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Bcl-2 protein family expression pattern determines synergistic pro-apoptotic effects of BH3 mimetics with hemisynthetic cardiac glycoside UNBS1450 in acute myeloid leukemia

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Resistance to apoptotic cell death¹ due to overexpression of anti-apoptotic Bcl-2 family proteins including Bcl-2, Bcl-xL or Mcl-1 is considered an interesting druggable target for the treatment of hematological malignancies including acute myeloid leukemia. In fact BH3 mimetics² like ABT-199 (Venetoclax)³ reverse the inhibitory function of anti-apoptotic Bcl-2 proteins⁴. Dependency on Bcl-2 family protein expression requests BH3 profiling to efficiently stratify patients potentially benefiting from ABT-199 therapy.⁵ Most often, Mcl-1 is considered a main resistance factor² and recently a first class of selective Mcl-1 inhibitors was characterized.⁶ As an alternative to functional inhibitors, we previously described proteasome-dependent downregulation of Mcl-1 expression^{7, 8} by cardiac glycoside UNBS1450⁸⁻¹⁰. We hypothesize here that a combination of UNBS1450 with a BH3 mimetic would affect AML subtypes especially "addicted" to Mcl-1. To provide a targeted therapeutic approach, we describe the synergistic anti-leukemic effect of ABT-199 with UNBS1450 in cell lines, colony formation assays, zebrafish xenografts and validate results in primary cells from 23 *de novo* AML patients.

Figure 1A shows the expression pattern of major anti-apoptotic Bcl-2 proteins of selected AML cell lines suitable for the assessment of single/combinational strategies. First, we assessed the sensitivity of these cells against ABT-199, ABT-263 and UNBS1450 as a single agent, by determining IC₅₀ values after 24h (Figure 1B). A multiple linear regression for each drug on the three proteins (Figure 1C) confirmed the significant positive correlation between IC₅₀ values and Bcl-2 expression and, *vice versa*, a negative correlation with Mcl-1 expression for ABT-199. IC₅₀ values positively correlated with Mcl-1 expression and negatively with Bcl-xL expression for UNBS1450, in agreement with previous data.⁸ For ABT-263, we could not reach significance based on this panel; analysis of the raw data indicates a positive correlation with Bcl-2 and Bcl-xL expression and a negative one with concomitant Mcl-1 expression. Generally, effects with ABTs were obtained with micromolar concentrations, prone to trigger resistance.

We then selected U937 and TF-1 cell lines as models to investigate the synergistic potential of combination treatments. Mcl-1 inhibitor A-1210477 previously allowed characterizing Bcl-2 protein co-dependency involving Mcl-1.¹¹ Combination of A-1210477 and ABTs provided evidence of the co-requirement of Bcl-2/Mcl-1 expression and Bcl-xL/Mcl-1 in U937 and TF-1 cells, respectively (Suppl. Figure 1). A-1210477 primed TF-1 cells (highly co-expressing Bcl-xL/Mcl-1) to apoptosis only

when combined with ABT-263. We then replaced A-1210477 by UNBS1450. In U937 cells, we documented a strong synergistic effect when UNBS1450 was combined with both ABTs (CI=0.14-0.18; Figure 1D; Suppl. Figure 2). In TF-1 cells, synergism was observed only when combining UNBS1450 with ABT-263, targeting Bcl-2, Bcl-xL and Bcl-w, but not with ABT-199, selective for Bcl-2, undetectable in these cells (CI=0.05-0.15; Figure 1E).

Colony formation was strongly reduced when U937 cells were treated with a combination of UNBS1450/ABT-199 (Suppl. Figure 3) whereas tumor mass formation was completely abrogated in a zebrafish xenograft model, whereas individual treatments did not, validating our results (Figure 2A).

We confirmed differential toxicity by a combined treatment (20nM UNBS1450; 0.1μM ABT-199) that led to 40% induction of cell death (Figure 2B; Suppl. Figure 3A), but of 80% with ABT-263 (Suppl. Figure 4B) in CD34⁺ cells from cord blood of healthy donors, compared to 100% in leukemia cells.

As platelets were strongly affected by ABT-263^{2,3}, we tested different concentrations of UNBS1450 alone or in combination with ABT-199 without impacting the viability of leucocyte-depleted platelets pool from healthy donors.^{3, 12} ABT-263, used as reference, deteriorated viability (Figure 2C and Suppl. Figure 4C).

Next, we analyzed UNBS1450 alone and combined to ABT-199 on 23 *de novo* diagnosed AML patients (Figure 2, Suppl. Figure 5, Suppl. Table 1). UNBS1450 dose- and time-dependently reduced viability of primary AML cells (Figure 2D). A sub-group of 14 AML patients moderately responded to UNBS1450 or ABT-199 alone but were sensitized to death in co-treatments (Figure 2E). Analysis of CD34⁺CD38⁻ sub-populations confirmed these results (Figure 2D-E). In the same AML samples, no significant impact on healthy lymphocytes was observed (Figure 2F). Analysis of expression patterns of major anti-apoptotic Bcl-2 family members in the panel of AML patients revealed the presence of a second band for Mcl-1 in many specimens, which is compatible with the reported 32 kDa short isoform. This band was not or barely detectable in established AML cell lines (Figure 1A; Suppl. Figure 8). A multiple correspondence analysis (Suppl. Figure 7) based on the expression level of Bcl-2, Bcl-xL and both detectable Mcl-1 bands (40 and 32 kDa) showed that patient samples that are sensitive to ABT-199 (Pt # 1, 6, 9, 18) exhibit a high expression level of Bcl-2, as expected, but also of Mcl-1 32 kDa, besides Bcl-xL. A group of AML patient cells highly susceptible to UNBS1450 (Pt# 4, 5, 8, 11, 19)

generally present a reduced level of Bcl-xL and high expression levels of Mcl-1 32 kDa. We could not associate any typical profile with samples positively responding to the co-treatment; however, most samples belonging to this group concomitantly express Mcl-1 and Bcl-2 proteins at various levels (Pt# 3, 12, 14, 16, 17) (Figure 2G). Our results can provide the basis for future clinical trials with UNBS1450 used as single agent or in combination with ABTs in AML. Bcl-2 protein expression patterns, especially Mcl-1, could become an essential biomarker allowing AML patient stratification and response prediction. Results also prompt to explore both role and/or origin of the different Mcl-1 isoforms in drug response, a novel and emerging topic. A few studies focus on the modulatory roles of short isoforms and the actual biological functions of the 32 KDa Mcl-1 short isoform were recently investigated^{13, 14}. Moreover, from a mechanistic point of view, UNBS1450-induced degradation of Mcl-1 occurs *via* proteasome-dependent, Noxa-independent degradation of pre-existing Mcl-1 rather than by a transcriptional modulation of Mcl-1 expression⁸. Further studies are required to validate sub-type dependency on Bcl-2 protein expression more likely to benefit from a combination treatment. Recent application of BH3-profiling on patient samples allows establishing Bcl-2 dependency and predicting ABT response profile.¹⁵ The same approach can be applied to predict patients responsive to combination treatments.

So far, our analysis was carried out on *de novo* AML. We will extend our investigations to relapsed forms. Moreover, the patient panel tested so far does not allow correlations with specific factors including age, sex or cytogenetic mutations. An exploratory study to correlate specific mutations to drug response indicates that FLT3-ITD mutation has not the same effect for all treatments as opposed to FLT3-WT (Suppl. Figure 6): it decreases susceptibility to ABT-199 (-16%) and combination treatments (-12%), while increasing susceptibility to UNBS1450 (+20%). Even though none of these effects is significant in our limited panel, however, this preliminary evidence encourages further studies on effects of UNBS1450 alone or in combination with ABTs on FLT3-ITD AML patients' sub-groups.

Importantly, we observed here that sub-toxic single treatments by ABT compounds do not change anti-apoptotic Bcl-2 protein expression. Moreover, UNBS1450 downregulates Mcl-1 without a compensatory overexpression of other Bcl-2 family proteins (Figure 1D).

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Correlation between cell-type specific expression of anti-apoptotic Bcl-2 family proteins and synergistic combinational effects of ABTs and UNBS1450 in AML cells. **(A)** Analysis of major anti-apoptotic Bcl-2 proteins in AML cell lines. **(B)** Susceptibility to ABTs and UNBS1450 after 24h incubation. IC_{50} values determined by MMP loss and quantification of apoptotic nuclear morphology. **(C)** Effect of significant protein expression levels (β -actin ratios) on IC_{50} for ABT-199 (μ M) and UNBS1450 (nM). The larger the size, the larger IC_{50} . Coefficients estimated from multiple regression equations (right), ns: not significant, *: $p < 0.05$, **: $p < 0.01$. **(D)** Synergistic effects of sub-toxic concentrations of ABTs (0.1 μ M) and UNBS1450 (20 nM) in U937 cells assessed as described, together with WB analysis of caspase cleavage in parallel to modulation of anti-apoptotic Bcl-2 proteins. **(E)** TF-1 cells treated at indicated concentrations of UNBS1450. Combinational index was estimated by CalcuSyn software. Data are the mean of at least three independent experiments \pm SD. Significance was estimated by using two-way ANOVA test (post-hoc analyses Dunnett). Significance is reported as ****/#### $P < 0.0001$.

Figure 2. Impact of UNBS1450 alone or with ABTs. **(A)** Effect of UNBS1450 and ABT-199 single/combination treatments on tumor formation in a zebrafish xenograft model with U937 cells injected after an 8-hour UNBS1450/ABT-199 pretreatment. Analysis of differential toxicity in healthy donor CD34⁺ **(B)** and platelets **(C)**; by Annexin-V and Cell Titer Glo® assays, respectively. Apoptogenic potential of UNBS1450 alone or in combination on AML patient samples (Suppl. data): **(D)** analysis of UNBS1450 alone (by Annexin-V assay or MitoTracker® Red staining); **(E)** combination of UNBS1450 (30 nM; 48h pre-incubation) and ABT-199 (0.01 μ M; 18h of incubation). **(F)** Same analysis in AML patient lymphocytic sub-population. **(G)** WB analysis of anti-apoptotic Bcl-2 protein expression. Synergy was estimated by using the “Response Additivity” approach. Corresponding Combinational index of significant interactions were computed. Statistical analyses were performed in GraphPad Prism. Significance is */ $P < 0.05$; **/ $P < 0.01$, ***/ $P < 0.001$; ****/ $P < 0.0001$ (two-way ANOVA; repeated measures; post-hoc analyses Dunnett; Sidak).

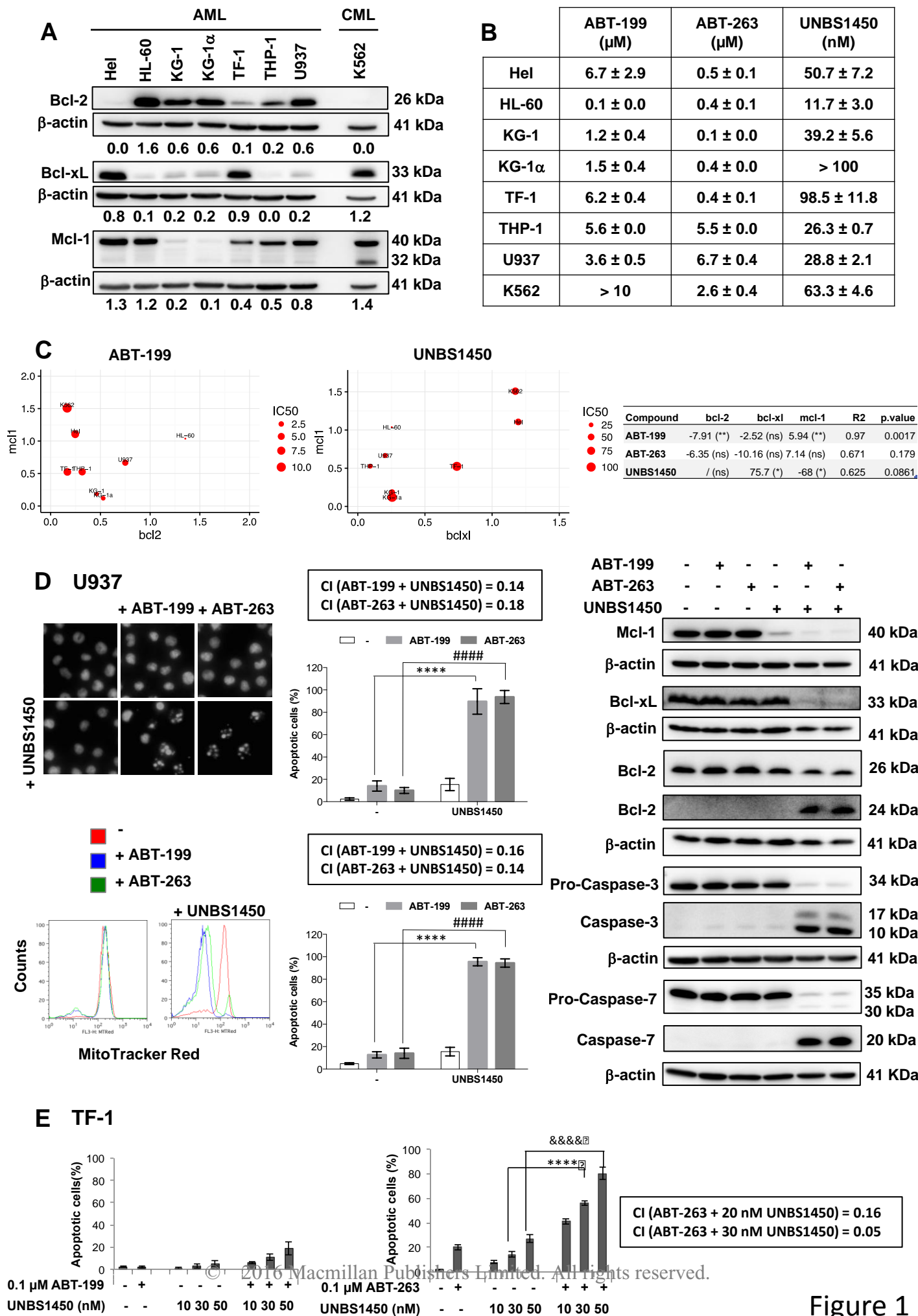


Figure 1

