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A Nitrogen Mustard Induces Formation of DNA–Histone Cross-Links in Nucleosome Core Particles

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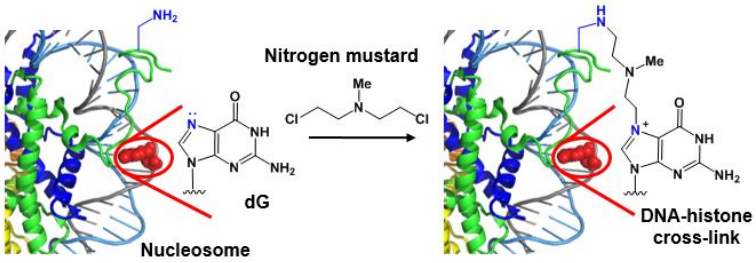
TOC



Abstract

Nitrogen mustards have long been used in cancer chemotherapy, and their cytotoxicity has traditionally been attributed to the formation of DNA interstrand cross-links and DNA monoalkylation. Recent studies have shown that exposure to nitrogen mustards also induces the formation of DNA–protein cross-links (DPCs) via bridging between N7 of a deoxyguanosine residue in the DNA and the side chain of a Cys residue in the protein. However, the formation of nitrogen mustard–induced DNA–histone cross-links has never been observed. Herein, we demonstrate that treating reconstituted nucleosome core particles (NCPs) with the nitrogen mustard mechlorethamine results in the formation of DNA–histone cross-links, in addition to DNA monoalkylation and interstrand cross-link formation. The yields of these three types of DNA lesions in the NCPs decreased in the following order: DNA monoalkylation >> DNA interstrand cross-links > DNA–histone cross-links. Mechanistic studies involving tailless histones and competitive inhibition by a polyamine demonstrated that Lys residues in the N- and C-terminal tails of the histones were the predominant sites involved in DNA–histone cross-link formation. Given that NCPs are the fundamental repeating units of chromatin in eukaryotes, our findings suggest that nitrogen mustard–induced formation of DNA–histone cross-links may occur in living cells and that DPC formation may contribute to the cytotoxicity of nitrogen mustards.

INTRODUCTION

Nitrogen mustards are bifunctional alkylating agents that are used in cancer chemotherapy.[1, 2] Their cytotoxicity is attributed mainly to their ability to induce DNA monoalkylation and interstrand cross-link formation (Figure 1A).[3-5] DNA monoalkylation occurs via formation of a highly active aziridinium intermediate and subsequent nucleophilic attack by the N7 of a deoxyguanosine (dG) residue.[6] DNA interstrand cross-link formation induced by nitrogen mustards shows sequence propensity, with the predominant cross-links bridging two dG residues at the duplex sequence 5′-d(GNC).[7-9]

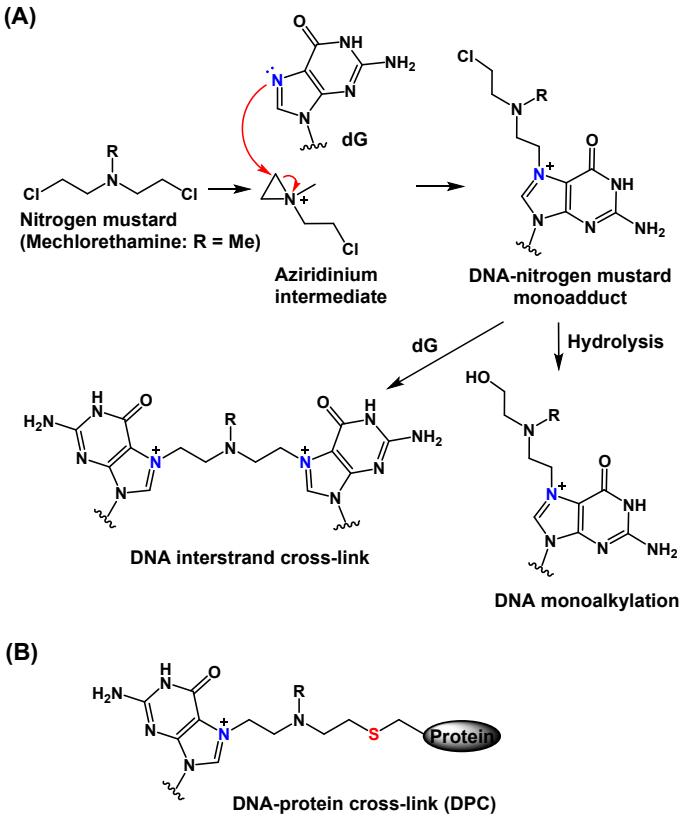


Figure 1. Nitrogen mustard–induced DNA monoalkylation, interstrand cross-link formation, and DPC formation.

In addition to inducing DNA monoalkylation and interstrand cross-link formation, nitrogen mustards also induce the formation of DNA–protein cross-links (DPCs).[10, 11] Tretyakova et al. showed that the DNA repair protein *O*6-alkylguanine DNA alkyltransferase can be readily cross-linked to DNA in the presence of nitrogen mustards.[12] The cross-link is formed between the N7 of a dG residue in the DNA and a Cys residue of the protein (Figure 1B). These

investigators also captured proteins from nuclear protein extracts by using 5′-biotinylated dsDNA together with nitrogen mustards and subjected the captured proteins to mass spectrometry–based proteomics analysis, which showed that a wide variety of nuclear proteins are cross-linked to DNA by nitrogen mustards. However, DNA–histone cross-links were not observed.[13] More recently, DeCaprio used model peptides to demonstrate that nitrogen mustards also react with Lys and His to form stable adducts, although these reactions are much less efficient than the reaction with Cys.[14]

In eukaryotes, approximately 83% of genomic DNA is packaged in nucleosome core particles (NCPs),[15, 16] which consist of 145 bp of DNA wrapped around a histone octamer core. Histones are Lys-rich, especially in their flexible N-terminal tails, which protrude from the core of the particle. Epigenetic modifications of these relatively exposed Lys residues play important roles in regulating DNA transcription.[17, 18] Recently, several research groups showed that Lys residues in histones can react with electrophilic DNA damage intermediates to form transient or stable DPCs in NCPs.[19-25] These DPCs form more readily in NCPs than by simply mixing histones with damaged DNA. This is attributed to the fact that the DPC formation in NCPs is actually an intramolecular reaction and thus kinetically more favorable than the intermolecular reaction in the latter case.

In light of this previous work, we hypothesized that treatment of NCPs with nitrogen mustards might yield DNA–histone cross-links via intramolecular addition of a Lys residue to a DNA–nitrogen mustard monoadduct. It is worth noting that Hopkins et al. studied DNA damage by nitrogen mustards in reconstituted NCPs almost two decades ago[26] and reported that treatment of NCPs with nitrogen mustards leads to both DNA monoalkylation and interstrand cross-link formation. However, these investigators did not look for DNA–histone cross-link formation. In this study, we demonstrated that the nitrogen mustard mechlorethamine can induce the formation of essential DNA–histone cross-links in reconstituted NCPs, in addition to inducing DNA monoalkylation and interstrand cross-link formation.

MATERIALS AND METHODS

Materials

Mechlorethamine hydrochloride was obtained from Selleck Chemicals. Expression of histones and reconstitution of histone octamer were carried out as previously reported.[19, 27, 28] Tailless histones were kindly provided by Professor Marc Greenberg of Johns Hopkins University. Gels were visualized with an Amersham Typhoon Gel and Blot Imaging System at excitation and emission wavelengths of 488 and 526 nm, respectively.

Preparation of dsDNA

We prepared '601' dsDNA (145 bp) by PCR amplification of plasmid pGEM-3Z-601.[29] A 5′-FAM-labeled forward primer was used, and therefore the obtained '601' dsDNA contained a 5′-FAM label in one strand.

We prepared '601' dsDNA modified with an abasic site (AP) (designated '601' dsDNA-AP) as described previously.[19, 30] The strand containing the AP modification was 5'-FAM-labeled.

Reconstitution of NCPs

The 5′-FAM-labeled '601' dsDNA (50 pmol), salmon sperm DNA (23.5 μg), and wild-type histone octamer (325 pmol) were combined in a Slide-A-Lyzer MINI Dialysis Unit (3500 MWCO, Thermo Scientific, prod. no. #69550) in a aqueous solution containing 2 M NaCl (final volume, 100 μL). The dialysis unit was placed inside a dialysis bag, filled with ~20 mL of high-salt buffer (2 M NaCl, 10 mM HEPES pH 7.5, 0.1 mM PMSF). The dialysis bag was placed in 2 L of low-salt buffer (10 mM HEPES, pH 7.5, 0.1 mM PMSF) and dialyzed overnight at 4 ºC. The dialyzed sample was incubated at 37 ºC for 2 h, and any precipitate that formed was pelleted by means of a 10 min spin at 2,000 *g*. The supernatant was then transferred to a fresh siliconized tube. The resulting NCPs were used directly in subsequent experiments, without adjustment of the concentration after reconstitution.

For determination of the extent of reconstitution, a small aliquot was removed and analyzed by 5% native PAGE (10 × 8 × 0.1 cm, acrylamide/bisacrylamide, 59:1, 0.6 × TBE buffer, run at 4 ºC with 0.2 × TBE buffer). The gel was run under limiting voltage (150 V) until the bromophenol blue band migrated to the middle of the gel. All the reconstituted NCPs were stored at 4 ºC and used directly in the following studies.

Tailless NCPs were reconstituted by means of the same procedure, except that tailless histone octamer was used instead of wild-type octamer.

12% SDS PAGE analysis of mechlorethamine-induced DPC formation in NCPs

Freshly prepared mechlorethamine hydrochloride aqueous solution (4 μL) was added to reconstituted NCP solution (10 mM HEPES, pH 7.5, 60 mM NaCl, 0.1 mM PMSF, 16 μL); the final concentration of mechlorethamine varied from 1 to 300 μM. The resulting mixture was incubated at 37 °C for 3 h. Two aliquots were then withdrawn; one was analyzed by 12% (w/v) SDS PAGE (10 × 8 × 0.1 cm, acrylamide/bis(acrylamide) 29:1 [w/w], 5% [w/v] stacking layer), and the other was analyzed by 5% native PAGE (10 × 8 × 0.1 cm, acrylamide/bisacrylamide, 59:1, 0.6 × TBE buffer, run at 4 ºC with 0.2 × TBE buffer).

Time course of mechlorethamine-induced DPC formation in NCPs

Freshly prepared mechlorethamine hydrochloride aqueous solution (14 μL, 500 μM) was added to reconstituted NCP solution (10 mM HEPES, pH 7.5, 60 mM NaCl, 0.1 mM PMSF, 56 μL); the final concentration of mechlorethamine was 100 μM. The mixture was incubated at 37 °C for the duration of the time-course experiment. Aliquots were periodically removed and analyzed by 12% (w/v) SDS PAGE.

Effect of spermine on time course of mechlorethamine-induced DPC formation in NCPs

Freshly prepared mechlorethamine hydrochloride aqueous solution (14 μL, 500 μM) and freshly prepared spermine aqueous solution (14 μL, 5 mM) were added to reconstituted NCP solution (10 mM HEPES, pH 7.5, 60 mM NaCl, 0.1 mM PMSF, 42 μL). The mixture was incubated at 37 °C for the duration of the time-course experiment. Aliquots were periodically removed and analyzed by 12% (w/v) SDS PAGE.

In another experiment, freshly prepared mechlorethamine hydrochloride solution (14 μL, 500 μM) was combined with 42 μL of reconstituted NCP solution. After incubation at 37 °C for 3 h, freshly prepared spermine solution (14 μL, 5 mM) was added. The mixture was incubated at 37 °C for the duration of the experiment, and aliquots were periodically removed and analyzed by 12% (w/v) SDS PAGE.

Preparation of AP-DPCs

The 5′-FAM-labeled '601' dsDNA-AP (final concentration, 0.1 μM; the AP exists as a photolabile precursor in the dsDNA[19, 30]), a specific histone (final concentration, 0.2 μM), and

$NaBH_3CN$ (final concentration, 10 mM) were mixed in a test tube. The mixture was photoirradiated at 350 nm for 7 min and then incubated at 37 °C for 10 h. The resulting sample was analyzed by 12% (w/v) SDS PAGE.

Quantification of DPCs, DNA interstrand cross-links, and DNA monoalkylation in NCPs

Freshly prepared mechlorethamine hydrochloride aqueous solution (14 μL) was added to reconstituted NCP solution (10 mM HEPES, pH 7.5, 60 mM NaCl, 0.1 mM PMSF, 56 μL); the final concentration of mechlorethamine was 10, 30, or 50 μM. After incubation at 37 °C for 3 h, the sample was split into three portions. The first one was analyzed by 12% (w/v) SDS PAGE. To the second portion was added 1 μL of proteinase K (10 μg/μL), and the resulting solution was incubated at room temperature for 10 min and then analyzed by 8% (w/v) denaturing PAGE. To the third portion was added 1 μL of proteinase K (10 μg/μL), and the resulting solution was incubated at room temperature for 10 min. Then an equal volume of 1 M piperidine (final concentration, 500 mM) was added, and the solution was incubated at 90 °C for 30 min. After removal of piperidine by means of a SpeedVac, the sample was analyzed by 8% denaturing PAGE.

As a control, 5′-FAM-labeled '601' dsDNA was treated the same way in parallel.

RESULTS AND DISCUSSION

Mechlorethamine-induced DPC formation in reconstituted NCPs

To determine whether DPC formation could be induced by the nitrogen mustard mechlorethamine, we used '601' NCPs[27, 31] that had been reconstituted with 145 bp '601' dsDNA (labeled with 5′-FAM at one end) and recombinant histone octamer. Incubation of the NCPs with mechlorethamine under physiologically relevant conditions (10 mM HEPES, pH 7.5, 60 mM NaCl, 0.1 mM PMSF) and subsequent 12% SDS PAGE analysis revealed two groups of new products (Groups I and II in Figure 2A). These products contained the 145 bp dsDNA but showed greatly reduced mobility in the gel relative to that of the pure 145 bp dsDNA. Treatment of these products with proteinase K transformed them to free DNA that co-migrated with the 145 bp dsDNA. These observations indicate that both groups of products were DPCs. In addition, an electrophoretic mobility shift assay (EMSA) of the

mechlorethamine-treated NCPs showed that neither NCP disassembly nor NCP dimerization or oligomerization took place (Figure S1), indicating that mechlorethamine-induced DPC formation occurred via an intramolecular process.

The yield of DPCs depended on the mechlorethamine concentration. The overall yield of DPCs was 1% after incubation with 1 μM mechlorethamine at 37 °C for 3 h (Figure 2B); but under the same conditions, the DPC yield increased to 38% when the mechlorethamine concentration was increased to 300 μM. The Group I DPCs were the major products, and their yield was approximately 5 times that of the Group II DPCs. Analysis of the time course of DPC formation at a mechlorethamine concentration of 100 μM revealed that the reaction followed first-order kinetics (Figures 2C and S2).

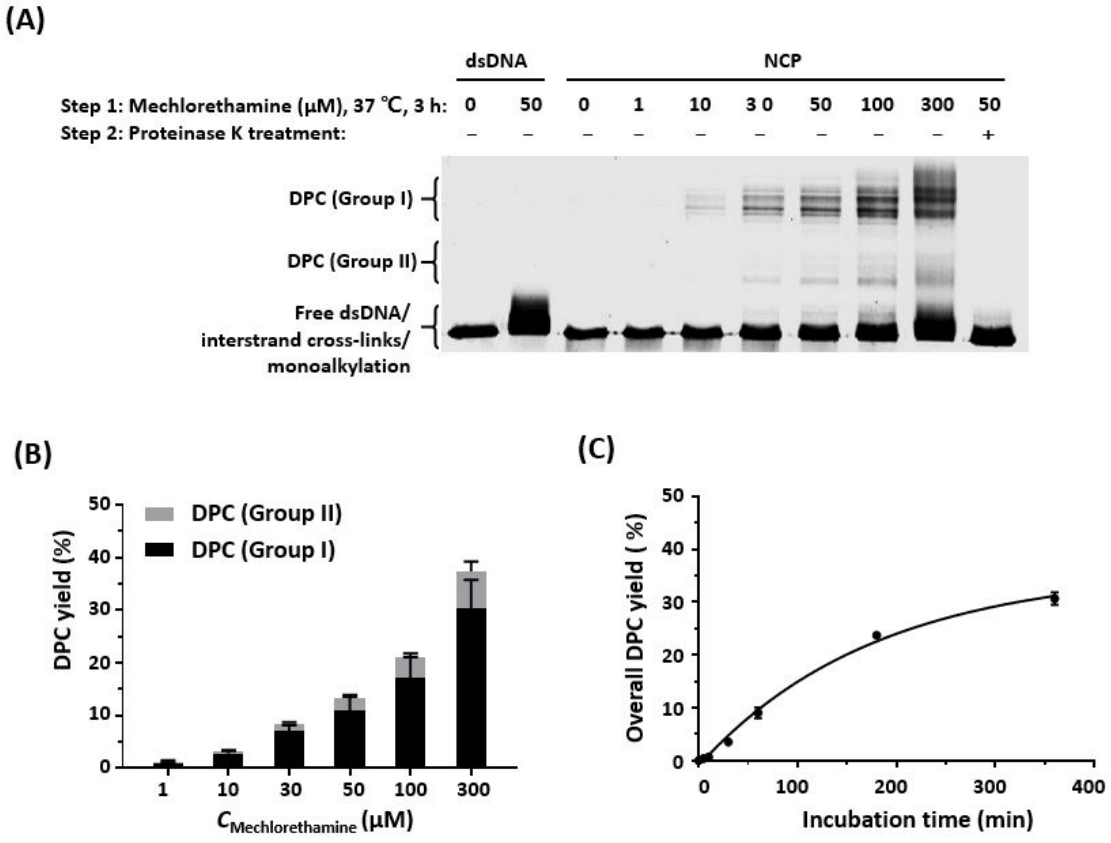


Figure 2. DPC formation induced by treatment of NCPs with mechlorethamine. (A) 12% SDS PAGE showing DPC formation after incubation of NCPs with various concentrations of mechlorethamine at 37 °C for 3 h. (B) Yields of DPCs after incubation of NCPs with various concentrations of mechlorethamine at 37 °C for 3 h. (C) Kinetics of DPC formation upon

treatment of NCPs with 100 μM mechlorethamine. Error bars indicate mean ± standard deviation of at least triplicate measurements.

Characterization of DPCs generated by treatment with mechlorethamine

To characterize the DPCs generated by treatment of the NCPs with mechlorethamine, we prepared a series of DNA–histone conjugates by '601' dsDNA-AP with different histones in the presence of $NaBH_3CN$. We previously demonstrated that in the presence of this reagent, Lys residues of histones react with the AP to form two different types of DNA_1–$histone_1$ cross-links (The subscript 1 means the cross-link is formed by conjugating one histone molecule to one dsDNA molecule), designated DPC_{cl} and DPC_{un} (Figure 3A).[19] AP^{89}-DPC_{un} conjugates formed by reactions of dsDNA-AP^{89} with four different histones showed slightly different migration rates in 12% SDS PAGE analysis, and they co-migrated with the Group I DPCs that formed when the NCPs were treated with 100 μM mechlorethamine (Figure 3B). These results suggest that the Group I DPCs were a mixture of four different DNA_1–$histone_1$ conjugates.

Using the same strategy, we also prepared AP^{137}-DPCs (Figure 3C). In the AP^{137}-DPCs, the histone was conjugated to one end of the dsDNA, whereas in the AP^{89}-DPCs, the histone was conjugated to the middle of the dsDNA. Although the AP^{137}-DPCs and the AP^{89}-DPCs have exactly the same molecular weights, the AP^{137}-DPCs migrated much faster than the AP^{89}-DPCs in 12% SDS PAGE analysis. Interestingly, we found that the AP^{137}-DPCs migrated at the same rate as the Group II DPCs generated by treatment of the NCPs with mechlorethamine. Therefore, we suggest that the Group I and Group II DPCs are DNA_1–$histone_1$ conjugates and that all four histones could be cross-linked to DNA in the NCPs by treatment with the nitrogen mustard. Notably, increasing the mechlorethamine concentration to 300 μM resulted in new products that migrated even slower than the Group I DPCs (Figure 2A). These products could be DNA_1–$histone_n$ conjugates that were formed by cross-linking multiple histones to the DNA ($n \leq 8$ because there are only 8 histone molecules in one NCP).

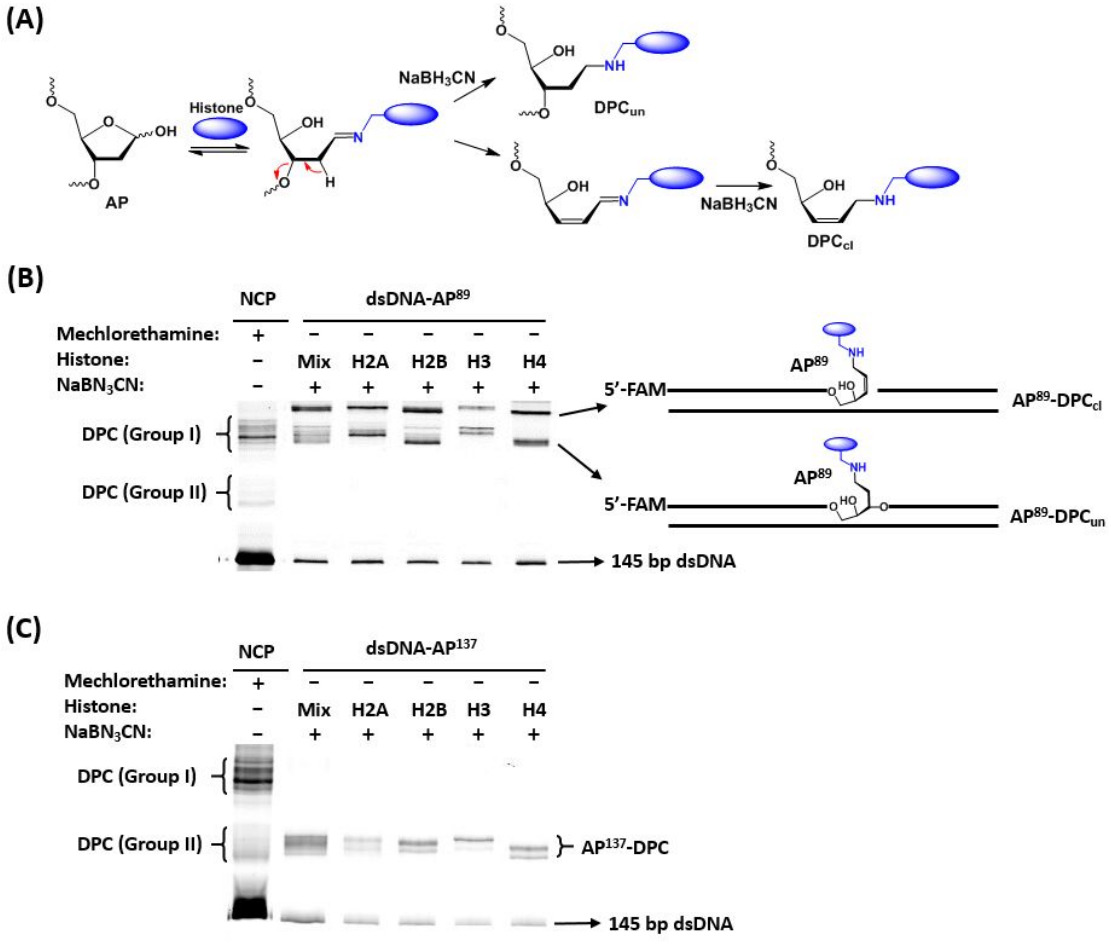


Figure 3. Characterization of DPCs generated by treatment of NCPs with mechlorethamine. (A) Mechanism of DPC_{un} and DPC_{cl} formation by reaction of AP with histones in NCPs. (B) 12% SDS PAGE showing that Group I DPCs generated by treatment of NCPs with mechlorethamine co-migrate with AP^{89}-DPC_{un} conjugates. (C) 12% SDS PAGE showing that Group II DPCs generated by treatment of NCPs with mechlorethamine co-migrate with AP^{137}-DPC_{un} conjugates. Mix: mixture of H2A, H2B, H3, and H4.

Identification of amino acid residues involved in mechlorethamine-induced DPC formation in NCPs

The above-described experiments demonstrate that treatment of reconstituted NCPs with nitrogen mustards leads to substantial DNA–histone cross-link formation. The reactive residue involved in DPC formation is unlikely to be Cys because there is only one Cys residue (H3-Cys110) among all four histone proteins, and it is buried inside the NCP. DeCaprio et al.

showed that nitrogen mustards react not only with Cys residues but also with His and Lys residues, albeit with lower yields.[14] Because the N-terminal tails of histones are flexible and Lys-rich (Figure 4A), we hypothesized that Lys residues in the N-terminal tails were the main sites involved in mechlorethamine-induced DPC formation. To test this hypothesis, we prepared tailless NCPs in which the four wild-type histones were replaced by corresponding tailless mutants: H4-del 1–20, H3-del 1–37, H2A-del 1–15, H2B-del 1–31 (Figure 4A). After incubation with mechlorethamine (100 μM at 37 °C for 3 h), the total yield of DPCs in the tailless NCPs (8%) was significantly lower than that in the wild-type NCPs (Figures 4B and C). This result confirms that mechlorethamine-induced DPC formation occurred mainly as a result of cross-links between DNA and residues in the N-terminal tails of the histones in the NCPs. There are 5, 8, 4, and 12 Lys residues in the N-terminal tails of histones H4, H3, H2A, and H2B, respectively; but there is only one His, which is located in the N-terminal tail of H4 (Figure 4A). Therefore, Lys residues in the N-terminal tails of the histones can be expected to have been the predominant sites involved in mechlorethamine-induced DPC formation in the NCPs.

The distribution of the types of DPCs generated in the tailless NCPs also differed from the distribution in the wild-type NCPs. Group II DPCs were not observed in the former, and only one Group I DPC was generated (Figure 4B), which was identified as a dsDNA–H2A conjugate, as indicated by its migration rate in the gel and MS analysis (Figure S3). H2A is an unique histone in that it, unlike other three histones, has a flexible C-terminal tail (residues 117–129). The C-terminal tail contains five Lys residues and no other nucleophilic residues such as Cys and His (Figure 4A). Therefore, the H2A-del 1–15 mutant could still form a DPC in the tailless NCPs via attack on mechlorethamine by Lys residues in the C-terminal tail.

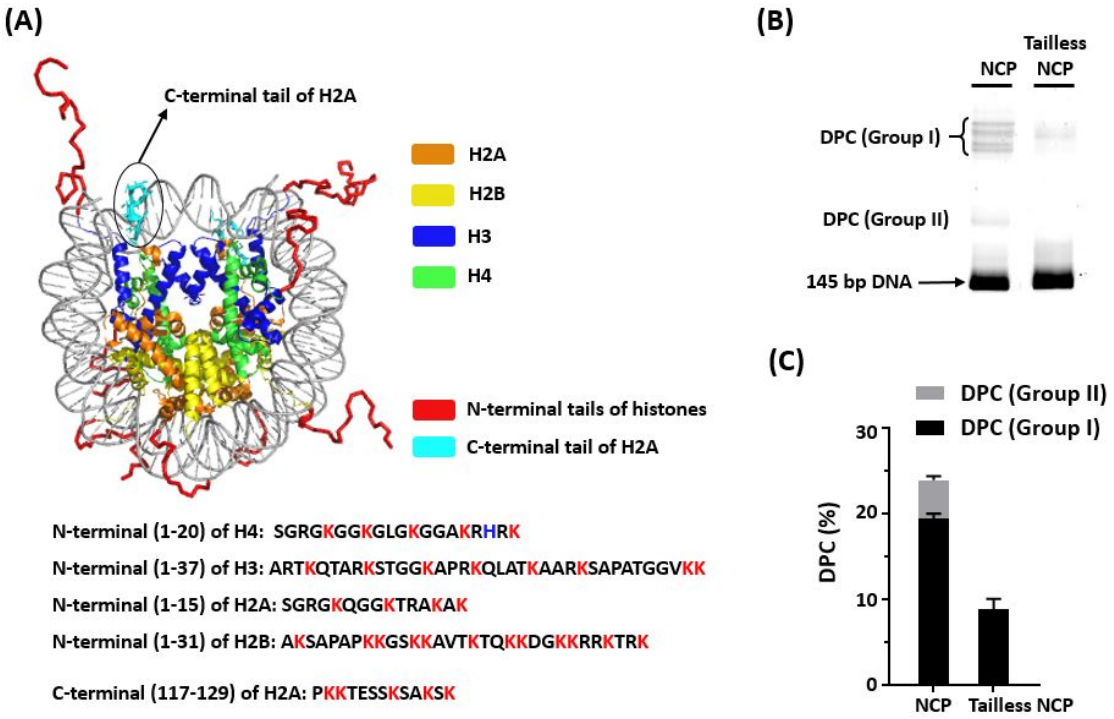


Figure 4. Amino acid residues involved in mechlorethamine-induced DPC formation in NCPs. (A) X-ray crystal structure of an NCP (from PDB 1KX5) showing the flexible histone tails. (B) 12% SDS PAGE analysis of DPC formation in tailless NCPs induced by mechlorethamine (100 μM at 37 °C for 3 h). (C) DPC formation yields in tailless NCPs and wild-type NCPs induced by mechlorethamine (100 μM at 37 °C for 3 h). Error bars indicate mean ± standard deviation of at least triplicate measurements.

The above-described results indicate that Lys residues in the flexible N- and C-terminal tails of histones were the predominant sites for DPC formation in the NCPs. In principle, cross-link formation occurs via nucleophilic attack on mechlorethamine by the ε-amino group of Lys. Therefore, we speculated that spermine, a polyamine present at millimolar concentrations in the nucleus, might interfere with mechlorethamine-induced DPC formation in NCPs. The pK_a of spermine is similar to that of the ε-amine of Lys[32] and reactions between spermine and mechlorethamine thus might compete with mechlorethamine-induced DPC formation. We have previously shown that NCPs remain intact in the presence of 1 mM spermine.[21] Thus, the mechlorethamine-induced DPC formation in NCPs was examined in the presence of 1 mM spermine. The overall DPC yield was approximately 1/4 of that in the absence of spermine

(Figure 5). However, if the NCPs were first incubated with mechlorethamine at 37 °C for 3 h to allow DPC formation and then treated with spermine (1 mM), the DPC yield did not decrease upon further incubation (Figure S4). This result indicates that the DPCs generated by treatment with mechlorethamine were stable toward spermine and that the lower DPC yield in the presence of spermine was due to competitive inhibition of DPC formation by spermine. These results have two implications: (1) they confirm that alkyl amines can react with nitrogen mustards under physiologically relevant conditions (pH 7.5), and (2) they indicate that inside cells, mechlorethamine-induced DPC formation can be counteracted by the presence of polyamines in the nucleus. It is worth noting that the reaction of spermine with nitrogen mustard may lead to one new type of DNA lesion, DNA-nitrogen mustard-spermine adduct, which may also contribute to the toxicology of nitrogen mustards inside cells.

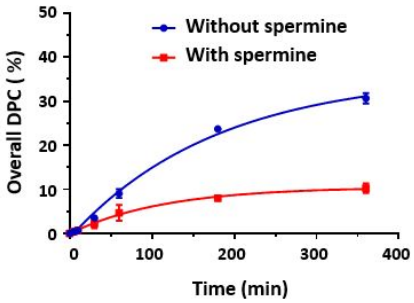


Figure 5. Kinetics of mechlorethamine-induced DPC formation in reconstituted NCPs in the presence and absence of spermine (1 mM). Error bars indicate mean ± standard deviation of at least triplicate measurements.

Comparison of mechlorethamine-induced formation of DNA lesions in NCPs and in free dsDNA

Hopkins and co-workers showed that treatment of NCPs with nitrogen mustards results in DNA monoalkylation and interstrand cross-link formation.[26] Having demonstrated that DPCs are also formed, we next tried to quantify the three types of DNA lesions generated by mechlorethamine in NCPs and in free dsDNA. For this purpose, we treated reconstituted '601' NCPs and 145 bp '601' dsDNA with mechlorethamine at three different concentrations at 37 °C for 3 h. DNA interstrand cross-links and total DNA alkylation in free DNA were quantified

by means of Hopkins's strategy.[26] That is, interstrand cross-links were directly quantified by means of 8% denaturing PAGE analysis, in which they migrated much slower than free dsDNA and monoalkylated products (Figures 6A and S5). Treatment of the sample with 0.5 M piperidine at 90 °C for 30 min resulted in a DNA strand break at the dG-N7-alkylated site;[26] and subsequent 8% denaturing PAGE analysis allowed us to quantify the overall yield of DNA damage, including both DNA monoalkylation and interstrand cross-link formation (Figure S6). Samples of the NCPs were first treated with proteinase K to digest the histones, and then the yield of interstrand cross-links and the overall yield of DNA damage (monoalkylation, interstrand cross-links, and DPCs) were quantified by means of the strategy described above (Figures 6A, S5, and S6). The yields of DPCs in the NCPs were directly quantified by means of 12% SDS PAGE analysis (Figure S7).

In the NCPs, the overall yields of DNA damage were much higher than the yields of DPCs and DNA interstrand cross-links (Figure 6B), indicating that DNA monoalkylation was the predominant mode of damage. Thus, the yields of the different types of DNA lesions in the NCPs decreased in the following order: DNA monoalkylation >> DNA interstrand cross-links > DPCs. The yields of DPCs were slightly lower than the yield of DNA interstrand cross-links.

In the presence of 10 μM mechlorethamine, the yields of interstrand cross-links and overall DNA damage in the NCPs were less than those in dsDNA (Figure 6B), suggesting that NCPs protect DNA against damage by nitrogen mustards. Therefore, although assembly of dsDNA into NCPs gives rise to the risk of DPC generation, a type of DNA damage that is unlikely to occur in dsDNA, this adverse effect is counteracted by a reduction in overall DNA damage. Whereas in the presence of 50 μM mechlorethamine, the extent of overall DNA damage became comparable in NCPs and dsDNA (Figure 6B), suggesting that the protective effect of NCP is marginal in the presence of high-concentration nitrogen mustard.

In addition to providing the yield of overall DNA damage, 8% denaturing PAGE analysis of samples treated with hot piperidine also revealed the overall pattern of DNA alkylation by mechlorethamine (Figure S6). Alkylation occurred exclusively at dG, and the sequence propensity for the NCPs was found to be exactly the same as that for free DNA. This result, which is consistent with what Hopkins and co-workers observed,[26] indicates that the rotational

position of dG in the NCPs had only a marginal effect on alkylation by mechlorethamine and that all dG bases were accessible to mechlorethamine.

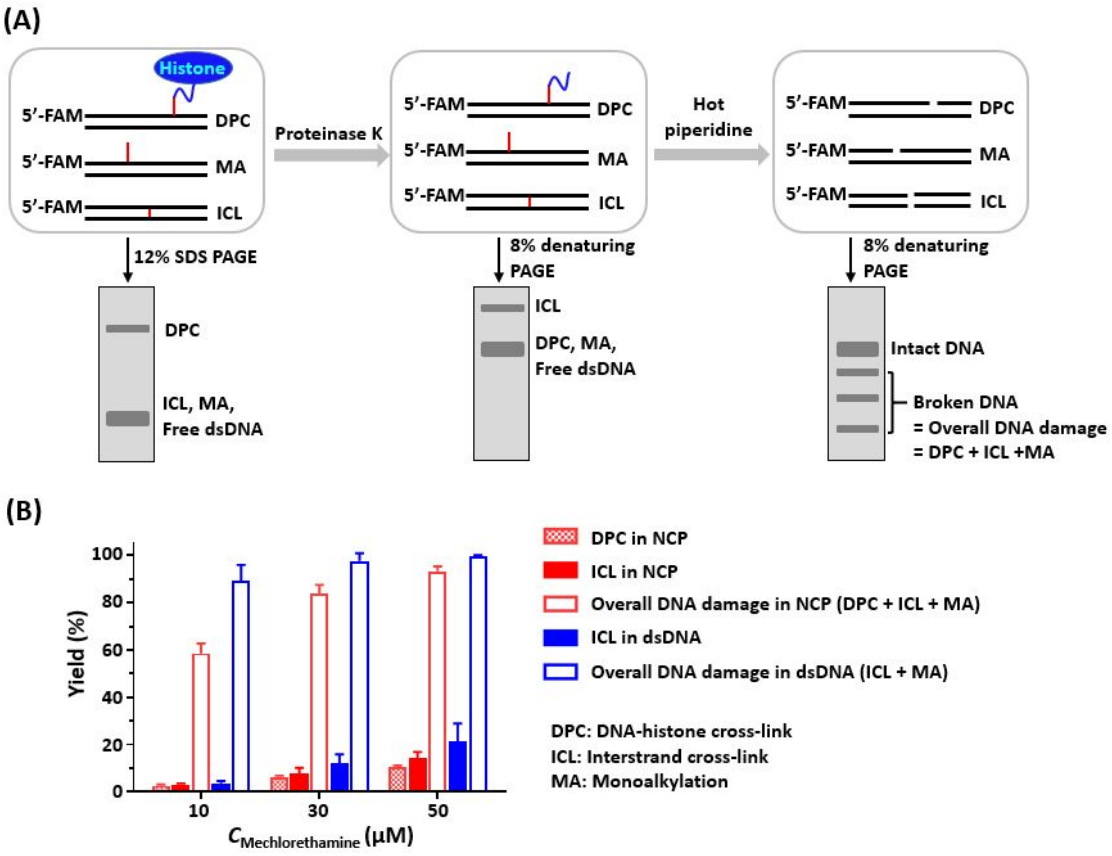


Figure 6. Comparison of DNA damage by mechlorethamine in NCPs and in free dsDNA. (A) Strategies used for quantification of different types of DNA damage induced by mechlorethamine in NCPs. (B) Yields of different types of DNA damage in NCPs and in free DNA upon treatment with various concentrations of mechlorethamine at 37 °C for 3 h. Error bars indicate mean ± standard deviation of at least triplicate measurements.

Proposed mechanism of nitrogen mustard–induced DPC formation in NCPs

The above-described experiments have demonstrated that the nitrogen mustard mechlorethamine can induce DPC formation in NCPs. The reaction occurs at dG nucleotides in DNA, and the predominant conjugation sites in histones are Lys residues in the flexible N- and C-terminal tails of the proteins. In principle, nitrogen mustard–induced cross-links are

formed via two successive nucleophilic substitution reactions, and the first is much faster than the second.[6, 11] To form DPCs in NCPs, mechlorethamine can react first either with DNA or with a histone to form a DNA–nitrogen mustard monoadduct (Figure 7A) or a histone–nitrogen mustard monoadduct (Figure 7B), respectively. The DNA–nitrogen mustard monoadduct then undergoes hydrolysis to give a DNA monoalkylation product, or reacts intramolecularly with a dG residue to generate a DNA interstrand cross-link, or reacts intramolecularly with a histone to afford a DPC (Figure 7A). In contrast, further transformation of histone–nitrogen mustard monoadducts in NCPs leads to a DPC, a protein–protein cross-link, or protein monoalkylation. We examined the reactions between dsDNA and free histones in the presence of mechlorethamine. DPC formation was observed, but not for protein–protein cross-link (Figure S8). We have demonstrated that after incubation of NCPs with mechlorethamine, the yields of different DNA lesions in the NCPs decreased in the following order: DNA monoalkylation >> interstrand cross-links > DPCs; and this result reveals that N7 of dG is much more reactive toward nitrogen mustards than the Lys residue in the histones is. Therefore, formation of a DNA–nitrogen mustard monoadduct (Step 1 in Figure 7A) is more favorable than the intermolecular reaction between nitrogen mustards and histones (Step 1 in Figure 7B). However, once a DNA–nitrogen mustard monoadduct forms, its reaction with histones in NCPs becomes feasible because it is now an intramolecular process (Step 2 in Figure 7A); and as a result, we did observe DPC formation. Taken together, our results indicate that nitrogen mustard–induced DPC formation in NCPs likely occurs via the following pathway (Figure 7A): the nitrogen mustard reacts with nucleosome DNA to generate a DNA–nitrogen mustard monoadduct, which undergoes intramolecular attack by a histone Lys residue to afford a DNA–histone cross-link.

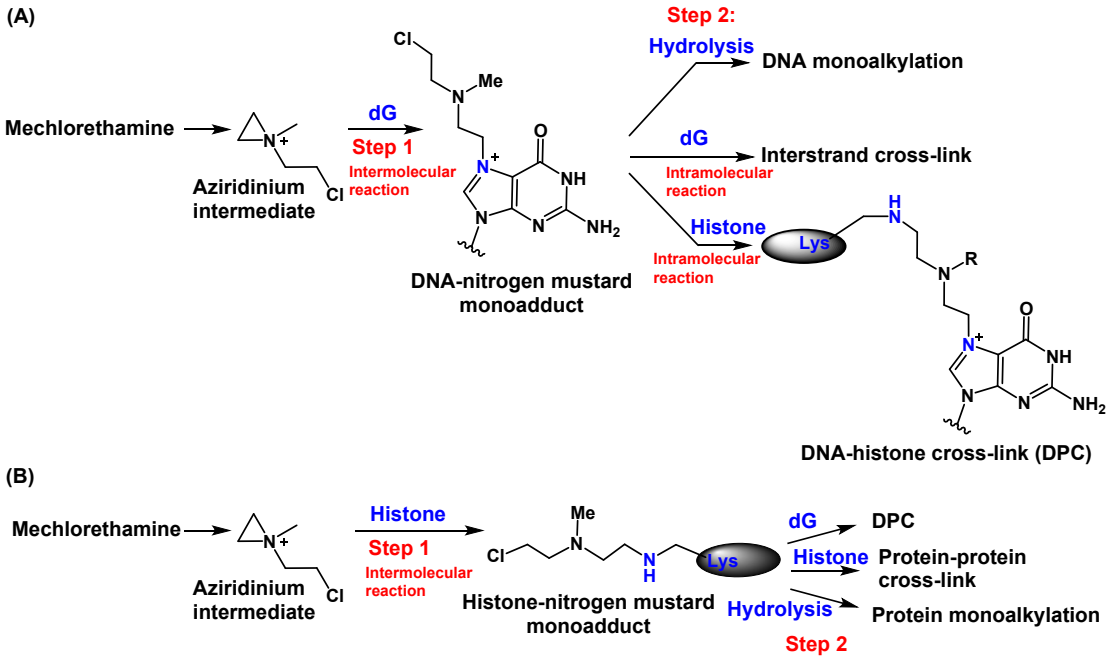


Figure 7. Proposed mechanism of nitrogen mustard–induced DPC formation in NCPs.

Conclusion

The cytotoxicity of nitrogen mustards was historically thought to arise via the formation of DNA interstrand cross-links. A few later studies showed that exposure to these compounds also leads to the formation of DPCs via bridging of the N7 of dG in DNA and the side chain of a Cys residue in a protein. However, nitrogen mustard–induced DNA–histone cross-link formation had not been reported. In the present study, we demonstrated that treating reconstituted NCPs with the nitrogen mustard mechlorethamine induces the formation of DNA–histone cross-links in addition to DNA monoalkylation and interstrand cross-link formation. Lys residues in the N- and C-terminal tails of histones are the predominant sites involved in mechlorethamine-induced DPC formation.

Upon treatment of NCPs with mechlorethamine at various concentrations, the yields of DNA–histone cross-links are only slightly lower than the yields of DNA interstrand cross-links. Although the addition of a polyamine to the reaction mixture reduces the yields of DPCs, their formation was still observed. Our results indicate that nitrogen mustard–induced formation of DNA–histone cross-links may occur in living cells. Compared with other DNA lesions, DPCs are more difficult to repair and persist longer in the cell cycle.[33] The presence of DPCs inside cells may have deleterious effects on essential biological processes such as DNA replication,

transcription, and repair and epigenetic regulation.[34, 35] On the basis of our results, we speculate that DNA–histone cross-link formation may contribute to the cytotoxicity of nitrogen mustards. It is worth noting that several DPC repair pathways, such as nuclease-dependent MRN[37] and protease-dependent SPRTN,[38, 39] have been revealed recently.[36] Whether those pathways can repair the nitrogen mustard-induced DPCs and compromise the chemotherapy effect of nitrogen mustards need to be addressed in the future.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge via the Internet at http://pubs.acs.org.

Supplementary figures (PDF)

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Notes

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ABBREVIATIONS

AP, abasic site; dsDNA, double-stranded DNA; DPC, DNA–protein cross-links; EMSA, electrophoretic mobility shift assay; NCP, nucleosome core particle

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