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Anti-Tat and anti-HIV activities of trimers of *n*-alkylglycines

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Abbreviations:
AIDS, acquired immunodeficiency syndrome
AZT, azidothymidine
CDK, cyclin-dependent kinase
CTD, C-terminal domain of the RNA polymerase II
HIV-1, human immunodeficiency virus
LTR, long terminal repeat promoter
Tat, Trans-activator of transcription
TAR, transactivating response element
VSV, vesicular stomatitis virus

ABSTRACT

Transcription of human immunodeficiency virus (HIV-1) is activated by viral Tat protein which regulates HIV–LTR transcription and elongation. In the present report, the evaluation of the anti-Tat activity of a combinatorial library composed of 5120 *N*-trialkylglycines is reported. The antiviral activity was studied through luciferase-based assays targeting the HIV-1 promoter activation induced by the HIV-1 Tat protein. We identified five peptoids with specific anti-HIV-1 Tat activity; none of these peptoids affected the binding of HIV-1 Tat protein to the viral TAR RNA. Using a recombinant-virus assay in which luciferase activity correlates with the rate of HIV-1 transcription we have detected that one of the five selected peptoids, NC37-37-15C, is a potent inhibitor of HIV-1–LTR transcription in both primary T lymphocytes and transformed cell lines. The inhibitory effect of NC37-37-15C, which is additive with azidothymidine (AZT), correlates with its ability to inhibit CTD phosphorylation and shows a suitable profile for development of novel anti-HIV-1 drugs. Likewise, the structural simplicity of *N*-alkylglycine oligomers makes these peptidomimetics amenable to structural manipulation, thus facilitating the optimisation of lead molecules for drug-like properties.

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1. Introduction

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus, type 1 (HIV-1), which is a retrovirus that enters permissive cells through cell surface receptors and following viral entry, its RNA genome is reverse transcribed into a double-stranded DNA molecule that enters the nucleus and integrates into the host chromatin [1,2]. The post-integration phase of the viral cycle preferentially occurs in activated cells and is regulated by the collaborative action of the viral regulatory protein Tat (Trans-activator of transcription) and cellular factors on the long terminal repeat promoter (LTR), which determines the extent of HIV-1 gene transcription and the level of viral replication in the infected cells [3,4]. The HIV-1-LTR promoter is approximately 640 nucleotides long and has binding sites for many cellular transcription factors and a *cis*-activating stem-loop RNA structure called transactivating response element (TAR), that represents the main binding site for the HIV-1 Tat protein [4,5]. Through interaction with TAR, Tat recruits the positive transcriptional elongation factor (p-TEFb), which phosphorylates the C-terminal domain of the RNA polymerase II [6–8]. Recruitment of p-TEFb to TAR has been proposed to be both necessary and sufficient for activation of transcription elongation from the HIV-1–LTR promoter [9–11].

Efforts to find an effective anti-HIV chemotherapy have been mainly focused on the development of chemicals targeting viral proteins, which are essential for HIV-1 replication [12]. This current antiviral therapy presents important limitations [13–15] and, therefore, the development of new anti-HIV-1 agents is focusing on novel structures and/or new action mechanisms. Thus, new molecules impairing the function of either viral or cellular proteins required for efficient HIV-1 replication should be considered in the search of new anti-HIV-1 agents. Among those proteins, the HIV-1 Tat regulatory protein represents an important target. It has been shown that small organic molecules that inhibit the HIV-1 Tat-TAR interaction, such as analogs of amino acids or nucleotides, or arginine-conjugated aminoglycosides, block HIV-1 replication in infected cells [16–19]. More recently, Lind et al. using a computational approach have screened a large chemical library for binding to TAR RNA and identified around 500 compounds that do not share common structural features [20]. Among them, phenothiazines showed anti-Tat activities at micromolar concentrations in functional cellular assays.

Peptoids rescued from libraries constructed by the split-and-mix format have been identified as ligands for membrane receptors [21–23] and have exhibited specific protein-binding activity [24]. Furthermore, the inherent conformational flexibility of these oligomers has led to their use in the disruption of protein–protein, protein–nucleic acid and ligand–receptor membrane interactions [25–27]. In this sense, the synthesis of the first library containing more than 10,000 individual *N*-alkylglycine trimers constructed under the positional scanning format was previously reported [28]. It was shown that this library was useful to identify both TRPV-1 channel and NMDA receptor open-channel blockers [29], and to discover peptoids with ''in vivo'' neuroprotectant activity [30]. A second and optimized library of trialkyl-glycines was developed [31]. This new library, containing over 5000 peptoids, was characterized by the use of primary amines bearing additional tertiary amino groups exclusively in the third amination step to circumvent possible side-reactions. The screening of this library has led to the identification of two novel and potent modulators of the multidrug resistant phenotype [32].

Here, we have aimed to identify new molecular scaffolds that define anti-Tat activity by screening a mixture-based, *N*-trialkylglycine combinatorial library composed of 5120 compounds. We identified five peptoids with specific anti-HIV-1 Tat activity being one of them a potent inhibitor of HIV-1–LTR transcription in both primary T lymphocytes and transformed cell lines.

2. Materials and methods

2.1. Synthesis of trialkylglycines-based combinatorial mixtures and of individual compounds

An optimized library of 5120 peptoids in 52 controlled mixtures was synthesized by using the positional scanning format on solid phase [31]. The mixture positions were incorporated by coupling a mixture of 22 or 16 selected primary amines with the relative ratios adjusted to yield equimolar incorporation [28]. Briefly, starting from Rink amide resin (0.7 mequiv./g; Rapp Polymere, Tuebingen, Germany), the eight-step synthetic pathway involved the initial release of the Fmoc protecting group. Thereafter, the successive steps of acylation with chloroacetic acid and diisopropylcarbodiimide, followed by the corresponding amination of the chloromethyl intermediate using the selected individual amine or the mixture of amines, were conducted. Thereafter, the products were released from the resin by using trifluoroacetic acid/dichloromethane/water cocktail, solvents were evaporated, and the residues were lyophilized and redissolved in 10% dimethyl sulfoxide at a concentration of 5 mg/ml for screening. A focused, mixture-based combinatorial library made of trimers of *N*-alkylglycines in a positional scanning format was screened to identify anti-Tat compounds. The library consisted of three sublibraries, each having a single position defined with one of the 20 (OXX) or 16 (XOX, XXO) primary amines used as chemical diversity source (Fig. 1), and the remaining two positions had an equimolar mixture of these amines. The rationale of using two different sets of amines was due to side reactions that occur when primary amines bearing an additional tertiary amino moiety were used for the internal or/and C-terminal positions of the trimer. Thus, none of the amines used in the design of the library for the internal and C-end positions had these additional amino groups, whereas four additional amines containing the tertiary amino moiety were added to the set used for the *N*-terminal position [31]. Individual peptoids were prepared by simultaneous multiple solid-phase synthesis following the same synthetic sequence. The purity and identity of the most active individual oligo *N*-alkylglycine compounds were determined by analytical high-performance liquid chromatography, mass spectrometry, and ^{1}H and ^{13}C NMR. Synthesized peptoids were N15-10-15C ([*N*-(2-(2′,4′-dichlorophenyl)ethyl)glycyl]-[*N*-(2-phenylethyl)glycyl]-[*N*-(2-(2′,4′-dichlorophenyl)ethyl]glycinamide); N15-37-15C ([*N*-(2-(2′,4′-dichlorophenyl)ethyl)glycyl]-[*N*-(2-(4′-

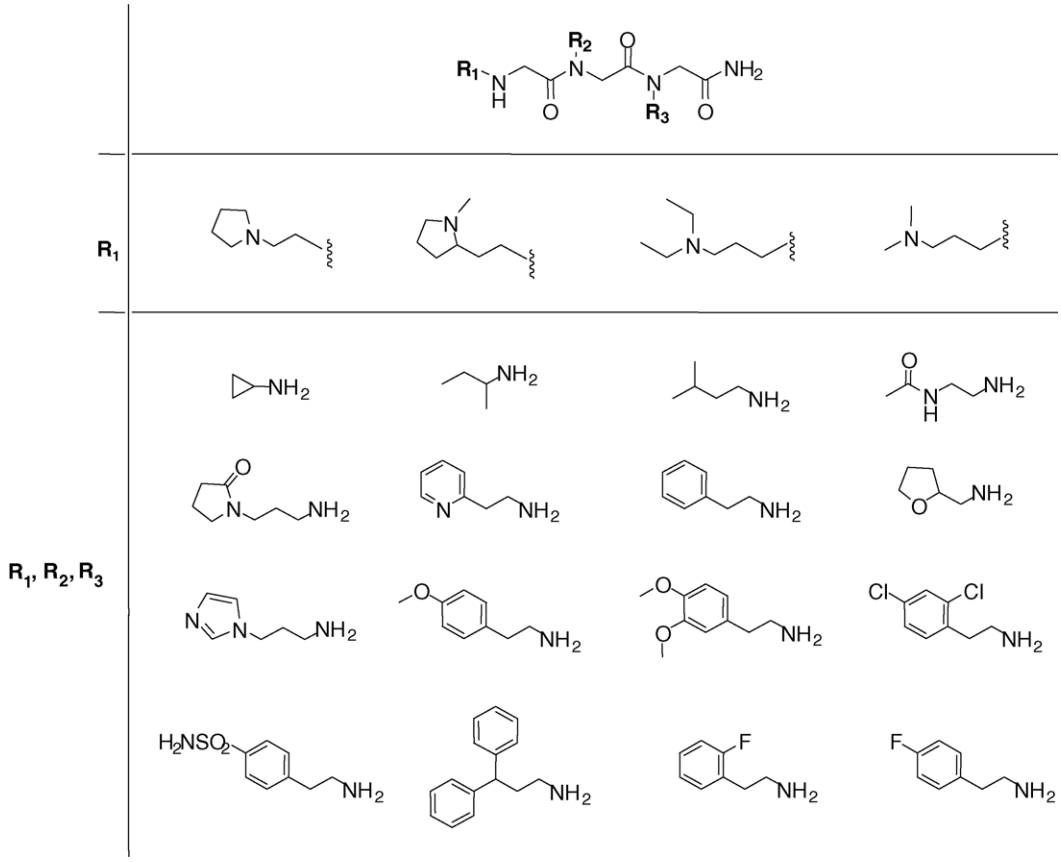

Fig. 1 – Set of amines used for the synthesis if the *N*-trialkylglycine-based combinatorial library.

fluororophenyl)ethyl)glycyl]-[*N*-(2-(2′,4′-dichlorophenyl)ethyl]-glycinamide), N37-10-15C ([*N*-(2-(4′-fluororophenyl)ethyl)glycyl]-[*N*-(2-phenylethyl)glycyl]-[*N*-(2-(2′,4′-dichlorophenyl)ethyl] glycinamide; N37-10-20C ([*N*-(2-(4′-fluororophenyl)ethyl)glycyl]-[*N*-(2-phenylethyl)glycyl]-[*N*-[3,3 (diphenyl)propyl]glycinamide); and N37-37-15C ([*N*-(2-(4′-fluororophenyl)ethyl)glycyl]-[*N*-(2-(4′-fluororophenyl)ethyl)glycyl]-[*N*-(2-(2′,4′-dichlorophenyl)ethyl]glycinamide).

2.2. *Cell lines*

Jurkat cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Invitrogen, Barcelona, Spain), containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml), and were maintained at 37 °C in a 5% CO_2 humidified atmosphere and were maintained in exponential growth. The Hela-Tat-Luc is a HeLa derived cell line stably transfected with the plasmids pLTR-Luc and pcDNA3-Tat and was described previously [33]. The construction of the Hela-Tet-On-luc cell line that has been previously reported [34] was maintained in DMEM complete medium in the presence of hygromycin and G418 (100 μg/ml).

2.3. *Luciferase assays*

In Hela-Tat-Luc cells the HIV-1–LTR is highly activated as a consequence of high levels of intracellular Tat protein. Cells (10^5 cells/ml) were seeded the day before the assay and treated either with the CDK9 inhibitor DRB, as a positive control, or with the selected compounds. After 16 h, the cells were washed twice with PBS and then lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. The lysates were spun down and the supernatants were used to measure luciferase activity using an Autolumat LB 9510 (Berthold, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The results are represented as the percentage of activation (considering the untreated cells 100% activation). For the Hela-Tet-On luciferase assay, the cells (10^5 cells/ml) were seeded the day before the assay, and then stimulated with doxycycline (2 μg/ml) in the presence or absence of the compounds for 6 h. Then, the cells were washed twice in PBS, lysed and the luciferase activity measured as described.

2.4. *Isolation and activation of peripheral mononuclear cells*

Human peripheral blood mononuclear cells (PBMC), from healthy adult volunteer donors were isolated by centrifugation of venous blood on Ficoll-Hypaque® density gradients (Amersham Biosciences). Cells (2.5×10^6/ml) were treated with SEB for 72 h and then collected and used for recombinant virus infection assays as previously described [33].

2.5. Plasmids

The vector pNL4-3.Luc.R$^-$ E$^-$ (AIDS Research and Reference Reagent program, NIAID, National Institutes of Health) from N. Landau was previously described [35]. This vector contains the firefly luciferase gene inserted into the pNL4-3 nef gene and two frameshifts (5′ Env and Vpr amino acid 26) render this clone Env$^-$ and Vpr$^-$. The pcDNA$_3$-VSV plasmid contains the cDNA encoding the vesicular stomatitis virus (VSV) G protein and was obtained from Dr. Arenzana-Seisdedos (Institute Pasteur, Paris, France).

2.6. Production of VSV-pseudotyped recombinant viruses and infection assays

High titer VSV-pseudotyped recombinant virus stocks were produced in 293 T cells as previously described [33]. Briefly, the cells were co-transfected with the pNL4-3.Luc.R$^-$ E$^-$ plasmid along with the pcDNA$_3$-VSV plasmid by the calcium phosphate transfection method. Supernatants, containing virus stocks, were harvested 48 h post-transfection and centrifuged 5 min at 500 × *g* to remove cell debris, and stored at −80 °C until use. Cell-free viral stock was tested using an enzyme-linked immunoassay for antigen HIV-1 p24 and cultures were infected with 200 ng of HIV-1 gag p24 protein as follows; Jurkat cells or isolated PMBC (10^6/ml in 24-well plates) were pre-treated with the compounds for 30 min and then inoculated with the virus stocks. After 24 h, the cells were lysed and the luciferase activity measured as described above. Results are represented as the percentage of HIV-1 activation (considering the infected and untreated cells 100% activation). Results represent mean ± standard deviation (S.D.) of four independent experiments.

2.7. CTD-kinase and electrophoretic mobility shift assays

For the CTD kinase assay preinitiation complexes (PICs) from nuclear extracts of HeLa and HeLa-Tat cells were isolated using biotinylated templates as described previously [36]. The HIV-1 long terminal repeat template (nucleotides −104 to + 172) was amplified by PCR from the plasmid pLTRxLUC with the forward primer 5′-biotinylated GACTTTCCGCTGGGGACTTTC-3′ and the reverse primer 5′-TTATGTTTTTGGCGTCTTCCAT-3′. The PCR product was purified by phenol/chloroform and precipitated prior to use. CTD Kinase assay was performed in 100 μl reaction volume containing the purified PICs, 40 μM ATP, 5 μCi of γ-32P-ATP and the substrate protein GST-CTD bound to sepharose beads in kinase buffer (20 mM HEPES pH 7.4, 1% Nonidet P-40, 20 mM $MgCl_2$, 2 mM DTT, 10 mM NaF, 1 mM PMSF, 10 mM β-glycerophosphate, 1 μg/ml leupeptin and 0.5 μg/ml aprotinin) in the absence or presence of the peptoid N-37-37-15C. The mixture was incubated for 2 h at 30 °C and washed three times with RIPA buffer (50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF 0.5%, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml aprotinin). After extensive washing the reaction mixture was resuspended in RIPA buffer, mixed with Laemmly buffer and electrophoresed in 10% SDS-polyacrylamide gel. The gel was fixed, dried and exposed to X-ray at −80 °C. For the Tat-TAR binding assay, an RNA probe containing the 5′ bulge of TAR [26] was end-labeled with [γ-^{32}P]AT and incubated with 20 nM recombinant GST-Tat protein in EMSA buffer (0.5 μg poly (dI-dC), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml BSA and 4% Ficoll) for 30 min at 4 °C in the presence or absence of the selected peptoids, and RNA-protein complexes were separated by a 6% nondenaturing polyacrylamide gels, dried, and exposed to x-ray film at −80 °C.

2.8. Cytotoxicity assays

Hela-Tat-Luc cells (10^5/ml) were seeded in 24-well plates in complete medium and treated with the indicated doses of the compounds for 16 h. Samples were then diluted with 300 μl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 μg/ml). After incubation, cells were immediately analysed by flow cytometry.

3. Results

3.1. Screening of a N-trialkylglycine-based combinatorial library to identify novel anti-Tat compounds

The library was organized as an array of 52 separate mixtures. Each mixture contained either 256 (OXX) or 320 molecules (XOX, XXO), and the library chemical diversity comprised 5120 individual trimers. The set of amines included aliphatic and aromatic groups to increase the probability of finding anti-Tat compounds, to enhance membrane permeability, and to improve the bioavailability of the active *N*-trialkylglycines.

Peptoid mixtures were assayed in the Hela-Tat-Luc clone that was constructed for the screening assays of anti-Tat compounds. The Hela-Tat-Luc contains the luciferase gene driven by the HIV-1–LTR promoter and the Tat gene regulated by the CMV promoter. Therefore, the HIV-1–LTR is highly activated in this cell line as a consequence of high levels of intracellular Tat protein and the luciferase activity is in the order of 10^7 R.L.U./10^5 cells (considered 100% activation). The cells were incubated with each mixture of peptoids (100 μg/ml) or with the CDK9 inhibitor DRB, as a positive control and 16 h later both the cellular viability and the luciferase activity were measured. In the first round of the screening we detected 20 peptoid-mixtures that inhibited the luciferase activity in Hela-Tat-Luc cells to the same extent than DRB (40% inhibition compared)(Fig. 2A–C). However, some of peptoid-mixtures were found to be highly cytotoxic as detected by propidium iodide uptake (data not shown) and were discarded for further analysis. To further confirm the specificity of the non-cytotoxic peptoid-mixture on the anti-Tat activity we tested again in both Hela-Tat-Luc and in Hela-Tet-On-Luc cells. First, we identified the IC50 inhibitory activity of each peptoid-mixture in the Hela-Tat-Luc assay (data not shown) and then these concentrations of each mixture were re-tested again in the Hela-Tet-On-Luc clone. We found that the luciferase inhibitory effects of the OXX (A2, A12, A16), XOX (A7, A15, A16), and XXO (A10, A12, A14, A15, A16) peptoid-mixtures (Fig. 3A) were not due to an interference with the transcriptional machinery or with the in vitro activity of the luciferase enzyme, since the inducible expression of luciferase mediated by doxycycline in Hela-Tet-On-Luc cells was not affected by any of the mixtures (Fig. 3B).

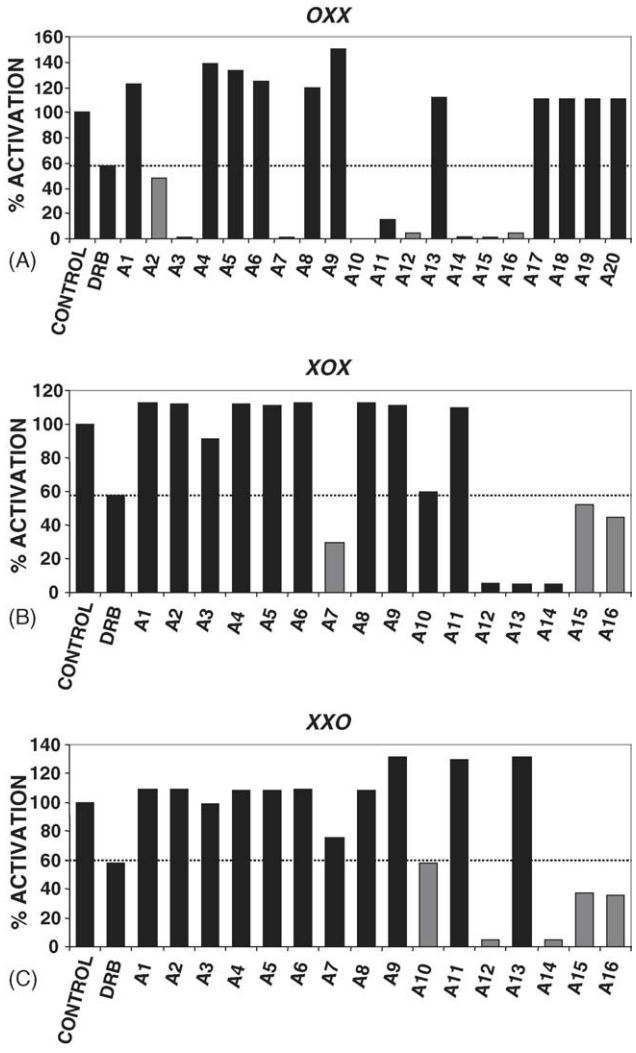

Fig. 2 – Screening of an oligo *N*-substituted glycine combinatorial library to identify anti-Tat compounds. HeLa-Tat-Luc cells were incubated with either DRB (50 μM) or each mixture peptoid (100 μg/ml) and 18 h later Tat-induced LTR-luciferase activity was measured. Untreated cells were considered as the 100% of activity and the results are representative of three different experiments. Grey bars represent those compounds showing more than 40% inhibition in HeLa-TAT-Luc in a specific manner.

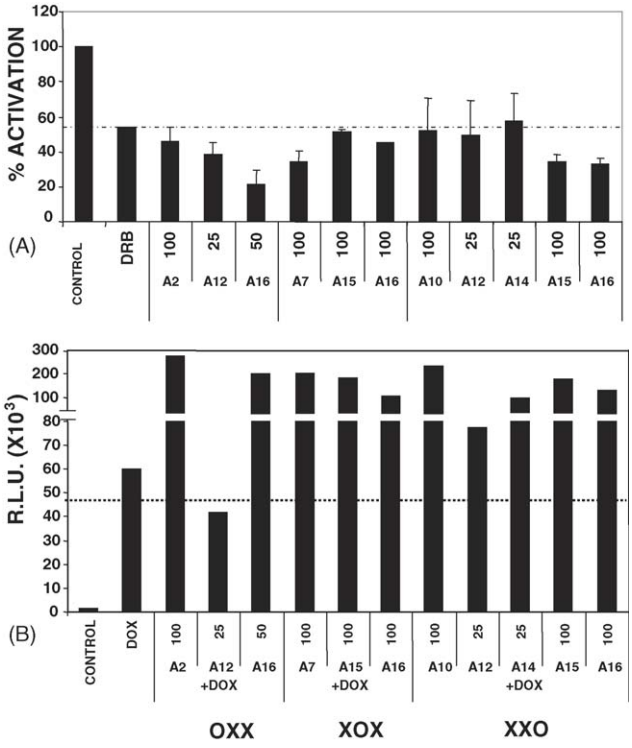

Fig. 3 – Dose-dependent inhibition of Tat-induced HIV-1–LTR transactivation of the selected mixtures of peptoids. HeLa-Tat-Luc cells were incubated with increasing doses of each mixture of peptoids for 18 h and the luciferase activity measured as indicated. In panel A) it is shown the minimum concentration of each mixture (in μg/ml) that neither induces cytotocixity nor inhibits the luciferase activity driven by an artificial promoter (panel B). Luciferase activity was measured and the results are the means ± SE of three determinations expressed as the percentage of inhibition compared to untreated cells.

3.2. *Isolated peptoids from the N-trialkylglycine-based library are potent inhibitors of Tat-induced HIV-LTR transactivation*

The data derived from the primary screening and the corresponding deconvolution process suggested the chemical identity of the bioactive peptoids in the library [28,37]. Therefore, a set of five compounds derived from the more potent and specific anti-Tat peptoid mixtures (Mixture OXX: compounds A2, A12 and A16; Mixture XXO: compounds A15 and A16) were independently synthesized and their structures are shown in Fig. 4. As expected, these individual *N*-trialkylglycines showed potent anti-Tat activity in the HeLa-TAT-Luc assays (Fig. 5A). Among them the peptoid N37-37-15C was the more potent and specific inhibitory compound from this family (Fig. 5 B). The dose–response relationship for this peptoid resulted in an inhibitory IC50 of 3.9 ± 0.17 μM. Next, the biochemical mechanism of the N37-37-15C anti-Tat activity was investigated. Gel retardation is an effective method for determining whether TAR forms a complex with HIV-1 Tat. In the presence of TAR, which contains the 5′ bulge and central loop, recombinant Tat protein forms a lower mobility complex that was detected on polyacrylamide gels (Fig. 6A, lane 1). The Tat/TAR complex was not detected when an anti-Tat antiserum was included in the binding reaction (Fig. 6A, lane 2) demonstrating the specificity of this assay. We found that neither N37-37-15C nor the other peptoids were able to disrupt the binding of HIV-1 Tat protein to the LTR TAR element at the concentration tested (Fig. 6A). Tat binding to TAR is required to recruit P-TEFb to the preinitiation and elongation complexes. P-TEFb, which is composed of CycT1 and CDK9, induces the CTD hyperphosphorylation of the large subunit of RNAP-II leading to Tat-mediated transactivation of the HIV-LTR promoter. Accordingly, we detected in nuclear extracts from Hela-Tat cells an increase in CTD kinase activity

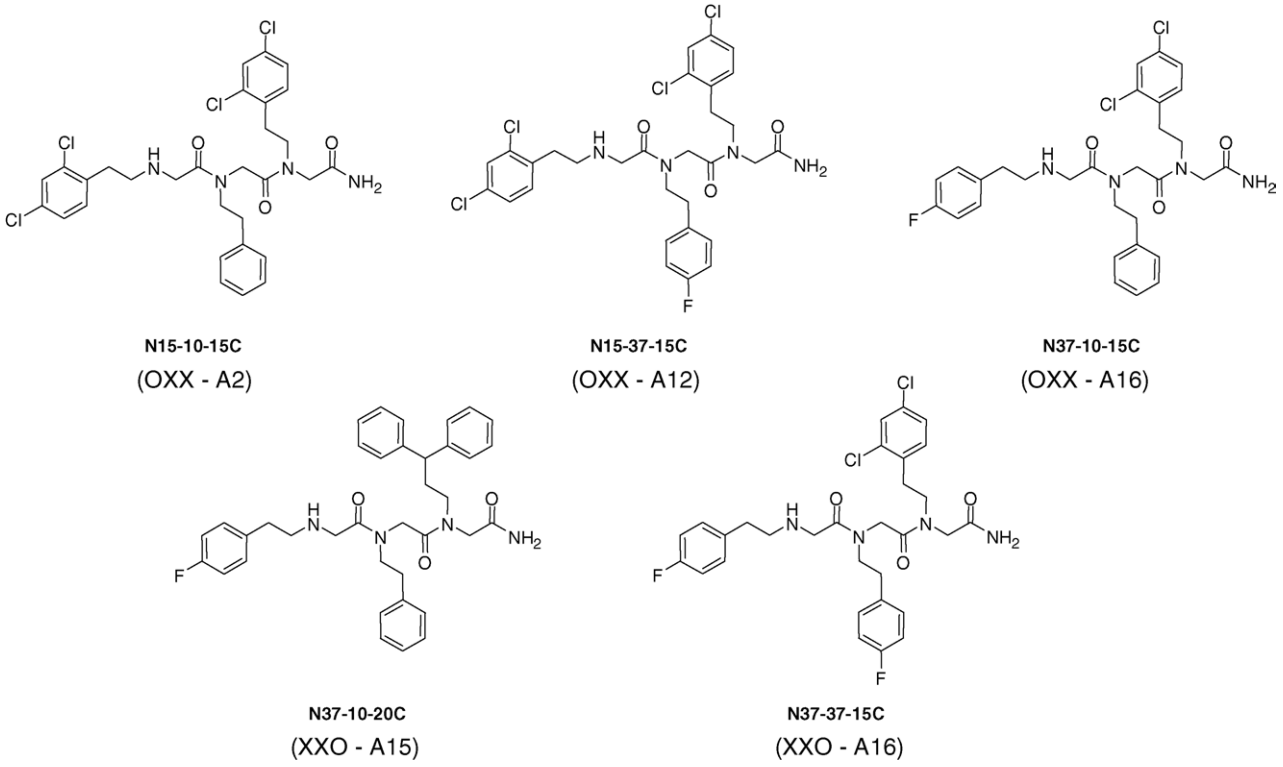

Fig. 4 – Molecular structures of the peptoids N15-10-15C, N15-37-15C, N37-10-15C. N37-10-20C and N37-37-15C.

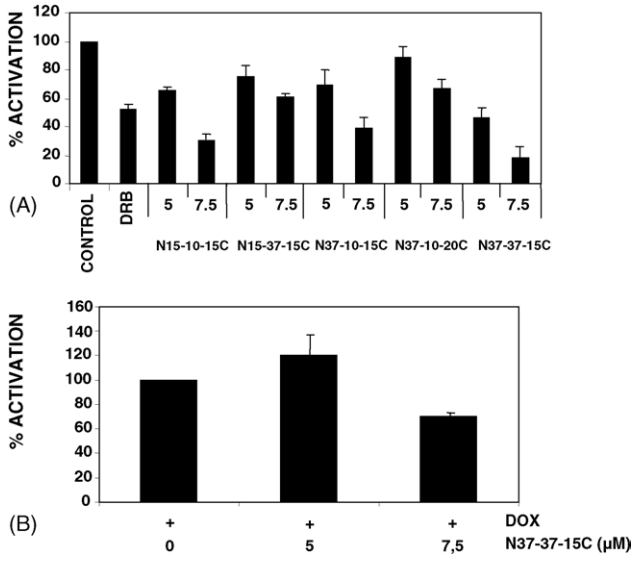

Fig. 5 – Effects of the individual *N*-trialkylglicines on Tat-induced HIV-1-LTR transactivation. (A) HeLa-Tat-Luc cells were incubated with the indicated concentrations of the compounds for 18 h and the luciferase activity was measured as in Fig. 2. (B) Effect of N37-37-15C on doxycycline-induced luciferase activity. Luciferase activity was measured and the results are the means ± S.E. of three determinations expressed as the percentage of inhibition compared to untreated cells.

when compared to nuclear extracts from the parental Hela cells. Interestingly, this kinase activity was greatly inhibited by the presence of N37-37-15C in the kinase reaction assay (Fig. 6B).

3.3. *Effects of N37-37-15C in HIV-1–LTR transcription in lymphoid cells*

In order to study the anti-HIV activity of the peptoid N-37-37-15C we infected Jurkat cells (Fig. 7A) or peripheral blood mononuclear cells (Fig. 7B) with the pNL4-3 HIV-1 clone pseudotyped with the VSV envelope, which bypasses the natural mode of HIV-1 entry into these cells that support robust HIV-1 replication [33,38]. Upon integration into host chromosomes this recombinant virus expresses the firefly luciferase gene and consequently luciferase activity in infected cells correlates with the rate of viral transcription. Thus, high luciferase activity levels were detected 24 h after cellular infection with the VSV-pseudotyped HIV-1 clone, and pre-treatment of the cells 30 min prior infection with either AZT or N37-37-15C resulted in a clear inhibition of luciferase activity. The anti-HIV activity of N37-37-15C was more evident in peripheral mononuclear cells that in the Jurkat cell line. Since AZT and N37-37-15C seem to inhibit HIV-1 replication by targeting different steps in the viral life cycle either a synergy or an additive effect between both compounds could be expected. We show that a clear additive anti-HIV effect (≅ 90% inhibition) was found with a combination of both compounds that again was more evident in primary cells.

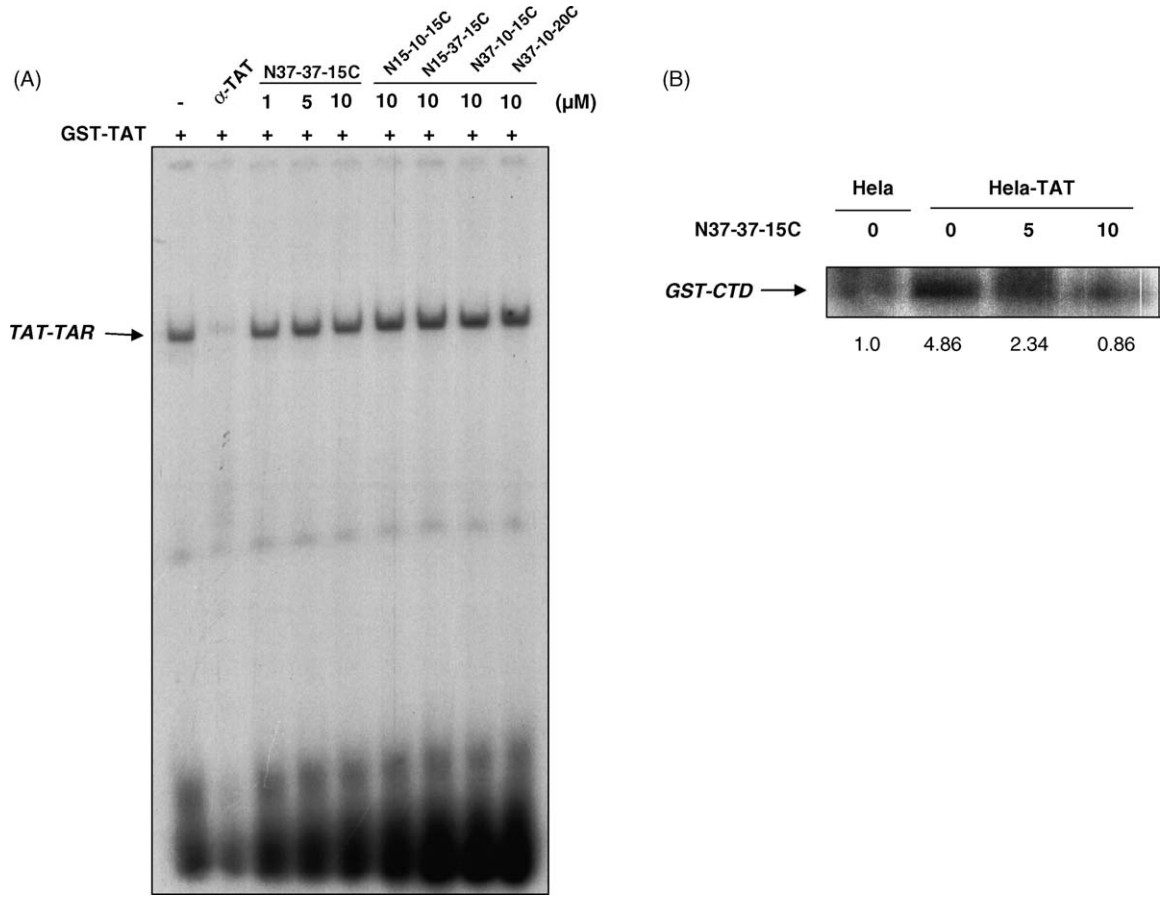

Fig. 6 – Effects of the synthesized *N*-trialkylglicines on Tat–TAR binding activity and CTD phosphorylation. (A) Recombinant GST-Tat protein (20 nM) was incubated with [γ-^{32}P] ATP-labelled RNA probe containing the 5′ bulge of TAR in EMSA buffer in the presence or absence of the peptoids at the indicated doses. The RNA-GST-Tat complexes were resolved by electrophoresis in 6% polyacrylamide gel. (B) Isolated preinitiation complexes (PICs) from HeLa and HeLa-Tat cells were isolated and assayed for "in vitro" CTD kinase activity.

4. Discussion

In this study, we have screened an *N*-trialkylglycine-based library composed of 5120 compounds to find new anti-Tat and anti-HIV-1 molecular entities. We found that peptoid N37-37-15C is a potent inhibitor of Tat-mediated HIV-1–LTR transcription and also inhibits the CTD-kinase activity that was increased in HeLa-Tat cells. The N37-37-15C anti-Tat activity correlates with the anti-HIV-1 activity found in a model of VSV-pseudotyped recombinant virus infection.

According to the recent reports, the global AIDS pandemic has killed 28 million people and infected an estimated 42 million people worldwide. Second to the identification of HIV-1 as the causative agent for AIDS, the most impressive scientific advances have occurred in the development of effective antiretroviral drugs for treating individuals infected with HIV-1. Efforts to find an effective anti-HIV-1 chemotherapy have been mainly focused on the development of chemicals that target viral proteins, which are essential for HIV replication. To date, the combination of drugs targeting the viral proteins retro-transcriptase and protease constitutes the so called highly active anti-retroviral therapy (HAART). However, drug-resistance and the serious side effects that appear during HAART therapy are major limitations to this therapy. Moreover, HAART has no direct effect on the proviral burden [13] and therefore HIV-1 latency is still an unresolved problem.

The use of natural or synthetic compounds targeting cellular proteins involved in HIV-1 replication has opened new research avenues in the management of AIDS [39]. For instance, CDKs, which represent key molecules involved in the regulation of cell cycle, are good candidates for mutation-insensitive antiviral drugs. Although the evidence for the role of CDK9/CyclinT1 in Tat-mediated HIV-1 transcription is overwhelming, other CDKs such as CDK7 and CDK2 have been involved in the Tat response [8,36]. We have found that peptoid N37-37–15C, although do not prevent the binding of Tat to the TAR region, clearly inhibited the CTD kinase activity in nuclear extracts of HeLa-Tat cells. At the present moment, we cannot discriminate which CDK kinase is inhibited by N37-37-15C. However, we observed that N37-37-15C does not affect the cell cycle progression in Jurkat cells and therefore is unlikely that CDK2 is inhibited by this compound at the concentrations tested. In our experiments, we detected that N37-37-15C is more effective to inhibit HIV-1 transcription in

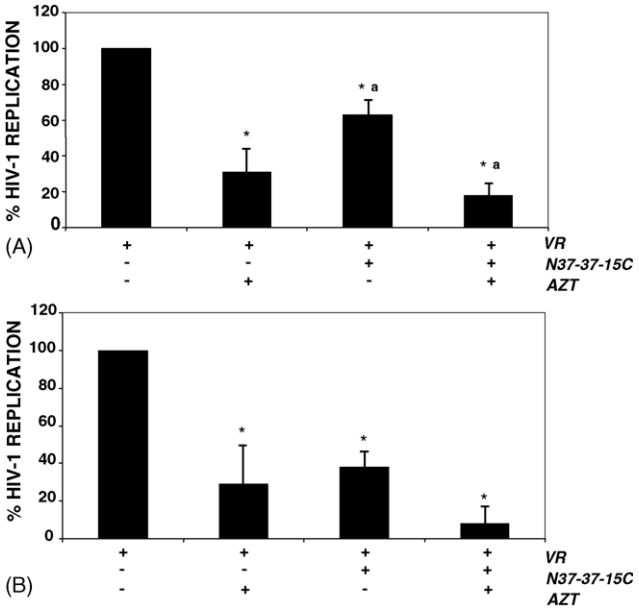

Fig. 7 – Effects of N37-37-15C on recombinant virus LTR transcription. (A) Jurkat T cells (10^6/ml) were pretreated with AZT, N37-37-15C separately or in combination for 30 min and then infected with VSV-pseudotyped-pNL4-3.Luc.R$^-$E$^-$ (200 ng p24) for 24 h. Luciferase activity in cell extracts was determined and results represented as percentage of activation ± S.D. compared to non-treated infected cells (100% activation). (B) SEB-activated PBMC for 48 h (10^6/ml) were pretreated as above and then infected with the VSV-pseudotyped-pNL4-3.Luc.R$^-$E$^-$ (VR (VSV recombinant virus)) for 24 h. Results are represented as R.L.U. ± S.D. of three different experiments. (*$P < 0.05$; Student's *t*-test (compared to control); [a]$P < 0.05$; Student's *t*-test (compared to AZT treatment).

primary T cells than in transformed cells, in which in addition to CDK9 other kinases can also play a redundant role in Tat-mediated LTR transcription. By the contrary, primary T cells have a lower rate of HIV-1-LTR transcription and therefore it is likely that CDK9 is the major kinase involved in the HIV-1 gene transcription in these cells. Alternatively, it is also possible that N37-37-15C could inhibit the phosphatase PP2A that is required for CDK9 dephosphorylation prior to its association with the transcription initiation complex [40]. Therefore, further research to identify the CTD kinase and mechanisms targeted by N37-37-15C in both primary and transformed T cells is warranted.

Trimers of *N*-alkylglycines is a family of peptidomimetics that has been successfully used to identify anti-inflammatory, analgesic and neuroprotectant compounds with "in vivo" activities [29,30], indicating that these molecules may present a good pharmacological profile. Oligomers of N-substituted glycines provide a class of small, non-natural molecules that are proteolytically stable and have potent biological activities (31). A major advantage of using short oligomers is that low molecular mass molecules (≤600 Da) usually display acceptable tissue penetration properties and better pharmacological conformities [41–42].

We have demonstrated in the present report that these compounds also target the transcriptional step of the HIV-1 cycle and synergise with reverse transcriptase inhibitors in blocking HIV-1 full transcription. Novel antiviral compounds interfering with HIV-1-LTR promoter regulatory proteins are of special interest since these drugs, if borne useful for patients, are unlikely to generate resistant HIV strains. Thus, synthetic trimers of *N*-alkylglycines such as N37-37-15C might have a potential therapeutic role in the management of AIDS most probably in combination with other anti-HIV drugs. Likewise, the structural simplicity of *N*-alkylglycine oligomers makes these peptidomimetics amenable to structural manipulation, thus facilitating the optimisation of lead molecules for drug-like properties. Specifically, the design of conformationally restricted analogues of compound N37-37-15C could amplify both its activity and selectivity. Work addressed to this objective is in progress in our laboratories.

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