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Targeting BCL-2 as a Therapeutic Strategy for Primary *p210BCR-ABL1*-positive B-ALL Cells

MICHELE MASSIMINO[1,2*], ELENA TIRRÒ[1,2*], STEFANIA STELLA[1,2*], MARIA STELLA PENNISI[1,2], SILVIA RITA VITALE[1,2], ADRIANA PUMA[1,2], CHIARA ROMANO[1,2], SANDRA DI GREGORIO[1,2], MARIA ANNA ROMEO[3], FRANCESCO DI RAIMONDO[3] and LIVIA MANZELLA[1,2]

[1]*Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy;*
[2]*Center of Experimental Oncology and Hematology, A.O.U. Policlinico Vittorio Emanuele, Catania, Italy;*
[3]*Division of Hematology and Bone Marrow Transplant, A.O.U. Policlinico Vittorio Emanuele, Catania, Italy*

Abstract. *Background/Aim: Philadelphia-positive acute lymphoblastic leukemia (Ph$^+$ B-ALL) is caused by the malignant transformation of lymphoid cells induced by BCR-ABL1 constitutive catalytic activity. BCR-ABL1 tyrosine kinase inhibitors (TKIs) are effective against chronic myeloid leukemia (CML) cells, inducing durable hematological, cytogenetic and molecular responses. However, in Ph$^+$ B-ALL - as in CML progressing to blast crisis - TKIs fail to maintain disease remission. We, therefore, wanted to investigate if dual targeting of BCL-2 and BCR-ABL1 would be more effective in killing Ph$^+$ B-ALL cells. Materials and Methods: p210-B-ALL CD34-positive cells were used to evaluate the BCR-ABL expression and pharmacological targeting of BCL-2, by venetoclax, alone or in combination with BCR-ABL1 inhibition. Results: We demonstrated the cytotoxic effect of BCL-2 inhibition and that dual targeting of BCL-2 and BCR-ABL1 with venetoclax and nilotinib further increases this cytotoxicity. Conclusion: BCL-2 is a key survival factor for primary Ph$^+$ B-ALL cells and its inhibition - alone or in combination with a BCR-ABL1 TKI - should be further investigated as a potential therapeutic strategy for these patients.*

Approximately 30% of adult Acute Lymphoblastic Leukemia (ALL) patients display neoplastic blasts expressing the Philadelphia chromosome (Ph$^+$ ALL), *i.e.* the product of the reciprocal t(9;22) translocation which generates the *BCR-ABL1* chimeric oncogene (1). The ensuing BCR-ABL1 oncoprotein is characterized by constitutive tyrosine kinase activity that transforms hematopoietic stem cells altering multiple biological mechanisms involved in their survival and proliferation (2-4). Different *BCR-ABL1* fusion transcripts can be generated depending on the *BCR* and *ABL1* exons involved (5-8). More common *BCR-ABL1* isoforms include e1a2 (p190), e13a2 and e14a2 (both p210) and, while most ALL patients express p190 *BCR-ABL1* in B lymphoid precursors (9), previous data have shown that some of these patients may also express p210 *BCR-ABL1* or co-express p190 and p210 (10). Furthermore, while B-ALL is generally considered an aggressive disease, patients expressing *p210BCR-ABL1* usually display an inferior prognosis compared to those displaying the p190 isoform (10). The introduction of the first-generation (1G) tyrosine kinase inhibitor (TKI) imatinib (IM) (11), has radically improved the hematological, cytogenetic and molecular responses of *BCR-ABL1*-positive individuals. However, most individuals diagnosed with Ph$^+$ ALL ultimately develop IM resistance because of BCR-ABL1-dependent or -independent mechanisms (12-17). To overcome this resistance, both second- (nilotinib, dasatinib, bosutinib) (18-22) and third-generation (ponatinib) (23, 24) TKIs have been developed and introduced in clinical practice. However, as these drugs are unable to offer a complete disease remission (10), stem cell transplantation (SCT) still represents the golden-standard approach for Ph$^+$ ALL, with long-term survival rates ranging between 35% and 55%. Recently, allosteric inhibition of the BCR-ABL1 oncoprotein through the small-molecule asciminib (25) has led to eradication of CML xenograft tumors (26) when the compound was combined with nilotinib (NIL). However, to date, the efficacy of this approach on Ph$^+$ B-ALL blasts has not been investigated.

The BCL-2 family of proteins plays a critical role in mitochondrial-mediated apoptosis (27) and leukemic stem cell survival (28). Indeed, the activity of the BCL-2 anti-apoptotic protein is deregulated in *BCR-ABL1*-positive cells

*These Authors contributed equally to this study.

Correspondence to: Michele Massimino, Ph.D., Center of Experimental Oncology and Hematology, A.O.U. Policlinico - Vittorio Emanuele, *Via* Santa Sofia 78, 95123 Catania, Italy. Tel: +39 095 3781952, Fax: +39 095 3781949, e-mail: michedot@yahoo.it

Key Words: BCL-2, venetoclax, nilotinib, asciminib, CML, ALL.

Table I. *Patient characteristics at diagnosis.*

Diagnosis	B-ALL; Ph+ p210
Immunophenotype	
Blasts 40%	CD19+; CD22±; CD33+; CD34+; HLA-DR+; CD38+; TDT+; CD79a±; CD45±
Cytogenetic Analyses	
Karyotype	100% Ph+ metaphases 46, XX, t(9;22)(q34;q11)
Fusion Transcript	
BCR-ABL1	e14a2/e13a2

Immunophenotype of ^{p210}B-ALL blast crisis cells performed by flow cytometric analysis. Cytogenetic analysis shows the percentage of Ph+ metaphases. Fusion transcript indicates the coexistence of both e14a2 and e13a2 BCR-ABL isoforms.

showing a further increase in Ph+ blast cells (29). In CML stem/progenitor cells obtained from patients that have progressed to the acute phase of the disease, dual targeting of BCL-2 and ABL1 strongly reduces the number of vital leukemic blasts (29-31). Hence, we wanted to investigate the efficacy of a dual therapeutic approach combining BCL-2 (venetoclax) and BCR-ABL1 (asciminib and nilotinib) targeted therapies on Ph+ B-ALL blasts. We found that BCL-2, but not BCR-ABL1, inhibition is critical for the survival of Ph+ B-ALL leukemic cells.

Materials and Methods

RNA extraction and multiplex RT-PCR. White blood cells (WBCs) derived from peripheral blood (PB) were used to perform RNA isolation and multiplex RT-PCR, as previously reported (32).

Isolation and expansion of CD34-positive cells. CD34-positive cells were immuno-magnetically separated from the bone marrow employing the CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (33). CD34+ progenitors were then grown in StemSpan Serum-Free Expansion medium (SFEM) supplemented with recombinant human (rh) cytokines at low concentrations, *i.e.* Flt-3 ligand 5 ng/ml; rh stem cell factor 5 ng/ml; rh interleukin-3 1 ng/ml and rh interleukin-6 1 ng/ml (all from Stem Cell Technologies, Köln, Germany).

Trypan blue exclusion, colony forming unit (CFU) assays, apoptosis assessment and drug treatment. A total of 300×10^3/ml CD34-positive cells were left untreated or exposed to NIL (2 μM) (34), ASC (1.2 μM) (35) (all provided by Novartis) and VEN (400 nM) (Selleckchem, Munich, Germany) (36). Alternatively, cells received a combination of NIL plus VEN or ASC plus VEN for 24 hours. At this time, 10 μl of the cell culture were diluted in 10 μl of 0.4% Trypan Blue solution mixed and counted in a hemocytometer to determine their proliferation rate, while CFU assay and apoptosis assessment were performed as previously published (37, 38).

Statistical analysis. Statistical significance was calculated using the Prism Software version 6.0 applying the unpaired *t*-test. Results were considered statistically significant at a $p<0.05$.

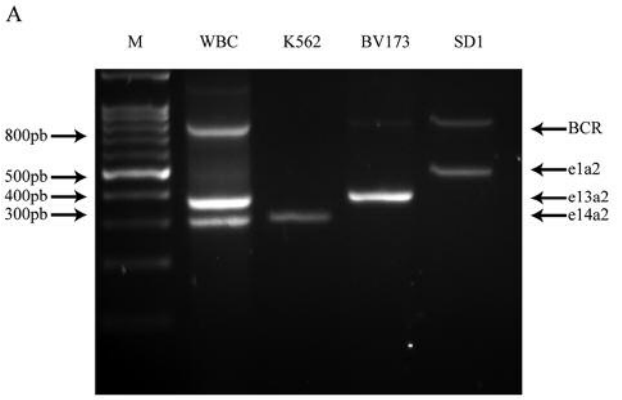

Figure 1. *Co-expression of e13a2 and e14a2 BCR-ABL1 fusion transcripts in Ph+ B-ALL cells. Results of a multiplex RT-PCR performed using total RNA extracted from WBC of a Ph+ B-ALL patient and from K562, BV173 and SD1 were used as controls.*

Results

Co-expression of e13a2 and e14a2 BCR-ABL1 fusion transcripts in Ph+ B-ALL primary cells. Flow cytometry and cytogenetic analyses performed at diagnosis suggested the presence, at diagnosis, of BCR-ABL1-positive B-ALL blasts (Table I). To confirm this finding, we performed a multiplex RT-PCR using mRNAs extracted from SD1 (e1a2 *BCR-ABL1*), BV-173 (e13a2 *BCR-ABL1*) and K562 (e14a2 *BCR-ABL1*) cell lines as reaction controls. We detected co-expression of e13a2 and e14a2 *BCR-ABL1* fusions in the patient's blasts (Figure 1).

Venetoclax - alone or in combination with nilotinib reduces the proliferation and clonogenesis of primitive Ph+ B-ALL cells. To investigate the cytotoxic effect of individual and combined targeting of BCL-2 and BCR-ABL1, we exposed Ph+ primitive blasts to venetoclax (VEN), nilotinib (NIL) or asciminib (ASC) or to VEN combined with NIL or ASC, respectively. Initially, we evaluated the effect of this therapeutic approach on proliferation of Ph+B-ALL^{p210} primitive blast cells. We found that VEN – alone 82% ($p<0.01$) or combined with NIL 95% ($p<0.01$) – significantly reduced cell proliferation compared to the untreated control (Figure 2A). We next investigated the effect of the two-drug combinations and observed that, while VEN decreased clonogenesis (68.5%, $p<0.01$) compared to NIL or ASC alone, only co-treatment with NIL further enhanced this cytotoxic effect inducing a CFU reduction of 93.45% ($p<0.001$) (Figure 2B).

Ph+B-ALL^{p210} primitive blast cells are dependent on BCL-2 for their survival. We wanted to investigate if the reduction in proliferation and clonogenesis induced by VEN, alone or in combination with NIL, was dependent on higher apoptotic

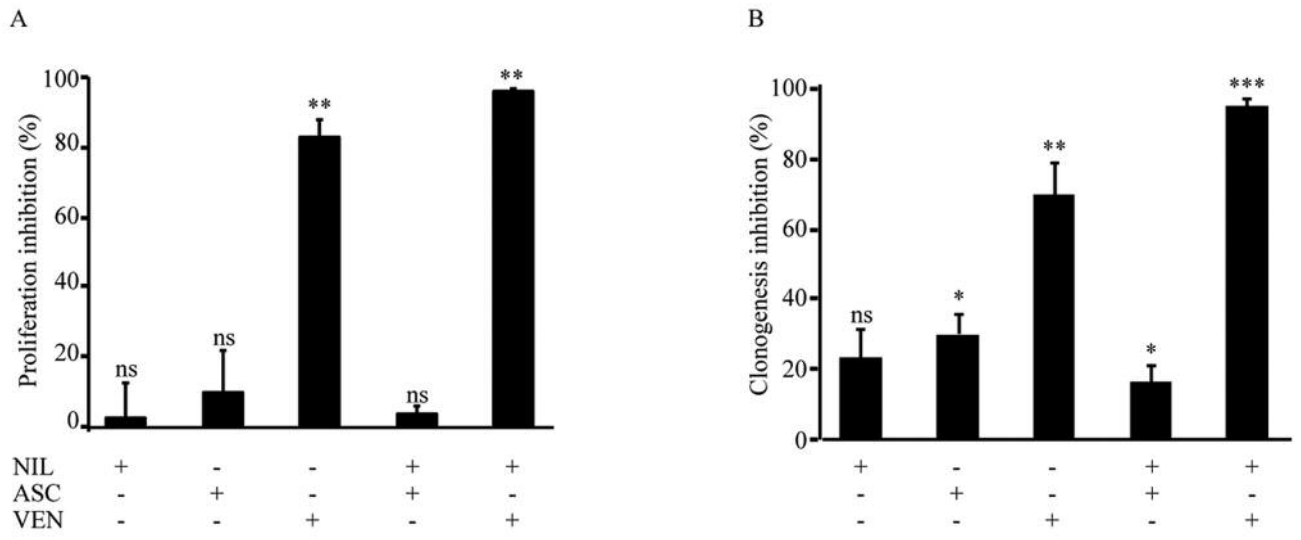

Figure 2. *Pharmacological targeting of BCL-2 - alone or in combination with BCR-ABL1 TKIs reduces the proliferation and clonogenesis of Ph+ B-ALL blasts. Histograms depict the proliferation (A) and clonogenesis (B) of Ph+ B-ALL cells exposed to the specified treatment. Values were obtained by arbitrarily setting the untreated condition at zero. Bars indicate the standard deviation of two independent experiments. Unpaired t-tests were used to calculate statistical significance using the Prism Software (*p<0.05, **p<0.01, ***p<0.001). p-Values were obtained comparing each condition to untreated cells.*

rates. To this end, we exposed Ph+ B-ALL primitive blasts to these compounds and stained the cells with Annexin V and 7-AAD. We found that single-agent VEN induced a massive (95.7%) increase in apoptosis (Figure 3A and B). When we combined VEN with NIL we observed similar cell death rates (96.5%), indicating that BCL-2 inhibition, but not suppression of BCR-ABL1 kinase activity is critical for the survival of Ph+ ALL blasts.

Discussion

The concept of precision medicine postulates that effective treatment strategies should be tailored to the individual variability of both the patient and his disease. Indeed, in several neoplastic diseases targeted therapy has increased survival compared to conventional strategies (25, 39-45). Several FDA-approved TKIs are available for the treatment of CML and Ph+ B-ALL. However, an ever-increasing number of these patients exhibit TKI resistance requiring alternative therapeutic approaches (46). We found that VEN-mediated BCL-2 inhibition reduces both the proliferation and clonogenic potential of Ph+ B-ALL-naïve cells and that these effects are increased when the drug is combined with NIL. We also found that ABL-directed inhibition by NIL or ASC is unable to generate the same effect.

Two previous studies have evaluated the efficacy of BCL-2 inhibition in TKI-resistant *BCR-ABL1*-positive cells derived from the blast crisis of CML patients previously in chronic phase. Both reports demonstrated that single agent VEN displayed modest activity but could overcome TKI resistance if associated with a BCR-ABL1-selective inhibitor (31, 36). As our findings significantly differ from those of Ko *et al*. and Bing and colleagues, we hypothesize that while morphologically and phenotypically similar, CML-derived blast crisis and Ph+ B-ALL are two biologically distinct diseases. Of course, selection of TKI-insensitive clones is likely to further increase these differences.

In summary, we report that BCL-2 selective inhibition displays a strong anti-leukemic activity in Ph+ B-ALL p210 and that this effect is increased by NIL. Our data support additional evaluation of combined BCL-2 and BCR-ABL1 pharmacological inhibition for the treatment of patients with this disease.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

MM drafted the manuscript; MM, ET and SS were responsible of study concept, designed and performed the experiments; MM, ET, SS, MSP, SRV, AP, CR, SDG analyzed and interpreted the data; MAR made a critical revision of the paper and managed the patient; FDR supervised the project; LM conceived the original idea and supervised the project. All Authors have read and approved the manuscript to be published.

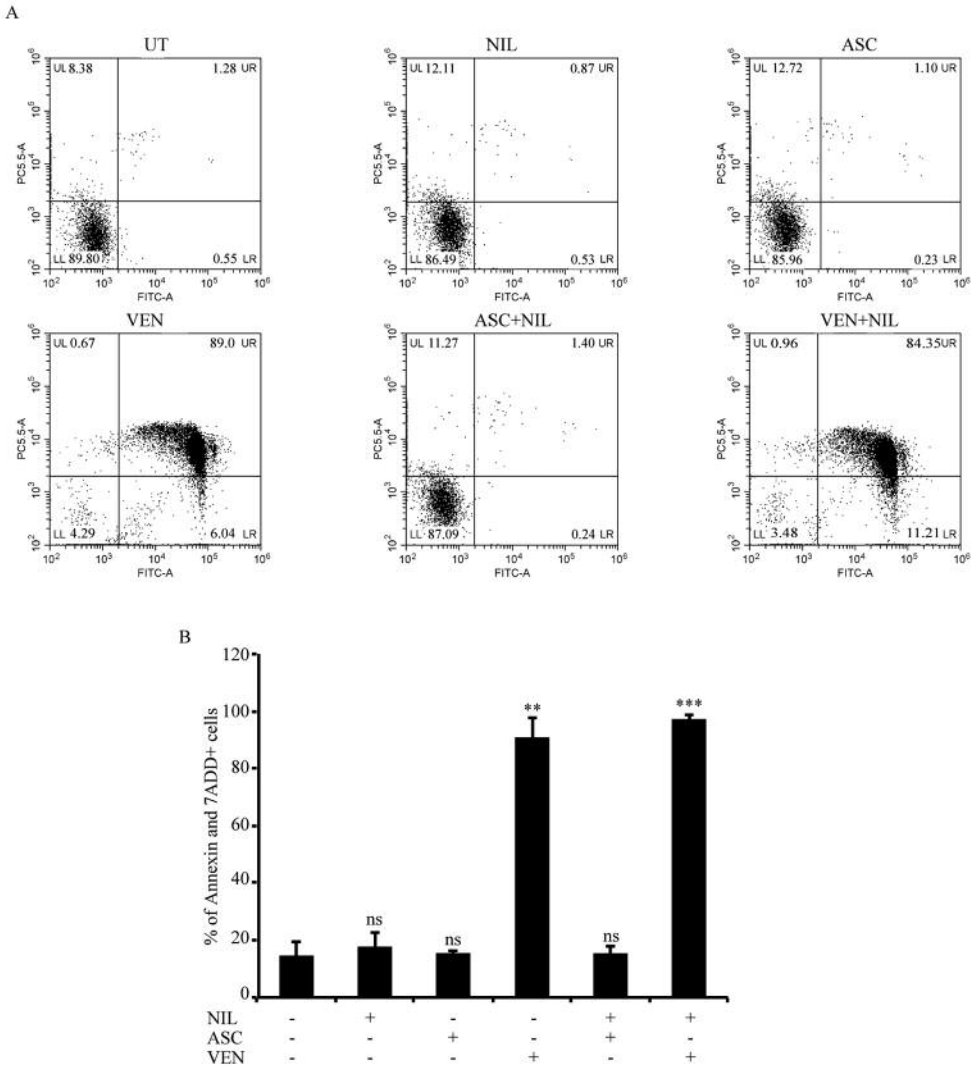

Figure 3. *BCL-2 is a critical regulator of Ph+ B-ALL survival. (A) Dot plots indicate a representative experiment displaying apoptotic rates detected in Ph+ B-ALL cells. The indicated percentages refer to the distribution of necrotic (upper left), early (lower right) and late (upper right) apoptotic cells in the untreated condition or after exposure to the specified drugs. (B) Histograms representing average Annexin V- and 7AAD-positive cells left untreated or exposed to the reported drugs. Bars indicate standard deviations from two independent experiments. The unpaired t-test was used to calculate statistical significance as previously indicated. p-Values were obtained comparing each condition to untreated cells.*

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