Protein kinase C inhibitor Sotrastaurin selectively inhibits the growth of CD79-mutant diffuse large B-cell lymphomas

Running title: Sotrastaurin inhibits CD79 mutant DLBCL

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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-kB pathway and oncogenic lesions have been identified in its regulators including CARD11/CARMA1, A20/TNFAIP3, and CD79A/B. In this study, we offer evidence of therapeutic potential for the selective PKC 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-kB 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-kB pathway inhibition and were mediated by induction of G1 phase cell cycle arrest and/or cell death. We found that STN produced significant anti-tumor 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 that harbor CD79 mutations, also illustrating the necessity to stratify DLBCL patients according to their genetic abnormalities.

PRECIS

Diffuse large B cell lymphomas that harbor mutations in a certain regulator of the NF-kappaB pathway may render them therapeutically susceptible to PKC inhibitors.
Introduction

Diffuse large B-cell lymphoma (DLBCL) represents the most common subtype of malignant lymphoma and is diagnosed in over 20,000 patients each year in the US. DLBCL is heterogeneous with respect to morphology, biology, and clinical presentation (1). By gene expression profiling, at least three molecular subtypes of DLBCL can be distinguished termed Germinal center B-cell-like (GCB) DLBCL, activated B-cell-like (ABC) DLBCL, and primary mediastinal B-cell lymphoma (PMBL) (2-4). The molecular DLBCL subtypes differ not only 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 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 nuclear factor-κB (NF-κB) pathway, which occurs predominantly via the CBM (CARD11/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 motifs) 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, which initiates downstream signaling through BTK (Bruton’s tyrosine kinase) and PLCγ (phospholipase C gamma) 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 (Caspase recruitment domain-containing protein 11, also known as CARMA1) (9, 10). Once activated at the plasma membrane, the CBM complex facilitates the activation of the IKK (I kappa B kinase) complex, which phosphorylates IκBα 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 physiological 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 appear 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, demonstrating a clear dependence on NF-κB pathway signaling (6, 7, 15). These results contrast another study which 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 sotrastaurin (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 IMDM with 20% FBS, and OCI-Ly10 in IMDM with 20% human serum. Cell lines were authenticated by SNP profiling (fingerprinting). For the RNAi experiments, cell lines were engineered to express the murine ecotropic retroviral receptor for efficient retroviral transductions and the bacterial tetracycline repressor for doxycycline-inducible shRNA expression as described (7). The targeting sequence of the PKCb shRNA was CGACCAACACTGTCTCCAAAT.

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 or UBC promoter (pLenti6-CMV, 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, BrdU 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...
(FACS) and BrdU incorporation (Roche). Caspase 3/7 activity was measured using CaspaseGlo (Promega). Detailed protocols are available in supplemental Materials and Methods.

TaqMan mRNA Expression, NF-κB nuclear translocation and IL6/IL10 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 IL6/IL10 secretion levels in supernatants were assessed using Trans-AM (Active Motiv) ELISA plates and Quantikine ELISA kits (R&D systems), respectively. Detailed protocols are available in supplemental Materials and Methods.

Western blotting, and Gene Expression analysis

For Western blotting, 30ug of protein from total cell lysate were loaded onto 4-12% Bis-Tris gradient gels (Invitrogen). The following primary antibodies were used: α-CARD11, α-cRel (Cell Signaling), α-p65 α-PARP, α-PKCb (Santa Cruz) and GAPDH (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 supplemental 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 mm$^2$ (21 days post tumor implantation). Sotrastaurin 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 x Width$^2$/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 two ATP-competitive PKC inhibitors, the pan-PKC inhibitor sotrastaurin (STN) (18, 20-22) and the PKCα/β-selective compound BHA536 (Novartis, unpublished, see structure and selectivity data in Supplementary Figure 1), on the proliferation of a panel of DLBCL cell lines. As control compounds we included 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 (IC$_{50}$) greater than 10μM in SU-DHL4 and DB cells (Figure 1A-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 (Figure 1B), but their response to pharmacological PKC inhibitors varied strongly (Figure 1A). OCI-Ly3 and SU-DHL2 cells were insensitive to PKC inhibition, with IC50 values greater than 10μM in the growth assays with STN and BHA536 (Figure 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 Figure 2) provides a potential molecular rationale for their insensitivity. U2932 cells, which displayed intermediate sensitivity to PKC inhibitors (Figure 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, and OCI-Ly10 cells were very sensitive to both PKC inhibitors, with IC50 values ranging from 0.2-1.0 μM (Figure 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 (Figure 1C, Supplementary Figure 3A, and Table 1). These findings demonstrate 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 et al. identified mutations in the BCR co-receptor CD79A/B that lead to chronic activation of BCR signaling (14). We confirmed that each of the three ABC DLBCL cell lines that were sensitive to PKC inhibition harbored mutations in the ITAM motif of CD79A/B (Supplementary Figure 4 and data not shown). Consistent with the short-term growth assays, CD79 mutant cell lines (HBL1 and TMD8) also
showed significant growth inhibition in a clonogenic assay, whereas the CARD11 mutant cell line (OCI-LY3) was not affected by sotrastaurin (Supplementary Figure 5).

To exclude the possibility that CD79 mutant cell lines are just generally hypersensitive to pharmacological agents, we tested a variety of ‘cytotoxic’ agents, such as Cisplatin, Taxol, and Velcade across our lymphoma cell line panel. Importantly, none of these drugs showed preferential or enhanced killing of HBL1, TMD8, and OCI-Ly10 cells compared to the other B-cell lymphoma lines (Supplementary Figure 3C, Table 1, and data not shown), further supporting the notion that the differential growth inhibition in response to PKC inhibitors is a specific effect.

During our evaluation of additional PKC inhibitors, we surprisingly found that the PKC inhibitor enzastaurin, which is in clinical development for several cancers including DLBCL (25), had only a modest effect on the growth of CD79 mutant ABC DLBCL cell lines (Supplementary Figure 3B). While enzastaurin caused a limited inhibitory effect at 5-10 μM, those higher concentrations also affected the proliferation of several GCB cell lines without significant discrimination of CD79 mutant cell lines (Supplementary Figure 3B). These findings suggest that sotrastaurin and BHA536 have superior activity in CD79 mutated cell lines compared to enzastaurin.

PKCβ is thought to be the major PKC isoform that mediates BCR signaling. We therefore reasoned that the growth inhibitory effect of STN and BHA536 in CD79 mutated cells is mediated through inhibition of PKCβ rather than other PKC isoforms. To test this hypothesis, we examined the effect of a PKCβ selective shRNA across a panel of ABC and GCB lymphoma lines. Strikingly, knockdown of PKCβ was toxic to CD79 mutant ABC DLBCL cells, but did not affect the proliferation of CARD11 mutant or that of GCB DLBCL cell lines (Figure 1D), closely recapitulating the effects of the pharmacological PKC inhibitors. Collectively, our...
findings demonstrate that CD79 mutated ABC DLBCL cell lines are specifically sensitive 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. Sotrastaurin (STN) treatment of HBL1 and TMD8 cells inhibited the nuclear translocation of both p65 and c-Rel (Figure 2A-B), two NF-κB transcription factor subunits that have been implicated in mediating NF-κB pathway activation in ABC DLBCL (26). To monitor the transcriptional response to PKC inhibition, we treated the CD79 mutant cell line HBL1 with sotrastaurin and assessed relative changes in gene expression at several time points by whole genome expression arrays (Figure 2C-D). Application of two independent NF-κB pathway signatures to the gene expression profiling data revealed that sotrastaurin 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 (Figure 2C-D, and Supplementary Figure 6A-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 sotrastaurin treatment in our gene expression profiling studies, with the most pronounced downregulation at the 6 and 12h timepoints (Supplementary Figure 6C). We therefore used IL-6...
and IL-10 as markers to study NF-κB pathway modulation by PKC inhibitors in more detail. Sotrastaurin treatment resulted in a dose-dependent decrease in IL-6 mRNA expression in the CD79 mutant cell lines OCI-Ly10, HBL1 and TMD8 (Figure 3A). In contrast, IL-6 mRNA expression was unaffected in the CARD11 mutant cell line OCI-Ly3 (Figure 3A), which is consistent with the fact that CARD11 functions downstream of PKC (Supplementary Figure 2). Treatment of TMD8 cells with two 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 (Figure 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 (Figure 3C). The PKC inhibitors sotrastaurin and BHA536 also strongly inhibited IL-6 and IL-10 secretion in TMD8 and HBL1 cells (IC₅₀ < 0.2μM), but again did not affect their levels in the CARD11-mutant cell line OCI-Ly3 (Figure 3D and Supplementary Figure 7). 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 concordance with the insensitivity of this cell line in growth assays. Collectively, these findings demonstrate 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 sotrastaurin 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 may obviate the requirement for PKCβ-mediated activation of the CBM complex (Supplementary Figure 2). Indeed, we confirmed in an engineered cellular system that in contrast to PMA/ionomycin-induced pathway activation, NF-κB pathway activation in response to CARD11-L244P is not sensitive to PKC inhibition (Supplementary Figure 8). We therefore wanted to test if 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 control of the CMV or UBC promoter. Expression of the mutant CARD11 allele was confirmed by Western blot and sequencing of the reverse transcribed mRNA transcripts (Figure 4A-B). The expression levels of exogenous CARD11-L244P in the CMV construct was comparable to that of endogenous CARD11 (Figure 4A-B), and only 2-3 fold above endogenous when expressed from the UBC promoter (Figure 4A). Strikingly, expression of CARD11-L244P rendered HBL1 cells to be resistant to the growth inhibitory effects of sotrastaurin (Figure 4C). Similar rescue of sotrastaurin sensitivity was observed in TMD8 cells (Supplementary Figure 9C). A CARD11 mutant construct lacking the CARD domain, which is crucial to facilitate CBM complex assembly (31), was unable to confer resistance to PKC inhibitors (Supplementary Figure 9A). The CARD11-L244P expression lines, however, were still responsive to IKKβ inhibitors (Figure 4C and Supplementary Figure 9B), thereby excluding the possibility that the resistance to PKC inhibitors was due to emergence of a non-specific resistance mechanism.
Next we analyzed whether the growth rescue by CARD11-L244P expression was due to restoration of downstream NF-κB signaling. While IL-6 secretion was strongly inhibited by sotrastaurin in the parental HBL1 cells, IL-6 secretion was no longer modulated in cells engineered to express CARD11-L244P (Figure 4D). Cells expressing the CARD11 constructs, however, still downregulated IL6 in response to IKKβ inhibitor treatment (Figure 4D). Together, these ‘rescue’ experiments further demonstrate that the growth inhibitory effect of sotrastaurin 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 sotrastaurin treatment induces cell cycle arrest or apoptosis in sensitive lines. Treatment with sotrastaurin (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 (Figure 5A-B, Supplementary Figure 10). The G1 arrest was further confirmed using a BrdU assay, where STN caused a dose-dependent inhibition of S-phase entry in HBL1 and TMD8 cells (Supplementary Figure 11). Importantly, the cell-cycle arrest occurs at concentrations that correlate well with the growth-inhibitory IC50s. While G1 arrest appeared to be the predominant response of CD79 mutated cells to STN, we noted the emergence of a sub-G1 peak in TMD8 cells, especially at later timepoints (48h) (Figure 5B and Supplementary Figure 10), 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 but not HBL1 cells (Supplementary Figure 12C). Expression of CARD11-L244P in TMD8 cells was able to rescue both the G1 arrest (Figure 5C) and cell death (Figure 5D), strongly indicating that these effects of sotrastaurin are on-target and mediated by inhibition of CBM-NFκB 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 to strong apoptosis inducers such as the DNA damaging agent Camptothecin (Supplementary Figure 12A-B). These findings suggest that the cell death observed in TMD8 cells at later time points is likely a secondary effect of prolonged G1 arrest rather than acute apoptosis induction. Collectively, our data indicates that the major response of CD79 mutated DLBCL cell lines to STN appears to be G1 cell cycle arrest.

**Sotrastaurin inhibits tumor growth of CD79 mutated DLBCL in vivo.**

To address whether CD79 mutated ABC DLBCL are also sensitive to PKC inhibition in an *in vivo* setting, we established a subcutaneous TMD8 xenograft model. We optimized the dosing regimen for sotrastaurin to achieve sustained NF-κB pathway inhibition as judged by downregulation of IL10 mRNA over the entire dose period (Figure 6B, data not shown). Daily oral dosing of sotrastaurin (80mg/kg, tid) resulted in statistically significant inhibition of tumor growth compared to vehicle-treated animals, corresponding to 17% T/C (Figure 6A). This dosing regimen was well tolerated with less than 5% reduction of mouse body weight (data not shown).
Together, these results demonstrate an *in vivo* dependence of CD79 mutated tumors on PKC signaling and supports the potential therapeutic value of sotrastaurin 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 options. By screening several selective PKC inhibitors including sotrastaurin 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 et al. 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 demonstrate that the selective killing of CD79 mutant ABC DLBCL cells by sotrastaurin is due to on-target PKC inhibition. First, selective inhibition of growth is observed with several different PKC inhibitors, and the cellular IC$_{50}$ of PKC-NF-κB pathway inhibition strongly correlates with the anti-proliferative 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 sotrastaurin. 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 PKCα/β-selective inhibitor that does not significantly inhibit the novel or atypical PKC isoforms (Supplementary Figure 1), 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 exhibit 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 et al (14) clearly demonstrate that only a genetically defined subpopulation of DLBCL, those carrying CD79A/B mutations, is sensitive to pharmacological 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 et al., 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 anti-proliferative effects observed at 5-10μM enzastaurin do not appear 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 anti-proliferative 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 sotrastaurin has a superior activity compared to
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-κB 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 demonstrates 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 sotrastaurin and other PKC inhibitors. In addition, expression of the constitutive active CARD11mutant -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-κB signaling A20 are currently unclear (34). A20 is frequently inactivated in different subtypes of lymphoma (12, 13). A20 is thought to predominantly function in a negative feedback loop to dampen NF-κB-pathway signaling, but it is unclear whether loss of A20 by itself is sufficient to drive oncogenic NF-κB signaling or still requires upstream signaling. Interestingly, the only cell line (SU-DHL2) in our lymphoma panel that harbors biallelic loss of A20, was insensitive to SYK, BTK, and PKC inhibitors. Several putative A20 substrates, such as NEMO and MALT1, are thought to function downstream of
PKCβ (34, 35). Thus, it is possible that complete loss of A20 function can override the need for upstream pathway activation.

BCR signaling is complex and leads to the activation of several downstream effector pathways, including the PI3K-mTOR, Ras-Raf-Mek-ERK, and CBM-NF-κB pathway. The BCR signaling network therefore presents several opportunities for rationale combination therapies, such as ‘horizontal combinations’ of PKC and PI3K-mTOR pathway inhibitors (36). Consistent with this notion, preliminary studies suggest that sotrastaurin shows significant synergy when combined with the mTOR-selective inhibitor RAD001 (data not shown, (37, 38) ). In addition, to maximizing the anti-tumor 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 PKC inhibitors in preclinical and clinical studies. In conclusion, our study provides a strong rationale for investigating the efficacy of sotrastaurin 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 based on their molecular NF-κB pathway lesions to maximize response rates for PKC inhibitors in DLBCL.
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Disclosure of Conflicts of Interest

TN, HT, AL, LC, YY, WS, MW, and FS are employees of the Novartis Institutes for BioMedical Research.

Author Contribution

TN, HT, BAR, AL, LC and AE performed research and analyzed data. PL performed statistical analyses. WS contributed vital new reagents. BD, YY and MW provided intellectual input and helped write the paper. FS, TN, and GL designed the research, analyzed data, and wrote the paper.
Reference List


Table 1: PKC, BTK, and SYK inhibitors selectively inhibit the proliferation of CD79 mutant ABC DLBCL cell lines. 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. (ND – not determined, NR – not reported)

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Figure Legends:

**Figure 1: PKC inhibitors and PKCb 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 Sotrastaurin (STN), the PKCα/β-selective inhibitor BHA536, the IKKβ inhibitor (B) AFN700, or (C) BTK inhibitor PCI-32765. Relative cell growth (average of at least 3 independent experiments) was measured by Cell Titer Glo and normalized to DMSO treated cells. (D) shRNA mediated knockdown of PKCb is toxic to CD79A/B mutant ABC DLBCL but not to ABC DLBCL with mutant CARD11 or GCB DLBCL cell lines. PKCb 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 PKCb.

**Figure 2: The PKC inhibitor Sotrastaurin (STN) attenuates NF-κB signaling in CD79 mutant ABC DLBCL cell lines.** The CD79 mutant ABC DLBCL cell lines HBL1 and TMD8 were treated with the PKC inhibitor STN (5uM) or DMSO for 24 hours. (A) Cytoplasmic (cyto) and nuclear (nuc) fractions from cell lysates were separated on SDS-PAGE gels and immunoblotted with antibodies to cRel and p65. (B) The activity of the NF-κB transcription factors p65 and c-Rel in response to STN treatment was determined from total protein lysates using a oligonucleotide capture ELISA assay. DNA binding is expressed relative to lysates from DMSO treated cells. (C) Gene expression profiling of the ABC DLBCL cell line HBL1 after...
treatment with the IKKβ inhibitor MLN120B and the PKC inhibitor Sotrastaurin at the indicated time points (6, 12, 24, and 48 h). Gene expression changes were assessed using DNA microarrays and depicted according to the color scale shown. A gene was selected as an NF-κB target gene in HBL-1 cells, if MLN120B decreased the expression of the gene by at least 50% at three of the four time points. This gene signature was subsequently applied to the gene expression data following treatment with PKC inhibitor STN. Note that a two color array was used to normalize compound treated samples to control treated (DMSO) samples. (D) Decrease in the NF-κB target gene signature following incubation with the IKKβ inhibitor MLN120B and the PKC inhibitor STN. Gene expression measurements for the component genes in the NF-κB signature (panel C) were averaged for each of the indicated conditions.

**Figure 3: The growth inhibitory effect of the PKC inhibitor sotrastaurin (STN) correlates with NF-κB pathway inhibition.** (A) STN treatment (16h) leads to a dose-dependent reduction of IL-6 mRNA levels in CD79 mutant cell lines (HBL1, TMD8, OCI-Ly10), but not in the CARD11-mutant cell line OCI-Ly3. IL-6 mRNA expression was determined by Taqman qPCR assays using the delta-delta C_t method relative to DMSO treated cells. TBP was used as the endogenous control gene. (B) IL-6 mRNA levels are reduced in a dose-dependent manner by the treatment with the PKC inhibitors BHA536 and LY333531. (C,D) The secretion of IL-6 is inhibited by the IKKβ inhibitor MLN120B (C), and the PKC inhibitor STN (D) in dose-dependent manner. IL-6 secretion was determined 48 hours post-treatment with the Quantikine ELISA assay using supernatant from treated cells (normalized to DMSO treated cells).
Figure 4: Expression of activated CARD11 renders HBL1 cells insensitive to Sotrastaurin.

HBL1 cells were stably transduced with constructs that express activated CARD11 (L244P) under the control of either a CMV or UBC promoter. (A) Expression of exogenous CARD11-L244P was confirmed by sequencing the reverse transcribed mRNA of engineered HBL1 cells (TCT->TCC encodes the L244P codon change). OCI-Ly3, which harbor homozygous mutation of CARD11-L244P were used as a control. (B) Western blot to compare CARD11 expression in parental and engineered HBL1 cells. Note that exogenous CARD11-L244P is untagged and will migrate similarly to endogenous CARD11.

(C) Parental HBL1 and engineered HBL1-CARD11 mutant cells were treated for 5 days with the PKC inhibitor sotrastaurin (STN, left graph) (0-20μM) or the IKKβ inhibitor MLN120B (0-40μM, right graph). Relative cell growth was determined using the Cell Titer Glo assay and is expressed as a percentage of DMSO-treated cells. Error bars denote standard deviation. (D) Parental HBL1 and engineered HBL1-CARD11 mutant cells were washed and then immediately treated for 48 hours with the PKC inhibitor sotrastaurin (STN, left graph) or the IKKβ inhibitor MLN120B (right graph). IL-6 levels in cell supernatants were determined by Quantikine ELISA assay and are expressed as a percentage of IL-6 secretion from DMSO-treated cells. Error bars denote standard deviation.

Figure 5: The PKC inhibitor Sotrastaurin (STN) induces a G1 arrest and/or cell death in CD79 mutant ABC DLBCL cell lines. (A) HBL1 and (B) TMD8 cells were treated with 5μM STN for 48 hours and cell cycle distribution was assessed by measuring DNA content of PI-stained cells (flow cytometry). 2N and 4N denote the DNA content of diploid cells in G1 and G2
phase, respectively. (A,B) STN treatment induces G1 arrest (HBL1) and apoptosis (TMD8) in a dose-dependent manner. The graph displays the percentage of cells in each cell cycle phase (G1, S, G2/M, S, and sub-G1) relative to DMSO treated cells. (C,D) Parental TMD8, engineered TMD8-GFP-CARD11 mutant (CARD*) and TMD8-GFP cells (control) were treated for 5 days with 1.25μM STN. Constitutive CARD11*(L244P) expression (C) restores cell cycle progression through G2/M and S phase and (D) rescues cell death induced by STN as determined by flow cytometry.

Figure 6: Treatment with the PKC inhibitor Sotrastaurin (STN) results in significant inhibition of in vivo tumor growth. (A) In a subcutaneous TMD8 xenograft model in SCID mice, STN treatment (80mg/kg tid) resulted in inhibition of tumor growth (T/C=17%) relative to animals treated with vehicle. Data are expressed as mean +/- SEM. (B) mRNA expression of IL-10 was measured by Taqman qPCR at 1 and 8 hours after the first STN dose.
Figure 1, Naylor et al.
Naylor et al, Figure 2

A

B

C

D

HBL1 TMD8

Cyto Nuc Cyto Nuc 5μM STN

p65 c-Rel

TMD8 HBL1

Rel. DNA binding

DMSO 5μM STN

c-Rel p65 c-Rel p65

Bcap inhibitor treatment

Sorbaquin treatment

NF-κB signature expression

p-value
Naylor et al, Figure 5

A

HBL1 control

HBL1 + 48h STN (5μM)

Events

Events

% 100

% 100

Sub-G1 G1 S G2/M

Sub-G1 G1 S G2/M

B

TMD8 control

TMD8 + 48h STN (5μM)

Events

Events

% 100

% 100

Sub-G1 G1 S G2/M

Sub-G1 G1 S G2/M

C

TMD8 rescue

D

TMD8 rescue

Percent cells

Percent cells

G2/M S phase

G2/M sub-G1

parental GFP CARD11*

parental GFP CARD11*
Figure 6, Naylor et al.

(A) Tumor volume (mm³) mean ± SEM

- Vehicle
- STN

(B) % IL10 mRNA

Vehicle 1h 8h

STN
Protein kinase C inhibitor Sotrastaurin selectively inhibits the growth of CD79-mutant diffuse large B-cell lymphomas

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