Application of In Vitro Chemosensitivity Testing for Evaluation of New Cytotoxic Drugs in Chronic Lymphocytic Leukaemia

BY

ANNA ÅLESKOG
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Despite major advances in the understanding of the biology of chronic lymphocytic leukaemia (CLL), progress in improving its treatment has been limited and it still remains an incurable disorder. In the present research, we have performed in vitro drug sensitivity testing of primary CLL cells for preclinical evaluation of cytotoxic drugs, using the fluorometric microculture cytotoxicity assay (FMCA).

The tumour type-specific activities of 14 standard drugs, evaluated in vitro on tumour cells from patients with CLL and acute leukaemias, were in good agreement with their known clinical activities. A correlation between drug treatment and development of cellular drug resistance was demonstrated in CLL, but not in the acute leukaemias. Moreover, the nucleoside analogues fludarabine, cladribine, cytarabine and gemcitabine, as well as the anthracycline idarubicin, were highly active in CLL cells. A new cytotoxic drug candidate, CHS 828, was evaluated in primary cell cultures from a broad spectrum of tumours. CHS 828 was highly active against haematological malignancies in vitro, especially CLL, but also against some solid tumours. The drug appeared to be non cross-resistant with standard drugs.

In addition, the relationship between drug sensitivity in vitro and a recently described prognostic factor in CLL, the mutational status of the immunoglobulin variable heavy chain (IgVH) gene, was evaluated. Interestingly, cells with unmutated IgVH genes were more chemosensitive than the mutated cells.

In summary, our results indicate that in vitro studies on tumour cells from leukaemia patients may yield considerable information regarding the activity, mechanisms of action and cross-resistance of cytotoxic drugs, as well as concerning the relationship between drug sensitivity and prognostic factors, which can be useful in the preclinical evaluation of new cytotoxic drugs. Furthermore, the results suggest that the pyrimidine analogues cytarabine and gemcitabine, as well as the anthracycline idarubicin, may have a role in the treatment of CLL. The new cyanoguanidine CHS 828 is highly active in CLL cells and appears to be non cross-resistant with standard drugs. The poorer prognosis in patients with CLL cells with unmutated IgVH genes can not be explained by increased chemoresistance.

Key words: Chronic lymphocytic leukaemia, cytotoxicity, in vitro assay, cytotoxic drug development, IgVH mutation status.

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To my family
LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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Cell samples and cell preparation

Tumour samples

Normal lymphocytes

Cell preparation

Cell lines

Reagents and drugs

The Fluorometric Microculture Cytotoxicity Assay (FMCA)

Method outlines

Quality control

Quantification of FMCA results

IgV_{H} gene analysis

Survival analyses

Statistical analysis

RESULTS AND DISCUSSION

Detection of tumour type-specific activity of newly introduced cytotoxic drugs (Papers I-III)

Tumour type-specific activity

Cross-resistance pattern

Acquired drug resistance

Detection of tumour type-specific activity of a novel cytotoxic drug candidate (Paper IV)

Tumour type-specific activity

Therapeutic window

Cross-resistance

Relationship between drug sensitivity in vitro and IgV_{H} gene mutation status (Paper V)

SUMMARY AND CONCLUSIONS

ACKNOWLEDGEMENTS

REFERENCES
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelocytic leukaemia</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CHS 828</td>
<td>N-(6-(4-chlorophenoxy)hexyl)-N’-cyano-N’’-4-pyridylguanidine,</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>dCK</td>
<td>Deoxycytidine kinase</td>
</tr>
<tr>
<td>DiSC</td>
<td>Differential staining cytotoxicity</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>IC50(30)</td>
<td>Concentration resulting in 50% (70%) cell survival</td>
</tr>
<tr>
<td>IgVH</td>
<td>Immunoglobulin variable heavy chain</td>
</tr>
<tr>
<td>LD</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance associated protein</td>
</tr>
<tr>
<td>MTT</td>
<td>Methyl-thiazol-tetrazolium</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NHL</td>
<td>Non Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pgp</td>
<td>Permeability glycoprotein 170</td>
</tr>
<tr>
<td>Rho</td>
<td>Spearman’s rank correlation coefficient</td>
</tr>
<tr>
<td>REAL</td>
<td>Revised European-American Lymphoma classification</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S/H</td>
<td>Solid/haematological</td>
</tr>
<tr>
<td>SI</td>
<td>Survival Index</td>
</tr>
</tbody>
</table>
BACKGROUND

Introduction

Chronic lymphocytic leukaemia of the B-cell type (B-CLL) is caused by the proliferation and accumulation of immune-incompetent B lymphocytes arrested at an intermediate stage of their differentiation. Clinical features reflect the accumulation of leukaemic cells in bone marrow and lymphoid organs and the immune disturbances that accompany the disease. The prognosis in patients with B-CLL varies. Some patients survive for a long time, while others have a rapidly progressive clinical course. In the last decades, important advances in the understanding of the biology of B-CLL have been made. Despite this, only limited progress has been made in improving its treatment, which still fails to cure the disease, with the possible exception of allogenic stem cell transplantation. Thus, new treatments need to be developed. It will become increasingly important to incorporate laboratory studies into the design of clinical trials in order better to understand the reasons for clinical success or failure of new laboratory findings.

In the present studies we have performed in vitro drug sensitivity testing of primary B-CLL cells, using the fluorometric microculture cytotoxicity assay (FMCA), for preclinical evaluation of newly introduced cytotoxic drugs and a novel cytotoxic drug candidate, CHS 828. We have compared the new drugs with established cytotoxic drugs and investigated the development of resistance following treatment. We have also compared the drug sensitivity profiles in B-CLL with those in other malignancies and have investigated the tumour type-specific activity of each drug. Finally, we have examined the relationship between drug resistance in vitro and the mutational status of the immunoglobulin variable heavy chain (IgVH) gene, a recently described prognostic factor in B-CLL.
Chronic lymphocytic leukaemia

**Epidemiology and aetiology**

Chronic lymphocytic leukaemia (CLL) is one of the most common leukaemias in adults in Western countries, accounting for 25 % of all leukaemias, in contrast to less than 5% of the leukaemia cases in Asia (1). The incidence in Europe among men ranges from 2.2-3.4 and among women from 0.9-1.5 per 100,000 per year (2). In the western countries 95% of CLL cases have the B-cell phenotype, while in Asia, T-cell CLL predominates (1). B-CLL (referred to hereafter as CLL) is rare in people under the age of 40, but the incidence increases dramatically with age (3). The median age of the patients at diagnosis is about 65 years (3).

The aetiology of CLL is unknown (1). The increased prevalence of CLL, other cases of leukaemia, lymphoproliferative diseases and autoimmune disorders in relatives of CLL patients suggests a genetic component in the disease (3). Environmental factors such as ionizing radiation, chemicals and drugs have shown no apparent relationship to development of CLL (4).

**Classification**

According to the revised European-American lymphoma classification (REAL) (5) and the newly introduced World Health Organization (WHO) classification (6), CLL is a non-Hodgkin’s lymphoma (NHL) (Table 1). Previously it was supposed that a high proportion of large, immature cells conferred a more aggressive clinical course, and all NHL histopathological entities were therefore divided into two groups, with low- and high-grade malignancy. It is now recognised, however, that the morphological appearance of malignant cells does not necessarily predict the clinical outcome (7). Hence, Harris at al. have suggested that the expression of “grade” should relate to the histopathological findings, and that the clinical course should instead be termed indolent, moderately aggressive, aggressive or highly aggressive (5).
Table 1. B-cell lymphoma according to the REAL classification
(adapted from N. L. Harris et al. (5)).

| Chronic lymphocytic leukaemia / prolymphocytic leukaemia/
small lymphocytic lymphoma |
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Lymphoplasmacytoid lymphoma / immunocytoma</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>Follicle center lymphoma, follicular, grade I-III</td>
</tr>
<tr>
<td>Follicle center lymphoma, diffuse</td>
</tr>
<tr>
<td>Splenic marginal zone lymphoma</td>
</tr>
<tr>
<td>Marginal zone B-cell lymphoma</td>
</tr>
<tr>
<td>extranodal (MALT-B-cell lymphoma)</td>
</tr>
<tr>
<td>nodal (monocytoid)</td>
</tr>
<tr>
<td>Hairy cell leukaemia</td>
</tr>
<tr>
<td>Plasmocytoma / myeloma</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>High-grade B-cell lymphoma, Burkitt-like</td>
</tr>
<tr>
<td>Precursor B-lymphoblastic lymphoma</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
</tr>
</tbody>
</table>

The immunophenotype of B cells in CLL

The observation that CLL is a disorder of the B lymphocytes was made almost simultaneously by two groups of investigators in 1972 (8). This was soon followed by characterization of the phenotypic profile of CLL lymphocytes and the recognition of CLL as a monoclonal disorder in which the leukaemic cells are arrested at the intermediate stage of differentiation (8). Further advances in immunology and flow cytometry resulted in establishment of a phenotypic profile of monoclonal B lymphocytes, which today is considered characteristic and therefore diagnostic of CLL. Thus, the B cells in CLL typically express the pan-B cell antigens CD 19, CD 20 and CD 23 and co-express on the surface the aberrant CD 5 molecule and faint levels of immunoglobulins (4).

Molecular biology

CLL is a model for failed programmed cell death or apoptosis. The bcl-2 family proteins, key regulators of apoptosis, are overexpressed in 90% of CLL cells, although in the vast majority of cases no translocation involving the bcl-2 gene has been detected (4). The clonal slow-growing B lymphocytes accumulate in the body, predominantly in the G0 phase of the
cell cycle. The imbalance in the ratio of major pro- and anti-apoptotic bcl-2 family proteins, such as bax and bak (induction of apoptosis), bcl-2 (anti-apoptotic), bad, bik and hrk (anti-apoptotic inhibitors) may play an important role in the behaviour and treatment response of CLL (4). Mutations in the tumour suppressor gene p53 and increased levels of expression of the cyclin-dependent kinase inhibitor p27 are other factors that may be involved in the behaviour of the disease (4).

Cytogenetics

Conventional cytogenetic analysis in CLL has been hampered by the low mitotic activity of the leukaemic cells in vitro (9). The development of techniques for studying chromosomes by fluorescent in situ hybridization (FISH) has increased the possibility of detecting numerical and structural chromosome abnormalities. Using the FISH technique, chromosomal aberrations are detected in 82% of the CLL cases (9). There is no single cytogenetic abnormality in CLL (1). The most common cytogenetic alteration is a deletion in 13q (55%), followed by a deletion in 11q (18%), trisomy 12q (16%) and a deletion in 17p (7%). Complex abnormalities may be present. Patients with a deletion in 13q as the sole genetic abnormality have a better prognosis than those with a normal karyotype and trisomy 12. Patients with a deletion in 11q or 17p have more advanced disease than the other groups, patients with a deletion in 17p having the shortest median survival (9).

Immunoglobulin variable heavy chain gene

One of the most important recent advances contributing to the understanding of the immunopathology of CLL was made when reports were published simultaneously in 1999 from two different laboratories, one by Damble et al. in the United States (10) and the other by Hamblin et al. in the United Kingdom (11). CLL lymphocytes had previously been considered to originate from immunologically naïve B cells. These two research groups demonstrated that there are two types of CLL, one with somatically hypermutated IgVH genes of leukaemic cells, suggesting that these cells were transformed at the postgerminal centre stage, and a second type without such mutations, suggesting that the leukaemic cells in these patients originated from the pregerminal centre stage. Patients with somatic mutations had significantly better overall survival and a more benign clinical course than their counterparts without mutations. These findings also applied
to patients with Binet stage A disease (see below). Expression of CD 38 has recently been suggested as a surrogate marker for the two subtypes (12). However, the results are discordant in approximately 30% of the cases. Multivariate analyses have shown that both IgVH gene mutation status and CD38 expression are independent prognostic factors and thus do not identify the same subgroups. Nevertheless, CD38 expression may have prognostic significance in patients whose IgVH gene status is known (12).

**Clinical features**

About 40-60% of patients with CLL are diagnosed in an asymptomatic phase (4). Frequently the presence of abnormal lymphocytosis during a routine medical examination is the only reason to consider the diagnosis (1). In symptomatic patients, the most frequent findings are generalized lymphadenopathy, fatigue, night sweat, weight loss, fever with or without infections and autoimmune diseases (4). Manifestations of bone marrow involvement, particularly by significant anaemia (haemoglobin <100 g/l) or thrombocytopenia (platelet count <100 x 10^9/l), are present at diagnosis in 15% of the patients (4). A positive direct antiglobulin test (DAT) has been reported in about 8-35% of patients (3). Autoimmune haemolytic anaemia occurs in 10 to 25% of patients and may be triggered by the cytotoxic drugs used to treat the disease (3). Immune thrombocytopenia is observed in 2% of the cases (3). Infections are primarily due to hypogammaglobulinaemia, although other immune defects, such as granulocyte dysfunction, may play a role (3). About 15% of the patients undergo a transformation to a more aggressive disorder, most often prolymphocytic leukaemia or diffuse large-cell lymphoma (Richter’s syndrome) (4).

**Diagnostic criteria**

The National Cancer Institute-Sponsored Working Group (NCI-WG) has published guidelines for diagnosis of CLL and criteria for its response to treatment (13). Peripheral blood lymphocytosis (absolute lymphocyte count > 5 x 10^9/l) with small mature-appearing lymphocytes with a characteristic immunophenotype of monoclonal B cells, as described above, is the minimum diagnostic requirement for CLL. If bone marrow examination is performed, the bone marrow aspirate must show > 30% of all nucleated cells to be lymphoid (4).
Prognostic factors in CLL

CLL is characterized by a highly variable clinical course. Many patients may survive for decades without requiring treatment, whereas others die from disease-related complications within a few months of diagnosis, despite appropriate therapy. The 5-year overall survival is 70.5% (2). Although prior investigators recognized the importance of clinical findings in predicting the outcome in patients with CLL, it was not until 1975 that a simple, reproducible and clinically applicable staging system was introduced by Rai et al. (8, 14). The original system consisted of five stages (0 to IV), but was modified to a three-stage method in 1987, as the survival curves of a large population of patients indicated three distinct categories – low-risk, intermediate-risk, and high-risk (8). Binet et al. in 1981, described a three-stage system in which stage A is the low-risk, stage B the intermediate-risk and stage C the high-risk category (15). Although several other staging systems were also described during this period those proposed by Rai et al. and Binet et al. are the most commonly used in CLL (Table 2) (1). Clinical staging systems are still the most useful prognostic parameters. However, neither system accurately separates those patients in early stages who will progress from those who will remain indolent (4).

Other unfavourable clinical prognostic factors, besides clinical stage, are age (>55 years), male sex, black race and poor performance status. However, there are no differences between older and younger CLL patients in presenting features, response rates or duration of response (4).

Because of the clinical heterogeneity within each clinical stage, additional prognostic markers were developed. Laboratory prognostic factors predicting a poor outcome are a rapid lymphocyte doubling time (> 12 months), diffuse bone marrow histology, high peripheral blood lymphocytosis, increased serum levels of lactate dehydrogenase, β2-microglobulin and soluble CD23, certain cytogenetic abnormalities, atypical morphology, atypical immunophenotype, unmutated IgVH genes, loss of p53 gene, expression of CD38 and a high bcl2/bax ratio (16). In a recent study, the prognostic significance of several of the above factors was evaluated in 205 CLL patients. Multivariate analysis showed clinical stage, IgVH gene mutational status and loss or mutation of the p53 gene to be independent prognostic factors (17).
Table 2. The Rai and Binet staging systems (adapted from S. Molica (16)).

<table>
<thead>
<tr>
<th>Staging system</th>
<th>Stage</th>
<th>Modified three-stage system</th>
<th>Clinico-haematological features</th>
<th>Median survival (yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai</td>
<td>0</td>
<td>Low-risk</td>
<td>Lymphocytosis</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>Intermediate-risk</td>
<td>Above + lymphadenopathy</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>Above + splenomegaly and/or heparomegaly</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
<td>Above + anaemia (haemoglobin &lt; 110 g/l)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>High-risk</td>
<td>Above + thrombocytopenia (platelets &lt; 100x10^9/l)</td>
<td></td>
</tr>
<tr>
<td>Binet</td>
<td>A</td>
<td></td>
<td>Lymphoid areas involved &lt; 3</td>
<td>&gt; 10</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td></td>
<td>Lymphoid areas involved ≥ 3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td>Above + anaemia (haemoglobin &lt; 100 g/l) and/or thrombocytopenia (platelets &lt;100x10^9/l)</td>
<td>2</td>
</tr>
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</table>

Treatment of CLL

The principal aim of therapy in CLL patients is relief of disease-related symptoms. This is frequently achieved. With new drugs higher rates of complete remission (CR) have been obtained. This has led to a variety of therapeutic approaches also in early phases of the disease, especially in young patients with a poor prognosis, with the aim of inducing long-standing remissions and prolonging survival (18).

The alkylating agents chlorambucil and cyclophosphamide were shown to be active in CLL in the mid-1950s. At about the same time, corticosteroids were evaluated and found to have clinical activity. In the last decades, important advances have been made in the treatment of CLL with the development of new cytotoxic drugs (mainly fludarabine) and monoclonal antibodies (i.e. rituximab and campath). Although these new regimens have resulted in a higher response rate and longer duration of remission, no effect on the overall survival of patients with CLL has so far been observed (19).
Various combinations of these new treatments (such as fludarabine + rituximab) are currently being evaluated in clinical studies (20, 21).

**Indications for treatment**

Thirty per cent of the patients have indolent or smouldering CLL characterised by early-stage (Binet A, Rai 0), non-diffuse bone marrow histology, haemoglobin >130 g/l, blood lymphocytes <30 x 10⁹/l and a lymphocyte doubling time of >1 year. Their life expectancy is similar to that of a population without CLL. Only 15% of these patients are likely to exhibit progressive CLL (4). It is recommended that initial therapy be deferred until required by disease progression (18). Large randomized trials have demonstrated that early treatment with chlorambucil in a continuous or an intermittent schedule does not prolong survival in CLL patients with a low tumour burden (18).

Factors that generally prompt the initiation of therapy are:

1. Disease-related symptoms.
2. Bone marrow failure.
3. Massive and/or progressive lymphadenopathy or splenomegaly.
4. Recurrent infections.
5. Autoimmune haemolytic anaemia or thrombocytopenia.
6. High blood lymphocyte count (>300-500 x 10⁹/l).
7. Advanced clinical stage (Binet B, C; Rai III, IV).

**First-line treatment**

Alkylating agents

Alkylating agents and purine analogues are the most important drugs available for treatment of CLL. Chlorambucil (with or without prednisolone) has been the most frequently used first-line drug in the treatment of CLL in the past four decades. It is available in oral formulation and its absorption by the gastrointestinal tract is almost complete (4). A commonly used schedule is 15 to 30 mg/m² every 2 weeks. Regimens of 20-40 mg/m² every 4 weeks or a daily schedule of 4-8 mg/m² for 4-8 weeks are also used (4). The response rate among previously untreated patients, varies between 30 and 70% although complete remissions are rare and cure is not achieved (19). A combination of chlorambucil and prednisolone does not appear to be
superior to chlorambucil alone (18, 19). No benefit has been observed from maintained therapy after maximal response (19).

Cyclophosphamide appears to have similar activity to chlorambucil, but it is generally used only when chlorambucil is poorly tolerated and in combination regimens (19). The optimum dose and schedule of administration of chlorambucil or other alkylating agents has not been defined (18).

Purine analogues
Recently, fludarabine has been shown to be the most active agent for first-line therapy in CLL (19). The currently recommended schedule of administration of fludarabine is an intravenous bolus of 25 mg/m² daily for 5 consecutive days once a month (19). Patients failing to respond to two or three courses should be considered for alternative treatment. Patients achieving complete remission probably do not warrant additional courses of therapy (19). In patients with a partial response, treatment may be continued to the best response plus two additional courses, not exceeding a total of one year of therapy because of concerns of cumulative myelotoxicity (19). The oral bioavailability of fludarabine is 50-60% (19), and an oral formulation was recently approved. Fludarabine induces complete remissions in about 30% of previously untreated patients, with an overall response rate of more than 70% (19).

In addition to fludarabine, cladribine and pentostatin have been found to be effective in CLL, but the experience with these drugs is not as extensive as with fludarabine, and no randomized studies have been completed to show whether one of these purine analogues is to be preferred (18). However, like other drugs, purine analogues do not appear to be curative (18).

A randomised comparison of fludarabine with chlorambucil for patients with previously untreated CLL has shown a statistically significantly higher overall response rate in the fludarabine group compared with the group treated with chlorambucil (63% vs. 37%). The response duration was significantly longer after fludarabine treatment, but no difference in overall survival was seen. Bone marrow toxicity was higher in the fludarabine group (22). One main objection to this study has been that chlorambucil was used in too low a dose (18).

Combination regimens
Among previously untreated patients, combination chemotherapy, including such drugs as prednisolone, vincristine or anthracyclines, has produced
responses in 10 to 80%, with very few CRs (4). In randomized multicentre trials, a benefit from fludarabine as primary therapy compared with combination therapy such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) or CAP (cyclophosphamide, doxorubicin and prednisolone) has been observed in terms of tolerance and treatment response but not of survival (18). Thus, combination chemotherapy as primary treatment has not shown any advantage over the use of single drugs (18).

Second-line treatment

When relapse occurs after single drug treatment, retreatment with the same drug will often induce a new remission. However, the proportion of patients responding declines each time chlorambucil or any other single agent is readministered (18). Among patients who were initially treated with fludarabine and experienced a response that lasted one year or longer, retreatment with fludarabine was associated with an overall response rate of more than 50% (4). Patients with a shorter duration of response should be considered for other treatment options (4).

In patients showing progression on single alkylating agents, the purine analogues or various combinations of cytotoxic drugs, mostly CHOP, frequently induce tumour remission (18). Fludarabine is mostly regarded as the drug of choice for patients experiencing relapse after treatment with or refractory to an alkylating agent (4).

Fludarabine in combination with cytotoxic drugs such as cyclophosphamide, mitoxantrone and biological agents such as rituximab is currently being evaluated (20, 23).

In patients with advanced CLL who fail to respond to fludarabine or CHOP, the prognosis is poor. None of the salvage regimens reported has produced durable remissions (18). A clinical trial of a novel agent could be of benefit to these patients.

New therapies

In addition to new cytotoxic drugs, novel approaches to treatment of CLL include the use of monoclonal antibodies and stem cell transplantations.
Monoclonal antibodies

Rituximab is a chimeric anti-CD20 monoclonal antibody which is approved for the treatment of relapsed follicular low-grade lymphoma patients. Approximately half of the treated patients respond (24). In contrast, only limited activity has been observed in previously treated CLL patients (25). This may reflect the reduced surface density of CD20 on the CLL B lymphocytes (4). Rituximab in combination with fludarabine as primary therapy for CLL patients is currently being evaluated (20, 21).

Campath is a humanized anti-CD52 antibody that binds to normal and malignant mature B and T lymphocytes. Tumour regression is seen most notably in the peripheral blood, less in the bone marrow and spleen, and rarely in lymph nodes (4). An overall response was achieved in 87% (33 of 38) by previous untreated patients with CLL (26). In phase II trials with refractory patients, campath produced an overall responses in 33-42% (27-29). However, the serious side effects, mainly opportunistic infections, limit its clinical use (4).

Stem cell transplantations

The effect of high-dose chemotherapy with allogenic stem cell transplantation has been evaluated in young patients with CLL. Prolonged disease-free survival (cure?) has been observed in some patients. A graft-versus-leukaemia effect might be of importance for the outcome of allogenic transplantations. However, the risk of transplantation-related mortality is still considerable. Consequently, non-myeloablative regimens have been introduced in an attempt to reduce early transplantation-related mortality (18).

Autologous stem cell transplantation has been evaluated in young patients. Although relapse is common, this treatment has resulted in long disease-free survival in some patients. Benefit from purging autologous stem cells has been proposed, but evidence is lacking (18).

Cytotoxic drug resistance

Failure of cytotoxic drug treatment in the clinic can be due to many factors, including 1) low cellular sensitivity to cytotoxic drugs - cellular drug resistance, 2) low systemic exposure to the cytotoxic drug - pharmacokinetic resistance, and 3) an increase in the proliferative potential of the tumour
cells between courses of chemotherapy - *regrowth resistance* (30). This thesis is focused on cellular drug resistance.

Cellular multi-drug resistance (MDR) is a condition in which cells show intrinsic resistance at diagnosis or acquired resistance after treatment to the cytotoxic action not only of single drugs but also to groups of chemically related and unrelated drugs. Cellular drug resistance may be conferred to the leukaemia cell by one or more mechanisms.

**Plasma membrane transport**

The best characterized mechanism of resistance involving altered membrane transport is mediated by a transmembrane 170-kd permeability glycoprotein (Pgp), a product of the MDR1 gene ("classical" MDR). This protein belongs to the superfamily of adenosine triphosphate-binding cassette (ABC) proteins specialized in transmembrane transport (31). Pgp probably functions as an energy-dependent drug efflux channel for a variety of mechanistically and structurally unrelated cytotoxic drugs, mainly anthracyclines, vinca alkaloids and epipodophyllotoxins (32). All anthracyclines are subject to Pgp-mediated resistance, although some evidence has indicated that idarubicin has greater cellular retention and is less susceptible to Pgp-mediated efflux (33-35).

Multi-drug resistance associated protein (MRP) is also a member of the ABC superfamily and is capable of efflux and intracellular sequestration in conjugation or cotransport with glutathione (GSH). MRP induces a cross-resistance pattern similar to that produced by Pgp (36).

Although the lung resistance protein (LRP) is not an ABC transporter, it is frequently included in the discussion of drug resistance, as it is expressed in high levels in drug-resistant cell lines and some tumours. The function of LRP is probably associated with nuclear-cytoplasmatic transport (37), and the spectrum of resistance of cell lines expressing LRP covers the classical MDR phenotype as well as melphalan and platinum compounds (31).

**Resistance modifiers**

Non-cytotoxic agents have been shown to reverse MDR *in vitro* by inhibiting Pgp. Unfortunately, drugs such as verapamil, quinine and amidarone produce excessive toxicity with the doses required to reverse
MDR *in vivo*. Cyclosporin A (CsA) has been shown to be one of the most potent inhibitors of Pgp *in vitro* and PSC-833, an analogue of CsA appeared to be even more potent than CsA *in vitro* and less toxic *in vivo*. Unfortunately, despite promising preclinical experimental results, clinical trials with CsA and PSC-833 have not shown any significant improvement in outcome or the drugs have produced excessive toxicity (36).

**Decreased drug activation**

To be effective, some important cytotoxic agents require activation after entering the cell. A decrease in enzyme activity responsible for this conversion may thus lead to resistance. Deficiency of functional deoxycytidime kinase (dCK), which converts cytarabine to its active triphosphate, has been reported for cytarabine-resistant cell lines and for blasts from chemotherapy-resistant patients with acute myelocytic leukaemia (AML) (38).

**Increased drug inactivation**

Cellular mechanisms for inactivation of cytotoxic drugs involve increased activity of glutathione S-transferase (GST) and/or increased levels of GSH. GSH-utilizing enzymes play important roles in detoxification by scavenging free radicals and by conjugating drugs with GSH, rendering them inactive. GSTs are a family of enzymes that are also involved in drug detoxification. Alteration of the GSH/GST system has found to be correlated with development of resistance to several cytotoxic drugs, including alkylating agents and doxorubicin (39, 40).

**Altered target molecules**

Topoisomerase II is the cellular target of anthracyclines and epipodophyllotoxins, and decreased levels of topoisomerase II correlates with decreased sensitivity to these agents in cell lines (41).
Apoptosis deregulation

Most cytotoxic drugs exert their cytotoxic effect through induction of apoptosis (42). Both deregulated expression of anti-apoptotic factors and inactivation of pro-apoptotic factors, including those of the bcl-2 family, seem to be of prime importance in the regulation of apoptosis and thus as determinants for drug sensitivity (43). Mutations of the tumour suppressor gene p53 (induction of apoptosis) has also been associated with drug resistance in vitro (44).

Cellular drug resistance in CLL

Most CLL patients respond to initial treatment with cytotoxic drugs, but complete remissions are rare and usually drug resistance develops after one or more treatment cycles. The molecular events that underlie resistance of CLL cells to cytotoxic drugs are yet to be defined. Deregulation of apoptosis contributes not only to the pathogenesis of the different diseases but also to the development of resistance to cytotoxic drugs (45), and it may be especially important in CLL, which is predominantly an accumulative disease. The role of the bcl-2 family of proteins in drug resistance has been evaluated extensively in CLL cells using in vitro models and it is still a matter of dispute whether individual members of the bcl-2 family influence the drug sensitivity in this disease in vitro (43). CLL cells have consistently been shown to over-express the anti-apoptotic factor bcl-2, but the correlation of this over-expression with resistance to cytotoxic drugs in vitro is not clear (43, 46, 47). A reduced level of the pro-apoptotic factor bax has shown a correlation with in vitro cellular resistance to several drugs in CLL in some studies (43), but not in others (46). Some studies suggest that a high bcl-2/bax ratio correlates with resistance to cytotoxic drugs such as prednisolone, fludarabine and chlorambucil in vitro, but there are conflicting reports also on this issue (46-48). Mutation of the p53 gene has also been associated with drug resistance in vitro in CLL (44).

Other cellular drug resistance mechanisms that have been discussed in connection with CLL are different mechanisms of drug efflux. CLL cells frequently express Pgp at diagnosis and display functional drug efflux in vitro (32, 49). CLL cells have also been shown to over-express MRP and LRP (32). Consoli et al found in their study on 100 CLL patients that Pgp, MRP and LRP were frequently expressed in CLL cells. However, they did not find any correlation to stage or previous treatment (32). Others have
found that the functional expression of Pgp increases with advancing stage and prior treatment (49, 50).

Drug detoxification with GST has been associated with previous treatment and a poor response to therapy in CLL, but here again the results are conflicting (40, 51). Furthermore, altered topoisomerase II activity has been implicated in association with drug resistance in CLL, but the results are not consistent (40, 52, 53).

Recently it has been suggested that the level of DNA-dependent protein kinase, a nuclear kinase that functions in DNA double-strand break repair, may be a determinant in the cellular response to cytotoxic drugs in CLL (54, 55).

**Cytotoxic drug development**

The development of cancer chemotherapy evolved in the mid 1940s, with the introduction of alkylating mustard gas analogues for treatment of lymphomas. Today about 45 cytotoxic drugs are used in the clinical treatment of cancer in Sweden (56). These drugs are classified into main groups according to their mechanisms of action. The groups include alkylating agents, compounds active on microtubuli, antimetabolites, platinum compounds and topoisomerase I and II inhibitors. Among recently introduced drugs, the time frame for the process of development of a new drug was 10-15 years, extending from discovery of the active agents to approval for use in the patients. At best, a few drugs per year are added to the chemotherapeutic armamentarium (57).

**Identification of new anticancer drugs**

Identification of new compounds with antitumour activity can be made with different approaches. These include a) empirical observations and experimental follow-up, b) screening, c) analogue synthesis and d) application of a rational or targeted design (58).

The observation during World War II of the toxic effect of nitrogen mustard on normal lymphoid tissue, which led to experimental follow-up by testing in transplantable animal tumours and eventually to clinical testing, is an example of an empirical observation (58).
Large-scale screening of compounds for cytotoxic activity has been carried out at the National Cancer Institute (NCI) since 1955. Today, the NCI has established a large-scale anticancer drug-screening programme based on drug dose-response experiments on 60 human tumour cell types grown and treated in monolayer cultures (59). However, only a few of the commercially available anticancer drugs today have resulted from extensive screening programmes. Paxlitaxel is perhaps the most successful result of this strategy (58).

The development of analogues of known, active compounds with a goal of improving antitumour properties, pharmacokinetics or pharmaceutical characteristics, or to diminish toxic effects, has contributed to the modern arsenal of cytotoxic drugs. Most of the anticancer drugs that were found active in phase II trials in 1970-1985 were analogues of known drugs (60). Idarubicin, an analogue of daunorubicin, is an example of analogue development.

Comparatively few anticancer drugs have been developed by targeted synthesis of substances based on a rational molecular design and biochemical reasoning. Rare examples are some of the antimetabolites (58). Progress in molecular biology has recently led to the identification of numerous molecular targets of cancer therapy, and new potential anticancer drugs have been developed. Examples of promising anticancer drugs developed using this strategy are antibodies, tyrosine kinase inhibitors and farnesyl transferase inhibitors (61-63).

Irrespective of how the antitumour activity is observed, the subsequent development relies on testing of the compounds in preclinical systems. Extensive information needs to be collected before the clinical trials begin. According to guidelines used for development of anticancer drugs in Europe, investigations of the following are recommended: a) the activity profile and mechanism(s) of action, b) the mechanisms of resistance, c) the exposure time and cell-cycle dependency, d) the tumour type-specific activity, e) the \textit{in vivo} activity, f) the toxicity and g) interactions (64). Some of these points will be addressed in this thesis.

**Activity profile and mechanism(s) of action**

By determination of the activity of a new drug at different concentrations in a human tumour cell line panel and identifying the IC50 concentration for each cell line, a drug-specific activity profile can be obtained. This approach
was adopted by the NCI in 1985 and they routinely use 60 human tumour cell lines derived from patients with different malignant diagnoses (65).

The exact mechanisms of action of many cytotoxic drugs are still unclear. In the NCI cell line panel, activity patterns of new compounds can be compared with patterns produced by drugs with known mechanisms of action, and the activity of the new drug can be classified as similar or unrelated. If the activity profile of the new compound shows low or no correlation to known drugs, the compound may have a new mechanism of action (65).

Results from our laboratory indicate the feasibility of using a panel with only 10 cell lines for preliminary classification of new compounds (66). Cell lines representing different tumour types and selected for different types of resistance were used to classify standard cytotoxic drugs, and the overall performance was good (66).

**Mechanisms of resistance**

In preclinical drug development, possible mechanisms of resistance to a new compound can be investigated. The NCI has characterized their cell line panel with respect to different mechanisms of resistance such as transport proteins, and molecules regulating the apoptotic pathway. The activity pattern of a new compound can thus be investigated in this cell line panel (67). Assessment of the activity of standard drugs in parallel with that of the new compound can reveal the possible existence of cross-resistance. Observed resistance can also be investigated for its possibility of circumvention by resistance modulating agents.

The cell line panel used at our laboratory, also includes cell lines expressing different types of resistance (66).

**Prediction of tumour type-specific activity**

There has been a lack of good models for predicting diagnoses in which a new drug is likely to become effective. Thus clinical phase I-II evaluation has mostly been conducted in the form of screening among patients from a broad array of diagnostic groups, often at a late stage of the disease. The predictive value of the NCI cell line panel with respect to clinical tumour type-specificity seems questionable (68, 69). Neither does the activity of a drug on different rodent tumours in vivo seem to mirror the clinical activity
pattern of known drugs. (70). Moreover, agreement between the response in human tumour xenografts and clinical tumour type-specific activity seem uncertain (71).

Thus, other preclinical in vitro and in vivo models with improved clinical relevance are desired. Since the established cell lines may have lost some of their original tumour characteristics, the use of in vitro assays based on cells prepared from patients’ tumours might be an alternative. The clonogenic assay has been extensively investigated with respect to its suitability for this purpose, but correlation between the in vitro activity pattern of different tumour cells and clinical data has not been conclusively demonstrated (68). Several in vitro assays based on the concept of total cell-kill during short-term culture are available for these tests. These assays have been evaluated in primary tumour cell cultures and the activity pattern of known drugs seems to reflect the clinical activity, although no formal statistical analysis has been presented (72). Studies at our laboratory, using another short-term culture assay, the FMCA (see below), have indicated that these assays may be useful for detecting tumour type-specific activity both with respect to standard and investigational drugs (73, 74). This type of study is feasible and fairly easy to perform with the FMCA. A large number of samples from patients with different diagnoses, disease progression and treatment histories may be tested and the results may identify diagnoses and patients suitable for clinical studies.

In vitro cytotoxicity assays

Development of laboratory tests for measurement of sensitivity and resistance to cytotoxic drugs in tumour cells dates from the beginning of the twentieth century (30). Basically, the leukaemia cells are exposed in vitro to different cytotoxic drugs and the effect of the drugs on the survival or proliferation of the cells is investigated. The different assays can be divided into three main groups: a) clonogenic assays, b) cell proliferation assays and c) assays based on the concept of total tumour cell kill (30). The assays are potentially suitable for guiding the design of clinical studies and as tools in clinical practice.
Clonogenic assays

Clonogenic assays are based on single cell culture in soft agar and subsequent counting of formed colonies, 2-3 weeks after exposure to cytotoxic drugs. The assay relies on the proliferative capacity of a small fraction of cells, presumed to be stem cells (30). However, the method has several limitations (30). The test is laborious and time-consuming. The drug effects are measured only in a small fraction of proliferating cells, and drug effects on resting cells, which might have proliferative potential in vivo, are not measured. Some clones may be derived from contamination from normal cells. Clonogenic assays are also of limited value for lymphatic malignancies, owing to their low colony formation. Despite these drawbacks, the assay is sometimes used in comparison with newer techniques.

Cell proliferation assays

Cell proliferation assays are short-term assays (1-7 days) based on the incorporation of radioactive precursors of DNA and RNA in the proliferative fraction of cells. Inhibition of radionuclide incorporation is used as a parameter of the effect of drugs (75). These assays have higher reported technical success rates than clonogenic assays (30). The major drawbacks with this type of method are that drug effects are dependent on the degree of DNA synthesis and, as for the clonogenic assay, effects in resting cells may not be adequately measured (30).

Total cell kill assays

The total cell kill assays are based on the concept of total tumour cell kill in the whole, mainly non-dividing, tumour cell population. Total cell kill assays are short-term assays (3-4 days) and are today the most commonly used cytotoxicity assays. The different total cell kill assays are in many aspects comparable (e.g. the culture procedure), but the main differences are the various techniques used to estimate the proportion of viable cells after incubation with cytotoxic drugs, and the different drug exposures used.

The differential staining cytotoxicity (DiSC) assay measures live cells and dead cells by means of two different dyes (fast green and nigrosin) observed against an internal standard (duck erythrocytes) (76). The test relies on the intactness of the cell membrane in living cells as opposed to dead cells after
incubation with drugs. The assay has a high technical success rate and a main advantage is that malignant cells can be morphologically separated from non-malignant ones (30). However, the DiSC assay is laborious and requires subjective assessment by a skilled observer for the slide counting (30).

The methyl-thiaxol-tetrazolium (MTT) assay is based on the ability of living cells to convert a soluble tetrazolium salt into a coloured insoluble product. After the salt crystals have been dissolved, the colour change can be measured spectrophotometrically (30). The MTT assay has shown good correlations with the DiSC assay and is more objective and less laborious. However, the MTT assay requires a fairly pure tumour cell population, since it cannot distinguish between a drug effect in normal and malignant cells (30).

Another assay based on metabolic activity within the cell is the ATP assay which measures the cellular ATP content (30). The amount of ATP is relatively constant in a specific cell type. ATP is rapidly degraded by ATPase leading to prompt depletion if the glycolysis and/or the respiratory cycle are disturbed. Thus, ATP levels can be used as an indirect method for measuring cell growth and cell death. The ATP assay has shown good correlation with the DiSC and clonogenic assays (77).

In Uppsala, the fluorometric microculture cytotoxicity assay has been developed and used. The FMCA is based on measurement of fluorescence generated from non-fluorescent fluorescein diacetate (FDA), which rapidly enters cells with an intact plasma membrane and is hydrolyzed to its fluorescent derivative, fluorescein. It has been used on more than 3,000 samples from patients with haematological malignancies and solid tumours. The intra- and inter-assay variations have been estimated to be less than 5% and less than 10-15%, respectively (78, 79). The FDA fluorescence was linearly related to the number of living cells and the assay showed good correlation with the MTT and DiSC assays (78). The FMCA is a sensitive assay capable of detecting small numbers of cells. The assay is easy to perform and has a high capacity (78).

**Drug exposure**

Selection of drug concentrations for *in vitro* cytotoxic drug resistance assays is an important issue. There are at least three ways to approach the issue of appropriate drug exposure under assay conditions. One alternative is to find
drug concentrations that mimic the *in vivo* situation, by means of comparison of intracellular uptake *in vivo* and *in vitro* (80). In the case of some drugs, however, this will result in only small differences in cell kill between resistant and sensitive samples. In addition, this approach is not applicable in early drug development, before pharmacokinetic data become available (81). Alternatively, a full concentration-effect curve can be aimed at, and the estimated IC 50 value, can be used as the measure of activity (82). This will result in a better estimation of the difference between resistant and sensitive samples, but for appropriate testing, an increased number of cells will be required. The concentration obtained in this way will frequently lead to supra-pharmacological concentrations (81).

In the present studies an approach was chosen which aimed to reduce the number of cells needed, while retaining the largest possible inter-sample variability. For a new drug a number of cell samples are tested at a wide range of drug concentrations and the concentration giving rise to the largest scatter of cell survival is then chosen for further testing (30). Concentrations derived in this way for testing will again frequently lead to supra-pharmacological concentrations, as indicated by crude estimates of *in vitro* exposure derived from data on activity-decay over time (81).

*Apoptosis and in vitro drug resistance testing*

Most cytotoxic drugs induce apoptosis in malignant cells both *in vitro* and *in vivo*. Some of these drugs may also induce necrotic death in cell lines and fresh tumour samples *in vitro*, if too high concentrations are used. If induction of apoptosis and if resistance on the pathways leading to apoptosis are of importance for drug activity *in vivo*, this might be a critical point if under certain conditions necrotic cell death is measured by drug resistance assays. In most total cell kill assays cell death is measured as loss of cell membrane integrity, which is the end result of cell death both from apoptosis and necrosis. An alternative way in the development of *in vitro* drug sensitivity assays would therefore be to use apoptosis as the end point instead of membrane integrity. However, the proportion of cells in the process of apoptosis appears to depend on such factors as the methodology used, the timing of the measurement, the duration of drug exposure and the type of drug being tested (83). In spite of these facts there have been attempts to develop new assays based on apoptosis. The refined DiSC methodology, whereby drug-induced apoptotic cell death is observed, has shown good correlation to the clinical outcome in CLL (84).
Prediction of individual patient response

Human cancer is a heterogeneous disease with a diversity of pathological processes, cytotoxic drug sensitivities and resistance mechanisms, and also a considerable variation in tumour-cell sensitivity to cytotoxic drugs between patients with similar histological tumour types. It is consequently difficult to select the optimal treatment for an individual patient on an empirical and epidemiological basis. Many groups have investigated the total cell kill assays as a method of improving the disease management in individual patients and the results have correlated well with the subsequent patient response and survival for a variety of diseases and drug regimens (78, 85-90). A meta-analysis based on published papers from 1983-1997, reporting data on 1596 correlations between drug sensitivity in vitro and clinical response, concluded that the total cell kill assays performed well, with an overall sensitivity and specificity of about 0.9 and 0.7, respectively (81). The results also indicate that there are no major differences in overall results between the different assays. Also in CLL, results from in vitro drug resistance tests have been reported to predict the clinical response and survival (84, 91, 92). Although the feasibility of the testing has been established for more than 20 years, few prospective studies have been published. In those studies the response rates seemed to be better with the chemotherapy regimens selected in vitro than with empirical therapy, but no prospective clinical trial has demonstrated a significant improvement in patient survival with assay-directed therapy (93). The issue of predicting the response to cytotoxic drugs in individual patients will not be discussed further in this thesis.

Other studies have shown a relationship of in vitro drug resistance both with disease status and with cell biological features. Prognostic factors may reflect the inherent drug resistance of leukaemia cells, and in ALL in vitro cellular drug resistance has been correlated to age at presentation (94) and chromosomal abnormalities (95, 96). Furthermore, the poorer prognosis in childhood AML than in ALL has been found to be related to resistance to a large number of drugs (97).

In the present studies the in vitro assay was not used for predicting clinical drug sensitivity in the individual patient, but for elucidating the drug sensitivity pattern in different groups of patients, i.e. patients with different diagnoses, treatment statuses or prognoses.
Drugs of interest in the present studies

**Purine and pyrimidine nucleoside analogues**

Nucleoside analogues compete with their physiological counterparts for incorporation into nucleic acids and have earned an important place in the treatment of haematological malignancies (Fig. 1). The clinical success of the pyrimidine nucleoside analogue cytarabine as a highly effective agent in the treatment of AML prompted chemists to synthesize new nucleoside analogues. The purine nucleoside analogues fludarabine and cladribine have improved the treatment options in several types of indolent lymphomas. These drugs as well as the newly introduced pyrimidine analogue gemcitabine will be discussed in this thesis (Paper I).

**Figure 1.** The chemical structures of the nucleoside analogues discussed in this thesis.
Mechanisms of action

Their mechanisms of clearance distinguish the purine and pyrimidine nucleoside analogues. Cytarabine and gemcitabine are cleared metabolically by deamination by the enzyme adenosine deaminase (ADA). The first-pass clearance rapidly eliminates the parent nucleosides, with a half-life of 10 to 20 minutes. In contrast, fludarabine and cladribine are resistant to the enzyme ADA and their clearance occurs mainly by renal excretion. After entry into the cell and phosphorylation by the enzyme deoxycytidine kinase (dCK), it is generally the triphosphate of these nucleoside analogues that is the active metabolite (98). The triphosphates are incorporated into cellular DNA by replication and/or DNA repair machinery and thus cause inhibition of DNA synthesis and of DNA repair, and have inhibitory effects on RNA and protein synthesis. Although the nucleoside analogues act at multiple cellular targets, the incorporation into cellular DNA is a critical event in triggering an apoptotic response (99). The purine analogues fludarabine and cladribine can attain high intracellular concentrations because of their resistance to ADA. Consequently, cells such as lymphocytes with high dCK activity and low activity of 5’nucleotidases (specific enzymes involved in dephosphorylation of the analogues) accumulate toxic concentrations of nucleotides. It has been suggested that this accumulation may be another factor that triggers apoptosis (99).

Mechanisms of resistance

The mechanisms of resistance to the nucleoside analogues are not fully understood. In vitro studies have suggested several possibilities, including deficient dCK, induction of cytidine deaminase (for cytarabine and gemcitabine), inhibition of nucleoside transporters and increased triphosphate pools, but their relevance to resistance in human leukaemia is still uncertain (100).

Clinical data

Despite their similar chemical structures, clinical data suggest that certain nucleoside analogues have greater activity in some diseases than in others. Fludarabine has become an effective agent not only in the treatment of CLL (101), but also in many other low-grade lymphomas (102). Furthermore, it has high activity in AML in combination with cytarabine (103).
Cladribine is the drug of choice in the treatment of hairy cell leukaemia (104). It has also been found to be highly active in other indolent lymphomas, including CLL, although the exact role of cladribine in the treatment of these diseases is still under investigation (105-107). In addition, response is seen in AML in combination with cytarabine (108).

Cytarabine is one of the most effective agents in the treatment of AML. It is incorporated into almost all standard induction regimens for this disease, generally in combination with an anthracycline, and it is also used in the consolidation regimens. Cytarabine is also active in other haematological malignancies, including large cell NHL, ALL and chronic myelocytic leukaemia (CML), but has little activity as a single agent in solid tumours (100).

Its high activity in a variety of solid tumours distinguishes gemcitabine from the foregoing nucleoside analogues. Gemcitabine has become standard first-line therapy in patients with advanced pancreatic cancer and has an important place in the treatment of non–small cell lung cancer. It is also used in advanced bladder cancer and metastatic breast cancer (100). Lately, gemcitabine has shown activity in haematological malignancies (lymphomas) as well (109-112).

The major toxic effects associated with fludarabine and cladribine are myelosuppression and immunosuppression. In consequence, infections including those of the opportunistic type are frequently observed. For fludarabine, neurotoxicity is dose limiting and for cladribine, renal toxicity, but the lower doses conventionally used rarely produce these side effects (100). Both drugs have been linked to the onset of autoimmune haemolytic anaemia (113, 114).

Myelosuppression and gastrointestinal toxicity are the major complications related to cytarabine therapy. High dose cytarabine treatment is associated with conjunctivitis and neurological toxicity, mostly central, ataxia being predominant. In the case of gemcitabine, myelosuppression and gastrointestinal toxicity are common but mild (100).

**Idarubicin and other anticancer antibiotics**

Classic anticancer antibiotics all share the characteristic of being natural products of microbial metabolism. Most of them were initially isolated from various *Streptomyces* species. The anthracycline antibiotics doxorubicin and
daunorubicin, initially discovered over 30 years ago, are among the most widely used cytotoxic drugs in current clinical practice. A large number of analogues have been brought to clinical trials in the hope of finding a compound with lower cardiac toxicity and a broader spectrum of antitumour action (Fig. 2). These include the daunorubicin analogue idarubicin, the doxorubicin analogue epirubicin and the anthracenedione mitoxantrone (100). With the focus on idarubicin, the anticancer antibiotics are discussed in this thesis (paper II).

**Figure 2.** The chemical structures of the anthracyclines.

**Mechanisms of action**

The focal point of the cytotoxicities of anticancer antibiotics is DNA. By virtue of its structure, antibiotics can become intercalated between base pairs of DNA, bind to DNA and generate toxic oxygenfree radicals, which cause single- or double-strand DNA breaks. From this primary DNA damage,
some of the numerous observed cytotoxic effects of these agents derive, such as inhibition of DNA-directed RNA synthesis, protein synthesis and glutathione synthesis, defective mitoses, inhibition of catalytic activity of DNA topoisomerase II, and stimulation of apoptosis (100).

Mechanisms of resistance

Several mechanisms of resistance to anticancer antibiotics have been reported, including multidrug resistance mediated by MDR1 (Pgp) or MRP, topoisomerase II mutations and an altered apoptotic response (100). Some studies have indicated that idarubicin, in contrast to daunorubicin, is cytotoxic in Pgp-positive cells in vitro (34) and in vivo (115).

Clinical data

Doxorubicin and daunorubicin are especially active against haematological malignancies, such as the acute leukaemias, lymphomas and multiple myeloma, but also against some solid tumours, e.g. carcinoma of the breast, lung, ovary and stomach, sarcomas of the bone, and various childhood malignancies. Doxorubicin is currently used mainly for the treatment of solid tumours, especially breast cancer, and for lymphomas, whereas daunorubicin is routinely included as part of the induction programmes for AML and ALL. The doxorubicin analogue epirubicin is similar to the parent compound with respect to its spectrum of antitumour efficacy, but is significantly less potent. It is approved for treatment of breast cancer (100).

The newest anthracycline, the daunorubicin analogue idarubicin, has significant activity in the treatment of AML but is less active against solid tumours. Studies in AML suggest that induction chemotherapy regimens based on idarubicin are associated with superior remission rates and better overall survival as compared to regimens based on daunorubicin (116). In several phase II clinical trials, idarubicin in combination regimens has shown activity in low-grade NHL (117, 118).

In the search for analogues of the anthracyclines, another promising related class of compounds, the anthracenediones, has been synthesized. The most active compound tested was mitoxantrone. Although this agent is less toxic than the anthracyclines, the narrow spectrum of responding malignancies, that is breast cancer, prostate cancer, leukaemia and lymphomas, has limited
the possibility of replacing doxorubicin with mitoxantrone in clinical practice (100).

With anthracyclines, myelosuppression is common and generally dose-limiting. The cumulative dose-limiting toxic effect is cardiomyopathy. Severe local tissue damage after extravasations, and nausea and vomiting, are also common. Both idarubicin and mitoxantrone cause less cardiac toxicity and show diminished potential for extravasations injury than daunorubicin or doxorubicin. With mitoxantrone nausea and vomiting are also less common (100).

CHS 828

In a search for new antihypertensive agents acting on potassium channels, Leo Pharmaceuticals synthesized a number of cyanoguanidines in the early 1990s. Using a primary screening programme based on human cell lines in vitro and rodent tumour cells in vivo, one of the analogues, CHS 828, showed interesting antitumour activity and was selected for preclinical development (119) (Fig. 3). Further studies to characterize the drug and its behaviour have been initiated, both at Leo Pharmaceuticals and in our laboratory.

![Figure 3. N-(6-(4-chlorophenoxy)hexyl)-N’-cyano-N’’-4-pyridylguanidine, CHS 828](image)

In these studies CHS 828 has been found to be active against many tumour cell lines in vitro and in xenograft models in vivo (120). The drug has shown differential patterns of antitumour activity and no correlation to known mechanisms of drug resistance (120). The tumour type-specific activity of CHS 828 was investigated in primary cell cultures from different human tumours and results from that study are presented in this thesis (paper IV). Activity similar to that found in the latter study was also demonstrated in a
hollow fiber model in vivo using primary human tumour cells from patients (121), and in rats the toxicity was low irrespective of the dosing schedule used (122). These results were used in the planning of the clinical phase II study which targeted patients with CLL.

Further in vitro studies on the mode of cell death induced by CHS 828 indicated that the cell death caused by this agent was an active process although it did not conform to the typical morphology of classical apoptosis (123). Inhibition of the mitochondrial respiration, leading to extracellular acidification has been noted but this effect did not seem sufficient to explain the cytotoxic activity of CHS 828 (124). A recent study has shown that CHS 828 is able to increase the p53 level in mouse fibroblasts, but whether this is also the case in human tumour cells remains to be established (125). Further investigations to reveal the exact mechanism of action of CHS 828 are in progress.

Two clinical Phase II studies of CHS 828 in patients with solid tumours have been concluded. In the Swedish study CHS 828 was administered daily for five days in cycles of 28 days, an accelerated study design was employed and a phase II dose was set. Dose limiting toxicities were diarrhoea, oesophagitis, constipation, thrombocytopenia and thrombosis. No tumour responses were observed, but some patients showed stable disease (126). The other Phase I study, conducted by EORTC (European Organisation for Research and Treatment of Cancer) used a single day dosing schedule, and there was only one dose-limiting toxic effect, haematuria. No tumour responses were observed (127). A third Phase I study, as well as a Phase II study in patients with CLL, has recently closed and the results are not yet available. One important question is whether the concentrations needed to produce the promising antitumour effects seen preclinically will be achieved and tolerated in patients.
AIMS OF THE PRESENT STUDIES

The main objective of these studies was to find ways to improve chemotherapy in CLL by chemosensitivity testing on primary tumour cells in vitro, using the FMCA.

Specific aims of the studies were:

• to evaluate the cytotoxic activity of drugs in primary human tumour cells from patients with CLL and other haematological malignancies, with special focus on some newly introduced drugs with a potential role in the treatment of CLL, namely the purine analogues fludarabine and cladribine, the pyrimidine analogue gemcitabine and the anthracycline idarubicin. The goals were
  o to predict clinical tumour type-specific drug activity
  o to study cross-resistance between drugs
  o to assess the correlation between prior treatment and the development of in vitro cellular resistance

• to investigate the in vitro activity pattern of the novel cyanoguanidine CHS 828 for prediction of tumour type-specific drug activity in order to identify diagnoses suitable for early clinical trials.

• to compare the in vitro sensitivity of tumour cells from two different prognostic subgroups of CLL patients – those with unmutated and those with mutated IgVH genes - to different cytotoxic drugs.
MATERIAL AND METHODS

Cell samples and cell preparation

Tumour samples

Most of the work described in this thesis was performed on primary tumour cells from cancer patients. Tumour samples were obtained from surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. In some cases, tumour sampling was carried out for in vitro drug sensitivity testing only. The research ethical committee at Uppsala University Hospital approved the sampling for the FMCA.

Studies I and II (papers I and II) included all low-grade NHL and acute leukaemia samples from adult patients sent to the laboratory between 1992 and 1996. Totally, samples from 60 patients with low-grade NHL, according to the REAL classification (5), 99 patients with AML and 38 patients with ALL were included. At that time the number of samples from patients with CLL was small (n=42 patients), and the CLL samples were therefore included together with samples from the rest of the low-grade NHL group. Study III (paper III) comprised all adult leukaemia patient samples sent to the laboratory between 1992 and 2001. Totally, samples from 66 patients with CLL according to the REAL classification (5), 212 patients with AML and 80 patients with ALL were included. Both fresh and cryopreserved samples were used. Thus, samples from study I and II were also included in study III. In the summary of studies I-III given in this thesis, the samples from study III are presented. In study IV (paper IV), samples from 156 patients with both haematological malignancies and solid tumours were used. Some of the cells originated from samples sent to the laboratory during the study period, but to get a balanced distribution of cells from different diagnoses, cryopreserved tumour cells were also used. Study V (paper V)
was performed on cryopreserved cells from 46 previously untreated CLL patients.

**Normal lymphocytes**

Normal peripheral blood mononuclear cells (predominantly lymphocytes), from eight healthy donors, were used in some experiments in study IV.

**Cell preparation**

Mononuclear cells from bone marrow or peripheral blood were isolated by density gradient centrifugation (78). Solid tumour tissue was minced to a size of 1 mm$^3$ and the cells were then isolated by collagenase dispersion and density gradient centrifugation (128). Viability was determined by the trypan blue exclusion test and the proportion of tumour cells was judged by inspection of May-Grünwald-Giemsa stained cytocentrifugate preparations on day 0 and day 3. All experiments were performed in culture medium, RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 µg/ml streptomycin and 100 µg/ml penicillin. Cells were cryopreserved in culture medium containing 10 % DMSO and 90 % fetal calf serum and stored at -150°C. Before use, these cells were thawed and washed twice with culture medium.

**Cell lines**

The activity of idarubicin (paper II) was studied in a cell line panel containing 10 different human cell lines representing different histological subtypes and different mechanisms of drug resistance. The cell lines have been described in detail previously and drugs belonging to the same mechanistic class have been shown to have similar activity patterns in the panel (66). Among these cell lines, the doxorubicin-resistant subline 8226/Dox40 of the myeloma cell line 8226/S has been shown to express Pgp, and the difference in sensitivity between 8226/S and 8226/Dox 40 has been utilized to determine whether a drug is a Pgp substrate (66).
Reagents and drugs

All cytotoxic drugs used were tested at a concentration associated with a large scatter of survival indices. This choice was made purely on the basis of in vitro data and aimed at optimizing the conditions for differentiation between sensitive and resistant tumour cell samples. Cell survival at this concentration has previously been shown to correlate well with the activity pattern in the clinic (73, 74). Samples from CLL patients were often more sensitive than samples from the acute leukaemias, and lower concentrations of cytarabine, cladribine, fludarabine and idarubicin were chosen in the last study, in which only CLL samples were included. In the summary the lower concentrations of cytarabine and idarubicin are also presented. When the concentration-response relationship was determined, six different concentrations obtained by serial dilution were used (paper IV). Experimental 96-well microtitre plates were prepared with 20 µl/well of drug solution in triplicate at 10 x the desired final concentration. The plates were stored frozen at -70°C for up to 2 months until further use. Repeated testing of the plates indicated no loss of drug activity during storage.

The Fluorometric Microculture Cytotoxicity Assay (FMCA)

Method outlines

The FMCA is a total cell kill assay, based on the ability of cells with intact cell membranes to convert non-fluorescent fluorescein diacetate (FDA) to fluorescent fluorescein. At day one 180 µl of the tumour cell preparation (5 - 100 x 10^3 cells for patient cells, 5 - 20 x 10^3 for the cell lines) was added into 96-well microtitre plates. The culture plates were incubated for 72 h at 37°C, after which the plates were centrifuged (200 g, 5 min) and the medium was removed. After one wash with phosphate buffered saline (PBS), 100 µl/well of hepes buffered saline containing FDA (10 µg/ml) was added column-wise. The plates were incubated for 40 min and the fluorescence was then read in the fluorometer (exciting light at 485 nm for fluorescein, emitted fluorescence at 538 nm). The fluorometer was blanked against wells containing PBS including the dye, but without cells.
Quality control

Standard quality criteria for a successful assay included a fluorescence signal in control cultures of >5 x mean blank values and a coefficient of variation (CV) in control cultures of <30%. Furthermore, the fraction of tumour cells as assessed by microscopic examination after incubation should be >70%. The overall success rate in the assay is approximately 75% for haematological malignancies and 60% for solid tumours (74), with a low proportion of tumour cells in the cell preparation and a low fluorescence signal in controls being the most common causes of assay failures. Only successfully analysed samples are reported in this study.

Quantification of FMCA results

The results obtained are presented as a survival index (SI) defined as fluorescence in test wells in per cent of that in control cultures, with blank values subtracted. Low numerical values indicate a high cytotoxic effect.

Evaluation of disease-specific activity

In study I and V the mean SI were compared in cells from different diagnoses, different treatment statuses or different mutational statuses. In studies II and III the median SIs were used. Response rate was defined as the fraction of samples with an SI < 50% (paper IV).

In study IV activity of CHS 828 was expressed as IC\textsubscript{30} (concentration reducing survival index to 70 %) and IC\textsubscript{50} (concentration reducing survival index to 50 %). The values were calculated from the mean concentration-response curve in each diagnosis. From these curves the maximum effect (lowest survival = SI\textsubscript{min}) was also estimated.

To mirror the relative activity in solid tumours and haematological malignancies, an S/H ratio was introduced. This was defined as the ratio between the fraction of responders (showing a SI<50%) among the solid tumour samples and the corresponding fraction among the haematological samples (paper IV). Thus, high and low ratios indicate relatively high activity in solid tumours and haematological malignancies, respectively.
Cross-resistance pattern

Cross-resistance was investigated by analysing the degree of linear relationship between SI for two different drugs at fixed drug concentrations in cultures of patient tumour cells (papers I, II, IV) and in the cell line panel (paper II).

Evaluation on drug interactions

In study II the decrease in cell survival occurring with the use of a combination of a resistance modifier (cyclosporin A) and a cytotoxic drug was used to get some indications of the influence of Pgp. For the drug interaction analysis the multiplicative concept was used, in which the effect of an additive combination is expected to be equal to the product of the effects of its constituents; when the effect is greater (SI values lower) than this product synergy is observed (129). The ratio of the observed SI value to that expected from this additive interaction model was then calculated and observed/expected ratios <0.8 were defined as synergistic interactions.

IgVH gene analysis

In study V, IgVH gene analysis was performed (130). High molecular-weight genomic DNA was extracted from cryopreserved CLL cells using standard protocols. The Ig gene rearrangements were amplified using six IgVH gene family-specific primers and one JH primer. The amplification was performed with initial enzyme activation for 5 min at 95°C followed by 45 cycles of denaturation (94°C for 90 s), annealing (61°C-65°C for 30 s), extension (72°C for 80 s) and a final extension step of 5 min at 72°C using a thermocycler. For each PCR, a control with no added template was used to check for contamination. In the majority of samples, clonal PCR products were sequenced directly using a terminator cycle sequencing reaction kit. Each PCR product was sequenced in both directions in separate reactions employing IgVH primers identical to those used in the initial PCR reaction. In 5 cases, direct sequencing was unsuccessful and the sequence was determined by cloning of the PCR products. Between three and six colonies from each PCR product were thereafter sequenced. All samples were analysed using an automated sequencer. The sequences were aligned to the closest published germline IgVH gene using different databases. IgVH gene
sequences deviating more than 2% from the corresponding germline gene were defined as mutated.

Survival analyses

Survival data were obtained from the Swedish cancer registers in Uppsala (paper V). Overall survival was calculated from the date of diagnosis to death or last day of follow-up.

Statistical analysis

In studies I, IV, V and in the studies described in this thesis, parametric statistical analyses were used and in studies II and III, non-parametric analyses. The survival curve was estimated by the method of Kaplan-Meier, and the log-rank test was applied for comparisons (paper V). For further details see the respective papers.
RESULTS AND DISCUSSION

Detection of tumour type-specific activity of newly introduced cytotoxic drugs (Papers I-III)

Tumour type-specific activity

In study III the tumour type specificity of 14 cytotoxic drugs was investigated in samples from patients with leukaemia. The activities of the drugs were studied at concentrations associated with a large scatter of survival indices. The activities of 9 of these drugs in the different diagnoses are illustrated in Figure 4.

Marked differences between individual patients were found within the three diagnoses. CLL cells were more sensitive in vitro than AML and ALL cells to most of the drugs, and this difference was especially pronounced for the nucleoside analogues cytarabine, gemcitabine, fludarabine and cladribine, as well as for vincristine and cyclophosphamide. There was no difference between CLL and ALL cells in sensitivity in vitro to prednisolone, whereas AML cells were less sensitive to this drug. Etoposide is the only drug tested to which CLL cells were less sensitive than the AML cells.

In studies I and II the FMCA was used to evaluate the activities in vitro of the newly introduced purine nucleoside analogues fludarabine and cladribine and the pyrimidine nucleoside analogue gemcitabine (paper I) and also of the anthracycline idarubicin (paper II). The results are in accordance with those of study III, although the cells from the pure CLL group were more chemosensitive to most of the drugs than the cells from the mixed low-grade NHL group.

In contrast to the situation in the acute leukaemias, where tumour cell proliferation is extensive, the clonal expansion seen in CLL appears to be caused by extended survival of the malignant clone rather than by increased proliferative activity (47). The limited ability of all cells to proliferate under
assay conditions might be one explanation for the higher sensitivity of CLL cells despite their low proliferative nature. Thus the results may reflect the intrinsic sensitivity of CLL cells rather than their sensitivity due to proliferation. Indeed, CLL does show initial responsiveness to most cytotoxic drugs. The long-term clinical outcome may be determined by subclones not being accurately detected by the present method, in which cell death is measured in the whole cell population.

Figure 4. Cell survival after exposure of samples from patients with CLL, AML and ALL to the indicated drugs. The median survival index (SI) is indicated.
Low-grade NHL encompasses a variety of clinico-pathological entities with different degrees of response to chemotherapy and different outcomes (6). The material in paper I and II, included not only samples from CLL patients, but also samples from patients with other low-grade NHL, including mantle cell lymphomas (n=9) and prolymphocyte leukaemias (n=2). These subtyped histologically as indolent lymphomas, but clinically more aggressive.

An good correspondence overall between the in vitro cellular drug sensitivity profiles and known clinical activity of the drugs (56, 131) was observed. The clearest example is the lower prednisolone, vincristine and cyclophosphamide sensitivity of AML cells compared with CLL and ALL cells, and the lower etoposide sensitivity of CLL cells compared with the acute leukaemia cells. The high sensitivity of CLL cells to the purine nucleoside analogues fludarabine and cladribine is also in accordance with clinical data, whereas the high sensitivity of these cells to the pyrimidine analogues cytarabine and gemcitabine, as well as to the anthracycline idarubicin, is not in conformity with the clinical use. However, new studies on cytarabine, gemcitabine and idarubicin indicate that they also have clinical activity in CLL (see below).

There has been a lack of good models for predicting the diagnoses in which a new drug is likely to be effective. The predictive value of the NCI cell line panel (68, 69), of rodent tumours in vivo (70) and of human tumour xenograft models (71), with respect to clinical tumour-type specificity seems uncertain. Relatively few studies of the ability of in vitro cytotoxicity assays to detect tumour type-specific activity of drugs in tumour cells from patients have been performed (68, 69). However, the FMCA has previously shown an ability to predict tumour type-specific activity in mixed materials from patients with both haematological malignancies and solid tumours (73, 74), and the present studies indicate this ability among the haematological diagnoses as well.

The activity of cytarabine as a single drug in CLL or other low-grade NHL is not well established. A search in the literature yielded only one published study on this issue, where cytarabine was used as a single drug in 27 patients with low-grade NHL, with an overall response rate of 33 % (132). In addition a case report on a patient with concomitant CLL and AML in whom complete remission of CLL was achieved with high-dose cytarabine was found (133).

Gemcitabine is a pyrimidine analogue that is structurally similar to cytarabine (Fig. 1), and this and other in vitro studies have shown that the activity pattern also resembles that of cytarabine, with high activity against
haematological samples (134, 135). Gemcitabine has displayed clinical activity against several solid tumours, including pancreatic, ovarian, breast, lung and bladder cancers (112). In haematological malignancies, gemcitabine has shown activity in the treatment of patients with relapsed or refractory Hodgkin’s disease and high-grade NHL (109-111). The first trial in which gemcitabine was evaluated as a single agent in patients with refractory or relapsed low-grade NHL was published in 2001 (112). In accordance with our *in vitro* study, these results suggest that gemcitabine has significant antitumour activity in different forms of low-grade NHL.

Results from studies II and III indicate that idarubicin may be as active against low-grade NHL, including CLL, as against acute leukaemia. In fact an idarubicin concentration of 0.5 µg/ml was found to be more active *in vitro* against both low-grade NHL (including CLL) and acute leukaemias as compared with the same concentration of doxorubicin, epirubicin and mitoxantrone. Clinically, also idarubicin seems to be more potent than the other anthracyclines, but the question of which doses can be considered to be equivalent is still being discussed (116).

In several clinical trials, idarubicin in combination regimens has shown activity in low-grade NHL (117, 118). In a phase II study in 19 treated and untreated patients with CLL, oral idarubicin as a single agent was found to be well tolerated but of limited effectiveness; however, different doses and schedules are to be evaluated (136).

Thus, the FMCA may offer a potential in the development of new cytotoxic drugs in the selection of suitable diagnoses and patients for clinical phase II trials. The results from studies I-III indicate that the pyrimidine analogues cytarabine and gemcitabine, as well as the purine analogues fludarabine and cladribine, may have a role in the treatment of low-grade NHL especially CLL. The results also suggest a potential benefit of the anthracycline idarubicin in the treatment of these patients.

**Cross-resistance pattern**

Table 3 lists the correlation between the percentage survival indices for 9 of the cytotoxic drugs included in the present investigation, when tested on the material of CLL samples in study III (gemcitabine, unpublished data). As the CLL cells generally were more chemosensitive than the acute leukaemia cells, a lower concentration of cytarabine (0.5 µg/ml) and of idarubicin (0.1 µg/ml) was used than in the original studies (papers I-III) to obtain a larger
scatter of survival indices. Drugs with similar mechanisms of action, such as fludarabine and cladribine, and idarubicin and doxorubicin, show a high correlation coefficient. Notably the correlations of both vincristine and prednisolone with the rest of the drugs are low (Table 3).

**Table 3.** Relationship between survival indices (%) values for individual drugs expressed as correlation coefficients for 17-61 correlations on samples from patients with CLL. Modified from paper III.

<table>
<thead>
<tr>
<th></th>
<th>AraC</th>
<th>Gem</th>
<th>CdA</th>
<th>Fluda</th>
<th>Ida</th>
<th>Dox</th>
<th>Mixx</th>
<th>Vcr</th>
<th>Pred</th>
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<tr>
<td>AraC</td>
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<td>0.83*</td>
<td>0.68*</td>
<td>0.47*</td>
<td>0.56*</td>
<td>0.56*</td>
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<tr>
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<td>0.59*</td>
<td>0.30</td>
<td>0.07</td>
<td>0.58*</td>
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<td>0.19</td>
<td></td>
</tr>
<tr>
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<td>0.90*</td>
<td>0.59*</td>
<td>0.62*</td>
<td>0.74*</td>
<td>0.13</td>
<td>0.04</td>
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<td>50</td>
<td>0.47*</td>
<td>0.63*</td>
<td>0.62*</td>
<td>0.35*</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Ida</td>
<td>39</td>
<td>17</td>
<td>38</td>
<td>30</td>
<td>0.71*</td>
<td>0.71*</td>
<td>0.13</td>
<td>0.20</td>
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<tr>
<td>Dox</td>
<td>57</td>
<td>28</td>
<td>56</td>
<td>50</td>
<td>36</td>
<td>0.72*</td>
<td>0.51*</td>
<td>0.10</td>
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<tr>
<td>Mitx</td>
<td>36</td>
<td>17</td>
<td>40</td>
<td>31</td>
<td>38</td>
<td>38</td>
<td>0.10</td>
<td>-0.02</td>
<td></td>
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<tr>
<td>Vcr</td>
<td>61</td>
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<td>59</td>
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<td>35</td>
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</table>

Ara C, cytarabine; Gem, gemcitabine; CdA, cladribine; Fluda, fludarabine; Ida, idarubicin; Dox, doxorubicin; Mitx, mitoxantrone; Vcr, vincristine; Pred, prednisolone.

*p<0.05

Among the nucleoside analogues, gemcitabine structurally resembles cytarabine and cladribine resembles fludarabine. As can be anticipated from the chemical structure, in this material of CLL samples gemcitabine showed the highest correlation with cytarabine (R=0.78), whereas cladribine showed the highest correlation with fludarabine (R=0.90) (Table 3). This is in accordance with the results of study I in the material of low-grade NHL samples (R=0.90 and R=0.84 respectively). Relatively high correlations were obtained between all nucleoside analogues in both materials.

Few data have been published regarding the issue of cross-resistance among the purine analogues. As in our study, Bromidge et al. demonstrated cross-resistance *in vitro* between the two purine nucleoside analogues fludarabine and cladribine (137). The findings in most clinical studies conform with these results and indicate clinical cross-resistance (138-140). O’Brien at al. reported the largest experience with cladribine in patients refractory to fludarabine therapy. The overall response rate in 28 patients in their study was 7%, and no complete remissions were observed (140). In our study only a few samples were resistant to one drug and sensitive to the other, which is in accordance with the clinical observations.
There was a high correlation between the two anthracyclines and mitoxantrone tested in the CLL material (Table 3). In addition, the patient samples were grouped according to treatment status. In the CLL material SI values for idarubicin correlated well (R=0.76-0.85) to those for doxorubicin and mitoxantrone in samples from previously untreated patients (n=10), whereas the correlation was weaker (R=0.64-0.66) in samples from previously treated patients (n=26-28) (unpublished data). In the low-grade NHL material the difference was even more pronounced; Rho=0.81-0.90 in samples from previously untreated patients and Rho=0.24-0.42 in samples from previously treated patients (paper II). This was in contrast with the other anthracyclines tested, among which there was good correlation between the drugs in cells from both previously treated and from untreated patients (paper II).

Both experimental (34) and clinical studies (141) have provided evidence of incomplete, if any, cross-resistance between idarubicin and doxorubicin or daunorubicin. Our study confirmed these findings, only in cells from previously treated patients. One possible reason for this is that idarubicin may be less sensitive to mechanisms of acquired resistance, such as Pgp-mediated transport, than the other anthracyclines and mitoxantrone (33, 115, 142) and may thus retain its effect in cells from previously treated patients – cells that may have more characteristics of MDR (143).

The present results suggest that cross-resistance studies on new drugs, on fresh tumour cells from patients, if assessed in parallel with standard drugs, can not only provide mechanistic information on the new drug, but also reveal any cross-resistance and give some information on mechanisms of resistance. Cross-resistance analysis may also provide useful data when combination therapy is being considered, since a low level of cross-resistance may then be a desirable feature of the component drugs. If verified in clinical studies, the results of investigations of these kind may be useful in the preclinical evaluation of a cytotoxic drug.

**Acquired drug resistance**

To investigate the possible development of cellular drug resistance during drug treatment, we compared *in vitro* drug sensitivity in samples from previously untreated patients with that in samples from patients who had received cytotoxic therapy (papers I-III). Figure 5 shows a comparison of drug sensitivity, expressed as survival index, to 11 of the investigated cytotoxic drugs, in samples from both untreated and treated CLL patients.
included in study III (gemcitabine, unpublished data). As for the correlation analysis, for analyses presented in this thesis we used a lower concentration of cytarabine (0.5 µg/ml) and idarubicin (0.1 µg/ml), than in the original papers.

![Graph showing sensitivity to various drugs](image)

**Figure 5.** Comparison of the sensitivity, expressed as mean survival index (SI) (%) ± SEM of samples from untreated and treated patients with CLL to the indicated drugs. *P<0.05.

In agreement with previous findings (47, 144, 145), cells from previously treated patients with CLL were more resistant to most cytotoxic drugs than cells from treatment-naïve patients with this disease, with the exception of prednisolone and vincristine (Fig. 5). The results are in accordance with studies I-III except for the influence on treatment status of idarubicin activity, which was not found in study II and III. However, there was a large overlap in response between cells from untreated and previously treated...
patients. The pleiotropic drug resistance developing with treatment in CLL could be a result of a combination of many different mechanisms, such as drug efflux due to Pgp and alteration of the GSH/GST system (144), but also over-expression of anti-apoptotic proteins (47). In accordance with Bosanquet and Bell, we found that the sensitivity to prednisolone and vincristine persisted independently of drug treatment in samples from CLL patients (144).

In contrast to the retained effect in CLL cells after exposure to 0.5 µg/ml idarubicin, seen in study II, cells from treated patients with CLL showed higher median survival after exposure to a lower concentration of idarubicin (0.1 µg/ml) compared to cells from untreated patients. The reason for this may be that a higher concentration of idarubicin is too toxic, masking a true difference. However, daunorubicin at the same concentration (0.5 µg/ml), a drug that roughly has similar degree of cytotoxicity in vitro as idarubicin, was found to be more active against samples from untreated patients indicating a less strong influence of drug treatment on idarubicin sensitivity. Alternatively, the mechanisms that protect the cells from the cytotoxic effects of idarubicin and other anthracyclines may differ quantitatively rather than qualitatively.

The reason for a partially retained effect of idarubicin but not of the other drugs in previously drug-treated cells is obscure, but it is in accordance with the clinical experience that Pgp-related resistance can be overcome in heavily pretreated acute leukaemia patients, with idarubicin alone (115). In vitro, also, and in contrast to daunorubicin, idarubicin is cytotoxic in Pgp-positive cells (34, 142). One proposed explanation for these observations is that idarubicin is strongly lipophilic, and is thus transported to a lesser extent by Pgp (115). In our study, samples from previously treated patients seemed to express mechanisms of drug resistance that were more cyclosporin A sensitive than samples from previously untreated patients (paper II). Our results indicate a potential benefit of idarubicin, especially in previously drug-treated patients with low-grade NHL (CLL).

In contrast to the findings in CLL, no relationship between previous treatment and in vitro drug resistance was detected in AML and ALL samples (paper III). This may suggest that the adverse impact of previous treatment in AML and ALL is attributable to other factors than cellular drug resistance. Clinical drug resistance can also be caused by regrowth of neoplastic cells. In an in vitro study by Lövenberg et al., AML cell regrowth was shown to bear prognostic significance with respect to the short as well as long-term clinical outcome (146). In a study by Norgaard et al. the
regrowth capacity as evaluated with the MTT assay was of significance for the probability of obtaining complete remission (147). Thus, the proliferative potential of the leukaemic cell is a factor that might be of greater importance than cellular drug resistance in the acute leukaemias. CLL, on the other hand, is a disease which in general is characterized by a low proliferative rate, and thus acquired cellular drug resistance may be more important.

The results from studies I-III suggest that in vitro chemosensitivity testing of tumour cells from patients using the FMCA may yield considerable information regarding not only tumour type-specific drug activity, but also mechanisms of action, cross-resistance patterns and development of resistance, and therefore may be useful in the preclinical evaluation of a new cytotoxic drug. The results prompted us to use the method in an evaluation of the novel cytotoxic drug candidate, CHS 828 (paper IV).

Detection of tumour type-specific activity of a novel cytotoxic drug candidate (Paper IV)

This was an in vitro study using the FMCA to investigate the relative activity profile of CHS 828 in different malignant diagnoses. Primary tumour samples from 156 patients with different haematological malignancies and solid tumours were investigated.

Tumour type-specific activity

The relative activity of CHS 828 in different malignant diagnoses is presented in Figure 6. The activity of CHS 828 was high in all haematological malignancies, especially in samples from patients with CLL and other NHL, where most samples showed a 50% or greater decrease in SI. In several solid tumour diagnoses, the number of tested samples was small, and the estimates of relative activity should be viewed as very preliminary. Despite this, the high activity in a fraction of the samples from ovarian cancer and non-small cell lung cancer and the perhaps expected low activity in colon cancer are worthy of note.
Figure 6. Cell survival after exposure to 1 ug/ml CHS 828 in a total of 156 samples presented as survival index (SI). Fifty per cent survival is indicated; samples showing lower cell survival were arbitrarily designated as responders.

The higher activity of CHS 828 in haematological compared to solid malignancies is a characteristic in common with most conventional anticancer drugs. Actually, this differential activity, expressed as the S/H ratio as compared with other drugs tested in parallel, was less pronounced for CHS 828 than for cytarabine and cladribine, but in the same range as for vincristine and melphalan, which are well known to be active agents against haematological malignancies.

The use of valid in vitro models for prediction of tumour type-specific activity of new cytotoxic drugs is of great value in the preclinical phase of drug development. The FMCA is feasible and fairly easy to perform in studies of this type. A large number of samples from patients with different diagnoses, disease stages and treatment histories can be tested and the results may identify diagnoses and patients suitable for clinical studies. Since the FMCA measures cell damage in the whole tumour cell population, consisting of largely non-dividing cells, purely antiproliferative drugs may not be accurately detected by this assay (148). It should also be emphasized that indication of high drug activity in vitro in a specific tumour type is not
the same as prediction of clinical utility of the drug in question, which also depends on the achievable plasma concentration of that particular drug. In vivo it may not be possible to achieve cytotoxic concentrations of the drug either in the plasma or in the tumour, because of dose-limiting toxicity, pharmacokinetic factors or factors associated with the biology of the tumour itself (68).

**Therapeutic window**

To some extent, cytotoxicity assays may be used for preclinical evaluation of dose-limiting toxicity by comparing tumour cell responses with those of normal cells (149). In this study (IV) the efficacy of CHS 828 in CLL samples differed considerably from that in the normal counterpart (8 samples of normal lymphocytes from healthy donors). CLL cells showed 10 times higher sensitivity in terms of mean IC50 and 100 times higher in terms of mean IC30. This result indicates that there could be a therapeutic window for CHS 828 in CLL.

**Cross-resistance**

A correlation analysis was performed between the activity of CHS 828 and that of some standard drugs. The correlation was weak to moderate with most standard drugs, and the highest correlation (R=0.70) was obtained with vincristine, which might indicate some mechanistic similarity to the tubulin-active drugs. However, when the unresponsive solid tumours were removed from the analysis, the correlation were much weaker (R=0.47). Furthermore, the correlation between the tubulin-active vinorelbine and vincristine in the present material of haematological malignancies was considerably higher (R=0.93) than that between CHS 828 and vincristine. These findings clearly argue against a direct mechanistic link between CHS 828 and this group of compounds.

The results from this study were used in the planning of the clinical phase II study on CHS 828 which focused on patients with CLL. The study has recently closed and the results are not yet available. Further studies to characterize this drug and its behaviour are ongoing at our laboratory and at Leo Pharmaceuticals in Denmark.
Relationship between drug sensitivity \textit{in vitro} and IgVH gene mutation status (Paper V)

The aim of this study was to determine whether the difference in overall survival between the CLL cases with somatically mutated and those with unmutated IgVH genes (10, 11, 150) could be explained by a difference in cellular drug sensitivity. The \textit{in vitro} response to 9 cytotoxic drugs in unmutated and mutated CLL cases was investigated using the FMCA. The IgVH genes and \textit{in vitro} drug sensitivity were successfully analysed in 46 cases. At the time of sampling both groups (mutated and unmutated) included patients with a low tumour burden (Binet stage A, Rai stage 0, I) as well as with advanced disease (Binet C, Rai III, IV). Somatic hypermutation was present in 21 of the 46 cases (46 \%) and 25 cases (54 \%) had unmutated genes. Kaplan-Meier analysis showed a significant difference in survival between the mutated and unmutated groups (p=0.03, log rank test), hence confirming the prognostic significance of the IgVH gene mutation status. The median survival in the mutated group was 83 months, as compared to 65 months in the unmutated group.

\textit{Figure 7.} Mean survival indices ± SEM for the indicated drugs in CLL patients with unmutated (UM) (n=25) and mutated (M) (n=21) IgVH genes.
CLL cells with unmutated IgV_{H} genes tended to be more chemosensitive than cells from mutated cases, and for cytarabine and prednisolone the differences were statistically significant (p≤0.01) (Fig. 7). This novel finding indicated that the difference in prognosis between unmutated and mutated CLL could not be explained by divergent profiles of cellular drug sensitivity. This implies that other factors, such as acquisition of genetic aberrations may be more important for the clinical outcome than drug resistance as observed in vitro. Further studies on proliferation, cell differentiation and apoptosis are needed to reveal the biological basis of the difference in sensitivity to cytarabine and prednisolone between CLL cases with unmutated and mutated V_{H} genes.
SUMMARY AND CONCLUSIONS

- *In vitro*, CLL cells are more sensitive than AML and ALL cells to most standard cytotoxic drugs tested.

- In leukaemias, the tumour type-specific activity, predicted from *in vitro* sensitivity testing of standard drugs, are in good correspondence with the known clinical activity of these drugs.

- Cells from previously treated CLL patients are more resistant to most cytotoxic drugs than cells from previously untreated patients. In the acute leukaemias, drug resistance *in vitro* is not related to previous chemotherapy.

- The *in vitro* activity of the newly introduced pyrimidine analogue gemcitabine is strongly correlated to that of its analogue cytarabine, with high activity in haematological samples, particularly in CLL. Thus, cytarabine and gemcitabine may have a role in the treatment of CLL.

- The purine analogues cladribine and fludarabine show high cross-resistance *in vitro*.

- The *in vitro* activity of the anthracycline idarubicin indicates that the drug may have a role in the treatment of CLL, especially in previously treated patients.

- The new cyanoguanidine CHS 828 has high relative activity *in vitro* in haematological malignancies, especially CLL. The drug appears not to be cross-resistant with standard drugs.

- In previously untreated CLL, the difference in prognosis between patients with unmutated and mutated IgVH genes
could not be explained by divergent profiles of *in vitro* cellular drug resistance.
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REFERENCES


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45. Daniel PT. Dissecting the pathways to death. Leukemia 2000;14(12):2035-44.


