Betulinic acid and doxorubicin drug combination induced cell death in AML MOLM-13 cell lines via ROS stimulation

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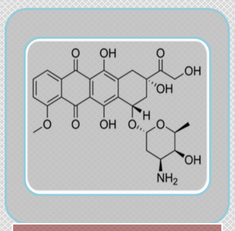
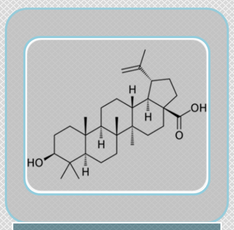
Introduction

Acute myeloid leukaemia (AML) has one of the highest mortality rate among the major leukaemia types[1]. Unfavourable clinical outcome of AML may be due to the high relapse rate of the disease and although not as common as in other cancers, this could lead to acquisition of additional mutations[2, 3]. Overexpression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) AML may contribute to difficulties in eradicating these cells[4,5]. Conventional AML treatment drugs such as doxorubicin (Dox) are very potent but also toxic to healthy non-cancerous cells[6].



Doxorubicin

- ✓ Standard chemotherapy
- ✓ Effective in a wide range of cancers
- ✗ Very toxic at higher dose (cardiotoxicity)
- ✗ Cancer develops resistance



Betulinic Acid

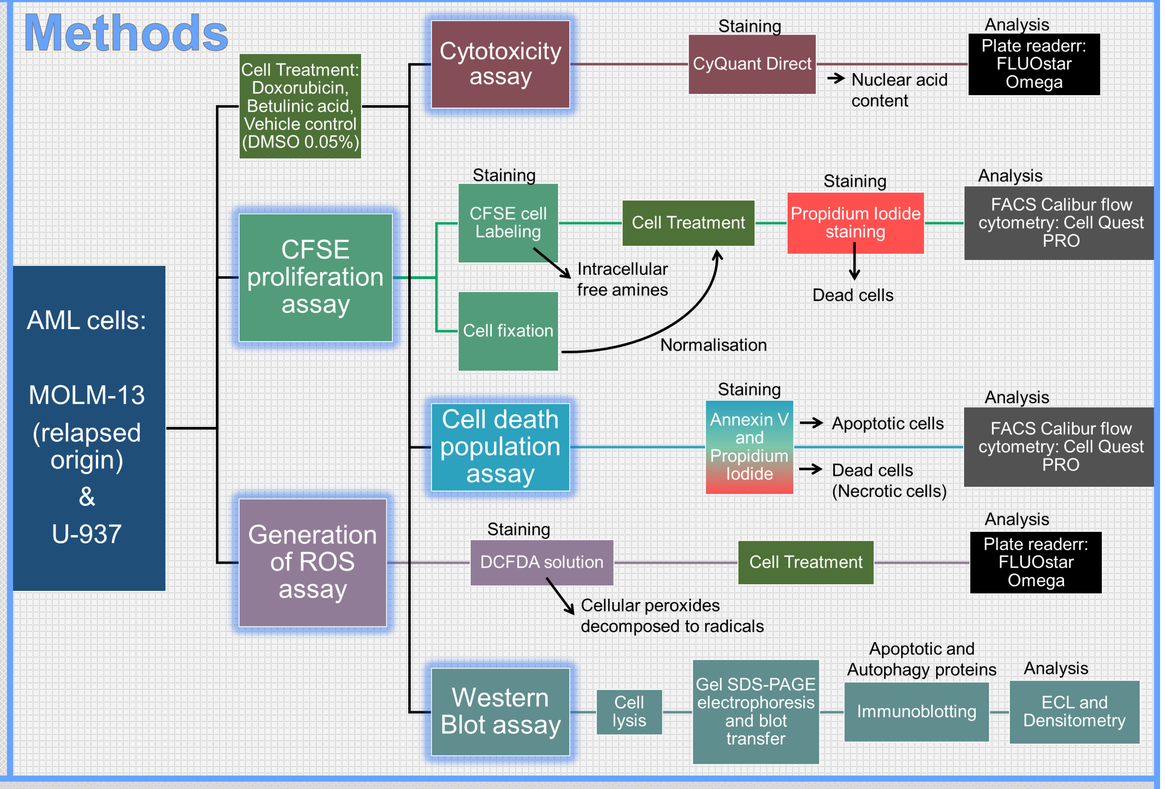
- ✓ Cytotoxic effect in various cancer cells
- ✓ Selective cytotoxic to cancer cells
- ✓ Sensitise cancer to chemotherapy drugs
- ✓ Stimulates cell death by affecting mitochondria

Compounds that can selectively target cancer through regulation of cellular processes such as apoptosis and/or autophagy may underpin cancer death induction. Betulinic acid (BetA) has been shown to cooperate with other chemotherapy drugs to sensitize cancers of epithelial origin to apoptotic death[7].

Aims

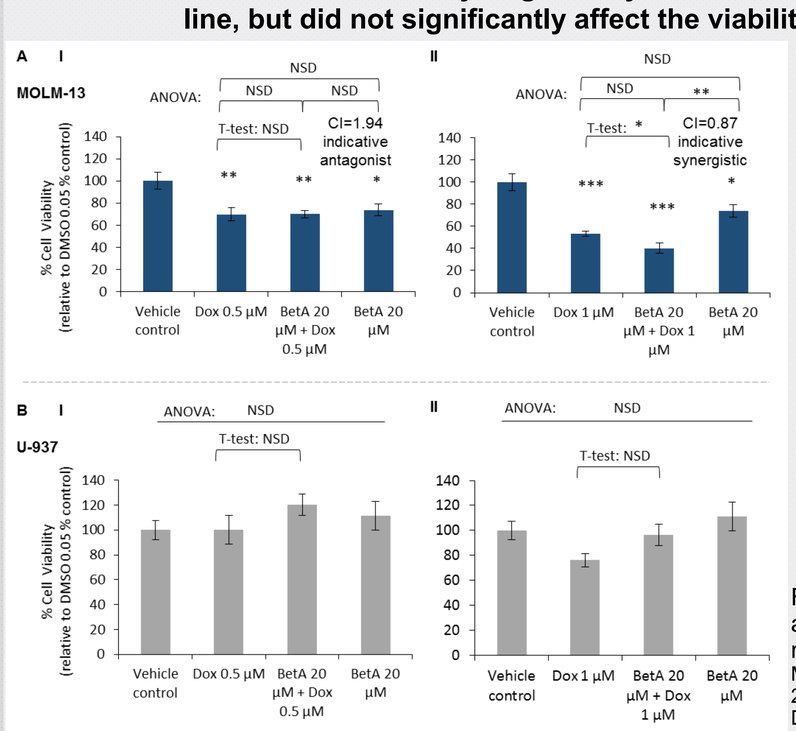
- ♦ To evaluate if BetA, in combination with Dox induce selective apoptotic death in AML cell lines
- ♦ To study the mechanism of cell death induced by the compounds singly and in combination
- ♦ To investigate the regulation and interaction between apoptotic and autophagy proteins by the treatments

Methods



Results

Combination of BetA and Dox synergistically reduced cell viability in MOLM-13 cell line, but did not significantly affect the viability of U-937 cells



$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2}$$

D is the concentration of single drugs and Dx, a dose of D alone, which gives x inhibition.
Chou-Talaya method: Combination Index (CI) using CompuSyn.
Additive effect: CI = 1
Synergism: CI < 1
Antagonism CI > 1

Figure 1. Effect of betulinic acid and doxorubicin combination treatments on cell viability of AML cells. MOLM-13 (A) and U-937 (B) treatments at 24 h. Cell viability measured by CyQuant Direct

BetA did not negatively affect the anti-proliferative effect of Dox in MOLM-13 cells

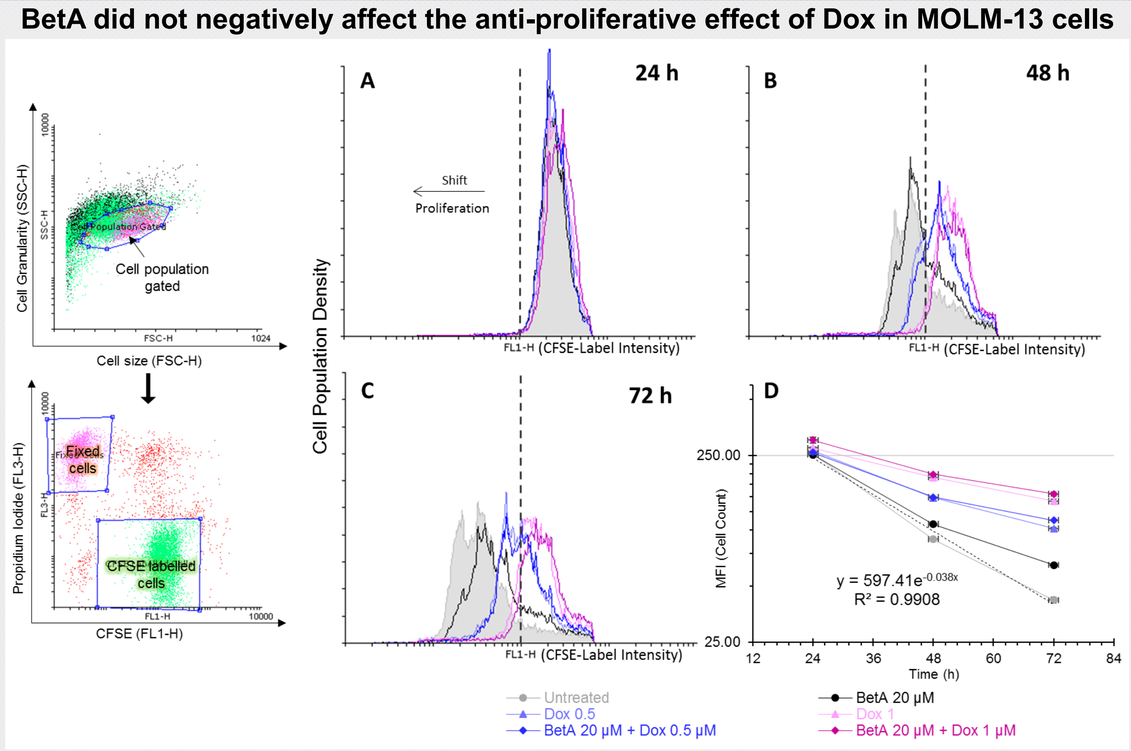


Figure 2. The anti-proliferative effect of betulinic acid and doxorubicin alone and in combination on AML cell line MOLM13 for 3 days. Cell proliferation was measured by CFSE . Flowing software was used for histogram overlay of treatments at (A) 24 h , (B) 48 h, and (C) 72 h. (D) The MFI values of MOLM13 after the treatments was plotted to generated the equation for doubling time.

Drug combination induced more death by late apoptosis in MOLM-13 cell line, while hindering apoptotic death of Dox in U-937 cells

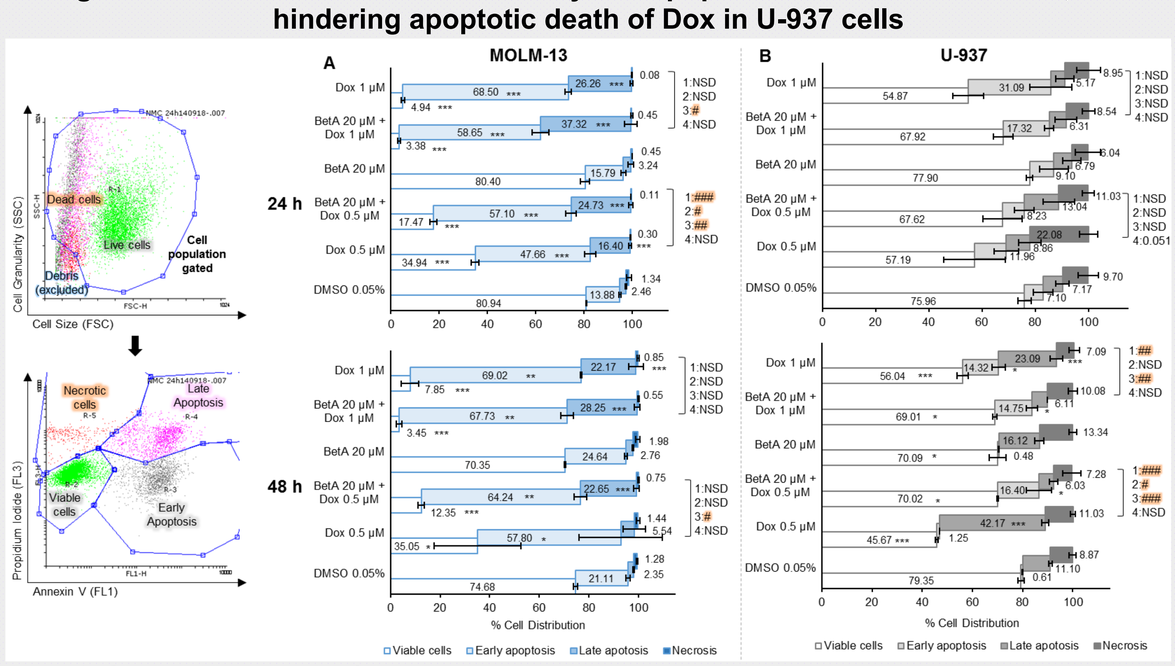
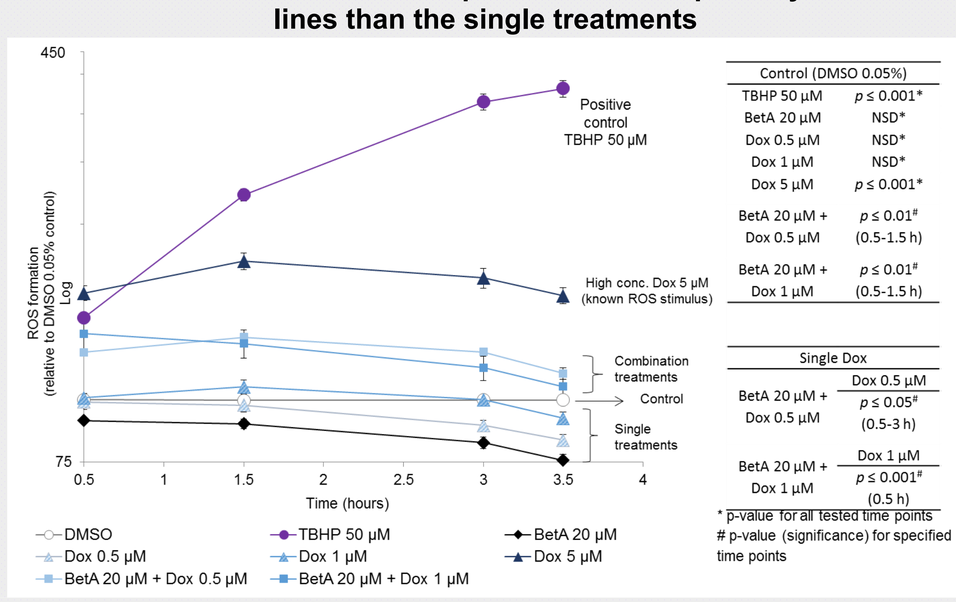


Figure 3. Cell death population of AML cells treated by single drugs and combination.
Leukaemic MOLM-13 (A) and U-937 (B) were double stained by Annexin V and Propidium Iodide.
* ($p \leq 0.05$)/** ($p \leq 0.01$)/*** ($p \leq 0.001$)Statistical difference between treatments and control (DMSO 0.05%).
# ($p \leq 0.05$)/## ($p \leq 0.01$)/### ($p \leq 0.001$)Statistical difference between single Dox and combination treatment.
1— Viable cells, 2—Early apoptosis, 3—Late apoptosis, 4—Necrotic cells

Combination treatments induced ROS production more potently in MOLM-13 cell lines than the single treatments



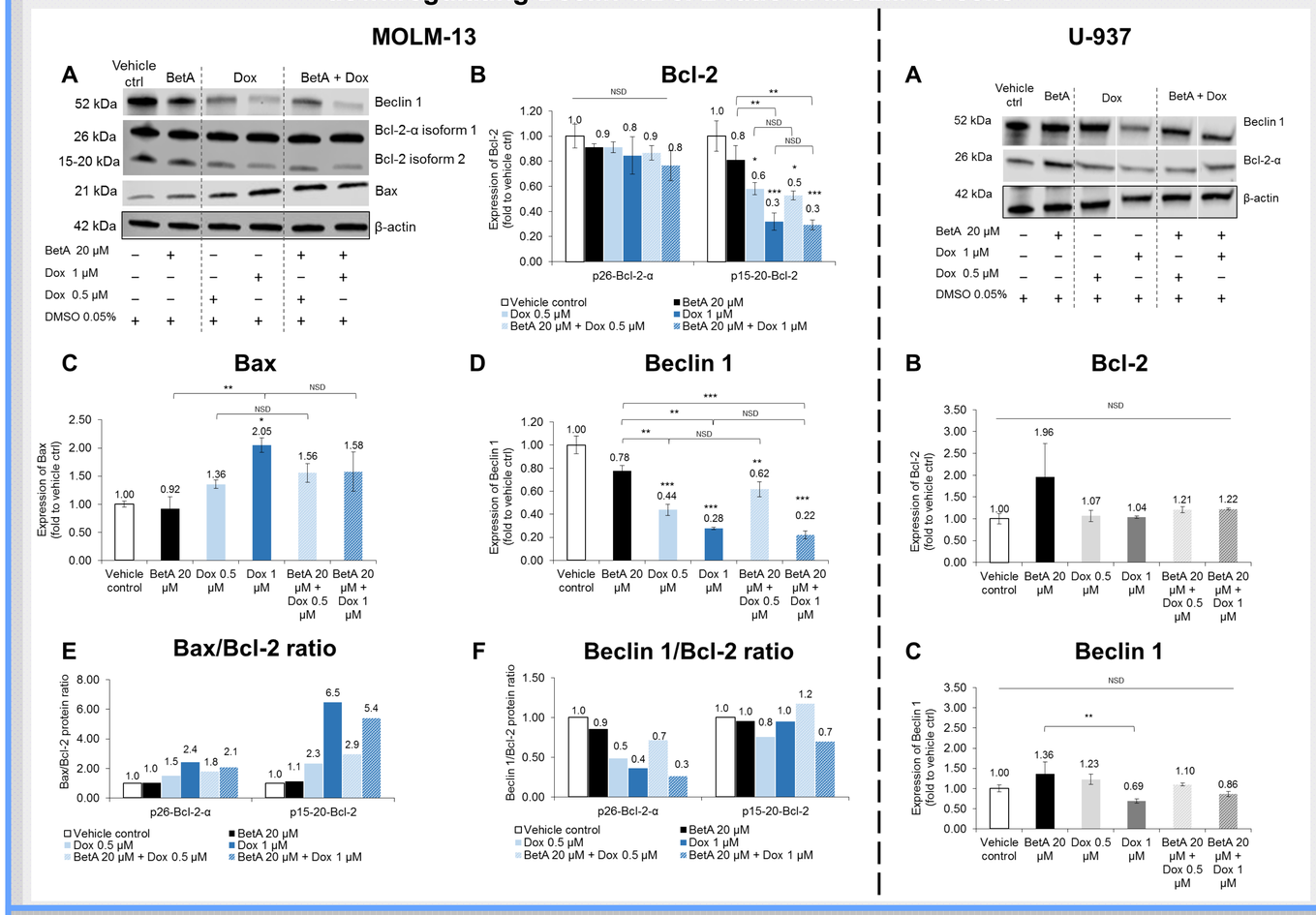
Control (DMSO 0.05%)	
TBHP 50 µM	$p \leq 0.001$*
BetA 20 µM	NSD*
Dox 0.5 µM	NSD*
Dox 1 µM	NSD*
Dox 5 µM	$p \leq 0.001$*
BetA 20 µM + Dox 0.5 µM	$p \leq 0.01$# (0.5-1.5 h)
BetA 20 µM + Dox 1 µM	$p \leq 0.01$# (0.5-1.5 h)

Single Dox	
	Dox 0.5 µM
BetA 20 µM + Dox 0.5 µM	$p \leq 0.05$# (0.5-3 h)
	Dox 1 µM
BetA 20 µM + Dox 1 µM	$p \leq 0.001$# (0.5 h)

* p-value for all tested time points
# p-value (significance) for specified time points

Figure 4. Reactive oxygen species formation over time in MOLM-13 after treatment with betulinic acid, doxorubicin and combination treatments. ROS formation up to 3.5 h was measured by DCFDA dye. TBHP and Dox 5 µM, known ROS inducers, were used as positive controls.

Dox alone and in combination with BetA elevated Bax/Bcl-2 ratio while downregulating Beclin 1/Bcl-2 ratio in MOLM-13 cells



Main findings

- ♦ The inhibition effect of BetA combined with Dox 1 µM was indicative synergistic (CI < 1) in suppressing the viability of MOLM-13 at 24 h incubation (Fig. 1 A II).
- ♦ The anti-proliferative effect of Dox in AML cell lines was not notably affected by combination with BetA (Fig. 2)
- ♦ More cells resided in irreversible late apoptotic stage after drug combination (BetA + Dox) treatment compared to the single Dox treatments ($p<0.05$) in MOLM13 cells (Fig.3 A)
- ♦ BetA suppressed Dox-induced apoptotic death in U-937 cells ($p<0.05$) at 48 h treatments (Fig. 3 B)
- ♦ BetA combined with Dox stimulated ROS formation in MOLM-13 cells between 0.5-1.5 h incubation period while individual drugs did not significantly alter ROS levels (Fig. 4)
- ♦ MOLM-13 cell death by single Dox and drug combination was found to be associated with an increase in Bax/Bcl-2 ratio (increase in apoptosis) and decrease Beclin 1/Bcl-2 ratio (modulating autophagy) (Fig. 5).

Figure 5. The effect of BetA, Dox and combination on apoptotic and autophagy protein expression on AML cell lines. Protein expression of whole cell lysate of MOLM-13 and U-937 cell lines by Western blotting. Expression of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax and autophagy protein Beclin 1

References: **1.** Cancer Research UK (2016)Leukaemia (all subtypes combined) statistics. **2.** Hassan *et al.*, (2017) 'Genetic and epigenetic heterogeneity and the impact on cancer relapse'. **3.** Ding, *et al.*, (2012) 'Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing'. **4.** Bensi *et al.*,(1995) 'Bcl-2 oncoprotein expression in acute myeloid leukemia'. **5.** Zinkel *et al.*,(2006) 'BCL2 family in DNA damageand cell cycle control'.**6.** Li *et al.*, (2016) 'Doxorubicin Blocks Cardiomyocyte Autophagic Flux by Inhibiting Lysosome Acidification'.**7.** Fulda & Debatin (2005) 'Sensitization for anticancer drug-induced apoptosis by betulinic acid'