The cytotoxic effects of bendamustine in combination with cytarabine in mantle cell lymphoma cell lines

Carlo Visco, Silvia Castegnaro, Katia Chieregato, Martina Bernardi, Elena Albiero, Cristina Zanon, Domenico Madeo, Francesco Rodeghiero

Department of Cell Therapy and Hematology, San Bortolo Hospital, Vicenza, Italy

Abstract

Bendamustine is clinically useful in mantle-cell lymphoma (MCL). Its favorable toxicity profile in-vivo favors its combination with other cytotoxic drugs. Cytarabine is a key drug in the treatment of younger patients with MCL. The current study investigated the in-vitro cytotoxic effect of bendamustine and cytarabine, alone or combined, on two MCL cell lines representing the classic and blastoid variant of the lymphoma subtype (JEKO-1 and GRANTA-519). Cell lines were exposed to each drug alone, or simultaneously and consecutively to both drugs, for different time schedules. Apoptosis was measured by flow cytometry. Mitochondrial damage, cell proliferation/metabolic activity, and cell cycle analysis were also assessed. The synergistic, additive, or antagonistic effects of the drugs were calculated with the combination index (CI) method. Bendamustine and cytarabine alone exhibited relevant cytotoxic activity on both cell lines. Both drugs induced cell cycle arrest in S phase. Drug combinations were associated with significantly higher cytotoxic effects than each drug alone. Among the combination schedules, the consecutive incubation of bendamustine followed by cytarabine was associated with the lower CI, being 10–100-fold lower than with simultaneous incubations. The cytotoxic effect of the consecutive combination was prominent on both cell lines, indicating a very strong and highly significant synergy in inducing apoptosis. Similar results were obtained measuring mitochondrial damage or the decline of the metabolic activity in all cell lines. The strong synergistic effect of bendamustine and cytarabine on MCL cells provides a rationale for developing schedules combining these agents in the treatment of MCL.

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Introduction

Mantle cell lymphoma (MCL) is a relatively rare disease, accounting for approximately 6% of all non-Hodgkin lymphoma (NHL) cases [1]. In the USA, however, there has been an increasing trend in the incidence of MCL, with the age-adjusted incidence rate per 100,000 increasing from 0.27 to 0.69 for the period 1992–2004 [2]. Patients have a median age of 60–65 years and typically present with generalized lymphadenopathy [3], and with advanced stage disease, the spleen and the gastrointestinal tract being the most frequently involved sites. While the overall response rate (ORR) to chemo-immunotherapy is usually good, relapses are frequent and the prognosis is poor [4].

Evolving intensive treatment regimens for MCL have led to improvements in prognosis [5–7], but only in the subset of younger patients. All these induction regimens have included high-dose cytarabine (also known as cytosine arabinoside, or Ara-C), which is considered as a key chemotherapeutic agent in this setting [3]. However, the benefits obtained by adding cytarabine to first-line regimens in terms of the rate, quality and duration of treatment response are limited by its relevant toxicity. Given that most patients are aged >60 years at the time of MCL diagnosis, and frequently affected by co-morbid conditions, many of them are not able to tolerate such aggressive therapies.

A number of novel agents have shown promising results in the management of older or frail patients. Among them is bendamustine, which has shown relevant clinical efficacy together with an excellent toxicity profile in the treatment of indolent lymphomas [8,9], chronic lymphocytic leukemia (CLL) [10,11], multiple myeloma [12], solid malignancies [13–15], and MCL [16–18].

Due to its structure bendamustine may interact as an alkylating agent as well as a purine analog, but little is known about its cross-resistance or interactions with other cytotoxic drugs. Pre-clinical models have shown that purine analogs increase the cytotoxic effect of cytarabine, favoring its incorporation into DNA strands, and it is well known that fludarabine and cladribine (2-CdA) augment the intracellular level of cytarabine triphosphate (ara-CTP), thereby increasing the inhibitory effect of the drug on DNA synthesis [19,20]. Many effective regimens used worldwide for treating acute myeloid leukemia (AML) are based on the association between fludarabine and cytarabine [19]. Preliminary reports have shown that, similarly...
to other nucleoside analogs, bendamustine elicits potent modulation of cytarabine metabolism in blasts of AML in vitro, increasing ara-CPT levels to higher amount than fludarabine, and thus enhancing the apoptotic effect of cytarabine [21]. However, no previous study has investigated the therapeutic synergy between bendamustine and cytarabine in lymphoma cells [22]. The aim of the current study was to investigate the putative synergistic effects of bendamustine and cytarabine in modifying apoptotic pathways and cell proliferation in two MCL cell lines (JEKO-1 and GRANTA-519). Demonstration of such cytotoxic effects would provide the rationale for the development of this combination in the treatment of patients with MCL.

Methods

Cell lines and cultures

Two MCL cell lines [JEKO-1 and GRANTA-519; DSMZ — Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany] were cultured and analyzed. Both lines were established from the peripheral blood of patients with MCL. JEKO-1 consisted of a classical MCL at diagnosis, while GRANTA-519 represented a relapsed blastoid variant of MCL. The culture media were 90% Dulbecco’s Modified Eagle Mem 4.5 g/l glucose (DMEM, Sigma-Aldrich, St Louis, MO, USA) for GRANTA-519 cells and 90% RPMI (Sigma-Aldrich) for JEKO-1. Both cultures were supplemented with 2 mM l-glutamine (Sigma-Aldrich) and 10% fetal bovine serum (FBS, STEMCELL Technologies, Vancouver, Canada).

Drugs doses and incubation schedules

The doses of bendamustine and cytarabine were established by testing a range of variably cytotoxic concentrations on each cell line (1 μg/mL to 1000 μg/mL for bendamustine, 0.10 μg/mL to 20 μg/mL for cytarabine). Doses that were found to give a linear dose–response curve after logarithmic transformation were chosen to be investigated along the study. In order to evaluate the synergistic effect of the two drugs, only the doses of the single drugs with an apoptotic effect equal or inferior to 75% of tumor cells were adopted, thus excluding doses associated with extensive cytotoxicity. For the JEKO-1 cell line the bendamustine concentrations chosen were 5, 10, 20 and 40 μg/mL, while the corresponding concentrations for cytarabine were 20-fold lower (0.25, 0.5, 1 and 2 μg/mL). Similarly, drug concentrations for GRANTA-519 cell line were 10, 50, 100 and 250 μg/mL for bendamustine, and 0.25, 0.5, 1 and 2 μg/mL for cytarabine. Cells were cultured with bendamustine and cytarabine at the calculated concentrations to obtain a linear log/log dose–response curve for each cell line.

The two drugs were incubated as single drugs, simultaneously and consecutively. The first two schedules were tested for 24 h and 48 h. The consecutive incubation scheme reflected previously reported drug associations [19–21], and consisted of bendamustine (B) for 2 h, followed by twice washing and culture for 21 h in the absence of the drug. Cytarabine (A) was then added for the following 24 h (B+A schedule). The same timing and scheme was used when the drug order was reversed (A+B schedule). After each drug incubation schedule, cells were collected and washed with PBS (Phosphate Buffer Saline, STEMCELL Technologies) to be used for the different tests.

Measurement of apoptosis by flow cytometry (7-AAD) and morphological analysis

Apoptosis was quantified by flow cytometry using the 7 aminoactinomycin D (7-AAD; Invitrogen, Carlsbad, CA, USA) test, which is based on the capability of 7-AAD dye to penetrate the altered membranes of apoptotic/dead cells, and on its subsequent intercalation between guanine-cytosine base pairs [23]. To perform this test, samples of 0.5×10^6 cells were washed with ice-cold PBS, supplemented with 1% FBS and then resuspended in 100 μL of PBS. Ten microliters of 7-AAD dye (0.1 mg/mL) was added to the cell suspension. After 15 min of incubation in the dark, 400 μL of PBS was added and mixed gently. Cell preparation was then analyzed using a FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm argon laser and a 637 nm HeNe laser. Assays were performed in duplicate. A morphological analysis was also performed to visualize the induction of cell death. Cells were collected after the established incubation schedules, spun down on a glass slide and stained with May-Grunwald Giemsa, according to standard methods.

Cell cycle analysis (7-AAD)

The cell cycle analysis is based on the ability of the 7-AAD dye to stain cellular DNA in a stoichiometric manner after permeabilisation of the membrane [24]. The amount of the stain is directly proportional to the amount of DNA within the cell and describes the phase of the cell cycle in which the agent may act. Cells were collected in polypropylene tubes, washed with PBS and resuspended in 300 μL PBS containing 50% of FBS, keeping the tubes on ice. Three volumes (300 μL each) of 80% ice-cold acetone were added to the suspension and mixed gently with every addition. After an incubation of 1 h at 8°C, cells were washed twice with a washing/staining solution (composed by PBS, 1% FBS and 10 μg/mL of 7-AAD) and incubated with 500 μL of the same solution for 30 min up to 16 h at room temperature. Following this, cells were measured by FC500 flowcytometer for cell cycle analysis. Relative DNA content, after excluding cell doublets, was illustrated in a single-parameter FL4 (area) histogram with linear x-axis. DNA content histograms were analyzed using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA) [25].

Measurement of mitochondrial damage (JC-1)

Evaluation of mitochondrial damage was used to assess the involvement of this pathway in the apoptotic process, and was determined by measuring the loss of mitochondrial membrane potential (Δψm). 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide dye (JC-1, Invitrogen) was used due to its capacity of selectively entering mitochondria. In the presence of healthy cells, JC-1 aggregates and emits red and green fluorescence, due to the high Δψm of the cells. When the cells loose mitochondria integrity, Δψm is low and JC-1 does not aggregate, emitting exclusively green fluorescence [26]. JC-1 was dissolved with DMSO (Sigma) at a final concentration of 2.5 mg/mL. The JC-1 stock solution was then diluted with PBS at 2.5 μg/mL and centrifuged at 700 g for 7 min to remove JC-1 aggregates and obtain a clarified supernatant, which became the working solution. Cell suspension (0.5–1.0×10^6 cells) was washed with PBS and incubated with 500 μL of JC-1 working solution for 20 min at room temperature in the dark. After incubation, cells were washed, suspended in 300 μL of PBS, and analyzed by flow cytometry.

Measurement of cell metabolic activity (WST-1)

The reagent WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedi sulfonate, Roche), a tetrazolium salt, was used to measure metabolic activity in the cells [27,28]. Active dehydrogenases in healthy cells cleave WST-1 to formazan dye, and hence the level of enzyme activity is related to the amount of formazan dye formed, which in turn correlates with the number of metabolically active cells in the culture. Therefore, the cytotoxic effect of the drug is proportional to the decrease in the amount of formazan dye, compared with a control consisting of the same cells without the drug. To perform this test 50 μL of cell suspension were
transferred to a 96-well plate, then 50 μL of fresh medium and 10 μL per well of WST-1 were added. After incubation for 4 h, the amount of formazan was quantified by a spectrophotometer (SpectraCount, Packard Bioscience Company) measuring the absorbance (abs) of the dye at 450 nm. Results were expressed as percentage of viability reduction according the following formula: 100 × [(abs untreated cells − abs cells exposed to drugs)/abs untreated cells]−1.

### Statistical analysis

The cytotoxic effect of bendamustine and cytarabine as single drugs was compared, in terms of percentage of affected cells (minimum 0%, maximum 100%), with the simultaneous and consecutive incubations, respectively. The t-student test was used to compare results, which were considered to be statistically significant when P<0.05.

The presence of a synergistic or antagonistic effect of bendamustine and cytarabine when tested in different combinations was analyzed with the Calcusyn computer software (Biosoft, Cambridge, UK) [29]. This software allowed determination of the correlations of single agent dose–response curves with the equivalent curves of the two drugs in combination through the combination index (CI). The CI is the quantitative measure of the interactions among drugs. A CI value <0.1 reflects a 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 on both the cell lines. The software allowed also to easily visualize the correlations between drug doses and their effect on cell mortality in a graph called median/endpoint plot, following a linear equation: Log (fa/fu) = m log (D) − m log (Dm). Fa represents the fraction of cells affected by the dose (D), fu is the fraction unaffected (1-fa), m the slope of the curve or potency of the drug, and Dm the dose of the drug affecting 50% of the cells (fa = fu).

The experiments were repeated for a minimum of four times in duplicate, and the mean value was taken.

### Results

**Effect of bendamustine and cytarabine as single drugs**

The effect of bendamustine and cytarabine as single drugs was measured in terms of apoptosis induction (7-AAD), mitochondrial damage (JC-1), and metabolic activity (WST-1), and visualized by means of morphological analysis. With the 7-AAD test bendamustine presented a linear dose-dependent cytotoxic effect on both the cell lines, which is shown in Fig. 1, as obtained with the Calcusyn software. The graph represents a median-effect plot that correlates the drug dose with its effect through a logarithmic equation. The cytotoxic effect of the drug was prominent when incubated for 48 h at the highest concentrations, with the JEKO-1 cell line showing a more rapid and not time-dependent decrease of WST-1. Similarly, cell cycle analysis revealed that both drugs induced an accumulation of cells in S phase after 24 h of incubation with an increase of the debris peak, indicating DNA fragments of apoptotic bodies (Fig. 2). These effects were dose- but not time-dependent on the two cell lines. Bendamustine induced the typical alterations of cell morphology with nuclear fragmentation that occurs during apoptosis, as shown in Fig. 3.

**Simultaneous incubation schedules**

The simultaneous incubation of bendamustine and cytarabine at any of the drug doses, for 24 h or 48 h, resulted in a constantly higher cytotoxic activity than that induced by each of the two drugs alone, that was particularly relevant with JEKO-1 cell line, but much less with GRANTA-519. With the 7-AAD test, the CI of both simultaneous schedules varied between 0.96 and 1.0 × 10⁻⁴ (from slight synergistic to very strong synergistic effect), depending on the drug doses and on the cell line (Table 1). In particular, the lowest CI (1.0 × 10⁻⁴), indicating the strongest synergy, was observed on JEKO-1 cells with the 24-hor incubation. Accordingly, in terms of percentage of apoptotic cells, the simultaneous incubations on JEKO-1 cells were clearly more effective than the two drugs alone both after 24 and 48 h (Fig. 4a). In contrast, the synergistic effect of the simultaneous incubation of the two drugs was less prominent with GRANTA-519 cells, with CI varying between 0.15 and 0.96 (from strong synergism to nearly additive), as shown in Table 1. Similar results were obtained in terms of percentage of apoptotic cells, where the cytotoxic effect of the simultaneous combinations on GRANTA-519 cells appeared weak (Fig. 4b).

Similarly, the simultaneous schedule induced a significant higher decrease in Δψ with the JEKO-1 cell line than the two drugs alone, but, again, this synergistic effect was less impressive on GRANTA-519. The simultaneous incubations were strongly to very strongly synergistic on JEKO-1 cells (CI from 0.009 to 0.04), but resulted less synergistic on GRANTA-519 cells (CI from 0.19 to 1.1). In terms of cellular metabolic activity, a decrease of enzymatic activity was observed both on JEKO-1 and GRANTA-519 cell lines, that was...
Consecutive incubation schedules (A + B and B + A)

Cytarabine and bendamustine were incubated consecutively (A + B and B + A) with the two cell lines. Results were compared with the data obtained with the single drug incubations, and with the simultaneous schedules. A significant increase in the proportion of apoptotic cells was observed with the consecutive schedules at any drug concentration compared to the drugs alone on both cell lines, as shown in Table 1. Similar effects were observed with GRANTA-519 cells, with CI ranging between 0.14 and $6 \times 10^{-5}$, although not proportionally to dose escalation. Overall, after normalizing for drug dose and cell line, the synergy of the consecutive schedules was 10 to 100 times greater (with CI 10 to 100 times lower) than the values observed with the simultaneous schedules. The more pronounced synergy was detected on JEKO-1 cells after incubation of bendamustine followed by cytarabine (A + B scheme), with CI ranging between $1.5 \times 10^{-5}$ and $8.4 \times 10^{-5}$.

The consecutive incubation of the drugs on JEKO-1 cells also induced marked mitochondrial disruption, which was three-fold greater than that induced by bendamustine alone, and two-folder greater compared with cytarabine alone in terms of percentage of disrupted cells. This synergism was confirmed by CI values that were significantly higher than the results obtained with the two drugs alone at any of the drug doses.

Table 1: Combination index (CI) values obtained by Calcusyn analysis in all investigated combinations of the two drugs in JEKO-1 and GRANTA-519 cell lines, according to the 7-AAD test.

<table>
<thead>
<tr>
<th>Drug doses (µg/mL)</th>
<th>Mix 24 h</th>
<th>Mix 48 h</th>
<th>B + A</th>
<th>A + B</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEKO-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 + 0.25</td>
<td>$2.2 \times 10^{-1}$</td>
<td>$6.5 \times 10^{-1}$</td>
<td>$1.5 \times 10^{-2}$</td>
<td>$8.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>10 + 0.5</td>
<td>$4.0 \times 10^{-2}$</td>
<td>$7.3 \times 10^{-2}$</td>
<td>$3.0 \times 10^{-3}$</td>
<td>$2.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>20 + 1</td>
<td>$5.0 \times 10^{-3}$</td>
<td>$7.0 \times 10^{-3}$</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$2.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>40 + 2</td>
<td>$1.0 \times 10^{-4}$</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$1.0 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>GRANTA-519</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 + 0.25</td>
<td>$9.1 \times 10^{-1}$</td>
<td>$7.1 \times 10^{-1}$</td>
<td>$8.0 \times 10^{-3}$</td>
<td>$1.7 \times 10^{-2}$</td>
</tr>
<tr>
<td>50 + 0.5</td>
<td>$1.5 \times 10^{-1}$</td>
<td>$1.7 \times 10^{-1}$</td>
<td>$1.0 \times 10^{-2}$</td>
<td>$2.0 \times 10^{-2}$</td>
</tr>
<tr>
<td>100 + 1</td>
<td>$1.7 \times 10^{-1}$</td>
<td>$2.6 \times 10^{-1}$</td>
<td>$6.0 \times 10^{-3}$</td>
<td>$1.4 \times 10^{-1}$</td>
</tr>
<tr>
<td>250 + 2</td>
<td>$9.1 \times 10^{-1}$</td>
<td>$1.0 \times 10^{-1}$</td>
<td>$3.5 \times 10^{-1}$</td>
<td>$1.0 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

CI ≥ 0.1 = very strong synergism; CI 0.1–0.9 = strong to slight synergism; CI 0.9–1.1 = nearly additive; CI = 1.1 = antagonism. Mix 24 h and mix 48 h correspond to the simultaneous incubations for 24 and 48 h respectively. B + A and A + B correspond to the consecutive schedules. B = bendamustine; A = cytarabine. The reported CI value was the result of four repeats for each experiment.
In this study, we found that both bendamustine and cytarabine produced potent cytotoxic effects when incubated with MCL cell lines. While these effects were apparent when each drug was incubated separately with the cell lines, the effects became more pronounced and significantly higher when the drugs were incubated simultaneously or consecutively. The highest cytotoxic activity was achieved when the drugs were incubated consecutively: this resulted in the strongest synergistic effect for inducing apoptosis, which was 10 to 100-fold greater than the maximum effect obtained with the simultaneous incubation of the two drugs together. Moreover, differently from the simultaneous schedule, the consecutive incubation of the drugs induced a remarkable cytotoxic effect also on the blastoid cell variant of MCL (GRANTA-519). After normalizing for drug dose and cell line, the best incubation schedule in inducing apoptosis of both MCL cell lines consisted of bendamustine followed by cytarabine (B+A). Similar findings were observed in terms of mitochondrial damage and decline of metabolic activity on both cell lines. This is the first study to demonstrate such a synergistic effect for bendamustine and cytarabine in lymphoma cell lines, and indicates that the combination of bendamustine and cytarabine might be promising as a treatment for patients with MCL.

The clinical course of MCL is sometimes indolent or moderately aggressive when first diagnosed, but the disease becomes more clinically aggressive and chemotherapy refractory with time, and has the worst long-term survival among the B-cell lymphoma subtypes [30]. Such an aggressive clinical course would benefit of an aggressive treatment approach. However, given the advanced median age at diagnosis of patients with MCL, and epidemiological data showing that approximately 80% of patients with cancer aged >65 years have two
or more co-morbidities [31], a large and increasing proportion of patients with MCL are not able to tolerate intensive treatments [5–7]. Several new and less toxic therapies are showing encouraging single-agent activity in relapsed or resistant MCL. These include (with reported response rates) bortezomib (33%) [32], lenalidomide (41–53%) [33,34], and temsirolimus (22–41%) [35–37]. The combination of rituximab and bendamustine has recently been investigated in two prospective clinical trials including cohorts of patients with MCL [38,39]. In both studies the combination of the two drugs has been shown to produce impressive response rates (75–92%) and relevant disease-free intervals, with manageable toxicity profile.

Although its precise mode of action has yet to be fully elucidated, bendamustine displays a distinct pattern of cytotoxicity, compared with other alkylating agents [40]. Some in-vitro studies on lymphoma cells have addressed the effect of bendamustine as monotherapy and in combination with other agents, including rituximab [41], purine analogs [42], and anthracyclines [42]. The monoclonal antibody rituximab has been reported to sensitize lymphoma cells to the effect of bendamustine, enhancing the cytotoxic effect of the drug [41]. Chow et al. [42] have investigated the in-vitro activity of bendamustine in combination with other established cytotoxic drugs, including anthracyclines and purine analogs (2-cda and fludarabine), using two follicular lymphoma cell lines (DOHH-2, WSU-NHL) and mononuclear cells (MNC) from patients with leukaemic low-grade B-cell NHL, T-NHL and CLL. Similar tests of our study were used to quantify the synergy between the different drugs. The authors found that the combination of bendamustine with anthracyclines resulted in antagonistic effects, while a remarkable synergistic in-vitro activity was observed for the combination of bendamustine and 2-cda [42], or fludarabine [43]. Similarly, a study on leukemic blasts of AML reported observed for the combination of bendamustine and 2-cda [42], or anthracyclines and purine analogs (2-cda and fludarabine), using two follicular lymphoma cell lines (DOHH-2, WSU-NHL) and mononuclear cells from patients with leukaemic low-grade B-cell NHL, T-NHL and CLL. Similar tests of our study were used to quantify the synergy between the different drugs. The authors found that the combination of bendamustine with anthracyclines resulted in antagonistic effects, while a remarkable synergistic in-vitro activity was observed for the combination of bendamustine and 2-cda [42], or fludarabine [43]. Similarly, a study on leukemic blasts of AML reported observed for the combination of bendamustine and 2-cda [42], or anthracyclines and purine analogs (2-cda and fludarabine), using two follicular lymphoma cell lines (DOHH-2, WSU-NHL) and mononuclear cells from patients with leukaemic low-grade B-cell NHL, T-NHL and CLL. Similar tests of our study were used to quantify the synergy between the different drugs. The authors found that the combination of bendamustine with anthracyclines resulted in antagonistic effects, while a remarkable synergistic in-vitro activity was observed for the combination of bendamustine and 2-cda [42], or fludarabine [43]. Similarly, a study on leukemic blasts of AML reported observed for the combination of bendamustine and 2-cda [42], or anthracyclines and purine analogs (2-cda and fludarabine), using two follicular lymphoma cell lines (DOHH-2, WSU-NHL) and mononuclear cells from patients with leukaemic low-grade B-cell NHL, T-NHL and CLL. Similar tests of our study were used to quantify the synergy between the different drugs. The authors found that the combination of bendamustine with anthracyclines resulted in antagonistic effects, while a remarkable synergistic in-vitro activity was observed for the combination of bendamustine and 2-cda [42], or fludarabine [43].

The blastic variant of MCL (GRANTA-519), which is associated with several molecular alterations involving cell cycle regulators and is usually more resistant to chemotherapy, was less influenced by equivalent doses of bendamustine and cytarabine compared to JEKO-1 cells in our study, but was significantly affected by the consecutive administration of the two drugs (see Table 1 and Fig. 5b). These schedules demonstrated a significant impact both on the fraction of apoptotic cells and on the loss of mitochondrial membrane potential, confirming the importance of consecutive schedules of the two drugs [20,21,41,45]. These schedules would allow a greater cytotoxicity based on the individual mechanism of action of the two drugs, avoiding the saturation of common pathways. The two agents, that we

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Drug scheme</th>
<th>Cell sources</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staub [21]</td>
<td>1999</td>
<td>B + cytarabine</td>
<td>PBMCN of patients with AML</td>
<td>B potentiates the effects of cytarabine augmenting intracellular levels of ara-CTP, to a lower extent than 2-cda, but more than fludarabine and gemcitabine.</td>
</tr>
<tr>
<td>Chow [42]</td>
<td>2001</td>
<td>B + mitoxantrone, B + doxorubicin, B + 2-cda</td>
<td>PBMCN of patients with CLL, B- and T-cell lymphoma, follicular lymphoma cell lines (DOHH-2 and WSU-NHL)</td>
<td>B + mitoxantrone and B + doxorubicin always antagonists; B + 2-cda synergistic in all cell sources.</td>
</tr>
<tr>
<td>Chow [41]</td>
<td>2002</td>
<td>B + rituximab</td>
<td>PBMCN of patients with CLL and B-cell lymphoma, follicular lymphoma cell lines (DOHH-2 and WSU-NHL), Burkitt’s lymphoma cell line (RAJI)</td>
<td>Rituximab sensitizes lymphoma cells to the effect of B in all cell sources.</td>
</tr>
<tr>
<td>Schwanen [43]</td>
<td>2002</td>
<td>B + fludarabine</td>
<td>PBMCN of patients with CLL</td>
<td>B + fludarabine is a highly synergistic combination.</td>
</tr>
<tr>
<td>Chow [20]</td>
<td>2003</td>
<td>B + cytarabine</td>
<td>AML cell lines (HL60 and HEL)</td>
<td>B + cytarabine have additive to antagonistic effect for synergistic and consecutive combinations.</td>
</tr>
<tr>
<td>Roué [49]</td>
<td>2008</td>
<td>B + fludarabine, B + gemcitabine</td>
<td>PBMCN of patients with MCL and CLL</td>
<td>B + fludarabine are synergistic on CLL cells irrespectively of their response to fludarabine alone; B + gemcitabine are synergistic on MCL cells; Cells with p53 alterations from both sources show the highest response to the drug combinations.</td>
</tr>
</tbody>
</table>

B = Bendamustine; PBMCN = Peripheral blood mononuclear cells; AML = Acute myeloid leukemia; ara-CTP = cytarabine triphosphate; 2-cda = cladribine; CLL = Chronic lymphocytic leukemia.
have shown to induce a block of the cell cycle in the S phase (Fig. 2), may both cause cross-links and DNA strand breaks, avoiding further DNA synthesis. Cells that escaped the cell cycle arrest or repaired the DNA damage after bendamustine action, would then be subject to the effect of higher amounts of ara-CTP. In case of reversal order of the two drugs, cells blocked in the S phase would instead initially incorporate ara-CTP into DNA facilitating the subsequent damage related to the exposure to bendamustine. This mechanism would parallel what observed for fludarabine in acute myeloid leukemia [19], where cytarabine is commonly administered following this drug with great clinical efficacy, especially in the relapsed setting (FLAG regimen). Specific studies are needed to address or confirm these theories. Other in-vitro studies show that bendamustine is a potent activator of p53 induced apoptosis [40], induces cell apoptosis with short exposure time [47], causes apoptosis-resistant cells to undergo mitotic catastrophe [48], and is active in cells resistant to other agents [40]. Although bendamustine activates p53 signalling, it is also cytotoxic in p53-deficient cell lines, indicating that this pathway can be bypassed by its mechanism of action. In a recent study by Roué et al. [49] on 13 CLL and MCL cell lines and primary tumor cells from patients with MCL and CLL cultured for up to 24 h with bendamustine, the cytotoxic activity of the drug was independent of p53 gene status. These findings confirm that the mechanism of action of bendamustine is more complicated than previously thought, and might give a rationale for the use of the drug in different lymphoid malignancies, alone or in association. Further studies are required to determine the mechanisms for the synergic effects of bendamustine and cytarabine in vitro. However, the data from our study suggest that the combination of these two agents is highly synergistic, both in the classic and the blastoid variant of the tumor, opening up the prospect of new and promising regimens for the treatment of patients with MCL. Drug combinations that have the potential advantage of treating older patients with comorbidities, who are not otherwise able to tolerate aggressive therapy, are warranted in the everyday practice. Given the positive results obtained with the combination of bendamustine and rituximab in MCL in vitro and in vivo, rituximab might represent another preferred candidate for the association, also due to its favorable toxicity profile. We are now conducting a phase 2 trial to test the clinical activity of the combination of rituximab, bendamustine and cytarabine (R-BAC study) in older patients with MCL. (ClinicalTrials.gov NCT00992134) [50]. Future studies should explore the combination of these agents, since they all exhibit individual and unique mechanisms of action in MCL, and a synergistic or additive effect might be expected when they are used in combination.

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