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Induction of apoptosis by monosaccharide butyrate stable derivatives in chronic lymphocytic leukemia cells

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ABSTRACT

Background and Objectives. **Different therapeutic approaches are needed to restore apoptotic mechanisms in CLL cells, as present ones are not successful. We assessed the apoptotic effects of stable butyrate derivatives on CLL lymphocytes: in these molecules a mannose molecule is bound as an ester to 1-5 butyrate moieties, conferring pharmacologic stability to the pro-drugs which are able to induce apoptosis in primary AML blasts.**

Design and Methods. **Peripheral blood samples obtained from 17 patients with typical B-CLL were cultured in the presence of 0.5-1mM D1 (O-n-butanoyl-2,3-O-isopropylidene-α-D-mannofuranoside), F1 (1-O-n-butanoyl-2,3-O-isopropylidene-D,L-xylitol) and G1 (1-O-n-butanoyl-D,L-xylitol) derivatives for 4 days and equimolar sodium butyrate as comparison. After culture, apoptosis was evaluated by cell morphology, cellular DNA content, pattern of DNA fragmentation, annexin V exposure on cell membrane, and cell cycle parameters. Bcl2, bax, and fas oncogene expression were also evaluated by the APAAP method.**

Results. **The addition to cell cultures of D1 or F1 or G1 butyrate monosaccharides as well as sodium butyrate 0.5 and 1 mM determined different increases in the percentage of apoptotic cells in all CLL samples, depending on the method and butyrate molecule added to the culture. Heterogeneity in CLL cell sensitivity to the three butyrates was observed. Up to 60-68% apoptotic bodies were present in treated cultures after exposure to D1 0.5-1 mM, 60-72% after F1 0.5-1 mM and 48-60% after G1 0.5-1 mM. Comparison of untreated versus treated cultures yielded important significance ($p < 0.001$). At DNA content analysis, analyzed by flow cytometry, apoptotic events accounted for up to 70-77% of D1-treated and 68-74% of F1-treated CLL cells at 0.5 and 1 mM concentrations ($p = 0.0001$, vs controls 0-39%), and for 72-81% of G1 (0.5-1 mM) treated cells (overall, $p = 0.005$). Cell cycle parameters were not altered by addition of butyrates, but expression of Annexin V was greatly enhanced. In a limited number of CLL cases fas, bcl2/bax ratio was analyzed and found unmodified.**

Interpretation and Conclusions. **Monosaccharide butyrate stable derivatives are potent inducers of primary CLL cell apoptosis, both in untreated and alkylating agent pre-treated cases. Our results suggest that the apoptotic pathways elicited by butyrate in CLL lymphocytes are direct, specific and most probably do not involve bcl2/bax. Pro-apoptotic agents like the stable monosaccharide butyrate derivatives here studied could give more insights into CLL biology and resistance to apoptosis, and possibly give rise alternative treatments for CLL.**

Key words: chronic lymphocytic leukemia, apoptosis, monosaccharide butyrate derivatives

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Chronic lymphocytic leukemia (CLL) is a disease characterized by accumulation of lymphocytes of nearly mature phenotype which have lost the capacity to undergo apoptosis. Attempts to treat and eradicate CLL are thus focused on forcing leukemic cells towards the restoration of programmed cell death. Purine analogs have obtained a great success in this sense and their use in the treatment of CLL has radically changed the natural history of the disease.[1] With longer follow-up of fludarabine-treated CLL patients, we know that nearly all of them will eventually relapse, and a conspicuous proportion of patients will develop acquired resistance to fludarabine.[2] The pathways leading to cell apoptosis and the factors modulating fludarabine acquired resistance have not yet been elucidated. Efforts are ongoing to evaluate different therapeutic approaches in CLL resistant patients and their mechanisms of induction of apoptosis in CLL cells.[3-5]

We recently showed that D1 (O-n-butanoyl-2,3-O-isopropylidene-α-D-mannofuranoside), F1 (1-O-n-butanoyl-2,3-O-isopropylidene-D, L-xylitol) and G1 (1-O-n-butanoyl-D,L-xylitol) were able to induce apoptosis in primary AML cell cultures, together with maturation and apoptosis.[6] In these compounds a mannose molecule is bound as an ester to 1-5 butyrate moieties, conferring pharmacologic stability.[7] It has been very recently reported that induction of programmed cell death by sodium butyrate in HL 60 cells is accompanied by phosphorylation of proteins of molecular weight 37 and 97 Kd. The addi-

tion of sodium vanadate, a phosphatase inhibitor, was able to enhance the apoptotic effect of sodium butyrate.[8] These observations gave further support to the notion that butyrates act specifically to induce apoptosis. Butyrates act through deacetylation and subsequent transcriptional activation.[9,10] It may be possible that neoplastic cells treated with monosaccharide butyrates are induced to restore the activation of their pro-apoptotic genes, otherwise shut down. Transcriptional activation during apoptosis of CLL cells is a field to explore fully.[11] The persistence of apoptotic activity of D1, F1 and G1, pro-drugs of butyric acid, hydrolyzed exclusively inside the tumor cell, prompted us to evaluate whether these newly synthesized butyrate monosaccharide derivatives could still be effective in a different biological system, and thus induce apoptosis *in vitro* of primary human CLL cells.

Design and Methods

Patients and cells

Heparinized peripheral blood samples were obtained from 17 patients with CLL, after informed consent had been obtained. Fourteen patients were untreated, at diagnosis and 3 had previously received treatment with intermittent chlorambucil, discontinued at least 3 months before entering this study. Clinical and laboratory characteristics of the patients are given in Table 1. All 17 patients fulfilled the National Cancer Institute's (NCI) criteria[12] for the diagnosis of low-risk CLL. Immunophenotyping by dual parameter-flow cytometry showed co-expression of CD 5 with B-cell antigens and isotypic light chain expression in all cases (Table 1). White blood cell (WBC) count ranged from 15 to $50\times10^3/\mu L$. After Ficoll-Isopaque centrifugation and washing, CLL cells were depleted of adherent cells following incubation in serum-free medium in plastic culture flasks (250 mL, Greiner, Germany) and then cultured.

Chemicals

We employed three stable butyrate monosaccharide esters (Fr. pat. FR 94/09348, PCT FR 95/00743) in this study and compared their effects to those of equimolar concentrations of sodium butyrate. These newly synthesized molecules are formed by a mannose or a xylitol bound as an ester to 1-5 butyrate moieties. Details of the chemistry, synthesis and structure of these molecules are published elsewhere.[6,7,13] Butyrate derivatives were prepared freshly immediately before experiments and used in cell cultures at the final concentrations of 0.5 and 1 mM.

Cell cultures

Cells were cultured in 250 mL plastic flasks (Greiner, Germany) in RPMI 1640, 10% FCS at 37°C, in 5% CO_2 and exposed to D1, F1 and G1 0.5 and 1 mM for 4 days.

Table 1. Characteristics of the patients.

Case	Sex	Age (yrs)	Rai stage	WBCx10³/L (lymphocytes %)	CD3%	CD5/CD20%	Therapy
1	F	57	I	35.6 (86)	12	78	none
2	F	68	II	29.6 (92)	16	84	C+P*
3	F	76	I	35.7 (63)	17	77	C+P*
4	M	69	0	40.7 (81)	23	70	C+P*
5	M	76	I	24.3 (70)	21	75	none
6	F	73	II	33.4 (65)	13	85	none
7	M	52	0	33.1 (78)	17	76	none
8	F	55	0	15.5 (70)	14	83	none
9	F	66	0	29.4 (89)	10	87	none
10	F	72	I	47.7 (74)	10	88	none
11	M	64	0	23.6 (75)	12	83	none
12	M	73	I	18.6 (73)	13	82	none
13	F	63	0	14.9 (71)	24	70	none
14	F	78	0	36.5 (56)	15	80	none
15	F	51	0	49.7 (86)	10	85	none
16	F	73	I	48.6 (89)	13	85	none
17	M	67	0	26.4 (74)	18	77	none

C+P: chlorambucil 0.07 mg/kg/day for 7 days every 4 weeks, prednisone 0.5 mg/kg/day for 7 days every 4 weeks.*

Morphology

Prior to and after culture, cytospin preparations of CLL cells were stained with May-Grünwald-Giemsa (MGG). Morphology of cells was then examined by light microscopy using 100× lens and immersion oil. At least 200 cells per slide were counted in duplicate. Nucleated cell number and viability was determined by counting with Türck solution and the Trypan blue dye exclusion test. Cell shrinkage, pyknosis of nuclei, cytoplasmic and chromatin condensation, and appearance of apoptotic bodies were scored as specific signs of apoptosis.

Cell kinetics and apoptosis

After incubation, CLL cells not treated and treated with butyrates were washed thoroughly, re-suspended in PBS/ethanol 1:3 vol/vol and kept overnight at 4°C. After fixation, cells were incubated in propidium iodide (PI) 50 mg/mL plus Nonidet 0.01%, RNAse 62 µg/mL. DNA content was measured by a FACScan flow cytometer (Becton Dickinson, San Josè, CA, USA). Analysis of the data was accomplished by the use of CellFit program, applying the MANL statistic program which quantifies the consistency of the *pre-G1* apoptotic peak, present in the region of channel 100.

DNA fragmentation

Cellular DNA was obtained from CLL cells, cultured with or without butyrates in the same conditions as for cell cycle determination (see above). DNA was obtained by lysis of the nuclear membrane and then by phenol/chloroform extraction. Equal amounts of purified DNA were then loaded on to a 2% agarose gel and electrophoresis was performed at low voltage (60 V) for 5 hours. The appearance of the characteristic DNA digestion fragments of 200 base pairs or multiples and the ladder-like DNA smear were considered as indices of apoptosis.

Annexin V expression

Exposure of phosphatidyl serine (PS) on cell membrane was quantified by surface annexin V staining as described elsewhere.[14] Cells were stained with PI and then incubated with annexin V-FLUOS (Boeheringer-Mannheim, Germany) according to manufacturer's instructions. Cells were analyzed by flow cytometry (FACScan, Becton Dickinson).

Immunoalkaline phosphatase procedure (APAAP)

Triplicate cytospins of each sample were fixed in acetone for 10 minutes at room temperature and then subjected to the APAAP technique, as previously described.[15,16] Briefly, the samples were incubated for 30 minutes with anti Bax MoAb (Immunotech), anti Fas MoAb (Calbiochem, S.Diego, CA, USA), and anti-bcl2 polyclonal mouse antibody. The alkaline phosphatase reaction was revealed using the new fucsin substrate solution at a final concentration of 1 mM. Cytocentrifugates were counter-stained with Gill's hematoxylin for 5 minutes.

Statistical analysis.

Data were analyzed by univariate analysis using Student's t test for paired data. Bonferroni's method was used for multiple comparisons. A probability value of less than 0.5 was considered statistically significant.

Results

Morphology

In all cases before culture more than 95% of isolated CLL cells were viable (Trypan blue negative).

The presence of apoptotic bodies was scored from May-Grünwald-Giemsa stained slides of cell cultured in the presence and in the absence of butyrate derivatives. Four-day control cultures showed different percentages of spontaneous apoptosis (Figure 1) (from 0% in case #10 and 11 to 40% in case #15). The addition of all butyrate monosaccharides at 0.5 and 1 mM determined an increase in the percentage of morphologically apoptotic cells. The apoptotic effects of the butyrates were only partially increased with dosage. Although we recorded a stronger apoptotic effect of butyrate derivatives at 1 mM concentration in 6 CLL cases (#7,10,11,12,13), a clear dose-response relationship was not demonstrated in all the cultures.

In Figure 1 the apoptotic effect of 0.5 mM butyrate monosaccharide derivatives on CLL lymphocytes are presented, as evaluated by cell morphology and scoring of apoptotic bodies. The addition of D1 0.5 mM to cultures provoked the appearance of up to 60% apoptotic bodies (case #15). Figure 1a presents the effects of D1 0.5 mM on CLL cells. In 11/17 cases (#1, 7, 9,10,11, 12, 13, 14, 15, 16 and 17) the apoptotic effects of D1 were dramatic, and overall results were highly significant ($p< 0.00001$). F1 was also effective in determining day 4 apoptosis both at 0.5 (Figure 1b) and 1 mM concentrations. The number of apoptotic bodies was significantly increased, with respect to the spontaneously occurring number, in 16/17 cases ($p< 0.00001$) and reached 36-70% of scored cells in case #7 and 34-61% and 42-71%, respectively in cases #16 and #17 at the two doses. After 4 days of incubation with G1 0.5 and 1 mM, apoptosis occurred in 8/17 cases at significant levels ($p=0.001$), whereas in cases #2, 3, 4, 5, 8, 9, 12, 13, 15 G1 0.5 mM did not induce a significant increase in apoptotic bodies (Figure 1c), but G1 1 mM did, so that when the entire cohort of cases studied was compared to untreated controls, significance was high ($p=0.001$). The efficacy of sodium butyrate was inferior to that shown by D1 and F1, but its activity in provoking the appearance of apoptotic bodies in culture was still significant ($p< 0.001$) (Figure 1d).

Apoptosis and cell kinetics

The percentage of hypodiploid cells with a decreased DNA content and distributed in A^0 pre-G1 apoptotic peak was evaluated by flow cytometry (Figure 2). The rate of spontaneous apoptosis was heterogeneous among different CLL cases, ranging from 0% (cases #10 and 11) to 39% (case #14) of the cell population, which was consistent with the results determined by morphology. The apoptotic effects of monosaccharide butyrates 0.5 mM are presented in Figure 2. The addition of D1 0.5-1 mM induced a significant increase in programmed cell death in 14/17 cases, maximally in case #1, with 88% of total cell population being apoptotic after exposure to D1 1 mM (data not shown). The results obtained after incubation of CLL cells in the presence of 0.5 mM D1 are

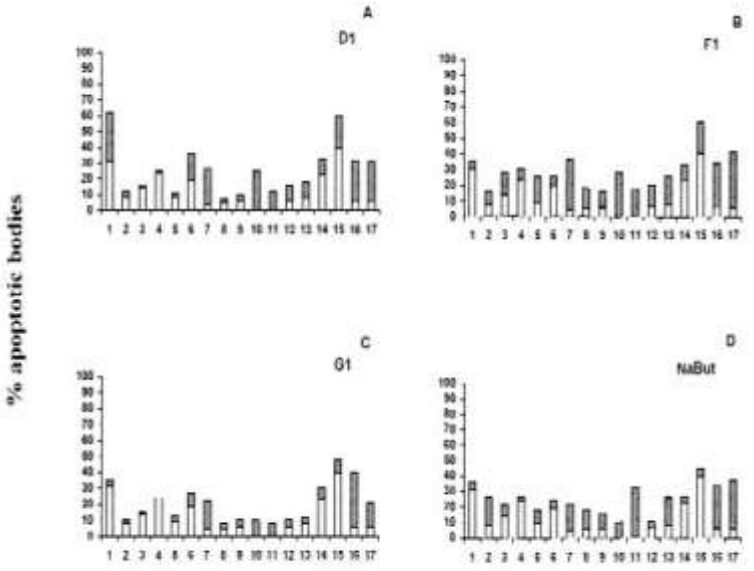

Figure 1. ***Induction of apoptosis by butyrate monosaccharide derivatives in CLL cells.*** **Morphologic apoptosis (cell shrinkage, pyknosis of nuclei, cytoplasmic and chromatin condensation and appearance of apoptotic bodies) was scored in May-Grünwald-Giemsa stained cytospins of CLL cells after 4 days of culture in the presence or absence of butyrate monosaccharide derivatives. White bars indicate the percentage of cells which underwent spontaneous apoptosis, black bars indicate CLL cells in apoptosis after exposure to butyrate derivatives or sodium butyrate. In Figure 1a the presence of apoptotic bodies in cultures of CLL cells exposed to D1 (O-n-butanoyl-2,3-O-isopropylidene-α-D-mannofuranoside) 0.5 mM is indicated by black bars. Significance (*p*<0.0001). The activity of F1 (1-O-n-butanoyl-2,3-O-isopropylidene-D,L-xylitol) 0.5 mM on CLL is represented in Figure 1b (*p*<0.0001). Figure 1c and 1d shows the percentages of apoptotic CLL cells (black bars), after treatment with G1 (1-O-n-butanoyl-D,L-xylitol) (*p*=0.001) and sodium butyrate (*p*<0.0001).**

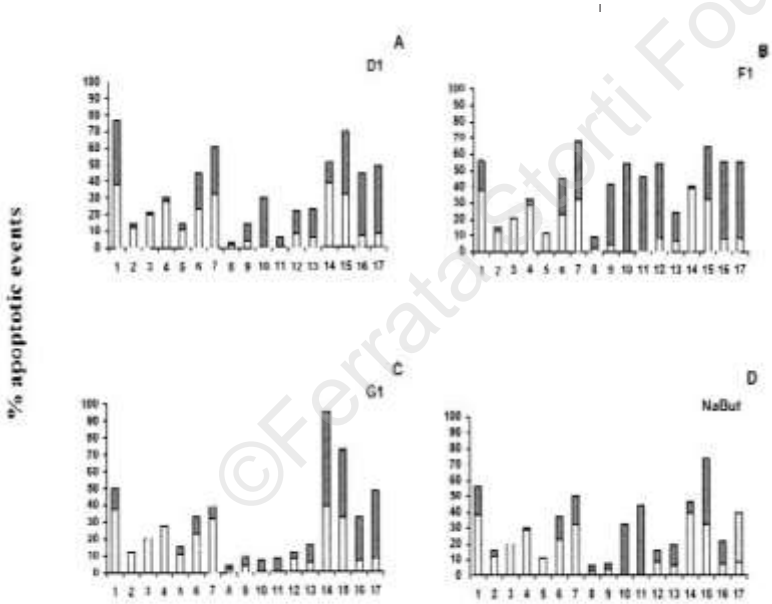

Figure 2. ***Induction of apoptosis by butyrate monosaccharide derivatives in CLL cells. DNA content.*** **Apoptosis was assessed by propidium iodide staining and subsequent cytofluorimetric analysis. The presence of a pre-G1 peak was analyzed and quantified by the CellFit program and FACSscan cytofluorimetry. White bars indicate the percentage of cells whoch have undergone spontaneous apoptosis, black bars indicate CLL cells in apoptosis after exposure to butyrate derivatives or sodium butyrate. In Figure 2a the presence of apoptotic bodies in cultures of CLL cells exposed to D1 (O-n-butanoyl-2,3-O-isopropylidene-α-D-mannofuranoside) 0.5 mM is indicated by black bars (*p*=0.0001). The activity of F1 (1-O-n-butanoyl-2,3-O-isopropylidene-D,L-xylitol) 0.5 mM on CLL is represented in Figure 2b (p <0.0001). Figure 2c and 2d shows the percentages of apoptotic CLL cells (black bars), after treatment with G1 (1-O-n-butanoyl-D,L-xylitol)(*p*=0.005) and sodium butyrate (*p*=0.001).**

presented in Figure 2a. Overall, apoptosis was highly significantly induced by D1 at both concentrations (*p*=0.0001). F1 0.5-1mM was extremely effective in inducing CLL cell apoptosis (*p*=<0.0001). Figure 2b represents the effects of F1 0.5 mM graphically. Equimolar concentrations of G1 were also able to provoke significant apoptosis at day 4 (*p*=0.005), but to lesser extent than D1 and F1 (Figure 2c). When the efficacy of sodium butyrate was compared to that of monosaccharide derivatives, it was found that, although able to provoke CLL cell apoptosis, this butyrate salt was less active than D1 and F1 (*p*=0.001). Spontaneous apoptosis did not correlate with response or resistance to butyrates. Cases # 2, 3, 4, 5 were less susceptible to monosaccharide-butyrate-induced apoptosis, irrespective of the molecule added to cultures. Although the percentage of apoptotic events scored differed, overall data obtained after PI

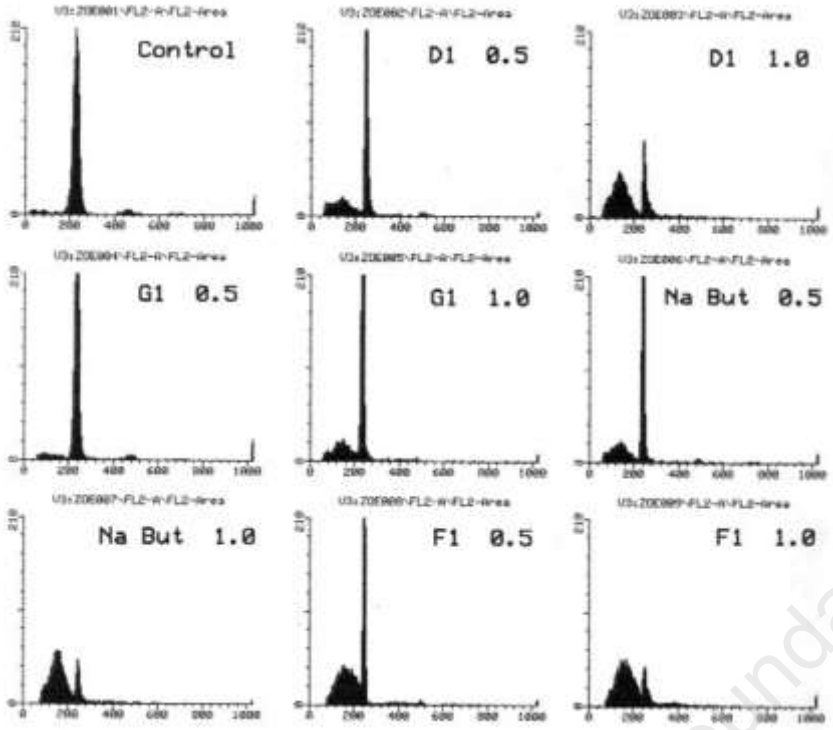

Figure 3. Cell cycle and apoptosis in CLL case #13 after monosaccharide butyrate derivative treatment. DNA content and cell cycle parameters were measured in a FACSscan cytofluorimeter after incubation of the cells with propidium iodide. Analysis of the data after 4 days of culture showed an increase of the pre-G1 apoptotic peak after exposure to D1, G1, sodium butyrate and F1, at the concentrations 0.5 and 1 mM, as indicated in the figure.

staining of CLL cells were consistent with morphologic data.

Cell cycle parameters were not modified by the addition of butyrates to cultures (data not shown). CLL cells were, as expected, exclusively distributed in the G1 phase of the cell cycle (Figure 3).

DNA fragmentation

Figure 4 shows the typical appearance of endonucleosome DNA digestion after butyrate derivative treatment (case #10). Similar laddering of DNA was present in agarose electrophoresis performed as the control for induction of apoptosis in all the samples included in this study.

Annexin V expression

The expression of phosphatidylserine on the cell surface was evaluated by Annexin V in 5/17 CLL cases. Figure 5 shows two representative cases (cases #10 and 13). The percentage of apoptotic cells (Region R2) determined at day 4 with this method was significantly increased by D1 0.5mM (range 20-44%), F1 0.5 mM (range 24-33%), G1 0.5 mM (range 19-29%) as well as sodium butyrate 0.5 mM (range 10-35%) with respect to control untreated CLL samples (range 7-19%). Necrotic cells (Region R3) were present in significantly higher proportions in D1 (range 20-43%), F1 (range 35-67%), G1 (range 30-61%) and sodium butyrate (range 16-36%) treated CLL samples with respect to control untreated cells (range 10-27%).

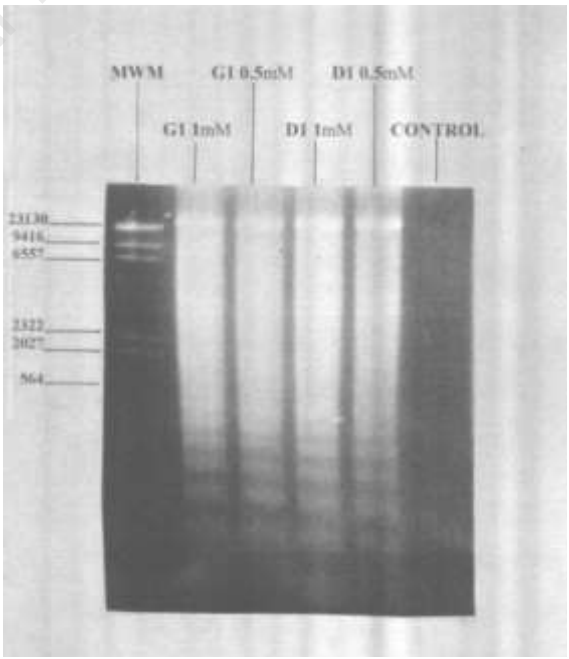

Figure 4. DNA fragmentation after monosaccharide butyrate derivative treatment (case #10). Incubation with monosaccharide butyrates provoked endonucleosomic digestion of DNA, resulting in the typical ladder-like image at agarose electrophoresis. Lane 1: DNA obtained from untreated CLL leukemic cells (control); lane 2: DNA of CLL cells after 4 days of incubation *in vitro* with D1 0.5 mM; lane 3: after D1 1 mM; lane 4: after G1 0.5 mM; lane 5: after sodium butyrate 1mM; lane 6: molecular weight marker II (Boehringer Mannheim).

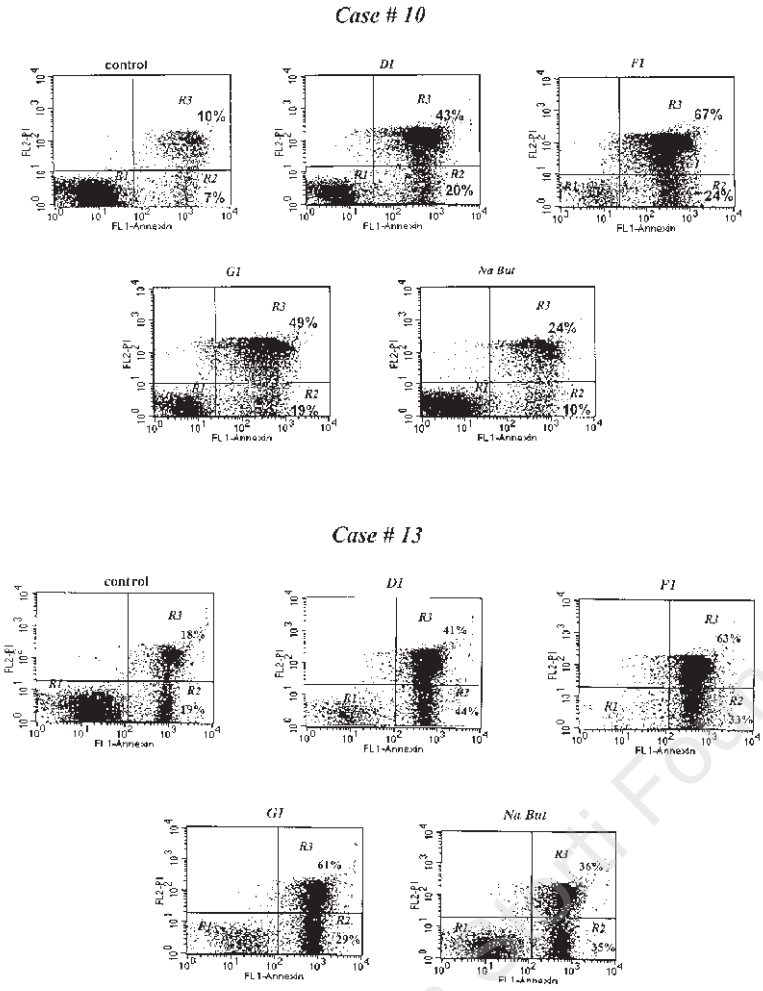

Figure 5. Annexin V expression after monosaccharide butyrate derivative treatment (cases #10 and #13). Plots in this figure show the cytometric evaluation of different expressions of annexin V on the cell surface of CLL lymphocytes treated with different monosaccharide butyrate derivatives or with sodium butyrate as indicated in single dot-plots. Region R3 identifies necrotic cells, positive for annexin V and for PI; region R2 identifies apoptotic cells, stained with annexin V-FLUO; region R1 viable cells.

Bcl2, Bax and Fas expression (APAAP)

In 5/17 cases (cases #7,9,10,11,13) the expression of bcl2, bax and fas proteins was evaluated immunohistochemically after 4 days of incubation of CLL cells in the presence or absence of D1 and G1 0.5 mM. In 3 out of 5 cases the addition of D1 or G1 to cultures provoked a decrease in the expression of bcl2. Bax expression was not modified by butyrates in any of the cases studied. The bcl2/bax ratio was therefore unaltered and remained very high. When detectable, Fas was not modified by butyrates (data not shown).

Discussion

Although fludarabine has changed the natural history of CLL,[1] a still huge proportion of CLL patients will relapse after initial response to the purine analog, or will develop resistant disease.[2,17] The mechanisms underlying this phenomenon are not well understood, but alternative therapeutic approaches to CLL are required. Few data are available on the efficacy of alternative regimens in CLL. It is therefore important to investigate this possibility, considering the use of drugs other than chemotherapeutics. Combinations of fludarabine with other agents e.g. mitoxantrone, cyclophosphamide, but also monoclonal antibodies are under active investigation, while novel agents such as flavopiridol and As_2O_3 hold promise.[18,19]

In this view, we judged it worthwhile to evaluate the efficacy of three stable monosaccharide derivatives of butyrate[7] in inducing specific apoptosis in CLL cells. We have shown previously that these monosaccharide derivatives are able to induce maturation and apoptosis in human acute myeloid leukemia cell lines and primary cells.[6] In this study we present evidence of the efficacy of these stable compounds in inducing apoptotic cell death in primary CLL cells cultured in the absence of any stimulation. CLL cells were derived from 17 Rai stage 0-II patients. Only 3 of these patients had received one course of alkylating therapy and then discontinued it. The other 14 had never been treated. None of the patients had symptoms or signs of disease progression at the time of enrollment into this study. The partially purified B-lymphocytes, with a negligible T-cell contamination, were cultured for 4 days in the presence of 0.5 and 1 mM D1 (O-n-butanoyl-2,3-O-isopropylidene-α-D-mannofuranoside), F1 (1-O-n-

butanoyl-2,3-O-isopropylidene-D,L-xylitol), and G1 (1-O-n-butanoyl-D,L-xylitol), pro-drugs of butyric acid, which have been shown to be pharmacologic stable and have low toxicity *in vivo*.[13,20] The doses we employed were those most effective in our previous AML study.[6] The apoptotic effects of butyrate on B-CLL lymphocytes are presented here for the first time to our knowledge. In 8/17 cases, spontaneous apoptosis was present. Although a correlation between spontaneous apoptosis and Rai stage of CLL has recently been demonstrated,[21] we were not able to draw any conclusions from our data because of the extremely limited number of patients included in the study. In the great majority (14/17) of CLL cases, significant apoptosis occurred in cells cultured in the presence of the monosaccharide derivatives of butyric acid, as well as in the presence of sodium butyrate. Programmed cell death was evaluated by several methods, to ensure that the elicited phenomenon was actual and specific. In fact, it has become evident from recent observations that different biochemical events contribute to apoptosis in CLL and that they may be disassociated (i.e. phosphatidylserine exposure and caspase activation occurring separately from DNA fragmentation).[22] All three monosaccharide derivatives were able to induce apoptosis in CLL, a dose-response curve was present for D1 and F1. Generally, F1 1mM induced more CLL cells into programmed cell death than other butyrates, but also provoked important cell necrosis (data not shown). The apoptotic effects of monosaccharide butyrates were evident, although to different levels, in all CLL cases; only 3/17 cases had marginal responses. Both 0.5 and 1 mM concentrations were active, but the higher dose provoked significant cell necrosis. We therefore thought that the optimal concentration for testing butyrate activity *in vitro* on CLL cells was 0.5 mM. Interestingly, no correlation with CLL stage or previous therapy and sensitivity to butyrates was noted.

The activity of G1 was more evident when analyzed in terms of annexin V expression as the other parameters studied indicated a lower number of apoptotic cells. This contributes to reaffirming a concept stressed before,[6] i.e. that the complexity of biological events leading to apoptosis is such that different methodologic approaches are needed to draw conclusions on drug effects. The rate of butyrate-induced apoptosis in CLL was so significant that we attempted to determine which pathway had been elicited. We used the APAAP procedure to investigate the involvement of the fas and bcl2/bax families in 5 CLL cases after butyrate-induced apoptosis. In 3/5 studied cases, bcl2 levels decreased slightly without a concomitant bax increase, thus failing to yield definitive evidence. The involvement of the bcl-2/bax family in mediating apoptotic resistance in CLL is in fact controversial.[23-27] Other pathways have been claimed to be responsible for induction of apoptosis in CLL, such as inactivation of NFkB-dependent genes[11] and endonuclease and proteasome activity.[27,28] Our observations seem to suggest bcl2/bax and/or fas are not involved in apoptotic pathways elicited by butyrates.

Recently, butyrates have been re-evaluated as precious tools in the therapy of hematologic malignancies because of their strong activity in histone acetylation and subsequent induction of gene expression.[9] Moreover, differentiating agents such as As_2O_3[19] have been shown to determine *in vitro* apoptosis in CLL cells, suggesting alternative clinical uses of differentiation molecules, but also strongly indicating that molecular pathways in CLL do not privilege a unique signal transduction.[29] The presence of a contaminating T-cell population in the samples analyzed should not be sufficient to modify CLL cell response. In fact, it has been demonstrated[30] that butyrates down-regulate T-cell proliferative and functional responses only indirectly, via antigen-presenting cells. T-cells are clearly resistant to butyrate induction of cell death and, therefore, when present in CLL peripheral blood, cannot contribute significantly to the percentage of apoptotic cells. Investigations on the *in vitro,* and possibly *in vivo,* efficacy of drugs such as monosaccharide butyrate derivatives will give more insights into CLL biology and resistance to apoptosis. We should reflect upon the different biochemical mechanisms of programmed cell death in CLL,[31] as they could be target of new drugs and thus have important clinical implications.

Contributions and Acknowledgments

VS designed the study, processed the results and wrote the paper. AG, BS and EG contributed to the preparation of the paper, performed the laboratory work and analyzed the results. RC performed cytometric analysis and interpretation of data. LR and RA performed and interpreted immunohistochemical analysis. AZ and AG contributed to the writing of the manuscript and the follow-up of the patients. PRF contributed with discussion and suggestions to the final interpretation and structure of this work. We thank Prof. M.Keating for his continuous support and helpful discussions.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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