IMPROVING GLUCOCORTICOID THERAPY IN
CHRONIC LYMPHOCYTIC LEUKEMIA

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Medical Biophysics
University of Toronto

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Improving glucocorticoid therapy in Chronic Lymphocytic Leukemia

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Master of Science
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2013

Abstract

Glucocorticoids (GCs) are commonly used in the clinic as a treatment for Chronic Lymphocytic Leukemia (CLL). The exact mechanism of GC action remains unclear and patients eventually develop resistance to this group of agents. Our findings show that GC-cytotoxicity in circulating CLL cells is caused by bioenergetic restriction resulting from the down-regulation of a key glycolytic enzyme, pyruvate kinase, muscle isozyme 2 (PKM2). Conversely, GCs were shown to promote fatty acid oxidation instead by up-regulating the expression of peroxisome proliferator activated receptor α (PPARα). These findings establish PPARα and fatty acid oxidation as novel mediators of GC resistance in CLL. Our findings also demonstrate that GCs enhance the cytotoxic effects of membrane-damaging agents such as ionophores and complement-mediated cytotoxicity. A clinically relevant agent known to intercalate in the cell membrane, Danazol was also found to have activity against CLL and can be combined safely with GCs for enhanced treatment efficacy.
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<th>Description</th>
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<tr>
<td>$\gamma_c^{\text{null}}$</td>
<td>IL-2R\gamma complete null mutation</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>3'-UTR</td>
<td>3'-untranslated region</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>ACM</td>
<td>Adipocyte conditioned media</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein 1</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CCND3</td>
<td>Cyclin D3</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cES</td>
<td>Embryonic stem cell media</td>
</tr>
<tr>
<td>CHK6</td>
<td>Cyclin-dependent kinase 6</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyl transferase 1</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>Dan</td>
<td>Danazol</td>
</tr>
<tr>
<td>Dex/DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DiOC$_6$(3)</td>
<td>3,3'-dihexyloxacarbocyanine iodide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DON</td>
<td>6-diazo-5-oxo-L-norleucine</td>
</tr>
<tr>
<td>DSS</td>
<td>4,4-dimethyl-4-silapentane-1-sulfonic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAO</td>
<td>Fatty acid oxidation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCR</td>
<td>Fludarabine-cyclophosphamide-rituximab</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GLS</td>
<td>Glutaminase</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid receptor element</td>
</tr>
<tr>
<td>HDGCs</td>
<td>High dose glucocorticoids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HDL</td>
<td>High-density-lipoproteins</td>
</tr>
<tr>
<td>HDMP</td>
<td>High dose methylprednisolone</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthineguanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IgV&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Immunoglobulin heavy-chain variable gene</td>
</tr>
<tr>
<td>I-κB</td>
<td>Inhibitor of nuclear factor kappa B</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density-lipoprotein</td>
</tr>
<tr>
<td>LDT</td>
<td>Lymphocyte doubling time</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MAG-L</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MEM-α</td>
<td>Minimum essential media α</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-essential fatty acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Non-obese diabetic/Severe combined immunodeficient</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Proliferation center</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase 4</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>P-GR</td>
<td>Phosphorylated-glucocorticoid receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase muscle isozyme 2</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator activated receptor α</td>
</tr>
<tr>
<td>PPARδ</td>
<td>Peroxisome proliferator activated receptor δ</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>Propionyl coenzyme A</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium-dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SRC1</td>
<td>Steroid receptor co-activator-1</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal transducer and activator of transcription 5</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SWItch/Sucrose NonFermentable</td>
</tr>
<tr>
<td>TAGs</td>
<td>Triacylglycerides</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline plus 0.05% Tween-20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low-density-lipoprotein</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>ZAP70</td>
<td>70-kDa-zeta-associated protein</td>
</tr>
</tbody>
</table>
CHAPTER 1:

Introduction
Glucocorticoids (GCs) are one of the oldest agents that have been utilized for the clinical treatment of a variety of immunological diseases such as autoimmune diseases and hematological malignancies. The cytotoxic effects of GCs against lymphocytes have been studied extensively and yet the exact mechanism of action remains uncertain. Cytotoxicity appears to result from numerous genomic, non-genomic and tissue-specific effects of GCs. B cell leukemias such as chronic lymphocytic leukemia (CLL) are commonly treated with GCs as a single agent or in combination with other treatment modalities including immunotherapy and chemotherapy. GCs have been shown to be an important element of highly effective regimens for specific subsets of CLL patients including high-risk patients and patients who develop resistance to first-line chemotherapy (Thornton et al., 1999; Thornton et al., 2003; Xu et al., 2010).

Understanding the precise mechanism of action of GCs against CLL cells may allow for the development of strategies to further enhance the efficacy of these drugs. The limiting factor of this treatment is the eventual development of GC resistance. Various mechanisms of resistance have been suggested however few have presented promising targets for the development of clinically-relevant agents. Results presented in this thesis comprise of investigations into the mechanism of action and resistance of GCs in circulating CLL cells. Furthermore, data on a new agent, Danazol, with clinical activity against CLL as a treatment option in combination with GCs will be presented.

1.1 Chronic Lymphocytic Leukemia (CLL)

CLL is the most common form of leukemia in North America with an incidence of 1.5 in 100,000 people and is typically found in patients over the age of 40 (Gribben et al., 2011). The diagnosis of CLL requires a circulating B-cell count of at least 5,000 B cells/µL as outlined in the International Workshop on CLL guidelines (Hallek et al., 2008). A distinguishing characteristic of CLL is its unique immunophenotype, which is the co-expression of the cell surface markers CD5, CD19, CD20 and CD23. However, in comparison to normal B cells, CLL cells’ expression levels of surface immunoglobulin, CD20 and CD79b are low (Ginaldi et al., 1998).

CLL cells are thought to arise from CD5+ B cells that have been transformed by genetic damages sustained at sites of inflammation (Landgren et al., 2007; Klein et al.,
2010). These CLL cells divide in proliferation centers (PCs) located in secondary lymphoid organs such as the bone marrow, spleen and lymph nodes, and eventually enter the blood stream (Ponzoni et al., 2011). CLL cells divide in PCs in response to inflammatory microenvironmental factors, such as antigens, chemokines, cytokines and Toll-like receptor (TLR)-ligands (Chiorazzi et al., 2005). Our lab (Tomic et al., 2011) and others (Chiorazzi et al., 2005) have shown that responses to these immunoreceptor-mediated signals are altered to support cell proliferation to promote malignancy of B cells.

1.2 CLL clinical staging

While the clinical course of CLL is highly variable, clinical staging can provide important prognostic information and guide treatment decisions. Traditionally there are two widely accepted staging methods, the Rai system (Rai et al., 1975) and the Binet system (Binet et al., 1981), which both rely on physical examinations and standard laboratory testing without the need for imaging modalities.

Rai classification defines low-risk disease as patients who exhibit lymphocytosis (increase in the number of lymphocytes) with leukemia cells in the blood and/or marrow (formerly Rai stage 0). Intermediate-risk disease is categorized as patients with lymphocytosis, enlarged nodes in any site and splenomegaly and/or hepatomegaly (formerly Rai stage I or II). Finally, high-risk disease is defined as patients with disease-related anemia (formerly Rai stage III) or thrombocytopenia (formerly Rai stage IV) (Hallek et al., 2008). Binet staging system uses letters A to C to stage CLL disease according to how many areas are involved as indicated by the presence of enlarged lymph nodes (>1cm in diameter) and whether there are signs of anemia (low red blood cell count) or thrombocytopenia (low platelet count) (Hallek et al., 2008).

It is becoming clear that the molecular profile of CLL provides important insights into disease pathogenesis and prognosis. Currently, fluorescence in situ hybridization (FISH) is used to categorize CLL into 5 main sub-types based on cytogenetic abnormalities (Döhner et al., 2000). According to Döhner et al., cytogenetic abnormalities are detected in 80% of CLL patients. Normal cytogenetic profiles are detected in around 20% of patients. Deletions in chromosome 13q (13q- deletions) occur
in roughly 55% of patients and are associated with a loss of microRNAs (miR-15 and miR-16) (Calin et al., 2002). Deletions of chromosome 11q (11q- deletions) and trisomy 12 (T12) can be found in 18% and 16% of patients respectively. Chromosome 11q contains the locus for the ataxixia telangiectasia mutated (ATM) gene, which encodes for a kinase involved in p53-mediated DNA damage repair (Schaffner et al., 1999). T12 results in the increased expression of genes on chromosome 12, including MDM2, which is an inhibitor of p53 (Watanabe et al., 1994). The most uncommon abnormality is the deletion of chromosome 17p (17p- deletions), which encompasses the p53 locus and occurs in only 10% of patients. p53 is an important mediator of the cellular response to DNA damage. CLL patients with T12, 11q- or 17p- deletions tend to demonstrate more aggressive forms of disease, which can be attributed to a malfunction in the p53 DNA damage pathway (el Rouby et al., 1993; de Viron et al., 2012). 17p- deletion patients tend to have inferior prognosis and greater resistance to standard chemotherapy. In comparison, CLL patients with only 13q- deletions tend to have a better prognosis, with some patients never requiring treatment (Döhner et al., 2000). These cytogenetic changes can evolve over time and it is important to reassess these markers appropriately to determine treatment options.

Other common prognostic biomarkers include the mutational status of the immunoglobulin heavy-chain variable gene (IgVH), use of IgVH and expression of 70-kDa-zeta-associated protein (ZAP70) and CD38 (Gribben et al., 2011). In addition, there are also serum markers that can predict survival or progression-free survival in CLL patients, such as CD23, thymidine kinase and β2-microglobulin (Hallek et al., 2008).

1.3 CLL therapy

Upon initial diagnosis of CLL, treatment may not always be necessary. This practice is due to evidence that has shown that there is no benefit to early or immediate therapy at least until there is clinical indication that warrants it. For early-stage, asymptomatic CLL patients (Rai 0, Binet A) who are categorized as low risk, a wait-and-watch approach is used until the patient starts developing symptoms (Gribben et al., 2011). Patients are categorized with active disease if they fulfill one of the following criteria: progressive bone marrow failure (development or worsening of anemia and/or thrombocytopenia),
splenomegaly, lymphadenopathy, progressive lymphocytosis (lymphocyte doubling time (LDT) < 6 months), autoimmune anemia and/or thrombocytopenia that is poorly responsive to corticosteroids, or the development of constitutional symptoms such as weight loss, extreme fatigue, high fevers or night sweats (Hallek et al., 2008). For patients who are symptomatic with active or advanced CLL disease, treatment needs to be started immediately. Currently, the standard treatment for CLL is combination chemoimmunotherapy, which consists of fludarabine, cyclophosphamide and rituximab (FCR) (Gribben et al., 2011). Fludarabine is a purine analog that interferes with DNA synthesis and has largely replaced the alkylating agent, chlorambucil as the initial, single agent treatment for patients with symptomatic CLL (Rai et al., 2000). Cyclophosphamide is an alkylating agent and rituximab is a monoclonal antibody against CD20. FCR treatment is associated with higher complete remission (CR) rates and improved overall survival (OS) compared to single agent treatments or FC treatment (Hallek et al., 2010; Keating et al., 2005; Tam et al., 2008). Even with initial treatment of FCR, some patients fail to respond and are deemed to have primary resistance. Other patients who do respond to FCR treatment eventually experience disease relapse and become resistant to fludarabine-based regimens, which portends extremely poor prognosis (Zenz et al., 2012).

Complications also arise when the patient’s prognosis is categorized as high-risk, particularly CLL patients with 17p- deletions. There are debates as to whether starting these patients on treatment earlier in their disease course is beneficial however there is still no conclusive evidence to support it (Gribben et al., 2011). In addition, the response rate of 17p- deletion patients to front line chemotherapy and chemoimmunotherapy is very poor therefore it is questionable whether FCR is a suitable treatment option (Hillmen, 2011).

For high-risk 17p- deletion patients and fludarabine-refractory patients, there are limited treatment options available. In these cases, non-chemotherapeutic agents play an important role in CLL therapy. Some of the non-chemotherapeutic agents utilized for treatment include monoclonal antibodies, like Alemtuzumab (anti-CD52 antibody) and Ofatumumab (fully humanized anti-CD20 antibody), cellular immunotherapy, immunomodulatory agents (Lenalidomide) and high dose glucocorticoids (HDGCs)
Finally, allogeneic stem cell transplantation provides a potential cure for CLL however this treatment is only suitable for a very small subset of patients (younger, physically fit patients with high-risk or refractory CLL disease) and there is the prevalent risk of mortality (Toze et al., 2012).

1.4 Glucocorticoids (GCs)

Glucocorticoids (GCs) are naturally occurring steroid hormones produced in the adrenal cortex under the control of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (Smith et al., 2010). GCs have a variety of physiological functions in the body, including alteration of metabolism, regulation of immune responses, cell growth and proliferation, development, and reproduction (Kfir-Erenfeld et al., 2010). Generally, GCs mediate their functions via signaling through the GC receptor (GR) pathway.

The GR is a member of the steroid/thyroid hormone receptor superfamily that is ubiquitously expressed, ligand-dependent transcription factor. The gene expressing the GR is found on chromosome 5 (5q31) in humans and contains nine exons (Theriault et al., 1989). There are three characteristic domains found in the GR protein: the N-terminal domain, internal DNA binding domain and the C-terminal domain.

The N-terminal domain contains a transactivation domain that is involved in transcriptional activation of target genes (Hollenberg et al., 1988). The internal DNA binding domain contains two zinc finger domains that are highly conserved as a result of its function for binding to glucocorticoid response element (GRE) sequences of target genes such as nuclear factor-κB (NF-κB) (Tao et al., 2001). This domain also contains a nuclear localization sequence (NLS1) (Picard et al., 1987). Finally, the C-terminal domain houses the ligand-binding domain, which is required for binding heat shock proteins (HSPs) and for GR dimerization (Giguere et al., 1986). It also contains a second nuclear localization signal (NLS2) (Picard et al., 1987).

The GR is primarily expressed in the cytoplasm however a cell membrane-associated receptor has been described as well (Gametchu et al., 1993). As a result of alternative splicing, there are multiple isoforms of the GR protein that are expressed differentially in the various tissue types, GRα, GRβ, GRγ, GR-A and GR-P (Hollenberg
The GRγ isoform is largely expressed in lymphocytes (Rivers et al., 1999).

The unliganded GR protein exists in the cytoplasm as a multi-protein heterocomplex containing chaperones like HSP90, stabilizing proteins like p23 and various other molecules (Pratt et al., 1997; Cheung et al., 2000). GCs bind to the inactive form of the GR in the cytoplasm, which initiates a conformational change in the receptor that releases it from its chaperones (Elbi et al., 2004). The GR then undergoes multiple phosphorylation events and subsequent homodimerization (Frankfurt et al., 2004). The ligand bound, dimerized receptor translocates into the nucleus via the interaction between its NLS and importins in the nuclear membrane (Freedman et al., 2004).

Once in the nucleus, the dimerized GR can exert its transcriptional effects by binding to GC response elements (GREs) found in the promoter regions of target genes to induce either transactivation or transrepression. The consensus GRE sequence consists of two hexamer half-sites separated by three random nucleotides (Smith et al., 2010). The most commonly found hexamer half-site sequence is TGTCTC. The number and location of the GREs within the promoter region of the target genes impacts the intensity of the transcriptional response (Freedman et al., 1993).

Upon binding to the GRE, the GR can recruit basal transcriptional machinery and transcriptional co-activators to the start site; such co-activators include cyclic adenosine monophosphate (cAMP) response element-binding (CREB)-binding protein (CBP), steroid receptor co-activator-1 (SRC1), p300 and SWI/SNF (Adcock, 2001; Wallberg et al., 2000). These co-activators induce histone acetylation allowing for transactivation of target genes. The GR can also induce transrepression of target genes by directly binding negative GREs within the promoter region (Sakai et al., 1988). On the other hand, GR can also regulate transcription via direct protein-protein interaction with other nuclear transcription factors such as NF-κB, AP-1, STAT5 and STAT3, thus affecting the transcription of genes controlled by these factors (McKay et al., 2000; Ray et al., 1994; Stocklin et al., 1996; Yang-Yen et al., 1990; Zhang et al., 1997).

One main mechanism of GC action is mediated through its inhibition of NF-κB. NF-κB is a heterodimeric transcription factor that mediates transcription of cytokines, cytokine receptors, chemotactic proteins and adhesion molecules, all of which are
involved in cell survival, inflammation and various other cellular processes. There are a few hypotheses that have been suggested regarding the mechanism of GC-mediated NF-κB inhibition. The first theory is that the GR can directly interact with subunits of NF-κB (p65) thus inhibiting its activation (Adcock et al., 1999; Liden et al., 1997; Nissen et al., 2000). Secondly, the GR is also capable of inducing the expression of I-κB, a negative regulator that sequesters NF-κB in the cytoplasm to prevent nuclear translocation (Auphan et al., 1995; Scheinman et al., 1995). GCs are also capable of stimulating the activity of phosphatases involved in the regulation of NF-κB (Shipp et al., 2010). The variability with which GRs can mediate their transcriptional effects could account for the cell-specific effects of GCs that have been observed over the years.

1.5 Clinical application of GCs

For the purposes of therapeutic usage, synthetic GCs, such as dexamethasone (DEX), prednisolone and prednisone have been manufactured to be far more potent than endogenous GCs like cortisol. These synthetic GCs are widely used in the clinic as immunosuppressive drugs for inflammatory and autoimmune disease, and are also used for the treatment of various hematological malignancies due to their well-known ability to induce apoptosis in lymphocytes (Dougherty et al., 1943; Pearson et al., 1949).

Apoptosis is a mode of programmed cell death that regulates the elimination of cells that are no longer required, have developed improperly or have been genetically damaged. Apoptosis is a unique process whereby dead cells are broken down into apoptotic bodies that are removed by phagocytosis without releasing their cytotoxic cellular contents to the surrounding microenvironment thereby avoiding an inflammatory response (Sankari et al., 2012). The mechanism for GC-mediated apoptosis in lymphocytes is postulated to be a combination of both genomic (GR regulation of gene transcription) and non-genomic (mitochondrial translocation of GR and activation of signal transduction pathways) effects, however the exact mechanism is unclear and may vary depending on cell-type (Kfir-Erenfeld et al., 2010).

The first reported use of GCs in CLL treatment dates back to 1949 (Pearson et al., 1949) and since then, synthetic GCs (especially prednisone and prednisolone) have been
commonly used as single agents or in combination with other chemotherapeutic agents. However, standard-doses of GCs with chemotherapy have not been shown to be beneficial to the survival of CLL patients (Catovsky et al., 1988; Keating et al., 1998; O’Brien et al., 1993) and therefore were confined to palliative uses, treating autoimmune complications and improving cytopenias (Hamblin, 2006; Zent et al., 2010). The first use of HDGCs in CLL was reported in 1995, whereby refractory CLL patients were treated with high-dose methylprednisolone (HDMP). HDGC treatment is defined as a 1g/m$^2$ dose of GCs administered for 5 days (Bosanquet et al., 1995).

Since then, multiple studies have shown that HDGCs alone or in combination with chemotherapeutic agents can generate better responses for refractory and 17p-deletion CLL patients (Thornton et al., 1999; Thornton et al., 2003). Overall, HDGCs were well tolerated with some minor side effects. The rationale for utilizing HDGCs in the treatment of 17p-deletion patients relates to the p53-independent mechanism through which GCs induce apoptosis (Johnston et al., 1997). Interestingly, recently published findings indicate that HDMP treatment can induce complete remission (CR) in fludarabine-refractory CLL patients (Xu et al., 2010).

Another successful avenue for HDGC therapy in CLL is in combination with immunotherapy. Combining HDGCs with monoclonal antibodies is logical due to their different mechanisms of action. It has been shown that the chimeric anti-CD20 antibody, Rituximab and DEX have synergistic antiproliferative and apoptotic effects in vitro (Rose et al., 2011). This combination was tested in vivo and proven to generate better response rates than seen with either agent alone in both fludarabine-refractory and 17p-deletion patients (Bowen et al., 2007; Dungarwalla et al., 2008; Castro et al., 2008). Another anti-CD20 antibody clinically available is Ofatumumab, a fully humanized monoclonal antibody that targets a different epitope on the CD20 molecule than Rituximab (Du et al., 2009). Preclinical studies have shown that Ofatumumab is more efficient than Rituximab in inducing complement-induced cell death, which is a major mechanism of action of monoclonal antibodies (Beum et al., 2008). In vivo, Ofatumumab has been found to be active against CLL as a single agent and in combination with HDGCs in both fludarabine-refractory and 17p-deletion patients (Castro et al., 2010; Teichman et al., 2011; Spaner et al., 2011; Wierda et al., 2010). There have also been successful reports of
combining Alemtuzumab (anti-CD52 antibody) with HDGCs for treatment of high-risk 17p- deletion patients (Pettitt et al., 2006; Pettitt et al., 2012).

Unfortunately, a major problem for HDGC therapy is the eventual development of therapeutic resistance. Another issue with HDGC therapy is the development of adverse side effects such as hypertension, protein catabolism, cushingoid features, adrenal suppression, myopathy, diabetes, obesity, osteoporosis and cataract (Kfir-Erenfeld et al., 2010). However, it is important to note that a majority of these side effects are either avoidable or treatable if patients are monitored carefully.

1.6 Metabolic effects of GCs

Traditionally, GCs are thought to modulate metabolic processes in the body to conserve glucose during stress or starvation periods (Vegiopoulos et al., 2007). To do this, glucose uptake is inhibited in muscle and adipose tissue and fat is broken down in adipose tissues thereby releasing fatty acids for the production of energy (Vegiopoulos et al., 2007). The notion that GCs’ effects on glucose metabolism could be responsible in part for their therapeutic effects on malignant lymphocytes has seldom been addressed.

GC-induced apoptosis is known to induce a loss of mitochondrial membrane potential in human leukemic cells, both established cell lines and primary cells (Eberhart et al., 2011; Tiefenthaler et al., 2001). The synthetic GC, DEX was found to induce changes in mitochondrial membrane properties, reduce expression of mitochondrial transporters of substrates and proteins, which lead to repressed mitochondrial respiratory activity and lower cellular adenotriphosphate (ATP) levels, which contributed to apoptosis (Eberhart et al., 2011). Similarly, HDGCs were shown to interfere with the sodium and potassium transport processes across the plasma membrane therefore leading to a restriction of cellular ATP supply in activated lymphocytes (Buttergereit et al., 2000). This bioenergetic restriction was deemed responsible for the therapeutic effects of HDGCs in activated lymphocytes, such as those found in autoimmune diseases (Buttergereit et al., 2000). Interestingly, autoimmunity is a common complication in CLL disease, which is also conventionally treated using HDGCs (Hamblin et al., 2006; Zent et al., 2010).
Recently, it has also been shown that an alternative form of cell death, autophagy was responsible for GC-mediated cell death in ALL and CLL cells as opposed to the traditional apoptotic pathway (Grandér et al., 2009). Autophagy is a form of cell death induced when cells undergo starvation and resort to degrading cellular components using lysosomal machinery to maintain cellular energy levels (Banergi et al., 2012). This mechanism of GC-mediated cell death ties in with the energy restriction effects that were established earlier both with GCs and HDGCs (Tiefenthaler et al., 2001; Eberhart et al., 2011; Buttergereit et al., 2000).

More recently, it was discovered that the synthetic GC, DEX has a profound effect on glucose metabolism and uptake in lymphoid and leukemic cells, very similar to the role they play in skeletal muscle tissue (Buentke et al., 2011). It was shown that DEX managed to reduce glucose consumption, utilization and uptake in ALL cells (Buentke et al., 2011). The mechanism of DEX-mediated decrease in glucose uptake was via decreased expression levels of plasma membrane-associated glucose transporter 1 (GLUT1) (Buentke et al., 2011). More importantly, the inhibition of glucose uptake was found to correlate with DEX-mediated cell death and lowering glucose concentrations resulted in increased DEX-mediated cell death (Buentke et al., 2011). All this information fuels the emerging idea that the metabolic effects of GCs could be linked to their cytotoxic effects in lymphocytes.

1.7 Resistance to GC therapy

The major issue with GC treatment in the clinic is the development of resistance, which stands in the way of achieving CR in hematological malignancies. Thus far, various mechanisms of GC resistance have been proposed that mostly involve the GR signaling pathway and apoptotic machinery, such as altered expression of GR isoforms, GC-induced alterations in GR expression, mutations in the GR, aberrant expression of anti-apoptotic proteins such as Bcl-2, failure to induce expression of pro-apoptotic proteins, like Bim, and interactions with the kinome (Kfir-Erenfeld et al., 2010; Melarangi et al., 2012; Smith et al., 2010). However, mutations of and alterations to the expression of the GR are not thought be important in GC resistance in leukemia (Haarman et al., 2003;
Tissing et al., 2005). In addition, few mechanisms have offered promising targets for drug development to overcome GC resistance.

Recent discoveries have found links between glucose metabolism and GC-resistance in leukemia. B-ALL cells are known to acquire altered glucose metabolism, higher expression of GLUT1 and sensitivity to the glycolysis inhibitor, 2-deoxy-D-glucose (2-DG) (Boag et al., 2006). It was demonstrated that GC resistance in precursor B-ALL is associated with increased expression of genes involved in glucose metabolism (Holleman et al., 2004) and the glycolytic pathway was confirmed as a modulator GC resistance in ALL cells (Hulleman et al., 2009). There have also been claims that modulation of glucose levels influences the effectiveness of GC treatment in ALL (Buentke et al., 2011). A key factor involved in the regulation of glycolysis is the serine/threonine kinase, Akt, which is known to regulate glucose uptake and induce expression of glucose transporters, GLUT1 and GLUT3. Akt is a pro-survival factor that is part of the mammalian target of rapamycin (mTOR) pathway (Schultze et al., 2012). Phosphorylation events in the mTOR pathway are also known to regulate glycolysis. Evidence of the mTOR inhibitor, rapamycin treatment overcoming GC resistance in ALL cell lines implicates a role for the Akt/mTOR pathway in GC resistance (Wei et al., 2006; Zhang et al., 2012).

It has also been shown that there is an association between the upregulation of glycolytic signaling pathways and GC-resistance in T-ALL cell lines (Beesley et al., 2009). Similar findings have also been discovered in multiple myeloma (MM) whereby responses to DEX were determined to be glucose-sensitive (Friday et al., 2011). All together, these findings present strong evidence for the role of metabolism, specifically glycolysis, in GC-resistance in leukemia.

1.8 Fatty acid oxidation

Free fatty acids (FFAs) are a major source of fuel for oxidative metabolism aside from glucose and glutamine, particularly in cardiac and skeletal muscles. They also play additional roles in membrane function and structure such as prostaglandin synthesis (Macfarlane et al., 2008). Fatty acids are carboxylic acids typically categorized based on
two aspects of the molecule: the length of the aliphatic chain and the presence of carbon-carbon double bonds (saturated or unsaturated).

FFAs need to be complexed to albumin or esterified with glycerol to triacylglycerides (TAGs) and packaged into lipoprotein particles for transport in the circulation (Spector, 1975). In vivo, the majority of fatty acids are derived from dietary sources and then stored as TAGs in adipose tissue. When required, TAGs packaged in lipoprotein or chylomicrons are released into the circulation from the liver and the gut. These TAGs are then hydrolyzed by lipoprotein lipase (LPL) located on the luminal surface of the capillary endothelium (Linder et al., 1976), which then allows the FFAs to be taken up into the cell either via a passive or facilitated process (Garfinkel et al., 1976; Kalant and Cianflone, 2004). There are two pathways that can process FFAs inside the cell, the first is β-oxidation in the mitochondria and the second is re-esterification to TAGs (Macfarlane et al., 2008).

Mitochondrial β-oxidation is the process whereby FFAs are broken down to generate acetyl-CoA for the production of cellular energy (Eaton et al., 1996). The first step in β-oxidation is the activation of the FFAs catalyzed by the enzyme, long chain acyl-CoA synthetase (McGarry and Brown, 1997). The FFA reacts with ATP and Coenzyme A to generate fatty acyl-CoA ester and AMP. The activated fatty acid is then transported into the mitochondria via carnitine palmitoyl transferase 1 (CPT1) (Bremer, 1983; McGarry and Brown, 1997). Once inside the mitochondria, two carbon units (acetyl-CoA) are released with every cycle of β-oxidation (Eaton et al., 1996). This continues until the entire chain is converted into acetyl-CoA. These acetyl-CoA molecules can then enter the tricarboxylic acid (TCA) cycle, which produces ATP. This process is typical for even numbered saturated fatty acids, however the final product is a propionyl-CoA molecule for odd numbered saturated fatty acids. The propionyl-CoA molecule can also enter the TCA cycle but it needs to be converted into succinyl-CoA beforehand (Raph et al., 2010).

The Warburg hypothesis has largely been used to describe the aberrant metabolism that fuels the growth of most cancer cells. The hypothesis proposes that cancer cells originate from non-neoplastic cells that acquire a permanent respiratory defect that bypasses the Pasteur effect (the inhibition of anaerobic fermentation by
oxygen). These cancer cells mostly produced energy via a high rate of glycolysis followed by lactic fermentation in the cytosol, regardless of the presence or absence of oxygen (Warburg, 1956). In the presence of oxygen, normal non-proliferating cells typically undergo a low rate of glycolysis followed by pyruvate oxidation in the mitochondria to maintain efficient energy production.

An alternative hypothesis was proposed that suggested that the increased dependence of cancer cells on glycolysis stemmed from their inability to synthesize ATP in response to the mitochondrial proton gradient (Lynen, 1951; Ronzoni and Ehrenfest, 1936). Following work demonstrated that mitochondrial uncoupling (inability to synthesize ATP in response to mitochondrial proton gradient) results in a shift in metabolism to the use of non-glucose carbon sources, such as fatty acids, to maintain mitochondrial function (Belfroy et al., 2008; Pecquer et al., 2008; Rossmeisl et al., 2000; Samudio et al., 2008).

Interestingly, there is a substantial amount of evidence that implicates fatty acid oxidation (FAO) as a metabolic hallmark of cancer. Increased FAO has been shown to promote chemoresistance in tumour cells (Harper et al., 2002). The expression of various genes involved in lipid metabolism was found to be increased in tumour cells (Hirsch et al., 2010). An enzyme that releases FFAs from lipid stores, monoacylglycerol lipase (MAG-L) was found to promote cancer pathogenesis (Nomura et al., 2010) Other studies have also shown that pharmacologic inhibition of FAO is capable of sensitizing leukemic cells to apoptosis (Samudio et al., 2010). Recently, the expression of FAO genes such as carnitine palmitoyltransferase 1C (CPT1C) (Zaugg et al., 2011) and peroxisome proliferator activated receptor α (PPARα) (Spaner et al., 2012) were found to confer a survival advantage to tumour cells under conditions of metabolic stress. Given the metabolic role GCs play in stimulating FAO, it is worthwhile to consider the contribution of FAO to GC resistance in tumour cells.
1.9 Danazol

Danazol is a derivative of the synthetic androgen, ethisterone (Dmowski et al., 1971). Danazol was initially created for the purposes of treating women with endometriosis, an estrogen-dependent proliferative disease with extrauterine endometrium-like tissue formation (Friedlander, 1973). The main mechanism by which Danazol prevents endometriosis is by inhibiting endogenous estrogen production and acting like a progesterone-like steroid hormone (Barbieri, 1977).

Danazol was subsequently found to be effective in treating various autoimmune and hematologic disorders such as hereditary angioedema, autoimmune hemolytic anemia and immune thrombocytopenic purpura (Ahn et al., 1983; Ahn et al., 1985). There has also been evidence to suggest that Danazol has activity against myelodysplastic syndromes, which are disorders of the stem cells in the bone marrow (Fontana et al., 2011; Sadek et al., 2000). The mechanism of action of Danazol in immune cells still remains a subject of investigation. However, various mechanisms have been suggested such as decreased production of inflammatory cytokines, plasma cell membrane intercalation and decreased expression of serum soluble CD23 from the membranes of activated B cells (Horstman et al., 1995; Matalliotakis et al., 2000; Odukoya et al., 1995; Tanaka et al., 2009). The therapeutic activity Danazol has against B-lymphocytes suggests that it could be utilized in the treatment of hematological malignancies, such as CLL.
1.10 Hypothesis

Glucocorticoid (GC) treatment of chronic lymphocytic leukemia (CLL) can be improved by targeting metabolic aspects of resistance.

1.11 Thesis objectives and organization

1. In an effort to improve GC treatment, the relationship between the metabolic and cytotoxic effects of GCs against CLL cells was investigated. In addition, metabolic strategies to overcome GC resistance were studied. The results of these studies are presented in Chapter 2.

2. The role of a clinically relevant agent, Danazol, was also studied as a potential combination option with GC treatment of CLL. The results of these studies are presented in Chapter 3.
CHAPTER 2:

PPAR-alpha and fatty acid oxidation mediate glucocorticoid-resistance in Chronic Lymphocytic Leukemia

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2.1 Abstract

Glucocorticoids (GCs) are an important treatment for Chronic Lymphocytic Leukemia (CLL). However, resistance to GCs inevitably develops and the mechanisms of action of GCs in CLL are not well understood. The synthetic GC dexamethasone (DEX) was found to decrease pyruvate kinase M2 (PKM2) expression and metabolic activity of CLL cells, indicated by loss of mitochondrial membrane potential and decreased levels of pyruvate and its metabolites. This metabolic restriction was associated with decreased size and resulted in the tumor cell death that was increased by concomitant damage to plasma membranes. However, the nuclear receptor Peroxisome proliferator activated receptor (PPAR)-alpha, which regulates fatty acid oxidation, was also increased by DEX and adipocyte-derived lipids, lipoproteins, and propionic acid protected CLL cells from DEX-mediated death. PPARalpha and fatty acid oxidation enzyme inhibitors increased DEX-mediated killing of CLL cells in vitro and clearance of CLL xenografts in vivo. These findings suggest that GCs prevent tumor cells from generating the energy they need to repair membrane damage, fatty acid oxidation is a mechanism of resistance to GC-mediated cytotoxicity, and PPARalpha-inhibition is a strategy to improve the therapeutic efficacy of GCs.
2.2 Introduction

The outcome of high-risk CLL patients whose tumor cells harbor deletions in the region of chromosome 17 that encodes p53 or who relapse shortly after completing first-line therapy with fludarabine, cyclophosphamide, and rituximab (FCR) is poor (Zenz et al., 2012). Despite the advent of newer agents (Wiestner et al., 2012), high-dose synthetic glucocorticoids (GCs) such as methylprednisolone or dexamethasone (DEX), with or without monoclonal antibodies such as Ofatumumab, remain among the most effective treatments for these patients (Spaner, 2011). Unfortunately, GC-based regimens are not curative and resistance inevitably develops with extended use. Considering their long clinical history (Hench et al., 1949), knowledge of how GCs exert their therapeutic effects is surprisingly incomplete. A better understanding of the mechanism of action and nature of resistance are needed to improve the efficacy of GC therapy for CLL patients.

GCs are steroid hormones made by the adrenal cortex that affect a variety of cell functions. They inhibit glucose metabolism and promote fatty acid oxidation during starvation and also have potent immunosuppressive properties (Sapolsky et al., 2000). GCs bind to glucocorticoid receptors (GRs), which are members of the nuclear receptor family of ligand-dependent transcription factors. Activation by ligands causes GRs to become phosphorylated and translocate to the nucleus where they bind GC-response elements (GREs) and mediate gene transcription. Ligand-activated GRs transactivate genes such as IkB that inhibit signaling pathways (Flammer et al., 2011) and also transrepress signaling through direct binding to kinases (Glass et al., 2010). GCs cause CLL cells to undergo apoptosis (Kfir-Erenfeld et al., 2010) but the processes that lead from GR-phosphorylation to cell death are not fully understood. The studies here were designed to provide more insight into the mechanisms of action of GCs in CLL.
2.3 Materials and Methods

**Antibodies and reagents.**

7-aminoactinomycin D (7-AAD) was obtained from Pharmingen (San Francisco, CA). Fatty acid free bovine serum albumin, 2-mercaptoethanol (2-ME), Mifepristone, 3,3’dihexyloxacarbocyanine iodide (DiOC₆(3)), propionic acid, ionomycin, GW6471 (PPARα-antagonist), and β-actin antibodies were from Sigma (St. Louis, MO). RPMI-1640, lipid-rich bovine albumin (AlbuMAX®II), and Mitotracker® Red and Green were from Invitrogen (Carlsbad, CA). Clinical grade dexamethasone sodium phosphate (Omega, Montreal, Quebec) was purchased from the hospital pharmacy. Ofatumumab was obtained from GlaxoSmithKline (London, UK). Low-Tox®-M Rabbit Complement was purchased from Cedarlane (Burlington, ON, Canada). Antibodies against phospho-(Ser²¹¹)glucocorticoid receptor and pyruvate kinase muscle isozyme (PKM2) were from Cell Signaling Technology (Beverly, MA), as were secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies (Cat. Nos. 7074 and 7076, respectively). PPARα antibodies were from Cayman Chemical Co. (Ann Arbor, MI). High-, low- and very-low-density lipoproteins were from EMD Chemicals (San Diego, CA). MK886 and Compound A (PPARα-antagonists) were generous gifts from Inception Sciences (San Diego, CA) and CVT-4325 (GS449794, β-oxidation inhibitor) was a generous gift from Gilead Sciences (Foster City, CA).

**CLL cell purification.**

CLL cells were isolated as previously described by negative selection from the blood of consenting patients (Tomic et al., 2011), diagnosed by a persistent monoclonal expansion of CD19⁺CD5⁺IgM⁺ lymphocytes and untreated for at least 3 months. Patient characteristics are described in Supplementary Table 1. Protocols were approved by the Sunnybrook Ethics Review Board.

**Cell culture.**

Unless otherwise specified, purified CLL cells were cultured at a concentration of 1 x 10⁶ cells/mL in RPMI-1640 medium supplemented with Transferrin and 0.02% AlbuMAX II in 6- or 24-well plates (BD Labware) at 37°C in 5% CO₂ for the times indicated in the figure legends.
Preparation of adipocytes and adipocyte conditioned medium (ACM).

OP-9 cells were maintained in OP-9 propagation medium consisting of MEM-α (Minimum Essential Media α), 20% FBS (Fetal Bovine Serum; BioWhittaker, Walkersville, MD), 100 U/ml penicillin, and 100 mg/ml streptomycin (Wisent USA). The cells were re-plated every 3 days upon growing to confluence. Adipocytes were obtained by the method of Wollins et al. (Wollins et al., 2006). OP-9 cells were grown to confluence in propagation media which was then replaced by stem cell medium (cES) (Garcia-Gonzalo et al., 2008), consisting of Advanced-DMEM-F12, 15% KnockOut™ Serum Replacement, 1% Glutamax-1 (all from Invitrogen), 1% MEM nonessential amino acids (Wisent USA), and 0.1 µM 2-ME. Within 3-4 days, more than 90% of the OP-9 cells become filled with large lipid droplets that were visible in a light microscope (Fig.10C). To obtain ACM, OP-9 adipocytes were washed and incubated with fresh cES for 3 days. ACM was collected and heat-inactivated at 60°C for 30 min.

Flow cytometry.

Cell viability was measured by washing cells in phosphate-buffered saline (PBS) and then incubating with 3.5 µL of 7-AAD for 10 min. Mitochondrial membrane potential was measured by staining with 0.2 nM of 3,3’-dihexyloxacarbocyanine iodide (DiOC₆(3)) for 15 min at room temperature followed by washing with PBS. Mitochondrial mass was measured by staining with 2 nM of MitoTracker Green FM for 15 min at 37°C followed by washing with PBS. Ten thousand viable counts were analyzed with a FACScan flow cytometer using Cellquest software (Becton Dickinson). Standardization of the flow cytometer was performed before each experiment using SpheroParticles (Spherotech Inc., Chicago, IL, USA).

Immunoblotting.

Protein extraction and immunoblotting were performed as previously described (Tomic et al., 2011). Proteins were resolved in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P transfer membranes (Millipore Corp., Billerica, MA, USA). Western blot analysis was performed according to the manufacturers’ protocols for each antibody. Chemiluminescent signals were created with SupersignalWest Pico Luminal Enhancer and Stable Peroxide Solution (Pierce, Rockford, IL, USA) and detected with a Syngene InGenius system (Syngene,
Cambridge, UK). For additional signal, blots were stripped for 60 min at 37°C in Restore Western Blot Stripping Buffer (Pierce) and washed twice in TBS-T (Tris-buffered saline plus 0.05% Tween-20) at room temperature and then re-probed as required. Densitometry was performed using Image J software. The densitometry value for each patient sample was normalized against the value obtained for β-actin to obtain the intensities for P-GR, PKM2 and PPARα reported in the figures.

**Real-time PCR.**

RNA was prepared with the RNeasy mini kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized from 2 µg of RNA using Superscript III reverse transcriptase (Life Technologies, Invitrogen), according to the manufacturer’s instructions. PKM2, PPARα, PPARδ, pyruvate dehydrogenase kinase 4 (PDK4), and hypoxanthineguanine phosphoribosyl transferase (HPRT) transcripts were amplified with the following primers: PKM2 forward, GTCGAAGCCCCATAGTGAAG; reverse, ATGTCTTTCTCCGACACAGC, PPARα forward, CTGGAAGCTTTGGCTTTACG; reverse, ACCAGCTTGAGTCGAATCGT, PPARδ forward, CTCTATCGTCAACAAGGACG; reverse, GTCTTCTTGATCCGCTGCAT, PDK4 forward, CATACTCCACTGCACCAACG; reverse, CCTGCTTGGGATACACCAGT, and HPRT forward, GAGGATTTGGAAAGGGTGTT; reverse ACAATAGCTCTTCAAGGTGTGA. Polymerase chain reactions were carried out in a DNA engine Opticon System (MJ Research, Waltham, MA, USA) and cycled 34 times after initial denaturation (95°C, 15 min) with the following parameters: denaturation at 94°C for 20 sec, annealing of primers at 58°C for 20 sec, and extension at 72°C for 20 sec. mRNA abundance was evaluated by a standard amplification curve relating initial copy number to cycle number. Copy numbers were determined from two independent cDNA preparations for each sample. The final result was expressed as the relative fold change of the target gene to HPRT.

**Sample preparation for NMR.**

To measure intracellular metabolite levels, 5x 10^7 CLL cells were pelleted, resuspended in 1 mL of ice cold PBS, transferred into a microfuge tube and pelleted again. Pellets were extracted with methanol 3 times by adding 1 mL of -80°C, 80% methanol, vortexing, incubating on ice for 30 min, vortexing again, and then centrifuging for 30 min
at 10000 rpm at 4°C. The supernatants were collected into 2 mL microfuge tubes and the solvent was removed with a centrifugal evaporator (SpeedVac) at room temperature. Metabolite extracts were stored at -80°C until NMR analysis.

**1H NMR.**

The extracts were taken up in 120µL of NMR buffer (50mM Na₂HPO₄, 0.1% NaN₃ in D₂O pH 7.0; uncorrected). DSS (0.5mM) was added as an internal reference standard and the sample was transferred to a 3mm NMR tube. All NMR spectra were run on a Bruker Avance II 800MHz spectrometer equipped with a triple resonance cryoprobe. One dimensional (1D) ¹H NMR spectra were collected at 298K with 128 scans using a 90° proton pulse and recycle delay of 2 sec. A total of 32K points were collected over a spectral width of 12.8kHz (16ppm). Metabolites were identified and quantified using the Chenomx 7.1 NMR software suite (Chenomx Inc., Edmonton, AB). Metabolite concentrations are reported as mean values ± standard deviation.

**CLL xenograft model.**

NOD-SCIDγ null mice were sublethally irradiated (245 Rads) and then injected intraperitoneally (ip) with 2.5 x10⁸ thawed CLL splenocytes that had been obtained at the time of therapeutic splenectomy. Subsequently mice were injected bi-weekly ip with 700 µl of plasma pooled from 8-10 patients with white cell counts greater than 100x10⁶ cells/ml. Plasma injections continued until engraftment was established in the spleen and the peritoneal cavities (6 weeks). Single cell suspensions from spleens and peritoneal cavities (PCs) were counted in a hemocytometer and analyzed by multi-color FACS to assess engraftment of CLL cells, as before. (Spaner et al., 2012; Wong et al., 2012)

**Statistical analysis.**

The Student’s t-test and paired t-tests were used to determine p-values.
2.4 Results

**Dexamethasone induces cell death and decreases the size of circulating CLL cells**

DEX (30 µM) killed purified CLL cells within 48 h in serum-free culture conditions involving RPMI-1640 media with 0.02% lipid-rich albumin. An example (Fig.1A) and results for 52 patient samples (Fig.1B) are shown. The dose of DEX was chosen to approximate plasma levels that result when patients are treated with high-dose GCs (Spaner et al., 2012). Cell death was prevented by the GR antagonist Mifepristone (Agarwai et al., 1996) (Fig.4A, bottom panel), suggesting it was a direct, transcriptional effect of DEX.

The number of CLL cells killed by DEX exhibited inter-patient variability (Fig.1B), which could not be correlated with the clinical characteristics of the patients. In all cases studied, the GR was phosphorylated by DEX, suggesting this variability was not due to differences in activation of the GR (Fig.1C). Despite the fact that many DEX-treated cells remained alive, as measured by their ability to exclude the DNA-dye 7-AAD, in all cases they decreased in size, as measured by the forward scatter parameter of flow cytometry (Fig.1D).
Figure 1. Effect of Dexamethasone on circulating CLL cells. A. CLL cells were purified and cultured for 48 h with or without dexamethasone (DEX) (30 μM) in serum free media (RPMI-1640 with Transferrin and 0.02% Albumax) in the presence or absence of the GR antagonist Mifepristone (1 μM). After 48 h, percentages of viable 7AAD− cells that exclude 7AAD were determined by flow cytometry and shown in the right upper corners of the dot-plots. Mifepristone prevented DEX-induced cell death. B. Results for 52 different CLL patient samples are shown. Each line represents percentages of 7AAD+ cells after 48 h in the presence or absence of DEX. C. Circulating CLL cells from the indicated patients were cultured for 4 h with or without DEX. Levels of Ser211 phosphorylated glucocorticoid receptor (P-GR) were measured by immunoblotting with β-actin as a loading control and quantified by densitometry. D. Summary of forward scatter median measurements at 48 h by flow cytometry indicating that DEX significantly decreased the size of circulating CLL cells. Paired t-tests were used to determine p-values. **, p<0.01
**Dexamethasone decreases mitochondrial membrane potential and intracellular metabolites**

Since size reflects metabolic activity (DeBerardinis et al., 2008), the smaller size suggested that DEX might be acting to restrict the metabolic activity of CLL cells. Cellular metabolism is supported by mitochondria and requires the generation and maintenance of a mitochondrial membrane potential that can be measured by flow cytometry with the cell-permeable, green-fluorescent dye 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)). As early as 4 h after DEX treatment, CLL cells exhibited a significant decrease in mitochondrial membrane potential (Fig.2A, left) that was sustained for at least 18 h (Fig.5A, right). The decrease in membrane potential could not be attributed to decreased mitochondrial mass (Supplementary Fig.7A) but could be prevented by Mifepristone (see Fig.5F), suggesting it was a transcriptional effect of DEX.

Levels of small-molecule intracellular metabolites were then measured by nuclear magnetic resonance (NMR) spectroscopy. Consistent with restricted metabolism, a number of metabolites were decreased by DEX. Intracellular glutamate, alanine, and lactate levels were significantly lower (Fig.2B). Pyruvate, succinate, and glutamine levels were also decreased while acetate was relatively preserved (Fig.2B). Pyruvate is a product of glycolysis and glutaminolysis that gives rise to lactate and alanine. Glutamate is a product of glutaminolysis that provides Tricarboxylic Acid (TCA) cycle intermediates, such as succinate. (DeBerardinis et al., 2008) Acetate is a product of fatty acid oxidation (Lin et al., 1996). Note that these changes in metabolite levels were measured at 18 h, prior to any loss of membrane integrity associated with cell death (Fig.2B) and could not be attributed simply to cytotoxicity.

**Dexamethasone downregulates pyruvate kinase expression**

The decrease in pyruvate, lactate, and alanine levels suggested that genes associated with pyruvate generation were decreased by DEX. Pyruvate kinase muscle isozyme-2 (PKM2) catalyzes the last step of the glycolytic pathway and transfers a phosphate group from phosphoenolpyruvate to adenosine diphosphate to make pyruvate and ATP (Tamada et al., 2012). DEX-treated CLL cells down-regulated PKM2 mRNA and protein levels within 4 h (Fig.2C,D).
Figure 2. Effect of DEX on mitochondrial membrane potential, intracellular metabolites, and PKM2 expression. A. Cells from 8 different CLL patients were cultured with or without DEX. At the indicated times, the cells were stained with DiOC₆(3) to measure mitochondrial membrane potential. Median fluorescence intensities (MFIs) are shown for each sample. B. 5 x 10⁷ cells from 5 different CLL patients were cultured for 18 h in the presence or absence of DEX and then analyzed by 1D ¹H NMR spectroscopy. The averages and standard errors for the 5 samples are shown. Glutamate, alanine and lactate levels were significantly lowered by DEX, while acetate levels were relatively preserved. C. CLL cells from 9 patients were cultured with or without DEX. After 4 h, PKM2 mRNA transcripts (relative to HPRT transcripts) were measured by quantitative PCR. D. After 18 h of culture, phospho-GR and PKM2 expression were measured in DEX-treated cells from 4 different patients by immunoblotting and quantified by densitometry, with β-actin used as a loading control. PKM2 mRNA and protein were both decreased by DEX. Student’s t-tests and paired t-tests were used to determine p-values. **, p<0.01; *, p<0.05. NMR spectroscopy data courtesy of Rob Laister.
**Concomitant membrane damage enhances DEX-mediated cytotoxicity**

The above results suggested that DEX restricted cellular metabolism in CLL cells in part by down-regulating PKM2. However, increased metabolic activity is needed to generate the substrates and energy required to repair cellular damage. Accordingly, DEX may inhibit the ability of CLL cells to repair membrane damage and subsequently increase killing by membrane damaging agents. To address this hypothesis, CLL cells were treated with Ofatumumab and/or complement in the presence or absence of DEX. In the absence of complement, Ofatumumab did not change basal killing by DEX in serum-free conditions (Fig.3A). Ofatumumab binds to CD20 and causes complement to deposit on the cell surface, forming the membrane attack complex and resulting in membrane damage and cell lysis (Rose et al., 2002). Death of CLL cells from complement damage was increased significantly by DEX (Fig.3A).

Ionomycin is a calcium ionophore that creates pores in plasma membranes, causing electrochemical gradient disturbances that require significant energy to repair. (Krauss et al., 2001) Consistent with the hypothesis that DEX restricts the metabolic activity needed to repair such damage, ionomycin-mediated cell death was increased significantly by DEX (Fig.3B). The serum-free conditions used in these studies would also be expected to damage plasma membranes through detergent-like effects of the fatty acids bound to lipid-rich albumin (Glatz et al., 2010; Holzer et al., 2011). Removing this source of membrane disruption should then decrease killing of CLL cells by DEX. Consistent with this idea, DEX-mediated cell death was significantly reduced when Albumax was replaced by fatty acid free albumin (Fig.3C). Note that the decrease in cell size was still observed (not shown).
Figure 3. Enhancement of cytotoxicity from membrane damage by DEX. A. Purified CLL cells were cultured with or without DEX, the monoclonal CD20 antibody Ofatumumab (7 µg/ml), or rabbit complement (1:40 final dilution). Cells were stained with 7AAD after 48 h and analyzed by flow cytometry. Specific death is the difference between the percentages of 7AAD\(^+\) cells in control samples and treated samples. The averages and standard errors of the results for the indicated numbers of samples are shown. B. Similar experiments were carried out in the presence or absence of the indicated doses of ionomycin. C. Purified CLL cells from 52 different patients were treated with DEX in RPMI-1640, Transferrin, and 0.02% lipid-rich Albumax or with RPMI-1640, Transferrin, and 0.02% fatty acid free (FAF) albumin to remove free fatty acids that could potentially damage cell membranes in culture. Specific death was determined after 48 h and was significantly lower in FAF albumin. **, p<0.01; *, p<0.05
**DEX increases PPARα expression**

The results in Figure 2 suggested that the capacity of CLL cells to generate pyruvate was compromised by DEX. However, the relatively preserved acetate levels (Fig.2B) suggested that genes associated with fatty acid oxidation might be increased in DEX-treated CLL cells. PPARα and PPARδ are nuclear receptors involved in the regulation of fatty acid oxidation (Harmon et al., 2011; Spaner et al., 2012). DEX up-regulated PPARA and PPARD mRNA and PPARα protein expression (Fig.4A,B,D). The changes in PPARA mRNA occurred later than the down-regulation of PKM2.

Among the genes that are regulated by PPARα and PPARδ is pyruvate dehydrogenase kinase 4 (PDK4). PDK4 phosphorylates and inactivates pyruvate dehydrogenase (PDH), preventing pyruvate from being oxidized in mitochondria and promoting fatty acid oxidation (Schulze et al., 2011). Consistent with the increase in PPARα and PPARδ, PDK4 mRNA expression was also increased by DEX (Fig.4C).
Figure 4. Effect of DEX on PPARα expression. CLL cells from the indicated patient samples were treated with or without DEX for 18 h. PPARA (A), PPARD (B), and PDK4 (C) transcripts (relative to HPRT) were then measured by quantitative PCR. D. Expression of phospho-GR and PPARα proteins were also measured in 4 patients by immunoblotting, with β-actin used as a loading control. PPARα protein and mRNA were both increased by DEX at this time.
**Adipocyte-derived lipids, lipoproteins and propionic acid reverse the effects of DEX**

The decreased expression of PKM2 coupled with increased expression of PPARα, PPARδ, and PDK4 suggested that DEX might cause CLL cells to depend primarily on fatty acid oxidation as a metabolic strategy. Accordingly, they might be able to use fatty acids to generate energy, support mitochondrial activity, and resist the cytotoxic effects of GCs. To investigate this possibility, cultures of DEX-treated cells in serum-free conditions were supplemented with various forms of fatty acid oxidation substrates.

Adipocytes are a major source of fuel for fat-burning tissues and are often found in lymphoid microenvironments where CLL cells reside *in vivo* (Pond *et al.*, 2005). To test whether adipocyte-derived lipids could confer resistance to DEX, CLL cells were co-cultured with OP-9-derived adipocytes (Wollins *et al.*, 2006). OP-9 is a mesenchymal stem cell line that differentiates rapidly into adipocytes in serum-free conditions (Supplementary Fig. 7C). CLL cells were highly resistant to DEX in the presence of adipocytes, compared to CLL cells in serum-free conditions alone (Fig. 5A).

Soluble factors from adipocytes were mainly responsible for the enhanced survival of DEX-treated CLL cells since conditioned media from OP-9-derived adipocytes also prevented DEX-mediated killing of CLL cells (Fig. 5B). Much of the protective effect survived heat inactivation, suggesting it was due to lipid factors.

Lipoproteins transport lipids to tissues that use fatty acid oxidation to generate energy. Very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) increased the survival of DEX-treated cells (Fig. 5C) while high-density lipoproteins (HDL), with lower triglyceride content, had little effect (not shown). Addition of long chain (>12 carbon) fatty acids increased the death of DEX-treated CLL cells, perhaps because of additional detergent-like effects in serum-free culture, as described above (not shown). However, short-chain fatty acids and ketone bodies also failed to increase the survival of DEX-treated CLL cells, despite their capacity to be used as fuel by fat-burning tissues without causing membrane damage (not shown). Given that an intact TCA cycle is required to oxidize fatty acids in mitochondria, even-numbered short-chain fatty acids may not be able to be oxidized in DEX-treated cells because of the impaired TCA cycle (Fig. 5). However, odd-numbered fatty acids can be used for both anaplerosis (i.e. to restore depleted TCA cycle intermediates) and as a fuel source. (Brunengraber *et
Propionic acid is a short chain 3-carbon fatty acid that is first converted to propionyl coenzyme A (propionyl-CoA) and then to succinyl-CoA, an intermediate of the TCA cycle. Supplementation with propionic acid maintained the mitochondrial potential of DEX-treated cells, as measured by DiOC6(3) staining, and increased their viability (Fig.5D,E,F).

Note that supplementation with glucose or pyruvate did not increase survival of DEX-treated CLL cells (Supplementary Fig.10E), consistent with a reduced ability to oxidize glucose (Fig.2,4). Supplementation with glutaminolysis metabolites, including glutamine, glutamate, or alpha-ketoglutarate also failed to rescue DEX-treated CLL cells (Supplementary Fig.7D).
Figure 5. Effect of fatty acid oxidation substrates on DEX-mediated death. A. Adipocytes and adipocyte conditioned media (ACM) were prepared as described in the materials and methods. 6 x 10⁶ CLL cells were co-cultured with OP-9-derived adipocytes (A) or in ACM (with or without heat-inactivation) (B) in the presence or absence of DEX for 48 h. Specific death was then determined by staining with 7AAD and flow cytometric analysis. C. CLL cells from 10 different patients were cultured with or without DEX in the presence or absence of low-density lipoproteins (LDLs) or very-low-density lipoproteins (VLDLs) (1:200 and 1:100 final concentrations, respectively). The averages and standard errors of the results for each treatment are shown. D. CLL cells from 15 individual patients were cultured with or without DEX in the presence or absence of propionic acid (1 mM), an odd-numbered fatty acid that provides both acetyl-CoA and succinate to support the TCA cycle. Percentages of viable 7AAD⁻ cells were measured 48 h later by flow cytometry. Specific death is the difference between the percentages of 7AAD⁺ cells in control and DEX-treated samples. E. Mitochondrial membrane potentials of DEX-treated CLL cells supplied with propionic acid were determined after 18 h by staining with DiOC₆(3) and flow cytometric analysis. As a control, DEX-treated CLL cells were also treated with the GR antagonist Mifepristone (1 µM). An example is shown. F. Summary of DiOC₆(3) MFI measurements for 8 different patient samples, indicating that propionic acid and Mifepristone both restore mitochondrial membrane potential in DEX-treated CLL cells. **, p<0.01; *, p<0.5
PPARα and fatty acid oxidation mediate GC resistance

The findings that PPARα was upregulated by DEX (Fig.4) and fatty acid oxidation substrates prevented DEX-mediated death (Fig.5) suggested that PPARα and fatty acid oxidation might mediate resistance of CLL cells to GCs. A previously described CLL cell-line model was then used to determine the effect of increased PPARα-expression on sensitivity to DEX (Spaner et al., 2012). CD5+ Daudi cells were transfected with human PPARA and a clone was obtained with high PPARα expression (Fig.6A) (Spaner et al., 2012). DEX activated the GR in both the PPARα-expressing cell line and its empty vector control, as determined by increased phospho-GR levels (Fig.6A). However the PPARα-expressing cell line was found to be resistant to DEX treatment in comparison to the empty vector control (Fig.6B).

Conversely, circulating CLL cells treated with the small molecule PPARα-inhibitors MK886 (Spaner et al., 2012), GW6471 (Xu et al., 2002) or Compound A (Shearer et al., 2007) became more sensitive to DEX. (Fig.6C) In addition, the fatty acid oxidation inhibitor CVT-4325 (Elzein et al., 2004) also enhanced killing of CLL cells by DEX (Fig.6C, bottom right panel).

**PPARα antagonists enhance clearance of CLL cells by DEX in vivo**

The ability of MK886 to improve the efficacy of DEX against CLL cells in vivo was then studied in a xenograft model involving transfer of spleen cells from patients who have undergone therapeutic splenectomies into NOD-SCIDγcnull mice along with injections of CLL plasma to support tumor engraftment (Spaner et al., 2012; Wong et al., 2012). Human T and CLL-B cells are found mainly in spleens and peritoneal cavities of tumor-bearing mice by 6 weeks after adoptive transfer (Wong et al., 2012). Groups of reconstituted mice were then given 4 daily ip injections of either saline, MK886 at 10 mg/kg, which was shown previously to clear CLL cells from peritoneal cavities but not spleens (Spaner et al., 2012), DEX at 4.6 mg/kg (the dose in the high-dose GC regimen for CLL (Spaner et al., 2011)) or both MK886 and DEX. DEX alone did not clear CLL cells from spleen. However, the combination of MK886 and DEX decreased splenic tumor burdens better than either agent alone (Fig.6D).
Figure 6. Effect of PPARα and fatty acid oxidation inhibitors on DEX-mediated cytotoxicity in vitro and in vivo. A. CD5⁺ Daudi cells engineered to over-express PPARα and vector-control cells were treated with DEX for 48 h. Expression of PPARα and the activated, phosphorylated GR were then determined by immunoblotting and quantified by densitometry, using β-actin as a loading control. B. Percentages of viable Daudi cells that excluded 7AAD were determined by flow cytometry after 48 h. Despite strong activation of GR, DEX-treated PPARα hi cells were resistant to DEX. C. CLL cells were cultured in the presence or absence of DEX with or without the indicated concentrations of the PPARα antagonists MK886, GW6471, or Compound A and the fatty acid oxidation inhibitor CVT-4325 (GS449794). After 48 h, specific death was determined by the difference of the percentages of viable 7AAD⁻ cells in control and treated cultures as measured by flow cytometry. PPARα and fatty acid oxidation inhibitors significantly increased specific death of DEX-treated CLL cells. Averages and standard errors of the results for each inhibitor are shown. D. NOD-SCIDγc null mice engrafted 6 weeks earlier with CLL splenocytes were treated with 4 consecutive injections of MK886 (10 mg/kg), DEX (4.6 mg/kg) or both MK886 and DEX. Four days after the last injection, human CD5⁺CD19⁺ CLL cells were measured in spleen cell suspensions by flow cytometry. **, p<0.01; *, p<0.05. In vivo CLL xenograft data courtesy of Karrie Wong.
**Supplementary Figure 7.** A. Summary of MitoTracker Green MFI measurements of control and DEX-treated CLL cells performed at 4 and 18 h, indicating no significant difference in mitochondrial mass between DEX-treated and control samples. B. Viability of DEX-treated CLL cells used for intracellular metabolite measurements by NMR spectroscopy. Viability is reported as percentage of 7AAD- cells measured by flow cytometry. C. Pictures (11x) of OP9 cells taken after 5 days in differentiation media. Adipocytes are characterized by the presence of large intracellular lipid droplets. D, E. DEX-treated CLL cells were supplemented with various concentrations of L-glutamine (D) or glucose (14mM) (E). The results show that these fuel substrates could not reverse the cytotoxic effects of DEX.
2.5 Discussion

The mechanism of action of high-dose GCs in CLL is not clear. GCs alter signaling processes important for tumor growth by increasing the expression of regulatory molecules such as I-kB and phosphatases (Sapolsky et al., 2000; Flammer et al., 2011; Glass et al., 2010) or directly altering the expression of apoptotic genes (Kfir-Erenfeld et al., 2010). It is widely known that many of the complications of GC therapy, such as diabetes and hyperlipidemia, are metabolic in nature and that a major function of endogenous GCs is to turn off glucose usage while at the same time to turn on fatty acid oxidation to allow cells to survive an overnight fast (Sapolsky et al., 2000). Despite these well-known facts, the possibility that the therapeutic activity of GCs in CLL is due to metabolic effects is not widely appreciated.

The findings in this paper suggest: 1. GCs alter the expression of metabolic genes (Fig.2C, 4) and restrict metabolism in CLL cells (Fig.2). 2. GCs may prevent tumor cells from being able to activate the bioenergetic programs needed to respond to membrane damage (Fig.3). 3. GCs increase the dependence of CLL cells on fatty acid oxidation by altering their expression of PPARα and PDK4 (Fig.4). Use of fatty acids to support metabolism can alleviate restrictions imposed by GCs and thereby constitutes a resistance mechanism to GC-mediated cytotoxicity (Fig.5). 5. PPARα inhibitors may offer a strategy to improve the therapeutic efficacy of GCs in CLL (Fig.6).

It has been shown that GCs cause leukemia cells to undergo autophagy in some conditions, which is a response to energy deprivation (Grander et al., 2009). However, we could not find evidence for autophagy, such as lipidation of LC3, in the serum-free conditions employed here (not shown). Previous studies have also shown that GCs inhibit glycolysis in acute leukemia cells by restricting glucose uptake and consumption (Buentke et al., 2011). However, the effect of GCs on pyruvate kinase expression (Fig.2) have not been previously reported and may possibly be unique to CLL cells. Regardless, the concept that GCs restrict the metabolic activity of leukemia cells is appealing in that it helps explain why GCs can enhance the therapeutic effects of cytotoxic drugs in combination chemotherapy regimens.

The mechanism(s) whereby GCs down-regulate pyruvate kinase and increase PPARα expression are not clear. The effects appear to be due to transcriptional
regulation by GCs because they can be prevented by Mifepristone (Fig.1,5). GCs may act
directly at the promoters of metabolic genes to positively or negatively regulate their
expression and may also regulate the expression of microRNAs (miRs) that control
metabolic gene expression (Harada et al., 2012).

Fatty acid oxidation has been recently described as a mechanism used by cancer
cells to generate energy and survive under conditions of metabolic stress (Spaner et al.,
2012; Samudio et al., 2010; Schafer et al., 2009; Zaugg et al., 2011; Carracedo et al.,
2012). The results reported here are in keeping with these observations and suggest that
PPARα-mediated fatty acid oxidation also allows CLL cells to survive the metabolic
stress imposed by GCs. Concomitant administration of GCs and PPARα-antagonists such
as MK86, which has been used before in humans (Spaner et al., 2012), appears feasible
(Fig.6) and constitutes a novel strategy to improve the therapeutic efficacy of GC therapy
in high-risk CLL patients.
CHAPTER 3:

A role for Danazol in Chronic Lymphocytic Leukemia

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3.1 Introduction

The clinical management of chronic lymphocytic leukemia (CLL) patients with fludarabine-resistant disease or tumor cells with 17p deletions remains problematic (Gribben et al., 2011). High-dose glucocorticoids (HDGCs) are among the most effective treatment approaches for such patients, as well as elderly patients who cannot tolerate conventional chemotherapy. However, HDGCs are not without significant side effects and are only palliative in nature (Castro et al., 2009). Identification of non-toxic agents that could improve the therapeutic efficacy of HDGCs would help significantly in managing these patients. We report a case that suggests the weak androgen Danazol (Fontana et al., 2011) may be such an agent.
3.2 Results

Clinical activity and in vitro toxicity of Danazol against CLL

A 61 year old female with slowly progressive Rai Stage II CLL, diagnosed 6 years earlier and characterized by the 11q deletion with high CD38 expression (44%), was being considered for first-line chemotherapy on the basis of increased fatigue and lymphocyte doubling time (LDT) (Fig.8A). The patient also had episodal angioedema from an acquired C1 esterase inhibitor deficiency related to CLL (Cicardi et al., 2003). Prior to a dental procedure, her allergist prescribed Danazol 300 mg PO BID for one week to raise C1-inhibitor levels in order to prevent possible airway obstruction from oral angioedema (Morvacallo et al., 2010). Coincidentally, she was seen in the CLL clinic a week later where her white blood cell (WBC) count had dropped from 76 to 42 x 10⁶ cells/ml (Fig.8A) and she reported improvements in subjective symptoms. Another dental procedure was scheduled for the following month and the patient consented to give blood before and after the prophylactic course of Danazol. Remarkably, the WBC count, which had increased in the time off Danazol, decreased significantly again in response to Danazol, although it increased again in the following weeks (Fig.8A). Similar declines in circulating WBC counts have been observed in 2 male patients treated with Danazol (400 mg PO BID) for 2 months (not shown).

This apparent clinical activity of Danazol prompted laboratory studies of its effects on CLL cells. Danazol was found to be toxic to CLL cells above 3 µM (Fig.8B) although it was generally less toxic than the synthetic glucocorticoid Dexamethasone (Dex) (Fig.8C). Danazol killed both male and female CLL cells although the former appeared somewhat more sensitive (Fig.8D).
Figure 8. Effect of Danazol on CLL cells in vivo and in vitro. A. WBC counts over 12 months for the patient described in the text. One week of Danazol caused a drop in the WBC count which was confirmed by the response to a second treatment. B. CLL cells obtained from consenting patients who were untreated for at least 3 months, and with local REB approval, were purified by negative selection and cultured in serum-free media in different concentrations of Danazol (Sigma, St. Louis, MO). Exclusion of 7AAD by flow cytometry was used to measure cell viability after 48 h. Danazol-specific death is the difference in %7AAD+ cells between Danazol-treated cells and control cells. Subsequent experiments used 10 µM of Danazol. C. Example of flow cytometric analysis. Numbers in the dot-plots represent percentages of viable 7AAD cells. D. Danazol-specific death after 48 h for 6 female and 5 male patient samples. The P value for the difference between sample means was calculated using a two sample t-test.
**Danazol and Glucocorticoids have enhanced efficacy in CLL**

We considered that Danazol might have off-target effects on the glucocorticoid receptor (GR) to explain its cytotoxic activity against CLL cells. Indeed, Danazol induced phosphorylation of the GR (indicating activation of the receptor) in CLL cells although it was weaker than Dex (Fig. 9A). However, Danazol-mediated killing was not blocked by the GR antagonist Mifepristone (Johanssen et al., 2007), in contrast to Dex (not shown). Consistent with a GR-independent mechanism, Danazol had additive effects on Dex-induced cytotoxicity (Fig.9B).

We then examined the effects of Danazol specifically on CLL cells with 17p deletions (Fig.9C, bottom left panel). At a dose of 10 μM, Danazol remained capable of killing such cells, especially in combination with Dex. Compared to CLL cells without this cytogenetic abnormality, 17p- tumor cells appeared less sensitive to Danazol, with or without Dex, although the differences were not statistically significant (Fig.9C, right panel). Note the results for Fig. 9C were obtained with cryopreserved cells and the higher levels of spontaneous death following thawing account for the lower specific death levels in comparison with Fig. 9B which were obtained with fresh tumor cells.

While Danazol and low doses of glucocorticoids could potentially compete for the GR, the possibility of combining Danazol with HDGCs is supported by the example of an 85 year old female with symptomatic Rai Stage IV CLL and trisomy 12 tumor cells. Fludarabine- and alkylator-based regimens were felt to be contraindicated because of the patient’s congestive heart failure and she was treated with HDGCs (1500 mg Prednisone PO x 5 days) but suffered side effects of fluid retention and profound fatigue. Based on previous use of Danazol as a steroid-sparing agent in autoimmune disorders, the prednisone dose was lowered to 1000 mg PO daily for 4 days and Danazol (200 mg po BID) was added to the next 2 treatment cycles. The treatments were better tolerated and the clinical response was maintained and possibly even enhanced (Fig.9D).
Figure 9. Effect of Danazol and Glucocorticoids on CLL cells in vitro and in vivo. A. Purified CLL cells from 3 different patients were cultured alone, with Dexamethasone (Dex) (Sigma) (30 µM), Danazol (Dan) (10 µM) or both. After 4 h, cell lysates were collected (Tomic et al., 2011) and examined by immunoblotting with antibodies to the phosphorylated glucocorticoid receptor (pGR) (Cell Signaling, Beverley, MA), using β-actin as a loading control. The results indicate that Danazol can weakly phosphorylate and activate the GR in CLL cells. Similar results were seen with 4 other samples. B. Summary of cell viability results, measured with 7AAD-staining and flow cytometry after 48 h of culture, for 13 different patient samples. C. Cryopreserved cells from 11 additional patients, with (n=5) or without (n=6) 17p deletions determined by fluorescence in situ hybridization (FISH), were thawed, rested overnight, and examined for cell viability following treatment with Dex, Danazol, or both. Specific deaths for individual patient samples are shown in the left panels and summarized in the right graph. P-values for the differences between sample means were calculated using a single factor ANOVA test. * p<.01; ** p<.02. D. Changes in the WBC count of the patient described in the text, indicating that addition of Danazol to HDGCs can potentially enhance efficacy without increased toxicity.
3.3 Discussion

These observations suggest that Danazol has clinical activity in CLL and can be used safely with HDGCs. Danazol has been prescribed for almost 40 years (Fontana et al., 2011) and has a well-known toxicity profile. It causes virilization in young women with endometriosis, which is not necessarily a problem for older male or post-menopausal female CLL patients. Danazol is otherwise relatively safe but patients must be monitored for thrombosis and lipid and liver abnormalities (Shephed et al., 1995). Along with its use in autoimmune disorders (Cicardi et al., 2003; Boruchoy et al., 2007), Danazol is thought to have activity in myelodysplastic syndromes (Fontana et al., 2011) and can prevent Interferon-induced thrombocytopenia (Alvarez et al., 2011). Over 2500 papers on Danazol are listed in PubMED but, to our knowledge, its activity in CLL has not been reported before.

The mechanism of action of Danazol in CLL is not clear. Blood levels of CD23 were lowered by Danazol in endometriosis (Matalliotakis et al., 2000), suggesting a direct effect on activated B cells which are considered the normal counterparts of CLL cells (Tomic et al., 2011). However, we could not detect an effect of Danazol on CD23 expression by CLL cells in vitro (not shown). Danazol has been reported to decrease the production of inflammatory cytokines, including IL-6 and TNF-α, which may limit their ability to cause proliferation of CLL cells in vivo (Tanaka et al., 2009). Danazol can also intercalate into cell membranes (Horstman et al., 1995) which may disrupt growth-promoting signaling processes and account for a mechanism of action apparently independent of glucocorticoid, estrogen, and androgen receptors (Fig.8D).

The observations reported in this letter suggest clinical trials to determine if the anti-CLL effect of Danazol can be exploited to improve the clinical efficacy of HDGCs. Conventional oral doses of Danazol have been reported to achieve erythrocyte membrane levels that approach the µM levels that appear to be needed for cytotoxicity (Fig.8,9). Initial clinical studies will focus on determining if optimal dosing of Danazol in CLL patients can be achieved through the oral route (Horstman et al., 1995) or if intravenous administration will be required.
CHAPTER 4:

Discussion and Future Perspectives
In order to improve GC treatment for CLL, a better understanding of its mechanisms of action and resistance is required. The findings of this thesis demonstrate a novel mechanism of GC action that is based on its metabolic-altering effects (Chapter 2). GC cytotoxicity is mediated by changes to the metabolic programs of circulating CLL cells, which allow the CLL cells to switch from glucose oxidation to fatty acid oxidation in order to resist cytotoxicity. In order to explore new avenues to overcome GC resistance, fatty acid oxidation inhibitors were studied in combination with GCs to assess their efficacy against CLL cells in vitro and in vivo. In addition, a clinically relevant agent, Danazol was also investigated for its clinical activity against CLL cells as a single agent and in combination with GCs (Chapter 3). In this final chapter, I will discuss the importance and relevance of these findings will be presented and outline the future directions of research in this area will be discussed.

4.1 GC treatment for proliferating CLL cells

The relationship between the metabolic and cytotoxic effects of GCs in lymphocytes still remains an incomplete picture. The findings of this thesis suggest that the metabolic state of CLL cells plays an important role in GC sensitivity. I have found that in circulating CLL cells, GC treatment causes bioenergetic restriction that is responsible for mediating cell death (Chapter 2). As previously mentioned (Chapter 1), CLL disease exists in two compartments in the body: proliferation centers (PCs) in lymphoid organs and the circulation. Generally, non-dividing circulating CLL cells can be effectively cleared by conventional cytotoxic treatments. The clearance of these circulating cells results in a drastic drop in the WBC count however this does not cure the disease.

Circulating CLL cells are known to originate from PCs in lymphoid organs. In PCs, CLL cells are driven to proliferate by external signals provided by the microenvironment (Herishanu et al., 2011). These signals can originate from T cells and stromal cells found in PCs that can produce soluble factors like IL-2 (Tomic et al., 2006) and activating ligands like fibroblast growth factors (Xia et al., 2012) respectively. Since CLL cells from PCs are the source of CLL relapse following conventional treatment and GC therapy, future studies would focus on understanding and targeting the metabolic impact of GCs on proliferating CLL cells in order to improve GC treatment.
The need to eliminate tumour cells in PCs requires novel treatments to be evaluated in this compartment. However, due to the inaccessible nature of these proliferating cells in vivo modeling PCs of CLL disease in vitro is required. In general, such models include culturing circulating CLL cells with antigen mimetics, cytokines, costimulatory molecules and other cells that are encountered in vivo in PCs (Cols et al., 2012; Ding et al., 2010). Our group has shown that IL-2 along with a TLR-7/8 agonist, Resiquimod can induce CLL cells to proliferate in vitro (Tomic et al., 2006). We have also developed a stromal cell-line from a patient’s spleen that supports proliferation by secreting soluble factors that activate signaling pathways like STAT3. Based on this information, culturing CLL cells in conditioned media from this stromal cell-line along with IL-2 and Resiquimod could function as an in vitro PC model.

Our findings show that GCs invariably induce atrophy in circulating CLL cells, however the proliferating CD5+ Daudi Vector leukemic cell-line (Chapter 2) was largely unaffected. The resistance of proliferating tumour cells may explain why GCs are mostly palliative to high-risk CLL patients. Pro-survival and growth-stimulating signaling pathways are turned on in proliferating CLL cells in vitro such as phosphoinositol-3-kinase (PI3K), Akt/protein kinase B (PKB), extracellular signal-regulated kinases (ERK) and nuclear factor-κB (NFκB) (Tomic et al., 2006). Activation of these signaling pathways results in increased metabolic activity to sustain the induced proliferation. This increased metabolic activity could be sufficient to resist the energy restriction mediated by GC treatment and to maintain the TCA cycle. Congruently, GC resistance in B-acute lymphoblastic leukemia (B-ALL) is associated with increased glycolysis (Holleman et al., 2004; Hulleman et al., 2009) and treatment with the glycolysis inhibitor, 2-deoxy-D-glucose (2-DG) conferred GC sensitivity (Boag et al., 2006).

Using the in vitro PCs model, the sensitivity of proliferating CLL cells to GC treatment can be assessed. It is expected that the activating signals provided to the CLL cells will render the cells resistant to GC treatment due to their enhanced metabolic activity. Therefore, treating the proliferating CLL cells with glycolysis inhibitors (2-DG) is anticipated to overcome GC resistance. Other promising targets for inhibition include the signaling pathways that up-regulate glycolysis, including PI3K and Akt.
4.2 Role of glutaminolysis in GC-mediated cytotoxicity

Based on the findings in this thesis, I have established that bioenergetic restriction is a major mechanism of GC-mediated cytotoxicity in circulating CLL cells (Chapter 2). I have shown that the primary lesion involves the down-regulation of a crucial glycolytic enzyme, PKM2. The down-regulation of PKM2 and in turn of glucose metabolism in the mitochondria leads to a compensatory up-regulation of fatty acid oxidation gene programs including, PPARα, PPARδ and PDK4. This thesis has demonstrated the key role PPARα-mediated fatty acid oxidation plays in GC resistance and established the major role metabolism plays in GC-mediated cytotoxicity in malignant lymphocytes.

Another major source of cellular energy is glutamine. Glutamine is the most abundant amino acid in the human circulation and can contribute to the TCA cycle via glutaminolysis (Newsholme et al., 1985). Glutaminolysis is a series of biochemical processes that degrades glutamine to substrates that can support the TCA cycle. Glutamine is converted to glutamate, which is then converted to α-ketoglutarate, an important anaplerotic compound that maintains the TCA cycle in the mitochondria (Daye et al., 2012).

Glutaminolysis occurs in proliferating cells like lymphocytes and tumour cells (Newsholme et al., 1985). Most tumour cells rely on glutaminolysis in addition to glycolysis to fulfill their high-energy requirements for growth (Maruzek and Eigenbrodt, 2003). Theoretically, the upregulation of glutaminolysis gene programs could allow proliferating leukemic cells to utilize alternate fuels to overcome the GC-mediated energy restriction. The contribution of glutaminolysis to proliferating CLL cells’ metabolism and sensitivity to GC treatment can be assessed using the in vitro PCs model.

A key glutaminolysis enzyme, glutaminase catalyzes the conversion of glutamine to glutamate. There are two isozymes of glutaminase: glutaminase (GLS), the liver isozyme, and glutaminase 2 (GLS2), the mitochondrial isozyme. GCs are known to up-regulate GLS expression in human lymphocytes and intestinal epithelial cells (Dudrick et al., 1993; Sarantos et al., 1994). It has been shown inhibiting mitochondrial GLS activity prevents oncogenic transformation (Wang et al., 2010). GC-regulation of the expression of these two GLS isozymes can be determined by Q-PCR and Western blotting. There are GLS inhibitors available such as 6-diaz-o-5-oxo-L-norleucine (DON) (Willis and
Seegmiller, 1977), which can be tested to see if they can overcome GC resistance in proliferating CLL cells.

4.3 Rationale for combinatorial GC treatment of CLL disease

GCs are commonly combined with other therapeutic modalities like chemotherapy and immunotherapy for the treatment of CLL disease. The efficacy of monoclonal antibodies such as Rituximab, Ofatumumab and Alemtuzumab, is significantly increased when combined with HDGCs (Bowen et al., 2007; Spaner et al., 2011; Pettitt et al., 2012). The work in this thesis shows that circulating CLL cells are sensitive to GC treatment in the presence of membrane-damaging agents (Chapter 2).

GC treatment causes bioenergetic restriction, which prevents cells from repairing the membrane damage caused by processes such as complement-mediated cytotoxicity. This observation provides an explanation for the enhanced efficacy of both chemotherapy and immunotherapy treatment modalities that have been successfully combined with GCs for the treatment of leukemias. Also, considering that the GC mechanism of action is metabolic in nature and there does not appear to be any correlation between cytogenetic abnormalities and GC efficacy, this suggests that GC treatment is a valid option for all CLL patients regardless of cytogenetic status.

4.4 Mechanism of GC-mediated down-regulation of PKM2

Following my findings that GC treatment causes bioenergetic restriction in circulating CLL cells, I also determined that GC treatment resulted in a rapid down-regulation of the enzyme, pyruvate kinase muscle isozyme 2 (PKM2) (Chapter 2). PKM2 is a kinase that is responsible for transferring a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP) to generate pyruvate and adenosine triphosphate (ATP) (Mazurek, 2011). Pyruvate feeds into the tricarboxylic acid (TCA) cycle in the mitochondria and goes on to generate acetyl coenzyme A. The TCA cycle is responsible for the generation of ATP, which is the major source of cellular energy (DeBerardinis et al., 2008). Given that the mRNA and protein expression level of an enzyme may not necessarily reflect its enzymatic activity, it would be important to directly measure
PKM2 activity in response to GC treatment. Measuring PKM2 enzymatic activity can be achieved using an enzymatic cycling microplate-based system developed for the quantitative determination of low levels of mammalian metabolic enzymes (Janke et al., 2010).

Another important aspect for consideration is the mechanism of GC-mediated down-regulation of PKM2. Down-regulation of PKM2 mRNA transcript was detected early following GC administration, thereby implicating a direct effect. GCs can differentially regulate the expression of metabolic genes, like PKM2 by directly interacting with the promoters of target genes. GCs are also known to regulate the expression of microRNAs (miRs), which can inhibit the expression of a variety of genes (Harada et al., 2012; Smith et al., 2010). miR-mediated repression of target gene expression is a quick process, which corresponds with the early event of GC-mediated PKM2 down-regulation. Future studies will investigate the involvement of miRs with the GC-mediated down-regulation of PKM2. Additionally, a potential mechanism of action for proliferating CLL cells’ GC resistance (Section 4.1) could be the prevention of PKM2 down-regulation, which will also be investigated in future studies.

4.5 Involvement of miR-17 in GC-induced cell death

miRs are a class of small, non-coding RNAs around 22 nucleotides in length that negatively regulate gene expression by either inducing degradation or translational inhibition of target mRNAs (Babashah and Soleimani, 2011). miRs are initially transcribed and processed in the nucleus before being exported into the cytoplasm where they undergo further processing to generate mature miRs (Figure 13). Mature miRs recognize and target the 3’-untranslated region (3’-UTR) of specific mRNAs that contain a complementary target site. Depending on the degree of complementarity between the mRNA target sites and the nucleotide sequence from position 2-8 at the 5’ end of the miRs (the seed region), there are two potential mechanisms of regulating target gene expression: Ago-catalyzed cleavage of target mRNA when the miR has perfect or near perfect complementarity or repression of translation when the miR has imperfect complementarity to the target mRNA (Hutvagner and Zamore, 2002).
The aberrant expression of miRs has been found in various types of solid tumours and leukemias (Calin and Croce, 2006; Babashah et al., 2012). The first evidence of the involvement of miRs in hematological malignancies was actually described in CLL. The miR-15a/miR-16-1 cluster resides on chromosome 13q14.3, a genomic region frequently lost or down-regulated in CLL, which was found to be associated with the indolent form of disease (Calin et al., 2002). Interestingly, some of the direct targets of the miR-15a/miR-16-1 cluster are mRNAs encoding gene products that are involved in regulating cell proliferation and apoptosis such as cyclins (CCND1 and CCND3), cyclin-dependent kinases (CDK6) and B-cell lymphoma-2 (Bcl-2) (Cimmino et al., 2005; Klein and Dalla-Favera, 2010). Other miRs that are known to be involved in CLL disease include miR-155, miR-34a, miR29b, and miR-181b (Dijkstra et al., 2009; Fulci et al., 2007; Pekarsky et al., 2006).

miRs are involved in controlling several cellular processes altered in cancer, such as proliferation, differentiation and apoptosis (Babashah et al., 2012). Considering the ability of GCs to induce apoptosis in lymphocytes (Dougherty et al., 1943; Pearson et al., 1949), recent studies have started investigating the relationship between GCs and miRs particularly with regards to leukemia. It has been shown that several miRs were regulated by GCs such as the myeloid-specific miR-223 and the apoptosis and cell cycle arrest-inducing miR-15a/miR-16-1 cluster in ALL cell lines (Rainer et al., 2009).

Other groups have also shown that miRs are severely repressed and the expression of miR processing enzymes are significantly reduced during GC-induced apoptosis (Harada et al., 2012; Smith et al., 2010). Specifically, the conserved miR-17-92 cluster was found to be a prime target of GC-induced repression, which mediated subsequent cell death in ALL cell lines (Harada et al., 2012; Smith et al., 2010). Inhibition of the level of miR-17 expression was shown to increase GC sensitivity and overexpression of miR-17 was found to induce GC resistance (Harada et al., 2012). The target of miR-17 was determined to be the pro-apoptotic protein, Bim. These findings suggest a model of GC-induced cell death that is mediated by the sequential down-regulation of miR-17 and up-regulation of Bim.

No specific miR response has been determined in GC-treated CLL cells. However, based upon the quick down-regulation of PKM2 in response to GC treatment
(Chapter 2), it is possible that GCs could up-regulate the expression of miR-17 in CLL cells. Future studies would focus on determining the effects of GCs on miR-17 expression in CLL cells. The potential miR-17 binding site on the PKM2 mRNA transcript needs to be identified and verified using a luciferase reporter assay. The CD5^+ Daudi cell line would also need to be engineered to overexpress miR-17 in order to assess the effects of miR-17 on PKM2 expression and GC-induced cell death. In addition, there are commercially available miR inhibitors (Qiagen miScript miRNA inhibitors) that can be used to inhibit miR-17 expression in primary CLL cells to verify its effects PKM2 and GC-induced cell death. To assess the in vivo effects of miR-17 on CLL cells, there are miR mimics available from Qiagen that can be injected into the CLL mouse model used in these studies.

4.6 Concluding remarks

Based on the findings presented in this thesis, emphasis needs to be placed on exploring metabolic inhibitors as a valid option for enhancing the effects of GC therapy in CLL. These metabolic inhibitors would allow for more efficient bioenergetic restriction by preventing tumour cells from harnessing energy from alternative sources, which would prevent GC resistance and enhance GC efficacy. More specifically, fatty acid oxidation is emerging as a common metabolic strategy of resistance in tumour cells. In the case of CLL, PPARα has emerged as a mediator of resistance against metabolic and cytotoxic stresses (Spaner et al., 2012) and therefore a promising therapeutic target especially with regards to GC treatment (Chapter 2).
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