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A small molecule that induces assembly of a four way DNA junction at low temperature†

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Small molecules that induce the formation of higher order DNA structures have potential therapeutic and nanotechnology applications. Screening of a click library has identified the first compound to induce the formation of a Holliday junction structure at room temperature without the need for a high temperature annealing step.

In 1997, Hurley and Neidle described the inhibition of telomerase through stabilization of a G-quadruplex in telomeric DNA sequences with a small molecule.[1] Since then, there has been an explosion of interest in targeting higher order DNA structures, particularly as G-rich sequences have been identified in the promoter regions of oncogenes[2] and targeting these structures has been shown to modulate gene expresion.[3] A number of other higher order DNA structures also exist and have been the subject of investigation.[4] The most well studied structure is the triple helix, in which a third strand of DNA or RNA binds in the major groove and molecules have been designed with an extended surface to target these by intercalation.[5] DNA three way junctions can be targeted by organometallic tube-like structures and have been shown to have interesting biological activity.[6] We and others have demonstrated that small molecules can also bind to the four way junction known as the Holliday junction (HJ). Peptides that bind to the junction were originally described by Segall and co-workers[7] and may have potential as anti-bacterials. We have shown that a compound that was originally designed as a threading DNA bisintercalator can reach across the junction and displace the adenine from an AT base pair.[8] This novel mode of binding prompted us to search for other compounds that bind to a HJ and that may have potential as biological probes for HJ structures *in vivo* or as additives in nanotechnology applications. Herein, we describe the first example of a molecule that promotes the formation of a four way junction *via* both thermal annealing and at room temperature.

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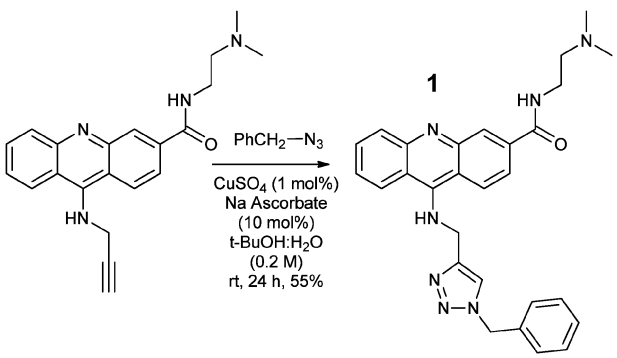
The prototype HJ binding molecule from our previous studies consisted of a symmetrical dimer of 9-aminoacridine-4-carboxamide. We have previously shown that it is possible to generate both 3- and 4-carboxamides using click chemistry with an azidoethylamino-group at the 9-position of the acridine.[9] Compound **1** was made through similar click reactions but this time utilizing a 9-propargylamino- substituent on the acridine chromophore.[10] Reactions of this type are somewhat more limited due to the unstable nature of the starting acridine substituted with an alkyne but compound **1** was isolated in 55% yield (Scheme 1).

Four oligonucleotides, labelled b, h, r and x (for sequences see the ESI†) were designed to form an X-stacked four way junction under standard annealing conditions in the presence of divalent metal ions.[11] In aqueous solution and following annealing in the absence of divalent metal ions, the oligonucleotides exist as an equilibrium mixture of single stranded DNA and the "open" form of the junction (Fig. 1). On the addition of divalent metal ions such as Mg^{2+} and high temperature annealing, the HJ is converted to the X-stacked form.

With oligonucleotide x doubly labelled with FAM (6-carboxyfluorescein) and TAMRA (tetramethyl-6-carboxyrhodamine), it was possible to visualize the formation of the HJ on a non-denaturing polyacrylamide gel following high temperature annealing in the presence of increasing concentrations of Mg^{2+} (Fig. 2a). It was clear that at a concentration of around 1 mM the HJ was formed with some small amount of single stranded DNA remaining even at 5 mM concentration. When the same oligonucleotides were annealed in the presence of



Scheme 1 Synthesis of compound **1** under click conditions.

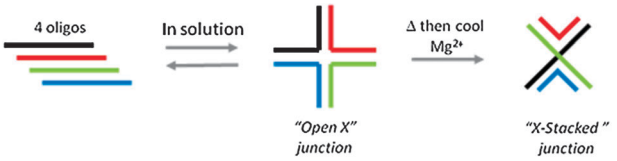


Fig. 1 Annealing in the presence of Mg^{2+} leads to the X-stacked (or closed) form of the junction.

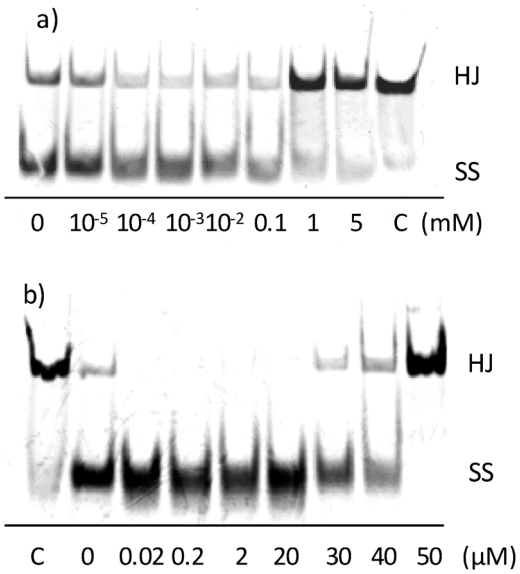


Fig. 2 Formation of the HJ detected by native polyacrylamide gel electrophoresis. (a) PAGE FAM image showing the formation of the HJ on addition of increasing concentrations of $MgCl_2$ (for conditions see ESI†). Lane C is a control lane where oligonucleotides b, h, r and x are annealed in 450 mM NaCl, 24 mM Na citrate pH 7.0 and 2mM $MgCl_2$. (b) PAGE FAM image showing the effect of increasing concentrations of compound **1** on the formation of the HJ. Lane C as for (a).

compound **1**, the HJ began to form at around 30 μM concentration and there was no evidence of single stranded DNA at a 50 μM concentration, suggesting that the compound promoted formation of the HJ at least 100 fold more efficiently than divalent metal ions (Fig. 2b).

An interesting observation in both gels is that at lower concentrations both the divalent metal ions and compound **1** appeared to inhibit the formation of the HJ. This may be due to the high affinity of both **1** and Mg^{2+} for the X-stacked form of the four way junction and suggests that compound **1**, at low concentrations, prevents the formation of the open form.

Acridines are well known fluorescent molecules and we utilized this property to probe binding dissociation constants by fluorescence titrations with the same HJ oligonucleotides without the fluorophores and with a double helical control formed from oligonucleotide x and its complementary strand (see ESI†). Compound **1** showed moderately higher affinity for HJ (K_d = 0.46 ± 0.13 μM) compared to duplex DNA (2.44 ± 0.07 μM) and a stoichiometry plot indicated a 1 : 1 binding mode.

To further explore the properties of **1** and its interaction with the HJ we employed circular dichroism spectroscopy to study whether **1** could affect the folding of the DNA. The CD spectrum of HJ in the presence of 10 mM tris·HCl, pH 7.00 shows a negative signal at 250 nm and a positive signal at 277 nm. The CD spectrum of HJ annealed in the presence of 10 mM tris·HCl, pH 7.00 and 10 mM of $MgCl_2$, however, demonstrates more intense signals at 250 nm (negative) and 277 nm (positive), indicative of the spectral difference between the open and closed HJ structures (Fig. 3a).[12] HJ annealed in 10 mM tris·HCl, pH 7.00 and 100 μM of ligand **1** (and no added $MgCl_2$) shows enhanced signals at −250 and +277 nm, analogous to the effect observed when annealed in the presence of $MgCl_2$. This supports the results from the gel-based assay and indicates that ligand **1** stabilizes the closed form of the junction in a manner analogous to divalent metal ions. Taken together, the evidence from the gels and CD experiments indicate that ligand **1** promotes formation of the four way junction during thermal annealing and does not require the presence of divalent metal ions.

This led us to consider whether **1** could promote the formation of four way junctions at room temperature *without* a high-temperature annealing step. To explore this hypothesis, we utilised CD titrations at room temperature. Compound **1** was titrated into pre-annealed solutions of HJ in the presence of 10 mM tris·HCl, pH 7.00, but in the absence of $MgCl_2$ (*i.e.* in the open form), mixed thoroughly and a spectrum acquired immediately. On titrating **1** into the solution of HJ, a subsequent increase in ellipticity was observed at 250 nm (negative) and 277 nm (positive), consistent with the effect observed when the HJ sample was annealed in the presence of Mg^{2+} (Fig. 3b). A similar effect was also observed when **1** was titrated into HJ annealed in the presence of 10 mM Mg^{2+} (see ESI†). We considered that the changes in ellipticity may arise from **1** intercalating with the duplex "arms" of the structure rather than at the junction itself. To rule this out we repeated an analogous CD experiment with double helical

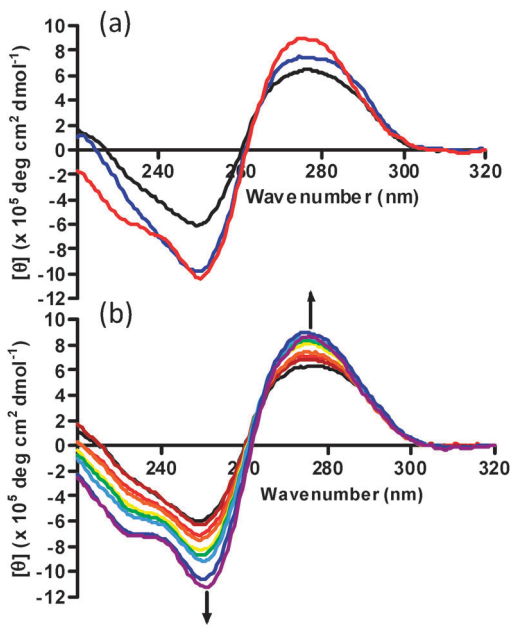


Fig. 3 CD spectra of HJ annealed in the presence of 10 mM tris·HCl pH 7.00 at 20 °C: (a) annealed in the absence of $MgCl_2$ (black); annealed in the presence of 10 mM of $MgCl_2$ (blue); annealed in the presence of 100 μM of ligand **1**. (b) Titration with ligand 1 (0–150 μM).

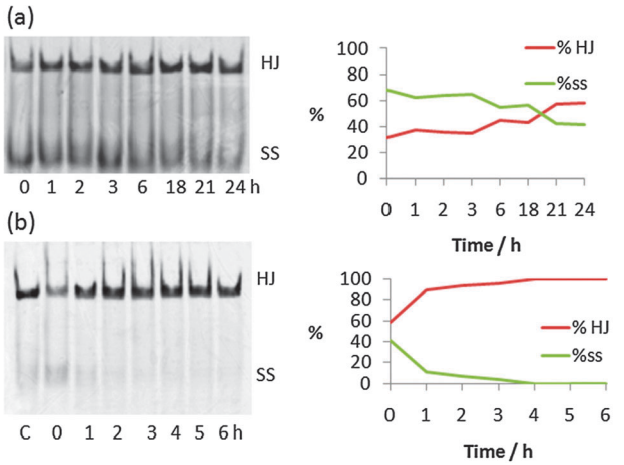


Fig. 4 Gels and densiometry plots showing the percentage of HJ *versus* single stranded oligonucleotide over the designated time period. (a) Oligonucleotides b, h, r and x were mixed together at room temperature in the presence of 2.0 mM $MgCl_2$ and left in the dark for the specified time (final HJ concentration 1.25 μM). (b) Oligonucleotides b, h, r and x were mixed together in the presence of **1** (50 μM) at room temperature and left in the dark for the specified time (final HJ concentration 1.25 μM).

DNA formed from x and its complementary sequence. Titration of **1** with the double helical "control" resulted in no changes in ellipticity (see ESI†), indicating the changes observed in the HJ samples are the result of a specific interaction with the junction as opposed to duplex intercalation. This is also consistent with the 1 : 1 binding mode indicated by the stoichiometry plot.

Finally, we investigated the ability of the ligand to induce the formation of a HJ *without* prior annealing using a gel assay. Mixing the four oligonucleotides in solution at 20 °C in the presence of 2 mM Mg^{2+} led to the formation of around 60% HJ over a 24 h period (Fig. 4a), demonstrating that even in the presence of high concentrations of divalent metal ions, a high temperature annealing step is required for the formation of the HJ. Notably, after 6 h, around 40% of the four way junction is present in solution. Carrying out the same experiment with 50 μM of compound **1** in both the absence and presence of Mg^{2+} ions (2 mM) led to the rapid formation of the HJ structure even at 20 °C (Fig. 4b shows the experiment in the presence of the divalent ions). Within 4 h all of the oligonucleotides in solution had formed the four way junction.

The ability of a small molecule to promote the formation of a four-way DNA junction is unprecedented. Within higher order DNA studies, several groups have previously described molecules that appear to promote the formation of a G-quadruplex structure, although usually within a defined intramolecular oligonucleotide sequence.[13] Here, compound **1** is not just perturbing the equilibrium between the single strands and open-X form of the junction but, from the CD data, is promoting the formation of the X stacked form in a similar way to divalent metal ions. This suggests that the compound is promoting the intermolecular assembly of oligonucleotide sequences. Fluorescence and CD spectroscopy also demonstrate that the molecule has a lower affinity for and different effects on the structure of duplex DNA.

The biological potential of a molecule that promotes the formation of the HJ is not known. Compound **1** is one of the few 9-aminoacridine-3-carboxamide structures that we have studied that has potent antitumour activity (9.1 μM in human leukaemia HL60 cell line) although the modest difference in affinity for the HJ *versus* duplex DNA may suggest that this derives from classical topoisomerase inhibition rather than effects on DNA repair processes. However, studies on the ability of **1** and analogs to target other HJ forming sequences and their effects on enzymes binding to four way junctions are underway.

This research also highlights the potential of compounds that can promote the formation of higher order DNA structures in nanotechnology. It is likely that screening for compounds with this ability will allow the formation of DNA nanostructures without the requirement for a high temperature annealing step and may change the physicochemical properties of the resulting structures.

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