Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia

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Abstract

The BCL2 inhibitor, venetoclax, induces high rates of durable remission in patients with previously treated chronic lymphocytic leukemia (CLL). However, despite continuous daily treatment, leukemia recurs in most patients. To investigate the mechanisms of secondary resistance, we analyzed paired pre-venetoclax and progression samples from 15 patients with CLL progression enrolled on venetoclax clinical trials. The novel Gly101Val mutation in BCL2 was identified at progression in seven patients, but not at study entry. It was first detectable after 19-42 months of therapy, and its emergence anticipated clinical disease progression by many months. Gly101Val reduces the affinity of BCL2 for venetoclax by ~180-fold in surface plasmon resonance assays, thereby preventing the drug from displacing pro-apoptotic mediators from BCL2 in cells and conferring acquired resistance in cell lines and primary patient cells. This mutation provides new insights into the pathobiology of venetoclax resistance and provides a potential biomarker of impending clinical relapse.

Statement of significance

Why chronic lymphocytic leukemia recurs in patients who achieve remission with the BCL2 inhibitor venetoclax has been unknown. We provide the first description of an acquired point mutation in BCL2 arising recurrently and exclusively in venetoclax-treated patients. The mutation reduces venetoclax binding and is sufficient to confer resistance.
Introduction

Venetoclax is a potent and highly selective BCL2 inhibitor(1) approved for treatment of patients with previously treated chronic lymphocytic leukemia (CLL), as monotherapy(2-5) or in combination with rituximab(6,7). Venetoclax is taken continuously once daily until disease progression. Clinical responses are rapid with ~80% of patients achieving substantial cytoreduction(2,3). Complete remissions are seen in ~20% of patients with monotherapy and 20%-50% when combined with rituximab(2,3,6-8). Further, a minority achieve remissions which are measurable residual disease [MRD]-negative by highly sensitive multi-color flow cytometry (MFC)(7,9). However, disease relapse ultimately occurs in the majority of patients who were heavily pre-treated prior to commencing venetoclax. The median response duration in patients with deletion 17p CLL receiving venetoclax monotherapy is 33.2 months, highlighting the significant challenge secondary resistance poses(9).

Disease progression on venetoclax may manifest as transformation to diffuse large B-cell lymphoma (Richter transformation [RT]) in a small proportion of patients, but usually presents as a recurrence of CLL (CLL-type progression) without morphological or immunophenotypic change(10,11). The underlying biological mechanisms leading to CLL progression in venetoclax-treated patients remain largely unknown. Acquired abnormalities in BTG1, TP53, CDKN2A/B, SF3B1 and BRAF have been reported in a small cohort of eight patients with deletion 17p CLL progressing on venetoclax, four with RT(12). However, a causal relationship has yet to be established, particularly for lesions which are common in CLL (e.g. TP53 and SF3B1), and the mechanisms that result in CLL-type progressions on venetoclax therapy may well be distinct from those emerging as RT.

Venetoclax inhibits BCL2’s function by binding its critical hydrophobic groove, the same site which sequesters its physiological ligands, BH3-domain containing pro-apoptotic proteins(13). If unconstrained by BCL2, these BH3-domain proteins (e.g. BIM, BAX, BAK) are free to drive apoptosis. Acquired mutations in the BH3-binding groove of BCL2 have been reported in a murine cell line
where venetoclax-refractoriness was induced through continuous drug exposure (Bcl2 Phe101Cys and Phe101Leu, analogous to human BCL2 Phe104Cys and Phe104Leu)(14). However, BCL2 mutations have yet to be described in patients treated with venetoclax.

Using a cohort of 67 patients(10) with relapsed CLL treated with venetoclax on three early phase clinical trials, we have performed a focussed genomic evaluation in those with CLL-type progressions specifically excluding those with RT (where genomic heterogeneity is well-recognized). Among the CLL-progressions on venetoclax, we have discovered and functionally characterized a recurrent novel BCL2 mutation (NM_000633.2:c.302G>T, p.(Gly101Val)) present at disease progression but not at treatment initiation. Gly101Val markedly reduces the affinity of venetoclax for BCL2 and confers acquired resistance in vitro and in vivo in patients with CLL.

Results

Twenty-one patients treated with venetoclax in the study cohort(10) have experienced CLL-type progression after achieving an initial response, while 18 developed RT. CLL-type progression manifested clinically as a gradual increase in morphological CLL burden over months to years, typically after a period when subclinical disease was initially detected in the bone marrow (and subsequently blood) by MFC. The median time on venetoclax to CLL-type clinical progression in this cohort was 36 (range 6.5-73) months.

Genomic assessment of patient samples pre- and post-venetoclax treatment

Fifteen of 21 patients with CLL-type progression had suitable pre-venetoclax and progression specimens available for analysis. Targeted amplicon next generation sequencing (NGS) was performed on paired samples assessing the entire coding region of BCL2. In four patients (CLL2, CLL3, CLL5 and CLL12), a single nucleotide variant was detected in BCL2 (NM_000633.2:c.302G>T, p.(Gly101Val)) in samples at, or following, CLL-type progression (Figure 1A,B). This variant was undetectable in paired pre-venetoclax samples. Digital droplet PCR (ddPCR) was used to characterize
the emergence of the mutation in serial archived samples from these patients (Figure 1C) alongside bone marrow disease burden measured by MFC. Gly101Val was first detected at low variant allele frequency (VAF) after 19-42 months on venetoclax, up to 25 months earlier than when standard disease progression criteria were met.

CD19⁺-selected tumor cells of 92.8% purity collected at CLL progression from CLL3 had a Gly101Val VAF of 32.49% by ddPCR. No copy number loss or acquired uniparental disomy at the BCL2 locus was observed (Supplementary Figure 1A). This is consistent with Gly101Val being present in the heterozygous state in the majority (~70%) of CLL cells in this patient at the time of progression. Expression of the Gly101Val mutation was confirmed in this patient by RNA-sequencing (Supplementary Figure 1B). Using sample CLL burden (determined by MFC) and ddPCR VAF we estimated the proportion of CLL cells carrying Gly101Val in patients CLL2, CLL5 and CLL12 (assuming heterozygosity) to be approximately 26%, 46% and 60%, respectively (Supplementary Table 1).

The observation of subclonality and detection of Gly101Val in pre-progression samples at frequencies lower than detectable by targeted amplicon NGS led us to test whether it could be detected in other CLL-type progressors using ddPCR. Gly101Val was detected in three other patients (CLL6, CLL8, CLL14) at low VAF (0.01%-0.4%) in post-venetoclax samples (Supplementary Table 1). The CLL disease burden assessed by MFC in these post-venetoclax samples ranged from 3.3% to 57.5% of leukocytes. Therefore, the estimated proportion of CLL cells carrying the Gly101Val mutation in these patients at progression was 1.4%-4.3%. Importantly, Gly101Val was not detected in paired pre-venetoclax samples from any of the seven Gly101Val mutated patients using ddPCR (limit of detection 0.01%). After cessation of venetoclax, a stable proportion of CLL cells bearing Gly101Val persisted for at least 6 months in five patients who received BTK inhibitor therapy (Supplementary Figure 2).

Targeted amplicon NGS of 32 other genes commonly mutated in CLL and lymphoid malignancy was also performed on pre- and post-progression samples (Supplementary Table 1). In 8/15 patients
with CLL-type progressions, different sequence variants were detected (not including \textit{BCL2} Gly101Val) at progression compared to baseline. Five had \textit{TP53} variants at baseline which were not detected at CLL-type progression, three had new \textit{TP53} variants at progression; variants in \textit{NOTCH1}, \textit{KRAS} and \textit{BIRC3} were lost in single patients at progression and there were acquisitions of \textit{SF3B1} or \textit{KRAS} variants in single patients.

\textit{BCL2} Gly101Val was not found in searches of either general population or cancer patient public databases (\textit{Figure 1D}). Importantly, review of \textit{BCL2} sequencing data from 96 patients with venetoclax-naïve CLL (63 untreated, 33 with relapsed/refractory disease; 23 with previous fludarabine-based treatment) did not reveal any cases bearing the Gly101Val mutation (\textit{Figure 1D}), and it was not observed in the limited number of biopsies analyzed from patients who progressed with RT (\(n=5\)). Further, Gly101Val was not detected in targeted amplicon sequencing data from 301 patients with other B-cell malignancies. Nor did we detect any cases with the \textit{BCL2} Phe104Leu mutation analogous to that reported to confer venetoclax resistance in murine cell lines(14). Given the observation of acquisition of Gly101Val only in the setting of CLL-type progression on venetoclax treatment, we hypothesized that this variant contributes to venetoclax resistance.

\textit{Patient CLL cells with Gly101Val at progression and Gly101Val expressing cell lines are intrinsically less sensitive to venetoclax}

Freshly collected CLL cells from progression on venetoclax for the four patients with significant proportions of leukemic cells harbouring Gly101Val were much less sensitive to venetoclax killing \textit{in vitro} than their CLL cells collected at study entry (\textit{Figure 2A}). A similar acquisition of reduced sensitivity was observed when the cells were exposed to ABT-737, a dual BCL2/BCL-xL inhibitor with a similar chemical scaffold(15) (\textit{Supplementary Figure 3}).

To determine whether the Gly101Val mutation alone is sufficient to confer resistance to venetoclax, the mutant was overexpressed in two human B-lineage cell lines. RS4;11 Gly101Val cells were approximately 30-fold less sensitive to venetoclax than RS4;11 wild-type (WT) BCL2 cells (\textit{Figure C}).
and similar results were observed using KMS-12-PE (Figure 2C,D). The Phe104Leu mutation also induced similar resistance in these cell lines (Figure 2B,C).

**BCL2 pro-survival function is maintained despite the Gly101Val mutation**

We next tested whether the pro-survival function of BCL2 was impacted more generally by the Gly101Val mutation. High levels of BCL2 are associated with resistance to DNA-damaging cytotoxics and other anti-cancer drugs, as demonstrated when WT BCL2 was overexpressed in RS4;11 cells (Figure 2E). Gly101Val similarly reduced *in vitro* sensitivity to each of etoposide, cytarabine, fludarabine and dexamethasone, as did Phe104Leu (Figure 2E). Following the same pattern, Gly101Val overexpression was as effective as WT BCL2 overexpression in KMS-12-PE cells at protecting against killing by bortezomib (Supplementary Figure 3B). Thus, the normal function of BCL2 to block apoptosis is preserved despite the Gly101Val mutation.

**Venetoclax binding to BCL2 is markedly reduced by the Gly101Val mutation**

Our functional studies in cell lines and the detection of the Gly101Val mutation exclusively in venetoclax-treated patients developing progressive CLL, imply that the effect of this mutation is likely to be highly specific for the inhibition of BCL2 by venetoclax. To test this, we examined how the Gly101Val mutation impacts upon the ability of BCL2 to bind either venetoclax or its physiological ligands, the key pro-apoptotic proteins such as BIM, BAX and BAK.

The Gly101Val mutation did not significantly impact the ability of BCL2 to bind a BIMBH3 peptide (Figure 3A,B), consistent with our findings that the mutant broadly retains wild-type pro-survival activity (Figure 2E). However, we saw a striking difference when we assessed the ability of venetoclax to compete with BIM for BCL2 binding (Figure 3D,E). Competition surface plasmon resonance (SPR) experiments showed venetoclax binds avidly to WT BCL2, preventing BIM binding. This is markedly reduced (~180-fold) with the Gly101Val mutant, as indicated by the flattening of the sigmoidal curves towards the hyperbola in the case of the mutation compared to WT protein (Figure 3E). A modest reduction in BAXBH3 binding was also observed (Figure 3A,C). Although the
magnitudes varied, similar trends were observed with the Phe104Leu mutant. Given that the principal impact of these mutations is reduction in venetoclax binding to BCL2, we predicted that venetoclax would be less able to displace pro-apoptotic molecules from BCL2 Gly101Val in cells.

To investigate this, we assessed the ability of venetoclax to free pro-apoptotic BIM, BAX or BAK already bound to BCL2 in cells. BIM binding to BCL2 (both WT and mutant) was very tight and was largely unaffected by addition of venetoclax (Figure 4A, Supplementary Figure 4). In contrast, venetoclax could free BAX and BAK from WT BCL2, but was ineffective when these pro-apoptotic molecules were bound to the Gly101Val or the Phe104Leu mutants. Thus, in the biochemical and cellular assays, we found venetoclax to be less effective against the Gly101Val mutant and this probably accounts for the reduced sensitivity of the cells to venetoclax. Interestingly, we also observed increased binding of BAK to the Gly101Val mutant, and reduced BIM and BAX binding in cells. Potentially, the impact of the mutation on the propensity of BCL2 to bind the various BH3-domain containing pro-apoptotic proteins might also contribute to the resistance observed.

The Gly101Val mutation confers a selective advantage during continuous exposure to sublethal concentrations of venetoclax

As our biochemical and short-term cellular studies highlight how the Gly101Val mutation impairs the sensitivity of BCL2 to venetoclax, we next explored the consequences of continuous exposure to venetoclax (as used clinically) in long term cultures. In the absence of venetoclax, the WT and Gly101Val expressing cells grew at similar rates. However, in the presence of a sub-lethal dose of venetoclax, the latter out-competed WT cells. This occurred rapidly in KMS-PE-12 cells and more slowly in RS4;11 (Figure 4B), reflecting the 25% shorter doubling time and greater sensitivity to venetoclax of parental KMS-PE-12 cells compared to RS4;11.

We next sought to understand how the presence of the Gly101Val mutation might interact with exogenous factors that also protect CLL cells from killing by venetoclax in vivo. Using an established model of the CLL microenvironment(16), we co-cultured CLL cells from progression on CD40 ligand-
expressing stromal cells. For comparison, we co-cultured CLL cells with exclusively WT BCL2 from three venetoclax-naïve patients. As shown in Figure 4C, freshly collected CLL3 cells were substantially less sensitive than venetoclax-naive CLL cells prior to co-culture. After one week on stroma, venetoclax-naive CLL cells were less sensitive to venetoclax, displaying similar sensitivity to fresh CLL3 cells prior to co-culture. With one week of co-culture, cells from CLL3, CLL2 and CLL5 became markedly resistant to venetoclax, even at concentrations between 1-10µM, the latter higher than achievable clinically.

Additional disease resistance mechanisms can co-exist with the Gly101Val mutation

Unlike cell line cultures, the patient samples showed significant subclonality for the Gly101Val mutation (e.g. 70% of malignant cells in CLL3, 25% in CLL2). Cytotoxicity experiments in mixed populations of BCL2 WT and BCL2 mutant cell lines confirmed that the presence of subclones can shift the observed LC50 of the whole population (Supplementary Figure 5B) by similar extents to that observed between study entry and progression for three patients (CLL3, CLL5, CLL12; Figure 2A). However, for CLL2, where the Gly101Val mutation-containing subclone comprised only 25% of the resistant leukemic population, the LC50 shift was ~450-fold, markedly higher than observed in either the cell lines or the other three patients. We investigated pro-survival BCL2 family expression in CLL-progression cells from this patient by mass cytometry and observed bimodal expression of another pro-survival protein, BCLxL, not observed in the other patient samples (Figure 4D, Supplementary Figure 6). Cells with high BCLxL expression had much lower BCL2 expression than in either the BCLxL-low subpopulation or other CLL samples. When cells from CLL2 were flow-sorted based on BCLxL expression, the Gly101Val mutation was almost exclusively confined to the population with low BCLxL expression, indicating that distinct mechanisms of resistance were operating in these two populations (Figure 4E). Notably, CLL2 was observed to be more sensitive to a BCLxL-specific inhibitor (A-1331852(17)) than other CLL samples. Modelling of the data was consistent with the presence of two subpopulations, one sensitive to BCLxL inhibition and the other
insensitive (Figure 4F). Mass cytometry did not identify significant alterations in MCL1 or BCLX-L expression in CLL cells in the other patient samples tested (CLL3, CLL5, CLL12).

Discussion

We have identified and functionally characterized a novel recurrent BCL2 mutation (Gly101Val) emerging in a cohort of patients with CLL-type progressions treated with venetoclax. Our data demonstrate that the Gly101Val mutation impairs binding of venetoclax to BCL2, confers resistance to venetoclax in both patient leukemia cells and engineered cell lines and provides a selective growth advantage over wild-type cells when maintained in the presence of the drug in vitro.

CLL cells are universally addicted to the high levels of BCL2 which keep pro-apoptotic relatives such as BIM, BAX and BAK in check by direct sequestration through its BH3-binding groove, thereby permitting their inappropriate survival(13,18). By binding BCL2 tightly in the same groove, venetoclax effectively displaces these pro-apoptotic proteins, freeing them to trigger apoptosis of the CLL cells in vitro and in vivo(1,19). Through competitive binding experiments we showed that venetoclax has markedly decreased binding (~180-fold) to Gly101Val, thereby compromising its ability to displace pro-apoptotic proteins such as BAX (Figure 4A) and hence, the induction of apoptosis (Figures 2A-C; Figure 4G). Since BCL2 binding to pro-apoptotic BIM and BAX was only marginally compromised, the ability of the Gly101Val mutant to function normally to protect cells from apoptosis in the absence of venetoclax was unaffected (Figure 2E). Further investigation is required to formally establish whether Gly101Val has any modest gain or loss of function when assessed under different physiological or pathological circumstances.

Thus, the Gly101Val mutation specifically impacts on the action of venetoclax (Figure 4E) and compounds with a similar chemical scaffold, such as ABT-737 and navitoclax(13) (Figures 2B,C; Supplementary Figure 3A). Surprisingly, venetoclax does not directly contact Gly101, at least in the
available structure of a venetoclax analog bound to BCL214 (Figure 1B). This residue is highly conserved among BCL2 pro-survival family members, and the binding groove is malleable such that it can accommodate multiple ligands including a variety of BH3 domains and BH3-mimetic drugs(15,20). We speculate that the mutation must indirectly impact upon the binding groove to selectively impair venetoclax binding. Gly101Val represents an early example of a mutation that induces resistance to a targeted therapy by modifying a protein:protein interaction site, rather than an enzyme active site (e.g. mutations in BCR-ABL1(21) and BTK(22)).

Whilst our experimental data indicate that the Gly101Val mutation is sufficient to confer resistance to venetoclax and outgrowth of mutation-bearing clones in the presence of continuous venetoclax exposure in vitro and in patients, our data also indicate that other mechanisms for development of resistance are clinically important. The wide range of subclonality of Gly101Val in our cohort (1.4%-70%) suggests that other acquired changes must confer resistance in non-BCL2 mutation-bearing subclones. Further research is required to identify these alternative mechanisms. However, in one patient with cryopreserved cells available for analysis, we were able to demonstrate increased BCLxL expression in a major subclone without the Gly101Val mutation. Increased BCLxL expression has been described recently as a mechanism of resistance to venetoclax in mantle cell lymphoma(23,24) and appears to be a major contributor to resistance in patient CLL2. Whether changes in expression of BCLxL or other BCL2 family proteins are common in CLL progression on venetoclax remains to be established. Of broad relevance, we also demonstrated that exogenous microenvironmental support amplified the intrinsic resistance of Gly101Val mutation-bearing CLL cells in all samples available for testing. This combined effect probably explains the scenario observed in our patients where BCL2-mutant clones with LC50s of 100nM-1µM accumulated despite ongoing exposure to the typical low micromolar (approximately 1-3µM) steady-state concentrations of venetoclax observed in vivo with continuous therapy(2).
Like others(12), we observed genomic changes between treatment commencement and progression, particularly acquisition and loss of TP53 variants, but no compelling candidates that could directly account for resistance to venetoclax. We have previously shown that venetoclax-mediated CLL cell death in vitro is independent of TP53 mutation and function(19). Most likely the association of TP53 aberrations with venetoclax resistance is functionally indirect, reflecting the underlying genomic instability in CLL cells from this heavily pre-treated, relapsed/refractory cohort of patients(10). Transcriptomic or epigenomic profiling of suitable clinical specimens when they become available may assist in the identification of additional causes of venetoclax resistance, as may broader genomic profiling with whole exome and genome sequencing. Whilst further research is required into the development of RT as an avenue to clinical resistance to venetoclax, we have not observed BCL2 mutations to be contributory. Reassuringly, recent clinical data suggest that RT is infrequently observed on venetoclax in less heavily pre-treated patients(7).

Our data support the concept that venetoclax resistance in CLL-type progression is a heterogenous phenomenon, both between and within individual patients. As we have observed disease progression in every case where Gly101Val was found and that this mutation could be detected before overt clinical relapse in patients through sensitive allele specific ddPCR, this discovery may have clinical application. Detection of the Gly101Val could potentially serve as an early biomarker of impending disease progression allowing early therapeutic intervention such as the addition of another drug with a different mechanism of action.

Methods (see also Supplementary Materials for additional details)

Clinical cohort

The patient cohort of the first 67 consecutive patients with relapsed CLL/SLL treated with venetoclax on clinical trials at our two institutions has been previously described(10). All provided written informed consent and the studies were conducted in accordance with the Declaration of Helsinki and after Human Research Ethics Committee approval. Patients were enrolled June 2011-March
2015, and were reviewed at a minimum every three months until progression, death or discontinuation from trial. Outcome data were updated to June 1st 2018.

**Next generation sequencing**

Sequences of targeted regions within 33 genes (listed in **Supplementary Methods**) were analyzed. Indexed, amplicon-based libraries were prepared using Access Array methodology (Fluidigm, South San Francisco, CA, USA) and sequenced to a depth of ~1000x on a MiSeq instrument using v2 chemistry (Illumina, San Diego, CA, USA). Alignment, variant calling and annotation were performed using an in-house pipeline. Variants were evaluated using multiple functional and quality filters to identify likely pathogenic variants. Sanger sequencing was used to confirm selected variants. Sample CLL3 was sequenced with a hybridization-based (Agilent SureSelect) NGS panel targeting 363 genes with mean target read depth ~600x, as described previously (25). Whole transcriptome sequencing was performed using TruSeq RNA Library Prep Kit v2 on patient CLL3 using RNA extracted from CD19+ selected tumor cells.

**Digital droplet PCR**

A ddPCR assay to detect the BCL2 NM_000633.2:c.302G>T, p.(Gly101Val) variant using forward and reverse oligonucleotide primers with locked nucleic acid probes against wild-type and mutant sequence was designed. PCR reactions were carried out with 1x ddPCR Supermix for Probes (no dUTP), 2µL of 360 GC enhancer (Applied Biosystems), primers (900nM), probes (250nM) and 50ng of DNA template to a final volume of 25µL. PCR cycling conditions were activation (95°C, 10 minutes), 40 cycles of denaturation (94°C, 30 seconds), annealing/extension (60°C, 1 minute) and enzyme deactivation (98°C, 10 minutes). All steps had a ramp rate of 2°C/sec. Droplets were generated using the Automatic Droplet generator QX200 AutoDG (Bio-Rad) and read on the QX200TM Droplet Reader with data acquired and analyzed by QuantaSoft™ software (Bio-Rad).

**Cell biology**

**Cell Lines**
KMS-12-PE (sourced from DSMZ in 2013; Cat#ACC606) and RS4;11 (sourced from ATCC in 2014; Cat#CRL-1873) were cultured with HTRPMI/10% fetal bovine serum. Early passages (P5-P7) after purchase were cryopreserved and thawed for the experiments. Cells beyond passage 15 were not used. Monthly tests for mycoplasma were consistently negative (Mycoplasma detection kit; Lonza, GA, USA). In vitro killing assays (19) and co-culture (16) were performed as previously described, with minor adjustments (see Supplementary Materials).

Plasmids, retrovirus production and infection

WT BCL2 construct was reported previously (26); the mutants were introduced by PCR using primers with the desired mutation. cDNAs encoding either WT or mutant FLAG-tagged BCL2 were inserted into the MSCV-IRES-hygromycin retroviral construct; retroviral production and generation of cell lines performed as previously described (27).

Cell viability assays

Sensitivity of the engineered cell lines to cytotoxics were determined in cell viability assays (CellTiter-Glo assay [Promega, Cat#G9241]) as previously described (27). For the competition experiments, WT (GFP\textsuperscript{+ve}) or Gly101Val BCL2 (BFP\textsuperscript{+ve}) cells were mixed in a 95:5 ratio and their growth in 100nM venetoclax or DMSO monitored by flow cytometry.

SPR binding experiments

Experiments were performed using a BIACore 4000 using a SA sensor chip (GE healthcare) immobilized with biotinylated BIMBH3 or BAXBH3 peptides, with BIMBH3-4A peptide as a non-binding reference. Peptide affinities were determined by direct binding with BCL2 (0-63nM) as the analyte. Venetoclax affinity was determined by competition against immobilized BIMBH3 peptide, using BCL2 (0-250nM) pre-mixed with Venetoclax (0, 20, 40, 60nM) as the analyte. Direct binding experiments were fitted to a 1:1 binding site model and competition to a steady-state competition model, see also Supplementary Methods.
Co-immunoprecipitation and mass cytometry

FLAG-tagged WT or mutant BCL2 was immunoprecipitated from equivalent lysates prepared from venetoclax-treated or control cells with the rat anti-FLAG antibody (clone 9H1, WEHI). The co-precipitated proteins were detected with antibodies to BIM (clone 3C5, WEHI), BAX (clone 21C10, WEHI) or BAK (clone 7D10, WEHI).

For mass cytometry, fresh of thawed cryopreserved patient cells were incubated with cisplatin to allow detection of non-viable cells, then fixed with paraformaldehyde (PFA: Electron Microscopy Sciences, Hatfield, PA, USA), pelted, washed and stored at -80°C.

Cell samples for batch analysis were barcoded using the 20-plex palladium barcoding kit according to manufacturer’s instructions (Fluidigm, South San Francisco, CA, USA). Following barcoding, cells were pelleted, washed then permeabilized at 4°C with methanol for 10 min. After washing cells were incubated with specific antibody-metal conjugates (see Supplementary Methods for specific listings) then washed and stained with 125nM $^{191}$Ir/$^{193}$Ir DNA intercalator (Fluidigm, South San Francisco, CA, USA) overnight. After washing, cells were resuspended with EQ normalisation beads immediately before analysis using a Helios mass cytometer (Fluidigm, South San Francisco, CA, USA). Throughout the analysis, cells were maintained at 4°C and introduced at a constant rate of ~300 cells/sec. Data concatenation, normalization and debarcoding are done with the Helios software, version 6.7.1014 (Fluidigm, South San Francisco, CA, USA). Single cells were gated using the Flowjo (version 10.5) and Cytobank software (http://www.cytobank.org) based on event length and $^{191}$Ir/$^{193}$Ir DNA contents to avoid debris and doublets. Following single cell gating, CD5^CD19^ live non-apoptotic cisplatin^low^ cells were analyzed for levels of the various metal conjugates.
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References


Figure Legends

Figure 1 Detection of the BCL2 Gly101Val mutation in patients with progressive CLL on venetoclax, but not in venetoclax-naïve patients

(A) Genomic structure of BCL2 locus indicating the position of the point mutation (left panel). The right panel shows Sanger sequencing traces from DNA extracted from CLL3 before venetoclax treatment (top) and at time of CLL-type progression (bottom) showing acquisition of NM_000633.2; c.302G>T, p.(Gly101Val).

(B) Ribbon representation of α-helices 1-8 that form the BCL2 binding groove, indicating the location of Gly101 (red) and Phe104 (purple) on the α2 helix. Gly101 is highlighted in the black sphere representation and a representative valine side-chain at this position indicated by the red stick. The structure is that of a venetoclax analog (orange) bound to BCL2 (PDB: 4MAN)(1)

(C) Time course of CLL response and subsequent re-emergence during venetoclax therapy. The CLL burden was measured by multiparameter flow cytometry in serial bone marrow aspirates from four patients from the initiation of venetoclax until the clinical diagnosis of progressive disease. Each achieved a clinical response: complete remission for CLL2, 3 and 12, partial remission for CLL5. The variant allele frequency (VAF) of BCL2 Gly101Val in bone marrow samples measured by droplet digital PCR is overlaid. (ND – not detected). BCL2 Gly101Val VAF is indicated in red. The mutation was first detected in bone marrow after 33, 42, 31 and 19 months on venetoclax for patients CLL2, CLL3, CLL5 and CLL12, respectively.

(D) Summary of the incidence of BCL2 Gly101Val mutation and BCL2 Phe104Leu mutation in CLL (venetoclax-treated, venetoclax-naïve) and other B cell malignancies in this study; and in publicly available cancer databases or a general population.


Figure 2 Cells bearing BCL2 Gly101Val have attenuated sensitivity to venetoclax

(A) Leukemic cells from patients CLL2, CLL3, CLL5 and CLL12 are markedly less sensitive to venetoclax at progression. Mononuclear cells prepared prior to the patients commencing venetoclax (black) or at CLL progression (red) were incubated for 24h with venetoclax in vitro (0-4µM). Data represent means ± 1SD of triplicate measurements in single experiments for viable (PI−) CLL cells (CD5+ CD19+); the fold-change in LC50s are indicated.
(B) Expression of BCL2 Gly101Val or BCL2 Phe104Leu in the RS4;11 cell line reduces sensitivity to venetoclax. Each of these mutants or WT BCL2 were equally expressed (see FACS profiles on left), and the in vitro sensitivities to venetoclax (0-10µM; middle) or to navitoclax (0-10 µM; right) measured 24h later. The fold reductions in LC50s with the Gly101Val mutation are indicated.

(C) Similar to (B), but in another B-lineage cell line, KMS-12-PE.

(D) Summary of the LC50s (B, C) for WT BCL2 and each of the mutants.

(E) Like WT BCL2, the BCL2 mutants afforded protection against killing by other anti-cancer drugs. Control RS4;11 cells, or ones expressing WT or mutant BCL2 were incubated with etoposide (0-100µM, 24h), cytarabine (0-100µM, 24h), fludarabine (0-100µM, 24h) or dexamethasone (0-10µM, 48h), and the LC50s calculated. The expression of WT BCL2 significantly inhibited killing by these drugs; the mutants (Gly101Val or Phe104Leu) were as potent as the WT protein.

Data in (B)-(E) represent means ± 1SD of three independent experiments.

Figure 3 Impact of the Gly101Val mutation on the ability of BCL2 to bind BH3 ligands

(A) Table summarizing the binding affinities of BH3 peptides derived from BIM or BAX for WT or mutant BCL2 as determined by direct binding assays shown in (B) and (C). Data represent means ± SD of three independent experiments.

(B) BIMBH3 binding. 0-62.5nM WT or mutant BCL2 were used as analytes against the BIMBH3 peptide immobilized on a SPR (surface plasmon resonance; BIACore) sensor chip, the top panel illustrating the experimental conditions used. The raw response (RU) curves (colored curves) from a representative experiment were fitted to a one site specific kinetic model (black curves) to derive on and off rates, and hence to calculate $K_D$ values for interactions with WT BCL2 (2nd panel), Gly101Val (3rd panel) and Phe104Leu (4th panel).

(C) Similar to (B), with the same BCL2 concentrations indicated in (B), but using a BAXBH3 immobilized sensor chip to determined BAXBH3 binding affinities.

(D) Reduced affinity of venetoclax for the Gly101Val mutant. Table summarizing the steady state binding affinities of venetoclax determined in competition assays (E). Protocols to immobilize venetoclax, unlike the BIMBH3 peptide, to sensor chips have not been optimized, hence precluding affinity determination by direct binding. Data represent means ± SD of three independent experiments.
Solution competition assays to determine venetoclax binding affinity. WT or mutant BCL2 (0-250nM) was pre-incubated with 0, 20, 40 or 60nM of venetoclax, before flowing the analyte over a BIMBH3 sensor chip. The data were fitted to a steady-state competition equation to derive $K_I$ for venetoclax (summarized in D) and the fitted curves for a representative experiment shown below. As venetoclax bound avidly to WT BCL2, the reduced amount of free BCL2 available to bind BIMBH3 on the sensor chip was clearly evident by a drop in the steady-state response ($\text{RU}_{(ss)}$) at low BCL2 concentrations proportional to venetoclax concentration (black arrow), whereas this was reduced with the Gly101Val mutant (red arrow) reflecting the weak affinity of venetoclax for the mutant.

**Figure 4 Consequences of the BCL2 Gly101Val mutation**

(A) Venetoclax is less able to compete endogenous BH3 ligands off mutant BCL2 in cells. Equivalent lysates prepared from KMS-12-PE cells ectopically expressing FLAG-tagged WT or mutant BCL2 (Figure 2C) were immunoprecipitated with the FLAG antibody. The amount of FLAG-tagged BCL2 proteins was comparable and expression of the binding partners BIM, BAX or BAK were unaffected (corresponding input lanes). Association of BIM and BAX with the Gly101Val mutant was reduced (compare red with black boxes), probably because of increased BAK binding to the mutant (compare red with black asterisks). In the WT BCL2 cells, BIM binding was very tight and was largely unchanged by venetoclax treatment, but bound BAX and BAK was reduced by venetoclax treatment (see black arrows). However, BAX or BAK remained tightly bound to the Gly101Val or Phe104Leu mutants. Data shown is from a representative of three experiments; control: FLAG immunoprecipitation from parental KMS-12-PE cells.

(B) To varying degrees, cells expressing the Gly101Val mutant out-grow ones expressing WT BCL2 when exposed to sub-lethal concentrations of venetoclax. KMS-12-PE cells (left) or RS4;11 cells (right) expressing WT (GFP labeled) or mutant (BFP labeled) BCL2 were mixed in a 95:5 ratio and grown for the indicated periods in 100nM venetoclax (filled symbols) or under control conditions (DMSO; open symbols). The relative percentage of the GFP$^+$ (WT BCL2) or BFP$^+$ (Gly101Val) cells were monitored by flow cytometry every 3-4 days. Data represent means ± 1SD of three independent experiments.

(C) CLL cells from patients who progressed on venetoclax became highly resistant to venetoclax when cultured under conditions mimicking the stroma. Samples from three venetoclax-naïve patients (black lines) became less sensitive when grown for one week on human CD40 ligand expressing feeder cells supplemented with recombinant human IL-21 and anti-IgM: compare continuous black line with dashed black line. Starting from a higher baseline, CLL3 patient
sample (red lines) also showed a marked attenuation in venetoclax sensitivity when cultured under these conditions, such that it became almost completely resistant. This was not due to selection for Gly101Val-expressing cells since the variant allele frequency (VAF) was unchanged. Data represent means ± 1SD of triplicate measurements in single experiments for viable (PI−) CLL cells (CD5+ CD19+) in each sample. The table summarizes the impact of culturing samples from three patients who had progressive CLL while on venetoclax.

**(D)** Histograms of mass cytometric analysis of BCLxL expression in viable (Cisplatinlow) CD5+CD19+ PBMC from three patients at study entry (CLL3) and upon relapse (CLL2, 3, 12). See also Supplementary Figure 6.

**(E)** The high BCLxL-expressing subclone in CLL2 does not bear the BCL2 Gly101Val mutation, while the BCL2 mutant clone expresses low levels of BCLxL. Thawed post-progression PBMC from CLL2 were permeabilised to enable flow cytometric intracellular measurement of BCLxL, then sorted for CD19+ cells with either high or low BCLxL expression (45% and 38% of cells respectively). The VAF for Gly101Val mutation was measured for each fraction by ddPCR, and is tabulated to the right. The VAF of the input CD19+ cells was 13.78%, indicating that approximately 27.5% of input CLL cells carried the mutation.

**(F)** *In vitro* sensitivity of progressive CLL to the BCLxL inhibitor, A1331852. Cell samples from patients after progression on venetoclax were incubated with varying concentrations of A1331852 for 24 hours. Left panel: cells from patients CLL2,3,5 and 12. Only CLL2 showed substantial sensitivity to this drug. However, visual inspection of the shape of the CLL2 curve suggested the presence of more than one population of cells. Right panel: Improved curve fit when CLL2 data were re-analyzed in a two population model (Prism). The data are consistent with the presence of two populations, one sensitive (a) and the other insensitive (b) to A1331852. The calculated LC50 for each is noted on the graph. Data represent means ± 1SD of triplicate measurements in single experiments for each patient sample.

**(G)** Model of how the Gly101Val mutation affected the induction of apoptosis by venetoclax. The predominant effect is likely to be the marked reduction in ability of venetoclax to bind to mutant BCL2.
Figure 1

A

Exon 1 — Exon 2 — BCL2 - NM_000633 — Exon 3

c.302G>T
p.(Gly101Val)

B

BCL2-WT

α1 — α2 — α3 — α4 — α5 — α6 — α7 — α8

90°

Val
Gly101

C

%CLL (flow cytometry)

0 5 10 15 20 25 30 35

ND

ND

ND

0.05%

1.33%

1.42%

CLL 2

ND

ND

0.04%

1.92%

12.02%

CLL 3

ND

0.01%

CLL 5

ND

0.03%

0.11%

0.90%

CLL 12

Months on venetoclax

D

<table>
<thead>
<tr>
<th>Population</th>
<th>Number Assessed</th>
<th>BCL2 Gly101 Val Detected (%)</th>
<th>BCL2 Phe104Leu Detected (%)</th>
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<tbody>
<tr>
<td>Venetoclax-naïve CLL</td>
<td>96</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<td>CLL-type progression on venetoclax</td>
<td>15</td>
<td>7 (46.7%)</td>
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<td>Other B-cell malignancies</td>
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<td></td>
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<tr>
<td>- Follicular lymphoma</td>
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<td>- Mantle cell lymphoma</td>
<td>28</td>
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<td>- Diffuse large B-cell lymphoma</td>
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<td>0 (0%)</td>
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<td>- Lymphoplasmacytic lymphoma</td>
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<td>- Multiple Myeloma</td>
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<td>Cancer Database (COSMIC)</td>
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<td>Population Database (gnomAD®)</td>
<td>30,836</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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Figure 2

A

Viability CD5+CD19- cells % untreated

B

RS4;11

KMS-12-PE

C

D

E

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<th>VEN LC50s (µM)</th>
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<td>RS4;11</td>
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<tr>
<td>WT</td>
<td>0.24±0.092</td>
<td>0.27±0.13</td>
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<tr>
<td>Gly101Val</td>
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<td>Phe104Leu</td>
<td>&gt;10</td>
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Figure 3

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<th>BimBH3 K_{D} (nM ± SD, n=3)</th>
<th>BaxBH3 K_{D} (nM ± SD, n=3)</th>
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<tr>
<td>WT</td>
<td>0.29 ± 0.17</td>
<td>1.4 ± 0.2</td>
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<td>Gly101 Val</td>
<td>0.84 ± 0.04</td>
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<td>Phe104 Leu</td>
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<th>BCL2 protein</th>
<th>VEN K_{I} (nM ± SD, n=3)</th>
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<td>WT</td>
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<tr>
<td>Gly101 Val</td>
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<td>Phe104 Leu</td>
<td>0.46 ± 0.12</td>
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Acquisition of the recurrent Gly101Val mutation in BCL2 confers resistance to venetoclax in patients with progressive chronic lymphocytic leukemia

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