ChemComm

Binding of anthracycline antibiotic nogalamycin to the site of a DNA single strand break engineered between two co-axially stacked hairpins

Michelle L. Colgrave,[ab] Huw E. L. Williams[a] and Mark S. Searle*[a]

[a] *School of Chemistry, University Park, Nottingham, NG7 2RD. E-mail:mark.searle@nottingham.ac.uk; Tel: 0115 951 3567; Fax: 0115 951 3564*

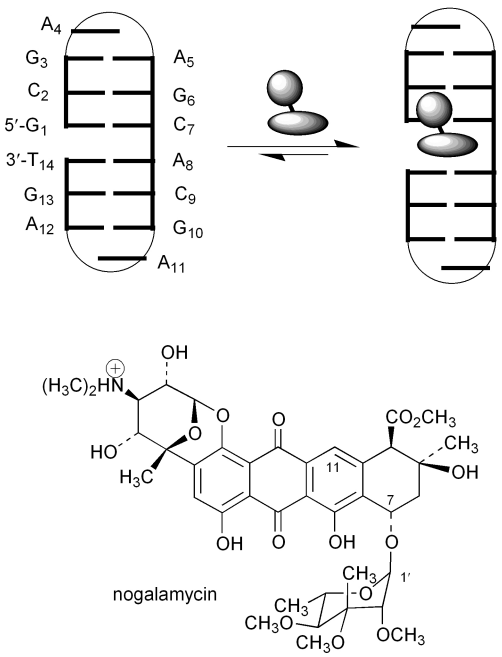
[b] *Department of Chemistry, University of Wollongong, New South Wales 2522, Australia*

Received (in Cambridge, UK) 12th December 2000, Accepted 12th January 2001
First published as an Advance Article on the web 31st January 2001

We show by NMR that the DNA sequence 5′-GCGAAG-CACGAAGT folds to form an intramolecular double hairpin structure containing 5′-GAA loops with co-axial stacking of the two hairpins significantly increasing their stability; the anthracycline antibiotic nogalamycin is able to intercalate between the two hairpins (5′-TG step) despite the break in the phosphodiester backbone.

Hairpins are a common feature of RNA folding and consist of a single-stranded loop region closed by a base paired stem. In contrast, there have been relatively few observations of stable DNA hairpins,[1] however, a number of reports have detailed the extraordinary stability of hairpins containing a 5′-GAA loop.[2–7] High resolution NMR spectroscopy has shown that the structure is folded back between the two As in the loop and is stabilised by a non-Watson–Crick G–A base pair with which there are extensive base stacking interactions.[3]

We have designed a novel intramolecular DNA structure that folds from a single strand of DNA (5′-GCGAAGCACGAAGT) to form a double hairpin structure with 5′-GAA loops (Scheme 1). One half of the structure consists of a hairpin identical to that studied by Hirao *et al.*,[3] 5′-GCGAAGC, whilst the other half consists of a novel hairpin containing an A–T base pair in the stem, 5′-ACGAAGT. Co-axial stacking of the two hairpin structures generates a double-stranded stem region with an effective single strand break in the middle of the duplex region (Scheme 1). We have designed the double hairpin sequence to contain a 5′-TG base step at the strand break site (between the 5′- and 3′-terminal nucleotides; see Scheme 1) and have used this novel structure to investigate (i) the extent to which the two hairpin components are associated through co-axial base stacking and how this affects the stability of the individual hairpin components, and (ii) the role of the phosphate backbone in sequence-specific recognition by the anthracycline antibiotic nogalamycin (Scheme 1) which binds selectively to the 5′-TG step. Nogalamycin is a threading intercalator with sugar moieties interacting in both the major and minor groove of DNA.[8–16]



Scheme 1

We first examined the stability of the designed double hairpin sequence by NMR to establish that the sequence was folded into two hairpin conformations and to what extent these hairpins are co-axially stacked (Scheme 1). While the isolated hairpin sequence 5′-GCGAAGC has already been characterised, the hairpin sequence 5′-ACGAAGT has not. We synthesised 5′-ACGAAGT and the full length double hairpin 5′-GCGAAG-CACGAAGT using solid phase methods described previously,[12] and carried out a full NMR assignment.[17] The NMR studies previously reported on the hairpin d(GCGA$_4$AGC) showed that the deoxyribose H4′ of A$_4$ undergoes a large up-field shift as a consequence of stacking interactions in the loop region.[3] We used this shift as one of a number of indicators that the hairpin is folded. Indeed, we see that A$_4$H4′ of 5′-AC$_2$GA$_4$AGT and A$_{11}$H4′ of 5′-GCGAAGCAC$_9$GA$_{11}$AGT are up-field shifted by >2 ppm compared with other H4′ signals. Residues in the stem region also show large shift perturbations as a consequence of stacking of the G–A base pair; C$_2$H2′ in the hairpin and C$_9$H2′ in the double hairpin are located up-field at 1.56 and 1.57 ppm in the two structures. A number of other anomolous chemical shifts, together with the pattern of NOEs close to the loop regions suggest that the loops are well formed. However, the observation of highly exchange broadened imino proton resonances for Watson–Crick hydrogen bonded base pairs suggest that the stem region of 5′-ACGAAG$_6$T is not particularly stable, with the G$_6$NH only visible at low temperature. In contrast, all four imino protons in the stem region of the double hairpin are readily resolved at 25 °C, with that of the terminal thymine (T$_{14}$) showing some evidence of exchange broadening (Fig. 1). We conclude that co-axial stacking of the two hairpins considerably enhances the stability of the less stable 5′-ACGAAGT sequence. Hirao *et al.*,[3] had previously determined the T_m of the isolated hairpin 5′-GCGAAGC as ≈ 76 °C. UV melting studies on 5′-ACGAAGT in isolation under similar salt concentrations (0.30 M NaCl and 30 mM NaH$_2$PO$_4$) gave a broad transition around 48 °C. Thus, substitution of an A–T for a G–C base pair in the stem region significantly reduces the T_m, consistent with the imino proton data. UV melting studies on the intact double hairpin show a broad melting transition with a T_m of 65 °C. The broadness of the transition does not enable us to determine whether this is a biphasic process involving melting of the two individual hairpins in separate transitions, or a single co-operative melting. However, the absence of the low temperature transition seen for the isolated hairpin 5′-ACGAAGT provides further evidence for stabilisation through co-axial stacking between hairpins. Detailed analysis of NMR data for the isolated hairpin 5′-ACGAAGT and the same sequence within the double hairpin

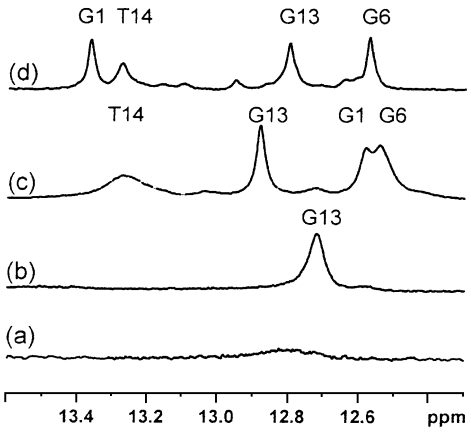


Fig. 1 Portions of the 500 MHz ^{1}H NMR spectra of 5′-ACGAAGT at (a) 15 °C and (b) 5 °C, (c) 5′-GCGAAGCACGAAGT at 15 °C, and (d) 1 : 1 5′-GCGAAGCACGAAGT–nogalamycin complex at 15 °C, all recorded in 90% H_2O solution under identical sample conditions. Imino proton resonances between 12 and 14 ppm are illustrated with assignments (see Scheme 1).

5′-GCGAAGCACGAAGT reveals some differences in chemical shifts of up to 0.2 ppm for nucleotides involved in end-stacking. Further, in NOESY data collected at 15 °C we observe internucleotide NOEs across the break site between G_1 and T_{14} (G_1H8 ↔ T_{14}H1′ and G_1H8 ↔ T_{14}H6), which provide convincing evidence for a significant population of co-axially stacked hairpins.

We subsequently examined the binding of nogalamycin to the 5′-TG site containing the single strand break to examine the role of the phosphate backbone in sequence-specific recognition. Titrating the drug into a solution of the double hairpin sequence, and monitoring the interaction by NMR, showed the emergence of a new set of DNA resonances alongside those of the unbound species. The free and bound DNA signals are in slow exchange at 25 °C, with the change in complexity of the NMR spectrum indicative of the formation of a single bound species. A complete assignment of the 1 : 1 complex has enabled some 30 drug–DNA NOEs to be identified. Fig. 2 shows a number of drug–DNA NOEs that define unambiguously the position and orientation of the bound ligand. In the complex we do not see NOEs across the intercalation site between G_1 and T_{14}, as observed for the free DNA; however, the drug acts as a surrogate base pair with the aromatic proton H11 giving NOEs to both G_1H1′ and T_{14}H1′, consistent with intercalation between the two hairpins. A number of other key NOEs to A_8H1′ at the intercalation site are also highlighted (Fig. 2), while NOEs from drug methyl groups on the nogalose sugar show specific hydrophobic interactions with, for example, G_1H1′ and C_2H1′ on the floor of the minor groove; these are analogous to those observed for nogalamycin binding to the 5′-TG site in duplex DNA.[12] These characteristic NOEs indicate that the drug–base pair stacking geometry is independent of the restraining influence of the phosphodiester backbone. In addition, the chemical shifts of the nucleotide resonances of G_1, C_7, A_8 and T_{14} were most affected by drug binding, consistent with nogalamycin intercalating at the 5′-TG step. UV melting studies on the 1 : 1 complex showed a more co-operative transition than for the free double hairpin, although the T_m of 74 °C is not significantly higher. Examination of the temperature-dependence of imino proton line widths showed that the base pairs forming the intercalation site are highly stabilised at 15 °C (Fig. 1d), indicating that they wrap around the drug to optimise van der Waals interactions, resulting in a highly stable complex templated by stacking and hydrophobic interactions with the bound drug. We can speculate that access of the drug to the intercalation site is greatly facilitated by the single strand break and that the association rate, which is slow for intact duplex DNA due to the requirement for local melting, may be significantly enhanced.

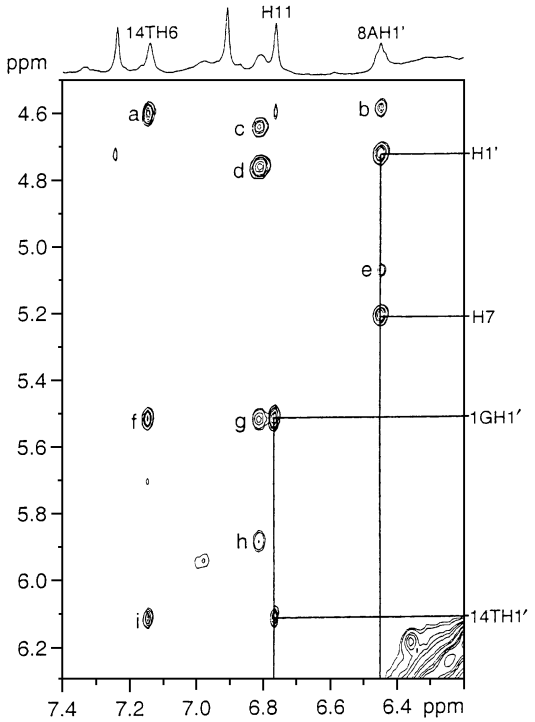


Fig. 2 Portion of the NOESY spectrum (100 ms mixing time) of the 1 : 1 nogalamycin: 5′-GCGAAGCACGAAGT complex recorded in D_2O solution at 15 °C highlighting key drug–DNA NOEs that identify the position and orientation of the bound intercalator at the 5′-TG step (drug H11, H7 and H1′ are marked on the axes), other DNA–DNA cross-peaks are labelled as follows: (a) T_{14}H3′–T_{14}H6, (b) A_8H1′–A_8H4′, (c) C_2H6–C_2H3′, (d) C_2H6–C_2H5, (e) A_8H1′–A_8H3′, (f) G_{13}H1′–T_{14}H6, (g) G_1H1′–C_2H6, (h) C_2H1′–C_2H6, (i) T_{14}H1′–T_{14}H6.

We thank the EPSRC (studentship to H. E. W. L.) for funding this work. M. L. C. thanks the Australian Research Council, Boehringer Ingelheim Fonds and Professor Margaret Sheil for the opportunity to travel to the UK. We are grateful to John Keyte in the School of Biomedical Sciences (Nottingham) for oligonucleotide synthesis.

Notes and references

1 S. Yoshizawa, G. Kawai, K. Watanabe, K. Miura and I. Hirao, *Biochemistry*, 1997, **36**, 4761.
2 I. Hirao, Y. Nishimura, T. Naraoka, K. Watanabe, Y. Arata and K. Miura, *Nucleic Acids Res.*, 1989, **17**, 2223.
3 I. Hirao, G. Kawai, S. Yoshizawa, Y. Nishimura, Y. Ishido, K. Watanabe and K. Miura, *Nucleic Acids Res.*, 1994, **22**, 576.
4 I. Hirao, Y. Nishimura, Y. Tagawa, K. Watanabe, Y. Arata and K. Miura, *Nucleic Acids Res.*, 1992, **20**, 3891.
5 G. Varani, *Annu. Rev. Biophys. Biomol. Struct.*, 1995, **24**, 379.
6 S. Yoshizawa, T. Ueda, Y. Ishido, K. Miura, K. Watanabe and I. Hirao, *Nucleic Acids Res.*, 1994, **22**, 2217.
7 Z. Chraibi, M. Refregiers, B. Jolles and A. Laigle, *J. Raman Spectrosc.*, 2000, **31**, 481.
8 J. F. Fisher and P. A. Aristoff, *Prog. Drug Res.*, 1998, **32**, 411.
9 S. K. Arora, *J. Am. Chem. Soc.*, 1983, **105**, 1328.
10 M. S. Searle and W. Bicknell, *Eur. J. Biochem.*, 1992, **205**, 45.
11 C. K. Smith, J. A. Brannigan and M. H. Moore, *J. Mol. Biol.*, 1996, **263**, 237.
12 H. E. Williams and M. S. Searle, *J. Mol. Biol.*, 1999, **290**, 699.
13 X. L. Zhang and D. J. Patel, *Biochemistry*, 1990, **29**, 9451.
14 Y. G. Gao, Y. C. Liaw, H. Robinson and A. H. J. Wang, *Biochemistry*, 1990, **29**, 10 307; Y. C. Liaw, Y. G. Gao, H. Robinson, G. A. van der Marel, J. H. van Boom and A. H. J. Wang, *Biochemistry*, 1989, **28**, 9913.
15 K. R. Fox and M. J. Waring, *Biochim. Biophys. Acta*, 1984, **802**, 162; K. R. Fox and M. J. Waring, *Biochemistry*, 1986, **25**, 4349.
16 K. R. Fox and Z. Alam, *Eur. J. Biochem.*, 1992, **209**, 31.
17 W. J. Chazin, K. Wuthrich, S. Hyberts, M. Rance, W. A. Denny and W. Leupin, *J. Mol. Biol.*, 1986, **190**, 439.