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Inhibition of retrovirus-induced syncytium formation by photoproducts of a brominated 1,8-naphthalimide compound

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Abstract

A major disadvantage of conventional phototherapy is the requirement for the in situ delivery of stimulating photoenergy *subsequent* to the binding of photochemicals to target malignant cells, or virus-infected cells, or viruses. This drawback has resulted in considerable limitation in the use of photochemicals in photomedicine. To circumvent this problem, we have investigated the antiviral efficacy of a brominated 1,8-naphthalimide photocompound, termed LY66Br [3-bromo-4-(hexylamino)-*N*-hexyl-1,8-naphthalimide], which upon exposure to visible light at 420 nm generates independently of oxygen one or more stable antiviral molecular photoproducts (e.g., is 'preactivated'). Human cell lines infected with the human immunodeficiency virus type 1 (HIV-1), or with the human T-lymphotropic virus type-1 (HTLV-I) exposed to photochemical products of LY66Br (P-LY66Br) completely lost their ability to form syncytia in vitro. Photoproducts of P-LY66Br retain full antiviral activity for at least 3 and 6 weeks when stored at room temperature and at −80°C, respectively. Concentrations of P-LY66Br, effective in inhibiting syncytium formation mediated by HIV-1 and HTLV-I, were nontoxic to normal red cell components of whole blood (red blood cell 2,3-diphosphoglyceric acid, adenosine triphosphate, osmotic fragility or blood type antigens). Additionally, no evidence of acute toxicity was demonstrated in mice following an intravenous bolus inoculation to achieve plasma concentration

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of 600 μM of P-LY66Br. These findings represent the first demonstration of inhibition of retrovirus-induced syncytium formation by a photochemical product, and justify further investigation of the preactivation process of photochemicals in the treatment of systemic viral infections such as the acquired immunodeficiency syndrome (AIDS), in cancer therapy, and in sterilization of banked blood products.

Keywords: Photochemical; Anti-HIV-1; Anti-HTLV-I; Syncytium inhibition; AIDS therapy; Blood banking

1. Introduction

The antiviral properties of several photochemicals have been investigated for the purpose of decontamination of blood products and for therapy of systemic viral infections. The ability of cyanine dyes and furocoumarins (8-methoxypsoralen, 8-MOP) to effect viral inactivation following light stimulation has been reported (Gaffney et al., 1990; Hudson and Towers, 1991). The photochemical α-terthienyl has been reported to inactivate the in vitro infectivity of cell-free human immunodeficiency virus type-1 (HIV-1) (Hudson et al., 1993a, b, c). A widely studied photochemical is the hematoporphyrin derivatives (Hpd), which includes a complex mixture of ringed tetrapyrroles derived from the natural product hematoporphyrin (Dougherty, 1983). Our laboratories have previously demonstrated the ability of Hpd enriched with dihematoporphyrin ether (DHE), activated by laser light with a wavelength of 630 nm, to inactivate cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1) and measles virus (Skiles et al., 1985), and to neutralize cell-free HIV-1 particles in culture medium (Matthews et al., 1988) and in whole normal human blood (Matthews et al., 1991). Light-activated DHE had no adverse effects on the normal components of whole human blood (e.g., 2,3-diphosphoglyceric acid [DPG], adenosine triphosphate [ATP] and whole blood potassium concentrations), and on red blood cell (RBC) osmotic fragility and blood type antigens (Matthews et al., 1991).

A major limitation of conventional phototherapy is the requirement of in situ delivery of stimulating light energy following the binding of photochemicals to target viruses or virus-infected cells. Consequently, photomedicine is generally applicable only in instances where target sites are easily accessible or where special manipulations such as extracorporeal photopheresis or fiber optics are feasible (Bisaccia et al., 1990). To circumvent this limitation, we have been investigating the possibility of using the photochemical products (or photoproducts) obtained by activating photochemicals *prior* to their addition to targets, a process we termed 'preactivation' or 'dark kill'. We have recently demonstrated the neutralization of HIV-1 and simian immunodeficiency virus (SIV) (Chanh et al., 1992) by merocyanine 540 (MC 540) treated by exposure to laser light at a wavelength of 514 nm. The photoproducts of MC 540 fully retained antiviral property for as long as 30 days at −135°C, and for 3 days when stored at room temperature (Gulliya et al., 1990).

Although the photochemicals mentioned above are efficacious in neutralizing cell-free HIV-1 and SIV, they are relatively ineffective in inhibiting viral spread from infected cells to normal target cells in the process of syncytium formation (T. Chanh, unpublished results). In this report, we describe the chemical synthesis of a new class of

brominated naphthalimide compounds, which could be preactivated by light energy at 420 nm, and which efficiently inhibited syncytium formation mediated by HIV-1 and HTLV-I.

2. Materials and methods

2.1. Viruses and cell lines

The HTLV-IIIB strain of HIV-1, the HTLV-I-infected MT-2 and the HTLV-IIIB-infected H-9 cell lines were obtained from the AIDS Research and Reference Reagent Program (AIDS RRRP, Rockville, MD). The rat sarcoma XC cell line which served as target cells for HTVL-I-induced syncytia (Palker et al., 1989) was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The $SupT_1$ human T-cell line was a gift from Dr. Jonathan Allan (Southwest Foundation for Biomedical Research, San Antonio, TX).

2.2. Chemical synthesis of LY66Br

The active preactivable dye, 3-bromo-4-(hexylamino)-*N*-hexyl-1,8-naphthalimide was prepared by the methods described previously (Chang et al., 1993). The reaction between excess n-hexylamine and 4-chloro-1,8-naphthalic anhydride proceeds smoothly under reflux to afford 4-hexylamino-*N*-hexyl-1,8-naphthalimide as a fluorescent yellow solid. Bromination of the imide with bromine occurs regiospecifically to afford only the 3-bromo isomer; the reaction was monitored by t.l.c. to avoid the formation of the N-dealkylated product which forms on over-oxidation of the initial bromination product by elemental bromine. The preactivable compound crystallizes as a fluorescent yellow solid with a metallic sheen. The synthesis and spectroscopic data of the two key compounds are described below.

Melting points were determined using a hot-stage microscope and are uncorrected. Infra-red spectra were obtained by diffuse reflectance from KBr using a BioRad FTS 60-A Infrared Spectrometer. Nuclear magnetic resonance spectra were obtained as $CDC1_3$ solutions at 200 MHz for ^1H or 50 MHz for ^{13}C using a Varian Associates Gemini 200 Spectrometer; peak positions are reported as (δ) ppm down field from internal Me_4Si as standard for ^1H, and relative to the center peak of $CDC1_3$ (77.1 ppm) for ^{13}C. Mass spectra were obtained using electron impact at 70 eV or fast atom bombardment from a glycerol-DMSO matrix and were recorded using a Kratos MS25 RF mass spectrometer. 4-Chloro-1,8-naphthalic anhydride was obtained from Janssen Chimica and was recrystallized from toluene prior to use; 1-hexylamine and solvents were distilled prior to use.

4-(Hexyl)amino-N-hexyl-1,8-naphthalimide. Freshly recrystallized 4-chloro-1,8-naphthalic anhydride (4.32 g, 18.5 mM) was dissolved in 1-hexylamine (120 ml), and the solution was heated under reflux for 18 h. The resultant red solution was allowed to cool, and the hexylamine was removed by evaporation under reduced pressure to afford the product as an oily solid. Re-crystallization of the crude product from methanol

afforded 4-(hexyl)amino-*N*-hexyl-1,8-naphthalimide (5.72 g, 81%) as a bright yellow solid, m. 59–60°C. FTIR (V_{max}): 3341, 1690, 1636 cm^{-1}. ^{1}H-6 NMR (δ): 8.59 (1H, d $J = 7.3$ Hz, **H-7**), 8.47 (1H, d $J = 8.4$ Hz, **H-2**), 8.08 (1H, d $J = 8.4$ Hz, **H-5**), 7.62 (1H, dd $J = 7.3$, 8.4 Hz, **H-6**), 6.72 (1H, d $J = 8.4$ Hz, **H-3**), 5.25 (1H, br. s, **NH**), 4.15 (2H, dd $J = 7.6$, 7.8 Hz, imide N-C**H**$_2$), 3.40 (2H, t $J = 7.3$ Hz, NHC**H**$_2$), 1.85–1.25 (22H, complex, C**H**$_2$) 1.0–0.75 (6H, complex, C**H3**) ppm. ^{13}C-NMR (δ): 165.2, 164.6, 149.8, 134.8, 131.4, 130.2, 126.6, 126.0, 125.0, 123.7, 104.5, 104.6, 43.9 40.3, 31.7, 31.6, 29.1, 28.2, 26.9, 22.6, 14.1, 14.0 ppm. FAB-MS (m/z): 381 (M + H, 37), 297 (100), 225 (72).

*3-Bromo-4-hexylamino-N-hexyl-1,8-naphthalimide.*To a solution of 4-(hexyl)amino-*N*-hexyl-1,8-naphthalimide (0.531 g, 1.40 mM) in carbon tetrachloride (20 ml), bromine (0.240 g, 1.5 mM) was added. The solution was stirred for 3.5 h at ambient temperature, after which time t.l.c. indicated that all the starting material had been consumed. The solvent and excess bromine were removed by evaporation under reduced pressure. Recrystallization of the crude product from methanol afforded 3-bromo-4-(hexyl)amino-*N*-hexyl-1,8-naphthalimide (0.435 g. 66%) as bright yellow needles, m. 58–60°C. FTIR (V_{max}): 3450, 3334, 3233, 1705, 1694 cm^{-1}. ^{1}H-6 NMR (δ): 8.64 (1H, s, **H-2**), 8.59 (1H, dd $J = 7.3$, 1.1 Hz, **H-7**), 8.45 (1H, dd $J = 8.6$, 1.1 Hz, **H-5**), 7.65 (1H, dd $J = 7.3$, 8.6 Hz, **H-6**), 4.14 (2H, dd $J = 7.4$, 7.7 Hz, imide N-C**H**$_2$), 3.66 (2H, t $J = 7.0$ Hz, NHC**H**$_2$), 1.8–1.2 (22H, complex, C**H**$_2$) 1.0–0.80 (6H, complex, C**H**$_3$) ppm. ^{13}C-NMR (δ): 164.1, 163.0, 145.8, 136.2, 135.5, 131.4, 130.9, 126.6, 126.0, 125.5, 123.6, 123.4, 40.5, 31.6, 31.4, 31.3, 28.1, 26.9, 26.8, 26.5, 22.6, 22.5, 14.1, 14.0 ppm. EIMS (m/z): 460 (100, M + [^{81}Br]), 458 (100, M + [^{79}Br]).

2.3. Photochemical treatment of LY66Br

Working stock of the photoproduct of LY66Br was prepared with the dye dissolved in a micellar Cremophor EL® suspension in phosphate-buffered saline (PBS), pH 7.2, Microcrystals of the dye were dissolved in Cremophor EL® (ethoxylated triricinoleate, average molecular weight = 2514 Da) using pulsed sonication for 10 min at a temperature of 65°C. PBS was added to give a final concentration of 80% PBS and 20% Cremophor, and the concentration of the dye was confirmed by spectroscopy. A further dilution in PBS provided a 10 mM solution. Aliquots (2.5 ml) of 10 mM dye were transferred to 35-mm covered petri dishes and irradiated with 450 ± 35 nm light filtered from a 1-kW Xenon lamp and directed with a mirror to be incident upon the top of the dish. Exposure was to approximately 6000 J/cm^2 using typically an irradiance of 97 mW/cm^2 sufficient to cause completion of the photochemical 'preactivation'. Verification of completion was ensured by fluorescence measurements showing $\geq 98\%$ photobleaching of the original fluorescence emission intensity of LY66Br ($\lambda_{ex} = 425$ nm, $\lambda_{em} = 520$ nm). The P-LY66Br solution was used immediately or stored at room temperature and/or -75°C or -80°C for later testing. Measurements of antiviral activity of P-LY66Br showed no decrease upon storage for 3 and 6 weeks, respectively, at room temperature and -80°C. Also, continuing comparison of the efficiency of killing of infected H-9 cells with fresh P-LY66Br solutions stored for up to 3 months at 4°C and at -75°C have shown no significant loss of cytotoxic activity.

2.4. P-LY66Br in vitro cytotoxicity

The in vitro cytotoxicity of P-LY66Br for a number of human cell types was assessed by incubating 5×10^4 cells/0.1 ml with different concentrations of P-LY66Br for 2 h at 37°C in the dark. Following incubation, the cells were washed three times with RPMI 1640 (Mediatech, Herndon, VA) supplemented with 15% fetal bovine serum (RPMI/15% FBS), and resuspended in 0.5 ml of RPMI/15% FBS. The treated cells were incubated at 37°C in the dark for 24 h. In some experiments, cells were treated without washing, and were cultured for 6 days before toxicity determination. Care was taken to avoid exposure to ambient light by wrapping the cultures in aluminium foil. Cell viability was determined by addition of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) as previously described (Robertson et al., 1988). In some experiments, cell viability was assessed by the trypan blue exclusion method. The percent viability was calculated as [mean optical density ($OD_{570\ nm}$) of treated samples/mean $OD_{570\ nm}$ of untreated controls] $\times$ 100.

2.5. Testing of normal blood components

All subsequent experiments described included negative controls consisting of the appropriate percentage of Cremophor EL® without P-LY66Br. Blood samples from nine normal human volunteers (four male, five female) were collected in citrate in three aliquots, mixed with P-LY66Br at concentrations ranging from 0 μM–800 μM, and incubated at room temperature for no less than 30 min and not more than 180 min, depending on the speed at which processing could be accomplished. One aliquot received no added photoproducts, one received LY66Br (the parent dye) but no light (dye control), and another sample received P-LY66Br.

Micromodifications of several methods were used in our tests (Matthews et al., 1991). The method of Michal was used to assay for ATP levels (Michal, 1974). To assay for 2,3-DPG, the method of Lamprecht and Trautschold (1974) was employed. The effect of different concentrations (100–600 mM) of LY66Br and P-LY66Br on osmotic fragility was determined essentially as described elsewhere (Dacie and Gordon-Smith, 1975a, b). Reproducible values of K^+ concentration could not be obtained in plasma samples containing P-LY66Br. Presumably, physico- and/or electrochemical reactions at the ion-specific electrode used in the analysis interfered with reproducibility of the results. Experiments using other analytical techniques are required in order to determine effects of P-LY66Br on red cell sequestration of the potassium ion.

Immunoglobulin (Ig) receptor tests (Coombs test) were conducted on RBCs by varying the concentrations of LY66Br and P-LY66Br from 0 μM to 800 μM, and also included untreated controls.

2.6. Acute small in vitro animal toxicity tests

Female BALB/C mice were inoculated intravenously via the dorsal tail vein with a bolus of P-LY66Br dispensed in Cremophor EL® in phosphate-buffered saline to give a maximum plasma concentration of approximately 600 μM. The animals were observed

continuously over the first hour and then hourly for the following 23 h, then daily for the ensuing 35 days for behavioral evidence of acute toxicity. Blood smears obtained via tail-end snips at 0, 1, 2, 3, 4 and 24 h and on days 4, 12, 18, 21, 28 and 35 were stained with Wright's stain to obtain differential white blood cell counts and for qualitative estimates of red blood cell count.

2.7. Inhibition of HIV-1-mediated syncytium formation

In vitro syncytium formation assay for HIV-1 was performed essentially as described (Nara et al., 1987). To assess for inhibition of syncytium formation, HIV-1-infected H-9 cells (5×10^4 cells/0.1 ml) were incubated with various concentrations of P-LY66Br (0.1 ml) in the dark for 1 h at 37°C. Following incubation, the cells were washed three times with RPMI 1640 supplemented with 15% FBS. After washing, treated and untreated HIV-1-infected H-9 cells (5×10^4cells/0.2 ml) were added to 96-well flat-bottom tissue culture plates containing 1×10^5 $SupT_1$ cells/0.05 ml/well in RPMI 1640/15% FBS. The plates were incubated in the dark at 37°C for 18–24 h. Syncytium formation was scored by two individuals independently using an inverted microscope. All determinations were done in triplicate, and the results were expressed as the mean of the two individuals' scores. The effects of P-LY66Br on the target $SupT_1$ cells in the syncytium formation process were also determined by treatment of the target cells with various concentrations of P-LY66Br. Following treatment, the treated $SupT_1$ cells were washed and the HIV-1-infected H-9 cells were added.

2.8. Inhibition of HTLV-I-mediated syncytium inhibition

In vitro syncytium formation assay for HTLV-I using the rat sarcoma XC cells as targets was done as described elsewhere (Palker et al., 1989). To assess for the ability of P-LY66Br to inhibit syncytium formation, HTLV-I-infected MT-2 cells (10^6 cells/ml) were left untreated or treated with different concentrations of P-LY66Br as described above. After treatment and washing, 5×10^3/0.1 ml of MT-2 cells in RPMI 1640/15% FBS were added to 3×10^4/0.1 ml indicator rat sarcoma XC cells, mixed thoroughly and dispensed in 96-well flat bottom tissue culture plates. After 18–24 h of incubation at 37°C in the dark, the wells were washed in serum-free RPMI 1640, and stained with a 1:1 ratio of 0.5% methylene blue, 0.125% basic fuchsin in methanol for 10 min. Cells were destained in distilled water and syncytia were counted using an inverted microscope ($\times 200$). Syncytium is defined as giant cell containing at least four visible nuclei. The total number of syncytia in triplicate wells was enumerated independently by two individuals. The percent inhibition of syncytium formation was determined by counting the numbers of syncytia in 12 fields ($200 \times$), and was calculated as [mean no. of syncytia in untreated controls − mean no. of syncytia in P-LY66Br-treated samples/mean no. syncytia in untreated controls] $\times 100$. The effects of P-LY66Br treatment of XC target cells in HTLV-I-mediated syncytium formation were also determined as described above for HIV-1-induced syncytia. Negative controls included RPMI 1640/15% FBS containing the appropriate concentration of the base solvent Cre-mophor EL®.

3. Results

3.1. Lack of toxicity on normal blood components

Whole normal human blood treated with as high as 800 μM of P-LY66Br showed no significant differences in ATP (Fig. 1A), 2,3-DPG (Fig. 1B), or red cell hemolysis (Fig. 1C, D). In addition, no significant reactions were observed on RBC Ig receptors in nonactivated controls and photoactivated samples (data not shown), except for a very mild (+/−) reaction at the higher P-LY66Br (800 μM) concentrations, which was within the limits observed with a saline control.

3.2. Lack of acute in vivo animal toxicity

Optical microscopy (1000 ×) of blood smears (Wright's stain) obtained from treated mice ($n = 2$), each given a single i.v. bolus of P-LY66Br to attain 600 μM plasma concentration (≈ 1000 mM in whole blood), obtained at 0, 1, 2, 3, 4, and 24 h, and on days 4, 12, 18, 21, 28 and 35 days post-treatment, disclosed no discernible changes in red or white cell morphology nor in the ratio of the total number of white to red cells per microscopic field, nor in the ratios of white cell types per field (10 fields per smear). No evidence of acute toxicity was observed during the study period, as based on the lack of

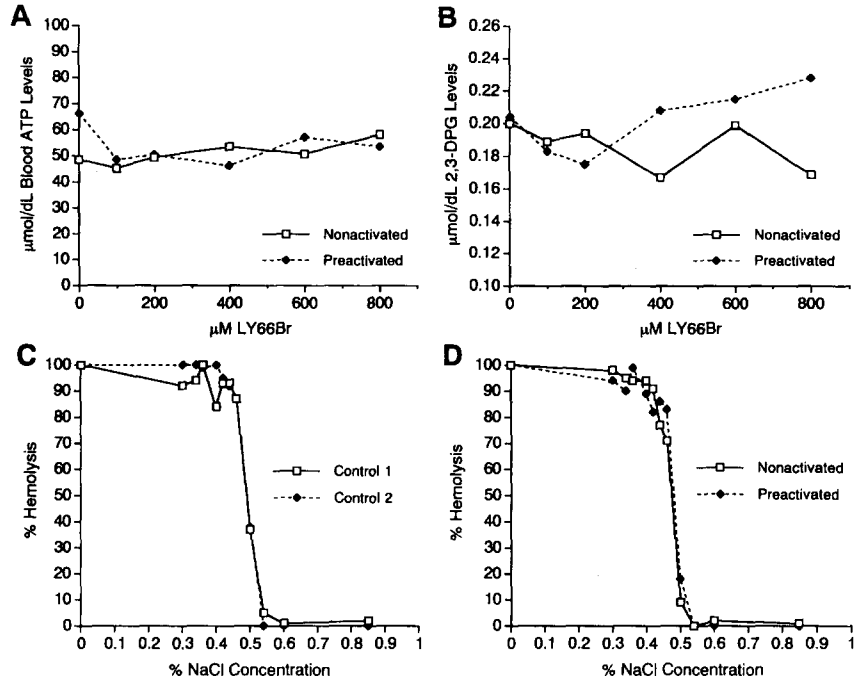

Fig. 1. Lack of effects of P-LY66Br on normal blood components. ATP levels (A); 2,3-DPG levels (B); and RBC hemolysis (C, untreated and D, treated with nonactivated and P-LY66Br).

Table 1
P-LY66Br cytotoxicity on normal human PBMC and various human cell lines

P-LY66Br (μM)	Cells				
	Normal human PBMC	$SupT_1$	Rat XC	MT-2	HIV-1-H-9
0	0.677 [a]	0.323	0.790	0.613	0.885
	(100) [b]	(100)	(100)	(100)	(100)
600	0.274	0.045	0.396	0.160	0.092
	(40.5)	(13.9)	(50.1)	(26.1)	(10.4)
300	0.717	0.059	0.506	0.266	0.168
	(100)	(18.3)	(64.1)	(43.4)	(19.0)
150	0.751	0.168	0.659	0.352	0.554
	(100)	(50.0)	(83.4)	(57.4)	(62.6)
75	0.771	0.198	0.769	0.491	0.840
	(100)	(61.3)	(97.3)	(80.1)	(94.9)
37.5	ND	0.240	0.807	0.546	1.010
		(74.3)	(100)	(89.1)	(100)
18.7	ND	0.330	0.792	0.603	0.980
		(100)	(100)	(98.4)	(100)

Cytotoxicity assays were done by treating cells with different concentrations of P-LY66Br. Viability was determined in triplicate samples using the MTT dye assay as described in Section 2.
[a] Mean optical density of triplicate determinations.
[b] Percent viability determined as (OD of treated cells/OD of untreated cells) × 100.

observable neurologic effects (tremor, convulsions, irritability, startled response, pilo-erection, salivation, excessive mouth and tongue movement, hyperventilation, lethargy, lack of coordination). In addition, food and water consumption were unchanged, body weight increased, and urine and fecal excretion was normal. A complete toxicological survey of all systems is planned as consequences of preliminary data on antiviral efficacy.

3.3. In vitro cytotoxicity of P-LY66Br

The toxicity of P-LY66Br was tested on normal human peripheral blood mononuclear cells (hu PMBC), uninfected human $SupT_1$, HTLV-I-infected human MT-2, and HIV-1-infected human H-9 cells, and the uninfected rat sarcoma XC cells as described in Section 2. Significant toxicity was observed with all cell types treated with the highest concentration of P-LY66Br (600 μM) used in this experiment (Table 1). The order of susceptibility to 600 μM P-LY66Br toxicity was as follows: HIV-I-H-9 > $SupT_1$ > MT-2 > hu PBMC > XC. A more selective toxicity was observed with a concentration of 300 mM of P-LY66Br. Whereas normal hu PBMC treated with 300 μM of P-LY66Br exhibited 100% viability, significant cytotoxicity was observed with the human $SupT_1$ cell line (18.3% viability), HIV-1-infected H-9 cells (19% viability), and with HTLV-I-infected MT-2 cells (43.4% viability) treated with the same concentration of P-LY66Br. The percent viability of treated cells increased with decreasing concentrations of P-LY66Br used. Linear regression analysis revealed TD_{50} (50% toxic doses) values of

Table 2
Inhibition of HIV-1-mediated syncytium formation by P-LY66Br

P-LY66Br (μM)	Viability of HIV-1--infected H-9	Syncytium inhibition [a]
150.0	62.6	+ +
75.0	94.9	+
37.5	100.0	+/−
7.5	100.0	−

[a] Syncytium inhibition was determined by counting syncytia in 12 fields at 200× magnification (see Section 2). + +, 90%–100% inhibition; +, 50%–90% inhibition; +/−, 20%–50% inhibition; and −, < 20% inhibition.

118 μM, 672 μM, 218 μM, and 183 μM for $SupT_1$, rat XC, MT-2, and HIV-1-H-9 cells, respectively. Similar cytotoxicity experiments were carried out in which cells were cultured with the same range of P-LY66Br concentrations for 6 days. Concentrations of P-LY66Br equal to or greater than 300 μM were highly toxic (viability < 5%) to all cell types tested, except for normal human PBMC (data not shown). Whereas 1-week treatment of normal human PBMC with 600 μM of P-LY66Br resulted in approximately 15% viability, treatment with 300 μM of P-LY66Br was significantly less toxic (89% viability). Treatment with doses of P-LY66Br lower than 150 μM for 6 days resulted in percent viability values similar to those presented in Table 1.

3.4. P-LY66Br inhibits HIV-1-mediated syncytium formation

To assess for the ability of P-LY66Br to inhibit syncytium formation between HIV-1-infected H-9 and indicator $SupT_1$ cells, HTLV-IIIB-infected H-9 cells were treated with different concentrations of P-LY66Br ranging from 7.5 μM to 150.0 μM (Table 2). The treated and washed infected cells were added to $SupT_1$ and syncytia were scored 18–24 h later (Fig. 2). Treatment of HIV-1-infected H-9 cells with 150 μM of P-LY66Br (a concentration which resulted in 62.6% viability of the infected cells) completely inhibited syncytium formation (Fig. 2C), as compared to untreated HIV-1-infected H-9 cells (Fig. 2B). Significant syncytium inhibition was also observed with 75 μM of P-LY66Br (Fig. 2D). The latter concentration of P-LY66Br was not cytotoxic as approximately 94.9% of the treated HIV-1-infected H-9 cells remained viable. A lesser degree of inhibition of syncytium was obtained with 37.5 μM P-LY66Br (Fig. 2E), whereas treatment with 7.5 μM of P-LY66Br basically had no effect on syncytium formation (Fig. 2F). The effects of P-LY66Br on target $SupT_1$ cells were also assessed by first treating the $SupT_1$ cells with 150 μM, 75 μM or 37.5 μM of P-LY66Br. The treated $SupT_1$ cells were washed and added to untreated HIV-1-infected H-9 cells. Treatment of indicator $SupT_1$ cells with P-LY66Br did not result in any observed inhibition of syncytium formation (data not shown).

3.5. LY66Br inhibits HTLV-I-mediated syncytium formation

To determine the ability of P-LY66Br to inhibit HTLV-I-induced syncytium formation, HTLV-I-infected human MT-2 cells were similarly treated with various concentra-

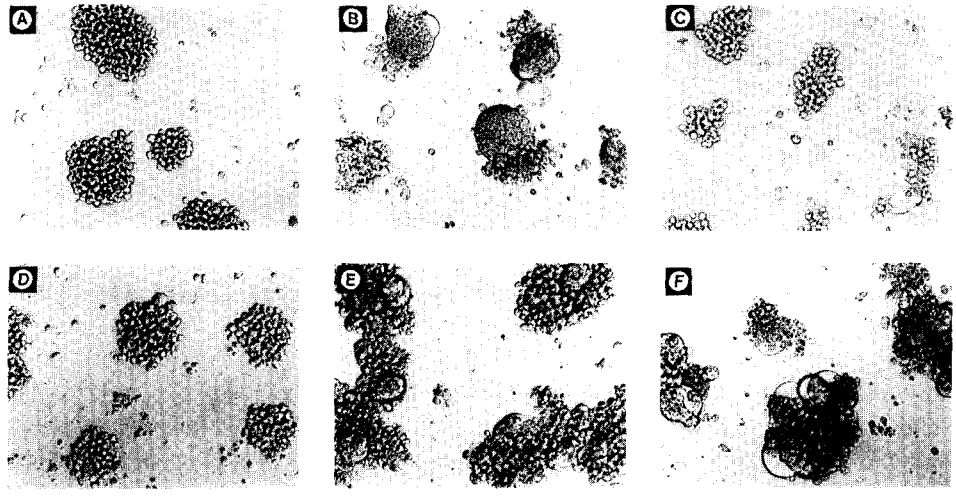

Fig. 2. Inhibition of HIV-1-induced syncytia by different concentrations of P-LY66Br. $SupT_1$ cells alone (A); $SupT_1$ + HIV-1-infected H-9 cells (B); $SupT_1$ + HIV-1-infected H-9 cells treated with 150 μM of P-LY66Br (C); or 75 μM of P-LY66Br (D), or 37.5 μM of P-LY66Br (E), or 7.5 μM of P-LY66Br (F).

tions of P-LY66Br, washed and added to the indicator rat XC cell monolayers (Palker et al., 1989). When HTLV-I-infected MT-2 cells were treated with a concentration of 150 μM of P-LY66Br which resulted in approximately 57.4% viability of the treated MT-2 cells (Table 1), a 95.4% inhibition of syncytium formation was observed (Table 3 and Fig. 3C). At P-LY66Br concentrations of 75 μM (Fig. 3D) and 37.5 μM (not shown) which resulted in 80.1% and 89.1% MT-2 cell viability, respectively, the observed percent inhibition of syncytium formation was still pronounced (80% and 70% inhibition, respectively). As the concentrations of P-LY66Br decreased, a concomitant decrease in the percent inhibition of syncytium formation was observed, reaching a low of 2.8% inhibition with 0.3 μM of P-LY66Br (Fig. 3E and Table 3). The ED_{50} (effective dose that inhibits 50% of HTLV-I-mediated syncytium formation) estimated by linear regression analysis was approximately 13.3 μM. Treatment of the indicator rat XC cells with P-LY66Br did not result in any inhibition of syncytium formation (data not shown).

Table 3
Inhibition of HTLV-I-mediated syncytium formation by P-LY66Br

P-LY66Br (μM)	Viability of HTLV-1--infected MT-2	% Syncytium inhibition [a]
150.0	57.4	95.4
75.0	80.1	80.0
37.5	89.1	70.0
7.5	100.0	23.5
1.5	100.0	6.7
0.3	100.0	2.8

[a] The percent inhibition of syncytium formation was determined as described in Section 2.

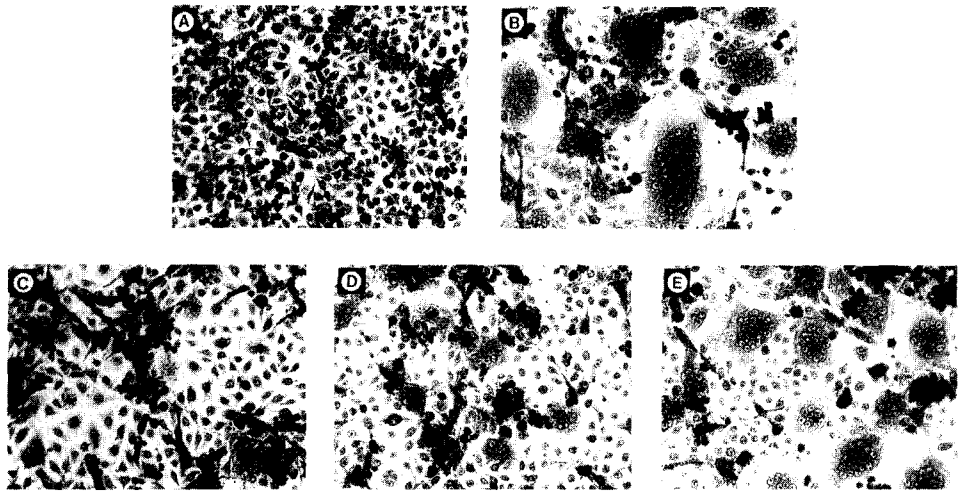

Fig. 3. Inhibition of HTLV-I-induced syncytia by different concentrations of P-LY66Br. Rat XC cells alone (A); XC cells + HTLV-I-infected MT-2 cells (B); XC cells + HTLV-I-infected MT-2 cells treated with 150 μM of P-LY66Br (C), or 75 μM of P-LY66Br (D), or 0.3 μM of P-LY66Br (E).

Controls consisting of RPMI 1640/15% FBS and the same concentrations of the solvent Cremophor EL® as found in P-LY66Br samples had no effects on either HIV-1- or HTLV-I-induced syncytium formation (data not shown).

4. Discussion

The antiviral properties of a number of photochemicals have been documented previously (Gaffney et al., 1990; Hudson and Towers, 1991; Hudson et al., 1993a, b, c). We have previously demonstrated the efficacy of DHE activated with a wavelength of 630 ± 5 nm in inactivating enveloped viruses including CMV, HSV-1, measles virus, HIV-1 and the simian immunodeficiency virus (SIV) (Skiles et al., 1985; Matthews et al., 1988; Chanh et al., 1989). Because of the inherent limitations imposed by the requirement of stimulating light energy delivery subsequent to binding of the photochemicals to targets in conventional phototherapy, we have been investigating the possibility of light-activating photosensitizing chemicals prior to addition to target cells, a process yielding photoproducts. Using this process, we demonstrated that merocyanine 540 (MC 540) exposed to laser light with a wavelength of 514 nm for 18 h efficiently neutralized HIV-1 and SIV suspended in culture medium or in whole normal blood (Chanh et al., 1992). P-MC 540 retained its antiviral activity for at least 3 weeks when stored at -80°C.

Recently, we reported on the synthesis of a new 1,8-naphthalimide photoactive compound, 1,14-bis-(*N*-hexyl-3′-bromo-1,8′-naphthalimide-4′-yl)-1,4,11,14-tetraazatetradecane-5,10-dione,or diED66Br). Activation of DiED66Br to an excited state by visible light (420 nm) following addition to HIV-1 or HIV-1-infected cells efficiently

neutralized the virus and inhibited HIV-1-induced syncytium formation, respectively (Chanh et al., 1993). Herein, we report that photoproducts of LY66Br, which represents the monomeric species of the above 1,8-naphthalimide dimer (DiED66Br), was capable of inhibiting HIV-1- and HTLV-I-induced syncytium formation. P-LY66Br retains its antiviral properties up to at least 3 and 6 weeks when stored at room temperature and at −80°C, respectively (data not shown). Conventional phototherapy with DiED66Br was more efficient than P-LY66Br in inhibiting HIV-1-induced syncytium formation. Whereas treatment of HIV-1-infected cells with 3.9 μM of DiED66Br was sufficient to effect complete inhibition of syncytium formation (Chanh et al., 1993), a concentration of 150 μM of P-LY66Br was required to obtain a similar degree of inhibition of syncytium formation induced by HIV-1-infected cells (Table 2). We have not derived photoproducts of DiED66Br because of potential complications associated with the presence of two reactive groups which provide an avenue for non-productive photochemistry. We believe that preactivation of the dimer which is strongly dependent on the conformation of the linking chain can only occur when the dimer is fully extended.

The structures of the photoproducts are unknown at this time. The compounds are known to be metastable. All attempts at isolation by conventional extraction methods have returned only unchanged starting materials. Spectroscopic characterization (^{1}H and/or ^{13}C NMR) has been complicated by the presence of low levels of free radical by-products. Our efforts directed toward characterization of the active photoproducts are continuing. The participation of single oxygen in the photochemistry has been precluded on the basis of photochemical bleaching experiments involving the brominated naphthalimide and tryptophan. The rate of photochemical bleaching of the naphthalimide is unaffected by the presence or absence of tryptophan. Photochemical bleaching of tryptophan in the presence of naphthalimide occurs at the same rate as naphthalimide bleaching after an induction period (40 J/cm^2 total irradiance). Any mechanism involving singlet oxygen should result in rapid loss of tryptophan fluorescence prior to bleaching of the naphthalimide until all oxygen in the system is consumed.

Studies with DiED66Br suggest selected antiviral activity against enveloped viruses (HSV-1 and vesicular stomatitis virus), but not nonenveloped viruses (echovirus and adenovirus). Because LY66Br is the monomeric form derived from DiED66Br, it is reasonable to speculate that P-LY66Br also selectively affects neutralization of enveloped viruses only, although direct experimental evidence is lacking. DiED66Br was shown to inactivate HIV-1 infectivity by interfering with the binding of HIV-1 envelope glycoprotein gp120 to its CD4 cellular receptor expressed primarily on the surface of T-helper lymphocytes (Chanh et al., 1993). The precise mechanism of viral neutralization by these 1,8-naphthalimides is not clearly understood. These compounds are highly lipophilic and bind to liposomes of β-oleyl-γ-stearoyl-L-α-phosphatidylcholine with biphasic kinetics (Chanh et al., 1993; Lewis et al., 1993a, b). We proposed that there is a rapid initial entry of the dye molecule into the hydrophobic core of the viral envelope followed by the formation of a donor-acceptor complex (e.g., a charge transfer of π complex) with a good single-electron donor group (Lewis et al., 1993). Excitation of the complex leads to a symmetry-allowed [1,3] sigmatropic shift of hydrogen which renders the dye molecule a much stronger electron acceptor. Single electron transfer from the protein side chains to the dye molecule results in the formation of a radical anion and

may lead to the modification of the tertiary structure of the protein. This modification may in turn lead to the inability of DiED66Br-treated HIV-1 to bind to CD4. It is reasonable to conceive a similar mechanism of viral inactivation by P-LY66Br, which presumably interferes with the membrane integrity of the virus-infected cells, preventing their binding to cellular receptors expressed on normal cells during the process of syncytium formation.

The results presented here on the ability of this new class of photochemicals to neutralize HIV-1, and to inhibit syncytium formation induced by HIV-1 and HTLV-I, support further investigations of these compounds as safe and effective therapeutic agents for the treatment of systemic viral infections such as AIDS, and the sterilization of banked blood products.

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References

Bisaccia, E., Berg, C. and Klainer, A.S. (1990) Extracorporeal photopheresis in the treatment of AIDS-related complex: a pilot study. Ann. Intern. Med. 113, 270–275.

Chang, S.-C., Archer, B.J., Utecht, R.E., Lewis, D.E., Judy, M.M. and Matthews, J.L. (1993) 4-alkylamino-3-bromo-*N*-alkyl-1.8-naphthalimides: New photochemically activatable antiviral Compounds. BioMed. Chem. Lett. 3, 555–556.

Chanh, T.C., Allan, J.S., Matthews, J.L., Sogandares-Bernal, F., Judy, M.M., Skiles, H., Leveson, J., Marengo-Rowe, A. and Newman, J.T. (1989) Photodynamic inactivation of simian immunodeficiency virus. J. Virol. Methods 26, 125–132.

Chanh, T.C., Allan, J.S., Pervaiz, S., Matthews, J.L., Trevino, S.R. and Gulliya, K.S. (1992) Preactivated merocyanine 540 inactivates HIV-1 and SIV: Potential therapeutic and blood banking applications. J. Acq. Imm. Def. Synd. 5, 188–195.

Chanh, T.C., Lewis, D.E., Allan, J.S., Sogandares-Bernal, F., Judy, M.M., Utecht, R.E. and Matthews, J.L. (1993) Neutralization of HIV-1 and inhibition of HIV-1-induced syncytia by 1,8-naphthalimide photoactive compound. Aids Res. Human. Retrovir. 9, 891–896.

Dacie, J.V. and Gordon-Smith, E.C. (1975a) Laboratory methods used in investigation of heamolytic aneamias. II. Hereditary haemolytic aneamias. In: J.V. Dacie and S.M. Lewis (Eds.), Practical Haematology, pp. 32–33. Churchill-Livingstone, New York.

Dacie, J.V. and Gordon-Smith, E.C. (1975b) Laboratory methods used in investigation of heamolytic anaemias. II. Hereditary heamolytic aneamias. In: J.V. Dacie and S.M. Lewis (Eds.), Practical Haematology, pp. 202–204. Churchill-Livingstone, New York.

Dougherty, T.J. (1983) Photoirradiation therapy-clinical and drug advances. In: D. Kessel and T.J. Dougherty (Eds.), Photosensitization, pp. 3–13. Plenum Press, New York.

Gaffney, D.K., Schober, S.L. and Sieber, F. (1990) Merocyanine 540-sensitized photoinactivation of leukemia cells: role of oxygen and effects on plasma membrane integrity and mitochondrial respiration. Exp. Hematol. 18, 23–26.

Gulliya, K.S., Chanh, T., Newman, J., Pervaiz, S. and Matthews, J.L. (1990) Preactivation - a novel antitumor and antiviral approach. Eur. J. Cancer 26, 551–553.

Hudson, J.B. and Towers, G.H. (1991) Therapeutic potential of plant photosensitizers. Pharmacol. Ther. 49, 181–222.

Hudson, J.B., Harris, L., Marles, J.R. and Arnason, J.T. (1993a) The anti-HIV activities of photoactive terthiophenes. Photochem. Photobiol. 58, 246–250.

Hudson, J.B., Harris, L., Teeple, A. and Towers, G.H. (1993b) The anti-HIV activity of the photochemical alpha-terthienyl. Antiviral Res. 20, 33–43.

Hudson, J.B., Harris, L. and Towers, G.H. (1993c) The importance of light in the anti-HIV effect of hypericin. Antiviral Res. 20, 173–178.

Lamprecht, W. and Trautschold, I. (1974) Determination with hexokinase and glucose-6-phosphate dehydrogenase. In: H.U. Bermeyer (Ed.), Methods of Enzymatic Analysis, Vol. 4, pp. 2101–2110. Academic Press, New York.

Lewis, D.E., Utecht, R.E, Chang, S.-C., Umback, N.J., Costello, L.J., Chanh, T.C., Judy, M.M., Newman, J.T. and Matthews, J.L. (1993a) Phototautomerization-s.e.t.: a new oxygen-independent mechanism for the membrane-based photochemical inactivation of enveloped viruses. Photochem. Photobiol. (submitted).

Lewis, D.E., Utecht, R.E., Judy, M.M., Matthews, J.L. and Chanh, T.C. (1993b) Photochemical neutralization and inhibition of HIV-1-induced syncytium formation by halogenated 1.8-naphthalimides. The Spectrum. 6(3), 8–14.

Matthews, J.L., Sogandares-Bernal, F., Judy, M.M., Marengo-Rowe, A.J., Leveson, J.E., Skiles, H., Newman, J.T. and Chanh, T.C. (1991) Preliminary studies of photoinactivation of human immunodeficiency virus in blood. Transfusion 31, 636–641.

Matthews, J.L., Newman, J.T., Sogandares-Bernal, F., Judy, M.M., Skiles, H., Leveson, J.E., Marengo-Rowe, A.J. and Chanh, T.C. (1988) Photodynamic therapy of viral contaminants with potential for blood banking applications. Transfusion 28, 81–83.

Michal, G. (1974) D-glycerate-2,3-diphosphate. In: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Vol. 3, pp. 1433–1438. Academic Press, New York.

Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A. and Fischinger, P.J. (1987) Simple, rapid, quantitative syncytium-forming microassay for the detection of human immunodeficiency virus neutralizing antibody. Aids Res. Hum. Retrovir. 3, 238–302.

Palker, T.J., Tanner, M.E., Scearce, R.M, Streinlein, R.D., Clark, M.E. and Haynes, B.F. (1989) Mapping of immunogenic regions of human T cell leukemia virus type 1 (HTLV-I) gp46 and gp21 envelope glycoproteins with env-encoded synthetic peptides and a monoclonal antibody to gp46. J. Immunol. 142, 971–978.

Robertson, G.A., Kostek, B.M., Schleif, W.A., Lewis, J.A. and Emini, E.A. (1988) A microtiter cell-culture assay for the determination of anti-human immunodeficiency virus neutralizing antibody activity. J. Virol. Methods 20, 195–202.

Skiles, H., Judy, M.M. and Newman, J.T. (1985) Photodynamic inactivation of viruses with hematoporphyrin derivatives. Am. Soc. Microbiol, Las Vegas, Nevada (Abstract A38, p.7).