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Binding affinity and inhibitory potency of neomycin and streptomycin on the Tat peptide interaction with HIV-1 TAR RNA detected by on-line acoustic wave sensor

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The binding of two aminoglycoside antibiotics, neomycin and streptomycin, to a segment of the transactivation responsive region (TAR) RNA of the human immunodeficiency virus, and their inhibitory potency to disrupt the interaction of the RNA with a regulatory Tat protein-derived peptide, have been studied using a flow-through acoustic wave detector system. Binding affinity is directly correlated with the inhibitory potency of these molecules and the acoustic wave detection system shows that neomycin exhibits at least a ten-fold greater affinity for TAR RNA and that it is also a more potent inhibitor than streptomycin. These results are in agreement with previous studies. However, unlike the time-consuming batch-based assays, use of the flow-through format offers considerable potential for the rapid screening of the chemistry of relatively small-molecule–nucleic acid binding events.

Introduction

The human immunodeficiency virus type 1 (HIV-1) Tat protein belongs to the class of RNA-binding proteins that contain an arginine-rich basic motif, which attaches to the target nucleic acid.[1] The protein is 86 amino acids long and regulates HIV gene expression by binding to the transactivation responsive region (TAR) on mRNA transcripts, resulting in a stimulation of the efficiency of transcription.[2] The HIV-1 TAR RNA has a conserved hairpin structure that contains double stranded regions interrupted by a trinucleotide bulge and hexanucleotide loop.[2] The arginine-rich region (residues 49–59) of the Tat protein is the RNA-binding domain and short peptides that contain this region bind to TAR with affinity and specificity similar to those of the protein.[3] In view of the important regulatory role that the protein plays in the life cycle of the virus, the TAR–Tat interaction constitutes a potential target for the development of an anti-viral drug.

The HIV-1 TAR–Tat interaction can be inhibited by aminoglycoside antibiotics.[4] These molecules are polycationic saccharides that are potent antibiotic therapeutics against bacterial infections.[5] The amino groups of aminoglycosides are positively charged at physiological pH, and recognition of RNA occurs by a three-dimensional projection of these moieties towards functional groups in the RNA binding site. These molecules do not make highly specific contacts and bind by a three-dimensional electrostatic complementarity with the negative electrostatic potential of RNA. The multi-ring structure of the aminoglycosides confers a great deal of conformational flexibility, which allows a facile match to the binding domain of the RNA.[6,7]

Aminoglycosides interact with TAR RNA and/or the TAR–Tat complex and not with the Tat protein due to their polycationic nature. These molecules inhibit TAR–Tat interaction by inducing a conformation in TAR that is unsuitable for peptide binding, and by facilitating the dissociation of the preformed TAR–Tat complex through an allosteric mechanism.[8] The drugs stabilize the free TAR conformation by simultaneously interacting with the lower and upper stem regions. Furthermore, they can induce dissociation of the peptide by binding to the preformed TAR–Tat complex since the double-helical stem region, which is the site for aminoglycoside binding, is not greatly affected when the RNA undergoes conformational changes instigated by binding to the protein.[9] Among the aminoglycoside antibiotics, neomycin has the greatest inhibitory effect on the binding of the Tat protein (IC_{50} = 0.92 μM), followed by streptomycin (IC_{50} = 9.5 μM) and gentamicin (IC_{50} = 45 μM).[4]

The detection of nucleic acid–ligand binding by biosensor technology offers significant advantages over traditional techniques, such as gel-shift assays, since radioisotope labels are not required and real-time data can be obtained. The thickness-shear mode (TSM) acoustic wave biosensor is based on the propagation of transverse waves in AT-cut piezoelectric quartz crystals. When these devices are employed in the liquid phase, the shear wave extends into the liquid beyond the crystal surface and decays exponentially.[10] Thus, any perturbation in the propagation of the acoustic wave due to deposited mass, structural changes of surface species, slip, and/or interfacial coupling causes changes in acoustic parameters.[10–12] The response of the device is measured by acoustic network analysis, which generates multidimensional data (resonant frequency, phase angle, impedance and equivalent circuit elements) for each frequency sweep.

In the present paper, we report the application of the on-line TSM configuration to the study of TAR RNA–drug binding at the sensor–solution interface. The disruption of TAR–Tat-derived peptide (Tat-20, Fig. 1) binding by two aminoglycoside antibiotics, neomycin and streptomycin (Fig. 1), as well as the potency of these molecules in inhibiting the above interaction, is examined.

Results and discussion

The on-line immobilization of a 31-base TAR RNA sequence (Fig. 1) onto one electrode of TSM devices was effected using neutravidin–biotin chemistry. The avidin–biotin interaction is very stable under most conditions ($K_d = 10^{-15}$); thus, the biotinylated RNA remains attached to the surface during the course of flow-through experiments. Subsequent to a downward shift in series resonance frequency of around 200 Hz instigated by the adsorption of the protein to the gold electrode of the TSM, immobilization of the nucleic acid on the device surface is confirmed by a further permanent reduction in frequency of 50 Hz. Binding of the drug molecules to TAR RNA produces a frequency change when either a dispersion of neomycin or streptomycin solution is allowed to flow over the immobilized nucleic acid. We have shown in previous work that neomycin binds reversibly to TAR RNA and multiple injections of the drug into the flow-through configuration can be performed on the same device, without the need for any regeneration

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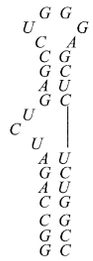
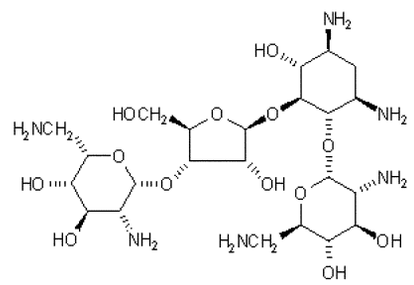
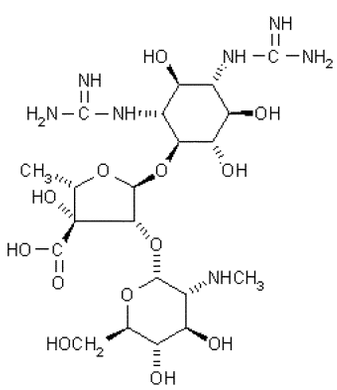


Tat-20 *Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Ser-Gln-Thr-His-Gln*



Streptomycin



neomycin

Fig. 1 Structures of HIV-1 RNA sequence (upper), Tat-20 peptide (centre), streptomycin and neomycin. Peptide represents a 20 amino acid sequence of Tat from the RNA binding domain as discussed previously.[13]

steps other than a simple wash with buffer.[13] Analysis of the frequency–time curve from real-time biosensor data yields a K_D value of 12.4 μM,[14] which is of the same order of magnitude as values obtained from non biosensor-based assays.

The fact that TAR RNA displays a ten-fold greater affinity for neomycin than streptomycin is reflected in the signals shown in Fig. 2. In this experiment, solutions of the two aminoglycosides are added sequentially (streptomycin first) at the same concentration to the same surface (nucleic acid in place), with a buffer wash being employed between injections of each dispersion. These results demonstrate the ease of assessment of RNA–ligand binding in the on-line protocol. Moreover, it is noteworthy that, like the short Tat-derived peptide of 12 amino acids,[13] these drugs cause a reversible frequency increase. This is the reverse of the situation expected from the classical picture of acoustic wave propagation. Clearly, the attachment of the small molecules to the nucleic acid results in an enhancement of energy storage of the acoustic system.

It has been reported previously that the ability of the aminoglycosides to inhibit the binding of Tat-derived peptides to TAR RNA is a direct function of their affinity for the nucleic acid.[4] An examination of this process using the acoustic wave

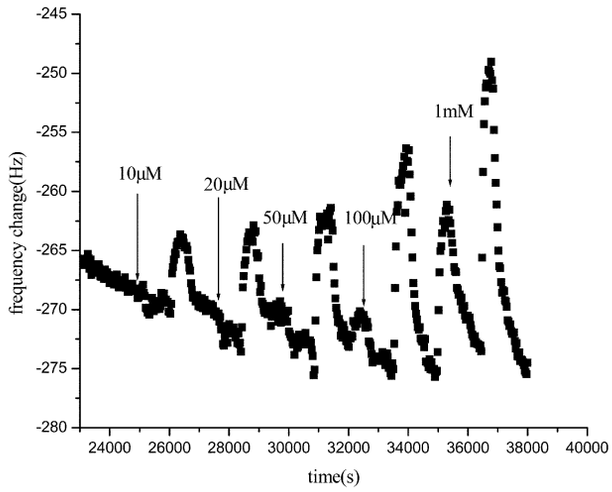


Fig. 2 Frequency–time plots for alternate introduction of streptomycin and neomycin to the same RNA-treated surface. Arrows indicate where streptomycin is injected into the on-line system.

device involves the formation of the TAR RNA–drug complex by subjecting the immobilized nucleic acid to a dispersion of a particular drug, followed by a peptide solution with *no wash steps* in between. Investigation of the non-competitive nature of the inhibition process is achieved through a reversal of this protocol. In this case, the pre-formed TAR–Tat-20 complex is subjected to a solution of selected aminoglycoside. Experimentally, introduction of a solution of neomycin to an on-line pre-formed TAR–Tat-20 complex results in a further increase in the series resonant frequency caused by drug binding superimposed on the positive signal produced by the attachment of the peptide, signaling the formation of a ternary TAR–Tat–neomycin complex (Fig. 3). It should be noted that similar introduction of a second dispersion of peptide solution to the pre-formed RNA–Tat-20 complex (not shown) yields no signal. Together with the former observation, this result confirms that the binding site for the drug is different from that of the peptide and that the signal generated by the aminoglycoside is associated with binding to a specific RNA site. The site for neomycin binding is the stem region of TAR RNA and the conformation of this element remains largely unaffected by the Tat peptide. Accordingly, neomycin is able to bind to the TAR–Tat complex and produce the observed change in series resonance frequency of the TSM device.

Introduction of a solution of Tat-20 peptide to the sensor surface, on which a pre-formed TAR–neomycin complex was in

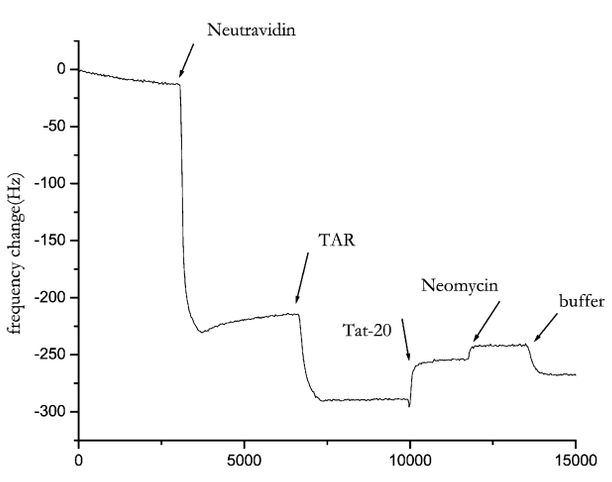


Fig. 3 Complete frequency response plot for the adsorption of neutravidin, attachment of the RNA, and interaction of neomycin with the RNA–Tat-20 complex.

place, does not produce a significant shift in frequency (Fig. 4). This illustrates that the binding of the peptide is inhibited by the presence of neomycin. Interestingly, a reproducible momentary dip in the response occurs that is likely connected to a partial removal of the drug caused by introduction of the peptide solution in the flow-through format. However, the signal returns to the previous level and no net change in frequency is observed. This result is in agreement with other studies which showed that neomycin changes the RNA conformation to one unsuitable for peptide binding, and inhibits the formation of the TAR–Tat complex in the range 0.1–1 mM.[15] The lowest concentration of this range was employed in the present study and is found to be effective in disrupting TAR–Tat binding.

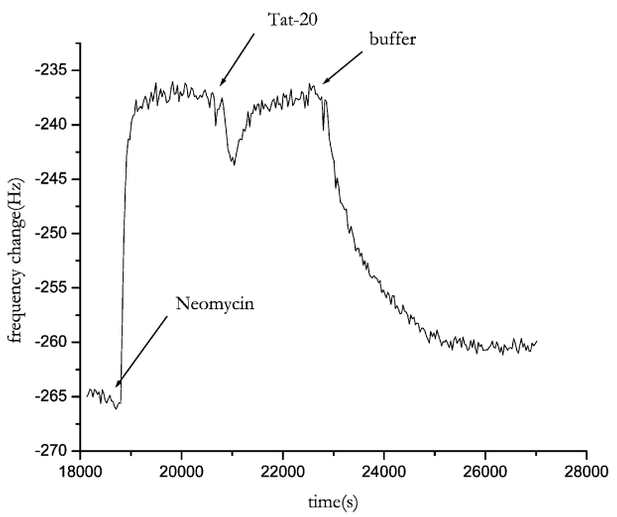


Fig. 4 Frequency–time plot for the inhibition of Tat-20 binding to a TAR–neomycin complex on the sensor surface.

Streptomycin is ten-fold less effective than neomycin in inhibiting TAR RNA–Tat binding.[4] Analogous experiments to those outlined above involving this drug do not generate the response level that was observed for neomycin. This aminoglycoside does not appear to form a ternary complex with TAR RNA–Tat-20 at the drug concentration employed (100 μM), which is a strong indication of its lesser affinity for both the nucleic acid and the RNA–Tat-20 complex. Moreover, it is ineffective in inhibiting the formation of the TAR RNA–Tat complex even at very high concentrations of up to 2 mM. Tat-20 peptide is observed to attach to the pre-formed TAR–streptomycin complex, unlike for the neomycin case where inhibition results. As depicted in Fig. 5, Tat-20 peptide binds to the TAR RNA–drug complex, producing a significant positive change in the series resonant frequency. Concentrations above 2 mM were not employed since the bulk viscoelastic properties of the surrounding medium are altered which, in turn, affects the acoustic response. The experimental protocol at such a high drug concentration no longer reflects selective interfacial chemistry. However, the range of concentrations employed was sufficient to enable comparison of the effectiveness of the two drugs, especially since neomycin is found to be a potent inhibitor at 20 times lower concentration.

Conclusions

The present work demonstrates that acoustic wave physics at the liquid–solid interface offers a particularly sensitive approach to the discrimination of biochemical macromolecule–small molecule interactions. The flow-though format, which is highly compatible with acoustic wave sensor technology, provides a significant advantage over other more batch-oriented methods for the medium-throughput screening of such binding events.

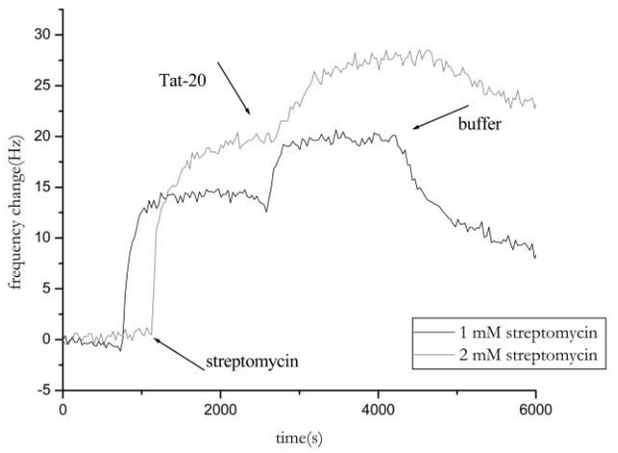


Fig. 5 Frequency–time plot for the inhibition of Tat-20 binding to a pre-formed TAR–streptomycin complex on the sensor surface.

Experimental

Piezoelectric quartz disks (9 MHz operating frequency; 0.178 mm thick with a diameter of 13.8 mm) were cleaned with acetone, ethanol and water and dried under a stream of nitrogen gas before use. The devices are sandwiched between two halves of a Plexiglas flow cell with one face being exposed to air. Measurement of the series resonance frequency by acoustic network analysis and general operation of the flow-through configuration were as described previously.[13] All measurements were taken during a continuous flow, and the pump was only stopped momentarily in order to switch between solutions. A 500 μL solution of neutravidin in Tris-buffer (1 mg ml^{-1}) was injected after the devices had been equilibrated with buffer and a stable frequency was obtained. Following a wash with buffer, the RNA was immobilized by flowing through a 500 μL solution of 3′-biotinylated TAR RNA (1 μM in Tris-buffer). This was followed by injection of 200 μL of a particular analyte solution (Tat-20 peptide, aminoglycoside) in the specified order.

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