Although killing cancer cells is a high-priority goal in cancer therapy, predicting responses to therapy is an imprecise art. Even in the era of targeted therapies, the connections between inhibiting a target and cellular demise are still poorly understood. Detailed knowledge of the regulation of mitochondrial outer membrane permeabilization (MOMP) during apoptotic cell death has led to much-improved forecasting of the efficacy of a class of targeted agents, BH3 mimetics, in several types of cancer. As demonstrated by Pan and colleagues in this issue (1), BH3 profiling assays couple a mechanism-based readout of mitochondrial disintegration, tightly linked to apoptotic death, with validation of on-target effects. Application to acute myelogenous leukemia (AML) provides strong evidence that BCL-2–targeted BH3 peptides mimetics have a role in this disease.

BCL-2 and the related mitochondrial antiapoptotic proteins (BCL-X<sub>L</sub>, MCL-1, and BCL-W) provide a barrier against MOMP by binding proteins essential for apoptotic pore formation in the outer mitochondrial membrane—BH3-only activators (BIM, BID, and PUMA) and their multidomain targets, BAX and BAK (Fig. 1). The BH3 helical domains of the proapoptotic proteins interact with hydrophobic grooves on the antiapoptotic proteins. Each antiapoptotic protein has a profile of different BH3-only sensitizer proteins capable of displacing their pore-forming ligands, thus effectuating MOMP and releasing cytochrome c and other mitochondria-resident proteins that activate cytoplasmic caspase proteases. For example, the BH3-only sensitizer protein HRK binds with high affinity only to BCL-X<sub>L</sub>, whereas NOXA selectively binds to MCL-1. Healthy cells may have low to absent levels of proapoptotic proteins bound to the BCL-2 survival protein family, but various exogenous or endogenous stresses lead to mitochondrial loading of proapoptotic proteins. This is referred to as a “primed” state, as the reserve of empty BH3-binding sites is depleted, and additional insults frequently lead to apoptosis. Primed cells are also susceptible to drugs designed to displace the bound proapoptotic factors from their sequestration at BCL-2–related antiapoptotic proteins.

BH3 mimetic drugs, such as navitoclax, resemble BH3 domains from sensitizer proteins (BAD in the case of navitoclax) and preserve their binding specificities to the antiapoptotic proteins. The first BH3 mimetic compounds, ABT-737 and ABT-263 (navitoclax), have subnanomolar binding affinities for BCL-2, BCL-X<sub>L</sub>, and BCL-W. Initial reports demonstrated preclinical single-agent activity in cancer cell lines derived from B-cell lymphomas and small cell lung carcinomas (two tumor types notable for BCL-2 expression), but not other types of solid tumors (2). In clinical trials, this lack of target selectivity proved to be a disadvantage, as on-target BCL-X<sub>L</sub> inhibition resulted in severe thrombocytopenia. Structure-based redesign of navitoclax led to the second-generation agent ABT-199, which, despite a higher binding affinity for BCL-2 (K<sub>i</sub> < 0.01 nmol/L), has >800-fold lower affinity for BCL-X<sub>L</sub> and minimal effects on platelet counts (3). In several studies, resistance to these BH3 mimetics, observed in many solid tumors, was mediated by expression of MCL-1, a related antiapoptotic protein that is poorly bound by ABT-737, ABT-263, and ABT-199 (4, 5).

By adding BH3 peptides from specific sensitizer proteins to permeabilized tumor cells or purified mitochondria, the identity of any antiapoptotic proteins bound to the pore-forming protein machinery can be inferred from the profile of BH3 peptides that elicit MOMP (measured as cytochrome c release or mitochondrial membrane depolarization). In its current format, digitonin-permeabilized cells in microtiter plates are loaded with a fluorescent probe of mitochondrial membrane potential, ΔΨ<sub>mit</sub>, and fluorescence followed over time after addition of BH3 peptide. This provides a remarkably simple functional readout to discern whether cancer cells are primed for apoptosis (i.e., BCL-2 and related antiapoptotic proteins are engaged in binding pore-forming components), and which proteins are involved. As MOMP and cytochrome c release are often viewed as points of no return in apoptosis, there is a high correlation between these responses and the efficacy of targeting specific antiapoptotic proteins. With this information in hand, the response to a BH3 mimetic drug is predictable.

Expression of BCL-2, BCL-X<sub>L</sub>, and MCL-1 in AML is highly variable, and not consistently linked to chemotherapy response (6). In contrast, mitochondrial priming, as measured using the BH3 profiling assay, correlates with clinical response in AML (7). This result is not entirely surprising, as mitochondrial priming integrates expression, subcellular localization, and protein–protein interactions of pro- and antiapoptotic proteins, details that are critical for apoptosis but require
intensive effort to interrogate as individual components. Notably, Konopleva and colleagues (4) previously reported single-agent activity of ABT-737 in three of five AML cell lines and eight of nine primary AML blasts, suggesting that BCL-2 is primed and has an important survival function in a significant subset of AML. The hierarchy of normal hematopoiesis is partially preserved in AML, and the expression of apoptotic regulators is known to also vary with developmental stage. BCL-2 is overexpressed in quiescent human AML stem cells characterized by low levels of reactive oxygen species, and ABT-737 and ABT-263 selectively kill these cells (8).

In the article by Pan and colleagues (1), preclinical results for the second-generation, BCL-2–selective ABT-199 in AML are presented. ABT-199 had single-agent cytotoxic activity in six of 12 AML cell lines and 20 of 25 patient samples with diploid cytogenetics and mutations in FLT3, NRAS, and NPM1 genes. In contrast, patient AML samples with complex cytogenetics, (8;21) and JAK2 mutations were largely insensitive (1 of 9 samples). Several features of ABT-199–induced apoptosis were consistent with an on-target mechanism, including the rapidity of onset (~1.5 hours), associated mitochondrial depolarization, and a significant positive correlation with BCL-2 expression levels. Experimental manipulation of BCL-2, BCL-XL, and MCL-1 expression modulated responses to ABT-199 as expected. AML cells selected for resistance to ABT-199 expressed lower BCL-2 levels. These results are highly encouraging, and a phase II study of ABT-199 in relapsed/refractory AML is currently enrolling (NCT01994837).

BH3 profiling was also conducted in primary AML myeloblasts, demonstrating a statistically significant correlation of BAD BH3 peptide-induced cytochrome c release with in vitro sensitivity to ABT-199. In vivo responses to ABT-199 in two primary human AML xenografts were also correlated with in vitro BH3 profiling for BAD peptide. Somewhat surprisingly, the correlation of BAD BH3 profiling results with ABT-199 cytotoxicity for 12 AML cell lines was weaker, even trending as an inverse correlation. By isolating the BCL-2 response to BAD BH3, as a corrected BH3 profiling metric with subtrac-

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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