4378–4382 Nucleic Acids Research, 1995, Vol. 23, No. 21

Sulphur mustards inhibit binding of transcription factor AP2 *in vitro*

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Received July 21, 1995; Revised and Accepted October 6, 1995

ABSTRACT

The bifunctional sulphur mustard (bis-(2-chloroethyl)sulphide, HD) and its monofunctional analogue (2-chloroethyl ethyl sulphide, CEES) are both vesicants. In this study, both mustards were shown to rapidly alkylate the AP2 consensus binding sequence incorporated in a 26mer oligonucleotide. The reaction was essentially complete within 10 min under the conditions employed in this study and ~95% of the oligonucleotides were alkylated at least once using 500 μM HD and 1 mM CEES. Progressive alkylation of the consensus sequence was parallelled by a decrease in transcription factor binding. Under reaction conditions which alkylated ~95% of the oligonucleotides at least once, the binding of cloned human AP2 was reduced by 93 and 76% by HD and CEES, respectively, compared with control values. The interference with binding is a result of alkylation of the DNA and not damage to the transcription factor by mustard or its hydrolysis products. Interference with transcription factor binding would be expected to have a profound influence on the ability of the cell to function normally and to respond to DNA damage and may contribute significantly to the skin damage produced by these compounds.

INTRODUCTION

Sulphur mustard (bis-(2-chloroethyl)sulphide; HD) is a highly lipid soluble molecule (Fig. 1a) which rapidly penetrates human skin and is capable of alkylating a range of biomolecules. Skin injuries produced by mustards are distinguished by inflammation, destruction of epithelial tissues and severe blistering. With vesicating doses of HD, blister formation begins after a characteristic delay following exposure, and results from cleavage between the basal cell membrane and the basement membrane (1,2). Sulphur mustard is both mutagenic and carcinogenic and the toxicology of this compound has been summarised recently (3). The monofunctional analogue of sulphur mustard (2-chloroethyl ethyl sulphide; CEES) is also a vesicant (Fig. 1a).

Human cells exposed in culture to HD show concentration dependent decreases in cell proliferation, DNA synthesis, protein synthesis and NAD^+ and ATP levels (4,5). DNA alkylation by mustards is well documented and thought to be a critical event underlying these disturbances. The major alkylation site of sulphur mustard is guanine-N^7. The reaction products are either 7-alkylguanine from monofunctional alkylations or di(guanin-7′-yl-ethyl)sulphide from crosslink formation (6–9). Alkylation is also observed at adenine-N^3 and, in the case of CEES, at guanine-O^6 (8,10).

Although the exact mechanism of action of sulphur mustard is not understood (3) the type of DNA damage inflicted by this compound would be expected to have a profound effect on the ability of DNA to function as a template for both DNA and RNA synthesis. Studies *in vitro* have shown that the mustards interfere with the progression of *Escherichia coli* RNA polymerase and *Taq* DNA polymerase and affect the interaction between *E.coli* RNA polymerase and the *lac* UV5 promoter (11,12). D'Incalci and co-workers have shown that O^6 methylation of guanine and alkylation by nitrogen mustard also interfere with the binding of transcription factors to DNA (13–15).

The bifunctional HD is considered to be more toxic than its monofunctional analogue, and the ability of HD to form crosslinks is invoked as an explanation of this difference (3). However, both compounds are toxic and cause vesication and this suggests that some of the important consequences of alkylation which lead to these effects should be common to both, and independent of crosslink formation.

Nitrogen mustard (bis(2-chloroethyl) methylamine; HN2) and quinacrine mustard both inhibit the binding of NFκB to its GC rich recognition sequence (14). This sequence contains a 5′-GAC nitrogen mustard cross-linking site (16,17). In contrast, HN2 did not inhibit the binding of OTF-1 to an AT rich oligonucleotide lacking a cross-linking site (14). However, the octamer binding site (5′-ATGCAAAT) does contain a 5′-GC sequence which may potentially be alkylated (18). There are several possible reasons for the different effects. First, GC is a relatively poor alkylation site (19). However, alkylation by nitrogen and sulphur mustard does occur at these sites at concentrations significantly lower than the 2 mM used by Fabbri and co-workers (12,14). Secondly, the distortion induced by crosslink formation by mustards may be responsible for inhibiting transcription factor binding, and alkylation of the lone GC may have no effect on OTF-1 binding. If this is so, then only those mustards capable of forming crosslinks will displace the transcription factors and the displacement will only occur within those binding sequences containing crosslinking sites.

The aim of this study was to determine if interference with transcription factor binding is a potential factor in the vesicant action of sulphur mustards. Both bifunctional and monofunctional sulphur mustard were tested to determine if crosslink formation is a requirement for this interference. The transcription factor AP2 was chosen for this study because the recognition

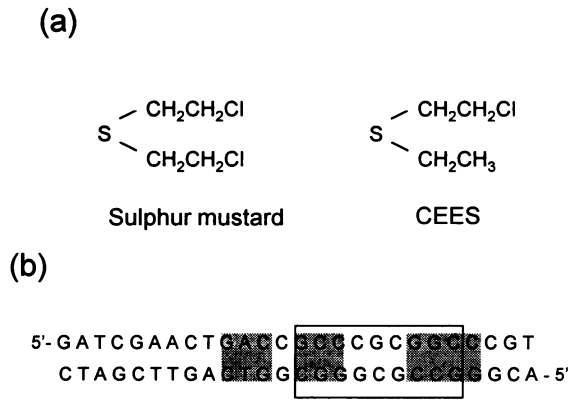

Figure 1. (**a**) Structures of the alkylating agents used in this study: sulphur mustard (bis-(2-chloroethyl)sulphide) and CEES (2-chloroethyl ethyl sulphide). (**b**) AP2 consensus oligonucleotide. The binding site is outlined and the crosslinking sites are shaded.

sequence of this protein is GC rich and contains several potential crosslinking sites (Fig. 1b).

MATERIALS AND METHODS

Materials

Purified, cloned human AP2 and its consensus oligonucleotide and T4 polynucleotide kinase were purchased from Promega Corporation. Sulphur mustard was synthesised at Aeronautical and Maritime Research Laboratory, DSTO and 2-chloroethyl ethyl sulphide (CEES) was purchased from Aldrich Chemical Company, Inc. The two sulphur mustards were >98% pure as assessed by ^{1}H NMR. [γ-^{33}P]ATP was from Amersham Australia. Urea, ammonium persulfate, TEMED and DTT were obtained from BioRad as electrophoresis purity reagents. Acrylamide (40%, 19:1 acrylamide/bis-acrylamide) and glycogen were from Boehringer and electrophoresis grade Nonidet P-40 and piperidine were from Sigma. NENSORB 20 columns were obtained from Dupont. All other chemicals were of analytical grade and all solutions were prepared using distilled, deionised and filtered water from a 'Milli-Q' 4-stage water purification system (Millipore).

Oligonucleotide alkylation and piperidine cleavage

The transcription factor consensus oligonucleotide was 5′ end-labelled using T4 polynucleotide kinase and [γ-^{33}P]ATP and the labelled DNA was purified using a NENSORB 20 column, dried and then dissolved in 100 µl of TE buffer.

The sulphur mustards were dissolved initially in ethanol and then sub-diluted to the required concentration in TE buffer. The final ethanol concentration in the alkylation reaction was 0.5% v/v. After alkylation for the appropriate time, the reaction was stopped by adding 5 µl of alkylated DNA to 32 µl of a 'stop' solution (75 µl H_2O, 18 µl 3 M sodium acetate and 375 µl ethanol) and 1 µl glycogen. After incubation at –20°C for 30 min, the DNA was precipitated by centrifugation at 4°C for 30 min and the pellet washed with 100 µl of 70% ethanol and dried under vacuum. The dried pellet was resuspended in 100 µl 10% (v/v) piperidine and heated to 90°C for 30 min. The solution was then dried under vacuum, washed twice with 10 µl H_2O and the resulting pellet dissolved in 10 µl of a solution containing 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue in 98% deionised formamide.

Transcription factor binding

The oligonucleotides were alkylated by combining equal volumes of alkylating agent in TE buffer, pH 8.0, (prepared as described above) and 5′ end-labelled consensus oligonucleotide and incubating at 37°C for 60 min. Following alkylation, the DNA–AP2 complex was formed by adding together 1 or 2 µl of the transcription factor solution, 2 µl of the alkylated DNA, 2 µl 5× AP2 reaction buffer and adjusting the volume to 10 µl with sterile water. The 5× reaction buffer contained 50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 20% glycerol and 2.5 mM DTT. The final ethanol concentration in the binding reaction was 0.1% v/v. After incubation at 37°C for 30 min the bound and free complexes were resolved electrophoretically as described below.

Electrophoresis and quantitation

Transcription factor binding was assessed by loading 4 µl samples onto a 4% [19:1 acrylamide/bis-acrylamide] non-denaturing submarine gel in TBE buffer. The gels were prepared using a BioRad sub-cell PAGE caster and contained 2 mM $MgCl_2$ and 0.01% NP-40. Electrophoresis was carried out at 100 V for 1 h in an electrophoresis buffer of TBE containing 2 mM $MgCl_2$ and 0.01% NP-40.

Piperidine cleavage samples were loaded onto a 38 × 50 × 0.4 cm 12 or 15% denaturing gel [19:1 acrylamide/bis-acrylamide] containing 7.5 M urea and pre-heated to ~45°C . Electrophoresis was carried out in TBE at 2000 V until the xylene cyanol had moved 25 cm down the gel. After electrophoresis the gels were washed for 20 min in an aqueous solution of 10% methanol and 10% acetic acid, dried and exposed to a Molecular Dynamics phosphor storage screen. Band intensity was quantitated using a Molecular Dynamics Model 400B PhosphorImager and ImageQuant software.

RESULTS AND DISCUSSION

The experimental approach used to determine the effects of the bifunctional and monofunctional sulphur mustards on the interaction between AP2 and its recognition sequence entailed two stages. In the first stage, the conditions of mustard concentration and exposure time required to alkylate the oligonucleotide were established using piperidine cleavage, and in the second stage the effects of the bifunctional and monofunctional sulphur mustards on AP2 binding were quantitated using gel retardation.

Oligonucleotide alkylation

The oligonucleotide containing the AP2 recognition site was alkylated by exposure to a range of HD and CEES concentrations at 37°C for 30 min, precipitated and then subjected to piperidine cleavage (Fig. 2a). The mustard concentrations were chosen on the basis of preliminary studies which showed that CEES produced approximately half the alkylation generated by an equivalent concentration of HD. Increasing alkylation was manifested as both a decrease in the intensity of the bands corresponding to the full length oligonucleotide and progressive loss of radiolabel from the alkylated oligonucleotide (i.e., the total radioactivity obtained by

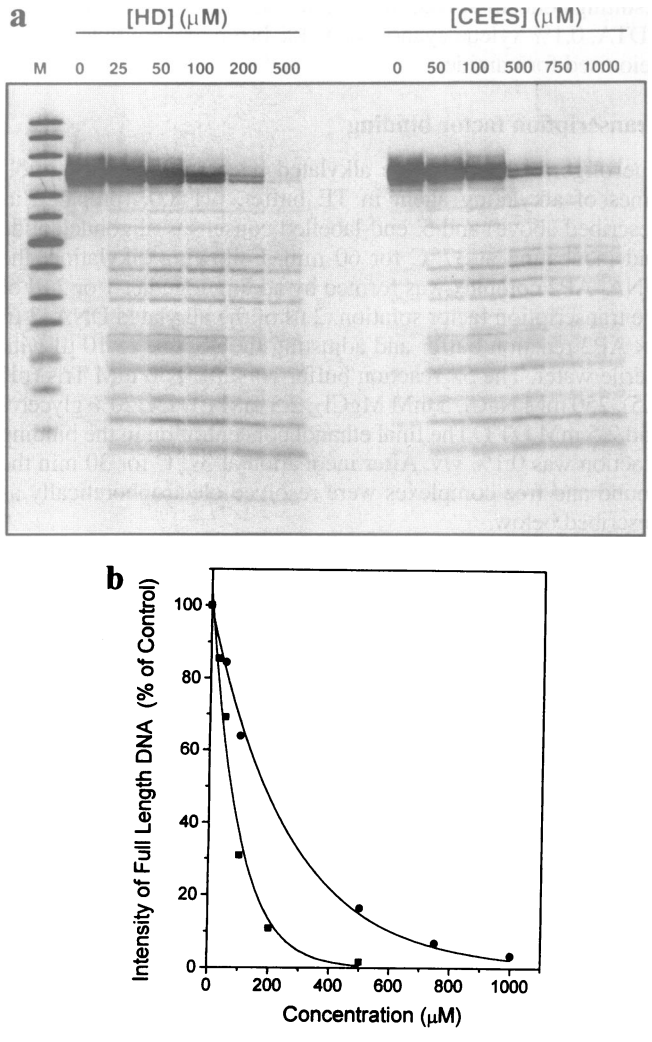

Figure 2. Effect of sulphur mustard concentration on alkylation of the AP2 consensus oligonucleotide. (**a**) AP2 oligonucleotide was alkylated with a range of concentrations of HD and CEES for 30 min, subjected to piperidine cleavage and the resulting fragments separated on a 12% denaturing gel. M is an oligonucleotide sizing marker. (**b**) The decrease in full length oligonucleotide is shown as fraction of the control (unalkylated) DNA (lane 0) for HD (■) and CEES (●).

summation of all bands in each electrophoresis lane decreased with increasing alkylation). The total amount of radiolabel remaining in all bands was reduced to ~11% of the control value after treatment with 500 μM HD and ~19% after treatment with 1 mM CEES (Fig. 2a) and reflects the susceptibility of both 5′ terminal nucleotides (A and G) to alkylation.

Because of this loss of label, the extent of alkylation is expressed in Fig. 2b as the decrease in the amount of full-length oligonucleotide and represents the occurrence of at least one alkylation per oligonucleotide. Alkylation of the oligonucleotide by HD and CEES increased with increasing concentration up to 500 μM and 1 mM, respectively. Approximately 95% of the oligonucleotide was alkylated at at least one site with 500 μM HD and 93% was alkylated with 1 mM CEES (Fig. 2b).

The alkylation rate was measured using 200 μm HD and 500 μM CEES. These concentrations were chosen because they produced approximately the same overall level of alkylation when the reaction was allowed to proceed to completion (Fig. 2b). The reaction rate was determined by removing 5 μl aliquots from the alkylation reaction, quenching the reaction with the 'stop' solution and subjecting the DNA to piperidine cleavage. The alkylation reaction was rapid and ~90% of the oligonucleotide was alkylated at least once within 10 min exposure to either 200 μM HD or to 500 μm CEES (data not shown). By comparison, Figure 2b shows that after 30 min exposure to the same concentrations the amount of oligonucleotide alkylated at at least one site was 90 and 84% for HD and CEES, respectively. The locations of the alkylation sites are essentially the same for both HD and CEES (data not shown).

The standard reaction condition for the transcription factor binding studies chosen on the basis of these results was alkylation at 37°C for 60 min. This ensures that the alkylation is complete and that any unreacted sulphur mustards have hydrolysed before addition of the transcription factor.

Transcription factor binding

The AP2 consensus oligonucleotide was alkylated at 37°C for 60 min using 0–500 μM HD and 0–1 mM CEES. These conditions, as shown by previous results, cover the range of 0 to ~95% alkylation for both sulphur mustards. Figure 3 shows the effect of HD on AP2 binding. The binding decreased with increasing HD concentration (Fig. 3a) and is expressed in Figure 3b as the percentage of total label located in the retarded band. For HD, inhibition of the binding of the transcription factor paralleled the alkylation profile and was essentially abolished (~7% of the control binding remaining) under conditions (500 μM for 1 h) that ensured alkylation of 95% of the oligonucleotides at at least one site. CEES also reduced the binding of AP2 to its consensus oligonucleotide with ~24% of the control value remaining under conditions which alkylated 95% of the binding sites (Fig. 4).

It is possible that either residual HD and CEES or their hydrolysis products interfere with the binding of AP2 by interacting directly with the protein. In order to eliminate this possibility, the oligonucleotide was alkylated with 200 μM HD or 500 μM CEES, then precipitated and washed to remove any mustard residues. The binding to the HD alkylated site was reduced to 18% of the control value, while CEES reduced the binding to 48% (data not shown). These results are consistent with the reduction to 22 and 52%, respectively without purification of the alkylated oligonucleotide as shown in Figures 3 and 4.

The results of the alkylation studies show that both HD and CEES rapidly alkylate the AP2 consensus oligonucleotide. The alkylation reaction was essentially complete within 10 min under the conditions employed in this study and ~95% of the oligonucleotides were alkylated at least once using 500 μM HD and 1 mM CEES. HD caused more extensive alkylation, by a factor of ~2, than CEES at equimolar concentrations. This difference in extent of alkylation reflects the existence of two active groups in HD and only one in CEES and the very rapid hydrolysis rate of CEES (20). Both HD and CEES are hydrolysed rapidly in aqueous solution, with half lives for hydrolysis of all reactive chloroethyl groups of ~44 s for CEES (20) and ~8.3 min for HD (21). The piperidine cleavage results also showed that the distribution of alkylation sites was essentially the same for both agents. Both HD and CEES react via a sulphonium ion intermediate and would be expected to alkylate DNA at the same sites (19). For the AP2 binding experiments, the alkylation

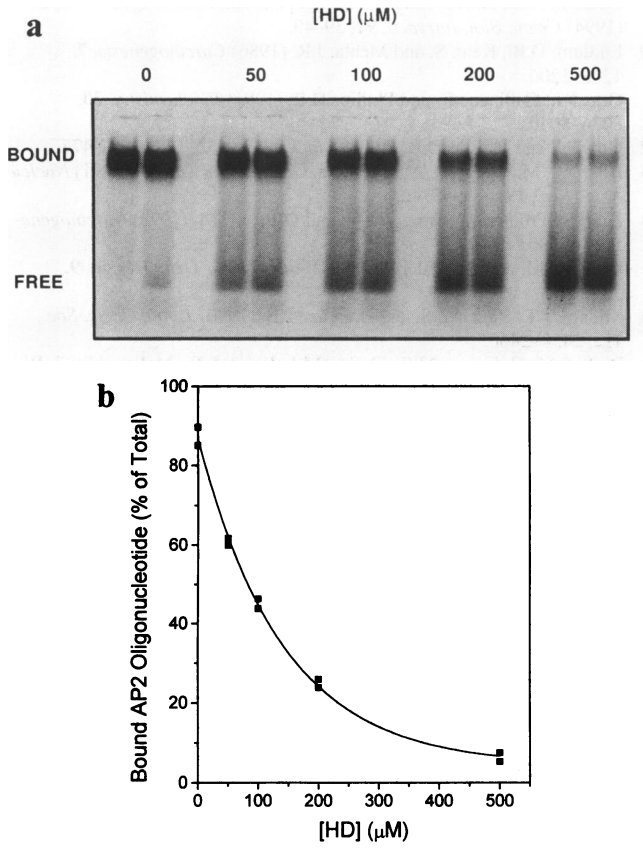

Figure 3. Bifunctional sulphur mustard inhibition of AP2 binding. (**a**) AP2 oligonucleotide was exposed to a range of concentrations of HD for 60 min. Following exposure, human AP2 was added and after incubation for 30 min the bound and free transcription factors were separated using a 4% submarine gel. (**b**) The bound AP2 is expressed as a fraction of the unalkylated control (lane 0). The curve is fitted to the average of the duplicate measurements shown (■).

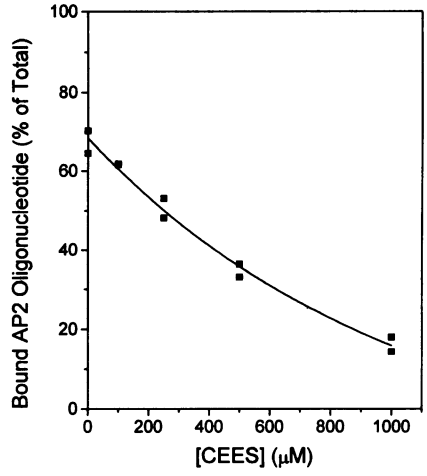

Figure 4. Monofunctional sulphur mustard inhibition of AP2 binding. The bound AP2 is expressed as a fraction of the unalkylated control and the curve is fitted to the average of the duplicate measurements shown (■).

reaction was incubated for 60 min to allow complete hydrolysis of any residual, unreacted mustard.

Alkylation by both the monofunctional and bifunctional mustards decreased AP2 binding, however, the oligonucleotide alkylated by CEES retained a greater ability to bind AP2 than oligonucleotide alkylated by HD.This finding suggests that there are at least two elements which contribute to disruption of the binding. One factor is the physical presence of mustard which blocks the binding site, while a second factor is distortion of the binding site by the formation of a crosslink. HD is able to fulfil both these functions while CEES is unable to form crosslinks. Depurination is unlikely to be significant during the time course of the reaction (22).

HD and CEES each give rise to two hydrolysis products. The hydrolysis products of HD are thiodiglycol and HCl and CEES hydrolyses to 2-hydroxyethyl ethyl sulfide and HCl (20,23). Disruption of AP2 binding by HD and CEES was essentially the same in the presence and absence of any hydrolysis products, which were removed by precipitation and washing of the alkylated DNA. This finding also indicates that damage to AP2 by residual free mustard or hydrolysis products is not a factor in diminishing the binding.

Vesication is a process that occurs at relatively high concentrations of mustard (>50 μM; 24) and considerable effort has been expended in trying to understand the process. Human cells exposed in culture to vesicating doses undergo G1/S phase blockage and do not recover (24) whereas cells treated with sub-vesicating doses are blocked at G2 and are able to resume normal cycling after a period of time (24). The change in the response of the cell with increasing exposure to mustard may reflect the general accumulation of DNA damage beyond the repair capacity of the cell, or alternatively may be due to damage to critical sites in the genome. Transcription factor binding sites may constitute such a class of sites critical in the vesication process and disturbance of their function may manifest itself in a number of ways deleterious to the cell. First, damage would be expected to interfere with the orderly regulation of normal transcription. Complex effects may result from inhibition of the binding of transcription factors which act synergistically with other factors (25). Secondly, DNA damage also induces specific genes which have protective or repair functions (26). Some of the primary DNA damage-response genes are transcription factors (26), and interference with the binding of such factors may prevent the cell from initiating the correct response to the damage.

The observation that AP2 binding is disrupted by alkylation with the two vesicants, HD and CEES, is a strong argument for a potential role for this process in vesication. However, the consequences of alkylation of transcription factor binding sites may vary between transcription factors. For example, OTF-1 tolerates base substitutions in its recognition site and this flexibility may make it less susceptible than other transcription factors to interference by alkylation (27,28). In addition, when trying to define the role of transcription factor binding in vesication it is also important to understand other effects of alkylation on the template function of DNA. In prokaryotic systems *in vitro*, alkylation disrupts both the initiation and elongation of transcription as well as the progress of prokaryotic DNA polymerases. The same detailed information is not available for eukaryotic systems at vesicating doses of mustards. Two questions which remain unresolved therefore are: (i) does interference with transcription factor binding represent a specific effect of these compounds at

vesicating doses or simply general dysfunction of the DNA template and (ii) is interference with transcription factor binding relevant under high dose conditions in which DNA and RNA synthesis are substantially inhibited? These questions are now being addressed in order to gain further insight into the mechanism of vesication produced by these compounds.

ACKNOWLEDGEMENTS

We thank Drs D. R. Leslie and I. Tilley for synthesis, purification and analysis of the mustards, and Dr D. R. Phillips for use of the PhosphorImager.

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