Discovery of a Small Molecule Tat-trans-Activation-responsive RNA Antagonist That Potently Inhibits Human Immunodeficiency Virus-1 Replication*

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Antiretroviral therapy to treat AIDS uses molecules that target the reverse transcriptase and protease enzymes of human immunodeficiency virus, type 1 (HIV-1). A major problem associated with these treatments, however, is the emergence of drug-resistant strains. Thus, there is a compelling need to find drugs against other viral targets. One such target is the interaction between Tat, an HIV-1 regulatory protein essential for viral replication, and trans-activation-responsive (TAR) RNA. Here we describe the design and synthesis of an encoded combinatorial library containing 39,304 unnatural small molecules. Using a rapid high through-put screening technology, we identified 59 compounds. Structure-activity relationship studies led to the synthesis of 19 compounds that bind TAR RNA with high affinities. In the presence of a representative Tat-TAR inhibitor (5 μM TR87), we observed potent and sustained suppression of HIV replication in cultured cells over 24 days. The same concentration of this inhibitor did not exhibit any toxicity in cell cultures or in mice. TR87 was also shown to specifically disrupt Tat-TAR binding in vitro and inhibit Tat-mediated transcriptional activation in vitro and in vivo, providing a strong correlation between its activities and inhibition of HIV-1 replication. These results provide a structural scaffold for further development of new drugs, alone or in combination with other drugs, for treatment of HIV-1-infected individuals. Our results also suggest a general strategy for discovering pharmacophores targeting RNA structures that are essential in progression of other infectious, inflammatory, and genetic diseases.

To date the United States Food and Drug Administration has approved only 16 anti-HIV1 drugs. These anti-HIV agents fall into three broad categories: 1) nucleoside inhibitors of reverse transcriptase, 2) non-nucleoside reverse transcriptase inhibitors, and 3) protease inhibitors. Although the use of multidrug cocktails has significantly reduced age-adjusted death rates from AIDS in developed countries, AIDS therapies still face many constraints, including some inadequate therapeutic responses and frequent intolerable drug toxicity. Another serious drawback of these therapies is that all of the current drugs induce single-drug- or multidrug-resistant mutant viruses. In view of these realities, there is a compelling need to find new drugs and/or treatment strategies that are not limited to targeting HIV reverse transcriptase and protease.

HIV-1 is a complex retrovirus that encodes six regulatory proteins, including Tat and Rev, essential for viral replication. Inhibition of Tat and Rev functions provides attractive targets for new antiviral therapies. Both Tat and Rev are RNA-binding proteins that require specific interactions with RNA structures called TAR (for trans-activation-responsive) and RRE (for Rev response element), respectively, for their functions.

After the integration step in the HIV life cycle, the HIV proviral genome is transcribed by human cellular machinery, including RNA polymerase II and other transcription accessory factors. Transcription from the HIV-1 long terminal repeat (LTR) promoter is a complex process and is elegantly regulated at the elongation stage of transcription (Fig. 1). HIV-1 encodes a transcriptional activator protein, Tat, which is expressed early in the viral life cycle and is essential for viral gene expression, replication, and pathogenesis (for reviews, see Refs. 1–6). Tat enhances the processivity of RNA polymerase II elongation complexes that initiate in the HIV LTR region (Fig. 1). Mutational analysis of HIV-1 Tat protein has identified two important functional domains: an arginine-rich region that is required for binding to TAR RNA, and an activation domain that mediates its interactions with cellular machinery (7, 8).

Recent studies showed that Tat transactivation function is mediated by a nuclear Tat-associated kinase (TAK) (1, 2, 4). The transactivation domain of Tat interacts with TAK (9, 10), which was recently shown to be identical to the kinase subunit of the positive transcription elongation factor complex, P-TEFB (11, 12). Tat interacts with the cyclin T1 (CycT1) subunit of P-TEFB and recruits the kinase complex to TAR RNA. Recruitment of P-TEFB to TAR has been proposed to be both necessary and sufficient to activate transcription elongation from the HIV LTR promoter.

The abbreviations used are: HIV, human immunodeficiency virus; TAR, trans-activation-responsive; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; LTR, long terminal repeat; Fmoc, N-fluorenylmethyloxycarbonyl; DCM, dichloromethane; DCM, dichloromethane; DMF, N,N-dimethylformamide; HOBT, 1-hydroxybenzotriazole; DIPC, N,N-diisopropylcarbodiimide; HF, Hewlett-Packard; TAK, Tat-associated kinase; GC/ECD, electron capture gas chromatography; μ-ECD, microelectron capture detector; FRET, fluorescence resonance energy transfer.
HIV-1 long terminal repeat promoter (13). Recent fluorescence resonance energy transfer studies, using fluorescein-labeled TAR RNA and a rhodamine-labeled Tat protein, showed that CycT1 remodels the structure of Tat to enhance its affinity for TAR RNA, and that TAR RNA further enhances interaction between Tat and CycT1 (14).

HIV-1 Tat protein acts by binding to the TAR RNA element, a 59-base stem-loop structure located at the 5’ ends of all nascent HIV-1 transcripts (15). TAR RNA contains a 6-nucleotide (nt) loop and a 3-nt pyrimidine bulge that separates two helical stem regions (16). TAR RNA was originally localized to nucleotides 1–80 within the viral LTR region (17). Subsequent deletion studies have established that the region from nucleotide 19 to 42 incorporates the minimal domain that is both necessary and sufficient for Tat responsiveness in vivo (18).

RNA-protein interactions are involved in many cellular functions including transcription, RNA splicing, and translation. In addition to its primary structure, RNA has the ability to fold into complex tertiary structures consisting of such local motifs as loops, bulges, pseudoknots, and turns (19, 20). Because RNA-RNA and protein-RNA interactions can be important in viral and microbial disease progression, it would be advantageous to have a general method for rapidly identifying synthetic compounds that target specific RNA structures (for recent reviews, see Refs. 21–23). A particular protein-binding RNA structure can be considered as a molecular receptor not only for the protein with which it interacts but also for synthetic compounds, which may prove to be antagonists of the protein-RNA interaction. Tat-TAR interactions provide an ideal target to test this concept of developing drugs that target specific RNA structures. Consequently, a number of innovative strategies including synthetic, computational, and structural design methods have been successfully employed in identifying Tat-TAR inhibitors (24–34).

Here we have used an encoded combinatorial library approach to discover small molecules that target TAR RNA in vitro and inhibit HIV-1 replication. We synthesized an encoded unnatural small molecule combinatorial library of 39,304 possible members by using solid-phase chemistry. Using on-bead screening technology, we identified 59 ligands that bind TAR RNA. Based on structural analysis, two parallel libraries were synthesized and the binding affinities of these unique molecules for TAR RNA were determined. Finally, 19 compounds (K<sub>d</sub>, less than 200 nM for TAR RNA) were synthesized on a large scale and tested for inhibition of HIV-1 replication. We have identified a set of molecules that are effective inhibitors of HIV-1 replication by disrupting Tat-TAR interactions, providing a new scaffold for further development of antiviral drugs.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Photocleavable tags were synthesized as previously described by Still and colleagues (35). RNA phosphoramidites were purchased from Glen Research (Sterling, VA). TentaGel S-NH<sub>2</sub> resin (34 mg, 0.18–0.22 mmol/g) was purchased from Peptides International (Louisville, KY). All other reagents were obtained from Aldrich.

**Monomer Synthesis**

Structures and labels of the 34 monomers used to synthesize the library are shown in Fig. 2. Carbamate monomers, 31–34, were synthesized from 31-CHCl<sub>3</sub>/H<sub>2</sub>, 70 °C, 10 ml (550 mg). One or two beads were placed in a test tube and dried under a stream of nitrogen. The red pellet was desilylated in 3% piperidine/DMF for 1 h at room temperature.

**Preparation of Encoded Combinatorial Library**

**Encoding Step**—TentaGel S-NH<sub>2</sub> resin (34 mg) was divided into 34 equal portions, and each reaction vessel was individually reacted with specific binary tags for 20 min at room temperature. After solvent filtration, 1-hydroxybenzotriazole (HOBT; 2.5 mg, 500 μmol) and the corresponding binary tag compounds (440 μl of 2 mM solution in DMF) were added to the swelled resin in 5% N,N-dimethylformamide (DMF)/DMF (6 ml) and stirred for 20 min at room temperature. N,N′-diisopropylcarbodiimide (DIPDCI; 2.8 μl, 44 μmol) was added to this reaction suspension and stirred overnight at room temperature.

**Unnatural Amino Acid Coupling**—The encoding step was monitored by electron capture gas chromatography (GC/ECD). A Hewlett-Packard (HP) 6890 series gas chromatography system, equipped with a micro-electron capture detector (μ-ECD), and a HP Chemstation operating system were used for all decoding analyses. The GC was operated in splitless inlet mode, using helium as carrier gas. A HP Ultra 1 column (35 mm × 0.2 mm inner diameter × 0.33 μm film thickness) was used with a temperature program of 1 min isothermal at 200 °C, followed by heating at 20 °C/min to 320 °C. The μ-ECD make-up gas was nitrogen.

If a GC/ECD test was positive for encoding, the encoded resin was suspended in 5% DMF/DMF (6 ml), stirred for 20 min at room temperature, and HOBT (30 mg, 550 μmol) and the corresponding monomer (550 μmol) were added. After stirring the resulting reaction mixture for 20 min at room temperature, 1,3-diisopropylcarbodiimide (86 μl, 550 μmol) was added, and the mixture stirred overnight at room temperature. The reaction resin was washed with DMF (10 ml × 5 min × 3) and DCM (10 ml × 5 min × 2), and one or two beads were placed in a test tube to monitor free amino groups by Kaiser test.

**Carbamate Coupling**—The encoded resin was suspended in 5% DMF/DMF (6 ml), stirred for 20 min at room temperature, and the corresponding carbamate monomer was added, and the resulting mixture stirred for 20 min at room temperature. N,N′-diisopropylcarbodiimide (250 μl) was added, and the mixture was stirred overnight at room temperature.

**Peptoid Coupling**—The encoded resin was suspended in 5% DMF/DMF (6 ml), stirred for 20 min at room temperature, and bromoacetic acid (1 ml, 2.2 m in DMF) and 1 N HOBT (1 ml) were added. The resulting reaction mixture was stirred for 20 min at room temperature, and DIPDCI (200 μl) was added, and the mixture stirred overnight at room temperature. The reaction mixture was washed with DCM (10 ml × 2), and 1 or 2 beads were placed in a test tube for Kaiser test. The bromoacetyl resin was suspended in DMF (4 ml), the corresponding primary amine (3 ml, 3 mM solution in DMF) was added, and the resulting mixture stirred overnight at room temperature.

**Fmoc Deprotection**—After the first coupling cycle, the individual reaction products were combined and treated with 5% piperidine/DMF for 10 min at room temperature, the solvent drained, and the resin was treated with 5% piperidine/DMF for another 20 min at room temperature. The resin was extensively washed (with DMF, MeOH, and DCM), dried under reduced pressure, and split into 34 portions by weight. A mixture of six binary tags was used to encode the 34 reaction vessels were reacted with the corresponding monomers as described above, and pooled for Fmoc deprotection. After deprotection, the combined resin was washed extensively (with DMF, MeOH, and DCM), and a third split synthesis was carried out.

**Deprotection of Side Chain Functional Groups**—The trimmer resin (3 g) was treated with methylene chloride (10 ml), thianisole (2 ml), ethanedithiol (0.65 μl), and trifluoroacetic acid (10 ml) for 1 h at room temperature. After stirring, the reaction beads were filtered and washed with DMF (10 ml × 2), 2-isopropyl alcohol (10 ml × 2), and DCM (10 ml × 5). The swelled resin was dried under reduced pressure overnight and stored at −20 °C.

**Preparation of Red Dye-labeled TAR RNA**

RNAs were prepared by chemical and enzymatic methods. Modified TAR RNA was synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer using 2-cyanoethyl phosphoramite chemistry (Scheme 1). All monomers of 2-cyanoethyl phosphoramite were obtained from Glen Research (Sterling, VA). Red dye-conjugated 2-cyanoethyl phosphoramidite was synthesized from 2-cyanoethyl diisopropylchlorophosphoramidite (1 g, 4.23 mmol) using the dye, disperse red 1 (1.1 g, 3.52 mmol) and 2-isopropyl alcohol (2.5 ml, 14.98 mmol). Cleavage and deporation of red dye-labeled RNA was carried out in methanic ammonia (1 ml) for 16 h at room temperature. After cleavage from the resin, the methanic supernatant was transferred to an Eppendorf tube and dried under a stream of nitrogen. The red pellet was desily-
luted in 50% triethylamine trihydrofluoride in dimethyl sulfoxide (0.5 ml) for 16 h at room temperature. The crude red dye-labeled RNA was precipitated by adding isopropyl alcohol (1 ml) at −78 °C for 1 h. The desired RNA was purified on 20% acrylamide, 8 M urea denaturing gels.

On-bead Colorimetric Screening Assay of TAR RNA-binding Ligands

A suspension of trimer unnatural peptide library (60 mg, ~4 copies of library) in TK buffer (400 μl; 50 mM Tris-HCl (pH 7.4), 20 mM KCl, 0.1% Triton X-100) was incubated with bovine serum albumin at room temperature for 1 h to reduce nonspecific binding. The aqueous phase was removed, the resin washed with TK buffer (500 μl, 3×), and TK buffer (600 μl) and M1 RNA (25 μM) were added. The resulting suspension was incubated at 4 °C for 24 h, and the buffer drained. To the beads were added TK buffer (600 μl), red dye-labeled-TAR RNA (3 μM), and M1 RNA (2.5 μM). After 2 days of stirring at 4 °C, the supernatant was drained, and the remaining beads were washed with water. Red beads were picked and individually placed in capillary tubes. Each bead was washed with DMF (4 μl × 3), and DMF (2 μl) was added. Capillary tubes were UV-irradiated (366 nm) for 4 h to cleave tags, and 1.5 μl of DMF solution was silylated with N,O-bis(trimethylsilyl)acetamide (0.1 μl) in a microsyringe. The trimethylsilyl derivatives of tag alcohols were analyzed by GC/ECD.

GC/ECD Decoding Analysis

An encoded bead was placed in a 25-μl microcapillary tube with DMF (2 μl) and washed with DMF (5 μl × 4). After draining the solvent, the bead was resuspended in DMF (2 μl), and the microcapillary was centrifuged for 4 min and sealed by flame. The capillary containing a bead was UV-irradiated (366 nm) for 4 h and then centrifuged for 5 min. After opening the capillary, the cleaved tag alcohols were silylated with N,O-bis(trimethylsilyl)acetamide in a microsyringe. The trimethylsilyl derivatives were analyzed by GC/ECD.

Parallel Synthesis and Typical Solid-phase Binding Assay

Based on structures selected from the colorimetric screening assay, a parallel library was designed and synthesized as shown in Tables II and III. Each ligand was manually synthesized according to standard solid-phase synthesis methods previously described (36). After side chain deprotection, the ligand attached to the resin (10 beads) was placed in an Eppendorf tube and washed with TK buffer (200 μl × 3). The beads were resuspended in TK buffer (500 μl) and incubated with TAR RNA (1.11 μM) overnight at 4 °C. The suspension was centrifuged, and the equilibrium concentration of unbound RNA in the supernatant was determined by measuring its absorbance at 260 nm with a Shimadzu UV-1601 spectrometer. Knowing the initial concentrations of ligand and RNA, and assuming simple bimolecular receptorsubstrate binding, the dissociation constant ($K_d$) was calculated from straightforward equations as described previously (29).

Inhibition of HIV-1 Replication

Cells (either Jurkat or MT4) were seeded in T-75 flasks at $5 \times 10^5$ ml in a total of 5 ml of complete RPMI 1640 medium and pretreated at 37 °C with or without test and control inhibitors for 15 min prior to infection. HIV NL4–3 virus stocks were prepared and quantified by reverse transcriptase (RT) assays. For our experiments, Jurkat cells were infected at either $1 \times 10^3$ or $1 \times 10^4$ tissue culture infectious units/ml, and MT4 cells at 1000 infectious units/ml. Test inhibitors were used at a concentration of 5 μM and zidovudine (control) was used at 1 μM. Following incubation with HIV-1 for 1 h at 37 °C, cells were pelleted, washed in medium to remove free virus, and resuspended in 5 ml of complete medium with or without inhibitor. At various times after infection, aliquots (2 ml each) were removed from each flask and replaced with an equal volume of fresh medium plus minus inhibitor to maintain a final inhibitor concentration of 5 μM. Aliquots (100 μl) of the collected supernatants were stored at −20 °C for RT assay.

Reverse Transcriptase Assay

RT assays were performed as previously described (38). Briefly, 5 μl of supernatant was added to 50 μl of assay mixture (50 mM Tris-Cl (pH 7.5), 63 mM KCl, 4.2 mM MgCl2, 0.1% Nonidet P-40, 0.08% EDTA, 4.2 μg/ml poly(A), 0.13 μg/ml oligo(dT), 4 μM dithiothreitol, and 40 μM [32P]TPTP) and incubated for 1 h (MT4 cells) or 2 h (Jurkat cells) at 37 °C. After incubation, 5 μl of each reaction was spotted onto DE-81 paper (Whatman), washed twice for 5 min each in 2× SSC (0.3 M sodium chloride, 0.05 M sodium citrate (pH 7.0)) to remove unincorporated counts, rinsed once in methanol, and air-dried. Visualization (Fujifilm Image Reader) and quantitation (Fujifilm Image Gauge) of RT incorporation were performed by phosphorimaging.

Toxicity Testing

Cytotoxicity of inhibitors in cultured cells was tested using a standard microscopic visualization of their ability to exclude 0.1% trypan blue in phosphate buffered saline. In vivo toxicity of inhibitors was investigated in mice under an animal study protocol approved by the NIAID animal use committee.

Fluorescence Resonance Energy Transfer (FRET)

FRET from fluorescein to rhodamine was measured on a Photon Technology International fluorescence spectrophotometer. The emission spectrum was recorded from 500 to 650 nm for each scan. The slits were set to 4 nm for both excitation and emission lights. All experiments were carried out at room temperature.

Samples were placed into a plastic microcuvette with a starting volume of 300 μl. The initial concentration of F-TAR was 13 nM, and an equal amount of Rh-Tat (47–59) was added to form 1:1 Tat-TAR complexes. Each addition of TR87 to solutions was followed by a 5-min incubation before the fluorescence spectrum was recorded. The stock concentration of TR87 for titration was 100 μM. The fluorescence intensities were calibrated for the background of TKT buffer (TKT: 50 mM Tris-HCl (pH 7.4), 20 mM KCl, and 0.02% w/v Tween 20).

Inhibition of Tat Transactivation in Vitro

The pWT2 plasmid derived from p10SLT containing HIV-1 5′–LTR was used in the cell-free transcription experiment (39, 40). Template DNAs were linearized with BamHI to generate 530-nucleotide runoff transcripts. Transcription reactions containing 0.5 μg of template DNA,
12 μl of HeLa nuclear extract, 50 mM KCl, 6 mM MgCl₂, 6 mM sodium citrate, 1 mM dithiothreitol, 10 mM phosphocreatine, 200 μM ATP, 200 μM GTP, 200 μM CTP, and 10 μCi of [α-32P]CTP (800 Ci/mmol, ICN; final concentration 0.5 μM) were carried out at 30°C for 60 min with 35 ng of Tat. Increasing amounts of TR87 (ranging from 0.1 to 400 μM) was added to the transcription reaction mixture. After incubation, 200 μl of stop solution (0.3 M Tris-HCl (pH 7.4), 0.3 M sodium acetate, 0.5% SDS, and 2 mM EDTA) was added. RNA transcripts were isolated by phenol extraction, precipitated with ethanol, and analyzed on 8% polyacrylamide, 7 M urea gels. Efficiencies of transactivation were determined by a phosphorimage analysis (Image Gauge version 3.4, Fuji).

RESULTS AND DISCUSSION

Synthesis of Encoded Combinatorial Library by Split-pool Strategy—We used a set of 34 monomers and three consecutive cycles of split-pool synthesis to synthesize an encoded combi-
As shown in Fig. 2, the 34 building blocks, which were selected to cover a fairly broad range of "chemical space," varied in charge, aromaticity, hydrogen bonding potential, flexibility, size, length of side chain, and hydrophobicity.

TentaGel resin has good swelling properties in a broad range of organic solvents as well as in aqueous media. Because of the long polyethylene glycol spacer on TentaGel beads, ligands on the beads behave kinetically as though they were in solution. Thus we decided to synthesize the library using TentaGel resin as a solid support. The library had the general structure, NH₂-M³-M²-M¹-N(CH₂)₂-O-TentaGel. The synthetic strategy used to create this library relies on standard Fmoc solid-phase synthetic methods (see "Experimental Procedures" for details). The library was encoded with a set of 18 photocleavable tags, which were protected by carrying out all reaction steps in the dark. For each cycle of monomer coupling, HOBT and DIPDCI were added to the beads. For carbamate coupling, the encoded beads were reacted with carbamate monomer, HOBT, and N,N-diisopropylethylamine. Each cycle of peptoid monomer coupling consisted of an acylation step and a nucleophilic displacement step. The peptoid backbone was formed via acylation with bromoacetic acid, and its side chain was introduced by nucleophilic displacement of the bromo group by a primary amine.

**On-bead Selection of TAR RNA-binding Unnatural Small Molecules**—Combinatorial chemistry has been previously used to identify new ligands that block the Tat-TAR interaction, but previous studies have relied on a variety of complex methods that are labor-intensive or require expensive robotic equipment (25). For the most part, these methods originated in the study of individual protein-nucleic acid interactions. Moreover, in some cases time-consuming deconvolution strategies are also needed to identify individual compounds responsible for properties found in a mixture of compounds tested together (26).

To identify small molecules that target specific RNA structures, we developed a rapid high through-put screening technology. Our approach entails covalent attachment of a dye molecule, disperse red, to the 5′ end of TAR RNA (Scheme I) and incubation in a suspension of library beads prepared by the split-synthesis method (29). Although low molecular weight receptors can diffuse rapidly into a bead of TentaGel resin, we were not sure if a macromolecule such as a protein or large nucleic acid could diffuse to the bead interior where the bulk of the ligand molecule is displayed. Our results demonstrate that the dye-TAR RNA conjugate entered the beads and bound in a
structure-dependent manner. The red color of the dye-TAR conjugate was clearly distributed throughout the translucent bead (Fig. 3). A mutant TAR RNA sequence lacking the trinucleotide bulge was used in excess to block nonspecific RNA-small molecule interactions. Nonspecific binding was also minimized by using a small amount of detergent and a low RNA concentration (250 nM). In addition to the relative simplicity of the combinatorial library method, our method offers the advantage of employing chemically encoded library beads. Once a dye-stained bead is selected, identification of the compound structure is rapid and straightforward. The beads produced by the encoded split synthesis strategy act as assay vessels for thousands of unique compounds, allowing multiple RNA-binding experiments to be performed simultaneously.

**Compound Structure Analysis**—Upon incubating the dye-TAR RNA conjugate with the library, we found that 59 beads took on a deep red color (Fig. 3). We picked these beads and decoded the structures of RNA-binding ligands (Table I). Analysis of ligand structures revealed four major patterns or motifs, which were used to group these structures. The largest number \((n = 21, 36\%)\) of screened beads had motif 1: M3-M2-L-LysCar. The next largest number \((n = 16, 27\%)\) had motif 2: M3-M2-D-LysCar, and another 13 \((22\%)\) had motif 1: M3-M2-L-Lys. Minor sequences were represented in motif 4: M3-M2-DL-ArgCar and M3-M2-5APP.

**Parallel Library Synthesis and Determination of RNA Binding Affinities**—To study structure-activity relationships and to

### Table 1

Small molecules selected by colorimetric assay with TAR RNA

| Identification no. | M3 (N-terminal) | M2 | M1 (C-terminal) | Frequency |
|--------------------|----------------|----|----------------|-----------|
| Motif 1: M3-M2-L-LysCar | 21 |
| TR0042 | d-LysCar | t-LysCar | t-LysCar | 3 |
| TR0080 | l-Lys | t-LysCar | t-LysCar | 1 |
| TR0047 | l-Lys | d-LysCar | t-LysCar | 2 |
| TR0054 | l-Lys | d-LysCar | t-LysCar | 1 |
| TR0064 | d-Lys | d-LysCar | t-LysCar | 2 |
| TR0046 | l-Lys | t-Lys | t-LysCar | 1 |
| TR0053 | l-Lys | t-Lys | t-LysCar | 1 |
| TR0043 | l-Lys | 7AHP | t-LysCar | 2 |
| TR0055 | 7AHP | 7AHP | t-LysCar | 2 |
| TR0044 | DMAB | 5APP | t-LysCar | 1 |
| TR0070 | d-Ala | 5APP | t-LysCar | 1 |
| TR0065 | d-Gln | 4ABP | t-LysCar | 1 |
| TR0048 | d-Ala | DMAB | t-LysCar | 1 |
| TR0052 | l-LysCar | d-Gln | t-LysCar | 1 |
| TR0068 | l-LysCar | d-Pro | t-LysCar | 1 |
| Motif 2: M3-M2-D-LysCar | 16 |
| TR0051 | d-Ala | l-Lys | d-LysCar | 1 |
| TR0009 | l-Lys | t-LysCar | d-LysCar | 1 |
| TR0067 | d-LysCar | l-LysCar | d-LysCar | 3 |
| TR0076 | l-Lys | d-LysCar | d-LysCar | 1 |
| TR0092 | l-Lys | d-LysCar | d-LysCar | 1 |
| TR0084 | 4ABP | l-Lys | d-LysCar | 1 |
| TR0085 | d-Lys | d-Lys | t-LysCar | 1 |
| TR0073 | 7AHP | 7AHP | t-Lys | 1 |
| TR0078 | 7AHP | 5APP | t-Lys | 1 |
| TR0062 | d-Gln | 5APP | t-LysCar | 1 |
| TR0072 | l-LysCar | DMAB | t-LysCar | 1 |
| TR0058 | d-Lys | d-Pro | t-LysCar | 1 |
| TR0087 | d-Asn | d-His | t-LysCar | 1 |
| Motif 3: M3-M2-L-Lys | 13 |
| TR0041 | l-Lys | l-Lys | l-Lys | 2 |
| TR0061 | d-LysCar | l-Lys | t-Lys | 1 |
| TR0066 | l-Lys | l-Lys | t-Lys | 1 |
| TR0056 | l-Lys | d-Lys | t-Lys | 1 |
| TR0081 | d-LysCar | l-LysCar | t-Lys | 1 |
| TR0060 | d-LysCar | 5APP | l-Lys | 1 |
| TR0045 | d-Ala | 4ABP | l-Lys | 1 |
| TR0049 | d-Lys | MePiperazine | t-Lys | 1 |
| TR0063 | d-LysCar | MePiperazine | t-Lys | 1 |
| TR0050 | 7AHP | DMAB | t-Lys | 1 |
| TR0071 | l-Lys | d-Arg | t-Lys | 1 |
| TR0086 | d-Tyr | d-His | t-Lys | 1 |
| Motif 4: M3-M2-M3 | 9 |
| TR0075 | Isonicotinic | l-LysCar | t-ArgCar | 1 |
| TR0083 | l-LysCar | d-LysCar | t-ArgCar | 1 |
| TR0070 | l-Lys | d-LysCar | d-ArgCar | 1 |
| TR0057 | 4ABP | d-LysCar | 5APP | 2 |
| TR0077 | l-LysCar | l-LysCar | 5APP | 1 |
| TR0074 | l-LysCar | l-LysCar | 5APP | 1 |
| TR0069 | l-ArgCar | d-HPhAla | 4ABP | 1 |

Small Molecule Tat-TAR Antagonist Inhibits HIV-1 Replication

Sequences are arranged according to various structural motifs.
identify the best ligands for TAR RNA, we analyzed the monomer distribution of 59 ligands and synthesized a parallel library. As shown in Tables II and III, we synthesized two sets of libraries with the motifs M3-M2-L-LysCar and M3-M2-D-LysCar. For the M2 position of the M3-M2-L-LysCar parallel library, we used a set of five monomers as shown in Table II, and for the M3-M2-L-LysCar parallel library we used six monomers (Table III). At the N-terminal position (M3 monomer), we used a set of nine structures: L-LysCar, D-LysCar, L-Lys, D-Lys, 5APP, 7AHP, 4ABP, L-ArgCar, and D-Gln. After three consecutive coupling reactions using the parallel synthetic method, we generated a total of 45 distinct M3-M2-L-LysCar parallel library compounds (Table II) and a total of 54 distinct M3-M2-D-LysCar parallel library compounds (Table III).

To evaluate the relative binding affinities of the parallel library compounds to TAR RNA, we measured their dissociation constants using a solid-phase assay as previously described (29). It is important to note that the \( K_D \) values presented in this report should not be considered as absolute binding affinities under the same experimental conditions. As shown in Table II, five ligands from the M3-M2-L-LysCar parallel library had dissociation constants (\( K_D \)) lower than 200 nM. Motifs L1 and L5 of the parallel library had moderate binding affinities, with dissociation constants ranging from 256 to 6136 nM. Motif L2 (M3-L-LysCar-L-LysCar) had two ligands, TR12 (M3/L-Lys/11005-L-Lys) and TR15 (M3/L/Lys/11005), with \( K_D \) values of 130 and 147 nM, respectively. The isomeric motif of L2, motif L1, had moderate binding affinity, with dissociation constants ranging from 301 to 981 nM. Motifs L3 (M3–4ABP-L-LysCar) and L4 (M3–5APP-L-

| Motif L1: M3-n-LysCar-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|---------------------------------|-------------------|-----|-------------|
| TR100                           | L-LysCar          | 460 |
| TR101                           | L-LysCar          | 603 |
| TR102                           | L-Lys             | 567 |
| TR103                           | L-Lys             | 567 |
| TR104                           | 5APP              | 301 |
| TR105                           | 7AHP              | 567 |
| TR106                           | 4ABP              | 865 |
| TR107                           | L-ArgCar          | 981 |
| TR20                            | L-LysCar          | 1256|
| TR21                            | L-LysCar          | 1111|
| TR22                            | L-Lys             | 130 |
| TR23                            | L-Lys             | 491 |
| TR24                            | 5APP              | 235 |
| TR25                            | 7AHP              | 147 |
| TR26                            | 4ABP              | 678 |
| TR27                            | L-ArgCar          | 2353|
| TR28                            | L-ArgCar          | 981 |

| Motif L2: M3-l-LysCar-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|---------------------------------|-------------------|-----|-------------|
| TR11                            | L-LysCar          | 1256|
| TR12                            | L-LysCar          | 1111|
| TR13                            | L-Lys             | 130 |
| TR14                            | L-Lys             | 491 |
| TR15                            | 5APP              | 235 |
| TR16                            | 7AHP              | 147 |
| TR17                            | 4ABP              | 678 |
| TR18                            | 7AHP              | 2353|
| TR19                            | L-ArgCar          | 981 |

| Motif L3: M3–4ABP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR20                        | L-LysCar          | 491 |
| TR21                        | L-LysCar          | 147 |
| TR22                        | L-Lys             | 718 |
| TR23                        | L-Lys             | 567 |
| TR24                        | 5APP              | 3926|
| TR25                        | 7AHP              | 256 |
| TR26                        | 4ABP              | 523 |
| TR27                        | L-ArgCar          | 183 |
| TR28                        | L-ArgCar          | 301 |

| Motif L4: M3–5APP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR30                        | L-LysCar          | 256 |
| TR31                        | L-LysCar          | 5113|
| TR32                        | L-Lys             | 567 |
| TR33                        | L-Lys             | 120 |
| TR34                        | 5APP              | 31639|
| TR35                        | 7AHP              | 235 |
| TR36                        | 4ABP              | 789 |
| TR37                        | 4ABP              | 235 |
| TR38                        | L-ArgCar          | 603 |

| Motif L5: M3–7AHP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR40                        | L-LysCar          | 1111|
| TR41                        | L-LysCar          | 678 |
| TR42                        | L-Lys             | 567 |
| TR43                        | L-Lys             | 130 |
| TR44                        | 5APP              | 235 |
| TR45                        | 7AHP              | 774 |
| TR46                        | 4ABP              | 6136 |
| TR47                        | L-ArgCar          | 460 |
| TR48                        | L-ArgCar          | 3151|

| Motif D1: M3-n-LysCar-n-LysCar | Identification no. | M3 | \( K_D \) nM |
|-------------------------------|-------------------|-----|-------------|
| TR70                         | L-LysCar          | 639 |
| TR71                         | L-Lys             | 523 |
| TR72                         | L-Lys             | 217 |
| TR73                         | L-Lys             | 2079|
| TR74                         | 5APP              | 1111|
| TR75                         | 7AHP              | 491 |
| TR76                         | 4ABP              | 403 |
| TR77                         | L-ArgCar          | 460 |
| TR78                         | L-ArgCar          | 603 |

| Motif D2: M3-l-LysCar-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|-------------------------------|-------------------|-----|-------------|
| TR60                         | L-LysCar          | 4313|
| TR61                         | L-Lys             | 1716|
| TR62                         | L-Lys             | 2717|
| TR63                         | L-Lys             | 183 |
| TR64                         | 5APP              | 183 |
| TR65                         | 7AHP              | 120 |
| TR66                         | 4ABP              | 1494|
| TR67                         | L-ArgCar          | 2079|
| TR68                         | L-ArgCar          | 376 |

| Motif D3: M3–4ABP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR70                        | L-LysCar          | 147 |
| TR71                        | L-Lys             | 165 |
| TR72                        | L-Lys             | 147 |
| TR73                        | L-Lys             | 325 |
| TR74                        | 5APP              | 89 |
| TR75                        | 7AHP              | 165 |
| TR76                        | 4ABP              | 403 |
| TR77                        | L-ArgCar          | 147 |
| TR78                        | L-ArgCar          | 460 |

| Motif D4: M3–5APP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR80                        | L-LysCar          | 256 |
| TR81                        | L-Lys             | 183 |
| TR82                        | L-Lys             | 183 |
| TR83                        | L-Lys             | 678 |
| TR84                        | 5APP              | 147 |
| TR85                        | 7AHP              | 431 |
| TR86                        | 4ABP              | 2527|
| TR87                        | L-ArgCar          | 202 |
| TR88                        | L-ArgCar          | 202 |

| Motif D5: M3–7AHP-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR90                        | L-LysCar          | 930 |
| TR91                        | L-Lys             | 718 |
| TR92                        | L-Lys             | 403 |
| TR93                        | L-Lys             | 3151|
| TR94                        | 5APP              | 183 |
| TR95                        | 7AHP              | 2191|
| TR96                        | 4ABP              | 235 |
| TR97                        | L-ArgCar          | 5991|
| TR98                        | L-ArgCar          | 639 |

| Motif D6: M3-l-Lys-l-LysCar | Identification no. | M3 | \( K_D \) nM |
|----------------------------|-------------------|-----|-------------|
| TR100                       | L-LysCar          | 202 |
| TR101                       | L-Lys             | 1418|
| TR102                       | L-Lys             | 403 |
| TR103                       | L-Lys             | 678 |
| TR104                       | 5APP              | 202 |
| TR105                       | 7AHP              | 183 |
| TR106                       | 4ABP              | 130 |
| TR107                       | L-ArgCar          | 215 |
| TR108                       | L-ArgCar          | 639 |
Small Molecule Tat-TAR Antagonist Inhibits HIV-1 Replication

TABLE IV
Molecules selected for viral replication inhibition experiments

| Identification no. | M3 (N-terminal) | M2 | M1 (C-terminal) | $K_D$ ($\text{nM}$) |
|-------------------|----------------|----|----------------|-----------------|
| TR12              | L-Lys          |    | L-LysCar       | 120             |
| TR15              | 7AHP           |    | L-LysCar       | 130             |
| TR21              | L-LysCar       | 4ABP| L-LysCar       | 147             |
| TR27              | L-ArgCar       | 4ABP| L-LysCar       | 147             |
| TR33              | L-LysCar       | 5APP| L-LysCar       | 183             |
| TR55              | 7AHP           |    | L-LysCar       | 120             |
| TR56              | 4ABP           |    | L-LysCar       | 130             |
| TR63              | L-Lys          |    | L-LysCar       | 183             |
| TR64              | 5APP           |    | L-LysCar       | 183             |
| TR65              | 7AHP           |    | L-LysCar       | 120             |
| TR80              | L-LysCar       | 4ABP| d-LysCar       | 147             |
| TR81              | n-LysCar       | 4ABP| d-LysCar       | 165             |
| TR82              | L-Lys          |    | d-LysCar       | 147             |
| TR84              | 5APP           |    | d-LysCar       | 89              |
| TR85              | 7AHP           |    | d-LysCar       | 165             |
| TR87              | L-ArgCar       | 4ABP| d-LysCar       | 147             |
| TR91              | L-ArgCar       | 5APP| d-LysCar       | 183             |
| TR94              | n-LysCar       | 5APP| d-LysCar       | 147             |
| TR104             | 5APP           |    | d-LysCar       | 183             |

LysCar) were similar in their M2 backbone structures, and ligands TR21, TR27, and TR33 had dissociation constants of 147, 183, and 120 nM, respectively.

In the case of the M3-M2-n-LysCar parallel library (Table III), a total of 14 ligands had high binding affinities, with dissociation constants lower than 200 nM. The most favorable motif was motif D3 (M3–4ABP–n-LysCar), with $K_D$, ranging from 89 to 460 nM. Among the ligands having this motif, TR50 (M3 = L-LysCar), TR51 (M3 = d-LysCar), TR52 (M3 = L-Lys), TR54 (M3 = 5APP), TR55 (M3 = 7AHP), and TR87 (M3 = L-ArgCar) bound tightly to the target molecule with dissociation constants lower than 200 nM. The highest affinity ligand, TR84, with $K_D$ of 89 nM, had the sequence 5APP–4ABP–D-LysCar. Replacing the M2 monomer with 5APP–4ABP–D-LysCar. Replacing the M2 monomer with 5APP (motif D4) or a longer side chain, 7AHP (motif D5), resulted in ligands (TR91, TR94, and TR104) with dissociation constants lower than 200 nM. In the n-LysCar parallel library, the position M2 monomer prefers the flexible peptoid-based monomer with a 4-aminobutyl side chain, which may account for its high binding affinity.

Inhibition of HIV Replication—To test whether these small molecules could be used to inhibit HIV-1 replication, we synthesized 19 ligands from Tables II and III that bind TAR RNA with high affinities ($K_D < 200$ nM) on a large scale (Table IV). The compounds were synthesized on the solid support as described earlier (36). After synthesis, the compounds were cleaved from the resin by trifluoroacetic acid treatment, purified by reverse phase HPLC, and the identity of the compounds was confirmed by matrix-assisted laser desorption ionization and fast atom bombardment mass spectrometry (29, 36). The kinetics of HIV-1 NL4–3 replication in Jurkat or MT4 cells was monitored by sampling culture supernatants every 3 or 4 days after infection and quantifying virus production by RT assay (41). Several compounds (TR27, TR56, TR64, TR65, TR82, TR85, TR87) showed an anti-HIV-1 activity, reducing virus production in infected cells by 5–20-fold or more during a 1–2-week course of infection (data not shown). The lack of activity of 12 compounds could be caused by insufficient cellular uptake or by the stability of these molecules upon entering the cell. An example of results using compound TR87 is shown in Fig. 4. Compared with mock treatment (no inhibitor), incubation of Jurkat cells (Fig. 4A, left panel) or MT4 cells (Fig. 4A, right panel) with 5 μM TR87 significantly suppressed HIV-1 NL4–3 replication over 24 or 12 days, respectively.

The sustained anti-HIV-1 effect observed with TR87 in Jurkat cells was specific, and treatment of cell cultures with this compound at 5, 50, or 100 μM produced no cytotoxicity. In trypan blue exclusion assays, the percentage of non-viable cells in TR87 treated cultures (~2%) was indistinguishable from that in parallel mock-treated cultures (Fig. 4B). Cell toxicity was also measured using a modified MTT assay. MTT absorbance in live cells measured for TR87-treated cultures was indistinguishable from mock-treated cells (Fig. 4C), indicating further that TR87 was not toxic to cells in culture. We then verified the benign properties of TR87 with an in vivo toxicity study in Taconic outbred albino mice. Two groups of five mice were injected intraperitoneally once daily for 5 weeks with either saline or TR87 to achieve a final body volume distribution of 5 μM. All 10 mice thrived equally; neither group on daily physical examination showed any changes in apparent health or body weight. Histological examination of vital organs from representative control and TR87 mice sacrificed after 5 weeks confirmed a lack of toxicity in the TR87-treated animals (Fig. 4D).

Our anti-HIV results compare favorably with those of other novel compounds that target the Tat-TAR interaction. For example, a recently described neomycin arginine conjugate (at 2.5 μM) in a limited 3-day course of infection reduced HIV-1 production modestly by ~45% in infected H9 cells (42). By comparison, TR87 provides a considerably more potent inhibition of HIV-1 replication. Additionally, whereas neomycin-based moieties are known to have in vivo neurotoxicity at drug levels near 250 μM (43) and other novel agents such as the diketoacid inhibitors of HIV-1 integrase (44) could also be toxic, our in vivo assays in mice indicate that daily infusions of TR87 over a 5-week course are impressively nontoxic (Fig. 4D and E).

Inhibition of Tat-TAR Complex Formation in Vitro—Having established TR87 as an inhibitor of HIV replication, we wanted to determine whether this inhibition was caused by TR87 specifically interrupting interactions between Tat and TAR. To examine the interactions between Tat and TAR in the presence and absence of TR87, we developed an in vitro competition assay based on FRET that monitors the interaction between RNA and peptides. FRET, in which a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule (45), is a powerful spectroscopic technique for visualizing the phenomenon associated with the distance change between donors and acceptors. TAR RNA was labeled with donor fluorescein at its 5’ end (F-TAR), which is...
excited at 490 nm, whereas Tat-(47–58) peptide was labeled with acceptor rhodamine (Rh-Tat-(47–58)). FRET from fluorescein to rhodamine was measured by their respective fluorescent intensities with a fluorescence spectrophotometer. The initial concentration of F-TAR was 13 nM, and an equal amount of Rh-Tat-(47–58) was added to form 1:1 Tat-TAR complexes.
Quenching of the fluorescein signal intensity signified FRET had occurred (Fig. 5A), indicating that F-TAR was interacting with the Rh-Tat-(47–58) peptide ($K_d = 2 \text{nM}$).\(^2\) TR87 was then titrated incrementally to the mixture, and each addition was followed by a 5-min equilibration before the fluorescence spectrum was recorded. As shown in Fig. 5A, quenching of the fluorescence signal intensity was relieved upon titration of increasing amounts of TR87, indicating that there was a decrease in FRET and reflected that TR87 directly competes with Rh-Tat-(47–58) at the beginning of the titration.

Data points were collected from fluorescence spectra at ~515 nm.

The relative binding affinity ($K_r$) of TR87 and Tat to TAR was obtained by fitting data to Equation 1.

$$C = A_0 - \frac{K_r(A_0 + I_{tot}) - \frac{(A_0 + I_{tot})^2}{2(K_r - 1)}}{2I_{tot}K_r}$$

(Eq. 1)

$C$ is Tat-TAR concentration, which can be converted from the relative fluorescence intensity, $A_0$ is initial concentration of TAR, and $I_{tot}$ is the total concentration of TR87 added. The dissociation constant of TR87 bound to TAR ($K_r$) can be obtained by Equation 2.

$$K_r = \frac{K_d}{K_i}$$

(Eq. 2)

$K_d$ is the dissociation constant of Rh-Tat-(47–58) bound to F-TAR. As shown in Fig. 5B, the concentration of Tat-TAR complexes decreased with increasing amounts of TR87. The solid curve shows the best fit for Equation 1 under the assumption that all F-TAR was bound to Rh-Tat-(47–58) at the beginning of the titration with TR87 and provides a $K_r = 0.0011$; therefore, a $K_i = 1.8 \mu M$ was calculated for the competitive binding of TR87 to F-TAR.

In this equation, $s$ is the slope, $y$ is the percentage of inhibition of Tat transactivation, and $x$ is the concentration of TR87. The calculated $K_r$ value was 1.0 $\mu$M, which was consistent with the competitive binding results described above. These results indicated that TR87 was significantly inhibiting Tat transactivation in a concentration-dependent manner, suggesting that TR87 specifically inhibited functions associated with Tat-TAR interactions in vitro.

We then determined whether TR87 was specifically affecting Tat-TAR functional interactions in vivo by evaluating Tat transactivation using a luciferase reporter assay to measure transcription from the 5'-LTR in living cells as described previously (47). HeLa cells were transfected with 100 $\mu$g of HIV-LTR-luciferase plasmid and 100 $\mu$g of a Tat expression vector, and TR87 was added to cells at various concentrations at the time of transfection. 48 h after transfection, luciferase activity of cell lysates was quantitated, acting as an indicator of Tat transactivation. As shown in Fig. 6B, luciferase activity increased significantly upon transfection with the Tat expression vector alone, but decreased as cells were treated with increasing amounts of TR87. Complete inhibition of Tat transactivation was seen using 100 $\mu$M TR87 that was comparable with the luciferase activity seen in control experiments when the HIV-LTR-luciferase plasmid was transfected into cells alone (Fig. 6B, compare lanes 1 and 6). These results showed that TR87 was inhibiting functional interactions between Tat and TAR in vivo in a concentration-dependent manner, as seen in the above in vitro studies. Altogether, these in vitro and in vivo studies strongly suggested that TR87 inhibition of HIV replication was resulting directly from the functional consequences of inhibiting Tat-TAR interactions.

Conclusions—According to the World Health Organization, an estimated 42 million people worldwide (38.6 million adults and 3.2 million children younger than 15 years) were living with AIDS as of the end of 2002. Despite remarkable medical advances, HIV-1 infections continue to increase. Anti-HIV agents used currently target only reverse transcriptase or protease. However, because of the emergence of viral resistance to protease and reverse transcriptase inhibitors and related toxic effects, there remains a need for more potent and less toxic therapies against other viral targets. The molecules we de-
scribe here targeted the Tat-TAR interaction, which comprises the essential first step of HIV-1 gene expression. TAR RNA is an attractive target because mutations in this element in the evolution of resistant viruses would render HIV-1 replication incompetent. In an effort to develop new antiviral methodologies, we developed a successful and rapid high-through-put methodology. Our efforts led to the discovery of several high-affinity small molecule TAR-binders that had sustained anti-HIV-1 activity but did not exhibit in vivo toxicity. We also compellingly analyzed one of these new HIV replication inhibitors, TR87. In the presence of TR87 (5 μM), we observed potent and sustained suppression of HIV replication in cultured cells over 24 days. The same concentration of this inhibitor did not exhibit any toxicity in cell cultures or mice, indicating the strong potential for this molecule to be used as an antiviral drug. The favorable characteristics of TR87 should be amenable to refinement in future studies with the aim of deriving anti-HIV activity while remaining non-toxic to cells. In conclusion, the anti-Tat-TAR compounds synthesized and identified herein and compounds optimized in the future as a result of these studies should be an important addition to the current armamentarium of drugs that abrogate viral entry, reverse transcription, and polyprotein processing.

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