

Functional Precision Profiling: The Way Forward for Personalized Medicine

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List of Abbreviations

AML: Acute Myeloid Leukemia; BAD: Bcl-2 associated agonist of cell death; BAK: Bcl-2 homologous antagonist killer; BAX: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Bcl-W: B-cell lymphoma-w; Bcl-xL: B-cell lymphoma-extra-large; Bfl-1: Bcl-2-related gene expressed in fetal liver; BH: Bcl-2 Homology; BIK: Bcl-2 Interacting Killer; BIM: Bcl-2-like protein 11; BMF: Bcl-2 Modifying Factor; CCND1: Cyclin D1; CD: Cluster of Differentiation; CLL: Chronic Lymphocytic Leukemia; FDA: Food and Drug Administration; HRK: Harakiri; JC-1: 1st J-aggregate-forming cationic dye; Mcl-1: Myeloid cell leukemia 1; MM: Multiple Myeloma; MOMP: Mitochondrial Outer Membrane Permeabilization; NOXA: Latin for damage; PCL: Plasma Cell Leukemia; PUMA: p53 Upregulated Modulator; sPCL: Secondary Plasma Cell Leukemia; tBID: Truncated BH3 interacting-domain death agonist

Introduction

Multiple Myeloma (MM) is a malignancy of the antibody-producing plasma cells found in the bone marrow [1]. In recent years, we have witnessed significant improvements made in both the diagnostic criteria and novel therapies for MM, resulting in the prolonged survival of MM patients. Approved novel therapies include proteasome inhibitors (bortezomib and carfilzomib), immunomodulatory drugs (thalidomide, lenalidomide & pomalidomide), monoclonal antibodies (daratumumab, elotuzumab & isatuximab) and B-cell maturation antigens (Belantamab) [2,3]. Recently, CAR T cell therapy has been FDA approved for MM treatment [4]. Despite these advancements, MM remains an incurable cancer with suboptimal overall survival,

with many patients developing relapsed/refractory MM. Plasma cell leukemia (PCL) is a rare and aggressive variant of MM. PCL is classified as either primary PCL, which develops *de novo*, or secondary PCL, that can arise in the late and advanced stages of MM [5]. The present diagnostic criteria for PCL include the number of circulating plasma cells exceeding $2 \times 10^9/L$ and/or $>20\%$ plasma cells in the total leucocyte count [6]. In both forms, PCL clinically resembles late-stage MM, with patients experiencing anemia, bone marrow failure, recurring bacterial infections renal insufficiency and hyperviscosity. Secondary PCL is a rare occurrence that is seen in approximately 1% of all MM, however that incidence is increasing as a result of the prolonged survival of MM patients [7]. Secondary PCL has a poor prognosis, is unresponsive to treatment and has a median survival of 1.3 months [7]. The underlying mechanisms that are involved in the transformation of MM to secondary PCL remain elusive. Owing to the rarity of this disease, data is limited with regards to therapeutic options. This underlines the importance of individual case reports and small case series for secondary PCL, as they aid the advancement of therapeutic treatment options for this difficult and challenging disease.

Bcl-2 Family Proteins

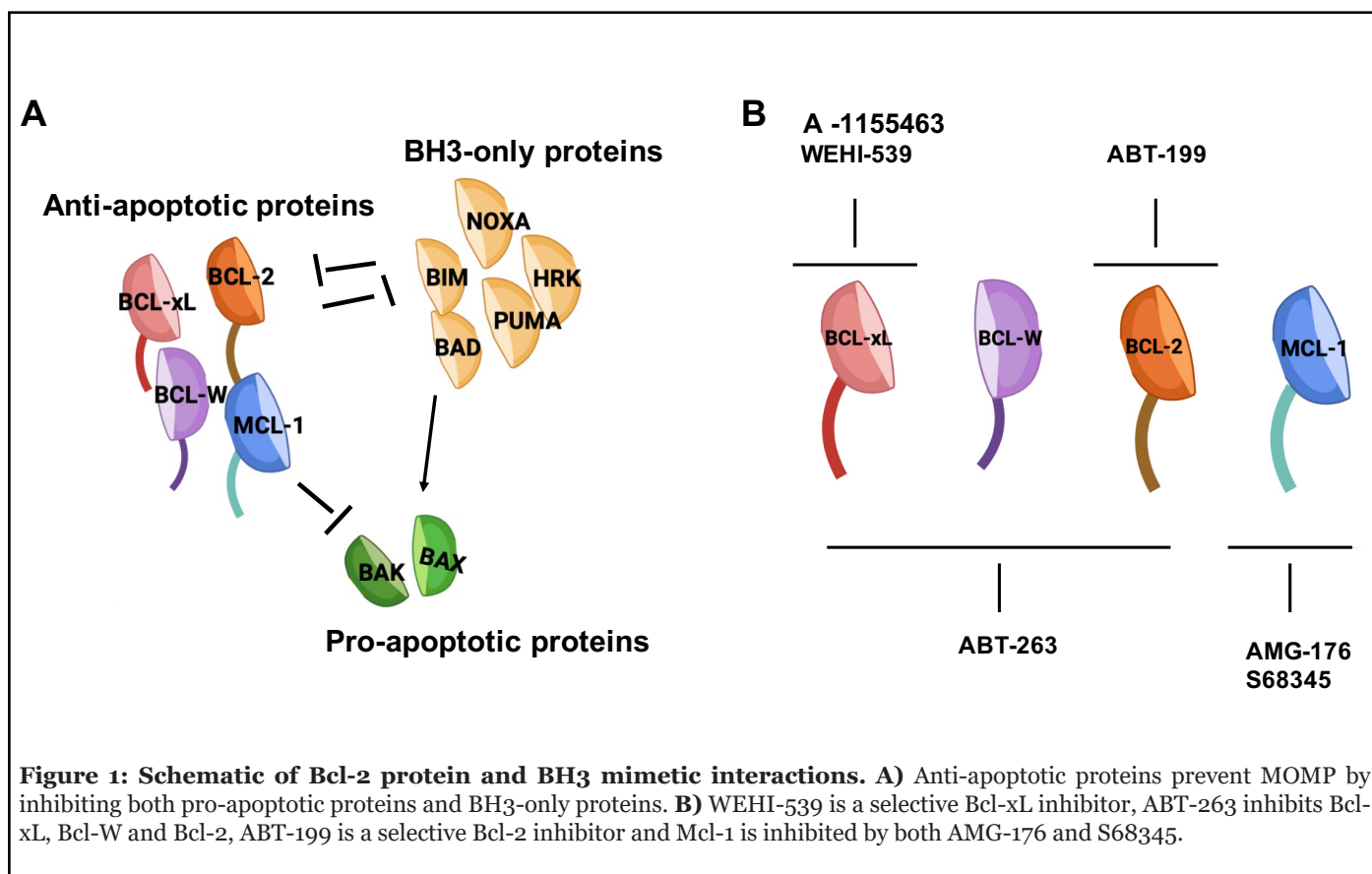
Human MM cells are reliant on Bcl-2 family proteins for survival [8,9]. Bcl-2 proteins are regulators of the mitochondrial apoptotic pathway. The anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, Mcl-1, Bfl-1) prevent mitochondrial outer membrane permeabilization (MOMP) by binding and inhibiting pro-apoptotic proteins [10-15]. Upon activation, the pro-apoptotic BAX and BAK proteins homo-oligomerise and insert in to the outer mitochondrial

membrane and form pores, which allows the release of cytochrome into the cytosol [16-18]. Lastly, are the pro-apoptotic BH3-only proteins which are further divided into activators and sensitizers. The activators (BIM, tBID, full length PUMA and NOXA) promote apoptosis through directly activating BAX and BAK [19-24]. The sensitizers (NOXA, HRK, BMF, BAD, BIK) bind to the pro-survival proteins (Bcl-2, Bcl-xL, Mcl-1), thereby inhibiting their anti-apoptotic function [25-30].

Development of BH3 Mimetics

The development of small molecule inhibitors to selectively target Bcl-2 proteins took over two decades of research, likely due to the difficult nature of disrupting protein-protein interactions. Ultimately, BH3 mimetics were developed using technical innovations such as structure-activity-relationship by nuclear magnetic resonance [31,32]. To be classified as a BH3 mimetic, the compound needs to not only display a high affinity to the targeted Bcl-2 protein, but also be able to induce cell death in a BAX/BAK dependent manner [33]. The first BH3 mimetic to be developed, ABT-737, bound to Bcl-2, Bcl-W and Bcl-xL in a manner analogous to the BAD BH3 domain [32,34]. The limiting factor with ABT-737 was the lack of bioavailability, which resulted in the development of ABT-263, or Venetoclax. ABT-263 inhibited both Bcl-2 and Bcl-xL and showed promising results in early clinical

studies. Unfortunately, ABT-263 caused a dose-limiting thrombocytopenia due to on-target inhibition of Bcl-xL in platelets [35,36]. To overcome this adverse side effect, a selective Bcl-2 inhibitor was developed. ABT-199 is the first BH3 mimetic to be approved by the FDA first for the treatment of chronic lymphocytic leukemia (CLL) and then for the treatment of acute myeloid leukemia (AML) in combination with demethylating agents [37-39] (Figure 1). In MM, Bcl-2 dependence was initially identified in a subset of patients, characterized by the presence of a translocation in cyclin D1 (CCND1) t(11;14) [40]. Sensitivity to ABT-199 is associated with t(11;14), which was confirmed across a panel of MM cell lines and patient samples [41,42]. In a Phase I study in relapsed/refractory MM, with ABT-199 as a single agent, the overall response rate in t(11;14) patients was 40%, which was exceptionally impressive for a single agent study in the relapsed/refractory setting [43]. There is very limited data on therapeutic options for patients with sPCL, due to the lack of prospective clinical trials specific for patients with PCL. However, our recent report is a single case study demonstrating a rapid and deep response in one patient with ventoclax [44]. However, further data is needed to support the use of this agent in larger numbers of PCL patients. It demonstrates the potential use of venetoclax as a single agent oral treatment, where therapeutic options are extremely limited.



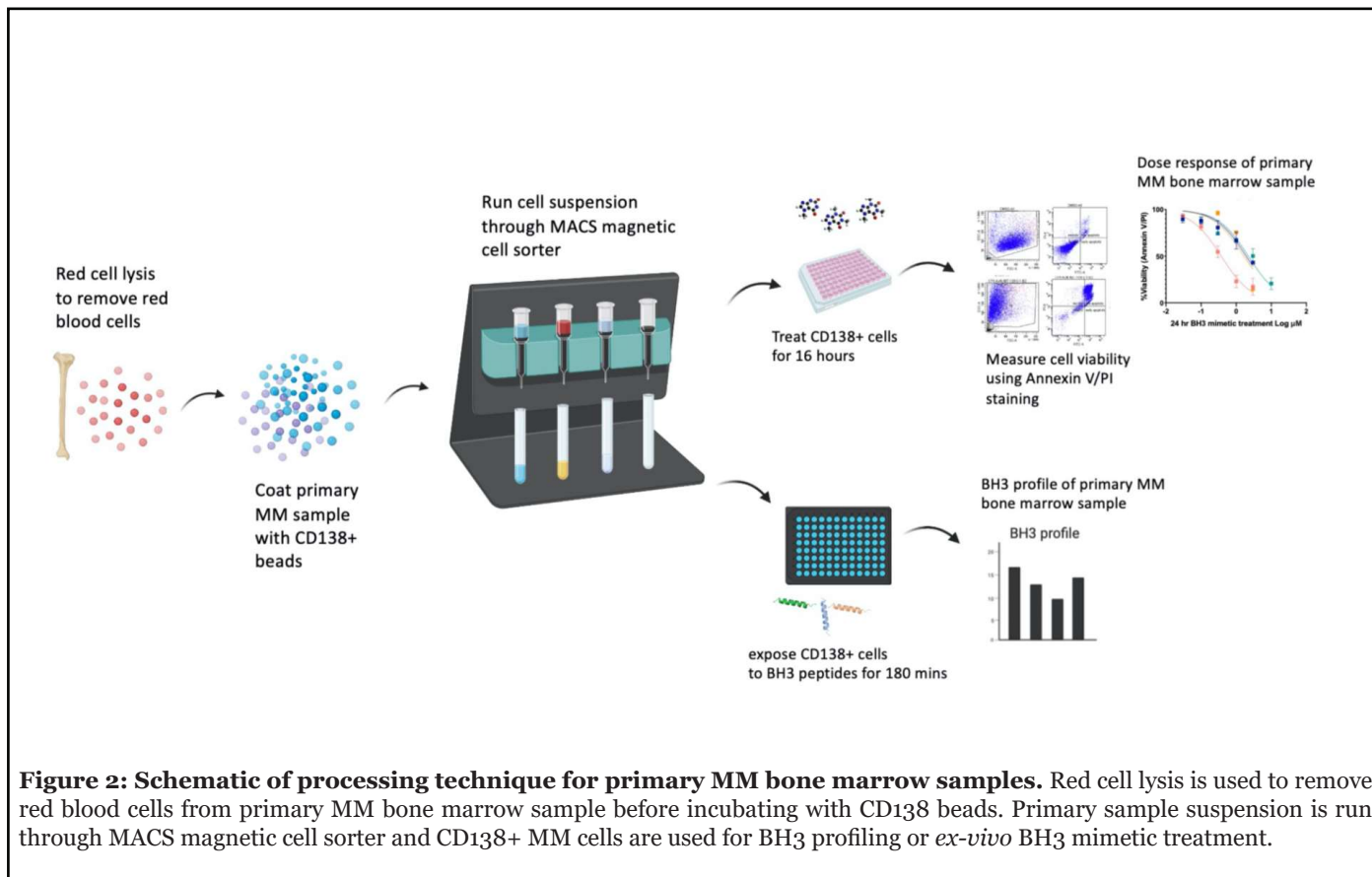
BH3 Profiling as a Potential Biomarker

Since the development of ABT-199, we now have selective BH3 mimetics that are being developed that target Bcl-xL (WEHI-539) and Mcl-1 (AMG-176) [45,46]. Additionally, there are CDK9 inhibitors that can target Mcl-1 [47]. The challenge now is to try and match the right targeted BH3-mimetic to the right patient, in a personalized approach. In the Phase III BELLINI trial, evaluating the combination of venetoclax (ABT-199), dexamethasone and bortezomib in relapsed/refractory MM, the patients with the t(11;14) translocation were particularly sensitive [48]. However, a proportion of patients without the t(11;14) translocation were also sensitive, suggesting that Bcl-2 dependence may exist outside of this cytogenic subgroup. This highlights the importance of developing a biomarker that can identify patients who will benefit from ABT-199 treatment, which is extremely relevant to avoid toxicity and maximize therapeutic benefit. To try to tackle this issue we conducted a study at RCSI and Beaumont Hospital, where we have combined the use of *ex-vivo* BH3 mimetic treatment of primary MM bone marrow samples, along BH3 profiling to identify anti-apoptotic dependence in primary MM samples. BH3 profiling is a state-of-the-art functional assay that was co-developed in the Dana Farber Cancer Institute [49,50]. The basis of BH3 profiling is to expose the mitochondria to known concentrations of BH3

peptides derived from the BH3 domains of pro-apoptotic BH3-only proteins of the Bcl-2 family. The mitochondria are exposed to these synthetic BH3 peptides for a defined length of time and the resulting MOMP is measured using flow cytometry or by plate reader [51]. Through the use of careful controls, comparisons can be made between the different cell lines and tissues towards sensitivity to intrinsic apoptosis and anti-apoptotic dependence [52,53]. The BAD peptide binds to Bcl-2/Bcl-xL/Bcl-W, while the HRK peptide binds selectively to Bcl-xL and NOXA peptides binds only to Mcl-1. Therefore, the selective binding of the synthetic BH3 peptides to the different anti-apoptotic proteins enables survival dependencies to be uncovered [54].

Methods

In our recent publication entitled “Secondary plasma cell leukaemia treated with single agent venetoclax” [44], we assessed if precision functional profiling correctly identified Bcl-2 dependence in MM cells (Figure 2). Primary myeloma patient bone marrow samples were collected following written informed consent and with local ethics committee approval. The primary MM bone marrow samples were processed using CD138 microbeads (Miltenyi Biotech) to isolate the myeloma cells as per standard protocol. Following this, the CD138 MM cells



are seeded and treated *ex-vivo* with BH3 mimetics or used in the BH3 profiling assay. The primary MM cells are treated for 16 hours with a panel of BH3 mimetics (ABT-199; selective Bcl-2 inhibitor, ABT-263; selective Bcl-2 and Bcl-xL inhibitor, WEHI-539; selective Bcl-xL inhibitor, AMG-176; selective Mcl-1 inhibitor). The cells are then stained with Annexin V/PI and cell viability post BH3 mimetic treatment is measured. The cells that are put aside for the BH3 profiling get stained and permeabilized with JC-1 and digitonin, before they are exposed to BH3 peptides and loss of MOMP is measured by a plate reader. CD138+ MM cells are processed immediately following bone marrow collection and analyzed within 24 hours to maintain cell viability. The volume of experiments that can be performed using primary patient MM samples are limited by the number of CD138+ cells we can isolate from a patient's sample. To perform BH3 profiling we need at least 1.5×10^6 cells, while *ex-vivo* BH3 mimetic treatments require 1×10^6 cells, therefore the downstream processing is highly dependent on the number of isolated CD138+ cells.

Results

By comparing the pre-clinical data generated from both the BH3 profile and *ex-vivo* BH3 mimetics, we can learn a considerable amount from the patient sample. Our case report was the first to demonstrate that ABT-199 induced a rapid hematologic and clinical response in a patient with hyperviscosity syndrome that otherwise would have undoubtedly been rapidly fatal. In this case, BH3 profiling of the sPCL sample demonstrated a response to the BAD peptide and ABT-199, consistent with BCL-2 dependence. This Bcl-2 dependence was also confirmed by assessing sensitivity to *ex-vivo* BH3 mimetic treatment by flow cytometry. This assay outperformed cytogenetics at our institute in terms of turn-around time and aided in clinical decision making. Even before the cytogenetic analysis in the clinic had revealed that this sPCL had a t(11;14) translocation and potentially was dependent on Bcl-2, the pre-clinical data had already demonstrated using two different techniques that this would be the case. For rare cancers such as sPCL, assessing the sample *ex-vivo* using techniques such as BH3 profiling and *ex-vivo* BH3 mimetic treatment could offer incredible insight into what treatment would best suit the patient. This is especially important when there is no standard therapeutic regimen, and the patient may have already received and be refractory to the standard anti-myeloma therapies.

Conclusion and Recommendation

This case report demonstrates the importance of clinicians and scientists working in collaboration. The pre-clinical data serves as proof of concept that BH3 profiling and *ex-vivo* BH3 mimetic sensitivity may be used as a

biomarker in predicting patient response to therapy in real time. In this particular case, the t(11;14) translocation was present and indicated that there would be a potential Bcl-2 dependence, which was confirmed by both BH3 profiling and *ex-vivo* BH3 mimetic sensitivity. However, had that translocation not been present, there would not have been an indication from genetics as to which, if any, anti-apoptotic protein the patient sample was dependent on. Therefore, the functional assay BH3 profiling is an incredibly useful technique, as it can distinguish the anti-apoptotic dependence and demonstrate which BH3 mimetic the sample may be sensitive to. BH3 profiling will also be highly relevant in the case of resistance to ABT-199, as it can also identify switching of anti-apoptotic dependence in a patient's sample [55]. There is evidence in acute lymphocytic leukemia from patients treated with venetoclax and navitoclax of switching from Bcl-2 dependence to other anti-apoptotic proteins such as Bcl-xl and Mcl-1 following treatment [56,57]. As more BH3 mimetics receive FDA approval to be used in the clinic, it becomes apparent that a fast effective biomarker is required to offer new therapeutic options to sPCL patients. BH3 profiling has the potential to predict the patient's response to treatment and help tailor the treatment to the specific patient to allow us to treat the right patient, at the right time with the right drug. Individual case reports aid in the advancement of therapeutic options for difficult and challenging diseases such as sPCL, where there are no clinical trials.

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