Are meningeal cells also involved in specific homing of ALL cells into the CNS? Is adhesion-mediated chemoresistance of ALL cells in contact with meningeal cells simply a cause of decreased proliferation and therefore diminished vulnerability to chemotherapeutic agents or is an active process involved (e.g., regulation of drug transporters)? Furthermore, it would be interesting to determine whether adhesion capacity of ALL cells to the CNS microenvironment could be used to improve CNS diagnostics. A recent report suggests that surface expression of α5-integrins on ALL cells is associated with the number of ALL cells in the cerebrospinal fluid detectable by diagnostic lumbar puncture.[18] Finally, there is a need to consider that mobilizing dormant ALL cells by breaking adhesive bonds with meningeal cells may also confer potential risks. Re-awakening leukemic cells may cause a resumption of proliferation and therefore overt CNS disease, an aspect which will have to be clarified in the future.

The recent study by Jonart *et al.* shapes a sharper image of the complex mechanisms of both CNS infiltration and CNS relapse, and may ultimately contribute to improved strategies for targeted treatment of CNS leukemia in ALL.

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BCL2 dependency in diffuse large B-cell lymphoma: it's a family affair

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Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma, accounting for approximately 25% of all lymphomas.[1] DLBCL is highly heterogeneous, so responses to standard therapy, R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) are mixed.[2] The response, as well as mechanisms of resistance to therapy, are associated with a cell's apoptotic threshold.[3] Therefore, determining the molecular basis for a tumor's ability to survive can provide insights into drug resistance as well as opportunities for precision medicine. In this issue of *Haematologica*, Smith *et al.* demonstrate the importance of the BCL2 family of anti-apoptotic proteins BCL2, BCLXL, and MCL1 in the survival of DLBCL, potentially revealing new treatment strategies.[4]

Inappropriate activation of oncogenes can result in cell death through the activation of pro-apoptotic proteins of the BCL2 family. Therefore, to survive the transformation process, tumor cells become more dependent on their anti-apoptotic BCL2 proteins (e.g., BCL2, BCLXL, and MCL1) than their normal counterparts.[5-7] This dependency is the result of binding and neutralizing the pro-apoptotic family members (e.g., BIM, BAK, and BAX) and is often referred to as mitochondrial priming, as increased

priming results in a lower apoptotic threshold.[5-7] Thus priming of anti-apoptotic BCL2 family proteins in cancer leads to increased sensitivity to therapy, as well as making BCL2 proteins excellent targets for therapy.[5-7]

Venetoclax (ABT-199) is a potent and selective inhibitor of BCL2.[8] The importance of BCL2 in DLBCL is well established. The t(14;18) translocation, occurring in approximately 20% of DLBCL, juxtaposes BCL2 to the immunoglobulin heavy chain gene enhancers resulting in overexpression.[9] While this translocation is most frequently found in the germinal center B-cell subtype of DLBCL, amplification and transcriptional upregulation of BCL2 are typically found in the activated B-cell molecular subtype.[10] Despite this, the clinical success of venetoclax in DLBCL has been disappointing, with an overall response rate of only 18%, regardless of BCL2 expression level.[11]

These findings led Smith *et al.* to propose a role for other anti-apoptotic proteins in DLBCL survival. To investigate this possibility, they employed venetoclax as well as A-1331852 and S63845, selective inhibitors for BCLXL and MCL1, respectively, as tools to determine the role of each anti-apoptotic protein in DLBCL survival. Using primary cells isolated from patients' samples, as well as a panel of cell lines representing the main subtypes of DLBCL, they demonstrated that all three inhibitors displayed activity in both the patients' samples and the cell lines. About half of the cell lines were preferentially sensitive to only one inhibitor, suggesting sole dependence on that anti-apoptotic family member for survival.

However, four of the 18 cell lines and at least two of the seven patients' samples showed sensitivity to two inhibitors, suggesting co-dependence on more than one anti-apoptotic protein for survival, a characteristic reported in other hematologic malignancies.[12] Resistance to all three mimetics was seen in six of the 18 cell lines; however, the mechanism of resistance was not explored. To further support a role for MCL1 and BCLXL in DLBCL the authors showed that silencing the anti-apoptotic proteins with short interfering (si)RNA was sufficient to induce apoptosis in cell lines sensitive to S63845 and A-1331852, respectively.

Based on the sensitivity data, the authors determined the expression levels and ratios of the BCL2 proteins and found that expression was highly variable between cell lines. Concluding that expression alone could not account for sensitivity to the inhibitors they moved on to examine protein interactions. Previous studies in other hematologic malignancies had demonstrated that the binding pattern of pro-apoptotic proteins to anti-apoptotic proteins is also a predictor of sensitivity to these small molecule

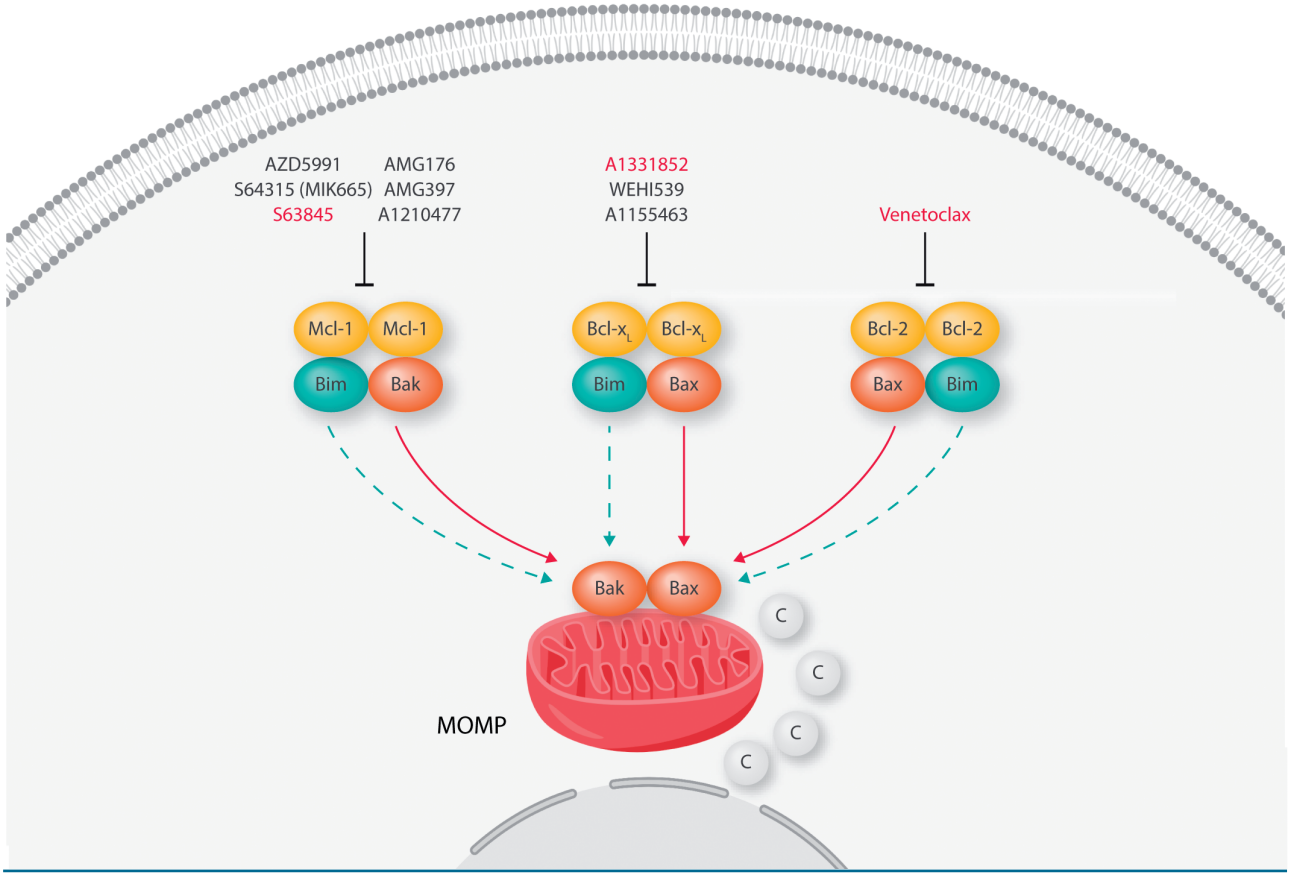

Figure 1. Schematic of the mechanism of action of selective BCL2 family inhibitors. Inhibitors disrupt interactions between anti-apoptotic BCL2 family members (green) and either the BH3-only activator protein BIM (orange) or the pro-apoptotic effectors BAX and BAK (red). This results in the release of activated effectors (solid arrows) or BIM, which can activate the effectors (dashed arrows) resulting in mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. The inhibitors in red were the ones used in the study by Smith *et al.*[4]

inhibitors, and this was found to be true in DLBCL as well.[12] Using co-immunoprecipitation, the authors showed BIM and BAX bound to BCL2 in BCL2-dependent/venetoclax-sensitive cell lines and BAX release upon drug treatment. In BCLXL-dependent/A-1331852-sensitive DLBCL lines, BIM, BAX, and BAK were bound to BCLXL and all three were released following treatment. The pro-apoptotic proteins BIM and BAK were bound to MCL1 and both displaced upon treatment with S63845 in MCL1-dependent cell lines.

BCL2 family expression data in the six resistant lines showed that five expressed some degree of all three anti-apoptotic proteins and one line expressed two. Furthermore, all six lines expressed BAK and/or BAX, with some variability in BIM expression. Co-immunoprecipitation assays performed in two of the resistant lines showed BIM binding to at least one anti-apoptotic protein but minimal binding of BAX or BAK. Displacement data following drug treatment in all six lines are necessary to gain a better understanding of the protein interactions driving this resistance. However, it remains unclear how a cell could be resistant to all three inhibitors. One possibility is that resistant cells utilize more than one BCL2 family member, requiring multiple inhibitors to be used in combination. Alternatively, the cells could be dependent on an anti-apoptotic BCL2 protein that was not tested. For example, BCL-w was recently reported to be overexpressed in DLBCL and investigating the role of this anti-apoptotic protein or that of BCL2A1 (A1/Bfl1) may reveal that it is implicated in cell survival in DLBCL.[13] While there is not currently a selective inhibitor for BCL-w, testing cell lines that are resistant to venetoclax and A-1331852 with ABT-737, which was reported to inhibit BCL-w, may provide indirect evidence of dependency.[14]

The authors used both siRNA and CRISPR to discern the contribution of the displaced proteins to the initiation of apoptosis. BIM is necessary for S63845-induced apoptosis in the two cell lines tested, whereas its role in venetoclax- and A-1331852-induced apoptosis is cell-line dependent. BAK and BAX are involved to some degree in the apoptotic response to all three inhibitors, however the contribution of each in initiation *versus* amplification of the apoptotic signal is not entirely clear.

The authors suggest that activated BAX released from BCL2 directly activates BAK; however, previous studies indicate that BAX is a poor activator of BAK.[15] An alternative explanation would be that displaced BIM is responsible for activating BAK. In the two BCLXL-dependent cell lines examined only one was protected from A-1331852-induced apoptosis when BAX was silenced and the apoptotic response was not altered in either in response to the silencing of BAK. Furthermore, CRISPR knockout of BAK appeared to have a minor, A-1331852 dose-independent, effect on apoptosis in the one cell line shown. Repeating these experiments on a larger panel of cell lines is necessary to understand the relative importance of these proteins in A-1331852-induced apoptosis. The BAX siRNA data support the authors' assertion that BAX is required for S63845-induced apoptosis; however, it remains unclear what protein(s) released from MCL1 are required to activate BAX. It should be noted that incomplete silencing of genes occurred in some experiments, which could influence the interpretation of the results. Performing the mechanistic experiments with CRISPR knockouts of BIM and BAX, along with BAK, would provide further insight into the role of each of these proteins in BH3-mimetic-induced apoptosis in DLBCL. Regardless, the studies clearly point to the importance of several BCL2 family members in the survival of DLBCL cells and provide insights into a potential means of targeting these vulnerabilities.

The potential of *ex vivo* testing as a means to deliver precision medicine based on functional testing instead of genotype has been reported in multiple myeloma with venetoclax and also in acute myeloid leukemia.[16,17] Given the data presented here, one could envisage a way this type of assay could be used in DLBCL. While venetoclax was not effective as a sole agent in DLBCL, it is being tested in combination with current therapeutics. Recently published data from the CAVALLI phase Ib trial demonstrate the benefit of combining venetoclax with R-CHOP or G-CHOP (obinutuzumab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) in BCL2-positive, MYC-positive DLBCL, with seven of eight patients (87.5%) reaching complete remission.[18] Additionally, a clinical trial evaluating the MCL1 inhibitor S64315 (MIK665) in MYC-positive DLBCL is currently recruiting (NCT02992483).[19] *Ex vivo* testing of patients prior to therapy initiation or enrollment on a clinical trial could provide guidance on treatment and spare the patient from ineffective therapy.

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Insights into vitamin K-dependent carboxylation: home field advantage

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Vitamin K-dependent (VKD) proteins play critical roles in blood coagulation, bone metabolism, and other physiologic processes. These proteins undergo a specific post-translational modification called gamma (γ)-carboxylation which is critical to their biologic function.[1] The reaction, which occurs in the endoplasmic reticulum (ER) and requires reduced vitamin K, carbon dioxide and oxygen as co-factors, is catalyzed by γ-glutamyl carboxylase (GGCX). GGCX converts several glutamic acid residues (Glu) on its protein substrate [e.g. prothrombin, FVII, FIX, FX, PC, PS, PZ, and bone Gla protein (BGP)] to γ-carboxy-glutamic acid, otherwise known as Gla.[2] How does this enzyme pick its protein substrate and modify specific glutamic acid residues? In work spanning over 30 years, researchers identified a critical sequence called the propeptide region that is N-terminal to the mature protein (Figure 1). GGCX binds the propeptide and directs carboxylation of 9-13 Glu residues on the so-called Gla domain in a processive fashion.[2] The signal sequence and propeptide region are removed by peptidases prior to secretion of the mature VKD protein (Figure 1). For the VKD coagulation factors, the enhanced net negative charge following carboxylation in the Gla domain allows for high affinity divalent metal ion binding.[3] This changes the structural conformation of the Gla domain which facilitates binding to anionic phospholipids and localizes these proteins to the site of vascular injury.[3,4] Defects of VKD protein carboxylation cause bleeding disorders, and inhibition of this pathway is the basis of warfarin anticoagulation.[2]

Acquiring mechanistic information about GGCX and deciphering how the propeptide influences carboxylation has been challenging. Since GGCX is an integral membrane ER protein (Figure 1), extracting it in a functional state is difficult and requires artificial conditions to study it. Early work used crude microsomal extracts or detergent-solubilized liver microsomes following warfarin treatment or vitamin K-deficient animals which contained the enzyme and small amounts of endogenous protein substrate (e.g. prothrombin).[1] Advancements to this system incorporated artificial peptide substrates for GGCX such as FLEEL (residues 5-9 of rat prothrombin).[5] In the late 1980s, it was recognized that the propeptide sequence is critical for VKD protein carboxylation.[6] This insight led to the development of GGCX substrates that contained a propeptide sequence and portions of the Gla domain which are superior when compared to FLEEL alone.[7,8] These and other substrates have been used to demonstrate the importance of propeptide affinity in substrate recognition using either crude preparations or purified forms of GGCX and increased our understanding about the enzyme.[9] Further insights into the importance of the propeptide came from studies using mutant peptides and identification of naturally occurring mutations in the propeptide region of FIX.[10,11] However, this knowledge about the function of GGCX was obtained outside of its natural environment under artificial conditions. To better understand VKD carboxylation in its native milieu, Tie and Stafford developed a cell-based reporter assay to study γ-carboxylation and the entire VKD cycle.[12] In this system, a chimeric reporter-protein, FIXgla-PC is used, in which the PC backbone was replaced at the N-terminus with the FIX Gla domain.[12,13] This allowed for an ELISA-based quantification of carboxylated reporter protein using a capture antibody that recognizes only a fully carboxylated FIX Gla domain and an antibody against PC. The advantage of the system is that it allows for functional assessment of the VKD cycle enzymes, including GGCX, in an environment that requires the enzymes to interact with their physiologic substrates, a departure from systems previously employed.

In this issue of Haematologica, Hao *et al.* use this cell-based assay to study the role of the propeptide in directing carboxylation of VKD proteins.[14] Previous studies indicate that the propeptide region of VKD coagulation factors show considerable variation in their affinities for GGCX with FX, FIX and PC showing high (K_d ~1 nM), intermediate (K_d ~5 nM), and low affinity (K_d ~20 nM), respectively (Figure 1).[15] It is thought that these disparate affinities contribute to the heterogeneity in carboxylation in mammalian expression systems. Furthermore, it is thought that there is likely an optimal propeptide affinity that best directs carboxylation. To better understand how GGCX interacts with its protein substrates *via* propeptide binding in its natural environment, the authors created a series of chimeric