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ORIGINAL ARTICLE

Bisphosphonates induce apoptosis in CLL cells independently of MDR phenotype

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Abstract
Purpose The anti-tumoral activity of bisphosphonates (BPs) has been reported in leukemia. In this study, we attempted to evaluate the apoptotic effects of the BPs pamidronate (PAM) and zoledronic acid (ZOL) in chronic lymphocytic leukemia (CLL) samples, and to correlate it with clinical parameters and multidrug resistance (MDR) phenotype (*P*-glycoprotein and multidrug resistance-related protein expression and/or their functional activity).
Results Both BPs were able to induce apoptosis significantly. No correlation was observed between BP-induced apoptosis and clinical parameters or MDR phenotype.
Conclusion Our data suggest that concurrent or sequential administration of BPs with conventional chemotherapeutic drugs may have significant therapeutic potential for CLL patients.

Karina Lani Silva and Deborah Vidal Vasconcellos were contributed equally to this paper.

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Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disease characterized by the accumulation of CD5+ monoclonal B cells in the peripheral blood, bone marrow and peripheral lymphoid organs. CLL is the most frequent form of leukemia in western countries and predominates in individuals aged above 50 years (reviewed in [1]). Nowadays, CLL treatment strategies include alkylating agents, purine analogs and monoclonal antibodies, which are capable of reducing leucometry and tumor burden. Potentially curative therapy is bone marrow allogeneic transplant. However, only younger patients or those with higher stages or with adverse prognostic factor may benefit from this therapy [1, 2]. Thus, CLL remains as an incurable disease for most patients.

During the disease progression, an increasing resistance to chemotherapeutic drugs is observed, leading these patients to death. In fact, the overexpression of multidrug resistance (MDR) proteins such as *P*-glycoprotein (Pgp) and multidrug resistance-related protein (MRP) is well documented in CLL cells [3–6]. These proteins are efflux pumps capable of catalyzing the fast efflux of cytotoxic drugs from the cells and act as anti-apoptotic molecules by reducing chemotherapy-induced apoptosis [7]. Recent studies have shown an association between Pgp [8] or MRP [6] expression with adverse prognostic factors, reforcing the important chemoresistant role played by these proteins in CLL. Therefore, despite advances in the discoveries of new therapeutic drugs for CLL treatment, refractory disease is still common, and the search for innovative protocols for CLL treatment ought to be a matter of constant concern.

Bisphosphonates (BPs) are metabolically stable analogues of pyrophosphate and were established as therapeutic options for treating bone diseases such as Paget disease, post-menopausal osteoporosis and tumor-associated osteolysis caused by bone metastasis secondary to breast cancer, prostate cancer and multiple myeloma [9–12]. Recent studies suggest that the efficacy of BPs in the clinical oncology area could also be due to their direct anti-tumoral effect besides their anti-osteoclastic activity [13]. Actually, the anti-tumoral activity of BPs has been reported in several cell lines, including hematological malignancies [14–18].

The anti-tumoral effect of BPs in CLL cells has not been reported until this moment. Physicians from the Hematology Department of the National Cancer Institute (Brazil) observed that some CLL patients who were under treatment with BPs not only had their bone pain related to bone lesions alleviated, but they also had tumor burden and peripheral white blood cell (WBC) count reduction. This fact called our attention to study the effect of two BPs, pamidronate (PAM) and zoledronic acid (ZOL), both of which are frequently used in the clinical practice, in CLL samples in order to verify if BFs were able to induce apoptosis in cells from CLL patients exhibiting MDR phenotype.

Materials and methods

Patients and cells

Peripheral blood cells from 23 CLL patients registered at the Hematology Department, National Cancer Institute, Brazil, were studied prospectively. Their diagnoses were based on the criteria recommended by the National Cancer Institute (NCI). Only samples with more than 70% of malignant B cells were included in this study. For all patients, initial clinical evaluation included clinical examination, laboratory tests, bone marrow biopsy and immunophenotype analysis. The Ethical Committee of the National Cancer Institute (Brazil) has approved this study.

All samples were collected into heparinized tubes, and mononuclear cells were separated from blood by centrifugation in a Ficoll-Hypaque gradient (Sigma) and ressuspended in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/mL streptomycin and 2 mM glutamine. Finally, CLL cells were depleted of adherent cells by plating for 1 h in plastic culture flasks, resuspended and then cultured, as previously described by our group [19].

Apoptosis assay

The Annexin V-FITC conjugated/propidium iodide (PI) assay (Apoptosis Detection Kit, Genzyme Corporation, Cambridge, MA, USA) was used to verify the apoptotic effect of BPs (PAM and ZOL) on both normal lymphocytes and CLL cells. As described in previous study [19], lymphocytes (5×10^5 cells per well) were cultured in 200 µL of RPMI-1640 medium with 10% FBS at humidified atmosphere containing 5% CO_2 at 37°C for 12 h in absence or presence of 50 or 100 µM of either PAM (Fauldpami®, Fauldding, Australia) or ZOL (Zometa®, Novartis AG, Switzerland). ZOL was kindly provided by Novartis. These concentrations were selected based on previous studies, which showed that both PAM and ZOL were capable of inducing apoptosis in cell cultures at these concentrations [17, 20–23]. Lymphocytes isolated from three healthy donors were submitted to the same process and used to evaluate the induction of apoptosis by ZOL in normal lymphocytes. This technique was performed according to manufacturer's instructions. Emission of fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson) using CellQuest software 3.1 version (Becton Dickinson). The index (%) of apoptosis (annexin-V and PI staining) was determined by subtracting the percentage of non-treated cells from BP treated cells. A cut-off point of 5% was used to segregate positive and negative BP-induced apoptosis and a cut-off point of 10% was used to define sensitive and resistant samples to apoptosis induced by PAM and ZOL [24, 25]. We only included in this study the samples in which the percentage of living cells was above 60% after 12 h of culture.

Detection of Pgp and MRP expression by flow cytometry

Flow cytometric analysis was carried out to detect the expression of Pgp and MRP proteins in CLL samples. The detection was performed as described before by our group [26]. Briefly, 10 µL of anti-Pgp monoclonal antibody (clone 4E3-Dako) or anti-MRP monoclonal antibody (Monosan) was used as primary antibody. For each sample, as non-specific staining controls, 10 µL FITC-conjugated secondary antibody (IgG_1–FITC, Dako) was used. The results were expressed as the ratios of the mean fluorescent intensity (MFI) of cells treated with primary and secondary antibodies divided by MFI of cells treated only with the second antibody.

The cell lines K562 (Pgp negative), K562-Lucena (Pgp positive), GCL4 (MRP negative) and GLC4-ADR (MRP positive) were used as positive or negative controls. Considering that K562 and GLC4 are MDR negative cell lines, we defined MDR negative samples as a ratio ≤ 1.1 based on experiments performed by some groups [3, 27] and our previous results [24, 26].

Detection of functional MDR status by flow cytometry

Efflux pump function was investigated as described [24, 26]. Briefly, cells (5×10^5) were first incubated with 200 ng/mL

Rhodamine-123 (Rho-123) plus 200 ng/mL cyclosporine A (CSA) for 45 min at room temperature. CSA is able to block Pgp or MRP efflux pumps and the intracellular fluorescence increases [28]. Cells were washed in ice-cold PBS and incubated with CSA in dye free medium for further 45 min at room temperature. Cells without dye or CSA were used to evaluate cell autofluorescence (control).

The data were calculated as the ratio of the MFI of cells treated with Rho-123 and CSA, divided by the MFI of cells treated with Rho-123 alone after subtracting the MFI representing the autofluorescence. The same cut-off ($\leq$1.1 = MDR negative; >1.1 = MDR positive) was used for function analysis of MDR [24, 26].

Statistical analysis

Comparisons of spontaneous and drug-induced apoptosis values and comparisons among PAM and ZOL concentrations were done using the Wilcoxon rank sum test. In order to test correlations between variables Spearman's correlation coefficient was calculated. To evaluate differences among groups non-parametric Mann–Whitney *U* test, Chi-square (χ^2) and ANOVA Kruskall–Wallis test was applied. Statistical analysis was performed using SPSS software (version 9.0, SPSS, Chicago, IL, USA). A *p* value of less than 0.05 ($p < 0.05$) was considered as statistically significant.

Results

Clinical and laboratorial characteristics of the patients

The clinical and laboratorial characteristics of the CLL patients included in this study are listed in Table 1. The mean age of the patients was 65.4 years (range 33–88). There were 8 females and 15 males. The WBC ranged from 8,280 to 89,290 (median 28,000). Nine patients were in advanced Binet stage, 7 in B and 7 in A Binet stage. Twelve patients had not received a chemotherapy schema before this study. The others had received either monochemotherapy with chlorambucil (associated or not to prednisone) or protocols including three or four different chemotherapeutic drugs. Four patients were treated with fludarabine.

Non-cytotoxic effect of bisphosphonates in normal lymphocytes

First, to verify whether BPs were capable of causing a cytotoxic effect in non-malignant lymphocytes, cells from three healthy donors were cultured for 12 h with different concentrations of ZOL and the annexin V assay was performed. Interestingly, ZOL was not capable of inducing apoptosis in normal lymphocytes in any concentration tested ($p > 0.05$) (Fig. 1).

Table 1 Summary of clinical data from CLL patients

Sample	Gender	Age	WBC × 109/L	LDT	Binet stage	Previous treatment
1	F	74	31,400	>12	A	No
2	F	74	31,000	D	B	No
3	M	88	40,800	D	C	No
4	F	59	33,600	>12	A	No
5	M	50	21,500	D	A	No
6	M	74	19,900	>12	A	No
7	M	70	89,290	D	C	No
8	M	73	45,410	D	C	No
9	M	70	49,230	>12	A	No
10	M	67	23,600	D	B	No
11	F	60	40,200	>12	B	No
12	M	62	26,737	D	B	No
13	F	61	22,500	<12	A	CLB + FLU + COP + CHOP + MAB
14	M	46	8,280	>12	C	CLB + PDN + FLU
15	M	67	28,000	<12	C	CLB + PDN + COP
16	M	33	21,000	<12	B	CHOP
17	M	59	36,000	>12	C	CLB
18	M	64	28,400	<12	B	COP + CLB + PDN + FLU
19	M	70	40,700	<12	C	CLB + PDN + COP
20	F	70	27,600	<12	A	CLB + PDN + FLU
21	M	74	16,970	>12	C	CLB + PDN
22	F	62	11,130	<12	C	CLB
23	F	79	22,800	<12	B	CLB + PDN

F female, *M* male, *WBC* white blood cell count, *LDT > 12* lymphocyte doubling time > 12 months, *LDT < 12* lymphocyte doubling time < 12 months, *D* at diagnosis, *CLB* chlorambucil, *FLU* fludarabine, *COP* cyclophosphamide + vincristine + prednisone, *CHOP* cyclophosphamide + adriamycin + vincristine + prednisone, *MAB* mabthera, *PDN* prednisone

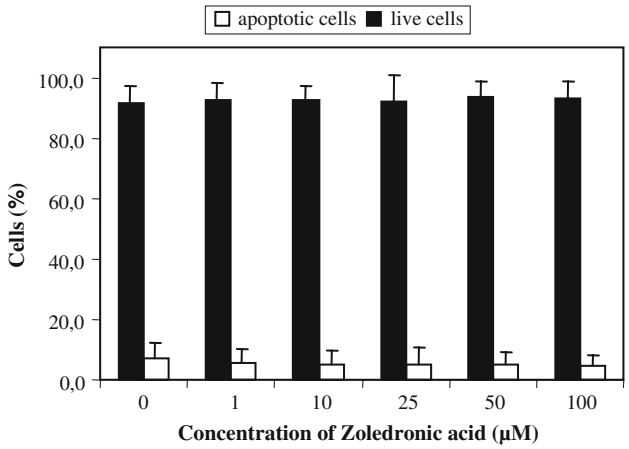

Fig. 1 Effect of zoledronic acid in non-malignant lymphocytes. Cells from three healthy donors were cultured for 12 h at different concentrations of zoledronic acid. There was no difference between the percentage of spontaneous apoptosis (0 μM = control, untreated cells) and zoledronic acid-induced apoptosis. Data represent mean ± standard deviation

Spontaneous and bisphosphonates-induced apoptosis

Spontaneous and PAM- ($n = 23$) or ZOL- ($n = 18$) induced apoptosis in CLL samples was analyzed by annexin V assay. The index of spontaneous apoptosis ranged from 0.19 to 40.0% (media = 14.39%; SD = 11.1). The index of apoptosis induced by PAM at 50 μM for 12 h of incubation ranged from 1.6 to 55.85% (median = 24.49%; SD = 11.7) while the index for PAM at 100 μM ranged from 0 to 46.6% (median = 26.95%; SD = 11.16%). PAM was not capable to induce apoptosis in three samples. ZOL at 50 μM was capable to induce an index of apoptosis ranging from 11.7 to 37.95% (media = 21.01%; SD = 7.57) while 100 μM of ZOL incubation caused apoptosis index ranging from 10.47 to 36.93% (media = 21.87%; SD = 8.24). Statistical analysis (Wilcoxon signed rank test) showed that BPs, PAM and ZOL, were capable of inducing a statistically significant increase in the index of cell death in CLL cells ($p < 0.001$) (Fig. 2). No statistically difference was observed between the index of apoptosis caused by PAM 50 μM versus PAM 100 μM, and ZOL 50 μM versus ZOL 100 μM. The effect of both PAM and ZOL according to the induction of apoptosis in malignant lymphocytes was similar ($p > 0.05$).

Considering the cut-off point of more than 5% for positive BP-induced apoptosis, PAM was capable to induce apoptosis in 15 out of 23 samples (65.2%) while ZOL induced apoptosis in 10 out of 18 samples (55.5%), in both concentrations tested. When we considered the cut-off point of more than 10% to segregate sensitive samples from BP-resistant samples we observed that 9 out of 23 samples (39.1%) and 7 out of 18 samples (38.9%) were sensitive to PAM and ZOL at 100 μM, respectively.

No correlation was found among spontaneous or BP-induced apoptosis associated to age, gender, WBC, lymphocyte doubling time (LTD), Binet stage or previous treatment. It is interesting to call attention to the fact that even those patients who have already received a heavy chemotherapeutic protocol showed an index of apoptosis induced by BPs similar to non-treated patients.

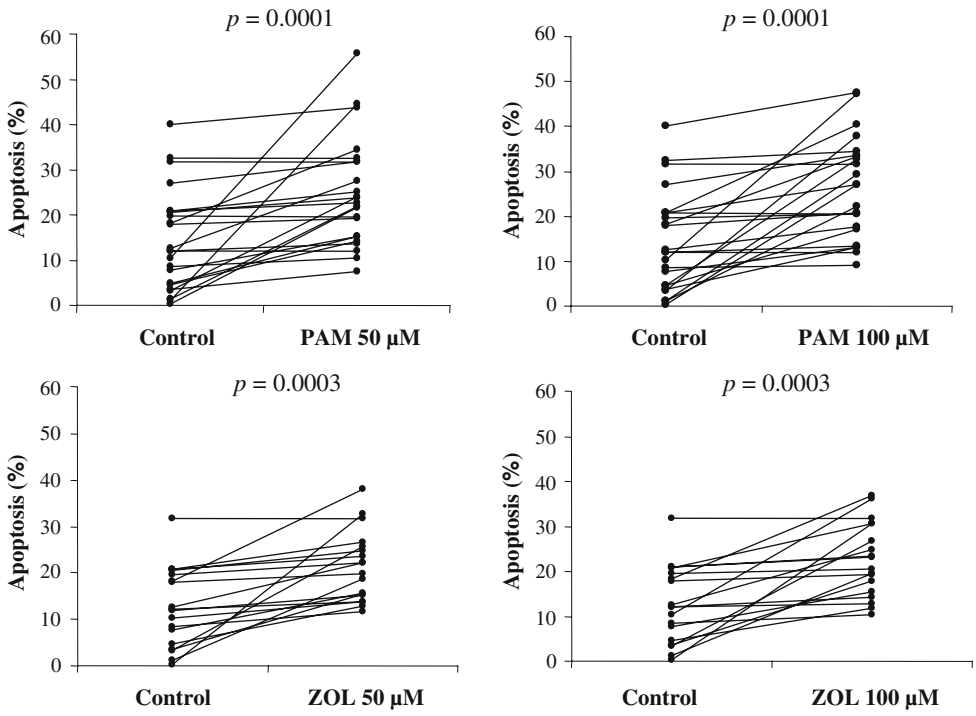

Fig. 2 Differences between spontaneous and bisphosphonates-induced apoptosis. CLL cells from 23 samples (pamidronate, *PAM*) or 18 samples (zoledronic acid, *ZOL*) were cultured for 12 h in presence or absence of different concentrations of bisphosphonates and submitted to Annexin V assay. Wilcoxon test shows that the difference between PAM- or ZOL-induced apoptosis and spontaneous apoptosis was significant ($p < 0.05$). No statistical difference was observed between PAM or ZOL concentrations. Control = untreated cells

MDR phenotype of CLL samples

The expression of Pgp was verified in 19 CLL samples and ranged from 1.0 to 13.8 (mean = 2.82; median = 1.36; SD = 3.07). Fourteen samples (73.7%) were positive for Pgp expression. Meanwhile, the expression of MRP analyzed in 22 samples, varied from 1.0 to 6.2 (mean = 2.1; median = 2.4; SD = 1.45) and the expression of MRP was positive in 14 samples (66.3%). The co-expression of Pgp and MRP was observed in 9 out of 18 samples (50%). The presence of active efflux pumps was identified in 13 out of 19 samples tested (68.4%).

MDR phenotype and BP-induced apoptosis

To evaluate the influence of MDR phenotype in BP-resistance, we compared the index of apoptosis caused by PAM and ZOL at 100 μM with the different levels of Pgp and MRP expression and Rho-123 efflux index. Interestingly, either PAM or ZOL were capable of inducing apoptosis significantly in CLL cells, independently of MDR phenotype, even in those patients that showed overexpression for both Pgp and MRP proteins (Table 2).

Table 2 Comparison between MDR phenotype patterns and the apoptotic effects of bisphosphonates in CLL cells

MDR phenotype and apoptotic status	PAM 100 μM	ZOL 100 μM
Pgp + Apoptosis+	7/19	6/15
Pgp + Apoptosis−	7/19	6/15
Pgp-Apoptosis+	4/19	2/15
Pgp-Apoptosis−	1/19	1/15
MRP + Apoptosis+	9/22	7/17
MRP + Apoptosis−	5/22	5/17
MRP-Apoptosis+	5/22	2/17
MRP-Apoptosis−	3/22	3/17
Rho-123 + Apoptosis+	9/19	7/15
Rho-123 + Apoptosis−	7/19	6/15
Rho-123-Apoptosis+	2/19	1/15
Rho-123-Apoptosis−	1/19	1/15
Pgp + MRP + Apoptosis+	5/9	3/9
Pgp + MRP + Apoptosis−	4/9	6/9
Pgp + MRP-Apoptosis+	1/3	0/2
Pgp + MRP-Apoptosis−	2/3	2/2
Pgp-MRP + Apoptosis+	2/3	2/2
Pgp-MRP + Apoptosis−	1/3	0/2
Pgp-MRP-Apoptosis+	2/2	1/1
Pgp-MRP-Apoptosis−	0/2	0/1

Pgp, MRP and Rho-123 were considered positive when the mean fluorescent intensity was > 1.1. The index of apoptosis induced by PAM or ZOL > 5% was considered as positive

Pgp *P*-glycoprotein, *MRP* multidrug resistance-related protein, *Rho-123* rhodamine-123, *PAM* pamidronate, *ZOL* zoledronic acid

Discussion

Based on our clinical observations that some CLL patients treated with BPs for bone lesions demonstrated tumor burden and leucometry reduction (personal communication), we decided to evaluate the apoptotic effect of two BPs, PAM and ZOL, in CLL cells in vitro. The choice of these drugs relied on their extensive use in the clinical setting. Moreover, a study performed by Kuroda et al. [17] showed that ZOL is less toxic to normal hematopoietic cells than to leukemic cells. In fact, in the present study, we also evaluated the cytotoxic effect of ZOL in normal peripheral blood lymphocytes and no or minimal apoptosis was observed, making the study of cytotoxic effects of these BPs on CLL cells potentially relevant when applied to clinical practice.

Interestingly, both PAM and ZOL were capable to induce apoptosis in CLL cells significantly ($p < 0.05$) at both concentrations tested (50 and 100 μM). According to previous pharmacokinetics studies, the concentration of BPs in plasma decline rapidly after infusion and ranges from 1 to 3 μM for only few hours [29], indicating that sufficient serum concentrations for anti-leukemic activity may not be readily obtained. Nevertheless, the concentrations of BPs in bone tissue are able to reach 100–1,000 μM [30]. Moreover, BPs incorporated in bone osteoclast disrupt osteoclasts and release BPs in bone marrow surrounding, promoting high concentrations of BPs for tumor cells present in this tissue. Thus, it is supposed that BPs are capable of directly promoting apoptosis in bone marrow tumor cells. In fact, several studies have shown the apoptotic effect of BPs on breast cancer, prostate cancer and multiple myeloma cell lines [20, 22, 31]. In these studies high concentrations of BPs were used (ranging from 10 to 300 μM in the majority of cell lines) to visualize the apoptotic effect of these drugs. Even those studies that used chronic myeloid leukemia cell lines or leukemic cells from patients were able to observe apoptotic cell death in high concentrations of ZOL [17, 23]. Thus, the concentrations used in our study are in agreement with the literature.

Despite the fact that some studies have shown that ZOL acts more efficiently in inducing cytotoxicity in myeloma cell lines [20], breast cancer cell lines [21] and myeloid leukemic cell lines [17] than PAM, our results showed no significant difference between the index of apoptosis induced by PAM and ZOL in CLL samples. Studies developed in cancer cell lines have shown that ZOL induces cell death in a dose-dependent manner [17, 20]. However, no statistically significant difference in the index of apoptosis induction was observed between the concentrations of 50 and 100 μM for both PAM and ZOL. Perhaps, intrinsic

characteristics of malignant lymphocytes might be determining this lack of sensitivity to different concentrations of BPs.

Some features found in CLL patients, such as age ≥55 years, masculine gender, LDT less than 12 months and advanced Binet stage, are considered as poor prognostic factors [32, 33]. In our study, no prognostic factor analyzed influenced in the PAM or ZOL-induced apoptosis rates. Despite low number of samples studied, no tendency of resistance to BP-induced apoptosis was observed ($p > 0.05$).

Increasing resistance to chemotherapy is fatal and the issue on MDR in CLL has attracted attention in recent years [5, 8]. The best well-known MDR-mechanism is the overexpression of the efflux pumps Pgp and MRP, which plays a physiologic role of pump catalyzing the rapid efflux of xenobiotics from the cells. These proteins may act as anti-apoptotic proteins since they can reduce the quantity of chemotherapeutic drugs inside the cells hence, inhibiting apoptosis [7]. It was shown before that CLL cells commonly express Pgp and MRP at diagnosis and display functional efflux pumps [34]. Actually, our results showed that approximately 79% (15/19) of CLL samples were positive for Pgp expression as already described by other authors [4]. In a previous study performed by our group in 30 CLL samples, 63% of patients expressed Pgp, which was not correlated with previously treated or non-treated patients [26]. In agreement with other studies, we also did not see any correlation between Pgp expression and clinical parameters such as previous treatment or disease stage [5, 26, 35]. Since Pgp and MRP are commonly expressed in CLL cells and the positivity for Pgp or MRP expression sometimes is not related to their functionality, we aimed to evaluate the Pgp or MRP activity by the Rho-123 efflux assay [26]. Alike Pgp or MRP expression, no correlation was observed between Rho-123 efflux and clinical or biological prognostic factors. Thus, it is correct to infer that MDR phenotype is an inherent characteristic of CLL cells. Although Pgp expression and/or functionality is not related to any prognostic factors, some drugs (such as vincristine), used in CLL treatment, for being Pgp substrates, might develop MDR phenomenon. In an attempt to investigate whether BPs were able to induce apoptosis in Pgp overexpressing cells, Kuroda et al. evaluated the cytotoxic effect of ZOL in a leukemic cell line overexpressing Pgp and in its parental cell line having found similar cytotoxic effects in both cell lines [17]. Likewise, we performed studies using PAM and ZOL with K562 and K562-Lucena, a Pgp overexpressing cell line [24]. No differences in cell death induction were observed between the two cell lines for both drugs (data not shown). In relation to the importance of MRP overexpression for BPs resistance, only one study was published recently. The study showed that ZOL per se or in combination with vinblastine induced fewer apoptotic rates in a HEK (human embryonic kidney) 293 cell line transfected with *MRP* gene (293 MRP) as compared to its parental cell line [36]. However, in our work when we evaluated the BPs-apoptosis induction in CLL cells in relation to MDR phenotype, no correlation was observed, i.e., despite the high expression of the efflux pump activity, both PAM and ZOL were able to induce apoptosis in a significant way. Also, of particular interest in our work was that the BPs were effective in killing cells from CLL patients exhibiting both Pgp and MRP1, simultaneously. In this situation, the co-expression of two MDR efflux pump proteins could contribute, at least in a theoretical point of view, to a highest resistance to chemotherapy. Therefore, it is true to state that BPs are not involved in the MDR system in CLL cells and may represent a promising means of overcoming MDR phenomenon.

In conclusion, our data demonstrated that BPs were able to induce apoptosis in CLL cells regardless of their Pgp and/or MRP overexpression. Interestingly, concomitant use of BPs with other chemotherapeutic drugs showed synergic effects in vitro in leukemic cell lines [17, 18], suggesting that concurrent or sequential administration of BPs with conventional chemotherapeutic drugs may have significant therapeutic potential for CLL patients.

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