2** without photoirradiation (Fig-

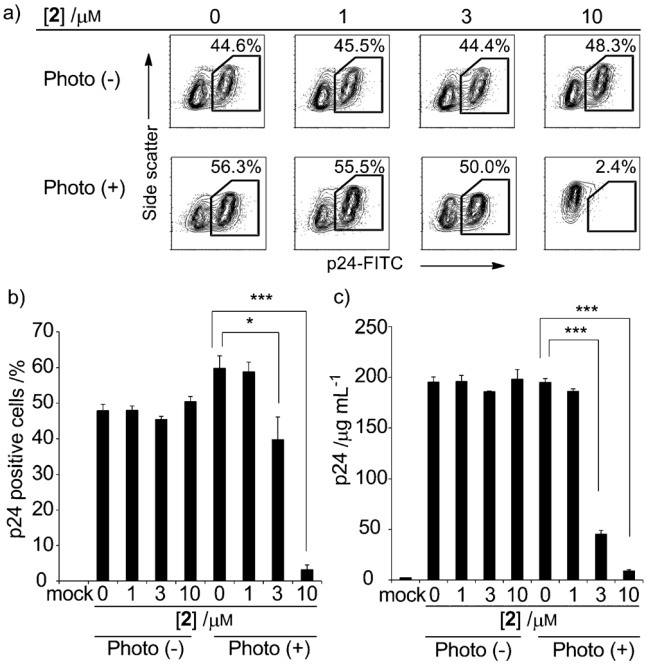

Figure 5. Inhibition of HIV-1 replication in Molt-4 T cells by **2**. Molt-4 cells were exposed to HIV-1 NL4-3 (50 ng mL^{-1} p24) by a spinoculation method.[12] Various concentrations of **2** (0–10 μM) were added after 48 h, and the cells were either exposed for 2 h or not exposed to visible light from a lamp (diffuse sunlight, 100 W) placed 45 cm from the cell culture. Viral replication was determined based on expression of the intracellular HIV-1 gag protein p24, determined by flow cytometry. a) Representative data from three independent experiments are shown. The numbers in the dot plots indicate the percentage of cells expressing HIV-1 p24. b) Bar graph showing the means and standard deviations from three independent experiments done in triplicate. c) The amount of p24 gag produced in Molt-4 T cells. *$p < 0.05$, ***$p < 0.001$.

ure 5a). Moreover, the amount of p24 in the supernatant was also decreased in the presence of **2** upon photoirradiation (Figure 5c).

Finally, we also examined the inhibitory activity of **2** on the replication of HIV-1 using peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA, Figure 6). As expected, **2** exhibited significant inhibitory activity of viral replication against PHA-stimulated PBMCs upon visible-light irradiation. Treatment with **2** reduced HIV-1 replication under photoirradiation conditions, while no inhibition was observed without photoirradiation. These results indicate that **2** inhibits HIV-1 replication in Molt-4 cells and PBMCs infected with HIV-1.

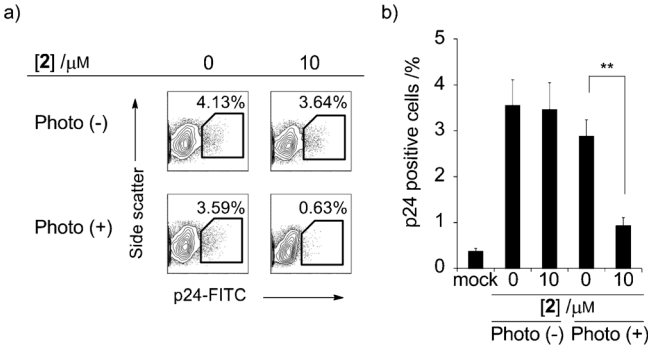

Figure 6. Inhibition of HIV-1 replication in PHA-stimulated PBMCs by **2**. PHA-stimulated PBMCs were exposed to HIV-1 NL4-3 (50 ng mL^{-1} p24). After 72 h, **2** (10 μM) was added, and the cells were either exposed for 2 h or not exposed to visible light from a lamp (diffuse sunlight, 100 W) placed 45 cm from the cell culture. Viral replication was determined based on expression of the intracellular HIV-1 gag protein p24, determined by flow cytometry. a) One representative data set from three independent experiments is shown. The numbers in the dot plots indicate the percentage of cells expressing HIV-1 p24. b) Bar graph showing the means and standard deviations from three independent experiments done in triplicate. $^{**}p < 0.01$.

In conclusion, it was found that fullerene–sugar hybrid **2** degrades HIV-1 protease upon irradiation with long-wavelength UV light or visible light in the absence of any additives and under neutral conditions. Moreover, we have developed a new method for effective inhibition of HIV-1 protease by photoirradiation using **2**. Although the inhibitory activity of **2** toward HIV-1 replication in living cells upon photoirradiation is not comparable with the photodegradation activity in vitro, probably due to a low cellular uptake, **2** showed significant light-selective inhibitory activity toward HIV-1 replication in living cells. The results presented here will contribute to the molecular design of novel protein photodegrading agents and agents for controlling the functions of proteins involved in diseases or infections.

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