Short communication

Embelin potentiates venetoclax-induced apoptosis in acute myeloid leukemia cells

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Running title: Embelin-venetoclax combined effects in acute leukemia cells

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Abstract

Acute myeloid leukemia (AML) belongs to a group of hematological cancer whose relapse cases are often associated with chemoresistance, impairing treatment success and contributing to a poor outcome. For this reason, there is an urgent need for the development of new therapeutic strategies Herein, we explore the combination of venetoclax, a BCL2 inhibitor, and embelin, an XIAP inhibitor, in the AML cell lines. Combinatory treatment of venetoclax and embelin potentiated cytotoxic effects of these drugs, demonstrating that both in combination present lower IC_{50} values than single treatment either with venetoclax or embelin alone in both cell lines analyzed. The combinatory treatment further increased the apoptosis-inducing properties of both compounds. Computer simulations suggest that embelin binds to both BIR2 and BIR3 domains of XIAP, reinforcing this inhibitory apoptosis protein as embelin target. Although all AML cell lines presented similar basal levels of XIAP, the combinatory treatment effectively inhibited XIAP expression in OCI-AML3 cells. In conclusion, the inhibition of both apoptosis inhibitory players, BCL2 and XIAP, by venetoclax and embelin, respectively, potentiated their cytotoxic effects in AML cell lines.

Key words: acute myeloid leukemia; embelin; venetoclax; apoptosis

1. Introduction

Acute myeloid leukemia (AML) is characterized by clonal expansion of myeloid progenitors in the bone marrow and peripheral blood. It is a heterogeneous disease, whose genetic alterations are frequently associated with poor chemotherapeutic responses and disease relapse. Thus, it is necessary to search for new compounds and targets in order to overcome AML resistance (Kirtonia et al. 2020). Venetoclax (ABT-199) is an oral bioavailability drug that selectively inhibits the anti-apoptotic protein BCL2. It was approved by the FDA, in 2016, for the treatment of chronic lymphocytic leukemia, presenting positive results in 70% of treated patients, especially as a first-line treatment. Recently, clinical trials indicated that venetoclax therapy displays effective results in unfavorable risk or elderly AML patient groups (DiNardo et al. 2020, DiNardo et al. 2019). Venetoclax activates intrinsic apoptosis, mediated by caspase 9 (Konopleva et al. 2006). Despite the promising results, both chemoresistance and relapse have been reported (Pei et al. 2020) and this is linked to positive regulation of anti-apoptotic proteins, including those of the Inhibitory Apoptosis Proteins (IAP) family (Chen et al. 2020).

Embelin (2,5-dihydroxy-3-undecyl-1,4- benzoquinone), a natural product isolated mainly from some species of Primulaceae, has been described to inhibit X-linked inhibitor of apoptosis (XIAP) (Nikolovska-Coleska et al. 2004, Ogawa et al. 1968). Among the IAPs, XIAP, also known as BIRC4, is recognized for its inhibitory activity of both initiator and executioner caspases. XIAP has three characteristic functional domains in N-terminal known as zinc finger baculoviral IAP repeats (BIRs), that bind to active caspases 9, 3 and 7 (Reis-Silva et al. 2020). Embelin binds to the BIR3 domain of the protein preventing its association with caspase 9 resulting in suppression of cell growth, proliferation, and migration in different tumor models (Prabhu et al. 2017). Its antitumor activity has been demonstrated for a variety of cancer types, including leukemia (Coyle et al. 2019).

Since the mechanisms that suppress the activation of caspases (*i.e.* XIAP) may attenuate the effects of venetoclax, contributing to the observed chemoresistance in treated patients, here we investigate whether embelin potentiates the effects of venetoclax in AML cellular models.

2. Material and methods

2.1. Cell culture and reagent chemicals

Acute myeloid leukemia cell lines, MOLM13, MV4-11, OCI-AML3, and Kasumi 1, were kindly provided by Prof. Eduardo Magalhães Rego (University of São Paulo, Ribeirão Preto, Brazil). Cell culture conditions were performed in accordance with the recommendations of the American Type Culture Collection (ATCC) and Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ). Venetoclax (ATB-199) was purchased from TargetMol (Target Molecule Corp., Boston, MA, USA) and prepared as a 50 mM stock solution in dimethyl sulfoxide (Me_2SO_4; DMSO) (Figure 1A). Embelin was isolated from the fresh ripe fruits of *Myrsine umbellata* Mart. (Primulaceae) collected at São José dos Alpes, Campos do Jordão (7°486'063" S, 452°648" W), Sao Paulo, Brazil (December 2014) as previously described with some modifications (Bisrat et al. 2014). The species was identified by Dr. Inês Cordeiro from Instituto de Botânica do Estado de São Paulo, São Paulo, Brazil, a voucher was deposited at the herbarium of the same institution (SILVA-3). Briefly, 252 g of dried fruits were extracted with 70% ethanol in a turbo-extractor. The resulting extract was filtered, and the solvent evaporated under reduced pressure at 40°C, obtaining crude hydroethanolic extract. It was then successively partitioned between MeOH-water mixture (1:9) and *n*-hexane. The solution was completely evaporated to give the corresponding dried fraction. It was subjected to column chromatography on silica gel using step gradients of *n*-hexane-ethyl acetate to obtain twelve main fractions (Fh-1 to Fh-12). Fraction Fh-3 was purified by preparative TLC (*n*-hexane-ethyl acetate. 1:1, v/v) to give embelin (Figure 1A).

2.2. Cell viability assay

The effects venetoclax and/or embelin on cell viability were investigated by methylthiazoletetrazolium (MTT) assays. Briefly, a total of 2×10^4 cells per well were plated in a 96-well plate and exposed to increasing concentrations of the venetoclax (Ø; 0.1; 0.5; 1; 5; 10 and 50 µM) or embelin (Ø; 8.5; 17; 34; 85; 170 µM) for 48 h. For combined treatment analysis, Kasumi 1 and OCI-AML3 cells were treated with graded doses of venetoclax (0.1; 0.5; 1; 5; 10, 25 or 50 µM) and embelin (8.5; 17; 34; 85 or 170 µM) alone or in combination with each other for 48 hours (Supplementary Table 1 and 2) and data were illustrated using multiple experiment viewer (MeV) 4.9.0 software (Saeed et al. 2003). Then, after incubation, 10 µL MTT solution (5 mg/mL) (Thermo Fisher Scientific, San Jose, CA, USA) was added and incubated at 37ºC, 5% CO_2 for 4 h. The reaction was stopped by using 100µL of 0.1N HCl in anhydrous isopropanol.

Cell viability was evaluated by spectrophotometry measuring the absorbance at 570 nm (Thermo Fisher Scientific, USA). The inhibitory concentration of 50% (IC_{50}) values was calculated using non-linear regression analysis on GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

2.3. Western blot analysis

Total protein extraction was performed using a buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 2 mM PMSF, 10 mM Na_3VO_4, 100 mM NaF, 10 mM Na_4P2O_7, and 4 mM EDTA. Equal amounts of protein (30µg) were used from total extracts followed by SDS-PAGE and Western blot analysis with the indicated antibodies and was carried out using a SuperSignal TM West Dura Extended Duration Substrate System (Thermo Fisher Scientific, San Jose, CA, USA) and a G:BOX Chemi XX6 gel doc system (Syngene, Cambridge, UK). Antibodies directed against XIAP (#2042), caspase 3 (#9665), cleaved-caspase 3 (#9661), PARP1 (#9542) and α-tubulin (#2144) were from Cell Signaling Technology (Danvers, MA, USA). Antibody directed against γH2AX (sc-51748) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was obtained using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and a SybrGreen System for the following expressions: *XIAP* (FW: GACAGTATGCAAGATGAGTCAAGTCA; RV: GCAAAGCTTCTCCTCTTGCAG), *HPRT1* (FW: GAACGTCTTGCTCGAGATGTGA; RV: TCCAGCAGGTCAGCAAAGAAT), and *ACTB* (FW: AGGCCAACCGCGAGAAG; RV: ACAGCCTGGATAGCAACGTACA). *HPRT1* and *ACTB* were used as reference genes. Relative quantification values were calculated using the $2^{-\Delta\Delta CT}$ equation (Livak et al. 2001). A negative 'No Template Control' was included for each primer pair.

2.5. Apoptosis assay

A total of 1×10^5 cells per well were seeded in a 24-well plate in the presence of vehicle (Ø) or venetoclax (Kasumi 1: 1µM; OCI-AML3: 10µM) and/or embelin (Kasumi 1: 85

and 170 μM; OCI-AML3: 8.5 and 34 μM) for 48 hours. Next, the cells were washed with ice-cold PBS and resuspended in a binding buffer containing 1 μg/ml 7AAD and 1 μg/ml APC labeled annexin V. All specimens were analyzed by flow cytometry (FACS Calibur; Becton Dickinson) after incubation for 15 min at room temperature in a light-protected area. Ten thousand events were acquired for each sample.

2.6. Computational docking

Computational docking was modeled with Protein Energy Landscape Exploration (PELE) using a global and a refinement local sampling. The Global sampling is to find possible binding sites. For this, PELE uses larger ligand translations and rotations and starts multiple simulations (128 in this case) with the ligand covering the entire protein surface. The local one takes the best binding sites found in the global search and performs a local refinement, where the ligand is asked to perform smaller translations and rotations, which are coupled to more thorough side chain predictions and minimization of the whole complex.

Herein, embelin bindings were modeled to two different BIR domains of XIAP protein. Therefore, a BIR2 conformation (PDB – 4WVS structure) with 1.4 Å resolution and a BIR3 conformation (PDB – 3CLX structure) with 1.4 Å resolution were used. Moreover, to confirm the binding site, the BIR3 domain was also modeled in its tetramer conformation (PDB – 3CLX structure). Default global and local protocols, as shown in the PELE web server, https://pele.bsc.es/pele.wt, were used.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). For comparisons, ANOVA and Bonferroni post-test were used. A p value <0.05 was considered as statistically significant.

3. Results

3.1. Venetoclax and embelin promote different cytotoxic effects on leukemia cells

In the present study, we confirmed that venetoclax was active against leukemia cell lines MOLM13, MV4-11, and Kasumi 1, with IC_{50} values ranging from lower than 0.1 to 5 μM. By contrast, the OCI-AML3 cell line was resistant to venetoclax treatment,

with IC_{50} value of 40 µM (Figure 1B), corroborating with Lima *et al.* (2021). For this reason, Kasumi 1 and OCI-AML3 cells were selected for drug combination assays.

Embelin treatment did not promote cytotoxicity in any cell line evaluated, with IC_{50} values superior to 170 µM. Even though it is possible to point out some cell lines that had a minimum drop in the cell viability, such as MOLM13 and OCI-AML3, the cell viability has not been compromised by 50% (Figure 1C).

The baseline XIAP expression was evaluated in AML cell lines used in the present study. In the mRNA assay, it was seen that although all of them express XIAP at different levels, the differences were not statistically significant (Supplementary Figure 1A). Western blot assay also revealed that all AML cell lines expressed high XIAP levels (Supplementary Figure 1B).

3.2. Venetoclax plus embelin combination potentiates cytotoxic effects on Kasumi 1 and OCI-AML3 cells through induction of apoptosis

In the combinatory treatment for Kasumi 1 cells, after 48h, IC_{50} values decreased from 6.9 µM for venetoclax alone to 4 µM for venetoclax when combined to 85 µM embelin (Figure 2A). Upon a higher concentration of embelin (170 µM), the IC_{50} of venetoclax was further reduced to 1.8 µM, demonstrating that although embelin did not have cytotoxic properties, when the cells were treated with venetoclax, it increased the cytotoxicity of the BCL2 antagonist. Similar results were obtained with OCI-AML3 cells, where the IC_{50} values reduced from 31.2 µM to 26.2 µM and 21.7 µM when venetoclax was combined to embelin at 8.5 µM and 34 µM, respectively (Figure 2B). The results obtained with OCI-AML3 were even more exciting because of the non-responsive nature of the cell.

To evaluate whether combinatory treatment promotes apoptosis in OCI-AML3 and Kasumi1 cell lines, we treated cells with venetoclax at 10 µM combined with embelin at 8.5 or 24 µM for OCI-AML3 cells and venetoclax at 1.0 µM combined with embelin at 85 or 170 µM for Kasumi 1 cells for 48h. The concentration was chosen based on the cell viability assay. There was no increase in the number of apoptotic and/or necrotic cells in the presence of embelin based on the analysis of annexin-V externalization assay for both cell lines. Venetoclax, on the other hand, increased annexin V positive cells, which indicated the presence of an increasing number of apoptotic cells compared to the control in both tested cell lines.

While the lowest concentration of embelin (8.5 µM for OCI-AML3 cells and 85 µM for Kasumi 1) did not change apoptosis-inducing effects of venetoclax, combined treatment with the highest concentration of embelin (34 µM for OCI-AML3 cells and 170 µM for Kasumi 1) further increased apoptosis in the presence of venetoclax, indicating potentiating effects for both cell lines (Figure 2C-D).

3.3. Embelin potentiates venetoclax effects through down-regulating XIAP and promoting DNA damage and apoptosis in OCI-AML3 cells

Considering that in OCI-AML3 cells, the potentializing effects of embelin in venetoclax-induced apoptosis were more prominent, these cells were selected for molecular analysis. XIAP expression was reduced and γH2AX expression was induced upon the single treatment with embelin and venetoclax. However, embelin plus venetoclax induced a strong reduction of XIAP expression, caspase 3 and PARP1 cleavage and H2AX phosphorylation, confirming the observed apoptosis induction (Figure 3A).

3.4. Embelin binds to BIR2 and BIR3 domains of XIAP

To better understand the binding between embelin and XIAP, we performed induced-fit docking studies using two BIR domains of XIAP, BIR2 (PDB 4WVS) and BIR3 (3CLX) domains. These BIR domains were used due to the previous related activity of embelin in modulating XIAP (Prabhu et al. 2017). While affinity ranges are not too different, our simulations indicate better binding energy profiles along with more localized local minima for the BIR2 domain. The minimum interaction energy with predicted affinity is shown in Figure 3B, where we observe the global search in dark blue and the local refinement in cyan, where it is possible to measure the distance between the C1 carbon of embelin to the LEU 307 alpha carbon. The best interaction energy observed for BIR2 simulation was around -40kcal/mol, while the best interaction energy for BIR3 simulation was around -35Kcal/mol (circled in orange for both simulations). The simulation with best interaction energy for BIR2 is pointed at Figure 3C. Other simulation using different values were performed demonstrating similar results regarding the binding site of embelin (Supplementary Figure 2). Moreover, since the 3CLX structure is a tetramer, a global simulation on the tetramer was performed, showing no major alteration regarding the binding modes with that of the BIR3 monomer (Supplementary Figure 3).

4. Discussion

Venetoclax, also named as ABT-199 or GDC-0199, was the first potent and highly selective BH3 mimetic antagonist of BCL2 (Souers et al. 2013). Since its approval for the treatment of chronic lymphocytic leukemia, venetoclax has been extensively studied, with over 150 clinical trial underway, mainly for the treatment of acute myeloid leukemia patients (www.clinicaltrials.org). Despite the promising results, survival rates for AML remains low, that is mainly caused due to resistance to chemotherapy and high rates of relapse. Combinatory therapy has been extensively studied and demonstrated better results (Liu et al. 2019, Nishi et al. 2020, Samra et al. 2020). Altogether, due to the multiplicity of phenotypes for AML, combinatory strategies have been exploited, and herein we described the positive combinatory effects of venetoclax and embelin, an XIAP inhibitor.

Embelin itself benefits for AML treatment were already observed in Yang *et al.* (Yang et al. 2015) that demonstrated embelin sensitized AML cell to TRAIL through the repression of NFκB signal pathway, moreover, combined therapy of Ad-TRAIL and embelin was attractive for clinical application in the treatment of AML. In the current study, we demonstrated that embelin alone did not induce cytotoxicity, however, when combined with venetoclax, even in resistant-venetoclax cell lines, embelin potentiated the cytotoxic effects. This feature may be explained by the downregulation of XIAP induced by embelin. XIAP inhibitors, such as embelin and dequalinium chloride promoted an impaired clonogenic capacity of AML stem cells (Moreno-Martinez et al. 2014), suggesting that XIAP is a promising target for AML. In addition, XIAP downregulation activated genes encoding proteasomal components, leading to the inhibition of proteasome degradation in AML cells. Both events, activation of caspases by targeting antiapoptotic proteins such as XIAP and inhibition of proteasome activity crosstalk, cooperate to the final antineoplastic effect in AML models (Carter et al. 2013). Present results suggested a direct interaction of embelin with both BIR2 and BIR3 domain of XIAP proteins, both already recognized as promising targets for cancer therapy (Cossu et al. 2019, Lee et al. 2016, Schimmer et al. 2009).

In summary, we found that venetoclax and embelin combination reduces the viability of AML cells by triggering apoptosis, XIAP down-regulation and induced DNA damage. Due to the low toxicity of embelin, this drug can be used in combination to reduce doses of venetoclax, which could minimize side effects with maintenance of

the therapeutic response or even reverse resistance mechanisms in AML patients. Our findings further highlight IAP-targeting drugs as a putative anticancer option for AML.

Author's contribution

C.M.S.R.S. execution of experiments, data analysis and interpretation, and manuscript writing. P.C.B. data analysis and interpretation, and manuscript editing. K.L. data analysis and interpretation, and editing. F.L.S. and P.R.H.M. embelin purification, and manuscript writing and editing. V.G. molecular docking analysis, and manuscript writing and editing. L.V.C.-L. conceptualization, supervision, manuscript writing and editing. J.A.M.-N. conceptualization, formal analysis, supervision, manuscript writing and manuscript writing. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Figure legends

Figure 1. Cell viability upon treatment with venetoclax and embelin in acute myeloid leukemia cell lines. (A) structures of embelin and venetoclax are illustrated. MOLM13, MV4-11, Kasumi 1 and OCI-AML3 cells were exposed to increasing concentrations of venetoclax (C) and embelin (D) for 48 hours. The values expressed represent the percentage of viable cells for each condition compared to the control (cells treated with vehicle [Ø]). The bar graphs represent the mean ± SD of at least four

independent experiments. The IC50 values are indicated in the graphs. The p-values and cell lines are shown in the graphs; * p <0.05, ** p <0.01, *** p <0.0001; ANOVA and Bonferroni post-test.

Figure 2. Embelin potentiates venetoclax effects in Kasumi 1 and OCI-AML3 cells. Dose-response cytotoxicity for combined treatment were analyzed by methylthiazoletetrazolium (MTT) assay for Kasumi 1 (A) and OCI-AML3 (B) cells treated with graded concentrations of embelin and venetoclax alone or in combination with each other for 48 hours, as indicated. Values are expressed as the percentage of viable cells for each condition relative to untreated controls. Results are shown as the mean of at least three independent experiments. Note that the IC50 in Kasumi 1 for treatment with venetoclax alone was 6.9 μM while in the treatment combined reduced to 4.0 and 1.8μM. Similarly, the IC50 in OCI-AML3 in treatment with only venetoclax was 31.2 μM while in the combined treatment reduced to 26. 2 μM and 21. 7μM. Apoptosis was detected by flow cytometry in Kasumi 1 (C) and OCI-AML3 (D) cells treated with embelin and/or venetoclax for 48 hours using an annexin V/7AAD staining method. Representative dot plots are shown for each condition; the upper and lower right quadrants (Q2 plus Q3) cumulatively contain the apoptotic population (annexin V+ cells). Bar graphs represent the mean±SD of at least three independent experiments quantifying apoptotic cell death. The p values and cell lines are indicated in the graphs; * p<0.05, ***p<0.0001; ANOVA test and Bonferroni post-test.

Figure 3. Embelin increases venetoclax-induced XIAP reduction and caspase 3 activation in OCI-AML3 and docking analysis between XIAP domains BIR2 and BIR3 and embelin. (A) Western blot analysis for the treatment in monotherapy and combination of embelin and venetoclax in OCI-AML3 cell line. The analysis was performed evaluating XIAP, α-tubulin, procaspase 3, cleaved-caspase 3, yH2AX, PARP1 in total cells extract from OCI-AML3 treated with embelin and venetoclax in concentrations of 34 μM and 10 μM for 48 hours. Membranes were reprobed with the antibody for the detection of α-tubulin. Note that embelin plus venetoclax strongly induced molecular markers of apoptosis and DNA damage in OCI-AML3 cells. Binding between embelin and XIAP BIR 2 and 3 domains using PELE. (A) Plotted graphic demonstrating the global search in dark blue and the local refinement in cyan. In the x axis, as a reference, it is the distance of the C1 (middle) carbon of embelin to the

LEU307 alpha carbon which was chosen due to the close proximity to the small molecule in the crystal. (B) Docked pose of embelin to the BIR2 domain (PDB- 4WVS) BIR3 (PDB- 3CLX) XIAP structure.

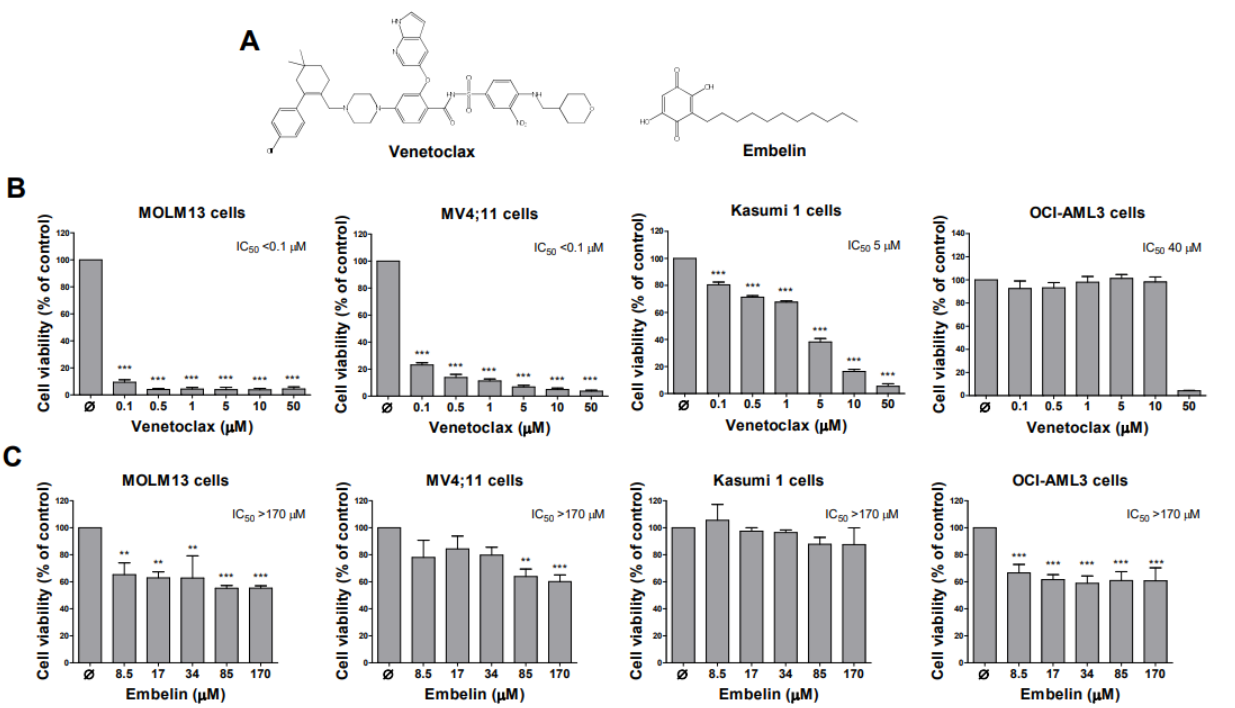
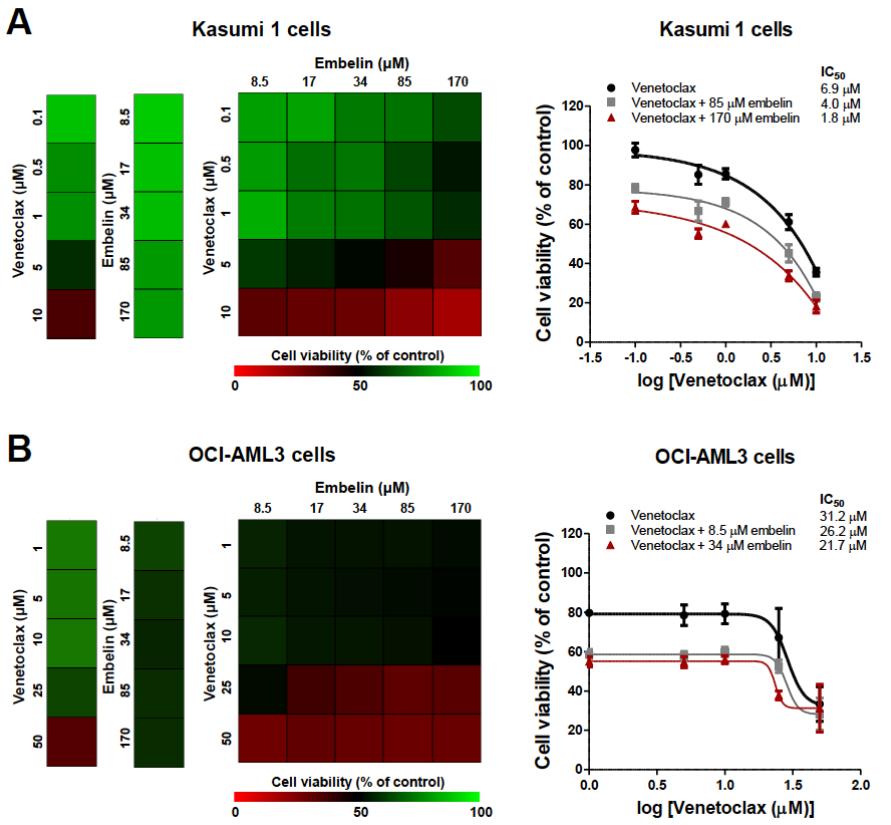

A
Venetoclax
Embelin
B
MOLM13 cells
IC50 <0.1 µM
MV4;11 cells
IC50 <0.1 µM
Kasumi 1 cells
IC50 5 µM
OCI-AML3 cells
IC50 40 µM
Cell viability (% of control)
Venetoclax (µM)
C
MOLM13 cells
IC50 >170 µM
MV4;11 cells
IC50 >170 µM
Kasumi 1 cells
IC50 >170 µM
OCI-AML3 cells
IC50 >170 µM
Embelin (µM)

Figure 1



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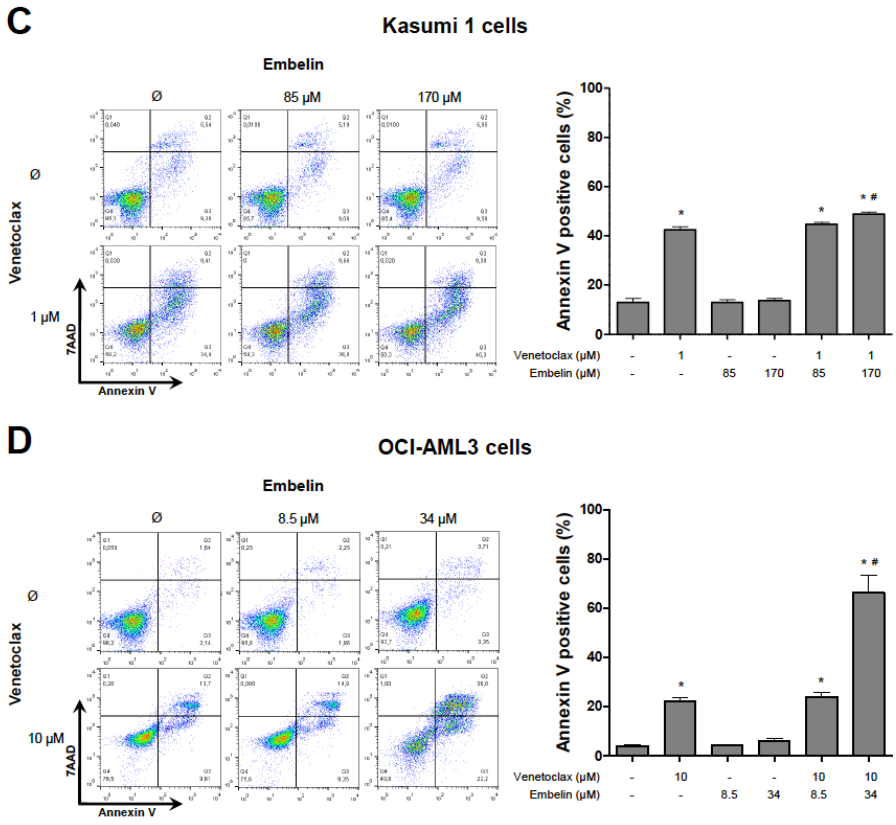

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464 Figure 2

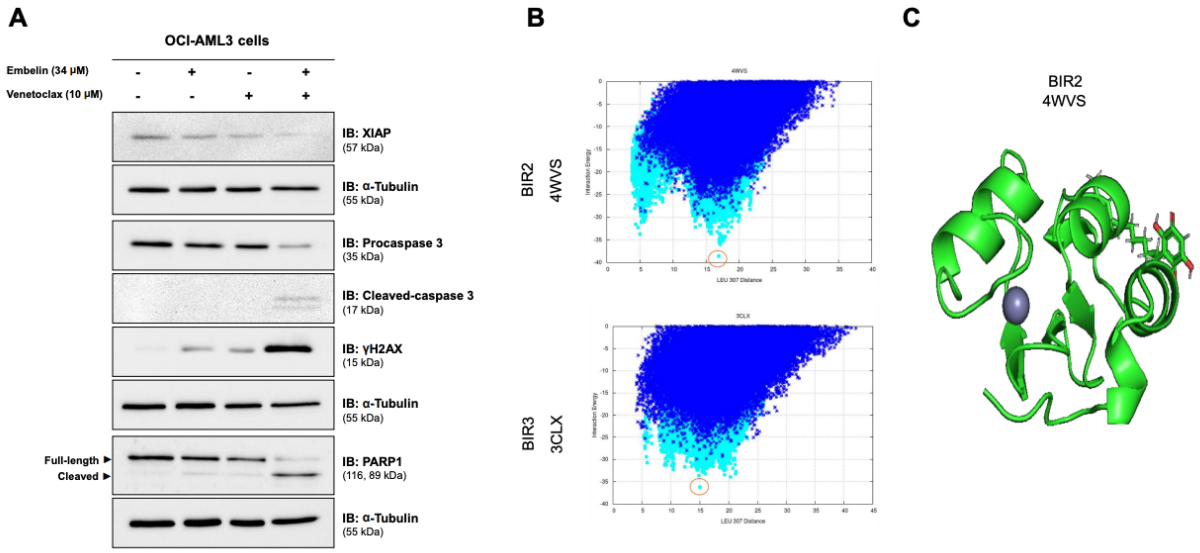
A
OCI-AML3 cells
Embelin (34 μM)
Venetoclax (10 μM)
IB: XIAP
(57 kDa)
IB: α-Tubulin
(55 kDa)
IB: Procaspase 3
(35 kDa)
IB: Cleaved-caspase 3
(17 kDa)
IB: γH2AX
(15 kDa)
IB: α-Tubulin
(55 kDa)
Full-length
Cleaved
IB: PARP1
(116, 89 kDa)
IB: α-Tubulin
(55 kDa)
B
BIR2
4WVS
BIR3
3CLX
C
BIR2
4WVS

Figure 3