The binding mode of drugs to the TAR RNA of HIV-1 studied by electric linear dichroism

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ABSTRACT

For the first time, the interaction between a series of small molecules and the TAR RNA of HIV-1 has been investigated by electric linear dichroism (ELD). The compounds tested include the DNA intercalating drugs proflavine and ethidium bromide and an amscarine-4-carboxamide DNA-threading intercalator as well as the AT-specific DNA minor groove binders netropsin, Hoechst 33258, berenil and DAPI. In all cases except for netropsin, negative reduced dichroism signals were measured in the drug absorption band. In agreement with previous studies, the results indicate that both classical and threading intercalation can occur with the TAR RNA. The ELD data show that the mode of binding of the drugs Hoechst 33258, berenil and DAPI to the TAR RNA is similar to their binding mode in GC-rich regions of DNA and likely involves intercalation into the A-form TAR RNA helix. The wide and shallow minor groove of the TAR RNA is apparently not accessible to DNA minor groove binding drugs such as netropsin. The ELD technique appears uniquely valuable as a means of investigating the interaction of drugs with the TAR RNA.

INTRODUCTION

Linear dichroism provides a rapid and sensitive method to study the structure and function of nucleic acids as well as for determining the orientation of drugs upon binding to DNA (1–3). Applied to DNA, this technique is probably the most direct method to evaluate intercalating versus groove binding drugs. Recently, we have shown that linear dichroism is uniquely valuable to investigate the sequence-dependent recognition of DNA by drugs (4,5). In particular we have shown that the drugs Hoechst 33258, berenil and DAPI (Fig. 1) which bind strongly to the minor groove of AT-rich sequences can also interact with GC-rich sequences in DNA via a non-classical intercalation process (4–8).

The continuous spread of AIDS and the increasing need for a more efficient retroviral chemotherapy have prompted us to extend the linear dichroism methodology to the study of RNA–drug interactions. So far, very little attention has been devoted to the recognition of RNA by drugs although there are now clear indications that RNA is a potential therapeutic locus for drugs such as the antitumour antibiotics bleomycin and the enediyne neocarzinostatin (9–12). A better knowledge of the mode and sequence-selectivity of binding of drugs to RNA, in particular the RNA genome of HIV-1, would be very useful for the design of new categories of anti-HIV-1 drugs.

The trans-activation-responsive region (TAR) RNA of HIV-1 provides a privileged candidate as potential substrate for anti-HIV drugs. It is now well established that activation of HIV gene transcription results from the direct specific interaction between the HIV Tat protein and its cognate TAR RNA sequence (13,14). Although the three-dimensional structure of the Tat-TAR complex has not yet been fully elucidated, indirect methods have allowed identity of the interaction down to the atomic group level of TAR RNA (15–17). These studies show that only a small, well structured region of TAR forms a binding pocket upon folding of the RNA suggesting that small molecular weight molecules interacting with TAR RNA could act as decoy to the interaction or ‘lock’ the flexible structure of TAR in a conformation which would not be recognized by the protein. There is little doubt that effective inhibitors of protein–TAR interactions might turn out to be highly effective antiviral agents (18). In recent years, the TAR RNA which presents a folded structure with an apical loop and a bulge with flanking Watson–Crick base-pair stabilized double-stranded regions (Fig. 2), has been used as a substrate for studying drug–RNA interactions as well as for designing anti-HIV compounds (19–21). A significant correlation between RNA affinity and anti-HIV activity has been observed for a series of quinoline and quinazoline intercalators (22). On the other hand, it has been shown that both DNA intercalators and groove binders can bind strongly and sometimes preferentially to the RNA polymer poly(A)·poly(U) compared with the corresponding DNA polymer poly(dA)·poly(dT) (23–25). These observations together with the recent discovery that the TAR RNA sequence is a good substrate for an ethidium bromide derivative (20) led us to question whether or not (and how) drugs such as Hoechst 33258, berenil and DAPI, which bind differently to AT and GC sequences in DNA (8,26–29), might interact with the TAR RNA.

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**MATERIALS AND METHODS**

**Drugs**

Hoechst 33258 [2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperaziny)-2,5′-bis-1H-bisbenzimide], berenil [1,3-bis(4-phenylamidinium)triazene] and DAPI (4′,6-diamidino-2-phenylindole) were purchased from Sigma Chemical Co. Netropsin was purchased from Serva. Ethidium bromide and proflavine hemisulphate were from BDH. The amsacrine-4-carboxamide derivative SN16713 was obtained by courtesy of Prof. W. A. Denny (University of Auckland, NZ). The drugs showed good aqueous solubility and were used as supplied without further purification. Ligand concentrations were determined spectroscopically in 10 mm pathlength quartz cuvettes using the following molar extinction coefficients (M$^{-1}$ × cm$^{-1}$): 21 500 at 296 nm for netropsin; 42 000 at 338 nm for Hoechst 33258; 34 400 at 370 nm for berenil; 27 000 at 342 nm for DAPI; 5700 at 480 nm for ethidium bromide; 41 000 at 444 nm for proflavine and 12 900 for SN16713 at 442 nm. All other chemicals were analytical grade reagents, and solutions were prepared with doubly distilled sterile water to prevent nuclease contamination. Tubes and tips were treated with diethylpyrocarbonate (DEPC from Sigma).

**In vitro transcription of TAR RNA**

A synthetic oligonucleotide corresponding to the wild-type TAR sequence was cloned between HindIII and EcoRI sites of the pUC19 plasmid (30). After digestion with EcoRI, TAR RNA was transcribed as a run-off product of 60 nt from the T3 RNA polymerase promoter. The transcript includes an additional G residue on the 3′-end derived from the EcoRI 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 DTT, 20 U RNasin (Promega) and 1 mM NTPs. The reaction was initiated by addition of 100 µg linearized plasmid DNA template and 40 µg T3 RNA polymerase and incubated for 2 h at 37°C. Nucleic acids were purified by extraction with phenol, precipitated with ethanol and then fractionated on a 10% (w/v) polyacrylamide gel containing 8 M urea in 0.5× TBE buffer (45 mM Tris–borate pH 8.3, 10 mM EDTA). After electrophoresis, the RNA was eluted in water for 18 h at 4°C and then extracted with ethanol prior to ethanol precipitation. The RNA was resuspended in DEPC-treated water to give a 500 µM stock solution ($\varepsilon_{260}$/phosphate = 10688 M$^{-1}$ × cm$^{-1}$).

**Absorption spectroscopy and circular dichroism**

Absorption spectra were recorded on a Perkin–Elmer Lambda 5 spectrophotometer using a 10 mm optical pathlength. Circular
dichroism (CD) measurements were recorded on a Jobin–Yvon CD6 dichrograph interfaced to a PC microcomputer. Solutions of drugs and/or RNA were scanned in 1 cm quartz cuvettes. Five scans were accumulated and automatically averaged.

**Electric linear dichroism (ELD)**

This electrooptical method exploits the fact that, under the influence of a short electric field pulse, the DNA or RNA molecule becomes oriented, rendering the solution optically anisotropic. The ELD measurements were performed with a computerized optical measurement system built by C. Houssier (31). The procedures previously outlined were followed (1,5). The optical set-up of a high sensitivity T-jump instrumentation equipped with a Glan polarizer was used under the following conditions: bandwidth 3 nm, sensitivity limit 0.001 in ΔA/A, response time 3 µs. The rectangular electric field pulses in the range 0–13 kV/cm were applied to the samples in a 10 mm optical pathlength Kerr cell with a distance between the platinum electrodes of 1.5 mm. The pulse duration was carefully adjusted to reach the steady-state orientation of the molecule (50–100µs, depending on the electric field strength). The structure of the TAR RNA is not disturbed by short electric pulses (32). Linear dichroism ΔA is defined as the difference between the absorbance for light polarized parallel $A(∥)$ and perpendicular $A(⊥)$ to the applied field at a given wavelength. The reduced dichroism is $ΔA/A = (A(∥) - A(⊥))/A$, where A is the isotropic absorbance of the sample measured in the absence of field at the same wavelength and under the same pathlength. Because of axial symmetry around the electric field direction, the changes in absorbance and under the same pathlength. Because of axial symmetry around the electric field direction, the changes in absorbance and absorbance of light polarized parallel $(A(∥))$ and perpendicular $(A(⊥))$ to the applied field at a given wavelength. The reduced dichroism is $ΔA/A = (A(∥) - A(⊥))/A$, where A is the isotropic absorbance of the sample measured in the absence of field at the same wavelength and under the same pathlength. Because of axial symmetry around the electric field direction, the changes in absorbance and under the same pathlength. Because of axial symmetry around the electric field direction, the changes in absorbance and absorbance of light polarized parallel $(A(∥))$ and perpendicular $(A(⊥))$ to the applied field at a given wavelength. The reduced dichroism is $ΔA/A = (A(∥) - A(⊥))/A$, where A is the isotropic absorbance of the sample measured in the absence of field at the same wavelength and under the same pathlength. Although the TAR RNA contains only 24 bp (among which are 13 G·C pairs), the reduced dichroism of the TAR oligomer oriented in an electric field of 13 kV/cm at low ionic strength can be accurately measured. Negative reduced dichroism values of about −0.010 were measured with good reproducibility in the 250–290 nm region. The CD spectrum of the TAR RNA in the low ionic strength buffer required for the ELD measurements is shown in Figure 3. The typical shape of the spectrum (a weak negative band at 235 nm adjacent to a large positive band centered at 265 nm) attests that under the low ionic strength conditions the TAR RNA is folded into an A-form helix as expected for such a double-stranded RNA.

Prior to investigating the binding of the drugs Hoechst, berenil and DAPI to the TAR RNA, we carried out a series of measurements with well-established intercalating drugs in order to determine whether the ELD technique can give reliable information about drug–RNA interactions. Both ethidium bromide and proflavine which are among the best characterized DNA intercalating drugs, exhibit negative reduced linear dichroism signals upon binding to the TAR RNA. As depicted in Figure 4, the reduced dichroism values measured with these two drugs are comparable, or even superior, to the value determined for the TAR RNA alone at 260 nm suggesting thus that the base pairs and the drug chromophore are more or less parallel, as expected for intercalating drugs. Strongly negative signals were also obtained with the amscarine–4-carboxamide derivative SN16713 which has been characterized previously as a DNA-threading intercalating drug (38–40). The larger amplitude of the dichroism of the bound drug may arise from a local stiffening of the RNA structure around the intercalation sites, as is the case with DNA. The ELD values obtained with the three intercalating drugs vary significantly (Fig. 4). The same differences between ethidium, proflavine and SN 16713 have been noted previously when using a series of natural DNA and polynucleotides (4). We believe that the variations originate in the different effects of the drugs on the nucleic acid structure. It is known that intercalating drugs unwind and stiffen the DNA double helix to various extents and that these structural perturbations can influence the degree of orientation of the DNA in the electric field. It is possible that a similar effect occurs with the TAR RNA. This first set of results are in perfect agreement with studies showing that (i) both classical and DNA-threading intercalators generally bind to the RNA polymer
poly(A)·poly(U) by the same mode (23,24) and (ii) an ethidium derivative effectively intercalates into the TAR RNA (preferentially at the bulge segment) (20,21). Therefore, the results obtained with the intercalating drugs support the validity of the ELD method for evaluating the binding mode of RNA ligands.

The AT-specific DNA minor groove binder netropsin was also tested for its capacity to bind to the TAR RNA but practically no signal could be detected. Netropsin, which exhibits antiviral properties but which is inactive towards HIV, apparently does not interact with the TAR RNA, just as it fails to interact with GC sequences in DNA. Distamycin, which is an analogue of netropsin, also fails to interact with RNA (23). Parenthetically, we may mention here that we also tested the topoisomerase I inhibitor camptothecin with the idea in mind that the single-stranded loop and bulge regions of TAR may somehow mimic an open structure in the DNA–topoisomerase I complex. But under the conditions used camptothecin does not bind to the TAR RNA, nor does it bind to DNA in the absence of topoisomerase I (data not shown).

Next we investigated the mode of binding to the TAR RNA of the sequence-dependent DNA binding ligand 4,6-diamidino-2-phenylindole, DAPI. Negative reduced dichroism values were measured in the drug absorption region (Fig. 5). The amplitude of the signals is very similar to that measured with the Hoechst 33258–TAR RNA complexes. To sum up, it is clear from the ELD data that DAPI interacts with GC sequences of DNA (8,29). The reduced dichroism values obtained with the Hoechst 33258–TAR RNA complexes are negative as with DAPI and Hoechst 33258 but the values are considerably smaller (Fig. 4). The amplitude of the ELD signal measured at 370 nm is about half that obtained with the TAR RNA alone indicating that berenil does not bind to the TAR RNA with the same geometry as DAPI or Hoechst 33258. A combination of intercalation plus groove binding (as with DNA; 42) would satisfactorily account for the ELD signals obtained with the TAR RNA–berenil complexes.

Negative ELD signals were measured for complexes between TAR RNA and the bisbenzimidazole derivative Hoechst 33258 (Fig. 4). It is well-established that Hoechst 33258 binds strongly to AT sequences in the minor groove of DNA and we have previously proposed that Hoechst can intercalate into GC sequences of DNA (6). By analogy with the acridines, ethidium and DAPI, we are forced to conclude that Hoechst 33258 can also intercalate into the TAR RNA. However, viscometric titrations that the bulky piperazine and hydroxyphenyl substituents attached at both ends of the extended bis-benzimidazole chromophore of Hoechst 33258 hinder the approach to the intercalation site—be it at the bulge as for ethidium (20) or within the duplex region of the RNA. Recent studies with dicationic diphenylfuran derivatives bis-substituted with different groups of various sizes have revealed that the compounds with the smallest substituents intercalate into RNA whereas those bearing the largest substituents (including six-membered rings as found in Hoechst 33258) cannot intercalate into RNA and may bind to the major groove of the A-form RNA helix (41). However, the high negative reduced dichroism values obtained with the Hoechst 33258–TAR RNA complex are incompatible with a tight fitting of the drug along the groove. The data can only be explained by invoking an intercalation of Hoechst into the RNA structure, or eventually a stacking of the drug in the groove parallel to the base pair.

A recent investigation of the binding of berenil to both DNA and RNA has shown that this (bis-phenyl)triazene derivative exhibits intercalative as well as minor groove binding properties when it binds to both DNA and RNA polymers (42). The reduced dichroism values measured for the TAR RNA–berenil complexes are negative as with DAPI and Hoechst 33258 but the values are considerably smaller (Fig. 4). The amplitude of the ELD signal measured at 370 nm is about half that obtained with the TAR RNA alone indicating that berenil does not bind to the TAR RNA with the same geometry as DAPI or Hoechst 33258. A combination of intercalation plus groove binding (as with DNA; 42) would satisfactorily account for the ELD signals obtained with the TAR RNA–berenil complexes.
presented in Figure 4 that Hoechst 33258, berenil and DAPI do not bind to the shallow and wide minor groove of the TAR RNA. By analogy with the results obtained with the three intercalating drugs ethidium, proflavine and SN16713 as well as the results previously reported with GC DNA polymers, it is realistic to hypothesize that these three drugs form intercalation complexes with the TAR RNA. Further studies are needed to confirm this hypothesis. One may suggest that the major groove of the TAR RNA, which is deep and narrow, may offer opportunities for drug binding: the more so since the major groove of RNA share common structural features with the minor groove of DNA (43). Although the major groove is fairly inaccessible in canonical A-form RNA, it possesses the ensemble of hydrogen bond donors and acceptors most suited for sequence-specific recognition (44,45). We cannot yet totally exclude this possibility but we believe that the binding of Hoechst 33258, berenil and DAPI to the major groove of the TAR RNA is unlikely because it would give rise to positive ELD signals. Moreover, if the drugs were to bind to the major groove of the TAR RNA, there is no reason why netropsin could not also bind.

Two major conclusions can be deduced from the ELD study reported here. First, the results indicate that the mode of binding of the drugs to the TAR RNA is similar to the mode of binding determined with GC-rich DNA and the RNA polymer poly(A)·poly(U). The ELD data strongly suggest that Hoechst 33258, berenil and DAPI as well as ethidium, proflavine and the ansacrine-4-carboxamide derivative SN16713 intercalate into the TAR RNA. Secondly, and this is important for future studies, the results show that the utility of the electric linear dichroism technique can be extended to the study of the interactions between drugs and small RNA such as the TAR RNA. A systematic investigation of the reversible binding of small molecules to a series of TAR RNA derivatives (with and without the bulge and/or the loop) has been initiated. The challenge, revived by the increasing resistance of the HIV to drugs such as Zidovudine (AZT), is to assist the development of new anti-HIV agents.

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