The novel anti-MEK small molecule AZD6244 induces BIM-dependent and AKT-independent apoptosis in diffuse large B-cell lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults accounting for nearly 35% of all non-Hodgkin lymphomas (NHL). Significant advances have been in the treatment of DLBCL, particularly with immunotherapy, however approximately 30%-40% of patients still die from this malignancy. In addition, short- and long-term toxicities of chemotherapy, including secondary malignancies and leukemias, continue to adversely impact the long-term prognosis of patients. Continued investigations of novel targeted therapeutic agents in DLBCL are warranted.

The RAS/RAF/MEK/ERK signaling pathway has been largely unexplored as a potential therapeutic target in lymphoma. The novel 2nd generation anti-MEK small molecule, AZD6244, down-regulated its direct downstream target, phospho-ERK (pERK) in germinal center and nongerminat center diffuse large B-cell lymphoma (DLBCL) cell lines and primary cells. Similar decreased pERK levels were noted despite constitutive activation (CA) of MEK. Consequently, several lymphoma-related ERK substrates were down-regulated by AZD6244 including MCT-1, c-Myc, Bcl-2, Mcl-1, and CDK1/2. AZD6244 induced time- and dose-dependent antiproliferation and apoptosis in all DLBCL cell lines and fresh/primary cells (IC50 100nM-300nM). Furthermore, AZD6244 resulted in significantly less tumor compared with control in an in vivo DLBCL SCID xenograft model. Cell death was associated with cleaved PARP, caspases-8, -9, and -3, and apoptosis was caspase-dependent. In addition, there was stabilization of FoxO3a, activation of BIM and PUMA, and a significant decrease in c-Myc transcripts. Moreover, siRNA knockdown of BIM abrogated AZD6244-related apoptosis, while shRNA knockdown of ERK minimally sensitized cells. Finally, manipulation of AKT with transfection of OCI-LY3 cells with CA-AKT or through chemical inhibition (LY294002) had minimal effect on AZD6244-induced cell death. Altogether, these findings show that the novel anti-MEK agent, AZD6244, induced apoptosis in DLBCL and that cell death was BIM-dependent.

Introduction

The majority of preclinical, and especially clinical trial data studying MEK inhibitors to date have emerged largely from solid tumor studies.10-14 We recently showed in preclinical studies that inhibition of ERK1/2 phosphorylation by 1st generation MEK and ERK inhibitors correlated with significant cell death in a lymphoma tumor model,15 while others showed that sublethal concentrations of a 1st generation MEK antagonist potentiated the effect of sorafenib in lymphoma cells.16 Furthermore, we recently demonstrated that MCT-1, an oncogene directly downstream of MEK/ERK, is over-expressed in the majority of primary DLBCLs.15 MCT-1 is known to colocalize with ERK1/2, while phosphorylation of MCT-1 protein by ERK is critical for stabilization of MCT-1 protein and for its functional ability to promote cell proliferation.15,17 ARRY-142886 (AZD6244, selumetinib; Astra Zeneca) is a selective nonATP-competitive 2nd generation oral MEK inhibitor studied primarily in solid tumor studies with reported nanomolar activity against purified MEK1 enzyme.18-23 Furthermore, phase 1 and phase 2 solid tumor clinical trials have shown this agent to be well-tolerated and have encouraging clinical efficacy.24-27 To our knowledge, minimal data are available on newer generation MEK inhibitors in lymphoma and moreover, this anti-MEK agent has never been examined in lymphoma. We sought to examine the mechanisms of action and cytotoxic effect of the novel 2nd generation MEK small molecule antagonist, AZD6244, in lymphoma cell lines, primary cells, and in vivo human DLBCL xenograft model.

The online version of this article contains a data supplement.

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1052 BLOOD, 28 JULY 2011 • VOLUME 118, NUMBER 4
Methods

Cell culture and treatment

DLBCL germinal center cell lines (SUDHL4, SUDHL6, SUDHL10, and OCI-LY19) and the nongerminall cell line, OCI-LY3, were grown in RPMI 1640 (Invitrogen) containing 10% FBS. The 2nd generation MEK inhibitor AZD6244 was supplied from Astra Zeneca.

MTT proliferation analysis

In a 96-well flat bottom plate, approximately 10^4 cells/100uL were plated and treated for 24, 48, or 72 hours with vehicle or increasing concentrations of AZD6244 (50nM–400nM). After treatment 20 µL of MTS/PMS (Promega Cell Titer 96 Aqueous Non-Radiative Cell Proliferation assay), solution was added to each well and incubated for 4 hours at 37°C. Plates were then analyzed at 490 nm wavelength. Data were plotted as growth percentage of control. This value was determined by comparing the absorbance reading of each set of control wells to which no drug was added.

Apoptosis assays

DLBCL cell lines were seeded at equal density and then treated with AZD6244 in complete RPMI 1640 medium. Forty-eight hours after treatment, cells were harvested and apoptosis was analyzed by flow cytometry using the annexin V/PI staining kit (BD Biosciences). The significance of differences between experimental conditions was determined using the Student t test.

Soft agar colony-forming assay

SUDHL4 and OCI-LY3 cells were incubated with nanomolar concentrations of AZD6244 in the soft agar to form colonies as previously described.15 The number of colonies in each well was counted. Colony formation (> 50 cells) was examined under phase-contrast microscopy. Images were taken at room temperature using a Nikon Eclipse TE-2000S microscope. Each experiment was conducted at least 3 times and the significance of differences between experimental conditions was determined using the Student t test.

Primary DLBCL cells

After approval by the Northwestern University Institutional Review Board (IRB) and written informed consent in accordance with the Declaration of Helsinki, peripheral blood was drawn from 3 patients with leukemic phase of DLBCL. Each of these 3 patients had relapsed disease after prior rituximab/centotoxic chemotherapy. Two of the 3 patients had a CD10+ germinal center phenotype, while the third appeared to have a nongerminall center DLBCL subtype (ie, CD10-negative and Bcl-6-negative). All patients had CD19+ B cells, typical of DLBCL. Each of these 3 patients had relapsed disease after prior rituximab/centotoxic chemotherapy. All patients had CD19+ B cells, typical of DLBCL.

In vivo human lymphoma xenograft model

Female severe combined immunodeficient (SCID) beige mice were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. SUDHL6 cells (2 × 10^6) were suspended in 100µL PBS and then mixed with an equal volume of Matrigel. The mixture was injected subcutaneously into the left and right dorsal flanks of 5- to 7-week-old female SCID mice. When the tumor reached the size of 60–160 mm³, the drug (AZD6244) was administered by intraperitoneal injection every other day at a dose of 10 mg/kg of body weight for a total of 3 weeks. Injection of the vehicle alone (5% DMSO in 0.05M PBS) was used as a control. The significance of differences between treatment arms was determined using the Student t test.

Cell-cycle analysis

Distinct phases of the cell cycle were distinguished by DNA staining with the fluorescent dye propidium iodide and measured by flow cytometry. Cells were washed in ice cold PBS, fixed in 70% ethanol, and stained for 30 minutes at 37°C with propidium iodide (50µg/mL propidium iodide in hypotonic sodium citrate solution containing 50 µg/mL RNase) followed by flow cytometric analysis. The percentages of cells in G1, S, and G2/M phases were determined using the cell-cycle analysis program Modfit LT 3.2 (Verity Software House).

Western blot analysis

Cells were centrifuged, washed with cold PBS, and lysed on ice for 30 minutes in lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad). Total protein (50 µg) was electrophoresed on 12% SDS polyacrylamide gels and transferred to nitrocellulose membranes, blocked for 1 hour with 50mM Tris buffer, pH 7.5 containing 0.15M NaCl, 0.05% Tween 20 (TBST) and 5% (wt/vol) nonfat dry milk and probed overnight at 4°C with TBST containing primary antibodies. After three 10-minute washes in TBST, the filters were incubated HRP-conjugated secondary antibody in the blocking buffer for 1 hour. After three 10-minute washes in TBST, proteins were detected by enhanced chemiluminescence detection reagents (Amersham Biosciences). The following antibodies used for immunoblotting were purchased from Cell Signaling Technology: pERK1/2, ERK1/2, MEK2, Bcl2, McI, BIM, PUMA, pFOXO3a, FoxO3a, caspases 3, 8, 9, and PARP, c-Myc antibody was purchased from BD Biosciences, and MCT-1 antibody was purchased from Research Genetics Inc. Blots were stripped and reprobed with β-actin (Santa Cruz Biotechnology) used as the loading control.

Plasmids and transfections

ERK1, ERK2, and BIM were knocked down using GIPZ lentiviral shRNA from Open Biosystem. After transduction, cells were selected in puromycin-containing media for 14 days. Stably transfected cells that were viable after knockdown were selected for further analysis. This included transfection with Bcl-2 (Addgene plasmid 8768) or Mcl-1 (Addgene plasmid 25375) expressing plasmids. Wild-type MEK2 (WT MEK2) and constitutively active MEK2 constructs have been previously described.26 Constitutively active MEK1 (plasmid LIE-1) was obtained from Addgene. Cells were transfected with empty vector, wild-type MEK2, and constitutively active MEK1 or MEK2 constructs using Amaxa Nucleofector kit V (Amaxa) and program M013. After transfection cells were selected in puromycin or hygromycin, respectively. Stably transfected cells were used for further studies. Constitutively active AKT construct or Myr-AKT (plasmid 1036) was purchased from Addgene. OCI-LY3 cells were transfected with Myr-AKT or empty vector using Amaxa nucleofection kit L (Amaxa) and program A20. After transfection cells were selected in neomycin. Viable stably transfected cells that expressed constitutively active AKT were selected for further analysis. BIM and AKT siRNA were purchased from QIAGEN. Cells were transiently transfected with control, BIM or AKT siRNA using Amaxa nucleofection kit L (Amaxa). After 24 hours of transfection, cells were treated with AZD6244 for 48 hours. The c-Myc promoter-luciferase reporter plasmid containing 2100 bp of human c-Myc promoter sequences was a kind gift from Dr Bayar Thimmapaya (Northwestern University). DLBCL cells were incubated with AdM4 Myc reporter or mutant Myc reporter adenovirus for 4 hours. After 24-hour transfection, cells were incubated with AZD6244 for 24 hours followed by luciferase assay.

Results

AZD6244 inhibits ERK phosphorylation levels in DLBCL cells

Because ERK is the only known direct substrate for MEK, we examined whether inhibition of MEK with AZD6244 affected ERK phosphorylation. Figure 1A shows prompt (as early as 3 hours) and marked reduction of ERK phosphorylation in SUDHL6, SUDHL10, OCI-LY19, and OCI-LY3 DLBCL cells after exposure to 200nM AZD6244. Figure 1B shows dose-dependent reduction in pERK phosphorylation in SUDHL6 cells.
and MCT-1 after incubation with nanomolar concentrations of AZD6244. We further examined the effect of AZD6244 on fresh/primary DLBCL cells. A notable decrease in pERK level was observed with as little as 50nM AZD6244, while there was only a slight decrease in MCT-1 expression with 200nM AZD6244 in DLBCL primary cells (Figure 1C). Interestingly, minimal decrease of MCT-1 was also noted in OCI-LY19 (Figure 1B) as well as OCI-LY3 cells (data not shown).

AZD6244-induced apoptosis is mediated through MEK/ERK inhibition

To further examine the specificity of AZD6244 and the MEK/ERK signaling pathway, we determined the effect of AZD6244 on ERK phosphorylation in Raji cells transfected with wild-type or constitutively active MEK (CA MEK). As expected, CA MEK up-regulated pERK expression. Of note, AZD6244 overcame the effect of constitutively active MEK; in Raji cells transfected with CA MEK, ERK phosphorylation remained markedly diminished (Figure 1D).

Furthermore, we corroborated our small inhibitory molecule inhibition findings employing a complementary genetic approach. ERK2 was knocked down in OCI-LY3 cells using lentivirus-based ERK2 shRNA, which was confirmed by Western blotting (Figure 1E). OCI-LY3 cells were treated with 200nM or 300nM of AZD6244 for 48 hours after transduction of ERK2 shRNA. AZD6244-induced apoptosis was increased in the presence of ERK2 shRNA at both concentrations compared with control, however this difference was not significant ($P > .05$; Figure 1F). We further investigated cell death after knockdown of both ERK1 and ERK2 concurrently in OCI-LY3 and OCI-LY19 cell lines. Knockdown with ERK1 and ERK2 together appeared to cause slightly increased apoptosis in these cells compared with either construct alone (OCI-LY3 more so than OCI-LY19), however these results were not significant (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Disruption of cell-cycle progression, growth inhibition, reduction in clonogenic capacity, and induction of apoptosis

Flow cytometric analysis revealed significant changes in the cell-cycle distribution profile of DLBCL cells after exposure to AZD6244, which occurred in a concentration-dependent manner. The treatment of cells with AZD6244 resulted in G0/G1 arrest with an associated decrease in G2/M and S phase cell population (Figure 2A). We hypothesized that disruption of MEK/ERK pathway would result in growth inhibition of DLBCL cells. To test this hypothesis, we treated cell lines with increasing concentrations of AZD6244 for 24 to 72 hours and measured cell growth inhibition. As shown in Figure 2B, there is significant growth inhibition in all cell lines after 48 hours. In addition, we noted a statistically significant decrease in the colony formation of SUDHL4 and OCI-LY3 cells after 200 nm ($P < .01$) or 300 nm ($P = .006$) AZD6244 exposure (data not shown).
We next examined induction of apoptosis in DLBCL cell lines after AZD6244 treatment. Figure 2C shows > 70% apoptosis in SUDHL4 and > 40% apoptosis in SUDHL6 with 200nM, while OCI-LY19 and OCI-LY3 show approximately 60% apoptosis with 300nM AZD6244. DLBCL patient peripheral blood monocytes were also exposed to increasing concentrations of AZD6244 (ie, 25nM to 400nM) for 24 and 72 hours (Figure 2D). AZD6244 induced apoptosis in primary cells at concentrations as low as 100nM. Altogether, the half maximal inhibitory concentration (IC50) for in vitro and primary cells was 100nM-300nM. Notably, there were no differences in apoptosis noted based on germinal center versus nongerminal center DLBCL subtype (data not shown).

**Antitumor efficacy of AZD6244 in a DLBCL SCID xenograft model**

We examined whether AZD6244 inhibits lymphoma growth in a SCID xenograft DLBCL mouse model. As shown in Figure 2E, 10mg/kg of AZD6244 effectively inhibited tumor growth in a SUDHL6 xenograft. At this drug dose, no lethal toxicity or significant weight loss were observed among treated animals compared with control mice (data not shown). From 28 weeks on, mice treated with AZD6244 had significantly decreased average tumor volume compared with control (P < .05).

**Drug inhibition and genetic manipulation of MEK**

We assessed pERK levels in OCI-LY3 cells transfected with constitutively active MEK1 followed by treatment with the first generation MEK inhibitor, PD098059, or AZD6244. Unlike AZD6244-treated cells, appreciable pERK levels were detectable with PD98059 exposure in the presence of constitutively active MEK1 in OCI-LY3 cells, albeit less than untreated cells (Figure 3A). Furthermore, constitutively active MEK1 transfection resulted in partial inhibition of AZD6244-induced apoptosis. Interestingly, PD98059 had minimal apoptotic effect in Raji or OCI-LY3 cells (Figure 3B) as well as other cell lines (data not shown). We also transfected OCI-LY19 and OCI-LY3 cells with dominant negative MEK1. Dominant negative MEK1 resulted in increased levels of apoptosis compared with vector control (Figure 3C).
Caspase-dependent cell death

To assess the role of caspase activation in AZD6244-induced apoptosis, caspases and PARP were measured. As shown in Figure 3A, increasing concentrations of AZD6244 induced cleavage of caspases 9 and 8 with associated decreases in full-length caspases. In addition, cleavage of caspase 3 and PARP were observed in cell lines (Figure 4A). In primary/fresh DLBCL cells, caspases 3 and 8 and PARP were cleaved (Figure 4B). To further examine the importance of caspase activation in AZD6244 induced cell death, cells were preincubated with the caspase inhibitors. In OCI-LY3 cells (Figure 4C), there was partial inhibition of apoptosis with caspase 9 and pan-caspase inhibition, while in SUDHL6 cells (Figure 4D), apoptosis was blocked most prominently with the pan-caspase inhibitor Z-VAD. Collectively, these data suggest that AZD6244-induced apoptosis is in part caspase-dependent regulated through the extrinsic and moreso intrinsic pathway.

AZD6244 modulates the expression of cell-cycle and apoptosis regulating proteins

We next analyzed the effect of AZD6244 on the expression of key regulators of cell-cycle progression and apoptosis by immunoblot analysis. Figure 5A shows a significant decrease in c-Myc, Bcl-2 and Mcl-1 after AZD6244 treatment in all DLBCL cell lines. Consistent with the observed G1 accumulation, protein expression of cyclin D1 and CDK1/2 was strikingly decreased in AZD6244-treated cells. Similar to all DLBCL cell lines, we noted down regulation of antiapoptotic proteins (Mcl-1 and Bcl-2) and c-MYC in primary DLBCL cells after AZD6244 exposure (Figure 5B). Conversely, the cyclin-dependent kinase inhibitor p27KIP1 accumulated in AZD6244 treated cells in a time-dependent fashion (Figure 5C). To determine whether AZD6244 could reduce c-Myc activity in DLBCL cells, we quantified the c-Myc transcriptional activation using AdM4, a Myc-reporter virus, after AZD6244 treatment. As shown in Figure 5D, Myc reporter activity was reduced 3- to 10-fold compared with control. Further, AZD6244 induced more prominent down-regulation of Myc in the germinal center cell line, SUDHL6, compared with the nongerminal center line, OCI-LY3.

MEK blockade modulates FoxO3a, PUMA, and BIM

To elucidate further the mechanisms of cell death, AZD6244-related apoptotic proteins were investigated by Western blot. Based in part on prior observations that FoxO3a may be down-regulated by ERK,29-31 we were interested in examining whether FoxO3a is a target for AZD6244-mediated cell-cycle arrest and apoptosis. Indeed, we found that AZD6244 enhanced down-regulation of FoxO3a phosphorylation in AZD6244-treated cancer cell lines (Figure 6A) resulting in stabilization of FoxO3a. Furthermore, time-dependent up-regulation of FoxO3a target proteins, p53-up-regulated mediator of cell death (BIM), and Bcl-2–interacting mediator of cell death (BIM) were observed after AZD6244 treatment (Figure 6B). An increase in PUMA and BIM expression was also observed in primary DLBCL cells after AZD6244 exposure (Figure 6C).
Furthermore, to confirm the role of BIM in AZD6244-induced apoptosis, we knocked down BIM in SUDHL4 and OCI-LY3 cells using BIM siRNA. As shown in Figure 6D (OCI-LY3) and E (SUDHL4), BIM knockdown diminished the apoptotic effect of AZD6244. In addition, to assess the contribution of Mcl-1 and Bcl-2 in the cell death process, BIM shRNA DLBCL cells were transfected with Mcl-1 or Bcl-2 plasmids. Figure 6F shows that overexpression of Mcl-1 in BIM knockout cells enhanced cell survival, while Bcl-2 overexpression had no significant effect on survival compared with BIM knockdown alone.

Inhibition of AKT minimally sensitzes DLBCL cells to AZD6244

To determine the extent, if any, that AKT is involved in the regulation of MEK-induced cell death, we analyzed the effect of AZD6244 on AKT activation. Treatment of cells with AZD6244 showed reduction in AKT activation at 300nM (Figure 7A). To investigate further the potential of AKT-mediated resistance to AZD6244-induced cell death, OCI-LY3 were transfected with siRNA against AKT. Knockdown of AKT minimally sensitized cells to AZD6244-induced apoptosis (Figure 7B). Similar results were obtained by inhibiting the PI3K-AKT pathway using the chemical inhibitor, LY294002 (Figure 7C). In addition, we further confirmed AKT-mediated resistance by transfected OCI-LY3 cells with constitutively active AKT construct followed by incubation with AZD6244. Cells transfected with constitutively active AKT showed a slight reduction in AZD6244-induced apoptosis (Figure 7D). These findings showed that AKT activation has minimal effect on anti-MEK AZD6244-induced apoptosis.

Discussion

Altogether, these data show that the 2nd generation MEK small molecule inhibitor, AZD6244, induced significant cell death at nanomolar (and clinically achievable) concentrations in multiple DLBCL cells lines, primary DLBCL cells, and in a preclinical human lymphoma xenograft model. This was associated with markedly decreased phosphorylation of ERK, the only known substrate downstream of MEK. Apoptosis was caspase-dependent and cell death was associated with cell-cycle dysregulation. Blockade of the MAP kinase MEK/ERK pathway resulted in stabilization of FoxO3a and hence down-regulation of p27, BIM, and PUMA. This was associated with cell-cycle arrest and induction of apoptosis. Moreover, BIM siRNA knockout blocked AZD6244-related apoptosis, while manipulation of ERK or AKT minimally affected cell death. In interpreting these findings, several factors should be considered.

The MAP kinase RAS/RAF/MEK/ERK signal transduction cascade plays a prominent role in the regulation of cell growth and
The MEK/ERK signaling pathway is stabilized or up-regulated in a large number of cancers, including lymphoma. ERK1 and ERK2, also known as p44/42 MAPK, are intimately involved in transducing signals from growth factor receptors and cytokine receptors after ligand binding. We and others have shown that ERK is constitutively activated in the majority of B-cell lymphomas. Furthermore, several studies from our group have shown that an oncogene directly downstream of MEK/ERK, MCT-1, is involved in cell proliferation, suppression of apoptosis, enhancement of cell survival signaling, and enhanced G1 cyclin/CDK kinase activity. Previous work from our group showed in a large-scale immunohistochemical (IHC) screen that MCT-1 protein was strongly expressed in 85% of DLBCL samples (weak and strong expression > 95%) compared with only 6% of follicular lymphoma cases. Moreover, we showed that genetic knockdown of MCT-1 resulted in apoptosis and tumor regression. There are currently no clinically available specific small inhibitor molecules that can directly modulate MCT-1 or ERK1/2 function, however several drug candidates, to target MEK to cripple the MEK/ERK pathway, have been developed. Small-molecule MEK inhibitors represent the most specific and effective strategy tested to date to suppress MAPK activity. PD184352 (CI-1040) was the first MEK inhibitor studied in oncology trials, however drug-related toxicities have precluded further clinical development of this agent. Two 2nd generation oral MEK inhibitors, which are more potent and better tolerated, have been clinically developed (ie, PD0325901 and AZD6244). AZD6244 is a benzimidazole and selective 2nd generation MEK inhibitor with reported activity at nanomolar concentrations against purified MEK1 enzyme in preclinical solid tumor studies. Further, AZD6244 is a noncompetitive MEK inhibitor with preclinical antitumor activity in solid tumor models including hepatocellular, colon, myeloma, thyroid, pancreatic, melanoma, and breast cancers and tested clinically in phase 1 and phase 2 trials of advanced, refractory colorectal, melanoma, and lung cancer. AZD6244 has been examined in leukemia and myeloma models, however to our knowledge, has never been tested in lymphoma.

We showed that nanomolar concentrations of AZD6244 resulted in marked down-regulation of pERK in DLBCL cell lines (ie, germinal center and nongerminal center). MCT-1, a substrate downstream of ERK was variably affected by AZD6244. However, several other target substrates of MEK/ERK were effectively down-regulated including c-Myc, Mcl-1, and Bcl-2, including in primary DLBCL cells. The precise contribution of MCT-1 in AZD6244-induced cell death is an area of active investigation in our laboratory. It is noteworthy that AZD6244 had a profound effect on c-Myc transcriptional activity; further, c-Myc activity was preferentially decreased in germinal center DLBCL cells, however the effect was significant in nongerminal centers as well.

At the cellular and molecular level, the Bcl-2 family represents a critical checkpoint for the hierarchical regulation of apoptosis. Others have shown that cell death of acute myelogenous leukemia cells with the 1st generation MEK
inhibitor PD184352 (CI-1040) in combination with other agents (eg, perifosine\textsuperscript{48}) was dependent in part on the Bcl-2 family. Meng et al showed that BIM in particular was key to AZD6244-induced apoptosis in a lung cancer model.\textsuperscript{48} We found here that AZD6244 resulted in up-regulation of several proapoptotic mediators (ie, PUMA and BIM) and down-regulation of antiapoptotic proteins (ie, Mcl-1 and Bcl-2). Knockdown of BIM in germinal center (SUDHL4) and nongerminal center (OCI-LY3) DLBCL cells diminished the effect of AZD6244-induced apoptosis. Furthermore, Mcl-1 appears to be an important component for DLBCL cell survival as overexpression of Mcl-1 with BIM shRNA knockout decreased apoptosis, while Bcl-2 over expression did not.

An exciting finding in this study was that forced AKT activation through a constitutively active construct or inhibition of AKT (through genetic silencing or chemical inhibition) had no significant effect on AZD6244-induced apoptosis. However, other pathways we found to be affected by AZD6244 included FOXO3a, p27\textsuperscript{kip1}, and c-Myc. Continued examination of these and other signaling pathways will be important in further delineating the cell death mechanisms for this and other novel anti-MEK small molecule agents as well as to aid in the identification of potential rational combinations. In addition, clinical study of AZD6244 in DLBCL is warranted, while the overall role of the MEK/ERK signaling cascade in lymphomagenesis should continue to be investigated.

Acknowledgments

The authors thank the flow cytometry core facility at Northwestern University, as well as the RNAi core facility for providing lentivirus/constructs and DNA/RNA delivery cores for providing lentivirus for ERK2 shRNA.

This work was supported in part by a K23 CA109613 award from the National Cancer Institute (A.M.E.), a Merit Review award from the Department of Veterans Affairs (R.B.G.), and R01 AA017972 (R.B.G.) from the National Institutes of Health.

Authorship

Contribution: S.B. designed and performed research, analyzed data, and wrote the paper; A.M.E. designed and performed research, analyzed data, and wrote the paper; B.D. performed research and analyzed data; S.P. performed research and analyzed data; L.I.G. designed research and analyzed data; and R.B.G. designed and performed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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V/PI staining followed by flow cytometry. After selection in neomycin for 14 days, positively selected cells were treated with indicated concentration of AZD6244 for 48 hours. Apoptosis was measured by annexin V/PI staining followed by flow cytometry. (D) Constitutive activation of AKT. OCI-LY3 cells were transfected with either constitutively active AKT (Myr-Akt) or vector (pcDNA) alone. After selection in neomycin for 14 days, positively selected cells were treated with indicated concentration of AZD6244 for 48 hours. Apoptosis was measured by annexin V/PI staining followed by flow cytometry.

Figure 7. Effect of AKT signaling. (A) Western blotting showing p-AKT and AKT expression. SUDHL4 and OCI-LY3 cells were treated with indicated concentrations of AZD6244 for 24 hours. Whole cell lysates were used to determine protein expression by Western blotting using specific antibodies against p-AKT and AKT. (B) AKT knockdown using AKT siRNA. OCI-LY3 cell were transfected with AKT siRNA or scrambled siRNA using Amaxa nucleofection kit. Knockdown of AKT is shown by Western blotting. After 24 hour transfection, cells were treated with AZD6244 for 48 hours. Apoptosis was measured by annexin V/PI by flow cytometry. (C) Chemical blockade of the PI3/AKT pathway. OCI-LY3 cells were pretreated with 20 \( \mu \)M of LY294002 (LY) for 1 hour followed by incubation with 200nM AZD6244 for 48 hours. Apoptosis was measured by annexin V/PI staining followed by flow cytometry. (D) Constitutive activation of AKT. OCI-LY3 cells were transfected with either constitutively active AKT (Myr-Akt) or vector (pcDNA) alone. After selection in neomycin for 14 days, positively selected cells were treated with indicated concentration of AZD6244 for 48 hours. Apoptosis was measured by annexin V/PI staining followed by flow cytometry.

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