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Modulation of DNA structure formation using small molecules

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Abstract

Genome integrity is essential for proper cell function such that genetic instability can result in cellular dysfunction and disease. Mutations in the human genome are not random, and occur more frequently at “hotspot” regions that often co-localize with sequences that have the capacity to adopt alternative (i.e. non-B) DNA structures. Non-B DNA-forming sequences are mutagenic, can stimulate the formation of DNA double-strand breaks, and are highly enriched at mutation hotspots in human cancer genomes. Thus, small molecules that can modulate the conformations of these structure-forming sequences may prove beneficial in the prevention and/or treatment of genetic diseases. Further, the development of molecular probes to interrogate the roles of non-B DNA structures in modulating DNA function, such as genetic instability in cancer etiology are warranted. Here, we discuss reported non-B DNA stabilizers, destabilizers, and probes, recent assays to identify ligands, and the potential biological applications of these DNA structure-modulating molecules.

Keywords

non-B DNA; genetic instability; small molecule ligands; small molecule DNA probes; DNA stabilizers; DNA destabilizers

There has been an explosive growth in structure-based drug design in the past decade, in part due to the progress in molecular and structural biology techniques, making 3D structures of macromolecular targets at an atomic-level readily available, and the rapid advancement in computer-aided drug design [1–3]. Based on the detailed information of the interactions between the targets and ligands, this approach has resulted in the identification of new therapeutic agents and/or the optimization of existing drugs.

The vast majority of these efforts have been focused on targeting proteins because their structural features and biological activities can often be recognized and targeted in a specific fashion. Nucleic acids have received less attention as potential targets, though they have clearly defined structural features and biological functions [4]. This is reflected in the

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dwindling number of FDA-approved drugs that target DNA. Before 1982, ~31% (12 of 38) of FDA-approved drugs were associated with DNA damage mechanisms, which has dropped to ~2% of new drugs (1 out of 47) during 2010–2015 [5].

DNA arguably remains to be an effective therapeutic target for genetic-instability-related diseases such as cancer. Recent investigations have shown that certain repetitive DNA sequences with the capacity to adopt alternatively-structured DNA (i.e. non-B DNA) often co-localize with “mutation hotspots” implicated in genetic instability-related diseases [6–9]. Naturally occurring non-B DNA structure-forming sequences can stimulate genetic instability [10–16], although the fundamental mechanisms are still unclear.

Beyond the Watson-Crick B-DNA helix

Watson and Crick first described the canonical B-form structure of the DNA duplex structure over 60 years ago. However, since this first description, more than a dozen alternative DNA structures have been characterized. These include left-handed Z-DNA, three-stranded triplex or H-DNA, 4-way junction-containing cruciforms, self-annealing hairpins, slipped DNA, guanine quartet-containing G4-DNA, A-DNA, cytosine-rich i-motifs, etc. [17–19].

These structures require certain sequence elements for their formation. For example, inverted repeat sequences can adopt hairpin or cruciform structures, where intra-strand annealing occurs on one or both strands due to the self-complementary feature of both strands. Simple repeats can form loop-out structures if the two strands are not perfectly re-aligned after unwinding [20]. If the looped-out regions are self-complementary, then duplex stem regions can be formed, such as in CNG triplet repeats [11, 21, 22]. Alternating purine-pyrimidine tracks can form left-handed Z-DNA structures (Figure 1), where the alternating stacking of bases in the *syn*- and *anti*- conformations results in a zigzag pattern of the sugar-phosphate backbone [23]. The Z-DNA conformation results in a double-stranded helix with ~12 base pairs (bp) per turn, compared to the ~10 bp per helical turn in canonical B-form DNA. A polypurine/pyrimidine region with mirror symmetry can form an intramolecular triplex structure (H-DNA, Figure 1) where the purine-rich strand in one half of the symmetry-containing duplex folds back and binds to the duplex in the other half of the mirror repeat *via* Hoogsteen hydrogen bonding in the major groove [24, 25]. Because initial studies showed that protonation of cytosine is required for Hoogsteen hydrogen bonding to the guanine in a GC Watson-Crick bp, the structure is named H-DNA. G4-DNA or G-quadruplex DNA can form at tandem repeats of guanine (G) sequences with a contiguous run of three or more guanine bases [26–28]. Four Gs associate in a square-planar cyclic array, stabilized by Hoogsteen H-bonds to form a G-tetrad that can stack and thereby form a G-quadruplex (G4-DNA, Figure 1) structure stabilized by monovalent cations, such as K^+ or Na^+. Because the formation of non-B DNA structures requires specific sequence elements, we and others have designed algorithms to search for such structure-forming sequences in genomes/databases of interest [29–31].

In addition, RNA can also form alternative structures, either intramolecularly or intermolecularly with other RNA strands or DNA strands. For example, RNA G-

quadruplexes have been reported to impact transcription, translation, RNA localization and splicing. Intermolecular G-quadruplex structures formed between two copies of HIV-1 genomic RNA at their 5′ end dimerization-linked sequence domains, can anchor the virus for dimerization, which is important for viral recombination, translation, and encapsidation (for reviews see [32, 33]). Both RNA*DNA-DNA triplexes and RNA*RNA-RNA triplexes can alter transcription and splicing [34]. Further, RNA can also form a left-handed alternative conformation, *i.e.*, Z-RNA, which can interact with Zα domain family proteins and regulate immunity *in vivo* [35, 36]. However, the focus of this review is on non-B DNA structures.

Although most non-B DNA structures exist in higher energy states compared to the B-DNA structure, negative supercoiling generated by the unwinding of DNA from histone cores behind the replication and transcription machinery, or other chromatin remodeling processes, can drive the B-DNA to non-B DNA transition and maintain non-B structures. Importantly, non-B DNA is involved in wide range of biological processes, such as DNA replication, transcription, recombination and telomere maintenance [7, 19, 37, 38], such that modulation of and/or alterations in DNA conformation could lead to significant biological outcomes (Figure 2).

DNA structure-induced genetic instability

While triplet repeat-forming hairpins have been implicated in a number of neurological disorders, largely via expansion of the repeats, here we will focus on Z-DNA, H-DNA and G4-DNA structures (Figure 1) that have been implicated in cancer etiology.

In human genomes, G4-DNA, H-DNA, and Z-DNA-forming sequences often occur near, and are enriched at chromosomal mutation hotspots in disease-related genes, implicating them in genomic instability and disease [6, 39, 40]. For example, in c-*MYC* translocation-induced lymphomas and leukemias, a major breakage hotspot region in the promoter overlaps with sequences capable of adopting H-DNA, Z-DNA, and G-4 DNA [41–49]. We have discovered that an H-DNA-forming sequence within this region is susceptible to genetic instability and stimulates the formation of DNA double-strand breaks (DSBs), deletions, and point mutations in mammalian cells both *in vitro* and *in vivo* [10, 13, 16]. Taking advantage of the accumulating sequencing data of mutations in human cancer genomes, and search engines used to identify sequences that have the potential to adopt non-B DNA structures, we and others have found that many types of non-B DNA-forming sequences are significantly enriched at cancer mutation hotspots, suggesting a biological role of DNA structures in cancer etiology [6, 10, 11, 50]. Z-DNA-forming sequences map near breakpoints in *c-MYC* [51, 52], and a 5’ breakage hotspot in the *BCL-2* gene [53, 54]. Z-DNA-forming sequences are also mutagenic and lead to DSBs, deletions, rearrangements and point mutations in mammalian cells *in vitro* and *in vivo* [13, 15]. In yeast, the introduction of G4-DNA-forming sequences leads to genome instability [55–58], and in human epidermal keratinocytes, intrinsic DSB sites are enriched within G4-forming sequences [59]. G4-forming sequences are also found near sites of mutation hotspots, such as the mutationally activated *hTERT* promoter in melanoma [60–62]. Thus, naturally occurring Z-DNA, H-DNA, and G4-DNA-forming sequences can stimulate mutations in

mammalian cells and in mouse tissues supporting a role for non-B DNA structure in genetic instability in the form of point mutations, chromosomal deletions, translocations, and rearrangements, leading to disease etiology [7, 11, 15, 16, 39, 63–69].

Interestingly, studies have suggested that some single nucleotide polymorphisms (SNPs) within non-B DNA-forming sequences can affect their structure-forming potential, and thereby impact biological outcomes. For example, replacement of an A with a G in a short palindromic sequence can reduce its hairpin-forming potential, and has been associated with a higher risk of ß-thalassemia in Indian populations [70]. Another example includes the promoter of the *GRIN1M* gene that has been shown to adopt G-quadruplex structures *in vitro*, where a one base change (−855 G to C) can alter the G-quadruplex structure formation and has been associated with schizophrenic patients [71]. A +2985 (T to C) polymorphism in the *APOE* gene has been associated with Alzheimer's disease, and such a change can also convert a hairpin structure of a "T" allele to a G-quadruplex *in vitro* [72]. Further, a bioinformatics study showed that SNPs in quadruplex-forming sequences were significantly associated with the expression of the corresponding genes [73]. Because a one base-pair alteration can affect non-D DNA formation and impact the associated phenotypes, modulating DNA conformation by small molecules may have a wide variety of applications (Figure 2).

Based on many studies of the roles of DNA structure in DNA transcription, replication, chromatin structure, and genetic instability, it has become evident that DNA structures are functional genomic elements. Thus, DNA is more than just an inert source of genetic information; it has conformational features that modulate genetic processes, some of which have been implicated in disease etiology, genome plasticity, and evolution. Moreover, the specific structural features of non-B DNA could overcome the presumed lack of specificity in targeting DNA, potentially providing selective targets for drug design. The structural formation of non-B DNA can be predicted given that the required sequence elements are known for the formation of various types of such alternative DNA structures [74, 75]. Thus, DNA structural features represent important targets of investigation, with non-B-DNA structure formation and stability as site-specific key targets of DNA functional regulation and/or disease intervention.

Modulation of DNA structures *in vitro* and *in vivo*

Non-B DNA-forming sequences are dynamic in nature. The propensity of these structures to form in genomic DNA is dependent upon many variables, including the stabilizing effect of super-helical stress in the form of negative supercoiling, protein interactions, chromatin structure, and intracellular conditions (i.e. cations, and polyamines such as spermine and spermidine). An example of structure-altering cellular conditions and protein interactions include the presence of helicases such as the RecQ family members, some of which can unwind these mutagenic structures to preserve genomic integrity [76–78]. There are also exogenous small molecule DNA-interacting ligands that can stabilize or destabilize these structures, and can be used as tools in the study of non-B-DNA structure formation and processing, which is the focus of this review.

Small molecules as tools to study DNA structure and function

Because of their size, small molecules can often reach their targets *in vivo* more efficiently than larger compounds. Thus, the use of small molecules has provided an approach to advance studies of biological mechanisms, evaluate therapeutic targets underlying diseases, and reveal novel therapeutic avenues [79–81]. As such, many small molecules have been found to interact with non-B DNA in a conformation-specific manner (see below). Many of the challenges associated with studies of non-B DNA structure-induced genetic instability could also benefit by the use of small molecules that have the potential to stabilize or destabilize these mutagenic structures. By using structure-specific recognition agents, precise temporal and spatial modulation of non-B DNA structure formation could be performed leading to a better understanding of the recognition and processing of a particular non-B DNA structure. In addition, fluorescent probes that can detect specific non-B DNA structures may be used to visualize non-B DNA structures, and therefore assist in revealing their cellular loci and possible biological functions. While there have been antibodies generated to visualize a number of non-B-DNA structures [82–90], there have been concerns regarding their use. For example, permeabilization of cells prior to antibody probing could potentially alter the conditions that are critical for DNA conformation.

There are many examples of the use of DNA structure-specific ligands and probes in the literature. Here, we review recent findings regarding small molecule non-B-DNA structure modulators (stabilizers and destabilizers) as well as probes, *focusing on Z-DNA, H-DNA, and G4-DNA structures*. We refer to previous or current reviews where applicable, and highlight salient points of known ligands relevant to discussion (e.g. studies with a focus on biological outcomes). We also present a brief overview on the recent techniques and analyses developed to enable successful identification of DNA structure-specific ligands.

Z-DNA

Among the known non-B DNA structures, Z-DNA is the only entirely left-handed form. Its nucleobases alternate between the *syn* (purines) and *anti* (pyrimidines) positions, resulting in the phosphate groups being closer together (compared to the canonical B-DNA form) in a zigzag fashion. With the close proximity of the phosphate groups in this structure, steric hindrance and anionic repulsion is generated [91, 92]. This also leads to a deepening and narrowing of the minor groove [93]. Thus, while non-B-DNA structures are generally transient and in high-energy states, Z-DNA is arguably the structure most heavily dependent on extrinsic factors for its formation and stability *in vivo* and *in vitro* [92, 93]. This is reflected in the literature where most Z-DNA-related small molecule studies describe *inducers* (i.e. promote the formation) of Z-DNA structures as opposed to ligands that only recognize the structure [91, 92, 94, 95].

Z-DNA stabilizers –

Pivotal to Z-DNA recognition is ‘handedness’ and the ability to counteract repulsive factors (phosphate-phosphate proximity and steric hindrance), which are largely provided for by chiral molecules and/or metal complexes. For example, the ruthenium complexes developed by the Barton group, e.g., $Ru(phen)_3^{2+}$, $Ru(DIP)_3^{2+}$, and $Ru(bpy)_2dppz^{2+}$ (phen, 1, 10-

phenanthroline; DIP, 4,7-diphenylphenanthroline; dppz, dipyridophenazine) whose left-handed lambda (^) enantiomers bind preferentially to the left-handed Z-DNA by intercalation [96–98]. It was previously thought that intercalation through the rigid Z-DNA backbone was not permissive until these complexes indicated otherwise [99, 100]. Moreover, their binding to Z-DNA was accompanied with marked enhancement in their visible and luminescence spectra, thus also serving as probes for Z-DNA structure [96–98]. This chiral recognition of Z-DNA by a left-handed enantiomer (left-handed recognizing left-handed) is again demonstrated in more recent publications where an (M)-chiral, 1,14-dimethyl[5]helicene-spermine ligand selectively recognized and stabilized Z-DNA over B-DNA [101, 102]. A zinc (II) porphyrin (ZnTMPyP4) complex also selectively bound Z-DNA, and prevented the Z-DNA to B-DNA transition, as demonstrated by several independent studies [103–106]. The metal complex-Z-DNA interaction was indicated by a strong induced circular dichroism (CD) spectrum representative of a Z-DNA signature, and a positive wavelength-dependent linear dichroism (LD) signal. Parsing the kinetics of Z-DNA binding revealed two coexisting binding modes: one from the electrostatic interaction between the Z-DNA phosphate groups and the porphyrin positive charges, and another from the axial coordination of the central Zn(II) of the complex to the exposed N7 of guanine in the Z-DNA [105]. A sulfonated nickel porphyrin from the same group also demonstrated spectroscopic discrimination between the B-DNA and the spermine-induced Z-DNA structures [107]. The strong induced CD signal from binding with the spermine-induced Z-DNA structure, was not observed in the presence of the B-DNA, nor spermine alone [107].

Z-DNA destabilizers –

Many of the studied Z-DNA destabilizers have shown preferred binding to the canonical B-DNA structure, and were able to revert Z-DNA spectroscopic signals back to those indicative of the B-DNA form. Among them are known intercalators, initially thought not to be easily accommodated in the rigid structure of Z-DNA [99, 100]. For example, netropsin [108], distamycin, daunomycin [109, 110], adriamycin [111], ethidium bromide (EB) [112–115], dipyrandium [112], actinomycin D [113,116] tilorone [117] and thiazole orange (TO) [118] facilitate the Z-DNA to BDNA conversion when added to poly(dG-dC) or poly(dG-m^5dC) in the Z-DNA form. The anticancer drug (+)daunorubicin, binds selectively to B-DNA and was found to be an allosteric effector. It converted a $[poly(dGdC)]_2$ in the Z-DNA conformation to its intercalated B-DNA form [119, 120]. More recently, quinacrine and 9-amino-acridine were observed to bind strongly to B-DNA and decrease the rate of transition from BDNA to Z-DNA, in addition to converting the left handed poly(dGm^5-dC) back to the right-handed B-DNA form [121]. The tetrapeptide, lysyl tryptophenyl glycyl lysine O-tert butyl ester (KWGK) was also shown to convert poly(dG-m^5dC) Z-DNA to its B-DNA form under low salt conditions as determined by CD spectroscopy. Although, this tetrapeptide seemed to have a stronger affinity for the Z-DNA compared to the B-form, and for alternating (over non-alternating) GC sequences, due in part to the intercalation of the tryptophan moiety to the DNA [122].

As for metal complexes, a similar induction of the poly(dGm^5-dC) in the Z-form to the B-form was seen for both the inversion-labile $Fe(phen)_3^{2+}$ and inversion-stable $Ru(phen)_3^{2+}$ complexes, even when no binding specificity was detected [123]. Unlike its zinc counterpart,

the copper (II) porphyrin (CuTMPyP4) was able to convert the Z-poly(dG-dC) to the B-form [124].

The antibiotic elsamicin A was found to inhibit both the rate and extent of the B- to Z-transition of poly(dG-dC) or poly(dG-m^5dC) DNA. It was also shown to convert Z-DNA to the intercalated B-form even under conditions that otherwise favored Z-DNA formation [125].

Z-DNA probes –

While there are many examples of the use of spectroscopy to monitor Z-DNA binding (almost exclusively changes in CD patterns), probes and techniques that can be used to visualize the cellular loci of Z-DNA formation are still lacking. The use of fluorescence resonance energy transfer (FRET) technology provides an alternative approach at a molecular level that allows for the monitoring and visualization of Z-DNA formation and binding that may be more sensitive and applicable to high-throughput screens. This was first demonstrated by using a tethered proto-Z-DNA-forming sequence [$d(m^5CG)_{10}$] flanked by a fluorescein (donor) and a Cy3 (acceptor) containing rigid DNA sequence motifs [126]. The swivel of the proto-Z-DNA-forming sequence upon its Z-DNA formation resulted in a concomitant change in the distance between the donor and acceptor and hence, FRET efficiency. Several laboratories have used the Cy3 (donor) and Cy5 (acceptor) pairs in similar successive studies [127, 128]. Examples include a study in combination with magnetic tweezers, in real-time monitoring of Z-DNA formation under controlled tension and superhelical density [127], and another study that observed the B-DNA to Z-DNA transition under low-salt conditions and Z-DNA-binding protein association and dissociation events [128].

It is notable that because the DNA conformations are dynamic and can transition between B-DNA and non-B forms, using probes to detect/monitor Z-DNA (and other types of non-B DNA) can potentially affect such equilibrium. Thus, biases can arise from the probe-induced or stabilized non-B-DNA formation.

Z-DNA ligands and biological activity –

Studies concerning biological outcomes as a result of modulating Z-DNA formation through ligands are limited. In a study investigating the mechanism by which EB exerts its anti-trypanosomal effect, Chowdhury, *et al.* (2010) reported that low concentrations of EB blocked replication initiation of the trypanosomic minicircle DNA (and at higher drug concentrations, nuclear replication) [129]. Interestingly, 16–24 hour exposure of trypanosomic minicircle DNA to EB produced highly supertwisted minicircle fractions containing Z-DNA (probed with an anti-Z DNA antibody), which were otherwise fully relaxed in the absence of EB [129]. Whether the replication blockage was due to Z-DNA structure formation, however, was not explored.

H-DNA

In a polypurine-polypyrimidine-containing mirror-repeat sequence, an intramolecular triplex serves as a major structural element of H-DNA resulting from the association of the DNA

strand (serving as the 3rd strand) from the symmetric half of the mirror-repeat with the major groove of the purine-rich strand of the underlying duplex [130, 131]. The triplex can be of the divalent cation-dependent R*RY type (R: purine, Y: pyrimidine, *Hoogsteen/reverse Hoogsteen H-bonds) if the purine-rich tract serves as the third strand, or an acidic pH-dependent Y*RY type if the pyrimidine-rich strand serves as the third strand. This triad/triplex association is mediated either by Hoogsteen (Y third strand, e.g. T*AT or C^+*GC) or reverse Hoogsteen (R third strand, e.g. A*AT, G*GC) H-bonding. Hoogsteen and reverse Hoogsteen interactions also run in parallel and antiparallel directions, respectively, with respect to the purine-rich strand of the underlying duplex. RNA strands or combinations of RNA and DNA strands can also form triplex structures [25, 34]. Thermodynamic studies *in vitro* have demonstrated that RNA*DNA:DNA triplexes are as stable as three DNA strands [132].

Another mode of triplex formation is through an intermolecular mechanism where the third strand is provided by an exogenous short DNA strand, i.e., a ‘triplex-forming oligonucleotide’ (TFO). TFOs have been studied for their specific DNA duplex targeting characteristics as potential gene regulatory and therapeutic agents, for example, in the regulation of gene expression, stimulation of DNA repair, site-specific mutation, etc. [131]. Thus, most studies on TFO-derived intermolecular triplexes have been focused on *inducing* the formation of triplex DNA. Many modifications have been made to TFOs to enhance their duplex binding specificity and affinity, including alterations to the base, sugar, 5’- and/or 3’-ends, and the phosphate backbone. These have been extensively reviewed elsewhere [131, 133–136]. However, small molecule ligands, utilized to stabilize or destabilize the triads of the intermolecular triplex are considered herein.

Triplex formation occurs with inherent instabilities, which small molecule ligands can help alleviate. For example, the interaction of a DNA third strand with a DNA duplex results in high local negative charge density, leading to unfavorable electrostatic repulsion that must be overcome to enable and maintain stable triplex formation. As such, charge-neutralizing factors that counteract this repulsion such as multivalent cations (e.g. Mg^{2+}) and polyamines, have been well-documented [131, 132, 137, 138]. Additionally, the hydrogen donor and acceptor groups from the third strand must be available to maintain two stabilizing Hoogsteen H-bonds between the 3rd strand nucleobase and the duplex purine; thus, the formation of a Y*RY triplex such as those involving C^+*GC triads, requires acidic pH for stabilization [138]. These Hoogsteen-duplex interactions are also critical to allow for base stacking among the triads [25]. Thus, triplex-specific recognition can be modulated by small molecule ligands through electrostatic interactions, Hoogsteen hydrogen bonding, third strand binding, base stacking and therefore the shape of the polyaromatic ring system.

Triplex stabilizers –

Triplex stabilizers often consist of an extended aromatic ring system and surface areas analogous (and preferably overlapping) with that of the triad, enabling strong stacking interactions. This renders specificity to the triplex structure as binding to the duplex could be disfavored due to the presence of the larger surface area. The polyaromatic ring system can be fused and planar, typical of intercalators, or unfused and non-planar linked by a torsional

bond that allows flexibility for matching the propeller twist of the base triplet [25, 137]. The torsional freedom of the latter can also allow for a twist in the molecule that enables groove binding in the triplex region. Lastly, cationic moieties, either as protonated ring heteroatoms or side chains that mediate electrostatic binding to the grooves tend to be triplex-stabilizing [25, 95, 137–140], but may not always be applicable for C^+*GC-containing triads due to electrostatic repulsion. Aliphatic nitrogen atoms, generally protonated below pH 8.0, have been widely used as side chain functional groups of some intercalating agents [141].

Triplex intercalators ——An early report of a molecule binding to a poly(dA)•2poly(dT) triplex, albeit weakly, was ethidium bromide [142]. However, its binding with C^+*GC-containing triplexes was found to be destabilizing. This is presumably due to electrostatic repulsion to the protonated cytosines of the triad, providing evidence for its T*AT specificity [136, 142]. The characterization of benzo[e]pyridoindole (BePI) and its derivatives followed, which may be the first compound shown to interact preferentially with triplex DNA over duplex DNA [143–146]. Other intercalators that have been shown to have triplex-stabilizing properties include the unfused aromatic napthylquinolines [146, 147], LS-08 and MHQ-12 [148–150] and their self-dimers [151]. Although favoring T*AT triads, the napthylquinolines can also stabilize a triplex with a GA strand at acidic pH due to the protonation of the chromophore, which is advantageous for electrostatic binding [146, 149]. Other triplex stabilizers include: midazothioxanthones [152]; coralyne, which has a very high affinity for T*AT triplexes such that it can disproportionate a poly(dA)•poly(dT) duplex into a poly(dA)•2poly(dT) triplex and a free poly(dA) strand [153–155]; dibenzophenanthroline derivatives [156, 157]; 2,6-disubstituted anthraquinones whose neutral scaffold may preclude the requirement of T*AT triads for binding [146, 158]; acridines [159, 160]; SN-18071, a bis-quaternary ammonium (BQA) heterocycle that binds via electrostatics in the T*AT minor groove, reportedly more effectively than spermine [161, 162]; benzoindoloquinolone derivatives whose electron-withdrawing substituents can improve triplex formation and selectivity [163]; quinacridines, which have been shown to have photocleaving properties [157]; NB-506 [164]; and 3,3’-diethyloxadicarbocyanine (DODC) [165].

Diazoniapolycyclic salts, because of their extended aromatic system, have been investigated for triplex binding [137, 166, 167]. They have been shown to afford a marked 10–30°C stabilization for the T*AT triplex-to-duplex transition with minimal effect on the duplex-to-single strand transition (1–2°C stabilization) [166]. However, they have also been shown to increase the thermal melting of AT duplexes as well as calf thymus DNA. This highlights the importance of having separate structural and or sequence controls, rather than relying on the effects of the ligands on changes only in the transitions of the triplex structures.

U*AU triplex binding by the coralyne family of isoquinoline alkaloids [154], such as berberine [168] and palmatine has been recently explored [169, 170]. Berberine and palmatine showed triplex stabilization via thermal melting (by monitoring the triplex to duplex transition, without the use of separate duplex controls) but not to the same extent as coralyne [169, 170]. This was further reflected in their binding affinities, which were an order of magnitude lower compared to coralyne for triplex DNA substrates [170]. Based on fluorescence quenching and viscosity measurements, the authors inferred that coralyne

bound via full intercalation, whereas berberine and palmatine only partially intercalated into the triplex DNA structures [170]. Similarly-shaped naturally occurring indoquinoline alkaloids, cryptolepine and neocryptolepine, were tested against an extended panel of substrates [171]. Cryptolepine showed a 10°C stabilization for a poly(dA)•2poly(dT) triplex. However, for the shorter (T,C), (G,A) and (G,T)-containing triplexes, no change in the thermal melting profiles were detected in the presence of 6 μM cryptolepine. In addition, competition dialysis revealed that cryptolepine had modest affinity for GC-rich structures (e.g. duplex DNA and G4-DNA) [171].

Further, 4,9-dimethoxy-11-phenyl-substituted indoloquinoline analogs have been synthesized and characterized for their interactions with intramolecular triplex substrates containing varying lengths of T*AT tracts, as well as an antiparallel GT-containing triplex (Tap), duplex, and single-strand controls [172]. Interestingly, the analog with a singly-charged aminoethylamine side chain demonstrated Tap preference with a pH-dependent stabilization and affinity, despite the modest T*AT affinity. The stronger stabilization and binding of the protonated ligand at lower pH was associated with a more exothermic binding process [172]. A similar requirement for protonated amines (aside from the napthylquinolines) to afford triplex stabilization was also observed with the recently studied benzimidazoles [173]. To determine the number and type of interactions of triplex-selective indoloquinolines with parallel triplexes, NMR studies were performed using short intermolecular triplexes specifically labeled with 3'–^{15}N thymidine probes as substrates [174]. This study, which may be the only reported ligand-triplex NMR structural determination to date, reported that several co-existing species were observed. But a general observation was that the 5'-triplex-duplex junction was the most favorable intercalation site, in particular when flanked by a T*AT base triad. The triplex-duplex junction, as a strong site of intercalation, has been demonstrated by other intercalators such as acridine, phenanthroline, ellipticine, and ethidium bromide [175–178].

Extending the studies on substituted quinolines, the phenanthroline-containing derivatives were able to stabilize a T*AT triplex by as much as 28°C without any effect detected on the AT duplex [147, 179]. Other 2-arylquinolin-4-amines were studied in which a 4-hexamethylenediamine substituent resulted in a 44°C increase in triplex thermal melting with no effect detected on the duplex melting [137].

The importance of the complementary shape between the triads and an intercalator to promote binding efficiency was highlighted in the development of benzo[*f*]quino[3,4-b]quinoxaline (BQQ), which is possibly one of the most triplex-selective and effective ligands to date. BQQ, a product of rational design after molecular modeling of benzo[*f*]pyrido[3,4-b]quinoxaline (BfPQ), showed that better overlap (i.e. better stacking interactions) with the purine nucleobase/strand of a T*AT triplex can be achieved if the aromatic system can be extended by adding a new ring [180]. From a functional perspective, the use of BQQ and its conjugates to direct triplex-directed double-strand cleavage of plasmid DNA has been demonstrated [181].

Triplex-stabilizing groove binders ––—Even in the absence of the aromaticity that provides base stacking stabilization, the polyamine/carbohydrate structure of

aminoglycosides, neomycin in particular [182, 183], proved to be effective in specifically stabilizing a T*AT triplex DNA structure. The change in thermal melting by as much as 25°C, at a neomycin/base triplex ratio of ~2, without any effect detected on the DNA duplex substrate, may be the highest stabilization among triplex groove binders [184]. Neomycin has a novel and specific "Watson-Crick groove recognition" (see discussion below) that precludes duplex binding even in the presence of salt. Also, though it preferentially stabilized T*AT triads, it was also able to accommodate C^+*GC triads [182, 185], despite its polycationic feature. However, it was also shown to induce the formation of hybrid DNA-RNA-DNA and DNA-RNA-RNA triplexes [184].

Combining the triplex-selective intercalator (BQQ) with the triplex-selective groove binder (neomycin), a BQQ-neomycin conjugate was synthesized, which proved to be more effective in stabilizing poly(dA)•2poly(dT) and poly(rA)•2poly(rU) than either BQQ or neomycin alone [186–188]. Its 2.7×10^8 M binding affinity to poly(dA)•2poly(dT) was nearly 1000-fold greater than that of neomycin alone, increasing the melting temperature by 46°C [188]. Other intercalators (e.g. naphthalene diimide, anthraquinone, pyrene) were also conjugated to neomycin, and similarly increased the stability of the T*AT triads over that of neomycin alone, but were still second to the BQQ-neomycin conjugate [188]. Whether these intercalator-neomycin conjugates are specific and can induce the formation of hybrid triplexes similarly to neomycin alone is still an open question. Other ligands that bind triple helices via groove binding include Hoechst 33258, berenil, DAPI, distamycin A and its analogs [189–191].

Some of the triplex stabilizers have been shown to induce the formation of hybrid triplex structures that otherwise would not have formed in the absence of the ligand. In the presence of poly(rA) and poly(dT) strands and at low ionic strength (~18 mM Na^+), berenil, DAPI, netropsin, and ethidium bromide induced the formation of dT*rAdT triplexes, whereas the structurally similar berenil and DAPI induced the formation of rA*rAdT triplexes [192, 193]. For reference, a dT*rAdT triplex was shown to form in the presence of high Na^+ (2.5 M) [194]. There is also an intercalator, oxazine 170, which can induce the formation of a hybrid poly rA:$(poly\ dT)_2$ triplex [195].

It should be noted that most of these studies utilized poly(dA)•2poly(dT) triplex substrates and other Y*RY triplets [146]. The presence of the positive charge on the aromatic portion of the ligand can prevent interactions near the C^+*GC triads. This was shown by the broader affinity of a neutral 2,7-disubstituted anthraquinone to (TC)n, (CCT)n, (TTC)n and (CCTT)n-containing third strands, whereas the positively-charged napthylquinoline only stabilized (TTC)n and (CCTT)n [146]. Some triplex-stabilizing ligands can bind to triplexes containing C^+*GC triads but with lower affinities than to poly(dA)•2poly(dT) triplexes [136]. On the other hand, the use of R*RY triplexes as substrates of ligand interactions have been limited [149, 160, 172, 196, 197]. Some factors that may have contributed to this limitation include observations that antiparallel R*RY triplexes often possess G-rich sequences that may form competing G4-DNA structures, complicating the structural studies [198, 199]. Also, in our studies, an intramolecular R*RY triplex-forming sequence from a chromosomal breakpoint hotspot in the human *c-MYC* gene [200], formed a very thermally stable intramolecular triplex structure, such that further thermal stabilization effected by a

potential stabilizing ligand would be difficult to observe by the currently available techniques.

Triplex destabilizers –

Historically, some groove-binding agents have been shown to destabilize triplexes, largely due to their higher affinities for the B-DNA/duplex structure. This then either prevented the binding of the third strand to the major groove of the duplex or resulted in the displacement of the bound third strand. The binding of a third strand to form the triplex creates new groove structures. Using the denotations used by Escude and Sun [136] for the triplex-forming strands, the oligopyrimidine- and oligopurine-rich strands of the duplex are referred to as the Watson and Crick strands, respectively, and the third, Hoogsteen strand. The three new grooves are referred to as the Watson-Crick (highly similar to the B-DNA/duplex groove [201, 202]), Watson-Hoogsteen, and the Crick-Hoogsteen grooves. With exceptions, most classical duplex minor groove binders have an affinity for the Watson-Crick groove (i.e. B-DNA/duplex structure) even in the presence of the third strand in the major groove. This Watson-Crick minor groove-preferred binding generates an intergroove modulation that can result in the displacement of the third strand [140, 159, 203, 204]. Thus, H-bond mediated binding to the Watson-Crick minor groove can destabilize triplexes. Conversely, while the Crick-Hoogsteen groove is very narrow, the Watson-Hoogsteen groove can accommodate the binding of ligands, for example, neomycin, which is triplex stabilizing [182, 183].

There are only a handful of triplex destabilizers reported in the literature [138, 139, 189, 205]. Although most are minor groove binders, the details of destabilization are quite varied and can be condition specific. Mithramycin binding was shown to inhibit the formation of an R*RY triplex in the human *c-Ki-ras* promoter. Its binding in the minor groove also resulted in the displacement of the major groove-bound TFO [206, 207]. Using the same intermolecular triplex in the human *c-Ki-ras* promoter, the anti-trypanosomal agent, berenil, destabilized the triplex but did not cause TFO binding inhibition or TFO displacement like that seen with mithramycin [206]. In addition, prior investigation showed that although berenil bound both poly(dA):2poly(dT) DNA triplexes and poly(rA):2poly(rU) RNA triplexes without displacing the third strand, it thermally destabilized a T*AT triplex at $[Na^+] \geq 0.125$ M, whereas it was thermally stabilizing at $[Na^+] \leq 0.08$ M. Berenil also affected the thermal stability of the RNA triplex to duplex equilibrium depending on the [base triplet]/[total berenil] ratio, with weakly destabilizing effects at [base triplet]/[total berenil] ratios >5, while thermally stabilizing this equilibrium at [base triplet]/[total berenil] ratios <5 [204, 208]. Netropsin has been shown to have a higher affinity for duplex DNA [209, 210], it bound to T*AT triplets in the minor groove, and destabilized them relative to duplexes, and yet did not displace the major-groove bound pyrimidine-rich third strand [203]. Molecular dynamics simulations suggested that near saturation of the minor groove with ligand is needed to completely dissociate the third strand [211].

As predicted by molecular modeling, 2,6-diamidoanthraquinones (see stabilizers above) can stabilize T*AT triplexes due to the extended planarity of anthraquinone that can significantly stack with the triads and side chains that are well-oriented along the grooves. The 1,4-

diamidoanthraquinones, on the other hand, have steric requirements that precluded an effective triad overlap and groove accessibility by the side chains [158, 212]. Effectively competing for the third strand, the 1,4-diamidoanthraquinones showed preferential binding to duplex DNA and prevented the formation of triplex structures as evidenced by the absence of a triplex footprint [158, 212]. SN-6999, another BQA derivative, is unlike SN-18071 (see stabilizers above) in its ability to form H-bonds in the poly(dA):2poly(dT) minor groove, which can destabilize the triplex structure [161]. Other destabilizing groove binders include Hoechst 33258 [190, 213], and distamycin A [207, 214].

A more recent report, which investigated the interactions of poly(rA)·2poly(rU) triple strands with proflavine (PR) and its proflavine *cis*-platinum derivative (PRPt) showed that they both thermally destabilized the RNA triplex structure [215]. The destabilizing effect of PRPt was greater than that of PR (as much as 22°C destabilization versus only 8°C for PR) since its occupation of the major groove prevented the full intercalation of PR and also destabilized the binding of the third strand.

Triplex probes –

The most well-characterized triplex ligands, BePI [144] and coralyne [154], lose their fluorescence upon binding to their triplex substrates [139]. Further, the fluorescent staining of a rationally-designed H-DNA/triplex intercalator, BQQ, showed non-specific interactions with DNA [180, 216]. Thus, ligands that can specifically probe for the presence of triplex formation in real-time and provide an approach to visualize H-DNA loci in *vivo* are still lacking. While most triplex stabilizers are made up of extended aromatic ring systems, very few, if any, reports have fully addressed and investigated the spectroscopic changes that accompany binding of ligands to triplexes/H-DNA structures. The family of ruthenium complexes was first tested against Z-DNA, of which the derivatives $Ru(phen)_2dppz^{2+}$ and $Ru(bpy)_2dppz^{2+}$ were noted for their “light switch” effect upon nucleic acid binding [217]. However, they were found to attain their highest level of luminescent emission upon interacting with T*AT triplexes [217]. Luminescence data and modeling studies supported an intercalative model where substantial overlap and stacking occurred between the dppz ligand and the triplex bases [217]. Subsequent studies incorporating a larger intercalative ‘wing’, $Ru(phen)_2bdppz^{2+}$ [218], showed similar spectral properties between duplex or triplex DNA substrates confirming the triplex intercalative model, and further indicated that the binding occurred from the minor groove. More recent Ru^{2+}-based triplex studies have been tested against a U*AU RNA triplex substrate, and with varying complexities of coordinating aromatic intercalators. The intercalative mode of interaction and subsequent stabilization of the RNA triplex still holds, albeit without demonstration of specificity (e.g. duplex versus triplex, DNA triplex versus RNA triplex) [219–221].

Ligands that can stabilize intermolecular triplexes, a number of which have reported increases in fluorescence intensities, have been characterized. A bisintercalator YOYO was used in the recognition of a mixed DNA sequence by a homologous single-strand oligonucleotide [222]. The free and bound YOYO absorbed at 458 and 490 nm, respectively, which precluded signal interference from a number of biological molecules. YOYO also increased its fluorescence ~40-fold when intercalated in DNA, compared to the fluorescent

free YOYO. However, when its relative quantum yield was estimated, it was found to be similar when it interacted with duplex or triplex DNA [223]. Similarly, a pyrene moiety tethered to the 3'-end of a triplex- forming homopyrimidine oligonucleotide exhibited strong fluorescence when triplex formation occurred [224]. The probe alone was spectroscopically silent but was increased to as much as 45-fold in fluorescence quantum yield depending on the thermal stability of the triplex substrate. Thiazole orange (TO), a synthetic cyanine dye, which has shown a >1000-fold increase in fluorescence quantum yield when intercalated into duplex DNA [225], may have higher affinity and selectivity for G*GC triplexes and G4-DNA over duplex DNA [226]. A TO displacement assay has been developed based on its affinity to G4 DNA structures. The assay relies on the displacement of G4-bound TO to test the affinity and selectivity of putative G4-specific ligands [227–230]. A related cyanine dye, Cyan 40, which absorbs at ~435 nm as a free dye, was found to be specific for G*GC triplexes relative to G4-DNA [231]. DMT (methyl-2,6-[2-(4-methyl-sulfanyl-phenyl)-vinyl]-pyridine) is one of the more recently reported triplex-probing synthetic small molecules; however, it showed only moderate fluorescence enhancement and little structure selectivity [232].

Wang *et al.* (2015) tested a panel of flavonoids for their interactions with DNA triplex structures, and fisetin (7,3′,4′-flavon-3-ol), a plant polyphenol, was identified as a T*AT triplex-specific probe [233]. When bound to a triplex substrate, it exhibited a bright green fluorescence emission that was observable by the naked eye under UV illumination. This emission was reported to be the result of a process termed 'excited state intramolecular proton transfer' (ESIPT) between its exocyclic 3-hydroxyl and 4-carbonyl groups. Fisetin was shown to stabilize the triplex structure by ~14°C, without affecting the duplex control. With ESIPT requiring aprotic solvents, the use of fisetin may be limited to low pH-requiring C^+*GC triplexes. It will be interesting to see whether the strong emission also occurs in the presence of the R*RY triplexes. Another natural product, chelerythrine, was isolated from a panel of natural isoquinoline alkaloids, and found to be fluorescent (~120-fold) upon its interaction with T*AT triplexes, increasing the triplex to duplex thermal transition by ~7°C [234].

Triplex ligands and biological activity –

Despite the development of small molecules as modulators of triplex/H-DNA structures, cellular studies that demonstrate biological effects of such modulation are still underexplored. BePI was used in one of the first few studies that demonstrated that a ligand binding to H-DNA resulted in a biological outcome [235]. Here, a 55-bp polypurine-polypyrimidine sequence able to adopt a Y*RY H-DNA triplex structure, was inserted into a plasmid (pIbla69) between the *bla* promoter and the coding start site of the *β-lactamase* gene. In the presence of BePI, a stabilizer of H-DNA, chloroacetaldehyde modification showed increased hyperreactivity of the adenines on the 3'-side of the R strand consistent with the folding of the 5' half of the Y strand to form H-DNA. H-DNA structures can cause replication arrests [236–238] during *in vitro* replication, and in the presence of increasing BePI concentration, dose-dependent arrests (to as much as 90% in the presence of 2 μM BePI) of the elongation products of *E.coli* DNA polymerase I were observed. Using *Taq* polymerase at high temperature, the presence of BePI resulted in 55% and 40% inhibition of

replication at 52°C and 62°C, respectively. In contrast, in the absence of BePI, very few replication arrests were detected at 52°C, and no arrests were observed >52°C.

H-DNA-forming sequences have also been shown to cause transcription arrest [238]. Thus, using the same plasmid system described above, a decade later, the effect of the BQQ ligand on the H-DNA insert and subsequent *β-lactamase* expression (via β-lactamase activity) was assessed [239]. The expectation was that an H-DNA-forming sequence positioned upstream of the open reading frame would reduce *β-lactamase* gene expression. Indeed, there was decreased β-lactamase activity in the cells with the H-DNA-containing plasmid (pIbla69). Addition of increasing concentrations of BQQ (0.5 – 4 μM) led to a further dose-dependent reduction of β-lactamase activity with as much as 48% reduction in the presence of 4 μM BQQ. Conversely, the presence or absence of BQQ did not affect the enzymatic activity of the cells harboring the parental control plasmid (pBR322). The authors did not detect a marked difference in the quantity of plasmid DNA in the presence or absence of BQQ. From this, they eliminated the possibility of inhibition of plasmid DNA replication, and attributed the repression of transcription due to BQQ stabilization of H-DNA as the cause of reduced β-lactamase activity.

(GAA•TTC)n repeats implicated in Friedreich's ataxia (FRDA) were shown to form 'sticky DNA' arising from the association of two R*RY triplexes in negatively supercoiled plasmids at neutral pH [240]. These triplexes can stall RNA polymerases, leading to the inhibition of transcription and subsequent reduced frataxin (FXN) protein levels in FRDA patients [205, 241, 242]. Low FXN levels led to progressive neurodegeneration and cardiomyopathy, characteristic of FRDA [243]; thus, destabilizing the formation of the triple helical structure may restore FXN levels [205, 244–246]. One approach has been to use a short, low molecular weight oligonucleotide that can bind to the 'third' strand of the triplex structure. The oligonucleotide competed with the formation of the triplex structure, which led to a specific and concentration-dependent increase in the full transcript of the *FXN* gene [244]. Another approach was to use a linear β-alanine-linked polyamide FA1 (ImPyβImPyβImβDp, where Py=pyrrole, Im-imidazole, β=β-alanine, Dp=dimethylaminopropylamine), which is a duplex minor groove binder. By stabilizing the duplex, strand separation and subsequent triplex formation was disfavored [245]. Its use also increased FXN transcription by ~3-fold at both the mRNA and protein levels in an FRDA lymphoid cell line [245]. Pentamidine, another minor groove binding agent identified via a competition dialysis method, increased the levels of FXN by 2-fold in patient cells [246].

G4-DNA

G4-DNA structures can form at tandem repeats of guanine (G)-rich DNA or RNA sequences [32, 33, 247]. The four guanines associate with each other via Hoogsteen hydrogen bonding in a planar, cyclic array (i.e. a G-quartet or G-tetrad), either intramolecularly (single-strand folding upon itself) or intermolecularly (two or more strands) with parallel or antiparallel strand directionality. The large planar surface of two or more G-tetrad, in part due to strong Van der Waals attraction, can stack on top of each other to form the G4-DNA structure. G4-DNA structures can be stabilized by monovalent cations in the order of $K^+ > Na^+ > NH4^+ > Li^+$ [248], although some divalent cations have also been reported to stabilize G4 structures

[249]. The cations can counterbalance the high electronegative potential created by the carbonyl groups of the guanines, which are directed toward the interior of the G-tetrad [28]. This 'central' coordinating role of cations was calculated to be an important stabilizing factor in addition to the Hoogsteen H-bonding and stacking interactions of the G-tetrads [249, 250]. This is in addition to the known roles of cations in neutralizing phosphate backbones that are also in close proximity in G4-DNA. As dictated by their ionic radii, smaller Na^+ ions can either be in-plane of a G-tetrad or equidistant from two successive G-tetrads, while K^+ ions are exclusively found between two adjacent G-quartets [251].

G4-DNA structures have four grooves with dimensions that vary with the phosphodiester groups, their dimensions dictated by topology and the nature of the loops. The topology and loop conformation of the G4-DNA structures are known to be highly polymorphic [reviewed in [28, 247, 252–257]]. They are regulated by the nucleic acid sequence (length and composition; e.g., the number of loop nucleotides) [258], orientation (parallel versus antiparallel) and stoichiometry (one to four) of strands, the glycosidic conformation of the G nucleosides (*syn* versus *anti*), and solution conditions (e.g. cations, co-solutes) [249, 259, 260].

Thus, the structural features of G4-DNA amenable for ligand modulation include the G-quartets, the central channel of the G-quadruplex typically occupied by metal ions, the phosphate groups, the loop nucleobases, and the grooves at the surface of the G4-DNA stack.

G4-DNA stabilizers –

Much work has been published on G4-DNA ligand interactions, with the majority of the studies focusing on G4-DNA stabilization. We would like to direct the readers to reviews generated from the last ~5 years covering organic compounds and metal complexes as G4-DNA ligands [252–254, 261–267].

Studies on G4-DNA binding showed that an intercalative mode of binding in between G-tetrads is highly unfavorable because it may require displacing a cation within a very strong stacking environment [252, 263]. Only a few reports have indicated that intercalation may have taken place between G-quartets, and these were performed in the absence of K^+ and with TMPyP4 as a ligand [268–270]. Thus, the most common modes of stabilizing G4-DNA interactions are via: 1) stacking into one of the end G-quartets of G4-DNA (end-stacking); 2) groove/loop; and 3) combined end-stacking and groove/loop binding.

Small molecules that can end-stack with G4-DNA include planar aromatic surfaces that mimic the large planar surface of the G-quadruplex. Fused aromatic polycyclic systems, macrocycles, and non-fused aromatic systems have been cited as three major families of G4-DNA interactive molecules that target the external G-tetrads [263, 271]. Some of these include anthraquinones, cationic porphyrins, acridines [272] and others, which are derived from duplex DNA binders, and therefore often do not offer high specificity for G-quadruplexes. TMPyP4, for example, is a porphyrin-based macrocycle, that also binds duplex and triplex DNA [273, 274]. However, it was one of the first ligands to have been studied for its potential to stabilize a G4-DNA-forming sequence in the *c-MYC* promoter

(via partial end-stacking, as assessed by footprinting studies) with associated suppression of transcriptional activation [275]. A possible exception is Phen-DC3, a bisquinolinium compound, which despite its interaction with a c-*MYC* intramolecular G4-DNA-forming sequence via extensive π-π end stacking [276], has also displayed exceptional selectivity for G4-DNA relative to duplex DNA [228, 277]. It is also very potent in that it can enhance G4-DNA thermal stability (ΔTm) up to ~30°C [228, 277]. Thus, it has been used in several assays to probe genetic instability from stabilized G4-DNA formation in yeast and mammalian cells [56, 278, 279]. Further, as end-stacking only requires a G-tetrad (i.e. a planar aromatic surface) to stack with, the binding of these molecules may not require a specific G4-DNA topology, and thus, may lack discrimination among the various G4-DNA conformations, and may even promote ligand-mediated G4-DNA structure formation [266].

Alternatively, the groove and loop regions of G4-DNA differ from the canonical DNA duplex and other non-B DNA structures, and even among G4-DNA structures, and thus may provide a better approach for structural selectivity in binding [reviewed in [280]; [252, 254, 266]]. Finally, recent reviews have suggested that the most thermally stabilizing and selective G4-DNA ligands often have the combined features of end-stacking and groove/loop binding. Specifically, these include aromatic ligands with a U-(or V) shape, exemplified by fused aromatic polycyclic and macrocyclic ligands, with electron withdrawing atoms/groups (e.g. protonable nitrogens, halogens) that can be locked in a planar conformation (i.e. with constrained flexibility) for effective G-tetrad stacking, and with basic side chains that target the loops and grooves for better selective G4-DNA binding and stabilization [263]. Pyridostatin (PDS) is such an example. PDS is a G4-selective stabilizing ligand whose design was intuited from previous studies on a potent macrocyclic oligoamide derivative [281]. Müller, *et al.* (2012), designed PDS as a flexible molecule, yet able to adopt a flat conformation such that it can participate in π-π interactions with the G-tetrad. It also contains amino groups as side chains to participate in electrostatic interactions [281]. As discussions below indicate, PDS has been useful in studies of co-localization [282] and in mapping the genome-wide distribution of potential G4-DNA-forming sequences [283].

G4-DNA destabilizers –

In contrast to the number of G4-DNA stabilizers that have been characterized, there have only been a limited number of reports that pertain to G4-DNA destabilizers. Bioavailable molecules such as polyamines at >1 mM concentration can destabilize G4-DNA structures [284], whereas 20 wt% urea resulted in an ~11°C T_m decrease for a human telomeric G4-DNA sequence in the presence of K^+ [285]. Many of the reported G4-DNA destabilizing ligands include cationic porphyrins and their derivatives. While TMPyP4 has stabilizing effects toward telomeric G4-DNA structures [286], it was found to be destabilizing toward a bimolecular d(CGG)n and intramolecular r(CGG)n G4-DNA-forming trinucleotide repeats [286, 287]. The formation of secondary structures in these trinucleotide repeats has been implicated in the silencing the *FMRI* gene in Fragile X syndrome [241, 243]. Excess TMPyP4 in the presence of $d(CGG)_7$ and 20 mM K^+ resulted in a 15°C decrease in thermal melting [286], whereas TMPyP4 slowed the electrophoretic migration of a $r(CGG)_{33}$-containing plasmid and enhanced the translation efficiency *in vitro* of $(CGG)_{99}$ firefly luciferase mRNA [287]. In a complex with platinum, PtTMPyP4 was found to be weakly

destabilizing (T_m decrease of 3°C) to a parallel bimolecular G4-DNA from the $d(TAGGG)_2$ sequence in the presence of 0.1 M K^+[288]. While the TMPyP3 isomer was reported to have destabilizing effects on the antiparallel human telomeric G4-DNA structure-forming sequence, $d(TTAGGG)_4$, decreasing its thermostability by 8°C [289]. A derivative bearing spermine pendants in the four *meso* positions of the porphyrin scaffold, H_2TCPPSpm4, led to a 30°C decrease in melting temperature of the tetramolecular G4-DNA-forming DNA aptamer $(TGGGAG)_4$ when added at a 2 μM concentration in a 1:1 molar ratio [290].

Lanthanide metallacrown complexes (MCs) have been shown to decrease the melting temperature of human telomeric G4-DNA (22Htel) by 16–20°C at a 5:1 complex:DNA ratio [291]. The destabilizing effect was also seen via CD spectroscopy studies where addition of the MCs led to the disappearance of the signature G4-DNA CD bands without the appearance of new bands. The destabilizing effect was ascribed to either the large MC plane that caused unfavorable steric interactions with the G4-DNA, or to the presence of Cu^{2+} ions, which also comprise the MCs, which can interact/coordinate with N7 of guanines [291].

A triarylpyridine with three side chains (TAP1) has been shown to have a moderate affinity (K_d ~11 μM) and a high stabilizing potential (ΔT_m ~23°C) for a G4-DNA-forming sequence from the promoter of the *c-kit* proto-oncogene [292]. Yet CD and NMR studies have revealed that TAP1 can significantly disrupt and weaken the G-quartets. Further support came from gene expression studies where human HGC-27 cancer cells overexpressing *c-kit* were incubated with different concentrations of TAP1. Previous studies showed that stabilization of G4-DNA led to reduced *c-kit* gene expression [275, 293–295], and treatment with TAP1 resulted in a dose-dependent increase (~90% increase with 5 μM treatment) in *c-kit* expression relative to control genes, as measured by quantitative real-time PCR [296].

A synthetic pyrrole-inosine nucleoside was designed to form a specific extended three-point Hoogsteen H-bonding interaction with guanines [297, 298]. From ^{1}H NMR ($CDCl_3$, CD_2Cl_2 as solvents) and ESI-mass spectrometry studies, this competitive interaction was shown to disrupt guanosine dimerization and G4-DNA formation from the parallel tetramolecular $[dTG_4T]_4$ and the intramolecular $d(T_2G_4)_4$ sequences [297]. While this represents a promising approach to destabilize G4-DNA, it may not be G4-DNA structure-specific and thus, may also disrupt other guanine-rich sequences in the genome.

G4-DNA ligands and biological outcomes –

There have been a large number of studies published utilizing ligand-mediated G4-DNA structure modulation to interrogate biological activities, and potential therapeutic outcomes. These include (but are not limited to) telomeric-end processing, effects on gene expression via transcriptional control, and genetic instability. These reflect telomeres and gene promoters as major loci of G4-DNA-forming sequences, the former being currently exclusive to G4-DNAs. As a result, some G4-DNA-specific ligands have entered human clinical trials. Quarfloxin/CX3543 [299] completed Phase I/II clinical trials for neuroendocrine and carcinoid tumors, whereas CX5461 [300, 301] is currently in late Phase II clinical trials for breast cancer patients with BRCA1/2 or homologous recombination deficiency (HRD) germline aberrations.

This rapid growth of ligand-G4-DNA investigation was, in part, stimulated by initial findings that sequences from the human telomere can form G4-DNA [302–304] and inhibit telomerase activity [305, 306]. Human telomeres are comprised of nucleoprotein complexes that can be found at the end of chromosomes, the extended maintenance of which has been implicated in cancer etiology [26]. The telomeric DNA is composed of a double-stranded region terminating into a single-stranded, G-rich 3'-overhang with a unit sequence of $(TTAGGG)_n$ whose G4-DNA formation [302–304] was shown to inhibit telomerase activity [305–307] by restricting access of the telomerase RNA template to the overhang. It has also been suggested that G4-DNA folding protects the overhang from replication protein A (RPA) binding, and thereby suppressing DNA damage signals [308]. Telomerase has been shown to be upregulated by as much as 85% in some human cancer cells [309], and is thought to promote the lifespan of the cancer cells by further synthesizing telomeric DNA [310, 311]. This gave rise to a biochemically informative assay to probe ligand interactions with telomeric G4-DNA, known as the telomeric repeat amplification protocol (TRAP) assay [312]. In the TRAP assay, telomerase activity appears as extension products, but in the presence of a G4-DNA stabilizing ligand, telomerase activity will be inhibited and prevent the formation of the extension products. Thus, ligand-mediated G4-DNA stabilization has become a potential anticancer approach through its interference with telomere function and/or inhibition of telomerase (and other telomere-related proteins), providing an impetus to identify telomeric G4-DNA interactive agents [261, 263, 264, 266, 267].

G4-DNA-forming sequences have been found to be overrepresented in biologically active regions of the genome, including gene regulatory and promoter regions [313]. Interestingly, while estimates predict that ~40% of human gene promoters contain G4-DNA-forming sequences [314], proto-oncogenes are reportedly more enriched in these sequences compared to tumor suppressor and housekeeping genes [315, 316]. Thus, ligand-mediated structure modulation of G4-DNA in gene promoter regions may represent an approach to control deleterious gene expression. Studies to stimulate G4-DNA formation and stabilization have demonstrated potential for transcriptional control, either gene expression upregulation or downregulation, depending on the interactions of G4-DNA with proteins involved in the regulation of transcription [263, 264, 267, 317]. A number of reviews are available related to ligand targeting of promoter-specific G4-DNA formation [261, 266, 317]. On the other hand, G4-DNA structure destabilization, while relatively underexplored, is another means to control gene expression. This was exemplified by the increase in translation efficiency *in vitro* of $(CGG)_{99}$ firefly luciferase mRNA through treatment with the TMPyP4 ligand [287].

G4-DNA probes –

The abundance of characterized G4-DNA-stabilizing ligands provides ligands that can be used as potential probes of G4-DNA structure, from organic fluorophores to metal complexes [263, 264, 318–322]. In addition to the "light up" and "light off" probes [321, 323], which show gain or loss of spectroscopic/fluorescent signals upon G4-DNA binding, high-affinity ligands 'tagged' with fluorophores have also been investigated [323, 324]. Some ligands with promising biophysical characteristics have advanced to testing by imaging in cells at the chromosome level [321, 325, 326]. While selective staining in the

nucleus or nucleoli was observed in a few studies, the results may not able to be unequivocally interpreted as the occurrence of G4-DNA structures *in vivo*, as there could be non-specific binding to other biomolecules (e.g. RNA with other 3D topologies, etc.). Further, cellular localization of some ligands were noted to vary, whether in living or fixed cells, or depending on the mode of crosslinking or fixation used. Only by the use of orthogonal assays, such as crosslinking, pulldown, or sequencing (or a combination), using live cells, would such staining be confirmed as an actual visualization of G4-DNA structures *in vivo*.

Methods to identify small-molecule modulators of DNA structure

With increasing evidence of the involvement of non-B DNA structures in regulating biological processes, this review aims to highlight the current status of the recognition of non-B DNA (Z-DNA, H-DNA, and G4-DNA) by structure-specific ligands. This approach takes advantage of distinct structural features of a particular non-B DNA structure compared to the canonical B-form DNA (and other non-B DNA structures) to obtain small-molecule ligand recognition. Using methods that span from biophysical and biochemical assays to functional cellular investigations - the binding selectivity, binding affinity, and subsequent biological outcomes of structure-specific binding can be defined. The methods that have been employed to investigate non-B DNA-ligand interactions are essentially similar to those used to identify B-DNA-ligand interactions [327–330], but under experimental conditions (e.g. buffer, pH, wavelength, signatures, etc.) that are suited for the formation/detection of a particular non-B DNA structure. For example, to probe optical thermal melting for short sequences of G4-DNA structures, the temperature-dependent absorbance at 295 nm (A_{295}) characterized by a distinctive hypochromic shift, is monitored [331, 332], rather than absorbance at 260 nm (A_{260}), which is used to detect canonical B-DNA and H-DNA structures [333–335]. Examples of methods to detect and evaluate non-B DNA-ligand interactions are summarized in Table 1 and have been previously reviewed [91, 138, 336–341].

To better define the mechanistic details of non-B DNA and ligand binding interactions, *in vitro* analyses employing short non-B DNA structure-forming sequences have often been used [329, 330]. Such *in vitro* analyses can be used to address many questions, including: 1) *how* is the ligand binding to the non-B DNA-forming sequence (e.g. binding mode, binding affinity, kinetics, stoichiometry, energetics); 2) *where* is the ligand binding the non-B DNA-forming sequence (nucleotide-level binding site); 3) *how specific* is the ligand binding to the non-B DNA structure of interest (versus duplex DNA or other non-B DNA structures or structure-related sequence preferences); and 4) *what* is the effect of the ligand binding to the non-B DNA-forming sequence (stability, i.e., stabilizing or destabilizing). Consequently, the answers to these questions are better defined from combined results using different techniques, rather than from a single type of experiment.

On the other hand, *in vitro* biochemical- and molecular biology-based assays, able to accommodate longer non-B DNA-forming sequences (e.g. incorporated into plasmid DNA), can further define the *functional* effects of ligand binding to non-B DNA. By demonstrating that ligand-modulated structure formation affects the function and/or enzymatic processing

of the DNA substrate, the putative ligand-non-B DNA interaction and its stabilizing or destabilizing effect(s), can be further validated.

Lastly, cell/genome-based investigations using small molecule ligands have been employed to identify biologically-relevant functions of non-B DNA-forming sequences and to determine their loci in cells [338]. For example, footprinting-based studies have been used to detect non-B DNA structures in the genome [316, 342], and have revealed the presence of non-B DNA in promoter regions of developmental regulatory genes and oncogenes [316], and in human precursor mRNA [342]. Co-localization studies that involved treatment of live cells with G4-DNA-stabilizing ligands such as PDS, TMPyP4, and telomestatin showed increased numbers of G4-DNA-antibody foci, suggesting that either the ligands trapped the G4-DNA structures when they formed [86, 89], or that they induced the formation of G4-DNA. Similarly, telomestatin treatment of Fanconi anemia group J (FANCJ)-depleted chicken DT40 cells further increased the numbers of G4-DNA-antibody foci, which the authors claimed to be supportive of the existence of G4-DNA structures in mammalian cells and their processing by helicases, such as FANCJ [89].

While not intended to investigate mechanistic or functional effects of ligand binding, next generation sequencing (NGS) has been utilized with G4-DNA-specific ligands to investigate the genome-wide distribution of G4-DNA loci [343, 344]. A biotin-tagged Schiff-base catechol derivative was used to crosslink G4-DNA structures in murine melanoma cells [345], after which the G4-DNA-forming sequences were isolated using streptavidin magnetic beads and subjected to NGS. However, out of the 1,294 sequences isolated (mostly from gene promoter regions), only 120 (~10%) were putative G4-DNA forming sequences [345]. Another approach, G4-seq, combined the G4-DNA-stabilizing effects of PDS or PhenDC3 ligands with the polymerase stop assay followed by Illumina NGS [346] to profile G4-DNA structures in purified human genomic DNA [248]. The technique relied on ligand-mediated, G4-induced polymerase stalling, which introduced sequencing errors and mismatches at sequences of G4-DNA start sites. In comparison to the sequencing data obtained under conditions that disfavor G4-DNA formation, the exact position, as well as the putative G4-DNA-forming sequences can be elucidated [346]. G4-seq identified ~700,000 putative G4-DNA-forming sequences in the human genome, which is twice that estimated by standard G4-DNA predictive algorithms [344, 346] and orders of magnitude more than the sites revealed by chromatin immunoprecipitation (ChIP)-sequencing methodologies [283, 347]. A model study involving a cyclic hepta-oxazole compound with a biotin-affinity tag was reported to have isolated G4-DNA forming sequences from a mixture of G4-DNA and non G4-DNA forming sequences [348].

Recent developments in experimental methods of ligand-non-B DNA interactions have involved the use of high-throughput screens (HTS), alone or in combination with other techniques. Screening for triplex ligands from natural plant extracts using peak area-fading ultra high-performance liquid chromatography (HPLC) coupled with Orbitrap mass spectrometry (MS) [349], and a combination of HPLC-MS (ESI) [350] has been reported. An evaluation that involved ESI-MS, tandem mass spectrometry (MS/MS) and molecular modeling was performed on benzopyridoindole and benzopyridoquinoxaline triplex ligands [351]. Rosu *et al.* (2007) utilized molecular modeling data in combination with tandem mass

spectrometry data [which calculated the energy to dissociate 50% (CE50) of the bound ligand], to parse binding interactions of ligands with duplex or triplex DNA [351]. The combined analyses indicated that the ligands intercalated via the minor groove of the Watson-Crick duplex, and the benzopyridoindoles, in particular, interacted with the triplex via the duplex [351].

Thermal melting measurements [333–335] have been a standard method to determine stabilizing or destabilizing effects of ligands on nucleic acids. The low-throughput sampling of this approach has been alleviated through melting mixtures of poly- or oligonucleotides containing the structure-forming sequences of interest [352]. In this approach, addition of the ligand at low molar ratios will shift the thermal melting temperature (T_m) of the preferred structure or sequence [352]. High-throughput FRET-based thermal melting assays using fluorophore-quencher (FQ) combinations [128, 353], have also been described [354–356]. An in-depth testing of the ‘two-state’ model [334, 357], often assumed during thermal melting experiments, was performed by analysis of multidimensional ‘3D’ melting curves for G4-DNA [358]. This 3D-melting involves the recording of the entire spectra (absorbance, CD, and fluorescence) as a function of temperature, as opposed to the common practice of recording temperature-dependent spectral responses at a single wavelength. The 3D melting data was then subjected to analysis using singular value decomposition that led to better characterization of the unfolding of G4-DNA by revealing intermediate states previously unknown [358].

The use of FRET in the context of molecular beacon (MB)-based methodology has been explored for triplex ligand screening with T*AT triads [359]. Inspired by the MB technology and other published assays utilizing fluorophore-quencher (FQ) combinations [353–356, 360, 361], our group has developed a FRET-based assay that can identify both triplex stabilizers and destabilizers, simultaneously, in a facile HTS-compatible approach using a biologically-relevant triplex-forming sequence [362].

Due to their high extinction coefficients, absorption in the 500–700 nm range, and unique aggregate optical properties [363], gold nanoparticles (AuNPs) and their interactions with DNA have been utilized for facile colorimetric assays to screen for triplex-binding ligands. Whether the AuNP was functionalized with DNA [364, 365] or was label-free [363], the assay depended on the visible color change of the AuNP as a result of the ‘aggregation’ concomitant with triplex formation in the presence of a binding ligand.

Recently, the use of chemometric approaches through the application of principal component analysis (PCA) and hierarchical cluster analysis (HCA) on large data sets derived from G4-DNA fluorescent intercalator displacement (FID) assays was reported [366]. PCA and HCA were used as tools to provide information on the often complex relationship between binding affinity and binding selectivity [366].

Lastly, computational studies concerning non-B DNA structures continue to advance, with regards to molecular dynamic simulations of structures, and their thermodynamic stability [367–369]. And as virtual ligand screening still serves as an economical tool to identify

possible ligand candidates, the best approaches and platforms have been reviewed [370], and used in conjunction with an oligo affinity support assay for G4-DNA structures [371].

Challenges and perspectives

Challenges still remain to be addressed in optimizing ligands as tools to study non-B DNA structure formation and function. There is a need to study ligand-binding interactions using biologically-relevant structure-forming sequences as model targets to increase therapeutic relevance and increase the likelihood of an effective biological outcome. Also, there has been considerable progress made in identifying G4-DNA-interacting ligands as exemplified by the development of cell-based and genome-based functional methodologies that employ G4-DNA-interacting molecules in an effort to locate genome-wide G4-DNA loci, and the advance of G4-DNA-specific ligands, such as Quarfloxin/CX3543 [299] and CX5461 [300, 301] to Phase II clinical trials. However, there are fewer examples of Z-DNA- and H-DNA-specific ligands, and additional studies to identify non-B DNA-specific ligands are warranted. With regards to use of experimental methods, there is advantage in incorporating biochemical or *in vitro* functional assays, and when possible, cell and genome-based functional assays in studies to determine non-B DNA-ligand interactions. Consequently, facile and accessible assays to monitor biochemical or cellular responses (e.g. interference with processing of helicases or DNA damage responses) upon treatment with non-B DNA-interacting ligands are warranted. The development of such assays will likely improve as more information regarding the mechanistic processing of non-B DNA is obtained.

Because non-B DNA can stimulate genetic instability, the precise temporal and spatial modulation of a non-B DNA structure and its mutagenic outcome might be achieved by the use of DNA structure-specific small-molecule ligands. Though the majority of non-B DNA-specific small molecule ligands identified to date stabilize the DNA structures, it is also of importance to identify non-B DNA destabilizers. For example, non-B DNA destabilizers may prove useful in preventing genetic instability induced by these mutagenic structures. However, both non-B DNA stabilizers and destabilizers can serve as molecular tools to interrogate mechanisms involved in DNA structure-induced genetic instability, to probe structure formation *in vivo* and thus, have potential toward diagnostic and/or therapeutic applications.

Structure-destabilizing ligands are predicted to be a synthetic approach to decrease or even prevent the genetic instability associated with mutagenic non-B DNA structures. Thus, they may be impactful toward therapeutic approaches to prevent and/or treat disorders arising from DNA structure-induced genetic instability. An example is the recovery of frataxin (FXN) protein levels by destabilization of the triplex structure that inhibited the transcription of the *FXN* gene, characteristic of Friedreich's ataxia [205, 241, 242] as cited in the 'Triplex ligands and biological outcome' section of this review. Destabilization of the triplex was achieved through the use of an oligonucleotide [244], polyamide [245] or a pentamidine ligand [246]. Only a handful of non-B DNA-destabilizing ligands are known and this bottleneck is, in part, due to the shortage of efficient methods to assay for structure-destabilizing molecules. To effectively identify ligands that can destabilize mutagenic non-B DNA structures, the development of additional facile assays or screens is needed.

On the other hand, the use of effective and selective non-B DNA stabilizers will prove useful for mechanistic or diagnostic investigations, by allowing a better understanding of structural features, structure formation, location, and the biological functions of non-B DNA *in vivo*. Further, there are genomic regions that contain multiple and overlapping potential non-B DNA-forming sequences [19], or more than one topology of a particular non-B DNA structure [267]. Direct and conclusive methods to detect specific DNA structures within a region of putative non-B-DNA forming sequences *in vivo* are still lacking. Confounding such complexity is the accessibility limits posed by chromatin organization within the context of chromatin in a cell. Thus, there is a need for tools such as structure-specific fluorescent probes derived from stabilizers that can visualize and report on the conformational dynamics, cellular loci, and processing of non-B DNA structures. However, it should be noted that super resolution microscopy, which can provide better monitoring of biomolecular processes in live cells (compared to other forms of microscopy) [372], is fluorescent-label dependent, and has not yet achieved the resolution to visualize individual/monomeric non-B DNA structures [323]. Its current resolution is at 10–70 nm [373], which still needs improvement to resolve single-molecule non-B DNA structures. Thus, even in the case of a specific non-B DNA structure ligand, only large domains with a high density of non-B DNA structures can be imaged by microscopy [323]. Under what conditions such domains exist in the context of chromatin in a cell remain to be elucidated. As such, ligand-induced staining, which has been attempted for G4-DNA structures (see G4-DNA Probes discussion above), needs independent validation for the sequences and loci of non-B DNA structure formation [323]. To observe conformational dynamics of non-B DNA formation *in vivo*, parallel developments in super-resolution imaging, in conjunction with improved small molecule fluorophores, is required. Thus, in the search for non-B DNA ligands, consideration should be given to the features of ligand/fluorophores that would be compatible with or further improve the current limit of super-resolution imaging. For example, bright, photoswitchable (or photoactivatable), photon-emissive, photostable fluorophores [373–375], should be considered, in addition to the minimum ligand requirements such as non-B DNA selectivity (versus canonical B-DNA and other biomolecules), high affinity, and biocompatibility.

Another concern with the use of ligands as biological probes is their propensity to *induce* (versus detect) DNA structure-formation. Until new methods become available to monitor DNA conformation without impacting the DNA structure, researchers should carry this potential bias in mind to avoid over-interpretation of the results obtained.

With the involvement of non-B DNA structures in various biological processes and genetic instability, they may be considered as potential druggable targets, but how can non-B DNA structures be *validated* as such targets? Unlike proteins, non-B DNA structures do not have defined ‘active sites’ nor particular enzymatic functions. Instead, they contain unique structural features arising from known structure-forming sequences, as well as mutagenic potential as a result of error-prone processing. More information is being obtained with respect to their recognition and processing. As the field moves toward unraveling the mechanistic processing of non-B DNA, as well as their *in vivo* loci, the information provided will be invaluable in exploring non-B DNA structures as valid targets for the prevention and/or treatment of disease.

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Highlights:

- Genomic mutations co-localize with sequences that can form non-B DNA structures.
- Non-B DNA impacts transcription, replication, and genetic instability.
- Small molecules can be used as tools to study non-B DNA structure formation.
- Stabilizers/destabilizers of DNA structure, assays and applications are discussed.

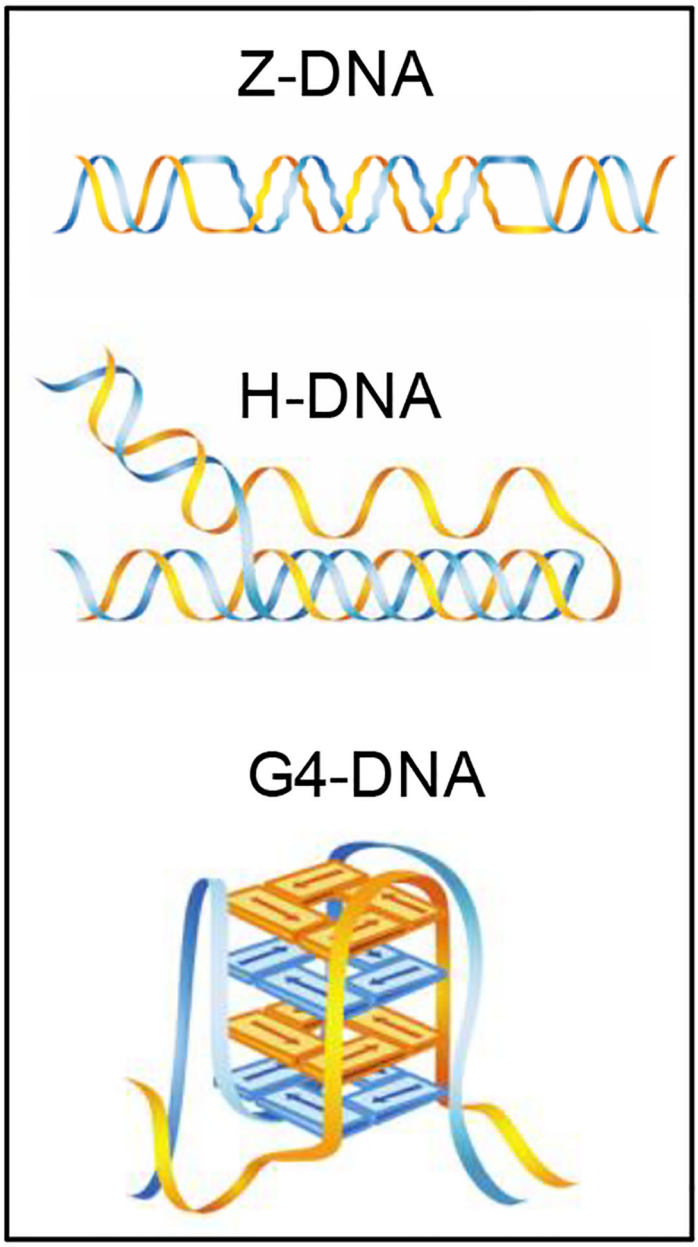

Figure 1.
Non-B DNA structures discussed in this review. Figure adapted with permission from Zhao, *et al.*, Nature Springer, *CMLS*, 2010 [7].

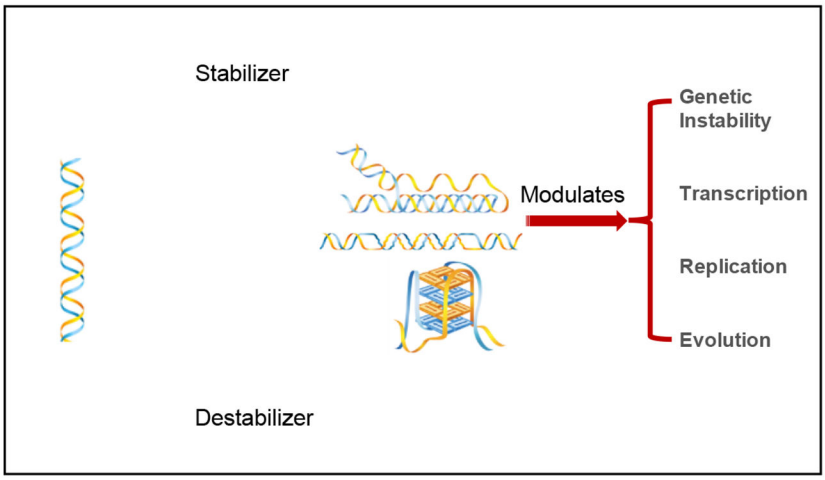

Figure 2.
Modulating DNA structure-related metabolism using small molecules.

Table 1.

Various methods to detect and evaluate non-B DNA-ligand interactions.

Analysis of *in vitro* binding	**Information on ligand-DNA binding interactions**
Spectroscopy	
UV-Vis	Concentration dependence/titration: affinity, stoichiometry Temperature dependence/thermal melting: stabilizing/destabilzing effect
Fluorescence	Concentration dependence/titration: affinity, stoichiometry Temperature dependence/thermal melting: stabilizing/destabilzing effect Competition/fluorescent intercalator displacement (FID): relative affinity, structural specificity (*vs.* competing non-B DNA structures), structure-related sequence preference (*vs.* other structure-forming sequences) Förster resonance energy transfer (FRET) with thermal melting: stability Quenching experiments: mode of binding
Circular/Linear Dichroism (CD/LD)	Comparison of CD/LD signatures: structural/topological changes, mode of binding Temperature dependence/thermal melting: stability
Nuclear Magnetic Resonance (NMR)	3D Structure, structural/topological changes, mode of binding
Surface Plasmon Resonance (SPR)	Concentration dependence/titration: affinity, stoichiometry, kinetics of binding
Electrospray ionization-mass spectrometry (ESI-MS)	Stoichiometry, relative binding affinity, relative abundance of ligand-DNA complexes
Viscosity/hydrodynamic measurement	Mode of binding
Isothermal titration calorimetry (ITC)	Binding energetics, stoichiometry
Electrophoretic mobility-shift assay (EMSA)	Stabilizing effect on a destabilized structure, binding affinity, specificity, kinetics of binding
Footprinting assay	Structural/topological changes, mode of binding
Competition equilibrium dialysis	Structural specificity (*vs.* competing non-B DNA structures), structure-related sequence preference (*vs.* other structure-forming sequences)
Capillary electrophoresis	Relative binding affinity
X-ray crystallography	3D Structure, structural/topological changes, mode of binding
Atomic force microscopy/optical tweezers	Stability, structure
***In vitro* functional assays**	**Information on ligand-DNA binding interactions**
DNA polymerase stop assay	Efficacy of ligand stabilizing (or destabilizing) effect that stalls (or improves) enzymatic processing of substrate
Reverse transcriptase stalling (RTS) stop assay	Efficacy of ligand stabilizing (or destabilizing) effect that stalls (or improves) enzymatic processing of substrate
G4 TRAP assay	Efficacy of ligand stabilizing (or destabilizing) effect that stalls (or improves) enzymatic processing of substrate
DNA polymerase extension assay	Efficacy of ligand stabilizing (or destabilizing) effect that stalls (or improves) enzymatic processing of substrate
Replication/transcription arrest assay	Efficacy of ligand stabilizing (or destabilizing) effect that stalls (or improves) enzymatic processing of substrate
Cell/genome-based functional assays	**Information on ligand-DNA binding interactions**
Footprinting assay	Structural/topological changes, binding loci
Co-localization assay	Visualization of non-B DNA-ligand loci, specificity of loci