Unusual Single-Stranded Polyribonucleotides as Potent Anti-HIV Agents

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Polyribonucleotides (PMTG and PMTI) containing 1-methyl-6-thioguanosine or 1-methyl-6-thioinosine, respectively, as the sole nucleoside component are shown to be potent inhibitors of various strains of HIV-1 and HIV-2 in a number of human lymphocyte and macrophage cell lines in tissue culture as well as in fresh human peripheral blood lymphocytes and macrophages. PMTI and PMTG exhibit potencies in the range of 10^{-7}–10^{-8} M in these systems. The polynucleotides are active against virus strains resistant to AZT and pyridinone derivatives. Both PMTI and PMTG are synergistic with AZT and with ddI, and both inhibit HIV reverse transcriptase at nanomolar concentrations. The polymers show little or no toxicity in human cell lines at the highest doses tested (100 μg/mL, or about 0.2–1 μM). This class of compounds represents a new lead in AIDS therapeutic drug discovery.

Introduction

As human acquired immunodeficiency syndrome (AIDS) moves into its second decade as a recognized human disease, it is becoming a devastating pandemic. By the end of the century, it is estimated that some 10 million adults and 15 million children worldwide will have contracted the disease.[1] Many compounds of diverse structure and mechanism of action are in preclinical study or clinical trial; however, only four drugs are currently approved by the Food and Drug Administration for use as primary AIDS therapeutics in the United States. These are AZT (3′-azido-3′-deoxythymidine), ddI (2′,3′-dideoxyinosine), ddC (2′,3′-dideoxycytidine), and d4T (2′,3′-didehydro-2′,3′-dideoxythymidine), all of which act as potent inhibitors of the human immunodeficiency virus (HIV) reverse transcriptase (RT) and as DNA chain terminators.[2] Each of these drugs has shown significant toxicity in chronic use, and viral resistance has been identified in clinical isolates from patients treated with these drugs.[3]

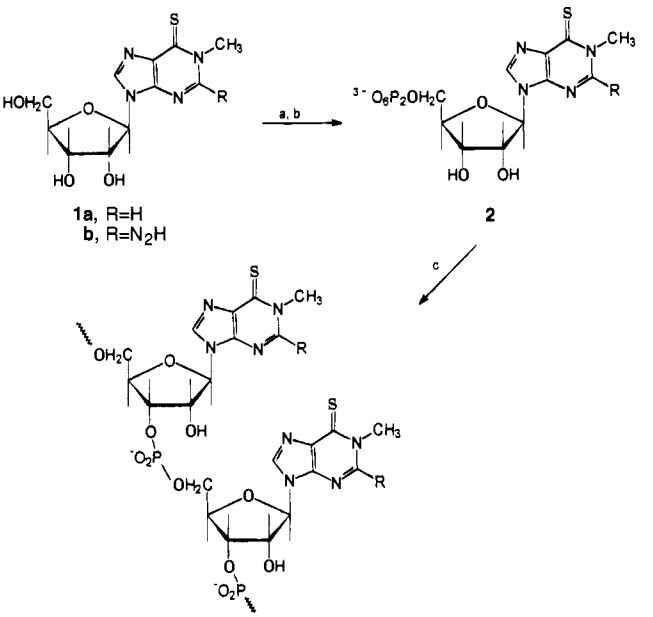
There is a pressing need for new AIDS therapeutics which possess unique mechanisms of action, are effective against a range of naive and drug-resistant viruses, may possess a reduced liability for the development of clinical resistance, and exhibit synergy with drugs in current clinical use. Two such molecules, both unusual polyribonucleotides, are the subject of this report.

Chemistry

The nucleosides required for this study, 1-methyl-6-thioinosine (MTI, **1a**) and 1-methyl-6-thioguanosine (MTG, **1b**) were prepared according to the general procedure of Ueda[4] as subsequently modified for large-scale preparation.[5] Conversion of these nucleosides to the required 5′-diphosphates was carried out using the phosphorylation procedure of Yoshikawa[6] and the diphosphate synthesis of Hoard and Ott[7] (Scheme 1). Polymerization was conducted as previously described.[8,9]

The polynucleotides, poly(1-methyl-6-thioinosinic acid) (PMTI) and poly(1-methyl-6-thioguanylic acid) (PMTG), are large, somewhat polydisperse molecules; the number

Scheme 1[a]



[a] (a) $POCl_3$, $PO(OEt_5)$;[6] (b) carbonyl diimidazole, $(nBuNH_3)^+$-$OPO_3H_2^-$, DMF;[7] (c) *M. luteus* polynucleotide phosphorylase.[8,9]

average molecular weights are estimated to be about 5 × 10^5 (PMTG) and 10^5 (PMTI). These estimates were originally based upon ultracentrifugation comparisons with poly(I) standards obtained from Pharmacia.[8,9] The availability of the Pharmacia standards, some RNAs of defined length obtained from Sigma, and a set of oligonucleotides synthesized in this laboratory in connection with other studies made it possible to evaluate the use of HPLC in size estimation.

It was found that a BioRad SEC 125 size exclusion chromatography column afforded excellent separations of oligonucleotides in the range of roughly 8–120 base pairs (Figure 1, Table 1). For PMTI and PMTG, this technique only allows for the determination that the molecule is longer than 120 nucleotides; for shorter molecules, the correlation can provide good estimates of molecular size. This was of interest because determination of the relationship of PMTI chain length to biological activity is essential.

Both polymerization and phosphorolysis components

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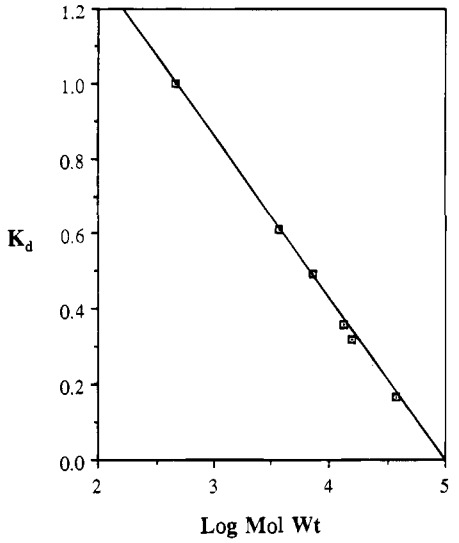

Figure 1. Relationship between retention time and log molecular weight of polynucleotides on an SEC 125 column.

Table 1. Relationship of Molecular Weight to Retention Time

no. of bases	molecular weight	log molecular weight	av t_R (SD)[a]	K_d[b]
1	458	2.661	13.18(0.03)	1
12	3635	3.561	10.68(0.05)	0.611
24	7270	3.862	9.92(0.04)	0.492
36	13428	4.128	9.06	0.358
51	15555	4.192	8.797(0.02)	0.317
120	38400	4.580	7.83(0.05)	0.167
1776	588320	5.755	6.76	0

[a] t_R (SD) = retention time (standard deviation from the mean of triplicate measurements). [b] K_d values are related to retention times by the relationship $K_d = (t_R - t_o)/t_i$ where t_R is the retention time for the peak in question, t_o is the void volume time (equal in this case to the elution time of the 1776-base RNA standard), and t_i is the inclusion volume time (maximum retention time based upon elution of the monomer minus the void volume time).

of *Micrococcus luteus* polynucleotide phosphorylase (PNPase) have been considered to be highly processive, with the former leading to high-molecular weight, relatively homogeneous polynucleotides[10] and the latter phosphorolyzing one chain at a time to 5′-diphosphates.[10,11] More recently, Benkovic and co-workers have shown that the synthetic reaction may be processive only above a certain size, giving rise to polymers ranging from 1 to 30 kilobases in length.[12]

The polymerization of MTIDP (**2a**) and MTGDP (**2b**) proceeded quite slowly but gave rise to polynucleotides much less polydisperse than those reported by Benkovic,[12] as judged by a single band centered at $s_{20,w}$ ~6 upon ultracentrifugation. The processivity observed here contrasts with that from Benkovic's study, suggesting a dependence upon such factors as enzyme source, reaction conditions, and substrates.

In order to determine whether a shorter chain length of PMTI would retain biological activity, the phosphorolytic component of polynucleotide phosphorylase action was evaluated. The yield of a long chain polymer was found to be optimal (about 33% isolated yield) after an 81 h incubation at 37 °C. As the incubation period was extended, the number average molecular weight as judged by HPLC decreased, such that at 108 h the average chain length appeared to be approximately 36 bases as judged by comparison with an oligoribonucleotide 36-mer (made available as a gift from Dr. Rich Meyer, MicroProbe Inc.). In contrast to the synthetic

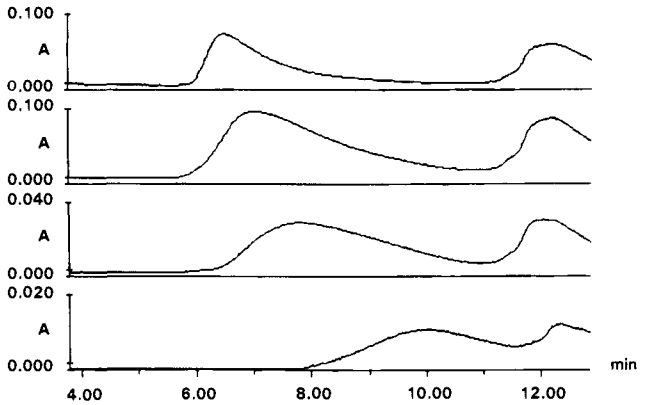

Figure 2. Phosphorolysis of PMTI by polynucleotide phosphorylase monitored by HPLC on an SEC 125 column.

Table 2. Inhibitory Activity (IC_{50}, μM[a]) against the HIV-1-Induced Cytopathic Effect in Various Cell Lines

cell line	PMTG	PMTI	AZT
CEM-SS	0.01 (5.2)[b]	0.08 (8.5)	0.002 (0.0006)
MT2	0.16 (82.2)	0.80 (77.6)	0.006 (0.0015)
PBL	0.03 (15.5)	0.03 (3.2)	0.02 (0.0050)
macrophage	0.002 (1.0)	0.15 (15.0)	0.4 (0.1)

[a] Concentrations were determined using the estimated number average molecular weights of 500 000 for PMTG and 100 000 for PMTI. [b] Numbers in parentheses are in micrograms per milliliter.

reaction, the phosphorolysis appeared not to be particularly processive. As the reaction was followed by HPLC (Figure 2), the average polymer size decreased steadily without the appearance of the biomodal distribution that could be expected from a processive degradation of one chain at a time to very small oligomers. The peak appearing between 12 and 13 min was shown to be MTI nucleotides by comparison with MTIDP.

As previously demonstrated,[8,9] both PMTI and PMTG undergo cooperative, highly hyperchromic UV melting transitions and a similar several-fold enhancement of circular dichroism ellipticities. Since PMTI, in particular, has no base protons available for hydrogen bonding, these changes are thought to result from random coil to stacked helical arrays. A more detailed analysis of the physicochemical properties of these unique polymers is in progress and will be reported elsewhere.

The integrity of these molecules was ascertained by HPLC homogeneity, UV absorption, and complete digestion to the starting nucleosides using snake venom phosphodiesterase and alkaline phosphatase[8,9] which gave rise only to the starting nucleosides.

Biological Data

The anti-HIV activity of PMTG and PMTI was assayed using a number of virus isolates and human cells. Microtiter antiviral assays were performed to determine the effect of high-molecular weight polynucleotides on the ability of HIV-1 to initiate a productive, cytopathic infection in CEM-SS cells. PMTG, PMTI, and AZT were examined in parallel for anti-HIV activity. PMTG and PMTI were able to protect CEM-SS cells from HIV-1-induced cytopathicity with IC_{50} values ranging from 0.01 to 0.08 μM (Table 2). The compounds were nontoxic up to concentrations of 100 $\mu g/mL$ (0.2−1.0 μM), indicating that they possess a high selectivity index.

Microscopic examination of infected cells protected by effective doses of the two compounds confirmed the complete absence of giant cell formation and any other

Table 3. Inhibitory Activity (IC_{50}, μM^a) against the Cytopathic Effect of Various Virus Strains in CEM-SS Cells

	PMTG	PMTI	AZT
	HIV-1		
III_B	0.01 (5.2)[b]	0.08 (8.5)	0.002 (0.0006)
RF	0.05 (24.9)	0.17 (17.2)	0.03 (0.0070)
LAV	0.03 (13.0)	0.04 (20.4)	0.004 (0.0015)
	HIV-2		
ROD	0.03 (16.2)	0.25 (25.4)	0.005 (0.0017)
MS	0.03 (18.9)	14.0 (0.14)	0.03 (0.0069)
G910-6 (AZT-res)	0.02 (10.0)	0.1 (11.8)	>1.0
A17 (pyridinone-res)	0.08 (42.9)	0.6 (57.8)	0.005 (0.0017)

[a] Concentrations were determined using the estimated number average molecular weights of 500 000 for PMTG and 100 000 for PMTI. [b] Numbers in parentheses are in micrograms per milliliter.

evidence of HIV-induced cytopathic effects. Confirmation of the anti-HIV activity of the two polynucleotides was obtained by analyzing the reverse transcriptase activity, the p24 core protein content, and the quantity of infectious virus particles in cell-free supernatants removed from each well of the microtiter plate. Reductions in RT activity, p24, and infectious particles were detected in all protected wells (data not shown), demonstrating that the ability of the polynucleotides to protect cells from death was directly related to their ability to inhibit HIV replication.

The polynucleotide anti-HIV agents and AZT were tested in parallel for their ability to inhibit the replication of phenotypically diverse strains of HIV-1 and HIV-2. Both compounds were able to protect CEM and MT2 cells infected with all isolates of HIV-1 and HIV-2. The IC_{50} values obtained for the two compounds ranged from approximately 0.01 to 0.25 μM for the inhibition of several isolates of HIV-1 and for two isolates of HIV-2 (Table 3). The two compounds were equally effective when tested against the AZT-resistant (AZT-res) strain G910-6 or against the pyridinone-resistant (pyridinone-res) strain A17. These results indicate that the polynucleotide compounds exhibit broad antiviral activity against immunodeficiency virus strains.

Further experiments testing the ability of the polynucleotide compounds to inhibit HIV replication in different human cell lines were performed. The compounds were effective in human T cell lines CEM and MT2 (Table 2), the human B cell line AA5, and the human macrophage–monocyte cell line U937 (data not shown). The compounds were tested in fresh human peripheral blood lymphocytes and macrophages infected with clinical strains of HIV-1 and were found to be effective inhibitors of HIV replication. The compounds exhibited IC_{50} values ranging from 0.01 to 0.8 μM in lymphocyte cultures and 0.002 to 0.15 μM in macrophage cultures. These results suggest that the compounds will effectively inhibit HIV in a variety of human cell lines. Toxicity was not observed in any of these assays at concentrations of the polynucleotides less than 0.2–1.0 μM.

Experiments were performed with the polynucleotides in parallel with AZT in which multiplicity of infection or time of drug addition was varied. In each experiment, the polynucleotides performed in a manner similar to that of AZT, showing progressively reduced protection with increasing virus titer. Pretreatment of cells with the compounds prior to infection for any time period did not significantly increase the anti-HIV activity of the polynucleotides. The anti-HIV activity diminished with drug addition at increasing time postinfection, suggesting inhibition at early stages of virus replication. At 24 h postinfection, drug addition was no longer protective to cells; similar results were also obtained with AZT. Reverse transcriptase inhibition assays utilizing a ribosomal RNA heteropolymer template system determined that the compounds were acting, at least in part, as reverse transcriptase inhibitors. The ID_{50} determined for each polymer in these experiments was less than 0.1 μM. Syncytium reduction assays carried out with PMTI suggest that the compound may also be active as a binding inhibitor. A short term assay virus control (no drug) gave rise to 87 syncytia, whereas PMTI treatment at 100 μg/mL (1.0 μM) yielded 14 syncytia, an 84% reduction. AZT has also been shown to reduce syncytium formation in this system at one to two logs higher concentration than that required for the inhibition of the virus-induced cytopathic effect.[13]

Polynucleotides were tested for anti-HIV activity in combination with AZT utilizing the standard microtiter assay. Eight concentrations of the polynucleotide compounds were tested in all combinations with five concentrations of AZT. Data analysis was performed according to the three-dimensional model of Prichard and Shipman[14] with the assumption that the compounds were acting at the same site. This assumption makes the analysis of the data more rigorous if the compound combination is synergistic.

Results of these assays demonstrate that the combined antiviral activity of the polynucleotides and AZT were greater than expected on the basis of additivity at nearly all concentrations, indicating siginificant levels of synergy. Appropriate controls were performed to demonstrate that AZT in combination with itself was additive and AZT in combination with ribavirin was antagonistic.

Concentrations of PMTG and AZT yielding maximal protection can be visualized in the contour plot provided (Figure 3). This antiviral synergy plot reveals striking synergy over a wide range of concentrations. Similar data were obtained using combinations of PMTG and ddI and PMTI with both AZT and ddI (data not shown).

It is important to note that the low-molecular weight PMTI resulting from prolonged exposure to PNPase, as noted above, was essentially devoid of anti-HIV activity and, indeed, showed some toxicity at 3–10 μg/mL. The basis of the relationship among polymer size, activity, and toxicity is unclear at the moment. It is, however, apparent that these polynucleotides must be of high molecular weight in order to elicit a potent anti-HIV response with a favorable therapeutic index. The nucleoside 1-methyl-6-thioinosine itself is neither active against nor toxic to Hep2 human epidermoid carcinoma cells at 100 μg/mL nor toxic to mice at 400 mg/kg (data from NCI antitumor screens).

Experimental Section

General. Chromatographic separations using HPLC were carried out on a Hitachi L6200 pump equipped with an L3000 photo diode array detector. Reverse-phase chromatography utilized a Rainin Microsorb MV C18 column, and size separations were carried out using a BioRad SEC 125 or SEC 250 column. UV spectra and melting curves were determined with a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Peltier variable temperature device.

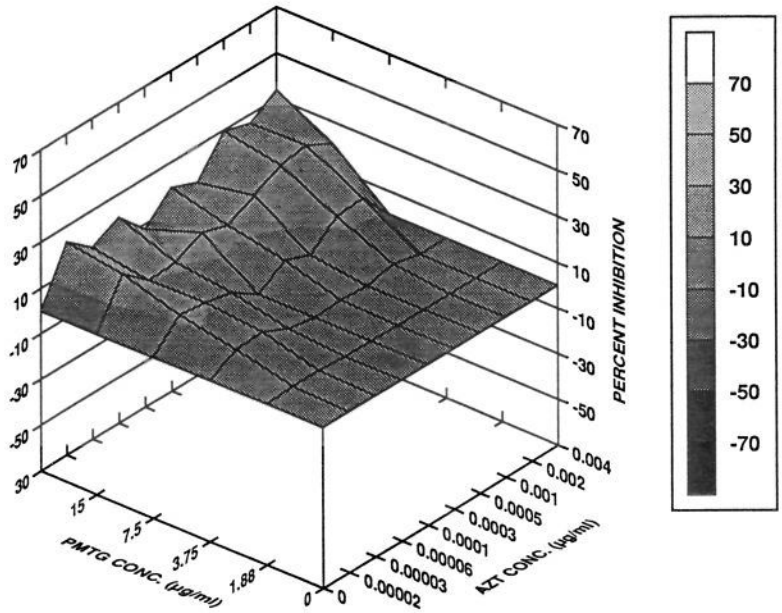

Figure 3. Antiviral synergy plot. The 95% confidence limit effect of PMTG on the anti-HIV activity of AZT.

Synthesis of polynucleotides was carried out using *M. luteus* polynucleotide phosphorylase obtained from Midland Certified Reagent Co., Midland, TX. The enzyme was provided in frozen solution containing 380 IU/mL. Degradation of polynucleotides to nucleosides was carried out using snake venom phosphodiesterase and alkaline phosphatase, both obtained from Sigma.

Molecular Weight Estimation. Estimation of the molecular size for full-length polymers was done by ultracentrifugation as previously described. The molecular weight of short chain PMTI was estimated by size exclusion HPLC using a BioRad SEC 125 column, 300 mm × 7.8 mm. Isocratic elution with an aqueous solution containing NaH_2PO_4 (50 mM), Na_2HPO_4 (50 mM), NaCl (150 mM), and NaN_3 (10 mM) with pH 6.8 at a flow rate of 1 mL/min gave distribution coefficients (K_d) which were linearly associated with log molecular weight (Table 1, Figure 1). K_d values are related to retention times by the relationship $K_d = (t_R - t_o)/t_i$ where t_R is the retention time for the peak in question, t_o is the void volume time (equal in this case to the elution time of the 1776-base RNA standard), and t_i is the inclusion volume time (maximum retention time based upon elution of the monomer minus the void volume time).

The 120- and 1776-base standards were obtained from Sigma. The 32-mer was a gift from Dr. Rich Meyer of MicroProbe Inc., and the remaining standards were oligodeoxyribonucleotides prepared in this laboratory for other projects. Data from Table 1 and Figure 1 were analyzed by a linear regression program (ANOVA) with and without the K_d corresponding to 1776 bases, giving r^2 values of 0.931 and 0.999, respectively. Exclusion of the 1776-base polymer from the standard curve is justified since it is much larger than the exclusion limit of the SEC 125 support.

Chemistry. PMTG was prepared as previously described.[9] PMTI was prepared on a larger scale by a modification of the earlier reported method.[8]

PMTI (Long Polymer). A solution made from the following components was incubated for 81 h at 37 °C: 4 mL of Tris·HCl (pH 9, 2 M); 2 mL of $MgCl_2$ (0.1 M); 2 mL of EDTA (0.01 M); 24 mL of water; 3200 IU of PNPase (*M. luteus*) in 8 mL of buffer containing 0.02 M Tris·HCl, 0.1 M NaCl, 0.001 M $MgCl_2$, and 0.001 M mercaptoethanol at pH 8; and 501 mg of MTIDP.

After incubation, the reaction mixture was diluted to 150 mL and adjusted to pH 7.5 with a solution of Na_2HPO_4 and NaH_2PO_4 (0.28 M, pH 6.8). The solution was extracted with 9 × 50 mL of chloroform/isoamyl alcohol (5.2) to give a clear aqueous solution. The aqueous layer was dialyzed against water (32 L) for 3 days. Lyophilization of the aqueous solution afforded PMTI (173 mg, 33%) as a pale yellow, fluffy solid: UV λ_{max} 318 nm, ϵ_{max} 14 100 (0.1 M NaCl); $s_{20,w}$ 6. HPLC analysis as described above gave a broad peak; t_R = 7.6 min (K_d = 0.13).

PMTI (Short Polymer). Incubation of MTIDP was carried out exactly as described above except the scale was one-tenth of that described above and the incubation time was 108 h. The white powder resulting from lyophilization was dissolved in deionized water (2 mL) and chromatographed on a Sephadex G-25 column (65 cm × 2.5 cm) using deionized water as eluent. The fractions showing HPLC retention times of 9 min were combined and lyophilized to give PMTI as a pale yellow, fluffy solid: yield 7.5 mg (15%); UV λ_{max} 318 nm, ϵ_{max} 15 100 (H_2O).

Biology: Cell-Based Assays. The CEM-SS cell line was utilized in all of the cell-based assays and was maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 2 mM glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/mL). Other cell lines utilized include the human T cell lines H9, MT2, Molt-4, Jurkat, the human B cell line AA5, and the macrophage–monocyte cell line U937. These cells were obtained from the NIAID AIDS Research and Reference Reagent Program and were cultured in the medium described. Fresh human cells were obtained from the American Red Cross.

Peripheral blood lymphocytes and macrophages were isolated following Ficoll–Hypaque centrifugation as described.[20] HIV-1 isolates included the common laboratory HIV-1 strains III_B, LAV, MN and RF as well as a panel of HIV-1 isolates cultured from peripheral blood lymphocytes of patients at Duke University Medical Center (Durham, NC) and the University of Alabama at Birmingham. The biological and biochemical properties of these isolates have been previously described.[21,22] HIV-2 isolate ROD was obtained from Dr. Luc Montagnier. The HIV-2 isolate MS and the matched pair of AZT-resistant and AZT-sensitive virus isolates were obtained from the AIDS Research and Reference Reagent Program. The pyridinone-resistant isolate A17 was obtained from Dr. Emilio Emini at Merck, Sharp & Dohme Laboratories.

Antiviral Assays. Antiviral assays were performed utilizing XTT measurement of virus-induced cytopathicity as described[23] with some modifications. CEM cells were added to each well of a 96-well microtiter plate at 5 × 10^3 cells per well.

Cells were infected with virus at a multiplicity of infection previously determined to give complete cell killing at 6 days postinfection in virus control wells. The panel of virus isolates were pretitered to induce equivalent infections (based on cell killing) in these assays. Serial half-log dilutions of test

compounds were added to appropriate wells in triplicate to evaluate their ability to inhibit HIV infection. Controls for each assay included drug calorimetric control wells (drug only), drug cytotoxicity control wells (cells and drug), virus control wells (cells and virus), and cell viability control wells (cells only). AZT was run in parallel as a positive control drug.

Following 6 days of incubation at 37 °C, the viability of the cells in each well was determined spectrophotometrically. XTT (Diagnostic Chemicals, Oxford, Canada) solution (1 mg/mL) containing phenazine methosulfate at 6.12 μg/mL was added to each well in a volume of 50 μL, and the plates were incubated for 4 h at 37 °C. Viable cells metabolized the tetrazolium salt to a soluble, colored formazan product. The results of each assay were determined by reading the optical density of each well at 450 cm using a Molecular Devices ν_{max} plate reader.

Samples of virus containing supernatants were removed from each well of the microtiter plate prior to staining with XTT. These samples were analyzed for virus content by reverse transcriptase activity assay, p24 ELISA (Coulter), and CEM-SS infectivity assay. RT and CEM-SS assays were performed as previously described.[22,24] The p24 ELISA was performed according to the manufacturer's recommendations (Coulter).

Syncytium Assays. CEM cells chronically infected with HIV-1 were plated in duplicate using serial 10-fold dilutions from 10^5 to 10^6 cells per well in a volume of 100 μL in one 96-well microtiter plate. Uninfected cells (1×10^5) were immediately added to each well in a volume of 100 μL. The compounds were added to appropriate wells in serial half-log dilutions. Following 24 h of incubation at 37 °C, the number of syncytia were quantitated by direct microscopic observation. The number of syncytia in wells containing varying concentrations of antiviral agents in control wells containing no compound were used to calculate the percent reduction in syncytium formation. AZT was run in parallel for comparison.

Drug Combination Assays. Analysis of drug combination assays were performed utilizing the XTT assay methodology according to the method of Prichard and Shipman.[14] Optical density measurements were performed as described, and the results were imported into the combination analysis programs (95% confidence interval, same-site analysis).

Conclusions

Although other polynucleotides have shown anti-HIV activity (notably, ampligen,[15] "thiolated" polynucleotides,[16] and poly(adenylic-uridylic acid)[17]), PMTG and PMTI are the first homopolyribonucleotides devoid of Watson–Crick hydrogen-bonding sites to show potent HIV inhibition. We hypothesize that these polymers bind to and fill the RNA-binding site[18,19] on HIV reverse transcriptase, thus serving the role of "antitemplates" as defined by Bardos.[16] If this is true, one might predict that resistance would develop with difficulty, since even mutant enzymes would require an effective template-binding region. These compounds serve both as potential human therapeutics in their own right and as leads for new approaches to drug discovery.

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