**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.² 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 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.**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age</th>
<th>Cytogenetics</th>
<th>FISH</th>
<th>TCL1A-expression</th>
<th>Consensus Dx</th>
<th>Tumor cell content (%)</th>
<th>Post-thawing viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>73</td>
<td>Complex karyotype</td>
<td>44,X,-15,-22,der(6)t(6:22)(p21q14),idic(6p33),</td>
<td>idic(8)(q11),del(11)(q14),der(12)t(12;22)(q21;q14),der(13)t(13;14)(q?;q?),dup(15)(q22),der(15),t(15;18)(q?;q?),</td>
<td>Negative</td>
<td>TCL1 positive T-PLL: WBC &gt;5x10⁹/L, TCL1 rearrangement, complex karyotype, TCR rearrangement</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>58</td>
<td>Complex karyotype</td>
<td>NA</td>
<td>Negative</td>
<td>TCL1 negative T-PLL: WBC &gt;5x10⁹/L, complex karyotype, TCR-rearrangement</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>41</td>
<td>Complex karyotype</td>
<td>NA</td>
<td>Positive</td>
<td>TCL1 positive T-PLL: WBC &gt;5x10⁹/L, TCL1 expression, complex karyotype, TCR rearrangement</td>
<td>&gt;90</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>76</td>
<td>NA</td>
<td>TCL1A rearrangement</td>
<td>Negative</td>
<td>TCL1 positive T-PLL: WBC &gt;5x10⁹/L, TCL1 rearrangement, complex karyotype, TCR rearrangement</td>
<td>93</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>81</td>
<td>Complex karyotype</td>
<td>TPS3_del MYB_del</td>
<td>Negative</td>
<td>TCL1 negative T-PLL: WBC &gt;5x10⁹/L, complex karyotype, TCR-rearrangement</td>
<td>98</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>67</td>
<td>Normal</td>
<td>TCL1A rearrangement</td>
<td>Positive</td>
<td>TCL1 positive T-PLL: WBC &gt;5x10⁹/L, TCL1 rearrangement, complex karyotype, TCR rearrangement</td>
<td>99</td>
<td>89</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>64</td>
<td>NA</td>
<td>TCL1A rearrangement</td>
<td>Positive</td>
<td>TCL1 positive T-PLL: WBC &gt;5x10⁹/L, TCL1 rearrangement, complex karyotype, TCR rearrangement</td>
<td>90</td>
<td>NA</td>
</tr>
</tbody>
</table>

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.
venetoclax, whereas cisplatin appeared the most antago-
nistic (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. In addition, independent annexin
V/Hoechst assays of primary T-PLL cells on NK-Tert stro-
mal 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 activ-
ity on the intracellular mediator of T-cell receptor signal-
ing 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. 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).^3

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.3 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 initia-
ted 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
T-PLL 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 Bcl-2-dependent apoptotic priming expression of phospho-ITK, ITK and BH3 family mem-
bers 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 + PCM [Hurdarabine, cyclophosphamide and mitoxantrone], rechallenged with
alemtuzumab monotherapy). He presented with dysp-
nea, a white blood cell (WBC) count of 519 x 10^9/L, ele-
vated lactate dehydrogenase (LDH; 5,230 U/L), fever,
absolute lymphocytosis (472 x 10^9/L) and neutrophilia
(42 x 10^9/L) (Figure 2A, Online Supplementary Figure S3A),
and splenomegaly (diameter: 26 cm). Fluorescence in situ
hybridization analyses of interphase nuclei revealed mul-
tiple cytogenetic aberrations (TCL1 and TCRA/D translo-
cations, 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 300 x 10^9/L
and LDH above 3,000 U/L after 2 weeks. When co-treat-
ment 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 respira-
tory support resulting in discontinuation of anti-T-PLL
therapy. After resolution of the pneumonia, T-PLL thera-
py was reinitiated, however treatment adherence dropped,
when the patient returned to his local treatment
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 (cyclophos-
phamide, doxorubicin, vincristine and prednisone) and
was refractory to alemtuzumab. She presented with dys-
pea, pleural effusion, a WBC count of 300 x 10^9/L, elevat-
ed LDH (581 U/L), and absolute neutrophilia (27 x 10^9/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 oral once daily before venetoclax
administration was started at 10 µM and increased daily 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 sub-
arachnoidal hemorrhage (grade 2). Ibrutinib was with-
held but venetoclax continued. As serum levels of ibruti-
hib dropped to undetectable levels, a concomitantly
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. 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. 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. 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. 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.

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. We acknowledge strong single-agent potency of other compounds such as the histone 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. Alfonso et al. treated one patient with venetoclax plus pentostatin who achieved complete remission for 10 months. This combination, however, did not demonstrate synergism in our screen (Figure 1B), but future studies could determine a putative benefit for T-PLL patients.

Out 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 vitro 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.

Christoph Kornauth,1 Charles Herbaux,2 Bernd Boidol,3 Chantal Guillemete,1 Patrick Caron,1 Marius E. Mayerhöfer,5 Stéphanie Foulant,6 Olivier Tournilhac,7 Tea Pemovska,1 Stephen J.F. Chong,2 Emiel van der Kouwe,1 Lukas Kazianka,1 Georg Hofinger,1 Daniel Heintel,8 Roland Jäger,10 Markus Raderer,7 Ulrich Jäger,1 Ingrid Simonitsch-Klupp,12 Wolfgang R. Sperr,1 Stefan Kubrick,3 Matthew S. Davids2 and Philipp B. Staber4

CK, CH and BB contributed equally as co-first authors.

1 MSD and PBS contributed equally as co-authors.

1 Department of Medicine I, Division of Hematology and Hemasociology, Medical University of Vienna, Vienna, Austria; 2 Department of Medical Oncology, Dana-Faber Cancer Institute, Harvard Medical School, Boston, MA, USA; 3 Center for Molecular Medicine (C3MM), Austrian Academy of Sciences, Vienna, Austria; 4 Centre Hospitaller Universitaire de Québec – Université Laval and Faculty of Pharmacy, Université Laval, Québec, Canada; 5 Department of Biomedical Imaging and Image-Guided Therapy, Medical University of Vienna, Vienna, Austria; 6 UMR CANTHER, INSERM 1277-CNRS 9202 UMRS 12, University of Lille, Hematology Laboratory, Biology and Pathology Center, CHU de Lille, Lille, France; 7 Service d’Hematologie Clinique et de Therapie Cellulaire, CHU, Universite Clermont Auvergne, EA7453 CHELTER, CIC1405, Clermont Ferrand, France; 8 3rd Medical Department, Centre for Oncology and Haematology, Kaiser Franz Josef-Spital, Vienna Austria; 9 4th Austria Medical Department, Center for Oncology and Hematology, Wilhelmshospitaal Vienna, Vienna, Austria; 10 Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria; 11 Department of Medicine I, Division of Oncology, Medical University of Vienna, Vienna, Austria and 12 Clinical Department of Pathology, Medical University of Vienna, Vienna, Austria

Correspondence: PHILIPP B. STABER philipp.staber@meduniwien.ac.at


Received: September 8, 2020.
Accepted: February 11, 2021.
Disclosures: UJ and PBS have received honoraria and advisory board fees from Abbvie and Janssen. MSD has provided consultancy or scientific advisory board services for AbbVie, Adaptive...
Biotechnologies, Ascentage, AstraZeneca, Beigene, Celgene, Genentech, Janssen, MEI Pharma, Pharmacyclics, Research to Practice, Syros Pharmaceuticals, TG Therapeutics, Verastem, and Zentalis, and has received institutional research funding from Ascentage, AstraZeneca, Genentech, MEI Pharma, Pharmacyclics, Surface Oncology, TG Therapeutics, and Verastem. The other authors have no conflicts of interest to disclose.

Contributions: CK, CH, BB, CG, PC, SJFC, LK and EK performed research; SP, OT, RJ, MEM and ISK provided material and performed expanded diagnostics on patients’ samples; GH, WRS, DH, MR, UJ and PBS managed and treated patients; CK, CH, TP, SK, MSD and PBS analyzed the data; CK, TP, PBS wrote the manuscript; MSD and PBS supervised the study.

Acknowledgments: our screening compound libraries are from the NIH clinical collection, gifts from F. Bracher, T. Nielsen, S. Nijman, J. Bradner, The Broad Institute, and Haplogen GmbH.

Funding: we acknowledge funding from the Austrian Science Fund (FWF) TRANSCAN-2 grant ERANET-PLL I 4156B, FWF TRANSCAN-2 grant EuroTCLym I 4154B, FWF grant P27532-B20 (to PBS), Vienna Science and Technology Fund (WWTF) grant LS16-034 (to UJ) and the Anniversary Fund of the Oesterreichische Nationalbank (OeNB) grant P15936 (to PBS). We further acknowledge funding from the Canadian Institutes of Health Research (CIHR; FRN-152986 and FRN-408093 (to CG), and the Canada Research Chair Program. CG holds a Canada Research Chair in pharmacogenomics (Tier 1).

References