Changes and functional interactions among Bcl-2 members in response to ibrutinib and venetoclax

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Abstract:
Chronic lymphocytic leukemia (CLL) cells cycle between lymph node (LN) and peripheral blood (PB) and display major shifts in Bcl-2 family members between those compartments. Specifically, Bcl-XL and Mcl-1, which are not targeted by the Bcl-2 inhibitor venetoclax, are increased in the LN. Since ibrutinib forces CLL cells out of the LN, we hypothesized that ibrutinib may thereby affect expression of Bcl-XL and Mcl-1 and sensitize CLL cells to venetoclax. We investigated expression of Bcl-2 family members in patients under ibrutinib or venetoclax treatment combined with dissecting functional interactions of Bcl-2 family members in an *in vitro* model for venetoclax resistance.

In the PB, recent LN emigrants had higher Bcl-XL and Mcl-1 expression than cells immigrating back to the LN. Under ibrutinib treatment, this distinction collapsed, and significantly, the pre-treatment profile reappeared in patients who relapsed on ibrutinib. In response to venetoclax however, Bcl-2 members displayed an early increase, underlining the different modes of action of these two drugs. Profiling by BH3 mimetics was performed in CLL cells fully resistant to venetoclax due to CD40-mediated induction of Bcl-XL, Mcl-1 and Bfl-1. Several dual or triple combinations of BH3 mimetics were highly synergistic in restoring killing of CLL cells. Lastly, we demonstrated that pro-apoptotic Bim interacts with anti-apoptotic Bcl-2 members in a sequential manner: Bcl-2>Bcl-XL>Mcl-1>Bfl-1. Combined, the data indicate that Bcl-XL is more important in venetoclax resistance than Mcl-1 and provide biological rationale for potential synergy between ibrutinib and venetoclax.

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Changes in Bcl-2 members in response to ibrutinib or venetoclax uncover functional hierarchy in determining resistance to venetoclax in CLL

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Key points:

- Bcl-2 family members undergo shifts in expression in response to ibrutinib and venetoclax, which revert in case of ibrutinib relapse
- Bcl-XL is dominant over Mcl-1 in CLL venetoclax resistance when both are present

Abstract (239 words)

Chronic lymphocytic leukemia (CLL) cells cycle between lymph node (LN) and peripheral blood (PB) and display major shifts in Bcl-2 family members between those compartments. Specifically, Bcl-XL and Mcl-1, which are not targeted by the Bcl-2 inhibitor venetoclax, are increased in the LN. Since ibrutinib forces CLL cells out of the LN, we hypothesized that ibrutinib may thereby affect expression of Bcl-XL and Mcl-1 and sensitize CLL cells to venetoclax. We investigated expression of Bcl-2 family members in patients under ibrutinib or venetoclax treatment combined with dissecting functional interactions of Bcl-2 family members in an *in vitro* model for venetoclax resistance.

In the PB, recent LN emigrants had higher Bcl-XL and Mcl-1 expression than cells immigrating back to the LN. Under ibrutinib treatment, this distinction collapsed, and significantly, the pre-treatment profile reappeared in patients who relapsed on ibrutinib. In response to venetoclax however, Bcl-2 members displayed an early increase, underlining the different modes of action of these two drugs. Profiling by BH3 mimetics was performed in CLL cells fully resistant to venetoclax due to CD40-mediated induction of Bcl-XL, Mcl-1 and Bfl-1. Several dual or triple combinations of BH3 mimetics were highly synergistic in restoring killing of CLL cells. Lastly, we demonstrated that pro-apoptotic Bim interacts with anti-apoptotic Bcl-2 members in a sequential manner: Bcl-2>Bcl-XL>Mcl-1>Bfl-1. Combined, the data indicate that Bcl-XL is more important in venetoclax resistance than Mcl-1 and provide biological rationale for potential synergy between ibrutinib and venetoclax.

Introduction

CLL is a B cell malignancy with considerable heterogeneity in clinical course and a prime example of a malignancy that is highly dependent on interactions with the microenvironment. Whereas quiescent CLL cells accumulate in the PB, active CLL cells grow in the LN, spleen and bone marrow. In the lymphoid microenvironment, CLL cells are provided with external signals from surrounding cells, such as CD40L-presenting T helper cells[1], myeloid and stromal cells. The result is an upregulation of anti-apoptotic Bcl-2 members Bcl-XL, Mcl-1 and Bfl-1 which renders these CLL cells less sensitive to therapeutic drugs[2-4]. Novel therapeutics that either target these microenvironmental signals or Bcl-2 members have entered clinical practice[1,5].

The B cell receptor (BCR) signaling pathway has emerged as an important therapeutic target for B-cell malignancies including CLL. Bruton's tyrosine kinase (BTK), a critical component of BCR signaling, plays a role in the survival, proliferation, adhesion and migration of CLL cells[6-9]. The BTK inhibitor ibrutinib shows strong clinical activity and allows for treatment without chemotherapy. Ibrutinib is now approved for both relapsed/refractory and first-line CLL irrespective of 17p deletions. Importantly, as ibrutinib inhibits CLL cell adhesion and migration, the interaction of CLL cells with their LN microenvironment is blocked, causing rapid redistribution of CLL cells from the LN into the PB. While kept in the circulation, CLL cells are deprived of pro-survival stimuli and will eventually die[10]. However, resistance to ibrutinib may occur in a subset of patients, particularly in heavily pre-treated patients and in high-risk CLL[11,12]. Resistance to ibrutinib correlates with aggressive disease that is difficult to treat and is often associated with acquisition of mutations in primary target BTK or downstream effectors like Phospholipase C-gamma-2.

Another successful strategy in CLL is to target the apoptosis machinery directly by so-called BH3 mimetics. The Bcl-2-specific inhibitor ABT-199, also known as venetoclax, is highly cytotoxic for CLL cells achieving a 79% overall response rate in R/R CLL[13]. Venetoclax provides profound reductions in circulating CLL cells in the majority of patients, but LN responses are less complete[13]. Although definitive evidence is lacking that residual LN sites after venetoclax treatment are the direct source of resistant disease, it is reasonable to assume they contribute to this process. While resistance to ibrutinib has been strongly associated with specific gene mutations in BTK and PLCG2, genetic causes of treatment resistance towards venetoclax, such as the G101V mutation in BCL2, are found in approximately 50% of patients under prolonged venetoclax treatment[14]. Additional venetoclax resistance mechanisms, including Bcl-XL overexpression, were observed, showing that alternative mechanisms play a role in venetoclax resistance. Moreover, Blombery et al. recently reported presence of multiple BCL2 mutations as well as simultaneous aberrations in Bcl-XL and Mcl-1, which further support a model where multiple, independent molecular mechanisms may underlie clinical relapse on venetoclax[15].

We hypothesize that changes in expression and shifts in functional interactions among pro-survival Bcl-2 members and their interactions with pro-apoptotic Bim, are key to clinical response and refractory disease. In order to investigate this, we developed three specific objectives.

First, as expression patterns of the different Bcl-2 members play a crucial role in evasion of cell death, which is a hallmark of resistance, it is important to investigate expression changes in response to treatment. Therefore, by measuring the expression of Bcl-2 members in patient samples before and during ibrutinib or venetoclax treatment, we aim to relate Bcl-2 member expression to clinical response.

Second, as outlined above, pro-survival signals in the LN upregulate other Bcl-2 members such as Bcl-XL and Mcl-1 that are not targeted by venetoclax. Therefore, we analyzed the contribution and interdependency of distinct Bcl-2 members in resistance to venetoclax in CLL in an *in vitro* CLL model to examine where resistance could be counteracted using a combination of BH3 mimetics targeting other Bcl-2 members such as Bcl-XL and Mcl-1.

Finally, we aimed to dissected the hierarchy of Bcl-2 members[16] in CLL to understand the mechanisms of venetoclax resistance. Therefore, we performed Bim co-immunoprecipitation experiments to investigate shifts in functional interactions of pro-survival Bcl-2 members with pro-apoptotic Bim in response to treatment in order to define a hierarchy of pro-survival Bcl-2 members and their relative importance in venetoclax resistance in CLL.

Materials & Methods

Patient material

After informed consent, patient blood was obtained during diagnostic or follow-up procedures at the Amsterdam UMC, the University of Southampton, the Peter MacCallum Cancer Centre & St. Vincent's Hospital in Melbourne and the Dana-Farber Cancer Institute in Boston. This study was approved by the AMC Ethical Review Board and conducted in agreement with the Declaration of Helsinki. The samples from the University of Southampton were collected as part of the ethically approved UK real-world observational study *A longitudinal observational study with phenotypic, functional and molecular characterization of the tumor lymphoid cells in patients with mature lymphoid malignancies in the United Kingdom* (REC reference 16/SC/0030; IRAS ID 186109). The samples from the Peter MacCallum Cancer Centre & St. Vincent's Hospital in Melbourne were collected under protocol 13/36 as part of the study *A prospective study to evaluate tumor biomarkers and host immunity in patients with CLL/SLL*, approved on 27th June 2013. At the Dana-Farber Cancer Institute in Boston, all patients had been previously consented to a serial tissue banking protocol approved by the Institutional Review Board and providing linked clinical information. Blood mononuclear cells of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech) were cryopreserved and stored as previously described. Expression of CD5 and CD19 (BD Biosciences, San Jose, CA, USA) on leukemic cells was assessed by flow cytometry (FACS Canto; BD Biosciences). CLL samples included in this study contained 85-99% $CD5^+/CD19^+$ cells.

Reagents

ABT-199 was purchased from Active Biochem (Bonn, Germany). A-1331852 was purchased from Chemietek (Indianapolis, IN, USA). S-63845 was purchased from Chemgood (Glen Allen, VA, USA). Q-VD-OPh Hydrate was purchased from APExBIO (Houston, TX, USA).

Cell culture and detection of apoptosis

Lymphocytes of CLL patients were co-cultured with NIH3T3 fibroblasts stably transfected with human CD40L or negative control as described before[17,18]. After 24 hours, CLL cells were detached and incubated with or without drugs for an additional 24 hours. CLL cell viability was measured by flow cytometry using DiOC6 and TO-PRO-3 viability dyes. Specific apoptosis is defined as [% cell death in treated cells] – [% cell death in medium control] / [% viable cells medium control] x 100.

Flow cytometry

Single-cell suspensions were stained with the following antibodies: anti-CD3, CD19 (BD Biosciences), CD184 (CXCR4), CD5 (Biolegend). Cells were subsequently permeabilized and stained with the

following antibodies: anti-Bcl-2 (Biolegend), Bcl-XL, Mcl-1 (Cell Signaling). Stained cells were analyzed on a FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA). Gating of LN emigrants versus immigrants was performed on plots of CD19$^+$/CD5$^{dim/high}$/CXCR4$^{dim/high}$ populations and setting the optimal 10 percentiles corresponding to the two populations. The proportions of immigrants and emigrants shifted in some patients during treatment as has been reported before[19], but this did not affect interpretation of the gating strategy (Supplemental Figure 1).

Western blot analysis

Western blot analysis was performed using standard techniques. Membranes were probed with the following antibodies: anti-Bcl-XL, Bcl-2 (Cell Signaling), Mcl-1 (Abcam), actin (Santa Cruz Biotechnology), Bim (StressMarq) and anti-A1/Bfl-1 was a kind gift of Prof. Dr. J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Odyssey Imager (Li-Cor Biosciences) was used as a detection method according to the manufacturer's protocol. When PB samples contained below 90% purity of CLL cells, they were purified using CD19 beads (Miltenyi) (data not shown).

Bim co-immunoprecipitation

Lymphocytes of CLL patients were co-cultured with NIH3T3 fibroblasts stably transfected with human CD40L or co-cultured with negative control NIH3T3 fibroblasts transfected with empty vector. After 24 hours, CLL cells were detached and incubated with or without BH3 mimetics in combination with caspase inhibitor QVD for an additional 24 hours. Treated CLL cells were lysed with NP-40 lysis buffer and subjected to immunoprecipitation using Dynabeads protein G immunoprecipitation kit (catalog no. 10007D, ThermoFisher). Rat-anti-Bim (3C5) monoclonal antibodies were coupled to protein G Dynabeads in a 1:50 dilution for 20 min on a rotator at room temperature. NP-40 lysates were incubated with antibody-coated beads for 20 min on a rotator at room temperature. All other steps were performed according to the manufacturer's protocol.

Statistics

The paired sample t-test was used to analyze paired observations. The one-way ANOVA was used to analyze differences between groups.

Results

Levels of Bcl-XL and Mcl-1 collapse in LN emigrants upon ibrutinib treatment

CLL cells can be divided into distinct fractions on the basis of inverse surface expression of CXCR4 and CD5. Separation and intraclonal analyses of these fractions in PB samples suggest a spectrum of CLL cells from recently divided and robust cells that are recent lymphoid tissue emigrants (CXCR4^{dim}/CD5^{high}), to older and less vital cells that need to immigrate back to lymphoid tissue to survive (CXCR4^{high}/CD5^{dim})[20]. Since CLL cells upregulate Mcl-1 and Bcl-XL in the LN[3,4], we hypothesized that LN emigrants have higher Mcl-1 and Bcl-XL expression than LN immigrants. To this end, we analyzed PB samples and no other compartments were studied. By gating the outer 10% fractions corresponding to these two populations, CLL cells emigrating from the LN indeed showed higher Mcl-1 and Bcl-XL expression than CLL cells immigrating back to the LN (Figure 1A; Supplemental Figure 2). Under ibrutinib treatment, levels of Mcl-1 and Bcl-XL in the LN emigrant population gradually reduced to the levels of the LN immigrant population. In some patients the proportion of the immigrant versus emigrant population shifted upon ibrutinib treatment over time, as has recently been reported[19], whereas in other patients this appeared to be stable over the period examined. Relative pairwise comparisons were made by setting the expression levels of the LN immigrant population at baseline to 100% (see Supplemental Figure 1 for an example). This demonstrated a significant reduction in the LN emigrant population of both Mcl-1 and Bcl-XL upon ibrutinib treatment (n=14). Bcl-2 levels were affected in some patients, but on average the changes were not significant (Figure 1B). These findings were not influenced by previous treatment regimens (see Supplemental Table 1). Although the difference in Bcl-2 family proteins is expected and observed to be the greatest between the LN immigrant and emigrant populations as gated by flow cytometry these findings could in some cases be confirmed in the total fraction of CLL cells via Western blotting of PB samples from patients undergoing ibrutinib treatment. The data obtained by Western blot technique were compared with flow cytometric data of the total CLL population obtained after CD40 stimulation in selected cases, and proved to correlate well (Supplemental Figure 3). It should be pointed out however that the Western blot technique, which analyzes the complete CLL population, cannot be expected to show the same clear differences as the specific LN immigrant and emigrant populations shown in the flow cytometric analyses. Reductions of Mcl-1 and Bcl-XL levels were observed in patients upon both short-term (Figure 1C) and long-term (Figure 1D) ibrutinib treatment compared to baseline samples (Supplemental Figure 4-5). In summary, levels of Bcl-XL and Mcl-1, which are known to be responsive to microenvironmental signals, collapse in response to ibrutinib treatment in PB CLL cells, indicating that the lack of microenvironmental signals extends into that compartment.

Levels of Bcl-2 members reappear upon ibrutinib relapse

We next investigated whether Bcl-2 member expression would reappear in patients relapsing on ibrutinib therapy. PB samples were collected at multiple time points during ibrutinib treatment where T1 represents an early time point and T2 represents a later time point upon ibrutinib treatment (Supplemental Table 1). In this group of patients, significant reductions of all Bcl-2 members were observed in the LN emigrant population upon ibrutinib treatment (Figure 2A) (n=16). Subsequently, patients were divided into remission (n=7) and non-responding/relapsing patients (n=9). At T2, Mcl-1 and Bcl-XL expression were significantly different between patients in remission versus non-responding/relapsing patients. When sufficient material was available, Bcl-2 members were analyzed by Western blot (Figure 2B, Supplemental Figure 6). Although the increase in Mcl-1 and Bcl-XL expression between T1 and T2 was variable between patients, their expression for each given patient was correlated (Supplemental Figure 7). All non-responding/relapsing patients were sequenced for mutations in BTK and PLCG2 and 1 patient contained a BTK mutation. In summary, these observations show that Bcl-XL and Mcl-1 levels reappear upon ibrutinib relapse, irrespective of the distinct time points when relapse occurred.

Levels of Bcl-2 members increase upon venetoclax treatment

The same analyses were applied to samples from patients treated with venetoclax. Samples were collected twice during ramp-up phase when the dosage of venetoclax is gradually increased and there are still detectable CLL cells present in the PB (Supplemental Figure 8). Significant increases of all Bcl-2 members could be observed in both LN immigrants and emigrants after the first sampling with further increases after the second sampling (Figure 3A). Samples were also compared by Western blot (Figure 3B; enriched for CD19+ cells to achieve >95% purity of CD5+CD19+ CLL cells). Upon 2 weeks of the venetoclax ramp-up, an increase of Bcl-2 could be observed in all patients whereas increase of Mcl-1 and Bcl-XL could be observed when PB was collected 4 weeks after starting the venetoclax ramp-up (Supplemental Figure 9-10). Quantitative PCR analyses to probe for changes in RNA expression of the various Bcl-2 members showed incidental and varying changes but not a consistent pattern (data not shown). In order to establish whether this venetoclax-associated increase in Bcl-2 member protein levels *in vivo* was due to short-term effects on Bcl-2 members, CD40-stimulated CLL cells were treated *in vitro* with venetoclax (ABT199) and other BH3 mimetics targeting either Mcl-1 (S-63845) or Bcl-XL (A-1331852) (Figure 3C). Upon CD40-mediated activation of CLL cells using a co-culture of CLL cells with CD40L-expressing 3T3 fibroblasts (3T40L)[21,22], upregulation of both Mcl-1 and Bcl-XL could be observed, similar to the situation in the LN[4]. Only for Mcl-1 could the *in vivo* effect be reproduced after *in vitro* incubation with venetoclax or S-63845. Mcl-1 is unstable and known to be stabilized at the protein level by BH3 protein binding[23-25],

suggesting that the observed increase in Mcl-1 after venetoclax treatment may reflect its binding to Bim *in vivo*. Altogether, these results show that levels of Mcl-1, Bcl-XL and Bcl-2 increase upon venetoclax treatment.

Functional profiling with BH3 mimetics to dissect venetoclax resistance

Upon starting venetoclax treatment, CLL cells rapidly disappear from the PB[13]. Since CLL cells upregulate Bcl-XL, Mcl-1 and Bfl-1 in the lymphoid microenvironment[3,4], these stimulated CLL cells are theoretically less sensitive to single Bcl-2 inhibition by venetoclax treatment. As this population could potentially survive under prolonged therapy, this may result in outgrowth of venetoclax-resistant subclones. This premise fits with the observation that patients with remaining enlarged LNs under venetoclax treatment have a shorter progression free survival[13], even when combined with Rituximab[26]. In order to dissect the functional contribution of other pro-survival Bcl-2 members to venetoclax resistance, we applied functional profiling using combinations of BH3 mimetics[27] targeting either Bcl-2 (ABT199), Bcl-XL (A-1331852) and/or Mcl-1 (S-63845) in an *in vitro* model where CLL cells are stimulated via co-culture with CD40L-expressing fibroblasts (as shown in Figure 3C) and become fully resistant to single agent venetoclax[27,28]. Combined inhibition of Bcl-2 and Bcl-XL (Figure 4A) or Mcl-1 (Figure 4B) diminished resistance. Targeting Bcl-XL seemed slightly more effective than targeting Mcl-1, resulting in a 4-fold difference in IC_{50} between the two combinations (See Supplemental Table 2). Interestingly, combined Mcl-1/Bcl-XL inhibition in absence of venetoclax was also able to restore killing (Figure 4C). Triple combination of BH3 mimetics in 3T40-stimulated CLL almost completely restored killing comparable to unstimulated PB CLL cells (Figure 4D). Further reduction of the dose of the triple combination however showed there was still a difference in sensitivity between CD40-activated CLL and control cells (Figure 4E). Since in this situation Bcl-2, Bcl-XL and Mcl-1 are inhibited, a role for Bfl-1, which is also induced after CD40 stimulation[2], is implied. Results were similar between mutated and unmutated CLL samples and IC_{50} values were calculated using a nonlinear regression model. The combination index of the different BH3 mimetic combination treatments was calculated (Supplemental Figure 11) and showed clear synergy between the dual and especially triple combinations (Figure 4F). Altogether, these data demonstrate that full resistance against single BH3 mimetic drugs after CD40 stimulation can be significantly reversed by dual BH3 mimetic combinations, and that targeting Bcl-XL contributes strongly in this reversion.

Functional hierarchy among Bcl-2 members in determining resistance to venetoclax

Of the pro-apoptotic BH3-only proteins expressed in CLL, Bim is expected to play a major part in killing cells upon venetoclax treatment[28]. Therefore, Bim was immunoprecipitated from primary CLL cells treated with BH3 mimetics in order to observe shifts in interactions of Bim with anti-apoptotic

Bcl-2 proteins (Figure 5A). These experiments were performed in the presence of the pan-caspase inhibitor QVD to prevent cell death. In resting CLL cells, where Bcl-2 is the most prominent pro-survival Bcl-2 member, Bim only interacted with Bcl-2 (Figure 5B). Upon CD40-mediated activation and subsequent upregulation of Bcl-XL and Mcl-1, Bim was also bound to Bcl-XL. When cells were then treated with venetoclax, Bim shifted further to Mcl-1, confirming that Bim has a preference for Bcl-XL over Mcl-1 when both are present. Based on data from Figure 4E, it was predicted that Bfl-1 could also play a role, and this was confirmed using the triple combination of BH3 mimetics (Figure 5C). Under these conditions, Bim abrogated binding to Bcl-XL and Mcl-1 and switched back to Bcl-2 and now also engaged Bfl-1 (Figure 5D). Although venetoclax was present in this condition, Bcl-2 was still capable of binding Bim when both Bcl-XL and Mcl-1 were blocked by other BH3 mimetics. Although Bcl-2 family member expression differed from patient to patient, a similar pattern was found in multiple patient samples (Supplemental Figure 12-13). In samples with high Bcl-XL expression, interactions with Bim could already be observed without 3T40 activation. In samples with high Mcl-1 expression, interactions with Bim could still be observed even after using the triple combination of BH3 mimetics. Finally, Bfl-1 could not always be observed in the co-immunoprecipitation. Overall, these data show that Bim interacts with anti-apoptotic Bcl-2 members in a sequential manner in the order of Bcl-2, Bcl-XL, Mcl-1 and lastly Bfl-1, portraying a functional hierarchy of anti-apoptotic Bcl-2 members in determining resistance to venetoclax.

Discussion

Here we report the first analyses in CLL of the effects of ibrutinib and venetoclax *ex vivo* and *in vitro* on pro-survival Bcl-2 members. Significant and opposite effects were found for Bcl-2 members in response to ibrutinib compared to venetoclax. Furthermore, we dissected the contribution of the Bcl-2 members in venetoclax resistance and found a dominant role for Bcl-XL and a functional hierarchy in binding Bim.

Due to the microenvironment-driven upregulation of Mcl-1 and Bcl-XL in the LN[2-4] and the absence of such signals in the PB[20], LN emigrants and immigrants differ in expression of these Bcl-2 members. As ibrutinib forces CLL cells out of the LN into the PB and prevents their return, we hypothesized that this distinction collapsed upon treatment. Gating specifically for LN immigrants and emigrants by flow cytometry showed a significant collapse of Bcl-XL and Mcl-1 upon ibrutinib whereas these effects could not always be reproduced by blotting total PB. Even though these two separate techniques show slightly different aspects, the available data showed that the decreased expression mediated by ibrutinib was true for Bcl-XL and Mcl-1.

Although previous studies did not find consistent differences in the expression of Bcl-XL and Mcl-1 upon ibrutinib therapy[29], this may be explained by the different techniques used as well as the duration of ibrutinib treatment. For Bcl-2 no significant differences were observed, which fits with the notion that Bcl-2 is overexpressed independently of the microenvironment in CLL. Yet, some patients studied here displayed a decrease in Bcl-2 upon ibrutinib treatment, although this did not correspond to IgHV mutation status as suggested in a preliminary report[30]. Further evaluation in larger patient cohorts will hopefully clarify which environmental or intrinsic signals may affect Bcl-2 upon ibrutinib treatment. Significantly, the Bcl-2 member pre-treatment profile reappeared in patients who relapsed on ibrutinib. Mcl-1 and Bcl-XL increased most likely due to CLL cells being able to recharge again in the LN, whereas increase of Bcl-2 may be due to BTK inhibition initially selecting clones expressing higher levels of Bcl-2[30]. Notably, of the 9 relapsing patients that were included, only 1 patient had a BTK mutation, indicating that other mechanisms than BTK or PLCG mutations were involved with the reappearance of Bcl-2 member expression during relapse in this group of patients.

In contrast to ibrutinib, venetoclax induced an increase of Bcl-2 members Mcl-1, Bcl-XL and Bcl-2. Mcl-1 also increased upon venetoclax or S-63845 treatment *in vitro* (Figure 3C*)*, and we propose that Mcl-1 is not upregulated but rather stabilized upon interaction with Bim or S-63845, which are previously reported effects[23-25,31]. Increase of Bcl-2 or Bcl-XL are most likely not due to stabilization since they have a much longer half-life than Mcl-1 and Bfl-1[32]. Notably, increase of Bcl-2 or Bcl-XL was not observed after short-term treatment *in vitro*. The apparent increase in Bcl-2 may be a result of preferential elimination of cells with low Bcl-2 protein during venetoclax ramp-up. Apart from a

theoretical possibility of longer half-life upon binding Bim, the increase of Bcl-XL *in vivo* remains unexplained, but may be a potential resistance mechanism. Indeed, increased Bcl-XL expression has been described as a mechanism of resistance to venetoclax in CLL and mantle cell lymphoma[33,34]. In addition, Blombery et al. identified the G101V Bcl-2 mutation in a cohort of CLL patients treated with venetoclax and showed that additional disease resistance mechanisms such as high Bcl-XL expression can co-exist with the G101V Bcl-2 mutation[14]. In order to establish the potential of Bcl-2 member expression as a biomarker for (non)response in the treatment of ibrutinib and/or venetoclax, patients should be tested in a prospective study in correlation with response parameters, such as MRD status in trials involving venetoclax[35].

Using an *in vitro* CLL model of CD40 stimulation, it was shown before that CLL cells with upregulation of Bcl-XL, Mcl-1 and Bfl-1 are fully resistant to single agent venetoclax[22]. We demonstrate that dual or triple targeting with BH3 mimetics restored efficient killing of 3T40-activated CLL cells. Triple combination of BH3 mimetics restored killing of 3T40-activated CLL cells to almost the same level of PB CLL cells. Although such combinations may not be directly translatable to clinical application, they allow assessment of the contributions of the various Bcl-2 members in CLL cells to venetoclax resistance. In a recent study with primary AML cells, synergy between Bcl-2 and Mcl-1 was observed[36]. Previous research, predominantly performed with cell lines, has also suggested that Mcl-1 can play a prominent role in determining resistance to venetoclax or navitoclax (ABT-737)[37-41]. A recent study using loss and gain-of-function screens in cell lines also confirmed a prominent contribution of Mcl-1 in venetoclax resistance[42]. Previous data using BCR-stimulated CLL cells also indicated a role for Mcl-1 in lowering venetoclax sensitivity[43]. Our data reported here using BH3 mimetics and Bim interaction studies, as well as previous siRNA approaches[22], indicate that in primary CLL cells Bcl-XL plays a more prominent role in venetoclax resistance than Mcl-1 when both are upregulated following CD40 stimulation. Combined, the available data establish that both Bcl-XL and Mcl-1 can confer resistance to venetoclax and can be expected to be selected for overexpression during prolonged venetoclax treatment. As direct targeting of multiple pro-survival Bcl-2 members by combinations of BH3 mimetics in patients may result in toxicity, future studies should be aimed at dissecting the signaling pathways that contribute to venetoclax resistance in relapsing patients. Combined, the BH3 mimetic and Bim interaction studies allow us to establish a hierarchy of Bcl-2 members in binding to Bim: Bcl-2>Bcl-XL>Mcl-1>Bfl-1, when all four are present. When all available BH3 mimetics were applied, Bim interacted with Bfl-1, confirming that Bfl-1 can also bind Bim in primary leukemic cells and thus potentially could play a role in venetoclax resistance in CLL[2,44].

Our data are compatible with combination treatment with ibrutinib and venetoclax, which is currently being tested in trials, as ibrutinib forces CLL cells out of the LN which then sensitizes these cells to venetoclax as levels of Mcl-1 and Bcl-XL collapse.

Author contribution statement

Marco Haselager performed research, analyzed data and wrote the paper. Karoline Kielbassa, Hanneke Ter Burg and Danique Bax performed research and analyzed data. Stacey Fernandes provided patient samples. Jannie Borst provided vital reagents and has read and corrected the paper. Constantine Tam, Francesco Forconi and Jennifer Brown provided patient samples and have read and corrected the paper. Giorgia Chiodin and Julie Dubois analyzed patient clinical characteristics. Arnon Kater provided patient samples and wrote the paper. Eric Eldering designed the research and wrote the paper.

Disclosures

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

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Legends

Figure 1. Levels of Bcl-2 members collapse upon ibrutinib treatment. (A) FACS analysis showing overlays of Mcl-1, Bcl-XL and Bcl-2 expression between LN immigrants and emigrants. These are the data of a representative patient at baseline and on 4 and 10 weeks of ibrutinib treatment. (B) Summary of Bcl-2 member expression in LN emigrants before and after ibrutinib treatment (n=14). gMFIs were normalized by setting the baseline LN immigrant population at 100%. Black lines indicate unmutated IgHV CLL samples, red lines indicate mutated IgHV CLL samples and gray lines indicate patient samples of which the mutation status was not determined (ND). Paired sample t-test was used for statistical analyses. (C-D) Western blots of peripheral blood collected from 8 patients treated with ibrutinib. Samples were collected at baseline (B) and after 6-17 days (d) of ibrutinib (patients #1-2) or after 3 months (mo) of ibrutinib treatment (patients #3-8). Protein lysates were probed for Mcl-1, Bcl-XL and Bcl-2 and actin as loading control.

Figure 2. Levels of Bcl-2 members reappear upon ibrutinib resistance. (A) Summary of Bcl-2 member expression in LN emigrants before and during ibrutinib treatment (n=16). Ibrutinib T1 represents an early time point showing clinical response to ibrutinib and T2 represents a later time point upon ibrutinib treatment. Red lines indicate patients who relapsed on ibrutinib (n=9) whereas black lines indicate patients who stayed in remission (n=7). gMFIs were normalized by setting the baseline LN immigrant population at 100%. Paired sample t-test was used for statistical analysis for all patients to compare expression at baseline to expression upon ibrutinib. Subsequently, patients were divided into remission or relapse groups after which one-way ANOVA was used for statistical analysis to compare Bcl-2 member expression between groups during follow-up. (B) Western blot of peripheral blood collected from 2 patients who relapsed on ibrutinib. Samples were collected at baseline (B), after 0.5-1.5 years (y) of ibrutinib treatment and during relapse on ibrutinib after 4 (patient #9) and 1 (patient #10) years (y) of ibrutinib treatment. Protein lysates were probed for Mcl-1, Bcl-XL and Bcl-2 and actin as loading control.

Figure 3. Levels of Bcl-2 members increase upon venetoclax treatment. (A) Summary of Bcl-2 member expression in LN emigrants and immigrants before and after venetoclax treatment (n=5). Venetoclax T1 and T2 represent time points 1 and 2 i.e. the first and second sampling of PB during venetoclax ramp-up (median time of 3 weeks in between time points). Paired sample t-test was used for statistical analyses between baseline and venetoclax T2. (B) Western blot of peripheral blood collected from 4 patients treated with venetoclax. Samples were collected at baseline (B) and upon 2-4 weeks (wks) of venetoclax treatment. Protein lysates were probed for Mcl-1, Bcl-XL and Bcl-2 and actin as loading control. (C) CLL cells of 2 patients were cultured on 3T3 or 3T40L for 24 hours. After

detachment, cells were treated with BH3 mimetics venetoclax (ABT-199), S-63845 (S63) or A-1331852 (A13) for an additional 24 hours. Protein lysates were probed for Mcl-1, Bcl-XL and Bcl-2 and actin as loading control.

Figure 4. BH3 mimetic profiling to dissect venetoclax resistance. CLL cells were cultured on 3T3 or 3T40L for 24 hours. After detachment, cells were treated with indicated BH3 mimetics for an additional 24 hours. The respective drug concentrations were determined in preliminary experiments. Viability was measured by flow cytometry using DiOC6/TO-PRO-3 staining. (A-D) Summary of viability data of CD40-stimulated CLL cells treated with BH3 mimetics (n=16). (E) Summary of viability data of CD40-stimulated CLL cells treated with low-dose BH3 mimetics (n=5). (F) Table showing synergy indexes of BH3 mimetics combination treatments (n=16)

Figure 5. Functional hierarchy of Bcl-2 members in determining resistance to venetoclax. (A-B) CLL cells were cultured on 3T3 or 3T40L for 24 hours. After detachment, cells were treated with 5µM QVD and 100nM venetoclax for an additional 24 hours and then lysed with NP-40 lysis buffer. Shown are the protein lysates (input), the proteins in the lysates that were pulled down (Bim IP) and the proteins still present in the lysates after the IP (cleared lysate) (A) as well as the co-immunoprecipitated proteins of the Bim IP (B). (C-D) CLL cells were cultured on 3T40L for 24 hours. After detachment, cells were treated with 5µM QVD and 100nM venetoclax supplemented with or without 100nM S-63845 and 300nM A-1331852 (Triple) for 24 hours before lysis. Shown are the protein lysates (input) (C) as well as the co-immunoprecipitated proteins of the Bim IP of 2 different patient samples (D).
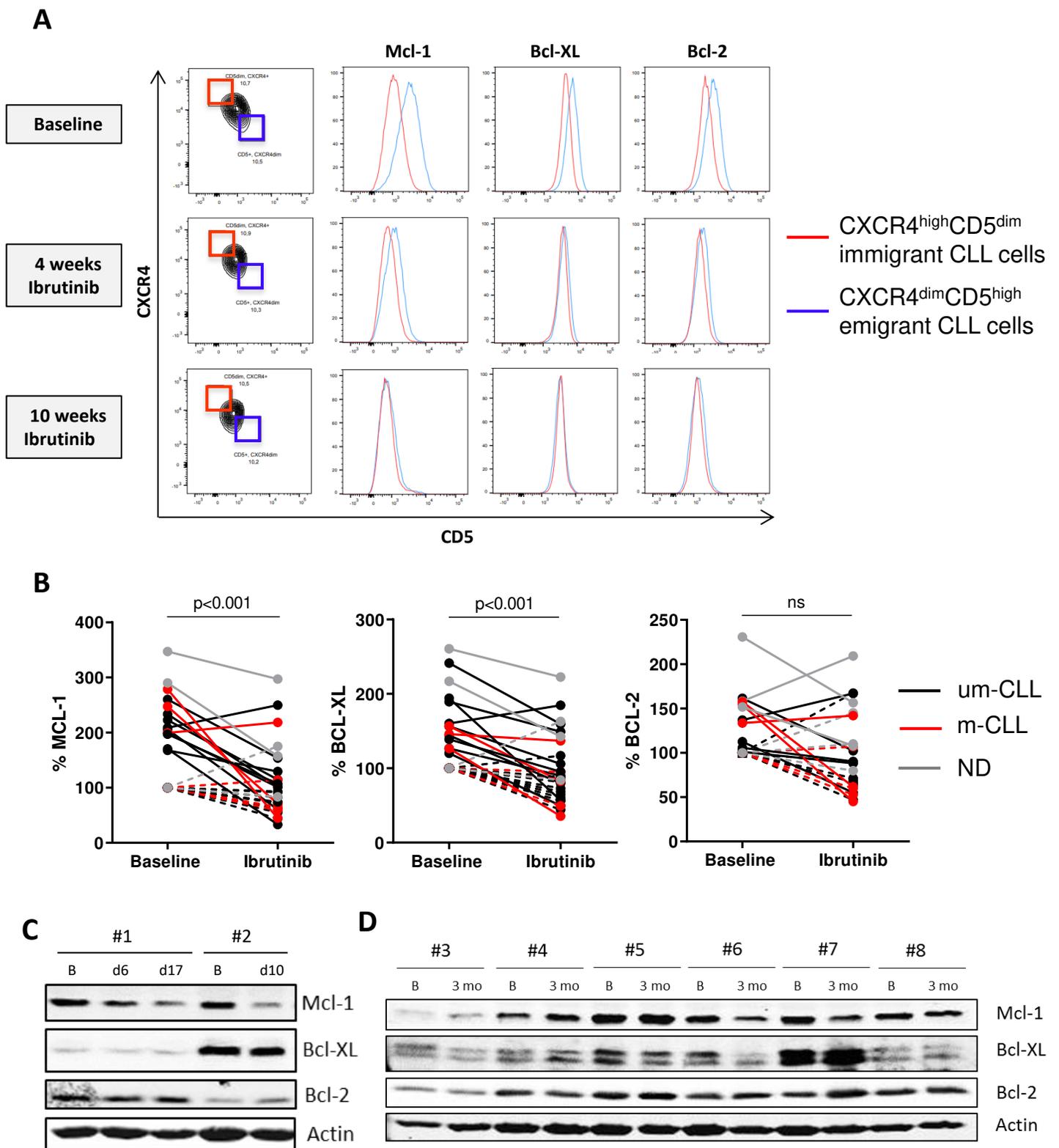
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A

B
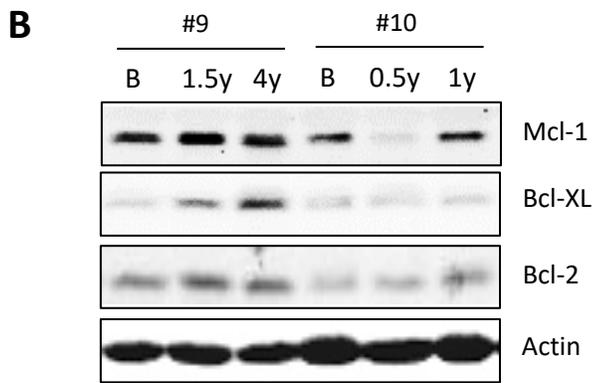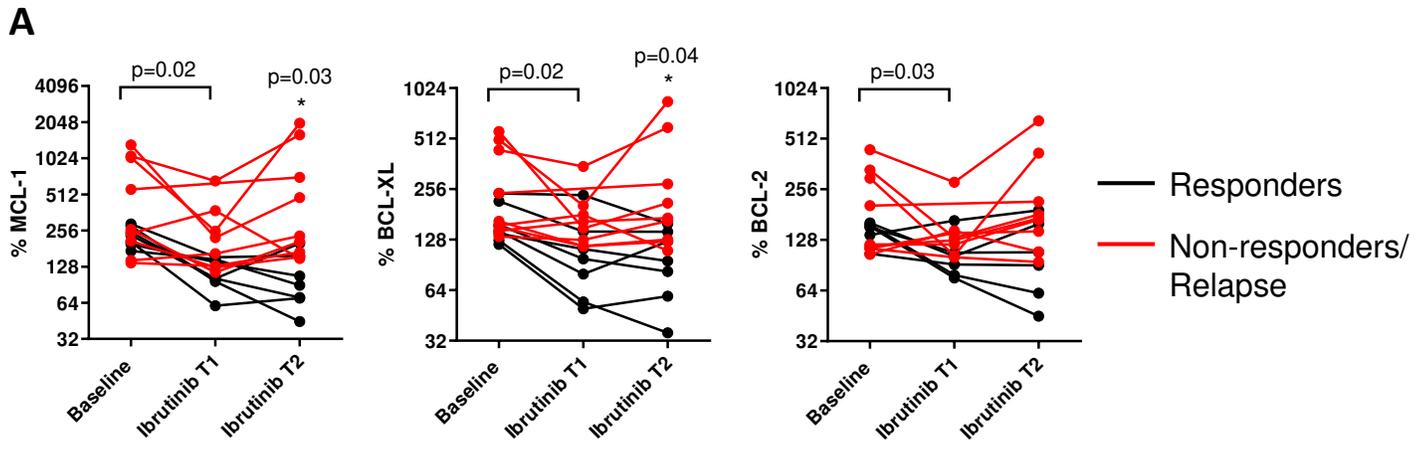
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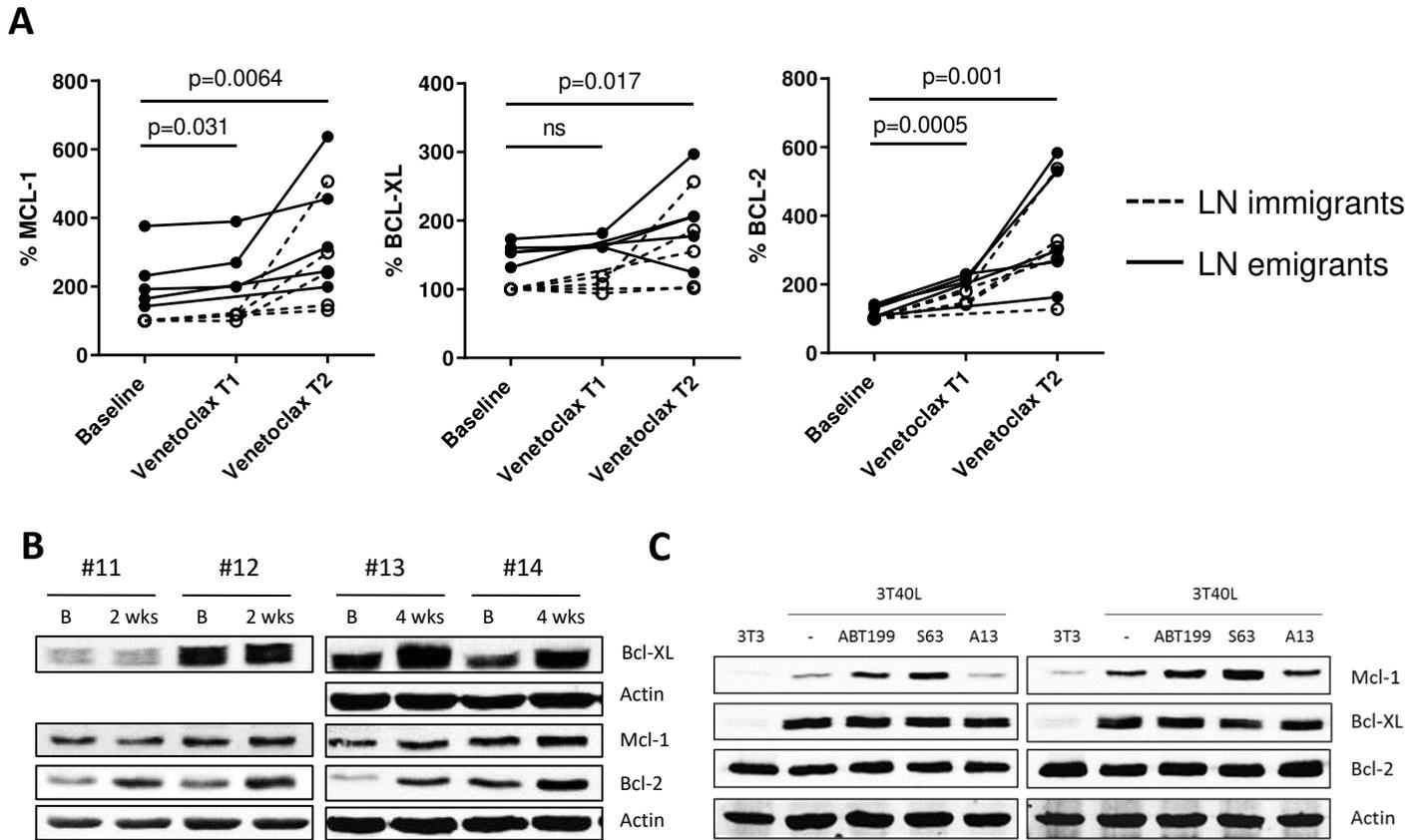
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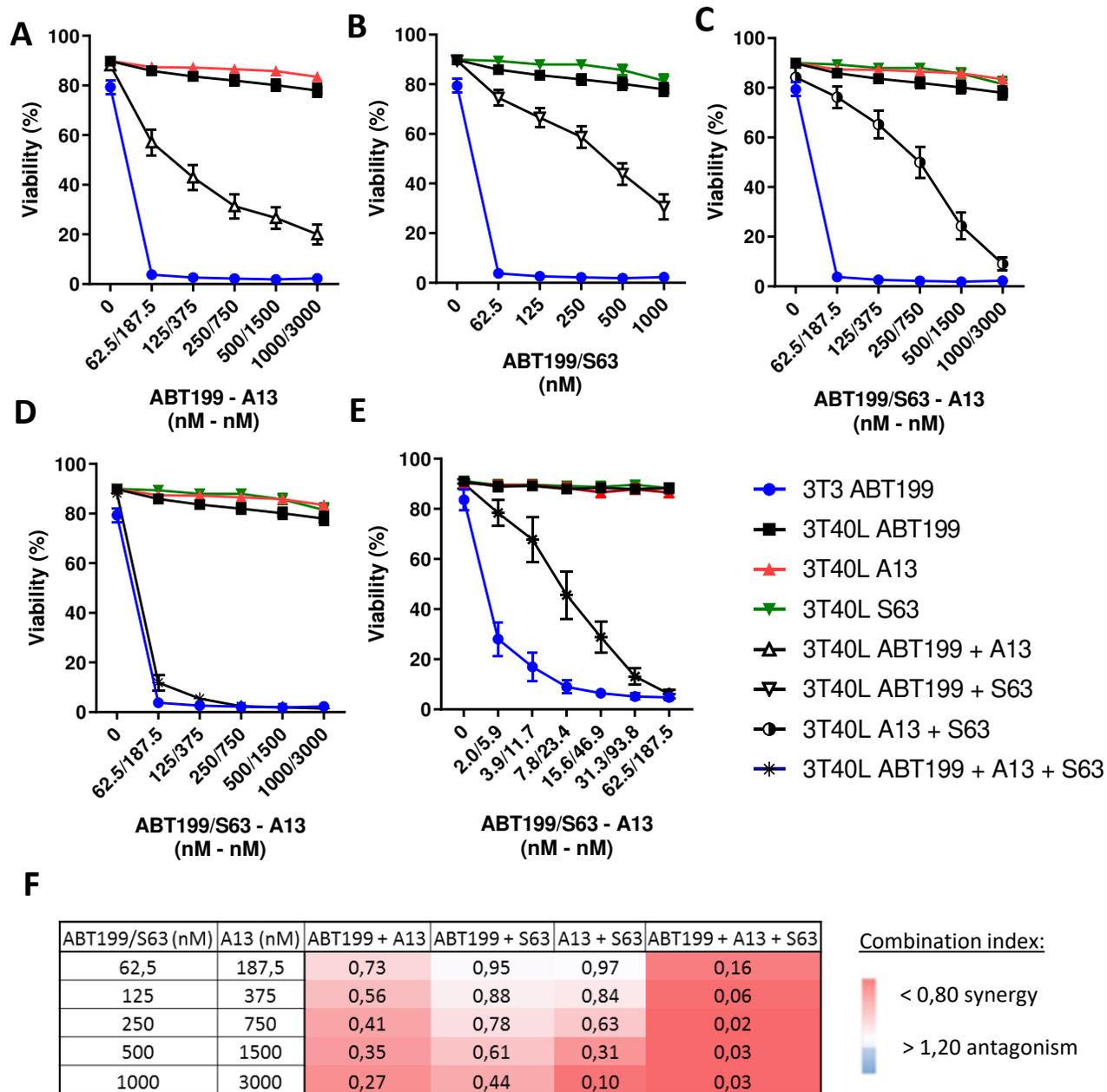
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