Rational combination of dual PI3K/mTOR blockade and Bcl-2/-xL inhibition in AML

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ACUTE MYELOID LEUKEMIA (AML) is diagnosed in more than 250,000 adults worldwide each year, the majority of whom are elderly (111). Induction chemotherapy with “7+3,” consisting of cytarabine and an anthracycline such as idarubicin or daunorubicin (or the anthracyclene mitoxantrone), first described over 40 years ago (140), has remained the standard approach to initial treatment and is usually followed by consolidation chemotherapy once morphologic remission is achieved, with bone marrow transplantation playing a potentially curative role in selected cases (9, 59). Despite substantial progress in recent years in advancing our understanding of the biology of AML, in large part due to the availability of mutational screening and whole genome/exome sequencing (12, 95), there has been little improvement in therapy, with cure remaining elusive for the large majority of patients (10). The outlook is particularly dismal for elderly patients (29) and those with relapsed/refractory disease (35). The inability of traditional chemotherapeutics to kill the quiescent, self-renewing leukemic stem cell (LSC) population (68) is potentially a major reason why cure has not been consistently achieved. It is believed that novel strategies for targeting AML must target this LSC population (86). Recent insights into the nature of oncogene addiction (115) have also revealed that multiple survival signaling pathways cooperate to promote transformed cell survival (116). A corollary of this concept is that multiple pathways, rationally selected for each tumor type and ideally personalized to an individual patient’s cancer, must be coordinately disrupted to optimize transformed cell killing (58, 75). An attempt was recently made (101) to target three such critical and interdependent survival pathways in AML cells [the Bcl-2 (B-cell lymphoma 2) family of antiapoptotic proteins, and the PI3K/Akt/mTOR (phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin) and MEK/ERK (mitogen-activated protein kinase kinase/extracellular signal-regulated kinase) signaling pathways] in an effort to prime them for apoptosis (132).
The Bcl-2 family proteins control the central, evolutionarily conserved pathway for regulating programmed cell death, i.e., the “intrinsic,” or mitochondrial pathway of apoptosis (106). In essence, the Bcl-2 family of proteins consists of the “multidomain” antiapoptotic proteins [Bcl-2, Bcl-xL, Bcl-w, Bfl-1 (A1), and Mcl-1 (myeloid cell leukemia-1)] and the apoptosis “effectors” Bax and Bak, and the “activator” (Bim, Bid, and perhaps Puma) or “sensitizer” (Bad, Bik, Noxa, Hrk, Bmf, Puma) “BH3-only” proteins (24). Bax and Bak directly participate in the formation of pores in the mitochondrial outer membrane, triggering mitochondrial outer membrane permeabilization (MOMP), the central event that controls cellular commitment to death via apoptosis, and the activator BH3-only proteins interact with and activate Bax and Bak directly to this end (24). The antiapoptotic Bcl-2 family proteins prevent MOMP by sequestering the apoptosis activators and effectors by binding to their BH3 domain, a phenomenon that is competitively inhibited by the proapoptotic sensitizer BH3-only proteins (24). The Bcl-2 family proteins operate as nodal points at the convergence of multiple pathways (106), and their central role in triggering the final common pathway of apoptosis, characterized by caspase activation, makes them particularly attractive drug targets (67). Multiple studies have implicated the role of Bcl-2 family proteins in AML pathogenesis (20, 42, 50), prognosis (27, 48, 131), and resistance to chemotherapy (11, 66, 69, 118). Small-molecule “BH3-mimetics” that inhibit the antiapoptotic functions of Bcl-2 and Bcl-xL have, therefore, been developed (91, 121, 129). Significantly, however, these drugs do not inhibit Mcl-1, which may be more critical for the development and maintenance of AML than Bcl-2 and Bcl-xL (42), and an essential effector of FLT3-ITD (fms-like tyrosine kinase - internal tandem duplication)-mediated drug resistance (60) and stem cell survival (144) in AML. Not surprisingly, numerous studies have demonstrated Mcl-1, a short-lived protein critically dependent upon active transcription and translation for its maintenance (55), to be a key determinant of resistance to the BH3-mimetic ABT-737 (63, 72, 126, 130, 141). Mcl-1 is an inducible protein (38) that also functions as a stress “sensor” to coordinate apoptosis and autophagy (41). Another Bcl-2 family protein involved in determining “Bcl-2 dependence” of certain leukemias [e.g., chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia] and, hence, their sensitivity to ABT-737 is the proapoptotic BH3-only protein, Bim (25, 26). Furthermore, complex interactions between, and not simply expression patterns of Bcl-2 family proteins, particularly those involving Bim, which binds to all three major antiapoptotic proteins (16, 43), have been shown to be critical factors underlying sensitivity or resistance to ABT-737 (18, 87). Several studies have used the aforementioned principles, viz. downregulation of Mcl-1 and upregulation of Bim, to design strategies that synergistically enhance the lethality of ABT-737 and other BH3-mimetics toward AML cells (17, 64, 102, 146), thus simultaneously targeting multiple arms of the apoptotic regulatory machinery (23).

**PI3K/AKT/mTOR SIGNALING PATHWAY**

The PI3K/AKT/mTOR pathway is a cellular growth, proliferation, motility, and survival signaling axis (13) that represents one of the most frequently dysregulated pathways in cancer (22), including AML (83), where activation of the pathway has been shown to be required for cell survival (138, 139). Three classes of PI3K exist, of which class IA PI3Ks are most clearly implicated in human cancers and targeted by current pharmacologic PI3K inhibitors (31). Class IA PI3Ks are heterodimers that consist of a p85 regulatory and a p110 catalytic subunit (22, 31). In response to extracellular cues [usually growth factor stimulation via receptor tyrosine kinase (RTK) signaling], the p85 regulatory subunit binds to RTKs and/or adaptors, relieving its inhibition of the p110 catalytic subunit and recruiting class IA PI3Ks to the plasma membrane, where their substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), resides (22, 31). PI3K then phosphorylates PIP2 at the 3-hydroxyl position to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), while the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) terminates PI3K-dependent signaling by catalyzing the reverse reaction (22, 31, 112). PIP3 brings two serine/threonine kinases, Akt/protein kinase B and phosphoinositide-dependent kinase 1 (PDK1), into close proximity on the cell membrane, upon which Akt is activated by PDK1 by phosphorylation at Thr308, while full activation requires Ser473 phosphorylation by mTORC2 (mTOR containing protein complex 2) (22, 31). Akt in turn regulates, through [generally inhibitory (13)] phosphorylation, a wide range of target proteins that control cell proliferation, survival, growth, and other processes: these include the proapoptotic Bcl-2 family proteins Bim, Bad, and Bax; forkhead box O (FOXO) transcription factors (which mediate apoptosis by activating the transcription of pro-apoptotic genes such as *Fasl* and *Bim*); murine double minute 2 homolog, the negative regulator of the tumor suppressor p53; GSK3 (glycogen synthase kinase 3) isoforms (which downregulate cyclin D1 and Myc); procaspase-9; I-kappa B kinase, the negative regulator of the prosurvival transcription factor NF-kB; TSC2 (tuberous sclerosis complex 2) and proline-rich Akt substrate of 40 kDa, critical negative regulators of mTORC1 (mTOR containing complex 1) signaling; the endogenous cyclin-dependent kinase (CDK) inhibitor p27kip1; and the DNA damage checkpoint kinase Chk1 (74, 78).

**RATIONAL FOR DUAL PI3K AND mTOR BLOCKADE IN AML**

Dysregulation of the PI3K/Akt/mTOR pathway in AML stems from multiple abnormalities, including PTEN loss (112), RTK activation [e.g., FLT3-ITD (7), KIT (88), Ras, insulin-like growth factor-1 and its receptor (IGF-1/IGF-1R) (113)], or activating mutations or overexpression of PI3K (51) or Akt (83). In AML, Akt activation (phosphorylation at Thr308/Ser473) variably occurs in 50–80% of patients, despite the rarity of activating mutations of Akt or PI3K or of PTEN loss (39, 82, 85, 122). Consequently, there is considerable interest in therapeutically targeting the PI3K/Akt/mTOR pathway in AML (34, 81). Both PI3K (47) and mTOR inhibitors have been shown to inhibit LSC survival (142, 143) and the latter to block Mcl-1 translation (6, 97), making them attractive candidates for AML therapy. Importantly, mTORC1 is a major downstream effector of Akt that is often not only under the control of PI3K/Akt signaling; GSK3 and AMP-activated protein kinase (AMPK), besides the two effector kinases downstream of RAS,
ERK and ribosomal protein S6 kinase (RSK), can also phosphorylate TSC2 and thus effectively promote mTORC1 activity (4, 76, 77, 125). This argues for a therapeutic advantage for dual PI3K/mTOR inhibitors over agents that inhibit only PI3K (31). Conversely, currently approved mTOR inhibitors (temsirolimus, everolimus) often lead to feedback activation of PI3K/Akt and MEK/ERK through induction of IRS-1 (insulin receptor substrate 1) and Grb10, a phenomenon that may compromise their antitumor activity (14, 53, 92, 110, 145).

This has been demonstrated preclinically to be the case in AML and provides a rationale for therapeutic inhibition of both PI3K/Akt and mTOR (127). Indeed, the dual PI3K/mTOR inhibitors PI-103 (94) and NVP-BEZ235 (15) display substantial activity against AML cell lines and primary AML samples. Finally, through a poorly understood mechanism (5, 30, 61), PI3K inhibitors may disrupt the complementary Ras/Raf/MEK/ERK survival signaling pathway, activated in >80% of AML samples (107), in AML cells (104, 124), in sharp contrast to its activation in other tumor types by PI3K (114) and mTOR inhibitors (14, 62), and by the Akt inhibitor perifosine in AML (103) and multiple myeloma cells (49). The combination of PI3K or mTOR inhibitors with MEK inhibitors to overcome this paradoxical activation is, in fact, a well-described synergistic strategy (32, 45, 117, 120, 134). It appears that, at least in AML cell lines, dual PI3K/mTOR inhibitors might be able to accomplish the task of cotargeting the PI3K/Akt/mTOR and MEK/ERK survival signaling pathways without the need for a MEK inhibitor.

RATIONALE FOR COMBINING DUAL PI3K/MTOR INHIBITORS WITH BH3-MIMETICS IN AML CELLS

The PI3K/Akt/mTOR and MEK/ERK survival signaling pathways extensively regulate the Bcl-2 family proteins and are thus intimately linked to the apoptotic pathways. Akt upregulates Bcl-2 and Mcl-1 via cyclic adenosine monophosphate response element binding protein (CREB) (80, 98, 133). The disposition of both pro (e.g., Bad, Bim)- and antiapoptotic (e.g., Mcl-1) proteins is regulated not just by Akt but also by ERK1/2 (54, 73, 117). For example, phosphorylation at Ser55/65/102 (by ERK1/2) and Ser87 (by Akt) leads to Bim (-EL) degradation (96, 99). Highly synergistic interactions have been reported between Akt and MEK inhibitors in promoting AML cell death through Bim- and Mcl-1 (but not Bad)-dependent mechanisms (100). Notably, Mcl-1 and Bim expression are tightly regulated by both the PI3K/Akt/mTOR and MEK/ERK pathways. While PI3K drives Mcl-1 transcription through a CREB-dependent process, mTOR regulates its translation, and the Akt/GSK3 axis and the E3 ubiquitin ligase SCF(FBW7) (SKP1-cullin-1-F-box complex that contains FBW7 as the F-box protein) control its posttranslational proteasomal degradation (52, 56, 133, 135). Similarly, ERK may activate Mcl-1 transcription through SRF/Ek-1, while also slowing turnover of the normally rapidly degraded Mcl-1 protein by phosphorylating it at Thr163 (28, 128). As discussed above, regulation of Bim expression and function by the PI3K/Akt and MEK/ERK pathways occurs at the transcriptional level through FOXO3a (33, 123) and particularly at the posttranslational level through phosphorylations by Akt (99) and ERK (46). Finally, it is important to consider the interplay between the MEK/ERK and PI3K/Akt/mTOR pathways: thus, as alluded to earlier, ERK phosphorylation and inactivation of TSC2 remove the latter’s inhibitory effects on mTOR signaling, cell proliferation, and oncogenic transformation (76).

It follows from the above discussion that the combination of a dual PI3K/mTOR inhibitor with a BH3-mimetic that antagonizes the antiapoptotic functions of Bcl-2 and Bcl-xL represents, at least theoretically, a particularly potent synergistic strategy against AML cells, especially those that are FLT3-ITD+, including LSCs. First, such a strategy would interrupt two major and complementary signaling pathways (viz., PI3K/Akt/mTOR and Ras/Raf/MEK/ERK) considered critical for AML cell survival while avoiding undesirable feedback activation of PI3K/Akt and ERK1/2. Second, PI3K/mTOR inhibitors would be expected to be particularly effective at downregulating Mcl-1, central to AML development and maintenance, and the main determinant of resistance to ABT-737, given that they act at three separate levels (viz., transcriptional, translational, and posttranslational, via GSK3-mediated degradation) to accomplish this. Based on this strong theoretical foundation (Fig. 1), the effects of concomitant PI3K/mTOR and Bcl-2/Bcl-xL inhibition were recently examined in AML cell lines, patient-derived blasts, and xenograft models of AML (101). The findings of these studies are summarized below.

Studies conducted on AML cells using tet-inducible short hairpin RNA constructs directed against Akt or Bcl-2 and Bcl-xL individually or together, as well as employing pharmacologic inhibitors (e.g., NVP-BEZ235/PI-103 and ABT-737) revealed that the PI3K/Akt/mTOR pathway and Bcl-2/Bcl-xL play important coordinate roles in protecting leukemia cells from lethality. Specifically, genetic or pharmacologic interventions simultaneously inhibiting the PI3K/Akt/mTOR pathway and disabling Bcl-2/Bcl-xL dramatically enhanced leukemic cell death, with minimal toxicity toward normal CD34+ hematopoietic progenitors. These findings were recapitulated in vivo in an AML xenograft model with little toxicity. Mcl-1
downregulation resulted from PI3K/mTOR inhibition, at least in part through GSK3 activation, which promotes the proteasomal degradation of Mcl-1. Although NVP-BEZ235 alone downregulated Mcl-1, it did not significantly trigger cell death, underscoring the importance of concomitant Bcl-2/Bcl-xL inhibition, as all three major antiapoptotic proteins sequester the apoptosis effectors Bax and Bak. Significantly, release of Bax and Bak from Mcl-1, Bcl-2, and Bcl-xL was observed. Another novel finding was a marked increase in Bim binding to Bcl-2 and Bcl-xL in response to PI3K/mTOR inhibition, a phenomenon effectively abolished by ABT-737. Presumably Bim, liberated from these two antiapoptotic proteins, as well as Mcl-1 (due to downregulation), triggered apoptosis by activating Bax and Bak. A particularly intriguing observation was an apparent correlation between responses to combined treatment with PI3K/mTOR inhibitors and ABT-737 and baseline Akt activation in primary AML blasts. Specifically, four of six specimens analyzed responded to the treatment, and all exhibited basal Akt phosphorylation. In contrast, basal Akt phosphorylation was not detected in nonresponding specimens or in normal CD34+ cells. This raises the possibility that the former cells were addicted to the PI3K/Akt/mTOR pathway and hence vulnerable to its inhibition. Definitive answers to this question will require analysis of a considerably larger number of specimens.

A major challenge with inhibitors of the PI3K/Akt/mTOR pathway has been delineation of their pharmacodynamic effects; i.e., determining whether or not the administration of these agents leads to target inhibition in vivo (109). Biomarkers that have been used to this end have included phosphorylation of Akt and its substrate Akt1S1 (also known as PRAS40), phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), and phosphorylation of RPS6, the proliferation marker Ki-67, and the apoptosis marker TUNEL (109). Of note, however, RPS6 and 4EBP1 can also be regulated by Ras/Raf/MEK/ERK/mTORC1 signaling, thereby confounding the assessment of the true pharmacodynamic effects of PI3K/Akt/mTOR pathway inhibitors. In the studies discussed above (101), Western blot analysis conducted on excised tumor tissue from treated mice revealed that NVP-BEZ235 alone or in combination with ABT-737 markedly decreased Mcl-1 protein levels and Akt phosphorylation, analogous to in vitro observations, thus confirming on-target pharmacodynamic activity of this novel drug combination in vivo in murine xenograft models of AML.

CLINICAL CONSIDERATIONS

Various Bcl-2 family and PI3K/mTOR inhibitors have been studied in clinical trials, including in patients with AML (Table 1), either alone or in combination with standard chemotherapy. To date, however, agents from these classes have not been studied in combination in AML. Among the Bcl-2 family inhibitors, GX15–070 (obatoclax, Teva), a pan-BH3 mimic that is no longer in development, was shown to induce apoptosis in AML cells (57, 65). However, significant infusion-related central nervous system toxicity, including euphoria, somnolence, and ataxia, led to this agent being discontinued (57). G-3139 (oblimersen, Genta), an anti-sense oligonucleotide against Bcl-2 mRNA, failed to obtain regulatory approval despite promising results in the phase III setting in CLL (89, 90). A phase III randomized controlled trial of induction therapy with cytarabine and daunorubicin followed by consolidation therapy with high-dose cytarabine with or without oblimersen in older, previously untreated patients with AML failed to meet any of its endpoints (improvements in complete remission rate, overall survival, disease-free survival from date of complete remission, and event-free survival) (2, 79). Development of ABT-263 (navitoclax), the orally available analog of ABT-737 (129), despite evidence of promising single-agent clinical activity, e.g., in CLL (108), has been discontinued owing to concerns regarding dose-dependent thrombocytopenia (40, 137) secondary to on-target inhibition of Bcl-xL, critical for platelet survival (84). Initial reports on the successor molecule, ABT-199 (GDC-0199), a Bcl-2-specific BH3-mimetic that spares platelets (121), in CLL, a highly Bcl-2 dependent malignancy (25), suggest robust activity (1). In an ongoing phase I trial (1) in patients with CLL/small lymphocytic lymphoma, the most common adverse effects reported were diarrhea (43%), neutropenia (37%), fatigue (33%), upper respiratory tract infection (33%), and cough (22%). A relatively high incidence of tumor lysis syndrome early in the trial was successfully addressed by lowering the dose of this very potent apoptosis inducer and by adopting a more cautious dose-escalation strategy (1). This agent has recently been shown to be remarkably effective in AML cell lines, primary patient samples, and murine primary xenograft models of AML.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacturer</th>
<th>Class</th>
<th>Phase</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEZ235</td>
<td>Novartis</td>
<td>dual PI3K and mTOR inhibitor</td>
<td>1</td>
<td>information not available</td>
<td>not applicable</td>
</tr>
<tr>
<td>G3139 (Oblimersen/Genasense/Augmerosen)</td>
<td>Genta</td>
<td>anti-sense oligonucleotide against Bcl-2</td>
<td>3</td>
<td>no differences in CR rate (48% vs. 52%; P = 0.75) or OS (P = 0.83) between groups with or without oblimersen therapy</td>
<td>79</td>
</tr>
<tr>
<td>GX 15-070 (Obatoclax)</td>
<td>Teva</td>
<td>small molecule BH-3 mimetic against Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bfl-1/A1, and Bcl-B</td>
<td>2</td>
<td>no CRs</td>
<td>105</td>
</tr>
<tr>
<td>ABT-199 (GDC-0199)</td>
<td>Abbvie/Genentech</td>
<td>small molecule BH-3 mimetic against Bcl-2</td>
<td>2</td>
<td>information not available</td>
<td>not applicable</td>
</tr>
</tbody>
</table>

PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; Bcl-2, B-cell lymphoma 2; AML, acute myeloid leukemia; CR, complete remission; OS, overall survival.
ABT-199 (GDC-0199), with results comparable to those observed in CLL cells, despite its selectivity for Bcl-2, raising hopes that clinically meaningful efficacy might be achieved even without Bcl-xL blockade in this disease (93). Regardless, rational combinations, such as with dual PI3K/mTOR inhibitors, are likely to prove necessary to further augment the activity of selective Bcl-2 antagonists like ABT-199 (GDC-0199) in AML for maximum therapeutic benefit.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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