A Novel Mcl-1 Inhibitor Synergizes with Venetoclax to Induce Apoptosis in Cancer Cells

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Abstract

Background

Evading apoptosis by overexpression of anti-apoptotic Bcl-2 family proteins is a hallmark of cancer cells and the Bcl-2 selective inhibitor venetoclax is widely used in treatment of hematologic malignancies. Mcl-1, another anti-apoptotic Bcl-2 family member, is recognized as the primary cause of resistance to venetoclax treatment. However, there is currently no Mcl-1 inhibitor approved for clinical use.

Methods

Paired parental and Mcl-1 knockout H1299 cells were used to screen and identify a small molecule named MI-238, which could specifically kill Mcl-1 proficient cells. Molecular docking, fluorescence polarization (FP) and Isothermal titration calorimetry (ITC) were employed to study MI-238 binding to Mcl-1 protein. Immunoprecipitation (IP) and flow cytometry assay were performed to analyze the activation of pro-apoptotic protein Bak. Annexin V staining and western blot analysis of cleaved caspase 3 were employed to measure the cell apoptosis. Mouse xenograft AML model using luciferase-expressing Molm13 cells were employed to evaluate in vivo therapeutic efficacy. Bone marrow samples from newly diagnosed AML patients were collected to evaluate the therapeutic potency in clinical AML patients.

Results

Here, we show that MI-238, a novel and specific Mcl-1 inhibitor, can disrupt the association of Mcl-1 with BH3-only pro-apoptotic proteins, selectively leading to apoptosis in Mcl-1 proficient cells. Moreover, MI-238 treatment also potently induces apoptosis in acute myeloid leukemia (AML) cells. Notably, the combined treatment of MI-238 with venetoclax exhibited strong synergistic anti-cancer effects in AML cells in vitro, MOLM-13 xenografts mouse model and AML patient samples.

Conclusions

This study identified a novel and selective Mcl-1 inhibitor MI-238 and demonstrated that development of MI-238 provides a novel strategy to improve the outcome of venetoclax therapy in AML.

Introduction

Apoptosis, one of the programmed cell death, occurs normally during development and aging, and it plays critical roles in maintaining tissue homeostasis[1]. Two major types of conserved signaling pathways, including intrinsic and extrinsic pathway have been established to execute apoptosis[2]. Intrinsic apoptosis is associated with mitochondria outer membrane permeabilization (MOMP), which subsequently causes the release of cytochrome C from mitochondria into cytosol to activate cascade of caspases cysteine proteases[3, 4]. While, extrinsic apoptosis is regulated by death receptors binding to its ligands, such as TNFR1/TNF[5, 6]. The intrinsic mitochondria apoptosis is controlled by Bcl-2 family proteins, which share structural homology in one to four conserved regions named Bcl-2 homology (BH)

domain[7]. In response to apoptotic stimuli, pro-apoptotic Bcl-2 proteins, such as Bax and Bak, oligomerize at the mitochondrial outer membrane and trigger MOMP[8–10]. Meanwhile, anti-apoptotic Bcl-2 proteins, such as Bcl-2, Mcl-1 and Bcl-xL, reside in the mitochondrial outer membrane and prevent pro-apoptotic protein mediated oligomerization and MOMP[7, 11].

The major anti-apoptotic Bcl-2 family members including Bcl-2, Mcl-1 and Bcl-xL have been observed to be overexpressed in various cancer cells, which confer cancer cell resistance to apoptosis[7]. Therefore, targeting these anti-apoptotic proteins to induce apoptosis has been utilized as a useful strategy to treat and prevent cancers[12, 13]. The anti-apoptotic Bcl-2 family members possess structural similarity to bind on BH3 domain of pro-apoptotic proteins, and this hydrophobic surface binding pocket within anti-apoptotic Bcl-2 proteins is named BH3 binding pocket, which is required to its anti-apoptotic function[14]. Synthetic peptides that fit into this pocket have been shown to induce apoptosis in vitro and in vivo[15]. Through structural-based drug design, small-molecule BH3 mimetics such as ABT-199, ABT-737 and ABT-263, which could bind to the hydrophobic pocket, have been developed[16–18]. These small-molecule BH3 mimetics induce apoptosis in various cancer cells and possess potent anti-tumor efficacy[17]. Through long period of clinical trials, ABT-199 (trade name venetoclax) was finally approved as monotherapy or combination therapy for the treatment of hematologic malignancies including chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML)[19].

However, venetoclax selectively binds to Bcl-2 and has limited efficacy against cancer cells that depends on other anti-apoptotic proteins for survival such as Mcl-1[20]. Treatment of venetoclax has been reported to increase the binding of Mcl-1 to pro-apoptotic proteins, such as Bax, Bim and Bak[21]. Therefore, it is well-established that Mcl-1 plays critical roles in venetoclax resistance, and combination treatment of venetoclax with Mcl-1 inhibitors induces synergistic anti-tumor activity and eradicates venetoclax-resistant cancers[19, 21]. Therefore, development of specific Mcl-1 inhibitor could be used not only as apoptosis inducer, but also in venetoclax combination therapy. Although several Mcl-1 inhibitors have been developed and exhibited promising anti-tumor efficacy in pre-clinical leukemia or solid tumor animal models[19, 22, 23], there is still a lack of Mcl-1 inhibitor approved for clinical use. In present study, we show that a novel small molecule named MI-238 could bind to Mcl-1 and selectively induced apoptosis in Mcl-1 proficient cells, but not in Mcl-1 deficient cells. The combined treatment with venetoclax and MI-238 induced synergistic anti-tumor effects in AML cells *in vitro*, xenograft mouse model and patient samples.

Materials And Methods

Cell lines, plasmids and antibodies

Parental and Mcl-1 knockout (KO) H1299 cells were maintained in RPMI 1640 medium supplemented with 10% FBS as our previously described[24]. Wild type (WT) and Mcl-1 KO MEF (mouse embryonic fibroblast) cells were cultured in DMEM medium supplemented with 10% FBS as ourpreviously described[24]. Anti-Mcl-1 (#94296), anti-cleaved caspase 3 (#9661) and anti-Bim (#2933) were purchased from Cell Signaling Technology (MA, USA). Anti-Bcl-2 (sc-7382), anti-Bax (sc-7480), anti-PARP1 (sc-8007)

and β-Actin (sc-8432) were obtained from Santa Cruz Biotechnology (CA, USA). Anti-Cytochrome C (ab133504), Anti-Bcl-xL (ab32370) and Anti-Bak (ab32371) were purchased from Abcam (Cambridge, MA).

Isothermal titration calorimetry (ITC)

The binding affinity of MI-238 with GST-Mcl-1 was examined by isothermal titration calorimetry (ITC) assay as described[24]. Briefly, ITC assay was carried out in the auto-iTC200 instrument (MicroCal, GE) and Mcl-1 protein was loaded into a 96 Deepwell PP plate. MI-238 compound was then titrated stepwise into the protein for a total of 16 injections. Reference power and initial delay were set as 5 μCal/sec and 60s, respectively. A string speed of 750 rpm was used for the ITC measurements. The binding constant (Kd) value was determined by fitting the titration curve to a one-site binding mode.

Immunoprecipitation and GST pull-down assay

Cells were lysated in ice-cold EBC buffer (0.5% NP-40, 50 mM Tris-HCl, pH 7.6, 120 mM NaCl, 5mM $CaCl_2$, 5mM $Mgcl_2$ and 1 mM β-mercaptoethanol) with protease inhibitor cocktail (TargetMol, China) by sonication. After centrifuge, the cell lysates were incubated with anti-Mcl-1, anti-Bcl-2, or anti-Bcl-xL antibody and Protein A/G-agarose beads (Santa Cruz, CA) overnight at 4℃ with rotation. After washing, beads were boiled in 30 μl SDS-PAGE loading buffer for 6 minutes and subjected to SDS-PAGE and analyzed by Western blotting. For GST pull-down assay, GST-fused Mcl-1 proteins were incubated with glutathione sepharose 4B beads (GE healthcare) in TBS buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) with protease inhibitor cocktail at 4℃ for 4 hours. After washing, the beads coated with GST-Mcl-1 proteins were incubated with recombinant Bak protein in TBS buffer in presence of increasing concentrations of MI-238 at 4℃ overnight. After washing, the samples were subjected to SDS-PAGE and analyzed by Western blotting.

Annexin-V staining assay

Cells were treated with MI-238 or venetoclax for 48 hours before apoptosis analysis. The percentage of apoptotic cells were measure using annexin-V apoptosis detection kit (BD Biosciences, NJ, USA) according to the manufacturer's instruction. Briefly, 1×10^6 of drug treated cells were incubated with annexin-V-FITC in binding buffer for 45 minutes in dark at room temperature. Then, 50 μg/ml of propidium iodide (PI) was added before analyzing by flow cytometry.

Flow Cytometry assay of Bak activation

For the detection of conformational changes of BAk, 2×10^6 of Molm13 cells were fixed with 2% paraformaldehyde (Sangon Biotech) for 30 minutes on ice, followed by permeabilization with 0.5% triton X-100 (Sangon Biotech) for 30 minutes at room temperature and blockingwith 5% goat serum for 30 minutes at room temperature. Cells were then labeled for 30 minutes with 1 mg/mL of antibodies against

the active form of Bak (clone G317-2; BD Pharmingen). After incubation with FITC-conjugated anti-mouse secondary antibody (Cell Signaling Technology), cells were analyzed by flow cytometry.

Immunohistochemistry (IHC)

Mouse bone marrow was harvested for Immunohistochemistry to identify AML burden. Briefly, Mice were anesthetized by inhaling isoflurane.The stripped tibia or femur was fixed in 4% paraformaldehyde, and decalcified in EDTA regent for two weeks, embedded in paraffin. Longitudinal sections (4 μm) of tibia or femur were prepared. After antigen retrieval, the slices were incubated in 3% (V/V) H_2O_2 at room temperature for 25 minutes to block endogenous peroxidase activity. Nonspecific staining was further blocked with BSA at room temperature for 30 minutes. The sections were incubated with anti-hCD45(Cell Signaling Technology) antibody at 4 ℃ overnight. The corresponding secondary antibody incubated the tissue for 50 minutes at room temperature. Then, the peroxidase reaction was observed with DAB peroxidase substrate. After counterstaining with hematoxylin, the slides were dehydrated, mounted, and visualized with Leica light microscope (Leica, Wetzlar, Germany).

Patient samples

Bone marrow samples from newly diagnosed AML patients were used to evaluate the anti-leukemia response of MI-238. AML patients and donor volunteers involved in this study signed a consent form. Bone marrow mononuclear cells were isolated by Ficoll gradient centrifugation. Then, primary AML cells were cultured in α-MEM medium supplemented with 20% FBS, 1% penicillin/streptomycin, and 10ng/ml recombinant human cytokine, including SCF, TPO, FLT-3 ligand, IL-3 and IL-6. All cytokines were purchased from Peprotech (NJ, USA). The primary AML cells were cultured with different concentrations of MI-238 or in combination with venetoclax for 48 hours before apoptosis analysis by flow cytometry.

Xenograft model

Cell line-Derived xenograft(CDX)was implemented to confirm the anti-leukemia effect of MI-238 in vivo as previously described[17]. Female NCG (NOD/ShiLtJGpt-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Gpt) mice, 8–10 weeks old, were inoculated with 7×10^5 (in 200μl PBS)luciferase-expressing Molm13 cells through the tail vein. After the AML mouse model was successfully constructed, MI-238 (70mg/kg) or vehicle was intraperitoneally injected, and venetoclax (50mg/kg) or vehicle was administered by daily gavage for two weeks. On days10, 17 and 24, AML burden was determined by fluorescence imaging. On day 24, the proportion of hCD45/hCD33 (Biolegend, 368504 and 366618) cells in peripheral blood was detected by flow cytometry. The above experimental protocol was approved by the ethics committee of Jinan University.

Statistics

All data were presented as mean ± standard deviation (SD) from at least three independent replicates. Statistical comparisons of two samples were performed using two-tailed student's t-test and $p < 0.05$ was

considered as statistical significance. Kaplan–Meier method was performed to analyze differences in the animal survival.

Results

MI-238 is a novel and potent Mcl-1 inhibitor

As an important pro-survival protein, Mcl-1 is over-expressed in various types of cancer. However, no specific Mcl-1 inhibitor is currently available for clinical use. Through cytotoxicity screening using the paired H1299 parental and Mcl-1 knockout (KO) cells, we discovered a small molecule that selectively inhibited the viability of H1299 parental cells, but not Mcl-1 KO cells (Figure S1), and we named this compound MI-238 (Fig. 1A). MI-238 was docked into Mcl-1 BH3 binding groove and proximal to the BH1 domain of Mcl-1 (Fig. 1A). To directly measure MI-238/Mcl-1 binding, we prepared GST tagged Mcl-1 recombinant protein (Fig. 1B). MI-238 exhibited a Ki of 0.45 ± 0.05 μM to human Mcl-1 protein in FP (fluorescence polarization) assay (Fig. 1C). Meanwhile, Isothermal Titration Calorimetry (ITC) assay also showed that MI-238 binding to GST-Mcl-1 with Kd of 0.23 μM, meanwhile, MI-238 did not bind with GST protein (Fig. 1D, E). Mcl-1 exerts its pro-survival function through binding with BH3-only pro-apoptotic proteins, such as Bak. To test whether MI-238 binding to Mcl-1 could disrupt Mcl-1/Bak association, purified Mcl-1 and Bak proteins were incubated in presence of increasing concentrations of MI-238. As shown in Fig. 1F, MI-238 disrupted Bak/Mcl-1 interaction in a dose-dependent manner *in vitro*. Collectively, these results demonstrated that MI-238 directly binds to Mcl-1 and inhibits Mcl-1 anti-apoptotic function.

MI-238 selectively induces apoptosis in Mcl-1 proficient cells.

To measure whether MI-238 induced apoptosis depends on Mcl-1, we generated H1299 Mcl-1 KO cells[24]. Knockout of Mcl-1 in H1299 and MEF cells did not significantly affect the expression Bcl-2 protein. However, BH3-only proteins including Bak, Bim and Bax were shifted to Bcl-2 and Bcl-xL in Mcl-1 deficient cells, which indicating Mcl-1 KO cells relied more on Bcl-2 and Bcl-xL for survival (Fig. 2A, C, Figure S2). The Annexin V apoptosis assay revealed that 20 μM of MI-238 treatment induced apoptosis in 50.1 ± 1.1% of parental H1299 cells, but intriguingly, 20 μM MI-238 failed to induce apoptosis in Mcl-1 deficient H1299 cells (Fig. 2B). Cleavage of caspase 3 initiates apoptotic DNA fragmentation and is recognized as an apoptosis hallmark[1]. Consistent with Annexin V assay, MI-238 treatment caused caspase 3 cleavage in H1299 parental cells, but not in Mcl-1 KO cells (Fig. 2E). Similarly, we found that MI-238 treatment also induced apoptosis in wild type (WT) mouse embryonic fibroblast (MEF) cells, but not in Mcl-1 KO MEF cells (Fig. 2D, F). These results demonstrated that MI-238 induced apoptosis is dependent on proficient Mcl-1.

Mi-238 Effectively Kills Aml Cells

Induction of apoptosis through targeting Bcl-2 anti-apoptotic proteins is an effective therapeutic strategy for hematologic malignancies, and Bcl-2 inhibitor venetoclax is widely used to treat CLL and AML[19]. To

test the therapeutic efficacy of MI-238 in AML cells, we treated a variety of AML cells with increasing concentrations of MI-238 and found that the IC_{50} of MI-238 against AML is around 5–30 μM (Fig. 3A). In addition, the IC_{50} of MI-238 was inversely proportional to the Mcl-1 protein levels among AML cells (Fig. 3B, C). We then examined the PARP1 and caspase 3 cleavage, the well-known apoptosis markers in Molm13 and MV-4-11 cells after MI-238 treatment [1]. As shown in Fig. 3D, MI-238 induced PARP1 and caspase 3 cleavage in a dose-dependent manner, which indicating MI-238 potently induces apoptosis in AML cells. We further employed Annexin V staining to measure the apoptosis frequency in Molm13 and MV-4-11 cells after MI-238 treatment, and found that 40 μM of MI-238 caused 60 ± 0.4% and 35 ± 1.5% apoptotic cell death in Molm13 and MV-4-11 cells respectively (Fig. 3E, F). Taken together, these results demonstrated that MI-238 effectively induces apoptosis in AML cells.

Mi-238 Treatment Induces Activation Of Bh3-only Proteins

Mcl-1 inhibits apoptosis through sequestering pro-apoptotic BH3-only proteins, such as Bax, Bak, Bim and Puma[23]. To check whether MI-238 treatment could release BH3-only proteins from Mcl-1, we performed immunoprecipitation (IP) assay using antibodies against anti-apoptotic proteins including Mcl-1, Bcl-2 and Bcl-xL, and the result showed that Mcl-1 mainly binds to Bak, Bim, and Puma, but not Bax in Molm13 cells (Fig. 4A). Meanwhile, treatment of MI-238 could disrupted Mcl-1 association with BH3-only pro-apoptotic proteins including Bak, Bim and Puma (Fig. 4A). Whereas, MI-238 failed to interrupt the binding of BH3-only proteins to Bcl-2 and Bcl-xL (Fig. 4A), suggesting MI-238 treatment specifically inhibits Mcl-1, but not Bcl-2 and Bcl-xL. Bak release from Mcl-1 causes its conformation change and homo-oligomerization to initiate apoptosis. We then employed flow cytometry analysis of Bak activation after MI-238 treatment by staining with activation-specific antibody. Consistent with IP assay, MI-238 treatment induced Bak activation in Molm13 cells in a dose-dependent manner (Fig. 4B).

Activation of BH3-only proteins results in release of cytochrome C from mitochondria into the cytosol, which in turn triggers apoptosis[25]. We then performed cell fractionation analysis to examined cytochrome C level in mitochondrial and cytosol after MI-238 treatment. As shown in Fig. 4C, we observed a significant decrease of mitochondrial cytochrome C level and increase cytosol cytochrome C after MI-238 treatment, indicating MI-238 could induce cytochrome C translocation from mitochondrial to cytosol.

Mi-238 Synergizes With Venetoclax To Induce Apoptosis In Aml Cells

Given that Mcl-1 is the primary venetoclax resistant protein[19], we then tested whether MI-238 could sensitize AML cells to venetoclax treatment. As shown in Fig. 5A-B, 10 μM of MI-238 induced 34.8 ± 1.2% apoptosis, 0.02 μM of venetoclax caused 26.1 ± 1.3% apoptosis in Molm13 cells,while, their combination induced 87.4 ± 0.3% apoptosis. Besides, synergistic effects of MI-238 and venetoclax on apoptosis induction in Molm13 cells have also been detected on different combinations (10μM + 0.1μM, 5μM +

0.02μM, and 5μM + 0.1μM). (Fig. 5C, D, E, F and Figure S3). Consistent with annexin V staining assay, combination treatment of MI-238 and venetoclax induced greater caspase 3 cleavage compared with treatment of MI-238 only or venetoclax only (Fig. 5G). In addition, MI-238 and venetoclax combination induced significantly greater activation of Bak compared with MI-238 or venetoclax treatment alone (Figure S4). These results indicate that MI-238 could sensitize AML cells to venetoclax treatment and combination of MI-238 and venetoclax induces synergistic anti-tumor effects.

Mi-238 And Venetoclax Have A Synergistic Effect In Aml Xenografts

To evaluate the therapeutic efficacy of MI-238 and venetoclax *in vivo*, mice were intravenously (i.v) injected with Molm13 cells stably expressing luciferase (Molm13-Luc) to generate Molm13 AML xenograft model. We started treatment at 10 days after cell implantation, and monitored the cancer progression once a week by the bioluminescence imaging (Fig. 6A). At the beginning of the treatment (10 days after Molm13 implantation), we clearly detected luciferase signal in all mice, that is proportional to amount of leukemic cells (Fig. 6B). Bioluminescence images obtained after drug treatment (17 and 24 days) showed a significant reduction of leukemia burden in response to MI-238 alone, while, the greater suppression of leukemia progression was seen in the combination treatment compared with MI-238 or venetoclax alone (Fig. 6B).

Meanwhile, the percentage of Molm13 cells in the murine peripheral blood was quantified by flow cytometry using anti-human CD45 (hCD45) and anti-hCD33 monoclonal antibodies, since hCD45/hCD33 double positive was recognized as the human AML marker.[26] As shown in Fig. 6C and D, MI-238 alone treatment significantly reduced the hCD45+/hCD33 + cells in the peripheral blood compared with vehicle-treated mice (11.7 ± 3.4% vs. 24.7 ± 3.9%). Although, venetoclax alone also decreased percentage of hCD45+/hCD33 + cells (10.5 ± 2.1%), venetoclax in combination with MI-238 could decrease hCD45+/hCD33 + leukemia cells to 3.0 ± 1.7% (Fig. 6C, D, Figure S5). Similarly, immunohistochemical (IHC) analysis of hCD45 + cells in bone marrow also proved that MI-238 treatment alone could significantly decrease tumor burden, while MI-238 in combination of venetoclax induced greater reduction in the tumor burden (Fig. 6E, F). In addition, survival analysis revealed that MI-238 alone or in combination with venetoclax could significantly prolong survival of tumor bearing mice (vehicle treated mice = 24.6 days, vs MI-238 treated mice = 28 days, vs venetoclax treated mice = 29.8 days, vs combination treated mice = 35.8 days) (Fig. 6G).

Mi-238 Treatment Alone Or In Combination With Venetoclax Is Effective In Aml Patient Samples

In order to further validate the therapeutic efficacy of MI-238 and its combination with venetoclax, primary patient AML cells were analyzed. Mononuclear bone marrow cells from 3 different AML patients were

treated with increasing concentrations of MI-238 and the apoptosis were analyzed by annexin V staining. Consistent with AML cell line, MI-238 treatment induced apoptosis in AML patient samples in a dose-dependent manner and more than half of bone-marrow mononuclear cells underwent apoptosis in presence of 40μM MI-238 treatment in all three patient samples (Fig. 7A, B, C, FigureS6). Similarly, we detected increasing cleavage of caspase 3 after treatment of MI-238 (Figure D, E, F), which confirmed that MI-238 potently induced apoptotic cell death in tumor cells from AML patient samples. Meanwhile, 20μM of MI-238 treatment failed to induce apoptosis in bone-marrow mononuclear cells from healthy donor (Figure S7). Then, we treated patient AML cells with MI-238, venetoclax or their combination to test whether MI-238 could sensitize AML patient samples to venetoclax. As shown in Fig. 7G-I, we detected significantly greater apoptosis in patient AML cells treated MI-238 plus venetoclax compared with cells treated MI-238 or venetoclax alone. Besides, greater cleavage of caspase 3 was detected in patients AML cells treated with MI-238 and venetoclax combination, which further demonstrated that MI-238 is effective in primary patient AML cells (Fig. 7J, K, L). Besides, a significantly synergistic effects of MI-238 and venetoclax on apoptosis induction in primary AML patient samples (Fig. 7M, N, O). Collectively, these data demonstrated that MI-238 alone or its combination with venetoclax efficiently induce apoptosis in the bone marrow samples of AML patient, further supporting its therapeutic efficacy to treat AML.

Discussion

Mcl-1 is a Bcl-2 anti-apoptotic family member with unique properties[27]. Mcl-1 has a short half-life and its expression is regulated by a variety of survival signals[28]. Multiple E3 ligases and deubiquitinases have been identified to control ubiquitination and proteasome mediated degradation of Mcl-1[29–32]. In addition, Mcl-1 is structurally different from other Bcl-2 anti-apoptotic members at the long amino terminus, which contains two PEST domains, rich in proline(P), glutamic acid(E), serine(S) and threonine(E) amino-acid residues[24]. This PEST domain bears many residues which could be phosphorylated by protein kinase such as GSK-3, and subsequently results in Mcl-1 degradation.[28] Furthermore, the BH3 binding pocket of Mcl-1 is distinct from that of Bcl-2 and Bcl-xL, which restricts the development of high affinity Mcl-1 inhibitor, and the known BH3 mimetics ABT-737 could effectively inhibit Bcl-2/Bcl-xL, but not Mcl-1[20]. Meanwhile, like other Bcl-2 anti-apoptotic members, high frequency of Mcl-1 gene amplification has been observed in various human cancers and the elevated Mcl-1 protein level has also been validated in cancer tissues, which underline the importance of Mcl-1 for cancer cell survival[33]. And, multiple strategies targeting Mcl-1, including small molecule BH3 mimetics[23] synthetic peptides fit into Mcl-1 BH3-binding groove[34], covalent allosteric inhibition[35], proteolysis targeting chimera (PROTAC) mediated Mcl-1 degradation[36], interfering Mcl-1 transcription[37], have been proved to possess promising anti-cancer efficiency. Here, we show a novel small molecule named MI-238, which could effectively inhibit Mcl-1's anti-apoptotic function *in vitro* and selectively induce apoptosis in Mcl-1 proficient cell. The discovery of MI-238 provides a novel drug candidate to target Mcl-1 for future cancer treatment.

In our present study, we confirmed that MI-238 has promising therapeutic efficacy in AML cells, animal model and patients' samples. And we did not detect observe adverse effects in mice after administration

of MI-238(70 mg/kg), however, pharmacokinetics and safety of MI-238 needed to be further evaluated. In light of the importance of Mcl-1 in cancer cell survival, developing Mcl-1 inhibitors have been extensively studied and a number of Mcl-1 inhibitors have been developed[19, 23, 36]. Recently, the phase I clinical results of Mcl-1 inhibitor AMG-176 in 26 patients with relapsed multiple myeloma (MM) were disclosed. Major side effects including neutropenia, anemia, nausea and diarrhea were observed[38]. Meanwhile, significant cardiac side effects were seen in another Mcl-1 inhibitor AMG-397 trial[39]. Since, Mcl-1 is implicated in normal cardiac myocyte functions[40], these cardiac adverse effects are probably resulted from on-target activity of these compounds and this on-target related side effect may limit therapeutic window of these Mcl-1 inhibitors. Besides the reported Mcl-1 inhibitors, MI-238 in our present study could not induce apoptosis in Mcl-1 deficient cells, demonstrating its high specificity. Nevertheless, further studies needed to be conducted to test whether MI-238 possess favorable safety profile and clinical efficacy.

Although Bcl-2 inhibitor venetoclax is highly effective in hematologic malignancies, especially in CLL, the acquired and intrinsic resistance still cause treatment failure[19]. Mcl-1 is considered as the primary venetoclax resistant factor and inhibition of Mcl-1 could reverse venotoclax resistance in various hematologic cancers[19]. In our present study, we validated that combined treatment of MI-238 and venetoclax exhibited synergistic anti-cancer efficacy in AML cell line, animal model and patients' samples. These results further confirmed that Mcl-1 inhibition is the primary strategy to overcome venetoclax resistance and our study provided a novel leading compound which could be utilized as venetoclax sensitizer.

Conclusion

In summary, our study provides a novel and selective Mcl-1 inhibitor, MI-238, which specifically induces apoptosis in Mcl-1 proficient cells, but not in Mcl-1 deficient cells. MI-238 treatment alone or its combination with venetoclax effectively kills AML cells *in vitro*, AML mouse model and primary AML patient samples.

Abbreviations

AML: acute myeloid leukemia; CLL: chronic lymphocytic leukemia; ITC: Isothermal Titration Calorimetry; FP: fluorescence polarization; MEF: mouse embryonic fibroblast; KO: knockout; IP: Immunoprecipitation; CI: combination index; MM: multiple myeloma; IHC: Immunohistochemistry

Declarations

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Authors' contributions

G.C. and H.Z. designed the study. T.M.Z, H.E.Z, S.R.X. performed major experiments. C.J., S.B.L., L.T., F.S.L. performed the rest of the necessary experiments. G.C. and T.M.Z wrote and edited the manuscript. G.C. and H.Z. read and approved the final manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are presented within article

and its additional files.

Ethics approval and consent to participate

Animal experiments were carried out at Institute of Laboratory Animal Science, Jinan University (approval number: 20201027-11). Bone marrow samples from healthy donor or AML patients were obtained from the Hematology of the First Affiliated Hospital, Jinan University. The procedures were approved by IRB of the First Affiliated Hospital of Jinan University (approval number: KYk-2021-029).

Consent for publication

Not applicable

Competing Interests

The authors declare no conflict of interest.

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Figures

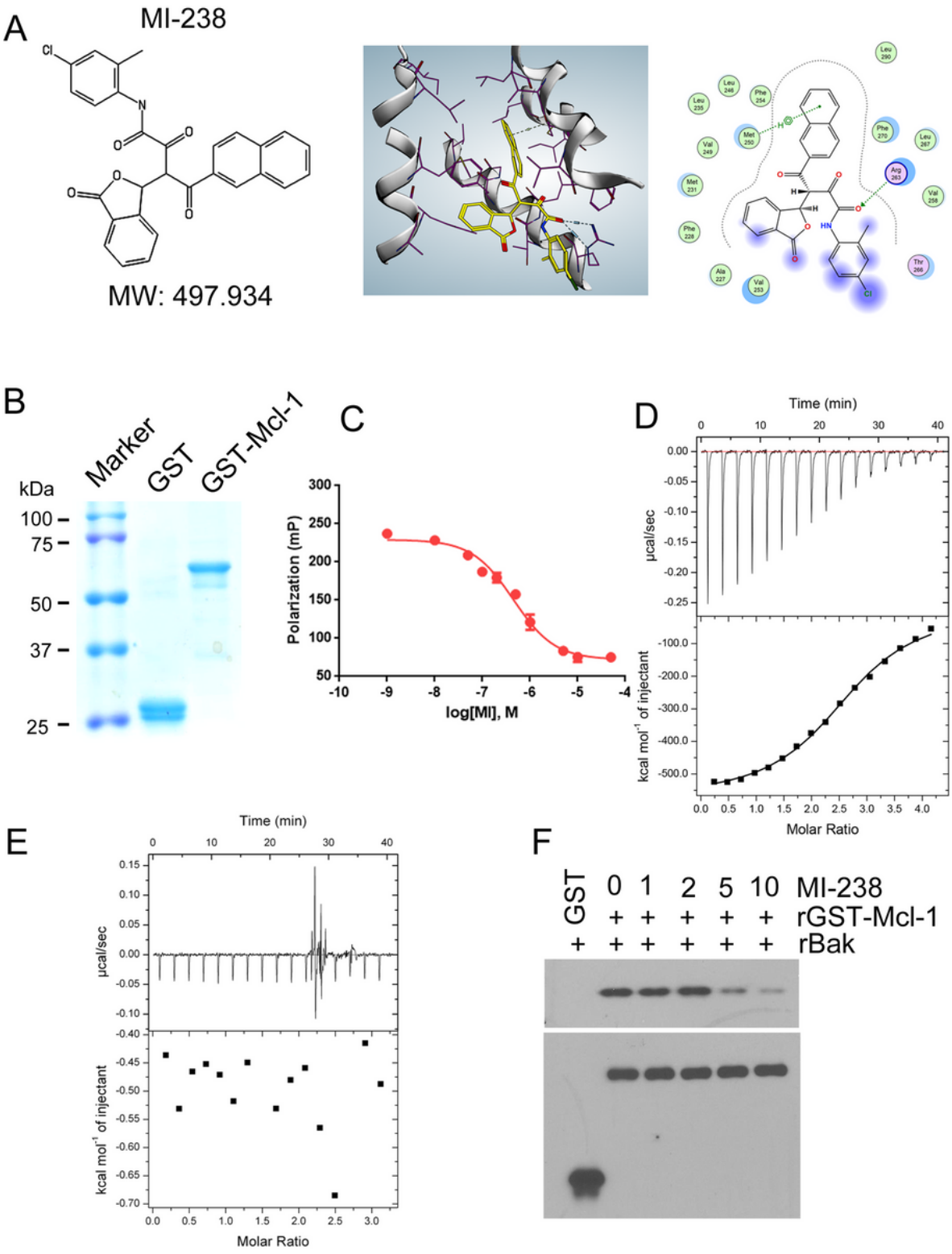

Figure 1

MI-238 targets Mcl-1 and disrupts Mcl-1/Bak association. (A) Chemical information and structure modeling of MI-238 in the BH3-binding pocket of Mcl-1 (PDB ID: 4HW3). (B) Purification of recombinant GST or GST-Mcl-1 proteins. (C) Fluorescence polarization (FP) assay was performed to measure the binding of MI-238 to recombinant GST-Mcl-1 protein. Data are represented as mean ± SD, n=3. (D-E) The binding affinity of MI-238 with GST-Mcl-1 (D) or GST (E) protein was examined by isothermal titration

calorimetry (ITC) assay. The binding constant (KD) value was determined by fitting of the titration curve to a 1-site binding mode. (F) GST pull down assay was conducted to analyze the association between recombinant GST-Mcl-1 (rGST-Mcl-1) and recombinant Bak (rBak) in presence of increasing concentrations of MI-238.

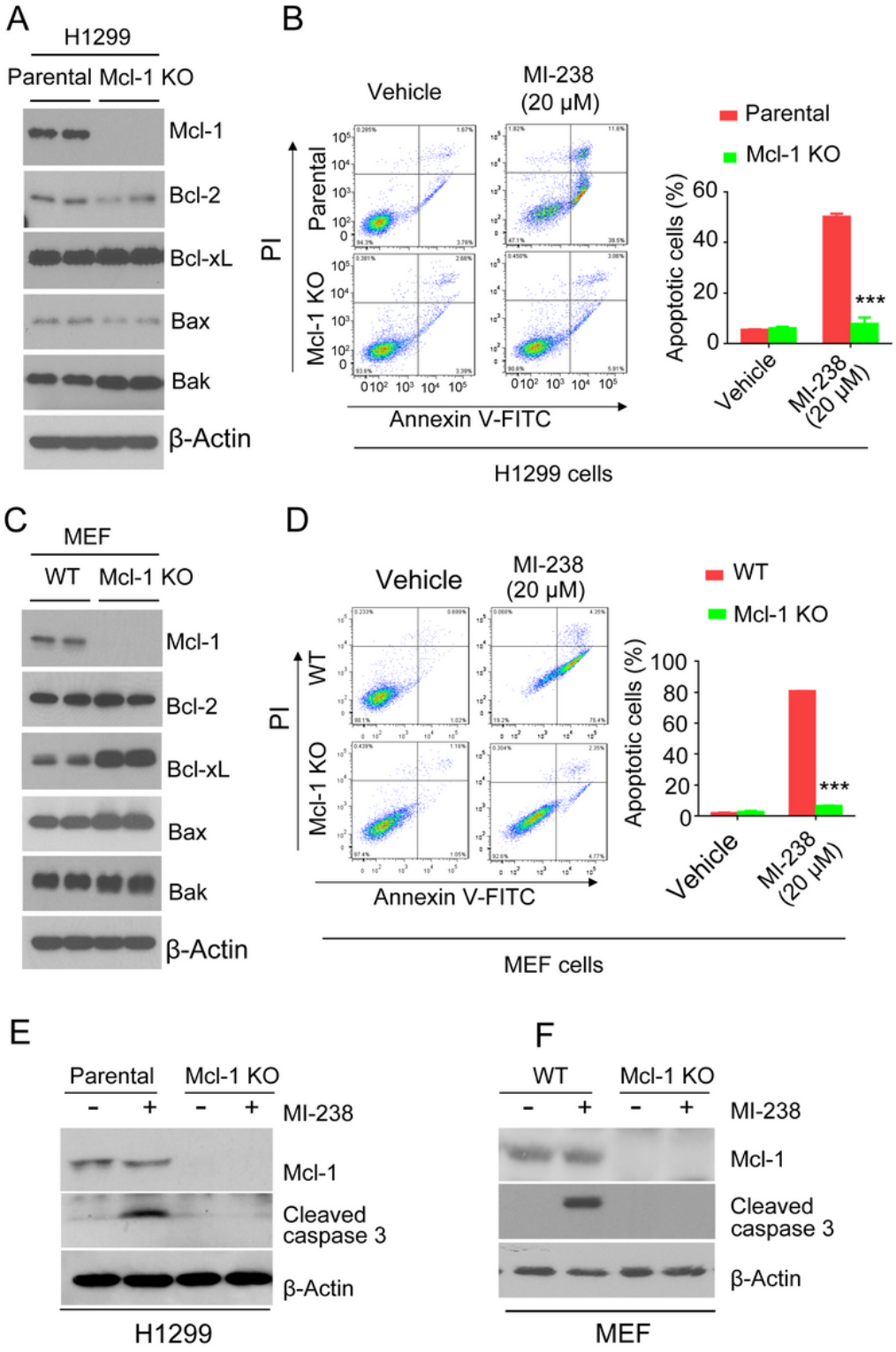

Figure 2

MI-238 selectively induces apoptosis in Mcl-1 proficient cells. (A) Western blotting analysis of indicated protein expressions in cell lysate derived from H1299 parental or Mcl-1 knockout (KO) cells. (B) H1299 parental or Mcl-1 KO cells were treated with or without 20 μM MI-238 for 48 hours, and the cell apoptosis were analyzed by annexin V staining. (C) Western blot analysis as above were performed in MEF wild-type (WT) or Mcl-1 KO cells. (D) Apoptosis analysis as above were performed in MEF wild-type (WT) or Mcl-1 KO cells. (E-F) Cell apoptosis were analyzed by caspase 3 cleavage in H1299 (E) or MEF (F). Data are represented as mean ± SD from three independent replicates, *** p<0.001 by two-tailed t-test.

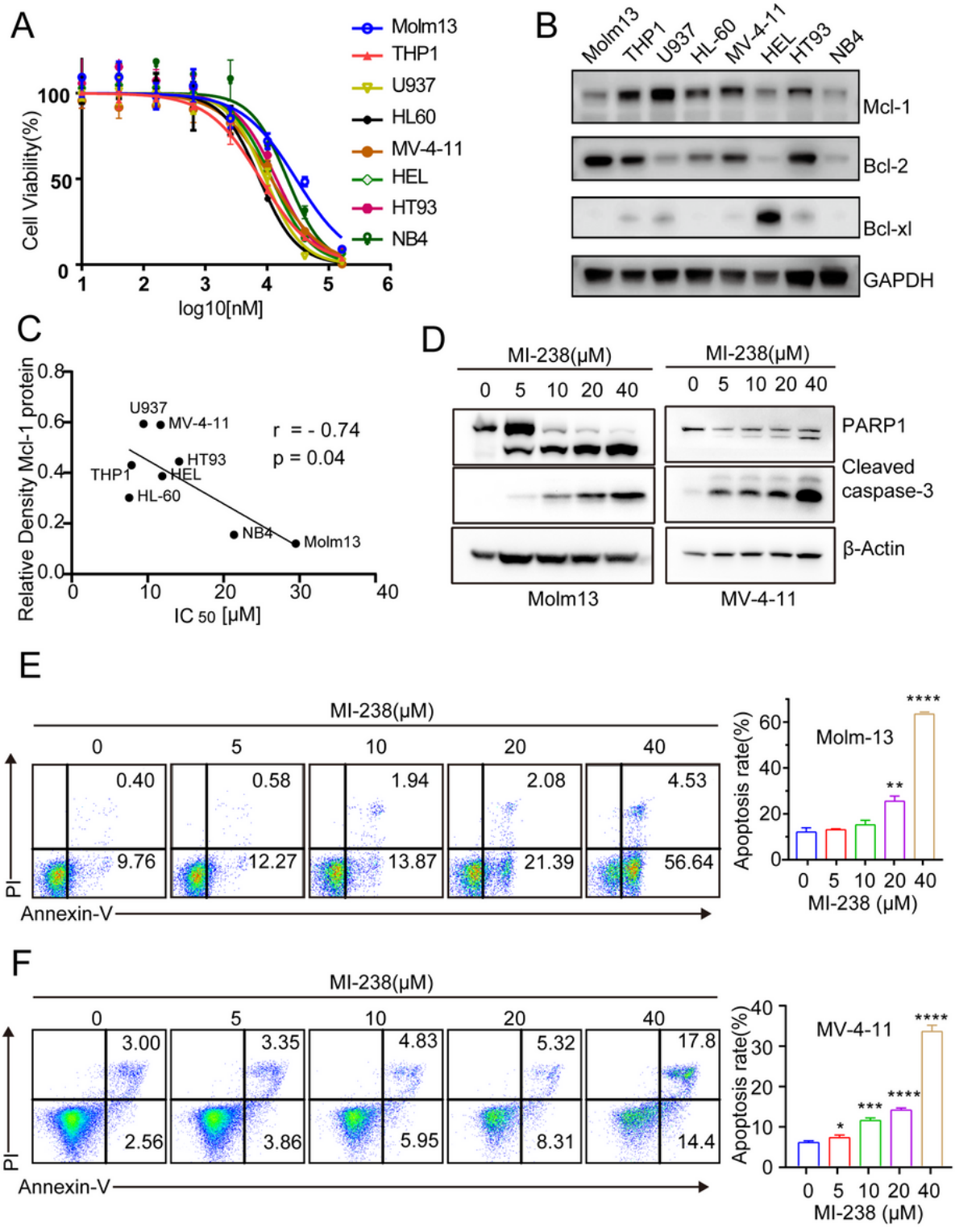

Figure 3

MI-238 effectively induces apoptosis in AML cells. (A) The cell viability of indicated 8 AML cell lines in presence of increasing concentrations of MI-238 were analyzed by cell counting kit-8 (CCK8) assay and the half-maximal inhibitory concentration (IC_{50}) IC_{50} were determined. (B) Western blot analysis of the expression of Mcl-1, Bcl-2 and Bcl-xL in AML cell lines. (C) Correlation of Mcl-1 protein level and the IC_{50} of MI-238 were determined. (D-E) Molm13 and MV-4-11 cells were treated with increasing concentrations of MI-238 for 48 hours, and the cell apoptosis was analyzed by western blot analysis of PARP1 and caspase 3 cleavage (D) and flow cytometry of annexin V staining (E-F). Data are represented as mean ± SD from three independent replicates, * p<0.05, **p<0.01 and *** p<0.001 by two-tailed t-test.

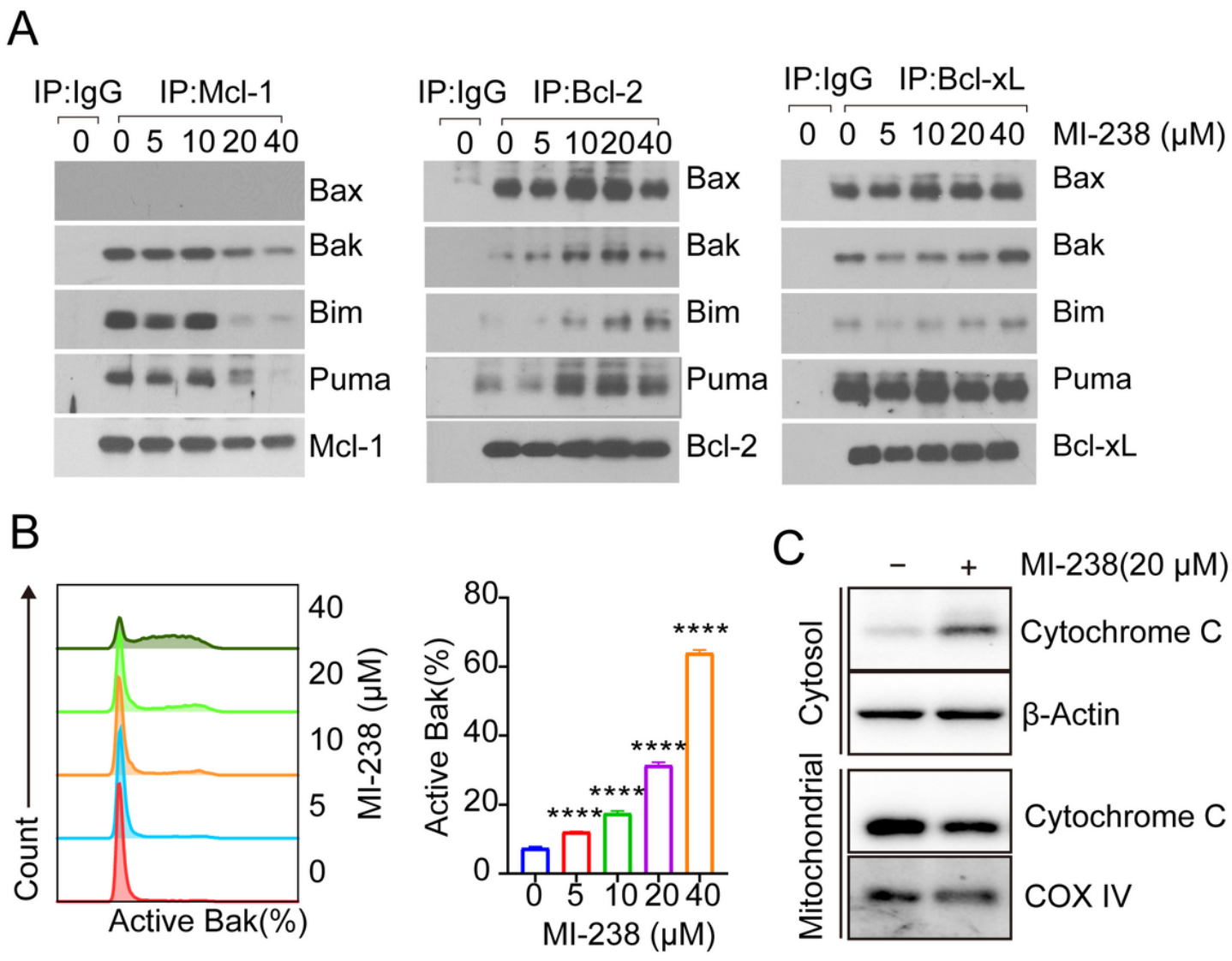

Figure 4

MI-238 disrupts the association of Mcl-1 with BH3-only proteins. (A) Molm13 cells were treated with indicated concentrations with MI-238 for 24 hours, followed by Immunoprecipitation (IP) assay using anti-Mcl-1, anti-Bcl2 and anti-Bcl-xL antibodies and western blot analysis with indicated BH3-only

proteins. (B) Molm13 cells were treated with indicated concentrations of MI-238, followed by flow cytometry analysis of Bak activation using the antibody (clone: G317-2) specifically recognized activated Bak. Data are presented as mean ± SD from three independent replicates, *** p<0.001 by two-tailed t-test. (C) Molm13 cells were treated with or without 20 μM MI-238 for 24 hours. Then, subcellular fractionation was subsequently performed and the cytochrome C level in mitochondria and cytosol were analyzed by western blot.

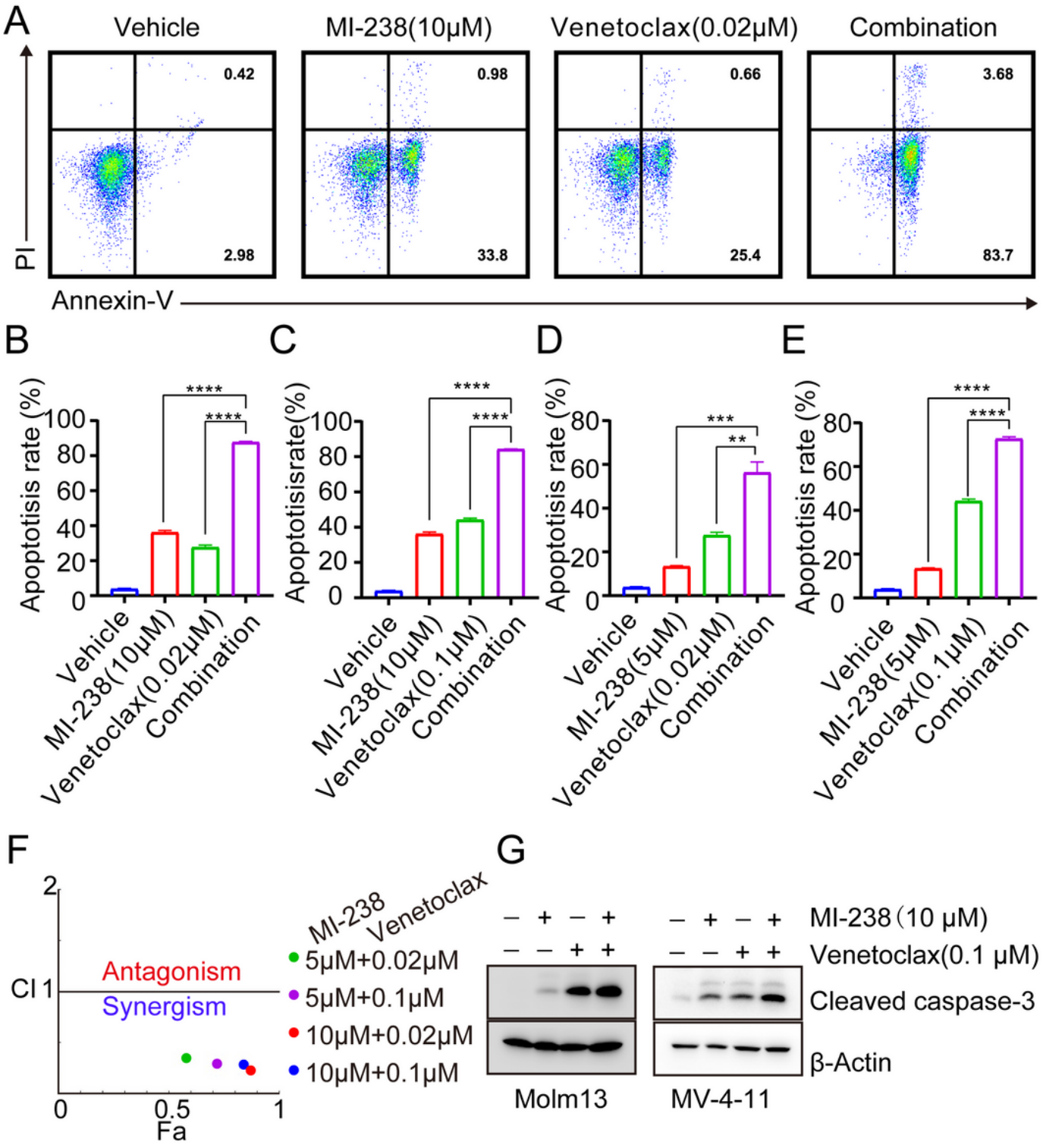

Figure 5

MI-238 synergizes with venetoclax to induce apoptosis in AML cells. (A-E) Molm13 cells were treated with indicated concentrations of MI-238, venetoclax, or their combination for 48 hours, followed by apoptosis assay by annexin V staining. Data are represented as mean ± SD from three independent replicates, **p<0.01 and *** p<0.001 by two-tailed t-test. (F) The combination index (CI) was calculated by CompuSyn software. (G) Molm13 and MV-4-11 cells were treated as indicated, and the caspase 3 cleavage was analyzed by western blot.

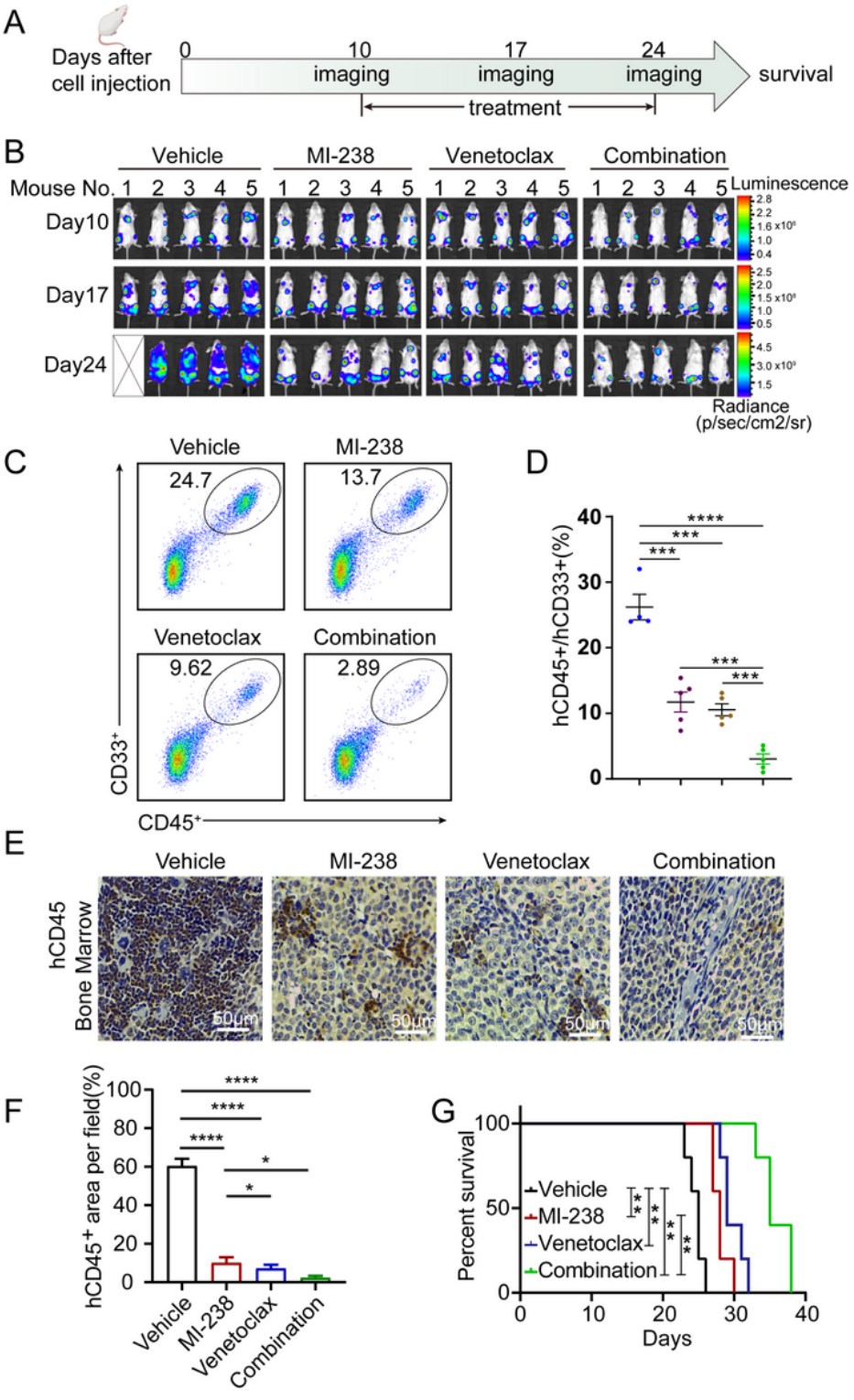

Figure 6

The combination of MI-238 and venetoclax potently inhibited the development of AML in murine model. (A) Schematic diagram of the experimental design showing the timeline for the treatment and imaging. (B) Representative bioluminescent images of the Molm13 tumor burden in mice treated with vehicle, MI-238 (70 mg/kg), venetoclax (50 mg/kg) or their combination. (C-D) The percentage of human CD45 (hCD45) and human CD33 (hCD33) positive cells were analyzed by flow cytometry to measure the

Molm13 tumor burden. The representative flow cytometry plots(C) and quantification of hCD45 and hCD33 double positive (hCD45+/hCD33+) cells(D) were shown. Data are represented as mean ± SD from three independent replicates. ***p<0.001 by two-tailed t-test. (E-F) Immunochemistry (IHC) analysis of hCD45 expression in bone marrow from experimental mice. Representative staining (E) and quantification (F) were shown. Data represent mean ± SD from three independent replicates, **p<0.01 and ***p<0.001 by two-tailed t-test. (G) Kaplan–Meier analysis showed MI-238 in combination with venetoclax resulted in a survival benefit in Molm13 AML xenograft mice. **p<0.01 by log-rank (Mantel–Cox) test (n=5).

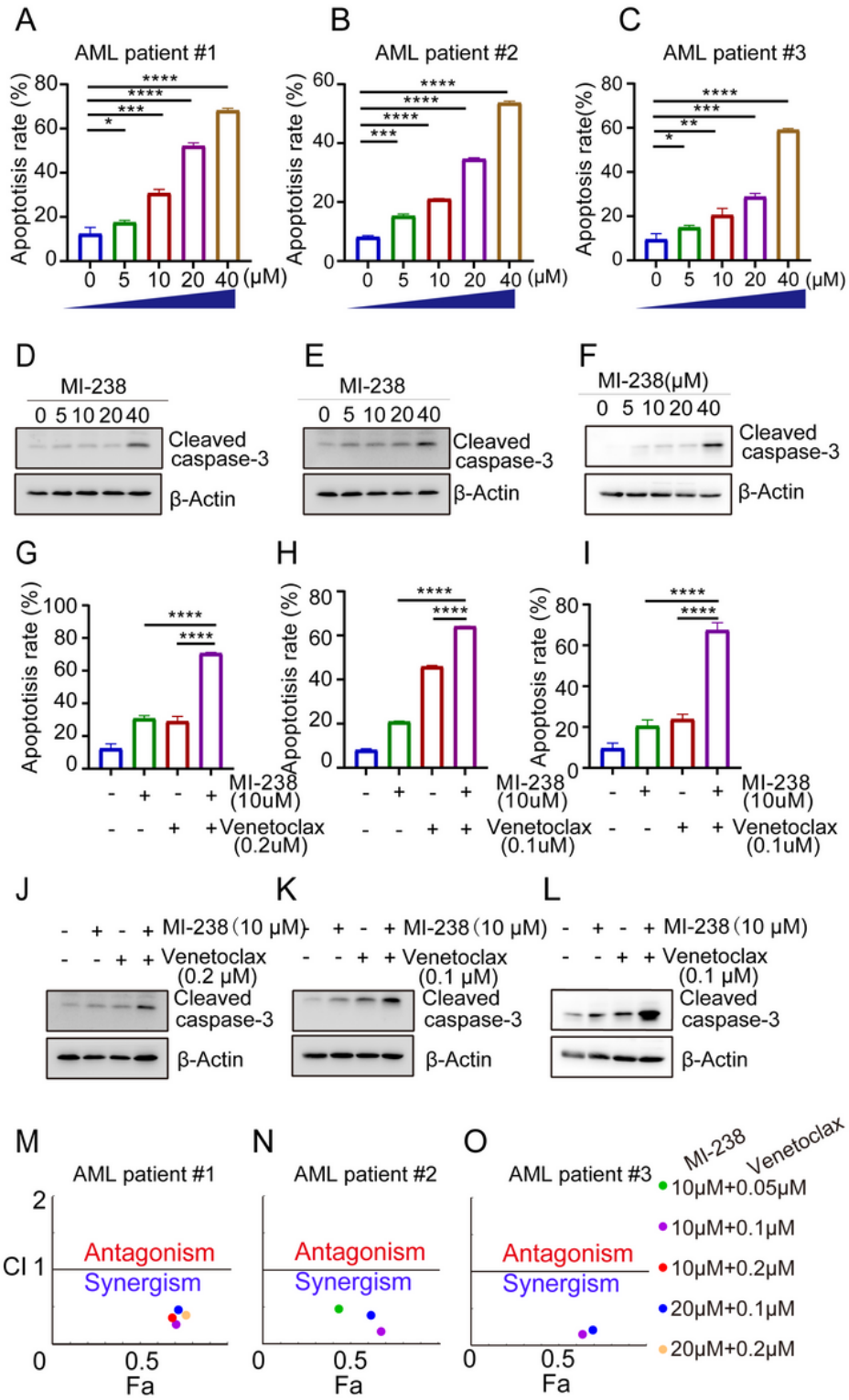

Figure 7

MI-238 treatment alone or in combination with venetoclax effectively induces apoptosis in primary patient AML cells. Bone-marrow mononuclear cells from three different AML patients were treated as indicated for 48 hours, and the apoptosis were measured by annexin V staining (A-C; G-I) or western blot analysis of caspase 3 cleavage (D-F; J-L). The combination index (CI) was calculated by CompuSyn software (M-O).

Supplementary Files

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