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Binding of hybrid molecules containing pyrrolo [2,1-*c*][1,4]benzodiazepine (PBD) and oligopyrrole carriers to the human immunodeficiency type 1 virus TAR-RNA

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Abstract

The binding properties of a set of four hybrids, prepared combining from one to four polypyrrole minor groove binders and pyrrolo [2,1-*c*][1,4]benzodiazepine (PBD), have been studied using as target molecule the HIV-1 TAR-RNA. We found that these hybrids bind to TAR-RNA and inhibit TAR/protein(s) interactions. The anti-proliferative activity of the hybrids has been tested *in vitro* on HL3T1 cells and compared to the anti-proliferative effects of the natural product distamycin A and PBD. The effects on HIV-1 LTR directed transcription were studied using the chloramphenicol-acetyltransferase gene reporter system, and structure–activity relationships are discussed. The results obtained demonstrate that the hybrids **22**–**25** exhibit different TAR-RNA binding activity with respect to both distamycin A and PBD. In addition, a direct relationship was found between number of pyrrole rings present in the hybrids **22**–**25** and anti-proliferative effects. It was found that increased length of the polypyrrole backbone leads to an increased *in vitro* anti-proliferative effect, i.e. the hybrid **25**, containing the four pyrroles distamycin analogous, is more active than **22**, **23** and **24** against cell proliferation. With respect to inhibition of HIV-1 LTR-driven transcription, it was found that the hybrids **23**–**25** containing two–four pyrroles are active. Therefore, when anti-proliferative effects are considered together with the inhibitory effects of HIV-1 LTR driven transcription, our results suggest that the hybrid **23** is the more interesting, since it exhibits low anti-proliferative activity and inhibits HIV-1 LTR driven transcription both *in vitro* and in *ex vivo* experiments.

Keywords: Distamycin; Anthramycin; TAR-RNA; Transcription; HIV-1; AIDS

1. Introduction

The HIV-1 genome, in addition to structural genes, encodes a set of regulatory proteins that modulate viral gene expression in infected cells [1]. Among them, Tat is required to induce high level transcription of the HIV-1 genome after binding to a structured TAR-RNA [2,3]. TAR-RNA is encoded by nucleotides spanning the region extending from +1 to + 57 and folds into a stem-loop structure composed of two domains: a three-nucleotide bulge and the loop sequence [2]. The ternary complex of human cyclin T1, interacting with the loop, and Tat, interacting with the major groove in the bulge region of TAR, causes cyclin-dependent kinase 9 to hyperphosphorylate the COOH-terminal domain of RNA polymerase II, allowing efficient processive transcription of the viral genome [4,5]. For this reason, inhibition of protein/

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Abbreviations: HIV-1, human immunodeficiency virus, type 1; AIDS, acquired immunodeficiency syndrome; LTR, long terminal repeat; PBD, pyrrolo[2,1-*c*][1,4]benzodiazepine; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyl transferase; PAGE, polyacrylamide gel electrophoresis.

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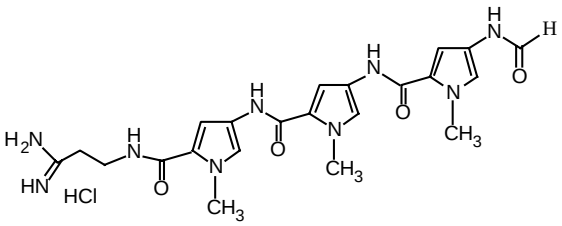
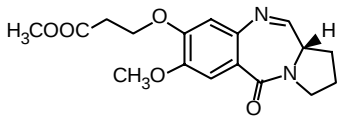
TAR-RNA complex formation may represent a novel strategy for new anti-HIV compounds. At least in theory, DNA-binding drugs could also be considered putative RNA-binding drugs, as recently reported by our research group in a paper showing that DNA-binding aromatic polyamidines interact with structured TAR-RNA and modulate Tat-induced HIV-1 transcription [6].

In this respect, distamycin analogs could be of great deal. Distamycin A is a naturally occurring antibiotic, characterized by the presence of an oligopeptide *N*-methylpyrrolecarbamoyl frame ending with an amidino moiety. This compound form a strong reversible complex by non-intercalative binding to nucleotide sequences consisting of 4–5 adjacent AT base pairs placed in the minor groove of double-stranded B-DNA. Electrostatic forces, van der Waals interactions and hydrogen bonds are involved in the stabilization of the complex between these compounds and DNA [7]. As far as design and synthesis of distamycin-analogs, hybrid molecules composed of a distamycin-like frame and structurally different bioactive molecules have been described [8–13]. For instance, the PBD group [14], which includes the natural compounds anthramycin and DC-81, was recently considered because it is capable of covalently binding to the C2–NH_2 of guanine residues in the minor groove of DNA. X-ray and footprinting studies on covalent DNA-PBD adducts have demonstrated a high



Distamycin



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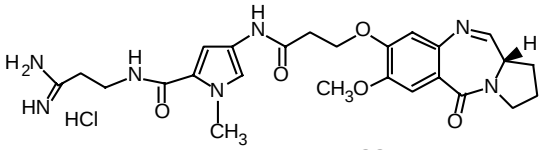

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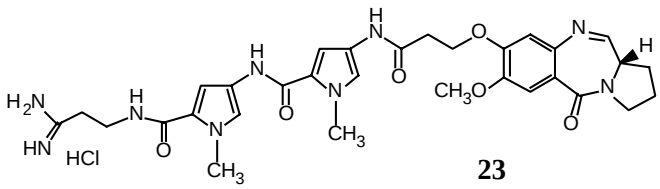

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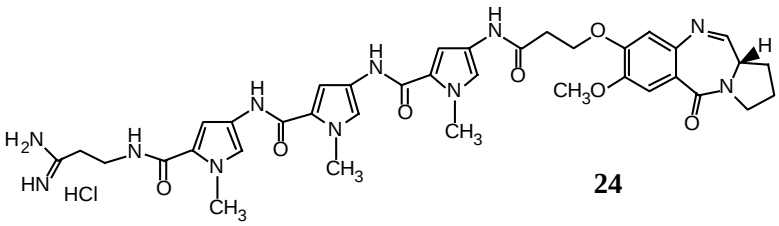

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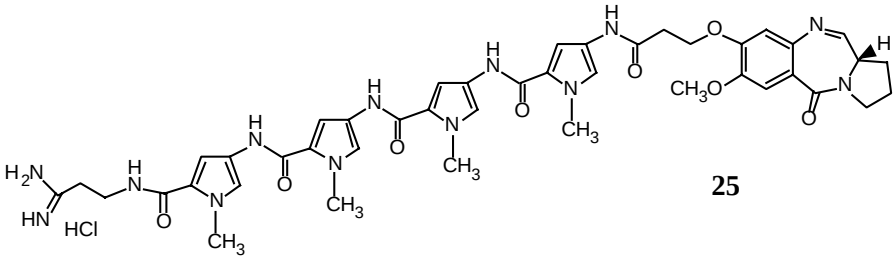

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Fig. 1. Chemical structures of the PBD-oligopyrroles hybrids used in this study.

sequence-specificity for GC rich DNA regions, in particular for X–G–X triplets (X = purine) [15]. Recently, we reported the synthesis of novel hybrids **22–25** (see Fig. 1 for chemical structures), consisting respectively of one, two, three or four pyrrole amide units linked to the PBD group **12** [16]. The rationale that led to the synthesis of these compounds was to tether the minor groove binder distamycin A frame to the minor groove alkylating moiety of PBD **12**, with the aim to obtain new derivatives which potentially bind nucleic acids with higher strength than the parent compounds. We demonstrated that PBD-distamycin hybrids inhibit DNA binding to transcription factors [17].

In the present paper we describe the interaction of PBD-oligopyrroles hybrids **22–25** with structured TAR-RNA of HIV-1. In order to determine whether these compounds bind to RNA in a sequence-selective manner, structured AU-rich or GC-rich RNAs have been used. Furthermore, the effects of PBD-oligopyrroles hybrids **22–25** on protein/TAR-RNA interaction have been tested (a) *in vitro* by EMSA and filter binding performed using nuclear extracts and Tat and (b) in *ex vivo* experiments using the HL3T1 cell line as a cellular system to study Tat-induced HIV-1 LTR driven transcription. The data herein reported demonstrate that PBD-oligopyrroles hybrids can be proposed as RNA-binding drugs.

2. Material and methods

2.1. PBD-oligopyrroles hybrids

The synthesis, characterization, purification of the PBD-oligopyrroles hybrids have been described elsewhere [16]. The chemical structures are reported in Fig. 1. Briefly, most of the reactions were carried out under an inert atmosphere of dry nitrogen. TLC or silica gel (precoated F254 Merck plates) visualized with aqueous $KMnO_4$ routinely monitored reaction progress and product mixtures. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents indicated in the procedure. Infrared spectra were recorded on a Perkin-Elmer 257 spectrophotometer with the solvent indicated in the procedure. Mass spectra were performed with Maldi-Toff Hewlett Packard G 2025 A instrument. Melting points were determined on a Buchi-Tottoli apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer polarimeter 241. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. All commercially available compounds were used without further purification. Organic solutions were dried over anhydrous $MgSO_4$. Methanol was distilled from magnesium turnings; dioxane was distilled from calcium hydride; triethylamine was dried over molecular sieves of 3 Å; and dry DMF was distilled from calcium chloride and stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used. HPLC separations were conducted with Waters Delta Prep 3000 A reversed-phase column (30 cm × 3 cm; 15 μm). The compounds were eluted with a gradient of 0–60% B in 25 min at a flow rate of 30 mL/min and the mobile phases were solvent A (10%, v/v, acetonitrile in 0.1% TFA) and solvent B (60%, v/v, acetonitrile in 0.1% TFA). The pure products were converted into the corresponding hydrochloride forms by addition of 0.1 N HCl aqueous solution to the mobile phase containing the pure product. Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument using a Vydac 218 TP 5415 C18 column (250 mm × 4 mm, 5 μm particle size) and equipped with a Bruker LC 313 UV variable-wavelenght detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10). All the compounds tested were dissolved in DMSO at 12 mg/mL immediately before the use and diluted just before addition to the cell culture.

2.2. Cell culture conditions

The human T-lymphoid Jurkat cells were cultured at 37° in 5% CO_2 humidified atmosphere in RPMI-1640 medium supplemented with 10% FBS. HL3T1 cells were cultured in αMEM medium supplemented with 10% FBS. Cell culture media were supplemented with 50 U/mL penicillin and 50 mg/mL streptomycin antibiotics. Log phase growing cells were collected by centrifugation at 650 *g* for 3 min. Cell pellets were washed in PBS. Nuclear extracts were prepared according to the standard Dignam procedure [18].

2.3. Electrophoretic mobility shift assay (EMSA) conditions

Synthesis and labeling of TAR-RNAs were performed by *in vitro* transcription as previously described using the SP6 or the T7 RNA polymerases and linearized plasmids [6]. Purified labeled RNA was quantified and 1 ng was incubated for 15 min with PBD-oligopyrroles hybrids at room temperature in binding buffer (5% glycerol, 20 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.01% Triton X-100) in a final volume of 25 μL. After this time, RNA/drug complexes were resolved by loading onto 6% polyacrylamide gel and low ionic strength running buffer (0.25 × TBE buffer = 22 mM Tris-borate, 0.5 mM EDTA).

When nuclear extracts were used, 1 ng of labeled TAR-RNA was pre-incubated with the specific drug in binding buffer containing 1 μg of poly(dIdC)·poly(dIdC) for 15 min and, after this time, 5 μg of Jurkat cells nuclear extracts were added for additional 15 min at room temperature. Protein/RNA complexes were fractionated from

free RNA probe by EMSA. The gel was vacuum-dried at 80° and exposed to Kodak XAR-5 film. Phosphor Imager (Bio-Rad Laboratories, Richmond, CA) was used to detect radioactivity contained in the retarded band.

2.4. Filter binding

In the filter binding experiments, 1 μg of Tat2E (wild type 86 amino acids) was spotted in replicated strips of nitrocellulose membrane. The production of Tat was performed as previously described [19]. Filter-bound Tat was incubated for 30 min at room temperature in 500 μL binding buffer containing 1 ng of radiolabeled wild type TAR-RNA. Alternatively, other spots containing Tat were incubated in 500 μL binding buffer, containing TAR-RNA/drug complexes pre-assembled for 15 min at room temperature in the presence of different concentrations of the appropriate drug to be tested. After 1 hr of incubation, the strips were separately washed in binding buffer (three times, 10 min each) and exposed to Kodak XAR-5 film at −80°. Ponceau Red staining of nitrocellulose strips was performed to verify the presence of equal amount of protein in replicated spots.

2.5. Cell proliferation assay

The effects of PBD-oligopyrroles hybrids on cell proliferation were tested on HL3T1 cells by the CellTiter 96 Cytotoxicity Assay kit (Promega), following the indicated instructions. Briefly, cells were seeded in 100 μL of complete cell culture medium and grown for 72 hr in the presence or in the absence of increasing amount (0–0.75–1.5–3–6–12–25–50 μM) of PBD-oligopyrroles hybrids. One well was for medium without cells or drugs (blank). After this time, 25 μL of staining solution were added to each well and the 96-well plate incubated at 37° for 2 hr. After addition of 100 μL lysis solution, the plate was incubated over-night at 37° for stain development. Color quantification was performed at 550 nm (OD_{550}) and 620 nm (OD_{620}). The obtained values were compared to the value obtained in the absence of drug, expressed as percent of control and graphed as function of drug concentration.

2.6. Transfections and CAT assay

HL3T1 cells, containing integrated copies of LTR-CAT retroviral construct, were seeded into 1 mL of OPTIMEM serum-free medium (GIBCO BRL-Life Technologies) the day prior to drug administration so that each well of a 6 well plate was 50–70% confluent at the time of drug treatment. After an over-night incubation, PBD-oligopyrroles hybrids were added to the cell culture medium at 0, 1, 5 and 10 μM final concentration. After an additional over-night incubation, HIV-1 Tat bound to lipofectin (GIBCO BRL-Life Technologies) was added directly into the cell culture medium. After 4 hr, 1 mL of RPMI-1640 plus 20% fetal calf serum was added and the cells were harvested 72 hr later. Cytoplasmic extracts were prepared, and similar protein amounts were assayed for CAT activity.

3. Results

3.1. Binding of distamycin, PBD and PBD-oligopyrroles hybrids to structured RNAs

The binding of distamycin A, PBD **12** and PBD-oligopyrroles hybrids **22**–**25** to ^{32}P-labeled wild type TAR-RNA was assayed by EMSA (Fig. 2B) using increasing concentrations of drug (0, 0.03, 0.1, 0.3, 1, 3, 10 μM). Binding reactions were fractionated by electrophoresis on a native polyacrylamide gel and RNA/drug complex was easily identified as a retarded band migrating at slower speed than free RNA. Our data demonstrate that PBD-oligopyrroles hybrids **22**–**25** bind TAR-RNA since they slowed down the migration of radiolabeled HIV-1 TAR-RNA. By contrast, distamycin and PBD **12** are completely inactive on the RNA migration. When compounds **22**–**25** are used, a large decrease in the mobility at high concentrations of the compounds was obtained, possibly due to aggregation, trapping the radioactive RNA in the gel well. It should be noted that other DNA- and RNA-binding drugs exhibit this behavior (reviewed in Gambari and Nastruzzi) [20]. The experiment reported in Fig. 2B demonstrates that hybrids composed of PBD and oligopyrroles acquire a new characteristic (the RNA-binding property) with respect to PBD and distamycin.

In order to verify whether the stem region sequence of a structured RNA may influence the binding to PBD-oligopyrroles hybrids **22**–**25**, AU-rich or GC-rich RNAs (for structures see Fig. 2A) were used in an EMSA approach similar to that described above (Fig. 2C). It was found that binding of PBD-oligopyrroles hybrids **22**–**25** to the AU-rich or GC-rich RNAs was less efficient when compared to wild type TAR-RNA. Again, neither distamycin nor PBD **12** retarded the migration of AU-rich or GC-rich RNAs.

Furthermore, in order to ascertain whether PBD-oligopyrroles hybrids **22**–**25** bind to wild type TAR-RNA by covalent bonds, the RNA/PBD-oligopyrroles hybrid complex contained in the retarded band was excised and, after denaturation, dissociated components were loaded onto denaturing urea-containing polyacrylamide gels (Fig. 3). In a first experiment, exposure of radiolabeled TAR-RNA to compounds **22**–**25** at 3 μM concentration was carried out for increasing length of time, spanning from 15 to 120 min (Fig. 3A). Autoradiography of the gel demonstrates that, when compounds **22** and **23** were used and for all the time periods considered, the ^{32}P-labeled TAR-RNA that was present in the drug/RNA complex migrates with an apparent molecular weight that is the same shown by free TAR-RNA that was not

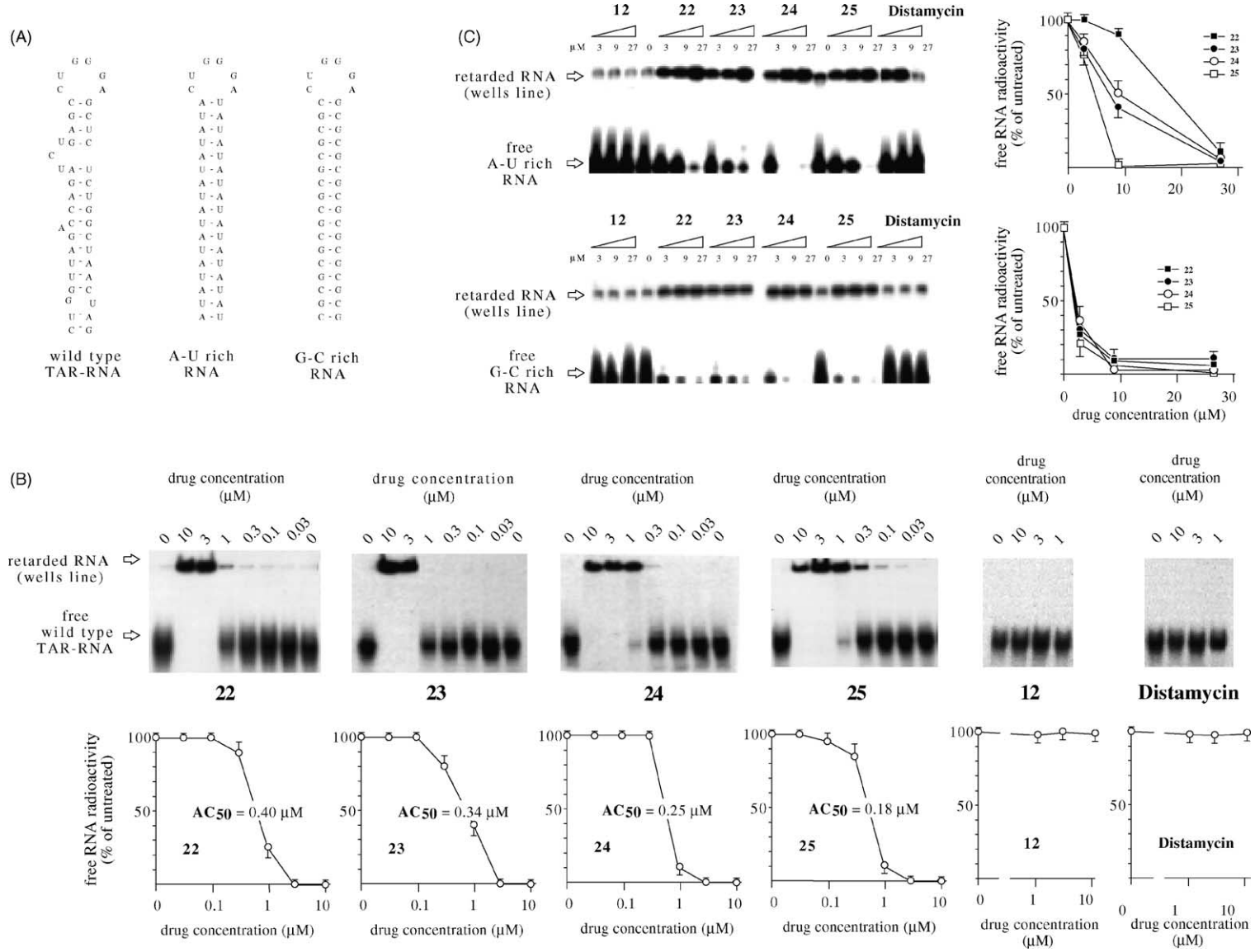

Fig. 2. (A) Stem-loop structures of the wild type TAR-RNA, the A–U rich RNA and the G–C rich RNA used as probe in the EMSA experiments. Synthesis and labeling of wild type TAR-RNA or RNAs carrying A–U rich or G–C rich stems were performed by *in vitro* transcription using run-off transcription of linearized plasmids in the presence of radiolabeled ribonucleotides. (B) Electrophoretic mobility shift assay. One nanogram of purified RNA was incubated with the indicated drug for 15 min at room temperature, before the fractionation onto native polyacrylamide gel. Upper side of the panel: authoradiography of representative experiments; lower side of the panel: quantitative data representing the average values of the free TAR-RNA band obtained in three independent experiments. The concentration of drug arresting the migration of 50% of the RNA probe (AC_{50}) is indicated. (C) Electrophoretic mobility shift assay performed as in panel B but using as probe the A–U rich RNA (upper side) or the G–C rich RNA (lower side).

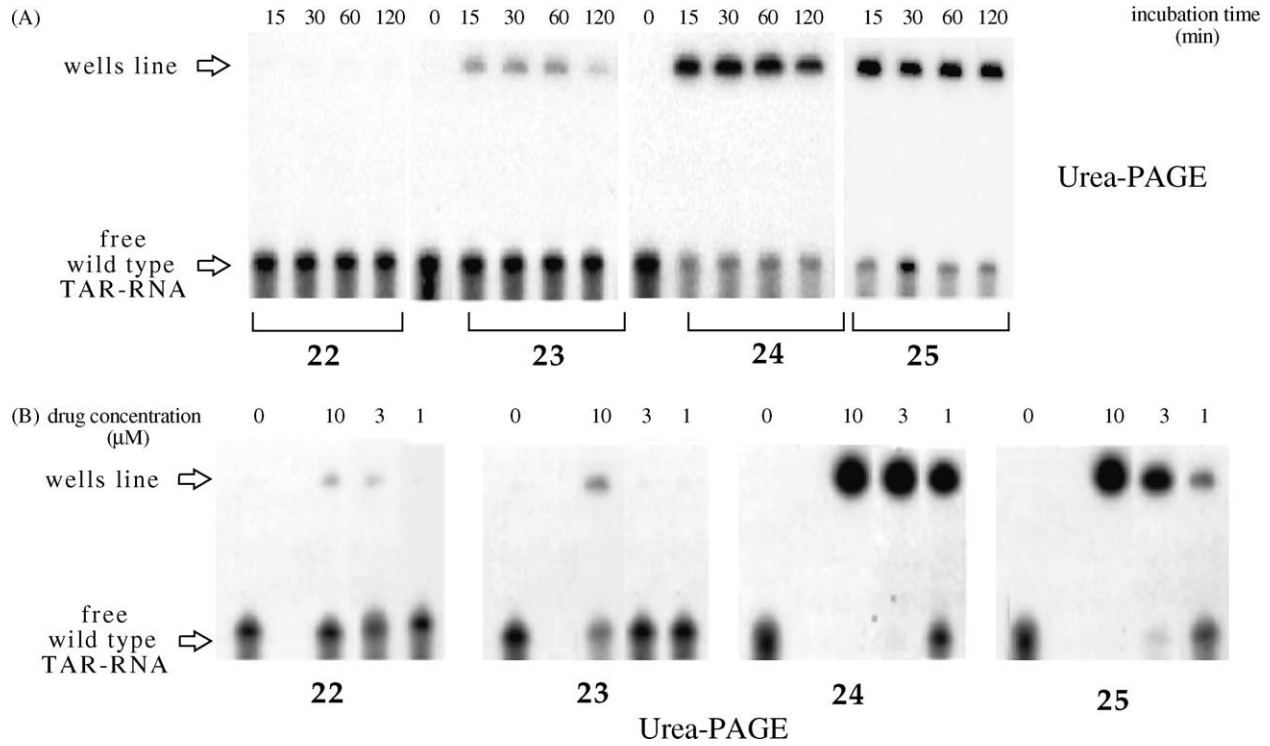

Fig. 3. Denaturing gel electrophoresis of the wild type TAR-RNA/PBD-oligopyrroles hybrid complexes. (A) Time-dependence assays. One nanogram of the radiolabeled wild type TAR-RNA probe was incubated with 3 μM of the indicated PBD-oligopyrroles hybrid for different time lengths. Subsequently, complexes were denatured by boiling and fractionated by urea–PAGE. Cross-linking of all the drugs to the TAR-RNA generates a band unable to migrate in the gel. (B) Dose-dependence assays. One nanogram of the radiolabeled wild type TAR-RNA probe was incubated with increasing concentrations of the indicated PBD-oligopyrroles hybrid for 15 min. Subsequently, the complexes were denatured by boiling and fractionated by urea–PAGE.

incubated with drug. We concluded that, in this case, the interaction of wild type TAR-RNA with these compounds does not involve (in the case of compound **22**) or moderately involves (in the case of compound **23**) the generation of covalent bonds (Fig. 3A, left side of the panel). By contrast, the molecular complexes between TAR-RNA and compounds **24** and **25** were not reverted by the denaturing conditions, suggesting that stable interactions (possibly covalent interactions) occur in this case (Fig. 3A, right side of the panel).

Next, we performed a similar experiment in order to determine the existence of dose-dependent relationship between irreversible binding and drug concentration (Fig. 3B). In this experiment, the wild type TAR-RNA was incubated for 15 min at different concentrations of compounds **22**–**25**. As expected from the results shown in panel A, most of the drug/RNA complexes obtained with compounds **24** and **25** still remain entrapped on the top of the gels. On the contrary, when compounds **22** and **23** are used, most of the drug/RNA complexes were confirmed to be reversible, except in the case of the experiment conducted with 10 μM compounds (Fig. 3B). The general conclusion from these experiments is that the increase of the number of pyrrole rings in the PBD-oligopyrroles hybrids is correlated with stability of the generated complexes with RNA, most of which are irreversible in the case of hybrids containing three and four pyrroles.

When the results shown in Figs. 2 and 3 are considered together, we reasoned that (a) PBD-oligopyrroles hybrids **22**–**25** acquire a characteristic RNA-binding property different from PBD and distamycin; (b) the interactions between the hybrids **22** and **23** and TAR-RNA are reversible, unlike interactions involving compounds **24** and **25**; (c) PBD-oligopyrroles hybrids **22**–**25** display a preferential affinity to the wild type TAR-RNA sequence than that to similarly structured AU-rich and CG-rich RNAs. Further experiments are necessary employing other functional RNA sequences, in order to verify sequence-selectivity of RNA binding activity of this class of molecules.

3.2. Inhibition of cellular nuclear proteins/TAR-RNA interactions and Tat/TAR-RNA interactions by PBD-oligopyrroles hybrids

Next, we studied the effect of distamycin, PBD and PBD-oligopyrroles hybrids on cellular proteins/TAR-RNA interactions by EMSA. ^{32}P-labeled TAR-RNA was pre-incubated with the specific drug and then incubated with nuclear extracts from the CD4+ cell line Jurkat. After the binding, protein/RNA complexes were separated from free RNA by native PAGE. The results shown in Fig. 4A demonstrate that distamycin and PBD were unable to inhibit protein/TAR-RNA interactions, whereas all the PBD-oligopyrroles hybrids capably blocked the formation of such protein/RNA complexes displaying different

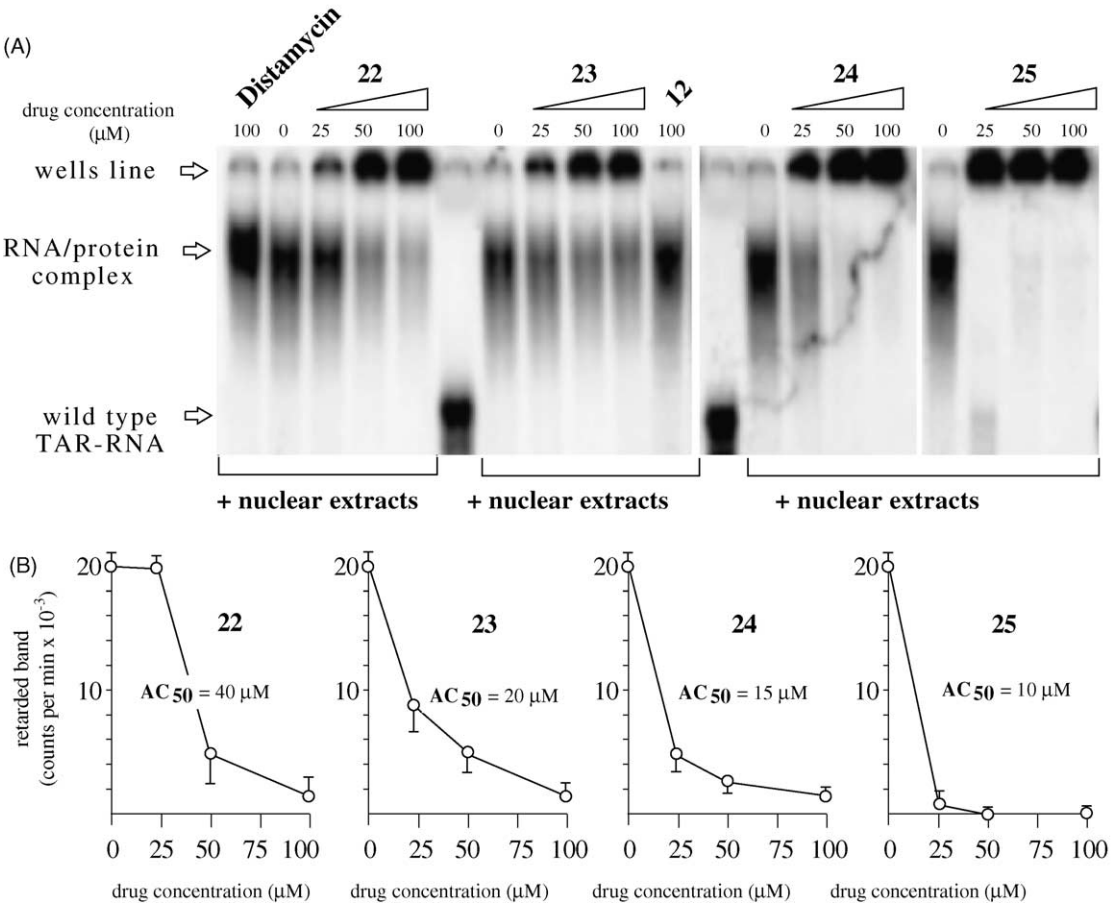

Fig. 4. Effects of the PBD-oligopyrroles hybrids with the binding of Jurkat nuclear proteins to the wild type TAR-RNA. (A) A representative EMSA experiment. In this experiment, 1 ng of the wild type TAR-RNA probe was pre-incubated with the drug at the indicated concentration, in the presence of 1 μg of poly(dIdC)·poly(dIdC) for 15 min at room temperature. After this time, 5 μg of Jurkat cells nuclear extracts were added for additional 15 min. RNA/protein complexes were fractionated from free RNA probe by native polyacrylamide gel electrophoresis. (B) Quantitative analysis of the radioactivity entrapped in the RNA/protein complexes as function of the drug concentration. The data represent the mean value of three independent experiments performed as outlined in (A).

efficiencies. In this respect, increasing the number of oligopyrroles in the hybrid augmented the inhibitory effect of PBD-oligopyrroles compounds (Fig. 4B). In fact, hybrid **22**, carrying only one pyrrole unit, shows the highest AC_{50} value. In our experiments, the AC_{50} value is the concentration of drug which putatively inhibit the formation of 50% of the protein/RNA complexes after 15-min incubation with 1 ng of labeled RNA and 5 μg of nuclear extracts, in a final volume of 25 μL at room temperature. By contrast, hybrid **25**, carrying four pyrrole units, shows the highest inhibitory activity (AC_{50} = 10 μM). Hybrids **23** and **24**, carrying two and three pyrrole units, respectively, show intermediate activities (20 and 15 μM, respectively). The fact that the decrease of the RNA/protein complexes is not accompanied by an increase of free wild type TAR-RNA is not surprising, since these compounds generate aggregates when incubated with target RNA (see Fig. 2). Therefore, in order to confirm that compounds **22**–**25** inhibits RNA/protein interactions it is imperative to reach the same conclusions with a different approach.

Accordingly, we studied the inhibitory effects of PBD-oligopyrroles hybrids **22**–**25** on Tat/RNA interactions using a filter-binding assay. Here, wild type HIV-1 Tat (Tat2E) spotted onto a nitrocellulose membrane was hybridized with ^{32}P-labeled wild type TAR-RNA pre-incubated in the presence or absence of drugs. The results of three independent experiments are reported in Fig. 5A and B, and show that PBD-oligopyrroles hybrids **22**–**25** inhibit the interaction of Tat with the TAR-RNA in a dose dependent fashion (AC_{50} values are shown in Fig. 5B). By contrast, PBD **12** and distamycin, two compounds that we demonstrated to be unable to bind TAR-RNA, do not inhibit the interaction of TAR-RNA with Tat. As expected, no significant binding of TAR-RNA with mutated Tat was observed, confirming that interaction of TAR-RNA with the Tat protein spotted on the solid support occurs in the expected manner (data not shown). In a second experiment employing compound **24**, we determined whether it is able to disassemble a pre-formed TAR-RNA/Tat complex. In this experiment, TAR-RNA was incubated with filter-bound Tat protein, and the resulted complexes, after a brief washing step in binding buffer, were incubated with increasing concentrations of compound **24**. Despite the fact that compound **24** inhibits, as expected, the binding of

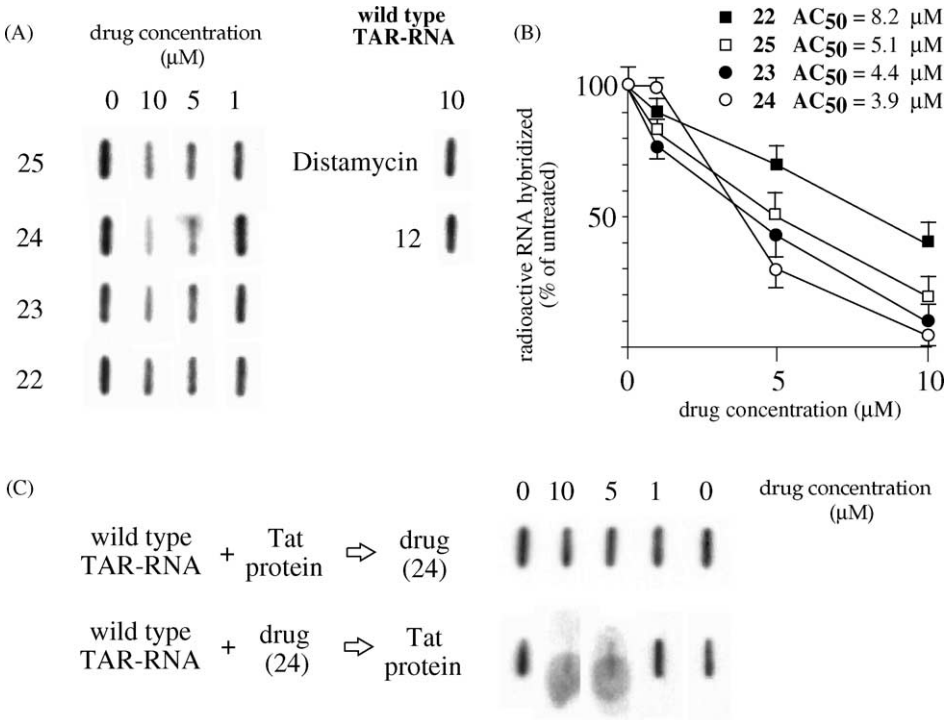

Fig. 5. Effects of the PBD-oligopyrroles hybrids on the binding of HIV-1 Tat protein to the wild type TAR-RNA. (A) Filter-binding experiments. One microgram of Tat per well was spotted on the nitrocellulose membrane. The filter-bound Tat was incubated in binding buffer at 4° with ^{32}P-labeled wild type TAR-RNA or with pre-assembled wild type TAR-RNA/PBD-oligopyrroles hybrid complexes obtained using the indicated concentration of drug. After 1 hr of incubation, the strips were separately washed in binding buffer (three times, 10 min each) and exposed to Kodak XAR-5 film at −80°. (B) Quantitative analysis of the radioactive TAR-RNA captured by the filter-bound Tat as a function of the drug concentration. The data represent the mean value of three independent experiments performed as outlined in the panel (A). The concentration of drug arresting the binding to Tat of 50% RNA molecules (AC_{50}) is indicated. (C) Filter binding experiment performed by two different approaches. In the first, the filter-bound Tat was incubated in binding buffer at 4° with ^{32}P-labeled wild type TAR-RNA or with pre-assembled wild type TAR-RNA/compound **24** complexes obtained using the indicated concentration of drug (lower side). In the second, the filter-bound Tat was incubated in binding buffer at 4° with ^{32}P-labeled wild type TAR-RNA for 15 min and, after this time, the indicated concentration of the compound **24** was added to the Tat/TAR-RNA complexes (upper side). After 1 hr of incubation, the strips were separately washed in binding buffer (three times, 10 min each) and exposed to Kodak XAR-5 film at −80°.

Tat to TAR-RNA (Fig. 5C, lower side of the panel), only minor effects were observed on pre-formed Tat/TAR-RNA complexes (Fig. 5C, upper part of the panel). Taken together, these data provide evidences that PBD-oligopyrroles hybrids **22–25** can prevent the binding of Tat to the HIV-1 TAR-RNA.

3.3. Cytotoxicity and effects of PBD-oligopyrroles hybrids on the Tat-induced HIV-1 LTR-driven transcription

The possible inhibitory effects of PBD-oligopyrroles hybrids on *ex vivo* Tat-induced HIV-1 LTR-driven transcription were studied by using as model the HL3T1 cell line which contains integrated copies of an HIV-1 LTR-CAT plasmid. Initially, we determined the cytotoxic effects of drugs on this cell line. Cells were grown in complete medium in the absence or in the presence of increasing concentration of drug and cell viability was quantified using the MTT assay. The concentration of drug leading to 50% cytotoxic effect was termed IC_{50}. We found that PBD-oligopyrroles hybrids **24** and **25**, containing the higher number of oligopyrroles are more cytotoxic (Fig. 6). Cytoxicity decreases when the number of oligopyrroles attached to the PBD group is reduced.

Subsequently, the effects of PBD-oligopyrroles hybrids on Tat-induced HIV-1 LTR-driven transcription were assessed using HL3T1 cells cultured in the absence or in the presence of 1, 5 and 10 μM of PBD-oligopyrroles hybrids **22–25**. As control, cells were treated with 10 μM PBD **12** or distamycin. After the addition of the HIV-1 Tat protein, the cells were harvested 72 hr later for CAT assay. The results reported in Fig. 7 demonstrate that PBD-oligopyrroles hybrids **23–25** inhibit Tat-induced HIV-1 transcription at 5–10 μM concentration. By contrast, hybrid **22** inhibited Tat-induced HIV-1 transcription much more weakly than hybrids **23–25**. PBD **12** and distamycin were entirely ineffective on Tat-induced transcription at 10 μM concentration. It should be underlined that compound **12**, which at 10 μM suppresses cell growth (see Fig. 6) has no effect on HIV-1 LTR driven CAT activity; on the other hand, compound **23**, which shows lower antiproliferative activity when compared to **24** and **25**, inhibits CAT activity, suggesting that the effects described in Fig. 7 are not due to cytotoxicity. Since Tat-induced HIV-1 transcription depends on the assembly of proteins/TAR-RNA

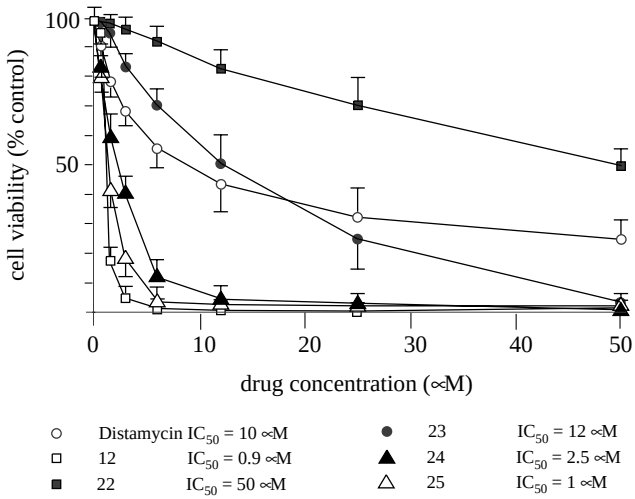

Fig. 6. Cell viability assay. The HL3T1 cells were seeded in complete medium and grown for 72 hr in the presence or absence of increasing amounts (0–0.75–1.5–3–6–12–25–50 μM) of PBD-oligopyrroles hybrids. After this time, the number of viable cells was determined by MTT staining. Cell viability is expressed as percent with respect to viable cells grown in the absence of drug and graphed as a function of drug concentration. The reported IC_{50} values were obtained by extrapolation of the curves.

complexes and basal transcription depends on the binding of transcription factors to DNA sequences, we reasoned that inhibition of the assembly of protein/TAR-RNA complexes could be the crucial step in the modulation of HIV-1 transcription by PBD-oligopyrroles hybrids **22–25**. While PBD **12** and distamycin are well-known for their DNA-binding property and for their efficient inhibitory effects on cognate DNA-binding by transcription factors TFIID [20] and Sp1 [17], it is intriguing that they were, even at 10 μM concentration, wholly ineffective on Tat-induced RNA-binding dependent transcription.

4. Discussion

Distamycin A and anthramycin are well known DNA-binding drugs able to interact with A–T or G–C rich sequences, respectively. Here, we show that both drugs failed to bind structured RNA (Fig. 2B) and do not modulate Tat-induced HIV-1 LTR-driven transcription (Fig. 7). Thus, these two compounds cannot be considered efficient RNA-binding drugs. Surprisingly, we found that the hybrid molecules, obtained by linking the distamycin-like oligopyrrole minor groove binder with the pyrrolo PBD, acquire the novel property of being able to bind structured RNAs (Fig. 2B). Moreover, the synthesized PBD-oligopyrroles hybrids **22–25**, even if bind A–T and G–C rich structured RNAs, seemed to prefer wild type HIV-1 TAR-RNA, suggesting their potential utility in AIDS therapeutics. This behavior suggests that the binding to structured RNAs could be due to recognition of structure rather than sequence. Furthermore, the binding of hybrids **22** and **23** to TAR-RNA could be completely dissociated under denaturing conditions (Fig. 3), thereby indicating that such interactions with TAR-RNA occurred without covalent bonding. This is not a trivial consideration since PBD group owes its DNA-interactive ability to a N10–C11 carbinolamine/imine moiety in the central B-ring which is capable of covalent binding to the C2–NH_2 of guanine residues in the minor groove of DNA [14]. Accordingly, compounds **24** and **25** generates irreversible drug/RNA complexes (Fig. 3).

Tat-induced transcription of the HIV-1 genome is strongly enhanced by the interaction of the human cyclin T1 with the loop region of structured TAR-RNA [4,5]. For this reason, inhibition of this complex assembly may represent a new target for anti-HIV compounds. Accordingly, we studied the effects of PBD-oligopyrroles hybrids on the binding of CD4+ Jurkat cellular nuclear proteins to TAR-RNA. EMSA data suggest that PBD-oligopyrroles hybrids **22–25** inhibited well nuclear protein/TAR-RNA interaction (Fig. 4). This inhibition was dose-dependent and proportional to the number of pyrroles linked to PBD. Separately, our filter-binding assay (Fig. 5) demonstrated that a pre-formed PBD-oligopyrroles hybrid/^{32}P-TAR-RNA complex was not accessible for binding by Tat. This finding lends support to the effectiveness of PBD-oligopyrroles hybrid/TAR-RNA interaction.

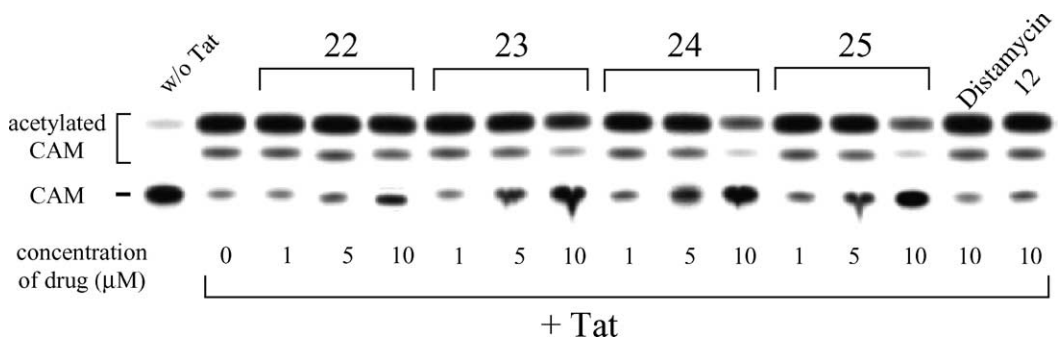

Fig. 7. Effects of PBD-oligopyrroles hybrids on Tat-induced HIV-1 LTR-driven transcription. The HL3T1 cells, containing integrated copies of LTR-CAT retroviral construct, were seeded at 50–70% confluence in serum-free medium and PBD-oligopyrroles hybrids were added to the cell culture medium at 0, 1, 5 and 10 μM final concentration. After 16 hr liposomes loaded with HIV-1 Tat were added and the cells were grown for an additional 72 hr in medium containing 10% FCS. Cytoplasmic extracts were prepared, and normalized protein amounts were assayed for CAT activity.

Finally, we have studied the effects of PBD-oligopyrroles hybrids **22–25** on Tat-induced HIV-1 LTR-driven transcription. In this respect, the *ex vivo* experiments performed on HL3T1 cells, as a model for the activation of an integrated provirus, demonstrate that PBD-oligopyrroles hybrids **23–25** were selectively capable of modulating Tat-induced transcription from the CAT reporter gene (Fig. 7). These results suggest that PBD-oligopyrroles hybrids **23–25** could be considered as lead compounds for developing potentially useful new anti-HIV-1 drugs. We note that among the active molecules, PBD-oligopyrroles hybrid **23** appears to be of interest, since it exhibits the lowest cytotoxic profile (Fig. 6).

In conclusion, we have shown that PBD-oligopyrroles hybrids are efficient RNA-binding compounds, which can interrupt protein/TAR-RNA interactions and Tat-induced LTR-driven HIV-1 transcription. We propose that such compounds be usefully considered for future experiments aimed at determining exact RNA binding sites and possible *in vitro* anti-viral activity. These experiments will clarify whether some of the molecules here presented will be useful in future development of chemotherapeutics for AIDS.

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