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Sequence Specificity for DNA Interstrand Cross-linking Induced by Anticancer Drug Chlorambucil

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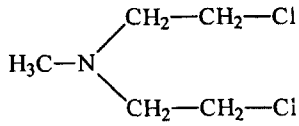
Chlorambucil is known to alkylate primarily N7 of guanine and N3 of adenine to induce DNA monofunctional adducts and interstrand cross-links (ISC). We have investigated the sequence specificity for DNA ISC induced by chlorambucil using duplex oligomers containing a defined cross-linkable sequences 5'-A*TT, 5'-G*TT, or 5'-G*CC in which asterisk indicates the potential cross-linking site and underlined base indicates the potential cross-linking site on the opposite strand. An analysis of 20% denaturing polyacrylamide gel electrophoresis showed that chlorambucil was able to induce DNA ISC in the duplex oligomers containing a sequence 5'-GCC. The formation of DNA ISC was not observed in the duplex oligomers containing sequences 5'-ATT or 5'-GTT. These results indicate that chlorambucil induces guanine-guanine DNA ISC but not guanine-adenine or adenine-adenine DNA ISC. In addition, we have tested the ability of chlorambucil to induce DNA ISC within 5'-GNNC or 5'-GC sequences using duplex oligomers containing the sequence 5'-$G^4G^3G^2C$. The result of DNA strand cleavage assay showed that DNA ISC was formed at the 5'-GGC sequence (an 1,3 cross-link, G_1-G_3) but not at 5'-GGGC (an 1,4 cross-link, G_1-G_4) or 5'-GC sequence (an 1,2 cross-link, G_1-G_2).

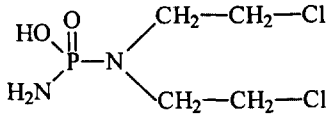
Key words : Anticancer drug, Sequence specificity, Chlorambucil, DNA interstrand cross-links

INTRODUCTION

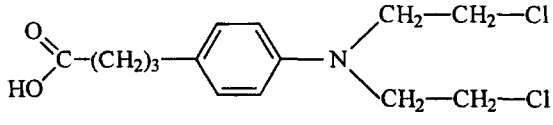
Chlorambucil (Fig. 1), a derivative of nitrogen mustard, is one of the antitumor agent that is widely used in clinical treatments of leukemia, lymphoma, and ovarian carcinoma. It is well known that nitrogen mustards alkylate DNA preferentially at the position of N7 guanine (Kohn *et al.*, 1987; Mattes *et al.*, 1986; Hartley *et al.*, 1990). DNA interstrand cross-links (ISC) can arise through bifunctional alkylation of N7 position guanine residues in opposite strands of DNA. The ability of chlorambucil to cross-link the two strand of duplex DNA is widely believed to account for its antitumor activity since DNA ISC prevent the separation of the DNA helix required for DNA replication and transcription (Kohn *et al.*, 1966; Gale *et al.*, 1981). Some studies of mechlorethamine and phosphoramide mustard with duplex DNA have suggested that 5'-GNC (an 1,3 cross-link) is the sequence for the mechlorethamine-induced DNA cross-link (Millard *et al.*, 1990; Rink *et al.*, 1993; Ojwang *et al.*, 1989; Dong *et al.*, 1995). While it has been known that nonaromatic nitrogen mustards induce DNA ISC at the 5'-GNC sequence, very little work has been done on DNA ISC induced by the aromatic analogue such as chlorambucil.



Mechlorethamine



Phosphoramide Mustard



Chlorambucil

Fig. 1. Chemical structures of nitrogen mustard and its analogues. The common two chlorethyl groups are the alkylating moieties. While mechlorethamine and phosphoramide mustard are nonaromatic nitrogen mustards, chlorambucil is the aromatic nitrogen mustard.

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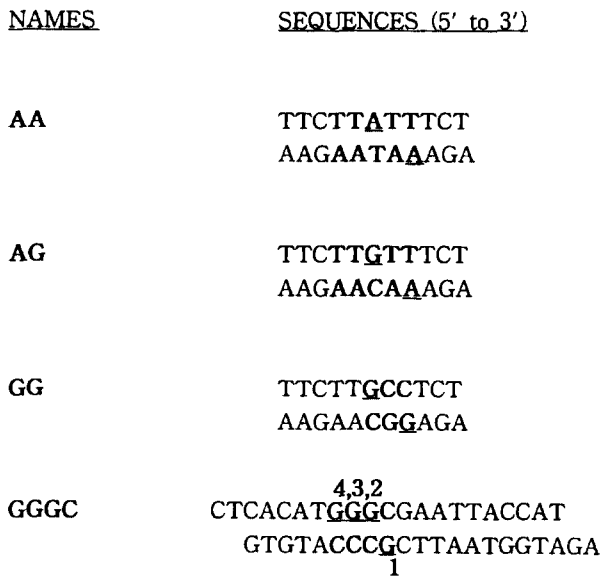

Fig. 2. Sequences of duplex oligonucleotides used in this study. Potential cross-linking sequences are shown in bold and potential cross-linking bases are underlined.

It has been found that chlorambucil alkylates N3 of adenine in addition to N7 of guanine (Wang *et al.*, 1991; Pieper and Erickson, 1990). It is, thus, interesting to determine whether adenine is the potential base involved in the formation of DNA ISC. For this purpose, we have designed three kinds of 11 bp duplex oligomers containing a defined cross-linkable sequences 5'-A*TT, 5'-G*TT, or 5'-G*CC (asterisk indicates the potential cross-linking site and underlined base indicates the potential cross-linking site on the opposite strand) (Fig. 2) and investigated the sequence specificity for the formation of DNA ISC induced by chlorambucil by a gel electrophoresis method. In addition, we have determined whether chlorambucil induces an 1,2 cross-link, an 1,3 cross-link, or an 1,4 cross-link using 21 bp duplex oligomers containing the 5'-G^4G^3-G^2C sequence (Fig. 2).

MATERIALS AND METHODS

Chemicals and reagents

Chlorambucil, acrylamide, dimethyl sulfate, and piperidine were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP was purchased from Amersham (Cleveland, OH). T4 polynucleotide kinase was from Promega (Madison, WI) and Spin X filter units (0.22 mm nitrocellulose) was from Costar (Cambridge, MA). A series of oligonucleotides (Fig. 2) were obtained from Korea Biotec which were fully deprotected with hydroxyl groups on both the 5'- and 3'-ends.

End-labeling and purification of oligonucleotides

Individual top or bottom single-stranded oligonucleotide was 5'-end labeled with 7 units T4 polynucleotide kinase and 40 μCi of [γ-^{32}P]ATP. After removal of unincorporated [γ-^{32}P]ATP by ethanol precipitation, DNA was purified by running on a 20% denaturing polyacrylamide gel until xylene cyanol marker dye migrated 10 cm. After the gel was exposed to x-ray film, labeled DNA was excised, crushed, soaked, filtered through Spin X centrifuge filter unit, and precipitated with ethanol and sodium acetate.

Determination of the DNA ISC formation induced by chlorambucil

An equal amount of unlabeled complementary strand was added to 5' end-labeled oligonucleotide. The mixture in 30 μl of 60 mM NaCl and 6 mM Tris-HCl, pH 8.0 was put in 70°C water and then slowly cooled to room temperature to form an annealed duplex. Three μl of 100 mM chlorambucil was added to the annealed duplex and incubated at 37°C for 3 hr. After ethanol precipitation of drug treated DNA, each sample was resuspended in 15 μl of tracking dye containing 80% formamide and 1 mM EDTA and then subjected to a 20% denaturing polyacrylamide gel electrophoresis. The DNA ISC formation was visualized by autoradiograpy and purified using the crush and soak procedure described above.

DNA strand cleavage assay to determine a base involved in the DNA ISC formation

Aliquots of isolated DNA ISC were heated in 40 μl of freshly diluted 1 M piperidine at 92°C for 20 min to convert sites of guanine N7 alkylation into strand breaks (Mattes *et al.*, 1986).

Maxam-Gilbert sequencing reaction

For the purine specific reaction, 30 μl of 88% formic acid was added to 30 μl of purified single-stranded DNA in distilled water and the mixtures were incubated at 37°C for 20 min. This reaction was terminated with ethanol precipitation. The chemically modified DNA duplex was resuspended in 40 μl of 1 M piperidine, and heated at 92°C for 30 min, and then lyophilized overnight (Maxam and Gilbert, 1980). The guanine-specific reaction was performed as described in the purine-specific reaction except that 30 μl of dimethyl sulfate was added instead of formic acid.

Sequencing gel electrophoresis

Samples were lyophilized and resuspended in 10 μl of tracking dye containing 80% formamide, 1mM EDTA, and xylene cyanol. After heating at 90°C for 2 min and quick cooling in ice-water bath, an equal amount

of DNA samples was loaded onto a 20% denaturing polyacrylamide gel (mono:bis acrylamide ratio=29:1, 8 M urea). Electrophoresis was carried out at 2800 V until the xylene cyanol migrated 8 cm. Drug-induced cross-linking sites were identified by reference to Maxam-Gilbert purine-specific chemical reactions on unmodified single-stranded DNA.

RESULTS AND DISCUSSION

Determination of chlorambucil-induced DNA ISC formation within 5'-ATT, 5'-GTT, and 5'-GCC sequences by a denaturing gel electrophoresis

Since chlorambucil has been found to alkylate adenine in addition to guanine, we tested the ability of chlorambucil to induce DNA ISC at adenine-adenine, adenine-guanine, or guanine-guanine sites. For this purpose, three kinds of 11 bp duplex oligomers containing only a single cross-linkable site 5'-A*TT, 5'-G*TT, or 5'-G*CC were prepared (AA, AG, and GG, respectively, in Fig 2). Each 11 bp oligomer was 5' end-labeled either on the top or the bottom strand and annealed with the unlabeled complementary strand to make a duplex oligomer. After duplex oligomers were reacted with chlorambucil, the reaction was terminated by ethanol precipitation and the mixture was analyzed using a 20% denaturing polyacrylamide gel electrophoresis. Since DNA ISC cannot be denatured and show reduced electrophoretic mobility than a DNA monofunctional adduct (MA) and single-stranded DNA (ssDNA), DNA ISC can be separated from DNA MA and ssDNA as shown in Fig. 3. The results showed that when either the top or the bottom strand was end-labeled, DNA ISC were observed in the oligomers containing 5'-GCC sequence (lane GG), not in the oligomers containing 5'-ATT sequence (lane AA) or 5'-GTT sequence (lane AG). It can, thus, be concluded that the formation of monofunctional adduct at the position of adenine does not necessarily induce the formation of adenine-adenine DNA ISC. This could be due to the fact that the efficiency of adenine alkylation by chlorambucil might be considerably lower than that of guanine alkylation.

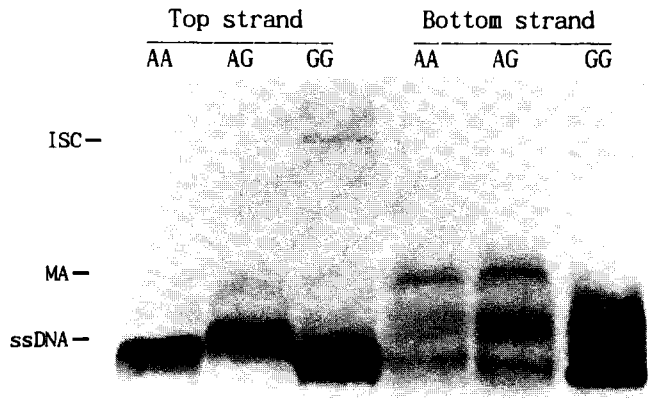

Fig. 3. Autoradiogram of a 20% denaturing acrylamide gel showing that chlorambucil induces DNA ISC only in duplex oligomers GG containing the sequence 5'-GCC.

Determination of the cross-linking site of chlorambucil within the 5'-GCC sequence

To determine the specific position of base involved in the DNA ISC formation in the oligomers containing 5'-GCC sequence, DNA ISC labeled on either the top or bottom strand of duplex DNA were purified and subjected to strand cleavage assay as described in Materials and Methods. The resulting DNA fragments were then resolved on a 20% sequencing gel in parallel with the Maxam-Gilbert sequencing reactions (Fig. 4). When the top strand was labeled (left), the cross-linking site (arrow) was the asterisk guanine in the 5'-TG*CCT sequence. When the bottom strand was labeled (right), the cross-linking site (arrow) was the asterisk guanine in 5'-AG*GCA sequence. Taken together these two results, it can be concluded that chlorambucil induces DNA ISC at 5'-G*CC sequence (an 1,3 cross-link).

Determination of the cross-linking site of chlorambucil within the 5'-GGGC sequence

To test the ability of chlorambucil to induce an 1,4 cross-link (5'-GNNC) or an 1,2 cross-link (5'-GC) in addition to an 1,3 cross-link (5'-GNC), we have used

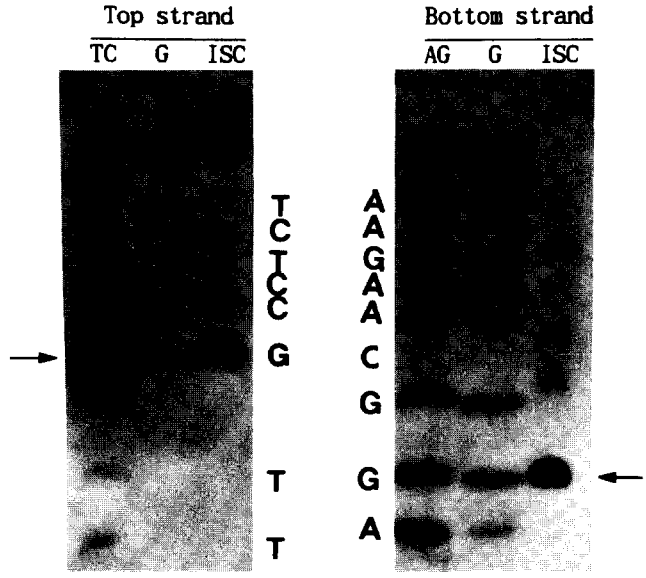

Fig. 4. Determination of the cross-linking site in duplex oligomers GG containing the sequence 5'-GCC. The arrow indicates the cross-linking site. Gel-purified DNA ISC were heated at 92°C for 20 min in the presence of 1M piperidine (lane ISC). TC, AG, and G lanes represent sequences obtained after Maxam-Gilbert sequencing reactions.

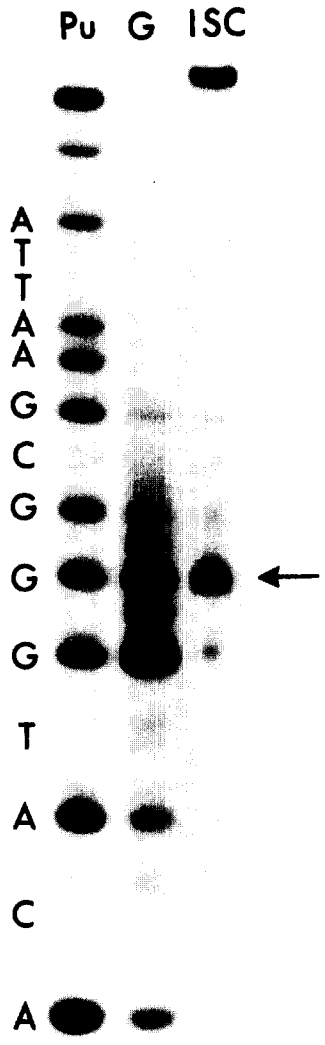

Fig. 5. Determination of the cross-linking site in duplex oligomers GGGC containing the sequence 5'-GGGC. The arrow indicates the cross-linking site. The top strand labeled DNA ISC were purified and heated at 92°C for 20 min in the presence of 1 M piperidine (lane ISC). Pu and G lanes are Maxam-Gilbert sequencing reactions.

21 bp duplex oligomers containing the 5'-$G^4G^3G^2C$ sequence (Fig. 2). After end-labeling of the top strand of duplex DNA, chlorambucil-induced DNA ISC were purified and subjected to a DNA strand cleavage assay. The resulting DNA fragments were then analyzed on a 20% sequencing gel in parallel with the Maxam-Gilbert sequencing reactions. As shown in Fig. 5, the cross-linking site was observed at the position of G3 in 5'-$G^4\underline{G}^3G^2C$ sequence (see arrow in ISC lane). The cross-linking site of the bottom strand of DNA was G1 at the 5'-G^1CCC sequence (data not shown). These results indicate that chlorambucil does not induces an 1,4 cross-link or an 1,2 cross-link but induce an 1,3 cross-link within the 5'-GGGC sequence.

An early computer modeling study suggested that 5'-GC was the favorable DNA sequence for nitrogen mustard-induced DNA cross-link (Hausheer *et al.*, 1989). Results of our present study, however, show that 5'-GNC (an 1,3 cross-link) sequence is required for the formation of chlorambucil-induced DNA cross-link. This is consistent with the other experimental evidences that nonaromatic nitrogen mustards (mechlorethamine and phosphoramide mustard) induce an 1,3 cross-link formation (Dong *et al.*, 1995; Millard *et al.*, 1990; Rink *et al.*, 1993; Ojwang *et al.*, 1989).

Molecular dynamic simulations of chlorambucil adducts and non-covalent complexes with DNA suggest that the initial, noncovalent interaction of chlorambucil with DNA induces the conformational changes required to form the 1,3 cross-linking (Remias *et al.*, 1995). Computational studies of phosphoramide mustard have shown that the geometric proximity of the binding sites favored the 1,3 cross-linking over the 1,2 cross-linking (Dong *et al.*, 1995). It can be considered that the structural basis for chlorambucil-induced 1,3 cross-linking would be very similar to that for the phosphoramide mustard-induced 1,3 cross-linking. It is not known at this time that how the 1,3 cross-link induced by chlorambucil relates to the cytotoxicity and antitumor activity.

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