Identifying the optimal corticosteroids to target acute myeloid leukemic stem cells

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August 2021

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract

Acute myeloid leukemia (AML) is a cancer of the blood characterized by an increase in the number of immature blood cells (blasts) in the bone marrow, which cause hematopoietic insufficiency. Chemotherapies used for AML have mostly stayed the same for the last 40 years and involve extremely intense toxic treatment, yet approximately 40% of AML is resistant to initial treatment or eventually relapse. The survival rate 5 years after diagnosis of pediatric AML patients is poor (approximately 60%) and accounts for almost half of the leukemic deaths in children. This is partially due to the chemoresistant nature of the leukemic stem cells (LSCs) that sustain the disease. Compounds that specifically target LSCs while sparing normal hematopoietic stem cells (HSCs) may significantly improve patient outcome and lower toxicity across a broad leukemia spectrum in adolescent and young adult (AYA) and pediatric patients. After conducting an initial drug screen, our lab was able to identify the glucocorticoids mometasone, halcinonide and budesonide as compounds that can target LSCs while sparing HSCs. Corticosteroids have been shown to trigger apoptosis in some AML. However, we observed that these glucocorticoids effectively eliminated LSCs by driving them to terminally differentiate. In an effort to find the optimal corticosteroids to target LSCs, I examined a broad set of 24 corticosteroids using an *in vitro* drug screen and high-throughput flow cytometry to identify those that can target LSCs more effectively than the three glucocorticoids from the initial drug screen. The results showed that the most effective compounds were from the same chemical structure groups as the three glucocorticoids from the initial screen. This demonstrated a correlation between the molecular structures of these corticosteroids and their anti-LSC activity. Further examination of their molecular structures showed that compounds with increased anti-LSC activity possessed the C1-C2 double bond, C6/C9 fluorine, C16 methyl group, C16-C17 acetonide group and C17 ester.

These structures have been shown to increase binding affinity for the glucocorticoid receptor and corticosteroid potency. From the 24 corticosteroids examined, fluticasone propionate was identified as the ideal compound for targeting LSCs. It showed superior anti-LSC activity compared to the three corticosteroids from the initial screen and its molecular structure possesses all the necessary structures to increase anti-LSC activity. Altogether these findings suggest that the mechanism of action of corticosteroids in LSCs is through the glucocorticoid receptor and that corticosteroids can possibly be an effective therapy against AML LSCs.

Résumé

Les leucémies aigues myéloïdes (LAM) sont des cancers du sang caractérisés par une augmentation en nombre des cellules sanguines immatures (blastes) dans la moelle osseuse, ce qui cause une insuffisance hématopoïétique. La chimiothérapie contre les LAM n'est pas trop changé durant les quatre dernières décennies. Alors qu'il s'agit d'un traitement intensif et extrêmement toxique, 40% des cas sont résistants et rechutent éventuellement. Le taux de survie à 5 ans après diagnostic pour les LAM pédiatriques est faible (~60%), et corresponds à la moitié des décès liés aux leucémies chez les enfants. Cela est partiellement dû à la chimiorésistance des cellules souches leucémiques (CSL) qui maintiennent la maladie. Des molécules ciblant les CSL d'une manière spécifique, tout en épargnant les cellules souches hématopoïétiques (CSH) normales, pourraient améliorer la réponse des patients et diminuer la toxicité au travers un spectre large des leucémies des adolescents et jeunes adultes (AJA) ainsi que les enfants. À la suite d'un criblage initial de drogues, notre laboratoire a identifié les glucocorticoïdes mometasone, halcinonode et budesonide comme molécules qui peuvent cibler les CSL sans affecter les CSH normales. Il était montré que les corticostéroïdes peuvent déclencher l'apoptose pour certains LAM. Néanmoins, nous avons observé que ces glucocorticoïdes peuvent éliminer les CSL, d'une manière efficace, grâce à une induction de leur différentiation terminale. Pour identifier les corticostéroïdes optimaux contre les CSL, j'ai examiné une liste de 24 corticostéroïdes en utilisant un criblage de drogues in vitro, couplé à une analyse cytométrique à haut-débit, dans le but de trouver des candidats ciblant les CSL d'une manière plus efficace que les trois composés initialement identifiés. Les résultats ont montré que les composés les plus efficaces appartiennent aux mêmes groupes structuraux que les glucocorticoïdes trouvés par le criblage initial. Cela montre une corrélation entre les structures moléculaires de ces

corticostéroïdes et leur activité anti-CSL. Une examination plus profonde a montré que les composés ayant une forte activité anti-CSL possèdent les modifications suivantes : une double-bande C1-C2, un fluor en C6/C9, un groupe methyl en C16, un groupe acétonide en C16-C17, ainsi qu'un ester en C17. Ces structures ont été montrées d'augmenter l'affinité de liaison au récepteur des glucocorticoïdes ainsi que la puissance des corticostéroïdes. Parmi les 24 corticostéroïdes examinés, le propionate de fluticasone a été identifié comme composé idéal contre les CSL ; il a montré une activité anti-CSL supérieure à celles des 3 corticostéroïdes identifiés par le criblage initial, et ça structure moléculaire possède toutes les structures nécessaires pour augmenter l'activité anti-CSL. Enfin, ces résultats suggèrent que le mécanisme d'action des corticostéroïdes contre les CSL implique le récepteur des corticostéroïdes, et que les corticostéroïdes pourraient être utilisés pour une thérapie efficace contre les CSL des LAM.

Acknowledgements

I would like to thank my supervisor Dr. Kolja Eppert for allowing me to join his lab and for giving me the opportunity to advance my education and knowledge of science. Thank you for your endless guidance and support throughout my project. I would also like to thank my academic advisor Dr. Inés Colmegna and thesis committee members Dr. Bertrand Jean-Claude and Dr. Michael Sebag for their questions, feedback and guidance. In addition, I am grateful to Dr. Bertrand Jean-Claude and Dr. Chris Williams for their assistance with the molecular modeling part of this project. I would also like to acknowledge Dr. Guy Sauvageau and his lab at the Institute for Research in Immunology and Cancer of the Université de Montréal for providing us with the corticosteroids used in this project.

To the past and present members of the Eppert lab Andrea, Meaghan, Heather, Patricia, Hassan and especially Isabella thank you for taking the time to assist me with training, experiments and presentations. It was a pleasure getting to know you and being able to work with you. You guys were such a strong source of support, and I could not have done this without you.

Thank you to my close friends for their support and kind gestures. And above all a special thank you to my family, my wife Angela, son Ethan, daughter Leila and mother Pamela who have stood beside me and supported me continuously on this journey. Thank you for your words of encouragement in those moments when I needed it most and for bringing joy into my life. Finally thank you to my father the late Errol Best for teaching me the value of education and for nurturing my interest in science, I know that he would have been proud to see me complete this master's degree.

Contribution to original knowledge

Corticosteroids have been previously shown to target bulk acute myeloid leukemia cells by apoptosis and differentiation. In this thesis project I was able to identify corticosteroids that can target leukemic stem cells more effectively than previously identified corticosteroids. I was further able to identify the key structural groups of the corticosteroids which allows them to induce their anti-LSC effect through differentiation. I also demonstrated that the leukemic stem cell differentiation is through the glucocorticoid receptor and that it is not simply an off-target effect. This was shown by the correlation between the corticosteroid functional groups that cause the anti-LSC effect and the functional groups that are required for glucocorticoid receptor binding. In addition, I demonstrated that the difference in effectiveness of the corticosteroids on leukemic stem cells is due to their ability to bind the glucocorticoid receptor. These findings provide insight into the mechanism of action of corticosteroids in leukemic stem cells, which was previously unknown. They also represent the first step in identifying the optimal corticosteroid for targeting leukemic stem cells, which can be further developed in the future.

Contribution of Authors

Chapter 1: The literature review and writing of chapter 1 was done by LeRon Best. Editing of chapter 1 was done with the assistance of Kolja Eppert.

Chapter 2: The experimental design for the *in vitro* drug screen was done by LeRon Best, Kolja Eppert, and Isabella Iasenza. Dr. Bertrand Jean-Claude conceived the idea for the structural-functional relationship of the corticosteroids. The experimental design to determine the structural-functional relationship for the corticosteroids was done by LeRon Best. Chapter 2 was written by LeRon Best. Editing of chapter 2 was done with the assistance of Kolja Eppert and Hassan Dakik.

Chapter 3: The *in vitro* drug screen of the corticosteroids was performed by LeRon Best. Data analysis of results was performed by LeRon Best and Kolja Eppert. Data analysis figures were constructed by LeRon Best. The structural-functional relationship analysis of the corticosteroids was performed by LeRon Best. Molecular modeling and molecular modeling figures were done by Dr. Chris Williams. Chapter 3 was written by LeRon Best. Editing of chapter 3 was done with the assistance of Kolja Eppert.

Chapter 4: The literature review and writing of the discussion for chapter 4 was done by LeRon Best and edited by Kolja Eppert.

List of Abbreviations

5hmC: 5-Hydroxymethylcytosine

5mC: 5-methylcytosine

ABCC1: ATP Binding Cassette Subfamily C Member 1

ADCC: Antibody-dependent cellular cytotoxicity

AF1: Activation function 1

AF2: Activation function 2

AML: Acute myeloid leukemia

Allo-HSCT: Allogeneic hematopoietic stem cell transplant

AP-1 Activator protein 1

ASXL1: Additional Sex Combs Like 1

CBC: Complete blood count

CBL: Casitas B-lineage lymphoma gene

CEBPA: CCAAT enhancer binding protein alpha

CH: Clonal hematopoiesis

CHIP: Clonal hematopoiesis of indeterminant potential

CLL-1: C-type lectin-like molecule-1

CML: Chronic myeloid leukemia

CO2: Carbon dioxide

CSC: Cancer stem cell

DNA: Deoxyribonucleic acid

DNMT3A: DNA (cytosine-5)-methyltransferase 3A

EC 50: Effective concentration 50

ELN: European LeukemiaNet

FACS: Fluorescence activated cell sorting

FDA: Food and drug administration

FLT3: FMS-like tyrosine kinase 3

G-CSF: Granulocyte colony stimulating factor

GNAS: Guanine nucleotide binding protein, alpha stimulating activity polypeptide

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

HSC: Hematopoietic stem cell

IC50: Inhibitory concentration 50

IDH1: Isocitrate dehydrogenase 1

IDH2: Isocitrate dehydrogenase 2

IL: Interleukin

ITD: Internal tandem repeat

JAK2: Janus kinase 2

Ki: Binding affinity

KRAS: Kirsten rat sarcoma viral oncogene homolog

LBD: Ligand binding domain

LIC: Leukemia initiating cell

LRP: Lung resistance-related protein

LSC: Leukemic stem cell

MDS: Myelodysplastic syndrome

MOE: Molecular operating environment

MPN: Myeloproliferative neoplasm

MPP: Multipotent progenitor

MRD: Minimal residual disease

MRP: multidrug resistance-related protein

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

nM: Nanomolar

NK: Natural killer

NOD/SCID: Nonobese diabetic/severe combined immunodeficient

NPM1: Nucleophosmin 1

NR3C1: Nuclear receptor subfamily 3 group c member 1

NRAS: neuroblastoma RAS viral oncogene homolog

NTD: N-terminal transactivation domain

PBS: Phosphate buffered saline

PDB: Protein Data Bank

PHF6: Plant homeodomain-like finger 6

PPM1D: Protein phosphatase 1D

RMSD: Root-man-square distance

RNA: Ribonucleic acid

RUNX1: Runt-related transcription factor 1

SCF: Stem cell factor

SCID: Severe combined immunodeficiency

SF3B1: Splicing factor 3b subunit 1

SFEM II: Serum-free expansion medium II

SIRP1- α: Signal regulatory protein α

SR1: StemRegenin 1

SRSF2: Serine and arginine rich splicing factor 2

TDZD-8: 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione

TET2: Ten-eleven translocation 2

TP53: Tumor protein p53

TPO: Thrombopoietin

TRAF 1: TNF receptor-associated factor 1

TRAF 2: TNF receptor-associated factor 2

VAF: Variant allele frequency

WHO: World health organization

WT1: Wilms tumor 1

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Chapter 1: Introduction

1.1 Overview

Acute myeloid leukemia is a severe, heterogeneous, hematological malignancy characterized by an increase in the number of immature blood cells of myeloid lineage (myeloblasts) in the bone marrow and peripheral blood. These cells are proliferative and clonal in nature and cause hematopoietic insufficiency leading to neutropenia, anemia, thrombocytopenia and subsequently death [1-4]. AML is the most common form of acute leukemia in adults and is associated with decreased survival. It is also the second most common acute leukemia in children constituting approximately 20% of all pediatric acute leukemias and accounts for approximately half of the leukemic deaths in this population [4-6]. Over the last 30 years the incidence of AML has risen from 18.0% of the total leukemia cases worldwide in 1990 to 23.1% in 2017 and will likely continue to rise [7]. This is in part due to an increasing aging population in developing countries and improved cancer detection and diagnosis. The likelihood of being diagnosed with AML increases with age, adults aged 65 years and older have the highest incidence rates and generally the poorest prognosis [4, 7, 8].

Drugs used in the standard treatment for AML have essentially remained the same for the past 40 years. They consist of intense cytotoxic induction chemotherapy with cytarabine and anthracyclines (daunorubicin or idarubicin) [8]. This is then followed by consolidation therapy that utilizes further rounds of chemotherapy to eradicate any minimal residual disease that may cause relapse. These treatments are highly toxic not only to the leukemia cancer cells but also to the patient causing serious adverse side effects. Although most patients do achieve remission with current treatment, up to 70% of adults and 30% of children have refractory disease or

eventually relapse and do not survive beyond five years after their initial response [3, 5, 9]. This relapse is caused by underlying minimal residual disease that is to some extent driven by the chemo-resistant nature of the leukemic stem cells that sustain the disease [10-12]. These cells are relatively resistant to standard therapies such as daunorubicin and cytarabine and this is partially due to their increased expression of multidrug resistance genes, for example ATP Binding Cassette Subfamily C Member 1/lung resistance-related protein (ABCC1/LRP) and their quiescent state, that reduce the effects of cytotoxic agents that normally target rapidly replicating cancer cells [13-15].

While modern molecular testing has guided the development of new therapies that can specifically target gene mutations and cell survival pathways in bulk AML cells, very few therapies specifically target LSCs that are the underlying cause of AML relapse. One difficulty in finding a therapy that can target LSCs is that these cells are similar to the normal HSCs that sustain hematopoiesis. This similarity creates challenges for the development of drugs that target LSCs for elimination without concomitantly targeting HSCs. Using computational analysis and *in vitro* screening with AML 8227, a heterogenous leukemia with mutations in p53, RUNX1 and FLT3-ITD, our lab was able to identify the corticosteroids mometasone furoate, budesonide and halcinonide as compounds that can target LSCs and spare HSCs. Our results showed that these corticosteroids were not only able to decrease the number of LSCs but also increase the number of blast cells. These results suggest that corticosteroids may be useful as part of a treatment regimen which can possibly prevent AML relapse by eliminating LSCs. This research project represents the next step in preclinical optimization of corticosteroids to determine the ideal compound for treatment of AML.

1.2 Leukemia

Leukemia is a heterogenous group of malignancies of the white blood cells. These malignancies originate from dysfunctional proliferation of developing white blood cells (leukocytes) in the bone marrow [16]. They can be classified as either acute or chronic according to the degree of cell differentiation and as myelocytic or lymphocytic according to the predominant type of cell involved [16]. Acute leukemias are characterized by a rapid increase in the number of immature blood cells called “blasts”. These blasts crowd the bone marrow, impeding the production of normal healthy blood cells. This results in anemia from low erythrocyte production, bleeding due to low platelets and infection from dysfunctional leukocytes. Having more than 20% blasts in the blood or bone marrow is required for a diagnosis of acute leukemia [16, 17]. Chronic leukemias are characterized by a gradual increase in the number of more mature leukocytes that are not completely normal. These leukemias may take months to years to develop and may be diagnosed incidentally as patients are usually asymptomatic [18]. Chronic leukemia can however, present with anemia, thrombocytopenia, and leukopenia; bruising and bleeding are less common than in acute leukemias [16]. Myelocytic leukemias are derived from cells of the myeloid lineage which include neutrophils, basophils, eosinophils, monocytes, erythrocytes, and megakaryocytes. Lymphocytic leukemias are derived from cells of the lymphoid lineage which consist of T cells, B cells, and natural killer cells [16].

AML is the most common acute leukemia in adults and accounts for almost half of the leukemia cases diagnosed in children and young adults [16]. In AML, chromosomal translocations, rearrangements, and gain or loss of chromosomes can lead to mutations and abnormal production of myeloblasts [16]. To diagnose AML various phenotypic markers can be used to identify myeloblast via immunophenotyping using flow cytometry, along with cytogenetic testing. The

markers used depend on the specific subtype of AML to be identified and the associated genetic mutations, they include but are not limited to CD4, CD7, CD11, CD13, CD14, CD15, CD33, CD34, CD36, CD38, CD45, CD56, CD61, CD64, CD117 and CD163 [19].

Acute lymphocytic leukemia (ALL) is the most common pediatric leukemia. It occurs when lymphocytes fail to develop into mature B cells and T cells leading to an increase in the production of lymphoblast. Abnormalities in chromosome number or structure are found in approximately 90% of children and 70% of adults with ALL [4]. Approximately 85% of ALL cases are of the B-lineage and express CD10, CD13, CD19, CD22, CD33, CD34 and CD79a. T-ALL accounts for 15–20% of cases and express CD1a, CD2, CD3, CD4, CD5, CD7, CD87 and CD34 [4]. Besides the use of these markers, cytogenetic testing such as fluorescence in situ hybridization (FISH) can be used to diagnose ALL.

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults, affecting older adults around age 65 [20]. It is characterized by the accumulation of mature clonal B lymphocytes in the blood, bone marrow and secondary lymphoid tissue. These lymphocytes express CD5, CD19, CD23 and low levels of CD20. Expression of this immunophenotypic profile is diagnostic of CLL if all these markers are expressed [21]. The diagnosis can also be made if there are at least 5 x 10^9/L clonal B lymphocytes in the peripheral blood [20].

Chronic myeloid leukemia (CML) is characterized by the excessive proliferation of mature myeloid cells (granulocytes) in the bone marrow. This proliferation is due to the occurrence of the Philadelphia chromosome which is the result of a translocation t(9;22)(q34;q11) between the long arms of chromosomes 9 and 22 with the derivative chromosome 22, der(22)t(9;22), being the fusion gene called BCR-ABL1 (Philadelphia chromosome) [22]. Identification of the

Philadelphia chromosome by cytogenetic testing is diagnostic of CML. Most cases of CML occur between ages 25 to 60 [16].

The treatment of leukemias depends on the leukemia subtype, cytogenetic and molecular findings, patient age, and comorbidities. For acute leukemias chemotherapy is usually the mainstay of most regimens; however, radiation therapy, monoclonal antibodies, and stem cell transplantation may be used [16, 18]. Early stage CLL may be monitored without treatment, while its active or late stages may be treated with chemotherapy or stem cell transplantation [18, 21]. CML is treated with tyrosine kinase inhibitors e.g imatinib, that inhibit the tyrosine kinase enzyme coded for by the fusion gene BCR-ABL1 which causes the uncontrolled cellular proliferation. While this treatment is not curative, it does offer long term control of the disease without the adverse effects of chemotherapy [18].

The prognosis of AML, ALL and CLL is variable and depends on the leukemia subtype, the genetic mutation present, the patients age and existing comorbid conditions. However, for CML the use of tyrosine kinase inhibitors gives this leukemia a good prognosis [4, 21, 22].

1.3 Acute myeloid leukemia

1.3.1 Etiology of acute myeloid leukemia

Leukemogenesis in AML is not entirely understood; however, what is known is that hematopoietic stem cells (HSCs) and or progenitor cells undergo oncogenic transformation from acquired mutations to form a leukemic clone that is capable of self-renewal and proliferation leading to the development of AML[4, 23]. AML can develop de novo (as occurs with the majority of cases) or it can be acquired secondarily where it has a worse prognosis. De novo development is associated with acquired genetic abnormalities, including cytogenetic changes

and somatic mutations [4]. Acquired chromosomal abnormalities account for approximately 50–55% of de novo AML cases [24]. Somatic mutations are acquired during clonal hematopoiesis and commonly occur in DNMT3A, ASXL1, and TET2 genes [25]. Other mutations include DNA methylation genes (IDH1, IDH2), tumor suppression genes (TP53, WT1, PHF6), signal transduction genes (FLT3, KIT, KRAS/NRAS), nucleophosmin (NPM1) and myeloid transcription factors genes (RUNX1, CEBPA) [26]. Somatic mutations in HSCs increase with age and have been linked to an increased risk of developing hematologic malignancies, cardiovascular disease, and all-cause mortality [27].

Secondary AML develops from an antecedent hematologic disorder, for example myelodysplastic syndrome, myeloproliferative neoplasms or myelodysplastic/myeloproliferative neoplasms (chronic myelomonocytic leukaemia). Aplastic anemia and paroxysmal nocturnal hemoglobinuria can also develop from a similar process as clonal hematopoiesis and are associated with AML [28, 29]. Prior exposure to cytotoxic therapies for the treatment of a primary malignancy is another cause of secondary AML. This type of AML termed “therapy-related AML (t-AML)” can be induced by alkylating agents (e.g cisplatin), radiation therapy and topoisomerase II inhibitors (e.g doxorubicin). Exposure to alkylating agents or radiation therapy can lead to progression to myelodysplasia or AML after approximately 4-7 years. This type of exposure is associated with a high incidence of cytogenetic abnormalities involving chromosomes 5 (−5/del(5q)) and 7 (−7/del(7q)). Topoisomerase inhibitors exposure has a latency period of 2-3 years and presents as overt acute leukemia. This type of exposure is associated with balanced translocations involving chromosome bands 11q23 or 21q22 [30].

Environmetal factors also play a role in developing AML. Exposure to benzenes, pesticides, organic solvents and ionizing radiation as well as lifestyle factors such as smoking, and obesity

have also been associated with increased risk. These factors cause DNA damage and associated genetic changes that have been associated with AML [4].

1.3.2 Leukemogenesis and clonal hematopoiesis

Over the years advancements in genomic sequencing have made it easier to detect genetic and epigenetic mutations. Large-scale studies have now identified the common mutations in hematologic malignancies and have further motivated the search to determine the cause of pre-cancerous lesions in hematopoiesis at the cellular and molecular level [25]. There is increasing evidence, that as aging occurs cells acquire somatic mutations or encounter environmental factors that induce mutations [29, 31, 32]. These mutations may have several effects causing downregulation, upregulation or no change at all in gene function. Initially acquired mutations which upregulate gene function, may increase cell growth leading to propagation with resulting clonal expansion. Clones can originate from pluripotent HSCs or more committed myeloid or lymphoid progenitor cells [33]. When an HSC or its progenitors acquires a somatic mutation that promotes increased self-renewal, proliferation, and/or reduced cell death, it gives these cells a fitness advantage allowing them to gain the capacity to expand at a disproportionate rate compared to other stem cell or progenitor clones which have not acquired the mutation. This fitness advantage relative to other clonal lineages is the defining feature of clonal hematopoiesis (CH) that can be defined as the expansion of a clonal population of blood cells from a single stem cell or progenitor with one or more somatic mutations [25, 29]. While many acquired mutations that occur in CH do not progress to malignancy, its occurrence may represent an antecedent permissive state for leukemic development, where secondary and/or tertiary mutations may result in a full-blown acute myeloid leukemia [29, 33-38].

Two models to explain leukemogenesis have been proposed. The first is by the HSC acquiring an initial transformation mutation that produces a self- renewing preleukemic clone and, in some cases, CH. These preleukemic clones consist of cells that have self-renewal and differentiation properties as well as more differentiated cells that lack these abilities. The self-renewing clones then acquire additional mutations and give rise to what is known as the leukemic stem cells also termed leukemia-initiating cells (LICs) because of their ability to initiate leukemia after transplantation. In the second model a multipotent progenitor cell (MPP) acquires a mutation that allows the MPP cell to regain its self-renewal program. It then acquires further mutations to become a LSC [39, 40]. Like normal hematopoiesis where the HSC sits at the apex of the hierarchy and gives rise to progenitors that later differentiate into more mature progeny. The LSC has a similar hierarchical structure with the capacity to give rise to leukemic progenitors that differentiate into leukemic blast that characterise AML.

Evidence of clonal hematopoiesis was first demonstrated by Busque *et al*. in a study that looked at the incidence of skewing (preferential inactivation) in X chromosome inactivation ratios using peripheral blood from healthy females. This study found that the incidence of skewing increased with age, occurring in 38% of healthy women over the age of 60 years and was less frequent in younger females [41]. The results also suggested that besides being age related, CH could be a premalignant state where mutated cells could acquire somatic mutations causing them to gain an advantage that could later progress to a malignant state if further mutations are acquired [33]. Further to this Busque showed that some women with skewed X chromosome inactivation also carried TET2 mutations which are common in patients with myeloid malignancies [33, 42].

Exome sequencing of peripheral blood cells of 30000 individuals from three large cohort studies has shown that CH increases with age [27, 43, 44]. Somatic mutations have been found in 18%

of people over the age of 90 years and 10% of people greater than 65 years, however they are rare in people under 50 years [33]. The reported frequency of CH however increases when more sensitive methods are used to reveal gene mutations at very low variant allele frequencies (VAFs) [45, 46]. To determine clonal hematopoiesis a VAF of at least 2% is required [47, 48]. Where there are detectable somatic mutations, but no presence of a hematological malignancy CH is known as clonal hematopoiesis of indeterminate potential (CHIP).

The most commonly identified mutated genes in CH are epigenetic regulation genes. These include DNMT3 and TET2 which regulate DNA methylation and ASXL1. Mutations in these three genes account for two thirds of the driver mutations that cause clonal hematopoiesis [25]. DNA (cytosine-5)-methyltransferase 3A (DNMT3A) is a DNA methyltransferase responsible for de novo DNA methylation, it transfers methyl groups to form 5-methylcytosine (5mC) at specific CpG sites in DNA [49]. Ten-eleven translocation-2 (TET2) is a methylcytosine dioxygenase that converts 5mC to 5 -hydroxymethylcytosine (5hmC), as an initial step in DNA demethylation [50]. Mutations causing loss of function in DNMT3A are associated with hypomethylation, while loss of TET2 is associated with DNA hypermethylation [29, 51, 52]. Investigations in mouse models have shown that deficiency in DNMT3A induced HSC expansion, increased self-renewal and impaired differentiation [33, 53, 54]. However additional mutations in NPM1 or FLT3 are required to cause leukemia [33, 55]. TET2 mutations also lead to clonal expansion of HSCs and self-renewal however the exact underlaying mechanism is unknown [29, 33, 56-58]. Additional Sex Combs Like 1 (ASXL1) regulates epigenetics and transcription through its interaction with the Polycomb repressive deubiquitinase complex (PR-DUB) that mediates histone methylation and ubiquitination resulting in transcriptional repression [25, 59, 60]. In mouse models loss of ASXL1 has contributed to stem cell expansion and this is

similar to what occurs in patients with recurrent nonsense and frameshift mutations in exon 11 and exon 12 of ASXL1 in secondary AML, myelodysplastic syndrome (MDS), and myeloproliferative neoplasm (MPN) patients [29]. Other mutations include SF3B1, SRSF2, JAK2, TP53, PPM1D, GNAS and CBL, which have been identified as drivers in AML, MDS and MPNs [25, 61-65] . Genes BCOR and BCORL1 have also been found to be associated with CH and are linked to aplastic anemia [29, 66].

1.3.3 The leukemic stem cell

LSCs possess unlimited self-renewal capacity and are experimentally defined as able to initiate a new leukemia when transplanted into a new host such as severely immunocompromised mice [39]. The origin of the LSC is a widely debated topic with models that suggest either the HSC or a downstream progenitor as the cell of origin. What is evident is that HSCs and LSCs share similar stem cell or stemness properties such as self-renewal and the ability to produce differentiated progeny [67]. AML LSCs follow the cancer stem cell (CSC) model where the cancer is organised hierarchically with a distinct subpopulation of CSCs at the apex that can sustain themselves through self-renewal and also give rise to progeny that form the rest of the cancer [68]. Evidence of a LSC population included *in vivo* examination of leukemia blast proliferation kinetics in human AML that showed that the majority of leukemic blasts were postmitotic and needed to be constantly replenished from a relatively small proliferative fraction. Further investigation showed that two proliferative fractions exist a large, fast cycling fraction with a 24-hour cell cycle and a smaller, slow cycling quiescent fraction that can take from weeks to months to cycle. It was noticed that the slow cycling fraction gave rise to the fast cycling fraction, and it is this slow cycling fraction that represents LSC population [68, 69]. Further to this studies have shown that AML LSCs demonstrate plasticity, oscillating between stem-like

and non stem-like cellular activity, slow cycling cells can convert in to fast cycling cells and vice versa [70].

In order to isolate LSCs, AML samples can be sorted into fractions / populations using fluorescence activated cell sorting (FACS). Phenotypically LSCs have been characterized by the expression of CD34 and CD38 on their cell surface [68]. While most LSCs can be found in the CD34+CD38- fraction, studies conducted by sorting and xenotransplant assay have indicated that they can also be found in other fraction combinations of CD34 and CD38 such as the CD34+CD38+ fraction, indicating their heterogeneity [67]. Studies done by Dick *et al.* showed that it is the CD34+CD38- fraction that when transplanted into severe combined immunodeficiency (SCID) mice is capable of engraftment and generation of leukemia [68]. Expression of CD38 and CD34 are important markers for lineage commitment and therefore the CD34+CD38- phenotype defines immature human cells in the bone marrow [71]. Use of cell surface markers CD34 and CD38 to identify LSCs is limited by the variability of their expression in patient samples [72]. Furthermore these markers only identify the enriched fraction which contain LSCs but do not specifically identify the LSC as a single cell. The markers are also used to identify normal HSPCs which, similar to LSCs, are CD34+CD38-, this makes it difficult to specifically identify LSCs in patient samples without the use of additional markers such as CD123 and C-type lectin-like molecule-1 (CLL-1) [73-75]. LSC frequencies in human AML are measured using limiting dilution assays in immunodeficient mice and can vary between different leukemia samples, ranging from 1 in 10000 to less than 1 in 5 million [39]. Variations in LSC frequency can be seen in AML patient samples taken at diagnosis and after relapse. There is a significant 9 to 90 fold increase at relapse compared to at diagnosis [10], suggesting that LSCs can further evolve after initial treatment. The unique biology of LSCs has been suggested as one

of the main reasons for treatment resistance, disease relapse and poor outcomes in patients. Clinical outcome in AML patients have been correlated with LSCs using gene expression signatures. Eppert *et al* generated LSC and HSC gene expression signatures that showed that a higher expression of stem cell expression signatures directly predicted poor patient survival and disease outcome. These expression profiles indicated a high frequency of LSCs [67]. Subsequently a highly prognostic 17 gene LSC signature that accurately predicted initial AML therapy resistance was later created by Ng *et al.* Patients who showed a higher expression of the LSC17 signature had poorer outcomes with current treatments including allogeneic stem cell transplantation [76]. The findings show the important role that LSCs play in driving the disease and determining treatment response.

Long-term chemotherapy resistance in AML has been attributed to LSCs. This is due to the quiescent nature of these cells that allows them to persist in the G0 phase making them less sensitive to standard chemotherapies such as cytarabine and daunorubicin that target rapidly replicating cells in contrast to the LSCs that are slow cycling. Studies done by Costello *et al* have shown that the CD34+CD38- population has reduced drug influx and chemotherapy sensitivity when compared to the CD38+ population. LSCs have also been found to express multidrug resistant genes such as MRP/ LRP and ABCC1 that increase their resistance [14, 39, 77]. With more investigation into the biology and mechanisms involved in LSC survival, new therapies can be created that specifically target LSCs.

1.3.4 Diagnosis and classification of AML

The presentation of signs and symptoms such as fatigue, fever or bleeding in a patient will generally prompt the need to order a complete blood count (CBC) which may give the first indication of diagnosing AML. The CBC may show an increased white cell count (leukocytosis)

due to an increase in myeloblast or it may show leukopenia suggesting bone marrow failure. Typically the diagnosis of AML is made by the presence of >20% myeloblast in the blood and or bone marrow. Cytogenetic, molecular diagnostic and immunophenotyping testing are also done to identify various chromosomal abnormalities and genetic mutations as well as to classify AML by prognosis and subtypes. These tests also allow for risk stratification of patients and determination of treatment response.

The French-American-British classification in 1976 was the first classification to be used for AML. It classifies AML from M0 to M7 based on cell morphology and cytochemical staining to determining whether blasts had the appearance of myeloblasts, monoblasts, erythroblasts, promyelocytes, or megakaryoblasts [4]. This classification however lacked clinical and biological relevance and was followed by the World Health Organization classification in 2001, that included morphology as well as immunophenotyping, cytogenetics, molecular studies and clinical features to be more clinically relevant and better predict prognosis and treatment [78, 79]. The WHO classification was updated in 2016 to include a wide range of new disease entities based on clinical information and advance laboratory test [80]. It incorporates both de novo and secondary causes of AML as well as myeloid sarcoma, myeloid proliferations related to Down's syndrome, Blastic plasmacytoid dendritic cell neoplasm, and AML not otherwise categorized. Another commonly used classification is the European LeukemiaNet (ELN) classification 2017. It groups patients into favorable, intermediate, and adverse categories by incorporating cytogenetic and molecular abnormalities into risk stratification of AML [4]. The main purpose of this classification was to standardize the reporting of genetic abnormalities particularly for correlations with clinical characteristics and outcome [81].

The value of these newer classifications is that they help to stratify patients based on their risk of treatment resistance, treatment-related mortality and prognostic factors. This helps healthcare providers to make better treatment decisions as to which type of therapy is best to use in a patient, for example standard or increased intensity treatment, consolidation chemotherapy, allogenic hematopoietic stem cell transplant, or investigational therapies [82].

1.3.5 Treatment of acute myeloid leukemia

1.3.5.1 Standard therapy

The standard therapy for AML consists of chemotherapy and is divided into two phases, the first induction therapy and the second consolidation therapy. The purpose of induction therapy is to reduce the number of leukemic cells and induce complete remission. Induction therapy uses the 7+3 regimen which has been in use since the 1970s. It consists of 7 consecutive days of iv cytarabine given with anthracycline (daunorubicin or idarubicin) on days 1-3. Remission is induced when there are <5% blast with recovery of the peripheral blood count [1]. Because of the intense toxicity and risk of infection, induction therapy is not always offered to the elderly. These patients may be offered demethylating agents (eg, decitabine, azacitidine) instead. However, some elderly patients may benefit from induction therapy [83].

Once complete remission is achieved patients can then be offered consolidation therapy. This treatment is based on pre-treatment patient risk stratification. For those with a favorable prognosis further courses of high dose cytarabine can be given [84]. High and intermediate risk patient can be offered allogeneic hematopoietic stem cell transplant (allo-HSCT) if a suitable donor is available. Studies have shown that patients who receive allo-HSCT have significantly prolonged relapse free and overall survival [82].

1.3.5.2 Therapies targeting LSCs

As the treatment of AML evolves it has become apparent that targeting LSCs may be an effective option. Currently the combination therapy of venetoclax and azacytidine has shown success in targeting LSCs in AML de novo patients [85]. This combination targets LSC amino acid metabolism leading to decrease amino acid uptake, which in turn reduces oxidative phosphorylation leading to LSC eradication. It has been shown that LSC survival is dependent on amino acid catabolism which is used for oxidative phosphorylation [86, 87].

Several novel therapies to target LSCs are being tested at the preclinical stage and in clinical trials. Parthenolide is one such therapy, it is a small molecule inhibitor of NF-κB and proapoptotic regulator of p53 [88]. It selectively eliminates myeloid leukemia cells and causes apoptosis in primary human AML cells and blast crisis CML cells while sparing normal hematopoietic cells. Analysis of AML progenitor cells in *in vitro* colony forming assays and stem cells in a xenograft model with nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice showed that Parthenolide preferentially targeted both AML progenitors and LSCs in the CD34+CD38- population. Parthenolide, however, has poor solubility in water making it a less than ideal candidate compound. An analogue Dimethylamino Parthenolide has been developed which is 1000 fold more water soluble [88, 89].

TDZD-8 (4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione) also targets LSCs by NF-κB inhibition. Additionally it is a competitive inhibitor of glycogen synthase kinase-3 beta (GSK-3β). Preliminary data has shown that TDZD-8, similar to parthenolide, selectively induces cell death in primary AML progenitor cells. An overnight culture of AML progenitor cells expressing CD34+CD38- treated with TDZD-8 showed that progenitor survival was rapidly impaired by TDZD-8. In a xenograft model using NOD/SCID mice to assess AML stem cell potential,

TDZD-8 inhibited the engraftment of AML leukemic stem cells but did not significantly inhibit engraftment of normal hematopoietic stem cells. These results suggest that TDZD may be of use in targeting LSCs. TDZD-8 mechanism of action is currently being investigated [88].

Immunophenotypic markers specific to LSCs have also been used to develop therapies. Gemtuzumab ozogamicin is an immunoconjugate compound that links an anti-CD33 antibody to calicheamicin an antitumor antibiotic. The purpose of this conjugate compound is to directly delivery the toxin to LSCs which express CD33. Gemtuzumab ozogamicin has been approved by the FDA under the name Mylotarg for the treatment of newly diagnosed CD33+ AML in adults and children older than two years [90, 91]. Vadastuximab Talirine (SGN-33A; Seattle Genetics) is a previously investigated conjugate compound that also used CD33. It conjugated CD33 to the DNA binding agent pyrrolobenzodiazepine to induce cell death. This compound was however discontinued during a phase 3 clinical trial due to safety concerns [91]. CD123 is another target that can be found on the surface of AML blast and LSCs. SGN-CD123A is an anti-CD123 antibody conjugated to pyrrolobenzodiazepine. In preclinical studies SGN-CD123A has shown significant antineoplastic activity against a broad panel of primary AML samples [92]. Talacotuzumab an IgG1 monoclonal antibody also targets CD123, it does so preferentially via antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer cells (NKs). Talacotuzumab is currently in a phase 2 clinical trial, however, it has shown low efficacy as a single therapy [93]. Bispecific CD33 and CD123 antibodies linked to CD3 have also been developed, as have CD123 antibody linked to CD16. These conjugates deliver cells expressing these targets into the vicinity of T cells or natural killer cells, respectively to induce cell death. The CD33/CD3 conjugate is known as AMG330 and is currently being tested in clinical trials. Studies have also shown that CD47 can be used as a target by preventing it from interacting with

its cognate macrophage receptor SIRP1- α. This leads to the activation of innate immunity and macrophage-mediated destruction of LSCs [91, 94].

1.4 Corticosteroids

Corticosteroids are one of the most widely used drugs in patient care due to their anti-inflammatory and immunosuppressive effects. They are used in the treatment of various diseases such as allergic, respiratory, rheumatologic, dermatologic, endocrinologic, gastrointestinal, ophthalmological, renal, hematologic/oncologic disorders and organ transplant [95]. While corticosteroids do offer great benefit as a treatment option, they do cause numerous side effects that are dose dependent, some of which include diabetes, hypertension, osteoporosis, skin atrophy, abdominal obesity, glaucoma, cataracts, growth retardation, avascular necrosis and infection. Therefore it is essential that high dose and long-term use be closely monitored [96]. Also of concern when using corticosteroids is their increase resistance that limits therapy.

Corticosteroids are produced naturally as steroid hormones or can be made as synthetic analogues. The natural corticosteroids include cortisol, cortisone, corticosterone and aldosterone. These steroids are made from cholesterol in the adrenal cortex and can be divided in to two groups, glucocorticoids and mineralocorticoids [97]. Aldosterone is the most naturally secreted mineralocorticoid; its function is to regulate electrolyte and water balance through ion transport in the epithelial cells of the renal tubules [98]. While the term corticosteroid is a general one, it is usually used to refer to the glucocorticoids and their effect. Glucocorticoids derived their name from their effect on carbohydrate (glucose) metabolism [99]. In the body they are responsible for the metabolism of fat, proteins and carbohydrates as well as anti-inflammatory, immunosuppressive, anti-proliferative, and vasoconstrictive effects [98]. Cortisol is the major glucocorticoid produced in the body. It is secreted in a circadian manner and in response to

stress; and is controlled by a negative feedback mechanism involving the hypothalamic–pituitary–adrenal axis [99, 100].

The effects of glucocorticoids in the body can vary depending on the tissue or organ. In the liver glucocorticoids up regulate gluconeogenesis pathways and induce glycogen storage. Whereas in the skin, muscle, connective, lymphoid and adipose tissue decreased synthesis and increased degradation of protein and RNA is seen. [101]. These effects exhibited by glucocorticoids have been thought to be produced through activation of the glucocorticoid receptor (GR) by ligand binding. The anti-inflammatory effects of glucocorticoids are produced by blocking inflammatory mediators through transrepression and inducing anti-inflammatory mediators through transactivation [98]. The immunosuppressive effect of glucocorticoids is multifactorial and involves reduced proliferation of T cells, decreased B cell antibody production and inhibition of nuclear factor kappa B (NF-κB) and cytokine gene expression [102-104]. The ability of glucocorticoid to induce vasoconstriction is medicated by inhibition of nitric oxide, prostacyclin and various inflammatory mediators such as histidine [98, 105]. The anti-proliferative effect is a result of inhibition of cytokine expression [106].

1.4.1 Glucocorticoid receptor

Glucocorticoids exert their effect through the glucocorticoid receptor (GR), which is a member of the nuclear receptor family of ligand-activated transcription factors. It is encoded by the nuclear receptor subfamily 3 group c member 1 (NR3C1) gene which consist of 9 exons located on chromosome 5 (5q31.3) and is ubiquitously expressed in the body [107]. The GR is a modular protein made up of three functional domains an N-terminal transactivation domain (NTD) encoded by exon 2, a central DNA binding domain (DBD) encoded by exons 3 and 4 and a C-terminal ligand-binding domain (LBD) encoded by exons 5-9. The NTD is an unstructured

domain that becomes structured when it binds DNA and forms dimers. It is the least conserved and most variable domain among the nuclear receptor family [99, 108]. The NTD contains transcription activation function 1 (AF1) that is responsible for activating target genes in a ligand independent manner and is the principal site for posttranslational modifications [99]. The DBD contains two zinc finger motifs that bind the glucocorticoid response elements (GREs) which induce gene transactivation. The LBD contains the hydrophobic ligand binding pocket, that is the actual site of glucocorticoid binding. It is formed by 12 α-helices, 4 β-sheets, and another ligand-dependent activation function domain AF2 [100].

The GR has various isoforms that determine its function and effect. There are five GR splice variant isoforms GRα, GRβ, GRγ, GR-A, and GR-P. GRα is the predominant form of the GR, it results from splicing of exon 8 to the beginning of exon 9 and is present in the cytoplasm. GRβ is produced from splicing the end of exon 8 to the downstream sequences of exon 9 and presents in the nucleus. GRβ lacks helix 12 in the LBD ligand binding pocket and therefore cannot bind glucocorticoids. GRβ functions as a dominant negative of GRα inhibiting its function and because of this it has been hypothesized that changes in the expression of GRβ may underlie the development of glucocorticoid resistance [99, 109]. Pro-inflammatory cytokines and immune activators seem to have a role in increasing the expression of GRβ leading to glucocorticoid resistance by a reduction in the GRα:GRβ ratio. A reduced GRα:GRβ ratio has been associated with glucocorticoid resistance in several inflammatory diseases as well as in acute lymphoblastic leukemia, and chronic lymphocytic leukemia [109]. Studies performed by Kelly *et al* have shown that GRβ can act as a transcription factor where it is able to repress genes independent of its dominant negative activity on GRα [110]. The GRγ splice variant isoform occurs when exon 4 is alternatively spliced to exon 3. GRγ has about half of the activity of GRα for glucocorticoid

target genes and its expression in childhood acute lymphoblastic leukemia has been shown to correlate with resistance to glucocorticoid treatment [99, 111]. The GR-A isoform is acquired by splicing of exon 4 to exon 8, removing exon 5-7. However, little is known about the biological function of GR-A [99]. The GR-P isoform occurs due to a failure to splice at the boundary of exon 7-8. It has been shown to have a variable effect on the transcriptional activity of GRα in various cell types and is expressed in glucocorticoid resistant hematological malignancies such as and multiple myeloma, acute lymphoblastic leukemia and non-Hodgkin's lymphoma [99, 112].

There are also eight GRα translational isoforms GRα-A, B, C1, C2, C3, D1, D2 and D3 which are generated from initiation of translation at eight different AUG start codons in exon 2 in a single GR-mRNA. Each of these variants has a progressively shorter N-terminus, but they all possess similar glucocorticoid and GRE binding capabilities. Isoforms GRα-A, B, C can be found in the cytoplasm when inactivate and later translocate to the nucleus on glucocorticoid binding, while GRα-D resides in the nucleus. Lu *et al* found that each GRα variant possesses a distinct transcription profile and regulates a unique set of genes with only a few common genes regulated by all of the isoforms [113]. In another study conducted by Gross *et al*, osteosarcoma cells expressing isoforms GRα-A, B, C were sensitive to dexamethasone and underwent apoptosis whereas cells expressing GRα-D were resistant. In addition they showed that cells expressing GRα-A, B, C where also able to inhibit NF-κB whereas cells expressing GRα-D were not [114]. These studies highlight the importance of the GR isoforms and their impact on glucocorticoid resistance. While studies in AML are lacking further investigations may show the role of the GR isoforms in leukemia.

1.4.2 Mechanism of action of glucocorticoids

Glucocorticoids exert their mechanism of action through multiple pathways using the GR. Under a pathway that directly affects transcription, the GR, which resides in the cytoplasm in a multi-protein complex (facilitates a high-affinity steroid binding conformation), binds its ligand and undergoes a conformational change, resulting in the dissociation of the multi-protein complex [115]. The GR then undergoes structural reorganization and translocates into the nucleus through nuclear pores. On entering the nucleus, the GR binds to the glucocorticoid response element (GRE) in the promoter regions of a target gene and regulates gene expression. Binding of the GR to the GRE induces conformational changes in the GR which lead to coordinated recruitment of coregulator and chromatin-remodeling complexes that influence the activity of RNA polymerase II and activates gene transcription and repression [99]. A study conducted by Surjit et al showed that binding of the GR to negative glucocorticoid response elements (nGRE) could induce transrepression by recruiting corepressors (NCoR1 and SMRT) and histone deacetlyases (HDACs) [116]. This changed the idea that GR + GRE binding was primarily linked to transactivation and highlighted other mechanisms at work that induce transrepression other than GR direct tethering to transcription factors. Glucocorticoids are understood to express their anti-inflammatory effect through transrepression. Ligand-bound GR is recruited to chromatin by transcription factors NF-κB and activator protein-1 (AP-1), the GR then directly binds the Jun subunit of AP1 and the p65 subunit of NF-kB and affects the transcriptional activation of these proteins causing a decrease in the inflammatory response [99].

An alternative rapid mechanism of action is mediated through physiochemical interactions with the GR in the cytoplasm or the GR bound to the cell membrane. These effects do not require protein synthesis and occur rapidly after GR activation [99]. This mechanism was studied by

Groeneweg *et al* by investigating signal patterns induced by glucocorticoids in the brain [117]. It has been suggested that this rapid non-genomic function of the GR utilizes the activity of various kinases, such as AKT, phosphoinositide 3-kinase and mitogen-activated protein kinases (MAPKs) [99, 118]. And that accessory proteins such as c-Src that activates signalling cascades are released through GR ligand binding. The c-Src protein phosphorylates annexin 1, inhibits phospholipase A2 activity and impairs the release of arachidonic acid, processes that are involved in the anti-inflammatory response [99].

1.4.3 Development of corticosteroids

In 1930 the first evidence of a substance presumed to treat adrenal insufficiency (Addison's disease) was identified by Wilbur W. Swingle and Joseph J. Pfiffner, biochemists at Princeton University. The substance was believed to be the adrenal cortical hormone called "cortin" and was made from an extract of cattle adrenal glands. It was used in a clinical trial to treat 20 cases of Addison's disease and 20 other nonrheumatologic patients. The results of the trial showed only a transient effect in the treatment of Addison's disease [119, 120]. In 1941 Edward C. Kendall *et al* reported that extracts from the adrenals contained two types of steroid hormones. Those without an oxygen at C11 that affect electrolytes and those with an oxygen at that position that affect gluconeogenesis, one of which he called "compound E" [120, 121]. Compound E was later termed cortisone and was first used in the treatment of rheumatoid arthritis in 1948 at the Mayo Clinic by Philip S. Hench. In 1950 Kendall, Hench and Tadeusz Reichstein a scientist from Zurich who had been studying the adrenal cortex hormones received the Noble Prize for discovering the function and structure of cortisone and other adrenal cortex hormones [120, 122]. Along with "compound E" Kendall had also identified another compound "compound F" which was later called hydrocortisone in 1950 [120]. Many medicinal chemists then tried to find other

suitable starting materials and synthetic processes that could be used for producing large amounts of cortisone to supply an increase in demand. Placing an oxygen at C-11 created the greatest difficulty in synthesizing cortisone because no available starting material had an oxygen at C-11, and no practical method was known for adding the oxygen [123]. Then in 1952 Durey H. Peterson found a way to produce cortisone from fermentation of progesterone (derived from stigmasterol a steroid present in soybeans) with Rhizopus mold, this allowed the addition of the oxygen at C-11 [124].

In spite of the good treatment response to cortisone, patients treated with it developed side effects such as facial hirsutism, acne, facial puffiness and increasingly depressive and hostile ideation [125]. Because of these side effects scientist continued searching for ways to improve the therapeutic effect of corticosteroids while decreasing the side effects. Through much trial and error, they found that by adding or blocking functional groups on the main corticosteroid structure it was possible to manipulate the effects of the steroid. In 1954 it was discovered that by introducing a double bond between C1-C2 of cortisone or hydrocortisone, it was possible to increase the anti-inflammatory activity to 4-5 times that of cortisone. This discovery led to the development of prednisone and prednisolone, respectively. These new analogues also had reduced electrolyte imbalances such as sodium retention and potassium excretion. Next by introducing a fluorine atom (F) at C9 of hydrocortisone or prednisolone it was possible to create a corticosteroid with approximately 10 times the anti-inflammatory and glucocorticoid effects [120]. This new more potent corticosteroid was called fluorocortisone (fludrocortisone), it however caused a significant increase in fluid retention as it possessed high mineralocorticoid activity. To combat the fluid retention a hydroxyl group (OH) was then added at C16 in 1956. This blocked the sodium retention and slightly increased even further the anti-inflammatory

effect leading to the creation of triamcinolone. With improvement in laboratory techniques in 1958 it became possible to replacing the C16 hydroxyl group (OH) with a C16 methyl group (CH3), this led to further increase in anti-inflammatory activity and elimination of the sodium-retention effects caused by the C9 fluorine group; by doing this dexamethasone and betamethasone were developed [120, 123, 126]. Next due to an unexpected reaction C6F cortisol was created by placing a fluorine at the C6 position, this compound exhibited potency eight times that of cortisol. The introduction of the C6 fluorine atom increased anti-inflammatory activity by a factor of 10–20. Building on this scientist then made other combinations of the various C6 and/or C9 halogenations and C16 methyl substitution that eventually led to the development of paramethasone and flumethasone [123].

During the corticosteroid development process scientists also investigated topical use of these compounds to avoid systemic side effects. Developing topical formulations created new barriers to obtaining efficient corticosteroids as the steroids had to penetrate the skin, maintain an anti-inflammatory effect and be devoid of electrolyte imbalances. The ability to place an acetonide group at C16-C17 was an important step in the development of corticosteroids as a topical agent. Addition of this group increased skin penetrability properties due to it lipophilic properties, resulting in improved percutaneous absorption and reduced undesirable electrolyte imbalances caused by the C6 and/or C9 fluorine substitutions. The acetonide group made the steroid 10 times more active topically than its parent compound but equal systemically. The addition of the acetonide group brought about triamcinolone acetonide and fluocinolone acetonide [123]. In an effort to improve the topical corticosteroids further substitution of the hydroxyl group at the C21 position with an acetate produced fluocinonide, a corticosteroid with even higher potential [127]. Corticosteroids containing esters at C21 showed relatively more resistance to metabolism, longer

durations of actions and enhanced lipophilicity resulting in improved percutaneous absorption [123]. Newer corticosteroids are heterocyclic ester derivatives with functional groups at C17 and C21. Mometasone furoate for example patented in 1981 [128], has a furoyl ester at C17 and carries a chlorine on the C21 side chain. Further substitutions have created fluticasone propionate launched in 1993 which is a trifluorinated corticosteroid with fluorines at C6, C9 and C21 and a propionyl group at C17. These substitutions immensely increase topical potency and anti-inflammatory affects. Both mometasone furoate and fluticasone propionate are highly lipophilic and rapidly inactivated following absorption. Hepatic first-pass metabolism inactivates about 80% of the topical corticosteroids, but only 20% of the systemic corticosteroids, therefore minimizing systemic side effects that can be caused by the topicals [126].

1.4.4 Classification of corticosteroids

Corticosteroids in particular glucocorticoids can be classified by potency or by structure. When classified by potency, topical glucocorticoids are classified using the human vasoconstrictor assay developed by McKenzie and Stoughton [129]. This assay assesses and scores the degree of cutaneous vasoconstriction (blanching) caused by various concentrations of the corticosteroids on the anterior forearm of healthy volunteers. It has been demonstrated that a relationship exists between the ability to induce vasoconstriction and the ability to combat inflammation in therapeutic use [123, 130]. According to the United States classification of topical corticosteroids they can be divided into seven classes. Class I Superpotent, class II high potent, class III upper mid-strength, class IV mid-strength, class V lower medium-strength, class VI low-potency and class VII least-potent [131, 132]. The activity of the topical corticosteroid molecule, its concentration and nature of vehicle are also considered when classifying these compounds [133]. See Table 1.

Corticosteroids can also be classified based on their structure using the Coopman classification. This classification is bases on corticosteroid cross reactivity, where corticosteroids with similar structures have been shown to cause contact allergies in patients [134]. In this classification there are five groups. Group A hydrocortisone type with no substitution in the D ring except C17 and/or C21 acetate esters, Group B triamcinolone acetonide type with C16, C17 – cis-diol or ketal group, Group C betamethasone type with C16 methyl substitution and Group D divided in to D1 betamethasone dipropionate type (halogenated) less labile with C16 methyl substitution plus C17/C21 long chain ester and D2 methylprednisolone aceponate type (labile prodrug esters) with only C17 long chain ester or C21 possible side chain [131, 135]. See Table 1.

Table 1. Classification of corticosteroids by potency and structure

Structural Class Potency	Group A Hydrocortisone type	Group B Triamcinolone acetonide type	Group C Betamethasone type	Group D1 Betamethasone Dipropionate Type	GroupD2 Methylprednisolone Aceponate Type
	No substitutions in the D ring, except C17 and/or C21 acetate esters	C16, C17 – cis-diol or ketal group	C16 methyl substitution	C16 methyl substitution plus C17/C21 long chain ester	C16 – no methyl substitution C16 – no halogenation C17 – long-chain ester C21 – possible side chain
CLASS 7: LEAST POTENT	Hydrocortisone Hydrocortisone acetate Tixocortol pivalate Methylprednisolone, prednisolone				
CLASS 6: LOW POTENT		Desonide 0.05% C, F Fluocinolone acetonide 0.01% C, S Triamcinolone acetonide 0.025% C Triamcinolone diacetate 0.025% C		Alclometasone dipropionate 0.05% C, O Betamethasone valerate 0.1% C	
CLASS 5: LOWER MID-STRENGTH		Desonide 0.05% O Fluocinolone acetonide 0.025% C Triamcinolone acetonide 0.1% C, 0.025% O, L Triamcinolone diacetate 0.1% C		Betamethasone dipropionate 0.05% L Betamethasone valerate C, L Fluticasone propionate 0.05% C	Hydrocortisone buteprate 0.1% C, O, S Hydrocortisone butyrate 0.1% C, O, S Hydrocortisone valerate 0.2% C Prednicarbate 0.1% C
CLASS 4: MID-STRENGTH		Amcinonide 0.1% C Fluocinolone acetonide 0.01%, 0.025% O Halcinonide 0.025% C Triamcinolone acetonide 0.1% O Triamcinolone diacetate 0.1% O	Clocortolone pivalate 0.1% C Desoximetasone 0.05% C	Betamethasone valerate 0.12% F Clobetasone butyrate 0.05% Mometasone furoate 0.1% C, L	Hydrocortisone valerate 0.2% O
CLASS 3: UPPER MID-STRENGTH		Amcinonide 0.1% L Fluocinonide 0.05% Triamcinolone acetonide 0.1% C, O Triamcinolone diacetate 0.1% C, O		Betamethasone dipropionate 0.05% C Betamethasone valerate 0.1% O Clobetasone butyrate 0.05% Diflorasone diacetate 0.05% C Fluticasone propionate 0.005% O Mometasone furoate 0.1% O	
CLASS 2: HIGH POTENT		Amcinonide 0.1% O, L, C Budesonide 0.025% C Fluocinonide 0.05% C, O, G, S Halcinonide 0.1% C, O, S	Desoximetasone 0.25% C, 0.05% G	Betamethasone dipropionate 0.05% O,C Betamethasone valerate 0.1% Diflorasone diacetate 0.05%	
CLASS 1: SUPERPOTENT				Betamethasone dipropionate 0.05% G, O, L Clobetasol propionate 0.05% C, O, G, S, F Diflorasone diacetate 0.05% O	

C=Cream, G=Gel, L=Lotion, O=Ointment, S=Solution, F=Foam

Adapted from "Corticosteroid classes: a quick reference guide including patch test substances and cross-reactivity" by S.E. Jacob, Journal of the American Academy of Dermatology. 2006; 54(4):723-727. Copyright 2006 by Elsevier. Adapted with permission.

1.4.5 Structural activity and functional relationships of corticosteroids

The ability of the glucocorticoids to exert their effect depends on the activation of the GR. Activation of the receptor occurs on binding of its ligand to its ligand binding domain (LBD). For this to occur the ligand must be structurally compatible with the LBD, as it must fit into the ligand binding pocket. The development and evolution of corticosteroids has been based on adding or changing functional groups on the basic chemical structure of the glucocorticoids that is made of cyclopentaneperhydrophenanthrene, consisting of three 6 carbon rings (A, B, C) and a one 5 carbon ring [126]. See Figure 1. These modifications include the addition of a C1-C2 double bond, C6/C9 halogenation with fluorine or chlorine, C16-C17 acetonide group, C16 methyl substitution, and C17 and C21 esters. By changing or adding functional groups, the glucocorticoids (ligand) modify their affinity for the LBD which in turn modifies their anti-inflammatory, immunosuppressive and metabolic effects as well as their potency. In general, high potency is determined by a high affinity for the receptor [136]. He *et al* demonstrated this with an assay which compared the affinities of mometasone furoate, dexamethasone and cortisol for the GR. Their results showed that the order of GR binding affinity was mometasone furoate > dexamethasone > cortisol and that the binding affinity (Ki) values for mometasone, dexamethasone and cortisol were 0.7 nM, 8 nM and 91 nM, respectively. Indicating that the more potent compound had the higher affinity. These results were also consistent with transactivation and transrepression potency assay results for these glucocorticoids, which showed that the more potent compound had a better dose response curve with left axis deviation. And also suggested that transpression occurs at a higher potency and lower concentration than transactivation [136].

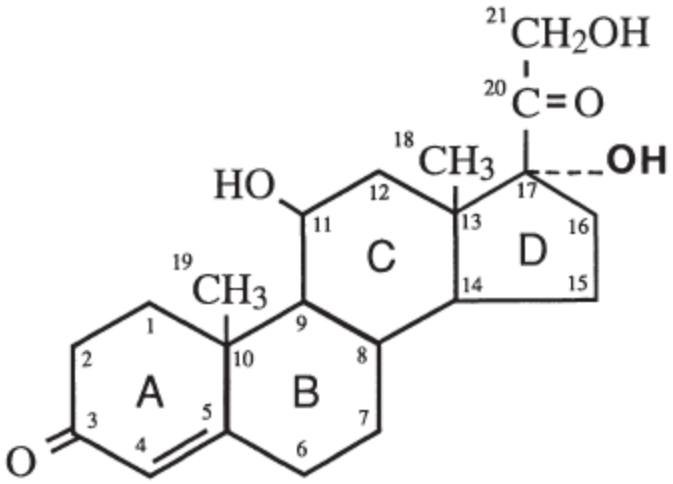

Figure 1. Structure of cortisol

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Cortisol was the first glucocorticoid discovered and its structure does not contain the C1-C2 double bond, C9 halogens or C17 side chain esters that can be found in the molecular structure of mometasone furoate or dexamethasone which have higher affinities. Furthermore, the difference in affinity between mometasone furoate and dexamethasone can be attributed to the C21 chlorine and lipophilic furoate ester group at C17of mometasone. These groups seem to play a major role in increasing mometasone's binding affinity and potency. Structural analysis and comparison of the cortisol-bound GR LBD and the dexamethasone bound GR LBD has revealed that the C1-C2 double bond of dexamethasone causes the steroid A ring and the C3 ketone group to become planar, allowing the C3 ketone to easily form hydrogen bonds with nearby amino acids that form the LBD pocket. In comparison, the C1-C2 single bond of cortisol is flexible, causing the steroid A ring to bend in order to form its hydrogen bonds with the amino acids, this leads to less

stability. Further to this, the C1-C2 single bond of cortisol oscillates between two conformations in its unbound form and requires a water molecule to form hydrogen bonds to hold it in the pocket. These findings account for the low affinity and potency of cortisol compared to dexamethasone[136].

Structural analysis and comparison of the dexamethasone bound GR LBD and the mometasone furoate bound GR LBD has shown that when dexamethasone occupies the LBD pocket, there is an empty hydrophobic cavity above its steroid D ring, which is due to dexamethasone's flat two-dimensional structure and orientation in the pocket. However, this empty cavity does not exist when mometasone furoate occupies the pocket, as it is filled by the C17 lipophilic furoate group which projects off the D ring at 90 degrees. The furoate group forms hydrophobic interactions with nearby amino acids which help to secure mometasone in place. This ability to adequately fill the entire pocket and form bonds increases mometasone's affinity for the GR 10-fold compared to dexamethasone, and increases its potency [136].

As mentioned, the development of the glucocorticoids had an evolutionary approach. Some of the more important structural modifications include the C1-C2 double bond that not only adds stability to the molecule but also increases the anti-inflammatory activity of these compounds and reduces their mineralocorticoid effects. In some instances, this double bond slows the metabolism of the glucocorticoid. Halogenation of C9 with a fluorine or chlorine increases the glucocorticoid (anti-inflammatory) and mineralocorticoid activity through electron-withdrawing from the C11 hydroxyl group. This mineralocorticoid effect can be decreased by moving the fluorine from C9 to C6 as in the case of paramethasone or by adding a C16 methyl group which also has the ability to increase the anti-inflammatory effect and potency of the compound. Addition of a C16 hydroxyl group can also reduce the mineralocorticoid activity as is the case

with triamcinolone. The introduction of the acetonide group at C16-C17 and the esterification of the hydroxyl group at C17 (for example betamethasone valerate, fluticasone propionate and mometasone furoate) or at C21 (betamethasome dipropionate) increases lipophilicity and topical anti-inflammatory activity while decreasing systemic effects. Substitution of the hydroxyl group at C21 with chlorine also provides increased topical anti-inflammatory activity [126, 138]. Other functional groups that have an impact on the structural activity are those that interact with the amino acids in the LBD. For example the C3 keto group of the steroid A ring of dexamethasone interacts with glutamine 570 and arginine 611, the C11 hydroxyl group of the C ring interacts with asparagine 564, and the side chain C-21 carbonyl group interacts with threonine 739. These groups form hydrogen bonds with the amino acids in the LBD pocket and hold the steroid backbone in position [136]. An important amino acid is glutamine 642 (Q642), this amino acid seems to be able to recognize and differentiate between high and medium to low potency glucocorticoids. When binding high potency ligands like mometasone furoate, Q642 is pushed away by the C17 lipophilic group, bending it almost 90 degrees. This leads to conformational changes which result in the characteristic of high potency. When binding a medium to low potency glucocorticoid such as dexamethasone or cortisol, Q642 forms a hydrogen bond with the C17 hydroxyl group to hold the ligand in position in the ligand binding pocket [136]. Altogether these functional groups and amino acid residues help to determine the potency and efficacy of the corticosteroid.

1.4.6 Corticosteroids use in acute myeloid leukemia

Glucocorticoid have been used in the treatment of leukemia in particular acute lymphocytic leukemia (ALL) since the 1950s when it was discovered that neoplastic white blood cells were sensitive to the cytotoxic effect of glucocorticoids, causing them to undergo apoptosis or

programmed cell death [101]. Dexamethasone and prednisone are used in ALL and have been largely responsible for the survival rates of approximately 90% of pediatric patients [139, 140]. In ALL corticosteroids inhibit cytokine production, change the expression of various oncogenes and induce cell-cycle arrest and apoptosis rather than induce cell differentiation [141]. There are two mechanisms by which corticosteroids can exhibit their effect on leukemic cells, the first being differentiation and the second apoptosis. Currently, for the treatment of AML corticosteroids are not used as part of standard treatment [142, 143]. In the past dexamethasone was used in older AML treatment regimens, but was discontinued because these regimens had high toxicity, most likely due to the other standard drugs used in combination with dexamethasone and the aggressive drug scheduling [140, 144, 145]. However in an effort to decrease the mortality rate of AML, investigations using glucocorticoids continued. In 1997 Miyoshi *et al*. demonstrated that dexamethasone could induced apoptosis in Kasumi-1 and SKNO-1 cells, both of which are AML cell lines with t(8;21), that result in an AMLl-MTG8(ETO) fusion transcript. This fusion plays a critical role in the abnormal proliferation and differentiation of myeloid leukemia cells [146]. This group also found that dexamethasone had a rapid effect with an effective concentration of less than 1 nM and that it did not cause differentiation in the Kasumi-1 and SKNO-1 cells [146]. Hiçsönmez, et al. in 2005 then showed that high dose methylprednisolone if given for a short duration of 3-7 days could induce differentiation and apoptosis of myeloid leukemic cells *in vivo* in children with different subtypes of acute myeloblastic leukemia (AML-M1, -M2, -M3, -M4, -M7). In addition to these morphological changes, surface marker analysis by flow cytometry showed a decrease in the expression of hematopoietic progenitor cell antigens (HLA-DR, CD117, CD 34) and an increase in cells expressing mature myeloid cell antigens (CD14 and CD15) indicating cell differentiation

[147]. Simon et al. 2017 also demonstrated sensitivity to glucocorticoids in RUNX1 mutated AML *in vitro*. They found that RUNX1 mutated AML was allele dosage dependent, meaning that loss of a RUNX1 allele resulted in sensitivity to glucocorticoids through apoptosis [148].

In our lab Laverdière et al. 2018 demonstrated through *in silico* analysis and an *in vitro* screen using AML 8227 that the glucocorticoids mometasone furoate, budesonide and halcinonide could decrease the CD34+CD38- LSC-enriched population while causing a concomitant increase in CD34-CD15+ blast cell population [77]. This suggested that these compounds could induce differentiation in LSCs. Furthermore our lab showed that when these compounds are used in combination with cytarabine their effect is additive, leading to eradication of both LSC-enriched populations and blast cells. In a retrospective study conducted by Bertoli et al. 2018, they found that the addition of dexamethasone to the treatment regimen of patients with hyperleukocytic acute myeloid leukemia was associated with a significant increase in disease-free and overall survival. They also investigated the impact of dexamethasone on what they called "leukemia initiating cells" and found that treatment of these cells with dexamethasone was able to reduce their frequency by 38±14% as compared to untreated primary AML cells. In addition they found a higher expression profile of the CD38 marker after treatment with dexamethasone, which suggests that differentiation may have occurred [149]. This study has led to a clinical trial investigating dexamethasone use in treating refractory or first relapsed AML [150]. Recently Gebru et al. 2020 demonstrated that the treatment of FLT3 internal tandem duplication (FLT3-ITD) AML cells with selective FLT3 inhibitors such as quizartinib, upregulates inflammatory genes in drug tolerant cells making them susceptibility to glucocorticoids [151]. Further to this they found that the combination of quizartinib and dexamethasone enhanced cell death of FLT3 mutant cells but not wild-type. The results from this study suggest that the combination of FLT3

inhibitors and glucocorticoids may potentially prevent minimal residual disease, mutational drug resistance, and relapse in FLT3-mutant AML [151].

The investigations introduced above all highly suggest that corticosteroids may have a role to play in the treatment of AML. These compounds could prevent disease relapse, decrease chemotherapy drug resistance and improve overall survival in patients who at high risk. While many of these studies do show that glucocorticoids can target blast cells and induce apoptosis or differentiation, they did not examine if glucocorticoids specifically target LSCs, which are the undelaying cause of this disease and the main cause of disease relapse. Further studies are therefore needed to identify the optimal glucocorticoid that can specifically target and eradicate LSCs while exposing patients to fewer side effects and the lowest toxicity.

1.5 Hypothesis and objectives

Therapy for AML has essentially stayed the same for the past 40 years. It involves extremely cytotoxic treatment yet approximately 70% of adults and 30% of children are refractory to initial therapy or relapse. This is partially due to the LSCs that are the underlying drivers of the disease. Novel therapies that can eradicate these cells without harming normal HSCs would greatly reduce the cytotoxic side effects caused by current therapies. They would also reduce relapse and improve survival. Through *in silico* analysis and *in vitro* screening our lab has identified the glucocorticoids mometasone furoate, budesonide and halcinonide as compounds that can eliminate LSCs and spare HSCs.

I hypothesize that by examining a broad set of an additional 24 steroid compounds, it will be possible to identify some that will target LSCs more effectively than the three from the small initial screen, gain insight into optimal compound design, and improve our understanding of the mechanism of action of steroids in LSCs.

The first objective was to establish the efficacy of the candidate compounds against primary human AML LSC and progenitor cells. This was done by an *in vitro* screen using AML 8227 which was cultured and treated with the candidate compounds for 6 days, followed by phenotypic analysis performed via flow cytometry to access cell viability and phenotype.

The second objective was to determine the critical structural components of these compounds and the structural activity. This was done by analyzing the molecular structure of each compound in association with molecular modeling.

Chapter 2: Materials and Methods

2.1 Cell culture

Cells were plated at 300,000 cells/well in a 24-well plate (Falcon®, non-treated clear flat bottom microplate) in StemSpan^{TM} SFEM II media (STEMCELL Technologies), supplemented with penicillin–streptomycin (Life Technologies), and LSC growth factors (Life Technologies): 10 ng/mL interleukin (IL)-3, IL-6 and granulocyte colony stimulating factor (G-CSF), 25 ng/mL thrombopoietin (TPO), 50 ng/mL stem cell factor (SCF) and FLT3 ligand (FLT3L). Cells were then incubated for 6 days at 37 °C with 5% CO2.

AML 8227 cells were collected into a 15 mL Falcon tube, and wells were washed twice with phosphate buffered saline (PBS). Cells were centrifuged at 400g for 10 minutes at 4 °C, then resuspended in StemSpanTM SFEM II media supplemented with penicillin–streptomycin (Life Technologies). Cells were counted under an optic microscope using trypan blue exclusion to estimate cell concentration and total number.

2.2 Corticosteroids

Mometasone furoate and dexamethasone were purchased from Tocris Bioscience and were used as positive controls for the experiments. The 24 corticosteroids used for the in vitro drug screen were generously provided by Dr. Guy Sauvageau's lab (Institute for Research in Immunology and Cancer of the Université de Montréal). For the retested corticosteroids beclomethasone was purchased from Sigma-Aldrich and hydrocortisone acetate, hydrocortisone base, triamcinolone, beclomethasone dipropionate and hydrocortisone-17-butyrate were purchased from Cedarlane.

2.3 In vitro drug screen (corticosteroids)

AML 8227 cells were plated at 50,000 cells/well in a Falcon® 96-well clear flat bottom non-treated microplate using StemSpanTM SFEM II supplemented with pen/strep and LSC growth

factors (as described above). Cells were then placed in an incubator at 37 °C with 5% CO2. The next day 8227 cells were exposed to varying doses of each corticosteroid (1 nM, 3 nM and 15 nM concentrations) in duplicate wells. Dimethyl sulfoxide (DMSO) (Fischer Scientific) was used as negative control. The cells were then returned to the incubator for 6 days. On day 6, cell phenotype and viability were assessed via flow cytometry using a LSR-Fortessa fitted with a high-throughput sampler (BD Biosciences). Anti CD34-APC, CD38-PE and CD15-FITC antibodies (Biolegend) were used to determine cell phenotypes CD34+C38- LSCs, CD34+CD38+ progenitors and CD15+CD34- blasts. SYTOX Blue (Life Technologies) was used to determine cell viability.

2.4 Data analysis

Flow cytometry data analysis was performed using FlowJo v10.7 for Windows to assess FACS profiles and obtain the percentage of LSC, progenitor and blast cell populations for the DMSO control and the various corticosteroid conditions. Corticosteroid dose response curve graphs were created using Prism version 9.0.0, GraphPad Software, to show the inhibitory effect of the corticosteroids on CD34+CD38- LSCs, CD34+CD38+ progenitors and CD34+ cells. Dose response curves were also created to show the agonist effect of the corticosteroids on CD15+CD34- blasts cells, CD34- cells and live cells.

2.5 Analysis of the structural-functional relationship of the corticosteroids

Corticosteroid molecular structures were obtained and adapted from PubChem. The structures were then analyzed to determine their functional groups and were classified into five groups A, B, C, D1 and D2 based on the functional groups (Coopman Classification)[134]. Observations were then made to assess the functional groups in each corticosteroid structure that corresponded

with effective elimination of LSCs and those that were less effective in each group. The key functional groups associated with anti-LSC activity were recorded.

2.6 Molecular modeling

The Protein Data Bank file for mometasone furoate (4P6W), triamcinolone acetonide (5UFS), dexamethasone (1M2Z) and hydrocortisone (6NWL) were obtained from the PDB database. Molecular operating environment (MOE) software was used to construct 3D images of the corticosteroids bound to the glucocorticoid receptor. These structures were overlaid to determine conserved structural features and differences in the alignment of the protein strands which form the helices of the glucocorticoid receptor, measurements were done using root-man-square distance. The position of the steroid core for each ligand within the binding pocket was also assessed. Hydrophobic areas were determined by MOE's electrostatic maps calculation. Percentage survival data for CD34+CD38- cells at 15 nM was used to determine ligand activity in relation to structural composition. Protein binding pocket dynamics analysis was performed using MOE software.

Chapter 3: Results

3.1 Efficacy of corticosteroids to target LSCs and cause differentiation

Three glucocorticoids that trigger loss of LSCs through differentiation were previously identified in our lab in an *in vitro* screen of an AML sample (mometasone, budesonide and halcinonide) [77]. Further analysis in our lab established that the commonly used glucocorticoid dexamethasone can also drive LSC differentiation, although at a higher dose. To identify which glucocorticoids may act at a low dose and the important structural-functional relationship of chemical groups on activity, I examined 24 corticosteroids (Table 2) in an *in vitro* LSC model system – AML 8227.

3.1.1 Screening strategy

To examine the efficacy of different subtypes of corticosteroids and to correlate structural subgroups with function, I screened 24 compounds from 5 different subtypes (Table 1 in the Introduction and Table 2). The assay was performed at three different doses: 1 nM (similar to the effective dose of mometasone), 3 nM (between the effective dose of mometasone and dexamethasone) and 15 nM (similar to the effective dose of dexamethasone) concentrations in order to be able to rank the compounds and correlate efficacy with structure. In each experiment mometasone and dexamethasone were included as positive controls.

Table 2. List of corticosteroids used for *in vitro* drug screen

Amcinonide	Betamethasone	Prednisolone acetate	Fluorometholone
Flunisolide	Flumethasone	Corticosterone	Beclomethasone
Fluocinonide	Dexamethasone Acetate	Isoflupredone Acetate	Rimexolone
Fludrocortisone Acetate	Beclomethasone Dipropionate	Triamcinolone	Fluticasone propionate
Hydrocortisone Acetate	Betamethasone Valerate	Triamcinolone Acetonide	Flumethasone Pivalate
Hydrocortisone Base	Diflorasone Diacetate	Flurandrenolide	Hydrocortisone17-Butyrate
Mometasone Furoate	Dexamethasone		

The 8227 *in vitro* LSC culture (Figure 2) is an aggressive, heterogenous patient-derived leukemia sample with mutations in p53, RUNX1 and FLT3-ITD [77]. Unlike most other AML samples, 8227 LSC population can remain undifferentiated *in vitro* and can be cultured for an extended period without the need for compounds that inhibit differentiation such as SR1 or UM729, making it ideal for an anti-LSC drug screen [77, 152]. It maintains an LSC-type hierarchy *in vitro*, with an LSC phenotypic marker profile (CD34 + CD38−) that can be quantified by flow cytometry. This allows for the assessment of differentiation to other cell populations in the sample such as the CD34+CD38+ progenitor and CD34+CD15- blast populations. Cultured 8227 LSC-enriched populations have a LSC gene expression signature that matches the LSC-signature in the primary sample. Furthermore, the 8227 LSC-enriched population undergoes differentiation in response to glucocorticoids, depleting it and making it an ideal system to test the efficacy of additional steroids [77].

In order to test the corticosteroids, I first cultured AML 8227 over several weeks to validate that it could stably grow and maintain these leukemic cells in cell culture (Figures 3 and 4). The cells

were cultured weekly in a 24 well non-treated clear flat bottom microplate using serum free expansion media II and growth factors, they were then incubated for six days at 37 °C with 5% CO2. The identity of 8227 cells was confirmed by their unique cytometry marker profile. The results of the culture showed that AML 8227 could stably maintain the CD34+CD38- LSC-enriched population over several weeks at sufficient levels to be used for an *in vitro* drug screen.

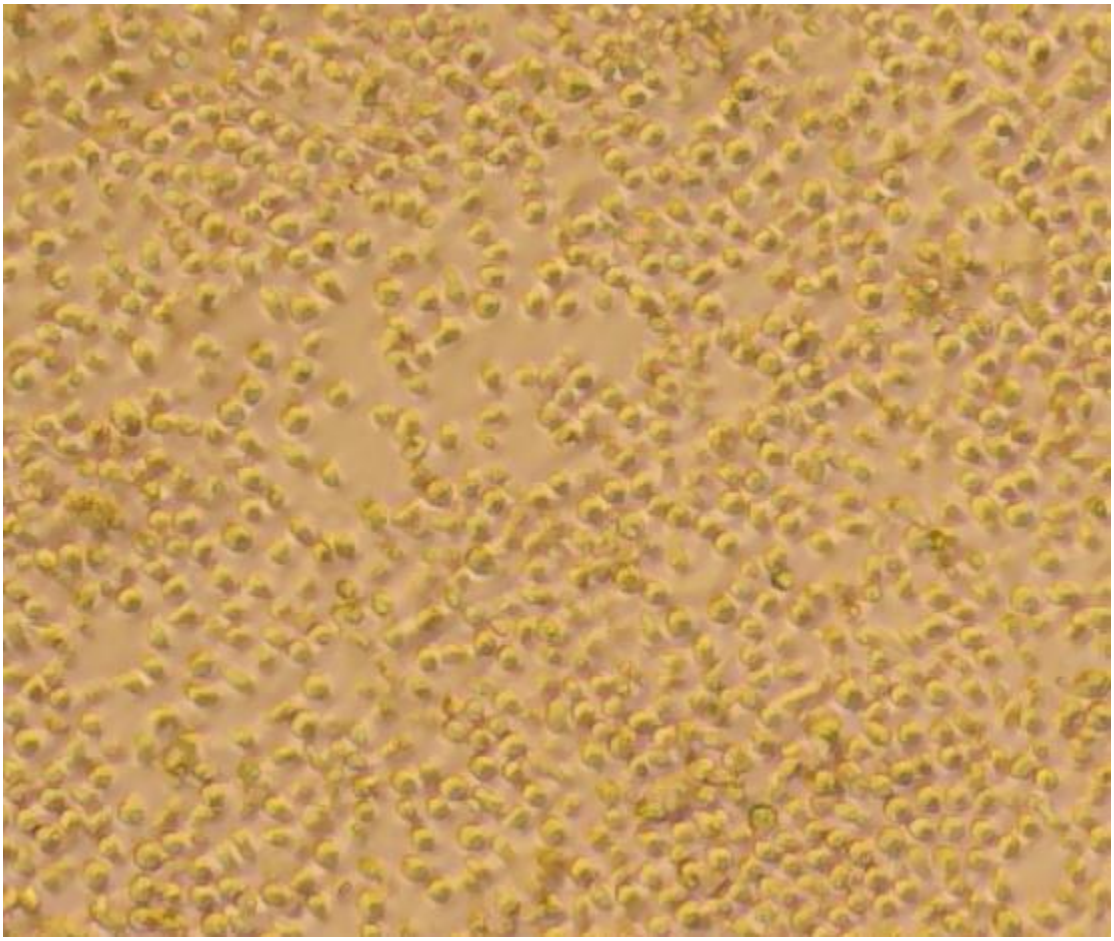


Figure 2: Microscopy of AML 8227 culture. Proliferation of abnormal myeloblast grown in suspension culture for 6 days. Myeloblast are characterised by a large nucleus to cytoplasm ratio.

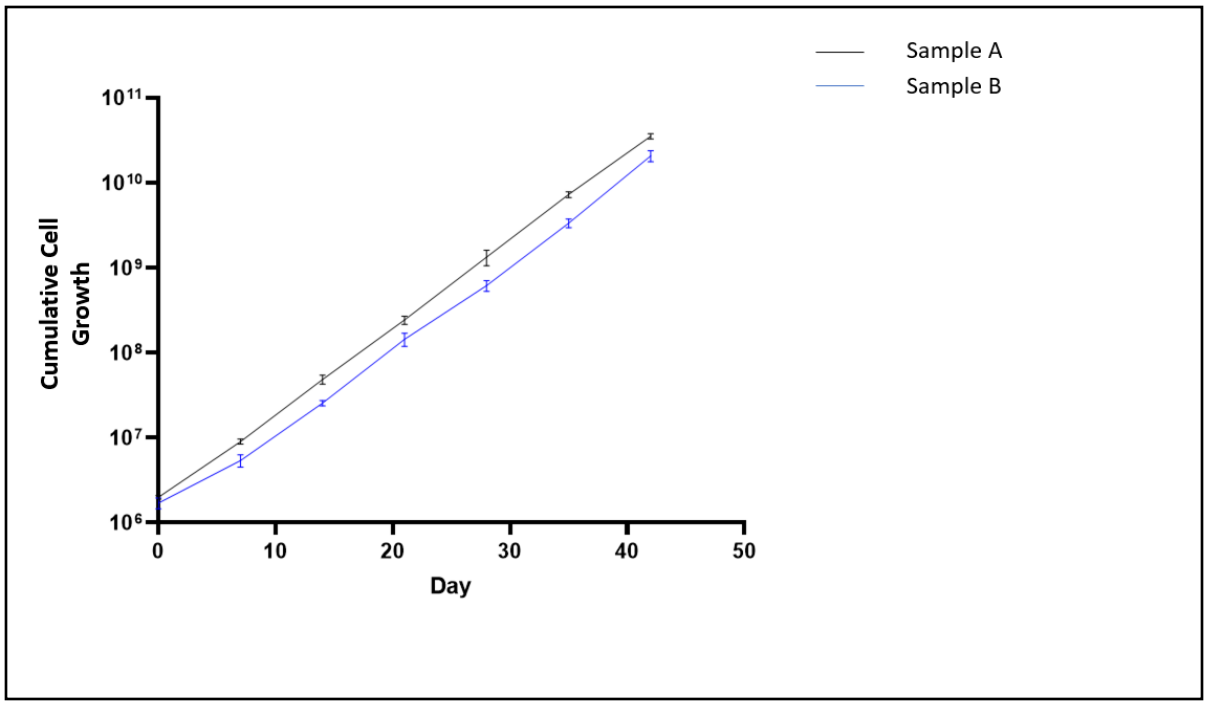


Figure 3: AML 8227 growth curve. Cumulative cell growth of AML 8227 showing stable growth of two samples A and B over six weeks.

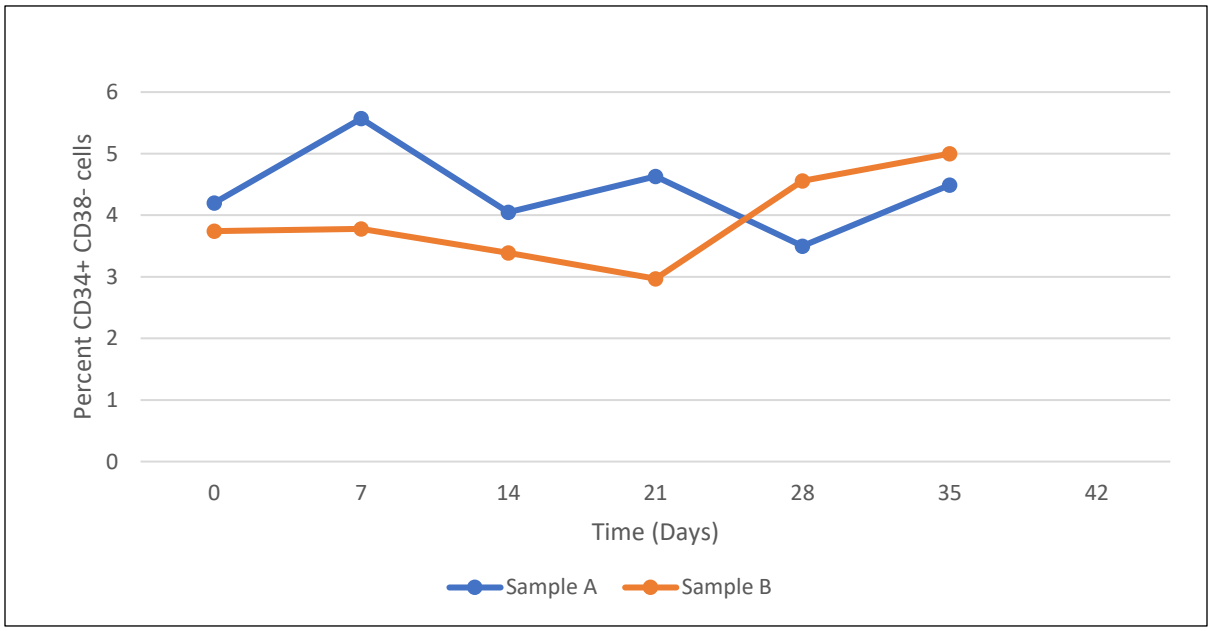


Figure 4: AML 8227 CD34+CD38- growth. Percentage of AML 8227 CD34+CD38- cells for two samples showing relatively stable growth over five weeks.

3.1.2 Group A-type glucocorticoids

I tested seven compounds of the Group A-type corticosteroids (Figures 5, 11). The hydrocortisone type had two compounds fluorometholone and fludrocortisone acetate that were the most effective at 15 nM. Both fluorometholone and fludrocortisone acetate moderately reduced the CD34+CD38- LSC-enriched population. However, fluorometholone was able to cause an expansion of the CD15+CD34- blast cell population, while fludrocortisone acetate did not trigger any expansion of CD34- cells. This indicates that the LSC differentiation triggered by the glucocorticoid compounds may be decoupled from the large production of CD34- cells, depending on the compound (Figure 5 red boxes). Furthermore, corticosterone, hydrocortisone base, prednisolone acetate, and isoflupredone acetate were found to be less effective as they only caused between 31 to 46% reduction in the CD34+CD38- LSC-enriched population at the 15 nM dose as compared to the controls (Figure 5 yellow boxes). Prednisolone acetate, and isoflupredone acetate showed minimal increase in the CD15+CD34- population, while corticosterone and hydrocortisone base had no increase in this population. Hydrocortisone acetate was found not to be effective at targeting AML 8227 when compared to others in this class. Overall, this data suggest that Group A-type corticosteroids are not highly effective at targeting LSCs and would require relatively high doses compared to mometasone or dexamethasone to achieve a significant response.

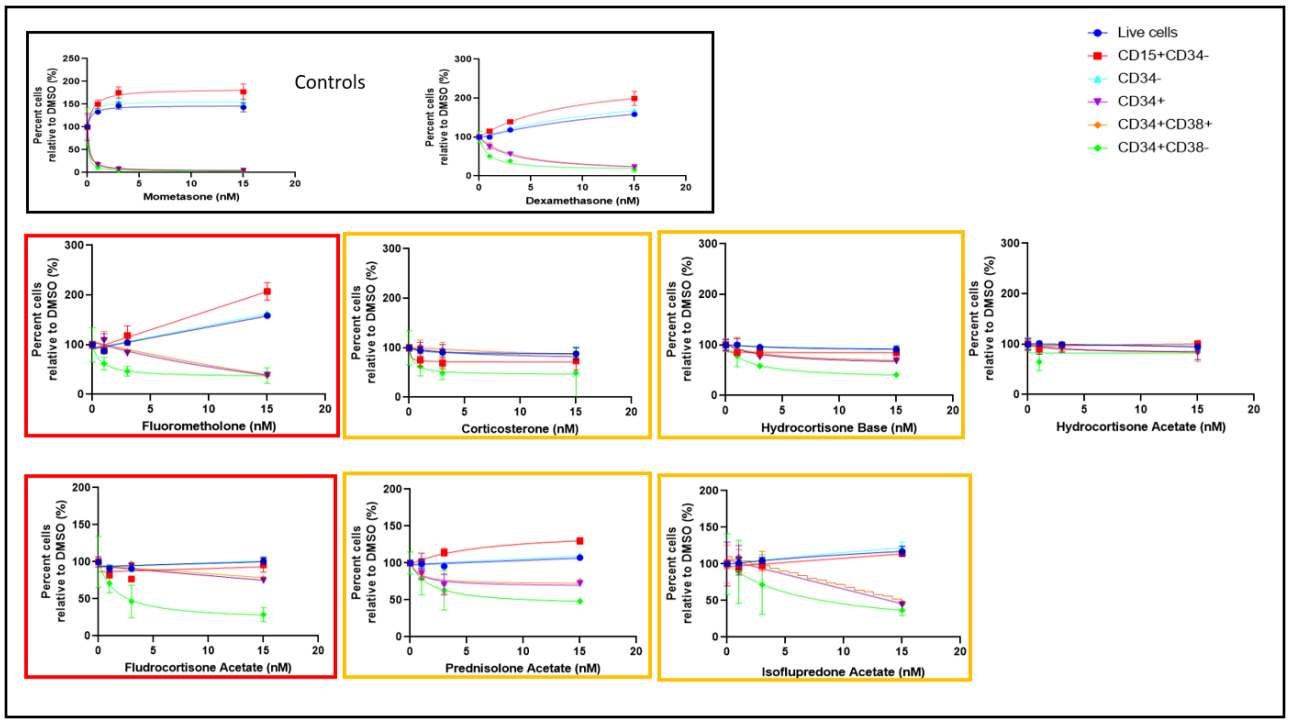


Figure 5: Group A corticosteroids dose response curves. Viability of AML 8227 treated with corticosteroids at 1nM, 3nM and 15nM concentrations for 6 days. Experiments were performed in duplicate and display the mean ± s.d. Dose response curves show the inhibitory effect of the indicated corticosteroid on CD34+CD38- LSC-enriched cells, CD34+CD38+ progenitors and all CD34+ cells. The agonist effect of the corticosteroid on CD15+CD34- blast cells, all CD34- cells and live cells is also shown. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds. Compounds that were not effective are not enclose in a box.

3.1.3 Group B-type glucocorticoids

Next, I examined six compounds of the Group B subtype (Figures 6, 12). This is the triamcinolone acetonide type and it contains the corticosteroids halcinonide and budesonide from the initial screen. This group had five compounds that were able to target LSCs through differentiation (Figure 6 red boxes). Amcinonide, triamcinolone acetonide and flucinonide

effectively decreased the CD34+CD38- LSC-enriched population at 3 nM and were able to cause an expansion of the CD15+CD34 – blast cell population. Of these three compounds amcinonide showed superior LSC targeting ability as it significantly reduced the CD34+CD38- LSC-enriched population by approximately 82% at the 3 nM dose and almost completely eliminated these cells at the 15nM dose. Flurandrenolide and flunisolide were more effective at the 15 nM dose in reducing the CD34+CD38- LSC-enriched population. Triamcinolone had less effect on AML 8227 than others in this class. These data establish that most Group B type compounds consistently eliminate LSCs at low nanomolar doses, indicating that the structural attributes that define this group contribute to anti-LSC activity. These data also suggest that other structural elements besides those that define the group may play a role in causing differentiation as there was variation in the effectiveness of the compounds.

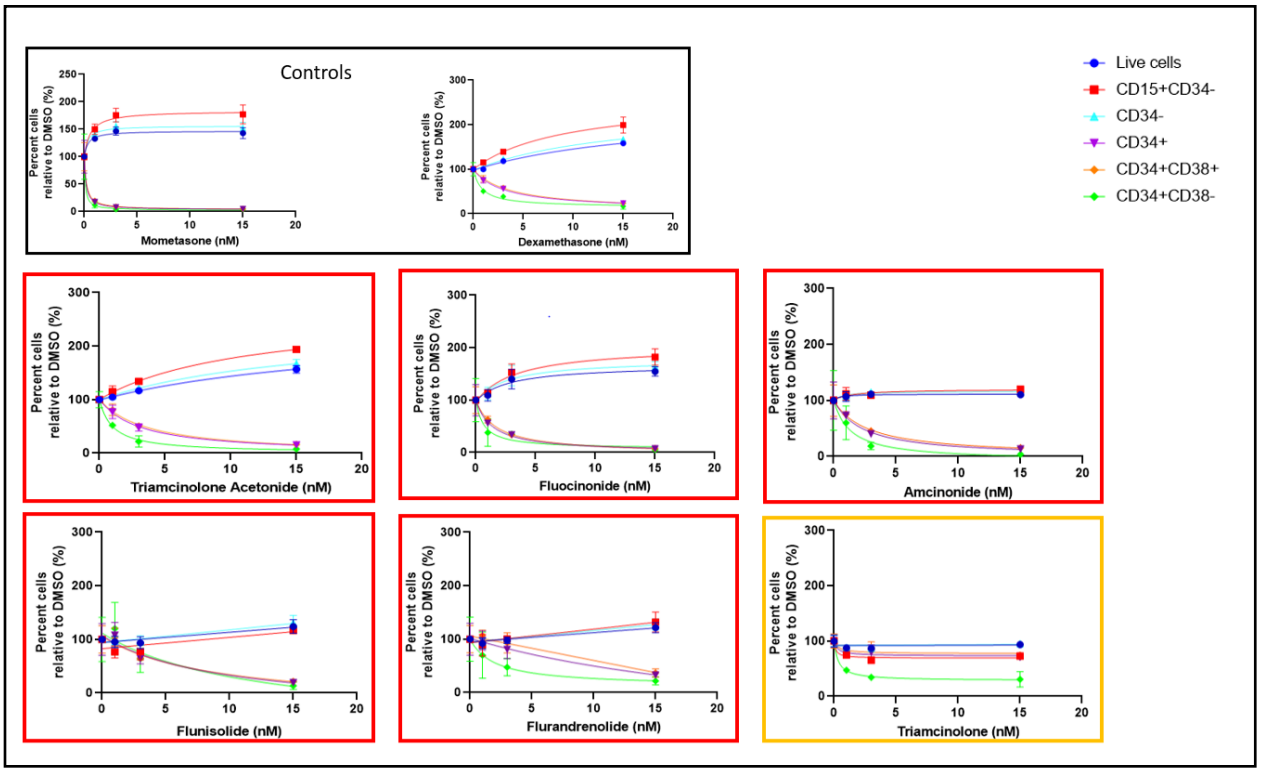


Figure 6: Group B corticosteroids dose response curves. Viability of AML 8227 treated with corticosteroids at 1nM, 3nM and 15nM concentrations for 6 days. Experiments were performed in duplicate and display the mean ± s.d. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds.

3.1.4 Group C-type glucocorticoids

I then tested five compounds of the Group C subtype (Figures 7, 13). These betamethasone-type steroids contain the corticosteroid dexamethasone that our lab had previously validated for differentiation. From this group flumethasone was the most effect corticosteroid, it targeted the CD34+CD38- LSC-enriched population at the 3 nM dose. Dexamethasone acetate, rimexolone and betamethasone effectively targeted the LSC-enriched population at the 15 nM dose. However, rimexolone was the least effective, its ability to target the CD34+CD38- LSC-enriched population was inferior to that of dexamethasone at all three doses. All four compounds were

able to induce differentiation resulting in an expansion of the CD15+CD34- population (Figure 7). Overall flumethasone, betamethasone and dexamethasone acetate targeted the LSC-enriched population more effectively than dexamethasone at the 3 and 15 nM doses. Beclomethasone was found not to be effective on AML 8227. These findings suggest that compounds which targeted the LSC-enriched population at a lower dose that dexamethasone may have other functional groups that increase their anti-LSC activity. As with Group B steroids, these findings further support that differences in effectiveness of the compounds may be linked to differences in the functional groups contained by each member of the subtype. The functional group that characterizes this subtype may not be the only driver of LSC differentiation.

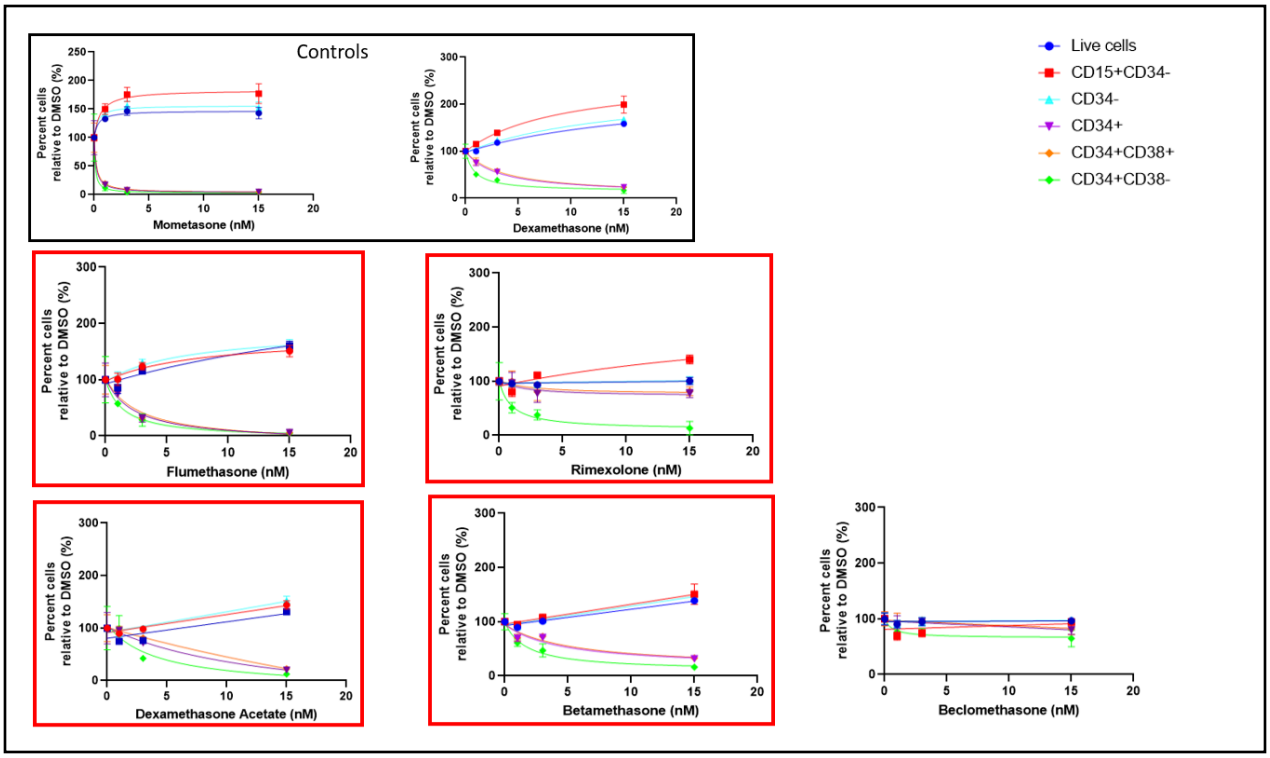


Figure 7: Group C corticosteroids dose response curves. Viability of AML 8227 treated with corticosteroids at 1nM, 3nM and 15nM concentrations for 6 days. Experiments were performed in duplicate and display the mean ± s.d. Red boxes indicate effective compounds, compounds that were not effective are not enclose in a box.

3.1.5 Group D1-type glucocorticoids

Next five compounds of the Group D1 subtype were tested (Figures 8, 14). The betamethasone diproprionate type (halogenated esters) contains mometasone furoate, which was the most effective compound from our previous anti-LSC screen [77]. Fluticasone propionate, flumethasone pivalate, betamethasone valerate, diflorasone diacetate and beclomethasone dipropionate are also in this structural group. From this group fluticasone propionate was found to be the most effective compound. It targeted the LSC-enriched population at the lowest dose of 1 nM, decreasing the CD34+C38- LSC-enriched population by approximately 94% and was superior to mometasone furoate at all three doses. In addition, fluticasone propionate concomitantly expanded the CD15+CD34- blast cell population (Figure 8). Flumethasone pivalate also effectively targeted the CD34+C38- LSC-enriched population, it worked at the 3 nM dose and was more effective than betamethasone valerate and diflorasone diacetate at this dose as well as at the 15 nM dose, where it was also superior to mometasone furoate. Diflorasone diacetate and betamethasone valerate were both effective at the 15 nM dose. However, diflorasone diacetate showed superior elimination of the CD34+C38- LSC-enriched population compared to betamethasone valerate. Beclomethasone dipropionate was found to have less effect on AML 8227 than the rest of the highly effective D1 compounds. These findings suggest that the Group D1 compounds may be the most effective corticosteroids to targeting LSCs at the lowest possible dose and that these compounds possess structural similarities that increase their anti-LSC activity and drive differentiation.

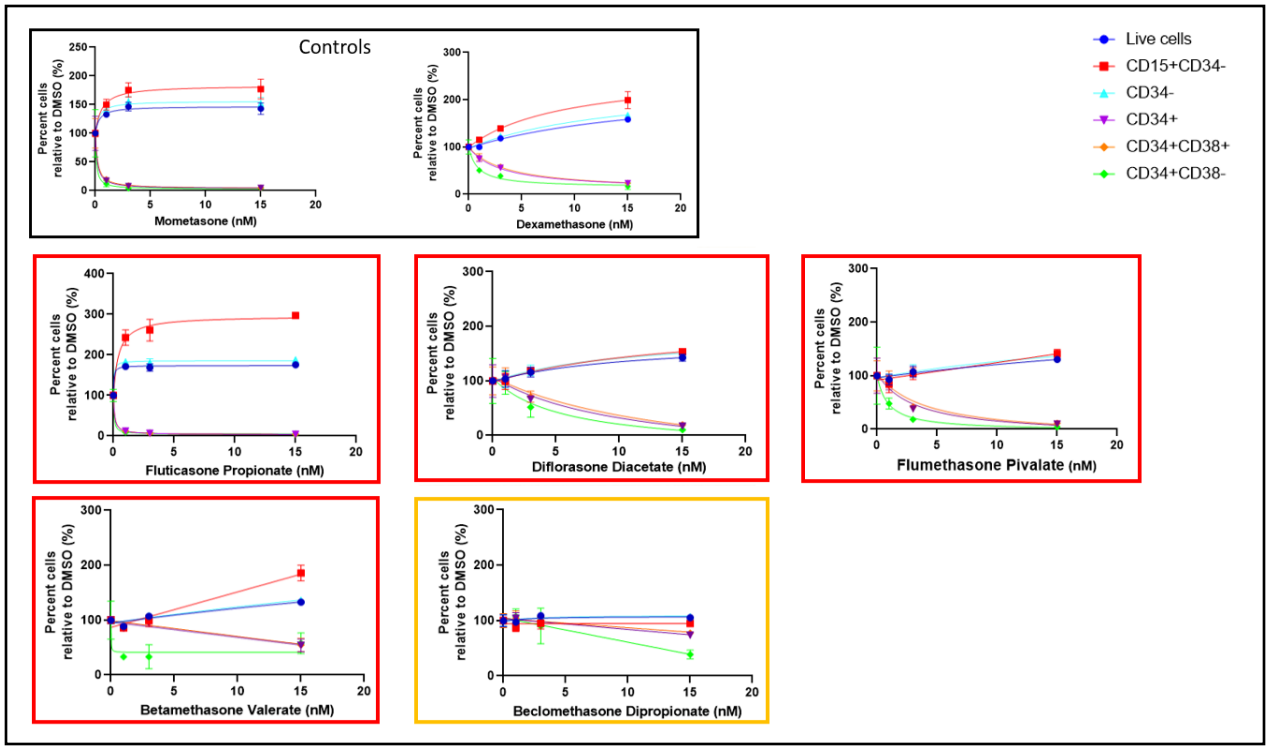


Figure 8: Group D1 corticosteroids dose response curves. Viability of AML 8227 treated with corticosteroids at 1nM, 3nM and 15nM concentrations for 6 days. Experiments were performed in duplicate and display the mean ± s.d. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds.

3.1.6 Group D2-type glucocorticoids

I then tested the Group D2 subtype (Figures 9, 15). The methylprednisolone aceponate type (labile prodrug esters) had one candidate compound hydrocortisone-17-butyrate that was tested. This compound was found not to be effective on AML 8227 (Figure 9), indicating that it lacks the structures necessary for anti-LSC activity.

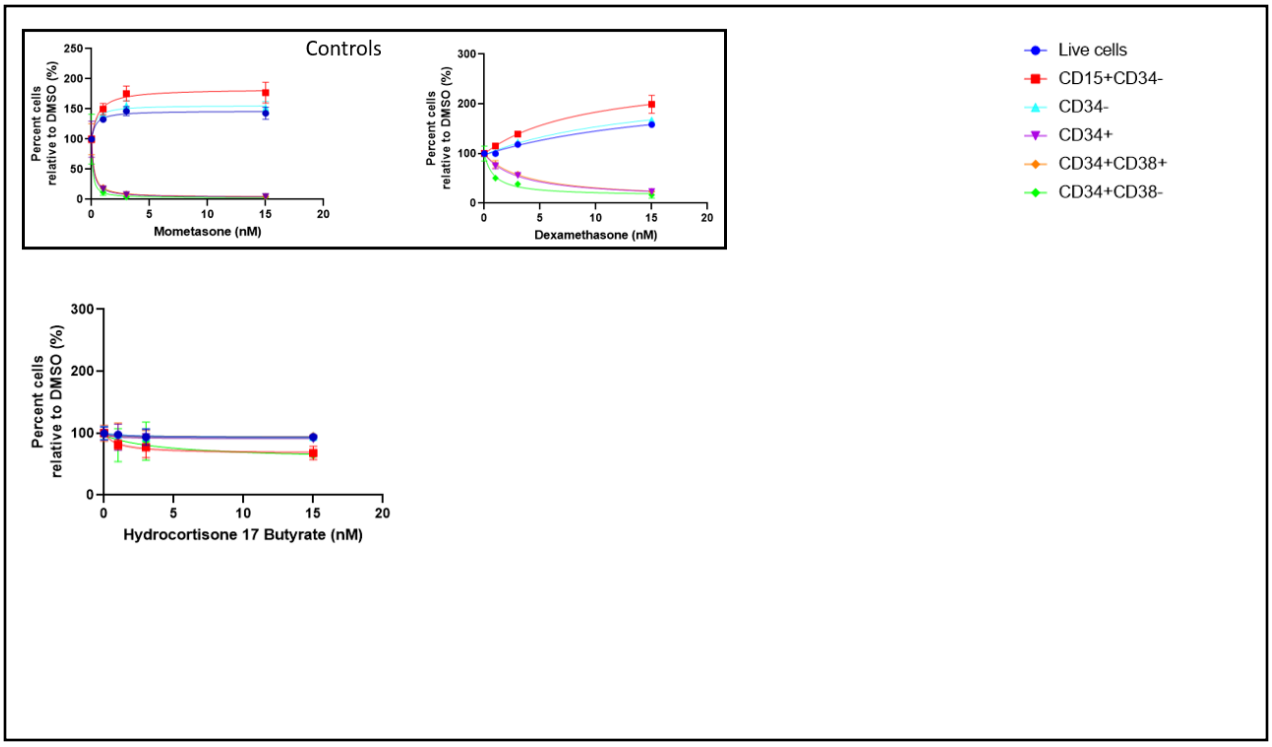


Figure 9: Group D 2 corticosteroids dose response curves. Viability of AML 8227 treated with Hydrocortisone-17-Butyrate at 1nM, 3nM and 15nM concentrations for 6 days. Experiment was performed in duplicate and displays the mean ± s.d.

3.1.7 Retesting of inactive corticosteroids

A number of compounds displayed low effectiveness, even across multiple replicate experiments. In order to verify that compounds with little effect against AML 8227 were truly inactive, they were reordered and retested to confirm their decreased activity and ensure that this was not due to compound degradation or impurity. The results of the retesting showed that while new, fresh preparations of hydrocortisone base, triamcinolone and beclomethasone dipropionate had some activity, they were still substantially less effective than other members of their groups. The other retested corticosteroids had similar dose response curves to prior testing. Results of the retested corticosteroids are shown in Figure 10 and the new dose curves were incorporated into Figures 5-10.

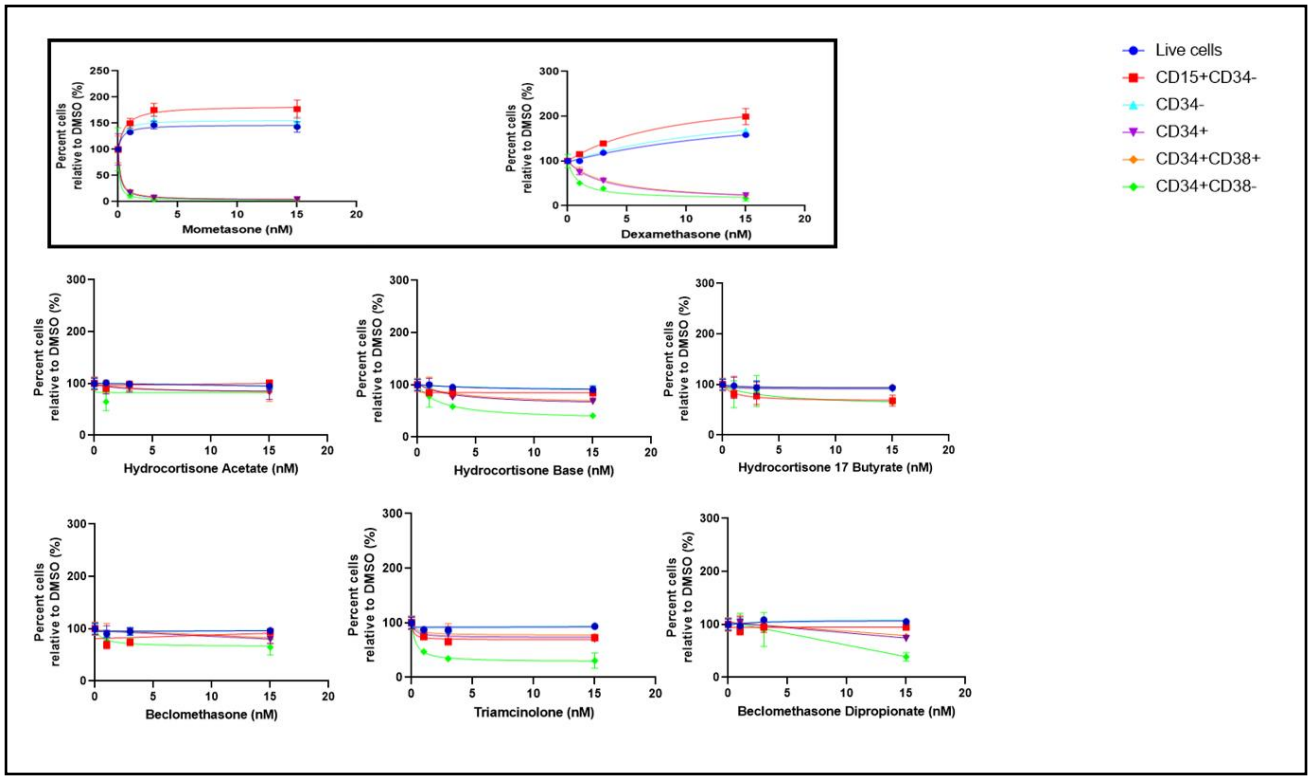


Figure 10: Results of the retested corticosteroids.

3.1.8 Summary of results of the in vitro drug screen

The *in vitro* screen of the 24 corticosteroids showed that 15 of these compounds were able to cause a significant decrease in CD34+CD38- LSC-enriched population. The top six corticosteroids which decreased the LSC-enriched population and increased blast cell count through differentiation were fluticasone propionate, flumethasone pivalate, triamcinolone acetonide, fluocinonide, amcinonide and flumethasone. Fluticasone propionate was the most effective compound of the *in vitro* drug screen it targeted the LSC-enriched population at a dose of 1 nM. Fluticasone propionate and flumethasone pivalate are in the same chemical structure group as mometasone furoate which was previously validated in our lab and found to cause cell differentiation. Flumethasone is in the same chemical structural group as dexamethasone, which

our lab has also validated to cause differentiation. While triamcinolone acetonide, fluocinonide and amcinonide were not as effective as fluticasone propionate, it was interesting to note that these three corticosteroids are from the same chemical structure group as budesonide and halcinonide which were also identified in the initial screen as having anti LSC-activity. The results of the *in vitro* drug screen are summarized in Table 3. Percentage survival for each corticosteroid at the various concentrations is shown in Table 4.

Table 3. Results of *in vitro* drug screen

1 nM	**3 nM**	**15 nM**	**Less Effective**	**Not Effective**
Fluticasone Propionate	Flumethasone Pivalate	Betamethasone	Hydrocortisone Base	Hydrocortisone Acetate
	Amcinonide	Flunisolide	Prednisolone Acetate	Beclomethasone
	Flumethasone	Dexamethasone Acetate	Isoflupredone Acetate	Hydrocortisone - 17-Butyrate
	Fluocinonide	Flurandrenolide	Corticosterone	
	Triamcinolone Acetonide	Rimexolone	Triamcinolone	
		Betamethasone Valerate	Beclomethasone Dipropionate	
		Diflorasone Diacetate		
		Fludrocortisone Acetate		
		Fluoromethalone		

Corticosteroids are listed according to the concentration at which they were found to be effective. Less effective and not effective compounds are listed accordingly.

Table 4. Corticosteroid response

Sample:	Dose	% Survival	Sample:	Dose	% Survival	Sample:	Dose	% Survival
FLUTI	1 nM	6.75635	FLUTI	3 nM	4.45361	FLUTI	15 nM	3.15696
MOM	1 nM	29.0625	MOM	3 nM	13.3892	AMC	15 nM	3.89294
FLU C	1 nM	38.5162	FLU P	3 nM	18.4915	FLU P	15 nM	4.37956
FA	1 nM	45.2555	AMC	3 nM	18.7348	FM	15 nM	5.58592
FLU P	1 nM	48.1752	FM	3 nM	25.1444	MOM	15 nM	6.51969
FM	1 nM	49.0493	FLU C	3 nM	36.1946	TRIAM A	15 nM	10.1903
AMC	1 nM	60.0973	TRIAM A	3 nM	38.1358	BETA	15 nM	13.3154
CORT	1 nM	61.5572	B VAL	3 nM	42.8224	FLU C	15 nM	14.0698
TRI	1 nM	61.6945	BETA	3 nM	43.2284	DA	15 nM	14.27
B VAL	1 nM	63.2603	FLU D	3 nM	45.362	FLU N	15 nM	14.9056
DEX	1 nM	64.0958	DEX	3 nM	49.0396	FLU M	15 nM	15.0852
BETA	1 nM	64.4407	DA	3 nM	50.5375	B VAL	15 nM	16.0584
TRIAM A	1 nM	65.8845	FLU N	3 nM	53.5698	DEX	15 nM	19.3878
FLU D	1 nM	66.022	FLU M	3 nM	54.9878	DD	15 nM	21.1395
RIM	1 nM	69.3431	DD	3 nM	58.0804	FA	15 nM	34.5499
HA	1 nM	74.0983	TRI	3 nM	63.1414	RIM	15 nM	35.0365
ISO A	1 nM	81.6402	FA	3 nM	70.5596	FLU D	15 nM	39.9398
BECLO	1 nM	84.3126	PRED	3 nM	72.0914	BD	15 nM	53.5923
PRED	1 nM	86.0745	BD	3 nM	72.3016	ISO A	15 nM	54.4288
FLU M	1 nM	94.8905	BECLO	3 nM	72.7117	TRI	15 nM	56.362
HYDROCORT	1 nM	95.2296	RIM	3 nM	75.6691	PRED	15 nM	58.9839
DA	1 nM	98.5884	CORT	3 nM	78.1022	BECLO	15 nM	61.7869
BD	1 nM	99.4132	HB	3 nM	80.584	HB	15 nM	64.2582
HB	1 nM	99.6436	ISO A	3 nM	81.4765	CORT	15 nM	69.8297
DD	1 nM	105.664	HA	3 nM	82.264	HYDROCORT	15 nM	86.7114
FLU N	1 nM	106.556	HYDROCORT	3 nM	97.5545	HA	15 nM	95.5644

Table 4 shows the percentage survival for each corticosteroid used in the in vitro drug screen at 1 nM, 3 nM, and 15 nM concentrations. Calculated based on the average of replicates after removing outliers. AMC (Amcinonide), B Val (Betamethasone Valerate), BD (Beclomethasone Dipropionate), BECLO (Beclomethasone), BETA (Betamethasone), CORT (Corticosterone), DA (Dexamethasone Acetate), DD (Diflorasone Diacetate), DEX (Dexamethasone), FA (Fludrocortisone Acetate), FLU C (Fluocinonide), FLU D (Flurandrenolide), FLU M (Fluorometholone), FLU N (Flunisolide), FLU P (Flumethasone Pivalate), FLUTI (Fluticasone Propionate), FM (Flumethasone), HA (Hydrocortisone Acetate), HB (Hydrocortisone Base), HYDROCORT/H17 (Hydrocortisone 17 Butyrate), ISO A (Isoflupredone Acetate), MOM (Mometasone), PRED (Prednisolone Acetate), RIM (Rimexolone), TRI (Triamcinolone), TRIAM A (Triamcinolone Acetonide)

3.2 Analysis the of structural-functional relationship of the corticosteroids

The *in vitro* drug screen results suggested that corticosteroids in the same chemical structure group may have similarities that allow them to target LSCs through differentiation. To further investigate this hypothesis, I obtained the chemical structures of the 24 corticosteroids from PubChem and analyzed them by their group subtypes to determine the structural-functional relationship of chemical groups on activity.

3.2.1 Group A-type glucocorticoids structural analysis

I first analyzed the seven chemical structures of the Group A subtype. The hydrocortisone type analysis revealed that the structures that carried the C9 fluorine such as fluorometholone [153], fludrocortisone acetate[154] and isoflupredone acetate[155] had the highest reduction in the CD34+CD38- LSC-enriched population when compared to structures without this functional group such as hydrocortisone base [156], prednisolone acetate [157], corticosterone [158] and hydrocortisone acetate [159], which had less effect on the LSC-enriched population (Figure 11). While isoflupredone acetate was classified as a less effective compound, its reduction in the CD34+CD38- LSC-enriched population was greater than that of hydrocortisone base, corticosterone and prednisolone acetate. The C1-C2 double bond was found in effective and less effective compounds in this group indicating that while it may play a role in the anti-LSC effect it is the C9 fluorine that increases the anti-LSC activity of these corticosteroids.

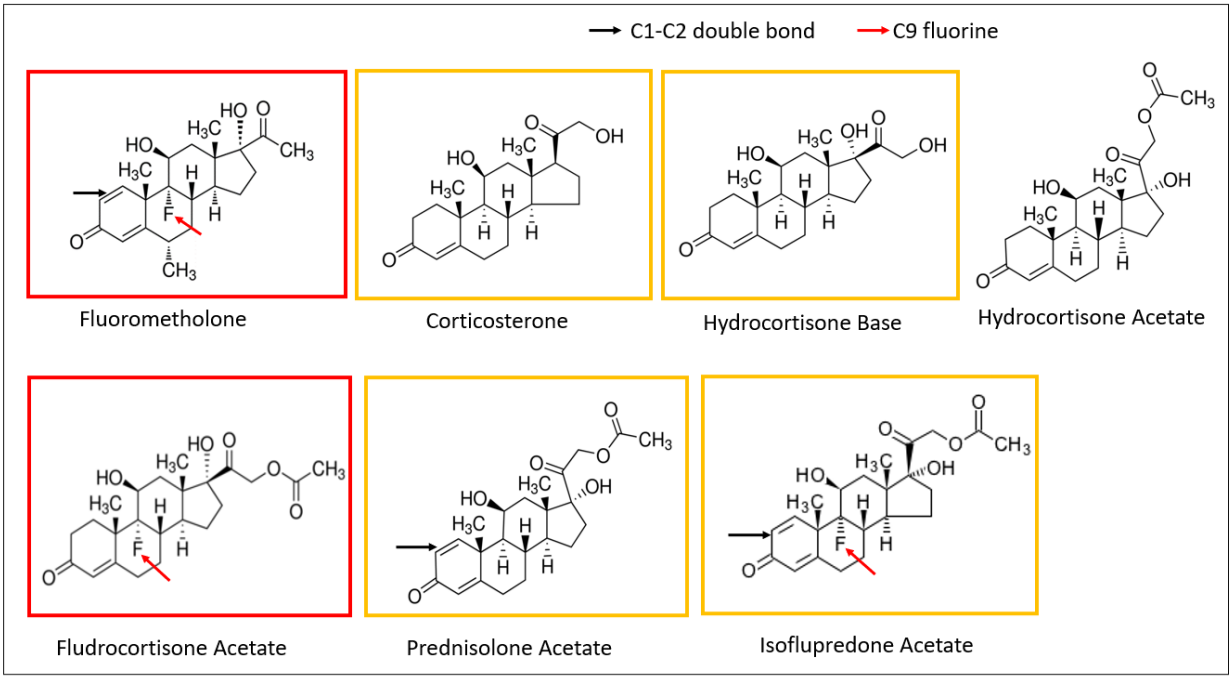


Figure 11: Group A corticosteroids chemical structures. Red arrows indicate the position of the C9 fluorine group that corresponds with an increase in anti-LSC activity. Black arrows indicate the position of the C1-C2 double bond. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds. Compounds that were not effective are not enclose in a box. Corticosteroid structures were obtained and adapted from PubChem.

3.2.2 Group B-type glucocorticoids structural analysis

Next, I analyzed the six chemical structures of the Group B subtype. The triamcinolone acetonide type analysis showed that structures that carried a C16-C17 acetonide group such as triamcinolone acetonide [160], fluocinonide[161], amcinonide [162], flunisolide [163] and flurandrenolide [164] had more efficacy than triamcinolone [165], which did not carry this group. Triamcinolone has a C16-C17 diol group instead and was found to have a reduced effect on CD34+CD38- LSC-enriched cells or CD15+CD34- blast cells (Figure 12). Budesonide and

halcinonide, which caused loss of LSC-enriched populations in the initial screen at low nM concentrations, are from Group B and both have acetonide groups. While the C1-C2 double bond and C6/C9 fluorine were present in the effective compounds in this group, they did not differentiate them from the least effective compounds indicating that it is the C16-C17 acetonide group that determines the anti-LSC activity.

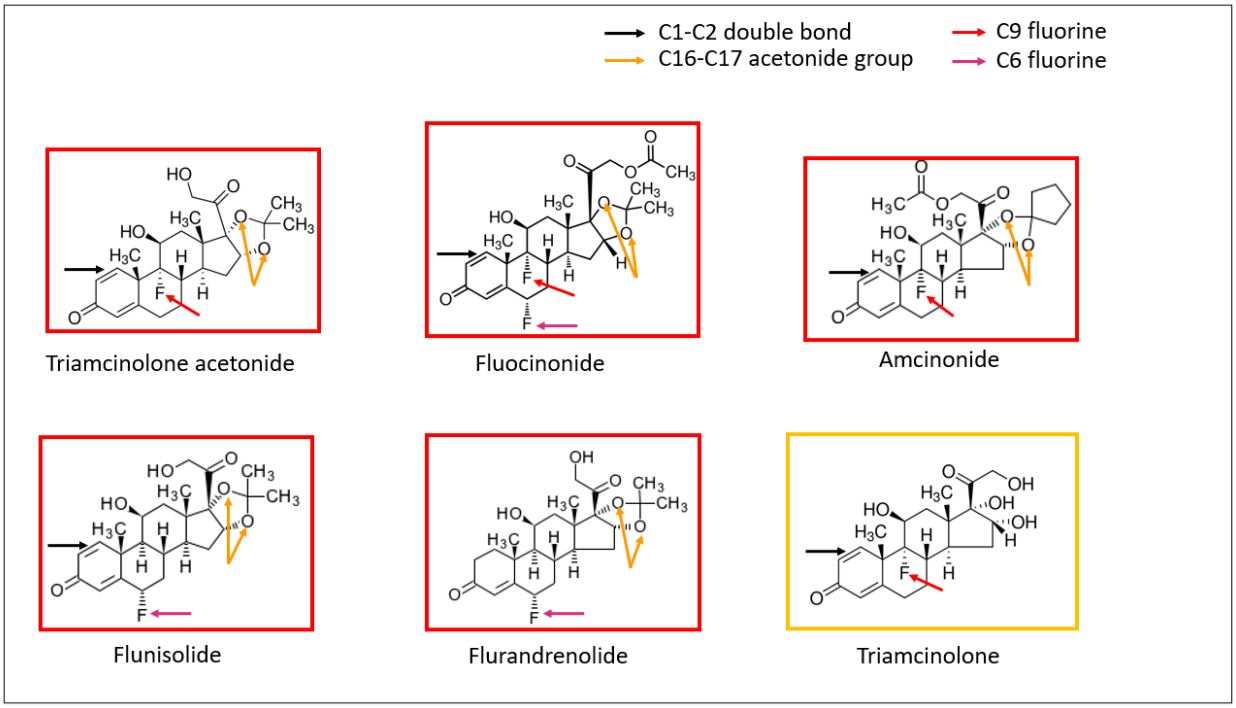


Figure 12: Group B corticosteroids chemical structures. Orange arrows indicate the position of the C16-C17 acetonide group which corresponds with an increase in anti-LSC activity. Black arrows indicate the C1-C2 double bond. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds. Corticosteroid structures were obtained and adapted from PubChem.

3.2.3 Group C-type glucocorticoids structural analysis

I then analyzed the five chemical structures of the Group C subtype. The betamethasone type most effective compounds had a C6 and/or C9 fluorine in addition to the C1-C2 double bond and a C16 methyl group which characterizes this group's subtype. The effective compounds included flumethasone [166], dexamethasone acetate [167], betamethasone[168] and rimexolone [169]. Compared to dexamethasone [170], the most effective compound flumethasone carried an additional C6 fluorine group that appears to increase its anti-LSC activity. Rimexolone was an exception as it did not have a fluorine group but did have an effect. Beclomethasone [171] does not possess a fluorine group and was found to not to be effective on AML 8227 cells (Figure 13).

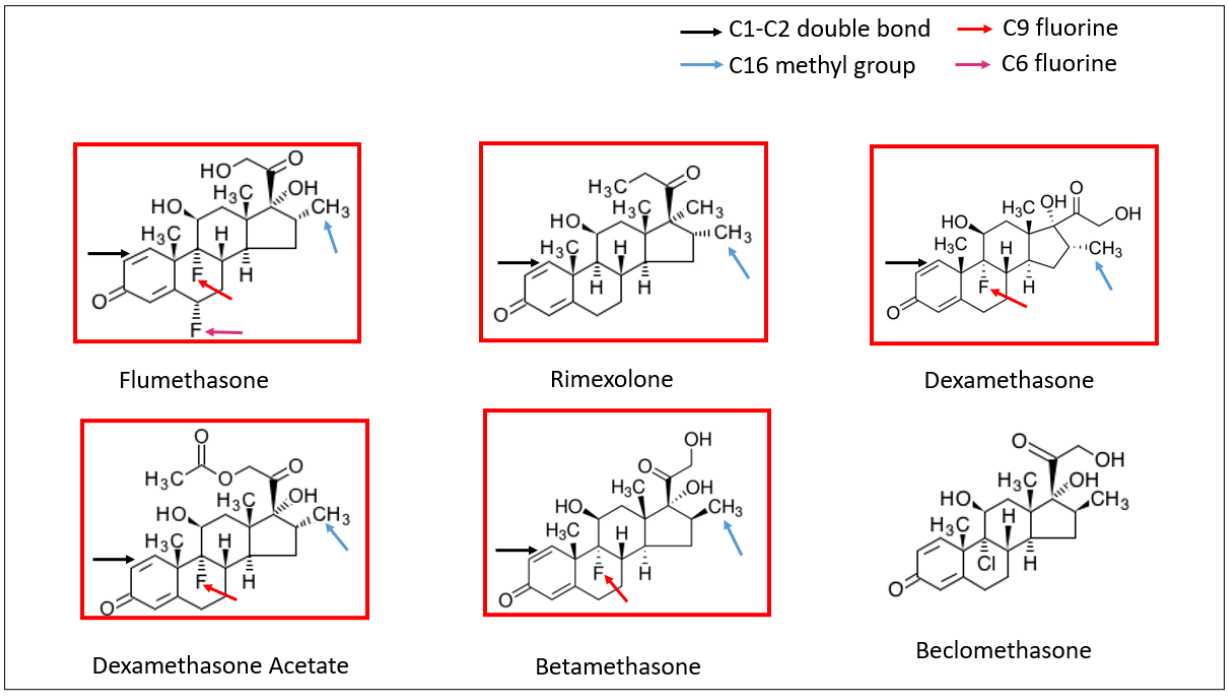


Figure 13: Group C corticosteroids chemical structures. The red arrows indicate the position of the C9 fluorine group, purple arrow indicated the C6 fluorine group, black arrows indicate the C1-C2 double bond and the blue arrows indicate C16 methyl group, all of which correspond with an increase in anti-LSC activity. Red boxes indicate effective compounds. Compounds that were

not effective are not enclose in a box. Corticosteroid structures were obtained and adapted from PubChem.

3.2.4 Group D1-type glucocorticoids structural analysis

Next, I analyzed the chemical structures of the five Group D1 subtype corticosteroids. The betamethasone diproprionate type (halogenated esters) analysis revealed that the effective compounds contained a C9 and/or C6 fluorine as well as a C1-C2 double bond and C16 methyl group. These are the same groups carried by the effective Group C compounds. In addition, the effective Group D1 compounds carried a C17 ester. The effective compounds included fluticasone propionate [172], diflorasone diacetate [173], flumethasone pivalate [174] and betamethasone valerate [175]. Beclomethasone dipropionate [176] carries a C16 methyl group and C17 ester but not a C9 or C6 fluorine and was found to be less effective (Figure 14). This suggests that the C9 and C6 fluorine play an important role in the occurrence of the anti-LSC effect. Mometasone furoate [177] is also in this group and while it does not carry a C9 or C6 fluorine it does carry a C9 chlorine which is a halogen like fluorine. This further suggests that halogenation at the C9 position is important for the anti-LSC effect. Furthermore, mometasone carries a C17 furoate ester suggesting that the anti-LSC effect may depend on the type of ester that occupies the C17 position.

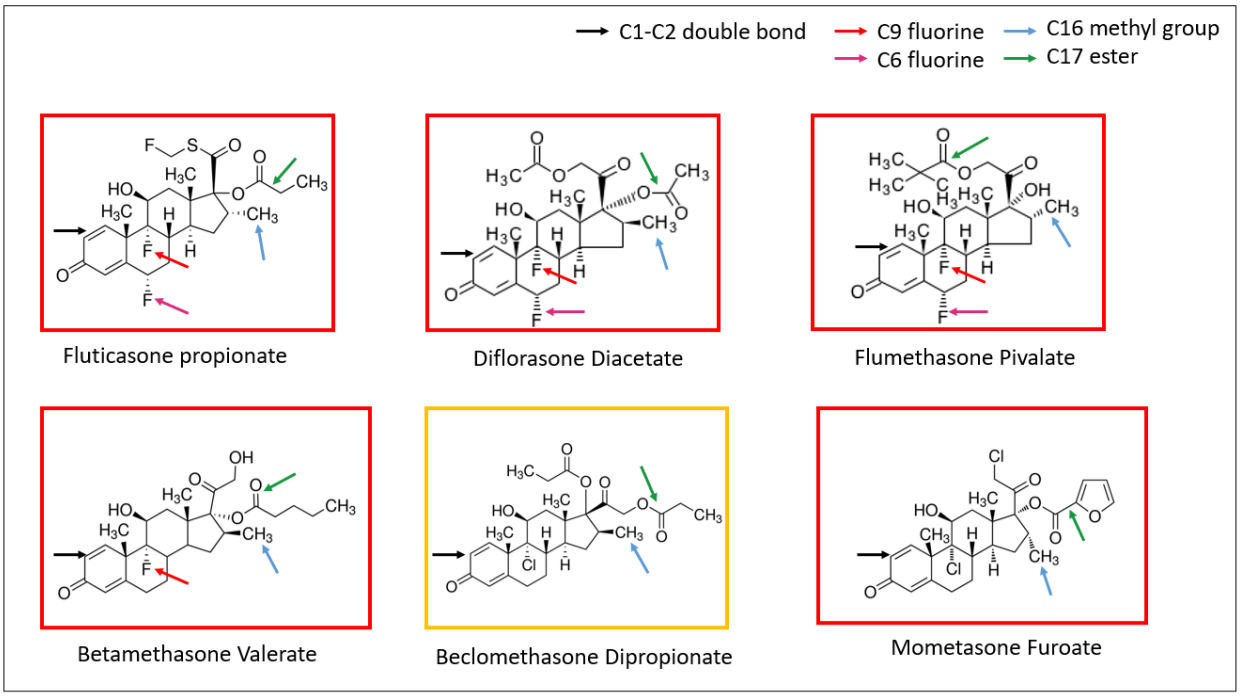


Figure 14: Group D1 corticosteroids chemical structures. The red arrows indicate the position of the C9 fluorine group, purple arrow indicated the C6 fluorine group, black arrows indicate the C1-C2 double bond, blue arrows indicate C16 methyl group and the green arrows indicate the C17 ester, all of which correspond with an increase in anti-LSC activity. Red boxes indicate effective compounds, yellow boxes indicate less effective compounds. Corticosteroid structures were obtained and adapted from PubChem.

3.2.5 Group D2-type glucocorticoids structural analysis

Finally, I analyzed the Group D2 subtype. The methylprednisolone aceponate type (labile prodrug esters) contains the corticosteroid hydrocortisone-17-butyrate [178], which was found not to be effective at targeting LSCs. Analysis of its chemical structure showed that it does not carry a C1-C2 double bond or C9/C6 fluorine. The lack of these structural groups corresponded with its inability to target LSCs (Figure 15).

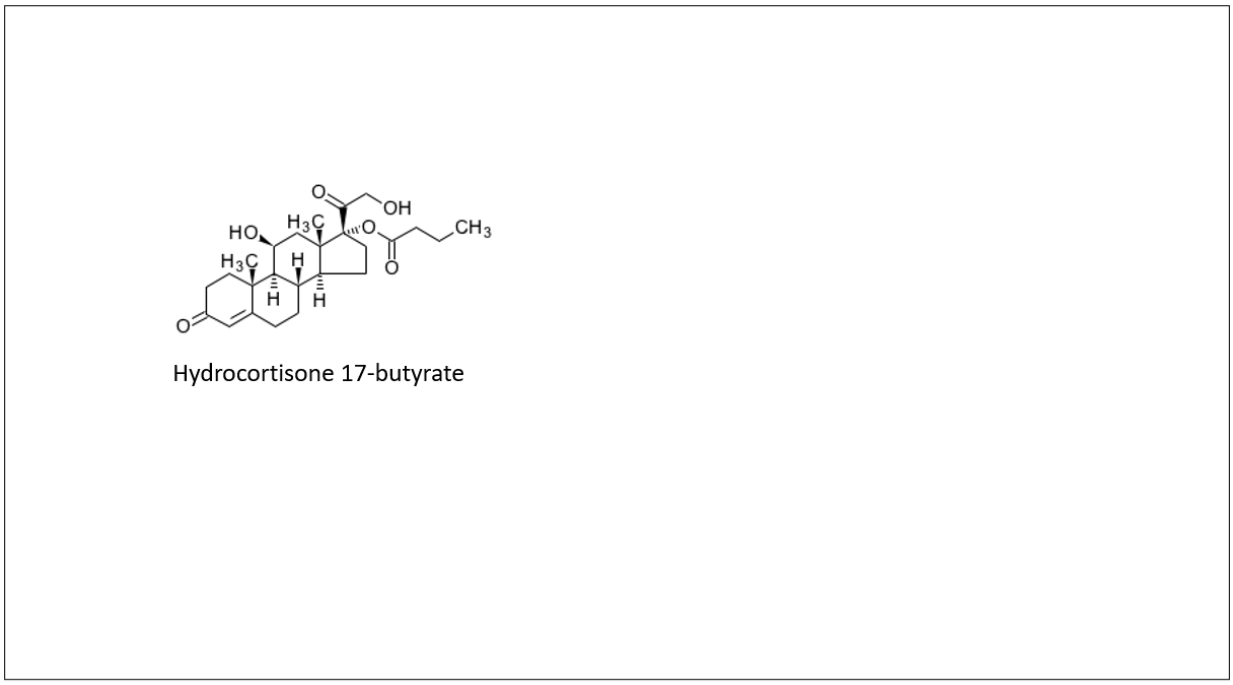


Figure 15: Group D2 corticosteroid chemical structure. Structure of Hydrocortisone-17-Butyrate which had an extremely reduced effect on AML 8227. Corticosteroid structure was obtained and adapted from PubChem.

3.2.6 Results of the glucocorticoids structural-functional relationship analysis

Analysis of the structural-functional relationship of chemical groups on activity showed that the C1-C2 double bond was present in the majority of effective compounds indicating that it plays a role in the mechanism by which corticosteroids target LSCs. Additional key subgroups are the C6/C9 flourine and at the C16 and C17 positions either a C16-C17 acetonide group, C16 methyl group or C17 ester group. For the Group A corticosteroids the C9 fluorine group was present in the compounds that had the highest reduction in CD34+CD38- LSC-enriched population in this group. For the Group B corticosteroids, the acetonide group was found in all of the effective compounds indicating its necessity for the anti-LSC effect. For Group C and Group D 1 subtype

corticosteroids the C16 methyl group while common to all the structures in those groups seemed to also play an important role in the mechanism by which they target the LSC-enriched population as 8 out of 10 of the corticosteroids with the C16 methyl groups were effective at targeting this population. For Group D1 and D2 subtypes, the presence of a C17 ester also seemed to play an important role as 4 out of 6 corticosteroids with this group were effective. The results of the structural-functional relationship analysis are summarized in Table 5.

Table 5. Results of structural-functional relationship analysis

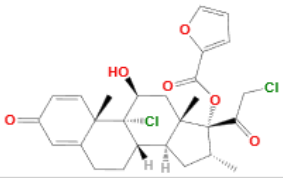
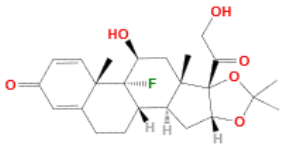
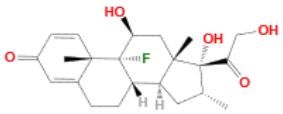
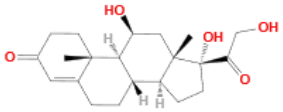
Anti-LSC structure	**No. of compounds with structure**	**No. of effective compounds with structure**
C1-C2 double bond	18	13
C16-C17 acetonide group	6	5
C6/C9 fluorine	16	14
C16 methyl group	10	8
C17 ester	6	4

3.3 Molecular modeling of corticosteroids

To further verify the structural-functional relationship of chemical groups on activity, molecular modeling was performed via computer aided drug design. The compounds hydrocortisone, triamcinolone acetonide, dexamethasone and mometasone furoate where analysed. These compounds were from the Group A, B, C and D1 subtypes respectively. The crystal structures of these compounds bound to the GR were obtained using their Protein Data Bank (PBD) files. The molecular structure of the corticosteroids was compared to the percentage survival of the CD34+CD38-LSC-enriched population at the 15 nM concentration. This showed that as the hydrophobicity and bulk on the five carbon D ring of the corticosteroids increased there was a corresponding decrease in the CD34+CD38- LSC-enriched population indicating that corticosteroids which had more or larger functional groups on their D ring had greater anti-LSC activity Table 6. This further explains why compounds such as mometasone furoate and

fluticasone propionate were able to induce greater reduction in the CD34+CB38- LSC-enriched population.

Table 6. Comparison of structures and percentage survival

PDB code	cmpd		%SURVIVAL (15nM)
4P6W	mometasone furoate		6.52
5UFS	triamcinolone acetonide		10.19
1M2Z	dexamethasone		19.38
6NWL	hydrocortisone		64.26

This table compares the corticosteroid structures and percentage survival of the CD34+CD38- LSC-enriched population at the 15 nM concentration for mometasone furoate (4P6W) [179], triamcinolone acetonide (5UFS) [180], dexamethasone (1M2Z) [181] and hydrocortisone (6NWL) [182].

3.3.1 Alignment and Superposition of PDB files

Next the PDB files used in Table 6 were aligned and superposed using molecular operating environment (MOE) software. The superposed structures in Figure 16 show ribbons that are

drawn as lines and are colored from green (low root-man-square distance - RMSD) to red (high RMSD). The ligands are shown in cyan. The steroid core of the ligands occupies the same position in all structures demonstrating that all the corticosteroid ligands occupy the same ligand binding domain. The ribbons are mostly green in the figure indicating there is little change in the backbone geometry of the GR as the protein binds these different ligands. These results suggest that GR activity depends on other factors besides the ligand simply binding to the receptor.

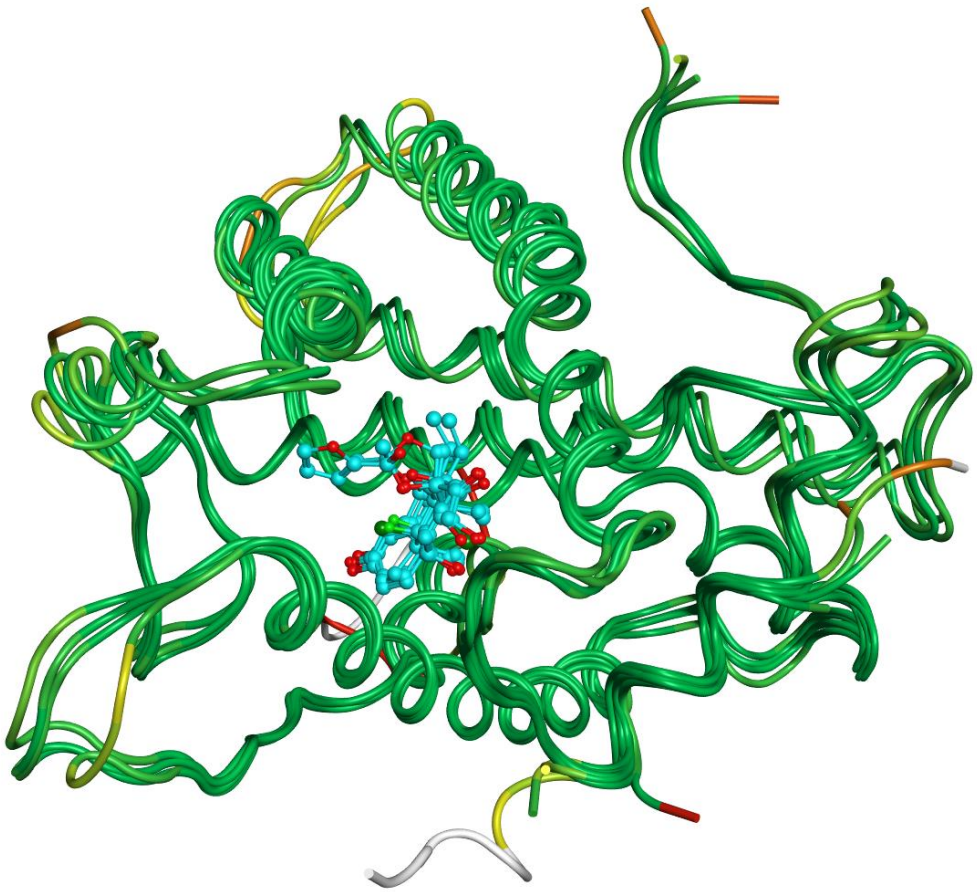


Figure 16: Superposed PDB files. Crystal structures of the corticosteroids hydrocortisone, triamcinolone acetonide, dexamethasone and mometasone furoate bound to the glucocorticoid receptor (GR) are shown superposed. Ribbons are representative of GR helices and their colour ranging form green to red indicate the root-man-square distance.

3.3.2 Residue motion in binding pocket

To further determine the effects of the corticosteroid ligand binding to the GR, residue motion in the GR ligand bind pocket was investigated. This showed that the most significant difference in binding pocket geometry between the four structures occurs at the glutamine residue Q111 side-chain. Figure 17 shows a close-up of the binding pocket for each of the four structures. The pictures in Figure 16 (A) and (B) show the Q111 side-chain rotated out of the pocket when these ligands bind. These are active ligands with 6.92 and 10.19 % survival rates respectively. In contrast, Q111 is rotated towards the pocket and makes a hydrogen bond interaction when the ligands in Figure 16 (C) and (D) bind. These ligands are less active with 19.4 and 64.26% survival rates respectively. These findings indicate that the GR ligand binding pocket residues specifically Q111, can differentiate between corticosteroid ligands with large or small functional groups on the D ring and that large function groups can cause specific confrontational changes in the ligand binding pocket that determines GR activity. Furthermore, this data also suggests that corticosteroids with smaller function groups on the D ring form weaker hydrogen bonds with binding pocket residues rather than more stable hydrophobic bonds that stabilize the ligand in the binding pocket. This explains why corticosteroids like hydrocortisone would be minimally effective as they would not form stable bonds to securely fit into the ligand binding pocket and effectively upregulate the GR.

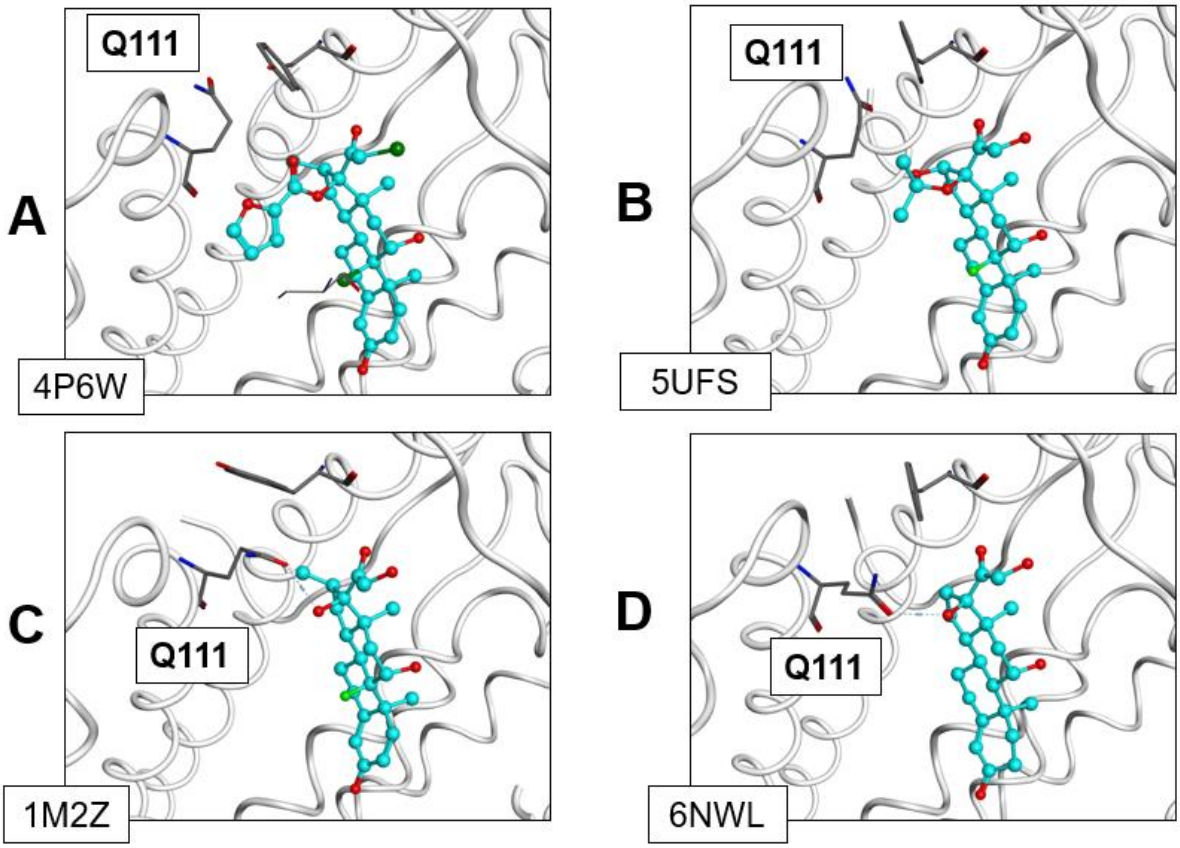


Figure 17: Motion of residue Q111. Close-ups of the binding pocket for each of the four structures with the orientation of the glutamine residue Q111 is shown. (A) Mometasone furoate. (B) Triamcinolone acetonide. (C) Dexamethasone and (D) Hydrocortisone.

3.3.3 Nature of perturbed binding pocket

In order to further understand the effect of the Q111 residue on the binding pocket. The binding pocket of 4P6W (mometasone furoate) was further studied and is shown in Figure 18. The figure clearly shows that displacement of Q111 causes a hydrophobic region to open up which is occupied by the bulky five carbon ring substituents. This illustrates that the movement of Q111 is dependent on the presence of the large functional group and that Q111 facilitates hydrophobic

bond formation with larger functional groups that secure the ligand in place leading to GR conformational changes that upregulate GR activity.

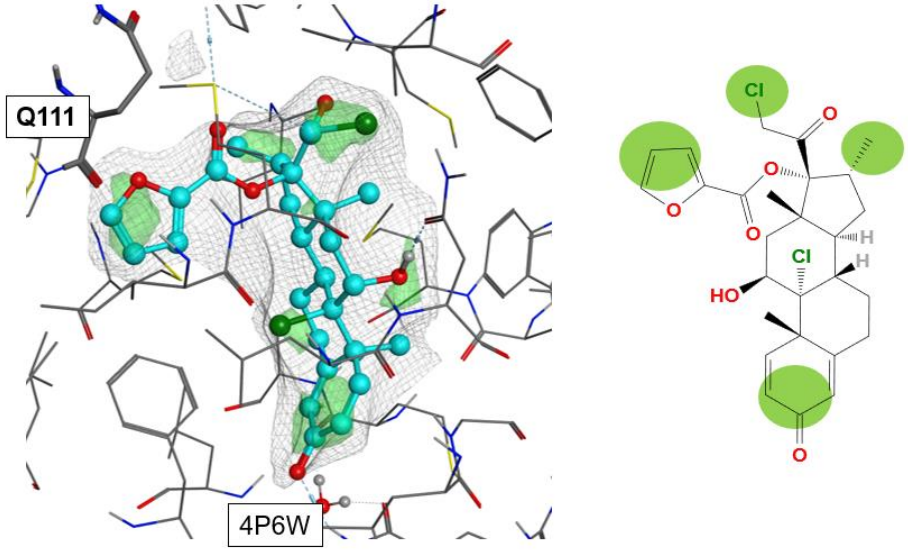


Figure 18: Perturbation of the ligand binding pocket. The ligand binding pocket of 4P6W is shown. The molecular surface is drawn in line mode, and hydrophobic binding hotspots as determined by MOE's electrostatic maps calculation are drawn as green patches. A 2D diagram is also shown that demonstrates which parts of the ligand are in contact with the hydrophobic patches.

Altogether these data suggest that high activity molecules tend to have more hydrophobic bulk on the five carbon D ring than the low activity molecules. This coincides with the C17 furoate and propionate esters found on mometasone and fluticasone respectively. Examination of the four PDB structures suggest that the bulky groups on the five-carbon ring of active molecules force Q111 to rotate outside of the pocket. In contrast, low-activity molecules make a hydrogen bond with Q111 which keep the sidechain rotate towards the pocket. Therefore, activity seems

to depend on the ability of the ligand to force Q111 to point away from the pocket. Bulky hydrophobic groups like the C17 esters help to achieve this by occupying the hydrophobic hot-spot patches that form when Q111 rotates outside of the pocket. Additionally, the position of the hydrophobic hot-spot patches corresponds with the position of the functional groups which appear to increase anti-LSC activity (2D diagram Figure 18), demonstrating that the same functional groups which bind to the GR are the groups which increase anti-LSC activity.

Chapter 4: Discussion

4.1 Significance of this project and its results

Current AML treatment is highly toxic and relatively ineffective, at least partially due to failure to eliminate LSCs. Finding novel, less toxic treatments with the ability to target and subsequently eliminate LSCs that cause the relapse of AML are indispensable in the treatment of patients with this disease. Corticosteroids may be an effective therapeutic against LSCs and may lead to substantially improved outcomes in patients. This project represents the next step in preclinical optimization of corticosteroids to determine the ideal compound for treatment of AML. Many drugs are currently being developed that can potentially treat AML, but these drugs may not specifically target LSCs which underlie AML relapse. Previous work done in our lab has shown that corticosteroids have the ability to specifically target LSCs and induce differentiation of these cells [77]. By identifying which corticosteroids can most effectively target LSCs and analyzing their chemical structure, I have been able to determine the important functional groups these compounds must have in order to carry out their anti-LSC effect. These findings contribute to elucidating the mechanism of action of corticosteroids in LSCs and coincide with findings from the group He *et al* who have shown that corticosteroids function through interaction of their functional groups with certain amino acid residues in the glucocorticoid receptor ligand binding domain [136]. The results of this project help to explain whether the LSC differentiation is through the glucocorticoid receptor or if it is an off-target effect and whether the difference in effectiveness of the corticosteroids is due to other elements or the ability to bind the glucocorticoid receptor.

The results from the *in vitro* drug screen identified many corticosteroids that were similar to those from the initial drug screen including dexamethasone. The data indicated that nine compounds flurandrenolide, flunisolide, dexamethasone acetate, betamethasone, rimexolone, betamethasone valerate, diflorasone diacetate, fludrocortisone acetate and fluorometholone were effective against LSC-enriched populations at a concentration similar to that of dexamethasone at around 15 nM. Five compounds amcinonide, triamcinolone acetonide, flucinonide, flumethasone pivalate and flumethasone were effective at a concentration between that of mometasone and dexamethasone at 3 nM and 15 nM, and one compound fluticasone propionate was effective at a concentration similar to mometasone at 1nM. Further testing is needed to establish the optimal concentration of the corticosteroids that worked at 1 nM and 3 nM, as their dose response curves suggested that they may work at lower concentrations than those used in the drug screen. A wide range of concentrations between 0.1 nM and 3nM should be used to calculate the IC50 value of the CD34+CD38- LSC-enriched cells and the EC50 valve of the CD15+CD34- blast cells of each corticosteroid.

The results of the *in vitro* drug screen also showed that the most effective compounds were from the same structural groups as the compounds that our screen had identified to cause differentiation of LSCs. Fluticasone propionate, which worked at 1 nM and flumethasone pivalate which worked at 3 nM are from the same structural group as mometasone furoate (Group D1), amcinonide, triamcinolone acetonide and fluocinonide, which worked at their lowest effective dose of 3 nM, are from the same group as budesonide and halcinonide (Group B) and flumethasone, which also was effective at 3 nM, is from Group C the same group as dexamethsaone. This suggests that corticosteroids that have specific functional groups have the propensity to target LSCs and induce differentiation. This appears to be due to the ability of

these functional groups to increase binding affinity for the glucocorticoid receptor [136], which appears to regulate differentiation. A decrease or absence of effectiveness against LSC-enriched cells was seen with corticosteroids that lacked the C1-C2 double bond, C16-C17 acetonide group and the C6/C9 fluorine. All of which increase interaction with the glucocorticoid receptor [136]. These findings along with those from our molecular modeling strongly suggest that the LSC differentiation we observed is through the glucocorticoid receptor and is not an off-target effect.

4.2 Implication of identifying the optimal corticosteroid to target AML LSCs

Identifying the optimal corticosteroid to target AML LSCs is a first step in finding a treatment that can eradicate AML at its source. This would decrease AML relapse and reduce the amount of intense cytotoxic therapy needed for further treatment, as occurs with consolidation therapy. Patients would therefore benefit from less toxic side effects and experience improved overall survival. The study conducted by Bertoli et al. included the addition of dexamethasone for the treatment of hyperleukocytic acute myeloid leukemia and resulted in increased disease-free and overall survival and highlights the benefit of corticosteroid use in treating AML patients [149]. Identifying the optimal corticosteroid to target LSCs could possibly enhance future results of similar studies. Research in our lab has also shown that the use of effective corticosteroid in conjunction with standard therapy (cytarabine) can increase the elimination of LSCs and bulk AML cells [77]. By finding the optimal corticosteroid it may be possible to improve the effectiveness of this combination therapy. Additionally, this gives insight into the structural components necessary to create the most effective corticosteroid.

Corticosteroids can cause severe negative side effects when used at high doses for long durations. Some of these side effects include diabetes, hypertension, adrenal atrophy,

osteoporosis, skin atrophy, peptic ulcers, glaucoma, cataracts, psychosis and infections [96]. For this reason there is hesitancy to use corticosteroid in AML patients. These patients have a greater risk of developing breakthrough infections as their immune system is compromised both from the disease and chemotherapy. By using the optimal corticosteroid at a low dose it may be possible to avoid these negative side effects. Results from this project suggest that fluticasone propionate may be an ideal candidate as it can be used at a low dose with the advantage of effectively targeting LSCs that drive the disease. Use of this corticosteroid as part of induction therapy may be beneficial as it could eliminate LSCs and prevent relapse. However, further testing will be required to determine if the pharmacokinetics of this compound would allow for its use as a therapy in AML patients.

4.3 Importance of structural-functional relationship in corticosteroid design

The results of the structural-functional relationship analysis showed that the C1-C2 double bond, C6 or C9 fluorine, C16-C17 acetonide group, C16 methyl group and the C17 ester were the most important groups for anti-LSC activity. These groups appeared to give the corticosteroids that carried them superior properties. Corticosteroid structural analysis done by He *et al.* has also linked these groups to the glucocorticoid receptor [136]. Understanding how the functional groups work is fundamental to designing corticosteroids with superior anti-LSCs properties. Functional groups can be used to design new corticosteroids with increase binding affinity for the glucocorticoid receptor. This would result in corticosteroids with increase potency that can be used at lower doses. He *et al.* investigated the structural-functional relationship of mometasone furoate, dexamethasone and cortisol in relation to their functional groups, binding affinity and potency. What they found was that mometasone furoate that contains the C1-C2 double bond,

C16 methyl group and C17 furoate group had the greatest binding affinity for the glucocorticoid receptor and the greatest potency when compared to dexamethasone that lacked only the furoate group, and cortisol that lacked all the groups. This was demonstrated by binding affinity and potency assays that measured the effect of potency on corticosteroid induced transactivation and transrepression, two important processes in the mechanism of action of corticosteroids [136]. Through computer aided drug design, they also discovered that the C1-C2 double bond and C17 furoate group allowed mometasone to form hydrophobic bonds more readily with amino acids in the glucocorticoid receptor ligand binding pocket. It is the C17 furoate group in particular, that gives mometasone furoate its superior glucocorticoid receptor binding ability. The furoate group secures the molecule in place by sticking out above it, occupying an empty cavity formed by the glucocorticoid receptor ligand binding pocket [136]. Our molecular modeling data showed similar results to those of *He et al* when we analyzed mometasone furoate bound to the glucocorticoid receptor. In our drug screen fluticasone propionate, which is from the same structural group as mometasone furoate, also appears to have the ability to fill the cavity with its projecting propionate group. This explains its ability to target LSC more effectively at the lowest screening dose. Corticosteroids that lack the furoate or propionate groups do not seem to be able to fill the empty cavity to form hydrophobic bonds. This causes them to have less binding affinity for the glucocorticoid receptor leading to decrease potency. Our molecular modeling data demonstrated this by showing that as hydrophobicity and bulk on the five carbon D ring increased, percentage survival of CD34+CD38- LSC-enriched cells treated at 15 nM decreased. Furthermore, our data also showed that areas of high hydrophobicity corresponded with the position of the functional groups which increase anti-LSC activity. These findings not only demonstrate the link between the functional groups and the glucocorticoid receptor but also show

that the effectiveness of the corticosteroids is due to their ability to bind the glucocorticoid receptor.

Other important properties of the functional groups, which are necessary for the design of effective corticosteroids, include the ability of the C1-C2 double bond to increase anti-inflammatory effects and reduces unwanted mineralocorticoid effects while adding stability to the corticosteroid molecule. The C6 and C9 fluorines both increase the anti-inflammatory and mineralocorticoid effects. However, the mineralocorticoid effects can be decreased by adding the C16 methyl group. The C16-C17 acetonide group and the C17 esters (furoate and propionate) increase lipophilicity and topical anti-inflammatory activity [120, 123]. The esters also contribute to the physicochemical characteristics of the molecule that impact solubility, dissolution rate and tissue affinity, these influence pharmacodynamic and pharmacokinetic properties [183]. The additional properties of the functional groups help to augment the glucocorticoid activity of the corticosteroids. By adding these groups to soluble corticosteroid core molecules (moieties) it may be possible to create corticosteroids with superior potency and increased anti-LSC effects.

From the drug screen fluticasone propionate was identified as a corticosteroid that possessed all the necessary functional groups for anti-LSC activity (C1-C2 double bond, C6 and C9 fluorine, C16 methly group and C17 ester). Its ability to target LSC-enriched cells at the lowest concentration of 1 nM correlated with having these functional groups. A structural comparison of fluticasone propionate and mometasone furoate revealed that mometasone furoate only carries a C9 chlorine and not a C6/C9 fluorine. This structural difference may be a reason why fluticasone propionate can target LSCs at lower concentrations than mometasone furoate. While fluticasone

propionate may seem ideal, the compound fluticasone furoate should be tested. This compound was designed to incorporate the superior glucocorticoid receptor binding ability of the furoate group with the core molecule of fluticasone which has all the desirable functional groups. Fluticasone furoate is known to be highly potent [136]. It may possibly work at even lower concentrations than fluticasone propionate and could be the best compound for targeting LSCs.

4.4 Corticosteroid potency and corticosteroid mechanism of action in LSCs

The mechanism of action of corticosteroids involves the processes of transactivation and transrepression. Transactivation occurs when the glucocorticoid receptor corticosteroid ligand complex translocates to the nucleus from the cytoplasm and binds to the glucocorticoid response elements at promotor regions of target genes, where it upregulates anti-inflammatory mediators such as lipocortin 1. Transrepression occurs when the complex tethers to transcription factors such as NF-κB and AP-1 down regulating their expression. Both processes are important pathways by which glucocorticoids produce their anti-inflammatory effects. These anti-inflammatory effects seem to play an important role in driving LSC differentiation. This project has shown that corticosteroids with functional groups that increase glucocorticoid receptor binding also induce LSC depletion, indicating that the mechanism of action of corticosteroids in LSC is through the glucocorticoid receptor. Therefore, transactivation or transrepression may be involved in the mechanism of action of corticosteroids in LSCs. An understanding of which pathway is activated in LSC differentiation would help to better explain the mechanism of action of corticosteroids in LSCs. He *et al.* found that corticosteroid potency can affect transactivation and transrepression differently. Potency is defined as the concentration of the drug required to reach half maximal activity (EC50) [136].Through transactivation and transrepression potency assays they found that transactivation potency was 10-fold lower than transrepression potency

for mometasone furoate, dexamethasone and cortisol. For example, at 0.1 nM, mometasone furoate reached 95% of transrepression efficacy but only 25% of transactivation efficacy [136]. This suggests that transrepression could be induced separately from transactivation and could mean that negative side effects which are mostly caused by transactivation could be avoided. It also suggests that transrepression may be the main pathway by which LSCs are depleted as a potent compound that induces LSC loss is more likely to cause transrepression. Another factor that suggests that transrepression may be part of the mechanism of action of corticosteroids in LSCs is transrepression's role in repressing NF-κB. NF-κB is known to be involved in the pathways of inflammation and cancer. In cancer it forms part of the cell survival pathway through induction of target genes TRAF1 and TRAF2 that inhibit components of apoptosis [184]. NF-κB has been shown to be elevated in patient AML blasts, AML CD34+ cells and LSCs [15, 185, 186]. Inhibition of NF-κB in CD34+CD38- LSC-enriched cells with a proteasome inhibitor MG-132 has been shown to induce apoptosis[15]. Therefore, it may be possible that transrepression of NF-κB instead of inhibition may be the mechanism of loss of LSCs through differentiation.

4.5 Glucocorticoid receptor isoforms and corticosteroid treatment response

Variations in glucocorticoid receptor isoforms seem to play a role in determining corticosteroid response. Being able to identify which isoform is dominant in a patient may help to better predict treatment response. Use of corticosteroids to target acute myeloid leukemic stem cells would only be beneficial if the right isoform is dominant. There are five splice variant isoforms of the GR. GRα is normally the predominant form, it has eight translational isoforms all of which bind glucocorticoids and glucocorticoid response elements similarly. However, each translational isoform regulates a unique set of genes [99, 113]. These isoforms will therefore vary in their

sensitivity and response to corticosteroids. For example in some cells isoforms GRα-A, B and C have been shown to be sensitive to dexamethasone leading to apoptosis whereas cells expressing GRα-D were found to be resistant [114]. Among the splice variant isoforms, GRβ lacks the ability to bind glucocorticoids, its dominance however can lead to disruption in the GRα:GRβ ratio leading to glucocorticoid resistance. This is due to GRβ's ability to inhibit GRα and act as a transcription factor allowing it to repress genes [99, 109, 110]. The GR-P splice variant isoform has been linked to glucocorticoid resistance in hematological malignancies and seems to affect the transcriptional activity of GRα [99, 112]. Variations in isoforms and their effects should therefore be considered when proposing to use corticosteroid for the treatment of chronic diseases such as AML. Next generation RNA sequencing may offer a way to detect patients who may possibly be non-responders such as those with elevated levels of splice variant isoform GRβ or GR-P. This would allow patients to avoid unnecessary corticosteroid treatments that cause negative side effects. The sequencing would also identify patients who would benefit the most form corticosteroid therapy.

4.6 Future directions

While this project has successfully identified which corticosteroid may optimally target LSCs. testing of the corticosteroid fluticasone furoate still needs to be done as this compound has all of the functional groups required for increase binding of the glucocorticoid receptor and high potency. Fluticasone furoate may show superior ability to targeting LSCs when compared to fluticasone propionate. Further testing to determine the optimal concentrations at which fluticasone propionate, fluticasone furoate, flumethasone pivalate, triamcinolone acetonide, fluocinonide, amcinonide, and flumethasone cause differentiation still needs to be determined.

IC50 values for the CD34+ CD38- LSC-enriched cells and EC50 values for the CD15+CD34- blasts should be calculated using at least eight concentration data points to determine the most accurate values.

The compounds that have been proven to target AML 8227 LSCs should also be tested in other AML subtypes. Previous testing of corticosteroids in our lab has shown that not all AML subtypes may be sensitive to corticosteroids. AML 9706 for example was used in a previous corticosteroid functional validation screen and found to be resistant to mometasone, it did not differentiate or reduce the CD34 + CD38− LSC-enriched population or increase the CD34- blast population [77]. Resistance in subtypes of AML could be due to the genetically heterogeneous nature of AML or the GR isoform carried by the subtype. Knowing which AML subtypes can be treated with corticosteroids would reveal mutations that are associated with corticosteroid sensitivity. To determine if LSCs are truly being eliminated when treating various subtypes, xenotransplantation of severely immunocompromised mice with treated cells should be done to assess bone marrow engraftment. We would expect that there would be no engraftment.

Further validation of the structural-functional relationships found in this project can be done using computer aided drug design. Using a structural-based drug design approach, the binding affinity (Ki) of the top candidate corticosteroids for the GR can be calculated. This gives an indication of which compounds have the highest affinity for the GR. From the results of this project fluticasone propionate would be expected to have the highest Ki as it possesses all the functional groups for increase binding. 3 D images of the corticosteroids in the GR ligand binging pocket can also be generated to look at the hydrophobic bonds created between the functional groups and the amino acid residues of the ligand binding pocket. Using 3 D imaging it

is possible to see how the corticosteroid ligand fits into the GR ligand binding pocket. This provides the opportunity to analyze the ligand and design analogs which can better bind the receptor. Structural-based drug design can be very useful in designing corticosteroid with superior potency and anti-LSC activity.

4.7 Conclusion

Acute myeloid leukemia is a severe blood cancer that affects adults and children. It has a high prevalence of relapse and is associated with extremely poor outcome. Standard treatment for this disease has remained essentially the same over the past 40 years and is highly toxic to patients. Therefore, there is a need for novel therapies that can treat this disease at its source, while causing less side effects and improving survival. Our lab had previously identified the corticosteroids mometasone furoate, budesonide, halcinonide and dexamethasone as compounds that can target the underlaying source of AML relapse the LSC, causing it to differentiate. In an effort to identify the optimal corticosteroid to target LSCs I performed an *in vitro* drug screen on 24 additional corticosteroid compounds. The results of my screen showed that the most effective compounds where from the same chemical structure groups as mometasone furoate, budesonide, halcinonide and dexamethasone. Suggesting that corticosteroids that have specific functional groups can better target LSCs. Further to the drug screen I performed structural functional relationship analysis on the 24 corticosteroids. This revealed that the most important functional groups for targeting LSCs were the C1-C2 double bond, C6/C9 fluorine, C16-C17 acetonide group, C16 methyl group and C17 ester. These groups have also been shown to increase affinity for the glucocorticoid receptor, suggesting that the mechanism of action of corticosteroids in LSCs is through the glucocorticoid receptor. Molecular modeling further showed that

corticosteroids that carried bulky groups on the five carbon D ring such as the furoate or propionate groups, have increase ability to target LSCs. Of the corticosteroids tested fluticasone propionate showed the highest efficacy working at the lowest screening dose of 1nM. This corticosteroid possessed all the important functional groups for anti-LSC activity. Further testing of its analog fluticasone furoate is necessary as this compound may show greater potency and better efficacy. Altogether this project has identified corticosteroids that can possibly target LSC more effectively than the three corticosteroids form the initial drug screen as well as dexamethasone. It also revealed the mechanism by which corticosteroids regulate LSC, giving us insight into its pharmacodynamics. These findings have taken us one step further in the development of novel therapies that can better treat AML.

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