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Synthesis and Virucidal Activity of a Water-Soluble, Configurationally Stable, Derivatized C_{60} Fullerene

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The bis(monosuccinimide) derivative of *p,p'*-bis(2-aminoethyl)diphenyl-C_{60} (compound 1), prepared by the fulleroid route, is active against human immunodeficiency virus type 1 (HIV-1) and HIV-2 (50% effective concentration [EC_{50}] averaging ~6 μM) in acutely or chronically infected human lymphocytes and is active in vitro against 3'-azido-3'-deoxythymidine-resistant HIV-1 (EC_{50}, ~3 μM). The virucidal properties of compound 1 were confirmed by virus inactivation assays. Compound 1 was noncytotoxic up to 100 μM in peripheral blood mononuclear cells and H9, Vero, and CEM cells. In cell-free assays, whereas the fullerene showed comparable activity against HIV-1 reverse transcriptase and DNA polymerase α (50% inhibitory concentration of ~5 μM), it demonstrated selective activity against HIV-1 protease.

The research activity on buckminsterfullerene (C_{60}) and other fullerenes in the last 3 years has been extraordinary. A host of physical and chemical properties of these materials have now been established, and their potential applications in several areas are now apparent (2, 10, 11). Fullerenes have recently been found in the geological environment of Shunga, a town in the lake region of Karwelia in Russia (4). To date, no pharmacological or other biological attributes of any fullerene have been reported. Nearly all the fullerenes characterized are nonderivatized homologs (spheroids, tubes, etc.) of C_{60}, and, like C_{60} itself, are highly hydrophobic and insoluble in aqueous media. A Swiss group recently reported the synthesis of a D-glucose derivative of C_{60}, but no biological data were provided (21). To examine the physical and chemical properties of fullerenes in biological systems, we sought fullerene derivatives that were not only soluble in water but also were solubilized by attachment of polar substituents, including ionic groups, situated in well-defined positions. That is, a premium was placed on preparing isomerically pure and configurationally stable covalently derivatized fullerenes. Recently, fullerene-cyclodextrin inclusion compounds (1) and fullerenes containing multiple covalently attached amine-derived substituents (7, 12, 13) have been demonstrated to have water solubility, but the lability of the former and configurational dynamism and complex isomerism of the latter compounds would preclude a ready and unequivocal evaluation of structure-activity data in biological systems. Polyhydroxylated, water-soluble fullerenes have also been prepared, but no single, fully characterized isomer has been isolated to date (5). Derivatives of the fullerenes which contain a functionalizable carbon atom have been labeled fulleroids.

For studies to inhibit retrovirus replication, it was desirable to have negatively charged functional groups at one end of the carbon cluster. We decided to construct a fulleroid derived from benzophenone since we were familiar with that reaction and since the synthesis of benzophenone derivatives is generally facile. The target fulleroid derivative was prepared according to the scheme shown in Fig. 1.

Compound 1, the bis(monosuccinimide) derivative of *p,p'*-bis(2-aminoethyl)-diphenyl-C_{60}, is prepared in a three-step sequence following the typical fulleroid synthesis (20, 22). The substituted diphenyldiazomethane was prepared in the usual manner from the substituted benzophenone hydrazone by oxidation with nickel peroxide. The bisacetamide was hydrolyzed with aqueous hydrochloric acid and was converted to the bissuccinamide 1 by treatment with succinic anhydride. Compounds 1 to 3 exhibit the usual fulleroid properties. The spectroscopic properties of water-soluble C_{60} derivative compound 1 were as follows: ^1H nuclear magnetic resonance (CD_3OD/CS_2) δ 8.10, 7.36 (2d, 8H, Arom. H), 3.44 (t, 4H, CH_2CH_2N), 2.86 (t, 4H, $PhCH_2CH_2N$), 2.55 and 2.43 (2t, 8H, $COCH_2CH_2CO$). Ir (KBr) 3,425 br, 2,925 m, 1,706 s, 1,650 s, 1,550, 1,427, 1,190, 590 m, 575 m, 557 m, 526 s. ^{13}C nuclear magnetic resonance ($DMSO_{d6}$, 125 MHz) δ 173.78, 170.78, 148.93, 145.73, 144.58, 144.52, 144.24, 144.09, 143.98, 143.60, 143.30, 142.36, 142.30, 141.66, 141.59, 139.97, 139.41, 137.13, 136.75, 130.94, 128.90, 79.37, 51.24, 34.87, 30.01, 29.14. UV-vis (λ_{max}, nm, CH_3OH/CS_2, solvent) 363, 433, 500 (sh). Mass spectroscopy (laser desorption time of flight) 1195 (M + Na)$^+$, 720 (C_{60}^+). Compound 1 is soluble and thermally stable in water at pH ≥7, making it an ideal substrate for evaluation of the physiological and pharmaceutical properties of a fulleroid.

Compound 1 was evaluated for antiviral activity in cells acutely and chronically infected with human immunodeficiency virus (HIV) (Table 1). Reverse transcriptase (RT) levels associated with virus were determined with virus concentrated from cell culture supernatants by high-speed centrifugation to reduce the chances of drug carryover in the enzyme assay (18). In human peripheral blood mononuclear cells (PBMC) infected with HIV type 1_{LAI} (HIV-1_{LAI}), compound 1 demonstrated activity with a median effective concentration (EC_{50}) (mean ± standard deviation) of 7.3 ± 5.9 μM (18). Surprisingly, this water-soluble fulleroid was also found to be effective in chronically infected H9 cells,

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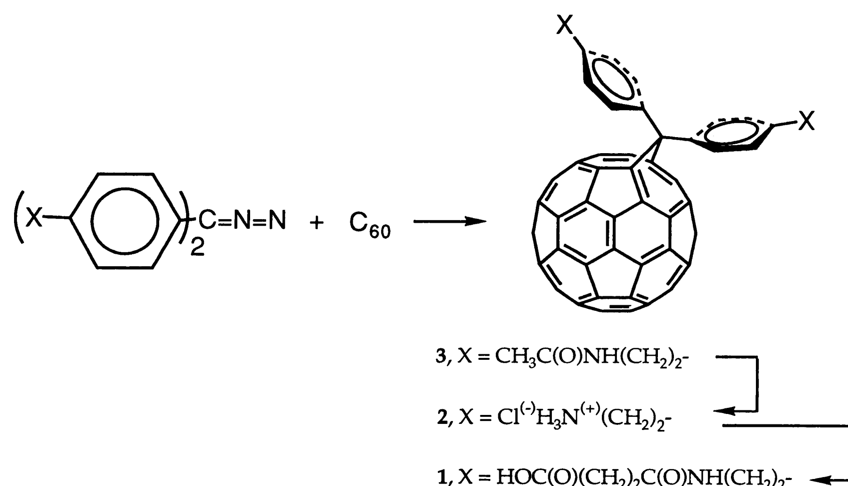

FIG. 1. Schematic representation of the synthesis of compound 1 and amine hydrochloride (2) and acetamide (3) intermediates.

with an EC_{50} of 10.8 ± 8.2 μM. Compound 1 appeared to be effective in human PBMC acutely infected with HIV-2_{ROD}, with an EC_{50} of 5.5 ± 3.8 μM. 3′-Azido-3′-deoxythymidine (AZT) used as a positive control had significant activity against HIV-1 and HIV-2 in acutely infected cells but no effect in chronically infected H9 cells (Table 1). However, in acutely infected cells, AZT was markedly more potent than compound 1. Since the fullerene may bind tightly to inactivate the viral RT, we also measured levels of HIV-1 p24 in supernatants of treated and untreated cultures. Clarified supernatants were treated with Triton X-100 and tested for HIV-1 antigen by immunocapture with a commercial kit (Abbott, North Chicago, Ill.). In PBMC acutely infected with HIV-1, the mean EC_{50} increased from 7.3 μM in an RT assay to 50 μM in a p24 assay, suggesting virus inactivation as the primary antiviral mechanism for this compound.

To confirm these findings, inactivation studies with HIV-1_{LAI} were performed. Briefly, in a Nalgene tube, 75 μl of virus (approximately 200 50% tissue culture infective doses) and 2 ml of compound 1 (5 to 25 μM) or medium without drug were incubated for 2 h at 37°C. After incubation, 3 ml of RPMI 1640 growth medium was added to each tube. Each tube was then centrifuged at 40,000 rpm for 30 min at 4°C in a Beckman 70.1Ti rotor. The supernatant was then removed, and the virus pellet was resuspended in fresh medium. The virions were added to human PBMC which had been stimulated for 2 days with phytohemagglutinin in 25-cm^2 flasks in a total of 10 ml of growth medium. On day 6, residual virus in the supernatant was quantitated by an RT assay, as described previously (18). Untreated virus-infected control had a mean RT activity of 1,140 kdpm per ml. When cell-free HIV-1 was incubated for 2 h with compound 1 and the virus was concentrated and inoculated into fresh mitogen-stimulated human PBMC as described above, virus infectivity was reduced by more than 95% relative to that of the untreated control at 5 to 25 μM.

The development of single agents to treat HIV-1 infections inevitably results in the emergence of drug-resistant virus (14). The availability of AZT-resistant and -susceptible viruses allowed us to evaluate the compounds for their susceptibilities to these viruses in acutely infected PBMC in two experiments. The susceptibility of the pretherapy isolate (HIV-1_{H112-2}) (EC_{50}, 2.8 μM) to compound 1 was compared with that of the posttherapy AZT-resistant virus (HIV-1_{G910-6}) (EC_{50}, 2.7 μM) in lymphocytes. For this virus pair, the AZT EC_{50}s increased more than 180-fold, from ≤0.001 to 0.18 μM. For these results, the variance from the means was less than 15%. With nevirapine (BI-RG587)-resistant and -susceptible HIV-1 (15), the EC_{50} for compound 1 increased from 0.53 to 8.7 μM, respectively, a 16-fold increase in resistance. In the same assay, the EC_{50} for TIBO [(+)-(5*s*)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo

TABLE 1. Summary of the activities of compound 1, phosphonoformate, and AZT

Compound	Antiviral activity (EC_{50} [μM]) in:			Cytotoxicity (IC_{50} [μM])[a] in:				IC_{50} polymerases (μM) in	
	PBMC infected with HIV_{LAI}[b]	PBMC infected with HIV-2_{ROD}[b]	H9 cells infected with HIV-1_{IIIb}[c]	PBMC	H9 cells	Vero cells (at day 3)	CEM cells	HIV-1 RT	DNA polymerase α
1	7.3	5.5	10.8	>100[d]	>100	>100	>100	4.6	4.9
PFA[e]	0.43	0.44	ND[f]	>640	ND	>100	>100	0.32	>100
AZT[g]	0.004	0.003	>100	>100	60	23	13	0.04	>100

[a] Measured by cell counts on day 6, except where indicated.
[b] Acutely infected. The virus RT level was determined on day 6 after infection (18). Values are means of quadruplicate experiments. The correlation coefficient for the data was >0.96. The variance from the means was less than 20%.
[c] Chronically infected. The virus RT level was determined after 6 days of treatment. Values are means of duplicate experiments.
[d] Measured by radiolabeled thymidine uptake on day 2 and by cell counts on day 6.
[e] PFA, phosphonoformate.
[f] ND, not determined.
[g] AZT-5′-triphosphate was used for the enzyme assays (3, 17, 18).

(4,5,1-jk)(1,4)benzodiazepin-2(1H)-thione] R82150 increased >500-fold (data not shown). The lack of cross-resistance with AZT suggests that combination studies of the fullerenes with AZT in vitro could be considered (16).

No cytotoxicity was demonstrable with compound 1 in uninfected, slowly dividing PBMC or rapidly dividing H9, Vero, or CEM cells (18). Few compounds have demonstrated selective antiviral activity in chronically infected cells (19). The apparent activity of compound 1 in acutely infected cells is comparable to that of the recently reported TAT inhibitor Ro 5-3335 developed by the Hoffmann La-Roche Co. (8). However, it is highly likely that residual drug may inactivate the virus directly, resulting in an overestimation of the potency of the fulleroid in chronically infected H9 cells. The compound was also evaluated for its inhibitory effect on recombinant p66/51 HIV-1 RT by using $poly(rA)_n \cdot oligo(T)_{12-18}$ as the template-primer (17). Compound 1 was active against this enzyme, with a median inhibitory concentration (IC_{50}) of 4.6 μM. This value was of the same order of magnitude as that noted for the antiviral assays (Table 1). The compound did not demonstrate selectivity against cellular DNA polymerase α (3). The finding that compound 1 inhibits DNA polymerase α in a cell-free system with an IC_{50} of 4.9 μM and also exhibits no cytotoxicity in various cells is consistent with the proposed virucidal mechanism, since one would anticipate some cytotoxicity if the compound were transported intracellularly. AZT-5′-triphosphate and phosphonoformate, used as positive controls, were effective and selective against HIV-1 RT. The collaborative group of Friedman et al. have garnered data indicating that compound 1 has some activity (IC_{50}, 40 μM) against HIV-1 protease but not to the degree that it has against HIV RT (6). We have confirmed the antiprotease activity of compound 1. Briefly, in an assay with 0.1 M sodium acetate buffer, pH 5.5, at 37°C and an enzyme concentration of 0.08 μM, compound 1 was found to have IC_{50}s of 2.0 μM against recombinant HIV-1 protease and 20 μM against pepsin (with 0.028 μM enzyme in 0.2 M sodium citrate, pH 2.0, at 37°C). The method used for the assay was similar to that described by Ido et al. (9). Inhibition was time dependent, and preincubation with the inhibitor resulted in greater enzyme inhibition, indicating a slow binding process (data not shown). Differences between our results and those of Friedman et al. (6) may be related to the final dimethyl sulfoxide concentration used in the assay as well as other factors. The results suggest that it may be possible to develop more specific and more potent protease inhibitors of this class of compound. Protease inhibitors are an important class of anti-HIV agents. The inherent problem with approaches aimed at inhibiting proteases is that the compounds must target the virion and penetrate the virus membrane or must be present in sufficient quantity near the cell membrane prior to virion budding. In addition, protease inhibitors must be resistant to degradative enzyme, have a low protein-binding affinity, and ideally should be orally bioavailable. The finding that the fulleroid demonstrates antiprotease activity in addition to virucidal properties suggests that a mechanism other than inhibition of RT may be responsible for the inactivation of virus.

In summary, we have demonstrated for the first time that a water-soluble fullerene has modest selective activity against HIV-1 in acutely and chronically infected cells. However, compound 1 was also shown to have virucidal properties, suggesting direct interactions between the fullerene and HIV-1. The virucidal properties of this compound probably account for the major viral inhibitory activity observed in vitro. Although additional studies of the mechanism of antiviral action of this compound are warranted, our results and those of Friedman et al. suggest that the compound inhibits HIV-1 RT and DNA polymerase α and selectively inhibits HIV-1 protease in cell-free systems (Table 1) (6). This compound can be viewed as a lead in the discovery of additional water-soluble fullerenes with greater virucidal and antiprotease activities and should also stimulate biochemical and pharmacological studies to determine the metabolic disposition of this novel class of compounds in living systems.

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