[CANCER RESEARCH 58, 5196–5200, November 15, 1998]

Nucleotide Excision Repair of Melphalan Monoadducts[1]

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ABSTRACT

The nucleotide-excision repair (NER) system removes bulky DNA adducts and is thought to be involved in resistance to chemotherapeutic drugs, which act by damaging DNA. In this study, we have investigated the ability of the NER system to recognize and excise melphalan monoadducts from a 140-mer DNA substrate. We show that rodent and human cell-free extracts (CFEs) excise 26–29-nt-long oligomers from a synthetic 140-mer containing centrally located melphalan adducts. CFEs from cell lines with mutations in xeroderma pigmentosum group *F* or *G* genes did not excise these alkylated oligomers; however, mixing the two CFEs restored excision activity to the level found with wild-type CFEs. These results demonstrate the ability of the NER system to excise melphalan monoadducts, and are consistent with the hypothesis that NER may be involved in resistance to melphalan chemotherapy.

INTRODUCTION

NER[3] is an excinuclease system made up of multiple proteins that interact to remove bulky adducts and other lesions from DNA. NER acts according to a defined series of steps consisting of: (*a*) damage recognition; (*b*) incision of the damaged strand both 5′ and 3′ to the damage; (*c*) excision of the oligonucleotide containing the damaged base(s); (*d*) DNA synthesis; and (*e*) ligation of the newly incorporated DNA to the original DNA strand. XP cell lines, which are deficient in NER, are sensitive to several alkylating agents used in cancer chemotherapy, including cisplatin and melphalan (1).

Melphalan is used for a variety of cancers and is the most effective chemotherapeutic agent used for treating multiple myeloma (2, 3). Optimal results are obtained when myeloablative i.v. doses of 200 mg/m^2 (2) are given in conjunction with hematopoietic stem cell support (4). Although most newly diagnosed multiple myeloma patients respond to some degree, complete remission is obtained in only about 40–50%, and many patients relapse due to the proliferation of surviving malignant cells (5). The mechanisms by which myeloma cells survive initial and subsequent courses of high-dose melphalan therapy are not known. Previous workers have exposed patient cells to melphalan *ex vivo* and found that DNA alkylation was 2–5-fold greater in cells from susceptible patients compared with cells from resistant patients. Because there was no difference between these cells in melphalan uptake, glutathione levels, or rates of adduct formation, the authors concluded that enhanced repair was the major mechanism involved in resistance (6). Several other reports have indirectly shown that DNA repair is involved in resistance to melphalan (7–10). DNA repair-deficient Chinese hamster ovary cell lines ERCC1 and ERCC4 were 24 and 32 times, respectively, more sensitive to melphalan compared with the wild-type control cell line (11), also implying that melphalan damage can be repaired by NER. Because melphalan forms both monoadducts as well as interstrand cross-links in target DNA (12–14), it is not clear what role NER might play in the repair process.

In this study, we have directly tested the ability of the NER system to remove melphalan monoadducts from DNA using a well-established *in vitro* assay system (15–18). In this system, a 140-bp linear DNA construct containing a centrally located DNA lesion is used as a substrate for NER enzymes (Fig. 1). NER activity is demonstrated by the release of the centrally located lesion in the form of 26–29-nt-long excision products (15, 16, 18–21). It has been shown previously that, after incubation with CFEs and resolution by gel electrophoresis, fragments in this size range contain excised thymine dimer (19), psoralen monoadduct (22), cholesterol adduct (20), and cisplatin GG diadduct (23). Our results extend these findings to melphalan, a clinically important interstrand cross-linking agent.

MATERIALS AND METHODS

For construction of the melphalan monoadduct substrate, 100 pmol of the central 12-mer shown in Fig. 1 was end-labeled with 130 pmol ATP plus 23 pmol γ-[^{32}P]-ATP (7000 Ci/mmol) and T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in 50 μl of reaction buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT] for 30 min at 37°C. This was then precipitated with ethanol and resuspended in 20 μl of alkylation buffer [100 mM NaCl, 1 mg/ml BSA, and 25 mM triethanolamine (pH 7.3)]. A stock melphalan (Sigma Chemical Co., St. Louis, MO) solution (10 mM) was prepared in 0.1 N HCl and stored in aliquots at −80°C. The stock melphalan solution was diluted on ice with an equal volume of 0.1 N KOH and 0.1 volume 250 mM triethanolamine (pH 7.3) just before alkylation reactions. The end-labeled 12-mer in alkylation buffer was treated with an equal volume of the diluted melphalan solution at 27°C for approximately 12 h and analyzed on a 10% denaturing gel as described (16).

After alkylation of the central 12-mer with 2.2 mM melphalan, a small aliquot was incubated with 1 M piperidine for 30 min and the products analyzed on a 20% sequencing gel to confirm the location and extent of melphalan alkylation.

For construction of the 140-mer duplex substrate for NER assays, we phosphorylated 230 pmol of the central 12-mer using 300 pmol γ-[^{32}P]-ATP (7000 Ci/mmol; 1 Ci = 37 GBq) as before, then alkylated this with melphalan (2.2 mM) for 12 h at 27°C. The monoalkylated 12-mer was then gel-purified from a 10% sequencing gel. Double-stranded DNA molecules were prepared by phosphorylation (using T4 DNA polynucleotide kinase), annealing and ligation of the six partially overlapping oligonucleotides (Fig. 1) using T4 DNA ligase and gel purification from a 6% denaturing gel as described (20).

The double-stranded Mel-140 substrate was then purified on a 5% nondenaturing gel and stored at −20°C in annealing buffer [50 mM triethanolamine (pH 7.3), 100 mM NaCl, and 10 mM $MgCl_2$] before use in excision assays as described (16, 24). Briefly, CFEs containing 50 mg of total protein were mixed with substrate DNA in 25 μl of reaction buffer and incubated at 30°C for the indicated times. DNA processing, gel electrophoresis, autoradiography, and quantitation of repair were as described (24), except a Molecular Dynamics (Sunnyvale, CA) phosphorimaging system was used for data analysis.

CFEs were prepared according to a well-established procedure (25) and stored at −80°C as described (24). Repair-proficient HeLa S3 cells were from the stock of Lineberger Comprehensive Cancer Center (Chapel Hill, NC). CHO cell lines were obtained from the American Type Culture Collection (Manassas, VA): repair-proficient AA8 (CRL1859), repair-deficient UV41 (CRL1860, ERCC4/XP-F), and UV135 (CRL1867, ERCC5/XP-G).

The oligomer containing the cholesterol lesion was obtained from the

Received 5/6/98; accepted 9/17/98.

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[1] Supported by NIH Grants GM3283 and CA55819-04S1.

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[3] The abbreviations used are: NER, nucleotide-excision repair; XP, xeroderma pigmentosum; CFE, cell-free extract; ERCC, excision repair cross-complementing; CHO, Chinese hamster ovary.

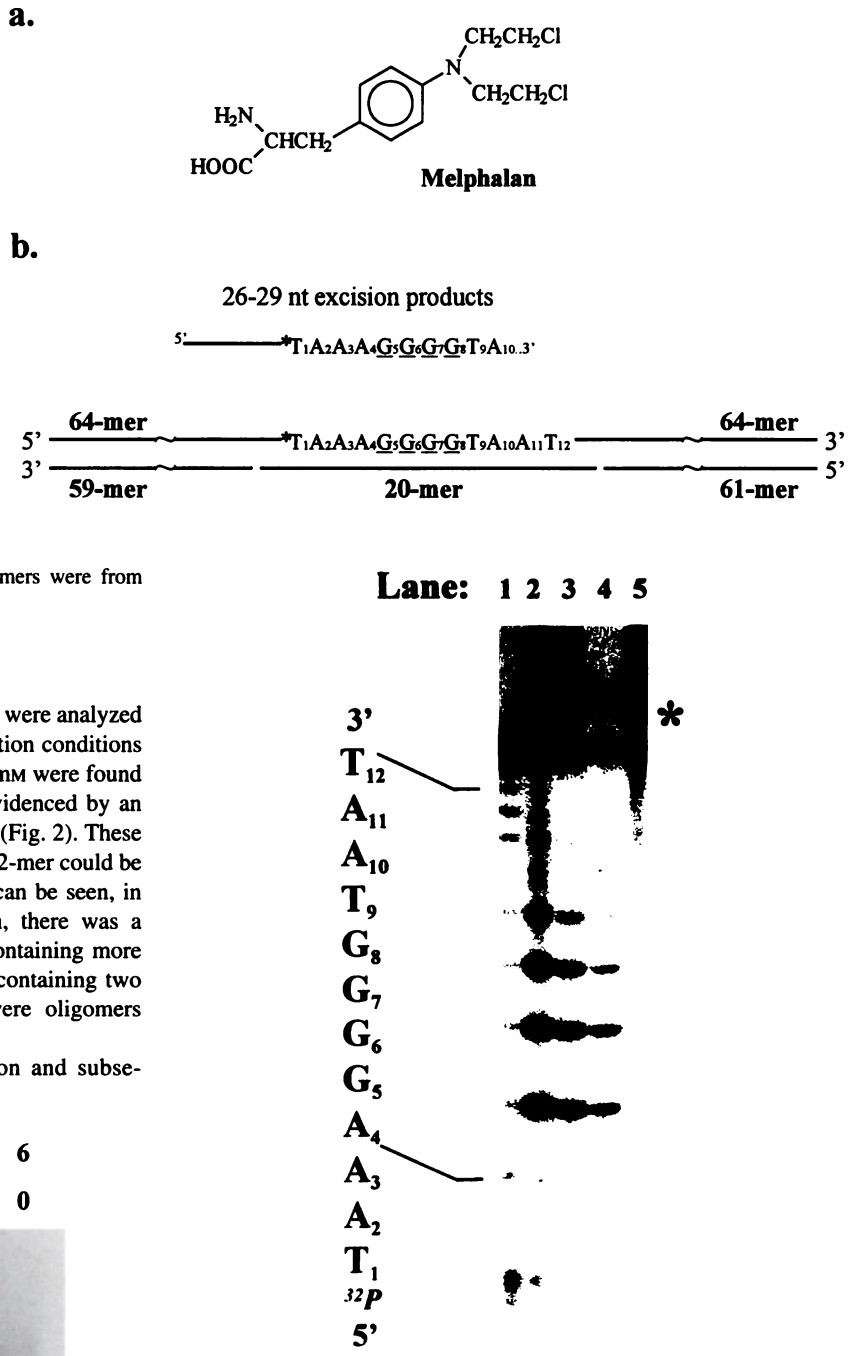

Fig. 1. *a*, structure of melphalan. *b*, schematic representation of the substrate used in the excision assays. The central 12-nt oligomer was 5′ end-labeled with ^{32}P then treated with melphalan at 27°C for 12 h. It was then annealed and ligated with five other oligomers to obtain the Mel-140 duplex substrate shown. *, position of the radiolabel. Potential alkylation sites are shown as underlined *G*. The main incision sites of human excinuclease produce 26–29 nt excision products containing the ^{32}P label as shown. The relative sizes of the fragments are not drawn to scale.

Midland Certified Reagent Co. (Midland, TX); other oligomers were from Operon Technologies (Alameda, CA).

RESULTS AND DISCUSSION

Reactions using various concentrations of melphalan were analyzed on a 10% denaturing gel to determine optimum alkylation conditions (Fig. 2). Melphalan concentrations between 1 and 2.2 mM were found to give the optimum yield of alkylated product, as evidenced by an increase in molecular weight of the 12-mer DNA band (Fig. 2). These results suggest that approximately 50% of the central 12-mer could be alkylated with melphalan under these conditions. As can be seen, in lanes containing higher concentrations of melphalan, there was a corresponding increase in the proportion of 12-mer containing more than one alkylation site/12-mer. However, oligomers containing two or more adducts were much less abundant than were oligomers containing just one adduct.

Alkylated guanines are susceptible to depurination and subsequent hydrolyzis of the sugar-phosphate backbone by piperidine (26). Our results (Fig. 3) show the near complete disappearance of the putative alkylation products and the appearance of bands corresponding to hydrolyzis at all four guanines after piperidine treatment. These results are consistent with the monoalkylated band (*, *Lane 5*) being composed of a mixture of a single alkylation event in each 12-mer at each of the four possible guanine alkylation sites (G_5-G_8), although some multiadducted 12-mer is

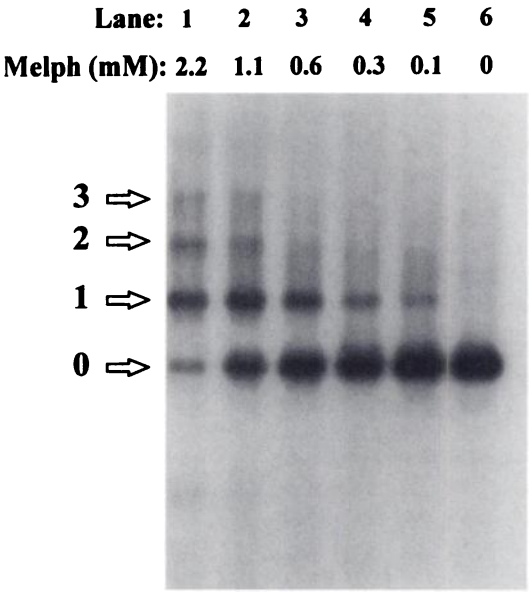

Fig. 2. Concentration-dependent alkylation of the central 12-mer with melphalan. Aliquots of the 5′ ^{32}P-end-labeled 12-mer (1 pmol) were alkylated for 12 h at 27°C in a final volume of 20 μl with varying concentrations of melphalan (shown across the top of the figure), then analyzed on a 10% denaturing gel. The numbers along the left edge of the figure indicate the number of putative melphalan adducts/12-mer.

Fig. 3. Piperidine cleaves the alkylated 12-mer at all four guanine residues. The synthetic 12-mer (sequence shown along the left side of the figure) was phosphorylated with γ-[^{32}P]-ATP, then alkylated with melphalan for 12 h at 27°C with 2.5 mM melphalan. *Lane 1*, Maxam-Gilbert reaction (G + A) on nonalkylated 12-mer showing the base sequence of the primer; *Lanes 2-4* contain 2.5, 1.25, and 0.63 pmol, respectively, alkylated primer treated with 1 M piperidine for 30 min at 90°C; *Lane 5*, 2.5 pmol alkylated primer treated for 30 min at 90°C without piperidine. *, position of the monoalkylated primer in *Lane 5*. *Lanes 2–4* provide an indication of the relative rates of alkylation of each individual guanine residue.

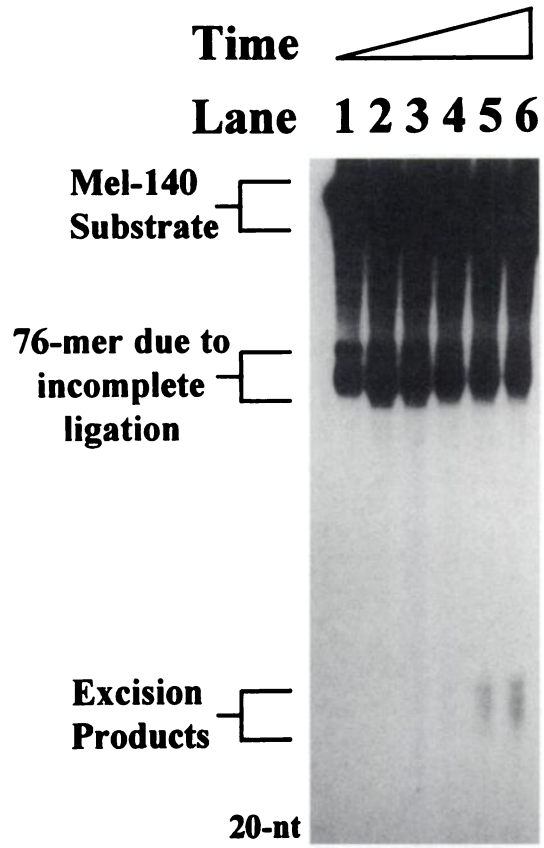

Fig. 4. Time-dependent excision of melphalan monoadducts from the Mel-140 substrate by CHO-AA8 CFE. Each reaction contained 50 μg of CHO-AA8 CFE, 2.2 fmol of Mel-140 substrate, and was incubated for 0, 5, 10, 20, 40, or 80 min at 30°C (*Lanes 1-6*, respectively). The Mel-140 substrate bands, contaminating 76-mer bands, excision product bands, and the 20-nt molecular weight marker are indicated. The 76-mer bands are most likely due to incomplete ligation during substrate preparation as well as nonspecific 3'-> 5' exonucleases, which are blocked by bulky adducts (20). These are also seen in the Chol-140 lanes shown in Fig. 5.

also clearly present in *Lane 5* (above the *). Alkylation of all four guanines within a single 12-mer would result in only the G_5 residue band remaining after piperidine hydrolyzis. The data suggest selectivity in the rate at which melphalan alkylates contiguous guanines, in agreement with previous reports (27), although as mentioned above, the pattern is biased by the small amount (~10%) of multiadducted 12-mer present.

To determine whether the alkylated Mel-140 was a substrate for NER, excision reactions were conducted for various times with CFEs from CHO-AA8 cells (25). The results show a time-dependent release of radiolabeled oligomers with sizes in the range of 26–29 nt (Fig. 4). No excision products are observed when undamaged DNA is used as the substrate (16, 28, 29). This experiment was then repeated using extracts from both the CHO-AA8 and human HeLa cell lines and compared with excision of a bulky cholesterol adduct (21). The results (Fig. 5) show that both HeLa and CHO-AA8 CFEs excise 26–29-nt oligomers from the Mel-140 and Chol-140 substrates, albeit with different efficiencies, as has been observed previously (18). Using a phosphorimaging system (Molecular Dynamics), we found that 0.9% and 0.7% of monoadducts in the Mel-140 substrate was excised in 90 min by 50 μg of CHO-AA8 and HeLa CFEs, respectively, compared with 17.7% and 7% for the cholesterol substrate.

To demonstrate that the excision was accomplished by the mammalian excinuclease, as defined by UV-sensitive human and rodent mutant cell lines, we conducted complementation assays with CFEs from two mutant cell lines: UV41 (ERCC-4/XP-F) and UV135 (ERCC-5/XP-G). The results (Fig. 6) show that CFEs from cell lines with mutations in *XPF* or *XPG* genes did not excise the 26–29-nt-long oligomers. However, mixing the two CFEs restored excision activity comparable with that seen with HeLa or CHO-AA8 CFE. Thus, we conclude that the mammalian excinuclease, as defined by the requirement for XP proteins, removes melphalan monoadducts by the specific dual-incision mechanism, which results in release of oligomers in the 26–29-nt size range.

We were surprised by the relative inefficiency of melphalan to alkylate single-stranded DNA *in vitro*, even when using exceedingly high concentrations of melphalan. We initially attempted to alkylate a central 12-mer containing a single G residue, but found that the amount of product obtained was too low to be useful for substrate preparation (data not shown). We also attempted to anneal the central 12-mer containing four contiguous Gs to a complementary 20-mer before alkylation, but this use of double-stranded DNA also did not significantly increase the yield of alkylated product (data not shown). Alkylation reactions without BSA in the alkylation buffer were slightly less efficient (results not shown). Bauer and Povirk (14) also found similar low efficiencies of alkylation of oligomers by melphalan *in vitro*. In addition, they found that melphalan intrastrand cross-links comprised <5% of the total adducts after reaction of melphalan with single-stranded DNA containing contiguous guanines. The inefficiency in alkylating single- or double-stranded DNA in solution is surprising considering that melphalan concentrations two to three orders of magnitude lower (2–20 μM) are cytotoxic to cells (8, 9, 30, 31).

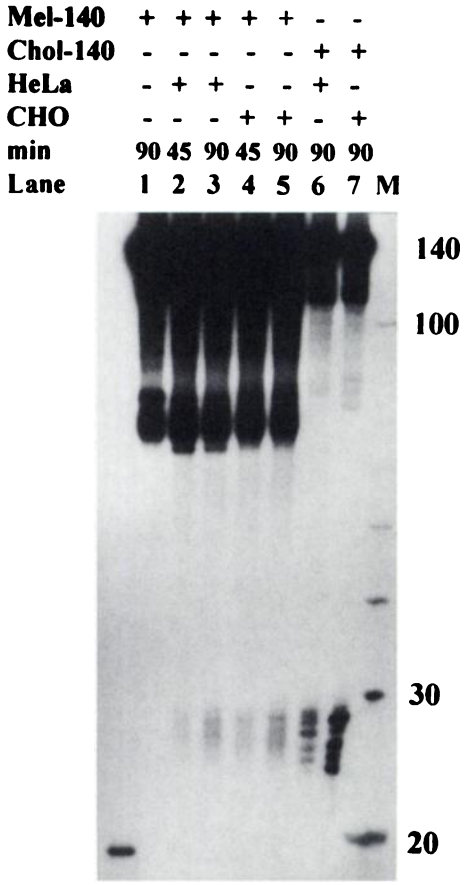

Fig. 5. Comparison of CFEs and substrates for excinuclease activity. The substrates used were Mel-140 or the previously characterized (21) cholesterol substrate (Chol-140), incubated for the indicated times with 50 μg of either HeLa or CHO-AA8 (CHO) CFE protein (M, 10-nt-ladder marker). Major bands are as described in Fig. 4.

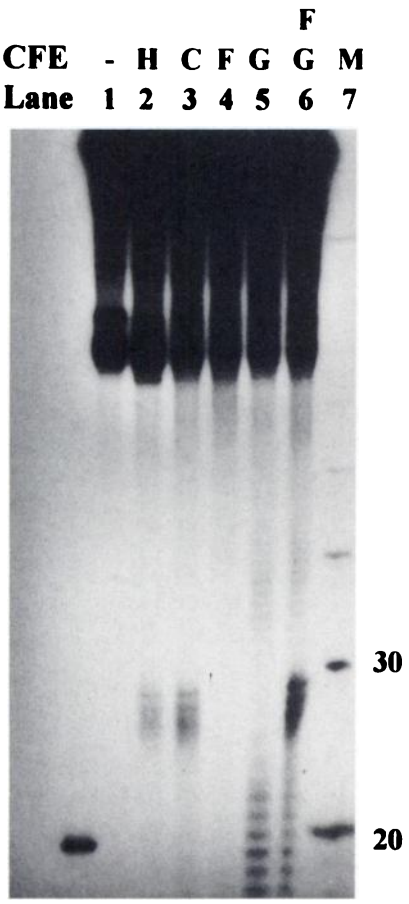

Fig. 6. Removal of melphalan monoadducts requires functional excinuclease. The Mel-140 substrate was incubated with CFEs from HeLa (H, *Lane 2*), CHO-AA8 (C, *Lane 3*), UV41-CHO (F, *Lane 4*), UV135-CHO (G, *Lane 5*), or UV41+UV135-CHO (FG, *Lane 6*) cell lines at 30°C for 2 h. Reactions (*Lanes 2–6*) each contained 50 μg of total protein. *Lane 1* contains the substrate incubated in buffer only, and *Lane 7* contains a 10-nt molecular weight marker. The low-molecular-weight species seen in *Lane 5* are generated by nonspecific nucleases in this particular preparation of UV135-CHO CFE. Other major bands are as described in Fig. 4.

The results presented here are consistent with previous indirect reports suggesting that melphalan adducts are excised by the NER pathway (9, 11). However, these experiments are the first to directly test this hypothesis using the well-defined NER assay system. When compared with the cholesterol substrate, melphalan monoadducts were removed at a significantly lower rate. Rates of NER removal of cisplatin intrastrand cross-links were shown to be approximately 3.3% in 90 min using 100 μg of CFE (16). After correcting for protein amount, this is approximately 2-fold greater than the rates we obtained using the melphalan substrate. This difference in excision efficiencies has been noted previously (16), but the mechanisms involved in determining these relative efficiencies are not yet understood.

Our results indicate a role for NER in the cellular response to melphalan, but do not rule out contributions from other repair pathways or cellular responses such as base excision repair. Indeed, the observed level of NER of melphalan adducts is quite low using this model system. Melphalan is a bifunctional alkylating agent, and available evidence supports the hypothesis that DNA interstrand cross-link formation is the cytotoxic lesion caused by melphalan (31–37). The repair of DNA interstrand cross-links is not well understood, but may involve a modified NER-mediated mechanism involving excision of undamaged DNA 5′ to the cross-linked base (22). Maximum interstrand cross-linking has been shown to occur from 6–24 h after initial exposure of cells to melphalan *in vitro* (12, 37, 38). This relatively long time between monoadduct formation and cross-link formation has been verified *in vitro* (14). Thus, repair of melphalan monoadducts before cross-link formation may play an important role in protecting cells from melphalan cytotoxicity and may be a significant factor leading to chemotherapy failure. If this is true, chemotherapeutic drugs that form DNA cross-links more rapidly may be less prone to NER-mediated resistance.

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