Blocking HIV replication by targeting Tat protein

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Background: A rapid development of viral drug resistance poses a serious limitation in the current drug development programs against HIV. In turn, this obstacle forms the basis for new efforts, which utilize alternative viral targets.

Results: By aiming at the Tat-driven process of HIV gene regulation, we discovered a new class of compounds as well as a novel target. The candidate compound acts on the one hand by classically inhibiting Tat/TAR complexation, however, without binding to nucleic acids.

Conclusions: Structure and molecular modeling/dynamics suggest that the stilbene derivative CGA137053 directly binds to Tat protein but not TAR RNA. As a completely new, second property, the compound also antagonizes a TAR-independent activity of free Tat protein by preventing the recently described upregulation of the HIV coreceptor CXCR4. With the stilbene CGA137053, we have identified a potent, double-hitting and chemically feasible Tat antagonist. The compound possesses high target specificity and low cytotoxicity, is not restricted to the Tat/TAR axis of HIV inhibition and highly active on HIV-infected, primary human cells.

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Introduction

The majority of therapeutically active chemicals target proteins, such as receptors or enzymes. Although a number of drugs targeting nucleic acids have been known, e.g. in the chemotherapy of cancer, only few attempts have been reported which directly target the interaction of a protein with a specific nucleic acid. With the rapid increase of knowledge about genes involved in establishment, progression or control of diseases, this new concept for therapeutic intervention is beginning to raise pharmaceutical interest. Successful examples have recently been described [1], which interfere with HIV-1 replication through a block of viral transcription. Specific disruption of protein/nucleic acid interactions, occurring directly on the viral long terminal repeat (LTR), was achieved using synthetic molecules. The interaction of cellular transcription factors with *cis*-DNA elements was blocked through the use of polyamide molecules designed to recognize and bind to the minor groove of those short DNA sequences [1]. Similarly, other studies reported de novo designed substances that were binding to the viral TAR element (Tat-responsive RNA), and prevented complex formation with the transactivator Tat thereby blocking HIV replication [2–4]. In both approaches, the described compounds bound the nucleic acid partner of the complex. Pharmaceutically, a nucleic acid target is highly delicate with respect to selectivity and reversibility of binding, and e.g. cancer chemotherapy, often utilizing DNA-binding agents, provides examples of deleterious side effects (mutagenicity, severe toxicity, etc.). One way of circumventing this drawback is to consider the facing partner of such a 'molecular tango'. Indeed, in the molecular recognition process between the nucleic acid-binding site of a protein and the protein-binding region of a nucleic acid, each can be seen as 'receptor' or 'ligand'. The finding of small molecular weight inhibitors should therefore not be restricted to one or the other. Corollary, appropriate assay design should allow identification of both types of molecules. Accordingly, a random screen, designed for a search of inhibitors of the Tat/TAR interaction in vitro, led to the discovery of a new class of potent compounds, which interestingly have no affinity for TAR RNA.

Results

In vitro competition of Tat/TAR complex formation

The high throughput screen (F.H. and T.K., manuscript in preparation) identified a peculiar, structurally related series of compounds. Briefly, in this assay, a biotinylated TAR-binding peptoid (described in Hamy et al. [2]), bound to streptavidin plates, was reacted with Eu-labelled TAR RNA. This interaction was competed by the respective compounds, and decrease of bound Eu label was scored.

The most active compound of the identified class was CGA137053 (Figure 1). As a remarkable feature, this structure does not have apparent homology with any of the previously reported Tat/TAR inhibitors [2–4]. The most striking difference to cationic RNA-binding inhibitors was that CGA137053 is negatively charged at physiological pH through the presence of two sulfonate groups ($pK_{a1} = -0.83$; $pK_{a2} = -1.57$). In the screening effort of the corporate library (> 200 000 entities, hit-rate of 0.02%), the vast majority of compounds was inactive, providing an easy starting point for the identification of appropriate negative controls. As shown in Figure 1, ba12330 reflects the one structurally closest to the active substance.

This compound pair was first profiled for inhibition of Tat/TAR complex formation by competition electromobility shift assay (EMSA). In this assay format, the amount of recombinant Tat protein was adjusted to shift 50% of the radiolabelled TAR (Figure 2a, lane '−Tat') into a complex, visible as gel-retarded form (Figure 2a, lane '0'). Increasing amounts of inhibitor (as indicated) were then added to this pre-formed complex and subjected to electrophoresis. As can be estimated from the autoradiograph in Figure 2a, CGA137053 was active with a CD_{50} (concentration of compound required for decreasing the retarded complex by half) in the low nanomolar range. The concentration range was found to be close to stoichiometric with the input amount of the reaction partners Tat (20 nM) and TAR (12.5 nM). In contrast, the CD_{50} value of ba12330 was at least 40-fold higher. It is also interesting to note from the autoradiographs that compound inhibition did not coincide with a detectable 'direct shift', which would have been an indicator for a direct contact between compound and TAR RNA. This phenomenon, attributed to a change of conformation of RNA upon drug-binding, was usually quite apparent for previously profiled TAR binders [2–4]. Using also other methodologies, no direct RNA-binding could be detected (RNase A foot-printing or measurements of differential melting temperature, data not shown).

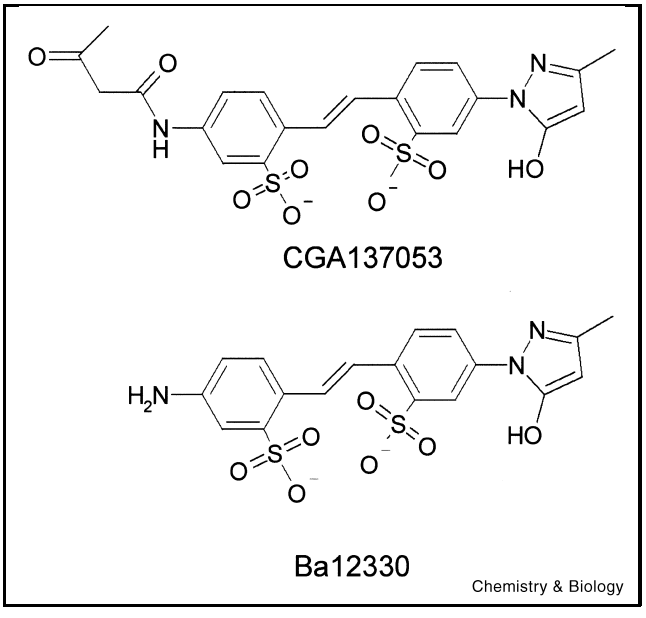


Figure 1. Structures of sulfonated stilbene compounds found to possess potent activity against HIV Tat (CGA137053) or to serve as related negative control (ba12330).

Our experimental setup involved only three reaction partners: purified recombinant Tat, synthetic TAR and the respective compound. Such an assay simplicity prompted us to further investigate the hypothesis that the negatively charged compounds could indeed interact with Tat.

Dynamics of Tat/TAR inhibition

These preliminary clues towards Tat-binding of the compound led us to investigate whether the order of addition of reactants would have an influence on the experimental outcome. For this, two of the three reaction partners (i.e. Tat plus inhibitor or TAR plus inhibitor) were premixed and incubated prior to the addition of the third (Figure 2b). Non-denaturing electrophoresis was performed immediately before the binding reaction had reached equilibrium (5 min at 0°C). A remarkable difference between the two conditions was observed: preincubation of Tat protein plus compound (Figure 2b, right-hand side) was > 10-fold more potent in blocking Tat/TAR complex formation than when compound was added after Tat/TAR complexation (Figure 2b, lanes on the left). In summary, compound concentrations as low as 3 nM abrogated the ability of Tat to form a complex with TAR.

Tat antagonism inside cells

Inhibition of Tat transactivation in cells

In order to prove that disruption of the Tat/TAR complex can translate into a specific inhibition of Tat-driven transactivation even inside the intact cell, a standard reporter cell assay [5] was performed. Briefly, in this stable LTR activation cell system (SX22-1 cells), native Tat protein is introduced via cocultivation with the constitutively HIV-1 producing lymphocyte line (Hut/4-3). The setup relies upon gp120/CD4-mediated virus entry. The HIV typical membrane fusion leads to the generation of giant, multinucleated fusion products (syncytia), which readily stain blue with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) only in the presence of effective LTR transactivation by Tat. Hence it strictly depends on the Tat/TAR interaction. The active compound CGA137053 induced white syncytia, demonstrating Env-mediated cell fusion but no LTR activation, as depicted in Figure 3b. The presence of extended syncytia was also a direct proof that the inhibitors did not act at the fusion level as generally seen for anionic entities [5] (oligonucleotides, dextran sulfate, etc.). In contrast, the control compound ba12330 did not have any

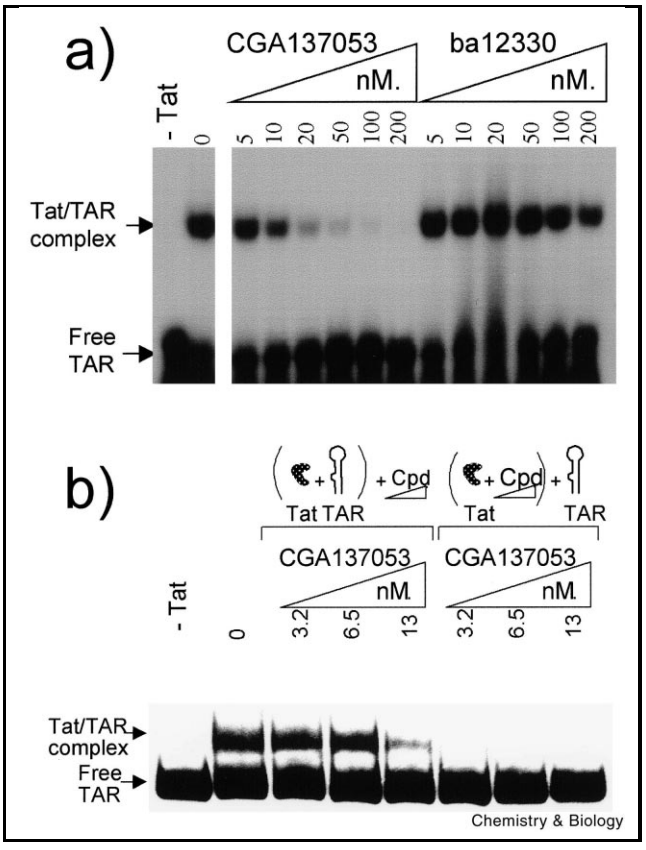


Figure 2. In vitro inhibition of Tat/TAR complex formation. **(a)** Radiograph of an EMSA with ^{32}P-labelled TAR and recombinant Tat in the presence of compound concentrations in nM as indicated. Lane '−Tat' indicates control lane without compound. **(b)** Dynamics of Tat/TAR complex formation. Mobility shift assay of Tat and TAT with or without CGA137053: 'order of addition' experiment in which the reaction partners are combined in the order as depicted above the graph (described in the text).

influence on β-galactosidase (β-Gal) induction (Figure 3a), and blue syncytia indicate that cell fusion was unaffected. Figure 3c shows a graphical representation of the quantified LTR activity through the β-Gal-mediated conversion of *ortho*-nitro-phenyl-galactopyranoside (ONPG) to *ortho*-nitro-phenol (ONP) (described in Klimkait et al. [5]).

Already at 3 μM concentration of CGA137053, a significant reduction of LTR activity was consistently observed, and at 10 μM more than 95% of the Tat activity was inhibited, proving that the compound had readily entered the cell. The control compound ba12330 showed no Tat-inhibitory activity. It is noteworthy that even at 100 μM none of the compounds showed any sign of cytotoxicity in the cell lines tested (not shown).

To further address the compound's specificity and cell entry, we analyzed HIV-inhibitory activity also following DNA transfection into SX22-1 cells of either the complete proviral clone pNL4-3 (Figure 3d, left panel) or the Tat expression plasmid pTat/Rev (Figure 3d, right panel). By microscopic inspection of blue events (not shown) and enzymatic quantification, Tat-specific inhibition was observed only with CGA137053, but no activity was seen with the ba12330 control (Figure 3d). When a constitutively active CMV-lacZ plasmid was transfected, no inhibition was observed with either compound up to the highest tested concentration of 30 μM. Moreover, also *trans*-regulated reporters such as androgen- or estrogen-responsive constructs (ARE-luc, ERE-tk-luc) remained unaffected by the stilbenes (not shown).

These cellular experiments match the biochemical data of activity and selectivity; they further demonstrated that CGA137053 can specifically block complex formation of Tat and TAR intracellularly and subsequently prevent HIV-1 gene activation.

Antagonism of free Tat protein

In addition to an infection-associated activity of Tat, a role of free Tat protein has been implicated in HIV-induced pathogenesis. Some of these extra activities have recently been characterized (reviewed in Gallo [6]), one of them in the induction of CXCR4 coreceptor expression [7]. We chose reverse transcriptase (RT)-PCR to probe for a possible antagonism also of this function of Tat. As shown in Figure 4, CXCR4 mRNA becomes readily detectable in fresh, unstimulated human peripheral blood lymphocytes (PBLs) after the exposure to recombinant Tat protein (100 ng/ml) (lane 2), or after stimulation with phytohemagglutinin (PHA) (1%) (not shown). The Tat-driven induction is suppressed by the addition of e.g. 10 μM CGA137053 (lane 3) but not with ba12330 at the same concentration (lane 4). Tat specificity of this inhibition is supported by the observation that PHA-stimulated CXCR4 expression was not affected by CGA137053 (lane 5). In addition, the endogenous RNA levels for β-actin remained constant even when Tat or PHA were added, irrespective of the presence of either stilbene (not shown).

Inhibition of HIV replication

In order to test whether the Tat antagonistic activity could further be extended to blocking viral replication in clinically relevant cells, we performed HIV-1 infection experiments of human PBLs from healthy donors. Figure 5a shows the graphical representation of viral replication profiles, which can be measured as HIV-specific RT activity in the culture supernatant, since this viral enzyme is strictly particle-associated. Again, CGA137053 clearly showed a dose-dependent inhibition of HIV-1 replication, and 30 μM achieved fully suppressive activity (Figure 5b). In in vitro differentiated macrophages, another clinically highly relevant infection system, again a dose-dependent inhibition was observed with suppression of $>95\%$ at 30 μM

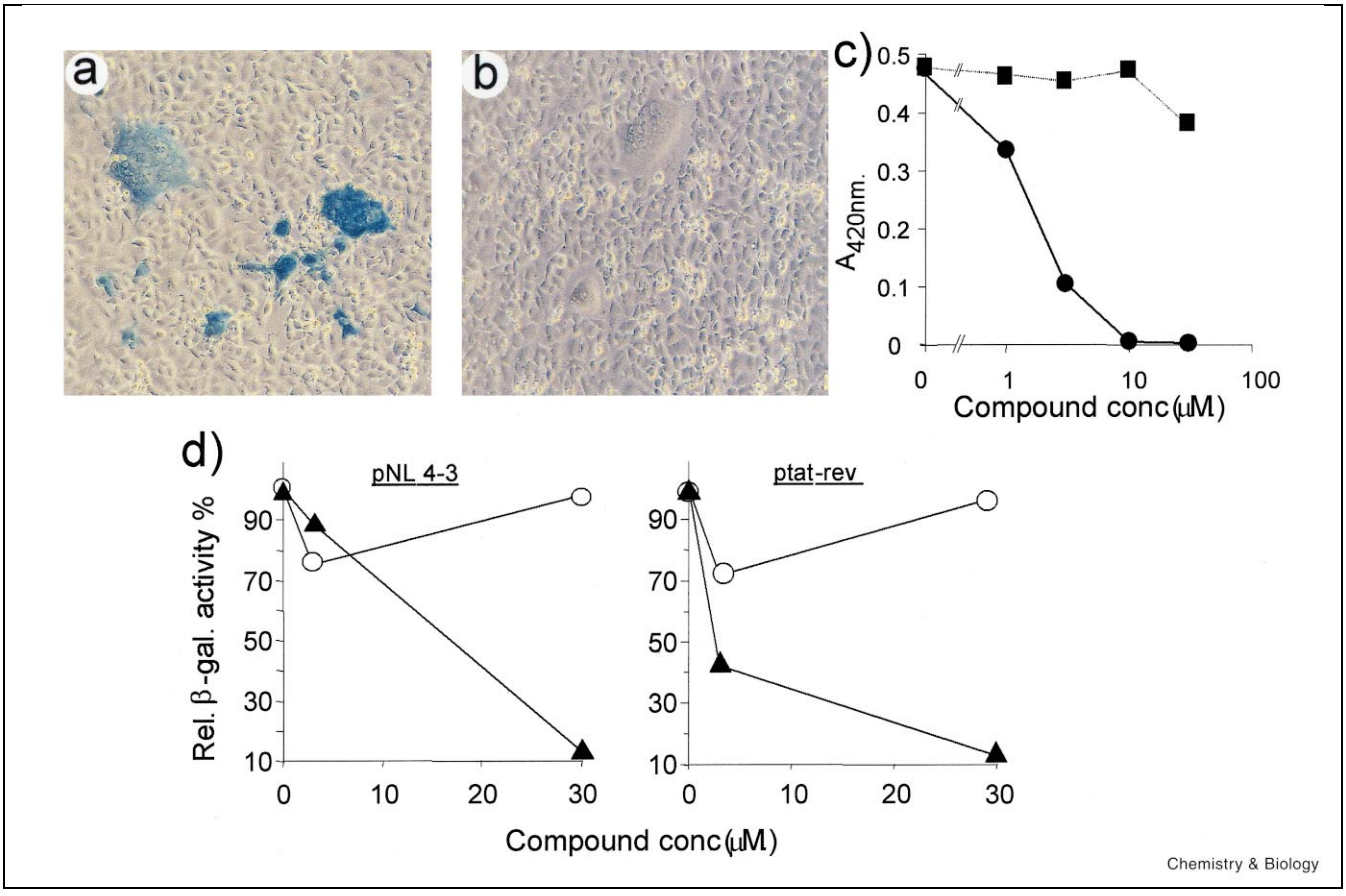


Figure 3. Cellular anti-Tat activity of CGA137053 and ba12330. Panel **(a)** depicts the coculture of indicator plus HIV^+ cells in the presence of 10 μM control compound ba12330 with numerous blue syncytia. Panel **(b)** shows the parallel culture treated with CGA137053 at 10 μM final concentration. Extended white syncytia are indicated by arrows. Panel **(c)**, the ONP-reading of cells cultured in ascending concentrations (0–30 μM) of the compounds: (●), CGA137053; (■), ba12330. Panel **(d)**, demonstration of Tat effect from 'within' cells. Indicator cells were transfected with DNA of either the complete proviral HIV-1 clone pNL4-3 or the Tat plasmid pTat/Rev as indicated. Graphs depict Tat-mediated LTR induction (relative β-Gal activity) as a function of compound concentration (0–30 μM): (▲), CGA137053; (○), ba12330.

concentrations of CGA137053 19 days after infection (Figure 5a). This block of viral replication was reproduced with various HIV-1 and even HIV-2 or SIV strains ($HIV\text{-}1_{IIIB,\ MN,\ RF,\ EHO}$, $HIV\text{-}2_{ROD}$, SIV_{mac251}) with EC_{90} ranging from 0.5 to 5 μM (details to be published elsewhere). The ba12330 control was inactive at 30 μM in all infection systems; the possibility that the compounds could have a direct impact on the assay read out was ruled out by adding the substances directly to the RT test itself (not shown).

Discussion

The compound CGA137053, identified by high throughput screening, showed to be highly potent in competing with Tat/TAR complex formation in vitro. The observed active concentration suggests that it occurs close to a 1:1 molar ratio of Tat or TAR in the reaction. This can be explained by the K_d for the compound towards its recognition partner (Tat) to be below the K_d of the authentic partner (TAR). Such an affinity was never observed for compounds that we had previously reported as strong and sequence-specific TAR binders [2–4].

Our findings regarding the dynamics of Tat/TAR inhibition in vitro show that activity of CGA137053 was enhanced by more than 10-fold when protein and compound were preincubated before an equilibrium with TAR RNA had occurred. This finding was further corroborated by the fact that several classical methods to assess TAR-binding inhibitors, such as melting temperature or linear dichroism or RNase A foot-printing, failed to show direct interaction of the compound with RNA (not shown). Thus the simplest explanation to the observed inhibitory profile of the compound is the unique finding of a direct (TAR-mimicking) interaction with Tat protein.

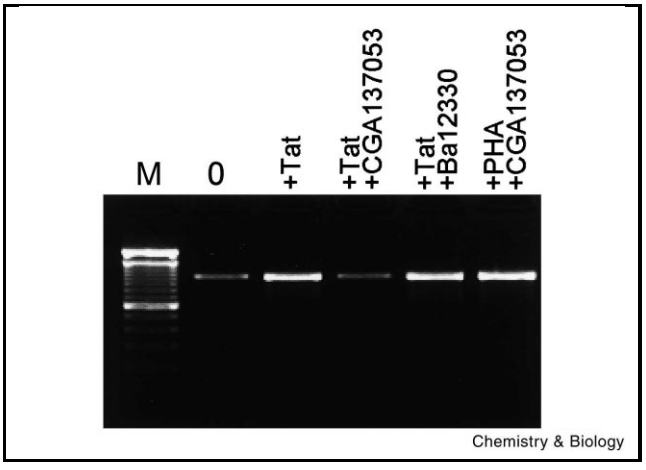
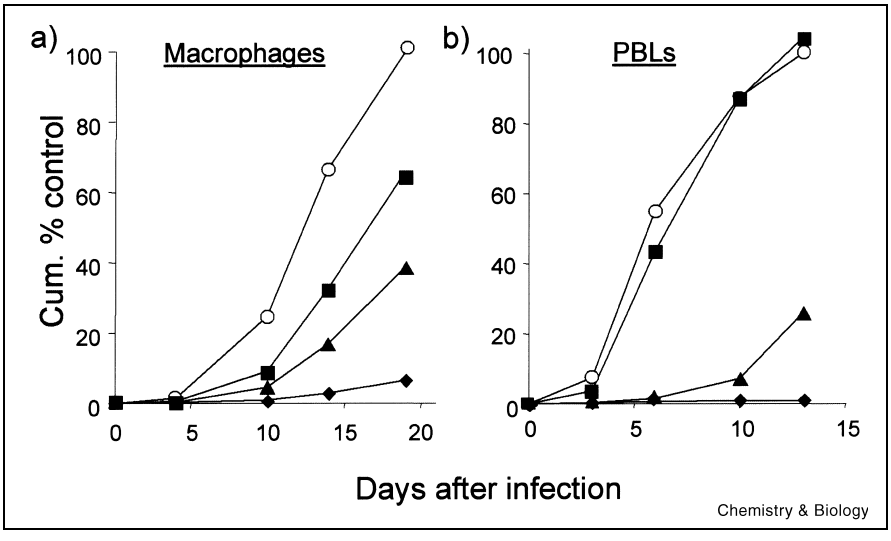


Figure 4. Inhibitory activity on Tat-mediated CXCR4 gene expression. RT-PCR was performed on mRNA from unstimulated PBLs in the absence of Tat and compound (lane '0'), in presence of 100 ng/ml Tat or 1% PHA and/or 10 μM compounds as indicated. 'M' indicates size marker lane.

Although the small series of related compounds available is undoubtedly too limited to allow specific conclusions about a structure/activity relationship, we analyzed for possible hallmarks in the structure of the compounds that could help to explain the observed activity. Active compound CGA137053 (Figure 1) can be defined as a di-sulfonated stilbene moiety linked to a pyrrazole ring on one side and to a mono-substituted amine on the other side. Substructure searches of the corporate compound library revealed that alterations in either one of the two negative charges or in the spacing between them yielded inactive compounds. The same was observed for substitutions of the pyrrazole moiety. Therefore (and prior to the availability of a comprehensive SAR analysis), we generated a speculative 'model', which provides the simplest explanation for our findings. As one important feature for molecular recognition is often contributed by electrostatic interactions, we first propose the two negative charges of the sulfonate groups and the strong nucleophilic pyrrazolic nitrogen to be candidates for the constitution of an electrostatic pharmacophore. A Monte Carlo search for a minimal energy conformation on CGA137053 yielded the structure depicted in Figure 6b. Distances and angles between the three atomic groups of the above-defined pharmacophore are summarized in Figure 6. As experimental data suggested that the compound mimics TAR RNA for the binding to Tat, we scrutinized the available three-dimensional structures of (arginine-bound) TAR to identify characteristics possibly matching the suggested pharmacophore. Figure 6a depicts the three-dimensional representation of the bulge region defining the Tat-binding site of TAR RNA, as published by Aboul-Ela et al. [8]. In this model, atomic groups previously reported as central actors of the Tat/TAR interaction [9,10] are highlighted: the heterocyclic N7 of guanine G26 and phosphate groups in the nucleic backbone of the unpaired residues. Distances and angles between these atomic groups were measured and averaged from the 20 different structures determined by nuclear magnetic resonance. We could find that nature and geometry of these could be precisely mimicked by distinct groups within CGA137053 with matching spatial display and electrostatic characteristics. This allowed us to speculate that (i) the nucleophilic/H-bond acceptor N7 (shown to interact with the guanido group of an arginine residue of Tat [8,10]) could be advantageously mimicked by the even more nucleophilic nitrogen of the OH-pyrrazole moiety, and (ii) the two sulfonate groups could possibly mimic phosphates of the RNA backbone (possibly P25 and P26, shown in Figure 6a). As the RNA-binding region of Tat

Figure 5. Antiviral activity of CGA137053 in primary human leukocytes (PBL) **(a)** and macrophages **(b)**. After viral adsorption, compound was added to the infected cultures at the following concentrations: untreated control (○), 3 μM (■), 10 μM (▲) and 30 μM (◆). Supernatant RT activity as a function of time post infection was determined at the indicated time points.



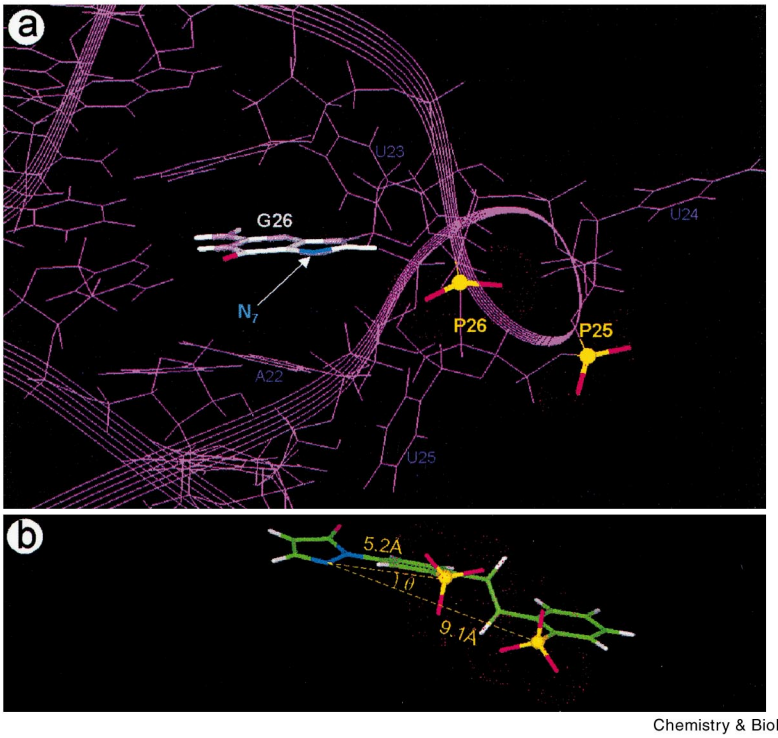


Figure 6. (a) Three-dimensional view of the major groove of bound TAR RNA (coordinates from [7]). Atomic groups critical for Tat-binding [9,10] are highlighted. **(b)** Energy-minimized conformation of CGA137053.

has been previously reported to have a certain degree of flexibility [11], we suggest that our class of compounds is readily recognized by Tat protein which is then titrated out of its interaction with TAR RNA. As a further extension to our hypothesis, the explanation why ba12330 is not active could possibly come from the presence of a primary amine, which may conflict in the interaction with the basic, Arg-rich TAR-binding domain of Tat.

Compounds with negative charges (e.g. sulfonated/phosphonated distamycin conjugates) have been reported to inhibit HIV replication in primary cells as well as LTR activation [12] in cellular settings similar to ours. These molecules had at that time been postulated to mainly act at the fusion level. Unfortunately, no data on any direct in vitro inhibition of Tat/TAR complex formation were reported. Our assays clearly demonstrate that the compounds described here act at the Tat/TAR level in a way which is distinct from an effect on fusion. Furthermore, we compared dextran sulfate, which shows a completely different activity profile in our assays in that we could not show any in vitro inhibition of Tat/TAR-binding, but consistently find a predominant effect on fusion (not shown). In turn we do not exclude that our compounds may have an additional beneficial activity outside the cell. Indeed, Tat has been demonstrated to possess diverse activities on cells, which do not involve the recognition of TAR (reviewed by Gallo [6]). Among other activities, extracellular Tat, possibly secreted from infected cells or released from cellular debris of disintegrating cells, has been discussed to promote HIV infection through an induction of HIV co-receptor expression, such as CXCR4 or CCR5 [7]. Since the population of activated and thereby infectable lymphocytes is only a minor fraction of the whole population, this second activity of Tat would provide a clever way to prepare the T-cells for efficient infection by the virus [13]. A simian anti-Tat vaccine has very recently been reported to work through inhibition of pathways presumably involving a role of free Tat [13]. Accordingly, the Tat-interacting stilbene compound described here could also have a favorable, inhibitory impact on these unwanted other activities of Tat. This way, the compound class may function as potent 'double-hitting' Tat antagonist, and those properties could undoubtedly make a great impact on novel anti-HIV therapy concepts.

Our observation of a very favorable cytotoxicity profile (up to mM concentrations) correlates well with the absence of toxic effects of sulfonated diaminostilbene derivatives in vertebrates [14].

Significance

For DNA- or RNA-binding drugs (kanamycin, adriamycin, *cis*-Pt, bleomycin, etc.), the therapeutic window is usually very narrow with high cytotoxicity, inherent to their mechanism of action, namely targeting genetic material. In turn,

the fact that the sulfonated stilbene compounds, described here, indeed bind to the protein partner (Tat) of a protein/nucleic acid complex may explain their surprisingly low toxicity in cellular systems. The presented profile of a 'lead compound' could serve as a starting point for further synthetic optimization by medicinal chemistry. As more disease-associated processes involving protein/nucleic acid interactions are emerging, new concepts as the one described above could open new drug strategies with implications far beyond anti-HIV therapies. Additional studies will be necessary to further precise the interaction of the identified compound class with Tat protein.

Materials and methods

Synthetic TAR sequences

TAR duplex was prepared by annealing the two synthetic oligoribonucleotides: T-14 (5′-GCUGCUCUCUGGCU-3′) and T-17 (5′-AGCCAGAUUUGAGCAGC-3′) (Genset, Paris, France). The crude oligoribonucleotides were purified on 20% polyacrylamide gels containing 8 M urea. The 14-mer strand of the synthetic duplex TAR RNA was labelled with T4 polynucleotide kinase using [γ-^{32}P]ATP and annealed in the presence of 1.5 equivalents of the unlabelled 17-mer by heating the mixture to 90°C for 3 min, followed by slow cooling to 0°C with a rate of 1°C per min.

For experiments on the dynamics of binding, full-length TAR RNA (60 nucleotides) was obtained from in vitro transcription as follows. Synthetic oligonucleotides corresponding to the wild-type TAR sequences were cloned between the *Hin*dIII and *Eco*RI sites of the pUC19 plasmid. After digestion with *Eco*RI, the RNA was transcribed as a run-off product of 60 nucleotides from the T3 RNA polymerase promoter. In each case, the transcript included an additional G residue on the 3′-end derived from the *Eco*RI cleavage site. Transcription reaction was performed in buffer containing 40 mM Tris–HCl, pH 7.4, 25 mM NaCl, 16 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 1 mM NTPs. The reaction was initiated by addition of 10 μg linearized plasmid DNA template and 40 U T3 RNA polymerase and incubated for 2 h at 37°C. Nucleic acids were then fractionated on a 10% (w/v) polyacrylamide gel containing 8 M urea in TBE buffer (89 mM Tris–borate pH 8.3, 10 mM EDTA). After electrophoresis, the RNA was eluted in water for 18 h at 4°C and precipitated with ethanol. The RNA was resuspended in water to give a 500 μM stock solution (ε^{260}/phosphate = 10 688 M^{-1} cm^{-1}). RNA was 3′-end-labelled with [^{32}P]cytidine biphosphate and T4 RNA ligase and then repurified from a 10% denaturing acrylamide gel.

EMSA

Binding reactions (25 μl) contained 500 fmol (10 000 cpm) of the labelled duplex TAR RNA, 20 nM of recombinant Tat protein (Actigen, Cambridge, UK) and varying concentrations of test compound in TK buffer (20 mM Tris–HCl, pH 8.0, 50 mM KCl) with 10 mM DTT, 0.1% Triton X-100. Order of addition and incubation conditions are described within the Results. The binding reactions were analyzed by electrophoresis on 8% non-denaturing polyacrylamide gels as described [15]. Following electrophoresis, the gels were either dried and exposed on X-ray film at −70°C for 16 h, or analyzed on a Phosphorimager (Molecular Dynamics).

Fusion-induced gene stimulation (FIGS)

Coculture. The HeLa CD4 cell line SX22-1 (carrying a lacZ gene under the control of the HIV-1 LTR) was cocultivated with the stably HIV-1-infected lymphocyte line Hut/4-3 at a ratio of 3:1 [5]. After overnight incubation, which permits syncytium formation and β-Gal synthesis, the cells were washed once with phosphate-buffered saline (PBS), fixed with glutaraldehyde/formaldehyde (0.1%/0.7%) in PBS for 5 min and stained for cell-associated β-Gal by using X-Gal as substrate. For photometric quantification, the fixed cultures were subsequently incubated with ONPG as β-Gal substrate, which is enzymatically converted to the chromophoric ONP and absorption was measured at 405 nm.

Transfection FIGS. Alternatively, seeded SX22-1 cells were transfected on the day following compound addition with plasmid DNA of either the complete, infectious HIV-1 clone pNL4-3, or with the deletion construct pTat/Rev (described in Klimkait et al. [5]). The latter, non-infectious plasmid contains deletions of *gag* between a unique *Spe*I site (1507) and *Eco*RI (5741), and of *env* between *Kpn*I (6344) and *Hin*dIII (8131), but preserves the splice sites for the *tat* and *rev* genes. Amounts of 1.2 μg were transfected using the Fugene (Boehringer Mannheim, Germany) or Transfast protocol (Promega, Madison, WI, USA). Two days after transfection, cells were fixed and assayed.

Infection of primary cells

Lymphocytes [16] were cultured in RPMI 1640 medium containing 10% fetal bovine serum; cells were activated for 2 days with 0.25 μg/ml PHA (Wellcome diagnostics, Temple Hill, Dartford, UK). After infection by HIV-1, the cells were resuspended in complete medium, supplemented with recombinant human interleukin 2 (Genzyme, Cambridge, MA, USA) at 100 U/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 by fresh medium every 3 days. Virus production was evaluated by determining RT activity in cell-free culture supernatants [17].

Tat induction of CXCR4 expression

Two micrograms of total RNA isolated from peripheral leukocytes from healthy human donors by using Trizol reagent (Gibco BRL, Paisley, UK) was primed with oligo dT and reverse transcribed into cDNA in a 20 μl reaction mixture containing Moloney murine leukemia virus RT ('superscript'-RT; Gibco BRL). One microliter of the cDNA reaction mixture was subjected to PCR amplification using 'Hotstar' Taq DNA polymerase (Qiagen, Hilden, Germany) in a thermocycler PE 9700 (PE/Biosystems, Rotkreuz, Switzerland). A 15 min hot start activation of the enzyme was followed by 30 cycles with denaturing at 95°C for 60 s, annealing at 60°C for 40 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 5 min. The following primers were used 5′-GTTACCATGGAGGGGATCAG-3′ and 5′-CAGATGAATGTCCACCTCGC-3′.

Molecular modeling

Coordinates for the structure of arginine-bound TAR RNA [8] were obtained from the Brookhaven protein data bank (pdb code = 1ARJ). Monte Carlo conformational search was performed on CGA137053 (2000 structures) using the Macromodel version 6.0 software package (Columbia University, New York, NY, USA) on an Octane workstation (Silicon Graphics, Mountain View, CA, USA). Graphics and measurements were done using the Insight II software (Molecular Simulation Inc., San Diego, CA, USA).

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