

**MECHANISMS OF RESISTANCE TO THE
ALKYLATING AGENTS IN CHRONIC
LYMPHOCYTIC LEUKAEMIA**

Qing Qiu Pu

The thesis submitted to the Faculty of Health Sciences,
University of the Witwatersrand, in fulfilment of the
requirements for the degree of

Doctor of Philosophy

Johannesburg, 1999

**MECHANISMS OF RESISTANCE TO THE
ALKYLATING AGENTS IN CHRONIC
LYMPHOCYTIC LEUKAEMIA**

Qing Qiu Pu

The thesis submitted to the Faculty of Health Sciences,
University of the Witwatersrand, in fulfilment of the
requirements for the degree of

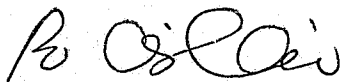
Doctor of Philosophy

Johannesburg, 1999

I declare that this thesis is my own work. No part of it has been submitted before for any degree or examination at any other university. The information used in this thesis was obtained while I was employed by the University of the Witwatersrand.

I declare that this thesis has the approval of the committee for Research on Human subjects and the number of the certificate of the approval is

M950117



Q. Q. Pu

26th Day of March 1999

DEDICATION TO

My Wife, Xin

Parts of this thesis have appeared in the following publications :

Pu Q.Q. and Bezwoda W.R. Induction of alkylator(melphalan) resistance in HL60 cells is accompanied by increased levels of topoisomerase II expression and function. *Mol Pharmacol* **56**: 147-153, 1999.

Pu Q.Q. and Bezwoda W. R. Interleukin-4 prevents spontaneous in-vitro apoptosis in chronic lymphatic leukaemia but sensitizes B-CLL cells to melphalan cytotoxicity. *Br J Haematol* **98**: 413-417, 1997;

Bezwoda W.R. and Pu Q.Q. In-Vitro assessment of melphalan induced interstrand-DNA crosslinks in lymphocytes provides a rapid method for assessment of alkylator sensitivity in patients with lymphoproliferative disorders. *Int J Oncol* **10**: 1061-1068, 1997.

ACKNOWLEDGEMENTS

I am indebted to Professor W.R. Bezwoda, who supervised this project, for his enthusiasm, guidance and encouragement during the course of my studies.

I would like to extend my sincere thanks to Mrs Margaret Bezwoda for her assistance and encouragement and friendship during past years.

It is with my appreciation that the following people are acknowledged :

The staff of Haematology/Oncology Research unit: Karin Amoils, Carol Crowther, Raquel Duarte, Terry Golombick, Lynne McNamara, Nazma Mansoor, Uta Schmidt and Luigi Zampieri for their valuable support and advice in many trying times.

Ms Pepita Bianchi at the Flow Cytometry Unit in the Department of Surgery.

The nursing staff in the Ward 495, Johannesburg Hospital for their valuable assistance in collecting samples.

All staff of Photo Illustration Unit, Wits Medical School, especially Mr Charles May and Mr Terence Borain for their efficient and immaculate preparation of pictures and graphs.

The staff of the Library of the Faculty of Health Sciences, University of the Witwatersrand.

Thanks to all those healthy donors and patients who voluntarily and gladly donated their blood without which these investigations would not have been possible.

During the performance of this work I was in receipt of a University of Witwatersrand Medical Faculty Research Endowment Fund in 1995 and a grant from Richard Ward Fund in 1995-1997.

Finally, I wish to thank my wife, Xin. Her encouragement, continual patience, interest in my work and the sense of humour have eased my task considerably and have been a constant source of inspiration.

TABLE OF CONTENTS

		Page
	DECLARATION	iii
	DEDICATION	iv
	PUBLICATIONS	v
	ACKNOWLEDGEMENTS	vi
	ABSTRACT	viii
	TABLE OF CONTENTS	xvi
	LIST OF TABLES	xxii
	LIST OF FIGURES	xxv
	LIST OF ABBREVIATIONS	xxxi
1.0	<u>INTRODUCTION-- Chronic Lymphocytic</u>	1
	<u>Leukemia(CLL)</u>	
1.1	Epidemiology	2
1.2	Etiology	3
1.2.1	Familial and Environmental Factors	3
1.2.2	Viruses	4
1.3	Biology	5
1.3.1	Clonality	5
1.3.2	Phenotypic Characteristics of the Neoplastic	6
	B Lymphocyte in CLL	
1.3.3	Normal Cellular Counterpart of the CLL B Cell	11

	Page
1.3.4 B Cell Differentiation, Antibody Production and Gene Expression	18
1.3.5 T and Natural Killer Cells	21
1.3.6 Hypogammaglobulinemia	23
1.3.7 Autoimmune Phenomena	24
1.3.8 Cytokines	27
1.3.9 Cytogenetic Abnormalities	28
1.3.10 Oncogenes	30
1.4 Clinical Features	35
1.4.1 Clinical Presentation and Laboratory Abnormalities	35
1.4.2 Diagnostic Criteria	37
1.4.3 Prognosis	41
1.4.4 Therapy	48
1.4.4.1 Treatment Goals	48
1.4.4.2 When to Treat	49
1.4.4.3 How to Treat	52
1.4.4.3.1 Patients with Low-Risk Disease	52
1.4.4.3.2 Patients with Intermediate-and High-Risk Disease	53
1.4.4.3.3 Patients with Cytopenias due to Immune Mechanism	55
1.4.4.3.4 Patients with Hypersplenism	56
1.4.4.3.5 Therapy of Systemic Complications	56
1.4.4.3.6 Disease Transformation	57
1.4.4.4 New Treatment Approaches	58

	Page
1.4.4.4.1 Purine Analogs	58
1.4.4.4.2 Biotherapy	61
1.4.4.4.3 Bone Marrow/Hemopoietic Stem Cell Transplantation	62
2.0 <u>Drug Resistance--INTRODUCTION</u>	64
2.1 Mechanisms of Resistance to Alkylating Agents-- Introduction	65
2.1.1 Biochemistry of Alkylating Agent Adduct Formation	65
2.1.2 Resistance due to Decreased Cellular Uptake of Agents	68
2.1.3 Intracellular Inactivation of Alkylating Agents	69
2.1.3.1 Glutathione and Glutathione-S-transferases	69
2.1.3.2 Metallothionein	73
2.1.3.3 Specific Metabolism	74
2.1.4 DNA Repair	78
2.1.5 Polymerases and DNA Repair	83
2.1.6 In Vivo Resistance to Alkylating Agents	86
2.2 Topoisomerases and Drug Resistance	87
2.3 Multidrug Resistance	90
2.3.1 Chromosomal Location and Genomic Organisation of the MDR Gene	91
2.3.2 Molecular Genetic Studies	93

	Page
2.3.3 Tissue Distribution of MDR Expression	93
2.3.4 Clinical Significance	94
3.0 <u>THE AIMS OF THIS THESIS</u>	97
4.0 <u>Materials and Methods</u>	100
4.1 Materials	100
4.2 Methods	100
5.0 <u>Cytotoxicity, Interstrand-DNA-Crosslinks and</u>	109
<u>Cell Cycle Analysis in the Melphalan-Sensitive B-</u>	
<u>Lymphocytic Cell Line(WIL2) and in the</u>	
<u>Melphalan-Resistant B-CLL Cell Line(WSU-CLL)</u>	
5.1 Introduction	109
5.2 Materials and Methods	111
5.3 Results	113
5.4 Discussion	122
6.0 <u>Melphalan Accumulation, Glutathione Levels, and</u>	131
<u>Glutathione-S-Transferase Activity In WSU-CLL</u>	
<u>and WIL2 Cell Lines</u>	
6.1 Introduction	131

	Page
6.2 Materials and Methods	132
6.3 Results	136
6.4 Discussion	142
7.0 <u>Increased Levels of Topoisomerase II Expression</u> <u>and Function in a Melphalan-Resistant B-CLL Cell</u> <u>Line (WSU-CLL) Which is Sensitive to</u> <u>Doxorubicin</u>	149
7.1 Introduction	149
7.2 Materials and Methods	151
7.3 Results	159
7.4 Discussion	179
8.0 <u>DNA Polymerase α Activity of in WSU-CLL Cells</u> <u>as Compared to WIL2 Cells</u>	186
8.1 Introduction	186
8.2 Materials and Methods	187
8.3 Results	189
8.4 Discussion	192

		Page
9.0	<u>Interleukin 4(IL 4) Prevents Spontaneous In-Vitro</u>	198
	<u>Apoptosis in Chronic Lymphatic Leukemia but</u>	
	<u>Sensitises B-CLL Cells to Melphalan Cytotoxicity</u>	
9.1	Introduction	198
9.2	Materials and Methods	199
9.3	Results	202
9.4	Discussion	209
10.0	<u>Summary</u>	212
11.0	<u>References</u>	219

LIST OF TABLES

No.	Heading	Page
1-1	Similarities and Differences Between B-CLL CD5 ⁺ B Cells and Normal CD5 ⁺ B Cells	12
1-2	CLDs of B-cell lineage: Morphologic Features in Differential Diagnosis	15
1-3	CLDs of B-cell lineage: Immunohistologic and Genetic Features in Differential Diagnosis	17
1-4	Chronic Lymphocytic Leukemia: Diagnostic Workup	38
1-5	Chronic Lymphocytic Leukemia: Diagnostic Criteria	39
1-6	Chronic Lymphocytic Leukemia: Rai Staging System	42
1-7	Chronic Lymphocytic Leukemia: Binet Staging System	43
1-8	Chronic Lymphocytic Leukemia: Other Prognostic Factors.	44
1-9	Smouldering Chronic Lymphocytic Leukemia: Diagnostic Criteria.	46
2-1	Nuclear DNA Polymerases	84
2-2	Expression of PGP Genes in Human, Mouse and Hamster	92
4-1	Materials used in the Experiments	101
5-1	The Rates of Removal of Melphalan-Induced Interstrand-DNA-Crosslinks	121

No.	Heading	Page
5-2	Cell Cycle Characteristics of WIL2 Cells and WSU-CLL Cells after 10 μ M Melphalan Treatment	124
6-1	BCH and the Absence of Sodium Inhibition of [^{14} C] Melphalan Uptake(3 min) in WIL2 Cells and WSU-CLL Cells	140
6-2	Glutathione Levels, and Glutathione-S-Transferase Activity	144
7-1	Effect of Melphalan, Doxorubicin or Camptothecin on WIL2 Cells and on Alkylator-Resistant Cells(WSU-CLL)	
7-1a	IC ₅₀ Concentrations for Melphalan, Doxorubicin and Camptothecin	160
7-1b	IC ₅₀ Concentrations for Melphalan in Alkylator-Sensitive Cells(WIL2) and Alkylator Resistant Cells(WSU-CLL), with and without Doxorubicin Pretreatment	163
7-2	Regression Analysis of the Kinetics of Interstrand-DNA-Cross-Links Formation and Removal	167
7-3	Topoisomerase II Catalytic Activity	170

No.	Heading	Page
7-4	The Affinity of Topoisomerase II for Native and for UV- Irradiated (Crosslinked) DNA	
7-4a	No Pretreatment with the Topoisomerase II Inhibitor Doxorubicin	173
7-4b	Following Pretreatment with the Topoisomerase II Inhibitor Doxorubicin	174
8-1	Activities of DNA Polymerase α and β in WIL2 Cells and WSU-CLL Cells	190
8-2	Effect of Aphidicolin on DNA Polymerase- α	191
8-3	IC ₅₀ Concentrations for Melphalan in WIL2 Cells and Alkylator Resistant WSU-CLL Cells; with and without Aphidicolin Pretreatment	195
9-1	Clinical and Hematologic Details of Patients with Chronic Lymphatic Leukemia	200

LIST OF FIGURES

No.	Legend	Page
2-1	Structures of Commonly Used Alkylating Agents	67
2-2	Structure and Constituent Amino Acids of Glutathione	70
2-3	The Oxazaphosphorine Alkylating Agents, the Active Metabolites of Which Are Inactivated by Aldehyde Dehydrogenase	75
2-4	The Metabolism of Cyclophosphamide	76
2-5	O-6 Chloroethylguanylic Acid and O-6 Benzylguanine, Substrates for O-6-Alkylguanine- Alkyltransferase.	79
5-1	Cytotoxic Effect of Melphaan on WIL2 Cells and WSU-CLL Cells	114
5-2	Percentage of Interstrand-DNA-Crosslinks($C_t\%$) in the WIL2 Cells and WSU-CLL Cells	116
5-3	The Relationship Between Surviving Fraction(SF) and Interstrand-DNA-Crosslinks($C_t\%$) in WIL2 Cells and WSU-CLL Cells.	117
5-4	The Time Course of the Formation and the Removal of Interstrand-DNA-Crosslinks ($C_t\%$) in WIL2 Cells and WSU-CLL Cells.	119

No.	Legend	Page
5-5	The Relationship Between Area Under the Curve for Interstrand-DNA-Crosslink Formation and Cytotoxicity in WIL2 Cells and WSU-CLL Cells.	123
5-6	Cell Cycle Analysis of 10 μ M Melphalan Treated WIL2 Cells and WSU-CLL cells	125
6-1	Melphalan Accumulation in WIL2 Cells and WSU- CLL Cells.	137
6-2	Lineweaver-Burk Plots of Melphalan Uptake Between 1-160 μ M in WIL2 Cells and WSU-CLL Cells.	139
6-3	Bar Graph Representation of Inhibition of Initial 3 min Uptake of 100 μ M Melphalan by 1 mM of Various Competitors	141
6-4	Efflux of Melphalan from WIL2 Cells and WSU-CLL Cells	143
7-1	Viable Cell Fraction (as a Proportion of Control) Following Treatment with Doxorubicin (Fig.7-1A), and Camptothecin (Fig. 7-1B) Against WIL2 Cells and WSU-CLL Cells	161

No.	Legend	Page
7-2	Interstrand-DNA-Crosslinks(C _i %) Following 60 Minutes Exposure to 40 μ M Melphalan(MLN) and then 24 Hours Drug Free Incubation	165
7-3a	Topoisomerase II Activity in Nuclear Extracts from WIL2 Cells and WSU-CLL Cells.	168
7-3b	Topoisomerase II Protein Levels, by Western Blot Analysis of Whole Cell Lysates from WIL2 Cells and WSU-CLL Cells.	168
7-4a	Competitive Binding of Unlabelled Topoisomerase II to WSU-CLL Native DNA and WSU-CLL UV-Irradiated DNA	171
7-4b	Time Course of Binding of ¹²⁵ I-Topoisomerase II to WSU-CLL Native DNA and WSU-CLL Crosslinked DNA.	171
7-5	Scatchard Analysis of the Binding of ¹²⁵ I-Topoisomerase II to WSU-CLL Native DNA(A) and WSU-CLL UV-Irradiated Crosslinked DNA (B).	175

No.	Legend	Page
7-6	The Effect of Doxorubicin (D) Pre-Treatment on Melphalan (M) Induced Interstrand-DNA-Crosslink Formation.	176
7-7	Doxorubicin-Induced dsDNA Breaks of WIL2 Cells and WSU-CLL Cells.	178
7-8a	Topoisomerase-I Activity in Nuclear Extracts from WIL2 Cells and WSU-CLL Cells	180
7-8b	Topoisomerase I Protein Levels by Western Blot Analysis of Whole Cell Lysates from WIL2 Cells and WSU-CLL Cells.	180
8-1	Viable Cell Fraction (as a Proportion of Control) Following Treatment with Aphidicolin Against WIL2 Cells and WSU-CLL Cells	193
8-2	Cytotoxic Effect of Melphalan (MLN) on WIL2 Cells (A) and WSU-CLL Cells (B) with or without 10 μ M Aphidicolin Pre-Treated for 24-hr.	194

No.	Legend	Page
8-3	Percentage of Interstrand-DNA-Cross-Links (C_i) Assessed After 60 Minutes Melphalan (MLN) Exposure Followed by 4 Hours Drug Free Incubation in WIL2 Cells and in WSU-CLL Cells with or without 10 μ M Aphidicolin Pre-Treated for 24-hr.	196
9-1	Effect of IL4 on Cell Viability Following 96 Hours Culture	203
9-2	DNA Fragmentation Assay. The Percentage of Soluble DNA was Measured in B-CLL Cells and in Normal B-Lymphocytes After 96 Hours Culture with or without Addition of IL4.	204
9-3	In-Vitro Cytotoxicity of Melphalan Against B-CLL Cells and Against Normal B-Lymphocytes in the Presence and Absence of (2 u/ml) IL4.	206
9-4	Percentage Interstrand DNA-Cross-Links (C_i %) Following Short Term (60 min) In-Vitro Exposure to Melphalan of Alkylator Resistant B-CLL Cells; with IL4 and without IL4	207

No.	Legend	Page
9-5	Percentage Interstrand-DNA-Cross-Links (C_t %) Following 4 Hours In-Vitro Exposure to Melphalan (10 μ M) of Alkylator Resistant B-CLL Cells with and without IL4	208

LIST OF ABBREVIATION

ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
AIHA	Autoimmune Hemolytic Anaemia
Ap	Aphidicolin
ATCC	American Type Culture Collection
AUC	Area Under the Curve
BCGF	B-Cell Growth Factor
BCH	DL- α -2-Aminobicyclo[2.2.1]Heptane-2-Carboxylic Acid
B-CLL	B-cell Chronic Lymphocytic Leukemia
bFGF	basic Fibroblast Growth Factor
BMT	Bone Marrow Transplantation
BSA	Bovine Serum Albumin
BSO	Buthionine Sulfoxime
CAP	Cyclophosphamide, Doxorubicin, Prednisone
CIG	Cytoplasmic Immunoglobulin on B lymphocytes
CLD	Chronic Lymphoproliferative Disorder
CLL	Chronic Lymphocytic Leukemia
C _i	Percentage of Interstrand-DNA-Crosslink Formation
Cys	Cystine
DNA	Deoxyribonucleic Acid

Continued:

dsDNA	Double Strand DNA
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EFA	Ethidium Bromide Fluorescence Assay
EGTA	Ethylene Glycol-bis(β -aminoethyl Ether)N,N,N',N'- Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FISH	Fluorescence in Situ Hybridization
GSH	Glutathione
GST	Glutathione-S-transferase
HCL	Hair Cell Leukemia
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HLA	Human Leukocyte Antigen
HTLV-I	Human T-cell Leukemia/Lymphoma Virus I
HTLV-II	Human T-cell Leukemia/Lymphoma Virus II
IC ₅₀	Drug Concentration Resulting in 50% Reduction of Cell Numbers
IFN	Interferon
IL	Interleukin
kDNA	Kinetoplast DNA
LDH	Lactate Dehydrogenase

Continued:

Leu	Leucine
LFA	Leukocyte Function Associated Antigen
MCL	Mantle Cell Lymphoma
Mel	Melphalan
MDR	Multidrug Resistance
MoAbs	Monoclonal Antibodies
mRNA	Messenger RNA
MTT	1,3[4,5 Dimethyl-1,2,4-triazole-2-yl]-3,5 Diphyl Tetrazolium Bromide
mU/ml	Milli Unit/ml
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NER	Nucleotide Excision Repair
NHL	Non-Hodgkin B-cell Lymphoma
NK Cells	Natural Killer Cells
O-6-AGAT	O-6-Alkylguanine-Alkytransferase
PGP	P-Glycoprotein
PHA	Phytohaemagglutinate
PLL	Pro-Lymphocytic Leukaemia
PMA	Phorbol Myristate Acetate
PMSF	Phenylmethylsulfonyl Fluoride
PRCA	Pure Red Cell Aplasia
R-HL60	Resistant HL60 Cell Line

Continued:

RNA	Ribonucleic Acid
SDS-PAGE	
Gel	Sodium Dodecyl Sulfate-Polyacrylamide Gel
SF	Surviving Fraction
SIG	Surface Immunoglobulin on B Lymphocytes
SLVL	Splenic Lymphoma with Circulating Villous Lymphocytes
SmIg	Surface Membrane Immunoglobulin
TNF	Tumour Necrosis Factor
Topo II	Topoisomerase II
TRIS.HCl	Tris [hydroxymethyl] Aminomethane Hydrochloride
Try	Tryptophan

CHAPTER 1

1.0. INTRODUCTION--Chronic Lymphocytic Leukaemia

Chronic Lymphocytic Leukaemia (CLL) is due to the proliferation and accumulation of immune-incompetent B-lymphocytes arrested at an early stage of their differentiation (Galton 1966; Dameshek 1967). It is the most common adult leukaemia in Western Europe and North America. Despite this relative frequency progress in understanding and treating CLL has been slow since the original clinical description in 1924 (Minot 1924). However, new insights into the biology of the disease, better diagnostic and staging criteria (Binet *et al* 1981; Rai 1990) and the recent development of more potent therapeutic agents are changing both the outlook for CLL patients and physicians' complacency toward this disease. Indeed, recent gains in remission rates and survival (Dillman *et al* 1989; Keating *et al* 1989; Juliusson *et al* 1993a; Keating *et al* 1993a, 1993b; Robertson *et al* 1993a) and the potential role of biotherapy and of bone marrow transplantation (BMT) raise hopes for the eventual cure of CLL (Bandini *et al* 1991; Michallet *et al* 1991). Because 95 percent of patients with CLL exhibit a clonal expansion of committed B lymphocytes (Fialkow

et al 1978). This review will be restricted to B-CLL.

1.1. Epidemiology

CLL accounts for approximately 30% of all adult leukaemias in Western Europe and North America. However, the incidence of CLL varies widely in different regions of the world, ranging from 2.5% of all leukemia in Japan (*Nishiyama 1969*) and other eastern countries and Africa to 38% in Denmark (*Brincker 1982*). The apparently increasing incidence of CLL over the last 50 years suggested by a Minnesota study (*Call et al 1994*) probably relates to improved diagnostic criteria (*Keating 1994*).

The incidence rate is age-dependent. The disease is rare before the fourth decade of life (*Spiers et al 1985*) but increases with each succeeding decade from 5.2 per 100,000 persons at age of 50 to 30.4 cases per 100,000 persons at 80 years (*Cancer Statistic Review 1987*). There is a male predominance, with a male-to-female ratio of 2:1.

1.2. Etiology

1.2.1. Familial and Environmental Factors

The etiology of CLL is unclear. Familial aggregates of CLL, usually together with other types of leukemia, lymphoproliferative diseases or autoimmune conditions, support the notion of a genetic factor in this disease (*Blattner et al 1979; Neuland et al 1983*). The excessive risk of leukemia in people with one or more first-degree relatives with CLL has been estimated as 2- to 7-fold greater than the risk in individuals without affected first-degree relatives (*Cuttner 1992*). However twin studies do not support a major genetic factor (*Brok-Simoni et al 1987*). HLA linkage studies in CLL have also been contradictory (*Finch 1992*).

An increased risk of CLL in people exposed to a number of chemicals (e.g., benzene, aromatic hydrocarbons) has been reported, although the evidence is weak (*Finch 1992*). In addition, some studies have reported a moderately higher incidence of CLL among farmers, and petroleum and wood workers than among other groups, suggesting an occupational exposure as a factor in the etiology of CLL (*Burmeister et al 1982; Arp et*

al 1983; Blair and White 1985; Finch 1992). Ionising radiation exposure does not appear to lead to this type of leukemia (Foon et al 1990; Dighiero et al 1991; Finch 1992; Cheson 1994). CLL is the only leukaemia not associated with occupational or accidental exposure to radiation or alkylating drugs.

1.2.2. Viruses

Numerous attempts have been made to assess the etiologic role of RNA and DNA viruses in CLL. The human T-cell leukemia/lymphoma virus I (HTLV-I), a type C RNA retrovirus implicated as the causative agent in adult T-cell leukemia (Gallo et al 1983) and persistent T lymphocytosis (Kinoshita et al 1985) is not associated with classical CLL notwithstanding early claims to the contrary (Garver et al 1984; Mann et al 1987). Likewise, reports of HTLV-I infections in subsets of Jamaican patients (Blattner et al 1981) whose uninfected CLL cells synthesized virus p24, envelope protein (Mann et al 1987; Mann et al 1988) and in West African CLL/lymphoma patients (Fleming et al 1983) most likely reflect an immune reaction to the virus in high incidence areas, rather than a leukemogenic role. Similarly, HTLV-II, which was initially thought to be

associated with T-variant hairy cell leukemia (*Kalyanaraman et al 1982; Wachsman et al 1984*) has not been convincingly linked to CLL (*Lion et al 1988*) or to other malignancies. Indeed, limited survey studies have suggested that the first known endemic HTLV-II infected population was at no increased risk of developing CLL, hairy cell leukemia or mycosis fungoides (*Hjelle et al 1991*).

Finally, the role of DNA viruses, such as Epstein-Barr virus or cytomegalovirus as etiologic agents in CLL remains highly speculative. Although CLL cells can be infected with the Epstein-Barr virus there is no direct evidence of a causative role.

1.3. **Biology**

1.3.1. **Clonality**

CLL results from an accumulation of clonal B-lymphocytes with a relatively mature phenotype. The clonal nature of the proliferating cells, initially suggested by the expression of a single Ig light chain (κ or λ) on the cell membrane, was confirmed by showing that B cells from a given

patient express a unique Ig idiotype specificity (*Pernis et al, 1974; Schoer et al 1974; Fu et al 1975*), a single pattern of glucose 6-phosphate dehydrogenase activity (*Fialkow et al 1978*), and clonal chromosomal abnormalities (*Solanki 1982; Juliusson 1986; Dighiero 1991; Foon et al 1990; Cheson 1994*). Moreover, unique Ig gene rearrangements were almost invariably observed, although in some cases heterogeneity of the hybridization pattern was seen, when probes for the heavy chain J segment were used (*Johnstone 1982; Korsmeyer 1985*).

1.3.2. Phenotypic Characteristics of the Neoplastic B Lymphocyte in CLL

In the early 1970s, several reports based on the presence of surface membrane immunoglobulin (SmIg) (*Eskeland et al 1971; Grey et al 1971; Preud'homme and Seligmann 1972; Ternynck et al 1974*), C3dR complement receptors (*Pincus et al 1972*) and receptors for the Fc fraction of Ig (*Dickler and Kunkel 1972*) clearly established that CLL is usually associated with expansion of a B cell clone.

The neoplastic B cell of CLL is currently known as the B1-B cell, in contrast to the normal B cell which is designated as the B2-B cell (*Kantor 1991*). In the neoplastic, CLL, lymphocyte surface membrane immunoglobulins (SmIg) are restricted to a single light chain and frequently express IgM or both IgM and IgD. In the latter case IgM and IgD have been shown to share idiotypic and antigenic specificities (*Pernis et al, 1974; Schoer et al 1974; Fu et al 1975*). There is controversy, however, as to whether the B lymphocytes in CLL display μ , δ and γ chains. Some studies have indicated that a heavy chain switch can occur in B-CLL; hence, some authors have suggested that there is a degree of maturation of malignant cells. Other studies have suggested that expression of other immunoglobulin types is extrinsic and that these are not synthesized by the malignant cell (*Johnstone 1982*).

In CLL B cells also express several antigens, including DR-related human leukocyte antigens and B cell specific antigens. B cells in most patients with CLL appear to react with CD19, CD20, CD24, CD37 and CD21 monoclonal antibodies (*Nadler 1986; Foon et al 1990; Dighiero 1991; Pinto et al 1991; Polliack et al 1993; Cheson 1994*). About 60% of patients with CLL are positive for CD23 (*Sarfati et al 1988*), whereas

membrane positivity with CD22 is infrequent. B cells in CLL and hairy cell leukaemia have been found to express a 69-kD glycoprotein which is not expressed by T and B lymphocytes in normal blood, by thymocyte-cultured T and B cell lymphoblastoid cell lines, or by acute lymphoblastic leukaemia cells (*Agree et al 1986*). In contrast to B cell polymphocytic leukaemia in which the cells constantly bind the FMC7 antibody this reactivity is not common in CLL (*Catovsky et al 1981*). Reactivity with CALLA(CD10) is almost constantly negative, whereas reactivities with subepitopes of CD2 (*Small et al 1987; Delia et al 1988; Merle-Béral et al 1989*) and CD11 (*Wormsley et al 1990*), as well as with CD6, CD7 and the TQ1 antigen, have been reported in some patients with B-CLL (*Keller et al 1987*). CLL cells also depict the common CLL antigen(cCLLa) (*Agree et al 1986*). Interestingly, myelomonocytic antigens have also been found to be expressed by CLL B cells (*Morabito et al 1987*). Contrary to most other B cell malignancies B lymphocytes in CLL are characterized by three particular phenotypic patterns: (1) they almost always express low amounts of SmIg, although increased amounts of intra-cytoplasmic Ig have been observed (*Ternynck et al 1974; Dighiero et al 1976, 1980*); (2) 31-95% of CLL B lymphocytes form rosettes with mouse erythrocytes (*Stathopoulos and Elliot 1974; Catovsky et al 1976*); (3) in most cases

they express the CD5 antigen, a 67kD antigenic determinant initially described as a pan-T marker (*Boumsell et al 1978; Royston et al 1980; Wang et al 1980*).

CLL is generally easy to distinguish from reactive lymphocytosis and other lymphoproliferative conditions. Five mature B cell malignancies should be distinguished from B-CLL on the basis of clinical features, cytology and phenotype (1) B-pro-lymphocytic leukaemia(PL) (*Melo et al 1986*), characterized by extreme leukocytosis($> 100 \times 10^9/L$) and splenomegaly with minimal or no lymphadenopathy. The pro-lymphocyte is the predominant cell in the peripheral blood($>55\%$, and usually $> 70\%$) and is characterized by its large size, prominent nucleolus and lower nuclear: cytoplasmic ratio than the small lymphocyte in CLL. The phenotype of prolymphocytic leukemia is also different from that of lymphocytes in CLL, with increased expression of surface Igs, low mouse rosette formation, low CD5 expression and positivity for FMC7. However, intermediate forms do occur. CLL-PL has features intermediate between those of classic CLL and classic PL, and includes patients with typical CLL that evolve to a 'pro-lymphocytoid' transformation as well as those with an increased proportion of pro-lymphocytes at diagnosis; (2)Hairy cell

leukaemia(HCL) which may be confused with CLL in those rare cases with leukocytosis and a high percentage of circulating hairy cells(so called HCL 'variant', which has morphological features intermediate between those of hairy cells and pro-lymphocytes) (*Bennett et al 1989*). The diagnosis of HCL can, however, usually be made by morphology, tartrate-resistant acid phosphatase positivity and the combination of CD25, LeuM5 and HC2 positivity; (3) Splenic lymphoma with circulating villous lymphocytes(SLVL) (*Melo et al 1987*) which is associated with massive enlargement of the spleen, moderate leukocytosis($10-30 \times 10^9/L$), small monoclonal bands in serum or urine (in approximately 60% of cases), circulating lymphocytes characterized by short cytoplasmic villi, and membrane markers similar to those of B-PL; (4) The leukaemic phase of non-Hodgkin's lymphoma('lymphosarcoma cell leukaemia'), generally with follicular or diffuse small cleaved cell histology (*Mintzer and Hauptman 1983; Bennett et al 1989*). Typical cells are often pleomorphic with nuclear clefting. The disease can also be distinguished from CLL by lymph node biopsy, histological pattern of bone-marrow infiltration and immunophenotypic features, including strong surface Igs, low percentage of mouse rosettes, FMC7 and often CD 10 positivity and a negative reaction with CD5; (5) Waldenström's macroglobulinaemia(in cases where

circulating lymphocytosis is present). The malignant lymphocytes generally have plasmacytoid features, however, and secrete substantial monoclonal IgM in the serum.

B-CLL should also be differentiated from mature T cell disorders, which are far less common than mature B cell disorders, (at least in Western countries), and have been classified into four main types, including T chronic lymphocytic leukaemia (or large granular lymphocytic leukemia), T pro-lymphocytic leukaemia, adult T cell leukaemia-lymphoma, and the Sézary syndrome (*Matutes et al 1988*).

1.3.3. Normal Cellular Counterpart of the CLL B Cell

A number of cellular and molecular features of B-CLL and normal CD5⁺ B cells are shown in table 1-1.

CD5⁺(B1-B) B cells constitute a small subpopulation of human B lymphocytes in the lymphoid organs and peripheral blood (10%-15%) of normal adults. The normal counterpart of the CD5⁺ B cells which

**Table 1-1 Similarities and Differences Between B-CLL CD5⁺ B Cells
and Normal CD5⁺ B Cells**

	CD5 ⁺ B- CLL B Cells	Normal CD5 ⁺ B Cells
Production of polyreactive auto-antibodies	Yes	Yes
Extensive somatic hypermutations	No	No
Cross-reactive idiotypes	Yes	Yes
Mouse erythrocyte rosettes	Yes	Yes
Myelomonocytic antigens	Yes	Yes
Low sIg levels	Yes	No
EBV transformability	No	Yes
Na ⁺ /H ⁺ antiporter activity	Low	High
Defective signal transduction	Present	Absent
Ca ⁺⁺ response	Defective	Normal
<i>Bcl-2</i> gene expression	Over expression	Low expression
<i>c-myc</i> gene expression	No	Yes

proliferate in CLL were initially located at the edge of the germinal centre in human lymph nodes (*Caligaris-Cappio et al 1982*). It was subsequently observed that a substantial number of B cells from 20-week-old fetal lymph nodes and spleen express the CD5 marker as well as μ and δ chains on the surface membrane. These fetal cells also appear to share the lectin non-responsiveness and the inability to cap SmIg characteristic of B lymphocytes (*Caligaris-Cappio et al 1993; Gale et al 1994*).

A high prevalence of CD5⁺ cells during early ontogeny has led some authors to assume that the CD5⁺ B lymphocyte in patients with CLL corresponds to expansion of an immature B cell clone arrested at a stage between pre-B and mature B cells (*Salmon and Seligmann 1974*). This hypothesis, however, does not provide a satisfactory explanation for the fairly high frequency of hypogammaglobulinaemia and autoimmunity directed against blood cell components in B-CLL (*Dighiero 1987, 1988*). These phenomena are only rarely observed in other B cell neoplasias. Alternatively, it has been postulated that CD5⁻ B cells could correspond to a separate B cell lineage. There is some evidence indicating that Ly1-B cells, the murine counterpart of human CD5⁺ B cells, constitute a discrete

B cell subset (*Manohar et al 1982; Hayakawa et al 1985; Freedman et al 1987*). However, no definitive evidence indicating that human CD5⁺B cells constitute a separate B cell lineage is presently available.

Chronic lymphoproliferative disorders (CLDs) of B-cell lineage represent a heterogeneous group of malignancies that include different histological and clinical entities. The use of morphological criteria alone does not allow a definitive characterization of different types of CLDs (Table 1-2). In the last 10 years the use of monoclonal antibodies recognizing a variety of surface molecules in most instances has identified the normal B-cell counterpart from which the malignant transformation originates (*Harris et al 1994*). These studies were the first to draw a distinction between B lymphocytes lacking or expressing the CD5 molecule, this antigen being consistently present in at least two B-cell disorders (i.e., B-CLL and mantle cell lymphoma), whereas CD5- B-cell malignancies represent a heterogeneous group of CLDs with discrete phenotype, including HCL, marginal zone NHL, and follicular-centre NHL, among others (*Foon et al 1986; Harris et al 1994*). These diseases may be differentiated from each other on the basis of the pattern of co-expression of several markers, including CD5, CD10, CD11c, CD23, and CD43 (Table 1-3).

Table 1-2 CLDs of B-Cell Lineage: Morphologic Features in Differential Diagnosis

Lymphoma	Pattern	Small Cells	Large Cells
B-CLL	Diffuse with pseudofollicles	Round(may be cleaved)	Prolymphocytes Paraimmunoblasts
HCL	Diffuse, interstitial	Lymphoid cell with 'hairy' projections	None
MCL	Diffuse, vaguely nodular, mantle zone, rarely follicular	Cleaved(may be round or oval)	None

Continued:

Lymphoma	Pattern	Small Cells	Large Cells
Centerfollicular NHL	Follicular +/- diffuse areas, rarely diffuse	Cleaved(centro cytes)	Centroblasts
Marginal zone NHL	Diffuse, interfollicular, marginal zone, occasionally follicular (colonization)	Heterogeneous: round (small lymphocytes), Cleaved (marginal zone/ monocytoid B cells), Plasma cells	Centroblasts Immunoblasts

Table 1-3 CLLDs of B-Cell Lineage: Immunohistologic and Genetic Features in Differential Diagnosis

Lymphoma Type	SIG	CIG	CD5	CD10	CD11	CD23	CD43	Chromosome Abnormality
B-CLL	+/-	-/+	+	-	-/+	+	+	Trisomy 12 (30%)
HCL	+	NA	-	-	+	-	NA	IgH-R and IgL-R**
Mantel cell	+	-	5	-/+	-	-	+	t(11;14)
Follicle centre	+	-	-	+/-	-	-/+	-	t(11;18)
Marginal zone NHL (extranodal)	+	40%+	-	-	+/-	-/+	-/+	Trisomy3

Abbreviations: +, 90% positive; +/-, >50% positive; -/+, <50% positive; -, <10% positive.

* Positivity may vary depending on antibody used.

**Ig heavy/light chain genes rearranged

1.3.4. B Cell Differentiation, Antibody Production and Gene Expression

Although earlier studies postulated that B-CLL lymphocytes were arrested at an early stage of differentiation considerable evidence has accumulated that these cells are able to differentiate. A number of studies (*Stevenson et al 1980; Caligaris-Cappio 1985a; Foon et al 1990; Dighiero et al 1991; Cheson 1994*), using anti-idiotypic reagents, showed that the pentameric form of SmIg was present in the serum of patients with CLL. Some studies have indicated that the heavy chain switch can occur in B-CLL, and hence, some authors have suggested that there is a degree of maturation of malignant cells (*Baldini et al 1985*). In vitro experiments with different mitogens, such as pokeweed, nocardia and phorbol esters, have succeeded in inducing differentiation of B-CLL lymphocytes (*Tötterman et al 1980; Okamura et al 1982; Gordon et al 1984; Miller and Gralow 1984*). With phorbol esters an increase in amount of RNA coding for secretory IgM was observed (*Tötterman et al 1980*). Interestingly, upon phorbol ester stimulation normal CD5- B cells expressed CD5 (*Miller and Gralow 1984*) and B-CLL cells developed tartrate-resistant phosphatase activity

and resembled hairy cells (*Caligaris-Cappio and Janossy 1985b*), whereas with lipopolysaccharide they were found to express increased levels of SmIg and FMC7 (*Caligaris-Cappio et al 1984; Caligaris-Cappio and Janossy 1985b; Freedman et al 1989*).

Antibody production by CLL cells has only recently been assessed by studying the antibody activity of Ig-containing supernatants obtained after stimulation of B-CLL lymphocytes with phorbol ester (*Bröcker et al 1988; Sthoeger et al 1989*), or by studying the antibody activity of hybridomas derived from B-CLL lymphocytes (*Borche et al 1990*). These reports all succeeded in demonstrating a high proportion of CLL B cells displaying natural autoantibody activity. Indeed, about half of the cells displayed rheumatoid factor activity and about 20% showed multispecific reactivity against autoantigens such as DNA and cytoskeleton proteins (*Borche et al 1990*). These results confirm that CD5⁺ B-CLL lymphocytes are frequently committed to the production of natural autoantibodies. However, CD5⁻ B lymphocytes from patients with follicular non-Hodgkin's lymphoma have also been found to be frequently committed to secretion of natural autoantibodies (*Dighiero et al 1990*).

As for Ig gene expression, CD5⁺ (B1-B) lymphocytes from CLL display a restricted expression of VH and VL genes. Ig VH genes of the relatively small VH subgroups, VH4 and VH5, and the single copy VH6 gene are rearranged at frequencies which are disproportionate to the relative sizes of these subgroups in the germline DNA. Humphries and co-workers (1988) reported that 30% of patients with CLL express VH251, which is one of the two germline members of the VH5 family. Logtenberg et al (1989) found that B-CLL lymphocytes express VH4 in 50% of patients, VH5 in 20% and VH6 in 15%. On the other hand, the largest VH gene subgroup, the VH3 gene family, seems to be under represented (Schroeder Jr et al 1994; Stewart & Schwartz 1994). Kipps et al (1988) found that a high proportion of B-CLL cells, expressing κ light chain on the membrane reacted with a murine anti-idiotypic antibody raised against a monoclonal IgM rheumatoid factor, expressed the Wa idiotypic. Analysis of κ light chain variable region genes expressed by leukaemic cells sharing the Wa idiotypic enabled these authors to demonstrate that the cells were expressing the terminal, unmutated Hum Kv 325 germ line gene. Similar restriction was found for VH genes, since the germ line VH1 51P1 gene, was found to be expressed in 20% of patients with CLL (Kipps et al 1989). Spatz and colleagues (1990) reported the nucleotide sequence of a clone derived from

a patient with CLL which was found to bind myelin associated glycerol(MAG) and denatured DNA. The patient was found to express a VKIIIa gene with 96% homology with the germ line counterpart and a VH3 gene whose germ line counterpart corresponded to the VH3-26gene.

Mdr genes and P-glycoprotein are variously expressed in CLL (*Michieli et al 1991; Shustik et al 1991; Sonneveld et al 1992; Wulf 1994*). Of the integrin family, the beta-1 integrin VLA4 is poorly expressed, while VLA3 is constantly present (*Baldini et al 1992; Möller et al 1992*). The beta-2 integrin LFA-1(CD11a/CD18) is not expressed (*Woessner et al 1994*).

1.3.5. T and Natural Killer Cells

In CLL a significant increase in T and natural killer(NK) cells has been reported in untreated patients (*Catovsky et al 1974; Kay et al 1979; Vuillier et al 1988*). Abnormalities such as decreased T helper cells (*Chiorazzi et al, 1979*), increased T cytotoxic-suppressor cells (*Kay 1981*) and inversion of the T4:T8 ratio have been reported.

The increase in these cells is probably polyclonal in nature, as indicated by the lack of chromosome abnormalities and heterozygosity for glucose 6-phosphate dehydrogenase (*Prchal et al 1979; Lucivero et al 1983*). The reason for this polyclonal increase in T and NK cells in CLL is unknown. However, one study claimed that 25% of patients with CLL displayed clonal rearrangements of the T cell receptor β from (*Wen et al 1990*). Whether these unexpected results favour the idea that, in some patients, the CLL target could correspond to a stem cell with both B and T cell differentiation ability and, in others, with myeloid differentiation potential (*Morabito et al 1987*), as attested by occasional expression of these makers, is as yet unclear. Functional studies of T cells from patients with CLL indicate that mitogenic responses to phytohaemagglutinate(PHA) are usually, but not always, normal whereas reactivity to autologous or allogeneic B cells is impaired (*Gale and Foon 1987*). Data concerning helper, suppressor, NK and ADCC cell function are contradictory and difficult to interpret (*Dighiero et al 1991*).

1.3.6. Hypogammaglobulinemia

Hypogammaglobulinemia occurs in 10-60% of patients with CLL, the frequency depending on the level used as the lower limit of normal (*Dighiero 1988*). Hypogammaglobulinemia is the major cause of infection in CLL. Patients with early stages of the disease tend to have defective specific antibody responses to infection or immunization (*Chapel 1987; Molica 1994*).

The pathogenesis of hypogammaglobulinemia is poorly understood as the phenomenon is rare in other B cell malignancies, including acute lymphoblastic leukemia, nodular and diffuse lymphomas, hairy cell leukemia, etc (*Dighiero 1988*).

Although regulatory abnormalities in T cells may play a role in induction of hypogammaglobulinemia data concerning helper and suppressor lymphocytes, and NK and ADCC activity are contradictory and fail to establish firmly the contribution of these cells to the development of hypogammaglobulinemia (*Dighiero 1987, 1988; Molica 1994; Schroeder Jr et al 1994*). Based on information presently available it

appears logical to assume that hypogammaglobulinemia is considered to be the result of a dysfunction of non-clonal B(B2-B) cells and correlates with tumor burden but not with previous treatment. Immune response to vaccines is considered to be hampered by hypogammaglobulinemia (*Molica 1994; Schroeder Jr et al 1994*).

Although a marked monoclonal(M) immunoglobulin component (usually IgM) is found in only approximately 5% of patients with CLL (*Miller et al 1987; Pangalis et al 1988*) a small M component can be detected in the serum or urine in 80% of patients by use of high-resolution techniques (*Beaume et al 1994*). In most cases this M component is secreted by non-neoplastic(B2-B) cells.

1.3.7. Autoimmune Phenomena

Associated autoimmune phenomena are frequently observed in CLL. The autotoxic manifestations are mainly directed against cells of the hematopoietic system (*Hamblin et al 1986*) and in most cases they reflect abnormalities in non-neoplastic(B2-B) cells. Nevertheless, some cases have been reported of antibodies produced by the neoplastic CD5⁺(B1-B) cell

clone (*Stoegher et al 1993*). A positive direct antiglobulin test has been reported in 7.7-35% of patients with CLL, depending on series and stage of disease (*Grey et al 1971; Keller et al 1987*). In the French Co-operative Group series a positive Coombs' test was found at diagnosis in only 1.8% (*Dighiero et al 1991*). This low prevalence is probably explained by the fact that the French Co-operative Group series included higher numbers of patients with early stage disease than was the case in other reports. Autoimmune haemolytic anaemia occurs in 20-25% of patients at some time during the course of the disease. Although one study described a case of cold agglutinin disease in which the autoantibodies were the product of CLL B cells (*Feizi et al 1983*) in most instances autoantibodies against red blood cells are polyclonal, warm, reactive, and of type IgG and display activity against monomorphic antigens of the rhesus system (*Hamblin et al 1986*). Immune thrombocytopenia is observed in about 2% of patients but higher frequencies of increased platelet-associated Igs have been reported (*Hamblin et al 1986*). Pure red cell aplasia and autoantibodies against neutrophils are observed only rarely and there is conflicting evidence concerning the frequency of other autoantibodies (*Hamblin et al 1986*). This pattern is similar to that observed in primary immunodeficiency syndromes in which immune thrombocytopenia, autoimmune haemolytic

anaemia and pure red cell aplasia are frequently observed (*Dighiero 1988*).

In CLL autoantibodies are not usually secreted by the malignant clone so it may be postulated that hypogammaglobulinaemia could induce a disturbance in the idiotypic network in such a way that anti-idiotypic antibodies designed to antagonize autoreactive clones are not produced (*Dighiero 1988*). Sultan and colleagues (*1984*) succeeded in suppressing production of anti-VIII autoantibodies by injecting intravenous Ig and speculated that anti-idiotypic suppression, mediated by injected Igs, could occur. However, no conclusive evidence concerning the role of intravenous Ig in the treatment of autoimmune-associated phenomena in CLL has so far been reported.

A high proportion of CLL B cells display natural antibody activity with about half of the CLL B cells having rheumatoid-factor activity and about 20% showing multispecific activity against autoantigens such as DNA and cytoskeleton proteins (*Borche et al 1990*).

1.3.8. Cytokines

The CLL cytokine network is complex. A variable pattern has been shown in the response to different cytokines such as Interleukin(IL)-2 and B-cell growth factors(e.g., BCGF, IFN alpha and gamma, and IL4). TNF alpha and possibly IL-1 and IL-6, either alone or in synergy, act as growth factors and IFN-gamma, IFN-alpha, and IL-4 inhibit apoptosis in CLL (*Hivroz et al 1986; Karray et al 1987, 1988; Foa et al 1990; van Kooten et al 1992; Hoffbrand et al 1993; van Kooten et al 1993*). These findings may be indicative of discrete stages of maturation and activation in patients with CLL. Among these cytokines IL-2 appears to be the most consistent activator, whereas IL-4 downregulates B-CLL lymphocytes in contrast to its effect on normal B cells (*Hivroz et al 1986; Karray et al 1987, 1988*). CLL cells also express IL-8 (*Di Celle et al 1994*).

Cytokines appear to be involved in CLL pathogenesis (*van Kooten et al 1992; Mainou-Fowler et al 1994; Trentin et al 1994; Chaouchi et al 1996; Mainou-Fowler et al 1996; Trentin et al 1996*). TNF- α , IFN- α , IFN- γ , IL-2, IL-4, IL-10, IL-13, IL-15, and basic fibroblast growth factor[bFGF] enhance survival of neoplastic B lymphocytes by acting

either as growth factors(e.g., TNF- α , IL-2,IL-15); by inhibiting apoptosis (e.g., IFN- α , IFN- γ , IL-4, bFGF); or by both mechanisms(e.g., IL-13). These effects take place through a number of different and complex pathways(e.g., IL-4 inhibits TNF- α -induced proliferation and also protects cells from IL-5-induced apoptosis) (*van Kooten et al 1992; Mainou-Fowler et al 1994*). However, these autocrine and paracrine pathways are far from being completely elucidated.

1.3.9. Cytogenetic Abnormalities

Chromosomal analysis of CLL has been facilitated in recent years by the use of mitogens able to stimulate CLL cells. These include the Epstein-Barr virus, phorbol esters, liposaccharide, and pokeweed mitogen. In addition techniques such as fluorescence in situ hybridization(FISH) have been used to enhance detection of chromosome abnormalities. Trisomy 12 is the most common abnormality detected, being found in 11% to 54% of patients with B-CLL (*Juliussen et al 1990; Perez-Losada et al 1991; Juliussen et al 1993b; Que et al 1993; Criel et al 1994; Witzig et al 1994*). The variable frequency of chromosome abnormalities appears to reflect differences in patient selection and in diagnostic criteria as well as

the sensitivity of the techniques used. For example, FISH identifies trisomy 12 more frequently than does conventional cytogenetic analysis (*Perez-Losada et al 1991; Juliusson et al 1993b; Que et al 1993*). The incidence of trisomy 12 in CLL is twofold to threefold higher when using the fluorescence in situ hybridization technique (*Escudier et al 1993*). Trisomy 12 has been variously reported to be associated with early (*Han et al 1984*), progressive (*Oscier et al 1988*), or advanced disease, as well as with poor prognosis (*Juliusson et al 1990; Escudier et al 1993*). In some reports, trisomy 12 appeared to be more frequent in 'atypical' CLL, with more prolymphocytes or with lymphoplasmacytoid differentiation, than in typical or classic CLL (*Que et al 1993; Criel et al 1994*). Other reports suggest that trisomy 12 alone is detected in early stages whereas trisomy 12 combined with other karyotypic abnormalities occur in patients with advanced disease (*Escudier et al 1993*). These findings have been interpreted as an indicating that trisomy 12 might represent the earliest karyotypic change whereas complex chromosome abnormalities result from clonal evolution or treatment (*Pittman et al 1984*). Other studies however reported no correlation with clinical variables, including stage (*Que et al 1993*) and prognosis (*Oscier et al 1988*) and stable chromosome abnormalities in most patients in contrast to what is observed in other

chronic hematologic diseases (Nowell *et al* 1988). These findings raise doubts about the role of trisomy 12 in the pathogenesis of CLL.

1.3.10. Oncogenes

Oncogenes are genes capable of conferring on host cells the property of unregulated growth. Although first identified as genes in retroviruses (Stehelin *et al* 1976), oncogenes are derived from cellular, 'proto'-oncogenes, their eukaryotic counterparts, and are found in normal cells of species ranging from sea urchins to humans. Proto-oncogenes encode for proteins that enable normal cells to respond to complex extracellular signals. There are considerable data to support the role of oncogenes, especially *myc*, *abl*, *src*, and *ras*, in human neoplasia, including leukemia (Neri *et al* 1988; Browett and Norton 1989). Abnormally activated proto-oncogenes are detectable either in mutated or amplified forms, or on a larger scale, as fusion proteins resulting from structural chromosomal abnormalities, including translocations and deletions. The best characterised chromosome abnormalities in hemopoietic malignancies are the *myc*- and *abl*-involving chromosome translocations in Burkitt's lymphoma and chronic myeloid leukaemias, respectively (Haluska *et al*

1987).

A variety of approaches have been used to study proto-oncogene expression in CLL, including chromosomal analysis and gene transfection, but none have produced convincing data linking CLL to oncogene abnormalities (*Erikson et al 1984; Butturini and Gale 1988; Adachi et al 1989; Ohno et al 1990; Zheng et al 1992*).

Although initial reports suggested involvement of the bcl-1 and bcl-2 genes in 5% to 15% of patients with CLL (*Butturini and Gale 1988; Foon et al 1990; Dighiero et al 1991; Raghoebar et al 1991; Newman et al 1993; Cheson 1994; Dyer et al 1994*), subsequent studies failed to confirm these data. In one analysis of 100 patients with well-diagnosed, 'classic' CLL, no alteration of the bcl-1 or bcl-2 oncogenes was detected (*Gaidano et al 1994*). This finding is in agreement with other reports (*Rechavi et al 1989*).

Although chromosome translocations are found infrequently in CLL, three cytogenetic abnormalities have been found which are of interest as providing possible etiologic clues. These include: t(14; 18), t(11; 14), and

t(14; 19).

- Translocation(14; 18), which juxtaposes the Ig heavy-chain gene locus on chromosome 14 to proto-oncogene bcl-2 gene product on chromosome 18, although characteristic of follicular lymphoma, is occasionally found in CLL (*Yunis et al 1987*). This juxtaposition has been reported in approximately 10% of cases of CLL without the t(14; 18) (*Adachi et al 1989*). Likewise t(11; 14), involving the J region of the Ig heavy chain on chromosome 14 translocated near the putative proto-oncogene bcl-1 on chromosome 11, is more frequently associated with intermediate-grade lymphoma (*Tsujimoto et al 1984; Medeiros et al 1990*) and prolymphocytic leukemia(PLL) than with CLL (*Erikson et al 1984*). In a few cases a t(14;19) has been reported with involvement of the bcl-3 gene (*van Krieken et al 1990; Ohno et al 1993*). Again, this abnormality seems to be extremely rare (*van Krieken et al 1990*).

Abnormal bcl-2 mRNA expression occurs in only 5% to 10% of CLL. In contrast to the follicular lymphomas, the translocation does not involve the 3' region of the bcl-2 gene but rather of the 5' region (*Adachi et al 1989; Dyer et al 1994*). The majority of data indicate that bcl-2 alone is not able

to transform B cells, but that cotransfection with c-myc confers oncogenic potential (*Fanidi et al 1992*).

Overexpression of the *c-myc* oncogene, deletions of the Rb1 gene, and mutations of the p53 suppressor gene have been reported in a small number of patients with CLL and small lymphocytic lymphomas showing clinical progression and morphologic transformation (*El-Rouby et al 1993*; *Mactolcsy et al 1994*).

Recurrent abnormalities involving chromosome 13q14, the site of the retinoblastoma(Rb1) suppressor gene, are present in one-third to one-fifth of the patients with B-CLL (*Juliussen et al 1990*; *Juliussen et al 1993b*). Other abnormalities include deletions of the long arm of chromosomes 6 and 11, with variable breakpoints (*Juliussen et al 1990*; *Juliussen et al 1993b*). Deletions of chromosome 6(q21q23) have been associated with a subset of lymphoid malignancies that exhibit larger, prolymphocytoid, cells in peripheral blood (*Offit et al 1994*).

Normal cell growth is also limited by growth-constraining genes called tumour-suppressor genes (*Weinberg 1991*). Inactivation of these genes leads to unimpeded cell growth. In certain tumour-suppressor genes mutation of one gene allele has no consequences and both alleles must be mutated to induce uncontrolled cell proliferation. This simple recessive mode of action is exemplified by the retinoblastoma gene (*Hollingsworth et al 1993*). Deletions of the Rb1 gene have been reported in 13%-31% of the cases of B-CLL (*Peterson et al 1992; Stilgenbauer et al 1993*), but biallelic deletions of the Rb1 gene have not been observed, and patients with monoallelic deletions have been shown to have normal Rb1 gene mRNA and protein.

A genomic region located telomeric to the Rb1 gene, identified by the D13S25 probe has been found to be homozygously deleted in leukemic cells from CLL patients. Further characterisation of this region may be of great importance. It is possible that the D13S25 recognizes a new tumour suppressor gene implicated in the pathogenesis of CLL (*Brown et al 1993; Hawthorn et al 1993*).

1.4. Clinical Features

1.4.1. Clinical Presentation and Laboratory Abnormalities

Patients with CLL present with a spectrum of symptoms and signs. Frequently, the diagnosis is made early in an asymptomatic individual following a routine complete blood cell count that exhibits lymphocytosis. In other patients, the diagnosis is made at the onset of constitutional systems (fever, night sweats, or weight loss), an increased susceptibility to viral or bacterial infections, or an episode of autoimmune hemolytic anaemia (AIHA). Likewise, physical findings range from no abnormalities to lymphadenopathy and/or organomegaly resulting from progressive infiltration by clonal lymphocytes. Small, palpable lymph nodes are initially localized, particularly to the cervical or supraclavicular areas. However, as the disease progresses symmetrical lymphadenopathy becomes widespread and lymph nodes enlarge and coalesce giving rise to large masses, particularly in the retroperitoneal and mesenteric spaces. Likewise, splenomegaly ranges from minimal to massive spleens that occupy most of the left hemiabdomen. In most advanced cases nonlymphoid organ infiltration also occurs, particularly in the prostate (*Worning and Dragssted*

1985), liver, and pleura, or, more rarely, other tissues (*Cresson and Sieagl* 1985; *Gogoi et al* 1989), resulting in organomegaly, effusions or other infiltrative manifestations. In contrast to the dermatotropism exhibited by T-lymphoid malignancies (*Warin and Roberts* 1979), lymphoid infiltration of the skin/s rarely observed in B-CLL and when it occurs is usually reactive rather than malignant in nature. Anaemia and thrombocytopenia, a hallmark of bone marrow failure, occur in the most advanced stages of the disease. Anaemia is usually normocytic and normochromic unless reticulocytosis associated with severe Coombs' positive haemolysis causes slight macrocytosis (*Pirofsky* 1978). Ig production abnormalities are frequent in CLL and include the following: panhypogammaglobulinemia, which though observed in early stages of the disease (*Faguet et al* 1992) progresses in frequency and severity with advancing disease (*Fairley and Scott* 1961) while monoclonal gammopathy, occurs in 4% to 31% of patients, depending on the sensitivity of detection methods used (*Bernstein et al* 1992), and occasionally leads to glomerulonephritis and nephrotic syndrome (*Moulin et al* 1992). Blood chemistries show no consistent abnormalities except for rare hypercalcemia (*Wang et al* 1978).

1.4.2. Diagnostic Criteria

The diagnostic workup of patients with CLL is shown in Table1-4. The National Cancer Institute/Working Group(NCI/WG)(*Cheson et al 1988*) and the International Workshop on CLL(IWCLL)(International workshop on CLL) have independently proposed criteria for diagnosing CLL (Table1-5). In the past, an absolute lymphocyte count of $15 \times 10^9/L$ was considered to be the threshold for defining CLL, but the NCI/WG and IWCLL lowered this threshold to $5 \times 10^9/L$ and $10 \times 10^9/L$, respectively. In fact, CLL can be diagnosed when there is an absolute increase in the number of lymphocytes in the blood which are morphologically and immunophenotypically consistent with the diagnosis. The typical phenotype of the neoplastic B lymphocytes in CLL is SmIg +/-, CD5+, CD19+, CD20+, CD23+, FMC7-, and membrane CD22 +/- (*Mattutes et al 1994*). Assessment of the degree of bone marrow infiltration, either by aspirate or biopsy, is more useful for predicting the outcome of the disease and in evaluating response to therapy than for diagnosis.

Table 1-4 Chronic Lymphocytic Leukemia: Diagnostic Workup

Physical Examination

Blood Cell Counts(including reticulocytes) and Morphology

Cell Markers(e.g., Smlg, CD5, Cd19, CD23, FMC7, CD4, CD8)

Biochemical Parameters(including LDH and beta-2 microglobulin

serum levels)

Coombs Test

Immunoglobulin Levels

Immunoelectrophoresis

Cytogenetics

Bone Marrow Aspirate and Biopsy

Chest Radiograph

Chest and Abdominal CT Scans

Table 1-5 Chronic Lymphocytic Leukemia: Diagnostic Criteria

-
1. Absolute Lymphocytosis in Peripheral Blood
 - > 5×10^9 /L (NCI/Working Group)
 - > 10×10^9 /L (IWCLL)
 2. The majority of lymphocytes should be small and mature in appearance
Morphologic Subtypes:
 - 2.1 Typical or classic CLL: $\leq 10\%$ atypical lymphocytes
 - 2.2 Atypical or mixed CLL; CLL/PL: prolymphocytes in blood between 11% and 54%
 3. Characteristic Immunophenotype: SmIg +/-, CD5+, CD19+, CD20+, CD23+, FMC7 -/+, CD22 +/-.
 4. Bone Marrow Infiltration
 - > 30% lymphocytes in bone marrow aspirate, or consistent pattern in bone marrow biopsy
-

CLL must be differentiated from other lymphoproliferative disorders, particularly splenic lymphoma with villous lymphocytes, lymphoplasmacytic lymphomas, and mantle-cell lymphoma which may resemble CLL both morphologically and immunophenotypically (*Mulligan and Catovsky 1991; Litz and Brunning 1993; Pangalis et al 1993*). In this regard, the analysis of the immunophenotype of the leukemic cells as well as their cytogenetic and molecular biologic features may be of help in diagnosis of difficult cases (table 1-2, 1-3). Likewise, cases of CD5-lymphocytosis should always raise the possibility of a disease other than CLL.

The French-American-British (FAB) Group has divided CLL into different morphologic variants on the basis of the proportion of atypical lymphoid cells in blood: (1). typical, in which most of the lymphocytes are small and mature in appearance with less than 10% of atypical ones; (2). mixed, CLL/prolymphocytoid (CLL/PL), when the proportion of prolymphocytes in blood is between 11% and 54%; and (3). a more vaguely defined form with a variable proportion of atypical lymphocytes but less than 10% prolymphocytes in blood (*Bennet et al 1989*); Whether this latter variety truly represents a form of CLL should be reconsidered in the light of the

existence of lymphomas which may be confused with CLL, as mentioned above.

1.4.3. **Prognosis**

The median survival of patients with CLL has increased from about 5 years in the early 1970s to about 8 years in recent years. This is due to the larger proportion of patients currently diagnosed when asymptomatic and in an early stage rather than to treatment progress. Some patients have a survival not different from that of the general population while others have a rapidly fatal course. Clinical stages (*Rai et al 1975; Binet et al 1981; International Workshop on CLL 1981*), bone marrow histopathology (*Rozman et al 1984; Geisler et al 1986; Pangalis et al 1987*), blood lymphocyte levels (*Baccarani et al 1982; Rozman et al et al 1982*), lymphocyte doubling time (*Montserrat et al 1986; Molica and Alberti 1987*), lymphocyte morphology (*Melo et al 1986; Vallespi et al 1991*), and cytogenetic abnormalities (*Juliussen and Gahrton 1993b*) are predictors of survival (Table 1-6, 1-7, 1-8). These prognostic factors apply to all patients with CLL regardless of their age (*Montserrat et al 1991a*).

Table 1-6: Chronic Lymphocytic Leukemia: Rai Staging System

			Median Survival (Years)
Low-Risk	Stage 0 -	Lymphocytosis alone	> 15
Intermediate-Risk	Stage I -	Lymphocytosis Lymphadenopathy	9
	Stage II -	Lymphocytosis Spleen and/or Liver Enlargement	5
High-Risk	Stage III -	Lymphocytosis Anemia(Hb < 11 g/dL)	2
	Stage IV -	Lymphocytosis Platelets < 100 x 10 ⁹ /L	2

Table 1-7 Chronic Lymphocytic Leukemia: Binet Staging System

		Median Survival (Years)
Stage A -	No anemia, No thrombocytopenia less than 3 enlarged lymphoid areas*	12
Stage B -	No anemia, No thrombocytopenia three or more enlarged areas	5
Stage C -	Anemia(Hb < 10g/dl) and or Platelets < $10 \times 10^9/L$	2

* Lymphoid areas considered are: cervical, axillary, and inguinal lymphadenopathies(whether uni- or bilateral), spleen, and liver.

Table 1-8 Chronic Lymphocytic Leukemia: Other Prognostic Factors

Parameter	Median Survival(years)
Bone Marrow Histopathologic Pattern	
Non-Diffuse	10
Diffuse	3-5
Number of Lymphocytes in Blood	
$\leq 50 \times 10^9/L$	6
$> 50 \times 10^9/L$	3-4
Doubling Time	
> 12 months	10
≤ 12 months	5
Lymphocyte Morphology in Peripheral Blood	
$\leq 5\%$ Prolymphocytes	5-6
$> 5\%$ Prolymphocytes	3-4
Cytogenetic Abnormalities	
Normal Karyotype	> 10
Multiple and Complex Abnormalities	5-6

N.B.: Number of lymphocytes and prolymphocytes in blood as well as doubling time behave as continuous variables: the higher the number of cells or the shorter the doubling time, the poorer the prognosis.

In most instances, these poor-risk factors are not found alone but accompanying advanced disease.

Clinical staging systems remain the most useful prognostic parameters. The two schemes most widely used are the ones proposed by Rai et al and by Binet et al (Table 1-6, 1-7). Other variables may add discriminant power to the clinical stages. For example, among patients in early stage, those with diffuse bone marrow histopathologic pattern and/or rapidly increasing lymphocyte counts are likely to progress whereas those with non-diffuse bone marrow involvement and low, stable blood lymphocyte levels tend to have an indolent and non-progressive disease. This is the so-called smoldering CLL, for which diagnostic criteria have been proposed (Montserrat et al 1988a; French Cooperative Group on Chronic Lymphocytic Leukemia 1990; Montserrat et al 1991b) (Table 1-9).

There are several karyotypic abnormalities that may be prognostic (Juliusson et al 1990; Juliusson and Gahrton 1993b). Patients with trisomy 12 as their sole abnormality appear to have a shorter survival than those with other single abnormalities, while patients with 13q⁻ anomalies do as well as those with a normal karyotype. The complexity of the karyotype and the proportion of cells with clonal abnormalities may have

Table 1-9 Smouldering Chronic Lymphocytic Leukemia: Diagnostic Criteria

Montserrat et al (1988a)

Stage A

Non-Diffuse Bone Marrow Histopathology

Haemoglobin $\geq$ 13g/dl

Blood Lymphocytes $\leq$ $30 \times 10^9/l$

Lymphocyte Doubling Time $>$ 12 months

French Cooperative Group on CLL(1990)

A-1

Stage A

Haemoglobin $>$ 12 g/dl

Blood Lymphocytes $<$ $30 \times 10^9/l$

A-2

Stage A

Haemoglobin $>$ 12 g/dl

Blood Lymphocytes $<$ $30 \times 10^9/l$

Lymphocytes in Bone Marrow Aspirate $<$ 80%

Number of Lymphoid Area* Involved $<$ 2

* Lymphoid areas considered are: cervical, axillary and inguinal lymphadenopathy(whether uni- or bilateral), spleen and liver.

prognostic significance (*Juliusson et al 1990*). Cytogenetic abnormalities have correlated with clinical stage in some investigations (*Juliusson and Gahrton 1993b*).

The prognostic value of the immunophenotype of neoplastic B lymphocytes is unclear, with many studies yielding inconsistent or even contradictory results. For example, in a large series of patients a high SmIg intensity or low CD 23 membrane expression were associated with poor prognosis (*Geisler et al 1991*), whereas in another study CD23 expression was found to be associated with other poor-prognostic indicators, namely, higher WBC count, advanced Rai stage, and lymphadenopathy (*Newman et al 1993*).

LDH, thymidine-kinase, beta-2 microglobulin, CD23, and CD25 serum levels have also been found to be of prognostic value in some studies (*Ellims et al 1981; Lee et al 1987; Lavabre-Bertrand et al 1994; Reinisch et al 1994*). Over-expression of *mdr-1* and *mdr-3* genes and p-glycoprotein detection on neoplastic cells have been correlated with

resistance to therapy and poor prognosis in some reports (*Shustik et al 1991; Sparrow et al 1993*).

Finally, response to therapy is associated with a better outcome (*Molica and Alberti 1987*).

1.4.4. **Therapy**

1.4.4.1. **Treatment Goals**

To prolong survival is the most important aim in CLL therapy. However, since there are subjects with CLL in whom the disease does not affect survival, treatment should not be decided without taking risk factors into consideration. In the majority of the patients with CLL needing treatment the most reasonable aim is to obtain the highest response with an acceptable toxicity. However, in young patients with poor risk factors experimental approaches with the cure of the disease as the objective are warranted.

1.4.4.2. When to Treat

Owing to their age, patients with CLL may also have other chronic diseases which may preclude intensive treatment approaches. On the other hand, age by itself should not be considered as criterion for treatment. A number of factors are generally acknowledged to justify therapy in CLL (*Montserrat and Rozman 1993; Rai 1993*). Almost none of the parameters listed below are found singly but rather occur altogether in the same patient.

1. *Disease-related symptoms* (i.e. weight loss, fever without infection, night sweats, weakness). Since systemic symptoms in uncomplicated CLL are rare infection or transformation of CLL to a more aggressive lymphoproliferative disorder [Richter's syndrome (Robertson et al 1993c), CLL/PL] should be excluded before assuming that these symptoms are due to the disease.

2. *Development of anemia or thrombocytopenia due to bone marrow infiltration.*

3. *Autoimmune hemolytic anemia or thrombocytopenia.* In such cases treatment should be initiated with corticosteroids alone and cytotoxic agents added only if no response is observed.

4. *Bulky lymphadenopathy and/or splenomegaly causing compressive problems.*

5. *High blood lymphocyte count.* The threshold for initiation of therapy is difficult to establish. Whereas hyperviscosity and/or leukostasis is very rare in CLL and has been reported only with lymphocyte counts higher than $500 \times 10^9/L$ (Baer et al 1985) blood lymphocyte counts above 30 to $50 \times 10^9/L$ are associated with shorter survival (Baccarani et al 1982; Rozman et al 1982). In the absence of other unfavorable factors or symptoms related to hyperleukocytosis treatment is not indicated unless the blood lymphocyte count is extremely high (e.g., above $500 \times 10^9/L$).

6. *Rapidly increasing blood lymphocytosis* (i.e., doubling time of less than 12 months).

7. *Hypogammaglobulinemia with increased susceptibility to bacterial infections.* Infection susceptibility usually correlates with immunoglobulin levels. Nevertheless, even in cases responding to therapy hypogammaglobulinemia is usually not corrected.

8. *Massive lymphocytic infiltration of bone marrow*(i.e., diffuse histopathologic pattern). This is usually associated with other poor-prognosis factors, particularly advanced clinical stage.

9. *Complex cytogenetic abnormalities.* In most cases cytogenetic abnormalities are associated with advanced clinical stage and other poor-prognosis features.

10. *Advanced clinical stage.* Clinical stage is the most important parameter for deciding upon therapy. Patients in early clinical stage(Binet A, Rai 0) usually have a long survival and should not be treated unless the disease progresses. On the other hand, patients with advanced clinical stage(Binet B, C; Rai III, IV) have a median survival of less than 5 years and usually require treatment.

To summarize, patients with no symptoms, early clinical stage, non-diffuse bone marrow infiltration, and low and stable blood lymphocyte counts have excellent prognoses and should not be treated unless the disease progresses.

By contrast, patients with symptoms due to the disease, advanced clinical stage, diffuse bone marrow involvement or high and rapidly increasing blood lymphocyte counts require therapy.

1.4.4.3. How to Treat

1.4.4.3.1. Patients with Low-Risk Disease

Treatment of patients in early stage (Binet A, Rai 0) has resulted in a delay in the rate of disease progression but no survival benefit (*Shustik et al 1988; French Cooperative Group on Chronic Lymphocytic Leukemia 1990; Catovsky et al 1991; Spanish Cooperative Group PETHEMA 1991; Montserrat and Rozman 1993; Brugiatelli et al 1995*). In fact, early treatment has been shown to have a detrimental effect on survival in some studies (*French Cooperative Group on Chronic Lymphocytic Leukemia 1990*). Therefore, patients with early and stable disease should be observed

at three- to six-month intervals and should be treated only in case of disease progression.

1.4.4.3.2. Patients with Intermediate- and High-Risk Disease

A proportion of patients in intermediate-risk stage(Rai I, II, Binet B) have an indolent disease. These patients may be followed with no therapy as are those with low-risk disease. Nevertheless, the majority of patients with intermediate stages of the disease and virtually all patients with advanced stage(Rai III, IV, Binet C), due to bone marrow infiltration, eventually require therapy.

Over the last two decades, chlorambucil(e.g., 0.4-0.8 mg/kg orally every two weeks) has been the treatment of choice (*Montserrat and Rozman 1993*). The combination of chlorambucil plus prednisone does not appear to be superior to chlorambucil alone (*Catovsky et al 1991; Spanish Cooperative Group PETHEMA 1991*). In a series of studies from the same group, high-dose chlorambucil(10-15mg daily until complete remission or toxicity) has given excellent results. In one of these trials response rates obtained with high-dose chlorambucil(89.5%) were significantly superior

to those achieved with a modified CHOP regimen(75%)($P < 0.001$), and survival was also significantly longer(median, 68 months vs. 47 months; $p < 0.005$) in patients treated with high-dose chlorambucil (*Jaksic and Brugiatelli 1988; Jaksic et al 1995*). These studies, besides showing a dose-response relationship for chlorambucil in CLL, have opened the door to further trials using this approach in CLL treatment.

Patients treated with combination chemotherapy regimens achieve higher response rates than those treated with chlorambucil at standard doses, but this does not translate into longer survival (*Montserrat et al 1985; Montserrat et al 1988b; Hansen et al 1991; Kimby and Mellstedt 1991; Raphael et al 1991; Spanish Cooperative Group PETHEMA 1991; Montserrat and Rozman 1993*). Nevertheless, frontline treatment with combination chemotherapy(e.g., CHOP) may be indicated in tumoral forms of the disease with compressive problems, a setting in which a rapid response is desirable. For that purpose, local radiotherapy may also be useful (*Newcomb et al 1995*). For patients failing frontline therapy purine analogs, particularly fludarabine(e.g., $25\text{mg}/\text{m}^2$ i.v. daily for five days every four weeks) and 2-CDA (e.g., $0.10\text{ mg}/\text{kg}$ i.v. daily for seven days every four weeks), are considered the treatment of choice, with response rates of

40%-60% (*Keating et al 1993b; Montserrat and Rozman 1993; O'Brien et al 1993; Tallman and Hakimian 1995*).

The number of previous treatments, prior response to therapy, patient age, general status, serum albumin, and β -2-microglobulin levels correlate with the response to fludarabine in previously treated patients (*Keating et al 1993b; Montserrat and Rozman 1993; O'Brien et al 1993; Tallman and Hakimian 1995*). At present, frontline therapy with fludarabine or other purine analogs should only be considered in the setting of clinical trials. The role of purine analogs in CLL therapy is further discussed below.

1.4.4.3.3. Patients with Cytopenias due to Immune Mechanism

These patients i.e., stage C(III, IV) immune-should be treated initially with corticosteroids with cytotoxic agents added only in case of no response after two to four weeks of treatment. In patients with autoimmune hemolytic anaemia not responding to or difficult to control with corticosteroids plus cytotoxic agents, high-dose immunoglobulin or

cyclosporin may be tried. A proportion of these patients, however, eventually require splenectomy or low-dose splenic radiation (*Aabo and Walbom-Jorgensen 1985*). Pure red-cell aplasia (PRCA) may occasionally be associated with CLL; good treatment results have been reported with cyclosporine (*Chikkappa et al 1992*).

1.4.4.3.4. Patients with Hypersplenism

In such cases, splenectomy or low-dose radiotherapy of the spleen may be of benefit.

1.4.4.3.5. Therapy of Systemic Complications

Hypogammaglobulinemia is frequent in CLL (40%-50% of patients) and is the major reason for infections, which are the most frequent cause of death and a significant cause of morbidity. In a placebo-controlled randomized study 400 mg/kg of immunoglobulin given intravenously at intervals of three weeks for one year was found to be effective in reducing the incidence of infections (*Cooperative Group for the study of*

Immunoglobulin in Chronic Lymphocytic Leukemia 1988). Cost-benefit considerations, however, make the routine use of immunoglobulin in all patients with hypogammaglobulinemia questionable (*Weeks et al 1991*). Lower doses of immunoglobulin (e.g., 18g every three weeks, 10g every three weeks, 250mg/kg every four weeks) might be as effective as higher doses (*Chapel et al 1994; Jurlander et al 1994; Boughton et al 1995*).

The role of oral antibiotics as prophylaxis has not been investigated. Vaccines are considered to produce a suboptimal response because of the impairment of the immune system. As in other settings, recombinant hemopoietic growth factors may overcome neutropenia related to treatment (*Hollander et al 1991*). Finally, erythropoietin may be useful to treat anaemia unresponsive to other measures (*Pangalis et al 1995*).

1.4.4.3.6. Disease Transformation

In 3%-10% of patients CLL evolves into a large-cell lymphoma (Richter's syndrome). The appearance of progressive lymphadenopathy or splenomegaly, fever, night sweats, and increased LDH levels should raise the possibility of disease transformation. Although the prognosis of such

an event is usually poor (median survival, less than six months), patients responding to combination chemotherapy regimens may have longer survival (*Robertson et al 1993a*).

1.4.4.4. New Treatment Approaches

1.4.4.4.1. Purine Analogs

Deoxycoformycin (Pentostatin), fludarabine (Fludara), and 2-chlorodeoxyadenosine (Cladribine) are related purine analogs with high activity in CLL (*French Cooperative Group on CLL 1994; Delannoy et al 1995; Rai et al 1995; Saven et al 1995; Tallman and Hakimian 1995; Johnson et al 1996*). Unfortunately there are no studies comparing the relative merits of the different purine analogs in CLL therapy. In single-arm, uncontrolled studies, high response rates have been reported with both fludarabine (*Keating et al 1993b; O'Brien et al 1993; Tallman and Hakimian 1995*) and 2-chlorodeoxyadenosine (*Delannoy et al 1995; Saven et al 1995; Tallman and Hakimian 1995*) both in patients with and those without prior therapy.

The preliminary results from a large randomized study comparing fludarabine vs. chlorambucil in previously untreated patients show a higher CR rate for fludarabine(40/120, 33.3%) than for chlorambucil(9/113, 8.0%) ($p < 0.0001$), with the proportions of PR being the similar(45/120, 37.5%, and 40/113, 35.4%, respectively)(*Rai et al 1995*). The follow-up of this series is still too short to see whether the higher CR rate obtained with fludarabine will translate into a longer survival. Likewise, in a reported trial in which fludarabine has been compared to CAP(cyclophosphamide, doxorubicin, prednisone) for the treatment of patients with intermediate- and high-risk stage CLL(Binet's B and C stage) a higher response rate to fludarabine was observed in both untreated(71% vs. 60%, $p=0.26$) and pretreated(48% vs. 27%, $p=0.036$) cases. In the latter group, however, remission duration and survival did not differ between treatment groups, with a median remission duration of 324 days after fludarabine and 179 days after CAP($p=0.22$) and median survival times of 728 and 731 days, respectively. In untreated cases, on the other hand, fludarabine induced significantly longer remissions than CAP, with the median not yet reached after fludarabine and a median of 208 days after CAP($p < 0.001$); this effect also translated into a trend toward a longer overall survival after fludarabine($p=0.087$)(*Johnson et al 1996*).

Taken together, these results are encouraging, but currently frontline therapy with fludarabine or other purine analogs should only be considered as part of clinical trials. Purine analogs are also being investigated in combination with other drugs(e.g., cyclophosphamide, platinum, epirubicin)(*Tallman and Hakimian 1995*).

The most important side effects of purine analogs are myelosuppression and infections; the latter seem to be more frequent in patients receiving concurrent corticosteroids in whom infections due to opportunistic organisms(e.g., *Legionella*, *Pneumocystis carinii*, *Toxoplasma*, and *Listeria*) may be observed (*Cheson 1995*). This is attributed to the decrease in CD4 lymphocytes caused by these agents. Many groups use some type of antibiotic prophylaxis(e.g., co-trimoxazole, inhaled pentamidine). In patients receiving purine analogs this is a reasonable approach although there is no formal proof of its effectiveness. Other side effects of concern are the triggering of autoimmune hemolytic anaemia (*Di Raimondo et al 1993; Myint et al 1995*) and the tumour lysis syndrome (*Frame et al 1992*). In addition, several cases of transfusional acute graft-versus-host disease have been reported in patients treated with fludarabine (*Maung et al 1994; Briz et al 1995*), which raises the point as to whether blood products should

be systematically irradiated in patients receiving purine analogs.

1.4.4.4.2. Biotherapy

Monoclonal antibodies (MoAbs), either alone (e.g., CAMPATH) or conjugated with toxin (B4-block ricin), cytotoxic agents, or radioisotopes (^{131}I) are being investigated. The response is usually partial and transient. MoAbs might be useful to eliminate residual disease in patients achieving good response after chemotherapy (Grossbard *et al* 1992; Stickney and Foon 1992; Levy 1993; Rabinowe *et al* 1993a). Interferon- α (IFN- α) has only demonstrated activity in patients with early disease and no prior therapy, although no CRs are obtained (Montserrat *et al* 1991c). Whether IFN- α prolongs responses achieved with fludarabine has not been explored in large randomized trials. Data reported up to now have given either negative results (O'Brien *et al* 1995) or some indication toward a beneficial effect of IFN- α in terms of remission duration (Zinzani *et al* 1994). This issue should be investigated further.

Interlukins (e.g., IL2, IL4, IL6) as well as antisense oligonucleotides are also under study (LeMaistre *et al* 1991; LeMaistre *et al* 1992; Peng *et al*

1995).

1.4.4.4.3. Bone Marrow / Hemopoietic Stem Cell

Transplantation

Transplantation both allogeneic and autologous, are increasingly performed in CLL patients (*Rabinowe et al 1993b; Khouri et al 1994; Michallet et al 1996*). In the most recent update of the cases collected by the European and International Bone Marrow Transplant Registries (*Michallet et al 1996*), the CR rate in 70 patients allografted was 76%, and in 29 patients autografted 83%, although in the latter group the CR rate of patients who were not transplanted in CR was only 27%. The relapse rates were 11% and 17% in the allografted and the autografted group, respectively. In turn, the five-year survival probability was 42% in patients submitted to allogeneic transplants and 52% in those receiving autotransplants. Of note was the finding that 50% of the patients allo-transplanted died as a consequence of treatment-related complications, whereas only 7% of the autografted patients did (*Michallet et al 1996*). The higher mortality of the allografted patients in this series as compared to that found in single-centre

studies most likely reflects differences in selection criteria (*Rabinowe et al 1993b; Khouiri et al 1994*). Interestingly, a likely graft-versus-CLL effect has been recently described (*Mehta et al 1996*). Cytofluorometry and/or molecular biologic techniques demonstrate that some of the remissions achieved after transplant are molecular, with no evidence of residual disease (*Sadoun et al 1994; Michallet et al 1996*).

Although the role of transplantation in CLL treatment has not yet been defined, the possibility of performing a transplant should be considered in any young patient with high-risk CLL. Since responses achieved with purine analogs may be not only complete but also molecular (e.g., without evidence of residual disease by cytofluorometry and molecular biology studies), autotransplants are increasing likely to be performed in patients with CLL. All transplanted patients should be reported to the International Registries for a meaningful analysis. In this context, it is also worth emphasizing that younger age by itself is not a criterion for transplantation.

CHAPTER 2

2.0. Drug Resistance--Introduction

Chemotherapy is curative in only a small proportion of adult tumours (e.g. Hodgkin's and non-Hodgkin lymphoma, acute leukemia, teratoma) and more frequently in childhood carcinomas. Apparent prolongation of survival by chemotherapy has been shown for small cell lung cancer, ovarian cancer and breast carcinoma. However, in the majority of patients with solid tumours there is a less than 20% response to chemotherapy and even curable tumours may relapse and become resistant. Resistance may be either de novo or acquired either as a stable change within the cell, or be rapidly inducible within the cell after drug administration. Several mechanisms have been described including altered drug uptake, an alteration in the amount or the activity of glutathione transferases, DNA repair and multidrug resistance, etc. Understanding these mechanisms may help to improve the therapeutic ratio and develop new approaches.

Mechanisms of drug resistance relating to pharmacokinetics can be considered to operate from the point of administration to achievement of cytotoxic concentrations at the target site. Important factors may include absorption, distribution and metabolism of drug. Numerous factors mediate bioavailability of drugs ranging from absorption to tumour blood supply. These factors will not be discussed in this review which will concentrate on some recent advances in understanding drug resistance at the molecular level.

2.1. Mechanisms of Resistance to Alkylating Agents-

Introduction

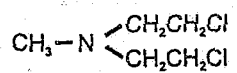
2.1.1. Biochemistry of Alkylating Agent Adduct Formation

Alkylating agents form unstable positively charged ions ($R-CH_2-CH_2^+$) that form covalent bonds with nucleophilic (electron rich) sites on DNA bases (Saffhill *et al* 1985). This interaction appears to be the major mechanism for both cell death and mutagenesis due to alkylating agents (Ludlum 1977). Ninety percent of interactions occur at the ring nitrogen N7-guanine position, and less commonly at the ring nitrogens of other

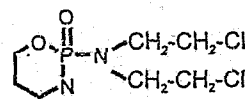
DNA bases.

The extra-ring oxygen atoms are also targets for alkylating agent attack, and the O6 atom of guanine appears to be frequently involved in these reactions. The site of interaction is important in determining what effects the adduct formation will have on the fate of the cell. For instance, adduct formation at the N7-guanine position is important for the development of intra- and interstrand crosslinks, with resultant DNA strand breaks and termination of DNA replication leading to chromosome loss or cell death. Adduct formation at the O6-guanine position appears more important for the stereotactic changes it causes in the guanine base, with resultant DNA mispairing and mutagenesis.

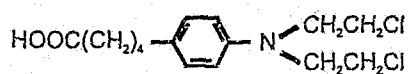
Structures of the commonly used alkylating agents are shown in Figure 2-1. With the exception of busulfan, they all share the common side chain, $-\text{CH}_2-\text{CH}_2-\text{Cl}$ as the active moiety. Melphalan, ifosfamide, nitrogen mustard, cyclophosphamide, chlorambucil, and the nitrosourea, BCNU, contain two of these groups and are considered bifunctional alkylators with the potential to form intra- and interstrand crosslinks (Colvin, 1990).



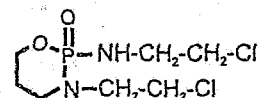
Nitrogen Mustard



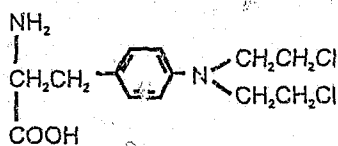
Cyclophosphamide



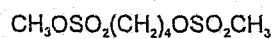
Chlorambucil



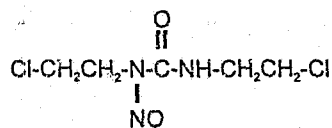
Ifosfamide



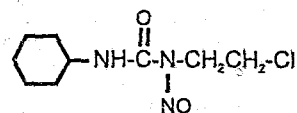
Melphalan



Busulfan



BCNU



CCNU

Figure 2-1: Structures of commonly used alkylating agents.

2.1.2. Resistance due to Decreased Cellular Uptake of Agents

Most of the alkylating agents are small, relatively nonpolar molecules, and enter cells by diffusion, rather than by active transport. No general mechanism of active extrusion, analogous to the MDR transport system, has been found for small molecules such as the alkylating agents.

Two alkylating agents have been found to enter cells by active transport, and cells resistant to these agents, by means of decreased uptake, have been described. Mustargen(HN_2 , nitrogen mustard) is a structural analogue of choline (Figure 2-1) and is actively transported into the cell by the choline transport system (Goldenberg *et al* 1971). Some researchers have described tumour cell resistance to mustargen on the basis of decreased activity of the choline transporter (Rutman *et al* 1968; Wolpert and Ruddon 1969; Goldenberg *et al* 1970). Melphalan is an analogue of the amino acid phenylalanine (Figure 2-1) and enters cells by at least two amino acid transport systems (Vistica *et al* 1978; Begleiter *et al* 1979). Resistance to melphalan due to decreased cellular uptake, associated with alterations in the amino acid transport systems in murine L1210 leukemia cells and Chinese hamster ovarian tumour cells has been described (Redwood & Colvin 1980; Dantzig *et al* 1984). High extracellular

concentrations of leucine and other amino acids that utilize the same transport systems will also diminish the antitumour effect of melphalan by competitively inhibiting the transport of melphalan into the cell (*Vistica et al 1978*).

2.1.3. Intracellular Inactivation of Alkylating Agents

Alkylating agents contain reactive electrophilic (electron-seeking) atoms and will spontaneously react with nucleophilic (electron-rich) atoms in biological molecules. Most of the alkylating agents undergo hydrolysis (alkylation of water), but since the concentration of water is essentially the same in all cells, and cellular pH is reasonably constant, the rates of this mode of inactivation should be the same for all cells.

2.1.3.1. Glutathione and Glutathione-S-Transferases

Glutathione (GSH, Figure 2-2) is a nucleophilic thiol containing tripeptide, which is present in variable concentrations in cells and is known to detoxify electrophilic toxins. A number of investigators have found a correlation between high cellular glutathione concentrations and resistance

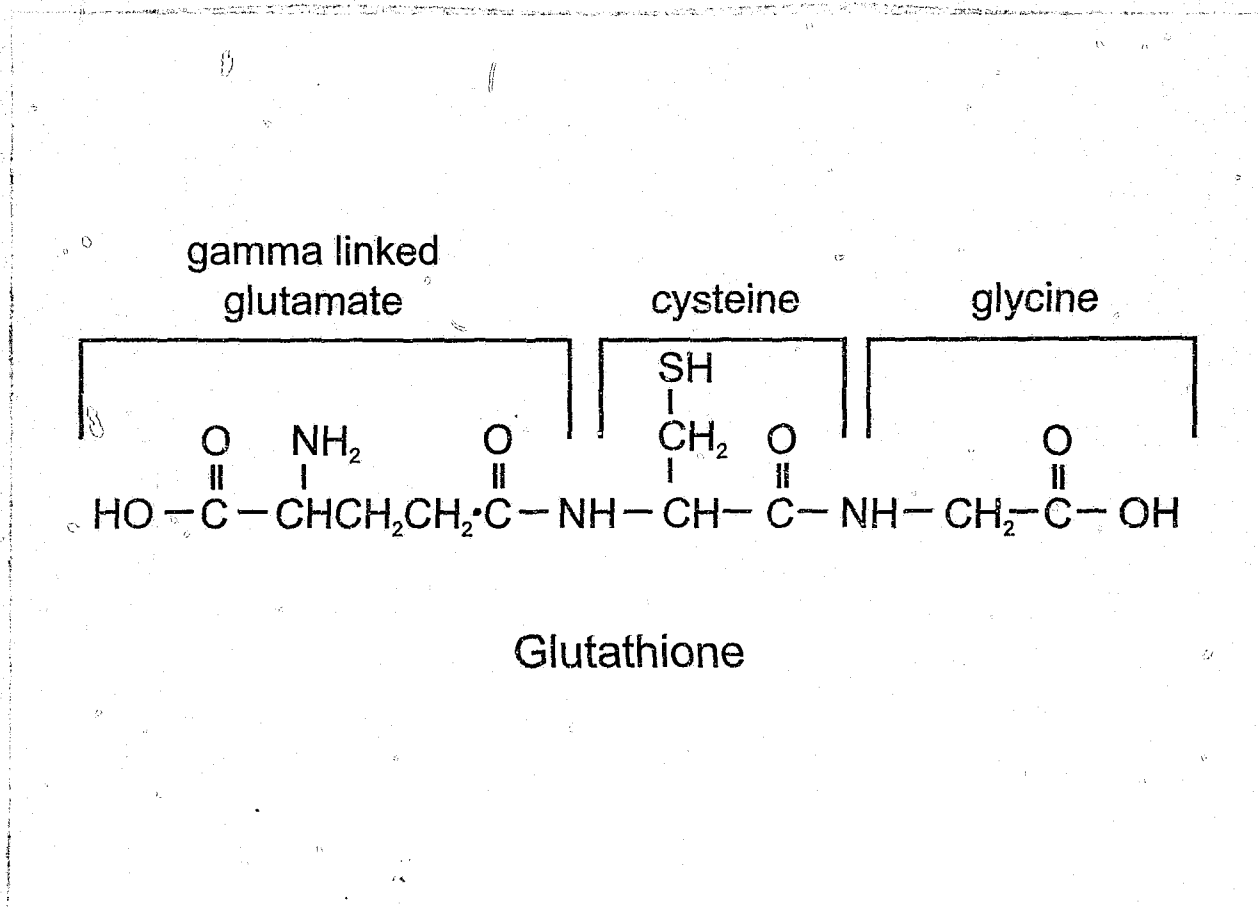


Figure 2-2: Structure and constituent amino acids of glutathione

of tumour cells to alkylating agents (*Calcutt G and Connors 1963; Suzukaka et al 1982; Bailey et al 1992; Friedman et al 1992*).

The reaction of glutathione with nucleophiles is enhanced by a family of enzymes, glutathione-S-transferases (GSTs), and elevated levels of GSTs have been associated with cellular resistance to alkylating agents (*Buller et al 1987; Robson et al 1987; Nakagawa et al 1990; Puchalski et al 1990*). In mammalian species the four major cytosolic GST families are designated α , π , μ , and θ . Increased expression of these various GST isoenzymes in cell lines is not associated with the same spectrum of drug resistance. GST- α seems to be implicated in nitrogen mustard (and chlorambucil, cyclophosphamide, melphalan) resistance, while GST- μ is associated with resistance to nitrosoureas and GST- π plays a role in a pleiotropic stress response and could be linked to the MDR phenotype (*Tew 1994*).

Buthionine sulfoxime (BSO), an inhibitor of gamma-glutamyl-cysteine-synthetase (*Dehmers & Meister 1981*), reduces cellular glutathione levels and sensitizes tumour cells to alkylating agents in vitro and in vivo (*Kramer et al 1987; Skapek et al 1988; O'Dwyer et al 1992*). As might be expected, BSO also sensitizes some normal cells to alkylating agents.

and in preclinical studies BSO administered in combination with melphalan or cyclophosphamide has been associated with increased bone-marrow, renal, gastrointestinal, and cardiac toxicity in rodents (*Ishikawa et al 1988; Smith et al 1989; Friedman et al 1990*). The combination of BSO and cyclophosphamide can be rapidly fatal in the rat, and studies have shown that this effect is due to cardiac toxicity, associated with profound depletion of cardiac glutathione (*Friedman et al 1990*). Depletion of glutathione in cells by cyclophosphamide in vivo (*Gurtoo et al 1981*) and by 4-hydroperoxycyclophosphamide in vitro (*Peters et al 1991*) has been previously demonstrated, and appears to be due to the reaction with glutathione of the acrolein released in the decomposition of 4-hydroxycyclophosphamide to phosphoramidate mustard, the reactive alkylating agent. Early clinical trials with the combination of BSO and melphalan indicate that the myelotoxicity of melphalan is enhanced, but it is too early to determine the therapeutic index of the combination (*Bailey et al 1994*).

The compounds ethacrynic acid and piriprost are inhibitors of glutathione-S-transferases and will sensitize rat breast cancer cells and human colon carcinoma cells to chlorambucil (*Tew et al 1988*). In a phase I clinical trial of the combination of ethacrynic acid and thiotepa, a sufficient dose of

ethacrynic acid could be administered to produce a decrease in the glutathione-S-transferase activity of peripheral blood mononuclear cells, and there appeared to be an increase in the hematopoietic toxicity of the thiotepa (O'Dwyer *et al* 1991).

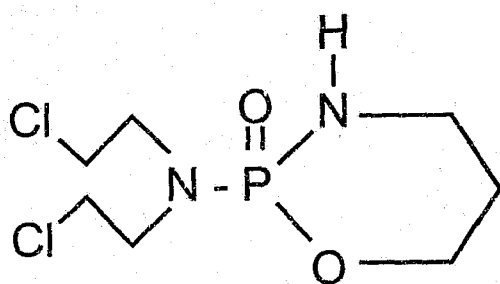
2.1.3.2. Metallothionein

Metallothioneins are thiol-containing proteins that bind metals and have been reported to be associated with resistance to alkylating agents. Cells rich in metallothionein were more resistant to cisplatin, chlorambucil, and prednimustine than cells low in metallothionein (Endresen & Rusgstad 1987). Kelley *et al* reported that tumour cell lines resistant to cisplatin after *in vitro* exposure to this agent expressed high levels of metallothionein and were also resistant to melphalan and chlorambucil (Kelley *et al* 1988). These authors also transfected tumour cells with a human metallothionein gene and demonstrated that the gene was expressed and that the transfected cells showed resistance to cisplatin, melphalan and chlorambucil. Chinese hamster ovarian tumour cells transfected with the human metallothionein gene showed resistance to N-methyl-N-nitrosourea, but the overall degree of DNA methylation was not affected, and the cells did not show enhanced resistance to methyl methane sulfonate or N-

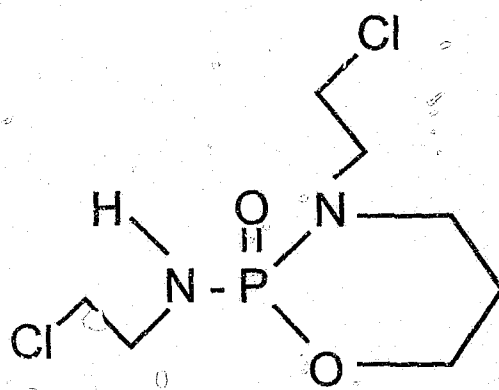
hydroxyethyl-N-chloroethylnitrosourea (*Kaina et al 1990*). Therefore, the association of metallothionein with alkylating-agent resistance may be complex.

2.1.3.3. Specific Metabolism

Alkylating agents may undergo metabolism that alters the reactivity and/or other properties of the agent. For example, cyclophosphamide and ifosfamide (**Figure 2-3**), are metabolically activated, and the active metabolites can be inactivated by further metabolism. The complex metabolism of cyclophosphamide is illustrated in **Figure 2-4**. Ifosfamide undergoes an analogous metabolic sequence (*Colvin et al 1990*). The parent compound is inactive and is activated by oxidation of a ring carbon by hepatic P-450 microsomal enzymes. The resultant 4-hydroxycyclophosphamide (or 4-hydroxyifosfamide) is in equilibrium with the open ring aldophosphamide which can spontaneously eliminate acrolein to produce phosphoramidate mustard, an active alkylating agent. While production of phosphoramidate mustard occurs extracellularly, this compound probably is a zwitterion at physiologic pH and enters cells slowly. However, the less polar 4-hydroxycyclophosphamide enters cells



Cyclophosphamide



Ifosfamide

Figure 2-3: The oxazaphosphorine alkylating agents, the active metabolites of which are inactivated by aldehyde dehydrogenase.

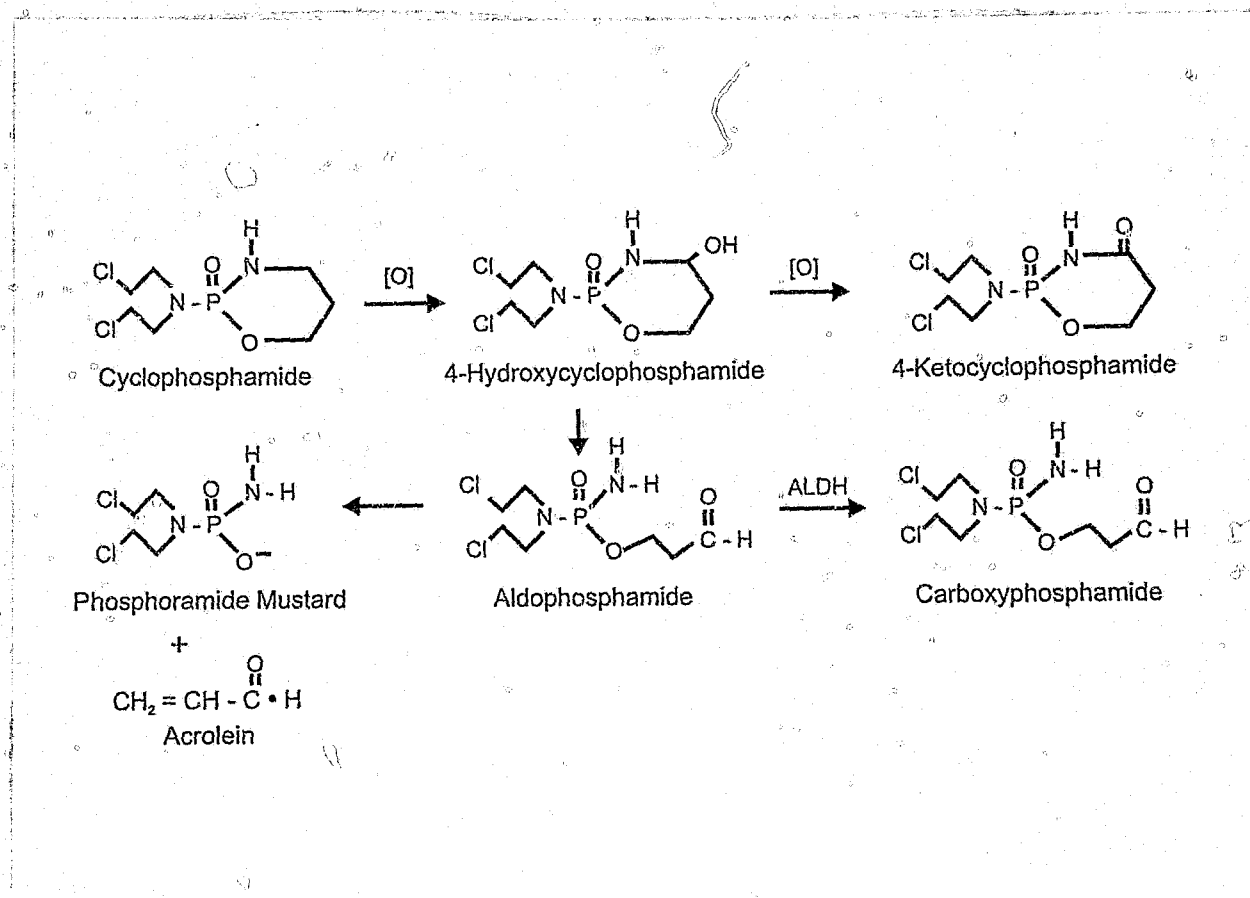


Figure 2-4: The metabolism of cyclophosphamide.

readily by diffusion and thus serves as a carrier to deliver phosphoramidate mustard(or ifosfamide mustard) into the cell.

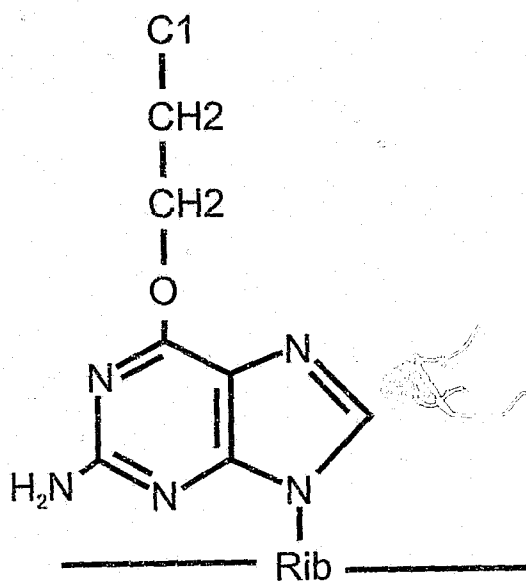
As illustrated in Figure 2-3, aldophosphamide is a substrate for the aldehyde dehydrogenase family of enzymes which metabolize aldophosphamide(or aldoifosfamide) to carboxyphosphamide. Carboxyphosphamide does not decompose to form phosphoramidate mustard, is not cytotoxic, and represents the major urinary excretion product of cyclophosphamide. It has now been established that bone marrow stem cells of several species and the epithelium of the small intestine of the mouse(the only species so studied) are rich in aldehyde dehydrogenase isozymes which inactivate aldophosphamide (*Kohn and Sladek 1985; Sahovic et al 1988; Russo et al 1989*). This evidence strongly suggests that the characteristic high therapeutic index of the oxazaphosphorines is due to protection of at least the bone marrow and gastrointestinal tract by aldehyde dehydrogenases. Aldehyde dehydrogenase has been shown to be a significant mechanism of resistance in murine lymphoid leukemia cells (*Hilton 1984; Sladek and Landkamer 1985*), rat acute myeloid leukemia cells (*Koelling et al 1990*), human acute myeloid leukemia cells (*Colvin et al 1988*), and human ovarian tumour cells (*Parsons et al 1990*). Elevation of aldehyde aldehydogenase

in cells should produce specific resistance to the oxazaphosphorines, such as cyclophosphamide and ifosfamide.

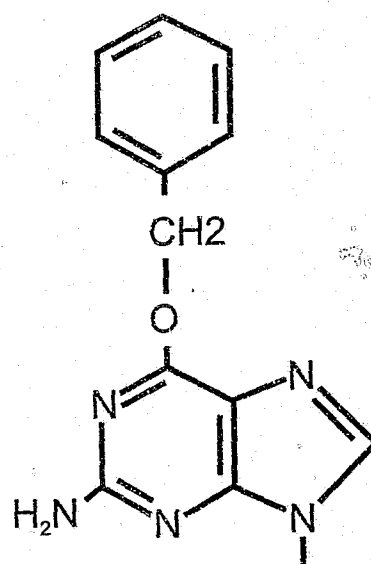
2.1.4. DNA Repair

There are extensive mechanisms for the repair of damage to DNA including alkylation damage in cells. Most of the better-characterized repair mechanisms have been those for the repair of radiation damage and alkylation by carcinogens.

The best characterized example of DNA-repair-based cellular resistance to alkylating agents is the role of O-6-alkylguanine-alkyltransferase (O-6-AGAT) in producing resistance to chloroethylnitrosoureas and other compounds that alkylate the O-6 position of guanylic acid (Figure 2-5). This enzyme removes alkyl groups from the O-6 position of guanylic acid units in DNA and thus prevents formation of DNA cross-links, or depurination and strand breakage produced by monofunctional O-6 alkylating agents such as procarbazine and dacarbazine. It is now established that both rodent and human tumors can be resistant to O-6 alkylating agents by this mechanism (*Erickson et al 1980; Brent et al*



Guanylic acid residue in
DNA chloroethylated at
O - 6 by BCNU



O - 6 Benzylguanine

Figure 2-5: O-6 chloroethylguanylic acid and O-6 benzylguanine,
substrates for O-6-alkylguanine-alkyltransferase.

1985; Bodell *et al* 1988; Pegg 1990), and this mechanism appears to be a major reason for the resistance of human gliomas to chloroethylnitrosoureas and procarbazine (Bodell *et al* 1988; Schold *et al* 1989; Ali-Osman *et al* 1990). The alkyl group removed by O-6-AGAT is bound covalently to the enzyme and results in the inactivation and metabolic degradation of the enzyme. The administration of a monofunctional O-6 alkylating agent, such as streptozotocin, will overcome resistance to subsequently administered BCNU (Pieper *et al* 1991). The mechanism of this resistance reversal appears to be as follows. The streptozotocin produces methylation of O-6 guanyl residues in DNA; these methyl groups are then removed by O-6-AGAT; the enzyme is saturated and depleted and the cells are then sensitive to BCNU until the O-6-AGAT levels are restored by synthesis of new enzyme. Since streptozotocin and other monofunctional alkylating agents may well have toxicities despite the presence of O-6-AGAT a perhaps more promising approach stems from the observation that O-6 alkylated guanine analogues will serve as substrates for the enzyme. A particularly promising compound to reverse O-6-AGAT-associated resistance is the compound O-6 benzyl guanine for which the enzyme has a high affinity (Dolan *et al* 1990, Mitchell *et al* 1992).

While removal of DNA cross-links from cells has been known to occur for some years, a role for a specifically enhanced cross-link repair mechanism in cellular alkylating agent resistance, such as increased activity of an excision endonuclease, has not been definitively demonstrated. However, a number of lines of evidence are converging to indicate how DNA repair may be enhanced in cells resistant to alkylating agents.

It has been known for some years that cells respond to DNA irradiation damage by arresting in both the G_1 and G_2 phases of the cell cycle. These cells are then able to repair the DNA damage before they undergo DNA replication or mitosis, and are thus more tolerant of the DNA damage than cells that continue in a proliferative phase. Undergoing DNA replication or mitosis with damaged DNA produces further damage to the DNA and chromosomes and enhances lethality. The wild-type p53 gene product is associated with the G_1 arrest following gamma irradiation damage to cells (*Kastan et al 1991; Kuerbitz et al 1992*).

Cells damaged by alkylating agents can respond with arrest in the G_2 phase, and during this arrest DNA repair can occur. Cells that respond to DNA damage with G_2 cell cycle arrest are more resistant to alkylating agents than cells that continue to proliferate and enter into mitosis with

alkylated DNA. A human lymphoma cell line with marked sensitivity to nitrogen mustard failed to enter G₂ arrest after nitrogen mustard treatment, as compared to a more resistant human lymphoma line (*O'Connor et al 1992*). The G₂ arrest in the more resistant lymphoma cells was associated with higher levels of hyperphosphorylated(inactive) cdc2 kinase in the resistant cells. It has been postulated that in the resistant cells the cell-cycle delay results from the inability of the cdc2 kinase to activate M-phase-promoting factor.

Caffeine and other xanthine derivatives have been shown to prevent cell-cycle delay in response to alkylating agents and to render cells more sensitive to alkylating agents (*Rao 1980; Fingert et al 1986; Teicher et al 1991*). Also, inhibitors of enzymes involved in DNA repair, such as aphidicolin (*Frankfurt 1991*), which inhibits repair synthesis, and novobiocin (*Eder et al 1987*), which inhibits topoisomerase II, will reverse this type of alkylating-agent resistance. Clinical trials have demonstrated that plasma concentrations of novobiocin that reverse alkylating agent resistance in vitro can be achieved in patients receiving high doses of thiotepa (*Eder et al 1991*).

2.1.5. Nucleotide Excision Repair(NER), Polymerases and DNA Repair

NER is an excinuclease system made up of multiple proteins that interact to remove bulky DNA adducts and other lesions from DNA XP cell lines, which are deficient in NER, are sensitive to several alkylating agents used in cancer chemotherapy, including cisplatin and melphalan. But there is no direct evidence that NER is involved in resistance to chemotherapeutic drugs, which act by damaging DNA(*Chaney and Sancar, 1996*). At least five mammalian DNA polymerases have been identified (**Table 2-1**). NER is sensitive to the drug aphidicolin, as shown by experiments with both cells and cell-free extracts (*Dresler and Frattini, 1986; Nishida et al 1988; Hunting et al 1991; Coverley et al 1992; Popanda and Thielmann 1992*). The known aphidicolin-sensitive DNA polymerases are Pol α (Pol I in yeast), Pol δ (poly III in yeast), and Pol ϵ (Pol II in yeast), whereas Pol β is not aphidicolin-sensitive (*Wang 1996*). There is good evidence that mammalian DNA polymerase α is not involved in NER. Studies of intact and permeabilized cells have taken advantage of other chemical inhibitors that can differentially inhibit polymerases, and have concluded that DNA polymerase δ or ϵ , but not α , is responsible for NER synthesis (*Dresler and Frattini 1986; Nishida et al 1988; Hunting et al 1991; Popanda and Thielmann 1992*). In line with this, monoclonal antibodies that neutralize the activity of DNA Pol α in solution do not significantly inhibit DNA-repair synthesis carried out by cell extracts (*Coverley et al 1992*). The main cellular function of DNA polymerases α

Table 2-1 Nuclear DNA Polymerases

DNA Polymerases	Molecular Weight(kd)*	Human chromosome map position	<i>S. cerevisiae</i> gene
Pol α (catalytic) subunit	165	Xp21.3	POL1
Pol α (middle subunit)	70		POL10
Pol α (primase subunit)	58		PRI1
Pol α (primase subunit)	49		PRI2
Pol β	39	8p11-p12	POL4
Pol δ (catalytic) subunit	124	19q13.3-q13.4	POL3
Pol δ (small subunit)	51	7	
Pol ϵ (catalytic) subunit	255(active fragment of 145)	12q24.3	POL2

(Continued)

DNA	Molecular	Human	<i>S. cerevisiae</i>
Polymerases	Weight(kd)*	chromosome	gene
subunit		map position	
Pol ϵ (small	55		
subunit)			
Pol ζ	173 (<i>S.</i>		REV3
(catalytic)	<i>cerevisiae</i>)		
Pol ζ (small	29(<i>S.</i>		REV7
subunit)	<i>cerevisiae</i>)		

* The molecular weight predicted from the open reading frame of the human gene is given, unless otherwise indicated. See reference(Wang, 1996) for further information and literature citations.

appear to be an essential role in semi-conservative DNA replication where the associated primase activity confers the unique ability to synthesize an RNA primer, and the first few nucleotides of each leading strand and Okazaki fragment (*Weinberg and Kelly 1989; Tsurimoto et al 1990; Gray and Wong 1992; Araki et al 1994*).

DNA polymerase β (Pol β) does not seem to play a direct role in cellular DNA replication (*Tsurimoto et al 1990; Matsumoto and Kim 1995*). A role for Pol β in base excision repair is supported by evidence demonstrating that cells deleted of both copies of the Pol β gene do not support base excision repair in vitro and that rat Pol β dominant-negative mutants inhibit base excision repair in *Sacharomyces cerevisiae* (*Clairmont and Sweasy 1996; Sobol et al 1996*). Pol β may also function in nucleotide excision repair in *Xenopus* oocytes (*Oda et al 1996*).

2.1.6 In-Vivo Resistance to Alkylating Agents

Resistance of tumour cells in vivo, with the same tumour cells remaining sensitive in vitro, has been described (*Teicher et al 1989*). Certainly the pH (*Jahde et al 1990*), state of oxygenation, and rate of activity of various

metabolic pathways in cells may vary considerably between the *in vitro* and *in vivo* settings, and between different locations *in vivo*. Poor vascular perfusion and decreased penetration of drugs into the centre of tumour masses have been demonstrated and may be responsible for the poor response of some *in vivo* tumours to alkylating agents. The presence of the *mdr* drug-resistance protein in the endothelial cells of brain capillaries has been described (Cordon-Cardo 1990; Nabors *et al* 1991). Since capillary endothelial cells in tumours proliferate under drug exposure along with tumour cells, these cells may express drug detoxification enzymes and play a role in drug resistance.

2.2. Topoisomerases and Drug Resistance

DNA topoisomerases are a unique class of enzymes that are responsible for controlling, maintaining and modifying the structure or topology of DNA during replication and translation of genetic material (Cozzarelli 1980; Gellert 1981; Liu 1983; Wang 1985; Wang 1987). Based on fundamental differences in their reaction mechanisms, DNA topoisomerases are classified into two types: DNA topoisomerase I and DNA topoisomerase II. Human DNA topoisomerase I is a 100 kD monomeric protein encoded by a single-copy gene located on human chromosome 20q12-13.2 (Liu and

Miller 1981; D'Arpa et al 1988; Juan et al 1988). Two different isozymes of topoisomerase I, with molecular weights of 100 and of 66kD, respectively, have been suggested to exist in drug resistant tumour cells (Pommier et al 1990).

Topoisomerase II (TopoII) is essential for DNA replication, chromosome segregation, transcription and perhaps DNA recombination (Wang 1987).

There are two isoforms of topoisomerase II in mammalian cells that are products of different genes (Chung et al 1989; Drake et al 1989; Jenkins et al 1992; Tan et al 1992; Austin et al 1993). These isoforms are termed α (170 kDa form) and β (180 kDa form) and have different patterns of expression, suggesting that they might perform different functions. The α isoform is produced primarily in late S-phase and during the G₂/M phase of the cell cycle (Woessner et al 1991). The gene encoding the α isoform has been mapped to chromosome 17q21-22 in humans (Tsai-Pflugfelder et al 1988). The β isoform is expressed throughout the cell cycle, with higher levels seen in non-proliferating cells (Woessner et al 1991) and is encoded on chromosome 3p24 in humans (Jenkins et al 1992; Tan et al 1992). The level of topoisomerase II β is relatively constant through the cell cycle (Woessner et al 1991). Most of the functions of topoisomerase II listed above are ascribed to the α isoform; the β isoform may function in rRNA

production (Wang 1987; Zini et al 1992).

Topoisomerase II α is a key target of several clinically important antineoplastic agents, including doxorubicin, epirubicin, mitoxantrone and etoposide (Liu 1989; Osheroff et al 1991; Capranico and Zunino 1992; Pommier 1993). Topoisomerase II α is a nuclear enzyme which alters DNA tertiary structure through transient double-stranded breakage of the DNA backbone and subsequent passage of a second intact DNA duplex through the break (Wang 1985; Austin and Fisher 1990; Osheroff et al 1991; Watt and Hickson 1994). The aforementioned drugs, as well as several other intercalating agents, including amsacrine (Nelson et al 1984), trap the enzyme in a covalently bound, reversible, complex with DNA, termed the cleavable complex. The stabilisation of this complex prevents religation of broken DNA and produces lesions which are thought to be cytotoxic by virtue of their ability to inhibit the passage of the replication fork, resulting in cell death via apoptosis (Liu 1989; Walker et al 1991; Lock 1992). There is evidence that the cellular level of topoisomerase II determines the extent of cleavable complex formation after drug treatment and, therefore, the degree of drug toxicity. Low levels of topoisomerase II are associated with the induction of a reduced number of DNA lesions and hence increased drug resistance (Beck et al 1993; Pommier 1993). The

converse relationship has been shown in mutant cell lines hypersensitive to topoisomerase II inhibitors (*Davies et al 1988*) and also in testicular teratoma cell lines as compared with bladder cell lines (*Fry et al 1991*). On the other hand, elevated levels of topoisomerase II have been associated with resistance to DNA-damaging agents like X-rays, alkylating agents, and cisplatin (*Tan et al 1987; DeJong et al 1991a, Eder et al 1993*). Treatment with topoisomerase II inhibitors may increase the cytotoxicity of DNA-damaging agents (*DeJong et al 1993, Eder et al 1989*), although the mechanisms of these effects need to be fully defined.

2.3. Multidrug Resistance

Multidrug resistance(MDR) is the phenomenon whereby exposure to one drug induces cross resistance to a variety of agents of different chemical class and often different mechanism of action to which the cell has never been exposed. Multidrug resistance usually includes resistance to adriamycin, vinca alkaloids and mitomycin C, but not platinum compounds, bleomycin and alkylating agents. The most intensively studied mechanism of MDR is the expression of P glycoprotein(PGP), a 170kD protein with six hydrophobic domains and a tandemly duplicated ATP binding domain (*Deuchars and Ling 1989; Endicott and Ling 1989*).

PGP acts as a transmembrane exporter of drugs but the normal substrates have yet to be identified.

2.3.1. Chromosomal Location and Genomic Organisation of the MDR Gene

In man the MDR1 gene is located on chromosome 7q21-31 and consists of an open reading frame of 1280 amino acids. There are 2 human and 3 rodent MDR genes (**Table 2-2**) (*Croop et al 1989; Chin et al 1993; Gottesman and Pastan 1993; Sikic 1993*). Control of expression of both human MDR genes occurs at the level of gene copy number, transcription, translation, and also post-translationally. Isolation and sequencing of a one kilobase portion of genomic DNA encoding the promoter region of the human MDR-1 gene shows a consensus CAAT box and two GC box like sequences but no TATA sequences. Although two potential promoter start sites have been found, only the downstream promoter was activated in the KB cells tested (*Ueda et al 1987; Chin et al 1993; Gottesman and Pastan 1993*).

Table2-2 Expression of PCP Genes in Human, Mouse and Hamster

Species	P-Glycoprotein Gene Class		
	I	II	III
HUMAN	mdr1		mdr3
MOUSE	mdr3	mdr1	mdr2
HAMSTER	pgp1	pgp2	

2.3.2. Molecular Genetic Studies

The gene encoding P-glycoprotein belongs to a multigene family. There are two *mdr* genes in humans. MDR1 encodes the P-glycoprotein responsible for conferring the MDR phenotype. The MDR2 gene has not been shown to convey resistance to cytotoxic agents (*Roninson et al 1986; van der Bliek et al 1987, 1988*). The cloning and sequencing of the mammalian MDR genes indicate that the P-glycoproteins are about 1280 amino acids in length with two highly homologous halves (*Endicott et al 1989; Nooter and Herweijer 1991; Pastan and Gottesman 1991; Roninson 1992*).

2.3.3. Tissue Distribution of MDR Expression

Although the normal physiologic function of the mammalian *mdr* genes remains unknown, these genes are differentially expressed in a variety of normal tissues, particularly along the apical surface of secretory epithelium of the jejunum and colon, bile canaliculi, proximal tubular epithelium of the kidney, pancreatic small ductule epithelium, and the glandular epithelium of the pregnant uterus (*Fojo et al 1987; Thiebaut et al 1987; Arceci et al 1988; Sugawara et al 1988; Cordon-Cardo et al 1989; Croop et al 1989; Thiebaut et al 1989; Georges et al 1990; van der Valk*

et al 1990). In addition, P-glycoprotein is expressed at high levels in the adrenal gland, placenta, capillary endothelium of the brain and testis, as well as in hematopoietic precursors and lymphocytes (*Cordon-Cardo et al 1989; Thiebaut et al 1989; Chaudhary and Roninson 1991; Chaudhary et al 1992; Drach et al 1992*).

2.3.4. Clinical Significance

There is evidence that the MDR1 P-glycoprotein acts a channel for the outward movement of adenosine triphosphate(ATP) under normal physiologic conditions (*Abraham et al 1993*) and as a volume-regulated chloride channel (*Gill et al 1992; Valverde 1992*). Evidence has also been presented which demonstrates that cortisol and aldosterone may be physiologic substrates (*Ueda et al 1992*). The expression pattern of MDR1 suggests that P-glycoprotein might be involved in the secretion of metabolites and toxic substances into the excretory paths of bile, urine, and the gastrointestinal tract (*Cordon -Ccardo 1989; Thiebaut et al 1989*). It has also been proposed that the MDR1 transporter could serves as secretory pathway for polypeptides lacking signal leader sequences (*Muesch et al 1990*).

Because of the clearly defined role of P-glycoprotein in determining the MDR phenotype in vitro, a logical expectation has been that this mechanism would also help to explain some forms of clinical resistance observed in human malignancies. This expectation has been further heightened by the discoveries of a large number of noncytotoxic compounds that are capable of reversing the drug efflux mechanism of P-glycoprotein (Goldstein *et al* 1992). Such agents include verapamil (Tsuruo *et al* 1981, 1982), cyclosporine A and several of its derivatives (Slater *et al* 1986a, 1986b; Twentyman and Bleehen 1991; Twentyman 1992), reserpine (Beck *et al* 1988), phenothiazines (Ford *et al* 1989), quinidine (Tsuruo *et al* 1984), the immunosuppressant FK506 (Epand *et al* 1984; Arceci *et al* 1992; Naito *et al* 1992), the antifungal rapamycin (Arceci *et al* 1992), progesterone (Yang *et al* 1989; Qian and Beck 1990), and tamoxifen (Ramu *et al* 1984). There is now considerable evidence that these MDR reversal agents are capable of interacting directly with P-glycoprotein and displacing the binding of cytotoxic drugs. Furthermore, reversal compounds have been used successfully to sensitize MDR1-positive tumours in the mouse model system (Tsuruo *et al* 1981; Formelli *et al* 1988; Hill *et al* 1988). For many of these drugs, widespread clinical use as MDR modulators has been precluded because of side-effects associated with their use at concentrations required to inhibit P-

glycoprotein(*Ferry et al 1996*). However, some of these modulators, such as verapamil and cyclosporine A, have shown clinical benefits in treatment of refractory haematopoietic malignancies (*Dalton, 1994*). Recently, some potent second-generation modulators have been developed and the most potent of these include PSC 833 (*Boesch et al 1991*), GF120918(GG918)(*Hyafil et al, 1993*) and XR9051 (*Dale et al 1998*). Some of these compounds are currently in clinical trials. Thus, these data have suggested that not only might the level of P-glycoprotein expression predict the response of individual tumours to specific cytotoxic drug combinations, but also provide an important cellular target for reversing MDR1-mediated drug resistance.

CHAPTER 3

Aim of the study

Treatment of CLL is usually initiated when the tumour burden or bone marrow failure warrants intervention. In the early stages of the disease, treatment with the alkylating agent (e.g. chlorambucil) may be effective in controlling lymphocyte proliferation for extensive periods. In advanced CLL, more aggressive doxorubicin-containing combination chemotherapy may be used. Although treatment is frequently effective initially, intrinsic or acquired resistance to cytotoxic drugs generally occurs as the disease and treatment progresses. Ultimately most patients become refractory to therapy, including therapy with alkylating agents.

Melphalan (L-phenylalanine mustard) is a rationally designed alkylating agent active against a variety of cancers. Since melphalan is the active agent, requiring no further metabolic activation, the drug has usually been used as a model for studying the mechanisms of alkylator resistance. In order to improve the therapeutic index of the alkylating agents, I hope to

- 1).to investigate the mechanisms of drug resistance to alkylating agents in chronic lymphocytic leukemia, using melphalan resistance as the model;
- 2).to find a way to overcome such resistance.

The methods used in this study were:

- 1). To determine the extent of interstrand DNA crosslinking, to investigate the relationship between melphalan cytotoxicity and formation of interstrand DNA crosslinking and cell cycle analysis in the melphalan-resistant B-CLL cell line(WSU-CLL) and in the melphalan-sensitive B-lymphocyte cell line(WIL2).
- 2). To determine melphalan accumulation and efflux as well as levels of glutathione and the important catalytic enzyme(GST) in the glutathione conjugation of alkylating agent thiotepa in WSU-CLL cells as compared to WIL-2 cells.
- 3). To determine topoisomerase I and II expression and activity in WSU-CLL cells and in WIL2 cells.
- 4). To determine DNA polymerase α and β in WSU-CLL cells and WIL-2

cells.

- 5). To investigate the effects of Interleukin 4 on malignant B cells from patients with B-chronic lymphocytic leukemia(B-CLL).

The aim of these investigations is to better define the factors determining alkylator resistance in CLL with the ultimate aim of finding methods of overcoming the problem of clinical alkylator resistance.

CHAPTER 4

Materials and Methods

4.1 Materials

Materials are shown in table 4-1 which were used in the experiments described in the following chapters:

4.2 Methods

This section describes some of the experimental methods common to the investigations described in the following chapters. Additional methods, peculiar to specific studies, are described in the appropriate chapters.

Table 4-1 Materials Used in the Experiments

Reagent Name	Manufactory Name
³² P-dATP	Amersham Life Science(USA)
¹²⁵ INa	
Contricon 100	Amicon, Inc. USA
Albumin	Boehringer Mannheim (Frankfurt, Germany).
Alkaline phosphatase-linked goat	
anti-mouse or anti-rabbit IgG	
antibody	
Aphidicolin	
5-bromo-4 chloro-3-indolyl	
phosphate Nitroblue tetrazolium	
chloride	
Proteinase K	
Recombinant Interleukin 4	
RNAse	
Dulbecco's phosphate buffered	Highveld Biologicals
saline	

Reagent Name	Manufactory Name
Fetal Calf Serum	Highveld Biologicals
RPMI 1640	(Johannesburg, S.A.)
[Chloroethyl- ¹⁴ C] melphalan(50 mCi/mmol)	Moravek Biochemicals Inc(USA)
Monoclonal anti-human topoisomerase II β p180 antibody	PharMingen(USA)
Activated calf thymus DNA	Sigma Chemicals St. Lo.(Mo USA)
Agrose	
Aprotinin	
BCH	
Benzamidine	
Choline chloride	
Cystine	
Deoxynucleotide bases	
Ficoll-Hypaque	
L-Leucine	
Leupeptin	
MTT	

Reagent Name	Manufactory Name
Pepstatin	Sigma Chemicals St. Lo.(Mo USA)
Phenol/Chloroform/Isoamyl	
Alcohol	
PMSF	
Propidium Iodide	
Soybean trypsin inhibitor	
Tryptophan	
Camptothecin	TopoGEN, Inc. (Columbus,USA)
Doxorubicin	
Monoclonal, antihuman topoisomerase II α p170 antibody	
Polyclonal antihuman topoisomerase I antibody	
Topoisomerase I assay kit	
Topoisomerase II assay kit	
Purified p170 kDa Topo II marker	
Melphalan	
	Wellcome (Pty Ltd)

4.2.1 Cell lines and Culture Conditions

WIL2 was obtained from ATCC, USA. It is a continuously growing B-lymphocyte cell line which was isolated from the spleen of a Caucasian male with hereditary spherocytosis.

WSU-CLL was a gift from Dr R .M.Mohammad at the Wayne State University, USA. It is a B-CLL cell line that was established from a 66-year-old black male who had been diagnosed as suffering from chronic lymphocytic leukemia with an autoimmune hemolytic anaemia. Initial treatment with cyclophosphamide and prednisone orally was switched to CVP(cyclophosphamide, vincristine, prednisone) due to poor response. The disease respond partially to this regimen for about a year then progressed. There was no response to subsequent treatment with fludarabine(2 cycles) and 2 cycles of VAD(vincristine, doxorubicin, dexamethasone). He died 2 months after the last cycle of VAD. WSU-CLL was establishes from a sample of the patient's peripheral blood during his terminal course.

Both cell lines are EBV-negative.

All in-vitro experiments were carried out in RPMI 1640 medium 10 % fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). Incubations were performed at 37 °C in a humidified atmosphere with 5 % CO₂.

4.2.2 Cytotoxicity Assays

Cell number was determined using the MTT assay, based on active mitochondrial reduction of 1,3[4,5 dimethylthiazole-2yl]-3,5 diphyl tetrazolium bromide. The assay used incorporated the modifications of Twentyman and Luscombe (Twentyman and Luscombe, 1987).

Cytotoxicity assays were performed using 5×10^5 cells in 96-well microtiter plates in 200 μ l of culture medium. Melphalan solutions were prepared daily in 70 % ethanol containing an equimolar concentration of hydrochloric acid. To minimize hydrolysis further dilutions were made in aqueous medium immediately prior to use. Cells were exposed to melphalan at concentrations ranging from 0-320 μ M, to doxorubicin or camptothecin at concentrations ranging from 0-5.0 μ M and to aphidicolin at final concentrations in the range of 0-15 μ M for up to 72 hours. Cells were then washed with fresh medium, followed by addition of 25 μ l MTT (4 mg/ml) in 200 μ l of medium and incubated for 4 hours. Medium was then aspirated and formazan solubilised in DMSO. Optical density was read within 30 minutes in an ELISA plate reader at 540 nm. Each experiment was performed in triplicate at each of the drug concentrations.

The surviving fraction of cells (SF) following treatment with each of the drugs, compared to control (untreated) cells, was calculated for each drug concentration. Cell number versus drug concentration was plotted and the IC_{50} value was calculated from dose response curves as the concentration of drug which reduced the number of viable cells to 50% of control.

4.2.3 Determination of Interstrand-DNA-Cross-Link

Formation

Cells were suspended in RPMI 1640 at 2×10^6 cells/ml. Cells were exposed to various concentrations of melphalan at $37^\circ C$ for 60 minutes after which the cells were centrifuged and washed once in fresh RPMI 1640 at room temperature, then resuspended in drug-free medium and re-incubated for up to 24 hours at $37^\circ C$. Viability of control lymphocytes (without melphalan exposure) was $\geq 90\%$ at the end of the 24 hour period. Interstrand-DNA-cross-linking by melphalan was detected by an ethidium bromide fluorescence assay based on the method of De Jong and co-workers (De Jong *et al*, 1986). Cells and incubation medium from test wells were added to 200 μ l of a lysing solution consisting of 4M NaCl,

50 mM KH_2PO_4 , 10 mM EDTA, and 0.1 % (w/v) Sarkosyl (pH 7.2). RNA-DNA and protein-DNA cross-links were digested by addition of 20 μl of bovine pancreas RNase (0.5 mg/ml) to the lysates, which were then incubated at 37 °C for 16 hours. Following the incubation, 25 μl of proteinase-K (0.5 mg/ml) and 25 μl heparin (500 IU/ml) were added for 30 min at 37 °C. Aliquots of the resulting lysates were denatured by heating at 100 °C for 5 min and were then rapidly cooled to 23 °C. Each of the resulting samples were added to 3 ml of a solution containing ethidium bromide (10 $\mu\text{g/ml}$), 20 mM K_2HPO_4 and 0.4 mM EDTA (pH 12.0) in test tubes wrapped in aluminum foil to prevent light-induced cleavage of DNA by ethidium bromide. Fluorescence was measured, both before and after denaturation, in 1 cm^2 cuvettes (at 23 °C) at an excitation wavelength of 525 nm and an emission wavelength of 580 nm in an LS 50 variable wavelength spectrofluorometer (Perkin Elmer, Norwalk, CT). The percentage of cross-linked DNA was determined by measuring the difference in fluorescence of denatured control cell lysates and the denatured melphalan-treated samples by the formula:

$$C_t \% = \frac{f_t - f_c}{1 - f_c} \times 100$$

Where $C_t\%$ = the percentage of interstrand cross-linked DNA in treated cells; f_t = fluorescence intensity after heat-denaturation divided by fluorescence intensity before heat denaturation in treated cells; f_c = fluorescence intensity after heat denaturation divided by fluorescence intensity before heat denaturation in untreated cells.

CHAPTER 5

Cytotoxicity, Interstrand-DNA-Crosslinks and Cell Cycle Analysis in the Melphalan-Sensitive B-Lymphocytic Cell Line(WIL2) and in the Melphalan-Resistant B-CLL Cell Line(WSU-CLL)

5.1. Introduction:

Alkylating agents remain an important class of chemotherapeutic drugs. These agents show a wide spectrum of clinical activity and high antitumour effectiveness (*DeVita et al 1993*). There is considerable evidence (*Erickson et al 1977; Ewig and Kohn 1977; Kohn 1977; Ewig and Kohn, 1978; Ross et al, 1978; Hansson et al 1987*) to suggest that interstrand cross-linking of cellular DNA by the bifunctionally alkylating chloro-ethyl carbonium ions constitutes the major cytotoxic event and consequently the major antitumour effects. Unfortunately, resistance to alkylating agents, either de-novo or acquired, does occur and such resistance remains a major

limitation in cancer chemotherapy. Several mechanisms of resistance to alkylating agents have been reported, including 1) alterations in drug transport (*Redwood and Colvin 1980; Begleiter et al 1983; Richon et al 1987; Teichner et al 1987; Waud 1987; Friedman et al 1988; Bungo et al 1990*); 2) elevated levels of secondary, non-essential, targets such as glutathione (GSH) (*Ahmad et al 1987; Richon et al 1987*) or metallothioneins (*McGown and Fox 1986; Kelley et al 1988*); 3) increased glutathione-S-transferase activity (*McGown and Fox 1986; Waxman, 1990*); 4) enhanced repair of DNA mono adducts or of DNA cross-links (*Bedford et al 1988; Bodell et al 1988; Eastman and Schulte 1988; Masuda et al 1988; Pegg et al, 1990*). An alternative mechanism by which alkylating resistant neoplastic cells escape drug targeting may be their ability to arrest their growth cycle in response to DNA damage. Cell cycle checkpoints present at G₁-S and G₂ allow the cell to assess DNA integrity before replication (*Hartwell and Weinert 1989*). Extensive DNA damage may lead to programmed cell death, known as apoptosis (*El-Deiry et al 1994*); however, lesser DNA damage can arrest cells in the growth cycle to allow time for assessment and repair of drug-induced damage (*Kastan et al 1991*).

Although the precise mechanisms involved in the development of resistance to the alkylating agents in the clinical situation are still the subject of much investigation it has been suggested that resistance is probably multifactorial in origin (*Hilton 1984; McGown and Fox 1986; Ahmad et al 1987; Richon et al 1987; Eastman and Schulte 1988; Masuda et al 1988; Batist et al 1989; Pegg 1990; Waxman 1990*).

In the present study, we investigated the cytotoxic activity and interstrand DNA-cross-linking effects of melphalan and cell cycle characteristics in the melphalan-sensitive B-lymphocytic cell line(WIL2) and in the melphalan-resistant B-CLL cell line (WSU-CLL) in order to elucidate possible mechanisms of resistance to alkylating agents.

5.2. Materials and Methods:

Cell culture, cytotoxicity assays and determination of interstrand DNA cross-linking which were used in this investigation are described in detail in Chapter 4.

Cell Cycle Analysis:

Cells were exposed to melphalan for one hour. After treatment, cells were washed three times with medium, fresh medium was added, and cells were re-incubated for various time(4 hr and 16 hr). Cells were washed and centrifuged. Cell pellets were resuspended in 300 μ l of ice-cold PBS containing 1% FCS and fixed by addition of 700 μ l of 100% ethanol for 15 min at 4 °C. Fixed cells were washed twice in ice-cold 1% FCS/PBS, resuspended in 500 μ l of PBS containing 1 mg/ml RNase (Boehringer Mannheim, Germany) and incubated at 37 °C for 15 min. Cells were washed and centrifuged. DNA content was determined after the addition of 50 μ g/ml propidium iodide(Sigma Chemical Co., USA) in PBS. Cell cycle analysis was performed using an EPICS XL-MCL Flow cytometer(Coulter Electronics, FL, USA). Data were analyzed using MultiCycle AV software(Phoenix, CA, USA).

Statistics: Differences were tested for statistical significance with Student's *t* test.

5.3. Results:

In-Vitro Cytotoxicity by Melphalan:

The relationship between melphalan concentration and cytotoxicity for WIL2 cells and WSU-CLL cells is shown in Figure 5-1. The median IC_{50} for WIL2 cells was $8.57 \pm 1.08 \mu M$. The median IC_{50} for WSU-CLL cells was $223.18 \pm 6.45 \mu M$. The IC_{50} values were obtained from ten independent experiments. Statistical analysis showed that WSU-CLL cells were approximately 26 times more resistant to the cytotoxic effects of melphalan than WIL2 cells ($p < 0.001$).

Cytotoxicity and Interstrand DNA Cross-links:

The amount of interstrand-DNA-cross-linking ($C_i\%$) produced following a 60 minute exposure to various concentrations of melphalan, measured after drug-free incubation for 4 hours, demonstrated a clear dose response relationship between increasing concentrations of melphalan and $C_i\%$ in

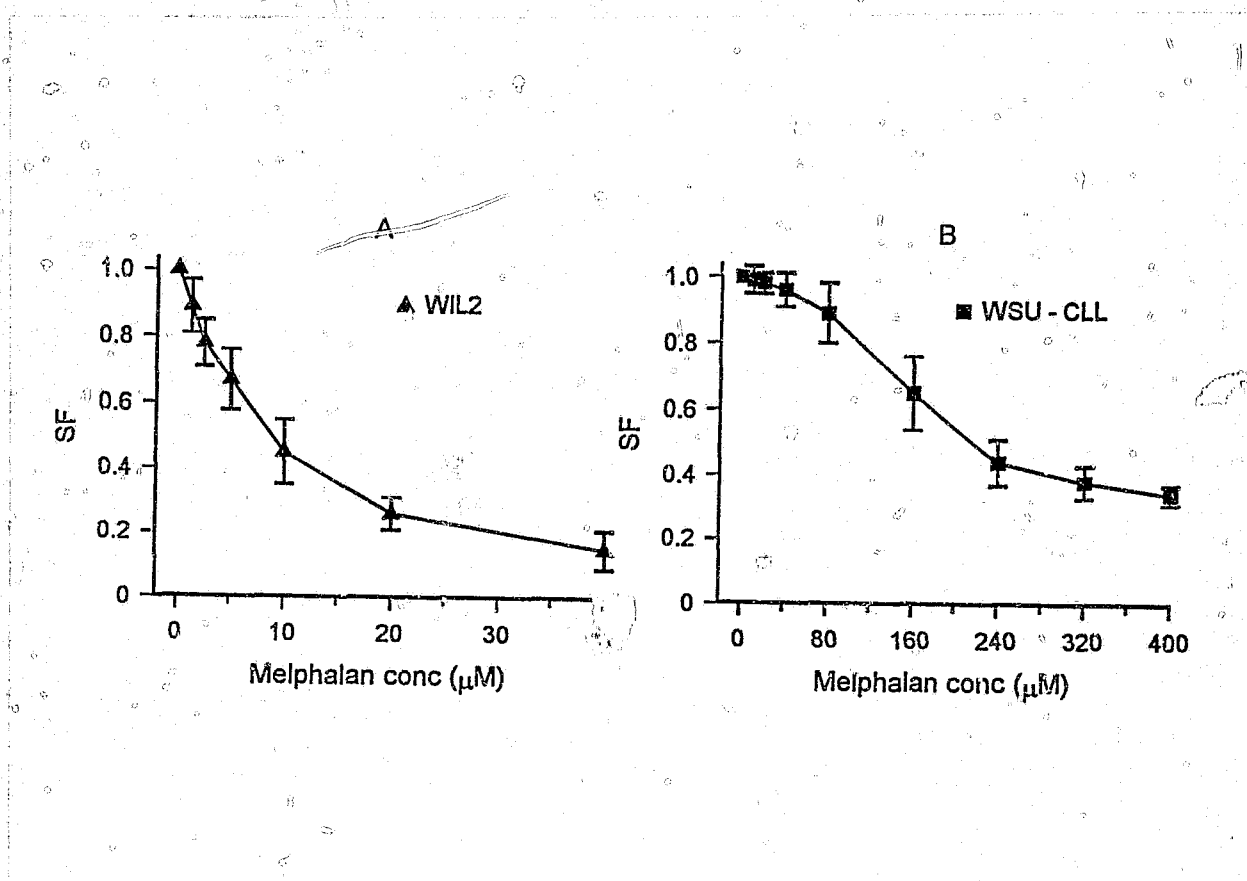


Figure 5-1: Cytotoxic effect of melphalan (MLN) on WIL2 cells(▲) and WSU-CLL cells (■). Points represent mean $\pm$ S.D of ten independent experiments. The significant difference was found when WIL2 cells and WSU-CLL cells were compared($p < 0.001$).

both cell lines. The percentage of interstrand-DNA cross-linking achieved at the end of 4 hours drug free incubation at any given concentration of melphalan was substantially lower in WSU-CLL cells as compared to that found in WIL2 cells ($p < 0.001$) (Figure 5-2). The relationship between cytotoxicity and $C_t\%$ after short term melphalan exposure is shown in Figure 5-3. There was a significant difference ($p < 0.01$) between WIL2 cells and WSU-CLL cells with a greater degree of toxicity at any given $C_t\%$ in WIL2 cells. The relationship between cytotoxicity and $C_t\%$ was influenced both by drug dose, and as will be demonstrated in the next section, by the kinetics of interstrand-DNA-crosslink formation and removal.

The Kinetics of Interstrand DNA cross-linking:

To better define the kinetics of the formation and removal of interstrand DNA cross-links, $C_t\%$ values were determined at 0, 4, and 24 hours following a 60 minute incubation with 40 μM melphalan. After melphalan exposure for 60 minutes the cells were washed and incubated in

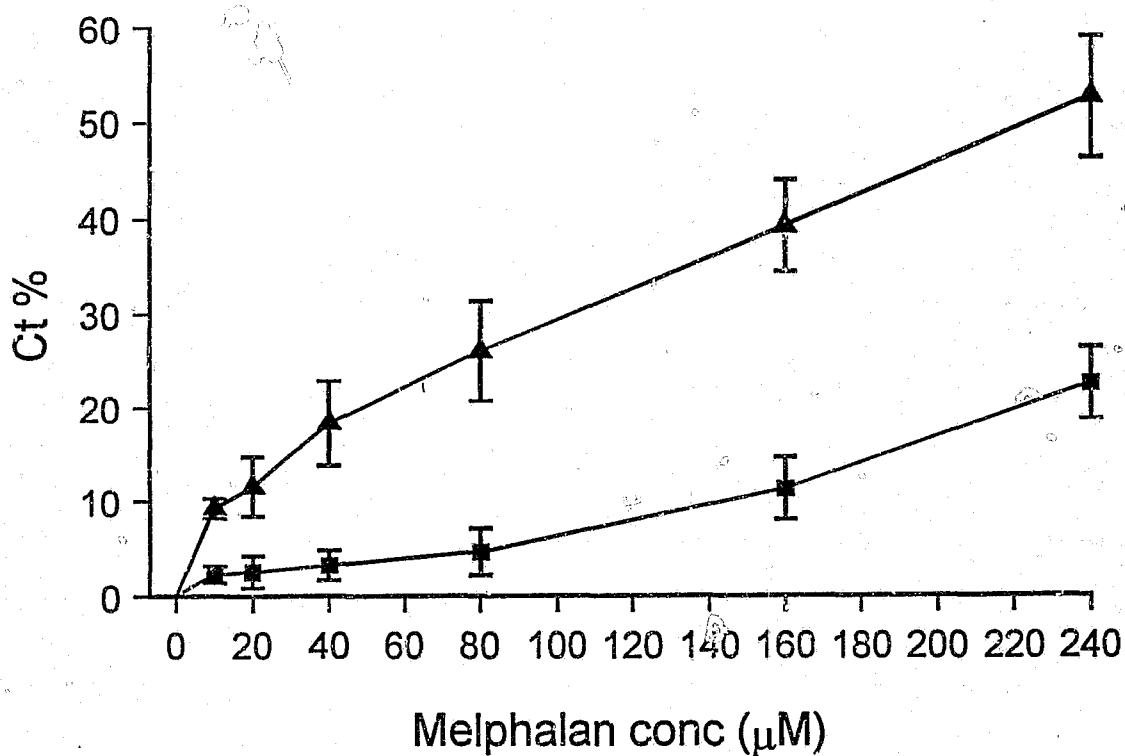


Figure 5-2: Percentage of interstrand-DNA-cross-links (Ct) assessed after 60 minutes melphalan (MLN) exposure followed by 4 hours drug free incubation in WIL2 cells(▲) and in WSU-CLL cells (■). Points represent mean $\pm$ SD of ten independent experiments. The significant difference was found when WIL2 cells and WSU-CLL cells were compared($p < 0.001$).

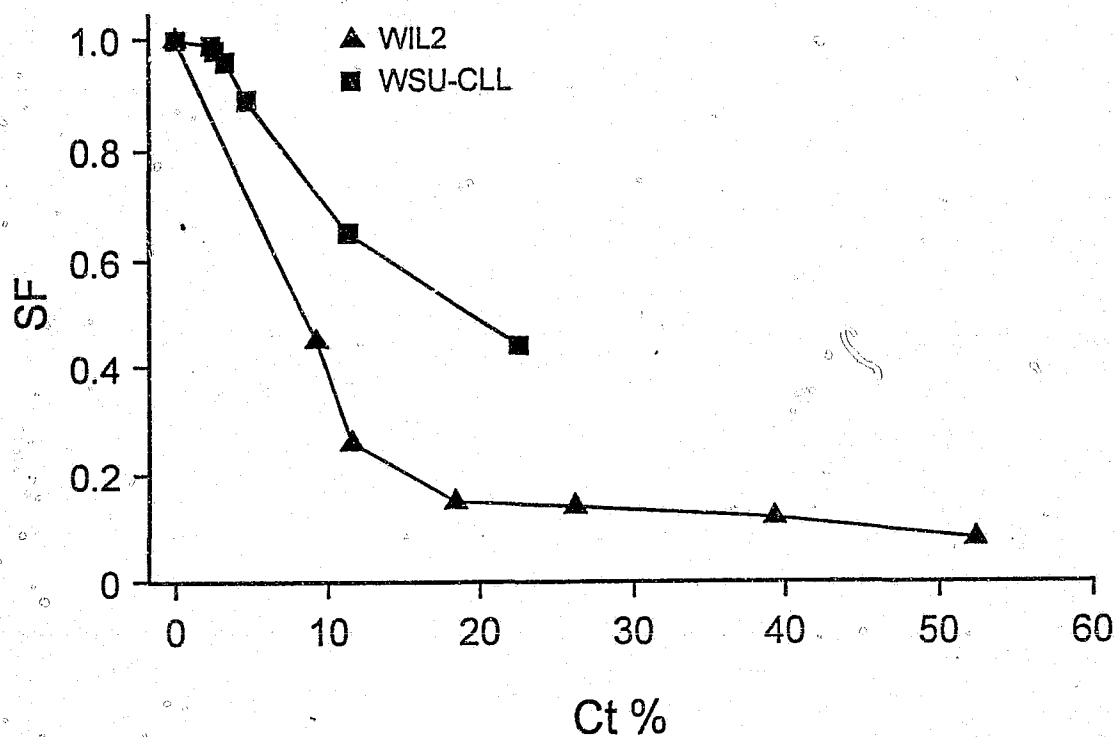


Figure 5-3: Surviving Fraction(SF) following 60 minute exposure to various concentrations of melphalan (MLN) and 72 hour drug free incubation in WIL2 cells (▲) and WSU-CLL cells (■). Interstrand-DNA-cross-links (Ct %) values obtained for WIL2 cells represent means at 0, 0.625, 1.25, 2.5, 5, 10 and 20; for WSU-CLL cells represent means at 0, 10, 20, 40 , 80, 160, and 240 μ M melphalan respectively and were measured after 1 hour drug exposure followed by washing and 4 hours incubation in drug free medium.

drug free medium for the stated times. Viability of lymphocytes was > 90 % at all test times.

Peak levels of interstrand-DNA-crosslink formation ($C_t\%$) were evident immediately following the 60 minute pre-incubation with $40\mu\text{M}$ melphalan in WSU-CLL cells. However, in WIL2 cell, peak $C_t\%$ levels were observed only at 4-hr following the start of drug-free incubation. Peak $C_t\%$ was much higher and the rate of disappearance of interstrand-DNA-crosslinks was prolonged in WIL2 cells compared to that occurring in WSU-CLL cells (Figure 5-4).

Exponential functions for the rate of removal of interstrand-DNA-crosslinks were calculated from the data for each concentration according to the equation: $Y = ae^{-ct}$, where Y = the percentage of interstrand-DNA-crosslinks remaining at t hour after the time of maximum cross-linking; a = the maximum amount of interstrand-cross-linking as estimated by linear regression analysis; and c = the rate constant for the removal of interstrand-DNA-cross-links. From these exponential functions the $t_{1/2}$ for removal of interstrand-DNA-cross-links was calculated. The $t_{1/2}$ values showed

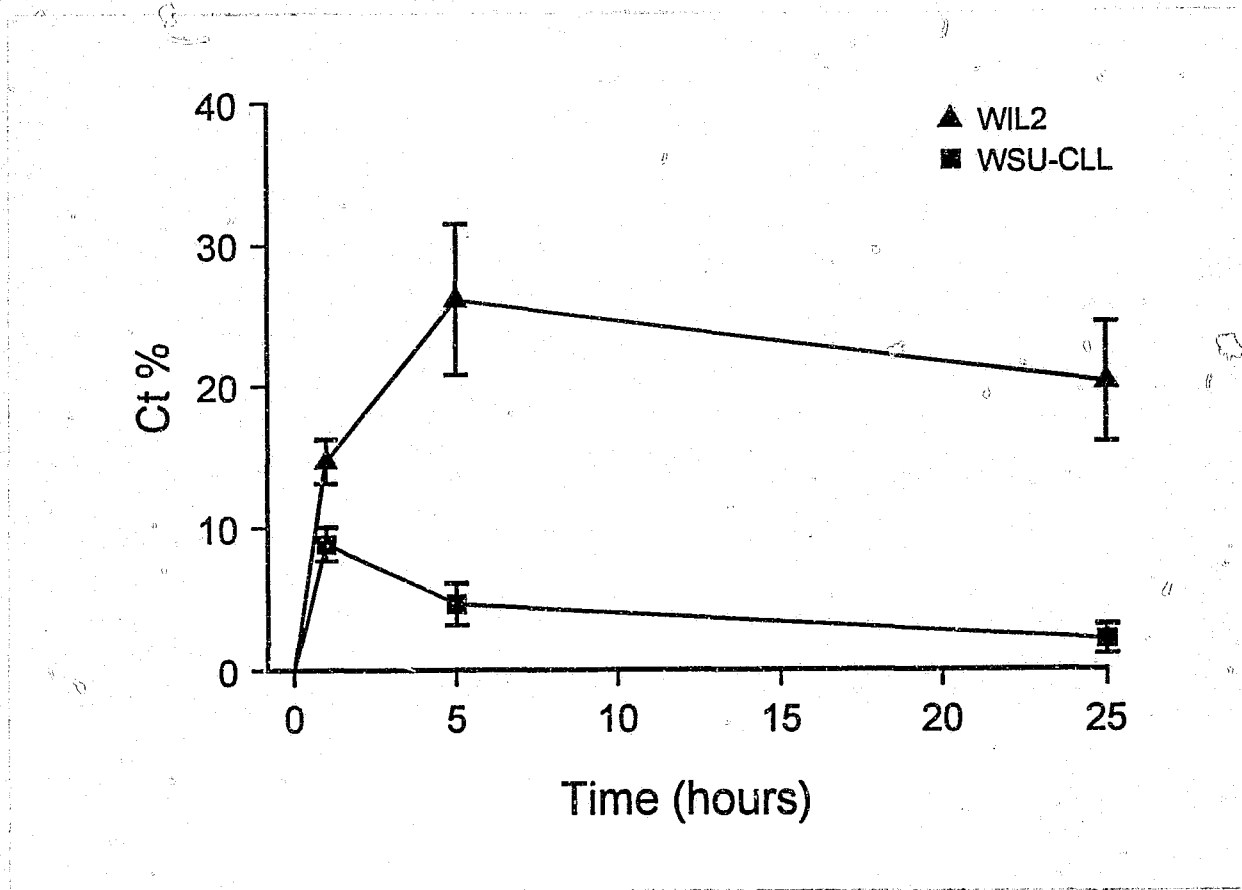


Figure 5-4: Interstrand-DNA-cross-links (Ct %) following 60 minute exposure to 40 μ M melphalan (MLN) and 24 hour drug free incubation in WIL2 cells ($\blacktriangle$) and WSU-CLL cells ($\blacksquare$). Time 0 represents the start of incubation with melphalan which was continued for 1 hour, followed by washing and then drug free incubation for up to 24 hours

significant differences for between two cell lines (Table 5-1).

In order to compare these relationship, the area under the curve (AUC) for interstrand-DNA-cross-links was calculated and plotted against cytotoxicity. The AUC for the exponential parts of the curves was derived by integrating the exponential function for interstrand-DNA-cross-link removal over time:

$$\int_0^{\infty} a e^{ct} (dt) = \frac{a}{c}$$

The AUC in melphalan resistant lymphocytes during the 60 minute of drug exposure was added, assuming a linear rate of formation of interstrand-DNA-cross-links from zero levels at the start of drug exposure. For the melphalan sensitive WIL2 cells, the AUC during drug exposure was calculated simply by drawing straight lines connecting the data points. These values were added to those under the exponential part of the curve. The relationship between the total AUC for interstrand-DNA-cross-links and cytotoxic effect was analyzed by linear regression. This analysis

Table 5-1: The Rates of Removal of Melphalan-Induced Interstrand-DNA-Cross-Links

MLN Conc.	WIL2 Cell Line		WSU-CLL Cell Line	
	Regression Equation	$t_{1/2}$ (h)	Regression Equation	$t_{1/2}$ (h)
40 μ M	$Y = 16.55e^{-6.008t}$	86.64	$Y = 10.67e^{-0.135t}$	5.13
80 μ M	$Y = 25.12e^{-0.006t}$	115.24	$Y = 13.23e^{-0.083t}$	8.35

$t_{1/2}$ is value for half-life of Interstrand-DNA-cross-links; MLN Conc. = melphalan concentration

Comparisons showed there are significant differences ($p < 0.01$) when results for WIL2 cells were compared to WSU-CLL cells.

demonstrated that the relationship was significantly different for WSU-CLL cells as compared to WIL2. There was a higher toxicity at a given AUC following melphalan exposure in WIL2 cells (Figure 5-5).

Cell Cycle Analysis of Melphalan Treated Cells:

Changes in the cell cycle progression of WIL2 and WSU cells after melphalan treatment were assessed by flow cytometric analysis of DNA content following propidium iodide staining. As shown in Table 5-2 and Figure 5-6, 10 μ M melphalan treatment resulted in the progressive arrest of the WSU cells in G₁ and G₂ phases as early as 4hr after melphalan treatment. Such cell cycle arrests lasted for at least 12hr after the treatment. WIL2 cells however continued to cycle. But apoptosis was initiated after 16hr drug-free incubation in WIL2 cells. Similar results were found at higher doses of melphalan (data not shown).

5.4. Discussion:

The reactions between alkylating agents and DNA have been studied extensively (*Erickson et al 1977; Ewig and Kohn 1977; Kohn 1977; Ewig*

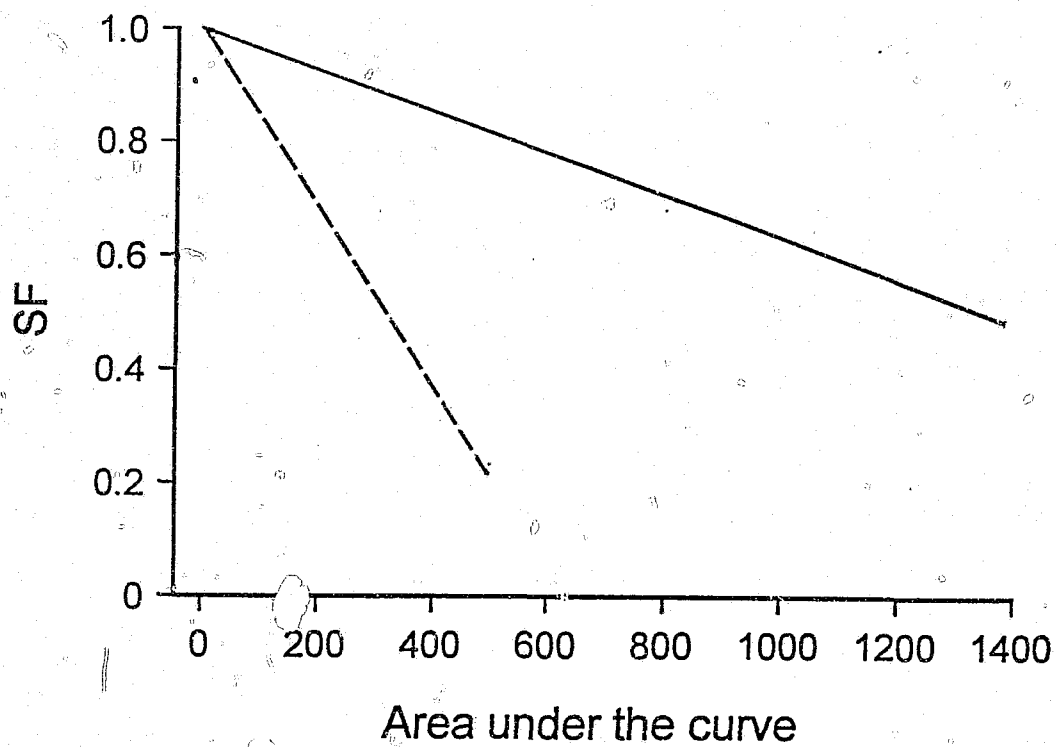


Figure 5-5: Relationship between area under the curve(AUC) for interstrand-DNA-crosslink formation and cytotoxicity. The slopes of the regression lines obtained between values (data from four time points: 0, 1, 5, 25hr) for WIL2 cells (--) and WSU-CLL cells (-) were significantly different ($p < 0.01$).

Table 5-2: Cell Cycle Characteristics of WIL2 Cells and WSU-CLL after 10 μ M Melphalan Treatment

Cell Lines/ Treatment	Phase/ G_0/G_1	Percentage of S	Cells G_2/M
<u>WIL2</u>			
Control	43.43 $\pm$ 3.19	47.80 $\pm$ 3.42	8.77 $\pm$ 1.14
4h Incubation	41.56 $\pm$ 4.88	47.14 $\pm$ 3.75	11.30 $\pm$ 1.15
16h Incubation *	40.32 $\pm$ 3.32	41.47 $\pm$ 4.42	8.86 $\pm$ 1.19
<u>WSU</u>			
Control	40.59 $\pm$ 1.19	57.49 $\pm$ 1.67	1.90 $\pm$ 0.39
4h Incubation	54.96 $\pm$ 3.84 ^a	38.91 $\pm$ 3.70	6.11 $\pm$ 0.61 ^d
16h Incubation	53.26 $\pm$ 4.32 ^{b, e}	40.67 $\pm$ 4.44	6.07 $\pm$ 0.66 ^e

Results are means of 5 independent experiments $\pm$ SD.

* Apoptosis(9.35 $\pm$ 1.28%) was initiated after 16hr drug-free incubation in WIL2 cells.

In WIL2 cells: Intergroup statistic analyses showed no significant difference.

In WSU cells:

a, b: $P < 0.01$ compared to G_0/G_1 control;

d, e: $P < 0.001$ compared to G_2/M control;

c, f: $P > 0.05$ compared to 4h incubation group.

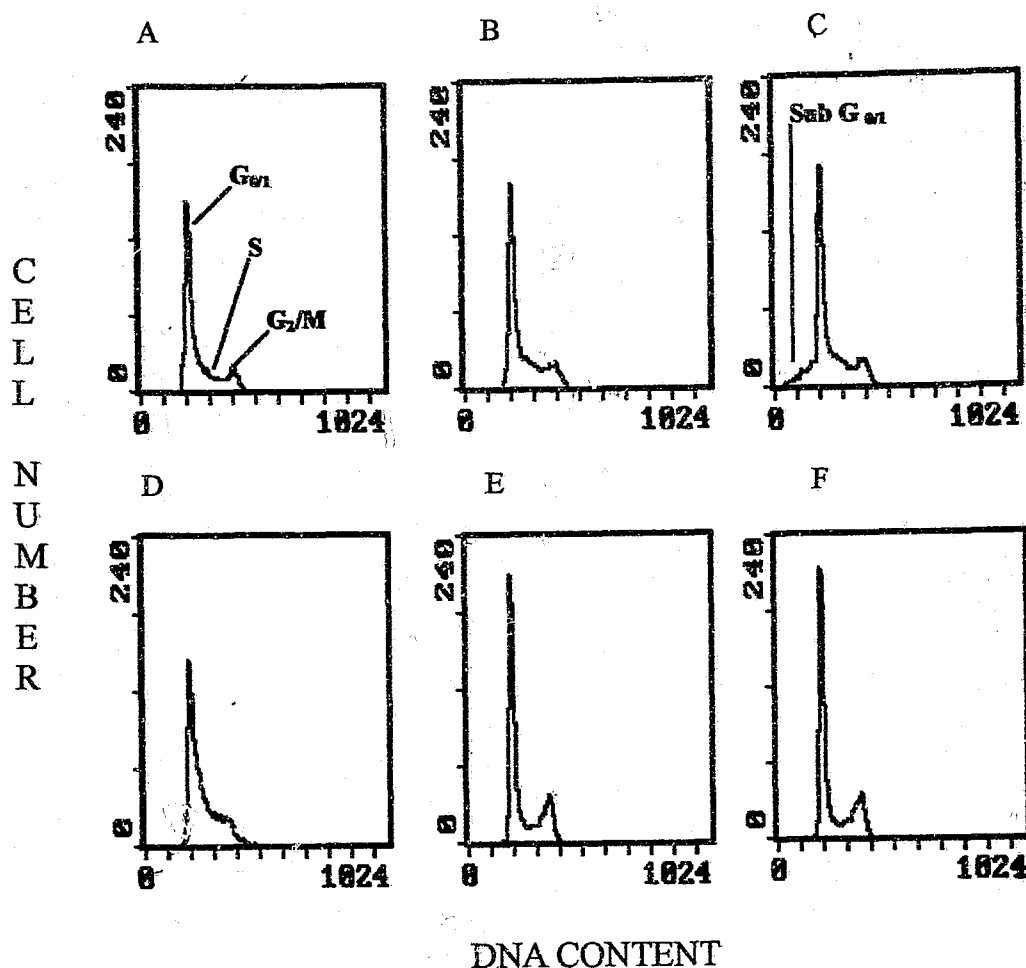


Figure 5-6: Cell cycle analysis of 10 μ M melphalan treated WIL2 cells and WSU-CLL cells. **A:** WIL2 cells control without treatment; **B:** WIL2 cells -- 1hr exposure to 10 μ M melphalan following 4hr drug-free incubation; **C:** WIL2 cells -- 1hr exposure to 10 μ M melphalan following 16hr drug-free incubation; **D:** WSU cells control without treatment; **E:** WSU cells -- 1hr exposure to 10 μ M melphalan following 4hr drug-free incubation; **F:** WSU cells --1hr exposure to 10 μ M melphalan following 16hr drug-free incubation.

and Kohn 1978; Ross *et al* 1978; Zwelling *et al* 1978; Zwelling *et al* 1979; Taylor *et al* 1984; Zwelling *et al* 1981; Hansson *et al* 1987). The common property of the alkylating agents is the ability to dissociate giving rise to a positively charged, electrophilic, alkyl group capable of reacting with negatively charged, electron rich, nucleophilic sites on biologic molecules (Ludlum DB 1977). With DNA a variety of adducts are formed involving mainly the N7, N1 and O6 positions of guanine, the N1, N2 and N3 position of adenine, the N3 position of adenine, the N3 position of cytosine and the O4 position of thymidine. While each of these reactions produces different lesions, including DNA strand breaks, DNA-RNA, DNA-Protein and DNA-DNA cross-links the most important lesion is probably interstrand-DNA-cross-linking (Zwelling *et al* 1978, 1979; Zijlstra *et al* 1986). One of the most common positions for interstrand-DNA-cross-linking is between guanines at the N7 position. The extent of interstrand-DNA-cross-linking correlates most closely with cytotoxicity (Zwelling *et al* 1979; Erickson *et al* 1981; Taylor *et al* 1984).

Various methods have been developed to measure the DNA lesions caused by alkylating agents. Of these, the fluorescence based methods which

measure predominantly interstrand-DNA-cross linking are among the most reliable and also the simplest technically (*De Jong et al 1986; Zijlstra et al 1986; Ali-Osman et al 1993*). The high specificity for double stranded DNA makes it possible to detect cross-links in DNA at alkaline pH, since cross-links in DNA can serve as a nucleation point for rapid denaturation. Their presence leads to a return of fluorescence enhancement. Apoptosis does not affect the proportion of cross-linked DNA determined by this method. Using this method the occurrence of DNA strand breaks, to the extent of up to 750 Rad-equivalents, have been shown not to cause interference with the measurement of interstrand -DNA-crosslinks (*De Jong et al 1986*).

Previous studies have also demonstrated that while the initial rate of interstrand-DNA-cross-link formation varies for different alkylating agents, the relationship between the Area Under the Curve (AUC) for interstrand-DNA-cross-links and cytotoxicity is the same for different alkylating agents (*Hansson et al 1987*).

The precise mechanism(s) for the differences in resistance to treatment with alkylating agents are not clear. The mechanisms of alkylator

resistance as defined in experimental systems suggest that various factors may be involved. These include alterations of drug transport, interaction with secondary non-essential targets, increased drug inactivation and altered DNA repair mechanisms (*Redwood et al 1980; Pera et al 1981; Richon et al 1987; Waud, 1987; Bedford et al 1988; Eastman and Schulte 1988; Kelley et al 1988; Masuda et al 1988; Pegg et al 1990*).

While a significant and consistent relationship between melphalan cytotoxicity and the extent of interstrand-DNA-cross-link formation was found in two cell lines, striking differences were, however, observed when WIL2 cells and WSU-CLL cells were compared. WSU-CLL cells were approximately 26 times more resistant to melphalan as compared to WIL2 cells. The finding that melphalan resistant WSU-CLL cells demonstrated rapid initial formation of interstrand DNA-cross-links, albeit at a lower level, following short time melphalan exposure, however, suggests that altered drug uptake is not a major resistance mechanism in human lymphoid cells. Both the maximum extent of DNA cross-linking as well as the rate of removal of interstrand DNA cross-links, as assessed by $C_t\%$, DNA interstrand crosslinks and $t_{1/2}$, were significantly different when

WIL2 cells were compared to WSU-CLL cells. While some of these findings may be consistent with decreased drug uptake or increased drug inactivation, either by interaction with non essential targets or by some other mechanism, a significant difference was found in the rate of removal of interstrand-DNA-crosslinks.

In the experimental system used, removal of cross-linked DNA could be the result either of elimination of cells containing interstrand-DNA-crosslinks by drug induced cell death(apoptosis) or due to repair of the DNA lesion by DNA repair mechanisms. Since the cytotoxic effect of melphalan is fairly slow to develop with no significant decrease of cell number, metabolic activity or viability for up to 24 hours following a 1 hour exposure to drug, the effect of melphalan on interstrand-DNA-crosslinks could be assessed for this period of time. The results showed that elimination of interstrand-DNA-crosslinks rather than elimination of DNA-damaged cells occurred, and that this was due to DNA repair.

It has been known that cells respond to DNA irradiation damage by arresting in the G₂ phase of the cell cycle (*Busse et al 1978; Lucke-Huhle 1982*). These cells are then able to repair the DNA damage before they

undergo DNA replication or mitosis, and are thus more tolerant of the DNA damage than cells that continue in a proliferative phase. Undergoing DNA replication or mitosis with damaged DNA produces lethality. These results demonstrate that WSU cells treated by melphalan can respond with arrest in the G_1 and G_2 phases, and during these arrests DNA repair can occur. However, WIL2 cells failed to enter G_1 or G_2 arrest after melphalan treatment, as compared to WSU cells.

In summary, more effective repair of alkylating-agent DNA damage can be responsible for resistance to alkylating agents, and that such repair can be facilitated by alteration of the cell cycle to allow effective DNA repair before the cell enters phases of the cell cycle in which the DNA damage will be lethal.

CHAPTER 6

6.0. Melphalan Accumulation, Glutathione Levels, and Glutathione-S-Transferase Activity in WSU-CLL and WIL2 Cell Lines

6.1. Introduction:

Alkylating agent therapy is central to the chemotherapeutic approach to most malignancies, yet relatively few mechanisms of alkylating agent resistance in CLL have been described. In particular, while transport-mediated resistance has been well-characterised for many antineoplastic agents, most notably the multidrug resistance phenotype associated with the drug efflux pump P-glycoprotein, little is known about mechanism of uptake, accumulation and efflux of alkylating agents. Most in vitro models of melphalan resistance have investigated glutathione-mediated pathways.

The present studies on melphalan transport, glutathione levels, and glutathione-S-transferase activity in WIL2 (the melphalan-sensitive cell

line, IC_{50} : 8.57 μ M) cells and WSU-CLL (the melphalan-resistant cell line.

IC_{50} : 223.18 μ M) cells were undertaken in an attempt to define which parameters might be involved in melphalan-resistance..

6.2. Materials and Methods:

Transport of [chloroethyl- ^{14} C] melphalan:

Melphalan uptake studies were performed as follows: During the exponential growth phase, WIL2 cells and WSU-CLL cells were washed three times with PAG transport medium (Dulbecco's phosphate buffered saline containing 6.8g/L albumin and 1g/L glucose) and were pre-warmed for 15 min at 37°C. [chloroethyl- ^{14} C]melphalan (50 μ M) was then added and cells were incubated further at 37°C. Studies with melphalan alone were performed over 0.5 to 35 min. Inhibition studies with 1 mM BCH, amino acids (added simultaneously with 100 μ M [chloroethyl- ^{14} C] melphalan) or sodium-depleted medium (Dulbecco's PBS with Na^+ replaced by choline, and Na_2HPO_4 replaced by K_2HPO_4) were performed over 3 min. For efflux studies, the 2 cell lines were preincubated at 37°C for 30 min with 50 μ M [^{14} C]-melphalan and drug uptake was measured and

corrected for surface binding. The incubation mixtures were layered over 0.5 ml solution of silicone oil and light mineral oil[84:16(v/v)] in 1.5 ml microcentrifuge tubes maintained at 37°C. Drug uptake was terminated by centrifugation of the cells through the oil layer at 12,000 x g for 2 min in a Beckman Microfuge B(Beckman Instruments, Inc., Palo Alto, Calif.). Cell pellets were solubilized in 1 N NaOH overnight at 4 °C and then neutralized with an equivalent volume of 1 N HCl. To these were added 10 ml liquid scintillation cocktail and the samples were counted in a Packard TriCarb Liquid Scintillation Analyzer (Model 2200 CA). Rapid non-specific surface binding of [¹⁴C]-melphalan to cells at 0°C was subtracted from [¹⁴C]-melphalan at 37°C at each time recorded time(such non-specific binding represented < 5% of the total radioactivity (Figure 6-1).

The drug uptake data were analysed by linear regression analysis, and the reciprocal initial velocities were plotted against reciprocal melphalan concentration to determine the apparent K_m and V_{max} for the transport process.

GSH Measurements:

Total glutathione levels were determined on cell cytosol by the cyclic reduction of oxidised glutathione with glutathione reductase and NADPH (Tietze 1969; Griffith 1980). Three solutions in stock buffer (125 mM sodium phosphate-6.3 mM, sodium EDTA, pH 7.5), were made as follows: (a) 0.3 mM NADPH; (b) 6 mM Ellman's reagent[5,5'-dithiobis(2-nitrobenzoic acid)]; (c) glutathione reductase, 50 units/ml. Cells were washed in Dulbecco's PBS, centrifuged at 1500 x g for 5 min, and resuspended in 0.4 ml H₂O pH 7.4, all at 4 °C. Cell lysis was accomplished by freeze/thawing once in methanol in dry ice. 100 µl 10 % sulfosalicylic acid was added(1/5 total volume), and the mixture was incubated at 4 °C for 10 min. Following centrifugation at 5000 x g for 5 min at 4 °C the supernatant was decanted and the volume was measured. The assay was performed by adding NADPH(700 µl), Ellman's reagent (100 µl), stock buffer(170 µl), and supernatant or stock buffer(20 µl) consecutively to a cuvet. Glutathione reductase(10 µl) was added to the mixture at room temperature and the cuvet was scanned on a Ultrospec 3000 UV/Visible spectrophotometer(Pharmacia Biotech Ltd, England) at 412 nM for 3 min; 5 µl of reduced glutathione(0.5 nmol) was then added to the sample cuvet

and it was rescanned. Sample glutathione levels were determined by comparison of the rate of 5,5'-dithiobis(2-nitrobenzoic acid) reduction to the internal glutathione standard(0.5 nmol). Glutathione levels were expressed per mg protein.

GST Measurements:

GST measurements were performed by using 1-chloro-2,4 dinitrobenzene as substrate (*Habig et al 1974*). Cells were washed in Dulbecco's PBS, centrifuged at 1500 x g for 5 min, and resuspended in 200 µl of 0.1 M potassium phosphate buffer/1 mM EDTA, pH 7.4, all at 4 °C. Following freeze/thawing twice in methanol in dry ice the suspension was centrifuged at 15,000 x g for 30 min at 4 °C. The assay was performed by adding water(700 µl), 10x 0.1 M KH_2PO_4 buffer(pH 6.5; 100 µl); 10 mM GSH (100 µl), and 10 mM 1-chloro-2,4-dinitrobenzene (100 µl) consecutively to a cuvet. Supernatant was added and the cuvet was scanned in a Ultrospec 3000 UV/Visible spectrophotometer(Pharmacia Biotech Ltd, England). Supernatant aliquots of variable volume were chosen until the rate of absorbance change was less than 0.05/min. One unit of GST enzyme activity is defined as the amount catalysing the conjugation of the substrate

with glutathione at the rate of 1 nmol per min per mg protein. Protein concentration was determined by the method of Bradford (*Bradford 1976*).

Statistics: Comparison of the statistical significance of difference of means was by Student's *t* test.

6.3. Results:

Melphalan Transport:

The cellular uptake of 50 μ M melphalan over a 35 min period is shown in **Figure 6-1**. This plot demonstrates that there is a significant difference in melphalan accumulation comparing WIL2 cells and WSU-CLL cells. Uptake appeared to be linear over the first 5 min but rapidly became nonlinear, and then reached a plateau by 25 min. Uptake of [^{14} C]-melphalan was strongly dependent upon temperature. At 0°C, no uptake could be demonstrated (**Figure 6-1**).

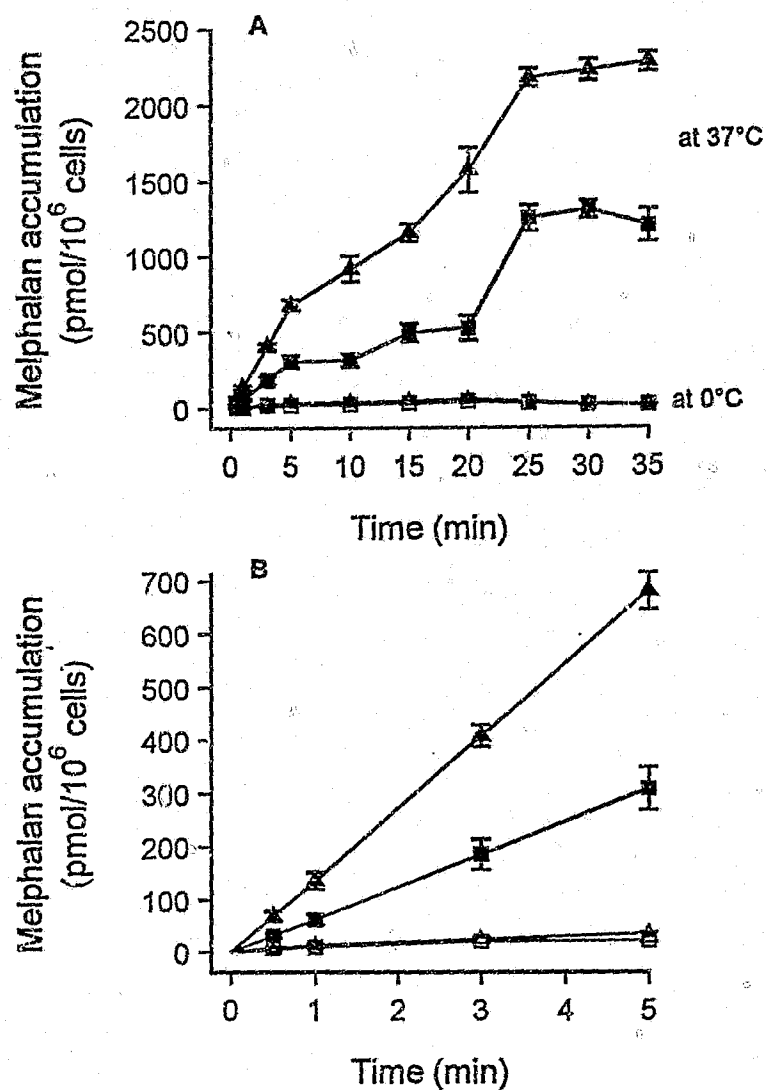


Figure 6-1: A: Melphalan accumulation in WIL2 cells(▲) and WSU-CLL cells(■). Uptake of 50 μ M melphalan was determined from 0.5 to 35 min in PAG transport medium at 37°C and 4 °C as described in the Methods section. The graph shows the mean $\pm$ SD of three separate determinations performed in triplicate. B: Melphalan accumulation in WIL2 cells(▲) and WSU-CLL cells(■) over first 5-min. Uptake appeared to be linear.

Lineweaver-Burke plots of melphalan uptake at 3 min by WIL2 cells and by WSU-CLL cells revealed linear relationships (correlation coefficients of 0.994 and 0.998, respectively) (**Figure 6-2**). The K_m and V_{max} for melphalan for each cell line was determined by linear regression analysis. The K_m was $26.72 \mu M$ and the V_{max} was $431.03 \text{ pmol} / 10^6 \text{ cell/min}$ for WIL2 cells. The K_m was $25.63 \mu M$ and the V_{max} was $123.61 \text{ pmol} / 10^6 \text{ cell/min}$ for WSU-CLL cells. Analysis of the K_m and V_{max} of Melphalan transport indicated that there was approximately 3.5-fold decrease of V_{max} in WSU-CLL cells compared with WIL2 cells.

The effects of BCH and sodium depletion on $[^{14}C]$ -melphalan uptake are shown in **Table 6-1**. The data shows that the sodium-independent amino acid transporter (L system) mechanism accounts for most of the instant melphalan transport both in WIL2 cells and in WSU-CLL cells. Melphalan uptake competition studies in the presence of excess unlabelled amino acids (**Figure 6-3**) demonstrated that the system L transporter plays the key role in melphalan uptake in the two cell lines. System L substrates, such as leucine and tryptophan were more effective in inhibiting initial melphalan

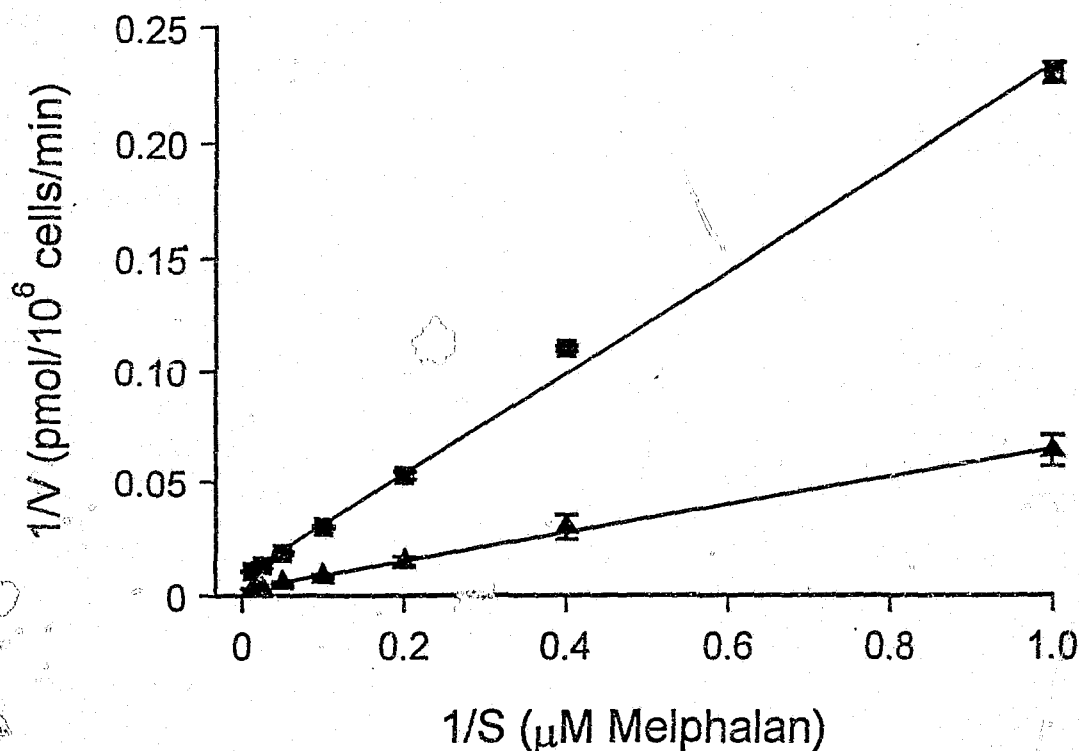


Figure 6-2: Lineweaver-Burk plots of melphalan uptake between 1-160 μM in WIL2 cells and WSU-CLL cells. Initial melphalan uptake was measured at 3 min at 37°C as described in the Methods section. The linear regression solutions are; (1). for WIL2 cells(▲): $Y = 0.002 + 0.062 X$ with a regression coefficient of 0.994; and (2). for WSU-CLL cells(■): $Y = 0.008 + 0.207X$ with a regression coefficient of 0.998; The graph indicates the mean $\pm$ SD of three separate determinations performed in triplicate.

Table 6-1

**BCH and the Absence of Sodium Inhibition of [¹⁴C] Melphalan
Uptake(3 min) in WIL2 Cells and WSU-CLL Cells**

Melphalan (μM)	Inhibitors	WIL 2 % of Control	WSU-CLL % of Control
10	BCH(1mM)	49.3 $\pm$ 6.7	41.5 $\pm$ 7.8
10	Absent Na ⁺	91.5 $\pm$ 9.2	84.3 $\pm$ 8.4
100	BCH(1mM)	38.4 $\pm$ 5.6	39.6 $\pm$ 4.3
100	Absent Na ⁺	89.4 $\pm$ 4.7	83.8 $\pm$ 6.8

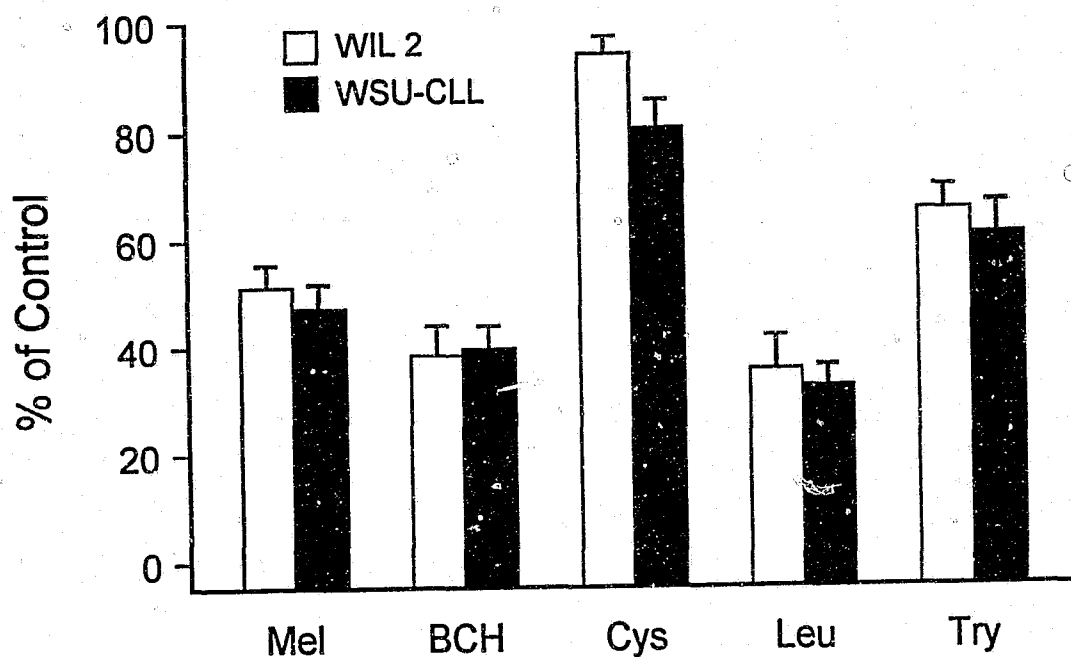


Figure 6-3: Bar graph representation of inhibition of initial 3 min uptake of 100 μ M melphalan by 1 mM of various competitors at 37°C. The bars show the mean $\pm$ SD of three separate determinations performed in triplicate. **Mel:** melphalan; **BCH:** DL- α -2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; **Cys:** Cystine; **Leu:** Leucine; **Try:** Tryptophan.

uptake than cystine which is a poor substrate for the L system transporter mechanism.

The time course for efflux of [^{14}C]-melphalan from WIL2 cells and WSU-CLL cells is illustrated in Figure 6-4. Results showed that there was no difference in melphalan efflux between the two cell lines after the initial loading period.

GST and GSH Measurements:

Levels of glutathione and its dependent enzyme(GST) in WIL2 cells and WSU-CLL cells are shown in table 6-2. There was no significant difference between the two cell lines in either glutathione content or glutathione-S-transferase activity.

6.4 Discussion:

The definition of transport parameters will facilitate identification of those mechanisms important in the generation of drug resistance.

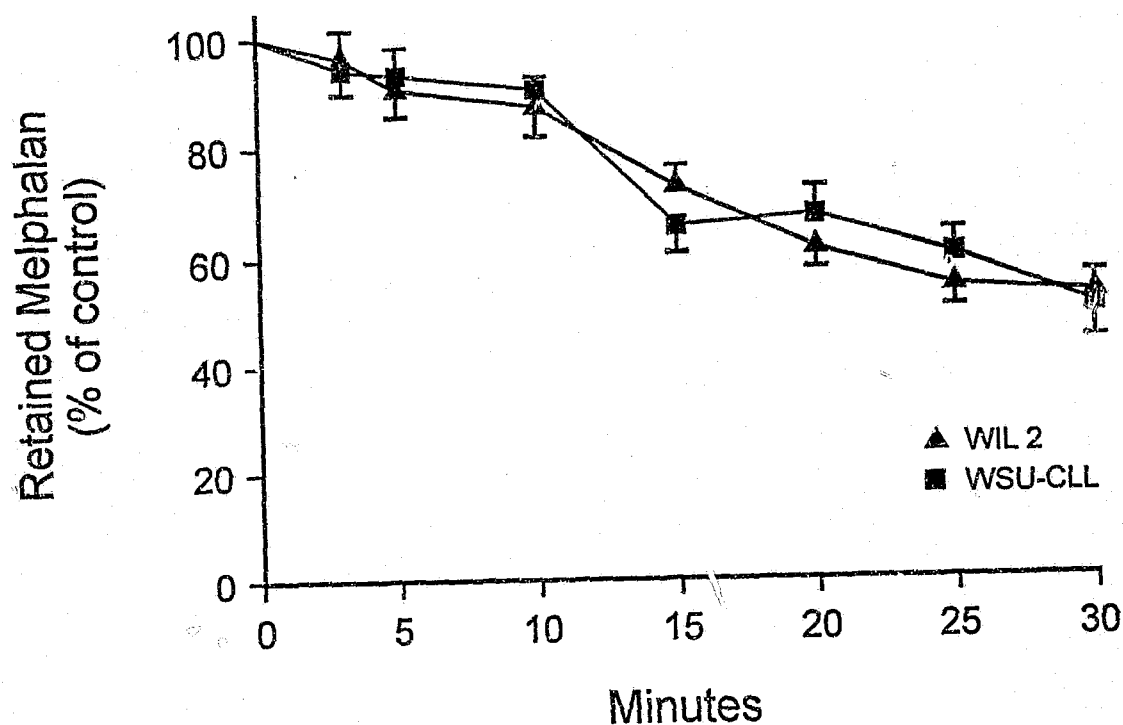


Figure 6-4: Efflux of melphalan from WIL2 cells and WSU-CLL cells. Cells were incubated in triplicate in 50 μ M melphalan in PAG transport medium at 37°C for 30 min. The medium was changed to PAG medium without drug and samples were examined over the time course for melphalan retention. Values are expressed as a percentage of retained melphalan relative to the intracellular melphalan present at the end of the loading period. The graph indicates the mean $\pm$ SD of three separate determinations performed in triplicate.

Table 6-2 Glutathione Levels, and Glutathione-S-Transferase Activity

	WIL2	WSU-CLL
GSH(nmol/mg protein)	36.5 ± 4.8	39.4 ± 6.1
GST(Units/mg protein)	62.4 ± 7.2	57.8 ± 9.7

Values are mean ± SD.

The present investigation confirms that melphalan transport in WIL2 cells and WSU-CLL cells is carrier mediated. Transport of melphalan into the two cell lines is an active process and is markedly temperature dependent with no accumulation of the drug observed at 0 °C.

Melphalan-resistant WSU-CLL cells demonstrated both a lower velocity of melphalan uptake and a lower intracellular accumulation of the drug compared to melphalan-sensitive WIL2 cells.

Previous studies of melphalan uptake have attributed melphalan uptake to two amino acid transport systems (*Goldenberg et al 1979; Vistica 1983*). Although biphasic Lineweaver-Burk plots were not seen between melphalan concentrations of 1-160 μ M, our findings support the presence of at least two carrier systems mediating drug uptake (similar if not identical to the BCH-sensitive Na⁺-independent L system and the BCH insensitive Na⁺-dependent ASC-like system) (*Goldenberg et al 1971; Goldenberg et al 1979; Vistica 1979*). Kinetic analysis at concentrations of 1 to 160 μ M melphalan indicates that there was approximately 3.5-fold decrease of V_{max} in WSU-CLL cells compared with WIL2 cells. Efflux studies of

[¹⁴C]-melphalan have revealed no difference in the efflux of the drug from WSU-CLL cells as compared to WIL-2 cells. Therefore, drug efflux does not appear to contribute to the decreased melphalan accumulation seen in WSU-CLL cells. This finding rules out the possibility that the drug resistance could be due to an enhanced efflux from these cells.

The demonstration that the melphalan-resistant WSU-CLL cells transport the drug less efficiently certainly mean that this alteration is one of the factors in the mechanism of resistance. However, considering our previous findings that WSU-CLL cells demonstrated rapid initial DNA crosslink formation, together with an increased rate of removal of interstrand DNA-cross-links, suggests that altered drug uptake is not the only or even the major resistance mechanism in these lymphoid cells.

A relationship between glutathione concentration and melphalan resistance has been described previously (*Green et al 1984; Ahmad et al 1987; Friedman et al 1988; Gupta et al 1989; Rosenberg et al 1989; Bellamy et al 1991; Schechter et al 1991; Bailey et al 1992*). Increased glutathione

levels have been observed in a variety of melphalan-resistant cell lines (Green *et al* 1984; Ahmad *et al* 1987; Rosenberg *et al* 1989; Bellamy *et al* 1991; Schechter *et al* 1991; Bailey *et al* 1992), but two other models of melphalan resistance have been reported in which no increase in glutathione content was found in the resistant cell lines (Friedman *et al* 1988; Gupta *et al* 1989). These latter findings are consistent with our result, which indicate drug resistance due to elevated GSH levels may be tumour cell line specific.

The relationship between glutathione-S-transferase and resistance to chemotherapeutic is still being defined. Our investigations with WSU-CLL cells are the first to measure melphalan transport, glutathione levels, and glutathione-S-transferase activity in the melphalan-resistant B-CLL cell line. The association between GSTs and models of melphalan resistance has been inconsistent, with an increase in GST activity reported in two cell lines (Gupta *et al* 1989; Schechter *et al* 1991) but not in others (Friedman *et al* 1988; Rosenberg *et al* 1989). These findings as well as the results of the present study indicate that glutathione level and glutathione-S-transferase activity are not involved in the alkylating resistance of WSU-

CLL cells. These findings suggest that glutathione-*S*-transferase level associated drug resistance may vary in different malignancies, as is the case with intracellular glutathione.

CHAPTER 7

Increased Levels of Topoisomerase II Expression and Function In a Melphalan-Resistant B-CLL Cell Line(WSU-CLL) Which is Sensitive to Doxorubicin

7.1. Introduction:

We have previously demonstrated that in haemopoietic cells a major mechanism for melphalan resistance is an increased rate of removal of DNA-interstrand-crosslinks (*Bezwoda and Pu, 1997*). Such increased rates of interstrand-DNA-crosslink removal may be due either to increased activity of DNA repair enzymes or due to alteration of DNA topology.

In eukaryotic cells, two major topoisomerases, topoisomerase I and topoisomerase II catalyse changes in the topological conformation of DNA by the concerted breakage of single or double strands. Topoisomerase II

action is important in DNA replication, transcription, recombination, and mitosis (Wang, 1987). Topoisomerase II is localized to AT-rich DNA regions, where it forms a significant part of the mitotic chromosomal scaffold (Earnshaw et al, 1985; Gasser et al, 1986; Nelson et al, 1986). Two isoenzyme forms of topoisomerase II, α and β , are expressed in mammalian cells. The two forms differ with respect to Molecular size (170KD versus 180KD, respectively), cleavage site, thermal stability, and catalytic capacity (Drake et al, 1987, 1989). The enzyme described first, topoisomerase II α , is expressed preferentially in proliferating cells (Heck and Earnshaw, 1986; Hsiang et al, 1988) and is cell cycle regulated (Heck et al, 1987). The topoisomerase II β enzyme, on the other hand, appears to be expressed at equivalent levels in proliferating and in quiescent cells (Woessner et al, 1990, 1991).

In this study, we investigated topoisomerase I and II expression and activity in melphalan resistant B-CLL cell line (WSU-CLL) and in melphalan-sensitive B-lymphocyte cell line(WIL2).

7.2. Materials and Methods

Cell lines and Cell Culture Methods; Cytotoxicity Assay; Determination of Interstrand-DNA-Crosslinks were described in detail in Chapter 4.

Preparation of nuclear extracts:

A procedure for isolation of nuclear and cytosolic fractions was developed based on the method of Chow and co-workers (*Chow et al, 1985; Matsumoto et al, 1993*). In brief, cells in the exponential phase of growth were treated with detergent buffer (1% Nonidet P-40, 30 mM HEPES, 200 mM sucrose, 40 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, PH 8.0), with constant swirling for 10-15 minutes. The cell suspension was centrifuged at 400×g for 10 min and the supernatant was saved as the cytosolic fraction. The sedimented nuclei were then washed 3 times by resuspending the pellet in buffer A (50 mM HEPES, 10% sucrose, 10 mM β-mercaptoethanol, pH 7.5), followed by repelleting. To the final pellet was added an equal volume of buffer B (50mM HEPES, 10% sucrose, 10mM β-mercaptoethanol, 0.7 M NaCl (final NaCl concentration 0.35M). After extraction for 60-min at 4°C the mixture was

centrifuged at 100000×g for 60 min, and the supernatant was saved as the nuclear extract. The cytosolic and nuclear extracts were dialysed against buffer C for six hours (Buffer C: 50% glycerol, 50 mM Tris-HCl, 0.5 mM DTT, 1 mM EDTA, 1 mM EGTA, 260 mM NaCl, pH 7.5). A combination of six protease inhibitors, including of soybean trypsin inhibitor (10 µg/ml), leupeptin (50 µg/ml), pepstatin (1 µg/ml), aprotinin (20 µg/ml), benzamidine (1 mM), and PMSF (1 mM), were prepared just prior to each experiment and added to each of the above-mentioned buffers just prior to each experiment. The protein concentrations in the extracts were determined by the method of Bradford (*Bradford, 1976*).

Topoisomerase I and II Assays:

Measurement of the topoisomerase II catalytic activity in cytosol and nuclear extracts was performed using the topoisomerase II decatenating method (Topogen, Columbus, Ohio, USA), which is modification of the method of Marini and co-workers (*Marini et al, 1980*). Photographic negatives of the ethidium bromide-stained agrose gels were scanned with a REP Scanning Densitometer (Helena Laboratories, USA) and the quantity of liberated minicircles was measured as a percentage of total kDNA.

Results are expressed in arbitrary units, and the ratio of activity in WSU-CLL cells to that in WIL2 cells was used for comparison.

Topoisomerase I activity was determined by using the topoisomerase I assay kit from Topogen (Columbus, Ohio, USA), essentially as described by Liu and co-workers (*Liu and Miller, 1981*). The open circular species (form II) to total DNA loaded, expressed as percentage of total DNA, was quantified using the same densitometer.

Western blot analysis:

Proteins from the nuclear extract and cytosol fractions of WIL2 and WSU-CLL cell lines were electrophoresed (100 μ g protein per lane) in 7.5 % SDS-polyacrylamide gels and transferred to nitrocellulose paper by the method of Harker and co-workers (*Harker et al, 1991*). The nitrocellulose strips were pre-incubated in blocking buffer (3 % BSA-5 % non-fat dry milk in PBS) overnight at 4°C. Transferred, immobilised, topoisomerase proteins were detected using purified mouse anti-human DNA topoisomerase II α p170 (Topogen USA), anti-topoisomerase II β

p180(PharMingen, USA) monoclonal antibodies or rabbit polyclonal, monospecific, anti-human topoisomerase I antibodies (Topogen, USA). Blots were incubated with primary antibody for 4h at 37°C, then washed with a wash buffer (1% BSA in PBS containing 0.2% Tween 20). The bound antibodies were visualized with alkaline phosphatase-linked sheep anti-mouse or anti-rabbit IgG, using 5-bromo-4 chloro-3-indolyl phosphate and nitroblue tetrazolium chloride substrates. Each experiment included controls for non-specific binding using non-immune rabbit serum in place of specific antibody and a negative control without primary antibody. The relative amounts of topoisomerase II α , II β and topoisomerase I proteins seen on Western blots were quantitated densitometrically. Specified regions of the film images of the Western blot were digitized by the scanner and the area and image intensities were calculated. The image intensity was calibrated against internal computer standards and values are expressed as arbitrary units relative to the standards. A linear relationship was present between the value of the integration units and the amount of protein extract loaded on the gel in the range; 30-250 μ g of protein (data not shown). 100 μ g of protein extracts were used for all comparative experiments.

Preparation of WSU-CLL native DNA:

A procedure for isolation of native DNA from WSU-CLL cells was developed based on the method of Gross-Bellard and co-worker (*Gross-Bellard et al*, 1972). Briefly, WSU-CLL cells was suspended in 1 vol digestion buffer(100mM NaCl, 10mM Tris-Cl, 25mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K, pH 8.0). Samples were incubated in a shaking incubator in tightly capped tubes for 12 to 18 hr at 50°C. An equal volume of phenol/chloroform/isoamyl alcohol was used for extraction followed by centrifugation for 10 min at 1700×g(3000rpm in Sorvall H1000B rotor). This organic extraction was repeated twice. The aqueous layer was transferred to a new tube and ½ vol of 7.5 M ammonium acetate and 2 vol of 100% ethanol were added. The solution was centrifuged for 2 min at 1700 x g. Organic solvents and salt were removed by two dialyses against 100 vol TE buffer for 24hr. Residual RNA was removed by adding 0.1% SDS and 1µg/ml DNase-free RNase and incubating for 1 hr at 37°C. The organic extraction and purification was repeated and the resultant purified DNA was resuspended in TE buffer at 1mg/ml.

Preparation of crosslinked DNA:

WSU-CLL DNA was suspended at 100 μ g/ml in TE buffer. Samples in a thin layer were irradiated at room temperature with UV light(360nm peak) 12cm below four FL-15BLB fluorescent lamps. The incident fluence rate was 30W/m², measured with a Model J221 Blak-Ray ultraviolet meter(Ultraviolet Products, San Gabriel, CA). The solution was extracted with phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the DNA was resuspended in TE buffer at 1mg/ml. The formation of crosslinks was determined by ethidium bromide fluorescence assay mentioned above. Crosslinked DNA accounted for 80% of total DNA after UV treatment.

Iodination of Topoisomerase II:

Topoisomerase II was iodinated by the Iodogen Method (*Salacinski et al 1981*), purified by contricon 100(Amicon, Inc. USA), and formulated in topoisomerase II storage buffer(15 mM sodium phosphate, pH 7.1, 700 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 50% glycerol) at the concentration of 1 Unit/ μ l(1unit will decatenate 0.2 μ g of kDNA in 30 min

at 37°C. The specific activity of the radiolabelled material is 600 cpm/ mU topoisomerase II.

Affinity of topoisomerase II for DNA:

5 μ g WSU-CLL cell DNA or WSU-CLL cell UV-irradiated crosslinked DNA was incubated with various concentrations of ¹²⁵I-Topoisomerase II in Topo II assay buffer (50mM Tris-Cl, 120mM KCl, 10mM MgCl₂, 0.5 mM dithiothreitol, 30 μ g BSA/ml pH 8) at 37°C for 1 hr. For some experiments the ¹²⁵I-Topoisomerase II was in the assay buffer with 5 μ M doxorubicin-preincubation for 30-min. Reaction mixtures were collected on DEAE-cellulose filters, washed with 5% trichloroacetic acid, and counted by the Packard 5330 γ -Ray Counter. Duplicate determinations were performed for each point. Non-specific binding was defined as bound ¹²⁵I-topoisomerase II that remained in the presence of a 100-fold excess of the unlabelled topoisomerase II, except for the competitive binding. The specific binding was determined by subtracting the non-specific binding from total binding. Scatchard analysis was employed to determine the maximal binding(B_{max}).

The Neutral Comet Assay of Double-Strand DNA Breaks:

The single cell comet electrophoresis assay was performed according to Olive's method (Olive *et al* 1991). Cells were suspended in PBS at a density of $5 \times 10^5/\text{ml}$ and treated with doxorubicin at a dose of $50 \mu\text{M}$ at 37°C for 3 hours. Following the treatment, 1.5 ml of 1% low gelling temperature agarose (Sigma) at 40°C were added to a tube containing 0.5 ml of cold cell suspension. The contents were quickly pipetted onto a frosted microscope slide and allowed to gel for about 1 min on a cold surface. And then slides were immersed for 5h at 50°C in 0.5% sodium dodecyl sulphate (SDS), 30 mM EDTA, pH 8.3. Following lysis, slides were thoroughly rinsed overnight in large volumes of 90 mM Tris-90mM boric acid-2mM EDTA buffer. Slides were placed in horizontal gel electrophoresis chamber containing 1 litre 90mM Tris-90mM boric acid-2 mM EDTA buffer at 0.6 V/cm for 30 min. DNA was stained by immersing slides in $2.5 \mu\text{g}/\text{ml}$ propidium iodide for 30 min.

A Olympus epifluorescence microscope attached to a camera and image analysis system was used to quantify the different parameters of the comets. Generally 100 comets were analysed per slide. Results were

expressed in terms of tail moment, which is defined as the product of the percentage of DNA in the tail multiplied by the tail length.

Statistical methods: Differences between two cell lines were tested for statistical significance by Student's *t* test.

7.3. Results:

Cytotoxicity and Interstrand-DNA Cross-link formation:

The effects of melphalan on growth and survival of WIL2 cells and WSU-CLL cells were already shown in Figure 5-1. The median IC_{50} for WIL2 cells was $8.57 \pm 1.08 \mu M$. The median IC_{50} melphalan for WSU-CLL cells $223.18 \pm 6.45 \mu M$. WSU-CLL cells were approximately 26 times more resistant to the cytotoxic effects of melphalan than WIL2 cells (Table 7-1a). The IC_{50} values were obtained from ten independent experiment. The effects of doxorubicin (Figure 7-1A), a topoisomerase II inhibitor and of camptothecin (Figure 7-1B), a topoisomerase I inhibitor, were also studied. Statistical analysis showed that WIL2 cells were significantly more sensitive to the cytotoxic effects of melphalan than WSU-CLL cells

Table7-1: Effect of Melphalan, Doxorubicin or Camptothecin on WIL2 Cells and on Alkylator-Resistant Cells(WSU-CLL)

(1a): IC₅₀ Concentrations for Melphalan, Doxorubicin and Camptothecin

IC ₅₀ (μ M)			
Drug	WIL2	WSU-CLL	P value
Melphalan	8.57 $\pm$ 1.08	223.18 $\pm$ 6.45	<0.001
Doxorubicin	4.35 $\pm$ 0.14	0.83 $\pm$ 0.09	<0.01
Camptothecin	2.75 $\pm$ 0.42	2.65 $\pm$ 0.68	>0.05

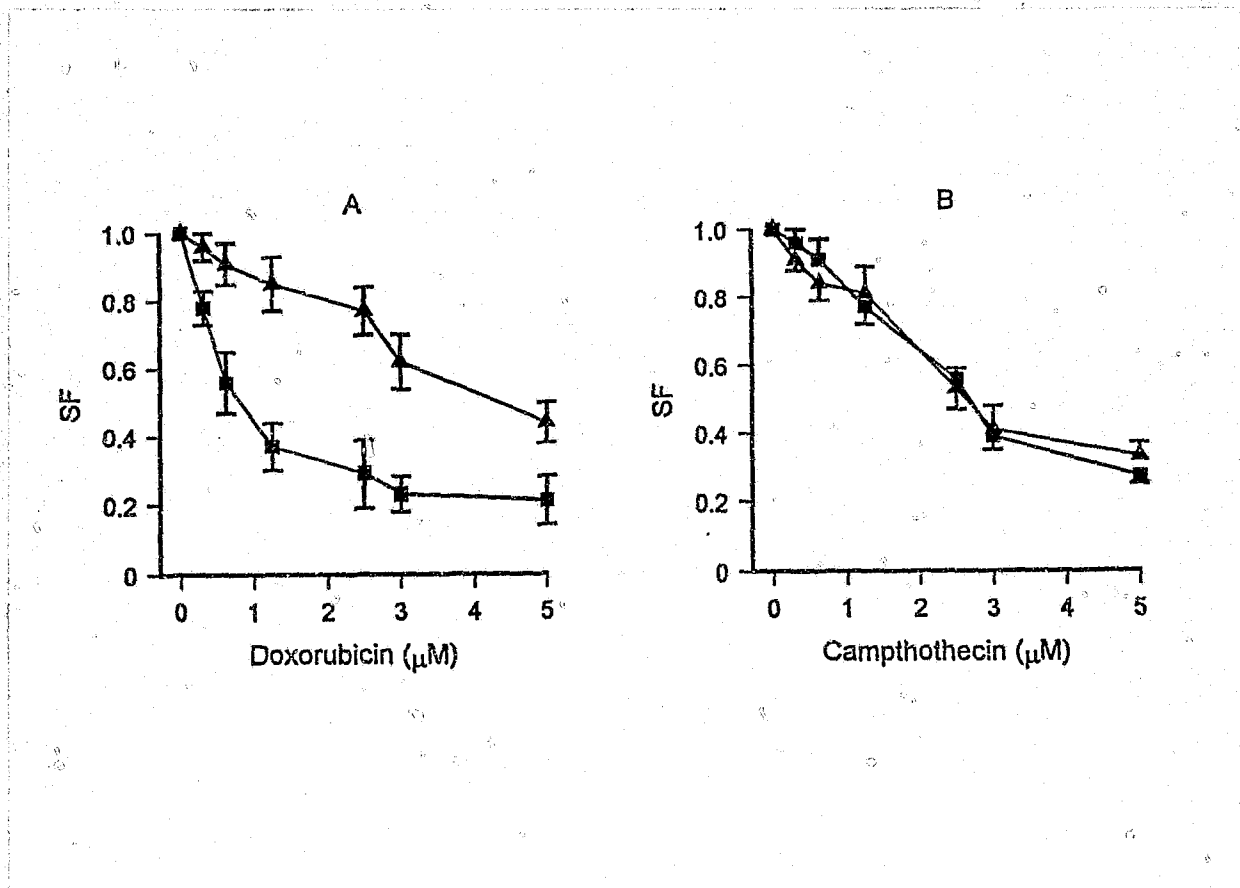


Figure 7-1: Viable cell fraction (as a proportion of control) following treatment with doxorubicin (A), and camptothecin (B) against WIL2 cells(▲) and WSU-CLL cells (■). Values are the mean $\pm$ SD of five independent experiments.

($p < 0.001$), but at the same time WIL2 cells were significantly more resistant to doxorubicin than WSU-CLL ($p < 0.01$). There was no difference in IC_{50} dose for camptothecin when WIL2 cells and WSU-CLL cells were compared (Table 7-1a). On the other hand, there was a significant difference in IC_{50} dose for melphalan between WSU-CLL and WSU-CLL with $0.01 \mu\text{M}$ doxorubicin pretreatment while there was no difference between WIL2 and WIL2 with $0.01 \mu\text{M}$ doxorubicin pretreatment (Table 7-1b).

Interstrand-DNA-crosslink ($C_t\%$) formation following a brief (60 minute) exposure to various concentrations of melphalan (measured after washing cells and a further drug-free incubation for 4 hours) demonstrated a clear dose response relationship between increasing concentrations of melphalan and $C_t\%$ in both cell lines studied. The percentage of interstrand-DNA-cross-link formation present at the end of 4 hours drug free incubation at any given concentration of melphalan was substantially lower in WSU-CLL cells as compared to that found in WIL2 cells (Figure 5-2).

**(1b): IC₅₀ Concentrations for Melphalan in Alkylator-Sensitive Cells
(WIL2) and Alkylator-Resistant Cells(WSU-CLL), with and without
Doxorubicin Pretreatment**

	IC ₅₀ (μM)
WIL2 cells	8.57 ± 1.08
WIL2 cells pre-treated with 0.01μM doxorubicin for 6-hrs	8.98 ± 1.43
WSU-CLL cells	223.18 ± 6.45
WSU-CLL cells pre-treated with 0.01μM doxorubicin for 6-hrs	48.73 ± 5.62

IC₅₀ = drug concentration resulting in 50% reduction of cell numbers as compared to controls(untreated).

The Kinetics of Interstrand DNA cross-linking:

To better define the kinetics of the formation and removal of interstrand-DNA-crosslinks, $C_t\%$ values were determined at 0, 4, and 24 hours following a 60-min incubation with $10\mu\text{M}$ melphalan. After melphalan exposure for 60 minutes the cells were washed and re-incubated in drug free medium for the stated times. Viability and metabolic activity of cells was monitored through this incubation and remained $> 90\%$ at all test times.

Interstrand-DNA-crosslink formation with melphalan was significantly different for each comparison, at any stated time except between WIL2 cells and WIL2 cells with 6hr $0.01\mu\text{M}$ doxorubicin-pretreatment (Figure 7-2).

Exponential functions for the rate of removal of interstrand-DNA-crosslinks were calculated from the data for each concentration according to the equation: $Y = ae^{-ct}$, where Y =the percentage of interstrand-DNA-cross-links remaining at t hour after the time of maximum crosslinking;

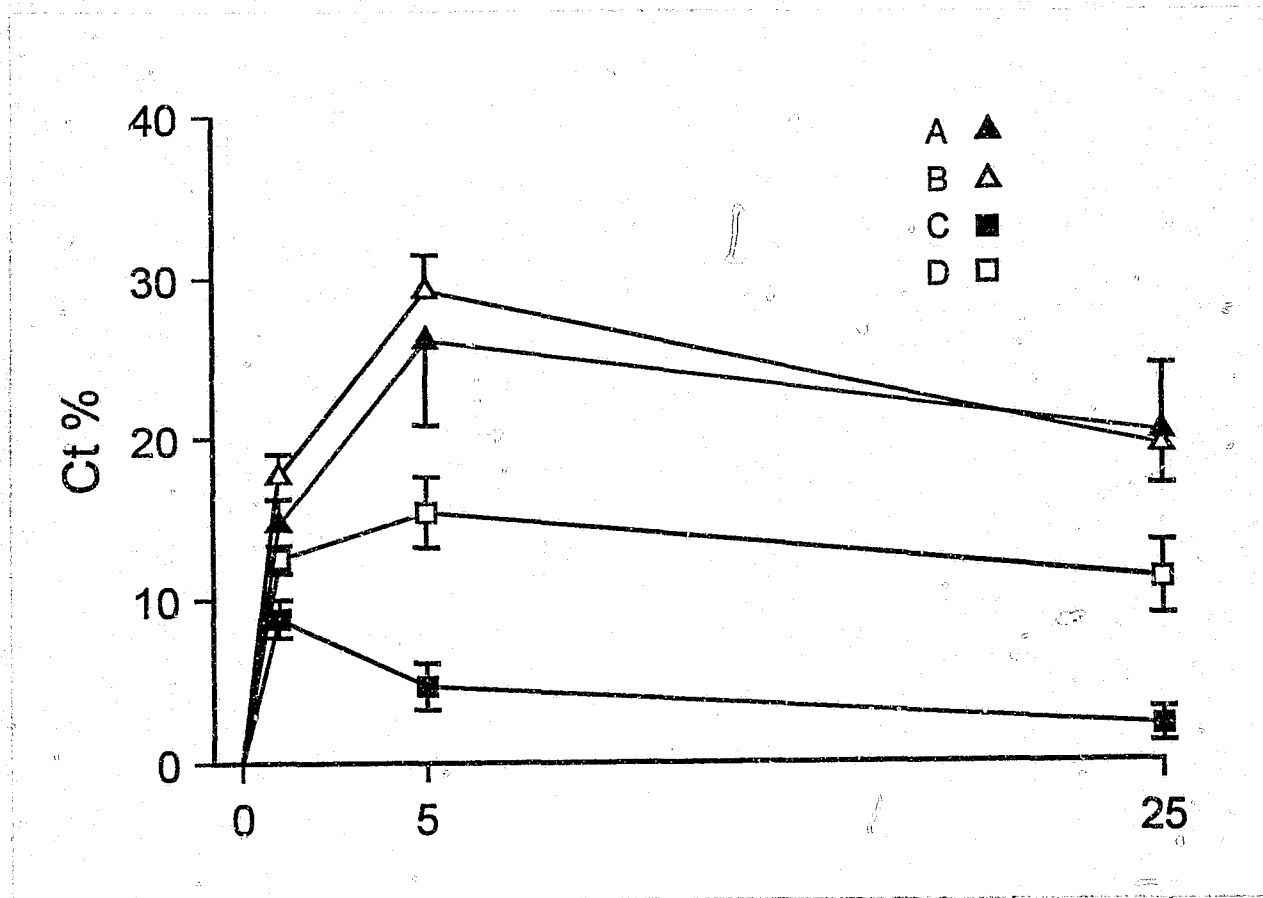


Figure 7-2: Interstrand-DNA-crosslinks(Ct%) following 60 minutes exposure to $40\mu\text{M}$ melphalan(MLN) and then 24 hours drug free incubation in: **A:** WIL2 cells; **B:** WIL2 cells with doxorubicin 6hr-pretreatment; **C:** WSU-CLL; **D:** WSU-CLL with doxorubicin 6hr-pretreatment. Time 0 represents the start of incubation with melphalan which was continued for 1 hour, followed by washing and then drug free incubation for another 24hr.

a = the maximum amount of interstrand-cross-linking as estimated by linear regression analysis; and c = the rate constant for the removal of interstrand-DNA-cross-links. From these exponential functions the $t_{1/2}$ for removal of interstrand-DNA-cross-links was calculated. The $t_{1/2}$ values showed significant differences for each of the groups except between WIL2 cells and WIL2 cells with 0.01 μ M doxorubicin 6hr-pretreatment (Table 3?).

Topoisomerase II activity and expression in WIL2 cells and WSU-CLL cells:

The strand-passing activity of topoisomerase II was measured by decatenation of kDNA. This reaction, resulting in release of double-stranded minicircles from the catenated kDNA network, is ATP dependant and specific for topoisomerase II (Figure 7-3). Figure 7-3A shows the results obtained using 0.35 M NaCl nuclear extracts at various dilutions. No decatenation activity was observed in WIL2 cells. Decatenation activity in WSU-CLL cells was much enhanced. In a set of five independent

Table 7-2:

**Regression Analysis of the Kinetics of Interstrand-DNA-Cross-Links
Formation and Removal**

	Regression	$t_{1/2}$
A	$Y = 16.55e^{-0.008t}$	86.64
B	$Y = 16.78e^{-0.008t}$	86.64
C	$Y = 10.67e^{-0.135t}$	5.13
D	$Y = 13.96e^{-0.018t}$	38.51

A: WIL2 cells

B: WIL2 cells pretreated with 0.01 μ M doxorubicin for 6-hour

C: WSU-CLL cells

D: WSU-CLL cells pretreated with 0.01 μ M doxorubicin for 6-hour

$t_{1/2}$ = half-life(hours) of interstrand-DNA-cross-links

Statistical comparisons showed:

Significant differences($p < 0.01$) for each comparison except between A and

B.

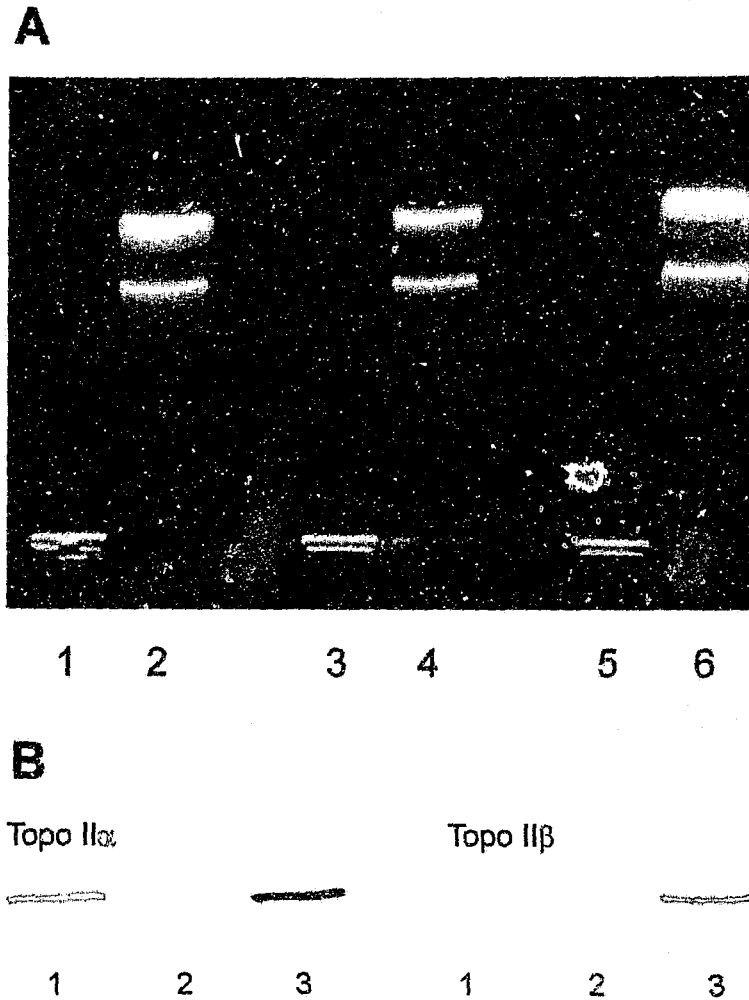


Figure 7-3A: Topoisomerase II activity in nuclear extracts from WIL2 cells and WSU-CLL cells. **Lane 1:** kDNA control; **Lane 2:** decatenated kDNA control; **Lane 3 and 5:** WIL2 nuclear extracts (containing 50 ng and 1000 ng protein per lane); **Lane 4 and 6:** WSU-CLL nuclear extracts (containing 50 ng and 100 ng protein per lane).

Figure 7-3B: Topoisomerase II α and II β protein levels, by Western blot analysis of whole cell lysates from WIL2 cells and WSU-CLL cells. **Lane 1:** the purified p170 kDa Topo II α marker (Topogen, USA); **Lane 2:** WIL2 cell extracts; **Lane 3:** WSU-CLL cell extracts. Each sample contained 100 μ g total cellular protein per lane. The blots were stained with monoclonal anti-topoisomerase II α p170 antibody (Topogen, USA) or monoclonal anti-topoisomerase II β p180 antibody (PharMingen, USA).

experiments a dramatic increase (mean 80.2%) in catalytic activity was found in nuclear extracts of WSU-CLL as compared to WIL2 cells(0) (Table 7-3).

Levels of topoisomerase II α and II β proteins in six independently prepared whole-cell extracts were determined by Western blot analysis using monoclonal anti-human DNA topoisomerase II α and II β antibodies. Densitometric analysis of the immunoblots (Figure 7-3B) showed that the levels of topoisomerase II α (60.36 ± 8.99) and II β (28.95 ± 5.23) proteins were also dramatically increased in whole-cell extracts of WSU-CLL cells compared with WIL2 cells(0).

The Affinity of topoisomerase II to DNA:

Figure 7-4A shows the ability of the unlabelled topoisomerase II to compete with ^{125}I -topoisomerase II for binding to DNA. The unlabelled topoisomerase II competed efficiently with ^{125}I -topoisomerase II in a dose-dependent manner. The time course analysis indicated that the maximal specific binding of ^{125}I -topoisomerase II to DNA was reached after 60 min of incubation at 37°C (Figure 7-4B). Scatchard analysis revealed that the

Table 7-3: Topoisomerase II Catalytic Activity

WIL2 CELLS	0
WSU-CLL CELLS	80.25 ± 9.23

Topoisomerase II catalytic activity was determined as a percentage of liberated minicircles to total kDNA. Results are expressed in arbitrary units. Values given are Mean ± SD of 5 separate experiments with five different nuclear extracts.

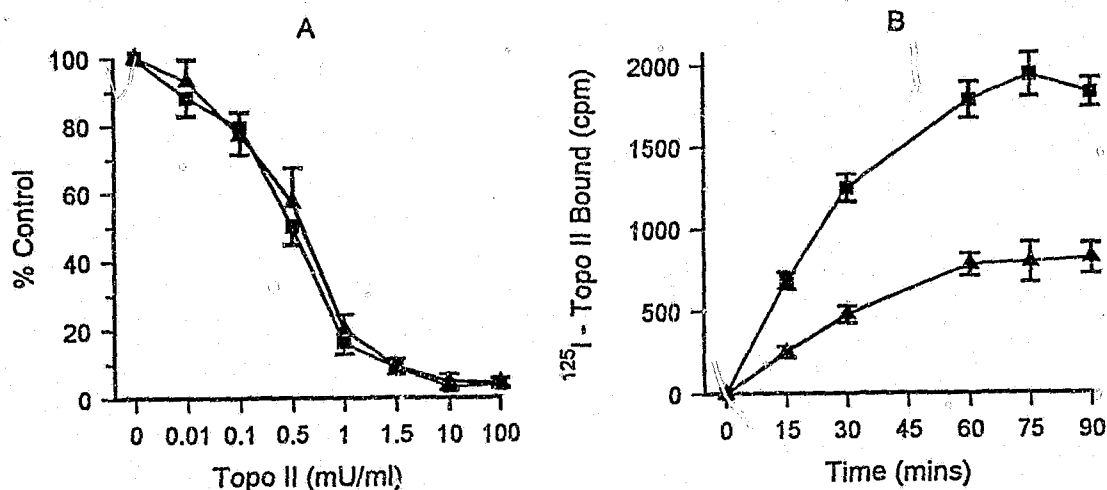


Figure 7-4: (A) Competitive binding of unlabelled topoisomerase II to WSU-CLL native DNA (▲) and WSU-CLL UV-irradiated, crosslinked DNA. DNA (■) was incubated with 10mU/ml ^{125}I -topoisomerase II for 1hr at 37°C in the topoisomerase II assay buffer. Specific binding was determined by subtracting the non-specific binding (in the presence of 1 U/ml unlabelled topoisomerase II) from total binding. Values shown represent the percentage of specific binding of ^{125}I -topoisomerase II alone. (B) Time course of binding of ^{125}I -topoisomerase II to WSU-CLL native DNA (▲) and WSU-CLL crosslinked DNA. DNA (■) was incubated with 10 mU/ml ^{125}I -topoisomerase II with or without a 100-fold excess of unlabelled topoisomerase II for the indicated times at 37°C. Points represent means $\pm$ SD. These determinations were repeated three time with similar results.

affinity of TopoII for UV-irradiated crosslinked WSU-CLL DNA was approximately 2.84-fold increased compared to that of native WSU-CLL DNA. Doxorubicin decreased Topo II binding to both native DNA and crosslinked DNA by 2.56-fold and 2.37-fold respectively (Table 7-4 and Figure 7-5A, 7-5B).

Effect of the topoisomerase II inhibitor, doxorubicin, on interstrand-DNA-crosslink formation in WIL2 and WSU-CLL cells:

A 6 hour exposure to a minimally cytotoxic concentration of 0.01 μM ($< 1\%$ growth inhibition of WIL2 and $< 5\%$ growth inhibition of WSU-CLL cells after 72 hour exposure) doxorubicin resulted in interstrand-DNA-crosslink formation of 0.29 % and 0.79 % in WIL2 and WSU-CLL cells respectively. Interstrand-DNA-crosslink formation was significantly enhanced in WSU-CLL cells pre-exposed to 0.01 μM doxorubicin for 6 hours followed by a 1 hour incubation with melphalar (40 μM) and then assayed at both 4 ($p < 0.05$) and at 24 hours ($p < 0.01$) as compared to WIL2 cells (Figure 7-6). Pre-exposure to 0.01 μM doxorubicin decreased

**Table 7-4: The Affinity of Topoisomerase II for Native and
for UV- Irradiated(crosslinked) DNA**

Table 4a: No pretreatment with the Topoisomerase II Inhibitor

Doxorubicin

		B_{max} (mU/ml)
WSU-CLL Native DNA		53.92 ± 3.16
WSU-CLL	UV-irradiated (crosslinked)DNA	153.11 ± 11.65

Table 4b: Following Pretreatment with the Topoisomerase II Inhibitor

Doxorubicin

		B_{max} (mU/ml)
WSU-CLL native DNA with		
1 μ M	Doxorubicin	21.59 $\pm$ 4.39
pretreatment		
WSU-CLL UV-		
irradiated(crosslinked) DNA with		64.41 $\pm$ 8.62
1 μ M	Doxorubicin pretreatment	

B_{max} is defined as the topoisomerase II concentration at which the DNA is maximally occupied by topoisomerase II.

There are significant differences of the affinity either between native DNA group and UV-irradiated(crosslinked) DNA or between same group with doxorubicin pretreatment and without doxorubicin pretreatment($p < 0.05$).

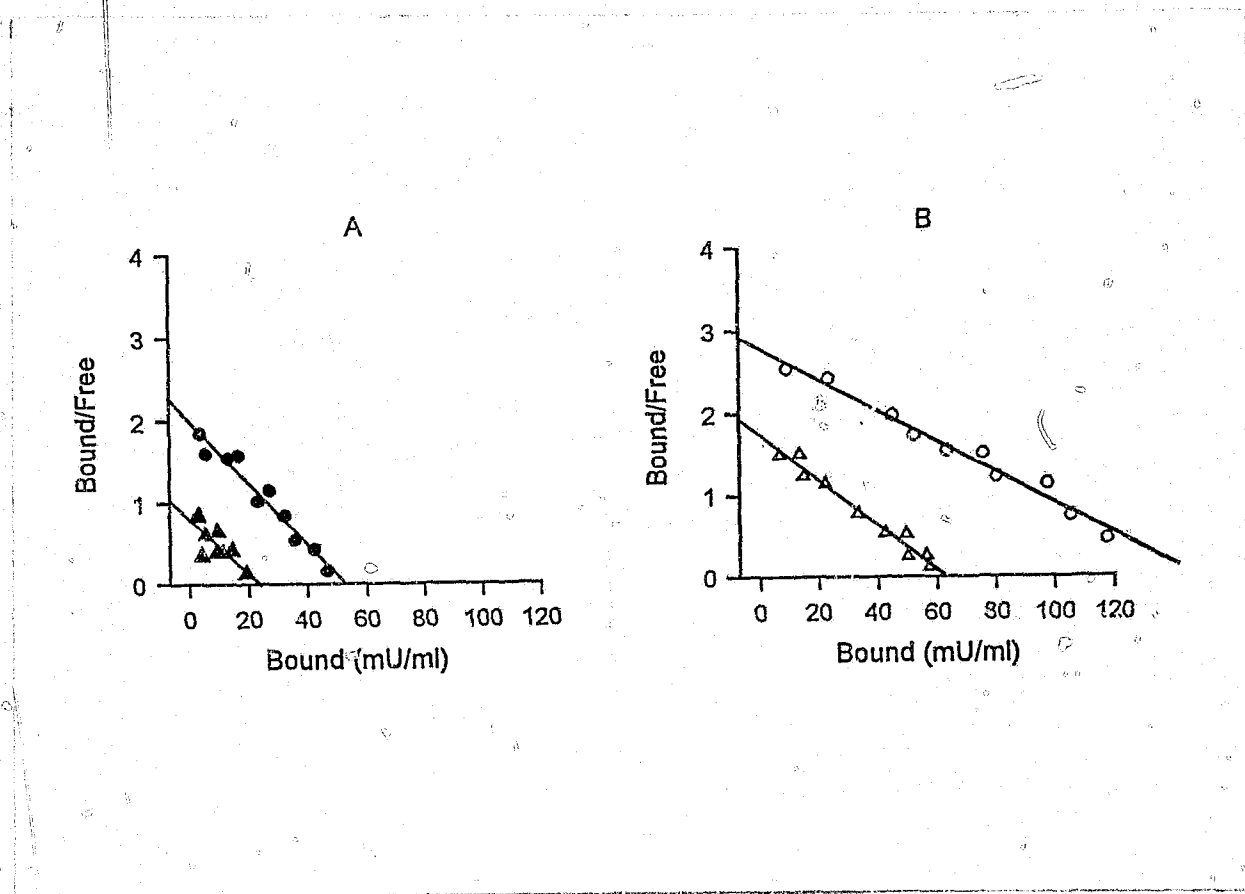


Figure 7-5: Scatchard analysis of the binding of ^{125}I -topoisomerase II to WSU-CLL native DNA(A) and WSU-CLL UV-irradiated crosslinked DNA(B). DNA was incubated with different concentrations of ^{125}I -topoisomerase II (10 mU/ml to 1 U/ml) with(▲, △) or without(●,○) pretreatment of 5 μM Doxorubicin for 30 min at 37°C in the presence or absence of a 100-fold excess of the unlabelled topoisomerase II for 1 hr at 37°C. The results in each panel represent data from one of three experiments, each of which gave the similar results.

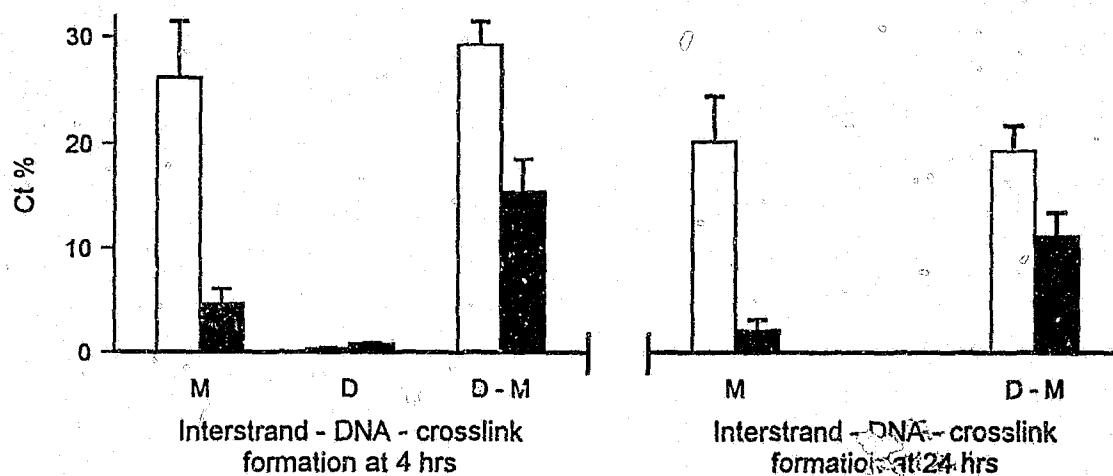


Figure 7-6: The effect of doxorubicin (D) pre-treatment on melphalan (M) induced interstrand-DNA-crosslink formation. D = 0.01 μ M doxorubicin for 6-hour; M = 40 μ M melphalan for 1 hour; D $\rightarrow$ M = preincubation with 0.01 μ M doxorubicin for 6-hour and then 40 μ M melphalan treatment for 1 hour. Cells were then washed and Ct (%) was measured after 4-hour and 24-hours drug-free incubation. Values are the mean $\pm$ SD of 10 experiments, each performed in triplicate.

the IC_{50} melphalan concentration for WSU-CLL cells from 223.18 ± 6.45 to $48.73 \pm 5.62 \mu M$.

***Effect of Doxorubicin on Double Strand DNA Breakage in HL60 and R-
HL60:***

DNA is able to migrate towards the anode in an electric field when cells are embedded in agarose and lysed. Individual cell DNA was visualized by fluorescence microscope. Each cell has the appearance of a "comet" with brightly fluorescent head and tail with an intensity that is related to the amount of ds DNA breakage sustained by the cell. The neutral comet assay only measures double and not single-strand breaks (Olive *et al*, 1991). WIL2 cells and WSU-CLL cells were examined for DNA double-strand induced by doxorubicin. The average tail moment of WSU-CLL cells was about 5.84-fold increased compared to that of WIL2 cells (Figure 7-7).

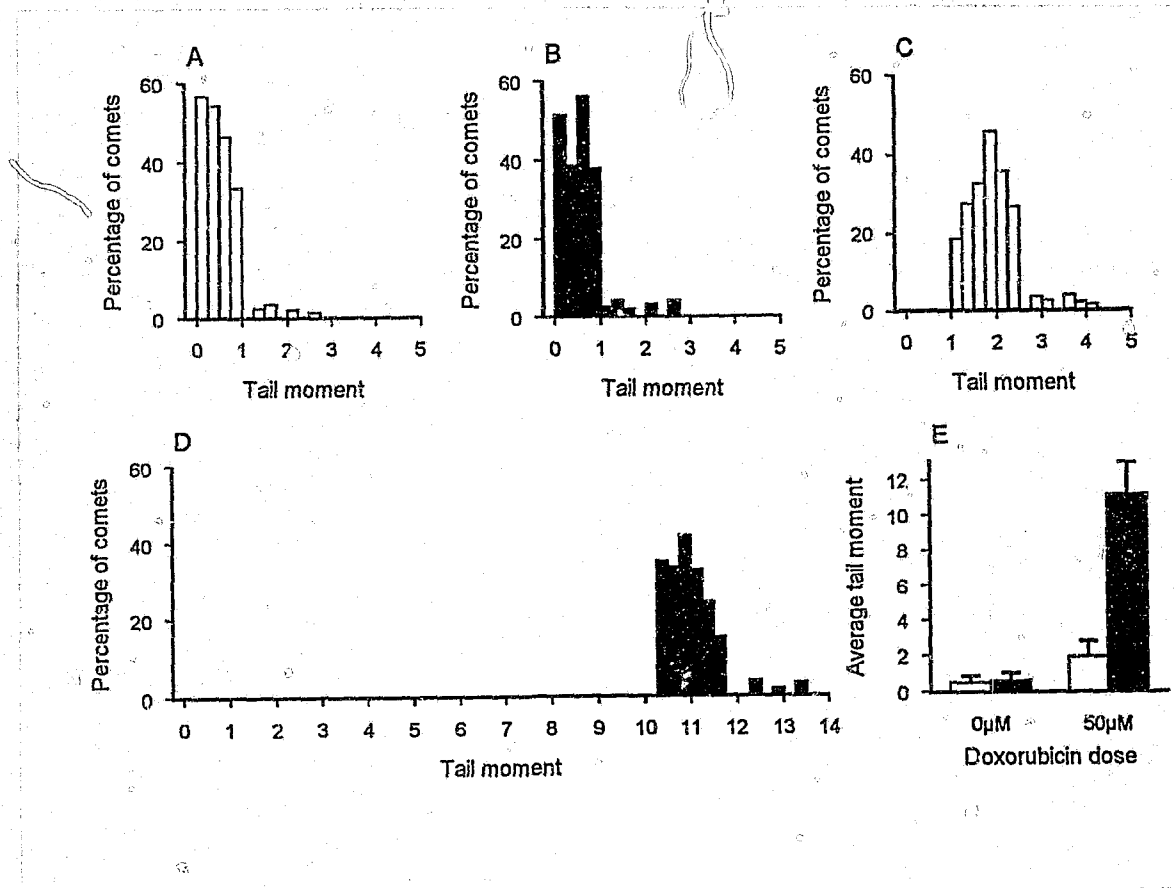


Figure 7-7: Doxorubicin-induced dsDNA Breaks of WIL2 cells and WSU-CLL cells. dsDNA breaks were measured by the neutral comet assay. Comets were analysed by calculating the "tail moment" for each cell. In A-D, Representative histograms from 200 comets are shown for A: WIL2 cells without doxorubicin pre-treatment; B: WSU-CLL cells without doxorubicin pre-treatment; C: WIL2 cells pre-treated with 50 μ M Doxorubicin; D: WSU-CLL cells pre-treated with 50 μ M doxorubicin; E. The means $\pm$ SD (bars) of tail moments for 200 comets were shown.

Topoisomerase I activity and expression in WIL2 and WSU-CLL cells:

The possibility that the increase in topoisomerase II activity might be balanced by decreased topoisomerase I activity was also investigated. Quantification of relaxed DNA forms (Figure 7-8A) in six independently prepared extracts of WIL2 and WSU-CLL showed a ratio of 1.25 ± 0.26 ($p > 0.05$) indicating similar topoisomerase I activity in both cell lines. The level of topoisomerase I protein was determined in five independent extracts by Western blot analysis (Figure 7-8B). Densitometric analysis showed no significant difference in the level of topoisomerase I protein expressed in the two cell lines. The ratio of topoisomerase I proteins in WSU-CLL extracts to WIL2 cells was calculated to be 0.98 ± 0.24 ($P > 0.05$).

7.4. Discussion:

Antineoplastic drugs that alkylate DNA are extensively used in cancer chemotherapy, either alone or in combination with other classes of chemotherapeutic drugs. One of the principal factors limiting the

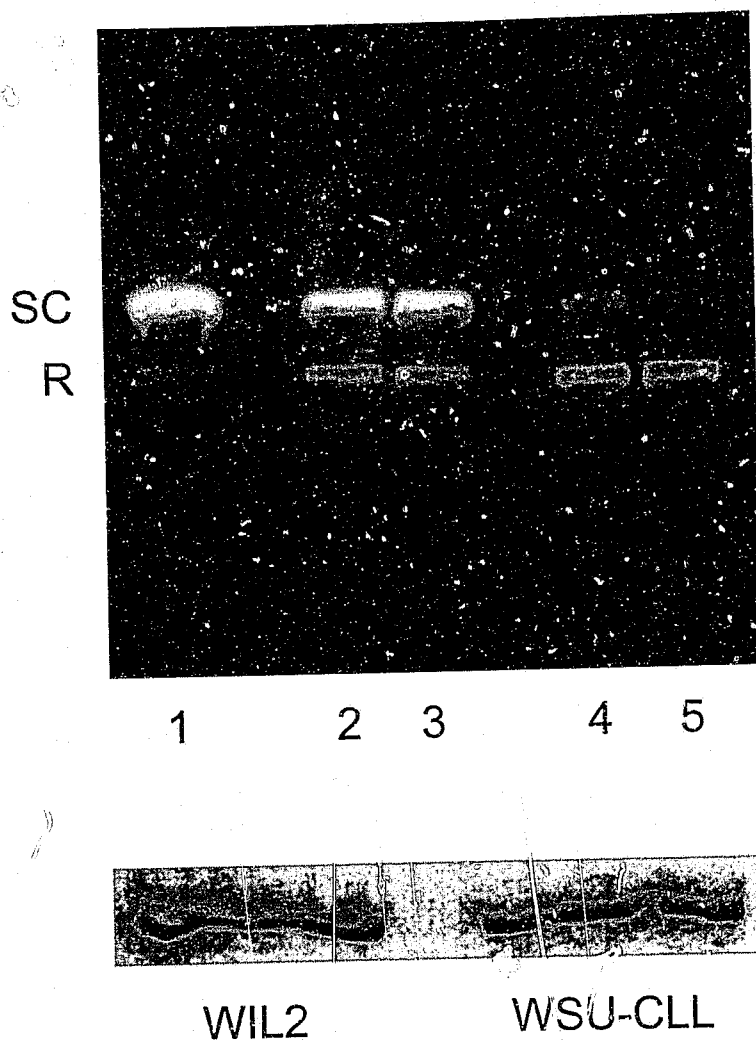


Figure 7-8A: Topoisomerase I activity in nuclear extracts from WIL2 and WSU-CLL cells. Lane 1: control; Lane 2 and 4: WIL2 nuclear extracts (containing 10 ng and 50 ng of protein per lane respectively); Lane 3 and 5: WSU-CLL nuclear extracts (containing 10 ng and 50 ng of protein per lane respectively). The locations of supercoiled (SC) and relaxed (R) topoisomer are indicated.

Figure 7-8B: Topoisomerase I protein levels by Western blot analysis of whole cell lysates from WIL2 cells and WSU-CLL cells. Samples contained 100 μ g total cellular protein.

effectiveness of alkylating agents is cellular resistance to the alkylating effect. There is considerable evidence from a number of in-vitro models including both human and animal tumour cell lines (*Louie et al, 1985; Frei et al, 1985; Robson et al, 1985*) that the cytotoxicity of these drugs results directly from interstrand DNA cross-link formation (*Kohn, 1977; Ewig and Kohn, 1978; Ross et al, 1978; Hansson et al, 1987; Erickson et al, 1997; Ewig and Kohn, 1997*). The alkylator resistant phenotypes are multifactorial and include decreased uptake, increased glutathione content, and increased DNA repair activity (*Bedford et al, 1988; Bungo et al, 1990; Pegg, 1990; Richon et al, 1990*). In addition to these previously defined mechanisms, the results of the current study demonstrate that alkylator resistance is critically determined by the capacity to repair interstrand-DNA-crosslinks, which in turn is determined by increased topoisomerase II activity, resulting from an increase in topoisomerase II protein expression.

Melphalan resistant WSU-CLL cells showed increased topoisomerase II activity and protein expression proportional to the degree of melphalan resistance. These findings taken in conjunction with the studies of kinetics of DNA crosslink formation in the sensitive and resistant cell lines suggest

that the enhanced DNA repair due to increased topoisomerase II activity with increased binding of topoisomerase II to crosslinked DNA in WSU-CLL is responsible for the resistance to alkylating agents. These melphalan resistant (WSU-CLL) cells were more sensitive to the cytotoxic effects of the topoisomerase II inhibitor doxorubicin, exposure to which resulted in substantially increased double strand DNA breakage in WSU-CLL cells. Inhibition of topoisomerase II activity by minimally cytotoxic concentrations of doxorubicin was able to significantly increase the amount of interstrand-DNA-crosslink formation following melphalan exposure in WSU-CLL cells, thus reversing the alkylator resistant phenotype.

Increased topoisomerase II activity and binding thus appears to be the rate limiting step for DNA repair. The specificity of this finding was suggested by the observation that there was no increase in topoisomerase I expression or alteration in sensitivity to the topoisomerase I inhibitor camptothecin, in WSU-CLL cells as compared to WIL2. These results are consistent with earlier studies which demonstrated increased topoisomerase II expression to be associated with resistance to mechlorethamine and cisplatin and that this resistance was associated with increased topoisomerase II binding to

cisplatin-damaged DNA (Eder *et al*, 1995). A possible correlation between topoisomerase II activity and cellular resistance to alkylating agents in the mechlorethamine resistant Raji-HN₂ cell line and in a cisplatin resistant human lung cancer cell line (Tan *et al*, 1987; DeJong *et al*, 1991b) has also been described.

In a previous study (Pu and Bezwoda 1999), human leukemic, HL60 cells, were selected for resistance to melphalan by stepwise exposure to increasing concentrations of melphalan. The resulting cell line (R-HL60) was 4-fold resistant (IC₅₀ melphalan 27.84±4.2 µM) to melphalan as compared to parental HL60 cells (IC₅₀ melphalan 6.9±1.78 µM). Nuclear extracts from R-HL60 cells possess an approximately 4-fold increase in DNA topoisomerase II activity as compared to parental HL60 cells. By Western blot analysis the level of topoisomerase II α protein expressed in R-HL60 cells was approximately 3-fold that of parental HL60 cells. But any difference was observed neither in the level of topoisomerase II β protein expression nor in the topoisomerase I activity and in the level of topoisomerase I protein expression was observed comparing the two cell lines. R-HL60 cells were five fold more sensitive than parental HL60 cells to the cytotoxic effect of the topoisomerase II inhibitor doxorubicin. The-

sensitivity to the cytotoxic effects of topoisomerase I inhibitor, camptothecin, did not differ in R-HL60 and parental HL60 cell lines.

Pre-incubation with doxorubicin significantly increased melphalan induced interstrand-DNA- crosslink formation and cytotoxicity in R-HL60 cells as compared to the parental HL60 cells.

The affinity of topoisomerase II for UV-irradiated-crosslinked HL 60 DNA was increased 2.47-fold as compared to that of HL60 native DNA. The affinity of topoisomerase II for both UV-irradiated (crosslinked) and for native DNA was significantly decreased after doxorubicin-pretreatment. We had very similar results as the present results. It indicates that this mechanism of melphalan resistance is not unique to B-CLL cell line (WSU-CLL).

Topoisomerase II has been identified as the putative cellular target of many clinically active antineoplastic agents, including the aminoacridines, anthracyclines, and epipodophyllotoxin (Glisson and Ross, 1987; Liu, 1989). Sensitivity to these agents in vitro appears to correlate with cellular topoisomerase II levels. Previous studies have shown that topoisomerase

II inhibitors also can partially reverse resistance to the alkylating agents (*Tan et al, 1987; DeJong et al, 1993*), although the mechanism of this effect has not been fully defined.

The results of the current study provide further insights into the mechanisms of alkylator resistance, as well as providing a mechanistic framework for the clinical use of alkylating agents in combination with topoisomerase II inhibitors such as doxorubicin and epipodophylotoxins. The findings provide a rationale for the clinical use of combination chemotherapy regimens based on alkylating agents and topoisomerase II inhibitors.

CHAPTER 8

DNA Polymerase α Activity in WSU-CLL Cells as Compared to WIL2 Cells

8.1. Introduction:

Knowledge about DNA polymerases in eukaryotes has increased considerably during recent years. In a comprehensive summary in 1985, relevant data regarding DNA-repair polymerases was available for two nuclear DNA polymerases, α and β (Friedberg 1985). Currently, there are five characterized nuclear DNA polymerases in eukaryotes (Wang 1996). A large body of evidence suggests an important role for DNA polymerases in the repair process, directly in excision repair pathways, and/or as part of a recombinational repair process (Hannawalt et al 1982; Friedberg et al 1987; Sancar and Sancar 1988; Perrino and Loeb 1990). DNA polymerases catalyze the synthesis of oligonucleotides for patching of gaps generated at sites of DNA damage by the action of endo- and exonucleases.

In this study, we examined the activities of DNA polymerase α and DNA polymerase β to determine whether there is any alteration in the activities

of these DNA repair associated proteins and whether the alterations were related to the observed changes in DNA repair capacity.

8.2. Materials and Methods:

Cell lines, culture conditions and cytotoxicity assays were detailed in Chapter 4.

Preparation of Cellular Extracts for DNA polymerase Assays:

Harvested cells were pelleted and resuspended in 100-200 μ l.(depending on cell number) extraction buffer(10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol) and disrupted by sonification. The homogenate was centrifuged at 15000xg for 30 min at 4 °C, followed by 30000xg for 90 min. The resulting supernatant was used for DNA polymerase assays (Laemmli 1970, Yamaguchi et al 1980).

Quantitation of DNA polymerase- α and DNA polymerase- β activities:

Activities of DNA polymerases- α and DNA polymerase- β in WIL2 cells and WSU-CLL cells were assayed essentially as previously described

(Knopf *et al* 1976; Yamaguchi *et al* 1980), with some modifications as follows: The reaction mixtures for both DNA polymerase- α and DNA polymerase- β assays consisted of 50 μ g bovine serum albumin(DNase-free), 50mM Tris/HCl, pH 8.5, 0.5 mM dithiothreitol, 7.5 mM $MgCl_2$, 0.1 mM each of dCTP, dGTP, dTTP and ^{32}P -dATP (600 cpm/pmol), and activated calf thymus DNA. In the mixtures for the DNA polymerase- α assay, 0.1 M KCl was added. In the DNA polymerase- β assay, 5 mM N-ethylmaleimide was used. Nuclease-free redistilled water was added to the reaction mixtures to a final volume of 100 μ l. The mixture was incubated for 1hr at 37°C and the reaction was stopped by adding 5 μ l 0.5M EDTA. Five replicate 10 μ l aliquots of each reaction mixture were collected to DEAE-Cellulose filters in a Millipore Multiscreen Assay System(Millipore Corporation, Bedford, Mass.); the filters were washed with cold 5% trichloroacetic acid, and counted by liquid scintillation.

One Unit of DNA polymerase- α or DNA polymerase- β was defined as the amount of enzyme that catalyzed the incorporation of 1 pmol of ^{32}P -dATP into newly synthesized DNA in 1 hour. Polymerase activity is expressed in units per milligram of proteins.

8.3. Results:

DNA polymerase- α and DNA polymerase- β Activities:

The activity of DNA polymerase- α in WSU-CLL cells has approximately 2.1-fold increased compared with that in WIL2 cells. DNA polymerase- β activity showed the no difference between two cell lines (Table 8-1). 10 μ M aphidicolin inhibited DNA polymerase- α to 65% of controls in WIL2 cells and 57% of controls in WSU-CLL cells (Table 8-2).

Cytotoxicity Assay and Interstrand DNA Cross-links:

Aphidicolin at all dosages tested produced no cytotoxicity as determined by MTT assay (Figure 8-1). The response of both cell lines to melphalan was not significantly enhanced by 10 μ M aphidicolin pre-incubation for 24 hours (Figure 8-2a,2b, Table 8-3). The amount of interstrand-DNA-crosslinking (C_i) produced following a 60 minute exposure to various concentrations of melphalan (measured after washing cells and a further drug-free incubation for 4-hr) demonstrated no differences between with

Table 8-1

Activity of DNA Polymerase α and β in WIL2 Cells and WSU-CLL Cells

Cell Line	DNA Pol- α (units/mg	DNA Pol- β (units/mg
	protein)	protein)
WIL2	27.82 ± 4.39	0.74 ± 0.09
WSU-CLL	58.43 ± 3.67	0.82 ± 0.11

Values are Mean $\pm$ SD of five independent experiments

Pol: polymerase

Table 8-2 Effect of Aphidicolin on DNA Polymerase- α

	DNA Polymerase- α (units/mg protein)	
	WIL2	WSU-CLL
Control	27.82 $\pm$ 3.39	58.43 $\pm$ 3.67
Aphidicolin (10 μ M)	18.08 $\pm$ 2.16	33.31 $\pm$ 3.14

DNA Polymerase- α was quantitated in WIL 2 cells and in WSU-CLL cells with or without 10 μ M Aphidicolin treatment for 24 hours at 37°C.

Values are Mean $\pm$ SD of five independent experiments.

and without 10 μ M aphidicolin pre-incubated for 24 hours in either WIL2 cells or WSU-CLL cells (Figure 8-3).

8.4. Discussion:

In this study, we demonstrated firstly that DNA polymerase- α level is elevated in the melphalan-resistant WSU-CLL cell line. This finding led to the further study designed to define the consequences on melphalan cytotoxicity of using aphidicolin to inhibit polymerase- α .

Aphidicolin is a tetracyclic diterpenoid derived from cephalosporium aphidicola and is a specific reversible inhibitor of DNA polymerase- α and DNA polymerase- δ (Ikegami et al 1978; Oguru et al 1979; Pedrali et al 1979; Huberman 1981; Goscin and Byrnes 1982). Incubation of the two cell lines with 10 μ M aphidicolin inhibited DNA polymerase- α to 65% in WIL2 cells and 57% in WSU-CLL cells, respectively compared with controls. However, the cytotoxicity of melphalan against two cell lines was not enhanced by the pretreatment with aphidicolin.

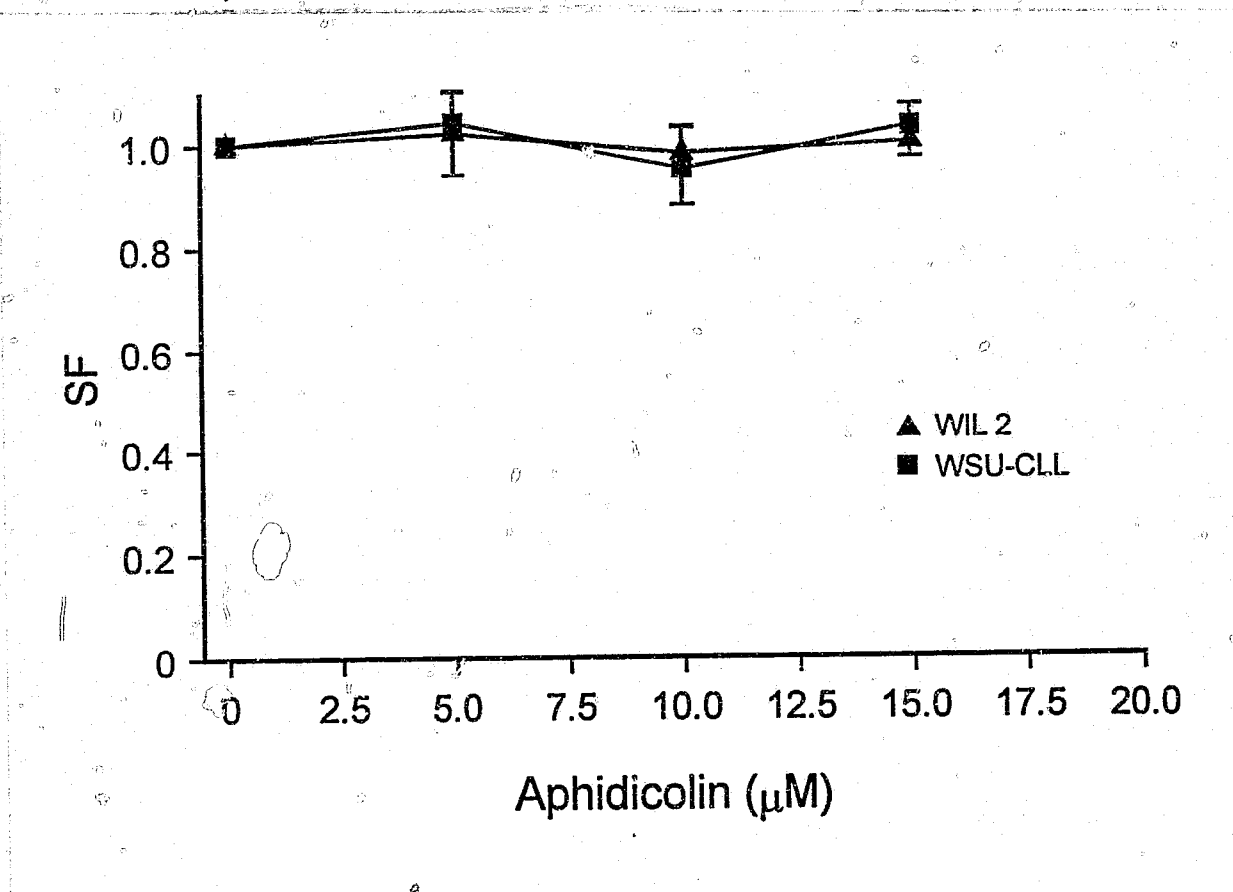


Figure 8-1: Viable cell fraction (as a proportion of control) following treatment with aphidicolin against WIL2 cells ($\blacktriangle$) and WSU-CLL cells($\blacksquare$). Values shown are the mean $\pm$ SD of five independent experiments.

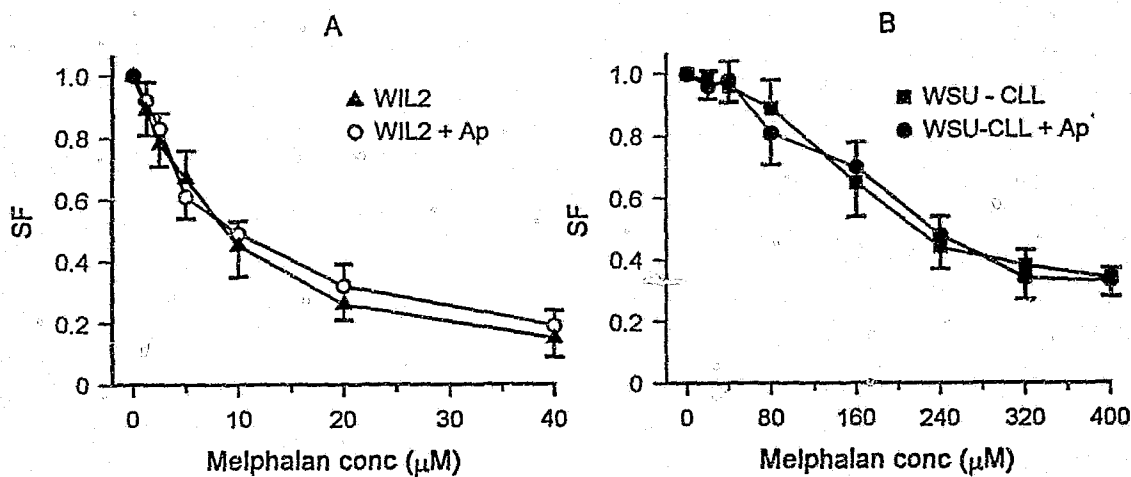


Figure 8-2: Cytotoxic effect of melphalan (MLN) on WIL2 cells(A: without Ap--▲; with Ap--○) and WSU-CLL cells (B: without Ap--■; with Ap--●) with or without 10 μM Ap(aphidicolin) pre-treated for 24-hr. Points represent mean $\pm$ S.D of ten independent experiments. The significant difference was found when WIL2 cells and WSU-CLL cells were compared($p < 0.001$). But there was no difference between with and without 10 μM Ap pre-treated for 24-hr.

Table 8-3:

**IC₅₀ Concentrations for Melphalan in WIL2 Cells and Alkylator
Resistant WSU-CLL Cells, with and without Aphidicolin Pretreatment**

	IC ₅₀ (μM)
WIL2 cells	8.57±1.08
WIL2 cells pre-treated with 10μM aphidicolin for 24-hrs	9.82±1.73
WSU-CLL cells	223.18±6.45
WSU-CLL cells pre-treated with 10μM aphidicolin for 24-hrs	231.42±5.21

IC₅₀ = drug concentration resulting in 50% reduction of cell numbers as compared to control(untreated).

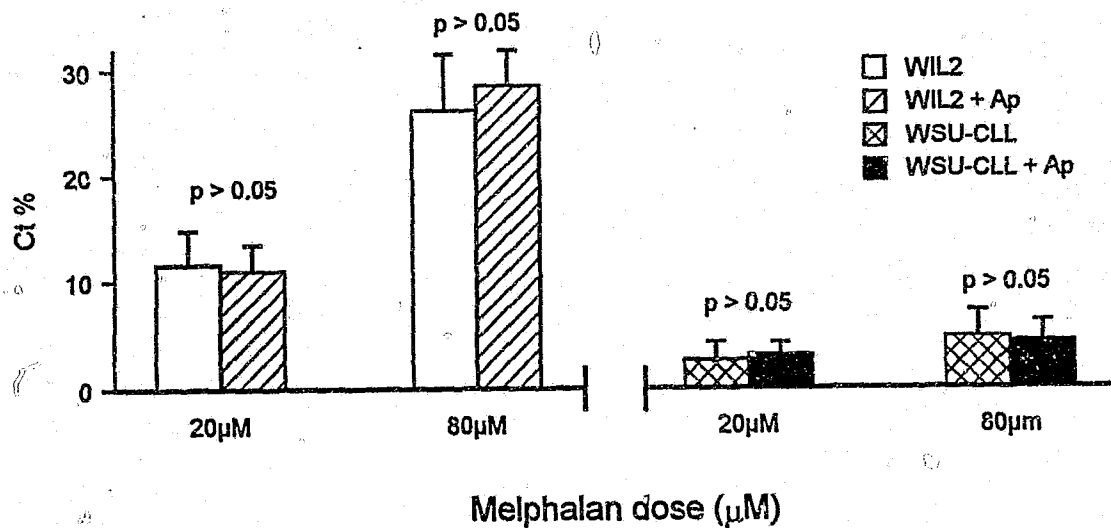


Figure 8-3: Percentage of interstrand-DNA-cross-links (C_t) assessed after 60 minutes melphalan (MLN) exposure followed by 4 hours drug free incubation in WIL2 cells (▲) and in WSU-CLL cells (■) with or without 10 μM Ap (aphidicolin) pre-treated for 24-hr. Points represent mean $\pm$ SD of ten independent experiments.

Previous studies demonstrated that DNA polymerase α is not involved in nucleotide excision-repair DNA repair, (*Dresler and Frattini 1986; Nishida et al 1988; Hunting et al 1991; Popanda and Thielmann 1992*). Coverley and co-worker used monoclonal antibodies that neutralized the activity of DNA Pol α in solution, which did not significantly inhibit DNA-repair synthesis carried out by cell extracts (*Coverley et al 1992*).

In line with those findings, the lack of increased sensitivity to melphalan with this approach suggests that melphalan resistance does not appear to involve DNA polymerase- α as an additional resistance mechanism.

CHAPTER 3

Interleukin-4(IL4) Prevents Spontaneous In-Vitro Apoptosis In Chronic Lymphatic Leukemia But Sensitises B-CLL Cells To Melphalan Cytotoxicity

9.1. Introduction:

B-CLL is characterised by a slowly accumulative expansion of CD5⁺ B-lymphocytes resulting from a decreased rate of spontaneous apoptosis (*Dameshek, 1967; Colins et al, 1989; Robertson et al, 1993b*). While in-vivo resistance to cell death characterises B-CLL, these cells show decreased in-vitro viability and an increased rate of apoptosis in the absence of specific maintenance factors such as IL4 (*Danescu et al, 1992; Manion-Fowler et al, 1993; Panayiotides et al, 1993*). IL4 is known to increase the expression of bcl-2 protein, an inhibitor of apoptosis, in B-CLL cells (*Panayiotides et al, 1993; Craig et al, 1993*). The bcl-2 product has also been associated with drug resistance in either spontaneously expressing or transfected cell lines with high levels of the protein (*Hockenbery et al, 1990; Korsmeyer S.J., 1992; Campana et al, 1992*). In

view of these observations, it might be expected that IL4 treatment of B-CLL cells would increase the resistance of these cells to chemotherapeutic agents. The present study demonstrates that, paradoxically, IL4 action on B-CLL cells is associated with enhanced sensitivity to alkylating agents and more specifically that it is B-CLL cells that demonstrate alkylator resistance in the absence of IL4 that are sensitised to the cytotoxic effects of the bifunctional alkylator, melphalan.

9.2. Materials and Methods:

Peripheral blood lymphocytes were obtained from 10 patients with B-CLL and 5 normal control subjects. Clinical details of the patients are shown in Table 9-1. Eight of 10 patients had had prior alkylator therapy and 6 had clonally restricted lymphocytes that were classified as alkylator resistant according to a previously defined LD₅₀ concentration of > 10 µM melphalan (Bezwdoda & Pu 1997). These patients had also shown clinical resistance to treatment with alkylating agents.

Table 9-1: Clinical and Hematologic Details of Patients with Chronic Lymphatic Leukemia

No.	Age	Sex	WCC (x10 ⁹)	Lymphocyte Percentage	LD ₅₀ (μM)	Prior Treatment
1	71	M	7.84*	53	3.25	+
2	69	M	127	34	4.25	-
3	51	M	4.37*	42	7.25	+
4	77	F	10.95*	75	8.75	+
5	78	M	9.20*	73	13.25	+
6	71	F	8.30*	54	16.50	+
7	71	M	22.31	77	17.50	+
8	69	M	42.20	92	18.25	+
9	57	F	25.50*	57	22.50	-
10	69	M	57.80	100	27.50	+

* Patients were investigated following treatment including either oral alkylating agent therapy (patients 1,3 and 5) or following second line treatment with fludarabine (patients 6 & 9). At the time of investigation patients were off treatment with stable leukocyte counts for at least 4 weeks.

Mononuclear cells were isolated by Ficoll-Hypaque centrifugation (*Boyum 1958*). Monocytes were removed by adherence to plastic and T-lymphocytes were removed by passage through by nylon wool (*Hudson & Hay, 1980*). The resulting cell population contained < 2 % T lymphocytes and > 95 % B-cells as assessed by flow cytometry using a panel of monoclonal markers including CD5, CD19, CD20 and CD3. In patients with B-CLL > 95 % of B-cells were demonstrated to be CD5⁺ and to have isotypic light chain restriction.

Cultures included IL4 at and/or melphalan at various concentrations as given in text and illustrations. For determination of cytotoxicity of melphalan, cells were pre-incubated for 24 hours with or without IL4, washed once with fresh medium and then cultured for up to 72 hours together with melphalan which was added at a final volume of 200 µl/well.

DNA fragmentation was assessed by analysis of soluble DNA in cell lysates as compared to total cell DNA (soluble plus precipitable, intact, DNA) using 33258 Hoechst according to the method of Cesarone et al (*Cesarone et al 1979*) and of Buschle and co-workers (*Buschle et al, 1993*).

Cell viability and metabolic activity was assessed both by trypan blue exclusion and by the MTT assay.

Statistical analysis of paired data used Student's *t* test.

9.3. Results:

IL4 did not stimulate proliferation of either B-CLL cells or normal lymphocytes. However, IL4 significantly increased the viability of both B-CLL cells as well as normal B-lymphocytes for periods of up to 96 hours. Differences were shown at all IL4 concentrations but the optimal concentration of IL4 required to maintain cell viability was 50 u/ml (Figure 9-1).

Spontaneous DNA fragmentation occurred in both normal lymphocytes and at a higher rate in B-CLL cells (Figure 9-2). DNA fragmentation consisted of typical 200 bp oligosomal multimers and was already evident after 24 hours incubation. IL4 significantly reduced DNA fragmentation in both cell types.

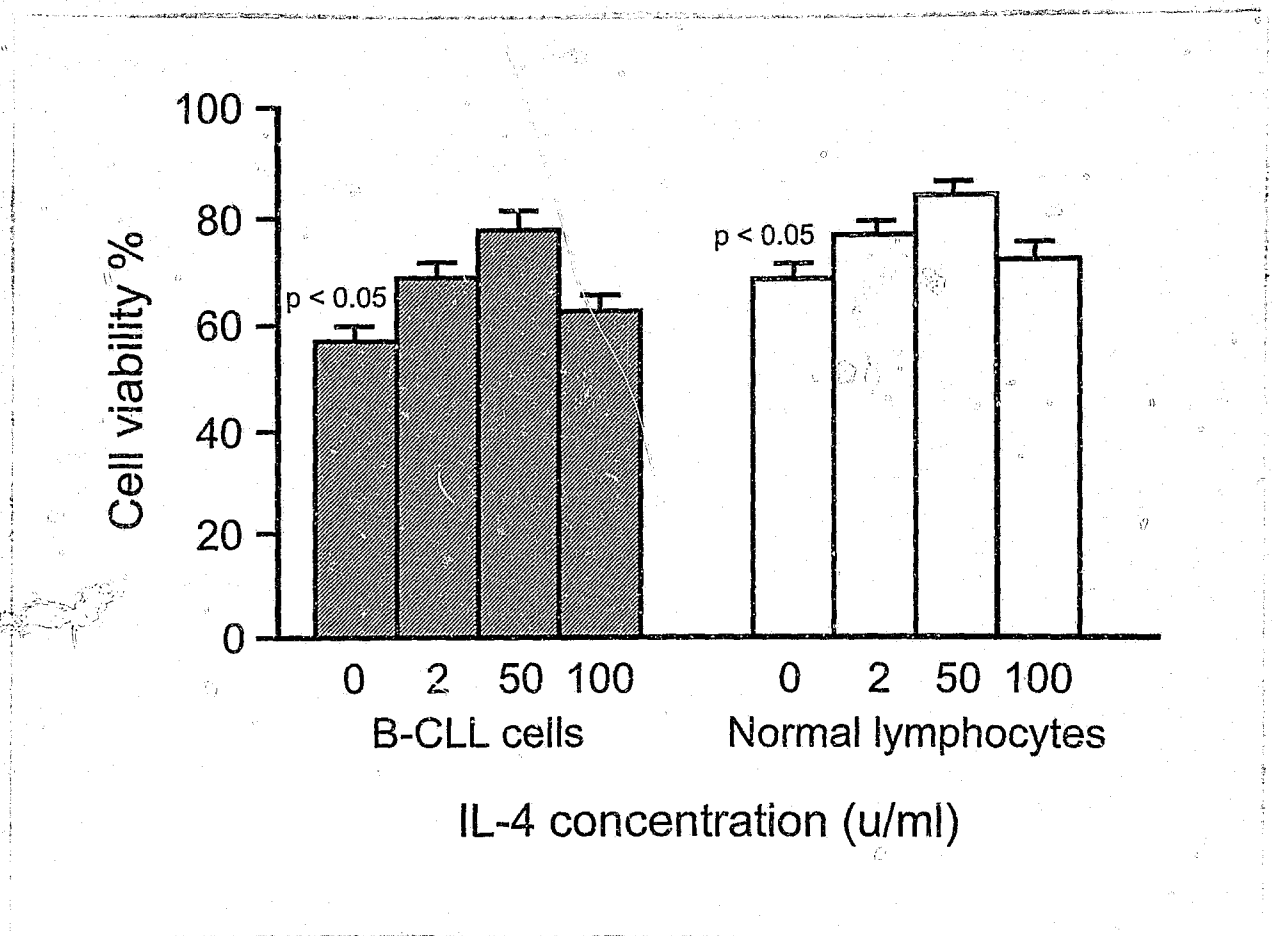


Figure 9-1: Effect of IL4 on cell viability following 96 hours culture.

Cell culture was in RPMI 1640 with 10 % fetal calf serum.

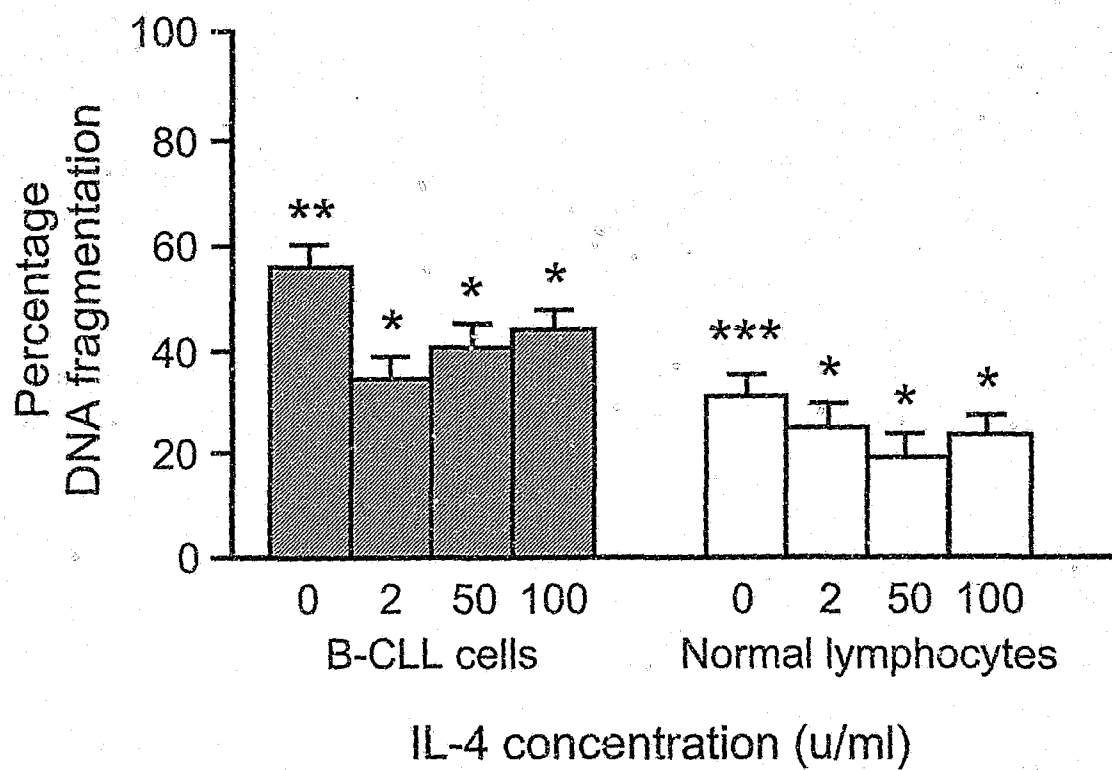


Figure 9-2: DNA Fragmentation assay. The percentage of soluble DNA was measured in B-CLL cells and in normal B-lymphocytes after 96 hours culture with or without addition of IL4.

- * Significantly different compared to control ($p < 0.01$).
- ** Significantly different comparing B-CLL cells and normal B-lymphocytes ($p < 0.01$).

Alkylator resistant ($LD_{50} > 10 \mu M$ melphalan in the absence of IL4) B-CLL cells exposed to IL4, were significantly sensitised to the cytotoxic effects of melphalan. The cytotoxic effect of melphalan against normal B-lymphocytes and against melphalan sensitive B-CLL cells was, however, not significantly altered. These findings are illustrated in Figure 9-3 at an IL4 concentration of 2 u/ml. Addition of increasing doses of IL4 up to 50 u/ml resulted in qualitatively similar findings, i.e. be specific sensitisation to the cytotoxic effects of melphalan confined to alkylator resistant cells. The enhancement of the cytotoxic effects of melphalan increased from 50 % at IL4 concentration of 2 u/ml to 80 % at IL4 concentration of 50 u/ml.

The percentage of interstrand cross-linked DNA (Ct %) following melphalan exposure was significantly higher at all times tested in IL4 treated alkylator resistant B-CLL cells than in cells not exposed to IL4 (Figure 9-4 & 9-5).

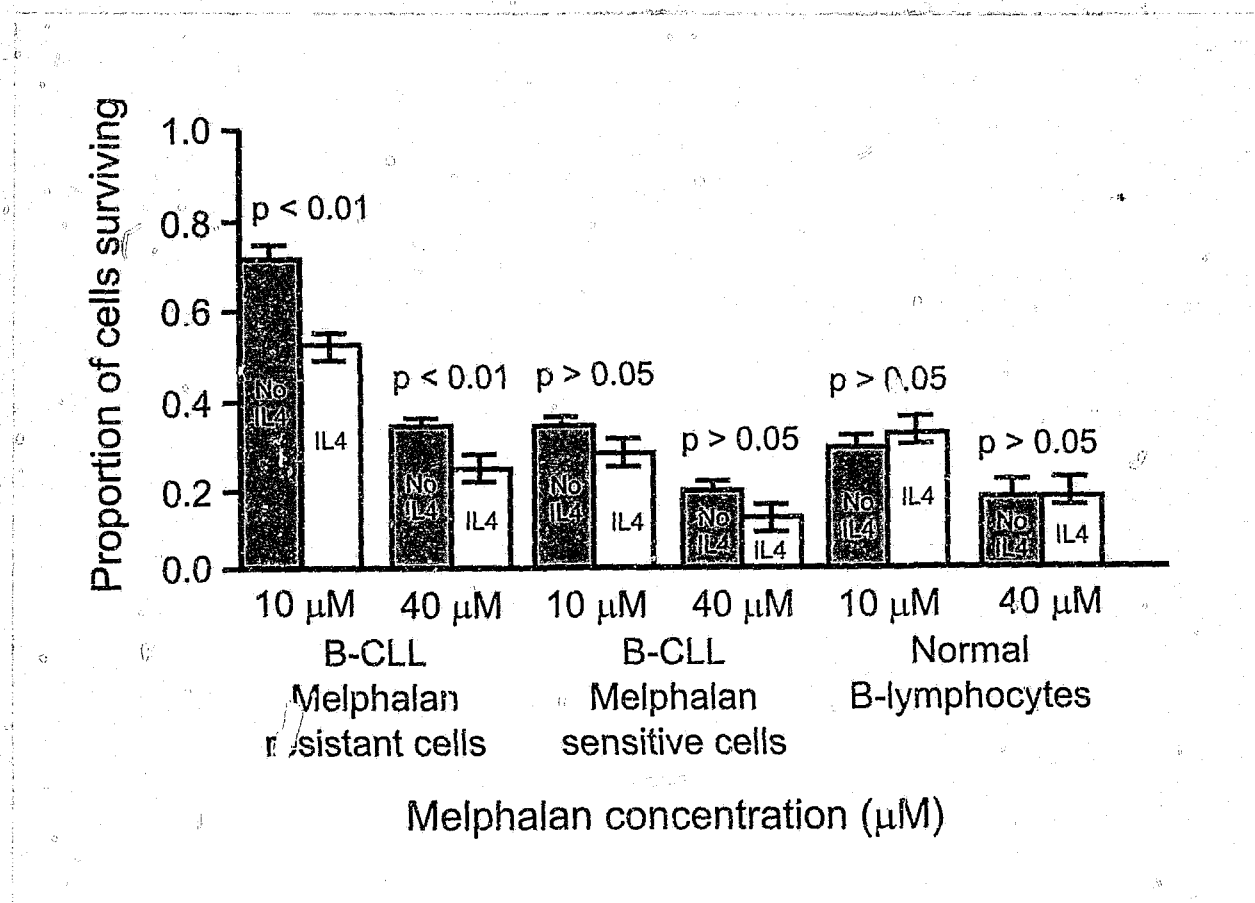


Figure 9-3: In-vitro cytotoxicity of melphalan against B-CLL cells and against normal B-lymphocytes in the presence and absence of (2 u/ml) IL4. Cells were pre-incubated for 24 hours in the presence or absence of IL4. Cytotoxicity was assessed by MTT assay after short term (60 min) exposure to melphalan.

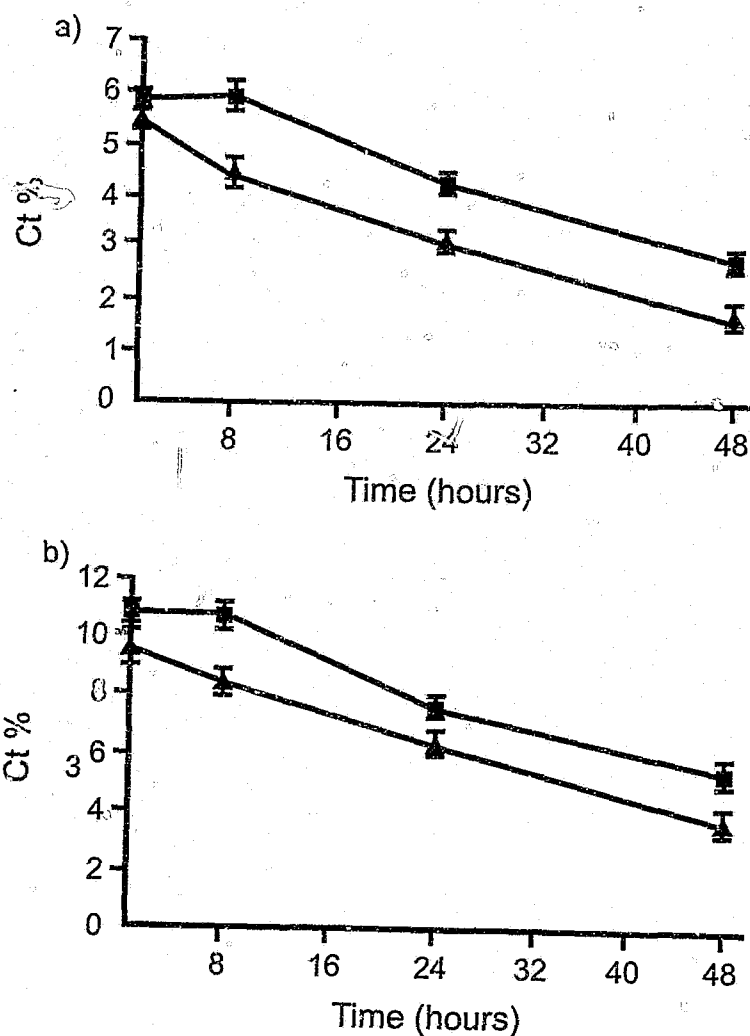


Figure 9-4: Percentage interstrand DNA-cross-links (Ct %) following short term (60 min) in-vitro exposure to melphalan of alkylator resistant B-CLL cells; with IL4 (■) and without IL4 (▲). Ct % was assessed at intervals for up to 48 hours following melphalan exposure. a) 10 μM melphalan; b) 40 μM melphalan.

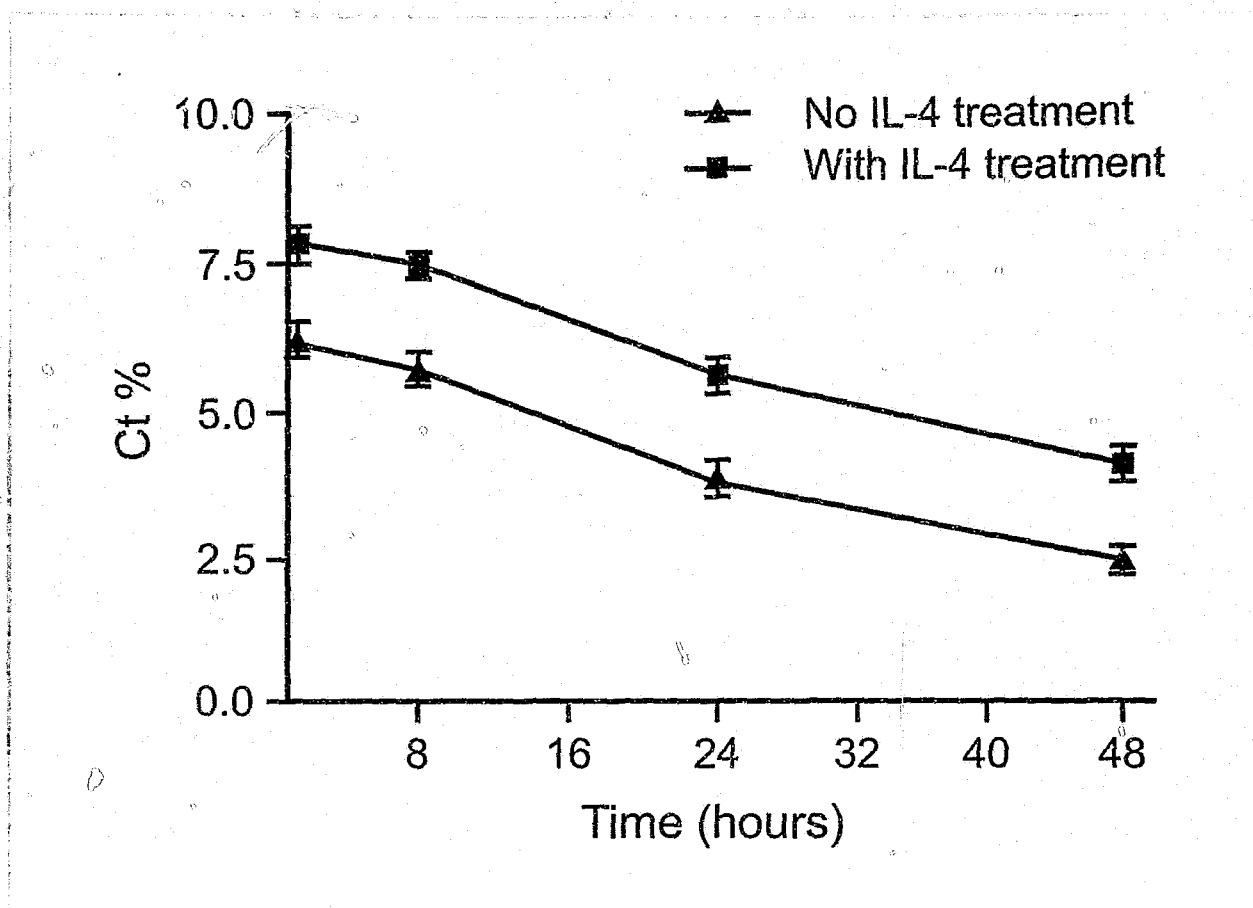


Figure 9-5: Percentage interstrand-DNA-cross-links (Ct %) following 4 hours in-vitro exposure to melphalan (10 μ M) of alkylator resistant B-CLL cells with IL4 (■) and without (▲). Ct % was assessed at intervals for up to 48 hours following 10 μ M melphalan exposure.

9.4 Discussion:

IL4 is a T-cell derived multifunctional cytokine, originally described as B-cell stimulatory factor, for normal preactivated human and mouse B-cells (De France et al, 1987). While IL4 has been shown to be required for maintenance of B-cell cells in-vitro, it is also a potent antiproliferative agent for B-CLL cells blocking the proliferative response of the leukemic B-cells to stimuli such as recombinant tumour necrosis factor α (TNF α), interleukin 2 (IL2) and interferon α (IFN- α) (Luo et al, 1991). The discrepancy between the prolonged survival of B-CLL cell in-vivo and their rapid death in-vitro suggests that humoral or cellular factors protect these cells in-vivo and lack of these factors in-vitro allows apoptotic cell death.

Both IL4 and phorbol myristate acetate (PMA) have been shown to be able to maintain bcl-2 protein levels in B-CLL cells (Panayiotidis et al, 1993). In the absence of these maintenance factors the levels of intra-cellular bcl-2 decline spontaneously in the majority of B-CLL cells in short term in-vitro culture. The maintenance of bcl-2 protein levels has been linked to failure of these cells to undergo apoptosis, although the exact mechanism by which bcl-2 prevents cell death is not clear. So far, the only agent described as

being capable of preventing apoptosis in both normal and leukemic B-cells, is PMA, an activator of protein kinase C (PKC) (*McConkey et al, 1991*). Whether the same mechanism is involved in IL4 stimulated cell maintenance is not, however, clear. The present study confirms previous reports that IL4 inhibits apoptotic cell death of B-CLL during in-vitro culture (*Manion-Fowler et al, 1993; Panayiotides et al, 1993; Craig et al, 1993; Hockenbery et al, 1990*). In addition, we found that IL4 can also inhibit apoptosis of normal B-lymphocytes from healthy volunteers.

Apart from the anti-apoptotic effect, bcl-2 expression has, in a number of instances, been shown to be associated with resistance of cells to the cytotoxic effects of a variety of chemotherapeutic agents (*Miyashita and Reed, 1993; Walton et al, 1993; Lotem and Sacks, 1993*). Bcl-2 expression has indeed been thought to provide another cellular mechanism for multidrug resistance apart from mdr1 and/or mdr2 expression (*Dive et al, 1992; Miyashita and Reed, 1992*). However, this study demonstrates that IL4 can enhance the cytotoxic activity of melphalan in B-CLL cells and does not have same effect on normal B-lymphocytes. This increased sensitivity to the cytotoxic effects of melphalan was associated with increased interstrand DNA cross-linking, the common mechanism of

alkylator induced cytotoxicity, which results in inhibition of normal DNA replication and transcription and ultimately in cell death (*Kohn et al, 1981; Zwelling et al, 1981; Hansson et.al, 1987*). These findings suggest that IL4 somehow increases the number of sites on DNA available for alkylation, possibly by altering DNA topology.

CHAPTER 10

Summary:

The investigations which make up the body of this thesis touch on various aspects of the alkylator resistance. Although many of the factors governing alkylator resistance have been elucidated specific problems remain to be defined in greater detail. The specific studies include:

The median IC_{50} for the B-lymphocyte cell line (WIL2) was $8.57 \pm 1.08 \mu M$. The median IC_{50} for the B-CLL cell line (WSU-CLL) was $223.18 \pm 6.45 \mu M$. WSU-CLL cells were approximately 26-fold resistant to melphalan as compared to WIL2 cells.

The relationship between in-vitro cytotoxicity and melphalan induced interstrand-DNA-cross-linking was studied in melphalan-sensitive WIL2 and in melphalan-resistant WSU-CLL. Interstrand-DNA-cross-link formation, as measured by ethidium bromide fluorescence assay (EFA) showed a highly significant correlation with in-vitro cytotoxicity in the two

cell lines. However, the melphalan concentration at which such interstrand DNA cross-linking occurred differed significantly when WIL2 cells and WSU-CLL cells were compared. The kinetics of interstrand-DNA-cross-link formation and removal following treatment with melphalan also differed significantly, with WSU-CLL cells, showing a much more rapid rate of removal of interstrand DNA cross-links as compared to WIL2 cells. Cell cycle analysis showed that melphalan treatment resulted in the progressive arrest of the WSU-CLL cells in G_1 and G_2 phases. But WIL2 cells failed to enter G_1 or G_2 arrest after melphalan treatment. These findings suggest an increased rate of DNA repair occurring in melphalan-resistant WSU-CLL cells and such repair can be facilitated by alteration of the cell cycle to allow effective DNA repair before the cell enters phases of the cell cycle in which the DNA damage will be lethal.

Melphalan transport was measured in WIL2 cells and in WSU-CLL cells. The study demonstrated decreased initial melphalan accumulation in WSU-CLL cells compared with WIL2 cells. The Lineweaver-Burk plots of the rate of initial melphalan uptake showed the follow kinetic parameters: WIL2 cells $K_m = 26.72\mu M$, the $V_{max} = 431.03 \text{ pmol}/10^6 \text{ cell/min}$;

WSU-CLL cells $K_m = 25.63 \mu\text{M}$, the $V_{\text{max}} = 123.61 \text{ pmol}/10^6 \text{ cell}/\text{min}$. There was an approximately 3.5-fold decrease of V_{max} in WSU-CLL cells compared with WIL2 cells. Melphalan transport was inhibited by both 2-amino-bicyclo[2,2,1] heptane-2-carboxylic acid(BCH) and by sodium ion depletion in both cell lines, indicating that both systems of amino acid transport (System L, which is sodium dependent and inhibited by BCH, and ASC, which is sodium dependent and unaffected by BCH) are functional in these two cell lines.

The finding that the melphalan-resistant WSU-CLL cells transport the drug less efficiently indicates that drug transport is a factor in the mechanism of resistance in the cell line. However the previous findings that WSU-CLL cells demonstrated a rapid rate of removal of interstrand DNA-cross-links and an increased rate of DNA repair suggests that altered drug uptake is not a major resistance mechanism in these human lymphoid cells.

Levels of glutathione and its dependent enzyme(GST) in WIL2 cells and WSU-CLL cells were also examined. There was no significant difference between the two cell lines in with regard to either glutathione content or glutathione-S-transferase activity. This finding differed from that reported

from some previous studies. The results demonstrate that glutathione and glutathione-S-transferase level associated drug resistance mechanisms appear to be tumour cell line specific.

Nuclear extracts from WSU-CLL cells possess a dramatic increase in DNA topoisomerase II activity as compared to WIL2 cells. By Western blot analysis the levels of topoisomerase II proteins expressed in WSU-CLL cells were also dramatically increased as compared to WIL2 cells. No difference in topoisomerase I activity or of the level of topoisomerase I protein expression was observed comparing the two cell lines. WSU-CLL cells were 5.24-fold more sensitive than WIL2 cells to the cytotoxic effect of the topoisomerase II inhibitor doxorubicin. The sensitivity to the cytotoxic effects of topoisomerase I inhibitor, camptothecin, did not differ in WSU-CLL and WIL2 cell lines. Pre-incubation with doxorubicin significantly increased melphalan induced interstrand-DNA-crosslink formation and cytotoxicity in WSU-CLL cells as compared to WIL2 cells. The affinity of topoisomerase II for WSU-CLL UV-irradiated-crosslinked DNA was increased 2.84-fold as compared to that of WSU-CLL native DNA. The affinity of topoisomerase II for both UV-irradiated(crosslinked)

and for native DNA was significantly decreased after doxorubicin-pretreatment.

Elevated topoisomerase II activity and the increased affinity of topoisomerase II for crosslinked DNA in melphalan resistant cells appears to contribute to alkylator resistance by changing DNA topology and thereby facilitating DNA repair.

Measurement of DNA polymerase α and DNA polymerase β revealed significant elevations in DNA polymerase α (58.82 ± 3.67 units/mg protein in WSU-CLL cells, as compared to 27.82 ± 4.39 units/mg protein in WIL 2 cells; $p < 0.01$) but not DNA polymerase β (0.82 ± 0.11 units/mg protein in WSU-CLL cells, compared to 0.74 ± 0.09 units/mg protein in WIL2, $p > 0.05$). Aphidicolin did not produce toxicity or demonstrate antineoplastic activity when given alone. The study showed that there was no change of melphalan cytotoxicity in either WIL2 cells or WSU-CLL cells after DNA polymerase α was inhibited using aphidicolin. The failure to increase cytotoxicity of melphalan using this approach suggests that the mechanisms of resistance do not involve DNA polymerase- α .

The effect of IL4 on cell viability, cell growth, apoptotic fraction, melphalan induced cytotoxicity and the degree of interstrand DNA cross-linking after alkylating agent exposure was investigated in peripheral blood B-CLL cells obtained from 10 patients suffering from chronic lymphocytic leukemia as well as in B-lymphocytes from 5 normal individuals. The addition of IL4 to culture medium maintained in-vitro viability and decreased spontaneous in-vitro apoptosis in both B-CLL cells and normal peripheral blood B-lymphocytes. IL4 did not, however, stimulate proliferation of either cell type. IL4 sensitised alkylator resistant B-CLL cells to the cytotoxic effects of melphalan (L-phenylalanine mustard) but had no influence on melphalan induced cytotoxicity against normal B-lymphocytes. The enhanced cytotoxicity against B-CLL cells was accompanied by an increase in the amount of interstrand-DNA-cross-linking in these cells following short term exposure to melphalan, which is the common mechanism of alkylator induced cytotoxicity, resulting in inhibition of normal DNA replication and transcription and ultimately in cell death. These findings suggest that IL4 somehow increases the number of sites on DNA available for alkylation, possibly by altering DNA topology.

Taken together these investigations demonstrated that resistance to alkylating agents is multifactorial. Understanding of these heterogeneous mechanisms of resistance to alkylating agents has been very helpful in developing strategies to overcome resistance. It has also contributed to major understanding of the normal tissue protection against carcinogens and environmental toxin and may be relevant to individual cancer risk and familial cancers. In future profiles of these resistance mechanisms may help select therapy for patients.

REFERENCES

Aabo K., & Walbom-Jorgensen S. Spleen irradiation in chronic lymphocytic leukemia(CLL): Palliation in patients unfit for splenectomy. *Am J Hematol* 19: 177-180,1985.

Abraham E., Prat A.G., Gerweck L., Seneveratne T., Arceci R.J., Kramer R., Guidotti G., & Cintiello H.F. The multidrug resistance(mdr1) gene product functions as an ATP channel. *Proc Natl Acad Sci USA* 90: 312-316, 1993.

Adachi M., Cossman J., Longo D., Croce C.M. & Tsujimoto Y. Variant translocation of the *bcl-2* gene in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 86:2771-2774, 1989.

Agree J.F., Gaver F.A., & Faguet G.B. An antigen common to chronic lymphocytic and hairy-cell leukemia cells not shared by normal lymphocytes nor by other leukemic cells. *Blood* 68: 62-66, 1986.

Ahmad S., Okine L., Le B., Najarian P. & Vistica, D.T. Elevation of glutathione in phenylalanine mustard-resistant murine L1210 leukemia cells. *J Biol Chem* 262: 15048-15053, 1987.

Ali-Osman F., Srivenugopal K., Berger M.S., & Stein D.E. DNA interstrand crosslinking and strand break repair in human glioma cell lines of varying (1,3-bis(2-chlorethyl)-1-nitrosourea) resistance. *Anticancer Res* 10: 677-682, 1990.

Ali-Osman F., Berger M.S., Rajagopal S., Spence A. & Livingston R.B. Topoisomerase II inhibition and altered kinetics of formation and repair of nitrosourea and cisplatin-induced DNA interstrand cross-links and cytotoxicity in human glioblastoma cells. *Cancer Res* 53: 5663-5668, 1993.

Araki H., Ropp P.A., Johnson A.L., Johnston L.H., Morrison A. & Sugino A. DNA polymerase II, the probable homology of mammalian DNA polymerase epsilon, replicates chromosomal DNA in the yeast *Saccharomyces cerevisiae*. *EMBO J* 11: 733-740, 1994.

Arceci R.J., Croop J.M., Horwitz S.B. & Housman D. The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. *Proc Natl Acad Sci USA* 85: 4350-4354, 1988.

Arceci R.J., Stieglitz K. & Bierer B.E. Immunosuppressants FK506 and rapamycin function as reversal agents of the multidrug resistance phenotype. *Blood* 11: 1528-1536, 1992.

Arp E.W., Wolf P.H. & Checkoway H. Leukemia and exposure to benzene and other solvents in the rubber industry. *J Occup Med* 25: 598-602, 1983.

Austin C.A. & Fisher L.M. DNA topoisomerases: enzymes that change the shape of DNA. *Sci Prog* 74: 147-162, 1990.

Austin C.A., Sng J.-H., Patel S. & Fisher L.M. Novel HeLa topoisomerase II is the II β isoform: complete coding sequence and homology with other type II topoisomerases. *Biochim Biophys Acta* 1172: 283-291, 1993.

Baccarani M., Cavo M., Gobbi M., Lauria F. & Tura S. Staging of chronic lymphocytic leukemia. *Blood* 59: 1191-1196, 1982.

Baer M.R., Stein R.S. & Dessypris E.N. Chronic lymphocytic leukemia with hyperleukocytosis. The hyperviscosity syndrome. *Cancer* 56: 2865-2869, 1985.

Bailey H.H., Gipp J.J., Ripple M., Wilding G. & Mulcahy R.T. Increase in gamma-glutamylcysteine synthetase activity and steady-state messenger RNA levels in melphalan-resistance DU-145 human prostate carcinoma cells expressing elevated glutathione levels. *Cancer Res* 52: 5115-5118, 1992.

Bailey H.H., Mulcahy R.T., Tutsch K.D., Arzoomanian R.Z., Alberti D., Tombes M.B., Wilding G., Pomplun M. & Springs D.E. Phase I clinical trial of intravenous L-buthionine sulfoximine and melphalan: an attempt at modulation of glutathione. *J Clin Oncol* 12: 194-205, 1994.

Baldini L., Mozzana R., Cortelezzi A., Neri A., Radaelli F., Cesana B., Maiolo A.T. & Polli E.E. Prognostic significance of immunoglobulin

phenotype in B cell chronic lymphocytic leukemia. *Blood* 65: 340-349, 1985.

Baldini L., Cro L., Calori R., Nobili L., Silvestris I. & Maiolo A.T. Differential expression of very late activation antigen-3(VLA-3)/VLA-4 in B-cell non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia. *Blood* 79:2688-2693, 1992.

Bandini G., Michallet M., Rossi G. & Tura S. Bone marrow transplantation for chronic lymphocytic leukemia. *Bone Marrow Transplant* 7: 251-253, 1991.

Batist G., Torres-Garcia S., Demuys J.M., Greene D., Lehnert S., Rochon M. & Panisci L. Enhanced DNA cross-link removal: The apparent mechanism of resistance in a clinically relevant melphalan-resistant human breast cancer cell line. *Mol Pharmacol* 36: 224-230, 1989.

Beaume A., Brizard A., Dreyfus B. & Preud'homme J.L. High incidence of serum monoclonal Igs detected by a sensitive immunoblotting technique in B-cell chronic lymphocytic leukemia. *Blood* 84:1216-1219, 1994.

Beck W.T., Cirtain M.C., Glover C.J., Felsted R.L. & Safa A.R. Effects of indole alkaloids on multidrug resistance and labelling of P-glycoprotein by a photoaffinity analog of vinblastine. *Biochem Biophys Res Commun* **153**: 959-966, 1988.

Beck W.T., Danks M.K., Wolverton J.S., Kim R. & Chen M. Drug resistance associated altered DNA topoisomerase II. *Adv Enzyme Regul* **33**: 113-127, 1993.

Bedford P., Fichtinger-Schepman A.M.J. & Hill B.T. Differential repair of platinum-DNA adducts in human bladder and testicular tumor continuous cell lines. *Cancer Res* **48**: 3019-3024, 1988.

Begleiter A., Lam H.Y., Grover J., Froese E. & Goldenberg G.J. Evidence for active transport of melphalan by two amino acid carriers in L5178Y lymphoblasts in vitro. *Cancer Res* **39**: 353-359, 1979.

Begleiter A., Grover J., Froese E. & Goldenberg G.J. Membrane transport, sulfhydryl levels and DNA crosslinking in Chinese hamster ovary cell mutants sensitive and resistant to melphalan. *Biochem Pharmacol* **32**: 293-300, 1983.

Bellamy W.T., Dalton W.S., Gleason M.C., Grogan T.M., & Trent J.M.
Development and characterization of a melphalan resistant human multiple
myeloma cell line. *Cancer Res* 51: 995-1000, 1991.

Bennett J.M., Catovsky D., Daniel M.T., Flandrin G., Galton D.A.,
Gralnick H.R. & Sultan C. Proposals for the classification of
chronic(mature) B and T lymphoid neoplasms. *J Clin Pathol* 42: 567-
584, 1989.

Bernstein Z.P., Fitzpatrick J.E., O'Donnell A., Han T., Foon K.A. &
Bhargava A. Clinical significance of monoclonal proteins in chronic
lymphocytic leukemia. *Leukemia* 6: 1243-1245, 1992.

Bezwoda W.R. & Pu Q.Q. In-vitro assessment of melphalan induced
interstrand-DNA cross-links in lymphocytes provides a rapid method for
assessment of alkylator sensitivity in patients with lymphoproliferative
disorders. *Int J Oncol* 10: 1061-1068, 1997.

Binet J.L., Auquier A., Digihiero G., Chastang C., Piguet H., Goasguen J.,
Vaugier G., Potron G., Colona P., Oberling F., Thomas M., Tchernia G.,
Jacquillat C., Boivin P., Lesty C., Duault M.T., Monconduit M., Belabbes

S. & Gremy F. A new prognostic classification of CLL derived from multivariate survival analysis. *Cancer* **48**: 198-207, 1981.

Blair A. & White D. Leukemia cell types and agricultural practices in Nebraska. *Arch Environ Health* **40**: 211-214, 1985.

Blattner W.A., Dean J.H. & Fraumeni J.F. Familial lymphoproliferative malignancy: Clinical and laboratory follow-up. *Ann Intern Med* **90**:943-944 1979.

Blattner W.A., Saxinger C. & Clark J. Human T-cell leukaemia/lymphoma virus-associated lymphoreticular neoplasia in Jamaica. *Lancet* **2**: 61-64, 1981.

Bodell W., Tokuda K. & Ludlum D. Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with N-(2-chloroethyl)-N-nitrosourea. *Cancer Res* **48**: 4489-4492, 1988.

Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P & Loor F. In vivo circumvention of P-glycoprotein-mediated multidrug resistance of tumour cells with SDZ PSC 833. *Cancer Res* **51**: 4226-4233, 1991.

Borche L., Lim A., Binet J.L. & Dighiero G. Evidence that chronic lymphocytic leukemia B lymphocytes are frequently committed to production of natural antibodies. *Blood* **76**: 562-569, 1990.

Bosch F., Jares P., Campo E., Lopez-Guillermo A., Piris M.A., Villamor N., Tassies D., Jaffe E.S., Montserrat E. & Rozman C. PRAD-1/Cyclin D1 gene overexpression in chronic lymphoproliferative disorders: A highly specific marker of mantle-cell lymphoma. *Blood* 84: 2726-2732, 1994.

Boughton B.J., Jackson N., Lim S. & Smith N. Randomized trial of intravenous immunoglobulin prophylaxis for patients with chronic lymphocytic leukemia and secondary hypogammaglobulinaemia. *Clin Lab Haematol* 17: 75-80, 1995.

Boumsell L., Bernard A. & Lepage V. Some chronic lymphocytic leukemia cells bearing surface immunoglobulins share determinants with T cells. *Eur J Immunol* 8: 900-904, 1978.

Boyum A. Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest* 21 (Suppl 97): 77-84, 1968.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.

Brent T.P., Houghton P.J. & Houghton J.A. O-6-alkylguanine-DNA alkyltransferase activity correlates with the therapeutic response of human rhabdomyosarcoma xenografts to 1-(2-chlorethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea. *Proc Natl Acad Sci USA* 82: 2985-2989, 1985

Brincker H. Population-based age and sex-specific incidence rates in the 4 main types of leukaemia. *Scand J Haematol* 29: 241-249, 1982.

Briz M., Cabrera R., Sanjuán I., Fores R., Díez J.L., Herrero M., Regidor C., Algora M. & Fernandez M.N. Diagnosis of transfusion-associated graft-versus-host disease by polymerase chain reaction in fludarabine-treated B-chronic lymphocytic leukemia. *Br J Haematol* 91: 409-411, 1995.

Brok-Simoni F., Rechavi G., Katzir N. & Ben-Bassat I. Chronic lymphocytic leukemia in twin sisters: Monozygous but not identical(Letter). *Lancet* 1: 329-330, 1987.

Bröker B.M., Klajman A. & Youinou P. Chronic lymphocytic leukemic(CLL) cells secrete multispecific autoantibodies. *J Autoimmun*

1: 469-481, 1988.

Browett P.J. & Norton J.D. Analysis of ras gene mutation and methylation state in human leukemias. *Oncogene* 4: 1029-1036, 1989.

Brown A.G., Ross F.M., Dunne E.M., Steel C.M. & Weir-Thompson E.M. Evidence for a new suppressor locus (DBM) in human B-cell neoplasia telomeric to the retinoblastoma gene. *Nature Genetics* 3: 67-72, 1993.

Brugiatelli M., Jaksic B., Planic-Peraica A., Kusec R., Ostojic S., Callea V., Iacopino P., Morabito F., Stelitano C. & Lutz D. Treatment of chronic lymphocytic leukemia in early and stable phase of the disease: Long-term results of a randomized trial. *Eur J Haematol* 55: 158-163, 1995.

Buller A.L., Clapper M.L. & Tew K.D. Glutathione-S-transferase in nitrogen mustard-resistant and -sensitive cell lines. *Mol Pharmacol* 31: 575-578, 1987.

Bungo M., Fujiwara Y., Kasahara K., Nakagawa K., Ohe Y., Irino S. & Saijo N. Decreased accumulation as a mechanism of resistance to cis-diamminedichloroplatinum (II) in human non-small cell lung cancer

cell lines: Relation to DNA damage and repair. *Cancer Res* 50: 2549-2553, 1990.

Burmeister L.F., Van Lier S.F. & Isacson P. Leukemia and farm practices in Iowa. *Am J Epidemiol* 115: 720-728, 1982.

Buschle M., Campana D., Carding S.R., Richard C., Hoffbrand A.V. & Brenner M.K. Interferon γ inhibits apoptotic cell death in B-cell chronic lymphocytic leukemia. *J Exp Med* 177: 213-218, 1993.

Busse P.M., Bose S.K., Jones R.W. & Tolmach L. J. The action of caffeine on X-irradiated HeLa cells. III. Enhancement of X-ray-induced killing during G₂ arrest. *Radiat Res* 76: 292-307, 1978.

Butturini A. & Gale R.P. Oncogenes in chronic lymphocytic leukemia. *Leuk Res* 12: 89-92, 1988.

Calcutt G. & Connors T.A. Tumor sulfhydryl levels and sensitivity to the nitrogen mustard merophan. *Biochem Pharmacol* 12: 839-843, 1963.

Caligaris-Cappio F., Gobbi M., Bofill M. & Janossy G. Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukemia.

J Exp Med 155: 623-628, 1982.

Caligaris-Cappio F., Janossy G. & Campana D. Lineage relationship of chronic lymphocytic leukaemia and hair cell leukaemia: studies with TPA.

Leuk Res 8: 567-578, 1984.

Caligaris-Cappio F., Pizzolo G., Chilosi M., Bergui L., Semenzato G., Tesio L., Morittu L., Malavasi F., Gobbi M. & Schwarting R. Phorbol ester induces abnormal chronic lymphocytic leukemia cells to express features of hairy cell leukemia. *Blood* 66: 1035-1042, 1985a.

Caligaris-Cappio F. & Janossy G. Surface markers in chronic lymphoid leukemias of B cell type. *Semin Hematol* 22: 1-12, 1985b.

Caligaris-Cappio F., Gottardi D., Alfarano A., Stacchini A., Gregoret M.G., Ghia P., Bertero M.T., Novarino A. & Bergui L. The nature of the B-lymphocyte in B-chronic lymphocytic leukemia. *Blood Cells* 19: 601-613, 1993.

Call T.G., Phylly R.L., Noel P., Habermann T.M., Beard C.M., O'Fallon W.M. & Kurland L.T. Incidence of chronic lymphocytic leukemia in Olmstead county, Minnesota, 1935 through 1989, with emphasis on changes in initial stage at diagnosis. *Mayo Clin Proc* 69: 323-328, 1994.

Campana D., Constan-Smith E., Manabe A., Buschle M., Raimondi S.C., Behm F.G., Ashmun R., Arico M., Biondi A. & Pui, C-H. Prolonged survival of B-lineage acute lymphoblastic leukemia cells is accompanied by overexpression of bcl-2 protein. *Blood* 81: 1025-1031, 1992.

Cancer Statistic Review, 1983-1987. NIH publication no. 90-2798, Bethesda, MD National Cancer Institute, Division of Cancer Prevention and Control Surveillance Program, 1987

Capranico G. & Zunino F. DNA topoisomerase-trapping antitumour drugs. *Eur J Cancer* 28A: 2055-2060, 1992.

Catovsky D., Milani E., Okas A. & Galton D.A.G. Clinical significance of T cells in chronic lymphocytic leukemia. *Lancet* 2: 751-753, 1974.

Catovsky D., Cherchi M. & Okas A. Mouse red-cell rosettes in B lymphoproliferative disorders. *Br J Haematol* **33**: 173-177, 1976.

Catovsky D., Cherchi M. & Brooks D. Heterogeneity of B cell leukemias demonstrated by the monoclonal antibody FMC7. *Blood* **58**: 406-408, 1981.

Catovsky D., Richards S., Fooks J. et al (MRC Working Party on Leukaemia in Adults). CLL trials in the United Kingdom. *Leuk Lymphoma* **5(Suppl)**: 105-112, 1991.

Cesarone C.F., Bolognes C. & Santi L. Improved microfluorometric DNA determination in biologic material using 33258 Hoechst. *Analytic Biochem* **100**: 188-197, 1979.

Chan V.T.W., Ng S.-W., Eder J.P. & Schnipper L.E. Molecular cloning and identification of a point mutation in the topoisomerase II cDNA from an etoposide resistant Chinese hamster ovary cell line. *J Biol Chem* **268**: 2160-2165, 1993.

Chaouchi N., Wallon C., Goujard C., Tertian G., Rudent A., Caput D., Ferrera P., Minty A., Vazquez A. & Delfraissy J.F. Interleukin-13 inhibits interleukin-2 induced proliferation and protects chronic lymphocytic leukemia B cells from in vitro apoptosis. *Blood* **87**:1022-1029, 1996.

Chaney SG & Sancer A. DNA repair: enzymatic mechanisms and relevance to drug response. *J Natl Cancer Inst* **88**: 1346-1360, 1996.

Chapel M.H. Hypogammaglobulinemia and chronic lymphocytic leukemia. In Gale RP & Rai K (eds) *Chronic Lymphocytic Leukemia: Recent Progress and Future Directions*. pp 383-391. New York: Alan R Liss, 1987.

Chapel H., Dicato M., Gamm H., Brennan V., Ries F., Bunch C. & Lee M. Immunoglobulin replacement in patients with chronic lymphocytic leukaemia: A comparison of two dose regimes. *Br J Haematol* **88**: 209-212, 1994.

Chaudhary P.M. & Roninson I.B. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* **66**: 85-94, 1991.

Chaudhary P. M., Mechetner E.B. & Roninson I.B. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral

blood lymphocytes. *Blood* **80**: 2735-2739, 1992.

Cheson B.D., Bennet J.M., Rai K.R., Grever M.R., Kay N.E., Schiffer C.A., Oken M.M., Keating M.J., Boldt D.H. & Kempin S.J. Guidelines for clinical protocol for chronic lymphocytic leukemia: Recommendations of the National Cancer Institute-Sponsored Working Group. *Am J Hematol* **29**: 152-163, 1988.

Cheson B.D. Chronic lymphocytic leukemia and hairy-cell leukemia. *Current Opinion Haematol* **1**: 268-277 & B91-93, 1994

Cheson B.D. Infectious and immunosuppressive complications of purine analog therapy. *J Clin Oncol* **13**: 2431-2448, 1995.

Chikkappa G., Pasquale D., Zarrabi M.H., Weiler R.J., Divakara M. & Tsan M.F. Cyclosporine and prednisone therapy for pure red cell aplasia in patients with chronic lymphocytic leukemia. *Am J Hematol* **41**: 5-12, 1992.

Chin K.V., Pastan I. & Gottesman M.M. Function and regulation of the human multidrug resistance gene. *Adv Cancer Res* **60**: 157-180, 1993.

Chiorazzi N., Fu S.M., Montazeri M., Kunkel H.G., Rai K.R. & Gee T.
T-cell helper defect in patients with chronic lymphocytic leukemia.

J Immunol 122: 1087-1093, 1979.

Chow K.C., Johnson T.L. & Pearson G.D. A novel method for the
detection and quantitation of eukaryotic topoisomerase I. *Biotechniques*
3: 290-296, 1985.

Chung T.D.Y., Drake F.H., Tan K.B., Per S.R., Crooke S.T. & Mirabelli
C.K.. Characterization and immunological identification of cDNA clones
encoding two human DNA topoisomerase II isoenzymes. *Proc Natl Acad*
Sci USA 86: 9431-9435, 1989.

Clairmont C.A. & Sweasy J.B. Dominant negative rat DNA polymerase
beta mutants interfere with base excision repair in *Saccharomyces*
cerevisiae. *J Bacteriol* 178: 656-661, 1996.

Collins R.J., Verschuer L.A., Harison B.V., Prentice R.L., Pope J.H. &
Kerr J.F.R. Spontaneous programmed death (apoptosis) of B-chronic
lymphocytic leukemia cells following their culture in-vitro. *Br J*
Haematol 71: 343-350, 1989.

Colvin M., Russo J.E., Hilton J., Dulik D.M. & Fenselau C. Enzymatic mechanisms of resistance to alkylating agents in tumor cells and normal tissues. *Advances in Enzyme Regulation* 27: 211-221, 1988.

Colvin M. & Chabner B.A. Alkylating agents. In *Cancer Chemotherapy: Principles and Practice*, edited by BA Chabner and JM Collins JB pp 276-313, J.B. Lippincott Company: Philadelphia, 1990.

Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia Intravenous immunoglobulin for the prevention of infection in chronic lymphocytic leukemia: A randomized controlled clinical trial. *N Engl J Med* 319: 9902-9907, 1988.

Cordon-Cardo C., O'Brien J.P., Casals D., Rittman-Grauer L., Biedler J.L., Melamed M.R. & Bertino J.R. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* 86: 695-698, 1989.

Cordon-Cardo C., O'Brien J.P., Boccia J., Casals D., Bertino J.R. & Melamed M.R. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 38: 1277-1287, 1990.

Coverley D., Kenny M.K., Lane D.P. & Wood R.D. A role for the human single-stranded DNA binding protein HSSB/RPA in an early stage of nucleotide excision repair. *Nucleic Acids Res* **20**: 3873-3880, 1992.

Cozzarelli N.R. DNA gyrase and the supercoiling of DNA. *Science* **207**: 953-960, 1980.

Craig V.A., Mainou-Fowler T. & Prentice A.G. Effect of Interleukin 4 (IL4) and dexamethasone on apoptosis and *bcl2* expression in B-chronic lymphocytic leukemia (B-CLL) in culture. *Exp Hematol* **21**: 1090a (Abst.), 1993.

Cresson D.H. & Sieagl G.P. Chronic lymphocytic leukemia presenting as an anal mass. *J Clin Gastroenterol* **7**: 83-87, 1985.

Criel A., Wlodarska I., Meeus P., Stul M., Louwagie A., Van Hoof A., Hidajat M., Mecucci C. & Van den Bergh H. Trisomy 12 is uncommon in typical chronic lymphocytic leukemia. *Br J Haematol* **87**: 523-528, 1994.

Croop J.M., Raymond M., Haber D., Devault A., Arceci R.J., Gros P. & Housman D.E. The three mouse multidrug resistance(mdr) genes are expressed in a tissue-specific manner in normal mouse tissues. *Mol Cell*

Biol **9**: 1346-1350, 1989.

Cuttner J. Increased incidence of hematologic malignancies in first-degree relative of patients with chronic lymphocytic leukemia. *Cancer Invest* **10**:103-109, 1992.

Dale IL, Tuffley W, Callaghan R, Holmes JA, Martin K, Luscombe M, Mistry P, Ryder H, Stewart AJ, Charlton P, Twentyman PR & Bevan. Reversal of P-glycoprotein(P-gp)-mediated multidrug resistance by XR9051, a novel diketopiperazine derivative. *Br J Cancer* **78**: 885-892, 1998.

Dalton WS. Is P-glycoprotein a potential target for reversing clinical drug resistance? *Curr Opin Oncol* **6**: 595-600, 1994.

Dameshek W. Chronic lymphocytic leukemia: an accumulative disease of immunologically incompetent lymphocytes. *Blood* **29**: 566-584, 1967.

Danescu M., Rubio-Trujillo M., Biron G., Bron D., Deiespesse G. & Sarfati M. Interleukin 4 protects chronic lymphocytic leukemia B-cells from death by apoptosis and upregulates bcl₂ expression. *J Exp Med* **176**: 1319-1326, 1992.

Dantzig A.H., Fairgrieve M., Slayman C.W. & Adelberg E.A. Isolation and characterization of a CHO amino acid transport mutant resistant to melphalan(L-phenylalanine mustard) Somat. *Cell Mol Genet* **10**: 113-121, 1984.

D'Arpa P., Machlin P.S., Ratrie H. III, Rothfield N.F., Cleveland D.W. & Earnshaw W.C. cDNA cloning of human DNA topoisomerase I: catalytic activity of a 67.7-kDa carboxyl-terminal fragment. *Proc Natl Acad Sci*

USA 85: 2543-2547, 1988.

Davis S.M., Robson C.N., Davis S.L. & Hickson I.D. Nuclear topoisomerase II levels correlate with the sensitivity of mammalian cells to intercalating agents and epipodophyllotoxins. *J Biol Chem* 263: 17724-17729, 1988.

De France T., Vandervliet B., Aubrey J.P., Takebe Y., Arai N., Miyajima A., Yokota T., Lee F., Arai K., De Vries J.E. & Banchereau J. B-cell growth promoting activity of recombinant human interleukin 4. *J Immunol* 139: 1135-1141, 1987.

De Jong S., Zijlstra J.G., Timmer-Bosscha H., Mulder N.H. & DeVries E.G.E. Detection of DNA cross-links in tumor cells with the ethidium bromide fluorescence assay. *Int J Cancer* 37: 557-561, 1986.

De Jong S., Zijlstra J.G., Mulder N.H. & DeVries E.G.E. Lack of cross resistance to fostreicin in a human small cell carcinoma cell line showing topoisomerase II-related drug resistance. *Cancer Chemother. Pharmacol* 28: 461-464, 1991a.

De Jong S., Zijlstra J.G., Timmer-Bosscha H., Mulder N.H. & De Vries E.G.E. Detection of DNA cross-link in tumor cells with the ethidium bromide fluorescence assay. *Int J Cancer* 37: 557-561, 1991b.

De Jong S., Timmer-Bosscha H., DeVries E. & Mulder, N.H. Effect of novobiocin on cisplatin cytotoxicity and DNA interstrand crosslink formation in a cisplatin resistant small cell lung carcinoma cell line. *Int J Cancer* 53: 110-117, 1993.

Delia D., Cattoretti G., Polli N., Fontanella E., Aiello A., Giardini R., Rilke F. & Della-Porta G. CD 1c but neither CD1a nor CD 1b are expressed on normal activated and malignant human B cells: identification of a new B cell subset. *Blood* 72: 241-247, 1988.

Delannoy A., Martiat P., Gala J.L., Deneys V., Ferrant A., Bosly A., Schieff J.M. & Michaux J.L. 2-chlorodeoxyadenosine (CdA) for patients with previously untreated chronic lymphocytic leukemia (CLL). *Leukemia* 9: 1130-1135, 1995.

Dethmers J.K. & Meister A. Glutathione export by human lymphoid cells: depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation. *Proc Natl Acad Sci USA*

78: 7492-7496, 1981.

Deuchars K.L. & Ling V. P-glycoprotein: multidrug resistance in cancer chemotherapy. *Semin Oncol* 16: 156-165, 1989.

DeVita V.T., Hellman S. & Rosenberg S.A. Cancer: Principles and Practice of Oncology, Ed. 4 Philadelphia: pp 401-409, J.B. Lippincott Company, 1993.

Di Celle P.F., Carbone A., Marchis D., Zhou D., Sozzani S., Zupo S., Pini M., Mantovani A. & Foa R. Cytokine gene expression in B-cell chronic lymphocytic leukemia: Evidence of constitutive interleukin-8(IL-8) mRNA expression and secretion of biologically active IL-8 protein. *Blood* 84:220-228, 1994.

Dickler H.B. & Kunkel H.G. Interaction of aggregated gammaglobulins with B lymphocytes. *J Exp Med* 136: 191-200, 1972.

Dighiero G., Follmer J.Y., Roisin J.P., Ternynck T. & Binet J.L. Comparison of normal and chronic lymphocytic leukemia lymphocyte surface Ig determinants in atypical lymphocytic leukemia. *Blood* 48: 559-567, 1976.

Dighiero G., Bodega E., Mayzner R. & Binet J.L. Individual cell-by-cell quantitation of lymphocyte surface membrane Ig in normal and CLL lymphocytes and during ontogeny of mouse B lymphocytes by immunoperoxidase assay. *Blood* 55: 93-100, 1980.

Dighiero G. Relevance of murine models in elucidating the origin of B-CLL lymphocytes and related immune-associated phenomena. *Semin Hematol* 24: 240-251, 1987.

Dighiero G. An attempt to explain disordered immunity and hypogammaglobulinemia in CLL. *Nouv Rev Fr Hematol* 30: 283-288, 1988.

Dighiero G., Hart S., Faur A., Borche L. & Binet J.L. Evidence that B-cell malignancies frequently involve autoreactive B-cells. *Blood* 76(supple): 346(abst.), 1990.

Dighiero G., Travade P.H., Chevret S., Fenaux P., Chastang C. & Binet J.L. (French Cooperative Group on CLL). B-cell chronic lymphocytic leukemia: Present status and future directions. *Blood* 78: 1901-1914, 1991.

Dillman R.D., Mick R. & McIntyre O.R. Pentostatin in chronic lymphocytic leukemia: A phase II trial of Cancer and Leukemia Group B. *J Clin Oncol* 7: 433-438, 1989.

Di Raimondo F., Giustolisi R., Cacciola E., O'Brien S., Kantarjian H., Robertson L.B. & Keating M.J. Autoimmune hemolytic anemia in chronic lymphocytic leukemia patients treated with fludarabine. *Leuk Lymphoma* 11: 63-68, 1993.

Dive C., Evans C.A. & Whetton A.D. Induction of apoptosis-new targets for cancer chemotherapy. *Semin Cancer Biol* 3: 417-427, 1992.

Dolan M.E., Moschel R.C. & Pegg A.E. Depletion of mammalian O-6-alkylguanine-DNA alkyltransferase activity by O-6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc Natl Acad Sci USA* 87: 5368-5372, 1990.

Drach D., Zhao S., Drach J., Mahadevia R., Gattlinger C., Huber H. & Andreeff M. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* 80: 2729-2734, 1992.

Drake F.H., Zimmerman J.P., McCabe F.L., Bartus H.F., Per S.R., Sullivan D.M., Ross W.E., Matteern M.R., Johnson R.K., Crooke S.T. & Mirabelli C.K. Purification of topoisomerase II from amsacrine-resistant P388 leukemia cells: Evidence for two forms of the enzyme. *J Biol Chem* **34**:16739-16747, 1987.

Drake F.H., Hofmann G.A., Bartus H.F., Mattern M.R., Crooke S.T. & Mirabelli C.K. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28**: 8154-8160, 1989.

Dresler S.L. & Frattini M.K. DNA replication and UV-induced DNA repair synthesis in human fibroblasts are much less sensitive than DNA polymerase α to inhibition by butylphenyl-deoxyguanosine triphosphate. *Nucleic Acids Res* **14**: 7093-7102, 1986.

Durkin W.J., Ghanta V.K., Balch C.M., Davis D.W. & Hiramoto R.N. A methodological approach to the prediction of anticancer drug effect in humans. *Cancer Res* **39**: 402-407, 1979.

Dyer M.J.S., Zani V.J. & Lu W.Z. *BCL-2* translocations in leukemias of mature B cells. *Blood* **83**:3682-3688, 1994.

Earnshaw W.C., Halligan B.C., Crooke C.A., Heck M.M.S. & Liu L.F.

Topoisomerase II is a structural component of the mitotic chromosome scaffold. *J Cell Biol* 100: 1706-1715, 1985.

Eastman A. & Schulte N. Enhanced DNA repair as a mechanism of resistance to *cis*-diamminedichloroplatinum (II). *Biochemistry* 27: 4730-4734, 1988.

Eder J.P., Teicher B.A., Holden S.A., Cathcart K.N. & Schnipper L.E. Novobiocin enhances alkylating agent cytotoxicity and DNA interstrand crosslinks in a murine model. *J Clin Invest* 79: 1524-1528, 1987.

Eder J.P., Teicher B.A., Holden S.A., Cathcart K.N.S., Schnipper L.E. & Frei E. III. Effect of novobiocin on the antitumor activity and bone marrow survivals of three alkylating agents. *Cancer Res* 49: 595-598, 1989.

Eder J.P., Wheeler C.A., Teicher B.A. & Schnipper L.E. A phase I clinical trial of novobiocin, a modulator of alkylating agent cytotoxicity. *Cancer Res* 51: 510-513, 1991.

Eder J.P., Chan V.T.W., Niemerko E., Teicher B.A. & Schnipper L.E. Conditional expression of a wild type topoisomerase II complements a

mutant enzyme in mammalian cells. *J Biol Chem* 268: 13844-13849, 1993.

Eder J.P., Chan V.T-W., Ng S-W., Rizvi N.A., Zacharoulis S., Teicher B.A. & Schnipper L.E. DNA Topoisomerase II α Expression is associated with alkylating agent resistance. *Cancer Res* 55: 6109-6116, 1995.

El-Deiry W.S., Harper J.W., O'Connor P.M., Velculescu V.E., Carman C.E., Jackman J., Pierenpol J.A., Burrell M., Hill D.E., Wang Y., Wimar K.G., Mercer W.E., Kastan M.B., Kohn K.W., Elledge S.J., Kinzler K.W. & Vogelstein B. WAF1/CIP1 is induced in p53-mediated G₁ arrest and apoptosis. *Cancer Res* 54: 1169-1174, 1994.

Ellims P.H., Gan T.E. & van der Weyden M.B. Thymidine kinase isoenzymes in chronic lymphocytic leukaemia. *Br J Haematol* 49: 479-485, 1981.

El-Rouby S., Thomas A., Costin D., Rosenberg C.R., Potmesil M., Silber R. & Newcomb E.W. p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood* 82: 3452-3459, 1993.

Endicott J.A. & Ling V. The biochemistry of P-glycoprotein mediated multidrug resistance. *Annu Rev Biochem* 58: 136-171, 1989.

Endresen L. & Ruscstad H.E. Protective function of metallothionein against certain anticancer agents. *Experientia Suppl* 52: 595-602, 1987.

Epand R.F., Epand R.M., Gupta R.S. & Cragoe E.J.J. Reversal of intrinsic multidrug resistance in Chinese hamster ovary cells by amiloride analogs. *Br J Cancer* 44: 4303-4307, 1984.

Erickson L.C., Bradley M.O. & Kohn K.W. Strand breaks in DNA from normal and transformed human cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea. *Cancer Res* 37: 3744-3750, 1977.

Erickson L.C., Laurent G., Sharkey N.A. & Kohn K.W. DNA cross-linking and monoadduct repair in nitrosourea-treated human tumor cells. *Nature* 288: 727-729, 1980.

Erickson L.C., Zwelling L.A., Ducore J.M., Sharkey N.A. & Kohn K.W. Differential cytotoxicity and DNA cross-linking in normal and transformed human fibroblasts treated with *cis*-diamminedichloroplatinum(II). *Cancer Res* 41: 2791-2794, 1981.

Erickson L.C., Bradley M.O. & Kohn K.W. Strand breaks in DNA from normal and transformed human cells treated with 1, 3-bis (2-chloroethyl)-1-nitrosourea. *Cancer Res* 37: 3744-3750, 1977.

Erikson J., Finan J., Tsujimoto Y., Nowell P.C. & Croce C.M. The chromosome 14 breakpoint in neoplastic B cells with the t(11; 14) translocation involves the immunoglobulin heavy chain locus. *Proc Natl Acad Sci USA* 81: 4144-4151, 1984.

Escudier S.M., Ferreira-Leahy J.M., Drach J.W., Weier H.U., Goodacre A.M., Cork M.A., Trujillo J.M. Keating M.J. & Andreeff M. Fluorescent in situ hybridization and cytogenetic studies of trisomy 12 in chronic lymphocytic leukemia. *Blood* 81: 2702-2707, 1993.

Eskeland T., Klein E., Inoue M. & Johansson B. Characterization of immunoglobulin structures from the surface of chronic lymphocytic leukemia cells. *J Exp Med* 134: 265-277, 1971.

Ewig R.A.G. & Kohn K.W. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis (2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res* 37: 2114-2122, 1977.

Ewig R.A.G. & Kohn K.W. DNA-protein crosslinking and DNA interstrand crosslinking by haloethylnitrosoureas in L1210 cells. *Cancer Res* 38: 3197-3203, 1978.

Ewig R.A.G. & Kohn K.W. DNA damage and repair in mouse leukemia L1210 cell treated with nitrogen mustard, 1, 3 bis (2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res* 37: 2114-2122, 1977.

Faguet G.B., Agee J.F. & Marti G.E. Clone emergence and evolution in chronic lymphocytic leukemia: Characterization of clinical, laboratory and immunophenotypic profiles of 25 patients. *Leuk Lymphoma* 6: 345-356, 1992.

Fairley G.H. & Scott R.B. Hypogammaglobulinaemia in chronic lymphocytic leukemia. *Br Med J* 2: 290-294, 1961.

Fanidi A., Harrington E.A. & Evan G.I. Cooperative interaction between *c-myc* and *bcl-2* oncogenes. *Nature* 359: 554-556, 1992.

Feizi T., Wernet P., Kunkel H.G. & Douglas S.D. Lymphocytes forming red cell rosettes in the cold in patients with chronic cold agglutinin disease. *Blood* 42: 753-758, 1983.

Ferry DR, Traunecker H & Kerr DJ. Clinical trails of P-glycoprotein reversal in solid tumours. *Eur J Cancer* 32A: 927-944, 1996.

Fialkow P.J., Najfeld V., Reddy A.L., Singer J. & Steinmann L. Chronic lymphocytic leukemia: Clonal origin in a committed B-lymphocyte progenitor. *Lancet* 2: 444-446, 1978.

Finch S.C. & Linet M.S. Chronic leukemias. *Baillière's Clin Haematol* 5: 27-56, 1992.

Fingert H.J., Chang J.D. & Pardee A.B. Cytotoxic, cell cycle, and chromosomal effects of methylxanthines in human tumor cells treated with alkylating agents. *Cancer Res* 46: 2463-2467, 1986.

Fleming A.F., Yamamoto N., Bhurnurmath S.R., Maharajan R., Schneider J. & Hunsmann G. Antibodies to ATVL(HTLV) in Nigerian blood donors and patients with chronic lymphocytic leukaemia or lymphoma(Letter). *Lancet* 2: 334-335, 1983.

Foa R., Massaia M., Cardona S., Tos A.G., Bianchi A., Attisano C., Guarini A., di Celle P.F. & Fierro M.T. Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: A possible regulatory role of TNF in the progression of the disease. *Blood* 76: 393-400, 1990.

Fojo A.T., Ueda K., Slamon D.J., Poplack D., Gottesman M.M. & Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci USA* **84**: 265-269, 1987.

Foon K.A. & Todd R.F.I. Immunologic classification of leukemia and lymphoma. *Blood* **68**: 1-31, 1986.

Foon K.A., Rai K.R. & Gale R.P. Chronic lymphocytic leukemia: New insights into biology and therapy. *Ann Intern Med* **113**: 525-539, 1990.

Ford J.M., Prozialeck W.C. & Hait W.N. Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. *Mol Pharmacol* **35**: 105-115, 1989.

Formelli F., Supino R., Cleris L. & Mariani M. Verapamil potentiation of doxorubicin resistance development in B16 melanoma cells both in vitro and in vivo. *Br J Cancer* **57**: 343-347, 1988.

Frame J.N., Dahut W.L. & Crowley S. Fludarabine and acute tumor lysis in chronic lymphocytic leukemia. *N Engl J Med* **327**: 1396-1397(Letter), 1992.

Frankfurt O.S. Inhibition of DNA repair and the enhancement of cytotoxicity of alkylating agents. *Int J Cancer* 48: 916-923, 1991.

Freedman A.S., Boyd A.W., Bieber F.R., Daley J., Rosen K., Horowitz J.C., Levy D.N. & Nadler L.M. Normal cellular counterparts of B cell chronic lymphocytic leukemia. *Blood* 70: 418-425, 1987.

Freedman A.S., Freeman G., Whiteman J., Segil J., Daley J. & Nadler L.M. Studies of in vitro activated CD5+ B cells. *Blood* 73: 202-208, 1989.

Frei E., Cucchi C. & Rosowsky A. Alkylating agent resistance: in vitro studies of human cell lines. *Proc Natl Acad Sci USA* 82: 2158-2162, 1985.

French Cooperative Group on Chronic Lymphocytic Leukemia. Effects of chlorambucil and therapeutic decision in initial forms of chronic lymphocytic leukemia(stage A): results of a randomized clinical trial on 612 patients. *Blood* 75: 1414-1421, 1990.

French Cooperative Group on CLL. Comparison of fludarabine(FDB), CAP, and CHOP in previously untreated stage B and C chronic lymphocytic leukemia (CLL): First interim results of a randomized clinical

trial in 247 patients. *Blood* 84(Suppl 1): 461a(Abstr), 1994.

Friedberg E.C. DNA repair. W.H. Freeman and Co. New York., 1985.

Friedberg E.C., Backendorf J., Burke A., Collins L., Grossman L., Hoeijmakers J.H.J., Lehmen A.R., Seeberg E., van der Schans G.P. & van Zeeland A.A. Molecular aspects of DNA repair. *Mutat Res* 184: 67-86, 1987.

Friedman H.S., Skapek S.X., Colvin O.M., Elion G.B., Blum M.R., Savina P.M., Hilton J., Schold S.C., Kurtzberg Jr. J. & Bigner D.D. Melphalan transport, glutathione levels and glutathione-S-transferase activity in human medulloblastoma. *Cancer Res* 48: 5397-5402, 1988.

Friedman H.S., Colvin O.M., Aisaka K., Popp J., Bossen E.H., Reimer K.A., Powell J.B., Hilton J., Gross S.S., Levi R., Bigner D.D. & Griffith O.W. Glutathione protects cardiac and skeletal muscle from cyclophosphamide-induced toxicity. *Cancer Res* 50: 2455-2462, 1990.

Friedman H.S., Colvin O.M., Kaufmann S.H., Ludeman S.M., Bullock N., Bigner D.D. & Griffith O.W. Cyclophosphamide resistance in medulloblastoma. *Cancer Res* 32: 5373-5378, 1992.

Fry A.M., Chresta C.M., Davies S.M., Walker M.C., Harris A.L., Hartley J.A., Masters J.R.W. & Hickson I.D. Relationship between topoisomerase II level and chemosensitivity in human tumour cell lines. *Cancer Res* 51: 6592-6595, 1991.

Fu S.M., Winchester R.J. & Kunkel H.G. Similar idiotype specificity for the membrane IgD and IgM of human B lymphocytes. *J Immunol* 114: 250-258, 1975.

Gaidano G., Newcomb E.W., Gong J.Z., Tassi V., Ner A., Cortelezzi A., Calori R., Baldini L. & Dalla-Favera R. Analysis of alterations of oncogenes and tumor suppressor genes in chronic lymphocytic leukemia. *Am J Pathol* 144:1312-1319, 1994.

Gale R.P. & Foon K.A. Biology of chronic lymphocytic leukemia. *Semin Hematol* 24: 209-229, 1987.

Gale R.P., Caligaris-Cappio F., Dighiero G., Keating M., Montserrat E. & Rai K. (International Workshop on CLL). Recent progress in chronic lymphocytic leukemia. *Leukemia* 8: 1610-1614, 1994.

Gallo R.C., Kalayanaraman V.S. & Sarngadharan M.G. Association of the human type C retrovirus with a subset of adult T-cell cancers. *Cancer Res* 43: 3892-3899, 1983.

Galton D.A.G. The pathogenesis of chronic lymphocytic leukemia. *Can Med Assoc J* 94: 1005-1010, 1966.

Garver F.A., Kiefer C.R. & Moscoso H. Characterization of a human retrovirus from cultured chronic lymphocytic leukemia B-cells. *Blood* 64 (Suppl 1): 202a (abstr), 1984.

Gasser S.M., Laroche T., Falquet J., Boy Dela Tour E. & Laemmli, U.K. Metaphase chromosome structure: involvement of topoisomerase II. *J Mol Biol* 188: 613-629, 1986.

Geisler C., Ralfkiaer E., Hansen M.M., Hou-Jensen K. & Larsen S.O. The bone marrow histological pattern has independent prognostic value in early stage chronic lymphocytic leukemia. *Br J Haematol* 62: 47-54, 1986.

Geisler C., Larsen J.K., Hansen N.E., Hansen M.M. Christensen B.E., Lund B., Nielsen H., Plesner T., Thorling K. & Andersen E. Prognostic importance of flow cytometric immunophenotyping of 540 consecutive

patients with B-cell chronic lymphocytic leukemia. *Blood* 78: 1795-1802, 1991.

Gellert M. DNA topoisomerases. *Annu Rev Biochem* 50: 879-910, 1981.

Georges E., Bradley G., Garipey J. & Ling V. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc Natl Acad Sci USA* 87: 152-156, 1990.

Gills D.R., Hyde S.C., Higgins C.F., Valverde J.A., Mintenig G.M. & Sepulveda F.V. Separation of drug transport and chloride channel functions of the human multidrug resistance P-glycoprotein. *Cell* 71: 23-27, 1992.

Glisson B.S. & Ross W.E. DNA topoisomerase II: a primer on the enzyme and its unique role as a multidrug target in cancer chemotherapy. *Pharmacol Ther* 32: 89-106, 1987.

Gogoi P.K., Stewart I.D., Keane P.F., Scott R., Dunn G.D. & Catovsky D. Chronic lymphocytic leukemia presenting with bilateral breast involvement. *Clin Lab Hematol* 11: 57-60, 1989.

Golden G.J., Lam H.-Y. P. & Begleiter A. Evidence for carrier-mediated transport of melphalan by L5148Y lymphoblasts in vitro. *Cancer Res.*, **37**: 755-760, 1977.

Goldenberg G.J., Vanstone C.L., Israels L.G., Ilse D. & Bihler I. Evidence for a transport carrier of nitrogen mustard in nitrogen mustard-sensitive and resistant L5178Y lymphoblasts. *Cancer Res* **30**: 2285-2291, 1970.

Goldenberg G.J., Vanstone C.L. & Bihler I. Transport of nitrogen mustard on the transport-carrier for choline in L5178Y lymphoblasts. *Science* **172**: 1148-1149, 1971

Goldenberg G.J., Lam H.-Y. P. & Begleiter A. Active carrier-mediated transport of melphalan by two separate amino acid transport systems in LPC-1 plasmacytoma cells in vitro. *J Biol Chem* **254**: 1057-1064, 1979.

Goldstein L.J., Pastan I. & Gottesman M.M. Multidrug resistance in human cancer. *Crit Rev Oncol Hematol* **12**: 243-253, 1992.

Gordon J., Mellstedt H., Aman P. Biberfeld P. & Klein G. Phenotypic modulation of chronic lymphocytic leukemia cells by phorbol ester: induction of IgM secretion and changes in the expression of B-cell

associated surface antigens. *J Immunology* 132: 541-547, 1984.

Goscin L.P. & Byrnes J.J. DNA polymerase delta: one polypeptide, two activities. *Biochemistry* 21: 2513-2518, 1982.

Gottesman M.M. & Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 62: 385-427, 1993.

Gray H. & Wong T.W. Purification and identification of subunit structure of the human mitochondrial DNA polymerase. *J Biol Chem* 267: 5835-5841, 1992.

Green J.A., Vistica D.T., Young R.C., Hamilton T.C., Rogan A.M. & Ozols R.F. Potentiation of melphalan cytotoxicity in human ovarian cancer cell lines by glutathione depletion. *Cancer Res* 44: 5427-5431, 1984.

Grey H.M., Rabelino E. & Pirofsky B. Immunoglobulins on the surface of lymphocytes. IV Distribution of hypogammaglobulinemia cellular immune deficiency and chronic lymphocytic leukemia. *J Clin Invest* 50: 2368-2377

Griffith O.W. Determinations of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**: 207-212, 1980.

Grossbard M.L., Press O.W., Appelbaum F.R., Bernstein I.D. & Nadler L.M. Monoclonal antibody-based therapies for leukemia and lymphoma. *Blood* **80**: 863-878, 1992.

Gross-Bellard M., Oudet P. & Chambon P. Isolation of high-molecular-weight DNA from mammalian cells. *Eur J Biochem* **36**:32-35, 1972.

Gupta V., Singh S.V., Ahmad H., Mwedh R.D. & Awashti Y.C. Glutathione and glutathione-S-transferases in a human plasma cell line resistant to melphalan. *Biochem Pharmacol* **38**: 1993-2000, 1989.

Gurtoo H.L., Hipkens J.H. & Sharma S.D. Role of glutathione in the metabolism-dependent toxicity and chemotherapy of cyclophosphamide. *Cancer Res* **41**: 3584-3591, 1981.

Habig W.H., Pabst M.J. & Jakoby W.B. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130-7139, 1974.

Haluska F.G., Tsujimoto Y. & Croce C.M. Oncogene activation by chromosome translocation in human malignancy. *Ann Rev Gene* 21: 321-345, 1987.

Hamblin T.J., Oscier D.J. & Young B.J. Autoimmunity in chronic lymphocytic leukemia. *J Clin Pathol* 39: 713-719, 1986.

Han T., Barcos M., Wmrich L., Ozer H., Gajera R., Gomez G.A., Reese P.A., Minowada J., Bloom M.L. & Sadamori N. Bone marrow infiltration patterns and their prognostic significance in chronic lymphocytic leukemia: Correlations with clinical, immunologic, phenotypic, and cytogenetic data. *J Clin Oncol* 2: 562-570, 1984.

Hanada M., Delia D., Aiello A., Stadtmauer E. & Reed J.C. *BCL-2* gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood* 82: 3452-3459, 1993.

Hannawalt P.C., Cooper P.K., Ganesan A.K., Lloyd R.S., Smith C.A. & Zolan M.E. Repair responses to DNA damage: Enzymatic pathways in *E. coli* and human cells. *J Cell Biochem* 18: 271-283, 1982.

Hansen M.M., Andersen E. & Birgens H. CHOP versus chlorambucil plus prednisone in chronic lymphocytic leukemia. *Leuk Lymphoma* 5(Suppl): 97-100, 1991.

Hansson J., Lewensohn J., Ringborg U. & Nielsson B. Formation and removal of DNA cross-links induced by melphalan and nitrogen mustard in relation to drug-induced cytotoxicity in human melanoma cells. *Cancer Res* 47: 2631-2637, 1987.

Harker W.G., Slade D.L., Drake F.H. & Parr R.L. Mitoxantrone resistance in HL60 leukemia cells: reduced nuclear topoisomerase II catalytic activity and drug induced DNA cleavage in association with reduced expression of the topoisomerase II beta isoform. *Biochemistry* 30: 9953-9961, 1991.

Harris N.L., Jaffe E.S., Stein H., Banks P.M., Chan J.K., Cleary M.L., Delsol G., De Wolf-Peeters C., Falini B. & Gatter K.C. A revised European-American classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood* 84: 1361-1392, 1994.

Hartwell L.H. & Weinert T.A. Checkpoints: controls that ensure order of cell cycle events. *Science* 246: 629-634, 1989.

Hawthorn L.A., Chapman R., Oscier D. & Cowell J.K. The consistent 13q14 translocation breakpoint seen in chronic B-cell leukemia(BCLL) involves deletion of the D13S25 locus which lies distal to the retinoblastoma predisposition gene. *Oncogene* 8:1415-1419, 1993.

Hayakawa K., Hardy R.R., Herzenberg L.A. & Herzenberg L.A. Progenitors for Ly1-B cells are distinct from progenitors for other B-cells. *J Exp Med* 161: 1554-1568, 1985.

Heck M.M.S., Earnshaw W.C. Topoisomerase II: a specific marker for cell proliferation. *J. Cell Biol.* 103: 2569-2581, 1986.

Heck M.M.S., Hittelman W.N. & Earnshaw W.C. Differential expression of DNA topoisomerases during the eukaryotic cell cycle. *Proc Natl Acad Sci USA* 85:1086-1090, 1987.

Hill A.B., Beck W.T. & Trent J.M. Cytogenetic and molecular characterization of tumors in nude mice derived from a multidrug-resistant human leukemia cell line. *Cancer Res* 48:393-398, 1988.

Hilton J. Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res* 44: 5156-5160, 1984.

Grillot-Courvalin C., Brouet J.C. & Seligmann M.
Her of responsiveness of chronic lymphocytic leukemic B cell
growth factor or interleukin 2. *Eur J Immunol* 16: 1001-1007, 1986.

Hjelle B., Mills R. & Swenson S. Incidence of hairy cell leukemia,
mycosis fungoides and chronic lymphocytic leukemia in first known
HTLV-II endemic population. *J Infect Dis* 163: 435-440, 1991.

Hockenbery D., Nuñez G., Millman C., Schreiber R.D. & Korsmeyer
S.J. *Bcl₂* is an inner mitochondrial membrane protein that blocks
programmed cell death. *Nature* 348: 334-336, 1990.

Hoffbrand A.V., Panayiotidis P., Reittie J. & Ganeshaguru K. Autocrine
and paracrine growth loops in chronic lymphocytic leukemia. *Semin
Hematol* 30: 306-317, 1993.

Hollander A.A.M.J., Kluin-Nelemans H.C., Haak H.R., Stern A.C.,
Willemze R. & Fibbe W.E. Correction of neutropenia associated with
chronic lymphocytic leukemia following treatment with granulocyte-
macrophage colony-stimulating factor. *Ann Hematol* 62: 32-34, 1991.

Hollingsworth R.E. Jr., Hensey C.E. & Lee W.H. Retinoblastoma protein and the cell cycle. *Curr Opin Genet Dev* 3: 55-62, 1993.

Hsiang Y., Wu H.-Y. & Liu L.F. Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* 48:3230-3235, 1988.

Huberman J.A. New views of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase alpha. *Cell* 23: 647-648, 1981.

Hudson L. & Hay C.F. Affinity chromatography of lymphoid cells. In: *Practical Immunology*, Sec. ed. pp 212-213, Blackwell Scientific Publications, Oxford 1980.

Humphries C.G., Shen A., Kuziel W.A., Capra J.D., Blattner F.R. & Tucker P.W. A new human immunoglobulin VH family preferentially rearranged in immature B-cell tumours. *Nature* 331: 446-449, 1988.

Hunting D.J., Gowans B.J. & Dresler S.L. DNA polymerase δ mediates excision repair in growing cells damaged with ultraviolet radiation. *Biochem Cell Biol* 69: 303-308, 1991.

Hyafil F, Vergely C, Du Vignaud P & Grand-Perret T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res* **53**: 4595-4602, 1993.

Ikegami S., Taguchi T., Ohashi M., Oguero M., Nagano H. & Mano Y. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-alpha. *Nature* **275**: 458-460, 1978.

International Workshop on CLL. Chronic lymphocytic leukemia: Proposals for a revised prognostic staging system. *Br J Haematol* **48**: 365-367, 1981.

International Workshop on CLL. Chronic lymphocytic leukemia: Recommendations for diagnosis, staging, and response criteria. *Ann Intern Med* **110**: 236-238, 1989.

Ishikawa M., Takayanagi Y. & Sasaki K. Effect of buthionine sulfoximine, an inhibitor of glutathione biosynthesis, on the selenium-induced lethality in mice. *Jpn J Pharmacol* **48**: 283-286, 1988.

Jahde E., Glusenkamp K.H. & Rajewsky M.F. Protection of cultured malignant cells from mitoxantrone cytotoxicity by low extracellular pH: a possible mechanism for chemoresistance in vivo. *Eur J Cancer* **26**: 101-106, 1990.

Jaksic B. & Brugiatelli M.X. High-dose continuous chlorambucil vs. intermittent chlorambucil plus prednisone for treatment of B-CLL. IGCI CLL-01 trial. *Nouv Rev Fr Hematol* **30**: 437-442, 1988.

Jaksic B., Brugiatelli M. & Krc I. Comparison of high-dose chlorambucil versus Binet's modified CHOP regimen in B-cell chronic lymphocytic leukemia in advanced phase: Results of an international multicentric randomized trial. *Blood* **86**: 607a(Abstr), 1995.

Jenkins J.R., Ayton P., Jones T., Vavis S.L., Simmons D.L., Harris A.L., Sheer D. & Hickson I.D. Isolation of cDNA clones encoding the β isozyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. *Nucleic Acids Res* 5587-5592, 1992.

Johnson S., Smith A.G., Loffler H., Osby E., Juliusson G., Emmerich B., Wyld P.J. & Hiddemann W. Multicentre prospective randomised trial of fludarabine versus cyclophosphamide, doxorubicin, and prednisone (CAP) for treatment of advanced-stage chronic lymphocytic leukemia. French Cooperative Group on CLL *Lancet* **347**: 1432-1438, 1996.

Johnstone A.P. Chronic lymphocytic leukaemia and its relationship to normal B lymphopoiesis. *Immunol Today* **3**: 343-345, 1982.

Juan C.C., Hwang J., Liu A.A., Whang-Peng J., Knutsen T., Huebner K., Croce C.M., Zhang H., Wang J.C. & Liu L.F. Human DNA topoisomerase I is encoded by a single-copy gene that maps to chromosome region 20q12-13.2. *Proc Natl Acad Sci USA* 85: 8910-8913, 1988.

Juliussen G. Immunological and cytogenetic studies improve prognosis prediction in chronic B-lymphocytic leukemia: a multivariate analysis of 24 variables. *Cancer* 58: 688-695, 1986.

Juliussen G., Oscier D.G., Fitchett M., Ross F.M., Stockdill G., Mackie M.J., Parker A.C., Castoldi G.L., Guneo A. & Knuutila S. Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. *N Engl J Med* 323:720-724, 1990.

Juliussen G. & Liliemark J. High complete remission rate from 2-chloro-2-deoxyadenosine in previously treated patients with B-cell chronic lymphocytic leukemia: Response predicted by rapid decrease of blood lymphocyte count. *J Clin Oncol* 11: 679-689, 1993a.

Juliussen G. & Gahrton G. Cytogenetics in CLL and related disorders. *Baillière's Clin Haematol* 6: 821-848, 1993b.

Author Pu Q Q

Name of thesis Mechanisms Of Resistance To The Alkylating Agents In Chronic Lymphocytic Leukaemia Pu Q Q 1999

PUBLISHER:

University of the Witwatersrand, Johannesburg

©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.