Review

Recognition of the unique structure of DNA:RNA hybrids

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Abstract

Targeting nucleic acids using small molecules routinely uses the end products in the conversion pathway of “DNA to RNA, RNA to protein”. However, the intermediate processes in this path have not always been targeted. The DNA–RNA interaction, specifically DNA:RNA hybrid formation, provides a unique target for controlling the transfer of genetic information through binding by small molecules. Not only do DNA:RNA hybrids differ in conformation from widely targeted DNA and RNA, the low occurrence within biological systems further validates their therapeutic potential. Surprisingly, a survey of the literature reveals only a handful of ligands that bind DNA:RNA hybrids; in comparison, the number of ligands designed to target DNA is in the thousands. DNA:RNA hybrids, from their scientific inception to current applications in ligand targeting, are discussed.

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Keywords: DNA:RNA hybrids; Ligand targeting; Aminoglycoside; Polyamide; Ethidium bromide

1. History

Six years after the report on the double-helical structure of DNA by Watson and Crick [1], the DNA:RNA hybrid was proposed to address the interaction between DNA and RNA [2]; a wonderful retrospective account can be found in the following reference [3]. The first synthetic DNA:RNA hybrid structure was formed in 1960 through the reaction of oligodeoxythymidylic acid with polyriboadenylic acid [4]. A mere year later, the first DNA:RNA hybrid helix was formed through the novel annealing of a RNA strand, with a complementary DNA strand [5,6]. X-ray fiber diffraction studies first appeared in 1967 [7], and confirmed CD spectroscopy observations that the conformation of the DNA:RNA hybrid was different from B-form DNA. The crystal structure of a DNA:RNA hybrid was solved in 1982 [8], while solution based conformational analysis, first used to study a DNA:RNA hexamer [9] was followed shortly by polymeric DNA:RNA hybrids in 1985 [10].

In the initial report where the presence of DNA:RNA hybrids was suggested [2], the mechanism of their formation, as a product of hybridization between a single-stranded DNA template strand and a newly formed RNA strand, was also hypothesized. Experimental data supported this hypothesis in 1960 [11]. This was 10 years before Temin [12] and Baltimore [13], independently, yet simultaneously, reported the use of reverse transcriptase in the synthesis of a new DNA strand, via RNA template strand. Originally proposed by Rich [2], this became known as reverse transcription, the second example of DNA:RNA hybrid formation. In 1975, the first example of DNA:RNA hybrid formation in the replication of DNA was discovered [14]. Preceded by their biological significance, DNA:RNA hybrids as therapeutic targets logically followed.

2. Importance

2.1. Transcription [15,16]

The DNA mediated synthesis of RNA is a finite process which initiates at promoter sites within the DNA template, proceeds in the 5’ to 3’ direction and terminates at terminator sites within the DNA template. RNA polymerase binds to the initiation site, unwinds a helical turn of duplex DNA and begins to incorporate ribonucleotides sequentially to the 3’ end
of the growing RNA strand. Ribonucleotide selection involves the interaction of the incoming base with the template DNA strand and the ability of the base to fit the active site of the enzyme [17]. Termination is less well understood; the termination process involves (i) cessation of RNA chain growth, (ii) the release of the RNA strand from the DNA template and (iii) the release of RNA polymerase from the DNA [18]. It has been suggested that termination concludes with the release of the RNA strand from an unstable DNA:RNA hybrid [19]. Furthermore, the lability of some hybrid sequences may aid the dissociation of the RNA strand from the DNA template [20,21].

2.2. Reverse transcription

The RNA mediated synthesis of DNA was originally proposed [22] and substantiated [23] as an integral part of the retroviral life cycle. Expanded to eukaryotic cells [25], as much as 10% of the eukaryotic genome is a direct product of reverse transcription [24]. Mechanistically similar to the transcription process, reverse transcription relies on the dual activity of reverse transcriptase (RT) [24,26]. DNA:RNA hybrid formation can be found following RT mediated DNA synthesis and prior to RT mediated RNase H cleavage of the template RNA strand. Since reverse transcription relies on the dual role of RT, the intermediate DNA:RNA hybrid form provides an enticing target for targeting by small molecules, prior to reattachment and subsequent cleavage by RT.

2.3. DNA replication [27]

The discussion of DNA replication may seem unnecessary when addressing DNA:RNA hybrid duplexes, however DNA:RNA hybrids play an important role in DNA replication. DNA replication begins with the unwinding of duplex DNA by DNA helicase followed by priming of the DNA strands by proteins and enzymes [18] and proceeds in: (1) a heavy (H)-strand origin (O H), 5' to 3', leading strand synthesis and (2) a light (L)-strand (O L), 3' to 5', lagging strand synthesis. The interaction of the incoming base with the template DNA strand and the ability of the base to fit the active site of the enzyme [17]. Termination is less well understood; the termination process involves (i) cessation of RNA chain growth, (ii) the release of the RNA strand from the DNA template and (iii) the release of RNA polymerase from the DNA [18]. It has been suggested that termination concludes with the release of the RNA strand from an unstable DNA:RNA hybrid [19]. Furthermore, the lability of some hybrid sequences may aid the dissociation of the RNA strand from the DNA template [20,21].

2.4. Mitochondrial DNA

Human mitochondrial DNA (mtDNA) is present in the mitochondria of the cell. Circular in conformation, mtDNA encodes for the remaining machinery of protein synthesis not encoded for in nuclear DNA [27]. The mechanism of replication is similar to that of nuclear DNA, however, the priming of mtDNA for DNA replication requires the sole use of RNA primers [30]. DNA:RNA hybrids are prerequisite to the initiation of transcription and serve as primers for DNA replication of the leading strand [18,31,32]. Similar use of RNA primers in the replication of DNA can be found in prokaryotes as well [33].

2.5. DNA:RNA hybrids of therapeutic interest

Despite reports of the existence of DNA:RNA hybrids of potential therapeutic importance (reports of the persistent existence of DNA:RNA hybrids in human cytomegalovirus have been made [34]); extensive studies reporting the targeting of these DNA:RNA hybrids as therapeutic strategies have not yet been examined, with the following notable exceptions.

The reverse transcription of HIV-1 has attracted substantial attention [27,35]. HIV-1 contains two identical polypurine tracts (PPTs). The first PPT only contains two pyrimidine bases and extends to 25 bases from the 3' long terminal repeat end of the viral RNA. The second PPT lies further towards the 5' end of the viral RNA and closer to the primer binding site used by tRNA to initiate minus strand DNA synthesis. Both of these PPTs are identical and 18 nucleotides in length [36]. Reverse transcription (minus strand DNA synthesis) of both PPTs occurs after DNA strand transfer by reverse transcriptase, providing a unique r(Pu):d(Py) type DNA:RNA hybrid [26]. This unique PPT DNA:RNA hybrid exists until cleavage of the RNA template strand (5' to the PPT) and cleavage of the tRNA and PPT. Targeting of DNA:RNA hybrids are discussed in subsequent sections of the manuscript.

Telomerase activity, seemingly synonymous with G-quadruplex formation, is a well understood process, of which DNA:RNA hybrid targeting is an equally viable approach for telomerase inhibition. Telomerase activity is associated with cellular immortality [37] and its potential as a universal cancer target has been proposed [38,39]. Telomeres exist as a protein coupled DNA complex with long repeats of a simple 6 base sequence, d(TTAGGG), which becomes shortened with each cellular division [40]. The 3' tail of the parent DNA telomere is longer than its compliment and forms a DNA:RNA hybrid upon binding to the active site of telomerase. Binding of small molecules to the DNA:RNA hybrid can potentially offer a therapeutic method for controlling telomerase activity, preventing telomere extension, by disturbing the substrate/enzyme interaction, or preventing dissociation of the enzyme from the substrate [41].

3. Stability and conformation

3.1. Stability

DNA:RNA hybrid composition can be isolated to homopurine and homopyrimidine strands, which gives rise to deoxyribo(pyrimidine):ribo(purine), d(Py):r(Pu), and deoxyribo(purine):ribo(pyrimidine), d(Pu):r(Py) type hybrids. The designation of pyrimidine (Py) and purine (Pu) bases is consistent through this manuscript, analogous to (Y) and (R), found in other accounts, respectively. Homopurine:homopyrimidine polymer and oligomeric structures decrease in stability in the order of r(Pu):r(Py) > r(Pr):d(Py):d(Pr) > r(Py):d(Py):d(Pr) > r(Pr):d(Pr):d(Pr), as suggested by Hung et al. [42,43]. However, the introduction of long stretches of
(A)ₙ:(T or U)ₙ reverses the trend, an observation made by Dubins et al. [44]. The polymeric duplexes containing stretches of (A)ₙ:(T or U)ₙ and at least one homoribonucleic acid strand follow the general hierarchy for stability DNA:DNA > DNA:RNA type d(Py):d(Pu) > RNA:RNA > DNA:RNA type d(Py):r(Pu).

Oligomeric DNA:RNA hybrids containing homopurine and homopyrimidine tracts, stabilized by a single G–C base pair at each end, d(CT₅G):r(GA₅C) and d(CA₅G):r(GU₅C), demonstrate the same hierarchy [19] as duplexes containing (A)ₙ:(T or U)ₙ. It should be noted that the stability of d(Py):r(Py) duplexes is low and although triplex formation is possible, the duplex structure does prevail [45]. Furthermore, Hall and McLaughlin [46] demonstrate that oligomers of mixed base sequences, not isolated to purine or pyrimidine strands, show exceptions to the previously described trends. In these exceptions, homoduplexes, d(Pu):d(Py) and r(Pu):r(Py), are more stable than heteroduplexes, d(Py):r(Pu) and d(Pu):r(Py).

An extensive survey and review of DNA:RNA hybrid stability while varying (1) the percentage of (A)ₙ:(T or U)ₙ content, (2) oligomeric length and (3) the percentage of (dPy) content in each of the strands, offers the most complete analysis of hybrid stability [47]. Lesnik and Freier demonstrate that in general: (i) an inverse relationship is displayed between the percentages of (A)ₙ:(T or U)ₙ content in the hybrid duplex and the thermal stability. (ii) Hybrid duplexes of equal d(Py) and d(Pu) content are thermally similar when comparing the d(Py):r(Py) and d(Py):r(Pu) forms. (iii) Decreasing oligomeric length decreases the thermal stability. (iv) Isolation of (A)ₙ:(T or U)ₙ content to continuous tracts results in a lower thermally stable duplex than when the same percentage of (A)ₙ:(T or U)ₙ content is dispersed throughout the sequence. (v) Continuous tract (A)ₙ:(T or U)ₙ content results in d(Py):r(Py) thermal stability lower than the corresponding d(Py):r(Pu) form, at physiological conditions, as expected.

It has also been suggested that DNA:RNA hybrid stability is related to the ability of the hybrid duplex to associate with a third single strand [43,48]. The d(Py):r(Pu) hybrid does not associate with a third strand of DNA, while d(Py):r(Pu) hybrids easily accommodate a third strand to form d(Py):r(Py):d(Pu) [43]. Furthermore, with the exception of long stretches of (A)ₙ:(T or U)ₙ, the stability of duplexes can also be attributed to the relative stability of the ribose chain. Unfavorable van der Waals contacts [49] have been observed between the sugar and pyrimidine nucleotides, resulting in the expectation that within a double helix, a pyrimidine nucleotide is less stable than a purine nucleotide.

### 3.2. Conformation

For the sake of this discussion, we examine DNA:RNA hybrid conformation by individually focusing on DNA:RNA hybrids of polymeric length as well as DNA:RNA hybrids of oligomeric length, further defined by homo ribose:deoxyribose oligomers and chimeric oligomers. For comparison purposes, the conformation of various DNA:RNA hybrids will be compared to conformationally distinct A and B form helices (Fig. 1).

### 3.2.1. Polymeric DNA:RNA hybrids

The first DNA:RNA hybrid structure [7] resisted conformational changes upon relative humidity changes, a technique commonly used to drive the A- to B- transition in duplex DNA. The observation, by Milman et al. [7], that DNA:RNA hybrids resembled A-form DNA while differing from the RNA duplex, coupled with solution studies of DNA:RNA hybrids [50,51], spurred a 20 year debate on the conformation and structure of DNA:RNA hybrids. X-ray fiber diffraction studies of poly(rA):poly(dT) suggest a structure similar to that observed for B-form DNA duplexes when the hybrid was in a highly solvated state [7] suggest a structure similar to that observed for B-form DNA duplexes. Parallel circular dichroism (CD) [52] and Raman [53] experiments refute a complete B-form model for the DNA:RNA hybrid. Further analysis by ³¹P solid state NMR suggests poly(rA):poly(dT) is capable of existing in an A-like conformation at relatively low humidity [54] while increasing the solvation from 87% to 92% yields a B-like conformation [55]. Equilibrium Raman spectroscopy further clarifies the conformational differences are a result of differences in furanose sugar conformation, unique to each respective ribose and deoxyribose strand [56], of which the global B-like conformation is dictated by the burial of the thymidine methyl [48] in the d(Py) strand [57]. Steely et al. suggest [10] that ‘the d(Py) strand contributes to the overall B-like conformation and concurrently stabilizes the duplex’. Currently, it is generally accepted that the conformation of poly(rA):poly(dT) is polymorphic, capable of undergoing an A- to B- type transition upon relative humidity changes [57,58].

Polymeric structures, type d(Pu):r(Py), poly(dA):poly(rU) and poly(dI):poly(rC) further broaden the conformational spectrum in which DNA:RNA hybrid structures exist. The model for poly(dA):poly(rU) suggests a ‘heteromeric’ structure [59]. This term was used to describe the overall conformation of the duplex in which the one strand, the d(Pu) strand, displays B-form characteristics while the other strand, the r(Py) strand, displays A-form characteristics. The global conformation of poly(dA):poly(rU) is strongly driven by the r(Py) strand and the global conformation of the hybrid is an A-type duplex closer in conformation to the A-form of RNA.
Mixed base polymeric DNA:RNA hybrids poly(dAC):poly(rGU) and poly(dGU):poly(rAC) have also been extensively studied. The duplexes are more similar to the analogous A-form RNA duplex. Furthermore, conformational differences between the individual heteroduplexes mirror differences observed in the polymeric analogs poly(dA):poly(rU) and poly(rA):poly(dT). The authors suggest poly(dGU):poly(rAC) is more A-like than poly(dAC):poly(rGU). These duplexes are also susceptible to dehydration driving the conformation to complete A-form conformation.

3.2.2. Oligomeric DNA:RNA hybrids

Pardi et al. used seven-mer oligomeric DNA:RNA hybrids containing an A5(T or U)5 stretch, sealed by terminal G-C base pairs as the first instance of oligomeric studies of DNA:RNA hybrids by NMR [60]. The authors suggest DNA:RNA hybrid conformation belonging to the A-form, is driven by the A-form conformation of the RNA strand, while the DNA strand is predominantly B-form. Unfortunately, partial triplex formation of d(CA3G):r(GU5C) precludes the assignment of this hybrid to A-form or B-form.

Oligomeric DNA:RNA hybrids, restricted in base composition to homopurine and homopyrimidine strands, have been studied by both solution [61–63] and crystal [64–66] techniques. Solution studies suggest a ‘heteromerous’ duplex containing A-form conformation in the RNA strand while the DNA strand conformation was closer to B-form [63]. However, crystal structure observations [64–66] seemingly dispel the notion that the conformation is ‘heteromerous’ by suggesting the conformation is homogeneous and closer to A-form.

The most complete analysis of the conformational relationships between homopyrimidine and homopurine DNA:RNA hybrids and their corresponding DNA and RNA duplexes was made comparing duplexes 5’-(GAAG)3-3’ and 5’-(CTTC)3-3’ [67]; thymidine bases were replaced by uracil in ribose strands. The DNA:RNA hybrid, d(Pu):r(Py) is closer in global conformation to B-form than d(Py):r(Pu); in fact the CD spectra closely resemble the CD spectra of d(Py):d(Pu). The opposite was true for the d(Py):r(Pu) hybrid; the CD closely resembles the duplex RNA analog r(Py):r(Pu), which further perpetuates the idea that d(Py):r(Pu) oligomeric hybrids are closer to A-form than analogous d(Py):r(Pu) duplexes. In fact, the authors suggest predictions of the global conformation of duplexes can be made based on whether or not the hybrid duplex contains a DNA or RNA purine strand.

A survey of mixed base DNA:RNA hybrids suggests the conformation varies depending upon the percentage of purine content in the DNA strand. Fedoroff et al. utilized a mixed base DNA:RNA hybrid and described its conformation as an intermediate between A-form and B-form [68]. The ‘heteromerous’ nature of DNA:RNA hybrids reprises in the DNA:RNA hybrid of 33% purine content in the DNA strand [69,70]. Increasing DNA purine content increases the debate in the conformational contributions from the respective DNA:RNA strands. One report suggests the DNA strand contributes B-form characteristics [71], while other reports suggest the DNA strand contributes A-form and B-form characteristics, a result of homologous sugar pucker [70,72,73] or distribution of independent sugar pucker in the DNA strand [74].

3.2.3. Chimeric DNA:RNA hybrids

Chimeric DNA:RNA hybrids are formed when DNA:RNA hybrid helix is joined to double-helical DNA. Studies on dG5:rC5dC5 confirmed the presence of a bend joining two distinct unique conformations within the polymer [75,76]. Crystallized in 1982, the chimera [r(GCG)d(TATACGC)]3 [8] suggests the global conformation adopted by the duplex is close to the A-form RNA double helix, although discrepancies exist [8,77]. Subsequent conformational treatments revealed that chimeric hybrids are polymorphic with conformationally distinct contributions from each portion [78]. Furthermore, the RNA influence from the ribonucleic strand in DNA:RNA hybrids is greatly diminished in chimeric duplexes [79]. Solution studies on chimeras reveal that regardless of flanking DNA:RNA hybrids with DNA duplexes or the contrary flanking of a central core DNA by DNA:RNA hybrids, the portion of the hybrid retains A-form characteristics that lie closer to B-form, while the DNA portion of the duplex exists as predominant B-form [80–82]. Even single junction hybrids composed of a DNA duplex and DNA:RNA hybrid duplex displayed the dual conformation characterized by a DNA:RNA hybrid:DNA bend [83–85].

3.3. Sugar conformation

The ribose sugar pucker is a major contributor to the helical parameters of nucleic acids. Ribose sugar pucker of ribonucleic acids are generally found in the C2v-endο (‘north’ or equivalent C2v-exo) conformation, an accommodation made by the sugar to facilitate the 2′-hydroxyl [55]. The C2v-endο ribose drives the conformation of homoribonucleic strands towards A-form, affecting most helical parameters, giving rise to the ‘heteromerous’ nature of DNA:RNA hybrids. Deoxyribose conformation is much more accommodating to a number of different conformations and generally assumes a C2v-endο (‘south’ or equivalent C2v-exo) conformation. This conformation is characteristic of B-form. Another sugar pucker, an intermediate between the north and south conformations, is C4v-endο and arises at various points in the subsequent discussion on the effect of sugar pucker on DNA:RNA hybrids helical parameters (see Figs. 2 and 3).

3.3.1. Polymeric DNA:RNA hybrids

Polymeric DNA:RNA hybrids generally follow two trends. Refinement of the secondary structure of poly(rA):poly(dT) suggests the sugar puckers, regardless of strand, are a slight variant of the C2v-endο conformation [10,54]. However, Gupta
and coworkers make the case that ‘in order to facilitate the 2'-hydroxyl, a displacement of the RNA bases from the helical axis is observed. This displacement results in a concomitant change in the tilt and twist of these bases as well as a change in a backbone torsion angle [10].’ However, there exists a ‘high energy barrier’ through which the ribose sugar of RNA must pass in order to convert to C2'-endo, a barrier significantly lower for DNA ribose [86]. Dehydration converts the global ribose sugar pucker to C3'-endo and results in parameters closer in agreement to A-form [55]. Unlike the case of d(Py):r(Pu), in which global conformational changes can be accommodated in both strands of the duplex, d(Pu):r(Py) type polymeric duplexes are not as forgiving. Driven strongly by the conformational stubbornness of the r(Py) strand to exist in an A-form state with C3'-endo sugar pucker, helical parameters tend to be more A-form in characteristic, with deviations arising from the d(Pu) strand [59,62,63].

3.3.2. Oligomeric DNA:RNA hybrids

Oligomeric DNA:RNA hybrids generally reflect the trends observed in polymeric hybrids. However, discrepancies arise in the conformation of the sugar pucker. C3'-endo sugar pucker is generally observed in the RNA strand, while variation in the interpretation of the deoxyribose conformation as an equilibrium between C3'-endo/C2'-endo or alternatively interpreted as C4'-endo [73,74]. Further assessment of sugar pucker suggests that the flexible DNA strand exists with a fraction of deoxyribose sugars in the C3'-endo form, increasing in the order of DNA:DNA duplexes, d(Pu):r(Py) and d(Py):r(Pu), which may be attributed to oligomeric d(Py):r(Py) type hybrids bearing more A-form characteristics [61,62]. Mixed purine/pyrimidine bases tend to assume the same general trends as observed in homopurine/homopyrimidine hybrids. Fedoroff et al. propose ‘DNA:RNA structure parameters (roll and tip) can be attributed to DNA strand propensity to roll about the long base pair axis while RNA susceptibility to rotate along the short base pair axis affects parameters such as tilt and inclination [74].’ Also, interpretation of DNA:RNA hybrid sugar pucker as global C3'-endo has been reported [64,65,67].

3.3.3. Chimeric DNA:RNA hybrids

Chimeric DNA:RNA hybrids are structurally unique, as already discussed. The extent of sugar pucker on the overall structure of the duplex is surprising. In the chimera, hybrid duplex flanked by DNA duplexes, the hybrid portion of the duplex adopts C3'-endo conformation in the ribose sugars, however with the exception of the junction, deoxyribonucleotides (which adopt C4'-endo conformation) assume a C2'-endo conformation exclusively [81]. DNA duplexes flanked by DNA:RNA hybrids have been suggested to have a global sugar pucker of C3'-endo with a conformation characteristic of A-form [88,89]. However, more recent studies have demonstrated that DNA residues adopt a conformation close in agreement to C4'-endo [83], as alluded to by Mellema et al. [79].
4. Targeting DNA:RNA hybrids

Although polymorphic in structure, as demonstrated in previous sections, DNA:RNA hybrids offer a unique structure to target and potentially inhibit biological functions. However, a survey of the literature reveals the existence of less than ten ligands which specifically bind DNA:RNA hybrids. In the course of this section, the binding of these ligands to DNA:RNA hybrids is examined. Attention is paid to ligand binding class as well as targeting biologically significant DNA:RNA hybrids. Structures, targets, binding constants and buffer conditions can be found in Table 2.

4.1. Ligand classes

An examination of various ligand binding modes to DNA:RNA hybrids is conducted in the subsequent section. Interestingly enough, DNA:RNA binding by small molecules has been achieved via all available modes: intercalation, groove binding as well as dual recognition. It should be noted that although the binding of antisense oligonucleotides to mRNA forms DNA:RNA hybrids, this therapeutic approach targets single-stranded RNA templates and is excluded from the following discussion, however, the use of antisense oligonucleotides is addressed in the section covering the PPT of HIV-1.

4.1.1. Intercalation

Using a technique developed by Muller and Crothers [90], Chaires et al. identified the intercalator, ethidium bromide, as a DNA:RNA hybrid, poly(rA):poly(dT), preferential binding ligand [91]. An ensuing report assayed 85 ligands against various nucleic acids and focused on binding to poly(rA):poly(dT). This assay confirmed the presence of five ligands: ellipticine, ethidium bromide, coralyne, propidium and TAS103, which recognized the poly(rA):poly(dT) structure. Of these ligands, ethidium bromide showed the highest preference for poly(rA):poly(dT). Complete thermodynamic profiles for ellipticine, propidium and ethidium bromide binding to poly(rA):poly(dT) yield binding constants for the three ligands (see Table 2). Inasmuch, an inverse relationship between the IC50 (inhibitory concentration giving 50% inhibition of poly(rA):poly(dT) degradation by RNase H) and the observed ligand binding constant[92] is observed. This assay was expanded to simultaneously provide the thermal stabilization afforded by the ligand on the preferentially targeted nucleic acid structure, while in competition with other nucleic acid structures [95]. Ethidium bromide competition dialysis findings were corroborated in this mixed melting experiment.

Actinomycin D (AMD) has demonstrated the ability to inhibit reverse transcriptase activity [96,97] through the inhibition of double-stranded DNA synthesis [96]. Comprised of an intercalating phenoxazome ring, substitution for the carbon at the 8-position of the planar ring system by nitrogen increases the selectivity of the compound for DNA:RNA hybrids [98]. Unfortunately, affinity of N8AMD (Table 2) for the DNA:RNA hybrid is slightly lower than the natural product. Of note, the authors suggest that AMD actually binds DNA:RNA hybrids, structures previously believed to be unable to bind AMD. However, the preference of AMD for DNA duplexes dominates. Similarly, substitution at the \(sp^2\) carbon at position 8, by fluorine increases the preference of

| Table 1 Helical parameters displayed by a wide array of DNA:RNA hybrid type duplexes |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| B-DNA             | Polymeric DNA:RNA hybrids |
|                  | poly(rA):poly(dT)  |
|                  | Hydrated           | B                | 33.7             | 3.37             | 10               | −5.9             | −   | [59,87,88]  |
|                  | 79% rel. humidity  | A                | 36.0             | 3.03             | 11.9             | −               | −   | [59]        |
|                  | poly(rI):poly(dC)  | A                | 35.6             | 2.97             | 12               | +.5.68           | −   | [49]        |
|                  | poly(dI):poly(rC)  | A                | 31.3             | −                | 10               | +15             | −   | [59]        |
|                  | poly(dA):poly(rU)  | A                | 33.7             | −                | 11               | +13             | −   | [59]        |
|                  | Polymeric DNA:RNA hybrids |
|                  | d(CTCTTTTCTC):r(GAAGAAGAG) | A    | 34.2             | 2.9              | 11.8             | +7.9             | −4.0 | [65]        |
|                  | d(GTGAACCTT):r(AAGUUCAC) | A    | −                | 3.2              | 11               | +17              | −4.5 | [71]        |
|                  | d(CAGTCCCTC):r(GAGGACUG) | A    | −                | 3.2              | 10.9             | +6.8             | −3.7 | [68]        |
|                  | d(CGCGCCCCCA):r(UUCGGGCGCC) | A    | −                | 2.9              | 10.9             | +13.9            | −3.3 | [72]        |
|                  | Chimeric DNA:RNA hybrids |
|                  | [d(CGCGCAUUAG):d(TTACCACG)]_2 | A/B  | −                | 3.1              | 11               | −1.3             | −1.8 | [81]        |
|                  | [r(GCGGdTATACGC)]_2  | A/B  | 33               | 2.6              | 10.9             | +20              | −2.0 | [8]         |
|                  | A-DNA              | A    | 28.2             | 2.56             | 11.0             | +20.0            | −   | [88]        |
|                  | A-RNA              | A    | 30.0             | 2.73             | 11.0             | +17.0            | −   | [89]        |


\( ^{a}x\)-displacement.

\( ^{b}\) Average values.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Target</th>
<th>$K_a$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>poly(dA):poly(dT): 7 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, 180 mM NaCl, 1 mM EDTA, pH 7.0—7.1</td>
<td>$1.1 \times 10^5$; fluorescence</td>
<td>[91]</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>poly(dA):poly(dT): 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM NaCl, pH 5.5</td>
<td>$3.3 \times 10^5$; fluorescence</td>
<td>[91]</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>poly(dA):poly(dT): 10 mM sodium cacodylate, 0.5 mM EDTA, 100 mM NaCl</td>
<td>$4.4 \times 10^5$; fluorescence</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>d(GGACGGGAC):r(CUCCGCUCC): 20 mM phosphate, 300 mM NaCl, pH 7.0</td>
<td>$3.4 \times 10^5$; UV-spectroscopic</td>
<td>[98]</td>
</tr>
<tr>
<td></td>
<td>5'-AUAAUGCAUUTTTTATATGCAATATAT-3'</td>
<td>$2.6 \times 10^5$; UV-spectroscopic</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td>5'-AUAAUGCUUCAUUTTTTATATGCAATATAT-3'</td>
<td>$1.6 \times 10^5$; UV-spectroscopic</td>
<td>[98]</td>
</tr>
</tbody>
</table>
\( R_1 = C; \ X = F \) (F8AMD)

\[ \begin{align*}
5'\text{-AUUAUGCAUAU TTATGCA TATAF-3'} \quad & 50 \text{ mM Tris–HCl, 10 mM MgCl}_2, 10 \text{ mM KCl, pH 7.5} \\
1.4 \times 10^5; \ & \text{UV-spectroscopic} \\
\end{align*} \]

\[ \text{d(CAAAGATTCCTC):(GTTTCTAAGGAG):} \quad 200 \text{ mM} \\
\text{Tris–HCl, 50 mM MgCl}_2, 1 \text{ mM DTT, 0.1 mM EDTA, pH 7.6} \\
5.4 \times 10^7; \ & \text{electrophoretic} \\
\]

\( X = N \) (MT-15)

\[ \begin{align*}
\text{d(CAAAGATTCCTC):(GTTTCTAAGGAG):} \quad & 200 \text{ mM} \\
\text{Tris–HCl, 50 mM MgCl}_2, 1 \text{ mM DTT, 0.1 mM EDTA, pH 7.6} \\
\sim 10^6; \ & \text{electrophoretic} \\
\end{align*} \]

\begin{align*}
\text{Paramomycin} \\
\text{(GCCACTGC):(GCAGUGGC):} & \quad 10 \text{ mM sodium calcodylate,} \\
& \text{0.1 mM EDTA, total Na}^+ 60 \text{ mM} \\
& \text{(pH 6.0)} \\
& \text{(pH 7.0)} \\
4.3 \times 10^6; \ & \text{calorimetric} \\
1.2 \times 10^6; \ & \text{calorimetric} \\
\end{align*}

\[ \begin{align*}
5'\text{-CGGGCGGCCACTGCTAGAG-3'}; \ & 5'\text{-GCCCGCGGUGACGAUCUC-3'} \\
& \quad 10 \text{ mM EPPS, 0.1 mM EDTA, total Na}^+ 100 \text{ mM, pH 7.5} \\
4.2 \times 10^5; \ & \text{calorimetric} \\
1.8 \times 10^5; \ & \text{calorimetric} \\
\end{align*} \]

\[ \begin{align*}
\text{Ribostamycin} \\
\text{(GCCACTGC):(GCAGUGGC):} & \quad 10 \text{ mM sodium calcodylate,} \\
& \text{0.1 mM EDTA, total Na}^+ 60 \text{ mM} \\
& \text{(pH 6.0)} \\
& \text{(pH 7.0)} \\
1.1 \times 10^6; \ & \text{calorimetric} \\
1.5 \times 10^5; \ & \text{calorimetric} \\
\end{align*} \]

(continued on next page)
<table>
<thead>
<tr>
<th>Structure</th>
<th>Target</th>
<th>$K_a$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>5'-CGGGCGCCACTGCTAGAG-3'; 3'-GCCCGCGUGACGAUUCUC-5'; 10 mM EPPS, 0.1 mM EDTA, total Na$^+$ 100 mM, pH 7.5 5'-CGGGCGCCACTGCTAGAG-3'; 3'-GCCCGCGUGACGAUUCUC-5'; 10 mM EPPS, 0.1 mM EDTA, total Na$^+$ 100 mM, pH 7.5 poly(rA):poly(dT): 10 mM sodium calcodylate, 0.5 mM EDTA, 100 mM NaCl, pH 6.8 poly(dA):poly(rU): 10 mM sodium calcodylate, 0.5 mM EDTA, 100 mM NaCl, pH 5.5</td>
<td>1.1 x 10^6;</td>
<td>[26]</td>
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<td></td>
<td></td>
<td>calorimetric</td>
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<td></td>
<td>1.4 x 10^5;</td>
<td>[26]</td>
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<td>calorimetric</td>
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<td>1.7 x 10^6;</td>
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<td>calorimetric/UV</td>
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<td>9.9 x 10^6;</td>
<td>[109]</td>
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<td></td>
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<td>calorimetric/UV</td>
<td></td>
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<tr>
<td>NM</td>
<td>poly(rA):poly(dT): 10 mM sodium calcodylate, 0.5 mM EDTA, 100 mM NaCl, pH 6.8 poly(dA):poly(rU): 10 mM sodium calcodylate, 0.5 mM EDTA, 100 mM NaCl, pH 5.5</td>
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<td>calorimetric/UV</td>
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<td>4.8 x 10^10;</td>
<td>[109]</td>
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<td>calorimetric/UV</td>
<td></td>
</tr>
</tbody>
</table>

*a* In all cases, bold denotes RNA nucleotides. Buffer conditions and method of binding constant determination are noted throughout.

*b* Unpublished results.
the formation of hybrid triplexes [100]. Later, paramomycin, stabilization for DNA:RNA hybrid duplexes and even induced significantly stabilize DNA:RNA hybrids [103]. Among all amino-acid major groove binders [100] and shown to significantly stabilize DNA:RNA hybrids [103]. Among all aminoglycosides studied, neomycin showed the maximum stabilization for DNA:RNA hybrid duplexes and even induced the formation of hybrid triplexes [100]. Later, paramomycin, an aminoglycoside, and its complexation with DNA:RNA hybrids was studied by Barbieri et al. [104]. Thermal stabilization of a mixed base RNA 8-mer duplex, afforded upon paramomycin complexation, is 6.3 °C, not surprising since aminoglycosides traditionally bind RNA. However, thermal stabilization of the DNA:RNA hybrid analog was on par with the RNA duplex at 6.2 °C. Furthermore, the binding of paramomycin to the hybrid duplex induces a shift in the hybrid duplex to a more A-form like conformation. Additionally, it was found that paramomycin binds the RNA duplex at higher binding affinities than the corresponding DNA:RNA hybrid (Table 2). Discrimination between paramomycin binding the RNA duplex and hybrid duplex is enthalpy driven. However, the difference between binding constants between the RNA duplex and DNA:RNA hybrid is diminutive in comparison to the analogous DNA duplex. Finally, the authors suggest the binding of paramomycin to the DNA:RNA hybrid inhibits both RNase H- and RNase A-mediated cleavage of the RNA strand.

4.1.2. Major groove binding

Aminoglycosides have been recognized as A-form nucleic acid major groove binders [100–103] and shown to significantly stabilize DNA:RNA hybrids [103]. Among all aminoglycosides studied, neomycin showed the maximum stabilization for DNA:RNA hybrid duplexes and even induced the formation of hybrid triplexes [100]. Later, paramomycin, an aminoglycoside, and its complexation with DNA:RNA hybrids was studied by Barbieri et al. [104]. Thermal stabilization of a mixed base RNA 8-mer duplex, afforded upon paramomycin complexation, is 6.3 °C, not surprising since aminoglycosides traditionally bind RNA. However, thermal stabilization of the DNA:RNA hybrid analog was on par with the RNA duplex at 6.2 °C. Furthermore, the binding of paramomycin to the hybrid duplex induces a shift in the hybrid duplex to a more A-form like conformation. Additionally, it was found that paramomycin binds the RNA duplex at higher binding affinities than the corresponding DNA:RNA hybrid (Table 2). Discrimination between paramomycin binding the RNA duplex and hybrid duplex is enthalpy driven. However, the difference between binding constants between the RNA duplex and DNA:RNA hybrid is diminutive in comparison to the analogous DNA duplex. Finally, the authors suggest the binding of paramomycin to the DNA:RNA hybrid inhibits both RNase H- and RNase A-mediated cleavage of the RNA strand.

4.1.3. Minor groove binding

Naturally occurring polyamides, distamycin and netropsin, have been investigated for their ability to bind DNA:RNA hybrid Okazaki fragments [105,106]. Lexitropsins were designed to bind AT stretches of DNA, displacing the spine of hydration, deep in the minor groove [107]. Distamycin derivatives were developed through various strategies [108], creating a number of distamycin dimers. Discussion of these compounds can be found in Section 4.2.2.

4.1.4. Dual recognition

Recently we have attempted to exploit the unique binding characteristics displayed by ethidium bromide and neomycin in the design of a ligand capable of binding DNA:RNA hybrids with high affinity [107]. Neomycin was covalently linked to 6-(4-carboxyphenyl)-3,8-diamino-5-methylphen-anthridinium chloride, a derivative of ethidium bromide, through the formation of an amide bond, to afford a neomycin-methidium chloride conjugate, NM (see Table 2). Competition melting experiments and competition dialysis experiments revealed a preferential binding of NM to DNA:RNA hybrid structures, of which d(Pu):r(Py) type, poly(dA):poly(rU), was preferred above d(Py):r(Pu) type, poly(rA):poly(dT). The thermal stability afforded by NM on the preferred hybrid, poly(dA):poly(rU), was 26 °C, much greater than the stabilization afforded by neomycin (7.8 °C), ethidium bromide (1.1 °C) or both neomycin and ethidium bromide 7.3 °C, at equivalent ratios.

Association constants, $K_{T25°C}$, values were determined utilizing UV, CD and calorimetry. Association constants demonstrate NM binds poly(dA):poly(rU) at higher affinities than neomycin or ethidium bromide. While the binding constant for neomycin closely resembles the binding of neomycin to the RNA backbone HIV-1 chimera [26], 9.93 × $10^6$ M$^{-1}$, and the binding of ethidium bromide was in close agreement with previously reported results [94] at 9.28 × $10^6$ M$^{-1}$, NM binds poly(dA):poly(rU) at a much higher association constant of 4.77 × $10^{10}$ M$^{-1}$. The binding constant of NM far exceeds neomycin or ethidium bromide clearly demonstrating the potential power of ligand conjugation. This conjugate provides an aminoglycoside based approach to the high affinity binding of DNA:RNA hybrids and is the highest binding constant observed for non-oligonucleotide based targeting of the DNA:RNA hybrid.

4.2. Targeting biologically significant DNA:RNA hybrids

This section examines reports of ligand targeting DNA:RNA hybrids of biological significance. If one considers the list of ligands capable of binding DNA:RNA hybrids as short, the list of accounts focusing on ligands binding biologically significant DNA:RNA hybrids is even shorter.

4.2.1. PPT of HIV-1

Targeting the polypurine tract, as previously mentioned, is an attractive method for inhibition of reverse transcription of HIV-1. Formation of a triplex can be achieved through the use of a single-stranded compliment designed to target the DNA:RNA hybrid present on the PPT of HIV-1. Triple helix formation can alternatively be formed using a duplex targeted at the single-stranded RNA strand of the PPT. Regardless, triplex forming oligonucleotides (TFOs) prevent RNase H cleavage of the RNA strand of the PPT and congruently inhibit initiation of plus-strand DNA synthesis in vitro [110]. It has been determined that a 25 mer TFO directed against the PPT is more inhibitory for RNA dependent DNA synthesis than antisense approaches directed at other regions of RNA [36]. Cell culture experiments indicate one TFO, forming an RNA:RNA:DNA hybrid triplex, is efficient in inhibiting retrovirus replication by preventing cleavage at the PPT site by RNase H, in turn inhibiting plus-strand DNA synthesis [110]. Furthermore, studies have suggested this unique DNA:RNA hybrid triplex can be induced and even stabilized by the presence of ligands such as 4′,6-diamidino-2-phenylindole (DAPI) [111] and the aminoglycoside, neomycin [100].
We first reported the ability of neomycin to stabilize DNA:RNA hybrids and even induce hybrid triplex formation [100]! This work was further expanded by Pilch and coworkers to include the aminoglycosides neomycin and ribostamycin, focusing on DNA:RNA hybrid chimeras. These chimeras were designed as constructs of the polypurine tract (PPT) of HIV-1, found at two unique steps in the reverse transcription of HIV-1 [26]. Containing the same DNA:RNA hybrid portion, these chimeras differ in the composition, RNA or DNA, of the complimentary strand. As reported earlier, RNase H cleavage of the DNA:RNA hybrid chimeras is inhibited by the presence of the aminoglycosides and a number of small molecule non-nucleoside reverse transcriptase inhibitors (NNRTIs) [112]. In fact, of the aminoglycosides surveyed, neomycin is far superior at inhibiting RNase H cleavage in both chimeras. Furthermore, thermal stabilization of the chimeras, afforded by the presence of aminoglycosides, reveals that neomycin increases the stability of both chimeras to a larger extent than paramomycin, which are both much greater than ribostamycin. Isothermal titration calorimetry derived binding constants for the aminoglycosides binding the two chimeras are located in Table 2. In both chimeras, the association constant for neomycin is greater than the other aminoglycosides studied. Furthermore, it is clear that the addition of ring four to the 2-deoxy streptose moiety is paramount to high affinity binding of aminoglycosides to DNA:RNA hybrid structures.

4.2.2. Okazaki fragments

A series of conformationally restrained bis-distamycin compounds, dimerized through the ortholpara or meta positions of benzene and pyridine (2,5- and 2,4- type respectively), were synthesized [108]. Ortholpara bis-distamycins were capable of binding both DNA duplexes as well as the Okazaki fragment at similar binding constants, ~10^8 M^{-1} and ~10^7 M^{-1} range, respectively. Surprisingly, mono-distamycins, linked to the benzene moiety, showed no binding to the duplexes. The meta pyridyl bis-distamycins were also studied, however, affinity for the Okazaki duplex is in the ~10^6 M^{-1} range.

4.2.3. Telomerase

DNA:RNA hybrid targeting with ethidium bromide has been applied to targeting telomerase through its DNA:RNA hybrid duplex. Friedman and coworkers have surveyed a number of compounds in an attempt to inhibit telomerase activity through DNA:RNA hybrid binding [41,113]. Surveying a number of intercalators, four ligands (ethidium bromide, rivanol, acridine orange and yellow) were found to inhibit telomerase activity at IC_{50} values in the low micromolar range. Furthermore, telomerase inhibition through the binding of these ligands to G-quadruplex structures, another substantiated method for achieving telomerase inhibition, was not observed [41]. Affinity chromatography isolated the high affinity DNA:RNA binding species from a homogeneous mixture of binding ligands. It was found that ethidium bromide uniquely binds a DNA:RNA hybrid duplex, derived from the hybrid formed during the catalytic cycle of telomerase, with the highest affinity, even in the presence of other binding ligands [113]. This work identified ethidium bromide as a lead compound for therapeutic approaches to DNA:RNA hybrid targeting of telomerases.

5. Concluding remarks

 Acting at the hub of biological information transfer, DNA:RNA hybrids provide a potential substrate for controlling gene expression. Conformationally distinguishable from more common nucleic acids, DNA and RNA, these hybrids necessitate more attention when studying nucleic acids therapeutics. Even more enticing, due to their relatively low occurrence within biological systems, when compared to DNA and RNA, DNA:RNA hybrids provide a unique substrate for targeting with small binding ligands. Despite their uniqueness, DNA:RNA hybrids have not received their share of attention. The attention DNA:RNA hybrids have received suggest that DNA:RNA hybrids can be targeted by small ligands. However, more work must be done to show that selective targeting of DNA:RNA hybrids can be attained in the presence of a much higher concentration of duplex DNA and RNA structures in the cell. Small molecule binding to DNA:RNA hybrids has shown the ability to disrupt the transfer of genetic information by disabling the proper function of enzymes. More work still needs to be done to develop highly specific ligands capable of binding the hybrids at association constants higher than naturally occurring enzymes. We have begun to outline the principles required for high affinity and high specificity binding of DNA:RNA hybrids by small ligands. Through the conjugation of an aminoglycoside to an DNA:RNA hybrid specific intercalator, DNA:RNA hybrids of distinctly different sequence can be distinguished from one another as well as other nucleic acid structures. More work is being done to develop more selective molecules such that DNA:RNA hybrid targeting can become a noteworthy therapeutic endeavor.

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References


