Specific oligonucleotides as inhibitors of feline immunodeficiency virus reverse transcriptase

Douglas G. McBroom

The University of Montana

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Specific Oligonucleotides as Inhibitors of Feline Immunodeficiency Virus Reverse Transcriptase

by
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Presented in partial fulfillment of the requirements for the Masters of Science degree in Biochemistry
The University of Montana
1995

Approved by

[Signatures]
Chairman of the Committee
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Date
December 7, 1995
Specific oligonucleotides as Inhibitors of Feline Immunodeficiency Virus Reverse Transcriptase

Director: Dr. Thomas W. North

Several RNA ligands that bind to feline immunodeficiency virus (FIV) reverse transcriptase (RT) were selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) with recombinant FIV RT. Two of these ligands, F20 #53, and F18 #60 were potent inhibitors of recombinant FIV RT. Inhibition of RT was competitive with respect to template-primer and noncompetitive with respect to deoxyribonucleoside 5'-triphosphate substrates. SELEX ligand F20 #53 inhibited FIV derived from virus better than recombinant RT. In contrast, SELEX ligand F18 #60 was less inhibitory with virion RT as compared to recombinant RT. SELEX ligand F20 #53 was also more inhibitory in reactions with heteropolymeric templates than in reactions with homopolymeric template. Ligand F20 #53 also inhibited RT from a mutant of FIV resistant to 3'-azido-3'-deoxythymidine (AZT).
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LIST OF ABBREVIATIONS

ABBREVIATIONS: AIDS, Acquired immune deficiency syndrome; AZT, 3'-azido-2'-deoxythymidine; ddC, 2',3'-dideoxycytidine; D4T, 2',3'-dideoxy-2',3'-didehydrothymidine; ddl, 2',3'-dideoxyinosine; DEPC, diethylpyrocarbonate; FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; PCR, polymerase chain reaction; PFA, phosphonoformic acid; RT, reverse transcriptase; SELEX, Systematic Evolution of Ligands by Exponential Enrichment; #53, SELEX ligand F20 #53; #60, SELEX ligand F18 #60.
ACKNOWLEDGEMENTS

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General Introduction

Human immunodeficiency virus 1 (HIV-1) is an RNA virus that belongs to the lentivirus family of non-oncogenic, cytopathic retroviruses (9). HIV-1 has properties similar to prototypic animal lentiviruses including simian immunodeficiency virus and feline immunodeficiency virus (FIV) which infect rhesus macaques and cats, respectively. These animal models may prove useful in the understanding and the treatment of acquired immunodeficiency syndrome (AIDS) (10).

Antiviral agents are usually targeted against an enzyme or other viral component that is instrumental in replication of the virus. Current efforts in AIDS chemotherapy are focused on interruption of the virus life cycle by inhibiting reverse transcriptase (RT), viral protease, or other viral proteins (35). However in clinical treatment of patients there is a rapid emergence of resistance to the drugs that inhibit RT (35). Kaplan et al. have also found viral resistance to protease therapy in vitro (15). This rapid emergence of drug resistance makes new strategies for drug design important. Systematic Evolution of Ligands by EXponential enrichment (SELEX) is a novel approach that may provide strategies to combat resistance (46). This process rapidly selects for ligands which have a high affinity for target enzymes. The purpose of this project was to characterize SELEX ligands which had been selected to bind to FIV RT. In order to study the modes of inhibition of RT by SELEX ligands, assays to be developed that utilize natural FIV RNA and DNA templates.

FELINE IMMUNODEFICIENCY VIRUS

Feline immunodeficiency virus is a lentivirus that has been shown to cause an AIDS-like syndrome in cats. This system provides a useful animal model for the study of AIDS (1, 29). The FIV system possesses economical
and practical advantages over other animal models, such as the low cost of animal care, ease of in vivo studies, and the safety of working with a virus that does not infect humans (1, 10, 29).

Pathogenesis. Like HIV-1 infection of humans, FIV infection of cats can be characterized by a series of five stages. The five stages for FIV infection are: acute infection, asymptomatic carrier, persistent generalized lymphadenopathy, AIDS related complex, and AIDS (28).

The primary phase of the FIV infection is characterized by variable degrees of fever, neutropenia, and generalized lymphadenopathy. After disappearance of the early clinical signs of disease, experimentally-infected cats enter a lengthy period of virus latency (no clinical signs of disease manifested) during which FIV can still be isolated from the blood. The third stage of infection is equivalent to the persistent generalized lymphadenopathy stage in HIV-1 infected humans. This stage is characterized by vague signs of disease in the absence of obvious infection. The fourth stage is reminiscent of the AIDS related complex (ARC) in humans. Cats with ARC-like illness have chronic secondary infections at one or more body sites. If the cat survives stage four (fewer than 10% do) then the cats exhibit a clinical picture that can be defined as AIDS-like. Cats with AIDS-like illness, similar to their human counterparts, often have weight loss (>20% lost) opportunistic infections in multiple body sites, and most are anemic and leukopenic (28).

Life cycle. The life cycle of FIV is typical of a retrovirus. Following attachment and entry into a cell, one or both copies of RNA are converted to double-stranded DNA by RT and this DNA is integrated into the hosts genome by another virus encoded-enzyme, integrase. This integrated viral DNA is called a provirus. The provirus is transcribed by cellular DNA-dependent RNA polymerases to generate structural genes, (gag, pol and env) and full length
Figure 1. The life cycle of a retrovirus.
RNA copies which are used as genomes for progeny virus (Fig 1). Viral gene expression requires the action of regulatory proteins such as Rev and Tat, which controls expression of viral genes. The proviral DNA remains a permanent component of the host cell genome and is passed to all daughter cells (45).

There are many points in the life cycle that offer targets for chemotherapy. Approaches to HIV-1 therapy have targeted a number of these, including attachment, enzymes such as RT, and protease (35), and regulatory the proteins, such as Tat and Rev (12).

The most successful target for AIDS therapy has been RT. All of the drugs that have been approved for AIDS therapy in humans target this enzyme. These include 3'-azido-3'-deoxythymidine, (AZT), 2',3'-dideoxyinosine (ddl), 2'3'-dideoxycytidine (ddC), and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T). (35, 37, 42). These nucleoside analogues have modifications at the 3' position on the deoxyribose. Incorporation of these analogues blocks 5'->3' elongation during polymerization and result in chain termination (31).

Biochemical features of RT. North et al. have purified and extensively characterized the FIV RT. FIV RT has been shown to be similar to HIV-1 RT in physical properties and in catalytic functions (22, 23, 25,). FIV RT, like HIV-1 RT has three catalytic functions. It has RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, and RNase H activities (7, 34). The FIV and HIV-1 RTs have a similar requirement for Mg²⁺ and similar template specificities. The p61-p55 heterodimer that is observed with HIV-1 RT is similar to that of the FIV RT. The RT activity of FIV, like HIV-1, is comprised of a heterodimer of two subunits which have common amino-termini (8). The larger subunit contains the RNase H domain of RT.

The genes encoding RTs of FIV and HIV-1 also exhibit considerable
sequence homology. When the predicted amino acid sequence of FIV RT was compared to that of HIV-1 RT, a 60% identity was observed (26, 44). These similarities make FIV RT an attractive model for the study of RT-targeted therapy of human AIDS.

**Kinetic mechanism of RT activity.** RT catalyzes the synthesis of DNA via an ordered kinetic mechanism in which template-primer binds to the enzyme before the first 2’-deoxynucleoside 5’-triphosphate is used for primer elongation (31). Phosphodiester bond formation proceeds with inversion of configuration at the α-phosphorus, indicating that the 3’-hydroxyl group of the primer terminus attacks the α-phosphorus of the 2’-deoxynucleoside-5’-triphosphate substrate without intervening formation of a covalent enzyme intermediate (31).

The steady-state and pre-steady-state kinetics of incorporation of nucleotides and nucleotide analogues into a defined RNA template sequence primed with DNA has been examined. The kinetic mechanism that explains the potent inhibition by chain terminating nucleotide analogues such as AZTTP, ddATP and ddCTP has been determined. The mechanism of AZTMP proceeds as follows: The steady state rate of AZTMP incorporation into the 3’-primer terminus is limited by the rate of dissociation of the chain-terminated template-primer from the enzyme. This slow rate of dissociation leads to the steady-state accumulation of RT chain-terminated template-primer complex and results in potent inhibition of the enzyme (31). Inhibition constants have been determined for several nucleotide analogues, such as, ddATP, and ddCTP, which act by the same mechanism as AZTTP for HIV-1 RT (5).

North et al. have shown substantial similarities in the kinetic constants for the inhibition of HIV-1 RT and FIV RT by nucleotide analogues, including, 2’,3’-dideoxythymidine 5’-triphosphate (ddTTP), AZTTP and 2’,3’-dideoxy-2’,3’-didehydrothymidine 5’-triphosphate (D4TTP) (6, 22, 24, 25). The nucleotide
analogues were all competitive with respect to variable substrate concentration. Inhibition constants for these nucleotide analogues with FIV RT and HIV-1 RT are given in Table 1.

**Table 1. Inhibition of FIV RT and HIV-1 RT by nucleotide analogues***.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mean Kᵢ ± SD (nM)</th>
<th>Mean K/Kᵢ ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FIV</td>
<td>HIV</td>
</tr>
<tr>
<td>AZTTP</td>
<td>6.2±1.2</td>
<td>6.5±1.8</td>
</tr>
<tr>
<td>dTTP</td>
<td>6.7±3.3</td>
<td>5.9±4.2</td>
</tr>
<tr>
<td>D4TTP</td>
<td>1.8±0.7</td>
<td>8.3±0.8</td>
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*values are from North et al. (6, 22, 24).

Inhibition constants for a non-nucleotide analog, phosphonoformic acid (PFA) have also been determined. PFA is an antiviral drug that inhibits DNA polymerases, including RT, by blocking the pyrophosphate exit site (40). PFA has been found to be a noncompetitive inhibitor of HIV-1 and FIV RTs with respect to a variable substrate concentration, and it yields similar inhibition constants with these two enzymes (24).

In order to compare activities of FIV and HIV-1 RT further, North et al. have examined template specificities which are summarized in Table 2. Both enzymes are able to utilize poly(rA)-oligo(dT)₁₀, poly(rl)-oligo(dC)₁₀ and poly(rC)-oligo(dG)₁₀. Both enzymes fail to utilize poly(rU)-oligo(dA)₁₀, and poly(rG)-oligo(dC)₁₀. The two most effective template-primers are poly(rA)-oligo(dT)₁₀, and poly(rl)-oligo(dC)₁₀ (23).
Table 2. Template specificity of FIV and HIV RT*.

<table>
<thead>
<tr>
<th>Template</th>
<th>FIV</th>
<th>HIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>% relative activity ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>poly(rA)-oligo(dT)$_{10}$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>poly(rU)-oligo(dA)$_{10}$</td>
<td>0.1</td>
<td>0.29</td>
</tr>
<tr>
<td>poly(rC)-oligo(dG)$_{10}$</td>
<td>46 ± 4</td>
<td>15 ± 1.5</td>
</tr>
<tr>
<td>poly(rG)-oligo(dC)$_{10}$</td>
<td>0.34 ± 0.6</td>
<td>0.16 ± 0.1</td>
</tr>
<tr>
<td>poly(rl)-oligo(dC)$_{10}$</td>
<td>109 ± 7.8</td>
<td>76 ± 1.8</td>
</tr>
</tbody>
</table>

* Values are from North et al (23).

The characterization of these templates has allowed studies of ddCTP, ddGTP, AZTTP, ddTTP, and PFA. These homopolymeric templates do not allow the study of dATP analogues such as ddATP. However, Cronn et al. and North et al. have studied heteropolymeric templates such as M13 and φX174, in order to study ddATP (7, 25). The activity of FIV RT with these DNA templates was only 10-20% of that obtained with poly(rA)-oligo(dT)$_{10}$ as template primers. None of the heteropolymeric RNA templates tested were efficiently used by FIV RT.
DRUG RESISTANCE

Drug resistance is a limiting factor in the treatment of AIDS. AZT was the first drug approved and used for treatment of AIDS (21), and clinical isolates of HIV-1 that are resistant to AZT are detected in patients treated six months or longer with AZT (17, 36). Subsequently, mutants of HIV-1 resistant to ddl and ddC have been reported (13, 35, 43). Mutants resistant to several other inhibitors such as ddGTP, (30) and protease inhibitors (15, 27, 35) have been selected in vitro. It is likely that resistance will arise with most, if not all, therapeutic approaches.

Resistance of HIV-1 to AZT, is due to mutations which map in the pol gene (35). Similarly, resistance to other drugs such as ddl and ddC, have also been mapped in the pol gene of HIV-1 (35). Our lab was the first to report the selection of an AZT-resistant mutant retrovirus in a cell culture system (32). As in HIV-1, AZT-resistant mutants of FIV have mutations in pol. FIV systems are more versatile than HIV-1 systems for the study of drug resistance because FIV mutants are selected more easily than HIV-1 mutants (35). FIV also is a safer virus to work with than HIV-1. These features make FIV an attractive model for studies of resistance for AIDS therapy.

Many strategies to combat drug resistance have been proposed. The major focus is on combination chemotherapy. However, even with the combination of AZT and ddl, or ddC resistant mutants of FIV and HIV-1 arise
Therefore it is desirable to find drugs or strategies to minimize resistance. SELEX is a novel approach which may offer an immediate strategy to combat resistance.

SYSTEMATIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT

SELEX is a novel method to rapidly select preferred binding ligands from a population of random RNA oligonucleotides. Inherent in the SELEX technique is the specificity of the RNA to the target enzyme (46). Another important advantage is that RNA may be less cytotoxic than the traditional nucleoside analogues, which are cytotoxic at high doses.

The SELEX method relies on the mechanism that is usually associated with evolution, that is, variation, selection, and replication (46). An overview of this procedure is given in Figure 2. First, a pool of RNAs that are completely randomized at specific positions are subjected to selection for binding to a target, in this case to purified recombinant FIV RT on nitrocellulose filters. Second, the selected RNAs are eluted and converted back to DNA by reverse transcription, and are amplified as double-stranded DNA by PCR. This DNA is then used for in vitro transcription by T7 RNA polymerase. The resulting DNA is enriched for those sequences which bind FIV RT. The selected RNA is then used to begin the next cycle. More stringent conditions can be used in subsequent cycles to select the ligands that bind with greatest affinity.
Figure 2. An overview of the SELEX selection procedure. (Starting at the top, going clockwise) Randomized template construction (46). *In vitro* transcription of the pool of oligonucleotides. Binding of oligonucleotide to target protein resulting in an enriched RNA population. cDNA synthesis by reverse transcriptase. Second strand synthesis and polymerase chain reaction (PCR) amplification. *In vitro* transcription to begin the next round of selection (46).
This approach has been employed to develop inhibitors of HIV-1 RT (47). The RNA ligands, although not kinetically characterized, have been shown to inhibit HIV RT specifically, and not RTs from avian myoblastosis virus, Maloney murine leukemia virus, or HIV type 2 (47). A gel-based assay was used in those studies to measure concentrations which inhibit RT activity by 50 % (IC\textsubscript{50}). The IC\textsubscript{50} for inhibition of HIV-1 RT by the RNA ligand was approximately 100 nM (47). In a collaboration with Dr. Larry Gold’s lab (University of Colorado), SELEX ligands have been made that inhibit the FIV RT (3).

Selection of ligands that bind FIV-RT. High affinity ligands were selected from an RNA repertoire containing 10\textsuperscript{14} unique species with a 40 base long random RNA sequence. These selections were carried out by Hang Chen and Larry Gold, University Colorado. The first ten rounds of selection were performed by nitrocellulose filter partitioning. The last eight rounds were performed by native gel shift assays. After 18 rounds of selection, the binding affinity of the selected RNA pool could not be further improved by continued selection. The FIV RT binding affinity of RNA from the eighteenth round of selection (Kd 7.7 nM) was at least 10\textsuperscript{3}-fold higher than that of the starting repertoire (Fig 3) (3).

RNA sequences were obtained from eighty clones derived from the eighteenth round of selection and these were analyzed by an RNA folding algorithm.
Figure 3. Binding of RNA from indicated rounds of SELEX to FIV RT. Open circles represent the starting pool of random RNA, Kd too high for accurate determination. Open squares represents RNA from round six, Kd too high for accurate determination. Closed circles represents round 10 with a Kd of >100. Closed squares represent round 14 with a Kd of 13.6. Open triangles, round 16, Kd is 8.7. Closed triangles represent round 18 with a Kd of 7.7 (3).
Class I

\[ 5' - \text{GUACCAGAUUG} - \text{CAG} - 3' \]
F1, \( K_d = 1.9 - 3.8 \) nM; Freq. 10/80

\[ 5' - \text{UUUUGUG} - \text{CAUUUUGCUAUAU} - 3' \]
F2, \( K_d = 5.8 \) nM; Freq. 23/80

\[ 5' - \text{AAUAAACG} - 3' \]
F3, \( K_d = 6.1 \) nM; Freq. 6/80

\[ 5' - \text{YYGACG} - \text{UAUAU} - 3' \]
F4, \( K_d = 24 \) nM; Freq. 5/80

Class II

\[ 5' - \text{CUUUGCGUCAAUAU} - 3' \]
F5, \( K_d = 4.2 \) nM; Freq. 3/80

\[ 5' - \text{CCUACCG} - 3' \]
F6, \( K_d = 5.7 \) nM; Freq. 5/80

\[ 5' - \text{YYGAAACG} - 3' \]
F7, \( K_d = 6.0 \) nM; Freq. 4/80

Class III

\[ 5' - \text{YGGGAG} - \text{UAUAU} - 3' \]
F8, \( K_d = 6.0 \) nM; Freq. 13/80,

\[ 5' - \text{YGGGAG} - \text{UAUAU} - 3' \]
F9, \( K_d = 6.5 \) nM; Freq. 11/80,

Figure 4. Predicted folding of the FIV RT-specific SELEX ligands. The secondary structure was predicted by folding programs (3).
The selected RNA molecules fell into three major classes (Fig 4)(3). Class I molecules form stem-loop structures or stem-loops with an internal bulge, and contain two U-tract consensus sequences present in the region predicted to be single stranded. Three subsets of class I ligands also have an ACG consensus in the loop. Class II consists of three subsets of species which could form stem-loops with internal bulges. Class III are A-rich and appear to be unstructured as predicted by the RNA folding program (50).

Seven representatives of the eighty FIV-specific ligands were obtained for further studies. A major goal of this work was to characterize the inhibition of FIV RT by those seven SELEX ligands.

**ENDOGENOUS TEMPLATES**

As will be discussed below, the inhibition of FIV RT by SELEX ligands is competitive with respect to template-primer. This is in contrast to the existing antiviral nucleoside analogues, whose 5’-triphosphates compete with a dNTP substrate. Virtually all work with RT has been with homopolymeric RNA templates, which has been adequate for studies of the nucleotide analogues. However, for the study of SELEX ligands that compete with template-primer for binding to RT (3), more biologically relevant, heteropolymeric templates are needed. There is very little information available on use of heteropolymeric templates for kinetic studies of RT. Characterization of SELEX ligands with such template-primer systems requires development of new assay systems for FIV RT.
In the past, only a few attempts have been made to use heteropolymeric templates. DNA from phages φX174 and M13 are two heteropolymeric templates that have been used in the kinetic studies of FIV RT (7, 25). RT had somewhat lower activities with these templates than with the standard homopolymeric template, poly(rA)-oligo(dT)$_{10}$ (7, 25). Attempts to use heteropolymeric RNA templates, including 23S rRNA primed with oligodeoxyribonucleotides, and poly(A)$+$mRNA primed with oligo(dT) were unsuccessful due to the low activities of RT [less than 2% of that with poly(rA)-oligo(dT)$_{10}$] with these templates (7).

Endogenous RNA and DNA templates have been made for some studies of HIV-1 RT (16, 31). Reardon used short oligonucleotides approximately 50 bases long in order to kinetically analyze the RNA-dependent and DNA-dependent activities of HIV-1 RT (31). Klarman et al. have made substantially larger templates, approximately 1.6 kb in length, in order to study template-directed pausing of DNA synthesis with HIV-1 RT (16). These authors found that certain sequences of HIV-1 RNA and DNA contain strong pause and stop sites for polymerization \textit{in vitro}. This may explain why some template-primer systems are not suitable for kinetic studies of FIV RT. Klarman et al. also showed that RT dissociates from the primer template at some pause sites and remains bound at others. These data suggest that when designing endogenous templates care has to be taken to find sequences that don’t contain strong pause sites (16).
All of the previous work that has been done with endogenous template systems has focused on studies of reaction mechanisms or the fidelity and processivity of RT (16, 31). There have been no previous studies of ligands that compete with the template-primer binding site on RT. A major goal of this work is develop and characterize endogenous templates that can be used in studies of RT-targeted antivirals, including SELEX ligands.

GOALS

The goals of this study were to evaluate seven SELEX ligands as inhibitors of FIV RT and study the kinetics of inhibition by the ligands that were the most potent inhibitors.
MATERIALS AND METHODS

MATERIALS

Enzymes. Recombinant FIV RT was purified by a method that has been
developed by North, et al., (25). Wild-type and AZTTP-resistant FIV RTs were
also purified from virions by a procedure described in North, et al. (23). T7 RNA
polymerase, human placenta RNase inhibitor, and RNase-free RQ1 DNase were
purchased from Promaga, Madison WI. *Thermus aquaticus* DNA polymerase was
purchased from Perkin Elmer Cetus, Norwalk CT.

Chemicals. Diethylpyrocarbonate (DEPC), phosphonoformic acid (PFA), 2',3'-
dideoxycytidine 5'-triphosphate (ddCTP), and Triton X-100 were purchased from
Sigma Chemical CO. St Louis, MO. AZTTP, the 5'-triphosphate of AZT was
provided by Wayne Miller, of Burroughs Wellcome Co., Research Triangle Park,
NC. Poly(rA)-oligo(dT)$_{10}$, and Poly(rI)-oligo(dC)$_{12,18}$ were purchased form
Pharmacia LKB, Piscataway, N.J. [Methyl-$^3$H]dTTP, and [5',5'-$^3$H]dCTP were
obtained from Dupont-New England Nuclear, Boston MA. *Geneamp PCR* core
reagents were purchased from Perkin Elmer Cetus, Norwalk, CT. All other
chemicals were reagent grade or better.

Preparation of SELEX Ligands

RNase free environment. All reagent and equipment were treated with DEPC
or baked at 100 °C whenever possible to ensure that RNase-free materials were
used.
PCR Amplification. Amplification of DNAs expressing SELEX ligands were performed using a Perkin Elmer DNA thermocycler 410. The cassette to be amplified (Fig 5) had the following sequence; 5'-GGGAGGAUGUUUUCUCAGA-CCGUAA-N40-UUGCAGGAUCGUGAAGGUAUCCGGG-3' where N40 represents the randomized portion of the SELEX ligand. The 5'-end is primed with primer designated as CH2 (5'-CCCAGCTTAATACGACTCACTATAGGGAGGATATT-CTCAGACC-3') and the 3' end is primed with primer designated as CH3 (5'-CCCGGCTCCTAGTTCCACGTGCTGCAA-3'). Each 100 µl reaction contained 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.75 mM MgCl₂, 0.05 mg/ml bovine serum albumin, 1 mM of each dCTP, dATP, dGTP, and dTTP (dNTP), 0.25 pmoles/µl of each primer CH2 and CH3, and 0.025U/µl Taq DNA polymerase. The PCR amplification was run for 25 cycles. Each cycle consisted of 94°C for 30 seconds for denaturation, 54°C for 20 seconds for annealing, and 72°C for 90 seconds for elongation (33). The DNA from the PCR amplification was used directly for in vitro transcription.

In vitro transcription. RNA ligands were transcribed from the amplified DNA in vitro. Reactions were run in a total volume of 100 µl that contained 40 mM Tris-HCl, pH 8.0, 12 mM MgCl₂, 5 mM dithiothreitol, 1 mM spermidine, 20% W/V polyethylene glycol (MW 8000), 0.01% V/V Triton X-100, 0.2 mM each of CTP, ATP, GTP, and UTP, 20U RNase inhibitor RQ1, 100 U T7 RNA polymerase, and 25µl (approximately 700 pM) of PCR product (20). The reaction was run at 37 °C for four hours.
A. **IN VITRO CASSETTE.**

GCTAATACGACTCACTATAGGGATAUUUUCUCAGACCGUAA-N^6-UUGCAGCAUCGUAAACUAGGAUCCGG-3'
3'-AACGTCGTAGCATTGATCCTAGGCCC-5'

B. **SELECTED RNA LIGANDS (ONLY THE VARIABLE REGION SHOWN)**

F20TH #60 5'-GUACCGAAUGUCUUUGGCGAUAUUUGGCCCUCGCAG-3' Kd=1.9 nM
F20TH #34 5'-AACUUUUGUGGCUUCACGACCACCAUAUUGUGUUUGUAA-3' Kd=6.1 nM
F20TH #82 5'-CUACUUCACCCACCCGAGCAUGAUCUCAUACGCAU-3' Kd=6.0 nM
F18TH #11 5'-CACUAAGCAGAUACCGAAAGACUUGCCCGCAACACGCA-3' Kd ND
F18TH #13 5'-GCGUGGACUGUCCAGCAUGACACUUGAUAUCCAC-3' Kd ND
F18TH #53 5'-UUGCGAAGGAAAAACCGACCUCUUCGCAUCACGUA-3' Kd=4.5 nM
F18TH #66 5'-ACUAAGAAUGUGGAAUGUAGUACACGUACAAUCA-3' Kd ND

Figure 5. *In vitro* cassette, and sequence of the selected RNA ligands. A) *In vitro* cassette. Underlined at the 5'-end is a T7 promoter sequence. The 3'-end is a PCR primer sequence. B) Sequence of selected RNA ligands.
Reactions were stopped by addition of, 100 μl 7M urea, TBE [0.0045 M Tris-Borate, 0.001 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0], containing 0.05% Xylene cyanate, and 0.05% bromophenol blue, and samples were heated at 90 °C for three minutes. The samples were then loaded onto a 7M urea, 10% polyacrylamide gel and the gels were run at 600 volts for 30 minutes. RNA was located by UV shadowing. RNA was eluted from the gel with two volumes of 2mM EDTA, and one volume of 3M sodium acetate. RNA was separated from gel material by filtration through a 0.22 μm acetate syringe filter. The filter was washed with 5 volumes of 100% ethanol. Samples were frozen in liquid nitrogen for 15 minutes, then thawed and the RNA was pelleted by centrifugation at 13,000 x g for 15 minutes. The pellet was dried by a speed vacuum and resuspended in DEPC-treated water. Concentrations were calculated by the following formula: [RNA] p moles/l = (1000 x OD_{260} x dilution factor x 33)/(320 x92)). Where 1000 is the unit conversion factor to p moles of RNA, one OD unit is 33 μg/ml, 320 is average molecular weight of a nucleotide and 92 is the length of the RNA ligand.

Preparation of RNA and DNA templates

PCR amplification. For preparation of DNA and RNA templates, the PCR reagents were the same as for SELEX PCR amplifications, except that different primers and DNA templates were used. These templates were made from the FIV molecular clone, 34TF10, which contains a cloned provirus of FIV inserted into the plasmid pUC119 (44). In order to enable synthesis of the RNA template
a primer designated as 24' with the following sequence was used; 5'-CCCAGCTTAATACGACTCACTATAGGAGTCC-AGAAGATAAATTACAGG-3'. This primer contains the promoter for T7 RNA polymerase (underlined), which allowed \textit{in vitro} transcription of the PCR product. The other primer used, designated as #14 was (5'-GGGGTCATAGTATCCTAGTTG-3'). These primers amplified a 316 (corresponding to nt. 2979-3295) base-pair region of the RT encoding region (44).

\textit{In vitro} transcription of RNA template. \textit{In vitro} transcriptions were performed under the same conditions as for \textit{in vitro} transcription of SELEX ligands.

\textbf{Gel purification of RNA template.} Gel purification of the RNA template was the same as used for the purification of SELEX ligands.

\textbf{Asymmetric PCR amplification for the preparation of DNA template.} To prime the 5' end of the DNA a primer designated as #24 (5'-CTCCAGAAGATAAATTACAGG-3') was used. Primer #14 concentration was 1/50 that of #24 in order to preferentially amplify only one strand.

\textbf{Single stranded DNA purification.} DNA from asymmetric PCR was purified by QIAquick spin PCR purification kit (Qiagen, Chatsworth, CA.). This procedure allows rapid purification with a 90% yield of single stranded or double stranded DNA PCR products.

\textbf{Primer annealing.} DNA and RNA templates to be used in RT assays, were
primed with PCR primer #14. This was done using a Perkin Elmer DNA thermocycler 410. The primer was added in a 2:1 molar ratio to the respective templates. The template primer mix was then heated at the following temperatures; 90 °C for 30 seconds, 60 °C for 15 minutes, 37 °C for 15 minutes, 25 °C for 15 minutes and 4 °C for 15 minutes. All template-primers were stored at -20 °C until used.

**RT assays.** RT was assayed essentially as described by North et al (23, 25). Reactions were carried out in a volume of 50 µl which contained 50 mM Tris-HCl, pH 8.5, 10 mM dithiothreitol, 0.05% Triton X-100, 250 µg of bovine serum albumin (nuclease free) per ml, 6 mM MgCl₂, 80 mM KCl, 20 µM [Methyl-³H]dTTP (used in reactions with 0.5 A₂₆₀ U with poly(rA)-oligo(dT)₁₀, endogenous DNA or RNA template-primers), or [5'-5-³H]dCTP (used in reactions with 0.5 A₂₆₀ U poly(rI)-oligo(dC)₁₀·₁₈] and approximately 5 U of RT enzyme. Aliquots of 40 µl were taken at the indicated times and spotted onto filters (no. 3, 2.3-cm diameter; Whatman, Inc., Clifton N.J.) that had been presoaked with 5% trichloroacetic acid-1% sodium pyrophosphate. Filters were then washed four times, at least 1 hour each, in ice cold 5% trichloroacetic acid-1% sodium pyrophosphate and once in 95 % ethanol, dried and counted in liquiflour (Dupont NEN research products, Boston MA). Kinetic parameters (Kₘ and Kᵢ) were determined in reactions with [dTTP] varied from 2.0 to 20 µM and [template] varied from 0.5 to 0.012 A₂₆₀ U. Intercept values calculated from double reciprocal plot were used to obtain kinetic parameters (22, 24). Values reported are averages from at least duplicate determinations.
RESULTS

Initial screening of the SELEX ligands. SELEX ligands were prepared by \textit{in vitro} transcription and gel purification as described in the Material and Methods section. The ligands were each tested for the ability to inhibit FIV RT, and Ki values were determined with variable concentrations of dTTP as substrate, poly(rA)-oligo(dT)$_{10}$ as a template-primer, and purified recombinant FIV RT as enzyme (Table 3). Two ligands F20 #60 and F18 #53 were potent inhibitors of FIV RT with Ki values of 108 and 89 nM, respectively. Their structures are shown in Figure 5. The other five ligands were less inhibitory and had Ki values greater than 1000 nM. A Lineweaver-Burke plot for inhibition of recombinant FIV RT by F18 #53 with variable dTTP is shown in Figure 6. All ligands tested were similar in that they were noncompetitive inhibitors of RT with respect to dTTP.

**TABLE 3. Ki DETERMINATIONS FOR SELEX LIGANDS***

<table>
<thead>
<tr>
<th>SELEX LIGAND</th>
<th>Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F20 #60</td>
<td>107 ± 18</td>
</tr>
<tr>
<td>F20 #82</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>F18 #11</td>
<td>&gt;1200</td>
</tr>
<tr>
<td>F18 #13</td>
<td>&gt;3200</td>
</tr>
<tr>
<td>F18 #53</td>
<td>89 ± 18</td>
</tr>
<tr>
<td>F18 #66</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>F18 #39</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

* All experiments were performed with poly(rA)-oligo(dT)$_{10}$ used as the template-primer. Each value represents a mean Ki value with 6 determinations per value.
Figure 6. Lineweaver-Burke Plot for inhibition of Recombinant FIV RT by SELEX ligand F18 #53. Concentration of F18 #53 were 200 nM (▼) 100 nM (▲) 50 (■) and no inhibitor (●).
**SELEX specificity.** All seven SELEX ligands were specific for FIV RT. They did not inhibit HIV-1 RT (data not shown), and did not bind to AMV and MMLV RTs (3).

**Inhibition of FIV RT by SELEX ligand F18 #53.** The SELEX ligand that had the lowest Ki value, F18 #53 (#53) was further characterized using three different FIV RTs. These were the purified recombinant RT (which was the enzyme the ligand was selected with), the purified RT from virions of wild-type FIV, and the RT from a AZT-resistant FIV, designated as virion-AZR-17c (33). Reactions were carried out with variable template-primer [poly(rA)-oligo(dT)\textsubscript{10}] and variable dTTP (Table 4). Competitive inhibition was seen with variable concentration of template-primer, and noncompetitive inhibition was observed with variable concentration of dTTP (Figure 7). Interestingly, the ligand #53 inhibited both wild-type RT and AZR-17C, (Ki values of 37 and 44 nM respectively) better than the recombinant RT (Ki value of 89 nM). It is important to note that there was no appreciable difference, between the AZTTP-resistant RT from AZR-17c and wild-type RT in susceptibility to this SELEX ligand.

**Inhibition of FIV RT by SELEX ligand F20 #60.** Ligand F20 #60 (#60) inhibited purified recombinant FIV RT with a Ki comparable to that of #53. This ligand was further characterized using variable template-primer (poly(rA)-oligo(dT)\textsubscript{10} and purified wild-type-virion FIV RT (Table 5).
Figure 7. Lineweaver-Burke plots showing modes of inhibition using SELEX ligand #53. Concentrations of #53 were 200 nM, (▲) 50 nM (●) and no #53 (●).

A) Noncompetitive inhibition with variable dTTP concentration and recombinant FIV RT.  
B) Competitive inhibition with variable poly(rA)-oligo(dT)$_{10}$ concentration and recombinant FIV RT.  
C) Noncompetitive inhibition with variable dTTP concentration and wild-type-virion FIV RT.  
D) Competitive inhibition with variable poly(rA)-oligo(dT)$_{10}$ concentration and wild-type-virion FIV RT.
TABLE 4.

**KI DETERMINATIONS FOR INHIBITION OF FIV-RT BY SELEX LIGAND #53***

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>VARIABLE</th>
<th>Ki (nM) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant FIV RT</td>
<td>dTTP</td>
<td>89 ± 18</td>
</tr>
<tr>
<td>Recombinant FIV RT</td>
<td>poly(rA)-oligo(dT)₁₀</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>Virion-wild-type FIV RT</td>
<td>dTTP</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>Virion-wild-type FIV RT</td>
<td>poly(rA)-oligo(dT)₁₀</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Virion-AZR-17c RT</td>
<td>dTTP</td>
<td>44 ± 4</td>
</tr>
<tr>
<td>Virion-AZR-17c RT</td>
<td>poly(rA)-oligo(dT)₁₀</td>
<td>26 ± 7</td>
</tr>
</tbody>
</table>

*All reactions were run with poly(rA)-oligo(dT)₁₀ as a template-primer. Each value represents a mean Ki value with six determinations per value.

The mode of inhibition is competitive with variable template concentration and noncompetitive with variable nucleotide concentration (data not shown). In contrast to #53, #60 inhibited virion-wild-type FIV RT about three fold less than recombinant FIV RT. Due to the poor inhibition of virion-derived RT, #60 was not further characterized.

In order to further characterize #53, because of the competitive type of inhibition with template-primer substrate, a more biologically relevant template-primer was needed.
TABLE 5.

**Ki DETERMINATIONS FOR INHIBITION OF FIV-RT BY SELEX LIGAND #60***

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>VARIABLE</th>
<th>Ki (nM) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant FIV RT</td>
<td>dTTP</td>
<td>107 ± 18</td>
</tr>
<tr>
<td>Recombinant FIV RT</td>
<td>poly(rA)-oligo(dT)₁₀</td>
<td>120 ± 7</td>
</tr>
<tr>
<td>Virion-wild-type FIV RT</td>
<td>dTTP</td>
<td>334 ± 4</td>
</tr>
</tbody>
</table>

* All reactions were run with poly(rA)-oligo(dT)₁₀ as a template-primer. Each value represents a mean Ki from six determinations.

**Construction of an endogenous RNA Template.** The RNA template was made by amplification of a region of the FIV genome from a plasmid containing 34TF10 which has the FIV provirus cloned into pUC119 (44). Primers that amplify a 316 base-pair portion, bases 2979-3295, of the RT-encoding region were used. A T7 RNA polymerase promoter sequence was added at the 5-end of the forward primer (24') to enable *in vitro* transcription of the PCR product (Fig 8). This provided an RNA template that could be used for RT assays. The primer used for RT assays with this template was primer #14 which was used for PCR amplification in the reverse direction.

Under the conditions stated in the Materials and Methods section ("*in vitro* transcription of RNA templates") approximately 2680 nmoles of RNA were
recovered. The RNA template was then primed with primer #14 (Fig 8) at various primer-template molar ratios to find the optimal conditions for RT activity. A template:primer ratio of 1:7 gave maximal RT activity (Fig 9 A). This template:primer ratio was used in all subsequent RT the assays.

**Construction of the DNA Template.** The DNA template was constructed by asymmetric PCR as described in Materials and Methods. A typical yield was 2500 nmoles. This template was primed with the PCR primer #14 (Fig 8) and the optimal template to primer molar ratio for RT activity was determined to be 1:9 (Fig 9 B).

**Characterization of reactions with endogenous RNA templates.** The velocity of FIV RT with the endogenous RNA template was first compared to that with poly (rA)-oligo(dT)\textsubscript{10}, the standard template primer used for FIV RT assays. The velocity of FIV RT with the endogenous RNA template was 100 % that of poly(rA)-oligo(dT)\textsubscript{10} template (Table 6).

The endogenous RNA template was characterized with AZTTP, ddCTP, PFA, and ddTTP as inhibitors in RT assays, as described in the Materials and Methods section. With AZTTP, ddCTP, and ddTTP the mode of inhibition was competitive with respect
1. T7 polymerase binding sequence (underlined).

5'-CCCAAGCTTAATACGACTCACTATAGGGAG-3' (25-MER)
3'-GGGTTCGAATTATGCTGAGTGATATCCCTC-5' (TEMPLATE)

2. Sense strand designated as #24.

5'-CTCCACAGATAAATTACAGG-3' (21 MER)

3. Primer 24' with the T7 polymerase binding sequence.

5'-CCCAAGCTTAATACGACTCACTATAGGGAGCTCCACAGATAAATTACAGG-3' (46 MER)

4. An overview of events.

(parent) 5'-
3'-CAACTAGGATACTATGACCC

(primer #14) 5'-
3'-GTTGATCTATGATACCTGGG

5'-CCCAAGCTTAATACGACTCACTATAGGGAGCTCCACAGATAAATTACAGG (primer 24')
3'-GAGGTATCTATTTAATGTGG-GTTGATCTATGATACCTGGG

Sequence after PCR

5'-CCCAAGCTTAATACGACTCACTATAGGGAGCTCCACAGATAAATTACAGG-CAACTAGGATACTATGACCC-3'
3'-GGGTTCGAATTATGCTGAGTGATATCCCTAGAGGTATCTATTTAATGTGG-GTTGATCTATGATACCTGGG-5'

Fig 8. Design of the genomic FIV RNA template. 1) represents the promoter sequence for T7 polymerase (underlined). 2) represents the primer (24) 5’-end of the amplified sequence. 3) represents the sequence of primer 24 with the T7 promoter sequence ligated to its 5’-end (24’). 4) is an overview of events during PCR amplification.
Fig 9. Effect of variable template:primer ratios on activity of RT with endogenous templates. A) Reactions with the RNA template B) Reactions with the DNA template.
to dNTP substrate, as with other homopolymeric template-primers (Fig 10). The mode of inhibition for PFA was noncompetitive, as it is with homopolymeric template-primers (Fig 10)(24).

The Michaelis Menton constant, $K_m$ for substrate (dTTP) was $2.0 \pm 0.1 \mu$M. The inhibition constants, $K_i$ values, for AZTTP, ddTTP, and ddCTP are summarized in Table 7. The $K_i$ values were between four-and five-fold higher than in reactions with homopolymeric RNA template-primers (24).

**Characterization of reactions with the DNA template.** The velocity of recombinant FIV RT with the single stranded DNA template-primer was only 13% of that obtained with poly(rA)-oligo(dT)$_{10}$ (Table 6). These data demonstrate that RT has a higher activity with the RNA than with a DNA template, at least with this nucleotide sequence. The DNA and RNA endogenous templates had the same primer and sequence (except U vs. T in RNA vs. DNA).

The inhibitors used to characterize the DNA template were AZTTP, ddCTP, ddTTP, and PFA. As found with other template-primers, the mode of inhibition for AZTTP, ddCTP, and ddTTP is competitive with respect to dNTP substrate (Fig 11). The mode of inhibition for PFA is noncompetitive with respect to dNTP for the endogenous DNA template (Fig 11).
### TABLE 6. TEMPLATE SPECIFICITIES OF RT ACTIVITIES

<table>
<thead>
<tr>
<th>TEMPLATE-PRIMER</th>
<th>PERCENT ACTIVITIES*</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly(rA)-oligo(dT)$_{10}$</td>
<td>100</td>
</tr>
<tr>
<td>poly(rC)-oligo(dG)$_{10}$</td>
<td>62.2</td>
</tr>
<tr>
<td>Endogenous RNA Template</td>
<td>100</td>
</tr>
<tr>
<td>Endogenous DNA Template</td>
<td>13.2</td>
</tr>
</tbody>
</table>

*100% is 4 pmoles of [³H]-dTTP incorporated per hour. Values represent the mean from two or more determinations. Recombinant FIV RT was used in each reaction.

### TABLE 7. INHIBITION CONSTANTS FOR RT INHIBITORS IN REACTIONS WITH ENDOGENOUS TEMPLATES.

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>RNA TEMPLATE</th>
<th>DNA TEMPLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZTTP</td>
<td>56.1 ± 9.0</td>
<td>90.1 ± 5</td>
</tr>
<tr>
<td>ddCTP</td>
<td>23.3 ± 5</td>
<td>58.7 ± 6</td>
</tr>
<tr>
<td>ddTTP</td>
<td>30.1 ± 0.5</td>
<td>21.5 ± 0.5</td>
</tr>
<tr>
<td>PFA</td>
<td>1485 ± 15</td>
<td>709 ± 15</td>
</tr>
</tbody>
</table>

* Each Ki value represents the mean value from at least six determinations. Recombinant FIV RT was used in each reaction.
Figure 10  Lineweaver-Burke plots for the inhibition of recombinant FIV RT by nucleotide analogues and PFA in reactions with the endogenous RNA template.

A) Competitive inhibition by AZTTP: 1000 nM AZTTP (△), 500 nM AZTTP (▼), 100 nM AZTTP (■) no AZTTP (●).

B) Competitive inhibition by ddCTP: 200 nM ddCTP (▼), 100 nM ddCTP (△), 50 nM ddCTP (■) no ddCTP (●).

C) Competitive inhibition with ddTTP: 500 nM ddTTP (▼), 100 nM ddTTP (△), 50 nM ddTTP (■) no ddTTP (●).

D) Noncompetitive inhibition with PFA: 25 μM PFA (▼), 10 μM PFA (△), 5 μM PFA (■) no PFA (●).
A

B

C

D

1/(CPM) (Times 10^5)

1/[dTTP] (μM)

1/(CPM) (Times 10^5)

1/[dTTP] (μM)
Figure 11. Lineweaver-Burke plots for inhibition of the recombinant FIV RT by nucleotide analogues and PFA in reactions with the endogenous DNA template. 

A) Competitive inhibition by AZTTP: 1000 nM AZTTP (▼), 500 nM AZTTP (▲), 100 nM AZTTP (■) no AZTTP (●). B) Competitive inhibition by ddCTP: 200 nM ddCTP (▼), 100 nM ddCTP (▲), 50 nM ddCTP (■) no ddCTP (●) C) Competitive inhibition with ddTTP: 500 nM ddTTP (▼), 100 nM ddTTP (▲), 50 nM ddTTP (■) no ddTTP (●). D) Noncompetitive inhibition with PFA: 25 μM PFA (▼), 10 μM PFA (▲), 5 μM PFA (■) no PFA (●).
The Michaelis Menton constant, $K_m$ for the endogenous DNA template was $2.0 \pm 0.1 \mu M$. The inhibition constants, $K_i$, for AZTTP, ddCTP, and ddTTP are four to five fold higher than with other DNA template-primers (Table 7) (24).

**Inhibition of recombinant FIV RT by SELEX ligand #53 in reaction with endogenous DNA and RNA template-primers.** SELEX ligand #53 was further characterized with the endogenous DNA and RNA template-primers. The $K_i$ values for inhibition of FIV RT by #53 in reactions with those template-primers are summarized in Table 8. Interestingly, #53 inhibited RT better with the endogenous templates (45 nM) than with to the homopolymeric templates (95 nM)(Table 4). The modes of inhibition were the same as seen with the homopolymeric templates, noncompetitive with respect to dTTP, and competitive with respect to template-primer (Fig 12).

**TABLE 8. INHIBITION CONSTANTS FOR LIGAND #53 IN REACTIONS WITH ENDOGENOUS DNA AND RNA TEMPLATES.**

<table>
<thead>
<tr>
<th>TEMPLATE</th>
<th>VARIABLE</th>
<th>$K_i$(nM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENDOGENOUS RNA</td>
<td>ENDOGENOUS RNA</td>
<td>45 ± 10</td>
</tr>
<tr>
<td>ENDOGENOUS RNA</td>
<td>dTTP</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>ENDOGENOUS DNA</td>
<td>ENDOGENOUS DNA</td>
<td>49 ± 8</td>
</tr>
<tr>
<td>ENDOGENOUS DNA</td>
<td>dTTP</td>
<td>35 ± 10</td>
</tr>
</tbody>
</table>

* Values represent mean from six or more determinations per value.
Figure 12. Lineweaver-Burke plots for inhibition of recombinant FIV RT by SELEX #53 in reactions with endogenous RNA and DNA templates. Concentrations were 200 nM #53 (▼), 100 nM #53 (▲), 50 nM #53 (■), and no #53 (●). A) Competitive inhibition in reactions with variable RNA template. B) Noncompetitive inhibition in reactions with variable dTTP and endogenous RNA template. C) Competitive inhibition in reactions with variable DNA template. D) Noncompetitive inhibition in reactions with variable dTTP and endogenous DNA template.
DISCUSSION

This study is the first kinetic characterization of the inhibition of RT by SELEX ligands. It has identified SELEX ligands that are potent inhibitors of purified FIV RT. Of seven SELEX ligands that were tested, only two ligands, #53, and #60, were found to be potent inhibitors of FIV RT. These ligands are comparable in potency to nucleotide inhibitors of FIV RT. AZTTP has a Ki of 54 nM using recombinant FIV RT, and the endogenous DNA template-primer where SELEX ligand #53 has a Ki of 45 under the same conditions.

These FIV-RT specific ligands, like the HIV-1 RT-specific RNA ligands reported by Tuerk et al. (47), were selected in such a way that facilitated but did not demand a pseudoknot structure. The pseudoknot structure is predicted for the FIV, and HIV-1 template-primer binding site (4). Interestingly, none of the FIV RT-specific SELEX ligands formed a psuedoknot. It is possible that some RNA molecules with pseudoknot structures are in the selected pool; however, the predominant ligands did not form pseudoknots. The two SELEX ligands that were identified as potent inhibitors fell into different classes based upon structure. Ligand #60 has a Class I structure which forms a stem-loop with an internal bulge. #53 has a class II structure, and also forms a stem-loop with internal bulge, and contains two U-tracts in the region which is predicted to be single stranded. Ligands #53, and #60, share the ability to form a stem-loop with an internal bulge. This structure may be important for inhibition of FIV RT.
The other five SELEX ligands were much less inhibitory to FIV RT than #53 and #60. Interestingly some of the dissociation constants of the other five ligands are lower than for #53. These data suggest that there is no correlation between dissociation constants and inhibition constants. All ligands were noncompetitive with respect to nucleotide concentration suggesting that they bind to RT at places other than the nucleotide binding site. Because SELEX ligands are oligonucleotides, it is not surprising that they compete with the template-primer, and that they are noncompetitive with respect to nucleotide concentration. These data (Fig 7, and Table 4) suggest that the #53 and #60 bind to the template-primer site.

All previous kinetic studies of RT with RNA templates, and initial parts of this work, were done with homopolymer templates. Homopolymeric templates are adequate for studies with nucleotide analogues which compete with dNTP substrate. However, the SELEX ligands compete with template-primer. Therefore it was desirable to study more biologically relevant, natural template-primers for further characterization of the ligands.

The templates that were developed included DNA and RNA templates corresponding to a 316 base-pair fragment of the FIV genome. With these templates, inhibition of FIV RT by nucleotide analogues and by PFA yielded Ki values similar to those obtained in reactions with other heteropolymeric DNA
templates previously used for kinetic characterization of RT inhibitors (6, 25). However, the RT activity with heteropolymeric RNA templates used in these studies was too low for kinetic analyses. RT activity with the endogenous DNA template-primer used in this study was similar to activity previously reported with φX174 and M13 DNA (6).

A few studies of HIV-1 RT have used endogenous templates, but those studies were focused on aspects of RT other than kinetics of inhibition by antivirals. Reardon (31) used short template-primers to study the kinetic mechanism of the RT. These were approximately 50 bases long, primed with a ten base oligonucleotide. Other studies with endogenous template-primers were used in order to study the fidelity of RT (16). These studies used long templates, approximately 2000-3000 bases in length, in order to study pause sites and stop sites in the HIV-1 genome (16). The work presented in this thesis is the first study of inhibition of RT by antivirals with endogenous templates.

Interestingly, SELEX ligand #53 was a more potent inhibitor of FIV RT in reactions with endogenous templates than with homopolymeric templates. Inhibition constants from reactions with endogenous templates were about half of those obtained with the homopolymeric templates. Thus, SELEX ligand #53 should be an even better inhibitor of viral DNA synthesis than was predicted with the initial studies with homopolymers. The characterization of SELEX
ligands with endogenous templates gives insight into how well the ligand may inhibit FIV replication in cells. However, the template used represents only a small portion of the FIV genome and the reaction with RT may vary considerably with sequences from other parts of the genome. Klarman et al. (16) observed strong pause and stop sites in the HIV-1 genome, and one can expect similar pause and stop sites in different sequences of the FIV genome. These sites will affect the enzyme velocity and may alter inhibition kinetics of SELEX ligands. It will be important to characterize activities of these inhibitors with template-primer from other parts of the FIV genome.

RT purified from virions of wild-type and AZT-resistant FIV were both more sensitive than recombinant FIV RT to inhibition by SELEX ligand #53. This is surprising because the ligand was selected with the recombinant RT. We do not know the reason for this but it may be due to modifications that occur during virus replication that do not occur in E. coli.

AZR-17c contains a point mutation at nucleotide 2939 that results in an amino acid change of a Glu to a Lys at amino acid 202 (33). This mutation in the FIV RT decreases sensitivity to the active form of AZT, AZTTP. However, this enzyme remains fully sensitive to inhibition by SELEX ligand #53. This is not surprising in view of the fact that the mutation responsible for AZT resistance is in a portion of the RT molecule located below the palm and away
from the template-primer binding site (as predicted from the crystal structure of HIV-1 RT and homology of FIV RT to the HIV-1 RT) (18, 44). It will be interesting to analyze other drug-resistant mutants, such as those resistant to ddi or ddC, which have mutations in the template-primer binding domain (2) with SELEX ligands.

A crucial step in developing SELEX ligands as drugs to use in patients will be providing a means of delivery. There are several possible ways that an RNA ligand can be delivered. These include modification of the RNA ligand to enable cellular uptake and protection from RNases, liposome delivery, and gene therapy. A problem in RNA modification is the likelihood of structural changes that may inactivate the ligand. Liposome delivery is limited by packaging problems, difficulties in mass production, instability, and problems in selective delivery (39). A gene therapy approach seems most promising because in recent years it has been used for the treatment of adenosine deaminase deficiency with some success (19). This success was achieved using virus vectors for delivery and has promoted our collaboration with NeXstar, to develop a Crandell feline kidney cell (CrFK), which has the DNA sequence to encode ligand #53 inserted behind strong pol III and pol II promoters within the vector genome.
Figure 13. Preliminary data on antiviral activity of SELEX ligand #53 expressed in CrFK cells. On the X-axis form left to right; CrFK (no ligand), FRT1-1, 1-2, 1-5, 1-7, 1-9 are all CrFk cell lines that express #53 as determined by northern blot analysis (data not shown). (preliminary results from LaCasse, Zhu and North).
In collaboration with Michael Lochrie of NeXstar we have constructed 36 CrFK cell lines which express ligand #53. Our laboratory has recently shown that some of these cell are highly protected (99%) from FIV infection (Fig 13). Our lab is continuing to characterize these cell lines and the antiviral state of cells expressing the SELEX ligand.

It will be important to use these cell lines that are resistant to infection by FIV to determine whether mutant FIV that are resistant to SELEX ligand #53 will arise. We predict that resistance will be less frequent than with nucleoside analogues because of the multiple interaction sites of the ligand with the enzyme.

If resistance to this ligand does arise, SELEX offers an immediate strategy to combat it. If a SELEX #53-resistant virus is found, the RT can be purified and then used for the selection of another SELEX ligand. By alternating the resistant enzyme and the wild-type enzyme it is conceivable that one can select a ligand that inhibits both the mutant and the wild-type RT. If further resistance arises additional ligands can be selected by SELEX. A long term goal will be to develop a ligand or a set of ligands that will protect against wild-type FIV and the major resistant mutants.

Results of this study clearly demonstrate that potent inhibitors of FIV RT can be obtained via the SELEX procedure. The preliminary studies mentioned above demonstrate that expression of these ligands in cultured feline cells protects against FIV infection. SELEX offers a promising approach to AIDS treatment with gene therapy that can be tested in cats.
REFERENCES


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