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ANTI-HUMAN IMMUNODEFICIENCY VIRUS, TOXICITY IN CELL CULTURE, AND TOLERANCE IN MAMMALS OF A WATER-SOLUBLE FULLERENE

Raymond F. Schinazi,[1*] Angela McMillan,[1] Amy S. Juodawlkis,[1] Julie Pharr,[1] Rint Sijbesma,[2] Gordana Srdanov,[2] Jan-Cornelis Hummelen,[2] F. Douglas Boudinot,[3] Craig L. Hill,[4] and Fred Wudl[2]

[1]*Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine/Veterans Affairs Medical Center, Decatur, GA 30033; [2]Institute for Polymers and Organic Solids and Departments of Chemistry and Physics, University of California, Santa Barbara, CA 93106; [3]Department of Pharmaceutics, School of Pharmacy, University of Georgia, Athens, GA 30602; and [4]Department of Chemistry, Emory University, Atlanta, GA 30322.*
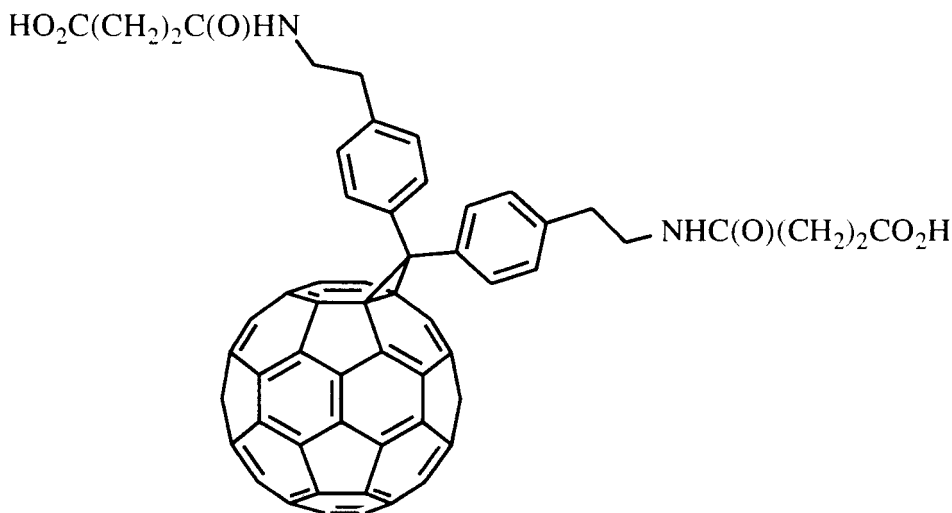
Abstract. The bis(monosuccinimide) derivative of *p,p*'-bis(2-aminoethyl)-diphenyl C_{60}, **1**, had substantial activity against HIV-1 and HIV-2 in acutely or chronically infected human lymphocytes, and activity against AZT-resistant HIV-1 *in vitro* (EC_{50} ~ 3-7 μM). This activity was primarily associated with virucidal and anti-protease properties of **1**. The fullerene had very low cytotoxicity (IC_{50} > 100 μM in PBMC, H9, Vero, and CEM cells) in various cells. Compound **1** did not interact with the $CD4^+$ receptor on human lymphocytes even at 100 μM. Similarly, no anti-fusion activity was noted at that concentration. No toxicity in mice was apparent when **1** was administered intraperitoneally at 50 mg/kg per day for 6 days.

INTRODUCTION

While the physics and chemistry of fullerenes have expanded at a remarkable rate in the last few years (1-3), the biology of this rapidly growing class of carbon clusters is in its initial stages of development. The physiological attributes and potential medical applications of fullerenes are limited in part by the fact that unfunctionalized fullerenes are hydrophobic and insoluble in water. With the advent of methanofullerenes, fulleroids (4) and other fullerene functionalization methodologies (5-15), several general approaches to derivatized water-soluble fullerenes are now defined. Preliminary reports on the application of a bis(monosuccinimide) derivative of *p,p*'-bis(2-aminoethyl)diphenyl C_{60}, **1** (Figure 1) to human immunodeficiency virus (HIV) have appeared (16-18). The concept for making water soluble fullerenes was reduced to practice when we discovered that another cluster class of compounds, namely polyoxometalates (POM), were found to inhibit HIV-1 protease (19). Since the size of certain POM fit the active site of this enzyme, we reasoned that fullerenes, if they could be solubilized in biological medium, should also inhibit this critical viral enzyme. At physiological pH, the terminal carboxylic acid groups of **1** are deprotonated rendering the complex water soluble. We report here further information on the antiviral activity, biochemical properties, antifusion/binding characteristics, and for the first time, mammalian tolerance of this complex.

EXPERIMENTAL

Chemistry. The title complex, **1**, was prepared and purified by the literature procedure (16). All other reagents were reagent grade and used as received.

Figure 1. Structure of a bis(monosuccinimide) derivative of
p,p'-bis(2-aminoethyl)diphenyl C_{60} (**1**).

Virology and cytotoxicity. The cell culture assays and protocols were described previously (16, 20, 21). For the inactivation studies with HIV-1$_{LAI}$, 75 μl of virus (approximately 200 TCID$_{50}$) and 2 ml of compound **1** (5 to 25 μM) or medium without drug (control) were incubated for 2 hr at 37°C in a Nalgene tube. After incubation, RPMI 1640 growth medium (3 ml) was added to each tube, then each was centrifuged at 40,000 rpm for 30 min at 4°C (Beckman 70.1Ti rotor). After removal of the supernatant, the virus pellet was resuspended in fresh medium. The suspension was added to 10 ml of growth medium containing 2 day phytohemagglutinin stimulated human peripheral blood mononuclear cells (PBMC) in 25 cm^2 flasks. On day 6, the amount of virus in the supernatant was quantitated by a reverse transcriptase (RT) assay and compared to untreated control (20). Untreated virus infected control had a mean RT activity of 1,140 kdpm/ml.

Effect of fullerene on anti-CD4 antibody binding in CEM or PBMC cells. The extent to which fullerene compounds will block the binding of anti-CD4 antibody in log phase CEM cells or PBMC (unstimulated) was determined using a fluorescent activated cell sorter (FACS). Cells (2 x 10^6) were incubated with **1** (1, 10, 25, 50 and 100 μM) in 10 ml RPMI-10% (or 20% for PBMC) inactivated fetal bovine serum for 3 hr at 37°C. The POM HPA-23 (100 μM) was used as a positive control. Untreated control flasks were also seeded (1 % DMSO and medium control). After incubation, the cells were centrifuged at 200 *g* for 10 min, washed with Dulbecco's phosphate buffered saline (PBS), and counted with a hemacytometer to determine the viability by dye exclusion. The cell pellet was resuspended in PBS (200 μl) and an aliquot (100 μl) was stained with mouse anti-CD3-FITC/anti-CD4-PE antibody (20 μl) (Becton Dickinson, San Jose, CA) for 30 min at room temperature in the dark. A negative control antibody (mouse anti-KLH-FITC/PE, Becton Dickinson) was used to stain the remaining aliquot (100 μl). After incubation with

antibody, the cells were centrifuged at 200 g for 4 min, washed with PBS (500 μl), recentrifuged for 10 min, resuspended in paraformaldehyde/PBS (1%, 500 μl) and stored at 4°C in the dark up to 72 hr prior to FACS analysis. FACS analysis was performed by acquiring 5000 events on a Becton Dickinson FACSort and gating on intact cells visualized by forward scatter *versus* side scatter. Gated cells were analyzed by histogram to obtain the red fluorescence (FL2) intensity mean channel number (MCN). Percent inhibition of binding was determined as follows:

$$\%I = 100(MCN_{medium}-MCN_{compound})/\ MCN_{medium}$$

Effect of fullerene on fusion of CEM and TF228.1.16 cells. Determination of the extent to which fullerene compounds inhibit the formation of syncytia between $CD4^+$ and gp 120^+ cell lines was performed using a modification of a previously reported technique (22, 23). TF228.1.16 cells, obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA), are non-infectious cells derived from BJAB cells transfected with a vector containing the full-length BH10 HIV-1 gp160. These cells stably express functionally active gp120-gp41 on its surface and like virus infected cells release low level gp120 into the media (23). CEM cells and TF228.1.16 cells (2.5×10^4 of each cell type) were mixed or aliquoted separately into 96-well plates in 180 μl of HY medium (GIBCO, Grand Island, NY) containing 10% heat inactivated fetal bovine serum. Compound **1** (1, 10, 50, and 100 μM) was added in duplicate (20 μl/well). HPA-23 (1, 10, and 100 μM) was used as a positive control. Negative untreated controls received only medium. Cells were incubated for 16-18 hr in a 5% CO_2 incubator at 37°C. Syncytia were counted in 5 fields using 100x magnification.

Toxicity studies in mice. Random-bred Swiss CFW mice (female, 4 to 5 weeks old) were obtained from Charles River Laboratory (Portage, MI). After acclimatization for 2 weeks, the mice were divided into 6 groups of 6 mice each and treated intraperitoneally (i.p.) with compound **1** at a dose of 15, 30, and 50 mg/kg per day (mkd) for 6 days. Control untreated, vehicle (2% DMSO) treated, and PBS treated groups were included. The compound was dissolved in 2% DMSO/PBS and 0.5 ml was administered. Mice were weighed individually on day 0, 1, 6, 8, 11, 15, and 22. Loss in weight, failure to gain weight, and/or ruffled fur were considered signs of distress or toxicity.

RESULTS AND DISCUSSION

Compound **1** was evaluated for antiviral activity in acutely and chronically HIV infected cells (Table 1). In human PBMC infected with HIV-1_{LAI}, **1** demonstrated activity with a median effective concentration ($EC_{50} \pm$ S.D.) value of 7.3 ± 5.9 μM. This water-soluble methanofullerene was also found to be effective in chronically infected H9 cells with an EC_{50} value of 10.8 ± 8.2 μM, and in acutely HIV-2_{ROD} infected human PBMC with an EC_{50} value of 5.5 ± 3.8 μM. 3'-Azido-3'-deoxythymidine (AZT) used as a positive control had potent activity against HIV-1 and HIV-2 in acutely infected cells, but no effect in chronically infected H9 cells (Table 1). When cell-free HIV-1 was incubated for 2 hr with compound **1** (5-25 μM) and the virus was then concentrated and inoculated into fresh mitogen stimulated human PBMC, virus infectivity was reduced by greater than 95% relative to untreated control, suggesting virucidal activity. No cytotoxicity was demonstrable with **1** in uninfected slowly dividing PBMC, and rapidly dividing H9, Vero or CEM cells. With the exception of protease and TAT inhibitors, few compounds have demonstrated selective antiviral activity in chronically infected cells (25).

The compound was also evaluated for its inhibitory effect on recombinant p66/51 HIV-1 reverse transcriptase (RT) using a poly(rA)$_n$·oligo(T)$_{12\text{-}18}$ as template-primer (24). 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). However, the compound did not demonstrate selectivity since it also inhibited cellular DNA polymerase α at a similar concentration. AZT-5'-triphosphate (AZT-TP) and phosphonoformate (PFA), used as positive controls, were effective and selective against HIV-1 RT.

Table 1. Summary of the effects of a water-soluble derivatized fullerene **1** on HIV production in acutely infected PBMC chronically infected H9 cells, its cytotoxicity in uninfected PBMC, H9, Vero and CEM cells, and effect on viral and cellular polymerases.

Compound	Antiviral activity (EC$_{50}$, μM) in:			Cytotoxicity (IC$_{50}$, μM) in:				Polymerases (IC$_{50}$, μM):	
	PBMC [a] HIV-1$_{LAI}$	PBMC[a] HIV-2$_{ROD}$	H9[b] HIV-1$_{IIIb}$	PBMC	H9[c]	Vero[c]	CEM[c]	HIV-1 RT	DNA pol α
1	7.3	5.5	10.8	> 100[d]	> 100	> 100	> 100	4.6	4.9
PFA	0.43	0.44	ND[e]	> 640[c]	ND	> 100	> 100	0.32	> 100
AZT	0.004	0.003	> 100	> 100[c]	60	23	13	0.04[f]	> 100[f]

[a] Acutely infected cells. Virus yield was determined on day 6 after infection (20, 21). Values represent means of quadruplicate experiments. The correlation coefficient for the data was > 0.96. Adapted from ref. 16.
[b] Chronically infected cells. Virus yield was determined after 6 days of treatment. Values are mean of duplicate experiments
[c] Measured by cell counts on day 6.
[d] Measured by radiolabeled thymidine uptake on day 2 and by cell counts on day 6.
[e] Not determined.
[f] AZT-TP was used for the enzyme assays (24).

Compound **1** was found to have an IC$_{50}$ of 2.0 μM against recombinant HIV-1 protease. The inhibition was time dependent and preincubation with the inhibitor resulted in greater enzyme inhibition indicating a slow binding process (data not shown). The method used for the assay was similar to that previously described (26). The collaborative group of Friedman, Kenyon, and Wudl have also reported that **1** has activity against HIV-1 protease with a K$_i$ of 5.3 μM (17, 18).

Table 2. Effect of the fullerene **1** on anti-CD4 antibody binding in CEM cells.

Compound	Class	Concentration, μM	% Inhibition[a]
1	Fullerene	100	23.0
HPA-23	POM	100	83.8

[a] Cells were stained with mouse anti-CD3-fluorescein isothiocyanate (FITC)/anti-CD4-phycoerythrin (PE) antibody. A negative control antibody [mouse anti-keyhole limpet hemocyanin (KLH)-FITC/PE] was also used.

Since compound **1** is a charged molecule, it was important to determine if it interacted with $CD4^+$ receptor on lymphocytes. It appears that **1** demonstrated a modest ability (23% at 100 μM) to block the binding of anti-CD4 antibody to $CD4^+$ CEM cells (Table 2). The compound was similarly evaluated in PBMC up to 100 μM; no appreciable inhibition (1.1%) was noted (data not shown). In a cell fusion assay between CEM and TF228.1.16 cells, compound **1** had no activity up to a concentration of 100 μM, whereas HPA-23 used as a positive control completely inhibited fusion at 1 μM. Taken together, these data suggest that **1** does not interact with either the viral glycoprotein gp120 nor with the cellular receptor $CD4^+$. However, these results do not rule out the possibility that other related fullerenes could have a different inhibitory profile.

The development of single agents to treat HIV-1 infections inevitably results in the development of drug-resistant virus (25, 28). The availability of AZT-resistant and susceptible viruses allowed us to evaluate the compounds for their susceptibility to these viruses in acutely infected PBMC. The susceptibility of the pretherapy isolate compared to post-therapy AZT-resistant virus in lymphocytes was not substantially different (Table 3). With the nevirapine (BI-RG587)-resistant and sensitive HIV-1 (27), the EC_{50} increased from 0.53 μM to 8.7 μM, respectively, a 16-fold increase in resistance. In the same assay, the EC_{50} value for the non-nucleoside RT inhibitor TIBO R82150 increased 506-fold (data not shown). The lack of cross-resistance with AZT suggests that combination studies of the fullerenes with AZT should be considered *in vitro* (29).

Table 3. Effects of a derivatized fullerene, **1**, against a pair of AZT-sensitive and resistant HIV-1 in acutely infected primary human lymphocytes.

Compound	AZT-susceptible HIV-1$_{H112-2}$ EC_{50}, μM	AZT-resistant HIV-1$_{G910-6}$ EC_{50}, μM	Fold increase in EC_{50} value
1	2.8	2.7	1.0
AZT	≤ 0.001	0.18	≥ 180

Because of concerns related to the potential carcinogenic effects of benzene and related compounds, Nelson et al. (30) were the first to study the effect of topical fullerenes in mammals. They demonstrated a lack of carcinogenic effects of C_{60} and C_{70} after acute and subchronic topical applications to mice. Since no systemic toxicity studies have been reported with any fullerene, we evaluated the intraperitoneal toxicity of compound **1** in mice treated daily for 6 days at 15, 30 and 50 mkd. A slight decline in weight was noted after the first dose in all the control and treated groups except for the untreated control group (Figure 2). Subsequently, all the animals gained weight over the period of observation and no animal died in any of the groups. There was no statistically significant difference in the weights of the treated groups *versus* control groups (p > 0.05). These results indicate that **1** was well tolerated up to a dose of 50 mkd (qd x 6d). The animals in this experiment continue to be monitored. As of 2 months after the initial treatment, none of the animals have died. Pharmacokinetic studies in rats with this compound are currently being performed and the results will be reported elsewhere.
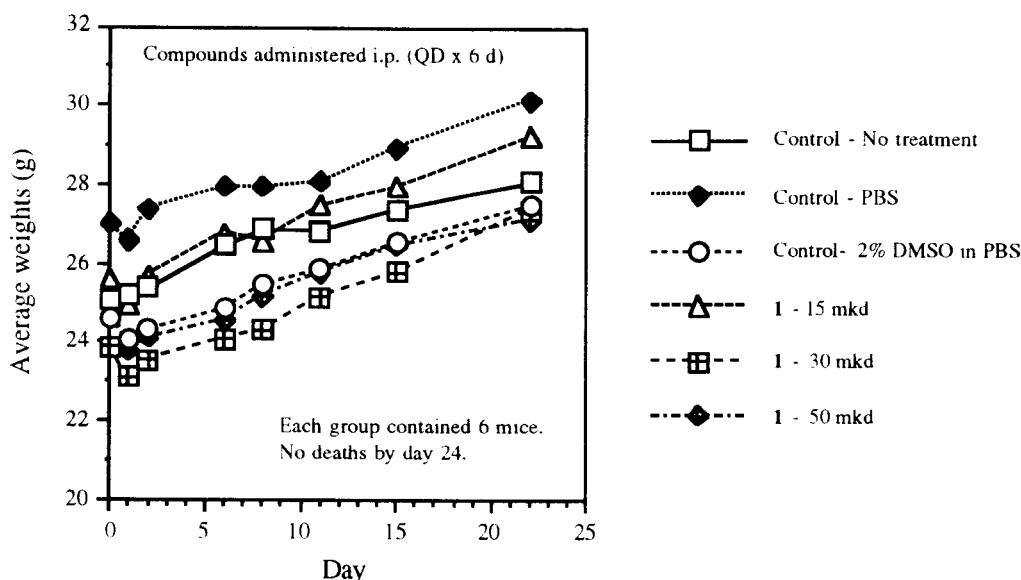
Figure 2. Effect of the water-soluble fullerene **1** in Swiss mice.

In summary, we have demonstrated for the first time that a water-soluble fullerene has potent and selective activity against HIV-1 in acutely and chronically infected cells. Compound **1** was also shown to have virucidal properties suggesting direct interactions between the fullerene and HIV-1. Although additional studies on the mechanism of antiviral action of this compound are warranted, results suggest that the compound can inhibit HIV-1 RT and protease. However, **1** had no significant antifusion properties and it did not interact with the $CD4^+$ receptor on lymphocytes (Tables 1 and 2). Of significance was the recent report that a fullerene carboxylic acid had photochemical properties and induced DNA cleavage predominantly at guanine residues (9). The single strand breaks were presumably generated by singlet oxygen. This physicochemical property could account in part for the virucidal property of **1**. The search for compounds that inactivate HIV in genital secretion without spermicidal properties or toxicity is a desirable medical goal. Such a compound may also have utility in inactivating HIV in blood and blood products (31). Compound **1** can be considered as a lead in the discovery of other fullerenes with greater virucidal activity. It is also encouraging that this class of compound is essentially non-toxic *in vitro* and is well tolerated in small mammals at high doses.

ACKNOWLEDGMENTS

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