The Combination of a Histone Deacetylase Inhibitor with the Bcl-2 Homology Domain-3 Mimetic GX15-070 Has Synergistic Antileukemia Activity by Activating Both Apoptosis and Autophagy

Yue Wei¹, Tapan Kadia¹, Weigang Tong¹, Ming Zhang², Yu Jia¹, Hui Yang¹, Yumin Hu¹, Francesco Paolo Tambaro¹, Jean Viallet³, Susan O’Brien¹, and Guillermo Garcia-Manero¹

Abstract

Purpose: Single-agent histone deacetylase inhibitors (HDACi) have limited clinical activity in human leukemia. Although the way HDACi exert their antileukemia effect is not fully understood, it is accepted that induction of apoptosis is important. We hypothesized, therefore, that combination of an HDACi with a proapoptotic agent, such as the Bcl-2 homology domain-3 mimetic GX15-070, could result in enhanced antileukemia activity.

Experimental Design: We analyzed the cellular and molecular effects of two different HDACi (MGCD0103 and vorinostat) in combination with GX15-070 in leukemia cell lines and primary acute myelogenous leukemia cells.

Results: We showed that the combination had synergistic antileukemia effect both in leukemia cell lines and in primary acute myelogenous leukemia cells. Using molecular markers and electron microscopy, we observed that in addition to apoptosis, autophagy accounts for the nonapoptotic decrease in cell viability, an effect that could be inhibited by chloroquine, an inhibitor of autophagy. Finally, we established a role for calpain activity in the induction of both autophagy and apoptosis by this combination.

Conclusions: The combination of an HDACi and GX15-070 has synergistic antileukemia activity, and the effect is mediated by induction of apoptosis and autophagy. The combination should be studied in clinical trials of leukemia and the role of autophagy in leukemia therapy needs to be better understood.

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Small-molecule inhibitors of histone deacetylases (HDAC) are a class of relatively novel therapeutic agents with clinical activity in both lymphoma (1) and leukemia (2, 3). Although the mechanisms of action of HDAC inhibitors (HDACi) are not completely understood, the clinical anticancer activity of HDACi has been traditionally thought to be mediated in part through induction of histone acetylation, which results in reactivation of aberrantly suppressed genes, leading to inhibition of proliferation, induction of apoptosis, and cell differentiation (4). Multiple HDACi are in clinical development (1, 5), and vorinostat, a hydroxamic acid derivative, is approved in the United States for patients with cutaneous lymphoma (6). MGCD0103, an isotype-specific small-molecule HDACi targeting HDAC isotypes 1, 2, 3, and 11, has been shown to be orally bioavailable with significant in vitro activity against a broad spectrum of human cancers, including leukemia (3). This agent has also been shown to be safe and have potential clinical activity in patients with advanced leukemia (3).

Historically, HDACi have been shown to have limited but significant single-agent clinical activity in leukemia (3). These results have led to the hypothesis that combination strategies may be the optimal way to use HDACi. GX15-070 (obatoclax) is a novel Bcl-2 homology domain-3 (BH3) mimetic that has been shown to induce apoptosis in acute myelogenous leukemia (AML) cells at micromolar concentrations by liberating proapoptotic proteins, such as Bak and Bim, from their antiapoptotic partners including Bcl-2 and Mcl-1 (7). Because induction of apoptosis plays an important role in the antileukemia effect of HDACi (4), we hypothesized that blocking antiapoptotic pathways with GX15-070 may enhance the antileukemia activity of HDACi. This is of clinical importance as GX15-070 has been recently reported to have clinical activity in chronic...
Histone deacetylase inhibitors (HDACi) have limited but significant clinical activity in human acute myelogenous leukemia. Although it is well established that HDACi can induce histone acetylation and gene expression, the mechanism by which these agents exert their antileukemia effect is not well understood. Because the antileukemia effect of HDACi is at least partially mediated via induction of apoptosis, we hypothesized that combining an HDACi with a proapoptotic agent could enhance their antileukemia activities. Here, we report that the combination of an HDACi with the Bcl-2 homology domain-3 mimetic GX15-070 results in synergistic antileukemia activity by inducing both apoptosis and autophagy. These results are of both biological and clinical significance, suggesting that this two-drug combination, especially the death-promoting form of autophagy induced by this combination, should be further studied in leukemia therapy.

**Translational Relevance**

Histone deacetylase inhibitors (HDACi) have limited but significant clinical activity in human acute myelogenous leukemia. Although it is well established that HDACi can induce histone acetylation and gene expression, the mechanism by which these agents exert their antileukemia effect is not well understood. Because the antileukemia effect of HDACi is at least partially mediated via induction of apoptosis, we hypothesized that combining an HDACi with a proapoptotic agent could enhance their antileukemia activities. Here, we report that the combination of an HDACi with the Bcl-2 homology domain-3 mimetic GX15-070 results in synergistic antileukemia activity by inducing both apoptosis and autophagy. These results are of both biological and clinical significance, suggesting that this two-drug combination, especially the death-promoting form of autophagy induced by this combination, should be further studied in leukemia therapy.

**Materials and Methods**

**Cell lines, primary AML samples, and reagents**

HL-60, THP1, and U937 cells were obtained from the American Type Culture Collection and were grown following standard conditions. Peripheral blood samples (n = 8) were obtained for in vitro studies from patients diagnosed with AML at the M.D. Anderson Cancer Center following institutional guidelines. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical Co.) density-gradient centrifugation. For cell proliferation analysis, AML cells were counted using trypan blue exclusion assays. GX15-070 was provided by Gemin X. MGCD0103 was provided by Methylgene, Inc., and vorinostat by Merck & Co., Inc. PD150606 was purchased from Calbiochem, chloroquine was from Sigma, and Z-LEVDFMK from Biovision. Antibodies used included active caspase-3 (eBiosciences), poly (ADP-ribose) polymerase (PARP; BD), Puma, calpain 2, LC-3, Grp78, Grp94, ATG12, and caspase-4 (Cell Signaling); Mcl-1, Bak1, Bax, Bcl-XL, and Noxa (Santa Cruz Biotechnology); Ac-H3 and Ac-H4 (Millipore).

**Analysis of apoptosis**

Apoptosis was quantitated by flow cytometry using propidium iodide/Annexin V FITC kit (BD Biosciences) following the manufacturer’s guidelines. Annexin V fluorescence was quantitated with a Becton Dickinson FACSCalibur or LSRII flow cytometer (BD Biosciences).

**Transmission electron microscopy**

This analysis was done at the Electron Microscopy Core Facility at the M.D. Anderson Cancer Center. Cells were harvested, pelleted, and fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L cacodylate buffer (pH 7.3), followed by wash and treatment with 0.1% Millipore-filtered cacodylate buffered tannic acid, 1% buffered osmium tetroxide, and 1% Millipore-filtered uranyl acetate. Samples were dehydrated at increasing concentrations of ethanol, infiltrated, and embedded in Spurr’s low-viscosity medium. Ultrathin sections were cut with a Leica Ultracut microtome, stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp.).

**Real-time reverse transcription-PCR**

Total cellular RNA was extracted using Trizol (Invitrogen) and was used for reverse transcription reactions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time PCR, primers and probes were purchased from Applied Biosystems and analyzed with TaqMan Universal PCR Master Mixture (Applied Biosystems) using an Applied Biosystems Prism 7000 Sequence detection system. Glyceraldehyde-3-phosphate dehydrogenase was used as internal control.

**Statistical analysis**

Analysis of the effect of the combination was done using a two-term tumor repopulation model (9–11). The synergistic effect of the drugs on primary leukemia cells was analyzed using the Bliss independence model (12, 13).

**Results**

**HDACi and GX15-070 synergistically inhibit cell proliferation in multiple AML cell lines**

We first evaluated the antileukemia effect of HDACi in combination with GX15-070 in leukemia cell lines. In human AML cell lines HL-60, THP1, and U937, 3 days of exposure to the combination of MGCD0103 (600 nmol/L) and GX15-070 (200 nmol/L) reduced cell viability more effectively than single-drug treatment (Supplementary Fig. S1A-C). We then analyzed the potential synergistic effect of MGCD0103 and GX15-070 in HL-60 and THP1 cells using a two-term synergy model (9). Analysis using this model requires cell viability to be calculated at multiple drug concentrations (as indicated in Table 1) and at multiple time points (24, 48, 72, and
HL-60 cells were treated with MGCD0103 (600 nmol/L) to induce apoptosis with the two-drug combination. Therefore, we analyzed the HDACi and GX15-070 synergistic cytotoxicity on AML cells. Combining HDACi with the BH3 mimetic GX15-070 results in a synergistic antiproliferative effect between MGCD0103 and GX15-070 (10, 11). Two-term synergy analysis was also done in HL-60 cells using another HDACi, vorinostat, and GX15-070. A synergistic effect (interactive index <0.4) at most of the concentrations analyzed was also observed (Table 1C). These results indicate that combining HDACi with the BH3 mimetic GX15-070 results in synergistic cytotoxicity on AML cells.

### Table 1. Summary of synergy analysis between HDACi and GX15-070 in AML cell lines using a two-term model

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<th>MGCD0103 (nmol/L)</th>
<th>GX15-070 (nmol/L)</th>
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<td><strong>A. HL-60 (interactive index)</strong></td>
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<td><strong>B. THP1 (interactive index)</strong></td>
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<td>0</td>
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<tr>
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<tr>
<td>600</td>
<td>0.17</td>
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<tr>
<td><strong>C. HL-60 (interactive index)</strong></td>
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<tr>
<td>Vorinostat (nmol/L)</td>
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NOTE: Interactive indexes were calculated using four time points for each combination. An index <0.4 indicates a synergistic effect. (A and B) Results for the combination of MGCD0103 and GX15-070 in HL-60 and THP1 cells, respectively. (C) Result for the combination of vorinostat and GX15-070 in HL-60 cells.

Combining HDACi and GX15-070 promotes autophagy in AML cells

Autophagy, also called type II programmed cell death, has been reported to contribute to the cytotoxicity mediated by single agents HDACi and GX15-070 in multiple malignancies (17–19). Because the role of autophagy in chemotheraphy-induced cell death is still controversial, it is of importance to examine if autophagy was induced by the two-drug combination in this study, and if so, to define the effect of autophagy on the cytotoxicity led by this specific drug combination in AML cells. First, we detected autophagy by observing enhanced conversion of microtubule-associated protein I light chain 3 (LC3) to its lower-migrating active form LC3-II (20, 21) in cells treated with the MGCD0103/GX15-070 and vorinostat/GX15-070 combinations (Fig. 2A). A low level of LC3-II induction compared with the effect in the two-drug combination–treated cells was also detected in cells treated with GX15-070 alone (Fig. 2A). We also observed increased levels of conjugated ATG5-ATG12, a protein complex involved in autophagosome formation (22), particularly in cells treated with the MGCD0103/GX15-070 combination (Fig. 2A).

We then used electron microscopy to examine the ultrastructural morphology of treated cells. Electron microscopy images confirmed increased apoptosis in cells treated with the MGCD0103/GX15-070 combination. More cells (~60% of total) had features of typical apoptotic morphology than cells treated with single drug (data not shown). We then focused on the nonapoptotic cells and observed that after treatment with the two-drug combination, approximately 40% of nonapoptotic cells carried small vesicles containing multiple cellular organelles in cytoplasm (Fig. 2B).

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about 60% of nonapoptotic cells after treatment with GX15-070 alone exhibited expanded endoplasmic reticulum (ER; Fig. 2B). Processing of LC3 to LC3-II can also be induced by ER expansion–associated but autophagy-independent mechanisms (24), which may explain the slight increase of LC3-II in cells treated with GX15-070 alone (Fig. 2A).

To evaluate the role of autophagy in the antileukemia effect of the combination of MGCD0103 and GX15-070, we analyzed the effect of chloroquine (CQ), a known inhibitor of autophagy (25). Whereas the addition of CQ to single agent MGCD0103 or GX15-070 increased relative cell viability by approximately 2-fold (Fig. 2C), the addition of CQ to the combination of MGCD0103 and GX15-070 increased relative cell viability approximately 5-fold (Fig. 2C). Furthermore, addition of CQ with the two-drug combination resulted in decreased levels of LC3-II (Fig. 2D). In contrast, the level of LC3-II in GX15-070 alone–treated cells was not reduced by the addition of CQ (Fig. 2D). These results suggest that autophagy is specifically induced by the two-drug combination and plays a positive role in the cytotoxic effect of the combination.

Calpain activity regulates HDACi/GX15-070 combination–induced apoptosis and autophagy

We then investigated potential molecular mechanisms involved in the synergistic cytotoxic effect observed between HDACi and GX15-070. The apoptosis-inducing effect of GX15-070 has been shown to be mediated through BH3 domain–containing proapoptotic proteins such as Bak and Bax, which are released by GX15-070 from interacting antiapoptotic partners (7). We therefore studied the effect of the MGCD0103/GX15-070 combination on the expression of several BH3 domain proteins and their known partners. Western blot analysis indicated that except for a slight increase of Bax, Bak, and Mcl-1, no significant difference at the protein levels was observed with the two-drug combination (Supplementary Fig. S4A). We did not observe further induction of histone H3 or H4 acetylation by the GX15-070/MGCD0103 combination compared with MGCD0103 alone (Supplementary Fig. S4B).

Calcium-dependent cysteine protease (calpain) has been implicated in both apoptotic and autaphagic cell death (24, 26, 27), including HDACi-induced apoptosis.
(28). To examine whether calpain activity contributes to the cytotoxicity induced by the two-drug combination, we studied the effect on cytotoxicity of the calpain inhibitor PD15060. Addition of PD15060 (25 μmol/L) increased cell viability more efficiently in MGCD0103/GX15-070 combination–treated cells compared with single agent–treated cells (Fig. 3A), suggesting that calpain activity plays a positive role in the synergistic cytotoxic effect of the two-drug combination. With the MGCD0103/GX15-070 combination treatment, addition of PD15060 caused a reduction of both cleaved PARP and LC3-II (Fig. 3B, lane 8 in comparison with lane 4), implying that calpain activity is involved in both apoptotic and autophagic processes. Because MGCD0103 is a HDACi and can potentially exert its cytotoxic effect through gene reactivation (4), we analyzed the expression of two forms of calpain: calpain 1 and calpain 2. Upregulation of calpain 2, but not calpain 1, was observed in cells treated with single

![Image](Fig. 2. The MGCD0103 and GX15-010 combination induces autophagy in HL-60 cells. Autophagy was analyzed in cells after 3 d of treatment (600 nmol/L MGCD0103 and/or 200 nmol/L GX15-070). A, Western blot analyses done after 48 h of drug treatment showed significant upregulation of LC3-II and ATG5-ATG12 conjugate in cells treated with MGCD0103/GX15-070 (top) and upregulation of LC3-II in cells treated with the vorinostat/GX15-070 combination (bottom). A slight increase of LC3-II was also observed in cells treated with GX15-070 alone. Equal loading was confirmed by blotting with an anti-actin antibody. B, autophagosomes were observed in the electron microscopy images of MGCD0103/GX15-070 combination–treated cells (top). The high-resolution (25,000×) image with arrows pointing to autophagosomes is the squared region in the lower-resolution (7,500×) image. Expanded ER was specifically observed in cells treated with GX15-070 alone (bottom). The high-resolution (25,000×) image for the ultrastructure of ER is the squared region in the lower-resolution (7,500×) image. Electron microscopy images were taken after 72 h of drug treatment. C, relative cell viability was calculated with control groups (CQ− and CQ+) as 100%, whereas fold change was calculated by comparing drug-treated groups to their control groups (CQ− or CQ+). Whereas the addition of CQ increased relative cell viability compared with the same treatments without CQ (black columns versus white columns), a more significant increase with the addition of CQ can be detected with the MGCD0103/GX15-070 combination than with MGCD0103 or GX15-070 alone. Trypan blue analysis was used to analyze cell viability after 72 h of drug treatment. D, Western blot analysis revealed reduction of LC3-II with the addition of CQ in MGCD0103/GX15-070 combination–treated cells. Equal loading was confirmed by blotting with an anti-actin antibody.)
The induction of calpain-2 expression was confirmed at the protein level by Western blot (Fig. 3D). Whether calpain upregulation is mediated by induction of histone acetylation of the calpain-2 promoter needs to be determined.

Results with PD15060 suggest that calpain activity played a more relevant role in the MGCD0103/GX15-070 combination-induced cytotoxicity than with single agent MGCD0103. Of interest, addition of GX15-070 to MGCD0103 did not enhance calpain expression compared with MGCD0103 alone. This implies that other GX15-070 mechanisms not related to calpain activation are operative in the cytotoxic effect of the combination.

When examining electron microscopy images of cells treated with single agent GX15-070, we noticed an expanded morphology of ER (Fig. 2B), a feature reflecting ER stress. ER stress has been shown to have the potential to progress to apoptosis or autophagy when the stress stimulus is protracted or more severe (29–31). We analyzed several ER chaperone proteins that are associated with ER stress and observed an increase in the glucose-regulated proteins (Grp) Grp78 and Grp94 in GX15-070– and two-drug combination–treated cells (with a slight stronger induction with the two-drug combination treatment), as well as increase of cleaved caspase-4, particularly in two-drug combination–treated cells.
been associated with Grp levels, the higher induction of Grp78 and Grp94 in the two-drug combination–treated cells (Fig. 3D) indicates that combining MGCD0103 with GX15-070 promotes the transition of ER stress to cell death. Because the calpain-caspase-12 and calpain-caspase-4 signaling pathways have been known to be pivotal for the ER stress to cell death transition in mouse and human cells (33–36), we hypothesized that calpain induction could activate caspase-12/caspase-4 signaling. This would promote the progression of GX15-070–induced ER stress to cell death. We analyzed the level of activated caspase-4 by Western blot and found it to be upregulated in MGCD0103/GX15-070 combination–treated cells, but not in single agent–treated cells (Fig. 3D). Furthermore, caspase-4 activation and induction of Grps with the two-drug combination were both repressed by the addition of the calpain inhibitor PD15060 (Supplementary Fig. 5D). These results strongly support our hypothesis and suggest that the calpain-promoted ER stress to cell death transition is an important mechanism for the synergistic cytotoxic effect observed between these two drugs. To further examine this hypothesis and the role of caspase-4, we studied the effect of the caspase-4 inhibitor Z-LEVD-FMK on the two-drug combination–mediated cytotoxicity. Addition of 10 μmol/L Z-LEVD-FMK caused a 2-fold increase in viability of cells treated with the MGCD0103/GX15-070 combination, whereas no obvious effect was observed in MGCD0103 or GX15-070 single drug–treated cells (Fig. 4A). Furthermore, the addition of Z-LEVD-FMK repressed the induction of active caspase-3 in two-drug combination–treated cells (Fig. 4B). Only a slight decrease of LC3 II was observed in these cells (Fig. 4B). These results support the importance of calpain-caspase-4 signaling in the synergistic cytotoxic effect of the two-drug combination.

**Combination treatment of MGCD0103 and GX15-070 in primary AML cells**

To study the clinical potential of the MGCD0103 and GX15-070 combination in AML, we evaluated its antiproliferative effect in primary mononuclear peripheral blood leukemia cells. Patient characteristics are shown in Supplementary Table S1. Using trypsin blue analysis, the MGCD0103/GX15-070 combination induced stronger cytotoxicity in AML primary cells compared with single-drug treatment (Fig. 5A). Of importance, the combination had no obvious cytotoxic effect on normal control primary cells using the same assay (Fig. 5B). Analysis of synergy using the Bliss model (12) indicated that the two-drug combination had synergistic cytotoxic effects in five of eight AML primary cells (#1, 2, 4, 6, and 7, Fig. 5A). Consistent with the cell line data, we observed a significant induction of apoptosis by the MGCD0103/GX15-070 combination in AML primary cells, accompanied by enhanced caspase-3 activation (Fig. 5C and D). Of importance, we also observed accumulation of LC3-II, indicative of autophagy (Fig. 5D).

**Discussion**

In this study, we show that the HDACi MGCD0103 or vorinostat in combination with the BH3 domain mimetic GX15-070 has synergistic antileukemia activity in AML. Although, individually, HDACi and GX15-070 have been reported to induce growth arrest and apoptosis in human cancer cells (3, 15), in this study a synergistic cytotoxic effect between these two types of agents occurred at concentrations in the nanomolar range. This is important as the IC50 values of MGCD0103 and vorinostat are in the micromolar range when used as single agents in vitro. It should be noted that there are limited data about the pharmacokinetic characteristics of MGCD0103. We have previously reported (3) the results of a phase 1 trial of this drug in leukemia. In this study, we could detect Cmax levels of 60 to 200 ng/mL at a dose of 80 mg/m2. This translated approximately to a concentration of 0.4 μmol/L. Therefore, the proposed levels could be achieved in humans with MGCD0103 in combination.
We also show that the synergistic antileukemia effect observed is accompanied by activation of both apoptosis and autophagy, and more importantly, both of them contribute to the synergistic cytotoxicity of the two-drug combination. Currently, the role of autophagy in cancer therapy remains controversial. Several studies have shown that the effect of autophagy on drug-treated cells is dependent on cell type, properties of drug, as well as duration of treatment. Here, we show that in cells treated with the HDACi/GX15-070 combination, there is evidence of autophagy accompanied by increased apoptosis and reduced cell viability in comparison with single-drug treatment. Although apoptosis accounts for the majority of cell death induced by the two-drug combination (based on the electron microscopy images and the effect of caspase-4), the death-promoting effect of autophagy observed in this study in leukemia indicates that the precise mechanism involved in induction of autophagy and how this leads to cell death need to be examined in more detail. Prior studies have shown that autophagy can specifically promote intrinsic pathway–induced apoptosis but not cell surface death receptor–activated apoptosis (37). Consistent with this, we observed upregulation of ER chaperone proteins (Grp) as well as caspase-4 in the MGCD0103/GX15-070

Fig. 5. Antiproliferative effect of the MGCD0103 and GX15-070 combination in primary cells from AML patients. A, cell viability was studied using trypan blue assays. Relative cell viability was calculated with the control group of each sample as 100%. In general, the combination of MGCD0103 with GX15-070 reduced cell viability more significantly in all eight patient samples compared with single-agent treatments. Synergistic effects, calculated using the Bliss model (12), were observed in patients 1, 2, 4, 6, and 7 (asterisk). B, using a similar approach as described in A, no obvious antiproliferative effect was detected in primary cells from normal controls. Columns, average from three control samples. C, stronger induction of apoptosis was observed in AML primary cells within 2 d of MGCD0103/GX15-070 combination treatment, but not in control primary cells. Relative levels of apoptosis were calculated with the levels of apoptosis from control treatments of patient and normal samples as 100%. D, increased levels of active caspase-3 and LC3-II were detected in AML primary cells within 2 d of MGCD0103/GX15-070 combination treatment. Equal loading was confirmed by blotting with an anti-actin antibody.
In ex vivo studies, we analyzed the effect of the combination against calpain 1 and calpain 2, which should be done.

Selective gene targeting studies, for instance using shRNAs, would be reasonable to hypothesize that GX15-070 can activate calpain, which could be implicated in the induction of cell death observed with these drugs. Because PD15060 is a pan-calpain inhibitor, specific effects on calpain 1 or calpain 2 cannot be elucidated in the experiments presented here. To analyze this in more detail, the effect of the combination was not analyzed in normal myeloblast counterparts (i.e., CD34+ cells), and therefore, this experiment should be considered as exploratory.

There are several limitations to the data presented here beyond what has already been discussed. The most important is the nonapoptotic but autophagic fraction of cells observed here. In our studies, we focused mainly on the morphologic aspects of the cells as detected by electron microscopy. That said, we did not directly analyze Annexin V-negative cells. Sorting and exclusion analysis could have provided access to that particular cell population. This would also have allowed investigating in more detail the role of ER stress in the transition to cell death. Finally, it would have been of interest to map the intracellular location of LC3-II. Analysis of this, for instance using GFP-LC3-II cells, would have added significant data for the analysis.

In summary, in the present study, we show that the combination of an HDACi with the BH3 domain mimic GX15-070 has synergistic antileukemia activity by inducing both apoptosis and autophagy. These results have implications for the clinical development of this type of combination in clinical trials in leukemia. Furthermore, the data presented here indicate that induction of autophagy, and not only of apoptosis, contributes to the activity of this type of combination. The role of autophagy needs to be more extensively studied in human clinical trials.

Disclosure of Potential Conflicts of Interest

J. Viallet: employee, Gemin X Pharmaceuticals. The other authors disclosed no potential conflicts of interest.

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References


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