

REVIEW

Bax expression correlates with cellular drug sensitivity to doxorubicin, cyclophosphamide and chlorambucil but not fludarabine, cladribine or corticosteroids in B cell chronic lymphocytic leukemia

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In B-CLL, non-proliferating B cells accumulate due to defective apoptosis. Cytotoxic therapies trigger apoptosis and deregulation of apoptotic pathways contributes to chemoresistance. Loss of the apoptosis-promoting Bax has been implicated in resistance to cytotoxic therapy. We therefore evaluated *ex vivo* drug sensitivity of CLL, producing chemoresistance data which are prognostic indicators for B-CLL, in particular in the case of purine nucleoside analogs. To analyze the underlying mechanisms of drug resistance, we compared endogenous Bax and Bcl-2 expression to *ex vivo* response to eight drugs, and to survival in 39 B-CLL patients. We found that reduced Bax levels correlated well with *ex vivo* resistance to traditional B-CLL therapies – anthracyclines, alkylating agents and vincristine (all $P < 0.04$). Surprisingly, no such relationship was observed for the purine nucleoside analogs or corticosteroids (all $P > 0.5$). Mutational analysis of p53 could not explain the loss of Bax protein expression. Levels of Bcl-2 were not associated with sensitivity to any drug. In contrast to the *ex vivo* data, neither Bax or Bcl-2 expression nor doxorubicin sensitivity were associated with increased survival whereas sensitivity to fludarabine correlated with better overall survival ($P = 0.031$). These findings suggest that the resistance to purine nucleoside analogs and corticosteroids in B-CLL is due to inactivation of pathways different from those activated by anthracyclines, vinca alkaloids and alkylating agents and may be the molecular rationale for the efficacy of purine analogs in this disease.

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Introduction

Deregulation of cell death pathways contributes not only to disease pathogenesis but also to the development of resistance to cytotoxic therapies.¹ Resistance to cell death programs is especially important in B cell chronic lymphocytic leukemia (B-CLL) cells which are predominantly non-proliferating G0/G1 quiescent cells that gradually accumulate because they are surviving too long. Both deregulated expression of anti-apoptotic factors and inactivation of pro-apoptotic factors, including those of the Bcl-2 family, have therefore been implicated in the pathophysiology of this disease. Compared with normal B cells, most B-CLL cells express higher levels of Bcl-2,² an apoptosis inhibitor, and lower levels of Bax, an apoptosis promoter, suggesting that they play an important role in the pathophysiology of B-CLL.^{3,4}

In this heterogeneous disease, the mechanisms responsible for the wide range of therapeutic responses documented are,

however, not yet clearly defined. The majority of patients do not achieve complete response despite the increased rates attributable to fludarabine (approaching 30% complete remissions) compared with alkylator-based therapies. In addition, the cellular immune deregulation induced by fludarabine can be very long lasting, thus limiting the use of other potentially promising therapies. This has prompted great efforts to elucidate levels of apoptosis-regulating proteins and their relationship to cellular resistance mechanisms and clinical response and survival.^{3–5}

Loss of pro-apoptotic factors in tumor cells is involved in the development of drug resistance. We previously observed a loss of Bax in a variety of carcinomas^{6–10} and in childhood acute lymphoblastic leukemia at time of relapse.¹¹ This loss of Bax was closely associated with resistance to therapy. In addition, loss of Bax is associated with shorter overall survival in a subgroup of patients with high grade lymphoma.¹² We also demonstrated recently, that the mitochondrial pathway, and not death receptor signalling, is the dominant pathway for induction of caspase activation and apoptosis by cytotoxic drugs in immature and mature B lymphoid tumor cells.¹³

In this work, we therefore investigated whether loss of Bax contributes to drug resistance in B-CLL. To this end, the *ex vivo* cellular sensitivity across a broad spectrum of commonly employed cytotoxic B-CLL therapies was determined and compared with the expression of Bax, Bcl-2 and the Bcl-2/Bax ratio in 39 B-CLL specimens. We have previously reported accurate predictive ability for this *ex vivo* apoptotic drug sensitivity assay when applied to freshly isolated B-CLL cells;^{14–16} assay results are an independent prognostic indicator in this leukemia.¹⁵

Our present findings suggest that low Bax protein expression correlates with agent-specific drug resistance. Low Bax was associated with resistance to doxorubicin, cyclophosphamide and vincristine but surprisingly not to fludarabine, cladribine and glucocorticoids. This suggests a different mode of action for fludarabine, cladribine and the steroids that is independent of the mitochondrial apoptosis pathway. This in turn may explain the activity of fludarabine and high-dose methylprednisolone¹⁷ in B-CLL refractory to conventional alkylating agent and anthracycline-containing therapies.

Materials and methods

Patients

Peripheral blood from B-CLL patients was sent for *ex vivo* drug sensitivity analysis by overnight delivery. If specimens arrived at the testing laboratories within 24 h of phlebotomy and

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yielded $>10^8$ cells, surplus lymphocytes were frozen for future protein analysis. Thirty-nine samples, analyzed for drug sensitivity to a panel of B-CLL drugs using fresh cells, were also analyzed for Bax and Bcl-2 protein expression using frozen cells from the same specimen. The thirty-nine patients included in this study (Table 1) had a median white cell count of 135.3×10^9 cells/l (interquartile range 84.5–221.3), mean age of 63.9 years (interquartile range 57.4–69.5 years), 31 were males and eight females. Patients with low leukocyte counts were not tested in this study to avoid false positive/negative results due to a higher contamination with non-malignant leukocytes in these samples. Approximately half the patients were untreated ($n=16$) and half previously treated ($n=23$) with two to six drug regimens. Pre-treated patients were a minimum of 7 days post-chemotherapy when blood was drawn for assay. Patients were staged according to Binet's classification and diagnosis of B-CLL was confirmed by morphology, a white cell count of $>15 \times 10^9$ cells/l and cell markers including CD5⁺ and CD23⁺.

Chemosensitivity assays

Circulating blood taken into EDTA was shipped at ambient temperature by overnight mail for processing the day after phlebotomy. Peripheral blood lymphocytes were obtained using density gradient centrifugation over HistoPaque 1077 (Sigma, Poole, UK). Cells from the interface were aspirated, washed twice in medium made up with RPMI 1640 supplemented with gentamycin (80 $\mu\text{g}/\text{ml}$) and L-glutamine (2 mM) and counted by hemocytometer. Median viability for specimens was 95% (interquartile range 92–97%), as determined by Trypan blue dye exclusion. All samples used in this investigation had a high white cell count, yielding sufficient lymphocytes for immediate drug sensitivity analysis and future protein expression experiments. For the latter, aliquots of lymphocytes (10^8) were pelleted and snap frozen in liquid nitrogen. For drug sensitivity testing, fresh cells (8×10^4) were incubated in duplicate in 0.6 ml polypropylene tubes in wash medium plus 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) or UltraCulture (BioWhittaker, Walkersville, MD, USA). Final volume of 90 μl included 10% phosphate-buffered saline (PBS) either without (control samples) or with added

cytotoxic drug. All classes of chemotherapy routinely used in B-CLL were tested: alkylating agents (chlorambucil and cyclophosphamide – as mafosfamide *in vitro*); purine nucleoside analogs (fludarabine phosphate and cladribine); corticosteroids (prednisolone and methylprednisolone); anthracyclines (doxorubicin); and a vinca alkaloid (vincristine). In order to induce cytotoxicity levels *ex vivo* comparable with those induced *in vivo*, the drugs were used at five concentrations encompassing the range of clinically relevant plasma levels, as previously reported.¹⁸ Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 92 h, allowing time for all drugs studied to exert their apoptotic effect.¹⁸ In culture, B-CLL cells remain in G0/G1 phase non-proliferative state and begin to die by apoptosis over time,⁴ and all drugs tested induced measurable drug induced apoptosis within 4 days.¹⁸ A mixture of fast green and nigrosin dyes in 10 μl PBS, and including fixed duck erythrocytes (50 000) as an internal standard, was added to the cell suspensions to stain dead cells black before cyto centrifugation on to microscope slides. The slides were air-dried and counter-stained with Romanowsky stain. Subsequent morphological evaluation (ie of cell shrinkage and other typical morphological signs of apoptosis) of slides by light microscopy facilitated the determination of drug efficacy at each concentration; to obtain the net effect of drug-induced apoptosis, live lymphocytes in the drug-treated samples were determined as a percentage of live lymphocytes in control cultures as described.¹⁸

Western blot

Lymphocytes frozen in liquid nitrogen were thawed, protein extracted and endogenous expression of Bax and Bcl-2 protein was measured by immunoblotting as described.¹¹ The protein concentration from each sample was measured with the BCA protein assay (Pierce, Rockford, IL, USA) using bicinchoninic acid¹⁹ and equal amounts of protein (15 μg) were subjected to SDS-polyacrylamide gel electrophoresis on 16% polyacrylamide gels following Laemmli's method.²⁰ Next, proteins were transferred to nitrocellulose membranes, which were stained using 0.1% Ponceau S in 1% acetic acid to verify equal loading of protein. The membranes were blocked in 1% casein Western blocking reagent (Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature, and incubated with respective primary antibody for 1 h (anti-Bax monoclonal mouse antibody, clone 4FM (Immunotech, Hamburg, Germany); anti-Bcl-2 monoclonal antibody, clone bcl-2/100/D5 at 1:100 (NovoCastr, Newcastle upon Tyne, UK)) at room temperature. Secondary antibody was applied for 1 h and bands were detected using the enhanced chemiluminescence (ECL) system from Amersham (Braunschweig, Germany). The integrated optical density of the resulting bands was determined by densitometric videoscanning²¹ and Scion Image 1.62 software (NIH, Bethesda, MD, USA). For standardization and inter-assay comparisons, 15 μg of protein extract from the human Burkitt-like lymphoma cell line BJAB were included on every blot as a positive control. Standard curves of the proteins were linear in the range of 12 to 20 μg total protein (data not shown¹¹). Interblot reproducibility of identical samples was checked and the samples showed a coefficient of variation of 9.0% for Bax and 2.0% for Bcl-2.

Table 1 Patient characteristics and Bcl-2 and Bax expression

Number of specimens	39
Previous regimens	
None	16
≥ 1	23
Gender	
Male	31
Female	8
Stage	
A	4
B	12
C	23
Age ^a	63.9 \pm 9.5 years
White cell count ^a	193 \pm 169 $\times 10^9/\text{l}$ (interquartile range 84.5–221.3, median 135.3)
Bcl-2 expression ^a	2.23 \pm 0.63 arbitrary units (range 0.98–3.71, median 2.13)
Bax expression ^a	2.13 \pm 0.95 arbitrary units (range 0.17–3.90, median 2.27)
Bcl-2/Bax ratio ^a	1.53 \pm 1.42 (range 0.59–6.74)

^aMean \pm s.d.

Mutation analysis for p53

DNA was extracted as previously described¹¹ and mutations in the p53 exons 5 to 8 were analyzed by genomic SSCP-PCR analysis as described.^{6,7} DNA was stored in 10 mM Tris HCl/0.1 mM EDTA buffer (pH 8.7). For the PCR reaction, we used 2% of the genomic DNA (from 10⁶ cells) in 2 μ l; 0.5 μ l of each sense and antisense primer (50 pmol). Primer sequences were as follows: exon 5a: CCA GTT GCT TTA TCT GTT CA and TGT GGA ATC AAC CCA CAG; exon 5b: CAA CTG GCC AAG ACC TGC and AAC CAG CCC TGT CGT CTC T; exon 6: CTC TGA TTC CTC ACT GAT TGC and GAG ACC CCA GTT GCA AAC CA; exon 7: TTG CCA CAG GTC TCC CCA A and AGG GTG GCA AGT GGC TCC; exon 8: CCT TAC TGC CTC TTG CTT C and CGC TTC TTG TCC TGC TTG C. 0.4 μ l of each dNTP (12.5 mmol of each dNTP in a total volume of 50 μ l), 1.5 μ l MgCl₂ (50 mM) in reaction buffer ((NH₄)₂SO₄) (16 mM) and 1.5 U of Taq polymerase (InViTAQ, Berlin, Germany) were added to the primers in water up to a total volume of 50 μ l. The amplification was performed by the use of a thermocycler 9700 (Perkin-Elmer/Cetus, Weiterstadt, Germany) according to the following protocol: exon 5a: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 20 s, 72°C for 15 s; exon 5b: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 65°C for 20 s, 72°C for 15 s; exon 6: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 60°C for 20 s, 72°C for 15 s; exon 7: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 58°C for 20 s, 72°C for 20 s; exon 8: 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 60°C for 20 s, 72°C for 15 s; final extension time of 7 min at 72°C. For SSCP analysis, the amplified DNA was diluted 1:1 in DNA loading buffer (82% formamide, 10 mM/l NaOH, 50 mM/l EDTA, bromophenol blue, xylene cyanole dye). The samples were denatured at 95°C (5 min), chilled on ice (4 min), loaded on a 10% non-denaturing polyacrylamide gel and finally electrophoresed at 500 V and 50 mA at 10°C in a Multiphor electrophoresis chamber (Pharmacia, Freiburg, Germany). Visualization was performed by silver staining. As a control for SSCP, DNA from SW620 cells which carry a p53 mutation in exon 7 was used as a positive control. LoVo cell DNA was used as a wild-type control.

Data analysis

Logistic curves were fitted to the cell count survival data; the log of survival probability was taken to be linear with respect to the logarithm of drug concentration.²² LC₉₀ doses, ie the concentration of drug to produce a 90% reduction in cell survival compared with control cells, were determined by calculating the log dose at which the fitted survival probability was equal to 0.1. Drug sensitivity indices were calculated as a percent rank of the 39 specimens tested, the most resistant scoring 0% and the most sensitive 100%.¹⁸

Associations between levels of Bax, Bcl-2 and the Bcl-2/Bax ratio and *ex vivo* response of B-CLL cells to eight cytotoxic drugs were determined using Pearson's correlation coefficient (SPSS version 8.0.0 for Windows95). The association of test results with patients responses were analyzed by univariate and multivariate logistic regression. Survival analysis was performed by Cox regression with backward elimination of non-significant covariates. All deaths were included in Kaplan-Meier curves; no qualitative differences were obtained when deaths due to causes other than B-CLL were classified as 'lost to follow-up'.

Results

Patients and specimens

In this study, we examined 39 B-CLL specimens. Patient characteristics are shown in Table 1. Both untreated and previously treated patients were studied: of the previously treated patients (*n* = 23), 14 had received chlorambucil, six fludarabine, six doxorubicin-containing combinations, five vincristine and 11 corticosteroids. Thus, this cohort of patients was typical of those found in routine clinics.

Bcl-2 and Bax expression

Bcl-2 and Bax were measured by Western blot analysis (Figure 1) and the results are presented in Table 1. We previously showed in childhood ALL that this method reliably allows quantification of Bax and Bcl-2 protein expression.¹¹ Endogenous Bax expression ranged from 0.17 to 3.90 relative units, median 2.27 (interquartile range 1.37–2.80). As expected in B-CLL cells,⁴ Bcl-2 levels were relatively high and ranged from 0.98 to 3.71 relative units, median 2.13 (interquartile range 1.84–2.54) being in line with the known increase of Bcl-2 protein expression in B-CLL as compared with normal B cells. A plot of Bcl-2 vs Bax expression shows a weak positive correlation between the expression of the two proteins (Figure 2) (Pearson's correlation coefficient, $r^2 = 0.142$, $P = 0.02$). Such a correlation can also be observed in high grade lymphomas.¹² There was no correlation found between patient age, stage or white cell count and Bcl-2, Bax or the Bcl-2/Bax ratio (Pearson χ^2 , all $P > 0.1$). In this context, high Bax levels were not a surrogate for treatment status, since similar numbers of pre-treated patients showed high or low Bax levels.

Since p53 has been described as a transcriptional activator of the human Bax gene and was also implicated in the inhibition of Bcl-2 expression, we analyzed the impact of mutation of p53 (exons 5 to 8, ie the DNA-binding domain) on the expression levels of Bax and Bcl-2. Figure 3 shows that there was no significantly lower Bax ($P = 0.13$, Mann-Whitney

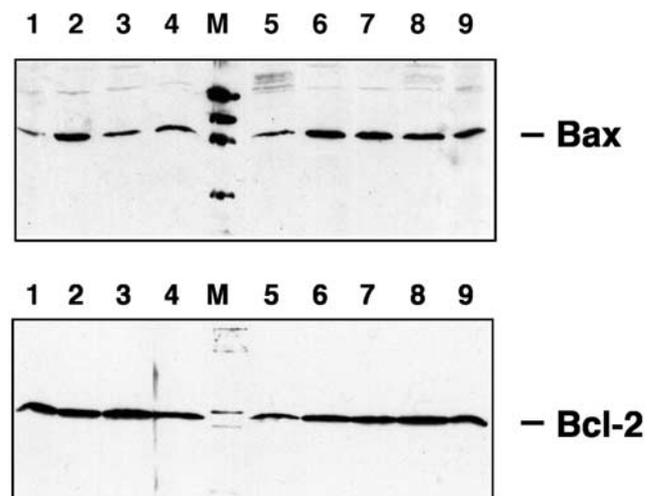


Figure 1 Western blot analysis for Bcl-2 and Bax protein expression in B-CLL. Representative samples of nine patients are shown. Note the more homogenous Bcl-2 expression as compared with Bax.

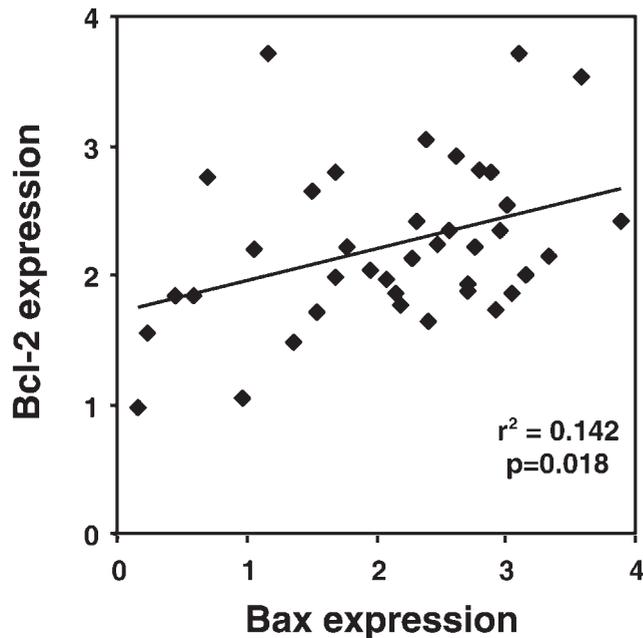


Figure 2 Correlation of Bcl-2 with Bax protein expression. Protein expression is expressed as OD as determined by videodensitometry (arbitrary units). Pearson linear correlation coefficient $r^2 = 0.142$, $t = 2.47$, $P = 0.0182$.

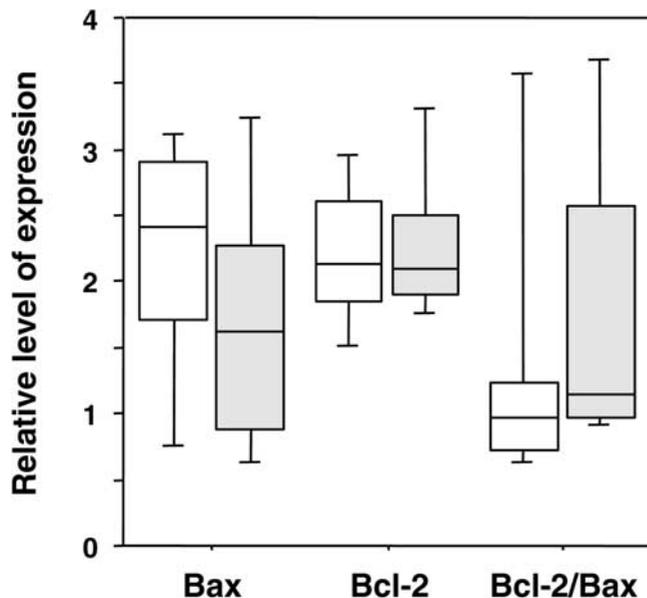


Figure 3 Impact of p53 mutation (exons 5 to 8) on Bax and Bcl-2 expression levels. Box plot analysis; grey boxes: p53 mutated samples ($n = 8$), white boxes: p53 wild-type samples ($n = 31$).

U test) or higher Bcl-2 expression ($P = 0.98$) in the p53 mutated ($n = 8$) as compared to the p53 wild-type CLL samples ($n = 31$). Nevertheless, p53-mutated samples showed a slightly lower Bax expression and, consequently, a higher Bcl-2/Bax ratio. This confirms our previous observation in breast cancer that not only inactivation of p53 but also additional genetic events are responsible for the loss of Bax protein expression.⁶

The mutational analysis of Bax in a variety of other cancers such as childhood B and T lineage ALL,¹¹ colorectal,⁷ esophageal squamous cell carcinoma²³ or breast cancer,⁶ showed

that Bax mutations are rare and restricted to mismatch repair deficient tumors. Thus, mutational inactivation of the Bax gene is not a very probable cause for the loss of Bax protein expression in drug-resistant B-CLL cells.

Ex vivo drug sensitivity

Drug sensitivity was assessed by the *ex vivo* apoptotic differential staining assay.²⁴ All chemotherapeutic drugs tested – fludarabine, cladribine, methylprednisolone, prednisolone, chlorambucil, cyclophosphamide (as mafosfamide *in vitro*), doxorubicin hydrochloride and the vinca alkaloid vincristine – induced apoptosis in a dose-dependent manner.

The sensitivities of the B-CLL cells expressed as LC_{905} showed considerable variability between the patients from as much as 120-fold below (for prednisolone) to 350-fold above (for cladribine) the median value (Table 2). For each drug, a range of sensitivities was observed, ranging from very sensitive to very resistant. These individual patient drug sensitivity profiles reflect the patient heterogeneity seen in the clinic.²⁴

Figure 4 demonstrates that the cellular sensitivity of B-CLL shows large inter- and intra-patient variations in drug response ranging from sensitivity to all drugs tested (Figure 4a) to resistance to all drugs. Some show extreme resistance to some drugs (eg fludarabine and cladribine in Figure 4b; steroids in Figure 4c) but sensitivity to other drugs tested. The comparison to Bax, Bcl-2 and the Bcl-2/Bax ratio shows in individual patients that there is no relation of resistance to purine analogs (Figure 4b), or corticosteroids (Figure 4c) to high Bax expression or Bcl-2 expression levels. In contrast, low Bax levels were associated with reduced sensitivity to doxorubicin, mafosfamide, chlorambucil and vincristine. Figure 4d is an example in which a low Bax level correlates with low sensitivity to several cytotoxic drugs (Figure 4d), especially with low sensitivity to doxorubicin (Figure 5).

As would be expected, cross-resistance was also observed between methylprednisolone and prednisolone; the cross-resistance between chlorambucil and mafosfamide was not as marked. Five patients had cellular resistance to all cytotoxic drugs included in this study.

Association of drug sensitivity with Bcl-2 and Bax expression

The associations of doxorubicin and fludarabine sensitivity with Bax, Bcl-2 and the Bcl-2/Bax ratio are shown in Figures 5 and 6, respectively.

A significant correlation of Bax expression levels and

Table 2 *Ex vivo* drug sensitivity

Drug	Abbreviation	Median	Range
Fludarabine	fl	0.296	0.047–83.1
Cladribine	cda	0.070	0.008–24.3
Methylprednisolone	mep	3.34	0.051–473.0
Prednisolone	pr	11.61	0.095–1350.0
Chlorambucil	chl	2.56	0.091–33.7
Cyclophosphamide	maf	0.893	0.142–6.17
Doxorubicin	dox	0.134	0.027–0.610
Vincristine	vc	0.598	0.014–18.24

Data are given as LC_{90} ($\mu\text{g/ml}$).

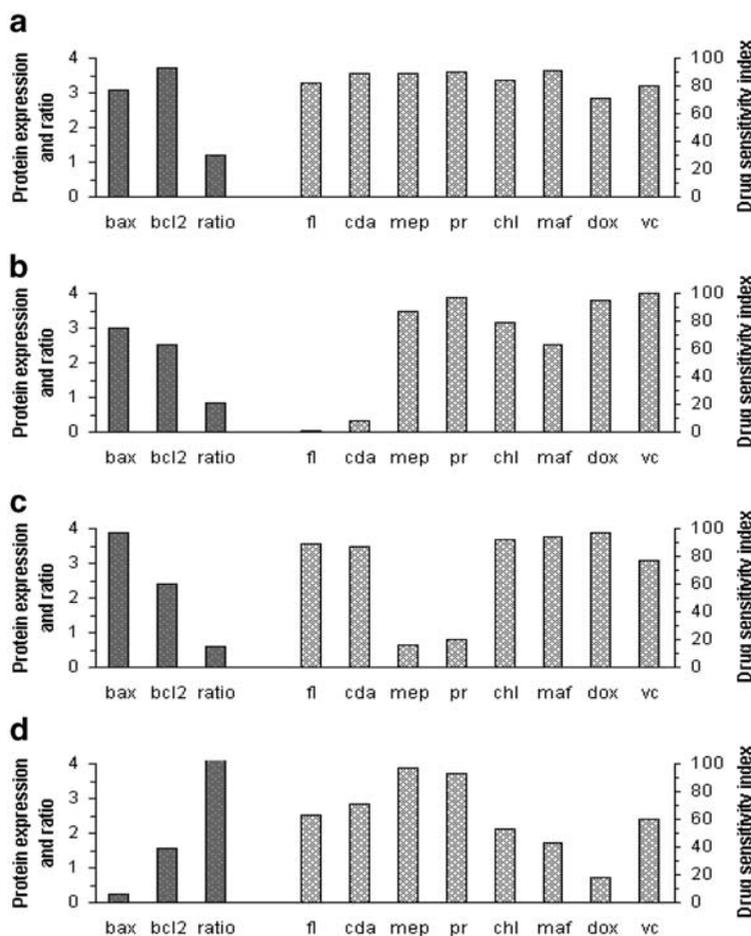


Figure 4 Four individual drug sensitivity profiles (a, b, c, d). Drug sensitivity index (bars on the right part of the figure) is calculated as the percent rank of the 39 specimens ranked from 0% (the most resistant tested) to 100% (the most sensitive tested). Thus, the specimen in (b) was the most resistant specimen tested against fludarabine but the most sensitive to vincristine. On the left side, the individual levels of Bax and Bcl-2 expression and the Bcl-2/Bax ratio are shown.

doxorubicin sensitivity is evident (Figure 5). Elevated levels of Bax protein as determined by quantitative Western blot analyses were associated with low doxorubicin LC_{90} values (Figure 5a). In the same line, low levels of Bax correlated with high doxorubicin LC_{90} values ($P=0.001$). Surprisingly, the levels of the Bcl-2 protein did not correlate with the sensitivity to doxorubicin-induced cell death (Figure 5b). Comparing the Bcl-2/Bax ratio to doxorubicin sensitivity showed only a weak but still significant correlation with doxorubicin sensitivity (Figure 5c).

Surprisingly, no association of either Bax or Bcl-2 protein or the Bcl-2/Bax ratio with *ex vivo* fludarabine sensitivity was observed (Figure 6a–c). This is in line with an earlier report which did show independence of nucleoside-induced apoptosis from Bcl-2.²⁵

A similar effect was observed when the sensitivities to additional drugs were compared with the Bcl-2 (Figure 7b) and Bax (Figure 7a) expression levels or the Bcl-2/Bax ratio (Figure 7c). Again, the level of Bcl-2 was not significantly associated with *ex vivo* cellular response to any drug tested (P mostly >0.2) (Figure 7b), although there is a suggestion of association with vincristine sensitivity ($P=0.052$). The Bcl-2/Bax ratio has been associated with progressive disease or treatment failure in B-CLL:^{26,27} in this study it was associated significantly only with *ex vivo* sensitivity to doxorubicin

($P=0.001$) (also with its epimer, epirubicin $P=0.001$, results not presented) but no other cytotoxic drug tested (Figure 7c).

The lack of association of Bax expression and sensitivity to purine nucleoside analogs and corticosteroids (all $P>0.5$) (Figure 7a) was in sharp contrast to a strong association with *ex vivo* drug response with established agents long used in B-CLL therapy: in particular, a strong association was seen for the anthracycline, doxorubicin ($P=0.001$). Bax was also significantly associated with *ex vivo* response to the alkylating agents chlorambucil ($P=0.034$) and cyclophosphamide (mafosfamide *in vitro*) ($P=0.017$) and to the plant alkaloid vincristine ($P=0.011$, Figure 7a).

Discussion

A major goal of this study was to determine whether expression of the apoptosis-regulatory proteins Bcl-2 and Bax and the Bcl-2/Bax ratio are associated with cellular drug sensitivity and/or survival across a broad range of chemotherapies used in B-CLL. In contrast to death receptor and death ligand-mediated signalling events,²⁸ the mitochondrial pathway of cell death represents a key switch in drug sensitivity of malignant B lymphoid cells.¹³ To this end, we analyzed the drug sensitivity profile of B-CLL cells by the use of an *ex vivo* apop-

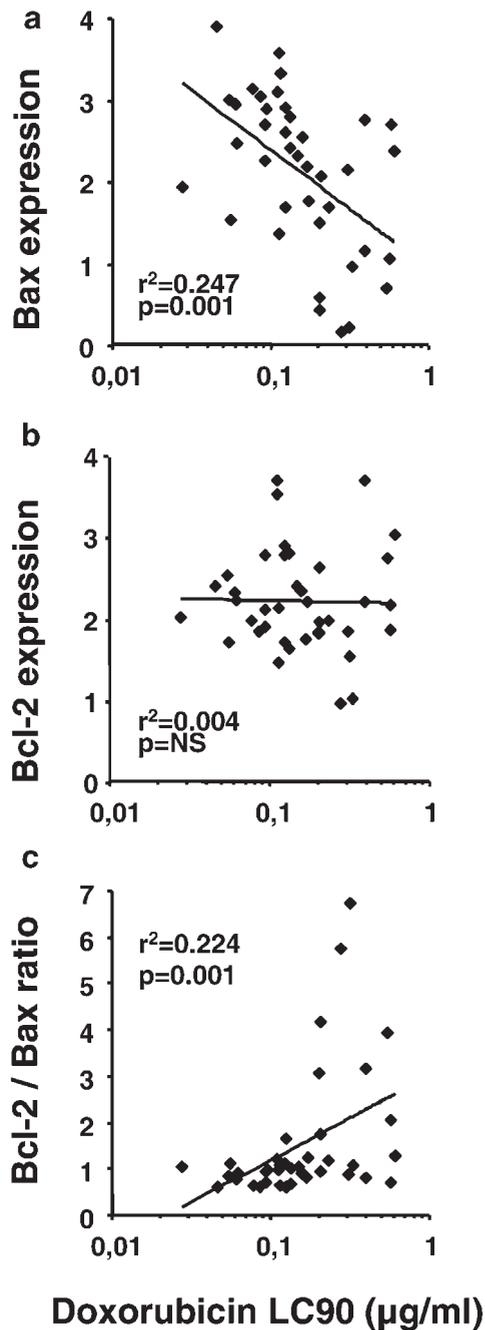


Figure 5 Doxorubicin sensitivity and correlation with Bax/Bcl-2 expression. Association of doxorubicin sensitivity with Bax (a), Bcl-2 expression (b) and the Bcl-2/Bax ratio (c). Pearson linear correlation coefficients (r^2) and significance are shown.

totic drug sensitivity assay that identifies response to therapy in B-CLL.^{14,15} This assay measures apoptotic cell death rather than inhibition of cell proliferation and is therefore suitable for B-CLL, the quintessential example of a neoplasm caused by cell accumulation of malignant cells resulting from apoptotic failure. It has been repeatedly demonstrated that apoptosis is the mode of tumor cell death promoted by chemotherapy.^{9,29-31} The biological basis for the widely differing therapeutic responses of B-CLL patients remains, however, largely unknown, and overall survival varies widely despite the large and expanding panel of drugs available to the clinician to treat this neoplasm.^{32,33}

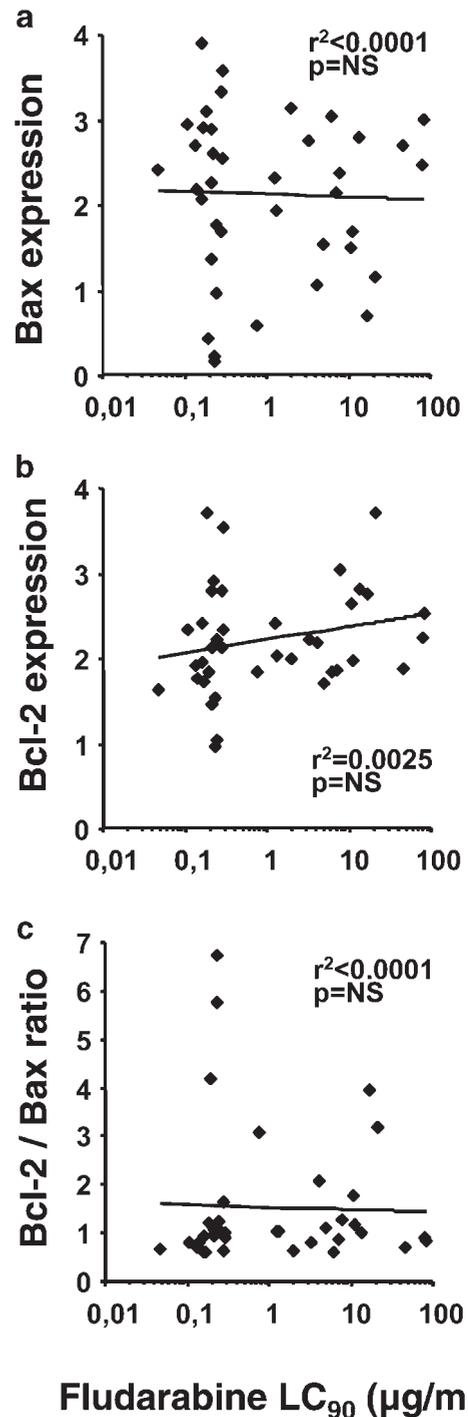


Figure 6 Fludarabine sensitivity and correlation with Bax/Bcl-2 expression. Association of fludarabine sensitivity with Bax (a), Bcl-2 expression (b) and the Bcl-2/Bax ratio (c). Pearson linear correlation coefficients (r^2) and significance are shown.

In the present study, we observed that high Bax expression correlates with *ex vivo* sensitivity to traditional B-CLL chemotherapy (the anthracyclines doxorubicin and epirubicin, the alkylating agents chlorambucil and mafosfamide, and the vinca alkaloid vincristine) but not to the purine nucleoside analogs cladribine or fludarabine, and corticosteroids. It should be noted, however, that the patients tested in this study displayed high peripheral lymphocyte counts. Thus, the con-

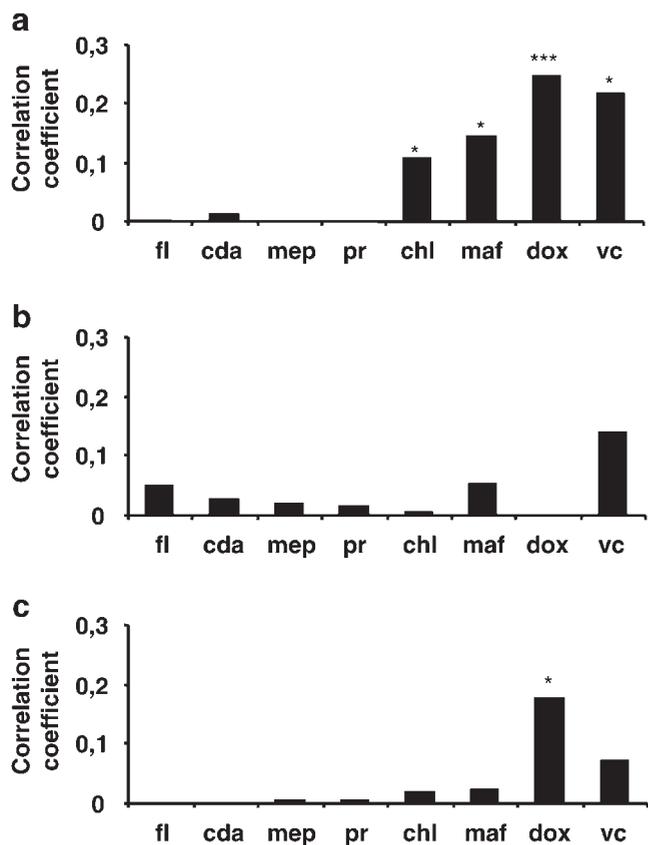


Figure 7 Association of Bax (a), Bcl-2 expression (b) and the Bcl-2/Bax ratio (c) with *ex vivo* drug sensitivity. Bars are Pearson linear correlation coefficients (r^2). Drug abbreviations are defined in Table 2. Significant associations are denoted by *, $P < 0.05$; ***, $P < 0.001$. $P = 0.052$ for vincristine LC₉₀ vs Bcl-2 expression. $P > 0.1$ for all other associations.

tamination with non-malignant leukocytes, which is a big problem in many gene expression profiling studies in acute and chronic leukemias, did not influence the measurements. On the other hand, while unlikely, the tumor biology of patients with lower leukocyte counts may differ.

Our data suggest that different drugs used for the treatment of B-CLL activate different cell death programs. The implied cross-resistance mediated by Bax, between the alkylating agents, anthracyclines and vincristine (Figure 7) may therefore be a factor limiting response to therapy. While anthracyclines, alkylating agents and vincristine seem to work via a Bax-dependent pathway, the nucleoside analogs might, according to these data, activate a Bax-independent death program. This is supported by recent reports showing that triphosphate metabolites of cladribine and fludarabine may act downstream of apoptosome, a cytosolic complex which is composed of apoptosis-activating factor-1 (APAF-1), cytochrome c, (d)ATP and procaspase-9, via activation of APAF-1 and the caspase cascade.^{34,35}

This suggests that a treatment strategy based on agents that induce response independently of Bax might improve response from traditional agents. Our results identify the purine nucleoside analogs and corticosteroids in this group. In fact, our present data show that fludarabine and cladribine induce cell death in B-CLL cells which are resistant to anthracyclines, alkylating agents and vincristine due to a decrease of Bax expression.

It is not clear why we did not observe a correlation of Bcl-2 levels with the chemosensitivity profiles in the present study. One reason might be that, compared with normal B cells, B-CLL cells constitutively express higher levels of Bcl-2 and lower levels of Bax.⁴ High expression of Bcl-2, an anti-apoptotic protein, can be associated with drug resistance in hematological malignancies.^{4,11,36–47} Thus, even the lower expression of Bcl-2 in some samples could provide an optimal anti-apoptotic effect. The role of Bcl-2 as a prognostic indicator remains controversial and it has therefore been suggested that Bcl-2 should be evaluated in parallel with other apoptotic markers. Such a calculation of the Bcl-2/Bax ratio in the present study showed a significant correlation, however, only with the *ex vivo* response to anthracyclines. In contrast, the correlations based on Bax alone showed a much clearer result which may reflect the fact that Bax may directly activate the cell death programme, independent from Bcl-2.^{48–50}

The role of the Bcl-2 family of proteins in chemoresponse has been evaluated extensively using *in vitro* models and it is still a matter of dispute whether individual members of the Bcl-2 family influence drug sensitivity of B-CLL cells *in vitro*.^{3,25} Previous studies support our finding that Bcl-2 is not an important determinant of response to purine nucleoside analogs in B-CLL^{51,52} perhaps because cladribine and fludarabine may directly activate the downstream apoptosis cascade,^{34,35} ie the caspases^{37,53} or other proteases^{54,55} which are related to apoptotic and other modes of cell death.⁵⁶ That fludarabine and cladribine do not make use of the Bcl-2/Bax pathway is consistent with their efficacy in patients resistant to the traditional therapies.^{14,15} This holds for the corticosteroids and we have previously reported efficacy for high-dose methylprednisolone in refractory B-CLL patients.¹⁷ Most work, to date, has concentrated on the relationship of protein expression to one or a few selected agents.

We found pronounced inter-patient variation in drug-induced apoptosis, agreeing with clinical experience. The data presented here reflect the complexities of apoptosis-regulatory gene expression and suggest that, although important players in regulatory apoptosis, they are not the only factors affecting drug-induced B-CLL cell death. Many other regulators participate to integrate the death signal generated upon drug exposure.^{1,49,50,57} However, our results highlight the significant association of Bax and the Bcl-2/Bax ratio with sensitivity to doxorubicin ($P = 0.001$). This is in agreement with work in tumor cell lines that have been transfected to express high levels of Bax and display enhanced sensitivity to doxorubicin.^{9,58,59} These results therefore provide convincing evidence that Bax expression is a key determinant of response to anthracycline-based chemotherapy in B-CLL.

The molecular events that underlie resistance of B-CLL cells to combination chemotherapy regimens are yet to be defined. Differences in the evolution and prognosis of B-CLL suggest a complexity of mechanisms that perhaps account for the heterogeneity seen in apoptotic cell death.⁶⁰ This may also be the reason why no prognostic role for Bax expression has yet been found in B-CLL.³ In this line, the levels of both Bax ($P = 0.69$) and Bcl-2 ($P = 0.41$) did not correlate with overall survival (data not shown). Nevertheless, while Bax expression did not correlate with survival, the *ex vivo* sensitivity to fludarabine showed such an association. The cellular resistance to fludarabine of the *ex vivo* tested B-CLL cells was associated with a shorter survival ($P = 0.031$), as previously described.^{14,15,24} There was, however, no association between survival and cellular sensitivity to doxorubicin ($P = 0.45$) and

the other drugs employed for the *ex vivo* testing (data not shown). This fits with the observation that Bax expression levels do not correlate with the *ex vivo* sensitivity/resistance for fludarabine or cladribine. Even when inactivation of specific apoptosis signalling components correlates with sensitivity to a specific treatment this might not necessarily result in a poor prognosis since different drugs may circumvent such signalling defects. This might be one of the reasons for the clinical success of the nucleoside analogs in the treatment of CLL as compared to anthracyclines or alkylating agents. Recent data show that nucleoside analogs may target the downstream apoptosis signalling cascade for drug-induced apoptosis, ie the caspase cascade.^{34,35} This is supported by our recent finding that the restoration or overexpression of procaspase-3 may overcome acquired drug resistance against a panel of different drugs employed in cancer therapy.²⁹

Thus, resistance to fludarabine may reflect a different cellular defect as compared with elevated Bcl-2 or decreased Bax expression levels or cellular resistance to doxorubicin. Furthermore, these results suggest that adenosine analogs could induce B-CLL cell apoptosis by activating intracellular pathways which are not common to those activated by anthracyclines, alkylating agents and vinca alkaloids. This may explain why resistance to fludarabine – as determined by *ex vivo* drug sensitivity testing – showed a correlation with the disease prognosis while the other parameters did not.

In future prospective studies it might therefore be worthwhile to include functional assays to measure the drug-induced mitochondrial step of apoptosis, eg by determination of the mitochondrial release of cytochrome c, determination of the mitochondrial membrane potential and the subsequent activation of the caspase cascade to further define the signalling events of apoptosis triggered by different drugs.

With the emergence of new drugs^{61–63} and classes of drug and novel combinations,⁶⁴ treatment options in B-CLL are widening exponentially, and increasingly complex therapeutic choices have to be made; the imperative to treat the heterogeneous patient population as individuals is increasingly evident.⁶⁵ De-selection of therapies – principally fludarabine and cladribine – in the minority of patients test-resistant to these drugs could improve outcome and may therefore improve survival.^{14,15} The molecular basis for resistance against these compounds remains, however, to be established.

The association of Bax expression with sensitivity to some but not all drugs is an example of the definition of molecular predictors for response to cytotoxic therapy since Bax expression was related to specific drug sensitivities. Such elucidation of an individual patient's response indicators to specific therapies could be instrumental in raising response rates and thereby improving survival.

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