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Binding and beyond: what else can G-quadruplex ligands do?

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Abstract: G-quadruplexes (G4) are four-stranded structures formed from guanine-rich oligonucleotides. Their defined 3D structures and polymorphic nature set them apart from classical nucleic acid morphology and suggest a range of potential applications in the development of functional materials. Meanwhile, the occurrence of G4 across the genomes of animals, plants and pathogens suggests roles for these structures in biology that may be exploited for therapeutic effect. Hundreds of G4 ligands are reported to bind these sequences with high specificity and affinity, but such ligands can also be engineered to do more than simply associate with G4 in a straightforward host-guest fashion. Ligands have been developed that can switch G4 topology, direct the selective covalent modification of nucleic acid structures, or respond to external stimuli to permit spatiotemoral control of their activity. Herein we survey the main themes of such "value-added" G4 ligands and consider the opportunities and challenges of their potential applications.

1. Introduction

Nucleic acids are well known for their ability to form a wide range of higher-order structures,[1–3] a property that allows these molecules to fulfil a wide range of fascinating and important functions. In biology, the DNA double helix provides the means of information storage for the whole of life.[4] Meanwhile, RNA is the basis of one of nature's most impressive machines, the ribosome, that so elegantly translates structure to function.[5] G-quadruplexes (G4) belong to this fascinating array of nucleic acid structures, having distinct properties of their own that have piqued interest across scientific disciplines interested in their chemistry, biology and applications.[6]

1.1 G-quadruplexes: structure and history

G4s are four-stranded structures formed from guanine rich nucleic acid sequences.[7] The underlying motif of this structure is the G-tetrad, formed from the self-association of four guanine residues in a square planar arrangement, stabilized by Hoogsteen hydrogen bonding and coordination to a central metal cation. The folding of the underlying oligonucleotide strand causes the

stacking of these tetrads to form the quadruplex (Figure 1). Notably, these structures exhibit polymorphism, with the folding conditions and the nature of the intervening loop sequences determining the overall topology. For example, the human telomeric G4 sequence (based on TTAGGG repeats) is observed to form parallel, antiparallel and hybrid conformations depending on the folding conditions, e.g. the buffer ionic strength, the identity of the metal cation, or the presence or absence of crowding agents.[8–10] Such structures can be elucidated by a variety of methods, though a particularly distinctive feature is the circular dichroism signature of the different types (Figure 1).[11] In each case, diagnostic bands arise from the *syn/anti* conformations of

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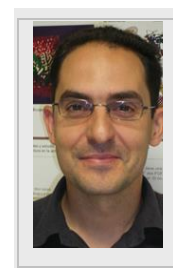
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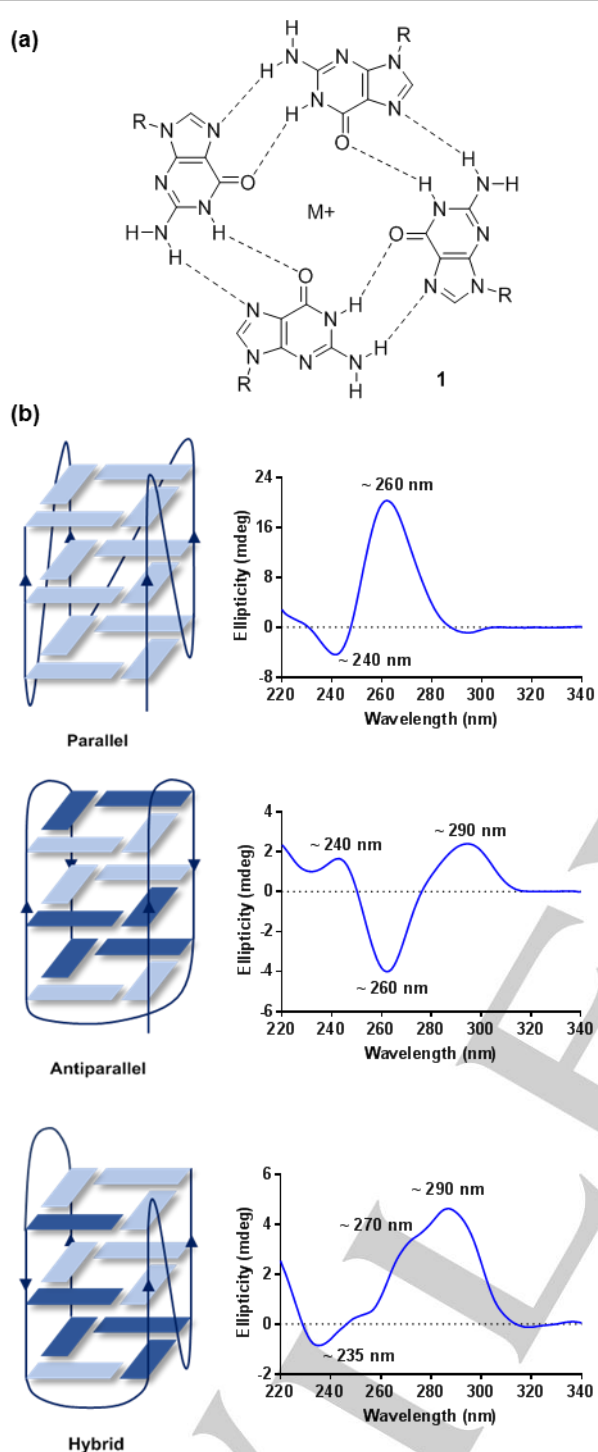
Figure 1: (a) Chemical structure of a G-tetrad (**1**) showing Hoogsteen hydrogen bonds between four guanine residues. The sugar-phosphate backbone is denoted R. (b) three topologies adopted by G4 nucleic acids (left) and their respective circular dichroism signatures (right). The central cations have been omitted for clarity. Color code of guanosine glycosidic bond conformations: pale blue = *anti*; dark blue = *syn*. CD spectra were collected by the authors using model G4 sequences in appropriate buffer.[46]

the glycosidic bond angles in the chiral structure. In the parallel structure all guanosines adopt an *anti* conformation whilst the antiparallel and hybrid topologies feature *syn* conformations at certain residues. Antiparallel G4s may be further divided into two types: the basket conformation (as in Figure 1b) characterized by two lateral loops (top tetrad) and one diagonal loop (lower tetrad). The alternative chair conformation contains three lateral loops.[12] A detailed examination of G4 polymorphism is beyond the scope of this article, but the interested reader is directed to many excellent reviews and studies on the topic.[7,11,13–17]

Clues to the existence of G4 were first uncovered during the 1970s and 1980s, when X-ray diffraction demonstrated the formation of helical structures from self-assembly of polyguanylic acid,[18,19] and Sen and Gilbert observed the self-association of G-rich DNA into four-stranded structures in monovalent salt buffer.[20] Even so, it was not until detailed structures were solved unambiguously by X-ray crystallography[21] and NMR[22,23] in the early 1990s that the field of G4 research took off in earnest. Since then, an explosion of interest in the structure and function of G-quadruplexes can be observed throughout the scientific literature which has been aided by the exponential progress in technological developments over the last two decades. Advances in structural biology, have allowed the intricacies of these folded structures to be explored in exquisite detail in solution[24–27] and even in live cells.[28] The maturity of bioinformatics allows the mining of whole genomes to locate potential G4-forming sequences, identifying hundreds of thousands of occurrences in the genomes of humans and other organisms,[29–32] whilst cutting-edge sequencing methods have validated the existence of such quadruplexes experimentally in a number of species.[33] Meanwhile, progress in biology has delivered ever increasing evidence for the role of these sequences in life, from organism development, through to epigenetic regulation and their role in health and disease.[34,35] Indeed, various therapeutic hypotheses have been proposed, such as targeting G4s to inhibit the transcription of oncogenes[36,37] (Figure 2) or by targeting the G4 sequence in chromosome telomeres.[38] But this does not confine interest in G4 to the biological community alone; the precise folding of such structures into well-defined shapes and their polymorphism, that can be controlled by different conditions and stimuli, point to exciting applications of these structures away from the biological milieu in the development of new responsive enzymes,[39–42] switches,[43,44] and functional materials.[45]

1.2. G4 as nanodevices

The polymorphic secondary structure of both the G4 and its sister strand, the cytosine-rich i-motif,[3] suggests a range of applications of these structures in the development of functional materials. Many strategies for exerting control of nucleic acid secondary structure have been explored. Systems have been designed to be responsive to a variety of stimuli including light,[47] pH,[48] redox processes[49,50] and the addition and sequestration of metal ions.[39] In particular, the sensitivity of the G4 to the presence of metal ions and the i-motif to pH have been exploited many times as the basis for the design of DNA derived switches, and progress in this area has been extensively reviewed.[43,44,51,52] In Section 2, we focus instead on the use of small molecule
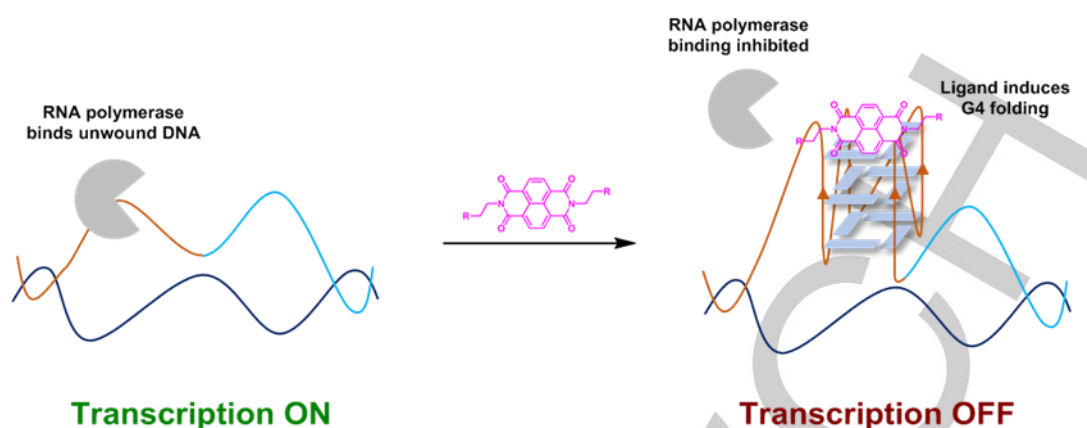
Figure 2: One of several possible mechanisms of oncogene suppression by targeting G4 DNA with small molecules. Induction of the G4 fold in the gene promoter by the stabilizing ligand inhibits the binding of RNA polymerase and transcription factors leading to downstream silencing of the gene. The folded G4 structure may also interfere with transcription factor binding, and examples of G4-targeting molecules that up-regulate certain genes are also known.[37]

ligands as triggers to control G4 structure and function. Towards the development of nanodevices, ligands are an interesting means to not only control the formation of G4, but also to toggle G4s between different conformations.[53] Since such small molecules can themselves be designed to be stimuli-responsive, they may themselves serve as fuels for the reversible manipulation of G4 systems.[54,55] In contrast to engineering the responsive functionality into the nucleic acid itself, an approach frequently adopted for the reversible control of biomolecules,[56] such supramolecular approaches have the advantage that pre-modification of the oligonucleotide is not necessary, allowing possible applications in situations, such as medicine, where this is not desirable or possible. In taking this approach, we do not mean to divert attention from the impressive body of work that achieves G4 regulation by the pre-incorporation of unnatural functionality, but we aim to provide a complementary picture and highlight the potential of ligand-driven approaches for regulation of G4.

1.3. G4 as therapeutic targets

The significant level of interest in G4 partly stems from its potential as a therapeutic target.[36,38,57–59] Indeed, G4-forming DNA and RNA sequences are not confined to the human genome but also observed in a host of other organisms including plants,[60] bacteria,[61] viruses[62–64] and parasites.[65–67] This provides many potential opportunities to exploit G4 for the development of new therapeutic strategies. Indeed, our own research groups and many others have reported work driven towards the identification of new G4 ligands as the basis of anticancer[68,69] and antiparasitic[65,70] therapies. The vast majority of G4 ligands employ rigid aromatic heterocyclic frameworks that are designed to π-stack onto the large surface of the G-tetrads.[71] Cationic character (for aqueous solubility and electrostatic interactions with negatively charged nucleic acids) is often introduced by conjugation of these cores to basic amino residues, to quaternized ammonium or pyridinium moieties, or to transition metal cations. Though the majority of G4 ligands contain this design feature, there are notable exceptions that are not cationic, such as the neutral macrocycle telomestatin (see Section 2.3) or N-methyl mesoporphyrin IX (see Section 2.2) which is negatively charged. Ligands may also be designed to target the G4 loops or grooves as well as the planar tetrads. Chemical structures and associated activity data for approximately one thousand ligands are currently curated in the online G4 ligand database, and new chemotypes are frequently reported.[72]. Progress towards the goal of G4-targeted therapy is frequently documented and it is not our intention to cover general G4 ligand design principles in this review, since they are covered comprehensively elsewhere.[58,71,73–79] However, over the course of our endeavors in G4 ligand chemistry we have become more deeply interested in the potential of ligands to do more than simply bind G4 with the aim of enhancing their stability or extending their lifetime *in vivo*. Indeed, the field has recently taken steps towards engineering G4 ligands that exert different kinds of control on G4 that could be exploited toward biological and therapeutic ends. Such approaches harness the selectivity of G4-binding chemotypes to trigger a further molecular event, such as modification of the G4 by alkylation[80] or oxidation,[81] or as the basis for the localized generation of singlet-oxygen by photosensitization.[82] Furthermore, a small number of responsive G4 ligands have also been described. In these cases, the activity of the ligand is unlocked in response to a stimulus, such as photoirradiation[83] or the redox environment.[84] Such approaches allow an additional level of spatiotemporal control over the system, and therefore potential to act as starting points for a new generation of smart drugs where activity can be controlled with greater precision to minimize off-target side effects.[85,86] In Sections 3 and 4 we aim to provide a snapshot of progress in these exciting areas of G4 ligand development.

1.4. Conclusions

The two applications discussed above, namely exploiting G4 in the development of new nanodevices and therapies, are united by a common theme: the need to *exert control* on the formation of the G4 structure and the potential of ligands to afford such control. In both cases, ligands that induce effects in G4 beyond simply binding to the native structure show potential to control G4 in interesting ways, particularly in cases where the activity of such ligands can itself be regulated by an external stimulus. In this report, we aim to provide a critical examination of developments in these areas to spark further interest in the development of such "value-added" G4 ligands. Our aim is to focus more on the different effects of the ligands, rather than to provide a comprehensive almanac of structures, biophysical results and thermodynamic data (though such data are quoted when appropriate). This is not to understate the importance of these parameters in G4 ligand design, and the interested reader will be able to find full details in the cited publications. But we hope that our account will help provide an overall picture of the state-of-the-art in this area and will allow more general considerations and effects to be reviewed in a holistic fashion. Several G4 sequences are referred to throughout the review, commonly employed as model systems by researchers investigating G4 ligand binding; those that feature in the text are shown in Table 1.

2. Ligand-driven switching of G4 folding – a pathway to novel nanodevices?

2.1. Introduction

The possibility of exploiting the self-assembly properties of nucleic acid structures as the basis of nanodevices has been around for some time, with pioneering examples exploiting hybridization strategies to exert control in DNA conformation employing complementary oligonucleotides as fuel.[88–90] In 2003, Alberti and Mergny reported the first example of a G4-based nanomachine, which relies on such a hybridization strategy.[87] A reversible folding/unfolding G4 device was produced that could be switched over eleven cycles without observable photofatigue

Table 1: Model G4 sequences referred to in this review

G4 model	Sequence
telo21	d[GGGTTAGGGTTAGGGTTAGGG]
telo22	d[AGGGTTAGGGTTAGGGTTAGGG]
telo23	d[TAGGGTTAGGGTTAGGGTTAGGG]
telo24	d[TTAGGGTTAGGGTTAGGGTTAGGG]
bcl-2	d[GGGCGCGGGAGGAATTGGGCGGG]
c-kit 1	d[GGGAGGGCGCTGGGAGGAGGG]
c-kit 2	d[GGGCGGGCGCGAGGGAGGGG]
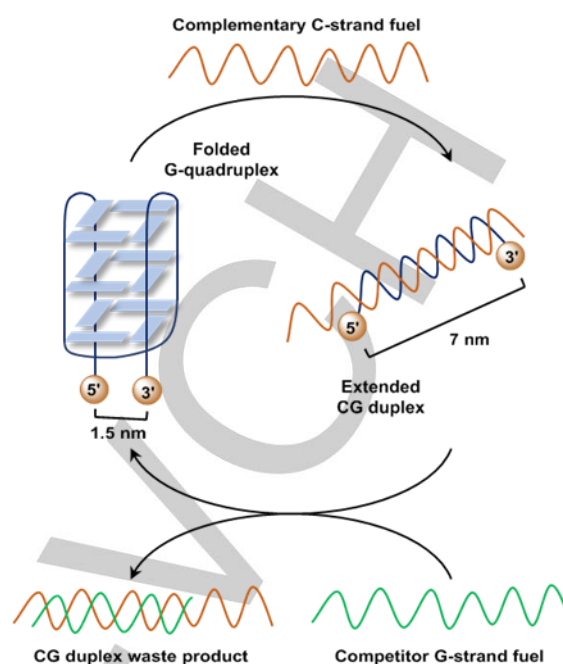
Figure 3: The G4-derived nanomachine developed by Alberti and Mergny. Addition of the C-strand fuel drives the unfolding of the G4 to allow hybridization to form the CG duplex. The process is reversed by adding G-strand fuel which displaced the G4-forming sequence from the C-strand to regenerate the original folded G4 and the CG duplex as a waste product.[87]

by addition of appropriate complementary DNA strands. The effective diameter of the G4 sequence changes from 1.5 nm in the folded state to 7 nm in the single stranded form (Figure 3). Such properties could possibly be exploited to position molecular cargo or exert force at the molecular level. However, the need for repeated additions of two different DNA fuels and the generation of the CG waste product are drawbacks of this approach. More recently, systems have been developed that circumvent the need for a chemical fuel by incorporation of responsive functionality in the DNA sequence itself, permitting the stimuli-driven changes in folding without need for external fuel. Ogasawara and Maeda have reported the incorporation of a photoresponsive nucleobase into an oligonucleotide sequence that allows the reversible folding and unfolding of G4 triggered by different wavelengths of UV-light.[91] More recently, the Heckel group have demonstrated the photoresponsive formation of an intermolecular G-quadruplex by incorporation of azobenzene functionality into the backbone of guanosine tetramers.[92] The Tucker group have reported a *bis*-anthracene-functionalised G4 thrombin-binding aptamer whereby photocrosslinking of the anthracene moieties can control the binding, and therefore activity, of thrombin.[93] Though many exciting applications of these elegant approaches to regulating G4 structure can be envisaged, the drawback in these cases is the need to rely on pre-incorporation of unnatural functionality into the oligonucleotide. Complementary applications, where such a modification of the underlying DNA sequence is undesirable, could be achieved by supramolecular ligand-driven approaches.[47] Since such ligands can be themselves designed
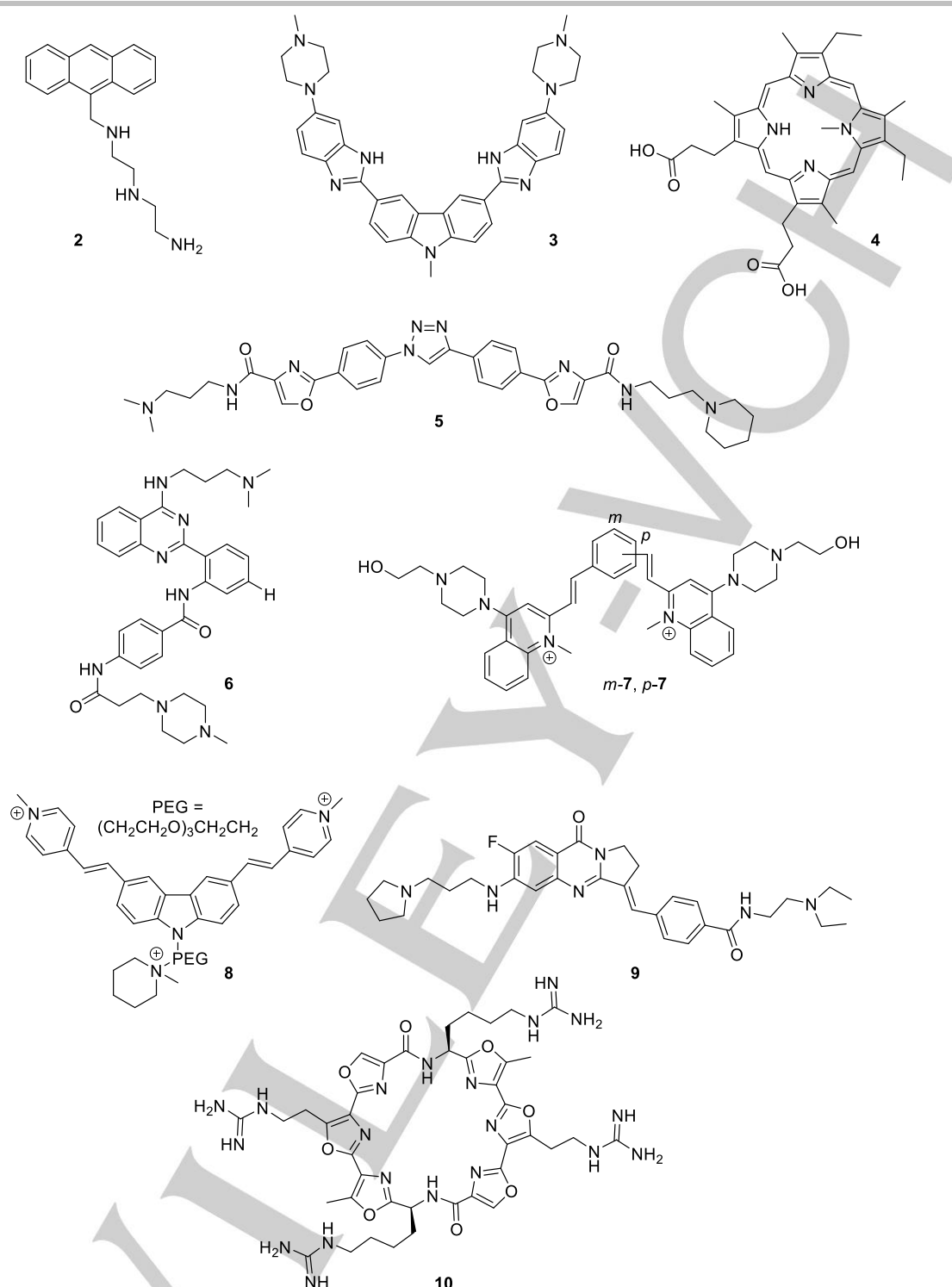
Figure 4: Ligands reported to induce switches to parallel G4 topology from antiparallel or hybrid structures

to be stimuli responsive, their effects can be controlled by a variety of triggers, affording additional levels of spatiotemporal control likely to be desirable in the development of functional materials.

As mentioned previously, we have chosen to focus specifically on small molecule ligands as G4 switching triggers. For a comprehensive review on other supramolecular triggers as the basis of G4 nucleic acids the reader is directed to several previous works.[43,44,51,52] For the most part, we have focused specifically on molecules that induce switches in pre-formed G4 topology in the presence of metal ions, rather than transitions between G4 and
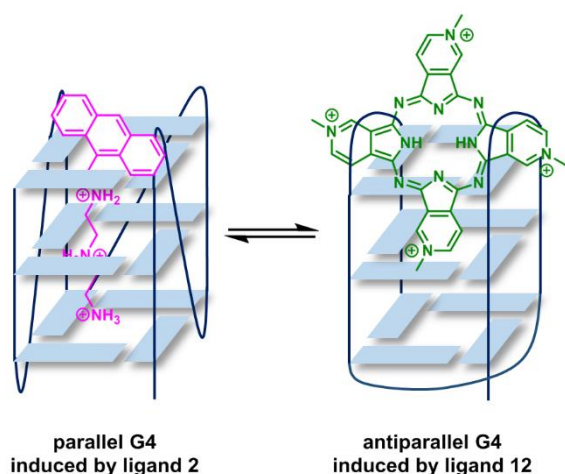
Figure 5: The reversible switching of telo24 DNA between parallel and antiparallel folds with anthracene derivative **2** and porphyrazine **12**.[94]

single stranded nucleic acids in metal-free conditions. Examples of the former appear to be more elusive, perhaps because of the additional barrier to override the conformational preference exerted by the metal cation. However, the latter is more relevant for applications in physical conditions, where the concentration of metal ions cannot be readily controlled. Given the great number of G4 ligands reported to date, we have chosen to provide a conceptual overview, focusing on key examples that switch G4 conformations between the three distinct types – parallel, hybrid and antiparallel. While there are no doubt more ligands than the ones we have highlighted that can induce such conformational remodelling, we believe the examples we have identified reflect the main concepts.
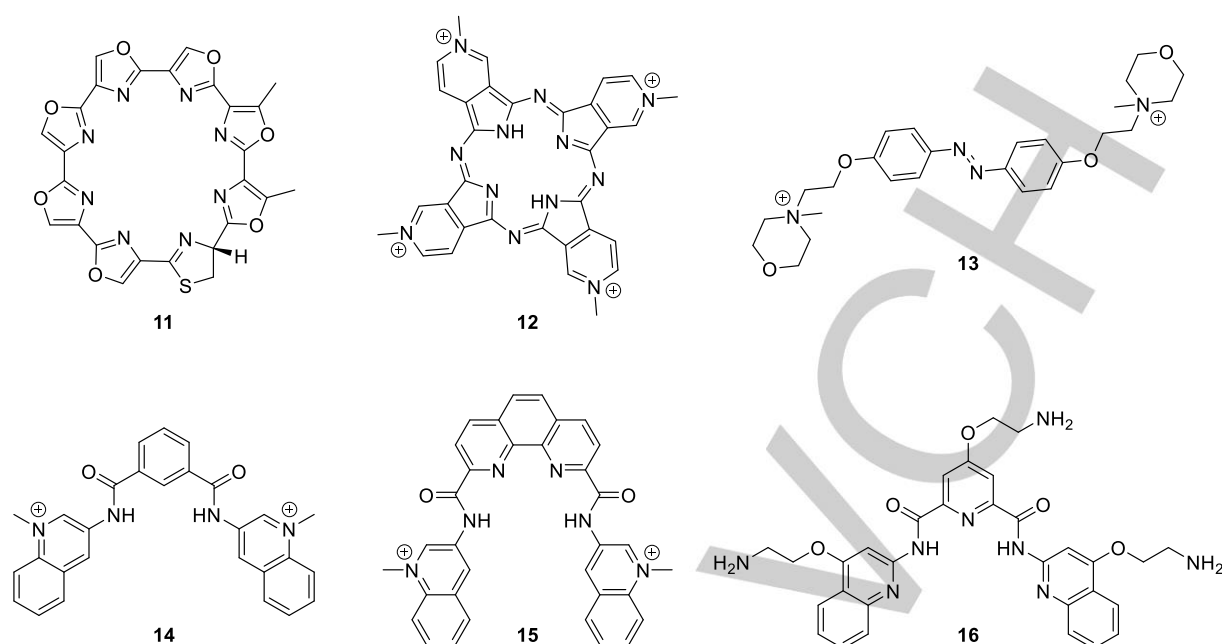
2.2. Ligands that induces switches to parallel G4 folding

One of the first examples of a ligand-driven conformational remodelling of G4 DNA was reported by Balasubramanian and co-workers in 2007.[94] Triamino-anthracene derivative **2** (Figure 4) was designed to bind G4 by simultaneous π-π stacking interactions with the surface G-tetrads and threading of the side-chain through the central channel of the G4 (Figure 5). The spacing of the amine groups was chosen to mimic the distance between K^+ ions in the native G4, allowing the amine moieties to act as the channel cations when protonated at physiological pH. Interestingly, this ligand induces a conformational switch of the telo24 G4 from an antiparallel structure in sodium buffer to a parallel form (monitored by distinctive changes in the circular dichroism spectrum) in about two hours. Furthermore, since the previously reported porphyrazine ligand **12** (Figure 7, see Section 2.3) preferentially stabilises the antiparallel form of telo24, the G4 conformation could be toggled between antiparallel and parallel forms by sequential addition of the two ligands. However, the conformational switch was only reversed once in this manner; possibly further cycles were inhibited by an overaccumulation of the ligand fuel.

Over the following decade, several further chemotypes capable of over-riding topological preference in favor of parallel G4 folds have been identified. For example, carbazole ligand BPBC (**3**, Figure 4) appears to induce telo22 to adopt a parallel fold in potassium buffer, rather than the usual hybrid-type G4 formed in such conditions.[95] For this effect to be observed however, it was necessary to co-anneal the ligand/DNA system (by thermal denaturation followed by cooling). Little conformational change was observed when the ligand was added to the pre-annealed structure as was observed for anthracene **2**. In this report however, the overall goal was the development of a fluorescent probe rather than the conformational switching of G4, with the conformational preference of the ligand appearing to be incidental rather than by design. Similarly, N-methyl mesoporphyrin IX (**4**, Figure 4), is able to induce the parallel structure in telo22 (amongst other telomeric sequences) when the sequence is annealed in the presence of ligand in potassium buffer, but not in sodium (antiparallel G4) or lithium (unfolded G4) conditions.[96] The same transition is observed if the ligand is added after the annealing step, though the process takes significantly longer (30h after annealing versus 12h prior to annealing). Cousins, Searle *et al.* disclosed DR4-47 (**5**, Figure 4), an oxazole-based ligand that strongly induces a parallel G4 signature in telo22 under potassium conditions when up to 5 equivalents of ligand are titrated into the pre-formed hybrid G4 without the need to re-anneal the DNA/ligand complex.[97] The same ligand also induces a striking switch from antiparallel to parallel G4 in Na^+ buffer (Figure 6), observed by attenuation of the positive features at 240 nm and 260 nm, and the emergence of a strong positive
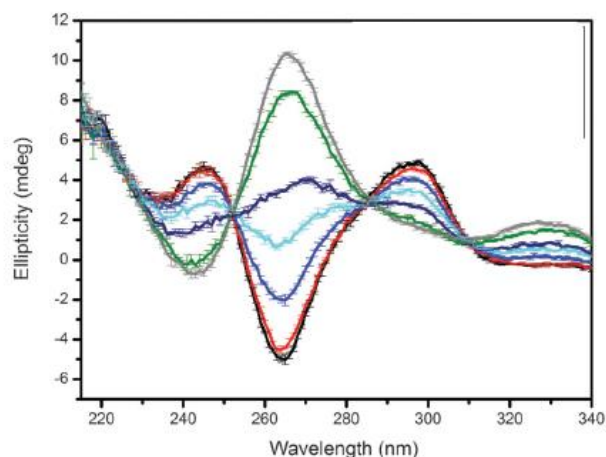
Figure 6: Circular dichroism spectral changes observed on titration of telo22 in sodium phosphate buffer with DR4-47 (**5**) showing apparent switch from the original antiparallel (black trace) to parallel topology. On adding increasing equivalents of ligand, the negative band at 260nm gradually disappears (red and blue traces) before a positive band at 265 nm (green) corresponding to the parallel fold appears. The final spectrum (grey) corresponds to addition of 5 equiv. ligand. Reproduced with permission from The Royal Society of Chemistry.[97]
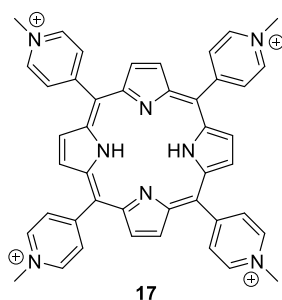
Figure 7: Ligands reported to induce switches to antiparallel G4 topology from parallel or hybrid structures.

band at 265 nm. Though the degree of conformational perturbation at equilibrium is impressive, kinetic studies (that would provide insight into the time required for the switch to be observed) were not reported. Meanwhile, the Huang group identified an interesting structure-activity relationship in a series of quinazoline derivatives. Ligands of type **6** (Figure 4) were capable of inducing shifts towards a parallel topology in potassium conditions, whilst ligands lacking the southern benzene ring were not capable of exerting such effects.[98] Further structural studies would be welcome in this case to understand the origins of the structural perturbation. Similarly, the *para* isomer of BQ-OHEtP (*p*-**7**) developed by the same group could induce antiparallel structure of telo21 to form the parallel structure in the presence of sodium ions, but the *meta* isomer (*m*-**7**) did not have this activity.[99]

Though the systems discussed above are of interest, the ability of the ligands to induce conformational switches in G4 DNA appears to have been discovered serendipitously in these cases, and the thermodynamic driving forces and mechanisms of the remodeling pathways are not fully understood. Furthermore, the new G4 topology in these earlier studies is assigned primarily from circular dichroism data. Whilst the changes observed are consistent with the diagnostic features of parallel G4s (i.e. a positive band at ~ 265 nm and negative band at ~ 240 nm),[11] this is not conclusive proof that a new topology has been generated. Indeed, the telo23 G4 has recently been shown to adopt the same hybrid G4 fold in both potassium and sodium conditions, despite significant changes in the respective CD spectra that are suggestive of different folding topologies.[100] Whilst comparison to reference spectra can be seemingly convincing, interpretation of CD data of G4/ligand complexes is complicated by the often overlapping absorbance spectra of the G4 and the ligand, meaning that induced dichroism in the ligand spectrum (by binding to the chiral

environment of the DNA) may perturb the apparent spectral signature of the nucleic acid structure. Care must therefore be taken before attributing a new folding topology to a G4 on the basis of CD data. These issues are addressed in work reported more recently by Wang and Chang, who employed a more rational approach towards the development of a ligand that induces a parallel G4 against the bias of the other folding conditions.[101] Here, the well-documented effect of polyethylene glycol (PEG) to induce parallel G4 folding, by molecular crowding effects, was applied in the design of a G4 ligand capable of exerting structural changes. A PEG moiety was conjugated to a G4-binding carbazole core bearing positively-charged pyridinium groups to create the ligand known as BMVC-8C3O (**8**, Figure 4), thereby translating the known solvent effect of PEG into a local ligand effect. The authors found that the ligand promotes a local dehydration effect induced by the close proximity of PEG to the G4 on ligand binding, disrupting the solvation of the G4 and favoring the parallel fold. Whilst no changes appeared to be induced in telo24 by ligand **8** at 25 °C, at physiological temperature (37 °C) the ligand induces conformational remodeling to the parallel structure in potassium buffer at the micromolar level in a matter of hours. As in previous examples, the changes detected by CD spectroscopy are convincing, but the authors provided further confirmation of their results through a more detailed structural study by NMR spectroscopy which proved the induction of parallel G4 by ligand **8**.[102] The mechanism of interconversion was also investigated, with hydrogen-deuterium exchange studies demonstrating a local rearrangement took place rather than global unfolding of the quadruplex. More recently, Tan *et al.* have discovered that isaindigotone derivatives (e.g. **9**, Figure 4) can induce parallel G4 folding in the telo21 sequence in potassium buffer.[103] Though the initial discovery appears to have been unexpected, the group

Figure 8: Porphyrin ligand TMPyP4, reported to induce switches to hybrid and antiparallel G4 structures and also unfold certain G4 DNA and RNA structures.

embarked upon a computational study to generate a pharmacophore model that predicts the ability of such derivatives to induce such conformational changes. Through this approach, a training set of twenty two derivatives from the group's compound library generated a model that identified ten further putative hits from the wider library of over 5000 compounds. Pleasingly, all ten compounds were found to induce significant remodeling of telo21 G4 to the parallel form in K+ rich conditions. Towards a similar end Ma, Nagasawa and co-workers recently employed a docking approach to identify compounds that bind more strongly to parallel form of G4, rather than the antiparallel form of the same sequence, with the aim of identifying ligands able to induce the parallel G4 fold against the inherent presence of the metal cation.[104] The team began with previously reported macrocyclic hexaconazole cores, known to strongly stabilize G4, and modified them to incorporate four side chains to target the four grooves of parallel G4. The lead compound (**10**, Figure 4) was demonstrated by CD spectroscopy to induce the parallel G4 fold in telo24, *bcl-2* and a thrombin aptamer in either sodium or potassium conditions. The switching process appears to be rather slow, requiring overnight incubation for the effect to be demonstrated with 5 equivalents of ligand, a factor that probably requires optimization before real applications can be realized.

## 2.3. Ligands that induce switches to antiparallel or hybrid G4 folding

In comparison to ligand-induced folding to parallel G4 structures, examples of ligands that drive switches to antiparallel or hybrid structures are seemingly rare. In 2005, the Hurley group reported that that telomestatin (**11**, Figure 7), able to induce the conversion of hybrid G4 to the antiparallel form in potassium buffer.[105] This appears to proceed without the need to re-anneal the DNA sequence in the presence of ligand. The porphyrazine derivative reported by Sanders (**12**, Figure 7) was previously introduced in Section 2.2 as a means to reverse the activity of anthracene **2** to regenerate an antiparallel G4 structure in telo24 DNA. Indeed, in a separate study, the ligand was shown to induce the formation of antiparallel G4 in the presence of potassium ions at 20 °C.[106] A similar effect was observed for azobenzene derivative **13** (see also Section 2.5) but again it is not clear how long this ligand takes to exert the conformational change.[107]

As mentioned in Section 2.2, care must be taken to avoid overinterpreting the CD data without more detailed structural information to validate these conformational switches. As well as NMR-based methods, mass spectrometry offers a complementary means of probing ligand/G4 complexes, since ligand and cation binding stoichiometries can be measured simultaneously. Gabelica and co-workers used these methods to identify that classical G4 ligands 360A (**14**), PhenDC3 (**15**), and pyridostatin (**16**, Figure 7) induce changes in G-quadruplex folding topology.[108] All three ligands exerted changes in cation stoichiometry in telo22, telo23, telo24, telo26 DNA, observed as a shift from two to one K+ cation per G4, implying the presence of only two contiguous G-tetrads. CD results added further insight to these observations, revealing spectral features consistent with the emergence of an antiparallel folded structure upon ligand binding.

Induction of hybrid G4 folding in metal-rich conditions appears to be even more elusive. Gray, Li and Chaires reported that porphyrin derivative TMPyP4 (**17,** Figure 8), a widely studied G4 ligand despite its poor selectivity for G4 over duplex DNA,[71] could induce the switching of telomeric DNA from basket to hybrid in sodium conditions as monitored through CD, though the switch was not driven to completion.[109] Meanwhile, the same ligand was shown to prompt changes in the CD spectrum of telo24 corresponding to induction of an antiparallel topology (from the hybrid topology) in K+ buffer by Zhang *et al.*[110]
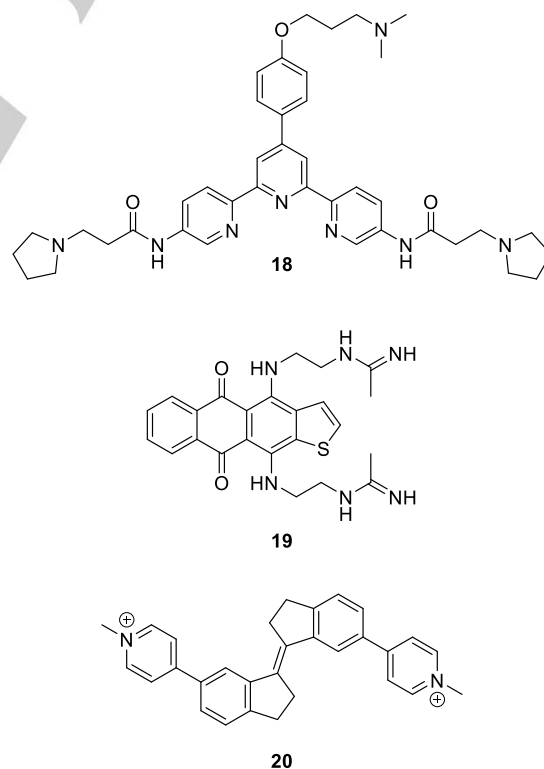
Figure 9: Ligands reported to induce unfolding of G4 structures, see also Figure 8.
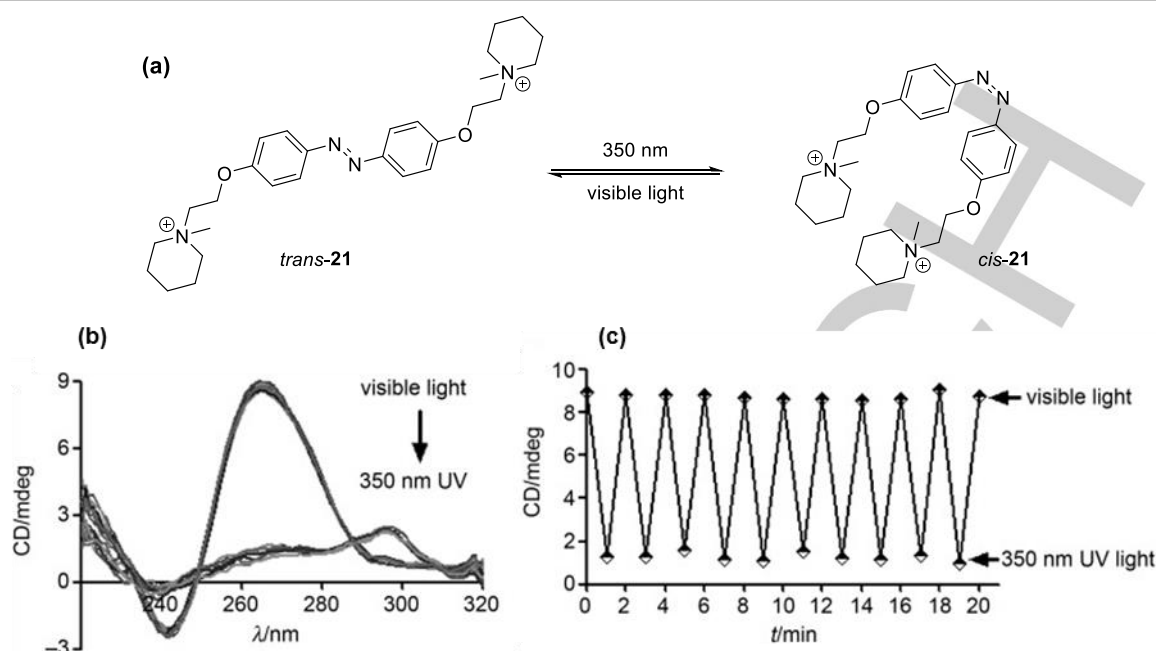
Figure 10: (a) Reversible *trans-cis* photoisomerization of azobenzene-derived G4 ligands (b) photoinduced folding and unfolding of telo24 G4 monitored by CD (c) evidence of robust photoswitching of G4 folding by ligand **21** over several cycles by monitoring ellipticity at 265 nm. Reproduced with permission.[54] Copyright 2010, Wiley.

## 2.4. Ligands that disrupt G4 folding

Each of the ligands discussed above, based on the data reported, appear to cause changes to G4 topology whilst preserving the overall quadruplex structure. A final class of structures warrants discussion at this point: ligands that destabilise G4 folding and cause a denaturation of the structure. Again, these ligands appear to have been discovered serendipitously, and more rational approaches towards molecules that can exert these effects would be a welcome addition to the G4-ligand literature.

The previously-mentioned cationic porphyrin TMPyP4 (**17**, Figure 8) was discovered to disrupt G4 structure in a bimolecular quadruplex formed by the Fragile X FMR1 gene containing d(CGG) repeats. Indeed, the ligand decreased the meting temperature of the d(CGG)$_7$ quadruplex by 14 °C at a concentration of 0.3 µM.[111] More recently, Basu and co-workers demonstrated, by NMR and CD spectroscopy, that the same ligand unfolds the otherwise stable G4 found in the MT3 endopeptidase mRNA sequence by NMR and CD spectroscopy at concentrations in the order of 10 µM. This effect appears to result in the upregulation of gene in HeLa cells.[112] Meanwhile, Waller, Balasubramanian and co-workers, discovered a triarylpyridine derivative capable of disrupting G4 in the *c-kit* 1 and 2 G4 sequences during a research programme examining the use of this scaffold for the design of G4-binding agents.[113] Interestingly, though many molecules of this class were able to confer stabilisation to these G4 structures,[114] compound **18** (Figure 9) was found to induced the unfolding the G4 at concentrations around 50 µM. This was initially detected by

attenuation of the G4 bands in the CD spectrum upon titration with **18**, further verified by NMR-methods and (in a subsequent study) by atomic force microscopy.[115] The destabilising effect on G4 of compound **18** also appears to translate into an up-regulation of expression of the corresponding gene in live HCG-27 cells, suggesting a use for such compounds to probe the role of G4 in gene expression. These results also contribute further evidence towards the hypothesis that G4 targeting molecules could be exploited as therapeutics by modulating gene expression.

Kaluzhny and colleagues later discovered an anthrathiophenedione derivative (**19**, Figure 9) that appears to unfold the sodium structure of telomeric DNA, since the perturbations observed in the CD spectrum of the G4/ligand complex match those generated when the sequence is unfolded by thermal denaturation.[116] For both **18** and **19**, the nature of the binding site precise mechanism of unfolding remains unclear, and further studies in this area would be welcome to provide valuable insight towards the more rational design of G4 stabilizing agents.

More recently, O'Hagan, Galan and co-workers identified a stiff-stilbene derivative (**20,** Figure 9) that induces the unfolding of the hybrid sodium form of telo23.[55] This was a particularly surprising finding given that the same ligand stabilizes the potassium form of the same sequence. Molecular dynamics and metadynamics simulations, in addition to NMR studies, were employed to identify the likely binding site and mechanism of unfolding, suggesting the ligand targets the G4 grooves before intercalating the G4 structure, which leads to eventual disruption of the hydrogen bond network of the G-tetrads. Whilst the finding that ligand **20** is
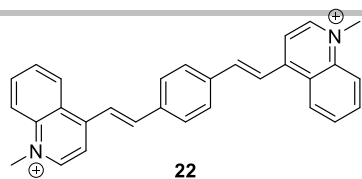
Figure 11: Arylstilbazolium ligands reported by Czerwinska and Juskowiak.[118]

capable of unfolding G4 structures under certain conditions was unexpected, it was deliberately designed with the intention of exploiting the photochemistry of the central stilbene scaffold as a means to control the spatiotemporal activity of the ligand using light as an external trigger, discussed in more detail in the following section.

2.5. Ligands that permit stimuli-responsive G4 folding

In the previous sections, we aimed to highlight the power of small molecules to go beyond simply stabilizing pre-formed G4 structures and exert changes in the folding arrangements. The refolding of the G4 can be thought of as a primitive mechanical system fueled by the added ligand, hinting at possible applications in the development of nanodevices. However, it might be difficult to imagine such applications for many of the previously described systems since in most reported cases the switch is a one-way process. Clearly, a much more useful system for such applications is one that can be switched back and forth multiple times, allowing more robust on-demand control of the "on" and "off" states. Furthermore, it would be ideal if the system could demonstrate this response without the repeated addition of chemical fuels, instead showing responsiveness exclusively to external triggers. In this section we explore progress made towards this goal.

2.5.1. Photochemical approaches

The first example of a stimuli-responsive ligand designed to influence G4 folding was reported by Wang, Zhou and colleagues in 2010. An azobenzene-derived ligand (*trans*-**21**, Figure 10a) was found to induce G4-folding in the absence of cations in water in the *trans* form, whilst the *cis* isomer did not have such an effect.[54] This allowed a very interesting application to be developed in which G4 confirmation could be controlled with light by exploiting the photoisomerisation properties of the ligand, since the *cis* isomer can be generated from the *trans* by illumination with UV light. Furthermore, this process proves to be reversible by the subsequent exposure of the system to visible light. This allowed the unfolding and refolding of G4 to be cycled at least ten times without any appreciable photo-fatigue (Figure 10b and 10c). In a follow-up study, the authors attempted to exploit this system in the presence of metal ions to mimic physiological conditions, those commonly employed in biophysical studies of G4s.[107] Unfortunately, ligand **21** was found to be ineffective in these studies. The authors do not specifically comment on why this system is adversely affected by the presence of metal salts, though perhaps the preference on G4 folding topology induced by the metal cation is more difficult to

overcome by ligand effects. This led the authors to develop further analogues of **21** (such as morpholino derivative **13,** Figure 7) which appear to allow the photo-regulation of G4 topology in ionic conditions to some extent. However, as in previous cases, though response observed in the CD spectra is consistent with topological switching and appears to be reversible, full details about the nature of the ligand-induced structures are unclear. Additionally, the reversibility is not as robust as the original system in the absence of metal cations, with only three switches in topology achieved in these later cases. Photoswitchable chiroptical azobenzene-G4 complexes were have also been reported by the Matczyszyn group.[117] Though in these cases the formation and dissociation of the complex could be controlled by the photoisomerization process, the compounds appear to exert little effect on G4 topology and also appear to interact significantly with duplex DNA, perhaps indicated that applications of azobenzene derivates may be confined to situations where a high level of quadruplex/duplex selectivity is not required.

Towards regulation of different G4 topologies in metal-rich conditions, Czerwinska and Juskowiak developed a series of photoswitchable arylstilbazolium G4 ligands (e.g. **22**, Figure 11).[118] Though these compounds were shown to bind with some selectively to different G4 topologies and isomerise between *E* and *Z* forms in sodium buffer, unfortunately the photoisomerization was inhibited in the presence of DNA. As introduced in Section 2.4, stiff-stilbene derivative **20** (Figure 9) induces the unfolding G4 DNA. Upon investigating the photochemistry of the ligand in these conditions, the authors found that a photooxidative reaction pathway dominates, rather than the *E-Z* isomerization observed for the Czerwinska ligands. This allowed the folded topology of the G4 to be regenerated by deactivation of the ligand by 400 nm light, and repeated cycling of unfolding/refolding was achieved by fueling the system with the photolabile ligand (Figure 12).[55] A key limitation of the system is of course the need for repeated additions of ligand fuel to drive the complementary photoresponse. Further efforts by the team are currently focused on optimizing the photochemistry of the ligand scaffold to produce a system that allows the full reversible photo-regulation of G4 folding.

2.5.2. Host-guest approaches

(a)

$K_a > 10^{12}$ M^{-1}

trans-**21**

24

$K_a = 2.6 \times 10^5$ M^{-1}

23

(b)
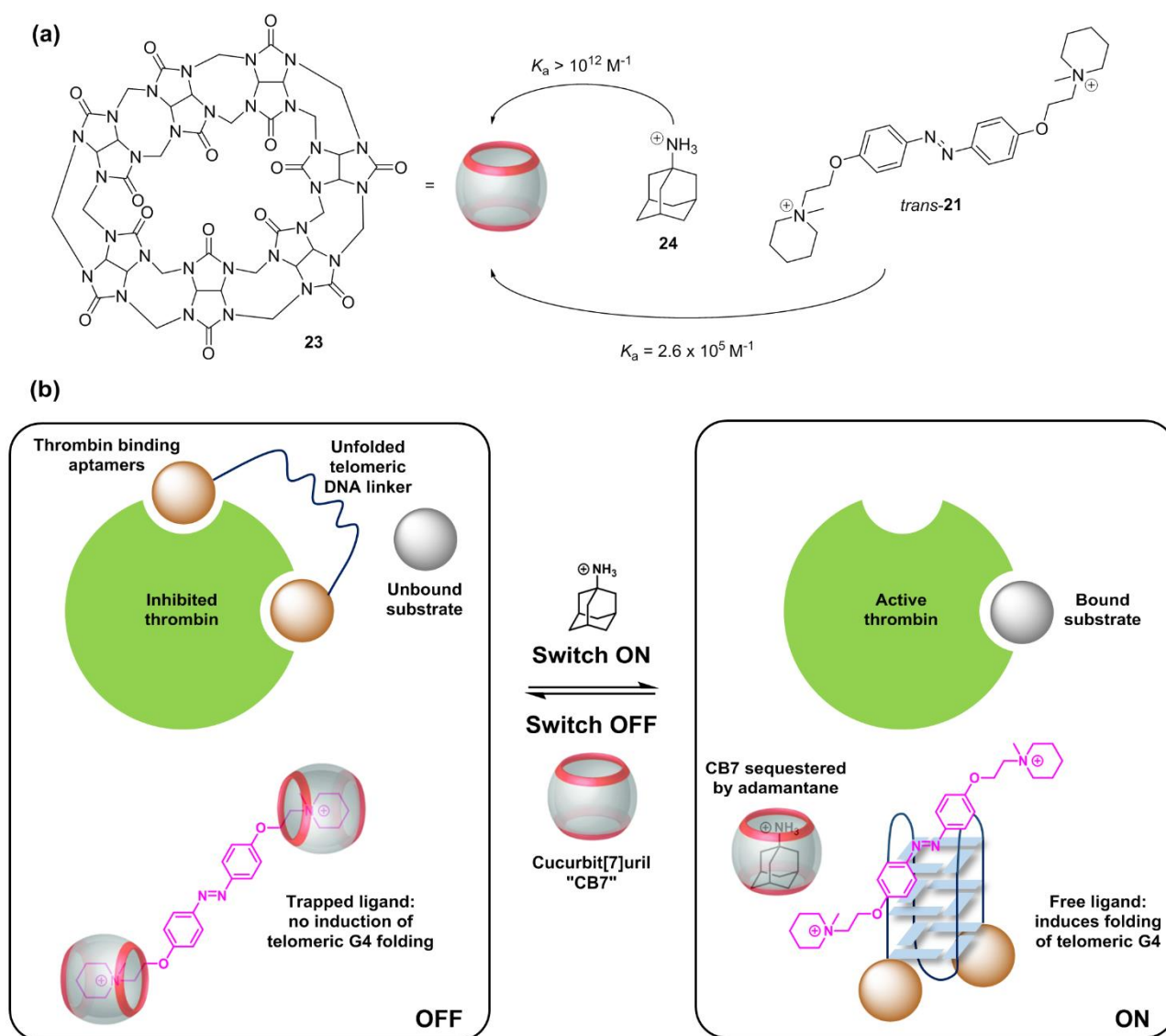
Figure 13: (a) Chemical structure and cartoon representation of cucurbit[7]uril, an efficient molecular host for ammonium cations such as *trans*-**21** and adamantylammonium **24**. **(b)** Switchable control of thrombin activity by exploiting the host-guest chemistry of G4 ligand **21** and CB7 **23**.[119]

The further systems that are worthy of discussion here employ host-guest approaches to control the effect of ligands on G4 folding. In the first, the Zhou group *et al.* employed the azobenzene derivative *trans*-**21** (Figure 10a), observing that the terminal piperidinium cations have good affinity for cucurbit[7]uril (CB7, **23**, Figure 13a) host.[119] Whilst free ligand *trans*-**21** induces the formation of G4 (see above), sequestration by CB7 causes dissociation of the ligand from the G4 and concomitant unfolding of the DNA secondary structure occurs. By fueling the system alternately with CB7 **23** and a competitive guest, (adamantylammonium **24**) 13 switches of G4 unfolding and refolding were achieved. This system was applied to control the activity of the thrombin enzyme (Figure 13b). In this case, a thrombin binding aptamer containing a G4-forming linker (based on telomeric TTAGGG repeats) binds to and inhibits thrombin activity. The addition of *trans*-**21** triggers the folding of the linker thereby causing the dissociation of the inhibitor, un-gating the
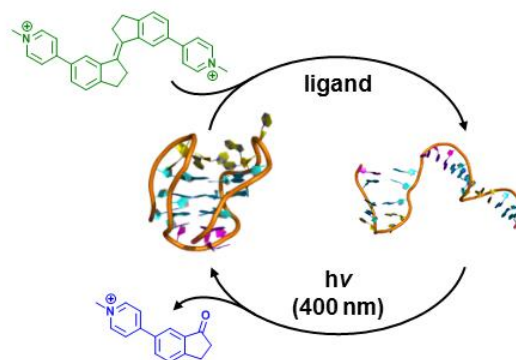
Figure 12: reversible unfolding of G4 DNA using the photoresponsive stiff-stilbene **20**. Reproduced with permission.[55] Copyright 2019, Wiley.

activity of the thrombin. The enzyme can be subsequently deactivated by adding CB7 (**23**) which sequesters the ligand, causing unfolding of the G4 and regeneration of the thrombin/inhibitor complex. In the same way as the original proof-of-concept outlined above, addition of adamantylammonium cation **24** reverses this process. Whilst the eventual utility of this system is perhaps restricted by the need for sequential addition of ligand fuels, it is an elegant proof-of-concept that supramolecular approaches can be used as a means to control G4-mediated processes and the possibility for further refinement is exciting.

In a different approach, Monchaud, Mergny and colleagues exploited the response of well-known G4 ligand 360A (**14,** Figure 7) to Cu^{2+} cations, which are also known to induce the unfolding of G4 DNA to a single-stranded form by preferential binding to the single-stranded structure.[120] The conformation of 360A can be switched from a crescent to linear geometry by the addition of Cu^{2+} cations (Figure 14), dramatically weakening the G4 affinity of the ligand. Whilst comparatively weaker ligands that do not display such an effect could successfully guard telomeric G4 against Cu^{2+} mediated unfolding, 360A was unable to do so despite being a much stronger G4 ligand. Therefore, the switch in geometry of the ligand induced by Cu^{2+} is ultimately responsible for the unfolding of the G4/360A complex. The folding can be reversed by adding the chelating ligand EDTA to sequester the copper and regenerate both the G4 and the active conformation of 360A. The Clever group also recently reported a Cu^{2+}/EDTA/ligand supramolecular system to control the binding of G4 DNA to thrombin.[121] In this case however it is necessary to pre-incorporate the required glycol-pyridine ligand into the oligonucleotide, rather than deliver it externally.

2.6. Conclusions

The work discussed above demonstrates the potential of G4 ligands to go beyond simply stabilizing a single G4 structure and exert control over G4 polymorphism. Interestingly, this survey shows a gradual emergence of studies concerned with the rational design of ligands capable of exerting such effects, for example by exploiting known solvent effects (e.g. BMVC-8C3O, **8**) or by using computational approaches (isoindigatone **9**). Furthermore, reports have recently appeared that demonstrate how ligands responsive to external triggers may allow the development of stimuli-driven G4 systems, where such reversible control may allow the spatiotemporal modulation of mechanical work or reactivity to be controlled by G4-folding processes. However, for real-world applications to be realized, more robust and reversible regulation of such systems, particularly in the presence of metal ions that drive G4-folding in physiological conditions, is likely to be required. Additionally, further evidence of the exact nature, mechanisms, and kinetics of conformational switching would be welcome to allow the refinement of these systems and enable new applications to be imagined.
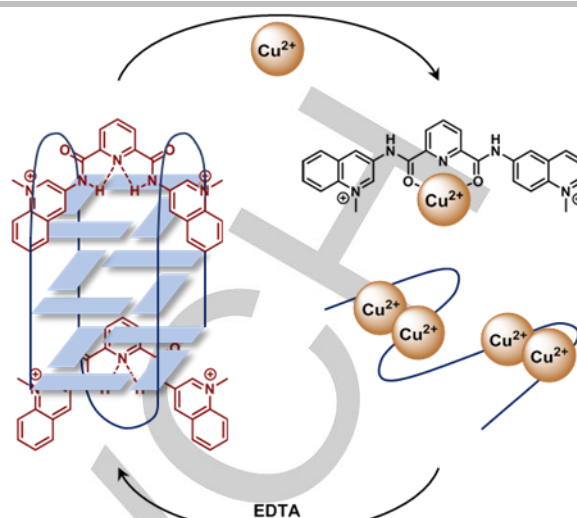

Figure 14: Supramolecular regulation of the activity of ligand **14** G4 binding affinity and G4 folding using Cu^{2+} and EDTA.[120]
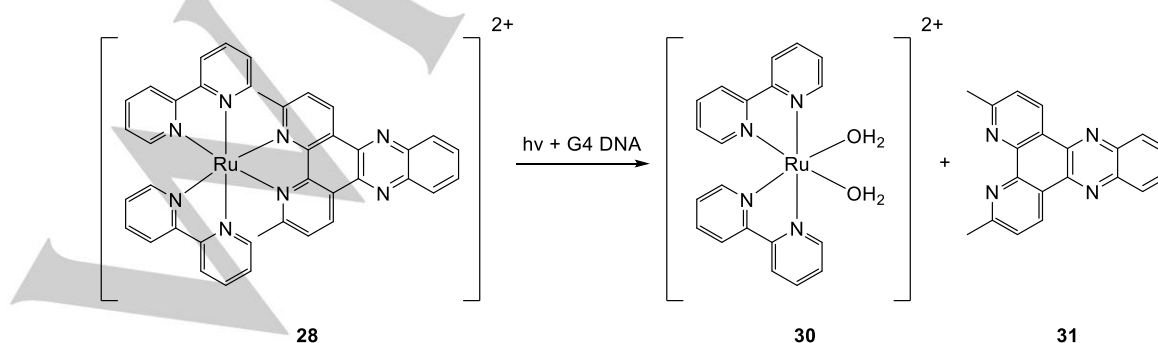
3. Ligand-driven modification of G4 structure – toward new biological probes and therapeutics?

All of the ligands discussed in the previous section rely on *non-covalent* supramolecular interactions with nucleic acids to exert their effects. In this section we consider a complementary strategy – the deployment of G4-binding chemotypes to direct the selective *covalent* modification of the oligonucleotide structures. It is well known that biology itself relies on such a strategy for the epigenetic regulation of gene expression.[122] Partly inspired by this feat of natural engineering, selective covalent modification of nucleic structures has commanded a significant amount of research attention, whether to develop new biological tools[123] or to induce nucleic acid damage for therapeutic effect, with the well-known anticancer drug cisplatin being a case in point.[124] However, obtaining selectivity for a particular DNA sequence is challenging, leading to unspecific labelling and off-target side effects.[124] The precise three-dimensional structures of G4s, which sets them well apart from single-stranded and duplex nucleic acids, provides an opportunity to overcome these issues, since their distinctive binding sites allow the selective targeting of these structures with small molecules. As summarized in the sections below, developments in the field of G4 ligand design have indeed permitted the design of several G4-targeting agents to modify nucleic acid in a more selective manner, for example by metalation,[125] alkylation[126] or scission.[127] Furthermore, since guanine is the most readily oxidized DNA base,[128] it is not surprising that efforts have also been made to utilize G4 ligands as a mean to direct the site-selective modification of nucleic acids in this way.[82] The following sections explore the progress made in these areas.
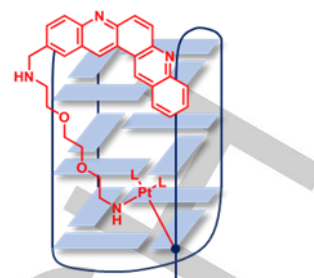
3.1. Metalation of G4

25

26

27

28 (R=H)
29 (R=Br)
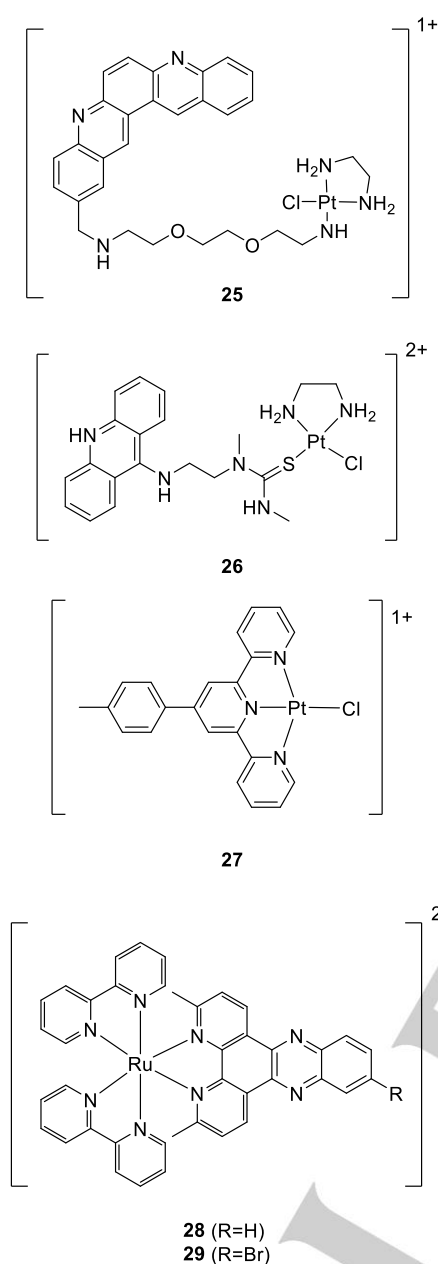
Figure 15: Ligands reported to direct the selective metalation of G4 nucleic acids.

Figure 16: Directed platination of terminal G-tetrad residues of telo22 by ligand **25**.[132]

A number of research programs have been directed towards the development of G4 ligands derived from metal complexes, where the highly electropositive character of the metal is able to confer strong binding affinity to the negatively charged DNA.[129–131] Given the known reactivity of nucleic acid bases with metal centers, such complexes also allow the possibility of covalent binding modes. Not only does this have the potential to strengthen the binding interactions themselves, but such a response could also be exploited to therapeutic ends, since the classical DNA damage induced by ligation to metals can be combined with the G4-specificity of the ligands over other nucleic acid structures to allow enhanced selectivity for particular nucleic acid sequences.

In 2007 the Teulade-Fichou group reported the first example of a dual non-covalent/covalent G4 ligand, derived from a platinum-quinacridine hybrid (**25**, Figure 15).[132] This molecule was designed to contain a G4-stacking motif with a linker that directs the Pt-warhead toward the opposite terminal tetrad or a loop residue (Figure 16). Indeed, by gel electrophoresis methods it was found that this compound platinates exclusively at the 5' tetrad of the antiparallel telo22 sequence in sodium buffer, at the G2, G10 and G22 residues.

28   **30**   **31**

Scheme 1: G4-gated photoinduced dissociation of ruthenium complexes to generate reactive metalating species.[125]

The denaturing gel assay found that the platinated adducts are stable, since they migrate through the gel in the complexed state. Whilst DNA platination usually occurs at the N7 site of guanine bases, these residues are protected in G4s by the Hoogsteen bonding arrangements of the G-tetrads, therefore suggesting that in this case metalation actually proceeds *via* the initial disruption of the terminal tetrad. Indeed, this hypothesis suggests that G4 loops can be targeted preferentially to the G-tetrads, a goal which has since been realized experimentally. Rao and Bierbach observed that Pt-ligand **26** (Figure 16) primary reacts at loops regions of telo22 and telo24, metalating adenine residues at N1, N3 and N7 sites.[133] Though some metalation of guanine N7 sites was still observed, kinetic experiments found that adenine residues are attacked more quickly and that reaction with G4 is approximately two-times faster than with duplex DNA. The authors acknowledge that the limitations of the system were likely due to the relatively poor G4 intercalating power of the acridine functionality (more commonly employed as a groove-binding chemotype) and suggest optimization of this feature may improve the efficiency and selectivity of the platination reaction.

More recently Bertrand, Teulade-Fichou and co-workers achieved exclusive platination of loop adenines in G4 with Pt-terpyrdine complexes.[134] With compound **27** (Figure 16), specific platination of A13 (35% yield) was observed in telo22 G4. In this case, the ligand is proposed to interact with the lower G-tetrad, thus directing platination to the adenine residue in the diagnonal loop. By monitoring the reactivity of the ligands with a duplex/G4 hybrid oligonucleotide model, the authors found that adenine platination dominated for G4 whilst guanine platination was more prevalent in duplex, with up to twelve-fold faster kinetics observed for the G4 sequence, a significant improvement on the previous systems.

Meanwhile, the Glazer group have developed ruthenium-derived complexes that promote the covalent metalation of G4, for example complexes **28** and **29** (Figure 15).[125,135] In these cases, the *ortho* methyl groups on the dipyridophenazine (dppz) functionality add steric strain to the system which promotes the photo-induced ligand dissociation to generate active metalating complex **30** and free ligand **31** (Scheme 1). Interestingly, whilst the ligand is relatively unreactive in water, it becomes an order of magnitude more reactive in the presence of DNA and is 3-fold more reactive when complexed to G4 DNA *versus* calf-thymus DNA. In these ligands, the lower energy "dark" state that arises from metal-to-ligand charge transfer to the phenazine portion of the dppz functinality, whilst the higher energy "bright" state results from charge transfer to the bipyridine portion of the ligand. Whilst aqueous environments lower the energy of the "dark" state, in the presence of DNA the bright state is more thermally accessible which essentially activates the observed photochemistry. Subsequently, the group reported halogenated analogues of type **29** were reported, which appear to have better selectivity for G4 over duplex DNA.[135]

3.2. Alkylation of G4

Though strategies for the covalent modification of nucleic acids have long been known,[137] the possibility of conjugating alkylation warheads to known G4-binding chemotypes to improve their selectivity has only been explored over the past decade or so as
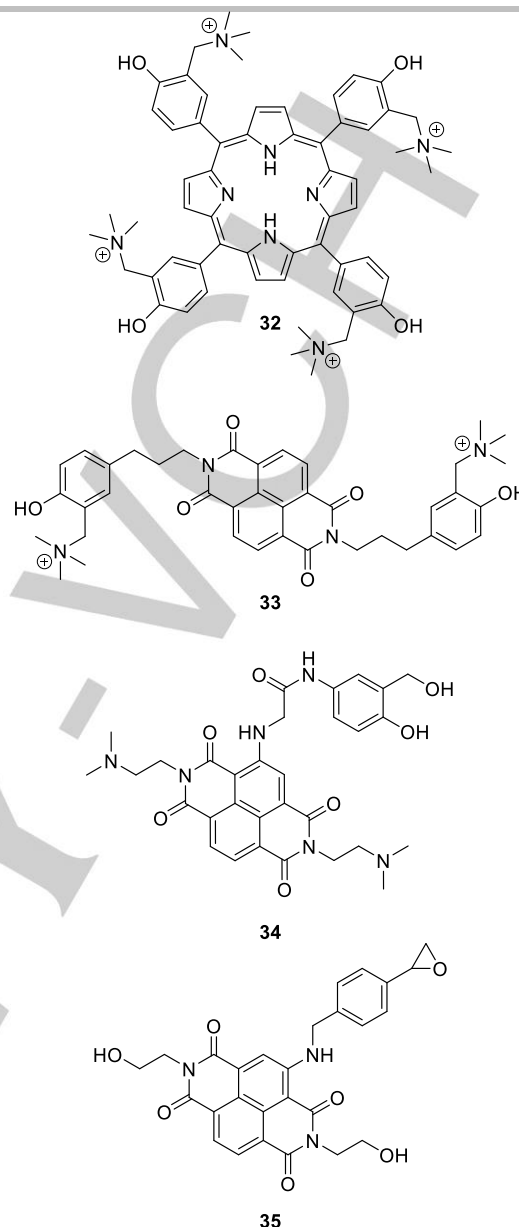
Figure 17: Ligands reported to alkylate G4 under thermal conditions

the field of G4 ligand design reached sufficient maturity to allow the more specific targeting of these structures. Interestingly, not only has selective alkylation of G4 in the presence of duplex DNA
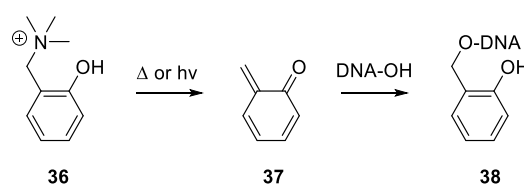
Scheme 2: General mechanism of DNA alkylation by *in situ* generation of quinone methides from aromatic Mannich bases.[136]

been achieved, which is clearly necessary for G4-targeted applications in biological circumstances, but certain classes of compound also allow for the stimuli-driven triggering of the alkylation events, allowing an additional level of spatiotemporal control.

3.2.1. Thermal alkylation

An early study hinting at the possibility of using G4 ligands as the basis to improve the selectivity of nucleic acid alkylating agents was reported in 2006 by Tan *et al.* The authors demonstrated the G4 selectivity of compound **32** (Figure 17) derived from porphyrin, a known G4-binding chemotype.[138] Previously the compound exhibited toxicity in cervical cancer (HeLa) and liver cancer (HepG2) cells under photoirradiation, an effect tentatively attributed to the photoinduced formation of quinone methides (**37**) and subsequent alkylation DNA (Scheme 2). However, it is unclear whether any such alkylation is G4 specific, or whether the toxic effects instead result from the generation of singlet oxygen (see Section 3.3).

Since then however, significant progress has been made towards the development of thermally activated G4-selective alkylating agents by the Freccero group. Following earlier work examining the potential of quinone methide precursors as general DNA alkylating agents,[139] the group exploited naphthalene diimide (NDI) derivatives to confer G4-selectivity to this warhead.[136] NDIs have themselves been extensively explored as G4 binding agents,[140] displaying impressive selectivity and, more recently, promising results against *in vivo* disease models.[141,142] Initially, the group reported compound **33** to be capable of alkylating telo22 G4. Generation of the active quinone methide required thermal activation (40 °C) in the buffered conditions required for the folding of the quadruplex. Under such conditions, 15% alkylation was observed (by denaturing gel electrophoresis) at 2 µM drug concentration after 24 h incubation and the products were determined to be stable up to 70 °C. Interestingly, the length of the alkyl linker between the NDI and the alkylating warhead was critical, with shorter or longer chains than the propyl chains in compound **33** resulting in comparatively lower alkylation yields. Most critically, the selectivity of the system for G4 DNA was demonstrated, with alkylation of duplex DNA or single stranded DNA being substantially lower under comparable conditions. In a subsequent study, a secondary generation of compounds were introduced, where the quaternary ammonium functionality was replaced by an additional alcohol group (e.g. compound **34**, Figure 17), since the former was considered unsuitable for cell-based applications owing to its permanent cationic charge.[143] In this case, a more electron-rich aromatic system was necessary to allow the compounds to generate the active quinone methide species at 40 °C, given the neutral nature of the leaving group. 16.8% alkylation of telo22 was achieved in 24h, comparable to the alkylation yields observed with compounds of type **33**. At higher ligand concentrations, two alkylation events per G4 could also be observed. Compounds of type **37** appeared to be more selective for G4 than those of the first generation, with the compound concentration required for alkylation to be observed being 100 to 1000 times lower for G4 than single stranded DNA (scrambled telo22) and duplex DNA respectively. Though putative alkylation sites were identified by enzymatic digestion,
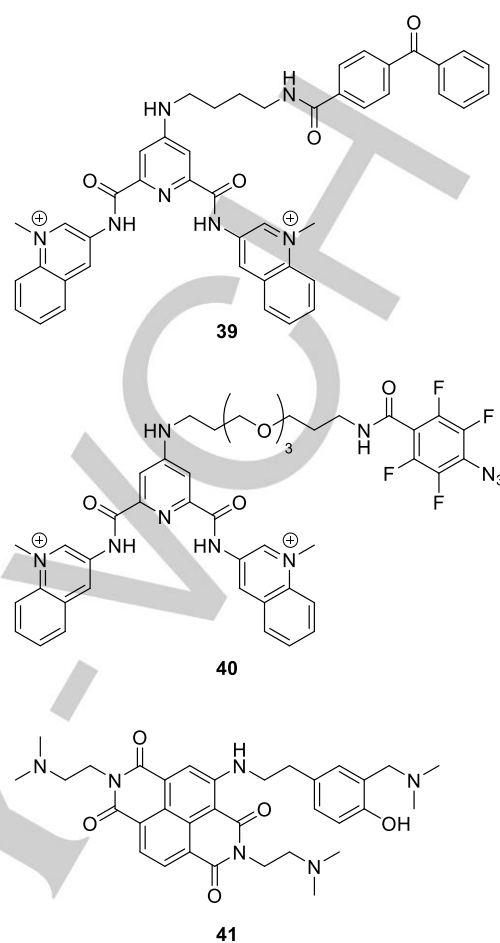
Figure 19: Ligands reported to alkylate G4 under photochemical conditions

unambiguous assignment remained elusive owing to the reversible nature of the adduct formation at higher temperatures.

These issues were later overcome by the development of a third generation of compounds (**35**, Figure 17), which exploit the electrophilic character of oxiranes to promote irreversible alkylation.[80] Not only does compound **35** exhibit high selectivity for G4 (16% adduct formation G4 versus < 2% alkylation of single-stranded and double-stranded DNA under comparable conditions), the adducts were shown to be significantly more stable than the quinone methide variants, allowing more detailed analysis of adduct formation by mass spectrometry. This revealed selectivity for the adenine residues of the G4, which contrasts with the usual selectivity for alkylation at guanine (Figure 18).
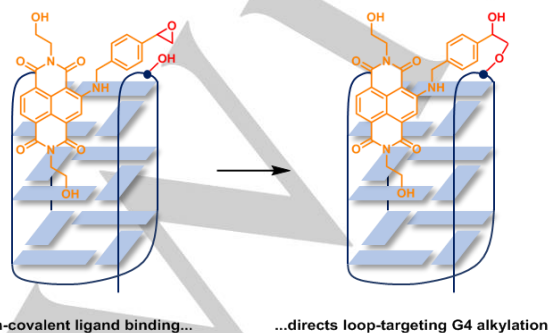
3.2.2. Photochemical alkylation

Both the quinone methide and oxirane approaches to G4 modification rely on thermal activation of the system to trigger alkylation events. Though this requirement has the potential to allow a degree of control on the system, the relatively mild temperature required is unlikely to serve as an ideal handle to fulfil this purpose. In contrast, light has several distinct advantages as a trigger for chemical processes, since it can be delivered with a much higher degree of precision in terms of location, intensity and time.

The Teulade-Fichou group recently deployed the high G4 selectivity of the bisquinolinium pyridodicarboxamide scaffold in the development of a series of photo-trigged G4 alkylating agents (e.g. compounds **39** and **40**, Figure 19).[126] Their strategy relies on the conjugation of a known G4-binding chemotype to a moiety that releases the alkylating species upon exposure to UV light. In the case of compound **39**, the photoexcited state of the benzophenone can react with unsaturated systems to produce adducts in a [2+2] fashion, whereas the azido derivative **40** generates a highly reactive nitrene species upon photoirradiation. Alkylation yields of 20-36% were achieved at a concentration of 25 μM ligand, depending on the nature of the G4 topology. As observed for the quinone methide compounds of Freccero,[136] alkylation yield was significantly dependent on the nature of the spacer between G4-binding core and alkylating warhead, with the authors arguing that a more hydrophilic spacer allows the ligand to interact more closely with the hydrated DNA structure in solution. However, a slightly increased in alkylation yield appears to come at the expense of selectivity. For example, compound **40** is a more effective alkylating agent of the K^+ quadruplex (36% yield) than compound **39** (26% yield), but whilst the presence of an excess of single-stranded DNA competitor significantly inhibits alkylation of G4 by azide **40** (by approximately 40%), the activity of benzophenone **39** against G4 is unperturbed by the addition of the same competitor. Through a combination of sequencing methods, the predominant alkylation sites were identified as the loop thymidines (T6 and T11) for compound **39** and external G-tetrad (G10 and G14) for compound **40**. All compounds displayed photo-triggered cytotoxicity against breast cancer (MCF7) and lung cancer (A549) cell lines, whilst remaining non-toxic in the dark.
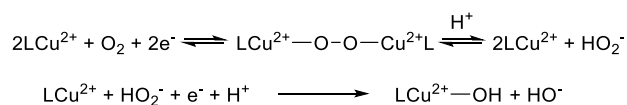

Figure 18: Irreversible alkylation of G4 with oxirane-NDI ligand **35**.[80]

Perhaps a key drawback to the application of compounds of type **39** and **40** in biological applications is the wavelength of light required to initiate the photochemical reactivity. For both compounds, UVA (330-365 nm) radiation is required, which is toxic to cells and also suffers from poor tissue penetration, limiting its use in *in vivo* settings. Towards addressing these limitations, Freccero *et al.* exploited the optical properties of the NDI core towards the development of a photoreactive G4 ligand that can be triggered by green light (**41**, Figure 19).[145] This approach relies on the absorbance of (λ_{max} = 532 nm) of the NDI chromophore and the subsequent generation of a highly reactive phenoxide radical by an intramolecular electron transfer process from the Mannich base to the triplet excited state of the NDI. Indeed, at 12.5 μM concentration of compound **41**, 63.7% alkylation yield was obtained, significantly higher than previously achieved, with only trace amounts of alkylation observed for non-G4 single-stranded or duplex DNA. In addition to mass spectrometry analysis, the group synthesized several mutant analogues of telo22 identify the precise modification sites in combination with digestion assays, demonstrating alkylation preferentially occurs at T12 and T6, in agreement with previous
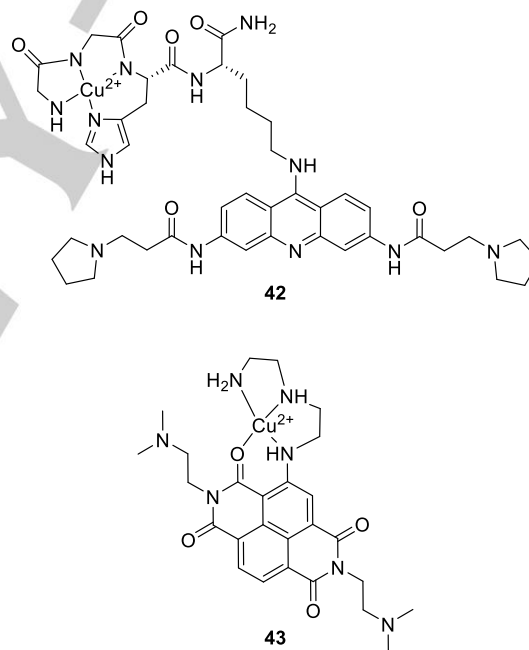
Figure 20: Ligands reported that promote the selective scission of G4.

$$2LCu^{2+} + O_2 + 2e^- \rightleftharpoons LCu^{2+}-O-O-Cu^{2+}L \xrightarrow{H^+} 2LCu^{2+} + HO_2^-$$

$$LCu^{2+} + HO_2^- + e^- + H^+ \longrightarrow LCu^{2+}-OH + HO^-$$

Scheme 3: Proposed mechanism of formation of reactive metal-bound hydroxyl radicals by Cu-ATCUN ligands.[144]

reports that phenoxide radicals preferentially target thymidine nucleobases.[146]

3.3. Scission of G4

The goal of the approaches discussed in the previous section is to essentially add non-native functionality to G4, whether metallic or organic species, with the ultimate goal of DNA damage or selective labelling. In contrast, the selective *cleavage* of G4 structures has also been demonstrated in a small number of cases. Yu, Han and Cowan conjugated a Cu^{2+} binding moiety, an amino-terminal copper/nickel binding ("ATCUN") to acridine-based G4 ligands to promote selective cleavage of G4 sequences (compound **42**, Figure 20).[147] Through mechanistic studies on related compounds in redox environments, it appears that the copper is involved in the generation of reactive oxygen species, such as the hydroxyl radical, with the distinction that they remain bound the copper rather than diffusing freely in solution.

A possible mechanism is shown in Scheme 3, in which two-electron reduction of O$_2$ generates hydrogen perioxide leading to a subsequent Fenton-like reaction that results in the generation of a copper-bound hydroxyl radical. This reactive species may abstract hydrogen atoms from the DNA backbone leading to subsequent scission of the oligonucleotide strand.[144] Such observations go some way towards explaining the selectivity of the acridine-ATCUN conjugates for specific DNA sequences, where the G4-targeting fragment is responsible for bringing the reactive warhead close to the target site. Predominantly telo22 was cleaved at A1 (12.8%), G2 (10.2%), T6 (13.7%) and A7 (7.6%) sites. Subsequently, Freccero *et al.* developed NDI variant **43** (Figure 20) which cleaves telo22 G4 specifically at A7−G9, A13−G15, and A19−G21 sites, whereas significantly less (and non-specific) scission was observed for single-stranded and duplex DNA under comparable conditions.[127] In this case, the authors undertook NMR and computational studies which found that ligand binding sites were consistent with cutting sites, potentially allowing the development of further analogues that could provide complementary scission patterns.
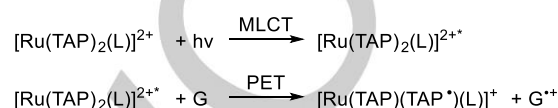
3.4. Oxidation of G4

Guanine has the lowest redox potential of the nucleic acid bases and is therefore the most susceptible to oxidation. The occurrence of such modifications *in vivo* leads to a variety of downstream effects. For example, the *syn* conformation of 8-oxo-guanosine may form Hoogsteen bonds to adenine rather than the usual Watson-Crick bonds to cytosine, leading to errors in DNA transcription.[128] Indeed, potential protective roles of G4s against such oxidative DNA damage have recently been explored.[150]

Given the over-representation of guanine in G4 forming sequences and the ability to target these sequences with ligands, it is unsurprising that groups have made efforts to develop tools for G4-targeted oxidation. Two possible pathways of oxidation of a guanine base are known.[128] Either singlet oxygen (generated by photosensitisation) may react directly with a guanine residue, generating 8-oxo-guanine **45** or spiroiminodihydantoin **46** (Scheme 4). Alternatively, the photoinduced metal-to-ligand charge transfer (MLCT) states of the complexes by may promote
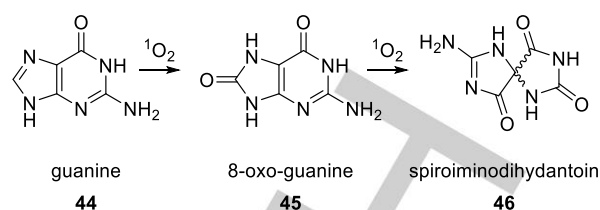
Scheme 4: Oxidation products of guanine generated by reaction with singlet oxygen.[128]

$$[Ru(TAP)_2(L)]^{2+} + h\nu \xrightarrow{MLCT} [Ru(TAP)_2(L)]^{2+*}$$

$$[Ru(TAP)_2(L)]^{2+*} + G \xrightarrow{PET} [Ru(TAP)(TAP^{\bullet})(L)]^{+} + G^{\bullet+}$$

Scheme 5: Mechanism of generation of guanine radical cations by photoinduced electron transfer from Ru-TAP complexes.[148]
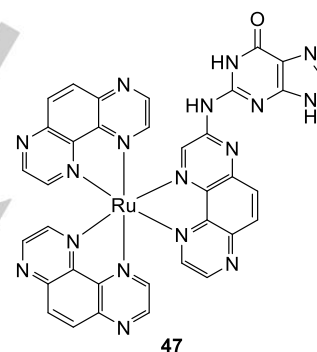

Figure 21: Adduct formed by the recombination of oxidized guanine radical cations and reduced Ru-TAP complexes.[149]

in photoinduced electron transfer (PET) and abstract an electron from the base to generate a guanine radical cation (Scheme 5) leading to subsequent adduct formation between the oxidized base and one of the metal ligands to generate products of type **47** (Figure 21). The following sections consider strategies for G4-targeted oxidation that exploit these complementary reactivities.

3.4.1. Photogeneration of singlet oxygen

Porphyrins are widely known to be efficient photosensitizers of oxygen.[151] Indeed, classical G4 ligand TMPyP4 (**17**, Figure 8) has been shown to promote the photocleavage of DNA sequences shown to prove more toxic to ovarian tumor cells irradiated with light than to cells kept in the dark, suggesting a mechanism of action involving the photogeneration of singlet oxygen and pointing to applications of G4 ligands in targeted photodynamic therapy.[152] However, since TMPyP4 is known to also bind duplex DNA sequences,[71] it is unlikely to target G4 genomic regions with the high specificity likely desired in therapeutic application. Furthermore, earlier studies suggested TMPyP4 induces photocleavage of the DNA strand meaning the reactive oxygen species presumably pursues more complex
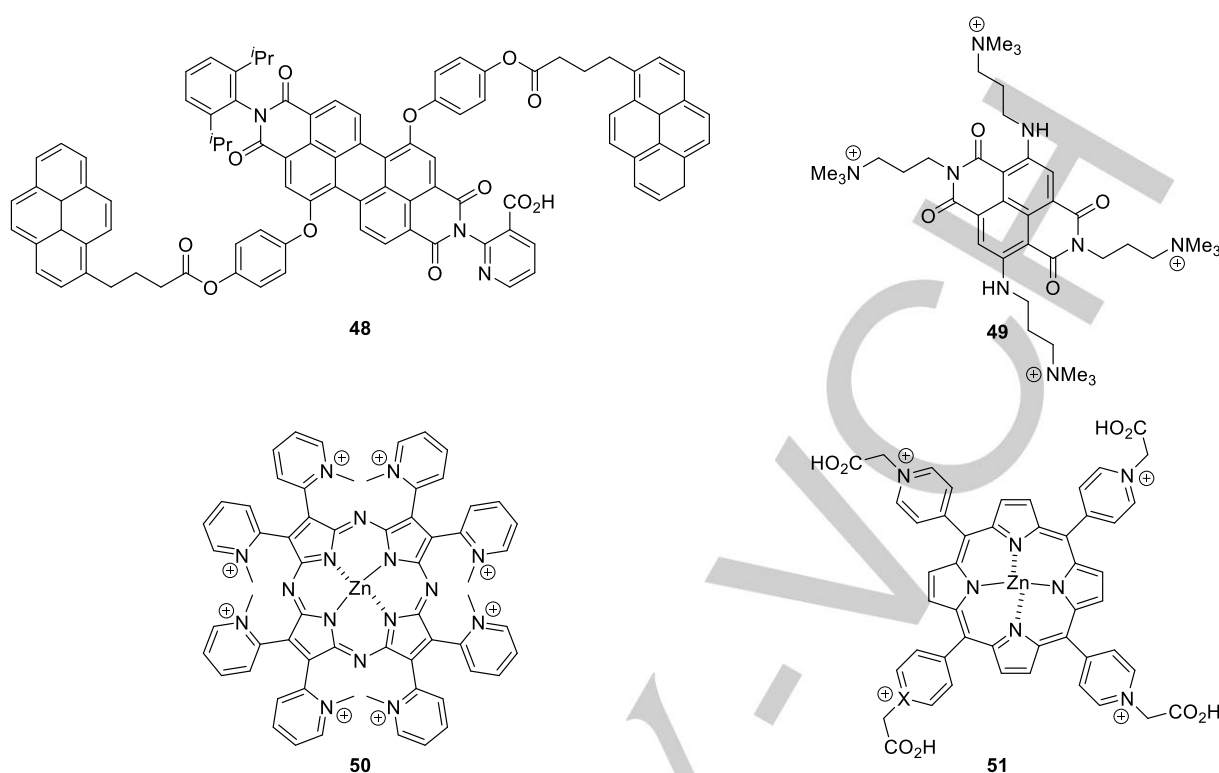
Figure 22: G4 ligands reported to promote the photogeneration of singlet oxygen.

pathways than those presented in Scheme 4.[153] Meanwhile, naphthalene and perylene diimides, both known to serve as effective scaffolds in selective G4 ligand design, have also been demonstrated to be effective photosensitizers of oxygen. Dinçalp *et al.* reported perylene/pyrene derivative **48** (Figure 22) which generates $^1O_2$ with a high (93%) quantum yield and binds G4 with good affinity ($K_a = 10^6$ M^{-1}), suggesting such compounds may allow G4-targeted photodynamic therapy.[154] Subsequently, Freccero *et al.* reported NDI analogues of type **49** could produce respectable quantum yields of $^1O_2$ in the phototherapeutic (600-800 nm) window.[155] Sciscione, Manet *et al.* also reported a series of magnesium(II) and zinc(II) porphyrazines **50** that bind G4 and generate singlet oxygen upon photoirradiation. However, in all of the above cases, the application of the molecules as tools to introduce G4-specific oxidative lesions does not yet appear to have been explored.

More recently however, Beniaminov, Kaluzhny and co-workers disclosed tetracarboxymethyl porphyrin **51** which binds with preference to potassium hybrid G4s and returns distinctive oxidation patterns of guanine bases upon photoirradiation.[82] Interestingly, the oxidation footprint is found to depend on the G4 conformation. For example, in the hybrid G4 form, oxidation of G9, G15, G21 and G3 dominates, whilst under conditions that promote the parallel fold oxidation primarily occurs oxidation at additional residues G11 and G17. This suggests that this system could be used as a tool to diagnose G4 folding topologies. Unlike other photosensitizers (e.g. TMPyP4, previously discussed), it appears the ligand primarily generates G-specific oxidative lesions (8-oxo-G and spiroiminodihydantoin) rather than non-discriminate strand cleavage. Interestingly the light-triggered

guanine oxidation was found to convert hybrid and parallel G4 topologies to the antiparallel structure based on distinctive changes in the CD data (see Section 2). Critically, the system exhibits a degree of selectivity, with both hybrid and parallel telo23 G4s found to be more susceptible to oxidation than double-helical DNA or the sodium G4 conformation.

3.4.2. Photoinduced electron transfer

The second possible pathway of guanine oxidation is exemplified by certain ruthenium complexes and shown in Scheme 5. The design of these ruthenium complexes differs from the compounds discussed in Section 3.1 (designed to generate nucleic acid metalating agents upon photoirradiation) in that they exploit the electronics of the tetraazaphenanthrene (TAP) ligand, rather than the classical phenanthroline ligands, to promote photoinduced electron transfer leading to the generation of guanine radical cations.[148] In these Ru-TAP complexes, the triplet excited metal-to-ligand (MLCT) charge transfer state is strongly oxidizing, sufficiently so to abstract an electron from a guanine base with concomitant generation of the reduced metal species. Subsequent recombination of the oxidized base and the reduced complex forms adducts of type **47**. This reactivity has been demonstrated with guanosine monophosphate, and later applied in attempts to engineer systems that target G4 DNA due the over-representation of guanine in these motifs.
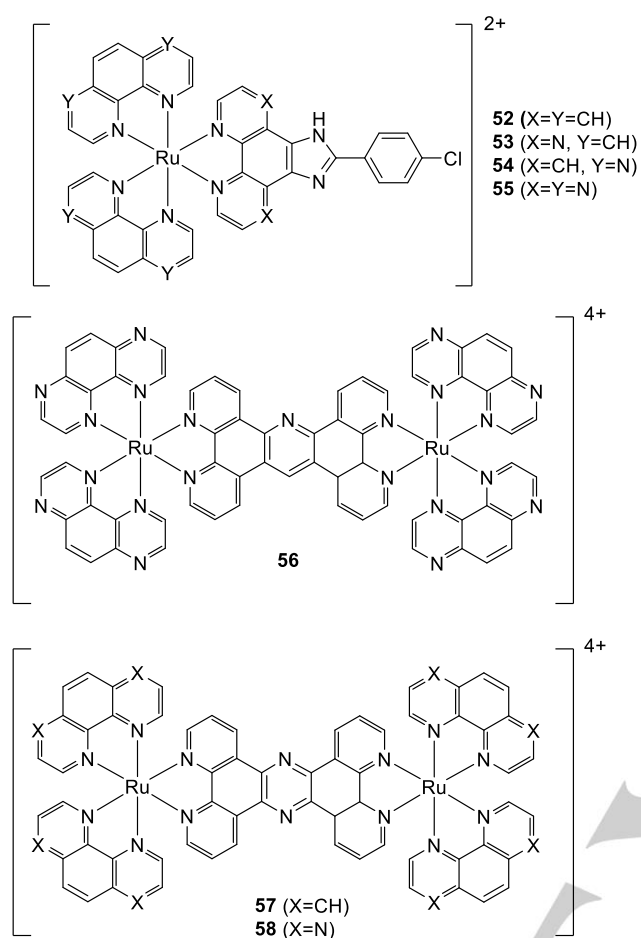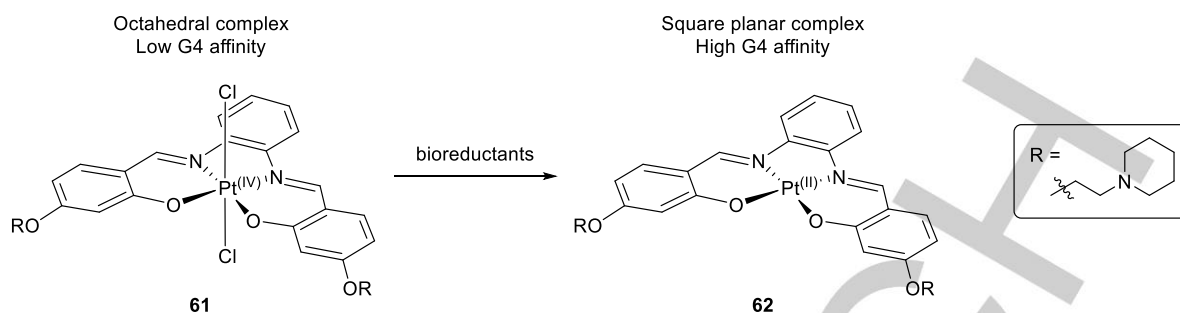
Figure 23: Ruthenium complexes reported to promote the oxidization of guanine bases by photoinduced electron transfer.

In effort to confer additional G4 selectivity to Ru-TAP complexes, the Elias group conjugated a known G4 binding chemotype, chlorophenylimizdazophenanthroline (CPIP), to the Ru-TAP center (**52**-**55**, Figure 23).[81] Several approaches to introduce the TAP functionality were pursued, including by modifying the CPIP ligand itself to include the pyrazine functionality (**53**), by employing TAP ancillary ligands (**54**), or by combination of both strategies (**55**). The control ligand (**52**), containing only phenanthroline ligands and no TAP functionality, did not promote oxidation, observed as an increase in ligand luminescence on DNA binding (indicating the excited state is not quenched by an oxidative process). Similar results were obtained when the TAP functionality was incorporated only in the CPIP ligand (**53**). However, when ancillary TAP ligands were used (**54**), the oxidative pathway was observed *via* quenching of the luminescence of the MLCT excited state. Pleasingly, the affinity of the compounds was 10-50 times higher for G4 than duplex DNA, suggesting adding known G4-targeting functionality successfully confers selectivity to these complexes. The complexes also demonstrated photocytotoxicity in cellular studies. However, whilst complexes **52**-**54** could possibly act *via* the PET pathway, compound **55** was also active, suggesting such photocytotoxicity may result from the generation of reactive oxygen species instead or, or in addition to, PET.

Complexes containing two metal centers offer the possibility of multiple oxidation events by the same ligand, which may lead to DNA cross-linking. This approach was proposed by Mesmaeker and co-workers as a means to "freeze" the G4 conformation, leading to the design of complex **56** (Figure 24).[149] This dinuclear ruthenium species demonstrated a similar affinity for G4 and duplex DNA, (K_a in the order of 10^6 M^{-1}) however photoreaction with telomeric G4 was more rapid. Electrophoresis methods demonstrated that ligand/G4 adducts were generated with up to 6:1 stoichiometry. Though further experiments including mass spectrometry on the purified adducts confirmed the nature of addition (analogous to Figure 21), it was not possible to demonstrate with certainty that crosslinking between two G bases had been achieved. In a more recent studies by the Thomas group, related pyrazine compounds were synthesized (**57** and **58**).[156] In this example, the phenanthroline variant (**58**) was non-emissive in water but bright MLCT luminescence was induced upon DNA binding. Again, by switching to TAP ligands on the metal (compound **58**) the ligand promotes the guanine oxidation pathway. The compound also displays high photocytotoxicity in cells. However, like the Mesmaeker compound (**56**), G4/duplex discrimination is poor, suggesting that incorporation of G4-targeting functionality (using a similar approach to Elias and colleagues) is key to realizing enhanced selectivity in these compound classes.
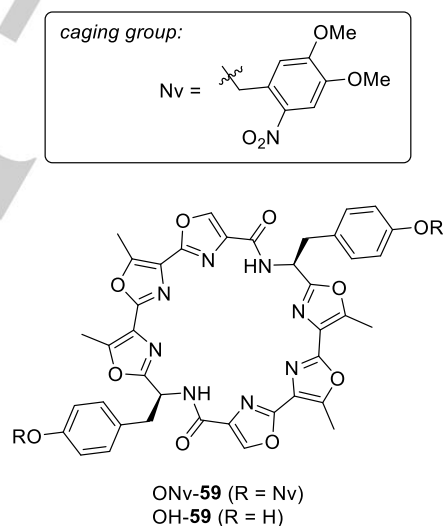
3.4.3 Conclusions

Octahedral complex
Low G4 affinity

Square planar complex
High G4 affinity

bioreductants

R =

61

62

Figure 25: A redox-activated G4 ligand. The Pt(IV) complex displays low G4 affinity but binding is restored by *in situ* reduction to the square-planar Pt(II) salphen species.[84]

The work discussed in this section clearly demonstrates that significant attention is devoted towards directing the specific covalent modification of G4 nucleic acids through a variety of strategies. Indeed, the hypothesis that G4-targeting ligands can be employed to confer selectivity to classical warheads (such as platinum complexes) appears to be attractive and shows significant promise. Some encouraging selectivity in nucleic acid modification has already been observed for such compounds, including between G4 and duplex/single-stranded DNA (e.g. NDIs **33**-**35**), between different DNA bases (e.g. **25**) and interestingly, between different topologies of G4 (e.g. **51**). Furthermore, it is also promising that the cytotoxicity in cells of several of these compounds can be triggered by exposure to light, in a photo-dependent manner. However, in many cases, the selectivity for particular nucleic acid sequences and structures likely requires further optimization to allows such molecules to serve as robust biological tools or therapeutic agents. A significant number of G4-targeting chemotypes remain to be explored for such purposes, leaving plenty of room for further development. The *in vivo* mechanism of action of such compounds would also benefit from further exploration to guide the development and optimization of G4-targeted photo-oxidative therapies.

4. Caged G4 ligands – *in situ* activation for targeted therapy?

Finally, we turn our attention to the concept of caged G4 ligands. These ligands are designed to remain dormant until their G4 binding is activated by a situational stimulus. Unlike the previous examples of responsive ligands considered in this review, these ligands have their G4 binding functionality masked from the outset and thus they are worthy of consideration in a separate section. These ligands offer interesting potential applications, such as in the design of G4-targeting prodrugs whereby binding to nucleic acids can be activated specifically in tumor cells with the aim of generating more specific pharmacologies.

4.1. Photocaged ligands

In 2012, the Nagasawa group reported the first example of a photocaged G4 ligand, the macrocyclic polyoxazole ONv-**59** (note

the similarity to the G4 binding drug telomestatin).[157] whereby the G4-binding activity is neutered by the presence of the nitroveratryl (Nv) group which can be removed in a quantitative yield in 30 minutes simply by photoirradation with 365 nm light. Indeed, FRET melting assays demonstrated the deactivation of G4 binding by the caging group, with ONv-**59** stabilizing G4 by only 4.0 °C and the unprotected form stabilizing by 16.6 °C. Indeed,

caging group:

Nv =

ONv-**59** (R = Nv)
OH-**59** (R = H)

NHNv-**60** (R = Nv)
NH$_2$-**60** (R = H)

Figure 24: Photocaged ligands for G4. The active form of the ligand is generated by *in situ* removal of the protecting group by irradiation with 365 nm light.

gel electrophoresis showed that no DNA ligand complex was

formed by ONV-**59** and telo21 DNA in absence of light, but photoirradiation of the system for 30 min generated a new band corresponding to the DNA ligand complex, evidencing the photoactivation of the drug. Compound ONv-**59** was also shown to be ineffective at inhibiting telomerase in the dark, but upon photoirradiation its activity was restored with potency comparable to the pure uncaged version. Significantly, whilst OH-**59** was toxic to a range of cancer cells at the sub-micromolar level after a two-day exposure, the caged version remained inactive unless administered in tandem with photoirradiation, though the results of the study do not unambiguously confirm the mechanism of action to be a result of light-activated G4 binding.

The Balasubramanian group employed a similar strategy to cage the activity of pyridostatin, using again the nitroveratryl group to block one of the terminal amine residues (NHNv-**60**).[83] Like the Nagasawa compound ONv-**60**, the ligand can be quantitatively deprotected with UV light in 30 minutes. Again, the FRET assay confirmed that the caged ligand does not affect the stability of G4, but that G4 binding is restored to a level comparable to uncaged pyridostatin after irradiation for 30 min. Photo-dependent cytotoxicity was also observed for the compound, with the growth inhibition being very poor for the caged compound, and toxicity increasing in a time-dependent fashion, reaching the same level as pyridostatin after 30 min irradiation. Pleasingly, the authors also found the ligand effectively suppresses the expression of several G4-containing genes in live cells upon photodeprotection, whilst the caged version did not have such an effect. This suggests the mechanism of action of this compound could indeed be related to inhibition of gene expression by G4 folding, in a manner analogous to that shown in Figure 2.

4.2. Redox caging

A final example of a caged G4 ligand, provided by Vilar and co-workers, is particularly interesting since it uses the redox and co-ordination state of the metal to activate its G4 binding activity. The team developed a novel synthesis of platinum(IV) salphen complex **61** by oxidizing the corresponding platinum(II) precursor (**62**) with a hypervalent iodine reagent.[84] In the oxidized state, compound **61** has poor affinity for G4, possibly because of disruption of stacking interactions by the axial chloride groups. However, the compound is readily reduced to the platinum(II) species by glutathione and ascorbic acid, both common reducing agents *in vivo*. Pleasingly, the square planar complex has good affinity (K_a in the order of 10^5 M^{-1}) for G4 DNA. These results suggest that analogues of **61** may display interesting toxicity for hypoxic cancer cells, owing to their reducing environment. It will be interesting to see whether this exciting application can be realized to achieve selectivity in different tumour types characterized by different redox environments.

4.3. Conclusions

Given the ever-growing quest to develop more specifically targeted therapies through pro-drug strategies, it is encouraging to see initial progress towards this area in the field of G4-ligand design, despite the fact that such molecules have yet to reach the stage of clinical application. Light has again emerged as a favored trigger for the activity of particular G4 ligands, however, whilst light does indeed offers significant advantages over chemical stimuli, the challenges associated with its clinical use, its deliverability *in vivo* remain to be fully demonstrated in a wide range of situations. In the examples described above, the wavelength of light required to uncage the ligand is incompatible with *in vivo* application due to poor tissue penetration. Thus, given these current limitations, it is encouraging to see the emergence of complementary strategies for the *in situ* activation of G4 ligands that could be exploited towards therapeutic ends in the Pt-salphen complexes that may be activated by reducing environments, suggesting applications in hypoxic tumors whereby the trigger is endogenous rather than externally administered. All of these systems can surely be optimized and no doubt many further opportunities to control the *in situ* release of G4-targeting pro-drugs will be developed in due course.

5. Summary and outlook

The past two decades have seen great strides in G4 ligand development. From Hurley and Neidle's seminal work on the discovery of a G4 ligand that efficiently inhibits the enzyme telomerase,[158] the first decade of G4 ligand development focused largely on optimizing G4 affinity and achieving discrimination against duplex DNA binding of such chemotypes. Impressive results have been obtained through these research programs leading to the emergence of several lead scaffolds that find wide application in biological studies and that represent starting points for further development. Work continues apace in this area, especially geared towards the hitherto elusive goal of achieving a G4-targeting therapy in the clinic. In the course of this review however, we have attempted to look beyond the use of G4 ligands simply to stabilize G4 structures and examine what else they might achieve towards realizing both clinical and non-clinical applications. This reveals significant progress over the past ten years that clearly demonstrates additional and wide-ranging opportunities for control of G4 by small-molecule ligands. We have considered how ligands are capable of over-riding innate topological preferences to perform mechanical work or control reactivity in response to external stimuli. Next, we have examined how G4 ligands can confer selectivity to warheads that promote the modification of nucleic acids, and finally we have summarized progress towards the development of G4-targeting pro-drug candidates. Through this perspective, we have highlighted the opportunities and challenges such approaches provide towards the functional applications of G4 ligands. Whilst significant progress has been made in these areas, further opportunities remain to optimize such systems to fulfil their potential applications as therapeutics and it is our hope that this review will encourage new researchers to enter these fields. Finally, we hope that by bringing together the key literature on these topics together, new approaches to deploying G4 ligands for a range of applications will be identified. We look forward to following the progress of this exciting field in the coming years.

Acknowledgments

Keywords: DNA • G-quadruplexes • nucleic acids • RNA • supramolecular chemistry

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Hybrid: telo23 (see Table 1), potassium.

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MINIREVIEW
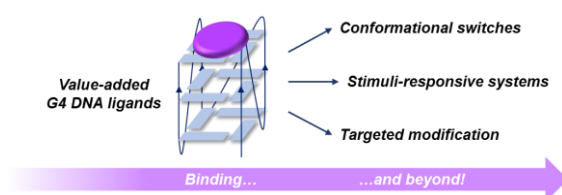
G-quadruplex (G4) ligands can do much more than simply associate with nucleic acids in a straightforward host-guest fashion. This review examines what else can be accomplished with G4-targeting molecules and considers the opportunities and challenges towards the development of potential applications in biology and nanotechnology.

G-quadruplexes

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Binding and beyond: what else can G-quadruplex ligands do?