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Biochemistry

An inhibitor of the Tat/TAR RNA interaction that effectively suppresses HIV-1 replication

(peptoid/RNA–protein interactions/TAR RNA structure)

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ABSTRACT **One of the first steps in HIV gene expression is the recruitment of Tat protein to the transcription machinery after its binding to the RNA response element TAR. Starting from a pool of 3.2×10^6 individual chemical entities, we were able to select a hybrid peptoid/peptide oligomer of 9 residues (CGP64222) that was able to block the formation of the Tat/TAR RNA complex *in vitro* at nanomolar concentrations. NMR studies demonstrated that the compound binds similarly to polypeptides derived from the Tat protein and induces a conformational change in TAR RNA at the Tat-binding site. In addition, 10–30 μM CGP64222 specifically inhibited Tat activity in a cellular Tat-dependent transactivation assay [fusion-induced gene stimulation (FIGS) assay] and blocked HIV-1 replication in primary human lymphocytes. By contrast, peptides of a comparable size and side-chain composition inhibited cell fusion in the FIGS assay and only partially inhibited HIV-1 replication in primary human lymphocytes. Thus, we have discovered a compound, CGP64222, that specifically inhibits the Tat/TAR RNA interaction, both *in vitro* and *in vivo*.**

After integration into the host genome, human immunodeficiency virus (HIV) remains quiescent until basal transcription produces a threshold level of the viral trans-activator protein, Tat. Tat increases viral mRNA production several hundredfold by increasing the elongation capacity of RNA polymerase (1–4).

Tat is brought into contact with the transcription machinery after binding the trans-activation-responsive (TAR) element, a 59-residue stem-loop RNA found at the 5′ end of all HIV-1 transcripts (5). *In vitro*, Tat forms a tight, specific complex with TAR RNA centered on a U-rich bulge found near the apex of the TAR RNA stem (6, 7). Recent NMR studies demonstrate that critical functional groups recognized by Tat are presented to the protein in a unique spatial arrangement created by a conformational rearrangement in TAR RNA that occurs during binding (8–10).

It seems very likely that small molecules that inhibit Tat binding to its recognition site on TAR RNA will also have antiviral activity. In this paper, we identify a low molecular weight compound that is able to reproduce the binding behavior of the protein and suppress Tat-mediated transactivation in cells.

MATERIALS AND METHODS

Combinatorial Peptoid Library. The library was synthesized with a split and mix process (11) on a 1% crosslinked polystyrene resin bearing the flourenylmethoxycarbonyl (Fmoc)-protected acid Rink amide linker (Novabiochem; 0.39 mmol/g of resin) (12). The variable regions were built up from 20 different building blocks (Fig. 1*B*). The side-chain protections were *tert*-butoxycarbonyl (Boc) for side chains 1 and 3, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for side chains 2 and 8, Boc for side chains 9 and 10, and *tert*-butyl (tBu) for side chain 15. The reagents for residues 1 to 8 were Fmoc-protected amino acids amenable to the same coupling procedure used for the assembly of the constant region. The reagent for residue 8 was synthesized as described (13). The structural elements with side chains 9 to 20 were incorporated according to the submonomer coupling procedure of Zuckermann *et al.* (14), where primary amines are allowed to react *in situ* on the solid support with previously coupled bromoacetic acid to form N-substituted glycines.

Synthesis of CGP64222. This compound was resynthesized starting from 85 μmol of Fmoc-D-Pro-amide of the Rink amide resin (Novabiochem). The crude product was purified with preparative high-pressure liquid chromatography on a Nucleosil 7C18 (5 μm) reverse-phase column (20 mm × 250 mm) and characterized by mass spectrometry and NMR.

Gel Mobility-Shift Assay. Recombinant Tat protein was prepared as described (4). TAR duplex was prepared by annealing two synthetic oligoribonucleotides: T-14, 5′-GCUGCUCUCUGGCU-3′; and T-17, 5′-AGCCAGAUUU-GAGCAGC-3′ (Genset, Paris). The 14-mer strand of synthetic duplex TAR RNA was labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP.

Binding reaction mixtures (25 μl) contained 500 fmol (≈10,000 cpm) of the labeled duplex TAR RNA, 20 nM recombinant Tat protein, and various concentrations of sublibraries in TK buffer (20 mM Tris·HCl, pH 8.0/50 mM KCl) with 10 mM dithiothreitol and 0.1% Triton X-100 (7, 15). The 20 sublibraries were initially tested at four different concentrations, and the three best sublibraries were reassayed at six different concentrations. After electrophoresis on nondenaturing 8% polyacrylamide gels, the gels were either dried and exposed on x-ray film at −70°C for 16 hr or measured by PhosphorImager analysis (Molecular Dynamics).

NMR Spectroscopy. NMR spectra were acquired at either 500 or 600 MHz on Bruker AMX-500 or DMX-600 spectrometers equipped with triple-resonance gradient probes. Nuclear Overhauser effect spectroscopy (NOESY) and total correlated spectroscopy (TOCSY) spectra were acquired in ^2H_2O using standard pulse sequences at mixing times of 100 and 300 ms (NOESY) and 50 ms (TOCSY). NOESY spectra were ac-

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Abbreviations: Boc, *tert*-butoxycarbonyl; FIGS assay, fusion-induced gene stimulation assay; Fmoc, flourenylmethoxycarbonyl; k, D-Lys; LTR, long terminal repeat; Nahg, *N*-6-aminohexylglycine; Narg, *N*-3-guanidopropylglycine; Nphe, *N*-benzylglycine; p*, D-Pro-amide; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; r, D-Arg; RT, reverse transcriptase; tBu, *tert*-butyl.

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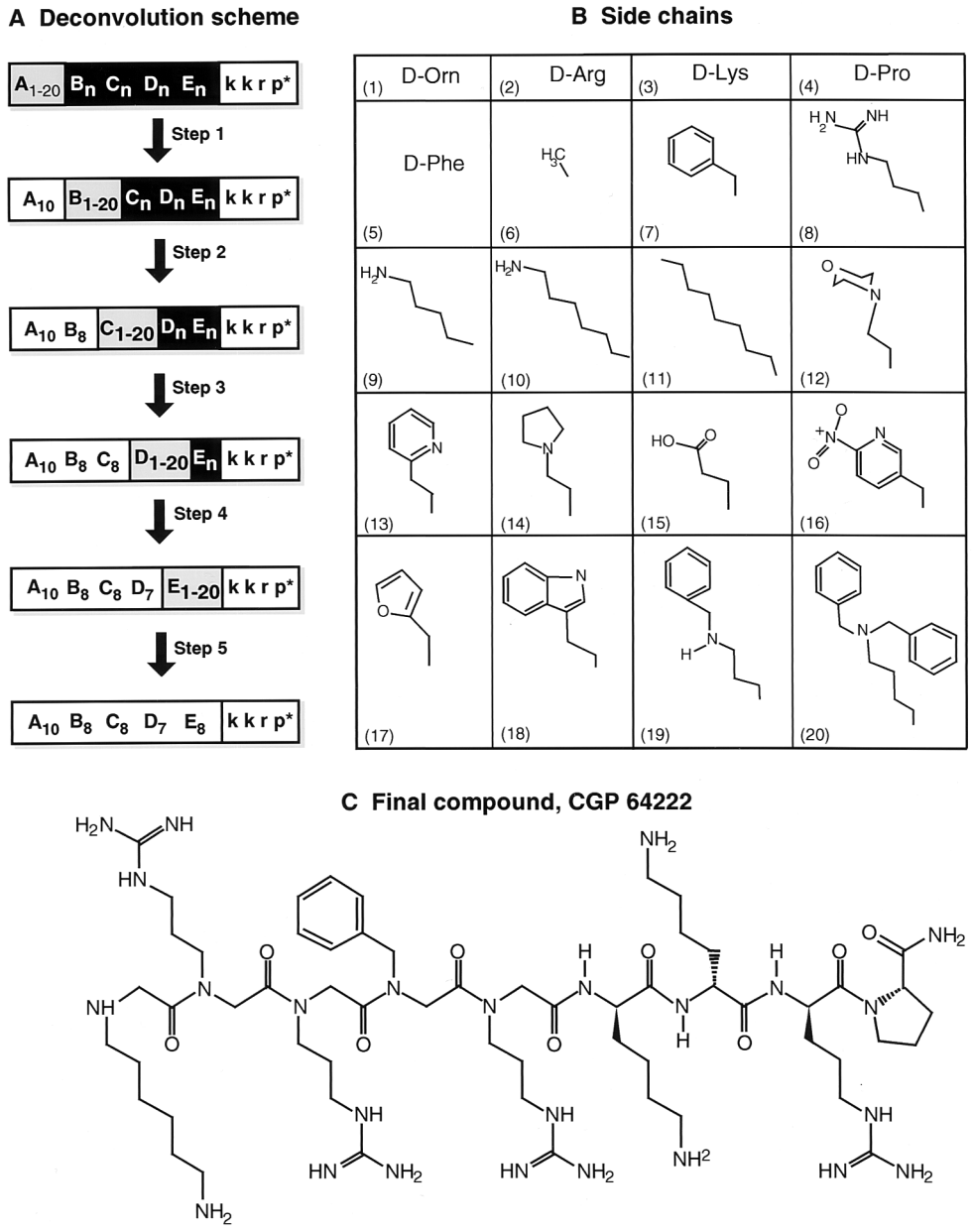

FIG. 1. (*A*) Scheme for the deconvolution of combinatorial libraries. Initially 20 sublibraries carrying unique residues in position A ($A_{1–20}$; gray box; subscripts refer to side-chain numbers shown in *B*) and a random collection of side chains in positions B, C, D, and E (X_n; black box) were synthesized. k k r p* indicates D-Lys-D-Lys-D-Arg-D-Pro-amide. The sublibrary with the greatest inhibitory activity against the Tat/TAR RNA interaction was detected by using a gel mobility-shift assay. After the optimal residue for position A had been selected, another set of 20 sublibraries was prepared with a fixed residue in position A (A_{10}, white box), unique residues at position B ($B_{1–20}$; gray box), and a random collection of side chains in positions C, D, and E (X_n; black box). This deconvolution process was continued until a single compound was identified. (*B*) Structure of side chains used to create the combinatorial library. (*C*) Structure of the final compound, CGP64222.

quired in H_2O at 150 ms mixing time using the jump-and-return solvent suppression scheme. Spectral assignments were obtained by standard methods starting from the full assignments of the free and Tat-bound forms of TAR RNA (8).

Molecular Modeling. Models for the peptoid–TAR complex were generated using X-PLOR Version 3.1 (16). Distance and dihedral angle restraints for TAR RNA were experimentally derived (8). Restraints were introduced within the peptoid backbone to ensure tetrahedral geometry for the proton-substituted carbon atoms and planar configurations for the side-chain-attached nitrogen. The models shown in Fig. 3 included the following model-building assumptions: (*i*) distance restraints for *N*-3-guanidopropylglycine (Narg) in position C were those used for an arginine residue in the Tat-derived peptide ADP-1-bound TAR RNA structure (8); (*ii*) center-averaged guanidinium protons from Narg residue B were restrained to within 6 Å of phosphates 22 and 23; (*iii*) center-averaged amino protons from *N*-6-aminohexylglycine (Nahg) at residue A were restrained to within 6 Å of phosphates 39 and 40; and (*iv*) the guanidinium protons from Narg at residue E were restrained to within 5 Å of phosphate 38. The structure shown in Fig. 3 is representative of a set of energy-minimized structures with very small violations of experimental constraints and model-building assumptions.

Cellular Trans-activation Assay. The HeLa-CD4 cell line SX22–1 [carrying a *lacZ* gene under control of the HIV long terminal repeat (LTR)] was cocultivated with HIV-1-infected $HUT_{4–3}$ lymphocytes at a ratio of 3:1 (17). After overnight incubation, to permit syncytia formation and *lacZ* synthesis, the cells were washed once with PBS, fixed with glutaralde-

hyde/formaldehyde (0.1%/0.7%) in PBS for 5 min, and stained for cell-associated β-galactosidase by using 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) as a substrate.

HIV Infection of Peripheral Blood Lymphocytes. Lymphocytes (18) were cultured in RPMI-1640 medium and activated for 2 days with 0.25 μg/ml phytohemagglutinin (Welcome Diagnostics, Temple Hill, Dartford, England). After infection by HIV-1, the cells were resuspended in complete medium supplemented with human recombinant interleukin 2 (Genzyme, Cambridge, MA) at 100 units/ml and plated at a density of 9×10^4 cells per well in U-bottom 96-well plates. Two-thirds of the culture medium was replaced every 3 days. Virus production was evaluated by determining reverse transcriptase (RT) activity in cell-free supernatants (19).

RESULTS

Combinatorial Peptoid Library Design. Synthetic peptides corresponding to the basic, arginine-rich, motif of Tat protein are able to bind directly to TAR RNA (6, 7, 20). Although arginine-rich peptides have significantly lower affinity (10- to 100-fold) and specificity for TAR than the native Tat protein (7, 21), the binding of both the peptides and Tat is mediated by an identical conformation change in TAR (8–10).

To discover low molecular weight inhibitors of the Tat/TAR RNA interaction, we adopted a combinatorial chemistry approach based on the synthesis of oligomers containing both peptoid and (all-D)-peptide residues (22). Peptoids are isomers of peptides in which all the side chains are carried by the backbone nitrogens (N-substituted glycines) (23). Peptoids are more flexible than peptides because intramolecular CO···HN hydrogen bonds are removed and the steric interactions that induce secondary structure are different. For pharmacological applications, peptoids have the advantage of being stabilized against enzymatic degradation.

The strategy for the synthesis and deconvolution of the peptoid library is illustrated in Fig. 1*A*. To limit the complexity of the library, and to maintain high concentrations of individual components in the sublibraries, the four C-terminal residues were always a sequence composed of D-amino acids, D-Lys-D-Lys-D-Arg-D-Pro-amide (k-k-r-p*). Compounds of this size (<2,000 Da) are large enough to produce specific RNA binding but remain small enough to make attractive drug leads.

Five positions (residues A to E) were randomized by introducing a set of 20 building blocks carrying a wide range of functional groups (Fig. 1*B*). The majority of building blocks carried a partial positive charge. This bias was introduced because electrostatic interactions between the peptoid and the

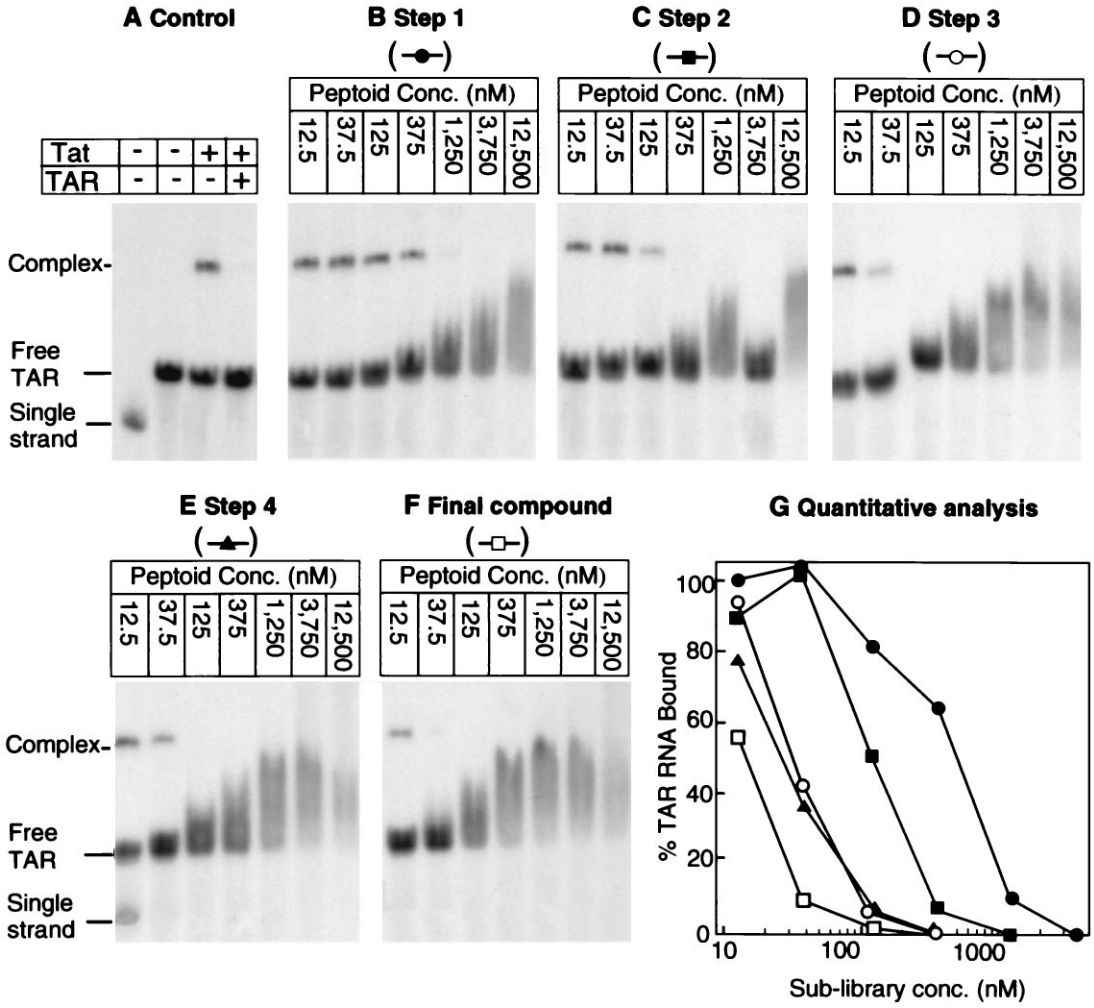

FIG. 2. Analysis of sublibraries by gel mobility-shift assay. (*A*) Control gel mobility-shift assays. First two lanes show the migration of ^{32}P-labeled 14-mer oligonucleotide and ^{32}P-labeled TAR RNA duplex. Second two lanes show Tat binding reaction mixtures containing 500 fmol of ^{32}P-labeled TAR RNA duplex and 20 nM recombinant Tat protein. The last lane contained 20 nM unlabeled TAR RNA competitor. (*B–F*) Competition binding assays. Binding reaction mixtures contained 500 fmol of ^{32}P-labeled TAR RNA duplex, 20 nM recombinant Tat protein, and between 12.5 nM and 12.5 μM each sublibrary. (*G*) Quantitative analysis of the binding data obtained by PhosphorImager analysis of gel shown in *B–F*.

phosphate backbone of the RNA are expected to be a major source of binding affinity. To maintain high chemical diversity, some aromatic residues, some neutral side chains, and a side chain with negative charge were also included in the libarary.

The libraries were also designed to explore whether structural features of the backbone chain could make a contribution to binding specificity. We therefore included five D-amino acids in the library (D-Orn, D-Arg, D-Lys, D-Pro, and D-Phe). We were particularly interested to learn whether the achiral analogue of D-Arg, *N*-guanidopropylglycine (Narg) (13) would have a selective advantage for Tat binding compared with D-Arg.

Library Deconvolution. The initial combinatorial library consisted of 3.2×10^6 (20^5) compounds divided into 20 sublibraries of 160,000 compounds each. To identify the compound with the highest affinity for TAR RNA in these highly complex mixtures, we followed a deconvolution scheme analogous to that described by Houghten *et al.* (22) (Fig. 1*A*). In the first step, 20 sublibraries, each of which contained a unique residue at the first position under investigation (the N-terminal residue, residue A) and randomized sequences at residues B to E. The sublibrary showing the highest TAR RNA-binding activity was identified using a gel mobility-shift assay. This defined the optimal side chain for residue A. A new set of 20 sublibraries was synthesized, each of which contained the optimal residue A, a unique sequence at residue B, and randomized sequences at residues C and D. Continuing with this approach, it was possible to progressively limit the complexity of the sublibrary and to identify optimal residues at each position. After the fifth deconvolution cycle, a single compound with high affinity for TAR RNA, CGP64222 (Fig. 1*C*), was identified.

Fig. 2 *B–F* shows the gel mobility-shift assay data obtained for the best sublibraries identified after each round of deconvolution. A quantitative analysis of the binding data is shown in Fig. 2*G*. Under our standardized starting conditions there was an excess of TAR RNA and sufficient recombinant Tat to bind 50% of the TAR RNA in the absence of competitor. Competition for TAR RNA binding resulted in a decrease in the amount of retarded Tat/TAR RNA complex. As shown in Fig. 2, after each selection step, significantly lower concentrations of the sublibraries are required to completely block the Tat/TAR RNA interaction. The final compound, CGP64222, was remarkably potent and required a concentration of only 12 nM to inhibit formation of the Tat/TAR RNA complex by 50%.

The selection experiment described above provides very strong evidence that there are strict sequence and steric requirements for the optimal interactions of peptidic compounds with TAR RNA. An aminohexyl residue was optimal at position A, Narg residues were optimal at positions B, C, and E, and an *N*-benzylglycine (Nphe) residue was the optimal residue at position D. The best compounds did not arise by indiscriminate insertion of positive charges at each position. For example, D-Arg was never selected in place of the Narg residue, not even as the second-best building block. However, the increase in binding due to the inclusion of the optimal side chain was not equal for each position in the sequence. For example, the inclusion of Nphe at position D produces only a small gain (1.4-fold) in competition activity compared with the preceding sublibrary. This suggests that position D tolerates broad structural changes without substantial loss of binding affinity to TAR RNA.

Molecular Modeling of the CGP64222–TAR Complex. NMR spectroscopy was used to probe the structure of the complex formed between the TAR RNA and CGP64222. Comparisons of the chemical shifts of the free and CGP64222-bound TAR RNA constitute a footprint of the CGP64222 molecule on TAR. The chemical shift changes unambiguously indicate that the CGP64222 interacts with the RNA only in the region encompassing the UCU bulge and the two base pairs on either side of the bulge.

The NMR data were used to model the interaction between CGP64222 and TAR RNA. The model was constructed to satisfy two key experimental observations: First, CGP64222 induces a conformational change in TAR resulting in a structure very similar to that of the ADP-1-bound TAR RNA (8). When the spectra of the CGP64222–TAR RNA and ADP-1–TAR RNA complexes were compared in detail, no significant differences were observed in the strand containing the bulge (residues A20 to G28), the region where the most significant conformational changes occur upon ligand binding. The conformational change is mediated by direct contacts between an Narg side chain and G26 and U23, as observed for the TAR–ADP-1 complex (8). Second, chemical shift changes in the pyrimidine-rich strand indicate that side chains from CGP64222 are in proximity to residues C37 to C41. Clear differences between peptide and CGP64222 interactions with TAR RNA were observed in the pyrimidine-rich strand opposite the bulge (C37 to C41). Since CGP64222 should be more flexible than ADP-1, it seems likely that recognition of the pyrimidine-rich strand by interaction with the phosphodiester backbone accounts for the enhanced binding properties of the new compound.

Since only the first six residues of CGP64222 are required for full activity, and the first five provide the majority of the binding energy (data not shown), the modeling of the compound was confined to the five selected peptoid residues. The model building showed that if the guanidinium group of residue C inserts into the binding pocket surrounding G26, the guanidinium group of residue B is able to reach the critical P22 and P23 phosphates (24). Residues A and E of CGP64222 were assumed to be responsible for the interactions with the pyrimidine-rich strand. Residue E was constrained to be in the proximity of the phosphates of residues C37 and U38, while the unusually long side chain of residue A permits direct contact to the distant U40-C41 phosphates. Since this contact would not be possible with a lysine side chain at this position, this

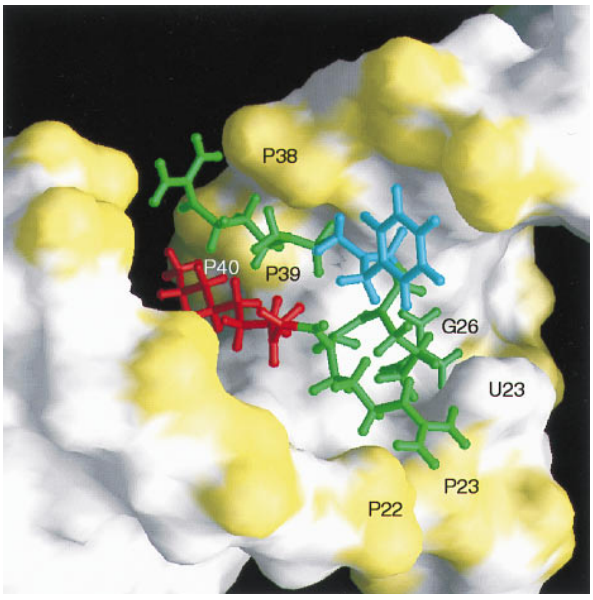

FIG. 3. Model for the peptoid–TAR RNA complex. The five essential residues of the peptoid bind the major groove of TAR RNA. The three Narg residues are in green, the Nphe residue is in blue, and the Nahg residue is in red. The TAR RNA structure is shown as a surface representation with the negative charges (primarily associated with the backbone phosphates) highlighted in yellow. Note the high occupancy of the groove, with the extended Nahg residue reaching across the duplex to make contact with phosphates opposite the bulge.

observation may explain why an aminohexyl side chain was selected at position A. Thus, the model shown in Fig. 3 demonstrates that it is both sterically possible and energetically favorable to fit CGP64222 into the major groove of TAR RNA.

Inhibition of Tat Activity in Cellular Trans-activation Assays. The antiviral activity of CGP64222 was tested in cellular assay systems designed to measure its ability to inhibit either viral membrane fusion activity and cell entry or HIV-LTR function. Fig. 4 shows the activity of CGP64222 and a control peptide carrying five L-Arg residues and the sequence of D-amino acids found in CGP64222 (R-R-R-R-R-k-k-r-p*; Arg5) in a novel assay called the fusion-induced gene stimulation (FIGS) assay (17). In the FIGS assay, an adherent CD4-positive indicator cell line (SX22–1) is cocultivated with HUT_{4-3} lymphocytes that are chronically infected by HIV-1. Mixing the two cell lines results in a rapid development of extended syncytia due to Env-CD4-mediated membrane fusion. The SX22–1 cell line also carries an integrated *lacZ* reporter gene under the control of the HIV-1 LTR. Blue syncytia, indicating foci of active HIV replication and Tat expression, can be easily detected after staining the cells with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside). The FIGS assay system is a very useful tool to profile inhibitors of HIV replication, since it permits inhibitors of HIV entry and inhibitors of LTR activation to be distinguished (17).

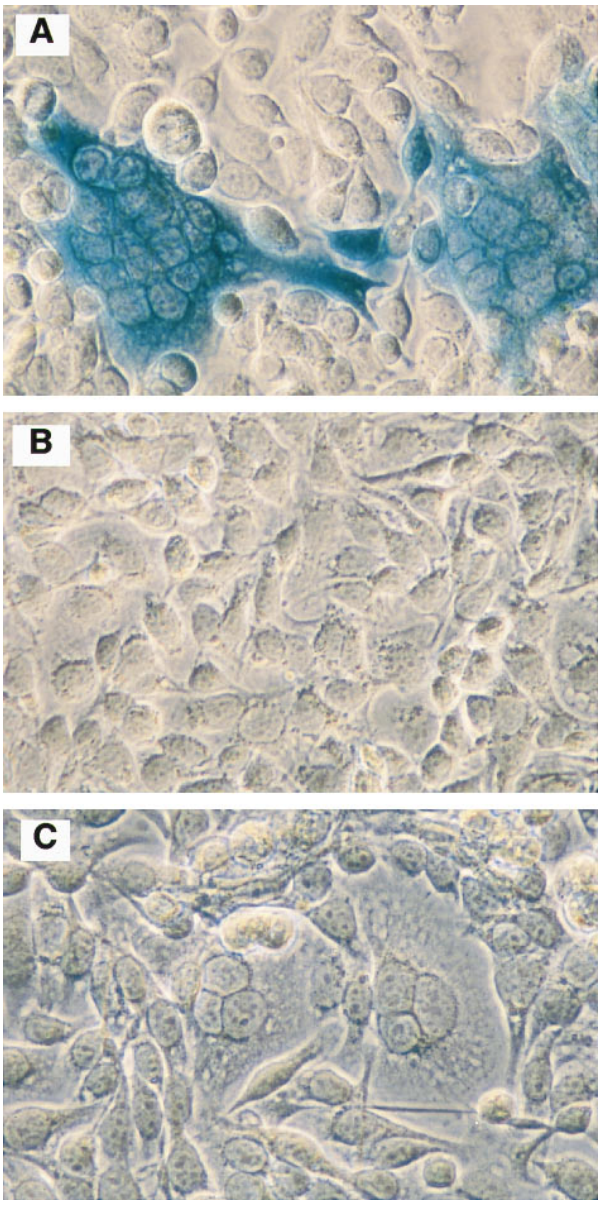

FIG. 4. Evaluation of CGP64222 in the FIGS assay. Tat activity was assessed 24 hr after coculture of chronically HIV-1-infected HUT_{4-3} cells with SX22–1 indicator cells. (×1000.) (*A*) Untreated control cells showing syncytium formation and the subsequent activation of the *lacZ* gene by Tat. (*B*) Cells treated with 10 μM Arg5 peptide. Note that fusion is inhibited. (*C*) Cells treated with 10 μM CGP64222. Note that generation of syncytia is normal, but Tat activity is completely suppressed.

As expected, untreated control cultures contained a high number of blue syncytia (Fig. 4*A*). Addition of the Arg5 peptide strongly inhibited cell fusion and resulted in a suppression of syncytia formation. Since Tat protein was not transferred from the infected donor cells to the reporter gene in the indicator cells, there was no induction of β-galactosidase (Fig. 4*B*). In contrast to the nonspecific inhibition of cell fusion by the Arg5 peptide, CGP64222 was able to prevent β-galactosidase synthesis in the extended syncytia. As shown in Fig. 4*C*, cultures treated with 10 μM CGP64222 contain numerous colorless syncytia with 10, or more, nuclei. The IC_{50} for CGP64222 in the FIGS assay is 3–5 μM. The compound remains an effective inhibitor of trans-activation at concentrations up 100 μM, although there is also some inhibition of cell fusion observed at concentrations greater than 30 μM. No cytotoxic or cytostatic changes were noted for CGP64222 at concentrations of up to 100 μM (data not shown). Since Tat

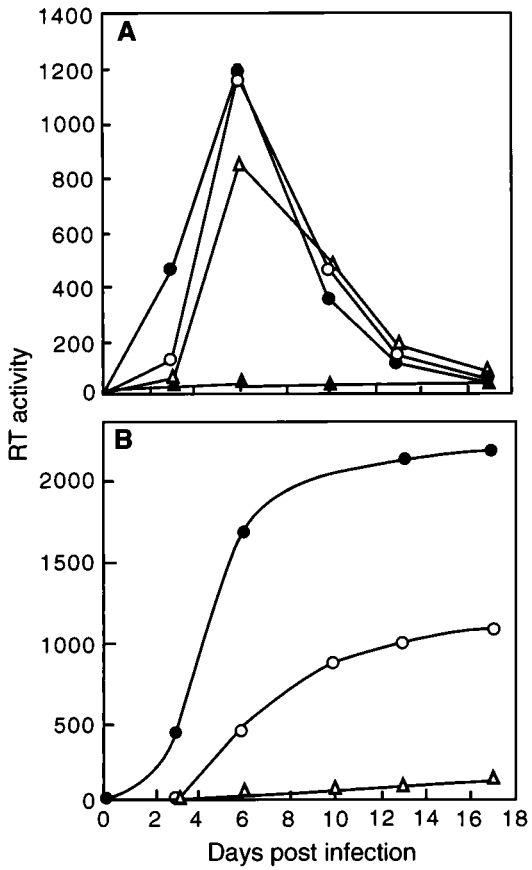

FIG. 5. (*A*) Effect of CGP64222 on HIV-1_{LAV} replication in human lymphocytes. Supernant RT activity was determined on days 3, 6, 10, 13, and 17. Each data point represents the mean of 6 replicates (variation <10%). ●, Untreated cells; ○, 3 μM CGP64222; △, 10 μM CGP64222; ▲, 30 μM CGP64222. (*B*) Effect of CGP64222 and the Arg5 peptide on HIV-1_{LAV} replication in human lymphocytes. Cumulative supernatant RT activity from days 3, 6, 10, 13, and 17 is shown. Data represent the mean values of 6 replicates (variation <10%). ●, Untreated cells; ○, 30 μM Arg5 peptide; △, 30 μM CGP64222.

protein is readily provided in this system by the donor cell, the failure to synthesize β-galactosidase must be due to inhibiton of Tat activity by CGP64222.

Effect of CGP64222 on HIV Replication in Human Lymphocytes. Fig. 5*A* shows the effect of CGP64222 on HIV-1_{LAV} replication in acutely infected cells. The compound was added at 3, 10, and 30 μM (without uptake enhancers, such as Lipofectin) to primary human lymphocytes that had been infected 6 hr previously with HIV-1_{LAV}. As shown in Fig. 5*A*, 30 μM CGP64222 totally suppressed the production of supernatant RT. Partial suppression of RT production was observed when the compound was added at the lower concentrations. At each of the concentrations tested, CGP64222 did not inhibit cell viability or the proliferation capacity of the lymphocytes (data not shown).

The control Arg5 peptide also showed an inhibitory effect on HIV-1 growth in primary lymphocytes. However, as shown in Fig. 5*B*, while the Arg5 peptide achieved approximately 50% inhibition of RT production at a concentration of 30 μM, complete suppression of HIV replication is observed with an equivalent concentration of CGP64222.

CONCLUSION

The search for drugs that can block trans-activation started several years ago and used a variety of cellular and *in vitro* screens, but none of the inhibitors identified in these screens blocked the Tat/TAR RNA interaction (25, 26). Using a combinatorial approach, we were able to identify a peptidic compound, CGP64222, that is able to effectively compete with Tat binding for TAR RNA. NMR analysis shows that CGP64222 binds directly to TAR RNA at the Tat-binding site. The peptoid derivative is a biologically stable lead that displays significantly higher potency than its peptidic analogue, Arg5.

Thus, CGP64222 is to our knowledge the first example of an antiviral compound that selectively inhibits a protein/RNA interaction. In addition to the Tat/TAR RNA interaction there are numerous viral and cellular processes that depend on critical protein/RNA interactions. As the chemistry for synthesizing small molecules capable of recognizing specific RNA sequences becomes more refined, RNA targets will find increasing importance in drug discovery.

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