Cytosine arabinoside potentiates the apoptotic effect of bendamustine on several B- and T-cell leukemia/lymphoma cells and cell lines

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Abstract

Bendamustine and cytosine arabinoside (ara-c) are commonly used cytotoxic agents with unique mechanisms of action. We have previously reported a striking additive cytotoxic effect of the consecutive combination of bendamustine and ara-c in mantle cell lymphoma (MCL) cell lines. In the present study, cell lines of follicular lymphoma (DOHH-2), chronic lymphocytic leukemia/lymphoma (EHEB), diffuse large B-cell lymphoma (SU-DHL-4), T-cell leukemia/lymphoma (JURKAT and KARPAS-299) and MCL (JEKO-1 and GRANTA-519) were exposed to the two single drugs or the drugs combined, given simultaneously and consecutively. Peripheral blood chronic lymphocytic leukemia (CLL) B-cells from five patients were also analyzed. Apoptosis, cell proliferation/metabolic activity and mitochondrial damage were evaluated. The combination index (CI) was used to assess synergy between the drugs. Bendamustine exhibited a relevant cytotoxic effect that was dose- and time-dependent, except for SU-DHL-4 and T-cell lymphoma cells. The addition of ara-c after bendamustine significantly potentiated the single-drug cytotoxic effect of bendamustine on all cell lines, including 17p − CLL B-cells, JURKAT and SU-DHL-4, the latter presenting the highest synergism (CI < 0.01). Bendamustine and ara-c are highly synergistic on T- and B-cell lymphoma cells and cell lines, similar to MCL, overcoming resistance to the single agents.

Keywords: Bendamustine, cytarabine, B-cell lymphoma, T-cell lymphoma, 17p − CLL, R-BAC

Introduction

Bendamustine (4-{5-[bis(2-chloroethyl)amino]-1-methyl-2-benzimidazolyl}butyric acid) is a bifunctional compound that has shown clinical activity against various human cancers including non-Hodgkin lymphoma [1,2], chronic lymphocytic leukemia (CLL) [3], multiple myeloma [4,5], breast cancer [6] and small-cell lung cancer [7,8].

Structurally, it comprises two main elements: a 2-chloroethylamine group and a benzimidazole ring. The 2-chloroethylamine alkylating group is shared by other alkylators such as cyclophosphamide, chlorambucil and melphalan. The benzimidazole central ring system is unique to bendamustine, and has antimitabolite properties typical of purine analogs. This ring structure may contribute to the unique antitumor activity of bendamustine and distinguishes it from conventional alkylators [9]. Both preclinical [10,11] and clinical [12] studies have shown that bendamustine is active in cancer cells that are resistant to conventional alkylating agents.

Cytosine arabinoside (ara-c) is a pyrimidine analog of cytidine with an arabinose as sugar moiety instead of ribose. The drug activity depends on phosphorylation of the prodrug ara-c to the active metabolite ara-c triphosphate (ara-CTP) [13,14]. Ara-CTP inhibits DNA polymerase and is incorporated into DNA, in turn inhibiting DNA replication during the S-phase of the cell cycle [15,16]. Ara-c is one of the more widely used drugs in the treatment of lymphoid malignancies and myeloid leukemia [17].

Preclinical models have shown that purine analogs increase the cytotoxic effect of ara-c, favoring its incorporation into DNA strands. Purine analogs such as fludarabine and cladribine augment the intracellular level of ara-CTP, thereby increasing the inhibitory effect of the drug on DNA synthesis. Many regimens used for treating acute leukemias are based on the association of these drugs [18]. Bendamustine, similar to nucleoside analogs, has been shown to elicit potent modulation of ara-c metabolism in leukemic blasts in vitro [18–20], enhancing the apoptotic effect of ara-c.

In the present study we investigated the effect of bendamustine, alone or combined with ara-c, in modifying apoptotic pathways and cell proliferation of aggressive and indolent
B- and T-cell leukemia/lymphoma cells and cell lines. By means of this study we aimed at providing a rationale for use of this combination in patients with lymphomas or CLL that are resistant to conventional drug combinations.

Materials and methods

Cell lines and mononuclear cells from patients

Seven lymphoma cell lines were cultured: two MCL (JEKO-1 and GRANTA-519, representing a classical MCL and a relapsed blastoid variant of MCL, respectively); a follicular lymphoma (DOHH-2); a diffuse large B-cell lymphoma (SU-DHL-4); a chronic lymphocytic B-cell leukemia/lymphoma (EHEB); and two T-cell lymphoma (JURKAT and KARPAS-299, representing a lymphoblastic T-cell lymphoma and CD30+ anaplastic large T-cell lymphoma, respectively) cell lines. All lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and had a doubling time ranging between 40 and 50 h.

Peripheral blood samples from five patients with B-CLL were analyzed. Informed consent was obtained from all subjects prior to any study procedure. One of them had high-risk CLL according to known biological prognostic factors (17p−, unmutated immunoglobulin heavy-chain variable-region [IgHV], zeta-chain-associated protein kinase 70 [Zap70] positive), while two patients had intermediate-risk CLL (13q− and normal karyotype, respectively, both with unmutated IgHV and Zap70+). The remaining two patients had favorable CLL (both 13q− with mutated IgHV).

This work was conducted in the research laboratories of the Department of Cellular Therapies and Hematology of the San Bortolo Hospital and of the Hematology Project Foundation.

Cell preparation, doses and incubation schedules

The media used were 90% Dulbecco’s modified Eagle’s medium (MEM) with 4.5 g/L glucose (Sigma-Aldrich, St Louis, MO; http://www.sigmaaldrich.com) for GRANTA cells, and 90% RPMI (Sigma-Aldrich) for the other six lines. Both media were supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 10% fetal bovine serum (PBS; STEMCELL Technologies, Vancouver, Canada; http://www.stemcell.com). Peripheral blood mononuclear cells were isolated using Ficoll–Hypaque (Sigma-Aldrich) gradient centrifugation and monocytes were depleted by plastic adhesion. B-CLL lymphocytes were cultured using the same methods as for the cell lines.

The doses of bendamustine (B) and ara-c (A) were established by testing a range of variably cytotoxic concentrations on each cell line (1–1000 μg/mL for bendamustine, 0.10–20 μg/mL for ara-c). Doses that were found to give a linear dose-response curve after logarithmic transformation were chosen to be investigated in the study. In order to evaluate the synergistic effect of the two drugs, we chose a more limited range of doses that gave an apoptotic effect of less than 75% of tumor cells. Doses ranged between 5 and 500 μg/mL for bendamustine, and between 0.025 and 10 μg/mL for ara-c, as specified in Table I.

Bendamustine and ara-c were incubated as single drugs, and simultaneously and consecutively, as follows: (a) single incubations: B or A as single drug for 24 h and 48 h at 37 °C; (b) simultaneous incubations: B and A together for 24 h and 48 h at 37 °C; and (c) consecutive incubations: B for 2 h, then washed twice and A added 21 h later for 24 h (B + A schedule) (the same scheme but with opposite order was adopted for the A + B schedule). After each drug incubation schedule, cells were collected and washed with phosphate buffered saline (PBS; STEMCELL Technologies) to be used for the different tests.

Table I. Apoptotic effect of bendamustine and ara-c on seven lymphoma cell lines and CLL B-cells obtained from three treatment-naïve patients.

<table>
<thead>
<tr>
<th>Drug concentration (min/max)</th>
<th>Range of 7-AAD + cells* (% ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 24 h</td>
<td>A 24 h</td>
</tr>
<tr>
<td>MCL (JEKO-1)</td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>20 μg/mL</td>
<td>2 μg/mL</td>
</tr>
<tr>
<td>MCL (GRANTA-519)</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>250 μg/mL</td>
<td>2 μg/mL</td>
</tr>
<tr>
<td>CLL (EHEB)</td>
<td>25 μg/mL</td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>Follicular lymphoma (DOHH-2)</td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>50 μg/mL</td>
<td>0.75 μg/mL</td>
</tr>
<tr>
<td>DLBCL (SU-DHL-4)</td>
<td>50 μg/mL</td>
</tr>
<tr>
<td>500 μg/mL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>T-cell leukemia (JURKAT)</td>
<td>12.5 μg/mL</td>
</tr>
<tr>
<td>100 μg/mL</td>
<td>0.2 μg/mL</td>
</tr>
<tr>
<td>T-cell lymphoma (KARPAS-299)</td>
<td>20 μg/mL</td>
</tr>
<tr>
<td>80 μg/mL</td>
<td>1.6 μg/mL</td>
</tr>
<tr>
<td>13q−, unmutated1 CLL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>250 μg/mL</td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>Normal karyotype, unmutated1 CLL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>250 μg/mL</td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>17p−, unmutated1 CLL</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>250 μg/mL</td>
<td>5 μg/mL</td>
</tr>
</tbody>
</table>

*Range of 7-AAD positive cells of seven tested cell lines in the presence of bendamustine (B) and ara-c alone (A) at minimum and maximum tested doses and in combination after 24 h of incubation. Mix 24 h: simultaneous incubation for 24 h; B + A and A + B: consecutive schedules. Each experiment was performed four times in duplicate. Results are expressed in terms of mean % ± standard deviation (SD).

1Unmutated refers to mutational status of immunoglobulin heavy-chain variable-region (IgHV) of three patients with B-CLL; all patients were Zap70 positive. CLL, chronic lymphocytic leukemia/lymphoma; 7-AAD, 7-amino-actinomycin D; MCL, mantle cell lymphoma; DLBCL, diffuse large B-cell lymphoma.
Analysis of apoptosis by flow cytometry (annexin V/7-AAD test) and cell cycle analysis
The quantification of apoptosis was measured 24 h and 48 h after any incubation schedule with an annexin V/7-aminoactinomycin D (7-AAD) kit (Invitrogen, Carlsbad, CA; http://www.invitrogen.com).

Cell cycle analysis was based on the ability of the 7-AAD dye to stain cellular DNA in a stoichiometric manner after permeabilization of the membrane with acetone. After fluorescence activated cell sorting (FACS) analysis, DNA content histograms were analyzed using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA; Dean–Jett model), as previously reported [21].

Measurement of mitochondrial damage (JC-1 test) and metabolic activity analysis (WST-1 test)
The loss of mitochondrial membrane potential (ΔΨm) was monitored using a flow cytometer (FC 500; Beckman Coulter) using 5,5,6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide dye (JC-1; Invitrogen). The water-soluble tetrachlorodium salt WST-1 was used for the measurement of cell metabolic activity and viability. To evaluate the cytotoxic effects of the drugs, we measured the decrease in level of the formazan dye compared to a sample of untreated cells, as previously reported [21]. The results were expressed as percentage reduction of viability.

Drug combination and statistical analysis
The cytotoxic effect of bendamustine and ara-c as single drugs was compared, in terms of percentage of affected cells, with simultaneous incubation after 24 and 48 h, respectively; the consecutive schemes were compared with single incubation for 24 h. Student’s t-test was used to compare results, which were considered to be statistically significant when \( p < 0.05 \).

The apoptotic effect of the two drugs alone and in combination was analyzed using CalcuSyn computer software (Biosoft, Cambridge, UK). This software elaborates data correlating the drug dose with its effect through the following linear equation:

\[
\log \left( \frac{f_a}{f_u} \right) = m \log (D) - m \log (Dm)
\]

where \( f_a \) represents the fraction affected by the dose, \( f_u \) the fraction unaffected \((1 - f_a)\), \( m \) the slope of the curve, \( D \) the dose of the drug and \( Dm \) the concentration of drug at which a 50% effect is obtained \((f_a = f_u\)). The slope of the curve represents the potency of the drug; the highest \( m \) corresponds to the greatest potency.

The combination index (CI) and dose reduction index (DRI) were used to establish whether a synergistic or antagonistic interaction between these drugs may exist. The CI is a quantitative measure of the degree of drug interaction, and the DRI can be defined as a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. A CI value < 0.1 reflects very strong synergism between the two drugs, while values between 0.1 and 0.9 reflect a strong to slight synergistic effect, and values > 1 represent antagonism between the two drugs. The CalcuSyn method was applied for quantifying the modification of the apoptotic pathways, the cell proliferation capacity, the decline in metabolic activity and the mitochondrial damage for all cell lines.

Experiments were repeated four times in duplicate for each condition, and the mean value was taken.

Results
Measurement of apoptosis by annexin V/7-AAD test and cell cycle analysis on seven cell lines
Two populations were observed by cytofluorimetric analysis after the first two tests for each of the cell lines. Specifically, viable cells were double-negative (low annexin V and low 7-AAD signal), and necrotic cells had high annexin V and high 7-AAD signals. No early apoptotic cell population was observed in any of the experiments (high annexin V and 7-AAD negative). Therefore, we considered the 7-AAD dye as an indicator of apoptosis in all subsequent analyses.

The replication phase of the cell cycle after single incubations of the two drugs was determined by flow cytometry. Both bendamustine and ara-c were associated with the accumulation of cells in S-phase, with an improvement of the debris peak for all cell lines. Results are summarized in Table I.

Bendamustine and ara-c as single drugs
Bendamustine presented a dose- and time-dependent cytotoxic effect on cell lines, except for SU-DHL-4 and T-cell leukemia/lymphoma cells, which were barely affected by the drug (maximum level of apoptosis 19 ± 10%, and 28 ± 10% and 24 ± 4%, respectively) (Figure 1). The follicular lymphoma DOHH-2 cell line was most sensitive to bendamustine (63 ± 4% after 24 h and 92 ± 5% after 48 h). The percentage of apoptotic cells after exposure to bendamustine in CLL EHEB cells was similar to the cell-death rate observed in MCL cells (43 ± 8% after 24 h and 62 ± 7% after 48 h).

Ara-c exhibited a cytotoxic effect that was not dose related, and poorly influenced by time of exposure. For MCL cells and follicular lymphoma DOHH-2 cells, exposure to ara-c caused apoptosis of half of the cells. Diffuse large B-cell lymphoma SU-DHL-4 cells, CLL EHEB cells and T-cell lymphoma cells

![](image) Figure 1. Median-effect plot of bendamustine after 24 h of incubation for seven cell lines. Figure shows linear relationship between cytotoxic effect \( \log \left( \frac{f_a}{f_u} \right) \): \( f_a \) is fraction affected by dose and \( f_u \) is fraction unaffected] and dosage \( \log (D) \): \( D \) is drug dosage. Reported values were obtained as median result of experiments performed four times in duplicate.
were poorly affected by ara-c, even at the highest concentrations (Table I).

**Simultaneous incubation schedules**
The simultaneous schedules induced an improvement in the apoptotic rate that was significantly higher compared to that observed for the two drugs alone in MCL JEKO-1 cells, CLL EHEB cells, follicular DOHH-2 cells and T-cell KARPAS-299 cells. Diffuse large B-cell lymphoma SU-DHL-4 cells and JURKAT cells were not sensitive to the simultaneous incubations, as observed with the single drug incubations.

**Consecutive incubation schedules**
The consecutive incubation schedules were associated with significant improvement of the cytotoxic effects in all cell lines, with the only exception of the A + B schedule on KARPAS-299 cells. The more powerful cytotoxic effect following consecutive administration of the two drugs compared with the cytotoxicity of the single drugs was observed on diffuse large B-cell lymphoma SU-DHL-4 cells (97 ± 1% of apoptotic cells with B + A at maximum doses). A relevant synergism was also observed on MCL JEKO-1, T-cell lymphoma JURKAT and CLL EHEB cells (Table I). The apoptotic effects of the different schedules and the single drugs at equivalent doses are reported in Figure 2.

**Measurement of apoptosis on peripheral blood lymphocytes from patients with B-CLL**
The apoptotic effect induced by bendamustine alone was dose- and time-dependent on all CLL B-cells. In two of the five samples, however, the effect of the drug was so strong (90% or more of cell mortality) that it prevented any further combination test. Both patients had favorable-risk CLL with mutated IgHV.

Therefore, only three CLL B-cell samples were used in the experiments with drug combinations. The three samples exhibited a similar behavior to CLL EHEB cells, showing a relevant and significant improvement of apoptosis when bendamustine and ara-c were combined in a consecutive manner, as graphically reported in Figure 2. The 17p-deleted CLL B-cells, which are known to be usually resistant to purine analogs, were significantly affected by the consecutive schedule (p < 0.01), as were other CLLs without the 17p deletion.

**Drug synergism according to CI**
Results were expressed in terms of additive effect, synergism or antagonism, as summarized in Table II. Consistent with previously reported cytotoxic effects, the diffuse large B-cell lymphoma SU-DHL-4 and T-cell lymphoma JURKAT cell lines were more influenced by the consecutive schedules in terms of CI (CI < 0.01). These cell lines exhibited a straightforward sensitivity to the B + A and A + B schedules when compared with the simultaneous or single incubations of the two drugs.

**Disruption of mitochondrial membrane potential (ΔΨm)**
Bendamustine caused a ΔΨm disruption in a dose-dependent manner on all cell lines, except KARPAS-299 T-cell lymphoma cells. After 48 h we measured an increase in the percentage

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**Figure 2.** Apoptotic effect (% of 7-AAD + cells) of bendamustine alone, B, and in combination with ara-c, A, on five B- and T-cell lines, mononuclear cells of three patients with B-CLL and two MCL cell lines. Mix: simultaneous incubation for 24 h; B + A and A + B: consecutive incubations. Each experiment was performed four times in duplicate. *p < 0.01, significant vs. bendamustine alone. **17p−** histograms represent percentage of cell death observed in peripheral blood lymphocytes of one patient with this abnormality. “Not 17p−” is mean apoptotic rate of cells of two patients without 17p−. Doses of B + A are listed under each cell type. MCL cell lines were used as a comparison.
CLL, chronic lymphocytic leukemia/lymphoma; MCL, mantle cell lymphoma; 

<table>
<thead>
<tr>
<th></th>
<th>Mix 24 h</th>
<th>B + A</th>
<th>A + B</th>
<th>Mix 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL (JEKO-1)</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
</tr>
<tr>
<td>MCL (GRANTA-519)</td>
<td>0.1–0.9</td>
<td>0.1–0.9</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
</tr>
<tr>
<td>CLL (EHEB)</td>
<td>0.3–0.7</td>
<td>$&lt;0.1$</td>
<td>0.1–0.7</td>
<td>0.3–0.7</td>
</tr>
<tr>
<td>Follicular</td>
<td>0.3–3.3</td>
<td>$&lt;0.1$</td>
<td>0.1–1.1</td>
<td>0.3–0.7</td>
</tr>
<tr>
<td>lymphoma</td>
<td>(DOHH-2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLBCL (SU-DHL-4)</td>
<td>0.3–1.2</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.01$</td>
<td>$&gt;1$</td>
</tr>
<tr>
<td>T-cell leukemia</td>
<td>0.2 &gt; 1</td>
<td>$&lt;0.01$</td>
<td>$&lt;0.01$</td>
<td>$&gt;1$</td>
</tr>
<tr>
<td>(JURKAT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-cell lymphoma</td>
<td>0.2 &gt; 1</td>
<td>0.1–0.6</td>
<td>0.6 &gt; 1</td>
<td>0.5–0.9</td>
</tr>
<tr>
<td>(KARPAS-299)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13q–, unmutated†</td>
<td>0.5–1.2</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
<td>0.5–0.9</td>
</tr>
<tr>
<td>CLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal karyotype, unmutated†</td>
<td>$&lt;0.1$–0.3</td>
<td>$&lt;0.1$</td>
<td>$&lt;0.1$</td>
<td>0.1–0.4</td>
</tr>
<tr>
<td>CLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17p–, unmutated†</td>
<td>$&lt;0.1$–0.5</td>
<td>$&lt;0.1$–0.5</td>
<td>$&lt;0.1$–0.5</td>
<td>$&lt;0.1$–0.4</td>
</tr>
</tbody>
</table>

Table II. Combination index (CI) of bendamustine and ara-c combinations in lymphoma cell lines and CLL B-cells obtained from three treatment-naïve patients.

CI calculated for seven cell lines and three CLL B-cell samples in all combination schedules. We reported results as range when CI varied depending on drug doses. CI $< 0.1$: very strong synergism; 0.1–0.9: strong to slight synergism; >1: antagonism. Bold type indicates stronger synergism (CI $< 0.1$) with respect to each cell line.

† Unmutated refers to mutational status of immunoglobulin heavy-chain variable-region (IgHV) of three patients with B-CLL; all patients were Zap70 positive. CLL, chronic lymphocytic leukemia/lymphoma; MCL, mantle cell lymphoma; DLBCL, diffuse large B-cell lymphoma.

of depolarized/altered mitochondrial function from about 20%, in the absence of bendamustine, to 63 ± 6% (EHEB), 79 ± 4% (SU-DHL-4), 88 ± 7% (DOHH-2), 79 ± 7% (GRANTA-519), 50 ± 2% (JEKO-1) and 70 ± 10% (JURKAT) at the highest concentrations.

On the two MCL, the follicular lymphoma DOHH-2 and the T-cell lymphoma JURKAT cell lines, ara-c alone caused remarkable $\Delta$ψm disruption in a dose- and time-dependent manner, varying from 52 to 80%. On the other hand, ara-c did not cause appreciable cytotoxicity mediated by the mitochondrial pathway on SU-DHL-4, EHEB and KARPAS-299 cells, as shown by a constant percentage of $\Delta$ψm reduction at the different tested doses.

Simultaneous incubations did not significantly affect the mitochondrial pathway compared with the drugs alone.

On all cell lines and CLL B-cells the consecutive incubations induced mitochondrial damage that was significantly higher than for the drugs alone (Figure 3 shows results for CLL EHEB cells as an example). This very strong synergistic effect was confirmed by CI values less than 0.1 with all drug doses.

Reduction of metabolic activity induced by bendamustine and ara-c

The decrease of enzymatic activity induced by bendamustine was relevant only after 48 h in the MCL cell lines, KARPAS-299 and JURKAT, while for the other lines and CLL B-cells a more rapid decline was observed (maximum level of about 80% in SU-DHL-4 at 100 $\mu$g/mL).

An effect of ara-c was observed after 48 h on DOHH-2, EHEB, GRANTA-519 and JURKAT cells, whereas for the mantle cell JEKO-1 and diffuse large B-cell lymphoma SU-DHL-4 cells the decrease of enzymatic activity was prominent after 24 h of incubation (55 ± 7% and 32 ± 4%, respectively). No effect was detected on the T-cell KARPAS-299 cell line. The simultaneous incubations led to a decrease of activity of 60–80% in all cell lines, even at the lower concentrations. In KARPAS-299 cells the simultaneous schedule affected the cell metabolism more than the consecutive schedules. For the other cell lines and CLL B-cells the consecutive schedules induced a decrease of enzymatic activity of 60–80%, independent of drug order.

Discussion

We have addressed the cytotoxic effect of bendamustine alone or combined with ara-c on several B- and T-cell leukemia/lymphoma cell lines, and on peripheral blood lymphocytes of three patients with B-CLL. Bendamustine had a linear dose- and time-dependent cytotoxic effect on all cell lines, with follicular lymphoma DOHH-2 cell line being the most sensitive to the drug, followed by MCL cells. In contrast, diffuse large B-cell lymphoma and T-cell lymphoma cells were barely affected by the drugs, irrespective of dose and time of incubation, even when bendamustine and ara-c were administered simultaneously. However, both lines achieved an impressive improvement of cytotoxicity when the drugs were incubated consecutively. The apoptotic rate that characterized the diffuse large B-cell lymphoma cell line corresponded to the strongest synergistic effect in inducing apoptosis compared to all cell lines, being more than five-fold greater than the maximum effect obtained with the single drugs. A similar synergistic effect was observed in T-cell lymphoma cell lines. All cell lines benefited from the consecutive schedules of incubation. The limited activity of bendamustine as a single drug on aggressive lymphoma cell lines resembles what is commonly observed in vivo, where high grade lymphomas are less responsive to the drug than low grade lymphomas and MCL [2,22].

Similar to what was observed by Schwänen et al. [23], we showed that bendamustine induced apoptosis in a dose- and time-dependent manner also in B-CLL lymphocytes isolated from peripheral blood, whereby an elevated dosage seemed to be more efficient than a prolonged incubation time. Results for CLL cells did not differ significantly between the CLL cell line and the isolated tumor cells, suggesting that the cell lines are representative of what might happen in vivo with tumor cells. In the above mentioned study [23], the authors combined bendamustine with fludarabine (a purine analog) in order to induce higher rates of apoptosis, and observed that this combination was highly synergistic.

Bendamustine, in combination with rituximab, is likely to become one of the treatments of choice in older patients with CLL [24], but its efficacy in patients with high-risk disease (i.e. 17p deletion) is low. One of the three tested patients had very unfavorable biological features including 17p deletion and unmutated IgHV. This form of CLL is usually refractory to treatment in the clinical setting. The CLL B-cells of this patient were significantly more sensitive to the consecutive combination of the drugs than to the single drugs (see Table I or Figure 2).

The plasma therapeutic levels of ara-c and bendamustine that are usually achieved in vivo in hematologic malignancies (0.24–0.5 $\mu$g/mL and 10–100 $\mu$g/mL, respectively) are similar
Bendamustine and ara-c in B- and T-cell lymphomas

However, contrary to what was observed with the 7-AAD assay, both diffuse large B-cell lymphoma SU-DHL-4 cells and T-cell JURKAT cells exhibited a remarkable sensitivity to bendamustine as single drug in terms of mitochondrial damage. This suggests activation of the intrinsic pathway of apoptosis involving the caspase cascade by bendamustine, which might predispose the cells to subsequent damage induced by ara-c, thus explaining the success of the consecutive schedules. On the other hand, the synergistic effect of bendamustine and ara-c could be related to the individual mechanisms of action of the two drugs, whose serial administration would avoid saturation of the common pathways. While ara-c is a pure antimetabolite, bendamustine also acts as a purine analog. For this reason the two agents, which are known to induce a block of the cell cycle in S-phase [21], may cause cross-links and DNA strand

to concentrations we used in our experiments (0.25–2 μg/mL and 5–250 μg/mL, respectively).

The synergistic cytotoxicity in vitro of purine analogs combined with ara-c has already been reported in the myeloid setting [18,20], with fludarabine and cladribine potentiating the cytotoxic effect of ara-c on several acute myelogenous leukemia cell lines and fresh isolated blasts. The modulation effect of ara-c by bendamustine on myeloid blasts and MCL lines has already been described [19], but to the best of our knowledge this is the first report describing a synergistic effect of the two drugs on several B- and T-cell lymphoma cell lines, and on CLL B-cells obtained from patients.

Our results in terms of rate of cytotoxicity were confirmed when we analyzed the decline of metabolic activity and mitochondrial damage, predictive of the release of cytochrome c and other proteins essential for the activation of procaspases and execution of apoptosis. However, contrary to what was observed with the 7-AAD assay, both diffuse large B-cell lymphoma SU-DHL-4 cells and T-cell JURKAT cells exhibited a remarkable sensitivity to bendamustine as single drug in terms of mitochondrial damage. This suggests activation of the intrinsic pathway of apoptosis involving the caspase cascade by bendamustine, which might predispose the cells to subsequent damage induced by ara-c, thus explaining the success of the consecutive schedules. On the other hand, the synergistic effect of bendamustine and ara-c could be related to the individual mechanisms of action of the two drugs, whose serial administration would avoid saturation of the common pathways. While ara-c is a pure antimetabolite, bendamustine also acts as a purine analog. For this reason the two agents, which are known to induce a block of the cell cycle in S-phase [21], may cause cross-links and DNA strand

Figure 3. Cytotoxicity on EHEB cell line induced by B + A consecutive incubation. (A) Morphological differences of cells exposed to different doses. Healthy cells (green) presented a normal size, whereas EHEB with partly or totally depolarized Δψm (red) showed a decreased cell size (FS) and increased granularity (SS), which indicates apoptotic cells. (B) Increasing drug doses, we detected a shift of JC-1 green fluorescence and decrease of red emission indicative of altered mitochondrial function.
breaks, providing an obstacle to further DNA synthesis [25]. Cells escaping the cell cycle arrest induced by bendamustine and trying to repair their damage would be prone to incorporating the metabolite ara-CTP into DNA, similar to that reported by Staib et al. [19] in acute myeloid leukemia cells. Furthermore, bendamustine itself may determine a deficiency of deoxyribonucleotide triphosphates, resulting in cumulative DNA damage.

The mechanisms involved in the strong synergy between bendamustine and ara-c still need to be fully elucidated. However, our study has shown that bendamustine and ara-c are highly synergistic, not only in MCL cell lines [21], but also in peripheral blood CLL B-lymphocytes and cell lines of aggressive and indolent T- and B-cell lymphomas. Confirming these findings, the preliminary results of a phase 2 trial that is ongoing at our institution in older patients with MCL treated with this combination were very encouraging [26]. The drug combination seems to overcome resistance to the single agents in aggressive T- and B-cell lymphomas and in 17p− CLL B-cells.

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