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REVIEWS

The manipulation of apoptosis for cancer therapy using BH3-mimetic drugs

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Preface/abstract:
Apoptosis is a form of programmed cell death that is regulated by the balance between pro-survival and pro-apoptotic BCL-2 protein family members. Evasion of apoptosis is a hallmark of cancer which arises when this balance is tipped to favour survival. Anti-cancer therapeutics, termed BH3-mimetics, have been developed to directly activate the apoptosis machinery in malignant cells. These drugs bind to and inhibit specific pro-survival BCL-2 family proteins, thereby mimicking their interaction with the BH3 domains of pro-apoptotic BCL-2 proteins. The BCL-2 specific inhibitor venetoclax/venclexta is approved by the FDA and many regulatory authorities worldwide for the treatment of chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML). BH3-mimetics targeting other BCL-2 pro-survival proteins have been tested in pre-clinical models of cancer and drugs targeting MCL-1 or BCL-XL have advanced into phase 1 clinical trials for certain cancers. As with all therapeutics, efficacy and tolerability need to be carefully balanced to achieve a therapeutic window whereby there is significant anti-cancer activity with an acceptable safety profile. Here we review the current state of BH3-mimetics targeting various pro-survival BCL-2 proteins and discuss emerging data regarding primary and acquired resistance to these agents and approaches that may overcome this. We highlight issues that need to be addressed to further advance the clinical application of BH3-mimetic drugs, both alone and in combination with additional anti-cancer agents, for improved therapy.

Introduction

The BCL-2 regulated pathway to apoptosis (Figure 1) is critical to remove damaged or superfluous cells (reviewed in[1-4]). This pathway becomes activated in response to diverse cellular stresses, including DNA damage, growth factor deprivation and oncogene activation. Such stimuli lead to upregulation of the pro-apoptotic BH3-only proteins (BIM, PUMA, NOXA, BID, BAD, BMF, BIK and HRK) by transcriptional and post-transcriptional mechanisms. These proteins bind via their BH3 domain into the hydrophobic pocket of pro-survival proteins (BCL-2, MCL-1, BCL-XL, A1/BFL1, BCL-W) (shown for MCL-1:BIM interaction in Figure 2a). This leads to the liberation and consequent activation of the pro-apoptotic effector proteins BAX and BAK that oligomerise and cause a breach in the mitochondrial outer membrane[5-11]. Cytochrome c and other apoptogenic factors are then released from inside the mitochondria, triggering formation of the apoptosome. This in turn activates caspase-9, initiating a cascade of effector caspases that cleave hundreds of proteins and thereby dismantle the cell in an orderly fashion (Figure 1). Several layers of selectivity in the pathway exist: different cellular stresses preferentially induce different BH3-only proteins (reviewed in[12]) and the different BH3-only proteins have selective binding to different BCL-2 family pro-survival proteins[13-15].

A wealth of data has demonstrated that defects in apoptosis signalling promote tumorigenesis and can render malignant cells resistant to diverse anti-cancer agents (Box 1 summarises the key discoveries in this field). BH3-mimetic drugs have been developed to directly induce apoptosis in malignant cells by binding and inhibiting select pro-survival members of the BCL-2 protein family (Box 2 describes the development of BH3-mimetic drugs; Table 1 provides the names and structures of the leading compounds). It is timely to review this rapidly evolving field. Firstly, there is an expanding range of indications for the BCL-2 inhibitor venetoclax approved by the FDA and many regulatory authorities worldwide, and several ongoing clinical trials with other BH3-mimetic drugs. Secondly data regarding resistance on venetoclax in patients with chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) are emerging. Finally, there is compelling evidence that BH3-mimetics targeting other pro-survival BCL-2 family proteins, either used as monotherapy or in combination with venetoclax or other anti-cancer agents, could be highly effective at inducing robust and durable regressions of diverse tumour types, provided on-target toxicities to healthy cells could be minimised. The latter has invigorated efforts to develop strategies to target BH3-mimetic drugs specifically to cancer cells. We focus our review on these emerging clinically focused issues, referring readers to other informative reviews describing apoptosis and the detailed rationale behind the development of BH3-mimetic drugs[2,3,16,17].

Targeting BCL-2 pro-survival proteins - pre-clinical studies

Early work using gene knockout (ko) mice predicted the cell types which might be impacted by on-target toxicities associated with therapeutic targeting of BCL-2 pro-survival proteins (Summarised in Box 3). Fortunately, observed toxicities from BH3-mimetic drugs are lower than predicted from these models. Even for MCL-1, which has an essential role in many tissues[15,18-20], there is evidence to suggest that a therapeutic window exists for some cancers, which can be exploited by MCL-1 inhibitors[21]. Specifically, it was shown that loss of a single allele of *Mcl-1* was sufficient to kill MYC-driven lymphomas *in vivo*, and importantly, healthy mice could tolerate loss of one allele of *Mcl-1*, even when administered with patient-relevant doses of chemotherapeutic drugs[22]. The disconnect between predicted and observed toxicities

could be explained by the transient and possibly incomplete nature of the inhibition of pro-survival proteins by BH3-mimetic drugs, compared with complete and irreversible protein loss in gene knockout mice. In order to identify cancers that may benefit from use of particular BH3-mimetic drugs, these compounds have been used to explore the dependency of a wide range of human cancer derived cell lines and mouse models on particular pro-survival proteins.

BH3-mimetics as monotherapy

Given the prevalence of high levels of BCL-2 expression among haematological malignancies, for example in follicular lymphoma (FL)[23,24] and CLL[25], BH3-mimetics were initially trialed as monotherapy in these diseases. Preclinical work with ABT-737, the precursor to navitoclax (targets BCL-2, BCL-XL, BCL-W with high affinity (K*i* <1 nM)), and the BCL-2 selective inhibitor venetoclax (binds with high affinity to BCL-2 (K*i* <1 nM) and much less avidly to BCL-XL (K*i* ~50 nM) and BCL-W (K*i* >200 nM)) found clear efficacy in CLL[26,27], AML[28,29] and myeloma harbouring t(11;14)[30]. Responses to single agent venetoclax have also been observed in certain other haematological cancers and solid malignancies, such as estrogen receptor-positive breast cancer[31], and some BCL-2 positive small cell lung cancers[32]. These findings have led to a multitude of clinical trials employing venetoclax. Servier have also developed an orally bioavailable BCL-2 selective inhibitor called S55746 that showed potent efficacy against primary cells from CLL and MCL patients *in vitro* and in xenograft models in preclinical studies[33]. S55746 and a related compound S65487 that is administered intravenously have progressed into clinical trials for several haematological malignancies.

The MCL-1 selective inhibitors S63845, AZD5991 and AMG 176 have shown promising results in cancer derived cell lines and mouse models of AML, multiple myeloma (MM), and non-Hodgkin lymphomas (NHL)[21,34,35]. MCL-1 inhibitors may also have a role in combination with venetoclax, particularly in AML[36-39] and B-cell acute lymphoblastic leukaemia (B-ALL)[40,41], for example by combatting drug resistance caused by upregulation of MCL-1 following venetoclax exposure. Xenografts of human cancer cells in mice are commonly employed to test the impact of BH3-mimetics *in vivo*, but one caveat when conducting preclinical studies of MCL-1 inhibitors in mice is that three of the leading compounds, S63845, AMG 176, and AZD5991, have a significantly higher affinity for human MCL-1 than mouse MCL-1 (6-fold, 1000-fold and 25-fold, respectively)[21,34,35]. This means both the efficacy of these compounds against cancer cells and damage to normal tissues is underestimated in murine studies. To improve the accuracy of preclinical studies using these MCL-1 inhibitors, mice carrying the human MCL-1 coding regions in place of murine MCL-1 have been generated[34,42]. Cells from these mice show greater sensitivity to MCL-1 inhibitors than their wildtype counterparts. Encouragingly, a therapeutic window for targeting cancer cells expressing human MCL-1 in these humanised MCL-1 mice could still be established[34,42].

BH3-mimetics targeting BCL-XL are effective in cell line studies for aggressive human NK/T-cell lymphomas, subsets of MM[43,44] and Hodgkin lymphoma[45] *in vitro* and *in vivo*[46]. Targeting BCL-XL may be more promising for killing many blood cancers than appreciated from *ex vivo* studies, due to signalling from stromal cells to the malignant cells in the lymph nodes and bone marrow, which can elevate expression of BCL-XL *in vivo*, for example through NF-kB pathway activation[47-49]. However, challenges remain with the *in vivo* use of BCL-XL inhibitors due to on-target toxicity to platelets[50].

BH3-mimetics in combination drug regimens

The survival of many solid cancer cells is safeguarded by both MCL-1 and BCL-XL[51], and a myriad of studies in human cancer cell lines and mouse models have highlighted the effectiveness of co-targeting these proteins, including for cervical cancer[52], paediatric solid tumours, including osteosarcoma and neuroblastoma[53], melanoma[54], lung squamous cell carcinoma[55], head and neck squamous cell carcinoma[56], malignant pleural mesothelioma[57] and colorectal cancer[58]. Combining MCL-1 inhibitors with navitoclax (thereby inhibiting MCL-1, BCL-XL, BCL-2 and BCL-W) showed promise for treatment of refractory melanoma[59] and cervical cancer[60]. Simultaneous deployment of BCL-XL and MCL-1 inhibitors *in vivo,* however, has not yet been achieved safely, with one attempt resulting in acute liver toxicity in mice[55], a predictable outcome based on studies of BCL-XL/MCL-1 conditional double knockout mice showing that these proteins are required for the survival of hepatocytes[61] (see also Box 3). Employing creative dosing schedules may assist in limiting toxicity, however, advancements in the delivery of BCL-XL and/or MCL-1 inhibitors to achieve preferential or even specific targeting of malignant cells to reduce toxicity will likely be required for this drug combination to achieve clinical potential[62,63].

BH3-mimetic drugs synergise with diverse standard-of-care anti-cancer agents, including chemotherapeutic drugs and oncogenic kinase inhibitors (OKIs). This synergy can be explained by these drugs causing increases in BH3-only proteins, such as BIM, PUMA and NOXA, that inhibit the pro-survival BCL-2 proteins that are present in malignant cells but not neutralised by the BH3-mimetic used[64-66]. For triple-negative or HER2-amplified breast cancer, the MCL-1 inhibitor S63845 synergised with docetaxel (microtubule stabilising chemotherapeutic), lapatinib (tyrosine kinase inhibitor), and trastuzumab (monoclonal antibody targeting HER2)[67], while targeting BCL-XL enhanced the effectiveness of BET inhibitors for killing of triple-negative breast cancer-derived cell lines[68]. The MCL-1 inhibitor S63845 could overcome TRAIL resistance in melanoma cell lines[69], and addition of venetoclax re-sensitised aggressive c-MYC-driven lymphomas with elevated BCL-2 expression to BET inhibitors, leading to a reduction in tumour burden and prolonged survival of DLBCL xenograft bearing mice[70]. Neutropenia is one of the established side effects of BCL-2 inhibition[71]; caution must therefore be used when combining BCL-2 inhibitors with chemotherapeutics that also impact this subset of immune cells.

OKIs target pathways essential for tumour cell survival and proliferation, resulting in stabilisation of malignant disease, but not necessarily regression. Addition of BH3-mimetics to drive apoptosis presents an attractive strategy to eliminate cancer cells after OKI treatment, which frequently induces cytostasis in sensitive cancer cells[72]. Combining MEK inhibitors with BH3-mimetics induces tumour regression in KRAS- and EGFR-mutant non-small cell lung cancers in genetically manipulated mice or human tumour xenografts[73,74] as well as in human BRAF-mutant colon, pancreatic and melanoma derived cell lines[75]. A more durable response to EGFR inhibitors was achieved in a human lung adenocarcinoma xenograft model through application of ABT-737 (inhibitor of BCL-XL, BCL-2 and BCL-W; tool compound)[76]. Furthermore, the combination of navitoclax (inhibitor of BCL-XL, BCL-2 and BCL-W that has entered clinical trials) and an Aurora kinase A inhibitor was synergistic for patient-derived xenografts of aggressive alveolar rhabdomyosarcoma[77]. The Ascentage Pharma BCL-2 inhibitor APG-2575 showed synergistic killing of human DLBCL cell lines with high BCL-2 expression when combined with BTK inhibitors or a novel MDM2 inhibitor[78], and this agent is currently being evaluated in clinical trials for a range of haematological malignancies as monotherapy (NCT03537482 and NCT04215809) and in combination with rituximab or acalabrutinib or voruciclib (NCT04215809).

BH3-mimetic drugs have also been paired with hypomethylating agents (HMAs), the current standard-of-care for myelodysplastic syndromes (MDS), a class of disorders that can progress into AML[79]. HMAs have been shown to reduce the levels of MCL-1 in primary AML cells[80] and can also induce the DNA damage response, leading to upregulation of BH3-only proteins, such as NOXA[81]. MDS-associated CD34+ myeloblasts express substantial BCL-2[82], providing a rationale for combining HMAs with venetoclax. Preclinical studies using primary patient MDS or AML samples found that this combination was synergistic *in vitro*[83,84].

It is clear from these studies that the ability to target pro-survival proteins, either only one member or two in combination, offers extensive opportunities for improving the effectiveness of a myriad of currently used anti-cancer drugs for a wide range of malignancies.

BH3-mimetics in the clinic

BCL-2 inhibitors

Whilst venetoclax is the furthest advanced in the clinic, the first *bona fide* BH3-mimetic to enter phase I clinical trials was navitoclax, the orally bioavailable small molecule with high binding affinity to BCL-2, BCL-XL and BCL-W[85], used initially in patients with relapsed and refractory (RR) non-Hodgkin lymphoma (NHL)[86] and CLL[87]. Overall response rates (ORR) of up to 22% for NHL and 35% for CLL were observed, all of which were partial remissions (PR) and translated into modest progression free survival (PFS) of 16 months[86] and 25 months, respectively[87]. Furthermore, combining navitoclax with rituximab in CD20 positive lymphoproliferative disorders[88] demonstrated a significantly superior ORR compared to rituximab monotherapy in CLL[89]. However, the clinical development of navitoclax was hampered by thrombocytopenia[86,87] due to on-target inhibition of BCL-XL in platelets which is crucial to their maintenance in the blood[50,90]. This limited dose escalation of navitoclax above 300 mg per day prevented full exploration of the clinical potential of this therapy. Despite this, the promising early phase clinical efficacy underpinned impetus for development of a BCL-2 selective inhibitor.

In 2013 the first BCL-2 selective inhibitor, venetoclax, was reported[27]. The efficacy of venetoclax is dependent on BAX and BAK, as evidenced by the inability of the drug to kill cells that lack these effectors of apoptosis[91]. Furthermore, markers of apoptotic death of CLL cells were observed in patients after dosing with venetoclax[91]. Venetoclax has shown activity in a range of malignancies and is approved by the FDA and other regulatory authorities for a number of haematological diseases with emerging evidence of activity in some non-haematological neoplasms (Table 2). BCL-2 inhibitors have also been developed by other pharmaceutical companies including, but not limited to, Servier (drugs termed S55746 and S65487) and are being tested in clinical trials (Table 2), but so far no results have been published (conference abstract[92]). These trials include S65487 as monotherapy for treatment relapsed refractory AML, NHL, MM, CLL (NCT03755154), S55746 as monotherapy for CLL, NHL, MM (NCT02920697), AML and MDS (NCT02920541) and S65487 in combination with azacytidine in AML (NCT04742101).

Relapsed and refractory (RR) chronic lymphocytic leukaemia (CLL)

Venetoclax was first tested in humans as monotherapy in RR CLL and NHL. Even among heavily pretreated CLL patients enriched for adverse prognostic markers this drug demonstrated ORR of 79% establishing its role in the treatment of high-risk RR disease[71]. These findings were subsequently verified in a cohort of patients with 17p deletion (with loss of the

tumour suppressor TP53)[93]. The most serious adverse event identified in the early phase studies of venetoclax was tumour lysis syndrome (TLS)[71,93] which in some cases was fatal and related to the rapid death of CLL cells in patients with significant tumour bulk[94]. Validated TLS mitigation strategies, such as dose ramp-up of venetoclax over a period of weeks, use of uric acid limiting agents, hydration, close monitoring of biochemistry and stratifying patients for intensified monitoring based on TLS risk, have significantly mitigated the risk of TLS[95]. Other toxicities associated with venetoclax treatment include gastrointestinal upset and cytopenias, especially neutropenia. Importantly, as anticipated, venetoclax treatment avoids the thrombocytopenia which hindered clinical progression of navitoclax (inhibitor of BCL-2, BCL-XL and BCL-W).

A phase Ib study of indefinite venetoclax in combination with six monthly doses of rituximab demonstrated deeper clinical responses than were seen in the phase I first in human study, with 57% of patients obtaining undetectable minimal residual disease (uMRD)[96]. The deep responses achieved with the use of this combination therapy led to the hypothesis that venetoclax could be given as time limited therapy, i.e. achievement of deep responses would enable prolonged disease-free survival off active therapy. This hypothesis was tested in the Murano study where patients were randomised to 24 months of venetoclax in combination with six months of monthly rituximab (VenR). Among patients who were uMRD at the end of treatment, 60/83 (72%) were progression free at a median follow up of 59.2 months, validating a time limited strategy (conference abstract[97]). A phase 1b study of venetoclax in combination with obinutzumab and ibrutinib also showed promising results for RR CLL [98]. Sequential cycles of each drug were used to minimise the risk of TLS. At the end of the fixed-duration regimen, all 12 patients achieved uMRD in either blood or bone marrow and the 24-month estimated PFS was 92%.

In older patients with treatment-naïve CLL and comorbidities, venetoclax in combination with obinutuzumab was superior to standard-of-care chemo-immunotherapy with chlorambucil-obinutuzumab[99]. Venetoclax alongside other targeted therapies has for the first time opened the possibility of cytotoxic drug-free treatment regimens for patients in whom traditional chemotherapeutics are associated with inferior outcome[100,101]. The Captivate study explored venetoclax-ibrutinib treatment in patients with previously untreated CLL (conference abstract[102]). Patients were treated with 3 cycles of ibrutinib, followed by 12 cycles of venetoclax-ibrutinib over 12 months. Patients with uMRD were then randomised to either ibrutinib monotherapy or placebo. No significant difference in disease-free survival was found at 12 months for the ibrutinib (100%) or placebo (95.3%) arms, suggesting that fixed duration therapy is feasible if patients achieve uMRD. Those patients who did not achieve uMRD were randomised to either ibrutinib monotherapy or venetoclax-ibrutinib; in both arms, PFS at 30 months was >95%.

Lymphoma

Venetoclax has been assessed as mono- or combination therapy for a range of lymphomas. As monotherapy, venetoclax is associated with an ORR of 75% in patients with RR mantle cell lymphoma (MCL)[103]. Venetoclax was also tested in combination with ibrutinib in 23 patients with RR MCL and 1 patient with treatment naïve MCL, including approximately 50% of patients with TP53 mutations. In this high-risk group, 67% of patients achieved uMRD by flow cytometry[104]. Two patients had TLS, but other adverse events were generally low grade. Waldenstroem's macroglobulinemia (a type of NHL) appears to be particularly sensitive to venetoclax with 30 RR patients achieving ORR of 87% to venetoclax monotherapy (conference abstract[105]). The vast majority of follicular lymphomas (FL) harbour a t(14;18) translocation

resulting in overexpression of BCL-2. However, venetoclax monotherapy in 29 patients with FL achieved an ORR of only 38%[103]. The CONTRALTO study investigated the efficacy of venetoclax and rituximab *vs* venetoclax in combination with both rituximab and bendamustine (BR) versus BR alone in 163 patients with RR FL[106]. While the CR rate was highest among patients given venetoclax in combination with BR, this group was also found to have increased haematological toxicity, requiring dose modification and in some cases cessation of treatment. Why a disease characterised by BCL-2 overexpression like FL would be relatively resistant to venetoclax remains an open question. One may hypothesise that if malignant cells not only express high levels of BCL-2 but also substantial levels of one or more additional pro-survival BCL-2 proteins (e.g. BCL-XL, MCL-1) they will be less dependent on BCL-2 and hence less sensitive to a BCL-2 inhibitor than cancer cells expressing substantial BCL-2 but only low levels of other pro-survival proteins (e.g. many cases of CLL). Another explanation is that the sensitivity to BCL-2 inhibition depends not only on BCL-2 levels but also the levels of critical pro-apoptotic BH3-only proteins, such as BIM[107].

In diffuse large B cell lymphoma (DLBCL) the venetoclax response rates are poor, with an ORR as monotherapy of only 18%[103]. When venetoclax was given in combination with standard of care DLBCL treatment, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP)[108], in a study of 206 patients there was no significant improvement in CR compared with an R-CHOP historical cohort. Furthermore, venetoclax enhanced the toxicity of R-CHOP with increased haematological toxicity and infection rates, although importantly there was no increase in mortality. The use of venetoclax in combination with rituximab is currently being investigated in high-risk DLBCL (NCT03984448). Given the limited efficacy of single agent venetoclax in DLBCL, it is likely that future studies will focus on its potential for additive benefit in BCL-2-driven DLBCL such as the very high-risk 'double hit' group that have two chromosomal translocations that drive over-expression of both c-MYC and BCL-2.

Multiple myeloma (MM)

Venetoclax as monotherapy in a cohort of heavily pretreated patients with RR MM[109] demonstrated an ORR of 21%, however, among those patients with the t(11;14) chromosomal translocation the ORR was 40%, suggesting that venetoclax may be a promising treatment for this subgroup. Venetoclax was also tested in combination with bortezomib and dexamethasone *vs* bortezomib and dexamethasone alone in a randomised phase 3 study of 291 patients with RR MM (conference abstract[110]) of whom 12% harboured a t(11;14) translocation, and 79% exhibited high BCL-2 by immunohistochemistry (BELLINI trial). While venetoclax significantly improved PFS compared with placebo (conference abstract[110]), treatment related mortality (often due to infections)[111] was significantly higher in the venetoclax arm, resulting in improved overall survival (OS) in the placebo arm. This has raised significant concerns about the safety of venetoclax in myeloma especially in combination with bortezomib. The reasons for the excess incidence of treatment related mortality in the venetoclax arm in myeloma in contrast to other indications requires further examination but may relate to significant levels of pre-existing immunosuppression among myeloma patients due to hypogammaglobulinemia and extensive pretreatment. Despite this, patients with the t(11;14) translocation who were treated with venetoclax, bortezomib and dexamethasone had a favourable risk-benefit ratio due to increased response rates in this subgroup[111]. This highlights the importance of identifying biomarkers that predict tumour response to venetoclax and therefore enable optimal selection of patients for treatment.

Acute myeloid leukaemia (AML)

Venetoclax monotherapy in RR AML demonstrated that the agent is well tolerated and associated with an ORR of 19%[112]. Venetoclax in combination with hypomethylating agents (HMA) decitabine or azacytidine in treatment naïve AML who were unfit for intensive chemotherapy [113] demonstrated 67% CR in the venetoclax-HMA arm. The phase 3 trial of venetoclax plus azacytidine as first-line therapy for previously untreated patients with AML[114] randomised 431 patients to either venetoclax-azacytidine or azacytidine along with a placebo. At a median follow-up of 20.5 months the median OS increased in the combination arm *vs* the placebo arm (14.7 *vs* 9.6 months; $p < 0.001$). This held true across all high-risk molecular subgroups analysed, including for AML with IDH1/2, FLT3 and TP53 mutations. Venetoclax has also been examined in combination with intensive therapy in a mixed cohort of patients with treatment naïve or RR AML (conference abstract[115]). An ORR of 84% was achieved, with 89% of newly diagnosed and 66% of RR patients achieving a composite CR.

Acute lymphoblastic leukaemia (ALL)

As an aggressive rapidly evolving leukaemia, venetoclax monotherapy for ALL has not been extensively evaluated, however good preclinical rationale exists for dual BCL-XL/BCL-2 inhibition for this indication[116]. Combining venetoclax with low dose navitoclax was hypothesised to allow targeting of BCL-XL without dose-limiting thrombocytopenia. A phase I trial of these BH3-mimetics in combination with chemotherapy was performed on 47 patients with RR ALL or lymphoblastic lymphoma[117]. A CR of 60% was achieved, with 57% achieving uMRD. Overall, the combination treatment was well tolerated by most patients, with the primary safety concern identified being delayed haematopoietic recovery. Another avenue being investigated is the combination of venetoclax with low dose chemotherapies. Preliminary results from a phase I trial found that this combination induced profound responses in elderly treatment-naïve ALL patients, with 9/10 patients achieving uMRD and no relapses recorded at a mean follow up of 11.3 months (conference abstract[118]). These promising results warrant further investigation with larger patient cohorts.

Non-haematological malignancies

The first study to examine venetoclax for use in solid tumours was a phase 1b study of venetoclax in combination with tamoxifen for treatment of ER/BCL-2-positive breast cancer[119]. 33 patients received this combination treatment, with no adverse events reported beyond expected on-target lymphopenia. Of the 24 patients who were treated with the recommended phase II dose, a 54% response rate was achieved, with a clinical benefit rate of 75%. Further trials of venetoclax in combination with additional agents for breast cancer are ongoing, including with palbociclib (a CDK4/6 inhibitor that blocks proliferation) and letrozole (a standard-of-care agent) for ER/BCL-2 positive breast cancer (NCT03900884) (conference abstract[120]) and with fulvestrant (a novel ER antagonist) for ER positive, HER2 negative breast cancer following CDK4/6 inhibition (NCT03584009) (conference abstract[121]). The rationale for these drug combinations is to simultaneously inhibit tumour cell proliferation and induce apoptosis for effective elimination of cancer cells.

MCL-1 selective inhibitors

MCL-1 targeting BH3-mimetic drugs have been slow to reach the clinic due to challenges in their development and reservations regarding predicted on-target toxicity to many healthy cell types, including cardiac cells[15,18-20,122]. Since 2016, six MCL-1 inhibitors, developed independently by several pharmaceutical companies, (AMG 176, AMG 397, S64315, AZD5991, ABBV-467, and PRT1419) have entered clinical trials for lymphoma, MM and

AML (Table 3). On September 12th 2019, the U.S. Food and Drug Administration (FDA) placed a clinical hold on a phase I study of the oral MCL1 inhibitor AMG 397 after finding a safety signal of cardiac toxicity with the agent. As a precaution, a voluntary hold was also placed on the intravenously delivered AMG 176 in patients with AML. Clinical trials with AMG-176 have since recommenced and are ongoing with other MCL1 inhibitors, such as MIK665 (S64315) and AZD5991. Whether potential cardiac toxicity will limit the clinical application of BH3-mimetics targeting MCL-1 remains to be determined. To date MCL-1 inhibitors are not registered for clinical use.

Resistance to BH3-mimetics

Despite the promise of BH3-mimetics across a diverse range of diseases they are not curative by themselves and over time relapse appears inevitable, limiting their long-term efficacy. As the clinical trials with venetoclax have progressed, data are emerging to suggest two patterns of venetoclax resistance: upfront (primary) resistance to therapy and, more recently, secondary resistance that emerges in patients on therapy. Primary resistance to venetoclax is rare in diseases that are known to respond well to this drug, with ORR in RR CLL close to 80% and RR MCL ~75%[71,103]. Primary resistance to venetoclax is more common in B cell lymphoproliferative disorders other than CLL, for example FL (ORR 38%) and DLBCL (ORR ~18%)[103] despite high levels of BCL-2 in both malignancies. Even though substantial response rates have been observed, venetoclax has never been shown to be curative and at least in the RR setting, secondary resistance inevitably emerges with time. The reasons for this are varied. Here we delve into the factors, both intrinsic and extrinsic, that are reported to confer resistance to BH3-mimetics.

Primary resistance

Insights into the modes of primary resistance to BH3-mimetic therapy have emerged from clinical trials of venetoclax in patients with lymphoid malignancies such as FL. The reasons for the poor responses despite high BCL-2 expression in certain malignancies have not been fully resolved but may reflect dependencies on multiple pro-survival proteins. There is evidence that the BCL-2/BIM ratio may be a more reliable predictor of response than high levels of BCL-2, with low ratios conferring sensitivity to venetoclax[107] and this is discussed further in the section on predicting responses to BH3-mimetics.

Recently evidence has emerged to indicate that genetic abnormalities that affect chromatin remodelling can contribute to primary resistance, possibly due to these defects leading to increased expression of BCL-XL. Whole exome sequence (WES) analysis of 5 MCL patient samples from the AIM clinical trial, that showed primary resistance to venetoclax combined with ibrutinib, revealed genomic abnormalities affecting the SWI-SNF chromatin remodelling complex (namely mutations in SMARCA4 and ARID2 and somatically acquired copy number deletions involving SMARCA2)[123]. These tumour samples displayed high levels of *BCL-XL* mRNA, possibly due to loss of accessibility of chromatin at the *ATF3* gene locus leading to decreased expression of ATF3, a repressor of BCL-XL expression[123,124]. This raises the possibility of testing approved agents known to affect chromatin remodeling, such as vorinostat and panobinostat, in combination with venetoclax.

Insights into the factors that contribute to primary resistance to BH3-mimetic drugs targeting other BCL-2 pro-survival proteins are currently limited to pre-clinical studies. There is evidence

that high levels of BCL-XL correlate with poor responses to MCL-1 inhibitors in a diverse range of malignancies[21,34], drawing parallels to the underlying mechanism of resistance observed in MCL patients treated with venetoclax.

Cell-intrinsic secondary resistance

Early indications that mutations within BCL-2 itself could confer resistance to venetoclax came from pre-clinical studies involving the continuous culture of a mouse leukaemia cell line to achieve venetoclax resistance. The resistant cell line had acquired two BCL-2 mutations (Phe101Cys and Phe101Leu, equivalent to human Phe104Cys and Phe104Leu)[125]. These particular BCL-2 mutations have not been detected in resistant tumour samples from patients treated with venetoclax. However, a different BCL-2 mutation has recently been detected in 7/15 patients treated with venetoclax that had progressed with a CLL-type disease, but excluding those with Richter's transformation (RT)[126]. Targeted amplicon NGS of the entire *BCL-2* coding region revealed a G101V mutation in 4 samples from patients that were given venetoclax, occurring at a frequency of 26-70% of CLL cells, yet undetectable in the paired patient pre-treatment samples. In three other relapsed CLL patient samples, the G101V BCL-2 mutation was detected at a lower frequency (1.4-4.3%) of CLL cells. This same mutation was found in a separate cohort of CLL patients that progressed on venetoclax therapy[127]. Of note, the BCL-2 G101V mutated CLL cells were more resistant to venetoclax but not to other anti-cancer agents, including etoposide, cytarabine, fludarabine, and dexamethasone. This indicates that this mutation confers specific resistance to BCL-2 targeting BH3-mimetic drugs but that the BH3-only proteins that are up-regulated in response to chemotherapy can still efficiently bind and neutralise this mutant BCL-2. Structural studies revealed that the mutated G101 residue is located in the alpha 2 helix of BCL-2 and its mutation displaces the adjacent Glu152 residue, from the alpha 5 helix into the base of the P2 pocket, thereby preventing venetoclax from binding deeply into the pocket (Figure 2d)[126,128]. Plasma surface resonance studies showed that the G101V mutation reduced the binding affinity of venetoclax to BCL-2 by ~180 fold, but did not affect mutant BCL-2 protein binding to the BH3 domain of BIM, which can function without inserting as deeply into the pocket. Therefore, the ability of BCL-2 to be inhibited by BH3-only proteins, such as BIM, is retained[128]. The finding that this mutation in BCL-2 is detectable after 19-42 months on venetoclax and prior to relapse may permit its use as a biomarker of disease recrudescence.

The finding that the BCL-2 G101V mutation is sub-clonal (1.4-70% of CLL cells in relapsed patient samples) indicates that co-existing resistance mechanisms contribute to disease progression on venetoclax. Accordingly, a second acquired BCL-2 mutation, Asp103Tyr, was found in a CLL patient sample bearing the G101V mutation but in separate CLL subclones. High BCL-XL expression was found in a CLL patient with the G101V BCL-2 mutation, again in separate subclones. Another BCL-2 mutant, Phe104Ile, that also decreases venetoclax binding by changing the p2 binding pocket[128] has been described in a patient with FL[129]. It is postulated that this mutation arose through somatic hypermutation of the translocated *BCL-2* gene in FL. It remains to be determined whether therapy with BH3-mimetics targeting other pro-survival BCL-2 family proteins, such as MCL-1 inhibitors, will also result in the emergence of therapy-resistant mutated variants of the targeted protein.

Mechanistically, it seems likely that resistance to BH3-mimetic drugs may arise through loss of expression of the targeted pro-survival protein, increased expression of non-targeted pro-survival BCL-2 proteins or reduced expression of critical pro-apoptotic proteins. Recent

analyses of patients on therapy and pre-clinical studies have provided evidence for this. MCL-1 overexpression was observed in samples from CLL patients that relapsed after venetoclax therapy, a finding confirmed by cellular models of several haematological cancers[130]. The derivation *in vitro* of venetoclax resistant cell lines of diverse leukaemia and lymphoma subtypes revealed frequent upregulation of MCL-1 or BCL-XL[131]. Furthermore, co-treatment with MCL-1 and/or BCL-XL inhibitors alongside venetoclax could delay development of acquired venetoclax resistance in human CLL cell lines[132]. Studies using MCL and NHL-derived cell lines revealed selection for rare lymphoma cells that had loss of the *BCL-2* amplicon[133]. Genome-wide GOF screens containing ORFs for almost 13,000 proteins in OCI-AML cells identified enrichment for increased BCL-XL, BCL-W, BCL-2 or MCL-1 expression after venetoclax treatment[130]. Whole genome-wide CRISPR LOF screens in AML and B lymphoma cell lines have identified enrichment for sgRNAs that target genes encoding the pro-apoptotic proteins BIM, NOXA, BAX and BAK after 14 days venetoclax treatment[130,134,135]. Similarly, *Bax* was identified as the top hit from a CRISPR LOF screen performed in MCL-1 dependent mouse *Eμ-Myc* lymphoma cells treated with the MCL-1 inhibitor S63845[136].

A less predictable finding is that *TP53* gene aberrations confer resistance to BH3-mimetic drugs. Since BH3 mimetics activate apoptosis downstream of TP53 (Figure 1), it was predicted that they would be efficacious for *TP53* mutated cancers that are often unresponsive to conventional DNA damaging therapeutics. Indeed, early studies were consistent with this notion, with deep initial responses to venetoclax monotherapy observed in patients with *TP53*-mutant CLL[71,91]. However, evidence is emerging that *TP53* dysfunction is associated with earlier relapsing disease in patients with CLL treated with venetoclax[137], and in patients with AML treated with venetoclax combined with either chemotherapy or a hypomethylating agent[33,138,139]. These findings from clinical studies were confirmed in non-biased pre-clinical studies where CRISPR whole genome LOF screens in AML cell lines identified loss of TP53 as a resistance factor to venetoclax treatment[134,135,140]. Similar work identified loss of *Trp53* as a resistance factor to MCL-1 targeting BH3-mimetics in mouse *Eu-Myc* lymphoma cell lines that are highly MCL-1 dependent[136]. The underlying mechanisms surrounding resistance to BH3-mimetic drugs conferred by TP53 dysfunction are still emerging, but delayed/reduced apoptosis, genomic instability, mitochondrial reprogramming and metabolic changes as a result of TP53 loss have been postulated to contribute.

WES analysis of 8 samples from CLL patients with TP53 loss or mutation that progressed on venetoclax (4 with RT to DLBCL) revealed recurrent non-synonymous mutations in *TP53*, *NOTCH1*, *BTG1* (2 patients with missense mutations), and non-synonymous mutations in *BRAF*, *SF3B1*, *RB1, BIRC3* (not validated) and *MLL3* in individual patients, plus homozygous deletion of *CDKN2A/B* (in 3 patients) and focal amplification of *CD274* encoding PD-L1[141]. All patient samples showed signs of increasing genomic instability and there was evidence of subclonal outgrowth during venetoclax treatment. The causality of these mutations remains to be determined. *BTG1* mutations, whilst rare in pre-treated samples, were found alongside CDKN2A/B loss in some resistant samples. *SF3B1* mutations were detected pre-treatment in 0.04% and 0.033% of CLL cells and were selected for during venetoclax treatment but were probably not the single cause of resistance since they were also found alongside CDKN2A/B loss. In studies using cell lines, the deletion of CDKN2A/B made no difference to venetoclax sensitivity, possibly explaining why this deletion is always found alongside other genomic alterations in patient samples.

Evidence suggests that the presence of TP53 mutant clones alone does not preclude robust clinical responses to BH3-mimetics, but the data support the notion that BH3-mimetics should be dosed as highly as can be tolerated to ensure deep responses, thus overcoming the selective advantage associated with TP53 mutated disease[142]. Evidence from the CAVEAT study

suggests that combining BH3-mimetics with chemotherapeutics does not improve survival outcomes of patients with TP53 mutant AML[138]. Pre-clinical data suggest increased therapeutic benefit may, however, be achieved by combining BH3-mimetics that target different BCL-2 pro-survival proteins, such as BCL-2 and MCL-1, rather than including drugs that rely on TP53 for action[136]. Non-DNA damaging agents, such as venetoclax, result in better outcomes for patients with high-risk TP53 dysfunctional disease compared with standard chemo-immunotherapy based approaches in CLL. Understanding and overcoming the adverse prognostic implications of this defect remains a key challenge (conference abstract[97]).

Further modes of resistance to BH3-mimetics have been indicated from studies of patient samples and pre-clinical models. A WES analysis of DNA from CLL cells before venetoclax treatment and at the time of relapse was compared to germline DNA and found marked clonal shifts in patient samples during treatment, including changes in subclones that have CLL driver mutations, such as in *TP53*, *ATM* or *SF3B1*. However, no somatic single nucleotide variations (sSNVs) of known CLL driver mutations were consistently selected for during emerging resistance, indicating that venetoclax resistance is not driven by any one sSNV or somatic copy-number variation (sCNV) but rather by multiple changes[130].

CRISPR LOF screens in AML cells treated with venetoclax identified selection for sgRNAs targeting NFKBIA (inhibitor of NF-KB activation), genes encoding lymphoid transcription factors (IKZF5, ID3, EP300, NFAI) and ubiquitin-related processes (OTUD5, UBR5)[130]. Of note, many of these genes are mutated in B cell malignancies, consistent with the notion that their loss enhances the survival and proliferation of malignant cells. Using GOF screens, factors conferring resistance to venetoclax were components of the PKA/AMPK signalling pathway, mitochondrial energy metabolism, vesicle transport/autophagy, ribosomal proteins or components of ubiquitination[130]. The importance of mitochondrial factors in conferring resistance to BH3-mimetic drugs was apparent from several pre-clinical studies. Lymphoid cell lines selected for resistance to venetoclax had higher basal and maximal oxygen consumption, most coupled to ATP production by OXPHOS, and an overall increased respiratory capacity and OXPHOS. These venetoclax resistant cells had more mitochondria compared to normally sensitive cells[130]. Genes involved in mitochondrial organisation and function were found to be depleted in a genome-wide CRIPSR/Cas9 screen to identify genes whose inactivation sensitises AML cells to venetoclax[135].

Cell extrinsic secondary resistance

Cell extrinsic factors are also known to play a role in protecting tumour cells from BH3-mimetic drug therapy. Culturing patient-derived primary MCL and CLL cells on CD40L-expressing cells with IL-10, both cell survival promoting cytokines that are produced in the tumour micro-environment, increases resistance to venetoclax, likely because these cells have an NF-kB driven increase in BCL-XL and/or MCL-1 expression[143,144].This supports the rationale to combine BH3-mimetic drugs with BTK inhibitors that can mobilise CLL cells from their niche in lymph nodes thereby removing them from the above mentioned survival promoting factors. Recent clinical data have shown that treating RR CLL patients that progressed on venetoclax with BTK inhibitors, such as ibrutinib or zanubrutinib, can provide durable control of disease[145]. Furthermore, the combination of venetoclax with ibrutinib has proven an efficacious strategy in a variety of diseases, including CLL and MCL[104] (AIM), with ongoing studies in Marginal Zone Lymphoma (AIM2).

Predicting responses to BH3-mimetics

As is evident from the preclinical and clinical data discussed, the response of a tumour to a particular BH3-mimetic drug cannot be predicted from the expression of pro-survival proteins alone. The concept of apoptotic priming – that is, how close a cell is to committing to cell death, plays an important role[146]. The primed status of tumour cells and the amount and type of the further pro-apoptotic signal that is required to trigger cell death can be examined by BH3 profiling. This technique exposes isolated mitochondria to a panel of peptides derived from pro-apoptotic BH3-only proteins that induce apoptosis by sequestering one or more specific pro-survival proteins[146]. By measuring MOMP in response to these BH3 peptides, the individual pro-survival proteins which are protecting the cell from apoptosis can be identified. BH3 profiling can be used to predict the clinical response of cancer cells to chemotherapies[147,148] and can be used to predict sensitivity of tumours to BH3-mimetic drugs[149,150]. Recent work has shown that the primed state of a tumour and therefore its pro-survival dependency can change during drug treatment[151,152]. This is consistent with the finding that BH3-mimetic drug resistance can arise through increased expression of non-targeted pro-survival proteins in tumour cells that have escaped initial waves of cell death[130-132]. Of note, acquired resistance to one therapy can sometimes result in hypersensitivity to another. A recent study using CRISPR/Cas9 knockout screens in AML cell lines found that cells which tended to acquire resistance to bromodomain inhibitors through upregulation of c-MYC were in turn sensitised to BCL-2 inhibition[153]. BH3 profiling of tumours before and during treatment may be useful to identify patients most likely to benefit from initial or continued treatment with BH3-mimetic drugs[112,154] but in cases where tumour cells from patients are available for testing it will be more direct to examine their response to BH3-mimetics by treating them with these agents in culture.

Novel approaches to target BCL-2 family proteins

Clinical progression of compounds targeting pro-survival proteins other than BCL-2 has been somewhat restricted largely due to on-target toxicity (e.g. killing of platelets with BCL-XL inhibition[50]). This has inspired the development of strategies to target this pro-survival protein selectively in cancer cells. One such approach is based on antibody drug conjugates linking BCL-XL-specific BH3-mimetics from the WEHI/ABBVIE BCL-XL selective series with antibodies specific for tumour surface markers. AbbVie disclosed proof-of-concept data in the patent literature with BCL-XL inhibitor/antibody conjugates (ADCs) targeting EGFR (US20160158377). ABBV-155/Mirzotamab Clezutoclax, a BCL-XL inhibitor coupled to an antibody targeting B7-H3 (CD276), is currently in phase 1 clinical trials in patients with advanced solid tumours (NCT03595059). Results of these studies are eagerly awaited to learn whether this strategy will realise the potential of BCL-XL inhibitors for cancer therapy.

Other technologies that improve tumour delivery of BH3-mimetic drugs have also been employed to reduce the toxicity of BCL-XL inhibitors. The Astra-Zeneca dendrimer-conjugated BCL-2/BCL-XL inhibitor AZD0464 allows enhanced accumulation and release of this inhibitor into tumours[155], resulting in decreased cardiovascular toxicity in rats and dogs. While AZD0464 still induced thrombocytopenia, this could be moderated by intermittent dosing to allow platelets to recover. The Ascentage Pharma BCL-2/BCL-XL targeting pro-drug APG-1252 is converted to a more active form inside cells but has reduced permeability into platelets (conference abstract[156]). Both AZD0646 (NCT04214093) and APG-1252 (NCT03080311) (conference abstract[157]) are in clinical trials for haematological and/or solid cancers.

An alternative approach to limit toxicity has been to exploit the "proteolysis-targeting chimera" technology, called PROTACs[158]. This strategy employs bifunctional compounds with one module targeting the protein of interest and the second targeting an E3 ubiquitin ligase or an E3 adapter protein, most commonly the von Hippel-Lindau (VHL) or cereblon (CRBN) proteins. PROTACs function by forcing a targeted protein into the proximity of the E3 ligase, leading to its ubiquitination and ensuing proteasomal degradation. VHL is poorly expressed in platelets, thus targeting BCL-XL using a VHL-based PROTAC could spare platelet viability, overcoming the on-target toxicity seen for BH3-mimetics targeting BCL-XL. VHL targeting PROTACs incorporating BCL-XL binding warheads have been reported based on both the ABT-263[62] and the WEHI/ABBVIE BCL-XL selective scaffolds[63]. Interestingly, while ABT-263 is a potent inhibitor of BCL-XL, BCL-2 and BCL-W, the degrader molecules based on this BH3-mimetic specifically target BCL-XL (but not BCL-2) for degradation and kill cancer cells that are dependent on BCL-XL for survival, including in xenograft models, with reduced toxicity to platelets[62,159]. One such BCL-XL-degrading PROTAC, DT2216, recently entered clinical trials for relapsed/refractory malignancies (NCT04886622). PROTACs that degrade MCL-1 have also been described[160], but it remains to be seen whether utilising the PROTAC-based approach will improve the therapeutic window over classical MCL-1 inhibitors. BH3-mimetic/PROTAC strategies may provide opportunities to expand BH3-mimetic therapies to settings in which they have yet to find application due to on-target toxicities, including the simultaneous targeting of two pro-survival BCL-2 family members, such as MCL-1 plus BCL-XL, that is otherwise highly toxic.

Targeting BH3-mimetics to tumours using nanoparticles might also facilitate the progression of BH3-mimetics, especially when they are used in combination. For example, co-targeting of BCL-2 and MCL-1 is already proving a powerful combination against a number of blood cell cancers, with one trial underway (NCT03672695), but this strategy may encounter the same safety challenges as single agent MCL-1 inhibitor treatments. Cancer targeted nanoparticles have been reported to improve both the efficacy and the tolerability of a BCL-2/MCL-1 inhibitor combination in pre-clinical models of DLBCL[161]. Enabling targeted delivery of this and other BH3-mimetic combinations with innovative and clinically-safe strategies will be another exciting development in the field.

Conclusions/perspective:

In some cancer types, the responses to BH3-mimetic drugs have been transformative but challenges remain. To improve long-term response rates in patients with resistant or relapsing disease, the employment of single cell technologies that permit the early identification and characterisation of resistant clones will be critical. Similarly, more accurate pre-clinical models of disease that can be utilised to test the therapeutic potential of combination therapies that could overcome resistance to BH3-mimetic monotherapy will improve the clinical application of BH3-mimetics. A major barrier that precludes maximal administration of BH3-mimetic drugs is the on-target toxicity associated with inhibiting critical pro-survival proteins required for normal function of many non-cancerous cells. This is particularly pertinent since there is accumulating evidence from pre-clinical studies that co-targeting two or more pro-survival BCL-2 proteins (e.g. BCL-2 plus MCL-1) with different BH3-mimetic drugs can achieve long and durable responses. In order to achieve therapeutic windows, future efforts to specifically target the delivery and/or activation of BH3-mimetics to cancer cells will be essential.

Competing Interests

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BOXES

Box 1: Blocking apoptosis promotes tumorigenesis and renders malignant cells resistant to diverse anti-cancer agents

The first observations that blocking apoptosis could enhance uncontrolled proliferation and accelerate oncogene-driven tumorigenesis were made through overexpression of BCL-2[162-164]. This was recapitulated with overexpression of other pro-survival proteins[165,166] or deletion of pro-apoptotic BCL-2 family proteins (e.g. BIM or PUMA)[167-169]. Accordingly, the apoptotic pathway is frequently deregulated in human tumour cells. This can occur through overexpression of pro-survival BCL-2 proteins, e.g. through chromosomal translocations as in follicular lymphomas where there are frequent *IgH; BCL-2* (t(14:18)) translocations[170-172], gene amplifications as is reported for *MCL-1* and *BCL2L1* (that encodes BCL-XL)[173] and hemizygous or homozygous loss of certain microRNAs (e.g. miRs-15a, -16-1 and -29) that negatively regulate the expression of pro-survival BCL-2 family members[174-176]. Alternatively, apoptosis can be deregulated by silencing of pro-apoptotic proteins through epigenetic mechanisms as is reported for *BIM* and *PUMA* in Burkitt Lymphoma[168,177] or gene deletions, such as at the *BIM* locus in mantle cell lymphoma (MCL)[178,179]. These perturbations result in decreased sensitivity to diverse anti-cancer drugs, including standard chemotherapeutics and inhibitors of oncogenic kinases[180]. BH3-mimetic drugs have been developed to directly activate the cell intrinsic apoptotic machinery for cancer therapy[16].

Box 2: Development of BH3-mimetic drugs

BH3-mimetic compounds are a complex and diverse set of molecules (Table 1) developed through leveraging knowledge of the structural features that underpin BCL-2 protein family interactions. These key pro-survival protein:BH3 domain interaction sites (Figure 2a) include: (1) four hydrophobic pockets, P1-P4, on the pro-survival protein that engage conserved hydrophobic residues on the interacting BH3 domain from a pro-apoptotic member (P2 and P4 may be particularly important[181]), and (2) a charged interaction between a conserved arginine (pro-survival)/aspartate (BH3) pair[181,182].

AbbVie spearheaded BH3-mimetic drug discovery with three milestone compounds, ABT-737 (Figure 2b), ABT-263/navitoclax (Figure 2c) and ABT-199/venetoclax (Figure 2d)[26,27,183,184]. ABT-737, the first BH3-mimetic tool compound, targets BCL-XL, BCL-2 and BCL-W[26,185,186]. Improvement of ABT-737's properties led to ABT-263/navitoclax (BCL-XL, BCL-2 and BCL-W), the first BH3-mimetic to reach the clinic[85,87,184]. Optimisation of selectivity, to alleviate on-target platelet toxicity by BCL-XL inhibition, led to the BCL-2 inhibitor ABT-199/venetoclax, now a marketed drug[50,27]. This class of molecules creates a deep pocket in P2 and fill P4. Selective BCL-2 inhibitors have also been disclosed by Servier[33], e.g. S55746 which engages P1, P2 and P3[128].

BCL-XL specific inhibitors have also been developed, notably WEHI-539 (Figure 2e)[187,188] and the optimised analogue A-1331852 (Figure 2f and Table 1)[189-192]. On-target platelet toxicity[50] has hampered their progression to the clinic, although targeted approaches may circumvent this issues (see main text). These compounds create a large cavity at P2 that likely provides potency and selectivity (Figure 2e and 2f)[187,192]. Other important interactions occur through hydrophobic groups sitting in P4, and between a carboxylate and the conserved arginine.

MCL-1 inhibitors hold therapeutic potential, but their development has lagged, primarily due to toxicity concerns[193]. Servier disclosed the first potent and selective MCL-1 inhibitor, S63845

(Figure 2g)[194,195], demonstrating efficacy in multiple *in vivo* tumour models and providing evidence for an achievable therapeutic window[21]. The optimised analogue S64315 has entered clinical trials (Table 3), as have inhibitors from Astra-Zeneca and Amgen (AZD5991[196] and AMG-176[34,35]). AZD5991, AMG-176 and S63845 all create a wide hydrophobic pocket around the P1/P2 region in MCL-1 (Figures 2g-i), and S63845 leverages additional interactions at P4. Additional MCL-1 inhibitors with similarities to the Astra-Zeneca series (VU-661013) have also been disclosed[197].

BOX 3: Insights from murine models into the role of pro-survival BCL-2 family members in non-transformed cells.

Toxicity to normal cells is a major limitation of targeting pro-survival BCL-2 proteins for cancer therapy. The cell types that may be affected by individual and combined BH3-mimetic drugs can be predicted from studies of gene deletion mice.

BCL-2 deficient mice die at 4-8 weeks due to polycystic kidney disease caused by apoptosis of developing kidney epithelial cells in the embryo[198]. In the adult, BCL-2 is required for survival of mature T and B cells and melanocytes[198-201].

BCL-XL deficient mice die by E13.5 due to apoptosis of neuronal and erythroid cells. BCL-XL is also required for survival of T and B cell progenitors[202,203]. Conditional deletion of *Bcl-X* in the haematopoietic system[204,205] and deletion of a single allele of *Bcl-X*[50] revealed a critical role for this protein in the survival of reticulocytes and platelets.

MCL-1 deficient embryos fail to implant and die at ~E3.5[206]. Conditional deletion studies showed that MCL-1 is essential for the survival of a range of cell types, including T and B lymphocytes[15], cardiomyocytes[19,20], haematopoietic stem/progenitor cells[18] and neuronal progenitors[122].

Mice lacking single alleles of *Mcl-1* and *Bcl-X* exhibit developmentally lethal craniofacial defects[207]. In adult mice, deletion of single alleles of *Mcl-1* and *Bcl-X* showed that hepatocytes are reliant on this combination of proteins for survival[61]. In contrast, deletion of single alleles of *Mcl-1* and *Bcl-2* or *Bcl-X* and *Bcl-2* in adult mice caused only minimal abnormalities and mice survived to late adulthood[207], suggesting that combinations of drugs targeting these proteins may be better tolerated than co-targeting of MCL-1 and BCL-XL.

FIGURES AND TABLES

Figure 1: Schematic of the intrinsic (BCL-2 regulated) apoptosis pathway, indicating where BH3-mimetic drugs act to induce apoptosis. Cytokine deprivation, DNA damage, and cellular stress caused by oncogene activation can activate the pathway leading to upregulation of pro-apoptotic BH3-only proteins that bind to pro-survival proteins. This results in activation of the pro-apoptotic effector proteins BAX and BAK that oligomerise and cause a breach in the mitochondrial outer membrane, leading to the release of cytochrome c and other apoptogenic factors, and triggering formation of the apoptosome. This activates caspase-9, initiating a cascade of effector caspases that cause the dismantling of the cell. BH3-mimetic drugs bind to select pro-survival proteins and inhibit their function, permitting pro-apoptotic proteins to induce apoptosis.

Figure 2: Binding mode of select BH3-mimetic compounds in complex with pro-survival proteins. A) Interaction of a pro-survival protein with a BH3 domain from a pro-apoptotic relative as exemplified by the MCL-1:BimBH3 complex[208]. The BH3 domains possess 4 hydrophobic residues that interact with 4 pockets (p1 red shading, p2 green shading, p3 blue shading, p4 yellow shading) within the BH3 binding groove on the pro-survival protein. Additional interactions (circled) occur between a conserved Asp on the BH3 domain with a conserved Arg (cyan in all panels) on the pro-survival protein. Interaction between (B) BCL-XL and ABT-737 (green)[209], (C) BCL-2 and Navitoclax (ABT-263) (light green)[27], (D) BCL-2 and Venetoclax (ABT-199) (magenta)[128]. Venetoclax engages BCL-2 by creating a deep pocket in the P2 region lined by a number of hydrophobic residues[27,128]. Key interactions in the P4 pocket with ASP103 and ARG107 are believed to drive selectivity, especially the former where the equivalent residue in BCL-XL is a glutamic acid (GLU96). While all these compounds bear an acidic moiety (acysulfonamide), these do not directly mimic the Arginine-Aspartate interaction key for BH3-only protein binding (dotted line on Figure 2a), (E) BCL-XL and WEHI539 [purple][187], (F) BCL-XL and compound 4 (grey) a close analogue of A1331852[210], (G) MCL-1 and S63845 (yellow)[21], (H) MCL-1 and compound 3 (mauve)[211] a close analogue AMG 176[34], and (I) MCL-1 and AZD5991 (blue)[35]. All compounds target the p2 pocket (green shading) of the BH3 binding groove, with a selection also targeting the p4 pocket (yellow shading) and the conserved Arg (as labelled with dotted lines). Interestingly, compounds targeting MCL-1 (S63845, AMG series and AZD5991) occupy a merged pocket that spans P1 (red shading) and P2 (green shading). In all images the pro-survival protein is displayed as a transparent surface with MCL-1, BCL-2 and BCL-XL shown as brown, pink and blue ribbons, respectively. **Structures are published as cited, but these images were produced by PC.*

Table 1: Validated BH3-mimetics in preclinical/clinical phase or approved.

Table 2: Progression of BCL-2 selective BH3-mimetics through clinical trials.

Table 3: Clinical trials initiated with BH3-mimetics targeting MCL-1.

Figure 1

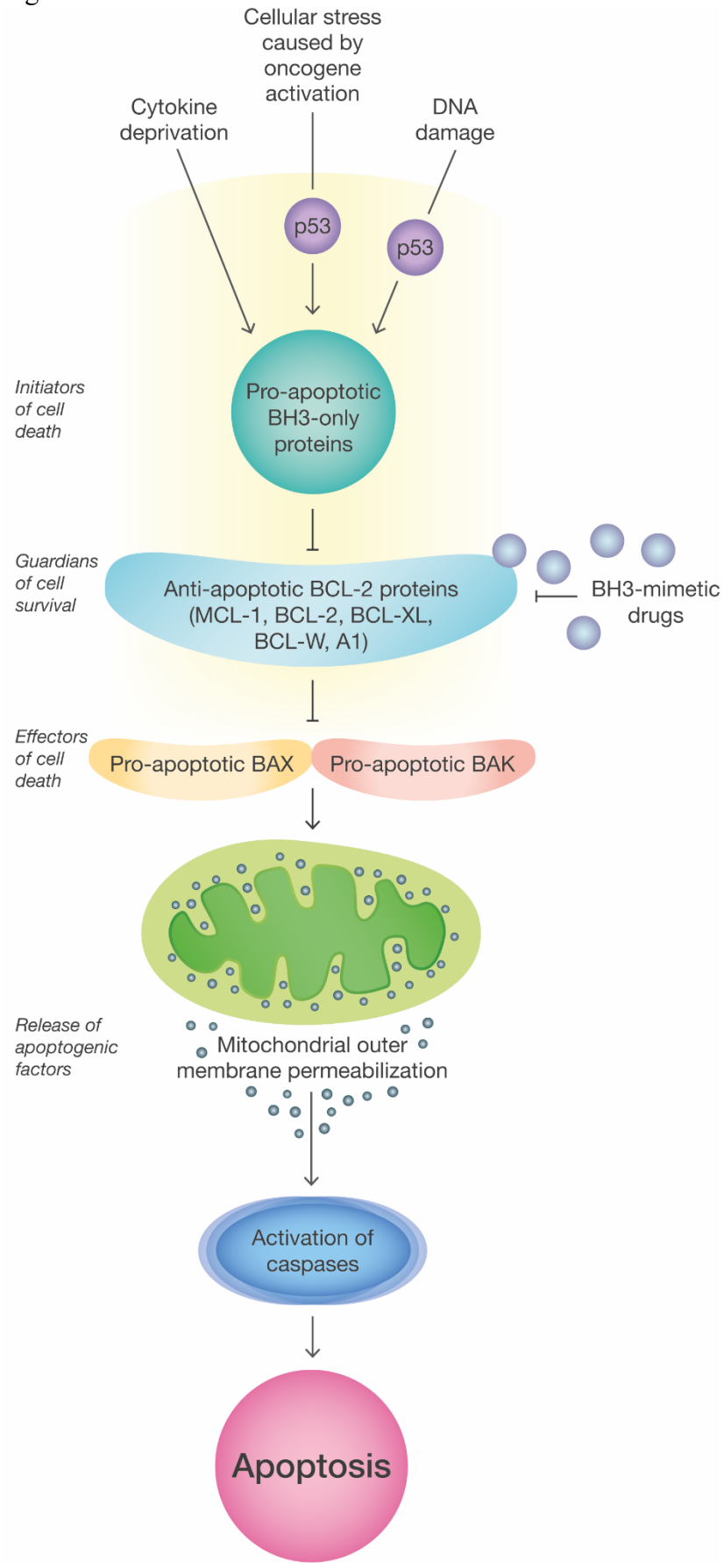

Cellular stress caused by oncogene activation
Cytokine deprivation
DNA damage
p53
p53
Initiators of cell death
Pro-apoptotic BH3-only proteins
Guardians of cell survival
Anti-apoptotic BCL-2 proteins (MCL-1, BCL-2, BCL-XL, BCL-W, A1)
BH3-mimetic drugs
Effectors of cell death
Pro-apoptotic BAX
Pro-apoptotic BAK
Release of apoptogenic factors
Mitochondrial outer membrane permeabilization
Activation of caspases
Apoptosis

Figure 2

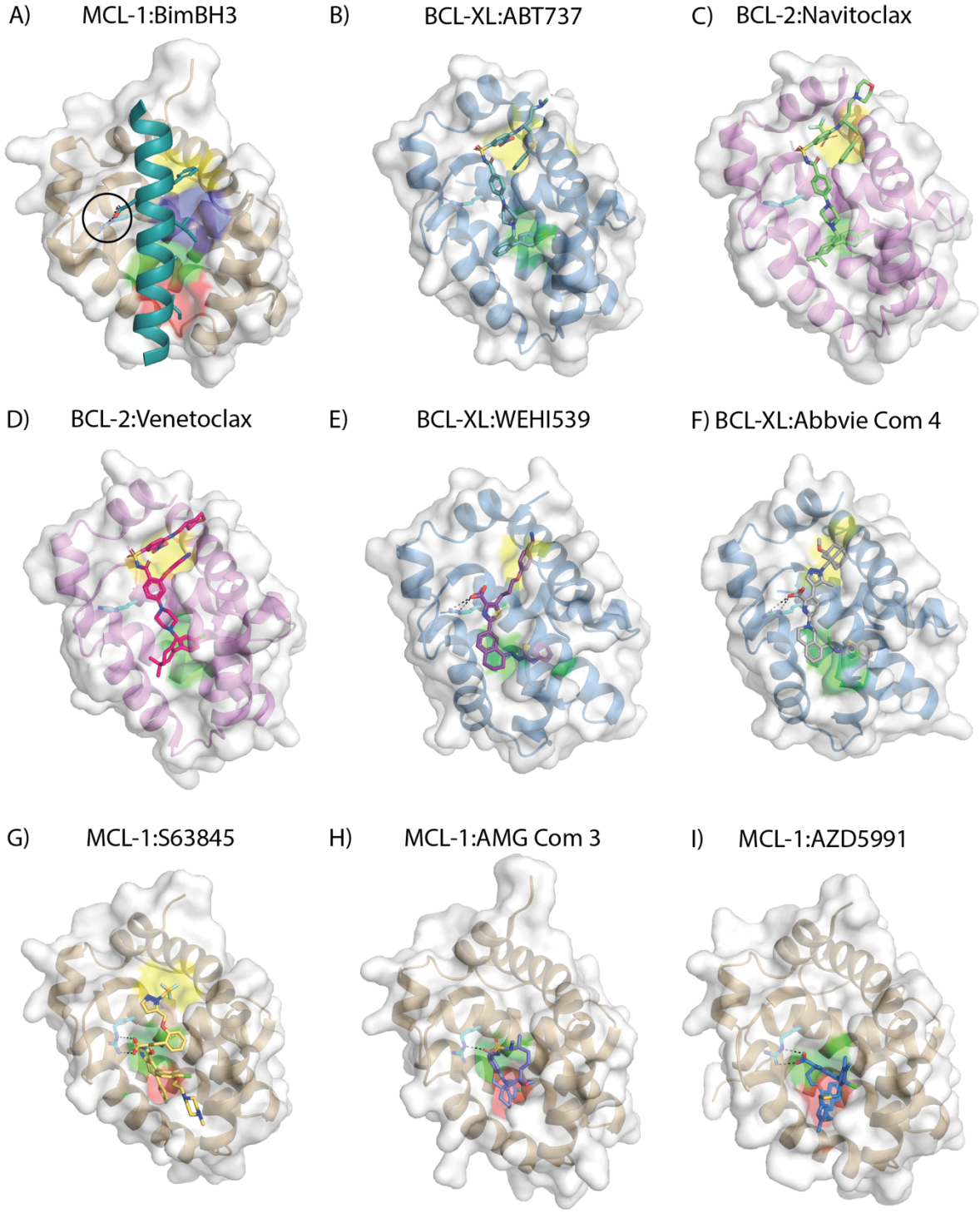
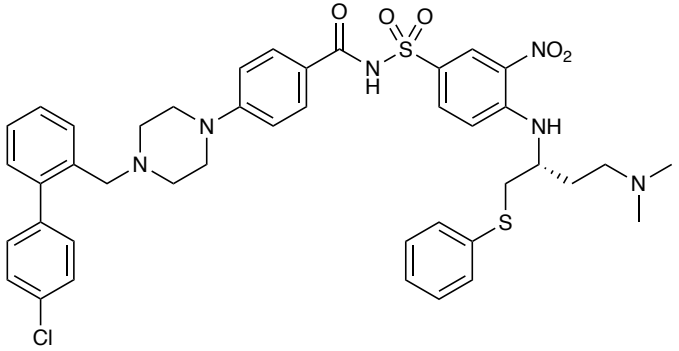
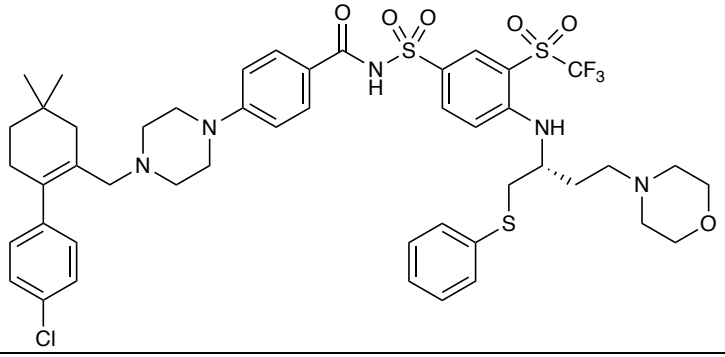
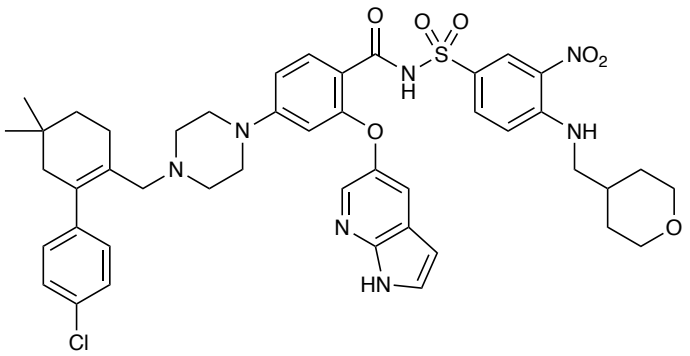
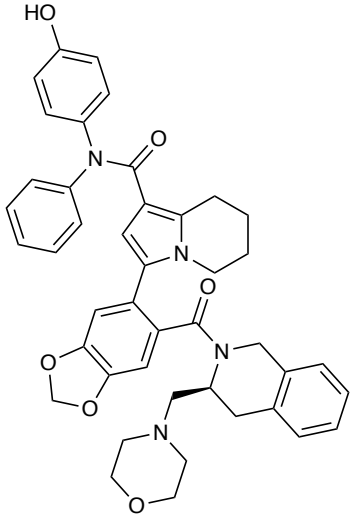
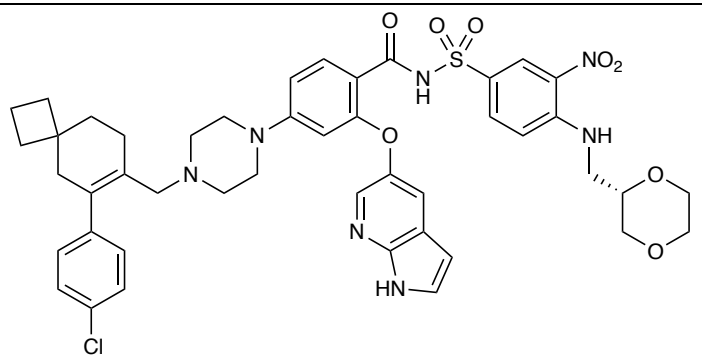
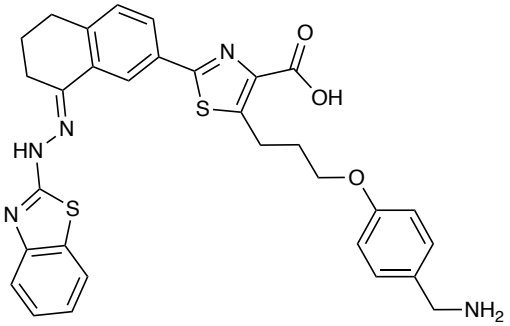
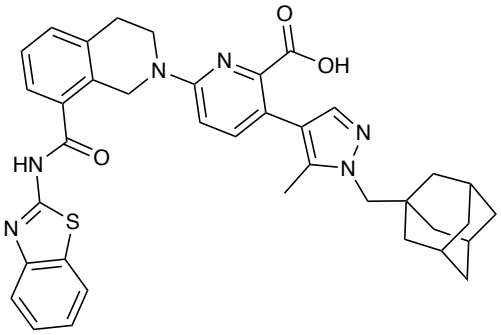
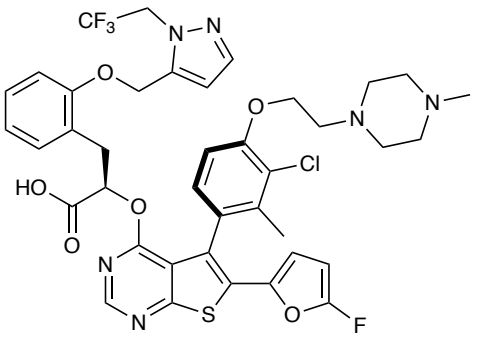
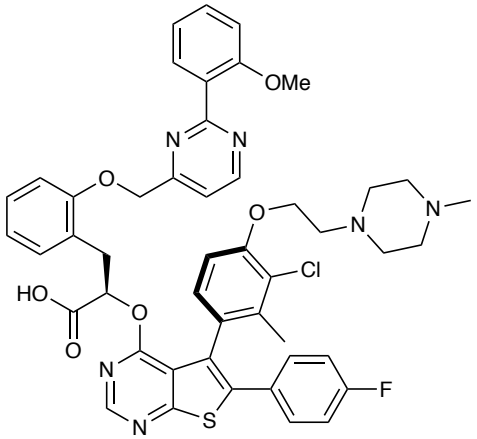
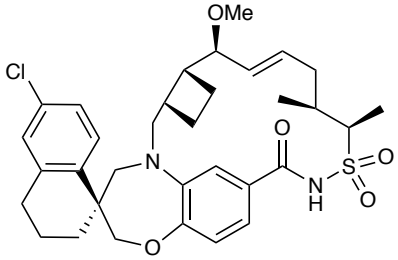

A) MCL-1:BimBH3
B) BCL-XL:ABT737
C) BCL-2:Navitoclax
D) BCL-2:Venetoclax
E) BCL-XL:WEHI539
F) BCL-XL:Abbvie Com 4
G) MCL-1:S63845
H) MCL-1:AMG Com 3
I) MCL-1:AZD5991

Table 1. Validated BH3-mimetics in preclinical/clinical phase or approved.

Target	Compounds	Structure	Originator	Status
BCL-2, BCL-XL, BC-W	ABT-737		AbbVie/Idun	Pre-clinical
	ABT-263/Navitoclax		AbbVie	Phase 1/2

BCL-2	ABT-199/Venetoclax		AbbVie/Genentech/WEHI	Approved
	S55746		Servier/Novartis	Phase 1

	APG-2575/Lisaftoclax		University of Michigan/Ascentage	Phase 1
BCL-XL	WEHI-539		WEHI/Genentech	Pre-clinical
	A-1881852		AbbVie/WEHI/Genentech	Pre-clinical

MCL-1	S63845		Vernalis/Servier	Pre-clinical
	S64315		Vernalis/Servier/Novartis	Phase 1
	AMG-176		Amgen	Phase 1 (on hold)

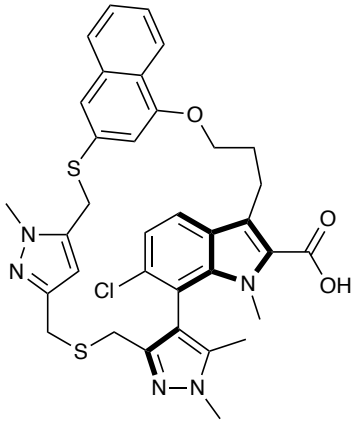
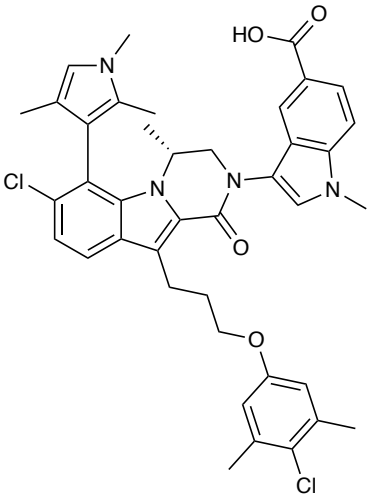
	AZD5991		Astra-Zeneca	Phase 1/2
	VU661013		Vanderbilt University	Preclinical

Table 2. Progression of BCL-2 selective BH3-mimetics in clinical trials.

Indication (FDA registration status)	**Treatment**	**Progression free survival (PFS)/Overall survival (OS)/Overall Response Rate (ORR)/Complete Response (CR)**	**References**
RR chronic lymphocytic leukaemia (**FDA approval:** Single agent for the treatment of adult patients with chronic lymphocytic leukaemia (CLL) or small lymphocytic lymphoma (SLL).)	Single agent venetoclax	Median PFS: 25 months PFS (15 months): 69% Estimated OS (2 years): 84%	[71] NCT01328626
	Single agent venetoclax in del17p population	PFS (24 months): 54% Estimated median PFS: 27.2 months OS (24 months): 73%	[212] NCT01889186
	Venetoclax + Rituximab	Estimated PFS (2 years): 82% Median OS: Not reached	[96] NCT01682616
	Venetoclax + Rituximab time limited therapy	PFS (4 years): 57.3% OS (4 years): 85.3%	[213] NCT02005471 MURANO
	Venetoclax + Ibrutinib	ORR: 89% CR: 51%	[214,215](conference abstract) EudraCT 2015-003422-14 ISCRTN13751862 CLARITY
	Venetoclax + Ibrutinib + Obinutuzumab time limited therapy	Estimated PFS (24 months): 92%	[98] NCT02427451
Upfront chronic lymphocytic leukaemia (**FDA approval:** Single agent for the treatment of adult patients with chronic lymphocytic leukaemia (CLL) or small lymphocytic lymphoma (SLL).)	Venetoclax + Obinutuzumab	Estimated PFS (24 months): 88.2% Median OS: Not reached	[99] NCT02242942
	Venetoclax + Ibrutinib	PFS (30 months): >95% OS: Not reported	[102](conference abstract) NCT02910583 CAPTIVATE

RR multiple myeloma	Single agent venetoclax	ORR: 21% (15% VGPR)	[109] NCT01794520
	Venetoclax + Bortezomib + Dexamethasone*	Median PFS: 22.4 months Median OS: Not reached	[110,216(conference abstract)] NCT02755597 BELLINI
Upfront diffuse large B cell lymphoma	Venetoclax + RCHOP	CR: 69%	[108] NCT02055820 CAVALLI
RR follicular lymphoma	Venetoclax + Rituximab, with or without Bendamustine	CR (Venetoclax + Rituximab): 17% CR (Venetoclax + Rituximab + Bendamustine): 75%	[106] NCT02187861 CONTRALTO
RR mantle cell lymphoma	Venetoclax + Ibrutinib	CR: 42% by CT, 71% by PET OS (18 months): 74% Estimated PFS (18 months): 57%	[104] NCT02471391 AIM
RR Waldenstroem's macroglobulinaemia	Venetoclax + Ibrutinib	ORR: 87% (17% VGPR, 63% PR)	[105(conference abstract)] NCT02677324
RR acute myeloid leukaemia	Venetoclax monotherapy	ORR: 19% CR: 13% Estimated OS (6 months): 26% Median PFS: 2.5 months	[112] NCT01994837
	Venetoclax + FLAG-IDA	ORR: 74% CR: 66% OS (1 year): 52% Median OS: 11 months	[115(conference abstract)] NCT03214562
Upfront acute myeloid leukaemia (**FDA approval:** In combination with azacytidine, or decitabine, or low-dose cytarabine for the treatment of newly diagnosed acute myeloid	Venetoclax + decitabine or azacytidine	CR: 67% OS: 17.5 months	[113] NCT02203773
	Venetoclax + azacytidine	CR: 36.7% OS: 14.7 months	[114] NCT02993523 *VIALE-A*
	Venetoclax + FLAG-IDA	ORR: 96% CR: 89% OS (1 year): 92% Median OS: Not reached	[115(conference abstract)] NCT03214562

leukaemia (AML) in adults 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy)			
RR acute lymphoid leukaemia	Venetoclax + Navitoclax	CR: 60%	[117] NCT03181126
Upfront/RR ER/BCL-2 positive breast cancer	Venetoclax + Tamoxifen	ORR: 54% Clinical benefit rate: 74% Median PFS not reached at 51 weeks	[119] NCT03584009
RR non-Hodgkin lymphoma, including diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma and marginal zone lymphoma	Single agent S55746	CR: 1/25 DLBCL patients PR: 2/25 DLBCL patients, 1/3 FL patients	[92] NCT02920697

*trial ceased early due to safety signal

VGPR = very good partial response; PR = partial response

Table 3. Clinical trials initiated with BH3-mimetics targeting MCL-1

Indication	MCL-1 Inhibitor	Treatment	Sponsors/Reference
RR acute myeloid leukaemia (AML), multiple myeloma (MM)	AMG 176	For MM, as single agent. For AML, as single agent or in combination with Itraconazole or Azacytidine.	Amgen NCT02675452
RR or intensive chemotherapy-excluded AML, myelodysplastic syndrome (MDS)	S64315/MIK 665	Single agent.	Institut de Recherches Internationales Servier NCT02979366
RR MM, lymphoma, diffuse large B cell lymphoma (DLBCL)	MIK 665/S64315	Single agent.	Novartis Pharmaceuticals NCT02992483
RR chronic lymphocytic leukaemia (CLL), AML, MDS, MM	AZD5991	For CLL, MDS and MM, as single agent. For AML, as single agent or in combination with venetoclax.	Astra-Zeneca NCT03218683
RR or intensive chemotherapy-excluded AML	S64315/MIK 665	In combination with venetoclax.	Institut de Recherches Internationales Servier NCT03672695
RR AML, non-Hodgkin's lymphoma (NHL), DLBCL	AMG 176	In combination with venetoclax.	AbbVie NCT03797261*
RR MM, AML, NHL, MDS	AMG 397	For NHL, as single agent. For AML and MDS, as single agent or in combination with azacytidine. For MM, as single agent or in combination with dexamethasone.	Amgen NCT03465540^
RR MM	ABBV-467	Single agent.	AbbVie NCT04178902
RR MM, AML, NHL, MDS	PRT1419	Single agent.	Prelude Therapeutics NCT04543305

*Trial suspended

10 ^Trial terminated

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