Conjugation of a Polyamine to the Bifunctional Alkylating Agent Chlorambucil Does Not Alter the Preferred Cross-Linking Site in Duplex DNA

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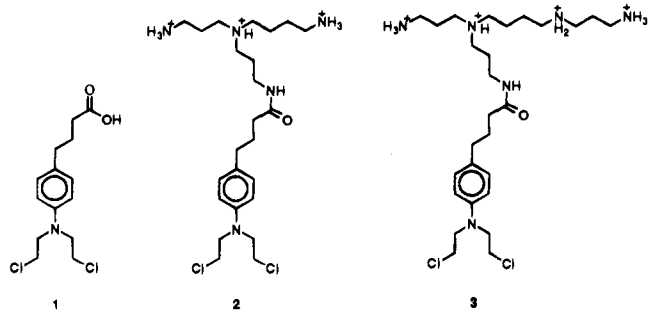
The aromatic nitrogen mustard chlorambucil **1** continues to be a clinically important chemotherapeutic agent against chronic lymphocytic leukaemia, ovarian cancer, Hodgkin's disease, and breast carcinoma.[1] The ability of chlorambucil and other bifunctional alkylating agents to cross-link the two strands in duplex DNA is widely believed to account for their antitumor activities, and indeed it has been demonstrated that cytotoxicity correlates well with cross-linking ability.[2] We recently reported



1 **2** **3**

the synthesis of the chlorambucil–spermidine conjugate **2**,[3] which was designed to exploit the polyamine uptake system that has been characterized in a wide range of cells, but particularly in a number of tumor cell lines.[4] The conjugate **2** was also expected to show a high affinity for DNA by virtue of the polycationic nature of the polyammonium moiety at physiological pHs. This expectation has been substantiated by the observation that **2** cross-links naked DNA *in vitro* more efficiently than chlorambucil by a factor of close to 10^4, although hitherto little is known about the nature of the interaction of **2** with DNA. Nitrogen mustards are known to alkylate DNA preferentially at N7 of deoxyguanosine residues, and until relatively recently it was assumed that the cross-linking reaction occurred between two adjacent deoxyguanosine residues.[5] We report here that chlorambucil introduces interstrand cross-links preferentially between two guanine residues in the sequence 5′-GNC, as has been observed for mechlorethamine and in line with the prediction of Millard *et al.*[6] We also report that the site of cross-linking is not perturbed when chlorambucil is conjugated to either spermidine or spermine to give conjugates **2** and **3**, respectively. The results have important implications for the nature of interaction between DNA and polyamines.

The potential for polyamine metabolism and regulation as a novel target for cancer chemotherapy has attracted considerable recent interest.[7] The most advanced approach has involved the development of inhibitors of the various enzymes involved in polyamine biosynthesis, since it has been shown that high levels of polyamines are required to maintain rapid cell proliferation. In an alternative approach, we have tried to exploit the fact that for some tumor cells, this high polyamine level is sustained by scavenging polyamines from extracellular sources by means of an efficient active uptake system. Through conjugation of cytotoxic agents to polyamines, we hoped to target tumor cells providing that these conjugates are recognized and transported by the polyamine uptake system. Furthermore, drugs whose intracellular target is DNA might be expected to become more potent on conjugation to a polyamine because of the high affinity of polyammonium cations for DNA. The preliminary results with the conjugate **2** have substantiated both expectations. We have shown that the conjugate is a good competitive inhibitor of the uptake system in ADJ/PC6 plasmacytoma cells and is ~35 times more cytotoxic than chlorambucil in these cells and ~200-fold more cytotoxic when the cells are depleted of polyamines by pretreatment with (difluoromethyl)ornithine (DFMO).[3,8] In contrast, chlorambucil cytotoxicity appears to be actually decreased on depletion of polyamines. Finally, of particular interest to this present study, **2** cross-links DNA *in*

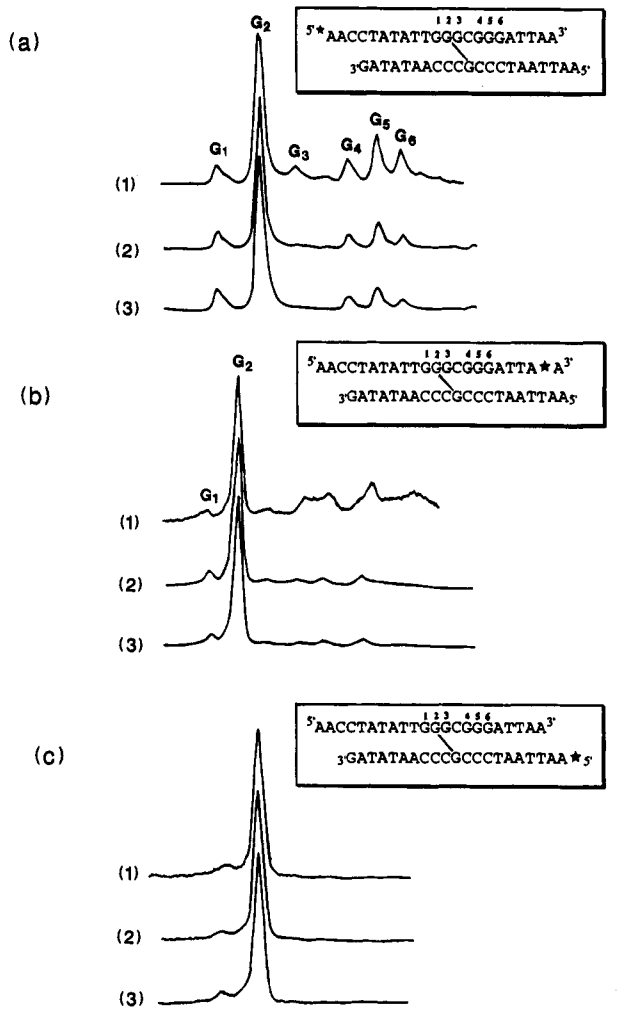


Figure 1. Phosphorimager gel scans of fragments derived from treatment of labeled duplex oligonucleotides with either chlorambucil **1** or one of the two conjugates **2** or **3**, followed by isolation of the cross-linked oligonucleotides by PAGE and treatment with piperidine to fragment the oligonucleotides at the site of cross-linking. The three sets of data show (a) sequencing of the top strand (sequence A) from the 5′-end, (b) sequencing of the top strand (sequence A) from the 3′-end, and (c) sequencing of the bottom strand (sequence B) from the 5′-end.

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vitro almost 10^4 times more efficiently.[3,8] We have shown that in terms of hydrolysis and reaction with simple nucleophiles, chlorambucil **1** and the conjugate **2** show similar intrinsic reactivities.[9] The increased cross-linking efficiency of **2** must therefore reflect the binding affinity of the polyammonium moiety as originally anticipated. *A priori*, it is not clear that the polyammonium moiety in **2** would necessarily facilitate or allow the same intrinsic base and sequence-selective reaction with DNA as for chlorambucil **1** itself, and indeed the binding of the polyamine to DNA might promote a competing cross-linking site. In order to address the origin of the increased cross-linking reactivity of **2**, we have determined the sequence specificity of chlorambucil **1** and the conjugates **2** and **3**.

The oligonucleotide sequences shown in Figure 1 were selected to allow direct comparison with mechlorethamine, for which the cross-linking sequence specificity is firmly established.[6] Exposure of the oligonucleotide to chlorambucil **1** led to a small percentage (~5%) of cross-linked oligonucleotide which could be identified and purified by denaturing polyacrylamide gel electrophoresis (PAGE).[10] The major discrete band obtained was isolated and heated in aqueous piperidine to convert the N7 alkylation sites into strand breaks. The 5′-labeled top strand of the oligonucleotide following this treatment showed that all derived fragments had been cleaved at G sites and that the single dominant alkylation site was G^2, Figure 1a, and this was confirmed with oligonucleotide 3′-labeled in the top strand, Figure 1b. The corresponding 5′-labeled bottom strand showed fragmentation at the single central G site, confirming that the interstrand cross-linking site was indeed between the guanines on the opposing DNA strands in the sequence 5′-GNC, as previously seen for mechlorethamine.[6] It has already been noted that the formation of such an interstrand cross-link appears to require considerable distortion of the duplex. The same oligonucleotides were used to determine the sequence specificity for cross-linking by the conjugates **2** and **3**. The spermine conjugate **3** has been recently synthesized to determine the influence of the polyamine moiety on the cross-linking efficiency and hence the chemotherapeutic potential, and indeed **3** is even more reactive in the cross-linking assay than the spermidine conjugate **2**.[11] Despite the presence of the polyamine moiety in **2** and **3**, the denaturing PAGE gels showed identical interstrand cross-linking sequence specificities.

The lack of perturbation of the cross-linking site despite considerable modification of the properties of the bifunctional alkylating agent is at first perhaps surprising. It has been suggested that the 5′-GNC sequence specificity seen for mechlorethamine may arise from the initial inherent specificity for the monoalkylation site.[6] If this hypothesis is correct, then the polyammonium moiety must not perturb this initial monoalkylation specificity. There has been considerable debate about the binding interaction(s) between polyamines and DNA, and contradictory conclusions concerning the site of spermidine or spermine polycation interaction include binding in the major groove,[12] in the minor groove,[13] and spanning the minor groove;[14] theoretical studies even predict that some sequence specificity may exist for polyamine binding to DNA.[15] This present study would support the conclusion that polyamines bind to DNA in an essentially nonspecific manner and that the association is likely to be principally electrostatic rather than specific hydrogen bonding.[16] These results also tend to suggest that there is no significant local perturbation of the DNA conformation caused by the binding of the polyammonium moiety. If the binding was specific or the DNA conformation significantly altered, some perturbation of the cross-linking reaction would certainly have been anticipated.

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Supporting Information Available: Original autoradiographs of the PAGE gels from which the data in Figure 1 are derived (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of this journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(10) Complementary oligodeoxynucleotides (A sequence, 5′-AACCTATATTGGGCGGGATTAA; B sequence, 3′-GATATAACCCGCCCTAATTAA) were synthesized (Applied Biosystems Model 394 DNA/RNA synthesizer) on a 1 μmol scale and were purified by denaturing PAGE (20%, 19:1 acrylamide/bisacrylamide solution, National Diagnostics, 7 M) (*Evaluating and Isolating Synthetic Oligonucleotides*; User Bulletin No. 13; Applied Biosystems: Warrington, U.K., 1992. Alley, S. C.; Brameld, K. A.; Hopkins, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 2734). DNA (0.05 OD) was 5′-end-labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Gibco BRL) DNA duplexes (Rink, S. M.; Solomon, M. S.; Taylor, M. J.; Rajur, S. B.; McLaughlin, L. W.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 2551) (0.05 OD) were 3′-end-labeled using [α-^{32}P]-ATP (Amersham) and DNA polymerase I, Klenow fragment (Pharmacia Biotech) (Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989). Reactions with **1** (1 mM), **2**, and **3** (typically both at 1 μM concentration) were performed with labeled DNA in 25 mM triethanolamine, 1 mM EDTA, pH 7.2, at 37 °C for 6 h. Reactions were terminated by the addition of equal volumes of 0.6 M sodium acetate, 20 mM EDTA, 100 μg/mL yeast tRNA (Gibco BRL), and 200 μM spermine, and the DNA was precipitated and resuspended in formamide/water (9:1). Cross-linked DNA was isolated by denaturing PAGE (20%) (*Oligonucleotide recovery from polyacrylamide gel with Micropure inserts and Microcon microconcentrators*; Publication 315; Amicon Ltd.: Gloucestershire, U.K., 1994), and cleavage reactions were carried out in 1 M piperidine for 1 h at 90 °C. The cleavage products were analyzed by 20% denaturing PAGE. Gels were dried onto one layer of DE81 filter paper and one layer of Whatman 3MM paper on a Speed Gel SG200 (Savant) drier and quantified by phosphorimagery (Molecular Dynamics laser PhosphorImagery system, Image Quant version 3.3); bands were assigned by reference to a Maxam–Gilbert G lane, (Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, *65*, 499).

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