



ILKKA KIVEKÄS

Drug Resistance in
Chronic Lymphocytic Leukaemia



ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, referred to in the next by their Roman numerals I–IV:

- I Kivekäs I, Vilpo L, Vilpo J (2002): Relationships of in vitro sensitivities tested with nine drugs and two types of irradiations in chronic lymphocytic leukemia. *Leukemia Research* 26: 1035-1041

- II Kivekäs I, Hulkkonen J, Hurme M, Vilpo L, Vilpo J (2002): CD80 antigen expression as a predictor of ex vivo chemosensitivity in chronic lymphocytic leukemia. *Leukemia Research* 26: 443-446

- III Kivekäs I, Tobin G, Thunberg U, Vilpo L, Sundström C, Rosenquist R, Vilpo J (2003): Ex vivo drug and irradiation sensitivities in hypermutated and unmutated forms of chronic lymphocytic leukemia cells. *Leukemia Research* 27: 337-341.

- IV Kivekäs I, Nagy B, Vilpo L, Knuutila S, Sinisalo M, Lehtimäki T, Vilpo J: Apoptosis-associated genes are not determinants for drug sensitivity in chronic lymphocytic leukemia. Submitted.

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ABBREVIATIONS

Bax	Bcl2-associated x protein
Bcl2	B cell CLL/lymphoma 2
Bcl2a1	Bcl2-related protein A1
Bcl2l1	Bcl2-like L1
BCR	B cell receptor
B2M	beta 2 microglobulin
CDA	chlorodeoxyadenosine
CIS	cisplatin
CLB	chlorambucil
CLL	chronic lymphocytic leukaemia
CD	cluster of differentiation
ID80	inhibition dose 80%
Dapk1	death-associated protein kinase 1
CYA	cyclosporin A
DOX	doxorubicin
DNA-PK	DNA-activated protein kinase
FISH	fluorescence in situ hybridisation
FLU	fludarabine
GAM	gamma-irradiation
IgHV	immunoglobulin heavy-chain variable
Mcl1	myeloid cell leukaemia sequence 1
M-CLL	mutated CLL
Myc	myelocytomatosis viral oncogene homolog

NER	nucleotide excision repair
NHEJ	non-homologous end joining
PCR	polymerase chain reaction
PRE	prednisolon
QRT-PCR	quantitative real-time PCR
TK	thymidine kinase
UM-CLL	unmutated CLL
UV	ultraviolet irradiation
VER	verapamil
VIN	vincristine
WHO	World Health Organization
Zap70	zeta associated protein 70

ABSTRACT

Background: Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia in the Western countries. Typically, it is a slowly progressing disease, and treatment by cytostatics is initiated after follow-up in a situation where the patient has an aggressive disease or develops general symptoms. The major obstacle in treatment is drug resistance and, moreover, multidrug resistance. Extensive research into the mechanisms or prognostic factors for chemo- or irradiation resistance has produced few clinically encouraging results.

Aims: To evaluate (I) multidrug resistance in CLL and to define the impact of (I) previous chemotherapy, (II) surface antigens, (III) the mutation status of the immunoglobulin variable region (IgHV) genes as well as (IV) programmed cell death, apoptosis, associated gene transcripts in drug and irradiation resistance in CLL.

Material and methods: Peripheral blood samples from a cohort of 36 CLL patients were collected and mononuclear cells, containing mainly CLL cells, were isolated. Nine drugs and two types of irradiation were selected according to their usefulness in CLL therapy or on the basis of their otherwise interesting mechanism of action. Doses causing 50 or 80% inhibition of living cells were measured from prednisolon (ID50) and from all other drugs and irradiations (ID80), respectively. In total, 2,376 duplicated cell cultures were performed to determinate these ID80 or ID50 values. Expressions of 34 surface antigens were analysed by means of flow cytometry. PCR assays were used to determine the mutation status of the IgHV genes. Apoptosis-associated gene mRNA expressions (anti-apoptotic: *Bcl2*, *Mcl1*, *Bcl2a1*, *Bcl2l1*; and pro-apoptotic: *Bax*, *Myc*, *Dapk1*) were analysed with QRT-PCR.

Results: Multidrug resistance was more common in the previously treated CLL group than the untreated group, but concordances between drug therapy and in vitro drug resistance were poor. Statistically significant correlation was confirmed between the sensitivities of the same-group drugs chlorodeoxyadenosine and fludarabine. Correlation between two P-glycoprotein-dependent drugs, vincristine and doxorubicin, was not demonstrated (I). There were no correlations between the investigated surface antigens and drug or irradiation sensitivities. The nucleoside analogues' (chlorodeoxyadenosine and fludarabine) responses could be divided in two groups by their CD80 expression; every patient expressing the CD80 surface antigen was sensitive to nucleoside analogues, whereas all resistant cases had low or negative CD80 expression (II). IgHV mutation status divided the CLL patients in two groups, unmutated (67%) and mutated (33%), but statistically significant differences could not be confirmed in the drug or irradiation sensitivities between these groups (III). A higher amount of cell-death-inductive pro-apoptotic gene transcripts did not induce drug or irradiation sensitivity, and a higher amount of cell-death-preventive anti-apoptotic gene transcripts did not induce drug or irradiation resistance (IV).

Conclusion: Chemotherapy induces drug and irradiation resistance. Drug resistance does not always develop against the used chemotherapeutic agent, as chemotherapy may also expose to drug resistance against some other agents. CD80 could be a surrogate chemosensitivity marker for nucleoside analogues. The IgHV genes' mutation status or apoptosis-associated genes are not determinants of drug or irradiation resistance in vitro.

TIIVISTELMÄ

Tausta: Krooninen lymfaattinen leukemia (KLL) on länsimaiden yleisin leukemia. Se on yleensä hitaasti etenevä tauti, ja hoidot solunsalpaajilla aloitetaan vasta seurannan jälkeen, jos tauti muuttuu nopeasti eteneväksi tai aiheuttaa yleisoireita. Lääkeresistenssi muodostuu yleensä hoidon suurimmaksi ongelmaksi, ja usein ilmenee monilääkeresistenssiä. Lääke- ja säderesistenssiä on tutkittu runsaasti, mutta kliinisesti merkittäviä tuloksia niin ennustetekijöistä kuin mekanismeista on vähän.

Tavoite: Tutkia KLL-solujen (I) monilääkeresistenssiä ja arvioida (I) aiemman lääkehoidon, (II) pinta-antigeenien, (III) immunoglobuliinien variaabelialueen (IgHV) geenimutaatioiden ja (IV) ohjelmoituun solukuolemaan, apoptoosiin, liittyvien geenien merkitystä lääkeresistenssin ennustajina KLL:ssa.

Aineisto ja menetelmät: 36 KLL-potilaan verinäytteistä eristettiin mononukleaariset solut, joista suurin osa edusti leukeemista kantaa. Yhdeksän lääkeainetta ja kaksi eri säteilylajia valittiin joko sen vuoksi, että niitä käytetään KLL:n hoidossa kliinisessä työssä, tai niiden mielenkiintoisten vaikutusmekanismien vuoksi. Lääkeaineista ja sädelajeista määritettiin annospitoisuus, jolla saavutetaan 80 %:n vähenemä soluissa (ID80). ID80-arvot määritettiin 2376 rinnakkaisella soluviljelmällä. 34 pinta-antigeenin pitoisuudet määritettiin virtaussytometrialla. IgHV-geenien mutatoitumisaste määritettiin PCR:llä. Apoptoosiin liittyvien geenien (anti-apoptoottiset: *Bcl2*, *Mcl1*, *Bcl2a1*, *Bcl2l1* ja pro-apoptoottiset: *Bax*, *Myc*, *Dapk1*) mRNA-pitoisuudet määritettiin QRT-PCR:llä.

Tulokset: Monilääkeresistenssi oli yleisempää aiemmin hoidetuilta KLL-potilailta otettujen solujen ryhmässä kuin hoitamattomassa ryhmässä. Hoidoissa käytettyjen lääkkeiden ja in vitro -lääkeresistenssin välillä ei näyttänyt olevan merkittävää yhteyttä. Saman lääkeryhmän lääkkeiden, klorodeoksyadenosiinin ja fludarabiinin, herkkyksien välillä nähtiin selvä korrelaatio, mutta P-glykoproteiiniiniriippuvien lääkkeiden, vinkristiinin ja doxorubisiinin, herkkyksien välillä ei näyttänyt olevan korrelaatiota (I). Lääke- ja säderesistenssin ja pinta-antigeenien välillä ei löytynyt tilastollisesti merkittävää riippuvuutta. CD80-pinta-antigeeni jakoi nukleosidianalogiryhmän lääkkeiden (klorodeoksyadenosiinin ja fludarabiinin) vasteet kahteen ryhmään: CD80:n ilmeneminen KLL-soluissa herkisti solut näille lääkkeille, kun taas kaikilla resistenteillä potilailla CD80:n ilmeneminen KLL-soluissa oli heikko tai negatiivinen (II). IgHV-mutatoitumisaste jakoi potilasryhmän kahteen joukkoon: mutatoitumattomiin (67 %) ja mutatoituneisiin (33 %). Näiden ryhmien välillä ei löytynyt tilastollisesti merkittävää eroa lääke- tai sädeherkkyksissä (III). Solukuolemaan johtavien pro-apoptoottisten geenien korkeampi ilmeneminen ei herkistänyt soluja lääkkeille tai sädetykselle. Myöskään solukuolemalta suojaavien anti-apoptoottisten geenien korkeampi ilmeneminen ei johtanut lääke- tai säderesistenssiin (IV).

Päätelmät: Lääkehoito altistaa lääke- ja säderesistenssille. Lääkeresistenssi ei aina kehity hoidossa käytettyä lääkettä kohtaan, vaan lääkehoito voi altistaa lääkeresistenssin kehittymisen myös muita lääkkeitä kohtaan. Nukleosidianalogien lääkevasteita voidaan ennustaa CD80:n ilmenemisen perusteella. IgHV-mutatoitumisasteella ja apoptoosiin liittyvillä geneilla ei näyttäisi olevan yhteyttä lääke- tai säderesistenssiin.

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia in the Western countries. In Finland, roughly 200 new cases are diagnosed yearly (Finnish Cancer Registry 2010). CLL mainly affects the older population – the average age of patients is 65 years at diagnosis. The clinical course of CLL varies to a great extent: some develop an aggressive disease and die within one year of diagnosis, while others survive longer than ten years (Chiorazzi et al. 2005). Therefore, prognostic factors have a significant role in the diagnosis of CLL. The clinical staging systems by Rai and Binet are powerful and easily achieved prognostic methods. The variation in the clinical outcome with these staging systems is still quite extensive, and more exact prognostic factors are needed. Different biological prognostic factors have been found, and the most remarkable of these are chromosomal aberrations (17p deletion and 11q deletion), surface antigens of the leukaemic cells (CD38), the expression of the zap70 protein and the mutation status of immunoglobulin variable (IgHV) region genes (Van Bockstaele et al. 2009).

The development of chemo- or irradiation resistance is another important determinant of patient outcome in CLL (Shanafelt 2009). The mechanisms of drug resistance have been extensively investigated, and in vitro sensitivity studies have been shown to correlate with the clinical outcome of chemotherapy (Bosanquet et al. 2009). However, the clinically encouraging results have been few. The main known resistance mechanisms are based on i) diminished drug concentration in the cell, ii) altered drug effect because of abnormal enzymatic activity, iii) altered drug target enzymes or proteins, iv) enhanced DNA-repairing mechanisms or v) disturbances in leading the cell to the programmed cell death (Kruh 2003).

The pathological basis of CLL is unknown, but dysregulation of programmed cell death, apoptosis, seems to explain at least a part of the disease progression (Buggins and Pepper 2010). The current opinion on the accumulation of lymphocytes in CLL is that it is caused by i) a

dysregulation of apoptosis and, specifically, an overexpression of the Bcl2 protein as well as ii) an increased proliferation of lymphocytes (Messmer et al. 2005).

When chemotherapy for is initiated for a patient, the sensitivity to the chemotherapeutic agent/agents of the leukaemic cells is not known. The current guideline for the treatment of CLL is based on a combination of chemotherapeutic agents (Dighiero and Hamblin 2008). The clinical results can be optimised by individualising the chemotherapy (Bosanquet et al. 1999a). There are few clinically relevant prognostic factors for chemotherapy available – these are chromosome 17p deletion and p53 protein mutation (Zenz et al. 2008a). These findings are quite rare in CLL and cannot be considered an explanation for chemoresistance in most of cases.

The purpose of the present study series was to evaluate the prognostic impact of apoptosis-associated genes, surface antigens and the mutation status of IgHV-region genes on the in vitro sensitivities of leukaemic cells from 36 CLL patients to nine drugs and two types of irradiation. Furthermore, the prevalence of cross-resistance and the impact of previous treatments on drug resistance were evaluated.

1 REVIEW OF THE LITERATURE

1.1. Chronic lymphocytic leukaemia

1.1.1. *Definition and diagnosis*

The term leukaemia comes from the Greek words “leukos” and “heima,” meaning white blood. John Benett and Rudolph Virchow were the firsts to describe leukaemia as a blood disease in 1845, and Virchow gave the condition its name, leukaemia (Hajdu 2003). In early 20th century, chronic lymphocytic leukaemia (CLL) was described as one subgroup of leukaemia. It was described as a disease where there should be an increase in lymphocytes in the blood and an enlargement of the spleen and the lymph nodes (Pinkus 1905). Since then, the definition of CLL has become more exact. The World Health Organisation classified the condition in 2008 as a mature B-cell neoplasm composed of small monomorphic B lymphocytes in the peripheral blood, bone marrow, liver, spleen and lymph nodes (Swerdlow et al. 2008). The diagnosis of CLL requires the presence of at least 5×10^9 B-lymphocytes/ L in the peripheral blood, and the typical clonality of the malignant cell should be confirmed by flow cytometry. The lymphocytosis should be present for at least 3 months, and the degree of lymphocytosis in the bone marrow is characteristically over 30%. If the degree of lymphocytosis is lower than 5×10^9 B lymphocytes/L and occurs in the absence of lymphadenopathy, organomegaly or cytopenias, the condition should be considered a low-stage CLL or monoclonal B lymphocytosis. Other lymphoproliferative diseases might be difficult to distinguish from CLL, which is why flow cytometry and bone marrow examination should be performed (Hallek et al. 2008).

1.1.2. Epidemiology

CLL is the most common type of leukaemia in Europe and North America, but it is rare in Asia. It comprises roughly one third of all types of leukaemia (Altekruse 2010). In Finland slightly more than 200 new cases are diagnosed every year (average in 2004–2008), 40% of them occurring in women and 60% in men (Finnish Cancer Registry 2010). More than 90% of CLL patients are over 50 years old at diagnosis, and the highest incidence is between the sixth and seventh decades of life. Women with CLL have a superior survival outcome when compared to men, and they experience a better clinical outcome in terms of progression-free survival, even if the difference is not large (Molica 2006).

Table 1. New CLL patients according to age groups in Finland in 2004–2008 (Finnish Cancer Registry 2010).

AGE GROUP	0–34	35–39	40–44	45–49	50–54	55–59	60–64	65–69	70–74	75–79	80–84	85>
MALE	1	1	5	24	34	58	81	86	99	96	66	62
FEMALE	1	3	7	19	14	41	46	39	45	63	60	88

1.1.3. Aetiology

The aetiology of CLL is poorly understood. Approximately 10% of individuals with CLL report a family history of CLL and genetic factors thus seem to be at least a part of the aetiology (Brown 2008). First-degree relatives of CLL patients have a 7–8.5-fold relative risk for developing CLL and a 2.6-fold relative risk for having any form of lymphoproliferative malignancy (Goldin et al. 2009). There are significant ethnicity-related differences in CLL incidence – the incidence of CLL in Asians is significantly lower than Europeans or Americans. Migrant studies in CLL support the hereditary component; low rates of CLL in Asians appear to remain stable with migration to the Western countries (Pang et al. 2002).

Some chemical exposures have been connected to excess incidence of CLL, and people employed in agricultural occupations as well as the pesticides, rubber and petroleum industries are over represented in CLL (Blair et al. 2007). There is no significant association between ionizing radiation and CLL, even though ionizing radiation has been clearly connected to other types of leukaemia (Schubauer-Berigan et al. 2007).

It has been postulated that infection agents could trigger CLL development. In recent studies an increased occurrence of respiratory infections has been found before the CLL diagnosis (Landgren et al. 2007; Anderson et al. 2009). The respiratory infections could also be a consequence of the altered immunity preceding CLL. Moreover, similarities in the B-cell receptors (BCR) of CLL patients support the idea of a common antigen trigger and support the hypothesis that an antigen-driven process contributes to CLL pathogenesis (Messmer et al. 2004; Widhopf et al. 2004).

1.1.4. Clinical features

The clinical course of CLL is subject to great variation: a third of the patients never need treatment, for another third an initial indolent phase is followed by disease progression, and the remaining third develop an aggressive disease and need to be treated from the beginning (Dighiero and Hamblin 2008). In most of the cases, CLL diagnosis is made by coincidence – leukocytosis is detected in a routine blood test, while the patient does not have any symptoms. Enlargement of the neck, axillary or inguinal lymph nodes may also represent the first sign of CLL, but an enlargement of the spleen or liver is rarely the first sign of disease. Fever, weight loss, night sweating and fatigue are normally markers of progressive disease.

The majority of complications in CLL are related to the impaired immune function, with most patients developing a reduction in immunoglobulin levels. Infections are the major complication in CLL, and most of them are bacterial respiratory infections. Recurrent and chronic

sinusitis, bronchitis and pneumonia are common in CLL. The response to vaccinations is altered due the altered immune function (Sinisalo et al. 2003). The risk of infection increases over time, especially in treated patients with active disease, and infections are usually the cause of death in advanced CLL (Dearden 2008).

Autoimmune complications are well-recognized in CLL, occurring in up to 25% of patients at some time during the course of their disease (Hamblin 2006b). The most common autoimmune complication is autoimmune haemolytic anaemia. Another possible autoimmune complication is immune thrombocytopenia. The increased risk of secondary malignancies seen in CLL is likely to be related to the underlying immune impairment (Dearden 2008).

1.1.5. Classification and prognostic factors of CLL

1.1.5.1. Clinical classification and its impact on prognosis

Clinical staging systems are based on lymphadenopathy, organomegaly, platelet and haemoglobin values. Binet and Rai have developed staging systems that have an important influence on treatment decisions and prognosis (Rai et al. 1975; Binet et al. 1981). These staging systems are simple and reproducible. Rai's system is based on five stages, whereas Binet's system is based on three classes. Both of them are in clinical use, the Rai system mainly in North America and the Binet system in Europe.

Table 2. The Rai staging system for CLL (Rai et al. 1975).

Stage	Risk group	Clinical findings	Median survival (months)
0	Low	Lymphocytosis $> 5 \times 10^9/l$	>150
I	Medium	Stage 0 + lymphadenopathy	101
II	Medium	Stage 0 + splenomegaly or hepatomegaly	71
III	High	Stage 0 + anaemia $<110g/l$	19
IV	High	Stage 0 + thrombocytopenia $<100 \times 10^9/l$	19

Table 3. The Binet staging system for CLL (Binet et al. 1981).

Class	Risk group	Clinical findings	Median survival (months)
A	Low	Lymphocytosis $>5 \times 10^9/l$ and < 3 sites lymphadenopathy*	not reached
B	Medium	$3 \geq$ sites lymphadenopathy	84
C	High	anaemia and/or thrombocytopenia	24

*Note: Five possible nodal sites: neck, axillary, inguinal, spleen, liver.

1.1.5.2. Morphological classification and its impact on prognosis

A typical CLL cell is a small lymphocyte, usually with a round nucleus and condensate chromatin. The mitotic activity is low. The proportion of prolymphocytes is normally less than 2%. A prolymphocyte count of higher than 2% (CLL/PL), or atypical cell morphology (CLL/mix), correlates with more aggressive disease. In a case where the prolymphocyte count is over 55%, the diagnosis is prolymphocytic leukaemia rather than CLL (Swerdlow et al. 2008).

Bone marrow involvements are defined as interstitial, nodular or diffuse. The life expectancy of patients with a diffuse bone marrow infiltration pattern is significantly shorter than that of those with non-diffuse patterns (Swerdlow et al. 2008).

1.1.5.3. Biological markers of CLL and their prognostic impact

Molecular and cellular markers that could predict disease progression have been identified. In particular, the mutational profile of immunoglobulin genes, surface antigens and some cytogenetic abnormalities show a strong prognostic value.

1.1.5.3.1. Classification and prognostic impact of chromosomal abnormalities

Fluorescence *in situ* hybridisation (FISH) has enabled a precise approach to evaluating genetic abnormalities in CLL. Chromosomal aberrations are common in CLL, as demonstrated in Table 4. Over 80% of the patients have aberrations in the genome, and as many as 29% of patients have multiple rearrangements (Döhner et al. 2000; Krober et al. 2002).

Table 4. The incidence of the most usual chromosomal aberrations in CLL patients (Döhner et al. 2000).

Chromosomal aberration	% of CLL patients
13q deletion	55%
11q deletion	18%
12q trisomy	16%
17p deletion	7%
6q deletion	6%
8q trisomy	5%
t(14q32)	4%
3q trisomy	3%

The most common aberrations are 13q deletion, which corresponds to a more favourable course of disease (Döhner et al. 2000). Two microRNA genes, miR-15a and miR-16-1, are located in the 13q region (Calin et al. 2002). These microRNA gene products inhibit *Bcl2* translation, and deletion in the 13q region therefore leads to the accumulation of Bcl2 (Calin et al. 2007).

The deletion of 11q corresponds with faster disease progression and extensive lymphadenopathy and splenomegaly (Döhner et al. 2000). Five-year survival in 11q deletion has been found to be roughly 70% compared to the 95% in 13q deletion (Döhner et al. 2000). The 11q deletions are associated to ATM mutations, which lead to more advanced disease (Austen et al. 2005).

The 12q trisomy is a quite frequent aberration in CLL, found in approximately 15% of patients (Döhner et al. 2000). Trisomy 12 does not increase the progression of the disease and the survival prognosis is good.

Patients with deletion in the 17p region suffer from more advanced disease than others. The estimated median survival time from the date of diagnosis is 32 months (Döhner et al. 2000; Krober et al. 2002). The patients are also more symptomatic: they are more likely to experience fever, night sweats and, weight loss and they have a lower blood count (other than lymphocytes) (Dohner et al. 2000). The gene of the p53 protein is located in chromosome 17p. Among CLL patients with 17p deletion, the majority (over 80%) also have a mutation in the p53 protein, leading to drug resistance and more advanced disease (Zenz et al. 2008a; Zenz et al. 2008b).

1.1.5.3.2. Classification and prognostic impact of leukaemic cell surface antigens

The B lymphocyte surface antigens are revealed by flow cytometry. The clonality of the CLL cells is investigated from the surface antigens: the CLL B lymphocyte represents either kappa or lambda immunoglobulin light chains, whereas a normal B lymphocyte expresses both (Hallek et al. 2008). The typical surface antigens in the CLL cells facilitate the differentiation of the CLL cell from normal B lymphocytes and other lymphoproliferative malignancies. The CLL B lymphocytes typically express the CD5, CD19 and CD23 antigens, with weak expressions also of surface immunoglobulin (Ig) M or D, CD20, CD22, FMC7 and CD79b (Swerdlow et al. 2008).

In clinical use, the most valuable prognostic surface antigen is CD38. It is a transmembrane glycoprotein and only part of the CLL cells express CD38. An increased proportion of CD38-positive CLL cells correlates with more aggressive disease, a higher need for chemotherapy and a shorter overall survival (Krober et al. 2002; Hock et al. 2009; Van Bockstaele

et al. 2009). The unsolved question is what level of CD38 expression should be regarded as positive – 5%, 7%, 10% or 30% (Hamblin 2006a).

Another potential prognostic surface antigen is adhesion molecule CD49d, which is observed in patients with advanced disease. It is associated with the need for treatment and shorter overall survival (Van Bockstaele et al. 2009).

1.1.5.3.3. Classification of immunoglobulin variable region mutations and their impact

The mutation status of the immunoglobulin heavy chain variable (IgHV) segment is the most powerful prognostic marker of CLL outcome, but the measurement is problematic. The mutation status of the IgHV segment is measured by sequencing the DNA and comparing it to international databases. This assay is both expensive and labour-intensive, precluding its routine use.

The B cell response to antigen stimulation is mediated through the B cell receptor (BCR). In normal B lymphocytes the diversity of the BCR is developed in germinal centres. BCR is combined from two heavy chains and two light chains, each of which are combined from variable (V), diversity (D) and joining (J) segments. During B cell maturation, the genes of these V, D and J segments are altered to somatic hypermutations in germinal centres, thus leading to a huge diversity of receptors.

This maturation can be measured from the proportion of mutated genes in the IgHV segments in comparison to the baseline database. If the IgHV genes differ from the baseline database by more than 2%, the definition is mutated IgHV, and if the IgHV genes differ by less than 2% from the baseline database, the definition is unmutated IgHV.

The IgHV mutation status divides CLL patients into two groups that are almost equal in size. The prognosis of the unmutated IgHV CLL (UM-CLL) patients is worse than that of mutated IgHV CLL (M-CLL) patients. The median survival for UM-CLL has been determined as

117 months and for M-CLL 293 months (Hamblin et al. 1999). An exception to this generalization is made by a small group of M-CLL patients who have a mutation in the IgHV3-21 region; this leads to aggressive disease (Tobin et al. 2002).

Researchers have searched for a surrogate for IgHV mutation status (as a prognostic marker in CLL), and intracellular tyrosine kinase ZAP-70 has been proposed as the most reliable candidate, although it is rather a supplemental factor than a surrogate one. Most of the mutated cases are ZAP-70-negative and most of the unmutated ones ZAP-70-positive (Wiestner et al. 2003; Orchard et al. 2004; Kipps 2007). On the other hand, ZAP-70-positive cases can be categorized into the high-risk group, ZAP-70-negative and IgHV-unmutated cases into the intermediate-risk group and ZAP-70 negative and IgHV-mutated cases into the low-risk group (Gripen 2008).

The ratio of lipoprotein lipase (LPL) as well as disintegrin and metalloproteinase 29 (ADAM29) mRNA has also been found to be a surrogate for IgHV mutation status. The LPL/ADAM29 ratio has been found to show 90% concordance with the mutation status (Oppezzo et al. 2005).

1.1.5.3.4. Apoptosis-associated proteins and microRNA

Accumulation of the B cells is typical for CLL progression. For many years, CLL was considered to be a disease of failed apoptosis, resulting in the accumulation of long-lived lymphocytes. However, a recent study has demonstrated that CLL is also a dynamic disease with significant amounts of proliferation and leukaemia cell turnover every day (Messmer et al. 2005).

The molecular mechanisms that regulate apoptosis in CLL are complex. The BCL2 protein family is one of most important and researched aspects of apoptosis in CLL. Overexpression of the BCL2 protein has been detected in CLL cells, leading to apoptosis resistance (Skena et al. 1992; Pepper et al. 1997).

Micro-RNAs (miRNA) are small non-coding RNA particles, which regulate mRNA translation. It has been shown that two miRNAs, miR-15 and miR-16, are deleted or down-regulated in many CLL patients – both of these miRNAs negatively regulate *Bcl2* at the post-transcriptional level (Nicoloso et al. 2007). Both of them are located in the chromosome 13q area, and the deletion 13q, which is common in CLL, is therefore related to the down-regulation of these miRNAs (Calin et al. 2002). Low expression of miR-29c and miR233 is associated with more aggressive disease and early treatments in CLL (Stamatopoulos et al. 2009). The miR-29 microRNA is connected to the negative regulation of the expression of the *Mcl1* and *Tcl1* oncogenes (Calin et al. 2007; Stamatopoulos et al. 2009).

The p53 protein is a tumour suppressor protein which causes cell arrest in the case of DNA damage until the damage is either repaired or leads the cell to the apoptosis if DNA damage cannot be repaired. Mutation in this protein leads to a resistance to apoptosis and causes multidrug resistance. The CLL p53 mutation is strongly associated with chromosome 17p deletion, which is a marker of poor prognosis (Zenz et al. 2008b).

1.1.5.3.5. Other biological prognostic markers

Lymphocyte doubling time (LDT) is defined as the number of months it takes the absolute lymphocyte count to double in number. Patients with an LDT of 12 months or less have a significantly shorter overall and treatment-free survival than patients with a LDT of more than 12 months (Swerdlow et al. 2008; Van Bockstaele et al. 2009).

Soluble surface antigens, such as soluble CD23 (sCD23), have been connected to more advanced disease (Van Bockstaele et al. 2009). Moreover, the short doubling time (less than one year) of the sCD23 level has been associated with more aggressive disease (Meuleman et al. 2008).

The lactate dehydrogenase (LDH) level is normally low in CLL, although it is commonly elevated in many haematological malignancies. Elevated LDH is a marker of advanced disease in CLL (Van Bockstaele et al. 2009).

Serum beta 2 microglobulin (B2M) is a laboratory parameter that correlates with clinical outcome, the bone marrow infiltration degree and bulky disease. High (>3.5 mg/L) B2M levels are associated with a worse prognosis (Shanafelt 2009).

Thymidine kinase (TK) is a cellular enzyme found in dividing cells that is absent in resting cells. Elevated serum TK levels are associated with disease progression. Serum TK level determination is performed with an immuno assay, which is why it has not been adopted in routine clinical usage (Van Bockstaele et al. 2009).

The prognosis for chemoresistant patients is poor. The chemoresistance mainly develops as a result of chemotherapy, but untreated patients may also be chemoresistant. The median survival for fludarabine-resistant patients has been found to be ten months (Keating et al. 2002).

1.1.6. Treatment and outcome

Although in the case of most cancers, early detection and thus early treatment are essential in improving outcome, CLL is rarely treated at diagnosis. Normally, treatments are initiated after the first symptoms appear or in the case of aggressive disease. This treatment model is mainly based on the results of investigations that have compared early and late treatment, demonstrating no benefit from early treatment (CLL Trialists' Collaborative Group 1999).

The aims for the treatment in early-stage disease are relative, and the decision to treat should be made individually. The period of watchful waiting provides more information on a patient's disease and assists in the decision-making regarding treatments. Patients in an early stage (Binet A or B) should not be treated without symptoms, whereas patients in an advance stage (Binet

C) should be treated. The main symptoms for treatment are i) enlargement of lymph nodes, such as cosmetically unpleasant lymph nodes in the neck, or enlargement of the spleen; ii) bone marrow failure, such as in the case of anaemia, neutropenia or thrombocytopenia; iii) progressive lymphocytosis, where the increase in lymphocytes exceeds the rate of 50% in two months or where the lymphocytic doubling time (LDT) is less than six months; and iv) disturbing general symptoms, such as fatigue, night sweating or weight loss (Hallek et al. 2008).

Over the past decades, chlorambucil with or without prednisolon has been widely used as a first-line therapy in CLL. Currently, chlorambucil is used for the older patients and those who are not candidates for more intensive treatment (Dighiero and Hamblin 2008). The nucleoside analogues fludarabine and chlorodeoxyadenosine are the most powerful drugs in CLL, and fludarabine is the most frequently used chemotherapy agent. In the investigations, fludarabine has been combined with cyclophosphamide with better results than monotherapy (Catovsky et al. 2007), even if the overall survival has not increased. Monoclonal antibodies are new therapeutics which are used as a concomitant therapy with fludarabine, with or without cyclophosphamide. The monoclonal anti-CD20 antibody, rituximab, combined with fludarabine and cyclophosphamide, has yielded encouraging results as a first-line therapy (Tam et al. 2008). Furthermore, the monoclonal anti-CD52 antibody alemtuzumab alone or combined with other chemotherapy agents has confirmed good results (Gribben and Hallek 2009). The side effects of these monoclonal antibodies include infections, specifically cytomegalovirus and herpes virus infections, as well as *Pneumocystis carinii* pneumonia, myelosuppression, fever, skin rash and hypotension (Motta et al. 2009).

Autologous and allogeneic stem cell transplantation can be used in a chemoresistance situation. Allogeneic transplantation might reach a curative result, but autologous transplantation is only a palliative treatment (Dreger 2009). Long-term progression-free survival can be achieved in

30%–60% of transplanted poor prognosis patients by means of allogeneic transplantation (Dreger 2009).

The common problem in every treatment modality is the drug resistance that is reached at least after chemotherapy.

1.2. Chemo- and irradiation therapy and drug resistance in chronic lymphocytic leukaemia

1.2.1. The used experimental drug and irradiation treatments and their mechanism of action

1.2.1.1. Chlorambucil

Chlorambucil is one of the first drugs used in the treatment of cancer. It is mainly used in CLL. In the past, chlorambucil was a cornerstone in the treatment of the CLL, but currently it has been mostly replaced by fludarabine as a first-line therapy (Dighiero and Hamblin 2008). It is a bifunctional alkylating agent of the nitrogen mustard type.

Mechanism: As an alkylator, chlorambucil is an agent that transfers an alkyl group to another molecule (Kundu et al. 1994). Bifunctional alkylating agents have two reactive alkyl groups that can make two reactions with other molecules, such as cells, DNA and proteins. Chlorambucil is transported into cells by carrier-mediated systems. The main mechanism of action or toxic action is thought to be due its ability to form cross-links between DNA strands, which prevents DNA replication and transcription and thus causes apoptosis. The main resistance mechanisms to chlorambucil are enhanced DNA repair mechanism (Panasci et al. 2001), inactivation by enhanced levels of glutathiones or metallothioneins and impaired apoptosis (Morrow et al. 1998).

1.2.1.2. Verapamil

Verapamil is a calcium channel blocker of the phenylalkylamine class. It is mainly used in the treatment of hypertension, angina pectoris, cardiac arrhythmia and cluster headaches, and as preventive medication for migraine.

Mechanism: Verapamil's mechanism of action is to block voltage-dependent calcium channels. It also has an ability to block P-glycoprotein, which is a selective drug efflux pump causing multidrug-resistance in cancer cells. Verapamil alone is toxic and potentiates the effect of vincristine and anthracyclines against chronic lymphocytic leukaemia in vitro (Vilpo et al. 2000a). Verapamil has also been shown to have cytoreductive potential in vivo (Berrebi et al. 1994). The Verapamil cell-killing mechanism may result from disturbed calcium metabolism (Vilpo et al. 2000a).

1.2.1.3. Cyclosporin A

Cyclosporin A is an immunosuppressant drug. It was discovered 1972 and it is produced by the two fungi *Thricoderma polysporum* and *Cylindrocarpon lucidum* (Laupacis et al. 1982). It is widely used in post-transplant medicine and the treatment of many autoimmune diseases. Cyclosporin A is not commonly used in CLL, but it might be used in the case of an autoimmune disease, such as autoimmune anaemia, to prevent drug efflux in multidrug resistance and to attempt to kill CLL cells (Koski et al. 2000).

Mechanism: In a normal immunological defence system, cyclosporin A has a very selective inhibitory effect on T lymphocytes, suppressing the early cellular response to antigenic and regulatory stimuli. The consequence of cyclosporin A binding to its receptor is the inhibition of calcineurin activity and suppression of calcineurin-stimulated effects. This leads to the inhibition of T-cell activation-associated genes, including the one for interleukin-2, certain proto-oncogenes, and those for cytokine receptors (Rao 1995). Cyclosporin A also prevents the P-glycoprotein-dependent

drug efflux pump, therefore potentiating the effect of vincristine and anthracyclines (Consoli et al. 2002).

1.2.1.4. Vincristine

Vincristine is a vinca alkaloid from the *Catharanthus roseus* plant. It is used in cancer chemotherapy.

Mechanism: The known mechanism of action for Vincristine has been related to the inhibition of microtubule formation in mitotic spindle, resulting in an arrest of dividing cells at the metaphase stage. Even if CLL cells are not mitotically active, the susceptibility of CLL cells to vincristine in vitro has been demonstrated. Interestingly, CLL cells are much more sensitive to vincristine than normal lymphocytes (Vilpo and Vilpo 1996; Vilpo et al. 2000b).

1.2.1.5. Fludarabine

Fludarabine is an antimetabolite which belongs to a nucleoside analogy group. It is used as a first-line therapy in CLL (Dighiero and Hamblin 2008).

Mechanism: Fludarabine is taken up by cells through the nucleoside transporter mechanism and converted by cellular enzymes to the active metabolite F-ara-ATP, the triphosphate of fludarabine. The rate-limiting enzymes for this phosphorylation are cellular kinases; hence, fludarabine accumulates mainly in cells with a high activity of these enzymes, such as lymphocytes. In proliferating cells, F-ara-ATP is actively incorporated into DNA by DNA polymerases during replication (Fidias et al. 1996). This incorporation causes the termination of the new DNA strand synthesis at the sites of drug incorporation, including the steps required in the DNA repair process, and it is closely connected with the drug's cytotoxic activity (Yamauchi et al. 2001). Fludarabine is also incorporated into RNA and causes premature termination of the RNA transcripts. Inhibition of

the RNA transcription may be the major mechanism of action in quiescent CLL cells (Huang et al. 2000).

1.2.1.6. Chlorodeoxyadenosine

Like fludarabine, chlorodeoxyadenosine (CDA) belongs to a nucleoside analogy group. They have structural, pharmacological and biological similarities. CDA is mainly used in the treatment of lymphoproliferative malignancies, such as CLL (Robak 2001), and it is mainly used in combination with cyclophosphamide.

Mechanism: CDA is transported into the cell by the nucleoside transporter mechanism and, in order to be activated, CDA is phosphorylated within the cell by nucleoside kinases, which are highly active in lymphocytes. CDA inhibits both cellular ribonucleotide reductase and DNA synthesis and is incorporated into cellular DNA during replication, causing the termination of new DNA strand synthesis at the sites of drug incorporation (O'Brien et al. 1994; Hentosh and Tibudan 1997; Yuh et al. 1998). CDA is able to inhibit DNA repair, which leads to the accumulation of DNA strand breaks and, moreover, to apoptosis.

1.2.1.7. Doxorubicine

Doxorubicine, adriamycin, is an anthracycline antibiotic. It was initially isolated from *Streptomyces caesius* bacteria in the 1950s. It is commonly used as an additional component of chemotherapy in the treatment of leukaemia and lymphoma but also many other cancers.

Mechanism: There is still some controversy about the mechanism of action with regard to doxorubicine. The anticancer activity or cytotoxic effects have been proposed to be due to the following mechanisms: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules; 2) generation of free radicals, leading to DNA damage or lipid peroxidation; 3)

DNA binding and alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding or DNA strand separation and helicase activity; 6) direct membrane effects; 7) initiation of DNA damage via inhibition of topoisomerase II; and 8) induction of apoptosis in response to topoisomerase II inhibition (Binaschi et al. 2001; Minotti et al. 2004).

1.2.1.8. Prednisolon

Prednisolon is a steroid hormone. It is widely used as an anti-inflammatory and immunosuppressant agent.

Mechanism: As a lipophilic, prednisolon diffuses across the cell membrane passively and adheres to the nuclear receptor in the cytoplasm. The receptor translocates to the nucleus upon ligand activation, where it can cause regulation of the gene expressions (De Bosscher et al. 2003; Lowenberg et al. 2008). Inflammatory mediators constitute the gene group that is the most regulated by prednisolon, but it can also cause the activation of apoptosis cascade. The ability of prednisolon to cause lymphocyte apoptosis p53 independently in CLL is the main reason for its usage, but it is also used to treat autoimmune cytopenias (Tsimberidou and Keating 2009).

1.2.1.9. Cisplatin

Cisplatin is a platinum-based compound. It is widely used in the treatment of many cancers, mostly solid tumours.

Mechanism: It diffuses across the cellular membrane and accumulates in the cell, where its chloride atoms are replaced by water molecules, thereby transforming cisplatin into a very reactive mode. The main mechanisms of action are either intrastrand or interstrand DNA strand cross-links. DNA and protein cross-links have also been described. The cytotoxic effect is thought to be mediated by platinum-DNA adducts that inhibit DNA replication and transcription. Several cellular

pathways are activated in response to DNA-cisplatin and DNA-protein interactions, which include repair proteins, translesion synthesis by polymerases, and induction of apoptosis. The apoptotic process is regulated by the activation of caspases, the p53 gene, and several pro-apoptotic and anti-apoptotic proteins. This kind of cellular processing eventually leads to an inhibition of the replication or transcription machinery of the cell (Ahmad 2010). Cisplatin is not commonly used in the treatment of CLL.

1.2.1.10. The use of gamma and UV irradiation

Gamma irradiation is widely used in cancer therapy. In CLL it is used as a concomitant therapy, such as decreasing size of the spleen or the lymph nodes.

Mechanism: Gamma irradiation causes cell damage by the formation of single- or double-strand DNA breaks (Elkind 1985), the former being repairable but the latter being lethal to the cell (Myllyperkio et al. 1999). The DSB/SSB induction ratios are roughly 1:20 for ionizing irradiation.

UV irradiation is not in clinical use as a cancer treatment, but rather in investigative use.

Mechanism: UV irradiation induces DNA cross-links and DNA single-strand breaks which are normally repaired by the nucleotide excision repair system. First, this repair system excises the damaged site and then creates a new strand. UV irradiation has been used to investigate these repair systems (Vilpo et al. 1995; Myllyperkio et al. 1999).

1.2.2. Mechanism of the drug resistance

Drug resistance is a major problem in cancer treatment. The mechanisms of drug resistance have been investigated extensively with poor clinical results. Insight into the cellular resistance mechanisms holds the promise of leading to better treatments for cancer patients. Resistance

mechanisms can be divided into two groups. The first of these appears in the case of A) an altered intracellular concentration of the drug, which is mainly meant to enhance their extrusion from the cell. The second group involves B) the required activation of the drugs once inside the cell – this can be i) impaired by the loss of specific enzymatic activities involved in their metabolic activation; but ii) certain agents can also be enzymatically inactivated; iii) cellular targets can be altered, either by increased or decreased expression levels, or by mutations; and, finally, iv) once the cellular target of the drug is damaged, resistance factors can attenuate the capacity of a cell to undergo apoptosis.

1.2.2.1. Drug-resistance-related proteins and enzymes

P-glycoproteins. In humans several P-glycoproteins have been identified. The most widely known P-glycoprotein, p170, is produced by the MDR-1 gene. P-glycoproteins are large transmembrane proteins that act as an energy-dependent efflux pump for physiological substrate steroids, hormones and bilirubin and cytotoxic agents. The main substrates in malignant cells are anthracyclines, vinca alkaloids and epipodophyllotoxins. Overexpression of p170 causes reduced intracellular concentrations of drugs that are substrates for p170, thus inducing multidrug resistance (MDR). It is frequently observed in leukaemia and lymphomas (Ambudkar et al. 1999; Ambudkar et al. 2003). In many haematological malignancies, the elevated expression of an MDR phenotype is a consequence of exposure to cytotoxic drugs (Webb et al. 1998). In CLL lymphocytes, the expression of p170 has been shown to be elevated (Burger et al. 1994).

The function of the P-glycoproteins can be modulated *in vitro* by Verapamil and Cyclosporin A (Vilpo et al. 2000a; Consoli et al. 2002). The problem is that effective concentrations required to modulate p-glycoprotein activity are easily above the normal therapeutic dose.

MDR-related proteins (MRP) are an ATP-dependent transmembrane transporter protein family. This family is comprised of nine transporters that are able to transport structurally

diverse lipophilic anions and function as drug efflux pumps. MRP has similar transport specificity to MDR, but drugs are frequently conjugated with glutathione and other anions, or they are co-transported with glutathione (Ambudkar et al. 2003). MRPs have been connected to resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins and methotrexate (Kruh and Belinsky 2003).

In CLL only a small group of patients express MPR proteins before treatment, but after anticancer drug therapy the proportion increases significantly (Webb et al. 1998).

Glutathione (GSH) is a major intracellular reducing agent. Two important pathways utilise glutathione: 1) free radical neutralisation, either spontaneous or catalysed by glutathione s-transferase which is important for xenobiotic detoxification, 2) the reduction of peroxides, such as hydrogen peroxides or lipid peroxides and disulphide bonds (Estrela et al. 2006). An increased level of glutathione may decrease the anticancer drug concentration in the cell and thus lead to drug resistance, whereas a decreased cellular concentration of glutathione potentiates the anticancer drug effect (Hagman et al. 2004). The intracellular concentration of glutathione has been demonstrated to be higher in CLL lymphocytes than normal lymphocytes (Ferraris et al. 1994). The depletion of GSH in sensitising cells to ionising radiation has been demonstrated in lymphoid cell lines (Dethmers and Meister 1981).

Glutathione s-transferases (GST) are multifunctional proteins that protect cells against carcinogens and many other toxins. GST conjugates glutathione with a wide variety of hydrophobic and electrophilic compounds, and this facilitates the elimination of the xenobiotics, since the resulting metabolites are more water-soluble (Hayes et al. 2005). Several antineoplastic drugs, particularly the reactive electrophilic alkylating agents, can form conjugates with glutathione both spontaneously and in GST-catalysed reactions, thereby detoxifying them (Dirven et al. 1994).

GST expression levels in normal and CLL lymphocytes vary on an individual basis, but there are no significant differences. Lymphocytes from chlorambucil-resistant patients have

been reported to be subject to a twofold increase in GST activity when compared to the lymphocytes of untreated patients (Schisselbauer et al. 1990). However, glutathione-conjugated chlorambucil is more water-soluble and therefore less permeable to the plasma membrane, which is why it needs to be transferred across the plasma membrane by an efflux mechanism (Morrow et al. 1998; Ballatori et al. 2009). Therefore, not only one mechanism alone but rather a complex of mechanisms achieves drug resistance.

Metallothioneins are a group of small intracellular proteins with the capacity to bind metal ions strongly, mainly zinc in physical conditions. Several functions of metallothionein have been described: free radical neutralisation, inflammation regulation, regulation of zinc-containing proteins (like p53), DNA damage and cell death regulation following a number of pathological conditions, and protection of cells against various forms of stress, such as oxidative injury, radiation, chemotherapy and metal toxins (Pedersen et al. 2009).

The metallothioneins' zinc ion can be easily displaced by metal ions that have a high affinity for thiolates. In this way, metallothioneins serve as a detoxifying protein for toxic metal ions that may enter the cell. Increased metallothionein levels seem to decrease active anticancer drug concentrations and therefore contribute to drug resistance (Hagrman et al. 2003). Increased expressions of metallothioneins have been reported in various cancers (Pedersen et al. 2009).

Topoisomerases are intranuclear enzymes that modify DNA strands during the transcription, replication and chromosome segregation. Topoisomerases are classified as type I or type II according to their ability to introduce transient breaks in the proteins linked in one or both strands of the double helix (Nitiss 2009a). Drugs targeting topoisomerase II are divided into two classes; A) an increased level of topoisomerase II-DNA covalent complexes which lead to DNA strand breaks and, therefore, apoptosis, and B) the elimination of the essential enzymatic activity of topoisomerase II, which leads to the catalytic inhibition of the cell and, therefore, apoptosis. Sensitivity to topoisomerase-II-targeting drugs depends on i) the levels of topoisomerase II protein

and ii) qualitative changes in topoisomerase II enzymes which decreases the topoisomerase-drug complex formation (Danks et al. 1987). Cells overexpressing topoisomerases are hypersensitive to topoisomerase-dependent drugs, while cells expressing low levels of topoisomerases are relatively drug resistant (Nitiss 2009b). Topoisomerase II is the target of certain classes of antineoplastic agents, including anthracyclines, epipodophyllotoxins, amsacrine, and other intercalating agents.

In CLL the expression level of the topoisomerase II protein, mRNA and catalytic active has been found very low (Potmesil et al. 1987; Beck et al. 1996; Valkov and Sullivan 1997). This suggests a natural resistance of CLL to topoisomerase-II-affecting drugs.

1.2.2.2. Apoptosis-associated proteins

If the chemotherapeutic agent eventually manages to damage DNA permanently, the programmed cell death cascade is activated, leading the cell to apoptosis. The apoptosis cascade is a complex pathway of a multitude of regulated processes. The p53 protein regulates the expression of many apoptosis associated genes, with the Bcl2 protein family in particular. The Bcl2 protein family, consisting of both pro- and anti-apoptotic proteins, regulates mitochondrial membrane potential and, moreover, regulates cytochrome C release to cytoplasm. Finally, cytochrome C activates the caspases cascade which leads to the splitting of the DNA and major cell structures and, finally, to apoptosis (Burz et al. 2009).

In CLL the expression of apoptosis-associated proteins is increased in comparison to normal lymphocytes. Especially the Bcl2 protein has been identified as up-regulated in CLL (Skena et al. 1992; Pepper et al. 1997).

Bcl2 is an anti-apoptotic protein and causes resistance to chemotherapeutic agents (Johnston et al. 1997; Pepper et al. 1997; Thomas et al. 2000). The **Bax** protein belongs to the Bcl2 protein family, but its effect is pro-apoptotic. Underexpression of Bax leads to delayed apoptosis and can cause drug resistance (Bosanquet et al. 2002). Even more so than Bcl2 or Bax alone, the

Bcl2/Bax ratio has been implicated as a marker of chemoresistance in CLL (Pepper et al. 1997; Pepper et al. 1998; Pepper et al. 2001).

Bcl2l1, Bcl2a1 and Mcl1 (myeloid cell leukaemia sequence 1) belong to the Bcl2-associated family and suppress apoptosis in a pathway common to Bcl2 (Burz et al. 2009). High Mcl1 expression has been connected to a failure to achieve a good treatment response in CLL (Saxena et al. 2004).

Death-associated protein kinase 1 (**Dapk1**). Most normal cells require adhesion to the correct extra-cellular matrix for survival, and inadequate or inappropriate cell–matrix interaction often causes apoptosis. One of the main negative mediators of these interactions is Dapk1. Activation of this kinase leads to the activation of a p53-dependent apoptosis cascade (Bialik and Kimchi 2006). The expression of Dapk1 is downregulated in CLL (Raval et al. 2007).

Myc is a DNA-binding phosphoprotein that regulates transcription, and it can regulate both proliferation and apoptosis. Myc drives cells into apoptosis via several mechanisms – e.g., via the action of P53 and Bax and by inducing susceptibility to tumour necrosis factor- α (TNF- α). Deregulation of the *Myc* oncogene has been shown to play a critical role in pathogenesis of many lymphomas (Klapproth and Wirth 2010). It is expressed normally in most of CLL cases, but Myc overexpression leads to aggressive disease (Huh et al. 2008).

The p53 protein acts as an activator of the apoptosis signalling pathway. It upregulates the proapoptotic genes Bax and Bak via the Puma protein. It is also able to downregulate the antiapoptotic Bcl2 protein family, with Bcl2 in particular. The p53 protein is inactivated in most human cancers and plays a certain role in drug resistance (Chari et al. 2009). The molecular alterations can result in either high levels of a mutated (inactive) p53 protein or a complete absence of the p53 protein. In CLL p53 is mutated in 15%–20% of patients (Cordone et al. 1998) and mutation is associated with chromosome 17p deletion, although mutations in the absence of 17p deletion are also seen. This mutation decreases the activity of the p53 protein and leads to a

resistance to apoptosis, thereby inciting more aggressive disease and drug resistance (Zenz et al. 2008b).

The ataxia telangiectasia mutated (ATM) is a protein kinase that coordinates an integrated cellular response to DNA damage in the form of double-stranded DNA breaks. The main target of the ATM is to activate the p53 protein (Shiloh 2003). Mutations in the ATM gene have been found in 12% of CLL cells, and reduced overall and treatment-free survival has been observed in patients with *ATM* mutations (Austen et al. 2005).

1.2.2.3. DNA repair molecules

Most of the chemotherapeutic agents exert their action via DNA damage – i.e. DNA strand breaks, cross links or incorporated nucleoside analogues. The repair of DNA damage before the initiation of signalling pathways that induce programmed cell death, apoptosis, represents a cause of drug resistance. The repair mechanisms can be divided into the following groups: i) direct reversal of damage, ii) base excision repair, iii) nucleotide excision repair (NER), iv) mismatch repair, v) repair of cross-links (NHEJ), and vi) double strand break repair (Puranen 2007).

The activity of the NER process in CLL lymphocytes has been found equal in chlorambucil-pretreated and untreated patients (Bramson et al. 1995). Furthermore NER activity has been demonstrated to be low in most of CLL lymphocytes (Barret et al. 1996), and the NER process after UV induction seems to be more active in normal lymphocytes than CLL lymphocytes (Myllyperkio et al. 2000). The DNA-PK protein is a key component in the NHEJ process. Low levels of DNA-PK have been associated with the sensitivity to chemotherapy in CLL cells, whereas increases in DNA-PK activity have resulted in an increased resistance to chlorambucil (Deriano et al. 2005; Amrein et al. 2007). Nucleoside analogues, fludarabine and chlorodeoxyadenosine, can be used as NER inhibitors and thus potentiate the effect of alkylating agents (Bellosillo et al. 1999; Flinn et al. 2007).

AIMS OF THE STUDY

In the present study series, the responses to nine drugs and two types of irradiation were evaluated in 36 CLL patients. The correlations of sensitivities were analysed and factors predicting the responses evaluated.

The specific aims were:

1. to evaluate the multidrug resistance in CLL patients and, specifically, to assess the significance of previous treatments in drug resistance, in addition to finding correlations between differently acting drugs **(I)**
2. to evaluate the impact of surface antigens as prognostics markers of chemo- and irradiation resistance **(II)**
3. to compare two groups of CLL patients, evaluating the effect of the IgHV mutation status on chemotherapy and irradiation responses **(III)**
4. to evaluate the significance of the expressions of apoptosis-associated genes for chemotherapy and irradiation responses **(IV)**

MATERIALS AND METHODS

3.1. Ethical considerations

The study protocols were approved by the ethical committee of Pirkanmaa Hospital District. The work was carried out according to the Helsinki Declaration. Written informed consent was obtained from all subjects.

3.2. Materials

3.2.1. Patients and samples

The samples for this study series (**I**, **II**, **III** and **IV**) were collected from 36 consecutive CLL patients referred to the CLL outpatient clinic at Tampere University Hospital. Some of the patients were newly diagnosed cases, and the rest were diagnosed earlier and referred due to changes in the disease status or for the startup of chemotherapy. Diagnosis and staging were based on standard clinical, morphological and immunophenotyping criteria. All patients had a CD19+/CD23+/CD5+ phenotype. All cases had a mature CLL, CLL/mix or CLL/PL morphology. Most patients had chromosomal aberrations but none indicating other types of chronic lymphoid leukaemia besides typical CLL. Nine of the 36 patients had previously been treated, and 27 were untreated (Table 5). Clinical responses were documented in 2010 from 25/36 patients; the rest of subjects were monitored in other hospitals and the information was not available. Out of these 25 patients, 6 did not require treatments and 19 were treated as described in Table 5.

Table 5. Information concerning the investigated patients; treatments before and after leukaemic cell isolation.

NO	MUT	YEAR	AGE	SEX	BINET	LEUK	TREAT	HIST	CYTOGENETICS	CLINICAL RESPONSE
1	0	1993	54	m	A	66	None	M	Del 11q, del 13q	99-02 LPx31-PR; 02 FLUx1-NR; 02-03 FLU+CYCx7-CR; 04 FLU+CYCx3+GAM-PR; 04 GAM-NR
2	1	N	64	m	A	72	LP	I	Del 7q, del 11q	N
3	0	1994	72	m	B	105	None	D	12q trisomy, 11q del	No treatment
4	0	1991	69	m	B	108	L	I	Normal	94 LP-CR; 97 LP-PR
5	1	N	61	f	A	90	None	I	Del 6q	N
6	1	1988	50	f	A	166	None	N	Del 13q	96-97 LP-CR; 00 LPx3-PR
8	0	N	58	m	C	323	LP, 6xCO	D	Del 17p, trisomia 12	N
9	0	N	68	m	B	73	None	I	Del 6q, del 13q	N
10	1	1992	68	m	A	56	None	M	Del 13q	No treatment
11	0	1990	68	f	C	90	L	D	Del 11q, del 13q	93 L-PR; 95 LP-PR
12	1	1992	64	m	C	135	LP	D	Del 13q	93 LP-PR
13	1	N	68	m	C	168	None	M	Del 11q, del 13q	N
14	0	N	66	f	C	190	LP, 7xCO	D	Add 1p, del 5q, del 10, del 17	N
15	1	1992	57	m	A	68	None	D	Del 13 q	97-99 LPx15-CR; 01-02 Lpx11-PR
16	0	1994	68	m	A	87	None	I	Del 13q	98 LPx43-CR
17	0	N	57	m	A	130	None	I	Del 13 q	96-98 LPx20-CR; 99 LPx 8-PR; 00-01 CHOPx17-PR; 01 GAM-PR; 02 FLUx5-CR; 02 RITx4-PR; 03 RADx2-PR; 03 FLU+CYC-NR
18	0	1995	73	f	C	87	None	M	12q trisomy	95-96 LPx8-PR; 97 CHOPx3+GAM-PR; 97 FLUx3-PR
19	0	1996	55	m	A	40	None	M	12q trisomy, 6p trisomy, 13 q del	96 LPx4-PR; 97-99 CHOPx15+GAM-PR; 02 FLU+CYCx2-CR
20	0	1996	53	m	B	72	None	D	Normal	No treatment
21	1		73	m	B	99	None	D	del 11q	No treatment
22	0	1996	55	m	B	67	None	I	del 6q, del 13q	99 FLU-PR; AUTO STC 00-CR; 04 RIT+CHOPx8+GAM-PR
23	0	1995	62	m	B	62	None	M	del 11q	97 LPx10-CR
24	0	N	79	f	B	97	4xCHOP	M	Normal	N
25	0	N	54	m	B	220	None	D	12q trisomy, del	N

										13q
26	1	N	48	m	A	93	None	I	Normal	N
27	1	1995	67	m	A	74	None	M	Del 13q	99-00 LPx10-PR; 01 LPx5-PR
28	0	1991	67	m	C	183	LP	D	del 11q	96 LPx6-PR; 97-98 LPx8-PR; 99 CHOPx2-PR
29	0	1997	78	m	B	231	None	M	Translocation 4;11	98-99 L-PR; 00 LPx5-PR
30	0	1996	69	f	A	75	None	M	del 4q, del 16, del 17, del 18	00 LP-NR; 00 CHOPx3-PR
31	1	1991	59	m	A	106	LP	M	Normal	94-95 L-PR; 01 LPx15-CR; 04 LPx6-PR; 05 LPx3-PR
32	0	N	70	m	A	147	None	D	Normal	N
33	N	N	57	m	A	190	None	M	Normal	N
34	N	1979	75	f	B	180	None	D	Normal	No treatment
35	0	1992	76	f	C	211	None	M	del 11q	98 Lpx3-PR
36	0	1989	78	f	A	158	None	I	Trisomy 16	No treatment
37	N	1998	63	m	C	198	None	I	Trisomy 12	98-99 LPx15-CR; 02 LPx3-NR; 02 CHOP x5 NR, 02 FLUx1-PR; 03FLU+CYCx4-PR; 03 FLU+CYC+RITx4-PR

Abbreviations: NO, patient number; MUT, IgHV mutation status, with 0 = no mutation, 1 = mutation, N = not evaluated; YEAR, year of diagnosis, with N = not known; AGE, age at diagnosis; SEX, m = male, f = female; BINET, clinical classification by Binet; LEUK, peripheral blood leukocytes; TREAT, treatment before leukaemic cell isolation, with LP = Leukeran (chlorambucil) and prednisolon, CHOP = cyclophosphamide- (hydroxydaunorubicin (doxomycin)-Oncovine(vincristine)-prednisolon; HIST, bone marrow histology, with I = interstitial, N = nodular, D = diffuse, M = mixed; CYTOGENETICS, cytogenetical variation; CLINICAL RESPONSE, clinical responses after sample collection, with CR = complete response, PR = partial response, NR = no response, FLU = fludarabine, GAM = gamma irradiation, RIT = rituximabi, CYC = cyclophosphamide, N = not known.

3.2.2. Isolation of leukaemic cells

Peripheral blood mononuclear cells were isolated from heparinised (Noparin, Novo Nordik, Dagsvaerd, Denmark) blood samples by means of centrifugation over a Lymphoprep layer (Nycomed, Oslo, Norway) at a density of 1.077 g/ml. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and once with a medium consisting of RPMI 1640 (20 mM Hepes, ICN Biochemicals, Costa Mesa, CA), 10% heat-inactivated foetal calf serum (Gibco BRL, Paisley, Scotland), 2 mM L-glutamine (Gibco BRL) and antibiotics (Gibco BRL, penicillin, 50 U/ml, streptomycin, 50 µg/ml). Cell counting was performed by using Technicon H1, H2 or H3 analyzers (Bayer Diagnostics). The density gradient centrifugation yields – with no other purification steps – were good (range 29%–92%; mean 61.7%; SD 16.2%, n = 36). This information is necessary for the interpretation of the results. The proportion of monocytes plus polyclonal T and B lymphocytes was 1%–13%, indicating that 87%–99% of the isolated cells represented the leukaemic population.

3.3. Methods

3.3.1. The experimental drugs and irradiation used

The following drugs and types of irradiation were used in the present study series (**I**, **II**, **III** and **IV**): **chlorambucil** (Sigma Chemical Co., St. Louis, MO); **2-chloro-2'-deoxyadenosine** (Sigma); **cyclosporin** (Cyclosporin A, Sandoz Pharmaceutical Co., Basle, Switzerland); **cisplatin(II)diammine-dichloride** (cisplatin, Sigma); **doxorubicin hydrochloride** (Adriamycin, 2 mg/ml, Pharmacia, Uppsala, Sweden); **2-fluoroadenine-9-beta-D-arabinofuranoside** (fludarabine, Sigma); **prednisolon sodium succinate**; Di-Adreson-F (prednisolone, N.V. Organon, Oss, the Netherlands); **vincristine** (Oncovin, 1mg/ml, vincristine sulfate, Lilly France SA, Fegersheim, France); **verapamil** (Verpamil 2.5 mg/ml, Orion Ltd., Espoo,

Finland); **gamma irradiation:** 0.007 Gy s⁻¹ (¹³⁷Cs source; Molsgaard Medical, Denmark); **UV-C irradiation:** 1 J m⁻² s⁻¹ (UV Unit; Gelman Sci-ences, Ann Arbor, MI).

3.3.2. Cytotoxicity assay by leucine incorporation

The cytotoxic effects of nine drugs and two types of irradiation against peripheral blood lymphocytes from 36 CLL patients were assessed using 4-day cultures by adding five different concentrations (0, 2, 10, 50, 250 and 1,250 nmol/l) of test drugs to the cultures in 96-well microplates. Alternatively, the cells were irradiated by five different doses of ionizing and UV irradiation. 200,000 cells per well were seeded in a volume of 100 µl (2 x 10⁶ cells per ml). The effects were monitored by assessing protein synthesis using ¹⁴C-leucine incorporation. ¹⁴C-leucine (specific activity 1.3 mCi/mmol, 0.5 µCi/ml) was added for the final 24 hours of culture. After incubation, the proteins were precipitated with 0.2 M perchloric acid and collected on fibreglass filters using a multiple cell harvester (LKB Wallac 1295-001, Turku, Finland). The radioactivity incorporated was measured in a liquid scintillation counter (Wallac 1410). ID80 values were calculated from dose-response curves using duplicate or triplicate assays with 5 drug/irradiation doses. The ID80 values are doses causing 80% inhibition in cell viability, as measured by means of ¹⁴C-leucine incorporation. Previously, good correlation has been demonstrated between the number of living cells and leucine incorporation in 4-day cultures *ex vivo*, using the same drugs and types of irradiation (Vilpo and Vilpo 1996; Vilpo et al. 2000b).

3.3.3. Surface antigens and immunophenotyping by flow cytometry

Immunophenotyping of the all samples was performed by means of flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry systems, California) using commercial mouse monoclonal

antibodies and respective immunoglobulin isotype controls. The antibodies used in Study II are summarised in Table 6.

Table 6. Investigated monoclonal antibodies.

Antigen (source) ^a	Label	Isotype control	Clone
CD2 (B)	FITC	γ2a	Leu-5b
CD4/CD8 (B)	FITC/PE	γ1/γ2a	Leu-3a/leu-2a
CD20 (B)	FITC	γ1	Leu16
CD45RA (B)	FITC	γ1	Leu-18
CD45RO (B)	PE	γ2a	Leu-45RO
CD122 (B)	PE	γ1	Anti-IL-2R-p75
CD19 (B)	PE	γ1	SJ25C1
CD5 (B)	FITC	γ2a	Leu-1
CD23 (B)	PE	γ1	Leu-20
FMC7 (I)	FITC	γ	FMC7
CD22 (B)	FITC	γ2b	Leu-14
CD34 (B)	PE	γ1	8G12
CD38 (B)	PE	γ1	Leu-17
CD10 (B)	FITC	γ2a	Anti-CALLA
CD124 (I)	PE	γ1	S4-56C9
CD126 (I)	PE	γ1	M91
CD11c (D)	FITC	γ1	KB90
CD14 (D)	FITC	γ2a	TUK4
CD21 (D)	FITC	γ1	1F8
CD79b (D)	FITC	γ1	SN8
mIgG (P)	FITC	γ1	G18-145
mIgD (P)	FITC	γ2a	IA6-2
mIgM (P)	PE	γ1	G20-127
CD95 (P)	FITC	γ1	DX2
CD40 (P)	FITC	γ1	5C3
CD27 (P)	FITC	γ1	M-T271
CD130 (P)	PE	γ1	VC041
CD25 (B)	FITC	γ1	Anti-IL-2R
CD154 (B)	PE	γ1	Anti-gp39
CD80 (B)	PE	γ1	Anti-B7
CD45/CD14 (B)	FITC/PE	γ1/γ2b	Anti-Hle-1/leu-3M
κ/λ (B)	FITC/PE	γ1/γ1	TB28-2/1-155-2

^a**Abbreviations:** B, Becton Dickinson; D, Dako; I, Immunotech; P, Pharmingen. For a more accurate description of antigen proteins, see, e.g.,

<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein&itool=toolbar>

3.3.4. Analysis of the immunoglobulin variable region gene mutation status by PCR

For Study **III**, the mutation status of IgHV genes was analysed by means of polymerase chain reaction (PCR). Because previously received chemotherapy modifies the in vitro chemosensitivity, analyses were performed only for untreated patients. Additionally, three patients were excluded because their IgHV gene mutation status could not be determined. The final count of patients included in the analysis was 24.

Variable (V) gene family-specific (PCR) amplification was performed using six family-specific V primers and one joining (J) primer. The PCR amplification and the PCR conditions have been detailed elsewhere (Li et al. 1999). To distinguish monoclonal PCR products from polyclonal ones, single-stranded conformation polymorphism analysis was performed using GenePhor system electrophoresis (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. Clonal PCR products from rearrangements were sequenced directly with Big-Dye Terminator Cycle Sequencing Reaction Kits (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). All sequence reactions were analysed using an automated DNA sequencer (ABI 377, Applied Biosystems, Foster City, CA, USA). The obtained sequences were aligned with the closest published germline genes using the IgBLAST, the V-BASE and the international IMGT databases. IgHV gene sequences deviating by more than 2% from the corresponding germline gene were defined as mutated, whereas sequences displaying less than 2% mutations were considered unmutated.

3.3.5. Analysis of the apoptosis-associated genes by quantitative RT-PCR

In Study **IV**, expressions of the apoptosis-associated genes were compared to drug and irradiation sensitivities. The expressions of the apoptosis-associated genes (*Bcl2*, *Bax*, *Bcl2l1*, *Bcl2a1*, *Mcl1*,

Dapk1, *Myc*) were analysed by means of quantitative real-time transcription polymerase chain reaction (QRT-PCR). Gene expression analysis failed in four cases, and the final patient count was therefore 32.

Total RNA was extracted using Trizol Reagent (Gibco BRL, Grand Island, NY, USA). The RNA samples were treated with DNase I (Boehringer Mannheim, Mannheim, Germany) to remove the genomic DNA contaminating the preparations, following the instructions of the manufacturer. The quality and integrity of the RNA were checked by means of electrophoresis using a 1% agarose gel with ethidium bromide staining and UV transillumination. RNA concentrations were measured by a spectrophotometer at 260 nm.

The primers and other analytical methods have been described in detail previously (Nagy et al. 2003). In brief, cDNA was synthesized using 0.5 µg total RNA. PCRs with gene-specific primers were performed in a LightCycler thermal cycler (Roche). For each cDNA sample, the PCR was run in duplicate. Standard curves were obtained by means of serial dilutions of the beta-globulin gene (DNA Control kit, Roche), according to the supplier's instructions. The concentration of each gene product was determined on the basis of a kinetic approach using LightCycler software (Roche). The levels of beta-actin were measured and used for the normalization of RNA quantity and quality differences in all samples.

3.3.6. Statistical analysis

Correlations were calculated using the Spearman's rank order correlation test. Multiple regression analyses were carried out using forward stepwise regression. The Mann-Whitney U test was applied for group comparisons. Statistical analyses were performed using the statistical software package STATISTICA for Windows (ver. Win 5.1D, 1996 StatSoft, Inc., Tulsa, OK).

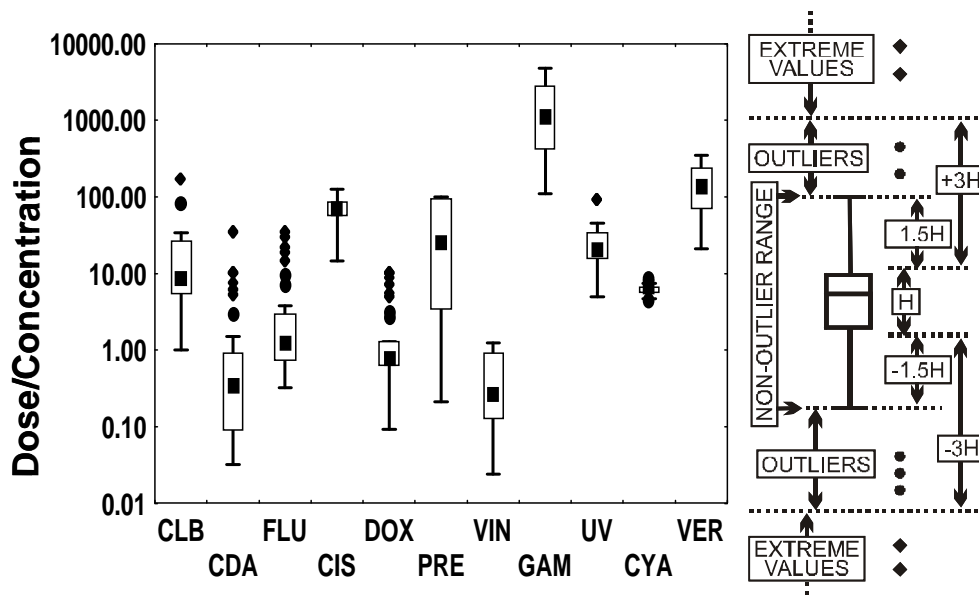
RESULTS

4.1. Multidrug resistance (Study I)

In Study I, sensitivities of nine drugs and two types of irradiation against the peripheral blood leukaemic cells of 36 CLL patients were compared. These results are based on 2,376 duplicated cultures, with six doses, eleven test agents and 36 patients.

From the tested drugs, chlorodeoxyadenosine and vincristine induced an 80% median inhibition in leucine incorporation by the lowest concentrations (Figure 1). Moreover, the drugs requiring the highest concentrations for a similar effect were verapamil and cisplatin. Cyclosporin A demonstrated the lowest and prednisolon the highest variation in the responses. Furthermore, the ID80 values for prednisolon could not be determined for all patients, which is why ID50 values were used. These sensitivity results are mostly in accordance with previously published ones (Bosanquet and Bell 1996; Bosanquet et al. 2009).

Figure 1. In vitro sensitivities of the peripheral blood leukaemia cells of 36 CLL patients to nine drugs and two types of irradiation.



Abbreviations: CLB, chlorambucil; CDA, chlorodeoxyadenosine; FLU, fludarabine; CIS, cisplatin; DOX, doxorubicin; PRE, prednisolon; VIN, vincristine; GAM, gamma irradiation; UV, UV irradiation; CYA, cyclosporine A; VER, verapamil. Median values, ranges, outliers and extremes are illustrated according to the statistics given on the right side of the figure. The ranges of outliers and extremes are illustrated in the classic box and whisker plot where the upper box value is the 75th and the lower box value the 25th percentile. The outlier coefficient is equal to 1.5 and the extreme coefficient equal to 3. The ID80 values are expressed in microM, with the exception of gamma irradiation in Gy and UV irradiation in J/M². The patients with outlier or extreme values are characterised further in Table 8.

The extreme and outlier values are separately illustrated in Figure 1. This was our statistical approach to determine relative cellular drug resistance. Individual cases are discussed in Table 7.

Out of the 36 patients, 14 were categorised as belonging to the outlier or extreme group of one or more drugs. Furthermore, 6/9 patients who had previously received chemotherapy and 8/27 previously untreated patients were categorised into this outlier or extreme group. This result indicates that the ratio of resistant/sensitive cases in the chemotherapy group was 4.8 times higher than among the previously and currently untreated patients ($P=0.0484$, χ^2 -test, $n=36$).

The concordance between previous or ongoing drug therapy and in vitro drug resistance was poor. In three out of the nine cases, the patients had received the same drug to which he or she had demonstrated drug resistance (Table 7). Subsequent clinical responses for these outlier and extreme patients were available in 10 of 14 cases (Table 5). In three out of ten cases, treatments were not needed, and three out of the remaining seven patients had received the same drug to which he or she demonstrated drug resistance. Patient no. 18 demonstrated in vitro resistance against fludarabine, and her clinical response to fludarabine was partial. Patients no. 19 and 22 demonstrated in vitro resistance against doxorubicine and their clinical responses to CHOP treatment were partial.

Table 7. Patients with extreme and outlier ID80 values in cytotoxicity testing.

Patient # (FAB type)	Drugs ¹		ID ₈₀ (μM)	Previous treatment ³
2 (CLL)	Fludarabine (O)	- ²	9.5	LP
8 (CLL/PL)	Chlorambucil (E)	+	80.4	LP, 6xCOP, 7xCHOP
	2-Chlorodeoxyadenosine (E)	-	35	
	Fludarabine (E)	-	22	
	Doxorubicin (E)	+	7.0	
10 (CLL)	UV-irradiation (E)	-	92	None
	Chlorambucil (E)	-	165	
	2-Chlorodeoxyadenosine (E)	-	10	
	Fludarabine (E)	-	34	
11 (CLL)	Doxorubicin (E)	-	8.5	L
	UV-irradiation (E)	-	92	
14 (CLL/PL)	2-Chlorodeoxyadenosine (E)	-	10	LP, 7xCOP, 10xCHOP
	Fludarabine (E)	-	30	
15 (CLL)	Doxorubicin (O)	-	2.6	None
18 (CLL, mix)	2-Chlorodeoxyadenosine (E)	-	5.1	None
	Fludarabine (E)	-	14	
19 (CLL/PL)	Doxorubicin (E)	-	5.3	None
	Cyclosporin A (O)	-	7.5	
20 (CLL/PL)	2-Chlorodeoxyadenosine (E)	-	7.1	None
	Fludarabine (E)	-	19	
21 (CLL, mix)	Doxorubicin (O)	-	3.1	None
22 (CLL/PL)	Doxorubicin (E)	-	10	None
24 (CLL, mix)	2-Chlorodeoxyadenosine (E)	-	6.0	4xCHOP, LP
	Fludarabine (O)	-	7.0	
	Doxorubicin (E)	+	5.0	
	Cyclosporin A (O)	-	4.4	
29 (CLL)	Doxorubicin (E)	-	10	None
31 (CLL)	2-Chlorodeoxyadenosine (O)	-	3.0	LP

Abbreviations: FAB, The French-American-British classification system; ¹ E = extreme and O = outlier (see Figure 1); ² - = not given previously, + = previously given; ³ L = Leukeran (chlorambucil), P = prednisolon, CH(O)P = cyclophosphamide-(hydroxydaunorubicin (doxomycin)-Oncovine(vincristine)-prednisolon.

Multidrug resistance was analysed by using the non-parametric Spearman's rank order correlation analysis. The results are illustrated in Table 8. There were strong correlations between i) chlorambucil and gamma-irradiation, ii) chlorodeoxyadenosine and fludarabine, iii) chlorodeoxyadenosine and vincristine, iv) chlorodeoxyadenosine and gamma-irradiation, v) fludarabine and cisplatin, vi) doxorubicin and gamma-irradiation, vii) prednisolon and cyclosporin

A, as well as viii) vincristine and verapamil. These correlations did not change remarkably if the nine treated patients were excluded. However, the correlations of treated patients were more variable: 5/9 demonstrated a stronger correlation, 1/9 a similar correlation and 3/9 a less stringent correlation than in the total patient group.

Table 8. Relationships between the cytotoxicity of nine drugs and two types of irradiation in vitro in cells from 36 CLL patients.

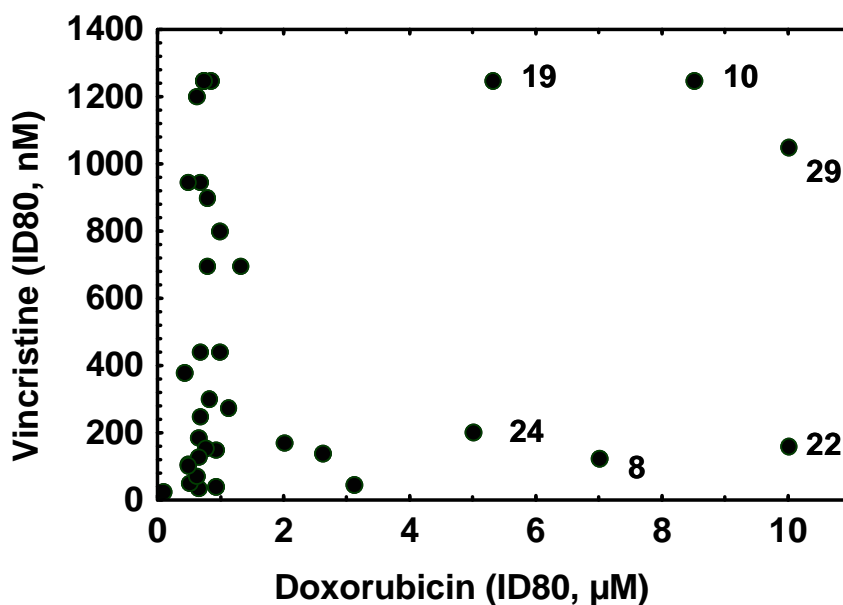
Drug	CLB8 0	CDA8 0	FLU8 0	CIS80	DOX8 0	PRE5 0	VIN80	GAM8 0	UV80	CYA8 0
CDA80	0.36 0.030									
FLU80	0 0.24 0.150	0.62 0.0000								
CIS80	9 0.27 0.116	0.29 0.0866	0.44 0.007							
DOX80	5 0.32 0.064	0.39 0.0209	0.11 0.520	0.28 0.107						
PRE50	0 0.04 0.819	0.20 0.2430	0.22 0.202	-0.02 0.921	0.21 0.2219					
VIN80	3 -0.01 0.945	0.42 0.0099	0.12 0.501	-0.11 0.522	0.26 0.1373	0.31 0.061				
GAM80	6 0.48 0.002	0.50 0.0020	0.15 0.391	0.03 0.852	0.59 0.0002	0.25 0.138	0.42 0.010			
UV80	8 -0.07 0.678	0.17 0.3304	0.38 0.023	-0.15 0.389	0.13 0.4575	0.26 0.121	0.15 0.397	0.09 0.6139		
CYA80	7 0.16 0.364	0.12 0.5040	-0.05 0.791	0.07 0.694	0.08 0.6483	0.47 0.003	0.34 0.041	0.14 0.4053	-0.16 0.351	
VER80	8 -0.17 0.316	0.24 0.1675	0.24 0.161	-0.16 0.347	0.26 0.1270	0.39 0.017	0.53 0.000	0.31 0.0662	0.41 0.013	0.14 0.4097
	1		1	8		8	9		9	

Note: ID80 values were used, with the exception of the ID50 values for prednisolon. Correlations were calculated by using Spearman's rank order regression analysis. The values shown are R-values, with the corresponding *p*-values below; statistically significant correlations (*p* < 0.01) are expressed in boldface. Abbreviations: ID80, inhibition dose 80% (prednisolon 50%); CLB, chlorambucil; CDA, chlorodeoxyadenosine; FLU, fludarabine; CIS, cisplatin; DOX, doxorubicin; PRE, prednisolon; VIN, vincristine; GAM, gamma irradiation; UV, ultraviolet irradiation; CYA,, cyclosporine A; VER, verapamil.

It is conceivable that the CLL/PL patients are less drug sensitive than is the case with the typical morphological forms of CLL. In our material 5/6 CLL/PL cases occurred in the drug resistance category (Table 7), which would suggest that the CLL/PL group seems to be more cross-resistant than the typical morphological form of CLL.

Two P-glycoprotein-dependent drugs were analysed separately, namely doxorubicin and vincristine. No correlation could be observed between these drugs (Figure 2). Three cases (patient nos. 10, 19, 29) clustered differently from the others, indicating a cross-resistance. Interestingly, none of the three belonged to the chemotherapy group, as could be assumed.

Figure 2. Relationships of vincristine and doxorubicin sensitivity of peripheral blood leukaemic cells from 36 CLL patients. The comparison concerns ID80 values which were determined as described in Methods section. The high values can be clustered in three different groups: i) vincristine resistant alone, ii) doxorubicin resistant alone and iii) cross-resistant.



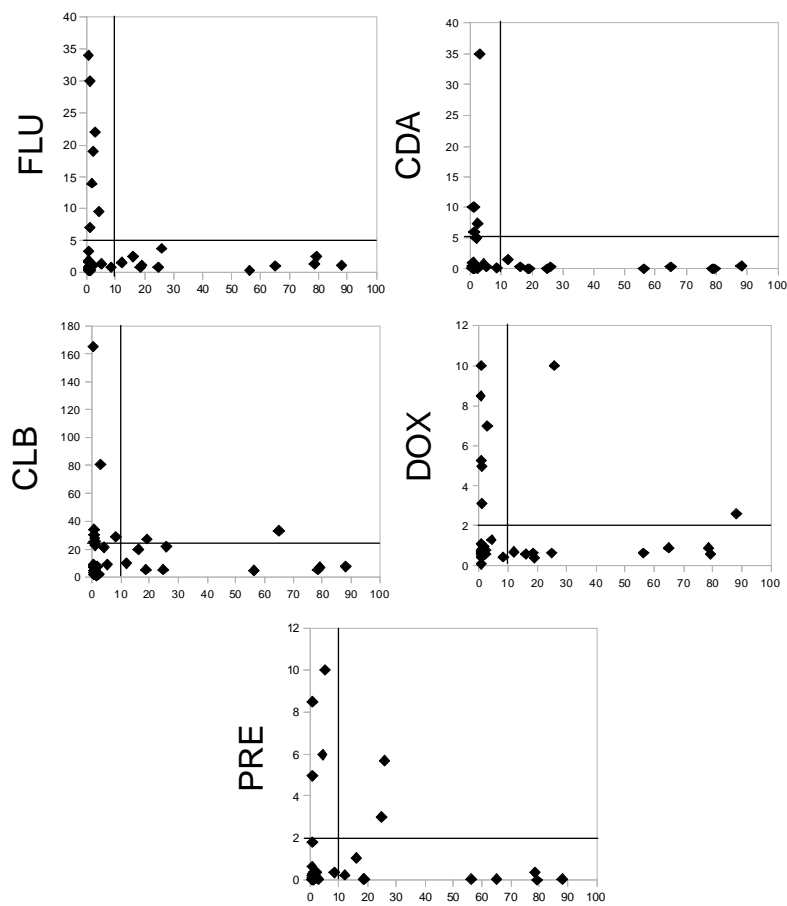
4.2. Prognostic value of surface antigens in drug and irradiation sensitivities (Study II)

In Study **II**, the prognostic value of surface antigens in drug and irradiation sensitivities were analysed.

No statistically significant correlations were found between any surface antigen expressions and drugs or irradiations sensitivities, when they were analysed by means of the Spearman's Rank Order Correlation test.

The expressions of CD80 divided the patients into two mutually exclusive groups as regards the sensitivity to the purine analogues 2'-chlorodeoxyadenosine ($p=0.0001$, χ^2 -test, $n=36$) and fludarabine ($p=0.001$, χ^2 -test, $n=36$). All resistant cases had a very low CD80 expression, as indicated by the percentage of positive cells or by geometric mean fluorescence (Figure 3). In other words, positive CD80 expression seems to sensitise a cell to purine analogues. A similar trend, although not exclusive, was observed with chlorambucil ($p=0.0017$, χ^2 -test, $n=36$), doxorubicin ($p=0.1335$, χ^2 -test, $n=36$) and prednisolon ($p=0.51$, χ^2 -test, $n=36$), but not with the other drugs or the two types of irradiation tested.

Figure 3. CD80 expression and the in vitro sensitivities of peripheral blood leukaemic cells from 36 CLL patients to fludarabine, chlorodeoxyadenosine, chlorambuil, doxorubicine and prednisolon. ID80 values were used with the exception of prednisolon, for which ID50 was applied. CD80 expression was examined by determining the percentages of positive cells.

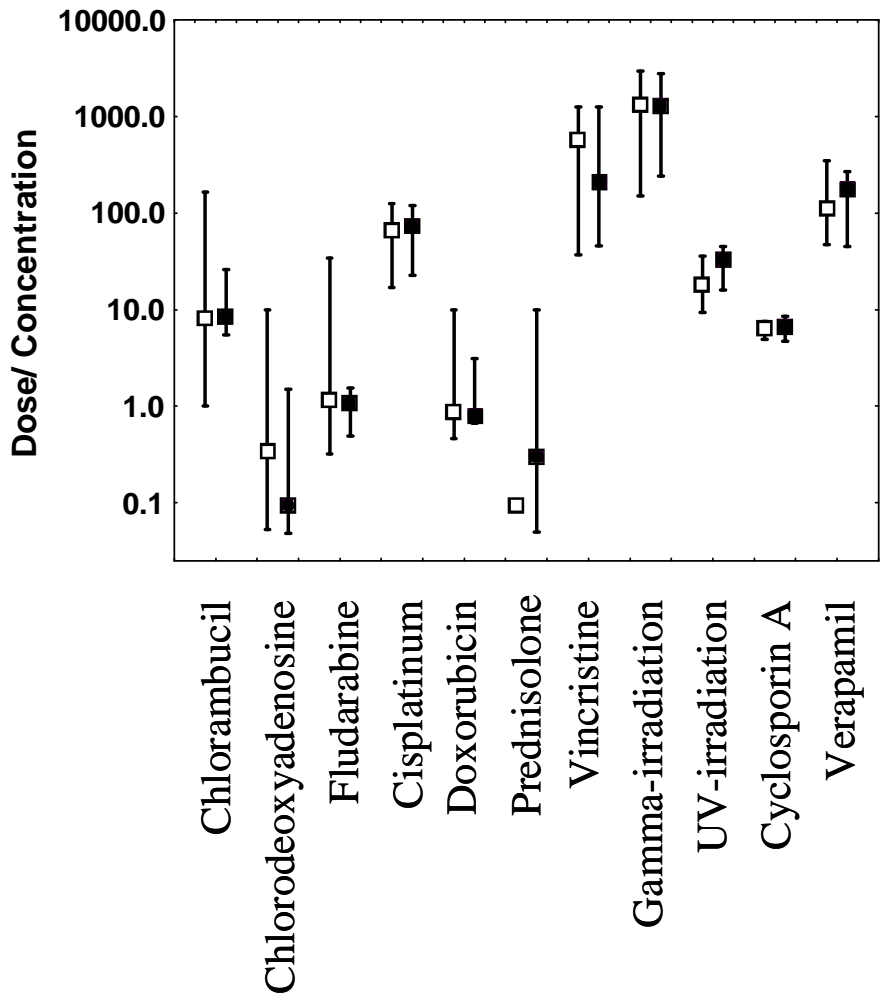


4.3. Effect of immunoglobulin variable region mutation status on drug and irradiation sensitivities (Study III)

The effect of IgHV mutation status on drug and irradiation sensitivities was analysed in Study **III**.

Out of the 24 patients, eight cases (33%) displayed mutated IgHV genes, whereas 16 (67%) cases were unmutated. The ID80 values were similarly distributed in both types of CLL; the results are illustrated in Figure 4. The only statistically significant difference was seen with regard to prednisolon, which had to be evaluated at the ID50 level, since ID80 was not achieved in all cases even when a high dose of the drug was tested. UM-CLL cases were, on average, slightly more sensitive than M-CLL cases ($p=0.041$).

Figure 4. Sensitivity in vitro of peripheral blood leukaemic cells of 16 UM-CLL patients and 8 M-CLL patients to nine drugs and two types of irradiation. The ID80 values (ID50 values for prednisolon) are expressed in micro-mol/L, with the exception of gamma irradiation in Gy and UV irradiation in J/m². Medians and total ranges are illustrated. Open symbols = UM-CLL; closed symbols = M-CLL.



We also evaluated whether IgHV mutation status affects the variation of drug/irradiation sensitivity in individual patients, so we assessed the distribution of heterogeneity of intra-individual sensitivity in cases of M-CLL versus UM-CLL. To this end, we also determined the ID20 values and adopted the ratio of ID80 per ID20 as a measure of that parameter. The results

illustrated in Table 9 clearly demonstrate that there were no significant differences in intra-individual heterogeneity of sensitivity to any of the toxic agents in these two groups of CLL.

Table 9. Heterogeneity of the sensitivity of UM-CLL and M-CLL cells to nine drugs and two types of irradiation.

Drugs and irradiation types	UM-CLL		M-CLL		Mann-Whitney U: p
	MEAN	SD	MEAN	SD	
Chlorambucil	14.8	12.7	15.4	7.7	0.4260
Chlorodeoxyadenosine	40.7	44.9	24.7	23.5	0.3425
Fludarabine	19.4	25.7	9.3	3.3	0.5815
Cisplatinum	9.7	5.1	7.2	3.2	0.0662
Doxorubicin	19.9	20.7	27.9	40.5	0.7383
Prednisolone	72.15	244.27	93.52	123.15	0.0758
Vincristine	47.4	49.1	74.3	141.0	0.8065
Gamma-irradiation	31.8	31.9	30.4	19.2	0.7595
UV-irradiation	6.1	2.4	8.4	3.7	0.1417
Cyclosporin A	4.5	3.5	2.3	1.5	0.0662
Verapamil	16.4	17.7	15.1	12.7	0.3913

Note: Values represent ID80/ID20 ratios with the exception of prednisolon, for which the ratio ID50/ID20 was determined.

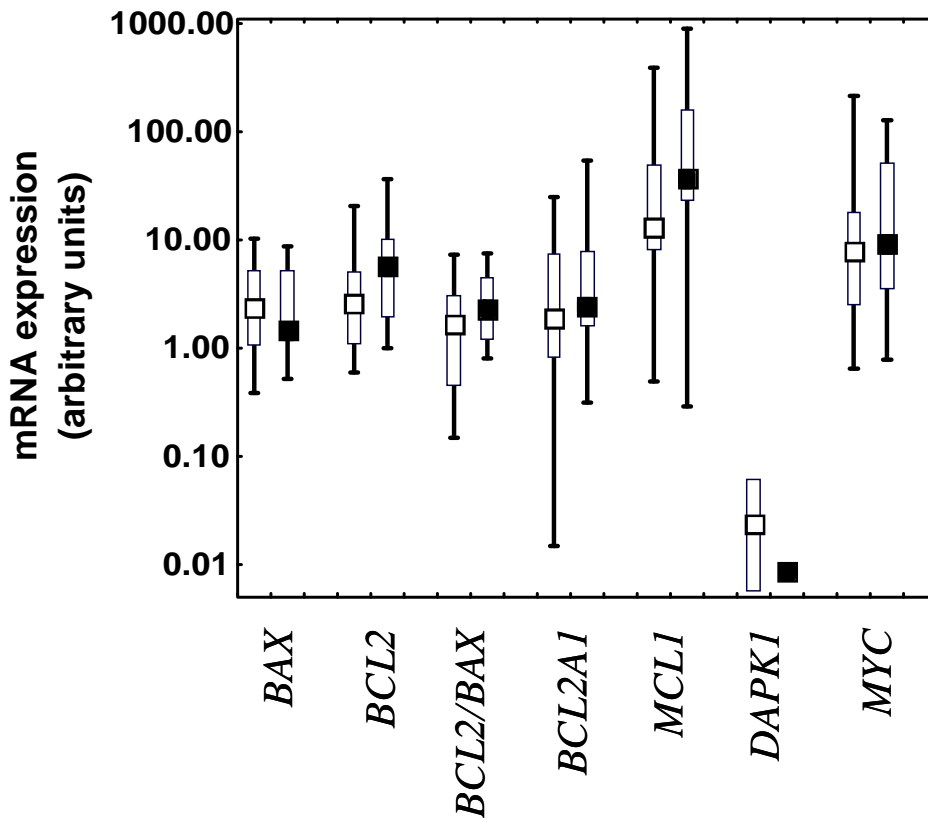
4.4. Effect of Apoptosis associated genes to drug and irradiation sensitivities (Study IV)

In Study IV the expression levels of apoptosis-associated genes were compared to drug and irradiation sensitivities.

Untreated (23) and previously treated (9) groups were compared with the Mann-Whitney U test. No statistically significant differences were observed in the expressions of apoptosis-related genes between these groups; results are illustrated in Figure 5. Owing to very low values, approaching the blank level, *Bcl2l1* was excluded from the analyses. Rank-order correlations were calculated between the expression levels of apoptosis-related genes and the ex vivo ID80 values obtained for nine drugs and two types of irradiation (Table 10). No statistically

significant positive correlations were demonstrated between chemo/radioresistance (large ID80 values) and the strength of the expression of anti-apoptotic genes (*Bcl2*, *Bcl2a1* and *Mcl1*). Similarly, the strength of the expression of pro-apoptotic genes (*Bax*, *Myc* and *Dapk1*) was not associated with increased sensitivity (small ID80 values) to the agents tested. On the other hand, several negative, albeit very weak, correlations between drug/irradiation resistance (large ID80 values) and the expression of anti-apoptotic genes were noted at the significance level of $p < 0.05$ (Table 10). These were between (i) *Bcl2a1* and doxorubicin, (ii) *Bcl2a1* and vincristine, (iii) *Bcl2a1* and gamma irradiation, (iv) *Mcl1* and gamma irradiation, (v) *Mcl1* and cyclosporin A, (vi) *Bcl2* and cisplatin, and (vii) the *Bcl2/Bax* ratio and chlorodeoxyadenosine.

Figure 5. Comparison of the expression at the mRNA level of apoptosis-related genes in 32 CLL patients. Open symbols represent patients who had not received chemotherapy (n=23) and closed symbols represent previously treated patients (n=9). Medians (squares), the 25th and 75th percentiles (columns) and total range (whiskers) are illustrated.



The ratios of *Bcl2/Bax* were also analysed and compared to the drug and irradiation sensitivities. There were no significant correlations between drug or irradiation sensitivities and *Bcl2/Bax* ratios. Furthermore, the ratio did not vary at a statistically significant level between untreated and treated groups.

Table 10. Correlations between the expression of apoptosis-related genes and the *ex vivo* cytotoxicity of nine drugs and two types of irradiation *in vitro* in cells from 32 patients with chronic lymphocytic leukaemia.

Drug	Apoptosis-associated gene expressions							
	Bax	Bcl2l1	Bcl2a1	Dapk1	Myc	Mcl1	Bcl2	Bcl2/Bax
CLB	0.00	0.30	0.01	-0.05	-0.03	-0.15	-0.33	-0.33
	<i>0.979</i>	<i>0.100</i>	<i>0.952</i>	<i>0.766</i>	<i>0.879</i>	<i>0.417</i>	<i>0.069</i>	<i>0.063</i>
CDA	0.05	-0.06	-0.28	0.15	0.20	-0.14	-0.20	-0.36
	<i>0.800</i>	<i>0.760</i>	<i>0.117</i>	<i>0.404</i>	<i>0.281</i>	<i>0.460</i>	<i>0.264</i>	0.045
FLU	-0.08	-0.27	-0.08	-0.02	0.02	0.08	0.00	0.00
	<i>0.654</i>	<i>0.142</i>	<i>0.659</i>	<i>0.917</i>	<i>0.895</i>	<i>0.678</i>	<i>0.986</i>	<i>0.996</i>
CIS	-0.13	0.13	-0.22	-0.22	0.03	-0.24	-0.41	-0.31
	<i>0.477</i>	<i>0.465</i>	<i>0.217</i>	<i>0.218</i>	<i>0.880</i>	<i>0.194</i>	0.021	<i>0.083</i>
DOX	0.11	-0.23	-0.42	0.17	0.28	-0.22	-0.21	-0.30
	<i>0.548</i>	<i>0.210</i>	0.019	<i>0.371</i>	<i>0.120</i>	<i>0.227</i>	<i>0.261</i>	<i>0.106</i>
PRE	0.04	-0.09	-0.23	0.32	0.10	-0.12	0.12	0.00
	<i>0.837</i>	<i>0.621</i>	<i>0.201</i>	<i>0.075</i>	<i>0.582</i>	<i>0.508</i>	<i>0.501</i>	<i>0.994</i>
VIN	0.02	-0.12	-0.41	0.24	0.16	-0.31	-0.30	-0.32
	<i>0.921</i>	<i>0.519</i>	0.018	<i>0.194</i>	<i>0.394</i>	<i>0.088</i>	<i>0.094</i>	<i>0.073</i>
GAM	0.03	-0.08	-0.38	0.14	0.34	-0.38	-0.28	-0.34
	<i>0.869</i>	<i>0.680</i>	0.033	<i>0.451</i>	<i>0.061</i>	0.033	<i>0.126</i>	<i>0.058</i>
UV	0.16	-0.33	-0.15	0.13	0.33	0.13	0.29	0.11
	<i>0.386</i>	<i>0.069</i>	<i>0.428</i>	<i>0.468</i>	<i>0.065</i>	<i>0.468</i>	<i>0.107</i>	<i>0.531</i>
CYA	-0.23	0.14	-0.21	0.16	-0.21	-0.40	-0.31	-0.09
	<i>0.213</i>	<i>0.440</i>	<i>0.241</i>	<i>0.385</i>	<i>0.260</i>	0.022	<i>0.090</i>	<i>0.621</i>
VER	0.10	-0.23	-0.18	0.28	0.17	-0.01	0.17	0.07
	<i>0.581</i>	<i>0.197</i>	<i>0.338</i>	<i>0.124</i>	<i>0.352</i>	<i>0.954</i>	<i>0.347</i>	<i>0.712</i>

Note: The values shown are R-values (above) with the corresponding p-values (below, in *italics*).

Correlations were calculated by using Spearman's Rank Order regression analysis. Statistically significant correlations ($p < 0.05$) are indicated in boldface. Abbreviations: CLB, Chlorambucil; CDA, Chlorodeoxyadenosine; FLU, Fludarabine; CIS, Cisplatin; DOX, Doxorubicin; PRE, Prednisolon; VIN, Vincristine; GAM, Gamma irradiation; UV, Ultraviolet irradiation; CYA, Cyclosporin A; VER, Verapamil.

Multiple regression analysis with a forward stepwise approach did not reveal any statistically significant associations between the investigated genes and cytotoxicity ID80 values.

DISCUSSION

5.1. Methodological considerations

Several methods have been used for cell counting in drug sensitivity studies. The Differential Staining Cytotoxicity (DiSC) assay is a manual and labour-intensive method, while the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a semiautomatic method. In the current study, the cells' viability was measured by the incorporation of radioactive ¹⁴C labelled leucine to CLL cells. Leucine is one of the essential amino acids that a cell cannot produce by itself, and measuring its radioactive labelled incorporation therefore reflects the strength of cell viability. Leucine incorporation has been shown to correlate with living cells in many studies (Vilpo and Vilpo 1996; Vilpo et al. 2000a; Vilpo et al. 2000b). In Study I we demonstrated good correlations between trypan blue excluding tests and the level of leucine incorporations. Koski has shown that a decrease in leucine incorporation reflects apoptosis-like cell death as measured by DNA fragmentation, annexin binding and propidium iodide permeability (Koski et al. 2000).

5.2. Multidrug resistance

The main interest for the treating physician is whether or not two drugs are equivalent in efficacy and, therefore, non-cross-resistant. Cross-resistant drugs would not generally be given together, nor would they be administered sequentially if the patient was clinically proven to be resistant to the first one. The chlorodeoxyadenosine sensitivity among the present 36 CLL patients demonstrated a close correlation with fludarabine, vincristine and gamma irradiation. The correlation between in vitro responses to chlorodeoxyadenosine and fludarabine has also been demonstrated by others (Bosanquet and Bell 1996; Bosanquet et al. 2009), and, furthermore, clinically relevant cross-resistance has been reported (O'Brien et al. 1994). An entirely unexpected finding of the present

study was the disclosure of the close relationships between the responses to drugs with very different mechanisms of action, such as chlorodeoxyadenosine and vincristine as well as several others. Although numerous mechanisms of drug resistance have been revealed, our results underline the fact that very little is known about the complexity of the cellular mechanism of drug sensitivity or resistance in a clinical context.

The present results clearly express a relationship between chemotherapy and drug resistance *ex vivo*. The enhanced *in vitro* resistance was not necessarily against the same compound as used in chemotherapy – this phenomenon has also been reported by others (Bosanquet and Bell 1996). For instance, six patients in the extreme/outlier group had been previously treated with chlorambucil, but only one of the six was chlorambucil-resistant.

The multidrug resistant phenotype of CLL cells has been demonstrated in a number of publications. While it is evident that the p-170 is expressed in a significant subset of CLL cases (Sparrow et al. 1993; Burger et al. 1994; Matthews et al. 2006), no clear picture has emerged as to whether their expression is associated with poor prognosis, an advanced stage or prior chemotherapy (Friedenberg et al. 2004). The results of the current work were in accordance with previously reported findings concerning the significance of MDR in CLL. No correlation could be discovered between vincristine and doxorubicin, which are both p-170-dependent agents. Only three patients (8.3%) were simultaneously resistant to both drugs, meaning that the p-170 mechanism was not relevant in rest of the cases. These findings are also in accordance with a previous study; it has been shown that CLL cells are 25 times more sensitive to vincristine than normal lymphocytes and the uptake and efflux in CLL versus normal lymphocytes are essentially similar (Vilpo et al. 2000b).

The pleiotropic drug resistance observed in the present study and other investigations strongly suggests that not only one but rather multiple resistance mechanisms are being induced by chemotherapy (Bosanquet and Bell 1996; Bosanquet et al. 2009). According to our results, some

drug resistance mechanism may still be undefined. The current study also provides new information on putative drug combinations for clinical usage – all non-correlating drug combinations could be putatively used.

The minimal residual disease (MRD) status has been shown to be a good clinical outcome predictor, since patients who achieved complete phenotypic remission as assessed by four- or six-colour flow cytometry had a superior response duration and survival when compared with patients who did not reach MRD (Varghese et al. 2010). Therefore, it is our opinion that ID80 values would reflect clinical outcome better than ID50 values, which have been used in many other studies.

5.3. Prognostic factors of drug and irradiation resistance

Since the development of modern medicine, one of the most significant purposes for a physician has been to provide the prognosis for the disease. There are many clinically relevant biochemical prognostic factors in CLL, such as the expression of the CD38 surface antigen, the mutation status of the IgHV genes, and chromosomal aberrations (Van Bockstaele et al. 2009). In CLL chemotherapy there are only a few clinically relevant prognostic factors, namely chromosome 17p deletion and mutation of p53 protein. If the p53 is mutated or chromosome 17p is deleted, the prognosis for most of the drug therapy is poor (Zenz et al. 2008b).

The surface antigens of the CLL cells are routinely defined at diagnosis. We went through 32 surface antigens of 36 CLL patients and found no statistically significant correlations between the expressions of surface antigens and drug or irradiation sensitivities. The expression of the CD80 antigen predicts partly ex vivo cellular response to fludarabine and chlorodeoxyadenosine. All of the cases with detectable expression of CD80 were sensitive to these drugs when cells were tested in vitro. The same phenomenon, although not as clearly detectable,

was observed with doxorubicin, chlorambucil and prednisolon. In most cases CD80 expression was low.

The expression of the CD38 antigen is the most clinically used prognostic factor of the surface antigens. The prognosis of the disease is poor, if the CD38 antigen expression of the CLL cells is high (Krober et al. 2002; Hock et al. 2009; Van Bockstaele et al. 2009). In our material, CD80 status did not correlate with CD38 expression. Expression of CD38 did not predict drug or irradiation resistance.

CD80 – otherwise known as B7-1 – is a transmembrane glycoprotein expressed mainly on activated antigen-presenting cells. It is also expressed on activated, but not on resting B lymphocytes (Azuma et al. 1993). CD80 is an important costimulatory factor for the B cell receptor (BCR) and in T cell stimulation (Collins et al. 2005). In most B cell lymphomas CD80 is expressed at a low level, and in small lymphocytic leukaemia (SLL), which is a very similar disease to CLL, it is expressed at a very low level or absent (Dorfman et al. 1997). It has been demonstrated that in CLL CD80 is expressed at a low level, as it is also in normal B lymphocytes, and the expression level does not differ between stage I and stage IV cases (Dai et al. 2009). Our results are in accordance with these findings.

The mutation status of the IgHV genes is the most powerful prognostic factor in CLL. The only problem in terms of its clinical usage is that the testing is very labour-intensive. Prognosis for the UM-CLL patient is clearly worse than for a M-CLL patient (Damle et al. 1999; Hamblin et al. 1999), and treatment-free time from diagnosis is also clearly shorter in the UM-CLL group than the M-CLL group (Wiestner et al. 2003). In our untreated CLL patients' data, the sensitivities to nine drugs and two types of irradiation were similar between the UM-CLL and M-CLL groups. In others studies the results have been controversial; in *in vitro* studies, UM-CLL patients have been more sensitive than M-CLL patients to cytarabine and prednisolon (Aleskog et al. 2004), whereas

clinical studies have demonstrated that UM-CLL patients more often require therapy and respond more poorly to chemotherapy, including fludarabine (Damle et al. 1999).

Apoptosis is very closely associated with CLL; one reason for the accumulation of lymphocytes in CLL is the dysregulation of apoptosis (Danilov et al. 2006), and another reason is the increased proliferation of lymphocytes (Messmer et al. 2005). Many of the chemotherapeutic drugs exert their cell-killing effect through apoptosis (Burz et al. 2009). Mutation of the p53 protein leads to drug resistance, but it is quite rare in CLL and thus cannot explain drug resistance in most cases (Zenz et al. 2008a). The Bcl2 protein family, and especially the Bcl2 protein, is the most investigated apoptosis-associated protein (Buggins and Pepper 2010).

In most studies overexpression of the Bcl2 protein has been connected to chlorambucil resistance (Pepper et al. 1997; Pepper et al. 1998; Pepper et al. 1999; Thomas et al. 2000; Pepper et al. 2001), but also negative results, as ours, have been found (Johnston et al. 1997; Bosanquet et al. 2002). Low expression of the Bax protein has been connected to, among other treatments, chlorambucil and fludarabine in CLL (Bosanquet et al. 2002; Pepper et al. 2008), whereas negative results have been found concerning, for example, fludarabine and chlorambucil (Johnston et al. 1997; Bosanquet et al. 2002). A high expression of Mcl1 is associated with rituximab and fludarabine resistance, and it has also been correlated with the stage of disease (Bannerji et al. 2003; Pepper et al. 2008). Low Mcl1 expression has been connected to complete clinical treatment responses in CLL (Kitada et al. 1998; Saxena et al. 2004), although negative results have been published in this regard as well (Grever et al. 2007).

The ratio of anti- and pro-apoptotic proteins, such as the Bcl2/Bax or Mcl1/Bax ratio, is believed to be more important than the expression level of individual proteins. A high ratio of Bcl2/Bax indicated the coincidence of a high expression of Bcl2 and a low expression of Bax. In most of studies, a high Bcl2/Bax ratio has been connected to chlorambucil and fludarabine resistance (Pepper et al. 2001), although negative results have also been observed with fludarabine,

chlorambucil, chlorodeoxyadenosine, cladribine and prednisolon (Johnston et al. 1997; Bosanquet et al. 2002).

The results of previous studies have been conflicted. In our current data we did not find any statistically significant and biologically relevant correlations between the expressions of apoptosis-associated genes and drug or irradiation sensitivities. Seven negative correlations between the expressions of the anti-apoptotic genes and drug resistance were observed, but these were very weak and remained without a biologically relevant explanation. Our results are not directly comparable with other studies, because others have examined the levels of apoptosis-associated proteins whereas we studied the expression levels of apoptosis-associated genes.

Gene expression in quiescent CLL cells is regulated at different levels: i) regulation of RNA transcription, ii) regulation of mRNA stability and iii) regulation of mRNA translation. In CLL the dysregulation of mRNA translation and decay has been investigated from at least two different perspectives. MicroRNAs (miRNA) are a class of small non-coding RNAs regulating mRNA translation and decay. *Bcl2* mRNA is negatively regulated by two miRNAs, miR-15a and miR-16-1. These two microRNAs are located in the chromosome 13q region, and they are deleted in major of cases in CLL (Nicoloso et al. 2007). Furthermore, microRNAs have been linked to the progression and outcome of CLL (Calin et al. 2007). Nucleolin is a cytosolic protein which stabilises *Bcl2* mRNA and thus leads to elevated translation of the Bcl2 protein. The level of nucleolin has been shown to be increased in CLL (Otake et al. 2007).

These regulation mechanisms may explain the different results between the current study and most other reports. Moreover, only the regulation mechanisms of *Bcl2* are known, and it could be expected that other genes also have some kind of as yet undetermined regulation mechanisms, which could explain the differences between the expression levels of genes and protein.

5.4. Limitations of the study and future directions

In the present study series, the number of patients investigated was quite small, and the results included data from both treated and untreated patients. On the other hand, this enables the comparison between untreated and treated patients. Multiple comparison tests have an internal tendency to reveal non-existing correlations. In our Study I, we used small P-values of <0.01 as an indicator for significant finding, and even when multiplied by 10 (corresponding to 10 comparisons in each family), only one of the P-values became higher than 0.05.

The combination of two or three drugs is currently the most common therapy in CLL. Combined chemotherapies are mostly based on fludarabine, which was included in our investigation. Fludarabine is commonly combined with cyclophosphamide (Catovsky et al. 2007) and in recent years also with biological agents such as Rituximab (Tam et al. 2008). Cyclophosphamide or biological drugs were not included in the current study series. Biological agents will play a more important role in future treatments, which is why they should be included in future investigations.

The expression of the CD80 surface antigen seems to predict the sensitivity of CLL cells to fludarabine and chlorodeoxyadenosine and, to a degree, also to doxorubicin, chlorambucil and prednisolon. Flow cytometry analysis is used in the diagnosis of CLL, but expressions of CD80 are not determined routinely. It would be easy to include it in future as a diagnostic tests.

MicroRNAs and their connections to different diseases have been extensively investigated in recent years. In CLL several microRNAs have been found to be connected to the pathogenesis of the disease – these include miR-15, miR-16, miR-29c and miR223. The significance of these microRNAs for drug resistance has not been investigated to date, and this would be an interesting area to explore.

The present sensitivity results were obtained from an in vitro analysis and, as such, do not necessarily directly reflect clinical outcome, although good correlations between in vitro assays

and clinical outcome have been demonstrated (Bosanquet et al. 1999a; Bosanquet et al. 1999b). For this reason, we collected data on the later clinical responses for this patient group. Only three cases from the outlier and extreme group had received the same drug in subsequent chemotherapy to which they had demonstrated in vitro resistance. All three patients had only a partial response against these drugs, reflecting directional connections between in vitro and clinical responses.

SUMMARY AND CONCLUSIONS

In the present study series, peripheral blood leukaemic cells of 36 CLL patients were subjected (?) to nine drugs and two types of irradiation. For the purposes of determining the sensitivity of the CLL cells to the investigated agents, ID80 values were measured. Cross-resistance as well as the impact of i) previous treatment, ii) surface antigens, iii) IgHV gene mutation status and iv) the expression of apoptosis-associated genes on drug resistance were analysed.

- I** Drug or irradiation resistance is more common after chemotherapy, but in most of cases the resistance was not to the same drugs as used in chemotherapy. Cross-resistance was noted between the same drug group agents fludarabine and chlorodeoxyadenosine, but also between very differently acting drugs such as chlorodeoxyadenosine and vincristine. The commonly accepted P-glycoprotein-dependent drugs, doxorubicin and vincristine, were not correlated, indicating that there must be multiple resistance mechanisms rather than one mechanism.
- II** The expression of the CD80 antigen seems to predict fludarabine and chlorodeoxyadenosine sensitivities in CLL patients. All of our study cases with a detectable expression of this antigen were sensitive when tested *in vitro*.
- III** The mutation status of IgHV genes did not predict the *in vitro* sensitivities of the investigated drugs or irradiations in the untreated CLL patient group.

IV Expressions of apoptosis-associated genes (anti-apoptotic: *Bcl2*, *Mcl1*, *Bcl2a1*, *Bcl2l1* and pro-apoptotic: *Bax*, *Myc*, *Dapk1*) were not determinants of the investigated drug or irradiation sensitivities in CLL.

Drug resistance seems to be more common after chemotherapy. Previous treatments may cause drug resistance against other drugs besides the ones used in the therapy. Detectable expression of the CD80 surface antigen seems to predict fludarabine and chlorodeoxyadenosine sensitivity in CLL patients. The mutation status of IgHV genes or expressions of apoptosis-associated genes are not determinants of drug or irradiation sensitivity in CLL.

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ORIGINAL PUBLICATIONS



Relationships of in vitro sensitivities tested with nine drugs and two types of irradiation in chronic lymphocytic leukemia

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Abstract

Extensive research into mechanisms of cytotoxic drug and irradiation resistance have produced few clinically encouraging results. In this report, we apply correlation analyses to drug and irradiation response results from a cohort of 36 classical B chronic lymphocyte leukemia (CLL) patients. Nine drugs and two types of irradiation were selected according to their usefulness in CLL therapy or on the basis of their otherwise interesting mechanisms of action. Part of the results concerning individual drugs have been previously published, but new correlation analyses are presented in this paper. Altogether 2376 duplicate cultures were performed in order to determine ID₈₀ values, i.e. doses causing an 80% inhibition in 4-day cultures when leucine incorporation was used as an indicator of cells vitality. Non-parametric Spearman's rank order correlation confirmed a tight relationship between 2-chlorodeoxyadenosine and fludarabine, as expected. Surprisingly, correlation between two *P*-glycoprotein-dependent drugs, vincristine and doxorubicin, was not demonstrable. A number of entirely unexpected correlations were identified between drugs with very different mechanisms of action: (i) chlorambucil and γ -irradiation; (ii) 2-chlorodeoxyadenosine and vincristine; (iii) 2-chlorodeoxyadenosine and γ -irradiation; (iv) fludarabine and *cis*-platin; (v) doxorubicin and γ -irradiation; (vi) prednisolone and cyclosporin A; (vii) vincristine and verapamil. Our findings emphasize: (i) the usefulness of fresh tumor cells instead of cell lines in cytotoxicity studies; (ii) the great variation in cytotoxicity in individual patients, i.e. tumor cell heterogeneity, as well as patient heterogeneity; and (iii) an entirely unexpected finding that there were tight relationships in drug and irradiation responses between substances supposed to act with very different mechanisms.

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1. Introduction

In cancer chemotherapy the major obstacle is drug resistance (for a review, see [1]). Since, the introduction and development of drug-resistant cell lines, mechanisms of drug resistance have been extensively investigated, but cell line models used to investigate these mechanisms may not directly pertain to the clinical resistance [2]. Many drug resistance mechanisms have been revealed in chronic lymphocytic leukemia (CLL). These include multidrug resistance associated with *P*-glycoprotein and several other mechanisms like multidrug resistance-associated protein, non-*P*-glycoprotein-mediated transport mechanisms, glutathione *S*-transferase and glutathione levels, topoisomerases, metallothioneins, *p53* gene mutation, and drug-specific resistance mechanisms (see Bosanquet and Bell [2]).

We have recently demonstrated with a cohort of 36 classical CLL patients that the ex vivo sensitivity of CLL cells to various chemotherapeutic substances is highly variable. This concerns both inter- and intra-individual variation, which has been demonstrated with: (i) γ -irradiation [3]; (ii) UV-irradiation [4]; (iii) cyclosporin A [5]; (iv) calcium antagonists alone and together with several classic antileukemia drugs [6]; (v) vincristine [7,8]; (vi) 2-chlorodeoxyadenosine and fludarabine [9]; as well as with (viii) prednisolone, doxorubicin, *cis*-platin and chlorambucil [10].

It is likely that the improvement of clinical results can be achieved by individualizing the anticancer therapy. This, on the other hand, means that more information of the relationship about the action of various therapy modalities should be available. For instance, previous correlation analyses by others [11] and by us [9] have demonstrated that the sensitivity spectra of 2-chlorodeoxyadenosine and fludara are very similar. In this paper, we report further correlation analyses concerning the same data and cohort of 36 CLL patients [3–10]. Unexpectedly, significant statistical correla-

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tions were noted in drug and irradiation responses between substances considered to act in very different ways.

2. Materials and methods

2.1. Patients and samples

Clinical specimens were obtained after informed consent from 36 consecutive CLL patients referred to the CLL out-patient clinic at Tampere University Hospital (Finland). Diagnosis and staging were based on standard clinical, morphological and immunophenotyping criteria. All patients had a CD19+/CD23+/CD5+ immunophenotype (the cohort has been reanalyzed in 2000) and mature CLL, CLL/mix or CLL/PL morphology [12]. Most patients had chromosomal aberrations but none indicated chronic lymphoid leukemias other than typical CLL [10].

Peripheral blood mononuclear cells were isolated from heparinized (Noparin, Novo Nordisk, Dagsvaerd, Denmark) blood samples by centrifugation over a Lymphoprep layer (Nycomed, Oslo, Norway) at a density of 1.077 g/ml. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and once with medium consisting of RPMI 1640 (20 mM HEPES, ICN Biochemicals, Costa Mesa, CA), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, Scotland), 2 mM L-glutamine (Gibco BRL) and antibiotics (penicillin 50 U/ml, streptomycin 50 µg/ml from Gibco BRL). Cell counting was performed by using Technicon H1, H2 or H3 analyzers (Bayer Diagnostica). Density gradient centrifugation yields—without other purification steps—were good (range 29–92%; mean 61.7%; S.D. 16.2%, $n = 36$). This information is necessary for interpretation of the results [13]. The proportion of monocytes plus polyclonal T- and B-lymphocytes was 1–13%, indicating that 87–99% of the isolated cells represented the leukemic population (reanalyzed in 2000).

Immunophenotyping was performed by flow cytometry (FACSCalibur, Becton Dickinson immunocytometry systems, CA) using commercial mouse monoclonal antibodies and, respective immunoglobulin isotype controls.

2.2. Chemo- and radio-sensitivity in vitro

2.2.1. Drugs and irradiation

Following drugs and types of irradiation were used. Chlorambucil (Sigma, St. Louis, MO); 2-chloro-2'-deoxyadenosine (Sigma); cyclosporine (cyclosporin A, Sandoz Pharmaceutical Co., Basle, Switzerland); *cis*-platinum(II) diammine-dichloride (*cis*-platin, Sigma); Doxorubicin hydrochloride (adriamycin 2 mg/ml, Pharmacia, Uppsala, Sweden); 2-fluoroadenine-9-β-D-arabinofuranoside (fludarabine, Sigma); prednisolone natrium succinate; di-adreson-F (prednisolone, N.V. Organon, Oss, The Netherlands); verapamil (verpamil 2.5 mg/ml, Orion Ltd., Espoo, Finland); γ-irradiation 0.007 Gy/s (¹³⁷Cs source; Molsgaard

Medical, Denmark); UV-irradiation 1 J/m²/s (UV Unit; Gelman Sciences, Ann Arbor, MI).

2.2.2. Cytotoxicity assay

The methodology has been described in more detail in the original papers [3–10]. In short, the cytotoxic effects of nine drugs and two types of irradiation against peripheral blood lymphocytes from 36 CLL patients were assessed using 4-day cultures by adding five different concentrations of test drugs to the cultures in 96-well microplates. Alternatively, the cells were initially irradiated by five different doses of ionizing and UV-irradiation. The 200,000 cells per well were seeded in a volume of 100 µl (2×10^6 cells per ml). The test substances were selected according to their usefulness in CLL therapy on one hand and on the basis of their individual mechanisms of action on the other. Effects were monitored by assessing protein synthesis using [¹⁴C]-leucine incorporation. The cells were cultured for 4 days. [U-¹⁴C]-leucine (specific activity 1.3 mCi/mmol, 0.5 µCi/ml) was added for the final 24 h of culture. After incubation, the proteins were precipitated with 0.2 M perchloric acid and collected on glass fiber filters using a multiple cell harvester (LKB Wallac 1295–001, Turku, Finland). The radioactivity incorporated was measured by a liquid scintillation counter (Wallac 1410). The ID₈₀ values were calculated from dose-response curves using duplicate or triplicate assays with 5 drug or irradiation doses.

2.3. Statistical treatment

Correlations were calculated using Spearman's rank order correlation test. The proportion comparison was performed by χ^2 -test. Statistical analyses were performed using a statistical package: STATISTICA for Windows (ver Win 5.1D, 1996 StatSoft Inc., Tulsa, OK).

3. Results

We have previously demonstrated a good correlation between the number of living cells and leucine incorporation in 4-day cultures *ex vivo* by using cyclosporin A, verapamil and vincristine [5,6,8,14]. Here, a very good correlation between cell numbers and leucine incorporation in 4-day *ex vivo* cultures is demonstrated with six other drugs and two types of irradiation (Fig. 1).

The results of the cytotoxicity testing of nine drugs and two kinds of irradiation against the peripheral blood leukemic cells of 36 CLL patients are illustrated in Fig. 2. These results are based on 2376 duplicate cultures. From the nine drugs tested, 2-chlorodeoxyadenosine and vincristine induced an 80% median inhibition in leucine incorporation by lowest drug concentrations. Correspondingly, the drugs requiring the highest concentrations for a similar effect were verapamil and *cis*-platin. Cyclosporin A demonstrated the lowest variation and prednisolone the highest variation

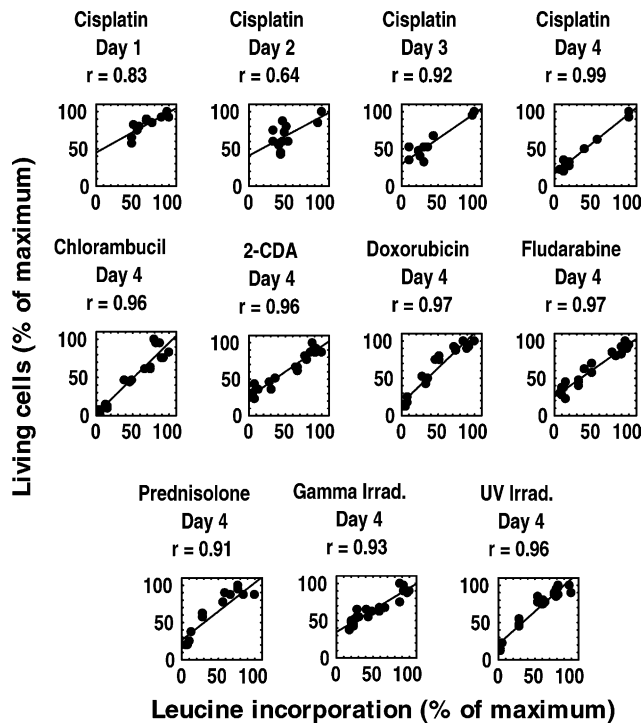


Fig. 1. Correlation between leucine incorporation (dpm per culture well; abscissa) and living cell concentration (trypan blue-excluding cells per ml; ordinate) in ex vivo cultures of randomly chosen example patients. In order to facilitate the comparison, all cases were scaled to a common (% of maximum) level. The upper panel demonstrates the progression of cell death during the culture period. The best correlation was observed on day 4 of culture. The lines represent the least-squares regression by Pearson's method. A corresponding correlation coefficient is given above each panel. Despite good linear correlation in all cases, sometimes leucine incorporation was more sensitive than the living cell counts (eg. 2-CDA, fludarabine and γ -irradiation).

among these toxic substances. Furthermore, the ID_{80} -values for prednisolone could not be determined for all patients. Hence, we used ID_{50} -values instead.

The extreme and outlier values are separately illustrated (Fig. 2). This was our statistical approach to determine relative cellular drug resistance. Individual cases are discussed in Table 2. Among the nine patients having received chemotherapy six had one or more outlying and extreme ID_{80} values. In contrast, only eight patients not having received chemotherapy belonged to the outlier/extreme group. This result indicates that the ratio of resistant:sensitive cases in the chemotherapy group was 4.8 times larger as compared with the previously and currently untreated patients ($P = 0.0484$, χ^2 -test, $n = 36$).

There was a poor concordance between previous or ongoing drug therapy and the in vitro drug-resistance. In three cases, the patient had received the same drug against which he or she demonstrated drug resistance. These were patient 8 showing chlorambucil and doxorubicin resistance and patient 24 showing doxorubicin resistance.

In addition to the inter-individual variation in the responses to toxic substances, there was a strong intra-individual variation, i.e. tumor cell heterogeneity. In many instances, the dose-response curves of individual patients spun over a 100-fold concentration difference [3–10]. The inter-relationships of different toxic agents were analyzed by using the non-parametric Spearman's rank order correlation analysis. As illustrated in Table 1, there was strong evidence ($P < 0.01$) about a statistical relationship of the sensitivities of CLL cells to: (i) chlorambucil and γ -irradiation ($P = 0.0028$); (ii) 2-chlorodeoxyadenosine and fludarabine ($P < 0.0001$); (iii) 2-chlorodeoxyadenosine and vincristine ($P = 0.0099$); (iv) 2-chlorodeoxyadenosine and

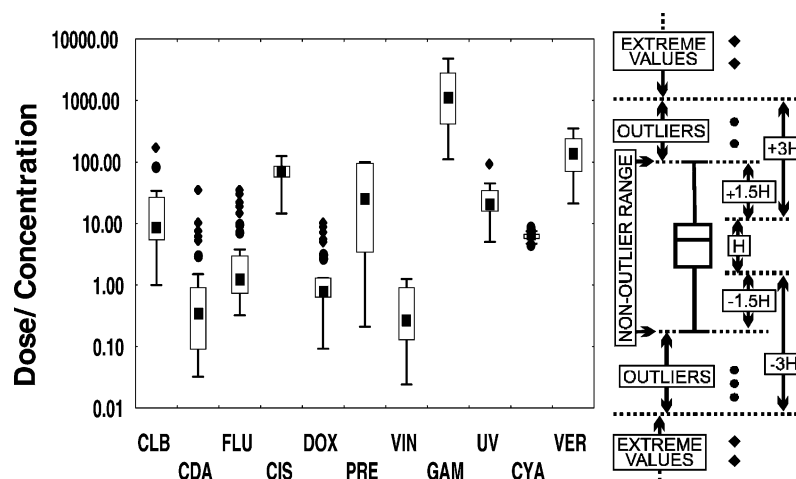


Fig. 2. Sensitivity in vitro of peripheral blood leukemia cells of 36 CLL patients to nine drugs and two types of irradiation. The abbreviations are: (CLB) chlorambucil; (CDA) 2-chlorodeoxyadenosine; (FLU) fludarabine; (CIS) cis-platin; (DOX) doxorubicine; (PRE) prednisolone; (VIN) vincristine; (GAM) γ -irradiation; (UV) UV-irradiation; (CYA) cyclosporin A; (VER) verapamil. Median values, ranges, outliers and extremes are illustrated according to the statistics given on the right side of the figure. The ranges of outliers and extremes are illustrated in the classic box and whisker plot where the upper box value is the 75%, the lower box value is the 25%. The outlier coefficient is equal to 1.5 and the extreme coefficient equal to 3. The ID_{80} values are expressed in μM , with the exception of γ -irradiation in Gy, and UV-irradiation in J/m^2 . The patients having outlying or extreme values are further characterized in Table 2.

Table 1
Relationships between cytotoxicity of nine drugs and two types of irradiation in vitro in cells from 36 patients with CLL^a

	CLB80	CDA80	FLU80	CIS80	DOX80	PRE50	VIN80	GAM80	UV80	CYA80
CDA80	0.36 (0.0300)									
FLU80	0.24 (0.1509)	0.62 (0.0000)								
CIS80	0.27 (0.1165)	0.29 (0.0866)	0.44 (0.0075)							
DOX80	0.32 (0.0640)	0.39 (0.0209)	0.11 (0.5205)	0.28 (0.1075)						
PRE50	0.04 (0.8193)	0.20 (0.2430)	0.22 (0.2021)	−0.02 (0.9219)	0.21 (0.2219)					
VIN80	−0.01 (0.9456)	0.42 (0.0099)	0.12 (0.5015)	−0.11 (0.5229)	0.26 (0.1373)	0.31 (0.0615)				
GAM80	0.48 (0.0028)	0.50 (0.0020)	0.15 (0.3917)	0.03 (0.8526)	0.59 (0.0002)	0.25 (0.1389)	0.42 (0.0107)			
UV80	−0.07 (0.6787)	0.17 (0.3304)	0.38 (0.0233)	−0.15 (0.3890)	0.13 (0.4575)	0.26 (0.1210)	0.15 (0.3977)	0.09 (0.6139)		
CYA80	0.16 (0.3648)	0.12 (0.5040)	−0.05 (0.7912)	0.07 (0.6943)	0.08 (0.6483)	0.47 (0.0036)	0.34 (0.0410)	0.14 (0.4053)	−0.16 (0.3511)	
VER80	−0.17 (0.3161)	0.24 (0.1675)	0.24 (0.1611)	−0.16 (0.3478)	0.26 (0.1270)	0.39 (0.0178)	0.53 (0.0009)	0.31 (0.0662)	0.41 (0.0139)	0.14 (0.4097)

^a The ID₈₀ (ID₅₀ for prednisolone) values were determined as described in Section 2. The correlations were calculated by using Spearman's rank order regression analysis. The values shown are: *R*-values, and the corresponding *P*-values (in bracket); statistically significant correlations (*P* < 0.01) are indicated (in bold).

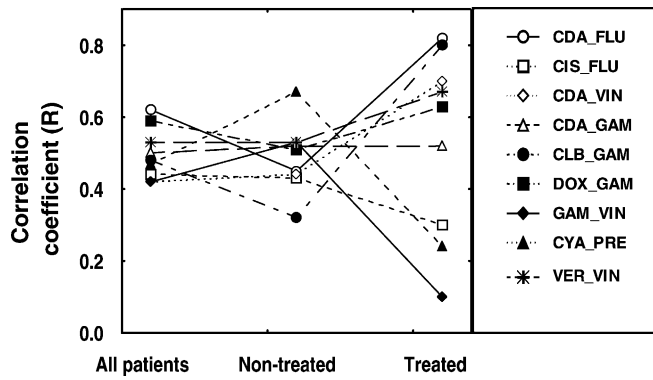


Fig. 3. Relationships of drug and irradiation sensitivities in three CLL patient groups. The drug/irradiation pairs were selected according to their best correlation as assessed by Spearman's rank order correlation test ($P < 0.01$ in any of the three patient groups illustrated): all patients $n = 36$, non-treated $n = 27$, treated $n = 9$; the abbreviations are similar to those used in Fig. 2.

γ -irradiation ($P = 0.0020$); (v) fludarabine and *cis*-platin ($P = 0.0075$); (vi) doxorubicine and γ -irradiation ($P = 0.0002$); (vii) prednisolone and cyclosporin A ($P = 0.0036$); and (viii) vincristine and verapamil ($P = 0.0009$). The number of statistically significant correlations was much

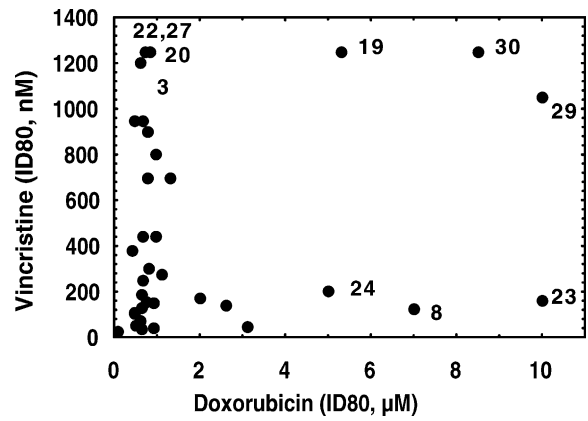


Fig. 4. Relationship of vincristine and doxorubicine sensitivity of peripheral blood leukemic cells from 36 CLL patients. The comparison concerns ID_{80} values which were determined as described in Section 2. The high values clustered in three different groups: (i) vincristine resistance alone, (ii) doxorubicine resistance alone, and (iii) double-resistance; the numbers of the most resistant patients are illustrated.

larger than that expected by a chance alone. Fig. 3 furthermore illustrates that these correlations did not remarkably change if the nine treated patients were excluded. However, the correlations of treated patients (Fig. 3) were different:

Table 2
Patients having extreme and outlier ID_{80} values in cytotoxicity testing

Patient # (FAB type)	Drugs ^a		ID_{80} (μM or J/m^2)	Previous treatment ^b
2 (CLL)	Fludarabine (O)	- ^c	9.5	LP
8 (CLL/PL)	Chlorambucil (E)	+	80.4	LP, 6 \times COP, 7 \times CHOP
	2-Chlorodeoxyadenosine (E)	-	35	
	Fludarabine (E)	-	22	
	Doxorubicin (E)	+	7.0	
	UV-irradiation (E)	-	92	
10 (CLL)	Chlorambucil (E)	-	165	None
	2-Chlorodeoxyadenosine (E)	-	10	
	Fludarabine (E)	-	34	
	Doxorubicin (E)	-	8.5	
11 (CLL)	UV-irradiation (E)	-	92	L
14 (CLL/PL)	2-Chlorodeoxyadenosine (E)	-	10	LP, 7 \times COP, 10 \times CHOP
	Fludarabine (E)	-	30	
15 (CLL)	Doxorubicin (O)	-	2.6	None
18 (CLL, mix)	2-Chlorodeoxyadenosine (E)	-	5.1	None
	Fludarabine (E)	-	14	
19 (CLL/PL)	Doxorubicin (E)	-	5.3	None
	Cyclosporin A (O)	-	7.5	
20 (CLL/PL)	2-Chlorodeoxyadenosine (E)	-	7.1	None
	Fludarabine (E)	-	19	
21 (CLL, mix)	Doxorubicin (O)	-	3.1	None
22 (CLL/PL)	Doxorubicin (E)	-	10	None
24 (CLL, mix)	2-Chlorodeoxyadenosine (E)	-	6.0	4 \times CHOP, LP
	Fludarabine (O)	-	7.0	
	Doxorubicin (E)	+	5.0	
	Cyclosporin A (O)	-	4.4	
29 (CLL)	Doxorubicin (E)	-	10	None
31 (CLL)	2-Chlorodeoxyadenosine (O)	-	3.0	LP

^a (E) extreme and (O) outlier (as shown in Fig. 1).

^b L, leukeran (chlorambucil); P, predniso(lo)ne; CH(O)P, cyclophosphamide-(hydroxydaunorubicin (doxomyacin)-oncovine(vincristine)-prednisolone.

^c (-) Not given previously; (+) previously given.

(i) 5/9 demonstrated a stronger correlation, (ii) 1/9 a similar, and (iii) 3/9 less stringent correlation than in the total or non-treated patient groups.

It is conceivable that the CLL/PL patients are less drug-sensitive than the typical morphological forms of CLL. This was the case and 5/6 CLL/PL cases occurred to belong to the drug resistant category (cf. Table 2). Accordingly, the omission of CLL/PL cases from Table 1 would decrease the number of significant correlations from 8 to 5.

We also wanted to analyze whether a correlation exists between two *P*-glycoprotein-dependent drugs vincristine and doxorubicine (cf Table 1). No such correlation could be observed between the ID₈₀-values of vincristine and doxorubicine (Fig. 4). Three cases (patient nos. 19, 29 and 30) clustered very differently from the others indicating a cross-resistance. Interestingly, none of the three belonged to the chemotherapy group.

4. Discussion

Several methods have been used in order to assess the chemosensitivity of CLL leukemia cells *ex vivo*. These include Differential Staining Cytotoxicity (DiSC) assay [2] and 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [15]. The former is a manual [2] and evidently a labor-intensive technique while the latter is a semiautomatic method [16] like that one used in the present investigation. All these assays use different end-points. Hence, the validation of the laboratory method is important. We have previously demonstrated that there are very good correlations between the numbers of living cells and leucine incorporation in 4-day cultures although very different toxic drugs, such as calcium channel antagonists, vincristine and cyclosporine were investigated [5,6,8]. Here, we extend this laboratory validation to concern all other toxic substances used in this investigation.

Extensive research into mechanisms of cytotoxic drug and irradiation resistance have produced few clinically encouraging results. This can partially be explained by an *in vivo*-induction of post-treatment pleiotropic resistance, the phenomenon, which has carefully been investigated with CLL cells: the conclusion was that the pleiotropic resistance observed in fresh CLL cells is most likely caused by the complex interplay of a number of resistance mechanisms, some of which may yet be undefined [2]. Similarly, the present investigation clearly revealed a relationship between previous or ongoing chemotherapy and drug resistance *ex vivo*. Surprisingly, the enhanced *in vitro* resistance was not necessarily against the same compound, e.g. chlorambucil, which had been used in clinical chemotherapy. This is in accordance with the previous results, according to which, for example treating patients with anthracyclines (MDR-associated drugs) induced more *ex vivo* resistance to steroids and platinumins while treating with cyclophosphamide induced resistance to all the MDR drugs except vincristine [2].

Begleiter et al. have previously demonstrated the cross sensitivity of CLL cells to 2-chlorodeoxyadenosine and fludara [11]. The assay strategy used in the present series of investigations has confirmed this finding, although cross-resistance cannot be assumed in all CLL patients [9]. An entirely unexpected finding of the present work was the disclosure of tight relationships between responses to drugs with very different mechanisms of action (cf. Table 1), such as 2-chlorodeoxyadenosine and vincristine and several others. This strongly emphasizes the fact that finally very little is known about the complexity of cellular mechanisms of drug sensitivity or resistance in a clinical context.

The current material represents a normal cohort of CLL patients referred to a university hospital. It is important to recognize special characteristics of minor groups, such as cases with CLL/PL morphology and pre-treated cases. These groups may have reduced drug sensitivity, as also demonstrated with this patient cohort.

The multidrug-resistant phenotype of CLL cells has been demonstrated in a number of publications. *P*-glycoprotein is frequently expressed at diagnosis [17] and functional drug efflux [18–20] has been demonstrated *in vitro*. Furthermore, it has been shown that CLL cells are resistant to anthracyclines [21]. The results of the recent and current work from our laboratory point to an entirely different conclusion about the significance of MDR in CLL. We have recently shown that, despite a 25-fold sensitivity of CLL cells to vincristine [7], the uptake and efflux in CLL versus normal lymphocytes are essentially similar [8]. This excludes any significant role for MDR in the tremendous *in vitro* sensitivity of CLL cells to vincristine. Furthermore, no correlation could be revealed between vincristine and anthracycline sensitivities in the present investigation. Only three patients (8.3%) were simultaneously resistant to both drugs meaning that MDR mechanisms were not detectable in the rest of the cases. Interestingly, the three cross-resistant cases belonged to the non-chemotherapy group and, hence, presented a simultaneous *de novo* resistance to these two drugs.

We have recently demonstrated that calcium channel antagonists verapamil and nifedipine as well as the immunosuppressive drug cyclosporin A are toxic against CLL cells *in vitro* [5,6]. In addition, verapamil and nifedipine potentiated the action of non-*P*-glycoprotein-dependent drugs [5,6]. With an exception of verapamil versus vincristine, the present investigation did not reveal correlation of either of these two types of compounds with any other modalities of *in vitro* treatment used in the present work. This may mean that the mechanism of action of these two drugs are very different from the others and deserve more scrutiny towards clinical chemotherapy in CLL. On the other hand, the tight correlation, even between very different drugs, may indicate similarities in the mechanisms of action. For instance, 2-chlorodeoxyadenosine sensitivity among 36 CLL patients correlated tightly, in addition to fludarabine, vincristine as well as to ionizing radiation. It is very likely that this kind of observational investigation, although enabling the confir-

mation of the statistical relationship, can never confirm the biological inter-relationship.

Multiple comparison procedures have an internal tendency to reveal non-existing correlations. The comparisons performed in this work represent 10 families of related tests, each of the toxic substances against all others. There are very few possibilities to accurately determine the *P*-values in this kind of situation. One very robust and conservative approach may be based on the Bonferroni-type inequality, generally used with variance analysis. If each *P*-values in Table 1 were multiplied by 10 (corresponding to 10 comparisons inside each family) only one of the *P*-values (CIS80 versus FLU80) would become larger than 0.05, the others still remaining 0.036 or below.

Even without accurate knowledge of the mechanisms, this new information about the toxic substances may be relevant in the clinical context. However, this remains to be investigated. If this similarity concerns an upstream (putative) component of the death signal, a cross-resistance may be expected. On the other hand, if the similarity is distal in the death signal pathway, the cancer cell damage may be additive or even synergistic. This is a novel area of cancer research and deserves further investigation.

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CD80 antigen expression as a predictor of ex vivo chemosensitivity in chronic lymphocytic leukemia

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Abstract

We investigated the correlation between expression of 31 surface membrane antigens and chemosensitivity of peripheral blood mononuclear cells from 36 patients with CLL. The sensitivity of CLL cells to nine drugs (2'-chlorodeoxyadenosine, cisplatin, chlorambucil, cyclosporin A, doxorubicin, fludarabine, prednisolone, verapamil and vincristine) and two types of irradiation (gamma and UV-irradiation) was determined from dose-response curves of 4-day cultures ex vivo. The results indicated that the CLL cases responding to purine analogs (2'-chlorodeoxyadenosine and fludarabine) can be identified according to CD80 expression: all resistant cases had low or negative CD80 expression. No other correlations were revealed. CD80 may be a surrogate chemosensitivity marker for purine analogs. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Chemosensitivity; Purine analogs; Cluster of differentiation; CD80; Chronic lymphocytic leukemia

1. Introduction

In cancer chemotherapy a major obstacle is drug resistance. CLL is not an exception. Chemosensitivity assays may bring specific guidance to avoid ineffective compounds and to potentially direct chemotherapy towards more successful treatment at each point of disease progression [1]. Cell surface markers are crucial for current diagnostics of CLL. Some markers, such as CD25, CD38 and serum-soluble CD23 are associated with a more adverse clinical course of CLL (for a review, see [2]). Here we report that surface membrane antigen expression was of low value in predicting the ex vivo irradiation or chemotherapy response. The main exception was CD80 expression, predicting the response of CLL cells to purine analogs.

2. Materials and methods

2.1. Patients and samples

Clinical specimens were obtained after informed consent from 36 consecutive CLL patients with a CD19+/CD5+/

CD23+ immunophenotype and mature CLL, CLL/mix or CLL/PL morphology (FAB classification). Most patients had chromosomal aberrations but none indicating chronic lymphoid leukemias other than typical CLL [3].

Peripheral blood mononuclear cells were isolated from heparinized blood samples by centrifugation over a Lymphoprep layer as described [3]. Density gradient centrifugation yields—without other purification steps—were good (range 29–92%; mean, 61.7%; S.D., 16.2%; $n = 36$). The proportion of monocytes plus polyclonal T- and B-lymphocytes was 1–13%, indicating that 87–99% of the isolated cells represented the leukemic population.

2.2. Immunophenotyping

Immunophenotyping was performed by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry systems, San Jose, CA) using commercial mouse monoclonal antibodies and respective immunoglobulin isotype controls. The antibodies used are illustrated in Table 1.

Staining was performed according to the suppliers' instructions. For cell surface immunoglobulin staining, the cells were first incubated in PBS (pH 7.4) at 37 °C for 30 min to remove heterophilic antibodies. The buffers used in all the steps did not contain serum.

Abbreviations: CD, cluster of differentiation; CLL, chronic lymphocytic leukemia

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Table 1
Monoclonal antibodies used

Antigen (source) ^a	Label	Isotype control	Clone
CD2 (B)	FITC	γ_{2a}	Leu-5b
CD4/CD8 (B)	FITC/PE	γ_1/γ_{2a}	Leu-3a/leu-2a
CD20 (B)	FITC	γ_1	Leu16
CD45RA (B)	FITC	γ_1	Leu-18
CD45RO (B)	PE	γ_{2a}	Leu-45RO
CD122 (B)	PE	γ_1	Anti-IL-2R-p75
CD19 (B)	PE	γ_1	SJ25C1
CD5 (B)	FITC	γ_{2a}	Leu-1
CD23 (B)	PE	γ_1	Leu-20
FMC7 (I)	FITC	μ	FMC7
CD22 (B)	FITC	γ_{2b}	Leu-14
CD34 (B)	PE	γ_1	8G12
CD38 (B)	PE	γ_1	Leu-17
CD10 (B)	FITC	γ_{2a}	Anti-CALLA
CD124 (I)	PE	γ_1	S4-56C9
CD126 (I)	PE	γ_1	M91
CD11c (D)	FITC	γ_1	KB90
CD14 (D)	FITC	γ_{2a}	TUK4
CD21 (D)	FITC	γ_1	1F8
CD79b (D)	FITC	γ_1	SN8
mIgG (P)	FITC	γ_1	G18-145
mIgD (P)	FITC	γ_{2a}	IA6-2
mIgM (P)	PE	γ_1	G20-127
CD95 (P)	FITC	γ_1	DX2
CD40 (P)	FITC	γ_1	5C3
CD27 (P)	FITC	γ_1	M-T271
CD130 (P)	PE	γ_1	VC041
CD25 (B)	FITC	γ_1	Anti-IL-2R
CD154 (B)	PE	γ_1	Anti-gp39
CD80 (B)	PE	γ_1	Anti-B7
CD45/CD14 (B)	FITC/PE	γ_1/γ_{2b}	Anti-Hle-1/leu-3M
κ/λ (B)	FITC/PE	γ_1/γ_1	TB28-2/1-155-2

^a B: Becton Dickinson; D: Dako; I: Immunotech; P: Pharmingen. For more accurate description of antigen proteins, see, e.g. <http://www.ncbi.nlm.nih.gov/PROW>.

The forward scatter channel was checked prior every analysis using unlabeled calibration particles (SPHERO Calibration Particles, blank 6.5–8 μm , Pharmingen). The instrument linearity was checked using commercial standard reagents (Immuno-Brite Standards Kit, Coulter, Hialeah, FL) prior to study. The fluorescence compensations were set prior to every analysis using calibrate beads (caliBRITE, Becton Dickinson) and FACSComp Software (Becton Dickinson). Instrument calibration was additionally checked using Immunobrite level II standard beads (Coulter). On the basis of the data thus obtained, the interassay variability in this study was 6.5% for FL1 channel and 7.6% for FL2 channel.

The percentages of cells were calculated on the basis of data obtained from two-color immunofluorescence dot blot. Analyses were carried out using CellQUEST Software (Becton Dickinson). Quadrant markers were set relative to negative immunoglobulin isotype controls in a such way that 99% (99.05 ± 0.04) of the cells labeled with the control antibodies were located in the left lower quadrant. The intensity

of antigen expression in the cell membrane was analyzed in a histogram data display using logarithmic scale. The number of CD45-positive cells was always $>98\%$ (99.79 ± 0.35). The mean proportion of CD14-positive cells in an analyzed gate was 0.77% (S.D., 1.11; range 0.05–6.82).

2.3. Chemo- and radiosensitivity in vitro

The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and once with complete medium consisting of RPMI 1640 (20 mM Hepes, ICN Biochemicals, Costa Mesa, CA), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, Scotland), 2 mM L-glutamine (Gibco BRL) and antibiotics (Gibco BRL, penicillin 50 U/ml, streptomycin 50 $\mu\text{g}/\text{ml}$). Cytotoxic effects were assessed in 4-day cultures, adding five different concentrations of test compounds to cultures in 96-well microplates. Alternatively, the cells were initially irradiated with five different doses of ionizing and UV-irradiation. A total of 200,000 cells per well were seeded in a volume of 100 μl (2×10^6 cells/ml). The test substances were selected according to their usefulness in CLL therapy on one hand and on the basis of their individual mechanisms of action on the other. Effects were monitored by assessing protein synthesis using [^{14}C]-leucine incorporation. We have demonstrated that with various toxic drugs, leucine incorporation is tightly correlated to the number of living CLL cells in this type of assay (see [3] and references therein). The ID₈₀ (80% decrease in leucine incorporation) values were calculated from dose-response curves representing duplicate or triplicate cultures of six different drug concentrations.

3. Results

Expression of CD80 divided the patients into two mutually exclusive groups as regards sensitivity to the purine analogs 2'-chlorodeoxyadenosine and fludarabine. All resistant cases had very low CD80 expression as indicated by the percentage of positive cells or by geometric mean fluorescence (Fig. 1). A similar trend, although not exclusive, was observed with chlorambucil, doxorubicin and prednisolone, but not with the other drugs or two types of irradiation tested. No statistically significant correlations were found between expression of any other surface antigen and drug sensitivity, when analyzed by using Spearman's rank order correlation test.

4. Discussion

Purine analogs are promising antileukemic compounds competing with chlorambucil in first line therapy in CLL. In fact, the results of a recent clinical trial justified the conclusion that fludarabine yields higher response rates and a longer duration of remission and progression-free survival

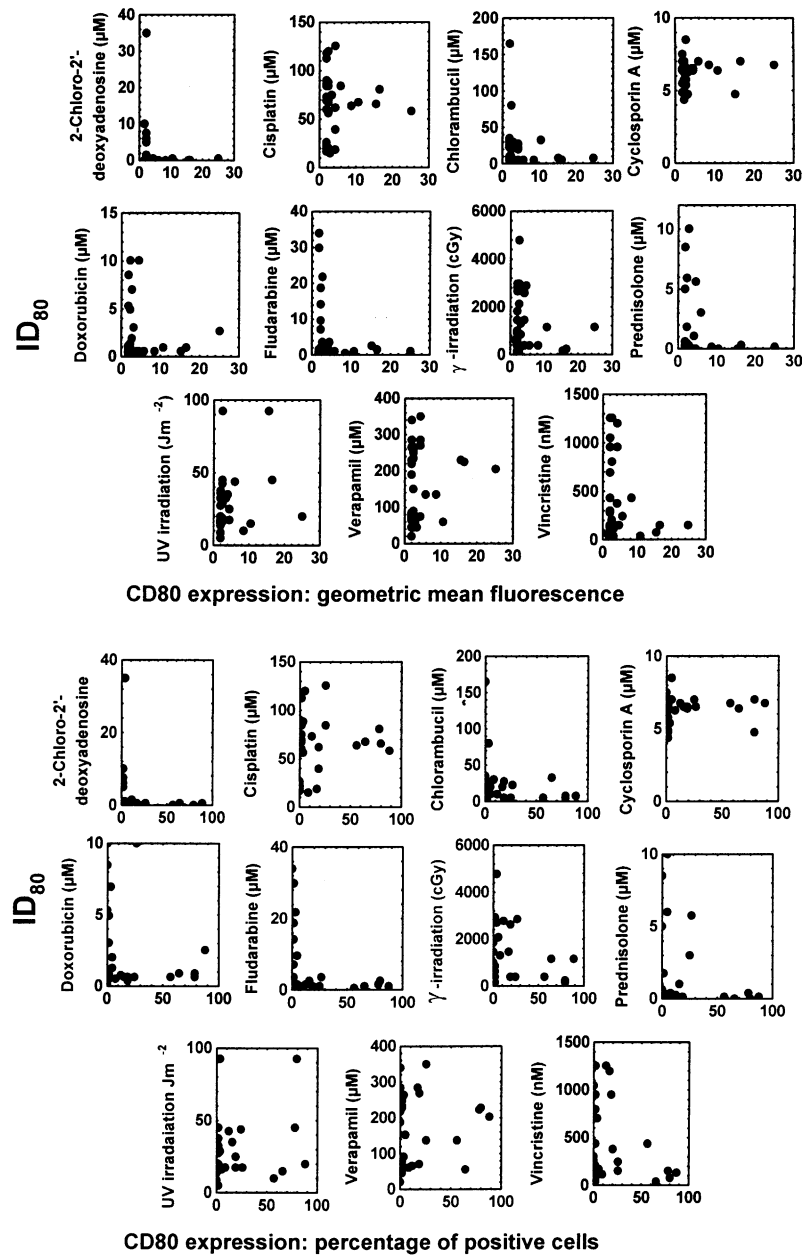


Fig. 1. CD80 expression and ex vivo sensitivity of CLL cells to nine drugs and two types of irradiation. Peripheral blood mononuclear cells from 36 CLL patients were examined. The ex vivo chemo- and radiosensitivities of CLL cells were determined in 4-day cultures by exposing the cells to six doses of the toxic agents. ID_{80} values were determined from the dose-response curves. These indicate the doses causing an 80% decrease in cell viability. Expression of CD80 was determined by using flow cytometry and fluorescent monoclonal antibodies. It was examined by determination of geometric mean fluorescence (upper panel) and the percentage of positive cells (lower panel). Similar results were obtained by both means.

than chlorambucil, but overall survival is not enhanced [4]. However, among 170 patients treated with fludarabine, 63 (37%) had stable or progressive disease. Hence, novel tools for individualizing chemotherapy are required. Our current data demonstrate that the ex vivo cellular response to fludarabine can be predicted using CD80 expression as a surrogate marker of chemosensitivity. None of the cases with detectable expression of this antigen was resistant when

cells were tested in vitro. The same feature was observed with 2-chlorodeoxyadenosine. High expression of CD80 also excluded the resistance of CLL cells to doxorubicin, chlorambucil and prednisolone. Interestingly, CD80 status did not correlate to CD38 expression, which has appeared to a versatile predictor of clinical outcome in B-CLL cases [5].

Very little is known about the biological role of CD80. It has been shown that in follicular lymphoma the priming

of malignant B cells with CD40L induces an increase in CD80/CD86 expression and a consequent recognition of these cells by normal T lymphocytes [6]. The data here indicate that the function of CD80 is not specifically related to purine analog pharmacodynamics. The usefulness of CD80 expression as a predictive marker of purine analog sensitivity deserves clinical validation. A better understanding of the biochemical function of CD80 may bring new light to this topic.

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Ex vivo drug and irradiation sensitivities in hypermutated and unmutated forms of chronic lymphocytic leukemia cells

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Abstract

Several investigators have now established that chronic lymphocytic leukemia (CLL) is not a uniform disease entity, since approximately half of the cases of CLL have undergone immunoglobulin V region (IgV) hypermutation, whereas the other half display unmutated Ig genes. The median survival time of mutated CLL (M-CLL) cases has been shown to be approximately twice as long as that for unmutated CLL (UM-CLL), but no clear explanation for this difference is currently available. In this work, we have investigated a cohort of previously untreated CLL patients, to see whether the ex vivo sensitivities of leukemic cells of 16 UM-CLL patients differ from those of 8 M-CLL patients, using nine different drugs and two types of irradiation. Our results demonstrated very similar ex vivo sensitivities and tumor cell heterogeneity of sensitivity of UM-CLL and M-CLL cells when tested against chlorambucil, 2-chloro-2'-deoxyadenosine, cyclosporin A, *cis*-platinum(II)diammine-dichloride, doxorubicin hydrochloride, 2-fluoroadenine-9- β -D-arabinofuranoside, prednisolone sodium succinate, verapamil, vincristine, γ -irradiation, and UV-irradiation. This indicates that de novo chemo/radiosensitivity cannot explain the survival difference observed between UM-CLL and M-CLL.

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Keywords: CLL; Chronic lymphocytic leukemia; Immunoglobulin gene; Somatic hypermutation; Drug sensitivity

1. Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries. This disease is characterized by accumulation of mature-looking but functionally compromised lymphocytes preferentially in the bone marrow, blood and lymphoid tissues [1].

During an immunological germinal center response, the immunoglobulin genes of normal polyclonal B cells are subjected to a hypermutation mechanism resulting in non-random single base changes in the immunoglobulin V region (IgV) genes [2]. It has gradually become obvious that this may also concern cells that have given rise to B lymphoid malignancies [3]. For example, CLL consists of two clinical entities with either somatically mutated (M-CLL) or unmutated (UM-CLL) V_H (heavy-chain variable-region) genes [3–5]. The two subtypes of CLL are biologically very interesting and their recognition is also of immediate

clinical relevance, since the two groups of CLL have been shown to have different prognoses with significantly shorter median survival for UM-CLL than M-CLL [6–8].

Apart from clinical staging, there are very few reliable prognostic markers predicting the outcome of CLL [9]. One natural clinical prognostic marker could be the sensitivity of CLL cells to chemotherapy. Tumor cell sensitivity in vivo, however, depends on drug pharmacokinetics as well as on the internal sensitivity of tumor cells to the chemotherapeutic agents administered. In this work we have investigated, by using nine different drugs and two types of irradiation, whether the sensitivities of UM-CLL versus M-CLL cells differ from each other ex vivo.

2. Materials and methods

2.1. Patients and samples

Clinical specimens were originally obtained after informed consent from 36 consecutive CLL patients referred

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to the CLL out-patient clinic at Tampere University Hospital (Finland). Twelve patients were excluded, as described below. Thus, the final number of patients included in this investigation was 24. Diagnosis and staging were based on standard clinical, morphological and immunophenotyping criteria. All patients had a CD19+/CD23+/CD5+ immunophenotype (the cohort was reanalyzed in 2000); immunophenotyping was performed by flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry systems, California) using commercial mouse monoclonal antibodies and respective immunoglobulin isotype controls [10]. All cases had a mature CLL, CLL/mix or CLL/PL morphology [11]. Most patients had chromosomal aberrations but none indicating chronic lymphoid leukemias other than typical CLL [12–15]. Others and ourselves have previously demonstrated that chemotherapy modifies the *in vitro* chemosensitivity of CLL cells [16,17]. Hence, of the original 36 patients, 9 cases were not included in this investigation because they had received chemotherapy. Additionally, three patients had to be excluded since their IgV_H mutation status could not be determined.

Peripheral blood mononuclear cells were isolated from heparinized (Noparin, Novo Nordik, Dagsvaerd, Denmark) blood samples by centrifugation over a Lymphoprep layer (Nycomed, Oslo, Norway) at a density of 1.077 g/ml. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and once with medium consisting of RPMI 1640 (20 mM HEPES, ICN Biochemicals, Costa Mesa, CA), 10% heat-inactivated fetal calf serum (Gibco BRL, Paisley, Scotland), 2 mM L-glutamine (Gibco BRL) and antibiotics (penicillin, 50 U/ml, streptomycin, 50 µg/ml, Gibco BRL). Cell counting was performed by using Technicon H1, H2 or H3 analyzers (Bayer Diagnostica). Density gradient centrifugation yields—without other purification steps—were good (range 29–92%; mean 61.7%; S.D. 16.2%, $n = 36$). This information is necessary for interpretation of the results [18]. The proportion of monocytes plus polyclonal T- and B-lymphocytes was 1–13%, indicating that 87–99% of the isolated cells represented the leukemic population (reanalyzed in 2000) [10].

2.2. V_H gene analysis in UM-CLL versus M-CLL

V_H gene family-specific PCR amplification was performed using six family-specific V_H primers and one J_H primer, as previously described [19]. The PCR amplification and the PCR conditions have been detailed recently [19]. To distinguish monoclonal PCR products from polyclonal ones, single stranded conformation polymorphism analysis was performed using GenePhor system electrophoresis (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions.

Clonal PCR products from rearrangements were sequenced directly by using BigDye Terminator Cycle Sequencing Reaction Kits (Perkin-Elmer, ABI, Foster City, CA, USA). All sequence reactions were analyzed using

an automated DNA sequencer (ABI 377, Applied Biosystems, Foster City, CA). The obtained sequences were aligned to the closest published germline genes using the IgBLAST (National Center for Biotechnology Information, USA), the V-BASE (MRC Centre for Protein Engineering, Cambridge, UK) and the international ImMunoGeneTics (<http://imgt.cines.fr:8104>; Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France) databases. V_H gene sequences deviating more than 2% from the corresponding germline gene were defined as mutated, whereas sequences displaying less than 2% mutations were considered unmutated. For D gene determination, a requirement of a minimum of seven matching nucleotides was used.

2.3. Chemo- and radiosensitivity *in vitro*

2.3.1. Drugs and irradiation

The following drugs and types of irradiation were used. Chlorambucil (Sigma, St. Louis, MO); 2-chloro-2'-deoxyadenosine (Sigma); cyclosporin (cyclosporin A, Sandoz Pharmaceutical Co., Basle, Switzerland); *cis*-platinum(II)-diammine-dichloride (cisplatin, Sigma); doxorubicin hydrochloride (adriamycin 2 mg/ml, Pharmacia, Uppsala, Sweden); 2-fluoro-9-β-D-arabinofuranoside (fludarabine, Sigma); prednisolone sodium succinate; di-Adeson-F (prednisolone, N.V. Organon, Oss, The Netherlands); verapamil (verpamil 2.5 mg/ml, Orion Ltd., Espoo, Finland); γ-irradiation: 0.007 Gy/s (¹³⁷Cs source; Molsgaard Medical, Denmark); UV-irradiation: 1 J/(m² s) (UV Unit; Gelman Sciences, Ann Arbor, MI).

2.3.2. Cytotoxicity assay

The methodology has been described in more detail in the original papers [16,20–25]. In short, the cytotoxic effects of the nine drugs and two types of irradiation against peripheral blood leukemic cells were assessed using 4-day cultures, by adding five different concentrations of test drugs to cultures in 96-well microplates. Alternatively, the cells were initially irradiated with five different doses of ionizing and UV-irradiation. A total of 200,000 cells per well were seeded in a volume of 100 µl (2×10^6 cells/ml). The test substances were selected according to their usefulness in CLL therapy on one hand and on the basis of their individual mechanisms of action on the other. Effects were monitored by assessing protein synthesis using [¹⁴C]-leucine incorporation. [U-¹⁴C]-leucine (specific activity 1.3 mCi/mmol, 0.5 µCi/ml) was added for the final 24 h of culture. After incubation, the proteins were precipitated with 0.2 M perchloric acid and collected on glass fiber filters using a multiple cell harvester (LKB Wallac 1295-001, Turku, Finland). The radioactivity incorporated was measured by a liquid scintillation counter (Wallac 1410). Values of ID₂₀ and ID₈₀ were calculated from dose–response curves using duplicate or triplicate assays with five drug/irradiation doses. ID₂₀ and ID₈₀ are equivalent to a concentration of a drug or a dose of irradiation which decreases ¹⁴C-leucine incorporation by

20 and 80%, respectively. We have previously shown in a number of experiments that there is a good correlation between ^{14}C -leucine incorporation and the number of living cells in a culture [16,20–25].

2.4. Statistical treatment

The Mann–Whitney U -test was used for group (median) comparisons: STATISTICA for Windows (Version Win 5.1D, 1996 StatSoft Inc., Tulsa, OK).

3. Results

3.1. V_H gene analysis in UM-CLL versus M-CLL

Detailed analysis of gene usage (V_H , D and J) and V_H mutation status will be published elsewhere (Vilpo et al., submitted for publication). Eight cases (33%) displayed mutated Ig V_H genes, whereas 16 (67%) cases were unmutated.

3.2. Sensitivities of UM-CLL versus M-CLL cells

The results of cytotoxicity testing of nine drugs and two kinds of irradiation against the peripheral blood leukemic cells of 8 M-CLL and 16 UM-CLL cases are illustrated in Fig. 1. The ID_{80} values, i.e. doses causing 80% inhibition in cell viability, as measured by ^{14}C -leucine incorporation, were surprisingly similarly distributed in both types of CLL. The large scatter of individual values demonstrates the great

individual variation of CLL cell sensitivity among different patients of both CLL types. Statistical analysis did not reveal any significant differences between these two types of disease ($P > 0.05$). The only exception was prednisolone, which had to be evaluated at the ID_{50} level, since ID_{80} was not achieved in all cases, even when a high dose of the drug was tested. UM-CLL cases were, on average, slightly more sensitive than M-CLL cases ($P = 0.041$).

In addition to evaluating variation between patients by using the ID_{80} values, we also evaluated whether V_H mutation status affects the variation of drug/irradiation sensitivity in individual patients, i.e. we evaluated the distribution of heterogeneity of intra-individual sensitivity in cases of M-CLL versus UM-CLL. To this end, we also determined the ID_{20} values and adopted the ratio of ID_{80} per ID_{20} as a measure of that parameter. The results illustrated in Table 1 clearly demonstrate that there were no significant differences in intra-individual heterogeneity of sensitivity to any of the toxic agents in these two groups of CLL.

4. Discussion

The present work was performed in order to establish whether different drug or irradiation sensitivities could contribute to the longer survival of patients with M-CLL versus UM-CLL, but this could not be confirmed.

Many prognostic factors for CLL have been proposed (for a review, see [9]), but so far few investigations concerning their occurrence in UM-CLL versus M-CLL have yet been published. Preliminary information suggests, for example, that 13q deletion as a single chromosomal aberration (a marker of good prognosis), is more frequent in M-CLL, whereas 11q deletion (a marker of bad prognosis) is preferentially associated with UM-CLL [26]. It remains to be shown, however, whether this or other kinds of prognostic indicator may explain the survival difference between patients with UM-CLL versus M-CLL. Intensive gene expression studies have revealed <30 gene expression differences when the expression of about 12,000 genes in UM-CLL versus M-CLL was compared [27].

The other conceivable explanation for the survival difference of UM-CLL versus M-CLL patients could be different chemo- and radiosensitivity in these two forms of CLL. The most reliable way to investigate this topic would be a clinical study. At the time being this is not possible, since (i) there are no such clinical chemosensitivity data available concerning Ig V_H -characterized patients, (ii) tumor cell concentrations of various drugs in vivo are not known, and (iii) a clinical trial comparing UM-CLL and M-CLL in a randomized study would be difficult to organize. The present work was performed in order to provide preliminary information about chemo- and radiosensitivity in the two forms of CLL. The cellular sensitivities ex vivo could be investigated without the interfering factors mentioned above. Overall, the two types of CLL demonstrated similar

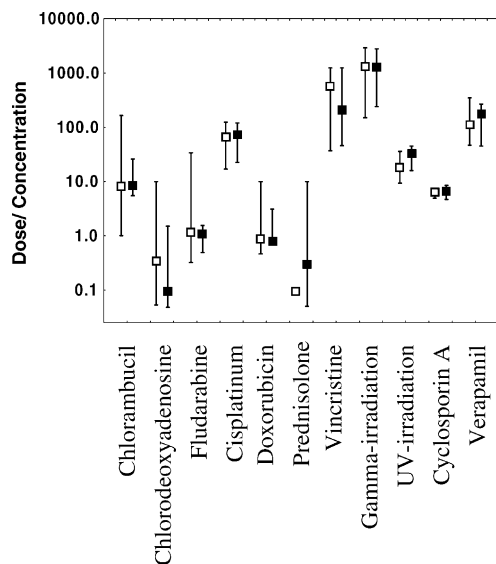


Fig. 1. Sensitivity in vitro of peripheral blood leukemia cells of 16 UM-CLL patients and 8 M-CLL patients to nine drugs and two types of irradiation. The ID_{80} values (ID_{50} for prednisolone, since ID_{80} was not achieved in all cases) are expressed in $\mu\text{mol/l}$, with the exception of γ -irradiation in Gy, and UV-irradiation in J/m^2 . Medians and total ranges are illustrated. Open symbols: UM-CLL; closed symbols: M-CLL.

Table 1
Heterogeneity of sensitivity of UM-CLL and M-CLL cells to nine drugs and two types of irradiation^a

Drugs and irradiation types	UM-CLL		M-CLL		Mann–Whitney <i>U</i> (<i>P</i> -value)
	Mean	S.D.	Mean	S.D.	
Chlorambucil	14.8	12.7	15.4	7.7	0.4260
Chlorodeoxyadenosine	40.7	44.9	24.7	23.5	0.3425
Fludarabine	19.4	25.7	9.3	3.3	0.5815
Cisplatinum	9.7	5.1	7.2	3.2	0.0662
Doxorubicin	19.9	20.7	27.9	40.5	0.7383
Prednisolone	72.15	244.27	93.52	123.15	0.0758
Vincristine	47.4	49.1	74.3	141.0	0.8065
γ-Irradiation	31.8	31.9	30.4	19.2	0.7595
UV-irradiation	6.1	2.4	8.4	3.7	0.1417
Cyclosporin A	4.5	3.5	2.3	1.5	0.0662
Verapamil	16.4	17.7	15.1	12.7	0.3913

^a The figures represent ID₈₀/ID₂₀ ratios with the exception of prednisolone, for which the ratio ID₅₀/ID₂₀ was determined.

cellular sensitivity to nine drugs and two forms of irradiation. M-CLL had a tendency to be slightly more sensitive to 2-chloro-2'-deoxyadenosine and vincristine than UM-CLL, whereas the opposite was shown for UV-irradiation, but no significant difference was shown between the subsets. The only statistically significant difference concerned prednisolone, for which UM-CLL cases were more sensitive than M-CLL ($P = 0.04$). Furthermore, the intra-individual or tumor cell heterogeneity of chemo/radiosensitivity in both groups was very similar. This indicates that de novo chemo/radiosensitivity cannot explain the survival difference observed between patient with UM-CLL versus M-CLL. Our results, on the other hand, do not exclude the possibility that UM-CLL cells might achieve resistance during clinical chemotherapy faster than do M-CLL cells. However, the observed tendencies of difference in cellular resistance for some of the single drugs between the mutated and unmutated CLL cases warrant further investigation in a larger patient material to make clear if the findings are significant.

In conclusion, the current pilot study demonstrated very similar ex vivo sensitivities and tumor cell heterogeneity of sensitivity of UM-CLL and M-CLL cells when tested against chlorambucil, 2-chloro-2'-deoxyadenosine, cyclosporin A, *cis*-platinum(II)diammine-dichloride, doxorubicin hydrochloride, 2-fluoroadenine-9-β-D-arabinofuranoside, prednisolone sodium succinate, verapamil, vincristine, γ-irradiation, and UV-irradiation.

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