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

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ABSTRACT

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, downregulated it’s direct downstream target, phospho-ERK (pERK), in germinal center and non-germinal center diffuse large B-cell lymphoma (DLBCL) cell lines. Similar decreased pERK levels were noted despite constitutive activation (CA) of MEK. Consequently, a number of lymphoma-related ERK substrates were downregulated by AZD6244 including MCT-1, c-myc, bcl-2, mcl-1, and CDK1/2. AZD6244 induced time- and dose-dependent anti-proliferation 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, however, apoptosis was caspase-dependent. Additionally, there was activation of BIM and PUMA, stabilization of FoxO3a, 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

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid malignancy in adults accounting for nearly 35% of all non–Hodgkin’s lymphomas (NHL). Significant advances have been in the treatment of DLBCL, particularly with immunochemotherapy, however approximately 30-40% of patients still die from this malignancy. Additionally, 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/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) plays a prominent role in cancer biology, including hematologic malignancies, in part through the regulation of cell growth and proliferation.1-5 Activating mutations in RAS and RAF lead to aberrant activation of their downstream target, MEK1/2. Directly downstream of MEK1/2, ERK1 and ERK2 are intimately involved in transducing signals from growth factor receptors and cytokine receptors following ligand binding.2 Further, ERK is the only known catalytic substrate of MEK.6 We and others have shown that the MEK/ERK signaling pathway is constitutively active in a large number of cancers, including hematologic malignancies.3,4,7-9 Moreover, the MEK/ERK signal transduction cascade has been shown to be susceptible to pharmacologic intervention. Thus, MEK has emerged as an attractive therapeutic target in cancer.

The majority of pre-clinical, and especially clinical trial data studying MEK inhibitors to date have emerged largely from solid tumor studies.10-14 We recently showed in pre-clinical 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 sub-lethal concentrations of a 1st generation MEK antagonist, sorafenib, was effective in lymphoma cells.16 Furthermore, we recently
demonstrated that MCT-1, an oncogene directly downstream of MEK/ERK, is overexpressed in the vast majority of primary DLBCLs.\textsuperscript{15} MCT-1 is known to co-localize 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.\textsuperscript{15,17}

ARRY-142886 (AZD6244, selumetinib; Astra Zeneca) is a selective non-ATP-competitive 2\textsuperscript{nd} generation oral MEK inhibitor studied primarily in solid tumor studies with reported nanomolar activity against purified MEK1 enzyme.\textsuperscript{18-23} Furthermore, phase I and phase II solid tumor clinical trials have shown this agent to be well-tolerated and have encouraging clinical efficacy.\textsuperscript{24-27} To our knowledge, minimal data are available on MEK inhibitors in lymphoma and moreover, this newer anti-MEK agent has never been examined in lymphoma. We sought to examine the mechanisms of action and cytotoxic effect of the novel 2\textsuperscript{nd} generation MEK small molecule antagonist, AZD6244, in lymphoma cell lines, primary cells, and an in vivo human DLBCL xenograft model.

**Materials and Methods**

**Cell culture and treatment**

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

**MTT proliferation analysis**

In a 96-well flat bottom plate, approximately 10\textsuperscript{4} cells/100\muL were plated and treated for 24, 48 or 72 hours with vehicle or increasing concentrations of AZD6244 (50nM to 400nM). After treatment 20\muL of MTS/PMS (Promega Cell Titre 96 Aqueous Non-Radioactive Cell Proliferation assay), solution was added to each well and incubated for...
4 hours at 37°C. Plates were then analyzed at 490nm 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's t test.

**Soft agar colony-forming assay**

SUDHL4 and OCILY3 cells were incubated with nanomolar concentrations of AZD6244 in the soft agar to form colonies as previously described. 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 several times and the significance of differences between experimental conditions was determined using the Student's t test.

**Primary DLBCL cells**

Following 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 three patients with leukemic phase of DLBCL. Each of these three patients had relapsed disease following prior rituximab/cytotoxic chemotherapy. Two of the three patients had a CD10+ germinal center phenotype, while the third appeared to have a non-germinal center DLBCL subtype (i.e., CD10-negative and Bcl-6-negative). All peripheral blood was diluted 1:1 with PBS (Ca^{2+} and Mg^{2+} free) and was layered on top
of Ficoll-Paque Plus (Sigma). Samples were then centrifuged at 150 x g for 20 min at room temperature; the buffy coat layer was removed and washed with PBS twice and subsequently placed in culture with RPMI medium.

**In vivo human lymphoma xenograft model**

Female severe combined immunodeficient mice (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 x 10^6) were re-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 to 160mm^3, the drug (AZD6244) was administered by intraperitoneal injection every other day at a dose of 10 mg per kilogram of body weight for a total of 3 weeks. Injection of the vehicle alone (5% DMSO in 0.05mol/L PBS) was used as a control. The significance of differences between treatment arms was determined using the Student's *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 50µg/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 (Verity Software House, Topsham, ME).

**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, Hercules, CA). Total protein (50μg) 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 4C with TBST containing primary antibodies. After three 10-minutes washes in TBST, the filters were incubated horseradish peroxidase-conjugated secondary antibody in the blocking buffer for 1 hour. After three 10 minutes washes in TBST, proteins were detected by enhanced chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, United Kingdom). The following antibodies used for immunoblotting were purchased from Cell Signaling Technology: pERK1/2, ERK1/2, MEK2, BCL2, MCL1, BIM, PUMA, pFox3a, FoxO3a, caspases 3, 8, 9, and PARP. C-MYC antibody was purchased from BD Biosciences (Rockville, MD), and MCT-1 antibody was purchased from Research Genetics, Inc. (St. Louis, MO). Blots were stripped and re-probed with β-actin (Santacruz Biotechnology) used as the loading control.

**Plasmids and transfections**

ERK1, ERK2, and BIM were knocked down using GIPZ lentiviral shRNA from Open Biosystem. Following transduction, cells were selected in puromycin-containing media for 14 days. Stably transduced cells that were viable after knock down 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. Constitutively active MEK1 (plasmid L1E-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. Following 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. Following 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. 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 transduction, cells were incubated with AZD6244 for 24 hours followed by luciferase assay.

Results

AZD6244 inhibits ERK phosphorylation levels in DLBCL cells

Since 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 following exposure to 200nM AZD6244. Figure 1B shows dose-dependent reduction in pERK and MCT-1 following 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, constitutively active MEK upregulated pERK expression. Of note, AZD6244 overcame the effect of constitutively active MEK; in Raji cells transfected with CA MEK, ERK phosphorylation was still diminished. (Figure 1D). Furthermore, we corroborated our small inhibitory molecule inhibition using a genetic approach. ERK2 was knocked down in OCI-LY3 cells using lentivirus-based ERK2 shRNA; ERK2 knockdown was confirmed by western blotting (Figure 1E). OCI-LY3 cells were treated with 200nM or 300nM of AZD6244 for 48 hours following 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>0.05) (Figure 1F). We further investigated cell death following knock down of both ERK1 and ERK2 concurrently in OCI-LY3 and OCI-LY19 cell lines. Knock down with ERK1 and ERK2 together appeared to cause slightly increased apoptosis in these cells compared to either construct alone (OCI-LY3 more so than OCI-LY19), however these results were not significant (Supplementary Figure 1).

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 following exposure to AZD6244, which occurred in a concentration-dependent manner. The treatment of cells with AZD6244 resulted in G0/G1 arrest with a 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. Additionally, we noted a statistically significant decrease in the colony formation of SUDHL4 and OCILY3 cells following 200nm (p<0.01) or 300nm (P=0.006) AZD6244 exposure (data not shown).

We next examined induction of apoptosis in DLBCL cell lines following AZD6244. 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 (i.e., 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 (IC_{50}) for in vitro and primary cells was 100nM-300nM. Notably, there were no differences in apoptosis noted based on germinal center vs non-germinal 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<0.05).

**Drug inhibition and genetic manipulation of MEK**

We also tested level of cell death following dominant negative MEK1 as well as with use of the MEK 1/2 inhibitor, PD09895, alone or in the presence of constitutively active MEK1. We assessed pERK levels following OCI-LY3 cells transfected with constitutively active MEK1 subsequently treated with 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 indicating more likely involvement of apoptosis through the extrinsic caspase pathway (Figure 4B). To further examine the importance of caspase activation in AZD6244 induced cell death, cells were pre-incubated 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 predominantly regulated by extrinsic pathways.

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, BCL2 and MCL-1 following 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 anti-apoptotic proteins (MCL-1 and Bcl2) and c-MYC in primary DLBCL cells following AZD6244 exposure (Figure 5B). Conversely, the cyclin-dependent kinase inhibitor p27^KIP1^ accumulated in AZD6244 treated cells in a time-dependent fashion (Figure 5C). To determine whether AZD6244 can reduce c-Myc activity in DLBCL cells, we quantified the c-Myc transcriptional activation using AdM4, a Myc-reporter virus, following AZD6244 treatment. As shown in Figure 5D, Myc reporter activity was reduced 3 to 10-fold as compared with control. Further, AZD6244 induced more prominent downregulation of Myc in the germinal center cell line, SUDHL6, compared with the non-germinal 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 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-upregulated modulator of apoptosis (PUMA), and BCL2-interacting mediator of cell death (BIM) were observed following AZD6244 treatment (Figure 6B). An increase in PUMA and BIM expression was also observed in primary DLBCL cells following 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 Figure 6E (SUDHL4), BIM knock down diminished the apoptotic effect
of AZD6244. Additionally, to assess the contribution of Mcl-1 and Bcl-2 in the cell death process, BIM shRNA DLBCL cells were transfected with Mcl1 or Bcl2 plasmids. Figure 6F shows that over expression of Mcl1 in BIM knock out cells enhanced cell survival, while Bcl2 over expression had no significant effect on survival compared with BIM knock down alone.

**Inhibition of AKT minimally sensitizes 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 transfecting 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 pre-clinical 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 followed by 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 proliferation. The MEK/ERK signaling pathway is stabilized or upregulated 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 following ligand binding. We and others have shown that ERK is constitutively activated in the majority of B-cell lymphomas. Furthermore, a number of 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 in order 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.\textsuperscript{11,12} Two second-generation oral MEK inhibitors, which are more potent and better tolerated, have been clinically developed (i.e., PD0325901 and AZD6244).\textsuperscript{10,14,18-23} AZD6244 is a benzimidazole and selective 2\textsuperscript{nd} generation MEK inhibitor with reported activity at nanomolar concentrations against purified MEK1 enzyme in pre-clinical solid tumor studies.\textsuperscript{18-23} Further, AZD6244 is a non-competitive MEK inhibitor with pre-clinical antitumor activity in solid tumor models including hepatocellular, colon, myeloma, thyroid, pancreatic, melanoma, and breast cancers\textsuperscript{19,20,41} and tested clinically in phase I\textsuperscript{24} and phase II trials of advanced, refractory colorectal, melanoma, and lung cancer.\textsuperscript{25-27} AZD6244 has been examined in leukemia and myeloma models,\textsuperscript{42-44} however to our knowledge, has never been tested in lymphoma.

In a spectrum of DLBCL cell lines (i.e., germinal center and non-germinal center), we showed that nanomolar concentrations of AZD6244 resulted in marked downregulation of pERK. MCT-1, a substrate downstream of ERK as discussed before, was variably affected by AZD6244. However, a number of 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 \textit{c-Myc} transcriptional activity; \textit{c-Myc} activity was preferentially decreased in germinal center DLBCL cells, however the effect was significant in non-germinal centers as well.

At the cellular and molecular level, the BCL-2 family represents a critical checkpoint for the hierarchical regulation of apoptosis.\textsuperscript{45-47} Others have shown that cell death of acute myelogenous leukemia cells with the 1\textsuperscript{st} generation MEK inhibitor PD184352 (CI-1040) in combination with other agents (e.g., 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 pro-apoptotic mediators (i.e., PUMA and BIM) and down-regulation of anti-apoptotic proteins (i.e., Mcl-1 and BCL-2). Knock down of BIM in germinal center (SUDHL4) and non-germinal 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 over expression of Mcl-1 with BIM shRNA knock out 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, p27KIP1, 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. 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.

Conflict of interest: There are no relevant financial conflicts of interest (all authors).

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References


Figure Legends

Figure 1. ERK and MCT-1 in DLBCL cell lines and primary cells. (A) SUDHL6, SUDHL10, OCI-LY19, and OCI-LY3 cells were treated with 200nM AZD6244 for the indicated periods of time. Cell lysates were subjected to western blotting using pERK and ERK antibodies. Actin was used as an internal control. (B) SUDHL4, SUDHL6, and OCI-LY19 cells were treated with indicated concentration of AZD6244 for 18 hours. pERK, ERK, and MCT-1 protein levels were measured by western blotting using the respective specific antibodies. (C) Western blot showing down-regulation of pERK and MCT-1 in primary DLBCL cells following AZD6244 exposure for 6 and 16 hours. Peripheral blood monocytes (PBMC) were obtained from three DLBCL patients (each with relapsed/refractory transformed DLBCL with leukemia involvement). PBMCs were incubated with indicated concentrations of AZD6244 for 6 or 16 hours. This patient/figure is representative of three primary DLBCL subjects. Cell lysates were subjected to western blotting using specific antibodies for pERK, ERK or MCT-1. (D) Over-expression of constitutively active (CA) MEK2 increased ERK and MCT-1 protein levels. Raji cells were transfected with wild type (WT) MEK2, CA MEK2 construct, or the vector control (V). After 24 hour transfection, cells were incubated with 200nM of AZD6244 for 24 hours. The cell lysates were subjected to Western blot analysis. (E) OCI-LY3 cells were transduced with scrambled (sc) shRNA or ERK2 shRNA by spin infection using GIPZ lentivirus system. After puromycin selection cells were subjected to western blotting to check the protein level. (F) OCI-LY3 cells after positive selection were treated with 200nM or 300nM AZD6244 for 48 hours, which was followed by Annexin V/PI staining and analyzed by flow cytometry.

Figure 2. AZD6244 induces cell cycle and growth arrest in DLBCL cell lines, primary cells, and in a SCID xenograft model. (A) All cell lines were treated with the
indicated concentrations of AZD6244 for 24 hours. Following fixation in 70% ethanol, cells were stained with propidium iodide (final concentration 50µg/ml) in hypotonic solution containing RNase (180 units/ml) for 30 minutes. Cells were analyzed by flow cytometry. The bar graph shows significant G1 arrest following AZD6244 treatment. **AZD6244 induces growth inhibition.** All cell lines were treated with varying concentration (50nM to 400nM) for 24, 48, and 72 hours. Cell growth was measured by MTT assay. P values are inserted for each cell line regarding concentration- and time-dependent comparisons. **(C) AZD6244 induces concentration-dependent apoptosis.** All cell lines were treated with indicated concentrations of AZD6244 for 48 hours. Apoptosis was measured by Annexin V/PI staining by flow cytometry. **(D) Apoptotic induction in primary/fresh DLBCL cells following AZD6244.** PBMCs were incubated with the indicated concentrations for 24 (left) or 72 hours (right). Apoptosis was measured by Annexin V/PI staining followed by flow cytometric analysis. Data was analyzed by FACS express software. **(E) SUDHL-6 cells were subcutaneously injected into left and right dorsal flanks of 7-week-old female SCID mice.** When the tumor reached the size of approximately 60-160mm³, AZD6244 was administered intraperitoneally every other day at a dose of 10 mg per kg body weight for a total of 3 weeks. Tumors were measured three times weekly. Abbreviations: Ctrl = control; Hr = hours. * indicates p <0.05; ** p <0.01, and *** p<0.001.

**Figure 3. MEK over expression and deletion.** **(A)** DLBCL cells were transduced with wild type or constitutively active MEK1 using retroviral delivery. After transduction of constitutively active MEK1, cells were treated with PD98059 (100µM) or AZD6244 (200nM for Raji and 300nM for OCI-LY3). Expression of pERK and ERK was determined by western blot analysis using specific antibodies in OCI-LY3 cells with constitutively active MEK1 in the presence of PD98059 or AZD6244. **(B)** Raji and OCI-LY3 cells were...
treated with PD98059 or AZD for 48 hr following transduction with MEK1-CA retrovirus. Apoptosis was measured by Annexin V/PI staining followed by flow cytometry. (C) OCI-LY3 and OCI-LY19 cells were transfected with vector alone or dominant negative (DN)-MEK1. After 72-hour transfection, apoptosis was measured by annexin V/PI staining and assessed by flow cytometry. Expression of DN-MEK1 was confirmed by western blotting using HA antibody. Abbreviations: CA = constitutively active; wt = wild type; AZD = AZD6244; PD = PD98059.

**Figure 4. AZD6244 induces caspase-dependent apoptosis.** (A) All cell lines were treated with the indicated concentrations of AZD6244 for 18 hours. Protein levels were measured by immunoblotting using specific antibodies. (B) Caspase and PARP cleavage in PBMCs from a DLBCL patient. These results are representative of three total DLBCL patients. Cells were incubated with the indicated concentrations of AZD6244 for 6 and 16 hours. Cell lysates was subjected to western blotting using caspase and PARP antibodies. (C) OCI-LY3 and (D) SUDHL6 cells were pre-incubated with 50μM caspase inhibitor for 2 hours followed by incubation with 200nM or 300nM for 48 hours. Apoptosis was determined by AnnexinV/PI staining followed by flow cytometric analysis.

**Figure 5. AZD6244 induces decrease in cell cycle regulatory and anti-apoptotic proteins.**

(A) Cells were incubated with 200nM and 300nM AZD6244 for 18 hours. Protein levels were measured in whole cell lysates by immunoblotting using indicated antibodies. (B) Down-regulation of MCL1, BCL2 and c-MYC in the whole cell lysate of PBMC following AZD6244 exposure for 18 hours. (C) Induction of p27 by AZD6244 in all the cell lines following incubation with 200nM AZD6244 for 18 hours. (D) SUDHL6 and OCI-LY3 cells
were incubated with AdM4 Myc reporter or mutant Myc reporter adenovirus for 4 hours. After 24-hour transduction, cells were incubated with 200nM AZD6244 in SUDHL6 and 300nM in OCI-LY3 cells for 24 hours followed by luciferase assay.

**Figure 6. FOXO3a and BIM expression and function.** (A) Reduction of FoxO3a activity. SUDHL4, SUDHL10, and OCI-LY3 cells were treated with indicated concentration of AZD6244 for 24 hours. Cell lysates were subjected to Western blotting using p-FoxO3a and FoxO3a antibodies. (B) Induction of pro-apoptotic proteins by AZD6244. SUDHL4 and OCI-LY-19 cells were treated with 200nM AZD6244 for indicated periods of time. PUMA and BIM proteins were detected by immunoblotting using specific antibodies. (C) Increase in pro-apoptotic proteins PUMA and BIM in PBMCs from DLBCL patients by western blotting following AZD6244 treatment for 18 hours. Actin was used as a loading control for all blots. (D) OCI-LY3 and (E) SUDHL4 cells were transfected with BIM siRNA or scrambled siRNA, using Amaxa nucleofection kit, followed by incubation with 100nM or 200nM AZD6244 for 48 hours. Knock down of BIM is shown by Western blot. Apoptosis was measured by AnnexinV/PI staining followed by flow cytometry. (F) OCI-LY3 cells were transduced with BIM shRNA using lentivirus system. Stably transduced cells that were subsequently transfected with Bcl-2 or Mcl-1 expressing plasmids. After 24-hour transfection, cells were treated with 200nM of AZD6244 for 48 hours and annexin V/PI staining and analyzed 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 knock down 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 AnnexinV/PI by flow cytometry. (C) Chemical blockade of the PI3/AKT pathway. OCI-LY3 cells were pretreated with 20µM of LY294002 (LY) for 1 hour followed by incubation with 200nM AZD6244 for 48 hours. Apoptosis was measured by AnnexinV/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 AnnexinV/PI staining followed by flow cytometry.
Figure 1

A

SUDHL6  SUDHL10

OCI-LY19  OCI-LY3

0 3 6 16 24 48 0 3 6 16 24 48

B

Control  AZD-100 nM  AZD-200 nM

Control  AZD-100 nM  AZD-200 nM  AZD-300 nM

SUDHL4  SUDHL6  OCI-LY19

C

Primary DLBCL

6 Hr 16 Hr

C 50 100 200 C 50 100 200 AZD(nM)

pERK  ERK

MCT-1  Actin

D

Primary DLBCL

AZD (200 NM)

P-ERK  ERK

MCT-1  Actin

E

E2 fRNA  ERK2 fRNA  ERK2 fRNA

pERK  ERK

MCT-1  Actin

F

% Apoptosis

Control-shRNA vector  ERK-shRNA

Untreated  AZD-200 nM  AZD-300 nM
Figure 2

A

SUDHL4

SUDHL6

SUDHL10

OCI-LY19

OCI-LY3

% Cells in G1

% Cells in G1

% Cells in G1

% Cells in G1

Control
AZD 100 nM
AZD 200 nM
AZD 300 nM

Control
AZD 100 nM
AZD 200 nM
AZD 300 nM

Control
AZD 100 nM
AZD 200 nM
AZD 300 nM

Control
AZD 100 nM
AZD 200 nM
AZD 300 nM

Control
AZD 100 nM
AZD 200 nM
AZD 300 nM

% Cells in G1

% Cells in G1

% Cells in G1

% Cells in G1

% Cells in G1
Figure 2

B

OCI-LY3

AZD6244

P=0.006-ctrl vs 400 nM
P=0.003-24 vs 72 hr

SUDHL4

AZD6244

P=0.005-ctrl vs 400 nM
P=0.002-24 vs 72 hr

OCI-LY19

AZD6244

P=0.005-ctrl vs 400 nM
P=0.048-24 vs 72 hr

SUDHL6

AZD6244

P=0.01-ctrl vs 400 nM
P=0.008-24 vs 72 hr
Figure 2

C

SUDHL4

SUDHL6

OCI-LY19

OCI-LY3

% Apoptosis

Control AZD-100nM AZD-200nM AZD-400nM

Control AZD-100nM AZD-200nM AZD-400nM

Control AZD 100nM AZD 200 nM AZD 300 nM AZD400 nM

Control AZD 100nM AZD 200 nM AZD 300 nM AZD400 nM

DLBCL - Primary cells

24 Hour

72 hr Hour

% Apoptosis

Control AZD-100nM AZD-200nM AZD-400nM

Control AZD-25 nM AZD-50nM AZD-75 nM AZD-100 nM

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Figure 3

A

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<td>PD-100μM</td>
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pERK

ERK

B

Raji

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OCi-LY3

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C

OCi-LY3

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OCi-LY19

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HA-tag

GAPDH
Figure 4

A

Control | AZD 300 nM | Control | AZD 300 nM | Control | AZD 300 nM | Control | AZD 300 nM

Caspase 9 (full length) | Cleaved Caspase 9 | Caspase 8 | Caspase 3 (full length) | Cleaved Caspase 3
PARP | Cleaved PARP | Actin

SUDHL4 | SUDHL10 | OCI-LY3

B

6 Hr | 16 Hr

C 50 | 100 | 200 | C 50 | 100 | 200 | AZD(nM)

Caspase 9 | Caspase 8 | Caspase 3 | Cleaved Caspase 3
PARP | Actin

Primary DLBCL

C

OCI-LY3

% Apoptosis

Control (BM6D) | LEHD | ETO | 2-WAD | AZD 300nM | LEHD | AZD 300nM+LEHD | AZD 300nM+ETO | AZD 300nM+2-WAD

D

SUDHL6

% Apoptosis

Control (BM6D) | LEHD | ETO | 2-WAD | AZD 300nM | LEHD | AZD 300nM+LEHD | AZD 300nM+ETO | AZD 300nM+2-WAD

**
Figure 5

A

B

C

D

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Figure 6

A

BimxL, BimL, BimS

Bim siRNA

SUDHL4, OCI-LY3

SUDHL4, OCI-LY19

DLBCL

p-Foxo3a (ser 253)
p-Foxo3a (Thr32)
Foxy3a
Actin

SUDHL4
SUDHL10
OCI-LY3

0 200 300
0 200 300
0 200 300
AZD(nM)

0 3 6 16 24 48
0 3 6 16 24 48
Hr

Puma
BimxL
BimL
BimS
ERK

Sc

Bim siRNA

Actin

OCI-LY3

D

E

SUDHL4

Sc

Bim siRNA

% Apoptosis

0 10 20 30
0 10 20 30

Control
AZD 100 nM
AZD 200 nM

% Apoptosis

0 10 20 30
0 10 20 30

Control
AZD 100 nM
AZD 200 nM

OCI-LY3

F

NS

Vector
Mot1
Bim
Mot1 + Bim
Mot1 + Bim + AZD
Bim + AZD

% Apoptosis

0 10 20 30
0 10 20 30

Control
AZD 200 nM
Figure 7

A

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

Savita Bhalla, Andrew M. Evens, Bojie Dai, Sheila Prachand, Leo I. Gordon and Ronald B. Gartenhaus