BCL2 Inhibitors: Insights into Resistance

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Abstract Over the last decade, improved understanding of the mechanisms and structures of proteins integral to apoptosis have enabled therapeutic targeting of BCL2 to become more specific, less toxic and ultimately more clinically effective. The first BCL2-selective inhibitor, venetoclax, is now approved for use in patients with relapsed and refractory chronic lymphocytic leukemia (CLL) in multiple countries. Early phase clinical trials demonstrated an 80% overall response rates in patients with relapsed/refractory CLL, independent of traditional risk factors, without undue toxicity. Venetoclax is also highly active in other lymphoid malignancies that express high levels of its target, BCL2, such as mantle cell lymphoma. However, there is a cumulative incidence of disease progression while on therapy. Ongoing follow-up of the early phase trials is only now enabling elucidation of the incidence and risk factors for disease progression and treatment failure. Preventing development of resistance to BCL2 inhibition requires further research aimed at delineating

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the genetic and epigenetic drivers of disease progression. This will facilitate targeting of resistance mechanisms through the use of rational drug combinations, help to prospectively identify patients most likely to benefit and abet early identification of emerging resistance. These therapies are improving outcomes for patients with previously poor prognosis disease.

Keywords BCL2 (B cell lymphoma 2) · Apoptosis · Venetoclax · Chronic lymphocytic leukemia

Abbreviations

AML	Acute myeloid leukemia
BCL2	B cell lymphoma 2
BCR	B cell receptor
BH	BCL2 homology
BTK	Burtons tyrosine kinase
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CR	Complete remission
CRi	Complete remission, incomplete count recovery
Del11q	Deletion 11q
Del17p	Deletion 17p
DLBCL	Diffuse large B cell lymphoma
DLT	Dose limiting toxicity
DOR	Duration of response
EC_{50}	Half maximal effective concentration
EFS	Event free survival
FFP	Freedom from progression
FL	Follicular lymphoma
G	Grade
HL	Hodgkin lymphoma
IDH	Isocitrate dehydrogenase
IGVH	Immunoglobulin variable region heavy chain
IHC	Immunohistochemistry
MCL	Mantle cell lymphoma
MLL	Mixed lineage leukemia
MM	Multiple myeloma
MRD	Minimal residual disease
MTD	Maximum tolerated dose
MZL	Marginal zone lymphoma
NA	Not applicable
NHL	Non-Hodgkin lymphoma
nM	Nano-molar

ORR	Overall response rate
OS	Overall survival
PD	Progressive disease
PFS	Progression free survival
PI3κ	Phosphoinositide 3 kinase
PR	Partial response
RP2D	Recommended phase 2 dose
RT	Richter's transformation
SLL	Small lymphocytic lymphoma
TLS	Tumor lysis syndrome
TTP	Time to progression
WM	Waldenstrom's macroglobulinemia

Introduction

The advent of rituximab and other monoclonal antibodies, in combination with standard cytotoxic chemotherapy, have heralded a new era of durable remissions in many challenging B cell lymphomas and leukemias [1, 2]. Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries and is traditionally considered an indolent disorder. Together deletion 17p (del17p) and deletion 11 q (del11q) CLL account for approximately 25% of patients and these individuals have a significantly inferior survival [3]. Other patients whose disease falls into a high-risk category include patients with complex cytogenetics [4], bulky nodal disease [5, 6], fludarabine refractory disease [5–9], and unmutated immunoglobulin variable heavy chain (*IGVH*) gene [10]. For such patients, approaches other than traditional chemo-immunotherapy are required due to the low probability of durable responses.

The last 5 years have seen the emergence of a number of novel targeted agents, for instance the Brutons' tyrosine kinase inhibitor (BTKi), ibrutinib, and the phosphoinositide 3 kinase (PI3κ) inhibitor, idelalisib. Both these agents achieve high response rates in relapsed and refractory CLL. However, the longer term durability of response varies [11, 12], and not all patients are able to tolerate chronic administration of these drugs [13, 14].

Another promising novel approach to treatment in non-Hodgkin lymphoma (NHL) and CLL is to enhance malignant cell death through therapeutic manipulation of the intrinsic pathway of apoptosis. While the concept of targeting this pathway is not new, it is only in recent years that specific and potent agents acting on this pathway have become available. To date the most promising of these is venetoclax, which entered clinical trials in 2011. This review examines its mechanism of action, summarizes key clinical data and explores emerging data about how resistance can emerge.

BCL2 and Cancer

Apoptosis, or programed cell death, is a stereotypical process ubiquitous to all eukaryotic organisms [15]. Apoptosis culminates in the activation of proteolytic enzymes (caspases) that irreversibly commit the cell to death. Failure of this process is a recognized hallmark of malignancy [16, 17] which drives not only the development of malignancy but also resistance to chemotherapy and radiotherapy [18–23].

There are two major pathways of apoptotic cell death: (1) the extrinsic pathway in which intracellular signaling cascades, that culminate in caspase activation, are triggered by extracellular death signals [24] and; (2) the intrinsic pathway which is the pathway most commonly perturbed in B cell malignancy.

Central to the intrinsic pathway of apoptosis is the BCL2 (B cell lymphoma 2) family of intra-cellular proteins that are characterized by conserved sequences in up to four BCL2 homology (BH) domains. This family is divided into three subgroups: pro-apoptotic BH3-only proteins; pro-survival proteins like BCL2; and apoptotic mediators. These three sub-groups interact specifically with one and other to trigger cell death (Fig. 1). In essence, it is the balance between the pro-survival and pro-apoptotic members of the BCL2 family that ultimately determines cellular fate [25].

The pro-apoptotic BH3-only proteins are activated by cellular stress signals including cytokine deprivation, oxidative stress, DNA-damage from chemotherapy or radiation, and proliferative stress. These proteins include BIM, BID, PUMA, and NOXA. When activated they bind selectively to [25] and inhibit the pro-survival BCL2 proteins. Under some circumstances they can also interact directly with the pro-apoptotic mediators to promote cell death.

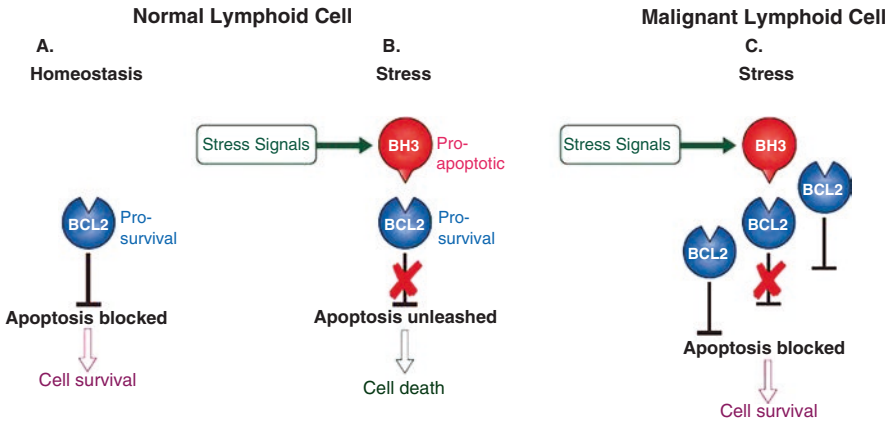

Fig. 1 In a normal healthy lymphoid cell the BCL2 pro-survival family of proteins block apoptosis and keep the cell alive (**a**) Under conditions of stress the BH3 only pro-apoptotic family of proteins are activated thus inhibiting the BCL2 family and unleashing apoptosis and cell death (**b**) In a malignant lymphoid cell however, BCL2 overexpression can overwhelm the capacity of the BH3 only proteins to trigger cell death resulting in inappropriate cell survival (**c**) *Figure created for Anderson, Seminars in Haematology, 2014* [24] (*Adapted from Chen et al. 2005* [25])

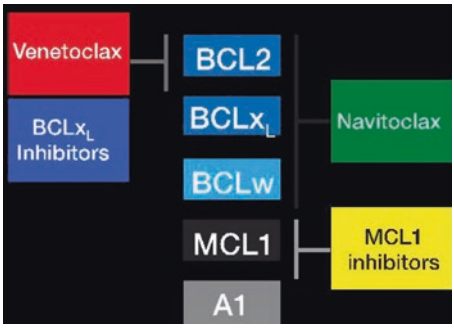

Fig. 2 Binding of BH3 mimetics to the BCL2 family (*Figure adapted from Chen, Cell, 2005* [25])

The pro-survival BCL2 proteins include: BCL2, BCLx$_L$, BCLw, MCL1 and A1. These proteins are bound by specific BH3-only family members. For instance, BAD binds to BCL2, BCLx$_L$ and BCLw, while NOXA binds to MCL1 and A1 and BIM binds to all BCL2 family members [25]. This selective BCL2 binding to the BH3-only proteins can now be therapeutically mimicked (Fig. 2). The BCL2 family acts to prevent cell death by binding to and inhibiting the pro-apoptotic mediators. The family members of this group are differentially expressed in various tissues throughout the body, and act to maintain tissue-specific cell survival. For instance, BCL2 is critical for the survival of lymphocytes at various stages in their development [26, 27], whereas BCLx$_L$ is critical to the survival of circulating platelets [28], and MCL1 is essential for plasma cell survival [29].

The final group of proteins that comprises the BCL2 family are the apoptotic mediators, which comprise the proteins BAK and BAX. These proteins are activated by removal of the BCL2 brake on their function. When active BAK and BAX trigger the mitochondrial outer membrane permeabilization is induced with cytochrome C release. Cytochrome C is an essential co-factor for caspase activation. The absence of BAK and BAX renders a cell resistant to apoptotic cell death via the intrinsic pathway [30, 31].

A direct consequence of BCL2 overexpression within a cell is quenching of the capacity of the BH3-only proteins to trigger apoptosis. Mechanistically, this underpins the inappropriate survival of cells in which BCL2 is overexpressed, and helps to explain why BCL2 overexpression can be such a powerful contributor both to malignancy and chemotherapy resistance. The role of BCL2 overexpression in malignancy was first elucidated in follicular lymphoma (FL) where the near universal presence of t(14;18), translocating the *IGVH* promoter to the *BCL2* gene, results in constitutive BCL2 overexpression [32, 33]. CLL has a high level of BCL2 expression [34], driven by the loss of mir15/16 mRNAs [35]. In CLL, the accumulation of BCL2 is thought to be central to the accumulation of malignant cells. While the critical role of BCL2 overexpression in driving resistance to apoptosis is best recognized in CLL and FL, BCL2 is commonly highly expressed in multiple B cell malignancies including multiple myeloma (MM) [36], mantle cell lymphoma (MCL) [37], Waldenstrom's macroglobulinemia (WM) [38] and diffuse large B cell lymphoma (DLBCL) [39].

BH3 Mimetics

Targeting BCL2 for the treatment of B cell malignancies has long been a goal of researchers as a way of enhancing the outcomes of chemotherapy among this group of patients. A true BH3 mimetic has been defined by Lessene et al. [40] as a drug that meets four key criteria: (*i*) apoptosis is via BAK/BAX with mitochrondrial disruption; (*ii*) the drug binds at least one BCL2 protein with high affinity; (*iii*) the drugs' activity correlates with its expression of relevant BCL2 family members and; (*iv*) relevant biomarkers are affected by the drug in animal models. The advent of nuclear magnetic resonance technology [41] greatly enhanced the structural understanding of the binding between the BH3-only proteins and the BCL2 family, this facilitated the development of the first true BH3 mimetic agent – ABT-737.

ABT-737

First described in 2005, ABT-737 is a small molecule prototype analogue for the BH3-only protein BAD [42] (Table 1). Like the physiologic intracellular protein BAD, ABT-737 binds to BCL2, $BCLx_L$ and BCLw with high affinity ($Ki < 1$ nM), with much lower binding affinity for MCL1 ($Ki > 500$ nM) [42]. The in vitro cytotoxicity of ABT-737 is dependent upon BAX and BAK [48], the sensitivity of malignant cells to the drug the correlates with expression of BCL2, $BCLx_L$ and BCLw [49] and it achieves both in vivo and in vitro efficacies against a range of B cell malignancies [42, 49–52]. However, ABT-737 was not suitable for oral administration due to solubility issues, and did not enter clinical trials.

Navitoclax

Navitoclax (also known as ABT-263) is an orally available analogue of ABT-737 [43] that entered clinical trials in 2007. Like ABT-737, navitoclax binds with high affinity to BCL2, $BCLx_L$ and BCLw ($Ki < 1$ nM) with minimal binding to MCL1 and A1 [43] and has pre-clinical evidence of efficacy against B cell malignancies [53].

The first-in-human trial of navitoclax amongst patients with relapsed/refractory B cell malignancies (including DLBCL, MCL, FL, CLL, peripheral NK/T cell lymphoma and Hodgkin lymphoma [HL]) demonstrated an overall response rate (ORR) of 22% (all partial responses [PRs]) with a median progression free survival (PFS) of 16 months [44]. However, there were few objective responses in diseases other than CLL/small lymphocytic lymphoma (SLL). When navitoclax was tested exclusively in patients with relapsed/refractory CLL/SLL it was associated with a 35% ORR (all PR) and a median PFS of 25 months [45].

Table 1 Comparison of BH3 mimetics

		ABT-737 nM [42]	Navitoclax nM [43–45]	Venetoclax nM [46, 47]
Target Binding (K*i*)	BCL2 $BCLx_L$ MCL1	<1 <1 460	<1 <1 >500	<0.0 48 >444
CLL Clinical Efficacy	ORR PR CR/CRi Median PFS	Did not enter clinical trials	35% 35% 0% 25 months	79% 59% 20% Median not reached; 69% (15 months)
NHL Clinical Efficacy	ORR PR CR/CRi Median PFS	NA	22% 22% 0% 16 months	44%[a] 13% 31% Varied according to subtype[b]
CLL G3/G4 Haem AEs	Neutropenia Thrombocytopenia Anemia	NA	28% 18% (G4)	41% 12% 12%
NHL G3/G4 Haem AEs	Neutropenia Thrombocytopenia Lymphopenia Anemia	NA	18% 29% 14%	11% <15% <15% 15%
Dose Limiting Toxicity (DLT)		NA	Thrombocytopenia	In CLL tumor lysis syndrome, tolerated with ramp up dose scheduling In NHL: no DLT identified In MM: no DLT identified

Table adapted from Anderson, Seminars in Haematology, 2014 [24]. *nM* nano molar, *ORR* Overall response rate, *PR* Partial response, *CR* Complete response, *CRi* Complete response with incomplete marrow recovery, *PFS* Progression free survival, *NA* not applicable, *G3* Grade 3, *G4* Grade 4 *AE* Adverse event, *CLL* Chronic lymphocytic leukemia, *NHL* Non-Hodgkin lymphoma, *MM* multiple myeloma

[a]Response rates varied with disease subtype: mantel cell lymphoma ORR 44%, CR 13%; follicular lymphoma ORR 38%, CR 14%; diffuse large B cell lymphoma ORR 18%, CR 12%

[b]The estimated PFS for all patients was 6 months by subtype it was: 14 months for mantle cell lymphoma; 11 months for follicular lymphoma; and 1 month for diffuse large B cell lymphoma

While these results were promising even better outcomes were achieved when navitoclax was used as combination therapy. When used in combination with rituximab in $CD20^+$ lymphoproliferative disorders there was a 75% ORR with 5 out of 12 patients achieving a complete response (CR) [54]. In a randomized trial of navitoclax plus rituximab versus rituximab alone for previously untreated CLL, unsuitable for cytotoxic therapy, single agent rituximab achieved a 35% ORR in comparison to the combination arm where there was a 70% ORR ($p = 0.03$) [55]. Similarly, enhanced results were achieved when navitoclax was used in combination with bendamustine and rituximab in patients with relapsed/refractory CLL with 35% CR rate and 44% PR rate [56].

In a subset of patients treated on the phase I trial of navitoclax as a single agent in CLL, BCL2 family members were measured at baseline using western blotting [45]. In keeping with the literature, BCL2 was highly expressed at baseline in most samples, whereas MCL1 was expressed only in some samples. Importantly, however, there was no correlation between the objective clinical response and the expression of MCL1 or BCL2 at baseline [45]. However higher MCL1 levels did predict for a lesser reduction in lymphocytosis and high BIM:MCL1 ratios were associated with patients achieving an objective clinical response (all PRs) [45]. However, it was evident from very early in its clinical development that navitoclax was consistently associated with dose-proportional reductions in platelet counts and that thrombocytopenia was dose-limiting [44, 45]. Associated translational research demonstrated that $BCLx_L$ is critical for the survival of platelets in the peripheral circulation [28, 57], and that inhibition of BCLxL by navitoclax caused the thrombocytopenia. Thrombocytopenia precluded dose escalation of navitoclax above 300 mg daily, thus prohibiting the exploration of whether incremental improvements in clinical outcome could be achieved with higher doses.

Nevertheless, the promising clinical and preclinical data suggesting that inhibition of the BCL2 family was an effective therapeutic measure led to work to develop a BCL2 selective inhibitor. Unencumbered by $BCLx_L$-mediated thrombocytopenia, a BCL2 selective inhibitor was anticipated to allow greater dose escalation and more potent BCL2 inhibition with a safer hematological toxicity profile.

Venetoclax

Biochemistry

Venetoclax (also known as ABT-199) was developed by reverse engineering navitoclax to produce a compound which binds with high avidity to BCL2 (Ki <0.01 nM) but has much less avidity for $BCLx_L$ (Ki 43 nM) and BCLw (Ki 245 nM) with no measurable binding to MCL1 (Ki >444 nM) [46]. Venetoclax meets all the Lessene criteria for a true BH3 mimetic. It has no effect on double knock out BAX/BAK negative mouse embryonic fibroblasts [46], and kills normal and malignant B cells in a BAX/BAK-dependent fashion [58, 59]. Venetoclax demonstrates effective killing in the BCL2-dependent RS4;11 cell line, but not in the $BCLx_L$-dependent H146 cell line [46]. In cell lines, the cytotoxic effect of venetoclax is accompanied by markers of apoptotic cell death including cytochrome C release, activation of caspase 3/7 and phosphatidylserine exposure which was blocked by a pan-caspase inhibitor [46, 60]. Furthermore, venetoclax mediated killing is associated with disrupted BCL2-Bim, but not $BCLx_L$-Bim, complexes [46]. Cell death due to venetoclax was also proportional to the BCL2 expression [46].

Pre-clinical Data

Venetoclax killed CLL cells ex vivo at least as effectively as navitoclax, however ex vivo platelets were much less sensitive to death from venetoclax than navitoclax [46, 60] (Table 2). This translated to less in vivo platelet toxicity in dogs when treated with venetoclax compared to those treated with navitoclax [46]. Furthermore, killing of CLL cells by venetoclax has been shown to be via induction of apoptosis both in vitro and in vivo in patients. Venetoclax-mediated cytotoxicity is independent of the TP53 pathway function [61].

Table 2 Preclinical efficacy data

Disease model	*In vitro*	*In vivo*
Chronic lymphocytic leukemia	Primary CLL cells LC_{50}[a] 1.9 nM [61]	No accepted CLL cell line or appropriate murine model
Mantle cell lymphoma	3/8 MCL cell lines LD_{50}[b] <200 nM 10 primary MCL samples LD_{50} <10 nM[62]	In granta-519 xenografts tumor growth is inhibited by venetoclax [46]
Waldenstroms macroglobulinemia	CXCR4 mutations make WM resistant to ibrutinib In CXCR4 mutated WM cells sensitivity to ibrutinib enhanced by venetoclax[63]	
Follicular lymphoma	5 cell lines EC50 0.05 – 11 μM [46]	Toledo xenografts which harbor t(14:18) showed decreased tumor growth [46]
Diffuse large B cell lymphoma	20 cell lines EC_{50} 0.003 – 34.3 μM [46]	
Multiple myeloma	Primary myeloma cells harboring t(11;14) particularly sensitive [64]	
Acute lymphoblastic leukemia	4 cell lines EC_{50} 0.008 – 9.2 μM [46] Most ALL requires some $BCLx_L$ inhibition, however in MLL ALL venetoclax alone sufficient to induce cell killing [65]	In RS4;11 xenograft tumor growth inhibition and tumor delay dose dependent [46] *In vivo* MLL-ALL xenografts responded to venetoclax [65, 66]
Acute myeloid leukemia	In 2 cell lines EC_{50} 0.16 – 0.76 μM [46] Primary AML samples sensitive with median IC_{50}[c] 10 nmol/L, with death occurring within 2 h [67] Primary cells with IDH1 and IDH2 mutations were more sensitive compared with wild type [68]	Murine xenografts were sensitive [67]

Summary of pre-clinical venetoclax results in a variety of hematological malignancies
[a]50% lethality concentration
[b]50% lethal dose
[c]50% inhibitory concentration
MLL Mixed lineage leukemia

The near uniform overexpression of BCL2 in FL conferred by the presence of t(14;18) made this disease an obvious target for venetoclax. However, FL cell lines show variable sensitivity to venetoclax with half maximal effective concentrations (EC_{50}) ranging from 0.05–11 μM [46]. FL xenografts showed decreased tumor growth with venetoclax [46]. The more variable BCL2 expression in DLBCL is reflected in the fact that the sensitivity of DLBCL cell lines to venetoclax is highly variable with EC_{50}'s among 20 different cell lines ranging from 0.003 to 34.3 μM [46].

In MCL, 3 out of 8 cell lines were sensitive to venetoclax although intriguingly all primary samples tested showed sensitivity to this agent, possibly due to the fact that the primary samples were detached from the stroma with its protective niche [62]. WM cells with CXCR4 mutations are relatively resistant to ibrutinib. However, WM cells with CXCR4 mutations showed increased cell death when exposed to either ibrutinib or idealisib in combination with venetoclax [63]. Primary myeloma cells harboring t(11:14) appear uniformly sensitive to venetoclax [64].

ALL cell lines showed variable sensitivity to venetoclax with EC_{50}s ranging from 0.008 to 9.2 μM and in the RS4;11 xenograft model there was dose-dependent reduction in tumor growth in response to venetoclax [46]. Mixed lineage leukemia (MLL) ALL was sensitive to venetoclax alone [65, 66] raising the possibility of tailoring BCL2 inhibition to the individual leukemia subtype. Among AML cell lines the sensitivity to venetoclax ranged from EC_{50} 0.16 to 0.76 μM [46]. Primary AML samples died promptly and at low concentrations in response to venetoclax [67], especially AML cells with IDH1 (isocitrate dehydrogenase) and IDH2 mutations [68]. Similarly, AML xenografts were sensitive to venetoclax [67].

Administration and Pharmacokinetics

The phase I first-in-human study of venetoclax was a dose-finding study and tested oral venetoclax in CLL and NHL patients at once daily doses ranging between 150 and 1200 mg [47, 69]. The major toxicity associated with venetoclax in CLL was tumor lysis syndrome (TLS), which could be ameliorated by the implementation of slow dose escalation along with routine xanthine oxidase inhibition and hydration [47]. No maximum tolerated dose (MTD) was identified. In CLL, the recommended phase II dose (RP2D) was 400 mg daily [47].

The peak plasma concentrations of venetoclax were found at 6–8 h post first dose and the half-life after a single 50 mg dose was 19 h [47]. The steady state exposure to venetoclax is proportional to dose. Importantly, the pharmacokinetics of venetoclax were not affected by co-administration with rituximab [70].

Clinical Outcomes

In the phase I first-in-human study of venetoclax among 116 patients with relapsed/refractory CLL or SLL treated with venetoclax monotherapy [47] the ORR across all risk subgroups was 79% despite being a study heavily enriched for poor prognosis

CLL [47] with a 20% CR rate and 5% of patients who achieved bone marrow minimal residual disease (MRD) negativity by flow cytometry. While these results are very encouraging the reported cumulative rate of disease progression after a median of 17 months was 35%, with 16% of patients experiencing a Richter's transformation (RT) (most commonly to DLBCL) [47]. The 2 year overall survival (OS) estimate was 84% and the 15 months PFS estimate at a dose of 400 mg daily was 69%, with a median PFS of 25 months (95% confidence interval [CI] 17–30 months) among the dose escalation cohort [47]. Despite equivalent overall response rates, progression appeared to be more common among patients with del17p. In patients with del17p the median PFS was only 16 months (95% CI 11–25 months) in contrast to those without del17p among whom the median PFS was not reached (estimated at 71% at 15 months) [47]. The duration of response was longer among those who achieved a CR compared with those whose best response was PR [47].

These findings were confirmed in a phase II open label study of venetoclax among relapsed/refractory CLL patients with del17p in which 107 patients were treated with 400 mg orally once a day [71]. There was a similar ORR and PFS amongst all prognostic categories with the response being unaffected by: refractoriness to prior therapies, proportion of cells with del17p, presence of *TP53* mutation and other poor prognostic markers [71]. Among all patients the median time on treatment was 12.1 months during which time 37 patients discontinued study drug. The reasons for discontinuation were disease progression in 24 (11 due to RT), adverse events in 9, withdrawal of consent in 2, non-compliance in 1 and allograft in three [71]. The estimated 12-month PFS was 72% with an estimated 12-month OS of 86.7%, an estimated 12-month event free survival (EFS) of 70% and a 12-month time to progression (TTP) of 77% [71]. Among the patients achieving CR, 100% continued to respond to venetoclax at 12 months on study. Among the 24 patients with progressive disease the TTP was shorter in the patients who progressed with RT (4.7 months) compared to the patients who progressed with CLL (6.3 months) [71]. Interestingly in a sub-group analysis of patients who had previously progressed on ibrutinib or idealisib, the majority of these patients responded to venetoclax [71].

Venetoclax (200–600 mg) has also been tested in combination with rituximab (monthly for 6 months) in a phase Ib dose finding study of 49 patients with relapsed/refractory CLL [70]. In this study, the ORR was 86% with improved rates of CR (51%) of whom 80% of patients were negative on MRD testing [70]. As with the single agent studies similar OR and CR rates were seen across all prognostic groups analyzed. In this study, disease progression on treatment was seen in 11/49 patients. Among these 6 progressed with CLL (all patients in whom the best response was PR) and 5 progressed with RT (all transformations were seen at less than 9 months on study) [70]. However, the deeper responses seen with combination therapy and were associated with more enduring disease control; for instance, the 2 year estimates for freedom from progression (FFP) and ongoing response were 82% and 89%, respectively [70]. The TTP was not reached in this study but the 2-year progression free estimate was 82% (95% CI

66–91 months). The two-year estimate for ongoing response was 89% (95% CI 72–96 months) with deeper responses being more durable [70]. For instance, the 2-year estimate for ongoing CR was 100% (95% CI 100–100) while those for ongoing PR or MRD positive disease response were 73% (95% CI 42–89) or 71% (95% CI 39–88), respectively.

Deep and enduring responses raise the possibility of prolonged remissions or even 'cure' with combination therapy. Hence, 13 CLL patients on the phase Ib study of venetoclax in combination with rituximab who achieved either CR or PR with bone marrow MRD negativity discontinued venetoclax. At the time of publication 11 patients who were MRD negative remained off treatment with no evidence of progression. The two patients who were MRD positive developed disease progression after 24 months off treatment but then responded to re-institution of therapy with venetoclax [70].

The phase I first-in-human study of venetoclax monotherapy also included an arm for 106 patients with relapsed/refractory NHL treated in dose escalation and safety expansion cohorts at doses from 200 to 1200 mg daily [69]. The study encompassed a diverse range of lymphomas including MCL, FL, DLBCL, DLBCL due to RT, WM and marginal zone lymphoma (MZL). The ORR for the study was 44%. However, the response rates varied with the NHL subtype, for instance: MCL ORR was 75% with 21% CR; FL ORR was 38% with 14% CR; DLBCL ORR was 18% with 12% CR; in RT DLBCL ORR was 43% with no CR; MZL ORR was 67% with no CR; and WM ORR was 100% with no CR [69].

In keeping with the CLL findings, among patients achieving CR the responses appeared more durable than those in patients whose best response was PR. Also in keeping with the findings from navitoclax testing in CLL, the strength of BCL2 expression by immunohistochemistry (IHC) did not correlate with resistance to venetoclax among NHL patients [69].

At the time of publication 87/106 NHL patients had discontinued the study due to: progressive disease (PD) (77), adverse events (3), change in management to allograft (3), withdrawal of consent (2) and non-compliance/investigator decision (2) [69]. Among these, 87 patients exiting study, 38 have subsequently died; 10 within 30 days of coming off study (all due to PD) and 28 at more than 30 days after coming off study (among whom 24 died of PD) [69].

In a phase I study of venetoclax in combination with the proteasome inhibitor bortezomib, which can indirectly inhibit MCL [72, 73], an ORR of 50% with a median DOR of 5–9 months (range 0–14.1 months) was observed among 41 patients with relapsed/refractory myeloma. Efficacy was largely restricted to patients who were either bortezomib naive or had responded to previous exposure to bortezomib [74].

In a phase Ib study of venetoclax in combination with decitabine or azacitidine in treatment naive elderly patients with AML, among 19 evaluable patients there were 14 CRs, 2 PRs and 3 resistant. During the follow-up period no relapses were seen among the patients who achieved objective responses [75].

Clinical Effect of Resistance

Our group has recently analyzed 67 treated patients with venetoclax for relapsed/refractory CLL/SLL [76]. Twenty-five (37%) patients progressed during a median follow up of 23 months of whom 17 had a RT; 14 DLBCL and 3 HL [76]. RT was manifested by B symptoms with or without cytopenias in 3 cases and by asymptomatic progressive lymphadenopathy in 14 cases [76]. In the majority of patients with RT PET scans revealed multifocal sites of FDG avidity and in all cases of DLBLC RT BCL2 overexpression was present on immunohistochemistry [76]. The median time to progression with RT was 7.9 months compared with 23.4 months for those who progressed with CLL ($p = 0.003$) [76]. On univariate analysis the highest risk for progression was seen among patients with either fludarabine refractory disease or complex cytogenetics [76]. Other high risk features such as advanced age, multiple lines of prior therapy, deletion 17p, deletion 11q and TP53 mutations were not associated with the risk of progression [76].

Six of 8 patients with progressive CLL/SLL on venetoclax were subsequently treated with ibrutinib and of these five achieved a PR with three remaining alive on therapy at 6, 6, and 9 months of follow up [76]. The treatments for RT included chemotherapy followed by consolidation with autograph (2), allograft (2) or radiotherapy (2) [76]. A further 10 patients with RT received chemotherapy alone and one patient was managed with palliative care alone [76]. Three patients with DLBCL RT who responded to salvage therapy subsequently progressed with CLL/SLL and remain alive on BTK inhibitors at 30, 34, and 38 months [76]. Median post progression survival for HL RT, DLBCL RT and progressive CLL/SLL was not reached, 10.9 and 8.6 months respectively [76].

In the phase I first in human study of venetoclax in relapsed/refractory NHL the estimated PFS for 106 patients was 6 months (95% CI 4–10 months). However, PFS varied by histology being longer in disease subtypes associated with deeper clinical responses; for example, the median PFSs were 14, 11, and 1 months for MCL, FL and DLBCL, respectively [69]. In WM, the DOR varied from 11.1–41.5 months and in MZL it varied from 2.3–23.6 months. Overall, the estimated 12 month OS was 70% but varied by subtype being 100%, 82% and 32% for FL, MCL and DLBCL, respectively [77].

Molecular Mechanisms of Resistance

In CLL, in vivo mechanisms of resistance to BCL2 inhibitors are not yet fully elucidated but there are emerging clinical data pertaining to a heterogeneous group of implicated molecular pathways (Table 3). Identifying the molecular drivers of resistance is an area of active genomic research. While point mutations in the drug-binding site of the target protein BTK are a common form of resistance to the drug ibrutinib, to date analogous mutations in the drug- binding interface of BCL2 have not been identified in patients treated with venetoclax.

Table 3 Potential mechanisms of resistance to BCL2 inhibiting BH3 mimetics

	Cell lines	Mouse models	Primary samples
BCL2 mutations	Mouse lymphoma cells with BCL2 mutations resistant to venetoclax [78]		
BAX mutations	Mouse lymphoma cells with BAX mutations resistant to venetoclax [78]		
Reduced BCL2 expression	Resistant FL cell lines down regulate BCL2 [79]		
Up-regulation MCL1 + $BCLx_L$ expression	MCL1 and $BCLx_L$ conferred resistance to venetoclax in MCL cells cultured on fibroblasts this was lost when cells were detached from fibroblasts [62] In myeloma cell lines resistance is mediated by MCL1 and sensitivity is correlated with high BCL2, low $BCLx_L$ and low MCL1 [80] In human tumor cell lines cyclin E depletion with CDK inhibitors decreased MCL1 protein levels restoring sensitivity to BH3 mimetics [81]	Multiple myeloma xenografts that co-expressed $BCLx_L$ or MCL1 with BCL2 were resistant to venetoclax [80] In a variety of ALL xenograft models there was increased killing with dual $BCLx_L$ and BCL2 inhibition compared with BCL2 inhibition with venetoclax alone [65]	MCL cells mobilized in patients treated with ibrutinib were highly sensitive to venetoclax [62] In primary CLL cells BCR signaling up-regulates MCL1 conferring resistance to venetoclax. This can be overcome by SYK inhibitors which prevent BCR mediated MCL1 induction [82]
Micro-environment mediated protection			CLL cultured on a stromal growth layer is resistant to venetoclax, this is overcome by co administration of anti CD20 antibodies [83] When MCL cells were cultured in a lymphoid like environment they become resistant to venetoclax; this can be overcome with co-treatment using the anti CD20 antibody obinutuzumab [84]

(continued)

Table 3 (continued)

	Cell lines	Mouse models	Primary samples
ERK activation	In FL cell lines activation of ERK protects against venetoclax induced apoptosis inhibition of PI3κ increased apoptosis due to venetoclax [79]		

Summary of representative published data for purported mechanisms of resistance to BH3 mimetics

In BCL2 mutations render mouse lymphoma cells resistant to venetoclax [78]. Acquired mutations in BCL2 family proteins have also been shown to confer in vitro resistance to venetoclax in lymphoma cell lines [78]. However, we have looked specifically for these mutations among our resistant CLL patients and to date we have been unable to demonstrate an association between mutations in BCL2 and venetoclax resistance (unpublished).

Previous studies utilizing IHC [69] and protein expression by western blotting [45] have failed to demonstrate a strong correlation between resistance to BH3 mimetics in patients and the protein expression of family members within the malignant cells. To date only preliminary ad hoc analysis of IHC is available at the time of CLL progression on venetoclax. In work by our group, (unpublished), all 14 patients with DLBCL RT were IHC positive for BCL2. This suggests that, at least in DLBCL RT, BCL2 down regulation is not the mechanism underlying disease progression.

Among NHL patients in the phase I study, 41/46 patients assessed had high BCL2 expression by IHC and this was not correlated with either best response or PFS [69]. High BCL2 IHC expression was seen in all NHL subtypes including MCL, FL, DLBCL and WM (all >75%) [69]. When both BCL2 and c-MYC expression were assessed by IHC among the DLBCL patients, the double expresser status did not predict for an objective response either [69].

Increased MCL1 and $BCLx_L$ expressions have been associated with resistance of MCL cells to venetoclax when cultured on fibroblasts; this resistance, however, is lost if the cells are detached [62]. Similarly, MCL cells mobilized in vivo by ibrutinib are sensitive to venetoclax [62]. In myeloma cell lines, MCL1 can mediate resistance to venetoclax and targeting MCL1 results in the death of 70% of myeloma cells [59]. In primary myeloma cells, sensitivity to venetoclax is correlated with increased BCL2, reduced $BCLx_L$ and reduced MCL1 [80]. Reducing MCL1 with CDK inhibitors can overcome resistance to BH3 mimetics in human tumor cells [81]. MCL1 expression may account for the relatively poor response of non t(11;14) multiple myeloma to venetoclax monotherapy [85] compared with the higher response rates seen when it is combined with bortezomib [74], which can down regulate MCL1. In vitro, increased MCL1 from B cell receptor signaling (BCR) in

CLL results in venetoclax resistance, which can be overcome by SYK inhibition, (which deceases BCR mediated MCL1 induction) [82].

In non MLL ALL, venetoclax alone resulted in fewer objective responses among xenografts compared to dual $BCLx_L$ and BCL2 inhibition [65]. Resistant FL cell lines have been associated with reduced BCL2 [79]. BAX mutations in mouse lymphoma can also result in resistance to venetoclax [78]. Among AML patients receiving venetoclax monotherapy BH3 profiling was able to predict for patients likely to be more sensitive to venetoclax, however, it did not predict for longer duration of resistance [86]. In this study, the best predictor of sustained response was reduced $BCLx_L$ and MCL1 functions [86]. BH3 profiling using Bim peptide as a measure of mitochondrial priming for apoptosis suggested that patients with increased mitochondrial priming had better in vivo responses to venetoclax [61] and this technique may emerge as a way of predicting for venetoclax resistance. However, protein expression studies have so far been unable to demonstrate an association between relative expression of MCL1, BCL2 or $BCLx_L$ and clinical outcomes in either CLL or NHL patients treated with BH3 mimetics [45, 69].

The microenvironment can also confer resistance to venetoclax and this appears to be overcome by anti CD20 antibodies in both CLL [83] and MCL [84]. In FL xenografts the acquired resistance to venetoclax can be overcome by the addition of rituximab [79]. Furthermore, in FL cell lines the activation of ERK protects from venetoclax-induced apoptosis and this can be overcome by PI3κ inhibitors [79]. Collectively, this suggests that at least some forms of resistance to venetoclax may be overcome by rational drug combinations.

Conclusion

Venetoclax represents a significant step forward in the management of high-risk CLL and potentially a number of other hematological malignancies. However, a percentage of patients continue to be primary refractory to this agent and even among responders the PFS can be limited. Understanding the mechanisms for clinical resistance will be critical to improving outcomes for patients. It is hoped that targeting multiple intracellular cancer pathways through rational drug combinations will improve both response rates and duration of response. Proof of this concept has already been shown in CLL with the combination of rituximab and venetoclax resulting in deeper and longer lasting responses compared to monotherapy with either agent alone [70]. Venetoclax combination studies are currently underway in a variety of disease subtypes and in a variety of combinations including: with ibrutinib in CLL and MCL (NCT02756897 and NCT02419560); with bortezomib in multiple myeloma [74]; and in combination with standard chemotherapy in NHL (NCT02055820). Identifying biomarkers for resistance will help to target which patients require combination therapies for optimal results.

Ongoing clinical trials are being undertaken to address all these questions. Enhanced molecular understanding of the genetic features of CLL determining

depth of response to venetoclax, and progression of disease while on treatment with this agent, will be necessary to identify which patients are most likely to benefit and to target combinations to optimize long-term outcome.

Conflict of Interests MAA and AWR are employees of the Walter and Eliza Hall Institute of Medical Research which receives milestone and royalty payments related to venetoclax.

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