Protein Kinase C Inhibitor Sotrastaurin Selectively Inhibits the Growth of CD79 Mutant Diffuse Large B-Cell Lymphomas

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Abstract

The activated B-cell–like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) correlates with poor prognosis. The ABC subtype of DLBCL is associated with constitutive activation of the NF-κB pathway, and oncogenic lesions have been identified in its regulators, including CARD11/CARMA1 (caspase recruitment domain-containing protein 11), A20/TNFAIP3, and CD79A/B. In this study, we offer evidence of therapeutic potential for the selective PKC (protein kinase C) inhibitor sotrastaurin (STN) in preclinical models of DLBCL. A significant fraction of ABC DLBCL cell lines exhibited strong sensitivity to STN, and we found that the molecular nature of NF-κB pathway lesions predicted responsiveness. CD79A/B mutations correlated with STN sensitivity, whereas CARD11 mutations rendered ABC DLBCL cell lines insensitive. Growth inhibitory effects of PKC inhibition correlated with NF-κB pathway inhibition and were mediated by induction of G1-phase cell-cycle arrest and/or cell death. We found that STN produced significant antitumor effects in a mouse xenograft model of CD79A/B-mutated DLBCL. Collectively, our findings offer a strong rationale for the clinical evaluation of STN in ABC DLBCL patients who harbor CD79 mutations also illustrating the necessity to stratify DLBCL patients according to their genetic abnormalities. Cancer Res; 71(7); 2643-53. ©2011 AACR

Introduction

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype of malignant lymphoma and is diagnosed in more than 20,000 patients each year in the United States. It is heterogeneous with respect to morphology, biology, and clinical presentation (1). By gene expression profiling, at least 3 molecular subtypes of DLBCL can be distinguished: germinal center B-cell–like (GCB) DLBCL, activated B-cell–like (ABC) DLBCL, and primary mediastinal B-cell lymphoma (PMBL; refs. 2–4). The molecular DLBCL subtypes not only differ with respect to their gene expression patterns but also have significantly different overall survival rates. GCB DLBCL and PMBL patients respond favorably to current standard-of-care combined therapy with the anti-CD20 antibody rituximab and CHOP [cyclophosphamide, hydroxydaunorubicin (doxorubicin), vincristine (Oncovin), prednisone] chemotherapy (R-CHOP). In contrast, ABC DLBCL represents the least curable subtype with 3-year overall survival rates of approximately 40% (5). A hallmark of the molecular pathogenesis of ABC DLBCL is the constitutive activation of the NF-κB pathway, which occurs predominantly via the CBM (CARMA1-Bcl10-Malt1) [CARD11 (caspase recruitment domain-containing protein 11, also known as CARMA1)/BCL10/MALT1] signaling complex that promotes cell proliferation, differentiation, and suppresses apoptosis (6, 7).

Physiologically, activation of the CBM complex in B cells occurs in response to B-cell receptor (BCR) stimulation. Antigen binding to the BCR induces receptor oligomerization and thereby promotes Lyn-mediated phosphorylation of ITAM (immunoreceptor tyrosine-based activation motif) domains in the B-cell coreceptors CD79A and CD79B (8). Phosphorylated ITAM domains recruit and activate the protein tyrosine kinase SYK (spleen tyrosine kinase) at the plasma membrane that initiates downstream signaling through BTK ( Bruton’s tyrosine kinase) and PLCγ (phospholipase Cγ) and ultimately leads to the activation of PKC (protein kinase C). In B cells, PKCβ is thought to be the predominant PKC isoform mediating BCR-NF-κB activation, at least in part, through the phosphorylation of CARD11 (9, 10). Once activated at the plasma membrane, the CBM complex facilitates the activation of the IKK (1 kappa B kinase) complex, which phosphorylates IκBζ targeting it for proteosomal degradation, and thereby allows NF-κB transcription factors to enter the nucleus and drive the expression of its target genes.
Until recently, it was unclear whether the constitutive NF-κB pathway activation in ABC DLBCL represents a primary pathogenetic event in lymphomagenesis or merely reflects the physiologic status of the tumor cell of origin. The identification of oncogenic CARD11 mutations provided the first evidence for genetic deregulation of this pathway (11). Moreover, recent studies have revealed somatically acquired genetic lesions in several NF-κB pathway regulators, including frequent loss-of-function mutations and deletions in the negative regulator A20 (12, 13) and genetic abnormalities in CD79A and CD79B (14). Thus, the vast majority of ABC DLBCLs seem to harbor genetic lesions that constitutively activate NF-κB pathway signaling. Previous studies showed that ABC DLBCL lines are sensitive to inhibition of CARD11, BCL10, MALT1, or IKKβ, showing a clear dependence on NF-κB pathway signaling (6, 7, 15). These results contrast another study that proposed that ligand-independent “tonic” BCR signaling is a more general feature of B-cell lymphomas that renders these cells dependent on downstream BCR signaling (16). To clarify the role of BCR signaling and assess the therapeutic potential of PKC inhibitors in the treatment of DLBCL, we analyzed the response of DLBCL cell lines to treatment with the selective PKC inhibitor sotraustatin (STN, also known as AEB071), which is currently in phase II clinical trials for psoriasis and solid organ transplantation (17–20).

Materials and Methods

Cell culture and cell line generation

TMD8, SU-DHL4, SU-DHL2, BJAB, U2932, K422, and HBL1 cells were grown in RPMI 1640 with 10% FBS, DB cells in RPMI 1640 with 20% FBS, OCI-Ly3 in Iscove’s modified Dulbecco’s medium (IMDM) with 20% FBS, and OCI-Ly10 in IMDM with 20% human serum. Cell lines were authenticated by single nucleotide polymorphism profiling (fingerprinting). For the RNA interference experiments, cell lines were engineered to express the murine ecotropic retroviral receptor for efficient retroviral transduction and the bacterial tetracycline repressor for doxycycline-inducible short hairpin RNA (shRNA) expression as described (7). The targeting sequence of the PKCβ shRNA was GCAGCAACACTGTCTCCAAAT.

HBL1 cells were engineered to stably express the activating CARD11-L244P mutation by transduction with a lentiviral vector that constitutively expresses CARD11-L244P driven by either a CMV (cytomegalovirus) or UBC (ubiquitin C) promoter (pLenti6-CMV and pLenti6-UBC; Invitrogen). TMD8 cells were engineered to stably express the activating CARD11-L244P mutation by retroviral transduction. The CARD11-L244P cDNA was inserted into a modified version of the inducible pRetroSuper dual–promoter vector. Retroviral transductions were performed as previously described (7).

Cell growth, flow cytometry, BrdUrd assay, and caspase 3/7 assay

Cell growth assays were performed using CellTiter-Glo Luminescent Cell Viability Assay Reagent (Promega). To determine cell-cycle distribution, cells were analyzed by flow cytometry [fluorescence-activated cell sorting (FACS)] and BrdUrd (bromodeoxyuridine) incorporation (Roche). Caspase 3/7 activity was measured using CaspaseGlo (Promega). Detailed protocols are available in Supplementary Materials and Methods.

TaqMan mRNA expression, NF-κB nuclear translocation, and IL-6/IL-10 secretion assay

In vivo tumor samples were harvested and snap frozen in liquid nitrogen. Tissue samples were homogenized and lysed in RLT buffer with reagent DX, using the TissueLyser (Qiagen), and mRNA expression was analyzed by TaqMan. NF-κB translocation and interleukin (IL)-6/IL-10 secretion levels in supernatants were assessed using Trans-AM (Active Motif) ELISA plates and Quantikine ELISA kits (R&D Systems), respectively. Detailed protocols are available in Supplementary Materials and Methods.

Western blotting and gene expression analysis

For Western blotting, 30 μg of protein from total cell lysate was loaded onto 4% to 12% Bis-Tris gradient gels (Invitrogen). The following primary antibodies were used: α-CARD11, α-c-Rel (Cell Signaling), α-p65 α-PARP, α-PKCβ (Santa Cruz), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; Sigma). Gene expression was measured using whole-genome Agilent 4 × 44K gene expression arrays (Agilent Technologies) following the manufacturer’s protocol. Detailed protocols are available in Supplementary Materials and Methods.

Tumor xenografts

Mice were maintained and handled in accordance with Novartis Biomedical Research Animal Care and Use Committee protocols and regulations. Treatment was initiated when tumor volume reached an average size of 160 mm² (21 days post–tumor implantation). STN solution was prepared weekly and dosed orally on a “tid” schedule. Tumor volume was determined by twice-weekly digital calipering and calculated using the formula: length × width²/2. Data were expressed as mean ± SEM, and differences were considered statistically significant at P < 0.05 by Student t test.

Results

CD79 mutant ABC DLBCL cell lines are sensitive to PKC inhibition

A recent study (16) proposed that the majority of DLBCL cell lines exhibit tonic BCR signaling that leads to dependence on downstream kinases including SYK for their proliferation and survival. This model suggested that cells with tonic BCR signaling may also be sensitive to PKCβ inhibition, as this kinase is a critical mediator of CBM complex activation downstream of SYK (9, 10). We therefore evaluated the effects of 2 ATP-competitive PKC inhibitors, the pan-PKC inhibitor STN (18, 20–22) and the PKCα/β-selective compound BHA536 (Novartis; unpublished, see structure and selectivity data in Supplementary Fig. S1) on the proliferation of a panel of DLBCL cell lines. We included as control compounds the IKKβ-selective inhibitors AFN700 (23) and MLN120B (24).
GCB cell lines were generally insensitive to both IKKβ and PKC inhibitors, with half-maximal growth inhibitory concentrations (IC50) greater than 10 μmol/L in SU-DHL4 and DB cells (Fig. 1A and B and Table 1), which is consistent with the notion that this subtype does not exhibit NF-κB pathway activation (6, 7). In contrast, most ABC DLBCL cell lines were sensitive to IKKβ inhibitors (Fig. 1B), but their response to pharmacologic PKC inhibitors varied strongly (Fig. 1A). OCI-Ly3 and SU-DHL2 cells were insensitive to PKC inhibition, with IC50 values greater than 10 μmol/L in the growth assays with STN and BHA536 (Fig. 1A). Notably, OCI-Ly3 and SU-DHL2 have reported mutations in CARD11 and A20, respectively, and the fact that these oncogenic lesions are thought to function downstream of PKCβ in CBM-NF-κB signaling (Supplementary Fig. S2) provides a potential molecular rationale for their insensitivity. U2932 cells, which displayed intermediate sensitivity to PKC inhibitors (Fig. 1A), were recently reported to harbor a TAK1 mutation, although the oncogenic nature of this mutation has not yet been confirmed experimentally (12). In contrast, HBL1, TMD8,

Figure 1. PKC inhibitors and PKCβ knockdown selectively impair the proliferation of CD79 mutant ABC DLBCL cell lines. CD79A/B mutant ABC DLBCL cell lines (blue; OCI-LY10, HBL1, TMD8), ABC DLBCL cell lines with downstream NF-κB pathway mutations (green; OCI-LY3, U2932, SU-DHL2), and GCB DLBCL cell lines (red; SU-DHL4, DB) were treated for 5 days with serial dilutions of (A) the pan-PKC inhibitor STN, the PKCα/β-selective inhibitor BHA536, (B) the IKKβ inhibitor AFN700, or (C) the BTK inhibitor PCI-32765. Relative cell growth (average of at least 3 independent experiments) was measured by Cell Titer Glo and normalized to dimethyl sulfoxide (DMSO)-treated cells. D, shRNA-mediated knockdown of PKCβ is toxic to CD79A/B mutant ABC DLBCL but not to ABC DLBCL with mutant CARD11 or GCB DLBCL cell lines. PKCβ was knocked down by retroviral vectors expressing an shRNA together with green fluorescent protein (GFP). Live GFP+ cells were enumerated by FACS on the indicated days post-retroviral infection and normalized to the value at day 2 following retroviral infection. Western blot analysis shows shRNA-mediated knockdown of PKCβ. Dox, doxycycline.
PKC, BTK, and SYK inhibitors selectively inhibit the proliferation of CD79 mutant ABC DLBCL cell lines

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NOTE: The table displays the half maximal growth inhibitory concentrations (IC50) for the indicated compounds across several DLBCL cell lines. IC50 values are expressed in micromolar concentrations and were derived from dose-response curves similar to the ones described in Figure 1.

Abbreviations: ND, not determined; NR, not reported.

and OCI-Ly10 cells were very sensitive to both PKC inhibitors, with IC50 values ranging from 0.2 to 1.0 μmol/L (Fig. 1A), suggesting that these cell lines are dependent on BCR signal transduction upstream of CARD11. To further test this hypothesis, we examined the sensitivity of the DLBCL cell line panel to small molecule inhibitors targeting other kinases that mediate signaling from the BCR to CBM complex. Strikingly, HBL1 and TMD8 cells were also the most sensitive lines in response to treatment with BTK and SYK inhibitors (Fig. 1C, Supplementary Fig. S3A, and Table 1). These findings show that HBL1, TMD8, and OCI-Ly10 cells are “addicted” to BCR/CBM/NF-κB pathway signaling and raised the possibility that these cells may harbor genetic lesions that activate the NF-κB pathway upstream of PKCβ. Indeed, during the course of our study, Davis and colleagues identified mutations in the BCR coreceptor CD79A/B that lead to chronic activation of BCR signaling (14). We confirmed that each of the 3 ABC DLBCL cell lines that were sensitive to PKC inhibition harbored mutations in the ITAM motif of CD79A/B (Supplementary Fig. S3A, and Table 1). These findings show that HBL1, TMD8, and OCI-Ly10 cells are “addicted” to BCR signaling inhibitors and identify PKCβ as a novel therapeutic target for this genetically defined subpopulation of DLBCL.

PKC inhibitors reduce NF-κB pathway signaling in CD79 mutant cells

Constitutive activation of the NF-κB pathway is a molecular hallmark of ABC DLBCL cells and is required for their proliferation and survival (2, 6, 7). We therefore evaluated whether the growth inhibitory effect in response to PKC inhibition is mediated through modulation of NF-κB pathway signaling. STN treatment of HBL1 and TMD8 cells inhibited the nuclear translocation of both p65 and c-Rel (Fig. 2A and B), which are NF-κB transcription factor subunits that have been implicated in mediating NF-κB path-
way activation in ABC DLBCL (26). To monitor the transcriptional response to PKC inhibition, we treated the CD79 mutant cell line HBL1 with STN and assessed relative changes in gene expression at several time points by whole-genome expression arrays (Fig. 2C and D). Application of 2 independent NF-κB pathway signatures to the gene expression profiling data revealed that STN treatment caused a significant downregulation of NF-κB target genes, strongly supporting the notion that PKC inhibition results in suppression of the NF-κB pathway (Fig. 2C and D, and Supplementary Fig. S6A and B).

NF-κB pathway activation leads to the induction of various cytokines, such as IL-6 and IL-10, which promote proliferation and survival of B cells (27–29). IL-6 and IL-10 also responded to STN treatment in our gene expression profiling studies, with the most pronounced downregulation at the 6 and 12 hour time points (Supplementary Fig. S6C). We therefore used IL-6 and IL-10 as markers to study NF-κB pathway modulation by PKC inhibitors in more detail. STN treatment resulted in a dose-dependent decrease in IL-6 mRNA expression in the CD79 mutant cell lines OCI-Ly10, HBL1, and TMD8 (Fig. 3A). In contrast, IL-6 mRNA expression was unaffected in
the CARD11 mutant cell line OCI-Ly3 (Fig. 3A), which is consistent with the fact that CARD11 functions downstream of PKC (Supplementary Fig. S2). Treatment of TMD8 cells with 2 additional PKC inhibitors [BHA536 and LY333531, a pan-PKC inhibitor also known as ruboxistaurin (30)] also reduced IL-6 mRNA expression in a dose-dependent manner (Fig. 3B), providing further evidence that IL-6 modulation is an on-target effect of PKC inhibition. The fact that IL-6 is secreted from cells allowed us to monitor IL-6 levels in cell culture supernatants. As expected, treatment with IKKβ inhibitors strongly reduced IL-6 secretion in all ABC DLBCL cell lines tested (Fig. 3C). The PKC inhibitor STN also strongly inhibited IL-6 and IL-10 secretion in TMD8 and HBL1 cells (IC50 < 0.2 μmol/L) but again did not affect their levels in the CARD11 mutant cell line OCI-Ly3 (Fig. 3D and Supplementary Fig. S7). Importantly, the concentrations required to inhibit NF-κB signaling (as measured by IL-6 secretion) in the sensitive cell lines closely correlated with the growth inhibitory IC50 values. Moreover, the lack of NF-κB pathway modulation by PKC inhibitors in OCI-Ly3 cells is in agreement with the insensitivity of this cell line in growth assays. Collectively, these findings show that PKC inhibitors can suppress NF-κB pathway signaling in cells with CD79 mutations and support the notion that downregulation of NF-κB pathway signaling is a major contributor to the growth inhibitory effect observed in these cells.

**Oncogenic CARD11 mutations confer resistance to PKC inhibitors**

Our cell line profiling experiments with STN indicated that OCI-Ly3 cells, which have an activating mutation in CARD11 (L244P), are insensitive to PKC inhibition. This finding suggested that the L244P mutation in the coiled-coil domain of CARD11 might obviate the requirement for PKCβ-mediated activation of the CBM complex (Supplementary Fig. S2). Indeed, we confirmed in an engineered cellular system that in contrast to PMA (phorbol-12-myristate-13-acetate)/ionomycin -induced pathway activation, NF-κB pathway activation in response to CARD11-L244P is not sensitive to PKC inhibition (Supplementary Fig. S8). We therefore wanted to test whether expression of CARD11-L244P in a CD79 mutant cell line confers resistance to PKC inhibitors. To this end, HBL1 cells were stably transduced with lentiviral vectors expressing CARD11-L244P under the CMV or UBC promoter. Expression of the mutant CARD11 allele was confirmed by Western blotting and sequencing of the reverse-transcribed mRNA transcripts (Fig. 4A and B). The expression levels of exogenous CARD11-L244P in the CMV construct was comparable with that of endogenous CARD11 (Fig. 4A and B), and only 2- to 3-fold above endogenous levels when expressed from the UBC promoter (Fig. 4A). Strikingly, expression of CARD11-L244P rendered HBL1 cells to be resistant to the growth inhibitory effects of STN (Fig. 4C). Similar rescue of STN sensitivity was observed in TMD8 cells (Supplementary Fig. S9C). A CARD11 mutant construct lacking the CARD domain, which is crucial to facilitate CBM complex assembly (31), could not confer resistance to PKC inhibitors (Supplementary Fig. S9A). The CARD11-L244P expression lines, however, were still responsive to IKKβ inhibitors (Fig. 4C and Supplementary Fig. S9B), thereby excluding the possibility that the resistance to PKC inhibitors was due to emergence of a nonspecific resistance mechanism.
Next, we analyzed whether the growth rescue by CARD11-L244P expression was due to the restoration of downstream NF-κB signaling. While IL-6 secretion was strongly inhibited by STN in the parental HBL1 cells, IL-6 secretion was no longer modulated in cells engineered to express CARD11-L244P (Fig. 4D). Cells expressing the CARD11 constructs, however, still downregulated IL-6 in response to IKKβ inhibitor treatment (Fig. 4D). Together, these "rescue" experiments further show that the growth inhibitory effect of STN in CD79-mutated cells is due to on-target inhibition and strongly suggests that NF-κB pathway downregulation is a critical mediator of the growth inhibitory effect.

PKC inhibition induces G1 arrest and/or cell death in CD79 mutant cells

To gain additional insights into the nature of the growth inhibitory effects in response to PKC inhibition, we next examined whether STN treatment induces cell-cycle arrest or apoptosis in sensitive lines. Treatment with STN induced a strong G1 arrest in both HBL1 and TMD8 cells, as evidenced by an increase in the G1 population and concomitant decreases in the fraction of cells in S and G2/M phase (Fig. 5A and B, Supplementary Fig. S10). The G1 arrest was further confirmed by a BrdUrd assay, where STN caused a dose-dependent inhibition of S-phase entry in HBL1 and TMD8 cells (Supplementary Fig. S11). Importantly, the cell-cycle
arrest occurs at concentrations that correlate well with the growth inhibitory IC_{50} values. While G_1 arrest seemed to be the predominant response of CD79-mutated cells to STN, we noted the emergence of a sub-G_1 peak in TMD8 cells, especially at later time points (48 hours; Fig. 5B and Supplementary Fig. S10), suggesting that TMD8 cells may be undergoing cell death upon prolonged STN treatment. We also detected an increase in PARP cleavage, a cellular marker of cell death/apoptosis, in TMD8 cells but not in HBL1 cells (Supplementary Fig. S12C). Expression of CARD11-L244P in TMD8 cells could rescue both the G_1 arrest (Fig. 5C) and cell death (Fig. 5D), strongly indicating that these effects of STN are on-target and mediated by inhibition of CBM-NF_{κB} signaling.

To further examine whether STN induces an apoptotic response in TMD8 cells, we monitored the activity of the key effector caspases (3 and 7) in response to compound treatment. STN treatment induced only a modest increase in caspase 3/7 activity, especially when compared with strong apoptosis inducers such as the DNA-damaging agent camptothecin (Supplementary Fig. S12A and B). These findings suggest that the cell death observed in TMD8 cells at later time points is likely a secondary effect of prolonged G_1 arrest rather than acute apoptosis induction. Collectively, our data indicate that the major response of CD79-mutated DLBCL cell lines to STN seems to be G_1 cell-cycle arrest.

**STN inhibits tumor growth of CD79-mutated DLBCL in vivo**

To address whether CD79-mutated ABC DLBCLs are also sensitive to PKC inhibition in an in vivo setting, we established a subcutaneous TMD8 xenograft model. We optimized the dosing regimen for STN to achieve sustained NF-κB pathway inhibition as judged by downregulation of IL10 mRNA over the entire dose period (Fig. 6B, data not shown). Daily oral dosing of STN (80 mg/kg, tid) resulted in statistically significant inhibition of tumor growth compared with vehicle-treated animals, corresponding to 17% tumor volume change, treated over control group (Fig. 6A). This dosing regimen was well tolerated with less than 5% reduction in mouse body weight (data not shown). Together, these results show an in vivo dependence of CD79-mutated tumors on PKC signaling and support the potential therapeutic value of STN in CD79-mutated ABC DLBCL.

**Discussion**

Despite recent improvements in the therapy of DLBCL, especially the introduction of the anti-CD20 antibody rituximab (32), a significant number of patients will still succumb to their disease and new therapeutic strategies for DLBCL patients are critically warranted. Our increasing molecular understanding of the signaling pathways that contribute to lymphoma pathogenesis provides several novel therapeutic
In addition, we genetically confirmed that PKC also strongly inhibits the proliferation of CD79 mutant cells. The novel or atypical PKC isoforms (Supplementary Fig. S1), PKC complex. Consistent with this notion, we found that BHA536, a isoform that relays the signal from BCR activation to the CBM leads to PKC and BTK dependency. (chronic signaling) but not tonic BCR pathway activation these findings strongly suggest that mutational activation tive to pharmacologic PKC and BTK inhibitors. Together, these findings strongly suggest that mutational activation (chronic signaling) but not tonic BCR pathway activation leads to PKC and BTK dependency.

options. By screening several selective PKC inhibitors including STN across a panel of B-cell lymphoma lines, we discovered that these compounds selectively inhibit a subset of ABC DLBCL cell lines, whereas most lymphoma cell lines screened were insensitive to PKC inhibition. Intriguingly, we identified that all cell lines with CD79A/B mutations exhibit strong dependence on PKC signaling. During the course of our studies and consistent with our results, Davis and colleagues found that CD79 mutations are associated with chronic active BCR signaling (14) and confer dependence on BTK and SYK. We provide several lines of evidence to show that the selective killing of CD79 mutant ABC DLBCL cells by STN is due to on-target PKC inhibition. First, selective inhibition of growth is observed with several different PKC inhibitors and the cellular IC50 of PKC/NF-xB pathway inhibition strongly correlates with the antiproliferative effect. Second, cell lines that harbor mutations in downstream signaling components, such as CARD11, are insensitive to selective PKC inhibitors. Moreover, expression of mutant CARD11 in the CD79 mutant HBL1 cell line renders these cells completely resistant to the growth inhibitory effects of STN. PKCβ is thought to be the major PKC isoform that relays the signal from BCR activation to the CBM complex. Consistent with this notion, we found that BHA536, a PKCδ/β-selective inhibitor that does not significantly inhibit the novel or atypical PKC isoforms (Supplementary Fig. S1), also strongly inhibits the proliferation of CD79 mutant cells. In addition, we genetically confirmed that PKCβ is the critical isoform, as shRNA-mediated knockdown of PKCβ was selectively toxic to CD79-mutated cell lines. A recent study showed that a substantial fraction of DLBCL cell lines exhibits tonic BCR signaling and proposed that tonic pathway activation confers dependency on downstream BCR signaling (16). However, our data and a recent study by Davis and colleagues (14) clearly show that a genetically defined subpopulation of DLBCL, such as those carrying CD79A/B mutations, is sensitive to pharmacologic PKC and BTK inhibitors. Together, these findings strongly suggest that mutational activation (chronic signaling) but not tonic BCR pathway activation leads to PKC and BTK dependency.

The results from a clinical trial of the pan-PKC inhibitor enzastaurin in DLBCL patients were recently reported (25). In the phase II study by Robertson and colleagues, only 7% of patients achieved a long-term remission. We surprisingly found that enzastaurin only modestly inhibits the growth of CD79 mutant ABC DLBCL cell lines. Moreover, the antiproliferative effects observed at 5 to 10 μmol/L enzastaurin do not seem to be specific for CD79 mutant cell lines, as enzastaurin inhibits the proliferation of many cancer cell lines, including GCB DLBCL cell lines, at these higher concentrations. Given the lack of selective antiproliferative effect, it is possible that the growth inhibitory effects at higher enzastaurin concentrations may be attributed to previously described off-target activities of this compound (33) rather than specific PKC inhibition. Together, these findings suggest that STN has superior activity than enzastaurin and may provide a larger therapeutic window for this genetically defined subpopulation of DLBCL. It is also important to note that patients in this phase II study with enzastaurin were neither stratified for molecular DLBCL subtype (ABC DLBCL or GCB DLBCL) nor screened for mutations in the NF-xB pathway. Given the fact that CD79 mutations occur in about 20% of ABC DLBCL and 10% of all DLBCL (including non-ABC subtypes), it is possible that the few observed complete responses may have occurred in patients harboring CD79 mutations or other genetic lesions that lead to chronic active BCR signaling.

Our study clearly shows the therapeutic potential of PKC inhibitors in patients with CD79 mutant DLBCL. However, future clinical trials in DLBCL should incorporate methods such as gene expression profiling and mutational screens to discern patients according to their genetic aberrations. Our data provide first insights into potential mechanisms of resistance that may emerge in patients treated with specific pathway inhibitors. We detected that the CARD11 mutant cell line OCI-Ly3 is insensitive to STN and other PKC inhibitors. In addition, expression of the constitutively active CARD11 mutant L244P rendered a CD79 mutant cell line resistant to PKC inhibitors. These findings indicate that patients with CARD11 mutations are unlikely to respond to PKC inhibitors.
or other upstream inhibitors. The implications of mutations of the negative regulator of NF-kB signaling A20 are currently unclear (34). A20 is frequently inactivated in different subtypes of lymphoma (12, 13). It is thought to predominantly function in a negative feedback loop to dampen NF-kB pathway signaling, but it is unclear whether loss of A20 by itself is sufficient to drive oncogenic NF-kB signaling or still requires upstream signaling. Interestingly, the only cell line (SU-DHL-2) in our lymphoma panel that harbors biallelic loss of A20 was insensitive to SYK, BTK, and PKK inhibitors. Several putative A20 substrates, such as NEMO and MALT1, are thought to function downstream of PKKβ (34, 35). Thus, it is possible that complete loss of A20 function can override the need for upstream pathway activation.

BRC signaling is complex and leads to the activation of several downstream effector pathways, including the PI3K/mTOR, Ras/Raf/Mek/ERK, and CBM/NF-kB pathway. The BCR signaling network therefore presents several opportunities for rationale combination therapies, such as "horizontal combinations" of PKK and PI3K/mTOR pathway inhibitors (36). Consistent with this notion, preliminary studies suggest that STN shows significant synergy when combined with the mTOR-selective inhibitor RAD001 (data not shown; refs. 37, 38). In addition to maximizing the antitumor activity, combination therapies may also provide a valuable strategy to delay or prevent the emergence of resistance (36). Several targeted therapies for the PI3K/mTOR pathway are currently in clinical development (39), and future studies should further evaluate the benefit of combining these agents with selective PKK inhibitors in preclinical and clinical studies. In conclusion, our study provides a strong rationale for investigating the efficacy of STN in CD79 mutant DLBCL in a clinical trial. Moreover, our findings illustrate the necessity to incorporate gene expression profiling and genetic sequencing to stratify patients on the basis of their molecular NF-kB pathway lesions to maximize response rates for PKK inhibitors in DLBCL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contribution

T.L. Naylor, H. Tang, B.A. Ratsch, A. Loo, N.J. Waters, L. Chen, and A. Enns performed research and analyzed data. P. Lenz performed statistical analyses. W. Schuler contributed vital new reagents. B. Dorken, Y-m. Yao, and M. Warmuth provided intellectual input and helped write the article. F. Stegmeier, T.L. Naylor, and G. Lenz designed the research, analyzed data, and wrote the article.

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