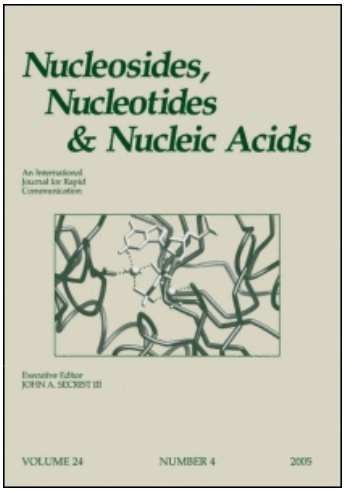

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Synthesis and Evaluation of a Novel Synthetic Phosphocholine Lipid-AZT Conjugate That Double-Targets Wild-Type and Drug Resistant Variants of HIV†

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ABSTRACT

INK-20, a synthetic phosphocholine lipid-AZT conjugate, was evaluated for antiviral activity against wild-type HIV-1, a matched pair of pre-AZT and post-AZT and multi-drug resistant clinical isolates. In addition, it was tested for activity against viruses resistant to nucleoside (AZT, 3TC) and nonnucleoside (nevirapine) reverse transcriptase and protease (saquinavir) inhibitors using the syncytial plaque reduction assay for infectious virus multiplication. The EC_{50} values were 0.004, and 0.005 μM against wild-type HIV-1 for INK-20 and AZT, respectively. INK-20 showed little or no cytotoxicity when assayed in CEM-SS cells and four other cell types including PBMC. This resulted in a selective index of > 25,000 and > 20,000 for INK-20 and AZT, respectively. When tested against a matched pair of pre-AZT and post-AZT clinical isolates, the EC_{50} values were 0.01 and 0.03 μM for INK-20 and 0.0005 and

†In honor and celebration of the 70th birthday of Professor Leroy B. Townsend.

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0.33 μM for AZT, respectively. INK-20 had moderate to good activity against two other AZT resistant variants and very good activity against a multi-drug resistant clinical isolate compared to marked resistance of these viruses to AZT alone. INK-20 retained significant activity against viruses resistant to 3TC, nevirapine, and saquinavir. The synthetic phosphocholine lipid-AZT conjugate INK-20 represents a novel class of anti-HIV compounds, which may provide new strategies for the treatment of HIV drug-resistant variants.

Key Words: Phosphocholine lipid analog conjugate; Drug resistant HIV; Anti-retrovirus drug conjugates.

INTRODUCTION

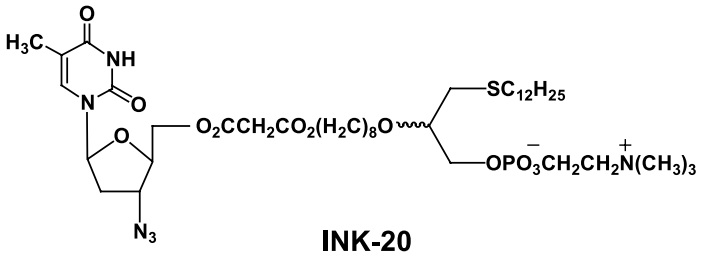
Current treatment of HIV/AIDS involves the use of physical combination therapies that include nucleoside and nonnucleoside reverse transcriptase inhibitors, protease inhibitors, and T-20, the newest class of entry/fusion inhibitor, to produce a regimen of ''highly active antiretroviral therapy'' (HAART).[1,2] Despite the potent antiretroviral activity of HAART, major problems have been associated with each of the currently approved classes of drugs including toxic side effects associated with long-term HAART treatment[3] coupled with human cases of HIV variants containing mutations that cause resistance to one or more drugs,[4] transmission of drug-resistant virus in humans[5] and poor pharmacokinetic properties. Better-tolerated drug combinations with novel mechanisms of action and activity against drug-resistant variants are urgently needed for the treatment of HIV/AIDS.

Initial studies with anti-HIV synthetic phosphocholine lipids alone were strongly encouraging and indicated potent activity against wild-type HIV with little or no cytotoxicity compared to AZT.[6,7] These lipid molecules alone displayed a dose-dependent inhibition of infectious virus production that was the likely result of inhibiting a late stage in virus replication, possibly at the level of assembly.[7–9] The molecules did not directly inactivate preformed extracellular virus indicating that their activity was not the result of a detergent-like effect on HIV infectivity.[7] Subsequently, it was observed that combinations of phosphocholine lipids and AZT resulted in an apparent synergistic or additive effect.[6] Recently, chemical combinations of a phosphocholine lipid (e.g., INK-18) with a nucleoside analog (e.g., AZT) resulted in a conjugate compound (e.g., INK-20) (Figure 1) that could ensure simultaneous delivery of two therapeutic agents (phosphocholine lipid plus nucleoside analog) to infected cells and double-target the HIV life cycle at different sites. Results presented in this paper indicate that the phosphocholine lipid-AZT conjugate (INK-20) has significant and selective antiviral activity against wild-type and several classes of drug-resistant HIV-1 variants.

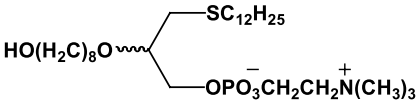
RESULTS

Synthesis

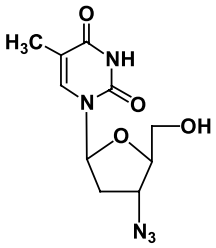
The dialkyl phosphocholine lipid (7) shown in Scheme 1 was prepared using previously established procedures.[10–15] In brief, 1-thioglycerol (**1**) was alkylated with



INK-20

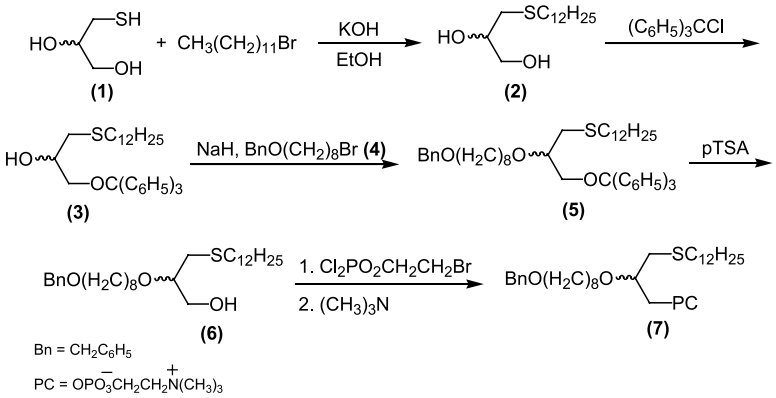


INK-18

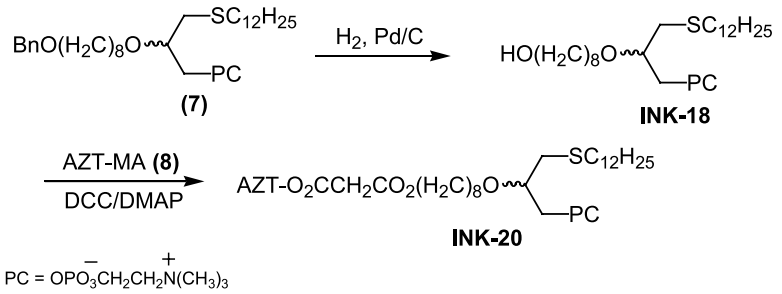


AZT

Figure 1. Structures of INK-20, INK-18, and AZT.



Scheme 1. Synthesis of dialkyl phosphocholine lipid backbone.



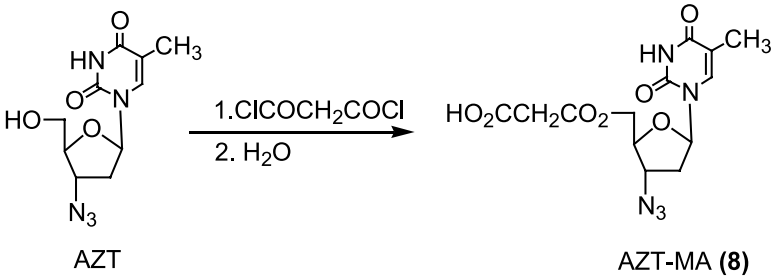
Scheme 2. Synthesis of phosphocholine lipid-AZT conjugate (INK-20).

1-dodecyl bromide in alcoholic KOH to give compound **2**. The primary hydroxyl of **2** was protected as the triphenylmethyl (trityl) ether using trityl chloride. The resulting compound **3** was then alkylated at the secondary hydroxyl using sodium hydride and 1-bromo-8-benzyloxyoctane (**4**). Compound **4** was prepared by reacting 1,8-dibromooctane with benzyl alcohol in the presence of NaOH and tetrabutylammonium hydrogen sulfate.[16] The protecting trityl ether of compound **5** was removed by acid hydrolysis using p-toluenesulfonic acid in MeOH/$CHCl_3$ (Scheme 1) to give the substituted thioglycerol lipid (**6**). The phosphocholine **7** was formed in a two-step sequence: reaction of the lipid primary hydroxyl with bromoethyl dichlorophosphate[17] followed by aqueous trimethylamine. The benzyl ether of **7** was removed by hydrogenolysis over Pd/C giving **INK-18**. Finally, the terminal hydroxyl was coupled with AZT-malonic acid (AZT-MA, **8**) in the presence of DCC and DMAP/pyridine to give **INK-20** (Scheme 2). AZT-MA (**8**) was prepared by reaction of AZT with malonyl dichloride (Scheme 3).

Biological Evaluation

Determination of Cytotoxicity in Uninfected and Antiviral Activity in Wild-Type HIV-1 Infected CEM-SS Cells

Initial experiments were done to determine the toxic concentration 50 % (TC_{50}) in uninfected CEM-SS cells. Results indicated that the TC_{50} in CEM-SS cells was > 100



Scheme 3. Synthesis of AZT-malonic acid (AZT-MA).

Table 1. Determination of TC_{50}, EC_{50} and selective index (SI) for INK-18, INK-20 and AZT in uninfected or HIV-1 infected CEM-SS cells.[a]

Compound	TC_{50} (μM)[b]	EC_{50} (μM)	SI[c]
INK-18	>100	0.61	>163
INK-20	>100	0.004	>25,000
AZT	>100	0.005	>20,000

[a]A syncytial plaque reduction assay was used to determine the EC_{50}. Five serial five-fold dilutions of compound starting at 10 μM were tested in triplicate to obtain an average % inhibition of HIV-1 (strain IIIB) syncytial plaques. The % inhibition at each concentration was used to calculate the EC_{50}. Results are representative of a typical experiment repeated at least once with similar results.
[b]The TC_{50} was determined using five serial five-fold dilutions of compound starting at 100 μM in triplicate. After 6 days treatment at 37°C, the cells in suspension culture were counted using Trypan blue exclusion and the % dead cells was measured to calculate the TC_{50}.
[c]$SI = TC_{50} \div EC_{50}$.

μM (100 μM was the highest concentration tested) for INK-18, INK-20, and AZT (Table 1). Similarly, the TC_{50} of the compounds was >100 μM in four other cell culture systems including human PBMC (Table 2). The anti-HIV activity of INK-18, INK-20 and AZT was determined in wild-type (IIIB) HIV-1 infected CEM-SS cells. The effective concentration 50% (EC_{50}) values were 0.61, 0.004, and 0.005 μM for INK-18, INK-20, and AZT, respectively, (Table 1). The phosphocholine lipid-AZT conjugate INK-20 had the best selective index (SI = >25,000) followed by >20,000 for AZT and >163 for INK-18. These results indicate that the phosphocholine lipid-AZT conjugate (INK-20) is well tolerated by human tissue culture cells and it has potent anti-HIV-1 activity.

Anti-HIV Activity Against Matched Pairs of Pre-AZT and Post-AZT Clinical Isolates

Since phosphocholine lipids alone are not inhibitors of reverse transcriptase but target infectious HIV-1 replication at a late stage in the virus life cycle, possibly

Table 2. Determination of cell cytotoxicity in different cell cultures.

	Cell cultures (TC_{50}, μM)			
Compound	PBMC[a]	MOLT-4[a]	MRC-5[b]	MRC-9[b]
INK-18	> 100	> 100	> 100	> 100
INK-20	> 100	> 100	> 100	> 100
AZT	> 100	> 100	> 100	> 100

[a]TC_{50} was determined by Trypan blue exclusion after 4 days incubation with compound.
[b]TC_{50} was determined by neutral red assay after 3 days incubation with compounds. Results are representative of a typical experiment repeated at least once with similar results.

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Table 3. Evaluation of compounds against a matched pair of HIV-1 pre-AZT and post-AZT clinical isolates.[a]

	EC_{50}, μM		
Compound	Pre-AZT H112-2	Post-AZT G910-6	Fold-increase
INK-18	0.21	1.2	6
INK-20	0.01	0.03	3
AZT	0.0005	0.33	660

[a]A syncytial plaque reduction assay with CEM-SS cells was used to determine the EC_{50}. Five serial five-fold concentrations of compound starting at 10 μM were tested in triplicate to obtain an average % inhibition of HIV-1 syncytial plaques. The % inhibition at each concentration was used to calculate the EC_{50}. Results are representative of a typical experiment repeated at least once with similar results.[9]

assembly,[7] we tested the antiviral activity of the phosphocholine lipid-AZT conjugate against matched pairs of pre-AZT and post-AZT clinical isolates. A syncytial plaque reduction assay was used to determine the effective concentration 50% (EC_{50}) for INK-20 against a matched pair of HIV-1 pre-AZT and post-AZT clinical isolates. Results (Table 3) indicated that between the pre-AZT and post-AZT clinical isolates the EC_{50} was 0.21 and 1.2 μM for INK-18, 0.01 and 0.03 μM for INK-20 and 0.0005 and 0.33 μM for AZT, respectively. The differences in EC_{50} between the pre-AZT and post-AZT isolates for INK-18 and INK-20 are probably not due to resistance because these changes are within the experimental variations of the assay. In contrast, the EC_{50} between the pre-AZT and post-AZT isolates for AZT was 0.0005 and 0.33 μM, respectively (Table 3). These results indicate that INK-20 has potent activity against both pre-AZT and post-AZT clinical isolates indicating activity against AZT resistant HIV-1 compared to AZT alone. Since the EC_{50} against the post-AZT isolate is lower for INK-20 than AZT alone, these data suggest that the enhanced anti-HIV activity seen with INK-20 is most likely due to a synergistic effect between the phosphocholine lipid (INK-18) and AZT moieties of the conjugate.

Anti-HIV Activity Against a Panel of Drug-Resistant Variants

To further assess the potential activity of the conjugate we tested INK-18, INK-20 and appropriate control drug against wild-type and representative protease (saquinavir), nucleoside analog (3TC, AZT), or non-nucleoside analog (nevirapine) drug-resistant variants. The fold-increase in EC_{50} relative to wild-type virus was determined by dividing the EC_{50} for the resistant virus by the EC_{50} for the wild-type virus. The results in Table 4 indicate that viruses resistant to saquinavir (variant 2948), and 3TC (variant 2970) remained sensitive to inhibition by both INK-18 and INK-20. Also, nevirapine resistant virus (variant 1392) was sensitive to INK-20, but it had increased resistance to nevirapine compared to wild-type virus (strain IIIB). Sensitivity of the nevirapine resistant virus (variant 1392) to INK-18 was not determined. Virus-resistant variants 2526 and 2529 that had a 26- and 90-fold increase in EC_{50} to AZT had an 11- and 40-fold increase to INK-18 and 1- and 10-fold increase to INK-20, respectively. Although

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Table 4. Antiviral activity of INK-18 and INK-20 against a panel of drug resistant variants of HIV-1 measured by plaque reduction assay.[a]

WT/Variant		Virus genotype	Compound	EC_{50}, μM	Fold-increase relative to wild-type
RF2803/		Wild-type	INK-18	0.08	
RF2803/		Wild-type	INK-20	0.01	
RF2803/		Wild-type	Saquinavir	0.003	
	/2948	G48V, L90M	INK-18	0.18	2
	/2948	G48V, L90M	INK-20	0.07	7
	/2948	Protease mutations	Saquinavir	0.29	97
LAIXXBRU/		Wild-type	INK-18	0.19	
LAIXXBRU/		Wild-type	INK-20	0.05	
LAIXXBRU/		Wild-type	3TC	0.06	
	/2970	M184V	INK-18	0.04	<1
	/2970	M184V	INK-20	0.01	<1
	/2970	RT mutation	3TC	> 0.4	>67
R5X4/		Wild-type	INK-18	0.16	
R5X4/		Wild-type	INK-20	0.05	
R5X4/		Wild-type	AZT	0.04	
	/2526	64N, 70R, 215F 219Q	INK-18	1.7	11
	/2526	64N, 70R, 215F 219Q	INK-20	0.06	1
	/2526	RT mutations	AZT	0.96	26
HXB2/		Wild-type	INK-18	0.05	
HXB2/		Wild-type	INK-20	0.003	
HXB2/		Wild-type	AZT	0.0001	
	2529/	74V, 41L, 106A 215Y	INK-18	10	40
	2529/	74V, 41L, 106A 215Y	INK-20	0.03	10
	2529/	MDR RT mutations	AZT	0.009	90
IIIB/		Wild-type	INK-20	0.008	
IIIB/		Wild-type	Nevirapine	0.24	
	/1392	Y181 to C	INK-20	0.003	<1
	/1392	RT mutation	Nevirapine	> 10	>42
5346/		Wild-type	INK-18	0.18	
5346/		Wild-type	INK-20	0.02	
5346/		Wild-type	AZT	0.001	
	/MDR5652	M41L, T69N, K70 R L74V, K103N, Y181C M184V, H208Y	INK-18	0.48	3
	/MDR5652	T215F A98S, D67N K219Q	INK-20	0.03	1
	/MDR5652	M461, 184V, L90M L101, K201, M361, L63P, A71V, G73S MDR RT mutations	AZT	0.443	443

[a]A syncytial plaque reduction assay with CEM-SS cells was used to determine the EC_{50}. Five serial five-fold concentrations of compound starting at 10 μM were tested in triplicate to obtain an average % inhibition of HIV-1 syncytial plaques. The % inhibition at each concentration was used to calculate the EC_{50}. Results are representative of a typical experiment repeated at least once with similar results.

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the mechanisms of action of phosphocholine lipids[7,8] and AZT[18] in tissue culture cells are not completely understood, both inhibitors can block virus maturation and disrupt syncytial formation. Therefore, a mutation involving virus maturation and syncytial formation might explain the fold-increase in EC_{50} for variants 2526 and 2529 to the phospholipid and AZT. Of great interest were results indicating that the MDR 5652 variant had only 3- and 1-fold increase to INK-18 and INK-20, respectively, compared to 443-fold increase to AZT (Table 4). In summary, the phosphocholine lipid-AZT conjugate (INK-20) has very good activity against several classes of drug-resistant variants.

DISCUSSION

Results presented in this paper demonstrate that the synthetic phosphocholine lipid-AZT conjugate INK-20 belongs to a novel class of anti-HIV compounds that in tissue culture cells has potent and selective activity against HIV-1 (Table 1), as well as drug-resistant variants (Tables 3 and 4) representing three major classes of drugs (nucleoside and non-nucleoside reverse transcriptase and protease inhibitors).

INK-20 was synthesized as a model compound that embodies two molecules (a phosphocholine lipid, e.g., INK-18, and a nucleoside analog, e.g., AZT) linked together through a malonate diester bond (Figure 1). Other phosphocholine lipids and nucleoside analogs could replace the lipid and nucleoside components, respectively. It is expected that the ester linkages will be cleaved in cells to release both components and thus target two different stages in the viral replication cycle (i.e., RT activity due to the nucleoside analog and virus assembly with the production of defective progeny virus due to the phosphocholine lipid).[7–9] Esterase activity could be modulated through alkyl or aryl substitution at the C-2 position of the malonate linker.

It is hypothesized that metabolism of the conjugate INK-20 releases the nucleoside AZT, which after phosphorylation to AZT-triphosphate, inhibits the HIV-induced reverse transcriptase.[19] In contrast, the phosphocholine lipid portion (INK-18) does not inhibit the reverse transcriptase activity. The evidence so far is that phosphocholine lipids are involved in assembly of defective virus-like particles within intracytoplasmic vacuoles.[7,20] These viral progeny lack functional gp120 expression on the virus surface and have reduced capacity to bind to CD4 + cells. This action does not appear to involve virus induced synthesis and processing of virus structural proteins in infected cells.[8] The hypothesis is that synthetic phospholipids may inhibit transport of gp120 from the golgi to the plasma membrane or the binding of gp120 and/or *gag* viral proteins to cell plasma membranes and lipid membrane rafts. There are accumulating data indicating the role of host cell proteins, cell membranes and membrane rafts in the entry and assembly of HIV-1 into cells.[21] Synthetic phospholipids are membrane interactive molecules that may have a profound effect on the lipid composition of membrane rafts and thus alter the proper binding of viral structural proteins and assembly of infectious HIV particles.

In summary, chemical combination of a synthetic phospholipid with another anti-HIV drug could lead to distinct advantages compared with a physical combination of several antiviral drugs (e.g. AZT and 3TC) including 1) increased half-life in vivo and prolonged duration of biological action due to protection of AZT from rapid

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glucuronide formation at the 5′ position, 2) reduced toxicity and increased tolerability in-vivo, 3) simultaneous delivery of constant amounts of multiple agents (e.g. phosphocholine lipid and AZT) to virus infected cells, 4) uptake of drugs into the CNS and lymphoid tissues which serve as HIV sanctuaries, 5) intracellular activation of the conjugate (see review by Morris-Natschke et al.,[9] and 6) activity of the phospholipid against drug resistant variants.

EXPERIMENTAL

Solvents were purchased from Acros and purified/dried as necessary by standard methods. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium hydride (NaH) and stored over Na ribbon, chloroform and dichloromethane were distilled from $CaCl_2$ and stored over molecular sieves type 4A, and pyridine was distilled from and stored over KOH. Reagents were purchased from Sigma Aldrich and used as such. NMR spectra were recorded on Varian 300 spectrometer and are reported in delta units with TMS resonance at 0 ppm used as reference resonance for ^1H spectra. The mass spectra were recorded on Micromass Quattro II Triple Quadrupole mass spectrometer equipped with Z−spray and nanospray sources. The melting points were obtained on Thomas-Hoover melting point apparatus and are uncorrected. Silica gel plates (MK6 F_{254}) were used for thin layer chromatography and spots visualized by UV_{254}, iodine, and NH_4SO_4 charring. Chromatographic purification was performed on ISCO CombiFlash 100c flash chromatograph using ISCO RediSep silica gel columns.

1-S-Dodecyl-3-O-trityl-rac-1-thioglycerol (3). 1-S-Dodecyl-3-O-trityl-rac-thioglycerol (**3**) was prepared by alkylation of 1-thioglycerol (**1**) followed by tritylation of the resulting thioether (**2**) as previously described.[10–14] Structures were confirmed by ^1H NMR.

1-Bromo-8-benzyloxyoctane (4). 1,8-Dibromooctane (272 g, 1 mol) was added to a mixture of NaOH (41 g), water (82 ml), tetrabutylammonium hydrogen sulfate (8.0 g) and benzyl alcohol (43 g, 0.34 mol).[16] This heterogeneous system was stirred vigorously for 22 h at room temperature (25°C). The reaction mixture was extracted with 500 ml and then 200 ml of hexane. The combined hexane layers were extracted with 100 ml of water and dried over magnesium sulfate overnight. After filtration and solvent evaporation in vacuo, the thick, brown oil (290 g) was flash chromatographed on silica gel using EtOAc gradient in hexane (0% → 10% EtOAc) to afford 62.2 g (61.2%) of product as a thick, light tan, lachrymating oil. TLC: 5% EtOAc:95% hexane, $R_f = 0.55$. Structure was confirmed by ^1H NMR.

1-S-Dodecyl-2-(8-benzyloxyoctyl)-3-trityl-rac-1-thioglycerol (5). Sodium hydride (60%, 12 g, 0.3 mol) was added to 300 ml of dry THF and chilled to 0°C in an ice-salt bath. The suspension was stirred 20 min and 108 g (0.208 mole) of compound **3** in 300 ml THF were slowly added over period of 1 h. Stirring was continued 30 min and 62.1 g (0.21 moles) of l-bromo-8-benzyloxyoctane (**4**) in 100 ml THF were added. After removing the ice bath, the reaction mixture was stirred 1 h at room temperature, then refluxed 42 h. Heating was discontinued and the reaction

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mixture cooled in an ice bath. Ice (50 g) was added slowly, solution stirred 1 h, and saturated sodium bicarbonate (100 ml) was added followed by 350 ml of saturated NaCl. The milky emulsion was extracted with 2 × 700 ml of CH_2Cl_2, and organic layers combined and dried over Na_2SO_4 for 20 h. Solvents were evaporated in vacuo and residual oil (230 ml) flash chromatographed on silica gel with EtOAc gradient in hexane (0% → 6% → 15% EtOAc) to give 53.8g (35.2%) of pure product (**5**) as light tan oil, together with 18.6 g of impure **4**, and 55.3 g of pure **3**. TLC: 10% EtOAc:90% hexane, R_f = 0.8. Structure was confirmed by ^{1}H NMR.

1-S-Dodecyl-2-(8-benzyloxyoctyl)-rac-1-thioglycerol (6). Compound **5** (35 g, 0.048 mol) was dissolved in a mixture of 250 ml anhydrous $CHCl_3$ and 125 ml anhydrous MeOH, containing 1.0 g of p-toluenesulfonic acid monohydrate and stirred 3 h at 40°C, at which time the reaction was complete. The reaction mixture was shaken repeatedly with 400 ml of saturated $NaHCO_3$ until carbon dioxide evolution ceased. After drying over Na_2SO_4 for 10 h, the solvent was removed at reduced pressure. Hexane (100 ml) was added and evaporated in vacuo. Hexane (25 ml) was added to the resulting oil to prevent crystallization of methoxy triphenylmethane and this mixture was flash chromatographed on silica gel with gradient of EtOAc in hexane (0% → 12% EtOAc). Product was obtained in 67% yield (15.8 g) as thick oil. TLC: 10% EtOAc:90% hexane, R_f = 0.2. The structure was confirmed by ^{1}H NMR.

1-S-Dodecyl-2-O-(8-benzyloxyoctyl)-rac-thioglycero-3-phosphocholine (7). A magnetically stirred mixture of anhydrous diethyl ether (50 ml) and THF (50 ml) was cooled to 0°C in an ice bath under argon atmosphere and 3 ml (5.35 g, 0.022 mol) of freshly distilled 2-bromoethyl dichlorophosphate (prepared as given in Ref. [17]) in 10 ml anhydrous pyridine were added dropwise. Compound **6** (4.93 g, 0.01 mole), dissolved in 50 ml of anhydrous diethyl ether was added, the reaction mixture allowed to warm to room temperature, then refluxed 4 h, and cooled to 0°C. Water (6 ml) was added and stirring continued 30 minutes. Solvents were removed in vacuo, and the resulting semisolid dissolved in 1:1 $CHCl_3$:MeOH (25 ml), mixed with 10 g of silica gel and the solvents evaporated. The residue was packed in an injection cartridge, then eluted onto 120 g silica gel column (ISCO RediSep) and eluted with a gradient of MeOH in $CHCl_3$ (0% → 15% → 30% → 40% MeOH). Fractions were analyzed on silica gel TLC developed with $CHCl_3$:MeOH:H_2O 75:25:4, and those containing the R_f = 0.65 component (fractions 62–71) were combined and solvent evaporated to afforded 3.1 g (45.5%) of chromatographically pure product as glassy light tan solid. The structure was confirmed by ^{1}H NMR. This intermediate (1.4 g, 0.002 mol) was dissolved in 25 ml of $CHCl_3$, and 25 ml of isopropyl alcohol was added followed by 40 ml of 40% aqueous trimethyl amine. The slightly turbid solution was stirred vigorously and refluxed (63°C) for 20 h. The reaction mixture was allowed to cool to room temperature (about 1 h) and 2.75 g (0.01 mole) of Ag_2CO_3 was added. Stirring and reflux were resumed and continued for 1.5 h. Therefore, heating and stirring were stopped and most of the black sediment was filtered using a sintered glass funnel (medium). $CHCl_3$ (25 ml) was then added and the mixture formed two layers. The lower (organic) layer was separated and evaporated to dryness, and the residue dissolved in MeOH (20 ml). This solution was mixed with 10 g of silica gel, evaporated to dryness, and packed in an injection cartridge which was then eluted on 10 g silica gel column

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(ISCO RediSep) and eluted with MeOH:$CHCl_3$ gradient (100% $CHCl_3$ followed by linear gradient to 80% MeOH). Product (**7**) was obtained in 66% (0.8 g) as a light tan glassy and extremely hygroscopic material. TLC: $CHCl_3$:MeOH:H_2O 75:25:4, R_f = 0.3. The structure was confirmed by ^{1}H NMR.[14]

1-S-Dodecyl-2-O-(8-hydroxyoctyl)-rac-thioglycero-3-phosphocholine (INK-18). The phosphocholine lipid **7** (0.97 g, 1.5 mmol, was dissolved in 150 ml of absolute EtOH and transferred into a Parr hydrogenation bottle under stream of argon. 10% Pd on activated carbon (1.0 g) was added and the bottle was placed into a Parr hydrogenation apparatus. The system was repeatedly evacuated and flashed with hydrogen and finally pressurized to 50 psi. Hydrogenation proceeded at room temperature (25°C) for 32 h until a constant pressure was reached for 8 h. The system was evacuated and flushed twice with argon. The catalyst was filtered off and washed with 25 ml of absolute ethanol, and the solvent was evaporated in vacuo. The glassy, hygroscopic residue (1.3 g) was dissolved in 15 ml of $CHCl_3$ containing 1% EtOH, injected on 10 g silica gel column (ISCO RediSep) and eluted with MeOH. Crude product (1.04 g) was obtained as thick oil, which was dried by repeated co-evaporation with 4% benzene in EtOH. The resulting glassy semisolid was placed in high vacuum at 40°C and dried 24 h to afford 0.81 g (95%) of chromatographically pure, hygroscopic, sticky solid. The structure was confirmed by ^{1}H NMR and mass spectrometry.

5′-Malonyl AZT (8). One gram of AZT was dissolved in 30 ml of anhydrous acetonitrile and added dropwise to a solution of 630 mg of malonyl dichloride in 20 ml of acetonitrile at 0°C. The reaction mixture was stirred for 2 h at 0°C then at 8–10°C for 4.5 h. Thin layer chromatography showed that the reaction was complete. Water (4 ml) was added. Solvents were removed in vacuo and the residue purified by silica gel chromatography eluting with $CHCl_3$:MeOH to give pure product in a 68% yield as a glassy solid. The structure was confirmed by ^{1}H NMR.

1-S-Dodecyl-2-O-[8-(5′-malonyl-AZT)octyl]-rac-thioglycero-3-phosphocholine (INK-20). Pyridine (200 ml) was freshly distilled from KOH and used in this experiment, which was performed entirely in an argon atmosphere. 5′-Malonyl AZT (**8**) (0.353 g, 0.001 mole) was dissolved in 6 ml of dry pyridine and evaporated to dryness. This procedure was repeated twice. The dry residue was then dissolved in 10 ml of pyridine, 0.62 g (0.003 mole) of dicyclohexylcarbodiimide was added, and the mixture evaporated to dryness. This process (addition of pyridine followed by evaporation) was repeated twice. p-Dimethylaminopyridine (0.12 g, 0.001 mole) in 10 ml of pyridine was added, and the solution then evaporated to dryness. This procedure was repeated twice with 10 ml of pyridine. Finally, 0.57 g (0.001 mole) of INK-18 in 10 ml of pyridine were added, evaporated to dryness (repeated twice), and then redissolved in 20 ml of pyridine. The flask was sealed and immersed in a 40°C water bath. The reaction mixture was stirred at this temperature for 18 h. Water (1 ml) was added, and stirring continued at 40°C for 1 h. The solvent was evaporated to dryness, the residue dissolved in 10 ml of MeOH: chloroform (1:2), mixed with 5 g of silica gel, evaporated to dryness and eluted onto a 35 g silica gel column (ISCO RediSep) with a gradient of MeOH in chloroform 0–66%. Chromatography gave 0.37 g of impure product, which was re-chromatographed on 4 g column (ISCO RediSep) with a MeOH:$CHCl_3$ gradient

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(0% → 80%) to yield 0.18 g (19.9%) of chromatographically pure INK-20. TLC: $CHCl_3$:MeOH:NH_4OH 75:25:5, $R_f \sim 0.3$. The structure was confirmed by ^1H NMR and mass spectrometry.

Virology

Virus stocks. HIV-1 (strain IIIB) was propagated in H9IIIB persistently infected cells as previously described.[7] HIV-1 pre-AZT (H112-2) and post-AZT (G-910-6; RT mutant at codon 215) clinical isolates and drug-resistant variants (2948, 2970, 2526, 2529, 1392) were obtained through the AIDS Research and Reference Reagent Program, NIH, Bethesda, MD, USA. The MDR 5652 resistant HIV-1 was received from Dr. Mark Wainberg, Lady Davis Institute, Jewish General Hospital, Montreal, Quebec, Canada. Stocks of drug resistant variants of HIV-1 were propagated in acutely infected CEM-SS cells using methods described by Krugner-Higby et al.[8]

Cell cultures. CEM-SS, PBMC and Molt-4 lymphoid cells were grown 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.[7] MRC-5 and MRC-9 human fibroblast cells were grown and maintained in Dulbecco's minimal essential medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 10% fetal bovine serum.

TC_{50} determinations. Test compounds were prepared as stock reagents by dissolving in absolute ethanol (INK-18, INK-20) or PBS (AZT) at a final concentration of 10 mM and stored at − 20°C. To test for cytotoxicity, the stock solutions were brought to room temperature (25°C) and serial dilutions were prepared in RPMI-1640 growth medium and added to 10,000 CEM-SS cells/well in triplicate wells 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 compound at 37°C. Trypan blue cell counts were made using a conventional light microscope to determine the viable cell count in treated and untreated CEM-SS, PBMC or MOLT-4 cell cultures. From the total cell counts, a TC_{50} for cytotoxicity was calculated as previously described by Chou et al.[22] and cited by Piantadosi et al.[6] A neutral red test was used to determine cell cytotoxicity in MRC-5 and MRC-9 cell monolayer cultures. The protocol was the same as described above except that instead of using Trypan blue cell counts, the overlay medium in each well was aspirated and each well received 200 μl of 0.01% neutral red (Sigma cat. #N-2880) in phenol red free D-MEM medium. After 2 hrs incubation at 37°C, the cells were washed with PBS to remove residual neutral red and then 200 μl lysis reagent containing 50% ethanol, 1% glacial acetic acid, 49% distilled water was added to each well to lyse the cells. In addition, 12 blank wells each received 200 μl of the lysis reagent. After 30 mins incubation at 37°C on a rotator platform the OD_{510} was read using an automatic ELISA plate reader. The % inhibition in OD_{510} in the test wells relative to the untreated control wells and minus the OD_{510} from the blank wells was used to calculate the TC_{50} by the method of Chou et al.[22] and cited by Piantadosi et al.[6]

EC_{50} determinations. The syncytium plaque reduction assay for infectious virus multiplication was used in the presence or absence of the phosphocholine lipid-AZT

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conjugate (INK-20) or control compounds (INK-18, AZT) as previously described by us.[7] The syncytium plaques were counted on day 5 or 6 post-infection and treatment and the percentage inhibition and effective concentrations 50% (EC_{50}) were calculated by the method of Chou et al.[22] and cited by Piantadosi et al.[6]

CONCLUSION

Phosphocholine lipid-nucleoside conjugates offer a novel approach to designing new strategies for double-targeting HIV infected cells simultaneously with two active drugs and attacking drug-resistant virus variants with a new compound (INK-20) that has a novel mechanism of action that may involve inhibition of virus structural protein binding (e.g. gag, gp120) to cell membranes and membrane rafts during the process of virus assembly.[21]

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