The Histone Deacetylase Inhibitor MGCD0103 Induces Apoptosis in B-Cell Chronic Lymphocytic Leukemia Cells through a Mitochondria-Mediated Caspase Activation Cascade

Victoria El-Khoury¹, Etienne Moussay¹, Bassam Janji¹, Valérie Palissot¹, Nasséra Aouali¹, Nicolaas H.C. Brons¹, Kris Van Moer¹, Sandrine Pierson¹, Eric Van Dyck¹, and Guy Berchem¹,²

Abstract

Clinical trials have shown activity of the isotype-selective histone deacetylase (HDAC) inhibitor MGCD0103 in different hematologic malignancies. There are data to support the use of HDAC inhibitors in association with other cancer therapies. To propose a rational combination therapy, it is necessary to depict the molecular basis behind the cytotoxic effect of MGCD0103. In this study, we found that MGCD0103 was substantially more toxic in neoplastic B cells relative to normal cells, and we described the death pathways activated by MGCD0103 in B-cell chronic lymphocytic leukemia (CLL) cells from 32 patients. MGCD0103 decreased the expression of Mcl-1 and induced translocation of Bax to the mitochondria, mitochondrial depolarization, and release of cytochrome c in the cytosol. Caspase processing in the presence of the caspase inhibitor Q-VD-OPh and time course experiments showed that caspase-9 was the apical caspase. Thus, MGCD0103 induced the intrinsic pathway of apoptosis in CLL cells. Moreover, MGCD0103 treatment resulted in the activation of a caspase cascade downstream of caspase-9, caspase-dependent amplification of mitochondrial depolarization, activation of calpain, and Bax cleavage. We propose a model whereby the intrinsic pathway of apoptosis triggered by MGCD0103 in CLL is associated with a mitochondrial death amplification loop.

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Introduction

B-cell chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal CD5⁺, CD23⁺, and dim CD20⁺ B lymphocytes in the peripheral blood, bone marrow, and secondary lymphoid organs (1). In CLL, the neoplastic cells have a prolonged survival due to failure of programmed cell death (2). Despite improvements in response rates using chemoimmunotherapy combinations, CLL remains incurable (aside from an allogeneic stem-cell transplant).

Histone tail lysine acetylation and deacetylation provide an important mechanism to modulate chromatin structure and regulate gene expression. Whereas acetylation marks generated by histone acetyltransferases are generally associated with transcriptionally active chromatin, loss of acetylation leads to chromatin compaction and transcriptional repression (3). Importantly, histone deacetylases (HDAC), which mediate the deacetylation of histones, are generally associated with transcriptionally active chromatin, loss of acetylation leads to chromatin compaction and transcriptional repression (3). Histone acetylation marks generated by histone acetyltransferases are generally associated with transcriptionally active chromatin, loss of acetylation leads to chromatin compaction and transcriptional repression (3). Importantly, histone deacetylases (HDAC), which mediate the deacetylation of histones, also catalyze the removal of acetyl groups from nonhistone proteins, including regulators of cell proliferation and cell death. The 18 known HDACs have been divided into four classes based on their structure and biological activity (3).

HDAC inhibitors are emerging as potent anticancer agents that can reactivate gene expression and restore the capability of malignant cells to undergo apoptosis. When evaluated in preclinical studies, some HDAC inhibitors exerted promising antitumor activity in CLL (4–8). However, HDAC inhibitors have varying degrees of specificity towards HDACs (3). Beyond their roles in cancer initiation and progression, HDACs control several key biological functions such as vascular and bone development, myogenesis, and cardiac growth (9),...
thus highlighting the need for isoform-selective HDAC inhibitors.

Recent findings point out the particular relevance of class I HDACs (HDAC1, 2, 3, and 8) as targets in the treatment of cancer (10). In this regard, MGCD0103 (MethylGene Inc.) seems very promising. It is a rationally designed, orally available, isotype-selective benzamide that targets HDACs 1, 2, 3, and 11 (11). MGCD0103 was shown to induce apoptosis and to have a more potent antiproliferative effect than the HDAC inhibitors SAHA and MS-275 in various human cancer cell lines (12). Moreover, it exhibited low toxicity and was more active than SAHA in mice with human tumor xenografts (12). MGCD0103 also showed clinical activity in hematologic malignancies, including myeloid leukemia and lymphoma (13). A phase I clinical study has shown that MGCD0103 administered orally three times per week is safe and active in patients with acute myelogenous leukemia and myelodysplastic syndromes (14). In phase II trials, MGCD0103 showed significant anticancer activity in relapsed/refractory Hodgkin and non-Hodgkin lymphoma (13). A recent phase II clinical trial evaluating MGCD0103 in patients with relapsed and refractory CLL concluded that the efficacy of MGCD0103 as a single agent was limited, and that the majority of patients could tolerate a maximum of only two cycles of drug treatment (15). However, MGCD0103 was clearly shown to reduce in vitro the viability of tumor cells from untreated patients (15). These results suggest that MGCD0103 efficacy may occur in previously untreated CLL patients or through combinational strategies.

Importantly, MGCD0103 displays favorable pharmacokinetic properties and sustained pharmacodynamic effect when compared with other HDAC inhibitors (14, 16, 17), thus allowing dose reduction and long rest intervals in combinational treatments. However, the optimum deployment of association strategies with MGCD0103 requires true knowledge of its induced cell death mechanism. Moreover, depicting the characterization of cell death processes induced by MGCD0103 might help to understand the molecular basis for MGCD0103 resistance in CLL. Thus, considering the small amount of data on the mechanism of MGCD0103-mediated cytotoxicity, we investigated the death pathways activated by MGCD0103 ex vivo in CLL cells from a cohort of 19 untreated and 13 previously treated patients.

Materials and Methods

**Patients, cell separation, and culture conditions.** After informed consent was obtained according to the Declaration of Helsinki, peripheral blood samples were collected from 32 CLL patients and 8 healthy donors at the Hospital Center of Luxembourg. Nineteen patients were not previously treated, and 13 were untreated for ≥1 month at the time of cell collection. The clinical features of the patient cohort are summarized in Table 1. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation (Lymphocyte separation medium, MP Biomedicals). PBMC and Mec-1 cells (DSMZ) were incubated at 1.5 to 3 × 10^6 cells/mL in Iscove’s Modified Dulbecco’s Medium (Lonza) containing 10% fetal bovine serum. MCF-7 cells (kindly provided by Chouaib S., INSERM U753, Villejuif, France), and JVM-2 and JVM-3 cells (DSMZ) were cultivated in RPMI 1640 medium containing 10% fetal bovine serum. The cells were incubated at 37°C in an atmosphere of 5% CO2. When caspase inhibitors were used, they were added 1 hour before MGCD0103.

**Reagents and antibodies.** MGCD0103, N-(2-amino-phenyl)-4-((4-pyridin-3-yl-pyrimidin-2-ylamino)-methyl)-benzamide, was provided by MethylGene Inc. (Quebec, Canada). Caspase inhibitors and anti-cytochrome c antibody were from R&D Systems. Anti-CD3-FITC, anti-CD5-FITC, and anti-CD19-phycoerythrin antibodies were from ImmunoTools. Annexin V-APC (allophycocyanin) was from BD Pharmingen. Propidium iodide (PI) was from Sigma-Aldrich. MitoProbe JC-1 Assay Kit was from Molecular Probes. Antibodies against poly(ADP-ribose) polymerase (PARP), cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP), cleaved lamin A/C, procaspase-6, Bcl-Xl, and caspase-3, -8, and -9 were from Cell Signaling Technology. Anti-procaspase-10 antibody was from Abnova. Antibodies against Bax, Bcl-2, and Mcl-1 were from DakoCytomation. Anti-Bim antibody was from Merck Chemicals Ltd. Anti-calcipain antibody was from Chemicon International. Anti-VDAC1 antibody was from Abcam. Anti-β-actin antibody was from Sigma-Aldrich.

**Determination of cell viability.** Cell viability was measured using Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) as recommended by the manufacturer.

**Analysis of phosphatidylserine externalization and cell permeability.** PBMC were stained with Annexin V-APC in Annexin V-binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2]. Anti-CD5-FITC, anti-CD3-FITC, and anti-CD19-phycocerythrin antibodies were added when necessary. Cells were resuspended in the same buffer containing 0.1 μg/mL of propidium iodide and analyzed on BD FACSCanuto flow cytometer. Fifty thousand events were collected per sample. BD FACS DIVA software was used for flow data analysis. The percentage of apoptosis corresponds to the percentage of Annexin V-positive/PI-negative cells and Annexin V-positive/PI-positive cells (early and late apoptotic cells, respectively).

**Mitochondrial membrane potential analysis.** Change in mitochondrial membrane potential (ΔΨm) was measured by flow cytometry using the cationic dye JC-1 and Annexin V double staining as recommended by the manufacturer. JC-1 was used at 6 μmol/L. Cells were analyzed on BD FACSCanuto flow cytometer. Thirty thousand events were collected per sample. BD FACS DIVA software was used for flow data analysis.
Immunoblotting analysis. For whole-cell lysate preparation, PBMC were lysed in radioimmunoprecipitation assay buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). To assess cytochrome c release and Bax translocation, cytosolic and membrane fractions were obtained as described before (18). Immunoblotting was done using standard procedures.

Statistical analysis. Statistical analysis was carried out using a two-sided t test. A P value <0.05 was considered to be statistically significant.

Results

MGCD0103 induces caspase-dependent apoptosis in CLL cells. To determine whether MGCD0103 induces apoptosis of CLL cells, phosphatidylserine externalization was investigated in PBMC incubated with increasing doses of MGCD0103, including a clinically relevant concentration of 0.5 μmol/L. This concentration was calculated based on a phase I trial of MGCD0103 in acute myeloid leukemia and myelodysplastic syndromes, given on a schedule of three times per week, which

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Abbreviation: NA, not available.

*Sample with >20% of CLL cells expressing ZAP-70 is considered ZAP-70*.

†IgVH gene with <98% homology with the corresponding germline gene is considered mutated.

‡Sample with >7% of CD38-positive CLL cells is considered CD38*.
recommended a phase II dose of 60 mg/m² (14). At this dose, the mean maximum concentrations of drug in plasma ($C_{\text{max}}$) ranged from 161 ng/mL on day 1 to 245 ng/mL at the “steady-state” on day 12, corresponding to MGCD0103 plasma concentrations of 0.4 μmol/L and 0.6 μmol/L, respectively (14). Therefore, a concentration of 0.5 μmol/L of MGCD0103 was considered as a clinically achievable concentration. As shown in Fig. 1A, MGCD0103 induced apoptosis in a time- and dose-dependent manner. Despite the increase in apoptosis observed 48 hours after exposure to 0.5 μmol/L of MGCD0103, levels of apoptosis suitable for analysis were only reached upon prolonged incubation at this concentration (72 hours, 51.93% ± 7.59%, mean value ± SE). Such levels were also obtained with 3 μmol/L of MGCD0103 after shorter times (Fig. 1A). Therefore, to avoid the increase of spontaneous apoptosis of CLL cells ex vivo (19) and the toxic effect of a prolonged incubation with fmk-based caspase inhibitors, MGCD0103 was used at 3 μmol/L for a maximal duration of 48 hours in the following experiments of the study. Interestingly, statistical analysis revealed that CLL cells from previously untreated patients were more sensitive to MGCD0103 effects than those from treated patients, with a significant difference observed after 24-hour treatment with 3 μmol/L drug (mean values ± SE, 41.72 ± 5.36 versus 18.21 ± 5.39, respectively; $P = 0.01$).

We next aimed to determine whether MGCD0103-induced apoptosis in CLL cells was dependent on caspase activity. Although the broad-spectrum caspase inhibitor Z-VAD-fmk partially restored cell viability, the more potent pan-caspase inhibitor Q-VD-OPh completely abrogated the apoptotic effect of MGCD0103 (Fig. 1B). Thus, MGCD0103-induced apoptosis is primarily caspase dependent.

The involvement of the different caspases in MGCD0103-induced apoptosis was investigated using specific inhibitors of caspase-1/4 (Z-YVAD-fmk), caspase-2 (Z-VDVAD-fmk), caspase-3/7 (Z-DEVD-fmk), caspase-6 (Z-VEID-fmk), caspase-8 (Z-IETD-fmk), caspase-9 (Z-LEHD-fmk), and caspase-10 (Z-AEVD-fmk; Fig. 1C). To exclude the influence of spontaneous apoptosis of CLL cells, the apoptotic rates are presented as “MGCD0103-specific apoptosis” using the following formula (20): [(% apoptosis in the drug-treated group − % apoptosis in the control group)/100] × 100. Inhibitors of caspase-6 and -10 significantly reduced MGCD0103-induced apoptosis whereas the other inhibitors did not. The caspase-9 inhibitor Z-LEHD-fmk increased the basal apoptosis level from 26.98% ± 18.99% to 51.5% ± 24.07% ($P = 0.05$), complicating the interpretation of the results obtained with the association Z-LEHD-fmk plus MGCD0103. The toxicity of Z-LEHD-fmk was probably dependent on the amino acids in the LEHD sequence as previously suggested in human leukemic cells (21). Combinations of caspase-3 and -6 inhibitors or caspase-8 and -10 inhibitors rescued cells from MGCD0103-induced apoptosis with a higher efficiency than either inhibitor alone. When combined with caspase-8 or -10 inhibitors, the toxicity of Z-LEHD-fmk was considerably reduced [apoptosis = 7.17% ± 7.09% ($P = 0.06$) and 25.94% ± 6.43%...
MGCD0103 activates the intrinsic pathway of apoptosis and a death amplification loop. The involvement of the intrinsic or the extrinsic pathway in MGCD0103-induced apoptosis was first assessed by caspase processing after a 24-hour treatment with 3 μmol/L of MGCD0103. Caspase-9 was cleaved to its p35 and p37 forms, resulting from its autoprocessing at Asp 315 and cleavage by caspase-3 at Asp 330, respectively (Fig. 2A). Caspase-8 was processed to its p43/41 and p18 fragments and procaspase-10 level decreased. The autoprocessing of caspase-3 to its active fragments p19/17 increased. Procaspase-6 level decreased and the cleaved form of lamin A/C, a caspase-6 substrate, accumulated. PARP was cleaved to its 89 kDa form, which is consistent with the activation of caspase-3 and -6 (Fig. 2A). The pan-caspase inhibitor Q-VD-OPh inhibited MGCD0103-induced cleavage of procaspase-8 and -10 but not the autoprocessing of caspase-9. Similar caspase processing profiles were observed in CLL cells incubated with MGCD0103 at 0.5 μmol/L for 72 hours (data not shown). These results suggest that procaspase-8 and -10 were not processed at the death-inducing signaling complex but by an active caspase, whereas procaspase-9 processing seemed to follow an “induced-proximity” model (22), indicating the activation of the intrinsic pathway of apoptosis. Consistent with this notion, MGCD0103 treatment did not alter the levels of c-FLIP (Fig. 2B), which is known to inhibit the autoprocessing of procaspase-8 and -10 in CLL cells (4, 22). MGCD0103-induced activation of the intrinsic cell death pathway was also observed in the mantle cell lymphoma cell line JVM-2, and in the CLL cell lines JVM-3 and Mec-1. In JVM-2 and JVM-3 cells, the extrinsic pathway seemed to be activated as well (Supplementary Fig. S1).

Time course experiments showed that in the primary CLL cells the increase of p35 caspase-9 level occurred after a 12-hour treatment, whereas the decrease of procaspase-10 level and the appearance of the p18 active form of caspase-8 occurred after 24 hours, its p43/41 forms being for the most part not active (ref. 23; Fig. 2C, lanes 1, 2, 5, and 6). These results confirm that caspase-9 was activated before caspase-8 and -10. The increase of caspase-3 processing was observed after 12 hours whereas caspase-6 was activated after 24 hours. The processing of caspase-9 to its p37 fragment was clearly increased after 24 and until 36 hours, in accordance with the accumulation of active caspase-9.

Figure 2. Caspase-9 is the apical caspase in MGCD0103-induced apoptosis of CLL cells. PBMC were incubated with MGCD0103 (3 μmol/L) alone or in the presence of caspase inhibitors, and the status of the indicated proteins was analyzed by immunoblotting, using actin as a loading control. A, the effect of Q-VD-OPh (10 μmol/L) on MGCD0103-induced caspase processing was assessed in PBMC after a 24-hour treatment. B, the effect of MGCD0103 on c-FLIP, expression was investigated in PBMC at the indicated times. C and D, the time course of MGCD0103-induced caspase activation was assessed in PBMC. See text for details. Representative blots from three (D) or six (A, B, C) independent experiments are shown in each panel.
forms of caspase-3 (Fig. 2C, lanes 2, 3, 6, and 7). The progressive decrease of the levels of cleaved caspases was likely due to an apoptosis-related degradation. The sequence of caspase activation suggests that MGCD0103 activates caspase-9, which cleaves procaspase-3. Active caspase-3 in turn cleaves procaspase-9 and likely procaspase-8. With delayed kinetics, procaspase-6 and -10 are then processed. In the experiment shown in Fig. 2C, it was not possible to determine which of procaspase-6 or -10 was cleaved first. However, sequential processing was clearly observed in other CLL samples (Fig. 2D), where caspase-6 was activated before caspase-10, in agreement with previous reports (24, 25). In conclusion, these results suggest that MGCD0103 activates a cascade of caspases downstream of caspase-9 that may function to amplify the apoptotic process.

To further analyze the activation of the intrinsic pathway in CLL cells, we investigated the mitochondrial alterations induced by MGCD0103. Treatment with MGCD0103 elicited severe mitochondrial depolarization, as illustrated by the decreased percentage of cells with high ΔΨm and the increase in the percentage of cells with low ΔΨm (Fig. 3A). Q-VD-OPh inhibited MGCD0103-induced apoptosis as assessed by phosphatidylserine externalization (Fig. 3A) but did not prevent mitochondrial depolarization as illustrated by the decreased number of cells with high ΔΨm. In the presence of Q-VD-OPh, however, MGCD0103 induced only partial mitochondrial depolarization, as shown by the accumulation of cells with intermediate ΔΨm (Fig. 3A). These results indicate that MGCD0103 induces an initial caspase-independent loss of ΔΨm which is amplified by caspase activation. Moreover, MGCD0103 induced a time-dependent translocation of Bax from the cytosol to the mitochondria (heavy membrane fraction) and a simultaneous release of cytochrome c from mitochondria (Fig. 3B), explaining caspase-9 activation. Interestingly, Bax was cleaved from its p21 form to a p18 fragment upon treatment with MGCD0103. This fragment, which was shown to be a more potent inducer of apoptosis than p21 Bax (26), appeared only in the heavy membrane fraction enriched for mitochondria. The mitochondrial perturbations observed after MGCD0103 treatment support the involvement of the intrinsic pathway of apoptosis and the presence of a caspase-mediated cell death amplification loop.

Activation of Bax is regulated by other Bcl-2 family proteins, comprising prosurvival and proapoptotic members. Thus, we examined the effects of MGCD0103 on the expression of three major antiapoptotic proteins abundant in CLL cells, Mcl-1, Bcl-2, and Bcl-XL (4, 8), and the proapoptotic Bim, which is involved in HDAC inhibitor-induced apoptosis in leukemic cells (7). No changes in Bim, Bcl-2, and Bcl-XL expression were observed after MGCD0103 treatment. However, we observed a dramatic decrease in Mcl-1 levels that did not result from a degradation process in relation with apoptosis or caspase-mediated cleavage because it was not inhibited by Q-VD-OPh (Fig. 3C).

**MGCD0103 induces caspase-3-dependent activation of calpain and cleavage of Bax at a late stage during apoptosis.** The activity of calpain, a calcium-activated cysteine protease, within mitochondria-enriched fractions...
is responsible for Bax cleavage (27). Thus, we raised the question of whether calpains were activated by MGCD0103. Autolysis of the 30 kDa subunit of calpain has been used as a marker for calpain activation (27). MGCD0103 (3 μmol/L) induced a substantial autolysis of calpain after 24 and 48 hours, which was correlated with cleavage of Bax (Fig. 4A). Accumulation of p18 Bax was observed after caspase-3 activation and PARP cleavage (Fig. 4A). Pretreating the cells with Q-VD-OPh completely abrogated calpain activation and Bax cleavage (Fig. 4B). Similar results were obtained when MGCD0103 was administered at the clinically relevant concentration of 0.5 μmol/L for 48 and 72 hours (data not shown). These data show that MGCD0103 treatment triggers caspase-dependent calpain activation and Bax cleavage that may amplify cell death at a late stage of the apoptotic program.

Previous data showed that caspases upregulate calpain activity through cleavage of the endogenous calpain inhibitor calpastatin (28). The major proteases responsible for this cleavage in vivo seemed to be caspase-3 and -7 (29). Therefore, we tested the contribution of both caspases in MGCD0103-induced calpain activation and Bax cleavage, using the caspase-3–deficient MCF-7 cell line. After 48-hour treatment with 3 μmol/L of MGCD0103, MCF-7 cells displayed substantial cleavage of PARP, which was inhibited in the presence of Z-DEVD-fmk or Q-VD-OPh (Fig. 4C). However, neither calpain activation nor Bax cleavage was observed (Fig. 4C, lanes 1 and 2), suggesting that caspase-3 was responsible for calpain activation in MGCD0103-treated cells.

**MGCD0103 preferentially kills neoplastic B cells.** To compare the toxicity of MGCD0103 in CLL and normal cells, a viability assay was done in PBMC from CLL patients and healthy donors. The average concentration of MGCD0103 that killed 50% of cells (LC₅₀) after a 48-hour treatment was 0.6 μmol/L for CLL and 4.6 μmol/L for normal cells (Fig. 5A). Next, the different PBMC populations within the same CLL patient blood sample were compared for their sensitivity to MGCD0103-induced apoptosis. In the representative experiment shown in Fig. 5B, a >3-fold increase in apoptosis was observed in the neoplastic B cells after a 24-hour treatment with MGCD0103. In contrast, the populations of T cells, monocytes, normal B cells, and other mononuclear cells were less affected by such treatment (Fig. 5B). These data show that MGCD0103 is substantially more toxic to neoplastic B cells relative to normal mononuclear cells.

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**Figure 4.** MGCD0103 induces caspase-3–dependent activation of calpain and cleavage of Bax in CLL cells. A, the time course of calpain activation and Bax cleavage was assessed in PBMC incubated with MGCD0103 (3 μmol/L) for the indicated times. Proteins were analyzed by immunoblotting, using actin as a loading control. B, the effect of Q-VD-OPh on calpain activation and Bax cleavage was investigated. PBMC were incubated with MGCD0103 alone or in the presence of Q-VD-OPh (10 μmol/L) for 24 hours. Proteins were analyzed as in A. C, the requirement for caspase-3 in MGCD0103-induced calpain activation and Bax cleavage was assessed. Caspase-3–deficient MCF-7 cells were incubated with MGCD0103 (3 μmol/L) alone or in the presence of Q-VD-OPh (10 μmol/L) or Z-DEVD-fmk (100 μmol/L) for 48 hours. Immunoblotting was done as in A. Proteins from MGCD0103-treated CLL cells were loaded as positive control for calpain activation and Bax cleavage. Representative blots from six independent experiments are shown in each panel.
Discussion

Despite the modest clinical activity observed with MGCD0103 in patients with advanced solid tumors (16) and patients with relapsed/refractory CLL (15), clinical activity with MGCD0103 was shown in patients with acute myelogenous leukemia, myelodysplastic syndromes (14), and Hodgkin and non-Hodgkin lymphoma.
Mechanism of MGCD0103-Induced Cell Death in CLL

suggests that the extrinsic pathway only plays a minor role in CLL cells (4, 22). This finding, together with the c-FLIPL, known to inhibit death receptor inhibitors (4, 8), MGCD0103 did not alter the levels of procaspase-8 and -10. Importantly, unlike other HDAC inhibitors (31). Although patients given fludarabine (30), and MGCD0103. Indeed, gene expression profiles are altered after incubation of CLL cells with fludarabine (30), and MGCD0103-induced cell death. Moreover, all 21 patients in this clinical study had previously received fludarabine, which may also have contributed to the development of resistance to MGCD0103. Indeed, gene expression profiles are altered after incubation of CLL cells with fludarabine (30), and some of these modifications (e.g., the upregulation of MKP1) have been previously correlated with resistance to HDAC inhibitors (31). Although patients given fludarabine may prove refractory to subsequent treatment with MGCD0103, preliminary combination studies indicate that MGCD0103 synergizes with fludarabine to induce apoptosis of CLL cells, suggesting that MGCD0103 may have a possible clinical benefit in association with fludarabine as first-line therapy. The characterization of the cell death pathways induced by such combination is the subject of current investigations.

MGCD0103-induced activation of the intrinsic pathway of apoptosis in CLL cells was supported by the following: (a) MGCD0103 decreased the expression of the antiapoptotic Bcl-2 family protein Mcl-1, and induced the translocation of Bax to the mitochondria, caspase-independent loss of ΔΨm, and the release of cytochrome c, necessary for procaspase-9 activation in the apoptosome (22); (b) autoprocessing of procaspase-9 was caspase independent and occurred before cleavage of procaspase-8 and -10. Importantly, unlike other HDAC inhibitors (4, 8), MGCD0103 did not alter the levels of c-FLIP, known to inhibit death receptor-mediated apoptosis in CLL cells (4, 22). This finding, together with the caspase-dependent cleavage of procaspase-8 and -10, suggests that the extrinsic pathway only plays a minor role, if any, in MGCD0103-induced cell death of CLL. However, we noted that JVM-2 and JVM-3 cells exhibited caspase-independent cleavage of both procaspase-8 and -9 following incubation with MGCD0103, suggesting that MGCD0103 may also activate the extrinsic pathway in cells where this pathway is not inherently inhibited. Similar results were observed in the breast cancer cell line MCF-7 (data not shown).

We showed that, once activated, caspase-3 cleaved caspase-9 to a p37 fragment in MGCD0103-treated CLL cells. Others have shown that this proteolytic event represents a feedback amplification loop that accelerates apoptosis (32). The fact that activation of caspase-6 and -10 occurs downstream of caspase-3 might have been interpreted as suggestive of only a minor contribution to apoptosis. However, the use of specific caspase inhibitors revealed that both caspases were needed for optimal induction of apoptosis (Fig. 1C). Because caspase-9 cannot process procaspase-6 (33) this observation suggests that caspase-3 mediated procaspase-6 cleavage. Surprisingly, the caspase-6 inhibitor significantly reduced MGCD0103-induced apoptosis in CLL cells, whereas the caspase-3 inhibitor did not (Fig. 1C). This discrepancy may result from an incomplete inhibition of caspase-3 activity by Z-DEVD-fmk. Moreover, we noted that the use of a caspase-6 inhibitor considerably reduced the processing of caspase-3 in MGCD0103-treated CLL cells (data not shown). This finding indicates that activated caspase-6 may be part of a loop that amplifies the processing of caspase-3, consistent with previous reports (34), and thus may explain the significant reduction of apoptosis observed with caspase-6 inhibitor. Furthermore, the processing of procaspase-10 occurred after caspase-6 activation (Fig. 2D) and was substantially reduced in the presence of caspase-3 or caspase-6 inhibitors (data not shown). These findings suggest that, once activated by caspase-3, caspase-6 activates caspase-10 (Supplementary Fig. S2). This is in agreement with the hierarchy of caspase activation previously described (24, 25). Considering the importance of caspase-6 and -10 in MGCD0103-induced apoptosis of CLL cells (Fig. 1C), it is tempting to speculate that in these cells, caspase-10 participates in the activation of caspase-6, as previously described for taxol-induced apoptosis in the CCRF-HSB-2 human lymphoblastic leukemia cell line (35). It has been shown that caspase-6 is the major activator of caspase-8 in the mitochondrial pathway (36). In the present study, procaspase-8 seems to be cleaved by caspase-3, as the p43/41 fragments of caspase-8 started to accumulate prior to caspase-6 activation. This is likely due to the fact that caspase-3 is also able to process procaspase-8 albeit less efficiently than can caspase-6 (23). However, the p18 fragment indicative of active caspase-8 was observed only when caspase-6 was active. Whether this cleavage was dependent on caspase-3 or caspase-6 activation remains unclear. Nevertheless our findings do not preclude a role for caspase-6 in caspase-8 activation. Taken together,

3 El-Khoury et al., unpublished data.
these results suggest a model whereby MGCD0103 activates an amplification loop primarily through signaling from caspase-9 to caspase-3 to caspase-6, -8, and -10 (Supplementary Fig. S2), p18 Bax, which has been proposed to facilitate the formation of death pores that release cytochrome c (26), may participate in this loop by amplifying the drop of $\Delta \Psi_m$ as suggested by our data showing that caspase inhibition decreased the extent of $\Delta \Psi_m$ disruption.

Our data further suggest that Bax cleavage induced by MGCD0103 is mediated by calpain. Bax is cleaved in various tumor cell lines (26, 27) and in CLL patients’ cells (37) following treatment with chemotherapeutic agents. To our knowledge, there is only one report of a treatment with a HDAC inhibitor eliciting cleavage of Bax (38). However, the authors did not clarify the mechanism and the importance of this cleavage in the induced apoptosis. Here, we showed that MGCD0103 induced the activation of calpain in CLL cells, which was consistent with the kinetics of Bax cleavage. Few studies have investigated the role of calpain in HDAC inhibitor–mediated toxicity and these were limited to the use of multiple myeloma cell lines (39, 40). Activation of calpain can be dependent or independent of caspase activity (41). Here, we clearly showed that MGCD0103-induced activation of calpain was caspase-3 dependent and that cleavage of Bax occurred at a late stage of apoptosis as previously described (27, 41). Thus, the release of the Ca$^{2+}$ stored in the mitochondria following the $\Delta \Psi_m$ drop together with caspase-3–mediated inactivation of calpastatin could provide a mechanism for MGCD0103-induced calpain activation. Therefore, we propose that calpain-mediated cleavage of Bax participates in the amplification of apoptosis initiated by the caspases following MGCD0103 treatment (Supplementary Fig. S2). In agreement with this, previous studies have proposed that p18 Bax may enhance cell death at a relatively late stage of apoptosis (42).

Bcl-2 family members are critical regulators of the intrinsic pathway of apoptosis. Overexpression of Bcl-2 antiapoptotic proteins such as Mcl-1, Bcl-2, and Bcl-X$_L$ has been associated with survival of CLL cells and their resistance to apoptosis (4, 8). Moreover, perturbations in the expression of Bcl-2 proteins by HDAC inhibitors have been previously reported in CLL cells (7). We showed that MGCD0103 decreased the expression of Mcl-1 in CLL cells. Interestingly, Mcl-1 overexpression is associated with a failure to achieve a complete response to CLL chemotherapy (including fludarabine) in vitro and in vivo, whereas its downregulation by small interfering RNA has been shown to potentiate the response of CLL cells to rituximab (43). Thus, we suggest that the down-regulation of Mcl-1 elicited by MGCD0103 may help improve the response to chemotherapeutic treatment in a combinational regimen.

In this study, we showed that MGCD0103-induced apoptosis in CLL involves members of the Bcl-2 family, as illustrated among others by the decrease in the levels of Mcl-1 observed following treatment with the drug. Thus, resistance to MGCD0103 might occur, for instance, in cells where the levels of Mcl-1 are abnormally high and/or refractory to MGCD0103-induced downregulation. Therefore, we propose that Bcl-2 inhibitors may help improve the response to MGCD0103 in combination regimens. Our observations may bear also direct relevance to the mechanisms leading to MGCD0103 resistance in CLL patients with poor prognostic markers (15). Indeed, higher levels of Mcl-1 and Bcl-2, and lower levels of Bax have been observed in these patients (43). Overexpression of antiapoptotic Bcl-2 proteins has previously been associated with resistance to HDAC inhibitors (44). Moreover, a common polymorphism in the BAX gene has been shown to result in low protein Bax expression in some CLL patients (43).

Our data provide evidence that, in CLL cells, MGCD0103 activates the intrinsic pathway of apoptosis and triggers an amplification loop involving caspasases and calpains. Several drugs could be considered for combination therapies with MGCD0103. These include drugs directed against the same targets as MGCD0103, such as inhibitors (obatoclax, ABT-263) or modulators (oblimersen, flavopiridol, rituximab) of antiapoptotic Bcl-2 family proteins, some of which are currently under investigation in clinical trials for the treatment of CLL. Importantly, the cyclin-dependent kinase inhibitor flavopiridol induces an early mobilization of intracellular calcium in CLL cells (45); whether this mobilization could further enhance the activation of calpain induced by MGCD0103 treatment remains to be determined. Moreover, drugs activating the extrinsic pathway in CLL may also be appropriate for combination with MGCD0103. Among those are proteasome inhibitors, which have been shown to induce cell death in CLL cells in vitro, at least in part through downregulation of c-FLIP and upregulation of TRAIL and its death receptors (46). Although clinical studies of proteasome inhibitors in CLL have yielded only modest single-agent activity (47), their association with MGCD0103 could induce a synergistic response, because this HDAC inhibitor induces by itself the activation of caspase-8 and -10 via the mitochondrial pathway. Relevant in this regard is a recent study showing that very low concentrations of the HDAC inhibitors romidepsin and belinostat and the proteasome inhibitor bortezomib synergistically killed primary CLL cells (48). It also seems that, despite the sensitivity of tumor cell lines, several primary tumor cells are resistant to death receptor–mediated apoptosis (49, 50). Thus, the potential of MGCD0103 to circumvent the inherent resistance of cancer cells to the induction of apoptosis by the death receptors may be clinically important. Finally, the activation of both extrinsic and intrinsic pathways of apoptosis in JVM-2, JVM-3, and MCF-7 cells support further investigation of MGCD0103 not only in CLL but also in other hematologic and solid cancers, alone and in combination regimens. Association strategies are currently under
investigation to identify synergistic responses with MGCD0103.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

The Histone Deacetylase Inhibitor MGCD0103 Induces Apoptosis in B-Cell Chronic Lymphocytic Leukemia Cells through a Mitochondria-Mediated Caspase Activation Cascade

Victoria El-Khoury, Etienne Moussay, Bassam Janji, et al.


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