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Chemomodulation of drugs involved in multidrug resistance in chronic lymphatic leukemia of the B-cell type

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Abstract. Reduced drug accumulation may be one reason for intrinsic drug resistance in chronic lymphatic leukemia of the B-cell type (B-CLL). Immunophenotyped leukemic human B-cells from 38 cases of B-CLL were characterized for P-glycoprotein (PGP) content. In all, 30 cases of B-CLL were additionally analyzed for further parameters: accumulation of daunorubicin (DNR, $n = 20$) and rhodamine 123 (Rh123, $n = 30$) in both the presence and the absence of verapamil (VRP). Also, 16 cases of B-CLL were analyzed for vincristine (VCR) accumulation with or without VRP. Concerning the relative expression of PGP, these 16 cases of B-CLL were representative for the whole group of 30 cases. Spontaneous accumulation of Rh123 and VCR varied over a wide range: accumulation of Rh123, by a factor of 11.8; accumulation of VCR, by a factor of 9.7; and accumulation of DNR, by a factor of 3.6. VRP modulated the accumulation of RH123 in 16/30 cases (53%), that of VCR in 69% of cases, and that of DNR in 11% of cases. The maximal VRP-mediated increases in accumulation amounted to factors of 1.3 for DNR, 2.3 for Rh123, and 7.8 for VCR. Spontaneous drug accumulation did not correlate with the extent of chemomodulation. The amount of PGP in B-CLL cells (all cases studied were PGP-positive) did not correlate with drug accumulation or with the extent of VRP-mediated chemomodulation. Thus, high expression of PGP is only partially responsible for defective drug accumulation in B-CLL. Only the degree of chemomodulation by VRP is predictive for the extent of the PGP-related functional drug accumulation defect. Thus, in B-CLL, PGP-independent drug accumulation defects seem to be as important as those mediated by PGP. The extent of this drug accumulation defect varies for the different drugs in the following order VCR > Rh123 > DNR. The relevance of PGP-mediated and -independent drug accumulation defects in vivo may depend to a large extent on the drug being used and on the individual cell type. Both types of defect in drug accumulation are of high importance when regimens include VCR a drug commonly used in second-line chemotherapy of B-CLL. Both defects in drug accumulation may be responsible for intrinsic VCR resistance in B-CLL.

Key words: Chemomodulation – Multidrug resistance – Lymphatic leukemia

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Introduction

Chronic lymphatic leukemia of the B-cell type (B-CLL) is characterized by cytologically and immunocytologically mature-appearing lymphocytes that proliferate and accumulate mainly in the bone marrow and lymphatic system. In most cases B-CLL is a clonal disorder. Nonrandom chromosomal aberrations occur in about 30% of cases. The natural history of the disease is very heterogeneous and characterized by progressive lymphocytosis, splenomegaly, and immunodeficiency syndrome. A number of independent prognostic factors for survival can be defined, such as lymphocyte count, lymphocyte doubling time, hemoglobin content, diffuse versus nodular bone marrow infiltration, and stage. Mainly two staging systems, Binet and Rai, have been proposed. To date the effects of treatment have been unsatisfactory and merely palliative. However, in a large cohort, multivariant analysis has shown the response to treatment to be an independent prognostic factor for survival [5]. Therefore, intrinsic drug resistance, which is determined genetically and is independent of prior therapy, seems to be a major problem and should be considered in the development of new treatment modalities [24].

Correlative studies have shown a close association between drug accumulation and in vitro resistance in patients with myeloma, lymphoma, and breast cancer [10, 32]. In the human cell lines used in these studies, drug accumulation was significantly reduced by P-glycoprotein (PGP)-mediated drug efflux.

We investigated whether B-CLL cells that constitutively express *mdr*1 and *mdr*3 gene products [21, 25, 37] exhibit

reduced accumulation of different drugs commonly used in the treatment of B-CLL, such as anthracyclines and vincristine (VCR). These drugs are used, for example, in the CHOP schedule (cyclophosphamide, Adriablastin, and prednisone), which is strongly supported by the French Cooperative Study Group on CLL as first-line therapy in patients with advanced B-CLL of stages B and C according to Binet [15]. Although it has become accepted that defective accumulation of drugs involved in PGP-mediated drug efflux is usually associated with the hyperexpression of PGP, model systems show that the mechanistic basis for multidrug resistance (MDR) or MDR in the absence of PGP remains to be further elucidated. Our study shows that besides PGP-dependent mechanisms, PGP-independent mechanisms may also play a crucial role in drug accumulation in B-CLL.

The functional importance of the MDR phenotype can be demonstrated by chemomodulation. PGP expression is a necessary correlate to differential sensitization with drug-efflux modulators such as verapamil (VRP). As VRP affects PGP-mediated drug efflux by direct inhibition of PGP, no VRP-mediated chemomodulation can be detected in PGP-negative MDR cell lines. The mechanism of chemomodulation is independent of the drug being modulated. Its effect on drug accumulation is dose-dependent and rapidly reversible.

Three chemically different MDR drugs were chosen for chemomodulation assays: daunorubicin (DNR), rhodamine 123 (Rh123), and VCR. These compounds differ in their intracellular binding sites as well as their binding characteristics to PGP. Rh123 is a supravital cationic dye with special affinity for mitochondrial membranes [8]. Binding sites for VCR are mainly tubulin molecules, whereas DNR binds unspecifically to many intracellular target molecules [33, 34, 45].

To assess the mechanisms regulating MDR-drug accumulation in B-CLL, we studied the PGP content of B-CLL as well as spontaneous and VRP-mediated drug accumulation to analyze the factors that modulate drug accumulation in B-CLL and correlated our results with the cell type and the binding characteristics of these drugs.

Patients and methods

Materials. VRP (Knoll AG, Ludwigshafen, Germany), Rh123 (Sigma Chemical Company, St. Louis, USA), daunoblastine (Farmitalia, Carlo Erba GmbH, Freiburg, Germany), [^{3}H]-VCR (Amersham Buchler GmbH, Braunschweig, Germany; specific activity, 226 MBq/mmol), silicone oils AR 20 and AR 200 (Wacker Chemie GmbH, München, Germany), silicone solution in isopropanol, trichloracetic acid (TCA), KOH (Serva, Feinbiochemica, Heidelberg, Germany), Lymphoprep (Nycomed AG, Oslo), fetal calf serum (FCS, Gibco BRL, Berlin, Germany), RPMI 1640, phosphate-buffered saline (PBS), $NaHCO_3$ (7.5%), glutamine solution (200 m*M*), bovine serum albumin (BSA, Sigma Chemie Deisenhofen, Germany), monoclonal antibody (mAb) C219 (Cis Isotopen Diagnostik GmbH, Dreieich, Germany), fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Dako, Hamburg, FRG), propidium iodide, RNase (Worthington), and spheres (calibration standard) comprised the materials used.

Patients and specimens. Lymphocytes were obtained from the peripheral blood of patients suffering from B-CLL. Low-density mononuclear cells were isolated by density centrifugation in Lymphoprep. Cells either were used fresh or were stored at –70° C in the presence of 10% dimethyl sulfoxide until use. Frozen samples were resuspended after rapid thawing in 5–10 ml preservative-free heparin-containing medium and allowed to hydrate for 2–3 h before further manipulation. No major difference was detected in VCR, Rh123, or DNR accumulation or antibody reactivity between the fresh and frozen cell samples. All samples were immunophenotyped. Cytospin preparations were done for cell staining according to Pappenheim.

Immunophenotyped leukemic human B-cells from 38 cases of B-CLL were characterized for PGP content. In all, 30 cases of B-CLL were analyzed for further parameters: total accumulation of DNR (n = 20) and Rh123 (n = 30) in the presence/absence of VRP. In addition, 16 cases of B-CLL were analyzed for VCR accumulation with or without VRP. These 16 cases of B-CLL were representative for the whole group of 30 cases in terms of the distribution of PGP levels.

Dye accumulation assays. The ability of VRP to inhibit DNR, Rh123, or VCR efflux and to enhance the cellular retention of these drugs via inhibition of PGP action was evaluated concurrently with the study of cellular PGP expression. The cell lines P388/ADR (PGP-positive) [41] and K562 (PGP-negative) were adjusted to a concentration of 10^6 cells/ml with RPMI/10% FCS. In modulation assays, VRP was added at a final concentration of 8 μ*M* to each sample at an incubation temperature of 37° C.

The excitation wavelength (EX) for DNR was 579 nm and the emission wavelength (EM) was 603 nm. For Rh123, the EX was 505 nm and the EM, 534 nm. Dye accumulation was established in RPMI (10% FCS) at 37° C in a shaking water bath. DNR was added to a final concentration of 10 μg/ml and Rh123, to a final concentration of 1 μg/ml. The incubation period used in this assay was chosen according to the time necessary for the cells to reach the maximal cellular concentration of the respective dye (60 min for DNR and 30 min for Rh123) [18, 38].

PGP-mediated drug efflux was demonstrated by adding the PGP inhibitor VRP (8 μ*M*) to the incubation mixture. After two washes, cells were incubated in 10 ml dye-free media for 3 h at 37° C or at 4° C with or without the PGP inhibitor VRP. After incubation, the cells were placed on melting ice and washed three times by centrifugation (300 *g* at 4° C). The pellet was resuspended with ice-cold PBS. The cells were then stained with propidium iodide and kept on melting ice until the performance of flow cytometry (FCM, within 2 h). Incubation of the cells with DNR or Rh123 was not associated with detectable cytotoxic effects.

[^{3}H]-VCR accumulation assay. The assay used has been described elsewhere [28]. Briefly, cells were suspended in RPMI/10% FCS and brought to 37° C in a water bath. Aliquots of 200 μl (1.05×10^6 cells) were treated as follows. Aliquots were mixed with 10 μl [^{3}H]-VCR (final concentration, 85 n*M*) in a siliconized 1.5-ml Eppendorf cup. Then, 200 μl (10^6 cells) was layered on a silicone-oil gradient and incubated at 37° C in a water bath. The Eppendorf cups were laid horizontally in the water bath and turned every 5 min according to the incubation time.

After an incubation period of 60 min, necessary for the cells to reach the maximal cellular VCR concentration, the cells were centrifuged for 30 s at 11,000 *g*. Afterward, all cells were collected in the KOH layer in the tip of the Eppendorf cup. The gradient cup was cut in the region of the silicone-oil gradient and the tip containing the cells in KOH was transferred into the scintillation vial. The KOH solution was neutralized with 250 μl H_2O and 50 μl TCA (5 *M*). The probes were incubated for 3 h at 60° C before analysis [28]. All accumulation studies were performed in duplicate with and without VRP.

Flow cytometry. DNR, Rh123, and FITC were excited by 488-nm light from an argon laser (FACS II flow cytometer). Fluorescence emitted by the dyes was detected using a photomultiplier. Two 520-nm long-pass filters blocked the scattered light. Gating bit maps were generated for all experiments on the basis of their forward- and right-angle light scatter. The intensity of forward- and right-angle light-scatter signals are related to cell size and granularity, respectively. Dead cells can be

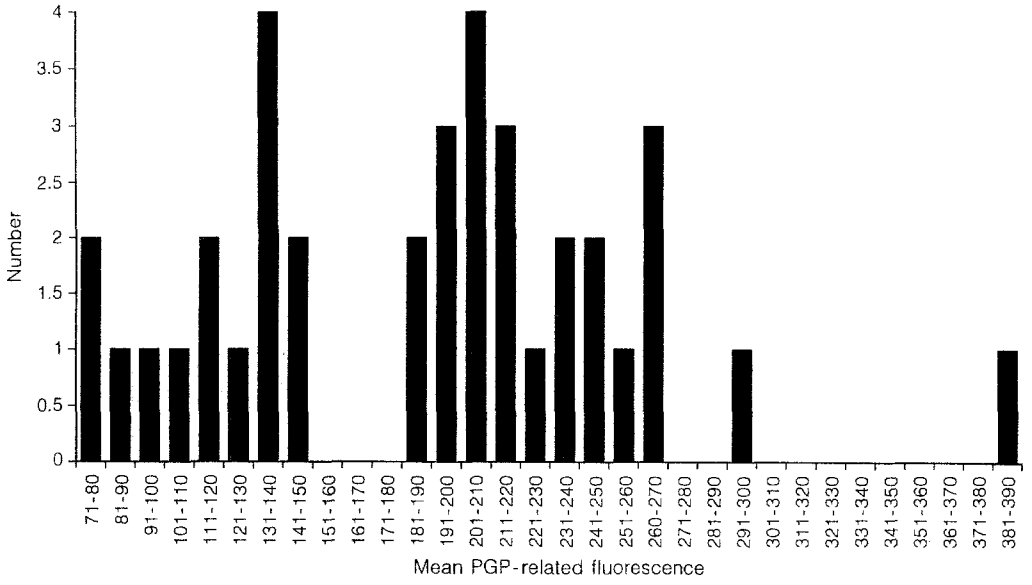

Fig. 1. Distribution of mean PGP-related fluorescence (mAb C219) in 38 cases of B-CLL

separated on the basis of high propidium iodide fluorescence, granularity, and cell size. For each sample, at least 10,000 cells were analyzed. All data were corrected for cellular autofluorescence and for unspecific binding of FITC IgG. The mean fluorescence is expressed in arbitrary fluorescence units, representing the mean fluorescence (MF) of the cells positively stained with Rh123, DNR, or PGP [10, 19, 33]. B-CLL cells were incubated with mAb C219 according to the description of Epstein et al. [10].

Modulation factor. The modulation factor is defined as the quotient of mean accumulation plus VRP/mean accumulation without VRP and describes the extent of inhibition of a steady-state drug accumulation by VRP.

Statistical analysis. The statistical analysis was performed using Student's *t*-test. A P value of <0.05 was considered significant. Variant analysis was done using the Kruska-Wallis model (distribution-free variant analysis).

Results

PGP expression

In all 38 cases 68%–98% of B-CLL cells were positive for mAb C219. The intensity of specific PGP-related fluorescence differed between 76 and 387 (mean fluorescence channel number of PGP-positive cells). Two groups of B-CLL could be separated according to their modulation factors (quotient of drug accumulation with VRP/without VRP), with the cutoff point at mean fluorescence being 160. In all, 15/38 cases of B-CLL (39%) expressed low levels of PGP and 23/38 cases expressed high levels (61%; Fig. 1).

Drug accumulation without VRP and VRP-mediated drug accumulation in the steady state

In most cases, steady-state accumulation of DNR, Rh123, and VCR was relatively low. Steady-state accumulation of Rh123 and VCR varied over a wide range (factors of 11.8 for Rh123 and 9.7 for VCR). This observation was in contrast to the variation found for DNR accumulation (factor of 3.6). On the other hand, VRP-modulated VCR accumulation was distributed heterogeneously over a wide range of intracellular VCR concentrations (Fig. 2). VRP-mediated VCR and Rh123 accumulation were significantly enhanced in comparison with the drug accumulation observed in the absence of VRP ($P<0.01$), whereas the corresponding accumulation values for DNR did not differ significantly.

The mean modulation factor (quotient of mean accumulation plus VRP/mean accumulation minus VRP) was as follows DNR $<$Rh123 $<$VCR = 1.0 <1.3 <2.5. The modulation factors obtained in each group (DNR, Rh123, VCR) differed significantly in univariate analysis ($P<0.01$). In addition, the number of cases of B-CLL in which MDR-drug accumulation could be modulated by VRP (by a factor of >1.2) was dependent on the drug being modulated: DNR, 3/26 (11%); Rh123, 9/30 (30%); and VCR, 11/16 (69%). Although the mean modulating capacity of VRP is drug-dependent and seems to be characteristic for each drug used, single cases differed from this pattern. These B-CLL cells showed higher modulation factors for Rh123 than for VCR (n = 4). In one case of B-CLL, the modulation factors were ≥ 1.2 for all three drugs being tested (modulation factors: DNR, 1.2; Rh123, 1.3; VCR, 3.7). In P388/ADR cells, the modulation factor for VCR (3.9 ± 1.2; range, 2.5–5.3) was very low as compared with the modulation factor for Rh123 (28.2 ± 7.1; range, 20.1–37.2). These modulation factors represent mean values $\pm$ SD for 30 assays.

Drug accumulation and PGP expression

No linear correlation could be demonstrated between PGP levels and VCR or Rh123 accumulation in the steady state with or without VRP. However, there was a linear correlation between PGP levels and modulation factors (corre-

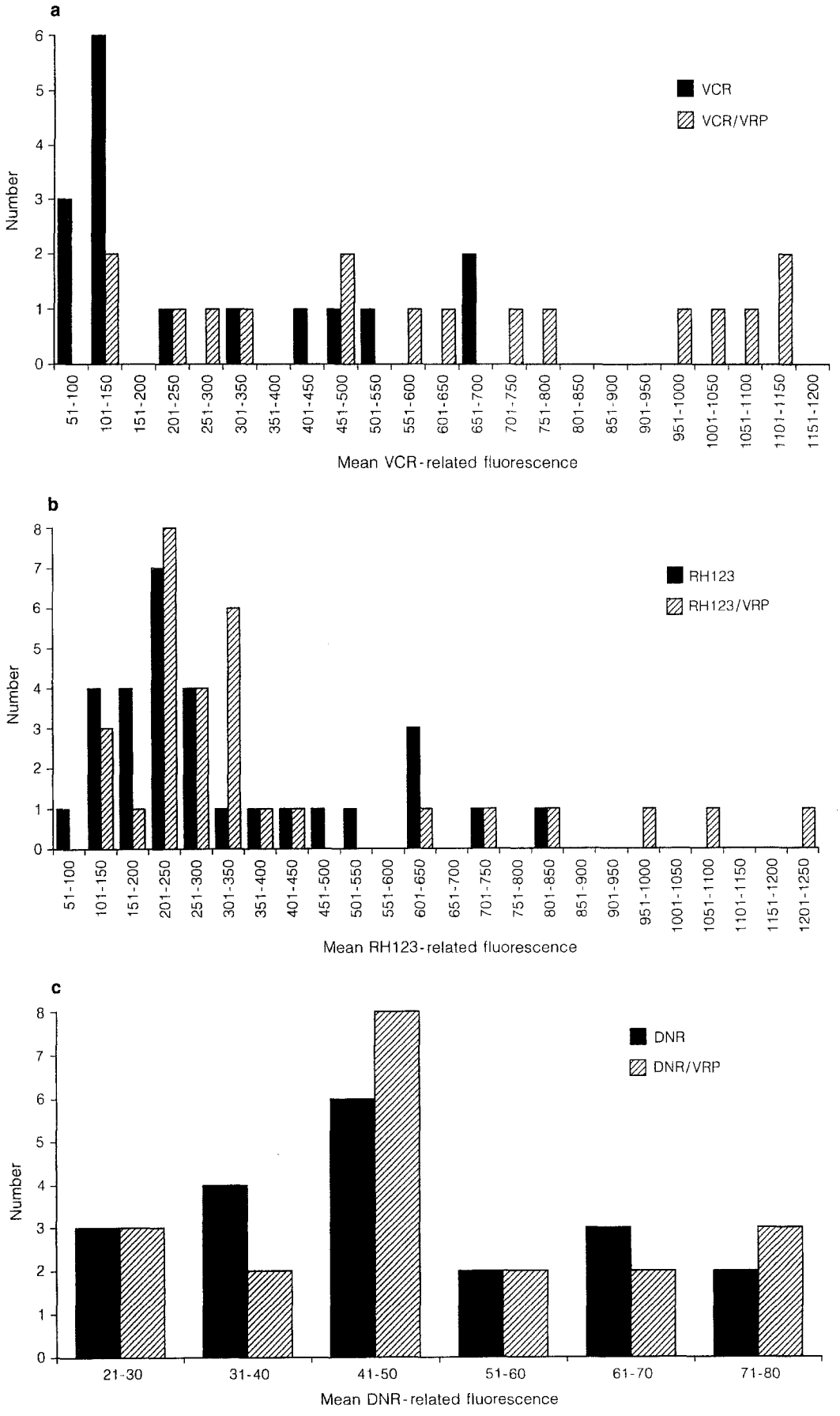

Fig. 2a–c. Distribution of **a** [^{3}H]-VCR accumulation ($n = 16$), **b** Rh123 accumulation ($n = 30$), and **c** DNR accumulation ($n = 30$) in the presence or absence of VRP

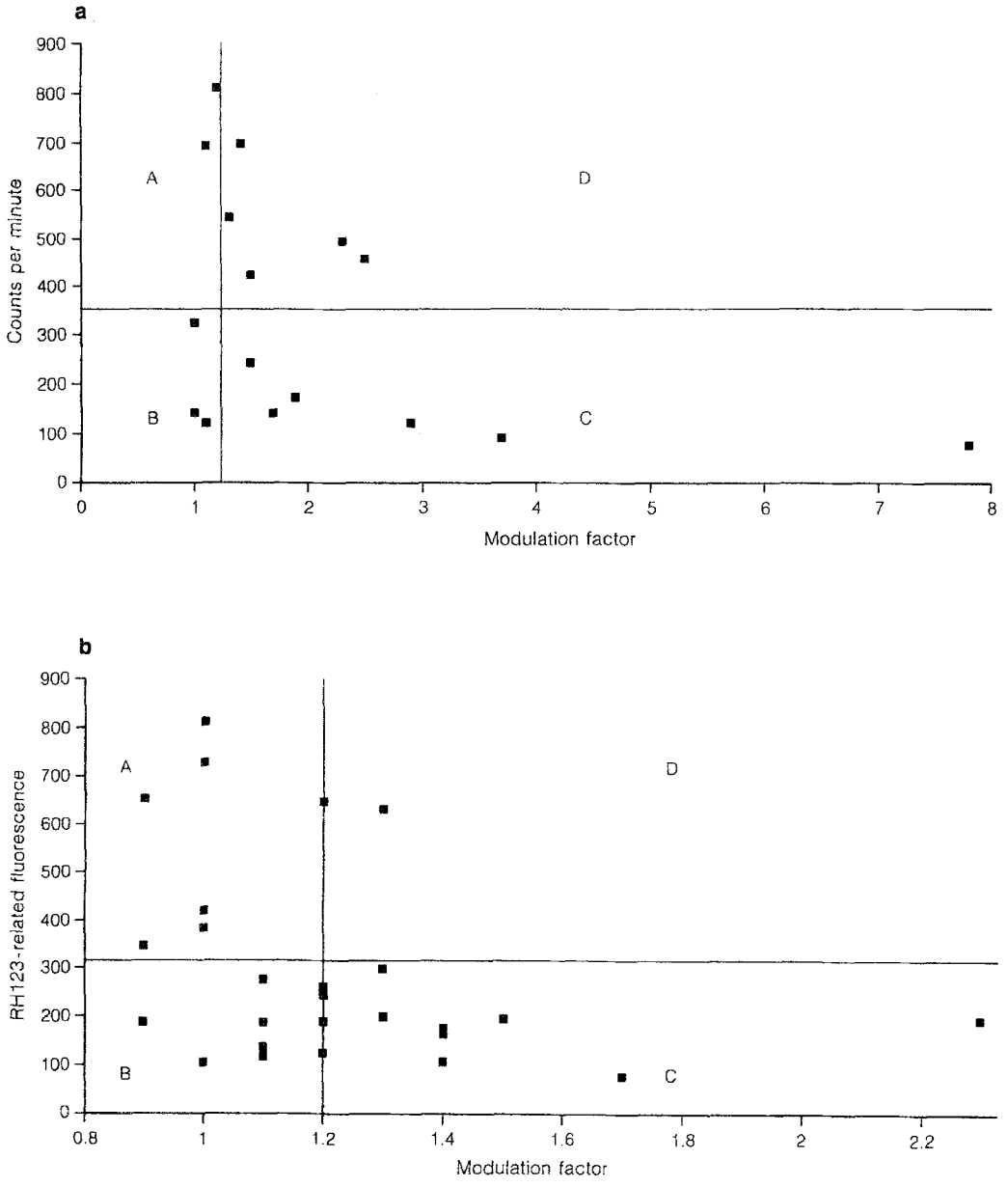

Fig. 3.a Spontaneous [^{3}H]-VCR accumulation (n = 16), **b** spontaneous Rh123 accumulation (n = 30) versus, and the corresponding modulation factors m (quotient of accumulation plus VRP and minus VRP)

lation coefficient: m_{VCR}, $r = 0.513$; m_{Rh123}, $r = 0.417$). PGP levels differed significantly between the arbitrary groups of VCR accumulation B versus D or B versus C in univariate analysis (Fig. 4b). The groups of Rh123 accumulation did not significantly differ in terms of PGP levels (Fig. 4a). Nevertheless, a tendency toward higher PGP levels was found in group C as compared with group B.

In the group of B-CLL cases in which VCR accumulation was measured, 38% of the cases expressed PGP at low levels (mean fluorescence (MF), $\leq$160), whereas 62% expressed PGP at high levels. This distribution was in correlation with a group showing modulation factors of $\leq$1.2 (31%) and with a group showing modulation factors of >1.2 (69%), respectively. The PGP levels measured in the group of B-CLL cases with modulation factors for VCR of >1.2 were significantly enhanced (MF >160) in comparison with those measured in the group with modulation factors of $\leq$1.2 (MF $\leq$160; Fig. 4b).

In the group of B-CLL cases in which Rh123 accumulation was measured, 37% of the cases showed low levels of PGP and 63% expressed high levels. In contrast to VRP-mediated VCR modulation, however, the group with modulation factors of $\leq$1.2 was larger (64%) than the corresponding group expressing low levels of PGP (37%). The degree as well as the frequency of VRP-mediated MDR-drug modulation was highest for VCR as compared with Rh123 or DNR (Fig. 3). Thus, only MDR drugs that are modulated by VRP to a high degree and frequency are suitable for the detection of functionally important PGP.

Univariate analysis showed that a high level of PGP expression in B-CLL cells (MF >160) is predictive for modulation factors of >1.2 ($P < 0.01$) if a drug such as

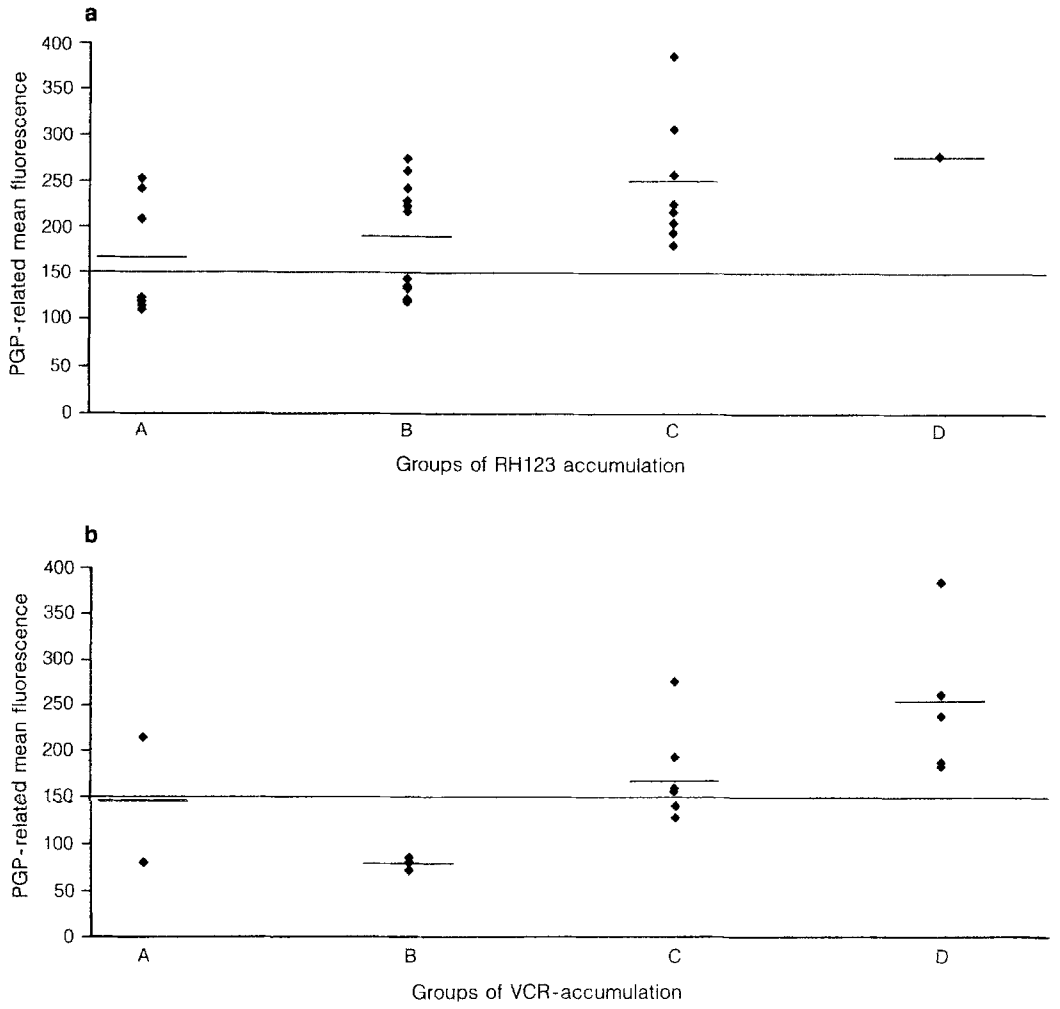

Fig. 4a, b. Mean PGP-related fluorescence (mAb C219) versus the corresponding modulation factors for **a** Rh123 and **b** VCR in groups A–D (Fig. 3)

VCR is used, which is modulated by VRP to a high degree and frequency. When Rh123 uptake is modulated by VRP, low PGP levels (MF $\leq$160) predict the modulation factor to be $\leq$1.2 ($P < 0.01$). VRP-mediated DNR modulation seems to be of less importance in B-CLL because of the generally low modulation factors found for DNR accumulation in the steady state.

These results show that B-CLL cases expressing low PGP levels are heterogeneous in terms of the potency of VRP in modulating chemically different MDR drugs (groups A and B, Fig. 4).

Spontaneous total drug accumulation versus modulation factor

Figure 3 demonstrates that spontaneous drug accumulation is not generally inversely related to modulation factors, as may be expected when the action of the PGP efflux pump is considered (correlation coefficient for linear regression: VCR, $r = -0.29$; Rh123, $r = -0.11$. Only 6/16 (VCR) and 8/30 (Rh123) cases showed high modulation factors and correspondingly low spontaneous accumulation of Rh123 or VCR.

Four arbitrary groups of B-CLL cases could be separated according to spontaneous VCR or Rh123 accumulation in the steady state and corresponding modulation factors m (Fig. 3). The cutoff point for the modulation factor was chosen to be 1.2, because this separates two groups of B-CLL cases according to the extent of PGP expression when VCR is modulated by VRP (Fig. 4b). The cutoff point for spontaneous accumulation arbitrarily separates lowly and highly accumulating B-CLL cases (Fig. 3): group A (A_{VCR}, 13%; A_{Rh123}, 23%), showing high levels of spontaneous drug accumulation and low modulation factors ($\leq$1.2); group B (B_{VCR}, 19%; B_{Rh123}, 47%), showing low levels of spontaneous drug accumulation and low modulation factors; group C (C_{VCR}, 30%; C_{Rh123}, 27%), showing low levels of spontaneous drug accumulation and high modulation factors ($>$1.2); and group D (D_{VCR}, 31%; D_{Rh123}, 3%), showing high levels of spontaneous drug accumulation and high modulation factors ($>$1.2). For both VCR and Rh123, the modulation factors obtained in group D were smaller than those attained in group C.

Defective drug accumulation in B-CLL

In all, 56% of the B-CLL cases showed a low degree of VCR accumulation (groups B and C, Fig. 3). Chemomodulation assays in our study demonstrated that drug accumulation was mainly PGP-independent in 31% of cases and mainly PGP-dependent in 69% of cases (Fig. 3). The mean VRP-mediated VCR or Rh123 accumulation recorded in group C was significantly lower than the drug accumulation without VRP noted in group A ($P \leq 0.01$). The results underline the importance of PGP-independent factors regulating drug accumulation.

Discussion

Factors modulating drug accumulation

In cases of reduced drug accumulation, the analysis of MDR using a chemomodulation assay with VRP may distinguish between PGP-dependent and -independent factors that result in different patterns of defective cellular accumulation of MDR drugs. The study of reduced drug accumulation using a chemomodulation assay promises to be a valid method for distinguishing between different mechanisms of defective drug accumulation and may thus provide a valuable predictive test for chemosensitivity. Our study shows that it is impossible to distinguish between different mechanisms of drug accumulation solely by measuring total drug accumulation.

B-CLL cells display an atypical accumulation profile for MDR drugs, may express high levels of PGP but may simultaneously show high levels of spontaneous drug accumulation (5/30 for VCR, 1/30 for Rh123). On the other hand, they may exhibit reduced drug accumulation (56% in the VCR group, 73% in the Rh123 group) by virtue not only of enhanced PGP-mediated efflux but also of decreased drug accumulation due to PGP-independent factors. In B-CLL, PGP-independent factors may be at least as important as PGP-related factors for a reduced accumulation of MDR drugs. Both factors, which determine drug accumulation in B-CLL, are expressed independently: low or high levels of spontaneous MDR-drug accumulation may be associated with either low or high modulation factors (Fig. 3). The modulation factors of group D were lower than those of group C, despite the relatively high PGP levels measured in group D (Figs. 3, 5). High spontaneous PGP-independent accumulation rates may override the low chemomodulatory effects of VRP. Therefore, the modulation factors in group D were measured too low.

Changes in membrane and cytoplasmatic protein apart from PGP have been described in cells displaying the MDR phenotype [14, 44]. The data presented demonstrate that patterns of defective drug accumulation in B-CLL may differ phenotypically from MDR as originally defined. Whereas PGP is cross-reactive with chemically quite different drugs, PGP-independent factors influencing drug accumulation are likely to be dependent on the drug used as well. The relevance of these findings is that different mechanisms regulating drug accumulation may be identified in the same cell type as being basis for defective drug accumulation, even though all three chemically different MDR drugs tested share a common accumulation-regulatory mechanism: PGP-mediated MDR-drug efflux. Thus, similar behavior of different MDR drugs with respect to drug accumulation must not necessarily be associated with common accumulation mechanisms, even if PGP is expressed at higher levels.

PGP-independent factors modulating MDR-drug accumulation

Characteristic distribution patterns of VRP-mediated accumulation rates of DNR, Rh123, and VCR indicate drug-related modulation mechanisms for PGP-independent accumulation. PGP-independent factors modulating DNR, Rh123, and VCR accumulation may not be cross-reactive with other MDR drugs.

The factors that influence VRP-mediated Rh123 and VCR accumulation are cell-type-related and may vary among B-CLL cases. In our study, four cases of B-CLL expressed cell-type-related alterations in VRP-mediated accumulation patterns for VCR and Rh123 (modulation factor: VCR <Rh123). Either these cells express functionally altered PGP molecules with altered affinity for Rh123 and VCR or PGP-independent factors that augment VCR or Rh123 accumulation vary interindividually among cases of B-CLL. PGP-independent factors that influence VRP-mediated VCR accumulation seem to be much more heterogeneous than those that affect Rh123 accumulation (Fig. 4).

The factors that augment VRP-mediated chemomodulation of MDR drugs and those that regulate spontaneous drug accumulation in mainly PGP-independent MDR-drug accumulating cells are likely to be identical, but the underlying mechanisms may be multifold: (1) altered intracellular target sites, (2) altered binding of the drug to target sites, (3) altered transmembrane potential [11], and (4) altered membrane characteristics [12].

The drug-related typical variation in VRP-mediated total drug accumulation indicates a possible heterogeneity in B-CLL for factors that modulate drug accumulation. Differences in the variation of VRP-mediated DNR, Rh123, or VCR accumulation are paralleled by qualitative differences in intracellular drug binding; DNR is bound to multiple diverse binding sites [38], Rh123 shows specific affinity for mitochondria [8], and binding of VCR to tubulin is even guanidine triphosphate (GTP)-dependent [2, 3].

Pharmacokinetics studies suggest that the distribution of VCR in vivo is influenced by the tissue tubulin concentration [45]. In the cell line KV11, reduced VCR binding was shown to be associated with reduced tubulin levels in these cells [42]. In addition, Tsuruo and Jida [40] demonstrated the importance of the cellular microfilament system for drug transport in tumor cells. In a recent study we have shown tubulin levels in B-CLL to be highly variable [27]. Increased tubulin content in B-CLL was associated with increased chemomodulation of VCR by VRP at constant PGP levels [27, 28]. Therefore, interindividually varying tubulin levels may be one important reason why

modulation factors for VCR do not generally parallel PGP levels.

Similar to the tubulin content, the number of mitochondria, which are binding sites for Rh123, may vary constitutively or cell-cycle-dependently. Rh123 retention is increased in carcinoma cells versus normal cells and is also cell-cycle-dependent. Dye uptake during lymphoblast transformation has been shown to be due mainly to an increase in the number of mitochondria per cell [8, 39]. High numbers of mitochondria and relatively low tubulin content may explain why some B-CLL cells similar to the P388/ADR cell line express stronger VRP-mediated chemomodulation of Rh123 uptake as compared with VCR uptake. Another explanation may be an altered affinity of these drugs for functionally or structurally altered PGP molecules.

DNR has multifold unspecific intracellular drug-target sites (many intracellular proteins, glycosaminoglycan, and DNA). In contrast to acute leukemia, B-CLL cases show a small interpatient spreading of DNR accumulation rates [31]. The rate of anthracycline uptake and retention differs with the drug structure and the cell type [8, 26]. As compared with other PGP-positive human tumor cell lines [30], VRP-mediated DNR modulation is of less importance for B-CLL. The importance of PGP-independent drug accumulation indicates that besides drug-specific PGP-drug interactions, intracellular drug-target sites are also very important for modulation of accumulation in MDR B-CLL cells.

Factors modulating PGP-mediated drug efflux are likely to be involved in the first steps of developing MDR that is not yet accompanied by an increase in PGP levels. In erythroleukemia cell lines, variants have been selected for MDR that express both decreased anthracycline uptake and VCR resistance but no increase in PGP levels [23, 29, 35]. In this paper we show that in B-CLL, PGP-independent factors are involved in drug accumulation simultaneously with accompanying PGP-related factors. Cellular changes in PGP-independent factors may be due to an increased "atypical" MDR phenotype with unchanged PGP levels [28]. PGP-independent factors such as changes in tubulin content may vary cell-cycle-dependently, thus leading to various degrees of the MDR phenotype. Furthermore, our data add evidence to the hypothesis that PGP-independent factors influencing PGP-dependent accumulation in B-CLL are drug- and cell-type-related and may not mediate cross-reactivity with chemically different MDR drugs.

A human plasma fraction has been shown to contain compounds that influence the PGP-related drug-efflux pump [16]. If these compounds play any physiological role in the regulation of the accumulation of substances involved in PGP-mediated drug efflux, accumulation of such substances may be additionally augmented by the presence of intracellular drug-specific binding sites as indicated by our study.

Reduced cytotoxicity of MDR drugs that is not due to decreased PGP-related drug accumulation has been originally described as "atypical" MDR [36]. Altered topoisomerase II activity was demonstrated in cell lines with "atypical" MDR. Non-PGP-related active transmembrane drug transport could play an additional role apart from that of the demonstrated PGP-dependent drug transport [36]. However, a quantitatively important drug transport by non-PGP-related transporters seems to be more unlikely. In 69% of the studied cases of B-CLL, VCR accumulation could be significantly enhanced by VRP. In addition, 7/16 (44%) B-CLL cases accumulated VCR to a high degree in the absence of VRP (Fig. 3a).

Characteristics of MDR in B-CLL

First, two PGP phenotypes can be distinguished in B-CLL: a low-level (MF, ≤ 160) and a high-level (MF, > 150) MDR phenotype (Fig. 4). The predictive value of PGP levels in terms of chemomodulation depends on the degree and frequency of modulation of the MDR drug by VRP. Second, accumulation of VCR can be modulated in the majority of B-CLL cases (69%); a cell-type-related alteration in accumulation may occur additionally. Third, as compared with the relatively uniform accumulation in the steady state, the corresponding drug-related characteristic distribution patterns of VRP-mediated accumulation indicate that PGP-independent factors are active as comodulators of PGP-dependent MDR drug accumulation augmenting VRP-mediated drug accumulation. Fourth, as judged from our study, only the accumulation of drugs with specific intracellular binding sites (Rh123, VCR) may be augmented by VRP-mediated chemomodulation. This observation underlines the importance of intracellular binding sites in drug accumulation. Fifth, low-level MDR in B-CLL is characterized by a lack of increased PGP levels and modulation factors of < 1.2. Finally, 63% of B-CLL cases express high levels of MDR. In this group, limited spontaneous MDR-drug accumulation seems to be due predominantly to high PGP levels. Nevertheless, VRP-mediated chemomodulation is also influenced by PGP-independent factors.

Our study shows that all cases of B-CLL studied express PGP to a variable extent, similar to normal peripheral-blood lymphocytes [6, 9]. The observed frequent expression of PGP or *mdr*1 mRNA in B-cell CLL is in agreement with recent results obtained by our group and other investigators [21, 25]. The functional importance of PGP expression in B-cell CLL is now additionally supported by the high frequency of VRP-mediated chemomodulation of VCR (69%).

For studying PGP expression with mAb C219 we used fixed cells, in contrast to other investigators doing similar studies [21]. This methodological difference probably explains the differences observed in the distribution of high or low proportions of PGP-positive cells among cases of B-CLL. Determination of mean fluorescence was chosen to correlate PGP levels with VCR modulation data because these cannot be expressed on a single-cell basis.

The two defined groups that express different levels of MDR implicate *mdr*1 gene expression to be either up- or down-regulated in B-CLL. Chemomodulation assays support these results: in a small percentage of B-CLL cases VCR accumulation either could not be modulated by VRP or was modulated merely to a small extent despite the low level of spontaneous MDR-drug accumulation (group B). On the other hand, in the group expressing a high level of the MDR phenotype, rather high modulation factors were

found. The distribution pattern of modulation factors, however, did not parallel the distribution pattern of PGP levels. Our study adds evidence to the hypothesis that PGP-independent factors either may override PGP-mediated accumulation (groups A, D) or may at least augment PGP-mediated MDR-drug accumulation. Therefore, a correlation of modulation factors with PGP levels is limited [27, 28].

Differences in the intensity and frequency of chemomodulation indicate different efflux capacities of PGP, depending on the chemical structure of the drug. These differences are paralleled by a qualitatively different binding of anthracyclines and VCR to PGP. PGP provides high-affinity binding sites for daunomycin in MDR Chinese hamster ovary (CHO) B30 cells. The selectivity for daunomycin binding is entirely reflected in the dissociation rates. However, VCR binding to membrane vesicles in EH R2 cells selected for DNR resistance has been shown to be adenosine triphosphate (ATP)-mediated. Similar associations between anthracyclines and PGP could not be demonstrated [4, 20, 22]. In addition, differences in vinca alkaloid and anthracycline efflux have been detected in many cell lines.

Recent results indicate that PGP expression in B-CLL is constitutive. A follow-up study of PGP expression in B-CLL demonstrated very constant PGP expression over long periods that was independent of the chemotherapy applied and independent of the Binet stage [25]. In the present study, PGP expression in B-CLL was shown to be either low or high, suggesting mechanisms that constitutively up- or down-regulate PGP expression. Tumor suppressor gene p53 is likely to be a candidate for modulating PGP expression [7]. Besides constitutive MDR expression, B-CLL may express constitutive PGP-independent accumulation-modulating mechanisms as well (group B). Thus, low-level MDR-drug accumulation may be intrinsic and multifactorial.

Prerequisites for the clinical use of MDR-drug modulators such as VRP are a constitutive PGP expression or a stable acquired MDR phenotype and a high degree and frequency of the modulation capacity of the MDR drug to be modulated. If PGP expression is used for the prediction of the modulating capacity of VRP, the following results have to be taken into consideration: (1) the extent of chemomodulation is cell-type-dependent, (2) the acquired degree and frequency of the modulation capacity of the MDR drugs being used is drug-dependent, and (3) interindividual differences within a cell type may occur. PGP levels in general do not predict the chemomodulating capacity of a chemomodulator such as VRP at low-level MDR.

VRP enhanced VCR accumulation in vitro by a factor of >1.2 in about 66% of the B-CLL cases investigated and by a factor of >2.0 in 33% of the cases. Thus, chemomodulation of VCR uptake seems to be of clinical importance. The degree of VRP-mediated VCR modulation in B-CLL cases with low-level MDR seems to be similar to that described for normal colon mucosa cells (modulation factor, 1.3) [17].

Our ongoing studies as well as a study by Twentyman et al. [43] show that increased VCR accumulation is associated with increased cytotoxicity. However, 44% of B-CLL cases accumulate VCR to a high degree independently of PGP levels. There remains a small group of B-CLL cases that show low levels of drug accumulation that are due to enhanced PGP levels. In these cases, only the modulation of PGP-independent factors would be useful in overcoming accumulation defects for MDR drugs.

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