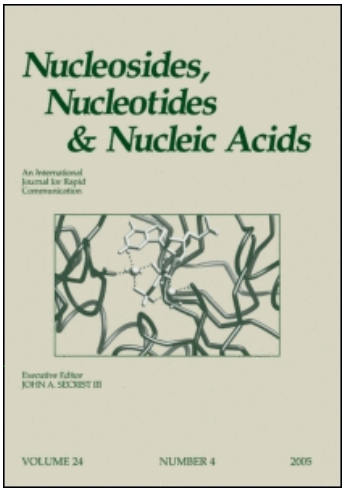

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"SENSELESS" ANTIVIRAL POLYRIBONUCLEOTIDES: POLY (1-PROPARGYLINOSINIC ACID)

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ABSTRACT

Previous work has shown that novel amphipathic oligo and polyribonucleotides exhibiting secondary structure in solution are potent inhibitors of HIV and HCMV replication and cytopathicity in tissue culture. It was hypothesized that the mechanism(s) of action for these compounds might be inhibition of retroviral reverse transcriptase (RT) and/or viral uptake by cells. Pursuit of the essential pharmacophore has led to the discovery of poly (1-propargylinosinic acid) (**10**), an HIV and HCMV-active polyribonucleotide lacking the secondary structure previously thought to be essential for the observed antiviral activity.

INTRODUCTION

The rate of new infections of human immunodeficiency virus (HIV) in the United States is slowing and showing signs of leveling[1]. However, worldwide, it was estimated in 2000 by the United Nations that the number of people living with HIV/AIDS as of December 2000 was 36.1 million; 21.8 million had already succumbed to the disease[2].

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Current HIV treatment strategies utilize fourteen FDA approved drugs that fall into three classes: nucleoside analogs (AZT[3], ddI[4], DDC[5], 3TC[6], D4T[7] and Abacavir[8]); nonnucleoside reverse transcriptase inhibitors (Viramune[9], Rescriptor[10] and Efavirenz[11]) and protease inhibitors (Invirase[12], Norvir[13], Crixivan[14], Viracept[15] and Amprenavir[16]). These three classes of drugs interfere at two sites within the HIV replication pathway; namely viral DNA formation and viral protein production. Current therapeutic strategy involves the combination of several drugs from either the same class or from different classes, the latter of which is referred to as Highly Active Anti-Retroviral Therapy (HAART). Though able to significantly lower viral load, in some cases to undetectable levels, these therapies are unable to provide curative results[17]. Failure is blamed upon many factors including low patient compliance, latent reservoir formation[18] and low drug potency.

The key problem in eradicating HIV stems from the infidelity of reverse transcriptase (RT). Mutation due to RT misincorporation occurs once every 10^4 replication[19]. At 10^{10} replication cycles per day[20], this allows for mutation at every site within the HIV genome. Many mutants are able to resist drug pressure, survive and proliferate[21]. Drug pressure without eradication eventually leads to resurgence of drug resistant HIV particles, the development of AIDS and eventual patient death. To combat this impressive mutation ability, additional drugs must be developed that have diverse structures and unique targets. For both first and second line management of HIV and HCMV infections, new drugs are needed that lack cross-resistance with existing agents and are slow to select resistant clones.

Human cytomegalovirus (HCMV) is an opportunistic infection observed in individuals with impaired immune system responses. Its prevalence in HIV infected individuals is widespread and leads to a significant number of deaths. HCMV retinitis is the leading cause of blindness in AIDS patients[22] Current therapeutic options are limited (ganciclovir, cidofover, foscarnet and fomivirsen), costly and toxic[23]. As is the case with HIV, development of new therapeutic agents is essential. The development of an agent having activity against HIV and HCMV simultaneously would provide a significant advancement towards the successful treatment of individuals infected with both diseases.

This laboratory has invested several years in the development of novel oligo- and polyribonucleotides as a new class of potent anti-HIV and HCMV drugs. In contrast to the well-known antisense, sense and nonsense terminology used to describe nucleic acid interactions, these compounds are referred to as ''senseless'' because of their complete lack of Watson-Crick hydrogen bonding sites. It was demonstrated earlier that PMTG (**1**), PMTI (**2**)[24], PTPR (**3**) and TTPR-32mer (**4**)[25], all have effective anti-HIV activity (Chart 1) with EC_{50} values ranging from 0.5 to 1.7 μM. Conversely, the polymers PATI (**5**)[26], PMI (**6**)[27] and TPR-32mer[25] (7), were devoid of any activity (Chart 2). Additional data has shown no resistance development against PMTI after 17 rounds of replication[28].

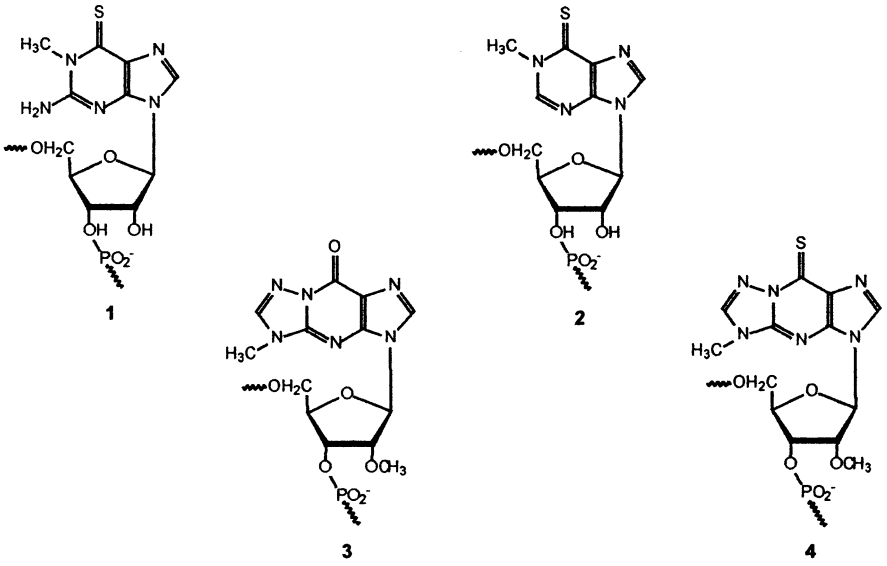


Chart 1. Anti-HIV active oligo- and polyribonucleotides.

SAR analysis reveals that the required structural elements for activity in this limited set of seven compounds are amphipathic character (hydrophobic base/hydrophilic backbone) and secondary structure (non-hydrogen bonded base-stacked array in solution). The difference in activity of PMTI vs. PMI and TTPR-32mer vs. TPR-32mer also indicates a preference for sulfur over oxygen at the purine 6 position. From this information, it was reasoned that the synthesis of a nucleoside incorporating an extension of the alkyl methyl group present in PMTG and PMTI to a propargyl group would build upon this SAR and lead toward the development of a more potent inhibitor. Initially, the plan was to synthesize the 1-propargyl-6-thiopurine nucleoside

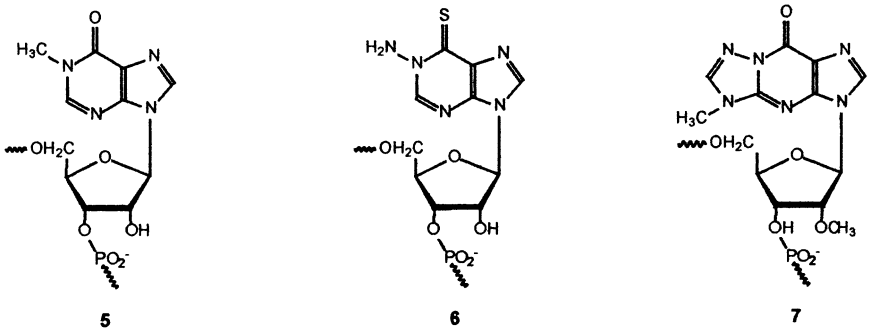
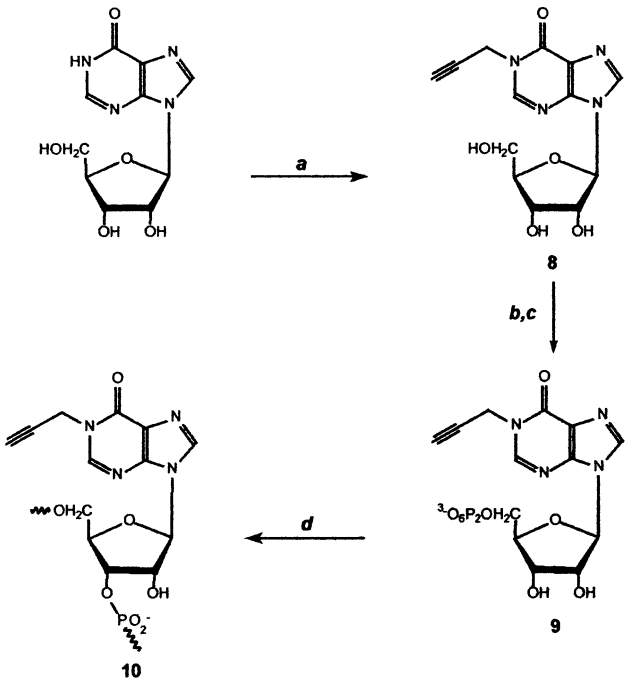


Chart 2. Oligo- and polyribonucleotides displaying no anti-HIV activity.

precursor. However, approaches which had succeeded in the earlier studies, including sulfohydrolysis of 1-propargyladenosine or direct thiation of protected 1-propargylinosine, failed to afford the desired product, presumably because of the instability of the propargyl group. The milder conditions required for 5′-diphosphate synthesis, however, were compatible with the propargyl functionality. Reported herein are the synthesis, characterization and biological activity of poly(1-propargylinosinic acid).

CHEMISTRY

The nucleoside, 1-propargylinosine, was prepared by alkylation of inosine with propargyl bromide in the presence of DBU. Conversion of this nucleoside to its 5′-diphosphate was accomplished by phosphorylation as described by Yoshikawa[29] and diphosphate synthesis as described by Hoard and Ott[30] (Sch. 1). The nucleotide was polymerized by modification of the procedure described earlier[24,26]. It was found that this substrate required 21 times less enzyme and 88% less time for formation of long chain polymer as



Scheme 1. Synthesis of poly (1-propargylinosinic acid) **10**. Reagents and Conditions: a. DMAC, DBU, propargyl bromide; b. $POCl_3$, $PO(OEt_5)$; c. DMF, carbonyl diimidazole, $(nBuNH_3^+)OPO_3H_2^-$; d. E. *coli* PNPase, Tris pH 9, 37°C.

compared to PMTI. The polymerization reaction was monitored by size exclusion HPLC and the reaction was determined to be complete when the ratio of peak area of the polymer and nucleotide remained unchanged. As observed previously, the PNPase enzymatic activity was processive, giving rise to a moderately polydisperse peak estimated to have an average molecular weight of 1×10^5. Size estimation was based upon earlier work describing the relationship between the log of the molecular weight and HPLC retention time with a standard curve of r^2 equal to 0.999[24]. The polymer was purified by a new ion exchange chromatography technique that proceeded with less difficulty than the traditional organic extraction methodology. No cooperative melting curve was observed (0.1 M PBS), suggesting the lack of any organized secondary structure in solution. The integrity of the polymer was verified by enzymatic digestion with snake venom phosphodiesterase and alkaline phosphatase[31,32] to 1-propargylinosine; the only compound detected by TLC.

BIOLOGICAL RESULTS AND DISCUSSION

The anti-HIV activity of **10** was evaluated against strains of HIV-1 (RF, III_B, SK1) and HIV-2 (ROD) in parallel with ddC in the CEM-SS human cell line. Although **10** is a 6-oxo analogue and demonstrates no secondary structure, it has potent anti-HIV activity, an observation that is inconsistent with the previously developed SAR. The EC_{50} values range from 0.0579 to 0.263 μM (Table I). Because cell viability remains at 90% or greater up to 0.620 μM for HIV-1 strains and 0.194 μM for HIV-ROD, the polymer has a high selectivity index. The inhibitory capability of the polynucleotide was further tested in the macrophage cell line and found to have an EC_{50} of 1.18 μM for BaI and 0.86 μM for ADA, demonstrating the ability to protect cells from the cytopathicity of HIV across cell types. The polynucleotide showed no inhibitory activity against DNA pol α or pol β (data not shown).

Table 1. Inhibitory Activity (EC_{50}, μM[a]) Against the Cytopathic Effect of Various Retroviral Strains of HIV in CEM-SS Cells

Virus	ddC	**10**
RF	0.039	0.131
III_B	0.0693	0.263
SK1	0.0254	0.0444
ROD	0.0621	0.0579

[a]Concentration of polynucleotide based upon the average molecular weight of 100,000.

Table 2. Inhibitory Activity (EC_{50}, μM) Against Reverse Transcriptase (RT) in a Cell Free Supernatant

Compound	RT, EC_{50}	CEM-SS (RF), EC_{50}
10	0.0223[a]	0.131
PMTI	0.00028	0.17[b]

[a]Concentration of **10** and PMTI based upon the average molecular weight of 100,000.
[b]Data reported earlier[24].

Table 3. Inhibitory Activity Against HCMV Strain AD-169 in the MRC-5 Cell Line

Compound	CD_{50}(g/ml)[a]	EC_{50} (g/ml)[b]	TI[c]
10	316 (3.16 μM)[d]	1.9 (19 nm)[d]	166
Ganciclovir	> 1000[e]	2.4	> 417

[a]50% Cytotoxic dose. [b]50% Effective dose. [c]TI = CD50÷ED50. [d]Concentration based upon the average molecular weight of 100,000. [e]Previous data.

Previous research has demonstrated that the ability of these polynucleotides to protect cells from the cytopathicity of HIV is directly related to their ability to inhibit the replication of HIV[14]. Though **10** is reasonably effective at inhibiting reverse transcriptase in a cell free supernatant (EC_{50} 0.0223 μM, Table 2), it is two orders of magnitude less potent than PMTI. It appears that PMTI acts by at least two mechanisms; inhibition of cellular viral uptake and inhibition of reverse transcriptase[33]. The similarity of the anti-cytopathic concentration of PMTI and **10**, despite their marked difference in reverse transcriptase inhibition, may reflect differences in cell uptake of the drug, a possibility currently under exploration.

The anti-HCMV activity of **10** was evaluated in parallel with ganiciclovir against HCMV strain AD-169 in the human diploid embryonic lung cell line (MRC-5). As shown in Table 3, the activity of **10** surpasses ganciclovir, however, it is more cytotoxic with cell viability of 80% at 1.0 μM and 60% at 3.16 μM[34]. The polynucleotide **10** is somewhat more potent against HCMV than HIV. This result demonstrates a phenomenon shown previously with **2**, **3** and **4**[25].

CONCLUSION

Until now, the focus of this research has been on N1 substituted, 6-thiopurine nucleotide polymers. These analogs have been difficult and many times impossible to synthesize because either the nucleoside cannot be

constructed or PNPase will not accept the nucleotide as substrate. The synthesis of **10** proved to be ideal. All synthetic steps proceeded with high yield and very little enzyme was required for polymerization which dramatically lowers the cost of production. The activity of **10** is exciting with potent inhibition of both HIV and HCMV. This research has opened up a new group of 6-oxo analogs to be explored for activity in future studies.

EXPERIMENTAL

Materials were purchased from Aldrich, Bio-Rad, Chem-Impex International, EM Science, Fisher, Selecto Scientific and Sigma and used without purification. Thin Layer Chromatography (TLC) was performed on silica gel coated Whatman aluminum backed flexible plates containing fluorescent indicator F_{254}. TLC spots were visualized with either short wavelength UV or by charring after treatment with a 5% sulfuric acid in methanol solution. Column chromatography was performed with silica gel, particle size 63–200. Conversion to the NH_4^+ form of the mono- and diphosphates was carried out using BioRad 50W-X8 (200–400 mesh) cation exchange resin. Cation exchange chromatographic purification of the polymer was performed with AG® MP-50, 100–200 mesh, hydrogen form resin using a Wiz pump, ISCO type 6 optical unit (254 nm) and an ISCO UA-5 absorbance monitor. HPLC chromatography was preformed with a Hitachi L6200 pump equipped with a L3000 photo diode array using a Bio Rad SEC-125 column. High resolution mass spectra were recorded with a MAT 95 spectrometer. Proton NMR were recorded with an IBM AF200 MHz FT-NMR. UV spectra and T_m measurements were recorded with a Hewlett-Pack 8452A diode array spectrometer equipped with a Peltier variable temperature controller.

1-Propargylinosine (8). Propargyl bromide (11.8 ml, 105.9 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (20.62 ml, 135.2 mmol) were added to a suspension of inosine (27.56 g, 102.8 mmol) in dimethylacetamide (590 ml). The reaction mixture was stirred at room temperature under argon overnight, at which time TLC in chloroform/methanol (85:15) demonstrated disappearance of starting material. The reaction was quenched by addition to 3.5 L of ether/hexanes (1:1); the resulting suspension was placed at $-15^{\circ}C$ overnight. While still chilled, solvent was decanted from the resulting gum, which was then dissolved in methanol and evaporated in vacuo (oil pump) onto silica gel. The product was isolated by silica gel chromatography using chloroform/methanol (95:5). Isolated yield was 94.6%. FABMS (glycerol), m/z 307 (MH^+); HRMS (glycerol) m/z Calcd. 307.10424 Obsv. 307.10502. ^{1}H-NMR (DMSO-d_6): 8.50 (1H, s, H2); 8.39 (1H, s, H8); 5.86 (1H, d, H1′); 5.50 (1H, d, 2′-OH); 5.22 (1H, d, 3′-OH); 5.05 (1H, t, 5′-OH); 4.84, 4.83

(2H, d, CH_2); 4.44 (1H, m, H2′); 4.11, (1H, m, H3′); 3.92 (1H, m, H4′); 3.62 (1H, m, H5′); 3.57 (1H, m, H5″); 3.41 (1H, t, CH).

1-Propargylinosine-5′-monophosphate (9a). The dry solid **8** (7.2 g, 23.53 mmol) was converted to its 5′-monophosphate as described earlier by Yoshikawa[15] yielding 10.2 g (83.2%). Samples for HRMS were obtained by cation exchange chromatography converting the compound into its NH_4^+ salt. HRMS (glycerol) m/z Calcd. 385.05493 Obsv. 385.05122 (M-3NH_3-H, negative ion mode).

1-Propargylinosine-5′-diphosphate (9b). The dry product **9a** (10.2 g, 19.6 mmol) was converted to its 5′ diphosphate as described earlier by Hoard and Ott[16] yielding 0.41 g (15.6%). Samples for HRMS were obtained by cation exchange chromatography conversion of the compound into its NH_4^+ salt. UV_{max} 250 nm, ε_{max} 7432 (0.1 M NaCl, 0.1 M phosphate buffer pH 6.8). HRMS (glycerol) m/z Calcd. 465.02126 Obsv. 465.02023 (M-3NH_3-H, negative ion mode).

Poly(1-propargylinosinic acid) (10). A solution comprised of the following components was incubated at 37°C for 3 hours with gentle rocking: 0.333 ml Tris HCl (pH 9.0, 2 M); 0.333 ml $MgCl_2$ (0.1 M); 0.333 ml 2-mercaptoethanol (2%); 1.332 ml H_2O; 15.0 IU of PNPase (E. *coli*) in 0.1 ml of buffer containing 50% glycerol, 5 mM Tris and 0.5 mM dithiothreitol at pH 8.0; and 49.2 mg of **9b**.

After incubation, the polymer was purified from the reaction mixture by cation exchange chromatography with BioRad AG® MP-50 (H^+) resin using 0.25 M Tris HCl (pH 5.5) as eluent. (0.25 M Tris HCl, pH 5.5)at a flow rate of 1.32 ml/min. All hardware required for column chromatography (i.e. column, fittings, tubing) had been sterilized by bathing in ethanol overnight. All fractions with 254 nm absorbance were pooled and the pH was adjusted to 7 with dilute NaOH. The aqueous solution was dialyzed against 0.1 M NaCl (12 L, 24 hours) and H_2O (24 L, 48 hours). Lyophilization of the aqueous solution gave poly (1-propargylinosinic acid) (13.9 mg, 28.3%) as a fluffy, pale yellow solid. UV_{max} 250 nm, ε_{max} 7 007 (0.1 M NaCl, 0.1 M phosphate buffer pH 6.8).

Degradation of polymer to starting nucleoside. To a solution of **10** in 0.1 M NaCl (100 µl, 2 mg/ml) was added 65 µl of a solution comprised of the following: 33 µl Tris HCl (2 M, pH 9.0); 44 µl $MgCl_2$ (0.1 M), 44 µl venom phosphodiesterase; and 22 µl alkaline phosphatase. The solution was incubated at 37°C with gentle rocking for 18 hours and then diluted to 5 ml with NaCl (0.1 M). TLC in SSE (4 EtOH:1 isPrOH:2H_20) confirmed degradation to starting nucleoside **8**.

Biological assays. Anti-HIV activity was conducted by the Southern Research Institute at the Fredrick Research Center with strains of HIV-1 and 2 in various cell lines and with cell free preparations of reverse transcriptase as previously described[24]. Anti-HCMV activity was conducted by Drs. R. Sidwell and J. Huffman at Utah State University with the AD-169 strain in the MRC-5 cell line as previously described[35].

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