THE COMBINATION OF A HISTONE DEACETYLASE INHIBITOR WITH THE BH3-MIMETIC GX15-070 HAS SYNERGISTIC ANTILEUKEMIA ACTIVITY BY ACTIVATING BOTH APOPTOSIS AND AUTOPHAGY

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TRANSLATIONAL RELEVANCE

Histone deacetylase inhibitors (HDACi) have limited but significant clinical activity in human acute myelogenous leukemia (AML). 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. Since 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 BH3 mimetic GX15-070 results in synergistic antileukemia activity by inducing both apoptosis and autophagy. These results are of significance as suggest that this combination should be tested in patients with leukemia and that the role of autophagy in leukemia therapy needs to be studied further.
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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 pro-apoptotic agent, such as the BH3 domain 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 AML cells.

Results: We demonstrated that the combination had synergistic antileukemia effect both in cell lines and primary AML cells. Using molecular markers and electron microscopy, we observed that in addition to apoptosis, autophagy accounts for the non-apoptotic decrease of 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 of this combination.

Conclusions: The combination of and HDACi and GX15-070 has synergistic antileukemia activity and effect is mediated both 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.
INTRODUCTION

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, resulting in reactivation of aberrantly suppressed genes, leading to inhibition of proliferation, induction of apoptosis and cell differentiation.\(^4\) Multiple HDACis are in clinical development,\(^1\)\(^,\)\(^5\) and vorinostat, a hydroxamic acid derivative, is approved in the US for patients with cutaneous lymphoma.\(^6\) MGCD0103, an isotype-specific small molecule HDACi targeting HDAC isotypes 1, 2, 3, and 11, has been demonstrated to be orally bioavailable with significant \textit{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 HDACis. GX15-070 (obatoclax) is a novel Bcl-2 homology domain-3 (BH3) mimic, that has been demonstrated to induce apoptosis in acute myeloid 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 anti-leukemia effect of HDACis,\(^4\) we hypothesized that blocking anti-
apoptotic pathways with GX15-070 may enhance the antileukemia activity of HDACis. This is of clinical importance as GX15-070 has been recently reported to have clinical activity in chronic lymphocytic leukemia (CLL)\(^8\) and potentially other leukemias. Therefore, we investigated the antileukemia activity of the combination of GX15-070 with MGCD0103, \(^2,3\) a class I specific HDACi, and with vorinostat, a paninhibitor of HDAC. \(^6\) We demonstrate a synergistic antileukemia effect between HDACis and GX15-070 in multiple AML cell lines, and that this effect involves induction of calpain-associated apoptotic and autophagic pathways. These results indicate that the combination of GX15-070 with HDACis effectively increases the antileukemia activity of these two drugs, and should be studied in human clinical trials.

MATERIAL AND METHODS

Cell lines, primary AML samples and Reagents HL-60, THP1 and U937 cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown following standard conditions. Peripheral blood samples (N=8) were obtained for \textit{in vitro} studies from patients diagnosed with AML at M.D. Anderson Cancer Center (MDACC) following institutional guidelines. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density-gradient centrifugation. For cell proliferation analysis, AML cells were counted using trypan blue exclusion assays. GX15-070 was provided by Gemin X (Malvern, PA). MGCD0103 was provided by Methylgene Inc. (Montreal, Quebec, Canada), and vorinostat by Merck & Co., Inc (Whitehouse Station, NJ). PD15060 was purchased from Calbiochem (Cambridge, MA), chloroquine was from Sigma (St. Louis, MI), and Z-LEVD-FMK from Biovision (Mountain View, CA). Antibodies used include A-caspase 3 (eBiosciences, San Diego, CA), PARP (BD, Franklin Lakes, NJ), Puma, Calpain 2, LC-3, Grp78, Grp94, ATG12 and caspase 4.
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(Cell Signaling, Beverly, MA), MCL1, BAK1, Bax, BclXL, and Noxa (Santa Cruz Biotech.), Ac-H3 and Ac-H4 (Millipore, Billerica, MA).

Analysis of apoptosis Apoptosis was quantitated by flow cytometry using PI/Annexin V FITC kit (BD Biosciences, San Jose, CA) following manufacturer guidelines. Annexin V fluorescence was quantitated with a Becton Dickinson FACS Calibur or LSR II flow cytometer (BD Biosciences, San Jose, CA).

Transmission Electron Microscopy This analysis was performed at the Electron Microscopy Core Facility at MDACC. Cells were harvested, pelleted, and fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1M 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 Spurre’s low viscosity medium. Ultrathin sections were cut in a Leica Ultracut microtome (Leica, Deerfield, IL), 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 80kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

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

Statistical Analysis Analysis of the effect of the combination was performed using a two-term tumor repopulation model. 9, 10, 11 Synergy detection of the drugs on primary leukemia cells was analyzed using the Bliss independence model.12, 13

RESULTS

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

We first evaluated the anti-leukemia effect of HDACis in combination with GX15-070 in leukemia cell lines. In human AML cell lines HL-60, THP1 and U937, three days exposure to the combination of MGCD0103 (600 nM) and GX15-070 (200 nM) reduced cell viability more effectively than single drug treatment (supplemental Fig. 1A, 1B and 1C). We then analyzed potential synergistic effect between 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 96 hr). For each combination at different concentration levels, the interactive index was calculated using cell viability data from four time points. Results from this analysis indicated that in HL-60 and THP1 cells, interactive indexes from all different concentration combinations were below 0.4 (Table 1A and 1B), indicating a strong synergistic antiproliferative effect between MGCD0103 and GX15-030. 10, 11 Two-term synergy analysis was also performed 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 BH3 mimetic GX15-070 results in synergistic cytotoxicity on AML cells.
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**Induction of apoptosis with the combination of HDACi and GX15-070**

Both GX15-070 and HDACis can induce apoptosis in leukemia cells.\(^3, 7, 8, 14\) Therefore we analyzed the induction of apoptosis with the two drug combination. HL-60 cells were treated with MGCD0103 (600 nM) and GX15-070 (200 nM) alone or in combination for 72 hrs. As shown in Fig. 1A, while MGCD0103 and GX15-070 induced modest levels of apoptosis in HL-60 cells, the two drug combination increased the fraction of apoptotic cells significantly. Similar results were observed in three independent experiments (Fig. 1B). Apoptosis at different time points (day 2, 3 and 4) was also monitored, and similar results were observed (supplemental Fig. 1D). Further, after 48 hrs of drug treatment, we observed enhanced activation of caspase 3 in MGCD0103/ GX15-070 combination treated cells (Fig 1C), accompanied by increased cleavage of Poly (ADP-ribose) polymerase (PARP) (Fig 1C).\(^15, 16\) Significant induction of apoptosis and caspase 3 activation were also observed in HL-60 cells treated with vorinostat/ GX15-070 combination (supplemental Fig. 2A and 2B). These results suggest that induction of apoptosis contributes to the HDACi and GX15-070 combination mediated antileukemia effect.

**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 agent HDACi and GX15-070 in multiple malignancies.\(^17, 18, 19\) Since the role of autophagy in chemotherapy-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
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treated with MGCD0103/ GX15-070 and vorinostat/ GX15-070 combination (Fig. 2A). A low level of LC3-II induction compared to the effect with 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, particularly in cells treated with MGCD0103/ GX15-070 combination (Fig. 2A).

We then used electron microscopy (EM) to examine the ultrastructural morphology of treated cells. EM 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 non-apoptotic cells and observed that after treatment with the two drug combination, approximately 40% of non-apoptotic cells carried small vesicles containing multiple cellular organelles in cytoplasm (Fig. 2B). Under higher magnification, we observed that those vesicles had double or multiple membrane boundaries (Fig. 2B), a feature of autophagosomes. In contrast, no autophagosome was observed in GX15-070 or MGCD0103 single drug treated cells (Fig. 2B and supplemental Fig. 3). Of importance, about 60% of non-apoptotic cells after the 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, 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. While the addition of CQ to single agent MGCD0103 or GX15-070 increased relative cell
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viability by approximate two 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, 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 positively 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, that are released by GX15-070 from interacting antiapoptotic partners. We therefore studied the effect of 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 Mcl1, no significant difference at the protein levels was observed with the two drug combination (Supplemental Fig. 4A). We did not observe either further induction of histone H3 or H4 acetylation by GX15-070/ MGCD0103 combination compared to MGCD0103 alone (supplemental Fig. 4B).

Calcium-dependent cysteine protease (calpain) has been implicated in both apoptotic and autophagic cell death, including HDACi induced apoptosis. To examine whether calpain activity contributes to the cytotoxicity induced by the two drug combination, we studied the effect...
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on cytotoxicity of calpain inhibitor PD15060. Addition of PD15060 (25 μM) increased cell viability more efficiently in MGCD0103/GX15-070 combination treated cells compared to 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 treatment of MGCD0103/GX15-070 combination, addition of PD15060 caused a reduction of both cleaved PARP and LC3-II (Fig. 3B, lane 8 in comparison to lane 4), implying that calpain activity is involved in both apoptotic and autophagic processes. Since MGCD0103 is an HDACi and can potentially exert its cytotoxic effect through gene reactivation, we analyzed the expression of two forms of calpain, calpain 1 and calpain 2. Up-regulation of calpain 2, but not calpain 1, was observed in cells treated with single agent MGCD0103 and two drug combination at the RNA level (Fig. 3C). 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 to 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 EM images of cells treated with single agent GX15-070, we noticed morphology of expanded 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. We analyzed several ER chaperon proteins that are positively associated with ER stress and
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observed increase of glucose-regulated protein (GRP) Grp78 and GRP94 \(^{32}\) in GX15-070 single agent treated cells, and a higher level of induction of these two Grps in MGCD0103/ GX15-070 combination treated cells (Fig. 3D). Similar induction of Grp78 in cells treated with vorinostat/ GX15-070 combination was also observed (Supplemental Fig. 4C). Grp upregulation confirmed the induction of ER stress by GX15-070. Since ER stress, and its progression into cell death, has been positively associated with Grp levels, the higher induction of Grp78 and Grp94 with the two drug combination treated cells (Fig. 3D) indicates that combining MGCD0103 with GX15-070 promotes ER stress to transit into cell death. Since calpain-caspase 12 and calpain-caspase 4 signaling has been known to be pivotal for ER stress cell death transition in mouse and human cells \(^{33, 34, 35, 36}\), we hypothesized that calpain induction could activate caspase 12/caspase 4 signaling. This would promote GX15-070 induced ER stress to progress to cell death. We analyzed levels 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, as well as induction of Grps, with the two drug combination were both repressed by the addition of calpain inhibitor PD15060 (Supplemental Fig. 4D). These results strongly support our hypothesis and suggest that 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 caspase 4 inhibitor Z-LEVD-FMK on the two drug combination mediated cytotoxicity. Addition of 10 \(\mu\)M of Z-LEVD-FMK caused 2-fold increase of viability in cells treated with 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
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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 MGCD0103 and GX15-070 combination in AML, we evaluated its antiproliferative effect in primary mononuclear peripheral blood leukemia cells. Patient characteristics are shown in supplemental Table 1. Using trypan blue analysis, MGCD0103/GX15-070 combination induced stronger cytotoxicity in AML primary cells compared to 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 indicated that the two-drug combination had synergistic cytotoxic effects in 5 out of 8 AML primary cells (#1, 2, 4, 6, and 7, figure 5A). Consistent with the cell line data, we observed significant induction of apoptosis by MGCD0103/ GX15-070 combination in AML primary cells accompanied by enhanced caspase-3 activation (Fig. 5C and 5D). Of importance, we also observed accumulation of LC3-II, indicative of autophagy (Fig. 5D).

DISCUSSION

In this study, we demonstrate that HDACi MGCD0103 or vorinostat in combination with the BH3 domain mimetic GX15-070 have synergistic antileukemia activity in AML. Although individually both HDACi and GX15-070 have been reported to induce growth arrest and apoptosis in human cancer cells, 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 of MGCD0103...
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and vorinostat are in the micromolar range when used as single agents in vitro. It should be noted that there is limited data regarding the PK characteristics of MGCD0103. We have previously reported 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 uM. Therefore the proposed levels could be achieved in humans with MGCD0103 in combination.

We also demonstrate that the synergistic antileukemia effect observed is accompanied by activation of both apoptosis and autophagy, and that both of them, including autophagy, 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 HDACi/GX15-070 combination there is evidence of autophagy accompanied by increased of apoptosis and reduced cell viability in comparison to single drug treatment. Although apoptosis accounts for the majority of cell death induced by the two drug combination (based on the observation from EM images and from 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 needs to be examined in more detail. Prior studies have shown that autophagy can specifically promote intrinsic pathway induced apoptosis but not cell surface death receptors activated apoptosis. Consistent with this, we observed upregulation of ER chaperon proteins (Grp) as well as caspase-4 in the MGCD0103/ GX15-070 combination treated cells. While these proteins are located in ER and are implicated in intrinsic apoptotic...
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inducing pathways, these results indicate that both autophagic death as well as induction of apoptosis by autophagy contribute to the synergistic antileukemia effect observed here.

In this study, we also demonstrate that calpain activity plays an important role in the synergistic cytotoxicity of HDACi/ GX15-070 combination. Calpain activity has been implicated at multiple levels in apoptosis and autophagy induction through a wide range of proteolysis substrates, in both caspase dependent and independent fashion. Particularly with the two drug combination our data indicates that one essential mechanism for the effect of calpain relates to the transition from GX15-070-induced ER stress to cell death. This is partially mediated by caspase 4. While calpain inhibitor PD15060 decreased both apoptosis (cleaved PARP) and autophagy (LC3-II) at a comparable level, caspase 4 inhibitor Z-LEVD-FMK had a more significant effect on repressing the major apoptotic signaling mediator caspase 3 than on repressing autophagy marker LC3-II. This result suggests that the major signals mediated by caspase 4 activation are pro-apoptotic. The signal for calpain activated ER stress to autophagy transition still needs to be determined. We also noticed that calpain inhibitor PD15060 could not completely restore cell viability, which implies that other calpain activity independent cell death mechanisms are induced by the two drug combination.

Of importance, ER stress has also been known to activate calpain activity through mechanism independent of expression induction. Therefore, although we have shown in this study that MGCD0103 induces calpain through upregulating expression of calpain 2, it would be also reasonable to hypothesize that GX15-070 stress can also have an effect on calpain 1 and calpain 2 activity. When combined, GX15-070 and MGCD0103 may cause synergistic activation
of calpain, which could be implicated in the induction of cell death observed with these drugs. Because PD15060 is a pancelpain inhibitor specific effects on calpain 1 or 2 cannot be elucidated in the experiments presented here. To analyze this in more detail selective gene targeting studies, for instance using shRNAs against calpain 1 and calpain 2, should be performed.

To further study the relevance of the in vitro data presented here, we analyzed the effect of the combination ex vivo in cells from 8 patients with AML. In concordance with the in vitro data, we observed that the combination was synergistic in a majority of the patient samples analyzed and that this was mediated by apoptosis and autophagy as represented by levels of caspase 3 and LC3-II in western blots. That said the analysis of synergism was performed using only one concentration with the Bliss model. More information regarding different concentrations and sequences are required to further elucidate the activity of this combination. Furthermore the effect of the combination was not analyzed in normal myeloblasts, i.e. CD34+ cells, counterparts 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 regards to the non-apoptotic but autophagic fraction of cells observed here. In our studies we focused mainly on morphological aspects of the cells as detected by EM. That said we did not directly analyzed 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 a GFP-LC3-II cell would have added significantly to the paper.

In summary, in the present study, we demonstrate that the combination of an HDACi with the BH3 domain mimetic GX15-070 has synergistic antileukemia activity by inducing both apoptosis as well as autophagy. These results have implications for the clinical development of this type of combination in clinical trials in leukemia. Furthermore, the data presented here indicates 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.

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FIGURE LEGEND

Fig. 1 HDACi and GX15-010 combination induces apoptosis in HL-60 cells. (A) Analysis of apoptosis was performed using annexin V flow cytometry assays in cells after 72 hr of treatment with drugs indicated (MGCD0103 600nM and/ or GX15-070 200nM). Upper-right and lower-right windows represent annexin V positive apoptotic cells. A significant increase of apoptotic cell population was observed in MGCD0103/ GX15-070 combination treatment. (B) Summary of apoptosis flow cytometry analysis with MGCD0103 and GX15-070 treatment. Numbers on the y-axis represent percentage of apoptotic cells. (C) Western blot analyses performed after 48hr of drug treatment confirmed the significant increase of apoptosis by showing higher level of active caspase 3 accompanied by elevation of cleaved form of PARP in cells treated with MGCD0103 and GX15-010 combination. Equal loading was confirmed by blotting with an anti-actin antibody. MGCD, MGCD0103; GX, GX15-070.

Fig. 2 MGCD0103 and GX15-010 combination induced autophagy in HL-60 cells.

Autophagy was analyzed in cells after 3 days of treatment (MGCD0103 600nM and/ or GX15-070 200nM). (A) Western blot analyses performed after 48hr of drug treatment showed significant upregulation of LC3-II and ATG5-ATG12 conjugation in cells treated by MGCD0103/GX15-070 (higher panel), and upregulation of LC3-II in vorinostat/ GX15-070 combination (lower panel). 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 MGCD0103/GX15-070 combination treated cells in EM images (upper panels). High resolution (25000X) image with arrows pointing to autophagosomes is from the squared region from the lower resolution (7500X) image. Expanded ER was
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specifically observed in cells treated with GX15-070 alone (lower panels). High resolution (25000X) image for the ultra structure of ER is from the squared region from the lower resolution (7500X) image. EM images were taken after 72 hr of drug treatment. (C) Relative cell viability was calculated using control groups (CQ- and CQ+ respectively) as 100%, while fold of changes was calculated by comparing drug treated groups to their control group (CQ- or CQ+). While the addition of CQ increased relative cell viability compared to the same treatments without CQ (black bar v.s. while bar), a more significant increase with the addition of CQ can be detected in MGCD0103/ GX15-070 combination than in MGCD0103 or GX15-070 alone. Trypan blue analysis was used to analyze cell viability after 72 hr 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.

MGCD, MGCD0103; GX, GX15-070; CQ, chloroquine.

Fig. 3 Calpain activity is positively involved in apoptosis and autophagy induction by MGCD0103/ GX15-010 combination. Cells were analyzed after 3 days of treatment (MGCD0103 600nM and/ or GX15-070 200nM). (A) Relative cell viability was calculated using control groups (PD15060- and PD15060+ respectively) as 100%, while fold of changes was calculated by comparing drug treated groups to their control group (PD15060- and PD15060+). While the addition of PD15060 increased cell viability compared to the same treatments without PD15060 (black bar v.s. while bar), a more significant increase with the addition of PD15060 can be detected in MGCD0103/ GX15-070 combination compared to MGCD0103 or GX15-070 alone. Trypan blue analysis was used to analyze cell viability after 72 hr of drug treatment. (B) Western blot analysis reveals reduction in cleaved PARP and LC3-II in MGCD0103/ GX15-070 combination treated cells with the addition of PD150606. Equal loading was confirmed by
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blotting with an anti-actin antibody. (C) Quantitative RT-PCR analysis showed that MGCD0103 and two drug combination increased calpain 2 RNA levels at a similar level. Average of 3 Q-PCR results is shown. (D) Western blot analyses showed increase of calpain 2 expression at protein level in MGCD0103 and two drug combination treated cells, increase of ER chaperon protein Grp78 and Grp 94 in GX15-070 and two drug combination treated cells (with a slight stronger induction with two drug combination treatment), as well as increase of cleaved caspase 4 specifically in two drug combination treated cells. MGCD, MGCD0103; GX, GX15-070; PD, PD15060.

Fig. 4 Caspase 4 activity positively associates with MGCD0103/ GX15-010 combination induced cytotoxicity. (A) Relative cell viability was calculated using control groups (Z-LEVD-FMK- and Z-LEVD-FMK+ respectively) as 100%, while fold of changes was calculated by comparing drug treated groups to their control group (Z-LEVD-FMK- and Z-LEVD-FMK+ respectively). While the addition of Z-LEVD-FMK increased relative cell viability for about 2 folds in cells treated with MGCD0103/ GX15-070 combination compared the same treatment without Z-LEVD-FMK (black bar v.s. white bar), no obvious effect can be detected in MGCD0103 or GX15-070 alone treated cells. (B) Western blot showed that while MGCD0103/ GX15-070 combination increased active form of caspase 3 and LC3II, the addition of Z-LEVD-FMK significantly repressed this two drug combination induced A-caspase 3, whereas the repressing effect of Z-LEVD-FMK on LC3II induction was less significant. MGCD, MGCD0103; GX, GX15-070.
HDACi and GX15-070 combination in leukemia

Fig. 5 Antiproliferative effect of 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 using control group of each sample as 100%. In general, the combination of MGCD0103 with GX15-070 reduced cell viability more significantly in all 8 patient samples compared to single agent treatments. By calculation using the Bliss model\textsuperscript{12}, synergistic effects were observed in patient 1, 2, 4, 6, and 7 (asterisk). (B) With similar approach as described in A, no obvious antiproliferative effect was detected in primary cells from normal controls. Averages of results from 3 control samples are presented. (C) Stronger induction of apoptosis was observed in AML primary cells with 2 days of MGCD0103/GX15-070 combination treatment, but not in control primary cells. Relative levels of apoptosis were calculated by using levels of apoptosis from control treatments of patient and normal samples as 100%. (D) Increase of active caspase 3 and LC3-II were detected in AML primary cells with 2 days of MGCD0103/GX15-070 combination. Equal loading was confirmed by blotting with an anti-actin antibody. MGCD, MGCD0103; GX, GX15-070.
REFERENCES


HDACi and GX15-070 combination in leukemia


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Table 1. **Summary of synergy analysis between HDACi and GX15-070 in AML cell lines using two-term model.** Interactive indexes were calculated using 4 time points for each combination. An index below 0.4 indicates a synergistic effect. (A) and (B) Results for the combination between MGCD0103 and GX15-070 in HL60 and THP1 cells respectively. (C) Result for the combination between vorinostat and GX15-070 in HL60 cells.

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

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HDACi and GX15-070 combination in leukemia

**Fig. 1**

A

B

C
HDACi and GX15-070 combination in leukemia

Fig. 2

A

B

C

D

Fig. 2

A

B

C

D
HDACi and GX15-070 combination in leukemia

Fig. 3
HDACi and GX15-070 combination in leukemia

Fig. 4

A

![Graph showing relative level of survival (percentage of control) for no Z-LEVD-FMK and Z-LEVD-FMK 10μM]

B

![Images of Western blots showing A-caspase 3, LC3-I, LC3-II, and β-actin]
HDACi and GX15-070 combination in leukemia

**Fig. 5**

(A) AML Patient Primary Cells

(B) Normal Cells

(C) AML primary cell

(D) Western blot analysis for A-Caspase3, LC3-I, LC3-II, and β-Actin.
Clinical Cancer Research

The Combination of a Histone Deacetylase Inhibitor with the BH3-Mimetic GX15-070 has Synergistic Antileukemia Activity by Activating Both Apoptosis and Autophagy

Yue Wei, Tapan Kadia, Weigang Tong, et al.

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