Bendamustine Is Effective in p53-Deficient B-Cell Neoplasms and Requires Oxidative Stress and Caspase-Independent Signaling

Gaël Roué,1 Mónica López-Guerra,1 Pierre Milpied,1 Patricia Pérez-Galán,1 Neus Villamor,1 Emili Montserrat,2 Elias Campo,1 and Dolors Colomer1

Abstract

Purpose: Chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL) are two incurable B-cell lymphoid neoplasms characterized by distinct clinical presentation and evolution. Bendamustine hydrochloride is a multifunctional, alkylating agent with a purine-like ring system that exhibits activity in multiple cancer models, including CLL and MCL, but whose mechanism is only partially described. Our aim was to analyze the apoptotic pathways activated by bendamustine in CLL and MCL together with the relevance of p53 mutation in determining the response to this drug.

Experimental Design: Thirteen CLL/MCL cell lines and primary tumor cells from 8 MCL and 25 CLL patients were cultured for up to 24 h with bendamustine followed by cytotoxic assays, flow cytometry, immunofluorescence, and Western blot analysis of p53 response pathway and apoptosis-related factors.

Results: Bendamustine displayed cytotoxic activity on most CLL and MCL primary cells and cell lines irrespective of ZAP-70 expression and p53 status. Bendamustine was found to act synergistically with nucleoside analogues in both CLL and MCL, this combination being effective in p53 mutated cases resistant to standard chemotherapy. Bendamustine cytotoxicity was mediated by the generation of reactive oxygen species and triggering of the intrinsic apoptotic pathway involving up-regulation of PUMA and NOXA, conformational activation of BAX and BAK, and cytosolic release of caspase-related and caspase-unrelated mitochondrial apoptogenic proteins.

Conclusions: Our findings support the use of bendamustine as a therapeutic agent, alone or in combination, for CLL and MCL with p53 alterations and describe the molecular basis of its activity in these entities.

Chronic lymphocytic leukemia (CLL) is the most common leukemia in western countries and is characterized by the accumulation of CD5+ monoclonal B cells (1). Prognostic markers include the mutational status of the immunoglobulin heavy chain genes, the expression of the ζ-chain-associated protein 70 kDa (ZAP-70) and the CD38 antigen as well as certain cytogenetic abnormalities (17p, 11q, and 13q deletions; ref. 2). Mantle cell lymphoma (MCL) represents about 5% to 10% of all B-cell lymphomas and is characterized by the expansion of mature B clonal lymphocytes harboring the t(11;14)(q13;q32) translocation, which induces overexpression of cyclin D1 and cell cycle deregulation. The prognosis for this entity is in most cases extremely poor, with a median survival of 3 to 4 years (3). Current treatments include anti-neoplastic agents, molecular targeted therapy, hematopoietic stem cell transplantation, radiotherapy, and/or a combination of these approaches (4). However, all these strategies are rapidly confronted with the onset of resistance.

Most cancer therapies work by activating the apoptosis program of tumor cells. The intrinsic apoptotic pathway is activated on the permeabilization of the outer mitochondrial membrane, which leads to the cytosolic release of mitochondrial apoptogenic proteins including cytochrome c and Smac/DIABLO, conducting to the activation of the caspase cascade and consequent oligonucleosomal DNA fragmentation. Alternatively, some forms of cell death involving mitochondria but independent of caspase activation have also been described, such as that involving the mitochondrial apoptosis-inducing factor (AIF), which translocates into the nucleus where it is participates in a partial, large-scale, DNA fragmentation and peripheral chromatin condensation (5). Regulation of mitochondrial integrity is controlled by the balance and interaction among members of the BCL-2 family of proteins. The BH3-only members (BIM, PUMA, NOXA, BAD, BID,
Translational Relevance

Bendamustine hydrochloride is a multifunctional cytotoxic agent, which has been used for years in the clinical treatment of B lymphoid neoplasms and solid tumors, but whose mechanism of action has been principally linked to p53 stabilization. In this study, we describe the mechanisms underlying its antitumoral activity in CLL and MCL, two entities characterized by frequent alterations of the p53 gene and rapid onset of resistance to current therapeutic strategies (antineoplastic agents, molecular targeted therapy, hematopoietic stem cell transplantation, radiotherapy, and/or a combination of these approaches). Using a series of CLL/MCL primary samples and cell lines, we observe that bendamustine exerts a cytotoxic effect and synergizes with nucleoside analogue therapy. We show that bendamustine fully activates the mitochondrial apoptotic machinery independently of p53 status, underlying its activity in malignant B cells resistant to common chemotherapy. Thus, our findings support the use of bendamustine as a therapeutic agent, alone or in combination, for CLL and MCL with p53 alterations and describe the molecular basis of its activity in these entities.

BIK, BMF, and HRK function as sensors of cellular well-being and, when activated by cytotoxic signals, engage the prosurvival mitochondria, BCL-2 family members, allowing BAX and BAK to release from antiapoptotic members, followed by their oligomerization and the consequent permeabilization of the mitochondrial outer membrane (6).

The efficacy of the therapies used for the treatment of lymphoid neoplasms is often limited to cases with functional p53, a tumor suppressor gene frequently mutated and/or deleted in these entities (7). Bendamustine is an antineoplastic drug designed to combine the properties of a purine analogue and an alkylating agent. It contains a bifunctional alkylating nitrogen mustard group and a benzimidazole nucleus. Its alkylating toxicity is based on the induction of single-stranded and double-stranded DNA breaks, although its lack of cross-resistance with other alkylating agents, especially linked to p53 stabilization. In this study, we describe previously (16). Cells were cultured at a density of 0.3 × 10^6 to 0.5 × 10^6/mL in RPMI 1640 supplemented with 10% FCS, 10 mmol/L glutamine, 50 μg/mL penicillin-streptomycin, and 100 μg/mL normocin (Amaxa), except for MEC-1 and Granta-519 cells, which were cultured in IMDM and DMEM (Life Technologies), respectively. For the Jeko-1 cell line, an additional 10% FCS was added to the culture medium. All cell lines were cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Treatments and cytofluorimetric assessment of apoptosis. Cells were incubated with different concentrations of bendamustine hydrochloride (kindly provided by Cephalon) and/or fludarabine (Schering) or gemcitabine (Lilly) for the times specified. When indicated, cells were preincubated for 1 h with the pan-caspase inhibitor benzoyl-x-carbonyl-Val-Ala-Asp-fluoro-methylketone (z-VAD.fmk; Bachem) at 50 to 100 μmol/L or with the antioxidant gluthathione-reduced ethyl ester (GSH; Sigma) at 2 mmol/L. Phosphatidylserine exposure was quantified by double staining with Annexin V conjugated to FITC and propidium iodide (Bender Medsystems), considering both Annexin V-positive/propidium iodide-negative and Annexin V-positive/PI-positive populations as apoptosis committed/dead cells. Changes in mitochondrial membrane potential and reactive oxygen species (ROS) production were evaluated by staining cells simultaneously with 20 nmol/L 3,3'-diexyloxacarbocyanine iodide (Molecular Probes) and 2 μmol/L dihydroethidium (Molecular Probes), respectively. BAX and BAX conformational changes and caspase-3 activation were determined by immunocytometry as described previously (17). Labeled cells were analyzed on a FACScan flow cyrometer (Becton Dickinson) using CellQuest and Paint-A-Gate software (Becton Dickinson). LD₅₀ was defined as the concentration of drug required to reduce cell viability by 50%. Combination index values were calculated in cells exposed to varying concentrations of both agent considered by using the Calcosyn software v2.0 (Biosoft).

Subcellular fractionation and Western blotting. Thirty million cells were washed once in PBS and cytosolic and mitochondrial fractions were obtained by incubating for 10 min with 800 μL ice-cold subcellular fractionation buffer mix complemented with 1 μmol/L DTT, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate according to the manufacturer’s recommendations (Takara Bio Europe/Clontech). Total protein extraction and Western blot analysis were done as described previously (18). Membranes were probed with the following primary antibodies: anti-p53 (Calbiochem); anti-NOXA and anti-Smac/ DIABLO (Alexis Biochemicals); anti-PUMA and anti-COX IV (Abcam); anti-β-actin, anti-α-tubulin, and anti-AIF (Sigma); and anti-cytochrome c (Becton Dickinson). Then, antibody binding was detected using horseradish peroxidase-labeled anti-mouse (Sigma), anti-rabbit (Sigma), or anti-rat (DAKO) antibodies and the enhanced chemiluminescence detection system (Amersham) coupled to a LAS3000 Fuji film device. Equal protein loading was confirmed with β-actin or α-tubulin detection.

4,6-Diamidino-2-phenylindole staining. Cells (5 × 10⁶) were washed once in PBS and fixed on poly-L-lysine-coated glass coverslips with 4% paraformaldehyde for 15 min at room temperature. Cells were then washed once in PBS and stained with 1 μg/mL 4,6-diamidino-2-phenylindole (Calbiochem). Coverslips were mounted on glass slides from each patient in accordance with the Ethics Committee of the Hospital Clinic. The characteristics of these patients are listed in Table 1. Peripheral blood mononuclear cells from CLL/MCL patients were obtained by Ficoll/Hypaque sedimentation (Seromed). In one case (MCL patient 7), tumor cells were obtained from splenectomy material after squiring with RPMI 1640 (Life Technologies) using a fine needle. Cells were cryopreserved in liquid nitrogen in the presence of 10% DMSO and 60% heat-inactivated FCS (Life Technologies). After thawing, mononuclear cells were cultured in X-vivo 10 medium (Cambrex Biosciences) at a density of 1 × 10⁶ to 2 × 10⁶/mL at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Materials and Methods

Isolation and culture of primary cells. Cells from 25 patients diagnosed of CLL and 8 diagnosed of MCL according to the WHO classification criteria were studied. An informed consent was obtained...
using Vectashield-Hard Set medium (Vector Laboratories) and preparations were viewed under an Olympus DP70 fluorescence microscope (Olympus) using DPManager software v2.1.1 (Olympus).

Intracellular antibody delivery.

Cells (1 × 10⁶) were transfected with an anti-AIF polyclonal antibody (Sigma; final dilution 1:2,500) complexed with 0.5 µL Chariot reagent (Active Motif) according to the manufacturer’s recommendations. Cells were then treated for 24 h with bendamustine and dead cells were quantified by flow cytometry analysis of propidium iodide incorporation.

Results

Bendamustine is cytotoxic and exhibits a synergistic effect with nucleoside analogues independently of p53 status in both CLL and MCL. Primary tumor cells from 25 CLL and 8 MCL patients and a panel of 12 cell lines (2 derived from CLL and 10 from MCL cells) were incubated with bendamustine doses ranging from 2 to 100 µmol/L, and cell viability were evaluated after 24 h by Annexin V staining. The LD₅₀ values of bendamustine in CLL and MCL primary cultures are listed in Table 1. Most of CLL/MCL samples (25 of 33; 75.7%) showed a LD₅₀ inferior to 100 µmol/L. Although the percentage of cases harboring p53 alterations was low, reflecting their occurrence in these malignancies, we observed that CLL/MCL sensitivity to bendamustine was not correlated to p53 status either in primary cultures (Fig. 1A, left) or in cell lines (Fig. 1A, right). Additionally, no significant correlation was found between sensitivity to bendamustine and ZAP-70 expression in CLL samples. Of note, primary cells purified from patients refractory to previous fludarabine- and alkylator-based therapy (CLL 1, 4, 5, and 25) or alkylator-based polychemotherapy (MCL 3 and 6) showed similar LD₅₀ to bendamustine than cells from untreated patients (Table 1).

Because bendamustine has been shown previously to act synergistically with the nucleoside analogue fludarabine in CLL

<table>
<thead>
<tr>
<th>Table 1. Characteristics of CLL and MCL patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>CLL 1</td>
</tr>
<tr>
<td>CLL 2</td>
</tr>
<tr>
<td>CLL 3</td>
</tr>
<tr>
<td>CLL 4</td>
</tr>
<tr>
<td>CLL 5</td>
</tr>
<tr>
<td>CLL 6</td>
</tr>
<tr>
<td>CLL 7</td>
</tr>
<tr>
<td>CLL 8</td>
</tr>
<tr>
<td>CLL 9</td>
</tr>
<tr>
<td>CLL 10</td>
</tr>
<tr>
<td>CLL 11</td>
</tr>
<tr>
<td>CLL 12</td>
</tr>
<tr>
<td>CLL 13</td>
</tr>
<tr>
<td>CLL 14</td>
</tr>
<tr>
<td>CLL 15</td>
</tr>
<tr>
<td>CLL 16</td>
</tr>
<tr>
<td>CLL 17</td>
</tr>
<tr>
<td>CLL 18</td>
</tr>
<tr>
<td>CLL 19</td>
</tr>
<tr>
<td>CLL 20</td>
</tr>
<tr>
<td>CLL 21</td>
</tr>
<tr>
<td>CLL 22</td>
</tr>
<tr>
<td>CLL 23</td>
</tr>
<tr>
<td>CLL 24</td>
</tr>
<tr>
<td>CLL 25</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female. ND, not determined.
† 17p13 deletion assessed by fluorescence in situ hybridization and p53 mutational status analyzed by direct sequencing.
‡ ZAP-70 status and % tumor cells were quantified by standard flow cytometry labeling of CD5+/CD19+ cells and light chain restriction.
§ Patients treated previously with FCM (CLL 1 and 25), CHOP (MCL 3), fludarabine + CHOP (CLL 4), FCM + CHOP (CLL 5), and CHOP + HyperCVAD (MCL 6).
ⅠBlastoid morphologic variant.
cells (19), we were interested in determining whether nucleoside analogue therapy was also capable of synergistic interaction with bendamustine in MCL cells. Because high doses of fludarabine are required to induce a cytotoxic effect in MCL cells (20), we compared the activity of bendamustine and the pyrimidine analogue gemcitabine (21). CLL and MCL primary samples were cultured for 48 h with 1 μg/mL (7.5 μmol/L) fludarabine or 20 μg/mL (66.7 μmol/L) gemcitabine in combination with a subtoxic dose of bendamustine (10 μmol/L; 3.6 μg/mL). As expected, bendamustine and fludarabine were found to exert a synergistic effect on CLL primary samples (combination index < 1), and this effect was irrespective of their initial response to fludarabine (Fig. 1B). Interestingly, this combination was highly synergistic (combination index = 0.42) in cells from a representative patient harboring p53 alterations, which showed no sensitivity to fludarabine or bendamustine alone (CLL 3). Similarly, the combination of bendamustine with gemcitabine also exerted a synergistic effect (combination index < 1) in MCL primary cultures (Fig. 1C). Again, cells from patients with nonfunctional p53 (MCL 3 and 8) showed the highest response to the combination versus each drug alone. In contrast, the differences between combination index values could not discriminate between samples from untreated (CLL 3, 6, and 10 and MCL 8) and chemotherapy-treated (MCL 3 and 6) patients. These results confirmed the synergistic interaction of bendamustine with chemotherapeutics currently used in the clinical practice.

**Bendamustine activates the mitochondrial cell death pathway in CLL and MCL cells with no functional p53.** To elucidate the role of p53 signaling pathway during bendamustine-induced apoptosis, Western blot analysis of p53 and its downstream target genes PUMA and NOXA was assessed. Figure 2A shows that bendamustine induced p53 stabilization and consequent up-regulation of PUMA and NOXA in cases harboring p53 wild-type (WT) gene (CLL 17, MCL 7, and EHEB and Z-138 cell lines). In contrast, in samples with mutated and basally stabilized p53 protein, NOXA accumulation was predominant over PUMA (CLL 25 and MCL 8 and HBL-2 cell line). Expression of PUMA could not be detected in HBL-2 cells and was found unchanged or even decreased in the other nonfunctional p53 cases when high cytotoxicity was reached (Fig. 2A). Flow cytometric analysis of mitochondrial apoptosis hallmarks showed that bendamustine induced activation of both BAX and BAK, depolarization of the mitochondria, processing of caspase-3, and generation of ROS in cells with p53 WT as well as in those with nonfunctional p53 (Fig. 2B). These results suggest that the ability of bendamustine to simultaneously induce p53-related activation of PUMA and
p53-independent up-regulation of NOXA may ensure the activation of mitochondrial apoptotic pathway in CLL and MCL cells regardless of p53 status.  

**Bendamustine-induced cell death involves both caspase-dependent and caspase-independent signaling and depends on ROS generation.** To further investigate the apoptosis executors involved in bendamustine signaling downstream mitochondrial depolarization, we checked the release of the apoptogenic factors cytochrome c and AIF from mitochondria to the cytosol using the same CLL/MCL cell lines as above. We observed that, in untreated EHEB, Z-138, and HBL-2 cells, cytochrome c and AIF were exclusively located in the mitochondria compartment, whereas a shift in these apoptogenic factors to the cytosolic fraction was observed after exposure to bendamustine (Fig. 3A, left). This phenomenon was correlated with the presence of apoptotic bodies typical of oligonucleosomal, caspase-mediated DNA fragmentation, in addition to nuclei with peripheral and partially condensed chromatin, typical of apoptosis-like, AIF-mediated cell death (Fig. 3A, right). Similarly, a shift from the mitochondrial punctuated staining of these factors observed in untreated CLL/MCL primary cells to a diffused cytosolic/nuclear pattern in bendamustine-treated cells could be observed by immunofluorescence labeling (Supplementary Fig. S1).

To assess whether such AIF activity was responsible for this particular apoptotic phenotype, cell lines were transfected with an anti-AIF polyclonal antibody before incubation for 24 h with bendamustine to neutralize the cytosolic form of AIF and inhibit its translocation into the nucleus. Anti-AIF-transfected cells presented a 30% to 40% reduction in cell death after bendamustine treatment when compared with control transfected cells (Fig. 3B). Immunofluorescence staining of DNA and AIF in the representative cell line HBL-2 confirmed that the protein was restricted to the cytosolic compartment in most of the cells containing the anti-AIF antibody (Supplementary Fig. S2). These results suggest that bendamustine-induced cell death may involve both apoptotic and apoptotic-like mitochondrial effectors in CLL and MCL.

**Fig. 2.** Bendamustine triggers p53-dependent and p53-independent activation of the mitochondrial apoptotic pathway in CLL and MCL. A, CLL/MCL samples with WT or mutated p53 status were incubated for 24 h with 100 μmol/L bendamustine, except for Z-138 (10 μmol/L), EHEB (50 μmol/L), and HBL-2 (50 μmol/L), and subjected to Western blot analysis of p53, PUMA, and NOXA expression. B, flow cytometry analysis of mitochondrial membrane potential (ΔΨm loss), conformational changes of BAX (BAX activation) and BAK (BAK activation), caspase-3 activation (CASP-3 activation), and ROS production in CLL/MCL cells treated as above, except for EHEB (100 μmol/L bendamustine). The indicated values correspond to the percentage of cells positive for each variable analyzed. Representative results from three independent experiments.

### Table 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>p53 Status</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHEB</td>
<td>wt</td>
<td>65</td>
</tr>
<tr>
<td>Z138</td>
<td>mut</td>
<td>30</td>
</tr>
<tr>
<td>HBL2</td>
<td>mut</td>
<td>34</td>
</tr>
<tr>
<td>CLL</td>
<td>no.17</td>
<td>72</td>
</tr>
<tr>
<td>MCL</td>
<td>no.17</td>
<td>23</td>
</tr>
<tr>
<td>CLL</td>
<td>no.7</td>
<td>92</td>
</tr>
<tr>
<td>MCL</td>
<td>no.7</td>
<td>53</td>
</tr>
<tr>
<td>CLL</td>
<td>no.25</td>
<td>93</td>
</tr>
<tr>
<td>MCL</td>
<td>no.25</td>
<td>61</td>
</tr>
<tr>
<td>CLL</td>
<td>no.8</td>
<td>42</td>
</tr>
<tr>
<td>MCL</td>
<td>no.8</td>
<td>79</td>
</tr>
</tbody>
</table>

ΔΨm loss, BAX activation, BAK activation, CASP-3 activation, and ROS level were measured by flow cytometry analysis. The indicated values correspond to the percentage of cells positive for each variable analyzed. Representative results from three independent experiments.

**Fig. 2.** Bendamustine triggers p53-dependent and p53-independent activation of the mitochondrial apoptotic pathway in CLL and MCL. A, CLL/MCL samples with WT or mutated p53 status were incubated for 24 h with 100 μmol/L bendamustine, except for Z-138 (10 μmol/L), EHEB (50 μmol/L), and HBL-2 (50 μmol/L), and subjected to Western blot analysis of p53, PUMA, and NOXA expression. B, flow cytometry analysis of mitochondrial membrane potential (ΔΨm loss), conformational changes of BAX (BAX activation) and BAK (BAK activation), caspase-3 activation (CASP-3 activation), and ROS production in CLL/MCL cells treated as above, except for EHEB (100 μmol/L bendamustine). The indicated values correspond to the percentage of cells positive for each variable analyzed. Representative results from three independent experiments.
To further compare the relevance of both caspase-dependent and caspase-independent signaling during bendamustine-induced cell death, CLL and MCL cells were pretreated for 1 h with the pan-caspase inhibitor z-VAD.fmk before incubation for 24 h with bendamustine. We observed that z-VAD.fmk partially reversed bendamustine cytotoxicity in primary cells (CLL 24 and MCL 7) as well as in EHEB and Z-138 cell lines (Fig. 4A). In parallel, pretreatment of the same primary CLL and MCL cells with 2 mmol/L of the antioxidant GSH partially rescued cells from bendamustine-induced apoptosis but to a lesser extent than that observed with z-VAD.fmk. Similar results were obtained when using N-acetylcysteine (10 mmol/L) as antioxidant (data not shown). Pretreatment of cells with both GSH and z-VAD.fmk reversed bendamustine cytotoxicity almost completely in both CLL and MCL primary samples (Fig. 4A, left) and cell lines (Fig. 4A, right). Accordingly, and as confirmed in Fig. 4B, ROS accumulation decreased substantially in primary cultures and cell lines pretreated with GSH. The ROS scavenger also induced a notable reduction of BAX and BAK conformational changes, mitochondrial membrane potential loss, and caspase-3 activation (Fig. 4B) as well as a delay in the induction of NOXA in bendamustine-treated cells (Fig. 4C). Thus, these results show that oxidative stress is an event occurring at the onset of bendamustine-induced cell death in malignant lymphoid B cells.

Discussion

Bendamustine hydrochloride is characterized by an original structure that allows it to act as an alkylating agent as well as a purine analogue. This drug has shown therapeutic activity in a variety of tumors, including MCL and CLL, either alone or in combination with other drugs (10, 12, 13, 22, 23). Bendamustine has shown little or no accumulation in the plasma of receiver patients, thereby facilitating its lack of cross-resistance with common alkylating agents (8, 24). In the present report, we describe that bendamustine induces cytotoxicity in the majority of cell lines and primary tumor cells from CLL and MCL patients, involving p53-dependent and p53-independent mechanisms, which lead to the activation of various cell death effectors. Because p53 alterations are frequently associated with CLL and MCL pathogenesis, the possibility that bendamustine...
can bypass this principal DNA damage sensor to exert its action is of particular interest. Although mitochondrial alteration has been suggested to occur in bendamustine-treated cells (9, 19), this crucial step of intrinsic cell death signaling has not been yet formally described in malignant lymphoid B cells. We show that bendamustine triggers cell death in CLL and MCL cells by activating the mitochondrial apoptotic pathway via the up-regulation of the proapoptotic proteins PUMA and NOXA. These two BH3-only members of the BCL-2 family of proteins have been described as essential regulators of cellular stress response in lymphoid disorders (25, 26). Although the synthesis of PUMA is considered to strictly depend on p53

Fig. 4. Bendamustine cytotoxicity depends on early production of ROS in CLL and MCL cells. A, representative CLL and MCL samples were pretreated with the pan-caspase inhibitor z-VAD.fmk (50 μmol/L) ± 2 mmol/L of the antioxidant (GSH) before addition of 100 μmol/L (CLL 24 and MCL 7), 50 μmol/L (EHEB), or 10 μmol/L (Z-138) bendamustine. After 24 h, cell viability was measured as stated previously. Mean ± SD of three independent experiments. B, CLL and MCL cells were incubated as in Fig. 3 in the presence or the absence of 2 mmol/L GSH and analyzed for ROS content and intrinsic apoptotic pathway hallmarks. Results are expressed as the increase in the percentage of cells positive for each hallmark, referred to the corresponding untreated or GSH-treated control cells. Mean ± SD of three independent experiments. C, Western blot analysis of NOXA expression in three representative CLL/MCL cell lines treated for 24 h with 50 μmol/L (Z-138) or 100 μmol/L (EHEB and HBL-2) bendamustine in the presence or the absence of 2 mmol/L GSH. Western blot pictures are representative results from three independent experiments.
transcriptional activity (27), accumulating data suggest that NOXA can be regulated by both p53-related and p53-unrelated mechanisms (26, 28, 29). In agreement with this, we show that bendamustine treatment up-regulates PUMA only in CLL and MCL cases with functional p53. This suggests the involvement of this BH3-only protein specifically in p53-dependent signaling. In contrast, the activation of NOXA in bendamustine-treated cells is not restricted to cases with p53 WT. Moreover, its up-regulation can be observed at lower bendamustine doses in cells carrying a nonfunctional p53 than in WT cells, a phenomenon already observed in other models (26, 30).

Our data provide evidences that the p53-independent up-regulation of NOXA and NOXA-mediated apoptotic signaling observed following bendamustine treatment may be regulated by the intracellular levels of ROS, because both events can be notably rescued by the ROS scavenger GSH. However, GSH has to be combined with the pan-caspase inhibitor z-VAD.fmk to completely restore cell viability. Thus, ROS production may be implicated in the caspase-independent process activated concomitantly with the caspase-dependent pathway in bendamustine-treated cells. Accordingly, caspase-independent cell death has been found in several cancer models to be associated with oxidative stress and ROS production following exposure to a broad range of stimuli including tumor necrosis factor, proteasome inhibitors, or genotoxic drugs (26, 31, 32). The increase in intracellular ROS levels can lead to cell cycle arrest in G2-M phase, involving the checkpoint kinase Chk1 that is in turn implicated in the activation of the p53 homologue p73 and the consequent transcription of NOXA, as a possible system to bypass the alterations in the DNA damage response in cancer cells with nonfunctional p53 (33). Accordingly, we observed that, in these cells, bendamustine preferentially provoked a G2-M cell cycle blockade associated to the apparition of tetraploid nuclei characteristic for mitotic defects (data not shown). Furthermore, it has been recently reported that activation of ataxia telangectasia mutated protein may control this cell cycle arrest in G2-M, in parallel to p53-mediated apoptosis commitment (34). Thus, we might hypothesize that bendamustine exerts cytotoxicity in nonfunctional p53 tumor cells due to the ability of ROS to activate a G2-M cell cycle checkpoint and the synthesis of NOXA by a p53-independent mechanism, which leads to mitochondrial depolarization. As a consequence, we show that bendamustine treatment leads to cytosolic release of the mitochondrial apoptogenic factors cytochrome c and AIF, which are able to activate caspase-dependent and caspase-independent signaling, respectively, leading to different forms of apoptotic chromatin condensation. Transfection of cells with a neutralizing antibody against AIF consequently reduces cell death following bendamustine treatment. This suggests that AIF may be the executor implicated in bendamustine-induced, caspase-independent signaling, a feature recently attributed to classic alkylating agents (35).

Most of these latest phenotypic features are concordant with one of the mechanisms recently proposed for bendamustine cytotoxicity in lymphoid cells, the so-called “mitotic catastrophe” (9). This atypical cell death pathway is generally triggered by mitotic failure caused by defective G2-M cell cycle checkpoints and the development of aneuploid cells that are doomed to die, following exposure to DNA damaging agents, as well as microtubule stabilizing or destabilizing drugs. Although it is accompanied by mitochondrial membrane permeabilization, this form of cell death does not necessarily depend on caspase activation (36).

The clinical activity of the nucleoside analogues used to treat patients with B-cell malignancies involves modulation of DNA damage response pathway, cell cycle arrest, and/or initiation of cell death. The essential role of p53 in the response makes its mutational status a crucial factor for a given therapy to be effective (37). Our results show that bendamustine might overcome this defect and therefore could have a therapeutic advantage over conventional therapies. Moreover, we show that the combination of bendamustine with fludarabine or gemcitabine is highly synergistic. These results concur with clinical studies conducted in refractory/relapsed B-cell malignancies in which the synergism between bendamustine and other agents makes it possible to overcome drug resistance (15, 38–40).

In conclusion, our results confirm the efficacy of bendamustine in CLL and MCL with defects in the DNA damage response pathway, describe its capacity to trigger simultaneously distinct apoptotic pathways, and support its inclusion in combination therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

15. Rummel MJ, Al-Batran SE, Kim SZ, et al. Bendamustine plus rituximab is effective and has a favorable


Bendamustine Is Effective in p53-Deficient B-Cell Neoplasms and Requires Oxidative Stress and Caspase-Independent Signaling

Gaël Roué, Mónica López-Guerra, Pierre Milpied, et al.


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/14/21/6907

Cited articles This article cites 40 articles, 19 of which you can access for free at: http://clincancerres.aacrjournals.org/content/14/21/6907.full.html#ref-list-1

Citing articles This article has been cited by 9 HighWire-hosted articles. Access the articles at: /content/14/21/6907.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.