UNIVERSITY OF CALIFORNIA, SAN DIEGO

G protein-coupled receptor expression and function in malignant B-cells: Therapeutic targets for Chronic Lymphocytic Leukemia

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2012

The thesis of Trishna Chetan Katakia is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2012

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LIST OF ABBREVIATIONS

cAMP = 3’5’ adenosine monophosphate

MC2R = Melanocortin 2 receptor

ACTH = Adrenocorticotropic hormone

VIPR1 = Vasoactive intestinal peptide receptor 1

PDE = phosphodiesterase

GPCR = Gprotein-coupled receptor

CLL = Chronic Lymphocytic Leukemia

PBMC = Peripheral Blood mononuclear cells

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ACKNOWLEDGEMENTS

I would like to acknowledge Paul Insel for his support as the chair of my committee. Throughout the past two years, Paul has given me countless opportunities to participate in novel research and has given me experiences that will be invaluable to me in my professional and personal life.

I would also like to acknowledge my advisor and mentor, Fiona Murray, for her unfailing guidance, encouragement, and support throughout the duration of my time in the lab. Her advice and insight into the direction of my project was key in allowing me to complete this study.

I would also like to acknowledge Lingzhi Zhang for her guidance and mentorship throughout the beginning stages of my project.

I would like to thank Nakon Aroonsakool and the rest of the Insel Lab for all the help and encouragement they have given me over the past 28 months.

Most importantly, I would like to thank my parents for their love, devotion, and incredible support throughout my years in graduate school.

Finally, I would like to acknowledge and thank Stuart Brody and Jayant Ghiara for taking the time to serve on my thesis committee.

ABSTRACT OF THE THESIS

G protein-coupled receptor expression and function in malignant B-cells:

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Master of Science in Biology

University of California, San Diego, 2012

Professor Paul Insel, Chair

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Chronic lymphocytic leukemia (CLL) is associated with the accumulation of B-cells due to decreased apoptosis. CLL is classified as aggressive (rapidly progressive) or indolent (slow-growing). This thesis sought to assess expression of G protein-coupled receptors (GPCRs), in particular GPCRs that regulate the synthesis of the second messenger 3'5'-cyclic adenosine monophosphate (cAMP), in samples from patients with CLL and in normal human B cells. Using a Taqman® GPCR array, we found that normal B-cells, indolent-CLL cells and aggressive-CLL cells express >117 GPCRs, many of which were differentially expressed in CLL-cells and in the two stages of CLL. Expression of the vasoactive intestinal polypeptide receptor 1 (VIPR1) was >700-fold greater in aggressive CLL cells than in indolent CLL-cells and normal B-cells; the

agonist (VIP, 1 μM) in combination with a phosphodiesterase 4/7 inhibitor (IR284, 100 nM) raised cAMP levels in both indolent and aggressive CLL-cells, but not in normal B-cells. In addition, VIP treatment (48 hr alone and together with IR284) induces apoptosis in aggressive CLL-cells. The melanocortin 2 receptor MC2R) was expressed in aggressive and indolent CLL-cells but not normal B-cells. Treatment with the MC2R agonist, adrenocorticotropic hormone (ACTH, 1 nM), in combination with IR284 induced apoptosis in aggressive CLL-cells, but not in indolent CLL-cells or normal B-cells. These results reveal that expression of particular GPCRs can provide stage-specific markers and identify novel therapeutic targets for the treatment of CLL. Moreover, the results identify a paradigm that may be useful in other disease settings.

1. Introduction:

1.1 Chronic Lymphocytic Leukemia

B-cell Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the Western world.[1] In the United States, the average incidence of CLL is 2.7 persons per 100,000, with the risk of developing CLL increasing progressively with age: CLL occurs twice as often in men compared to women.[1] CLL is characterized by the accumulation of mature B-cells, due to cell cycle arrest in the G_0/G_1 phase and reduced apoptosis [2] The progression of CLL is highly variable with patients surviving a few months to many years after the initial diagnosis.[2] In some cases, the disease displays indolent behavior, slow growing, and not requiring treatment, with patients dying from causes that are unrelated to the illness.[3] However, the disease can be aggressive, which requires immediate treatment and has a poor prognosis.[3] Although clinical stages are still the basis for determining CLL disease progression, the past few years of research have represented a shift in focus to the use biological factors, biomarkers, as prognostic markers for the disease.[3-4] For example, several serological markers such as thymidinkinase (TK), β 2-microglobulin, soluble CD23, and ZAP-70 expression have been used to gather information about tumor mass, the proliferative activity of CLL cells, as well as bone marrow infiltration, all of which provide information about the disease progression.[3-4] Levels of TK have been used to predict disease progression, while high levels of CD23 seem to correlate with faster doubling time and diffuse bone marrow infiltration.[3] An increase in ZAP-70 expression along with a specific gene mutation is indicative of a poor prognosis; ZAP-70 alone has been shown to be a useful biomarker.[3]

However, these biological factors do not provide insight into the actual mechanism of CLL. Biomarkers which are closely related to the mechanism of the disease, such as those that play a critical role in contributing to the reduced apoptosis, would also be of therapeutic potential.

1.2 Apoptosis

The molecular basis of controlling apoptosis in CLL is influenced by many factors that regulate cell death.[5] Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. Signals such as radiation, cytotoxic drugs, and cellular stress all activate the intrinsic cell death pathway, which disrupts the mitochondrial membrane.[5] The intrinsic pathway involves B-cell lymphoma (Bcl)-related family proteins that have anti-apoptotic (e.g., Bcl-2, BclXL) or pro-apoptotic (e.g., Bax, Bak, BAD, BIM, BclXS). When the membrane is damaged, cytochrome *c* is released from the mitochondrial intermembrane space and combines with cytosolic Apaf-1 and an inactive initiation caspase, procaspase 9. This multiprotein complex called the apoptosome activates caspase 9, which triggers a signaling cascade activating several other downstream caspases. One caspase in particular, caspase 3, "the effector caspase," triggers biochemical changes in the cell associated with cell death.[5]Alternatively the extrinsic pathway functions independently of the mitochondria and is activated by cell surface death receptors (e.g., Fas). These receptors activate the initiator caspase, caspase 8, within the death-inducing-signaling-complex (DISC), which initiates a caspase signaling cascade leading to cell death.[5] In CLL, the reduced rate of apoptosis could be

resulting from either decreased activity of the pro-apoptotic pathway or increased activity of the anti-apoptotic pathway.[6]

1.3 G protein-coupled receptors (GPCRs)

B-cells express a wide variety of receptors that facilitate the interaction of these cells with their extracellular environment. Hormones and neurotransmitters in the extracellular milieu play key roles in modifying B-cells under both physiological and pathophysiological conditions. G protein-coupled receptors (GPCR), the largest receptor family in the human genome, comprise ~3% of the genes and are the targets for >30% of prescribed drugs [36-38]. GPCRs are localized on the plasma membrane and are a major focus in drug discovery. GPCRs are guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins, whose α and βγ subunits dissociate upon ligand binding. G proteins are divided into four main classes according to their α subunit, Gαs, Gαi, Gαq/11, and Gα12/13, (Fig. 1): Gαs stimulates the membrane-associated enzyme adenylyl cyclase (AC), which catalyzes the synthesis of cAMP, and regulates Ca2+ channels; Gαi inhibits AC activity, decreasing cAMP, and also regulates K+ and Ca2+ channels; Gαq/11 stimulates membrane-bound PLCβ, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol (1,4,5)-triphosphate (IP3) and diacylglycerol (DAG); Gα12/13 regulates the small GTPase, RhoA. In addition, Gβγ can act as a heterodimer to activate both concordant and disconcordant signals, i.e. regulating PLCβ, AC, and PDZ domain-containing proteins.[12] A balance of these intracellular second messengers regulates apoptosis and modulates the phenotype of B-cells and therefore could influence the biology of CLL cells.

1.4 cAMP

The second messenger, 3': 5'-cyclic adenosine monophosphate (cAMP) is a ubiquitously expressed second messenger whose intracellular concentration can be increased by GPCRs.[8] In addition to the regulation of its synthesis by G-proteins and AC, as described above, cAMP is hydrolyzed by a group of enzymes called cyclic nucleotide phosphodiesterases (PDEs) [7,11.] The balance of ACs and PDEs control the intracellular levels of cAMP.[9] cAMP actions are mediated via protein kinase A (PKA) and Epac (Exchange protein directly activated by cAMP), which both play important roles in the differentiation, proliferation, and function of lymphoid cells.[49]

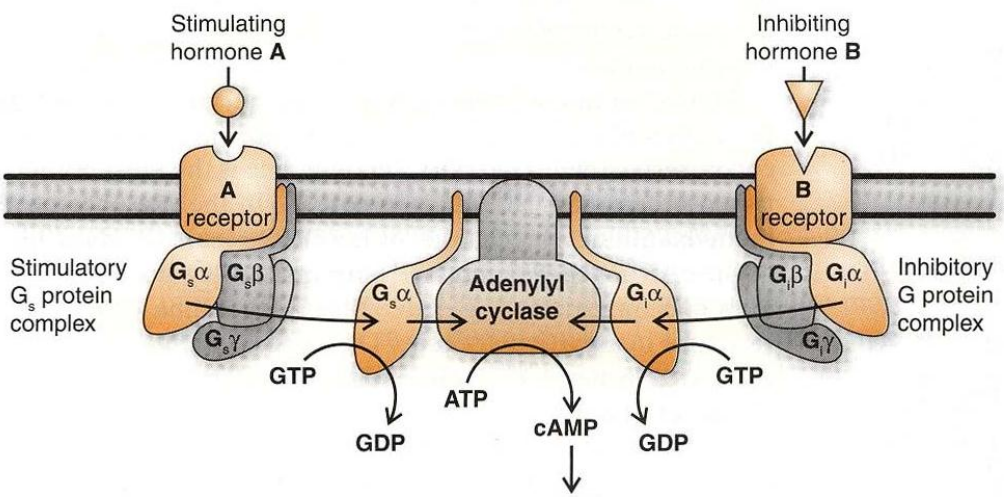

Figure 1.1. Schematic diagram showing the signaling pathway of G_s-coupled and G_i-coupled G protein-coupled receptors (GPCRs). Agonist binding to a G_s-coupled GPCR activates the stimulatory trimeric G-proteins (α_s,β,γ) and catalyzes the exchange of GDP for GTP on the $G_{\alpha s}$-subunit, causing it to dissociate from the $G_{\beta\gamma}$-subunits. The $G_{\alpha s}$-subunit activates adenylyl cyclase, which then facilitates the conversion of ATP to 3': 5'-cyclic adenosine monophosphate (cAMP). An agonist bound to a G_i-coupled GPCR activates the inhibitory trimeric G-proteins (α_i,β,γ) and catalyzes the exchange of GDP for GTP on the $G_{\alpha i}$-subunit, causing it to dissociate from the $G_{\beta\gamma}$-subunits. The $G_{\alpha i}$-subunit inhibits adenylyl cyclase, activity, which prevents the conversion of ATP to cAMP. In addition, the $G_{\beta\gamma}$-subunits from G_i heterotrimers can inhibit $G_{\alpha s}$-subunits to decrease AC activity.

1.5 Targeting cAMP in CLL

Cyclic AMP can stimulate or inhibit apoptosis, depending on the cell type.[39] Of note, cAMP-promotes apoptosis in certain lymphoid cells, including CLL-cells. The concentration of cAMP and activity of PKA are reportedly lower in lymphocytes of CLL patients compared to those of normal subjects, suggesting a disease-related defect in this pathway.[40,41]

The expression profile of PDE isoforms in the cells of patients with CLL differs from that of normal lymphocytes[10]. Specifically, CLL cells have an increased expression of PDE7B, a cAMP-selective isoform.[10]. Inhibition of PDE7 or of PDE4/7 (PDE 4 is also a cAMP-selective PDE isoform) promote apoptosis of CLL cells, but not normal B-cells, via a cAMP-PKA-mitochondrial-dependent process and is associated the decreased expression of the anti-apoptotic protein survivin.[10]. cAMP can also synergistically increase apoptosis in CLL-cells when used in combination with other drugs (e.g., glucocorticoids).[12] These data suggest that cAMP is an important therapeutic target for CLL and perhaps for other hematopoietic malignancies.[11] Furthermore, in addition to being therapeutic targets, PDEs are also biomarkers for CLL: CLL patients with PDE7B mRNA levels in the top quartile (i.e., greater than nine-fold elevation relative to normal controls) have a several-year shorter median time-to-treatment (TTT, 36 months) compared to that of patients whose CLL cells express lower levels of PDE7B mRNA (TTT, 77 months, p=0.001). PDE7B mRNA expression thus appears to be a clinically useful biomarker to predict the prognosis of patients with CLL.

In addition to PDEs, GPCRs, coupled to either Gαs or Gαi can alter cellular levels of cAMP. Previous studies have shown that at least one GPCR that is highly expressed in CLL cells relative to normal B-cells, CXCR4, is a potential therapeutic target for inducing apoptosis.[13] The CXCR4 chemokine receptor was one of the first GPCRs characterized in CLL and is expressed at high levels on the surface of CLL B cells when compared to normal subjects.[14] The expression of CXCR4 is down-regulated by its ligand, CXCL12, through receptor-mediated endocytosis; this ligand-GPCR interaction was shown to be a possible target in the treatment of CLL.[13-14] CXCR4 antagonists that inhibit protection of cells by CXCL12 induced pro-apoptotic effects through the activation of downstream signaling components, such as mitogen-activated protein kinases (MAPK) and signal transducer and activator of transcription 3 (STAT 3).[13] These data suggest that highly or uniquely expressed GPCRs on CLL-cells could be novel therapeutic targets for CLL.

Unbiased approaches have begun to define tissue expression of GPCRs. Regard et al., 2009, quantified RNA transcripts for 353 non-odorant GPCRs in 41 tissues from mice, uncovering possible new roles for a number of GPCRs in particular tissues.[43] Interestingly, many orphan (whose physiologic agonists are not known) and olfactory GPCRs are expressed in most tissues, although their function has not yet been determined.[44] Limited data are available regarding GPCR expression in individual cells, however recently it was found that taste receptor GPCRs, previously identified on the tongue, are highly expressed on bronchial smooth muscle cells and that agonists for these receptors relax airway smooth muscle more than β2-adrenergic receptor agonists, implying they could be a new targets to promote bronchodilation.[45] Taken together, these

data suggest that GPCRs involved in the regulation of malignant B-cells and that contribute to the pathophysiology of CLL may have been "missed" in prior studies. Profiling GPCR expression in CLL cells could uncover these receptors, which could be biomarkers and/or therapeutic targets for the disease.

1.6 Hypothesis and Goals

The overall hypothesis is that previously unrecognized GPCRs are expressed and functional in CLL cells and could be novel biomarkers and/or therapeutic targets for CLL

1.6.1 Hypothesis One

GPCR expression is disease-stage specific and can identify novel biomarkers for CLL

1.6.2 Hypothesis Two

GPCRs that are increased or uniquely expressed in CLL-cells, in particular those that regulate cAMP, can promote apoptosis and be novel therapeutic targets for CLL.

2. Materials & Methods

2.1. PBMC Isolation

Blood was collected from healthy donors and CLL patients (to isolate peripheral blood mononuclear cells [PBMC] and CLL-cells, respectively) following 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. The diagnosis of CLL was made by assessment of blood cell morphology and immunophenotyping. The patients' median age was 60 yr (range, 45-75 yr). PBMC were isolated by density-gradient centrifugation using Ficoll-Paque (Amersham Biosciences), washed, suspended in fetal-calf serum containing 10% DMSO then stored in liquid N_2 for subsequent use.

2.2. Cell Resuspension

Each 1 mL cell sample (both CLL and normal) was thawed and diluted in 10 ml of RPMI 1640media (with added 10% heat-inactivated fetal bovine serum [FBS], Atlanta Biologicals). A 25µl portion of cell/media solution was mixed with 25µl of 0.4% Trypan blue (Invitrogen, Carlsbad, CA), and 10µl of this solution was pipetted onto a Bright-Line Hemacytometer (Reichart, Depew, NY) for cell counting. The original cells were pelleted at 1200 rpm for 5 min at 25°C. Media was aspirated and the cells were resuspended at 0.5-5.0 million cells/ml, depending upon the experiment.

2.3 Real-time PCR

2.3.1 Primer Design

Primers for various GPCRs were designed using the NCBI Entrez search engine (http://www.ncbi.nlm.nih.gov/sites/entrez, Bethesda, MD). The gene sequence was then pasted into the Primer3 online primer-designing program (http://frodo.wi.mit.edu/, MIT, Cambridge, MA) using standard settings. Several primer pairs were chosen from the program's suggestions. All primers were obtained from ValueGene(San Diego, CA) and diluted with diethyl pyrocarbonate-(DPEC)-treated water to a stock concentration of 200μM.

2.3.2 Extraction and Real-time PCR Protocol

RNA was extracted from 2 million CLL-cells and normal PBMC using an extraction kit (Versagene [5 PRIME]) according to the manufacturer's instructions. cDNA was synthesized using a protocol of Applied Biosystems, per the manufacturer's instructions. Real-time PCR was performed using 8ng cDNA, 0.5 μM forward and reverse primers, and Qpcr Mastermix Plus for Sybr Green I (Eurogentec, San Diego, CA) and the Opticon 2 RT-PCR machine (MJ Research, Waltham, MA). The RT-PCR Program and RT-PCR primers used are shown in Table 2.1 and Table 2.2. Primer efficiency was calculated for each primer set before use and samples were compared using the relative cycle threshold (C_t) method, normalizing to 28S rRNA. For real-time PCR, the threshold for receptor expression was a ΔC_t value of

approximately 32. A GPCR with a ΔC_t value of above 32 was considered to be not expressed.

2.3.3 TaqMan® GPCR array

We initially defined GPCR expression in normal B-cells and CLL cells from patients with aggressive and indolent disease using a TaqMan® GPCR array (Invitrogen), according to manufacturer's instructions, with cDNA pooled from normal B-cells (n=10), indolent CLL-cells (n=10), and aggressive CLL-cells was isolated and placed into the 384-well micro fluidic card along with TaqMan® Universal PCR Master Mix. The plate was centrifuged to dispense the sample mix into the individual wells and run on a Real-Time PCR System.

To quantify GPCR expression, the expression of each GPCR was normalized to that of the housekeeping gene (18S), which is expressed in all cell types. The C_t value of 18S, approximately 13.6, was subtracted from the C_t value of the GPCR to obtain the ΔC_t, the normalized value representing GPCR expression. For this GPCR array, the threshold for receptor expression, the maximum ΔC_t value, was approximately 27. Any GPCR with a ΔC_t value greater than 27 was considered to be not expressed.

Table 2.1. Real-time PCR protocol

Step	Temperature (°C)	Time
1	50	2 minutes
2	95	10 minutes
3	95	15 seconds
4	60	30 seconds
5	72	1 minute
6	Plate read.	
Go to Step 3 34 more times.		
7	Construct melting curves for samples by heating the plate from 60°C to 95°C. Read plate every 0.2°C, holding the temperature for 1 second.	

Table 2.2. RT-PCR primers

GPCR	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	T_m
VIPR1	atgttcgccttctttccgg	cacgaacgagctcaaagacca	60°C
MC2R	catcctcagcctgtctgtga	agaacgtccagatgaccgta	60°C
CXCR4	cttcttatgcaaggcagtcc	cttttcagccaacagcttcc	60°C
GPR113	tctcaaacatgtcccatcac	atggaagtcgagccacatct	60°C
GPR63	cacaacaccagcagcattta	caaacaaccaagttcccaag	60°C
GPR120	ccagaacttcaagcaagacc	cctgcacagtgtcatgttgt	60°C
GPR4	catcctctactgcctggtca	ctgttcctcttggaggtgag	60°C
TAAR5	cgacagcctccttcacttta	cgaaaccactggtaggaaa	60°C
28S	gcctagcagccgacttagaa	aaatcacatcgcgtcaacac	60°C

2.4. cAMP Radioimmunoassay

One million CLL-cells or normal PBMC/ml in RPMI 1540 + 10% FBS were plated in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC) and incubated for 20 min at 37°C in air plus 5% $CO_2$with a dual PDE4/7 inhibitor [IR284, (4-(3-chloro-4-methoxyphenyl)-2-(1-morpholine-4-carbonyl) piperadin-4-yl)-4a, 5,8,8a-tetrahydrophthalazin-1(2*H*)-one, CSD Cancer Center Medicinal Chemistry Core]. After the incubation time, VIP (vasoactive intestinal peptide receptor agonist, Tocris Bioscience, Minneapolis, MN) or ACTH (a melanocortin 2 receptor agonist, GenWay, San Diego, CA) were added to the 12-well culture plate and incubated for 10 min at 37°C in air plus 5% CO_2. After the incubation time, the cells were centrifuged at 1200 rpm for 5 min at 4°C. The media was aspirated and 50µl of 7.5% trichloroicacetic acid (TCA) was added to each cell pellet.

Assay tubes were filled with 1ml of 10mM sodium acetate buffer (pH 4.75) and a standard curve was made by performing a serial dilution with exogenous cAMP (Millipore, Billerica, MA). An appropriate amount of sample was added to the assay tubes and the samples were acetylated with the addition of 20µl triethylamine (Sigma) and 10µl acetic anhydride (Sigma) and vortexed immediately after. 100µl of the 10mM sodium acetate buffer (pH 4.75) was added to each well of a 96 well filter plate and vacuumed out using Millipore apparatus. 50µl of diluted, acetylated sample was added to each well along with 25µl diluted antibody [6µl primary cAMP antibody (Millipore) in 6ml γ-globin buffer] and 25µl diluted ^{125}I radioactivity [16µl ^{125}I (PerkinElmer) in 3ml γ-globin buffer]. Actual amount of radioactivity that was added depended upon the date of

production. The 96 well filter plate containing the sample was incubated overnight at 4°C.

Following overnight incubation, 50µl secondary antibody [Biomag Goat anti-Rabbit IgG 8-4300D, Qiagen] was added to each well and the plate was incubated for 1 h at 4°C. 100µl of 12% polyethylene glycol in 10mM sodium acetate pH 6.2 added to samples and then removed. This wash step was repeated two more times. The base of the plate was then removed with the sample punched out into fresh assay tubes and then placed onto the WIZARD2 Automatic Gama Counter (PerkinElmer) for determining radioactivity.

2.5. Flow Cytometry

One million CLL-cells or normal PBMC/ml in RPMI 1540 + 10% FBS were plated in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC) and incubated for 48 hr at 37°C in air plus 5% CO_2 with a dual PDE4/7 inhibitor [IR284 (100nM), (4-(3-chloro-4-methoxyphenyl)-2-(1-morpholine-4-carbonyl) piperadin-4-yl)-4a, 5,8,8a-tetrahydrophthalazin-1(2*H*)-one, CSD Cancer Center Medicinal Chemistry Core]+/- VIP (1µM) or ACTH(1nM).

After 48 hr incubation the cells were pelleted by centrifugation at 1200rpm for 5 min at 4°C, washed in 1 ml of cold PBS with 1% FBS, washed in 1ml Hank's Balanced Salt Solution (HBSS) (Invitrogen), resuspended in 100 µl of the HBSS, and treated with 1 µl of annexin V/fluorescein isothiocyanate (FITC) (Becton Dickinson Biosciences, San Jose, CA). After 15 min incubation at 37 °C, the cells were treated with 200 µl of HBSS and 10 µl of 30 µg/ml propidium iodide to assess cell viability and apoptosis. Each 300

μl cell sample was then analyzed by flow cytometry using FACScan (Becton Dickinson Biosciences, San Jose, CA).

2.6. Protein Analysis

2.6.1. Protein Purification

100μl of lysis buffer (Novagen Cytobuster protein extraction reagent) was added to each cell pellet and incubated at room temperature for 15 min followed by sonication. The solution was centrifuged at 16,000 rcf for 5 min at 4°C and the supernatant containing protein extracted. Protein concentration was analyzed using the Pierce BCA protein assay kit according to the manufacturer's instructions.

2.6.2 Western Blots

9μl of 4X sample buffer (Invitrogen), 3.6μl 10X reducing agent (Invitrogen), and an appropriate amount of distilled water were added to purified protein samples. Samples were loaded onto pre-cast 4% to 12% gel (Invitrogen) and run for 1 hr 30 min at 200V, 40mA, and 25W. Gels were incubated in 10% methanol transfer buffer for 10 min and protein transferred to nitrocellulose membrane using the iBlot (Invitrogen). Membranes were then incubated in 4% milk (in PBST) for 1 hr and primary antibody [1:1000 dilution in 2% milk] added to membrane and incubated overnight. The following day, the membrane was washed 3x (10 min/wash) with PBST (phosphate buffered saline with tween). Secondary anti-rabbit antibody (1:5000 in 2% milk, AbCam) was added to the

membrane and incubated for 1 hr. The membranes were washed 3X (10 min/wash) and developed with ECL luminescence (GE Healthcare).

2.7. Statistical Analysis

Statistical significance was based on an unpaired Student t test or paired t test where appropriate. Values are expressed as mean ± SEM and $P< 0.05$ was considered statistically significant.

3. Results:

3.1. Quantification of GPCR Expression in normal B-cells, Indolent CLL, and Aggressive CLL

Using an unbiased approach, a TaqMan® GPCR array, we defined GPCR expression in normal B-cells and CLL cells from patients with aggressive and indolent disease. We sought to use this approach to identify GPCRs that are higher or uniquely expressed in CLL among the 384 genes that were analyzed (29 housekeeping genes + 355 non-chemosensory GPCRs). We found that normal B-cells (n=10) express more than 200 GPCRs (74 orphan receptors), indolent CLL cells (n=10) express more than 170 GPCRs (74 orphan GPCRs) and aggressive CLL cells (n=10) express more than 117 GPCRs (51 orphan GPCRs) (Table 3.1). The non-orphan expressed GPCRs in each cell type were classified further and separated according to their specific G protein-coupled signaling pathway (Gs/Gi/Gq, Table 3.2) by using the 2011 BJP (Britsh Journal of Pharmacology) Guide to Receptors and Channels and the IUPHAR Database of Receptors and Ion Channels as references.

We analyzed the expression of specific GPCRs identified by the GPCR array by determining (from the literature) their linkage to heterotrimeric G-proteins and expressed the results as the three highest expressed Gs-linked and Gi-linked GPCRs from normal B-cells and B-cells isolated from patients with indolent and aggressive CLL (Table 3.3/3.4). The numerical values are representative of the cycle threshold (C_t), such that the lower C_t values indicate higher expression, while higher C_t values indicate lower expression of a particular GPCR. The β_2-adrenergic receptor is the highest expressed Gs-

coupled receptor in normal, indolent, and aggressive B-cells (Table 3.3). The CXCR4 chemokine receptor, a Gi-coupled receptor, is one of the highest expressed receptors in normal, indolent, and aggressive B-cells (Table 3.4). Categorizing the receptor expression within each group allowed us to determine the expression of GPCRs that regulate cAMP and highlighted potential therapeutic targets, since increases in cAMP are predicted to promote apoptosis in CLL cell.[10]

Table 3.1.GPCR expression in normal B-cells (n=10), indolent CLL (n=10), and aggressive CLL (n=10).

Cell Type	Total GPCRs	Undetectable GPCRs	Expressed GPCRs	Expressed orphan GPCRs	Expressed non-orphan GPCRs
Normal B-cells	355	155	200	85	115
Indolent CLL	355	185	170	74	96
Aggressive CLL	355	238	117	51	66

Table 3.2. GPCRs in normal B-cells (n=10), indolent CLL (n=10), and aggressive CLL (n=10) classified by their G protein-coupling .

GPCRs- non-orphans			
Cell Type	Gs-coupled	Gi-coupled	non Gs/Gi-coupling
Normal B-cells	24	49	42
Indolent CLL	18	45	33
Aggressive CLL	16	28	22

Table 3.3. Three highest expressed G_s-linked GPCRs in normal B-cells (n=10), indolent CLL (n=10), and aggressive CLL (n=10). ΔC_t values were averaged and normalized with 18S (lower values represent higher expression).

Normal B-cells		Indolent CLL		Aggressive CLL	
GPCR	ΔC_t	GPCR	ΔC_t	GPCR	ΔC_t
β_2-Adrenergic	16.3	β_2-Adrenergic	14.6	β_2-Adrenergic	14.7
Prostanoid EP4	16.3	Prostanoid EP4	14.9	Prostanoid EP4	15.7
Adenosine A2B	17.8	Adenosine A2A	17.4	VIPR1 (Vasoactive intestinal peptide)	16.8

Table 3.4. Three highest expressed G_i-linked GPCRs normal B-cells (n=10), indolent CLL (n=10), and aggressive CLL (n=10). ΔC_t values averaged and normalized with 18S (lower value represents higher expression).

Normal B-cells		Indolent CLL		Aggressive CLL	
GPCR	ΔC_t	GPCR	ΔC_t	GPCR	ΔC_t
CXCR4	11.4	EBI2 (GPR183)	11.0	CXCR4	11.1
EDG1 (Sphingosine-1-phosphate-1)	13.3	CXCR4	11.3	EBI2 (GPR183)	12.5
EBI2 (GPR183)	13.3	CXCR5	13.2	CCR7	13.7

3.2. Differences in GPCR Expression between normal B-cells, Indolent CLL-cells and Aggressive CLL-cells

The difference in expression of GPCRs between clinically disparate disease states or between a normal state and diseased state can provide insight into the possible mechanisms of disease development and progression as well as defining possible targets for therapy. To identify such differences between normal and CLL cells, we made use of the C_t values of specific GPCRs provided by the TaqMan® GPCR array. We sought to identify particular GPCRs that showed the greatest difference in ΔC_t values between each cell type or GPCRs that were expressed in one cell type but not another (that is, uniquely expressed GPCRs). GPCR expression was compared between indolent CLL and normal B-cells, aggressive CLL and normal B-cells, and aggressive vs. indolent CLL cells.

Figures 3.1, 3.2, and 3.3 show Venn diagrams that depict the similarities and differences in GPCR expression between normal B-cells, aggressive CLL and indolent CLL cells separated on the basis of G protein coupling. The number in each circle indicates the GPCRs expressed by an individual cell type while the number in the overlapping area of both circles indicates the GPCRs that are expressed in both cell types. For example, analyzing the expression of Gs-coupled GPCRs between normal B-cells and indolent CLL cells in Figure 3.1, one sees that of the 18 Gs-coupled GPCRs expressed in indolent CLL cells, only two are uniquely expressed in indolent CLL when compared to normal B-cells. However, those results do not take into account differences that may occur in the level of expression among the different types of cells.

The three GPCRs that displayed the largest changes in expression within each group are noted in Table 3.5. Also listed is the difference in ΔC_t values of specific

GPCRs between the two cell types being compared ($\Delta\Delta C_t$). A higher $\Delta\Delta C_t$ indicates a greater difference in expression. The melanocortin 2 receptor (MC2R) was uniquely expressed in both indolent and aggressive CLL but was not expressed in normal B-cells. In order to quantify this unique expression pattern, the ΔC_t value of MC2R expression in normal B-cells is listed as 27.3, the baseline value for receptor expression that is detectable in the TaqMan® GPCR array (Table 3.5). Table 3.5 also shows the fold-changes in GPCR expression between cell types. A higher fold-change represents a greater difference in expression of a particular GPCR. The fold-change was calculated using the following equation: $2^{-(-\Delta\Delta Ct)}$. The $\Delta\Delta C_t$ values used are displayed above the fold change in Table 3.5. These results identified GPCRs that became the focus of further studies, namely VIPR1 and MC2R.

VIPR1 showed substantially greater expression in aggressive CLL cells compared to indolent CLL cells and normal B-cells, while MC2R was uniquely expressed in both stages of CLL. Both these GPCRs are Gs-coupled and therefore should increase cAMP and promote apoptosis, thereby making them attractive targets to study in CLL.

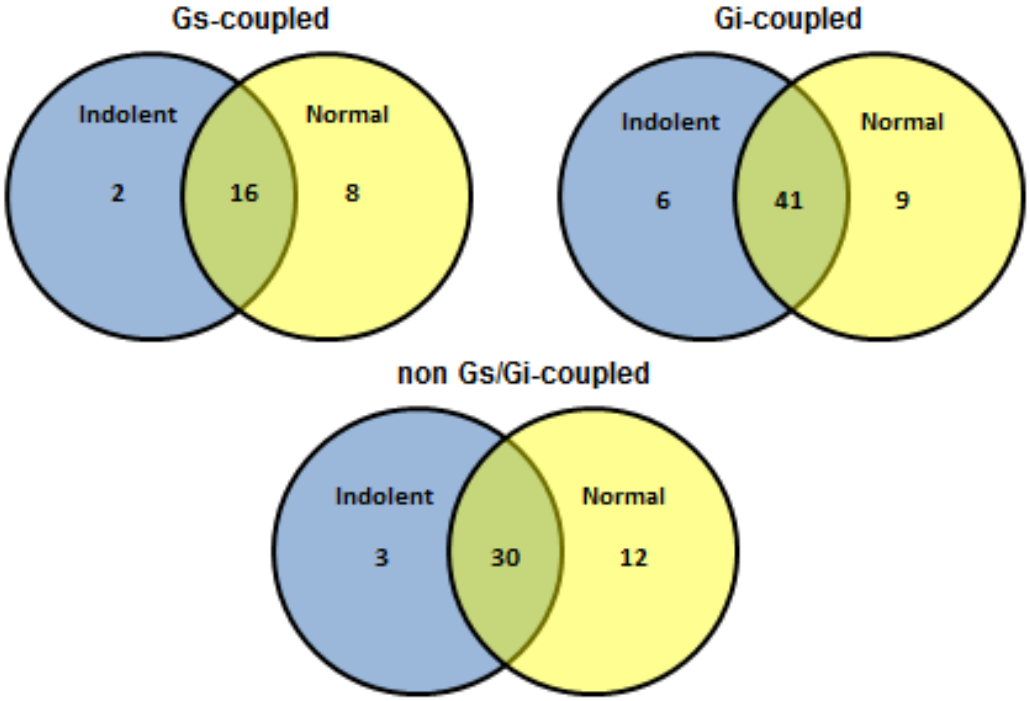

Figure 3.1. Comparison of GPCR expression between normal B-cells and indolent CLL. GPCR expression is categorized by Gα coupling (Gαs, Gαi, or non Gαs/Gαi).

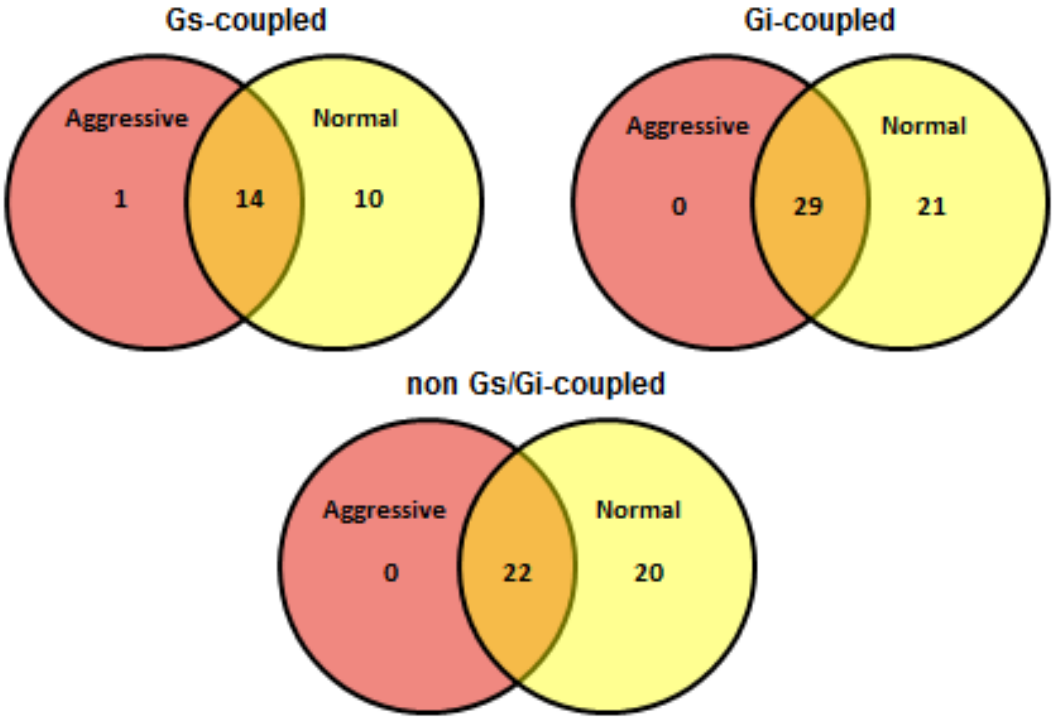

Figure 3.2. Comparison of GPCR expression between normal B-cells and aggressive CLL. GPCR expression is categorized by Gα coupling (Gαs, Gαi, or non Gαs/Gαi).

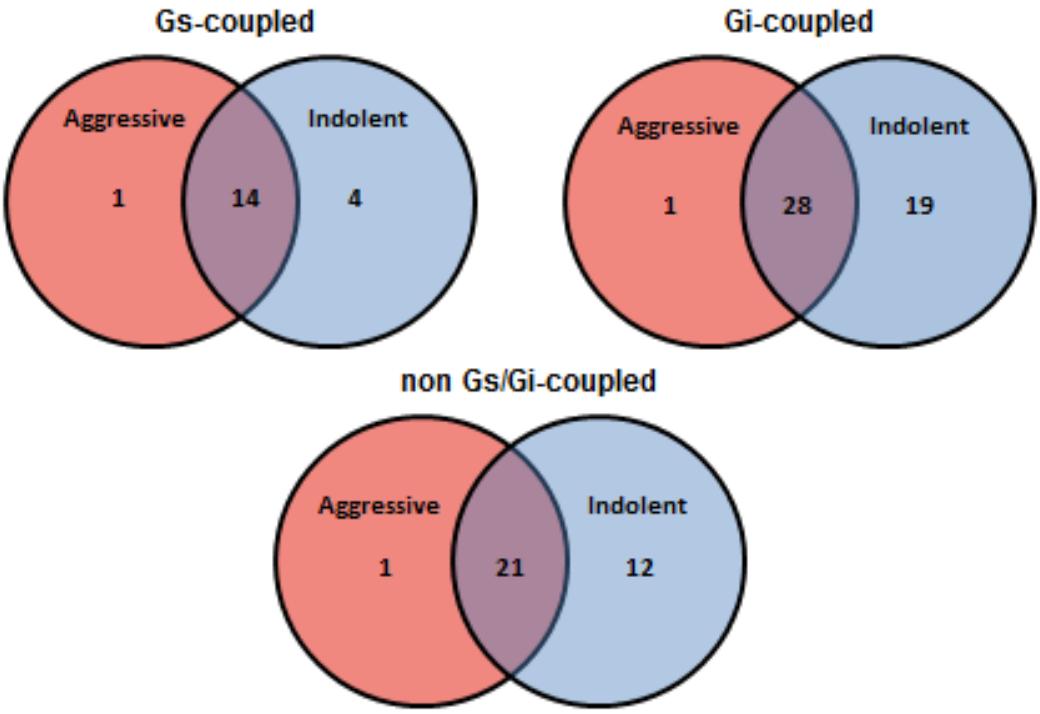

Figure 3.3. Comparison of GPCR expression between indolent CLL and aggressive CLL. GPCR expression is categorized by Gα coupling (Gαs, Gαi, or non Gαs/Gαi).

Table 3.5. GPCR expression between normal B-cells, indolent CLL-cells, and aggressive CLL-cells. The results shown represent GPCRs that have the greatest differences in expression ($\Delta\Delta C_t$ values) between the different groups and the relevant fold changes.

Indolent vs. Normal		Aggressive vs. Normal		Aggressive vs. Indolent	
GPCR	$\Delta\Delta C_t$	GPCR	$\Delta\Delta C_t$	GPCR	$\Delta\Delta C_t$
EDG7 (Gi) (LPA receptor 3)	**8.0** (262 fold-increase)	VIPR1 (G_S) (vasoactive intestinal peptide)	**4.5** (22 fold-increase)	VIPR1 (G_S) (vasoactive intestinal peptide)	**9.5** (706 fold-increase)
AGTRL1 (Gi) (angiotensin receptor-like 1)	**6.8** (114 fold-increase)	MC2R (G_S) (melanocortin 2 receptor)	**4.1** (17 fold-increase)	P2RY14 (Gi) (purinergic receptor P2Y 14)	**3.2** (9 fold-increase)
MC2R (G_S) (melanocortin 2 receptor)	**6.7** (110 fold-increase)	Cannabinoid CB_2 (Gi)	**3.2** (9 fold-increase)	FPRL2(Gi) (formyl peptide receptor-like 2)	**1.6** (3 fold-increase)

4. Validation of GPCR expression using real-time PCR

The results from the GPCR array in normal B-cells, indolent CLL cells, and aggressive CLL cells revealed VIPR1 and MC2R as potential therapeutic targets. Since the initial GPCR array data were pooled samples from 10 patients we sought to confirm these results by performing real-time PCR analysis on samples from individual patients. The PCR protocol and primer design are shown in Table 2.1 and Table 2.2 respectively

4.1. Akin to our results obtained with the pooled samples assessed by the GPCR array individual real-time PCR assays revealed that expression of VIPR1 mRNA is increased in samples from patients with aggressive CLL compared to indolent CLL and normal B-cells

Figure 4.1 demonstrates, by comparing normalized C_t values, that mRNA expression of VIPR1 was significantly increased in samples from patients with aggressive CLL (n=7, 19.5±5.2 fold-increase, $P<0.05$) compared to samples from patients with normal B-cells (n=4) and indolent CLL (n=5). The $\Delta\Delta C_t$ value between normal B-cells and aggressive CLL was 7.4, which represents a 169-fold increase in the samples from patients with aggressive CLL. Similarly, the $\Delta\Delta C_t$ value between samples from patients with indolent CLL and aggressive CLL cells was 7.0, which is a 128-fold increase in aggressive CLL. The mRNA expression of VIPR1 was similar in samples from patients with indolent CLL and normal B-cells. Thus, the results we obtained via real-time PCR confirmed those obtained from the TaqMan® GPCR array.

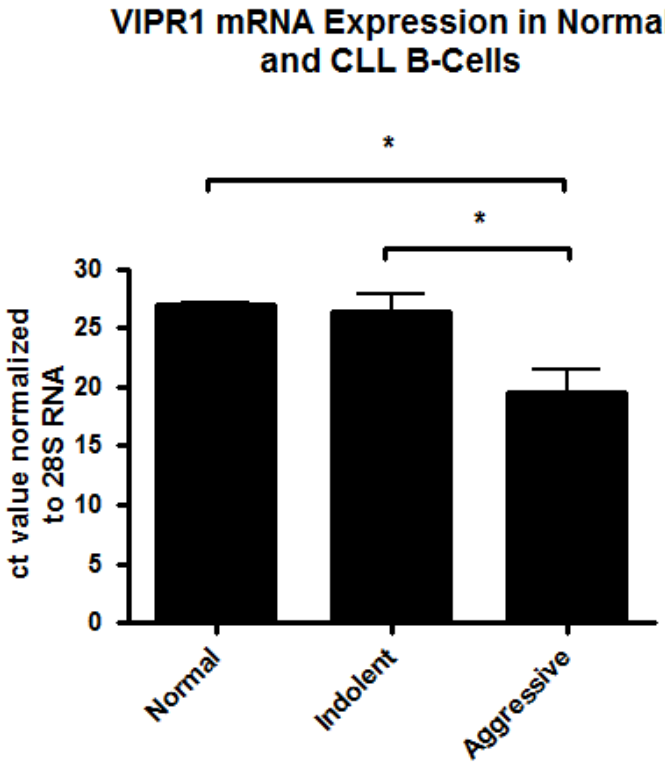

Figure 4.1. VIPR1 mRNA expression in normal B-cells (n= 4), indolent CLL (n= 5), and aggressive CLL (n=7), as determined by real-time PCR. Cycle thresholds (lower value denotes higher expression) normalized to 28S. Significance determined by Student's t-test is depicted by (*) for $P<0.05$.

4.2. Akin to results obtained with the GPCR array, MC2R is significantly increased in CLL-cells compared to normal B-cells

We used real-time PCR to determine the mRNA expression of MC2R in samples from patients with CLL cells (n=9) and normal B-cells (n=9). The PCR protocol and primer design are outlined in Table 2.1 and Table 2.2 respectively. As shown in Figure 4.2, mRNA expression, the C_t value, of MC2R is significantly increased in CLL cells (25.5±3.94, $P<0.001$, n=9). The $\Delta\Delta C_t$ value between the normal B-cells and CLL patient samples is 11.3, which is an approximate 2,521 fold-increase in CLL.

As noted above, results of the TaqMan® GPCR array using pooled samples revealed that MC2R was not expressed in normal B-cells. Similarly, real-time PCR analysis of six out of nine samples from normal subjects had undetectable levels of expression of MC2R. To quantitate the expression of these six samples, they were assigned a C_t value of 40, the baseline value for raw mRNA expression in our real-time PCR assay. Then, the C_t value of 28S, the housekeeping gene, was subtracted from the raw C_t value of 40 to obtain the ΔC_t. This value, approximately 28, was assigned to the non-expressed receptors and used for statistical analysis. The expression of MC2R mRNA, ΔC_t, in the other three samples was 16, 22.6, and. The results thus support the conclusion that MC2R mRNA is uniquely expressed in CLL

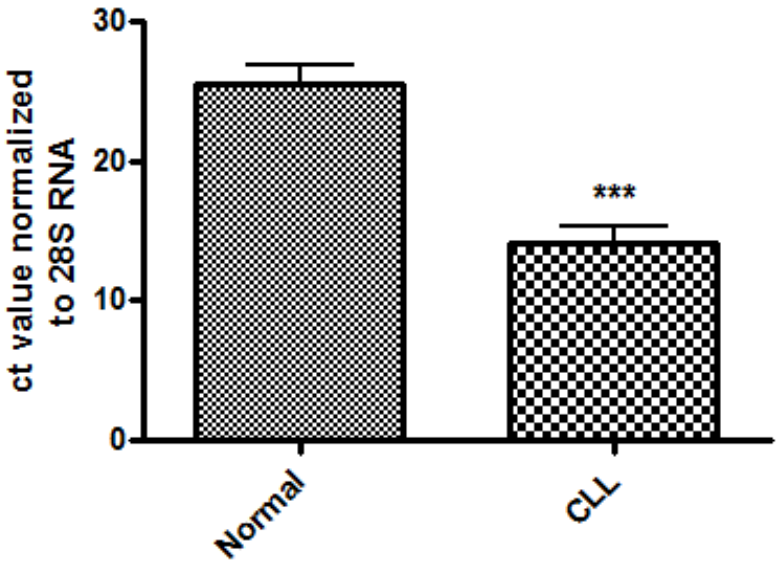

Figure 4.2. MC2R mRNA expression in normal B-cells (n=9) and CLL cells (n=9) as determined by real-time PCR. Cycle thresholds (lower value denotes higher expression) normalized to 28S. Statistical significance was determined by Student's t-test and is depicted by (***) for $P<0.001$ compared to normal.

5. cAMP production

Both VIPR1 and MC2R couple to Gs subunits and therefore simulate cAMP accumulation. Since previous studies have shown that increased intracellular cAMP promotes apoptosis in CLL, we sought to assess if VIPR1 and MC2R agonists, would increase cAMP in normal B-cells and CLL cells.[10] Differences in agonist-stimulated cAMP accumulation between normal B-cells and CLL cells would suggest that agonists directed at the VIPR1 and MC2R receptors might enhance apoptosis and thus be potentially useful as therapies for CLL.

5.1 Vasoactive intestinal peptide but not adrenocorticotropic hormone increased cAMP more than the PDE4/7 inhibitor in CLL cells but not in normal B-cells

Vasoactive intestinal peptide (VIP) was used as the agonist for VIPR1 while adrenocorticotropic hormone (ACTH) served as the agonist for MC2R. The accumulation of cAMP in CLL cells and normal B-cells was measured after treatment with 100 nM IR284 (a PDE4/7 inhibitor, which prevents the breakdown of cAMP) +/- 1 μM VIP or 1nM ACTH. The amount of cAMP produced is highly variable between patients and thus the effect of the GPCR agonist was expressed as a percentage increase above the level observed in cells only treated with IR284. Figure 5.1 shows that treatment of CLL cells with VIP+IR284 significantly increased (106%±30%, $P<0.05$, n=8) the level of cAMP accumulation when compared to IR284 alone. In contrast, treatment of CLL cells with ACTH+IR284 (13.7%±55%, n=9) did not significantly increase cAMP accumulation (Figure 5.1).

Figure 5.2 shows in normal B-cells (n=5), VIP+IR284 (27.8%±30%) or ACTH+IR284 (18.6%±29%) did not significantly increased cAMP levels above those of IR284 alone. Thus in CLL cells but not normal B-cells, VIP but not ACTH increases intracellular cAMP levels.

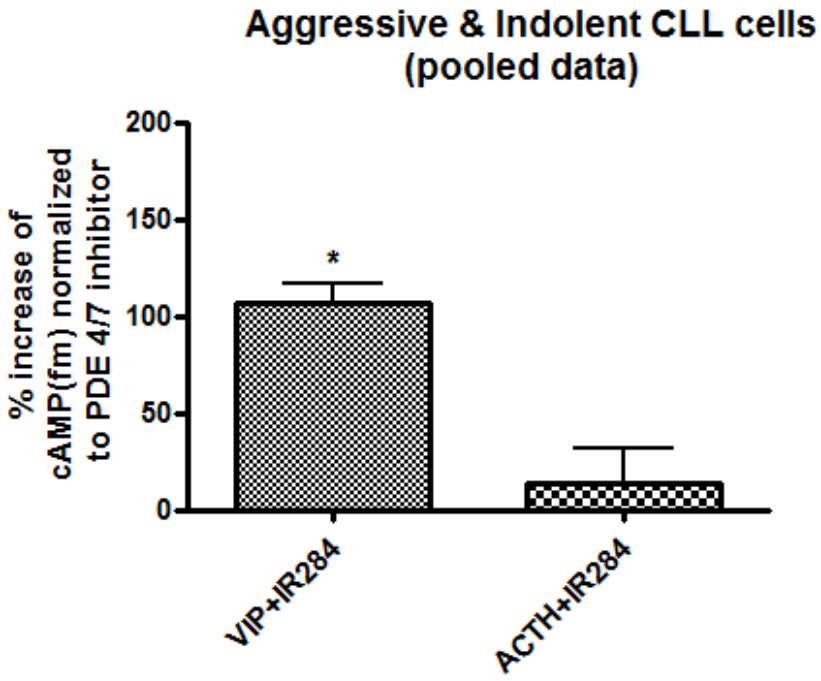

Figure 5.1. cAMP accumulation in CLL cells in response to treatment with 1 μM VIP + 100 nM IR284 (n=8) and 1 nM ACTH + 100 nM IR284 (n=9). The results are expressed relative to cAMP levels observed in cells treated only with 100 nM IR284, a PDE4/7 inhibitor. Statistical significance was determined by paired t test and is shown as (*) $P<0.05$ compared to IR284 alone.

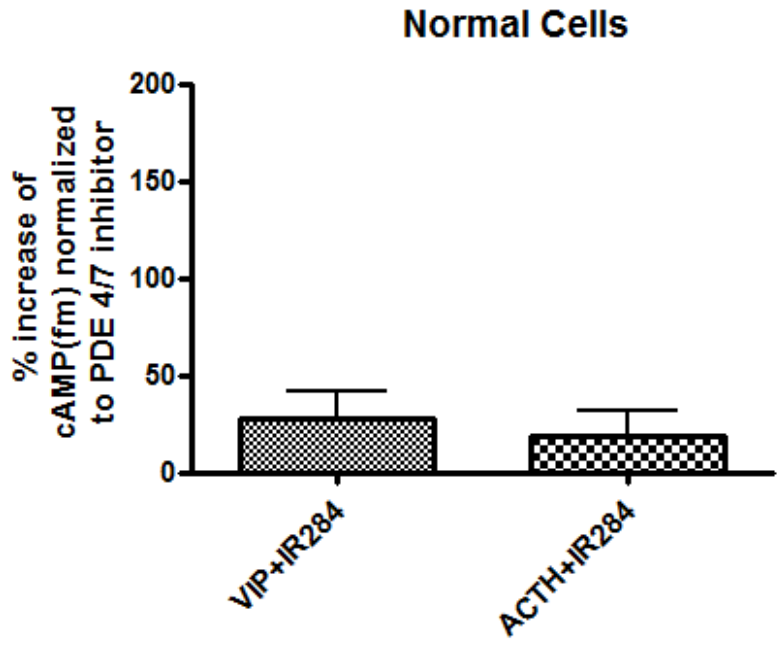

Figure 5.2. cAMP accumulation in normal B-cells (n=5) in response to treatment with 1 μM VIP + 100 nMIR284 and 1 nM ACTH + 100 nMIR284. The results are shown and analyzed as in Figure 5.1.

5.2. VIP increases cAMP accumulation in both indolent and aggressive CLL cells, but ACTH increases cAMP only in indolent CLL.

In Figure 5.1 above, indolent and aggressive CLL cells were grouped together. However, it is important to distinguish between patients who are in the indolent and aggressive stages of CLL, since cellular differences between these stages can provide clues into disease progression and possible therapeutic approaches. With this in mind, the patient samples from Figure 5.1 were separated into the indolent CLL and aggressive CLL categories, so that we could determine if the stage of the disease altered the ability of the agonist to increase intracellular cAMP.

Figure 5.3 shows that VIP+IR284 significantly increases cAMP accumulation in both indolent CLL cells (115%±34%, $P<0.05$, $n=3$) and aggressive CLL cells (101%±30%, $P<0.05$, $n=5$). In contrast, treatment with ACTH+IR284 only significantly increased cAMP in indolent CLL (68.0%±15%, $P<0.05$, $n=4$) but not in aggressive CLL (-29.7%±23%, $n=5$) (Figure 5.4).These data suggest that targeting VIPR1 may be therapeutically beneficial for both stages of CLL, while targeting MC2R would only be predicted to affect those with indolent CLL.

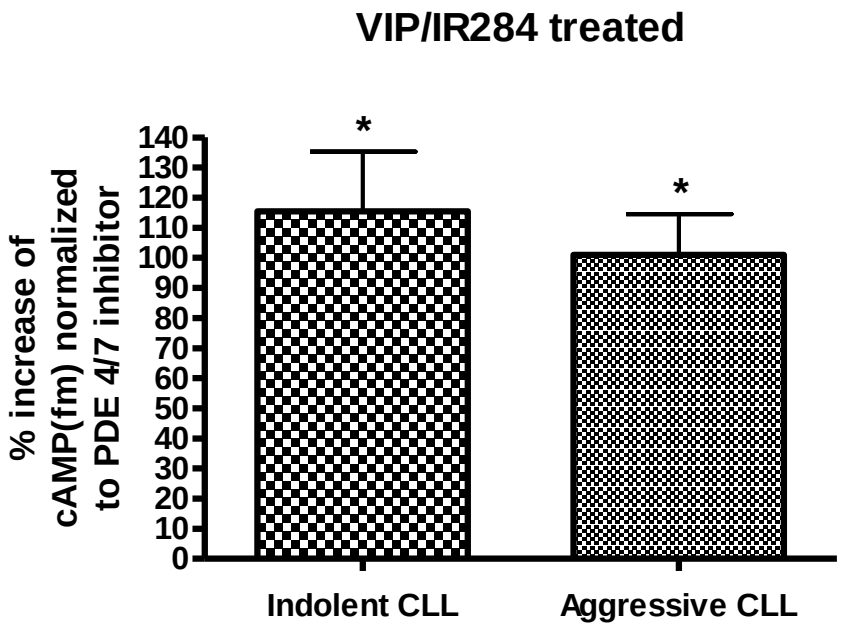

Figure 5.3. cAMP accumulation in indolent CLL cells (n=3) and aggressive CLL cells (n=5) in response to treatment with 1 μM VIP + 100 nM IR284. Statistical significance was determined by paired t test with (*) $P<0.05$ compared to IR284 alone.

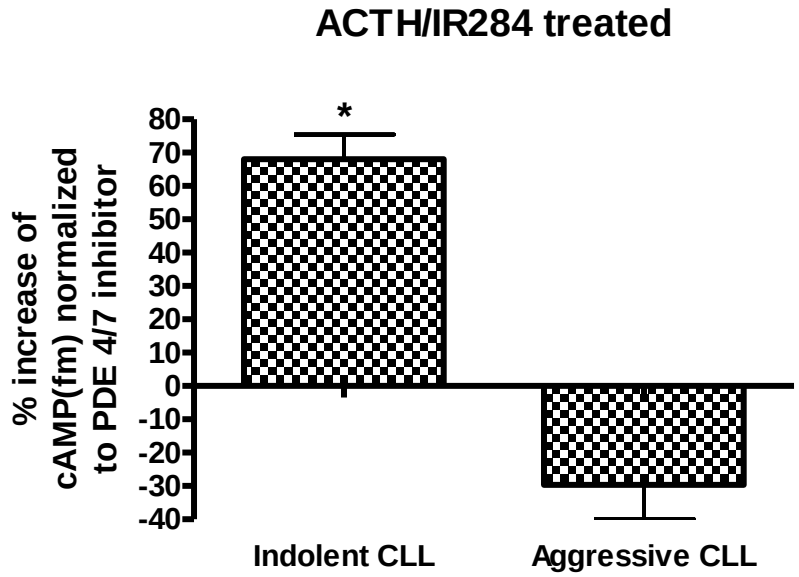

Figure 5.4. cAMP accumulation in indolent CLL cells (n=4) and aggressive CLL cells (n=5) in response to treatment with 1 nM ACTH + 100 nM IR284. Statistical significance was determined by paired t test with (*) $P<0.05$ compared to IR284 alone.

5.3 Correlation of cAMP accumulation in response to ACTH with the mRNA expression of MC2R

To investigate if the increased mRNA expression of MC2R in indolent CLL-cells (compared to aggressive CLL-cells) correlates with the ability of ACTH to increase cAMP accumulation, average cycle threshold values from the TaqMan® GPCR array were plotted against average cAMP accumulation generated in response to ACTH . We found that the increased mRNA expression in indolent-CLL cells was associated with larger amounts of ACTH-stimulated cAMP accumulation, while the lower expression of MC2R mRNA in aggressive-CLL cells was associated with lower levels of ACTH-stimulated cAMP accumulation (Figure 5.5). This relationship suggests that higher levels of mRNA expression yield more functional receptor protein, which stimulates greater intracellular levels of cAMP.

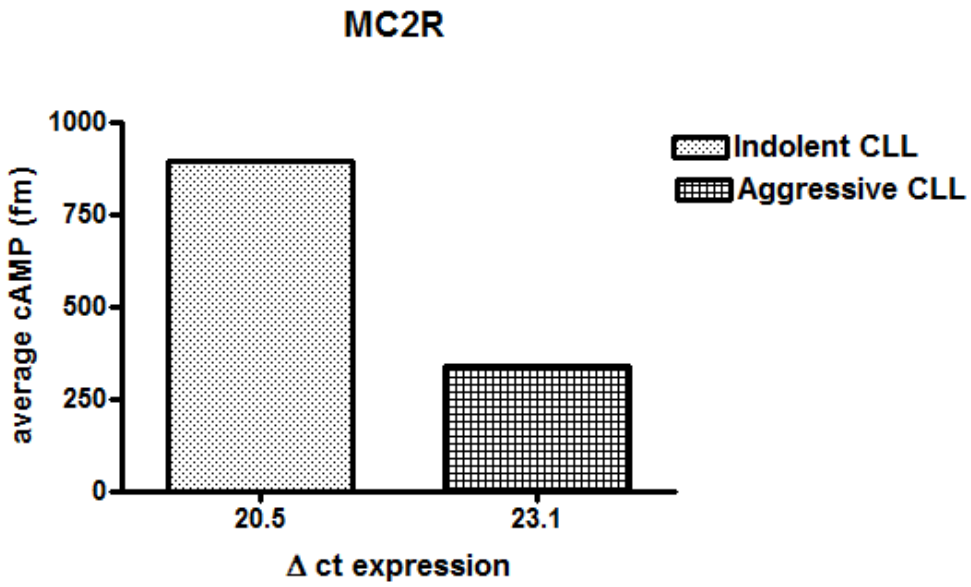

Figure 5.5. Relationship between mRNA expression of MC2R in aggressive CLL cells (n=10) and indolent CLL cells (n=10) with cAMP accumulation in aggressive CLL cells (n=3) and indolent CLL cells (n=3) after treatment with 1 nM ACTH + 100 nM IR284. ΔC_t values were obtained from the GPCR array.

6. Pro-apoptotic effects of VIP and ACTH in CLL cells and normal B-cells

The data shown thus far indicate that agonists of GPCRs expressed in CLL cells increase cAMP accumulation. We next set out to examine if this increase in cAMP could induce apoptotic cell death. As was done in the cAMP assays, we investigated apoptosis in combination with a PDE4/7 inhibitor (and which had previously been shown to increase apoptosis in CLL cells).[12]

6.1 VIP and ACTH induce apoptosis in aggressive CLL cells, but not indolent or normal B-cells

We incubated cells for 48 hr with 100 nM IR284, 1 μM VIP, or 1 nM ACTH each alone or with VIP and ACTH together with IR284.and assayed for apoptosis by FACS using annexin V-FITC and propidium iodide staining. Figure 6.1 shows that treatment of aggressive CLL cells with VIP alone (24.7%±5.9%, $P<0.05$, n=3) and in combination with IR284 (43.0%±11%, $P<0.01$, n=3) increased apoptosis compared to untreated control cells. Treatment of aggressive CLL cells with ACTH+IR284 (46.0%±11%, $P<0.05$, n=3) but not ACTH alone (10.7%±7.6%, n=3) significantly increased cell death relative to untreated cells (Figure 6.1). By contrast, incubation of indolent CLL cells with VIP (0.67%±1.2%, n=3), VIP+IR284 (11.7%±7.56%, n=3), ACTH (2.30%±1.2%, n=3), or ACTH+IR284 (12.0%±7.0%, n=3) did not significantly increase apoptosis (Figure 6.2). Similarly, as shown in Figure 6.3, neither VIP (7.33%±1.5%, n=3), VIP+IR284 (5.33%±4.7%, n=3), ACTH (12.3%±8.4%, n=3) nor ACTH+IR284 (9.00%±0.00%, n=3) killed normal B-cells.

These data thus show that VIP and ACTH are only pro-apoptotic in aggressive CLL cells and not in either indolent CLL-cells or in normal B cells. As in previous studies, the apoptosis observed with VIP and ACTH treatment of aggressive CLL cells was enhanced by PDE inhibition (i.e, use of IR284).[12] Our results suggest that normal B-cells and indolent CLL cells share certain similar properties as neither cell type underwent apoptosis when treated with VIP or ACTH. This observation also mimics the clinical appearance of many patients with the indolent form of the disease, who commonly do not display symptoms or characteristics of CLL but instead appear to be healthy.

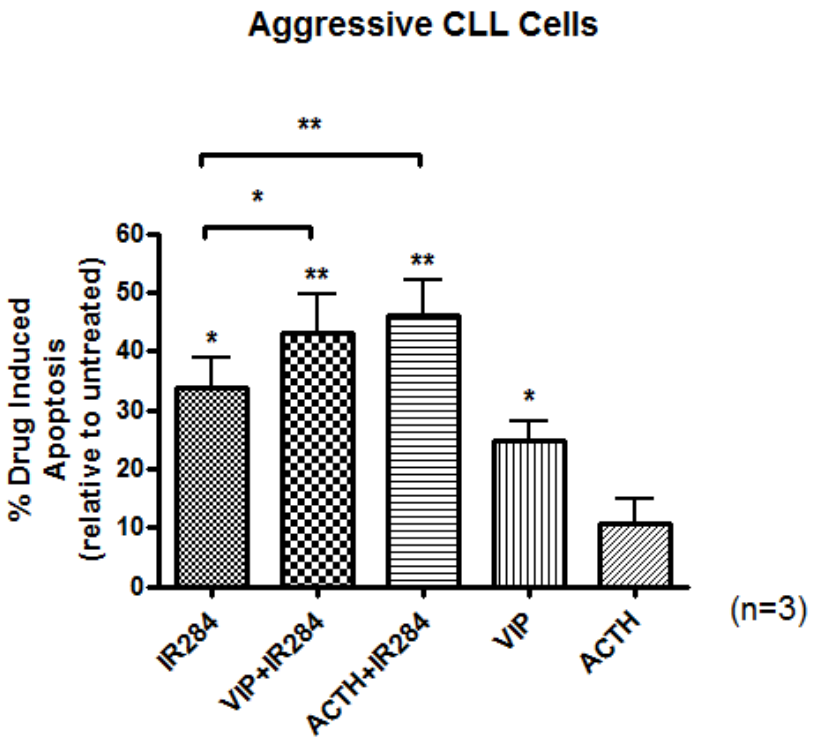

Figure 6.1. Apoptosis of aggressive CLL cells (n=3) in response to 100 nM IR284, 1 μM VIP+100nM IR284, 1 nM ACTH+100nM IR-284, 1 μM VIP, and 1 nM ACTH. Statistical significance was determined by Student's t-test and is depicted by (*) for $P<0.05$ and (**) for $P<0.01$ for samples that were compared to either untreated cells or cells treated with IR284 alone.

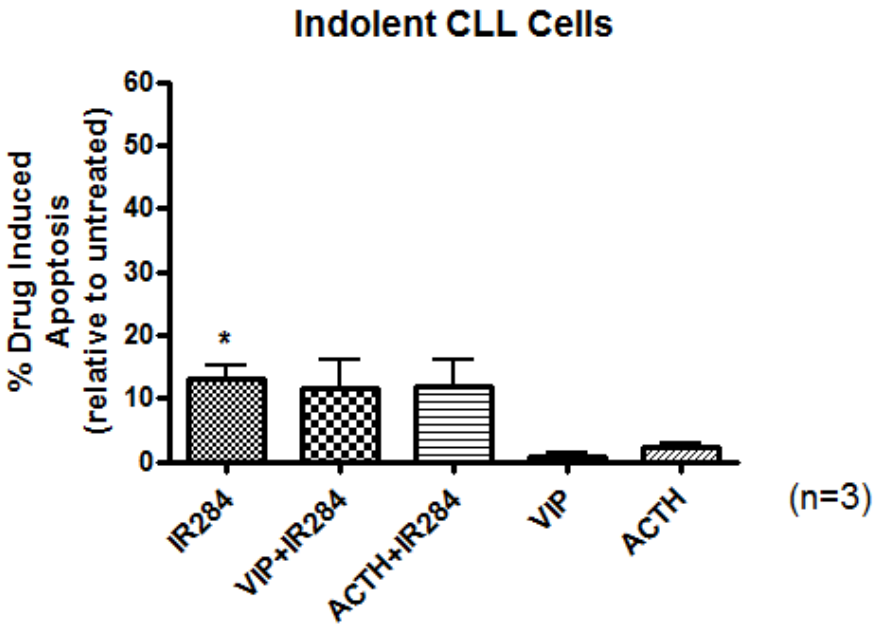

Figure 6.2. Apoptosis of indolent CLL cells (n=3) in response to 100 nM IR284, 1 μM VIP+100 nM IR284, 1 nM ACTH+100 nM IR-284, 1 μM VIP, and 1 nM ACTH. Statistical significance was determined by Student's t-test and is depicted by (*) for $P<0.05$ for samples compared to untreated cells.

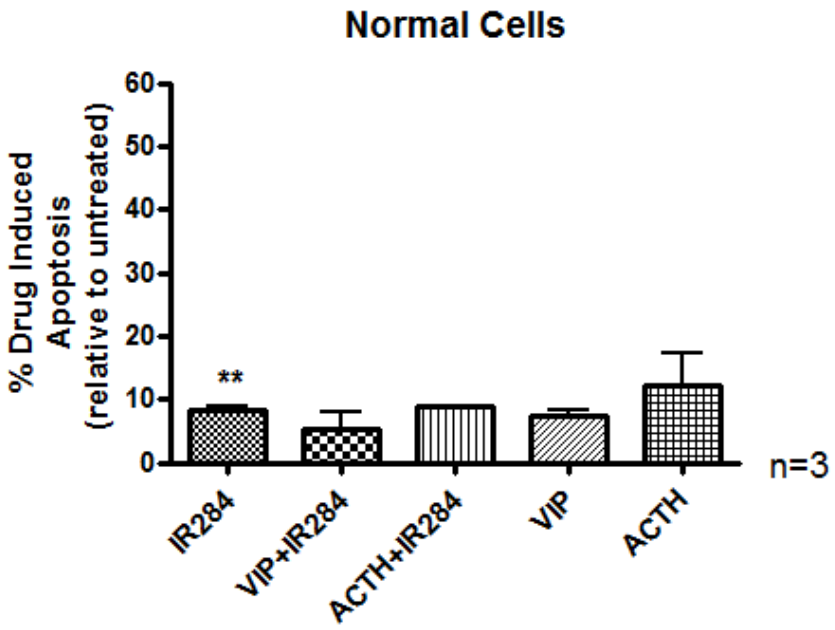

Figure 6.3. Apoptosis of normal B-cells (n=3) in response to 100 nM IR284, 1 μM VIP+100 nM IR284, 1 nM ACTH+100 nM IR-284, 1 μM VIP, and 1 nM ACTH. Statistical significance determined by Student's t-test is depicted by (**) for $P<0.01$ for samples compared to untreated cells.

6.2. VIP increases expression of pro-apoptotic proteins BIM and BID

To further investigate the pro-apoptotic effect of VIP, CLL cells were treated for 48 h with 1 μM VIP. Western blots were run to assess the expression levels of BID and BIM, two pro-apoptotic Bcl-family proteins, after VIP treatment and were protein expression was normalized to β-tubulin. Densitometric analysis (Figures 6.4 and 6.5) showed that the levels of BIM and BID increased in CLL cells after treatment with VIP (n=2), but these increases were not statistically significant. Obtaining statistically significant results will require the analysis of samples from additional patients.

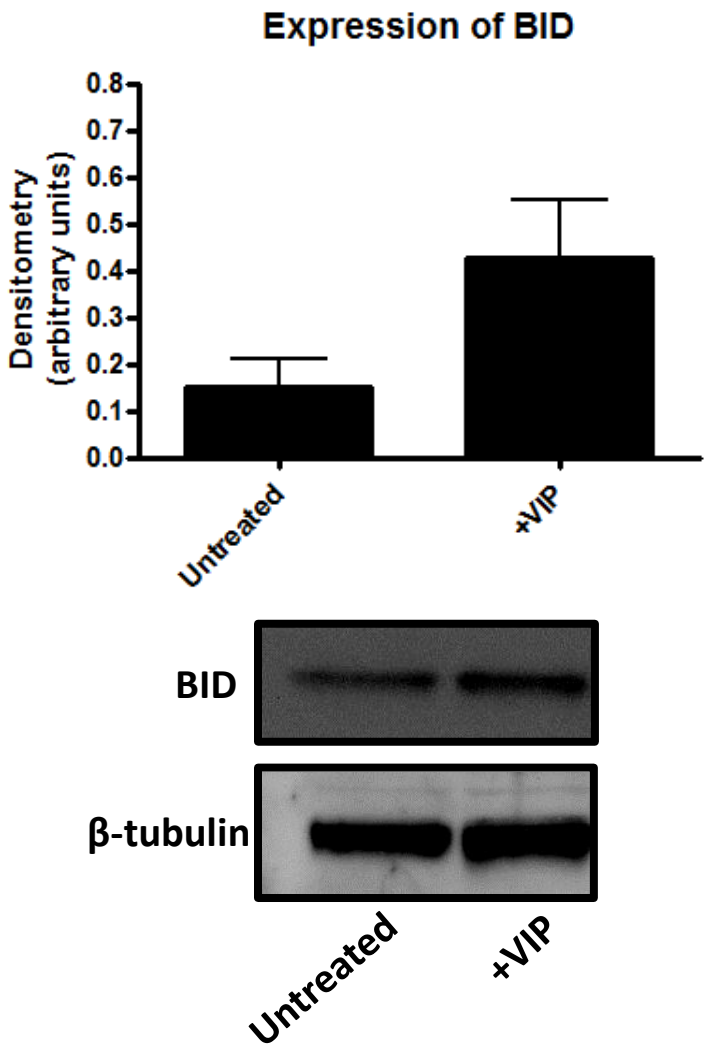

Figure 6.4.BID expression following treatment of CLL cells (n=2) with 1μM VIP. Protein expression normalized to the expression of β-tubulin, a housekeeping gene. The increase in BID protein after VIP treatment is not significant (P>0.05), according to Student's t-test.

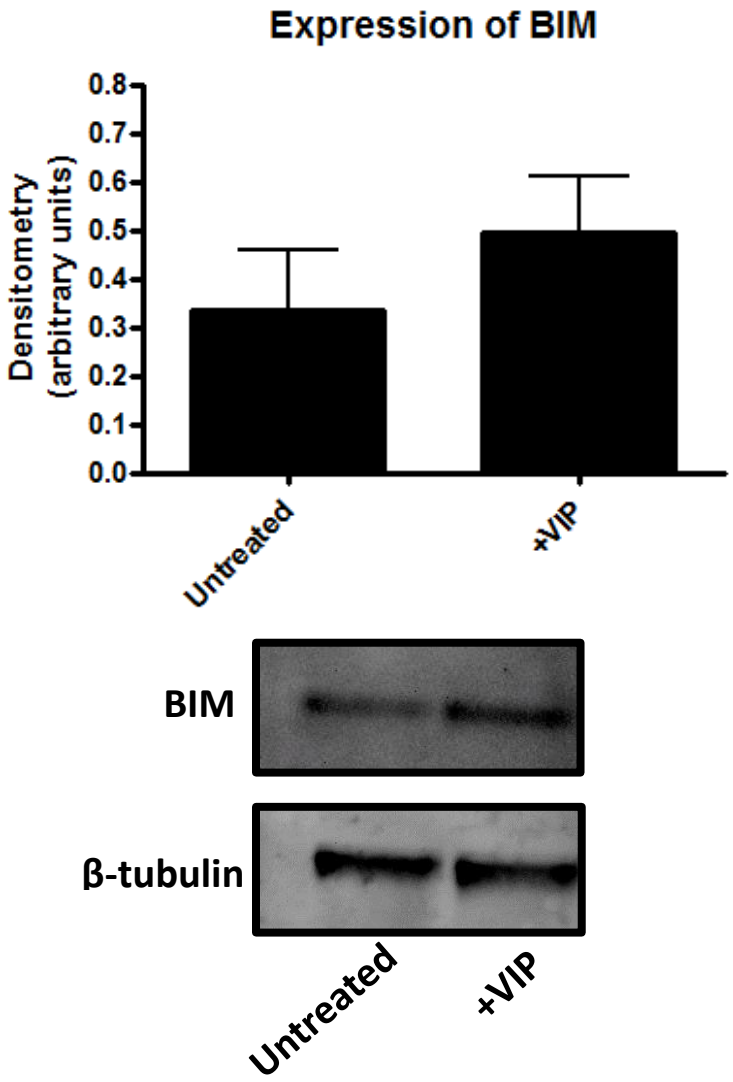

Figure 6.5.BIM protein expression following treatment of CLL cells (n=2) with 1μM VIP. Protein expression of BIM was normalized to that of β-tubulin. The increase in BIM protein expression after VIP treatment is not significant (P>0.05), according to Student's t-test.

7. Orphan GPCRs in CLL cells

The results shown thus far indicate that the GPCR microarray is an effective tool for identifying receptors that are differentially expressed between CLL and normal B-cells and between the two different stages of CLL. The data above focused on non-orphan GPCRs that display a unique expression patterns, since the role for receptors with known function and ligands are easier to determine. A fundamental, but unanswered, question is if orphan GPCRs display selective or unique expression patterns in CLL cells. We decided to begin answering this question by using the results of the GPCR array to determine the relative expression levels of mRNA for orphan receptors in normal B-cells, indolent and aggressive CLL cells.

7.1. Five orphan GPCRs are uniquely expressed in CLL cells,

Analysis of the data from the GPCR microarray revealed four GPCRs (GPR113, GPR120, GPR4, TAAR5) that are selectively expressed in CLL (both indolent and aggressive CLL), and one GPCR (GPR 63) that is expressed only in aggressive CLL. Table 7.1 shows that unlike the expression of GPR63, the four orphan GPCRs expressed in both indolent and aggressive cells show little difference in expression between the two stages of the disease. . These data suggest that GPR63 might contribute to the aggressive phenotype and is a potential therapeutic target for patients with the aggressive CLL.

7.2. As found with the GPCR array, independent real-time PCR analysis reveals that GPR113 mRNA is more highly expressed in aggressive CLL than in normal B-cells

We examined the mRNA expression of GPR113 in aggressive CLL (n=4), indolent CLL (n=3), and normal B-cell (n=4) using real-time PCR. The PCR protocol and primer design are outlined in Table 2.1 and 2.2 respectively. Figure 7.1 shows that mRNA expression of GPR113 is significantly increased in aggressive CLL when compared normal B-cells (23.7 ± 1.2, $P<0.01$, n=4). However, we observed no significant difference in the mRNA expression of GPR113 between indolent CLL-cells and normal B-cells. These data show that the results for the aggressive (not indolent) CLL patients obtained by real-time PCR analysis are consistent with those obtained from the GPCR array for GPR113.

Table 7.1. Expression of orphan GPCRs in aggressive CLL-cells, indolent CLL-cells, and normal B-cells, as determined by use of a GPCR array. ΔC_t values averaged and normalized with the expression of 18S RNA (lower value represents higher expression).

Orphan GPCRs	Expression in Aggressive CLL (ΔC_t)	Expression in Indolent CLL (ΔC_t)	Expression in Normal B-cells (ΔC_t)
GPR 113	21.9	22.3	no expression
GPR 120	22.1	21.3	no expression
GPR 4	21.5	22.3	no expression
TAAR 5	23.1	22.3	no expression
GPR 63	20.1	no expression	no expression

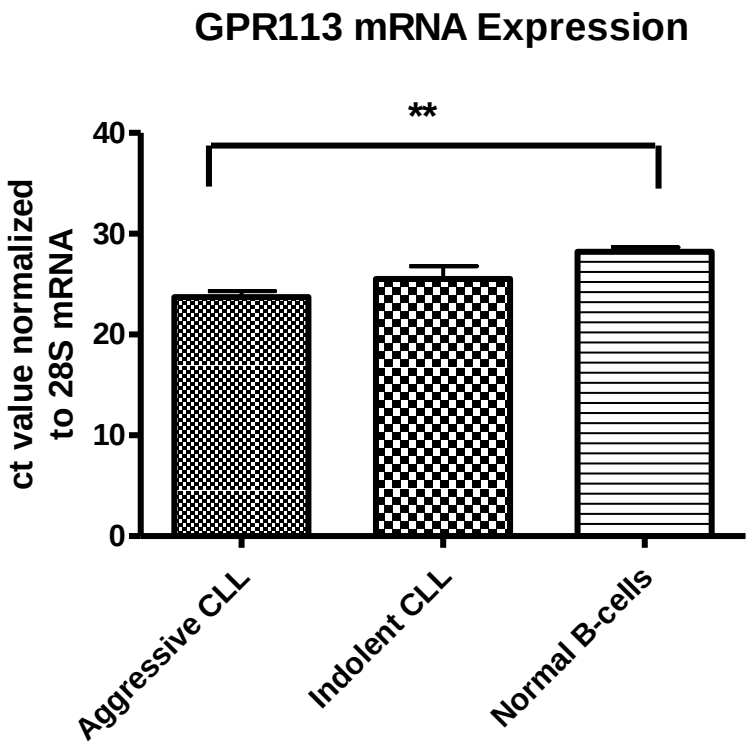

Figure 7.1. GPR113 mRNA expression in normal B-cells (n=4), indolent CLL cells (n=3), and aggressive CLL cells (n=4) as determined by real-time PCR of individual samples. Cycle thresholds (lower value denotes higher expression) normalized to 28S RNA. Statistical significance, determined by Student's t-test, is depicted by (**) for $P<0.01$ in aggressive CLL cells compared to normal B cells.

8. Discussion

Chronic lymphocytic leukemia (CLL) is most commonly characterized by the accumulation of B-cells in the blood and lymphoid tissues.[15] Signaling through the many receptors present on B-cells promotes the cell survival and proliferation of these leukemic cells.[16] However, the mechanism that causes CLL cells to exhibit a decreased rate of apoptotic cell death, has yet to be fully explained.[17] Recently, the transcription factor, E2A, which regulates B-cell survival and proliferation, has been shown to play a key role in CLL persistence.[18] E2A mRNA and protein levels were elevated in CLL cells compared to normal B-cells and E2A silencing significantly increased spontaneous apoptosis.[18] Previous work has also emphasized the importance of B-cell lymphoma-2 (BCL-2) proteins in blocking apoptosis and has encouraged the development of small molecule BCL-2 inhibitors.[17] Despite these recent findings, further characterization of B-cell receptor signaling pathways is needed to better understand the mechanisms of this disease and to develop the foundation for the design of new, effective therapies.

GPCRs, the largest family of membrane receptors, comprising of ~3% of human genes transduce extracellular signals into intracellular effector pathways.[36-38] Ligand binding to GPCRs leads to the activation/inactivation of signaling pathways that control [Ca2+], the production of second messengers, and gene expression. The accessibility of GPCRs on the plasma membrane, their tissue-selective distribution and role in regulating physiological functions make them excellent pharmacological targets: >30% of prescribed drugs act via GPCRs.[38] Despite the large number of physiological responses that are regulated by GPCRs and the wide number of approved drugs that target GPCRs,

only a small number are currently targeted in cancer and none are currently used as targets in the treatment of CLL.

Hypothesizing that GPCRs might regulate CLL cells and be possible therapeutic targets in this disease, we used an unbiased approach, a GPCR RT-PCR array (Applied Biosystems), to define the most abundantly and uniquely expressed GPCRs in CLL. We validated the data from the GPCR-array by designing our own primers and confirming expression by RT-PCR, investigating protein expression, and determining functional responses. The array served as a tool, which provided insight into the differing patterns of expression of GPCRs and identified receptors on which to focus our functional studies.

We were particularly interested in GPCRs that regulate cellular cAMP levels and whose expression is increased in patients with aggressive CLL. Since patients with aggressive CLL need treatment within a short period of time after diagnosis compared to those with the more stable, indolent form, the discovery of a biomarker or therapy for such patients would be an important breakthrough.[19]

Our use of the GPCR arrays revealed that expression of VIPR1 is significantly increased in aggressive CLL compared to both indolent CLL and normal B-cells (Table 3.5). The expression of VIPR1 was up-regulated 706-fold in aggressive CLL cells compared to indolent CLL cells and 22-fold compared to normal B-cells (Table 3.6). VIPR1 is a Gs-coupled GPCR, for vasoactive intestinal peptide (VIP), a neuropeptide that is involved in smooth muscle relaxation, exocrine and endocrine secretion.[46] Limited, previous data has shown that VIPR1 is highly expressed in CLL cells from patients who had a poor prognosis, presumably the aggressive form of CLL, although its functional significance was not determined.[20]

GPCRs that are unique to CLL and show little to no expression in normal B cells could provide insight into the cellular changes that occur in B-cells and that give rise to the characteristics of this disease. Comparing GPCR expression profiles of all three patient subtypes, we found that MC2R was uniquely expressed in CLL cells. MC2R displayed a 110-fold increase in expression in indolent CLL cells compared to normal cells and displayed a 6-fold increase expression in aggressive CLL cells (Table 3.6). MCR2 is a receptor for ACTH, which is expressed in thymocytes, is Gs-coupled and increases cAMP.[47,48]

A number of previous studies have used arrays to evaluate gene expression patterns in B-cell CLL patients.[21] One study used cDNA from 37 CLL patients to uncover a gene that allowed for CLL patients to be separated on the basis of immunoglobulin (Ig) mutation status.[21] These data showed that ZAP-70 was helpful to discriminate between cases of unmutated versus mutated Ig V gene type B-CLL and also later found that gene expression patterns differ between normal B-cells and CLL cells.[21] Furthermore, DNA microarrays led to the discovery that mutations in the variable region of the B-cell receptor heavy chain could be an indicator of CLL progression.[22] Thus, use of gene arrays can uncover unique biomarkers that can aid in the identification of events leading to disease progression and eventually, perhaps to therapeutic targets. Other data from our lab has indicated that large microarrays, such as ones marketed by Affymetrix, are not optimal for detecting the expression of GPCRs we used a specific TaqMan® GPCR array.

Although micorarrays are one of the leading methods to identify differentially expressed genes, their reliability in detecting differences in transcription hinges on

several factors.[23] Some of those factors include RNA extraction, probe labeling, hybridization conditions, as well as array production.[23] Due to these intrinsic limitations in reliability, the genes identified as differentially expressed on the gene array need to be validated with another method.[23] Therefore, results obtained from our GPCR array were validated with real-time PCR to ensure that the receptors that we identified and that we sought to further study did indeed show the apparent differences in expression between the various types of patients.[24] Real-time PCR was chosen as the supplementary method to measure gene expression because it is quantitative, uses less RNA, and provides rapid results.[23-24]

Using real-time PCR we validated the changes in VIPR1 and MC2R that we identified by using the GPCR array. Analysis of the real-time PCR data showed that, as shown by the results of the array data, VIPR1 was significantly up-regulated in aggressive CLL cells compared to indolent CLL-cells and normal B-cells. Although the general trends remained the same, the fold-changes between the different groups varied when comparing the GPCR array and the independent real-time PCR data on individual samples. These differences in expression between the cell types can most likely be attributed to the different patient samples used for each method as the patterns of gene expression in CLL patients are highly variable.[25] The GPCR array also detected that CLL cells express 17-110-fold more MC2R mRNA than do normal B-cells; real-time PCR revealed that CLL cells exhibit an even greater increase in MC2R expression compared to normal cells (2,500-fold) (Table 3.6).

After we validated the increased expression of VIPR1 and MC2R in CLL cells with real-time PCR, we conducted experiments to assess their function in CLL cells.

Since both VIPR1 and MC2R are Gs-coupled GPCRs, we hypothesized they would be good targets to induce apoptosis in CLL-cells by raising cAMP.[10] cAMP kills CLL cells but not normal B cells. We treated aggressive CLL cells, indolent CLL cells, and normal B-cells with the GPCR agonists together with IR248, a dual PDE4/7 inhibitor. Previous studies have explored the use of these cyclic nucleotide PDE inhibitors as a way of manipulating the immune response.[26] In addition, blocking the hydrolysis of cAMP with PDE inhibitors induces apoptosis of CLL cells.[26,27] We thus treated cells with a PDE4/7 dual inhibitor in addition to the GPCR specific agonist in order to augment the effects of these agonists thus making it easier to see increases in intracellular cAMP levels.[27]

Pooled data from indolent and aggressive CLL cells reveals that treatment with VIP+IR284 significantly increases levels of intracellular cAMP, while ACTH does not (Figure 5.1). In normal cells (Figure 5.2), neither agonist has an effect on cAMP accumulation. This result was expected because the expression levels of VIPR1 and MC2R are fairly low in normal B-cells. However, in the CLL cells, treatment with VIP, the VIPR1 agonist, significantly increased the levels of cAMP, which correlates with the high expression of the VIPR1 receptor (Figure 5.1). In contrast, ACTH, the MC2R agonist, did not increase cAMP accumulation even though there was a significant difference in the mRNA expression of MCR2 between normal B-cells and CLL cells. Figure 4.2).

To try to explain this unexpected result, we separated the pooled CLL samples based on the disease state to see whether agonist-induced cAMP accumulation differed in cells from patients who had the indolent or aggressive form of the disease. This was also important as it would provide some insight into differences between the two stages of the

disease and thus, might provide information with respect to possible therapeutic approaches.

We found that VIP increased cAMP in both aggressive CLL and indolent CLL cells (Figure 5.3).In contrast, ACTH only induced cAMP accumulation in indolent CLL cells, not in aggressive CLL cells (Figure 5.4). The indolent CLL cells showed increased mRNA expression of MC2R compared to aggressive CLL cells, thus these data shows that GPCR expression correlates with function (e.g., cAMP formation) and provide a proof-of-principle that our approach can identify physiologically relevant GPCRs.

To test if the VIP and ACTH agonist-induced increases in intracellular cAMP caused apoptosis, we carried out fluorescence activated cell sorting (FACS) assays to assess apoptotic cell death, based on the cell surface expression of annexin-V, which binds to phosphatidylserine marker displayed on cells undergoing apoptotic cell death.[28]

Figure 6.1 shows that VIP induced more apoptosis than observed in the untreated sample or in cells treated with the PDE 4/7 inhibitor alone. Similarly, ACTH induced apoptosis above the untreated control when used in combination with the PDE 4/7 inhibitor. Aggressive CLL cells treated with ACTH and IR284 displayed a level of cell death greater than that induced by IR284 alone (Figure 6.1). These data suggest that VIP and ACTH can be agonists that kill aggressive CLL cells. Importantly, these agonists did not increase apoptosis in indolent CLL cells or normal B-cells (Figure 6.2 and 6.3), thus suggesting that VIP and ACTH (or compounds that act on the same receptors) might be used to selectively kill aggressive CLL-cells while sparing normal B-cells.

One major limitation of measuring drug-induced apoptosis in CLL cells is the high level of basal cell death, which can be up to 70-80%, thus making it difficult to

observe additive effects relative to untreated samples when GPCR agonists and PDE 4/7 inhibitor that raise cAMP are added. CLL cells are more prone to apoptosis when they are cultured ex-vivo because of the lack of pro-survival signals that are normally present in the in-vivo environment.[29] Because of these constraints, "nurse-like cells" have been used in ex vivo studies in order to protect CLL cells from spontaneous and drug-induced apoptosis.[30] Future experiments aim to determine if VIP and ACTH induce apoptosis of CLL cells in the presence of nurse-like cells as such data would be useful for determining the therapeutic utility of the agonists.

Analyzing the expression of pro-apoptotic proteins also provides evidence of drug-induced apoptosis.[31] We performed western blots to test for protein expression of pro-apoptotic proteins BIM and BID. VIP-treated CLL samples displayed increased expression of BIM and BID although densitometry did not show this to be significant: studies with additional patient samples are needed to obtain more definitive results (Figure 6.4, 6.5). Future experiments are also needed to further dissect the pathway that mediates GPCR/cAMP-induced apoptosis. The actions of cAMP are largely mediated by protein kinase A (PKA) and exchange protein activated by cAMP (Epac). We have previously shown that PDE4/7 inhibitors promote apoptosis of CLL cells in a cAMP/PKA/survivin-dependent manner.[10] By contrast, increased expression of Epac-1 contributes to anti-apoptosis in CLL.[50] GPCRs that activate PKA but not Epac would be predicted to be particularly useful targets for CLL.

Together, the data in this thesis demonstrate that GPCRs discovered by an unbiased array of all non-chemosensory receptors can identify novel targets for aggressive CLL, the stage of the disease most in need of new therapies. Future

experiments are needed to determine if VIPR1 and MC2R agonists kill CLL cells in vivo and if targeting these receptors is a promising approach for the treatment of aggressive CLL.

In addition to identifying GPCRs with known G protein coupling, we also investigated the expression of orphan GPCRs as potential biomarkers or therapeutic targets for CLL. Since GPCRs are cell surface targets, perhaps antibodies could be developed to them as a novel approach for the treatment for CLL. Previous studies have used flow cytometry to identify cell surface molecules that are expressed only in circulating B lymphoma cells not on normal B-cells.[32] Subsequent studies have validated these discoveries by silencing those molecules and inducing apoptosis of CLL cells.[33]. Figure 7.1 shows that the GPCR array revealed four orphan receptors (GPR113, GPR120, GPR4, TAAR5) that are expressed in aggressive and indolent CLL, but not in normal B-cells. Of interest, GPR63 was only expressed in aggressive CLL and not indolent CLL cells or normal B-cells, thus implying that this receptor may be a biomarker or therapeutic target for aggressive CLL (Figure 7.1). Future experiments are needed to validate the array data regarding GPR63 and to use real-time PCR, immunoblotting and functional assays together with antibodies generated against this receptor. GPR63, which has been shown to be activated by lipid ligands, has potential as a biomarker for detection of aggressive CLL even independent of its utility as a therapeutic target.[51]

In regards to how these GPCRs can be targeted for therapeutic use, one of the recent methods has been the use of peptide-based therapeutics which would target cancer cells specifically and directly. Since peptides, in addition to their cleaved products, are often non-toxic, they are also regarded as very safe drugs.[34] There has been significant

progress made in the past few years to overcome major disadvantages of using this type of therapy. Problems with peptide design such as short half-life and rapid proteolytic cleavage were big obstacles to developing peptides aimed at targeting receptors on cancer cells.[34] Fortunately, advances in peptide development and innovative uptake strategies have made peptide-based therapies a viable option for treatment.[34] Another treatment strategy is therapeutic antibodies directed at specific GPCRs. Although antibodies are more expensive to develop than small molecules, i.e. peptides, they tend to have a longer duration of action than most peptides.[35] One study mentions that antibody therapeutics could be developed against approximately 80 GPCRs, some of which would require agonistic antibodies, with one of the major areas of opportunity being cancer.[35] Antibodies directed towards GPCRs can play a therapeutic role not only by affecting signaling pathways involved in proliferation and apoptosis, but also by indirectly allowing for the peptides to act as carriers for targeted toxin therapy.[35]

In conclusion, results in this thesis show that VIPR1 and MC2R may serve as important biomarkers to track disease progression in CLL and also may be targets for future therapies. Not only did VIPR1 display increased expression in aggressive CLL cells, but in addition, treatment of CLL cells with VIP in combination with IR-284 increased intracellular levels of cAMP and induced apoptosis in aggressive CLL cells. On the other hand, even though MC2R was expressed in indolent and aggressive CLL, it only raised cAMP levels in indolent CLL cells after treatment with ACTH in combination with IR284. Similar to VIPR1, treating the cells with ACTH and IR-284 only induced apoptosis in aggressive CLL. Together, these data show that previously unrecognized GPCRs, including orphans, are expressed and functional in CLL-cells and

could be novel targets and/or biomarkers for the disease. Also, targeting the cAMP signaling pathway via GPCR specific agonists can induce the apoptosis of cancer cells, in particular CLL cells.

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