Gene-set enrichment analysis unveils the mechanism for the phosphodiesterase 4B control of glucocorticoid response in B-cell lymphoma

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Running title: Inhibition of PDE4B reverses AKT/mTOR mediated GC resistance

Key Words: Phosphodiesterase, Lymphoma, Glucocorticoid, GSEA, cyclic-AMP

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Financial support: This work was supported in part by a grant from the National Cancer Institute (R21CA112043), by a CTSA grant (UL1RR025767), by a grant from the Cancer Prevention and Research Institute of Texas (CPRIT, RP110200) (all to R.C.T.A.). Core resources used in this study (Flow Cytometry, Pathology and Optical Imaging) are supported by a NCI Cancer Center Grant, 2P30CA054174-17.

The authors declare no competing interests
TRANSLATIONAL RELEVANCE:

Glucocorticoid (GC) resistance is a significant problem in the management of lymphoid malignancies. We have previously shown that modulation of intra-cellular cyclic-AMP via inhibition of phosphodiesterase 4B (PDE4B) induces apoptosis in diffuse large B-cell lymphomas (DLBCL). Here, we show that overexpression of PDE4B in DLBCL impinge on the same genes/pathways that are abnormally active in GC-resistant tumors. In agreement with these data, we demonstrated, in vitro and in vivo, that genetic and pharmacological targeting of PDE4B restores the GC sensitivity in B cell lymphomas, in association with cyclic-AMP-mediated inhibition of mTOR. A marked correlation between PDE4B levels and AKT-mTOR activity in primary DLBCLs confirmed the relevance of this interplay. Together, our data indicate that PDE4 inhibitors may be useful in the treatment of lymphoid malignancies, mechanistically explain how these agents could improve GC sensitivity, and demonstrate that insights into disease pathogenesis can be exploited to rationally overcome drug resistance.
ABSTRACT

**Purpose:** Resistance to glucocorticoid (GC) is a significant problem in the clinical management of lymphoid malignancies. Addressing this issue via a mechanistic understanding of relevant signaling pathways is more likely to yield positive outcomes.

**Experimental Design:** We used gene set enrichment analysis (GSEA), multiple genetic models of gain and loss of function in B-cell lymphoma cell lines, in vitro and in vivo, as well as primary patient samples, to characterize a novel relationship between the cyclic AMP/phosphodiesterase 4B (cAMP/PDE4B), AKT/mTOR activities and GC responses.

**Results:** Starting from the GSEA, we found that overexpression of the phosphodiesterase 4B (PDE4B) in diffuse large B cell lymphoma (DLBCL) impinge on the same genes/pathways that are abnormally active in GC-resistant tumors. Using genetically modified cell lines we showed that PDE4B modulates cAMP inhibitory activities towards the AKT/mTOR pathway and define GC resistance in DLBCL. In agreement with these data, pharmacological inhibition of PDE4 in a xenograft model of human lymphoma, unleashed cAMP effects, inhibited AKT and restored GC sensitivity. Finally, using primary DLBCL samples we confirmed the clinical relevance, and biomarker potential, of AKT/mTOR regulation by PDE4B

**Conclusions:** Together, these data mechanistically elucidated how cAMP modulates GC responses in lymphocytes, defined AKT as the principal transducer of the growth inhibitory effects of cAMP in B-cells, and allowed the formulation of genomics-guided clinical trials that test the ability of PDE4 inhibitors to restore GC sensitivity and improve the outcome of patients with B cell malignancies.
INTRODUCTION

In recent years, it has become apparent that the successful development of novel therapeutic approaches for the treatment of cancer needs to be guided by an improved understanding of disease pathogenesis. While these rational strategies have recently yielded important results(1), they are only rarely conceived with emphasis on overcoming acquired or innate resistance to pharmacological agents that are already part of the therapeutic armamentarium. In this context, a pressing issue is the identification and validation of strategies aimed at improving or re-establishing glucocorticoid (GC) sensitivity in lymphoid malignancies. This is an important goal because GCs remain an important agent in the treatment of these tumors and, despite all advances in the molecular classification of these entities(2-4), the in vitro and in vivo responses to GCs continues to be one of the determinants of clinical outcome, in particular in acute lymphoid leukemia and multiple myeloma(5-7).

Cyclic-AMP (cAMP) is a ubiquitous second messenger with marked growth inhibitory and pro-apoptotic properties in lymphocytes(8-11). At the termination point, the intracellular levels of cAMP are controlled by phosphodiesterases (PDE). In immune cells, members of the PDE4 family, particularly PDE4B in B-lymphocytes, account for most of the cAMP hydrolysis and inactivation(12). This feature, combined with pharmacological and structural principles that make phosphodiesterases excellent drug targets, prompted the testing of PDE4 inhibitors for inflammatory conditions such as asthma and chronic obstructive pulmonary disease (COPD)(13).
We previously showed that PDE4B expression was significantly elevated in patients with fatal diffuse large B-cell lymphoma (DLBCL)(4, 9). Subsequently, using in vitro genetic and pharmacological modulation of PDE4B activity, we confirmed that this enzyme abrogates the growth inhibitory effects of cAMP in DLBCL, explaining why elevated PDE4B expression contributes to the poor outcome of subsets of B cell tumors(9, 11). These data pointed to the potential of PDE4 inhibitors as anti-lymphoma agents, and highlighted the addiction of subsets of DLBCL to low cAMP levels. Importantly, in these initial investigations we uncovered an interplay between cAMP/PDE4B and the PI3K/AKT pathway in DLBCL, whereby cAMP-induced apoptosis was found to be dependent on the downmodulation of this growth promoting pathway.

Recently, an unbiased chemical genomic approach unveiled a critical role for the AKT/mTOR pathway in modulating GC resistance in acute lymphoid leukemia (ALL)(14). These observations led us to put forward the novel hypothesis that the cAMP-mediated downregulation of AKT could significantly influence GC response, and that PDE4B inhibition may restore GC sensitivity in malignant lymphocytes. Herein, we comprehensively examined this hypothesis and show that, i) high expression of PDE4B in primary DLBCL impinges on the same genes and pathways associated with GC resistance in ALL, ii) PDE4B expression directly correlates with GC resistance in B cell lymphomas, iii) genetic models of gain and loss of function for PDE4B confirmed the role of this enzyme in controlling GC effects in B cell lymphoma, iv) genetic modulation of AKT activity indicated that this kinase is the principal transducer of cAMP inhibitory effects in malignant B cells, v) pharmacological inhibition of PDE4 restored GC
sensitivity in B-cell lymphomas, in vitro and in vivo. Importantly, we showed that the correlation between PDE4B expression and AKT/mTOR activity was also present in primary DLBCL. Together, these data link PDE4B to AKT/mTOR mediated GC resistance, mechanistically explain how elevation of intracellular cAMP levels improves GC sensitivity, and provide further pre-clinical support for expanding the testing of PDE4 inhibitors in lymphoid cancers.

MATERIALS AND METHODS

Cell Lines, primary tumors and normal B cells. Human DLBCL cell lines (DHL6, DHL7, DHL10, Ly1)(15, 16), Ramos (human Burkitt lymphoma cell line), were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 100U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine, and 10mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer in a 5% humidified CO2 incubator at 37°C, as previously reported(11). The HEK293 cell line was cultured in DMEM supplemented as described above. The DLBCL cell line Ly3 was grown in IMDM supplemented with 20% human serum, as we described(9). The primary DLBCLs were obtained from our tumor bank and reported before(17); the detailed features of the samples used in this study are described in Supplementary Table 3. These studies were approved by the institutional review board of the University of Texas Health Science Center at San Antonio (UTHSCSA). Murine mature B cells were isolated and purified (>90% purity) from wild-type C57BL/6 mice spleens as we recently described(11), and according to approval from the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at San Antonio.
Gene Set Enrichment Analysis (GSEA). A gene collection containing 101 probe sets expressed at significantly higher levels in GC-resistant than in GC-sensitive ALL samples (14) were analyzed within the context of a cohort of primary DLBCL (n=56) previously investigated by gene expression profiling on microarrays (18). PDE4B expression below and above the median, as we determined previously by quantitative real-time RT-PCR (9), dichotomized the tumors in PDE4B-low and high, respectively.

Cell Proliferation and Apoptosis. The inhibitory activities of forskolin (2 - 40µM), 8-Br-cAMP (2mM), dexamethasone (1 - 1000nM), etoposide (2 - 40µM), staurosporine (.05-20nM), and rolipram (2 – 20µM) (all from Sigma-Aldrich, St. Louis, MO or A.G. Scientific, San Diego, CA) on cell proliferation and viability were determined with the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega, Madison, WI). The apoptosis rate was measured with Annexin V-PE staining followed by Fluorescence Activated Cell Sorting (FACS) analysis according to the manufacturer’s guidelines (Apoptosis Detection Kit I; BD Biosciences, San Diego, CA) and propidium iodide (Sigma-Aldrich) staining also followed by FACS, as we described (9). In these assays, the relevant cell lines were cultured either in FBS or human serum containing media, and data were collected at 48h, 72h or 96h. Specific details on culture conditions, time points and range of drugs concentration are indicated in the figures and figure legends.

Cyclic AMP Quantification. Intracellular cAMP levels were measured using the Parameter cAMP assay kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. To modulate cAMP concentrations, the relevant cell lines were treated with
forskolin, an adenylyl cyclase activator, and/or rolipram, a pan-PDE4 inhibitor (both purchased from Sigma). In brief, the PDE4B-high cell lines (Ly1, Ly3, and Ramos) were treated with forskolin (20-40µM) in the presence or absence of rolipram (20µM) for up to 72hrs. The genetically modified PDE4B-low DHL6 cell line (see below) were treated with forskolin (40µM) for 15 minutes.

Genetic modulation of PDE4B and AKT in DLBCL. The generation of DHL6 cell lines stably expressing a retrovirus construct (MSCV-eGFP) encoding the PDE4B-wild type (WT), PDE4B-phosphodiesterase inactive (PI), or constitutively active (CA)-AKT, has been reported previously(9). To create Ramos cell lines stably expressing PDE4B specific shRNA constructs, we cloned two previously validated targeting sequences (PDE4B#2, 5’-GCCUAAACAAUACAAGCAU-3’, PDE4B#5, 5’-GCAUCUCACGCUUUGGAGU-3’) into the pSilencer™ 4.1-CMV vector (Ambion, Austin, TX). These constructs, and a shRNA-control containing vector, were electroporated into Ramos cells using a Gene Pulser II System (Bio-Rad Laboratories, Hercules, CA) at 250 V and 950µF. Subsequently, puromycin resistant populations were obtained, and clones generated by limiting dilution. Confirmation of the PDE4B knockdown was performed with PDE4B-specific q-RT-PCR, as we described(9, 11). Expression of the glucocorticoid receptor (GR) in these cell models was determined by q-RT-PCR, at baseline and following exposure to forskolin, using primers that amplify all GR isoforms (GR Fw 5’ GGATCATGACTACGCTCAAC 3’ and Rv 5’ TGCAGTAGGGTCATTTGGTC 3’).

AKT and mTOR activity. The impact of PDE4B and cAMP levels on AKT/mTOR was determined by immunoblotting with anti-phospho-AKT (S473), phospho-S6K (T389), phospho-
4E-BP1 (T37/46) (all from Cell Signaling Technology, Danvers, MA) and anti-MCL1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Loading control antibodies included β-actin and α-tubulin (Sigma-Aldrich).

**Xenograft Model of Human B cell Lymphoma and In Vivo Imaging.** Ramos cells were transduced with a retrovirus encoding the luciferase gene and stable populations established with neomycin selection. Subsequently, two independent cohorts (n=20 and n=9) of 5 week-old nude mice (Harlan, Houston TX) were sub-lethally irradiated (400 cGy) and inoculated with 2 x10^6 cells in the right flank. Three days after cell implantation, mice were subjected to non-invasive bioluminescent imaging (IVIS Spectrum, Caliper Life Sciences, Hopkinton, MA), as we described(19), and randomized into the following treatment groups: vehicle (1% DMSO in distilled water), dexamethasone (15 mg/kg in distilled water), rolipram (10 mg/kg, 1% DMSO in distilled water) or combination of dexamethasone and rolipram, all administered daily in the peritoneal cavity. Treatment efficacy was monitored with weekly imaging and photon flux quantifications, and experiments terminated when tumors were larger than 1cm^3 or mice became moribund. These studies were approved Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at San Antonio.

**Statistics.** The statistical significance of all in vitro assays was determined with a two-tailed Student’s t-test or with one-way ANOVA with the Student-Newman-Keuls multiple comparisons test. The statistical values of the differences between the multiple treatment groups studied in vivo was determined with the Kruskal-Wallis test. In all instances *P*< 0.05 was considered significant. Data analyses were performed with Prism software (version 5.0; GraphPad) and
Excel software (Microsoft). Dose-effect curves were calculated with the CalcuSyn software (Biosoft, Cambridge, UK) and used to generate the combination index (CI), reflecting the synergistic activity of the drugs tested.

RESULTS

A gene expression signature shared by DLBCL expressing high levels of PDE4B, and ALL resistant to glucocorticoid. We showed that PDE4B activity controls PI3K/AKT signals in DLBCL(9) whereas Wei at al. independently determined that the AKT/mTOR pathway regulate GC sensitivity in ALL(14) Together, these data led us to hypothesize that PDE4B, by regulating the inhibitory effects of cAMP towards AKT, may play an important role in the resistance to GCs found in subsets of lymphoid cancers. We reasoned that if this hypothesis was correct, PDE4B expression should impinge on the same genes/pathways that control GC response in malignant lymphocytes, and that the gene signatures of GC resistance and PDE4B overexpression should be related, even if determined in distinct tumor types. To test this concept, we used gene set enrichment analysis (GSEA)(20) and found a significant enrichment of “GC resistance” genes in the expression signature of PDE4B-high DLBCLs (FDR=0.19) (Figure 1A and Supplementary Table 1).

PDE4B expression and cAMP levels associate with AKT/mTOR activity and glucocorticoid response in B cell lymphomas. The positive results obtained in the GSEA gave us the impetus to test the impact of PDE4B expression/activity on the response to GC found in lymphomas. Towards this end, we used a panel of B cell lymphoma cell lines expressing low/null or high
levels of PDE4B as defined by mRNA expression (Supplementary Figure 1A), which is highly correlated with previously defined protein expression(9). As we have reported before(9, 11), these cell lines present with low basal activity of adenylyl cyclase and, without proper stimulation, lack intracellular cAMP. Thus, to fully capture the relevance of PDE4B activity in this context, we experimentally increased intracellular cAMP with the adenylyl cyclase activator forskolin or with the cell permeable synthetic cAMP, 8-Br-cAMP.

Examining five independent B cell lymphoma cell lines, we found that in presence of cAMP, tumors expressing low levels of PDE4B were markedly more sensitive to dexamethasone-induced apoptosis than lymphomas expressing high PDE4B levels (p<0.05 one-way ANOVA test) (Figure 1B and Supplementary Figure 1B, measurements performed 48h after drug exposure with Annexin V and propidium iodide, respectively). Noticeably, at the concentrations used, neither forskolin (5µM) nor synthetic cAMP (2mM) significantly affected cell proliferation or viability indicating that in this context cAMP acted primarily as sensitizer of GC activities, in a PDE4B-dependent manner (Supplementary Figure 1C and 1D). Furthermore, in agreement with the important role of cAMP levels and PDE4B activity in regulating GC efficacy in lymphomas, we detected a progressive dexamethasone-induced growth inhibition when concomitantly augmenting the intracellular levels of cAMP (Supplementary Figure 1E).

To further define the relationship between cAMP/PDE4B, AKT/mTOR and GC sensitivity in B cell lymphomas, we also determined the phosphorylation levels of AKT and two mTOR-regulated proteins in our model. As shown in Figure 1C (and Supplementary Figure 1F), cAMP markedly inhibited the phosphorylation of AKT, S6K and 4E-BP1 (the latter two surrogate
markers for mTOR inhibition) in a PDE4B-dependent manner. Furthermore, we showed that the cAMP-mediated downregulation of this pathway is readily detectable in normal mature B cells (Supplementary Figure 1G), thus defining the physiologic relevance of these signals, and highlighting the role of the abnormally high PDE4B expression in blocking this growth inhibitory axis in B cell lymphomas.

In addition, as recent data have suggested a correlation between the pre-treatment levels of the anti-apoptotic protein MCL1 and GC response in MLL-translocated ALLs (21), we also measured the expression of this anti-apoptotic protein in our lymphoma models. In the group of cell lines analyzed, MCL1 baseline expression did not per se predicted GC response (Supplementary Figure 1H), although, as we will show below, MCL1 can be downregulated by cAMP in a PDE4B and AKT dependent fashion. Finally, c-MYC expression has been suggested to influence GC sensitivity in ALL (22). However, in the DLBCL cell lines studied here, c-MYC status (activated by a translocation t(8;14) in Ramos and DHL10, or wild-type in Ly1, Ly3, DHL6)(23, 24) appear to not segregate with the degree of GC sensitivity.

**PDE4B and AKT are central regulators of glucocorticoid sensitivity in DLBCL.** The data above defined an association between PDE4B expression and GC sensitivity, which related to cAMP-mediated inhibition of AKT/mTOR. To move beyond these correlative analyses, and firmly establish the roles of PDE4B and AKT in regulating GC response in B cell lymphomas, we generated DLBCL models stably expressing PDE4B (wild-type, WT, and phosphodiesterase inactive mutant, PI) or a constitutively-active (myristoylated) AKT protein (CA-AKT). We found that reconstitution of PDE4B-WT expression in the PDE4B-null and GC sensitive DHL6
cell line, rendered these cells significantly more resistant to dexamethasone (p<0.05, two-tailed Student’s t-test) than their isogenic counterpart expressing a phosphodiesterase inactive mutant, or the parental cell line (Figure 2A), thus indicating that the regulation of GC response in this context is related to the enzymatic activity of PDE4B, as confirmed by measurements of intracellular cAMP levels (Supplementary Figure 2A). In addition, ascertaining the central role of AKT in transducing cAMP effects and controlling GC responses in B cell lymphomas, we showed that the GC-sensitive DHL6 cell line stably expressing CA-AKT became resistant to dexamethasone to the same extent as their isogenic counterparts ectopically expressing PDE4B (Figure 2A). These results indicate that most, if not all, of the cAMP-mediated PDE4B-controlled GC responses observed in B cell lymphomas are transduced by AKT. In agreement with this concept, in the PDE4B-WT and CA-AKT expressing cells cAMP did not inhibited AKT/mTOR phosphorylation, whereas these effects were preserved in PI-mutant expressing cells (Figure 2B). Furthermore, we show that cAMP, in a PDE4B and AKT dependent manner also inhibits the expression of MCL1 (Figure 2B). These data agree with a previous observation that placed MCL1 downstream to AKT in regulating GC resistance in ALL(14), but they also highlight the novel role of cAMP/PDE4B in controlling these events. Together, our data suggest that high PDE4B expression in B cell lymphomas contributes to GC resistance by blocking cAMP inhibitory effect on the AKT/mTOR pathway and its downstream components.

Since AKT has broad activities, we found necessary to establish that in the present context its constitutive activation was primarily leading to a blockade in the transduction of cAMP signals, and not interfering with other relevant physiologic processes. To address this possibility, we first compared the intracellular levels of cAMP in DHL6-PDE4B-WT, -PI and CA-AKT cells. Upon
exposure to forskolin, cAMP levels were similarly raised in the PDE4B-PI and CA-AKT cells, despite the fact that the latter were resistant to cAMP-controlled GC sensitization, and in both cases at significantly higher levels than in the PDE4B-WT expressing cells (p<0.05, one way ANOVA test, Supplementary Figure 2A); these data demonstrate that AKT does not interfere with the generation of cAMP. Next, we examined whether the previously reported link between induction of glucocorticoid-receptor (GR) expression by cAMP and restoration of GC sensitivity (25, 26) accounted for our results. Here, we tested if CA-AKT expression was interfering with cAMP-mediated GR regulation. Our data showed that despite being resistant to cAMP-controlled GC sensitization, in the CA-AKT lymphoma cells GR expression was promptly induced by this cAMP (Supplementary Figure 2B). Finally, we showed that the GC resistance acquired by the CA-AKT expressing cells was not a general growth advantage phenomenon; in these assays we found that in the absence of cAMP, CA-AKT lymphomas proliferated at the same rate, and responded to dexamethasone in the same manner as their isogenic counterparts expressing PDE4B-WT and –PI cells (Supplementary Figure 2C).

Taken together, these results establish PDE4B as an important regulator of GC efficacy in lymphomas, indicate that a functional AKT pathway is critical for the cAMP/PDE4B-controlled restoration of GC activity, and demonstrate that induction of GR expression does not fully account for the improved GC response that follows PDE4B inhibition/cAMP elevation in lymphocytes.

**Genetic and pharmacological inhibition of PDE4B in B cell lymphoma restores glucocorticoid sensitivity.**
**In vitro:** Up to this point we have used genetic models of PDE4B gain of function to establish the role of this enzyme in controlling GC responses in B cell lymphomas. However, our ultimate objective is to test in the clinic the potential of pharmacological PDE4B inhibition as a rational strategy to reverse GC resistance in lymphoid malignancies. To start to address this translational aspect of our research, we examined the effects of rolipram, a prototypical PDE4 inhibitor(13), in three independent GC-resistant lymphoma cell lines that express high levels of PDE4B. In each instance, PDE4 inhibition with rolipram, in the presence of cAMP, significantly increased the anti-lymphoma effects of dexamethasone (Figure 3A-C, p<0.05, two-tailed Student’s t-test, and Supplementary Figure 3A) in a highly synergistic manner (combination index <0.1 in all three cell lines tested, as determined by isobologram analysis using the CalcuSyn Software, Supplementary Table 2 and Supplementary Figure 3B). Demonstrating the specificity of these effects, exposure to rolipram was accompanied by elevation of intracellular levels of cAMP (Supplementary Figure 3C) but did not impact on the effectiveness of two broad-spectrum chemotherapeutic agents (Supplementary Figure 3D). Finally, confirming the central role of PDE4B in regulating cAMP effects towards AKT/mTOR, and on the relevance of this pathway in controlling GC sensitivity in B cell lymphomas, rolipram treatment was accompanied by a decrease in the phospho levels of AKT, S6K and 4EPB1 (Figure 3D).

Our earlier data pointed to PDE4B as the critical phosphodiesterase in the controlling cAMP signals in malignant B cells(4, 9, 11). Thus, to confirm the specificity of the pharmacological observations derived from rolipram’s use, we created B cell lymphoma cell lines stably expressing two independent shRNA constructs targeting the *PDE4B* gene. These cells showed
downregulation of PDE4B, elevated intracellular cAMP levels (Supplementary Figures 3E-F) and more importantly became sensitive to dexamethasone activity (Figures 3E). Of note, using multiple RNAi strategies we were consistently unable to isolate stable polyclonal or monoclonal B cell populations displaying a substantial knockdown of PDE4B expression (e.g., > 80%), and only those cells with more modest downregulation of this gene (~40%) were rescued for downstream experiments. We attribute these findings to the addiction of B lymphoma cells to low level of cAMP, and loss of viability with continuous suppression of this gene. Nonetheless, even though in the cell populations that we analyzed the shRNA effects on PDE4B expression were only moderate (Supplementary Figure 3E), they readily led to increase in intracellular cAMP levels (Supplementary Figure 3F), and improvement in dexamethasone activity (Figure 3E).

**In vivo:** To determine whether PDE4 inhibition could also reverse GC resistance in a more relevant setting, we tested the efficacy of dexamethasone, rolipram or their combination for the treatment of a xenograft model of human lymphoma with non-invasive luminescent imaging capability. In these assays, the mice were inoculated subcutaneously with the aggressive B cell lymphoma cell line Ramos (PDE4B-high, GC-resistant) constitutively expressing the luciferase gene. Seventy-two hours after tumor implantation, the mice were imaged, assigned to distinct treatment group and followed weekly with luminescence imaging. Consistent with our in vitro results, within 2-3 weeks of treatment, mice from two independent cohorts which received combination of rolipram plus dexamethasone had a significantly better clinical response with a pronounced inhibition in tumor growth as compared with those receiving vehicle or single
agents, in association with downmodulation of phosphor AKT signals (Figure 4A and Supplementary Figure 3G, p<0.05 Kruskal-Wallis test).

**PDE4B expression and AKT activity in primary DLBCLs.** Using in silica investigations, and in vitro and in vivo studies in lymphoma cell lines, we mechanistically defined a novel interplay between PDE4B-controlled cAMP effects and AKT/mTOR activities, with major relevance for GC sensitivity. As a next step in our investigation, we aimed to confirm that this cross-talk is also present in primary lymphoma samples. This is also relevant because it could start to establish the potential of PDE4B measurements in association with AKT/mTOR activity, in the design of biomarker-guided trials aimed at restoring GC response in B cell malignancies. To achieve these goals, we recently identified a collection of primary DLBCL and obtained enough good quality matching RNA and protein from a subset of tumors (n=15). *PDE4B* expression was determined by quantitative real-time RT-PCR (q-RT-PCR) (Supplementary Table 3), a methodology that we have extensively shown to correlate with this enzyme’s activity (9, 11), and phospho-AKT and phospho-S6K levels were determined by Western blot. In agreement with our cell lines data, a significantly higher expression of these phospho-proteins was found in primary DLBCL expressing high levels of *PDE4B* (p<0.05, Mann-Whitney test for the densitometric values - normalized by two independent control proteins, β-actin and α-tubulin), thus suggesting that the cAMP/PDE4B axis could play a role in controlling this oncogenic pathway in primary DLBCL (Figure 5).
DISCUSSION

The mechanistic basis for GC resistance in B-cell tumors remains ill defined. Early investigations had tentatively linked this event to loss-of-function mutation, defective expression or specific promoter usage of the glucocorticoid receptor (GR) gene(27, 28). However, studies in large and well characterized ALL cohorts did not confirm these hypotheses(29, 30).

Interestingly, cAMP has long been known to modulate GR expression, and initial studies suggested that the benefits of this second messenger on GC sensitivity were related to GR induction(25). Still, we found that although GR expression in DLBCL is indeed induced by cAMP, it did not influence GC sensitivity. Rather, we demonstrated that the cAMP-mediated inhibition of AKT/mTOR is the central event needed to reinstate GC sensitivity in B-cell lymphomas. Additional studies will ascertain whether the cAMP-mediated induction of GR expression could also play role in the down-modulation of AKT/mTOR functions. In fact, our results agree with recent reports implicating this pathway in GC-resistant ALL(14), but refine the picture by showing that PDE4B is an important upstream regulator of AKT activity in this context. Downstream to AKT/mTOR, the anti-apoptotic protein MCL1 has been suggested to be a predictor of GC resistance in MLL-rearranged infant acute lymphoblastic leukemia(21). Our preliminary data in DLBCL did not find a correlation between baseline MCL1 levels and GC response, albeit cAMP lowered MCL1 levels in an AKT dependent manner. The reason for this discrepancy is presently unclear but it could reflect the distinct biology of immature (ALL) and mature B-cell malignancies (DLBCL), or indicate a differential contribution of effector proteins
downstream of AKT in these two tumor types, with MCL1 more relevant in specific subsets of ALL than in DLBCL. Further studies should help clarify this issue.

Our genetic models of gain or loss of function highlighted the principal role of the 4B member of the superfamily of phosphodiesterases in controlling cAMP levels in DLBCL. Nonetheless, other PDE families also have cAMP catalytic activity (e.g. PDE3, PDE7, PDE8) and a broader spectrum PDE inhibitor may be of additional value, as recently suggested(31). Of note, considering exclusively the PDE4s, a dominance of PDE4B activities may come with an unexpected benefit since PDE4D targeting has been linked to the most common side effect (emesis) found with the clinical use of broad PDE4 inhibitors(32). Thus, development of compounds with preferential activity to PDE4B versus other PDE4s, in particular PDE4D, should be sought as they may have improved tumor suppressing properties with more limited adverse effects.

Our pre-clinical data and examination of primary tumors also instruct on the design of clinical trials aimed at testing PDE4 inhibitors in B-cell cancers; these should be genomic-driven and perhaps restricted to patients with high levels of PDE4B expression and cAMP-responsive PI3K/AKT/mTOR signals, with wild-type status of PI3K, AKT, PTEN genes, amongst other regulators of this pathway. Further, based on our in vivo data it is possible that greater clinical benefit will be achieved when PDE4 inhibitors and glucocorticoids are combined with classical chemotherapy and/or additional rationally developed agents.
Together, our data show that modulation of cAMP levels, primarily via inhibition of phosphodiesterase, should be clinically tested for the treatment of lymphoid malignancies. In particular, our data highlight the role of PDE4 inhibitors in restoring GC sensitivity and demonstrate that insights into disease pathogenesis can be exploited not only to identify novel targets for treatment, but also to rationally overcome resistance to classical pharmacological agents. It is possible that further elucidation of the cAMP effects on B cells, in particular the events upstream to SYK and PI3K/AKT(9, 11), will improve our appreciation of the role of this second messenger in B cell biology, and create novel opportunities for the development of therapeutic strategies in malignant, inflammatory and auto-immune conditions.

ACKNOWLEDGMENTS:

We thank Dr. Kumaraguruparan Ramasamy for expert help with the animal studies. Authors contribution: S-W. K., designed assays, performed experiments, analyzed results; D.R., performed experiments; R.A., conceived the project, designed experiments, analyzed results, wrote the manuscript. The authors declare no competing interests.
REFERENCES

FIGURE LEGENDS

Figure 1. PDE4B expression correlates with glucocorticoid response in B cell lymphomas.
A) Enrichment plot from GSEA analysis performed with 101 probe sets expressed at high levels in GC-resistant ALL samples(14) in a collection of primary DLBCL dichotomized high vs. low PDE4B expression(9, 18). Statistically significant enrichment (FDR= .19) of “GC resistance” genes was found in DLBCL expressing high PDE4B levels when compared to tumors with low PDE4B expression. B) Annexin V staining demonstrate that in the presence of cAMP (forskolin, 5µM), DLBCL cell lymphoma cell lines expressing low-levels of PDE4B were markedly more sensitive to dexamethasone (100nM) than those B cell lymphomas expressing high PDE4B levels (p<0.05 one-way ANOVA test). Data shown were collected 48h after drug exposure, represent the mean of two independent experiments and were normalized by apoptosis rate of cells exposed exclusively to dexamethasone – normalization by forskolin yields largely the same result. C) Western blot analyses of PDE4B-low and high cell lines following rapid elevation of intracellular cAMP levels (forskolin, 40µM for 60 minutes) show significant inhibition of AKT (S473), S6K, and 4E-BP1 phosphorylation in a PDE4B-dependent manner. Actin immunoblotting confirms equal protein loading.

Figure 2. Interplay between PDE4B and AKT control glucocorticoid response in DLBCL.
A) Cell proliferation assays demonstrate that reconstitution of PDE4B-wild-type (WT) expression or a constitutive active AKT mutant (CA-AKT) equally induced dexamethasone resistance in the GC-sensitive DHL6 cell line (*, p<0.05, two-tailed Student’s t-test for WT vs. PDE4B-phosphodiesterase inactive mutant, PI, and CA-AKT vs. PI). PDE4B-PI cell line
response is similar to that of the DHL6 parental cell line. Growth inhibition curves include escalating doses of dexamethasone in the presence of forskolin (2µM); data were normalized by cells exposed solely forskolin (2µM), hence controlling for the effects of this small increment in cAMP levels on cell growth. Results shown are mean and SD of four independent experiments performed in triplicate, collected at 72 hours. B) Western blot based determination of the phosphorylation levels of AKT (S473) and 4E-BP1(T37/46), and total MCL1, show that isogenic cells expressing PDE4B-WT or CA-AKT, but not a PDE4B-inactive mutant, become resistant to the marked inhibitory effects of cAMP (forskolin, 40µM for 60 minutes) towards this pathway. B-actin immunoblots confirm equal protein loading.

Figure 3. Pharmacological and genetic inhibition of PDE4B improves glucocorticoid sensitivity in B cell lymphoma in vitro. A-C) In three independent B cell lymphoma cell lines (Ly1, Ly3 and Ramos) that express high levels of PDE4B, treatment with the PDE4-specific inhibitor rolipram significantly improved the anti-lymphoma activity of dexamethasone in a dose dependent manner (10-1000nM) (*, p<0.05, two-tailed Student’s t-test). In agreement with the high expression of PDE4B and low basal activity of adenylyl cyclases in these cells, forskolin (20-40µM) or rolipram alone (10-20µM) had limited effect on cell proliferation (see Figure S3A). Data shown are mean and SD of all data points from experiments performed in duplicate and collected at 72h (Ly1 and Ramos) or in triplicate and collected at 96h (Ly3), normalized by vehicle treated cells (no dexamethasone). D) Western blot analyses of the phosphorylation of AKT (S473), S6K (T389), 4E-BP1 (T37/46), and total MCL1 levels in three aggressive lymphoma cell lines show that PDE4 inhibition with rolipram (10-20µM) significantly inhibits this pathway. In lanes labeled (-) cells were exposed to forskolin (20-40µM) alone, and in (+)
forskolin and rolipram. E) Top: In the PDE4B-high, GC resistant B cell lymphoma cell line Ramos, stable expression of two independent PDE4B-specific shRNA constructs significantly increased GC sensitivity (dexamethasone, 100nM) (p<0.05, one-way ANOVA test), in comparison to cells expressing a shRNA control construct; all cells were also exposed to forskolin (10µM). Results shown are the mean and SD of data generated in triplicate, collected at 96 hours and are representative of three independent experiments. Bottom: Western blot detection of S6K phosphorylation (T389) in an aggressive B cell lymphoma stably expressing control- or PDE4B-shRNA confirm the role of this enzyme in regulating cAMP effects (forskolin 40µM, for 60 minutes) on the mTOR pathway and GC resistance.

**Figure 4. Pharmacological inhibition of PDE4B improves glucocorticoid sensitivity in B cell lymphoma in vivo** Bioluminescent imaging of a cohort of 20 mice inoculated with Ramos cells stably expressing the luciferase gene; Images shown are from pre-treatment (Day 0 – 72 hours post-inoculation) or at the 20th day of treatment with vehicle (1%DMSO in water, IP daily), dexamethasone (15mg/kg, IP daily), rolipram (10mg/kg, IP daily) or the combination or both agents. The panel on the right is a photon flux-based quantification of tumor size and spread and confirms the statistically significant improvement in GC activity following its rational combination with a PDE4 inhibitor (*, p<0.05, Kruskal-Wallis test). The combined analysis of two independent cohorts (n=29) is shown in Supplementary Figure 3G.

**Figure 5. AKT/mTOR activity correlates with PDE4B expression in primary DLBCL.** Western blot analyses of phospho-AKT (S473) and phospho-S6K (T389) was performed in
primary DLBCLs categorized by \textit{PDE4B} expression (see Supplementary Table 3). Densitometric analysis, normalized by two independent proteins (\(\beta\)-actin and \(\alpha\)-tubulin), is also shown and points to a correlation between PDE4B expression and activity of the AKT/mTOR pathway in the majority of primary DLBCL analyzed, and a significantly higher expression of these phospho-proteins in PDE4B-high DLCBL (\(p<0.05\), Mann-Whitney test for the densitometric values). Note that protein from sample #3279 was available for only one of the WB analysis.
Figure 1

A

B

C

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PDE4B-low

PDE4B-high

Research.
Figure 2

A

Absorbance 490nm (% ctrl)

0  1  2  5  10
Dexamethasone (nM)

- CA-AKT
- PDE4B-WT
- PDE4B-PI
- PDE4B-PI
- Parental

B

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Figure 3

A

Ly1

Absorbance 490nm (% ctrl)

Dexamethasone (nM)

B

Ly3

Absorbance 490nm (% ctrl)

Dexamethasone (nM)

C

Ramos

Absorbance 490nm (% ctrl)

Dexamethasone (nM)

D

Rollipram

-  +  -  +  -  +

p-AKT

p-S6K

p-4E-BP1

Actin

MCL-1

Ly1  Ly3  Ramos

E

Ramos

Absorbance 490nm (% ctrl)

sh-ctrl  sh-PDE4B#2  sh-PDE4B#5

cAMP

p-S6K

Actin

sh-ctrl  sh-PDE4B#2  sh-PDE4B#5
Figure 4

Day 0          Day 20

Vehicle

Dexamethasone

Rolipram

Rolipram + Dexamethasone

Photon flux

Day 0          Day 10          Day 20

Vehicle
Rolipram
Dexamethasone
Rolipram + Dexamethasone
Figure 5

**PDE4B**

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**pAKT**

**Actin**

**Densitometry (arbitrary units)**

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**pS6K**

**Tubulin**

**Densitometry (arbitrary units)**

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Clinical Cancer Research

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Sang-Woo Kim, Deepak Rai and Ricardo Aguiar

Clin Cancer Res Published OnlineFirst July 8, 2011.

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