Rationale for the combination of venetoclax and ibrutinib in T-prolymphocytic leukemia

T-prolymphocytic leukemia (T-PLL) is an aggressive mature T-cell neoplasm that responds poorly to conventional chemotherapy and has a dismal outcome.[1] Patients with active T-PLL present with an exponential rise of post-thymic T cells with prolymphocytic morphology, hepatosplenomegaly, skin rash, lymphadenopathy, and effusions.[1] T-PLL cells commonly demonstrate rearrangements involving T-cell leukemia/lymphoma 1 (TCL1) family genes *TCL1A, MTCP1* (mature T-cell proliferation), or *TCL1B* as molecular hallmarks.[2] The anti-CD52 antibody alemtuzumab has improved initial responses up to 90%; however, nearly all cases eventually relapse, and allogeneic stem cell transplantation remains the only curative treatment option for a small subset of patients.[3]

Recently, we and others have demonstrated *in vitro* activity and clinical efficacy of the Bcl-2 inhibitor venetoclax as a single agent in relapsed/refractory T-PLL (r/r-T-PLL).[4,5] Since clinical responses were transient, we set out to identify effective combination partners for venetoclax. We probed putative mechanisms and demonstrated clinical feasibility and activity of a putative combination by treating two patients with active, r/r-T-PLL.

We employed combinatorial drug screening to identify synergistic combination partners to enhance the efficacy of venetoclax in T-PLL patients. Twenty-four candidate compounds were selected based on their clinical approval status, literature data, and mechanisms of drug action. Venetoclax was used in pairwise combinations (Figure 1A) in primary T-PLL samples with a mean post-thawing viability of 93% and mean purity of 94% (Table 1). Drug screening was performed as previously described.[4] Here ibrutinib demonstrated the strongest synergism with

Table 1. Characteristics of patients included in the high throughput combinatorial drugs screen. Overview of clinical and molecular characteristics as well as sample quality information for patients included in the combinatorial drug screen.

Patient ID	Sex	Age	Cytogenetics	FISH	TCL1A-expression	Consensus Dx	Tumor cell content (%)	Post thawing viability (%)
1	M	73	Complex karyotype	44,XY,-18,-22,der(6),t(6;22)(p?;q?),idic(6p?), idic(8)(q11),del(11)(q14),der(12),t(12;22)(q?;q?), der(13), t(13;14)(q?;q?),dup(15)(q22),der(15), t(15;18)(q?;q?)[cp5]; TCL1A rearrangement	Negative	**TCL1 positive T-PLL:** WBC >5x10^9/L, TCL1 rearrangement, complex karyotype, TCR rearrangement	93	89
2	F	58	Complex karyotype	NA	Negative	**TCL1 negative T-PLL:** WBC >5x10^9/L, complex karyotype, TCR-rearrangement	98	95
3	M	41	Complex karyotype	NA	Positive	**TCL1 positive T-PLL:** WBC >5x10^9/L, TCL1 expression, complex karyotype, TCR rearrangement	>90	95
4	M	76	NA	TCL1A rearrangement	Negative	**TCL1 positive T-PLL:** WBC > 5x10^9/L, TCL1 rearrangement, TCR rearrangement, splenomegaly, effusion	93	96
5	F	81	Complex karyotype	TP53_del MYB_del	Negative	**TCL1 negative T-PLL:** WBC > 5x10^9/L, complex karyotype, TCR-rearrangement	98	NA
6	F	67	Normal	TCL1A rearrangement ATM_del MYC_amp	Positive	**TCL1 positive T-PLL:** WBC >5x10^9/L, TCL1 rearrangement, TCR rearrangement, trisomy 8, ATM abnormality	99	89
7	M	64	NA	TLC1A rearrangement	Positive	**TCL1 positive T-PLL:** WBC > 5x10^9/L, TCL1 rearrangement, TCR rearrangement, effusion	90	NA

M: male; F: female; FISH: fluorescence *in situ* hybridization; WBC: white blood cell count; TCR: T-cell receptor; NA: not available.
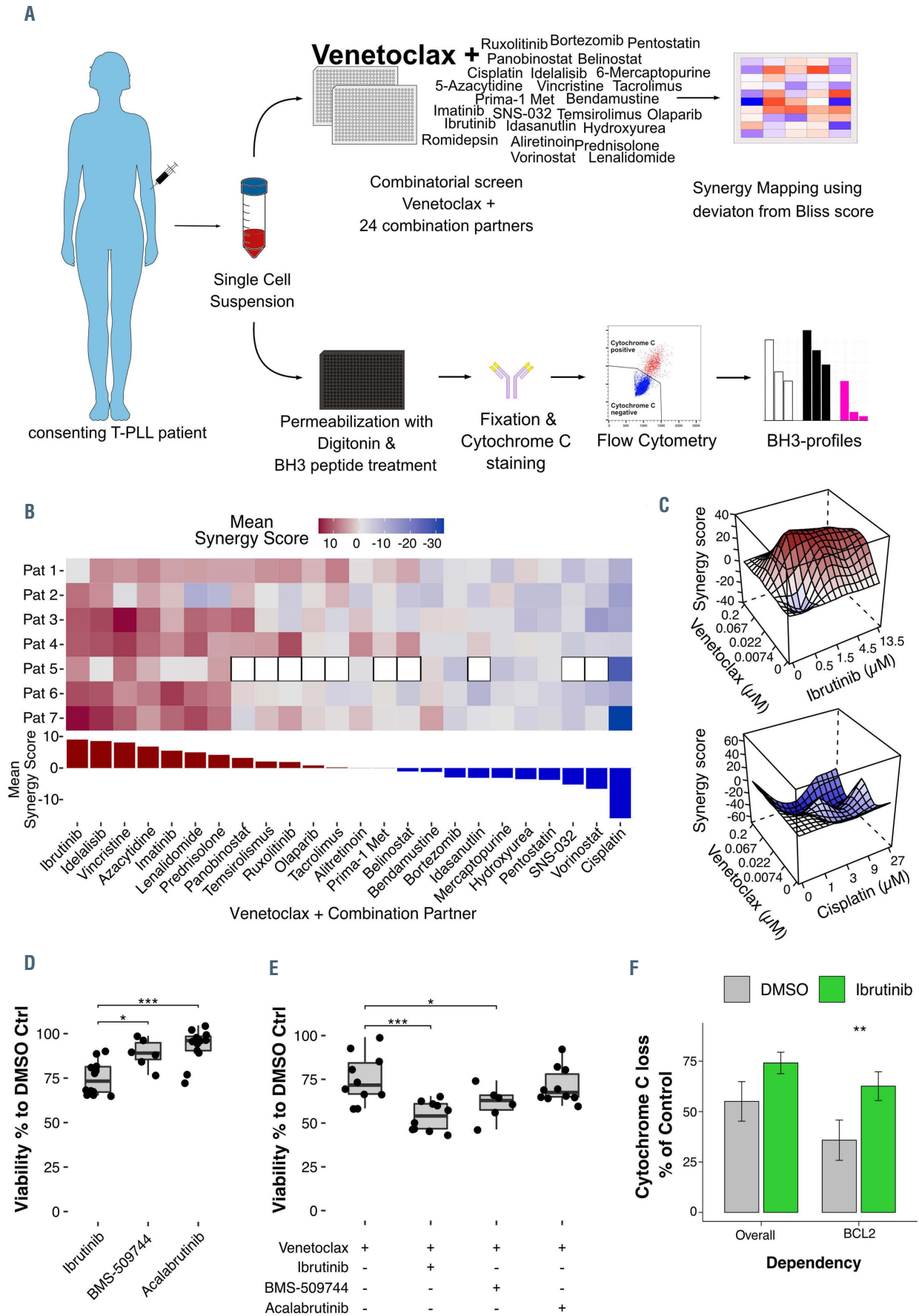
Figure 1. Legend on following page.

Figure 1. Ibrutinib synergizes with venetoclax in T-prolymphocytic leukemia via inhibition of ITK and enhances Bcl2-dependent apoptotic priming. (A) Single cell suspensions of blood or bone marrow samples were subjected to combinatorial drug screens with venetoclax and 24 potential combination partners. Deviation from the Bliss Independence score was evaluated for each combination. For BH3-profiling single cells were stained for cytochrome and analyzed by flow cytometry. (B) Heatmap demonstrating deviation from the Bliss independence score for each combination and individual patients (n=7). Synergy is denoted in red while antagonism is shown in blue. Clear boxes represent missing data for Patient 5 whose material was only screened for a subset of drugs due to sample availability. The bar plot in the lower half shows the synergy score as a mean over all patients. Drug synergy was calculated using the Synergy finder R package by integrating data of three independent runs (*Online Supplementary Figure S1B*). (C) A representative three-dimensional drug synergy plot (data from Patent 7) for ibrutinib and cisplatin with venetoclax. (D) Annexin V assay showing viability compared to that with a DMSO control for ibrutinib (n=12 different T-PLL patient samples), ITK-inhibitor BMS-509744 (n=6), and BTK-inhibitor acalabrutinib (n=12, Wilcoxon-Mann-Whitney test, ***$P<0.001$, *$P<0.05$, all compounds tested at 10 μM with drug exposure for 24 h under NKtert co-culture). (E) Annexin V assay demonstrating viability of T-PLL samples under NKtert co-culture treated with venetoclax alone (n=10), or in combination with ibrutinib (n=10), BMS-509744 (n=6), and acalabrutinib (n=10) compared to DMSO-Ctrl. (*t*-test,*$P≤0.05$, ***$P<0.001$, ibrutinib, acalabrutinib and BMS-509744 were used at a dose of 10 μM with drug exposure for 24 h, venetoclax was used at a dose of 100 nM with drug exposure for 4 h both as a single agent and in combination. After annexin V/Hoechst staining, cells were fixed with paraformaldehyde and analyzed using a BD Fortessa with a 96-well HTS plate-reader. NKtert cells were excluded using forward and side scatter parameters. Primary antibodies were directed against cytochrome c (Alexa Fluor 488-labeled, 6H2.B4/612308, Biolegend), CD19 (PE/Cy7-labeled, H13B19/302216, BioLegend), anti–CD5 (PE-labeled, UCHT2/300608, Biolegend). The analysis was performed on the CD5+CD19– cell fraction. (F) BH3-profiling in primary T-PLL samples treated with ibrutinib: cytochrome C release compared to control for overall mitochondrial priming and specific BCL2-dependence is shown for samples treated with either DMSO or ibrutinib 10 μM for 24 h (*t*-test,**$P≤0.01$). T-PLL: T-prolymphocytic leukemia; DMSO: dimethylsulfoxide; Ctrl. Control.

venetoclax, whereas cisplatin appeared the most antagonistic (Figure 1B and C, *Online Supplementary Figure S1B*). The activity of ibrutinib as a single agent was very modest (*Online Supplementary Figure S1A and B*), consistent with previous reports.[6] In addition, independent annexin V/Hoechst assays of primary T-PLL cells on NK-Tert stromal support demonstrated modest single-agent activity of ibrutinib (Figure 1D, *Online Supplementary Figure S2A*). Moreover, selective inhibition of Bruton tyrosine kinase (BTK) by acalabrutinib had no effect on viability (Figure 1D). In contrast, ibrutinib has substantial inhibitory activity on the intracellular mediator of T-cell receptor signaling IL-2-inducible T-cell kinase (ITK; half maximal inhibitory concentration [IC_{50}]: 2.2 nM), which has been reported to play a functional role in T-PLL.[5,7] In line with previous observations, single-agent treatment with BMS-509744, a specific ITK inhibitor, had no effect on viability in primary T-PLL samples (Figure 1D).[8]

To elucidate the mechanism of the combinatorial effect, we treated primary T-PLL samples with venetoclax alone and in combination with ibrutinib, BMS509744 or acalabrutinib. In contrast to BTK-specific inhibition, only drugs that inhibit ITK (ibrutinib, and BMS509744) enhanced Bcl2-inhibitor-induced cell death (Figure 1E). We performed dynamic BH3-profiling of primary T-PLL samples treated *ex vivo* with ibrutinib to elucidate changes in apoptotic priming. The assay measured cytochrome C release upon stimulation with BH3-mimetics as a readout for a cellular tendency towards apoptosis.[9] The data demonstrated enhanced overall mitochondrial priming for apoptosis and a shift to increased functional dependence on Bcl-2 for survival upon ibrutinib treatment (Figure 1F, *Online Supplementary Figure S2B*).

Based on our *in vitro* drug synergy findings, we initiated combined treatment with venetoclax and ibrutinib in two r/r-T-PLL patients after alemtuzumab-based therapy. Both patients presented with active disease after multiple treatment lines with no further standard treatment options available. We employed tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) to measure venetoclax and ibrutinib serum levels and *in vivo* BH3 profiling to evaluate overall and Bcl2-dependent apoptotic priming during treatment. The expression of phospho-ITK, ITK and BH3 family members was evaluated by immunoblotting of patients' cells obtained during treatment (Figure 2, *Online Supplementary Figure S3*).

Patient A (male, aged 78 years) was admitted with r/r-T-PLL after three previous treatment lines (alemtuzumab monotherapy, alemtuzumab + FCM [fluradabine,

cyclophosphamide and mitoxantrone], rechallenged with alemtuzumab monotherapy). He presented with dyspnea, a white blood cell (WBC) count of 519x10⁹/L, elevated lactate dehydrogenase (LDH; 5,230 U/L), fever, absolute lymphocytosis (472x10⁹/L) and neutrophilia (42x10⁹/L) (Figure 2A, *Online Supplementary Figure S3A*), and splenomegaly (diameter: 26 cm). Fluorescence *in situ* hybridization analyses of interphase nuclei revealed multiple cytogenetic aberrations (TCL1 and TCRA/D translocations, heterozygous deletions of several 6q and 13q loci, trisomies 8 and 12, duplication of the MLL locus as well as MYC amplifications), suggesting the presence of a complex aberrant karyotype. Venetoclax treatment was commenced with a daily ramp-up from 20 mg to 800 mg, which was well-tolerated. However, the clinical response was limited with the WBC count still above 300x10⁹/L and LDH above 3,000 U/L after 2 weeks. When co-treatment with ibrutinib at a dose of 420 mg was initiated on day 16, both the WBC count and LDH decreased steadily (Figure 2A). The patient's overall clinical condition improved, and the spleen size decreased to 22 cm after 20 days of co-treatment. Serum levels of venetoclax and ibrutinib were continuously monitored. Interruption of ibrutinib on day 24 was associated with a rise of WBC count that declined after ibrutinib was reintroduced. The course of patient A was complicated by influenza A infection and subsequent bacterial pneumonia requiring multiple admissions to hospital, and mechanical respiratory support resulting in discontinuation of anti-T-PLL therapy. After resolution of the pneumonia, T-PLL therapy was reinitiated, however treatment adherence dropped, when the patient returned to his local treatment team who eventually switched to best supportive care.

Patient B (female, aged 75 years) had r/r-T-PLL that relapsed after initial therapy with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) and was refractory to alemtuzumab. She presented with dyspnea, pleural effusion, a WBC count of 300x10⁹/L, elevated LDH (581 U/L), and absolute neutrophilia (27x10⁹/L) during alemtuzumab treatment (Figure 2B, *Online Supplementary Figure S3B*). The cytogenetic report demonstrated a complex karyotype including inv(14) and isochromosome 8q. Ibrutinib was started at a dose of 420 mg once daily before venetoclax daily at a dose increased from 20 mg to 400 mg. The combination of ibrutinib and venetoclax led to a rapid reduction of WBC count and LDH as well as an improvement of clinical status (Figure 2B). On day 26, the patient experienced a minimal subarachnoidal hemorrhage (grade 2). Ibrutinib was withheld but venetoclax continued. As serum levels of ibrutinib dropped to undetectable levels, a concomitant
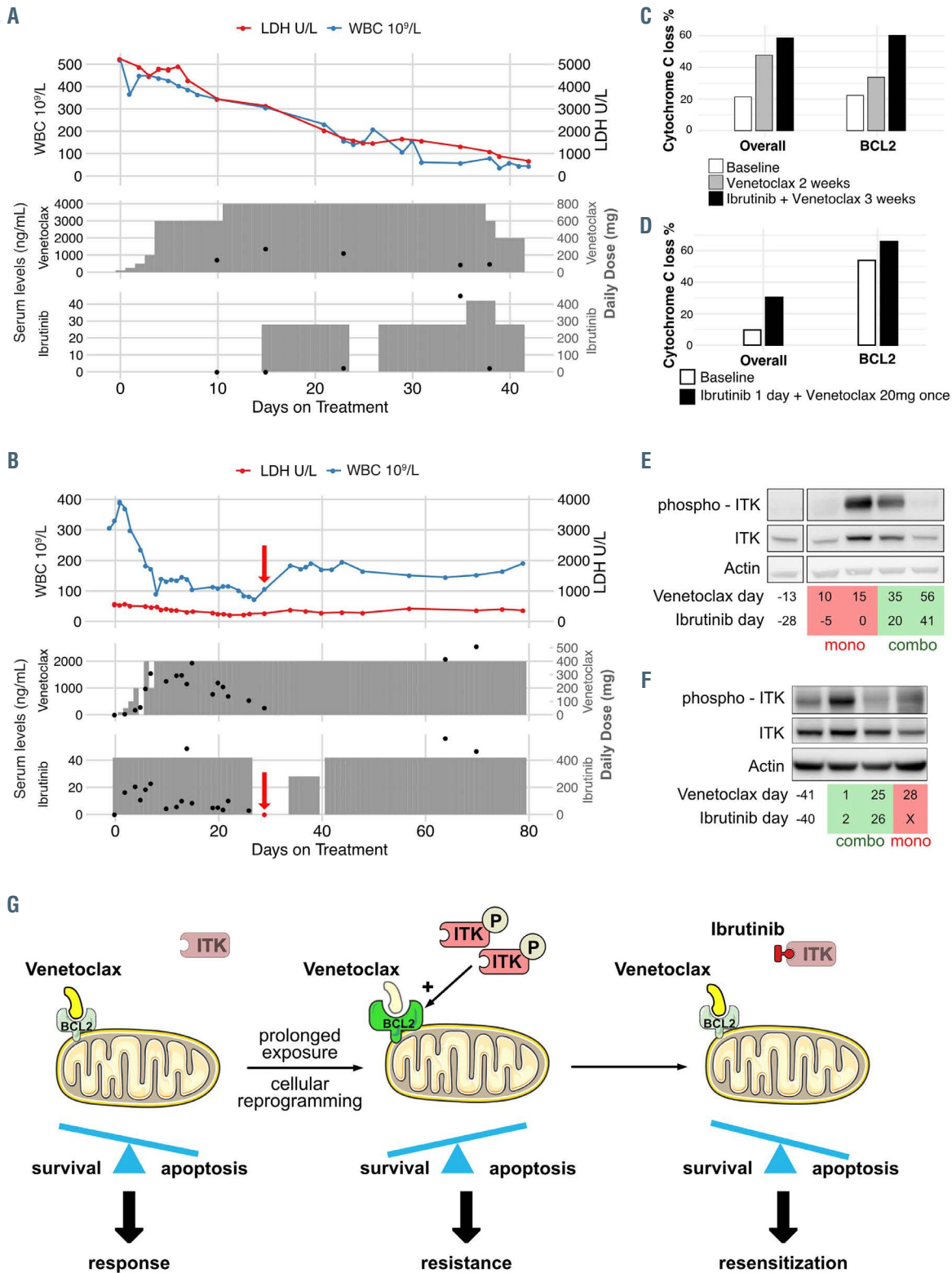
Figure 2. The combination of ibrutinib and venetoclax is clinically active in T-prolymphocytic leukemia. (A, B) Clinical follow-up of two patients treated with the combination of ibrutinib and venetoclax, patient A (A) and patient B (B). The WBC count and LDH concentration are plotted as blue and red lines, respectively. The lower part of each panel represents drug serum levels as black dots and drugs given as gray rectangles. Drug levels were determined by mass spectrometry. The red arrow denotes the time point at which the serum ibrutinib concentration dropped below the level of detection with a concomitant rise of WBC. (C, D) *In vivo* BH3 profiling of primary patients' samples during co-treatment: patient A (C) and patient B (D). (E, F) Western blot analysis of primary cells showing changes in protein levels of ITK and phospho-ITK during co-treatment of patient A (E) and patient B (F). Primary antibodies were directed against phospho-ITK (Tyr512, Life Technologies, #PA564523), ITK (Cell Signaling Technology, #2380S) and β-actin (Santa Cruz Biotechnology, #SC-47778) (G) Proposed mechanism. Monotherapy with venetoclax may lead to drug resistance via upregulation and activation of ITK and reduced apoptotic priming. ITK inhibition might increase Bcl-2-dependent apoptotic priming and restore the activity of venetoclax. WBC: white blood cell; LDH: lactate dehydrogenase.

increase of the WBC count was observed (Figure 2B). The hemorrhage resolved with supportive care, and ibrutinib was restarted, with stabilization of the WBC count. Three weeks later the patient died due to secondary bacterial pneumonia.

Despite high tumor burden neither patient showed signs of tumor lysis syndrome. In both patients, interruption of ibrutinib was associated with an increase of WBC count that declined (patient A) or stabilized (patient B) when ibrutinib was re-introduced. The clinical course of both patients was complicated by severe bacterial pneumonia which eventually led to treatment termination. The combination of venetoclax and ibrutinib had a tolerable safety profile but was associated with increased frequencies of neutropenia and respiratory infections in studies including patients with other hematologic diseases such as mantle cell lymphoma or chronic lymphocytic leukemia.[10,11] Both patients we treated had received alemtuzumab treatment as a re-induction attempt and experienced increased neutrophil counts (*Online Supplementary Figure S3A* and *B*). Thus, it is plausible that prior anti-CD52 could increase infectious complications of the combination.

In vivo dynamic BH3-profiling with samples from the two patients while on treatment showed a modest increase in overall apoptotic priming by ibrutinib, driven by an enhanced dependence on Bcl-2. This effect was further enhanced by venetoclax (Figure 2C and D), which is consistent with our *in vitro* data on T-PLL samples (Figure 1F). Immunoblotting analysis demonstrated that venetoclax treatment alone led to the induction of both phosphorylated and total ITK, an effect that was abrogated by the addition of ibrutinib as demonstrated in samples of patient A while on treatment (Figure 2E). In patient B, treatment with venetoclax was started 1 day after ibrutinib. ITK activity (phospho-ITK) decreased during treatment and regained activity upon interruption of ibrutinib treatment (Figure 2F). Intracellular T-cell receptor signaling via increased phospho-ITK expression is known to be associated with inferior prognosis in T-cell lymphomas, and ITK inhibition has been shown to prime apoptosis of malignant T cells by downregulating anti-apoptotic proteins, including Bcl-2, MCL-1, and Bcl-XL.[12] At the protein level, in patient A expression of Mcl-1 and Bcl-2 was induced during venetoclax monotherapy, but was reduced upon combinatorial treatment. In contrast, patient B showed a predominant induction of pro-apoptotic BH3 family members in response to combined ibrutinib and venetoclax treatment (*Online Supplementary Figure S3C* and *D*). However, previous research has shown that immunoblotting does not accurately reflect the clinical efficacy of BH3 mimetics.[9] Our previous report and data presented here suggest that exposure to venetoclax monotherapy leads to ITK activation and increased Bcl-2 and Mcl-1 expression, and Bcl-2 dependence with a suboptimal clinical response.[4] Co-treatment with venetoclax plus ibrutinib may reduce ITK activity, increase Bcl-2 dependence, and restore susceptibility to venetoclax (Figure 2G). Similarly, Mcl-1 inhibition has been shown to act synergistically with venetoclax in T-PLL cells.[13]

Our high-throughput screen identified ibrutinib as a synergistic combination partner for venetoclax in T-PLL. In this study, we favored synergy over potency, since we hypothesized that synergism would be associated with a more favorable clinical safety profile. Indeed, the combination of venetoclax and ibrutinib has been considered safe in other indications.[10,11] We acknowledge strong single-agent potency of other compounds such as the his-tone deacetylase inhibitor, panobinostat; however the added effect of its combination to venetoclax was negligible (*Online Supplementary Figure S1B*).

Recently, anecdotal cases of venetoclax combinatorial treatments of T-PLL patients have been published: In line with our findings Oberbeck *et al*. reported disease stabilization after short-term treatment of one patient with venetoclax plus ibrutinib, but progression after cessation of treatment.[14] Alfayez *et al*. treated one patient with venetoclax plus pentostatin who achieved complete remission for 10 months.[15] This combination, however, did not demonstrate synergism in our screen (Figure 1B), but future studies could determine a putative benefit for T-PLL patients.

Our *in vitro* studies demonstrated that the combination of venetoclax and ibrutinib increased T-PLL cell priming for apoptosis and Bcl-2 dependence. The combination produced clinical responses in two heavily pretreated patients with r/r-T-PLL and enhanced Bcl2-dependence *in-vivo* while reducing ITK activity. These results prompted the initiation of the first international multicenter clinical study in T-PLL, the phase II VIT-trial (NCT03873493) testing the combination of venetoclax and ibrutinib in r/r-T-PLL in a larger cohort of patients.

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