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DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards is preserved in intact cells

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ABSTRACT

Nitrogen mustard alkylating agents react with isolated DNA in a sequence selective manner, and the substituent attached to the drug reactive group can impose a distinct sequence preference. It is not clear however to what extent the observed DNA sequence preferences are preserved in intact cells. The highly reiterated sequence of human alpha DNA has been used to determine the sites of guanine-N7 alkylation following treatment of cells with three nitrogen mustards, mechlorethamine, uracil mustard and quinacrine mustard, known to react in isolated DNA with distinctly different sequence preferences. Alpha DNA from drug treated cells was extracted, purified, end-labeled, and a 296 base pair, singly end-labelled, fragment isolated. Following the quantitative conversion of alkylation sites to strand breaks the fragments were separated on DNA sequencing gels. Clear differences were observed between the alkylation patterns of the three compounds, and the selectivities were qualitatively similar to those predicted and observed in the same sequence alkylated *in vitro*. In particular the unique preferences of uracil and quinacrine mustards for 5′-PyGC-3′ and 5′-GT/GPu-3′ sequences, respectively, were preserved in intact cells suggesting that the pattern of sequence dependent reactivity is not grossly affected by the nuclear milieu.

INTRODUCTION

Mechlorethamine (nitrogen mustard) was the first clinically effective anticancer agent (1), and derivatives such as L-phenylalanine mustard (melphalan), cyclophosphamide and chlorambucil are still among the most clinically useful agents, despite their apparently non-specific chemical reaction mechanisms. Covalent binding may occur at many nucleophilic sites within nucleic acids and proteins, but DNA is probably the most important target with reaction predominantly at the N7-position of guanine. Two alkylating groups within the mustard molecule are required for antitumour activity, suggesting that the activity arises from the formation of crosslinks between macromolecular sites. DNA interstrand crosslinks and DNA-protein crosslinks have been observed in intact cells (2), and evidence for intrastrand crosslinks obtained indirectly (3).

A modification of the Maxam and Gilbert guanine-specific chemical cleavage technique for DNA sequencing (4) has allowed for a direct examination of the guanine-N7 reaction by akylating agents at the individual base level in purified DNA (5). Large variations in alkylation intensities existed among guanines in a DNA sequence following treatment with several classes of alkylating agents, including nitrogen mustards (6,7), and chloroethylnitrosoureas (8). The most striking finding was that most agents reacted preferentially in runs of contiguous guanines, the degree of preference being much greater than would be expected from the number of guanines alone, with weak reaction at 5′-GC-3′ sites. The sequence selectivity of these agents has recently been confirmed using a polymerase stop assay (9).

Although the reaction intensities of many mustards are closely correlated with that of mechlorethamine, some nitrogen mustards showed reaction patterns in purified DNA which were distinctly different from other mustards, indicating that the substituent attached to the reactive group could impose a distinct sequence preference for reaction. Most notably, uracil mustard shows an enhanced reactivity with 5′-PyGC-3′ sequences (Py = pyrimidine), and quinacrine mustard shows a strong preference for 5′-GTPu-3′, and 5′-GGPu-3′ (Pu = purine) sites (6,7). Models to explain these unique reactions have been proposed (7).

The sequence selective patterns were obtained from drug reactions with isolated DNA. The aim of the present study was to determine to what extent the sequence selectivities are preserved in cells. Using the highly reiterated 340 base pair sequence of human alpha DNA, which accounts for about 1% of the human genome (10), it was possible to examine sites of guanine-N7 alkylation in drug treated cells compared to the same DNA alkylated *in vitro*.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: mechlorethamine, Sigma Chemical Company; quinacrine mustard, Fluka Chemical Company; uracil mustard, Upjohn Company; T4 polynucleotide kinase and restriction endonucleases, Gibco-BRL Ltd; (γ-^{32}P)-ATP (50 Ci/mmol), Amersham International Ltd. All other reagents were of the highest purity available.

Cell culture and drug treatment of intact cells

The human myeloid leukaemic cell line, K562, was maintained in exponential growth in RPM1 1640 supplemented with 5% heat inactivated foetal calf serum (FCS) and 2mM L-glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Prior to drug treatment cells were pelleted and resuspended at 10^6 per ml in FCS-free medium. Drugs were dissolved at high concentration in DMSO so that the final concentration of DMSO was <1% when added to cells. Drug treatments were on 10^8 cells for 30 min at 37°C. Cells were then pelleted and washed by resuspending in phosphate-buffered saline.

Preparation of alpha DNA

Pelleted cells were lysed in 2ml lysis solution (2% SDS) and deproteinated by the addition of proteinase K (2.5 mg/ml, predigested for 30 min at 37°C) to a concentration of 0.06 mg/ml at 37°C for 30 min. This step was repeated. The mixture was then extracted once with phenol and twice with a mixture of chloroform:isoamyl alcohol (19:1). Genomic DNA was then ethanol precipitated, dried and resuspended in distilled water to which ribonuclease A was added to a final concentration of 0.1mg/ml. Incubation was for 1 hour at 37°C prior to a further ethanol precipitation.

Genomic DNA was digested with high activity EcoRI (50u/μl) and run on a 1.8% neutral agarose gel. Following staining with ethidium bromide (0.1 mg/ml) the 340 base pair alpha fragment was located under UV and excised from the gel. DNA was electroeluted out of the gel slice followed by purification down a Nacs column (Gibco/BRL). The purified alpha DNA was precipitated and stored dry until required.

Labeling of alpha DNA

Alpha DNA was 5′ end-labeled with T4 polynucleotide kinase and γ-^{32}P-ATP by standard procedures. It was then washed twice to remove unincorporated label prior to complete digestion with Hae III to give two singly end-labeled fragments. The 296 and 44 base pair fragments were separated on an 8% non-denaturing polyacrylamide gel. The wet gel was exposed onto autoradiographic film and the 296 base pair fragment located and excised. The DNA was removed from the gel slice by diffusion at 37°C overnight in 10mM Tris, 1mM $(Na)_2$EDTA, 0.15M NaCl followed by ethanol precipitation.

Drug treatments of isolated DNA

Purified alpha DNA was treated with drug in 25mM triethanolamine, 1mM EDTA, pH 7.2. Reaction was terminated by the addition of an equal volume of 0.6M sodium acetate, 20mM EDTA containing 100μg/ml tRNA. DNA was ethanol precipitated, washed by resuspension in 0.3M sodium acetate, 10mM EDTA, ethanol precipitated again, and dried by lyophilisation.

Sequence selectivity of guanine-N7 alkylation

Pelleted DNA was resuspended in 100μl of an ice-cold solution of 10% piperidine in water. Samples were heated at 90°C for 15 minutes to convert quantitatively the sites of guanine-N7 alkylation into single strand breaks (5). Following immediate freezing in a dry ice/ethanol bath the samples were lyophilised to dryness. Samples were then washed twice in 15μl water followed by lyophilisation to remove traces of piperidine. Washed samples were resuspended in deionised formamide dye mix containing 0.3 mg/ml bromophenol blue, 0.3 mg/ml xylene cylanol and 7.5 mg/ml $(Na)_2$EDTA. Following denaturation at 90°C for 2 min, samples were run on 80cm long, 4mm thick denaturing 6% polyacrylamide gels containing 7M urea at 55°C and 3000 V. Gels were dried onto filter paper (one layer Whatman 3mm, one layer Whatman DE81), and autoradiography performed at −70°C. Densitometric measurement was made using a LKB Ultrascan XL laser densitometer.

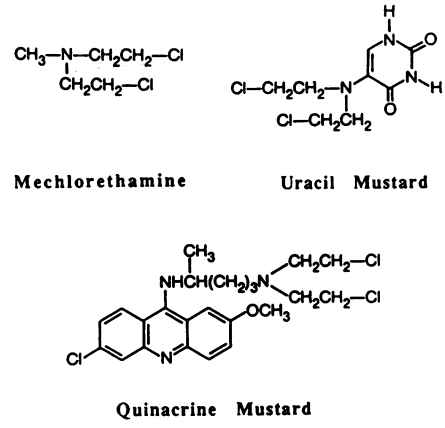

Figure 1. Structure of the nitrogen mustards used in this study.

RESULTS

The human alpha DNA sequence allows a direct comparison to be made of the sequence selective modification of guanine-N7 positions by alkylating agents in purified DNA and the same sequence in intact cells. Three agents were chosen in the present study, mechlorethamine, uracil mustard and quinacrine mustard (figure 1), which are known to have distinctly different patterns of alkylation in isolated DNA. Total cellular DNA was extracted from human leukaemic cells in culture and, following complete digestion with EcoRI, the 340 base pair alpha DNA purified by modification of existing procedures (10,11). The fragment was ^{32}P-end labeled at the 5′ ends and a 296 base pair singly end-labeled fragment was purified from polyacrylamide gels after a further digestion with the Hae III. The pattern of guanine-N7 alkylation, revealed on DNA sequencing gels, of alpha DNA drug treated at this stage was compared to that extracted directly from drug treated cells, following the quantitative conversion of sites of alkylation to single strand breaks by treatment with hot piperidine.

A typical DNA sequencing gel of the 296 base pair fragment is shown in figure 2. Some bands are observed in the control (lane A) which are only present following piperidine treatment of the DNA. These are, however, reproducible and in general not observed at guanine sites and therefore do not interfere with the qualitative comparison of guanine-N7 alkylation. Lanes B and C show the pattern of alkylation produced by mechlorethamine and uracil mustard, respectively. Bands are evident at all guanine sites by both drugs and some variation in the extent of alkylation at guanine sites is clearly seen.

In this way it was possible to compare the pattern of alkylation by the three drugs in isolated DNA and in intact cells. True quantitative comparisons of the extent of alkylation were difficult, but in general 10−20 fold more drug was required to give a

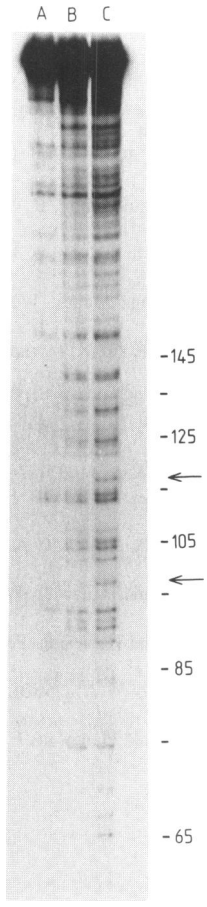

Figure 2. Pattern of guanine-N7 alkylation in the EcoRI-Hae III 296 base pair fragment of isolated human alpha DNA following treatment with mechlorethamine (100μM, lane B) or uracil mustard (100μM, lane C). Lane A is control unalkylated DNA. The base number is indicated and arrows indicate the two 5'-PyGC-3' sites within the sequence.

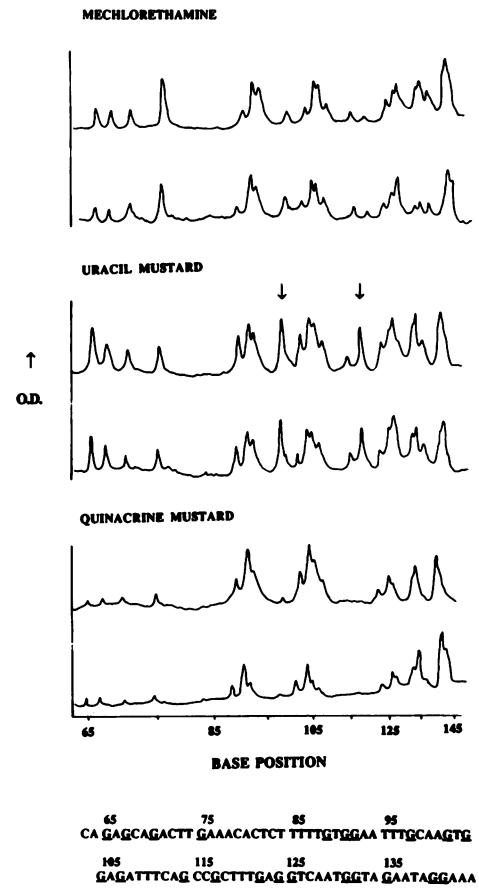

Figure 3. Densitometric traces showing the patterns of guanine-N7 alkylation for three nitrogen mustards either in isolated alpha DNA (upper traces) or in alpha DNA extracted from drug treated cells (lower traces). The doses of drug used in isolated DNA and intact cells, respectively, are mechlorethamine, 100μM and 2mM, uracil mustard, 100μM and 2mM, and quinacrine mustard, 10μM and 100μM. Each trace, following background subtraction, is scaled to the highest absorbance in the scan and the base sequence covered is indicated. Arrows correspond to the two 5'-PyGC-3' sites within the sequence.

similar overall extent of alkylation in cells. Qualitative comparisons of the patterns of alkylation were however possible because at the levels of drugs used the overall extent of alkylation is such as to give at most one alkylation per alpha DNA sequence. The results are summarised in figure 3 showing the densitometric traces of base positions 65 to 145 derived from the sequencing gel autoradiograms. Several important features are evident. Firstly in isolated DNA (upper traces) the three agents produce distinctly different (and reproducible) patterns of guanine-N7 alkylation. In particular, uracil mustard shows increased alkylation compared to mechlorethamine at base positions 98 and 118 (indicated by arrows in figures 2 and 3). These are 5'-TGC-3' and 5'-CGC-3' sequences, respectively, and cor firm the known preference of this agent for 5'-PyGC-3' sequences observed in isolated DNA previously (6,7). Quinacrine mustard shows a much greater discrimination between guanine sites and shows a distinct preference, as noted previously for this agent, for 5'-GGPu-3' and 5'-GTPu-3' sequences, e.g. base 91, 104 and 140 (all G-TA) and 132 (GTA).

Secondly the three drugs clearly alkylate the alpha DNA sequence in intact cells (figure 3, lower traces), and more importantly the patterns of guanine-N7 alkylation produced are qualitatively very similar to those produced in isolated DNA. In particular the unique preferences of uracil mustard for 5'-PyGC-3' (indicated by arrows) and quinacrine mustard for 5'-GG/TPu-3' sequences are preserved in alpha sequence within the intact cell.

DISCUSSION

The majority of work to date on the sequence selectivity of a variety of DNA interacting agents has been performed on defined sequences of isolated DNA. It is likely, however, that for many agents the extent and sequence specificity of damage will differ depending on whether purified DNA or cells are treated. In addition, studies on the mechanisms of repair of such lesions require analysis in cellular systems. The highly repeated alpha sequences contained in the mammalian genome provide a convenient subfraction of chromatin in which to compare DNA damage and repair with that in the bulk DNA, and has been used to show genomic repair heterogeneity following treatment with, for example, furocoumarins and aflatoxin B1 (12−14). It is clear from the present study that the alpha sequence can also be used to examine relevant chemical damage at the level of the individual nucleotide. The method employed is a modification of that described in detail by Haseltine (11), with several modifications.

In particular, the use of EcoRI* enzyme activity to produce a 92 base pair fragment of the alpha DNA was found to be very inefficient, and the presence of a Hae III site in the second 171 nucleotide imperfect repeat of the sequence was exploited to produce a workable 296 base pair singly end-labeled fragment.

The present study clearly demonstrates that with relatively simple chemical agents, such as the clinically used nitrogen mustard alkylating agents, the alpha DNA is a suitable system for examining guanine-N7 alkylation in cells. The results expand on those of Haseltine (15) in which alkylation by mechlorethamine was examined in the 92 base pair fragment. Clearly the cellular environment does not markedly alter the sequence selective reaction of these drugs with the alpha DNA sequence, and the unique specificities of different members of the same class of compound are preserved in the cell. This is highly significant considering the very complex environment of the DNA in the cell, and indicates that if a drug of this type can reach the DNA the chemistry of the alkylation reaction is similar to, and can be predicted by, the situation in isolated DNA. This has important implications in drug design, and demonstrates that simple systems employing isolated DNA can give a meaningful indication of the pattern of reactivity in cells.

As expected the total amount of DNA damage was decreased in intact cells relative to isolated DNA. Several factors could contribute to this, including limited diffusion of the drugs into cells, and the alkylation by these reactive compounds with other cellular components. In general, 10–20 fold drug was required in cells to give a similar extent of DNA alkylation to that observed in isolated DNA. True quantitative comparisons were difficult however, since at high doses in cells some background cleavage of genomic DNA was evident. This was particularly noticeable in the case of the highly reactive quinacrine mustard. At the doses employed, however, this background cleavage, which could be due to repair activity in the cells, or produced during the extraction of drug-damaged DNA, did not interfere with the subsequent analysis of the alpha DNA. The high levels of drug used in order to see a significant extent of alkylation are all supertoxic to the cells, but it is assumed that the pattern of alkylation observed reflects the situation at more pharmacologically and clinically relevant drug concentrations.

Although the alpha DNA provides a powerful tool for the analysis, at the individual base level, of DNA damage within intact cells it is not an ideal cellular target for study. The function of these unexpressed sequences is unknown, and analyses of damage and repair of more relevant genomic targets would be of biological interest, but are not suitable for analysis by such a technique. Although there have been many examples of heterogeneity of DNA damage and repair in the mammalian genome (16), studies at the individual nucleotide level remain few. A recently developed *Taq* DNA polymerase based assay, to measure the sequence selectivity of covalent modification by chemotherapeutic drugs on isolated DNA (9), may have potential for cellular damage and repair studies. The present study clearly indicates that studies on isolated DNA at the individual nucleotide level can give information relevant to the situation in the intact cell since the pattern of sequence dependent reactivity for nitrogen mustards is not grossly affected by the nuclear milieu.

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