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BETA-TUBULIN AND P-GLYCOPROTEIN: MAJOR DETERMINANTS OF VINCRISTINE ACCUMULATION IN B-CLL CELLS

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Abstract—Vincristine (VCR) accumulation in chronic lymphatic leukemia of B-cell origin (B-CLL) has recently been shown not to be inversely correlated to P-glycoprotein (PGP) levels. Therefore, we studied, in addition to PGP expression and accumulation of VCR, the cellular β -tubulin content in quiescent and rhIL-2 activated B-CLL cells. VCR mediates cytotoxicity by binding to tubulin. Constitutive β -tubulin levels in B-CLL cells varied considerably. Upon activation with rhIL-2, β -tubulin expression increased significantly. Therefore, tubulin levels could be correlated over a wide range to VCR accumulation. When the PGP-mediated drug efflux was blocked by verapamil (VRP), tubulin levels correlated linearly to VCR accumulation. All B-CLL cases expressed PGP at different levels. There was no linear correlation between PGP expression and VCR accumulation. A modulation factor m was defined as a quotient of VCR accumulation in the presence and absence of VRP to define the extent by which VRP inhibited a steady-state accumulation of VCR. The factor allowed discrimination between B-CLLs expressing low versus high PGP, irrespective of the levels of tubulin. However, PGP and β -tubulin levels together were predictive for VCR accumulation in steady state. There was no uniform accumulation defect for VCR in B-cell CLL because β -tubulin and PGP were expressed independently. Non PGP-mediated VCR transport seems to play a minor role in B-cell CLL. Leukemia-associated varying of cytoskeletal organization in B-cell CLL might be one reason for the diverse cellular responses to receptor-mediated signals.

Key words: Multidrug resistance, tubulin, vincristine, B-CLL.

Introduction

Microtubules are ubiquitous in eukaryotic cells and mediate important cellular functions, such as intracellular transport, cell shape, mitosis and meiosis, secretion or transport and transmembrane signalling [1–4]. Transmembrane signaling related to cell cycle traverse may be accompanied by microtubule polymerization [5]. Additionally, microtubules and microfilaments play an important role in human natural killer cell-mediated cell lysis [6]. Cytoskeletal organization seems to be altered in chronic lymphatic leukemia (CLL) cells of B-cell origin in comparison to normal peripheral blood lymphocytes [7–10].

In myeloperoxidase negative cells resistance to vincristine (VCR) is thought to be mainly due to reduced drug accumulation. Myeloperoxidase oxidatively degrades vinca derivatives [11]. Hyperthermia-induced heat shock proteins may also protect heat-treated cells from vincristine damage [12]. VCR mediates cytotoxicity by binding to a protein subunit

of microtubules, dimeric tubulin. Two major processes can be distinguished which may be involved in down regulating VCR cytotoxicity: (1) vinca alkaloids may be actively effluxed by the P-glycoprotein (PGP) efflux pump; and (2) binding to the target molecule tubulin may be altered [5, 13–15].

Multidrug resistance (MDR) is mediated by PGP and directed against chemically quite different drugs, such as vinca alkaloids, anthracyclines and semisynthetic epipodophyllotoxins. A substantial number of chemically unrelated drugs have been shown to reverse drug resistance related to an overexpression of PGP [16].

B-CLL cells have been demonstrated to frequently express the MDR phenotype [17]. Interestingly, no inverse correlation between VCR accumulation at steady-state conditions and cellular PGP content has been demonstrated [18]. Thus, we studied VCR accumulation in B-CLL by the addition of drug modulators, such as verapamil (VRP), in order to distinguish between the action of PGP-dependent and independent processes in VCR accumulation and to

assess the role of cellular β -tubulin [16]. The β -tubulin content was measured in resting as well as in cycling B-CLL cells.

Materials and Methods

Materials

The following materials and suppliers were used: verapamil (Knoll AG, Ludwigshafen, Germany), ^3H -vincristine (Amersham Buchler GmbH, Braunschweig, Germany), specific activity 226 MBq/mmol, silicon oil AR 20, AR 200 (Wacker Chemie GmbH, München, Germany), silicon solution in isopropanol, trichloroacetic acid (TCA), KOH (Serva, Feinbiochemica, Heidelberg, Germany), lymphoprep (Nycopred AG, Oslo), fetal calf serum (FCS) (Fa. Gibco BRL, Berlin, Germany), RPMI 1640, PBS, NaHCO_3 7.5%, glutamine solution (200 mM), bovine serum albumin (BSA), guanidinium thiocyanate, sodium citrate, mercaptoethanol, phenol, chloroform, isopropanol, formaldehyde, formamide, SSC, N-dodecylsulphate (SDS) (Sigma Chemie Deisenhofen, Germany), monoclonal antibody (MoAb) C 219 (Cis Isotopen Diagnostik GmbH, Dreieich, Germany), FITC-conjugated rabbit anti-mouse IgG (Dako, Hamburg, Germany), propidium iodide, RNase (Wortlington), spheres (calibration standard), recombinant human interleukin-2 (rhIL-2) (Eurocetus GmbH, Frankfurt, Germany), monoclonal antibody to β -tubulin (Monosan Cell Systems, Remagen, Germany).

Patients

Thirty-two consecutive patients with B-CLL were studied for PGP and β -tubulin expression and vincristine accumulation in steady state in the presence and absence of verapamil (VRP). In 16 of the 32 cases, B-CLL cells were stimulated with rhIL-2 prior to the VCR accumulation assays.

The median age of the patients (15 females, 17 males) was 65 years (range 47–83 years). At the time of lymphocyte collection, four patients were in stage I, eight patients in stage II, six patients in stage III and 14 patients in stage IV, according to Rai *et al.* [19]. The time from diagnosis ranged from 1 to 16 years (median 6.1 years). Since diagnosis, seven patients had received no specific therapy, 25 patients were treated with chlorambucil and prednisolone, in four patients chemotherapy was already escalated with vincristine and cyclophosphamide and six patients were additionally treated with anthracyclines.

Cells

Lymphocytes were obtained from the peripheral blood of patients suffering from B-CLL and were isolated by density centrifugation in lymphoprep. Cells were either used fresh or were stored at -70°C in the presence of 10% dimethyl sulphoxide until use. Frozen samples were resuspended in 5–10 ml preservative-free heparin containing medium after thawing and allowed to hydrate for 2–3 h before further experimenting. No major differences were detected in VCR accumulation or antibody reactivity of the peripheral blood cells from fresh or frozen samples. All samples were immunophenotyped (CD5+, CD19+, CD20+, monoclonal Ig (κ^+ or λ^+)). Cytospin preparations were performed for cell staining according to Pappenheim.

Immunophenotyping

The ability of VRP to inhibit VCR efflux via inhibition of PGP action was evaluated concurrently with the study of

cellular PGP expression. The cell lines P388/ADR (PGP positive) and K562 (PGP negative) were used as controls.

PGP staining was performed according Epstein [20]. Briefly, methanol-fixed cells were washed twice in PBS before staining. Cells (10^6) were resuspended in 100 μl of C219 mAb diluted to 10 $\mu\text{g}/\text{ml}$ in PBS. Following a 1 h incubation at 4°C with periodic gentle shaking and two washes with PBS and 1% BSA, cells were reacted for 1 h at 4°C with 100 μl of FITC-conjugated rabbit anti-mouse IgG diluted 1:50 in PBS. After the incubation, the cells were washed twice and finally incubated with propidium iodide to a final concentration of 20 $\mu\text{g}/\text{ml}$. For β -tubulin staining [21], cells ($10^6/\text{ml}$) were fixed in PBS containing 3.7% formaldehyde for 30 min. After washing the fixed cells in PBS cells were permeabilized by treatment with 10 $\mu\text{g}/\text{ml}$ of L- α -lysophosphatidylcholine in PBS for 30 min, washed with PBS twice and resuspended in PBS. Cells were then stained with mouse monoclonal antibodies against β -tubulin for 30 min followed by FITC-conjugated goat antimouse immunoglobulin antibody for 30 min.

FITC was 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 based on their forward angle and right angle light scatter. The intensity of forward and right angle light scatter signals is related to cell size and granularity, respectively. Dead cells can be 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. Mean fluorescence is expressed in arbitrary fluorescence units, representing the mean fluorescence (MF) of the cells positively stained with PGP or β -tubulin.

The standard deviation between triplicates concerning cellular levels of β -tubulin was $<22\%$, concerning levels of PGP it was $<23\%$.

Northern blot analysis

For the analysis of *mdr1* mRNA, total cellular RNA was extracted according to the method of Chomczynski and Sacchi [22]. Briefly, after washing the cells in PBS, they were resuspended in 4 mol/l guanidinium thiocyanate, 25 mmol/l sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 mol/l 2-mercaptoethanol at a concentration of 2×10^6 cells/ml, followed by phenol–chloroform extraction and two cycles of isopropanol extraction. The RNA pellet was suspended in northern blot sample buffer, resolved on 1% formaldehyde agarose gels and blotted on to Gene Screen Plus (New England Nuclear). Blots were prehybridized 4 h at 50°C in 50% formamide, $6 \times \text{SCC}$ ($1 \times \text{SCC} = 0.15$ mol/l NaCl and 0.015 mmol/l sodium citrate), $1 \times \text{Denhardt's}$ solution, 40 mmol/l Pipes, pH 6.5, 100 Rg/ml salmon sperm DNA, and 0.5% sodium dodecyl sulphate (SDS) and hybridized using nick-translated probes (10^6 cpm/ml). The pHDR5a probe for *mdr1* was ^{32}P labelled [23]. Individual ^{32}P bands were quantitated using a Pharmacia LKB ultrascan KL laser densitometer.

^3H vincristine accumulation assay

The assay being used has been described previously [24]. Briefly, cells were suspended in RPMI10% FCS at 37°C . Two hundred microliters (1.05×10^6 cells) were mixed with 10 μl ^3H -VCR (final concentration 85 nM) in a siliconized 1.5 ml Eppendorf cup. In modulation assays, VRP was added to each

Table 1. P-glycoprotein, β -tubulin expression and vincristine accumulation in steady state in 32 patients with B-CLL

No.	DD	Sex	Age	Stage	Prior treatment	PGP*	β -tubulin*	VCR accumulation
1	10/92	F	56	III	CBL, PRED	159	861	620
2	12/78	F	63	IV	CBL, PRED	112	1343	891
3	4/87	M	73	II	None	137	631	719
4	3/89	M	67	IV	CBL, PRED, VCR, ADR, CPM	141	512	702
5	5/87	M	61	IV	CBL, PRED, VCR, CPM	125	351	275
6	2/92	F	47	I	None	160	242	144
7	4/86	M	82	I	None	148	163	118
8	6/85	F	68	II	CBL, PRED	131	198	93
9	8/88	F	83	III	CBL, PRED	141	364	123
10	7/88	F	65	IV	CBL, PRED	171	181	91
11	10/89	F	59	IV	CBL, PRED, VCR, CPM	387	231	67
12	1/91	M	66	IV	CBL, PRED, VCR, CPM, ADR	341	357	80
13	5/92	M	47	IV	CBL, PRED, VCR, CPM, ADR, MITOX	239	179	89
14	8/89	F	77	II	CBL, PRED, VCR, CPM	192	193	41
15	11/81	M	56	III	CBL, PRED	216	238	48
16	3/86	F	60	III	CBL, PRED	305	259	57
17	1/89	M	69	II	None	172	315	119
18	12/90	M	53	II	CBL, PRED	289	281	45
19	4/93	F	62	III	CBL, PRED	263	387	72
20	5/84	M	75	IV	CBL, PRED	302	199	115
21	12/88	M	57	IV	CBL, PRED	244	244	69
22	1/89	M	74	IV	CBL, PRED, VCR, CPM	184	366	112
23	9/77	M	76	IV	CBL, PRED	191	235	93
24	7/93	F	69	I	None	189	531	186
25	6/93	F	48	III	CBL, PRED	234	406	142
26	8/89	F	72	II	CBL, PRED	173	871	305
27	4/85	M	58	IV	CBL, PRED, VCR, CPM, ADR	189	1113	401
28	2/87	M	51	I	None	317	507	140
29	8/84	F	63	II	None	301	489	133
30	8/79	M	73	II	CBL, PRED	287	433	211
31	6/87	M	81	IV	CBL, PRED, VCR, CPM, ADR, MITOX	186	711	216
32	2/90	F	79	IV	CBL, PRED, VCR, CPM	255	671	510

DD: date of diagnosis, CBL: chlorambucil, PRED: prednisolone, VCR: vincristine, CPM: cyclophosphamide, ADR: adriamycin, MITOX: mitoxantrone; *P-glycoprotein and β tubulin related mean fluorescence.

sample at a final concentration of 10 μ M. Two hundred microliters (10^6 cells) were layered on a silicon oil gradient and incubated at 37°C in a water bath. The Eppendorf cups were placed horizontally into the water bath and turned every 5 min. At the appropriate time points (2, 5, 10, 15, 30, 60 min) the cells were centrifuged for 30 s at 11,000 g. Afterwards, 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 silicon oil gradient and the tip containing the cells in KOH was transferred into the scintillation vial. The KOH solution was neutralized with 250 μ l H₂O and 50 μ l TCA (5 M). The probes were incubated for 3 h at 60°C before analysis. All accumulation studies were performed twice with and without VRP (Table 1).

Activation of B-CLL cells with rhIL-2

B-CLL cells were cultured for 4 days according to the description of Karray with final rhIL2 concentrations of 6, 60, 600 and 6000 U/ml (11 B-CLLs).

VCR accumulation assays were performed before and after stimulation with 6000 U/ml rhIL-2 ($n=16$) [25].

Modulation factor

The modulation factor is defined as a quotient of mean accumulation plus VRP/mean accumulation without VRP and describes the extent of inhibition of a steady-state drug accumulation by VRP [15].

Statistical analysis

The statistical analysis was performed using Students' *t*-test. A *P*-value <0.05 was considered significant. Variate analysis was carried out using the model of Kruska Wallis (distribution-free variant analysis).

Results

Immunophenotyped leukemic human B-cells from 32 B-cell CLL cases were characterized for β -tubulin, PGP content and accumulation of VCR in the presence and absence of VRP. In 16 of the 32 B-CLL cases, accumulation of VCR in the presence and absence of VRP was measured after stimulation with 6,000 U/ml

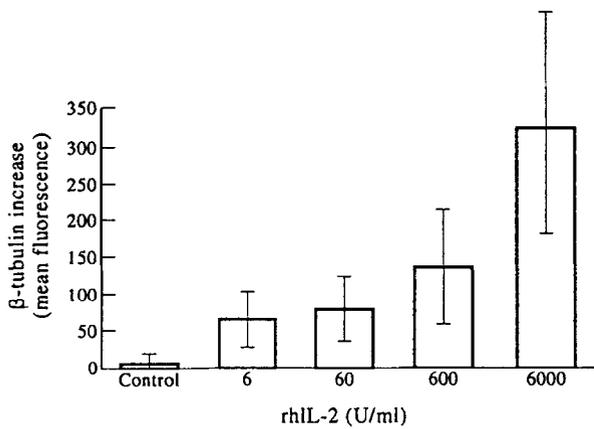


Fig. 1. Changes in cellular β -tubulin content after stimulation with increasing concentrations of rhIL-2 ($\Delta\beta$ tubulin).

rhIL-2. Additionally, β -tubulin levels were determined after stimulation with escalating concentrations of rhIL-2 (6, 60, 600, 6,000 U/ml).

Constitutive and IL-2-mediated expression of PGP and β -tubulin

All of the 32 B-CLLs studied expressed constitutively PGP as evidenced by the staining with the mAb C219. The mean intensity of specific fluorescence differed among the B-CLL cells from 112 to 387 (mean fluorescence channel number (MF) of PGP-positive cells), as reported in a recent paper [20]. PGP-related mean fluorescence levels did not significantly differ when mAb JSB was used (data not shown). Defining an arbitrary value of MF = 160 B-CLLs could be separated into two groups, with high levels of PGP expression ($n = 22, 69\%$) and low PGP levels ($n = 10, 31\%$).

To further substantiate our results, we also studied mRNA levels of *mdr1* in 16 of the 32 patients. The northern blots were probed for *mdr1*, scanned and the relative signal intensities calculated. The results are presented as indices in relation to the zero signal, the lowest signal, which was arbitrarily given an index of 1.

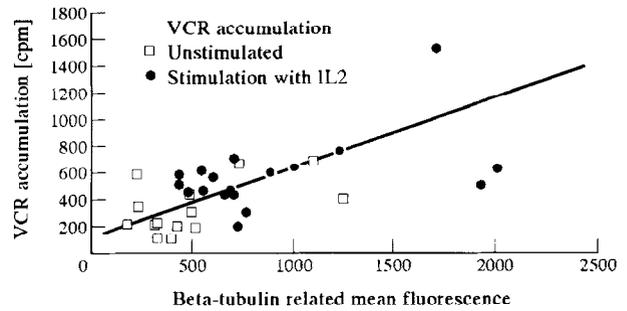


Fig. 2. Verapamil-mediated accumulation of vincristine versus β -tubulin content in unstimulated and rhIL-2-stimulated (6000 U/ml) B-CLL cells.

The mRNA signal for *mdr1* of 16 B-CLL cases was very heterogenous as indicated by a mean index of 3.9 (range 1–6.1). The mRNA indices for *mdr1* correlated with the corresponding PGP levels determined with the mAb C219 by flow cytometry in linear regression analysis ($r = 0.6931$).

When stimulated with rhIL-2 the cells responded with an increase in β -tubulin content in a dose-dependent fashion (Fig. 1). At a concentration of 6000 U/ml 23 of 27 B-CLLs tested had a significant increase ($P < 0.001$). The mean increase in β -tubulin content was 2.1 (range 1.3–2.8). After rhIL-2 stimulation, a great variation in the MF for β -tubulin could be observed (range 480–2010), as well as in unstimulated cells (range 163–1243). PGP levels did not change upon stimulation with rhIL-2 (data not shown). In linear regression analysis, no correlation could be found between PGP and β -tubulin expression.

VCR accumulation in steady state in the presence and absence of VRP

In unstimulated B-CLL cells, VCR accumulation was low and neither correlate with the expression of PGP nor β -tubulin (data not shown).

If PGP-mediated drug efflux was inhibited by the addition of VRP, VCR accumulation was significantly

Table 2. Vincristine accumulation and modulation factors versus tubulin and PGP content in unstimulated B-CLL cells

Group	Number of B-CLLs (%)	β -tubulin level*	PGP level†	VCR accumulation (10 μ M VRP) mean cpm [range]	Mean modulation factor [range]‡
I	4(12)	↑	↓	733[689–810]	1.0[0.9–1.1]
II	5(16)	↓	↓	151[112–331]	1.0[0.9–1.2]
III	14(44)	↓	↑	232[101–296]	3.4[2.1–7.8]
IV	9(28)	↑	↑	398[321–702]	1.6[1.3–2.4]

*Mean fluorescence channel number of PGP positive cells ≤ 160 (↓), >160 (↑). †Mean fluorescence channel number of β -tubulin positive cells ≤ 400 (↓), >400 (↑). ‡Mean accumulation plus VRP/mean accumulation without VRP.

increased ($n = 16$, $P < 0.001$) in quiescent cells (on average 1.39-fold, range 0.9–5.1), and even more ($P < 0.001$) in rhIL-2-stimulated cells (on average 1.79-fold, range 0.9–7.8) (Fig. 2). The mean VCR accumulation in quiescent cells was 219 cpm/ 10^6 cells (range 41–891 cpm, $n = 32$), in stimulated cells 512 cpm/ 10^6 cells (range 205–1653 cpm, $n = 16$). In spite of the significant increase in β -tubulin in rhIL-2-stimulated cells, this did not lead to a significant change in VCR accumulation without VRP (data not shown). Figure 2 demonstrates that VCR accumulation is strongly correlated with the levels of cellular β -tubulin content in cells where the PGP-mediated efflux is blocked by VRP (correlation coefficient $r = 0.791$ for unstimulated cells, $r = 0.772$ for rhIL-2-stimulated cells).

Modulation factors versus PGP and tubulin content

Modulation factors were ≤ 1.2 when corresponding PGP levels were low ($MF \leq 160$), independent of the tubulin levels (Table 2). In cases of high PGP levels ($MF > 160$) modulation factors were > 1.2 , independent of the β -tubulin levels. In spite of high modulation factors in group III most of the B-cell CLLs in group III have shown a lower VCR accumulation during chemomodulation with VRP than B-cell CLLs of group I without chemomodulation.

VCR accumulation versus β -tubulin and PGP levels in unstimulated B-CLL cells

Four arbitrary groups of B-cell CLLs (Table 2) could be separated according to high versus low PGP levels ($MF \leq 160$ versus $MF > 160$) and high versus low tubulin levels ($MF \leq 400$ versus $MF > 400$), group I (4/32) showing the highest VCR accumulation (high β -tubulin levels and low PGP levels), group II (5/32) with low tubulin and low PGP levels showing very low VCR accumulation similar to group III (14/32) with low tubulin levels and high PGP levels, and group IV (9/32) with high tubulin levels and high PGP levels, showing intermediate VCR accumulation. Low β -tubulin levels were significantly correlated with low VCR accumulation in steady state, independent of PGP levels. These cut-off points for tubulin and PGP levels were chosen to maximize differences between the four arbitrary groups.

In univariate analysis, only β -tubulin levels were predictive for VCR accumulation in the presence of VRP. In contrast, PGP levels or mRNA levels for *mdr1* did not predict VCR accumulation because of the leukemia-associated heterogenous expression of β -tubulin [26]. Therefore, the study design did not allow the presentation of PGP and β -tubulin levels as independent variables for the prediction of VCR accumulation. However, both variables together, β -tubulin levels and corresponding PGP levels and mRNA levels for *mdr1*, respectively, predicted the VCR

accumulation in steady state or the degree of VRP-mediated chemomodulation. In linear regression analysis the quotients of β -tubulin levels and corresponding PGP levels were correlated to the corresponding accumulation rates of VCR in steady state ($r = 0.7191$).

Discussion

Studying reduced drug accumulation by using a chemomodulation assay promises to be a valid method for distinguishing between different mechanisms of defective drug accumulation [24]. The results presented in this study demonstrate that B-CLLs display an atypical accumulation profile for VCR. Despite the expression of high levels of PGP, they show high spontaneous drug accumulation, but otherwise may exhibit reduced drug accumulation by virtue not only of enhanced PGP-mediated efflux but also by decreased drug accumulation due to PGP independent factors. The comparison of groups III and IV (Table 2) demonstrates the importance of high tubulin levels for VCR accumulation in B-CLL cells expressing higher levels of PGP. In spite of comparable PGP levels in both groups, high tubulin levels may functionally override PGP-mediated VCR efflux concerning net VCR accumulation. PGP expression is generally thought to be associated with a uniform accumulation defect for drugs involved in multidrug resistance. In this study we could demonstrate that there is no uniform accumulation defect for the MDR drug VCR in B-CLL because tubulin and PGP are shown to be expressed independently; both together they were predictive for VCR accumulation. Furthermore, the study shows that apart from PGP-mediated drug efflux β -tubulin content is a major determinant for VCR accumulation in B-CLL: when PGP-mediated VCR efflux is blocked by VRP, VCR accumulation is directly proportional to cellular β -tubulin content, both in unstimulated B-CLL cells and rhIL-2-stimulated cells. Following rhIL-2 stimulation the extent of β -tubulin increase directly parallels the increase in VCR accumulation. Thus, other cellular changes during cell activation may be less important for VCR accumulation in B-CLL.

The correlation of β -tubulin content and cellular VCR accumulation in steady state during VRP-mediated VCR accumulation indicates that intracellular drug concentrations depend on the cellular β -tubulin concentration. Therefore, the relative binding affinities of VCR to tubulin ($K(a)$ values) seem to be less important for determining the relative toxicity of VCR. This is in agreement with the observation concerning the VCR concentrations necessary to inhibit proliferation by 50% in different cell lines. The presence of about one tubulin-alkaloid complex molecule per 100 tubulin molecules is sufficient to produce 50% inhibition of

proliferation [13]. In mice, cellular β -tubulin content has been suggested as a major determinant of VCR accumulation in different tissues [27]. In the cell line KV11, reduced VCR binding was shown to be associated with reduced tubulin levels in these cells [28].

The role of β -tubulin is also demonstrated by the observation that VRP-modulated VCR accumulation in B-CLLs is heterogenous over a wide range of intracellular VCR concentrations in stimulated and unstimulated B-CLL cells, while low non-VRP-mediated VCR accumulation is a consistent finding [24]. Further VRP-mediated VCR accumulation is significantly enhanced in rHL-2-stimulated cells in comparison to unstimulated cells. This may be due to enhanced cellular β -tubulin levels.

Other factors which may influence VCR accumulation, such as altered membrane characteristics or altered transmembrane potential [29] or functionally enhanced VCR efflux [26], seem to be less important in modifying VCR accumulation during VRP-mediated chemomodulation. Non-PGP related drug transport seems to play a minor role: MRP expression was found to be intensive [30], whereas others demonstrated a low expression [31]. MDR3 expression is also commonly observed in B-CLL [32].

Differences in cellular β -tubulin content in B-CLL may have some implications on the function of the microtubule system, for example, on the microtubule polymerization during transmembrane signaling related to the cell cycle. Other cytokines, such as interferon α , have recently been shown to express modulating effects on the microfilament system by altering spectrin synthesis and organization [33]. Taken together, these results may suggest that cytokines may play an important role in organization of the cytoskeletal system of B-CLL cells. Cellular responses to receptor-mediated signals might be diverse because of the heterogeneity in the content and organization of cytoskeletal proteins in B-CLL.

It seems feasible to influence VCR accumulation by sequential activation of B-CLL cells followed by chemomodulation with VRP. In a recent study, we have shown that VCR cytotoxicity can be enhanced by increased VCR accumulation in activated B-CLL cells [18].

All B-CLL patients studied received vincristine-containing regimens as second-line chemotherapy. On the basis of our retrospectively evaluated response data, we cannot find any correlation between the extent of vincristine accumulation and the response rate. However, this might be mainly due to the combined use of other drugs in the COP or CHOP schedule.

In a previous study we have shown that PGP expression in B-CLL is not dependent on the actual stage or stage at diagnosis. Additionally, no correlation

could be observed between chemotherapy and PGP expression, even if *mdr*-drugs were used [34]. The independency of PGP expression upon actual stage, stage at diagnosis and previous chemotherapy, is further confirmed by the present study (Table 1). This is in agreement with other studies showing that MDR1 expression is not correlated with treatment [35–37]. Whether the extent of PGP expression may be a prognostic factor for outcome in B-CLL is under further investigation.

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