

## Original Article

# BCL-2 inhibitor synergizes with PI3K $\delta$ inhibitor and overcomes FLT3 inhibitor resistance in acute myeloid leukaemia

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**Abstract:** Inhibitors targeting the antiapoptotic molecule BCL-2 have therapeutic potential for the treatment of acute myeloid leukaemia (AML); however, BCL-2 inhibitors such as venetoclax exhibit limited monotherapy efficacy in relapsed or refractory human AML. PI3K $\delta$ /AKT signalling has been shown to be constitutively active in AML patients. Here, we demonstrate that the combination of BCL-2 and PI3K $\delta$  inhibitors exerts synergistic antitumour effects both *in vitro* and *in vivo* in AML. Cotreatment with venetoclax and the specific PI3K $\delta$  inhibitor idelalisib significantly enhanced antiproliferative effects and induced caspase-dependent apoptosis in a panel of AML cell lines. The synergistic effects were mechanistically based on the inactivation of AKT/4E-BP-1 signalling and the reduction of MCL-1 expression, which diminished the binding of Bim to MCL-1. Notably, compared with the parental FLT3-ITD-positive MV-4-11, the acquired FLT3 inhibitor quizartinib-resistant xenograft model carrying the F691L mutation, exhibited a markedly higher sensitivity to venetoclax. Furthermore, venetoclax combined with idelalisib led to tumour regression in all animals in this quizartinib-resistant AML model. Thus, these data indicate that combined inhibition of BCL-2 and PI3K $\delta$  may be a promising strategy in AML, especially for patients with FLT3-ITD and/or FLT3-TKD mutations.

**Keywords:** Acute myeloid leukaemia, BCL-2, PI3K $\delta$ , FLT3, synergistic lethality

## Introduction

Acute myeloid leukaemia (AML) is an aggressive heterogeneous clonal disease of haemopoietic progenitor cells [1-3]. In adults, AML is the most common form of acute leukaemia [4]. Until recently, therapy options for AML patients have been limited, it's largely due to the high heterogeneity and dynamics of AML, which is characterized by driver gene mutations acquired by multiple somatic cells, coexisting competitive cloning, and disease development over time [5]. Although many molecular targeted drugs have exhibited improved survival

rates [6], due to the genome instability of AML cells and the rapid emergence of drug resistance mutation, durable response with monotherapy for AML patients is often rarely seen [7]. Therefore, novel therapeutic strategies against specific targets have become an intensely important research area, and rational combinations are believed to be additional effective therapeutic options for AML patients.

The B-cell lymphoma 2 (BCL-2) protein is a critical regulator in the mitochondrial apoptotic pathway. Previous researches have shown that the expression of BCL-2 family proteins is

## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment

increased in AML mother cells, and the survival of most AML stem cells depends on the aberrantly high levels of BCL-2 [8, 9]. Abnormal overexpression of BCL-2 is related to tumorigenesis and increased chemotherapy resistance in multiple malignancies, including AML [8]. Venetoclax is a highly selective BCL-2 inhibitor and has been approved for use in chronic lymphocytic leukaemia [10]. However, venetoclax has limited and transient single-agent activity against AML in both preclinical and clinical investigations [11, 12]. Venetoclax-based combinations are under exploring, for example, cotreatment with intensive chemotherapy drugs [13] or other targeted inhibitors [14, 15]. In October 2020, the FDA has granted full approval for venetoclax in combination with azacytidine, decitabine or low-dose cytarabine for treating newly diagnosed AML [16, 17].

The class IA phosphoinositide 3-kinase (PI3K)/AKT pathway has been reported to be extensively hyperactive in AML cells, which prompts cell survival and proliferation [18, 19]. The catalytic subunits of class IA PI3K are subdivided into 4 isoforms: p110 $\alpha$ , p110 $\beta$ , p110 $\delta$  and p110 $\gamma$  [20]. The expression and activity of the p110 $\delta$  isoform of PI3K are consistently high in AML, and this isoform plays a critical role in disease progression, which makes it a potential therapeutic target [21]. However, monotherapy with idelalisib, a selective PI3K $\delta$  inhibitor that has been approved for use in relapsed chronic lymphocytic leukaemia and indolent lymphoma [22], exhibited limited effect against AML in a phase I dose-escalation study [23]. We previously reported that co-inhibition of PI3K $\delta$  and FMS-like tyrosine kinase-3 (FLT3) signalling exerted synergistic antitumour efficacy and overcame acquired drug resistance in FLT3-activated AML [24]. Although synergy between targeting BCL-2 and MCL-1 has been previously reported in AML, prior MCL-1 inhibitors have not been suitable for clinical development [25, 26]. Previous studies have shown that PI3K/mTOR pathway inhibition significantly diminishes MCL-1 protein level [27]. Furthermore, the acquired resistance of BCL-2 inhibitor develops due to increased activation of the PI3K/AKT/mTOR signalling pathway and upregulation of MCL-1 and BCL-XL that sequester Bim [27]. Collectively, PI3K $\delta$  inhibitor-based combinations may be a promising option for AML.

FLT3 mutations are the most common molecular alterations in AML, seen in approximately

30% of patients with newly diagnosed AML [28, 29]. FLT3-internal tandem duplication (ITD) mutation, which is the most common genetic alteration in AML [29]. In the venetoclax monotherapy trial, a small subset of FLT3-ITD<sup>+</sup> patients exerted no response and some patients relapsed with novel FLT3-ITD mutation [28]. Recently, many FLT3 inhibitors, including midostaurin and gilteritinib, have been developed and presented impressive preclinical and clinical activity in AML. However, drug resistance rapidly ensues following treating AML patients with FLT3 inhibitor, leading to modest durable responses and poor clinical therapeutic efficacy [30]. A potential strategy for overcoming acquired drug resistance in many tumour types is the usage of combination regimens [31]. In this study, we demonstrate that the combination of BCL-2 and PI3K $\delta$  inhibitors is synergistically lethal against AML, which is mediated by enhanced inhibition of AKT/4E-binding protein (4E-BP) and MCL-1. Importantly, venetoclax alone or in combination with idelalisib is highly potent in FLT3-ITD<sup>+</sup> AML carrying the F691L mutation, which acquires resistance to quizartinib. These findings provide the potential for combining venetoclax with PI3K $\delta$  inhibitors to lower the dosage of venetoclax and enhance its applicability in venetoclax- or quizartinib-resistant AML.

### Materials and methods

#### Materials

Venetoclax, quizartinib, crenolanib and gilteritinib were purchased from MedChemExpress (New Jersey, USA). Idelalisib, sunitinib, sorafenib and paclitaxel were purchased from Melone Pharmaceutical (Dalian, China).

Antibodies specific for PI3Kinase p110 $\delta$ , phospho-FLT3 (Tyr589/591), FLT3, STAT5, phospho-STAT5 (Tyr694), AKT, phospho-AKT (Ser473), phospho-4E-BP-1 (Thr37/46), phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-rpS6 (Ser235/236), rpS6, caspase-8, caspase-9, caspase-3, PARP, MCL-1, BCL-XL, BCL-2, Bim,  $\beta$ -Actin and GAPDH were all purchased from Cell Signaling Technology (Danvers, MA).

#### Cell lines

MV-4-11, THP-1, RS4;11, U937, and TF-1 cell lines were purchased from the ATCC (Manassas, VA). EOL-1, MOLM-13, SET-2 and HEL cell

## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment

lines were purchased from DSMZ (Braunschweig, Germany). Cell authentication was conducted by Geneing Biotechnologies Inc. (Shanghai, China). MV-4-11/quizartinib was generated by continuous incubating with gradually increased concentration of quizartinib.

### *Cell proliferation assay*

MTT (Sigma-Aldrich, St. Louis, MO) assay was used to measure the cytotoxicity after cells were incubated with gradient diluted concentrations of drugs for 72 h, and 50% inhibitory concentration (IC<sub>50</sub>) values were calculated using Prism 5 (GraphPad Software, San Diego, CA) curve-fitting software. The interaction between two drugs was evaluated via calculating the combination index (CI) values conducted in CalcuSyn (CalcuSyn, Inc., Paramus, NJ). Synergistic effects: CI < 1, additive effects: CI = 1, antagonistic effects: CI > 1.

### *Western blot and co-immunoprecipitation (IP)*

Whole-cell lysates were prepared in SDS sample loading buffer (Melone, Dalian, China) after treatment, and then separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After membrane closure and antibody incubation, the chemiluminescence intensity was determined by Tanon 4600 (Tanon, Shanghai, China).

For co-IP, whole-cell lysates were incubated with anti-Bim antibody and protein G agarose (Cell Signaling Technology, Danvers, MA), and the levels of immunoprecipitated proteins were subsequently analysed by Western blotting.

### *Survival and correlation analyses*

The survival analyses of PI3K $\delta$  and BCL-2 for AML patients and the correlation analyses of these two genes in the TCGA-LAML were performed via the Gene Expression Profiling Interactive Analysis web service (GEPIA, <http://gepia.cancer-pku.cn/index.html>).

### *Annexin V-fluorescein isothiocyanate (annexin V-FITC)/propidium iodide (PI) flow cytometry assay*

After treatment with venetoclax and/or idelalisib for 24 h, AML cells were collected and then using the Annexin V-FITC/PI Apoptosis Kit (BD Biosciences, San Jose, CA) to quantify cell

apoptosis according to the manufacturer's instructions.

### *Analysis of mitochondrial membrane potential*

Cells were collected at the end of treatment and their viability and membrane potential were determined using the Mitochondrial Membrane Potential Detection Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Fluorescence was acquired using CytoFLEX™ Flow Cytometer (Beckman Coulter, Inc., CA, USA). And the samples were visualized using Zeiss Axio Observer A1 fluorescence microscope (ZEISS, Oberkochen, Germany).

### *In vivo study*

5-6 weeks old female athymic BALB/c nude mice were purchased from Charles River Laboratories (Beijing, China). Nude mice were inoculated subcutaneously with cells to establish MV-4-11 and MV-4-11/quizartinib tumour xenografts. Mice were randomly assigned to the vehicle and treatment groups (n = 5-6/group) when tumours grew to a volume of 100-200 mm<sup>3</sup>. The treatment groups were administered by oral venetoclax, idelalisib, or their combination daily. Tumour volumes were monitored by callipers and calculated as (length $\times$ width<sup>2</sup>)/2. Tumours were dissected 3 h following the final dose and analysed by Western blotting or immunohistochemistry (IHC). All the animal-related procedures were canonically conducted according to the Institutional Animal Care and Use Committee guidelines of the Shanghai Institute of Materia Medica Chinese Academy of Sciences.

### *Statistical analysis*

Means, SD, and SEM were analysed using GraphPad Prism 5.0. Statistical differences were calculated by the Student's t-test between two groups or one-way ANOVA among three or more groups and considered significant if *P* values < 0.05.

## **Results**

### *The combination of BCL-2 and PI3K $\delta$ inhibitors exerts synergistic antiproliferative activity in AML cell lines*

First, we assessed the single-agent activity of the BCL-2 inhibitor venetoclax and the PI3K $\delta$

## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment

inhibitor idelalisib on the proliferation in a panel of AML cell lines with different expression levels of the BCL-2 family and PI3K $\delta$  proteins. Consistent with previous reports [12], overall, the level of BCL-2 correlated with sensitivity to venetoclax. However, the level of BCL-XL inversely correlated with venetoclax sensitivity (**Figure 1A, 1C**). MV-4-11, EOL-1, THP-1, RS4;11 and MOLM13 cells sensitive to venetoclax with IC<sub>50</sub> values ranging from 16 nM to 102 nM, exhibited high protein level of BCL-2 and low protein level of BCL-XL; in contrast, minimal cytotoxicity was noted in AML cells with low or absent protein level of BCL-2, such as U937, SET-2, HEL and TF-1. Idelalisib exhibited limited antiproliferative effects in all tested AML cells, even though PI3K $\delta$  was ubiquitously expressed (**Figure 1B, 1C**).

Next, we explored the effects of cotreatment with idelalisib and venetoclax in seven AML cell lines which displayed different sensitivities to idelalisib or venetoclax in monotherapy. As shown in **Figure 1D**, the combination clearly increased antiproliferative effects in both venetoclax-sensitive and venetoclax-insensitive cell lines, with CI values ranging from 0.09 to 0.54, except that antagonism was seen in the BCL-2-negative SET-2 cell line. Furthermore, the combination of venetoclax and idelalisib reached a strong synergism (CI < 0.3) in FLT3-ITD<sup>+</sup> MV-4-11 and MOLM-13 cells.

Additionally, we studied the roles of the BCL-2 and PI3K $\delta$  genes in the survival of patients with AML using the GEPIA database. The data suggested that increased BCL-2 was related to poor overall survival of AML (**Figure 1E**, P = 0.047 < 0.05). Although the expression of PI3K $\delta$  was not associated with patient survival, the BCL-2 level was positively correlated with PI3K $\delta$  by correlation analysis (**Figure 1F**, R = 0.41, P < 0.05). These results may provide some evidence for the potential clinical relevance of combining BCL-2 and PI3K $\delta$  inhibitors in AML treatment.

*The combination of BCL-2 and PI3K $\delta$  inhibitors synergistically induces caspase-dependent apoptosis in AML cell lines*

To measure the cell apoptosis induced by idelalisib and/or venetoclax in AML cell lines, the annexin V/PI assay was performed. Cotreat-

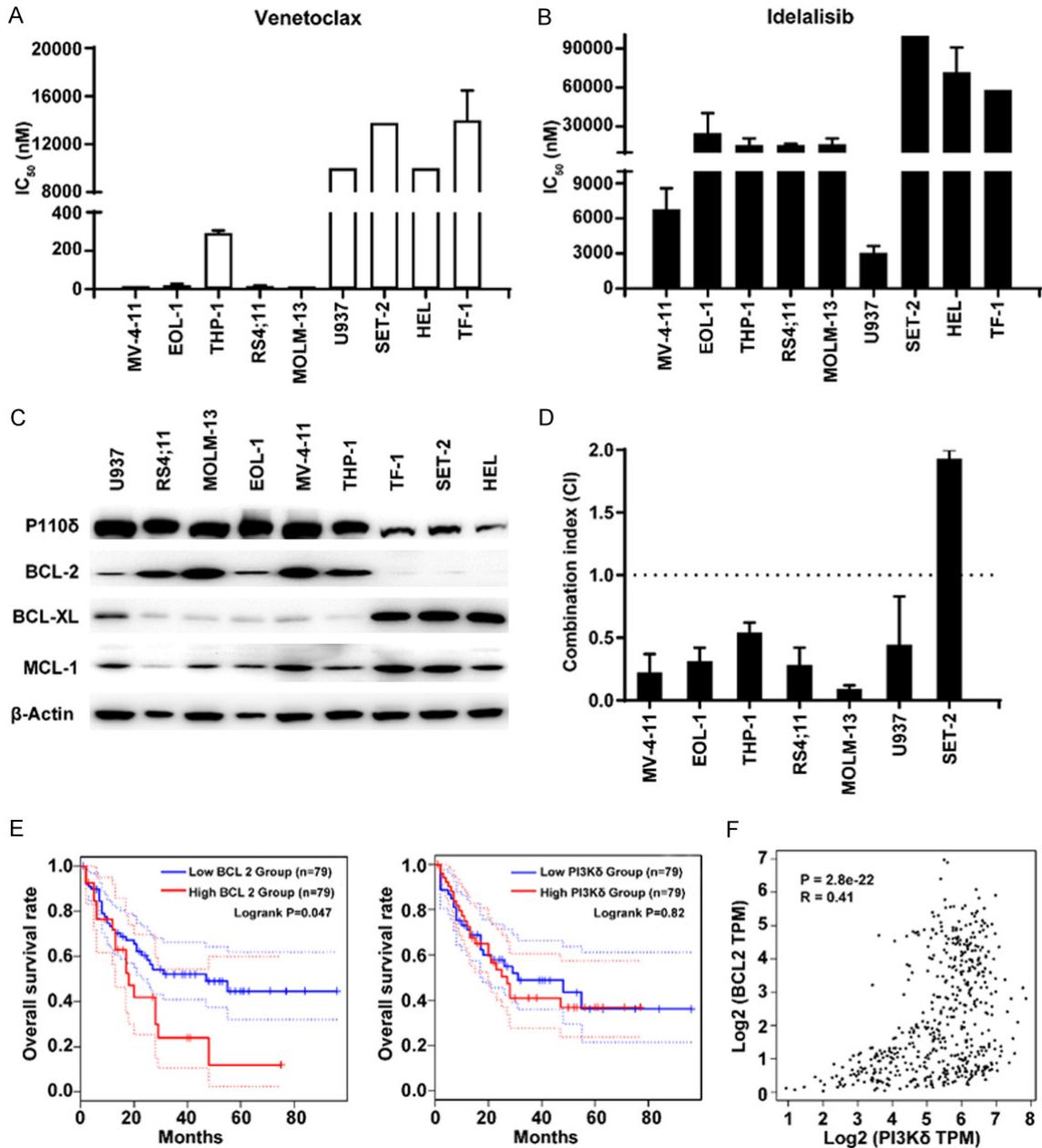
ment produced significantly enhanced cell apoptosis compared with single drug treatment (**Figure 2A**), which was accompanied by the increased cleaved caspase 9, caspase 8, caspase 3 and PARP levels in both MV-4-11 (FLT3-ITD<sup>+</sup>) cells and EOL-1 (FLT3-wild type activated) cells (**Figure 2B**). To further examine the effects of venetoclax and idelalisib on mitochondrial function, MitoTracker Red CMXRos assays were conducted. After pretreatment of MV-4-11 with the pan-caspase inhibitor Z-VAD-FMK, the amount of green fluorescent (Annexin V-FITC) was significantly decreased, together with increased red fluorescence, compared with venetoclax and idelalisib cotreatment, indicating that the caspase inhibitor rescued cell viability and increased the stability of the mitochondrial membrane potential (**Figure 2C, 2D**). In summary, these results suggested that the synergistic antiproliferative effect induced by co-inhibition of BCL-2 and PI3K $\delta$  mechanistically involved caspase-dependent apoptosis.

*Combination of BCL-2 and PI3K $\delta$  inhibitors downregulates the expression of MCL-1 mediated by AKT/4E-BP-1 inactivation*

Given that venetoclax monotherapy exhibits limited single-agent activity against AML with FLT3-ITD mutation in both preclinical and clinical investigations [11, 12], MV-4-11 and EOL-1, these two FLT3-activated AML cell lines were chosen to further identify the mechanism of synergistic apoptosis induced by the combination of venetoclax and idelalisib. Compared with either drug alone, venetoclax combined with idelalisib exhibited greater inhibition of MCL-1, but not BCL-2 or BCL-XL (**Figure 3A**). To determine the role of MCL-1 in the apoptosis induced by the combination, we performed co-IP experiment by Bim antibody and the interaction between MCL-1 and Bim was analysed. The combination of venetoclax and idelalisib markedly decreased the protein levels of MCL-1 bound to Bim in both MV-4-11 and EOL-1 cells (**Figure 3B**).

It has been shown that the PI3K/AKT/mTOR pathway regulates MCL-1 expression by 4E-BP-1/mTORC1-dependent translation in AML [32]. Next, we assessed the effect of cotreatment on the JAK/STAT, PI3K/AKT, and MAPK/ERK pathways in MV-4-11 and EOL-1 cells. Combination markedly inhibited the phosphorylation of AKT

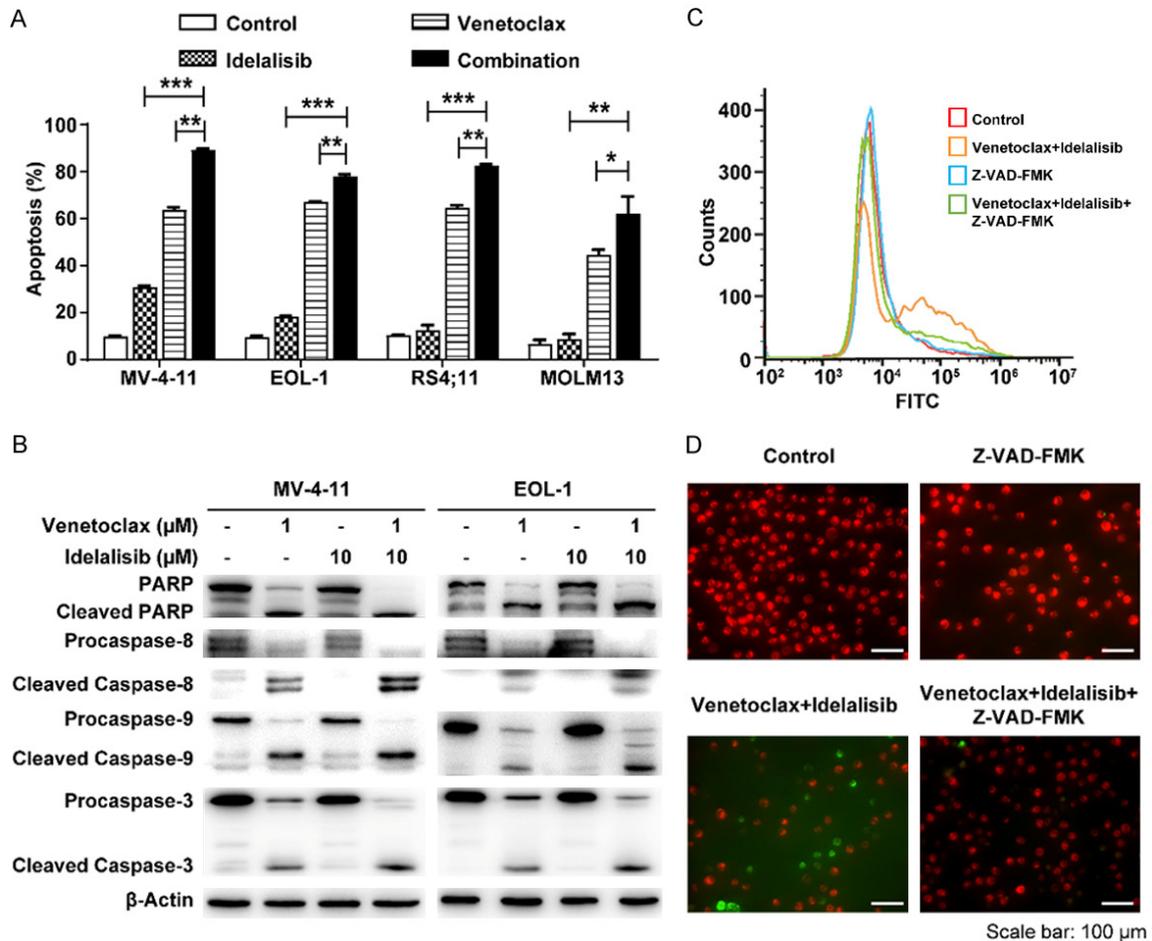
## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment



**Figure 1.** Antiproliferative activity of the co-inhibition of BCL-2 and PI3K $\delta$  in AML cell lines. (A, B) Antiproliferative activity of venetoclax (A) or idelalisib (B) in a panel of AML cell lines. After cells were treated with venetoclax or idelalisib for 72 h, MTT assay was used to assess cell viability. (C) Protein levels of PI3K $\delta$ , BCL-2, BCL-XL, and MCL-1 in a panel of AML cell lines were assessed by Western blotting. (D) Synergistic antiproliferative effects *in vitro*. After AML cells were treated with venetoclax and/or idelalisib for 72 h, MTT assay was used to assess cell viability. CI was calculated with CalcuSyn DemoVersion 2.0 software. Synergistic efficacy: CI < 1. (E) The differences in survival related to BCL-2 or PI3K $\delta$  mRNA expression were compared in each group (log-rank test). The dotted line indicated the 95% confidence interval. (F) Scatter plot showed the correlation between BCL-2 and PI3K $\delta$  mRNA levels in AML. Statistical analysis was performed using a two-tailed Student's t-test. R, Spearman's correlation coefficient.

and 4E-BP-1 compared with single drug treatment (Figure 3C). Collectively, these results indicated that combined inhibition of PI3K $\delta$  and BCL-2 leads to synergistic apoptosis th-

rough the inactivation of AKT/4E-BP-1 and the downregulation of MCL-1, which may diminish the binding of Bim to MCL-1 and lead to the activation of Bak/Bax.



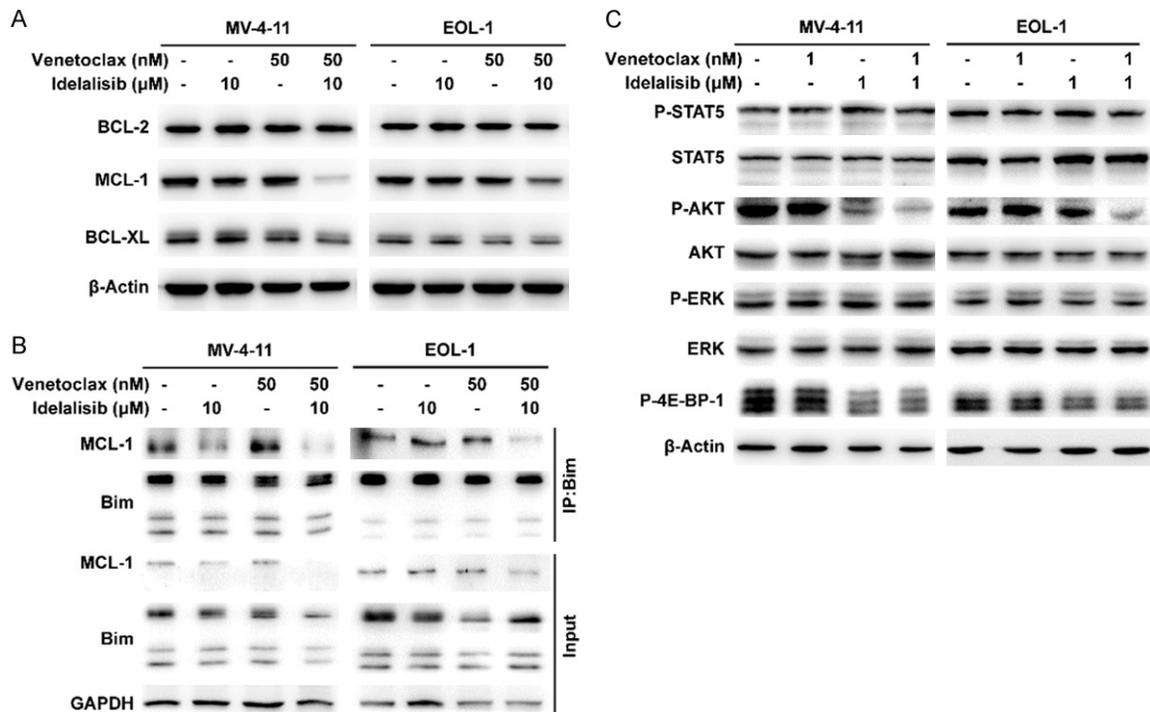
**Figure 2.** The combination of BCL-2 and PI3K $\delta$  inhibitors synergistically induces caspase-dependent apoptosis in AML cell lines. (A) AML cell lines were treated with idelalisib and/or venetoclax for 24 h, and then cell apoptosis was assessed by annexin V-FITC/PI staining and FACS analysis. The concentrations used in this study were 10  $\mu$ M idelalisib, 50 nM, 25 nM, 50 nM, and 10 nM venetoclax for MV-4-11, EOL-1, RS4;11 and MOLM13 cells, respectively. (B) Protein levels of PARP, Caspase 8, Caspase-9, Caspase-3 in MV-4-11 and EOL-1 cells were determined by Western blotting after treatment with idelalisib and/or venetoclax for 24 h. (C, D) MV-4-11 cells were treated with drugs for 24 h and then cell viability and mitochondrial membrane potential were measured by annexin V-FITC/MitoTracker Red CMXRos staining. FITC histograms were obtained by flow cytometry (C), and samples were visualized under a fluorescence microscope (D). The concentrations used in this study were 50  $\mu$ M Z-VAD-FMK, 5 nM venetoclax, and 10  $\mu$ M idelalisib. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001.

*The combination of BCL-2 and PI3K $\delta$  inhibitors overcomes acquired resistance to FLT3 inhibitors in AML cells*

Although many inhibitors for FLT3 have been explored and demonstrated impressive responses in AML, resistance often developed in most AML patients after prolonged FLT3 inhibitor monotreatment [30]. To examine whether the combination of BCL-2 and PI3K $\delta$  inhibitors could also overcome FLT3 inhibitors resistance, MV-4-11/quizartinib, the acquired quizartinib-resistant cell line was generated by continuous

incubating with gradually increasing concentration of the FLT3 inhibitor quizartinib. MV-4-11/quizartinib cells exhibited disparate sensitivities to multiple FLT3 inhibitors with resistance factors (RFs) of 1009.4, 1536.8 for quizartinib and sorafenib, whereas they showed less resistance to the type I FLT3 inhibitors crenolanib, gilteritinib and sunitinib, with RFs of 6.6, 31.8 and 5.6, respectively (Table 1). Additionally, MV-4-11/quizartinib-resistant cells retained sensitivity to both venetoclax and idelalisib, as well as the cytotoxic drug paclitaxel (Figure 4A; Table 1).

## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment



**Figure 3.** The combination of BCL-2 and PI3K $\delta$  inhibitors inhibits the AKT pathway and downregulates MCL-1, resulting in reduced binding of MCL-1 to Bim. (A) Protein levels of BCL-2, MCL-1 and BCL-XL in MV-4-11 and EOL-1 cells were determined via Western blotting after treatment with idelalisib and/or venetoclax for 3 h. (B) MCL-1/Bim interaction in MV-4-11 and EOL-1 cells was measured by co-IP experiment using Bim antibody after 3 h treatment with idelalisib and/or venetoclax, and the corresponding MCL-1 and Bim were analysed by Western blotting. (C) Phosphorylation levels of STAT5, AKT, ERK and 4E-BP-1 in MV-4-11 and EOL-1 cells were analysed by Western blotting after treatment with venetoclax and/or idelalisib for 3 h.

**Table 1.** Antiproliferative effects of compounds against MV-4-11 and MV-4-11/quizartinib cells

| Compound     | IC <sub>50</sub> (nM, Mean $\pm$ SD) |                     | RF     |
|--------------|--------------------------------------|---------------------|--------|
|              | MV-4-11                              | MV-4-11/quizartinib |        |
| Quizartinib  | 0.2 $\pm$ 0.1                        | 181.8 $\pm$ 15.5    | 1009.4 |
| Crenolanib   | 2.1 $\pm$ 0.1                        | 13.7 $\pm$ 0.9      | 6.6    |
| Gilteritinib | 1.5 $\pm$ 0.6                        | 47.0 $\pm$ 28.0     | 31.8   |
| Sunitinib    | 4.1 $\pm$ 0.1                        | 23.3 $\pm$ 0.8      | 5.6    |
| Sorafenib    | 0.7 $\pm$ 0.1                        | 1116.0 $\pm$ 152.7  | 1536.8 |
| Paclitaxel   | 0.4 $\pm$ 0.1                        | 0.2 $\pm$ 0.1       | 0.5    |

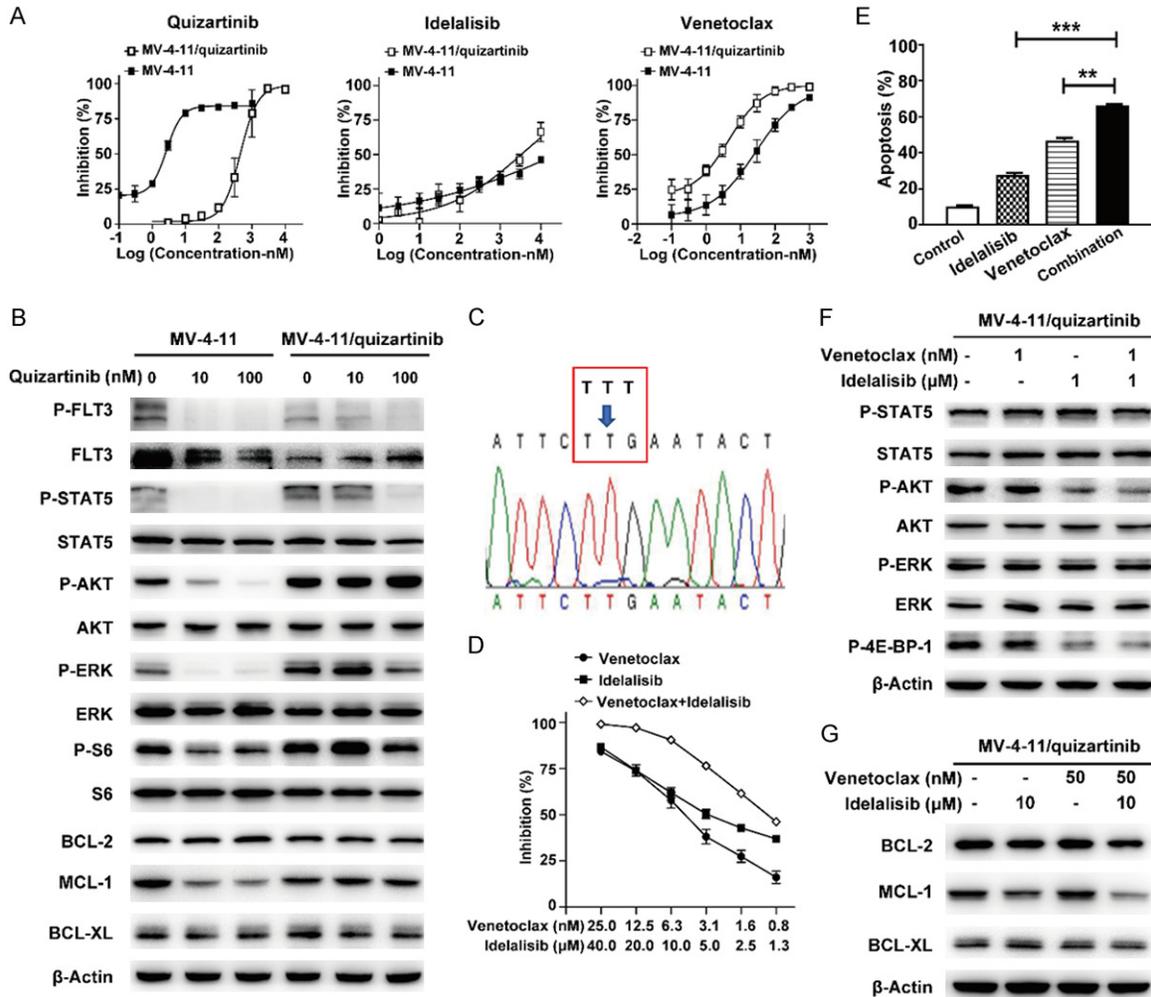
Notes: MV-4-11 and MV-4-11/quizartinib cells were treated with different concentrations of compounds for 72 h. Cell survival was analysed by MTT assay. Data are presented as the means  $\pm$  SD of three independent experiments. RF, resistance factor.

To investigate the mechanism of resistance to quizartinib in MV-4-11/quizartinib cells, the FLT3 signalling in parental and resistant cells was profiled. In MV-4-11/quizartinib, quizartinib still potently decreased the phosphorylation of FLT3 and STAT5, but to a lesser extent compared with parental MV-4-11 cells (Figure

4B). Nevertheless, it significantly suppressed the phosphorylation of AKT, ERK and S6 only in parental cells (Figure 4B). Additionally, quizartinib only suppressed the level of MCL-1 in parental but not in MV-4-11/quizartinib cells without affecting other antiapoptotic proteins of the BCL-2 family, including BCL-2 or BCL-XL. Next-generation sequencing of FLT3 in MV-4-11/quizartinib cells revealed substitution of F691L within the FLT3 tyrosine kinase domain (TKD) (Figure 4C), which was previously identified in AML patients who relapsed upon treatment with quizartinib [33].

Furthermore, the combination of idelalisib and venetoclax showed strong synergistic antiproliferative effects with a CI value of 0.08 and increased the quantity of apoptosis in MV-4-11/quizartinib cells (Figure 4D, 4E). Additionally, cotreatment greatly decreased the phosphorylation of AKT and 4E-BP-1 and the level of MCL-1 (Figure 4F, 4G). Together, these findings indicated the potency of the BCL-2 and PI3K $\delta$  inhibitor combination in refractory AML with FLT3-ITD/F691L dual mutations based on de-

## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment



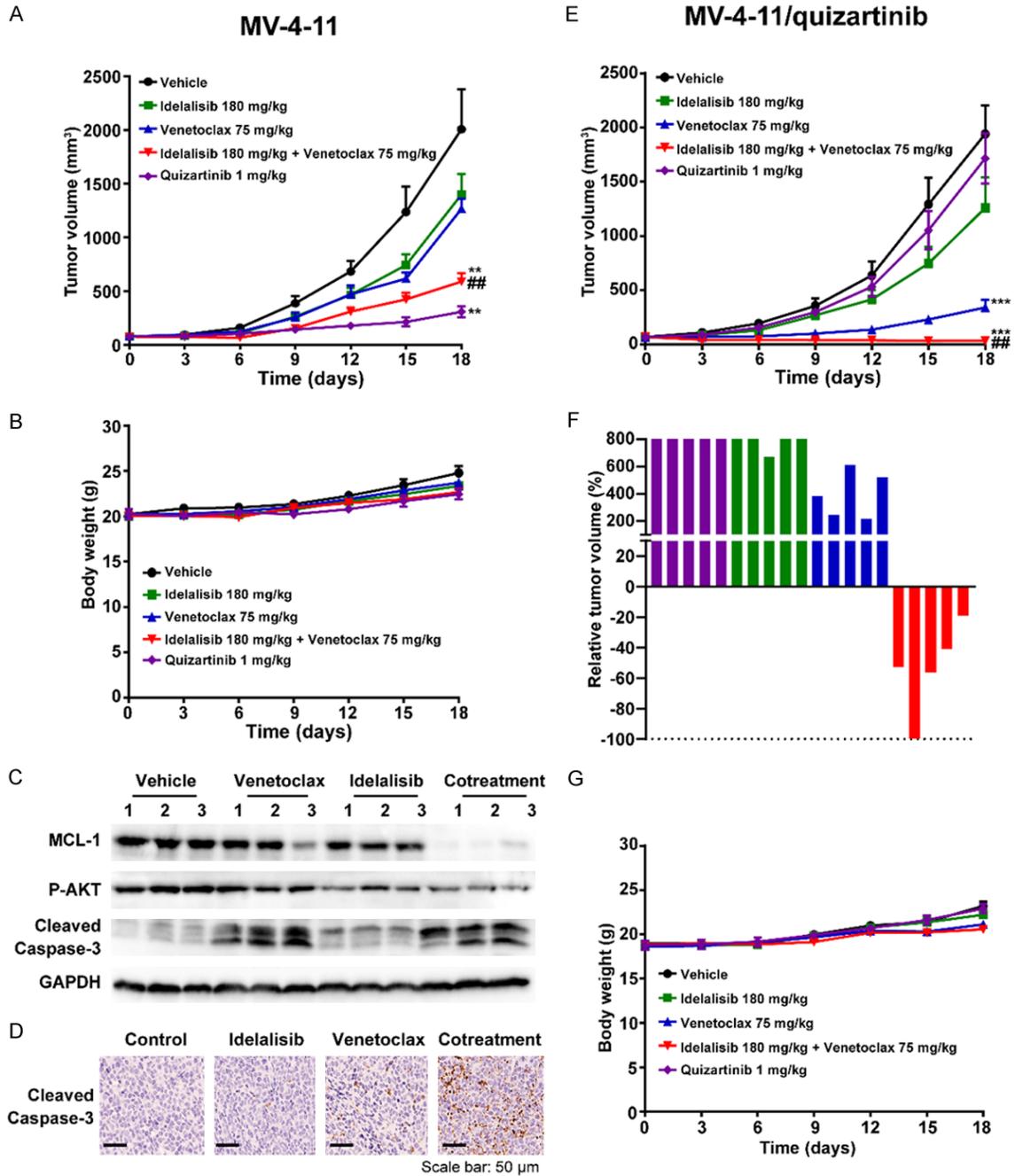
**Figure 4.** The combination of BCL-2 and PI3K $\delta$  inhibitors overcomes acquired resistance to FLT3 inhibitors in AML cells. (A) After MV-4-11 and MV-4-11/quizartinib cell lines were treated with quizartinib, idelalisib or venetoclax for 72 h, MTT assay was used to assess cell viability. (B) After 3 h treatment with quizartinib in MV-4-11 and MV-4-11/quizartinib cell lines, the phosphorylation status of FLT3 signalling protein and expression of BCL-2 family members were determined by Western blotting. (C) Sequencing of FLT3 in MV-4-11/quizartinib. The "T" to "G" replacement at the codon 691 led to a Phe-to-Leu exchange (F691L). (D) After MV-4-11/quizartinib cells were treated with venetoclax, idelalisib, or a combination for 72 h, MTT assay was used to assess cell viability. (E) Cells were treated with idelalisib (10  $\mu$ M), venetoclax (50 nM), or their combination for 24 h, and cell apoptosis was measured by annexin V-FITC/PI staining. (F, G) After MV-4-11/quizartinib cells were treated with venetoclax and/or idelalisib for 3 h, the lysates were detected by Western blot with the indicated antibodies. \*\*P < 0.01, \*\*\*P < 0.001.

creasing MCL-1 expression through the inactivation of AKT/4E-BP-1 signalling.

*The combination of BCL-2 and PI3K $\delta$  inhibitors has synergistic antitumour activity in AML xenografts*

Next, we investigated the *in vivo* antitumour efficacy of cotreatment with PI3K $\delta$  and BCL-2 inhibitors. Consistent with previous data [28], although MV-4-11 cells were sensitive to venetoclax *in vitro*, inherent resistance to venetoclax in MV-4-11 xenografts was observed. Ide-

lalisib (180 mg/kg) or venetoclax (75 mg/kg) alone showed weak antitumour activities with tumour growth inhibition (TGI) values of 38.3% and 31.4%, respectively, whereas cotreatment produced an enhanced antitumour efficacy with a TGI of 73.5% (Figure 5A). No obvious body weight loss was found when treated with drug alone or in combination (Figure 5B). Furthermore, compared with either drug alone, decreased expression of MCL-1 and an increased level of cleaved caspase 3 in tumour tissues from cotreatment groups were observ-



**Figure 5.** The combination of BCL-2 and FLT3 inhibitors exerts enhanced antitumor activity in mice xenografted with human AML. (A-D) MV-4-11 xenograft mice received vehicle, idelalisib (180 mg/kg), venetoclax (75 mg/kg), their combination or quizartinib (1 mg/kg). Tumour volumes were determined (A), and mouse body weights were measured (B). The mice were euthanized 3 h following the final dose, and then tumours were excised, lysed, and analysed by Western blotting (C) or IHC (D). (E-G) MV-4-11/quizartinib tumour-bearing mice were treated with vehicle or the indicated dosages of idelalisib, venetoclax, their combination or quizartinib. Tumour volumes were measured (E), relative tumour volumes were indicated as percent change from baseline at Day 18 (F), and body weights were shown as the means  $\pm$  SD (G). \*\*P < 0.01, \*\*\*P < 0.001, versus vehicle; ##P < 0.01 versus either single agent alone.

ed by Western blotting and IHC assays (Figure 5C, 5D).

Consistent with the *in vitro* data, MV4-11/quizartinib xenografts were resistant to quizar-

tinib with a TGI of 12.0% at a dosage of 1 mg/kg, compared with 88.1% in MV-4-11 xenografts. Unexpectedly, MV-4-11/quizartinib xenografts displayed preferable sensitivity to venetoclax with a TGI of 85.4% at a dosage of 75 mg/kg. Moreover, in comparison to each single agent, the combination led to further reduced tumour growth in the MV-4-11/quizartinib xenograft model ( $P < 0.01$ ) (**Figure 5E**). Importantly, tumour regression was observed in all mice from the cotreatment group, while no regression of tumours was observed when treated with single agent (**Figure 5F**). Furthermore, complete tumour regression was found in one of five mice in the cotreatment group. The combination treatment was generally well-tolerated, and no obvious loss of body weight was found in all groups (**Figure 5G**). In summary, these results indicated that venetoclax combined with idelalisib exhibits synergistic benefits in AML with FLT3-ITD and/or acquired TKD mutation.

### Discussion

BCL-2 overexpression is related to poor therapy response in AML, which makes it a theoretically promising treatment target. However, monotherapy with the BCL-2 specific inhibitor venetoclax shows limited efficacy at the relapse setting in AML [9]. Thus, there is a clinical rationale for exploring venetoclax-based combination regimens that maximize efficacy while minimizing toxicity. PI3K $\delta$ , which is constitutively activated in AML blast cells [21, 34], appears to be a suitable target of combination treatments against AML. In our study, we demonstrated that co-inhibition of PI3K $\delta$  and BCL-2 exerted synergistic lethality and overcame FLT3 inhibitors acquired resistance in AML through AKT/4E-BP-1 inactivation and MCL-1 down-regulation.

It has been shown that the combination of venetoclax and PI3K/mTOR inhibitors exhibits a synergistic effect. As PI3K $\delta$  is uniformly expressed and constitutively activated in AML, it may be more suitable for combination with a higher selectivity and lower toxicity. In the current study, the combination of venetoclax and a selective PI3K $\delta$  inhibitor idelalisib exhibited a synergistic antiproliferative effect and induced caspase-dependent apoptosis both in venetoclax-sensitive and venetoclax-resistant AML

cells. Since the complex interactions among BCL-2 family members, individual measurements of the various antiapoptotic proteins may be insufficient to provide comprehensive data on the evaluation of antiapoptotic dependencies in AML. FLT3-ITD mutation, which is one of the most common genetic alterations in AML, emerged at relapse after venetoclax monotherapy and a small subset of FLT3-ITD<sup>+</sup> patients showed no responses to venetoclax [12, 35]. Although FLT3-ITD<sup>+</sup> MV-4-11 cells have high expression of BCL-2 and are sensitive to venetoclax *in vitro*, as seen in our and others' previous studies [28], venetoclax exhibited weak *in vivo* antitumour activity in this xenograft model. However, the combination of venetoclax and idelalisib was synergistically lethal and exhibited significantly enhanced antitumour activity in FLT3-ITD<sup>+</sup> AML xenografts.

In addition to BCL-2, MCL-1 is frequently overexpressed and plays an important prosurvival role in AML [36]. Upregulated MCL-1 has appeared to be a common determinant of venetoclax resistance [37]. siRNA knockdown of MCL-1 in venetoclax-resistant cells resulted in improved efficacy of venetoclax [38], and ectopic overexpression of MCL-1 attenuated apoptosis induced by venetoclax [35]. A previous study showed that the PI3K/AKT/mTOR pathway regulated the expression of MCL-1 by 4E-BP-1/mTORC1-dependent translation in AML [32]. Another recent study suggested that MCL-1 disrupted intramolecular PH/KD interactions by its PEST domain with AKT at the PH domain, resulting in AKT activation in cancer cells [39]. In the present study, compared with either drug alone, venetoclax combined with idelalisib exhibited greater inhibition of AKT/4E-BP-1 phosphorylation and MCL-1 expression in venetoclax-sensitive AML cells. Additionally, the combination greatly diminished the level of MCL-1 protein bound to Bim both in MV-4-11 and EOL-1 cells. It is known that released Bim binding to MCL-1 is one of the mechanisms accounting for the intrinsic resistance to venetoclax [40]. Thus, PI3K $\delta$  inhibition, via down-regulating AKT, would lead to reduced MCL-1 expression, and the combination with the BCL-2 selective inhibitor venetoclax would prevent BCL-2 from sequestering free Bim, thereby activating Bim and allowing induction of apoptosis.

Certainly, the specific mechanism still requires more detailed experimental demonstrations.

FLT3 inhibitors have shown limited effect in AML due to the emergence of resistance, even the next-generation FLT3 inhibitor quizartinib [33, 41, 42]. Our results demonstrated that quizartinib-resistant MV-4-11 cells exhibited disparate sensitivities to multiple FLT3 inhibitors, with high-level resistance to both quizartinib and sorafenib, whereas they showed less resistance to the type I FLT3 inhibitors crenolanib, gilteritinib and sunitinib. Sequencing of FLT3 revealed the gatekeeper mutation of F691L within the TKD in quizartinib-resistant MV-4-11 cells. Accordingly, a previous study showed that AML patients with acquired point mutation at F691L developed resistance to the type II kinase inhibitor quizartinib but remained sensitive to the type I inhibitor [33]. Quizartinib markedly downregulated the phosphorylation of AKT and ERK, as well as MCL-1, only in parental MV-4-11 cells. However, the combination of venetoclax and idelalisib exhibited enhanced inhibition of the phosphorylation of AKT and expression of MCL-1 and synergistic efficacy in MV-4-11/quizartinib cells, as it did in the parental cells. Thus, MCL-1 may be one of the mechanisms contributing to intrinsic resistance to venetoclax and acquired resistance to quizartinib, which can be overcome by the combination of venetoclax and idelalisib.

It has been reported that FLT3-ITD<sup>+</sup> cells become BCL-2-dependent following quizartinib treatment [28]. Accordingly, in the present study, venetoclax alone was considerably more active against FLT3 inhibitor-resistant cell line MV-4-11/quizartinib than parental cell line MV-4-11 both *in vitro* and *in vivo*. The TGI values for venetoclax at a dose of 75 mg/kg were 31.4% and 85.4% in parental and quizartinib-resistant MV-4-11 xenografts, respectively. Although these results suggest that the shift of dependence to different BCL-2 family members may contribute to the potential antitumour effects of venetoclax in MV-4-11/quizartinib AML with the F691L mutation, it remains important to understand the mechanism underlying this regulation in the future. Moreover, the combination of venetoclax and idelalisib further improved the reduction of tumour burden in the MV-4-11/quizartinib xenograft than drug treatment alone ( $P < 0.01$ ) and induced tumour

regression in all mice from the cotreatment group. In addition, the cotreatment was generally well-tolerated, and no obvious loss in body weight was found during the experiments in all groups. Thus, co-inhibition of BCL-2 and PI3K $\delta$  may provide a new option against AML with higher selectivity and lower toxic effects and may also prevent the emergence of FLT3-mutated, venetoclax-resistant subclones in patients.

In summary, cotreatment with venetoclax and idelalisib exerts synergistic lethality against AML cells co-expressing BCL-2 and PI3K $\delta$  and overrides acquired resistance to quizartinib in FLT3-ITD<sup>+</sup> AML, both *in vitro* and *in vivo*, which is mediated by the greater attenuation of P-AKT and MCL-1 levels. Given the encouraging preliminary clinical results of venetoclax monotherapy in AML, our findings suggest that the combination of venetoclax and PI3K $\delta$  inhibitors is a promising strategy for the treatment of AML, particularly for FLT3-ITD<sup>+</sup> patients who develop quizartinib resistance because of FLT3-TKD mutations.

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### Disclosure of conflict of interest

None.

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## Combination of BCL-2 and PI3K $\delta$ inhibitors for AML treatment

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