Targeting the HIV *Trans*-Activation Responsive Region—Approaches Towards RNA-Binding Drugs

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Introduction

RNA–protein interactions are essential for many biological processes such as translation, RNA splicing, and transcription. For a long time, RNA was regarded mainly as a passive system for information delivery from DNA to proteins. Today it is known that the catalytically active part of the ribosome is its RNA component.[1] A considerable number of antibiotics bind to the ribosomal RNA and thus block protein synthesis in microorganisms. This finding suggests that RNA in general is a potential target for small molecules that could alter its biological function.[2] In the case of ribosomal RNA, however, the similarity between the human and procaryotic structures causes an intrinsic selectivity problem responsible for the well-known toxicity of aminoglycoside antibiotics.

One strategy towards developing less toxic RNA-binding antiinfectives is to target sequences specific for bacterial or viral replication. The *trans*-activation responsive region (TAR) of HIV-1, for example, is essential for viral gene expression but absent in uninfected human cells.[3] It was recognized early on that specific ligands for TAR have the potential to inhibit HIV-1 selectively.[4] During the past decade, numerous methods of structure determination and techniques for drug discovery have been applied to TAR. Most recently, regulatory elements have been identified in the untranslated regions of many mRNAs.[5] This discovery opens up fascinating opportunities for future drug development. Lessons learnt from well-characterized model systems such as TAR will be decisive for the successful treatment of these challenging problems.

The tat/TAR Complex

A key step during the replication of HIV-1 is the association of TAR RNA and the tat protein, which results in a drastic increase in the number of viral transcripts. TAR is a 59-base stem-loop structure located at the 5′ ends of all nascent HIV-1 transcripts (see below).[3b] Two helical stem regions are separated by a three-nucleotide bulge responsible for tat/TAR recognition, while the loop region is essential for *trans* activation and binding of the cyclinT1/tat complex.[6] By mutagenesis, chemical probing, and peptide binding studies it was found that U23, located in the bulge region, is a pivotal base for effective tat recognition. The remaining two bases, C24 and U25, clearly act mainly as spacers since they can be replaced by any other nucleotide. A further contribution to the binding of tat arises from the four base pairs flanking the bulge.[7] The tat protein consists of two important functional domains. One region is responsible for the interplay with the transcriptional machinery of the host. The other part is rich in basic amino acids like arginine and is accountable for transport into the nucleus and for binding to TAR (see below). To interfere with the tat/TAR association, a potential antagonist should possess a high and selective affinity for the bulge region of TAR. Herein we review the recent advances in tat antagonist development and the methods used to determine the efficacy of TAR RNA ligand binding.

Small Molecules

During the last decade, a limited number of nonpeptidic small ligands (FW $\leq$ 500 Da) have been reported to inhibit tat/TAR complex formation.

The first examples were benzodiazepines and epoxy steroids.[8] However, the mechanism of inhibition of viral replication by these molecules is not binding to TAR, but to the protein component tat.[9] In 1992 Frankel and Williamson proposed a structure for the TAR complex with arginine amide (**1**; Scheme 1) based on NMR data and biochemical experiments. Up to now arginine amide is the best-investigated TAR RNA ligand.[10] It binds at the bulge region, forms hydrogen bonds with G26, and induces a change of the RNA conformation characteristic for all tat-derived basic peptides. Four years later, Bailly et al. examined the TAR-binding properties of Hoechst 33258 (**2**)[11] by electric linear dichroism and suggested intercalation as the binding mode. Subsequent footprinting studies indicated that the GC-rich region G36–U40 is the predominant contact site (Scheme 2). In the same year, Mei et al. developed a high-throughput in vitro screening to identify drugs capable of inhibiting the tat–TAR interaction.[12] They started from 150 000 compounds and identified two promising candidates, quinoxaline (**3**) and tetraaminoquinozaline (**4**), with IC_{50} values of 1.3 μM and 10 μM, respectively. It is remarkable that the chemical structures of these compounds are related neither to typical intercalators nor to aminoglycoside antibiotics, and their TAR binding sites differ as well. Compound **3** binds in the bulge region, while **4** binds at the 3′ end of the TAR loop (Scheme 2).

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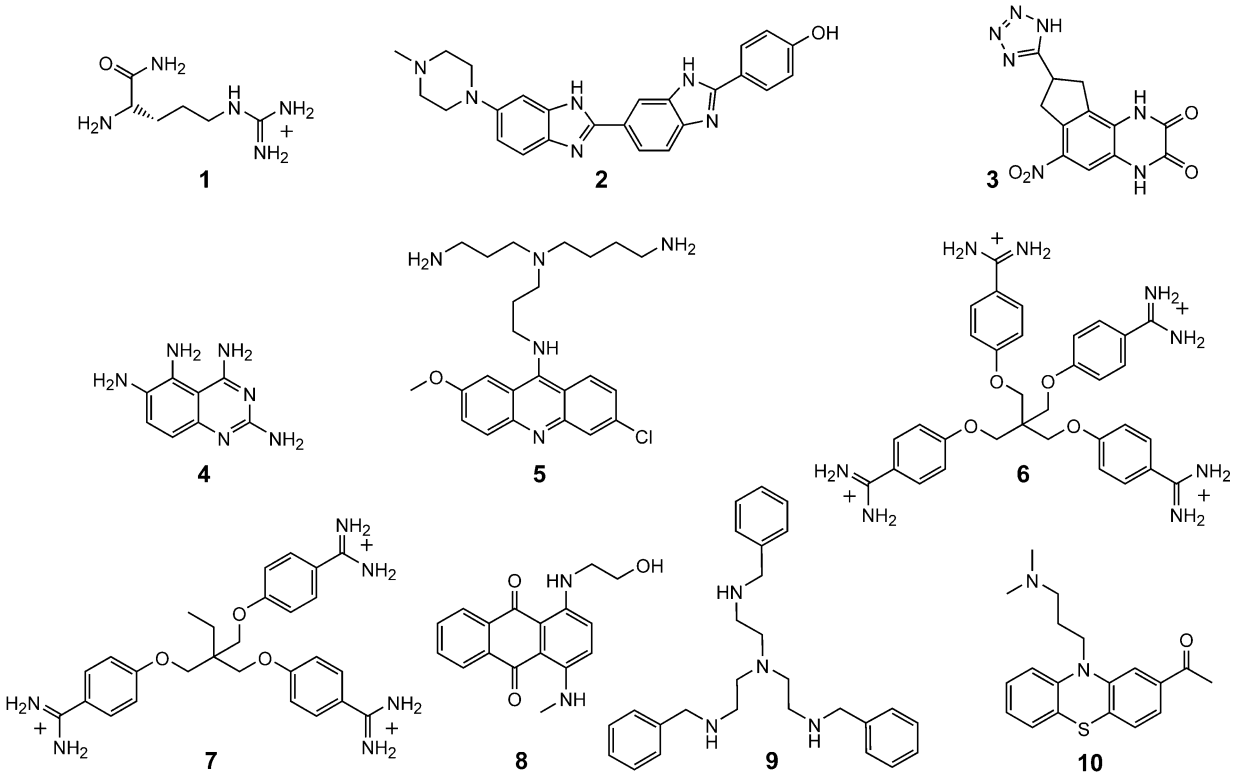
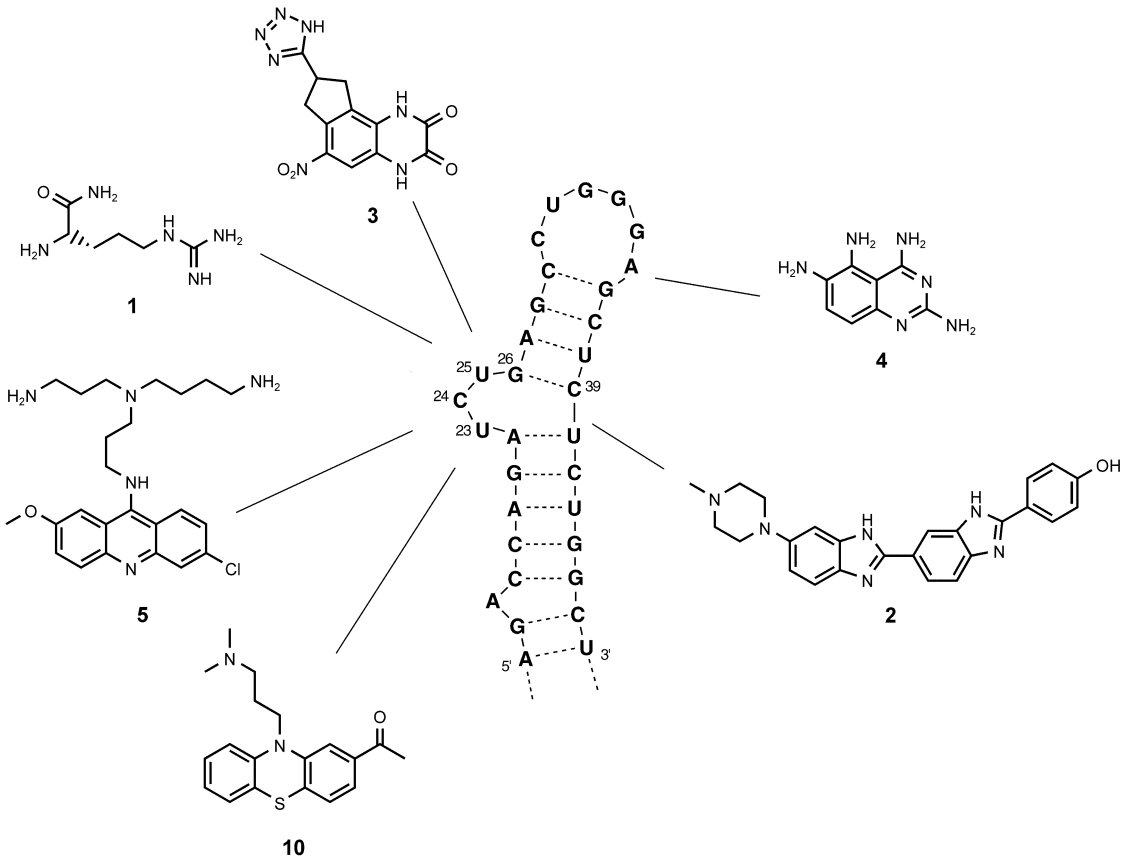


Scheme 1. *Small molecules shown to associate with TAR: arginine amide (**1**), Hoechst 33528 (**2**), quinoxaline (**3**), tetraminoquinozaline (**4**), CPG 40336A (**5**), TAPP (**6**), TAPB (**7**), anthraquinone (**8**; Available Chemicals Directory (ACD) code 00001199), trisaminederivative (**9**; ACD code 00192509), acetylpromazine (**10**).*



Scheme 2. *Binding sites of different ligands at TAR RNA.*

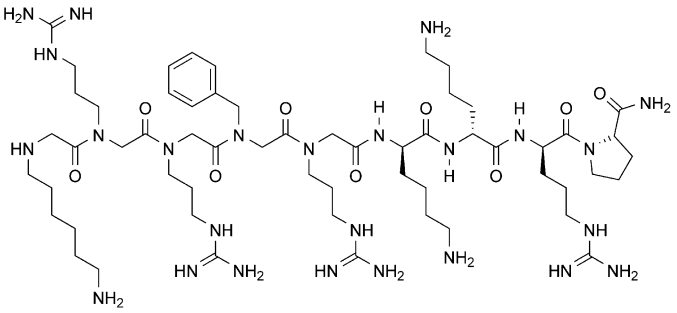
Independently, Hamy et al. synthesized a new class of HIV-1 antagonists, which they called "In-PriNts" (inhibitors of protein-ribonucleotide sequences).[13] This class of antagonists includes molecules with three different substructures: 1) an aromatic or heteroaromatic moiety with the potential for stacking interactions in the bulge, 2) a cationic residue providing electrostatic interactions with the phosphate backbone of the RNA, and 3) a linker between these two moieties. The most active compound among these inhibitors, CGP 40336A (**5**), exhibited an IC_{50} value of 0.022 μM in vitro and an IC_{50} value of 1.2 μM in a cellular assay. Footprinting and melting temperature experiments indicate an interaction with the G26·C39 base pair and a hydrogen bond between the acridine NH group and N7 of the guanine residue. A second hydrogen bond has been suggested to exist between the methoxy group of **5** and the NH_2 group of the cytosine residue. CGP 40336A (**5**) is the best TAR ligand found so far. Aromatic polyamidines like TAPP (**6**) and TAPB (**7**), another type of compound that inhibits tat-induced HIV-1 transcription, were investigated by Mischiati et al.[14] In an experimental model system with the HL3T1 cell line, TAPP and TAPB were found to inhibit HIV-1 transcription at concentrations of 18 μM and 22 μM, respectively. Furthermore, the introduction of one halogen atom (for example, a bromine atom) into the benzamidine rings strongly increases the RNA affinity of the molecule.

Well-established in silico techniques exist for the identification of protein ligands. These methods rely on detailed information about the target structure and have only recently been applied to RNA.[15] In 2000, James et al. reported virtual screening for TAR ligands.[16] The procedure starts with a fast rigid docking step followed by three steps of flexible docking using a pseudo-brownian Monte Carlo minimization in torsion angle space. From an initial 153 000 compounds from the Available Chemicals Directory, the best 30 000 ligands selected in the first step were redocked with tenfold increased sampling times per molecule. Finally, a ranking of 350 molecules with the highest predicted affinities to TAR could be obtained. This list included all aminoglycoside antibiotics already known as TAR ligands. Interestingly, many novel structures were suggested that have not been considered as RNA binders before. Two of them (Structures **8** and **9**) exhibited an IC_{50} value of approximately 1 μM in a scintillation proximity assay. In 2002 the same group obtained further important insights from virtual screening.[17] By starting with a database of 181 000 commercially available compounds, the computation described above was used to predict 500 potential TAR ligands, from which 50 were tested in vitro. Among these compounds, a most promising candidate is acetylpromazine (**10**), which represents a new class of TAR ligands with good bioavailability, a known pharmacological profile, and nanomolar binding affinity.[18] One-dimensional NMR spectra of the imino protons confirmed binding to the bulge of TAR, where the planar heteroaromatic ring system stacks between base pairs G26·C39 and A22·U40.

Peptidic Structures

Peptides or peptidomimetics that specifically target RNA structures are potential antagonists of RNA-binding proteins and are therefore capable of controlling cellular functions. During recent years several peptidic structures have been developed that specifically interact with the bulge region of TAR and reduce the replication rate of HIV-1 significantly.

In 1997 Hamy and coworkers[19] presented the peptide CGP 64222 (**11**), identified by deconvolution of combinatorial libraries. This peptoid/peptide nonamer is able to inhibit tat/TAR association in a cellular assay at concentrations of 10 – 30 μM. It also blocks HIV-1 replication in primary human lymphocytes. NMR studies of the TAR complex with CGP 64222 confirmed the specific interaction at the 3-nucleotide bulge region.



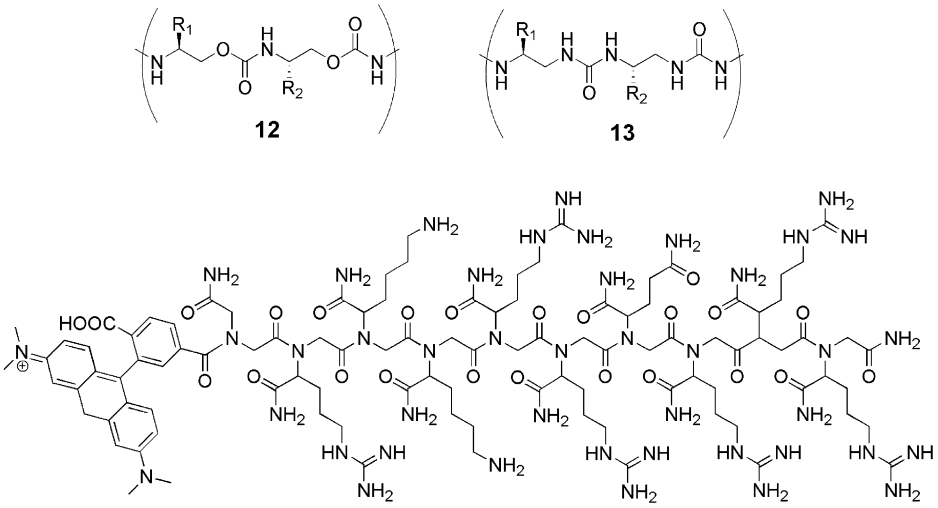
CGP 64222 (11)

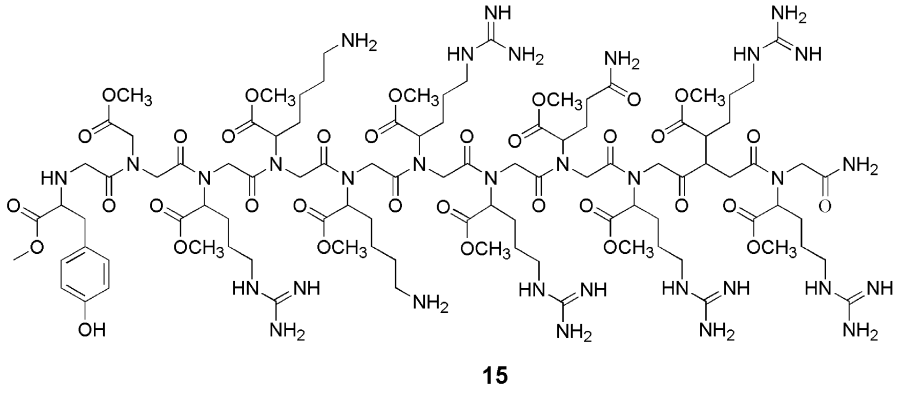
To investigate the influence of stereochemistry on RNA binding, Rana et al. synthesized D- and L-configurated shortened analogues of the tat protein (amino acids 37 – 72). Surprisingly, the D-tat peptide ($K_D = 0.22$ μM) binds with similar affinity to that of the L-tat peptide ($K_D = 0.13$ μM).[20] The same research group also synthesized peptide analogues based on carbamate and urea backbones (Scheme 3).[21a,b] The sequences of these molecules correspond to that of the basic region of tat. The dissociation constants, determined by electrophoretic mobility assays, indicate a higher binding affinity of the oligourea derivative ($K_D = 0.11$ μM) compared to the oligocarbamate ($K_D =$ 1.1 μM) and the natural L-peptide ($K_D = 0.78$ μM). Furthermore, the protease resistance of the compounds and their ability to control HIV-1 gene expression were determined by HL3T1 (CAT) cell assays. Again the effect of the oligourea compound ($IC_{50} \sim$ 0.5 μM) surpasses that of the carbamate analogue ($IC_{50} =$ 1 μM).[21c] To overcome protease degradation, oligopeptoids with additional ester or amide groups were synthesized and tested as potential HIV antagonists.[22] The ester peptoid containing the structural motif of the tat segment (47 – 57) shows a K_D value of 0.068 μM as detected by fluorescence quenching. A slightly lower affinity for TAR is found for the amide peptoid (Scheme 3).

To find out if even short peptides not related to tat may possess interesting affinities for TAR, a combinatorial library of tripeptides was prepared from D- and L-configurated standard amino acids. Out of 24 389 tripeptides, eight promising sequences were selected.[23] The best binders were NH_2-(L)Lys-(D)Lys-(L)Asn-OH ($K_D = 0.42$ μM) and NH_2-(D)Thr-(D)Lys-(L)Asn-OH ($K_D =$

^{47}Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg^{57}

tat peptide sequence 47–57

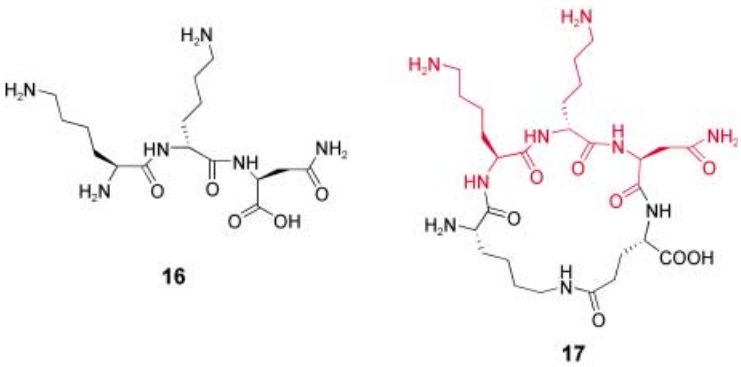




Scheme 3. *TAR-binding peptide analogues.*

0.56 μM), which indicates that X-Lys-Asn is a favored motif. The remaining peptides are diastereomers of NH_2-Lys-Lys-Asn-OH. These peptides show a drastic loss of affinity compared to the tat analogues, which indicates the importance of stereoselective effects upon binding. The efficacy of NH_2-(L)Lys-(D)Lys-(L)Asn-OH was further investigated in cell cultures (HL3T1). Tat-mediated transcriptional activation is reported to be suppressed by the peptide with an IC_{50} value of 0.05 μM.

To improve biological activity, the sequence (L)Lys-(D)Lys-(L)Asn (**16**) was incorporated into a cyclic peptide structure. The resulting pentapeptide (**17**, Scheme 4), however, does not show a significant increase in activity ($IC_{50} = 0.04$ μM) in comparison to the noncyclic molecule **16**.[24]

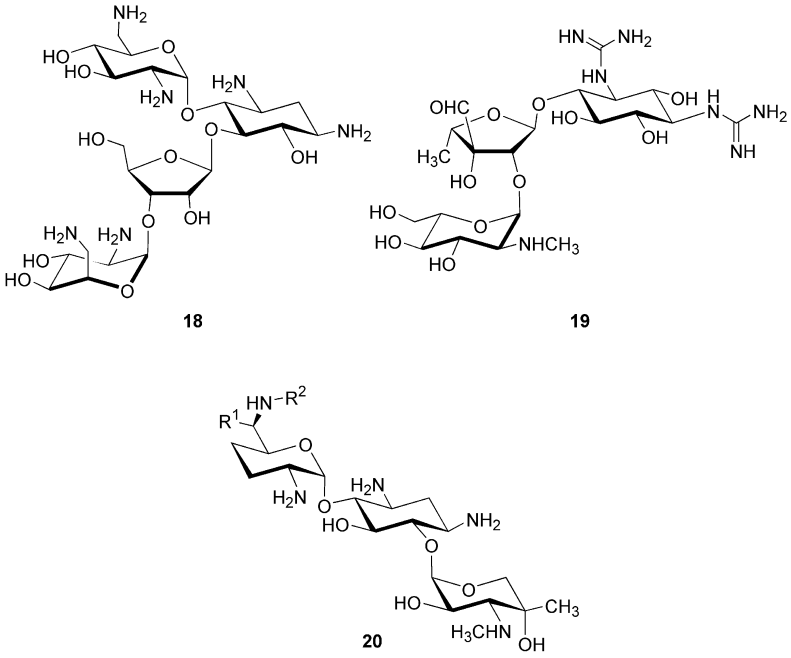


Scheme 4. *Incorporation of tripeptide **16** into a cyclic pentapeptide structure **17**.*

Aminoglycosides

In contrast to classical intercalators, which bind to DNA and RNA unselectively,[25] aminogylcosides show a promising preference for binding to RNA through electrostatic, nonintercalative interactions.[26] In 1995, TAR-binding affinities of neomycin (**18**), streptomycin (**19**), and gentamicin (**20**; Scheme 5) were determined by electrophoretic mobility shift assays. The results are summarized in Table 1.[27] The data indicate that the affinity for TAR is not strictly ruled by the magnitude of the positive charge. Streptomycin, for example, binds fivefold tighter than gentamicin in spite of a lower number of basic sites. Guanidinium groups may offer a specific advantage. In consequence, an augmentation of affinity can be achieved by conjugating several arginine residues to the aminoglycosides kanamycin A and gentamicin C1. The resulting polycationic compounds also exhibit significant biological activities in cell cultures.[28]

The binding sites of aminoglycosides have been characterized experimentally by footprinting techniques. Computational docking studies are a complementary approach, used by Westhoff and Hermann to investigate the complex of neomycin and TAR.[29] The computer experiment predicted different binding



*Scheme 5. Aminoglycosides with high affinity for TAR: neomycin (**18**), streptomycin (**19**), gentamicin (**20**).*

Table 1. Binding of aminoglycosides to TAR RNA.

Aminoglycoside	IC_{50} [μM]	Positive charges
neomycin	0.92 ± 0.09	6
streptomycin	9.5 ± 0.8	3
gentamicin	45 ± 4	5

sites for neomycin and tat, in agreement with biochemical data[12] and NMR spectroscopic evidence.[30] Neomycin inhibits tat/TAR association in a noncompetitive way by locking the RNA in the free protein conformation.

Mass spectroscopy is another possible way to study the stoichiometry of aminoglycoside – TAR interactions.[31] Published data indicate that one TAR molecule may be complexed by up to three neomycin molecules. In the presence of neomycin, a complex of tat and TAR shows two additional neomycin molecules. From tat, TAR, and neomycin, a complex with a 1:1:2 stoichiometry is formed.

Selected Methods to Quantify Complex Stabilities of RNA Ligands

After synthesis or isolation of potential ligands, characterization of the binding affinity with TAR is needed. Many traditional assays rely on radioactively labeled probes. Filter binding assays, for example, have often been used and can be adapted for high-throughput screening in microtiter plates.[7b, 12] A mixture of tat, RNA ligand, and ^{32}P-labeled TAR is equilibrated and then filtered through a nitrocellulose membrane that specifically retains tat and the tat/TAR complex. Free RNA is able to pass through the filter. By counting the radioactivity of the membrane and of the filtrate, the ratio of bound and nonbound RNA can easily be obtained. Good RNA ligands increase the amount of TAR in the filtrate.[12]

Another commonly used method is the electrophoretic mobility shift assay.[12, 32] Here again, the labeled TAR RNA (^{32}P) is incubated with tat. In a nondenaturing polyacrylamide gel, the mobility of the tat/TAR complex is reduced compared to that of free TAR or its complex with a small molecule.

A third method based on radioactive labels is the scintillation proximity assay.[33] The scintillant is embedded in streptavidin-coated solid beads. The beads are covered with biotinylated TAR RNA and incubated with ^{125}I-labeled tat peptides. Upon binding of tat, the radiolabel is fixed in direct proximity to the bead, and light emmission occurs.[12] Addition of ligands in various concentrations allows quantification of their affinities for TAR.

The disadvantage of the three techniques described above is the use of radioactivity. Fluorescence-based assays are a popular alternative. In 2000, Hamasaki et al.[34] reported the development of a fluorescence resonance energy transfer assay.[35] A 16mer tat peptide was labeled at its termini with a donor and an acceptor dye. In the absence of TAR, the peptide adopts random coil conformations, which leads to short mean distances between the dyes and to pronounced quenching effects. Binding to TAR results in more extended peptide conformations and thus increases the donor – acceptor distance and the quantum yield of fluorescence (Figure 1).

Electron paramagnetic resonance spectroscopy completes the broad scope of methods already applied to studies of TAR-binding ligands.[36] By chemical synthesis, Sigurdsson et al. introduced nitroxide spin labels into selected positions of the TAR sequence (2′-OH group of U23, U25, U38, or U40). Upon binding of guest molecules, characteristic changes of the signal forms occur. By systematic comparison of these effects, ligands can be assigned to groups with similar binding modes.

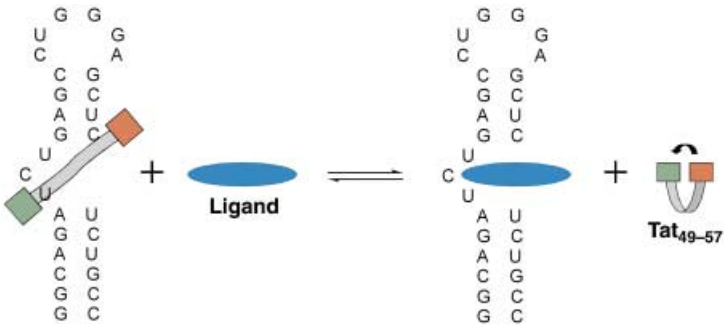


***Figure 1.** Ligands with affinity for the bulge region of TAR shift the equilibrium of tat/TAR complex formation in favor of free tat.*

Several other important nonradioactive techniques like surface plasmon resonance,[37] time-resolved fluorescence spectroscopy,[38] and fluorescence polarization[39] still wait to be adapted for use on the tat/TAR system.

Cell Assays

Over the last few years, several cell assays to examine the biological activities of tat antagonists have been established. These assays are frequently based on modified HeLa cells. HL3T1 cells containing a chloramphenicol acetyltransferase (CAT) reporter gene under the control of an HIV-1 long terminal repeat promoter, for instance, have been used to detect infective HIV particles and the effect of antiviral drugs.[40] A related technique is to cocultivate the HeLa cell line harboring the tat/TAR-dependent reporter gene (SX 22-1) with HIV-infected lymphocytes.[13]

Synthetic tat has been found to diffuse into HL3T1 cells and to upregulate the expression of CAT. This finding demonstrates that tat/TAR-dependent reporter gene assays can be established without the need to handle infective material.[41] Towards this aim, Rana et al. transfected HL3T1 cells with two plasmids. The first plasmid (pSV2-tat) encodes the production of tat to stimulate transcription of CAT. The second one (pAL) encodes luciferase as an internal control to detect nonspecific effects of TAR ligands.[21c] A similar approach utilizes permanently tat-expressing HeLa cells. When transfected with the plasmid pHIV*lac*Z, which contains TAR as a promoter and the reporter gene *lac*Z, β-galactosidase is expressed by the cells. Tat antagonists block this mechanism and thus reduce the level of the reporter protein.[12]

β-Galactosidase has also been used as a reporter in a yeast three hybrid assay. An RNA construct with two different protein binding sites brings two hybrid proteins into close proximity and thus activates the transcription of the reporter gene. Ligands interrupting one of the RNA – protein contacts will downregulate the concentration of β-galactosidase.[42]

Conclusions and Outlook

The tat/TAR system is one of the best-characterized RNA – protein complexes. Various strategies of drug discovery have been applied to identify low-molecular-weight inhibitors, with significant success. However, the pharmacological profile of these compounds is still insufficient for therapeutic use. Nevertheless, an important message is that RNA elements other than ribosomal RNA may be addressed by small molecules. Nature itself gives an excellent example in the form of the recently discovered "riboswitches": in bacteria, several vitamins are able to regulate their own production by interacting with the mRNA encoding one of the key enzymes involved in their synthesis.[5] These riboswitches are possible targets for the development of novel antibiotics to overcome the increasing problems of resistance.[43] Knowledge of the human genome will allow identification of many other RNA-based molecular switches of medicinal relevance in the near future. Pharmacological intervention at the stage of mRNAs could in theory offer fascinating opportunities, such as addressing isoenzymes selectively, controlling tissue-specific effects, etc. The bottleneck of research projects is still the shortage of selective high-affinity RNA binders that meet the requirements of modern pharmacology. This obvious challenge calls for a joint interdisciplinary effort. Chances are good that the work on RNA targeting drugs will become a major field of chemical biology in the near future.

Acknowledgements

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