DNA alkylation sites of nitrogen mustards conjugated to polyamines and their implications for polyamine–DNA interactions

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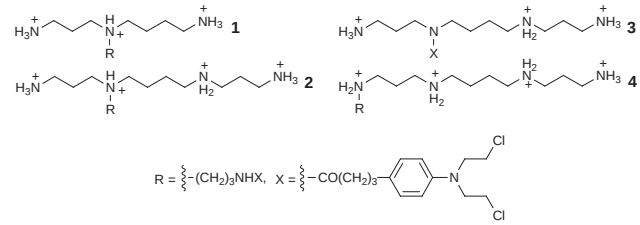
Polyamines conjugated to the nitrogen mustard chlorambucil increase the efficiency of DNA alkylation at N^7 of guanine by factors in the range 10^3 to 10^4; the sequence selectivity of this alkylation (the alkylation 'finger-print') is largely unchanged, which is consistent with flexible, electrostatic binding and incompatible with tight, sequence-specific binding of the polyamine moiety.

Polyamines are low molecular weight cations, essential for growth and differentiation,[1,2] that are present in high concentration in all cells. Polyamine–DNA interaction is associated with the physiological role of polyamines,[3–9] and there has been conflicting speculation concerning the nature and site of such interactions including reports of binding in the major[3] and minor groove,[4] spanning the minor groove[5,8] and theoretical studies that suggest interaction with the phosphate backbone or some sequence-specific interaction.[6] The crystal structure of a spermine–B-DNA complex[9] exhibits aspects of both charge–charge interactions with the phosphate backbone, and direct or water-mediated hydrogen bonds with bases and van der Waals interactions with hydrophobic regions. We previously determined the preferred cross-linking site on DNA for chlorambucil and both a spermidine– and spermine–chlorambucil conjugate.[10] The interstrand cross-link for each of these was at a 5'-GNC sequence within defined oligonucleotides, and this did not appear to be perturbed by the polyammonium moiety, despite a major enhancement of the efficiency of cross-linking. We interpreted this observation in terms of non-specific electrostatic interaction between the polyammonium cation and DNA. However, there may be a rather tight structural requirement for successful cross-linking which could be expressed in the second, interstrand alkylation step and which might potentially overwhelm the polyammonium ion binding preferences. Here we report the sequence specificities for mono-alkylation of DNA at N^7 of guanine shown by chlorambucil–polyamine conjugates and compare this to the known selectivity shown by chlorambucil itself.

The major alkylation site of DNA by chlorambucil is the N^7 of guanine bases.[11] The sequence selectivity in terms of guanine N^7 alkylation is demonstrated by converting these sites into strand breaks on treatment with hot piperidine and analysing the resulting fragments by denaturing polyacrylamide gel electrophoresis (PAGE). It has been shown that chlorambucil shows a significant sequence dependence in terms of the mono-alkylation sites,[11] with guanosine residues within runs of Gs being the most reactive.[12] In energetic terms, the enhanced reactivity in such regions as compared with isolated Gs represents a comparatively small difference, and a degree of alkylation at each G site can be detected on denaturing PAGE analysis of the cleavage products. In the case of the polyamine–chlorambucil conjugates it would be reasonable to expect that such small rate differences between the various alkylation reactions might be readily perturbed by the non-covalent binding interactions with the polyammonium moieties that are clearly promoting in some manner the eventual covalent step. We therefore anticipated that the alkylation selectivity could provide a potentially sensitive probe of the nature of the interaction between DNA and polyammonium cations. For example, it has been shown that the alkylation specificity of some aromatic nitrogen mustards is perturbed on conjugation to intercalators such as acridine, in a manner that reflects the acridine binding specificity.[13]

A DNA sequence of 276 base pairs was 5′-end-labelled and cleaved from linearised pBR322. Treatment of this double-stranded DNA with chlorambucil, or one of the polyamine–chlorambucil conjugates **1**–**4**,[14] followed by exposure of the



alkylated DNA to hot aqueous piperidine led to a family of DNA fragments that arise because of the lability of the DNA

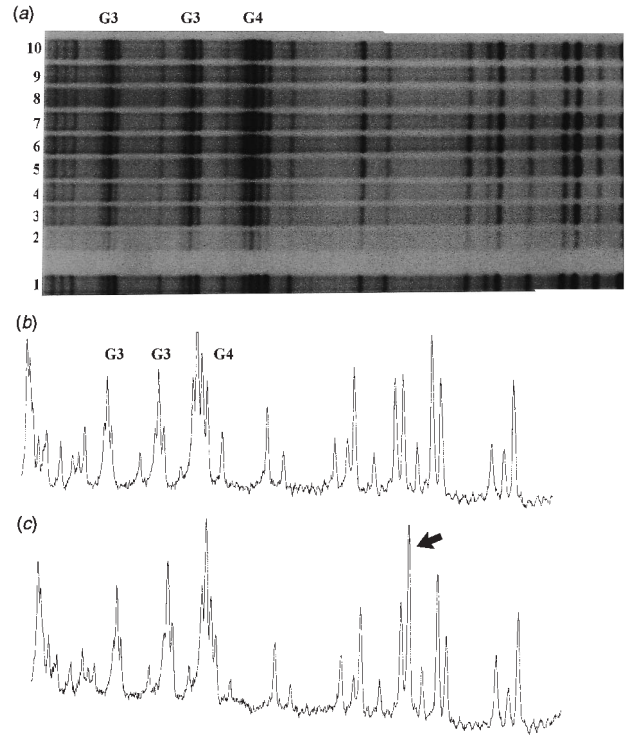


Fig. 1 (*a*) Autoradiograph of a denaturing PAGE gel showing sequence selective alkylation of guanine N^7 by chlorambucil–polyamne conjugates; lane 1: Maxam–Gilbert G track; lane 2: conjugate **1**; lane 3: conjugate **2**; lane 4: N^4–chlorambucil–spermidine conjugate (no linker); lane 5: conjugate **3**; lane 6: N^4–chlorambucil–norspermidine linked conjugate; lane 7: N^4–chlorambucil–homospermidine linked conjugate; lane 8: N^1–chlorambucil–spermidine linked conjugate; lane 9: conjugate **4** [0.1 μM; phosphorimager gel scan shown in (*c*)]; lane 10: DNA treated with chlorambucil [200 μM Phosphorimager gel scan shown in (*b*)]. DNA sequence: 5′-CGCGAGTACTCGGGCTTCACCGCTCGGGCTAG-AAGGGGTAGCCACTACAGCCGCTATATCCGCGGTCGTTGGCGT-GGACACCGC).

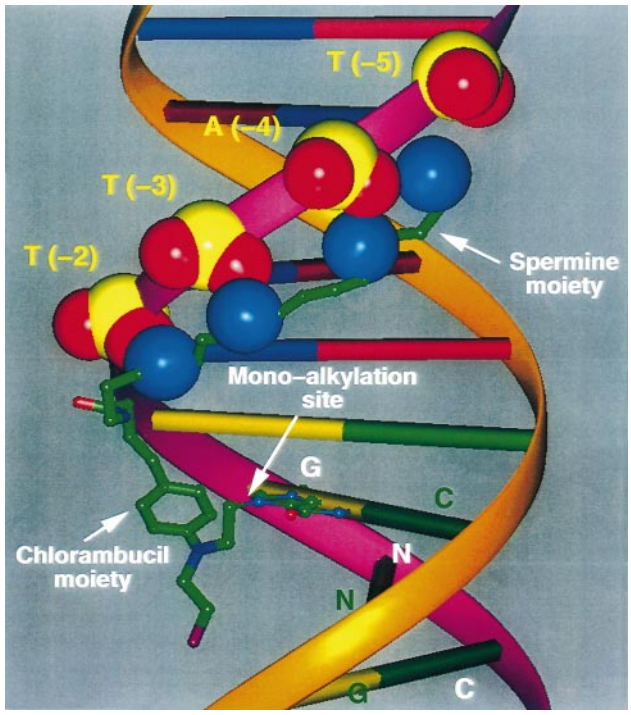


Fig. 2 Schematic representation of one of the molecular models for the B-DNA duplex d(CTATATTG**G**GCGGGATTAA)/d(TTAATCCCGCC-CAATATAG) monoalkylated by conjugate **4**, showing a low energy conformation in which the polyamine interacts with the phosphates 5′ to the alkylation site

alkylated at N^7 of the guanine bases. These cleavage products could be distinguished by denaturing PAGE (Fig. 1).[15] For chlorambucil the intensities of individual bands vary depending on the neighbouring sequence, confirming that guanine alkylation shows significant sequence selectivity, as reported previously[11] for a number of aromatic nitrogen mustards. More interestingly, all of the polyamine–chlorambucil conjugates show identical alkylation 'finger-prints', over a range of concentrations and molar ratios, but comparable levels of alkylation being observed with chlorambucil at 0.2 mM and conjugate **4** at 0.1 μM. Thus the presence of the polyamine moiety significantly enhances the alkylation by factors in the range 10^3 to 10^4 but does not alter the selectivity.

We have reported that chlorambucil and conjugate **1** hydrolyse and react with simple nucleophiles at the same rate and by the same mechanism.[16] The enhanced reactivity of the conjugates with DNA compared to chlorambucil is therefore a result of the polyamine–DNA interaction and in free energy terms this corresponds to about 23 kJ mol^{-1} (calculated for a 10^4-fold increased reactivity). The degree of enhanced reactivity, as with the observations on cross-linking efficacy, depends on the number of positive charges. The selectivity between different individual guanine bases is typically a factor of 2–3, which corresponds to an energy difference of 2–3 kJ mol^{-1}. The close similarity between the gel scans shown in Fig. 1, despite significant changes to the structure of the polyamine conjugate, must mean that the presence of the polyammonium moiety in the conjugate is able to enhance the reactivity at *all* of the guanine sites *and to the same extent.* A corollary to this is that although the polyammonium moiety has a high affinity for the DNA, it is not interacting at discrete sites and that there must therefore be significant mobility in the complex. This is consistent with a non-sequence selective, largely electrostatic interaction, and incompatible with sequence-specific H-bonded interactions. The similarities between the alkylation profiles is striking; however, close inspection of Fig. 1 does reveal a greater extent of alkylation by the polyammonium conjugate at a single site in the sequence GTCGTTG**G***CGTGGA [arrow in Fig. 1(*c*)]. This enhanced alkylation is reproducible but its structural basis is not evident.

Preliminary molecular modelling studies were performed to probe the nature of the interactions between DNA and these conjugates.[17] We have explored the covalent and non-covalent interactions of the spermine–chlorambucil conjugate by using B-DNA as the target and forming the first covalent link to N^7 of the more reactive guanine residue. Using this constrained adduct we have sought to probe three aspects: (i) whether the polyammonium moiety can make reasonable electrostatic interactions in such an adduct; (ii) whether the interactions are sensitive to the conjugate structure; and (iii) whether the cross-linking step involves much distortion of the conjugate or DNA structure. It is clear from the structure shown in Fig. 2 that in the adduct the polyammonium moiety can adopt a conformation that places the positive charges in good juxtaposition to the phosphate anions of the backbone. It is also clear that several alternative orientations of the polyammonium moiety are possible and of very similar energy. Similar interactions are possible for all of the conjugates **1**–**4** despite the structural differences, *e.g.* branched *vs.* non-branched. There is a trend in the strength of the interaction which increases with the number of positive charges. However, the differences in these values are close to the errors in the calculations and therefore should not be over-interpreted. It is very clear from the modelling that the formation of the cross-link requires appreciable distortion of the conjugate–DNA structure, and that this can be achieved whilst maintaining the interaction between the polyammonium moiety and the DNA. Thus the computational modelling is fully consistent with the conclusions drawn from the experiments.

Notes and References

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17 Molecular modeling and molecular dynamics refinements were performed on a duplex 19-mer canonical B-DNA, sequence d(CTA-TATTG**G**GCGGGATTAA)/d(TTAATCCCGCCCAATATAG) [where **G** is the first alkylation site], alkylated by **4** using InsightII and Discover (AMBER forcefield; MSI, San Diego, USA).

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