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ORIGINAL ARTICLE

Parthenolide has apoptotic and cytotoxic selective effect on B-chronic lymphocytic leukemia cells

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Summary
B-chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in the western world. It results from a relentless accumulation of small mature monoclonal lymphocytes. Following a recent demonstration of a significant increase in the proliferative pool of CLL cells in vivo, the gradual accumulation of malignant B-CLL cells seems to be primarily the consequence of their selective survival advantages relative to their normal B-cell counterparts. As the disease is mainly caused by defective apoptosis it is thus a good candidate for treatment by proapoptotic agents. Even though a large amount of research has been done during the last past years, the prognosis has not changed. Because of this, new therapeutic strategies are urgently needed, especially those that could switch on new apoptotic responses. In order to test the ability of parthenolide, a sesquiterpene lactone, to induce apoptosis and cytotoxicity of B-CLL cells *in vitro*, we cultured these cells in the presence of this substance. Incubations were continued for 3 days. Samples of cells were taken from cultures at 0, 24, 48 and 72 hours to measure apoptosis and cell viability. Peripheral Blood Mononuclear Cells (PBMCs) from five normal donors were submitted to the same techniques and served as control samples. In this study we show for the first time that parthenolide has a potent apoptotic and cytotoxic effect on B-CLL. It is noteworthy that this substance has almost no impact on normal PBMCs. This evidence suggests that parthenolide might be a promising therapy for B-CLL.

Keywords: parthenolide – apoptosis – B-CLL

INTRODUCTION

B-chronic lymphocytic leukemia (CLL) is the most common form of leukemia of adults in the western world (Diehl et al. 1999). This haematological disease represents an example of a human malignancy that appears to result primarily from one or more defects in programmed cell death regulation mechanisms (Kitada et al. 1998). It is assumed then, following a recent demonstration of a significant increase of the proliferative pool of CLL cells *in vivo* (Messmer et al. 2005), that the gradual accumulation of the malignant B-CLL cells is mainly the consequence of their selective survival advantages relative to their normal B cell counterparts. As the disease is mainly caused by defective apoptosis, B-CLL should also be a good candidate for treatment by proapoptotic agents.

A great diversity of still undefined compounds with antineoplastic properties can be obtained from botanical species. Some of these natural products could be effective in B-CLL by activating different cell death pathways. The active principles of a

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group of very well known herbs like *Tanacetum parthenium*, Feverfew, *Magnolia grandiflora* and others, have been previously described as components of different Japanese, Latin American or Chinese traditional medicine having recognized anti-angiogenic and/or anti-tumor properties (Song et. al 1989). It is an interesting issue that some of these herbs contain parthenolide (PTL) as one of their major active components (Ross et al. 1999). This last substance is a sesquiterpene lactone, a novel natural NF-kappa B inhibitor with antineoplastic properties (Yip-Schneider et al. 2005). In general, parthenolide is well tolerated by humans, making it a good candidate for further clinical testing as anti B-CLL agent.

Table 1. **B-CLL patients' data.**

Patient	Gender	Age	Rai Stage	CD38	WBC at diagnosis (mm3)	% of lymphocytes	% of lymphocytes CD5/CD19+	MD (months)	Previous Treatment
Nº 1	Female	49	II	neg.	35.000	72%	91%	12	No
Nº 2	Female	65	II	neg.	27.800	62%	88%	15	No
Nº 3	Male	72	II	neg.	18.600	67%	93%	18	No
Nº 4	Female	69	II	neg.	42.000	70%	92%	11	No
Nº 5	Male	58	II	neg.	38.000	74%	91%	8	No

WBC: White Blood Cells; Rai Stage, % of lymphocytes and CD5/CD19 positive cells were measured at time of patient inclusion in this work. MD: Months from diagnosis.

Table 2: **Impact of parthenolide on B-CLL Cells and normal PBMCs *in vitro*.**

Substance	Apoptosis (24 hours)			Cytotoxicity (72 hours)		
	B-CLL	Normal PBMCs	P value	B-CLL	Normal PBMCs	P value
Parthenolide	84.60 ± 7.69	13.7 ± 4.45	<0.05	91.3 ± 5.19	14.3 ± 2.60	<0.05

This table shows the apoptosis and cytotoxic process suffered by cells obtained from 5 B-CLL patients and 5 healthy donors; all cultures submitted to a concentration of 8μM of parthenolide.

OBJECTIVE

To test the ability of PTL to induce apoptosis and cytotoxicity of B-CLL cells *in vitro*.

MATERIAL AND METHODS

Heparinized peripheral blood samples from five B-CLL patients without previous treatment fulfilling the diagnostic criteria for Rai stage II (Table 1) and from five healthy donors were obtained after informed consent and used for all experiments and controls respectively. Samples were subjected to Ficoll-Hipaque (Pharmacia Biotech) density gradient separation to isolate peripheral blood mononuclear cells (PBMCs).The percentage of CD5+/CD19+ cells was determined by flow cytometric analysis in all samples. PBMCs from B-CLL patients and controls were seeded in duplicate 2 milliliter cultures at a cell density of 2 x 10^5 cells per culture in 15 milliliter Falcon test tubes. The culture medium was always RPMI 1640 with 10% fetal bovine serum and the test tubes were incubated under standard conditions (i.e. 37 ºC, 5% CO_2 and 80% humidity). Parthenolide (Sigma) was reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 0.2 M and further diluted in culture medium up to 10 to 1 μM

with the final DMSO concentration <1%. PTL was added with the cell seeding at different concentrations from 1 to 10 uM and the incubations were continued for 3 days. All cells were also tested for culture medium alone with and without 1% DMSO. Samples of cells were taken from the cultures at 0, 24, 48 and 72 hours and the *in vitro* cytotoxic as well as the apoptotic effect of PTL was measured. Percentages of viable cells were calculated by counting the living cells in relation to the total number of cells, using the trypan blue exclusion assay.

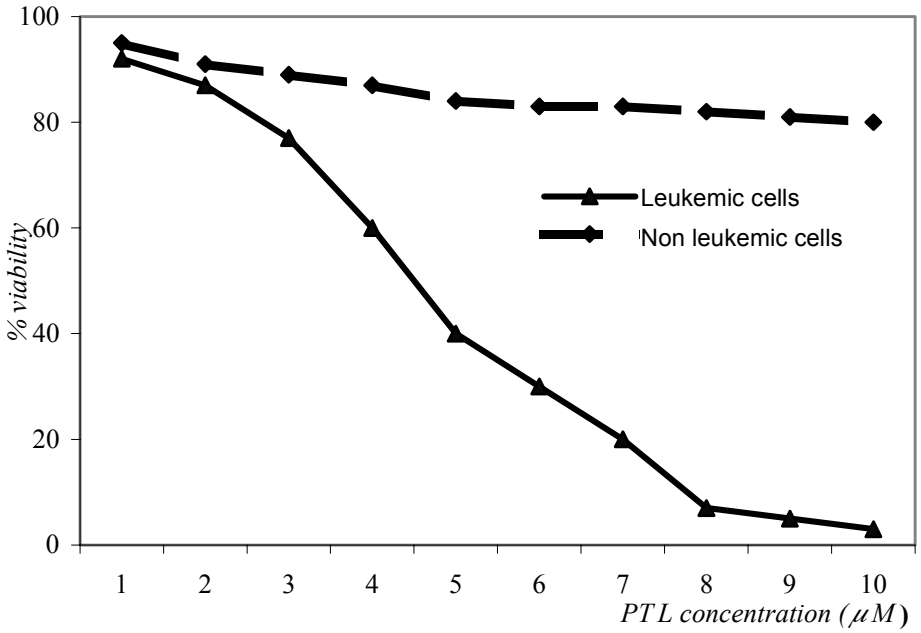

Fig. 1: **Viability of B-CLL and normal cells submitted to increasing PTL concentrations.** Mean percentages of cell viability after 72 hours of culture with increasing concentrations of parthenolide. Notice that at a concentration of 8 μM, there is no longer drastic improvement in leukemia cell mortality.

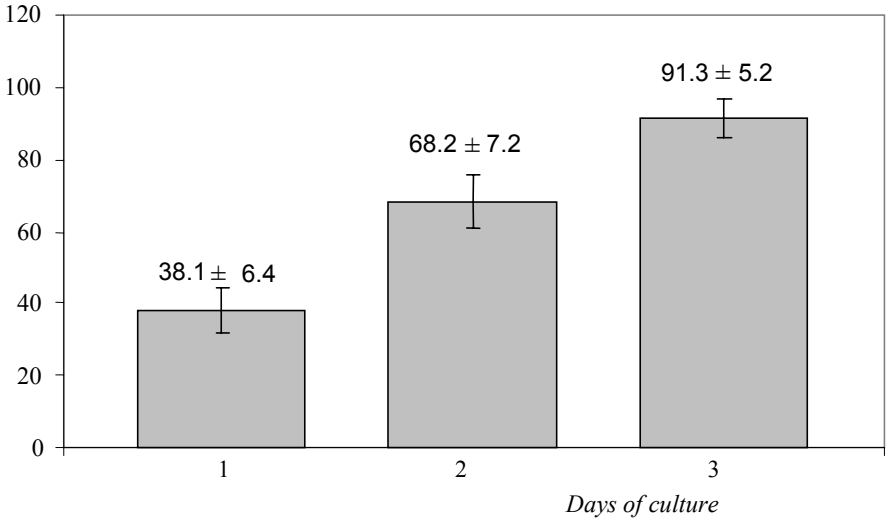

Fig. 2. **Cytotoxic effect of parthenolide on B-CLL cells.** Mean values of B-CLL dead cells in the presence of 8uM parthenolide showing a time dependent effect. Values are shown as an average of data obtained from ten assays each day (obtained from five B-CLL patients by duplicated)

For the apoptosis assays, cells were washed in 1x binding buffer by centrifugation and then resuspended in 200 µl of 1x binding buffer containing annexin V (0.1 µg/ml) and propidium iodide (0.5 µg/ml). After incubation at room temperature for 15 minutes, the cells were analyzed by flow cytometry for the presence of annexin V and propidium iodide. Apoptotic cells were identified as measured by annexin V-positive, propidium iodide-negative. The five patients and control healthy cells tested were measured at each time point/concentration by duplicate. All leukemia and control samples results were given as mean values ±SD. Statistical significance was determined by Student's t-test. A P value of 0.05 or less was considered significant.

RESULTS

No significant nonspecific loss of cells or apoptotic effect was observed either as a consequence of the culture media or DMSO at 1% (data not shown). The mean percentage of CD5+/CD19+ cells was 91±1.87% for B-CLL and 0% for normal PBMCs samples respectively. PTL displayed potent cytotoxic and apoptotic effects on B-CLL cells *in vitro*. B-CLL cells treated with PTL resulted in a dose and time dependent cytotoxicity. As shown in Fig. 1, PTL mediated cytotoxicity occurred at a concentration of 1 µM and above. A significant decrease in the cell viability of B-CLL cells obtained from all 5 patients was seen after one day of culture (38.1±6.37%) and at 72 h (90±5.19%) with a PTL concentration of 8 µM. (Fig. 2).

PTL displayed also a significant apoptotic effect on B-CLL cells *in vitro*. Again, these responses were dose and time dependent for PTL values from 1 to 10 uM. We found that parthenolide at 8 µM caused apoptosis in 84% of the B-CLL cells at 24 hours (Table 2). By contrast, this compound had little apoptotic or cytotoxic effect in PBMCs of healthy donors even at higher concentrations (Table 2).

DISCUSSION

Since B-CLL remains an incurable disease, more effective and safer strategies are urgently needed to treat this leukemia. Natural products with proapoptotic actions could be a good possibility. PTL, a sesquiterpene lactone obtained mainly from *Tanacetum parthenium*, *Magnolia grandiflora* and other plants, has recently demonstrated an interesting anti-tumoral activity against some solid tumors and acute myeloid leukemia (Yip-Schneider et al. 2005, Sweeney et al. 2005, Guzman et al. 2005), but it has still not been tried in CLL. In this study we show for the first time that PTL has a potent apoptotic effect on B-CLL cells without a great impact on normal PBMC. Our studies further showed that *in vitro* treatment with 8 uM of PTL up to for 72 hours did not induce significant apoptosis or cell death in normal PBMCs.

These results provide clues for interesting pathways involving different aspects of B-CLL cell apoptosis that could be exploited in therapies with this product. It could be speculated that parthenolide increased the amount of the NF-kappa B inhibitory protein, I kappa B-alpha, and decreased NF-kappa B DNA binding activity. All the evidence suggests that parthenolide may provide an anti- B-CLL effect and could be a potentially effective repertoire for chronic lymphocytic leukemia treatment. Even though all our patients were Rai II and CD38-, compromising mainly a potentially less aggressive category of disease, the results obtained in this work are more than satisfactory and probably could also be transfered to patients with a poor prognosis. As this compound had little cytotoxic *in vitro* impact on normal human PBMCs, side effects in the clinical setting could probably be minimized, and this is of course, a very important aspect to be considered in a chronic disease like B-CLL. Finally, it is also possible that a formulation combining parthenolide with some other natural molecules like honokiol or magnolol, obtained indeed from the Magnoliaceae family of plants, or the classical treatments might have a synergistically beneficial effect in B-CLL, being a potential promising strategy for the treatment of this hematological malignancy.

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