SOHO State of the Art Updates and Next Questions | Mechanisms of Resistance to BCL2 Inhibitor Therapy in Chronic Lymphocytic Leukemia and Potential Future Therapeutic Directions

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Abstract

Chronic lymphocytic leukaemia (CLL) constitutively overexpresses B-cell lymphoma 2 (BCL2) with consequent dysregulation of intrinsic apoptosis leading to abnormal cellular survival. Therapeutic use of BCL2 inhibitors (BCL2i, eg, venetoclax) in CLL, as both continuous monotherapy or in fixed duration combination, has translated scientific rationale into clinical benefit with significant rates of complete responses, including those without detectable minimal residual disease. Unlike with chemotherapy, response rates to venetoclax do not appear to be influenced by pre-existing chromosomal abnormalities or somatic mutations present, although the duration of response observed remains shorter for those with traditional higher risk genetic aberrations. This review seeks to describe both the disease factors that influence primary venetoclax sensitivity/resistance and those resistance mechanisms that may be acquired secondary to BCL2i therapy in CLL. Baseline venetoclax-sensitivity or -resistance is influenced by the expression of BCL2 relative to other BCL2 family member proteins, microenvironmental factors including nodal T-cell stimulation, and tumoral heterogeneity. With selection pressure applied by continuous venetoclax exposure, secondary resistance mechanisms develop in oligoclonal fashion. Those mechanisms described include acquisition of *BCL2* variants, dynamic aberrations of alternative BCL2 family proteins, and mutations affecting both BAX and other BH3 proteins. In view of the resistance described, this review also proposes future applications of BCL2i therapy in CLL and potential means by which BCL2i-resistance may be abrogated.

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Therapeutic BCL2-Inhibition

Chronic lymphocytic leukaemia (CLL) constitutively overexpress the BCL2 (B-cell lymphoma 2) protein and related family proteins, resulting in dysregulation of normal intrinsic apoptosis and ultimately in inhibition of cell death.[1,2] BCL2 family proteins comprised of anti-apoptotic proteins BCL2, BCL-XL, MCL1 amongst others; pro-apoptotic BH3-only proteins such as BIM, BAD, NOXA, PUMA; and the effector proteins of apoptosis BAX and BAK, have long held scientific and clinical interest.

The balance and interactions between these family member proteins determine whether apoptosis may occur.[3] Intrinsic cellular apoptosis may be induced in normal circumstances by the antagonistic binding of BH3-only proteins to anti-apoptotic BCL2 at the BH3-binding groove, which releases the restraints on BAX/BAK.[4] These effectors of apoptosis initiate mitochondrial outer membrane permeabilization (MOMP), culminating in caspase pathway activation via intermediary release of mitochondrial cytochrome c.[5] When BCL2 is over-expressed in CLL, the consequent sequestration of pro-apoptotic proteins tips the balance of BCL2 family proteins towards cell survival.

BCL2 is now an established therapeutic target in the treatment of CLL. Navitoclax, an oral BCL2 and BCL-XL inhibitor demonstrated promising clinical utility, however, its use as monotherapy has been limited by on-target platelet BCL-XL inhibition with consequent thrombocytopenia.[6,7] The selective BCL2 inhibitor venetoclax demonstrated considerable rates of overall response and complete responses in early phase monotherapy clinical trials treating lymphoid malignancies including CLL, importantly including a small number of minimal residual disease (MRD)-negative remissions.[8,9]

When venetoclax is administered in combination with anti-CD20 monoclonal antibodies or inhibitors of BTK (Bruton's tyrosine kinase), the rates of undetectable-MRD complete responses observed in CLL appear to be augmented.[10] Response rates are similarly high amongst high risk genetic subgroups for

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Table 1 Key studies evaluating venetoclax in treatment of CLL. A = acalabrutinib, B = bendamustine, C = cyclophosphamide, G = obinutuzumab, I = ibrutinib, R = rituximab, V = venetoclax, PFS = progression-free survival, MRD/uMRD = minimal residual disease, u = undetectable, DFS = disease-free survival, ORR = overall response rate, CR = complete responses, NR = not reached.

Completed studies	Population	Design	Main reported efficacy outcomes
Roberts et al., *NEJM*[8]	R/R CLL/SLL	Phase 1 dose-escalation study of continuous V monotherapy	ORR 79%; CR 20%; uMRD CR 5%; 15m PFS estimate 69% (400mg dose group)
Seymour et al., *Lancet Oncology*[10]	R/R CLL/SLL	Phase 1b dose-escalation study of VR	ORR 86%; CR 51%; uMRD CR 41%; 24m PFS estimate 82%
Stilgenbauer et al., *JCO*[76]	R/R 17p-deleted CLL	Phase 2, single-arm study of continuous V monotherapy	OR 79.4%
CLL14[13]	Untreated CLL with co-existing conditions	Phase 3, open label, randomised study of VG vs. ChI-G	PFS at median 39.6m f/up – HR 0.31, NR vs. 35.6m in favour of VG
MURANO[12]	R/R CLL	Phase 3, open label, randomised study of fixed-duration VR vs. BR	24m PFS 84.9% vs. 36.3% (HR 0.17) in favour of VR
CAPTIVATE[15,77]	Untreated CLL/SLL ≤70 years	Phase 2 study evaluating VI - fixed duration therapy and MRD-guided treatment discontinuation cohorts	*uMRD cohort:* Subsequent placebo vs ibrutinib – 1 y DFS no significant difference (95% vs. 100%) *Fixed duration cohort:* CR rate 55%; best uMRD rates 77% (PB) and 60% (BM); 24m PFS 95%; 24m OS 98%
Ongoing studies	Population	Design	Primary outcome measure(s)
NCT02950051 (GAIA/CLL13)	Untreated fit CLL without del17p or mutated *TP53This is not a pattern of 'ctgov' external object linking.*	Phase 3, open label, randomised, FCR or BR vs. VR vs. VG vs. GIV	uMRD rate (PB); efficacy by PFS
NCT05057494 (MAJIC)	Untreated CLL/SLL	Phase 3, open label, randomised, AV vs. VG	Efficacy by PFS
NCT04285567 (CRISTALLO)	Untreated fit CLL without del17p or mutated *TP53*	Phase 3, open label, randomised, VG vs. FCR or BR	uMRD rate by NGS
NCT03836261	Untreated CLL without del17p or mutated *TP53*	Phase 3, open label, randomised, multiarm study of AV(+/-G) vs CIT (FCR or BR)	Efficacy by PFS

whom responses to chemotherapy have been repeatedly demonstrated to be inferior, although the duration of responses are shorter.[11] Fixed-duration venetoclax combination therapy is now an emerging standard of care in the treatment of both frontline and relapsed/refractory CLL.[12-15] The completed and ongoing principal venetoclax studies in CLL are summarized in Table 1.

However, differential baseline sensitivity to venetoclax therapy between lymphoid neoplasms and indeed amongst CLL patients have been repeatedly observed. In addition, most patients who are continuously exposed to venetoclax following an initial response will eventually develop secondary resistance. This review seeks to describe the current understanding of intrinsic and acquired venetoclax-resistance mechanisms, and to suggest potential future therapeutic directions with use of BCL2-inhibition.

Intrinsic Sensitivity/Resistance to Venetoclax

Constitutional BCL2 Family Protein Expression

Perhaps the most fundamental factor conferring tumoral sensitivity to BCL2 inhibition is the degree of constitutional BCL2 expression and dependence upon the protein for survival. Constitutional BCL2 expression differs between tumour subtypes, and may result from a variety of mechanisms. CLL cells demonstrate aberrant expression of BCL2 protein due to a combination of hypomethylation of *BCL2,* upregulation of the transcription factor STAT3, and aberrations of chromosomal region 13q14 where *miR-15a* and *miR-16-1* may be lost.[16-18]

In general, malignancies of mature B lymphocytes exhibit upregulated BCL2 also exhibit dependence upon the protein. However, upregulation of BCL2 alone does not universally correlate with marked sensitivity to BCL inhibitor therapy as is demonstrated in the clinical experience treating follicular lymphoma (FL). Despite high levels of BCL2 secondary to the translocation between chromosomes 14 and 18, patients with FL demonstrated comparatively poor responses to venetoclax monotherapy, and the addition of venetoclax to chemotherapy thus far has not resulted in improved overall response or survival outcomes.[19] There are likely other mechanisms at play including dysregulation of other BCL2 family proteins such as BCL-W.[20]

Other BCL2 family proteins may also be overexpressed at baseline and demonstrate increased binding with BH3 pro-apoptotic proteins. Ratios of expression of BCL2 family proteins have been used to predict CLL cellular sensitivity to ABT-737 (an inhibitor of BCL2 and BCL-XL) and venetoclax in vitro.[21-23] Similarly, direct or indirect targeted inhibition of the alternative BCL2 family proteins has induced apparent venetoclax sensitivity in normally resistant cells.[21] Those venetoclax-treated CLL patients with exhibited elevated baseline levels of MCL1 suffered from worse progression-free survival.[24]

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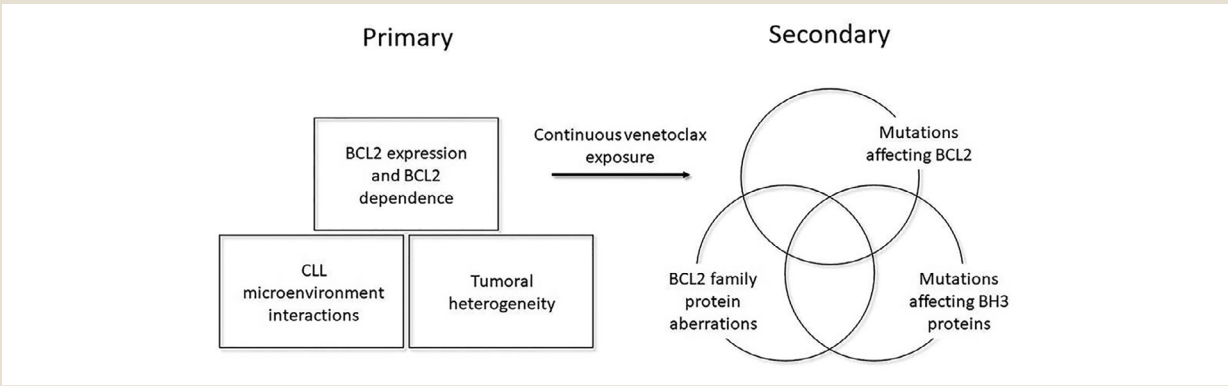
Figure 1 Summary of known factors affecting primary resistance to venetoclax and resistance mechanisms acquired after continuous venetoclax exposure in CLL.



These observations highlight the interplay between BCL2 family proteins and that differential dependence/expression of alternative BCL2 family proteins may also predict for BCL2 inhibitor response.

Microenvironmental Factors

CLL is recognized to comprise 2 compartments – the relatively inactive peripheral blood, and the active lymphoid tissue compartment. Interactions between CLL cells and the microenvironment likely contribute to intrinsic resistance within lymph nodes, spleen and other lymphoid tissue.[25]

Stimulation by microenvironmental agonists, including those via T-cell stimulation, likely contribute to upregulated NK-κB signaling with secondary baseline increase in antiapoptotic BCL2 family proteins such as BCL-XL and MCL1.[26,27] Those cells with higher baseline levels of BCL-X_L cells due to CD40 signaling are less sensitive to venetoclax, even when combined with anti-CD20.[28] The observation that those patients with higher volumes of residual nodal disease post-venetoclax therapy appear to have shorter progression-free survival may attest to the importance of the microenvironment in determining venetoclax resistance.

Similarly, pre-treatment bulky lymphadenopathy has been consistently associated with both reduced rates of complete response and reduced duration of response to venetoclax.[11] Supportive microenvironmental niches may further act to confer a survival advantage to resistant subclones amongst heterogeneous CLL cells residing in lymphoid tissue.[29]

Tumoral Genetic Risk and Heterogeneity

The pre-venetoclax mutational status of the CLL treated, including the presence of high-risk genetic features and increased tumour heterogeneity, likely have bearing on a shorter duration of response to the drug.

Considerable clonal diversity and evolution in CLL is well recognised.[29,30] Baseline chromosomal losses and somatic mutations, such as those affecting *ATM, TP53*, and *NOTCH1,* are well described and may evolve with both observation over time or with selection pressures applied by therapy. The evolutionary trajectory may be effectively time-mapped by the nature and pattern of clonal and sub clonal mutations present. Although response rates to venetoclax are preserved with high-risk genetic features in the front-line setting, the duration of response is seen to be shorter.[31,32]

Specific baseline genomic aberrations are also linked with overexpression of BCL2 family proteins. CLL harboring trisomy 12 are noted to have reduced levels of the transcriptional factor IRF4, elevated NOTCH2 expression, and consequently increased expression of MCL1.[33] In similar fashion, overexpression of BCL-XL in mantle cell lymphoma is observed as consequence of aberrations in the *SWI-SNF* chromatin remodeling complex.[34] Increased expression of alternative BCL2 family proteins is linked with venetoclax resistance. The role of acquired aberrations affecting BCL2 family proteins is discussed in a later section.

CLL tumoral heterogeneity may also comprise significant epigenetic modifications including DNA methylation, chromatin remodeling, and post-translational histone modification[29,35] Heterogeneity of these epigenomic changes has been associated with more aggressive disease biology. Methylation heterogeneity is associated with greater overall disease heterogeneity in the presence of established subclones, but the nature of the interaction between genomic and epigenomic instability is unclear.[36] The mechanisms of primary and secondary resistance to BCL2 inhibitors in CLL are summarised in Fig. 1.

Secondary Resistance - CLL-Type Progression

Mutations Affecting BCL2

The discovery of mutations within *BCL2* is considered to be a major advance in understanding the mechanisms of secondary venetoclax resistance. Whilst typical CLL-associated variants such as those affecting *SF3B1, TP53*, and *KRAS* may be acquired at any CLL-type progression, acquisition of *BCL2* mutations reflects the specific selection pressure of venetoclax therapy.

The first in-human venetoclax-induced *BCL2* variant discovered was Gly101Val. The discovery followed targeted amplicon next generation sequencing (NGS) of the entire *BCL2* gene from paired pre- and post-venetoclax patients' samples from early phase venetoclax clinical trials.[37] Seven patients demonstrated this variant at

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CLL-type progression on venetoclax therapy, present in up to 60% to 70% of the CLL tumour compartment as determined by droplet-digital polymerase chain reaction (ddPCR). For 3 of these patients the variant was detectable by ddPCR only, in as few as an estimated 1.4% of CLL cells present.

Modelling of Gly101Val mutated BCL2 demonstrates overcrowding of neighboring residues within a critical α helix comprising a portion of the BH3 binding groove.[38,39] Whilst Gly101Val mutated BCL2 retains pro-survival function by continued ability to bind BIM and BAX, it predicts for the inability of venetoclax to effectively displace the pro-apoptotic proteins from BCL2.[37] Acquisition of this variant induces resistance to killing from the drug in CLL cell lines, and subsequent continuous exposure to venetoclax provides a selective advantage for the mutated clone.[37]

Note was made of the high proportion of GC nucleotides within *BCL2*, creating challenges in variant detection.[40] Nevertheless, a number of *BCL2* mutations have now been described with further application of sequencing techniques, including within the original study cohort. They include changes at the Asp103 codon with amino acid substitutions to glutamic acid, valine, and tyrosine.[38,40] Additional point mutations Gly101Ala[41], Ala113Gly[40,41], Leu119Val[41], Arg129Leu[40], Val156Asp[40] have been described, as well as the in-frame insertion Arg107_Arg110dup,[40,41] affecting the sequence separating the α2 and α3 helices. All are implicated in venetoclax-resistance with consequent CLL-type progression.

The Asp103 residue resides within the P4 pocket of BH3 binding groove and is thought to bind to the indole ring of venetoclax. BCL2 and BCL-XL have structurally similar binding grooves; the Asp103Glu variant alters the BCL2 P4 pocket to more closely resemble that of BCL-XL.[40,42] As predicted the Asp103Glu variant cell lines demonstrated reduced sensitivity to venetoclax, but were seen to display markedly increased sensitivity to navitoclax.[40]

Acquired *BCL2* Phe101Cys and Phe101Leu variants were described in murine cell lines exposed to prior to the human studies described above.[43] They are considered analogous to human *BCL2* Phe104Cys and Phe104Leu, affecting the BH3 binding groove of BCL2 and conferring resistance to venetoclax. Whilst not detected in initial human studies, the Phe104Leu and Phe104Ser variants have now been identified alongside co-existent *BCL2* mutations in patient samples.[41]

Importantly, the original Gly101Val variant and subsequently identified variants have not been identified in pre-venetoclax patient samples, nor in large cohorts of venetoclax-naïve CLL and B-cell malignancies.[37,38] Review of serial archived samples taken prior to CLL-type progression demonstrated that *BCL2* mutations were seen to emerge up to 25 months prior to defined progressive disease[37] and up to a year before subsequent therapy was required.[38] In both studies, across all mutations analyzed, the mutational variant allele frequency (VAF) increased with time whilst on continuous venetoclax therapy until overt disease progression. The relative change in Gly101Val VAF within a singular patient was also seen to vary from anatomical compartment to compartment at different time points.[38]

Like initially modelled with Gly101Val, CLL cells demonstrating these variants ensure abnormal cell survival through essentially normal protein-protein interactions with BH3 proteins, whilst selectively reducing their affinity for venetoclax.[39] However, multiple observations made in these studies are suggestive of additional disease resistance mechanisms at play in what is a clonally complex landscape at relapse, discussed below.

Aberrations Affecting Alternative BCL2 Family Proteins

The survival of CLL cells appears to correlate with upregulation of BCL2 family proteins, particularly MCL1 and BCL-XL.[44] Baseline differential dependence upon particular BCL2 family proteins has been well described between lymphoid malignancies.[20] The balance and interactions between BCL2 family proteins is additionally affected by continuous venetoclax exposure and underpins an important feature of drug resistance.

Whilst venetoclax displaces BIM from BCL2 to potentially induce apoptosis, secondary alterations of BCL2 family proteins MCL1 and BCL-XL may increase alternative sequestration of BIM.[28,45] When BCL2 family genes are overexpressed they are proposed to similarly result in gain of function cellular resistance to venetoclax.[24] The resistant cells are seen to develop an alternative addiction to affected family proteins.[46-48]

Overexpression of BCL-XL

BCL-XL over-expression has been implicated as a mutually exclusive venetoclax-resistance mechanism with the Gly101Val variant.[37] In an instructive case of a patient who developed secondary venetoclax resistance in which a Gly101Val variant was identified in an estimated 25% of CLL cells only, further investigations identified a second clone exhibiting high BCL-XL, which likely accounts for venetoclax-resistance in the non-Gly101Val mutated cells.[37]

Cellular studies have confirmed that the dynamic upregulation of BCL-XL is implicated in venetoclax-resistance, and that subsequent co-treatment with a BCL-X_L inhibitor promotes re-sensitization to venetoclax.[26,28,49] Unlike MCL1, BCL-XL over-expression has been linked to the upregulation of both canonical, and subsequently, non-canonical NF-kB cell signaling. The upregulation of these pathways is at least in part tied to changes within the lymph node microenvironment, including increased follicular helper cell mediated CD40 signalling.[26,28,49-51]

Inhibition of both pathways by targeting NIK appears to abrogate venetoclax-resistance in CLL cell lines through dose-dependent down-regulation of BCL-XL.[49,52] Combination BCL2 and BCL-XL inhibition in anticipation of the secondary BCL-XL overexpression likely provides an interesting therapeutic angle.

Aberrations of MCL1

MCL1 is an inherently unstable protein, thought to be stabilized by BH3 protein binding.[53,54] Multiple variables at pre-transcriptional, transcriptional and post-transcriptional level are likely implicated in increasing levels and stability/persistence of the MCL1 protein.[45] As MCL1 stability is increased by BH3 protein binding, the increased levels of MCL1 seen following venetoclax therapy are thought to be consequence of increased binding of displaced BIM.

A number of CLL patient samples have demonstrated amplification of chromosome 1q [amp(1q)] after demonstrating venetoclax resistance, potentially implicated as a clonal resistance mecha-

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nism. This region contains *MCL1* and a gene (*PRKAB2*) encoding a regulatory subunit of AMPK; overexpression of MCL1 and increased AMPK signaling were confirmed by immunohistochemistry and seen to correlate with venetoclax-resistance.[24]

Like BCL-XL, CD40 stimulation also appears to result in overexpression of MCL1, although independently of upregulated NF-kB signalling.[49] This is thought to arise via upregulation of the PI3K-AKT-mTOR signaling pathway.[46,55,56] As proof of concept, the PI3K inhibitors idelalisib, copanlisib and duvelisib have demonstrated apparent synergy with venetoclax by reducing MCL1 expression, AKT-mediated BAX activation, and potentiation of apoptosis in lymphoid and broader haematopoietic malignancies.[47,57-59]

Additional transcriptional or post-transcriptional changes driving resistance in lymphoma that have been described include the potentiation of both ERK[46] and of MCL1 residues by phosphorylation.[55,60] Both result in increased MCL1 stability, persistence and likely enhanced binding to BIM. The cyclin-dependent kinase inhibitor dinaciclib is thought to antagonize MCL1 by both direct reduction of MCL1 mRNA transcription and by indirect CDK2/Cyclin-E-mediated MCL1 phosphorylation, and the compound flavipiridol likely reduces MCL1 levels via the former mechanism.[61] Concurrent BCL2 and MCL1 inhibition by venetoclax and dinaciclib respectively demonstrates synergistic apoptosis in CLL and lymphoma cells.[55]

Functional Implications of Aberrations of BCL2 Family Proteins

Whilst both proteins are upregulated and are seen to functionally contribute to venetoclax-resistance in vitro,[24,28,49] BCL-XL is likely more heavily implicated in venetoclax resistance than MCL1.

Direct co-inhibition of BCL-XL and BCL2 by combination BH-3 mimetics in venetoclax-resistant CLL cell lines is seen to abrogate venetoclax-resistance more effectively than co-inhibition of BCL2 and MCL1.[28] In addition, in the presence of BCL2 inhibition, BCL-XL appears to bind BIM preferentially to MCL1. These findings are collectively suggestive of a functional hierarchy amongst BCL2 family proteins under venetoclax therapeutic pressure.[28,49] It is also clear that further BCL2 family members are implicated in interactions with BIM in the context of venetoclax-resistance, including BFL1.[28]

However, the frequency with which alterations in BCL2 family proteins may occur in venetoclax-treated CLL patients has not been sufficiently defined to understand the clinical importance of these observations, and is under current investigations (NCT05246345).

Clonal Complexity at Disease Relapse and Evidence for Multiple Mechanisms of Resistance

The likelihood of co-existent distinctive molecular mechanisms of resistance first became apparent from observations of sub-clonal resistance mutations within venetoclax-resistant CLL cell populations.

The human CLL sample studies detailed above demonstrated considerably variable proportions of the CLL tumour compartment harboring *BCL2* variants at CLL-type progression, between minor subclone to the majority of tumour cells present.[37,38,40,41] Moreover, progressive CLL in singular patients expressed multiple *BCL2* variants at differential allele fractions emerging at different time points during venetoclax therapy, with considerable heterogeneity in the patterns observed. These observations are indicative of an oligoclonal pattern of resistance.

A single cell sequencing study of CLL patients who progressed on targeted agents (either BTK inhibitors, BCL2 inhibitors or both) has furthered understanding of the clonal interplay.[62,63] Importantly, multiple resistance mutations to the same agent arose in mutually exclusive clones at progression, consistent with convergent clonal evolution under therapeutic pressure.[63] Sequential therapy with both classes of agent is suggested to be capable of both inducing dual resistance mutations within the same clone, or within separate clones – the latter is indicative of additional, yet unexplained mechanisms of resistance. With subclonality, sequential application of therapies may induce significant clonal exchange at the time of second progression, and may provide a rationale for studies utilizing retreatment strategies based on clonal profile.[63]

Within the same study, typical CLL-associated mutations were seen to exist or evolve in independent clones or in clones also harboring new resistance mutations, providing evidence of ongoing clonal evolution within the resistant disease.[63] Polyclonal heterogeneity appears to consistently underpin venetoclax-resistance. In addition to co-existence with additional molecular mechanisms such as over-expression of BCL2 family proteins, these findings support the understanding that CLL-type progression on venetoclax therapy is likely driven by multiple independent molecular mechanisms.

BAX *Mutations and Clonal Myelopoiesis*

The unexpected discovery that *BAX* mutations likely occur commonly in the myeloid compartment, rather than neoplastic lymphoid compartment, in non-Hodgkin lymphoma patients treated with continuous venetoclax therapy is striking and describes significant genetic diversity under venetoclax treatment selection pressure.[64,65]

Mutations commonly implicated in clonal haematopoiesis or myeloid malignancy can be readily identified in CLL patients previously exposed to alkylators and/or fludarabine therapy, at allele fractions consistent with the myeloid compartment.[65] A reasonable proportion of patients (approximately 30%) subsequently exposed to long-term venetoclax, but not BTK inhibitor therapy, were seen to also harbor *BAX* mutations.

The *BAX* mutations described may co-exist and progress within the dominant myeloid clone with time on venetoclax therapy with other classical mutations defining clonal haematopoiesis, or persist at low level in non-progressive clones.[65] Myeloid *BAX* mutations may also be detected concurrently with *BCL2* mutations within the CLL compartment.[64] Those variants identified were enriched affecting C-terminus changes, resulting in loss of function of BAX protein due to structural change within key elements

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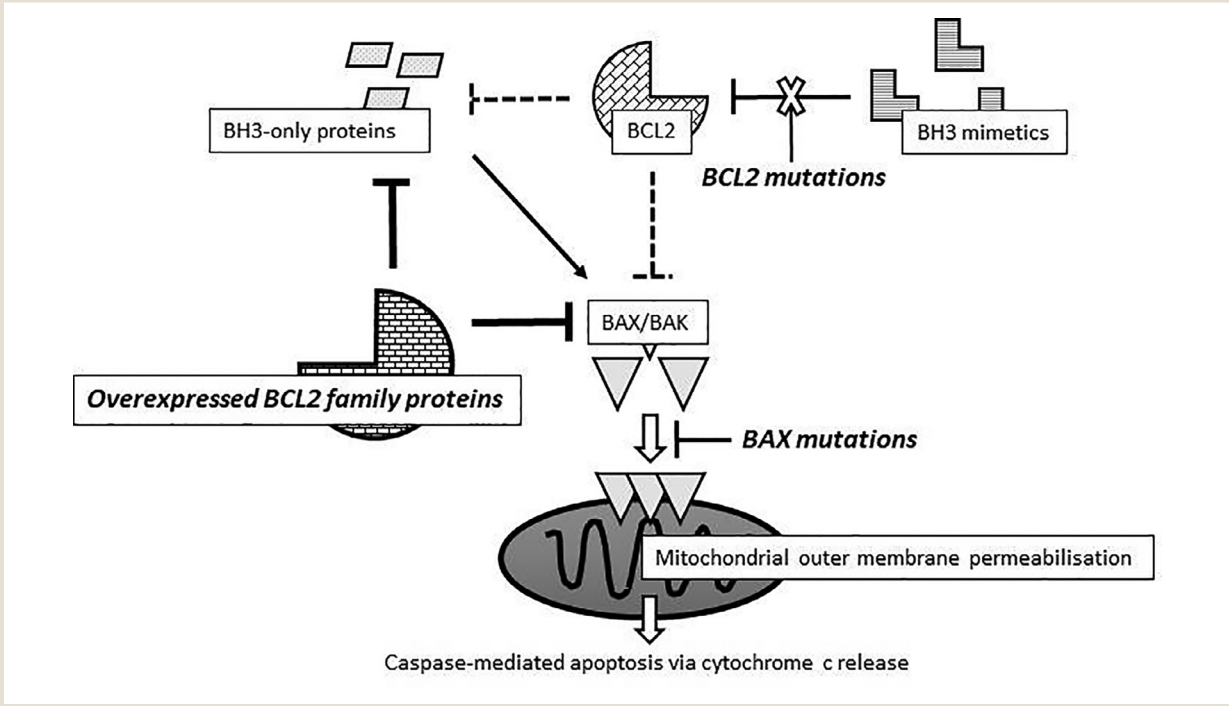
Figure 2 Secondary resistance mechanisms to BCL2 inhibition by BH3 mimetics such as venetoclax. Acquired BCL2 mutations affecting the protein binding groove impair usual binding of BH3 venetoclax, re-establishing anti-apoptotic activity as depicted by the dotted lines. Over-expression of BCL2 family proteins (eg, BCL-XL, MCL1) lead to increased sequestration of BH3-only proteins and effector proteins BAX/BAK. BAX mutations are thought to impair BAX oligomerization and hence mitochondrial membrane pore formation.



of the protein, including within the critical α9 helix. Similar mutations have been previously explored in cell lines which were seen to demonstrate consequential disruption of normal BAX function, and indeed have generated resistance to venetoclax in vitro.[43,64,66]

Clonal myelopoiesis with *BAX* mutations and/or mutations alike in alternative BCL2 family protein genes may prove to be the rule rather than the exception for patients treated with long-term venetoclax therapy due to the selective selection pressure applied. However, these mutations have no clear association at the present time with therapy-related myeloid neoplasms independently of those mutations associated with prior fludarabine-alkylator therapy.[64]

Mutations within other BCL2 family proteins have been previously described preclinically and recently also seen in resistant venetoclax-treated CLL.[63,66-68] Acquisition of *BAX, PMAIP1* (encoding NOXA) and *BCL2L11* (encoding BIM) mutations within the CLL compartment may coexist with *BCL2* mutations at differential allele fractions, suggestive of subclonally diverse mechanisms of potential resistance.[63,68]

However, despite preclinical predictions of conferred clinical drug resistance, mutations of BCL2 family genes outside of *BCL2* itself are not adequately described to be accepted as established venetoclax-resistance mechanisms at the present time. The interference of established secondary resistance mechanisms in ongoing BCL2 inhibition are depicted in Fig. 2.

Secondary Resistance - Richter Transformation

Less is known about the key mechanisms that underpin venetoclax-resistance in CLL manifested by Richter transformation (RT) during or shortly following therapy.

The *BCL2* mutations detected commonly in CLL-type progression have not been described to the same extent in RT, although fewer patients have been analyzed. Whilst the Gly101Val variant has not been reported, the Arg110dup variant has been seen at low VAF appearing at RT (0.4%).[41]

More broadly, the mutations previously seen commonly at RT of CLL may also occur at RT during venetoclax therapy. En route to RT, patient CLL samples have been observed to demonstrate increasing genomic instability with altered copy number changes and aneuploidy, and to acquire typical CLL-type mutations. Homozygous deletions of *CDKN2A/B* are an example of such recurring genomic changes, yet are insufficient in knock-down studies to affect resistance to venetoclax.[69]

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Table 2 Key ongoing novel venetoclax combination studies. DLTs = dose-limiting toxicities, AEs/SAEs = adverse events/serious adverse events, AML = acute myeloid leukaemia, MM = multiple myeloma, NHL = non-Hodgkin lymphoma.

Ongoing novel venetoclax combination studies	Population	Design	Primary outcome measures
NCT04214093	Advanced haematologic or solid tumours	First-in-human, phase 1 study of AZD0466 (BCL-XL/BCL2 inhibitor)	Incidence of DLTs; incidence of AEs
NCT03739554	R/R CLL	Phase 1 dose-escalation study of CYC065 (CDK inhibitor) in combination with venetoclax	Incidence of DLTs
NCT03672695	R/R or untreated AML without established treatment alternatives	Phase 1b dose-escalation study of S64135 (MCL1 inhibitor) in combination with venetoclax	Incidence of DLTs; incidence and severity of AEs including SAEs; frequency of dose interruptions and/or reductions; dose intensities
NCT04702425	R/R haematological malignancies - NHL, MM, AML	Phase 1b study of VOB560 (BCL2 inhibitor) in combination with MIK665 (MCL1 inhibitor)	Incidence of DLTs; incidence and severity of AEs including SAEs; frequency of dose interruptions and/or reductions; dose intensitiesThis is not a pattern of 'ctgov' external object linking.

Future Therapeutic Strategies to Optimize BCL2 Inhibition

Optimal Duration of Venetoclax Therapy and The Potential for Retreatment

It is probable that the duration of venetoclax selection pressure to a heterogeneous CLL tumour environment is an important determinant of emergence of *BCL2* resistance mutations described. In contrast to continuous venetoclax therapy, combinations with anti-CD20 monoclonal antibodies as used in the MURANO and CLL14 studies which allow effective fixed-duration therapy have not been seen to select for *BCL2* resistance mutations.

The Gly101Val variant was first seen at low VAF 19 to 42 months from continuous venetoclax treatment initiation.[37] The time to acquisition of other mechanisms of secondary resistance described, such as upregulation of alternative BCL2 family proteins, is unknown. Like *BCL2* mutations, it is reasonable to assume however that time-limited exposure may not necessarily select for resistant clones that acquire these changes. Similarly, the clonal haematopoietic changes defined by *BAX* mutations within the myeloid compartment have only been described with continuous venetoclax exposure. Time limited therapy potentially offers multiple benefits to patients and is likely the optimal way to use venetoclax.

The efficacy of venetoclax retreatment in the event of progression following completion of fixed-duration venetoclax is relatively unknown. A small number of patients from the MURANO study were retreated with venetoclax-rituximab combination or with venetoclax monotherapy, with an overall response rate of 55%.[70] Similarly, four patients who attained CR on monotherapy were previously retreated with monotherapy or venetoclax-rituximab following progression with successful disease responses observed. However, the characteristics of those patients who may best respond to retreatment are unknown.

Although the *BCL2* mutation status of the study patients who have been retreated has not been published, the presence of a significant *BCL2* mutant VAF may presumably predict for a poor response. The role of screening patients who may be considered for retreatment with venetoclax for *BCL2* variants is unknown, as is monitoring those who are currently receiving continuous therapy to anticipate loss of response and/or to subsequently intervene by adding an additional agent. Thresholds defining VAF of significance to predict clinical resistance are unknown.

An interesting observation in the era of targeting therapy is the potential for clonal evolution or exchange after exposure to a subsequent targeted agent. Clonal fractions harboring mutations of *BTK* or *PLCG2* following prior BTK inhibitor therapy may be seen to persist or increase during subsequent venetoclax exposure. This may be explained by the sequential acquisition of a venetoclax resistance mechanism within these clones, or suggest a role for BCR-signaling in the development of resistance.[41] Of interest, a patient was seen to undergo near-complete clonal exchange of numerous *BCL2* variants and a single *BAX* splice variant with *BTK*-mutated clones post-venetoclax progression on subsequent BTKi therapy.[63] Dynamic changes in clonal patterns may provide evidence for potential utility of venetoclax retreatment in selected patients.

Potential Combination Therapies to Overcome Resistance Mechanisms

Mechanistically, the venetoclax-killing effect may be enhanced in combination with a second agent and may allow for fixed-duration venetoclax exposure. Patients with detectable MRD at the end of fixed-duration therapy appear to have shorter progression-free survival, independent of other response observed.[71] Combination therapies appear to augment rates of complete response and importantly rates of undetectable MRD over those seen with continuous venetoclax monotherapy in early clinical trials.[12,13,15]

Baseline genetic risk features such as unmutated IGHV, complex karyotype, del17p, *NOTCH1* or *TP53* mutations; treatment factors such as prior BTK inhibitor, three or more prior treatment lines, or fludarabine-refractoriness; and disease burden with bulky lymphadenopathy have all been associated with less durable

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responses and/or progression on venetoclax therapy.[11,32] Compared with MRD-associated outcomes, it is less clear to what extent durations of response to venetoclax may be improved in combination with a second agent in high-risk groups.

The rationale for and efficacies of venetoclax combination therapies used for CLL, namely with anti-CD20 monoclonal antibodies, BTKi or both, and inhibitors of PI3K, have been well summarized elsewhere.[72] Broadly, there is a strong rational for therapeutic combinations in the setting of oligoclonal resistance mechanisms. BTKi therapy and anti-CD20 monoclonal antibodies demonstrate synergism with venetoclax,[15,73] due to posited increase in CLL cellular BCL2 dependence[45] and downregulation of microenvironmental signalling.[26] A large number of questions remain regarding the optimal combinations and sequencing of targeted therapies in CLL.

A less understood area of growing interest is the potential utility of targeting the mechanisms of venetoclax-resistance described, and even the microenvironment in which venetoclax-resistant clones may be selected for. Due to their roles in resistance described above, potential targets under evaluation include MCL1, BCL-XL, epigenetic modifiers, along with manipulation of NF-kB and PI3K/AKT/mTOR pathways. The preclinical data predicting utility for targeting of these proteins or pathways are summarized in the relevant sections above.

A small number of clinical trials have explored potentially resistance-abrogating combinations or are currently underway for treatment of various tumour types, summarized in Table 2. Following the efficacy of navitoclax against lymphoid malignancies limited by on-target thrombocytopenia, BCL2/BCL-XL combination inhibitors are being explored renew. The combination of venetoclax and low-dose navitoclax along with chemotherapy in a phase 1 study treating relapsed/refractory acute lymphoblastic leukaemia/lymphoma demonstrated a complete response rate of 60% with good tolerability and no significant thrombocytopenia.[74] The phase 1 data from treatment of multiple tumour types including advanced haematologic malignancies using the novel combination BCL2/BCL-XL inhibitor AZD0466 is awaited (NCT04214093).

The combination of venetoclax and a CDK2/9 inhibitor (resulting in indirect MCL1 inhibition) in relapsed/refractory CLL is under active evaluation (NCT03739554), and the combination inhibition of BCL2 and MCL1 is also under current evaluation in relapsed/refractory acute myeloid leukaemia (NCT03672695, NCT04702425). Safety signals of cardiotoxicity following direct MCL1 inhibition have however emerged and may hinder further development.[75]

In addition to these approaches seeking to abrogate resistance at treatment outset, a second yet untested approach could add an appropriate second agent as dominant resistant clones emerge under serial molecular evaluation. Note has been made of the navitoclax-sensitivity of the Asp103Glu *BCL2* mutant clones induced by venetoclax therapy.[37] Introduction of navitoclax upon such mutation acquisition which would offer an interesting, yet to be evaluated, dynamic therapeutic angle for this group of patients.

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