In vitro drug resistance in B cell chronic lymphocytic leukemia: a comparison with acute myelocytic and acute lymphocytic leukemia

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The aim of the study was to evaluate cellular drug resistance in B cell chronic lymphocytic leukemia (B-CLL) *in vitro*, and compare it with that in acute myelocytic leukemia (AML) and acute lymphocytic leukemia (ALL). *In vitro* drug resistance was analyzed by the fluorometric microculture cytotoxicity assay (FMCA) in all samples from patients with leukemia sent to our laboratory between 1992 and 2001. Up to 14 standard drugs were evaluated in samples from 66 patients with B-CLL, 212 patients with AML and 80 patients with ALL. B-CLL cells were found to be more sensitive than cells from both AML and ALL to cytarabine, cladribine, fludarabine, doxorubicin, idarubicin, vincristine and cyclophosphamide ($p < 0.05$). No difference in cellular drug resistance was found between B-CLL and ALL cells for prednisolone, whereas AML cells were more resistant ($p < 0.0001$). In B-CLL, cells from patients who had received previous chemotherapy were more resistant to almost all tested drugs as compared to cells from treatment-naive patients. In AML and ALL, *in vitro* drug resistance was not related to previous chemotherapy. For all drugs, there was a good agreement between the activity *in vitro* and the known clinical disease-specific activity. The study also demonstrated an acquired cellular drug resistance in B-CLL, but not in the acute leukemias.
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Introduction

B cell chronic lymphocytic leukemia (B-CLL) is a disease of long-lived accumulating B lymphocytes largely blocked in the G_0/G_1 phase of the cell cycle. Unlike in the acute leukemias, the clonal expansion seen in B-CLL appears to be caused by the extended survival of the malignant clone rather than by increased proliferative activity [1]. Typically, patients with B-CLL initially respond well to a number of cytotoxic drugs, but later develop drug resistance which continues to be a major obstacle to substantial improvements of prognosis in B-CLL.

Drug resistance is generally believed to be related to several different mechanisms, including cellular drug resistance, pharmacokinetics, and biological factors such as cell differentiation and leukemia regrowth [2,3]. The present study is focused on cellular drug resistance.

The molecular events that underlie resistance of B-CLL cells to cytotoxic drugs are yet to be defined. Deregulation of apoptosis contributes not only to disease pathogenesis, but also to development of resistance to cytotoxic drugs [4]. Both over-expression of anti-apoptotic factors like Bcl-2 and inactivation of pro-apoptotic factors like Bax have been discussed in association with drug resistance, but their role in drug resistance in B-CLL is not clear [5–7]. Other cellular drug resistance mechanisms that have been discussed for B-CLL are different mechanisms of drug efflux, like P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and lung resistance protein (LRP) [8,9]. Also, non-transport-mediated drug resistance, like altered topoisomerase II activity [10] and drug detoxification with glutathione S-transferases [11], have been discussed.

Short-term *in vitro* total cell kill assays have been used to study differences in *in vitro* cellular drug resistance between childhood acute myelocytic leukemia (AML) and acute lymphocytic leukemia (ALL) in relation to prognosis [12]. Moreover, the *in vitro* activity pattern of different tumor cells has been used for prediction of clinical diagnosis-specific activity in drug development [13–15]. Many research groups have also investigated these assays as a method of improving the disease management of individual patients. Cellular drug sensitivity correlates well with subsequent patient response and survival for a variety of diseases and drug regimens [16–18]. Results from *in vitro* drug resistance tests have

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been reported to predict response and survival in B-CLL [19–21]. Furthermore, *in vitro* drug resistance correlates with disease status as well as with certain cell biological features such as chromosomal abnormalities [22–24].

This study is an evaluation of the results from *in vitro* drug resistance testing performed on all leukemia samples sent to our laboratory between 1992 and 2001, using the total cell kill assay, the fluorometric microculture cytotoxicity assay (FMCA). The focus was *in vitro* cellular drug resistance in cells from patients with B-CLL as compared to cells from patients with ALL and AML. The activity pattern in the tumor cells was compared with known clinical diagnosis-specific activity of the cytotoxic drugs. The influence of patient chemotherapy to development of cellular drug resistance was also investigated.

Materials and methods

Patient samples

The study included all leukemia samples from adult patients sent to the laboratory between 1992 and 2001, and for which cell preparation and cell kill assay were successfully analyzed according to our standard quality criteria. The overall technical success rate was 73% for B-CLL, 75% for AML and 74% for ALL. The most common causes of assay failure were a too low proportion of tumor cells and a low fluorescence signal. Totally, samples from 66 patients with B-CLL (median age 64, range 43–94, years), 212 patients with AML (median age 56, range 16–86, years) and 80 patients with ALL (median age 31, range 16–81, years) were successfully analyzed. Samples were available from 12 AML patients and nine B-CLL patients both before and after chemotherapy. Samples were taken from peripheral blood, bone marrow or lymph nodes. Both fresh and cryo-preserved tumor cells were used. Cryo-preservation does not effect the sensitivity to any major extent [25]. The research ethical committee at Uppsala University hospital approved sampling for the FMCA.

Patient characteristics are shown in Table 1. In Sweden, first-line treatment of B-CLL has traditionally been chlorambucil with or without prednisolone. Refractory/relapsing patients are usually given fludarabine or (in the early days) an anthracycline-based regimen such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone). There was a considerable diversity in drug treatment history of the B-CLL patients included in the study. Thirty-four patients were untreated at the time of sampling, and 32 had received between one and six different regimens. Eleven different cytotoxic drugs had been used, including also high-dose methylprednisolone, CdA, daunorubicin, idarubicin, etoposide and mitoxantrone.

Table 1 Patient characteristics

B-CLL	
patients (*n*)	66
age (years) [median (range)]	64 (43–94)
treatment status	34 untreated
	32 previously treated
AML	
patients (*n*)	212
age (years) [median (range)]	56 (16–86)
treatment status	71 untreated
	141 previously treated
ALL	
patients (*n*)	80
age (years) [median (range)]	31 (16–81)
treatment status	29 untreated
	51 previously treated

Cell preparation

Mononuclear cells from bone marrow or peripheral blood were isolated within 24 h from sampling by 1.077 g/ml Ficoll-Isopaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation [26]. Tumor tissue from lymph nodes was minced to 1 mm^3 size, and cells were then isolated by collagenase dispersion and Percoll (Pharmacia Biotech) density gradient centrifugation [27]. Viability was determined by the Trypan blue exclusion test, and the proportion of tumor cells was judged by inspection of May–Grünwald–Giemsa-stained cytocentrifuge preparations on day 0 and day 3. All experiments were performed in culture medium, RPMI 1640 (Hyclone, Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone), 2 mM glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin. Cells were cryo-preserved in culture medium containing 10% dimethylsulfoxide (DMSO; Sigma, St Louis, MO) and 90% FCS by initial freezing for 24 h in –70°C followed by storage in liquid nitrogen.

Drugs

The cytotoxic drugs were selected based on clinical usefulness in B-CLL and acute leukemia treatment, and on diversity in mechanisms of action. The drugs were classified based on clinical use, as defined in a current textbook [28] and in *The Swedish Drug Compendium* [29] (Table 2). The drug concentrations used are presented in Table 3, and were selected based on experiments on series of hematological tumor samples in which several concentrations of the drug were tested and the concentration associated with the largest scatter of survival indices was selected (not shown). This concentration was chosen to optimize the conditions for differentiation between sensitive and resistant tumor cell samples *in vitro*, and has previously been shown to give a good correlation with the activity pattern in the clinic [13,26]. All drugs were obtained from commercial sources. Experimental plates were prepared with 20 μl/well of drug solution in triplicate at 10 times the desired final concentration with aid of a programmable pipetting robot (PROPETTE; Perkin-Elmer, Norwalk, CT). The experiments were performed using continuous drug exposure.

Table 2 Clinical use of the cytotoxic drugs in this study according to DeVita [28] and the approved use of the drugs in Sweden according to *The Swedish Drug Compendium* [29]

Drug	Recommended clinical use		
	B-CLL	AML	ALL
Cytarabine		×	×
Cladribine	×		
Fludarabine	×		
6-Thioguanine		×	×
Doxorubicin	×		
Idarubicin		×	
Amsacrine		×	×
Mitoxantrone		×	
Vincristine	×		×
Etoposide		×	
Melphalan			
Cyclophosphamide	×		×
Cisplatin			
Prednisolone	×		×

The FMCA

The FMCA is a total cell kill assay, based on the ability of cells with intact cell membranes to convert non-fluorescent FDA to fluorescent fluorescein. At day 1 180 µl/well of the tumor cell preparation (0.8–6 × 10^5 cells/ml culture medium) was added into 96-well microtiter plates (Nunc, Roskilde, Denmark). The culture plates were incubated for 72 h, after which the plates were centrifuged (200 *g*, 5 min) and the medium 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 before reading the fluorescence in a Fluoroscan II (exciting light at 485 nm for FDA, emitted fluorescence at 538 nm). The fluorometer was blanked against wells containing PBS with dye, but without cells. Standard quality criteria for a successful assay included a fluorescence signal in control cultures of greater than 5 times mean blank values and a mean coefficient of variation (CV) in control cultures of less than 30%. Furthermore, the fraction of tumor cells as assessed by microscopic examination after incubation should be greater than 70%. The results obtained were presented as survival index (SI) defined as fluorescence in test wells in percent of control cultures with blank values subtracted. Low numerical values indicate a high cytotoxic effect.

Quantification of FMCA results and statistical analysis

Non-parametric statistical analysis was used. The SI values for different drugs were expressed as median values with interquartile range. The Mann–Whitney *U*-test was used to compare the SI values generated from one drug in different diagnoses, and in samples from previously untreated and previously treated patients. Two-sided p values with a significance limit of 0.05 were used throughout.

Results

Results of *in vitro* drug resistance testing are summarized in Table 3 and Figure 1. Marked differences between individual patients were found within the three diagnoses. B-CLL cells were more sensitive ($p < 0.05$) *in vitro* than AML and ALL cells to cytarabine, cladribine, fludarabine, doxorubicin, idarubicin, vincristine and cyclophosphamide. When including samples from untreated patients only, the differences were even more pronounced. In response to amsacrine, B-CLL cells were more sensitive than AML cells ($p < 0.05$), whereas no difference between B-CLL and ALL cells was detected. There was no difference in cellular drug resistance between B-CLL and ALL for prednisolone, whereas AML cells were less sensitive ($p < 0.0001$). As for etoposide and cisplatin, B-CLL cells were less sensitive than those from patients with acute leukemia ($p < 0.05$). In addition to prednisolone, vincristine, melphalan, cyclophosphamide and cisplatin also demonstrated greater ($p < 0.05$) cytotoxicity in ALL than in AML.

As illustrated in Figure 1 and Table 3, the *in vitro* cellular drug resistance profiles is in rough accordance with the present clinical indications of the drugs. The clearest example comprised the lower prednisolone and vincristine sensitivity of AML compared with B-CLL and ALL cells, as well as the lower etoposide sensitivity of B-CLL cells compared with the acute leukemia cells. The high sensitivity of B-CLL cells to the purine nucleoside analogs, fludarabine and cladribine, is also in accordance with clinical data.

To investigate the possible development of cellular drug resistance during drug treatment, we compared the *in vitro* drug resistance in samples from previously untreated patients with samples from patients having received cytotoxic therapy. With the exception of prednisolone and vincristine, cells from patients who had received treatment with chemotherapy were more resistant than cells from treatment-naive patients with B-CLL (Table 3). In contrast, *in vitro* drug resistance was not related to previous drug treatment for any of the drugs tested in AML and ALL. Looking at *in vitro* resistance in the same patient before and after treatment with cytotoxic drugs showed the same tendency with acquired resistance in B-CLL, but not in AML (not shown).

Discussion

Most B-CLL patients respond to initial treatment with cytotoxic drugs, but drug resistance usually develops and complete remissions are less common. On the other hand, complete remissions are common in the acute leukemias. Unlike the acute leukemias, where tumor cell proliferation is extensive, the clonal expansion seen in B-CLL appears to be caused by an extended survival of the malignant clone rather than increased proliferative

Table 3 Drug resistance *in vitro*, expressed as median survival index values with interquartile range for the indicated drugs in samples from previously treated and previously untreated patients with AML, ALL and B-CLL (the Mann–Whitney *U*-test was used to compare the SI values generated from previously untreated and previously treated patients, respectively)

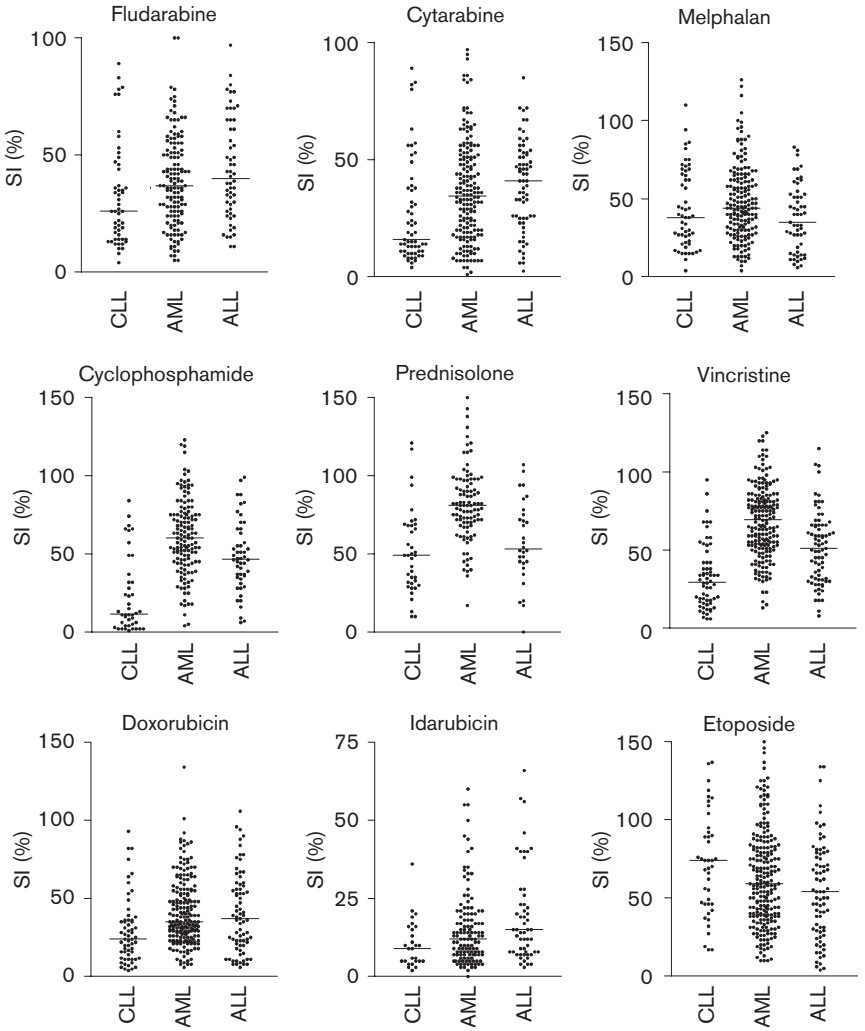
Drug		AML	*n*	ALL	*n*	CLL	*n*
Cytarabine 2.5 μg/ml	total	34 (19–51)	176	41 (25–53)	67	16 (11–38)	57
	untreated	33 (21–51)	55	45 (25–55)	21	14 (10–16)	30
	treated	35 (18–50)	121	41 (25–52)	46	28 (18–55)	27
	p	NS		NS		<0.01	
Cladribine 0.2 μg/ml	total	39 (21–54)	199	42 (25–63)	75	24 (13–48)	60
	untreated	38 (23–52)	69	41 (29–61)	25	14 (11–21)	31
	treated	39 (21–54)	130	44 (29–66)	50	38 (24–64)	29
	p	NS		NS		<0.001	
Fludarabine 2.5 μg/ml	total	37 (24–52)	137	40 (26–63)	56	26 (14–46)	53
	untreated	42 (28–56)	45	39 (26–64)	15	20 (14–33)	29
	treated	35 (23–50)	92	42 (28–62)	41	36 (23–67)	24
	p	NS		NS		<0.05	
6-Thioguanine 10 μg/ml	total	41 (29–58)	186	45 (32–71)	69	59 (49–70)	40
	untreated	36 (28–62)	63	55 (39–76)	25	46 (38–66)	12
	treated	43 (31–58)	123	44 (26–62)	44	62 (44–74)	28
	p	NS		NS		NS	
Doxorubicin 0.5 μg/ml	total	35 (26–54)	195	37 (20–58)	75	24 (12–36)	59
	untreated	32 (23–50)	67	41 (23–57)	27	18 (9–26)	29
	treated	38 (28–55)	128	29 (18–58)	48	35 (22–55)	30
	p	0.187		0.562		<0.001	
Idarubicin 0.5 μg/ml	total	12 (7–20)	127	15 (8–25)	55	10 (5–20)	27
	untreated	14 (8–20)	50	16 (10–39)	24	5 (5–9)	21
	treated	9 (7–20)	77	13 (7–22)	31	10 (5–16)	6
	p	NS		NS		NS	
Amsacrine 1 μg/ml	total	47 (30–70)	192	41 (28–58)	75	36 (21–61)	34
	untreated	39 (27–64)	65	44 (29–59)	28	20 (17–29)	10
	treated	50 (34–71)	127	36 (26–57)	47	40 (28–77)	24
	p	NS		NS		<0.01	
Mitoxantrone 0.5 μg/m	total	33 (16–36)	183	21 (11–31)	69	22 (10–38)	41
	untreated	22 (16–37)	62	25 (11–46)	22	9 (5–20)	11
	treated	24 (15–34)	121	19 (10–27)	47	26 (16–43)	30
	p	0.714		0.188		<0.01	
Vincristine 0.5 μg/ml	total	70 (53–84)	200	51 (32–66)	77	30 (17–42)	62
	untreated	68 (51–85)	69	54 (32–74)	28	28 (18–38)	30
	treated	72 (53–83)	131	49 (32–62)	49	30 (16–45)	32
	p	NS		NS		NS	
Etoposide 5 μg/ml	total	54 (37–77)	198	54 (29–78)	77	74 (46–94)	43
	untreated	46 (35–74)	66	60 (40–86)	28	46 (32–71)	12
	treated	58 (39–79)	132	50 (25–7)	49	57 (28–72)	31
	p	NS		NS		<0.01	
Melphalan 2.5 μg/ml	total	44 (30–62)	171	35 (17–52)	55	38 (22–68)	55
	untreated	44 (30–61)	58	32 (22–56)	16	33 (16–44)	28
	treated	46 (32–62)	113	40 (16–51)	39	57 (28–71)	27
	p	NS		NS		<0.01	
Cyclophosphamide[a] 2 μg/ml							
	total	60 (45–75)	137	46 (35–63)	50	11 (4–32)	42
	untreated	60 (44–75)	58	37 (30–52)	21	6 (3–14)	17
	treated	60 (45–75)	79	51 (40–68)	29	23 (10–51)	25
	p	NS		NS		<0.05	
Cisplatin 2 μg/ml	total	69 (53–88)	183	53 (40–65)	64	64 (60–91)	41
	untreated	71 (58–87)	60	53 (37–68)	19	67 (45–78)	11
	treated	69 (51–84)	123	53 (44–64)	45	79 (62–92)	30
	p	NS		NS		NS	
Prednisolone 10 μg/ml	total	81 (70–97)	102	53 (44–75)	30	49 (31–68)	3822
	untreated	81 (68–97)	49	50 (45–84)	12	50 (31–69)	22
	treated	81 (71–94)	53	58 (41–72)	18	40 (31–59)	16
	p	NS		NS		NS	

[a]*In vitro* as the active metabolite 4-hydroxycyclophosphamide.

activity [1]. Interestingly, we found that B-CLL cells were more sensitive than acute leukemia cells to several cytotoxic drugs despite the low proliferative nature of B-CLL. A possible explanation is an intrinsic sensitivity of B-CLL cells to cytotoxic drugs unrelated to proliferation. Indeed, B-CLL does show initial responsiveness to most cytotoxic drugs. Long-term clinical outcome in B-CLL may rather be determined by subclones not being accurately detected by the present method, which is based on measuring cell death in the whole cell population.

Despite recent progress in the treatment of B-CLL, there is still a need for discovery and development of new anticancer drugs. The cost of bringing new drugs to the clinic is considerable, and it is necessary to reduce the

Fig. 1



Cell survival after exposure to the indicated drugs in samples from B-CLL, AML and ALL. The median survival is indicated. For the number of patients (*n*) for each diagnoses and drug, see Table 3. *p* values determined by the Mann–Whitney *U*-test for statistically significant differences between the different diagnoses, see Results.

time and cost of their development. There has been a lack of good models to predict in which diagnoses a new drug is likely to be effective, which may lead to a rather random approach in the choice of diagnosis for phase II clinical studies. The FMCA has previously shown ability to predict tumor type-specific activity in solid tumors and hematological malignancies [13]. The present study extends this ability also in hematological diagnoses. Thus, the FMCA could aid in the planning of clinical trials and development of new cytotoxic drugs.

Although B-CLL shows initial responsiveness to a number of cytotoxic drugs, most patients become resistant to treatment. Cures are practically never achieved. In agreement with previous studies [1,30–33], the present results show that exposure to mechanistically unrelated cytotoxic drugs may induce cellular drug resistance. This can be detected *in vitro*, to several different drugs in B-CLL (Table 2). However, there is a large overlap in response between untreated and previously treated patients. The pleiotropic drug resistance developing with treatment in B-CLL could be a result of multiple drug resistance mechanisms like MDR associated with P-gp [30], but an over-expression of anti-apoptotic proteins in B-CLL cells could also lead to pleiotropic resistance and ultimately treatment failure regardless of the drug used [1].

In accordance with Bosanquet and Bell, we found that the sensitivity to prednisolone and vincristine was independent of prior treatment status in samples from B-CLL patients [30]. Bosanquet and Bell also found a sensitization *in vitro* to prednisolone by administration of chlorambucil [30]. In the current study, most of the previously treated B-CLL patients had received chlorambucil therapy and samples from treated patients tended to be more sensitive to prednisolone than samples from untreated patients.

In contrast to the results in B-CLL, no relationship between previous treatment and *in vitro* drug resistance could be detected in AML and ALL in this study (Table 2). This may suggest that the adverse impact of previous treatment in AML and ALL is attributed to other factors than cellular drug resistance. As mentioned, 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 short as well as long-term clinical outcome [34]. In a study by Norgaard *et al.*, the autonomous blast cell survival as evaluated with the MTT assay was of significance to the probability of obtaining complete remission [35]. Thus, the proliferative potential of the leukemic cell is a factor that could be of greater importance than cellular drug resistance in the acute leukemias. B-CLL, on the other hand, is a disease which is in general characterized by a slow proliferative rate and thus acquired cellular drug resistance may be more important.

In summary, the present study shows that in the FMCA, the diagnosis-specific activity of cytotoxic drugs in tumor cells from patients with B-CLL, AML and ALL correlated well with the clinical disease-specific activity pattern. The study also demonstrates a correlation between prior chemotherapy and development of cellular drug resistance for B-CLL, but not for ALL and AML, which suggests that factors other than cellular drug resistance are the major determinants of clinical resistance development for the acute leukemias. Although short-term drug-resistance assays have not yet moved to clinical implementation, studies using these assays have contributed to our understanding of drug resistance mechanisms in leukemia. The FMCA might aid in the design of clinical trials and development of new anticancer drugs.

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