STIMULUS REPORT

blood advances

Combined ibrutinib and venetoclax treatment vs single agents in the *TCL1* mouse model of chronic lymphocytic leukemia

Arnon P. Kater,[1-4] Erik Slinger,[1] Gaspard Cretenet,[1,*] Anne W. Martens,[1-4,*] Sriram Balasubramanian,[5] Joel D. Leverson,[6] and Eric Eldering[1-4]

[1]Department of Experimental Immunology and Hematology, [2]Cancer Center Amsterdam, [3]Amsterdam Institute for Infection and Immunology, and [4]Lymphoma and Myeloma Center, University of Amsterdam, Amsterdam, The Netherlands; [5]Janssen R&D, Spring House, PA; and [6]AbbVie Oncology Discovery, North Chicago, IL

Key Points

- Synergy of venetoclax and ibrutinib occurs in vivo via inhibition of proliferation by ibrutinib and increased cell death by venetoclax.
- Combined treatment with ibrutinib and venetoclax results in T-cell subset normalization in all compartments.

The covalent inhibitor of Bruton's tyrosine kinase ibrutinib and the specific Bcl-2 inhibitor venetoclax are both highly efficacious single-agent drugs in the treatment of chronic lymphocytic leukemia (CLL). Based on their complementary modes of action, ibrutinib and venetoclax are hypothesized to act in a synergistic fashion. Currently, it is unclear whether combined treatment is indeed superior to continuous single-agent treatment and what mechanisms underlie the resistance to combination treatment. In addition, the effects of such treatment on the skewed T-cell compartment characteristic of CLL are as yet unknown. In the murine Eμ-TCL1 adoptive transfer model resembling aggressive CLL, we found that combined treatment resulted in the deepest responses, with the longest duration related to a combination of decreased proliferation and increased induction of apoptosis. In addition, alterations in T-cell subsets were most prominent after combination treatment, with increased naive cells and reduced effector memory cells. Remarkably, effects of single agents but also combination treatment were eventually interrupted by relapse, and we found downregulation of BIM expression as a plausible cause of acquired drug resistance. Nevertheless, in this murine model, the combination of venetoclax and ibrutinib has increased efficacy over single agents, accompanied by a restoration of the T-cell compartment.

Introduction

Ibrutinib, the covalent inhibitor of Bruton's tyrosine kinase (BTK), is a highly efficacious single-agent drug for treating chronic lymphocytic leukemia (CLL). Most responses with continuous treatment are partial, with reductions in spleen and lymph node (LN) disease, but typically with persistence in the bone marrow.[1] In contrast, the BCL-2 inhibitor venetoclax results in deep clearance of peripheral blood (PB) and marrow but LN responses are often partial.[1]

Venetoclax and ibrutinib have distinct mechanisms of action. Ibrutinib inhibits CLL adhesion and migration, thereby blocking interaction with the LN tumor microenvironment (TME), which causes rapid redistribution from the LN into the PB.[2] If kept in circulation, CLL cells are deprived of prosurvival stimuli and eventually die. Ibrutinib also inhibits proliferation evoked by stimuli that mimic the LN TME, such as CD40L/IL-21 and CpG.[3] Venetoclax selectively binds to BCL-2, which leads to release of proapoptotic proteins such as BIM, resulting in activation of the intrinsic apoptotic pathway.[4] However, rebinding of BIM to other antiapoptotic proteins, especially BCL-X_L and MCL-1, when expressed, prevents the onset

Submitted 29 March 2021; accepted 21 July 2021; prepublished online on *Blood Advances* First Edition 23 September 2021; final version published online 10 December 2021. DOI 10.1182/bloodadvances.2021004861.

*G.C. and A.W.M. contributed equally to this study.

Presented in poster form at the 59th American Society of Hematology Annual Meeting in December 2017.

For original data, please contact the corresponding author (a.p.kater@amsterdamumc.nl).

The full-text version of this article contains a data supplement.

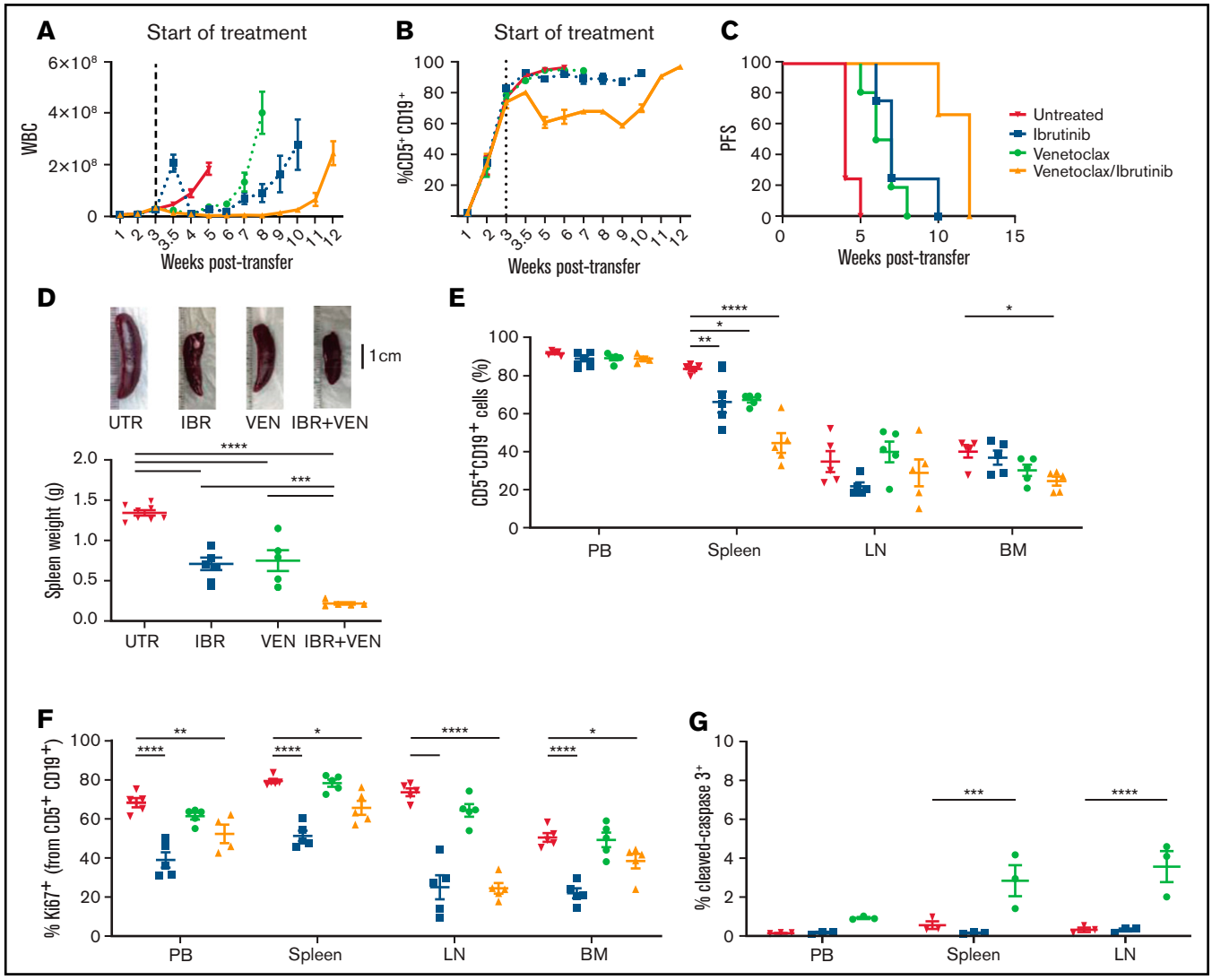

Figure 1. Combined venetoclax and ibrutinib vs single-agent treatment in the *Tcl1* mouse model of CLL (A-B) Mice were treated with ibrutinib (0.16 mg/mL in drinking water), venetoclax (once daily 100 mg/kg, intragastric), or a combination of both agents and assessed on a weekly basis for WBC count (A) and percentage of CLL cells (B). Means of WBC counts and percentage of CLL cells ± standard error of the mean (SEM) are presented. The figure is representative of 4 independent experiments, including 5 mice in each arm of the respective experiments. (C) Splenomegaly was partially reversed after 4 weeks of treatment and is more pronounced in the combination treatment. At this time point, WBCs were 1.18×10^8 in untreated, 1.26×10^7 in ibrutinib-treated, 6.2×10^6 in venetoclax-treated, and 1.3×10^7 in combination-treated mice. Photographs showing spleen size are representative of 3 experiments. Means of spleen weights ± SEM are presented; significant differences were determined by 1-way analysis of variance (ANOVA) with Tukey's multiple-comparisons test. ***$P < .001$; ****$P < .0001$. (D) Single-agent and combination treatment decreased CLL cell accumulation in mouse spleen and combination-treatment decreased CLL cell accumulation in bone marrow (BM). Means of the percentage of CLL cells ± SEM are presented. Significant differences were determined by 2-way ANOVA with Tukey's multiple-comparisons test. *$P < .05$; **$P < .01$; ****$P < .0001$. Reduced percentage of proliferating cells (E) and increase in apoptotic cells measured in PB, spleen, and LN (F) after 4 weeks of the indicated treatments. Mean percentage of $Ki67^+$ or of cleaved caspase 3^+ cells ± standard error of the mean (SEM) are presented; significant differences were determined by 2-way analysis of variance with Tukey's multiple-comparisons test. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

of an apoptotic response.[5] Expression of these antiapoptotic proteins is specifically increased within the LN TME through both transcriptional (BCL-X_L) and posttranscriptional (MCL-1) mechanisms.[6,7] Based on their distinct and complementary modes of action, ibrutinib and venetoclax are hypothesized to act synergistically. Indeed, preliminary phase 2 results of combination trials have demonstrated high overall response rates and undetectable minimal residual disease response rates in the first-line and relapsed settings.[8-10] So far, it is unclear whether combined treatment is indeed superior to continuous single-agent treatment, what the exact impact of combined vs single agent treatment is on the different compartments (lymph node/spleen, blood, and marrow) and what mechanisms underlie resistance to combination treatment. In addition, the effects of targeted treatment on the skewed T-cell compartment observed in CLL is currently not well characterized. Interest in T-cell function is currently increasing because of the exploration of autologous T-cell–based treatment options.

The *TCL1* transgenic mouse model recapitulates many aspects of aggressive human CLL disease, including overexpression of

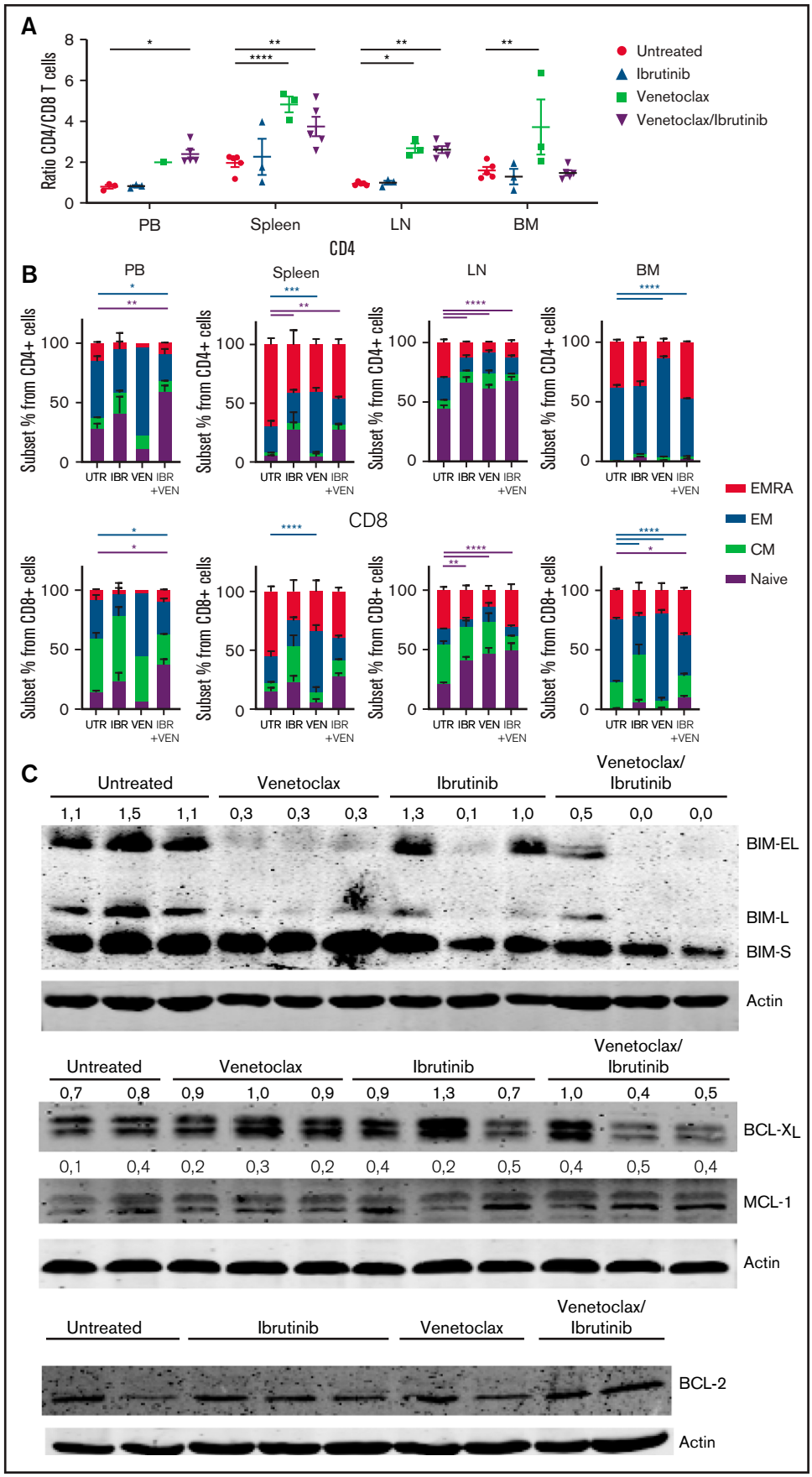
A
Ratio CD4/CD8 T cells
Untreated
Ibrutinib
Venetoclax
Venetoclax/Ibrutinib
PB
Spleen
LN
BM
CD4
B
PB
Spleen
LN
BM
Subset % from CD4+ cells
UTR IBR VEN IBR +VEN
CD8
Subset % from CD8+ cells
EMRA
EM
CM
Naive
C
Untreated
Venetoclax
Ibrutinib
Venetoclax/ Ibrutinib
1,1 1,5 1,1 0,3 0,3 0,3 1,3 0,1 1,0 0,5 0,0 0,0
BIM-EL
BIM-L
BIM-S
Actin
0,7 0,8 0,9 1,0 0,9 0,9 1,3 0,7 1,0 0,4 0,5
BCL-XL
0,1 0,4 0,2 0,3 0,2 0,4 0,2 0,5 0,4 0,5 0,4
MCL-1
Actin
Untreated
Ibrutinib
Venetoclax
Venetoclax/ Ibrutinib
BCL-2
Actin

Figure 2.

antiapoptotic BCL-2 family members, enlarged spleen sizes (as a proxy for LN disease, as in this model, spleens are heavily infiltrated in contrast to sparse infiltration of LN),[11] and shifts in T-cell subsets toward accumulation of pseudoexhausted effector memory cells.[12,13] We therefore used the *TCL1* model to study and compare single-agent ibrutinib or venetoclax treatment with combined treatment.

Methods

Immune-competent C57BL/6J mice received an adoptive transfer (AT) of *TCL1* splenocytes (20×10^6), obtained from full leukemic *TCL1* transgenic mice with the same genetic background. Three weeks after AT, or when CD5/CD19 reached >70% of all B cells, the mice were treated with cyclophosphamide (10 mg/kg; positive control), venetoclax (once daily 100 mg/kg, intragastric),[14,15] ibrutinib (0.16 mg/mL in drinking water, which is equivalent to 25 mg/kg per day and results in >90% inhibition of BTK),[16,17] or a combination of ibrutinib and venetoclax (n = 5 in all arms). The study was approved by the animal ethics committee of the Academic Medical Center, Amsterdam. For a table of antibodies and primer sequences, see the supplemental Tables 1 and 2.

Results and discussion

After AT, the mice developed overt leukemia, reflected by increased percentages of $CD5^+CD19^+$ cells (supplemental Figure 1A) and absolute white blood cell (WBC) counts (supplemental Figure 1B). The time to leukemia development varied per clone in all experiments, as previously described.[12]

Untreated mice experienced a rapid progression of disease, reaching a WBC count of $>2 \times 10^8$/mL and requiring euthanasia. The mice responded to treatment with cyclophosphamide, which indicates that the model is suitable for the study of therapy responses (supplemental Figure 1A-B). Within the first 5 days of treatment, mice receiving ibrutinib showed a rapid lymphocytosis (mean WBC counts at the start of treatment, 2.03×10^7/mL; peak mean at day 2 of treatment, 1.2×10^8/mL), followed by a reduction in WBCs (Figure 1A). Single-agent venetoclax as well as the combination treatment resulted in rapid reductions in WBCs (Figure 1A). The maximum drop in WBCs compared with baseline were as follows: ibrutinib 50% at week 6, venetoclax 70% at week 4, combination 91% at week 5 ($P < .001$). In contrast to absolute WBC counts, percentages of $CD5^+CD19^+$ cells remained >75% during single-agent treatment and dropped to ~50% only during combination treatment (Figure 1B). The mice receiving either monotherapy showed signs of relapse after 4 weeks of treatment, which was manifested by an increase in WBC counts, whereas those with the combination treatment did not relapse until week 9 (Figure 1A), resulting in significantly improved progression-free survival (Figure 1C). Using different *TCL1* clones, we found that development of leukemia after AT and the duration of the responses varied per donor, yet in all experiments, the deepest responses were observed with the combination treatment (supplemental Figure 1C-D).

Differences in responses per compartment were examined at the time of remission during single-agent and combination treatment at 4 weeks after initiation of treatment. Spleen sizes were reduced significantly in monotherapies (reduction: ibrutinib, 47%; venetoclax, 44%) and were even further reduced in mice that received combination therapy (percentage reduction, 84%; Figure 1D). Untreated mice displayed a disturbed splenic architecture with massively enlarged marginal zones and a loss of demarcation between B and T cells, which was partially restored only in combination treatment (supplemental Figure 1E). The percentage of infiltrating CLL cells in the spleen was significantly lower, only after combination treatment (45%; $P < .001$; Figure 1E). Proliferating splenocytes were reduced only in mice treated with ibrutinib (28%; Figure 1F), whereas cleaved caspase-3^+ B-lymphocyte levels, a marker for apoptosis, increased only in mice treated with venetoclax (Figure 1G).

Similar to human CLL, *TCL1* mice developed a skewed CD4/CD8 ratio (supplemental Figure 2A). In addition, percentages of effector memory cells increased at the expense of the naive compartment (supplemental Figure 2B).[18] Venetoclax or ibrutinib+venetoclax combination treatment resulted in normalization of the CD4/CD8 ratio in all compartments, which was not observed after single-agent ibrutinib (Figure 2A). Alterations in T-cell subsets were most prominent upon combination treatment with increased naive cells and reduced effector memory cells, in both $CD4^+$ and $CD8^+$ cells (Figure 2B). At relapse, the differences between different treatment arms were no longer apparent, and the subset distribution resembled untreated mice (supplemental Figure 2C).

Finally, expression levels of BCL-2, MCL-1, BCL-X_L, and BIM were compared upon disease relapse to understand whether shifts in these proteins could underlie development of disease resistance in our model. Although BCL-2, MCL-1, and BCL-X_L levels remained comparatively equal to those in untreated mice, disappearance of BIM-EL was observed in most of the mice that relapsed after single-agent venetoclax, as well as combination treatment (Figure 2C; supplemental Figure 2D). BIM-EL downregulation seemed to be partially transcriptionally regulated (supplemental Figure 2E), although the qPCR data did not correspond fully with the western blots.

These data support that the combination of venetoclax and ibrutinib has increased efficacy over single agents. Even in this short treatment time frame, there is a deeper and more durable response with the combination. Our model suggests that inhibition of proliferation by ibrutinib and increased cell death by venetoclax underlie the observed synergy. With the caveat that our studies were performed in an aggressive leukemia mouse model, the data suggest that combination treatment is not curative and results rather rapidly in outgrowth of apparently resistant cells. We have not been able to pinpoint a recurring mutation by whole-exome sequencing or RNA sequencing that might uniformly explain the resistance mechanism (data not shown). An intriguing possibility may be that BIM-EL expression is silenced, thereby interrupting an essential aspect of venetoclax action. Transcriptional/epigenetic or MAPK/Erk-related mechanisms that target

Figure 2. Combination of venetoclax and ibrutinib decreases T-cell compartment skewing and acts on BIM regulation in CLL cells from a *Tcl1* mouse model (A) Quantification of the ratio of CD4/CD8 T cells in the different arms of the experiment. Means of CD4/CD8 T-cell ratio ± standard error of the mean (SEM) are presented; significant differences were determined by a 2-way analysis of variance (ANOVA) with Tukey's multiple-comparisons test. *$P < .05$, **$P < .01$ ****$P < .0001$. (B) Percentages of phenotypes (naive, central memory, effector memory, and double negative) from $CD4^+$ and $CD8^+$ T cells based on CD44 and CD62L expression are presented as means ± SEM. Significant differences were determined by 2-way ANOVA with Tukey's multiple-comparisons test. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. (C) Western blot analysis of sorted *Tcl1* PBMC leukemia cells at time of relapse. Actin protein is presented as the loading control.

BIM as part of resistance pathways have been described in both solid cancers and acute lymphocytic leukemia.[19,20] It would be worthwhile to follow up this possibility in a samples of humans who have relapsed. In addition, normalization of the T-cell compartment specifically after venetoclax-based induction treatment, suggests that T-cell–based consolidation treatment may be effective and can be used to deepen responses and extend disease remission.

Acknowledgments

This work was supported by a research grant from AbbVie and Janssen. A.P.K. is funded by a Dutch Research Council grant (VIDI-91715337) and a European Research Council grant (2020 BOOT-CAMP). Whole-exome sequencing and RNA-sequencing were performed at the University of Heidelberg by Martina Seiffert and Mark Zapatka, funded by the ERA-NET on Translational Cancer Research (TRANSCAN 2) in support of the consortium Fighting Resistance in CLL (FIRE-CLL).

Authorship

Contribution: E.S. and G.C. performed the research, analyzed the data, and wrote the paper; A.W.M. performed the research; S.B. and J.D.L. codesigned the research and provided the drugs; and A.P.K. and E.E. designed the research and wrote the paper.

Conflict-of-interest disclosure: J.D.L. is an employee and shareholder of AbbVie; and S.B. is an employee and shareholder of Janssen. The remaining authors declare no competing financial interests.

ORCID profile: E.S., 0000-0002-4919-3419.

Correspondence: Arnon P. Kater, Department of Hematology, Amsterdam UMC, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; e-mail: a.p.kater@amsterdamumc.nl.

References

1. Burger JA, O'Brien S. Evolution of CLL treatment - from chemoimmunotherapy to targeted and individualized therapy. *Nat Rev Clin Oncol.* 2018;15(8):510-527.
2. Chen SS, Chang BY, Chang S, et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia.* 2016;30(4):833-843.
3. Slinger E, Thijssen R, Kater AP, Eldering E. Targeting antigen-independent proliferation in chronic lymphocytic leukemia through differential kinase inhibition. *Leukemia.* 2017;31(12):2601-2607.
4. Anderson MA, Deng J, Seymour JF, et al. The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. *Blood.* 2016;127(25):3215-3224.
5. Haselager MV, Kielbassa K, Ter Burg J, et al. Changes in Bcl-2 members after ibrutinib or venetoclax uncover functional hierarchy in determining resistance to venetoclax in CLL. *Blood.* 2020;136(25):2918-2926.
6. Thijssen R, Slinger E, Weller K, et al. Resistance to ABT-199 induced by microenvironmental signals in chronic lymphocytic leukemia can be counteracted by CD20 antibodies or kinase inhibitors. *Haematologica.* 2015;100(8):e302-e306.
7. Bojarczuk K, Sasi BK, Gobessi S, et al. BCR signaling inhibitors differ in their ability to overcome Mcl-1-mediated resistance of CLL B cells to ABT-199. *Blood.* 2016;127(25):3192-3201.
8. Jain N, Keating M, Thompson P, et al. Ibrutinib and venetoclax for first-line treatment of CLL. *N Engl J Med.* 2019;380(22):2095-2103.
9. Hillmen P, Rawstron AC, Brock K, et al. Ibrutinib plus venetoclax in relapsed/refractory chronic lymphocytic leukemia: The CLARITY Study [published correction appears in *J Clin Oncol.* 2019;37(30):2722-2729]. *J Clin Oncol.* 2019;37(30):2722-2729.
10. Niemann CU, Levin MD, Dubois J, et al. Venetoclax and ibrutinib for patients with relapsed/refractory chronic lymphocytic leukemia. *Blood.* 2021; 137(8):1117-1120.
11. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci USA.* 2002;99(10):6955-6960.
12. Bresin A, D'Abundo L, Narducci MG, et al. TCL1 transgenic mouse model as a tool for the study of therapeutic targets and microenvironment in human B-cell chronic lymphocytic leukemia. *Cell Death Dis.* 2016;7(1):e2071.
13. Dong S, Harrington BK, Hu EY, et al. PI3K p110δ inactivation antagonizes chronic lymphocytic leukemia and reverses T cell immune suppression. *J Clin Invest.* 2019;129(1):122-136.
14. Ackler S, Oleksijew A, Chen J, et al. Clearance of systemic hematologic tumors by venetoclax (Abt-199) and navitoclax. *Pharmacol Res Perspect.* 2015;3(5):e00178.
15. Pham LV, Huang S, Zhang H, et al. Strategic therapeutic targeting to overcome venetoclax resistance in aggressive B-cell lymphomas. *Clin Cancer Res.* 2018;24(16):3967-3980.
16. Hanna BS, Yazdanparast H, Demerdash Y, et al. Combining ibrutinib and checkpoint blockade improves CD8+ T-cell function and control of chronic lymphocytic leukemia in Em-TCL1 mice. *Haematologica.* 2021;106(4):968-977.
17. Ponader S, Chen SS, Buggy JJ, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood.* 2012;119(5):1182-1189.
18. Gorgun G, Ramsay AG, Holderried TA, et al. E(mu)-TCL1 mice represent a model for immunotherapeutic reversal of chronic lymphocytic leukemia-induced T-cell dysfunction. *Proc Natl Acad Sci USA.* 2009;106(15):6250-6255.
19. Sun C, Hobor S, Bertotti A, et al. Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3. *Cell Rep.* 2014;7(1):86-93.
20. Jing D, Huang Y, Liu X, et al. Lymphocyte-specific chromatin accessibility pre-determines glucocorticoid resistance in acute lymphoblastic leukemia. *Cancer Cell.* 2018;34(6):906-921.e8.