# Nitrogen mustard drug resistance in B-cell chronic lymphocytic leukemia

by

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## Abstract

Previous studies of nitrogen mustard drug resistance in B-cell chronic lymphocytic leukemia (B-CLL) indicated that resistance was a result of enhanced DNA repair associated with increased expression of two DNA repair genes, ERCC-1 and alkyl-N-purine DNA glycosylase. The aim of this thesis was to expand upon these observations and solidify the link between DNA repair and nitrogen mustard drug resistance. Contrary to our expectations, overexpression of ERCC-1 in CHO cells produced increased sensitivity to melphalan and cisplatin. No correlation was found between ERCC-1 expression and nitrogen mustard resistance in B-CLL, when analyzed in a larger cohort by both Northern and western blots, nor was there evidence of altered expression of a second nucleotide excision repair gene (NER), ERCC-2. Overexpression of alkyl-N-purine DNA glycosylase in CHO cells failed to produce melphalan resistance. Nitrogen mustard resistant B-CLL lymphocytes displayed cross-resistance to the bifunctional agents, mitomycin C and cisplatin, but not to UV or methyl methanesulfonate, supporting a role for enhanced crosslink repair in the resistant phenotype. Poly(ADP-ribose) polymerase (PARP) has been identified as a binding protein which can recognize melphalan damaged DNA. This binding appears to result from nicks induced by the melphalan treatment and can be inhibited if the DNA is alkylated with melphalan in the presence of methoxyamine. PARP expression was the same in both sensitive and resistant lymphocytes. When 3-aminobenzamide was used to inhibit PARP, synergy with melphalan was found in 4 of 7 samples we studied. When the DNA synthesis inhibitors, aphidicolin and ara-C, were used to modulate chlorambucil toxicity, synergy was found in both sensitive and resistant populations. There was also evidence for cross-resistance between chlorambucil and ara-C.

Thus, our studies indicate that nitrogen mustard resistance in B-CLL correlates with enhanced activity of a crosslink specific repair process. The observation that nitrogen mustard resistance in B-CLL is associated with cross-resistance to mitomycin C, cisplatin and ara-C, through a mechanism other than P-glycoprotein or glutathione, suggests that this model may represent a novel multi-drug resistant phenotype.

**Résumé**

Des études ultérieures ont démontré que la résistance de la leucémie lymphocytaire chronique type B (abrégée LLC-B), aux médicaments de la classe de moutarde azotée (melphalan, chlorambucil), est due à une augmentation de la réparation de l'ADN, et que cette résistance est associée avec une sur-expression de deux gènes de réparation: l'ERCC1 et l'alkyle-N-purine glycosylase (NPG). L'objectif de cette thèse est de poursuivre ces investigations afin de mieux clarifier la relation entre la réparation et la résistance aux médicaments de la classe de la moutarde azotée. Contrairement à ce que nous avions prévu, la sur-expression du gène ERCC1 dans les cellules CHO a entraîné une augmentation de la sensibilité au melphalan et au cisplatinum. L'analyse d'un nombre élevé de cas de LLC-B par des études de Northern et Western révèle l'absence de corrélation entre la résistance et l'exprèssion du gène ERCC1 et également du gène ERCC2. La sur-expression de la NPG n'a aucun effet sur la résistance. La résistance au melphalan dans les LLC-B est associée avec une résistance croisée aux agents bi alkylants y compris le cisplatinum et la mitomycin C mais non aux radiations UV et à l'agent mono-alkylant l'ethyl-methane sulfonate. Ceci suggère que l'augmentation des liaisons inter-/intra-brins est impliquée dans le phénotype de la résistance. Par ailleurs, nous avons démontré que l'enzyme poly (ADP-ribose) polymérase (PARP) est capable de se lier à l'ADN alkylé par le melphalan. Cette liaison semble être du à la présence de coupures dans les brins d'ADN, à la suite du traitement par le melphalan, puisque l'inhibition de ces coupures par le méthoxyamine inhibe les liaisons de l'enzyme PARP à l'ADN alkylé. Cependant, l'expression de l'enzyme PARP est identique dans les cellules LLC-B sensitives et résistantes. A la suite du traitement des cellules LLC-B avec un inhibiteur de l'enzyme PARP, le 3-aminobenzamine, une synergie a été observée avec le melphalan dans 4 des 7 échantillons testés. Des inhibiteurs de la synthèse de l'ADN tel que la cytosine arabinoside (Ara-C) et l'aphidicoline, ont également un effet synergitique avec le chlorambucil dans les cellules sensitives et résistantes. Enfin, une résistance croisée a été également observée entre le chlorambucil et l'Ara-C.

Nos études démontrent bien que la résistance aux médicaments de la classe de moutarde azotée est due à une augmentation de la réparation dans les LLC-B. Le fait que la résistance à ces médicaments soit associée avec une résistance croisée à la mitomycin C, le cisplatinum et l' AraC, indépendamment des mécanismes impliquant la glycoprotéine P et le glutathione, suggère que les LLC-B représentent un nouveau model du phénotype de la résistance multiple.

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## List of abbreviations

| | | |
|---|---|---|
| ANPG | : | alkyl-N-purine DNA glycosylase |
| AP site | : | apurinic/apyrimidinic site |
| ara-C | : | 1-β-D-arabinofuranosyl cytosine |
| B-CLL | : | B-cell chronic lymphocytic leukemia |
| BCNU | : | *bis*-chloroethylnitrosourea |
| BER | : | base excision repair |
| CHO cell | : | Chinese hamster ovary cell |
| cisplatin | : | *cis*-diamminedichloroplatinum (II) |
| CNU | : | chloroethylnitrosourea |
| CS | : | Cockayne's syndrome |
| DRP | : | damage recognition protein |
| DTT | : | dithiothreitol |
| EMSA | : | electrophoretic mobility shift assay |
| ERCC gene | : | Excision Repair Cross Complementing gene |
| f196 | : | 196 base pair DNA fragment |
| GSH | : | glutathione |
| GST | : | glutathione S-transferase |
| HEPES | : | N-2-Hydroxyethylpiperazine-N''-2-ethane sulfonic acid |
| HMG | : | high mobility group |
| HSSB | : | human single strand binding protein |
| MGMT | : | $O^6$-methylguanine DNA methyltransferase |
| MTT | : | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NAD | : | nicotinamide adenine dinucleotide |
| NER | : | nucleotide excision repair |
| PAGE | : | polyacrylamide gel electrophoresis |
| PARP | : | poly(ADP-ribose) polymerase |
| PBS | : | phosphate buffered saline |
| PMSF | : | phenylmethylsulfonyl fluoride |
| SDS | : | sodium dodecyl sulfate |
| UV | : | ultraviolet light |
| XP | : | Xeroderma pigmentosum |

# Chapter 1:

# Introduction

Cancer is a leading cause of death in our society. In the past year, there were 94 743 new cases of cancer diagnosed in Canada. In that same time period, 47 966 Canadians died of the disease. Much research is invested into studying cancer in order to understand the mechanisms of tumorigenesis and develop new strategies to fight it. Currently, chemotherapy is an important part of the first-line defense in the battle against this disease. Used widely in the treatment and management of many cancers, including breast and ovarian, chemotherapeutic agents have revolutionized the field of oncology since their introduction in the 1940s. Most of these drugs are extremely cytotoxic. The principle underlying chemotherapy is that tumor cells are proliferating at a faster rate than the rest of the tissues in the body. As a result, these cells are less able to recover from the damage incurred by chemotherapy before they enter the next phase of the cell cycle, causing cell death. The remaining tissues in the body, being largely non-proliferative, can repair the damage and survive, largely unscathed, with the exception of the bone marrow and mucous membranes. Myelotoxicity is the dose limiting factor of most chemotherapeutic agents. The highly proliferative nature of the hematopoeitic compartment makes it extremely sensitive to the effects of many of the drugs. One strategy designed to maximize the potency of chemotherapy involves autologous bone marrow transplantation in combination with high dose chemotherapy. In this way, drugs can be administered to the maximum tolerated dose by the individual, ablating the bone marrow. The blood system can then be reconstituted by infusion of hematopoetic stem cells taken from the host prior to treatment. Such regimes must be executed within the confines of a hospital, as the patient will be extremely susceptible to infection following the chemotherapy. Of course, other associated toxicities of these drugs must also be considered prior to treatment and that is why specialized personnel (medical oncologists) are required to administer and follow the progress of chemotherapy.

Medical oncology was born with the introduction of anti-tumor drugs in the 1940s. Nitrogen mustard, a derivative of mustard gas, and aminopterin, an anti-folate, exhibited signs of clinical usefulness and inspired hopes of improved treatment and cure for patients suffering

from cancer (Gilman and Philips, 1946; Farber, 1948). Since that time, modifications of the parent compounds have produced drugs which still exhibit much clinical usefulness. Nitrogen mustard is a member of an extremely potent class of anti-cancer drugs known as the alkylating agents. This group includes mitomycin C, busulphan, procarbazine, BCNU and cisplatin (although this is in fact a platinating agent, it is often included in the alkylating agent class because of its similar drug action). These agents share the common property of alkylating cellular molecules including DNA, RNA and proteins. The more potent of these compounds are bifunctional alkylating agents which possess two alkylating arms and can generate crosslinks following alkylation of two nucleophilic centers (Lawley and Brookes, 1961).

The objective of cancer chemotherapy is to eliminate the malignant cells. In order to do so, the kinetics of drug toxicity must be understood for the agents to be used effectively. The *fractional cell kill hypothesis* attempts to explain this phenomenon. It states that: "a given drug concentration applied for a defined time period will kill a constant fraction of the population, independent of the absolute number of cells" (Chabner, 1990). Therefore, the effectiveness of therapy will depend upon: (1) the size of the tumor, (2) the dose of drug administered and (3) the number of administrations. Currently, chemotherapy is given in cycles, such that if a given dose kills 99% of the cells, then repeated administrations will reduce the tumor burden in a logarithmic manner by 2 orders of magnitude each treatment, resulting in a multiplicative effect. However, there is an inherent flaw in this hypothesis. Many of the current agents are toxic only in cycling cells (phase specific), yet, as a result of the tumor anatomy, many tumors contain a large fraction of non-cycling cells which would be resistant to such drugs. Thus, the effects of a phase-specific agent may not reflect the fractional cell kill hypothesis because not all tumor cells are susceptible to killing. However, if an initial treatment is used to reduce the tumor burden prior to drug administration (surgery, radiation, or treatment with a non-phase specific drug), these non-cycling cells can begin to grow and enhance the potency of these phase-specific agents (Chabner, 1990).

Chemotherapy can be used as the only treatment or in an adjuvant/neoadjuvant setting in combination with surgical resection. While these methods have shown success in invoking remissions in many patients, this success is limited and recurrence is quite frequent. Often recurrence is associated with a more aggressive tumor which is resistant to the agent with which it was originally treated. This reemergence is believed to be a result of a subpopulation of resistant cells present in the original tumor which survived therapy. Goldie and Coldman (1979) proposed a model for drug resistance which is based on the inherent genetic instability of tumor cells. As a tumor grows, resistant clones will develop as a result of spontaneous mutation. The number of resistant clones in a tumor would then be a function of the tumor size, therefore, the smaller the tumor, the lower the probability of resistant clones, and the higher the likelihood of a cure. Adjuvant treatment is based on the belief that if the bulk of the tumor is removed by surgery, then the residual tumor cells can be killed off by chemotherapy. Chemotherapeutic regimes which cause DNA damage would increase the mutation rate, such that alkylating agent treatment would eventually produce resistant disease. This is observed in diseases such as chronic lymphocytic leukemia (CLL), where single agent nitrogen mustard therapy is the first-line treatment and almost all patients develop drug resistance.

Emergence of a resistant tumor can be explained by combining Goldie and Coldman's hypothesis with the fractional cell kill theory. As a given dose of drug can only kill a fraction of the number of cells in a tumor and in each tumor there exist resistant clones, then it is likely that the surviving fraction following drug treatment would contain the resistant cells. Thus, treatment actually becomes a means of selection for resistance, as each round of chemotherapy will enrich the population of resistant cells in the tumor. One way to avoid this problem is by using a combination of agents during the therapy. If multiple agents are used, each with a different mechanism of action, then even if a fraction of the population is resistant to one drug, it is unlikely that this same fraction would display resistance to the other drugs used [ A notable exception is multi-drug resistance (reviewed by Moscow and Cowan, 1988)]. Another advantage of the combinational therapy is that, if the drugs do not display overlapping

toxicities, each drug can be used at, or near, the maximum dose, further strengthening the impact of the treatment.

Another way to increase the potency of this therapy is to understand the mechanism of resistance, such that specific inhibitors could be developed to impair the resistance. In this way, combinations of drugs and inhibitor could be used to improve the fractional cell kill without significantly affecting associated toxicities. This has led to a great deal of research into the mechanisms of tumor resistance and models to simulate this phenomenon *in vitro* and *in vivo*. In our laboratory, we have been studying nitrogen mustards and the mechanisms by which cells become resistant to these drugs. To this end we have tried to develop clinically applicable models of drug action and resistance (Batist *et al.*, 1989; Dufour *et al.*, 1985; Panasci *et al.*, 1988). This thesis will deal specifically with my studies of nitrogen mustard drug resistance in the clinical model of B-cell chronic lymphocytic leukemia (B-CLL).

## 1. Nitrogen Mustards

The nitrogen mustards were the first alkylating agents to show significant anti-tumor activity in man. Nitrogen mustards represent a family of clinically effective drugs with a common functional group, *bis*-chloroethylamine. These agents were developed based on observations made during World War I, when sulfur mustard gas (*bis*-chloroethylsulfide) was used as a chemical weapon. Originally developed as a vesicant, it became apparent in the years following that mustard gas could be absorbed through the skin and exert toxic effects on a variety of organs ( reviewed by Gilman and Philips, 1946). Of particular interest was the observation that susceptibility to mustards correlated with proliferative activity and was most pronounced in the lymphoid system. Nitrogen mustards were developed during World War II as a result of renewed interest in chemical warfare and found to be less reactive than their sulfur counterparts. Researchers studying these agents noted the possible value of these chemicals in the treatment of cancer, specifically in lymphoid malignancies. Since initial trials

performed during the war period were classified, it was only upon declassification in 1946 that the data from these trials was presented in a statement by C.P. Rhoads (1946). It was clear from the results of these limited studies, that methyl-bis(β-chloroethylamine) [mechlorethamine] had shown significant activity against various lymphomas and chronic leukemias. Since that time, chemical modifications of mechlorethamine has led to the development of a class of agents with wide-spread application in the field of medical oncology.

## 1.1. Alkylation

The activity of these agents has been correlated with their ability to transfer an alkyl moiety to molecules, hence the title of alkylating agent. Alkylation by nitrogen mustards occurs by an Sn1 type reaction (figure 1.1). This reaction begins with initial loss of chlorine from one of the chloroethyl arms. This arm then cyclizes to generate a highly reactive aziridinium ring. This positively charged intermediate serves as a site for attack by a cellular nucleophile resulting in the formation of a monoadduct. When a similar reaction occurs with the remaining chloroethyl arm, the result is a crosslink between the two alkylated molecules (Colvin and Chabner 1990).

Early studies revealed that, while mustard treatment affected a wide range of cellular functions, including enzymatic reactions, these agents produced profound anti-mitotic, cytological and mutagenic effects, implicating DNA damage as a mechanism of action (reviewed by Wheeler, 1962). From studies in both *E. coli* and HeLa cells, DNA synthesis

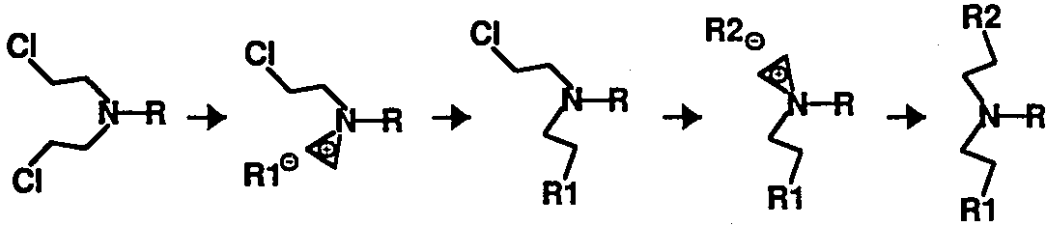



**Figure 1.1** Alkylation of two nucleophiles (R1 and R2) by a nitrogen mustard.

appeared to be uniquely sensitive to the effects of these agents at toxic doses, while at the same drug concentrations RNA and protein synthesis were largely unaffected (Lawley and Brookes, 1965; Crathorn and Roberts, 1966)

Alkylation studies using purified DNA showed that the $N^7$ position of guanine was the preferred site of alkylation by these agents and, in fact, the guanine moiety of DNA was more reactive than free guanine or guanylic acid (Lawley and Brookes, 1960; Wheeler, 1962). However, bulk alkylation of the DNA alone did not correlate with nitrogen mustard toxicity (Rutman *et al.*, 1961; Trams *et al.*, 1961). This led to investigations into the nature of the alkylated lesion, not just the quantity. It was noted that bifunctional mustards were more toxic than monofunctional mustards, this suggested that toxicity was a result of the crosslinking of two nucleophilic centers within the DNA (Loveless, 1950). More specifically, it was theorized that the lethal lesion would be a crosslink between adjacent DNA strands (an interstrand crosslink). A diguanyl derivative of nitrogen mustard was isolated by Brookes and Lawley (1961) and proof of interstrand crosslinking came in later reports (Kohn *et al.*, 1966; Lawley and Brookes, 1967). $N^7$-guanine was also the major lesion produced by nitrogen mustard treatment of chromatin and 25% of these lesions were crosslinks (Yerushalmi and Yagil, 1980).

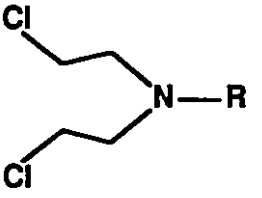
Bacteriophage were inactivated more readily using bifunctional than monofunctional alkylating agents, supporting the involvement of interstrand crosslinks in enhanced toxicity (Yamamoto *et al.*, 1966; Lawley *et al.*, 1969; Brakier and Verly, 1970). Mechlorethamine treatment of single stranded phage caused inactivation, while treatment with monofunctional agents did not, implicating a role for intrastrand crosslinks in toxicity (Yamamoto *et al.*, 1966). However, these crosslinks may have arisen in regions of double stranded DNA produced by folding of the phage DNA, effectively producing an interstrand crosslink. Also, double stranded bacteriophage was more readily inactivated than single stranded phage, suggesting a greater potency of the interstrand crosslink. In fact, more diguanyl crosslinks can be produced in double stranded DNA than either denatured DNA or native RNA,

indicating that interstrand crosslinks form more readily than intrastrand crosslinks (Brookes and Lawley, 1961). We, and others, have shown that in mammalian cells there is a correlation between interstrand crosslinking and *in vitro* toxicity of nitrogen mustards (Zwelling *et al.*, 1981; Ducore *et al.*, 1982; Hansson *et al.*, 1987; Torres-Garcia *et al.*, 1988). Thus, although the intrastrand crosslink can contribute to cytotoxicity, the primary toxic lesion appears to be the interstrand crosslink.

## 1.2. Mustard analogues and transport

Figure 1.2 lists four of the commonly used nitrogen mustard analogues in chemotherapy. The substituent (R) groups endow the compounds with various pharmacological differences. The presence of electron withdrawing groups in melphalan, chlorambucil, and cyclophosphamide greatly reduce the reactivity of these molecules in comparison to the parental compound mechlorethamine. Due to its short half life, mechlorethamine must be given intravenously, while the others can be administered orally. Toxicity of two nitrogen mustards, mechlorethamine and melphalan, could be correlated to their reactivity (Ross *et al.*, 1978; Hansson *et al.*, 1987). In this case, reactivity was measured by the speed with which these compounds produced crosslinks. In any case, the serum half-life of these agents is quite short (Figure 2), with the exception of cyclophosphamide which requires metabolism to become activated.

The introduction of the phenylalanine group in the development of melphalan, was done with the aim of increasing drug uptake into melanoma cells, where the production of melanin requires phenylalanine and tyrosine as substrates (Bergel and Stock, 1954). In fact, it has been demonstrated that mechlorethamine, melphalan, and cyclophosphamide are transported into cells by active transport or facilitated diffusion, while it appears that chlorambucil enters cells primarily by passive diffusion (Goldenberg *et al.*, 1970, 1977, 1974; Begleiter and Goldenberg, 1983 ). Further analysis, using specific competitors of uptake, demonstrated mechlorethamine uptake to be mediated by the choline transporter, while



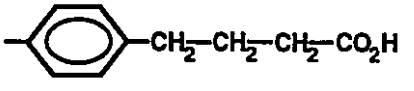
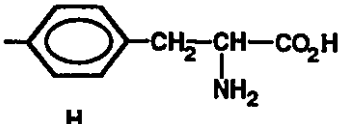
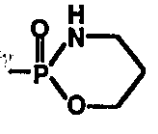

| Drug | Side Chain (R) | Plasma half-life [a] |
|---|---|---|
| Mechlorethamine | $-CH_3$ | minutes |
| Chlorambucil |  | 1.5 hours |
| Melphalan |  | 1.5 hours |
| Cyclophosphamide |  | 7 hours |

**Figure 1.2** Nitrogen mustard analogues

[a] Taken from Pratt et al., 1994

melphalan uptake involves the combined activities of the L and ASC amino acid transport systems (Goldenberg *et al.*, 1971; Begleiter *et al.*, 1979). It should be noted, however, that under physiological conditions, the transporter affinities for their natural substrates is much greater than the nitrogen mustards and, therefore, active transport represents a small fraction of the melphalan and mechlorethamine uptake. Vistica at al. (1978) demonstrated that L-leucine, at physiological concentrations, could reduce the cytotoxic effect of melphalan on L1210 cells *in vitro*, indicating that modulation of amino acid concentrations may affect transport of melphalan into tumor cells. Similarly, interference with choline transport by the agent 5-N,N-dimethylamiloride protects cells against mechlorethamine toxicity (Doppler *et al.*, 1988). Attempts to reduce the circulating levels of leucine as a means of increasing melphalan transport across the blood-brain barrier by the large neutral amino acid transporter have met with positive results, demonstrating that dietary restriction might serve to enhance the toxicity of melphalan (Groothuis *et al.* 1992).

Even though nitrogen mustard analogues, like melphalan, did not exhibit tumor selective toxicity, there did appear to be a distinct advantage of carrier-dependent drugs. Byfield and Calabro-Jones (1980) demonstrated that actively transported drugs displayed enhanced toxicity in proliferating cells. Using stimulated and non-stimulated human T cells, they found that drugs like mechlorethamine and melphalan had greater toxicity in the proliferating population, while the toxicity of non-transported drugs, like BCNU and mitomycin C, was not affected by proliferation. An earlier study using L5178Y cells established that transport and toxicity of mechlorethamine was greater in rapidly dividing cells than those in stationary phase (Goldenberg *et al.*, 1971). In an other report, a mouse myeloma cell line exhibited 70-fold greater sensitivity to melphalan than murine hematopoeitic stem cells, however this difference could not be attributed to proliferative state (Ogawa *et al.*, 1971). When a human myeloma cell line was compared to human bone marrow, no significant difference in toxicity was observed, although melphalan accumulation was greater in the myeloma line (Millar *et al.*, 1989). Begleiter *et al.* (1980) found that uptake of

melphalan into MCF-7 breast cancer cells *in vitro* was 4 times greater than that of peripheral human lymphocytes indicating a potential selectivity of this drug for breast cancer cells. Thus, melphalan still offers hope of some degree of tumor selectivity.

Cyclophosphamide is a rather unique congener, as it is inactive in its native state. This nitrogen mustard must first undergo various metabolic changes before it can generate an active alkylating species (Struck, 1974) . In this process, various other toxic by-products are generated which may also contribute to the toxicity of this agent (Hohorst, 1976). This is a widely used drug with a poorly understood mechanism of action. It appears to be actively transported into cells in its native form, however the critical metabolic changes required for activation of the alkylating group can only be performed in the liver (Goldenberg *et al.*, 1974). Oddly, mice bearing MOPC-315 plasmacytoma can be cured by a monofunctional derivative of cyclophosphamide, demonstrating that this agent has anti-tumor activity independent of crosslinking (Mokyr *et al.*, 1986). This curative ability was ascribed to an intrinsic immunomodulatory activity of cyclophosphamide. It should be noted, however, that the bifunctional form of cyclophosphamide, which produces crosslinks, was more effective.

Nitrogen mustards are used in the treatment of a variety of malignancies including CLL, breast and ovarian cancer, multiple myeloma, and lymphomas (Knospe *et al.*, 1974; Fisher *et al.*, 1986; Howell and Pfeifle, 1984; George *et al.*, 1972; DeVita *et al.*, 1972; Portlock *et al.*, 1987). Perhaps the most notable achievement of nitrogen mustards is the success of the MOPP regimen (**mechlorethamine**, vincristine, procarbazine, prednisone) in treating Hodgkin's disease which produces complete remission in 80% of the cases (DeVita *et al.*, 1972). One of the major limitations with the use of these drugs is the development of resistance by the tumor. It is, therefore, important that we understand this resistance so that improved regimens can be developed by which it can be circumvented.

## 2. Nitrogen Mustard Drug Resistance

In order to understand the development of resistance, resistant tumors have been generated experimentally by sequential exposure to increasing concentrations of drug. Resistance to alkylating agents is believed to arise from : (1) altered intracellular accumulation of drug, (2) increased levels of glutathione/glutathione-S-transferase, (3) increased levels of metallothionein, and (4) increased repair of DNA adducts. The following sections will deal with alkylating agent resistance in general and, wherever possible, the specific implications for these mechanisms in nitrogen mustard resistance will be discussed.

### 2.1. Transport alterations

As various nitrogen mustard analogs have demonstrated active transport into cells via specific transporters, a primary line of defense by the cell could be downregulation of surface transport and reduction of cellular drug accumulation. This was demonstrated by Rutman *et al.* (1968) when repeated *in vivo* exposure of Lettre-Ehrlich ascites cells to high doses of mechlorethamine rendered them resistant. Cell lines developed from these experiments displayed reduced DNA alkylation by mechlorethamine associated with a 2-3 fold decrease in accumulation of the drug. Another group produced an ascites tumor cell line which displayed 40 fold resistance to mechlorethamine, but no cross-resistance to either cyclophosphamide or chlorambucil, implicating alterations in mechlorethamine transport as a key factor (Doppler *et al.* 1988). While the $V_{max}$ for choline in the resistant line was unchanged, the $K_m$ was 8 fold higher as compared to the sensitive line.

A resistant L1210 cell line displayed reduced accumulation of melphalan associated with a 3 fold increase in the $K_m$ for melphalan transport (Redwood and Colvin 1980). MCF-7 cells (human breast adenocarcinoma) were rendered resistant to melphalan by culturing them in sequentially increasing drug concentrations (Moscow *et al.* 1993). Resistance in these cells was associated with both a reduction in the $V_{max}$ and melphalan affinity. In both lines (L1210

and MCF-7), the transport alterations were associated with the L system of melphalan uptake. Likewise, a CHO line selected for reduced L-leucine uptake displayed resistance to melphalan, as a result of decreased transport via this carrier (Dantzig *et al.* 1984). Decreased melphalan accumulation has also been noted using an *in vivo* model. A human rhabdomyosarcoma xenograft was developed which was resistant to melphalan (Lilley *et al.*, 1991). This resistance was associated with a 2-fold decrease in tumor-to-plasma ratio of melphalan in the resistant tumor compared to the sensitive one. An alternative way of reducing accumulation is to increase the rate of drug efflux. Although this is not common with nitrogen mustards, increased drug efflux has been reported in melphalan-resistant CHO cell lines (Begleiter *et al.*, 1983b). While the differences in rate of efflux seemed to correlate with reduced drug accumulation at 30 minutes, the small changes in accumulation (< 2 fold) were unable to account for the degree of resistance (4.5 to 15 fold).

Evidence of transport alteration being associated with nitrogen mustard resistance in the clinic is weak. This is primarily because it is very difficult to measure transport *in vivo*. In order to perform these studies *ex vivo*, a uniform cell suspension is required. This requirement precludes the use of solid tumors, as they tend to clump and are difficult to disperse by mechanical means. While enzymatic digestion may improve dispersion, such a process can puncture the membrane and skew the observations of uptake. Hematological neoplasms represent the most suitable model; unfortunately, the data is sparse.

## 2.2. Glutathione/glutathione-S-transferase

Glutathione (GSH) is an extremely abundant intracellular non-protein thiol. It is present throughout evolution in both prokaryotic and eukaryotic cells (Arrick 1984). This molecule is a tri-peptide (glutamate-cysteine-glycine) generated through a gamma peptide linkage from glutamate to cysteine and an alpha peptide linkage from cysteine to glycine. The production of this element is catalyzed in two steps by the sequential action of $\gamma$-glutamylcysteine synthase and glutathione synthetase. The functions of this tri-peptide are

numerous and its primary function appears to be the export of toxins from the cell and maintenance of the oxidative state. GSH acts through conjugation to toxic substrates rendering them less injurious. This conjugation reaction can be mediated by an enzyme called glutathione-S-transferase (GST). These enzymes are as numerous as they are complex (Tew 1989, Pickett and Lu 1989). The GSTs can be subdivided into groups based on their pI (class $\alpha,\pi,\mu$). While there is very significant homology among the GST families, there can be considerable difference in the active sites, which results in different isoforms having different specificities for their substrates.

It has become quite clear that GSH can be involved in the detoxification of nitrogen mustards. In theory, the highly reactive, positively charged aziridinium intermediate of nitrogen mustards should be an excellent target for the nucleophile glutathione. It has been shown *in vitro* that conjugation of GSH with nitrogen mustards occurs through this intermediate, both spontaneously and in a GST-enhanced manner (Ciaccio *et al.* 1990). A model has been proposed for the attenuation of CNU crosslinks in which GSH reacts with the monofunctional CNU-DNA adduct, preventing crosslink formation (Ali-Osman 1989). There is also an extensive literature describing cell lines which have developed resistance to nitrogen mustards associated with elevations of both GSH and GSTs (reviewed by Morrow and Cowan, 1990; Tew, 1989). Human cell lines from a variety of origins (myeloid, ovarian, rhabdomyosarcoma, prostate), as well as several rodent lines, display increased levels of GSH associated with nitrogen mustard resistance (Bellamy *et al.* 1991; Green *et al.* 1984; Rosenberg *et al.* 1989; Ripple *et al* 1993; Yang *et al* 1992; Schechter *et al.* 1991) . While the exact mechanism of this elevation is not clear (increased production or reduced turn-over), one study has shown that elevated GSH is associated with increased expression of the $\gamma$-glutamylcysteine synthase enzyme, which catalyzes the rate-limiting step in GSH production (Bailey *et al.*, 1992).

Buthionine sulfoximine (BSO), a potent inhibitor of $\gamma$-glutamylcysteine synthase (Griffith *et al.* 1982), has been used to reduce intracellular GSH and sensitize cells to nitrogen

mustards *in vitro* and *in vivo* (Hansson *et al.*, 1988; Mistry *et al.*, 1991; Kraemer *et al.*, 1987; Siemann *et al.*, 1993; Rosenberg *et al.*, 1989). A requirement for GSH in the wild type response to nitrogen mustards has been shown in a study in which a CHO line was developed which contained less than 30% of the parental GSH levels (Mandel *et al.*, 1991). These cell lines displayed hypersensitivity to melphalan, although the degree of hypersensitivity was not perfectly correlated with levels of GSH. Similarly, BSO-mediated depletion of GSH in a human melanoma line increased its sensitivity to melphalan and mechlorethamine, this increased sensitivity was related to an increase in the number of interstrand crosslinks produced by the drugs (the BSO treatment alone had no apparent adverse effect on the cells) (Hansson *et al.*, 1988). One study involving a melphalan resistant L1210 subline revealed that BSO treatment of this tumor, *in vivo*, resulted in a more rapid depletion of GSH than the parental, sensitive line. However this was not associated with enhanced therapeutic effect for the combination of BSO and melphalan over melphalan alone, as the combination appeared to be more toxic to the host (Kraemer *et al.*, 1987). Using the mouse KHT sarcoma model, Siemann and Beyers (1993) found that 16 hours following GSH depletion by BSO, GSH levels in the bone marrow returned to normal while tumor GSH was about 40% control, suggesting that timing of the BSO/nitrogen mustard administration could improve the efficacy of this combination. Ozols *et al* (1987) reported BSO potentiation of melphalan toxicity in an ovarian cancer cell line adapted for *in vitro* and *in vivo* (as a xenograft) growth. Moreover, they found that BSO pretreatment of human bone marrow cells had no significant effect on melphalan toxicity in the CFUc-GM assay. An interesting observation was made by Peters *et al.* (1991), who found that *in vitro* synergism between 4-hydroperoxy-cyclophosphamide (the activated form of cyclophosphamide) and cisplatin was dependent upon the depletion of GSH by cyclophosphamide. These results clearly demonstrate that GSH modulation can increase nitrogen mustard toxicity, and may prove to be beneficial in combinational therapy.

The rate of the GSH-nitrogen mustard conjugation can be enhanced by the GST enzyme. Although this reaction occurs spontaneously, it has been shown *in vitro* , using

mouse liver GSTs, that the α (basic) class enzyme catalyzes this reaction uniquely (although at high protein concentrations, the π and μ class can also enhance the reaction) (Ciaccio *et al.*, 1990, Bolton *et al.*, 1991). GST-α class enzyme expression has also been correlated with acquired resistance to nitrogen mustards in MatB and CHO cell lines (Schechter *et al.*, 1993; Lewis *et al.*, 1988). The resistant CHO line, CHO-Chl$^r$, exhibits high expression of a basic GST isoform not found in the parental line, which is capable of enhancing the GSH-melphalan adduct formation (Hall *et al.*, 1994). The association of GST α isoforms and nitrogen mustard resistance is most clearly demonstrated in the MatB cell line (Schechter *et al.*, 1991, 1993). Upon *in vitro* selection with melphalan, resistant clones were obtained which exhibited increased GST-α activity. Transfection of the rat Yc subunit (α class) conferred resistance to nitrogen mustard in the parental cell line, confirming GST α overexpression as having a primary role in resistance in this model. Transfection of the rat Ya subunit into mouse 10T1/2 cells rendered them resistant to chlorambucil and melphalan (Puchalski and Fahl, 1990). A similar result, although less pronounced, occurred when Ya was introduced into COS cells. Transfection of the human α class cDNA could produce nitrogen mustard resistance in yeast and NIH 3T3 cells (Black *et al.*, 1990; Lewis *et al.*, 1992). Likewise, expression of the rat Yc gene in NIH 3T3 cells conferred resistance to chlorambucil and mechlorethamine (Greenbaum, 1994). In contrast, transfection of the human GST-α into the human MCF-7 cell line failed to yield nitrogen mustard resistance (Leyland-Jones, *et al.* 1991). Transfection of GST-π conferred resistance to 10T1/2 cells, low level resistance to COS cells and no resistance in NIH 3T3 cells (Puchalski and Fahl, 1990; Nakagawa *et al.*, 1990).

Experiments involving modulators of GST activity indicate that, as with GSH, GSTs may represent a good cellular target for combinational treatments. Ethacrynic acid, a diuretic which inhibits GST activity, can inhibit all three classes of GST in a non-covalent, competitive fashion (Ploeman *et al.*, 1990). This competition is unaffected by conjugation of ethacrynic acid to glutathione. Ethacrynic acid or piripost (another GST inhibitor) treatment of various tumor cell lines (of human and rat origin) could reduce GST activity levels and enhance

toxicity of chlorambucil and melphalan (Tew *et al.*, 1988; Hansson *et al.*, 1991). However, prolonged treatment with ethacrynic acid in human lung cancer cell lines caused GSH levels to increase, which may be counter productive as detoxification by GSH can occur spontaneously (Rhodes and Twentyman, 1992). When a 2 hour exposure was used, which did not produce a significant effect on GSH, there was no evidence of potentiation of melphalan toxicity in any of the lines tested. It appears that the involvement of this drug detoxification pathway may be a matter of context, i.e., in certain systems it is responsible for resistance, while in others it is secondary. Each model must be investigated individually, as these effects cannot be generalized.

## 2.3. Metallothionein

Metallothioneins are cysteine-rich, low molecular weight proteins in the cell whose function appears to be heavy metal homeostasis. Multiple metallothionein genes exist and they are subclassified into two groups based on their electrophoretic properties : MT-I and MT-II. Exposure to heavy metals (Cd or Zn) can induce metallothionein expression and accumulation. When cells are designed to overexpress metallothionein, they display increased tolerance to heavy metals (Karin, 1983). It has been inferred from these studies that metallothioneins regulate the bioavailability of metals, such as Cd, Zn, and Cu, by direct binding to the protein (Karin, 1985). Normally found in trace amounts, these heavy metals can be extremely toxic. The regulation of metallothionein expression appears to be bi-directional, as metallothioneins are rapidly degraded in cells which lack Zn (Karin, 1981).

Due to the high thiol content of these proteins, theoretically, they might also serve as excellent scavengers of nucleophilic, reactive substances like the nitrogen mustards and cisplatin. It has been shown that melphalan and chlorambucil resistance can be induced in CHO lines which were cultured in escalating concentrations of heavy metals as a means of boosting metallothionein levels (Tobey *et al.*, 1982; Endresen *et al.*, 1983). It should be noted that, in addition to low molecular weight metallothioneins, heavy metal exposure also

induced the production of two proteins of higher molecular weight (70 and 100 kD), the relevance of these, or other proteins induced by heavy metals, to nitrogen mustard resistance is unknown (Levinson *et al.*, 1980). Exposure of CHO cells to Zn also caused increases in GSH and GST activity and, therefore, conclusions about the induction of nitrogen mustard resistance by prior exposure to heavy metals must be approached with caution (Seagrave *et al.*, 1983).

Ovarian cancer cell lines have been made resistant to Cd, by progressive escalation of $CdCl_2$ levels in the medium (Andrews *et al.*, 1987). These lines display 1.2- and 3.2- fold resistance to $CdCl_2$ and 3.3- and 4.1- fold resistance to cisplatin. It was noted that these cells also demonstrated increased levels of GSH, however BSO depletion to 17% of control levels had no effect on cisplatin resistance. Overexpression of metallothionein using a BPV expression vector rendered CHO-K1 cells resistant to the effect of monofunctional nitrosoureas but not to methylmethane sulfonate or N-hydroxyethyl-N-chloroethylnitrosourea (Kaina *et al.*, 1990). Interestingly Kelley *et al.* (1988) found that transfection of C127 cells with the BPV-metallothionein vector rendered them resistant to melphalan, chlorambucil, and cisplatin, yet these results were not reproduced when the same cells and vectors were used by Schilder *et al.* (1990). In the second series of experiments 5 different cytotoxicity assays were used with both 1 hour and continuous exposures of drug. The authors state that they did not use the same assay conditions as Kelley *et al.* and perhaps that is the reason for the discrepancy. Similarly, transfection of metallothionein into NIH 3T3 produced cell lines with resistance to cadmium, but no evidence of cisplatin resistance (Morton *et al.*, 1993)

The proposed mechanism for metallothionein-associated nitrogen mustard resistance was through direct binding of metallothionein to the reactive molecules, as had been shown for cisplatin (Bakka *et al.*, 1981; Kraker *et al.*, 1985). When metallothionein-overexpressing CHO cells were exposed to radiolabeled chlorambucil, 20 to 40% of the radiolabel was found to associated with metallothionein fractions in elution studies (Endresen *et al.*, 1983). More recently it has been reported that covalent alkylation of metallothionein by melphalan can be

detected by HPLC, confirming a direct interaction between the protein and the drug (Yu *et al.*, 1994).

In cell lines from various tissues selected *in vitro* for resistance to cisplatin, metallothionein content appeared to correlate with increasing resistance (Kelley *et al.*, 1988). Similar studies performed with ovarian cancer cell lines derived from patients with cisplatin responsive and non-responsive tumors failed to demonstrate any correlation between resistance and metallothionein expression/inducibility (Schilder *et al.*, 1990). Clinical data in support of these observations is limited. Murphy *et al.* (1991) found that metallothionein levels in ovarian tumors were higher than in normal ovaries. However, they found no difference between metallothionein levels in untreated specimens and those from patients who received therapy, both responders and non-responders. In the treated group, the metallothionein levels were not influenced by response, or any other criteria which is believed to correlate with clinical outcome. Chin *et al.* (1993) analyzed metallothionein expression in 33 primary testicular germ cell tumors by immunohistochemical analysis. In these studies, there appeared to be an association between tumor stage and metallothionein expression. However, there was no obvious relationship between metallothionein content and clinical response to chemotherapy. While the evidence from these experiments appears to be conflicting, so is the nature of much of the studies related to drug resistance.

## 2.4. DNA repair

Early studies of mustard action in prokaryotes and eukaryotes revealed that cells are capable of excising mustard lesions from their genome in a similar fashion to the DNA repair exhibited following UV or X-ray irradiation (Lawley and Brookes, 1965; Kohn *et al.*, 1965; Roberts *et al.*, 1968; Ewig and Kohn, 1977). A direct requirement for efficient repair in response to nitrogen mustard induced DNA damage comes from studies involving DNA repair deficient cell lines. These lines display hypersensitivity to nitrogen mustards and other crosslinking agents, demonstrating that toxicity is inversely related to repair proficiency.

Mutant analysis indicates that several repair systems may be involved in the response to nitrogen mustards. Cross-sensitivity between nitrogen mustards and UV light or methyl methanesulfonate indicate that both the nucleotide and base excision repair pathways are required for the wild type response to nitrogen mustard damage (Hoy *et al.*, 1985; Meyn *et al.*, 1991). Investigations of the genetic disease, Fanconi's anemia, indicate that a pathway, separate from the two aforementioned systems, may exist which specifically recognizes crosslinks, as cells from these patients display unique hypersensitivity to crosslinking agents (Ishida and Buchwald, 1982). By inference it can be deduced that repair of nitrogen mustard lesions involves 3 different pathways. Interestingly, all these mutant lines display cross-sensitivity to cisplatin, mitomycin C, and BCNU, indicating that there may be a common mechanism which responds to crosslinks in the DNA which relies on proteins which are key to each of the repair processes. In light of these observations, it is quite feasible to speculate that upregulation of the DNA repair process by a cell could render it resistant to these agents.

Early reports of enhanced repair of both nitrogen and sulfur mustard lesions came from a strain of *E. coli* with acquired resistance to sulfur mustard (Kohn *et al.*, 1965; Lawley and Brookes, 1968; Venitt, 1968). These results were then extended to mammalian cells when Walker and Reid (1971) reported that resistance to sulfur mustard in L-cells was associated with a 2 to 3-fold increase in excision of alkylated DNA. Similarly, resistant Yoshida sarcoma cells exhibited increased repair of interstrand crosslinks generated by nitrogen mustards and busulphan (Harrap and Gascoigne, 1976; Bedford and Fox, 1982). Resistance to cisplatin in L1210 cell lines was associated with increased release of cisplatin lesions from the DNA (Eastman and Schulte 1988). The resistant L1210 line also displayed enhanced capacity to repair a plasmid which was damaged by treatment with cisplatin prior to transfection (Sheibani *et al.* 1989). Cross-resistance to UV light in the L1210 cells suggested a role for nucleotide excision repair (NER) in the resistance (Jennerwein *et al* 1991). Unscheduled DNA synthesis following drug exposure (an indicator of repair synthesis) was higher in a cisplatin resistant ovarian cancer cell than the sensitive parent cells (Lai *et al.*, 1988; Masuda *et al.*, 1988). A

report from our group described a melphalan-resistant MCF-7 cell line which displayed a greater repair capacity for releasing melphalan induced lesions than the sensitive, wild-type MCF-7 line (Batist *et al.*, 1989).

The molecular events associated with enhanced repair are not clear. Enhanced repair of cisplatin lesions was dependent on DNA polymerase α in one ovarian cancer cell line, while others studies found increased levels of DNA polymerase β in cisplatin resistant ovarian carcinoma and P388 leukemia cells (Masuda *et al.*, 1988; Lai *et al.*, 1988; Scanlon *et al.*, 1989; Kraker and Moore, 1988). Extensive Northern blot analysis, including transcripts for DNA polymerase α and β, found no differences in expression of any genes between cisplatin resistant and sensitive L1210 cells, except for a slight (2 fold) increase in thymidine kinase (Sheibani and Eastman, 1990). Interestingly, there was also no increased expression of nucleotide excision repair genes, ERCC-1 and ERCC-2, even though these cells display enhanced resistance to UV light (Jennerwein *et al.*, 1991). In one study, samples were taken from a human glioma patient before cisplatin therapy and after the patient relapsed (Ali-Osman *et al.*, 1994). The latter samples displayed enhanced repair capacity associated with increased activity of DNA polymerase β and DNA ligase, demonstrating that such changes do occur *in vivo*. Similarly a melphalan resistant rhabdomyosarcoma xenograft displayed cross-resistance to a variety of bifunctional alkylating agents and exhibited increased activity of DNA polymerases α and β and increased topoisomerase II expression (Lilley *et al.*, 1991; Friedman *et al*, 1994). Elevated topoisomerase II has been reported previously in a mechlorethamine resistant Burkitt's lymphoma cell line (Tan *et al.*, 1987). Resistance in this cell line was associated with an altered chromatin structure as demonstrated by enhanced DNase sensitivity in the resistant line.

While these cell lines display enhanced repair capacity at the level of the entire genome, it may be even greater in actively transcribed regions. It has been demonstrated clearly in the literature that cells preferentially repair certain types of damage, including nitrogen mustard lesions, in transcribed regions as compared to non-transcribed regions (Hanawalt, 1991;

Larimnat *et al.*, 1993; Wasserman and Damgaard, 1994). Thus, a 2-3 fold increase in overall repair could translate to a much more profound increase in repair at the level of transcribed genes, as only 1% of the DNA is estimated to contain coding sequences. Enhanced preferential-repair of interstrand crosslinks in the active DHFR gene has been shown in cisplatin resistant lines (Zhen *et al.*, 1992).

As a result of poor understanding of the DNA repair systems, few inhibitors exist to modulate their activity. However, the final step of DNA repair is DNA resynthesis and inhibitors do exist which block this cellular function. Studies involving such inhibitors (ara-C, aphidicolin, hydroxyurea) indicate that they can be used to potentiate toxicity of crosslinking agents, such as nitrogen mustards and cisplatin (Swinnen *et al.*, 1989; Frankfurt *et al.*, 1993; Alaoui-Jamali *et al.*, 1994). It has also been shown that high concentrations of these inhibitors can actually prevent crosslink removal, therefore, such drugs may be useful clinically in combination with nitrogen mustards (Frankfurt, 1991).

In many of these models it appears that multiple systems are involved in the resistant phenotype. Enhanced excision repair has been noted in mechlorethamine resistant Ehrlich ascites cells, which exhibited a 50% decrease in drug accumulation yet a greater than 10-fold increase in resistance (Rutman *et al.*, 1968; Yin *et al.*, 1973). In many cases where BSO or ethacrynic acid potentiates toxicity, restoration of sensitivity is not complete. Moreover, the chemosensitizer often affects the sensitive cells as well, indicating that it may not be specific for the resistance factor (Tew *et al.*, 1988; Mistry *et al.*, 1991;Bellamy et al., 1991) . As regards DNA repair, it appears that GSH depletion has a negative effect on DNA repair, perhaps by inhibiting ribonucleotide reductase and limiting deoxyribonucleotide pools (Lai *et al.*, 1989). Thus, an unexpected interplay between two seemingly unrelated systems may serve to enhance the effect of chemosensitizers like BSO. Which pathway of resistance is more important is presently impossible to judge and, therefore, all potential mechanisms must be explored. However, the objective of this research is to identify resistance mechanisms

which can be targeted with other drugs. In that respect, if a resistant tumor cell can be sensitized using a specific inhibitor, even in the absence of complete restoration of sensitivity, this could still yield a clinical response and reduce the tumor mass. In fact, BSO combinational therapy with nitrogen mustards is currently in clinical trial.

A complication arises in extrapolating data obtained from tissue culture systems to the clinical situation. When xenograft models are used, in which resistant tumors are selected *in vivo*, cell lines developed from the resistant tumors have displayed different characteristics *in vitro* and *in vivo* (Teicher *et al.*, 1990, Schechter *et al.*, 1991). In fact, it has been shown that a line which was resistant *in vivo*, displayed no resistance *in vitro* (Teicher *et al.* 1990). Xenograft systems and cell lines which can be propagated *in vivo* may provide models with clinical relevance. Both solid tumor and leukemia models have been developed in mice using human and murine cell types. However, it is clear that mice and humans differ physiologically with respect to their tumor response. Another system which has been established is three-dimensional toxicity analysis. This system involves culturing the cells in agarose such that they grow as a 3-D mass similar to the tumor *in vivo* and has produced results comparable to that seen using the xenografts (Kobayashi *et al.*, 1993).

In our laboratory, we sought to establish clinically relevant mechanisms of resistance and, therefore, we have performed studies on samples obtained directly from patients. These tumors acquired resistance *in vivo*, so the properties we reveal should be clinically relevant. The difficulty associated with such an analysis of solid tumor tissue is the establishment of uni-cellular suspensions which are required for most laboratory manipulations. Early transport studies in the laboratory involved the analysis of bone marrow aspirates and various cancer samples from patients (Dufour *et al.*, 1986). Such problems of uni-cellular solutions can be overcome if the malignancy being sampled consisted of cells in suspension (i.e., leukemias). Thus, we (and others) have begun to develop B-cell chronic lymphocytic leukemia as a model for *in vivo* drug resistance.

## 3. B-Cell Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western hemisphere. Slightly more prevalent in men than women (ratio = 1.7 : 1), this disease generally affects the elderly, although it has been diagnosed in people as young as 35 years old (Rai, 1994). While the term applies to a variety of neoplasms of lymphatic origin (including prolymphocytic leukemia and hairy cell leukemia), CLL refers to a single disease (Gale and Foon, 1985). Leukemic cells can be of either B or T cell origin with B-cell CLL (B-CLL) being the most common, accounting for 95% of the cases. The disease manifests itself through the progressive accumulation of mature-appearing B-lymphocytes (Dameshek, 1967). B-CLL lymphocytes arise from a single malignant clone. Monoclonality has been established using a variety of techniques, including genetic (analysis of immunoglobulin gene rearrangements) and biochemical (glucose-6-phosphate dehydrogenase typing) tests (reviewed by Gale and Foon, 1985).

### 3.1 Clinical features

Patients are generally asymptomatic upon examination, lymphocytosis is often detected during routine blood tests. In cases of advanced disease, patients may experience night sweats, weakness/fatigue, weight loss, and increased incidence of fever (Rai, 1994). Early stage disease may not present any physical abnormalities. As the malignancy progresses, patients exhibit lymph node involvement and splenomegaly. Anemia and thrombocytopenia appear in advanced disease and death is generally a result of opportunistic infection resulting from bone marrow failure. There is a wide range of survival times among B-CLL patients. In some cases the disease follows an indolent course (smoldering CLL) and patients can live a long time, in other cases the course is much more rapid and death ensues within 2 years (Dighiero *et al.*, 1991; Rai, 1994 ).

In order to assist prognosis, staging systems have been established. The first system, devised by Rai *et al.* (1975), involves 5 groups: Stage 0, lymphocytosis in blood and bone marrow; Stage I, lymphocytosis and lymphadenopathy; Stage II, lymphocytosis and organomegaly (spleen or liver); Stage III, lymphocytosis and anemia (hemoglobin <11 g/dL; Stage IV; lymphocytosis and thrombocytopenia (platelets < 100 000/$mm^3$). Binet *et al.* (1981) modified the staging system based on lymph and organ involvement. They divided the disease into 3 groups: Group A, less than three involved areas (the five possible areas are: cervical, axillary and inguinal nodes, spleen and liver); Group B, three or more involved areas; Group C, thrombocytopenia and or anemia (as described for Rai staging). The median survival for patients classified by Binet is: Group A, >10 years; Group B, 7 years; Group C, 2 years. The success of the Binet system has led Rai (1994) to modify the original staging to three groups: low risk, Rai stage 0; intermediate risk, Rai stage I and II; high risk, Rai stage III and IV. Such staging systems are required by the physician in order to determine treatment. Patients with a good prognosis would be spared chemotherapy, while patients with poor prognosis could receive more aggressive therapy.

## 3.2 Biology

Microscopically, B-CLL lymphocytes appear to be mature lymphocytes, however at the molecular level they are quite different. These cells express low levels of surface immunoglobulin, generally consisting of mu heavy chains and, in some cases, there is coexpression of delta heavy chains. The light chains can be either kappa or lambda, but never both (Foon *et al.*, 1990). The most unique aspect of B-CLL lymphocytes is the presence of the CD5 antigen. CD5 is a T cell antigen, normally found on a very small population of B cells which appear to be involved in auto-immune syndromes (Hayakawa and Hardy, 1988). This may explain the incidence of auto-immune hemolytic anemia which occurs in 15% of B-CLL cases (Foon *et al.*, 1990). Most of the auto-antibodies produced by B-CLL cells have rheumatoid factor binding or binding activity for the Fc portion of human IgG (Kipps and

Carson, 1993). During B-cell development, Ig rearrangements recruit genes from several gene families in order to generate a complete Ig coding sequence. Often this is coupled with a phenomenon called somatic hypermutation which adds more diversification to the number of potential Ig sequences. However, in B-CLL certain variable region (V) genes are overrepresented and often in their germline form (Kipps, 1993; Dighiero, 1993). These genes are believed to produce low affinity, polyreactive Ig (Dighiero, 1993). This polyreactivity may be responsible for assisting these cells in escaping deletion during maturation by expressing some reactivity to non-self antigens (Kipps, 1993). The authors also postulate that continuous exposure of B-CLL cells, with auto-immune function, to self-antigen may assist in the neoplastic transformation.

B-CLL lymphocytes appear to be halted in their maturation, however, under the correct culture conditions, these cells can be stimulated to proliferate and produce Ig, so this block does not appear to be static (Fluckinger *et al*, 1992). *In vivo*, there is evidence that, in a small number of cases, patients with B-CLL may develop a high-grade lymphoma (Richter's syndrome; RS). While the RS lymphoma cells do not express CD5, they exhibit the same surface Ig and gene rearrangements as the B-CLL cells, strongly indicating that they have arisen from a common clonal origin (Cofrancesco *et al.*, 1992; Cherepakhin *et al.*, 1993; Traweek *et al.*, 1993). Although the origin of Richter's syndrome remains controversial, these results demonstrate that in some cases B-CLL cells can evolve *in vivo*. Therefore, factors must exist which are responsible for maintaining these cells frozen at this immature stage of differentiation.

When B-CLL lymphocytes are maintained in culture, they begin to apoptose within 30 hours (Collins *et al.*, 1989). This rapid induction of cell death is most likely caused by loss of serum factors required for survival. It has recently been shown that the presence of IFN-$\gamma$ in the culture medium can prevent this induction of apoptosis. Similarly, 70% of the B-CLL patients tested had high levels of IFN-$\gamma$ in their serum, while in the serum of normal controls this cytokine is undetectable (Buschle *et al.*, 1993). Phorbol esters have also been shown to

block apoptosis (McConkey *et al.*, 1991). IFN-γ transcription is strongly stimulated following phorbol ester treatment, suggesting an autocrine function for this protein in B-CLL cell viability (Buschle *et al.*, 1993). In other studies it has been shown that IL-4 can prevent apoptosis in B-CLL cells up to 3 weeks in culture (Danescu *et al.*, 1992). Inhibition of apoptosis was associated with increased expression of the *bcl-2* oncogene. Interestingly, IL-4 is not produced by B-CLL cells. The protooncogene *bcl-2* was first identified in follicular lymphoma and its expression can protect cells from apoptosis (Hockenberry *et al.*, 1990; Bissonnette *et al.*, 1992). Prevention of apoptosis is a feasible explanation for the appearance of this disease, which presents itself as an accumulation of long-lived, rather than proliferative, lymphocytes (Dameshek, 1967). A link between inhibition of apoptosis and oncogenesis has recently been hypothesized (Williams, 1991).

The fine line between quiescence (longevity) and cell death in B-CLL most likely reflects an intricate system of both stimulatory and inhibitory signals acting on the cell in both an autocrine (IFN-γ) and a paracrine (IL-4) fashion (reviewed by Hoffbrand *et al.*, 1993). Fisher (1994) suggested that induction of apoptosis is one way by which chemotherapeutic agents may kill cells. *In vitro* treatment with glucocorticoids or chemotherapeutic agents (chlorambucil, 2-chlorodeoxyadenosine, fludarabine) can enhance the onset of apoptosis in B-CLL lymphocytes, perhaps explaining their activity *in vivo* (McConkey *et al.*, 1991; Robertson *et al.*, 1993; Begleiter *et al.*, 1994).

### 3.3 Disease treatment

Treatment is generally withheld until the appearance of disease related symptoms (anemia, thrombocytopenia, painful lymphadenopathy or splenomegaly, or lymphocyte count > 150 000/μl) (Rai, 1994). Chlorambucil (sometimes cyclophosphamide) is the standard treatment of choice, given orally, either daily or monthly. Nitrogen mustard treatment invokes a response in about 60% of untreated patients (Foon *et al.*, 1990). Due to the lymphotoxic nature of glucocorticoids, they have been included in combination with nitrogen mustards,

however this combination does not improve survival (Dighiero, 1991). Another combination, COP (cyclophosphamide, vincristine, prednisone), also proved effective but not to any greater extent than chlorambucil alone. Likewise, the CHOP protocol (COP plus doxorubicin) was effective but no better than high dose chlorambucil (Dighiero, 1991). Perhaps the most promising new treatments for B-CLL involve the use of nucleoside analogs (fludarabine, 2-chlorodeoxyadenosine, and 2-deoxycoformycin). In particular, fludarabine has shown impressive activity in initial trials, achieving response in 70% of untreated patients (Keating *et al.*, 1989). Randomized trials are currently underway investigating the benefits of chlorambucil and fludarabine alone compared to the combination of chlorambucil and fludarabine. As with other malignancies, recurrence is frequent in B-CLL and continued treatment as a means of controlling the disease almost inevitably results in the development of resistance.

Therefore, B-CLL represents an excellent model for the study of nitrogen mustard resistance from several reasons:

(1) Extreme lymphocytosis results in very high concentrations of malignant B cells in the peripheral blood of the affected patients. As a result, we can obtain relatively homogeneous (>90% pure B lymphocyte) tumor cell populations from the peripheral blood of patients.

(2) Patients can live a long time with disease. This allows the researcher to obtain samples from the patient prior to and after therapy, when the drug resistance develops.

(3) Long term single agent treatment almost inevitably results in resistance. Thus, all B-CLL patients treated in this way will eventually become resistant.

## 4. Nitrogen mustard resistance in B-CLL

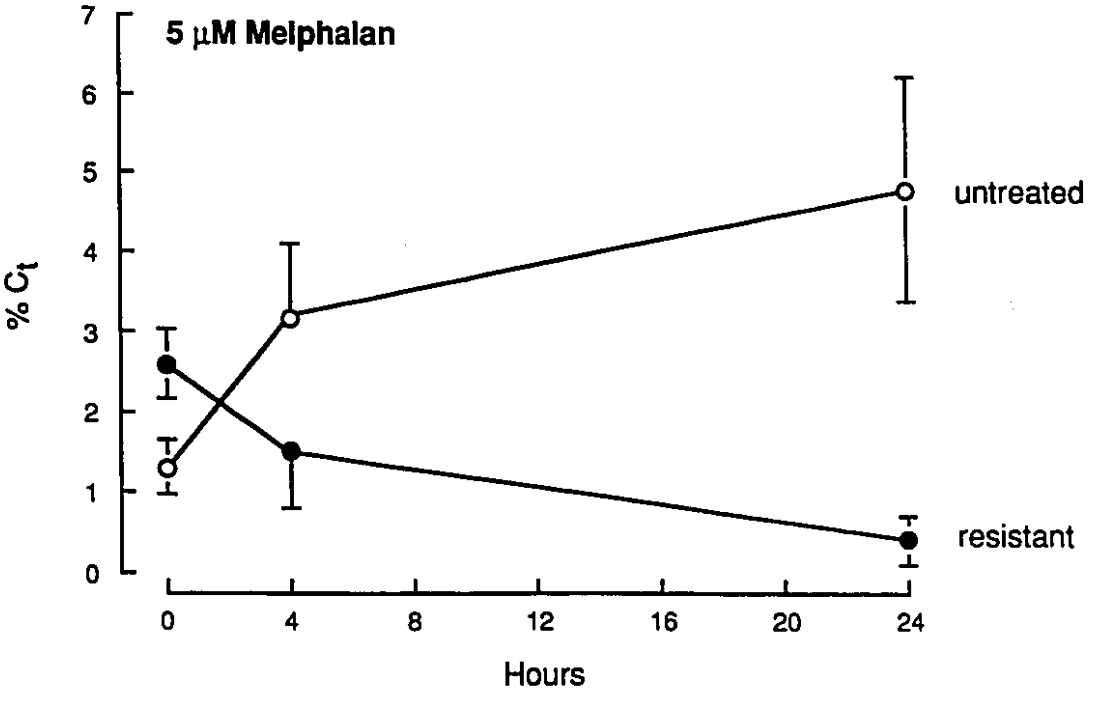
Initial studies of resistance in B-CLL by our lab involved analysis of melphalan transport into lymphocytes from both untreated and treated-resistant patients (Panasci *et al.*, 1988). These experiments failed to reveal any differences in either the transport kinetics of uptake or steady-state accumulation levels of melphalan in treated-resistant lymphocytes as compared to untreated lymphocytes. We were also unable to find evidence of any melphalan metabolites or the dihydroxy form of the drug in either untreated or treated-resistant patients' lymphocytes. Another group studying chlorambucil uptake in B-CLL found similar results; i.e. no difference in transport or metabolism (Bank *et al*, 1989). Schisslebauer *et al.* (1990) reported a 2 fold increase in overall GST activity levels in treated patients' lymphocytes as compared to those of untreated patients and normal controls, but there was no significant correlation between GST activity and resistance. Also, it was not clear what component of this elevated activity represented GST-$\alpha$ (the isoform implicated in nitrogen mustard resistance) and this isoform was only found in 63% of the samples. GST-$\pi$ was the major isoform present in both untreated and resistant lymphocytes. While it is not certain what role this isoform may play in nitrogen mustard resistance, it does not appear to be as efficient as GST-$\alpha$ in catalyzing the conjugation reaction nor does overexpression of this gene confer nitrogen mustard resistance. Johnston *et al.* (1990) demonstrated an inverse correlation between the product of GSH and GST and the formation of interstrand crosslinks in B-CLL lymphocytes. However, they were unable to demonstrate a correlation between resistance and interstrand crosslink formation. Another study found no evidence of GSH elevation nor increased activity of any of the GST isoforms in nitrogen mustard resistant B-CLL (Ribrag *et al.*, 1994). In a recent report, ethacrynic acid used in combination with chlorambucil produced a response in a B-CLL patient who was refractory to chlorambucil, however the toxicity of ethacrynic acid on its own was not determined (Petrini *et al.*, 1993). We have also screened for metallothionein expression in B-CLL using Northern blot analysis (unpublished data).

This gene was very weakly expressed in B-CLL lymphocytes and there was no evidence of differences in expression between the untreated and treated-resistant patients' lymphocytes.

Our analyses focused on the status of interstrand crosslinks in B-CLL lymphocytes following *in vitro* incubations with melphalan. In contrast to the observations of Johnston *et al.* (1990), we found that reduced crosslinking did correlate with resistance. It was discovered that 3.5 times more melphalan was required to generate 5% (an arbitrary value) crosslinking in resistant patients' lymphocytes as compared to untreated patients' lymphocytes (Panasci *et al.*, 1988; unpublished data). This level of resistance to crosslinking is similar to resistance found when inhibition of DNA synthesis following chlorambucil treatment is used as an endpoint (Bentley and Blackmore, 1992). In these studies, lymphocytes were stimulated to proliferate prior to chlorambucil exposure. Following drug treatment, $^{3}$H-thymidine was added to the cultures and the lymphocytes were incubated a further 24 hours. It was shown that chlorambucil could inhibit DNA synthesis in a dose dependent manner. These researchers found that non-responsive patients exhibited 3-fold resistance to the synthetic inhibition by chlorambucil and patients who were refractory (acquired-resistant) displayed 4 fold resistance. Further kinetic analysis of crosslink formation/removal, performed in our lab, revealed an enhanced rate of crosslink repair in the samples from resistant patients [see figure 1.3 (Torres-Garcia *et al.*, 1989)]. While untreated patients' lymphocytes accumulated crosslinks gradually to 4 hours and demonstrated little removal of crosslinks 24 hours later, resistant patients' lymphocytes acquired more crosslinks immediately following melphalan incubation and showed steady removal of crosslinks to 24 hours, when most, if not all, detectable crosslinks are repaired. The higher initial level of crosslinks in resistant lymphocytes correlates with the observation of increased accumulation of melphalan and chlorambucil in resistant B-CLL lymphocytes following *in vitro* incubation (Panasci *et al.*, 1988; Bank *et al.*, 1989). Enhanced crosslink removal indicated that resistance may be mediated by increased DNA repair. Analysis of 2 key DNA repair proteins (ERCC-1 and N$^{3}$-methyladenine DNA glycosylase), revealed that resistant lymphocytes seemed to overexpress these enzymes, supporting the role

**Figure 1.3 Analysis of crosslink formation and removal in B-CLL lymphocytes.**

This figure was adapted from Torres-Garcia *et al.* (1989). Lymphocytes were incubated *in vitro* with either 5 or 10 μM melphalan for 30 minutes at 37 ° C, followed by a further 24 hour incubation at 37 ° C in the absence of drug. Samples were taken at 0, 4, and 24 hours post drug incubation and analyzed for the presence of interstrand crosslinks. Crosslinking is expressed as $\%C_L$, which is an arbitrary value calculated as the ratio of non-denaturable DNA following alkylation to the total denaturable DNA in the absence of alkylation. The open circles represent sensitive patients and the closed circles represent resistant patients. The upper panel represents the mean of seven untreated and six resistant patients' lymphocytes, while the lower panel was compiled from the results of eight untreated and six resistant patients' lymphocytes.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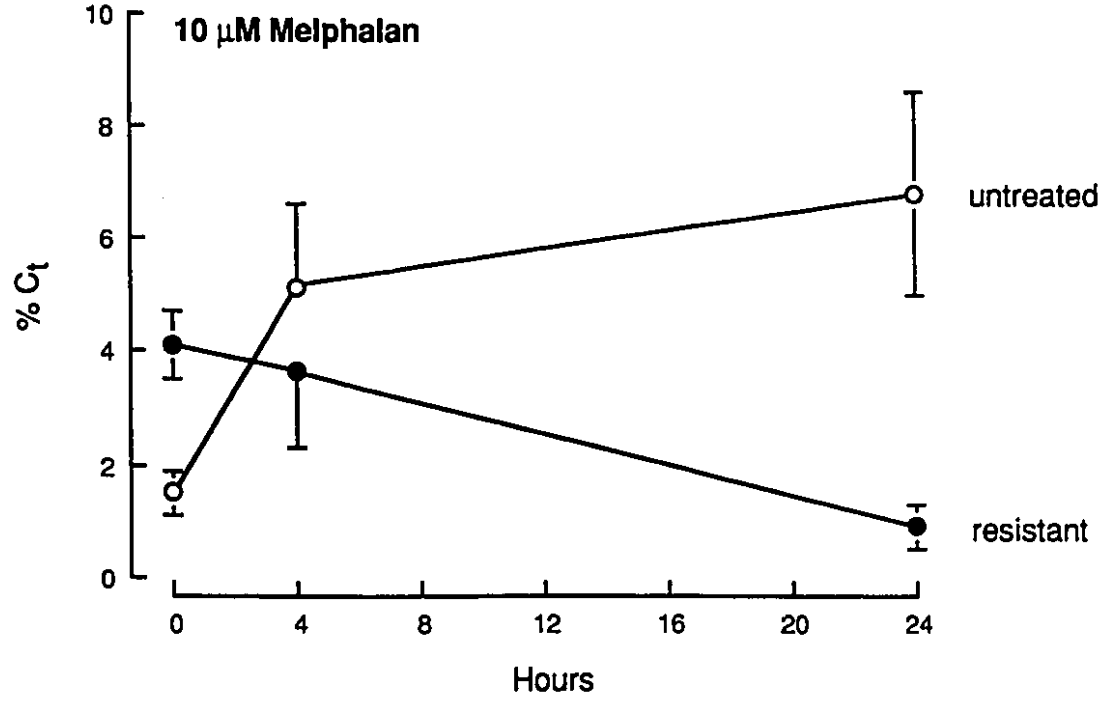
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of DNA repair in nitrogen mustard resistance in B-CLL (Geleziunas *et al.*, 1991). Begleiter *et al.* (1991) failed to correlate increased repair with resistance, however their criteria differed from ours. We define a patient as resistant if they initially respond but fail therapy following successive treatments, Begleiter *et al.* described a resistant patient as one whose lymphocyte count failed to be reduced by 40% following a single treatment. Thus, we believe our observation of enhanced DNA repair is consistent with acquired resistance, while the model of Begleiter *et al.* may correlate better with *de novo* resistance.

## 5. DNA Repair

The subject of DNA repair is complicated by the number of different repair systems present in the cell. DNA encodes "the blueprint of life" and, as a result, injury to the DNA can have profound effects upon the viability of a cell. More importantly, genetic alterations can cause cellular transformation and malignancy; this, in turn, can pose a serious threat to the life of the organism. In order to combat the effects of mutagens and carcinogens found not only exogenously, but endogenously as well, the cell has developed specialized repair systems which can respond to a variety of DNA damage. Interestingly, while these systems appear distinct with respect to natural substrates, there may be some overlap in the repair of nitrogen mustard and other crosslinking agent induced lesions. The subject of DNA repair and drug resistance is somewhat paradoxical in nature. Cancer is generally believed to result from an accumulation of mutations which can be modulated by the effectiveness of DNA repair. Resistance may then arise when the same cancer cell defies treatment by upregulating a system whose inefficiency possibly gave rise to the tumor in the first place.

The following section will deal with the topic of DNA excision repair. This pathway should not be confused with the mismatch repair pathway which has gathered much attention in the past few months. It has been shown that a heritable form of non-polyposis colon cancer is linked to two separate genes, both of which are key players in the mismatch repair system

(Fishel *et al.*, 1993; Leach *et al.*, 1993; Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994). As it is not clear whether common proteins are involved in mismatch and excision repair, this review will focus solely on excision repair. Excision repair has been classically defined in two forms, long patch and short patch (Regan and Setlow, 1974). The term "excision" stems from the observation that such repair is associated with release of the damaged bases from the DNA template. In short patch repair (resulting from methylating agent damage), only a few bases (1-3) are removed from the template (Dianov *et al.*, 1992), while in long patch repair (which responds to bulky lesions such as UV) up to 29 nucleotides have been shown to be removed (Huang *et al.*, 1992). Currently short-patch and long-patch repair are described as base and nucleotide excision repair (BER and NER, respectively), because short patch begins with release of the damaged base by a specific glycosylase, while long patch repair involves incision in the phophoribosyl backbone of DNA with resultant loss of oligomeric stretches of DNA (Duncan *et al.*, 1976). I will also present some information on a third repair pathway, crosslink repair, which has been very poorly characterized to date.

## 5.1 Base excision repair (BER)

Base excision repair begins with release of the damaged base from the sugar backbone, either by the enzymatic activity of a glycosylase or spontaneous depurination. A variety of glycosylases exist in the cell which can recognize altered bases such as 3-methyladenine, uracil, 8-hydroxyguanine, and thymine glycol (each is released by a separate glycosylase) (reviewed by Barnes *et al.*, 1993). The glycosylase initiates BER by cleaving the glycosidic bond between the base and the sugar backbone producing an abasic (apurinic/apyrimidinic site or AP) site. Spontaneous depurination, occurring at the level 2 -10 000 purines lost per cell per day, can also generate AP sites (Lindahl, 1993). These lesions are both non-instructional and cytotoxic, so they must be dealt with right away. While there is a certain level of spontaneous breakdown of these sites, this is an inefficient process as it results in the generation of 3' phosphate residues which must be removed prior to base replacement (Bailly

and Verly, 1988). There also exist specific proteins, known as AP endonucleases, which will cleave the phophoribosyl backbone adjacent to the AP site at the 5' end (Doetsch and Cunningham, 1990). Following this incision, another enzyme, DNA deoxyribophosphodiesterase, is thought to release the depurinated sugar residue by cleavage of the 3' end, leaving a 5' phosphate on the adjacent base (Price and Lindahl, 1991). The lost base is then replaced by a DNA polymerase (most likely polymerase β (Dianov *et al.*1992)) and the remaining nick is sealed by a DNA ligase. Figure 1.4 presents an example of methylation repair by the BER system in mammalian cells.

*Alkyl-N-purine-DNA glycosylase*

As mentioned above, nature has provided an array of glycosylase enzymes with which to initiate BER. I will focus on one specific glycosylase, the mammalian alkyl-N-purine DNA glycosylase (ANPG; or $N^3$-methyladenine DNA glycosylase). This enzyme is the specific glycosylase involved in the detection and repair of alkylated lesions in the genome. When DNA is methylated, by either exogenous (methylmethane sulfonate) or endogenous (S-adenosyl-L-methionine) means, the major products are $N^3$-methyladenine and $N^7$-methylguanine (Lindahl, 1979; Rydberg and Lindahl, 1982). Secondary lesions include $N^3$-methylguanine and $N^7$-methyladenine. $N^3$-methyladenine DNA glycosylase activity was first identified in *E. coli* and named *tag* (*t*hree methyl-*a*denine DNA *g*lycosylase) (Riazzudin and Lindahl, 1978; Lindahl, 1979). Interestingly, this enzyme showed absolute specificity for $N^3$-methyladenine lesions. No other damaged bases could be released from a methylated DNA substrate and the activity was inhibited by excess free $N^3$-methyladenine. A second glycosylase activity was identified in *tag*- mutants (Karran *et al.*, 1980). While this second glycosylase represents only 5-10 % of the activity in untreated *E. coli*, the activity increases 10 to 20-fold following exposure to methylating agents as part of a process known as "the adaptive response" (Karran *et al.*, 1982; Evensen and Seeberg, 1982; reviewed by Lindahl and Sedgwick, 1988). This second glycosylase, called *alkA*, displays a broad range of substrate

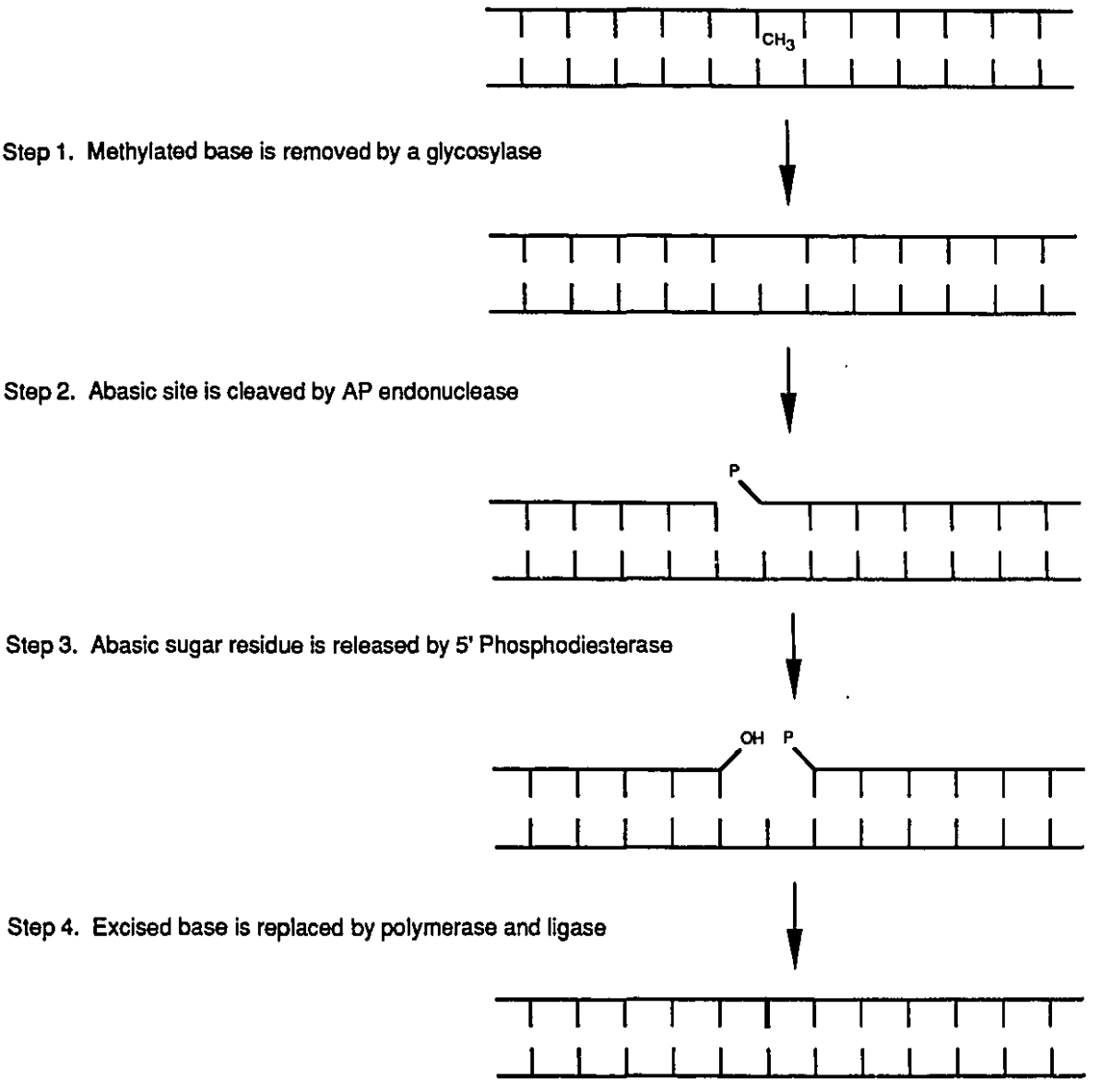



**Figure 1.4** Schematic diagram of the release of a methylated base by the base excision process.

specificity (including $N^3$-methyladenine, $N^3$-methylguanine, and $N^7$-methylguanine) and is not inhibited by free methylated bases.

Singer and Brent (1981) showed that human lymphoblasts contain glycosylase activity(ies), which could release $N^7$- and $N^3$-methyl and ethyl purines. Partially purified 3-methyladenine DNA glycosylase was also capable of releasing $N^7$-methylguanine and $N^3$-methylguanine (Gallagher and Brent, 1982). These 3 activities continued to copurify, even upon 6000-fold purification (Gallagher and Brent, 1984). As these glycosylase activities exhibited similar physical and biochemical properties, it appeared that a single glycosylase existed in human cells with a substrate spectrum similar to *E. coli* AlkA. Two groups successfully cloned a human cDNA encoding the 3-methyladenine DNA glycosylase (Chakravarti *et al.*, 1991, O'Connor and Laval, 1991). To achieve this goal, the teams exploited the highly conserved nature of the glycosylase and used a human cDNA expression library to functionally complement *tag*$^-$ *alkA*$^-$ *E. coli*. The bacteria were transformed with the expression library and selected in the presence of methylating agents. Surviving bacteria were grown up and the plasmid retrieved. Both groups isolated a single sequence and, upon further characterization, found that this cDNA produced a functional glycosylase with activity against $N^3$-methyladenine lesions, as well as $N^7$-methylguanine and $N^7$-methyladenine, supporting the hypothesis of a unique glycosylase. As a result of the multiplicity of activity exhibited by this protein it was renamed alkyl-N-purine DNA glycosylase, since its substrate specificity seemed to be N-alkyl purines (O'Connor and Laval, 1991). The mammalian glycosylases (human, mouse, and rat) exhibit very significant sequence homologies (>80%), yet they display no significant homology to the *S. cerevisiae* MAG gene or the *E. coli alkA* and *tag* genes, in spite of strong functional conservation (Engleward *et al.*, 1993).

ANPG can recognize ethyl lesions as well as methyl lesions (O'Connor and Laval, 1991). Nitrogen mustard lesions occur at the $N^7$-position of guanine and, as they are only 2 carbons long, might serve as substrate for this enzyme. It is quite clear that lesions produced

by CNUs can be substrates for this enzyme . Using both *in vivo* and *in vitro* systems, it has been shown that $N^7$ lesions produced by CNUs can be released by both the purified AlkA protein and the partially purified yeast and human ANPG (Habraken *et al.*, 1991; Matijasevic *et al.*, 1993a; Matijasevic *et al.*, 1993b). Expression of the yeast glycosylase in *tag⁻ alkA⁻ E. coli* can protect them from CNU toxicity (Matijasevic *et al.*, 1993b). Similarly, *E. coli tag-alkA-* mutants are hypersensitive to mechlorethamine and expression of the human ANPG in these cells can partially complement these bacteria (Appendix 2). Thus, this enzyme could have an important role in the repair of nitrogen mustard lesions in mammalian cells.

It is quite interesting that our knowledge of BER comes largely from prokaryotic studies which were then extrapolated to the eukaryotic arena. Many mammalian BER proteins are highly conserved and function quite well in *E. coli* and vice versa. Also interesting is the fact that no genetic diseases have been associated with mutant BER activity. No ANPG deficient mutant cell lines have been identified, although knock-out mice are currently in production in order to generate such a phenotype (Engelward *et al.*, 1993). This may indicate an essential role for the glycosylase in mammalian cells. Methylation can arise not only from exogenous sources but endogenous ones as well. S-adenosyl-L-methionine is a methylating molecule found ubiquitously in mammalian cells and it is estimated that 600 3-methyladenine lesions per cell are produced by this agent each day (Rydberg and Lindahl, 1982). These lesions cannot be tolerated as they are cytotoxic and block replication, indicating that a functional glycosylase may be required for everyday survival (Boiteux *et al.*, 1984). AP endonucleases also appear to play an important role in the cell. Besides its role in protecting the genome from the havoc of AP sites, it also appears to have a role in signal transduction. The C-terminal portion of the mammalian protein encodes an AP lyase activity, while the N-terminal portion contains a redox activity which seems to be required for regulating the DNA binding activity of many transcription factors including AP-1, CREB, and NF-kB (Xanthoudakis *et al.*, 1992; Walker *et al.*, 1993; Xanthoudakis *et al.*, 1994). This activity was

first purified and named ref-1. Upon cloning and sequencing, it was found that the ref-1 gene was identical to HAP-1 and APE-1, encoding the human AP endonuclease (Xanthoudakis *et al.*, 1992). Such a unique dual function may be happenstance, or it may reveal a more intimate role between repair proteins and the signaling cascade induced following DNA damage. It is well known that DNA damage elicits a response of DDI genes (DNA damage inducible), the AP endonuclease may represent a member of the pathway by which this occurs (Fornace, 1992).

## 5.2 Nucleotide excision repair (NER)

### 5.2.1. Mechanism

Nucleotide excision repair is perhaps the most widely known repair system. This repair pathway has been classically defined by virtue of its activity on UV induced DNA damage. Much insight into this pathway has been gained through studying *E. coli*, yeast, and cell lines from patients with genetic DNA repair defects which render them hypersensitive to UV light. Repair of UV lesions can be separated into two steps: (1) preincisional (damage recognition and incision) and (2) post-incisional (repair patch resynthesis) (Shivji *et al.*, 1992). Damage identification is believed to be mediated by specific repair proteins which locate the lesions and initiate the repair reaction. In *E. coli*, the step involves an active scanning mechanism in which the recognition protein translocates along the DNA along with a helicase until a damaged site is detected (Grossman and Thiagalingam, 1993). A similar mechanism is believed to occur in mammalian cells, where both damage recognition and helicase proteins have been identified (Hoeijmakers, 1993). As discussed previously, preferential repair of transcribed DNA indicates that the RNA polymerase complex may play a role in the active scanning process (Hanawalt, 1991). Once a damaged base has been identified, an ATP-dependent endonuclease cleaves the DNA 5' and 3' of the site, releasing an

oligonucleotide of 27-29 bases (Huang *et al.*, 1992; Svoboda *et al.*, 1993). The excised bases are then replaced by the sequential action of a DNA polymerase and DNA ligase.

### 5.2.2. Molecular genetics

Identification of key genes in the nucleotide excision repair system has been facilitated by the study of human repair-deficiency syndromes (reviewed by Hoeijmakers, 1993). Xeroderma Pigmentosum (XP) is a genetic disease which manifests itself as an extreme hypersensitivity to UV light and greatly increased incidence of skin cancer. XP can be subdivided into 7 separate complementation groups which display varying degrees of UV hypersensitivity. Another disease, Cockayne's syndrome (groups A and B), presents a milder form of repair-deficiency which appears to result from a defect in preferential strand-selective repair (Hoeijmakers, 1993; van Hoffen *et al.*, 1993). Attempts were made to clone the genes underlying these deficiencies through phenotype complementation of immortalized XP fibroblasts by DNA mediated gene transfection, using DNA from wild-type cells. Two genes were successfully cloned via this approach, XPA and XPC (Xeroderma pigmentosum group A and C complementing genes) (Tanaka *et al.*, 1989; Legerski and Peterson, 1992). Unfortunately, due to the low transfection efficiency of human cell lines, this route was at a technical disadvantage. Rodent cell lines demonstrated higher efficiency of transfection and UV hypersensitive mutant lines were developed in an effort to forge a system which would mimic the situation in XP. A total of 11 different UV sensitive CHO complementation groups were developed, 6 of these lines (ERCC-1,-2,-3,-4,-5,-11) displayed XP-like defects. In 1984, the first human nucleotide excision repair gene was cloned from the CHO cell line 43-3B (complementation group 1) (Westerveld *et al.*, 1984). Since that time, 4 more rodent lines have been complemented, yielding human excision repair genes designated ERCC (Excision Repair Cross-Complementing) -1,-2,-3,-5,-6 for the respective CHO complementation groups. ERCC-2, -3, and -5 have been determined to be the complementary genes for XP-D, XP-B, and XP-G, respectively (reviewed by Tanaka and Wood, 1994). A DNA binding

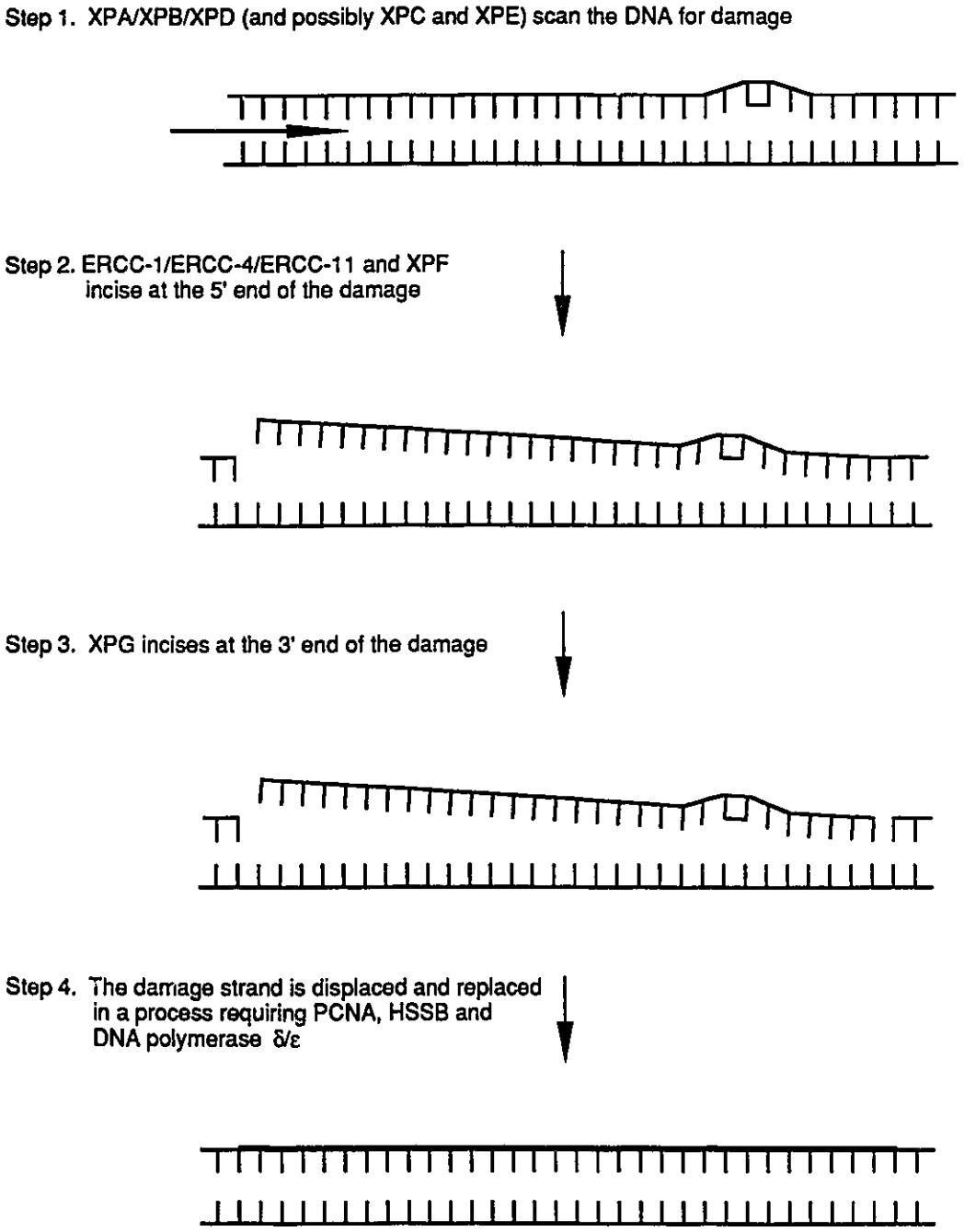



**Figure 1.5** Schematic diagram of the release of a thymine dimer by the nucleotide excision process.

factor shown to be complementary to XP group E (XPE-BF) cells has been purified and characterized, but the gene has not yet been cloned (Hwang and Chu, 1993; Keeney *et al.*, 1994). ERCC-6 failed to complement any of the known XP groups, but it was found to be complementary to Cockayne's syndrome type B cells. Interestingly ERCC-1 does not seem to underlie the defects in any of the disease groups (van Duin *et al.*, 1989). Deficiency in ERCC-1 results in the most extreme phenotype. ERCC-1 mutant lines display UV hypersensitivity equivalent to or greater than any of the other cell lines, but only rodent complementation groups 1 (ERCC-1) and 4 (ERCC-4) present significant hypersensitivity to bifunctional alkylating agents. In fact, while these 2 groups display 10 fold hypersensitivity to UV light, they demonstrate 40 - 100 fold hypersensitivity to agents such as nitrogen mustards, cisplatin, and mitomycin C (Hoy *et al.*, 1985; Hoeijmakers, 1994). So, it appears that these two NER proteins may be key players in the repair of nitrogen mustard damage in the DNA.

### 5.2.3. Biochemistry

Strong sequence homologies were found between human genes XPA, XPB, XPC, XPD, XPG, ERCC-1 and yeast proteins RAD14, RAD3, RAD4, RAD25, RAD2, RAD10, respectively (reviewed by Bardwell *et al.*, 1994). It appears that the activities of these proteins are very strongly conserved between yeast and mammals. RAD10 can partially complement the hypersensitivity of CHO complementation group 1 cells, which lack a functional ERCC-1 gene (Lambert *et al.*, 1988). RAD25, the yeast homologue of ERCC-3, is required for viability, as well as DNA repair (Park *et al.*, 1992). Recently, a RAD25 yeast mutant was engineered to contain a mutation similar to XP group B. These mutant yeast were viable and displayed a repair deficiency comparable to XPB, confirming the functional conservation of this protein (Sweder and Hanawalt, 1994). Sequence and biochemical analysis of the human and yeast genes has provided valuable information regarding protein function. ERCC-1 possesses a helix-turn-helix motif suggesting DNA binding capability (van Duin *et al.*, 1986). Its yeast homologue, Rad10, binds tightly to Rad1 and forms a single stranded endonuclease

(Sung *et al.*, 1993a; Tomkinson *et al.*, 1993). XPA is a zinc-finger DNA binding protein (Tanaka *et al.*, 1990; Jones and Wood, 1993). XPB and XPD contain sequences suggesting helicase activity (Weeda *et al.*, 1990; Weber *et al.*, 1990) and such activity has recently been confirmed biochemically (Schaeffer *et al.*, 1993; Sung *et al.*, 1993b ). XPC possesses no known functional domains and the activity of this protein remains unknown. XPG has been purified and shown to be a single-stranded endonuclease (O'Donovan *et al.*, 1994). The predicated protein sequence for ERCC-6 suggests that it is a helicase. As this gene restores preferential strand repair in CS-B cells, ERCC-6 may represent the coupling factor between transcription and NER (Troelstra *et al.*, 1992; Sweder and Hanawalt, 1993).

The deficiencies in the cell lines used for isolation of the above genes all demonstrate defects in the incisional step of nucleotide excision (Hoeijmakers, 1993; Masutani *et al.*, 1993). Recently, insights have been gained into the proteins required for the second step of repair (post-incisional). These proteins include PCNA and HSSB, two proteins required in the replication complex for polymerase δ/ε activity and appropriate synthesis activity, indicating that repair synthesis may be dependent upon polymerase δ or ε (Coverley *et al.*, 1991; Shivji *et al.*, 1992).

The link between transcription and excision repair has been strengthened by the discovery that ERCC-3 was one of the peptides contained in the transcription factor TFIIH complex (Schaeffer *et al.*, 1993). It has since been demonstrated that XPD/ERCC-2 and XPC also associate with TFIIH (Drapkin *et al.*, 1994; Schaeffer *et al.*, 1994). Two other subunits of TFIIH, p44 and p34, display significant homology to the yeast SSL1 protein, which appears to be involved in translation and DNA repair. It has been postulated that stalling of the RNA polymerase complex at damaged sites might serve as a signal for the repair machinery. This theory is supported by the evidence that the helicase and ATPase activities of the yeast XPD homologue, RAD3, are inhibited by UV lesions (Naegeli *et al.*, 1993). In combination with the recent observation that this protein can bind UV damaged DNA, the TFIIH complex

is strongly implicated in the damage detection/recognition step of repair (Sung *et al.*, 1994; Burtowski, 1993a, 1993b)).

Another protein complex in NER has been recently identified with the finding that ERCC-1 associates with ERCC-4, ERCC-11, and XPF (van Vuuren *et al.*, 1993; Biggerstaff *et al.*, 1993). It is not clear, at the moment, whether ERCC-11 and XP-F are the same protein, as their corresponding mutant lines both display similar mild UV sensitivity, but the genes have not been cloned and appropriate complementation analysis was not performed. It was found that immunoprecipitation of ERCC-1 could remove complementing activities for extracts from CHO complementation group 1, 4 and 11 cells, and XP group F cells. The complex of ERCC-1 and ERCC-4 was expected as their respective yeast homologues, RAD 10 and RAD 1, also combine in order to activate an intrinsic single stranded endonuclease activity. Thus, repair is believed to require at least 4 events at the molecular level. Step 1: XPA/XPB/XPD (possibly XPC and XPE) scan the DNA for damaged sites (Hoeijmakers, 1993). Step 2: Upon detection of damage, the ERCC-1/ERCC-4/ERCC-11/XPF complex incises the DNA at the 22nd phosphodiester bond 5' to the damage, producing a 5' "flap" (Huang *et al.*, 1992; Hoeijmakers, 1993; Harrington and Lieber, 1994). Step 3: XPG recognizes this "flap" and cleaves the DNA at the 6th phosphodiester bond 3' to the damage (Huang *et al.*, 1992; Harrington and Lieber, 1994; O'Donovan, 1994). Step 4: HSSB, PCNA, and DNA polymerase δ/ε displace the damage-containing oligo and resynthesize the DNA (Shivji *et al.*, 1992; Hoeijmakers, 1993). A current scheme for the NER process is provided in Figure 1.4.

### *ERCC-1*

This gene is of fundamental importance to the study of DNA excision repair. ERCC-1 was the first human NER gene cloned and is the smallest of all ERCC proteins. It was isolated through complementation of the UV and mitomycin C hypersensitivity of 43-3B cells (Westerveld *et al.*, 1984). The complementation groups established in CHO cells were

designed to mimic the complementation groups found in human XP cells. However, when ERCC-1 was tested in XP cells, it was not found to be complementary to any of the XP defects (van Duin *et al.*, 1989). Interestingly, ERCC-1 deficiency results in extreme hypersensitivity to UV and crosslinking agents, whereas most XP cells display UV hypersensitivity with minimal cross-sensitivity to crosslinking agents (Hoy *et al.*, 1985; Hoeijmakers, 1993). Sequence analysis of the gene revealed strong homology between the N-terminal of ERCC-1 and the yeast RAD10 gene (van Duin *et al.*, 1986). This homologous section spanned a putative DNA binding domain (helix-turn-helix), indicating an important functional role of this region. Transfection of RAD10 could partially complement the hypersensitivity of CHO complementation group 1 cells, strengthening the evolutionary conservation of this protein function (Lambert *et al.*, 1988). ERCC-1 was also found to contain certain domains found in the *E. coli* repair genes *uvr A* and *uvr C* (van Duin *et al.*, 1988). The *uvrA* homologous region overlaps with the end of the RAD10 homologous region. Disruption of this portion of the gene by linker insertion inactivates the protein (van Duin *et al.*, 1988). As ERCC-1 has not been linked to any genetic deficiency, a knock-out mouse was prepared to examine the effect of gene inactivation (McWhir *et al.*, 1993) . The homozygotes were non-viable, displaying primarily liver abnormalities. These animals died within 4 weeks of birth and were severely runted. The link between the DNA repair gene deficiency and these phenotypic effects is not clear, although patients with CS do exhibit some growth retardation (Hoeijmakers, 1993). Based on these studies it appears that this gene is essential for survival, explaining why ERCC-1 is not associated with any of the known genetic defects.

This gene is required for repair of bifunctional alkylating agent damage (Westerveld *et al.*, 1984; Hoy *et al.*, 1985). We have demonstrated a 2 fold increase in ERCC-1 expression in lymphocytes from patients with nitrogen mustard resistant B-CLL as compared to lymphocytes from untreated patients (Geleziunas *et al.*, 1991). Following our observation, another group studying cisplatin therapy of ovarian cancers found increased expression of

ERCC-1 in non-responsive tumors (Dabholkar *et al.*, 1992). Thus, further studies of this gene's role in bifunctional alkylating agent resistance is required in order to understand how ERCC-1 overexpression affects DNA repair.

## 5.3. Crosslink repair

### 5.3.1. Mechanism

This poorly defined pathway is known to exist but is rather intangible in terms of a molecular mechanism in mammalian cells. In early studies, it was noted that interstrand crosslink repair required two steps (Kohn *et al.*, 1965; Reid and Walker, 1971). In the first step, the crosslink is "unhooked", presumably by incision at one of the crosslinked sites. Release of the diguanyl-mustard derivative is only detected at later time, suggesting that the cell must complete repair of the first strand before beginning to repair the adjacent strand. A mechanistic model for crosslink repair was proposed by Cole (1973) and modified by van Houten (1990). In this model, incision occurs on one of the crosslinked strands producing a gap, which can then be widened by an exonuclease. This is followed by homologous pairing and recombination. Following the recombination step, the second alkylated site can be incised and the crosslinked DNA is removed. Figure 1.6 is a schematic representation of recombination repair of a crosslinked lesion.

### 5.3.2. Genetics and biochemistry

Most of what we know of the pathway in mammals comes from studies of the genetic disease Fanconi's anemia (FA). This disease is an autosomal recessive disorder which is characterized by "progressive pancytopaenia, a diverse assortment of congenital malformations, and a predisposition to the development of malignancies" (Strathdee and Buchwald, 1992). It was observed that cells from patients with FA displayed an increased

Step 1. Incisions are made on one of the damaged strands flanking the lesion.

Step 2. The incised strand is displaced and strand exchange is initiated.

Step 3. A Holliday junction is formed.

Step 4. Resolution of the Holliday junction transfers the gap from the crosslinked chromosome to the sister chromosome. Incisions are made flanking the remaining alkylated site.

Step 5. Repair synthesis restores all gapped regions.

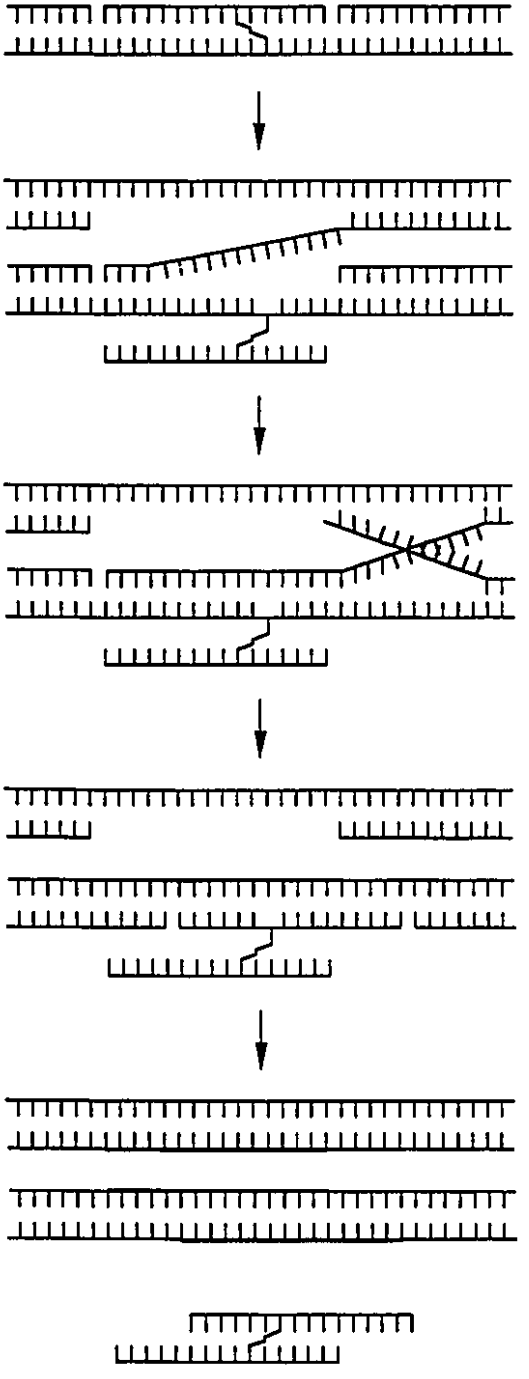


**Figure 1.6** Schematic representation of recombinational repair of interstrand crosslinks.

level of spontaneous chromosomal aberrations, suggesting a DNA repair defect. FA cells exhibit hypersensitivity to crosslinking agents like nitrogen mustards and mitomycin C, but appear normal with respect to UV, monofunctional alkylating agents, and X-rays ( Fujiwara *et al.*, 1977; Ishida and Buchwald, 1982; Fornace *et al.*, 1979). Thus, it appears that in these cells the BER and NER systems are intact. The defect seems to arise from some other aspect of bifunctional alkylating agent damage repair. FA cells appear to have a reduced capacity to perform incisions at the crosslink, resulting in longer duration of the interstrand crosslink and higher toxicity (Fujiwara *et al.*, 1977). Some FA cell lines were better able to repair crosslinks than others and the hypersensitivity was correlated to the repair capacity (Fujiwara *et al.*, 1977; Matsumoto *et al.*, 1989). Interestingly, Fornace (1979) reported a FA cell line which displayed low-levels of hypersensitivity to mitomycin C (2.5 fold), yet was normal with respect to incisional capability. FA has recently been separated into 4 complementation groups, indicating 4 unique genetic defects (Strathdee and Buchwald, 1992). As with XP, the different defects probably have varying effects on the cell. The previous studies were performed prior to the assignment of complementation groups and, therefore, it is not known which results belong to which groups. Only the gene complimentary to FA group C (FACC) has been isolated and the predicted amino acid sequence of this gene revealed no known motifs nor homology to any proteins (Strathdee *et al.*, 1992).

In *E. coli*, crosslink repair begins with incision by the same enzyme complex required for UV repair [the uvr(A)BC excinuclease], it is not yet clear whether the same holds true for mammals (Van Houten *et al.*, 1986). Since at least two genes, ERCC-1 and ERCC-4, are required for both UV and crosslink repair, it is quite probable that certain components of NER are required for both repair pathways. Similarly, the yeast homologues, RAD10 and RAD1, are required for both the incision step of UV damage repair and mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990). Following incision, the next step of crosslink repair is dependent upon Rec A in *E. coli* (Cole, 1973; Cheng *et al.*, 1991). This protein promotes the strand exchange and homologous pairing required to initiate

recombination and generation of the Holliday junction (reviewed by Cox, 1994). A number of eukaryotic RecA homologues have been isolated, including a human gene, and await further characterization (Shinohara *et al.*, 1993) The yeast Rec A homologue, RAD51, is required for mitotic recombination and repair and interacts directly with the RAD52 protein (the RAD52 pathway being the major recombination system in S. Cerevisiae) (Shinohara *et al.*, 1992; Milne and Weaver, 1993). RAD10 has also been shown to promote renaturation of homologous strands suggesting a potential involvement in the strand exchange reaction and further implicating a central role for ERCC-1 in crosslink repair (Sung *et al.*, 1992).

The next step of recombination requires resolution of the Holliday junction. In *E. coli*, this accomplished by at least 2 independent systems: (i) *ruv ABC* and (ii) *recG* (reviewed by West, 1994). The Ruv A and B proteins are required for translocation of the Holliday junction. Ruv C is responsible for junction resolution. RecG can substitute for Ruv A and B in the branch migration reaction. It is not clear whether RecG can also resolve Holliday junctions or whether an additional protein is involved. Resolvase activities have been identified in mammalian cells. Couture and Chow (1992) isolated a mammalian endo-exonuclease activity which could bind to and cleave a synthetic Holliday junction substrate. More recently, a mammalian resolvase activity has been purified which can substitute for RuvC *in vitro* (Hyde *et al.*, 1994).

It should be noted that recombinational repair is not limited to crosslink damage. Data from *E. coli* and yeast indicate that a recombinational process is required for post-replicative repair of UV lesions (Davies *et al.*, 1975; Sancar and Sancar, 1988). The recombination event is believed to occur concomitant with DNA replication. When the polymerase encounters the nucleotide adduct, it stops replicating and reinitiates replication at some region downstream, producing a gap in the daughter strand. This gap can then be filled through a homologous strand exchange mechanism which is *recA* dependent in *E. coli* (Sancar and Sancar, 1988). The lesion which remains on the parental strand can then be removed by the NER machinery. From this data, it seems that there exists a link between NER and recombinational repair, such

that the removal of interstrand crosslinks may simply be an alternative pathway within the NER repair system.

## 6. Damage Recognition Proteins (DRPs)

The rate limiting step in DNA repair may be the detection of damage and signaling of the repair machinery to the lesion. In the case of NER, it is quite conceivable that at least part of the damage recognition involves the transcription complex. As the repair genes seem to be intimately associated with the active transcription complex, damage repair may be signaled by perturbations in transcription (i.e. stalling of RNA polymerase at the site of damage). The transcription initiation factor TFIIH possesses 3 subunits which are required for efficient NER and it is possible that this initiation factor plays a key role in transcription-coupled DNA repair (Schaeffer *et al.*, 1994; Humbert *et al.*, 1994; Drapkin *et al.*, 1994; Buratowski, 1993a, 1993b). One drawback to this theory is the fact that the factors associated with TFII H are required for initiation of transcription, but not elongation. Thus, it is not yet clear how these initiation factors can be involved in active "scanning" during elongation, but it is possible that they assume another role during this process, effectively separating their functions as transcriptional initiators and excision repair proteins (Sweder and Hanawalt, 1993).

Of course, repair occurs in non-transcribed regions as well (Hanawalt, 1991), so transcription-coupled damage recognition is not the only way lesions on the DNA are repaired. Several of the cloned DNA repair proteins exhibit DNA binding activity and are likely involved in repair initiation. The RAD 1 and RAD 10 proteins (and presumably ERCC-4 and -1) can bind to single stranded DNA, but show no preference for damaged sites (Tomkinson *et al.*, 1993; Sung *et al.*, 1993). XPA and its yeast counterpart, RAD 14, demonstrate DNA binding activities with preference to sites of UV damage (Jones and Wood, 1993; Guzder *et al.*, 1993). Interestingly, the RAD3 helicase (and probably XPD/ERCC-2) also demonstrates UV-specific binding (Sung *et al.*, 1994). It seems likely from the data, that damage recognition

proteins such as XPA/RAD14 and RAD3 serve to direct other DNA binding proteins like the RAD1/RAD10 complex to the sites of the DNA damage.

More direct analysis of damage specific binding has uncovered a variety of proteins which appear to be involved in damage recognition. Most of the work in this field has been done using DNA probes which have been damaged with UV light and cisplatin. Early studies employing electrophoretic mobility shift assays (EMSA) identified a binding activity which could bind specifically to a UV treated probe (Chu and Chang, 1988). This binding activity could be competed from the UV damaged probe by using cisplatin treated DNA, indicating that the binding proteins could recognize cisplatin lesions as well. Investigations using extracts from patients with XP revealed that this binding activity was lacking only in patients from XP group E, thus the protein has been named XPE. This group also identified a DRP (damage recognition protein) which specifically recognized a cisplatin damaged probe and could not be competed by UV damaged DNA. Interestingly, in cell lines with acquired resistance to cisplatin, it appeared that resistance correlated with increased expression of the XPE binding factor but not the cisplatin DRP. (Chu and Chang, 1990). The XPA binding protein can also bind to cisplatin damaged DNA, but its molecular weight clearly distinguishes it from XPE (Jones and Wood, 1993). A Hela cell line selected for cisplatin resistance displayed cross-resistance to UV and increased expression of the UV DRPs (as determined by EMSA) (Chao *et al.* 1991b). These studies also described cisplatin DRPs, separate from the UV binding activities, and in the cisplatin resistant line both species of DRP were overexpressed (Chao *et al.*, 1991a,b).

Using a different approach, cDNAs encoding cisplatin binding proteins were isolated from an expression library using a cisplatin modified DNA probe (Toney *et al.*, 1989). Multiple cDNAs were isolated which produced proteins which bound to cisplatin damaged DNA. One protein (SSRP1) was found to contain a sequence motif with homology to HMG1 known as the "HMG box" (Pil and Lippard, 1992). EMSA analysis using purified HMG 1 protein found that this protein could bind to a specific cisplatin lesion, the intrastrand GG or

AG crosslink. The purified protein did not bind to the interstrand crosslink nor to the intrastrand GTG crosslink. Both HMG 1 and HMG 2 could bind to the cisplatin probe (as determined by Southwestern blot analysis). In addition to SSRP1, hUBF has recently been identified as a cisplatin binding protein (Treiber *et al.*, 1994). Both SSRP1 and hUBF contain "HMG box" domains. The GG/AG intrastrand crosslink produces a very distinct distortion in the DNA and it is this structural alteration which is believed to cause HMG binding (Bellon *et al.*, 1991; Donahue *et al.*, 1990; Grosschedl *et al.*, 1994). Thus, the binding of "HMG box" proteins to cisplatin damage in DNA may not be specific for DNA repair. In fact, the authors postulate that these artificial HMG binding sites created by cisplatin damage may actually sequester these proteins, which are otherwise associated with transcriptionally active regions of the genome. This "sequestration" would then lead to reduced transcription from these regions and add to the toxicity of cisplatin. (Pil and Lippard, 1992; Treiber *et al.*, 1994)

The various binding factors detected by EMSA appear to be different as determined by their substrate specificities. It was found that most of the DRPs detected using either UV or cisplatin probes also bound to single stranded DNA. This observation was believed to result from the unwinding of the DNA helix following the damage (Bellon *et al.*, 1991). Human single stranded binding protein (HSSB), which is required for both replication and DNA repair (Coverley *et al.*, 1991), can bind to cisplatin damaged probes and is present in one of the two binding complexes detected by EMSA using cisplatin damaged probes (Clugston *et al.*, 1992). There was no evidence of altered HSSB expression, as determined by western blot, between cisplatin sensitive and resistant cell lines.

The relationship between DRP binding activities and cisplatin drug resistance is not clear. In one case a UV DRP (which can bind to cisplatin damage) is found to be overexpressed in cisplatin resistant lines while the cisplatin DRP (which can not bind to UV damage) is not (Chu and Chang, 1990). Another group has found that both binding activities (cisplatin and UV DRPs) are overexpressed in cisplatin resistant Hela cells (by EMSA and Southwestern analyses) (Chao 1991a, 1991b, 1992). As mentioned above, HSSB does not

exhibit altered expression in cisplatin resistant cell lines. Cisplatin DRPs were identified in tumor specimens with no apparent difference between levels of binding activity in ovary, cervical and testicular tissues, in spite of the high incidence of cisplatin resistance in cervical carcinoma. This group also studied a panel of 12 ovarian carcinoma cell lines by Southwestern analysis but failed to demonstrate a correlation between binding and cisplatin resistance (Bissett *et al.*, 1993). No work has been reported with respect to nitrogen mustard DRPs [with the exception of a recent suggestion of XPE binding (Chu, 1994)], although it is very likely that they do exist.

*Poly (ADP-ribose) Polymerase*

Although it is one of the earliest identified DRPs, the role of poly(ADP-ribose) polymerase (PARP) in DNA repair remains enigmatic. This protein is activated by DNA damage and synthesizes poly(ADP-ribose), a reaction which appears to be required for DNA repair. PARP binds to single- and double-strand breaks via two zinc fingers present in the N-terminus of the protein (Menissier-deMurcia *et al.*, 1989). The first zinc finger is absolutely required for PARP activation, while deletion of the second zinc finger allows activation by double-strand breaks but eliminates activation by single-strand breaks (Ikejima *et al.*, 1990). Interestingly, it is the second zinc finger which is required for DNA binding and specific recognition of the strand breaks (Gradwohl *et al.*, 1990). Once bound to the DNA, PARP generates poly(ADP-ribose), using the cellular co-factor NAD (Cleaver, 1991). The NAD binding and polymerization domains are located in the C-terminal portion of the protein (Simonin *et al.* 1993). These (ADP-ribose) polymers are often found on chromatin associated proteins (histones) and on certain DNA repair enzymes (topoisomerase I), however the major ribosylated protein in this process is the enzyme itself (Cleaver 1991). The functional consequence of protein ribosylation is loss of DNA binding activity, presumably as a consequence of electrostatic repulsion [the (ADP-ribose) polymer is highly negatively charged and causes the ribosylated proteins to dissociate from the negatively charged DNA]. Auto-

modification of PARP causes it to dissociate from the DNA and consequently cease poly(ADP-ribose) synthesis. It has recently been reported that PARP actually binds DNA as a dimer and the auto-modification occurs inter-molecularly (Mendoza-Alvarez and Alvarez-Gonzalez, 1993).

How the auto-modification of PARP and the generation of poly(ADP-ribose) affects excision repair is not clear. Auto-modification may simply serve as a feedback regulatory process which turns off the polymerase activity. Initially, it was found that reduced PARP activity using 3-aminobenzamide, a potent inhibitor, caused an increase in the accumulation of single stranded breaks following treatment of cells with alkylating agents (Durkacz, 1980), indicating that poly(ADP-ribosyl)ation may be required for the ligation step. Treatment of L1210 cells with a monofunctional alkylating agent caused enhanced DNA ligase II activity without enhanced protein production (Creissan and Shall 1982), suggesting activation by post-translational modification [probably (ADP-ribosyl)ation]. Yoshihara *et al.* (1985) failed to corroborate this finding using assays which directly assessed the effect of poly(ADP-ribosyl)ation *in vitro*. In fact, it appeared that PARP inhibition blocked repair prior to the ligation step and the accumulation of single strand breaks was not a result of direct ligase inactivation but simply a consequence of alkylating agent damage (Ireland and Stewart 1987). In addition, some reports demonstrated enhanced ligation rates and DNA repair synthesis in the presence of PARP inhibitors (Miwa *et al.*, 1981; Althaus 1982a; Sims *et al.*, 1982). These contradictory findings are not easily explained, but may result from the different cells and culture conditions used by the various groups.

One model for the involvement of auto-modification in excision repair describes PARP as part of a histone shuttle mechanism (Althaus, 1992). Upon binding to a strand break present in the chromatin, the auto-modification of PARP would draw histone proteins from the DNA onto the (ADP-ribose) polymers. (ADP-ribose) residues can then be removed from PARP by the action of poly(ADP-ribose) glycohydrolase and the histones would be free to reassociate with the DNA and regenerate nucleosomes. It is clear that the presence of histones

on the chromatin template is inhibitory to the excision repair process, as well as other DNA related processes (like transcription) (Wang 1991; Laybourn and Kadonaga, 1991). Poly(ADP-ribose) synthesis has been shown to decondense chromatin *in vitro* (deMurcia *et al.*, 1986). In fact, poly(ADP-ribosyl)ation within nucleosomal core particles could cause dissociation of DNA from the nucleosomal core *in vitro* (Mathis and Althaus, 1987). Primary hepatocyte cultures could be stripped of chromatin-associated poly(ADP-ribosyl)ation and survive for up to 9 days in culture and still exhibit liver specific functions (Althaus *et al.*, 1982a,1982b). When such cells are treated with the carcinogen N-acetoxy-2-acetylaminofluorene, nucleosomal unfolding, which is normally associated with excision repair, appears to be completely inhibited (Mathis and Althaus, 1990). As a consequence, the release of the modified base (guanine) in the treated cells was much slower than in the control cells. The histone "shuttle" has been reconstituted *in vitro* using purified PARP, poly(ADP-ribose) glycohydrolase, histones and DNA (Realini and Althaus, 1992). PARP automodification was sufficient to cause dissociation of histone-DNA complexes. Subsequent degradation of the (ADP-ribose) polymers by the glycohydrolase released the histones and the histone-DNA complex was reformed. In support of the "histone shuttle" theory, it has recently been shown in a poly(ADP-ribose) binding assay, that histones are the major binding proteins in nuclear lysates (Panzeter *et al.* 1993). Moreover, the binding sites between histones and poly(ADP-ribose) seems to be localized to the histone "tails", which are believed to be responsible for nucleosomal folding.

A second theory suggests that PARP may act as a temporary inhibitor of repair. When a single nick is introduced into plasmid DNA, this nick could be sealed using cellular extracts from wild type cells (Satoh and Lindahl 1992). This activity could be enhanced by the addition of NAD to the extracts. In the presence of 3-aminobenzamide this repair activity is prevented implicating PARP in this process. Interestingly, when extracts were depleted of PARP, the repair process was no longer affected by the addition of NAD. Moreover, 3-aminobenzamide could no longer inhibit the repair reaction. Similarly, if mutant PARP

proteins are used in the *in vitro* assay, deletion of the DNA binding domain failed to cause repair inhibition (Smulson *et al.*, 1994). On the other hand, deletions within the NAD binding or auto-modification domains resulted in varying degrees of repair inhibition which could not be relieved by NAD. This led to the suggestion that PARP may serve a protective role in repair. Following binding to single stranded nicks activation of PARP results in the generation of long stretches of poly(ADP-ribose) (>30 residues), creating a region of negative charge in the vicinity of the break. These long polymers exist for only a short period of time and can be detected both *in vitro* and *in vivo* (Satoh *et al.*, 1994; Malanga and Althaus, 1994). Electrostatic repulsion would prevent DNA molecules from entering this region, effectively preventing spurious recombination. Thus, the transient appearance of long (ADP-ribose) polymers may protect the damaged site and allow time for entry of the repair machinery. Support for this theory comes from recent evidence that short (ADP-ribose) polymers are sufficient for efficient excision repair *in vitro* (Satoh *et al.*, 1994). The binding of PARP to breaks in the DNA is also inhibitory upon elongation of 3'-OH ends. (Smulson *et al.*, 1994). In the context of a cycling cell, the transient binding of PARP to nicks could inhibit progression of the replication fork in the region of the DNA damage, allowing time for DNA repair (deMurcia and deMurcia, 1994).

The repair processes which require PARP activity are also controversial. PARP deficient cell lines have been developed which possess less than 11% of the wild type levels (Chatterjee *et al.* 1989, Yoshihara *et al.* 1992). PARP deficient V79 cells display hypersensitivity to a wide range of DNA damaging agents including mono- and bifunctional alkylating agents and UV light (Chatterjee *et al* 1990, 1991). Surprisingly, the cells were only minimally hypersensitive to X-rays. Reduction of PARP activity using anti-sense vectors produced increased sensitivity to methyl methanesulfonate which resulted from impairment of the early step (within 30 minutes) of repair (Ding *et al.*, 1992; Ding and Smulson, 1994). Reports of PARP involvement in UV damage repair are conflicting (Cleaver *et al.* 1983; Hunting and Gowans, 1988). Most recently, PARP inhibition *in vivo* has been achieved

using a trans-dominant model in which the DNA binding domain alone was overexpressed by transfection or micro-injection (Küpper *et al.*, 1990; Molinete *et al.*, 1993). In this model, PARP inhibition blocks BER following treatment with monofunctional agents, but UV damage repair was unaffected (Molinete *et al.*, 1993). Similar results were obtained employing the cell free repair extract system as described in the previous paragraph. Repair of plasmid DNA damaged by γ-irradiation, bleomycin, and monofunctional alkylating agents was NAD dependent (Satoh *et al.* 1993). Removal of PARP released the repair machinery from this NAD dependence. It was noted that the bulk of UV damage repair was NAD independent in this system, but the small component of NAD promoted repair following UV damage was a result of pyrmidine hydrates (minor lesions which are released by BER). So it appears that with respect to excision repair, the involvement of PARP may be restricted to BER .

As regards drug resistance, one report describes a cisplatin resistant rat ovarian tumor cell line which displays increased PARP activity (Chen and Zeller, 1992). Administration of 3-aminobenzamide could increase toxicity of cisplatin in the resistant line but not the parental sensitive line. This enhanced toxicity appeared to be a result of inhibition of interstrand crosslink repair. How this observation fits in with the various models discussed above is not clear. What is clear from this data, is the complex role of PARP, not only in excision repair, but in the general regulation of chromatin and genomic integrity.

## 7. Specific Aims

It was the aim of my thesis project to expand upon the observations already made with respect to nitrogen mustard resistance in B-CLL. In order to do this, I endeavored to identify central components of the system in the following ways:

1. *DNA mediated gene transfection*

   Using high molecular weight DNA obtained from patients with nitrogen mustard resistant B-CLL, NIH 3T3 and CHO AA8 cells were transfected in an attempt to confer nitrogen mustard resistance. If the DNA transfection was successful in transferring resistance, the genes responsible for this phenotype could then be cloned.

2. *Transfection of ERCC-1 and ANPG expression vectors*

   CHO cells were transfected with expression vectors encoding ERCC-1 or ANPG in order to determine what effect gene overexpression had on the sensitivity of these cells to bifunctional alkylating agents.

3. *Identification of damage recognition proteins*

   Using a DNA probe damaged with melphalan, binding proteins were identified using Southwestern blot analysis of nuclear extracts from B-CLL lymphocytes.

The high molecular weight DNA mediated gene transfection was largely unsuccessful, the results are included in appendix 1. The other specific aims were successfully carried out and are reported in chapters 3,4, and 6. In chapter 5, I have used an *in vitro* cytotoxicity assay as a means of identifying the DNA repair mechanism most likely associated with resistance.

# Chapter 2:

# Materials and Methods

## Preface

This chapter describes the materials and methods used throughout this thesis. Northern blot analysis, tissue culture and plasmid purification was already performed in the lab prior to the initiation of this project. The candidate was responsible for developing subcloning techniques (for inserting cDNAs into expression vectors), transfection experiments, western blotting techniques, and NAD analysis.

## Bacterial transformation

Preparation of competent bacteria

Bacteria are grown in 50 mls of LB broth until they reach an $OD_{650}$=0.6. The culture is cooled on ice for 30 minutes and the bacteria are collected by centrifugation at 3000 rpm, 4°C. The cell pellet is then resuspended in 25 mls of 50mM $CaCl_2$ and incubated for an hour on ice. Bacteria are collected by centrifugation as before and resuspended in 5 mls of 50mM $CaCl_2$, 20% glycerol. After the suspension is aliquoted, the bacteria are stored at -70°C until needed.

Bacterial transformation

An aliquot of 20 - 100 ng of DNA is added to 50 µl of TNE [10mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA], followed by 130 µl of competent bacteria and 20 µl of 10X TCM [100mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM $CaCl_2$]. The transformation reaction is incubated on ice for 30 minutes followed by a 90 second "heat-shock" at 42°C, followed by additional incubation on ice for 2-5 minutes. The bacterial preparation is diluted with 1 ml of LB broth and incubated at 37°C on a shaker. An aliquot of 50 - 250 µl of the final mix is spread onto an LB/agar plate supplemented with the appropriate antibiotic.

## **B-CLL lymphocyte cell culture** (Chapters 5 and 6)

Patient criteria

Each patient had a diagnosis of B-cell chronic lymphocytic leukemia. Patients were classified into two groups, either untreated or treated-resistant. In general, patients with resistant B-CLL had received 4 - 6 mg of chlorambucil daily and/or, in some cases, 50 - 100 mg of cyclophosphamide daily for 3 months or more. A patient is considered resistant if their peripheral lymphocyte count failed to decrease to less then 30 000 cells/ µL following such

therapy. All of the patients with resistant disease had previously responded to nitrogen mustard therapy.

B-CLL lymphocyte isolation and culture

Culture medium and fetal calf serum were obtained from Gibco/ BRL (Life Technologies). Lymphocytes were isolated from the peripheral blood of B-CLL patients by sedimentation centrifugation on Ficoll-Paque (Pharmacia). An aliquot of 3 mls of whole blood was layered onto 4 mls of Ficoll-Paque in 15 ml polypropylene tubes (Falcon) and centrifuged at 1800 rpm for 40 minutes. The white blood cell layer was recovered, washed two times with MEM supplemented with 20mM HEPES, and resuspended in lymphocyte culture medium (RPMI 1640, 10% fetal calf serum, 20mM HEPES, 10 µg/ml gentamycin) and processed as required.

MTT cytotoxicity assay

The MTT assay was performed as described by Plumb *et al* (1989). B-CLL lymphocytes were diluted to a final concentration of 1.5 X $10^6$ cells/ml. The lymphocytes were then exposed to various DNA damaging agents and seeded into 96 well microculture dishes (Corning) in aliquots of 200µl. Following 72 hrs. of incubation at 37 °C, 5% $CO_2$, 50µl aliquots of 3 mg/ml MTT were added to each well and incubated an additional 4 hrs. The cells and formazan crystals were pelleted in the wells by centrifugation at 1500 rpm for 10 min. The supernatant was removed and the insoluble MTT-formazan crystals were resuspended in 200 µl DMSO followed by addition of 25µl Sorensen's glycine buffer (0.1M glycine plus 0.1M NaCl equilibrated to pH 10.5 with 0.1 N NaOH). The $OD_{570}$ was measured using an ELISA reader. A standard curve was prepared in a separate dish using lymphocyte concentrations ranging from 10 000 to 300 000 cells/well. The number of viable cells in the experimental wells were obtained from the standard curve and the $ED_{50}$ (effective dose producing 50% cell death) was established as the concentration of drug required to reduce the viability by 50%.

**CHO cell culture** (Chapters 3 and 4)

Culture conditions

Culture medium and fetal calf serum were obtained from Gibco/BRL, plasticware was obtained from NUNC (Life Technologies), supplements for MAXTA were obtained from Sigma Chemicals. CHO cells were cultured in α-MEM supplemented with 10% fetal calf serum and 10 μg/ml gentamycin. Transfected cells were maintained in MAXTA (standard culture medium plus 10 μg/ml mycophenolic acid, 25 μg/ml adenine, 250 μg/ml xanthine, 10 μg/ml thymidine, 2 μg/ml aminopterin) as a selection medium.

Clonogenic cytotoxicity assay

Aliquots of 150 cells were seeded into 60 mm petri dishes and given 4 hours to attach to the substratum prior to treatment. The cells were then treated with alkylating agents or UV light and incubated for 6 days. Colonies were fixed and stained in 10 % TCA, 0.2% Sulforhodamine B (Sigma) and washed with 1% acetic acid. Routinely the colony forming efficiency of the UV20, AA8, and transfected cells was 70-80%. During the assay period, all cell lines were cultured in α-MEM, 10% FBS. In each assay, a control line was included to account for any possible variations due to drug stock concentrations.

**Drugs and DNA damaging agents**

Melphalan, chlorambucil, aphidicolin, ara-C, and 3-aminobenzamide were obtained as powder from Sigma Chemical Company. Methyl methanesulfonate was purchased from Aldrich. Cisplatin and mitomycin C (Bristol-Meyers) were obtained from the hospital pharmacy dissolved in water at a concentration of 1 mg/ml. Melphalan was dissolved in 75% Ethanol, 0.032N HCl at a concentration of 6.55 mM. Chlorambucil and aphidicolin were dissolved in DMSO at a concentration of 18mM and 29.5mM, respectively. BCNU (Bristol-

Meyers) was dissolved in 10mM sodium citrate (pH 4) at a concentration of 15 mM. Ara-C and 3-aminobenzamide were prepared fresh in phosphate-buffered saline at a concentration of 50mM and 100mM, respectively. Cells were irradiated with UV light (254 nm) from a 60 Hz, 0.16 A UV lamp at a fixed distance of 19 cm.

**Glutathione and glutathione-S-transferase measurements**

GSH and GST measurements were made according to a protocol from Drs. G. Batist and M. Alaoui-Jamali. Extracts were prepared from $10^7$ B-CLL lymphocytes by hypotonic lysis in 0.9 mls $H_2O$. A 0.1ml aliquot of 30% sulfosalicylic acid is added and the sample is incubated on ice for 15 minutes. The lysate is cleared by centrifugation for 2 minutes at 14 000 rpm and the supernatant is stored at - 80°C until use.

Total GSH is measured by adding 100 µl of 1M triethanolamine (pH 8) to 100 µl of sample in a cuvette. To this, 700µl of 0.3mM NADPH in buffer A [0.125M NaH2PO4 (pH 7.5), 6.3 mM EDTA] and 100 µl of 6mM 5,5'-dithiobis-(2-nitrobenzoic acid) in buffer A is added. Finally, 10 µl of glutathione reductase is added (50U/ml in buffer A) and the $OD_{412}$ is measured for approximately 30 seconds to obtain the initial velocity for the glutathione reductase reaction. In order to quantify the concentration GSH in the sample, a standard curve is prepared at the same time using between 0 and 10 nmol GSH.

Total GST activity was measured by combining 650 µl of sample in phosphate buffer [0.1 M $KH_2PO_4$-KOH (pH 6.5)] with 50 µl of 20 mM GSH and 300 µl 3.33 mM 1-chloro-2,4-dinitrobenzene. The $OD_{340}$ is measured at 1 minute intervals for 7 minutes. The activity is calculated by the following formula:

$$\text{nmole / min / ml} = [(\Delta\ OD_{340} - \text{blank})/\ 6.6 \times 0.0096] \times \mu l.$$

All samples were normalized to protein concentration, which was determined using Lowry protein assay.

## Glycosylase Assays

The glycosylase assay was performed using a protocol from Peter Karran, Clare Hall Laboratories. Approximately $10^7$ cells were lysed in 150 µL of glycosylase extraction buffer [50mM Tris-HCl (pH 7.6), 1mM EDTA, 10 mM DTT, and 0.2% Triton X-100] on ice. The lysates were centrifuged at 12 000 g at 4°C for 10 minutes in a microfuge. Supernatants were stored at - 70°C.

For the glycosylase assay, M. lysodeitikus DNA (Sigma; 5 mg/ml in Na Cacodylate pH 7.0, 1mM EDTA) was alkylated with 5 mCi [$^3$H] dimethyl sulfate (New England Nuclear) by incubation at 37°C for 1 hour. The DNA was precipitated by the addition of 200µl 2M NaCl and 4 mls Ethanol, redissolved in 2 mls of 10 mM Tris-HCl (pH 8), 1mM EDTA (TE) and dialyzed against two changes of 500 mls of TE at 4°C. The glycosylase reaction was carried out in 100 µL of reaction buffer [70mM HEPES (pH 7.9), 1 mM EDTA, 1mM DTT, and $10^4$ CPM of [$^3$H] methylated DNA] plus 0 - 20 µL of cellular extract and incubated at 37 ° C for 60 minutes. The reaction was stopped by the addition of 10 µL of 2M NaCl, 10 µL salmon sperm DNA (2 mg/mL) and 300 µL ethanol. The DNA was precipitated at -20°C for 30 minutes and pelleted by centrifugation at 12 000 g for 10 minutes at 4°C. Then, 300 µL of supernatant was removed for scintillation counting. The protein concentration for each extract was determined using the Bio-Rad protein assay ( Bio-Rad Laboratories)

## NAD measurements (Chapter 6)

The protocol for this procedure was obtained from the lab of Dr. Guy Poirier at L'Université de Laval. Cells were treated under various conditions and at the appropriate time, samples were taken for analysis. An aliquot of $10^7$ B-CLL cells were washed twice in PBS, and lysed in 1 ml of 1 M Perchloric acid. The samples were vortexed vigorously and incubated at 4 °C for 15 minutes. The lysate was neutralized by the addition of 500 µl 2M

KOH, 0.33 M $KH_2PO_4$-$K_2HPO_4$ (pH 7.5), followed by further incubation on ice for 10 minutes. The pH was adjusted to 7.5 and the precipitate was removed by centrifugation at 3000 rpm for 10 minutes at 4°C. Aliquots of the supernatant were frozen off at - 20 °C and thawed only once.

NAD measurements were made using a microtiter assay using 96 well dishes (Corning). A standard curve was prepared in duplicate with 1,2,3,4,5,10,15,20 nmoles of NAD by diluting a 400 nM stock solution into water to a final volume of 50 µl. Similarly, duplicate aliquots of 5 and 20 µl of sample was diluted in 45 and 30 µl water. To each well, 100 µl of assay buffer was added [0.1 M Bicine (pH 7.8), 0.5M Ethanol, 4.17 mM EDTA, 0.83 mg/ml BSA, 0.42 mM MTT, 1.66 mM phenazine ethosulfate]. Alcohol dehydrogenase [0.335 mg/ml in 0.1M Bicine (pH 7.8) ] is then added as 20 µl aliquots. Due to the photosensitivity of the reaction, the plate is wrapped in tinfoil and incubated at 30°C for 30 minutes. At the end of the reaction period, the $OD_{570}$ of the wells is determined using a microplate reader. The NAD concentration of the samples are determined using the standard curve.

**Northern Analysis** (Chapter 3)

RNA preparation:

Total RNA was prepared from sub-confluent monolayers the day of each cytotoxicity assay using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Ausubel, 1989). Cells were grown to subconfluence on 60 mm petri dishes, washed twice with PBS, and lysed in 500 µL solution D (4 M guanidium isothiocyanate, 50mM Tris-HCl, pH 7.5, 25 mM EDTA) and the lysate was transferred into 1.5 ml microtubes. Fifty microliters of 2M sodium acetate (pH 4.0) was then added and the solution was vortexed vigorously. This was followed by the addition of 500 µl of water equilibrated phenol (mixed by vortexing) and 100 µl of chloroform (mixed by vortexing). The tubes were then chilled on ice for 5 minutes and

centrifuged at 9000 rpm at 4 ° C for 10 minutes. The aqueous layer was transferred into another microtube and mixed with an equal volume of isopropanol. The RNA was precipitated for 30 minutes at - 20 °C and recovered by centrifugation at 10 000 rpm at 4°C for 10 minutes. The RNA was redissolved in 300 µl solution D and reprecipitated with isopropanol. The final RNA pellet was rinsed several times with 70% ethanol and resuspended in 30 - 50 µl of RNase free water. Poly $A^+$ RNA was prepared from $10^9$ CLL lymphocytes using the Fast-Track kit (Invitrogen). The samples were then frozen at -70°C until needed.

Blotting and hybridization:

RNA was prepared for electrophoresis by adding RNA sample (10-15 µg total RNA, up to 30 µg poly $A^+$ RNA) to 4µl 10X MOPS running buffer [0.2 M MOPS (pH 7), 0.15 M Sodium acetate, 0.01 M EDTA (pH 8)], 7µl formaldehyde, 20 µl deionized formamide and water up to a final volume of 40 µl. Samples were mixed by vortexing and RNA was denatured by incubation at 55°C for 15 minutes. Ten microliters of loading buffer [1 mM EDTA (pH 8), 0.25% Bromophenol blue, 0.25% Xylene cyanol, 50% glycerol] was added to each sample prior to loading onto a denaturing gel (1.2 % agarose, 1.1% formaldehyde, 1X MOPS buffer). The samples were electrophoresed in 1X MOPS buffer until the xylene cyanol has migrated to the edge of the gel. The gel was rinsed several times in deionized water and soaked in 10X SSC (0.15M NaCl, 0.015M sodium citrate ) for 30 minutes. The RNA was transferred to a nylon filter (Biotrans+, ICN Biochemicals) by capillary action overnight and covalently bound by irradiation with $UV_{254\ nm}$ for 5 minutes. The filter was then baked at 80°C for 2 hours and stored at room temperature until use.

The blot was prehybridized for 4 hours at 42°C in 50% formamide, 5X SSC (0.75M sodium chloride, 0.075M sodium citrate), 5X Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrollidone, 0.1% BSA), 250 µg/ml salmon sperm DNA, 0.1% SDS.

Hybridization was performed in the same solution as prehybridization with the addition of 10% Dextran sulfate and 2 X $10^6$ CPM/ml of radio-labeled probe.

Radio-labeled cDNAs:

The cDNAs used to probe the Northern blots were prepared by restriction of cDNA-containing plasmids, followed by isolation of the appropriate DNA bands by visualization with ethidium bromide on a 0.7% low-melting point agarose gel. The band was cut out of the gel and melted at 65°C for 20 minutes. The agarose-DNA solution was diluted by the addition of three volumes of deionized water and frozen in aliquots containing 10-25 ng DNA in a maximum volume of 20 µl. The probe was then radio-labeled using the Multiprime kit (Amersham) and two radio-nucleotides, [$^{32}$P]-dATP and [$^{32}$P]-dCTP (New England Nuclear), according to the supplied protocol using the reagents provided in the kit.

**Nuclear extracts** (Chapters 4 and 6)

Chapter 4:

Nuclear fractions were prepared as described by Cool *et al.* (1989). Cells were washed twice with ice cold PBS and resuspended in hypotonic buffer [20 mM Tris-HCl (pH 7.1), 5mM KCl, 1mM $MgCl_2$ and 0.2 mM PMSF]. The cells were allowed to swell on ice for 10 minutes and then disrupted with 20 strokes using a pestle in a tight-fitting Dounce homogenizer. The cell suspension was centrifuged at 375 x g for 10 minutes. The pellet was resuspended in hypotonic buffer and centrifuged again. The final pellet was then lysed in glycosylase extraction buffer [50mM Tris-HCl (pH 7.6), 1mM EDTA, 10 mM DTT, and 0.2% Triton X-100] and the supernatant was used to assay for glycosylase activity.

Chapter 6:

Nuclear extracts were prepared using a modified Dignam protocol as described by Andrews and Faller (1991). An aliquot of $10^8$ cells was suspended in 400 µl of cold Buffer A

[10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF]. Following a 10 minute incubation on ice, the cells are vortexed for 10 seconds and the nuclei are pelleted by centrifugation in a microfuge at maximum speed and room temperature for 10 seconds. Nuclei are then suspended in 100 µl of cold buffer C [20mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 minutes. The extracts are cleared by centrifugation for 10 seconds as before. Lysates were pooled and dialyzed against buffer D [50 mM HEPES-KOH (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2mM EDTA, 0.5mM DTT, 0.5 mM PMSF] at 4 °C, then stored at -70°C until use.

**Plasmids and expression constructs** (Chapters 3,4 and 6)

Restriction enzymes were obtained from either Pharmacia or Promega and single restrictions were executed in the buffers supplied by the manufacturer. Double digestions were generally performed in 1 for All buffer (Pharmacia) or the most suitable buffer. Digestion reactions were generally carried out for 1-2 hours. Restriction enzymes were heat-inactivated at 75° C for 20 minutes. Overhanging 5' ends were made blunt by the addition of 1 U Klenow fragment (Pharmacia) and 50 µM dNTPs to the heat-inactivated restriction digest and incubating for an additional 30 minutes followed by heat-inactivation at 75° C for 20 minutes. If additional digestion was required, the reaction volume was increased and the appropriate amount of buffer and enzyme was added. If phosphatase treatment was required, this was done as a final step using Calf Intestinal Phosphatase (Pharmacia) for 1 hour at 37°C. The reaction products were separated by electrophoresis through 0.7% agarose gels (low-melting point, BRL) in 1 X TAE (40mM Tris-acetate, 1mM EDTA). The bands were visualized using long-wave UV light (318 nm) and cut out of the gel. Once the agarose was melted, the appropriate DNA molecules were mixed in a micro-centrifuge tube at 37 ° C, an equal volume of 2 X ligation buffer (BRL) containing 1 U of T4 DNA ligase (BRL) was

added and incubated for 16-24 hours at room temperature. The following day competent bacteria were transformed with 5 - 10µl of the ligation mixture. Bacterial colonies were chosen at random and plasmids were isolated by the micro protocol (see Plasmid purification). Plasmids which appeared to contain insert were then subjected to a more rigorous restriction analysis to identify appropriate clones for further use.

1. pcDE1 and pSVblank (Chapter 3):

The pcDE1 expression vector contains the complete human ERCC-1 cDNA under the control of the SV40 promoter. It was isolated by screening a cDNA library in the pcD vector and was graciously given to us by Dr. J.H.J. Hoeijmakers. I prepared the pSVblank vector by restricting pcDE1 with XhoI to release the ERCC-1 sequences, followed by religation of the remaining vector sequences.

2. pSVANPG (Chapter 4):

The full length human cDNA encoding the alkyl-N-purine DNA glycosylase enzyme was supplied by Dr. T. O'Connor as an Eco RI/Bgl II fragment. This fragment was subcloned into the pUC19 vector in BamHI/EcoRI sites yielding pUCANPG. From pUCANPG, an ANPG insert was prepared with a 3' blunt-end by cleaving with HindIII and filling in the overhang by Klenow. The insert was then released by restriction with EcoRI. The pSVK3 expression vector was prepared to receive the insert by cleaving with EcoRI and SmaI (a blunt cutter). This strategy allowed for the insertion of ANPG sequences in the correct orientation producing the final product, pSVANPG. This vector was tested for functionality by transient transfection in CHO cells and found to increase the glycosylase activity of the population by 5 fold at 24 hours post transfection.

3. pUC-f180 (Chapter 6):

A 180 base pair PvuII-AccI fragment was cut out of pSV2cat, spanning base pairs 340 to 520 of the plasmid. Following purification, the fragment was treated with Klenow in order

to fill in the overhanging ends. In order to produce a compatible site, the pUC19 vector was linearized with XbaI and rendered blunt-ended using Klenow and treated with a phosphatase to prevent religation of the vector ends. The fragment was then ligated to pUC19 vector in order to generate pUC-f180.

## Plasmid purification

### Micro protocol

An aliquot of 1.5 mls of confluent bacterial broth is placed in a micro centrifuge tube. Bacteria are pelleted by centrifugation at maximum speed for 30 seconds. The pellet is resuspended in 30 - 100 µl lysis buffer [10mM Tris-HCl (pH 8.0), 15% (w/v) sucrose, 2 mg/ml lysozyme, 200 µg/ml RNase A, 100 µg/ml BSA; stored at - 20 °C]. The suspension is incubated at room temperature for 5 minutes, boiled for 60 seconds and immediately chilled on ice for 60 seconds. Bacterial debris is then pelleted by centrifugation at maximum speed for 15 minutes. This preparation can be used for restriction digestion.

### Alkaline lysis technique

This plasmid purification technique can be used for both small and large preparations. For preparations from 5 mls (or less) of bacterial broth, the pelleted bacteria can be resuspended in 400 µl of solution P1 [50mM Tris-HCl (pH 8.0), 10mM EDTA]. Large volumes (500 mls or less) can be resuspended in 10 mls of P1. One volume of P2 [0.2N NaOH, 0.1% SDS] is then added, the solution is gently mixed, and incubated at room temperature for 5 minutes. One volume of P3 [3.0M Potassium acetate, pH 5.5] is then added and mixed gently, but quickly. The precipitate is then removed by centrifugation (maximum speed in a microcentrifuge for 5 minutes or 16 500 rpm in an SS34 for 30 minutes). The supernatant is removed and the DNA is precipitated by the addition of an equal volume of

isopropanol and pelleted by centrifugation. The pellet is washed one time with 75% ethanol and resuspended in an appropriate volume of TE [10 mM Tris-HCl (pH 8), 1 mM EDTA]. The DNA can be further purified by extraction with phenol:chloroform and reprecipitated using alcohol in the presence of 10mM $MgCl_2$ or 0.3M sodium acetate (pH 5.5). Alternatively, the DNA can be precipitated by adding 1/100 volume of 1M MgCl2 and 1 volume of 30% (w/v) polyethylene glycol.

**Poly(ADP-ribose) polymerase depletion**

Depletion was performed as described by Satoh et al. (1993). Briefly, 2 mg of nuclear extract was diluted in Buffer A [50 mM Tris-HCl (pH 8.0), 10 mM β-mercaptoethanol, 10% glycerol] to a final volume of 1 ml. The sample was then applied to a double stranded DNA cellulose column (Sigma), and the flow through was collected as fraction 1. Elution with 1ml of Buffer A containing 0.3M NaCl followed by 1 ml of Buffer A containing 0.4M NaCl yielded fractions 2 and 3, respectively. These two fractions were pooled with fraction 1 to constitute the PARP depleted extract. The pooled fractions were then dialyzed extensively against Buffer D (50 mM HEPES-KOH, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2mM EDTA, 0.5mM DTT, 0.5 mM PMSF) to remove excess salt. The dialyzed extract was concentrated on Centricon-3 filters (Amicon) at 7500 rpm in an SS34 rotor (Sorvall) for 2.5 hours at 4°C.

**Southwestern analysis** (Chapter 6)

Preparation of the f196 probe:

The probe was released from pUC-f180 (described in "plasmids and expression constructs") as a SmaI/HincII fragment of 196 base pairs with blunt ends (f196). The fragment contains multiple GAC sequences, which serve as sites for melphalan alkylation and crosslink formation. Following release of the f196 from the pUC19 vector, the fragment was

purified by PAGE (8% polyacrylamide) and the band containing the f196 was extracted overnight at 37°C in elution buffer ( 0.5 M ammonium acetate, 1mM EDTA). The fragment was end-labeled with ($\gamma$-$^{32}$P) dATP (ICN Radiochemicals) using T4 polynucleotide kinase (Pharmacia). Following the labeling reaction, the fragment was purified on Sephadex G-50 (Pharmacia) and ethanol precipitated. The radio-labeled f196 was resuspended in 50 mM HEPES (pH 7.4), 1 mM EDTA and treated with melphalan at various concentrations for 4 and 24 hours. In the indicated experiments, melphalan treatment was performed in the presence of 5 mM methoxyamine to prevent nick formation at apurinic sites (Liuzzi and Talpert-Borlé, 1985). The final products have been demonstrated to be a single band upon PAGE analysis [the melphalan treated fragment migrates with slower mobility as described for cisplatin treated probes(Bellon *et al.*, 1991)].

Southwestern blot:

Nuclear extracts were separated by SDS-PAGE (10% polyacrylamide) without prior boiling of the samples. The separated proteins were electrophoretically transferred onto a nitrocellulose filter at 4°C. For southwestern analysis, all manipulations were carried out at 4°C. The filter was soaked in HEPES-salt buffer [20mM HEPES (pH 7.9), 5mM $MgCl_2$, 50mM NaCl, 1mM DTT] for 15 min followed by Blotto-5% (HEPES-salt buffer with 5% Carnation powdered skim milk) for 1 hour. Subsequently, the filter was soaked overnight in Blotto-0.5% with the radio-labeled probe (1 x $10^5$ cpm/20 mls). The filter was washed in Blotto-0.5% once for 30 seconds and twice for 20 minutes. The final wash was in HEPES-salt buffer for 20 minutes. The filter was dried at room temperature and exposed to Kodak XAR-5 film for autoradiography.

## Statistical Analysis

As all comparisons used in these studies were based on two sets of independent variables, differences between the two groups were assessed using an unpaired t-test analysis.

The r values for the correlation curves were obtained using a simple linear regression analysis. All computations were performed using StatView 512 V.1.2, for the Macintosh.

**Transfection experiments** (Chapters 3 and 4)

Plasmids were prepared from transformed bacteria by alkaline lysis and purified on QIAGEN columns, using the protocol and solutions provided by the manufacturer. Cells were transfected using the calcium-phosphate technique as described by Weber et al.(1988). A DNA precipitate was prepared by adding a solution of 40 µg/ml DNA, 250 mM $CaCl_2$ to an equal volume of 2X HBS (280 mM NaCl, 10mM KCl, 1.5 mM $Na_2HPO_4$, 12 mM Dextrose, 50mM HEPES). Briefly, 2 x $10^6$ cells in a 100 mm petri dish were transfected with 18 µg of plasmid DNA and 2 µg of pSV2gpt overnight. The next day cells were washed and allowed to grow in non-selective medium for 24 hours. The cells were then harvested and seeded into MAXTA medium in 100 mm petri dishes at increasing dilutions to allow for individual colony formation. After approximately 10 days, colonies were visible and several colonies were selected at random using glass cloning rings (Bellco Biotechnology). The colonies were then expanded to cell lines and cryogenically stored for later use. Clones were screened for the appearance of novel transcripts by Northern analysis, indicating expression of the transfected cDNA. Stock cells were maintained under a constant selection of MAXTA and cells used in cytotoxicity assays were cultured in non-selective medium for 3 days as described.

**Western Analysis** (Chapter 3 and 6)

Protein preparation:

Samples were prepared by direct lysis in gel-loading buffer as described by Sambrook (1989). In the case of adherent cells, sub-confluent cells in 60mm dishes were washed twice with PBS and lysed by the addition of 60µl of 1X gel-loading buffer [50 mM Tris-HCl (pH

6.8), 100mM DTT, 2% SDS, 0.1% Bromophenol blue, 10% glycerol] at 80°C. The extract was scraped to the edge of the dish and transferred to an eppendorf tube. Next, the lysate was passed through a 26 gauge needle in order to shear the genomic DNA and boiled for 10 minutes. Centrifugation at 10 000 rpm for 10 minutes cleared the sample of non-solubilized material and the protein preparations were stored at -20°C until needed. Lymphocyte extracts were prepared by resuspending $10^8$ lymphocytes in suspension buffer [100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1mM EDTA, 0.6 mM PMSF] and lysing them by addition of an equal volume of 2X gel-loading buffer at 80°C. From this point, the protein extracts were processed in the same fashion as adherent cells. Protein concentrations were determined using the Bio-Rad assay kit (Bio Rad Laboratories) using the instructions provided by the manufacturer.

Western blot:

Approximately 10 µg of protein was separated by SDS-PAGE (acrylamide:*bis*-acrylamide = 29:1) with a 5% stacking gel, 12% resolving gel using the Mini-Protean II PAGE apparatus (Bio-Rad Laboratories). Following gel electrophoresis, proteins were transferred to nitrocellulose by electroblot (Bio-Rad Laboratories). The filters were then stained with Ponceau red in order to visualize the molecular weight markers. Several washes in TBS-T [10mM Tris-HCl (pH 7.5), 0.88% NaCl, 0.1% Tween-20] removed the stain. Non-specific binding sites were blocked by incubation in 5%-BLOTTO [5% skim milk powder (Epicerie Metro) in TBS-T with 10µg/ml gentamycin, 0.1% sodium azide] for 1 hour at room temperature. The blot was then hybridized to the primary antibody appropriately diluted in 5%-BLOTTO for 1 hour at room temperature. Alternatively, the blot was hybridized to the primary antibody overnight at 4°C. The blot is then washed in TBS-T at room temperature once for 15 minutes and twice for 5 minutes. The blot is incubated at room temperature in the presence of the secondary antibody for 1 hour, followed by 1 wash for 15 minutes and 4 washes for 5 minutes. Bound antibody can then be visualized using the Enhanced Chemiluminescence kit (ECL, Amersham) using the protocol described by the

manufacturer and exposed to X-ray film. After exposure the blots were stored in cellophane at 4°C. If a second protein was to be analyzed, the blots were stripped according to the ECL protocol at 50°C for 30 minutes in stripping buffer [100mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)].

Antibody probes:

ERCC-1 was detected using a polyclonal rabbit anti-serum kindly supplied by Dr. N.G.J. Jaspers. This polyserum was diluted at 1:1000 for the blotting procedure and the goat anti-rabbit antibody used for secondary hybridization was purchased from Bio-Rad.

Poly (ADP-ribose) polymerase was detected using a monoclonal antibody (C-2-10) graciously provided by Dr. G.G. Poirier. This antibody was diluted 1:1000 for the blotting procedure and the goat anti-mouse used in the second hybridization step was obtained from Promega.

# Chapter 3:

# ERCC-1 overexpression, nucleotide excision repair and nitrogen mustard resistance

## Summary

Previous studies of ERCC-1 gene expression in B-CLL and ovarian carcinoma tumor specimens indicated that increased expression may correlate with a lack of response to the alkylating agents, melphalan and cisplatin. In this chapter, the effect of ERCC-1 overexpression on tissue culture cells has been investigated. This was done by transfecting CHO cells with an expression construct encoding the human ERCC-1 gene. UV20 cells, which lack functional ERCC-1, exhibit hypersensitivity to melphalan. Expression of ERCC-1 in these cells can restore wild type levels of resistance, confirming a role for this gene product in the repair of melphalan lesions. Surprisingly, when ERCC-1 is overexpressed in wild type AA8 cells, they exhibit greater sensitivity to both melphalan and cisplatin, but not UV light.

Continued analysis of ERCC-1 expression in lymphocytes from patients with B-CLL, through both Northern and Western blot, analysis failed to confirm an association between enhanced gene expression and nitrogen mustard resistance. The transcript levels of a second nucleotide excision repair gene, ERCC-2, were also analyzed by Northern blot and found to be similar in both untreated and treated-resistant populations.

The results of these studies provide no evidence that enhanced ERCC-1 expression can cause nitrogen mustard drug resistance. In fact, overexpression of this gene induces sensitivity to certain DNA damaging agents. From this data, nucleotide excision repair does not appear to be limiting in nitrogen mustard resistance.

The data from the transfection experiments has already been published [**Bramson, J.** and Panasci, L. Effect of ERCC-1 overexpression on sensitivity of Chinese hamster ovary cells to DNA damaging agents. Cancer Res. 53: 3237-3240, 1993]. The data from the Northern blot analysis has been submitted to Leukemia in a separate manuscript.

## Introduction

Nucleotide excision repair (NER), which removes DNA lesions induced by UV light and bulky chemical adducts, is a dominant pathway in the repair of nitrogen mustard lesions on DNA (Hoy *et al.*, 1985). ERCC-1, a human excision repair gene, was cloned by complementation of the UV light hypersensitive CHO line 43-3B (UV complementation group 1) (Westerveld *et al.*, 1984). Another group 1 CHO mutant, UV20, is hypersensitive to UV and a wide variety of bifunctional DNA damaging agents including the nitrogen mustards (Hoy *et al.*, 1985). We have demonstrated that resistance to the nitrogen mustards in B-CLL correlates with increased removal of DNA crosslinks, implicating enhanced DNA repair in this process (Torres-Garcia *et al.*, 1989). Furthermore, there was a 2 fold increase in expression of the ERCC-1 1.1 kb (protein encoding) transcript in the lymphocytes from resistant B-CLL patients as compared to those of untreated B-CLL patients, suggesting that increased expression of ERCC-1 might be responsible for the nitrogen mustard resistance in B-CLL (Geleziunas *et al.*, 1991). ERCC-1 expression was also increased in tumors from ovarian carcinoma patients who did not respond to cisplatin (Dabholkar *et al.*, 1992). Thus, the increased expression of ERCC-1 seems to correlate with lack of response to bifunctional DNA damaging agents.

In order to evaluate the effect of ERCC-1 on DNA repair of nitrogen mustard damage, the human ERCC-1 cDNA was transfected into UV20 cells in order to demonstrate complementation of melphalan hypersensitivity. The same cDNA was then transfected into wild type CHO-AA8 cells to determine if overexpression of this protein can confer resistance to melphalan. The analysis of ERCC-1 expression in nitrogen mustard resistant B-CLL lymphocytes has been continued using both Northern and Western blot analysis. As well, the expression of a second NER gene, ERCC-2, has been analyzed by Northern blot.

## Results

Transfection of ERCC-1 into CHO cells

The involvement of ERCC-1 in the repair of nitrogen mustard lesions has always been inferred by the hypersensitivity of the UV20 cell line (UV complementation group 1, lacking a functional ERCC-1) to nitrogen mustard analogues (Hoy *et al.*, 1984). These cells demonstrate a 30 fold hypersensitivity to melphalan (Figure 3.2.A). While ERCC-1 complements the hypersensitivity of these cells to UV, mitomycin C, and bulky adducts (Westerveld *et al.*, 1984; Zdzienicka *et al.*, 1987), it has not yet been demonstrated for nitrogen mustards which alkylate a site on the DNA different from mitomycin C (N-7 guanine as opposed to N-2 guanine) (Tomasz *et al.*, 1987a ). This results in a distinctly different DNA lesion (Tomasz *et al.*, 1987b). The ERCC-1 gene demonstrated a strong evolutionary conservation, and therefore, one would expect the human gene product to be functional in the CHO cell line (van Duin *et al.*, 1986). Correction of the hypersensitivity to melphalan in UV20 cells using this cDNA confirms the functionality of the ERCC-1 expression vector. Two randomly selected UV20 clones transfected with ERCC-1 (UVIII-1 and UVIII-2) were examined for resistance to melphalan (figure 3.2.A). Both clones exhibited increased resistance but complementation was not complete. Complementation was also partial for mitomycin C and UV (Westerveld *et al.*, 1984).

Next, the wild type AA8 cell line was transfected in order to determine whether ERCC-1 overexpression would render cells with wild type DNA repair resistant to melphalan. Multiple clones were selected and screened for overexpression of ERCC-1. Two clones were chosen (CAIII-8 and CAIII-12) that demonstrated the presence of novel transcripts which coincided with those in the UV20 transfectants (figure 3.1.A). Subsequent western blot analysis confirmed a substantial overexpression of ERCC-1 in the transfected cell lines (figure 3.1.B). Surprisingly, these two clones displayed an approximately 2-fold increase in melphalan sensitivity (Figure 3.2.B). Several lines were developed from clones which

expressed only the endogenous transcript (example: CAIII-9, figure 3.1) and displayed wild type sensitivity to melphalan (data not shown). As a control for transfection, cells were transfected with a blank construct (pSVblank) which was identical to pcDE but the ERCC-1 cDNA was removed. Two lines were developed from this transfection, both of which exhibited wild type sensitivity, the data for one line, CONIII-1, is shown (figure 3.2.B).

The phenotype of the UV20 cell line suggests that ERCC-1 is a central protein in the repair of many different DNA lesions. The ERCC-1 transfected AA8 cell line was characterized for sensitivity to UV. No differences were found between the CAIII-12, CONIII-1 and AA8 cell lines (Figure 3.2.C). Thus, overexpression of ERCC-1 may affect a pathway specifically involved in the repair of crosslinks. The CAIII-12 cells were characterized for cross-sensitivity to cisplatin, another crosslinking agent. This agent is chemically distinct from melphalan yet functionally similar (it produces N-7 guanine lesions, primarily). The CAIII-12 cell line displayed similar hypersensitivity to this agent as well, indicating that the pathway of repair for this lesion is impaired (Figure 3.2.D).

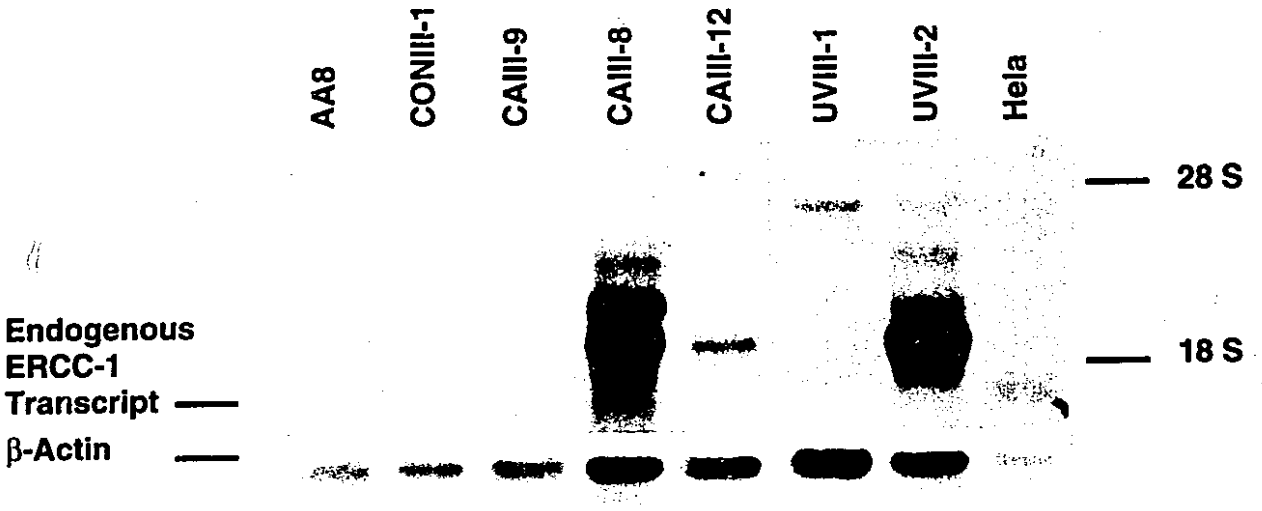
**Figure 3.1 Northern and Western blot analysis of transfectants.**

Panel A represents a Northern blot of total RNA from the various cell lines used in these experiments. RNA was electrophoresed through an agarose-formaldehyde gel and transferred to a nylon membrane by capillary action. The blot was then hybridized to a $^{32}$P-labeled human ERCC-1 cDNA, stripped, and hybridized with a $^{32}$P-labeled mouse β-actin cDNA.

Panel B represents a western blot analysis of total protein from the various cell lines used in these experiments. Proteins were separated by SDS-PAGE and transferred to nitrocellulose electrophoretically. ERCC-1 protein was detected using a rabbit polyserum against the C-terminal portion of the human ERCC-1 protein. Antibody bound proteins were visualized using a horseradish peroxidase conjugated goat anti-rabbit serum (Bio-Rad) and the ECL detection reagent (Amersham).

**AA8** = wild type; **CONIII-1**= pSVblank transfected AA8 cells;
**CAIII-8,-9,-12** = ERCC-1 transfected AA8 cells;
**UVIII-1,-2** = ERCC-1 transfected UV20 cells.

A


AA8
CONIII-1
CAIII-9
CAIII-8
CAIII-12
UVIII-1
UVIII-2
Hela
28 S
18 S
Endogenous
ERCC-1
Transcript
β-Actin

B

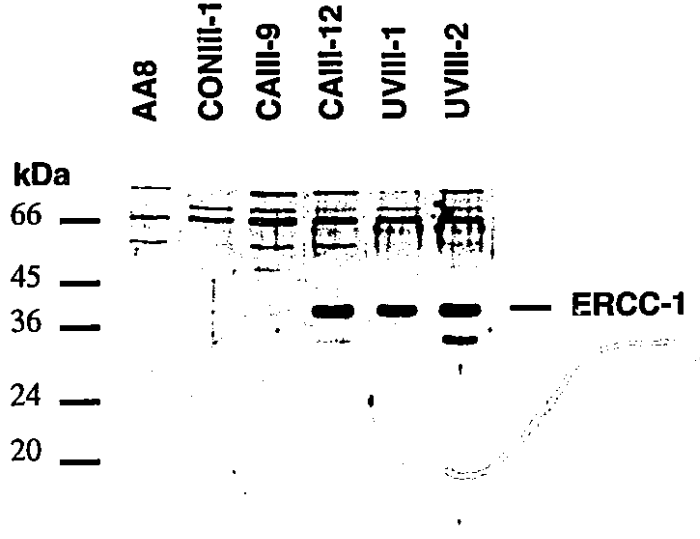

AA8
CONIII-1
CAIII-9
CAIII-12
UVIII-1
UVIII-2
kDa
66
45
36
24
20
ERCC-1

**Figure 3.2 Cytotoxicity analysis of experimental cell lines.**

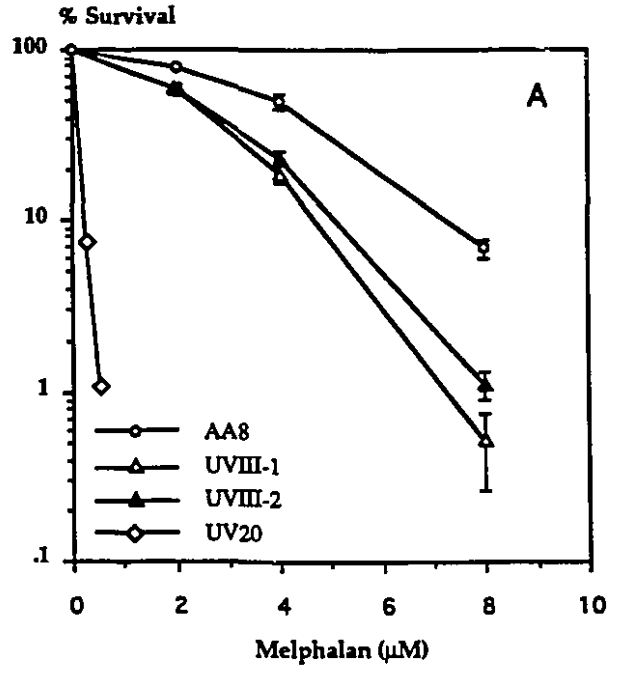
Cells were seeded into 60 mm petri dishes 4 hours before exposure to DNA damaging agents. In panels A and B, the cells were exposed to melphalan. In panels C and D, cells were treated with UV light and cisplatin, respectively. After a 6 day incubation, the colonies were fixed and stained with sulforhodamine B. The control plates routinely contained 90 - 100 colonies, cell death was expressed as % survival and calculated by dividing the number of colonies on the treated plates by the number of colonies on the control plates. *Points*, mean of 4 to 6 separate experiments performed in triplicate;*bars*, SE.

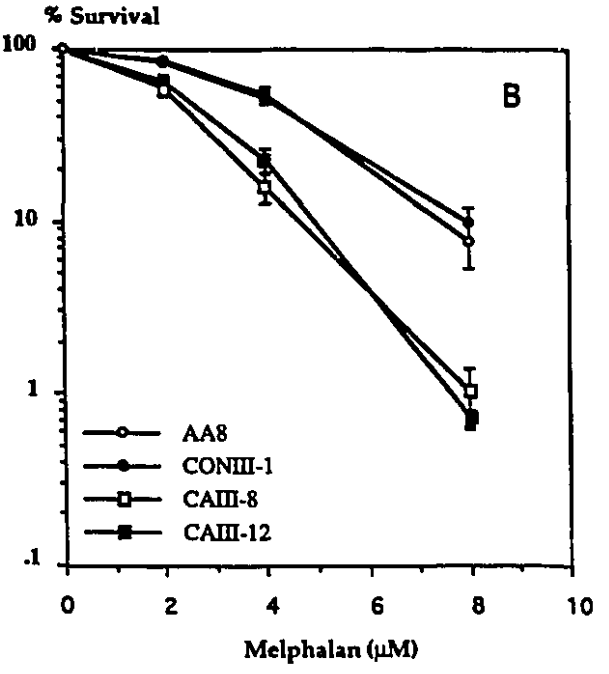
**UV20** = ERCC-1 deficient cell line

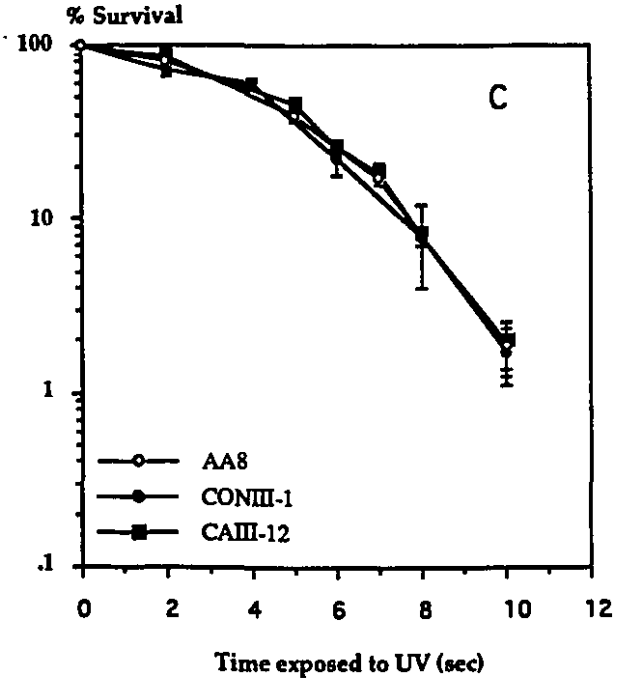
**UVIII-1,-2** = ERCC-1 transfected UV20 cells.

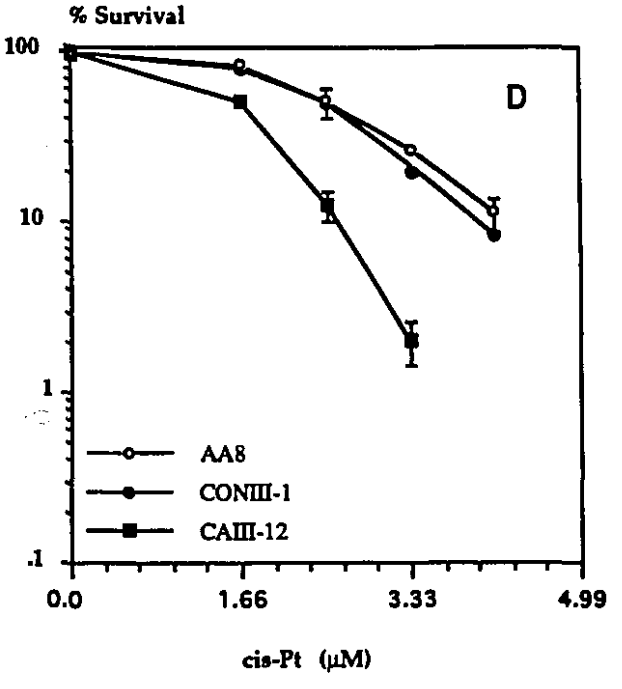
**AA8** = wild type; **CONIII-1**= pSVblank transfected AA8 cells;

**CAIII-8,-9,-12** = ERCC-1 transfected AA8 cells


% Survival
100
10
1
.1
A
AA8
UVIII-1
UVIII-2
UV20
0
2
4
6
8
10
Melphalan (μM)


% Survival
100
10
1
.1
B
AA8
CONIII-1
CAIII-8
CAIII-12
0
2
4
6
8
10
Melphalan (μM)


% Survival
100
10
1
.1
C
AA8
CONIII-1
CAIII-12
0
2
4
6
8
10
12
Time exposed to UV (sec)


% Survival
100
10
1
.1
D
AA8
CONIII-1
CAIII-12
0.0
1.66
3.33
4.99
cis-Pt (μM)

Analysis of ERCC-1 and ERCC-2 expression in B-CLL lymphocytes

The initial observation of increased ERCC-1 expression in nitrogen mustard resistant lymphocytes was made using a sample of 5 untreated and 5 treated-resistant patients' lymphocytes. This cohort has now been expanded to include 11 untreated and 12 treated-resistant patients, as determined by their clinical response to nitrogen mustards. Analysis of ERCC-1 was expression was continued using this larger cohort, and another NER gene, ERCC-2, was also examined. Three Northern blots were prepared and certain patients' samples were used twice on separate blots. Each blot contained an average of 5 untreated and 5 treated-resistant patients' samples (Figure 3.3). Each blot was analyzed individually using β-actin transcripts to normalize for loading differences. In order to perform inter-blot comparison the results for each patient were normalized to Hela cell mRNA which was included on each blot. In the case of patients who were screened on two separate blots, the mean of the two observations in the inter-blot comparison was included. The Hela RNA used for all three blots came from the same preparation.

In this larger cohort, there was no evidence of increased ERCC-1 or ERCC-2 expression (Table 3.1). As anti-serum was available against ERCC-1, western blots were employed to analyze the relative protein levels in sensitive and resistant lymphocytes. For this study, a separate group of 16 patients was used (8 sensitive and 8 resistant). The melphalan sensitivity of these patients was determined *in vitro* using the MTT assay and presented in the figure as $ED_{50}$ melphalan (Figure 3.4). There was no evidence of altered ERCC-1 expression by Western blot analysis (repeated two times), confirming our impression by Northern blot analysis.

**Figure 3.3 Example of Northern Analysis.** (opposite page)

This figure shows a representative of Northern analysis of ERCC-1, ERCC-2, and β-actin transcripts. Poly $(A)^+$ RNA from lymphocytes from patients with B-CLL (U=untreated; R= resistant) and Hela cells (He) was electrophoretically separated on a formaldehyde-agarose gel and transferred to nylon membranes by overnight capillary action. Blots were probed sequentially with $^{32}P$-labeled ERCC-1, ERCC-2 and β-actin. For these analyses, 3 Northern blots were prepared (the results are tabulated below).

**Table 3.1**

| | Average Expression , Number Patients | | |
|---|---|---|---|
| Transcript | Untreated | Treated-Resistant | P |
| ERCC-1 (1.1 kb only) | 0.76 ± 0.08, 11 | 1.02 ± 0.26, 12 | N.S. |
| ERCC-1 total | 1.06 ± 0.16, 11 | 1.16 ± 0.31, 12 | N.S. |
| ERCC-2 | 1.03 ± 0.26, 11 | 1.20 ± 0.57, 12 | N.S. |

These are the composite results of the Northern blot analyses. Each blot contained poly $A^+$ RNA from 5-6 untreated and 5-6 treated resistant patients and 1 lane with Hela cell RNA. Transcripts were normalized to β-actin and expressed relative to normalized Hela transcripts which were assigned the arbitrary value of 1 (mean ± s.e.). Comparison of individual blots revealed no significant differences between untreated and treated-resistant patients' lymphocytes, except for the ERCC-1 analysis described previously (Geleziunas *et al.*, 1991). Significance was determined using a two-tailed, unpaired t-test. N.S. = not significant

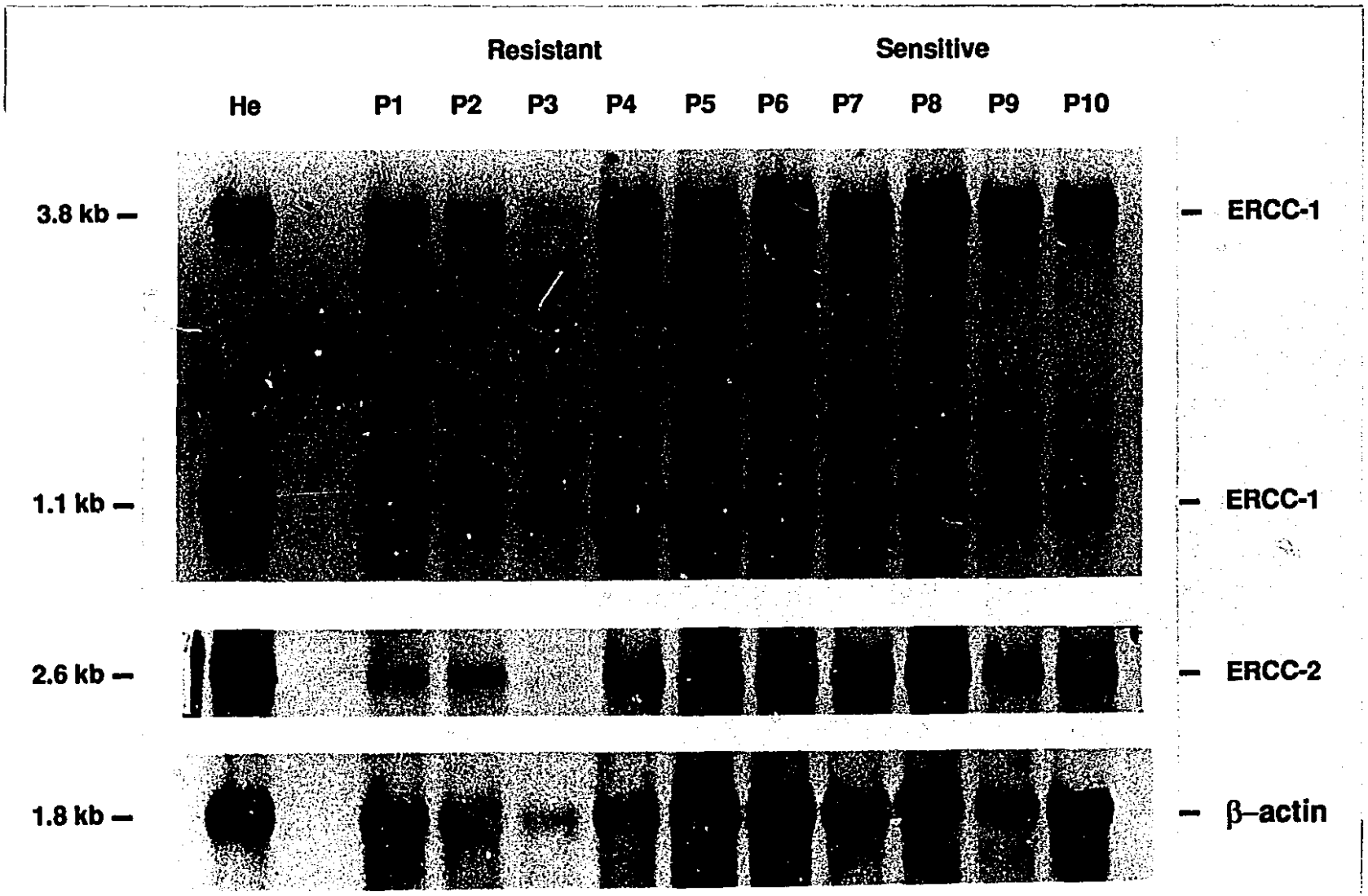
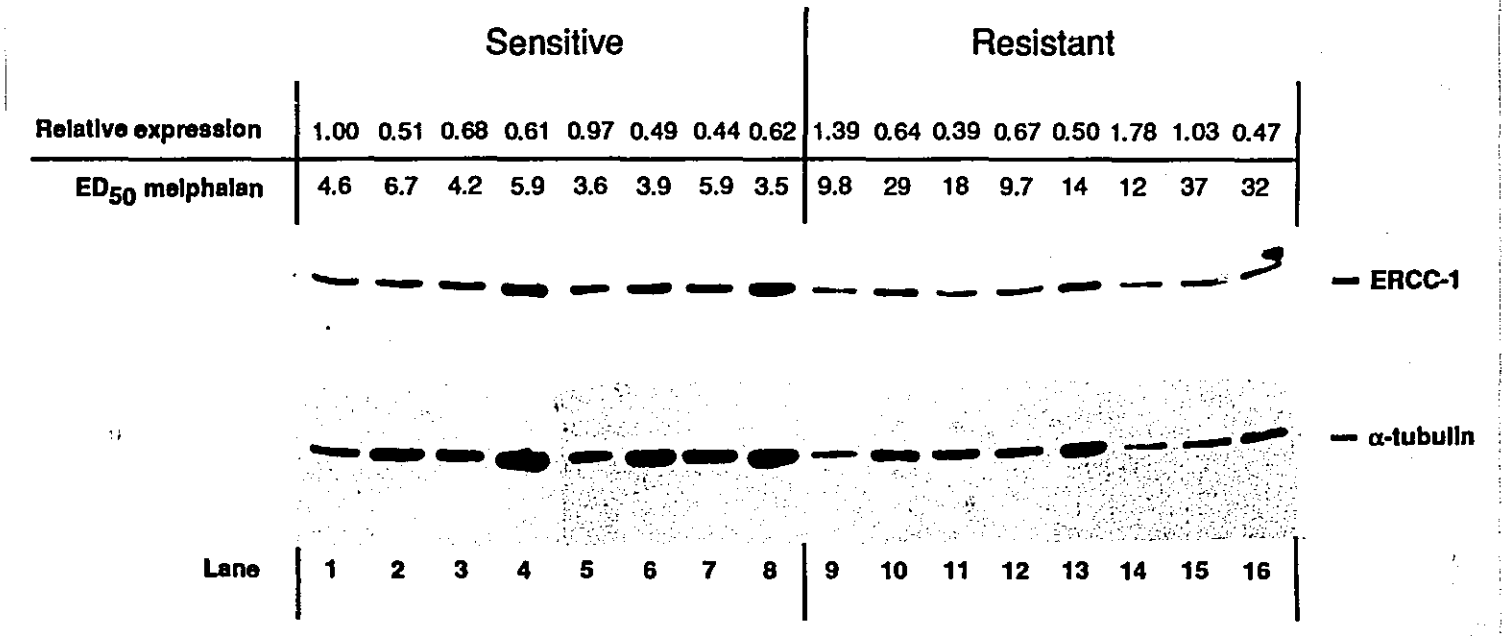

Resistant
Sensitive
He
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
3.8 kb
1.1 kb
2.6 kb
1.8 kb
ERCC-1
ERCC-1
ERCC-2
β–actin

**Figure 3.4 Western blot analysis of ERCC-1 expression in B-CLL lymphocytes.**

A western blot was prepared using extracts from 16 individual B-CLL patients' lymphocytes. Lanes 1 to 8 represent sensitive lymphocytes and lanes 9 to 16 represent the 8 resistant lymphocytes. Resistance was based on performance in the MTT cytotoxicity assay. The melphalan sensitivity of the lymphocytes as established *in vitro*, is listed in the figure as $ED_{50}$ melphalan (the dose of melphalan resulting in 50% cell death following 3 day *in vitro* exposure). ERCC-1 data was normalized to α-tubulin in order to account for loading differences. Relative expression was calculated by dividing the normalized results for lanes 1 through 16 by the result of lane 1. The mean relative expression for both sensitive and resistant groups is listed below the figure. Using an unpaired, two-tailed t-test, there was no evidence of a significant difference between the two groups. Moreover, when there was no significant correlation between the relative expression and $ED_{50}$ melphalan.




Mean relative expression:

mean sensitive (± s.d.) = 0.67 ± 0.21

mean resistant (± s.d.) = 0.86 ± 0.50

## Discussion

There is substantial evidence supporting an involvement of ERCC-1 in the response to nitrogen mustard damage. UV complementation group 1 CHO cells (which lack functional ERCC-1) are hypersensitive to nitrogen mustard (mechlorethamine) and its various derivatives (melphalan, chlorambucil, cyclophosphamide) (Hoy *et al.*, 1985). Previous studies in our lab have demonstrated enhanced DNA repair in the lymphocytes from patients with nitrogen mustard resistant B-CLL through assays which assess crosslink status intracellularly (Torres-Garcia *et al.*, 1989). A preliminary report indicated that increased expression of ERCC-1 may be associated with nitrogen mustard resistance in B-CLL (Geleziunas *et al.*, 1991). ERCC-1 demonstrates strong homology to the yeast RAD 10 gene which is required for mitotic recombination and survival following UV irradiation (van Duin *et al.*, 1986; Schiestl and Prakash, 1990). Crosslink repair is believed to involve a recombinational pathway which further implicates ERCC-1 in nitrogen mustard damage repair.

In order to demonstrate direct involvement of the ERCC-1 gene product in removal of melphalan lesions, UV20 cells ( UV complementation group 1) were transfected with an expression vector encoding the ERCC-1 gene. These cells regained an almost wild type resistance to melphalan as described previously for mitomycin C and ultraviolet irradiation (Westerveld *et al.*, 1984). We postulated that ERCC-1 may be a limiting factor in the pathway of DNA repair of melphalan lesions, so we transfected this gene into wild type AA8 cells in order to assess the effect of overexpression of this gene on cellular resistance to melphalan. Surprisingly, overexpression of ERCC-1 resulted in a hypersensitive phenotype. This hypersensitivity might represent a general inhibition of NER or may reflect a specific subpathway of the system that processes lesions induced by crosslinking agents.

There are other examples of DNA repair gene overexpression resulting in hypersensitivity to DNA damage. When the radC gene is overexpressed in *E. coli* the resultant transformant is more sensitive to UV light (Felzenszwalb *et al.*, 1992) and

overexpression of topoisomerase II in yeast sensitizes the cell to the effects of amasacrine and etoposide (Nitiss *et al.*, 1992). Belt *et al.* (1991) showed that overexpression of wild type human ERCC-1 had no effect on the cytotoxicity of UV light and mitomycin C in Hela cells but overexpression of a mutant ERCC-1 (non-functional in UV20 cells) sensitized the Hela cells to mitomycin C . These cells were never characterized for melphalan sensitivity. It has been postulated previously that different domains of the ERCC-1 protein may be involved in different types of repair (Lehmann, 1992). Certain sequences within these domains may not be conserved between the rodent and human genes. Such differences may disrupt repair of bifunctional DNA damaging agent induced crosslinks when the human ERCC-1 is overexpressed in a heterologous system.

While, initially, we believed that the negative effect of ERCC-1 overexpression may be due to some functional aspect of the protein, it now seems that this overexpression may simply be affecting the stoichiometry of the system. Over the past year, multiple reports have been released describing the interaction of the various repair proteins. It has now been demonstrated by two separate laboratories, that ERCC-1/ERCC-4/ERCC-11/XPF form one single protein complex (van Vuuren *et al.*, 1993; Biggerstaff *et al.*, 1993). In the analogous yeast system, the complex formed by RAD 1 and RAD 10 (ERCC-4 and ERCC-1 homologues, respectively) possess a single strand endonuclease activity, which does not belong to either protein individually (Sung *et al.*, 1993; Tomkinson *et al.*, 1993). Thus, subtle incompatibilities between the human and rodent proteins may disrupt the tight interaction of the complex and inhibit repair. Clearly the inhibition produced is not as severe as the case where ERCC-1 is totally absent. UV20 cells display 30 fold hypersensitivity to melphalan, while ERCC-1 overexpressing AA8 cells are only about 2 fold. So in this case, we observe a 7% inhibitory effect with ERCC-1 overexpression. As UV20 cells are only 6 fold more sensitive to UV, a similar 7% inhibition of repair would cause a 0.5 fold increase in sensitivity, which would probably go unnoticed.

While the genetic components of NER are still being defined, complementation analysis using mutant lines indicate that CHO groups 1-5 and 11 (representing ERCC-1 through 5) display the most extreme sensitivities to UV light (Hoeijmakers, 1993). ERCC-1 has also been implicated in the cellular response to a very wide range of DNA damaging agents including the bifunctional alkylators, melphalan, cisplatin, and BCNU, while ERCC-2 seems primarily required for UV damage repair (Hoy *et al.*, 1985). Preliminary observations of ERCC-1 expression in B-CLL revealed a 2 fold increase in expression levels of this gene in treated-resistant patients' samples (Geleziunas *et al.*, 1991). With a larger group of patients (12 treated-resistant and 11 untreated), there appears to be no increased expression of either ERCC-1 or ERCC-2. While mRNA levels are an indication of gene expression, post-transcriptional regulation can also affect protein levels. However, no alterations in protein expression were found using western blot analysis with ERCC-1 anti-serum and a separate group of 8 sensitive and 8 resistant patients (determined *in vitro*). Thus, we have screened a total of 19 untreated and 20 treated-resistant patients' lymphocytes and there is no evidence of increased ERCC-1 expression at either the transcript level or the protein level. The initial observations of increased ERCC-1 expression was most likely a result of the small sample number used in the original analysis. In a separate study, a group studying cisplatin response in ovarian carcinoma found an increase in ERCC-1 expression in non-responders (Dabholkar *et al.*, 1992). While this observation seems to contrast from our own, ovarian carcinoma is an epithelial tumor, and thus, the mechanism of alkylating agent resistance may differ from B-CLL.

*In vivo* models for resistance are required in order to test hypotheses developed from resistant cell lines *in vitro*. B-CLL appears to be an excellent *in vivo* model for the study of acquired resistance to nitrogen mustards. The results of this chapter results fail to provide evidence that NER (as defined by ERCC-1 and ERCC-2) is the limiting step in this phenomenon. The complex mechanisms involved in DNA repair in eukaryotic cells, and specifically repair of interstrand crosslinks, include NER, BER, and a crosslink specific

pathway. The exact mechanism(s) associated with resistance could potentially implicate several components of these systems and therefore more detailed understanding of these pathways and their regulatory elements will aid in investigating the phenomenon of resistance.

# Chapter 4:

# Overexpression of Alkyl-N-Purine DNA Glycosylase and Nitrogen Mustard Resistance

## Summary

Increased activity of alkyl-N-purine DNA glycosylase ($N^3$-methyladenine-DNA-glycosylase) has been correlated with resistance to both chloroethylnitrosoureas and nitrogen mustards. In order to determine how overexpression of the protein affects resistance to these bifunctional alkylating agents, wild type CHO-AA8 cells were transfected with an expression construct containing the human ANPG cDNA. Several clonally isolated lines were selected which expressed increasing levels of glycosylase activity. None of these lines displayed increased resistance to either *bis*-chloroethylnitrosourea or melphalan. In order to determine how overexpression of this protein affects cells in the absence of nucleotide excision repair, the mutant CHO-UV20 cell line was transfected with the same expression construct. This cell line lacks functional ERCC-1 protein and displays extreme hypersensitivity to bifunctional alkylating agents. Again, none of the UV20 transfectants displayed increased resistance. The results of these experiments indicate that overexpression of the glycosylase alone is not sufficient to confer resistance to bifunctional alkylating agents in this system.

The data presented in this chapter has been submitted for publication in Biochemical and Biophysical Research Communications.

## Introduction

Chloroethylnitrosoureas (CNUs) and nitrogen mustards are among the most commonly used chemotherapeutic drugs in the treatment of a wide variety of tumors. The limiting factor in clinical use of these agents is tumor resistance, either innate or acquired. Toxicity of these drugs has been correlated to their ability to form crosslinks in DNA and resistance has been associated with enhanced repair of alkylation damage. Such repair is displayed through either reduced crosslink formation or enhanced crosslink removal. Classically, most DNA alkylation is repaired by one of two pathways: (1) base excision repair (BER) or (2) nucleotide excision repair (NER). Both pathways have been implicated in resistance to CNUs and nitrogen mustards (Hoy *et al.*, 1985; Matijasevic *et al.*, 1991; Geleziunas *et al.*, 1991). The complexity of NER makes identification of genes key to alkylating agent resistance difficult (Hoeijmakers, 1993). BER involves fewer enzymes and therefore has been easier to characterize (Barnes *et al.*, 1993). Repair begins with excision of the alkylated base by the alkyl-N-purine DNA glycosylase (ANPG; or $N^3$-methyladenine DNA glycosylase) resulting in the formation of an abasic site. The site is then cleaved by the sequential action of a 3' endonuclease and a 5' deoxyribosephosphodiesterase which remove the depurinated sugar residue. The process is completed by "gap-filling" involving a polymerase and ligase.

The ANPG enzyme recognizes a wide variety of alkylated bases including $N^3$-methyladenine, $N^3$-methylguanine and $N^7$-methylguanine (O'Connor and Laval, 1991; Chakravarti *et al.*, 1991; O'Connor, 1993). This enzyme is also capable of recognizing ethyl lesions (O'Connor and Laval, 1991; O'Connor, 1993). Thus, it is conceivable that this enzyme could recognize the $N^7$ lesions incurred by nitrogen mustard and CNU alkylation. A mutant CHO cell line which is hypersensitive to nitrogen mustard (6.8 fold), also displays hypersensitivity to methylmethane sulfonate (10 fold), indicating that nitrogen mustard hypersensitivity may result from a defect in BER (Meyn *et al.*, 1991). While no ANPG

mutant mammalian cell lines are available, *E. coli* mutants (*tag- alk*A-) which are glycosylase deficient, exhibit hypersensitivity to nitrogen mustards and this sensitivity can be partially complemented by ANPG (Appendix 2). Furthermore, we have demonstrated an increased expression of ANPG in malignant B lymphocytes from patients with nitrogen mustard resistant CLL as compared to those from sensitive patients (Geleziunas *et al.*, 1991).

More direct evidence of the involvement of ANPG in CNU resistance comes from studies which have shown that the human ANPG can induce resistance to CNUs when overexpressed in *E. coli* (Matijasevic, 1993b; Appendix 2). CNU toxicity is believed to result from interstrand crosslinks which form between the $N^1$ position of guanine and $N^3$ position of cytosine (Erickson *et al.*, 1980). However, these crosslinks represent only 3% of total alkylation following treatment with CCNU, while up to 16% of the lesions occur at the $N^7$ position of guanine. Such $N^7$ lesions are believed to be toxic and these altered bases can be released by purified AlkA (the *E. coli* counterpart to ANPG) (Habraken *et al.*, 1991). Enhanced ANPG activity was associated with CNU resistance in a glioma cell line (Matijasevic *et al.*, 1991). This increased activity correlated with decreased accumulation of $N^7$-guanine-CNU lesions, indicating that removal of these lesions may be determinant in resistance (Bodell *et al.*, 1988).

As ANPG is responsible for the initial step in BER, it is likely that this enzyme represents the rate limiting step in repair of nitrogen mustard and CNU lesions by this pathway. It was therefore of interest to determine what effect overexpression of this enzyme would have on cellular sensitivity to the bifunctional alkylating agents, BCNU and melphalan. To this end, we have transfected wild type CHO-AA8 cells with an expression vector encoding the human ANPG. We have also transfected the NER deficient CHO line, UV20, with the same vector in order to assess the role of the glycosylase in the absence of NER.

## Results

While ANPG expression has been implicated in bifunctional alkylating agent resistance, this association has never been clearly demonstrated in mammalian cells. In order to do so, wild type CHO AA8 cells were transfected with an expression vector containing the full length human ANPG cDNA (pSVANPG). Previous results revealed that the human gene product is functional in CHO cells and recognizes both $N^3$-methyladenine and $N^7$-methylguanine lesions (Ibeanu *et al.*, 1992). Nuclear extracts were prepared from the transfected cells and activity profiles of nuclear preparations from the 3 clones used in these studies (AGLY-1,-2,-3) are shown in figure 4.1. Also shown is the activity profile of a control cell line which is transfected with the pSVK3 expression vector without the ANPG cDNA (A-CON). We screened our clones for resistance to the bifunctional alkylating agents, melphalan and BCNU. No increase in resistance was detected in any of the lines tested (Figure 4.2). There were no differences between the control transfected cells and the parental AA8 cells in sensitivity to either of the two agents (data not shown).

Both BER and NER have been implicated in resistance to alkylating agents. It is possible that in wild type CHO cells, NER is the dominant pathway and, thus, changes in the base excision pathway are undetectable. Therefore, we transfected the mutant UV20 cell line with the ANPG expression construct. UV20 cells lack a functional ERCC-1 gene and have an inhibited NER pathway. While these cells are 30 and 20 fold more sensitive to melphalan and BCNU, respectively, as compared to AA8 cells, they are only 1.2 fold more sensitive to methyl methanesulfonate indicating a functional BER system. UV20 cells were transfected with the ANPG expression construct and several lines were clonally derived. Figure 4.3 shows the activity profiles of nuclear extracts prepared from the UV20 clones used in this chapter (UVGLY-1,-2,-3), as well as a line transfected with pSVK3 (UV-CON). These cell lines displayed no differences in resistance to either melphalan or BCNU (Figure 4.4). The cell line UVGLY-2 seems slightly more resistant to the effect of BCNU. However, the cell

line, UVGLY-3, which expresses higher glycosylase activity, seems to be more sensitive. The inverse is true for melphalan sensitivity. Thus, these small differences in alkylating agent sensitivity can be attributed to clonal heterogeneity and not to glycosylase activity.

**Figure 4.1 Glycosylase activity in nuclear preparations from AA8 transfectants.**

Nuclei were prepared according to the method of Cool *et al.* (1989). Glycosylase extracts were prepared from the nuclei in the same fashion as whole cells. Glycosylase activity was measured using 2,5,10 and 20 µl of extract. Protein concentration was determined using the Bio-Rad assay. The activity was estimated from the initial slope of the curves and found to be 3.0, 9.0, 19, and 41 DPM [$^3$H] base released/hour/µg protein for A-CON, AGLY-1,-2, and -3, respectively. The results are representative of a single experiment performed in duplicate.

**A-CON** = pSVK3 transfected cells

**AGLY-1,-2,-3** = ANPG transfected cells

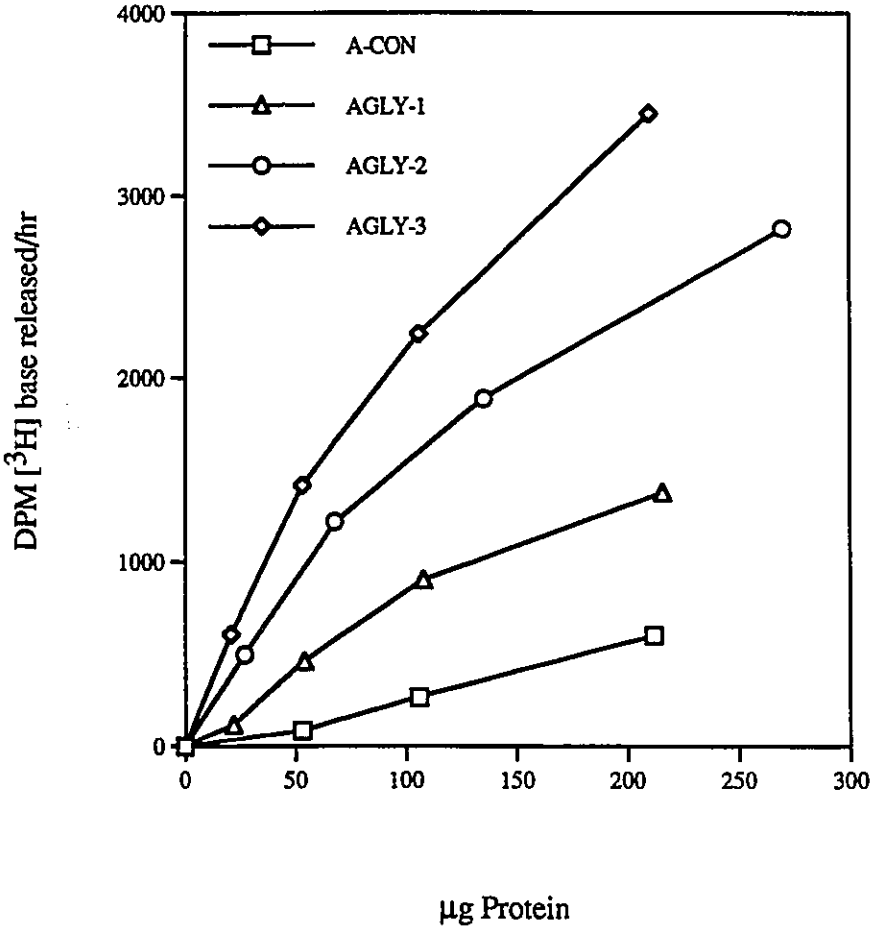
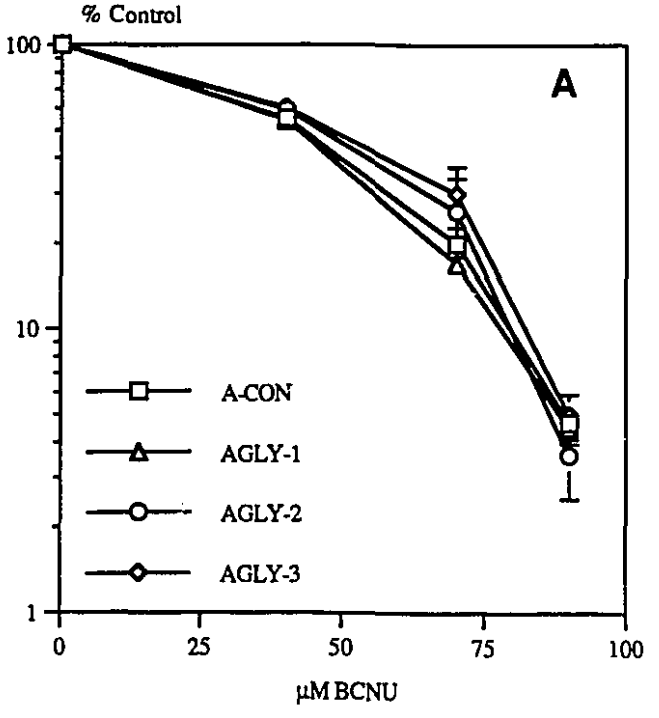

4000
3000
2000
1000
0
A-CON
AGLY-1
AGLY-2
AGLY-3
DPM [3H] base released/hr
0
50
100
150
200
250
300
µg Protein

**Figure 4.2 Cytotoxicity analysis of AA8 transfectants.**

Cells were seeded into 60 mm petri dishes 4 hours before exposure to DNA damaging agents. In panels A and B the cells were exposed to BCNU and melphalan, respectively. After a 6 day incubation, the colonies were fixed and stained with sulforhodamine B. The control plates routinely contained 90 - 100 colonies, cell death was expressed as % survival and calculated by dividing the number of colonies on the treated plates by the number of colonies on the control plates. *Points*, mean of 4 to 6 separate experiments performed in triplicate;*bars*, SE.

**A-CON**= pSVK3 transfected AA8 cells

**AGLY-1,-2,-3** = ANPG transfected AA8 cells


% Control
100
10
1
A
A-CON
AGLY-1
AGLY-2
AGLY-3
0
25
50
75
100
μM BCNU

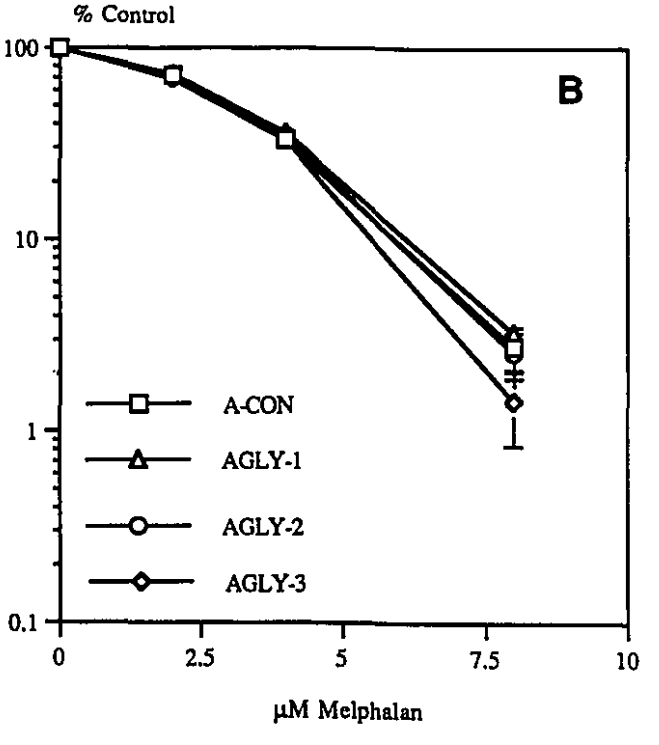

% Control
100
10
1
0.1
B
A-CON
AGLY-1
AGLY-2
AGLY-3
0
2.5
5
7.5
10
μM Melphalan

**Figure 4.3 Glycosylase activity in nuclear preparations from UV20 transfectants.**

Nuclei were prepared according to the method of Cool *et al.* (1989). Glycosylase extracts were prepared from the nuclei in the same fashion as whole cells. Glycosylase activity was measured using 2,5,10 and 20 µl of extract. Protein concentration was determined using the Bio-Rad assay. The activity was estimated from the initial slope of the curves and found to be 2.6, 10, 20, 40 DPM [$^3$H] base released/hour/µg protein for UV-CON, UVGLY-1, -2, and -3, respectively. The results are representative of a single experiment performed in duplicate.

**UV-CON** = pSVK3 transfected cells

**UVGLY-1,-2,-3** = ANPG transfected cells

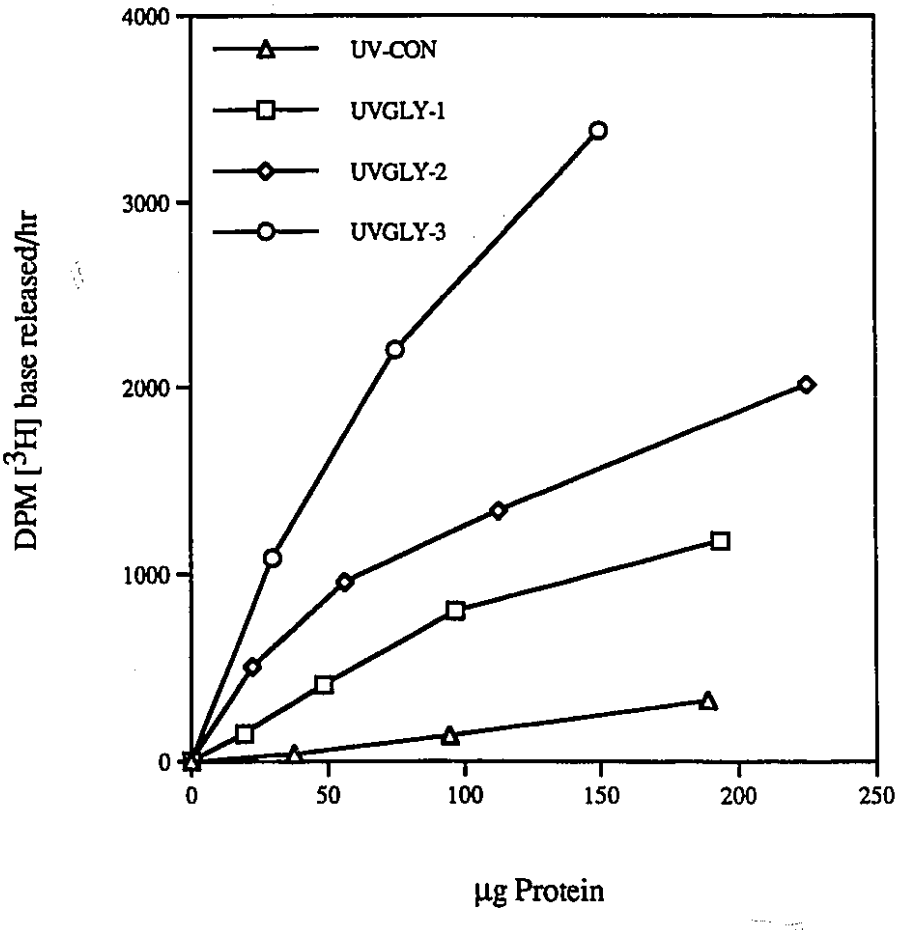
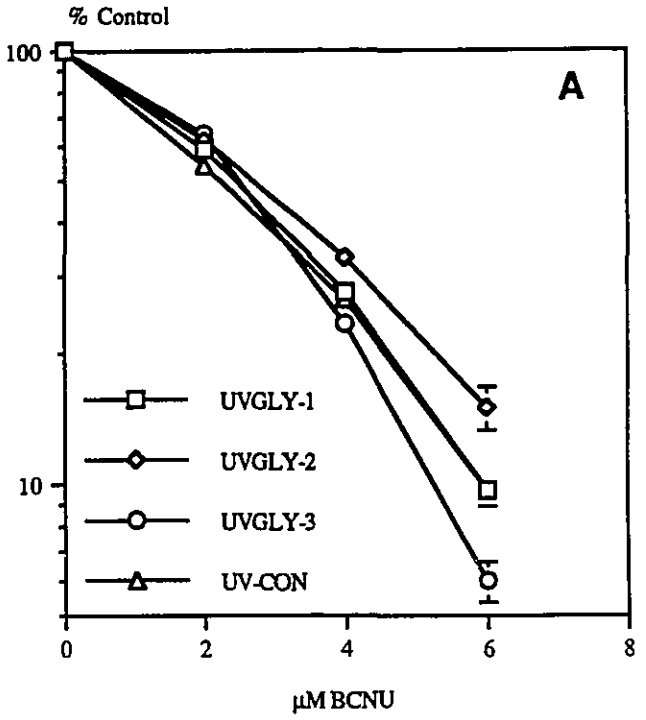

4000
3000
2000
1000
0
0
50
100
150
200
250
UV-CON
UVGLY-1
UVGLY-2
UVGLY-3
DPM [3H] base released/hr
µg Protein

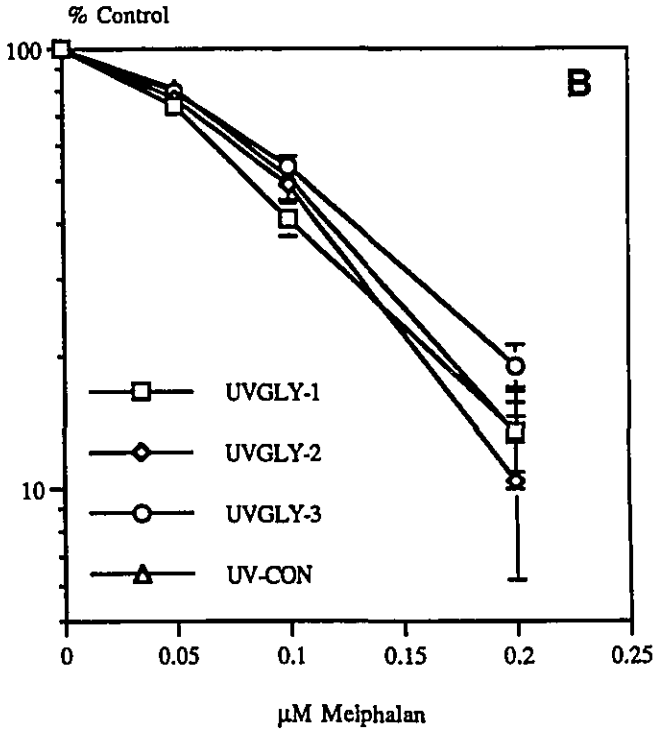
**Figure 4.4 Cytotoxicity analysis of UV20 transfectants.**

Cells were seeded into 60 mm petri dishes 4 hours before exposure to DNA damaging agents. In panels A and B the cells were exposed to BCNU and melphalan, respectively. After a 6 day incubation, the colonies were fixed and stained with sulforhodamine B. The control plates routinely contained 90 - 100 colonies, cell death was expressed as % survival and calculated by dividing the number of colonies on the treated plates by the number of colonies on the control plates. *Points*, mean of 4 to 6 separate experiments performed in triplicate; *bars*, SE.

**UV-CON**= pSVK3 transfected UV20 cells

**UVGLY-1,-2,-3** = ANPG transfected UV20 cells


% Control
100
10
A
UVGLY-1
UVGLY-2
UVGLY-3
UV-CON
0
2
4
6
8
µM BCNU


% Control
100
10
B
UVGLY-1
UVGLY-2
UVGLY-3
UV-CON
0
0.05
0.1
0.15
0.2
0.25
µM Melphalan

## Discussion

While enhanced DNA repair is responsible for resistance in certain tumors, the molecular mechanisms underlying this process are still undefined. The hypersensitivity of certain NER deficient cell lines to bifunctional alkylating agents have implicated NER in this process (Hoy *et al.*, 1985). No glycosylase deficient mammalian cells exist, therefore such an analogy is impossible. Glycosylase deficient bacteria display hypersensitivity to mechlorethamine and ANPG expression in *E. coli* confers resistance to BCNU implying a role for BER in repair of these lesions in prokaryotes (Matijasevic *et al.*, 1993; Appendix 2). It has been shown that purified AlkA (the *E. coli* homologue of ANPG) can release bases from DNA substrates treated with CNUs (Habraken *et al.*, 1991). We also have evidence that AlkA can release bases modified by nitrogen mustards (T. O'Connor, unpublished data). More importantly, human ANPG has been shown to release CNU specific lesions from DNA as well (Matijasevic *et al.*, 1993). With respect to drug resistance, ANPG activity is increased in B-CLL lymphocytes resistant to nitrogen mustards and in glioma cell lines resistant to CNUs (Geleziunas *et al.*, 1993;Matijasevic *et al.*, 1991). Recognition and release of the damaged base may be the key step in repair, therefore we examined the effect of ANPG overexpression on sensitivity to bifunctional alkylating agents in CHO cells.

We have transfected the NER proficient cell line, CHO-AA8, with an expression construct encoding the full length ANPG cDNA. The CHO cell lines were chosen for two reasons: (a) these cells are MER-, so the results with BCNU will not be affected by $O^6$-methylguanine-DNA-methyltransferase (MGMT) (Foote and Mitra, 1984), and (b) the availability of mutant lines allowed us to assess the role of the glycosylase in the absence of NER (Hoy *et al.*, 1985). We developed clonally selected cell lines which expressed between 3 and 13 times the normal ANPG activity. None of these cell lines displayed increased resistance to melphalan or BCNU. We believed that the NER pathway may predominate over BER in these cells and, therefore, we chose an NER deficient cell line (CHO-UV20) in order

to observe the effect of ANPG overexpression in the absence of NER. Again, the glycosylase transfected cells displayed no resistance to melphalan or BCNU.

In spite of these results, there remains strong evidence that BER may play a role in repair of bifunctional alkylating agent damage. The *E. coli* data argues strongly that BER can be determinant in resistance in prokaryotes. A CHO mutant line which displays hypersensitivity to nitrogen mustard and melphalan is also hypersensitive to methyl methanesulfonate (Meyn *et al.*, 1991). As methyl methanesulfonate lesions are repaired by BER, hypersensitivity to this agent indicates impairment in this repair pathway. This suggests that BER is required, at least in part, for wild type resistance to melphalan. The $N^1$-Guanine -$N^3$ Cytosine crosslink which results from CNU alkylation is believed to be responsible for the toxicity of these agents (Erickson *et al.*, 1980). This crosslink is formed via an initial alkylation at the neighbouring $O^6$ position of guanine. Expression of MGMT, which repair lesions at $O^6$-guanine, in CHO cells can render them resistant to CNU (Wu *et al.*, 1991). However, the existence of other lethal lesions has also been demonstrated (Wu *et al.*, 1992). *RecA- E. coli*, which exhibit hypersensitivity to both BCNU and ENU, can be adapted to nitrosourea exposure. The adapted cells are resistant to ENU, indicating proper repair of the $O^6$ lesion, however they are still hypersensitive to the effects of BCNU (Kacinski *et al.*, 1985). $O^6$ alkylation repair will prevent formation of the $N^1$-$N^3$ crosslink, thus other toxic lesions must also result from CNU alkylation. These lesions are likely to be $N^7$ guanine lesions, which represent the bulk of base alkylations following CNU treatment. Such $N^7$ lesions have also been shown to be substrates for the human ANPG enzyme (Matijasevic *et al.*, 1993a).

HPLC analysis of the cellular DNA from the CNU resistant glioma cells following BCNU treatment shows that both the $N^7$-lesions and $N^1$-$N^3$ crosslinks are reduced compared to the sensitive line, consistent with the observation of increased ANPG and MGMT activity (Bodell *et al.*, 1988; Matijasevic *et al.*, 1991). This suggests that, at least in gliomas, ANPG and MGMT may be members of a similar repair system that responds to alkylation damage.

Evidence of this comes from studies which show that MGMT and ANPG transcripts are both inducible upon challenge with mitomycin C or cisplatin, two DNA crosslinking agents (Laval, 1991; Lefebvre *et al.*, 1993). This evidence suggests that both ANPG and MGMT may act in a common pathway to render cells resistant to CNUs and nitrogen mustards.

Expression of the human ANPG in *tag- alkA- E. coli* strains can render these cells resistant to BCNU (Matijasevic *et al.*, 1993; Appendix 2). Furthermore, when *uvrA- tag- alkA- E. coli* (deficient in both NER and BER) are used, ANPG expression results in full complementation of BCNU sensitivity and partial complementation to mechlorethamine (Appendix 2). Thus, it appears that *E. coli* which lack functional crosslink repair can benefit from overexpression of ANPG. The lack of similar effect in the hypersensitive UV20 cell line indicates that there is an important difference in the regulation of BER in mammalian cells. In spite of a 13 fold increase in ANPG activity in the UVGLY-3 cells, there is no increase in resistance to BCNU. One explanation for this is that a second protein may be required in eukaryotic cells which is not required for enhanced repair in prokaryotes. Greater understanding of the BER process in mammalian cells will provide insight into the existence of such a protein. Unfortunately ANPG deficient cell lines have not yet been identified and therefore the involvement of ANPG in alkylating agent resistance is dependent upon the success of transfection experiments like the ones performed in this chapter.

# Chapter 5:

# Identification of the DNA repair pathway associated with nitrogen mustard drug resistance

**Abstract**

In order to identify the repair pathway responsible for resistance, lymphocytes were screened for cross-resistance to a variety of DNA damaging agents. Using the MTT assay, we have shown that B lymphocytes from patients with nitrogen mustard resistant B-CLL reflect their clinical status. Resistant lymphocytes were 5.6 and 4.1 fold more resistant to the nitrogen mustard analogues, chlorambucil and melphalan, respectively. This assay allowed us to classify lymphocytes as nitrogen mustard sensitive or resistant, based on *in vitro* observations. Resistant lymphocytes displayed no increased resistance to either methyl methanesulfonate or UV light, indicating that neither classical base nor nucleotide excision repair is involved in resistance. Resistant lymphocytes were 6.0 and 2.2 fold more resistant to the bifunctional agents, mitomycin C and cisplatin, respectively, suggesting enhanced crosslink repair. Neither glutathione nor glutathione-S-transferase levels correlated with resistance. Finally, we found that both aphidicolin and ara-C could potentiate the toxicity of chlorambucil in B-CLL lymphocytes, but this was not specific for the resistant cells. However, as resistance to ara-C correlated with resistance to chlorambucil and aphidicolin did not, it is our opinion that the combination of aphidicolin and nitrogen mustard could be investigated clinically.

The data from the cross-resistance analysis has been accepted for publication in Mutation Research. The aphidicolin and ara-C data has been accepted in Biochemical Pharmacology.

## Introduction

Accelerated removal of DNA crosslinks in nitrogen mustard resistant B-CLL suggests that DNA repair may be involved in nitrogen mustard resistance. Guanine bases in the DNA are believed to be the primary site of alkylation and interstrand crosslinks can form between 2 nearby guanines. This alkylation could be removed by either base excision repair (BER), nucleotide excision repair (NER), or a poorly defined pathway for crosslink repair. Previously, we reported increased expression of two DNA repair genes, ERCC-1 and Alkyl-N-Purine DNA Glycosylase (ANPG), associated with nitrogen mustard resistance in B-CLL (Geleziunas et al., 1991). As ERCC-1 is a central member of the NER pathway and ANPG is a key protein in BER, it appeared that enhanced DNA repair in nitrogen mustard resistant B-CLL may result from increased activity of either of the pathways individually, or in combination.

Overexpression of ERCC-1 did not render CHO cells resistant to melphalan (Chapter 3). Moreover, we have shown that the preliminary observation of ERCC-1 overexpression was not substantiated when a larger number of patients were sampled (Chapter 3). As well, overexpression of ANPG in CHO cells failed to increase the resistance of these cells to either melphalan or BCNU (Chapter 4). Thus, it is still unclear which DNA repair system underlies the nitrogen mustard resistant phenotype in B-CLL.

We have demonstrated previously that lymphocytes from patients with B-CLL demonstrate resistance to melphalan *in vitro* which correlates with their clinical status using the fast green dye assay (Appendix 3). However, that assay was quite time consuming and it was difficult to process multiple samples. In this chapter, we have used the MTT assay and shown that this assay is also an excellent indicator of *in vitro* resistance. Lymphocytes were screened for *in vitro* resistance to both melphalan and chlorambucil. As a means of identifying the repair process underlying nitrogen mustard resistance in B-CLL, we have screened lymphocytes for cross-resistance to the following DNA damaging agents : UV, methyl

methanesulfonate, cisplatin, and mitomycin C. Glutathione (GSH) and glutathione-S-transferase (GST) levels were assayed in the lymphocytes in order to determine whether this detoxification pathway may also be involved in nitrogen mustard resistance. Finally, we have investigated the ability of two DNA synthesis inhibitors, aphidicolin and ara-C, to potentiate chlorambucil toxicity in B-CLL lymphocytes.

| Patient | sex | Lymphocytes (cells / μl) | Rai Stage | % T lymphocytes | $ED_{50}$ Melphalan (μM) | |
|---|---|---|---|---|---|---|
| | | | **Untreated Patients** | | | |
| S1 | Male | 33 760 | I | 7.0 | 2.3 | |
| S2 | Female | 34 500 | I | 7.0 | 2.7 | |
| S3 | Male | 114 600 | III | 5.0 | 3.5 | |
| S4 | Male | 37 600 | II | 3.0 | 3.6 | |
| S6 | Female | 59 075 | I | 6.5 | 3.7 | |
| S7 | Female | 71 600 | II | 3.0 | 3.9 | |
| S8 | Female | 26 100 | 0 | 5.0 | 4.2 | |
| S9 | Male | 35 600 | I | 8.0 | 4.5 | |
| U1 | Male | 58 500 | II | 6.0 | 9.1 | |
| | | | **Treated-Sensitive Patients** | | | |
| S5 | Female | 97 800 | IV | 5.0 | 3.6 | Off treatment |
| S10 | Male | 75 400 | IV | 2.0 | 4.6 | Off treatment |
| S12 | Female | 28 500 | IV | 2.0 | 5.9 | On treatment* |
| S13 | Female | 77 600 | IV | 4.0 | 5.9 | On treatment* |
| S14 | Female | 27 400 | IV | 4.0 | 6.1 | Off treatment |
| | | | **Treated-Resistant Patients** | | | |
| R1 | Female | 104 000 | IV | 1.0 | 9.7 | On treatment |
| R2 | Female | 79 400 | 0 | 1.0 | 9.8 | On treatment |
| R3 | Male | 66 300 | I | 2.0 | 10.1 | On treatment |
| R4 | Male | 32 000 | IV | 3.0 | 12.0 | On treatment |
| R5 | Female | 59 400 | 0 | 3.0 | 13.0 | Off treatment |
| R6 | Female | 88 600 | 0 | 2.0 | 14.3 | On treatment |
| R7 | Female | 233 000 | III | 2.0 | 16.4 | On treatment |
| R8 | Female | 78 000 | 0 | 1.0 | 17.9 | On treatment |
| R9 | Male | 49 700 | IV | 5.0 | 19.4 | On treatment |
| R10 | Female | 49 400 | II | 3.0 | 32.0 | On treatment |
| R11 | Female | 96 700 | III | 4.0 | 36.7 | On treatment |

**Table 5.1 List of patients and clinical data.**

Staging is according to Rai et al (1975). % T lymphocytes was determined by FACS analysis using an anti-CD3 antibody. $ED_{50}$ is the concentration of drug which results in 50% lymphocyte death following a 3 day incubation. S= sensitive, U= status unknown, R= resistant. The mean T cell contamination in our B lymphocyte preparations was $3.8 \pm 0.4\%$ (s.e.). There was no correlation between the percentage of T cells and resistance to any of the drugs tested.

* Both S12 and S13 became resistant to low dose therapy. S12 responded when the dose was increased, while S13 was treated with fludarabine.

## Results

Cross-resistance analysis:

We have used the MTT assay in order to test B-CLL lymphocytes for sensitivity to nitrogen mustards *in vitro* (this cytotoxicity assay has already been validated for use with these cells (Hanson et al., 1991 ; Shen et al., 1992 )). We have screened the cells for sensitivity to melphalan and correlated the $ED_{50}$ (the dose resulting in 50% cell death) to the clinical data (Table 5.1). Untreated and treated-sensitive patients' lymphocytes are clearly distinguished from treated-resistant patients' cells by this assay. Using the treated-sensitive population to define the upper limits of melphalan sensitivity, sensitive lymphocytes were defined as those with an $ED_{50} \leq 6.1$ μM and resistant lymphocytes were those with an $ED_{50} \geq$ 9.7μM. Lymphocytes from patient U1 ($ED_{50} = 9.1$ μM) seem to be innately resistant, but as it is unknown whether the patient is clinically responsive, this patient was omitted from the group comparisons. We found a 4.1 fold difference in melphalan sensitivity between resistant and sensitive lymphocytes (17.4 μM vs. 4.2 μM, p=0.0001, Table 5.2).

While it has been accepted that resistance to one member of the nitrogen mustards in B-CLL implies resistance to all members of the family, no direct evidence existed to support this. We have tested lymphocytes for resistance to chlorambucil and compared the $ED_{50}$ for chlorambucil to that of melphalan. Chlorambucil is the most commonly used agent in the treatment of this disease. We found a very strong correlation between chlorambucil sensitivity and melphalan sensitivity (p=0.0001, figure 5.1). The fold resistance for chlorambucil is 5.6 (30.0 μM vs. 5.4 μM, p= 0.0034, Table 5.2).

While both BER and NER have been implicated in resistance to bifunctional alkylating agents, neither of these pathways has been directly correlated with resistance. In order to address the potential involvement of either of these repair systems in nitrogen mustard resistance, we screened the cells for cross-resistance to UV light and a monofunctional

alkylating agent, methyl methanesulfonate. Classically, NER is responsible for repair of UV light damage and BER is required for methyl methanesulfonate alkylation repair. However, the B-CLL lymphocytes displayed no cross-resistance to either of these agents (Figure 5.2.A and 5.2.B) indicating that neither system is rate-limiting for nitrogen mustard resistance in B-CLL.

Studies with lymphoblasts from patients with the genetic disease Fanconi's anemia indicate that a pathway separate from BER and NER is required for appropriate resistance to crosslinking agents (Ishida and Buchwald, 1982). In light of our results, it appeared that such a crosslink specific pathway may be involved in the enhanced DNA repair process observed in nitrogen mustard resistant B-CLL. We, therefore, tested the lymphocytes for resistance to two crosslinking agents, mitomycin C and cisplatin. We found a very significant correlation between resistance to melphalan and resistance to both mitomycin C and cisplatin ($p= 0.0001$, Figures 5.3.A and 5.3.B). The resistant patients were 6.0 fold (4.2 μM vs. 0.7 μM, $p= 0.0024$, Table 5.2) more resistant to mitomycin C, but only 2.2 fold more resistant to cisplatin (71.5 μM vs. 32.8 μM, $p= 0.0001$).

GSH and GST levels:

These results support our previous observations of enhanced removal of melphalan induced crosslinks in nitrogen mustard resistant B-CLL lymphocytes, indicating increased DNA repair (Torres-Garcia et al., 1989). It is not surprising that such enhanced repair should recognize lesions by other crosslinking agents. Other reports have suggested that the GSH/GST detoxification pathway may also be involved in nitrogen mustard resistance in B-CLL (Schisslebauer et al., 1990; Johnston et al., 1990). This detoxification system has also been implicated in resistance to both cisplatin and mitomycin C (Xu et al., 1992; Meijer et al., 1993). Therefore, we screened the cells used in our assays for GSH and GST. We found no correlation between either GSH or GST and melphalan resistance. The mean GSH for

sensitive lymphocytes was 26.6 ± 3.3 (n=11) nmole/mg protein and 22.5 ± 2.7 (n=10) for resistant lymphocytes (P = 0.348). GST activity in sensitive cells was 97.4 ± 14.6 (n=11) nmole/mg protein/min and 95.0 ± 11.6 (n=10) in resistant cells (P = 0.8977). We also assessed the activity of the GST α isoform which is believed to be specifically implicated in the metabolism of nitrogen mustards. Not all lymphocytes contained detectable activity and, in those that did, there was no correlation between resistance and activity levels (data not shown). Thus, it is unlikely that this detoxification system plays a role in crosslinking agent resistance in B-CLL.

**Figure 5.1 Correlation of $ED_{50}$ melphalan with $ED_{50}$ chlorambucil.**

Lymphocytes were screened for melphalan and chlorambucil resistance. Melphalan was dissolved in 75% ethanol, 0.032N HCl at a concentration of 2 mg / ml. Chlorambucil was dissolved in DMSO at a concentration of 4 mg / ml. Both drugs were diluted in PBS immediately prior to addition to the cells. The maximum final concentration of diluent in the cell suspension was 0.4%. Each point represents an individual patient and is the mean of 8 replicates.

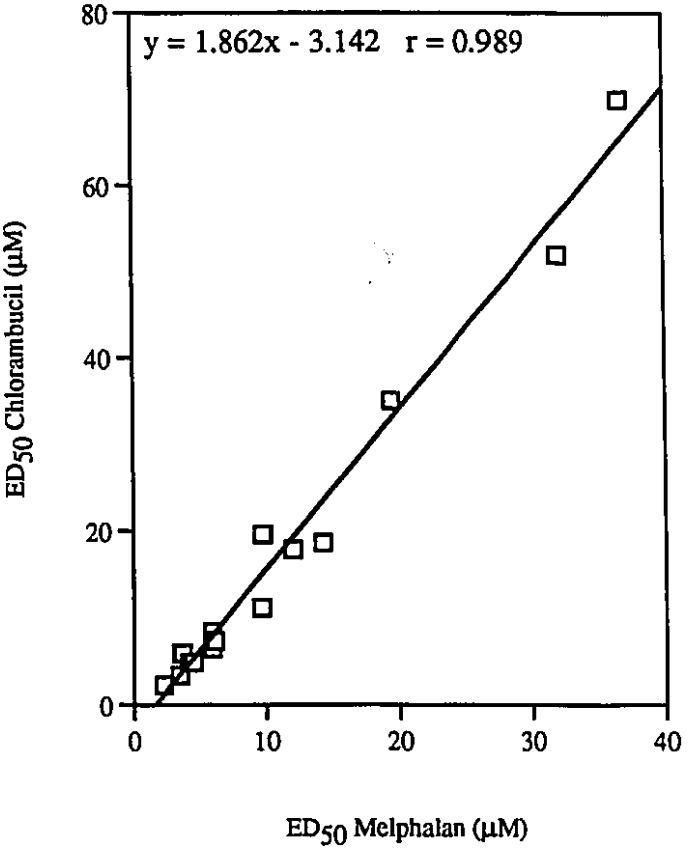
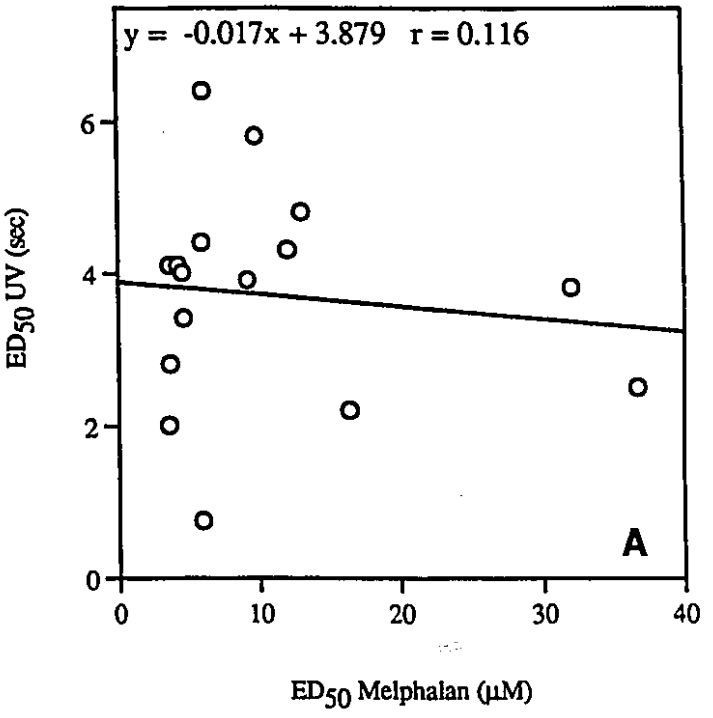

y = 1.862x - 3.142 r = 0.989
80
60
40
20
0
0
10
20
30
40
ED50 Chlorambucil (µM)
ED50 Melphalan (µM)

**Figure 5.2 Correlation of $ED_{50}$ melphalan with $ED_{50}$ UV and $ED_{50}$ methyl methanesulfonate.**

Lymphocytes were screened for resistance to melphalan, UV (panel A) and methyl methanesulfonate (panel B). Cells in 60 mm petri dishes were irradiated with UV light (254 nm) from a 60 Hz, 0.16 A UV lamp at a fixed distance of 19 cm. Methyl methanesulfonate was diluted in PBS prior to addition to the lymphocytes. Each point represents an individual patient and is the result of 8 replicates.


y = -0.017x + 3.879 r = 0.116
ED50 UV (sec)
ED50 Melphalan (µM)
A
0
2
4
6
0
10
20
30
40

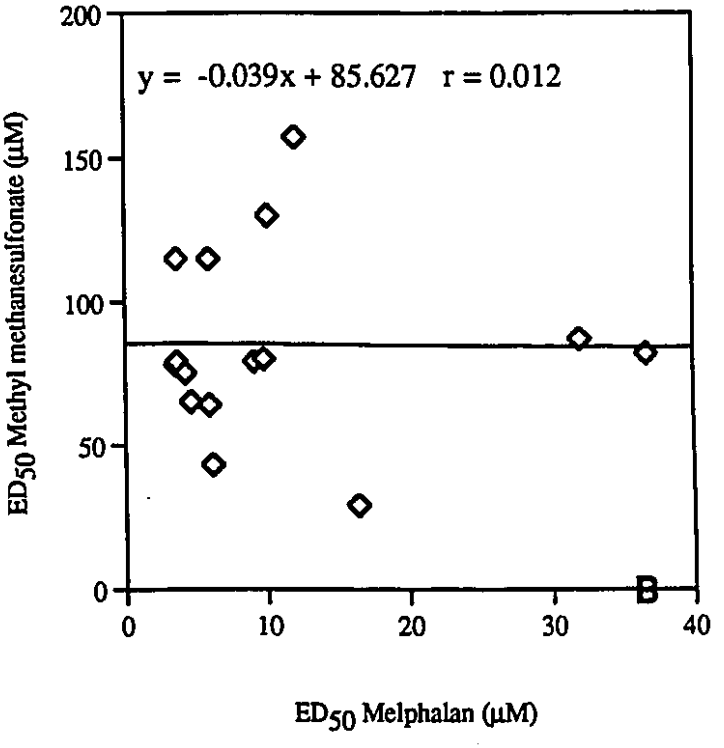
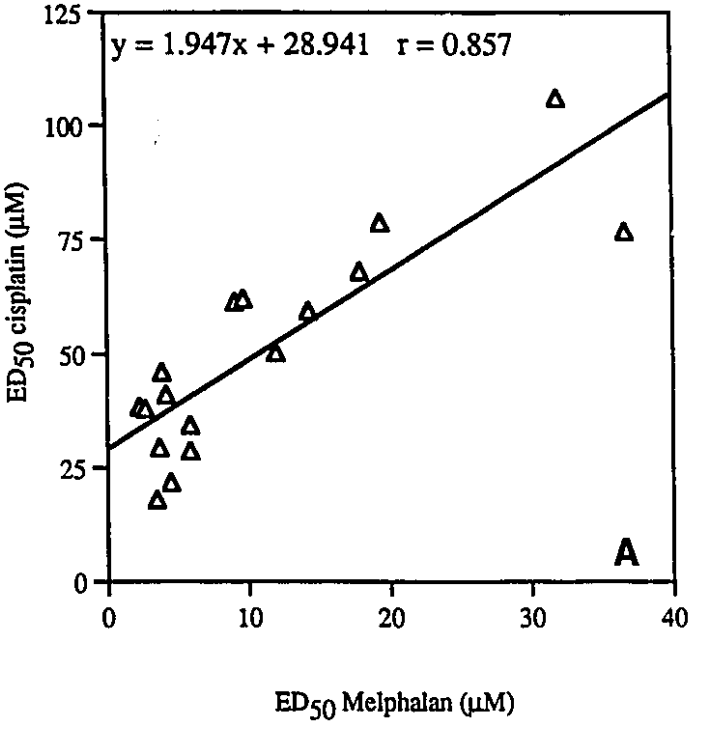
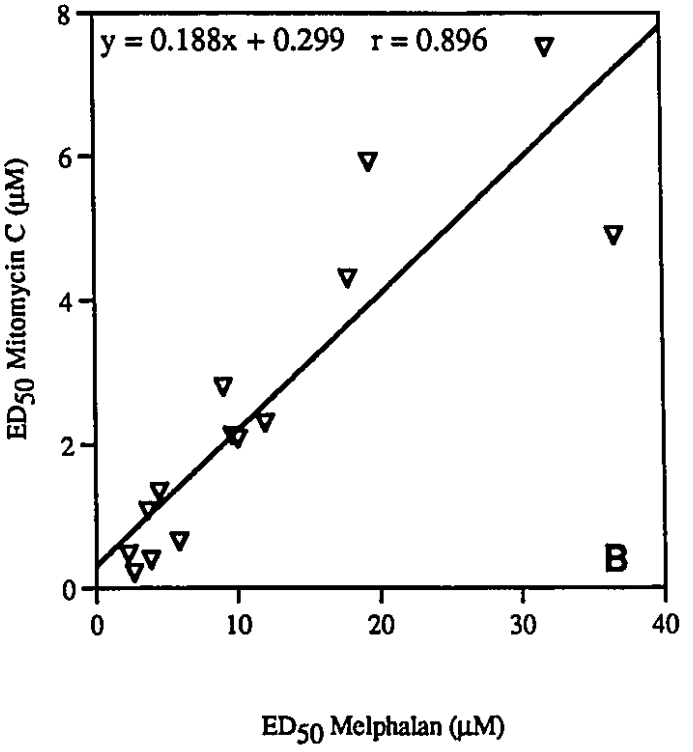

y = -0.039x + 85.627 r = 0.012
ED50 Methyl methanesulfonate (µM)
ED50 Melphalan (µM)
B
0
50
100
150
200
0
10
20
30
40

**Figure 5.3 Correlation of $ED_{50}$ melphalan with $ED_{50}$ cisplatin and $ED_{50}$ mitomycin C.**

Lymphocytes were screened for resistance to melphalan, cisplatin (panel A) and mitomycin C (panel B). Both cisplatin and mitomycin C were obtained from the hospital pharmacy in aqueous solution at a concentration of 1mg/ml. The drugs were diluted into PBS immediately before use. Each point represents an individual patient and is the result of 8 replicates.


y = 1.947x + 28.941 r = 0.857
125
100
75
50
25
0
0
10
20
30
40
A
ED50 cisplatin (μM)
ED50 Melphalan (μM)


y = 0.188x + 0.299 r = 0.896
8
6
4
2
0
0
10
20
30
40
B
ED50 Mitomycin C (μM)
ED50 Melphalan (μM)

| | Sensitive | | | Resistant | | | |
|---|---|---|---|---|---|---|---|
| **Drug** | **mean ± s.e.** | **(n)** | **Range** | **mean ± s.e.** | **(n)** | **Range** | **P** |
| Melphalan | 4.2 ± 0.3 µM | (13) | 2.3 to 6.1 µM | 17.4 ± 2.7 µM | (11) | 9.7 to 36.7 µM | 0.0001 |
| Chlorambucil | 5.4 ± 0.6 µM | (9) | 2.2 to 7.3 µM | 30.0 ± 7.4 µM | (8) | 11.2 to 70.0 µM | 0.0034 |
| UV | 3.6 ± 0.5 sec | (9) | 2.0 to 6.4 sec | 3.9 ± 0.6 sec | (6) | 2.2 to 5.8 sec | n.s. |
| MMS | 79.0 ± 8.8 µM | (8) | 43 to 115 µM | 94.0 ± 18.0 µM | (6) | 29 to 157 µM | n.s. |
| Cisplatin | 32.8 ± 3.0 µM | (9) | 18.0 to 46.0 µM | 71.5 ± 18.1 µM | (7) | 50.3 to 106 µM | 0.0001 |
| Mitomycin C | 0.7 ± 0.18 µM | (6) | 0.21 to 1.34 µM | 4.2 ± 0.8 µM | (7) | 2.1 to 7.5 µM | 0.0024 |

**Table 5.2 Cytotoxicity data sorted by lymphocyte status (sensitive or resistant).**

Lymphocytes were designated either sensitive or resistant based on clinical performance and $ED_{50}$ melphalan (determined *in vitro*); n, number of patient samples. P values were determined using an unpaired t-test; n.s., non-significant (P>0.05).

Potentiation of chlorambucil toxicity with aphidicolin and ara-C:

The main objective of the study of drug resistance is the development of improved therapies by which this resistance could be overcome. It is now clear from our studies that DNA repair is a central mechanism of resistance in B-CLL. However, it is also clear that this enhanced repair mechanism belongs to a undefined pathway and, therefore, will be difficult to target with the standard chemotherapeutic agents. DNA repair can be broken up into two steps: (I) incision and release of the damaged site and (II) resynthesis of the lost DNA. As we know very little about the repair mechanism in B-CLL, targeting step I would be difficult. We can, however, target step II using specific inhibitors of DNA polymerization (resynthesis).

The first inhibitor used was aphidicolin. Aphidicolin competes with dCTP for binding in the active site of DNA polymerase $\alpha$ (Spadari et al., 1985). Freshly isolated lymphocytes were incubated at 37 °C in the presence of various concentrations of aphidicolin for 30 minutes prior to the addition of chlorambucil. The aphidicolin was left in the culture throughout the 3 day incubation period. As the concentration of aphidicolin increased, so did the effect on chlorambucil cytotoxicity (Table 5.3.A). Strong synergy was obtained using this combination in resistant patients' samples (Table 5.3.B). It was our expectation that this combined effect of aphidicolin and chlorambucil would be more pronounced in resistant lymphocytes as compared to sensitive lymphocytes, since the resistant cells display greater crosslink removal. However, when sensitive lymphocytes were screened under the same conditions, similar observations were made (Table 5.3). So, while aphidicolin displays synergy with chlorambucil in our model, this effect is not specific to resistant lymphocytes.

The next DNA synthesis inhibitor used was ara-C. This agent has shown synergy when used in combination with cisplatin (Swinnen, 1989). Ara-C was chosen because this cytosine analog can compete with dCTP for binding in all polymerases tested *in vitro* (Spadari et al., 1985). As polymerase $\beta$ and $\delta$ have also been implicated in the repair reactions (Shivji et al., 1992), it is possible that this inhibitor will provide greater enhancement of toxicity. As

with aphidicolin, the cells were exposed to increasing concentrations of ara-C prior to chlorambucil treatment. The effect of ara-C in combination with chlorambucil was tested in both sensitive and resistant lymphocytes. While strong synergy was obtained (Table 5.4), the steep cytotoxicity curve and varying degrees of resistance made it difficult to obtain concentrations which were non-toxic in all patients' samples (Table 5.4). In fact, it appears that there is a correlation between resistance to ara-C and chlorambucil ($r=0.846$, $P=0.004$). There is no significant correlation between the toxicities of aphidicolin and chlorambucil ($r=0.497$, $P=0.1732$). Thus, while there may be synergy between ara-C and chlorambucil, cross-resistance to ara-C could defeat its usefulness in combinational therapy. It should be noted, though, that the two patients with low-level resistance (EST and EHA) did not display cross-resistance to ara-C. In fact, they exhibited greater sensitivity to ara-C than the sensitive population. It is possible that such patients could benefit from the ara-C/chlorambucil combination.

In the case of both aphidicolin and ara-C, the dose modifying factor was correlated to the toxicity of the inhibitor. In the case of aphidicolin, a comparison of aphidicolin toxicity and dose modifying effect revealed a correlation coefficient (r) of 0.891 ($P=0.0001$). For ara-C, the correlation was slightly weaker ($r=0.759$), but still highly significant ($P=0.0004$).

**Table 5.3.A Dose modification factor (DMF) of the aphidicolin/chlorambucil combination**

B-CLL lymphocytes were exposed to 0, 2, 5, and 12 μM aphidicolin for 30 minutes at 37 °C prior to treatment with chlorambucil. Cytotoxicity was measured using the MTT assay. Cytotoxicity profiles were also established for aphidicolin. The dose modifying factor (DMF) is calculated as the ratio of the $ED_{50}$ chlorambucil to the $ED_{50}$ of the chlorambucil/aphidicolin combination. Toxicity refers to the cytotoxicity of the aphidicolin alone. The data for each patient represents 8 replicates of a single experiment.

**Table 5.3.B Analysis of synergy between aphidicolin and chlorambucil.**

This table presents the analysis of synergy for the data in table 3A. Synergy was determined by the calculation:

$$I_X = \frac{ED_{50}\ C/I_X}{ED_{50}\ CLB} + \frac{Tox\ I_x}{ED_{50}\ I}$$

where $ED_{50}$ $C/I_X$ = the dose of chlorambucil required to produce 50% kill in the presence of aphidicolin at concentration X; $ED_{50}$ CLB= the dose of chlorambucil required to produce 50% kill in the absence of aphidicolin; Tox $I_X$ = the toxicity of aphidicolin alone at concentration X; $ED_{50}$ I= the concentration of aphidicolin required to produce 50% kill in the absence of chlorambucil. According to this formula when $I_X < 1$, then the interaction is synergistic. If $I_X = 1$ the interaction is additive and $I_X > 1$ indicates antagonism (Berenbaum, 1992).

**Table 5.3.A**

| aphidicolin | 2 μM | | 5 μM | | 12 μM | |
|---|---|---|---|---|---|---|
| Patient | DMF | toxicity | DMF | toxicity | DMF | toxicity |
| | | | ***sensitive*** | | | |
| JGO | 1.6 | 0 | 2.2 | 0 | 3.2 | 7 |
| SLA | 1.5 | 2 | 2.8 | 11 | 9.4 | 34 |
| RME | 1.7 | 0 | 2.1 | 7 | 3.4 | 16 |
| SSK | 1.3 | 0 | 1.5 | 0 | 3.0 | 6 |
| NNO | - | - | 2.0 | 3 | 2.4 | 14 |
| MLE | 1.4 | 0 | 1.8 | 0 | 2.2 | 0 |
| | | | ***resistant*** | | | |
| EST | 1.4 | 3 | 1.9 | 9 | 3.3 | 15 |
| PVA | 1.1 | 0 | 1.7 | 0 | 2.2 | 0 |
| EHA | 1.3 | 2 | 1.9 | 5 | 2.6 | 8 |
| AMO | 1.3 | 0 | 1.4 | 0 | 2.4 | 3 |
| FSA | 1.2 | 0 | 1.7 | 0 | 3.2 | 10 |
| SKR | 1.3 | 0 | 1.7 | 0 | 2.3 | 0 |
| RSL | 1.9 | 10 | 2.3 | 10 | 3.1 | 16 |

**Table 5.3.B**

| Patient | $ED_{50}$ Aph | $ED_{50}$ CLB | $I_2$ | $I_5$ | $I_{12}$ |
|---|---|---|---|---|---|
| | | ***sensitive*** | | | |
| JGO | 55 μM | 3.9 μM | 0.65 | 0.55 | 0.53 |
| SLA | 55 | 4.7 | 0.72 | 0.45 | 0.32 |
| RME | 91 | 4.8 | 0.63 | 0.53 | 0.42 |
| SSK | >100 | 5.7 | 0.81 | 0.73 | 0.45 |
| NNO | 132 | 7.4 | N.A. | 0.54 | 0.51 |
| MLE | 251 | 7.8 | 0.74 | 0.58 | 0.51 |
| | | ***resistant*** | | | |
| EST | 88 | 14 | 0.72 | 0.57 | 0.44 |
| PVA | 194 | 15 | 0.92 | 0.61 | 0.51 |
| EHA | 130 | 17 | 0.76 | 0.56 | 0.48 |
| AMO | 203 | 32 | 0.79 | 0.76 | 0.47 |
| FSA | N.A. | 38 | N.A. | N.A. | N.A. |
| SKR | >100 | 50 | 0.82 | 0.65 | 0.56 |
| RSO | N.A. | 75 | N.A. | N.A. | N.A. |

**Table 5.4.A Dose modification factor (DMF) of the ara-C/chlorambucil combination**

B-CLL lymphocytes were exposed to 0, 0.5, 1.0, and 2.0 μM ara-C for 30 minutes at 37 °C prior to treatment with chlorambucil. Cytotoxicity was measured using the MTT assay. Cytotoxicity profiles were also established for ara-C. The dose modifying factor (DMF) is calculated as the ratio of the $ED_{50}$ chlorambucil to the $ED_{50}$ of the chlorambucil/ara-C combination. Toxicity refers to the cytotoxicity of the ara-C alone. The data for each patient represents 8 replicates of a single experiment.

**Table 5.4.B Analysis of synergy between ara-C and chlorambucil.**

This table presents the analysis of synergy for the data in table 4A. Synergy was determined by the calculation:

$$I_X = \frac{ED_{50}\ C/I_X}{ED_{50}\ CLB} + \frac{Tox\ I_x}{ED_{50}\ I}$$

where $ED_{50}$ $C/I_X$ = the dose of chlorambucil required to produce 50% kill in the presence of ara-C at concentration X; $ED_{50}$ CLB= the dose of chlorambucil required to produce 50% kill in the absence of ara-C; Tox $I_X$ = the toxicity of ara-C alone at concentration X; $ED_{50}$ I= the concentration of ara-C required to produce 50% kill in the absence of chlorambucil. According to this formula when $I_X < 1$, then the interaction is synergistic. If $I_X = 1$ the interaction is additive and $I_X > 1$ indicates antagonism (Berenbaum, 1992).

**Table 5.4.A**

| Patient | 0.5 µM DMF | 0.5 µM toxicity | 1.0 µM DMF | 1.0 µM toxicity | 2.0 µM ara-C DMF | 2.0 µM ara-C toxicity |
|---|---|---|---|---|---|---|
| | | | ***sensitive*** | | | |
| SLA | 3.0 | 11 | 4.9 | 13 | 6.9 | 30 |
| RME | 1.6 | 5 | 2.2 | 13 | 9.6 | 45 |
| SSK | 2.7 | 5 | 7.7 | 28 | N.A. | N.A. |
| MLE | 1.8 | 0 | 2.3 | 4 | N.A. | N.A. |
| | | | ***resistant*** | | | |
| EST | 6.5 | 17 | 29 | 46 | N.A. | N.A. |
| EHA | 3.7 | 1 | 6.0 | 14 | N.A. | N.A. |
| AMO | 2.4 | 0 | 3.2 | 0 | 6.1 | 11 |
| FSA | 2.3 | 0 | N.A. | 0 | 4.1 | 6 |
| SKR | 1.6 | 0 | 2.3 | 4 | 4.2 | 25 |

**Table 5.4.B**

| Patient | $ED_{50}$ ara-C | $ED_{50}$ CLB | $I_{0.5}$ | $I_{1.0}$ | $I_{2.0}$ |
|---|---|---|---|---|---|
| | | ***sensitive*** | | | |
| SLA | 3.6 µM | 4.7 µM | 0.50 | 0.48 | 0.70 |
| RME | 3.2 | 4.8 | 0.78 | 0.77 | 0.73 |
| SSK | 2.9 | 5.7 | 0.54 | 0.47 | N.A. |
| MLE | 5.0 | 7.8 | 0.64 | 0.64 | N.A. |
| | | ***resistant*** | | | |
| EST | 1.2 | 14 | 0.57 | 0.87 | N.A. |
| EHA | 2.0 | 17 | 0.46 | 0.67 | N.A. |
| AMO | 6.6 | 32 | 0.48 | 0.46 | 0.47 |
| FSA | 7.3 | 38 | 0.51 | 0.55 | 0.52 |
| SKR | 11 | 50 | 0.67 | 0.53 | 0.43 |

## Discussion

As a means of studying alkylating agent resistance in a clinically applicable model, we have been studying nitrogen mustard resistance in lymphocytes from patients with B-CLL. Our previous studies demonstrated that resistance was associated with increased crosslink removal in lymphocytes from treated-resistant B-CLL patients (Torres-Garcia, 1989). We have, therefore, studied the expression of DNA repair enzymes in nitrogen mustard resistant B-CLL. Two, well defined repair pathways have been implicated in removal of nitrogen mustard crosslinks, base excision repair (BER) and nucleotide excision repair (NER). Our results have failed to demonstrate increased expression of the NER genes, ERCC-1 and ERCC-2 in resistant lymphocytes (Chapter 3). We have demonstrated a 1.7 fold increase in the activity of alkyl-N-purine DNA glycosylase in extracts from resistant patients lymphocytes (Geleziunas *et al.*, 1991). However, transfection of this gene into CHO cells in culture failed to increase the resistance of these cells to nitrogen mustards, in spite of a 13 fold increase in activity (Chapter 4). There is also a third pathway of repair which seems to be specific for DNA crosslinks and is best shown in the genetic disease Fanconi's anemia (Ishida and Buchwald, 1982). Lymphocytes from patients with this disease display hypersensitivity to the effects of mitomycin C and diepoxybutane, but wild type sensitivity to methyl methanesulfonate and UV light. While there are four distinct complementation groups in this disease, only the gene complementary to group C (FACC) has yet been cloned (Strathdee et al., 1992). Overexpression of this gene fails to render cells resistant to crosslinking agents (M. Buchwald, personal communication).

The large number of genes involved in repair of nitrogen mustard crosslinks makes analysis of each individual gene in a large group of patient samples quite difficult. Therefore, we endeavored to identify the repair pathway most likely involved in enhanced nitrogen mustard repair in B-CLL so that we could focus on specific genes involved in that system. To this end, we performed a cross-resistance analysis using DNA damaging agents for which the

repair processes have been well characterized. UV light damage is classically repaired by NER, lesions incurred by methyl methanesulfonate are repaired solely by BER, and mitomycin C has been used to demonstrate crosslink repair deficiency in Fanconi's anemia cell lines. In order to perform this study we needed a cytotoxicity assay which could be done quickly and allow for the processing of multiple samples, as B-CLL lymphocytes have a limited life span in culture. We have shown previously that lymphocytes from patients with B-CLL displayed *in vitro* resistance to melphalan, which correlated with their clinical status. The assay used in that study involved a differential staining technique to distinguish live from dead cells and was quite cumbersome (Bird et al., 1988). It had been reported that the MTT assay could also be used for B-CLL lymphocytes (Hanson et al., 1991; Shen et al., 1992). The results of our study with the MTT assay closely parallel the results of previous study and thus support the use of this microtiter assay for studying resistance *in vitro* (Appendix 3).

A major criticism with the use of melphalan as an indicator of nitrogen mustard resistance *in vitro* is that this drug is not used clinically in the management of the disease. Rather, it is chlorambucil which is most commonly used. We have shown in this paper quite clearly that resistance to melphalan is strongly correlated to chlorambucil resistance. This observation supports the clinical impression that resistance to one member of the nitrogen mustards implies resistance to other members. As well, this information suggests that resistance in B-CLL is raised against the functional group of the drug (*bis*-chloroethylamine). No correlation was observed between resistance to melphalan and resistance to either methyl methanesulfonate or UV light. Therefore, it appeared that neither classical BER nor classical NER were determinant in resistance. The lack of involvement of NER is consistent with the lack of increased expression of NER specific genes in nitrogen mustard resistant lymphocytes. The observation of increased glycosylase activity in the absence of increased resistance to methyl methanesulfonate is not altogether surprising. The glycosylase has been shown to be inducible following DNA damage (Laval, 1991; Lefebvre et al., 1993). Resistant patients' lymphocytes have been chronically treated with nitrogen mustards, and therefore, certain

inducible genes may become constitutively expressed. Also, we had shown previously that increased glycosylase activity correlated with increased [$^3$H] Thymidine incorporation (Geleziunas et al., 1991). The observation of increased glycosylase activity may simply be an epiphenomenon associated with nitrogen mustard resistance. Resistant lymphocytes did display strong cross-resistance to mitomycin C and less so to cisplatin, supporting the hypothesis that the resistant patients' lymphocytes have an increased capacity to repair DNA crosslinks.

Cisplatin, mitomycin C and nitrogen mustards are all structurally and chemically distinct. Nitrogen mustards and cisplatin form primarily N7 guanine lesions, whereas mitomycin C alkylates mainly the N2 position of guanine (Zwelling et al., 1981; Hansson et al., 1987; Szymkowski et al., 1992; Tomasz et al., 1987a). These N2 crosslinks form across the minor groove and result in very little distortion to the DNA (Tomasz et al., 1987b), while N7 lesions span the major groove and cause considerable distortion (as evidenced by altered mobility of melphalan and cisplatin treated DNA in native acrylamide gels (chapter 6; Bellon et al., 1991)). The high level of cross-resistance to mitomycin C in nitrogen mustard resistant B-CLL suggests a common pathway of resistance. The weak cross-resistance to cisplatin is also quite notable. Cisplatin can generate intra- and interstrand crosslinks, both of which have been implicated in cytotoxicity (Szymkowski et al., 1992; Zhen et al., 1992). In contrast, the toxicity of nitrogen mustards and mitomycin C has been correlated to the formation of interstrand crosslinks alone (Zwelling et al., 1981; Hansson et al., 1987; Tomasz et al., 1987b). The intrastrand crosslink represents the major lesion produced by cisplatin. Based on *in vitro* studies, it would appear that this lesion is repaired by the same pathway that processes UV damage. The cisplatin intrastrand crosslink induces conformational changes in the DNA which are similar to UV dimers and can be bound by the same proteins which recognize UV damage *in vitro* (Chu and Chang, 1990; Bellon et al., 1991). Moreover, studies with the *E. coli* (A)BC excinuclease revealed that this UV repair enzyme can also process cisplatin intrastrand crosslinks, but not interstrand crosslinks (Bellon et al., 1991). In

mammalian cells, it is likely that the response to cisplatin damage may require the combined activity of two repair systems, one which recognizes the intrastrand crosslink, and another which processes interstrand crosslinks. Our observation may reflect increased repair of the cisplatin interstrand crosslink but not the intrastrand crosslink; this is supported by lack of cross-resistance to UV. In light of these results, it appears that nitrogen mustard resistance in B-CLL correlates with resistance to other interstrand crosslinking agents.

A similar phenotype has been described for an *E. coli* strain which is resistant to psoralen crosslinks. These bacteria display cross-resistance to mechlorethamine and mitomycin C, but not to UV light or methyl methanesulfonate (Holland et al., 1991). Nitrogen mustard resistant B-CLL appears to be the mammalian counterpart to this *E. coli* model and provides a unique opportunity to investigate the mechanisms of crosslinking agent resistance and repair in human cells. The pattern of hypersensitivity to psoralen crosslinks in other mutants of *E. coli* suggest that genes involved in recombination are required for the repair of interstrand crosslinks (Holland et al., 1991). Thus, in combination with our previous observations (Panasci et al., 1988; Torres-Garcia et al., 1989), we propose that resistance to nitrogen mustards in B-CLL is associated with enhanced repair of interstrand crosslinks which may involve a recombination dependent system.

In the second part of the results section, the use of B-CLL as a model for the development of improved therapeutic strategies has been explored. It is clear from the data that both aphidicolin and ara-C can potentiate chlorambucil toxicity in sensitive and resistant lymphocytes. Clinical trials are currently underway using aphidicolin and ara-C so that dosing schedules and toxicities will soon be known. Aphidicolin provided the best synergism of the two inhibitors, indicating that this combination deserves further investigation. Previous trials have shown that a concentration of 0.5 μM ara-C is achievable *in vivo* and this dose is well tolerated (Donehower et al., 1986). In some cases, we found that 0.5 μM ara-C provided strong potentiation of chlorambucil toxicity (3-6 fold). The drawback of using ara-C is the severe myelosuppression associated with this drug. Moreover, the observation that ara-C

resistance correlated with resistance to chlorambucil further hinders the use of this combination. Therefore, this regime could prove useful in disease treatment, if lymphocytes from the patients could be screened for sensitivity before initiating therapy. Using the MTT assay with lymphocytes from resistant patients, the physician could establish the likelihood of positive outcome using clinically achievable doses of either aphidicolin or ara-C in combination with chlorambucil. Once more, our studies with B-CLL are helping to close the gap between the laboratory and the clinic.

# Chapter 6:

## Identification and analysis of a DNA damage recognition protein which can bind to melphalan damaged DNA

**Abstract**

As a means of identifying damage recognition proteins involved in repair of nitrogen mustard lesions in B-CLL, we performed southwestern analysis using a probe damaged with melphalan and protein extracts from B-CLL patients. We detected proteins of 116, 66, and 64 kD which bound the damaged probe with a higher specificity than the undamaged probe. The 66 and 64 kD proteins were determined to be degradation products of the 116 kD protein. The 116 kD protein was identified as poly(ADP-ribose) polymerase. The use of methoxyamine, an inhibitor of DNA strand breakage following depurination, significantly reduced binding of the melphalan damaged probe to poly(ADP-ribose) polymerase. Following depletion of poly(ADP-ribose) polymerase from the cell extracts, no other binding activity was discovered. Thus, poly(ADP-ribose) polymerase is the only demonstrable protein in B-CLL cells which can bind to a DNA probe damaged with melphalan.

Analysis of the poly(ADP-ribose) polymerase expression by western blot failed to reveal any alteration in expression between sensitive and resistant patients' lymphocytes. Furthermore, the effect of poly(ADP-ribose) polymerase inhibition on cell survival following melphalan exposure was inconsistent. While lymphocytes from some patients were sensitized to the effects of melphalan, others were not. These results indicate that poly(ADP-ribose) polymerase activity may be required for efficient repair of melphalan lesions and the extent of this involvement may be heterogeneous among the B-CLL population.

The data presented in figures 1 through 4 has already been published. [**Bramson, J.**, Prévost, J., Malapetsa, A., Noë, A.J., Poirier, G.G., DesNoyers, S., Alaoui-Jamali, M., and Panasci, L. Poly (ADP-Ribose) Polymerase can bind to melphalan damaged DNA. Cancer Res. 53: 5370-5373, 1993.]

## Introduction

The previous chapters have expanded upon the potential involvement of a crosslink specific DNA repair pathway as being determinant in nitrogen mustard resistance in B-CLL. Using the DNA synthesis inhibitors aphidicolin and ara-C, we have shown that chlorambucil toxicity could be potentiated in a non-specific fashion. Ideally, the inhibitors used in the combination therapies would be specific for the resistant cells. However, in order to produce such "resistance-specific" drugs, it will be necessary to define the key molecular events in the resistance mechanism. The complexity of the repair process makes analysis of the pathway of nitrogen mustard damage repair quite difficult. We postulated that damage recognition might represent a limiting step in crosslink repair and, therefore, endeavored to identify proteins associated with this event.

Over the past several years many groups have identified proteins which bind to sites of DNA damage. These include proteins which bind to cisplatin damaged and ultraviolet light damaged DNA (Chu and Chang, 1990; Donahue et al., 1990; Bissett et al., 1993). While, the exact role of the DNA binding proteins is unknown, they may serve as a signal for repair of the damaged site. Using cisplatin resistant Hela cells, one group identified a binding activity which was increased in the resistant cell line (Chu and Chang, 1990). However, others were unable to demonstrate correlation between the binding proteins and resistance levels in a panel of ovarian carcinoma cell lines (Bissett et al., 1993). We have detected a binding protein in B-CLL lymphocytes which recognizes a DNA probe damaged with melphalan. In this chapter, we present initial characterization of this binding protein, which has been identified as poly(ADP-ribose) polymerase. Using 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose) polymerase activity (Sims et al., 1982), we have examined the effect of this agent on lymphocyte survival following melphalan exposure *in vitro*.

## Results

Identification of binding proteins by Southwestern blot:

In order to investigate the potential role of DNA damage recognition proteins in the repair of melphalan lesions in B-CLL lymphocytes, we performed southwestern analysis using nuclear protein extracts from B-CLL patients' lymphocytes and a DNA probe treated with melphalan. Southwestern analysis has been used previously for the detection of proteins which bind to cisplatin damaged DNA (Chao et al., 1992). Melphalan alkylation of the DNA probe caused a mobility shift upon PAGE analysis as seen in Figure 6.1. We have incubated the probe in the presence of melphalan for both 4 and 24 hours. The 24 hour incubation was done to allow for reaction of all free chloroethyl groups. Initial studies employing gel shift analysis failed to detect any specific binding activity (data not shown). Using southwestern analysis, several proteins were detected at 116, 66, and 64 kD; the 116 kD protein being predominant. The lower molecular weight proteins were not apparent in each sample and the signal at 116 kD was always diminished in those samples where binding to the lower species did appear (data not shown). Thus, the lower molecular weight binding activities would seem to be degradation products of the higher one. While a certain amount of binding is observed when the untreated probe was used, binding increased as melphalan treatment increased (Figure 6.2). Thus, the binding appeared to be specific for the melphalan damage.

Identification of binding activities as poly(ADP-ribose) polymerase:

Poly(ADP-ribose) polymerase (PARP), a well characterized protein implicated in DNA repair, has an apparent molecular weight of 116 kD (Lamarre et al., 1988), similar to the binding activity we have identified. In order to investigate the possibility that the binding protein is PARP, we performed western and southwestern analysis using purified PARP,

samples from 2 B-CLL patients (P1 and P2) and a Hela cell protein extract. The proteins were separated on SDS-PAGE and transferred to nitrocellulose in duplicate. One blot was probed with a radio-labeled DNA probe and the other with a monoclonal antibody raised against PARP (Figures 6.3.A and 6.3.B). It is clear that not only can PARP bind to the melphalan damaged probe but the PARP antibody recognized the same proteins which bound to the melphalan damaged DNA probe. The antibody also detected the 66 kD binding protein in the extract from P1 (Figure 6.3.B, lane 3) confirming this protein as a degradation product of PARP. Therefore, it would appear that the activity observed in B-CLL lymphocytes is PARP.

The binding spectrum of PARP includes single and double DNA strand breaks, as well as single stranded DNA (Lautier et al., 1993). The binding to the melphalan treated probe may represent a novel binding activity or may be a result of structural changes induced by melphalan alkylation. It is well known that alkylation on the N7 position of guanine results in spontaneous depurination. The depurination is then followed by β- and δ- elimination reactions, which can occur spontaneously, breaking the phosphoribosyl backbone (Bailly and Verly, 1988). We sought to determine if melphalan induced nicks were the cause of PARP binding. Denaturing gel analysis of the probe was used to identify nicks induced by melphalan alkylation of the f196. There is significant nick formation following 24 hour treatment with melphalan. Concomitant treatment with methoxyamine, which has been demonstrated to protect apurinic sites from spontaneous nicking (Liuzzi and Talpaert-Borlé, 1985), eliminates breakdown of the melphalan treated probe (data not shown). Southwestern analysis also demonstrates reduced binding of the probe to PARP when alkylation was carried out in the presence of methoxyamine (Figure 6.4, lanes 1 and 4). Thus, binding seems to be largely a function of nicks induced by melphalan treatment, although a minor component of the increased binding may be secondary to melphalan alkylation of DNA.

Finally, in order to identify other proteins which may also bind to melphalan damaged DNA, the extracts were depleted of PARP prior to southwestern analysis. Western analysis of these samples indicated complete depletion of PARP (data not shown). As can be

seen in Figure 4, binding is reduced by the addition of methoxyamine, but it is eliminated in PARP depleted samples. However no other protein was detected. Therefore PARP seems to be the only demonstrable DNA binding protein in B-CLL cells which can recognize melphalan lesions. Western blot analysis of this protein (repeated twice) in B-CLL lymphocyte extracts fails to reveal any alterations in expression of this protein in sensitive lymphocytes as compared to resistant lymphocytes (Figure 6.5).

**Figure 6.1 Effect of melphalan treatment on DNA probe.**

The f196 DNA probe was end labeled with [$\gamma$-$^{32}$P]-ATP using T4 polynucleotide kinase, treated with increasing concentrations of melphalan (0-160 $\mu$M) for 4 or 24 hours and electrophoresed through an 8% acrylamide gel.

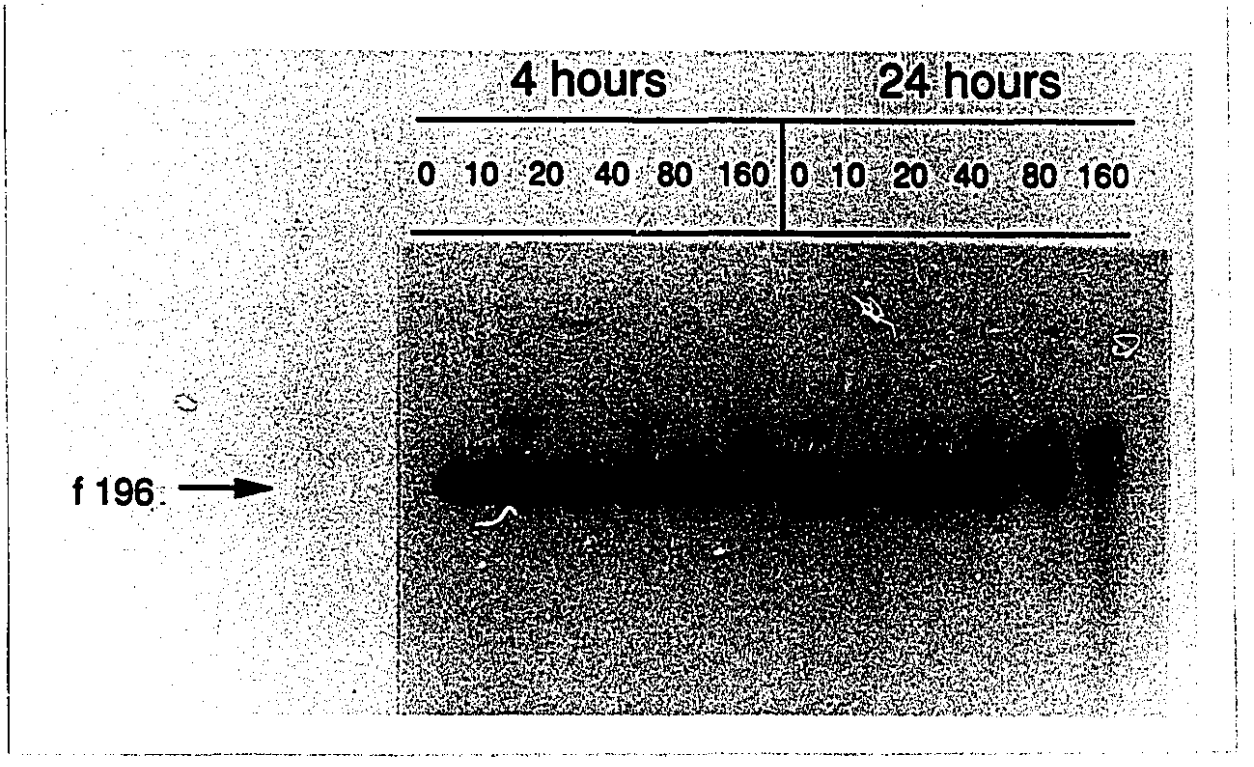

4 hours
24 hours
0 10 20 40 80 160 0 10 20 40 80 160
f 196

**Figure 6.2 Effect of increasing melphalan alkylation of DNA probe on southwestern blot.**

The f196 DNA probe was end labeled with [$\gamma$-$^{32}$P]-ATP using T4 polynucleotide kinase, treated with increasing concentrations of melphalan for 4 or 24 hours and tested for effect on binding by Southwestern analysis. The nuclear extract used for this blot were prepared from lymphocytes obtained from resistant CLL patient P1. Each lane contains 90 µg protein and samples were probed in duplicate.

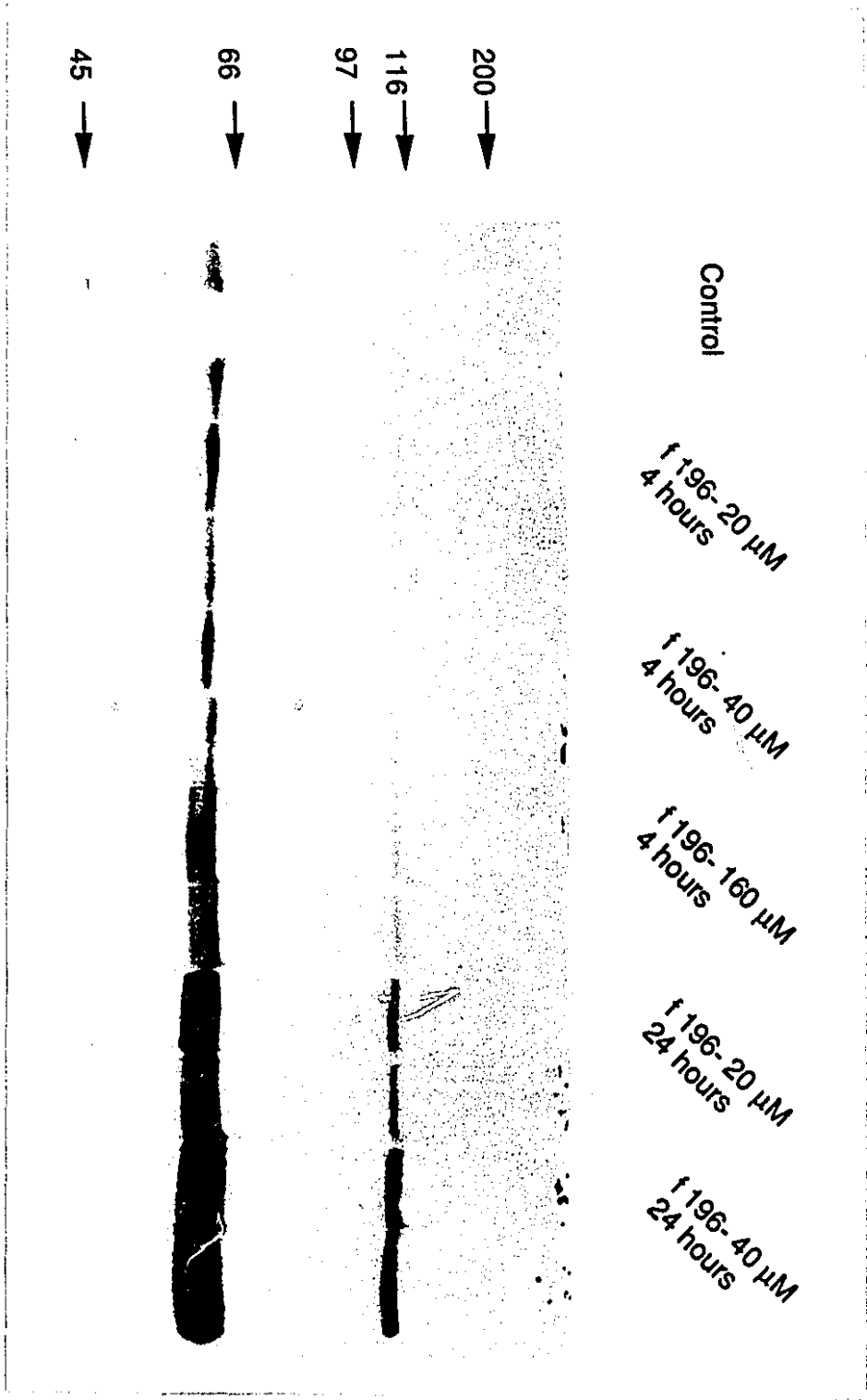

200
116
97
66
45
Control
f 196- 20 µM 4 hours
f 196- 40 µM 4 hours
f 196- 160 µM 4 hours
f 196- 20 µM 24 hours
f 196- 40 µM 24 hours

**Figure 6.3.A Southwestern blot with f196 treated with melphalan.**

The f196 DNA probe was end labeled with [$\gamma$-$^{32}$P]-ATP using T4 polynucleotide kinase and treated with 40μM MLN for 24 hours. H = nuclear extract from Hela cells (50 μg) , P2 = nuclear extract of lymphocytes from untreated CLL patient P2 (50 μg) , P1 = nuclear extract of lymphocytes from resistant CLL patient P1 (50 μg) , PARP = purified poly (ADP-ribose) polymerase (200 ng).

**Figure 6.3.B Western blot with monoclonal antibody against poly(ADP-ribose) polymerase.**

Refer to legend in figure 6.3.A. The western blot was probed with the mouse monoclonal antibody C-2-10.

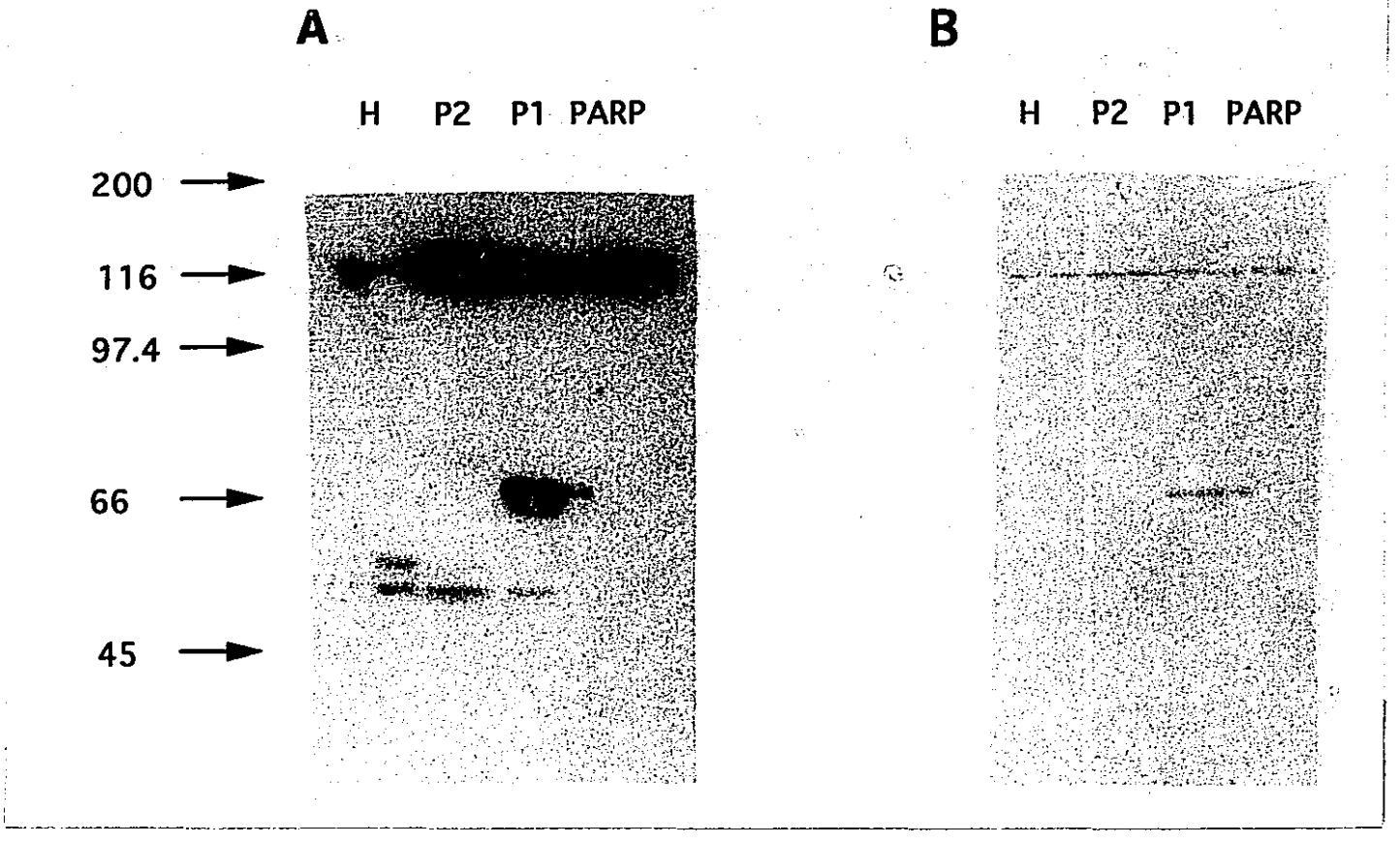

A
H
P2
P1
PARP
200
116
97.4
66
45
B
H
P2
P1
PARP

**Figure 6.4 Effect of poly(ADP-ribose) polymerase depletion on southwestern blot analysis of f196 following melphalan treatment in the absence or presence of methoxyamine.**

The f196 fragment was end labeled with [$\gamma$-$^{32}$P]-ATP using T4 polynucleotide kinase and treated with 40 μM MLN for 24 hrs with 5 mM methoxyamine (lanes 3, 4 and 5) or without (lanes 1 and 2). The extract from P3 was depleted of PARP in lanes 2 and 5. Each lane contains 60 μg of protein. CLL P3 = nuclear extract of lymphocytes from resistant CLL patient P3; Hela = nuclear extract from Hela cells.

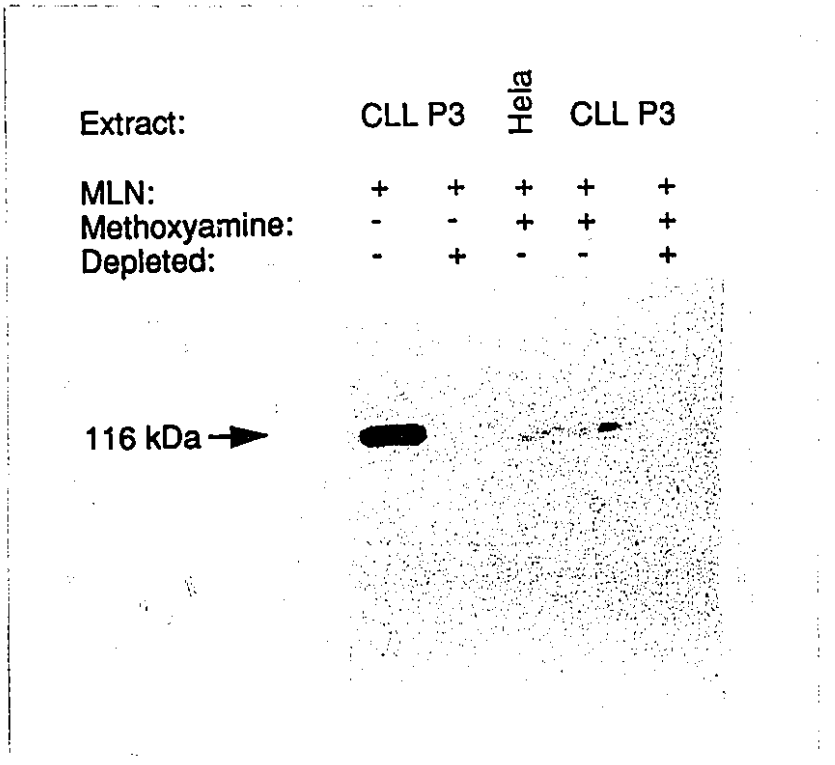
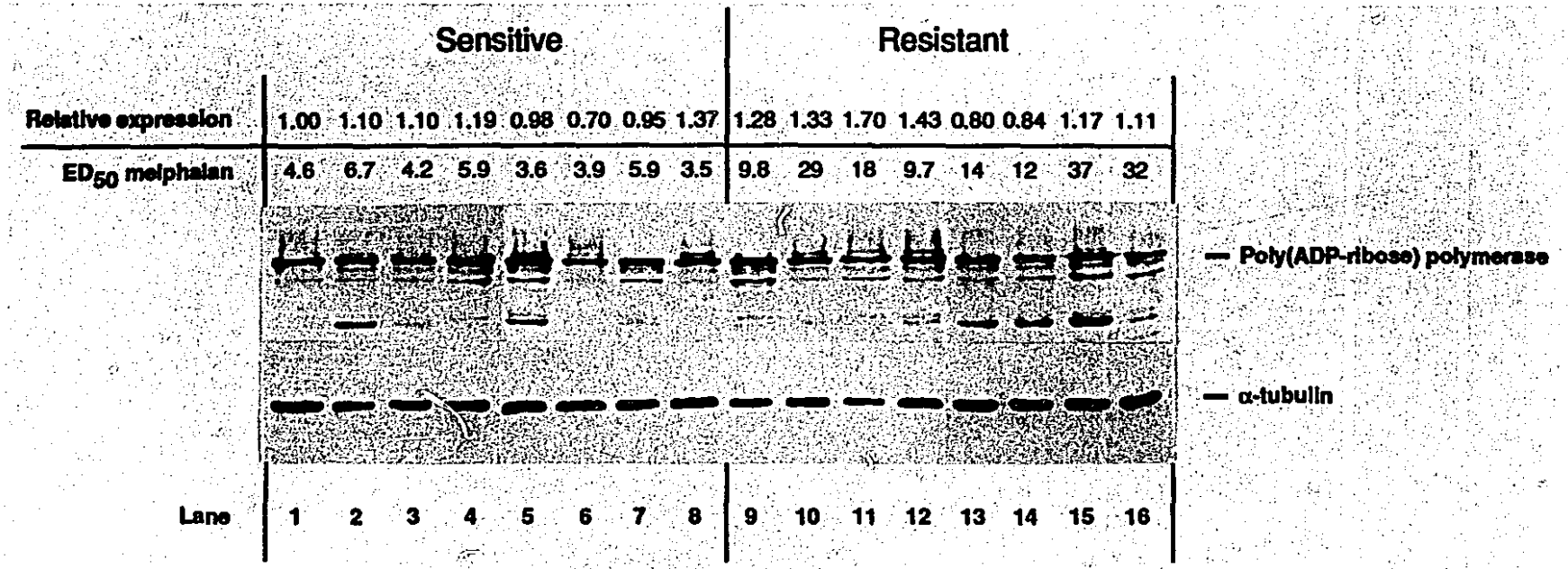

Extract:
CLL P3
Hela
CLL P3
MLN:
Methoxyamine:
Depleted:
+ + + + +
- - + + +
- + - - +
116 kDa

**Figure 6.5 Western blot analysis of PARP expression in B-CLL lymphocytes.**

A western blot was prepared using extracts from 16 individual B-CLL patients' lymphocytes. Lanes 1 to 8 represent sensitive lymphocytes and lanes 9 to 16 represent the 8 resistant lymphocytes. Resistance was based on performance in the MTT cytotoxicity assay. The melphalan sensitivity of the lymphocytes as established *in vitro*, is listed in the figure as $ED_{50}$ melphalan (the dose of melphalan resulting in 50% cell death following 3 day *in vitro* exposure). PARP data was normalized to α-tubulin in order to account for loading differences. Relative expression was calculated by dividing the normalized results for lanes 1 through 16 by the result of lane 1. The mean relative expression for both sensitive and resistant groups is listed below the figure. Using an unpaired, two-tailed t-test, there was no evidence of a significant difference between the two groups. Moreover, when there was no significant correlation between the relative expression and $ED_{50}$ melphalan.




Mean relative expression:

mean sensitive (± s.d.) = 1.05 ± 0.20

mean resistant (± s.d.) = 1.21 ± 0.30

Potentiation of melphalan cytotoxicity using 3-aminobenzamide:

The role of PARP in excision repair is cryptic. As a means of verifying PARP activation following melphalan treatment, B-CLL lymphocytes were exposed *in vitro* to varying concentrations of drug and then the intracellular concentrations of NAD was measured. It was clear that NAD was being depleted in a dose dependent fashion (Figure 6.6.A). Cells were then treated with melphalan in the presence of 3-aminobenzamide (a potent inhibitor of PARP) in order to establish a requirement for this enzyme in cell survival following DNA damage. The concentrations used (0.5, 1.0, and 2.0 mM) demonstrated almost complete inhibition of PARP activity (83 , 91, and 93 % inhibition, respectively) following treatment with 50 μM melphalan (Figure 6.6.B). The combination of melphalan and 3-aminobenzamide displayed synergy in 4 of 7 cases indicating that there is some requirement of PARP in at least 4 of the 7 lymphocyte populations studied (Table 6.1).

**Figure 6.6.A Effect of melphalan on NAD levels in B-CLL lymphocytes**

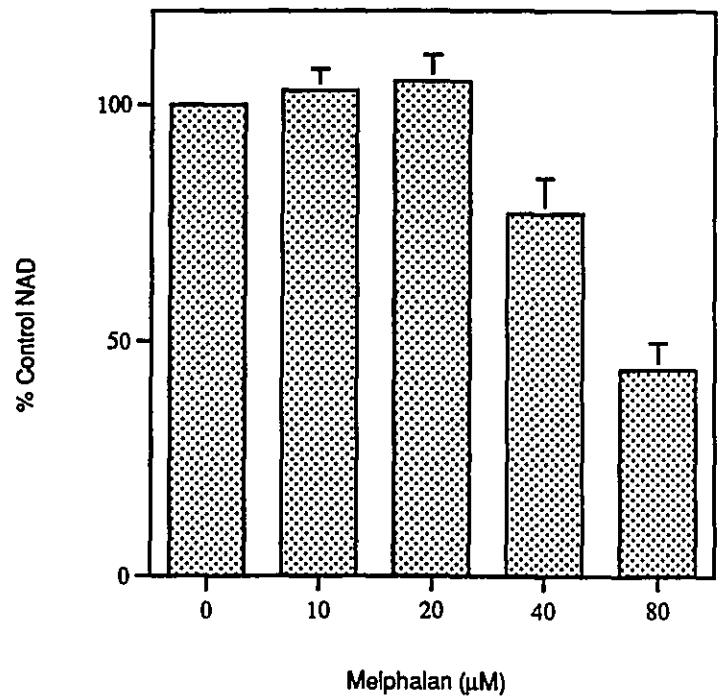
B-CLL lymphocytes were resuspended at a concentration of 3.33 x $10^6$ cells/ml and 3 mls of this stock was exposed to 0, 10, 20, 40, and 80 μM melphalan for 3 hours at 37 °C.

**Figure 6.6.B Inhibition of NAD depletion using 3-aminobenzamide**

B-CLL lymphocytes were resuspended at a concentration of 3.33 x $10^6$ cells/ml and 3 mls of this stock was exposed to 0, 0.5, 1.0, and 2.0 mM 3-aminobenzamide for 30 minutes at 37 °C. The cells were then exposed to 50 μM melphalan and incubated for an additional 3 hours at 37 °C.

Following the incubation at 37°C, the cells were collected by centrifugation and washed twice in PBS. The cell pellet was then lysed in 1 ml of 1M Perchloric acid and neutralized with KOH according to the protocol. The NAD concentration of the samples was measured using the microtiter assay described in the Materials and Methods chapter. These results represent the mean of 5 experiments (± s.e.)

A


100
50
0
% Control NAD
0
10
20
40
80
Melphalan (μM)

B

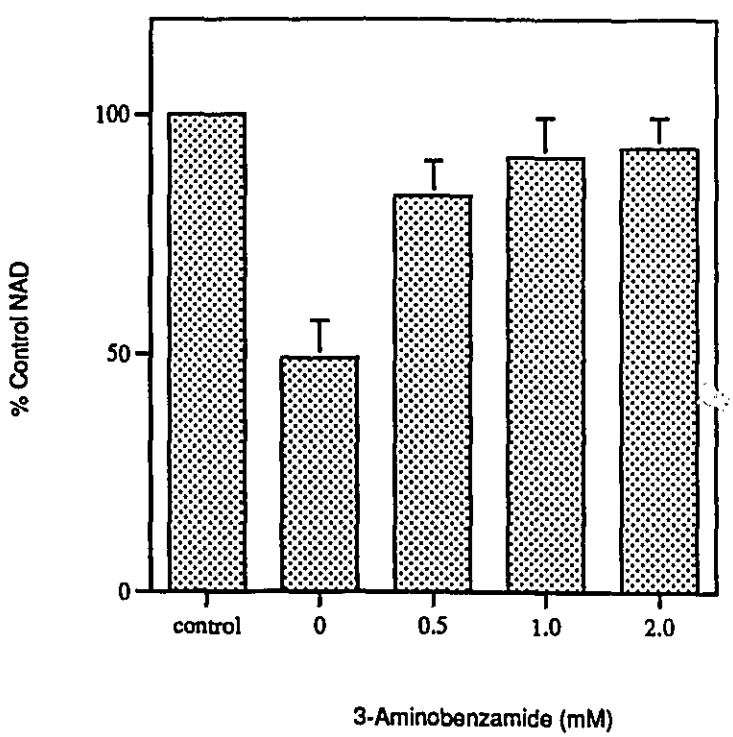

100
50
0
% Control NAD
control
0
0.5
1.0
2.0
3-Aminobenzamide (mM)

**Table 6.1.A Dose modification factor (DMF) of the 3-aminobenzamide/melphalan combination**

B-CLL lymphocytes were exposed to 0, 0.5, 1.0, and 2.0 mM 3-aminobenzamide for 30 minutes at 37 °C prior to treatment with melphalan. Cytotoxicity was measured using the MTT assay. Cytotoxicity profiles were also established for 3-aminobenzamide. The dose modifying factor (DMF) is calculated as the ratio of the $ED_{50}$ melphalan to the $ED_{50}$ of the melphalan/ 3-aminobenzamide combination. Toxicity refers to the cytotoxicity of the inhibitor alone at the specified concentration. The data for each patient represents 8 replicates of a single experiment.

**Table 6.1.B Analysis of synergy between 3-aminobenzamide and melphalan.**

This table presents the analysis of synergy for the data in table 1A. Synergy was determined by the calculation:

$$I_X = \frac{ED_{50}\ C/I_X}{ED_{50}\ MLN} + \frac{Tox\ I_X}{ED_{50}\ I}$$

where $ED_{50}$ $C/I_X$ = the dose of melphalan required to produce 50% kill in the presence of 3-aminobenzamide at concentration X; $ED_{50}$ MLN= the dose of melphalan required to produce 50% kill in the absence of 3-aminobenzamide; Tox $I_X$ = the toxicity of 3-aminobenzamide alone at concentration X; $ED_{50}$ I= the concentration of 3-aminobenzamide required to produce 50% kill in the absence of melphalan. According to this formula when $I_X < 1$, then the interaction is synergistic. If $I_X = 1$ the interaction is additive and $I_X > 1$ indicates antagonism (Berenbaum, 1992).

**Table 6.1.A**

| | 0.5 mM | | 1.0 mM | | 2.0 mM 3-AB | |
|---|---|---|---|---|---|---|
| Patient | DME | toxicity | DME | toxicity | DME | toxicity |
| CCO | 1.4 | 3 | 1.4 | 12 | 1.9 | 23 |
| MFI | 1.6 | 13 | 2.4 | 11 | 2.9 | 20 |
| ADE | 1.0 | 4 | 1.1 | 13 | 1.3 | 16 |
| PVA | 1.6 | 10 | 1.8 | 15 | 2.8 | 26 |
| EST | 1.4 | 0 | 1.6 | 5 | 2.1 | 13 |
| FSA | 0.84 | 4 | 0.84 | 6 | 0.84 | 17 |
| SKR | 0.83 | 0 | 0.86 | 0 | 0.92 | 0 |

**Table 6.1.B**

| Patient | $ED_{50}$ 3-AB | $ED_{50}$ MLN | $I_2$ | $I_5$ | $I_{12}$ |
|---|---|---|---|---|---|
| CCO | 5.1 mM | 3.7μM | 0.83 | 0.90 | 0.90 |
| MFI | 5.4 | 4.7 | 0.63 | 0.61 | 0.71 |
| ADE | 7.1 | 6.2 | 1.04 | 1.02 | 1.07 |
| PVA | 5.2 | 8.0 | 0.72 | 0.74 | 0.75 |
| EST | 5.5 | 10.0 | 0.78 | 0.80 | 0.85 |
| FSA | 5.6 | 17.0 | 1.27 | 1.36 | 1.54 |
| SKR | 13.0 | 24.0 | 1.30 | 1.24 | 1.23 |

## Discussion

Resistance to the nitrogen mustards in B-CLL is associated with increased DNA repair activity in B lymphocytes from these patients. Previous studies have detected proteins which bind to a variety of DNA lesions including cisplatin and UV damage. The exact function of these binding proteins is unknown, but they may be involved in the DNA repair process. In order to investigate the possible role of such a binding protein in the repair pathway associated with nitrogen mustard resistance in B-CLL, we have performed southwestern analysis utilizing protein extracts of B lymphocytes from B-CLL patients and a 196 bp DNA probe which is treated with melphalan. These experiments revealed the presence of several proteins which recognized the damaged probe. The binding was clearly dependent upon melphalan damage since the binding increased as the melphalan treatment increased.

We postulated that the binding protein might be poly(ADP-ribose) polymerase (PARP). Using purified protein and a monoclonal antibody, it was demonstrated quite clearly that the activity present in the B-CLL cells is PARP. Methoxyamine treatment, which protects apurinic sites from subsequent breakdown, reduced binding of the melphalan damaged probe. This indicated that the majority of the binding to PARP may result from melphalan induced nicks in the DNA probe. Finally, in order to identify other proteins which may bind the melphalan damaged probe, we depleted the extracts of PARP, but found no other binding. Thus, PARP appears to bind the f196 DNA probe largely as a result of nicks created by melphalan alkylation, although a minor component of the binding may be due to other melphalan induced alterations in the f196 DNA probe.

The significance of these finding are unclear. PARP deficient cell lines display increased sensitivity to nitrogen mustards suggesting an involvement of this protein in repair of these lesions (Chatterjee *et al.*, 1990). Nitrogen mustard treatment produces N7-alkylguanine lesions which are very labile and subject to spontaneous depurination. The subsequent β- and δ- eliminations that result in strand breaks also occur spontaneously and can

be enhanced by the presence of polyamines like histones (Bailly and Verly, 1988). These breaks are very likely to occur *in vivo* and may play a role in toxicity and repair. Once PARP is bound to melphalan damaged DNA, the resultant auto-modification would lead to a loss of DNA binding affinity. This may account for the fact that we did not find PARP binding in the gel shift assay where the *in vitro* incubation may lead to such auto-modification (unpublished data). PARP activation is believed to cause nucleosomal unfolding via histone shuttling off and back onto the DNA (Althaus, 1992). In DNA excision repair, the tight association of histones with DNA is locally disrupted (Mathis and Althaus, 1990). Thus, the binding of PARP to DNA damaged by melphalan may be the initial step in repair of at least part of this damage. More specifically, the nicks in melphalan damaged DNA may activate PARP which, in turn, allows DNA repair enzymes access to the DNA with consequential repair of the nicks and other DNA lesions incurred by melphalan.

There was no evidence of any alterations in PARP expression in resistant patients' lymphocytes as compared to those from sensitive patients. However, it is clear that this protein is activated by melphalan treatment *in vitro*. When lymphocytes were treated with 3-aminobenzamide prior to melphalan exposure, there was evidence of synergy in 4 out of 7 samples, thus PARP appears to be required in some aspect of the response to melphalan. It is difficult to interpret the results in the remaining 3 lymphocyte populations which were not affected by 3-aminobenzamide. It has been shown *in vitro* that very little poly(ADP-ribosyl)ation is required for excision repair (Satoh et al., 1994). At 2mM 3-aminobenzamide, the mean PARP inhibition is only 93%. Therefore, it is unlikely that PARP is being effectively inhibited by this agent. This would explain why PARP deficient cell lines display such extreme sensitivity to nitrogen mustard (Chatterjee et al., 1990), while the increased sensitivity seen here is less than 3 fold. A further understanding of the PARP involvement in the response to melphalan damage in B-CLL awaits the development of more efficacious inhibitors.

# Chapter 7:

# General Discussion

Drug resistance is one of the major limiting factors in the effective treatment and cure of malignancies. As a result, a great deal of effort has been invested, by both basic and clinical researchers, in trying to understand the mechanisms underlying this phenomenon. Using *in vitro* and *in vivo* models, a number of putative resistance mechanisms have been described (reviewed in chapter 1). However, the clinical relevance of many of these findings is unclear. In an effort to develop a clinically applicable model for resistance, our lab has chosen to study human tumor samples. A drawback to this approach is the technical challenges associated with the use of fresh tissue. Fortunately, leukemic cells can be easily obtained from the peripheral blood of affected patients, particularly when the tumor cells represent the bulk of the leukocyte population. Such is the case in B-CLL, and we have chosen this disease to serve as a model for nitrogen mustard drug resistance. It is our hope that the results of these studies can be extrapolated to other malignancies of both hematological and epithelial origin. The following chapter is not designed to merely repeat the discussions provided at the end of each chapter in the results. Rather, the aim is to summarize this dissertation as a whole, in order to provide a more complete understanding of how the various results complement each other and have furthered our knowledge of nitrogen mustard resistance in B-CLL.

## Nitrogen mustard resistance and overexpression of ERCC-1 and ANPG

Our initial studies indicated that the resistance phenotype in B-CLL was associated with increased repair of DNA crosslinks and enhanced expression of ERCC-1 and ANPG (Geleziunas *et al.*, 1991). As there is no evidence to suggest that these two proteins act in concert, the genes were investigated individually. This was done by transfecting expression constructs containing each gene into CHO cells in culture. Several clones were prepared from each transfection and screened for melphalan resistance.

ERCC-1 was first transfected into the mutant CHO line, UV20. These cells lack a functional ERCC-1 and are hypersensitive to a wide range of DNA damaging agents, including nitrogen mustards. As expected, ERCC-1 expression in these cells complemented their hypersensitivity to melphalan. Surprisingly, when ERCC-1 was then transfected into wild type CHO cells, it produced increased sensitivity to melphalan and cisplatin, but not UV. It is our belief that this observation is a result of repair inhibition resulting from an excess of ERCC-1 protein. Similar inhibition of the uvr(A)BC excinuclease is observed *in vitro* when UvrA is present in excess (Bertrand-Burggraf *et al.*, 1991). In fact, the recent observations that most of the NER proteins associate to form multiprotein complexes suggests that is unlikely that overexpression of any single gene could enhance repair activity (van Vuuren *et al.*, 1993; Biggerstaff *et al.*, 1993; Drapkin *et al.*, 1994; Schaeffer *et al.*, 1994). Unless, of course, this gene product could upregulate the expression of the other proteins it associates with or alter the efficiency of DNA repair in a manner not related to complex formation.

When ANPG was overexpressed in CHO cells, no effect on melphalan sensitivity was produced, either in the presence (AA8 cells) or absence (UV20 cells) of effective NER. There was also no evidence of increased resistance to BCNU. These results were quite surprising in light of the *in vitro* data demonstrating that the *E. coli* AlkA protein could release lesions produced by both mechlorethamine and CNUs (T. O'Connor, personal communication; Habraken *et al.*, 1991). It has also been shown that the human ANPG gene could partially complement the mechlorethamine sensitivity and fully complement the BCNU sensitivity in repair deficient *E. coli* (Appendix 2; Matijasevic, 1993). As enhanced ANPG activity is not sufficient to adequately increase the repair system, another protein is probably required either at the recognition step or the repair step.

In order to further investigate the role of ERCC-1 expression in nitrogen mustard resistance in B-CLL, Northern and western blot analyses of gene expression in both sensitive and resistant patients' lymphocytes were performed. However, contrary to the initial findings, there was no evidence of enhanced ERCC-1 expression in the resistant samples. This was

quite unexpected but, in light of the larger cohort used in this study, it appears the initial findings may have been erroneous as a result of the small sample size used in the original analysis. The expression of a second NER gene, ERCC-2, was also investigated by Northern blot (no antibodies are available for western blots), and found to be the same in both populations of cells.

These findings have failed to support a role for either ERCC-1 or ANPG in nitrogen mustard resistance. Moreover, as our results for ERCC-1 expression are negative, there is little evidence for the involvement of this gene or the NER system in nitrogen mustard resistance in B-CLL. A word of caution is required. The workings of the various repair pathways are quite complex, involving an intimate association with the chromatin template which may not require enhanced gene expression in order to produce increased activity. Thus, while single gene analysis could provide some insight into the genetics of the process, assays which measure changes in specific DNA repair pathways are needed in order to identify the central molecular events.

## Cross-resistance analysis

In order to gain some insight into the repair pathway which is upregulated in nitrogen mustard resistant B-CLL lymphocytes, a cross-resistance analysis was used. In this study, the lymphocytes were screened *in vitro* for resistance to DNA damaging agents with well characterized systems of repair. *In vitro*, nitrogen mustard resistant lymphocytes demonstrate 4.1 and 5.6 fold resistance to the nitrogen mustards, melphalan and chlorambucil, respectively. These cells display cross-resistance to mitomycin C (6 fold) and partial cross-resistance to cisplatin (2.2 fold). There was no difference between the resistant and sensitive populations in sensitivity to UV or methyl methanesulfonate. These results support a role for a crosslink-specific repair process underlying the nitrogen mustard resistant phenotype. Such a repair pathway has been described in *E. coli* for the removal of psoralen interstrand

crosslinks (Cole, 1973; van Houten, 1987). Both nitrogen mustards and mitomycin C produce interstrand crosslinks, explaining the strong cross-resistance (Zwelling *et al.*, 1981; Tomasz *et al.*, 1987b). As cisplatin toxicity has been related to both inter- and intrastrand crosslinks, the partial cross-resistance is probably reflective of only the interstrand crosslink repair (Zhen *et al.*, 1992; Szymkowski *et al.*, 1992).

Interstrand crosslink repair is believed to mediated by a recombination-dependent system. The molecular mechanisms of such a system in mammalian cells are unknown. As with NER, our hope is to gain insight into the genetics of crosslink repair through the study of repair-deficient mutants. In this vein, one gene complementary to the Fanconi's anemia group C cells has been cloned (Strathdee *et al.*, 1992). The function of this gene is a mystery and the recent finding that this protein is localized to the cytoplasm further confuses the issue (Yamashita *et al.*, 1994). However, another way to dissect the molecular mechanisms of this pathway is to study cells in which the system is upregulated. In this way, the rate-limiting steps could be identified and provide us with a greater understanding of the mechanics. An *E. coli* strain has been isolated which demonstrates cross-resistance to psoralen crosslinks, nitrogen mustard and mitomycin C, but wild type sensitivity to UV and methyl methanesulfonate (Holland *et al.*, 1991). Two genes potentially involved in this resistance phenotype were identified, but have yet to be cloned. While bacteria may serve as an excellent venue for the elucidation of repair mechanisms, it is clear from the genetics of NER that the events in mammalian cells are much more complex than those in bacteria (Hoeijmakers, 1991). A mammalian model is required for the study of crosslink resistance and nitrogen mustard resistant B-CLL lymphocytes may provide us with just such a tool. Thus, future studies of this disease could yield not only clinical information regarding drug resistance *in vivo*, but we could also learn how the cell controls its recombinational machinery in order to increase crosslink repair which requires complicated maneuvers, such as homologous recombination.

An interesting observation which can be made from these experiments concerns the toxicity of the crosslinking agents and cisplatin. From our studies, the $ED_{50}$ melphalan for

CHO cells is approximately 4μM, the $ED_{50}$ UV is 4.2 seconds, and the $ED_{50}$ methyl methanesulfonate is 60 μM. These results are similar to the mean of the sensitive lymphocytes ($ED_{50}$ melphalan = 4.2 μM; $ED_{50}$ UV = 3.6 sec; $ED_{50}$ methyl methanesulfonate = 79 μM). It is quite interesting that these DNA damaging agents have similar toxicities in the proliferative CHO cells and the non-proliferative B-CLL cells. This data supports the impression that nitrogen mustards, and most alkylating agents, are phase non-specific. Interestingly, cisplatin is very toxic in CHO cells ($ED_{50}$ = 2.5 μM), yet the $ED_{50}$ in sensitive B-CLL lymphocytes is 32.8 μM. Other cell lines studied in our lab [MCF-7 (breast carcinoma), SKMG-1 (glioma) and SKI-1 (glioma)] demonstrate sensitivities similar to the CHO cells. This observation suggests that part of cisplatin toxicity may be cycle specific. It has been shown that Chinese hamster cells in stationary phase are more resistant to cisplatin than proliferating cells (Fraval and Roberts, 1979) As interstrand crosslinks can be correlated with toxicity in both the proliferative and non-proliferative cells, it would appear that the cisplatin intrastrand crosslink might be responsible for the proliferation-specific toxicity. It has been shown *in vitro* , that several "non-repair" proteins can bind specifically to the cisplatin ApG and GpG intrastrand crosslink, including HMG1, HMG2, and hUBF (Chu, 1994). It has been suggested that one way cisplatin may act is by "high jacking" these proteins away from their proper binding site (Treiber *et al.*, 1994). One of these proteins, hUBF, is very crucial in proliferating cells as it controls rRNA transcription. Mauck and Green (1973) demonstrated that rRNA transcription increases 2.5 to 3.5 fold when quiescent cells are stimulated to proliferate, but the synthesis of heterogeneous nuclear RNA (precursor to mRNA) remains unchanged. The affinity of hUBF for its promoter is the same as the cisplatin damaged DNA. Following cisplatin, treatment the number of platinum-DNA complexes in the cell is in the range of 0.1 - 1 μM, while only 5 nM platinum complexes are required to fully inhibit the hUBF/promoter complex formation (Reed, 1993; Treiber *et al.*, 1994). In cycling cells, sequestration of hUBF by these platinum complexes would inhibit cell survival while in the quiescent B-CLL lymphocyte such seclusion would not be as detrimental. Thus, our observation of 15 fold reduced cytotoxicity

of cisplatin in B-CLL cells as compared to tissue culture cells supports the "high jacking" theory.

**Nitrogen mustard damage recognition proteins**

The observation that poly(ADP-ribose) polymerase (PARP) could bind to melphalan damaged DNA was quite exciting. Given the tight association between this protein and the chromatin machinery, it was quite plausible that PARP could be involved in the initial steps of repair of melphalan damage by relaxing the chromatin and easing access to the repair machinery. It was then demonstrated that the binding appeared to be largely a result of nicks induced by the melphalan treatment. If these nicks were prevented by the addition of methoxyamine, so was the binding of PARP. Similar observations were made using a probe treated with BCNU (Malapetsa *et al.*, manuscript submitted). Even though the main cytotoxic lesion produced by BCNU is the $N^1$-guanine-$N^3$-cytosine crosslink, $N^7$ lesions make up the bulk of the alkylation following treatment. It is these $N^7$ lesions which are unstable and subject to spontaneous depurination which gives rise to single strand breaks. Although these breaks probably induce a certain component of DNA repair, unless the alkylations in resistant lymphocytes are more prone to depurination and breakdown than the corresponding lesions in sensitive cells, this does not explain the observation of enhanced repair.

Nonetheless, when lymphocytes are treated *in vitro* with melphalan, the NAD stores are depleted in a dose dependent fashion, indicating PARP activation following melphalan alkylation. In view of this, we examined the effect of 3-aminobenzamide on melphalan cytotoxicity *in vitro*. We found synergy in 4 of 7 lymphocyte populations studied, suggesting some involvement of this protein in the response to melphalan. In the 2 most resistant patients, we found evidence of antagonism by the PARP inhibitors. Although a potent inhibitor, 3-aminobenzamide is inefficient, even at the maximum concentration (2mM) NAD depletion was only inhibited by 93%. Given that PARP requires only a small amount of

(ADP-ribosyl)ation in order to perform its repair function *in vitro* (Satoh *et al.*, 1994), it is unlikely we are producing enough inhibition to truly block the repair activity of PARP. Thus, further studies are required to fully understand the involvement of the protein in the DNA repair process *in vivo*.

## Potentiation of nitrogen mustard toxicity by DNA synthesis inhibitors

In chapter 6, the DNA synthesis inhibitors, aphidicolin and ara-C, were used as a means of enhancing chlorambucil toxicity. Both combinations produced substantial synergy, suggesting potential therapeutic benefit. One surprising finding from this study was the correlation between ara-C and chlorambucil resistance. Although we only examined a small population (9 patients), the statistics for the correlation indicated that it was highly significant ($P=0.004$ ). This is somewhat confusing because the resistant lymphocytes exhibit about 2 times the DNA synthesis activity of the sensitive cells and might be expected to be more sensitive to ara-C (Geleziunas *et al.*, 1991). However, ara-C resistance could arise from a number of sources including reduced deoxycytidine kinase activity (this enzyme converts ara-C to its toxic form ara-CTP) or increases in the dCTP pool (ara-C is a competitive inhibitor) (Meuth, 1994). It has been recently reported that chlorambucil treatment induces ribonucleotide reductase activity, which, in turn, can increase deoxynucleotide pools (Hurta and Wright, 1992). Therefore, the observed resistance to ara-C may result from cellular changes required for the DNA polymerization system to adapt to the enhanced repair process.

Aphidicolin is considerably less toxic than ara-C in the lymphocytes and demonstrated no significant correlation with resistance. The addition of aphidicolin to the chlorambucil treatment enhanced toxicity up to 3-fold at the concentrations we studied. This is enough to bring some resistant lymphocytes ($ED_{50}$ melphalan = 10-15 μM) into the sensitive range ($ED_{50}$ melphalan = 3-5 μM). As clinical trials are currently underway with aphidicolin, this combination should be considered for diseases currently receiving single agent nitrogen

mustard therapy (B-CLL, multiple myeloma). Also, ara-C was able to produce > 6 fold enhanced toxicity in one resistant patient. As regards therapy, if resistant cells were tested *in vitro*, the patients which would fair best with either aphidicolin or ara-C could be identified before therapy begins.

### Acquired versus *de novo* resistance

In total, the lymphocytes from 16 untreated and 13 resistant patients have been screened for *in vitro* resistance to nitrogen mustard and the resistant population always displayed melphalan sensitivity ≥ 10 μM. We had also screened 5 patients who had responded to treatment and these patients all had sensitivity ≤ 6.1 μM, but were slightly more resistant than the sensitive untreated patients. As a result, we believe that the *in vitro* MTT assay is an excellent tool for identifying resistant lymphocytes. However, of the 16 untreated patients, only 2 (U1 and SKR; Chapter 5) appeared to be *de novo* resistant with $ED_{50}$ melphalan of 9.1 and 24 μM, respectively. This represents 12.5% of the untreated population. According to a recent review of the clinical data, 40% of untreated patients show no response to chlorambucil (Foon *et al.*, 1990). However, in the experience of the Jewish General Hospital, only approximately 20% of the patients are found to be non-responsive. This difference may be a consequence of different criteria for response. Even so, it appears that such non-responsive tumors are underrepresented in our sample population. It is possible that the size of our cohort has limited us in detecting *de novo* resistance. Another possibility is that the lack of response in the clinic is not solely dependent upon lymphocyte resistance. As chlorambucil is administered orally, there are a number of ways that the patient may appear resistant, in spite of having sensitive B-cells. Reduced absorption of the drug, the presence of serum factors which inactivate the drug and increased drug metabolism are all possible mechanisms of resistance. Thus, as discussed in the first chapter, our findings are consistent with acquired resistance, however the mechanisms of *de novo* resistance may differ.

## Multi-drug resistance in B-CLL

Multi-drug resistance is a phenotype associated with cells which have acquired resistance to a specific drug yet display cross-resistance to other drugs which are often structurally or functionally distinct. P-glycoprotein is the classical protein associated with this phenotype. This surface protein can efflux a variety of natural product drugs and imparts a high degree of resistance to the cell associated with decreased intracellular accumulation of drug (Gottesman, 1993). As nitrogen mustard resistant lymphocytes display no evidence of decreased accumulation of either chlorambucil or melphalan (Panasci *et al.*, 1988; Bank *et al.*, 1989), we do not believe that this mechanism underlies the resistant phenotype. Another system which has been associated with the multi-drug resistance phenotype is the GSH/GST detoxification system (Moscow and Cowan, 1988). This system has been implicated in resistance to alkylating agents, like nitrogen mustards, mitomycin C, and cisplatin . A similar pattern of cross-resistance is seen in nitrogen mustard resistant B-CLL, however we have not been able to provide evidence of increased GSH or GST levels in resistant lymphocytes. These results have been confirmed in a separate study (Ribrag *et al.*, 1994). The nitrogen mustard resistant lymphocytes display cross-resistance to mitomycin C, cisplatin, and ara-C independent of P-glycoprotein and the GSH/GST detoxification pathway. Thus, it appears that nitrogen mustard resistant B-CLL is actually a novel multi-drug resistant phenotype.

## Concluding Remarks

The observation of increased crosslinking agent resistance in B-CLL supports our initial finding of enhanced excision repair associated with nitrogen mustard drug resistance. The study of crosslink repair is still in its early stage and the B-CLL model will prove an asset to that research. Resistant lymphocytes represent an ideal setting for the analysis of the

molecular changes associated with enhanced repair. Preliminary experiments might investigate the nature of the deoxyribonucleotide pools in both populations of B-CLL cells, as a means of substantiating the theory for ara-C resistance. Later experiments might involve *in vitro* analysis of crosslink removal using extracts from the sensitive and resistant lymphocytes, as has been done for NER (Wood *et al.*, 1988). Unfortunately, the complex nature of recombinational repair may require the higher order structure of the nucleus in order to coordinate the homologous strands of DNA. Other techniques could be used to look for differentially expressed genes between the sensitive and resistant population. In fact, such a technique is currently being used in our lab as a means of detecting differentially expressed transcripts. Improved culture conditions are extending the longevity of B-CLL lymphocytes *in vitro* and cell lines could probably be developed from patients with varying degrees of resistance (Buschle *et al.*, 1993; Kawata et al., 1993). Future studies should provide unique insights into the mechanisms of interstrand crosslink repair which could only be obtained from resistant cells.

The results of these studies clearly establish B-CLL as a quick, effective system for examining drug toxicity in a clinically relevant model of resistance. Using this strategy, a potential therapeutic effect has been established for the aphidicolin/chlorambucil and ara-C/chlorambucil combinations. By combining the *in vitro* cytotoxicity assay using B-CLL cells with a method for measuring toxicity in hematopoeitic progenitor cells (ex. CFU-C assay), a screening system could be developed to investigate new combinations of alkylating agents and chemosensitizers. This same system could then be used with individual resistant patients, to see who would best benefit from the treatment. The MTT assay has already been used to predict clinical response to chlorambucil in untreated patients with very promising results (Shen *et al.*, 1992; Costin *et al.*, 1992). In conclusion, nitrogen mustard resistant B-CLL is the first demonstration of a tumor which develops multi-drug resistance *in vivo* and can be studied readily *in vitro*. This model should prove useful in understanding the mechanisms of

*in vivo* drug resistance and developing strategies to circumvent resistance in B-CLL, as well as other lymphoid malignancies.

# Chapter 8:

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# Contributions to original knowledge

In fulfillment of the requirements listed in the Guidelines Concerning Thesis Preparation, provided by McGill University, " The thesis for the Ph. D. degree must display original scholarship expressed in good literary style and must be a distinct contribution to knowledge...Elements in the thesis that are to be considered as contributions to original knowledge must be clearly indicated...at the end of the thesis.". The following section will review such contributions from each chapter.

**Chapter 3:** The sensitivity of UV20 cells to melphalan could be corrected by introducing the human ERCC-1 gene. When this gene is overexpressed in wild type cells, it conferred sensitivity to melphalan and cisplatin, but not UV. Enhanced ERCC-1 expression was not associated with the resistant phenotype as determined by Northern and western blot analysis of samples obtained from both untreated and treated-resistant patients. The expression of ERCC-2 was also not related to resistance. These results, in contrast to our initial observations, do not support a role for nucleotide excision repair in the resistant phenotype in B-CLL.

**Chapter 4:** An expression construct containing the complete ANPG cDNA was prepared to produce cell lines which overexpress this gene. In wild type CHO cells, overexpression of ANPG (up to 13 fold) had no effect on cellular sensitivity to either melphalan or BCNU. Similarly, overexpression in the NER deficient UV20 cells, failed to confer resistance to melphalan and BCNU. This data indicates that overexpression of ANPG alone is not sufficient to confer drug resistance.

**Chapter 5:** The MTT cytotoxicity assay used in the study has been shown to clearly distinguish clinically resistant lymphocytes from the total population. Lymphocytes obtained from resistant patients displayed resistance to melphalan, chlorambucil, and mitomycin C and partial cross-resistance to cisplatin. There was no evidence of cross-resistance to either methyl methanesulfonate or UV light. There was no correlation between GSH or GST levels and

nitrogen mustard resistance. These results support our hypothesis of enhanced crosslink repair in nitrogen mustard resistant B-CLL. The toxicity of chlorambucil could be modulated by two DNA synthesis inhibitors, aphidicolin and ara-C. This modulation occurred with both sensitive and resistant B-CLL cells. While there seemed to be a correlation between nitrogen mustard resistance and resistance to ara-C and aphidicolin, only the correlation between chlorambucil and ara-C resistance was significant. The combination of nitrogen mustards and aphidicolin or ara-C should be considered for malignancies which are currently treated using single agent nitrogen mustard therapy, such as B-CLL and multiple myeloma. Since resistance to nitrogen mustards in B-CLL correlates with resistance to mitomycin C, cisplatin and ara-C, this model appears to represent a novel multi-drug resistant phenotype.

**Chapter 6:** Several proteins were detected by southwestern blots using protein extracts from B-CLL patients and a DNA probe damaged to increasing degrees with melphalan. Analysis of these bands identified the major band (116kD) as poly(ADP-ribose) polymerase. The lower molecular weight bands were shown to be degradation products of the 116 kD protein. Depletion of poly(ADP-ribose) polymerase from protein extracts prior to Southwestern blot analysis abolished binding of the melphalan treated probe. When the DNA probe was treated with melphalan in the presence of methoxyamine, binding was significantly reduced. There was no evidence of altered expression of poly(ADP-ribose) polymerase in resistant patients' lymphocytes as compared to sensitive lymphocytes. Melphalan treatment of B-CLL lymphocytes *in vitro* caused a marked reduction in NAD levels, which was dose dependent. Concomitant treatment with 3-aminobenzamide could block this depletion. When lymphocytes were exposed to melphalan and 3-aminobenzamide, melphalan toxicity was enhanced in a synergistic manner in 4 of the 7 samples examined. In 2 samples the interaction displayed antagonism, and 1 case displayed additive toxicity. Thus, the involvement of poly(ADP-ribose) polymerase in repair of nitrogen mustard induced damage may be heterogeneous among the B-CLL population.

# Appendix 1:

# High molecular weight DNA transfection

Based on the clinical observations of nitrogen mustard drug resistance in B-CLL, it appears that resistance is not a transient effect; i.e. when a patient acquires resistance, he/she remains resistant even in the absence of the therapy. Therefore, we postulated that the resistance factor represented a stable change in the lymphocyte which is most likely the result of a genomic alteration. If this were the case, then resistance should be transferable to a non-resistant cell, if this cell integrates the appropriate DNA sequence. Using this rationale, high molecular weight DNA from resistant B-CLL lymphocytes was transfected into rodent cell lines in an attempt to confer resistance. The advantage of using a cell line of a different species is that the DNA of each species contains specific elements, known as Alu sequences. It is estimated that there are $5 \times 10^5$ Alu copies in the mammalian genome, which is approximately 1 Alu sequence every 6 kilobases in human DNA. In a genomic library, human DNA fragments could be distinguished from rodent DNA using these Alu sequences as a probe, facilitating the identification of the fragment of interest.

The first step in this process is the identification of a resistant patient. For this, two patients were employed, 1 of which (R17 in appendix 3) appeared extremely resistant using an *in vitro* cytotoxicity assay. In the early stages of these experiments, I used NIH 3T3 cells. A melphalan cytotoxicity curve was prepared and a toxic dose was extrapolated from the curve. A dose very close to total kill was chosen because it was not clear what degree of resistance would be achieved using the human DNA. All positive clones would be screened separately in order to confirm resistance, thus, any false positives would be quickly detected and false negatives could be minimized. Cells were transfected by the protocol described in the materials and methods, using 18 μg of DNA and 2μg pSV2gpt per dish. This process was repeated 4 times, using a total of 34 plates. Given an approximate transfection efficiency of 0.2% (determined experimentally using high molecular weight salmon sperm DNA), approximately $1.36 \times 10^5$ cells were transfected. A few positive clones were obtained,

however, following the transfection, these clones were difficult to propagate and, shortly thereafter, work with these cells was stopped.

A new cell line was chosen, CHO-AA8, because the culture conditions for these cells following transfection had been well established. These cells were approximately 3 times more resistant to melphalan as compared to the NIH 3T3 line. Again, a similar approach was taken using a dose of melphalan which just produced the desired effect. This process was repeated 4 times, using a total of 60 plates. Given the approximate transfection efficiency of 0.1% (determined using high molecular weight human DNA), approximately $1.2 \times 10^5$ cells were transfected. As the DNA in question was >30 kilobases long, if each cell only incorporated 1 DNA fragment, then the equivalent of 1 haploid genome was screened. These cells most likely incorporated more than 1 fragment and therefore more than 1 genome equivalent was likely screened. From these experiments, 11 positive clones were obtained. Upon screening for melphalan sensitivity, none of these clones appeared more resistant than the parental line. Given the time span required for these experiments (started January 1991 - ended August 1992), the likelihood of success was low, in spite of a valiant attempt by the candidate. Therefore the work was abandoned in order to pursue other projects which appeared more positive.

# Appendix 2:

## ANPG expression confers resistance to BCNU and mechlorethamine in *E. coli*

The following data was provided by our collaborator in the ANPG studies, Dr. Tim O'Connor. The work was done in his lab and he has given permission for it to be reproduced as an appendix to this thesis.

The human ANPG cDNA was subcloned in a prokaryotic expression vector to yield pANPG30. Four strains of *E. coli.* were transformed with this plasmid and a control plasmid, pUC19. Transformants were then screened for resistance to mechlorethamine and BCNU. Cytotoxicity assays were performed according to the following protocol:

An overnight bacterial culture is prepared and used to innoculate 10 - 20 mls of fresh broth. The cultures are grown to an O.D. of 0.6 at 600 nm. Bacteria are collected by centrifugation and resuspended in M9 basic salt medium. Drugs are added and the cells are incubated at 37°C for 20 minutes with gentle agitation. Bacteria are pelleted and resuspended in 10mM MgCl2. Bacteria are plated out as dilutions using top agar, incubated at 37°C overnight, and colonies are counted.

Four cell lines were used in these studies:

**w.t.** = wild type

**NER-** = *uvrA-*

**BER-** = *tag- alkA-*

**NER-,BER-** = *tag-alkA- uvrA-*

In figure A.1, the bacteria were screened for mechlorethamine resistance. In figure A.2, they were screened for resistance to BCNU. The panels on the left represent the control experiments (pUC19) and the panels on the right are the results from experiments using the pANPG30 transformed bacteria.

Figure A.1. Partial complementation by the human ANPG in *E. Coli* mutants sensitive to mechlorethamine.

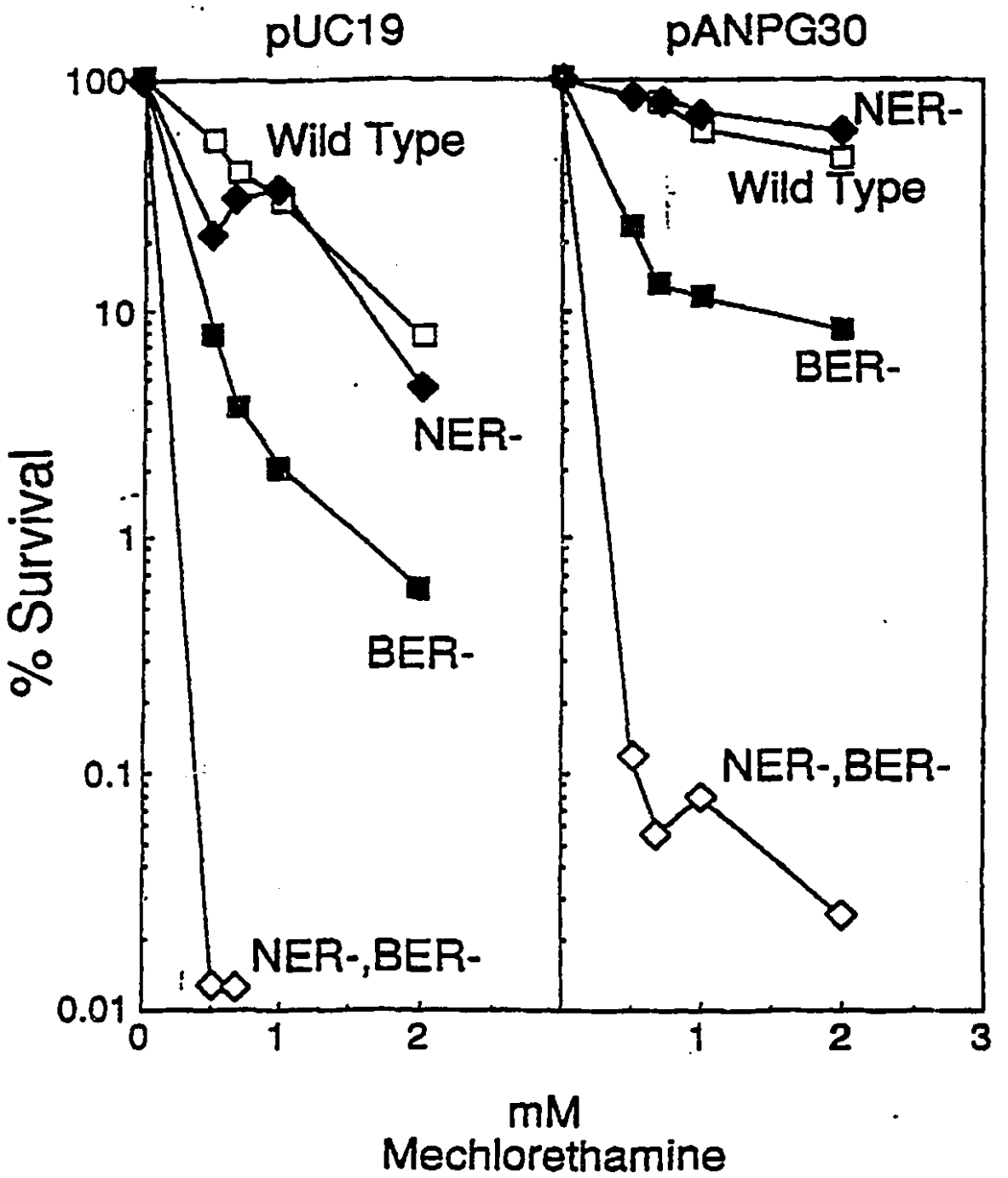
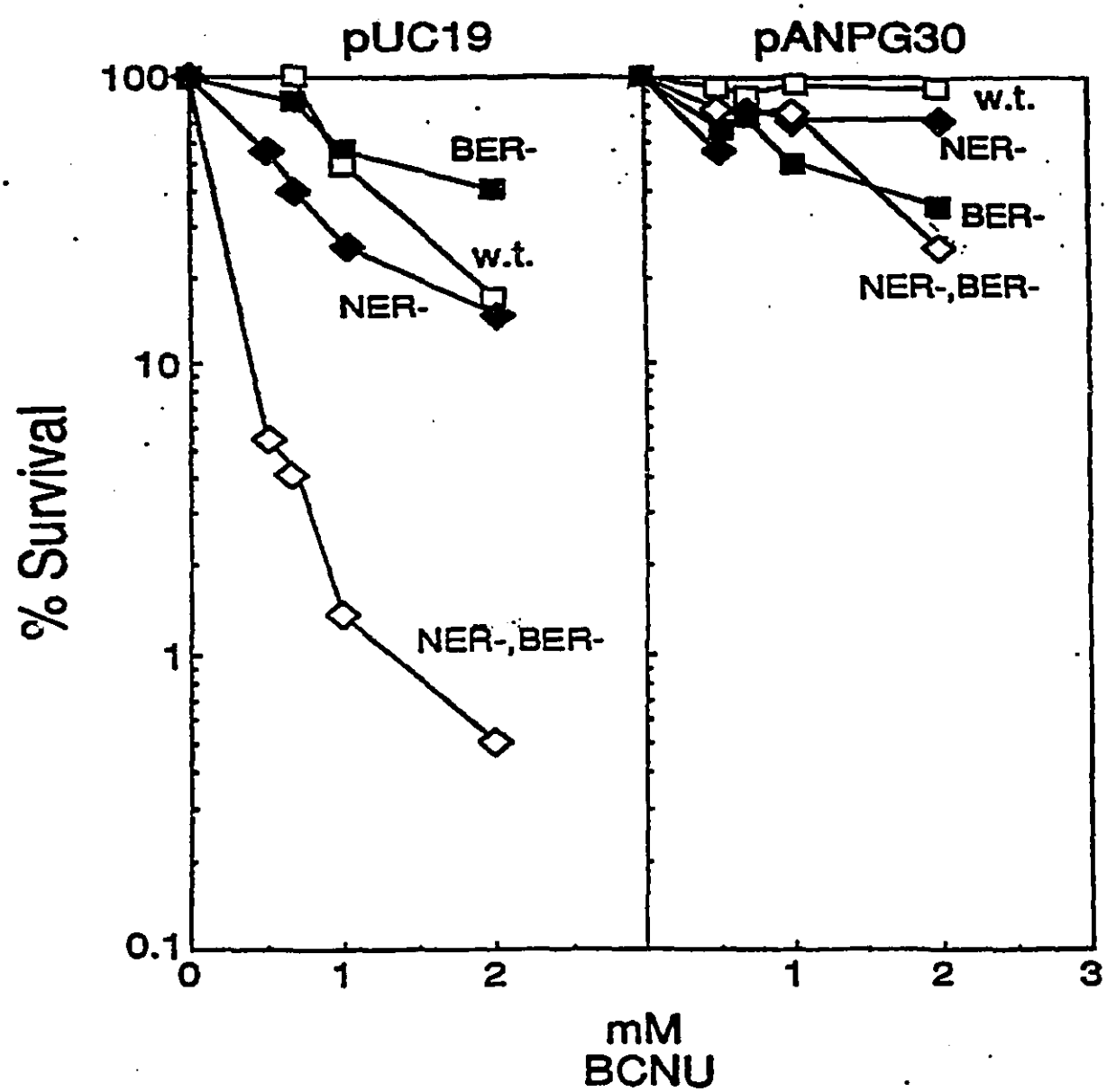


Figure A.2. Complementation by the human ANPG in *E. Coli* mutants sensitive to BCNU.



# Appendix 3:

## Analysis of melphalan toxicity in B-CLL lymphocytes using the DiSC assay.

The initial study of *in vitro* cytotoxicity was performed using a differential staining assay (DiSC). The experiments were performed by the candidate, A. McQuillan, M. Hutchinson and J. Mak.

The DiSC assay was performed according to the following protocol:

Freshly isolated lymphocytes were suspended at a concentration of $5 \times 10^5$ cells/ml in RPMI 1640, 10% FBS, 20mM HEPES, 10 μg/ml gentamycin. Aliquots of 450 μl of the cell suspension were distributed into 4-well dishes (Nunc) and MLN was added to the aliquots as 10X solutions in the same media. Plates are incubated for 3 days at 37°C. Cells were stained by addition of an equal volume of a 2% (w/v) Fast green dye solution (in complete medium) to each well and incubating at room temperature for 8 minutes. Aliquots of 500 μl were then concentrated onto glass slides by centrifugation (Cytospin 2, Shandon) and air-dried. Slides were counterstained using hematoxylin and eosin leaving dead cells green (21). Each concentration was performed in triplicate and a minimum of 100 cells were counted per slide and survivals are given as:

$$\frac{\text{\% live cells on experimental slide}}{\text{\% live cells on control slide}} \times 100$$

The results are summarized in the table on the following page.

**Table A.1**: Sensitivities of untreated and treated-resistant CLL patients' lymphocytes to melphalan *.

| Untreated | $ED_{50}$ | Treated-Resistant | $ED_{50}$ |
|---|---|---|---|
| U6 [a] | 3.6 μM | R2 | 16 μM |
| U9 [a] | 4 | R6 | 23 |
| U17 | 6 | R9 | 15 |
| U20 | 4 | R11 | 16 |
| U23 [a] | 3.5 | R16 | 16 |
| | | R17 | 40 |
| | | R18 | 26 |
| U8 [b] | 26 | R23 | 10 |
| **MEAN ± S.E.:** | **4.2 ± 0.46** | | **20.2 ± 3.3** |

* $ED_{50}$ is the concentration of melphalan which results in 50% lymphocyte death following a 3 day *in vitro* incubation. Cell death was determined by the fast green dye assay in which live cells exclude the dye.

[a] Patients were sampled on two separate occasions, the result is the mean of the two experiments.

[b] Patient was classified as untreated yet was resistant by the *in vitro* assay, not included in calculation of the mean.