A Monofunctional Derivative of Melphalan: Preparation, DNA Alkylation Products, and Determination of the Specificity of Monoclonal Antibodies That Recognize Melphalan−DNA Adducts

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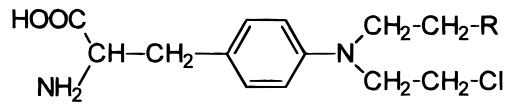
Bifunctional alkylating agents, such as those based on nitrogen mustard, form important parts of many anti-cancer chemotherapy protocols and are responsible for increased incidences of secondary tumors in successfully treated patients. These drugs generally form a majority of monofunctional DNA adducts, although the bifunctional adducts appear to be necessary for their powerful cytotoxic and antitumor effects. The relative importance of bifunctional as opposed to monofunctional adducts in the varied biological consequences of drug exposure has not been studied in detail, particularly in relation to the role and specificity of biochemical responses to therapy-related DNA damage. A simple method is described for the preparation of useful quantities of a pure monofunctional derivative of the nitrogen mustard-based drug melphalan. Monohydroxymelphalan was prepared by partial hydrolysis, purified by reversed phase chromatography, and characterized by MS, NMR, and HPLC. Contamination with melphalan was ≤0.2%. The heat labile DNA base adducts formed by monohydroxymelphalan were shown to contain undetectable levels of cross-linked species. The ratio of adenine to guanine adducts was 0.62, similar to the equivalent ratio for melphalan. The sequence-dependent pattern of alkylation of purified DNA was indistinguishable from that of melphalan, but required a higher dose to achieve comparable extents of reaction. The specificities of two monoclonal antibodies that recognize melphalan−DNA adducts were investigated using DNA alkylated with [^{3}H]monohydroxymelphalan. Adducts on this DNA showed similar immunoreactivities to adducts formed by melphalan. This shows clearly that neither antibody was specific for cross-linked adducts and that it is therefore possible to quantify adducts formed by both monohydroxymelphalan and melphalan with high sensitivities. The availability of monohydroxymelphalan in addition to melphalan, together with sensitive immunoassays for adducts on extracted DNA and in individual cells, constitutes a useful system for investigating cellular responses to the DNA modifications formed by a clinically relevant drug.

Introduction

Bifunctional alkylating agents, such as cyclophosphamide, melphalan (**1a**), and chlorambucil, are established human carcinogens (*1*) as well as important components of many multi- and single-agent cancer chemotherapy protocols (*2*, *3*).



1a R = Cl
1b R = OH

Recently, other nitrogen mustards have been identified as useful components of antibody- or gene-directed enzyme prodrug therapies for cancer (*4*−*8*). Sensitive measurements of the levels of DNA adducts formed by this class of drug in clinical and experimental samples facilitate investigations into the contribution that variations in drug−DNA interaction make to variations in efficacy or toxicity. Such measurements also underpin studies into biochemical responses to DNA adduct formation.

Monoclonal antibodies that recognize melphalan−DNA adducts (*9*, *10*) have permitted studies of drug−target interaction in DNA extracted from blood cells of patients undergoing therapy (*11*). The development of immunological techniques for analysis of melphalan−DNA adduct levels in individual cells (*12*) is permitting the analysis of clinical tumor and bone marrow biopsies. The monoclonal antibodies used in the above work were raised against drug-modified polymeric DNA because it was considered that this was the type of immunogen most likely to yield antibodies suitable for techniques such as

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cytological staining (*9*, *10*). However, this feature has made it difficult to determine the exact specificity of the antibodies because of the complex mixture of adducts formed when melphalan reacts with DNA (*13*−*15*). In particular, it has not been possible to determine if the antibodies, when applied to polymeric DNA, preferentially recognize bifunctional as opposed to monofunctional adducts. This question is important in regard to the interpretation of results obtained using these antibodies.

The heterogeneity of adducts formed by bifunctional mustards also complicates investigations into mechanisms of mutagenesis (*16*) and the specificity of the cellular functions that detect and trigger responses to DNA damage, such as the p53 response and induction of apoptosis. It is not clear if these responses show the same dependency on bifunctionality of alkylating agents as anti-cancer effectiveness or to what extent monofunctional drug−DNA adducts influence the way cells survive the simultaneous presence of cross-linkage.

Determination of the specificities of monoclonal antibodies to melphalan-modified DNA and also of cellular response mechanisms to DNA damage should be facilitated by the specific formation of monofunctional DNA adducts of melphalan through the use of a monofunctional derivative of this drug. Monofunctional nitrogen mustards were made during the drug development programs of the 1940s and 1950s, but studies with these agents were restricted to the assessment of antitumor effects in experimental animals (*17*−*21*). In general, the results showed a lack of antitumor action. One study illustrated the importance of elimination of contamination of monofunctional drug by its bifunctional counterpart (*21*). No studies appear to have investigated the nature, extent, or biochemical effects of DNA adducts formed by these agents.

This paper describes (1) a simple method for the preparation of hundreds of milligrams of monohydroxymelphalan (**1b**) free of the bifunctional drug, (2) confirmation of the structure and purity of this reagent, (3) biochemical and sequence-related characterization of the products for reaction of this reagent with DNA compared to those for the bifunctional drug, and (4) definition of the specificities of two previously described monoclonal antibodies using DNA alkylated with monohydroxymelphalan.

Materials and Methods

Hazardous Materials. Melphalan is a known human carcinogen (*1*), and monohydroxymelphalan is known to alkylate DNA in cells (*22*). Therefore, appropriate precautions must be taken to avoid human exposure.

Preparation of Monohydroxymelphalan. Melphalan (from Sigma) was partially hydrolyzed by incubation of a solution (5 mg/mL) in 11 mM HCl (37 °C), and reaction products were separated by chromatography using a C18 Sep-pak cartridge (Vac 35 cm^3, 10 g capacity, from Waters, Millipore Corp.). Thus, at time zero there was a single peak corresponding to melphalan which eluted with 100% solvent B. This peak progressively decreased in intensity and was replaced initially by one new peak, which eluted with a 9:1 mixture of solvents A and B and corresponded to monohydroxymelphalan. Eventually, an additional peak appeared which eluted with 100% solvent A and was assumed to correspond to dihydroxymelphalan. The peak corresponding to monohydroxymelphalan grew to a maximum and then declined, while the peak attributed to dihydroxymelphalan progressively increased to a plateau and then accounted for all the material present. A 3 h hydrolysis time was determined experimentally to give the maximum yield of the desired product. The reaction mixture at 3 h was passed through the Sep-pak cartridge at 5 mL/min. The cartridge was washed with solvent A (5 mL/min) until the A_{260} of the effluent returned to baseline. This eluted the substance assumed to be dihydroxymelphalan. Monohydroxymelphalan was then selectively eluted with a 9:1 mixture of solvents A and B. The cartridge was reused after elution of melphalan in 100% solvent B and re-equilibration with solvent A. Solvent A was aqueous trifluoroacetic acid (0.1% v/v), and solvent B was acetonitrile containing 0.1% (v/v) trifluoroacetic acid. All measurements of A_{260} of fractions were made after dilution of aliquots into 50 mM sodium phosphate buffer (pH 7), because of the low extinction coefficient of the hydrolysis products at pH 2. To facilitate the subsequent rotary evaporation step, the product was concentrated and water was removed in a second chromatographic procedure. For this, fractions containing the monohydroxymelphalan were diluted 5-fold with solvent A and reloaded onto a new C18 Sep-pak cartridge (10 mL/min). The desired product was eluted with 100% solvent B (5 mL/min), and the yield was estimated from the A_{260} of an aliquot diluted into 50 mM sodium phosphate buffer (pH 7) (A_{260} of 1.0 ≡ 15 μg/mL). A 20-fold molar excess of HCl was added to form the hydrochloride of the monohydroxymelphalan, and the solution was reduced to dryness under vacuum in a rotary evaporator, at about 40 °C. The product was finally dissolved in ethanol and stored at −80 °C. ^{3}H-labeled monohydroxymelphalan (specific activity of 3.1 MBq/mmol) was prepared by the same method except that [^{3}H]melphalan (Amersham, 34 GBq/mmol) was added to the nonradioactive melphalan at the start of the reaction. The final specific activity was determined by scintillation counting and measurement of A_{260}.

Mass Spectrometry. The mass spectrum of monohydroxymelphalan was recorded with a Kratos MS80 instrument operating in fast atom bombardment mode (glycerol matrix, isobutane reagent gas): *m/z* 287 (MH^+ for ^{35}Cl, 100% relative intensity), 289 (MH^+ for ^{37}Cl, 35% relative intensity).

NMR Spectroscopy. After the rotary evaporation step, a preparation of monohydroxymelphalan was dissolved in 10 mM DCl in D_2O. The ^{1}H NMR spectrum was recorded at 500 MHz with a Bruker instrument: δ 3.30 (1H, dd, J_{gem} = 14.5 Hz, J_{vic} = 7.2 Hz, benzylic H), 3.62 (2H, t, J = 6 Hz, NCH_2), 3.65 (2H, br t, NCH_2), 3.82 (2H, t, J = 6 Hz, CH_2OH), 4.06 (2H, t, J = 6 Hz, CH_2Cl), 7.54 (2H, br d, 2 × ArH), 7.58 (2H, br d, 2 × ArH).

TLC. Plates were made up of fluorescent silica gel 60A (Whatman). The solvent was butan-1-ol/acetic acid/water (7:2:1) or ethanol/water (2:1). Plates were sprayed with enhancer (Enhance, from NEN, Boston, MA) before autoradiographic exposure.

HPLC Analysis of Monohydroxymelphalan. This was performed using a Waters model 625E low-pressure mixing gradient system equipped with a model 996 photodiode array detector (Waters) and a Wisp 712 autoinjector. Solvent A was trifluoroacetic acid 0.1% (v/v) in water. Solvent B was trifluoroacetic acid 0.1% (v/v) in water/acetonitrile (1:1). A 3 μm ODS reverse phase cartridge column (4.6 mm × 80 mm) fitted with a 4.6 mm × 5 mm guard cartridge (Beckman) was equilibrated with solvent A at 1 mL/min. After injection of sample (10 μL, dissolved in solvent A containing ethanol at 2% v/v), elution was isocratic for 2 min followed by a linear gradient to 100% solvent B over 13 min.

Reaction of DNA with Monohydroxymelphalan. Native and heat-denatured highly purified calf thymus DNA (Merck, 600 μg/mL) were reacted with radioactively labeled monohydroxymelphalan (1.7 and 0.17 mM, respectively) using the same conditions that were used previously for reaction with melphalan (*9*). Native DNA was dissolved in 50 mM NaCl and 50 mM sodium phosphate; denatured DNA was dissolved in 5 mM sodium phosphate. The reactions were carried out at pH 7 and 37 °C for 1 h.

DNA was then separated from unbound drug by gel filtration chromatography, as described previously (*9*). The level of alkylation was determined from the specific radioactivities of the alkylated DNA and of the monohydroxymelphalan.

HPLC Analysis of DNA Base Adducts. DNA that had been reacted with [^{3}H]monohydroxymelphalan was hydrolyzed by heating (100 °C for 20 min at pH 7), and the products were analyzed by the ion-pairing HPLC method of Osborne and Lawley (*14*) except that the gradient was pumped for 5−40 min and 0.5 mL fractions were collected. Previously characterized (*14*) nonradioactive markers were mixed with the present radioactive samples before analysis and were detected by measurement of OD_{260}. The previous characterization of this method included a demonstration of the coincidence of UV-absorbing and radioactive peaks (*14*).

Sequence Specificity of Alkylation. The procedure employed was an application of a previously described method (*23*). Plasmid pBR322 DNA was linearized with *Bam*HI to provide a stop for *Taq* polymerase downstream from the primer. Drug−DNA reactions were performed in 25 mM triethanolamine and 1 mM EDTA at pH 7.2 and 37 °C for 1 h and terminated by addition of an equal volume of stop solution (0.6 mM sodium acetate, 20 mM EDTA, and 100 μg/mL tRNA). The samples were precipitated with 3 volumes of ethanol and washed with 70% ethanol. The appropriate oligodeoxynucleotide primer (5′-TATGCGACTCCTGCATTAGG-3′) was 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol, Amersham) and purified by elution through a Bio-Rad Biospin Column. Linear amplification of DNA was carried out in a total volume of 100 μL containing 0.5 μg of template DNA, 5 pmol of the labeled primer, each dNTP at 125 μM, 1 unit of *Taq* polymerase, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9), 0.01% Tween, 2.5 mM $MgCl_2$, and 0.05% gelatine. After an initial denaturation at 94 °C for 4 min, the cycling conditions were 94 °C for 1 min and 60 °C for 1 min for a total of 30 cycles. After amplification, the samples were ethanol precipitated and washed with 70% ethanol. Samples were dissolved in formamide loading dye, heated for 2 min at 90 °C, cooled in ice, and subjected to electrophoresis at 2500−3000 V for approximately 3 h on a 80 cm × 20 cm × 0.4 mm, 6% acrylamide denaturing sequencing gel (Sequagel, National Diagnostics). The gels were dried, and X-ray film was exposed to the gels (Hyperfilm, Amersham).

Immunoassay. Competitive ELISA[1] (enzyme-linked immunoadsorbent assay) experiments using monoclonal antibodies MP5/73 and Amp4/42 were performed essentially as described previously (*9*, *10*) except that the wells of the 96-well plates (high bind, from Greiner) were coated with DNA by incubation (overnight in a humidified box at 37 °C) with solutions of DNA in coating buffer [1 M NaCl and 50 mM sodium phosphate (pH 7), 50 μL/well]. Plates for the MP5/73 assay were coated with native DNA alkylated with melphalan (43 μmol of adducts/g of DNA, 2 μg of native DNA/mL). For the Amp4/42 assay, plates were coated with DNA alkylated at 49 μmol of melphalan adducts/g of DNA. Immediately before application to the plates, an aliquot of a solution of this DNA was incubated in 1 M NaOH (60 min at 37 °C) and then diluted 500-fold into coating buffer to give 0.22 μg of DNA/mL.

Prior to the MP5/73 assay, DNA samples were hydrolyzed with DNAase I (5 Kunitz units per μg of DNA, 1 h, 37 °C) as described previously (*11*) except that the DNAase was inactivated with EDTA and was not removed by ultrafiltration. Prior to the Amp4/42 assay, samples were incubated (60 min at 37 °C) in 0.25 M NaOH and then diluted at least 40-fold into 50 mM sodium phosphate and 50 mM NaCl (pH 7).

Results

Preparation of Monohydroxymelphalan. Monohydroxymelphalan was prepared by partial hydrolysis of melphalan followed by purification by reverse phase chromatography. The A_{260} of solutions of melphalan at pH 7 did not change during hydrolysis. However, at pH 2, the extinction coefficient (260 nm) of monohydroxymelphalan was approximately half of the value at pH 7, and at pH 2, the extinction coefficient (260 nm) of dihydroxymelphalan was essentially zero. These observations were attributed to a progressive increase, at each stage of hydrolysis, in the pK_a associated with the tertiary amine nitrogen.

A ^{3}H-labeled preparation of monohydroxymelphalan was made by adding ^{3}H-labeled melphalan to the unlabeled melphalan, resulting in a preparation with a specific activity of 3.1 MBq/mmol. TLC analysis of the products, using two different solvents, showed that all the detectable UV-absorbing material ran as a single band and all detectable radioactivity showed the same mobility as the UV-absorbing product.

NMR and MS Analyses. The ^{1}H NMR spectrum of monohydroxymelphalan, recorded at 500 MHz for the hydrochloride taken up in DCl/D_2O, showed the sample to be homogeneous. The spectrum was in accord with the assigned structure because four separate resonances (at δ 3.62 and 3.65 for the NCH_2 groups, 3.82 for the CH_2-OH, and 4.06 for the CH_2Cl) were observed for the methylene groups in the 2-chloroethyl and 2-hydroxyethyl arms. The structural assignment was confirmed by the fast atom bombardment spectrum of the monohydroxymelphalan, which showed a base peak at 287 mass units, corresponding to the protonated molecule containing ^{35}Cl. There was also a peak at 289 mass units, corresponding to the protonated molecular ion containing ^{37}Cl, which was one-third of the intensity of the ion at 287 mass units. No higher-mass ions were observed.

Freedom from the Bifunctional Drug. Monohydroxymelphalan was analyzed using an HPLC technique by which it is efficiently separated from melphalan (Figure 1a). Injection of 1600 ng of monohydroxymelphalan resulted in a small peak, coincident with melphalan but with a magnitude similar to other background peaks (Figure 1b). Through calibration of the area under the melphalan peaks, and assuming that the peak present in monohydroxymelphalan represents only melphalan, we estimated the maximum possible contamination of monohydroxymelphalan by melphalan to be 0.2%.

Adducts Formed by Reaction of Monohydroxymelphalan with DNA. Native DNA was reacted with ^{3}H-labeled monohydroxymelphalan to give an alkylation level of 15.1 μmol/g of DNA. A previously described HPLC procedure (*14*) permits separation of the major purine adducts formed by alkylation of DNA by melphalan, namely, monofunctional adducts of melphalan at guanine N7 and adenine N3 and cross-linked adducts involving cross-links from guanine N7 to guanine N7 and guanine N7 to adenine N3. Analysis of the bases liberated from the DNA that had been reacted with [^{3}H]-monohydroxymelphalan showed the expected products of monofunctional alkylation (Figure 2). There were no detectable peaks of radioactivity in the fractions corresponding to the two bifunctional adducts, and we estimate that each of these adducts therefore represented less than 2% of the total alkylation products. In two separate determinations, the ratios of adducts on adenine to adducts on guanine induced by monohydroxymelphalan were 0.68 and 0.56 (mean = 0.62) which are

[1] Abbreviations: ELISA, enzyme-linked immunoadsorbent assay; GMP, guanosine 5′-monophosphate.

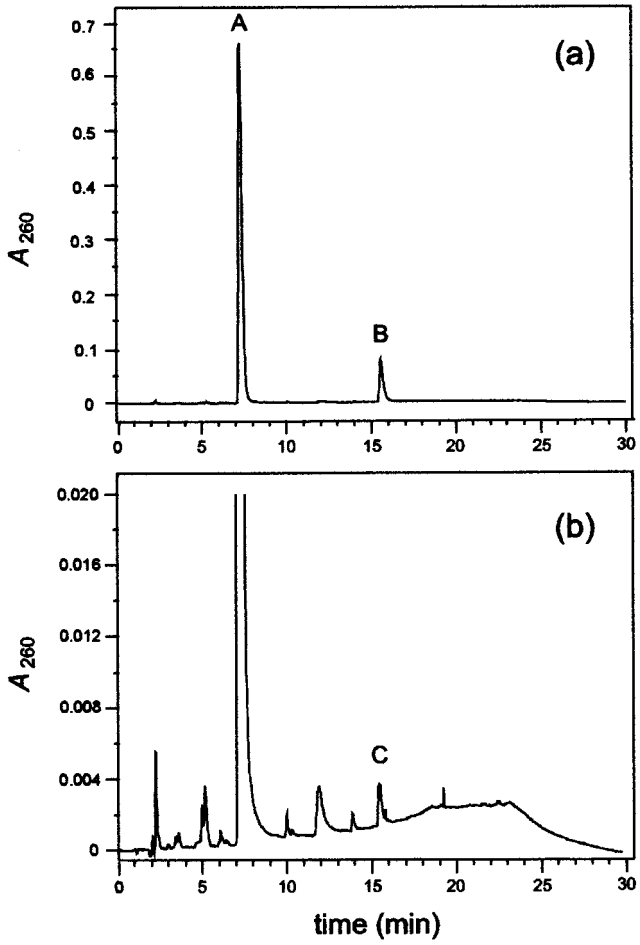

Figure 1. Determination of the level of melphalan in monohydroxymelphalan by HPLC. (a) Monohydroxymelphalan (1600 ng, peak A) and melphalan (50 ng, peak B) were injected. (b) Monohydroxymelphalan (1600 ng) was injected. The expanded ordinate scale reveals peak C eluting at the same time as melphalan.

similar to the ratio of 0.56 previously observed for the monofunctional adducts induced by melphalan (*14*).

Sequence Specificity of Alkylation by Monohydroxymelphalan. The sequence specificity of alkylation by monohydroxymelphalan and melphalan was compared in a GC-rich region of pBR322 DNA (265 base pairs) using the polymerase stop assay (Figure 3). The pattern of covalent adduct formation observed for melphalan was consistent with that observed previously where guanine N7 alkylation occurs preferentially at runs of contiguous guanines (*23*, *24*). No significant differences in the sequence preferences of melphalan and monohydroxymelphalan were observed, but the intensities of the bands indicated that the overall extent of alkylation by monohydroxymelphalan was lower than that resulting from the same concentration of melphalan, consistent with the expected 2-fold lower concentration of chloroethyl groups.

Specificity of Antibodies. DNA alkylated with ^{3}H-labeled monohydroxymelphalan or melphalan was hydrolyzed with DNAase I or submitted to alkali treatment, procedures which maximize the immunoreactivity of the adducts to antibodies MP5/73 (*11*) and Amp4/42 (*10*), respectively. Typical competitive ELISA data are shown in Figure 4 and illustrate the fact that the adducts formed by monohydroxymelphalan show immunoreactivities very similar to those of the adducts formed by melphalan. Results of experiments carried out to determine the

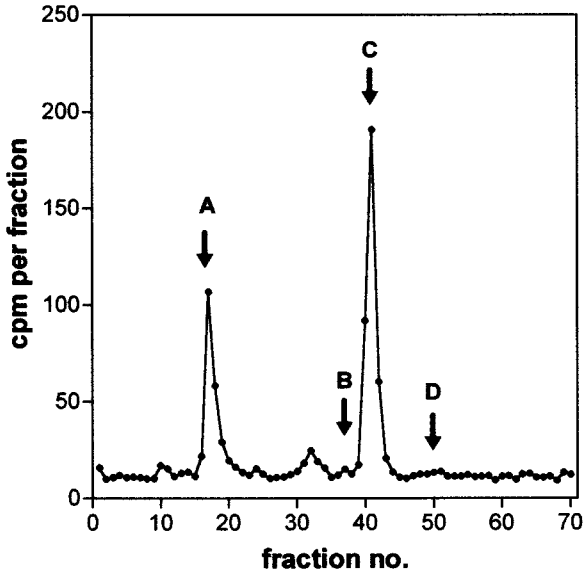

Figure 2. Ion-pair HPLC analysis of alkylated purines formed in DNA by [^{3}H]monohydroxymelphalan and released by heating. Arrows mark the elution times for the following characterized markers formed by reaction of melphalan with DNA: (A) monofunctional adducts at adenine N3, (B) cross-linked adduct involving guanine N7 and adenine N3, (C) monofunctional adducts at guanine N7, and (D) cross-linked adduct involving guanine N7 and guanine N7.

concentrations of adducts causing 50% inhibition of antibody binding are summarized in Table 1.

Discussion

An understanding of the factors that determine the effectiveness of bifunctional alkylating agents in cancer chemotherapy and of the associated toxicities requires knowledge of the levels of adducts formed following drug exposure and an understanding of cellular responses to the induced DNA damage. Previously, much attention has been given to interstrand cross-links because of their biological importance and because sensitive techniques for assaying these lesions have been available (*25*). More recently, the chemical structures of a number of adducts formed on DNA by melphalan and other nitrogen mustard-based drugs have been determined (*2*, *13*, *14*, *16*, *26*). Furthermore, immunological techniques have been developed that permit the assay of adducts in DNA extracted from patients' cells (*9*, *11*) as well as in individual cells (*12*). Cross-links, particularly interstrand cross-links, constitute a minority of the DNA adducts formed by these drugs, and the way cells cope with this type of damage may well be influenced by initial biochemical responses to the quantitatively predominant adducts. The preparation and characterization of a monofunctional derivative of the drug melphalan were undertaken to directly investigate the biochemical consequences of monofunctional DNA adducts formed by exposure of cells to a clinically relevant drug and also to further define the specificity of two immunoassays for melphalan−DNA adducts.

The association of anti-cancer effectiveness of alkylating agents with bifunctionality was discovered in the drug development programs of the 1940s and 1950s (*17*). With regard to comparison of the effects of mono- and bifunctional nitrogen mustards, most of the studies were restricted to in vivo rodent tumor models, and results

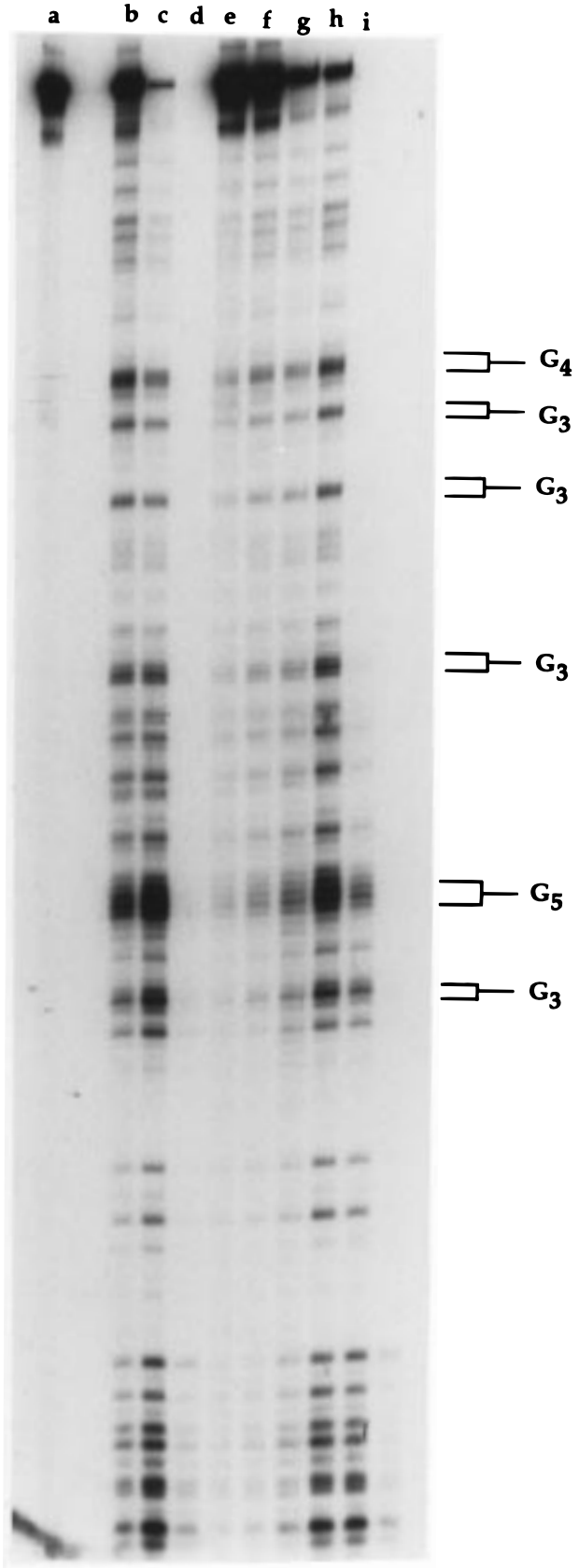

Figure 3. Sequence-specific alkylation produced by melphalan and monohydroxymelphalan determined using a polymerase stop assay: lane a, control unalkylated DNA; lanes b–d, melphalan at 10, 30, and 100 μM, respectively; and lanes e–i, monohydroxymelphalan at 1, 3, 10, 30, and 100 μM, respectively. Indicated are the runs of contiguous guanines present in the fragment of pBR322 DNA analyzed.

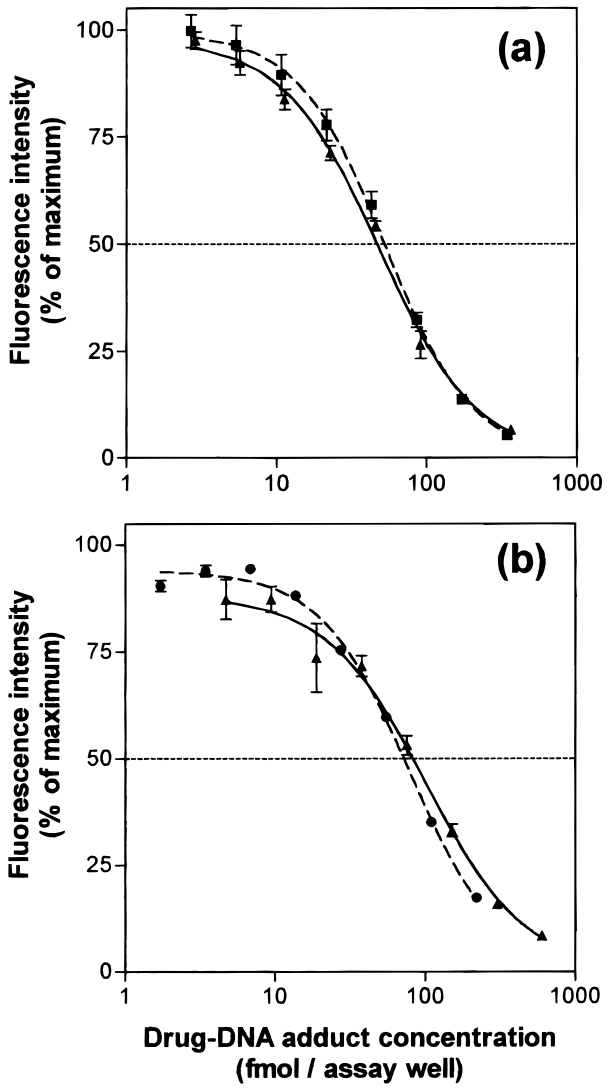

Figure 4. Comparison of immunological properties of DNA adducts formed by melphalan and monohydroxymelphalan using competitive ELISA. (a) Antibody MP5/73: (●) 2.4 μmol of melphalan adducts/g of native DNA and (▲) 15.1 μmol of monohydroxymelphalan adducts/g of native DNA. Samples were digested with DNAase I before assay. (b) Antibody Amp4/42: (●) 46.1 μmol of melphalan adducts/g of denatured DNA and (▲) 15.1 μmol of monohydroxymelphalan adducts/g of DNA. Samples were submitted to alkali treatment before assay. Each point represents the mean of four assay wells. SE lie within symbols unless shown by bars.

with monofunctional analogues were often not reported in detail (*18*, *27*). No studies have been reported in which modern biochemical techniques were used to compare the nature and extent of the DNA modifications formed or the responses of cells to equivalent mono- and bifunctional agents.

The simple procedure reported here for the preparation of a monofunctional derivative of the clinically important drug melphalan yields a product which exhibited the expected properties when analyzed by mass spectrometry and NMR spectroscopy. At pH 7, the chloroethyl group of monohydroxymelphalan showed a chemical reactivity similar to those to the chloroethyl groups in melphalan, as expected from previous reports of similar half-lives for the two stages of hydrolysis (*28*). UV absorption data at pH 2 were consistent with a progressive increase in pK_a with successive stages of hydrolysis. Since hydrolysis of melphalan was performed at pH 2, the increase in pK_a

Table 1. Abilities of Monoclonal Antibodies MP5/73 and Amp4/42 To Recognize DNA Adducts Formed by Monohydroxymelphalan

conformation of DNA at the time of alkylation	functionality of the drug	adduct level (μmol/g of DNA)	concentration of adducts causing 50% inhibition in competitive ELISA (fmol/assay well)[a]	
			MP5/73	Amp4/42
native	monofunctional	15.1	32 ± 8	79 ± 40
denatured	monofunctional	7.8	37 ± 14	64 ± 31
native	bifunctional	2.4	34 ± 7	
denatured	bifunctional	46.1		65 ± 13

[a] Mean ± SD of at least three determinations, each derived by fitting the logistic equation to a set of points, as illustrated in Figure 4.

following the first arm hydrolysis probably acted to reduce the rate of the second arm reaction, thereby increasing the yield of the desired product.

The level of contamination of the monofunctional drug with bifunctional melphalan was sufficiently low (≤0.2%) for it to be useful for a number of biological investigations. During exposure of cells to melphalan, monofunctional adducts can form either by the initial formation of monohydroxymelphalan or by hydrolysis of the second arm of melphalan that has bound to DNA monofunctionally (*14*). The data presented show that the overall ratio of alkylation of guanine and adenine by monohydroxymelphalan was indistinguishable from that observed with melphalan (*14*). Furthermore, the monohydroxymelphalan exhibited the same pattern of sequence dependency in its reaction with DNA as melphalan. Therefore, any differences observed in the biological effects of the two compounds will not be attributable to differences in the overall pattern of base alkylation.

As expected, analysis of the DNA base adducts formed by monohydroxymelphalan revealed undetectable proportions of cross-linked adducts, with the minimum detectable level estimated to be about 2% of the total adducts. This level of detectability was 7−9-fold lower than the levels of cross-linked bases reported previously for DNA alkylated in vitro with melphalan, in which adducts involving cross-links from guanine N7 to guanine N7 and guanine N7 to adenine N3 were present at 19 and 14%, respectively (*14*). However, from the purity of the monohydroxymelphalan, it is concluded that the actual level of cross-linked bases formed was considerably lower than the limit of detection of the above experiments.

The absence of detectable DNA interstrand cross-linkage in drug-treated cells has been confirmed by alkaline elution.[2]

Levels of alkylation of DNA reacted with monohydroxymelphalan were determined by use of a radiolabeled preparation of the compound. The resulting DNA preparations were recognized by two different antibodies that recognize melphalan−DNA adducts. MP5/73 recognizes initially formed guanine adducts (*9*, *13*), and Amp4/42 recognizes structures formed by alkali treatment of DNA alkylated with melphalan, probably the formamidopyrimidine products of ring opening of adducts at the N7 position of guanine (*10*). For both antibodies, the immunoreactivities of the DNA adducts formed by monohydroxymelphalan were similar to those of adducts formed by melphalan. The latter were in agreement with previously reported data (*9*, *10*). This demonstrates that both these antibodies recognize monofunctional DNA adducts at least as efficiently as bifunctional adducts. Bifunctional adducts constitute 30−40% of the total adducts on DNA alkylated with melphalan in vitro or in vivo (*14*, *15*). These results are consistent with the possibility that the antibodies recognize cross-linked adducts with an efficiency similar to that of monofunctional adducts.

Previous studies have concerned alkylated guanine nucleotides (*13*). Products in which two molecules of guanosine 5′-monophosphate (GMP) were cross-linked were considerably more immunoreactive to antibody MP5/73 than monofunctional melphalan−guanine adducts. The products in which both GMP moieties were cross-linked via the N7 of guanine and in which one molecule of GMP, alkylated via the guanine N7 position, was cross-linked to the phosphate of a second GMP caused 50% inhibition in the ELISA at 1.5 and 0.2 pmol/assay well, respectively. The possibility that MP5/73 preferentially recognized cross-linked adducts in polymeric DNA could not be distinguished from the alternative hypothesis that the overall conformation of isolated cross-linked nucleotides, particularly the N7−phosphate cross-linkage, most closely resembled the overall structure recognized by the antibody when a monofunctional adduct was present in polymeric DNA. These results prove that the latter hypothesis is correct and that antibody MP5/73 did efficiently recognize monofunctional adducts of melphalan on DNA. Recognition of melphalan−guanine adducts by the antibody was clearly influenced by the presence of adjacent nucleotides because adducts on polymeric DNA were considerably more immunoreactive (50% inhibition of the ELISA signal is typically caused by 30 fmol of adduct/assay well) than the alkylated guanine mononucleotides.

The availability of monohydroxymelphalan is permitting a number of new investigations, particularly regarding the specificity of biochemical responses of cells to DNA damage (*22*). The finding that the available antibodies recognize the adducts formed by this reagent means that it is possible to assay, with high sensitivity, levels of DNA damage in cells exposed to either the monofunctional or the bifunctional drug. In general, analysis of the responses of cells to DNA-damaging drugs benefits from an ability to quantify levels of DNA damage. This is illustrated by the finding that DNA adduct levels in cells exposed to monohydroxymelphalan were considerably higher than adduct levels induced by equal concentrations of melphalan (*22*).

Because of the availability of sensitive immunoassays for DNA adducts formed by both monohydroxymelphalan and melphalan, the melphalan−monofunctional melphalan system is particularly powerful for comparing the biological effects of mono- and cross-linked adducts formed by clinically used nitrogen mustards. This comparison of the DNA adducts formed by these compounds will be relevant to such studies, which could be of value in a number of research areas such as anti-cancer action, toxicity, mutagenesis, and DNA repair.

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[2] K. A. Gould et al., unpublished results.

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