Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia

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Cyclic nucleotide phosphodiesterase (PDE) isoforms can influence disease pathogenesis and be novel therapeutic targets. Because lower cAMP levels may contribute to the decreased apoptosis that occurs in chronic lymphocytic leukemia (CLL), we assessed the expression levels of PDE isoforms in peripheral blood mononuclear cells (PBMC) of healthy adults and patients with CLL. We found a unique PDE mRNA signature in CLL: higher levels than in normal PBMC of PDE7B (increased ~23-fold) and lower levels of PDE3B, 4D, 5A, and 9A mRNA (each decreased ~30-fold). Increased PDE7B mRNA in CLL correlates with a 10-fold-higher expression of PDE7B protein and results in an increased contribution of PDE7 to total PDE activity. Consistent with the higher level of PDE7B expression, inhibitors of PDE7 (BRL-50481, IR-202) and a dual PDE4/PDE7 inhibitor (IR-284) selectively increase apoptosis in CLL cells compared with normal PBMC or B cells. Apoptosis of CLL cells promoted by inhibitors of PDE7 and PDE4/7 is attenuated by PKA inhibition, occurs via a mitochondrial-dependent process, and is associated with increased cAMP accumulation and down-regulation of the antiapoptotic protein survivin and of PDE7B. The increase in PDE7B expression and PDE7 inhibitor-promoted apoptosis implicates PDE7B as a drug target in CLL. Our findings identify a unique PDE signature in CLL and illustrate the utility of broad analyses of PDE isoform expression in human disease.
Cells.

PDE7B Expression and PDE7 Enzymatic Activity Are Increased in CLL Cells. We assessed the expression of PDE7B mRNA in CLL cells from 60 CLL patients (Fig. 1A) and found that 95% of patients had 2-fold or greater expression of PDE7B mRNA in their CLL cells than did normal PBMC of healthy adults. Moreover, 37% of the patients had >18-fold higher PDE7B mRNA than did normal PBMC. Immuno blot analyses revealed that CLL cells have 10 ± 2-fold more PDE7B protein than do normal PBMC (Fig. 2A); 94% of the patients had cells with 3-fold or more PDE7B protein expression with 37% having at least 11-fold greater expression. Increased PDE7B protein expression correlated with the increase in PDE7B mRNA (r = 0.414, P < 0.05). The PDE7B protein localizes to the membrane and insoluble fractions of CLL cells, unlike PDE4B, which is predominantly cytosolic and primarily represents PDE4B2 (78 kDa) and PDE4B3 (100 kDa) (Fig. 2B and Fig. S2). Consistent with the RNA and protein expression data, studies with the PDE7 inhibitor BRL-50481 revealed that PDE7 contributes more to cAMP-PDE activity in CLL cells than in normal PBMC (Fig. 2C).

CLL Cells Are More Sensitive to the Cytotoxic Effects of PDE4 and PDE7 Inhibitors than Are Normal PBMC. Because cAMP levels can influence survival of leukemia cells (2, 14) and PDE7 selectively hydrolyzes cAMP, we examined whether PDE7 inhibitors induce apoptosis of CLL cells. We found that CLL cells are more sensitive than PBMC of healthy donors to proapoptotic effects of PDE7 inhibitors [BRL-50481, IC50 200 nM; and IR-202, IC50 85 nM, for inhibition of cAMP hydrolysis (16, 17)] but were not killed by inhibitors of PDE3 (milocinone) or PDE5 (T-0165) (Fig. 3). Both PDE7 inhibitors, used at concentrations below the IC50 for inhibition of other PDEs, selectively induced apoptosis of CLL cells (Fig. 3A) (16–18).

PDE7B is an abundantly expressed PDE in CLL cells, but PDE4B is the highest expressed PDE isoform (Fig. S1). Consistent with these data, and confirming previous work, we found that inhibitors of PDE4 (either rolipram or RO20-1724) induce apoptosis in CLL cells (3, 19). We hypothesized that combined inhibition of PDE7 and PDE4 would increase killing of CLL cells. In support of this hypothesis, we found that the selective PDE7 inhibitor BRL-50481 (30 μM), which itself kills CLL cells, enhanced apoptosis induced by the PDE4 inhibitor rolipram (3 μM) (Fig. 3B). Moreover, BRL-50481 enhanced

**Fig. 1.** mRNA expression of multiple PDE isoforms is altered in CLL. (A) QPCR analysis of the expression of PDE isoforms that show differences with CLL from 16 normal subjects (normal) and 25 CLL patients (CLL). Data are expressed as C, normalized to 28S rRNA (mean ± SEM). ***, P < 0.01. (B) QPCR showing the mRNA expression of PDE3B, 4D, 5A, 7B, and 9A from normal B cells (obtained by CD19-positive selection (n = 10) or negative isolation (n = 3)) and CLL cells (n = 25–60) compared with normal PBMC. *, P < 0.01. Data are expressed as fold change of each PDE isoform relative to the average expression in normal PBMC. In addition, CLL cells have significantly different expression of each of the PDE isoforms compared with normal B cells. #, P < 0.05.

**Fig. 2.** PDE7B protein expression and the contribution of PDE7 to total cAMP-PDE activity are increased in CLL cells. (A) Shown is a representative immunoblot of PDE7B (52 kDa) and β-actin (43 kDa) protein expression in 2 normal subjects (N1 and N2) and 5 CLL patients. Quantification of PDE7B protein expression normalized to β-actin showed a 10.8 ± 1.5-fold increase in CLL cells (n = 19) vs. normal PBMC (n = 5) (P < 0.01; samples from 2 other normal subjects had PDE7B protein levels that were below the level of detection). (B) Representative immunoblot of PDE7B (52 kDa) and PDE4B (100 kDa, PDE4B2) expression in cytosol, membrane, and insoluble fractions of CLL cells (data representative of n = 5; Fig. S2 shows all PDE4B isoforms detected). (C) The contribution of PDE7 to total cAMP-PDE activity was determined by using the PDE7 inhibitor BRL-50481 (30 μM, n = 9). Data are mean ± SEM. ***, P < 0.001 compared with normal.
with vehicle, 10 μM rolipram (Rolli), R020-1724, T-0156, or IR-202 or 30 μM BRL-50481 (BRL). Data are expressed as drug-induced apoptosis (%) for each PDE inhibitor. Each data point represents mean ± SEM of 3–8 normal subjects and 3–10 B-CLL patients. *P < 0.05; **P < 0.01 compared with vehicle. (A) Apoptosis of CLL cells incubated with 30 μM BRL-50481, 3 μM Rolli, or their combination for 48 h (n = 7). Data (mean ± SEM) are expressed as drug-induced apoptosis (%), **P < 0.01 for combination of BRL-50481 and Rolli vs. BRL-50481 alone; ##, P < 0.01 for combination of BRL-50481 and Rolli vs. Rolli alone. (C) Apoptosis in normal B cells (labeled using a PE-conjugated anti-CD19 antibody) and CLL cells after 48 h of incubation with vehicle, 10 μM rolipram (Rolli), 30 μM BRL-50481 (BRL), 10 μM IR-202, or 100 nM IR-284. Data (mean ± SEM) are expressed as drug-induced apoptosis (%) (n = 8–10), *P < 0.05; **P < 0.01 compared with vehicle. (D) Proliferation of EHEB cells incubated for 48 h with vehicle, 10 μM rolipram (Rolli), 30 μM BRL-50481 (BRL), 10 μM IR-202, or 100 nM IR-284. Data (mean ± SEM) are expressed as drug-induced decrease in proliferation (%) (n = 4), *P < 0.05; **P < 0.01 compared with vehicle.

rolipram-induced apoptosis of freshly isolated CLL cells, which have lower basal apoptosis (Fig. S3): BRL-50481 shifted the concentration–response curve for rolipram-induced apoptosis to the left (i.e., enhancing the apoptotic effect of low-dose rolipram) and increased the maximal response. These data provide further evidence for the potential of PDE7B as a drug target in CLL that can enhance the proapoptotic effect of low-dose PDE4 inhibitors. In accordance with these findings, we found that a dual PDE4/7 inhibitor, IR-284 (patent WO 02/085906, IC50 2.3 nM for PDE4-mediated and 23 nM for PDE7-mediated inhibition of cAMP hydrolysis), induces apoptosis of CLL cells: concentrations from 10 nM to 10 μM selectively kill CLL cells compared with normal PBMC (Fig. S3B).

PDE7 and Dual PDE4/PDE7 Inhibition Induce Significantly Less Apoptosis in Normal B Cells than Does Inhibition of PDE4. We tested killing of normal B cells by various PDE inhibitors and found that inhibition of PDE4, PDE7, or dual inhibition of PDE4/7 does not significantly kill these cells, in contrast to the proapoptotic effect observed in CLL cells (Fig. 3C). Normal B cells from 3 of 8 subjects showed apoptosis in response to rolipram whereas only one was sensitive to apoptosis in response to BRL-50481, IR-202, or IR-248. PDE7 and dual PDE4/7 inhibitors are thus more selective in killing of CLL than normal B cells, consistent with the higher PDE7B expression in CLL cells.

PDE4, PDE7, and Dual PDE4/PDE7 Inhibitors Decrease the Proliferation of EHEB Cells. Rolipram (10 μM), BRL-50481 (30 μM), IR-202 (10 μM), and IR-284 (100 nM) each inhibited the proliferation of EHEB, a CLL cell line (20), decreasing proliferation 32 ± 1%, 19 ± 1%, 21 ± 2%, and 48 ± 2%, respectively (Fig. S3D). IR-284 was more efficacious, perhaps because it inhibits both PDE4 and PDE7. EHEB cells are easy to maintain in culture and thus may be useful for identifying other PDE inhibitors with potential therapeutic utility in CLL.

Mitochondrial depolarization and the release of cytochrome c are key events in mitochondrial-dependent apoptosis (22). Rolipram, BRL-50481, IR-202, and IR-284 induced mitochondrial depolarization (25 ± 4%, 13 ± 4%, 17 ± 6%, and 39 ± 5% depolarization, respectively, vs. vehicle) and released cytochrome c from mitochondria into the cytosol (Fig. 4C), implying that PDE inhibitor-induced apoptosis of CLL cells occurs via a mitochondrial-dependent mechanism.

The proapoptotic and antiapoptotic proteins BIM and survivin, respectively, contribute to cAMP-promoted apoptosis in T cells (21). Two PKA-selective inhibitors [PKI (14–22, 5 μM) and Rp-cAMP (Rp-adenosine 3’,5’-cyclic monophosphorothioate triethylenamonium, 100 μM)] attenuated IR202-induced (54 ± 9% and 75 ± 8%, respectively) and IR284-induced (24 ± 6% and 28 ± 11%, respectively) apoptosis (Fig. 4B). Thus, a cAMP/PKA-dependent pathway likely mediates the killing of CLL cells promoted by PDE7 and PDE4/7 inhibitors.

Discussion
To our knowledge, these results provide the first comprehensive analysis of PDE isoforms in relation to human cancer; previous studies have examined expression of certain PDE isoforms in malignant cells (4, 13, 14, 24–27). We find that PBMC and normal B cells express PDE7B and that CLL cells selectively overexpress this isoform, highlighting PDE7B as a potential therapeutic target in CLL. Expression of other PDE isoforms (PDE3B, 4D, 5A, and 9A) is decreased in CLL cells. Because PBMC are enriched in T cells, whereas CLL cells are predominately B cells, differences in PDE isoform expression may result from differences in expression of the cell populations. However, we find large differences in expression of the PDE isoforms in CLL vs. normal B cells, implying that the PDE expression pattern in CLL cells is characteristic of the malignancy, perhaps as a result of clonal expansion of a subset of B cells. More generally,
the findings indicate that profiling PDE expression, which we have also used to identify novel increases in PDE1A and PDE1C with pulmonary hypertension (28), may prove useful for understanding pathophysiology and identifying therapeutic targets.

PDE7B, the isoform increased in CLL, together with PDE7A comprise the PDE7 family. After discovery of the PDE7 isoforms and their tissue expression (29–32), human B cells were noted to have PDE7 activity and high expression of PDE7 mRNA (33). PDE7A and 7B are high-affinity cAMP-specific PDEs (Kₐ for cAMP is <0.2 μM) (7, 29–32). PDE7A is abundant in brain, proinflammatory, and immune cells and is involved in T cell activation and proliferation; by contrast, PDE7B is highly expressed in brain, liver, and skeletal muscle and, based on its up-regulation by dopamine, is thought to contribute to dopaminergic signaling (18, 29, 31, 32, 34–37). PDE7A, but not PDE7B, is a dual inhibitor of cAMP signaling, increasing hydrolysis of cAMP and directly inhibiting PKA activity via a PKA pseudosubstrate site (38). The functional consequence of this site and relationship to transcriptional activation of PDE7B by cAMP/PKA via activation of the cAMP-response element binding (CREB) protein are unclear (31, 39). Investigation of the regulation of PDE7B in B cells should help define its function in these cells, mechanisms involved in its cellular expression, and its role in CLL.

The increase in PDE7B expression in CLL may be therapeutically relevant: We find that two different PDE7 inhibitors promote apoptosis of CLL cells and decrease proliferation of EHEB, a CLL cell line. Because PDE7 inhibitors are nonspecific we attempted to use siRNA to inhibit PDE7B as a means to determine its contribution to effects observed with such inhibitors; however, because of poor transfection efficiency and high levels of basal apoptosis of CLL cells, these experiments were not successful (data not shown). PDE7A and PDE7B are 70% identical and thus the design of isomser-specific inhibitors is a challenge, although their differences may be sufficient for identification of inhibitors with specificity for PDE7B (7, 31, 32).

PDE7B, the main PDE-PDE in PBMC and CLL cells, is the target of the PDE4-selective inhibitor rolipram (3, 4, 19). PDE7B inhibition enhances the proapoptotic effects of rolipram, which led us to synthesize and test IR-284, a PDE4/7 dual inhibitor. PDE4 inhibitors have dose-limiting cardiovascular, gastrointestinal, and central nervous system side effects; thus, combining a low dose of a PDE4 inhibitor with a PDE7 inhibitor or a dual PDE4/7 inhibitor may reduce side effects, increase efficacy for CLL cell killing, and perhaps be useful for the treatment of CLL (35, 40) by providing increased specificity but decreased toxicity of normal B or T cells (Fig. 3C) (16, 35). PDE7 and PDE4/7 inhibitors increase killing of CLL cells by dexamethasone (data not shown), suggesting that the PDE inhibitors may be a useful addition to current treatment regimes.

Activation of the cAMP/PKA-dependent pathway contributes to the proapoptotic effects of PDE7 and PDE4/7 inhibitors (Fig. 4). Such results and previous data imply that in CLL cells the activity of PDEs is important in governing cellular cAMP levels (4, 19). PDE7 and PDE4/7 inhibitors show greater killing relative to their ability to raise cAMP than does rolipram, suggesting that cellular compartmentation of cAMP and of PDE isoforms contributes to apoptotic response, perhaps facilitating interaction with signaling pathways (7, 8, 41). Unlike PDE4 inhibition or forskolin, PDE7 inhibition activates PKA but not Epac (exchange protein directly activated by cAMP) (15, 21), a downstream effector of cAMP. Because activation of Epac, but not PAK, is anticaspase in CL cell (21), PDE7 inhibitors would be predicted to be more effective than PDE4 inhibitors in enhancing cell killing. Moreover, PAK inhibition does not completely block the proapoptotic effects of IR-202 or IR-284 [akin to results obtained with theophylline- and rolipram-induced apoptosis (10, 21)], providing evidence that PDE inhibitors may act by non-PAK mechanisms to kill CLL cells.

PDE4 inhibition and, based on data shown here, PDE7 inhibition promote apoptosis of CLL cells by the intrinsic cell death pathway, releasing cytochrome c from mitochondria and cleaving caspases 3 and 9 (19). Consistent with evidence that the proapoptotic protein survivin contributes to cAMP/PKA-dependent apoptosis of murine lymphoma cells (23), IR-284 decreases expression of survivin (Fig. 4D), implicating survivin as a target for cAMP-promoted apoptosis, an idea that contrasts with other reports regarding survivin expression in CLL (42, 43).

We were surprised to find that PDE4/7 inhibition decreases PDE7B expression (Fig. 4D) because previous data show that PDE5 increases the expression of other PDEs in CLL cells (13,
Samples were compared by using the relative cycle threshold (Ct) method (3). Thermal cycling conditions were as follows: incubation 10 min at 95 °C, 30 s at 66 °C, and 30 s at 72 °C. Samples were compared by using the relative cycle threshold (Ct) method (3). Selective inhibition of PDE7 or dual PDE4/7 inhibition may provide a novel therapeutic approach for the treatment of CLL by enhancing killing and increasing specificity for CLL cells. More generally, the current data indicate that comprehensive analysis of PDEs in disease settings can reveal patterns of isoform expression that have the potential to serve as diagnostic markers and/or therapeutic targets.

Materials and Methods

Unless stated otherwise, all chemicals and cell culture reagents were purchased from Sigma-Aldrich and Gibco BRL, respectively. The PDE7 inhibitor BRL-50481 [3-(N,N-dimethylsulfonamido)-4-methyl-nitrobenzene] was from Tocris. IR-202 ([2]-methyl-4-(5-[3-hydroxy cyclohexylin]-4-ethyl-5, 4-dihydrodro-1) phenylcarbamate] and IR-284 ([4-(3-chloro-4- methoxyphenyl)-2-(1-morpholine-4-carbonyl) piperad-4)-yl]-4a, 5,8,9- tetrahydrophthalalin-1(2H)-one (patent WO 02/085906) were prepared by the University of California at San Diego Medicinal Chemistry Core as described (17). The PKA inhibitor PKI [myristylated PKI (14–22) amide] was from BIomol.

PBMC Isolation. Blood was collected from healthy donors (normal PBMC) and CLL patients (CLL cells) after informed consent, in agreement with institutional guidelines. All use of patient data and samples followed or exceeded the guidelines of the Health Insurance Portability and Accountability Act (46). CLL diagnosis was made by blood cell morphology and immunophenotyping. The patients’ median age was 61 yr (range, 44–75 yr), with a median WBC of 106 cells per milliliter (range, 44–75 yr), with a median WBC of 106 cells per milliliter (range, 15–460 cells per milliliter). PBMC were isolated by density-gradient centrifugation using Ficoll-Paque (Amer sham Biosciences), washed, suspended in FCS containing 10% DMSO, then stored in liquid N2 for subsequent use.

Isolation of B Cells. B cells were isolated from normal PBMC by using either Dynabeads CD19 pan B or a Dynal B cell negative isolation kit (Invitrogen), as assessed by flow cytometry.

EHEB Cell Culture. EHEB cells (from the German Collection of Microorganisms and Cell Cultures) were grown in 90% RPMI medium 1640 plus 10% FBS and maintained at 0.1–0.5 × 106 cells per milliliter at 5% CO2 at 37 °C.

Real-Time RT-PCR (QPCR). Total RNA was isolated from PBMC by use of the Versagen RNA Cell Kit (Genta; SuperScript II First Strand Synthesis System [Invitrogen]) was used to synthesize cDNA. Real-time PCR (MI Research Option 2 using Eurogentec QPCR Mastermix Plus SYBR Green Kit) was performed by using 8 ng of RNA per reaction and 100 nM sense/antisense primers. Two primer sets were designed for each PDE isoform, and the results were compared with the cycle threshold (Ct) method normalizing to 28S rRNA.


Fig. S1. mRNA expression of PDE isoforms in normal PBMC and CLL cells. Shown is quantitative PCR analysis of mRNA expression of PDE isoforms in PBMC from 7 normal subjects and 7 CLL patients. Data are expressed as cycle threshold (Ct) normalized with 28S rRNA (mean ± SEM). Expression of GAPDH and β-actin were similar in CLL cells and normal PBMC. The expression of PDE3B, 4D, 5A, 7B, and 9A are all statistically different (P < 0.01) in CLL compared with normal subjects.
Fig. S2. Dual PDE4/7 inhibition kills CLL cells. (A) Representative result for a patient in whom apoptosis was assessed in freshly isolated CLL cells treated with 30 μM BRL-54081 alone (BRL, filled square), 0.3–30 μM rolipram (Roli, solid lines), or a combination of 30 μM BRL-54081 and 0.3–30 μM rolipram (dashed lines) for 48 h. Data are expressed as drug-induced apoptosis (%). Studies of 4 patients yielded comparable results. (B) Apoptosis of CLL cells and normal PBMC induced by 1 nM to 10 μM IR-284. Data are expressed as drug-induced apoptosis (%). Data are mean ± SEM (n = 8). *, P < 0.05; **, P < 0.01 compared with vehicle.
Fig. S3. PDE4B is expressed in the cytosolic fraction of CLL cells. Shown is a representative immunoblot of PDE4B expression in CLL cells (n = 5). Two major immunoreactive bands were detected for PDE4B in the cytosolic fraction, representing PDE4B2 (78 kDa) and PDE4B3 (100 kDa). Weaker bands at 107 kDa and 66 kDa may represent PDE4B1 and PDE4B4, respectively.