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In vitro evaluation and characterization of newly designed alkylamidophospholipid analogues as anti-human immunodeficiency virus type 1 agents

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Our laboratories first reported two novel classes of complex synthetic lipids, including alkylamidophosphocholines (PC lipid; CP-51) and alkylamidophosphate ester-linked lipid–AZT conjugates (lipid–AZT conjugates; CP-92), with selective and potent activity against human immunodeficiency virus type 1 (HIV-1). To extend these observations, we synthesized additional PC lipids and lipid–AZT conjugates (INK and INK–AZT conjugate) to evaluate their structure–activity relationships by testing for selectivity against infectious wild-type (wt) and drug-resistant HIV-1 replication, virus fusogenic activity and toxicity for mouse bone marrow cells. PC lipid compounds with medium chain lengths at positions 1 and 2 gave an improved selective index (SI). INK-3, with 12 and 8 carbons and INK-15, with 10 and 12 carbons were among the most selective when evaluated in CEM-SS cells. INK-14, a lipid–AZT conjugate where AZT replaced the choline in PC lipid INK-3, gave the highest SI of >1250 against both infectious wt HIV-1 replication in CEM-SS cells and a clinical isolate in peripheral blood leukocytes. Notably, the PC lipid compounds INK-3 and INK-15, but not the lipid–AZT conjugate INK-14, were potent inhibitors of matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates. INK-3 also inhibited replication of HIV-2 and TIBO-resistant HIV-1, and inhibited HIV-1-mediated fusogenic activity by 78, 41 and 9% in a dose-dependent manner. The TC_{50} for mouse bone marrow cells was >100 μg/ml for INK-3 compared to 9.15–14.17 μg/ml for CP-51 and 0.142–0.259 μg/ml for AZT. These data suggest that optimum PC lipid compounds are significantly less toxic than AZT and have high potential as novel therapeutic agents for AIDS.

Keywords: alkylamidophosphocholine; phospholipid–nucleoside conjugate; HIV-1; AIDS

Introduction

Our laboratories first reported on two novel classes of complex synthetic lipids (alkylamidopropyl or alkylthioglycerol phosphocholines and phosphate ester-linked lipid–AZT conjugates) that have selective and potent activity against infectious human immunodeficiency virus (HIV) replication and pathogenesis *in vitro*. These compounds have a unique site of action involving alteration of HIV gp160/gp120 activity and production of defective virus particles (Kucera *et al.*, 1990a,b; Meyer *et al.*, 1991; Piantadosi *et al.*, 1991; Krugner-Higby *et al.*, 1995). A thiolipid–nucleotide conjugate (BM21-1290) has recently been introduced into Phase I/II tolerability and efficacy trials in ARC/AIDS patients by Boehringer Mannheim (Herrmann DBJ, Schleger C & Opitz HG; Antiretroviral activity of the novel candidate anti-AIDS drug BM21.1290 in the Friend-virus leukemia system in vivo; *VIth German AIDS Congress*, October 24–26 1997, Munich, Germany, Abstract V160; Herrmann DBJ, Opitz HG & Kucera LS; BM21-1290: evaluation of the antiretroviral activity of a new anti-AIDS drug in-vivo. *IVth Conference on Retroviruses and Opportunistic Infections*, January 22–26 1997, Washington, DC, USA, Abstract p92). Among the reported alkylamidopropyl phosphocholine (PC lipid) analogues synthesized and tested for anti-HIV-1 activity, the most selective was compound CP-51, 1-octadecanamido-2-ethoxypropyl-3-phosphocholine (Kucera *et al.*, 1990a). In order to extend these observations, we synthesized a series of CP-51 analogues to

evaluate their structure–activity relationships and thus to optimize the PC lipid compound for future evaluation as a potential therapeutic agent for AIDS. Results of this investigation indicated that among the PC lipid compounds tested in CEM-SS cells, compounds INK-3 and INK-15 with intermediate hydrocarbon chain lengths of 12 and 10, respectively, at position 1, and of 8 and 12, respectively, at position 2, gave the highest selectivity index (SI) against infectious HIV-1 replication. Selected PC lipid compounds were active against AZT-resistant clinical isolates, TIBO-resistant HIV-1 and wild-type HIV-2, inhibited HIV-1 fusogenic activity, and were less toxic to mouse bone marrow cells compared to AZT. Based on these interesting results, optimum PC lipid compounds may have great potential as novel therapeutic agents for AIDS, either alone or in combination therapy.

Materials and Methods: Chemistry

All chemicals were used as provided by the supplier without further purification unless otherwise indicated. AZT was obtained from Boehringer Mannheim (Mannheim, Germany). Column chromatography was performed with silica gel 60 (230–400 or 70–230 mesh). Melting points were obtained on a Hoover Meltemp apparatus and are uncorrected. Proton NMR spectra were obtained on Bruker 300 MHz or Varian 400 MHz spectrometer as solutions in $CDCl_3$ with Me_4Si as an internal standard. The final compounds were, in general, hygroscopic solids. FAB mass spectra were run on a VG 70S mass spectrometer. Thin layer chromatography (TLC) of phosphocholines in $CHCl_3$: MeOH : NH_4OH (70 : 35 : 7) and $CHCl_3$: MeOH (2 : 1) gave a single spot, which gave a blue colour with a modified Dittmer–Lester reagent (Ryu & MacCoss, 1979). A representative synthetic procedure is given for INK-3; the remaining phosphocholines were prepared from the appropriate reagents using analogous procedures as those detailed below and in our previous papers (Piantadosi *et al.*, 1991; Meyer *et al.*, 1991; Morris-Natschke *et al.*, 1993). Complete high resolution mass spectra results are available as supplementary data from the Editor-in-Chief.

3-Dodecanamido-1,2-propanediol

3-Amino-1,2-propanediol (42 g, 0.47 mol) was dissolved in pyridine (150 ml) and DMF (200 ml). Dodecanoyl chloride (47 g, 0.46 mol) in 150 ml DMF was added dropwise. After 24 h at room temperature, the product was removed by filtration, washed with H_2O and recrystallized from MeOH, then from $CHCl_3$. The amide was obtained in 28% yield (34.7 g, 0.013 mol, m.p. 94–96°C). ^{1}H NMR ($CDCl_3$): 0.90 (t, 3H, terminal CH_3), 1.25 [m, 16H $(CH_2)_8$], 1.6 (m, 2H, $NHCOCH_2CH_2$), 2.25 (t, 2H, $NHCOCH_2$), 3.45 (m, 2H, CH_2NH), 3.6 (m, 2H, CH_2OH), 3.8 (m, 1H, *CH*OH), 5.8 (t, 1H, NH).

3-Dodecanamido-1-triphenylmethoxy-2-propanol

The above diol (5.5 g, 0.020 mol) was dissolved in dry pyridine (50 ml), then a solution of trityl chloride (5.6 g, 0.020 mol) in pyridine (50 ml) was added dropwise at 50°C. The reaction mixture was stirred at this temperature for 10 h and then cooled. The pyridine was removed under vacuum, and water (100 ml) was added to the solid residue. After filtration and washing, the crude product was partitioned between water and $CHCl_3$. The water layer was extracted again with $CHCl_3$ and the organic layers combined and dried over anhydrous Na_2SO_4. After removing solvent, the resulting solid was dissolved in hexane : ethyl acetate (3 : 1); 1 g of starting diol was filtered. The mother liquor was concentrated and chromatographed on silica gel with a discontinuous gradient of hexane : EtOAc to give 7 g (0.014 mol, 70% yield) of pure product. ^{1}H NMR ($CDCl_3$): 0.90 (t, 3H, terminal CH_3), 1.25 [m, 16H $(CH_2)_8$], 1.6 (m, 2H, $NHCOCH_2CH_2$), 2.2 (t, 2H, $NHCOCH_2$), 3.2–3.4 (overlapping m, 3H, CH_2NH and CH_aCH_bOTr), 3.55 (m, 1H, CH_aCH_bTr) 3.9 (m, 1H, *CH*OH), 5.7 (t, 1H, NH), 7.3–7.5 (m, 15H, aromatic H).

3-Dodecanamido-2-octyloxy-1-triphenylmethoxypropane

The synthesized trityl ether (7.0 g, 0.013 mol) in 30 ml dry THF was added dropwise to an ice-cooled suspension of sodium hydride (1.0 g of 60% oil dispersion, 0.025 mol) in 100 ml THF under nitrogen. After warming to room temperature, heat was applied (60°C) for 1 h. 1-Bromooctane (2.9 g, 0.015 mol, neat) was added dropwise and heating continued for 10 h. After cooling, ice and then water were added slowly. The biphasic system was separated and the water layer extracted with EtOAc (3×50 ml). All organic fractions were combined and dried over anhydrous Na_2SO_4. After removing solvent *in vacuo*, the residue was chromatographed with a gradient of hexane : EtOAc (100 : 0 to 6 : 1) to give 3.6 g (38% yield) of pure product. Impure product was rechromatographed using the same conditions to give an added 3.0 g (31% yield) of product as a viscous oil. ^{1}H NMR ($CDCl_3$): 0.85 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.45 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 2.2 (t, 2H, $NHCOCH_2$), 3.2–3.6 (overlapping m, 7H, CH_2CHCH_2, OCH_2), 5.75 (t, 1H, NH), 7.3–7.5 (m, 15H, aromatic H).

3-Dodecanamido-2-octyloxy-1-propanol

Detritylation of the above compound (6.0 g, 9.5 mmol) was accomplished using p-toluenesulphonic acid (0.6 g, 3.1 mmol) in $CHCl_3$: MeOH (60 ml : 18 ml). The reaction

mixture was stirred at room temperature for 24 h then saturated $NaHCO_3$ solution was added and stirred for 30 min. The layers were separated and the $CHCl_3$ fraction dried over anhydrous Na_2SO_4. After concentration *in vacuo*, the residue was purified twice by column chromatography using $CHCl_3$: MeOH as eluent (100 : 0 and 15 : 1) to give 3.0 g (80% yield) of pure 3-dodecanamido-2-octyloxy-1-propanol (m.p. 42–43°C). ^{1}H NMR ($CDCl_3$): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.6 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 2.2 (t, 2H, $NHCOCH_2$), 3.2–3.7 (overlapping m, 7H, CH_2CHCH_2, OCH_2), 5.75 (t, 1H, NH).

3-Dodecanamido-2-octyloxypropyl 2-bromoethyl phosphate

3-Dodecanamido-2-octyloxy-1-propanol (1.0 g, 2.6 mmol) in 60 ml 2 : 1 anhydrous Et_2O : THF was cooled to 0°C. Pyridine (3.3 g) then 2-bromoethyl dichlorophosphate (2.4 g, 0.010 mol, prepared as described by Hansen *et al.*, 1992) were added dropwise. After warming to room temperature, the mixture was refluxed for 4 h, cooled and water (10 ml) added. After stirring for 30 min, the solvent was removed *in vacuo* and the residue dissolved in 100 ml $CHCl_3$: MeOH (2 : 1). The solution was extracted with water and back-extracted with 2×50 ml 2 : 1 $CHCl_3$: MeOH. The combined organic fractions were dried over anhydrous Na_2SO_4, evaporated and chromatographed on silica gel using a gradient of $CHCl_3$: MeOH (100 : 1 to 15 : 1). The desired bromoethylphosphate (633 mg, 1.1 mmol) was obtained in 43% yield. ^{1}H NMR ($CDCl_3$): 0.85 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.55 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 2.3 (t, 2H, $COCH_2$), 3.4–3.7 (overlapping m, 7H, $CHCH_2NHCO$, CH_2Br, OCH_2), 3.85 and 4.25 (two m, 4H, $CH_2OPO_3CH_2$), 6.8 (m, 1H, NH).

3-Dodecanamido-2-octyloxypropyl phosphocholine (INK-3)

The above phosphate ester (633 mg, 1.1 mmol) in 52 ml $CHCl_3$: isopropanol : DMF (5 : 3 : 5) was reacted with aqueous Me_3N (7.6 ml) at 65°C for 5 h. After cooling, Ag_2CO_3 (380 mg) was added and the heat reapplied for 1 h. After filtering the precipitated AgBr, the solvent was removed *in vacuo* and the residue purified by chromatography on silica gel using $CHCl_3$: MeOH (10 : 1 to 2 : 1) followed by $CHCl_3$: MeOH : NH_4OH (75 : 25 : 5) to give 275 mg pure (46% yield) and 140 mg impure phosphocholine. FAB MS showed a $[MH]^+$ ion at 551.418345 ($C_{28}H_{60}N_2O_9P$, 1.0 p.p.m.). ^{1}H NMR ($CDCl_3$): 0.87 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ $(CH_2)_5$], 1.5 (m, 4H, $NHCOCH_2CH_2$, OCH_2CH_2), 2.2 (t, 2H, $COCH_2$), 3.3 [s, 9H, $N(CH_3)_3$], 3.3–3.8 (overlapping m, 5H, CH_2CHOCH_2) (3.8–3.9, overlapping m, 4H, CH_2OP, CH_2NMe_3), 4.4 (m, 2H, PO_3CH_2), 7.0 (m, 1H, NH).

3-Dodecanamido-2-octyloxypropyl diphenyl phosphate

Diphenylchlorophosphate (0.4 ml, 1.7 mmol) in 10 ml diethyl ether was cooled to 4°C under nitrogen. 3-Dodecanamido-2-octyloxy-1-propanol (500 mg, 1.3 mmol) in 8 ml pyridine and 10 ml Et_2O was added dropwise. The solution was warmed to room temperature, then heated to around 52°C for 4 h and cooled to room temperature, diluted with 30 ml Et_2O, washed with 15 ml portions of water, 0.5 M HCl and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to an oil. Chromatography with a gradient of hexane : EtOAc (10 : 1 to 3 : 1) gave 556 mg (0.9 mmol, 69%) of pure product. ^{1}H NMR ($CDCl_3$): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.55 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 2.3 (t, 2H, $COCH_2$), 3.3–3.7 (overlapping m, 5H, OCH_2, $CHCH_2NHCO$), 4.25 (m, 2H, CH_2OP), 5.9 (m, 1H, NH), 7.2–7.8 (m, 10H, aromatic H).

3-Dodecanamido-2-octyloxypropyl phosphatidic acid

PtO_2 (81 mg) was placed in a Parr hydrogenation bottle. 3-Dodecanamido-2-octyloxypropyl diphenyl phosphate (589 mg, 1.0 mmol) in 118 ml EtOH was then added. After hydrogenation at 158.5 kPa (23 p.s.i.) for 4.5 h, the reaction mixture was filtered and checked for completion by TLC. To ensure complete conversion to the phosphatidic acid, hydrogenation was continued for another 6 h using 120 mg of fresh catalyst. The reaction mixture was then filtered through Celite and the EtOH removed *in vacuo*. The residue was chromatographed on silica gel using 4 : 1 $CHCl_3$: MeOH as eluent to obtain 233 mg (0.54 mmol, 54% yield) of pure phosphatidic acid. ^{1}H NMR ($CDCl_3$): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.55 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 2.3 (t, 2H, $COCH_2$), 3.3–3.7 (overlapping m, 5H, OCH_2, $CHCH_2NHCO$), 4.0 (m, 2H, CH_2OP), 6.8 (m, 1H, NH).

3′-Azido-3′-deoxy-5′-(3-dodecanamido-2-octyloxypropyl)-phosphothymidine (INK-14)

The procedure of Piantadosi *et al.* (1991) was followed to synthesize INK-14 in 22% yield from the above phosphatidic acid and AZT. FAB MS showed a $[MH+Na]^+$ ion at 737.403800 ($C_{33}H_{59}N_6O_9PNa$, –8.0 p.p.m.). ^{1}H NMR ($CDCl_3$): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H $(CH_2)_8$ and $(CH_2)_5$], 1.55 (m, 4H, $NHCOCH_2CH_2$ and OCH_2CH_2), 1.8 (s, 3H, thymine CH_3), 2.2 (t, 2H, $COCH_2$), 2.3–2.55 (m, 2H, 2′-CH_2), 3.3–3.6 (overlapping m, 5H, OCH_2, $CHCH_2NHCO$), 3.8–4.2 (overlapping m, 5H, $CH_2OPO_3CH_2$, 4′-CH), 4.5 (1H, 3′-CH), 6.15 (m, 1′-CH), 7.4 (m, 1H, thymine CH).

Materials and Methods: Virology

Virus stocks

HIV-1 (strain IIIB) was propagated in H9IIIB cells as previously described (Krugner-Higby *et al.*, 1995). Stocks of HIV-2 from R Weiss (Institute of Cancer Research, London, UK), TIBO-resistant HIV-1 (RT mutant at codons 103 and 181) from E Emini (Merck, Sharp & Dohme Research Laboratories, West Point, Pa., USA) and AZT-resistant HIV-1 isolates 1073 (RT mutant at codons 70 and 215) and 1074 (RT mutant at codons 70 and 215), and matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates G-762 (wild-type), G-691 (RT mutant) and H112-2 (wild-type), G-910 (RT mutant at codon 215) from D Richman (University of California, La Jolla, Calif., USA) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS Program, NIH, Bethesda, Md., USA). Stocks of viruses were propagated in acutely infected CEM-SS cells as previously described (Krugner-Higby *et al.*, 1995).

Cell cultures

All primary peripheral blood leukocytes (PBL) and cell lines were incubated and maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 20% fetal bovine serum (growth medium) as previously described (Krugner-Higby *et al.*, 1995). PBL were obtained from HIV-1-seronegative male and female personnel in the laboratory. Whole blood was separated into PBL using Isolymph (Gallard-Schlesinger Industries, Carle Place, N.Y., USA) according to a protocol supplied by the manufacturer. The separated PBL were activated for 3 days in the presence of phytohaemagglutinin (PHA; 5 μg/ml in growth medium), washed in growth medium, treated with polybrene (5 μg/ml in growth medium) and infected with HIV-1 in the presence of polybrene (1 μg/ml in growth medium). After a 2 h virus attachment, the cells were washed with growth medium to remove unattached HIV-1 and incubated (1×10^6 cells/ml of growth medium) for 96 h with or without added test compounds. Supernatant medium was harvested to measure virus replication by reverse transcriptase (RT) activity (Krugner-Higby *et al.*, 1995).

TC_{50} determinations

To measure the cytotoxicity in CEM-SS cells, test compounds were dissolved in 95% ethanol, methanol : chloroform (1 : 1) or RPMI 1640 plus 20% FBS growth medium, diluted in $\log_{10}$ or 0.5 $\log_{10}$ series in growth medium and tested for cytotoxicity in triplicate wells containing 10 000 CEM-SS cells/well of a 96-well plate. A viable cell count was made prior to the test to ensure more than 90% cell viability. Log phase cells were treated with test compound for 48 h at 37°C (approximately 2.5 cell generations in untreated control cultures) and pulse-labelled with 1 μCi [^{3}H]TdR (sp. act. 20 Ci/mmol) for 6 h before harvesting the cells using a multichannel cell harvester to measure total DNA synthesis in the presence or absence of compound. To measure cytotoxicity in PBL, the cells were activated with PHA (5 μg/ml) for 72 h, washed to remove the PHA and 20 000 activated PBL cells/well were cultured for 96 h in growth medium with or without test compound as described for CEM-SS cells. The treated PBL cells were labelled with 0.1 μCi [^{3}H]TdR (sp. act. 20 Ci/mmol) during the final 24 h culture period before harvesting. From the data, a TC_{50} for cytotoxicity was calculated as previously described by Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Syncytium plaque assay

The syncytium plaque assay for infectious virus multiplication was done in the presence or absence of PC lipid or lipid–AZT conjugate as previously described by us (Kucera *et al.*, 1990a). The syncytium plaques were counted on day 5 or 6 post-infection and treatment and the percentage inhibition and effective concentration$_{50}$ (EC_{50}) were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Fusogenic assay

Persistently HIV-1-infected H9IIIB cells were treated with compound for 96 h and cocultured with uninfected $CD4^+$ CEM-SS cells (ratio 1 : 100) in the presence of added compound for 48 h. The number of fusogenic cells was counted relative to untreated control cells to determine the percentage inhibition of fusogenic cells. The EC_{50} values were calculated by the method cited by Piantadosi *et al.* (1991).

Colony forming units–granulocyte macrophage (CFU-GM) assay

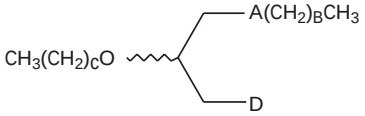
Mouse bone marrow granulocyte/macrophage cells were harvested and suspended in soft agar medium with or without added test compound (Herrmann DBJ, Kucera LS, Zilch H, Mertens A & Opitz HG; BM21.1290: in-vitro evaluation of a potential new anti-AIDS compound; *XIth International Conference on AIDS*, July 7–12 1996, Vancouver, Canada, Abstract p64). After incubation, the number of cell colonies was counted to determine the percentage inhibition of colony formation relative to a mock (PBS)-treated control. The 50% toxic concentration (TC_{50}) was calculated by the method cited by Piantadosi *et al.* (1991).

Results

Chemistry

Phospholipid and lipid–AZT conjugates were synthesized in our laboratories as previously described (Piantadosi *et al.*, 1991). Briefly, 3-amino-1,2-propanediol was reacted with an acyl chloride to form an alkylamidopropanediol. The primary alcohol was protected as the trityl ether by reaction with trityl chloride, then the secondary alcohol was alkylated with the appropriate alkyl halide using sodium hydride as the base. After removing the trityl ether with *p*-toluenesulphonic acid, the hydroxy group was converted either to a phosphocholine [reaction first with bromoethyl phosphodichloridate (Hansen *et al.*, 1982) then with aqueous trimethyl amine] or, in the case of INK-14, to the phosphatidic acid (reaction with diphenyl chlorophosphate then catalytic hydrogenolysis over PtO_2). The phosphatidic acid was coupled with AZT using dicyclohexylphosphodiimide as the condensing agent.

Table 1. Chemical structures of synthetic phospholipids



Compound	A	B	C	D*
CP-51	NHCO	16	1	PC
INK-1	NHCO	10	11	PC
INK-2	NHCO	10	9	PC
INK-3	NHCO	10	7	PC
INK-4	O	11	9	PC
INK-6	NHCO	10	5	PC
INK-7	NHCO	16	7	PC
INK-8	NHCO	10	7	$OPO_3(CH_2)_3N(CH_3)_3$
INK-13	NHCO	8	7	PC
INK-14	NHCO	10	7	OPO_3AZT
INK-15	NHCO	8	11	PC

*PC, $OPO_3CH_2CH_2N(CH_3)_3$.

Evaluation of compounds for cytotoxicity and anti-HIV-1 activity

To optimize the activity and/or SI, PC lipid and lipid–AZT conjugate compounds varying in hydrocarbon chain lengths at position 1 or 2 were synthesized (Table 1) and evaluated for cytotoxicity, anti-HIV-1 activity and SI in CEM-SS and PBL cells. Results of these experiments (Table 2) using CEM-SS cells indicated that the TC_{50} ranged from 27.6 (INK-2) to >100 μM (INK-13 and -14). The EC_{50} ranged from <0.08 (INK-14) to 1.02 μM (INK-8). The SI ranged from 53.2 (INK-8) to >1250 (INK-14) (Table 2). Compounds with a 12 or 10 carbon chain length at position 1, an 8 or 12 carbon chain length at position 2, and phosphocholine at position 3 of the three carbon backbone (INK-3 or INK-15) gave the highest SI (298.9 and 389.0, respectively) among the PC lipid compounds. INK-14, which has the same hydrocarbon chain lengths at position 1 and position 2 as INK-3 but is linked at position 3 through a phosphate ester bond to AZT in place of phosphocholine (see Table 1), gave the highest SI (>1250)

Table 2. Evaluation of PC lipid and lipid–AZT conjugate compounds for cell cytotoxicity, anti-HIV-1 activity and SI in CEM-SS and PBL cells*

	TC_{50} (μM)†		EC_{50} (μM)†		SI‡	
Compound	CEM-SS	PBL	CEM-SS	PBL	CEM-SS	PBL
INK-1	30.3±8.9	43.7	0.47±0.2	1.25	64.5	34.9
INK-2	27.6+5.7	48.4	0.15±0.1	>3	184.0	<16.1
INK-3	56.8±22.4	>50	0.19±0.1	>3	298.9	~16.7
INK-4	75.7±21.7	48.2	0.64±0.4	>3	118.3	<16.1
INK-6	40.2±12.8	>50	0.21±0.1	0.63	191.4	>79.4
INK-7	36.0	31.6	0.67	1.5	53.7	21.0
INK-8	54.3±14.3	>50	1.02±0.3	>3	53.2	~16.7
INK-13	>100±0.0	>50	0.77±0.7	2.5	>129.9	>20.0
INK-14	>100±0.0	>50	<0.08±0.08	0.62	>1250	>80.6
INK-15	38.9±2.9	42.1	0.10±0.06	>3	389.0	<14.0
CP-51§	14.3±2.3	ND	0.11±0.06	ND	130	–
AZT§	3.7±1.6	ND	0.009±0.008	ND	411	–

*The TC_{50} (cytotoxicity) and EC_{50} (anti-HIV-1 activity) data from CEM-SS cells represent averages ±SD of 2–4 independent experiments per compound except for compound INK 7, which was tested once. Data from PBL cells were from a single independent experiment. To determine the TC_{50}, five serial concentrations of each compound were assayed in triplicate for [^{3}H]TdR incorporation into total DNA. To determine the EC_{50}, three to five serial concentrations of each compound were assayed in duplicate. The syncytial plaque assay was used with CEM-SS cells infected with the prototype of HIV-1 (strain H9IIIB). A reverse transcriptase assay was used with PBL cells infected with a clinical isolate of HIV-1 (strain H112-2) as cited in Kucera *et al.* (1990a).
†TC_{50} and EC_{50} values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).
‡SI is the ratio TC_{50}/EC_{50}.
§Data from Piantadosi *et al.* (1991).

Table 3. Evaluation of PC lipids INK-3 and CP-51 and AZT against TIBO-resistant, AZT-resistant HIV-1 and wild-type HIV-2 measured by syncytial plaque assay

	EC_{50} (μM)*					
Compound	TIBO^r	1073	1074	G910	G691	HIV-2
INK-3	0.11	0.90	0.19	0.20	0.12	0.21
CP-51	0.10	0.19	0.20	0.64	0.33	0.32
AZT	ND	0.16	0.03	>2.5	>2.5	ND

*EC_{50} values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Table 4. Evaluation of INK compounds for activity against matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates

	EC_{50} (μM)		
	AZT-sensitive strain	AZT-resistant strain	Fold increase
Compound	G-762	G-691	res./sen.
INK-14	0.10	2.34	23.4
INK-15	0.06	0.08	1.3
AZT	0.003	2.20	673.3
	H112-2	G-910	
INK-14	0.01	4.69	469
INK-15	0.07	0.02	<1
AZT	0.004	0.30	75

Assays were carried out using CEM-SS cells and the syncytial plaque assay.
Abbreviations; res: resistant; sen: sensitive

among all the compounds evaluated. In PBL cells, the TC_{50} values were comparable to those from CEM-SS cells. However, the EC_{50} values were consistently higher in PBL than in CEM-SS cells (Table 2). These results were most likely due to differences in sensitivity of prototype HIV-1 (strain H9IIIB) and a clinical isolate (strain H112-2) to the test compounds.

Inhibition of HIV-2 and TIBO- and AZT-resistant HIV-1 strains as measured by syncytial plaque assay

Since selected PC lipid compounds (for example INK-3 and CP-51) have potent activity against prototype strains of HIV-1, we extended evaluation of their activity against HIV-2 and TIBO- and AZT-resistant HIV-1 clinical isolates. Results (Table 3) indicated that both INK-3 and CP-51 gave dose-dependent activity against HIV-2, with EC_{50} values of 0.21 and 0.32 μM and against TIBO-resistant HIV-1 with EC_{50} values of 0.11 and 0.10 μM, respectively. The EC_{50} values for INK-3 and CP-51 against AZT-resistant HIV-1 clinical isolates (1073, 1074, G910, G691) ranged from 0.12 to 0.90 μM and 0.19 to 0.64 μM, respectively (Table 3). The EC_{50} values for AZT against the same AZT-resistant HIV-1 clinical isolates ranged from 0.03 to >2.5 μM (Table 3). Note that the EC_{50} for AZT against a sensitive prototype HIV-1 strain was 0.009 μM (Table 2). Other results indicated that the PC lipid INK-15 had no significant fold increase in EC_{50} between the matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates (Table 4). However, the lipid–AZT conjugate INK-14 and AZT alone showed a significant fold increase in the EC_{50} ratio between the AZT-sensitive and AZT-resistant HIV-1 clinical isolates of 23.4, 469 and 673.3, 75, respectively. In summary, the PC lipids INK-3 and CP-51 have potent activity against HIV-2 and TIBO- and AZT-resistant HIV-1. There is no apparent cross-resistance between PC lipid and TIBO or AZT compounds. In contrast, the lipid–AZT conjugate (INK-14) did show some cross-resistance with AZT.

Inhibition of fusogenic activity between persistently HIV-1-infected H9IIIB cells and uninfected $CD4^+$ CEM-SS cells

Published data from our laboratories indicated that CP-51 significantly inhibited HIV-1-induced fusogenic activity (Krugner-Higby *et al.*, 1995). In similar experiments (Table 5), results indicated that INK-3 also has significant dose-dependent activity against HIV-1-induced fusogenic activity. The EC_{50} values were 0.08, 0.18 and >1.0 μM for INK-2, -3, and -1, respectively (Table 5). These results suggest that selected PC lipid compounds active against infectious HIV production (Table 2) also inhibited virus-induced fusogenic activity.

Toxicity against mouse bone marrow cells as compared to AZT

Two independent experiments were designed to compare the relative toxicity of CP-51 and selected INK compounds with AZT. Results of these studies indicated that INK-2, -3 and -6 had a TC_{50} value of >100 μg/ml (Table 6). In two experiments, the TC_{50} values for CP-51 were 9.15 and 14.17 μg/ml compared to 0.142 and 0.259 μg/ml for AZT (Table 6). These data are interpreted to suggest that selected PC lipid compounds are significantly less cytotoxic than AZT to mouse bone marrow cells.

Discussion

Most of our previously synthesized alkylamidopropyl phosphocholines and alkoxy- and alkylthioglycerol phosphocholines (PC lipids) were analogues of phosphatidylcholine and contained a long hydrocarbon chain length (C16–C18) at position 1 and a short chain length (C1–C2) at position 2 of the three carbon backbone. Many of these compounds exhibited potent anti-HIV-1 activity. The most promising analogue we have reported to date

Table 5. Effect of PC lipid compounds on fusogenic activity between persistently HIV-1-infected H9IIIB cells and uninfected $CD4^+$ CEM-SS cells

Compound	Concentration (μM)	Inhibition of fused cells (%)	IC_{50} (μM)
INK-1	1.0	18	
	0.1	27	
	0.01	17	>1.0
INK-2	1.0	86	
	0.1	53	
	0.01	17	0.08
INK-3	1.0	78	
	0.1	41	
	0.01	9	0.18

Persistently HIV-1-infected H9IIIB cells were treated with compound for 96 h and cocultured with uninfected $CD4^+$ CEM-SS cells (ratio 1 : 100) in the presence of added compound for 48 h. The number of fused cells was counted relative to untreated control cells to determine the percentage inhibition of fusion and the IC_{50}. The IC_{50} values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Table 6. *In vitro* cytotoxicity of PC lipid compounds in CFU-GM assays

Compound	TC_{50} (μg/ml) Experiment 1	Experiment 2
CP-51	9.15	14.17
INK-2	>100	>100
INK-3	>100	>100
INK-6	>100	>100
AZT	0.142	0.259

In control cultures treated with PBS there were 86 and 54 c.f.u./plate in Experiments 1 and 2, respectively.

(Kucera *et al.*, 1990a) was 1-octadecanamido-2-ethoxypropyl-3-phosphocholine (CP-51). This amidoalkyl derivative exhibited an EC_{50} of 0.11 μM and a SI of 130 (Table 2). Accordingly, we chose this analogue as our lead compound and proceeded to modify its structure to optimize its anti-HIV-1 inhibitory activity and/or SI. In addition, we wanted to ascertain whether a long hydrocarbon chain length at position 1 was essential for optimum anti-HIV-1 selectivity.

As seen from data in Table 2, numerous synthetic CP-51 analogues including INK-2, INK-3, INK-6 and INK-15 exhibited a higher SI than CP-51. The SI values for these analogues in CEM-SS cells were 184.0, 298.9, 191.4 and 389.0, respectively, whereas their EC_{50} values were comparable to that of CP-51. These results demonstrated that a long hydrocarbon chain length of 16–18 carbons is not essential for optimum selectivity in this series of PC compounds. Notably, data presented in Table 2 suggested that a shorter hydrocarbon chain length (Table 1) at position 1 (10 or 12 carbons) and a longer chain at position 2 (12 or 8 carbons) as seen in INK-15 and INK-3, respectively, improved the SI up to threefold (with a concomitant decrease in cytotoxicity) compared with the 18 and 2 hydrocarbon chain lengths in CP-51.

Previously, we synthesized a series of alkyletherglycerol phosphocholines (Meyer *et al.*, 1991). Like the alkylamido phosphocholine CP-51, these alkylether compounds had a long hydrocarbon chain (C16–C18, oxy- or thioether) at position 1 and a short chain (methoxy or ethoxy) at position 2. The EC_{50} values for these alkylether phosphocholines ranged from 0.3 to 1.4 μM and their SI were lower (4 to 69) compared to that of CP-51 (130). In view of the above data with the INK compounds, it was of interest to synthesize an alkylether analogue of INK-2 to determine whether the SI could be similarly improved in this series. This alkylether analogue, INK-4, with oxyether hydrocarbon chain lengths of 12 and 10 at the 1 and 2 positions, respectively, exhibited an EC_{50} of 0.64 μM, a TC_{50} of 75.7 μM, and a SI of 118.3. Notably, the TC_{50} value is significantly different from that of any previously reported alkylether phosphocholine (3 to 19 μM; Meyer *et al.*, 1991), again demonstrating that such hydrodrocarbon chain length modifications will lead to a less cytotoxic compound. In summary, these data indicated that hydrocarbon chain lengths in the range of 10 to 12 at position 1 and 8 to 12 at position 2 should result in compounds with less cytotoxicity than those with 16 to 18 hydrocarbon chain lengths at position 1 and 1 to 2 at position 2.

Also, the TC_{50} values from PBL cells were comparable to those from CEM-SS cells (Table 2). INK-13 and INK-14, with 8 and 10 carbons at position 1 and 7 carbons at position 2, respectively, were the least cytotoxic ($TC_{50} \geq 100$ μM) compounds in the series. At the present time, no specific experiments have been done to determine why INK-13 and INK-14 were the least cytotoxic in the series of compounds evaluated. In the case of the conjugate INK-14, the hypothesis is that the lipid component acts as a carrier for AZT. The lipid anchors the conjugate into the cell membrane and is metabolized to slowly release lower concentrations of AZT into cells compared to AZT treatment alone. The end result is reduced toxicity from AZT. Evidence in support of this hypothesis is the lower TC_{50} and higher SI for INK-14 compared to AZT (Table 2).

In the case of the phospholipid–AZT conjugates, INK-14 [3′-azido-3′-deoxy-5′-(3-dodecylamido-2-octoxypropyl)-phosphothymidine] and CP-92 [3′-azido-3′-deoxy-5′-(3-octadecylamido-2-ethoxypropyl)-phosphothymidine] had comparable SI values [>1250 (Table 2) and 1793 (Piantadosi *et al.*, 1991), respectively]. Both compounds had a higher SI than AZT alone (411, Table 2) and were three- to fourfold more selective than AZT. In addition, the cytotoxicity was lower; the TC_{50} for INK-14 was >100 μM in comparison to 3.7 μM for AZT.

The effects of lengthening the linkage between the phosphate ester and the quaternary ammonium functionality were also studied through the synthesis of INK-8, with three carbons separating the phosphate ester and the quaternary nitrogen. This modification increased the EC_{50} and decreased the SI values (Table 2). At the present time, it is difficult to draw any definitive conclusion relating to such modification.

Based on these promising *in vitro* data, selected PC lipid and lipid–AZT conjugates were further evaluated. The results indicated that CP-51 and INK-3 also were active against HIV-2 and TIBO-resistant HIV-1 (Table 3). We also examined the effects of CP-51 and INK-3 against AZT-resistant HIV-1 clinical isolates (1073, 1074, G910, G691). As seen from the data in Table 3, both CP-51 and INK-3 were markedly active against the AZT-resistant mutants. Both the nucleoside analogue AZT and the non-nucleoside TIBO analogue R82150 (nevirapine; White *et al.*, 1991) are known to inhibit HIV-1 RT (De Clercq, 1995). However, unlike AZT, which can also inhibit HIV-2 RT activity, the non-nucleoside RT inhibitors do not inhibit HIV-2 RT activity. This difference in antiviral spectrum may be due to the non-nucleoside analogue binding at an allosteric site of RT, in contrast to the active site like AZT (De Clercq, 1995). From our present data, PC lipids are active against both TIBO- and AZT-resistant strains of HIV-1, suggesting that PC lipids do not target the same site(s) in the HIV replication cycle as compared to TIBO derivatives and AZT.

Using matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates, results indicated that each pair of isolates tested was similarly inhibited by the PC lipid INK-15 (Table 4). These data are interpreted to suggest that PC lipids do not show cross-resistance with AZT. In contrast, these same pairs of clinical isolates did show increased resistance to the conjugate compound INK-14 and to AZT alone (Table 4), suggesting that the most active anti-HIV-1 component in the lipid–AZT conjugate compound is probably AZT.

Results in Table 5 indicate that PC lipids were also active in inhibiting fusogenic activity of HIV-1-infected cells with uninfected cells. Recent published evidence by other investigators (Feng *et al.*, 1997) indicated that HIV-1 enters cells via a co-receptor designated fusin (CXCR-4), a member of the G protein-coupled chemokine receptor family with seven member-spanning domains. McKnight *et al.* (1997) reported that a monoclonal antibody to fusin can block cell-to-cell fusion and cell-free virus infection of fusin-positive $CD4^+$ cells. Lipid analogues are known to accumulate at the cell plasma membrane (Storme *et al.*, 1985; van Blitterswijk *et al.*, 1987) and they serve as potent inhibitors of HIV-1-induced cell fusion (Krugner-Higby *et al.*, 1995). This inhibition was correlated with inhibition of gp160/gp120-specific monoclonal antibody with HIV-1 gp160/gp120 reactivity on the surface of HIV-1-infected and treated cells (Krugner-Higby *et al.*, 1995). It can be speculated that the presence of lipid analogues could cause steric hindrance of the $CD4^+$ receptor and fusin co-receptor in cell-to-cell fusion.

Published evidence indicates that the major antiviral effect of recombinant interferon α (rIFN-α) operates in the stages of virus assembly of gp120 and release (Hansen *et al.*, 1992; Smith *et al.*, 1991; Willey *et al.*, 1988). Syntheses of HIV-1-induced DNA, RNA and protein were minimally inhibited by rIFN-α but virus particles released from the treated cells were 100- to 1000-fold less infectious, owing to an assembly defect in gp120 (Hansen *et al.*, 1992). Our published data (Krugner-Higby *et al.*, 1995) indicated that anti-HIV PC lipids do not affect HIV-1-induced protein synthesis and processing. However, assembly of progeny virus particles made in the presence of antiviral PC lipids was shifted from the plasma membrane to intracytoplasmic vacuoles (Kucera *et al.*, 1990b), and this shift in assembly site was associated with a profoundly reduced capacity of progeny virus particles to bind to $CD4^+$ cells (Krugner-Higby *et al.*, 1995). Also, we observed that exposure of fresh $CD4^+$ cells to equivalent amounts of RT activity associated with HIV-1 particles from 1 μM PC lipid-treated cells resulted in a 40 to 68% inhibition in subsequent HIV-1 replication compared to HIV-1 particles from untreated control cells (LS Kucera, N Iyer, SL Morris-Natschke, SY Chen, F Gumus, K Ishaq & DBJ Herrmann, unpublished results). Data from Western blot analysis of virus particles from PC lipid-treated cells indicated that gp120 produced in infected cells was also present in virus particles recovered from PC lipid-treated cell supernatants (Krugner-Higby *et al.*, 1995). Incubation of PC lipid with preformed virions did not produce a virucidal effect (LS Kucera, N Iyer, SL Morris-Natschke, SY Chen, F Gumus, K Ishaq & DBJ Herrmann, unpublished results). Krugner-Higby *et al.* (1995) showed that HIV particles made in the presence of phospholipids had a reduced capacity to bind to $CD4^+$ cells. Taken together, our data suggest that the reduced capacity of progeny virus from PC lipid-treated cells to infect and replicate in fresh $CD4^+$ cells could be associated with an alteration in the assembly of gp120 on the surface of progeny virus particles. Thus, PC lipids may share a property in common with rIFN-α by operating at the stage of HIV gp120 assembly and release.

In summary, results from chemical synthesis and evaluation of compounds for anti-HIV-1 activity in this present investigation indicated that compounds with hydrocarbon chain lengths of 10 to 12 at position 1 and 8 to 12 at position 2 of a three carbon backbone have high SI against HIV-1 replication. PC lipid analogues have potent and

selective activity against HIV-1 and HIV-2 as well as against TIBO- and AZT-resistant strains of HIV-1 and virus-induced cell fusion. In view of the markedly reduced toxicity of PC lipid analogues against bone marrow cell growth *in vitro* compared to AZT, PC lipid analogues offer an innovative approach toward development of novel strategies for the treatment of HIV infections and AIDS.

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