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Junctions in DNA: underexplored targets for therapeutic intervention☆

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ABSTRACT

DNA has been a key target for cancer therapy, with a range of compounds able to bind and either impair its processing or induce damage. Targeting DNA with small molecules in a truly sequence specific way, to impair gene specific processes, remains out of reach. The ability of DNA to assume different structures from the classical double helix allows access to more specific ligand binding modes and, potentially, to new avenues of treatment. In this review, we illustrate the small molecules that have been reported to bind to three- and four-way junctions.

1. Introduction

Deoxyribonucleic Acid (DNA) conjures images of the B-form structure originally described by Watson and Crick,[1] Wilkins, Stokes and Wilson,[2] and Franklin and Gosling.[3] From a therapeutic perspective, this remains the principal target of many antitumour agents, from the intercalating topoisomerase inhibitor doxorubicin[4] to the DNA cleaving agent bleomycin[5] via the classical alkylating agents such as melphalan.[6] Other duplex DNA structures are well known: the A-form that was regularly seen in crystal structures until the work of Dickerson and Drew[7] and is the duplex form of ribonucleic acids (RNA); the left-handed Z-form that was discovered by Rich and co-workers.[8] The understanding of the genetic code that derived from the structural elucidation of DNA also led to the realisation that, while the classical antitumour compounds mentioned above interacted with nucleic acids in a relatively non-sequence specific way, the ability to target specific sequences could lead to gene-targeted drugs. The long-sought approach to targeting a specific sequence led to the design of oligonucleotides that could form a third strand in the major groove of DNA and effectively gave the first so called "higher-order" nucleic acid – the triplex or triple helix.[9] The constraints associated with targeting the groove (the need for positively charged cytosine (C), the limitations associated with targeting AT versus GC, the poor bioavailability of nucleic acids as drugs) mean that the success of the triplex approach to therapy has been limited, but it has led to the recognition that nucleic acid structures other than the duplex are available.

In 1997, Neidle, Hurley and their colleagues published the seminal paper on the targeting of the G-quadruplex.[10] The G-quadruplex had been known prior to this, the first crystal structure having been described by Rich.[11] Zahler and co-workers[12] had shown that the G-rich structures associated with telomeres structures could inhibit telomerase. As telomerase, the enzyme that extends telomeres, is active in cancer and as it could potentially be inhibited by the formation of a G-quadruplex structure, Neidle and Hurley surmised that compounds that stabilised the telomeric G-quadruplex could potentially have antitumour activity and subsequently showed this to be the case.[10,13] The presence of G-quadruplexes in oncogene promoters and in telomeres, and the possibility to use these as targets to block cell proliferation, generated interest in this alternative DNA structure.[13,14] In 1997, there were, in Pubmed, seven papers that referred to a G-quadruplex, in 2020, there were 723, demonstrating the interest in G-quadruplexes, their presence in the human genome, latterly in RNA sequences and as potential therapeutic targets.

If a G-rich sequence in a duplex is folded, what happens to the opposite strand? This question has, in part, led to the study of the i-motif, a C-rich strand that involves intercalated C structures, usually with one C partially-protonated to form a pseudo C—C^+ base pair, that was first described by Gehring and co-workers in 1993.[15,16] Similar to the G-quadruplex, later work has demonstrated that the i-motif can form at physiological pH and that molecules able to either stabilise or destabilise the i-motif can control the transcription of oncogenes and act as activators or repressors, depending on the specific promoter region and transcription factors.[17] These discoveries opened a clear path for the potential exploitation of i-motif targeting for cancer therapy.

A simple higher order DNA structure, that could potentially form very readily within a duplex DNA strand is a four-way junction (4WJ). There are extensive inverted repeat sequences (nucleotide sequences followed downstream by their reverse complement)[18] within the human genome, varying from small sizes of 6–9 base pairs through to very long sequences of over 40 base pairs. These sequences may be associated with

☆ In recognition and celebration of the work of our colleague Professor Stephen Neidle.

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transcription factor binding or conversely with the inhibition of transcription or could be a source of replication inhibition or genomic instability.[19] The four way junction is more widely known for its role in homologous recombination, suggested originally by Robin Holliday such that four way junctions are often known as Holliday junctions.[20] The process of homologous recombination is key to several bacterial processes, such that inhibitors of the process may have potential as antimicrobial agents *(vide infra)*.[21] Similarly, due to its role in double strand break DNA repair, homologous recombination has been suggested as a target for inhibition in cancer, perhaps in combination with inhibitors of single strand break repair processes such as PARP.[22,23]

Three-way junctions (3WJ) in DNA and RNA are also higher order DNA structures that may have potential as drug targets. These are constituted of complimentary sequences or inverted repeats which come together in a Y shape. Similarly to other non B-DNA structures, the ability of sequences to fold into non canonical structures depends on the stability of the sequence and the state of DNA. While B-DNA is usually the most stable form and is coiled around histones, unfolding, strand opening and supercoiling during DNA processing can facilitate 3WJ formation.[24] The DNA replication fork could be considered an archetypal 3WJ, although it is not complete at the junction.[25] The 3WJ junction has also been involved in DNA repair and recombination, including the Cre-Lox recombination path, an important biological tool for the manipulation of genomic DNA.[26] Although targeting of DNA is often associated with cancer therapy, the ability to selectively target specific DNA structures could function as a tool to investigate the role of 3WJs in other cellular and pathological mechanisms. An example could be triplet repeat expansion diseases. These diseases present slipped out repeats (e.g. (CAG)·(CTG)) which generate areas of duplex instability and can dynamically form 3WJs.[27–33]

In the following two sections, we will review the compounds which have been reported to bind either 3WJs or 4WJs, organised in classes.

2. Targeting four way junctions (4WJ)

2.1. The structures of 4WJs

In solution studies, the 4WJ has been found to adopt two main conformations - an open conformation, where a square-planar shape is formed in the middle of the junction and a more compact X-stacked form (Fig. 1). The open conformation is the structure usually adopted when the 4WJ is in a protein complex and has also been shown to occur in low salt concentrations, presumably due to the repulsion between negatively charged phosphate groups on the DNA backbone. The X-stacked conformation occurs in solution in a high salt concentration, where two pairs of DNA strands stack coaxially at a 60-degree angle to each other (Fig. 1). This can form due to the negative phosphates being shielded by the positive salt cations in solution. The two duplex strands come together antiparallel and one DNA strand from each pair forms a U-turn at the junction.[34]

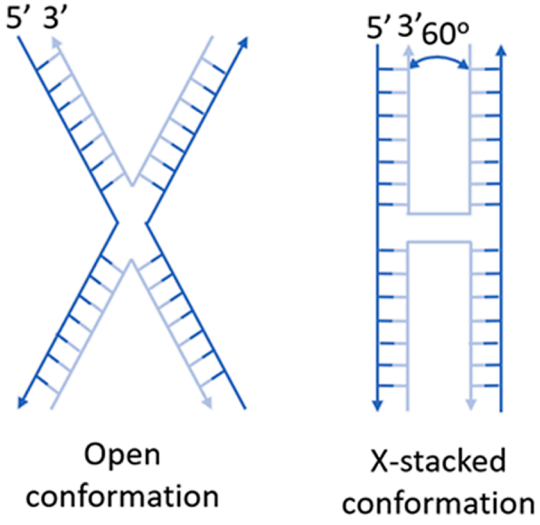


Fig. 1. 4WJ conformations.

2.2. Peptide ligands

2.2.1. Peptide WKHYNY

Segall's group pioneered the area of peptides targeting 4WJ, with an evolving set of peptides (Fig. 2) and mechanistic investigations into their binding and biology. After carrying out a large-scale library screen of hexapeptides, they found that the peptide WKHYNY was able to stabilise the 4WJ intermediate involved in Phage λ integrase (Int)-mediated recombination. This occurred when the junction was in its open conformation, preventing proteins from binding to, and resolving, the 4WJ. This compound was used as a biological tool when studying bacterial pathways. Although the peptide was unable to inhibit the overall growth of bacteria, they did find WKHYNY inhibited the bent-L pathway resulting in the build-up of 4WJ intermediates.[35]

To further understand the binding of WKHYNY to the 4WJ, a crystal structure was partially resolved, although the exact binding of the ligand remained unclear. This showed that the ligand bound to the centre of the square planar 4WJ pocket. This was further demonstrated by replacement of adenine bases at the centre of the 4WJ with 2-aminopurine (2AP) residues, a fluorescent analogue. Peptide binding was shown to quench the fluorescence of the solvent exposed residues at the centre of the 4WJ, due to the stacking of aromatic rings.[36]

2.2.2. Peptide WRWYCR

An additional screen, focussed on the inhibition of the excision pathway for site-specific recombination in Int, led to the discovery of the peptide WRWYCR, which had nanomolar potency against both free and protein bound 4WJ. WRWYCR binding was strongly inhibited by the presence of Mg^{2+}, showing the peptide binds to the open conformation. WRWYCR was found to have more than 50 times the potency in the excision pathway when compared to previously made hexapeptides.[37]

Further analysis showed that the formation of a dimer was required for WRWYCR to be active. This was confirmed by addition of the reducing agent dithiothreitol (DTT), which drastically reduced the potency of the peptide, by preventing disulphide bridge formation between the cysteine residues. The replacement of the cysteine residues by lysine also reduced the activity of the peptide. To confirm the formation of a dimer, two monomers of WRWYCR were cross-linked using bismaleimidohexane (BMH) (Fig. 2) to bind to both cysteine sulphide groups. Although the amount of HJ trapping reduced slightly, the addition of DTT did not affect activity.[37]

Modelling of the active dimer to a Cre-*Lox* 4WJ complex predicted that the dimer forms both intramolecular and intermolecular stacking interactions with DNA bases, and H-bonds with both deoxyribose and base residues (Fig. 3).[38]

Extensive studies to elucidate the biological effects of WRWYCR led to testing against the other three Int recombination pathways with good inhibition against the bent-L and integration pathways and less effective inhibition against the straight-L pathway. When testing WRWYCR against the Cre-*Lox* recombination complex, found in phage *P*1, the

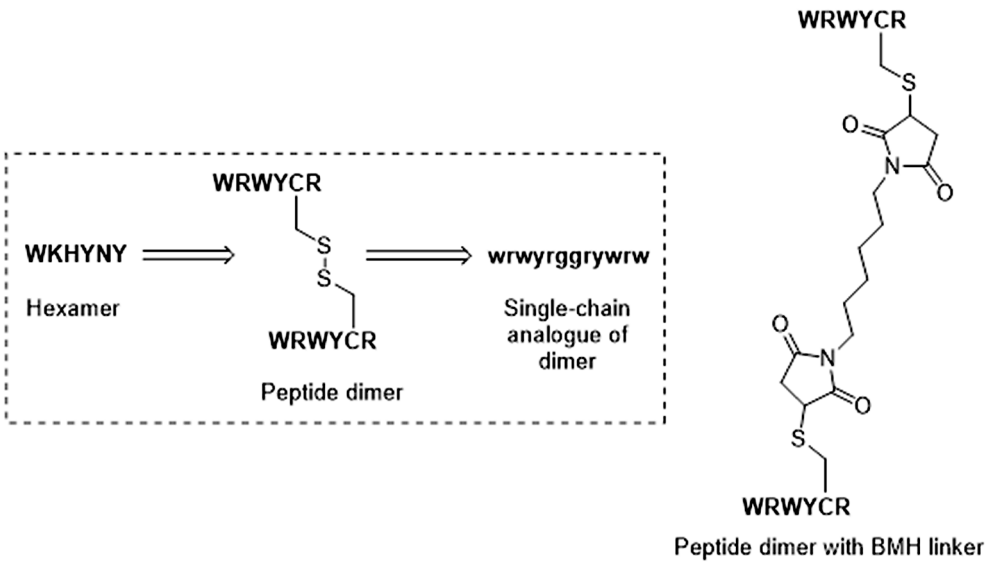


Fig. 2. Development of 4WJ peptide inhibitors.

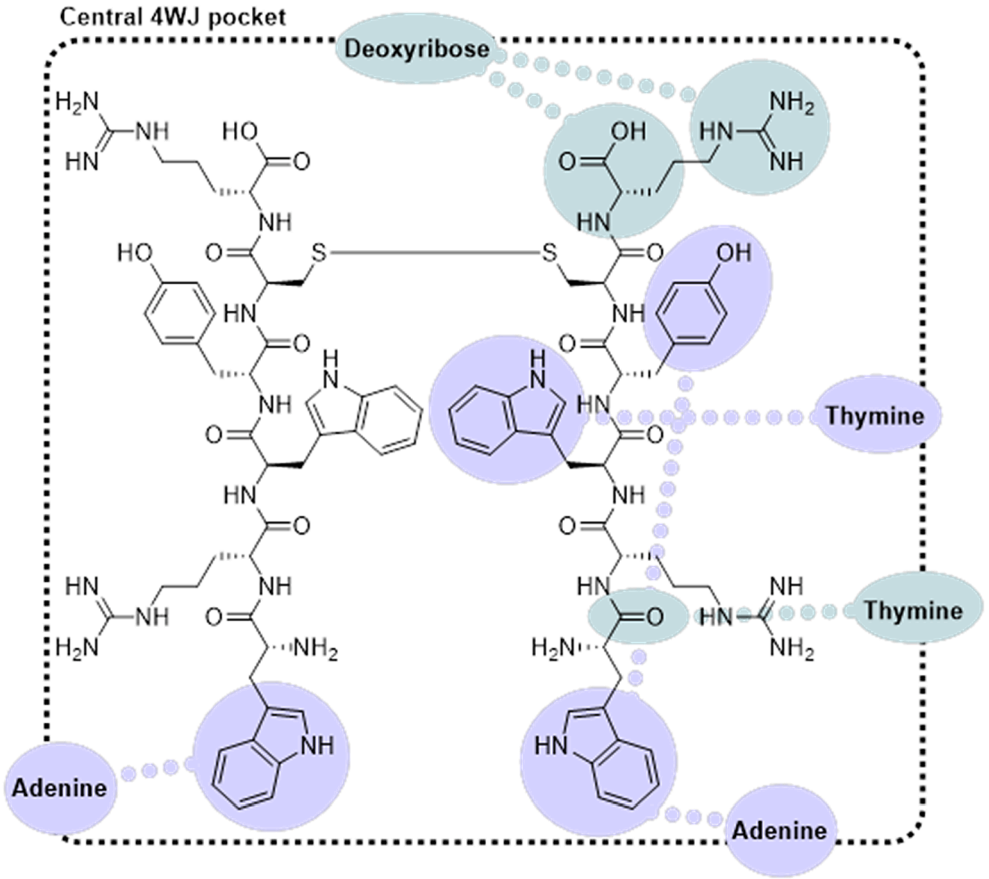


Fig. 3. Potential binding interactions between the Cre-Lox 4WJ and the WRWYCR dimer. π-π interaction are highlighted in purple and hydrogen bond interactions in green.

peptide was found to have a slightly reduced potency, when compared to the excision pathway of Int. Other peptides screened in the library of hexapeptides were found to have similar potency against both the Int pathways and Cre-*Lox* pathway.[37] This demonstrated how different peptides interact with each 4WJ complex distinctly.

WRWYCR was able to inhibit the binding of several proteins

including the RecG helicase and RuvABC complex of E. coli. WRWYCR acted as a competitive inhibitor of RecG-4WJ binding in competition assays using gel mobility shift.

The selectivity of WRWYCR for 4WJs over 3WJ replication fork substrates was demonstrated to be 4- to 9-fold. This was confirmed by the higher concentrations of WRWYCR required to quench the fluorescence of 2-AP bases at the centre of the 3WJ. In addition, WRWYCR has a 240-fold preference for 4WJs over non-specific ds-DNA in further competition assays.[39]

WRWYCR was found to have antimicrobial activity against both Gm + and Gm- bacteria, with increased activity against Gm + bacteria. The D-isoform wrwycr had slightly higher bacterial growth inhibition, with the growth of Bacillus subtilis fully inhibited for 8–10 h by 5 μM wrwycr compared to 8 μM of WRWYCR. This was speculated to result from the increased resistance of the D-isoform to cellular peptidase degradation compared to the L-isoform. The effect of wrwycr on the bacteria caused an SOS response and cell death due to the trapping of 4WJ interfering with the repair of collapsed replication forks. The ability of the peptide to target multiple pathways could help reduce antibiotic resistance.[40]

It has been shown that both the D- and L- forms of WRWYCR are able to enter the bacterial cell and are capable of in vivo inhibition of site-specific recombination. This was demonstrated by the inhibition of both excision and replication of the prophage Fels-1 in Salmonella from within the chromosome.[41] Both forms were also shown to inhibit the growth of Samonella cells in the presence of mammalian cells, reducing the number of bacterial cells recovered after 24 h by 100–1000 times. The peptides were shown to be non-toxic to murine cells at 50 μM, with no increase in cell death observed.[42] The ability of WRWYCR to exhibit selective antimicrobial effects makes it a promising starting point for developing novel antibiotics targeting DNA repair pathways.

2.2.3. Peptide WRWYRGGRYWRW

In 2013, the Segall group investigated making a single chain analogue of WRWYCR to develop a compound that does not have to form a dimer in the 4WJ, with the disulphide bond being susceptible to reduction in the cellular environment. This involved designing 10–12 amino acid analogues, resulting in the development of WRWYRGGRYWRW. This peptide is of a similar size to the dimer, has the same number of positive charges and avoids unwanted dimerization by the removal of cysteine groups.

WRWYRGGRYWRW was able to bind to the open conformation of the 4WJ with a similar affinity to WRWYCR, had similar potencies in excision pathways and remained active in the presence of DTT. Overall, it was found that the D-isoform of the single-chain peptide had more effective 4WJ binding and was expected to have increased resistance to proteolysis when compared to the L-isoform. When testing antimicrobial activity, wrwyrggrywrw had an equal or greater potency against both Gm + and Gm- when compared to wrwycr. However, wrwyrggrwrw had increased non-specific DNA binding. This may be caused by the increased number of positive charges and aromatic rings resulting in wrwyrggrywrw having increased non-specific interactions with different forms of DNA. This is reflected by the higher level of toxicity against eukaryotic cells. Despite this, when testing the peptide against eukaryotic cells the concentration necessary for bacterial growth inhibition remained sufficiently low to be non-toxic.[43]

2.2.4. Cyclic peptides

Following the discovery of WKHYNY, an investigation to produce cyclic peptides with increased specificity for 4WJs compared to other DNA isoforms was disclosed. In two separate studies, in 2003[45] and 2004,[46] small cyclic peptide libraries containing hexamers and octamers were produced (Fig. 4). The design of the cyclic peptides was focused around a 2-fold symmetry to mimic the symmetry of the 4WJ.

The initial screening of eight cyclic peptides showed that three of the compounds were able to trap 4WJs in the open conformation. These compounds contained the aromatic amino acids 2a-3a, 2a-3b and 2c-3b. The ability to trap the 4WJs was assessed by the blocking of RuvC endonuclease, in Escherichia coli (E. coli), from eliminating 4WJs, when at a 1 μM concentration of compound.

For the second screen, an effort was made to introduce hydrophilic, polar residues, to increase solubility and permeability. Eighteen compounds were synthesised with eleven tested against RuvC. It was found that all compounds containing tyrosine residues were able to trap 4WJs, showing the importance of this residue for 4WJ binding. It was also shown that both hexa- and octa-peptides could be accommodated, demonstrated by the more effective 4WJ trapping of the octamer with amino acids 4a-5a-6a when compared to the similar hexamer 4a-5d-6a (Fig. 4).

When the cyclic peptides were tested against both gram negative and positive bacteria, they were found to have no effect on cell growth. This could result from the compounds remaining too hydrophobic to cross bacterial cell walls, with the introduction of further hydrophilic residues required for anti-bacterial activity.[44]

2.3. Small molecule ligands

2.3.1. Early compounds

Early studies into the binding of metal complexes to an immobile 4WJ were carried out by Kallenbach et al.[50] They found that MPE-Fe^{II} (Fig. 5) was able to cleave a 4WJ within two residues of the central pocket, with intercalation occurring next to the branch point, and it appears likely that a similar behaviour is exerted on 3WJs.[51] Well-known DNA binding dyes, which appeared to bind in a different fashion in presence of 4WJs, were characterized using a combination of spectroscopy, competition, DNA cleavage and footprinting assays. It was suggested that the Stains-All dye and propidium iodide (Fig. 5) bind to the centre of the 4WJ.[48] On the other hand, tetrapyridyl porphyrins with varying metal groups and/or axial ligands, were suggested to bind near the branch point, although the binding mode has not yet been fully elucidated.[47]

2.3.2. Acridine dimers

Acridines feature aromatic systems and basic/cationic groups and have been studied in relation to 4WJ inhibition. These are compounds that have been known for their therapeutic properties since the 19th century.[52] Interest in the use of acridine molecules began in 1996, when Carpenter et. al. investigated the use of acridine and phenanthridine compounds, as reagents to probe DNA structures. This involved using various rigid linkers (Fig. 6) to bind two intercalating groups, that were then tested against 4WJs. Although the results are inconclusive, the most well discussed compound, acridine APEPA, binds to the 4WJ in a different way to its monomer AP (Fig. 6), and potentially at the junction.[53] Considering the following discoveries on acridine dimers, it would be interesting to see these compounds re-evaluated with other techniques.

Dimeric acridines were also investigated by Searcey et al. Compared to the previous structures, the linker is more flexible and the acridines carry an additional N,*N*-dimethylaminoethyl caboxamide in position 4. This type of substituted acridines are well-known cytotoxic compounds that act as topoisomerase inhibitors and have been shown to be active against tumour cells. Compound C6 (Fig. 7), with a six carbon flexible linker, was shown bind to a 4WJ in the central region. A crystal structure

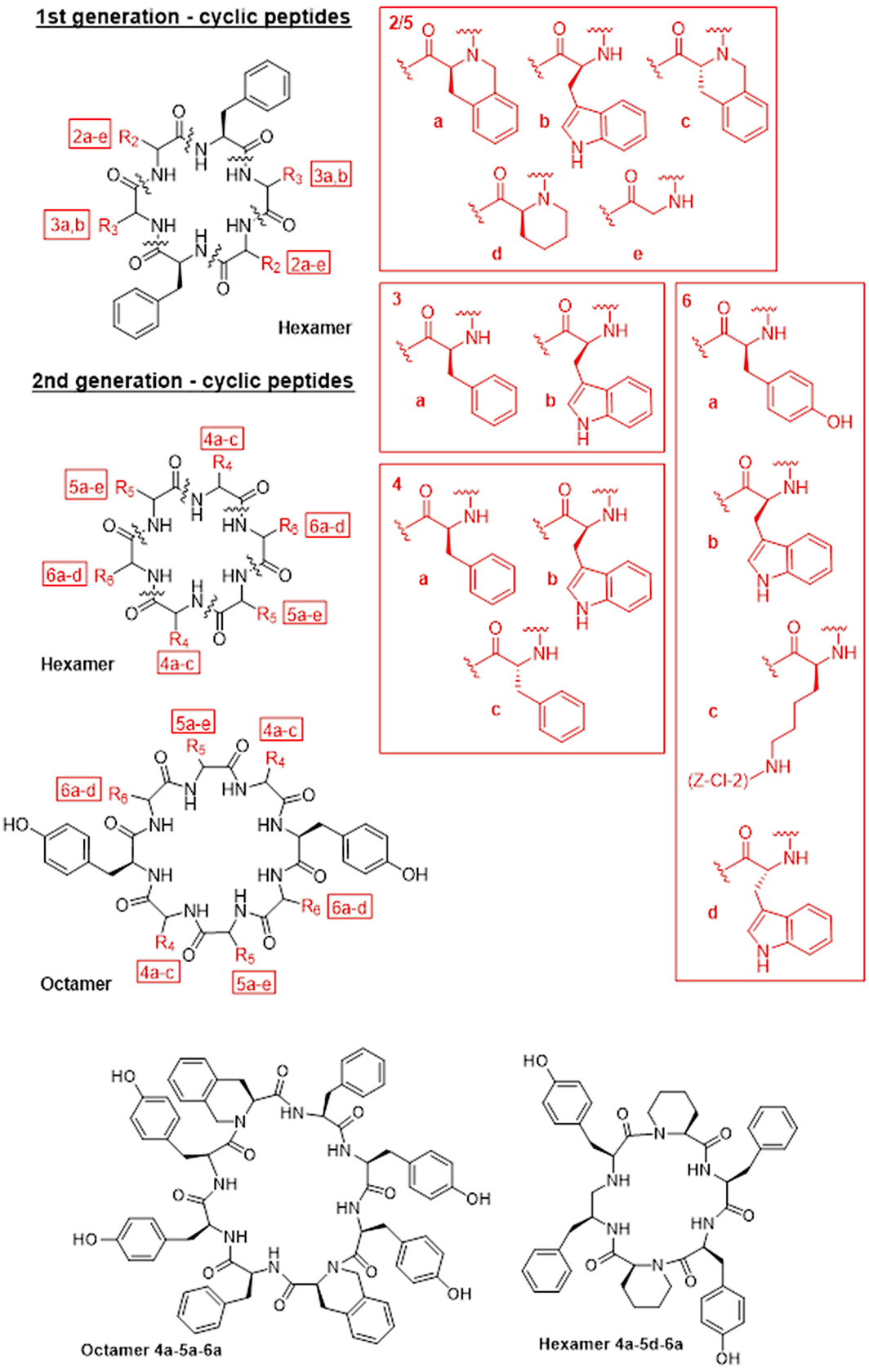


Fig. 4. Cyclic peptides.[44]

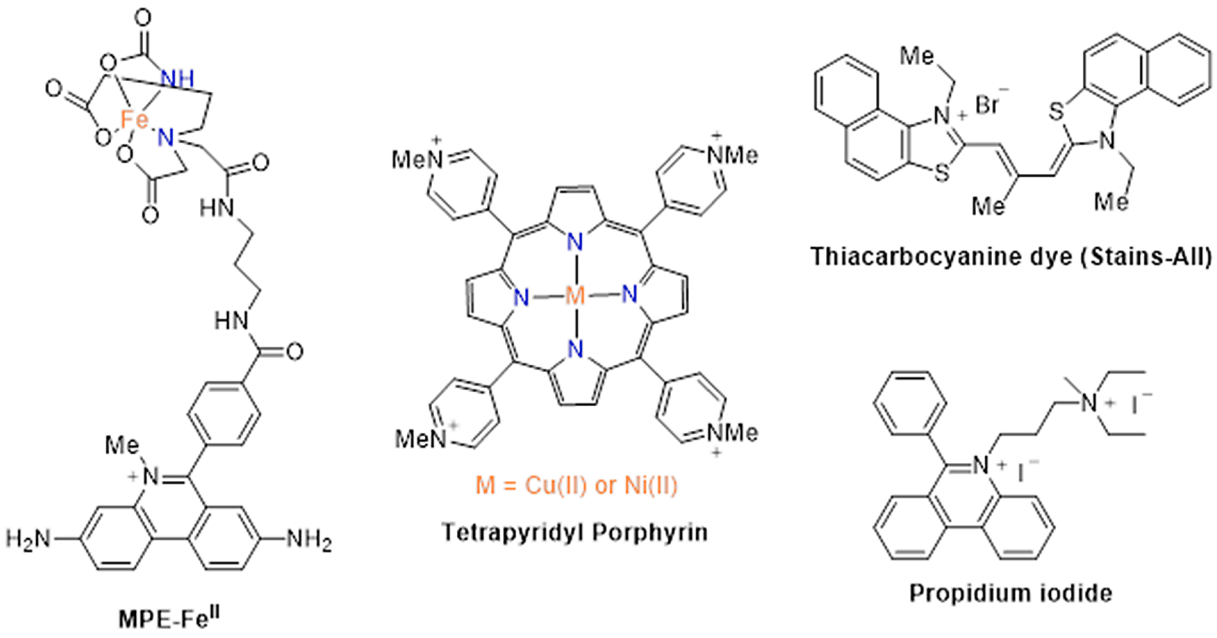


Fig. 5. Early compounds described by Kallenbach et al.[47–50]

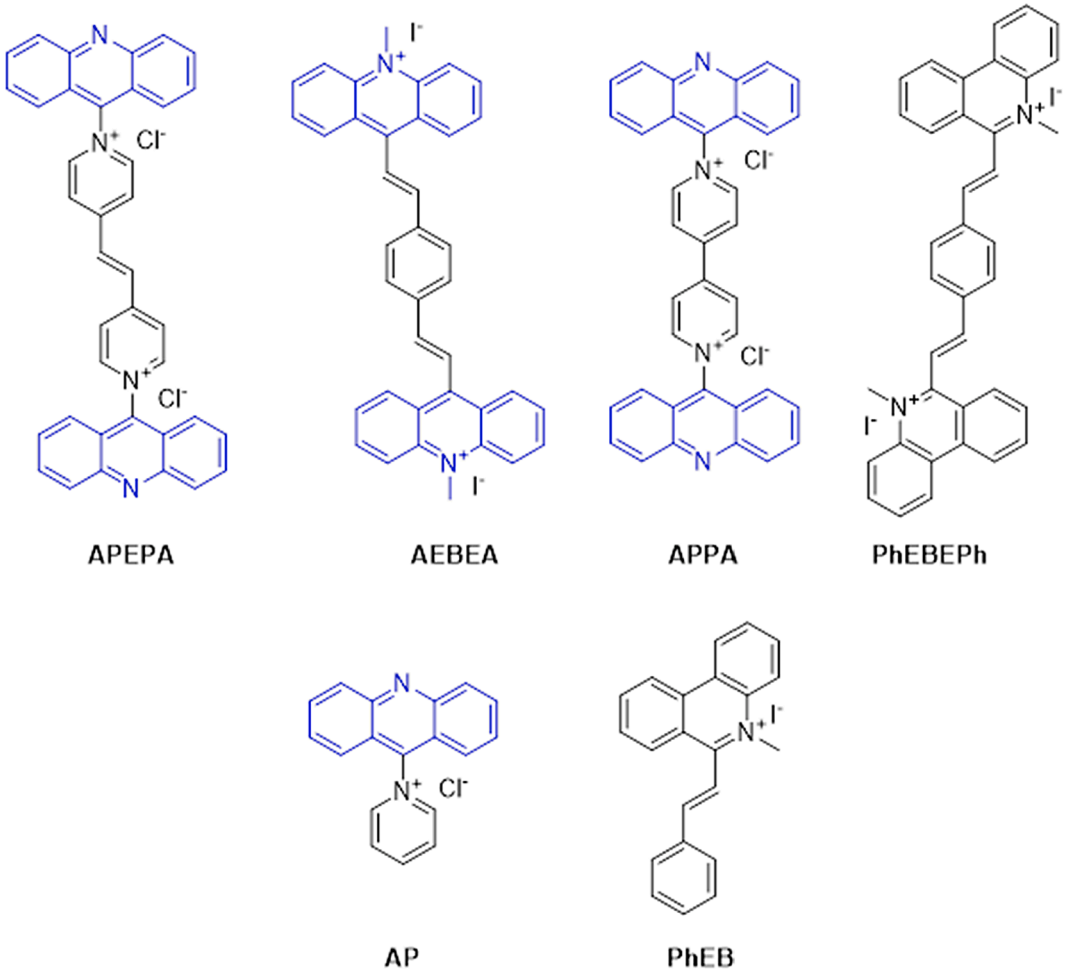
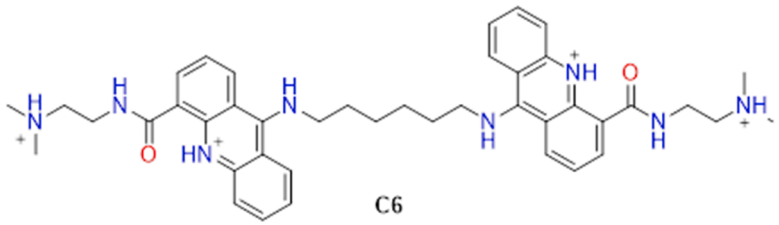


Fig. 6. Acridine dimers with rigid linkers. The compounds are named after the chemical groups they contain: A = acridine, B = benzene, E = ethylene, P = pyridinium, Ph = phenanthridinium.



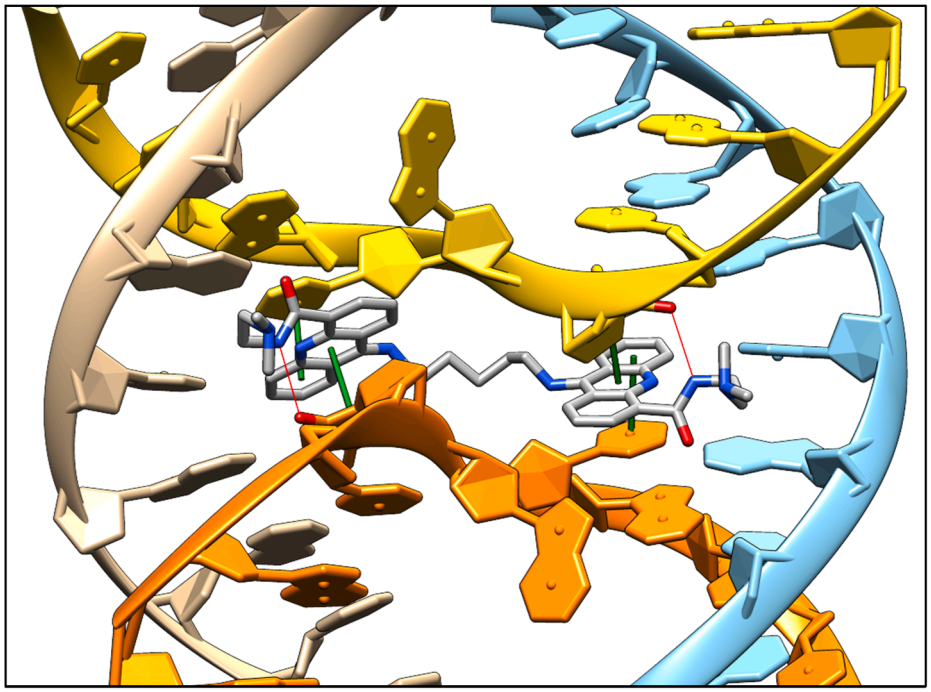


Fig. 7. Structure of C6 and its binding to a 4WJ. Each DNA strand is represented in a different color (yellow, orange, cyan and tan). C6 is represented as grey sticks with red oxygens and blue nitrogens. Green lines represent π-π interactions, hydrogen bonds are represented in red. Some atoms have been hidden for clarity. PDB: 2GWA.[54]

of the compound bound to a 4WJ (PDB code: 2GWA, Fig. 7) clarified this non-covalent binding mode across the centre of the 4WJ when in the X-stacked conformation.[54] It showed that the two acridine chromophores bound with a 2-fold symmetry through the centre of the 4WJ. Two adenine bases were flipped out to the major groove and replaced by the acridine groups, forming a pseudo-base pair with thymine. Extensive π-π interactions (Fig. 7, green lines) occur between the acridines and DNA bases. Additionally, H-bonding was observed between the amide hydrogen of the acridine side-chain and the oxygen of a cytosine base (Fig. 7, red lines). This results in the cytosine being misaligned with its base-paired guanine, causing hydrogen bonding between the base pairs to become longer than usual.[55]

A different set of acridine dimers (Fig. 8), with a short flexible chain and two aromatic groups between the monomers, was obtained via click chemistry. Of these, only BA2 and BA3 displayed 4WJ binding. Interestingly, compound BA3, is the only compound in the group which does not display antiproliferative activity, despite its ability to also bind dsDNA.[56]

2.3.3. *Clicked acridine monomers*

A key advance in 4WJ binding was the discovery of compound RTA (Fig. 9). Normally, folding of an open 4WJ into its X-stacked form requires high temperature annealing in the presence of Mg^{2+}. Compound RTA, unique among compounds discovered to date, not only targets 4WJs, but induces folding of the X-stacked form at room temperature. This effect was confirmed both via gel electrophoresis and CD. The compound was also found to be active against tumour cells, although this may result from the low level of selectivity of the compound for 4WJ

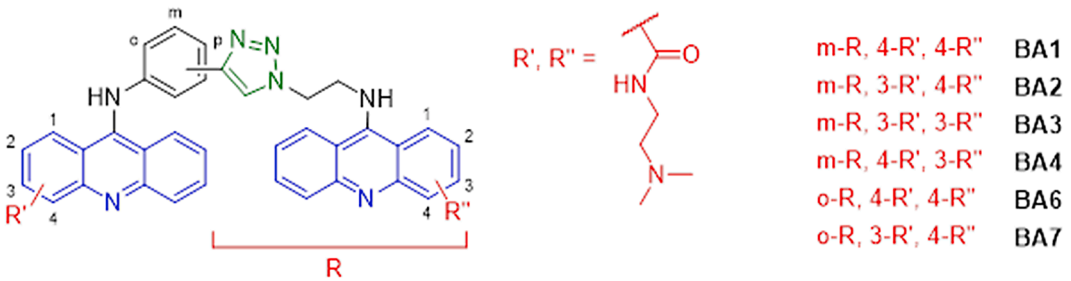


Fig. 8. Clicked acridine dimers.

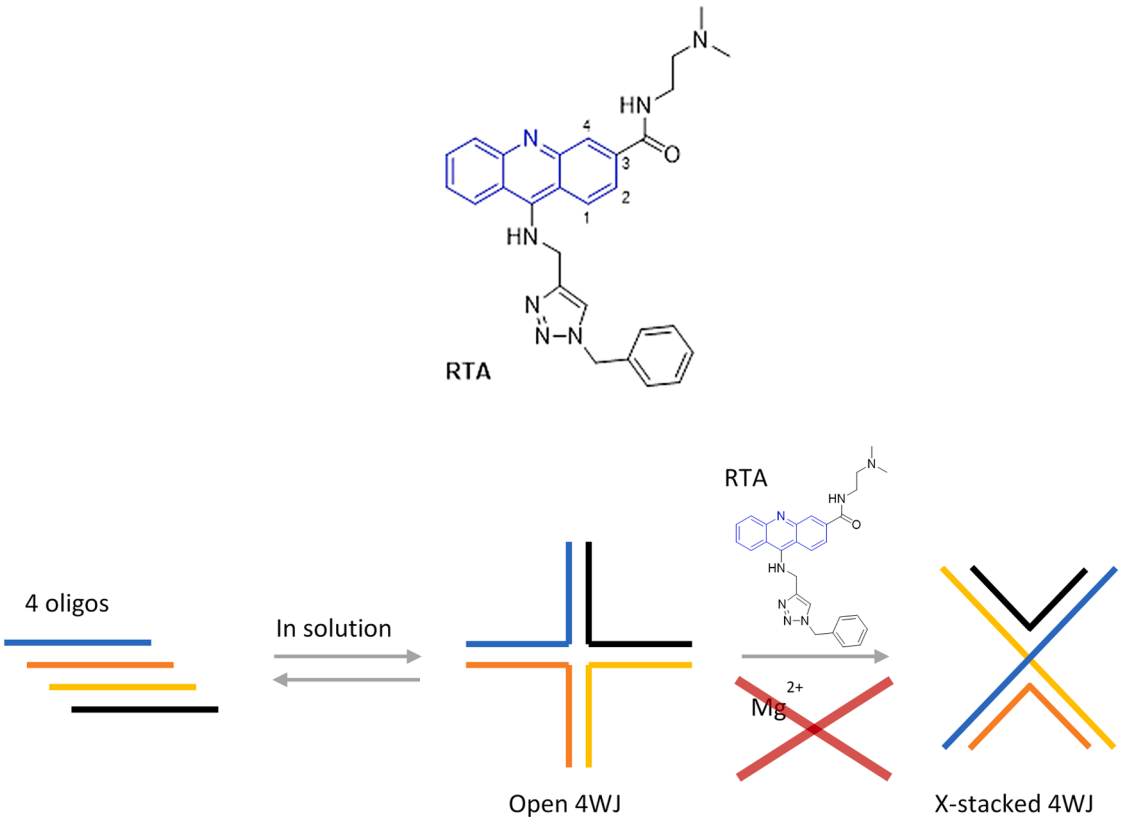


Fig. 9. RTA and folding of an X-stacked 4WJ through annealing in the presence of Mg2 + or in the presence of RTA.

and duplex DNA.[57]

Following attempts to synthesise compounds with the same ability to fold 4WJ (Fig. 10), three new structures with similar features, A14, A15 and A21, were identified. Similarly, to RTA, they all have the dimethylamino-side chain in position 3, and the distance between the aromatic groups is six atoms – 2 atoms shorter than the original C6 compound (6 carbons + 2 nitrogens). A14 and A15 also highlight how a small substituent in position 4 on the phenyl group is tolerated. It is interesting to note, on the other hand, that compounds A14 and A15 displayed antiproliferative activity, differently from A21, which is inactive. With the data available, it is difficult to draw a conclusion on the origin of their biological activity,[56] although it is possible that the lack of activity stems from poor cellular uptake by the acridine-3-carboxamides (unpublished observations).

Modulating folding at room temperature is a fundamental achievement and could allow regulation and folding in cells and material applications. The major limitation of acridines is their affinity for dsDNA, and advancement in their selectivity or discovery of new classes of compounds with the same folding ability would be a welcome addition in the field of 4WJ targeting.

2.3.4. *Triaminotriazine-Acridine conjugate*

Recently, the study of acridines in relation to 4WJ was continued by Chien et. al. when looking into a known DNA-binder; triaminotriazine-acridine conjugate (Fig. 11). This compound targets CTG trinucleotide repeats containing T:T mis-matches, a target that has been found to cause various neurological diseases. With the mechanism of binding remaining unknown, the team crystallised the compound with three CTG trinucleotides containing T:T mis-matches and found that a non-canonical 4WJ was formed (PDB: 6M4T, Fig. 16).

Instead of forming a standard X-stacked conformation they found that both duplex strands performed a unique U-turn when facing head-to-head with the other duplex. The two acridine-conjugate molecules sit at the centre of two overlapping -GCTGC- 5-mers. The conformation adopted by the ligands shows the acridine and triaminotriazine stack over each other, with the acridine further stacking with a guanine base. The insertion of the triaminotriazine moiety between DNA bases causes the flipping out of thymine's, resulting in the DNA backbone being able to bend more than 90° to form a 4WJ structure.[52] (see Fig 16).

2.3.5. *Pt complexes*

Extensive research has been carried out into the ability of *trans*-Pt complexes to bind to various DNA motifs. This started with the development of *cis*-platin (Fig. 12), a commonly used chemotherapeutic which targets duplex DNA. Following this, as detailed below in this review, Hannon and co-workers developed binuclear Pt complexes capable of binding to a 3WJ with DNA 6-mer oligomers of 5′-d(CGTACG)-3′ (Fig. 12). In 2019 Bonnet et. al. discovered a mononuclear platinum (II) complex, [Pt(Hbapbpy)]PF_6, that was able to cause the four oligomers of this same DNA sequence, 5′-d(CGTACG)-3′, to crystallise into a 4WJ-like motif (PDB: 6F3C, Fig. 16).

They found that two molecules of the Pt complex bound to form an X-stacked 4WJ, with the complexes sitting at the centre of the junction. The complexes adopted a distorted square planar structure when binding, with relatively flat structures, and were most likely to adopt a co-planar arrangement in relation to each other. The Pt complexes were found to form π-π interactions with the DNA bases and were stabilised by electrostatic interactions between the positively charged Pt atom and negatively charged DNA backbone. The two NH groups may be involved in H-bonding, although the longer length of the N…O contacts (2.6–4.1

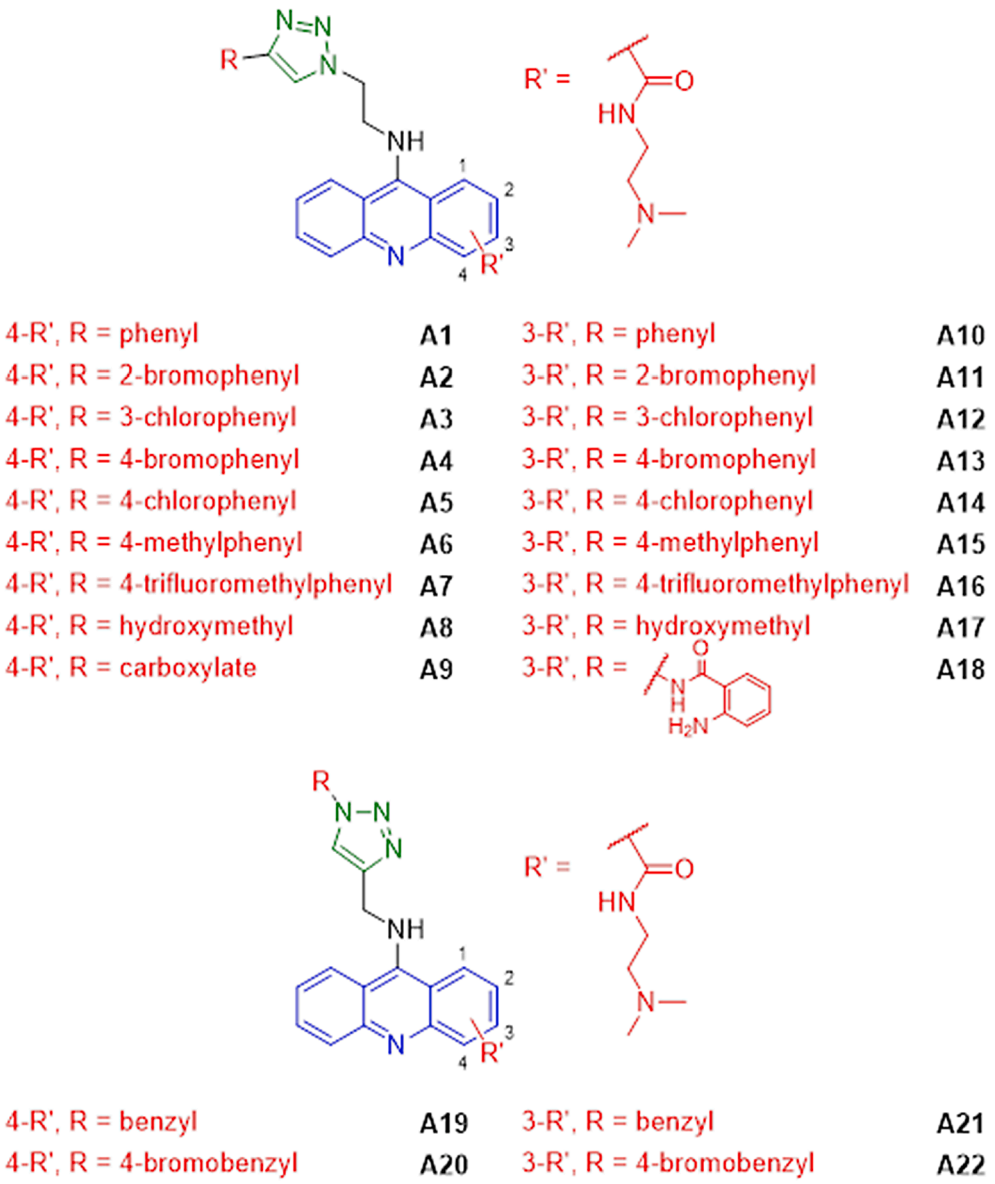


Fig. 10. Clicked acridine monomers.

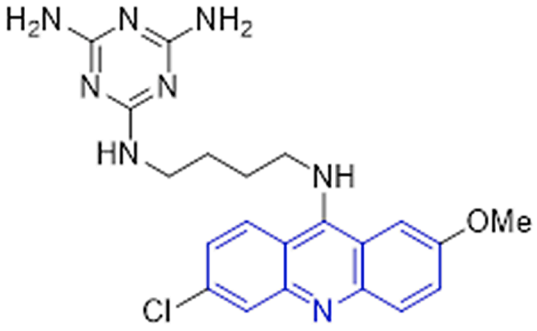


Fig. 11. Triaminotriazine-acridine conjugate.

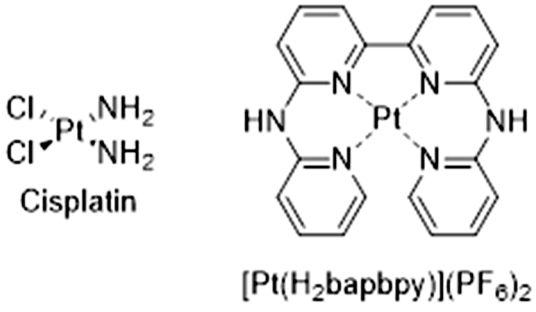


Fig. 12. DNA binding Pt complexes.

Å) indicates a weaker interaction.

When comparing the antiproliferative activity of this Pt complex to *cis*-platin in cancer cell lines, it was found that the new Pt complex was more potent than *cis*-platin and retained low micromolar potency against *cis*-platin resistant cells.[58] However, this could result from interactions of the complex with dsDNA, with further analysis required of the selectivity of the compound.

2.3.6. Ve-822

In late 2021 Wang et. al. established that the known drug VE-822 (Fig. 13), an ATR inhibitor in clinical trial for cancer with the

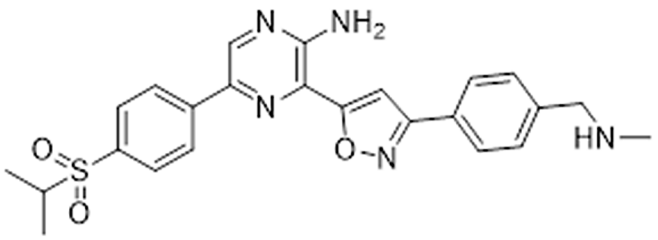


Fig. 13. Structure of VE-822.

commercial name Berzosertib, also promotes the formation of 4WJs. It was discovered in a screening of 160 compounds from a focussed library of DNA damage and repair compounds. By carrying out a CD assay and docking studies they confirmed that the compound converts a 4WJ to its X-stacked form. VE-822 has single digit micromolar potency for 4WJ formation and low levels of binding to ds-/ss-DNA. The group showed that VE-822 had significant anti-proliferation effects in siATR cells, which could indicate the stabilisation of 4WJ is contributing to the anti-cancer effects. The compound was shown to reduce levels of homologous recombination, triggering an increase in DNA damage, leading to potential apoptosis.

It has also been shown that VE-822 can bind to telomere-4WJs, albeit with 3-fold less potency when compared to 4WJs only. This could be of interest due to the alternative lengthening of telomeres (ALT) pathway containing 4WJ intermediates. This is a pathway that can lead to cancer cell immortality by allowing telomeres to lengthen and keep cells dividing. VE-822 has been shown to cause telomeric DNA damage by assembling telomere-4WJs. In osteosarcoma cells, known to rely on the ALT pathway, it was found that VE-822 was able to sensitise the cells to doxorubicin treatment. This could lead to a new approach for targeting telomeres.[59]

2.3.7. Cyclic thioureas and guanidines

In addition to their work on peptides, Segall's group investigated the development of small molecules for 4WJ inhibition. Initially the group carried out the screening of 9 million compounds with low molecular weights. They identified an *N*-methyl aminocyclic thiourea scaffold (**T1**, Fig. 14) able to trap 4WJs during site-specific recombination. However, due to low permeability, the compound did not exhibit antibiotic activity, as it was not able to enter the cell.[60]

Following this initial study, a more focused library was screened, involving compounds closer to the size and symmetry of the WRWYCR dimer. From this, a series of pyrrolidine bis-cyclic guanidine binders that affect DNA restriction were identified. Of these, only G1 (Fig. 14) was tested directly on protein free 4WJ and, compared to, WRWYCR, a lower binding affinity for 4WJ was observed, as well as lower inhibitory activity in RecG DNA repair. This may be caused by the reduced stability of the small molecules binding to 4WJs, caused by the absence of strong H-bonds. This is reflected by the reduction in flexible, basic groups when compared to WRWYCR.

Despite their lower 4WJ binding, the compounds were found to be more potent inhibitors of both gram-negative and gram-positive bacteria than the peptide hits. This probably resulted from the increased permeability of the small molecules. However, this series of compounds were found to inhibit eukaryotic cells, showing possible issues with toxicity. These promising compounds could be developed further with the addition of more basic functionalities to overcome difficulties with selectivity and stability.[61]

2.3.8. HMT

In 2001, Eichman et. al. found that 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT, Fig. 15) was able to induce the formation of a 4WJ. HMT is a type of Psoralen, a class of established photochemotherapeutics for the treatment of skin related diseases. To study the effects of cross-links formed by psoralens on DNA conformation, HMT was covalently cross-linked between two thymine bases in various DNA sequences and crystallised. It was found that the crystals formed a 4WJ as confirmed by X-ray crystallography (PDB code: 1FHY), with two HMT compounds bound to the 4WJ (Fig. 16, 1FHY).

HMT binding resulted in a naturally occurring 4WJ forming from four d(CCGGTACCGG) DNA sequences, with the ACC trinucleotide forming the stable centre of the junction. The binding of HMT to this 4WJ had little effect on the structure when compared to the unbound form. In contrast, the compound also induced a noncanonical 4WJ from four d(CCGCTAGCGG) DNA sequences. This is caused by the cross-linking of the HMT, which leads to the destabilisation of the dsDNA, allowing the exchange of strands and formation of the 4WJ. It could be conjectured that part of the therapeutic effects of psoralens may result from their ability to interfere with homologous recombination, potentially initiating cross-link repair in damaged cells.[62]

2.3.9. Comparison of different 4WJ-ligand crystal structures

With the emergence of multiple crystal structures containing X-stacked 4WJs bound to various ligands, Chien's team set out to compare the complexes formed. They found that the binding of each ligand

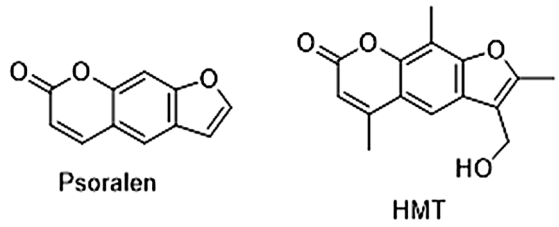


Fig. 15. Psorelan compounds.

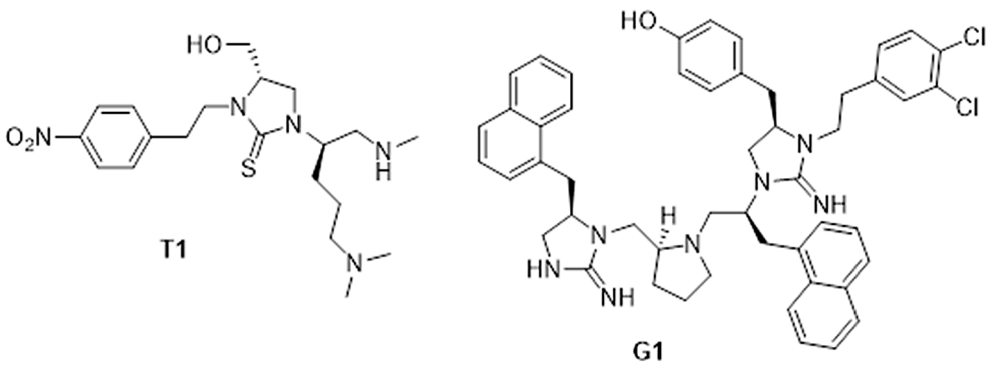


Fig. 14. Cyclic thioureas and guanidines.

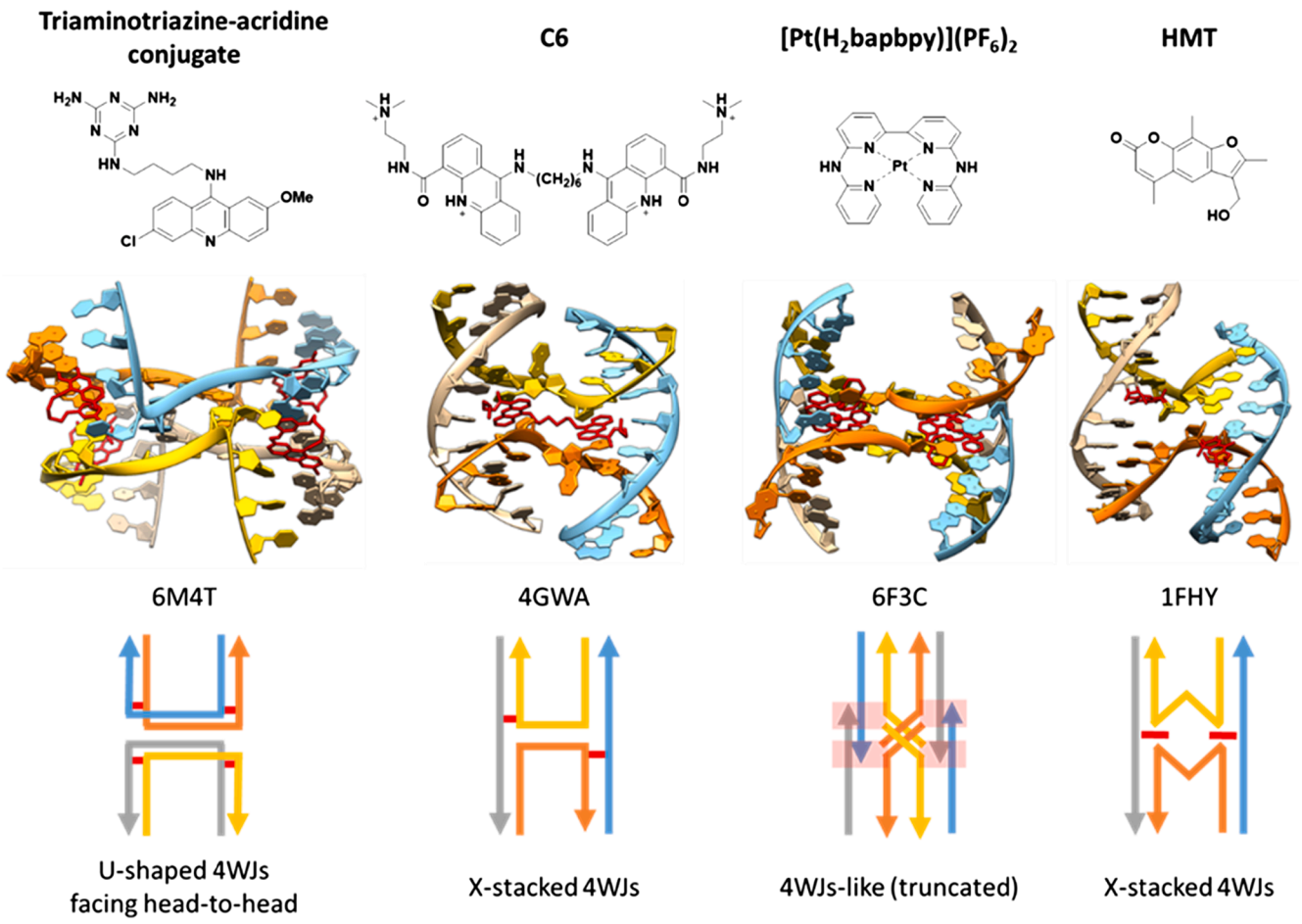


Fig. 16. Crystal structures or various ligands bound to 4WJs.[52]

results in a varying DNA topology Fig. 16. In addition, the Pt complex, acridine conjugate and HMT psoralen were able to form noncanonical 4WJs. The ability to induce the formation of specific higher-order DNA structures could have applications in the field of nanotechnology, allowing for the development of novel DNA sensors and superstructures.[52]

3. Targeting three way junctions (3WJ)

3.1. Helicates and the structure of 3WJs

Seminal work by Hannon and co-workers on helicates provided the first example of well characterised 3WJ binders. This class of

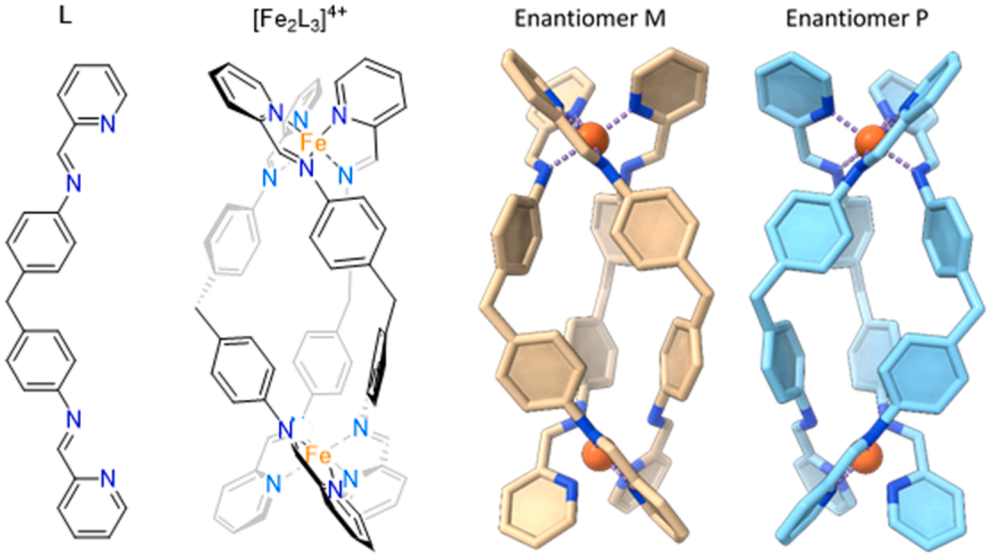


Fig. 17. $[Fe_2L_3]^{4+}$ helicate and enantiomer illustration.

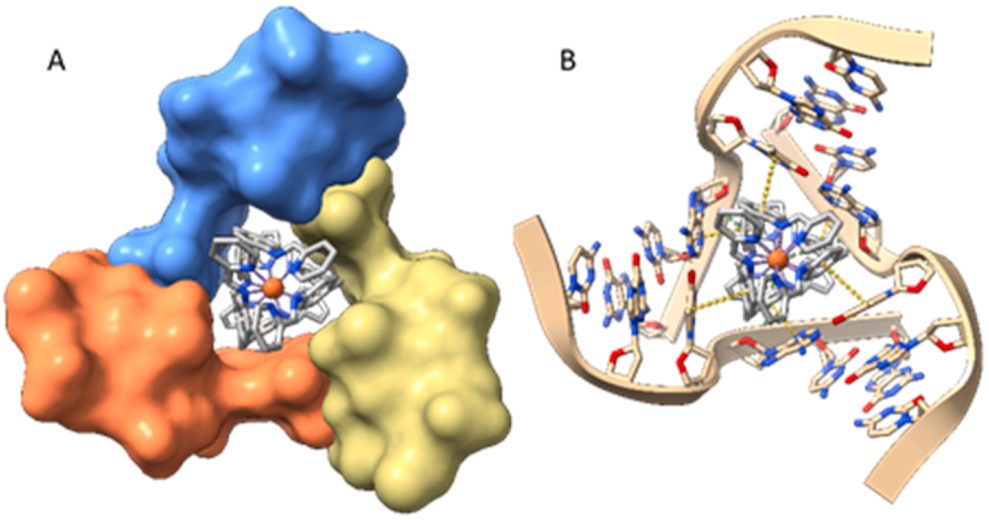


Fig. 18. Crystal structure of $[Fe_2L_3]^{4+}$, in grey, binding to a 3WJ. In A, the junctions is shown from the top and the three DNA strand surfaces are coloured in orange, blue and yellow. The same view, without surfaces, is shown in B. Yellow dashed bonds and green dashed bonds represent π-stacking and electrostatic interactions, respectively. PDB ID: 2ET0.

supramolecular structures are characterised by ligands with metal-chelating features, which can assemble as trimers, in complex with two divalent cations, in the form of $[M_2L_3]^{4+}$. The resulting complex has a helical structure, which is assigned an M or P enantiomer designation, depending on the handedness of the helix (Fig. 17).

Initial reports on helicates identified their ability to distort ct-DNA,[63] but they were later found to be excellent 3WJ ligands. A crystal structure of $[Fe_2L_3]^{4+}$ binding in the central junction of a 3WJ (Fig. 18) was reported as early as 2006.[64] The DNA junction displays a three-fold symmetry, with an almost-flat pyramidal arrangement. Neither the junction nor the ligand are distorted by the binding, with the ligand closely matching the size and symmetry of the junction. The B-form DNA opens at the junction with a 60° kink, closely followed by the turn in the helicate, allowing an almost perfect π-stacking between the two central phenyls of each ligand with the bases facing the junction (Fig. 18). Electrostatic interactions between the positively charged iron complex and the negatively charged phosphate backbone are also involved in the binding. Additionally, some short C—H—X interactions could also be

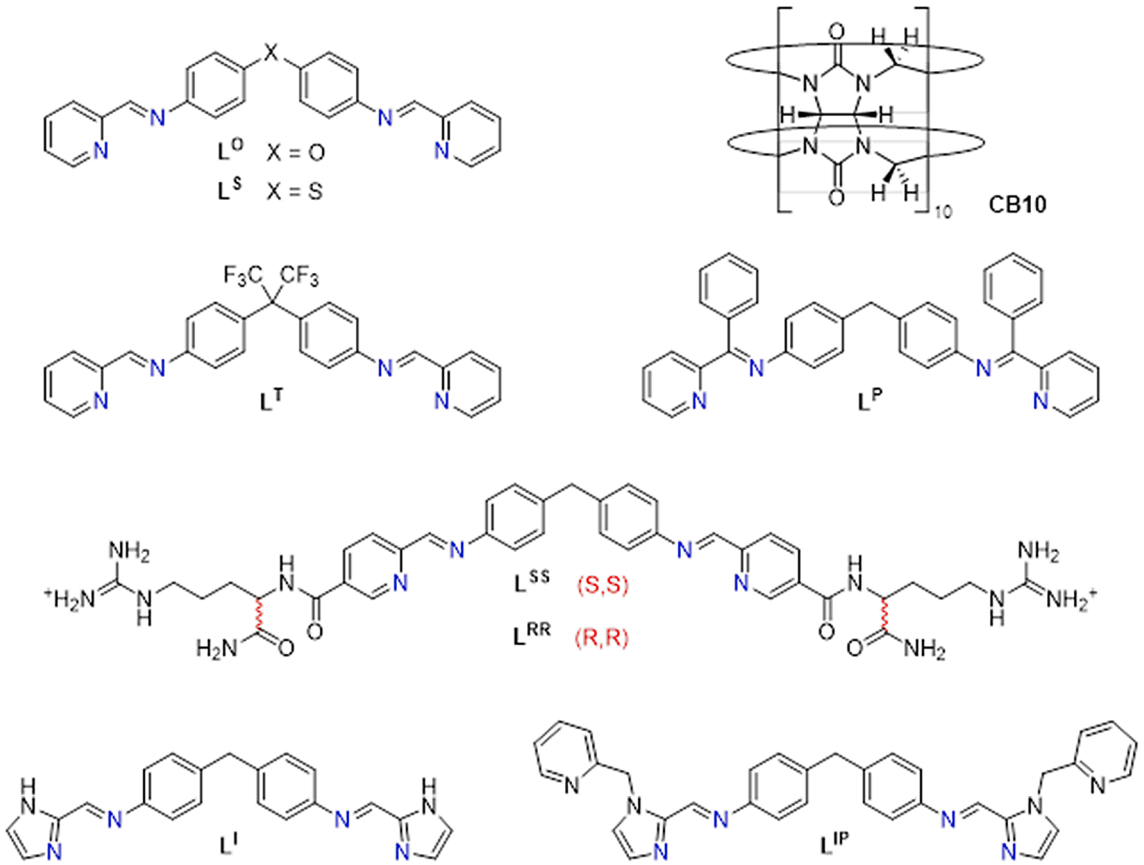


Fig. 19. Helicate ligands and rotaxane CB10.

contributing to the binding.[64] The crystal structure was formed from a racemic mixture, but the crystallization led to exclusive inclusion of the enantiomer M, suggesting this is a better binder; this was subsequently confirmed, with the P enantiomer displaying lower stabilization of the junction.[65] This interaction has subsequently been observed in solution with NMR studies, which confirmed the binding in the central junction and the same three-fold symmetry.[66] The interaction could also be confirmed by gel electrophoresis, which was also used to demonstrate how the interaction is not limited to palindromic DNA sequences, such as those used in the crystal structure, and it is also possible in the presence of GC pairs and unpaired bases.[65]

Modification of the helicate by introduction of trifluoromethyl groups on the central methine bridge (L^T, Fig. 19) or the addition of phenyl groups on the imine carbon (L^P, Fig. 19) are not tolerated,[65] likely due to simple steric hindrance. The replacement of the methine bridge with a sulphur (L^S, Fig. 19) or oxygen (L^O, Fig. 19) atom has only been investigated in relation to ct-DNA binding, but major effects would not be expected.[67] The sulphur containing helicates $[Fe_2L_3^S]^{4+}$ and $[Fe_2L_3]^{4+}$ were shown to bind to continuous and discontinuous 3WJs in crystal lattices, although these are more relevant to the field of material chemistry, rather than medicinal chemistry, due to their peculiar structure.[68]

Iron helicates built with ligands whose pyridine groups have been substituted with imidazoles, as in L^I (Fig. 19), or *N*-picolylimidazoles (L^{IP}, Fig. 19), retain their B-DNA and 3WJ binding properties, although their solution stability is lower compared to their nickel counterparts ($[Ni_2L_3^{I/IP}]^{4+}$). The ability of CB10 (Fig. 19) to wrap around $[Ni_2L_3^I]^{4+}$ is interesting, although the affinity is lower compared to DNA and 3WJ. On the other hand, permanently rotaxanated $[Ni_2L_3^{IP}\cdot CB10]^{4+}$, is not able to bind either DNA or 3WJ, highlighting that this strategy could be conveniently used to switch off binding to 3WJ or DNA if a rotaxanating compound with sufficient affinity could be found.[69]

The introduction of arginine residues on helicate ligands (L^{SS} and L^{RR}, Fig. 19) was investigated with the intent of engaging in additional interactions with DNA and impart a specific chirality to the resulting helicate. Indeed, $[Fe_2L_3^{SS}]^{10+}$ acquired a P helix configuration, while D-Arg imparted a M chirality to its corresponding helicate. As expected, $[Fe_2L_3^{RR}]^{10+}$ provides slightly better binding to a 3WJ compared to its enantiomer, as previously observed, and superior to $[Fe_2L_3]^{4+}$, which lacks the arginine residues, confirming that these are involved in the binding to DNA.[70]

Another approach to template the formation of helicates has been achieved by introducing a bipyridyl ligand on the *N*-terminus of bacteriophage T4 Fibritin foldon (T4Ff, Fig. 20).[71] This 27 amino acid sequence forms homotrimers with a β-propeller-like structure, which provides an optimal support to prearrange the ligands and form the helicate. Although the ligands are prearranged, and the presence of Fe^{2+} ions provides minor structural changes, the presence of Fe^{2+} is still essential for 3WJ binding, suggesting that a rigid organization of the helicate and/or electrostatic binding interactions may be involved in the binding. It is worth noting that the foldon does not appear to interact with DNA directly, and the helicate shows notable selectivity for 3WJs versus ds-DNA in the conditions tested, possibly as a result of the steric effect of the foldon itself.[71]

An alternative way to prearrange folding and stabilise the helicate is to tether the three ligands via short, bend-inducing amino acid sequences (Fig. 21). The introduction of arginine residues in these sequences allows the synthesis of helicates with good water solubility. While $[Fe_2L_3^H]^{8+}$ and $[Co_2L_3^H]^{8+}$ are dynamic complexes which move towards the folded helicate under thermodynamic control, the oxidation of the preformed Co^{II} helicate to its Co^{III} equivalent leads to a kinetically stable complex. On the other hand, this higher charged complex showed lower affinity to 3WJ compared to $[Fe_2L_3^H]^{8+}$ and non-specific binding to ds-DNA. The $[Fe_2L_3^H]^{8+}$ helicate, on the other hand, demonstrated affinity and selectivity for 3WJ. Its fluorescently tagged equivalent, $[Fe_2L_3^{HF}]^{8+}$, was used to directly determine its 1:1 binding stoichiometry to 3WJ with an apparent K_d of ≈ 0.45 μM.[72]

A similar class of compounds, defined as flexicates (Fig. 22), was developed in an attempt to obtain similar helical structures in a more accessible way from a medicinal chemistry point of view. These structures, like the previous helicates, are also bimetallic complexes with three ditopic bidentate ligands. However, their organization is not mechanically obtained because of the ligand rigidity, but it is predetermined by the chirality of the ligand itself. Flexicates maintain a C3 symmetry in solution, although the helicity of the complex appears less accented compared to the original helicates.[73] All Fe^{II} flexicates assembled from the ligands in Fig. 22 showed some degree of stabilization on 3WJ in melting assays with a standard, non-palindromic DNA sequence, in particular $[Fe_2L_3^{S1}]^{4+}$ and $[Fe_2L_3^{R1}]^{4+}$. In presence of unpaired bases at the junction, however, L^{S2-3} and L^{R2-3} displayed little to no activity. In gel electrophoresis assays, only $[Fe_2L_3^{S1}]^{4+}$ and $[Fe_2L_3^{R1}]^{4+}$ had significant ability to form 3WJ, with $[Fe_2L_3^{S1}]^{4+}$ being better than

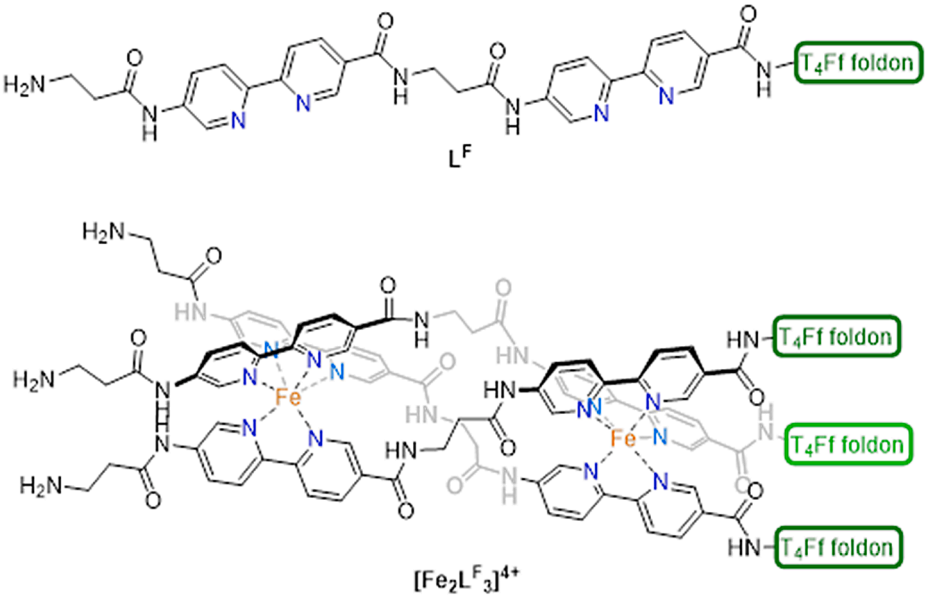


Fig. 20. T4Ff foldon-based helicate.

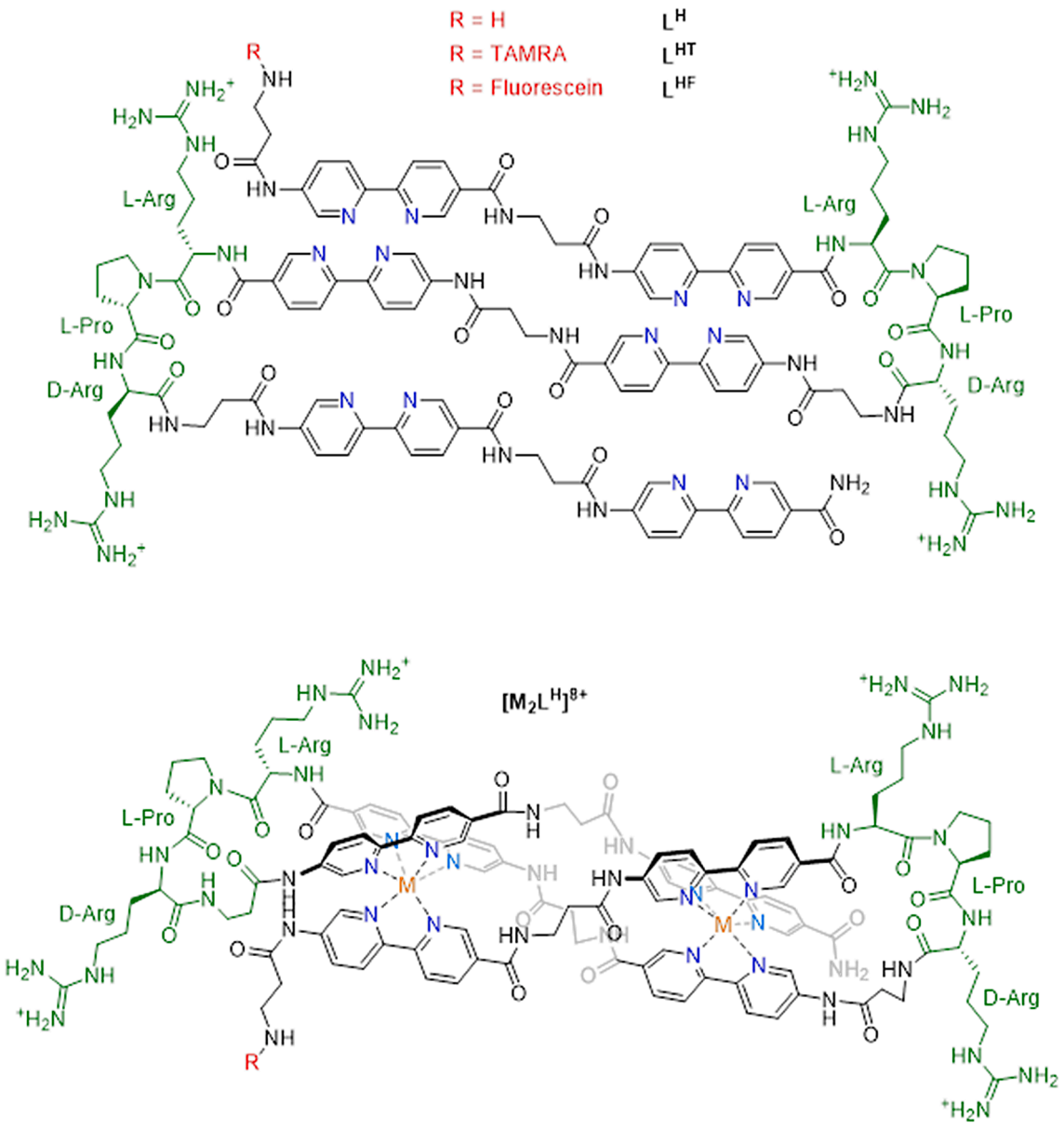


Fig. 21. Tethered helicates.

$[Fe_2L_3^{R1}]^{4+}$. These analyses also confirmed the 1:1 binding to 3WJ, suggesting a similar binding mode to the helicates, despite their ability to interact with other forms of DNA.[74]

In regard to biological activity, supramolecular cylinders have been shown to bind to DNA in cells and cause arrest of the cell cycle between phases G0 and G1, with subsequent death by apoptosis.[71,72,75] Despite the cytotoxicity, close to that of *cis*-platin, the mechanism does not appear to involve DNA damage.[75] It is also worth noting that this activity is not necessarily related exclusively to binding to 3WJs, considering the ability of helicates to bind to double stranded DNA and G-quadruplexes.[67,75–78] In certain cases, the toxicity could also be attributed to the possible release of ligand, such as in the case of ligands L^{S2-3} and L^{R2-3}.[74]

Overall, the seminal work on this class of compounds opened the path for the study of 3WJ binders and provided a structural basis for their interaction, facilitating the development and sparking the interest in the field.

3.2. Triazacyclononanes

A different class of complexes showing a C3 symmetry are derivatives of 1,4,7-triazacyclononane (TACN), such as those represented in Fig. 23.[79] In TACN-Q, the nitrogen atoms have the ability to coordinate both mono and dications, and impart a helical organization to the aromatic residues that participate in the chelation, providing a good match to the 3WJ branch point. Melting experiments showed how TACN-Q, in presence of lithium, can selectively stabilize a 3WJ compared to dsDNA. To assess the effect of the coordination on 3WJ affinity, the authors used TACN-A and TACN-N; both compounds showed a lower degree of stabilization in melting assays, with TACN-N being the least effective. This result was attributed to the lower helicity and the different effect of the aromatic residues. Different metal chelated TACN-Q derivatives were made and tested but they all showed stabilization intermediate between those of Li.TACN-Q and TACN-N. Li.TACN-Q was not able to displace the intercalating dye thiazole orange (TO) from dsDNA, indicating its inability to compete as an intercalator.

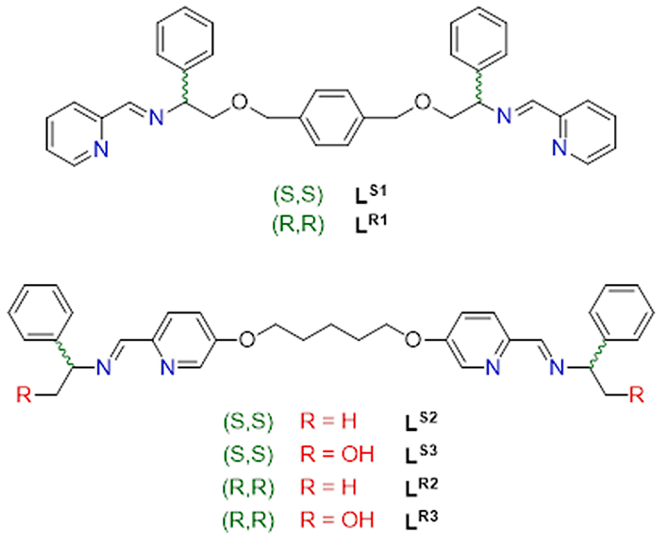


Fig. 22. Flexicate ligands.

Displacement of DT2C2 from a 3WJ, instead, was used to prove the ability to bind to 3WJs.[79] A following publication by Monchaud's group,[80] strongly supported the interaction between these compounds and 3WJs. Electrophoresis assays demonstrated the ability of TACN-Q to induce 3WJ assembly, and CD measurements, although with relatively weak binding, supported melting assay data. The selectivity against a DNA quadruplex was also assessed, collecting good evidence of their ability and selectivity as 3WJ binders.

TACN-Q has also been shown to inhibit cell proliferation in a melanoma cell model, although less effectively than azacryptands 2,7-TrisNP and 3,3′-TrisBP (see Paragraph 3.4).[80] It would be interesting to see further investigation to clarify the potential relationship between 3WJ binding inhibition and the effect on proliferation.

3.3. Triptycene derivatives

Triptycene derivatives (Fig. 24) are another class of compounds with a scaffold characterised by a threefold symmetry and a size comparable to the central cavity of 3WJs. Stabilization of the junction was demonstrated by melting experiments with Trip1-3 (Fig. 24), with Trip1 showing the best stabilization, while none of the compounds had any effect on dsDNA. Quite impressively, these triptycenes could displace a competing complimentary strand and drive the folding of a 3WJ at room temperature, both in fluorescence and gel shift assays.[81] This type of competition has also been demonstrated for 3WJs with slipped bases using triptycenes with amino acid sidechains Trip5-6, while arginine derivative Trip4 proved inactive.[33]

Only Trip1-3 have been tested in cells and, although IC50 values were not provided, Trip2 displayed the highest activity, while Trip3 proved inactive. This appeared to correlate with cellular uptake, which follows the same trend.[81]

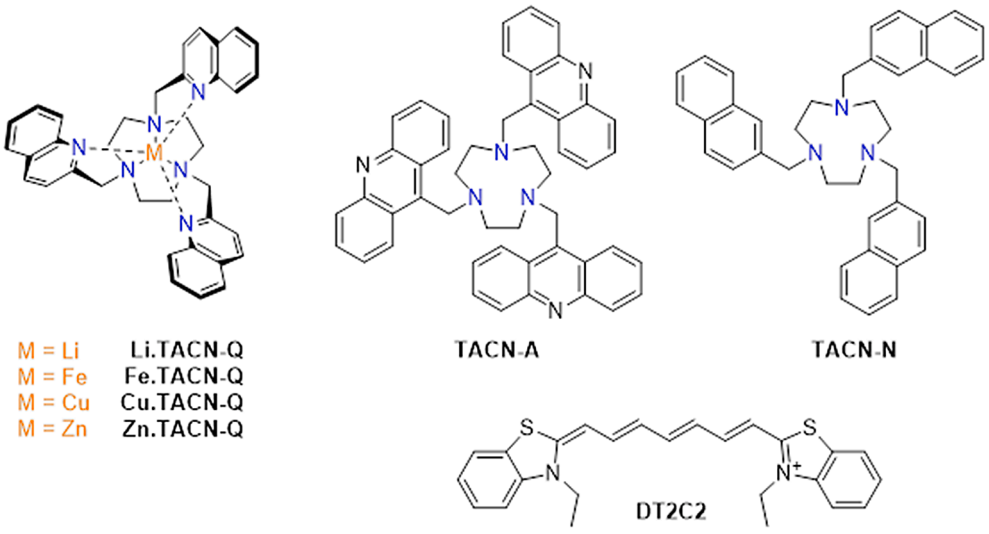


Fig. 23. Triazacyclononanes and cyanine dye DT2C2.

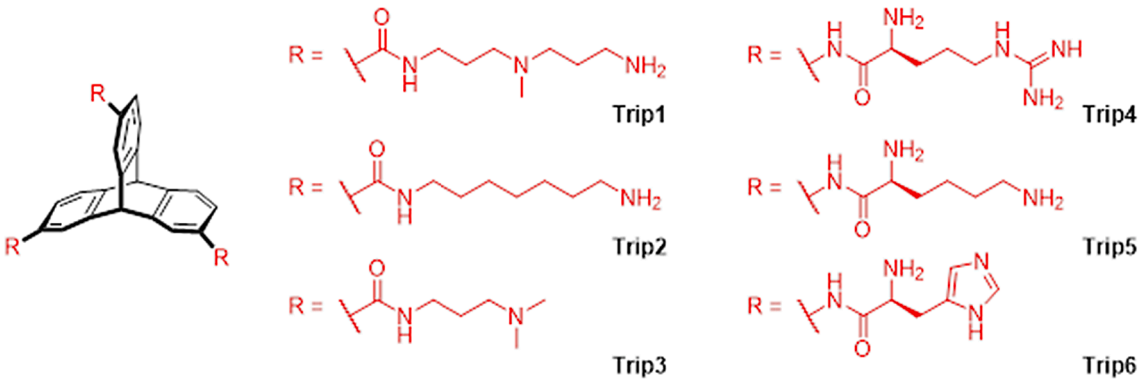


Fig. 24. Triptycene derivatives.

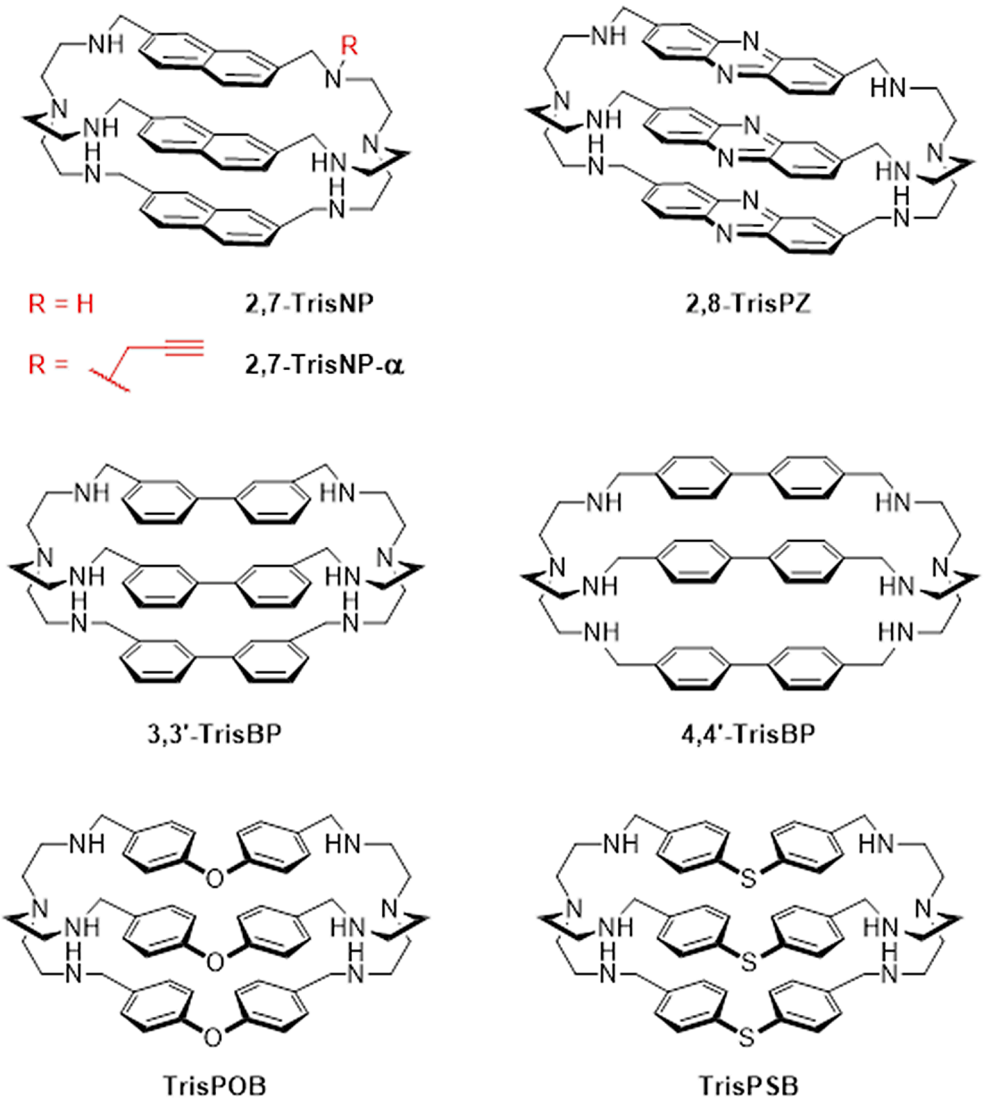


Fig. 25. Azacryptands.

3.4. *Azacryptands*

Differing from the previous two groups of compounds, azacryptands have a cylinder-like, less defined organization of their three aromatic arms (Fig. 25). This family of 3WJ ligands was initially reported in 2014,[80] and subsequently investigated in more detail, providing a new class of potent 3WJ binders and shedding light on some of their in-cell activity.[82,83]

The azacryptands 2,7-TrisNP, 2,8-TrisPZ and 3,3′-TrisBP displayed a much higher stabilization of a 3WJ in a FRET melting assay compared to TACN-Q, although their selectivity against dsDNA and a G-quadruplex proved slightly inferior. It is worth mentioning that 2,7-TrisNP and 3,3′-TrisBP were previously identified as good G-quadruplex binders, although with a different sequence than that used in this study.[84] Binding to the 3WJ was also assessed by CD and CD melting experiments, supporting the results from FRET melting. The ability to fold a 3WJ was also assessed by gel electrophoresis, which revealed that 2,7-TrisNP and 3,3′-TrisBP are very efficient, while 2,8-TrisPZ, despite its promising stabilizing effect in the previous melting assay, did not display appreciable folding, even lower than TACN-Q, which showed intermediate results.[80]

In a following publication,[82] 4,4′-TrisBP, TrisPOB and TrisPSB were also found to be excellent 3WJ stabilisers, with the ability to fold 3WJ both in gel electrophoresis studies and using a TWJ-screen, a specifically designed assay developed by Monchaud's group.[85] They also demonstrated superior stabilization of 3WJ in FRET-melting conditions compared to the previously discussed azacryptand 3,3′-TrisBP, providing a valuable set of binders.

The biological activity of these compounds was evaluated to gain insight into some of the underlying mechanisms of action of azacryptands.[82,83] 3,3′-TrisBP, 4,4′-TrisBP, TrisPOB, TrisPSB and 2,7-TrisNP have antiproliferative activities against MCF-7 cells with IC50 values in the low to sub-micromolar range, and lead to DNA damage, although it was also highlighted that 3,3′-TrisBP and TrisPSB could interfere with DNA damage signalling at higher concentrations.[82,83] Azacryptands TrisPOB and 2,7-TrisNP were assessed in more detail, also due to their ability to bind both 3WJ and G-quadruplexes, although with a clear preference for 3WJ.[83] Their synergic activity with a topoisomerase II catalytic inhibitor suggests that their activity is likely not due to their G-quadruplex binding properties, but more likely related to their 3WJ binding activity. The alkyne-labelled 2,7-TrisNP-α was used for in situ labelling and visualization with a chromophore, displaying binding to DNA and RNA; it did not, however, co-stain G-quadruplex foci identified by a G4-specific antibody. Both TrisPOB and 2,7-TrisNP also

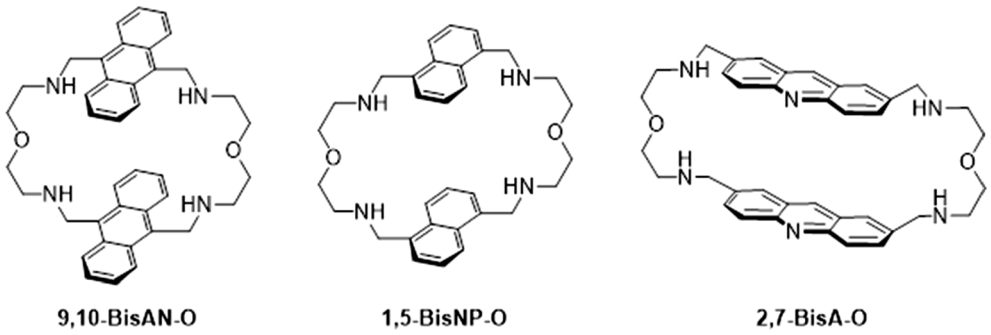


Fig. 26. CycloBis-Intercalators.

showed synergic effects with DNA damage repair inhibitors, substantiating their role in inducing DNA damage, mainly effected around the transition between phases G1 and S.[82,83] Differently from these, 3,3′-TrisBP and TrisPSB appeared to rely on different mechanisms in exerting their toxicity.[82] These reports[82,83] are a welcome opening route to clarify the activity of 3WJ binders, which, due to the lack of ideal biological tools, remains elusive and difficult to demonstrate with clarity.[83]

3.5. Cyclo-Bis-Intercalators

A group of compounds closely related to azacryptands is constituted of macrocycles with only two aromatic systems, as shown in Fig. 26. These compounds were identified by Monchaud's group using the TWJ-Screen, an elegant screening method they developed to identify 3WJ binders. This assay is operatively convenient, as it can be run in a common microplate reader, and provides a number of controls to exclude potential interfering compounds, on top of the possibility of running competition assays with other DNA structures.[85,86]

Compounds 9,10-BisAN-O and 1,5-BisNP-O shown in fig. 26 were identified in the first iteration of the screening, validated by PAGE and FRET melting assays, and were also found to be selective versus a ds-DNA and a G-quadruplex sequence.[85] A following iteration of the assay, also identified 2,7-BisA-O and brought further validation for these compounds.[86] It is worth mentioning that a few more compounds were identified in this last publication, but either PAGE or FRET melting did not confirm the binding, although possibly due to assay limitations, and we will not report them here.[86]

While both 9,10-BisAN-O and 1,5-BisNP-O displayed good toxicity against two breast cancer cell lines (MCF7 and MDA-MB-231), with low- (9,10-BisAN-O) and sub-micromolar (1,5-BisNP-O) activity, they also displayed a slightly higher activity on a normal fibroblast cell line (BJ-hTERT).

3.6. Calix[3]carbazole

The calix[3]carbazole represented in Fig. 27 is another example of compound endowed with a pre-organised 3-fold symmetry, similar to other compounds previously described herein. This compound has peculiar absorption–emission properties, which allowed the authors to identify the possibility of the compound binding to the central cavity of the 3WJ. This consideration was also supported by CD and NMR measurements and binding was also shown by UV/Vis melting and PAGE assays. The most interesting feature of this compound is its ability to fluoresce in presence of 3WJ, which could make it an interesting fluorescent probe. Unfortunately, however, although it displays some selectivity against a G-quadruplex, it suffers from non-specific binding to dsDNA via electrostatic interactions.[87] It would be interesting to see easily accessible and well characterised compounds with a turn-on fluorescence upon specific binding to 3WJs, as they would be excellent probes for competition assays.

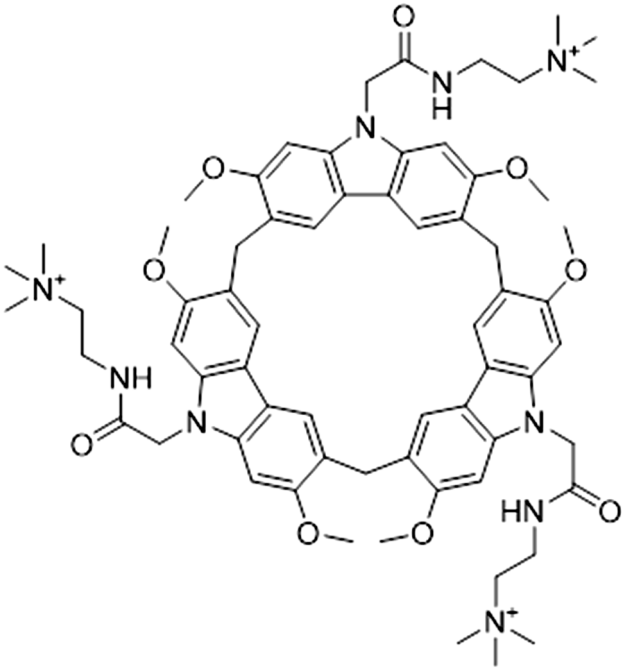


Fig. 27. Calix[3]carbazole.

4. Conclusions

G-quadruplexes and i-motifs are relatively complex, folded structures of DNA that require particular conditions to fold – the presence of divalent ions or local changes in pH (at least in vitro, if not in vivo). Three- and four-way junctions are much simpler structures and, particularly in the case of the 4WJ, have the potential to form in DNA strands under normal conditions, as well in processes such as replication or DNA damage repair.

From a biological perspective, compounds that bind to 4WJs have been shown to have both antitumour and antibacterial activity. Segall and co-workers have demonstrated the potential of this target in inhibition of homologous recombination, and it remains an under-researched area for the design of new antibacterial agents. The 3WJ compounds that have been described have been associated with DNA damage, although the exact mechanisms by which these compounds exert their effects have yet to be defined. This is something that will rely on selective inhibitors for these novel structures.

The simplicity of the structures may also be their downfall from a drug design and selectivity point of view – how does one target a stretch of double stranded DNA in a loop in the presence of mega- base pairs of duplex DNA? Targeting the junctions, the unique aspect of the structures, is the way most studies described above have approached this.

While the more complex structures of the G-quadruplex and i-motif perhaps offer more scope for designing selective small molecules; the junction structures also offer a unique target for ligand design that may be exploitable, as described above. It should not be overlooked too that where a G-quadruplex folds opposite an i-motif, if, indeed that is the case, then the space between them could be considered a variant of a 4WJ!

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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