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LYMPHOID NEOPLASIA

PKC-β as a therapeutic target in CLL: PKC inhibitor AEB071 demonstrates preclinical activity in CLL

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Key Points

- AEB071 demonstrates preclinical in vitro and in vivo activity against CLL independent of survival signaling and stromal cell protection.
- AEB071 can either inhibit or activate the WNT pathway emphasizing the importance of pharmacodynamic monitoring in its development.

Targeting B-cell receptor (BCR) signaling in chronic lymphocytic leukemia (CLL) has been successful with durable remissions observed with several targeted therapeutics. Protein kinase C-β (PKC-β) is immediately downstream of BCR and has been shown to be essential to CLL cell survival and proliferation in vivo. We therefore evaluated sotrastaurin (AEB071), an orally administered potent PKC inhibitor, on CLL cell survival both in vitro and in vivo. AEB071 shows selective cytotoxicity against B-CLL cells in a dose-dependent manner. Additionally, AEB071 attenuates BCR-mediated survival pathways, inhibits CpG-induced survival and proliferation of CLL cells in vitro, and effectively blocks microenvironment-mediated survival signaling pathways in primary CLL cells. Furthermore, AEB071 alters β-catenin expression, resulting in decreased downstream transcriptional genes as *c-Myc*, *Cyclin D1*, and *CD44*. Lastly, our preliminary in vivo studies indicate beneficial antitumor properties of AEB071 in CLL. Taken together, our results indicate that targeting PKC-β has the potential to disrupt signaling from the microenvironment contributing to CLL cell survival and potentially drug resistance. Future efforts targeting PKC with the PKC inhibitor AEB071 as monotherapy in clinical trials of relapsed and refractory CLL patients are warranted. (*Blood*. 2014;124(9):1481-1491)

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common types of adult leukemia and is currently incurable. Decades of research into the biology of CLL and other B-cell malignancies has brought forth B-cell receptor (BCR) signaling as a common required driving force in the survival and proliferation of these tumor cells. Several survival pathways involved in BCR signaling, including the phosphatidylinositol 3-kinase (PI3K), nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), are constitutively active in the lymph node and bone marrow compartment of CLL where disease expansion occurs.[1-3] Efforts to target BCR signaling with therapeutic agents which reversibly inhibit PI3K-δ[4,5] or irreversibly inhibit Bruton tyrosine kinase (BTK)[6-8] have shown significant clinical activity in CLL, including those with high-risk genomic disease, and are currently in phase 3 studies. Protein kinase C-β (PKC-β) is an immediate downstream target of BTK that has recently been shown to be overexpressed in CLL[9] and is essential to the in vivo development of CLL in Eμ-TCL1 mice.[10] In B cells, PKC-β is thought to be the predominant PKC isoform mediating BCR-dependent NF-κB activation.[11-13] Downstream of PKC-β, IκB kinase and caspase recruitment domain-containing protein 11 (CARD11 [also known as CARMA1]) are phosphorylated, leading to activation and transcription of NF-κB target genes such as *Mcl-1* and *Bcl-xl*.[13,14] Most recently, it has been shown that inhibition of PKC-β counteracts microenvironment-mediated protection of CLL cells by preventing activation of NF-κB and upregulation of its transcriptionally regulated genes as demonstrated in PKC-β knockout (*Prkcb*$^{-/-}$) mice.[15]

Another critical downstream target of PKC is β-catenin,[16,17] which (when active) binds to transcriptional cofactors such as the TCF/LEF family of proteins[18-20] and regulates the expression of genes involved in apoptosis and survival signaling, including *CD44*,[21,22] *c-Myc*,[19] and *Cyclin D1*.[23] Of note, PKC-β has been shown to increase β-catenin transcriptional activity through the phosphorylation and hence inactivation of glycogen synthase kinase 3-β (GSK3-β).[24] Active GSK3-β phosphorylates β-catenin on conserved serine and threonine residues subsequently leading to its ubiquitination and proteasomal degradation.[25]

PKC activity has been implicated in the regulation of malignant cell proliferation, apoptosis, and tumor invasiveness[26]; accordingly, a number of PKC inhibitors have been introduced into clinical trials for the treatment of human cancers. Sotrastaurin (AEB071) is a novel

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orally administered potent inhibitor of classical and novel PKC isotypes with strong and specific activity on PKC-α, PKC-β, and PKC-θ and lesser activity on PKC-δ, PKC-ε, and PKC-η. Although AEB071 affects primarily PKC, biochemical profiling of >200 kinases revealed both isoforms of GSK3, as previously unappreciated direct targets of AEB071.[27] Preclinically, AEB071 has demonstrated in vivo activity in an activated B-cell diffuse large B-cell lymphoma (DLBCL) model[28] and is currently being tested in a phase 1 clinical trial for patients with CD79-mutant DLBCL (ClinicalTrials.gov NCT01402440). Given its ability to suppress T-cell activation, AEB071 has been studied in phase 2 clinical trials for psoriasis and solid organ transplantation.[29-31] Results from these studies show that AEB071 is in general well tolerated, with the most frequently reported side effects being gastrointestinal in nature such as abdominal pain, constipation, diarrhea, nausea, and vomiting.

Herein, we describe a detailed study demonstrating that AEB071 promotes apoptosis, inhibits proliferation, and also prevents CLL cells from responding to survival stimuli provided by the microenvironment. AEB071 also antagonizes WNT signaling at low concentrations, a previously unrecognized mechanism of action of this compound. Collectively, these studies provide significant support for exploring the use of AEB071 as monotherapy in relapsed and refractory CLL patients.

Methods

Patient sample processing and cell culture

Blood was obtained from healthy subjects or CLL patients with informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the institutional review board of The Ohio State University (OSU; Columbus, OH). All patients examined had CLL as defined by the 2008 International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) criteria.[32] $CD19^+$ CLL cells were isolated and cultured as previously described.[33] For additional details, see supplemental Methods (available at the *Blood* Web site). 9-15c stromal cells were obtained from the RIKEN cell bank (Ibaraki, Japan) and HS-5 stromal cells were from ATCC and maintained in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS).

Reagents and antibodies

AEB071 was provided by Novaritis and OSU (Division of Medicinal Chemistry, College of Pharmacy) and exhibited similar results. See supplemental Methods for a detailed list of reagents.

Viability and flow cytometric studies

An MTS (3-[4,5 dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay, using the tetrazolium dye 3′(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, was performed to determine cytotoxicity as previously described.[4] In addition, cell viability was also measured using annexin V/propidium iodide (PI) flow cytometry (Beckman Coulter Cytomics FC500 cytometer).[4] Stromal coculture was done as previously described[4] by plating a 75 cm^2 flask (80%-100% confluent) per 12-well plate 24 hours before the addition of CLL cells. The surface expression of CD44 on $CD19^+$ CLL cells was evaluated in viable cells by staining with anti-CD44–phycoerythrin using Live/Dead near-infra red stain.

Lymphocyte depletion in whole blood

Whole blood from CLL patients or healthy subjects was incubated with AEB071 for 24 hours under constant rotation at 37°C in an atmosphere of 5% CO_2. Lymphocytes were then stained as previously described.[34] See supplemental Methods for details.

Proliferation assay

Cell proliferation was determined by tritiated thymidine incorporation as previously described.[7] See supplemental Methods for details.

Immunoblot analysis

Proteins extracted from whole-cell lysates (30-40 μg per lane) were separated on polyacrylamide gels and transferred on nitrocellulose membrane as previously described.[35] See supplemental Methods for a detailed list of antibodies.

Quantitative real-time PCR

Real-time polymerase chain reaction (PCR) was performed using complementary DNA prepared as described[33] using the following TaqMan gene expression assays: *CCND1* (ID: Hs00765553_m1), *c-MYC* (ID: Hs00905030_m1), *CD44* (ID: Hs00153310_m1), and Human *GAPDH* Endogenous Control (4352934E) from Life Technologies.

PP2A activity (nonradioactive assay)

The protein phosphatase activity of total cellular lysate was determined using the malachite green-phosphate complex assay (Upstate Biotechnology) as previously described.[36]

Statistical analysis

All analyses were performed using SAS/STAT software, version 9.2 (SAS Institute, Inc). See supplemental Methods for details.

Results

AEB071 induces selective cytotoxicity in CLL cells and inhibits proliferation

Given the importance of the PKC family in B-cell activation and survival, we sought to determine the in vitro activity of AEB071 against CLL patient cells. CLL cells from 11 patients were treated with increasing concentrations of AEB071 for up to 72 hours. AEB071 exhibited significant cytotoxicity in CLL cells in a dose- (Figure 1A, $P < .0001$) and time-dependent (Figure 1B, $P = .011$) manner as measured by MTS incorporation. Next, we tested whether AEB071-induced cytotoxicity was selective to B-CLL cells using a flow-based lymphocyte depletion whole-blood assay. Although AEB071 demonstrated a marked decrease in B-CLL cells ($P < .001$), no significant cytotoxicity was seen against T cells or natural killer (NK) cells (Figure 1C). Similarly, AEB017 lacked significant cytotoxicity toward normal B cells, T cells, and NK cells when tested on whole blood from healthy volunteers (Figure 1D). These data suggest that transformed B cells, which are reported to overexpress PKC-β compared with normal B cells,[9] are more sensitive to AEB071 treatment than normal B cells.

In addition to evading apoptosis, CLL cells proliferate in vivo in proliferation centers.[37] Therefore, we sought to determine whether AEB071 could effectively inhibit CLL cell proliferation following exposure to CpG oligonucleotides, a well-characterized promoter of CLL cell growth stimuli in vitro.[38] Interestingly, AEB071 antagonized CpG-induced AKT, GSK3-β, and ERK1/2 phosphorylation (Figure 1E). In addition, AEB071 sustained its cytotoxic effects in CpG-stimulated cells ($P = .0107$, Figure 1F). Of note, CLL cells without CpG stimulation have a very low tritiated thymidine uptake that is markedly enhanced after 5 days of treatment with CpG, and treatment of B-CLL cells with AEB071 effectively antagonizes the ability of CLL cells to proliferate (Figure 1G).

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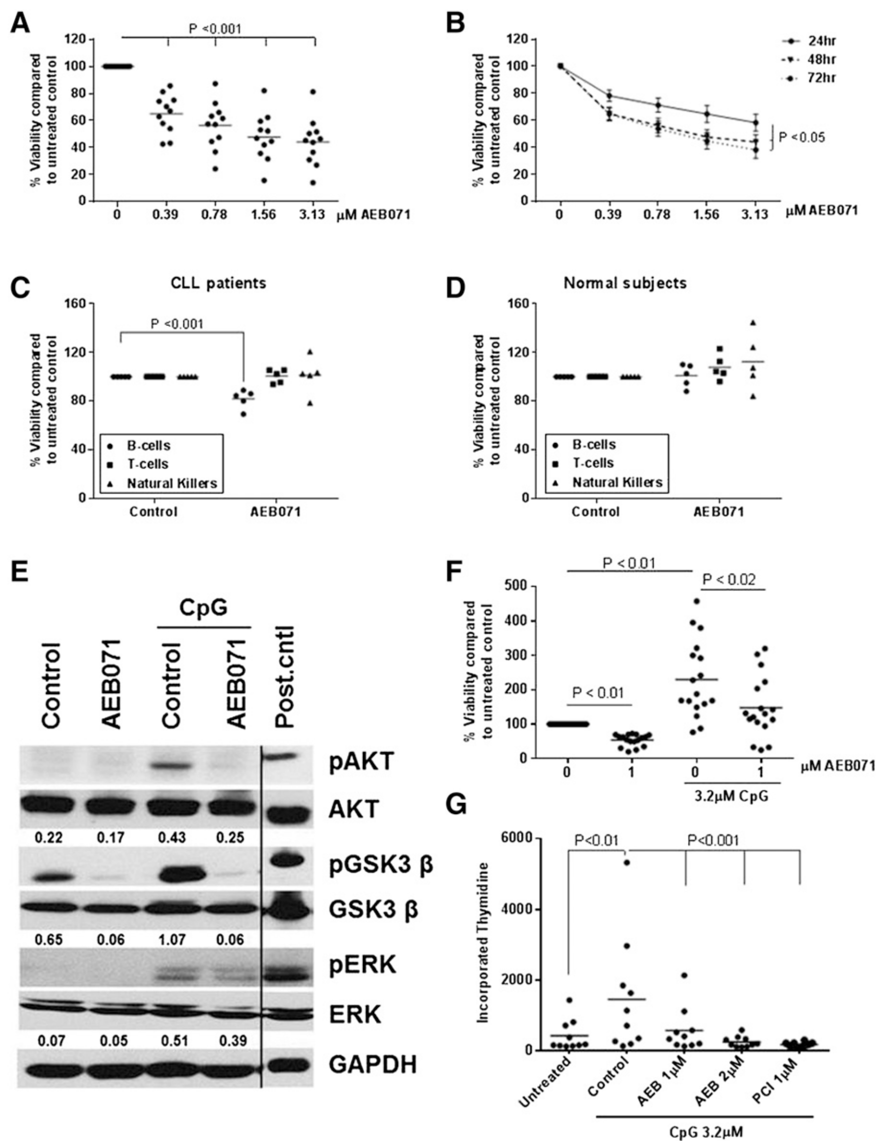

Figure 1. AEB071 induces selective cytotoxicity in CLL cells and inhibits proliferation. (A-B) CD19$^+$ cells from CLL patients (N = 11) were incubated with or without increasing concentrations of AEB071 for up to 72 hours. Viability was determined by MTS assay and was calculated relative to time-matched untreated controls. Dark lines represent averages. (C) Whole blood from CLL patients (N = 5) was incubated with AEB071 (2 μM) for 24 hours. Viability was determined by flow cytometry as described in "Methods." Dark lines represent averages. (D) Whole blood from normal subjects (N = 5) was incubated without AEB071 (2 μM) for 24 hours. Viability was determined by flow cytometry as described in "Methods." Dark lines represent averages. (E) CD19$^+$ cells from CLL patients (N = 3) were treated with AEB071 (2 μM) and stimulated with CpG685 oligonucleotides (3.2 μM) for 24 hours. AKT phosphorylation at Ser473, GSK3-β phosphorylation at Ser9, and ERK phosphorylation at Thr202/Tyr204 was assessed by immunoblot. A representative blot with band quantification is presented; black line indicates cropped regions wherein only relevant bands are shown. (F) CD19$^+$ cells from CLL patients (N = 17) were incubated with AEB071 (1 μM) and CpG685 (3.2 μM) for 72 hours. Viability was determined by MTS. Dark lines represent averages. (G) CD19$^+$ cells from CLL patients (N = 10) were incubated with CpG685 (3.2 μM) and treated with AEB071 (1 or 2 μM) or PCI-32765 (1 μM) and proliferation was assessed 120 hours later by tritiated thymidine. Dark lines represent averages.

This antiproliferative effect was comparable to that elicited by the BTK inhibitor ibrutinib (PCI-32765) (Figure 1G). These data demonstrate the ability of AEB071 to inhibit potent mitogenic signals as well as CLL cell proliferation after Toll-like receptor 9 signaling mediated by CpG.

AEB071 attenuates BCR-dependent activation in B-CLL cells

Targeting BCR downstream pathways is of therapeutic importance in eradicating CLL cells. Activation of BCR has been shown to lead to downstream activation of several survival pathways including the MAPK, PI3K, and NF-κB pathways.[1] Given the role of PKC in these signaling pathways induced by BCR engagement, we therefore sought to determine whether AEB071 could inhibit activation-induced signaling through these pathways. B-CLL cells were stimulated with immobilized anti–immunoglobulin M (IgM). As shown in Figure 2A-B, treatment with AEB071 resulted in a reduction of anti-IgM–induced phosphorylation of ERK1/2 (MAPK pathway), AKT and GSK3-β (PI3K pathway), and IκBα (NF-κB pathway). In addition, we observed a marked increase in CLL viability with anti-IgM stimulation that was significantly attenuated with AEB071 treatment as measured by annexin V/PI staining (Figure 2C). These data suggest that AEB071 treatment can alter survival signaling via BCR signaling involving the MAPK, PI3K, and NF-κB pathways thereby providing multiple mechanisms for altering CLL cell survival.

AEB071 antagonizes PMA-induced B-CLL survival and enhances apoptosis

Next, we sought to determine whether AEB071 could alter survival pathways induced by other stimuli. For this purpose, the tumor promoter phorbol 12-myristate 13-acetate (PMA) as a direct activator of PKC[39,40] was used. As expected, AEB071 antagonized PMA-induced ERK, AKT, GSK3-β, and NF-κB activation (Figure 3A-B), and attenuated PMA-induced CLL viability (Figure 3C).

Because the clonal excess of B cells observed in CLL disease results from decreased cell death rather than increased proliferation,[41] we sought to investigate the effect of AEB071 on apoptosis in stimulated B-CLL cells. B-CLL cells were stimulated with the potent mitogen PMA. AEB071 treatment markedly

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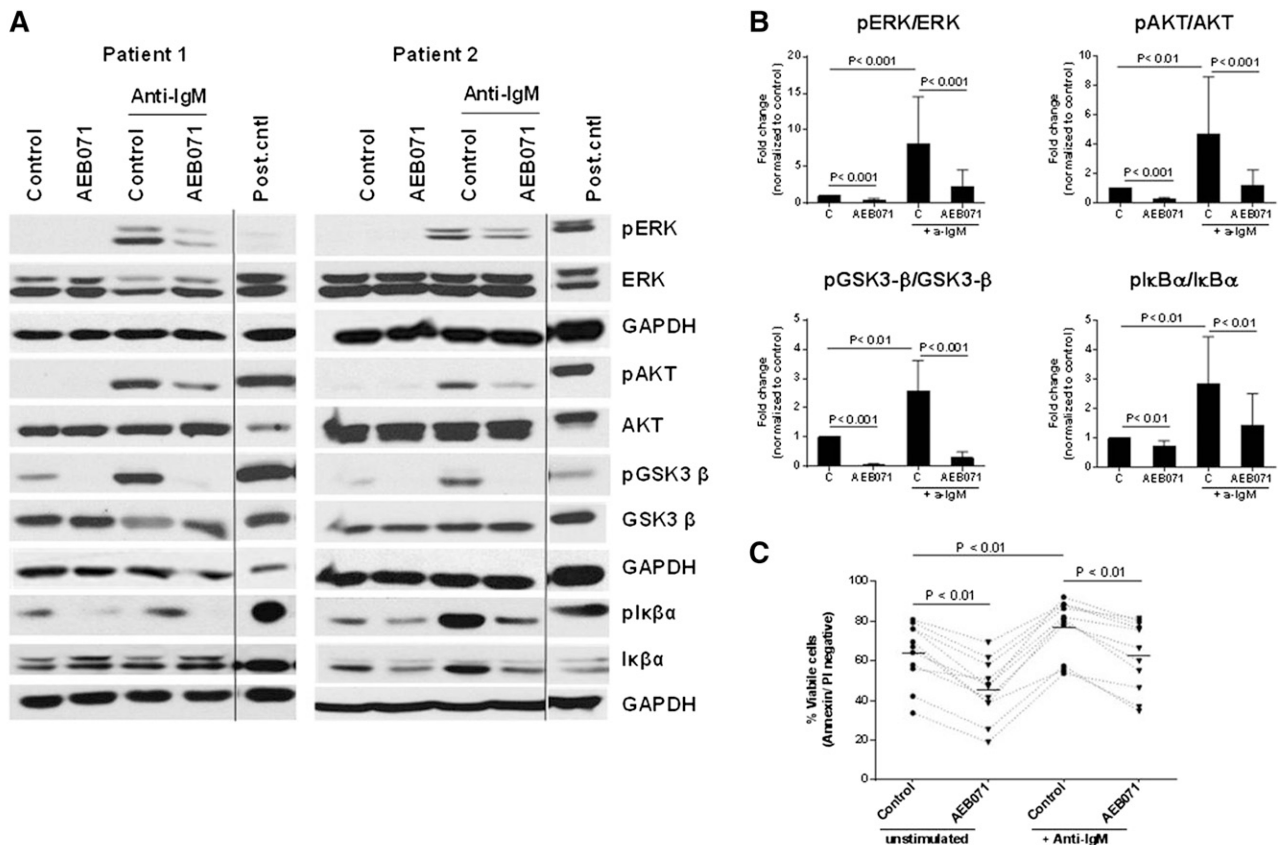

Figure 2. AEB071 attenuates BCR-dependent activation. (A-B) CD19$^+$ cells from CLL patients (N = 8) were treated with AEB071 (2 μM) and stimulated with immobilized anti-IgM for 24 hours. ERK phosphorylation at Thr202/Tyr204, AKT phosphorylation at Ser473, GSK3-β phosphorylation at Ser9, and IκBα phosphorylation was assessed by immunoblot. Results from 2 patients are presented; black line indicates cropped regions wherein only relevant bands are shown. Band quantification is represented as fold change from unstimulated control (mean ± standard deviation [SD]). (C) CD19$^+$ cells from CLL patients (N = 11) were stimulated with immobilized anti-IgM in presence or absence of AEB071 (2 μM) for 24 hours and cytotoxicity was measured by annexin/PI staining. Dark lines represent averages.

attenuated the expression of antiapoptotic proteins such as MCL1 (Figure 3D-E) and BCL2 (Figure 3F-G). In addition, flow cytometric analysis revealed a marked increase in annexin V–positive cells following AEB071 treatment comparable to that elicited by the BTK inhibitor ibrutinib (supplemental Figure 1A); PARP cleavage was also observed (supplemental Figure 1B). These data reflect the effect of AEB071 not only to enhance the apoptosis of B-CLL, but also to induce B-CLL apoptosis in the presence of survival stimuli.

Of note, our in vitro experiments were conducted using medium supplemented with FBS, so to evaluate the contribution of serum protein binding (matrix effect) brought about by human serum, B-CLL patient cells were cultured in medium supplemented with human serum and incubated with increasing concentrations of AEB071. In comparison with cells cultured in FBS-containing medium, we observed a reduced effect of AEB071 on multiple pathways essential for B-CLL survival including MAPK and PI3K (supplemental Figure 2). This is of importance and in line with the reported high serum protein binding capacity (>95%) of AEB071,[31] which would suggest the need to use higher doses of AEB071 to elicit biological effect(s) in patients.

AEB071 antagonizes critical microenvironment stimuli

Accessory signals from the microenvironment are fundamental for the survival, expansion, and progression of malignant B cells.[2,42] Given the importance of microenvironmental stimuli on survival of CLL cells and response to therapy, we sought to evaluate the ability of AEB071 to induce cytotoxicity of CLL cells in the presence or absence of soluble factors known to reduce spontaneous CLL cell apoptosis (CD40L and BAFF), induce activation of key signaling pathways (tumor necrosis factor-α [TNF-α] and interleukin 4 [IL4]),[43-46] or mediate homing within the bone marrow (CXC chemokine ligand 12 [CXCL12]).[47,48] As shown in Figure 4A-E, each of these factors enhanced CLL cell viability cells and cotreatment with AEB071 reduced this protection. These findings suggest that AEB071 can disrupt signaling from the microenvironment that contributes to in vivo CLL cell survival.

A major recognized mechanism of resistance is direct contact and the communications between CLL cells and bone marrow stroma.[48-50] PKC-β–dependent activation of NF-κB in stromal cells has recently been found to be pivotal for the survival of B-CLL cells in vivo.[15] Therefore, the efficacy of AEB071 in the presence of stromal protection was investigated using the murine marrow-derived mesenchymal cell line 9-15c. As expected, coculture of B-CLL cells for 48 hours on stromal cells resulted in a significant increase in cell viability ($P < .01$, Figure 4F). Treatment with AEB071 was able to overcome this stromal protection and induce cytotoxicity comparable to B-CLL cells cultured under normal suspension conditions (Figure 4F). Direct treatment of stromal cells with AEB071 for 48 hours had no effect on viability, hence ruling out the contribution of reduced stromal cell viability to the cytotoxicity effects of AEB071 on B-CLL cells (supplemental Figure 3A).

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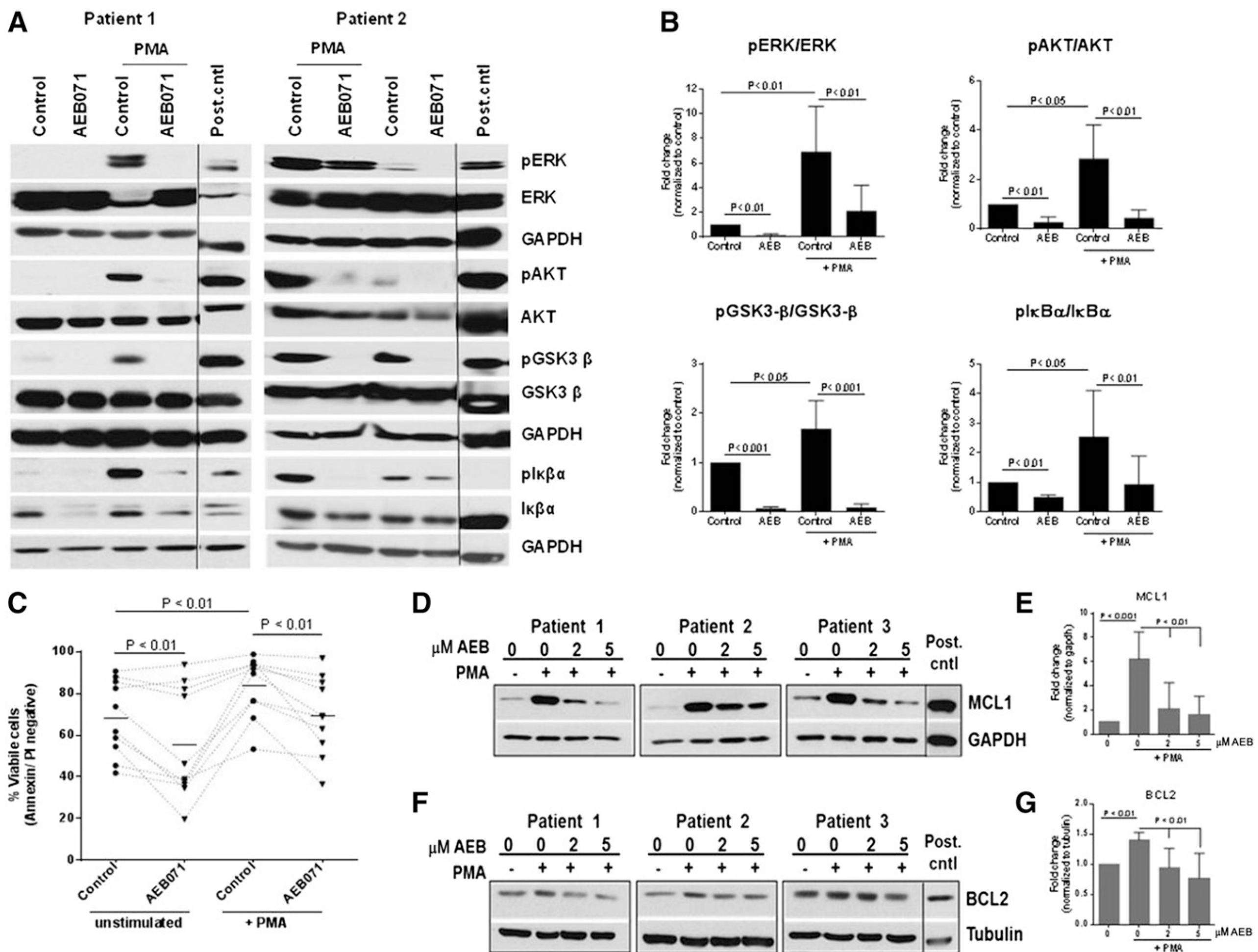

Figure 3. AEB071 antagonizes PMA-induced B-CLL survival and enhances apoptosis. (A-B) $CD19^+$ cells from CLL patients (N = 7) were treated with AEB071 (2 μM) and stimulated with PMA (250 ng/mL) for 24 hours. ERK phosphorylation at Thr202/Tyr204, AKT phosphorylation at Ser473, GSK3-β phosphorylation at Ser9, and IκBα phosphorylation was assessed by immunoblot. Results from 2 patients are presented; black line indicates cropped regions wherein only relevant bands are shown. Band quantification is represented as fold change from unstimulated control (mean ± SD). (C) $CD19^+$ cells from CLL patients (N = 10) were stimulated with PMA (250 ng/mL) in presence or absence of AEB071 (2 μM) for 24 hours and cytotoxicity was measured by annexin/PI staining. Dark lines represent averages. (D-G) $CD19^+$ cells from CLL patients (N = 6) were treated with AEB071 (2 or 5 μM) and stimulated with PMA (250 ng/mL) for 24 hours. Expression of MCL1 (D-E) and BCL2 (F-G) was assessed by immunoblot. Results from 3 patients are presented; black line indicates cropped regions wherein only relevant bands are shown. Band quantification is represented as fold change from unstimulated control (mean ± SD).

Similar results were observed when evaluating the cytotoxic effect of AEB071 using a different stromal cell line, namely the human marrow–derived fibroblast cell line HS-5 (supplemental Figure 3B). AEB071 treatment notably induced B-CLL cytotoxicity with no effect on stromal cell viability (supplemental Figure 3C), indicating that AEB071-induced B-CLL cytotoxicity is not a function of reduced stromal cell viability. PKC-β expression and the subsequent activation of NF-κB in bone marrow stromal cells have been shown to be a prerequisite to support the survival of CLL cells.[15] Together, these data suggest that the cytotoxic effect elicited by AEB071 will not be significantly diminished in the presence of a protective in vivo microenvironment.

GSK3-β as a key mediator in AEB071-induced effects in B-CLL cells

Although AEB071 primarily affects PKC, GSK3 isoforms were revealed by biochemical kinase profiling to be valid targets of AEB071.[27] Accordingly, we noted that after 30 minutes of anti-IgM stimulation, AEB071 could directly abolish GSK3-β phosphorylation without affecting upstream PKC phosphorylation, which was attenuated 24 hours poststimulation, indicating an early and perhaps direct effect of AEB071 on GSK3-β (Figure 5A-B). Protein phosphatase 2A (PP2A) dephosphorylates and has been shown to activate GSK3-β.[51,52] We next sought to investigate whether the effect of AEB071 on GSK3-β dephosphorylation is dependent upon activation of PP2A. For this purpose, okadaic acid (a known inhibitor of PP2A) was used in cell culture experiments. The ability of AEB071 to dephosphorylate GSK3-β in the presence of okadaic acid was reduced yet not completely abolished (Figure 5C-D). Moreover, when B-CLL cell viability was measured 48 hours later we noted that in the presence of the PP2A inhibitor, the cytotoxic effect of AEB071 was clearly attenuated (Figure 5E). In addition, upon measuring PP2A enzymatic activity, we observed that B-CLL cells treated with AEB071 demonstrated increased enzymatic activity (Figure 5F) similar to that observed with the immunosuppressive drug FTY720 previously reported to induce PP2A activation.[36] These data indicate that AEB071 mediates its cytotoxic effect(s) partially through PP2A activation hence suggesting a direct effect of AEB071 on GSK3-β in CLL cells.

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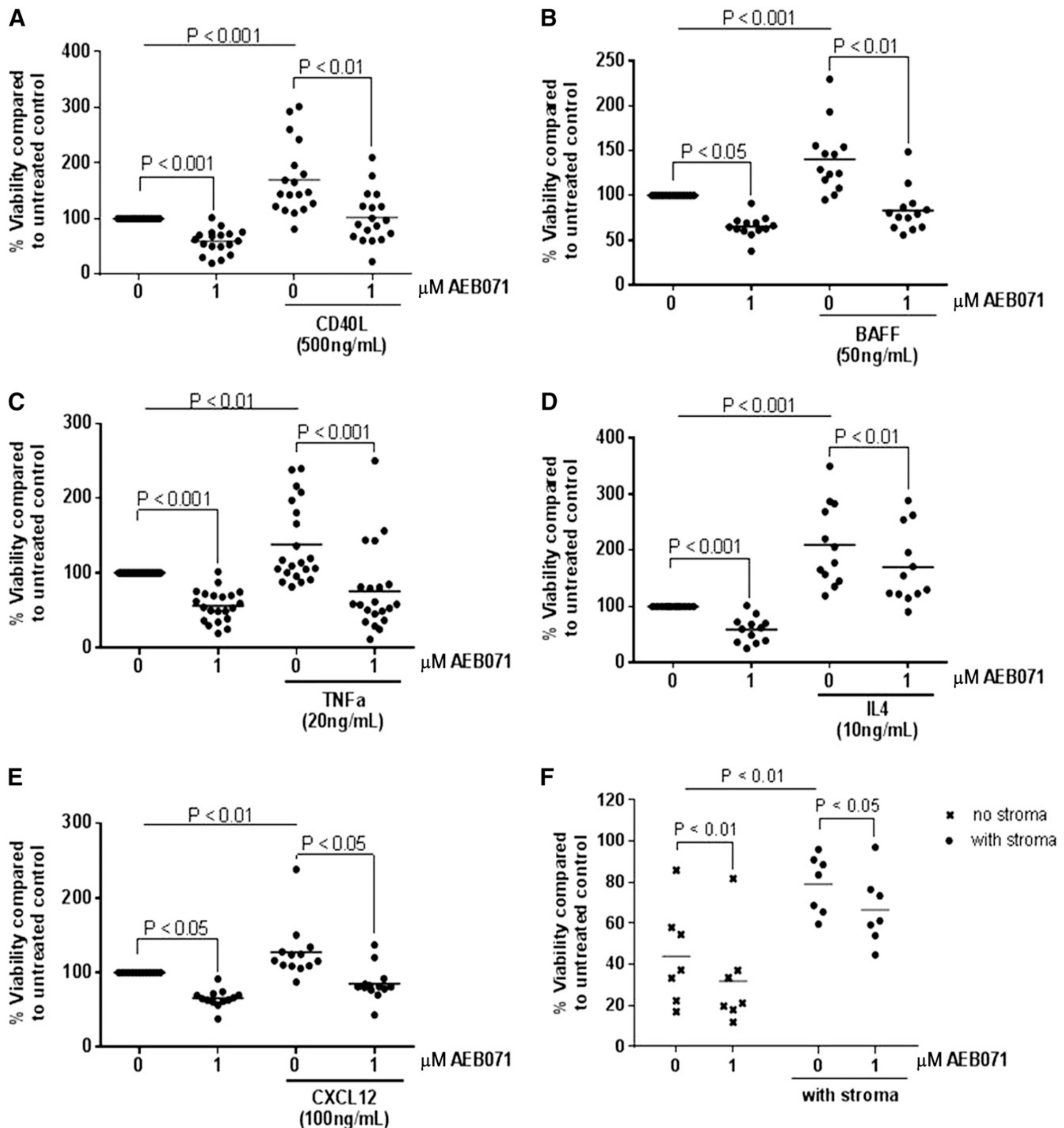

Figure 4. AEB071 antagonizes microenvironment stimuli and stromal-induced protection. CD19$^+$ cells from CLL patients (N = 12-21) were incubated with 1 μM AEB071 and 1 μg/mL CD40L (A), 50 ng/mL BAFF (B), 20 ng/mL TNF-α (C), 800 U/mL IL-4 (D), and 100 ng/mL CXCL12 (E). Viability at 72 hours was determined by MTS. Dark lines represent averages. (F) CD19$^+$ cells from CLL patients (N = 7) were isolated from peripheral blood and incubated with AEB071 (1 μM) in suspension or in coculture with 9-15c stromal cells for 48 hours. Viability was determined by annexin V/PI flow cytometry. Dark lines represent averages.

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AEB071 alters β-catenin expression in B-CLL cells

One critical downstream target of both PKC and GSK3-β is β-catenin, an important regulator of cell proliferation in various cells including malignant cancer cells. Stability of β-catenin is tightly controlled by GSK3-β, which phosphorylates β-catenin thus facilitating its subsequent ubiquitin-mediated degradation; however, in cancer cells, GSK3-β is inactivated by phosphorylation at serine 9. As AEB071 could significantly inhibit GSK3-β phosphorylation (Figures 2, 3, and 5), we sought to examine the effect of AEB071 on β-catenin expression in B-CLL in addition to downstream transcriptional targets of β-catenin as c-Myc, cyclin D1, and CD44. In PMA-stimulated B-CLL cells, AEB071 treatment mediated dephosphorylation of GSK3-β, which correlated with degradation of β-catenin and downmodulation of cMyc, cyclin D1, and CD44 on the protein level (Figure 6A-B). We also evaluated the transcript levels of these genes by real-time PCR; decreases in *c-Myc* and *Cyclin D1* transcript levels were observed in both unstimulated and PMA-stimulated B-CLL cells (Figure 6C-D),

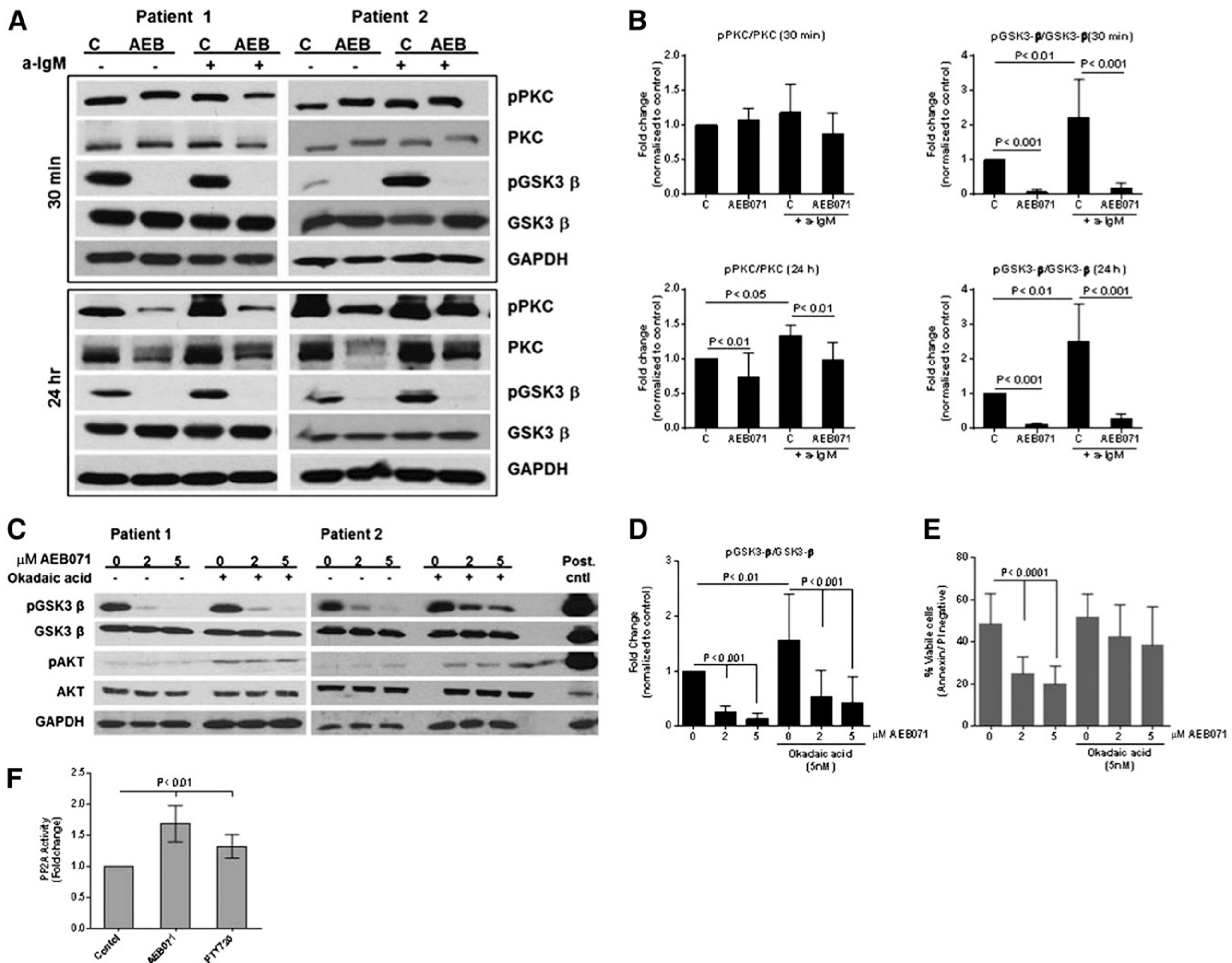

Figure 5. GSK3-β contributes to AEB071-induced effects in B-CLL. (A-B) CD19⁺ cells from CLL patients (N = 9) were treated with AEB071 (2 μM) and stimulated with immobilized anti-IgM for 30 minutes or 24 hours. PKC phosphorylation at Thr638/641 and GSK3-β phosphorylation at Ser9 was assessed by immunoblot. Results from 2 patients are shown and band quantification is represented as fold change from unstimulated control (mean ± SD). (C-D) CD19⁺ cells from CLL patients (N = 6) were pretreated with media or okadaic acid (5 nM) for 2 hours, followed by incubation for 3 hours with AEB071 (2 or 5 μM). GSK3-β phosphorylation at Ser9 and AKT phosphorylation at Ser473 was assessed by immunoblot. Results from 2 patients are shown and GSK3-β band quantification is represented as fold change from unstimulated control (mean ± SD). (E) CD19⁺ cells from CLL patients (N = 4) were pretreated with media or okadaic acid (5 nM) for 2 hours, followed by incubation with AEB071 for 48 hours. Cell viability was measured by annexin/PI staining and expressed as mean ± SD. (F) CD19⁺ cells from CLL patients (N = 3) were treated with AEB071 (2 μM) or FTY720 for 5 hours. Enzymatic activity of PP2A in the cell lysates was measured by a nonradioactive assay as described in methods and expressed as fold change from untreated control (mean ± SD).

whereas a significant decrease in *CD44* transcript was only observed in PMA-stimulated B-CLL cells (Figure 6E). These results indicate that AEB071, through dephosphorylating GSK3-β, induces β-catenin degradation as well as reduces various β-catenin downstream transcriptional targets important in B-CLL survival.

Interestingly, although loss of β-catenin was observed at lower AEB071 concentrations, increased concentrations of AEB071 (10 μM) correlated with increased levels of β-catenin (Figure 6F) whereas GSK3-β was still dephosphorylated (active form), suggesting GSK3-β–independent β-catenin regulation.[53] This is consistent with previous reports indicating that AEB071, at higher doses, is a direct activator of β-catenin signaling.[27,54] Moreover, treatment of B-CLL cells with higher doses of AEB071 resulted in an increase in cell survival starting at a dose of 6.25 μM (Figure 6G), which could be a reflection of our observed increase in β-catenin levels. Given the importance of Wnt/β-catenin signaling in hematological malignancy, our laboratory is currently undertaking further investigations to explore the effect(s) of AEB071 on the Wnt/β-catenin pathway. However, when cells were cultured in human serum-containing medium, we observed a reduced effect of AEB071 on β-catenin and its downstream target c-Myc (supplemental Figure 2) when compared with cells cultured in FBS-containing medium. This dose-dependent effect of AEB071 on β-catenin would suggest a potentially narrow therapeutic index of AEB071 where avoiding high doses of drug would be important to preventing activation of this pathway.

Discussion

CLL is characterized by aberrant activation of several signaling/transcriptional pathways that promote B-CLL cell survival (eg, MAPK, PI3K/AKT, Wnt/β-catenin, and NF-κB). Therefore, a therapeutic strategy simultaneously targeting multiple death and antioncogenic pathways disrupted in this disease may have broad application for many subsets of patients.

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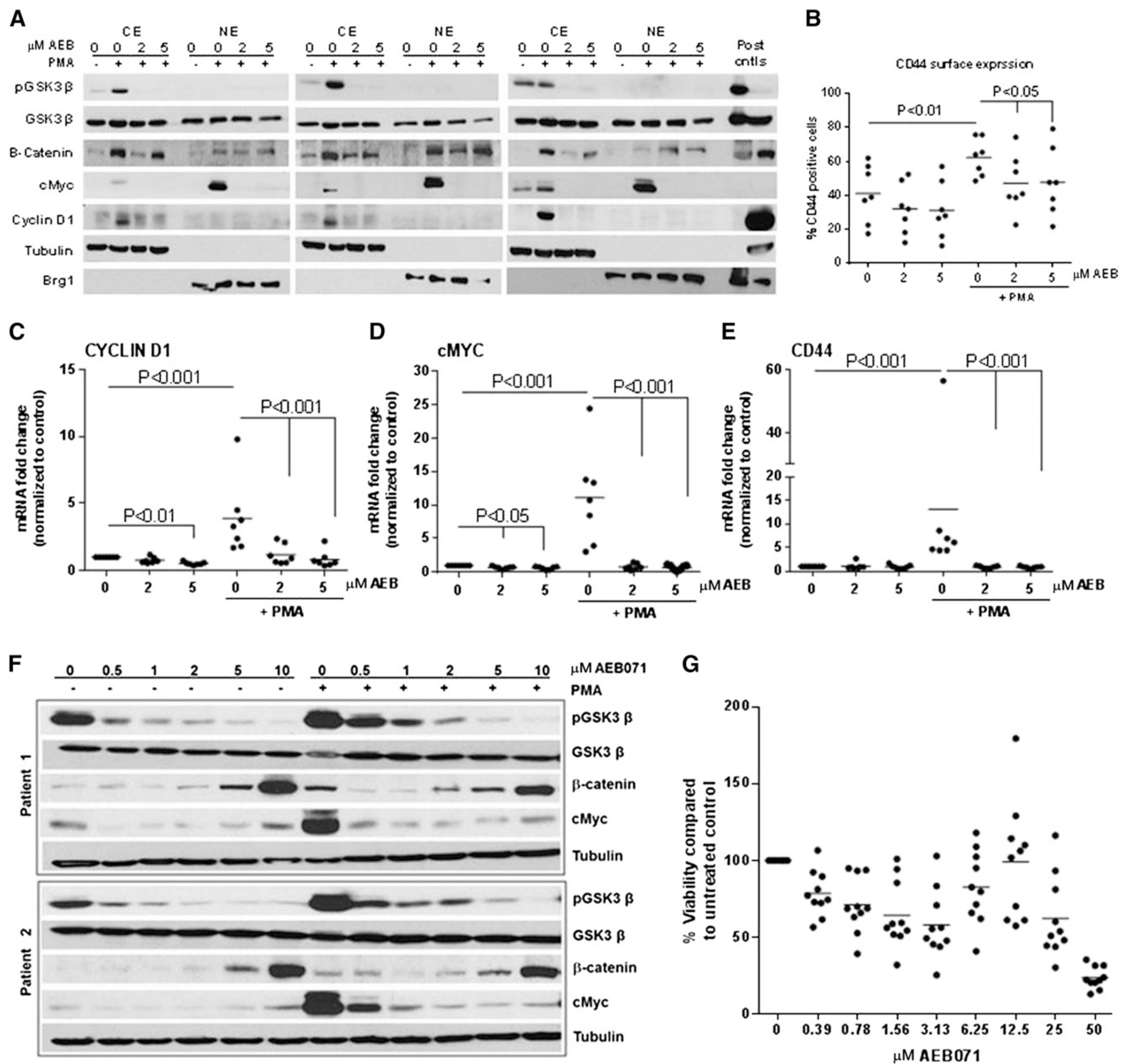

Figure 6. AEB071 targets β-catenin and its downstream transcriptional targets in B-CLL. (A) CD19+ cells from CLL patients (N = 3) were treated with AEB071 (2 or 5 μM) and stimulated with PMA (250 ng/mL) for 24 hours. GSK3-β phosphorylation at Ser9 and protein expression of β-Catenin, c-Myc, and Cyclin D1 was assessed in cytoplasmic (CE) and nuclear extracts (NE) by immunoblot. (B-E) CD19+ cells from CLL patients (N = 7) were treated with AEB071 (2 or 5 μM) and stimulated with PMA (250 ng/mL) for 24 hours, wherein surface expression of CD44 (B) was evaluated in viable cells by flow cytometry and transcript levels of *Cyclin D1* (C), *c-Myc* (D), and *CD44* (E) was estimated by real-time PCR. Dark lines represent averages. (F) CD19+ cells from CLL patients (N = 3) were treated with increasing concentrations of AEB071 and stimulated with PMA (250 ng/mL) for 24 hours. GSK3-β phosphorylation at Ser9 and protein expression of β-Catenin and c-Myc was assessed in whole-cell lysates by immunoblot. Results from 2 patients are presented. (G) CD19+ cells from CLL patients (N = 10) were incubated with increasing concentrations of AEB071 for 24 hours. Viability was determined by MTS assay and calculated relative to untreated control. Dark lines represent averages.

Efforts to target BCR signaling pathway with idelalisib[55-61] and ibrutinib[6,62-66] have shown significant clinical activity in CLL including those with high-risk genomic disease. However, despite the impressive durability of remissions obtained with ibrutinib in CLL, minimal residual disease-negative status has generally not been achieved. A recent study in ibrutinib refractory CLL patients has also identified mutations in BTK and its downstream target, phospholipase Cγ2 (PLCγ2),[67] which lead to the autonomous BCR signaling pathway and can potentially explain the acquired resistance of these patients to ibrutinib and further emphasize the need for alternate therapies for this subset of patients. Recently, our laboratory has identified an additional gain-of-function mutation in PLCγ2 (L845F) among ibrutinib-treated patients,[68] however, mutations in PKC were not detected.[68] Furthermore, in vitro treatment of cells from patients who experience persistent lymphocytosis following ibrutinib therapy showed that these cells could respond to a panel of targeted kinase inhibitors including AEB071.[69] PKC-β has been shown to be essential to CLL cell survival and proliferation in vivo. As PKC-β is an immediate downstream target of BTK and PLCγ2 at the confluence of the BCR pathway, the NF-κB cascade, and the RAS/RAF/MEK/ERK pathway, modulation of PKC-β has the potential to bypass mechanisms of resistance affected by mutations in these genes. Therefore, PKC inhibitors represent attractive therapeutic options in CLL patients including ibrutinib-refractory patients.

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Our study demonstrates that AEB071, a potent PKC inhibitor, shows selective cytotoxicity against B-CLL cells whereas no significant cytotoxicity was seen against normal B, T, or NK cells. Of note, AEB071 treatment not only enhanced the spontaneous apoptosis of B-CLL, but also attenuated the expression of the antiapoptotic proteins MCL1 and BCL2. As expected, PKC inhibition by AEB071 attenuated fundamental BCR-mediated survival pathways including MAPK, PI3K, and NF-κB pathways in stimulated B-CLL cells. In addition, our data show that AEB071 completely abrogated CpG oligonucleotide-induced proliferation and survival of CLL cells in vitro and was able to overcome the protective effect(s) elicited by various microenvironmental stimuli including CD40L, BAFF, TNF-α, IL4, and CXCL12. Similarly, coculture with stromal cells decreased the spontaneous apoptosis of CLL cells, wherein AEB071 treatment was able to abrogate stromal protection. Together, these data suggest that AEB071 acts both directly on CLL cells to induce apoptosis, and effectively blocks microenvironment-mediated survival signals in primary CLL cells, thereby providing multiple mechanisms for altering CLL cell survival.

Although AEB071 primarily affects PKC, GSK3 isoforms have also been shown to be targets of AEB071.[27] Interestingly, we could demonstrate an early and PKC-independent effect of AEB071 on GSK3-β that was clearly attenuated but not completely abolished upon PP2A inhibition. In addition, treatment with AEB071 increases PP2A enzymatic activity in B-CLL cells in ranges previously described for FTY720.[36] These findings indicate that, in addition to inhibiting PKC-β, AEB071 mediates its cytotoxic effect(s) partially through PP2A activation as well as through a direct effect on GSK3-β in B cells.

Apart from our mechanistic studies which clearly demonstrate the effect of AEB071 in attenuating BCR-mediated survival signaling pathways, we could also show an effect of AEB071 on β-catenin, a critical downstream target of both PKC and GSK3-β, which suggests that this inhibitor has alternative targets potentially relevant in CLL. This is of profound importance given the fact that the Wnt/β-catenin pathway is aberrantly activated in CLL and contributes to the antiapoptotic and mitogenic nature of B-CLL cells.[70-72] In our study, we showed that AEB071 treatment at lower doses mediated GSK3-β dephosphorylation, which correlated with reduced expression of β-catenin as well as its downstream transcriptional targets c-Myc, Cyclin D1, and CD44 at both protein and transcript levels. Given this novel finding, pursuing AEB071 as a therapeutic agent that can target Wnt/β-catenin pathway in CLL is worthwhile and may prove to have significant therapeutic benefit in CLL patients.

Given the fact that AEB071, at higher doses, was reported to activate β-catenin signaling,[27,54] it was important to evaluate this effect in B-CLL. Our studies revealed increased levels of β-catenin with 10 μM AEB071. Moreover, treatment of B-CLL cells with higher doses of AEB071 enhanced B-CLL cell survival, which could be a result of the increase in β-catenin levels that we observed. This is of importance given the reported high serum protein binding capacity (>95%) of AEB071,[31] which would suggest the need to use higher doses of AEB071 to elicit biological effect(s). Such a dose-dependent effect of AEB071 on β-catenin suggests a potentially narrow therapeutic index of AEB071 such that avoiding high doses of drug would be important to preventing activation of this survival pathway.

Lastly, considering the importance of PKC-β to CLL development in Eμ-TCL1 mice[10] and the preliminary efficacy of AEB071 in preclinical models of DLBCL,[28] our laboratory is currently pursing in vivo studies using TCL1 transgenic mice. Our preliminary results from 2 different CLL mouse models reveal beneficial antitumor effects of AEB071, wherein AEB071 treatment resulted in reduction of CD5/CD19-positive peripheral blood lymphocytes and spleen size in leukemic mice (supplemental Figure 4). Such findings support the use of AEB071 as a valuable therapeutic in CLL.

Taken together, our results indicate that targeting PKC-β has the potential to disrupt signaling from the microenvironment contributing to CLL cell survival and, potentially, drug resistance. To address limitations of currently used therapies in CLL, further studies are needed to elucidate the role of novel agents and their combinations in the management of CLL, catering to the individualized needs of patients. Future efforts targeting PKC with the PKC inhibitor AEB071 in clinical trials of relapsed and refractory CLL patients are warranted.

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Authorship

Contribution: D.E.-G. planned the research, performed experiments, analyzed data, drafted the first and subsequent drafts of the paper, and approved the final version of the paper; K.W., M.C., T.D.L., Y.Z., J.A.W., and E.W. contributed to components of the experimental work presented (biological and animal studies); J.S.B. provided scientific input and edited the manuscript; J.A.W., F.T.A., J.J., L.A., and K.M. accrued patients, reviewed drafts of the paper, and approved the final version of the paper; C.-H.W. and C.-S.C. provided necessary reagents, reviewed drafts, and approved the final version of the paper; A.L. and X.Z. performed all statistical analyses, reviewed drafts, and approved the final version of the paper; and R.L. and J.C.B. planned every aspect of the proposal, supervised the research, analyzed data, reviewed and modified drafts, obtained funding for the research work, and approved the final version of the paper.

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