DOPAMINE RECEPTOR TARGETING IN HUMAN ACUTE MYELOID LEUKEMIA

DOPAMINE RECEPTOR TARGETING IN HUMAN ACUTE MYELOID LEUKEMIA

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

Descriptive Note

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TITLE: Dopamine Receptor Targeting in Acute Myeloid Leukemia

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NUMBER OF PAGES: 222

ABSTRACT

Standard of care chemotherapy for acute myeloid leukemia (AML) has remained unchanged for decades and is associated with therapy failure and unsatisfactory survival rates. While several theories have been put forth to explain the issue of therapy failure in AML, their clinical relevance remains ambiguous and they have yet to advance therapy decisions. To date, the underlying basis of therapy failure remains unresolved, partly due to a lack of reliable surrogate models that authentically reflect the biology of human AML.

To dissect the unique biological basis of therapy failure in a clinically-relevant system, we developed a unique patient-derived xenograft model that simulated chemotherapy regimens *in vivo*. Using this model, we characterized residual leukemia populations immediately after chemotherapy exposure, and monitored their longitudinal growth kinetics towards relapse. Despite the prevailing hypothesis of therapy resistance that involves leukemia stem cells (LSCs), we found that LSC pools were profoundly depleted shortly after chemotherapy, as determined by a lack of LSC-related functional and transcriptional properties. Instead, the residual leukemia cells displayed a unique transcriptional profile that emerged prior to the unrestrained regeneration phase that led to overt relapse. With the goal of identifying novel therapeutic targets, we searched for druggable gene products within the unique transcriptional signature of these leukemia regenerating cells (LRCs), which revealed a member of the dopamine receptor (DRD) family. Functionally, AML recurrence was prevented

in mice treated with DRD antagonist thioridazine (TDZ) in combination with chemotherapy. Mechanistically, DRD modulation by small molecules and immunotargeting resulted in suppression of neoplastic self-renewal towards cellular maturation, exclusively in leukemia progenitor cells with no adverse impact on healthy hematopoietic cell function. These findings provided proof-of-concept for our targeting approach and defined a novel role for the DRD pathway in human AML biology.

These pre-clinical observations motivated a phase I clinical trial to evaluate the safety and efficacy of DRD antagonist TDZ in AML patients. In a cohort of older patients with relapsed/refractory AML, treatment with TDZ resulted in a reduction of leukemic blasts, predominantly in the peripheral blood. The suppressive effect of TDZ was selective to leukemia cells and was associated with patient-specific DRD expression levels. Collectively, the data presented in this thesis offer a novel perspective on human AML biology with a focus on targetable vulnerabilities of leukemia, derived from sophisticated xenograft systems and validated with clinical level data. Our findings describe a novel role for DRDs in regulating leukemic hematopoiesis and propose DRDs as a cancer-selective therapy target for AML.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Mick Bhatia for giving me a shot at this. The lab has been my home away from home, where research was one of many things I learned. I hope to express my gratitude by getting the work that you supported over the years, to the finish line. I would also like to thank my supervisory committee members, Dr. Hope and Dr. Xenocostas. Kristin, I look up to you and your work. Thank you for all the good advice. Dr. Xenocostas, we feel so fortunate to have such an engaged collaborator like yourself, giving us first-hand clinical insights.

I'd also like to thank my lab family: Allison, you are a fantastic friend and an exceptional scientist! I learn something from you every day and I look forward to what #Allilison can accomplish as a team. Ryan, thank you for being my personal interpreter, for sharing your Rydeas but withholding that Ryrage. Also, a big thank you to my wonderful fellow students: Kyle, Stefo, Sarah, Deanna, Mohammed, Jenn, Wendy and Justin, awesome post-docs: Yannick, Orlando of Torino, Borhane and Terry, and a great technical team: Monica, Zoya, Marie-Pierre, Linda, Jenn and Aline. Last but not least, thank you Eva for always looking out for me and for all of your support. It has been wonderful to get to know you and to work with you all.

Finally, I'd also like to thank my family for being so understanding of my constant absence and drawn-out student life. Thank you Baba Reza and Maman Soraya, your love and support means the world to me...my beautiful sister Sanaz, for taking such good care of me here... and my brother Romin, for sending me love from home. Thank you Ivan, Pedrum, Kaveh, Myrto and Shayan for being the perfect amalgamation of fun, insightful, thoughtful and reckless. Jonathan, thank you for being so patient and caring during the frenzy of these past few months, and for all the motivation you give me every day.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

ABBREVIATIONS

5-FU	5 fluorouracil
AC	Adenylyl cyclase
Allo-HSCT	Allogeneic hematopoietic stem cell transplant
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
AraC	Arabinose cytosine, Cytarabine
ATRA	All-trans retinoic acid
BM	Bone marrow
BCL-2	B-Cell lymphoma 2
BET	Bromodomain and extra-terminal protein
cAMP	Cyclic adenosine monophosphate
CB	Cord Blood
CCLE	Cancer cell line encyclopedia
CD	Cluster of differentiation
CEBPA	CCAAT/enhancer binding protein
CFU	Colony forming unit
CFU-S	Colony forming unit-Spleen
CFU-E	Colony forming unit-erythroid
CFU-G/M	Colony forming unit-granulocyte/macrophage
CFU-Mixed	Colony forming unit-mixed colony types
CML	Chronic myelogenous leukemia
CR	Complete remission
CR_i	Complete remission, incomplete recovery
CSC	Cancer stem cell
CXCR4	C-X-C ligand 12
DMSO	Dimethyl sulfoxide
DRD	Dopamine receptor
ECOG	Eastern Cooperative Oncology Group
ELN	European leukemia net
FAB	French-American-British
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLT3	Fms-like tyrosine kinase 3
GCSF	Granulocyte stimulating factor
GEO	Gene expression omnibus
GSEA	Gene set enrichment analysis
HSC	Hematopoietic stem cell

HSPC	Hematopoietic stem and progenitor cell
IMDM	Iscove's modified Dulbecco's medium
IV	Intravenous
IF	Intrafemoral
IL	Interleukin
LDA	Limiting dilution assay
Lin-	Lineage depleted
LRC	Leukemia regenerating cell
LSC	Leukemia stem cell
LSK	Lineage$^-$ Sca1$^+$c-Kit$^+$
LTC-IC	Long-term culture initiating cell
MFI	Mean fluorescence intensity
MDS	Myelodysplatic syndrome
MNC	Mononuclear cell
MPB	Mobilized peripheral blood
NOD	Non-obese diabetic
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
MCP1	Monocyte chemoattractant protein 1
MPN1	Nucleophosmin 1
MRD	Minimal residual disease
NSG	NOD/SCID/IL2Rγ^{null}
PB	Peripheral blood
PCA	Principle component analysis
PE	Phycoerythrin
RTK	Receptor tyrosine kinase
SAMHD1	sterile α motif and histidine-aspartic domain-containing protein 1
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SL-IC	SCID leukemia initiating cell
SRC	SCID repopulating cell
TDZ	Thioridazine
TPO	Thrombopoietin
TRM	Treatment-related mortality
WGS	Whole genome sequencing
WHO	World health organization

CHAPTER 1: INTRODUCTION

1.1 Overview

Stem cells were first discovered and experimentally interrogated in the hematopoietic system (Becker et al., 1963; McCulloch and Till, 1960; Till and McCulloch, 1961). Today, the phenotypic features of the hematopoietic hierarchy have been extensively delineated, affording the isolation of hematopoietic stem cells (HSCs) with almost single cell resolution (Notta et al., 2011). The depth of knowledge and command over HSCs has culminated in the procedure of BM transplantation, presenting the only established stem cells-based therapy in the clinic (Muller et al., 2016).

Similarly, the cancer stem cell concept was initially introduced in the context of neoplastic hematopoiesis in AML, laying the paradigm of tumor heterogeneity and fundamental functional assays that were adopted by other cancer types. This introductory chapter focuses on the origins of these assays, and the cellular subsets/properties they interrogate. This chapter also discusses the involvement of LSCs in current theories of therapy failure in AML, as well as the evolution of LSC-directed approaches in the clinical management of AML.

1.2 Healthy hematopoietic system

1.2.1 Discovery of hematopoietic stem and progenitor cells

Hematopoietic stem cells (HSCs) generate the entire spectrum of blood cells throughout an individual's lifespan. This property is sustained through a tight balance between the self-renewal capacity to preserve the HSC pools for life-long hematopoiesis, versus the differentiation potential to produce mature hematopoietic cells required for daily physiological functions (Seita and Weissman, 2010). Initial clues on the presence of hematopoietic stem cells were found in the 50s when scientists discovered that irradiation-induced hematopoietic deficiencies could be rescued by transplantation of cells recovered from the bone marrow (BM) of non-irradiated mice (Ford et al., 1956; Jacobson et al., 1951; Lorenz et al., 1952). These findings demonstrated the regenerative capacity within the hematopoietic system. However, the true hematopoietic ancestral cell was not discovered until a decade later, through the pioneering work of Canadian scientists Till and McCulloch. The two scientists carried out a series of BM transplantation experiments, through which they serendipitously discovered that intravenously transplanted BM cells from a donor mouse were capable of forming "regeneration nodules" in the spleen of recipient mice, giving rise to a variety of blood cell types (Till and McCulloch, 1961). Through the use of ionizing radiation, these scientists generated tractable chromosomal aberrations in the donor mouse BM cells, which allowed them to confirm that the splenic colonies were genetic descendants of the donor mouse BM. These findings were published in the Journal of Nature (Becker et al., 1963). The authors also discovered that while spleen colonies were genetically distinct from one another, the cells within

individual colonies were genetically homogenous, suggesting clonal expansion of a single ancestor, termed colony forming unit-spleen (CFU-S) (Till and McCulloch, 1961; Till et al., 1964). Frequency analysis demonstrated that 1 in 10,000 nucleated BM cells were capable of generating spleen colonies, representing a "rare population" (Till et al., 1964). The authors later described that in addition to morphological heterogeneity, the cells within colonies displayed functional heterogeneity, as only a subset of the cells were able to re-initiate secondary colonies after serial transplantation (Till et al., 1964). Collectively, these findings led to the discovery of HSCs and their cardinal features including self-renewal and differentiation into various blood cell types. Additionally, these scientists developed a series of powerful functional assays, such as the spleen colony assay, serial transplantation and HSC quantification assays, which established a template for the future studies of the hematopoietic system.

1.2.2 *In vitro* assays of primitive hematopoietic cells

While the development of the CFU-S model and serial transplantation assays advanced the knowledge on murine hematopoiesis, little was known about the biology of human hematopoietic system due to a lack of appropriate model systems. Concurrently, *in vitro* models were developed to study murine hematopoietic cells with colony forming capacity. In this assay, hematopoietic suspensions were deposited in semi-solid media, which physically confined cell movement and ensured that individual colonies are developed from single cells

with clonal capacity, termed colony forming units (CFU) (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). This assay was adopted to study primitive human hematopoietic cells with clonal properties *in vitro* (Pike and Robinson, 1970). Similar to the murine splenic colonies, genetic analysis of individual colonies reinforced clonal growth within colonies (Fauser and Messner, 1978). Also, much like their murine counterparts, individual human-derived colonies displayed morphological and functional heterogeneity, with a subset of cells capable of initiating secondary colonies upon re-plating (Ash et al., 1981). These experiments were critical, as they demonstrated that some level of primitive hematopoiesis could be functionally captured *in vitro*. However, several lines of evidence suggested that a more primitive ancestor must exist that was not supported by the CFU assay: 1) There were no signs of lymphoid cells in human colonies generated in the dish or in murine splenic colonies, denoting a lack of multipotency of the clonogenic cells. 2) Despite the elimination of clonogenic cells after exposure to chemotherapeutic agent 5-Fluouracil (5-FU), the surviving cells were able to give rise to CFU-S cells (Hodgson and Bradley, 1979), suggesting that a more ancestral cell may exist upstream of the CFU-S fraction. 3) There was evidence that genetic aberrations observed in lymphoid organs such as the thymus and lymph nodes were also detectable in the BM or spleen (Wu et al., 1968). As CFU assays had already demonstrated that lymphoid cells could not be generated downstream of myeloid progenitors, this finding suggested that a

common ancestor must exist between lymphoid and myeloid cells that could not be read out by the CFU assay.

As a result, effort was invested in the development of *in vitro* systems that were able to capture more primitive fractions than the clonogenic cells. In order to provide a more supportive environment, hematopoietic cells were exposed to non-hematopoietic adherent BM cells that provide cell-extrinsic cues in the form of *in vitro* co-culture systems (Dexter et al., 1973; Dexter et al., 1980; Sutherland et al., 1990). These assays led to the identification of long-term culture initiating cells (LTC-IC), which are upstream precursors of CFUs and were successfully maintained for an extended period of time compared to the CFU assay (Andrews et al., 1990; Fraser et al., 1992; Sutherland et al., 1990). Nevertheless, even the LTC-ICs could not be preserved beyond 8-10 weeks *in vitro* (Sutherland et al., 1990) and therefore failed to reflect the longevity of HSCs. Similar to the *in vitro* CFU assay, LTC-ICs did not capture lymphopoiesis, indicating that *in vitro* systems fail to capture the full spectrum of features associated with a true hematopoietic stem cell (HSC).

1.2.3 *In vivo* assays of primitive hematopoietic cells

As *in vitro* systems appeared inadequate in capturing the long-term and multi-lineage properties of HSCs, effort was invested in studying human hematopoiesis in xenograft-based *in vivo* assays. The premise of xenografting is to remove murine immune barriers in order to allow propagation of human hematopoiesis in

the recipient mouse.

First attempts to initiate human hematopoiesis *in vivo* were made in the 80s and early 90s, when human hematopoietic fragments were implanted in the renal capsule of recipient mice (McCune et al., 1988; Namikawa et al., 1990). As this injection site lacked the proper physiological cues for hematopoietic cells, the implanted cells simply persisted in these areas for several months but were unable to promote the true human hematopoietic reconstitution as determined by dissemination of the cells outside of the implanted area and into hematopoietic sites.

The severe combined immunedeficiency (SCID) mouse with defective adaptive immunity was one of the first xenograft models to be used. In this model, a mutation in the *prkdc* gene involved in the repair of DNA double strand breaks led to an inability to express rearranged antigen receptors by lymphocytes and prevented the rejection of human grafts (McCune et al., 1988). Non-obese diabetic (NOD) mice represented another immunodeficient model, established in Shionogi laboratories in Japan (NOD/ShiJic) (Makino et al., 1980). While the NOD mouse displayed reduced natural killer (NK) cell activity as well as other defects in innate immunity, the residual immunity in these mice caused a rejection of the injected human cells. Eventually, the NOD mice were shipped to Jackson Laboratories and were crossbred with *Prkdc*^{scid} to generate NOD/SCID mice that displayed defects in innate and adaptive immune systems (Shultz et al., 1995). This model enabled robust human chimerism and quickly became the

predominant model to detect and quantify HSCs. The impact of this model can be inferred by the term SCID repopulating cell (SRCs), which describes human HSCs capable of repopulating the NOD/SCID mouse (Bhatia et al., 1997; Lapidot et al., 1992; Larochelle et al., 1996).

Despite its popularity, the NOD/SCID model came with a number of limitations. One major issue was the residual natural killer (NK) cell activity, presenting an immune barrier. This issue was later mitigated by treatment with a monoclonal antibody against CD122, or depletion of NK activity in NOD/SCID/B2m-/- mice (Kollet et al., 2000). Also, NOD/SCID mice had a short life span due to a susceptibility to thymic lymphomas (Shultz et al., 1995), which limited the analysis of long-term HSCs.

Subsequent efforts to improve xenografting techniques included the crossing of NOD/SCID strain with *IL2Rgamma*^{null} mice, one with a null mutation and the other with an inactivated IL2Rgamma. These efforts led to the generation of NSG and NOG mice (Ito et al., 2002; Shultz et al., 2005). As IL2Rgamma-chain is required for cytokine signaling, its inactivation leads to severe defects in lymphocyte development and natural killer cell activity. As a result, these mice represent some of the most powerful xenograft models with almost a complete absence of host immunity. Furthermore, this model was not susceptible to lymphomas, affording long-term studies of human HSCs for up to 6 months.

Other advancements in xenotransplantation included modification of cell delivery methods from the intravenous (IV) to intrafemoral (IF) injection

(Mazurier et al., 2003; McKenzie et al., 2005), and the addition of human cytokines to generate "designer mice". This was accomplished by the injection or endogenous generation of human cytokines in the recipient mouse to provide a more supportive microenvironment for human hematopoietic cell development (Lapidot et al., 1992; Miller et al., 2013). An example includes the NSG-SGM3 model that harbors constitutive expression of hSCF, hGM-CSF, hIL3. These mice display enhanced myelopoiesis and enable reconstitution of a wider range of AML patient samples (Medyouf et al., 2014; Wunderlich et al., 2010).

1.3 Neoplastic hematopoiesis in acute myeloid leukemia

1.3.1 Epidemiology and clinical presentation

AML is a cancer of the hematopoietic system, characterized by uncontrolled proliferation of maturation-stalled myeloid progenitors, also known as blasts (Estey and Dohner, 2006). The infiltration of the bone marrow with these neoplastic blasts halts the maturation process of healthy hematopoietic cells, leading to fatal consequences such as infections, bleeding and ultimately BM failure (Estey and Dohner, 2006).

AML is the most common myeloid disorder in adults, with a median age of 70 at presentation (Estey and Dohner, 2006). The incidence starts with 2-3 per 100,000 in younger patients and rises to 13-15 per 100,000 in the seventh and eighth decade of life (Burnett et al., 2011). The five-year overall survival is 21% for all ages and 4.6% for patients 65 and older (Ferrell et al., 2016).

Few known risk factors are recognized to contribute to the pathogenesis of AML. AML may arise in association with hereditary or de novo germ line mutations such as trisomy 21 abnormality (Nickels et al., 2013; Xavier et al., 2009). AML may also occur as a secondary disease in roughly 30% of patients with myelodysplastic syndrome (MDS)(Ades et al., 2014), a group of clonal disorders with sub-leukemic blast levels and maturation defects in one or more hematopoietic lineage (Corey et al., 2007; Shih and Levine, 2011). Environmental risk factors that may lead to AML include exposure to ionizing radiation, benzene, smoking and previous exposure to cytotoxic chemotherapy mainly for solid tumors (Estey and Dohner, 2006). The latter is classified as therapy-related AML and comprises up to 10-15% of AML cases. Yet, the number of people who develop AML far exceeds those that are exposed to the environmental risk factors (Estey and Dohner, 2006), denoting that intrinsic mechanisms may play a greater role in the development of AML than the environmental factors.

1.3.2 WHO classification

The principle requirement for AML diagnosis is accumulation of neoplastic myeloid blasts in the BM and peripheral blood (PB) (Dohner et al., 2010). This criterion was the basis of the French-American-British (FAB) classification system, which considered a >30% blast content together with cytomorphology and cytochemistry features to confirm the diagnosis of AML (Bennett et al., 1976). Over the years, the importance of genetic factors in AML biology has

become more recognized, leading to the establishment of new diagnostic criteria under the World Health Organization (WHO) classification of tumors related to hematopoietic tissues (Vardiman et al., 2009). According to the new guidelines, a blast content of over 20% in the peripheral blood (PB) or BM is required for AML diagnosis, with the exception of certain genetic aberrations (t(8;21)(q22;q22), inv(16)(p13.1q22), t(16;16)(p13.1;q22) and t(15;17)(q22;q12)) that are sufficient to confirm AML, regardless of the blast content (Vardiman et al., 2009).

WHO classification was recently updated in 2016 to include a new category of "myeloid neoplasms with germline predisposition", in recognition of the increasing cases of AML that arise in association with inherited or de novo germline mutations (Aber et al., 2016; Dohner et al., 2017). This new addition helps provide proper genetic counselling to the affected patients and family members suspected of having an inherited myeloid malignant syndrome.

1.3.3 Diagnosis and prognosis of AML

Morphological assessments of the marrow specimens and blood smears constitute routine components of AML diagnostic procedure. A minimum of 200 leukocytes on a blood smear or 500 nucleated cells from marrow samples should be analyzed (Dohner et al., 2017). The blast count includes myeloblasts, monoblasts and megakaryotic blasts (Dohner et al., 2017). However, the morphology of leukemic blasts alone may not always be straightforward. Therefore, additional immunophenotypic characterization by flow cytometry may help determine the

lineage of the blasts (Dohner et al., 2015). Immunophenotypic features of the leukemic clone can also be utilized to quantify the minimal residual disease (MRD) after treatment (Dohner et al., 2010).

Cytogenetic analysis remains a mandatory component of the diagnosis of AML (Dohner et al., 2010). This is generally carried out by conventional karyotypic assessments in which a minimum of 20 metaphase cells are analyzed for chromosomal abnormalities. Other molecular cytogenetic analyses may include fluorescence in situ hybridization (FISH), molecular analyses for gene fusions such as *RUNX1-RUNX1T1*, *CBFB-MYH11*, *MLLT3-MLL*, *DEK-NUP214* by reverse transcription-polymerase chain reaction (RT-PCR), or analysis of acquired somatic mutations such as *NPM1*, *FLT3*, *CEBPA*, etc. (Dohner et al., 2017). Genomic profiling of AML holds the promise of uncovering novel leukemia-related genes with currently undetermined functional significance, providing insights into the molecular basis of AML and identifying novel therapy targets (Dohner et al., 2017; Lowenberg, 2008). While at an investigational stage (Burnett et al., 2011), these analyses are starting to be applied clinically for a more comprehensive sub-classification of the disease (Papaemmanuil et al., 2016).

AML prognostic factors are evaluated at diagnosis and post-induction. Risk factors at diagnosis are generally subdivided into patient-related and AML disease-related risk factors (Dohner et al., 2010):

Patient-related risk factors at diagnosis

Old age, patient performance status by Eastern Cooperative Oncology Group (ECOG) and presence of other comorbidities constitute patient-related risk factors at diagnosis. Of these, increasing age is considered an independent adverse risk factor and may be used to inform therapy decisions (Dohner et al., 2017; Dohner et al., 2010; Dohner et al., 2015).

AML-related risk factors at diagnosis

Major AML disease-related factors at diagnosis include white blood cell count (WBC), existence of prior myeloid neoplasms, previous exposure to cytotoxic chemotherapy for other cancers, as well as cytogenetic and molecular genetic factors (Dohner et al., 2017). Among these, cytogeneic and molecular genetic factors are described as the strongest prognostic factors and account for approximately two thirds of the variables that determine event-free survival (EFS) and overall survival (OS) rates (Dohner et al., 2017; Dohner et al., 2010). In addition to cytogenetics analysis at diagnosis, screening for certain genetic mutations affords a more precise determination of risk categorization. Specifically, normal karyotype AML can be classified as intermediate or favorable risk based on the presence or absence of FLT3 or NPM1 mutations (Dohner et al., 2010). FLT-3, NPM1 and CCAAT/enhancer binding protein a (CEBPA) mutations are becoming widely adopted following the

recommendations by European Leukemia Net (ELN) in 2010 (Dohner et al., 2010). Since then, the ELN criteria and other risk stratification systems have been widely adopted to standardize the reporting of genetic abnormalities and their correlation with clinical outcomes. Recently, the ELN system was updated as a 3-group system with favorable, intermediate and adverse categories (Dohner et al., 2017). The former version of this system included favorable, intermediate I, intermediate II and adverse risk groups. However, the intermediate-I and II groups were found to be prognostically indistinguishable in older patients, who in fact constitute the majority of AML cases (Dohner et al., 2017) and were therefore consolidated as "intermediate". In addition to the prognostic significance of these systems at the pre-treatment stage, prognostic characterization is also instructive for post-remission therapy decisions and particularly for consolidation therapy with BM transplants (Estey and Dohner, 2006; Lowenberg, 2008).

Post-induction risk factors

A lack of response to the first induction cycle or lack of early blast clearance are considered major predictors of poor outcome (Dohner et al., 2010). Also, presence of MRD presents an important risk factor. MRD is initially assessed to determine whether remission has been achieved and may be monitored continually beyond consolidation to predict the risk of relapse. Currently, MRD detection is carried out either by multi-parameter flow cytometry or by molecular techniques including real-time quantitative PCR (RT-qPCR) for leukemia-specific targets

(Dohner et al., 2017; Dohner et al., 2010; Dohner et al., 2015). While multi-parameter flow cytometry is potentially applicable to all patients, it remains less sensitive than the molecular techniques. Inclusion of 6-8 parameters in multi-parameter flow cytometry will likely improve the sensitivity but is not yet standardized. Precise MRD detection improves risk assessments and guides post-remission therapy decisions (Dohner et al., 2017; Dohner et al., 2010).

1.3.4 Clinical management of AML

Induction therapy

Clinical management of AML consists of two phases. The first phase starts preferably with minimal delay after diagnosis and aims to achieve a state of remission (Dohner et al., 2010; Dohner et al., 2015). With the exception of acute promyelocytic leukemia (APL), which is managed by a combination of all-trans retinoic acid (ATRA) and other agents such as arsenic trioxide (Coombs et al., 2015), all other AML subtypes generally receive a standardized induction therapy protocol known as "3+7". This regimen consists of a 7-day treatment with a cytosine analogue named cytarabine (AraC), which is considered the gold standard chemotherapeutic agent for the past 40 years (Reese and Schiller, 2013). An anthracycline agent, typically daunorubicin, is administered for the first 3 days of the 7-day regimen (Estey and Dohner, 2006). This treatment achieves complete remission (CR) in 60-80% of adults below 60 years of age, and in 40-60% of patients over 60 years of age (Dohner et al., 2017; Dohner et al., 2015).

Consolidation therapy

If remission is achieved with the first phase of treatment, consolidation therapy is applied to maintain remission states and to reduce the risk of relapse (Burnett et al., 2011; Dohner et al., 2010). Standard post-remission therapy typically consists of intensive conventional chemotherapy as well as hematopoietic stem cell transplantation (HSCT) (Dohner et al., 2017; Dohner et al., 2015; Estey and Dohner, 2006):

Conventional intensive consolidation therapy

In adults 60 years and younger, 2-4 cycles of high dose AraC is preferred (Dohner et al., 2015). Therapy outcomes are less satisfactory for older patients and may range from repetitive cycles of intermediate dose AraC to investigational treatments, depending on patients' risk profile and general health. For patients that are unfit to tolerate intensive chemotherapy, treatment options include supportive care with hydroxyurea, low dose AraC or hypomethylating agents such as decitabine or azacytidine, or clinical trials with investigational drugs (Dohner et al., 2017; Dohner et al., 2015).

Hematopoietic stem cell transplantation

Autologous HSCT may be considered for patients with favorable and intermediate risk cytogenetics (Dohner et al., 2010). However, this procedure has not achieved

convincingly superior outcomes compared to consolidation chemotherapy and in fact has been associated with higher mortality (Shipley and Butera, 2009).

Post-remission therapy with allogeneic HSCT (allo-HSCT) provides potentially curative outcomes due to the combined effect of cytoreductive pre-conditioning regimens and the graft-versus leukemia effect (Dohner et al., 2017; Dohner et al., 2015). In the recent years, the use of unrelated or haploidentical donors as well as cord blood sources for HSCT has made this treatment available to a larger number of patients (Dohner et al., 2017). However, this procedure may come at a substantial cost, as it is associated with significant transplant-related morbidity and mortality such as infections and graft-versus-host disease (Dohner et al., 2010; Lowenberg, 2008). As a result, allo-HSCT is only considered for medically fit patients with less than favorable cytogenetics (Dohner et al., 2017; Dohner et al., 2010; Lowenberg, 2008).

Therapy options for relapsed/refractory AML

For relapsed patients and those that are refractory to therapy, treatment strategies must carefully assess the benefits of further treatment, as prognosis in this setting is dismal (Dohner et al., 2017). Patients with induction failure can be considered for chemotherapy using salvage regimens followed by allo-HSCT. Refractory patients that are not eligible for allo-HSCT can be considered for investigational clinical trials (Dohner et al., 2010). For younger patients with relapsed AML, a prognostic index can be evaluated based on a number of factors including the

duration of remission, patient age and cytogenetic profile at diagnosis (Breems et al., 2005) (Dohner et al., 2010). This information helps guide further therapy with a curative objective, palliative care or clinical trials (Dohner et al., 2010). Older patients with relapsed disease may be unfit for high dose AraC and can be considered to receive low intensity therapy, supportive care or investigational drugs (Dohner et al., 2010; Dohner et al., 2015).

1.3.5 Evaluation of therapy outcomes

Response assessment after induction therapy with 3+7 is routinely performed between days 21-28 after the therapy start (Dohner et al., 2010). For investigational studies, response assessment may be required as early as 7-10 days after treatment to evaluate the anti-leukemic efficacy of the novel agent or to guide subsequent treatment. During follow-up periods, blood counts should be monitored every 1-3 months for the first 2 years, followed by every 3-6 months for up to 5 years. Repeat marrow aspirates are usually not required unless blood counts appear abnormal. However, follow up marrow aspirates are recommended every 3 months for the first 2 years in clinical trial settings and to assess for MRD (Dohner et al., 2017).

A number of response categories and evaluation criteria have been defined for AML (Dohner 2017), which can be grossly categorized as follows: Complete remission (CR) includes a number of criteria, the most important of which are reduction of BM blast count to below 5% and restoration of platelets and

neutrophil counts (Dohner et al., 2017). CR with incomplete hematopoietic recovery (CR_i) includes all CR criteria without the recovery of neutrophil or platelet counts. If no CR or CR_i is achieved after 2 courses of intensive induction therapy, patients are considered to have primary refractory disease. Relapse is defined as equal or greater than 5% blast in the BM, or reappearance of blasts in the peripheral blood or extramedullary organs (Dohner et al., 2017).

Additional response categories have been defined for clinical trials such as partial remission (PR), stable disease or progressive disease (PD). The goal of these new additions is to meet the specific objectives of investigational studies beyond the strict response evaluation criteria that are currently applied to standard of care induction therapy.

1.4 Therapeutic targeting of leukemia stem/progenitor cells

1.4.1 Cancer stem cells

Heterogeneity in the morphology of different tumor cells was already recognized in the 1800s (Kreso and Dick, 2014). Intra-tumor heterogeneity was discovered later when technological advancements disclosed the presence of multiple cell populations within a single tumor including melanomas, breast and colon cancers (Dexter et al., 1979; Dexter et al., 1978; Gray and Pierce, 1964). In addition to these morphological discoveries, functional heterogeneity was also identified when radiolabeled cancer cells with distinct morphologies showed disparate

proliferation kinetics in human leukemias (Clarkson et al., 1970; Gavosto et al., 1967).

Two predominating theories have been put forth to explain the heterogeneity of tumors. In the traditional view, individual cells are considered to have equal capacity or probability to accumulate mutations that impart tumorigenicity. The acquisition of mutations gives rise to the formation of sub-populations and genetically varied cancer cells (Nowell, 1976). In contrast to this model, an alternative theory suggests that tumorigenicity is a unique property and is restricted to cellular entities specialized in self-renewal, termed cancer stem cells (CSCs) (Hanahan and Weinberg, 2011; Kreso and Dick, 2014). CSCs are described as rare, functionally distinct cells at the apex of the tumor hierarchy that have the ability to initiate and sustain the tumor (Bonnet and Dick, 1997; Lapidot et al., 1994). These theories harbor critical clinical implications, as they inform whether anti-cancer therapies should be directed at the entire tumor or tailored towards the specialized CSC compartment.

Seminal work that led to the identification of CSC was initiated by John Dick's group in Toronto, who demonstrated that a subset of leukemia cells isolated from AML patient samples was capable of re-initiating the parent tumor in NOD/SCID mice (Bonnet and Dick, 1997; Lapidot et al., 1994). These cells were termed SCID leukemia-initiating cells (SL-IC) and were found in the CD34+CD38- fraction, whereas CD34+CD38+ or CD34- cells showed little to no tumorigenic potential (Bonnet and Dick, 1997; Lapidot et al., 1994). This finding

suggested that: 1) A hierarchical system of functionally heterogeneous cells exists among tumor cells. 2) CSCs can be prospectively isolated as distinct biological entities. 3) CSCs demonstrate remarkable phenotypic and functional similarity to their healthy counterparts. Using the popular NOD/SCID xenograft model at the time, SL-ICs were described to be as rare as <0.1% of the tumor cells (Bonnet and Dick, 1997; Lapidot et al., 1994).

Presence of CSCs was later described in other cancer types including breast (Al-Hajj et al., 2003), brain (Singh et al., 2004), colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) and melanomas (Schatton et al., 2008). Using the immunodeficient models that were available at the time, all of these studies described CSCs to be as infrequent as (0.0001-0.1%) (Eaves, 2008), consistent with the CSC model. However, this view was challenged when melanoma initiating cells were found to be as frequent as 1 in 5 cells using a more permissive xenograft model (Quintana et al., 2008), in stark contrast to the frequency of tumorigenic melanoma cells (0.0001%) reported in NOD/SCID mice (Schatton et al., 2008). Several interpretations were associated with this observation: 1) Not all tumors are hierarchically organized. 2) Tumors are hierarchically organized but some tumors have a shallow hierarchy, in which CSC comprise a larger fraction of the tumor (Kreso and Dick, 2014). 3) “Rare” cancer stem cells may be an artifact of the xenograft model and its inherent immune barriers or growth factor incompatibility. Nevertheless, studies from autologous transplantation of tumors in volunteering cancer patients reinforced the presence

and rarity of CSC, since over 10^6 cells were required to reinitiate the tumor, even in the absence of immune barriers (Southam, 1961). Despite the on-going controversy on CSCs, this view has reshaped the field of cancer and stem cell biology as it offers a new perspective on tumor behavior and treatment strategies. Further studies are required to firmly establish the relevance of CSCs in critical clinical parameters such as survival, therapy resistance and relapse.

1.4.2 Current theories of therapy failure in AML

While up to 80% of younger AML patients may initially respond to standard induction therapy, the majority of patients will relapse within 2 years of achieving remission (Shipley and Butera, 2009). Relapsed disease becomes notoriously more difficult to treat (Forman and Rowe, 2013) and is the leading cause of mortality in AML patients (Estey, 2012). Several hypotheses have been put forth to explain the phenomenon of relapse in AML. These include cell intrinsic or extrinsic LCS-related features that equip these cells to resist chemotherapy, as well as acquired genetic lesions that confer a Darwinian survival advantage to the leukemic clone.

LSCs may resist therapies in a cell extrinsic manner through protective niches that serve as a refuge for LSCs (Duan et al., 2014; Ishikawa et al., 2007). Intrinsically, LSCs are described to resist chemotherapy through cell cycle quiescence (Saito et al., 2010). Similar to their healthy counterparts, CSCs are programmed to divide rarely, making them resistant to anti-proliferative therapies (Essers and Trumpp, 2010). Long-term labeling studies have demonstrated that

the most primitive HSCs in mice are extremely dormant and divide approximately 5 times per the life span of the animal (van der Wath et al., 2009; Wilson et al., 2008). However, HSC dormancy is not a static feature, as damage to the hematopoietic system due to bleeding, toxins, chemotherapy or radiation exposure prompts dormant HSCs to rapidly turn into active HSCs, to generate dividing progenitors that compensate the loss of hematopoietic cells (Essers and Trumpp, 2010; Morrison et al., 1997). Concurrently, cycling HSCs become increasingly sensitive to chemotherapy (Jorgensen et al., 2006) and a similar dynamic has been proposed for LSCs (Saito et al., 2010). These observations shaped the theory that overcoming CSC dormancy through chemosensitization is a critical step towards cancer eradication.

Other theories of therapy failure include acquisition of genetic lesions over time, resulting in an intense focus on genomics research in the past few years. The premise is that resilient clones are naturally selected as a result of selective pressures induced by chemotherapy, or by accumulation of additional genomic lesions that confer resistance to the existing leukemic clone (Mullighan et al., 2008). Systematic studies on matched diagnosis/relapse AML samples by whole genome sequencing (WGS) demonstrated two predominant clonal evolution patterns: 1) The founding clone identified at diagnosis acquires additional genomic lesions and evolves into the relapse clone. 2) A sub-clone of the founding clone, present at diagnosis, gains additional mutations and expands into the relapse clone (Ding et al., 2012; Kronke et al., 2013; Parkin et al., 2013).

Other possibilities including emergence of a genetically distinct sub-clone at relapse that was undetectable at diagnosis have not been conclusively shown in AML. These patterns were consistent with studies on matched diagnosis/relapse samples in ALL (Mullighan et al., 2008). The acquired mutations in either case were reported to be a consequence of genotoxic chemotherapy, suggesting that chemotherapy may reshape the disease behavior by altering the genomic landscape (Ding et al., 2012). Nevertheless, the acquired mutations that were reproducibly detected across patients did not explain a survival/proliferative advantage or therapy resistance of a given clone in a straightforward manner (Ding et al., 2012). Consistently, a recent study reported few genomic alterations in 8 matched diagnostic/relapse AML samples, suggesting that genomic evolution is unlikely to fully explain therapy failure (Ho et al., 2016).

The possible interconnections between the clonal and LSC-based theories of disease relapse have become a recent area of focus. Klco et al. pioneered these studies in AML by using xenotransplantation in combination with WGS, to investigate the functional properties of leukemic sub-clones and specifically to examine the relationship between clonal dominance and LSCs (Klco et al., 2014). The authors reported that unlike the parent tumors, the xenografts were predominantly monoclonal and the engrafting sub-clone was not necessarily derived from the dominant clone within the patient sample. Also, no predictable patterns were found between the engrafting sub-clone and its evolutionary trajectory towards relapse (Klco et al., 2014). An independent study on the clonal

evolution of relapse reported that only half of the relapse cases originated from operationally defined LSCs (Shlush et al., 2017). In light of these pioneering studies, a few critical questions arise: What is the significance of clonal evolution in coordinating AML disease progression and therapy response if clonal composition or their genetic attributes rarely alter from diagnosis to relapse? In the absence of detectable acquired genetic lesions, what alternative mechanisms explain therapy resistance of a given clone at relapse? Can relapse be considered an LSC-driven phenomenon if relapse is not always driven by the engrafting clone? Deep sequencing efforts alone have not enabled the field to outline distinct genetic alterations that explain the source of resistant/recurrent disease. This indicates that alternative exploratory approaches including epigenomic assays or global gene expression analyses may be crucial to uncover the non-genomic basis of relapse and provide tangible therapy targets.

1.4.3 Clinical relevance of leukemic stem/progenitor cells

Since its inception, the LSC concept has been a topic of debate, from the existence of LSCs to their true clinical relevance. To date, LSCs can only be operationally defined through leukemia-initiation in xenograft models, while lack of an unequivocal phenotypic or morphological property has been a barrier to their effective therapeutic targeting. As a result, clinical management of AML continues to rely on cytotoxic-based chemotherapy that tackles the tumor as a

whole, and the potential benefit of LSC-based approaches remains generally undetermined.

To evaluate the true clinical relevance of LSCs, a number of studies have sought to examine whether properties that distinguish LSCs from non-LSCs, correlate with clinically relevant parameters such as prognosis and patient survival (Eppert et al, 2011; Gal et al., 2006; Pearce et al., 2006; van Rhenen et al., 2005). Specifically, a few groups systematically investigated whether an absence/presence of LSCs was correlated with tangible clinical measures. Initial work was independently generated in the Civin lab, Bonnet lab and Andreef lab, demonstrating that leukemia initiation capacity *in vivo* was associated with patient cytogenetics. These studies showed distinct overall survival rates between LSC containing versus non-LSC containing patients (Lumkul et al., 2002; Monaco et al., 2004; Pearce et al., 2006). Additionally, they reported that adverse cytogenetic features were found more frequently in engrafting- versus non-engrafting samples (Lumkul et al., 2002; Monaco et al., 2004; Pearce et al., 2006). This was an important finding as cytogenetic characteristics are the strongest predictors of patient outcomes in the clinic (Dohner et al., 2017; Grimwade et al., 1998).

The next set of studies went one step further and proposed that phenotypically defined (CD34+CD38-) (Gentles et al., 2010; van Rhenen et al., 2005), or functionally validated LSCs (Eppert et al, 2011) may offer a novel prognostic tool at diagnosis. This was based on correlations found between LSC frequencies at diagnosis and prognostic outcomes such as overall survival (Eppert

et al, 2011; Gentles et al., 2010; Pearce et al., 2006) or relapse free survival (Eppert et al., 2011; Gentles et al., 2010; van Rhenen et al., 2005). Through these studies, Eppert et al. described an LSC-related signature of 42 genes (LSC-42) that was generated based on differentially expressed genes in LSC-containing versus non LSC-containing leukemia fractions (Eppert et al., 2011). Critically, the LSC-42 gene signature overlapped with prognostic AML gene signatures that were generated in xenograft-independent studies (BullingerDohner et al., 2004; Metzeler et al., 2008), indicating an inherent value for LSCs in predicting patient outcomes. The LSC signature was updated by the same group in 2016, with a set of 17 genes (LSC-17) that provided superior prognostic accuracy when compared to existing LSC signatures (Eppert et al., 2011; Gentles et al., 2010; Levine et al., 2015).

The authors proposed to translate the clinical application of LSCs by developing a LSC-17 nanostring platform as a novel prognostic tool to prospectively identify therapy resistant patients who may not benefit from standard therapy and should therefore be enrolled in clinical trials (Ng et al., 2016). However, the practical value of this proposition remains unclear as currently no investigational therapy agent has demonstrated superior efficacy compared to standard chemotherapy. Also, under current clinical guidelines, standard chemotherapy should not be withheld and/or replaced by investigational agents unless the patient is considered unfit, has relapsed shortly after the first remission, or has already displayed refractory disease trends, which is inevitably

determined after 2 induction cycles (Dohner et al., 2017). As a result, even though this signature offers exceptional precision in predicting therapy outcomes, its ability to offer alternative therapeutic solutions remains limited.

Moreover, selective druggable targets have not been derived from the existing LSC-42 or LSC-17 signatures as the two signatures were developed from "stemness" features that overlap with HSC properties (Eppert et al., 2011; Ng et al., 2016). To overcome this limitation, Saito et al. focused on genes that were overexpressed in LSCs relative to HSCs, to identify potential selective therapeutic targets. This study identified a set of 25 differentially expressed genes. However, the efficacy or selectivity of this signature was not verified (Saito et al., 2010).

Overall, current LSC-based signatures offer prospective and precise prognostic stratification, which is considered a major advancement towards the integration of LSCs into clinical diagnostic and prognostic measures. However, the true clinical challenge remains in therapy failure, which has also been associated with LSC properties. Future studies are required to concretely determine whether relapse originates from LSCs and whether LSC-directed therapies can help overcome leukemia recurrence.

1.4.4 Preclinical models of AML

Approximately 85% of preclinical anticancer agents entering clinical trials fail to demonstrate sufficient efficacy and safety to gain regulatory approval (Gao et al., 2015). The high rate of failure has been largely attributed to a lack of translatable

pre-clinical models, highlighting the need for experimental systems that faithfully replicate human cancer biology.

AML serves as one of the oldest model systems for systematic drug discovery. The National Cancer Institute (NCI) screening platform initiated in 1955 was based on murine leukemia models (Rubio-Viqueira and Hidalgo, 2009). Today, preclinical models of AML have evolved, each delivering different information and opportunities.

In vitro assays with cell lines are generally viewed as a starting point as they offer high throughput analyses and a rapid, cost-effective assessment of novel agents. Cancer cell line encyclopedia (CCLE), a collaboration between the Broad institute and Novartis, offers over 900 genotyped cancer cell lines, including 34 AML cell lines with detailed genetic characterization (Barretina et al., 2012). However, cell lines are described to deviate from their parent tumor as a result of accumulated mutations and clonal bias upon passaging (Gao et al., 2015). Additionally, they are often regarded inadequate in reflecting the intra-tumor complexity associated with AML (Gao et al., 2015; Marx, 2014). Systematic studies have now demonstrated that the cell line approach fails to robustly predict therapy outcomes (Gao et al., 2015). Nevertheless, cell lines may offer a repertoire of genetic backgrounds for specific target mutations and a straightforward means to untangle the mechanism of action for novel agents that have already displayed efficacy in more reliable model systems. Primary AML cells provide an alternative to cell lines. *In vitro* propagation of primary cells has

been shown to conserve the polyclonal composition of the parent tumor for up to 7 days. Also, the growth advantage of individual clones in culture faithfully reflected the patterns of clonal dominance in the patient (Klco et al., 2014). This is an important feature as individual clones have been hypothesized to display distinct therapy responses. However, the disadvantage with primary AML cells is their poor growth rates *in vitro* (Clutterbuck et al., 1985; Sawyers et al., 1992). Alternatively, primary AML cells can be assayed for clonogenic capacity of progenitor fractions using the established CFU assay (Moore and Metcalf, 1973), which is demonstrated to replicate response to cytotoxic therapy (Browman et al., 1983; Griffin and Lowenberg, 1986; Preisler, 1980; Tehranchi et al., 2010). Overall, the *in vitro* assays of AML have been described to robustly read out the function of progenitor subsets and differentiated cells within the hematopoietic hierarchy. However, they are unable to assay the therapy response of LSCs as a full repertoire of LSC properties cannot be reflected *in vitro* (**Figure 1**).

In vivo repopulation assays are indispensable if the goal of drug screening is to assay LSCs (**Figure 1**). Also, *in vivo* models are generally considered superior to *in vitro* systems, as they provide a physiological context for drug studies and incorporate the niche, which may participate in drug response (Damia and D'Incalci, 2009). Among the *in vivo* systems, xenograft-based models derived directly from patient biopsies with minimal *in vitro* manipulation have demonstrated superior clinical relevance (Gao et al., 2015). A recent study demonstrated that *in vivo* compound screening on patient xenografts was able to

reproduce phase 2 clinical trial response rates, indicating the reliability of this system as a pre-clinical tool (Gao et al., 2015). However, only 50% of AML patient samples generate an *in vivo* leukemic graft with the existing gold standard AML xenograft model (Pearce et al., 2006). To overcome this limitation, increasingly immunocompromised hosts have been generated to allow the analysis of an extended number of AML patients *in vivo*. However in some cases, the xenografts generated in these next generation mouse models may display disparate phenotypic features from that of the parent tumor and other xenograft models (Klco et al., 2014). This may confound an authentic drug response and therefore, the surrogacy of these new mouse models needs to be cautiously evaluated.

1.4.5 Leukemia stem/progenitor cell targeting in the clinic

AML has been theorized to originate from malignant stem and progenitor cells that fuel the rapid production of leukemia blasts (Bonnet and Dick, 1997). This offers the promise to eradicate the leukemic disease by targeting these primitive cellular subsets that are considered the root of the disease. However, this notion has yet to inform therapeutic management of AML, which continues to rely on cytotoxic elimination of the tumor bulk while only few novel drugs have gained approval in the past few years (Burnett et al., 2011). A number of these alternative therapy approaches that impact LSC properties are described below.

Receptor tyrosine kinases (RTK) inhibitors for FLT3 and KIT receptors represent one of the most promising novel agents with a potential to impact LSCs. These receptors mediate critical pathways of hematopoietic stem cell function (Lyman and Jacobsen, 1998; Metcalf, 1993). As a single agent, FLT3 inhibitors showed activity in peripheral blood blast levels and to a lesser extent in the bone marrow (Stone et al., 2005). These agents were found more effective when combined with standard chemotherapy and are currently in Phase III clinical trials (Dohner et al., 2010). It should be noted that FLT3 inhibitors have been associated with a number of concerns including a lack of selectivity for leukemia cells and the development of FLT3 resistant mutations (Dohner et al., 2015).

An alternative approach includes targeting of aberrant LSC phenotypes. Early LSC-specific immunotherapies included CD123 targeting (Dohner et al., 2010; Dohner et al., 2015). CD123 is a transmembrane alpha chain of IL3 and was reported to be expressed on LSCs and leukemic blasts at frequencies higher than myeloid associated antigen CD33 or primitive cell marker CD34 (Gill et al., 2014). However, CD123 expression is not exclusive to LSCs and therefore HSC function was found to be compromised as a consequence of CD123 targeted treatment (Gill et al., 2014).

Other LSC-directed approaches include modulation of LSC-related metabolic features (Dohner et al. 2010). It has been reported that LSCs preferentially rely on oxidative phosphorylation. Accordingly, pharmacological inhibition of mitochondrial activity through B-Cell lymphoma 2 (BCL-2) resulted

in compromised LSC function (Lagadinou et al., 2013). BCL-2 inhibitors are currently under investigation in clinical trials in North America, Europe and Australia.

Targeting the LSC niche and epigenetic modulators represent other modes of novel therapies with an impact on LSC function (Dohner et al., 2010). CD44 is an adhesion molecule on the surface of hematopoietic precursors whose ligand is expressed in the bone marrow (Ghaffari et al., 1996; Jin et al., 2006). Antibody-based CD44 targeting disrupted leukemia cell homing and repopulation *in vivo* (Jin et al., 2006; Krause et al., 2006). Consequently, a phase I clinical trial was conducted to evaluate the safety of CD44-directed immunotherapy in Europe (Vey et al., 2016). Other niche directed therapies include antagonists for anchorage molecule CXCR4, which are in early phase of clinical development (Dohner et al., 2017). Epigenetic modulators including inhibitors of demethylases (LSD1 or KDM1) or bromodomain and extra terminal protein (BET) inhibitors also disrupt LSC function (Fong et al., 2015; Schenk et al., 2012) and are currently under investigation in clinical trials (Dohner et al., 2017; Dohner et al., 2015; Pollyea and Jordan, 2017).

1.5 Dopamine receptors in hematopoiesis

1.5.1 Dopamine receptor function, classification and structure

The neurotransmitter dopamine (DA) was first identified in the human brain in the 1950s and eventually earned the Nobel Prize for physiology and medicine in 2000

due to its role as a primary mediator of signal transduction in the nervous system. DA functions as an independent neurotransmitter as well as a precursor for other neurotransmitters including adrenaline and noradrenaline (Beaulieu and Gainetdinov, 2011; Iversen and Iversen, 2007).

All of the physiological functions of DA are mediated by 5 distinct, but closely related G protein-coupled receptors DRD1-5. All 5 DRDs are activated by DA, with binding affinities that range from nanomolar to micromolar concentrations (Beaulieu and Gainetdinov, 2011). In addition to these 5 receptors, two pseudogenes have been described for human DRD5 that encode truncated non-functional receptor forms (Grandy et al., 1991)

Function

DRDs have a broad expression pattern in the brain and peripheral organs such as the kidney, adrenal glands, gastrointestinal tract, blood vessels and the heart (Beaulieu and Gainetdinov, 2011). In the brain, DRDs mediate key physiological functions such as voluntary movement, feeding, reward mechanism, sleep, memory and learning. Outside of the central nervous system, DRDs regulate olfaction, sympathetic tone, immune system, renal functions, hormonal regulation, blood pressure regulation and gastrointestinal motility (Beaulieu and Gainetdinov, 2011)

Classification

DRDs are subdivided into 2 major categories; the D1 and D2 classes of DRDs (Andresen et al., 1990). This classification was historically based on the ability of

DRDs to modulate cyclic AML (cAMP) production through adenylyl cyclase (AC). The D1 family consists of DRD1 and DRD5 (Tiberi et al., 1991) and these receptors activate $Ga_{s/olf}$ family of G proteins that stimulate cAMP production through AC (Kebabian and Calne, 1979; Spano et al., 1978). The D2 family includes DRD2-4 (Andresen et al., 1990), which are coupled to $Ga_{i/o}$ family of G proteins that inhibit AC and consequently suppress the production of cAMP (Beaulieu and Gainetdinov, 2011). However, in addition to the historical basis of DRD sub-classification, DRDs also vary based on their structural, pharmacological and biochemical properties. Unlike genes that encode D2-class receptors, DRD1 and DRD5 do not contain introns in their coding region (Gingrich and Caron, 1993) (Beaulieu and Gainetdinov, 2011). Alternative splicing of an 87-base pair exon in DRD2 leads to the generation of short and long DRD2 variants termed D2S and D2L (Beaulieu and Gainetdinov, 2011; Giros et al., 1989). There is a difference of 29 amino acids in the third intracellular loop between these 2 splice variants that leads to distinct signaling properties (Beaulieu and Gainetdinov, 2011). Some less characterized splice variants have been reported for DRD3 and DRD4 as well (Pivonello et al., 2007).

Structure

DRDs are classified as seven transmembrane spanning receptors and belong to the superfamily of GPCRs. The N-terminal portion of DRDs displays considerable homology across all receptors. The C-terminus has been associated with membrane anchorage of the receptor (Pivonello et al., 2007). The third

intracellular loop has been implicated in interaction with G proteins and signal transduction. The hydrophobic transmembrane domains are involved in binding to dopamine as well as agonists and antagonists (Pivonello et al., 2007).

1.5.2 Dopamine receptor signaling

DRDs have been predominantly associated with the GPCR-mediated activation of cAMP through AC (Beaulieu and Gainetdinov, 2011). Stimulation of D1 family coupled to $Ga_{s/olf}$ leads to cAMP elevation through AC, while D2 family receptors coupled to $Ga_{i/o}$ inhibit AC and cAMP production (Kebabian and Calne, 1979; Kebabian and Greengard, 1971) (**Figure 2**). cAMP in turn regulates a complex network of signaling pathways downstream of DRDs (Beaulieu and Gainetdinov, 2011). One of the most recognized pathways downstream of cAMP includes the activation of PKA and its substrates such as cAMP-responsive element binding protein (CREB) (Beaulieu and Gainetdinov, 2011) (**Figure 2**). CREB is a transcription factor, whose activation initiates the expression of CREB target genes that regulate critical cellular mechanisms such as metabolic control, cell cycle and survival (Cheng et al., 2008; Shankar et al., 2005). While CREB has been previously implied to function as a proto-oncogene in AML (Shankar et al., 2005), its function in myelopoiesis and leukemogenesis has been predominantly studied in cell line models, transgenic mice, or through stable knock down or over expression systems (Cheng et al., 2008; Pellegrini et al., 2008; Shankar et al., 2005), which do not reliably represent the dynamic and transient nature of CREB-

mediated signaling (Beaulieu and Gainetdinov, 2011). Future studies are required to elucidate the role of CREB in human AML biology in a more physiologically relevant context.

In addition to cAMP-mediated DRD signaling, DRDs can pair up with Ga_q and regulate phospholipase C (PLC), leading to increased mobilization of intracellular calcium (Beaulieu and Gainetdinov, 2011; Lee et al., 2004). Also, dopamine receptors can directly interact with ion channels like the calcium channel (Beaulieu and Gainetdinov, 2011; Kisilevsky et al., 2008), or modulate Akt/GSK3 signaling through beta-arrestins (Beaulieu et al., 2011; Beaulieu et al., 2005; Beaulieu et al., 2004). Furthermore, DRDs may heterodimerize with their peers or other GPCRs to regulate an even more intricate set of cellular networks that enable DRD-mediated signaling to be regulated in a temporal and cell context-dependent fashion (Ahn et al., 2004; Beaulieu and Gainetdinov, 2011).

1.5.3 Dopamine receptor biology in human hematopoiesis

Immune cells encounter DA through dopaminergic innervations in the BM, the lymph nodes and in the blood (Levite, 2012). Also, certain immune cells such as neutrophils have been reported to endogenously synthesize DA (Bergquist et al., 1994; Cosentino et al., 1999), to mediate immune cell communication with other immune cells and with neuronal cells (Levite, 2008). DRD expression by radio-labeled ligands has been demonstrated in lymphocytes (Faraj et al., 1991; Ricci et al., 1998). Additionally, DRD1-5 expression at mRNA or protein level has been

reported in T and B lymphocytes, monocytes, neutrophils and natural killer cells (Boneberg et al., 2006; McKenna et al., 2002; Pereira et al., 2003; Sookhai et al., 1999).

Among immune cells, the biology of dopamine and DRDs is more elaborately characterized in T-cells, where DRDs mediate highly specialized T-cell functions such as activation of naïve T-cells (Levite et al., 2001), T-cell adhesion, trafficking and extravasation (Levite et al., 2001), production of cytokines (Besser et al., 2005) and chemotactic-mediated migration (Watanabe et al., 2006). In comparison to lymphocytes, the function of DRDs in myeloid cells is less understood. Flierl et al. reported that the mRNA expression of tyrosine hydroxylase, the rate limiting enzyme for DA synthesis, was upregulated in phagocytic macrophages and neutrophils after exposure to bacterial lipopolysaccharides (Flierl et al., 2007). Also, this study demonstrated the existence of the catecholamine machinery in myeloid cells as well as the functional significance of catecholamines in mediating inflammatory responses. Additional studies demonstrated that exposure to DA or DRD1/ 2 modulating small molecules led to an alteration of macrophage mediated cytokine production, Fc receptor expression and phagocytic behavior (Ali et al., 1994; Hasko et al., 1996; Sternberg et al., 1987). In neutrophils, DA exposure led to a suppression of cell adhesion and production of reactive oxygen species (Pinoli et al., 2017).

The expression of DRDs in the primitive cell subsets of the hematopoietic hierarchy has been controversial. While one study reported the presence of DRD3

and DRD5 in putative HSCs (CD34+CD38-)(Spiegel et al., 2007), a later study found little to no DRD expression in these cellular subsets (Sachlos et al. 2012). Instead, they noted a preferential expression of DRDs 1-5 in leukemic blasts (Sachlos et al., 2012). It should be noted that the most striking DRD levels reported by Spiegel et al. were found in mobilized hematopoietic cells (Spiegel et al., 2007). As DRDs are involved in mediating chemotaxis (Watanabe et al., 2006), exposure to mobilizing cytokines may have led to an overestimation of DRD levels that are otherwise noted in unstimulated primitive cell subsets. Overall, emerging evidence suggests that DA and DRDs participate in a variety of immune cell functions, giving DA a new "neuro-immuno-transmitter" title (Levite, 2012).

SUMMARY OF INTENT

While AML has been intensely studied for decades, the vulnerabilities of leukemic hematopoiesis remain poorly understood, resulting in ineffective therapies and unsatisfactory patient outcomes (Estey and Dohner, 2006). Cytotoxic-based chemotherapy with AraC, designed to debulk the tumor mass, was approved in 1969 (Rein and Rizzieri, 2014) and has remained the standard of care for decades. However, these regimens are associated with a high rate of therapy failure in the form of initial refractoriness or relapse, and are indiscriminately toxic to healthy hematopoietic cells (Venton et al., 2016). The identification of rare LSCs in the 90s offered a solution for existing therapy hurdles as well as a targeting focus (Bonnet and Dick, 1997; Lapidot et al., 1994). However, two decades later, the LSC concept has yet to impact the clinical management of AML. To date, the central signaling networks of leukemia cell biology that are targetable and of clinical relevance remain poorly understood. On the basis of these postulations, I focused my research on the following 3 central questions:

1) What are the unique biological pathways that instruct AML biology?
2) What are the underlying mechanisms that regulate these leukemogenic pathways?
3) Can these pathways be exploited for leukemia-selective therapies?

Several unique features of the hematopoietic system allowed me to tackle these questions in a feasible way: hematopoietic tissue can be readily and repeatedly

isolated from AML patients in large quantities, affording high throughput biological assays on clinically heterogeneous patient samples. This also provides the opportunity to explore leukemogenic pathways in primary hematopoietic cells instead of cell lines, which develop divergent biological properties (Gao et al., 2015). Also, while not readily available for the majority of other tissues, healthy control hematopoietic cells can be isolated from healthy donors, providing a unique platform to carry out parallel biological comparisons between healthy and leukemic cells. Equipped with these tools, *I hypothesized that human AML co-opts cellular pathways that are uniquely regulated compared to those that operate in normal hematopoiesis, which can be targeted for development of leukemia-selective therapies.*

To address my hypothesis, I defined the following specific objectives:

1) Investigate the cellular and molecular pathways that uniquely regulate leukemic hematopoiesis, in order to identify leukemia-specific candidate therapy targets.
2) Characterize the underlying mechanisms by which these candidate leukemogenic pathways function.
3) Evaluate the therapeutic value of the candidate target(s) in a clinical context.

Critical networks that uniquely coordinate leukemia cell properties such as chemoresistance or leukemia regeneration post-chemotherapy have remained under characterized as rare cellular subsets associated with these properties cannot

be unambiguously isolated and interrogated within AML patients (Eppert et al., 2011; Levine et al., 2015; Ng et al., 2016). However, species specific antigens in the context of xenograft assays enable high resolution tracing of human leukemia cells *in vivo*. As a result, we developed a unique xenograft model, which simulated chemotherapy regimens *in vivo* and modeled remission to relapse transitions within the original recipient animal, similar to the clinical scenario. Xenografted human AML cells were analyzed at various intervals including immediately post-chemotherapy to determine the features of residual leukemia populations, as well as at extended time frames to monitor regeneration kinetics contributing to disease relapse. Our findings indicated that in contrast to the theories of LSC chemoresistance (Jordan et al., 2006; Kreso and Dick, 2014; Saito et al., 2010), LSC pools suffered a dramatic reduction immediately after chemotherapy as determined by serial transplantation assays and LSC-related gene signatures (Eppert et al., 2011). Instead, these LSC-depleted residual leukemia cells demonstrated transient expression of a unique gene set prior to actively regenerating the leukemic hierarchy towards disease relapse. Next, we identified druggable targets within this gene signature, of which we prioritized DRD2, capitalizing on the expertise in our lab on the use of DRD2 antagonist drug thioridazine (TDZ) (Sachlos et al., 2012). *In vivo* TDZ treatment coupled with chemotherapy prevented leukemia relapse, providing proof-of-concept for our approach and proposing a novel role for DRDs in AML biology.

One of the major goals of this thesis was to identify therapies that unlike conventional chemotherapy, are leukemia-selective and preserve healthy hematopoietic cell functions. This is critical as the most common cause of death in AML patients is BM failure, an inability to produce healthy blood cells (Estey and Dohner, 2006). To functionally validate the leukemia-selectivity of DRD targeting approaches, we built on the body of work previously generated in our lab that showed *ex vivo* incubation with TDZ selectively abrogates leukemia initiation *in vivo* without affecting healthy human reconstitution (Sachlos et al., 2012). Our complementary findings demonstrated that clinically relevant doses of TDZ *in vivo* (Nagel et al., 2012) selectively diminish human leukemia levels with minimal adverse effects on healthy chimerism levels. Similarly, *in vitro* exposure to TDZ dramatically suppressed the function of leukemia progenitor cells whereas healthy progenitor functions remained intact. Collectively, these experiments indicated that the function of healthy stem and progenitor cells (HSPCs) was not adversely affected by TDZ. Consistent with functional sparing of healthy hematopoietic cells, protein level expression of DRDs was preferentially detected in putative leukemia stem and progenitor cells (LSPCs) compared to HSPCs.

DRDs have been primarily associated with mediating the functions of the nervous system (Beaulieu and Gainetdinov, 2011), and their role in coordination of neoplasticity is less understood. Our mechanistic assays with other DRD-directed small molecules and specific antibodies indicated that the cyclic AMP-mediated DRD signaling pathway is functional in human leukemia cells and can

be exploited to suppress leukemia progenitor cell activity by directing cellular maturation. Based on these findings, I propose DRDs as regulators of leukemic hematopoiesis, whose preferential expression pattern in leukemia versus healthy primitive cells affords a leukemia-selective therapy approach.

The overarching goal of the project was to identify novel targets that may serve to address the unmet clinical need of effective therapies against AML. As a result, we conducted a phase I clinical trial with TDZ in combination with chemotherapy, which allowed us to evaluate the safety and efficacy of DRD targeting in older AML patients with relapsed/refractory disease. The trial was designed to include a lead-in with TDZ as a monotherapy, which uniquely allowed us to examine the effects of DRD targeting on human leukemia cell biology in a clinical context. Remarkably, 8 of the 11 patients that received the 5-day monotherapy with TDZ displayed reduced blast levels and the 3 remaining patients showed progressive disease trends. Importantly, these blast level fluctuations post-TDZ were associated with patient-specific DRD2 expression levels, supporting a DRD-mediated effect for TDZ in a clinical setting, and corroborating our preclinical observations. Additionally, the DRD-mediated response patterns suggested that DRDs may serve as a screening bio-marker for guided therapies in the future.

Altogether, this thesis advances our understanding of the unique regulators of leukemogenesis that can be tangibly targeted for selective therapies, and describes a novel role for the DRD pathway in AML biology. Our findings offer

concrete value for the field as they demonstrate that authentic experimental models that faithfully reflect the biology of human AML are instrumental in the development of novel therapies that resolve unmet clinical needs (**Figure 3**).

Figure 1

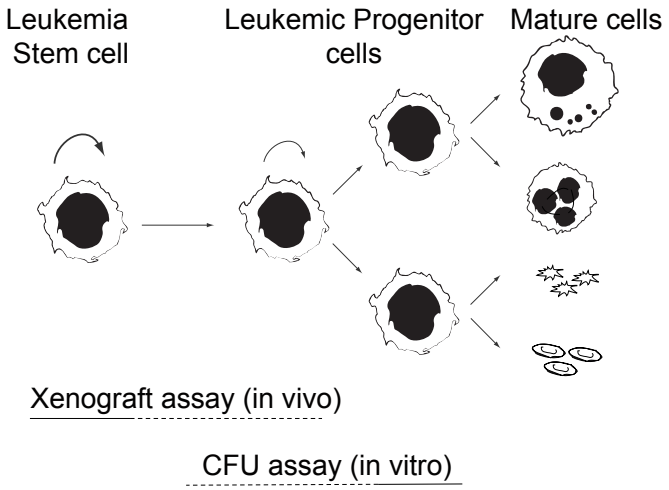

Figure 1. Functional assays of the leukemic hierarchy.
In vivo xenograft models are required to assay the function of the most primitive LSCs with long-term self-renewing potential. In this assay, human AML cells are transplanted into immunodeficient mice to propagate the disease. The clonogenic capacity of leukemic progenitors can be interrogated through *in vitro* CFU assays. The ability to self-renew and generate colonies is diminished upon cellular maturation and can no longer be assayed in differentiated progeny.

Figure 2

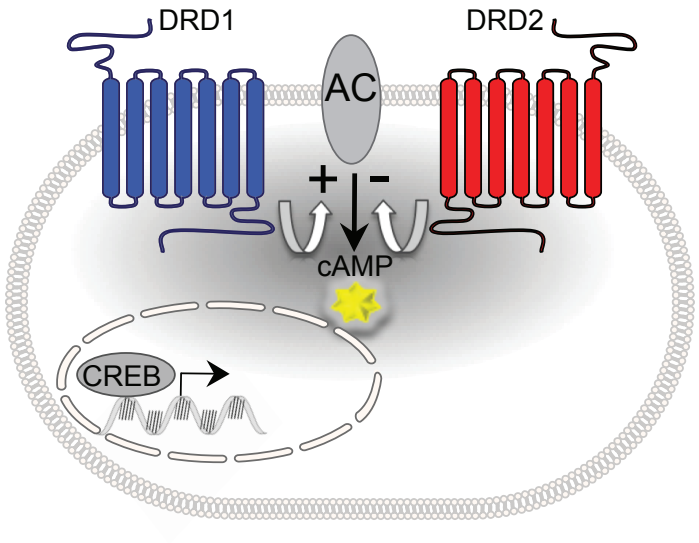

Figure 2. Dopamine receptor signaling
The prominent signaling pathway associated with DRDs is the modulation of cAMP through adenylyl cyclase (AC). DRD1 activation leads to the activation of AC and elevation of cAMP levels. In contrast, stimulation of DRD2 inhibits AC and consequently suppresses the induction of cellular cAMP. cAMP in turn activates CREB transcription factor, which leads to upregulation of CREB target genes.

Figure 3

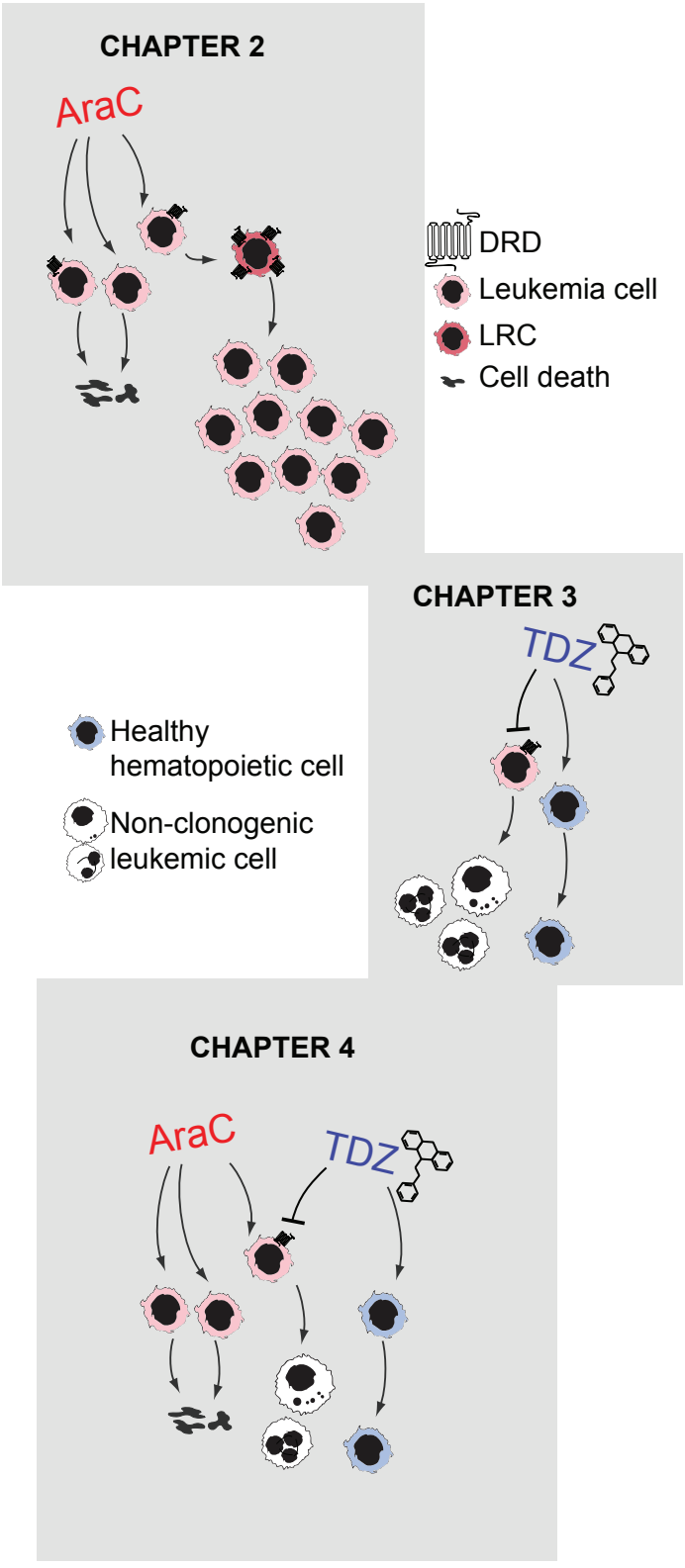
CHAPTER 2
AraC
DRD
Leukemia cell
LRC
Cell death
CHAPTER 3
TDZ
Healthy hematopoietic cell
Non-clonogenic leukemic cell
CHAPTER 4
AraC
TDZ

Figure 3. Summary of the thesis findings

The diagram illustrates the findings of the 3 chapters. In Chapter 2, we uncovered that AraC exposure leads cytoreduction, followed by aggravated leukemic regeneration. Leukemia regenerating cells (LRCs) displayed upregulated DRD2 expression levels. In Chapter 3, we demonstrate the functional mechanism of DRD targeting in human leukemia cells, which results in suppression of neoplastic features towards induction of cellular maturation. Healthy primitive hematopoietic cells remain unaffected due to minimal DRD expression level. To maximize these effects, we sought to combine the cytoreductive effect of AraC with the LRC-suppressive effect of TDZ. Therefore, we evaluated the safety of a combination therapy with TDZ and AraC in a phase 1 clinical trial in Chapter 4.

CHAPTER 2

Reoccurence of human AML disease develops from a state of therapeutic vulnerability

Preface

This chapter is prepared as an unpublished manuscript, in preparation for submission. The authors include:

Allison L. Boyd*, Lili Aslostovar*, Zoya Shapovalova, Jennifer Reid, Mohammed Almakadi, Kyle R. Salci, Anargyros Xenocostas, Ronan Foley, Brian Leber, Mickie Bharia

* These authors contributed equally to the work

I designed the study and wrote the manuscript with Dr. Mick Bhatia and Dr. Boyd. Experiments were performed in equal collaboration with Dr. Boyd. I played a more primary role in the design and execution of experiments related to DRD2 and Thioridazine, while Allison had a more primary role in designing and interpreting the kinetic characterization of AraC *in vivo*. Jennifer Reid assisted with the animal experiments. Dr. Shapovalova performed the bioinformatics analysis. Kyle Salci helped with cell purification experiments. Dr. Almakadi retrieved clinical history data for AML patients. Dr. Foley, Leber and Xenocostas provided the primary hematopoietic samples and Intellectual input.

This study was primarily driven to advance our understanding of the biological processes involved in leukemic relapse. Relapse has been associated with LSC subsets that are refractory to chemotherapy and are capable of re-establishing the leukemic hierarchy after remission. However, this notion has not been formally demonstrated as these rare residual cells cannot be readily isolated for experimental interrogation after the cytoreductive effect of chemotherapy. Also, due to a great amount of phenotypic overlap, these cells cannot be dissociated from their healthy counterparts at high enough resolutions for in-depth characterization. However, leukemia cell populations that propagate in immunodeficient animals can be unambiguously segregated from the murine cells, providing an opportunity to trace human leukemia populations *in vivo* and to physically isolate these cells for detailed characterization. As a result, we developed a customized xenograft model by introducing a clinically-relevant chemotherapy regimen *in vivo*, followed by monitoring the disease over extended time frames to replicate the short- and long-term events of leukemia cells after exposure to chemotherapy *in vivo*. This study represents a comprehensive account of leukemia cell dynamics in response to chemotherapy and provides novel insights for combination therapies that curb leukemic recurrence.

Reoccurrence of human AML disease develops from a state of therapeutic vulnerability

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ABSTRACT

For decades, Cytarabine (AraC)-based chemotherapy regimens have remained the standard of care treatment for acute myeloid leukemia (AML). Despite successful remission induction, most patients continue to suffer from aggressive recurrence of AML within 2-3 years(Estey and Dohner, 2006; Shipley and Butera, 2009). Rare subsets of leukemia stem cells (LSCs) have been proposed to preferentially resist chemotherapy, providing a cellular reservoir believed to form the basis for disease relapse (Bonnet and Dick, 1997; Lapidot et al., 1994). However, this theory has yet to advance to practical therapeutic management of AML patients, and limited direct evidence has tangibly addressed the features of leukemic cells that persist and re-grow following chemotherapy. Here we define the cellular and molecular hallmarks of leukemic regeneration by characterizing the recurrence process in a refined xenograft model of AML relapse and in human patients. During the process of remission induction, AraC chemotherapy proved to reliably collapse the hierarchical organization of AML disease, but this unexpectedly included the destruction of LSC pools. Disease recovery from cytoreductive treatment occurred by step-wise regeneration and reassembly of the hierarchical architecture of AML prior to complete disease recurrence. We define a unique molecular profile that emerges prior to AML regeneration in vivo that reveals leukemic regenerating cells (LRCs) that represent a leukemic state that extends beyond molecular definitions of LSCs (Eppert et al., 2011). This state can be pharmacologically targeted to interrupt leukemic recurrence in vivo thereby

defining a period of therapeutic vulnerability. Our study reveals that AML recurrence follows a complex and dynamic sequence that is conserved across genetically diverse patient subtypes and offers a unique therapeutic targeting approach to combat AML relapse.

MAIN

Rare human LSCs are believed to be responsible for disease initiation and chemotherapeutic resistance (Jordan et al., 2006). However, when we surveyed 47 genetically diverse AML patients including a subset with poor prognostic status (eg FLT3-ITD, and MLL mutations), we found that relapse rates are in fact independent of the presence or absence of LSCs at diagnosis (Fig. 1a and Extended Data Table 1). Alternatively, genetic risk classification has been suggested as a reliable predictor of therapy response(Döhner et al., 2010). However, even genetic sub-groupings lose prognostic power to stratify patients upon relapse, at which point outcomes become uniformly poor and few therapeutic options exist (Breems et al., 2005). As these leading paradigms used to explain relapsed disease have yet to deliver clear guidance for novel therapies to prevent recurrence, it is necessary to develop a more detailed understanding of the biology that drives therapy failure and AML regeneration post chemotherapy.

Relapsed leukemia originates from an ancestral genetic clone that does not represent a novel emergent disease (Ding et al., 2012; Kronke et al., 2013; Parkin et al., 2013). While the acquisition of additional genetic lesions in ancestral cells

is typical, these mutations are diverse across individual patients and have yet to clearly implicate specific pathways or cellular programs that distinctly characterize the driving processes of relapsed disease. In order to reflect combined genetic and epigenetic contributions to cellular states, global gene expression analysis was used to capture comprehensive molecular themes that uniquely define relapsed forms of AML. Leukemic blasts were profiled from 15 AML patients at the time of diagnosis and again at relapse (Extended Data Table 2). Unsupervised hierarchical clustering of these profiles partitioned global gene expression patterns into two major groups (Fig. 1b), respectively distinguished by genes associated with hematopoietic maturation (CD14, CD68, MPEG1) versus uncommitted primitive states (CD34, KIT, MSI2). These classifications were reinforced by the positioning of healthy mononuclear cells within the mature sub-group (Fig. 1b), as well as overlapping gene expression patterns between these groupings and transcriptional classifications previously reported to distinguish AML samples on the basis of maturation boundaries(Bullinger et al., 2004) (Extended Data Fig. 1a). However, unlike previously reported signatures of leukemia limited to gene subsets focused on hematopoietic tissue (Eppert et al., 2011), we reveal that the Primitive gene expression program that we have identified closely relates to primitive pluripotent stem cells as well (Extended Data Fig. 1b). This finding is reminiscent of studies of aggressive solid tumors, which also indicate that malignant features of primitiveness are not necessarily tissue- or lineage-specific (Ben-Porath et al., 2008), and are akin to stem cell

states shared with pluripotent cells. Using these definitions of primitive and mature disease states, AML patient gene expression signatures were equally distributed between mature and primitive states at diagnostic presentation (Fig. 1c). However, relapsed AML disease from the same set of patients reflected uniformity towards primitive signatures, independently of their initial mature vs. primitive heterogeneity at diagnosis (Fig. 1c). This dramatic convergence towards a unified primitive molecular state occurred only after exposure to cytoreductive chemotherapy treatment. As there are no existing models capable of delving into the formative cellular and molecular events that take place between treatment and overt disease recurrence, it remains unknown how chemotherapy exposure affects leukemic disease in such a consistent manner across patients.

To evaluate the causal influences of cytoreductive chemotherapy on human leukemia, it is necessary to develop controlled experimental systems that are validated in patients. To replicate the central clinical problem of disease recurrence in AML, we evolved the human AML-mouse xenograft model beyond its current use to detect human LSCs alone. Similar to a previous study (Farge et al., 2017), cytarabine (AraC) was used to develop this model as it represents the single common element across different chemotherapy regimens and is the only cytoreductive drug regularly administered as a monotherapy in AML treatment (Döhner et al., 2010). Xenografts bearing human AML were treated with optimized regimens of high-dose AraC (Wunderlich et al., 2013) (Extended Data Figure 2) and individual recipients were monitored by serial bone marrow (BM)

sampling, similar to clinical standards to assess chemotherapy response. This approach powerfully recreated both refractory and relapsed forms of recurrence that are seen and mirrored in AML patients' disease trajectories (Fig. 1d, e). Strikingly, AML disease reliably relapsed consistently across many individual mice, regardless of the apparent depth of remission states achieved (Fig. 1e). Although species-specific immunoprofiling afforded leukemia detection limits of well below standard morphological thresholds (from 5% to 0.01% BM blasts (Döhner et al., 2010; van Galen et al., 2014), AraC treatment was capable of briefly eliminating all evidence of human leukemia with limits of detection that are rarely achievable in the clinic (inset; Fig. 1e). Not only did our clinically inspired model closely recapitulate authentic therapy response kinetics, but recurrence in xenografts also mimicked molecular shifts towards primitive transcriptional states as previously seen in human patients (Fig. 1b, c; AML 16-18). These accurate cellular and molecular reflections of human patient disease establish a strong experimental foundation upon which to build a deeper understanding of the fundamental properties unique to recurrent AML despite achieving chemotherapy-induced remission.

Using this tractable *in vivo* model mimicking remission-relapsed evolution of AML, we were afforded the ability to further scrutinize the dynamics of leukemic regeneration at additional levels of precision not possible in the clinic. This model revealed that leukemic patterns of AML re-growth were unrestrained, and this response was in contrast to healthy human hematopoiesis that displayed

disciplined regeneration following chemotherapy exposure. Reproducibly across three distinct patients ranging from favorable to adverse genetic risk groupings, *in vivo* AraC-treated AML cells rapidly exceeded the threshold of disease burden established prior to chemotherapy (Fig. 2a). Although healthy human grafts also recovered promptly from AraC challenge, unlike AML they ultimately respected the boundary of their original levels of growth (Fig. 2a, Extended Data Fig. 3a), even following a prolonged observation period (Fig. 2a). Furthermore, growth rate quantification indicated that AraC directly exacerbated the growth characteristics of leukemic cells, but had little effect on the kinetics of healthy hematopoietic tissue regeneration. Following AraC treatment, healthy human cells resumed growth rates that were equivalent to their saline-treated counterparts (Extended Data Fig. 3b,c), whereas AraC reproducibly accelerated the velocity of leukemic expansion (Fig. 2b,c; Extended Data Fig. 3d,e). Critical to the management of AML in the clinic, where multiple scenarios cannot be tested for individual patients, we observed that this AraC-induced expansion occurred regardless of timing or transition through remission states (Fig. 2b,c). As a consequence, disease levels in AraC-exposed recipients ultimately approached and exceeded those of saline-treated controls (Fig. 2b), highlighting the insufficient efficacy of AraC cytoreduction in the long term, which correlates to eventual relapse in patients (Döhner et al., 2010). The remarkable consistency of AML recurrence responses across many individual mice and patient samples (Fig. 1d,e; Extended Data Fig. 3d) suggests that newly forged paths of genomic evolution are unlikely

to explain AraC-provoked changes in leukemic behavior seen in this model and noted clinically. Furthermore, xenografted AML typically manifests a uniform genetic landscape(Klco et al., 2014), providing little basis for sub-clonal selection events. Therefore, we proceeded to dissect the cellular architecture and biology that supports recurrent AML, instead of sub-clonal tracking analyses that have unproven therapeutic application (Ding et al., 2012; Shlush et al., 2014) or assays that require selective transplantation pressures (Sun et al., 2014) that may compromise the surrogacy of this model relative to the clinical setting.

AML disease is reliably composed of a complex cellular hierarchy that is sustained by the self-renewing behavior of rare LSCs and their descendant progenitors (Bonnet and Dick, 1997; Lapidot et al., 1994) (Fig. 2d). While both LSCs and progenitors principally reside within the CD34-expressing compartment, their specific cell surface phenotypes are inconsistent across patients (Bonnet and Dick, 1997; Eppert et al. 2011; Lapidot et al., 1994) and therefore functional assays are required for their accurate quantification (Fig. 2d; Extended Data Fig. 3f). We first applied these measures to characterize leukemic cells that withstand AraC treatment and thus represent initial surviving cells post chemotherapy. Serial transplantation of human AML cells recovered shortly after AraC treatment revealed eradication of operationally defined LSCs at this time point of treatment (Fig. 2e, Extended Data Fig. 3g), thus challenging the popular premise that LSCs are selectively chemoresistant (Ishikawa et al., 2007; Jordan et al., 2006). Similar to effects on LSCs capable of initiating disease, AraC

treatment also destroyed leukemic progenitors detected *in vitro*, as demonstrated by the lack of residual colony forming activity (Fig. 2f). These findings in our xenograft AML model were complemented by equivalent analyses of BM cells obtained from a human AML patient treated with AraC together with daunorubicin, an anthracycline agent commonly used in induction therapies. Here, therapy similarly abolished colony-forming progenitors (Fig. 2f) despite the persistence of residual AML disease. This patient's cells had no LSC capacity either before or after therapeutic treatment, confirming that these conclusions are applicable beyond the 44% of AML patients harboring LSCs (Extended Data Table 1). Longitudinal phenotypic assessment of both patient- and xenografted-AML corroborated our functional results, as CD34 expression sharply declined as a consequence of AraC-based therapy (Fig. 2g). Together these data suggest that unlike common conceptions (Ishikawa et al., 2007; Jordan et al., 2006), the uppermost tiers of the AML hierarchy are in fact profoundly impacted by chemotherapeutic intervention.

Given that molecular signatures of relapsed AML patient cells shift towards primitive states (Fig. 1), we expanded the scope of our cellular assays to monitor associated changes in functional biology over time. This chronology revealed that LSC and progenitor pools quickly reorganize, and compensate for AraC cytoreductive insult by acute proliferative expansion, ultimately exceeding their original pre-treatment proportions (Fig. 2h,i and Extended Data Fig. 3h,i). CD34 frequencies also recovered, but this occurred in a more gradual manner

(Fig. 2j and Extended Data Fig. 3j). Importantly, the massive rebound of functionally primitive cells occurred prior to any evidence of bulk leukemia re-growth (Fig. 2h,i and Extended Data Fig. 3h,i). As the peak outgrowth of these fractions coincided with the onset of overt leukemic regeneration (Fig. 2h,i and Extended Data Fig. 3h,i), this signifies a landmark period in the progression of recurrent disease and regeneration of AML that differs from *de novo* disease establishment processes related to LSC initiating properties (Jordan et al., 2006).

Based on these specific timelines of chemotherapy-induced remission and onset of regeneration, we reveal that highly clonogenic populations of AML cells are responsible for the regenerative process driving relapse post treatment. We termed this state of AML cells as a population representing Leukemic Regenerating Cells (LRCs). Highly clonogenic LRCs were reproducibly detected at this critical identified point via an independent set of AML-xenografts as well as a patient case of refractory AML (Fig. 3a). We therefore sought to molecularly characterize the LRC subset by capturing the gene expression profile of this unique leukemic state that represents the origins of disease re-emergence. Relative to matched therapy-naïve controls, cytoreductive therapy produced consistent global transcriptional shifts (Fig. 3b). This level of concordance among patients was impressive, as the patients profiled were widely heterogeneous, and included a clinically diverse range of genetic defects; from normal karyotypes to poor prognostic abnormalities (Extended Data Table 2). Furthermore, transcriptional consequences of AraC-based therapy were closely aligned between human patient

and xenograft measures (Fig. 3b), highlighting the robust and consistent nature of this acquired state and LRC development.

With the explicit goal of identifying optimal targets for LRC-directed therapy, we next resolved the specific and comparative transcriptional features that define LRC states. Transcriptional profiles of LRCs failed to reflect signatures characteristic of LSC-rich AML that have been characterized under chemotherapy-naïve, undisrupted conditions (Eppert et al., 2011) (Fig. 3c). This suggests that the LRC state is not represented by signatures that shape our current molecular understanding of AML-LSCs. Instead, a novel set of 248 protein-coding genes provided a distinguishing fingerprint to target the unique LRC molecular state (Fig. 3c,d). To elicit therapeutically targetable candidates within this list, we used the Drug–Gene Interaction database (Griffith et al., 2013) which further refined our selection to 16 druggable gene products (Fig. 3d and Fig. 3e; Extended Data Table 3). These genes were exclusively associated with transient LRC states and were not enriched at diagnosis or relapse, indicating the acute window for potential therapeutic intervention (Fig. 3f). Important to the context of previous reports (Bachas et al., 2015; Eppert et al., 2011; Pabst et al., 2016; Staber et al., 2004) these potential targets could not have been identified using molecular signatures of naïve LSCs or relapsed AML samples, supportive of the unique molecular condition of LRCs that emerge during chemotherapy.

Among the 16 druggable genes specific to LRC states, DRD2 was identified (Fig. 3e) and represents a cell surface receptor recently characterized in

human AML, as well solid tumors such as breast cancer (Sachlos et al., 2012). As DRD2 can be targeted using the drug thioridazine(Cheng et al., 2015; Mao et al., 2015; Sachlos et al., 2012; Seeman and Lee, 1975) (TDZ), we selected this drug to evaluate the utility of LRC targeting via combination therapy coupled with AraC. Flow cytometry analysis of three distinct AML patient xenografts confirmed elevated DRD2 protein levels at the point of LRC predominance following AraC treatment (Fig. 4a), validating our testable target based on our gene expression results. In contrast, *in vivo* administration of clinically relevant TDZ doses (Nagel et al., 2012) (Extended Data Fig. 4) caused a reduction of DRD2 expression (Fig. 4a). Incorporation of TDZ therapy into AraC treatment regimens successfully disrupted the aggressive growth trajectories otherwise provoked by AraC treatment alone (Fig. 4b,c). This objective growth quantification provides powerful evidence that LRC-targeted combination treatment achieves superior disease control relative to conventional AraC therapy that is exclusively cytoreductive (Fig. 2b and 4b). Accordingly, we suggest that our current model is able to project patient benefits in a meaningful way, as opposed to traditional readouts of leukemic progenitors and LSCs typically used in pre-clinical testing of AML that are susceptible to re-transplantation artifacts (Sun et al, 2014) and difficult to relate to AML patient health and survival.

To verify that this LRC targeting strategy operates by destabilizing the reassembly of AML hierarchies, we functionally interrogated leukemic populations exposed to TDZ + AraC treatment (Fig. 4d). In a case of a highly

clonogenic AML patient sample, TDZ co-treatment dramatically suppressed the AraC-driven recovery of leukemic progenitors, and completely abolished *in vivo* disease re-initiation capacity (Fig. 4d; AML 18). In replicate experiments from two additional independent AML patients, re-growth of AML was suppressed so deeply that residual leukemic cells were devoid of any progenitor capacity (Fig. 4d; AML 23 and 24). In a final patient sample, we demonstrated complete leukemia elimination in mice treated with AraC + TDZ (Fig. 4d; AML 25), indicating that co-treatment with TDZ decisively ensured AML eradication, in sharp contrast to AraC monotherapy that allows relapsed regeneration of disease (Fig. 4d; AML 25). With DRD2 targeting providing the initial proof of principle of our approach, our results validate the systematic molecular targeting of leukemic regenerating cells post chemotherapy to effectively eliminate AML recurrence. Our study integrates human clinical data and novel xenograft approaches to deliver a new model of disease recurrence in AML to better understand the molecular and cellular basis of relapsed disease (Fig. 4e). Following potent cytoreduction, the course of AML recurrence follows a dynamic path involving transitions through an intermediate LRC state that is unlike the final emergent disease (Fig. 4e). Critically, molecular determinants of LRCs are not found in naïve AML cells or LSC subsets (Eppert et al., 2011), and can be readily targeted to discourage AML re-growth, revealing a discrete window to strike residual AML cells during this newly identified period of vulnerability (Fig. 4e).

Although AML is recognized as a particularly heterogeneous disease (Döhner et al., 2010), we have uncovered unifying themes of therapy response that transcend patient classification schemes based on underlying genetics or LSC content alone. Our findings in AML complement emerging insights in solid tumor biology, suggesting that chemotherapy provokes repopulation events that directly contribute to therapy failure (Kurtova et al., 2014). As related opportunities for deliberate recurrence targeting are likely to be cancer- and tissue-specific, more extensive investigation of these concepts across diverse malignancies represents an important goal with immediate implications for patient care. Our results here have motivated a Phase I study, and remaining LRC targets represent valuable candidates to advance recurrence-targeted therapy for AML. Ultimately these findings will assist in applying this paradigm to identify analogous periods of vulnerability during chemotherapy in other cancers with successful remission induction regimes that are accompanied by high relapse frequencies (Goss and Chambers, 2010).

Acknowledgments

Supported by the Canadian Cancer Society Research Institute (to Dr. Bhatia) and Jan's Graduate Scholarships in Stem Cell Research (to Allison Boyd and Lili Aslostovar) as well as a graduate scholarship from the National Sciences and Engineering Research Council (to Allison Boyd). Dr. Bhatia is supported by the Canadian Chair Program and holds the Canada Research Chair in human stem cell

biology. We thank the patients and donors for providing the clinical specimens, Dr. Kennedy Makondo, Monica Graham, Jamie McNicol, Jennifer Russell, Claudia Hopkins and Irene Tang for technical support, Dr. Clinton Campbell, Dr. Ryan Mitchell and Dr. Kristin Hope for valuable comments, and the London Regional Genomics Center for performing the Affymetrix analysis.

METHODS

Primary human hematopoietic samples. Healthy human hematopoietic cells were isolated from BM and mobilized peripheral blood (MPB) of adult donors or from umbilical cord blood. Primary AML specimens were obtained from peripheral blood apheresis or BM aspirates of AML patients. The Labour and Delivery Clinic at the McMaster Children's Hospital provided healthy cord blood samples. Primary AML patient and healthy BM donor samples were provided by Juravinski Hospital and Cancer Centre, and London Health Sciences Centre (University of Western Ontario). All samples were obtained from consenting donors in accordance with approved protocols by the Research Ethics Board at McMaster University and the London Health Sciences Centre, University of Western Ontario. Details of AML patient samples are outlined in Extended Data Tables 1 and 2. Mononuclear cells (MNCs) were recovered by density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare) followed by red blood cell lysis using ammonium chloride solution (Stemcell Technologies). Lineage depletion of cord blood and MPB samples was carried out using EasySep

magnetic separation (Stemcell Technologies), according to the manufacturer's instructions.

Murine recipients and xenograft assays. Mice were bred and maintained at the McMaster Stem Cell and Cancer Research Institute animal barrier facility. All experimental procedures were approved by the Animal Council of McMaster University. NOD/Prkdc^{scid} (NOD/SCID) mice were used as xenograft recipients for AML recurrence modeling, and xenografts were initiated using previously described protocols (Boyd et al., 2014). For transplantation assays, 6-10 week old NOD/SCID mice were sublethally irradiated (200-350 Rads, using a 137Cs γ-irradiator) 24 hours prior to intravenous transplantation of primary human samples (Bonnet and Dick, 1997; Lapidot et al., 1994). Both male and female mice were used, however sex was controlled within individual experiments. Human chimerism in recipient mouse BM was defined as ≥0.1% hCD45$^+$, and hCD45$^+$CD33$^+$ for healthy and leukemic engraftment, respectively (Notta et al., 2011).

Longitudinal *in vivo* monitoring of human leukemic chimerism was carried out by serial BM aspiration as previously described (Verlinden et al., 1998). Briefly, 5-10 μl of BM cells were collected from femurs of anesthetized recipient mice; the procedure repeated at bi-monthly intervals on alternate femurs. Cellular growth rates were calculated as derived from the rate constant "k" of the fitted exponential growth model $Y=Y0^{(k*X)}$.

To quantify tumour initiating capacity indicative of LSCs, pooled BM cells from multiple xenografted primary recipients within the same group were serially transplanted into secondary animals by intravenous injection at multiple cell doses, normalized based on the number of injected human leukemia cells. For secondary progenitor assays, xenografted human leukemic cells were purified by fluorescence activated cell sorting (FACS) or by mouse cell exclusion using magnetic cell isolation (mouse CD45 and mouse Ter119; Miltenyi Biotec) and subsequently seeded in methylcellulose.

For drug delivery experiments, weekly weight measurements were used to ensure that an appropriate dose per weight ratio was sustained throughout the treatment. In combination regimens with AraC and thioridazine (TDZ), AraC (50 mg/kg/day) was administered daily between days 7-12 within the 21-day TDZ (22.5 mg/kg/day) regimen.

Myeloid progenitor assay. The clonogenic capacity of leukemic progenitors was evaluated by colony-forming unit (CFU) assays. Briefly, AML cells (5,000-50,000 cells/well) were seeded in semisolid methylcellulose media (Methocult GF #H4434; Stemcell Technologies) according to established protocols (Boyd et al., 2013; Tehranchi et al., 2010). A minimum of 40 cells was required for designation as a colony(Moore and Metcalf, 1973) (Extended Data Fig. 3f). CFU assays of xenografted leukemic cells were performed following human cell purification as described above. CFU images were acquired at 2x using the

Operetta High Content Screening platform (Perkin Elmer) by means of epi-fluorescence illumination and standard filter sets. Stitched whole well images were constructed in Columbus software (Perkin Elmer).

Fluorescence-activated cell sorting (FACS) and flow cytometry. Immunophenotyping for hematopoietic cell surface markers was carried out using the following antibodies: V450-conjugated anti-CD45 (2D1), APC-conjugated anti-CD33 (WM-33), and PE-conjugated anti-CD34 (563; all from BD Biosciences). DRD2 protein levels were measured using a mouse anti-human DRD2 primary antibody (B-10; Santa Cruz) followed by incubation with an Alexa fluor 647-conjugated donkey anti-mouse secondary antibody (Thermo Fisher). 7-aminoactinomycin D (Beckman Coulter) was used to discriminate live cells. When appropriate, fluorescence minus one controls were used to optimize gating strategies for target cell populations. In experiments that required cell sorting, FACS-purified cell populations were routinely checked for purity. FACS sorting and flow cytometry analysis were performed using FACSAria II sorter and LSRII Cytometer (BD), respectively. FACSDiva software (BD) was used for data acquisition and FlowJo software (Tree Star) was used for analysis.

RNA purification and gene expression profiling. RNA was isolated from healthy MNCs or FACS-purified leukemic blasts (based on side scatter and CD45 intensity(Vo et al., 2012)) using a total RNA purification kit (Norgen Biotek)

according to the manufacturer's instructions. Purified RNA was quantified on a Nanodrop 2000 Spectrophotometer (Thermo Scientific), and RNA integrity was assessed by a 2100 Bioanalyzer (Agilent Technologies). RNA was extracted and hybridized to Affymetrix Gene Chip Human Gene 2.0 ST arrays (London Regional Genomics Centre). Output data was normalized using the Robust Multichip Averaging algorithm with Genomics Suite 6.6 software (Partek Inc). Gene expression data from 11 paired diagnosis-relapse samples(Hackl et al., 2014) (GSE66525), and diagnostic AML patient samples with predominantly normal karyotypes (GSE425, Groups I and II (Bullinger et al., 2004)) were obtained from publically accessible data sets. Batch correction was performed on sources of technical variation (array technologies and/or isolation of human cells from patients versus xenografts). Expression values from all common gene symbols were used to create PCA plots. Pearson's correlation coefficient was used for hierarchical clustering to generate dendrograms. Gene set enrichment analysis (GSEA) was performed on normalized expression values of all common gene symbols between samples using GSEA software v2.1.0 (Broad Institute(Mootha et al., 2003; Subramanian et al., 2005)). Our microarray data is accessible at GEO (accession code GSE75086). Druggable gene targets within the list of 248 upregulated protein-coding genes after chemotherapy were identified using the Drug Gene Interaction Database (DGIdb v2.22).

Hematology analysis. Peripheral blood was collected from the superficial temporal vein and tail vein for WBC counts, which were measured using a Nexcelom Cellometer after acridine orange staining of diluted whole blood samples.

Statistical analysis. Summarized data are represented as mean ± standard error (s.e.m.). Statistical comparisons were analyzed using unpaired two-tailed Student's *t* tests, analysis of variance tests (ANOVAs) followed by Newman-Keuls post hoc tests, Fisher's exact tests or the Poisson distribution test. Prism software (version 5.0a; GraphPad) and ELDA(Hu and Smyth, 2009) were used for statistical analysis, and $P<0.05$ was considered statistically significant. Any deviations from normal distribution or homogeneity of variances were corrected by $\log_{10}$ transformation prior to parametric statistical tests. Experimental mice were only excluded from analyses if they failed to survive to pre-determined experimental endpoints. No blinding was used for animal or laboratory studies. Mice were allocated to drug treatment groups based on pre-treatment BM aspirates, to ensure similar starting levels of human chimerism across groups. If no initial assessment of chimerism was performed, mice were randomly allocated to experimental groups, assuring that cage mates were distributed across different

groups. Sample sizes were selected based on previous experience, and no formal power analysis was performed.

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Figure 1

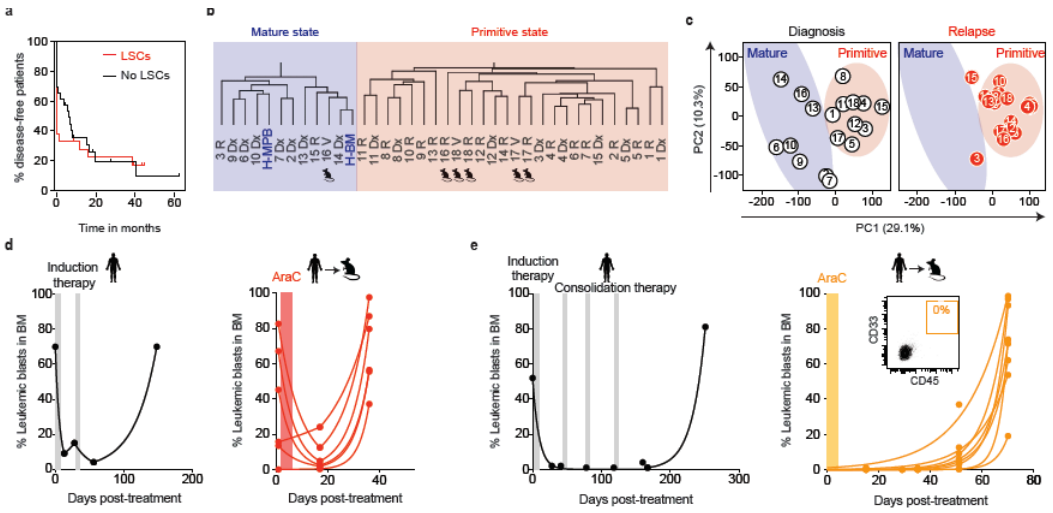


Figure 1. Recurrence xenograft modeling recapitulates clinical features of therapy failure. a, Kaplan-Meier analysis comparing time to disease recurrence in patient samples with (*n*=21) and without (*n*=26) LSCs based on human leukemic engraftment potential in immune-deficient mice (*P*=0.13). **b,** Unsupervised hierarchical clustering of global gene expression profiles of paired diagnosis (Dx) and recurrence (R) samples from 15 AML patients, as well as xenografted patient samples retrieved at recurrence following *in vivo* AraC treatment (R), or from saline-treated vehicle controls (V; AML 16-18 represent xenografted samples, as indicated by mouse silhouettes). Mononuclear cell (MNC) preparations from healthy donors were included as controls (BM and MPB). **c,** Principal component analysis (PCA) correlation biplot representing global gene expression for paired diagnosis and recurrence samples from *de novo* or xenografted AML samples presented in Fig. 1b, outlining a convergence towards primitive signatures at recurrence. Shaded ellipses identify the gene space defined by primitive and mature molecular signatures. The numbers within datapoints indicate patient/xenograft IDs (AML 16-18 represent xenografted samples). **d,** Human patient disease dissemination kinetics typical of refractory AML (left; AML 19) can be recreated in xenografted mice following *in vivo* cytoreduction with AraC (right; AML 16). Each curve represents an individual recipient mouse. **e,** The remission-relapsed disease scenario (left; AML 20) can also be captured in xenografted mice after *in vivo* AraC treatment (right; AML 16). Each curve represents an individual recipient mouse. The inset shows a representative flow cytometry plot of a follow-up BM aspirate performed at Day 29 post-treatment, confirming a state of remission.

Figure 2

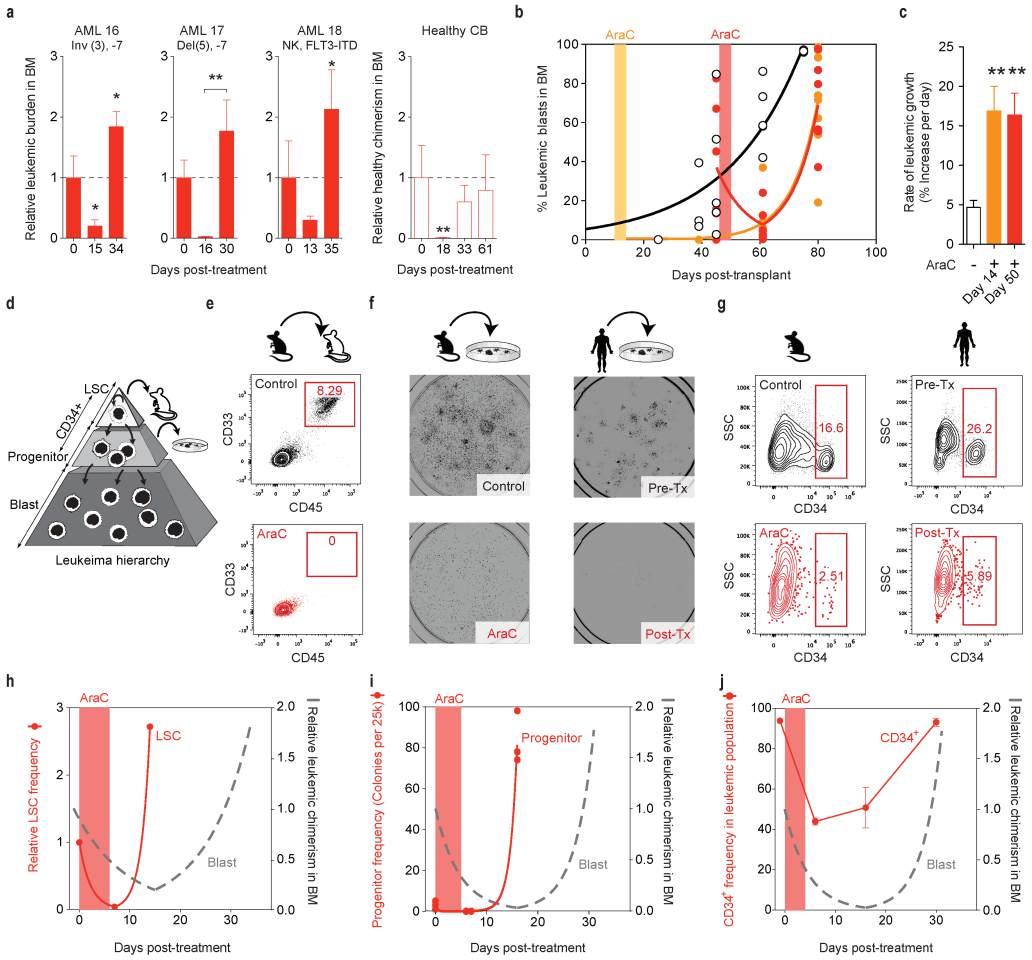


Figure 2. Cytoreductive chemotherapy fuels accelerated leukemic regeneration. a, Longitudinal analyses of leukemic or healthy human chimerism in xenograft BM following *in vivo* cytoreduction with AraC, normalized to pre-treatment levels. *n*=5 (healthy) or ≥6 (AML) mice per human sample at each time point. Patient genetic abnormalities are indicated above each graph. NK, normal karyotype; CB, cord blood. **P*<0.05, ***P*<0.01 by one-way ANOVA. **b,** Superimposed growth curves from AraC-treated xenografts presented in Fig. 1d-e, adjusted to reflect differences in the timing of AraC treatment between two independent experimental groups, as indicated by orange and red bars. Data points represent individual mice over time, and curves represent group averages. The black curve represents the average of vehicle-treated xenografts. **c,** Leukemic growth rates calculated for individual mice, based on growth curves presented in

b. $^{**}P<0.01$ relative to saline control, by one-way ANOVA. **d,** Schematic representing the leukemic cellular hierarchy, the functional assays (transplantation to secondary recipients and *in vitro* semi solid clonogenic assays), and putative phenotype corresponding to primitive cellular sub-fractions. **e,** Representative flow cytometry plots of a serial transplantation experiment performed with AML 16-xenografts, showing that AML-LSC capacity is reduced at Day 7 post-AraC initiation *in vivo*, relative to saline control. **f,** Representative whole-well CFU images showing that AML progenitor capacity is reduced during the initial stages following cytoreductive chemotherapy (within the first week following therapy cessation). Left, CFU image from AML 26-xenografts at Day 7 post-AraC initiation *in vivo* relative to saline control. Right, AML 21 patient cells showing loss of progenitor function at Day 14 following initiation of standard 3+7 induction chemotherapy ("Post-Tx"), relative to the diagnostic patient sample ("Pre-Tx"). **g,** Representative flow cytometry plots of CD34 expression frequencies during the initial stages post-cytoreduction (within the first week following therapy cessation). Left, CD34 expression in AML 16-xenografts at Day 7 following AraC initiation *in vivo* relative to saline control. Right, CD34 expression in the diagnostic ("Pre-Tx") AML patient sample versus at Day 14 after the initiation of standard 3+7 induction therapy ("Post-Tx") for AML 21. **h,** AraC-induced fluctuations in LSC frequencies as determined by serial transplantation of xenografted AML samples at multiple time points following therapy. Data represent calculated LSC frequencies of AML 16 and 18-xenografts normalized to saline treated control over time as detailed in Extended Data Fig. 3g,h. Grey dotted lines indicate total leukemic chimerism in BM relative to pre-treatment levels. **i,** Longitudinal analysis of progenitor frequencies within human leukemic xenografts (AML 18) in response to AraC treatment *in vivo*. Grey dotted lines indicate total leukemic chimerism in BM relative to pre-treatment levels. **j,** CD34 expression dynamics in human leukemic xenografts (AML 18) in response to *in vivo* AraC treatment over time. Grey dotted lines indicate total leukemic chimerism in BM relative to pre-treatment levels. All summarized data are expressed as mean ± s.e.m.

Figure 3

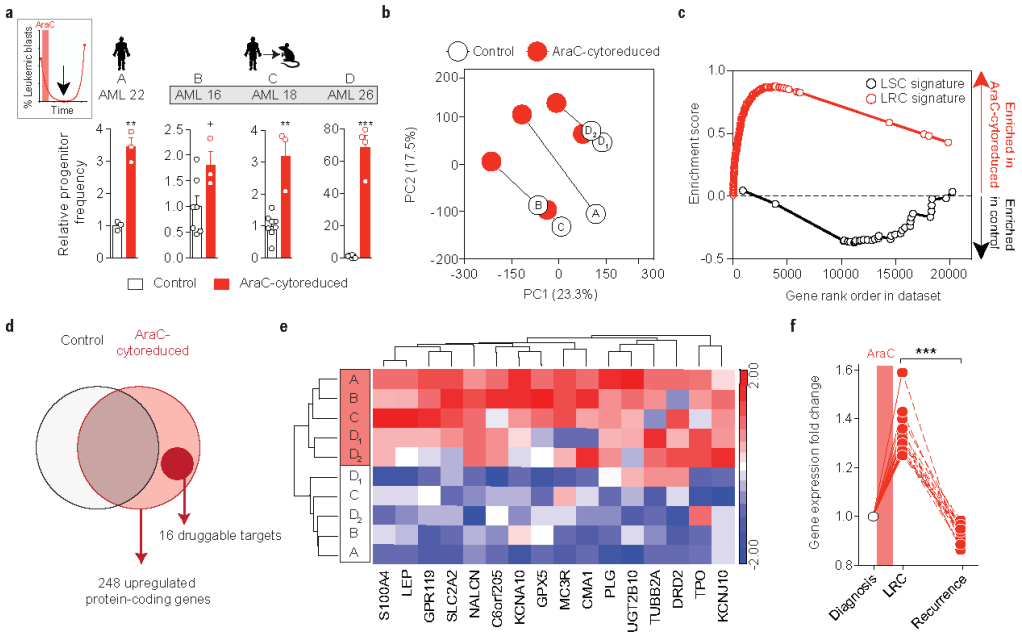


Figure 3. LRC gene signatures characterize a transient state of therapeutic opportunity. **a,** Relative progenitor frequencies in an AML patient sample and in AML xenografts, measured at regenerative phases of leukemic growth occurring at 3 weeks (human patient) and ≥1 week (xenografts) after chemotherapy withdrawal. Progenitor frequencies are normalized relative to matched controls unexposed to chemotherapy (saline controls or untreated diagnostic patient cells). The timing of the analysis is indicated by the arrow in the inset. Data are expressed as mean ± s.e.m. +P=0.05, **P<0.005, ***P<0.001, by unpaired t-test. **b,** PCA correlation plot of the global transcriptional shifts among purified human AML cells retrieved at the point of enhanced colony-forming capacity after cytoreductive therapy, versus matched controls. Samples correspond to those presented in **a**. **c,** Gene set enrichment analysis of samples presented in **a-b**, comparing gene signatures characteristic of naïve untreated LSCs (Eppert et al., 2011), versus newly defined LRCs, during states of low leukemic burden after cytoreduction (as illustrated in **a**). **d,** Venn diagram illustrating 248 up-regulated protein-coding genes following AraC-based cytoreduction. This list was further refined to identify 16 druggable candidates using DGIdb. **e,** Heat map highlighting expression levels of the 16 druggable genes unique to LRCs, across the AML samples shown in **a-b**. **f,** Relative expression levels of the 16 druggable

LRC genes over time indicate that these genes are only transiently up-regulated following chemotherapy treatment. Each data point represents an individual gene, measured during regenerative LRC phases (samples presented in **a-b**) and at overt disease recurrence (>1 month following chemotherapy treatment; samples presented in Fig. 1**b**). ***P<0.001, by unpaired t-test.

Figure 4

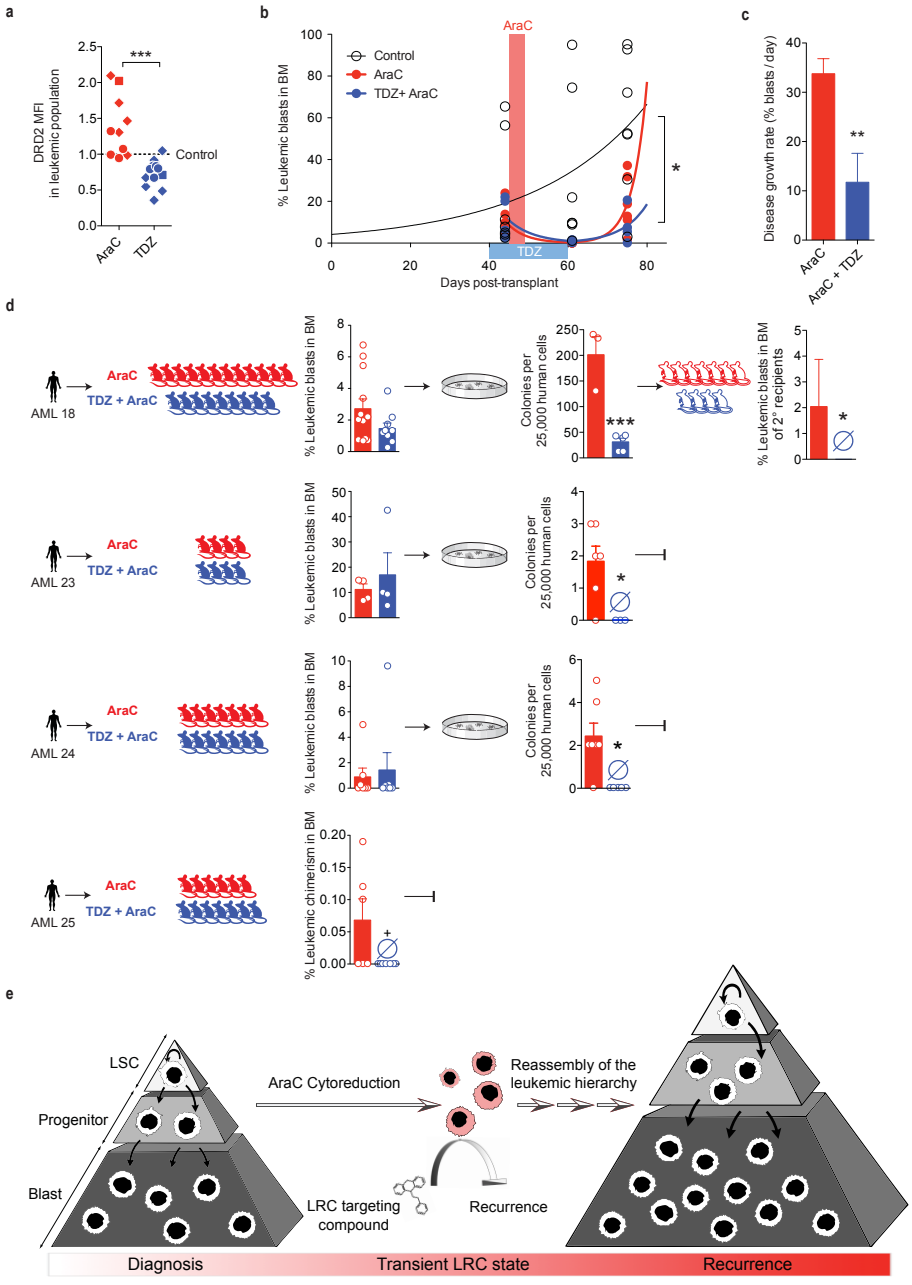
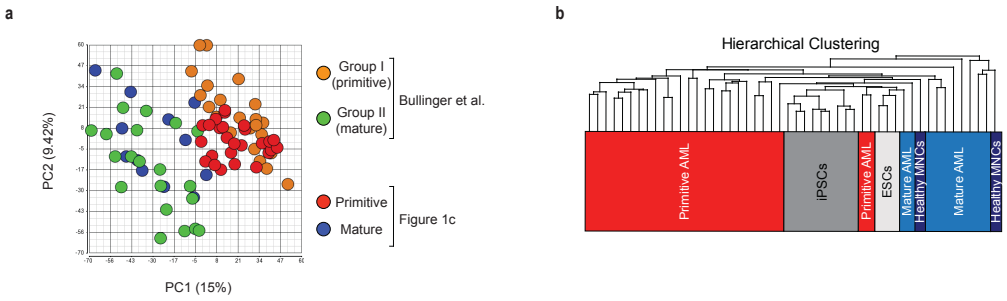


Figure 4. Novel combination therapy with LRC-targeted compounds interrupts AML recurrence. a, Mean fluorescence intensity (MFI) of DRD2 protein expression within AML patient xenografts treated with AraC or DRD2 antagonist TDZ, relative to mean expression levels within saline-treated controls

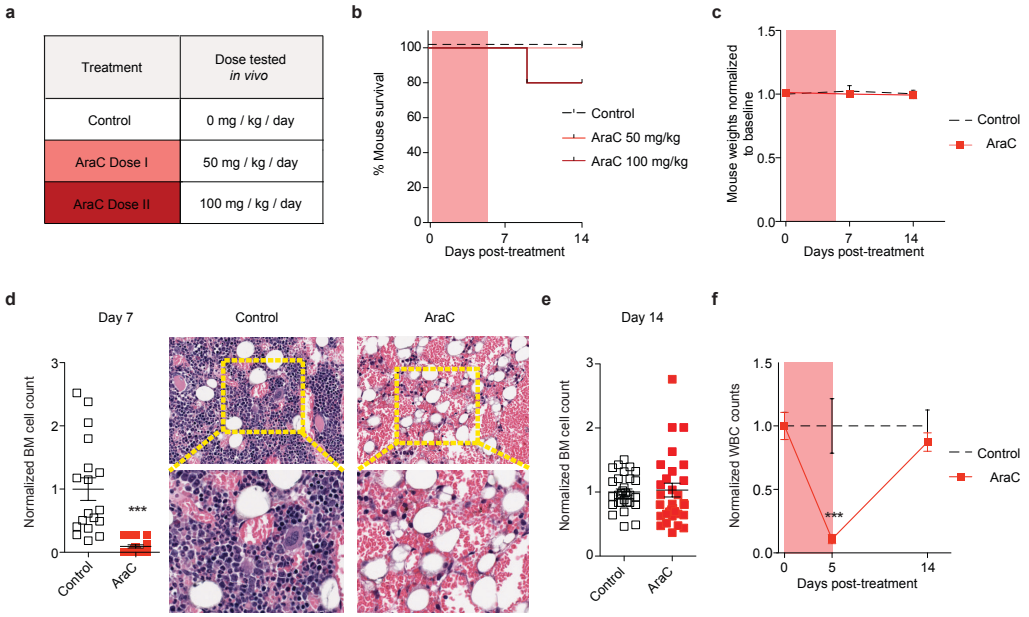
(dotted line). Each data point represents cells isolated from an individual mouse (diamonds, AML 18; circles, AML 23; squares, AML 24)**.** ***P<0.001, by unpaired t-test. **b,** Growth curves of AML 19-xenografts treated with AraC alone or in combination with TDZ. *P<0.05, final BM leukemic blast levels between vehicle control versus AraC + TDZ treatment groups, by one-way ANOVA. **c,** Addition of TDZ to *in vivo* AraC cytoreduction reduces leukemic growth rates, calculated for individual mice based on growth curves presented in **b**. **P<0.01 by unpaired t-test. **d,** Functional analyses performed on patient xenografts at the point of LRC emergence, following *in vivo* treatment with AraC alone or TDZ+AraC (Day 14 post-initiation of AraC treatment; Day 21 post-initiation of TDZ treatment). Treatment schedules were identical to **b**. Human leukemic cells recovered from xenografted mice were forwarded to CFU assays. Cells from AML 18-xenografts were also assayed for self-renewal capacity by serial transplantation. Mouse silhouettes indicate sample size, hollow silhouettes indicate secondary recipients that did not receive further therapy. Secondary assays were not performed with AML 25-xenografts, as no human leukemic cells were detected in any of the 7 mice treated with AraC + TDZ, whereas human leukemia persisted in 3 of 6 mice treated with AraC alone. "+"P=0.07, Fisher's exact test, *P<0.05, by Fisher's exact test, ***P<0.001 by unpaired t-test. **e,** Model of accelerated leukemic disease recurrence as a consequence of cytoreductive therapy. Reassembly of the leukemic hierarchy can be interrupted by targeted therapies during the transient LRC window. All summarized data are expressed as mean ± s.e.m.

Extended Data Figure 1



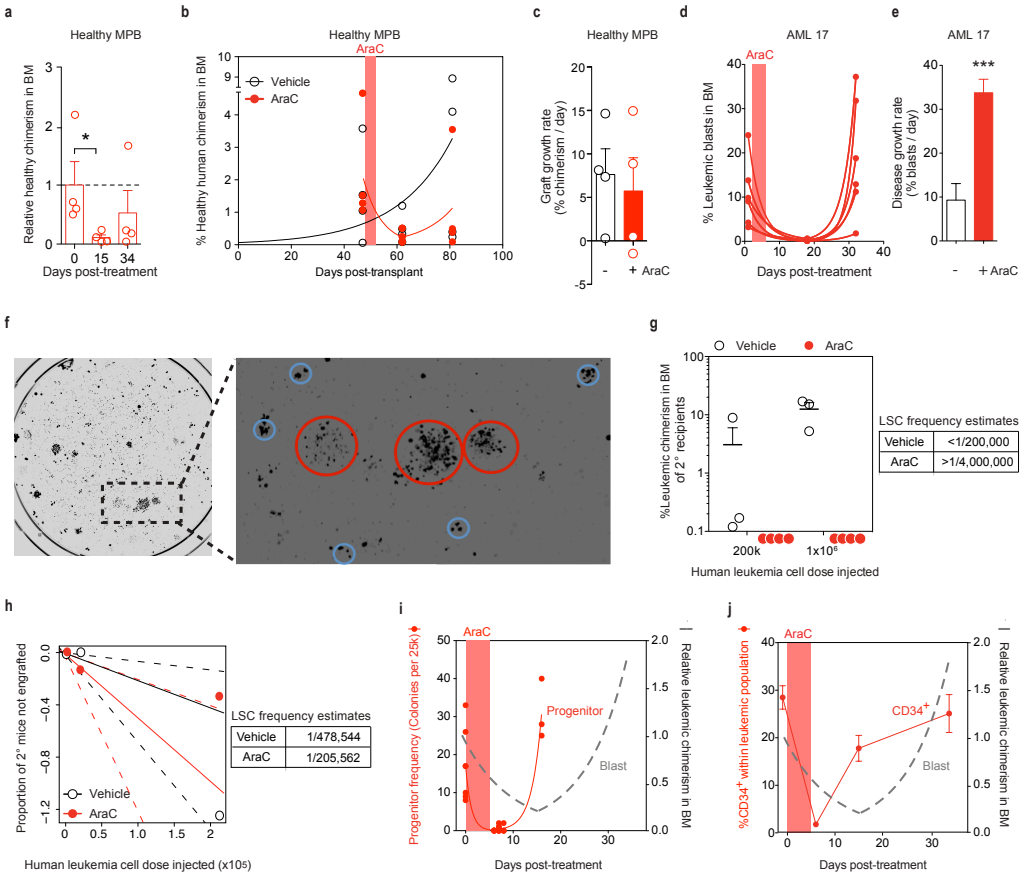
Extended Data Figure 1. Characterization of primitive versus mature transcriptional states of AML at diagnosis and recurrence. a, PCA correlation plot of paired diagnosis-recurrence AML patient samples presented in Fig. 1b-c, merged with diagnostic AML patient samples reported in Bullinger et al (Bullinger et al., 2004) (GSE425). Data point colors represent gene expression states as defined in Fig. 1b-c, or group membership defined in Bullinger et al (Bullinger et al., 2004), which were described to have primitive (Group I) or mature (Group II) characteristics. **b,** Unsupervised hierarchical clustering analysis of patient AML samples and healthy MNCs presented in Fig. 1b-c, in addition to non-hematopoietic human pluripotent stem cells of both embryonic origins (ESCs; *n*=3) and adult somatic cell-derived induced pluripotent stem cells (iPSCs; *n*=9). AML group membership matches that shown in Fig. 1b-c.

Extended Data Figure 2



Extended Data Figure 2. Determination of the maximum tolerable dose of AraC *in vivo*. a, Summary of treatment groups receiving AraC at 50 and 100 mg/kg/day. Control-treated mice received saline. *In vivo* AraC regimens consisted of daily subcutaneous injections for 5 consecutive days. **b,** Kaplan-Meier analysis showing survival of non-transplanted NOD/SCID mice in response to AraC treatment. **c,** Longitudinal monitoring of weekly mouse body weights following 5-day AraC treatment regimens. AraC at 50 mg/kg did not show deleterious effects on survival or body weights and was therefore selected for subsequent *in vivo* experiments. **d,** Left, viable BM cell counts of AraC-treated mice (50 mg/kg), presented relative to saline controls on Day 7 post-initiation of AraC treatment, as a measure of BM cellularity. Each dot represents an individual mouse, across two independent experiments. ***P<0.001, by unpaired t-test. Right, representative hematoxylin and eosin-stained BM sections of AraC- versus saline control-treated mice at Day 7 post-initiation of AraC treatment at 50 mg/kg. **e,** Recovery of BM viable cell counts from AraC-treated mice at Day 14 post-initiation of AraC treatment at 50 mg/kg. **f,** White blood cell (WBC) counts as a functional measure of BM output over time following AraC treatment at 50 mg/kg. ***P<0.001 by two-way ANOVA. All data are expressed as mean ± s.e.m. Red bars indicate the timing and duration of AraC administration.

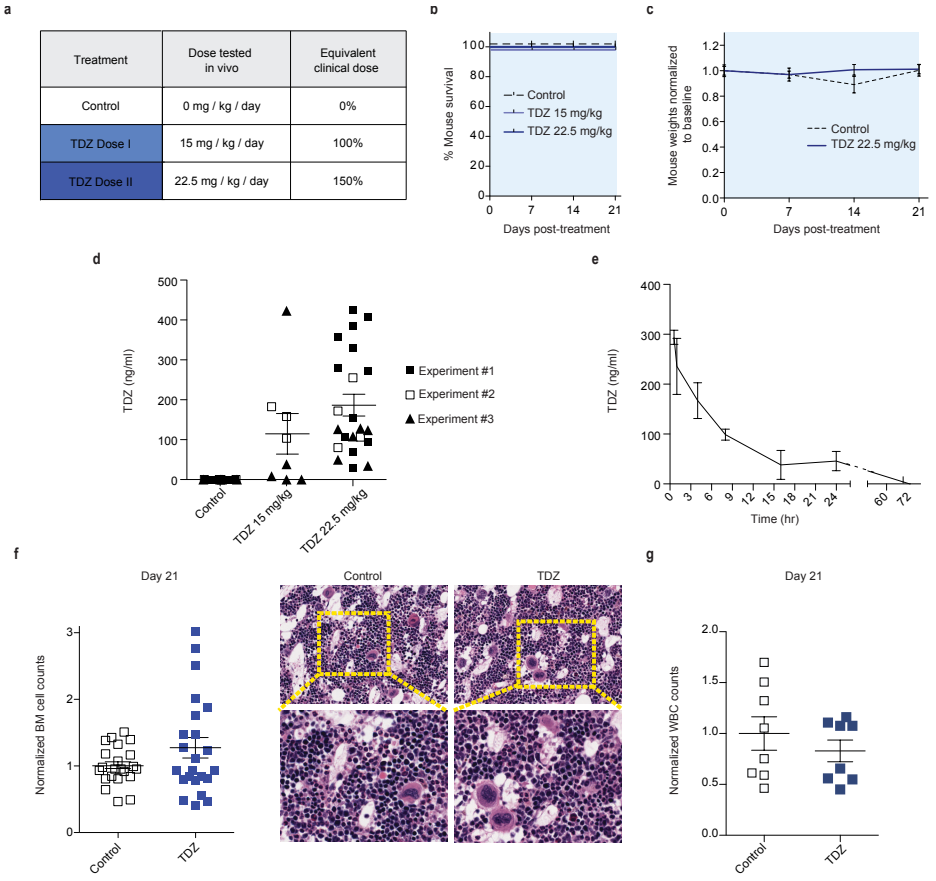
Extended Data Figure 3



Extended Data Figure 3. Phenotypic and functional AraC response dynamics in AML-xenografts. a, Longitudinal analyses of healthy human chimerism in MPB-xenografts following *in vivo* AraC treatment, normalized to pre-treatment levels. **P*<0.05, by one-way ANOVA. **b,** Growth curves of healthy MPB-xenografts, corresponding with data shown in **a**. Data points represent individual mice over time, and curves represent group averages. **c,** Growth rates of healthy hematopoietic xenografted cells after exposure to AraC, calculated for individual mice. **d,** Recurrence response dynamics following AraC treatment in AML 17-xenografts. Data represent flow cytometry measurements of human leukemic chimerism from serial BM aspirates and each curve represents an individual mouse. **e,** Leukemic growth rates of AraC-exposed AML 17-xenografts, as calculated for individual mice based on growth curves presented in **d**. ****P*<0.001, by unpaired t-test. **f,** A representative whole well image shows contrast-inverted calcein fluorescence of colonies arising from single progenitor

cells. The higher magnification image provides an example of our criteria used to score bona fide colonies for all experiments, based on a minimum requirement of 40 cells (Moore and Metcalf, 1973). Red circles indicate colonies that were counted, while blue circles indicate clusters that were not counted. **g,** LSC frequency estimates in human leukemic populations from AML 16-xenografts show a dramatic reduction in AraC- versus saline-treated mice at Day 7 post initiation of AraC treatment *in vivo*. **h,** LSC frequency estimates in human leukemic populations from AML 18-xenografts, calculated by limiting dilution(Hu and Smyth, 2009). By Day 14 after the initiation of AraC treatment *in vivo*, LSC frequencies exceed those of saline-treated controls. **i,** Longitudinal analysis of progenitor frequencies within human leukemic xenografts (AML 16) in response to AraC treatment *in vivo*. Grey dotted lines indicate total leukemic chimerism in BM relative to pre-treatment levels. **j,** CD34 expression dynamics in human leukemic xenografts (AML 16) in response to *in vivo* AraC treatment over time. Grey dotted lines indicate total leukemic chimerism in BM relative to pre-treatment levels. All summarized data are expressed as mean ± s.e.m.

Extended Data Figure 4



Extended Data Figure 4. Determination of a clinically relevant dose of TDZ *in vivo*. a, Summary of TDZ treatment groups. Non-transplanted NOD/SCID mice were administered TDZ at 15 or 22.5 mg/kg/day. Vehicle control-treated mice received 30% captisol. 15 mg/kg and 22.5 mg/kg TDZ doses represent weight-adjusted equivalents of 100% and 150% maximum clinical TDZ dose in humans (800mg/day). The *in vivo* TDZ regimen consisted of daily intraperitoneal injections for 21 consecutive days. **b,** Mouse survival during the 21-day TDZ treatment period, with no adverse effects at any doses tested. **c,** Weekly weight monitoring of recipient mice treated with TDZ *in vivo*. **d,** *In vivo* treatment with TDZ at 22.5 mg/kg achieves plasma TDZ levels that reach a clinically relevant range of plasma TDZ in humans at steady states (200-2000 ng/mL(Baumann et al., 2004)). Therefore, 22.5 mg/kg TDZ was selected for subsequent *in vivo* analyses. **e,** HPLC measurements of TDZ in murine plasma between 0-24 hr after TDZ administration at 22.5 mg/kg. Plasma TDZ levels peaked at 1-hour post

injection and declined to undetectable levels at 24 hours. **f,** BM cellularity as demonstrated by BM viable cell counts (left) and representative hematoxylin and eosin-stained BM sections (right) at Day 21 of *in vivo* TDZ treatment at 22.5 mg/kg. Each dot represents an individual mouse and the data are collected from 5 separate experiments. **g,** WBC counts at Day 21 of daily *in vivo* TDZ treatment at 22.5 mg/kg. All summarized data are represented as mean ± s.e.m. Blue shading indicates the timing and duration of TDZ delivery.

Extended Data Table 1. List of diagnostic AML samples evaluated for in vivo LSC content

Unique Patient ID (Experimental ID)	CG / Molecular	Tissue source	Leukemic engraftment	Number of mice tested
A683	NORMAL/NORMAL	PB	Yes	7
A037	NORMAL/NPM1 & FLT3D835	PB	Yes	4
A056	NORMAL/NPM1	PB	Yes	3
A787	45,X,-Y,t(9;11)(p22;q23)[23]/46,XY[2]	BM	Yes	4
A179 (AML14)	NORMAL/ NPM1, FLT3-ITD heterozygous	BM	Yes	8
A174	NA/NA/NO MLL BY FISH	BM	Yes	6
A040 (AML19)	NORMAL/FLT3 ITD	BM	Yes	6
A963	46,XX,del(7)(q11.2Q32)[3]/46, XX[22]	BM	Yes	2
A000 (AML18)	NORMAL/FLT3-ITD	BM	Yes	6
A256 (AML26)	48,XY,+8,+13[8]/46,XY[5]	PB	Yes	6
A939	NA	PB	Yes	5
A406 (AML24)	NORMAL/NA	PB	Yes	3
A289	NORMAL/NA	PB	Yes	15
A481	NORMAL/FLT3ITD	BM	Yes	6
A158 (AML17)	del(5) (q22q33), -7	PB	Yes	6
A150	NORMAL/CBFß/MYH11	BM	Yes	8
A566	NORMAL/FLT3 ITD	BM	No	6
A145	NA	PB	No	2
A947	47,XX,der(3)t(1;3)(q12;q29),+8[5]/46,XX[1]	BM	No	6
A472	NORMAL/NA	PB	No	2
A897	NORMAL/NO MLL BY FISH/NA	BM	No	7
A864	46,XY,del(4)(p15),-22[2]/NPM1/NO MLL	BM	No	13
A3051	NORMAL/ NPM1, FLT3-ITD heterozygous	PB	No	6
A534 (AML12)	NORMAL/NPM1 & FLT3-ITD	PB	No	2
A489	NORMAL/NPM1, FLT3-ITD	PB	No	5
A170	NA	PB	No	10
A526	46,XY, inv(16)(p13.1;q22)/CBFβ-MYH11	PB	No	4
A224	46,XY,t(10;11)(p15;q23)[18]/MLL present by FISH	BM	No	2
A355	NA	PB	No	4
A550	NORMAL/NA	BM	No	3
A250	47,XX,+8[20]/FLT3-ITD	PB	No	6
A760	46,XY,t(9;11)(p22;q23)[9]/MLL PRESENT/NORMAL	PB	No	6
A151	46,XY,inv(16)(p13.1q22)[20]/47,sl,+8[5]	PB	Yes	8
A157	NORMAL/NA	BM	Yes	5
A254	NORMAL/NA	PB	Yes	8
A295	47,XY,+i(21)(q10)[25]/46,XY[1]	PB	Yes	7
A302	NORMAL/NA	PB	Yes	7
A002	NORMAL/NA	NA	No	3
A009	46,XY,del(5)(q31)[3]/46,XY[21]	BM	No	2
A038	NA	NA	No	3
A051	NA	PB	No	3
A088	NA	NA	No	5
A238	NA	PB	No	5

Extended Data Table 2. Clinical details of AML patient samples

Patient ID	Disease stage	Tissue source	CG / Molecular	%Blast Diagnosis	%Blast Relapse	Source
1	Diagnosis / Relapse Pair	PB*	Normal / NA	83	57	GSE66525
2	Diagnosis / Relapse Pair	PB	Normal / NA	13	56	GSE66525
3	Diagnosis / Relapse Pair	PB	Normal / NA	85	81	GSE66525
4	Diagnosis / Relapse Pair	PB	Normal / NA	83	83	GSE66525
5	Diagnosis / Relapse Pair	PB	Normal / NA	47	16	GSE66525
6	Diagnosis / Relapse Pair	PB	Normal / NA	46	77	GSE66525
7	Diagnosis / Relapse Pair	PB	Normal / NA	93	79	GSE66525
8	Diagnosis / Relapse Pair	PB	Normal / NA	75	98	GSE66525
9	Diagnosis / Relapse Pair	PB	Normal / NA	66	90	GSE66525
10	Diagnosis / Relapse Pair	PB	Normal / NA	70	67	GSE66525
11	Diagnosis / Relapse Pair	PB	Normal / NA	46	87	GSE66525
12	Diagnosis / Relapse Pair	PB	Normal / NPM1, FLT3-ITD	58	63	Cryopreserved cells
13	Diagnosis / Relapse Pair	BM** / PB	Add1,-3, del3 (q21), del5 (q13q33), -7, -10, add11, del12 (p11.2p13), add13, add16, t(7;17)(p13;p13), 18, +21	50	40	Cryopreserved cells
14	Diagnosis / Relapse Pair	BM / PB	Normal / NPM1, FLT3-ITD heterozygous	>70	94	Cryopreserved cells
15	Diagnosis / Relapse Pair	BM / PB	NA / None detected	78	76	Cryopreserved cells
16	Progressed from MDS	PB	inv(3)(q21q26.2), -7	47		Cryopreserved cells
17	Diagnosis	PB	del(5) (q22q33), -7	89		Cryopreserved cells
18	Diagnosis	PB	Normal / FLT3-ITD	68		Cryopreserved cells
19	Multiple over time	BM	Normal / FLT3-ITD	Fig 2A	Fig 2A	Cryopreserved cells
20	Multiple over time	BM	Normal / NA	Fig 2B	Fig 2B	Cryopreserved cells
21	Diagnosis / post-induction Pair	BM / BM	Normal / NA	>50	>80	Clinical records
22	Diagnosis / post-induction Pair	PB	46-47,XX,del(5)(q13q33),del(13)(q12q14),+21,+22[cp26]	54	84	Clinical records
23	Diagnosis	BM	Normal / None detected	30		Cryopreserved cells
24	Diagnosis	PB	NA / None detected	68		Cryopreserved cells
25	Diagnosis	PB	NA / NA	92		Cryopreserved cells
26	Progressed from MDS	PB	48,XY,+8,+13[8]/46,XY[5]	35		Cryopreserved cells

* PB, peripheral blood

** BM, bone marrow

#NA, not available

Extended Data Table 3. List of druggable upregulated genes post-AraC

Gene Symbol	Full Gene Name	Fold change	p value
PLG	Plasminogen	1.59148	0.0015988
C6orf205	Mucin 21, Cell Surface Associated	1.45517	0.00904822
SLC2A2	solute carrier family 2 (facilitated glucose transporter), membe	1.43914	0.00358156
GPR119	G protein-coupled receptor 119	1.40593	0.0047744
S100A4	S100 calcium binding protein A4	1.36532	0.00567333
KCNJ10	potassium inwardly-rectifying channel, subfamily J, member 10	1.34991	0.0134538
KCNA10	potassium voltage-gated channel, shaker-related subfamily, m	1.32671	0.0123655
LEP	leptin	1.32108	0.00545013
CMA1	chymase 1, mast cell	1.31673	0.0146796
NALCN	sodium leak channel, non selective	1.30772	5.39E-05
MC3R	melanocortin 3 receptor	1.28131	0.037429
DRD2	dopamine receptor D2	1.27927	0.0352181
TUBB2A	tubulin, beta 2A class IIa	1.27229	0.0197661
TPO	thyroid peroxidase	1.26242	0.0195282
GPX5	glutathione peroxidase 5	1.26015	0.0242464
UGT2B10	UDP glucuronosyltransferase 2 family, polypeptide B10	1.25841	0.0299276

CHAPTER 3

Dopamine receptor targeting with Thioridazine allows selective suppression of progenitor activity in human acute myeloid leukemia

Preface

This chapter is an original article in preparation for submission. The authors include:

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I designed the study, executed the experiments and wrote the manuscript together with Dr. Mick Bhatia. Dr. Benoit performed the CREB experiments and gene expression analysis. Dr. Boyd assisted in the processing of the trial samples and provided intellectual input. Dr. Shapovalova and Jennifer Reid performed the bioinformatics analysis. Deanna Porras performed the cytospin preparations as well as the imaging. Dr. Tanasijevic performed gene expression analysis. Ryan Mitchell revised the manuscript. Dr. Foley and Leber provided the primary hematopoietic samples.

Based on our observations is (**Chapter 2**), transcriptional profiling of regenerating human AML cells identified a set of druggable targets, of which we prioritized DRD2 due to previous expertise in the lab with a DRD antagonist TDZ (Sachlos et al. 2012). These two studies independently pointed to a unique role for DRDs in regulating critical features of human AML biology. However, the mechanism by which DRD targeting leads to suppression of leukemogenicity is not elucidated. The studies in this chapter demonstrate the signaling circuitry downstream of DRDs in the context of human AML, through which the function of leukemic progenitor cells can be selectively and effectively harnessed in an *in vitro, in vivo* and clinical setting with no harm to healthy hematopoiesis. In addition to AML, analysis of public gene expression data sets demonstrated an elevated pattern of DRD expression in the neoplastic cells of other tumor types, suggesting that the therapeutic benefits of DRD targeting may apply to a wider range of cancer types.

Dopamine receptor targeting with Thioridazine allows selective suppression of progenitor activity in human acute myeloid leukemia

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Running head: DRD-targeting inhibits leukemic progenitors in AML

Keywords: Thioridazine, DRD, leukemic progenitor, Acute myeloid leukemia, cancer

Conflict of Interest: The authors of this paper declare no conflict of interest.

Key points:

- DRD-targeting by TDZ led to a suppression of leukemic progenitor activity *in vitro* and reduction of leukemogenesis *in vivo*.
- Clinical exposure to TDZ diminished leukemic progenitor function and alleviated the peripheral burden in a number of patients.
- Inter-patient variation in blast level changes in response to TDZ was associated with patient-specific DRD expression level.
- Preferential expression of DRDs in leukemic versus healthy progenitors offers a cancer-selective targeting approach.

ABSTRACT

Thioridazine (TDZ) has recently been identified to selectively target transformed cells including neoplastic cells of acute myeloid leukemia (AML). However, the precise nature of TDZ's effects on transformed hematopoiesis, including the target and mechanism of action are not fully understood. Systematic comparisons of leukemic cells side-by-side healthy human hematopoietic cells revealed that TDZ-induced preferential suppression of human AML occurs through dopamine receptors (DRDs) that are expressed in leukemic progenitor cells. Functional *in vitro* assays validated that TDZ suppresses leukemic progenitor activity in a diverse set of AML patient samples, independent of prognostic risk group. DRD2 antagonistm by TDZ, or DRD1 activation by immunotargeting, led to an elevation of cAMP and subsequent activation of CREB signaling, leading to a loss of clonogenic growth and induction of cellular maturation. *In vivo*, TDZ's selectivity for human AML was sustained in xenograft assays of primary cells, leading to reduction of disease burden with no adverse effects on healthy hematopoiesis. Clinical administration of TDZ in a Phase I clinical trial (NCT02096289) alleviated the peripheral leukemic burden in a number of relapsed/refractory AML patients. Inter-patient variation in blast level changes after exposure to TDZ was associated with DRD expression level. Collectively, our findings define a role for TDZ action via the DRD pathway in neoplastic hematopoiesis, and propose DRDs as attractive therapeutic targets for AML.

INTRODUCTION

Thioridazine (TDZ) has displayed efficacy in a wide range of human cancers including brain, breast, ovarian, colon and hematopoietic neoplasias (Byun et al., 2012; Cheng et al., 2015; Dolma et al., 2016; Huang et al., 2015; Ke et al., 2014; Rho et al., 2011; Sachlos et al., 2012; Zhang et al., 2016), representing candidacy as an anti-cancer drug. TDZ was previously regarded as an antipsychotic drug whose properties were mediated through dopamine receptors (DRD)(Seeman and Lee, 1975). DRDs are G protein-coupled receptors (GPCRs) and have been sub-categorized into D1- (DRD1 and DRD5) and D2-class (DRD2-DRD4) families based on structural distinctions and opposing effects on downstream cyclic AMP (cAMP)(Beaulieu and Gainetdinov, 2011). Predominantly studied in the context of the nervous system, DRDs are associated with mechanisms of learning, memory, locomotion, regulation of sympathetic tone, etc(Beaulieu and Gainetdinov, 2011). For TDZ's anti-neoplastic properties, various mechanisms of action have been described such as suppression of proliferation and invasion, induction of chemosensitivity and cell death (Cheng et al., 2015; Ke et al., 2014; Rho et al., 2011; Zhang et al., 2014; Zhang et al., 2016). However, it remains to be characterized if specific targets are uniquely associated with TDZ efficacy against various neoplasias in a tissue specific manner.

Recently, DRDs were detected in tumor cells of hepatic and breast origins (Borcherding et al., 2016; Lu et al., 2015) as well as neoplastic stem/progenitor cells of human acute myeloid leukemia (AML)(Sachlos et al., 2012). Notably,

these DRD-expressing cancer cells were reported to be responsive to TDZ treatment (Ke et al., 2014; Lu et al., 2015). Given the recurring presence of DRDs in multiple cancer cells, we sought to examine the involvement of DRDs in mediating neoplastic behaviour and whether their mode of action resembles that previously described in the nervous system. To formally interrogate the involvement of DRDs and other putative target receptors associated with TDZ, we used AML as a model system owing to available refined xenografting assays *in vivo* (Hope et al., 2004; Lapidot et al., 2004) that offer the closest available representation of patient heterogeneity (Damia and D'Incalci, 2009), and powerful *in vitro* assays of clonogenicity with established clinical relevance (Bonnet and Dick, 1997; Tehranchi et al., 2010). We demonstrate that the anti-leukemic effects of TDZ are reproduced by direct antibody-mediated targeting of DRD whose preferential expression in leukemic versus healthy hematopoietic progenitor cells afforded selective inhibition of leukemic clonogenicity.

METHODS

Primary Patient Samples and AML Cell Lines

Detailed clinical information on AML patient samples are outlined in Table1. Processing of primary hematopoietic samples mononuclear cells (MNCs) was carried out as previously described(Sachlos et al., 2012). Lineage depletion (Lin-) of CB was performed by magnetic cell separation using a lineage antibody

cocktail (#19309C, Stemcell Technologies). Leukemic cell lines OCI-AML3 and NB4 were purchased from DSMZ.

Fluorescence-activated Cell Sorting and Flow Cytometric Analysis

Cell surface immunopheontyping was carried out using V450-CD45 (#642275, BD Biosciences), PE-CD34 (#555822, BD Pharmingen) and APC-CD33 (#551378, BD Biosciences), PE-CD15 (#IM1954U, Beckman Coulter), Rabbit anti-human DRD1 antibody (#324390, EMD Millipore) and Rabbit anti-human DRD2 antibody (clone B-10, sc-5303, Santa Cruz). Anti-rabbit or mouse Alexa-Fluor-488 or 647 (Life Technologies) were used as the secondary antibody. For indirect staining, samples were blocked with 5-10% donkey serum (Jackson ImmunoResearch Laboratories) and human FC block (eBioscience). When appropriate, fluorescence minus one (FMO) controls were used for optimized gating strategy. 7AAD viability dye was used for live/dead cell discrimination (#A07704, Beckman coulter).

In Vitro Cell Culture

AML and CB samples were cultured in Stem Span (#9650, Stemcell Technologies), supplemented with 100 ng/ml stem cell factor, 100 ng/ml Flt-3 ligand and 20 ng/ml thrombopoietin (R&D systems). Patient HSPC and AML samples were treated with Thioridazine (#T-9025, Sigma) or 0.1% DMSO-vehicle for 24 hr prior to plating for a Colony Forming Unit (CFU) assay.

Methylcellulose Colony Forming Unit Assay and CFU Imaging

De novo AML patient MNCs at 25000-50000 cells and CB Lin- at 500-1000 cells were plated in semisolid methylcellulose media (#H4434; Stemcell Technologies) following established protocols (Salci et al., 2015). Human leukemic xenografts were recovered from the recipient mice and tested for re-plating capacity in methocult(Dick, 2008; Langenkamp et al., 2009; Somervaille and Cleary, 2006). For whole-well CFU analysis, images were acquired at 2x using Operetta High Content Screening (Perkin Elmer) by means of epi-fluorescence illumination and standard filter sets. Whole-well images were stitched in Columbus software (Perkin Elmer).

Murine Recipients and Xenograft Assays

NOD/Prkdc^{scid} and NSG mice (NOD/SCID IL2RG$^{-/-}$) were used as recipients using previously described protocols(Boyd et al., 2014). For transplantation assays, 6-10 week old immunodeficient mice were sublethally irradiated (315-350

rads) 24 hours prior to intravenous transplantation of primary human samples (Bonnet and Dick, 1997; Lapidot et al., 1994).

For *in vivo* treatments with TDZ, a daily dose of 22.5 mg/ kg/ day was administered intraperitoneally for 21 days. 30% captisol (Captisol technology) was administered in vehicle control-treated recipients.

RNA Purification, PCR and Affymetrix

Sorted CD34+ fractions of primary AML or CB Lin- samples were collected for RNA extraction. Total RNA purification was performed using RNeasy^{TM} Mini Kit (Qiagen), including DNase I on-column digestion step, following manufacturer's instructions. Purified RNA was quantified on a Nanodrop 2000 Spectrophotometer (Thermo Scientific), and cDNA was synthesized from 200 ng of total RNA using iScript^{TM} cDNA Synthesis Kit (BioRad). RT-PCR was performed using Recombinant Taq DNA Polymerase (Thermo Scientific). Random-primed Human Reference cDNA (Clontech) was used as a putative positive control. For DR2 amplification specifically, we performed a Touchdown PCR starting from 65C, with -Δ0.2C, for 40 cycles. Quantitative PCR were performed and analyzed as previously described (Mitchell et al., 2014; Pfaffl, 2001). List of primers is provided in Table 2.

In xenograft assays, RNA was isolated from purified human myeloid cells using a total RNA purification kit (Norgen biotek, ON, Canada) according to the manufacturer's instructions. RNA integrity was analyzed by 2100 Bioanalyzer

(Agilent Technologies) and hybridized to Affymetrix Gene Chip Human Gene 2.0 ST arrays (London Regional Genomics Centre, ON, Canada). Output data was normalized using the Robust Multichip Averaging (RMA) algorithm with Partek Genomics Suite 6.6 software (Partek Inc). Gene expression data for ovarian (GSE14407), lymphoma (GSE31312), brain cancers (GSE37418) were acquired from publically available datasets. Gene expression data of MB SHH (GSE37418 and GSE70576), healthy blood (GSE28359), and AML (GSE12326) were obtained from publically accessible data sets. All gene expression data sets were normalized using the RMA algorithm independently and data was merged altogether using shared gene symbol annotation. Batch correction was performed on sources of technical variation (3 array technologies used), which was also mitigated by utilizing the same array technology. Pearson's correlation coefficient was used for hierarchical clustering to generate dendrograms. Gene set enrichment analysis (GSEA) was carried out on normalized expression values of gene symbols using GSEA software v2.1.0 (Broad Institute; Subramanian et al., 2005). Curated gene set (C2) and Gene Ontology (GO, C5) gene set collections from Molecular Signatures Database (MSigDB) were used for GSEA analyses.

Cyclic AMP Measurement Assay

Primary samples and AML cell lines were treated with DRD antibodies or small molecules including (±)-SKF-38393 hydrochloride (#D047), R(+)-SCH-23390 hydrochloride (#D054), Thioridazine (#T-9025) (all sourced from Sigma) or Forskolin (#ab120058, Abcam) for 30 minutes in PBS, followed by wash and lysis in HCl 1N. The supernatant containing cAMP was collected and applied to cAMP direct immunoassay kit (#116811, Millipore/Calbiochem) as per the manufacturer's instructions. For control treatment, normal rabbit IgG (Sc-2027, Santa Cruz) and rabbit serum (869019, Millipore) were used interchangeably and produced comparable outcomes.

Western Blot Analysis

Proteins were extracted in SDS Laemmli buffer, separated by SDS-PAGE, and transferred onto nitrocellulose membranes as previously described (Benoit et al., 2009). Membranes were blocked in PBS containing 5% skim milk and 0.1% TWEEN 20 (BioRad). Rabbit polyclonal anti-phospho-CREB (S133) (CS204400, Millipore), anti-G9a (33065, Cell Signaling) and anti-total Histone H3 (ab1791, Abcam), as well as mouse monoclonal anti-CREB (9104, Cell Signaling Technology, MA, USA), anti-H3K9me2 (ab1220, Abcam) and anti-GAPDH (ab8245, Abcam) primary antibodies were used at 1:2000, 1:1000, 1:100000, 1:500, 1:2000 and 1:80000 dilutions respectively. Blot images were acquired using Chemidoc XRS system (Bio-Rad). For quantitative optical densitometry

analysis, bands intensities were assessed using Image J software (National Institutes of Health).

Measurement and Analysis of Dopamine in Human Plasma

Plasma was collected from the aqueous phase obtained during Ficoll separation of cells from human blood treated with anticoagulant and stored at -80^{o}C. The oxidation status of the plasma was stabilized with 20 mL per 1mL of a solution containing ethylene glycol-bis (2-amino ethylether)-N,N,N',N'-tetraacetic acid (0.2M) and glutathione (0.2M) at pH=7.5. The internal standard (3,4-Dihydroxybenzylamine) was added for further processing using solid phase extraction cartridges as per the manufacturer's recommendations (ChromSystems, Grafelfing, Germany). The samples were eluted into 120 mL and injected in triplicate within 24hr in a High Performance Liquid Chromatographic System (HPLC, Waters 2695) coupled to an Electro-Chemical Detector (Waters 2465). The HPLC system used an analytical reverse phase column (Atlantis dC18; 5 mm; 4.6x150mm; Waters) and an organic mobile phase (ChromSystems). The concentration of dopamine was calculated based on the area under the curve of the chromatograms with respect to the standards.

Statistics

Significant differences between groups were determined via unpaired two-tailed Student's t-test, two-way analysis of variance (ANOVA) or linear regressions.

Prism (version 5.0a; GraphPad) software was used for all statistical analyses, and the criterion for statistical significance was $p \leq 0.05$. In Figure S1B, a data point corresponding to one mouse within the TDZ treated group (n=6) was considered a significant outlier by Grubb's test ($p < 0.01$ two-sided, Z=2.038) and was removed.

RESULTS

TDZ selectively suppresses leukemic progenitor capacity

Ex vivo exposure to TDZ has been shown to selectively inhibit leukemia initiation *in vivo*(Sachlos et al., 2012). However, TDZ's efficacy against human AML has not been evaluated under more rigorous settings of established leukemic disease. To test this, we simulated the clinical scenario by establishing human leukemia *in vivo*, using 5 distinct AML patient samples, prior to treatment with TDZ (Table 1, AMLs 1-5) (Figure 1A, S1A). To ensure that the response is elicited exclusively from leukemia cells and not healthy hematopoietic cells within the clinical samples, we only selected samples from highly leukemia-infiltrated patients as determined by elevated blast levels (Figure S1B, Table 1). These samples were further screened *in vivo* to ensure that a strictly myeloid graft was generated (>99% hCD45+CD33+) (Figure S1C). This property has been associated with the absence of healthy hematopoietic stem/progenitor cells within patient samples (Eppert et al., 2011; Kennedy et al., 2013). Additionally, selected patient samples generated colonies that lacked the composition diversity noted with healthy donor

or remission samples, consistent with the over representation of the leukemic sub-population (Figure S1D).

Prior to the administration of TDZ *in vivo*, BM aspirate sampling was carried out to validate robust levels of human leukemia in recipient mice (Figure S1A). At this stage, xenografted recipients were assigned to treatment groups that harboured comparable levels of human chimerism at baseline and subsequently received TDZ (22.5mg/kg) or vehicle control (30% Captisol) for 21 days. TDZ dosing was optimized to achieve *in vivo* TDZ plasma levels, similar to that reported in humans receiving TDZ as an antipsychotic drug (Nagel et al., 2012) On day 21, leukemic xenografts were isolated from the BM of recipient mice and comparatively examined by functional, transcriptional and phenotypic profiling (Figure 1A-C, S1A).

TDZ treatment *in vivo* led to a 43% reduction of leukemic chimerism after 21 days (Figure 1A). Notably, in 4 of 5 xenografts that expressed the primitive cell bio-marker CD34(Bhatia et al., 1997), response to TDZ correlated with the CD34 content, suggestive of primitive leukemic cell targeting by TDZ (Figure S1E). This was functionally validated by *in vitro* analysis of leukemic progenitor cells within the TDZ-treated xenografts (Dick, 2008; Dolma et al., 2016; Langenkamp et al., 2009; Somervaille and Cleary, 2006), which revealed a substantial reduction of leukemic progenitor function by TDZ, ranging from 38% - 93.7% in 3 of 4 samples that harboured colony forming capacity (Figure 1B). AML5 was devoid of clonogenic potential at up to 150,000 cells interrogated and

therefore was not included in this analysis. Consistent with the functional suppression of human AML after exposure to TDZ *in vivo* (Figure 1A-B), transcriptional profiles reinforced the down regulation of hallmark AML transcriptional networks in TDZ- versus vehicle control-treated xenografts (Figure 1C).

Contrary to its potent effect on human AML, TDZ did not compromise healthy hematopoietic reconstitution or the clonogenic capacity of xenografted healthy progenitor cells after a 21-day treatment *in vivo* (Figure 1D-E). Furthermore, inspection of resident murine hematopoiesis uncovered that TDZ did not adversely affect white blood cell counts (Figure S2A), hematocrit levels (Figure S2B) or the primitive hematopoietic fraction marked by Lin- Sca1+ Kit+ (Figure S2C-D). These findings collectively indicate that TDZ at clinically relevant doses (Nagel et al., 2012) does not compromise transplanted or naïve hematopoiesis, yet it was capable of selective inhibition of leukemic primitive cells.

TDZ inhibits leukemic clonogenicity through induction of cellular maturation

In vivo delivery of TDZ demonstrated a selective suppression of leukemic progenitor function (Figure 1B). This observation was further validated *in vitro* with an expanded cohort of clinically diverse AML patient samples that were pre-screened to represent leukemia cell populations (n=8, Table 1) (Figure 1F)(Figure

S1B-D). These rigorous criteria were collectively applied to maximize the confidence in the leukemic nature of AML patient-derived colonies.

Primary AML samples were exposed to an *in vitro* dose of TDZ, optimized by functional sparing of healthy hematopoietic cells(Sachlos et al., 2012) (Figure S2E), which led to a significant abrogation of leukemic clonogenicity in 6 of 8 patient samples by ≥50% (Figure 1F). Notably, AMLs 1 and 2 that were also tested *in vivo*, displayed a strikingly similar response pattern to TDZ, regardless of the assay system used. This recapitulation of TDZ's activity on a patient-specific level suggested that the *in vitro* progenitor assay provides a reliable platform to characterize the effects of TDZ against human AML as observed *in vivo* (Figure 1B versus 1F). In addition to a reduction of the number of leukemic progenitor cells, TDZ also suppressed the proliferative capacity of progenitor cells, selectively in AML patient-derived colonies. This was measured by quantifying the number of the progeny contained within individual colonies (Figure S2F-H). These findings indicate that TDZ effectively and selectively compromises leukemic progenitor output.

Simultaneous with the suppression of leukemic primitive cell function, TDZ exposure also led to an acquisition of morphological features associated with cellular maturation (Figure 1G), as well as upregulation of a mature surface marker CD15 (Figure 1H) (Jin et al., 2006; Pabst et al., 2014). Notably, acquisition of CD15 expression was not observed in AML2, in line with its lack of functional response to TDZ *in vitro* or *in vivo* (Figure 1H vs. 1B, 1F).

Consistent with the acquisition of differentiated cell phenotypes, TDZ-exposed leukemic progenitors displayed a limited re-plating capacity, further supporting a loss of primitive cell function towards enhanced maturation (Figure 1I). These findings suggest that the TDZ-induced suppression of leukemic clonogenicity occurs through induction of cellular maturation and a loss of primitive cell features.

Dopamine receptors are preferentially expressed in leukemic primitive cells

TDZ has been historically described to function through DRDs for its antipsychotic properties(Seeman and Lee, 1975). Indeed, we found that TDZ response *in vivo* was correlated with the expression of DRD2 within the xenografts (Figure S3A). Nevertheless, numerous antipsychotic drugs exhibit context specific functions as well as target promiscuity(Mestres et al., 2008) and TDZ is likely no exception. Therefore, to systematically identify the target receptor(s) involved in TDZ's anti-leukemic properties, we consulted drug-protein interaction networks (STICH 4.0)(Kuhn et al., 2014) to identify receptors interacting with TDZ. This analysis revealed 19 putative targets that are predominantly associated with neural cell functions including dopaminergic, adrenergic, cholinergic, serotonin and histamine receptor families (Figure 2A). To refine our selection, we applied a filter to only capture the receptors that are expressed at the protein level within the hematopoietic context according to the Human Protein Atlas database (HPAD) (Uhlen et al., 2005) (Figure 2B). For

HTR6, ADRA1B and ADRA2A, protein expression profiles were unavailable and we instead queried HPAD for transcript level data (Figure 2B). From the dopamine receptor family, we selected DRD1 and DRD2 as representative members of D-1 and D-2 sub-families for future analyses, due to their enhanced binding affinity for TDZ compared to the other DRD members(Ekins et al., 2005). These filters refined our list to 8 receptors that were expressed within hematopoietic MNCs (Figure 2B). As TDZ was found to target the primitive cell fraction, we next examined the expression of these 8 candidate receptors within the CD34+ fraction, enriched for hematopoietic progenitor cells(Bhatia et al., 1997) (Figure 2C). DRDs were the only target receptors that were consistently detected within primitive leukemic populations (Figure 2C). These findings suggest that DRDs may be mediating TDZ's action in the hematopoietic context. Also, the preferential expression pattern of DRDs in leukemic versus healthy progenitors may explain TDZ's selectivity for AML.

TDZ-induced selective suppression of leukemic progenitor cells is mediated by dopamine receptors

In a recent report, TDZ was theorized to prohibit neoplastic proliferation of breast and monocytic cancer cell lines through histamine H1 receptor (HRH1) antagonism(Tuynder et al., 2004). However, we found minimal to no HRH1 protein expression in the CD34+ subset of healthy or AML patient samples by immunostaining (Figure 2D-E). Furthermore, functional HRH1 targeting with two

potent and selective HRH1 antagonists at concentrations reported to antagonize HRH1 in hematopoietic cells (Zappia et al., 2015) did not compromise leukemic clonogenicity in AMLs 6, 8 and 9 that had responded to TDZ (Figure 2F versus 1F), indicating that HRH1 is not likely mediating the response to TDZ in these samples. In contrast to HRH1, DRD protein was detected in the CD34+ subset of several AML patient samples (N=10, Figure 2G-H) and displayed a preferential expression pattern when compared to healthy CD34+ cells obtained from diverse tissues and developmental origins including CB, adult mobilized peripheral blood (MPB) and BM (Figure 2G-H, S3B-C).

Additionally, a recent study of medulloblastoma (MB) cells described an inhibitory effect for TDZ against the potassium channel EAG2 and its downstream partner KCNT2 (Huang et al., 2015). Comparison of transcription profiles revealed a sharp contrast in the expression intensities of these two genes between MB and AML patient samples, suggesting that the EAG2-mediated response to TDZ may be restricted to MB (Figure S3D). In contrast to potassium channels, DRDs displayed a clearly elevated expression pattern in human AML, approximating expression levels observed in neural cells for DRD1, 2 and 4. Additionally, this independent dataset corroborated our findings that DRDs are minimally expressed in healthy hematopoietic primitive subsets (Figure S3C-D).

Functional involvement of DRDs in regulating leukemic clonogenicity was validated with the clinically prescribed drug Domperidone(Reddymasu et al., 2007), which we employed as an alternative DRD antagonist in addition to TDZ

(Figure 2I). Based on these indications regarding the involvement of DRDs in regulating leukemic progenitor activity, we next interrogated the role of dopamine (DA) as the natural ligand for DRDs(Beaulieu and Gainetdinov, 2011). Analysis of circulating DA levels revealed significantly higher levels of DA in the plasma of AML patients (n=11) compared to healthy control samples (n=19) (Figure 2J). Furthermore, exposure of AML patient samples to physiological levels of DA (10^{-8}-10^{-7} M)(Levite, 2012) *in vitro* led to augmentation of leukemic clonogenicity in contrast to the outcome of DRD antagonism by TDZ (Figure 2K-L). These collective lines of evidence suggest that DRDs are of functional relevance to human AML biology.

A functional DRD circuitry exists in human AML and mediates TDZ-induced suppression of leukemic clonogenicity

Having established the functional relevance of DRDs in regulating neoplastic hematopoiesis, we then asked if DRD signaling in leukemic progenitors resembles that previously established in the neural context(Beaulieu and Gainetdinov, 2011), which is predominantly through adenylyl cyclase (AC)-mediated elevation of cAMP and subsequent activation of CREB (cAMP response element-binding protein)(Beaulieu and Gainetdinov, 2011). Due to its rapid and transient nature (Brown et al., 1977), we carried out assays of cAMP signaling *in vitro*. Initially, we tested the robustness of our cAMP tracing assay by exposing human AML cells to the widely used inducer of cAMP Forskolin (FSK), alone or in

combination with an inhibitor of AC, 2', 5'- dideoxyadenosine. cAMP was elevated in response to FSK and reverted to basal levels when exposed to 2', 5'-dideoxyadenosine (Figure S4A), validating that our assay reliably reflects cAMP nuances in response to established modulators. Using this assay system, TDZ exposure elevated cellular cAMP in AML1 as well as two AML cell lines OCI-AML3 and NB4 (Figure 3A). In addition to cAMP, simultaneous activation of CREB corroborated that the DRD-cAMP-CREB axis is functional in human AML cells (Figure 3B).

We further employed our cAMP tracing assay to resolve which member(s) of the DRD family is involved in the TDZ action. As previously described in the neuronal cell model, DRD1 is a positive regulator of AC, leading to cAMP generation; whereas activation of DRD2 negatively regulates AC, thereby preventing cAMP induction(Beaulieu and Gainetdinov, 2011). Using DRD antibodies, we separately targeted DRD1 and DRD2 (Figure S4B) and found that in contrast to TDZ, our screened DRD2-Ab led to cAMP level reduction (Figure 3C). However, DRD1-Ab showed a potent and dose dependent stimulation of cAMP levels (Figure 3D, S4C) and a subsequent phosphorylation of CREB (Figure 3E), indicative of DRD1 activation. The elevation of cAMP in response to DRD1-Ab was countered when DRD1-Ab was co-administered with DRD1 antagonist SCH23390, supporting that elevated cAMP was specific to DRD1 activation and not in response to a general cellular stress stimulus (Figure 3F). Collectively, these observations demonstrated that DRD1-Ab led to an elevation

of cAMP, similar to that observed with TDZ. Given that TDZ is a potent DRD2 antagonist (PDSP database)(Besnard et al., 2012), its observed induction of cAMP (Figure 3A) is likely due to DRD2 antagonism that relieves the DRD2-mediated repression of cAMP. Our findings suggest that the interplay between DRD1 activation or DRD2 suppression leads to elevation of cAMP, which may afford suppression of leukemic progenitor capacity.

Direct DRD1 targeting affords selective suppression of leukemic progenitors

As DRD1-Ab elevated cAMP levels, similar to TDZ, we next asked if DRD1-Ab also functionally reproduces TDZ's effect on AML. Exposure to DRD1-Ab *in vitro* led to a suppression of leukemic CFU output in a clinically diverse set of AML patient samples (n=7) (Table 1) (Figure 3G) without compromising healthy progenitor activity (n=3, Figure 3G inset). AML2 was consistently found refractory to DRD-targeted treatment with DRD1-Ab or TDZ *in vitro* and *in vivo* (Figure 3G, 1B, 1F, 1H). It is noteworthy that this sample expressed low levels of DRD (Figure 3G, S2B) and did not show a significant elevation of cAMP (Figure 3H), which may explain its lack of response to DRD-targeted approaches. Furthermore, while AML1 harboured a higher level of DRD1 relative to AML7 (Figure 3H), the induction of cAMP level for this sample was less dramatic than that noted for AML7 after exposure to DRD1-Ab (8 fold vs. 25 fold). This may suggest that response to DRD1-Ab cannot be exclusively explained by DRD1 frequencies, and nuances in the intensity of DRD signaling may also contribute to

the magnitude of response. Furthermore, the patient-specific pattern of progenitor suppression with DRD1-Ab was strikingly reminiscent to that observed with TDZ (Figure 3G versus 1F; Figure 3I), suggesting that both treatments may elicit an overlapping cellular response. In fact, combined *in vitro* treatment with TDZ and DRD1-Ab did not amount to a synergized effect on leukemic CFU ablation, further suggesting that both treatments may engage a shared signaling pathway (Figure 3J). In contrast to DRD1-Ab, DRD2-Ab did not abrogate leukemic progenitor activity (Figure S4D), in line with its inability to induce cAMP elevation (Figure 3C). To further validate the relevance of DRD-cAMP signaling in the context of leukemic clonogenicity, we exposed AML patient samples to DRD1-specific agonist (SKF38393), and FSK as a DRD-independent inducer of cAMP. Expectedly, both treatments led to a reduction of leukemic clonogenicity (Figure S4E-G). Conversely, DRD1 antagonism with SCH23390 reduced cAMP levels and lacked the suppressive effect on leukemic clonogenicity (Figures S4E-F). These findings collectively support that DRD1-mediated induction of cAMP leads to selective abrogation of leukemic clonogenicity, similar to that achieved with TDZ.

Based on the functional similarity between DRD1-Ab and TDZ *in vitro*, we next interrogated whether DRD1-Ab reproduced TDZ-induced suppression of xenografted human progenitor cells. However, it was not feasible to assess the efficacy of DRD1-Ab on established leukemic xenografts with a non-immunotherapy grade antibody due to the antibody clonality, risk of

immunogenicity in mice and lack of a customized Fc region(Chao et al., 2011; Gasiorowski et al., 2014; Jin et al., 2006; Jin et al., 2009; Kikushige et al., 2010). Therefore, we evaluated the efficacy of DRD1-Ab by *ex vivo* exposure of AML patient samples to the antibody followed by transplantation in recipient mice, as previously reported(Chao et al., 2011; Gasiorowski et al., 2014; Jin et al., 2006; Jin et al., 2009; Kikushige et al., 2010). The xenografts were isolated and evaluated for leukemic clonogenic potential, which demonstrated a suppression or elimination of leukemic clonogenicity in AML1 and AML9, respectively (Figure 3K). Reduction of progenitor output was not observed with AML7, which appeared unexpected given that this sample consistently responded to TDZ and DRD1-Ab *in vitro* (Figure 1F, 3G). However, compared to the de novo patient sample that was tested in the *in vitro* assays, we noticed a drastic drop in the DRD1 frequency in AML7-derived xenograft (Figure S4H). This is in contrast to AML1 that retained high levels of DRDs in the xenograft and responded more drastically to DRD1-Ab (Figure S4I). It is plausible that AML samples exhibit disparate levels of DRD dependence upon transplantation and therefore may vary in their magnitude of response to DRD-targeting. Collectively, these findings propose a novel role for DRDs in regulating the clonogenicity of transformed hematopoietic progenitor cells. Beyond AML, DRD over-expression has been reported in several neoplasias (Figure S5A), proposing a broader role for DRDs in mediating transformed cell behaviour.

TDZ as a monotherapy alleviates peripheral leukemic burden in a subset of relapsed/refractory AML patients in a DRD2-dependant manner

Our pre-clinical observations with TDZ motivated a phase I clinical trial (NCT02096289), evaluating the safety and efficacy of TDZ in combination with the chemotherapeutic agent AraC in older AML patients with relapsed/refractory disease. The trial was specifically designed to include a monotherapy phase with TDZ, which enabled us to evaluate the effect of TDZ on human AML biology in a clinical setting. During the monotherapy phase, TDZ was administered at 3 dose levels including 100, 200 and 400 mg/day for 5 days. Analysis of blast trends uncovered an alleviation of leukemic burden, predominantly in the peripheral blood of 8 out of 11 patients that completed the 5-day component (Figure 4A, Table 3). The 3 remaining patients showed progressive disease patterns, as determined by elevated peripheral blast levels. Notably, the inter-patient variation of blast levels after exposure to TDZ was associated with DRD2 levels in the CD34+ fraction (Figure 4A). Specifically, Patient 1, 3 and 8 displayed the lowest DRD2 expression levels in line with their lack of response to TDZ. Patients 4, 9 and 11 expressed an intermediate level of DRD2 and showed a 20% reduction of peripheral blast levels. Patients 2, 5, 6, 7 and 10 that experienced the most drastic reduction of peripheral blast (35-55%), displayed higher levels of DRD2 with the exception of Patient 2. This clinical level data is consistent with our experimental findings *in vivo* (Figure S3A), indicating that DRDs mediate TDZ response.

Moreover, patient-specific response patterns to TDZ were recapitulated when clinical trial samples acquired at baseline were subjected to TDZ followed by the *in vitro* CFU assay (Figure 4B). This was consistent with our observations *in vitro* that suggested leukemic progenitor activity is targeted by TDZ. To interrogate this further, we quantified the progenitor content pre- and post-clinical exposure to TDZ (day 1 versus 5) through limiting dilution assays (LDA). Among the 3 non-responsive patients, Patient 1 displayed a robust colony forming potential and was selected for progenitor assays (1 in 14.7 cells, versus >1 in 50000 cells for Patient 8, and 0 in 75000 cells for Patient 3). In line with its lack of clinical response to TDZ, Patient 1 showed no reduction of progenitor frequency between days 1 and 5 (Figure 4C, Table 3). In comparison, Patient 11 showed a 20% reduction of peripheral blast level between days 1 and 5 (Figure 4C, Table 3) and the progenitor content for this patient was diminished by over 2 folds after clinical administration of TDZ for 5 days (Figure 4C). The most drastic reduction of peripheral blast level was noted in patients 2, 5, 6, 7 and 10 (35-55%), of which Patients 6 and 10 were selected for functional analyses as they displayed robust colony formation (Table 3). In these patients, the higher extent of blast response was reflected in their significant and substantial ablation of progenitor content between days 1 and 5 (23 and 3.4 folds, respectively) (Figure 4C, S6A). Finally, while the observed clinical blast response could be resolved by progenitor dynamics for the majority of assayed patients, it did not unambiguously explain the response for Patients 4 and 9 (Figure S6A, Table 3).

Overall, these findings highlight the value of *in vitro* progenitor-level in recapitulating clinical outcomes, and provide preliminary data to support the clinical value of DRD-targeting approaches.

DISCUSSION

Our observations together with previous reports demonstrate that TDZ exhibits potent and cancer-selective effects against neoplasias of diverse tissue origins (Byun et al., 2012; Cheng et al., 2015; Huang et al., 2015; Ke et al., 2014; Rho et al., 2011; Sachlos et al., 2012), suggesting that similar pathways in various cancers may mediate the unified response to TDZ. Our combined experimental and clinical findings demonstrated that DRDs mediate TDZ's anti-leukemic capacity through downstream cAMP. The role of cAMP in induction of hematopoietic maturation has been previously reported (Brodsky et al., 1998; Tortora et al., 1989). In a previous study, a large-scale screen of chemical compounds led to the identification of 8 candidates capable of inducing functional myeloid differentiation, 5 of which were known inducers of cAMP (Stegmaier et al., 2004). However, other reports have described alternate signalling pathways for TDZ's anti-neoplastic mode of function such as calcium (Zhelev et al., 2004) and AKT signaling (Rho et al., 2011), suppression of NFkB target genes (Nagel et al., 2012) and regulation of voltage gated potassium channels (Einhorn et al., 1991; Perez et al., 2006). These seemingly contradictory modes of action may be explained by the versatile nature of DRDs to engage in a broad network of

signalling pathways (Beaulieu et al., 2011; Beaulieu and Gainetdinov, 2011; Del'guidice et al., 2011; Perez et al., 2006) in a cell context dependant manner. Particularly, given that TDZ is described to localize to the plasma membrane with the highest affinity relative to the cytosol or nucleus(Ekins et al., 2005), it is plausible that the aforementioned signaling pathways in response to TDZ are initiated by DRD as an upstream cell surface receptor that is shared among these cancer types.

Accordingly, human AML(Sachlos et al., 2012) as well as other TDZ-responsive neoplastic tissues such as breast (Borcherding et al., 2016), ovarian (Bowen et al., 2009), SHH MB cells (Robinson et al., 2012) and transformed embryonic cells (Werbowetski-Ogilvie et al., 2009) all displayed an upregulation of DRD members (Figure S5A). Also in glioblastomas, patients with higher levels of DRD4 and tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, exhibited worse survival outcomes compared to those with low DRD4 levels (Dolma et al., 2016). These observations suggest that in addition to their classic role as mediators of the nervous system, DRDs may engage in transformation events by contributing to neoplastic hallmarks such as proliferation (Lu et al., 2015), invasion (Spiegel et al., 2007) and metastasis (Borcherding et al., 2016). Importantly, the preferential DRD expression pattern in human AML as well as several other cancers may provide a therapeutic opportunity for cancer-selective targeting approaches (Figure S5A). Here, we evaluated this concept experimentally and clinically through the use of DRD antagonist TDZ against

human AML. While our initial observations *in vitro* and *in vivo* motivated a phase I clinical trial, the trial retrospectively provided a unique platform to validate the experimental findings with DRD-targeting by TDZ in the most relevant and authentic context of AML disease. Our data indicate that *in vitro* progenitor assays faithfully reproduce and/or prognosticated patient-specific therapy responses to novel anti-leukemic agents as previously suggested for a number of chemoterapeutic drugs(Browman et al., 1983; Griffin and Lowenberg, 1986; Preisler, 1980). Finally, we propose that in addition to AML, the therapeutic benefits of DRD targeting may apply to a wider range of cancers that exhibit an over-expression of the DRD family.

ACKNOWLEDGEMENTS

The authors are grateful to AML patients and healthy donors for providing the samples. Dr. Anargyros Xenocostas at the London Health Sciences Centre, University of Western, Dr. Michael Trus, Dr. Mohammed Almakadi and faculty members affiliated with Hematology at the Juravinski Hospital and Cancer Centre for providing patient and donor samples; the members of the Labour and Delivery Clinic at the McMaster Children's Hospital for supplying CB samples; Also, we thank Jamie McNicol, Aline Fiebig-Comyn, Monica Graham and Dr. Kennedy Makondo for technical support for flow cytometry; Dr. Eleftherios Sachlos and Dr. Kyle Salci for constructive advice; Dr. Ram Mishra and Asim H. Siddiqui in the Department of Psychiatry and Behavioral Neuroscience, McMaster

University, for facilitating initial dopamine analysis and Dr. Fanny L. Casado for assistance in running dopamine analysis from human plasma samples. Finally, we thank the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP), for providing the Ki values. This work was supported by Canadian Cancer Society Research Institute (to Dr. Bhatia) and Jan's graduate scholarship in stem cell research (to Lili Aslostovar). Dr. Bhatia is supported by the Canadian Chair Program and holds the Canada Research Chair in human stem cell biology.

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Figure 1

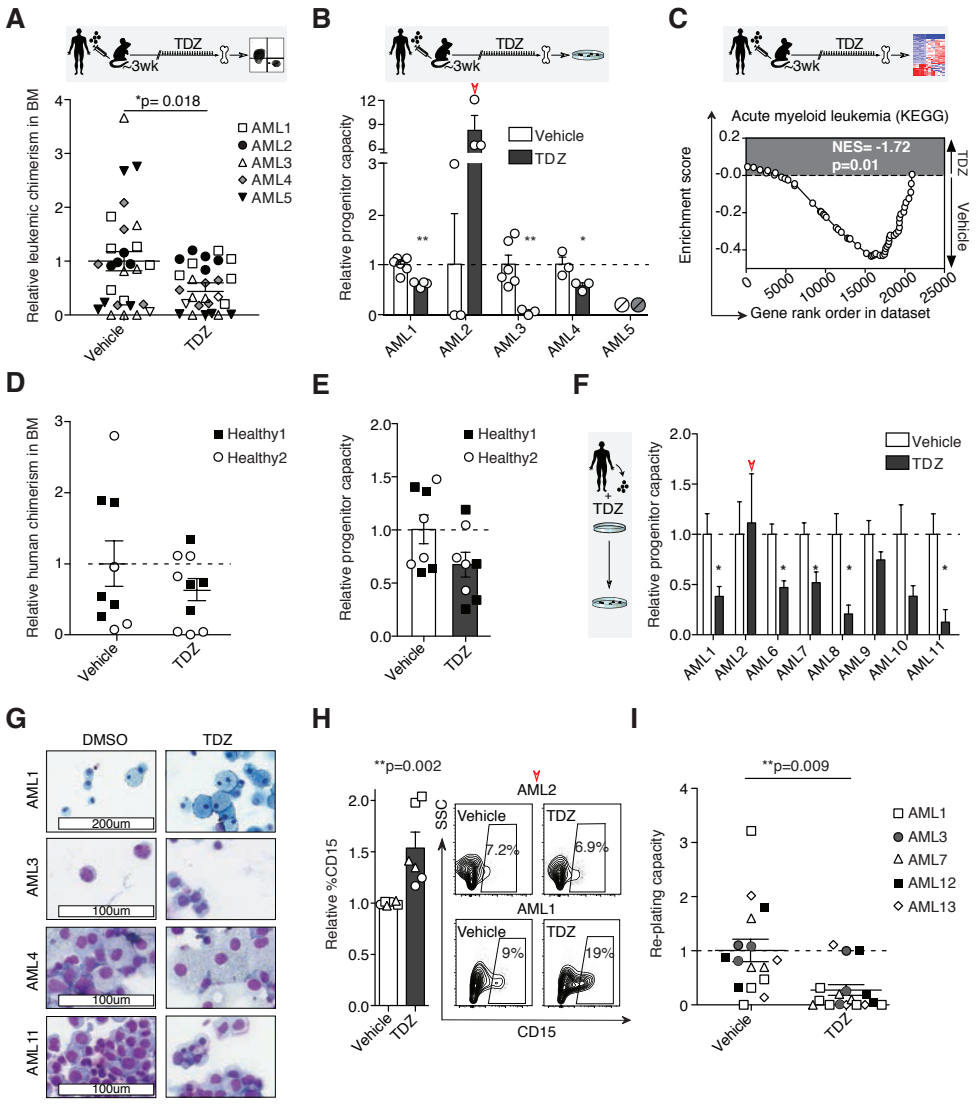


Figure 1. TDZ selectively suppresses leukemic progenitor activity by induction of cellular maturation (A) Schematic shows treatment of established human leukemic xenografts with a dose of TDZ (22.5 mg/kg) that achieves clinical levels of plasma TDZ in vivo. 3-5 weeks after transplantation of recipient mice with human AML patient samples, patient-derived xenografts were treated with TDZ or vehicle (captisol 30%) for 21 days. At the experimental endpoint, leukemia chimersim levels (hCD45+CD33+) were analyzed by flow cytometry. In vivo treatment with TDZ reduced human leukemia levels in the recipient mouse BM. Chimerism is normalized to vehicle control mice across 5 individual AML

patient-derived xenografts. Symbols represent individual recipient mice, *p=0.018 by unpaired t-test. (B) After in vivo exposure to TDZ for 21 days, leukemic xenografts were purified from recipient mouse BM and interrogated for progenitor capacity by seeding 25,000-100,000 human AML cells in semisolid media. AML5 did not harbour colony forming capacity at 150,000 cells interrogated. The red arrow over AML2 indicates that it did not respond to TDZ. Data are expressed as mean ± SEM relative to vehicle control, *p≤ 0.05, **p≤ 0.01 by unpaired t-test. (C) After in vivo exposure to TDZ for 21 days, human leukemia xenografts were purified and gene expression profiles were analyzed. Gene signature enrichment analysis (GSEA) from AMLs 1, 4 and 5-xenografts showed down regulation of AML-related signature genes after in vivo treatment with TDZ relative to vehicle control-treated xenografts. (D) In vivo treatment with TDZ for 21 days did not negatively impact healthy human hematopoietic chimerism (hCD45+) with 2 distinct Lineage depleted cord blood (Lin- CB) samples. Symbols represent individual recipient mice. Data is expressed as mean ± SEM relative to vehicle control. (E) Healthy human cells (hCD45+) were purified from the recipient mouse BM after in vivo exposure to TDZ for 21 days. 10,000 hCD45+ cells from 2 individual CB samples were purified from the recipient mouse BM and interrogated for progenitor function. Data is expressed as mean ± SEM relative to vehicle control. (F) Leukemic progenitor capacity was suppressed in 8 clinically diverse AML patient samples after treatment with TDZ (10uM) for 24 hrs in vitro. Consistent with its lack of response in vivo, AML2 did not respond to TDZ in vitro. Data is expressed as mean ± SEM relative to DMSO control (n=3-4 replicates, n=2 for AML 10 TDZ-treated group and this sample was not evaluated statistically), *p≤ 0.05 by unpaired t-test. (G) Giemsa-Wright stained cytospin preparations showed acquisition of a more differentiated cellular morphology after TDZ exposure in vitro. (H) Flow cytometry analysis showed acquisition of a mature cellular lineage marker (CD15) after TDZ treatment in vitro for 24hrs in AMLs 1 (square symbol), AML7 (triangles) and AML8 (circles). In accordance with its lack of functional response, AML2 did not show CD15 upregulation after exposure to TDZ (**p≤ 0.002 by Mann Whitney test). (I) AML patient samples were treated with TDZ or DMSO control for 24 hrs, seeded in semi-solid media and collected for assessment of re-plating capacity on day 14. Consistent with acquisition of cellular maturation features, re-plating capacity of leukemic progenitor cells was diminished after exposure to TDZ. Data is expressed as mean ± SEM relative to DMSO control (**p=0.009, two-way ANOVA).

Figure 2

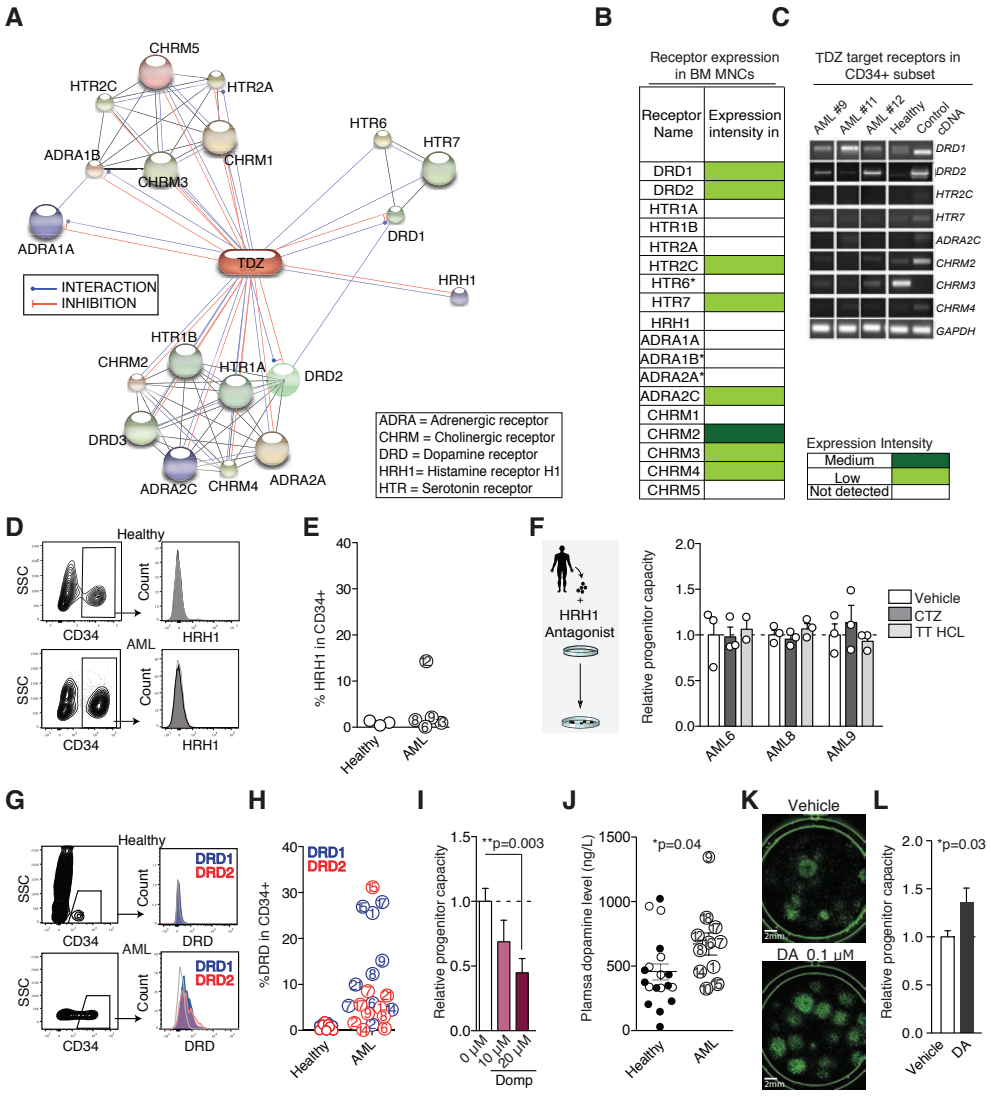


Figure 2. TDZ-induced suppression of leukemic clonogenicity is mediated by dopamine receptors (A) STITCH target interaction network (Kuhn et al., 2014) for putative TDZ target receptors including dopamine, adrenergic, serotonin, cholinergic and histamine receptors (version 4.0). (B) Heat map shows protein expression of putative TDZ target receptors (presented in Panel A) within human BM MNCs according to human protein atlas database (Uhlen et al., 2005). Asteriks indicate transcript levels where protein expression data was not available. (C) TDZ target receptors as filtered in Panel B were analyzed for transcript level in FACS-purified CD34+ progenitor-enriched cells of leukemic and healthy

primary hematopoietic samples. The experimental control predominantly consisted of human reference cDNA (HuRef) and/or a pool of multiple primary hematopoietic human samples when a given target gene was not highly expressed in the HuRef. (D) Representative FACS plots of HRH1 protein expression within leukemic and healthy CD34+ cells. (E) HRH1 protein expression in CD34+ cells of 5 AML patient samples and healthy donors (n=3 CB Lin-). Individual patient IDs are indicated within symbols. (F) Clonogenic potential of leukemic progenitors in response to treatment with HRH1 antagonism with cetirizine dihydrochloride (CTZ, 10 μM) and Trans-triprolidine HCL (TT HCL, 10 μM) (Zappia et al., 2015) for 24hrs in vitro. Data is expressed as mean ± SEM relative to DMSO control. (G) Representative FACS plots of DRD expression within leukemic and healthy CD34+ cells. (H) DRD protein expression in CD34+ cells of 10 AML (n=10) or healthy (n=7) samples. AML patient ID are indicated within symbols. (I) Relative progenitor capacity after in vitro treatment with domperidone (Domp) at 10uM (N=4 AML samples) and 20uM (N=1 AML sample) for 24 hrs. Data is normalized to DMSO control. N=3 replicates per sample, **p=0.003 by unpaired t-test relative to DMSO control. (J) Circulating dopamine (DA) levels in hematopoietic samples (n=8 healthy PB and 11 CB presented as hollow and black circles, respectively versus n=11 AML patient samples). Data is expressed as mean ± SEM, *p= 0.04 by unpaired t-test. (K) Representative stitched whole-well CFU images of DA at physiological levels (10^{-7} μM) versus DMSO control. (L) Increased leukemic progenitor output after treatment with physiological levels of DA (10^{-8}-10^{-7} μM) relative to DMSO control (n=6). Data is represented as mean ± SEM, *p= 0.03 by unpaired t-test.

Figure 3

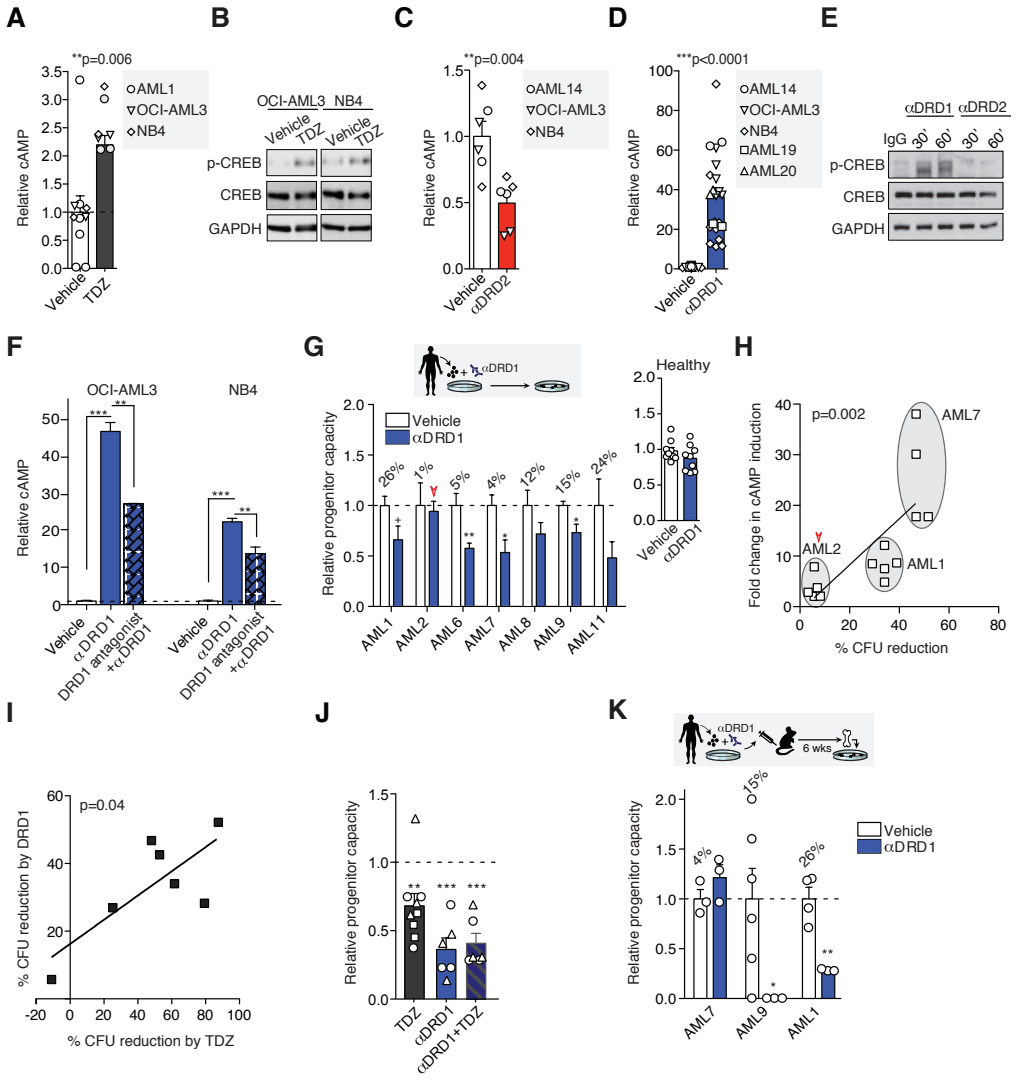


Figure 3. cAMP-CREB axis downstream of dopamine receptors mediates suppression of leukemic clonogenicity

(A) cAMP levels are elevated in response to a 30-minute *in vitro* treatment with TDZ (10uM) in AML1 and AML cell lines OCI-AML3 and NB4. Data is presented as mean ± SEM, **p=0.006 by unpaired t-test. (B) Western blot shows transient activation of CREB (p-CREB at Ser-133) in response to a 30-minute exposure to TDZ or DMSO control in OCI-AML3 and NB4 cell lines. (C) cAMP levels remained unchanged after treatment with DRD2-Ab *in vitro* for 30 minutes relative to IgG control. Data is expressed as mean ± SEM, **p=0.004 by unpaired t-test. (D) cAMP levels are elevated in response to DRD1-Ab treatment for 30

minutes relative to IgG control. Data is expressed as mean ± SEM, ***p≤ 0.0001 by unpaired t-test. (E) Consistent with elevation of cAMP after exposure to DRD1-Ab, CREB is transiently activated (p-CREB at Ser-133) in response to DRD1-Ab by western blot. (F) cAMP levels induced by DRD1-Ab were decreased after addition of DRD1 antagonist (SCH 23390, 10uM) in AML cell lines OCI-AML3 and NB4. Data is expressed as mean ± SEM, **p≤ 0.01,***p≤ 0.001 by one-way ANOVA. (G) Schematic demonstrates treatment of AML patient samples with DRD1-Ab *in vitro* for 30 minutes followed by seeding in semisolid media. Progenitor capacity is tested in 7 AML patient samples in response to *in vitro* treatment with DRD1-Ab or IgG control for 30 minutes (n=3-7 replicates). Data is expressed as mean ± SEM, *p≤ 0.05 and $^{+}$p=0.05 by unpaired t-test. DRD1 protein expression is reported for individual samples. Inset depicts colony forming potential of healthy progenitors after *in vitro* treatment with DRD1-Ab (n=3 CB Lin-). (H) correlation of cAMP induction in response to a 30-minute exposure to DRD1-Ab and functional response in CFU assay evaluated in AMLs 1 , 2 and 7. In accordance with its lack of functional response as shown in J, AML2 did not show a significant elevation of cAMP. Symbols represent individual replicates. (I) Response to DRD1-Ab as determined by % reduction of colony count is positively correlated with response to TDZ (p=0.04 by Linear regression). Symbols represent average of replicates per AML sample. (J) *In vitro* treatment with a combination of TDZ and DRD1-Ab for 30 minutes did not cause an additive effect on suppression of leukemic clonogenicity. Data is expressed as mean ± SEM, **p≤ 0.01,***p≤ 0.001 by one-way ANOVA. AML4, AML9 and AML11 replicates are presented as triangles, squares and circles, respectively. (K) Schematic demonstrates treatment of AML patient samples with DRD1-Ab *ex vivo* for 30 minutes followed by transplantation in recipient mice. Clonogenic capacity of the leukemic xenograft was assessed to DRD1-Ab relative to IgG control. Frequency of DRD1 protein expression is reported for individual samples. CFU capacity was abolished in AML9 after *ex vivo* exposure to DRD1-Ab, *P<0.05 by Fisher's exact test. **p≤ 0.01 by unpaired t-test.

Figure 4

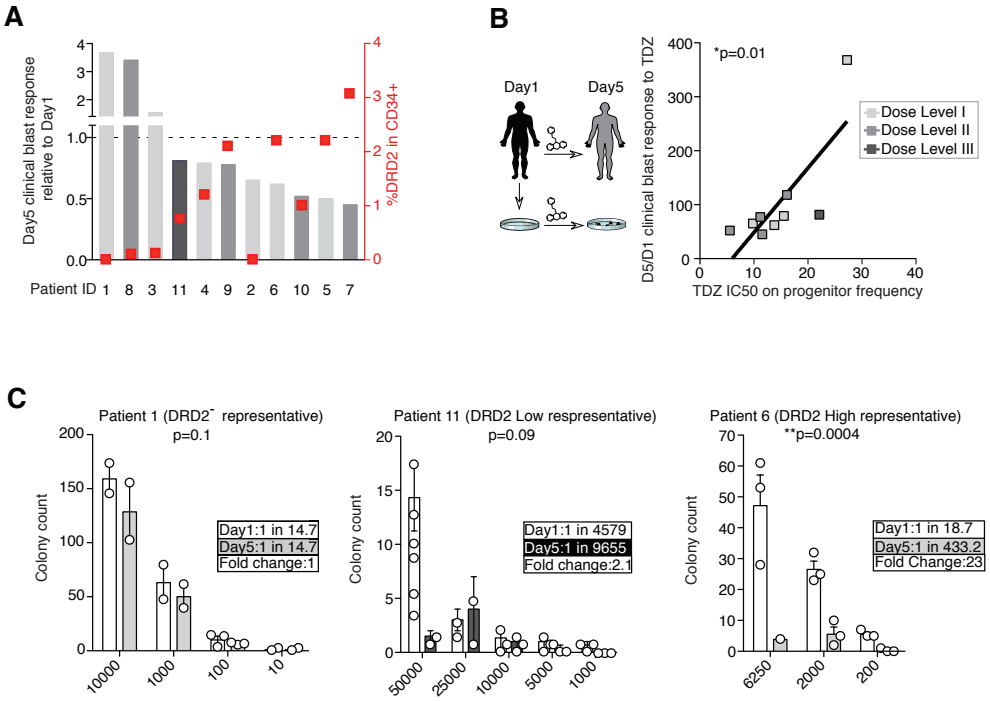
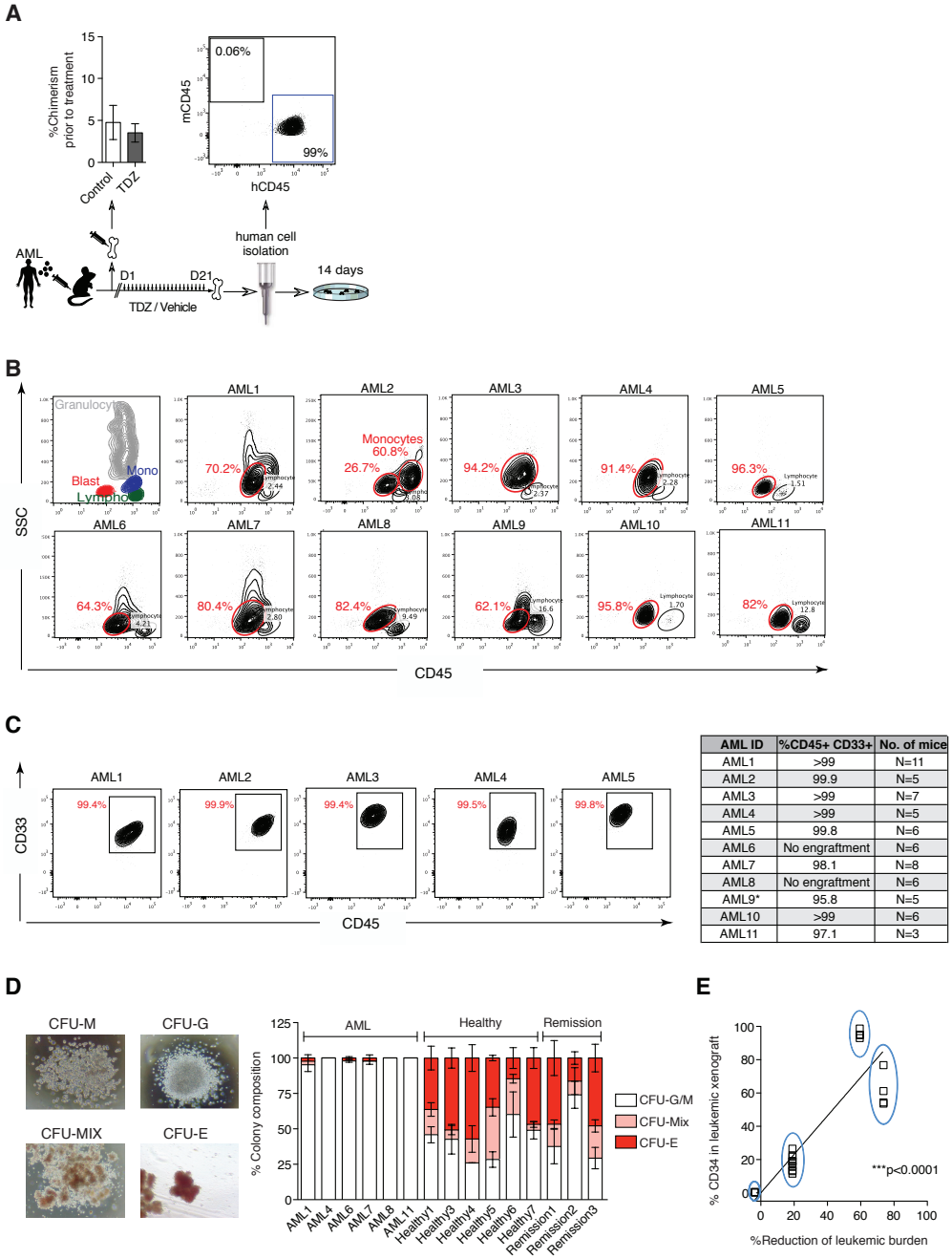


Figure 4. Patient-specific blast response to clinical administration of TDZ is mediated by DRD2 and is reproduced by functional progenitor assays (A) Change in the PB absolute blast counts (ABC) in the PB on Day5 relative to Day1 in response to clinical administration of TDZ as a monotherapy (left Y axis). For patients 2 and 9, response was evaluated based on BM blast% due to a lack of peripheral blasts. Colorcoding reflects clinical TDZ dose levels, ranging from 100mg/day (Dose I) as reflected in light grey to 200 (Dose II) and 400mg/day (Dose III) in dark grey and black, respectively. Right Y axis demonstrates TDZ target receptor DRD2 expression in CD34+ cells acquired prior to exposure to TDZ on Day1. (B) Sensitivity of leukemic progenitors to TDZ treatment *in vitro* reflects the clinical response of respective AML patients to clinical administration of TDZ as a monotherapy for 5 days. Patient response was defined by change in the ABC (BM blast% for patients 2 and 9). Progenitor IC50 was determined by treatment of trial patient samples obtained prior to exposure to TDZ on Day1 with up to 12 doses of TDZ for 24 hrs, followed by seeding in semisolid media. (C) Progenitor content of trial patients was interrogated prior to exposure to TDZ (Day1) and after 5 days of clinical TDZ administration (Day5) by limiting dilution assay. Progenitor output reflects the extent of patient-specific blast response to TDZ as demonstrated in 3 representative patients with varying DRD2 expression levels. Colour code reflects clinical TDZ dose level.

Figure S1

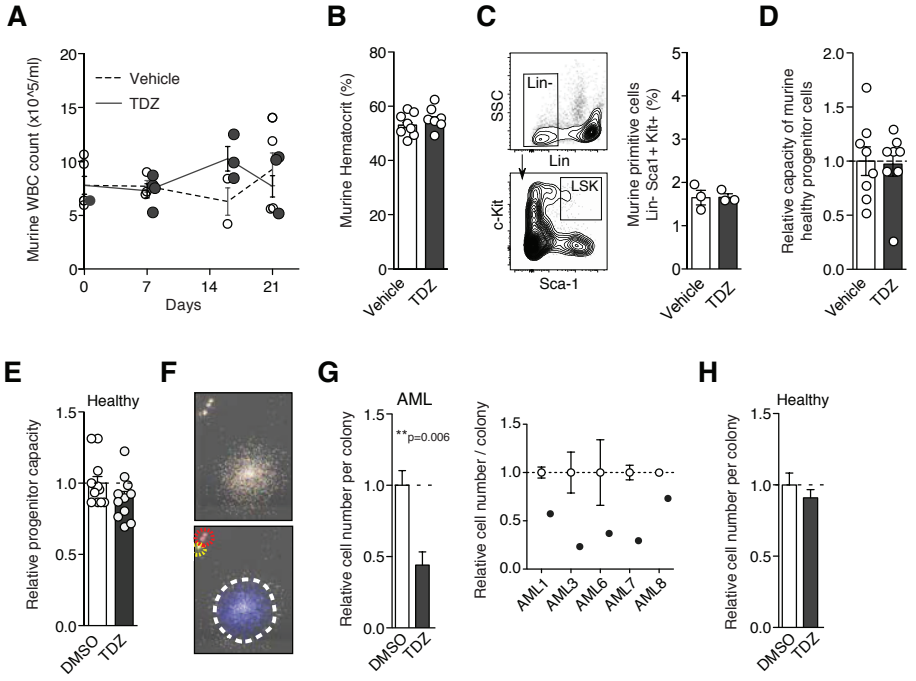


AML ID	%CD45+ CD33+	No. of mice
AML1	>99	N=11
AML2	99.9	N=5
AML3	>99	N=7
AML4	>99	N=5
AML5	99.8	N=6
AML6	No engraftment	N=6
AML7	98.1	N=8
AML8	No engraftment	N=6
AML9*	95.8	N=5
AML10	>99	N=6
AML11	97.1	N=3

Supplemental Figure 1. Functional characterization of the progenitor output of AML patient samples (A) Schematic shows analysis of AML xenografts after

TDZ treatment in vivo. Recipient mice were assigned to TDZ versus vehicle control groups based on comparable engraftment levels at baseline as quantified by BM aspirate sampling. At experimental end point, BM of the xenografted recipients was harvested and the leukemic xenografts were purified by exclusion of mouse cells (or FACS-isolation of human leukemia cells, not shown). FACS plot depicts the purity of recovered human leukemia cells prior to seeding in semisolid media. (B) FACS plots demonstrates the blast population (red gate) as defined by SSC vs. CD45 intensity (Vo et al. Cell, 2012) for AMLs 1-11. 8 of the 11 samples contain ≥80% malignant cells after Ficoll processing (Skrtic et al. Cancer Cell, 2011). The monocytic population in AML2 was clinically considered abnormal and was therefore included in the malignant blast gate. (C) FACS plots shows co-expression of the myeloid cell surface marker (CD33+) in the entire human xenograft (hDC45+), suggesting a lack of healthy stem cells in the xenografts derived from AMLs 1-5 when transplanted in NODSCID mice (Kennedy al., 2013; Eppert et al., 2011). The table shows a summary of the myeloid content of the 11 patient-derived xenografts used in functional assays. AML9 expressed an alternative myeloid marker (CD13). (D) (Left) Representative images of colony types including granulocytic (CFU-G) or monocytic (M), erythroid (CFU-E) or mixed colonies (CFU-MIX). (Right) Stacked bars represent the diversity of colony types derived from AML patient samples (or xenografts, for AMLs 3 and 9) vs. healthy hematopoietic cells from CB or MPB samples, or AML patient samples at remission. AML 5 is not included as it did not generate robust colonies (E) Reduction of leukemic burden in vivo is positively correlated with the CD34 content of the xenografts derived from AMLs 1-4 (N=4-7 mice per patient sample, p=0.01). %CD34 is averaged across replicate xenografts of the same AML sample. Xenografts derived from an individual AML patient are grouped together. AML5 did not express CD34 in the de novo AML patient sample or the xenograft.

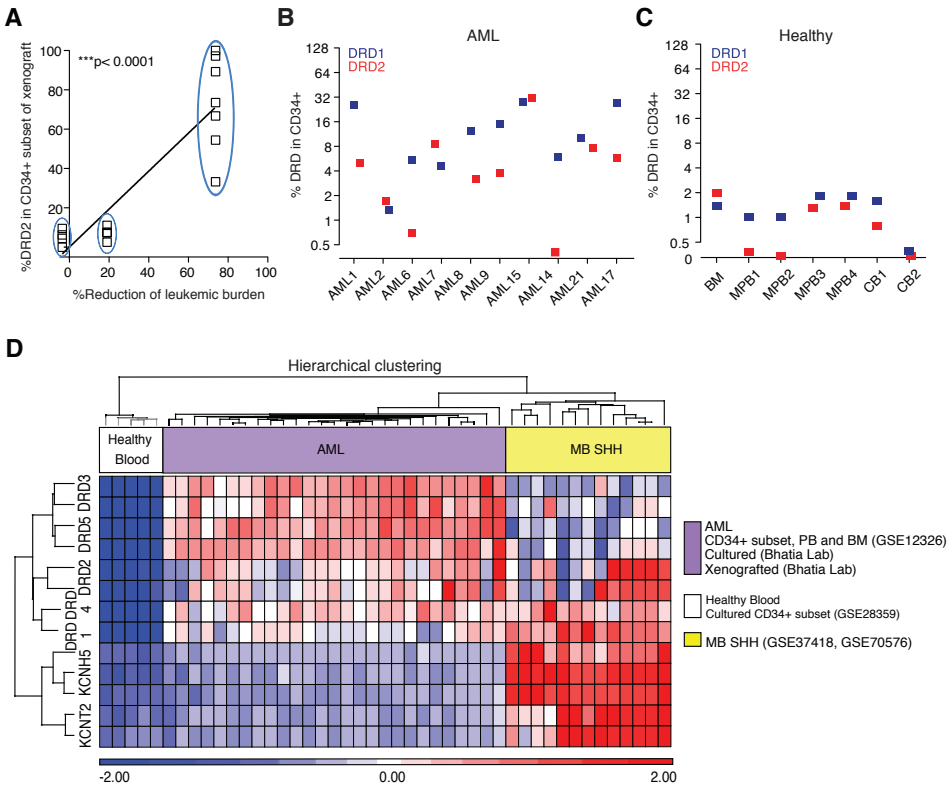
Figure S2



Supplemental Figure 2. TDZ does not compromise healthy hematopoietic progenitor function (A) Monitoring of healthy white blood cell count (WBC) in the recipient mouse peripheral blood over the course of TDZ treatment in vivo for 21 days showed no long-term suppression of WBC compared to vehicle control-treated animals. WBC was quantified by acridine orange staining for blood nucleated cells. Symbols represent average of 2 measurements per mouse. (B) Murine hematocrit remained unaffected after 21 days of in vivo treatment with TDZ. Symbols represent average of 3 measurements per mouse. (C) Lin- Sca-1+ Kit+ (LSK) frequency, representing the murine healthy primitive hematopoietic fraction, remained unchanged after in vivo treatment with TDZ for 21 days (N=3 mice). (D) Murine progenitor capacity is not compromised after in vivo exposure to TDZ. Progenitor function was determined by seeding of 5,000-25,000 mouse BM cells in semisolid media (N=2 mice per condition, n=8 replicates per condition). Data is normalized to vehicle control. (E) In vitro treatment with TDZ (10 μM) for 24 hrs did not compromise healthy hematopoietic clonogenicity relative to vehicle control (0.1% DMSO, n=3 CB Lin-). (F) Image demonstrates clustering of adjacent cells in semisolid media to identify an individual colony by custom scripts, followed by enumeration of the cells within colonyies as a measure of the proliferative capacity of individual colony forming units. (G)

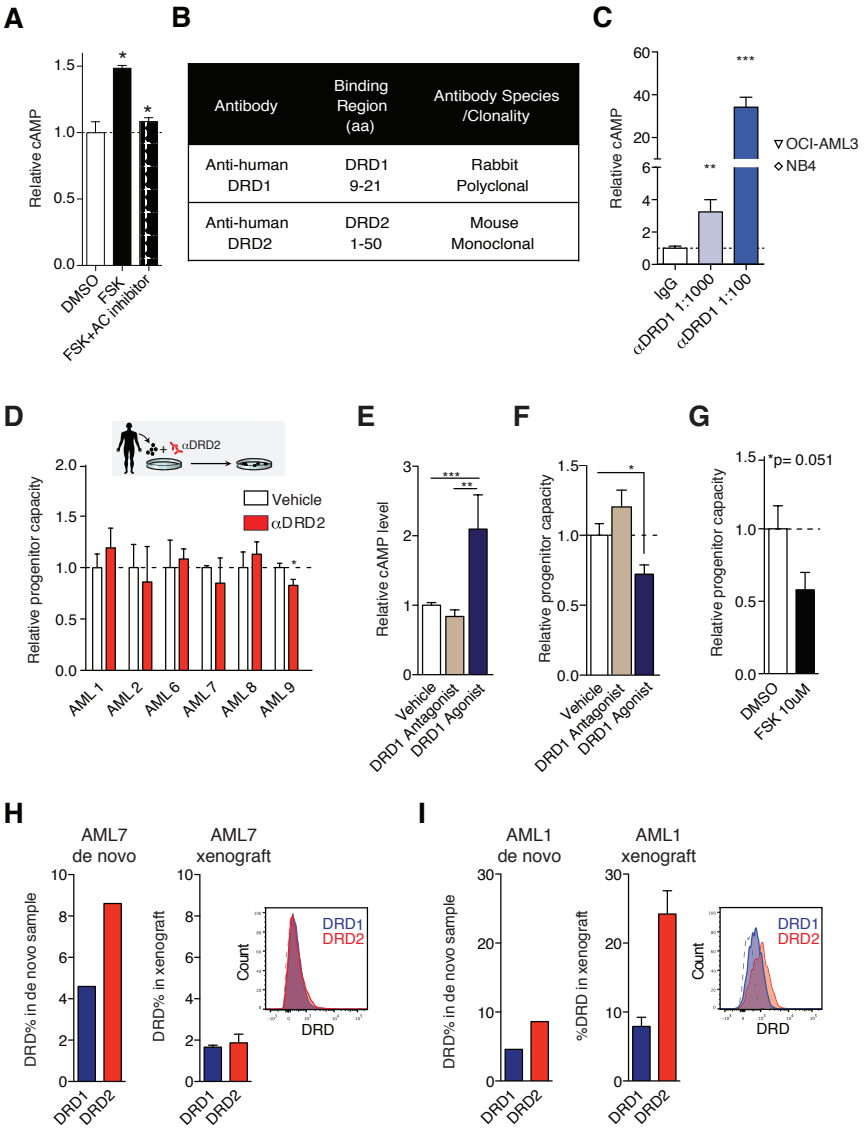
Average cell number per colony after a 24-hr treatment with DMSO or TDZ. TDZ induces a significant reduction in average cell number per colony (**p=0.006) for AMLs 1, 3, 6-8. The inset depicts individual AML samples. (H) Average cell number per colony remained unaffected after TDZ treatment in healthy hematopoietic colonies (n=2 CB and n=1 MPB sample).

Figure S3



Supplemental Figure 3. DRDs are preferentially expressed in primitive leukemic cells relative to their healthy counterparts (A) Reduction of leukemic chimerism in vivo was significantly correlated with the %DRD2 content of the xenografts derived from AMLs 1-3 (N=4-7 mice per patient sample, p=0.02 by linear regression). %DRD2 is averaged across replicate xenografts of the same AML sample. Xenografts derived from an individual AML patient are grouped together. %DRD2 was not analyzed in xenografts derived from AMLs 4 and 5. (B) DRD1-2 expression within CD34+ cells of AML patient samples. (C) DRD1-2 expression within healthy CD34+ cells obtained from MPB (n=4), CB (n=2) and BM (n=1). (D) Hierarchical clustering analysis of transcript expression of DRDs and potassium channels EAG2 (KCNH5) and downstream KCNT2 in hematopoietic cells in comparison to sonic hedgehog medulloblastoma (MB SHH) tissues. Gene expression data of MB SHH (GSE37418 and GSE70576), healthy blood (GSE28359), and AML (GSE12326) were obtained from public data sets.

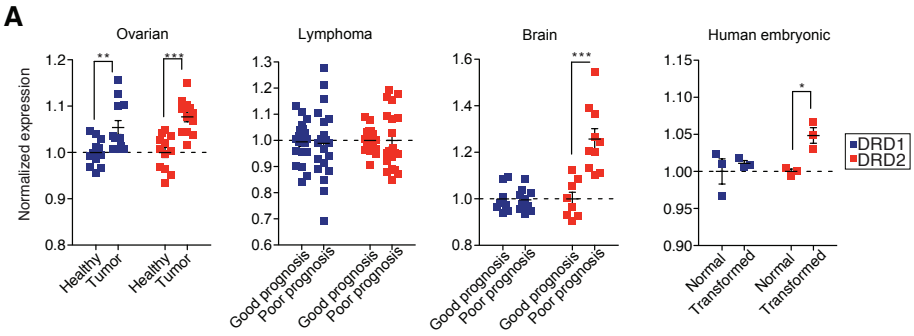
Figure S4



Supplemental Figure 4. Targeting DRD pathway in human AML (A) cAMP was induced when AML cell lines are exposed to 50uM Forskolin (FSK), inducer of cAMP. cAMP is reduced when AML cells are treated with FSK in combination with an inhibitor of adenylyl cyclase (AC) 2', 5'- dideoxyadenosine, validating the cAMP read out. Data is normalized to DMSO control (N=3 AML samples, n=3 replicates, *p<0.05 by one-way ANOVA). (B) Anti-human DRD antibodies used in cAMP assays, CFU screens and staining experiments. (C) Shows a dose-dependant induction of cAMP by a 30-minute treatment with DRD1-Ab in 2

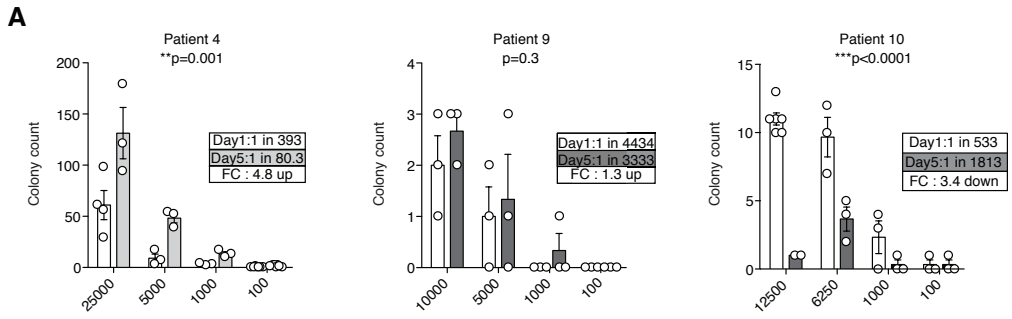
AML cell lines. N=4-8 replicates per treatment, ***p≤0.001 by unpaired t-test, relative to the IgG control. (D) Schematic shows treatment of AML patient samples with DRD2-Ab for 30 minutes in vitro followed by seeding in semisolid media. CFU output of 6 individual primary AML samples remained unaffected in response to DRD2-Ab or IgG control. Data is expressed as mean ± SEM, *p≤ 0.05 by unpaired t-test. (E) cAMP levels in response to DRD1 antagonist (SCH 23390, 10 μM) and DRD1 agonist (SKF 38393, 10 μM). N≥4 replicates for OCI-AML3 and NB4 cell line, **p≤0.01, ***p≤0.001 by one-way ANOVA. (F) Progenitor capacity after treatment with DRD1 agonist and antagonist. Data is expressed as mean ± SEM, N=4 AML samples, n=2-3 replicates per sample. *p≤ 0.05 by one-way ANOVA. (G) Progenitor capacity after treatment with FSK at 10uM. Data is expressed as mean ± SEM, p=0.051 by unpaired t-test. (H) %DRD1 and 2 in de novo AML7 patient sample versus lower DRD frequencies in AML7-derived xenograft. The inset shows DRD expression intensity in AML7 xenograft. (I) Appreciable levels of DRD1 and 2 can be detected in de novo AML1, as well as in AML1-derived xenograft. The inset shows DRD expression intensity in AML1 xenograft.

Figure S5



Supplemental Figure 5. DRDs are preferentially expressed in malignant cells of multiple neoplasias (A) Differential DRD expression between tumour cells versus tissues acquired from more favourable prognosis tumours or healthy control samples in 4 human neoplasias that responded to TDZ. The data were obtained from previous reports (Sachlos et al., 2012) and publically available datasets including GSE14407, GSE31312 and GSE37418.

Figure S6



Supplemental Figure 6. Assessment of functional response to TDZ by in vitro assays

(A) Progenitor content of trial patients was interrogated prior to exposure to TDZ (Day1) and after 5 days of clinical TDZ administration (Day5) by limiting dilution assay. Colour code reflects clinical TDZ dose level.

Table 1. Clinical details of the AML patient samples excluding Thoridal patients

AML Sample ID	Age / Sex	Tissue source	Disease stage	CG	Molecular	FAB subtype	Blast%	ELN prognosis
1	46 / F	PB	Diagnosis	Normal	FLT3 ITD+	M1	70.2+	Int-1
2	71 / M	BM	Diagnosis	Normal	ND	M5b	87.5*+	NA
3	52 / M	PB	Diagnosis	NA	Normal	M5b	94.2+	NA
4	69 / F	PB	Diagnosis	Complex	ND	M2	91.4+	Adverse
5	89 / F	PB	Diagnosis	NA	ND	NA	96.3+	NA
6	75 / M	BM	Diagnosis	Complex	ND	MDS to Aml	64.3+	Int-2
7	48 / M	PB	Relapse	Complex	ND	MDS to Aml	80.4+	Adverse
8	54 / F	PB	Diagnosis	Normal	NPM1+ Flt3ITD+	M2	82.4+	Int-1
9	64 / M	PB/BM	Diagnosis	Complex	ND	MDS to Aml	62.1+	Int-2
10	74 / M	PB	Diagnosis	NA	N.A	M2	96+	Int-1
11	63/M	PB	Relapse	Normal	NPM1+	M4	82+	Favorable
12	59/M	PB	Diagnosis	Normal	NPM1+	M5b	87*	Favorable
13	67/M	NA	Diagnosis	Normal	NA	NA	<24%	NA
14	54/F	PB	Diagnosis	Normal	Normal	M0	30	Int-1
15	75/M	BM	Diagnosis	Complex	NA	M4	80	adverse
16	38/F	PB	Diagnosis	ND	Normal	M5a	95	NA
17	53/F	BM	Diagnosis	Normal	NPM1+ FLT3-ITD Heterozygous	M5b	>70%	Int-1
18	51/M	BM /PB	Diagnosis	Complex	NPM1+	M5b	80/20	avderse
19	61/F	BM	Diagnosis	46,XX[14]	Normal	M1	39	Int-1
20	65/M	BM	Diagnosis	Normal	Normal	M2	30	Int-1
21	41/M	BM	Diagnosis	NA	CBFß/MYH11	M4	90	Favorable
22	NA	NA	NA	NA	NA	NA	NA	NA
23	56/F	PB	Diagnosis	Normal	NPM1+ FLT3 ITD+	M2	80	Int-1

+Values reflect post-Ficoll processing blast%

*The monocytic population was considered abnormal based on clinical notes and was included in the malignant population.

NA=Not available

ND=Not done

Table 2. Primers list

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
DRD1	CTGATCAGTGTGGCATGGAC	GTGGTGGTCTGGCAATTCTT
DRD2	GGTCTACATCAAGATCTACATTGTCCTCC	TGGCGAGCATCTGAGTGGCTTTCTTCTCC
HTR2C	AGATATTTGTGCCCCGTCTG	CACCTTTTCTTCGTCCCTCA
HTR7	AAAGTTGTGATCGGCTCCAT	TCTGCCTCACAGGGTATGTG
ADRA2C	TACTGGTACTTCGGGCAGGT	GATGCAGGAGGACAGGATGT
CHRM2	GTCAAGCGGACCACAAAAAT	GCAACAGGCTCCTTCTTGTC
CHRM3	CACTCATTTCGGCAGCTACA	AGGCCAGGCTTAAGAGGAAG
CHRM4	GCCCACTAATGAAGCAGAGC	CTGCTTCGTCACAATCTGGA
GAPDH	GAAGGTGAAGGTCGGAGTCA	AATGAAGGGGTCATTGATGG

Table 3. Clinical and experimental details of trial AML patients

Trial patient ID	Tissue source	Prognostic class	Day1 Blast	Day5 to Day1 Blast response (%)	Day1 progenitor frequency	Day1 DRD2% in CD34+
1	PB	Poor	11.6 (Bil/ml)	+368	1 in 14.7	0
2	BM	Intermediate I	46%	-35	1 in 1073	0
3	PB	Intermediate I	15.1 (Bil/ml)	+153	0 in 25000	0.12
4	PB	Intermediate II	24.8 (Bil/ml)	-21	1 in 435	1.2
5	PB	Intermediate I	1 (Bil/ml)	-50	0 in 40000	2.2
6	PB	Intermediate II	2.9 (Bil/ml)	-38	1 in 18.7	2.4
7	PB	NA	109.7 (Bil/ml)	-55	1 in 55000	3
8	BM	Poor	5.4 (Bil/ml)	+340	>1 in 50000*	<0.1
9	BM	Intermediate I	45%	-22	1 in 4434	2.1
10	PB	Intermediate I	2.5 (Bil/ml)	-48	1 in 533	1
11	PB	Intermediate II	12 (Bil/ml)	-19	1 in 4579	0.76

*For Patient 8 CFU assays, the BM samples was used instead of the PB due to a more robust colony formation capacity.

CHAPTER 4

A phase I clinical trial evaluating oral thioridazine in combination with intermediate dose cytarabine in acute myeloid leukemia patients 55 years of age and older, with relapsed/refractory disease

Preface

This chapter is an original article in preparation for submission. It is presented in its pre-submission format. The authors include:

Lili Aslostovar*, Allison Boyd*, Mohammed Almakadi*, Darryl P. Leong, Rommel G. Tirona, Richard Kim, Brian Leber, Mark N. Levine, Ronan Foley† and Mickie Bhatia†

I wrote the manuscript with Dr. Bhatia. Dr. Foley, Leong and Kim revised the manuscript. I analyzed the clinical data for short-term and long-term effects of TDZ on healthy hematopoiesis relative to leukemic blasts and executed the experiments related to DRD2 expression. Allison Boyd plotted and conceptualized the 5-day blast curves and provided input on the manuscript. Mohammed Almakadi retrieved the historical clinical data and provided input on the manuscript. Dr. Leong was the study cardiologist. Dr. Tirona and Kim performed and analyzed the pharmacokinetics and pharmacogenetics studies. Dr. Levine was the study director, and Dr. Foley, Leber and Bhatia were the principle investigators. The clinical trial (NCT02096289) was led by Ontario Clinical Oncology Group (OCOG).

The collective findings of our group (Sachlos et al. 2012, **Chapter 2** and **Chapter 3**) motivated a phase 1 clinical trial (NCT02096289) to evaluate the safety and feasibility of TDZ in combination with intermediate dose cytarabine in older AML patients with relapsed/refractory disease. As this patient population is in need of urgent cytoreduction due to the aggressively of the disease, the study was structured to include AraC for the majority of the regimen for maximum therapeutic benefit to the patients. However, we designed a brief lead-in with TDZ alone, which provided a unique opportunity to interrogate the safety and biology of human AML in response to TDZ alone, and together with AraC within a single clinical trial. With a safe dose of TDZ, we noted a level of efficacy in a number of AML patients that harboured higher levels of DRD2, providing preliminary evidence for the therapeutic value of DRD-directed approaches against human AML for the first time.

Abstract

We completed a phase I dose-escalation clinical trial to evaluate the safety of an oral dopamine 2 receptor (DRD2) blocking agent thioridazine in combination with cytarabine. A total of 13 patients 55 years and older with relapsed or refractory acute myeloid leukemia (AML) were enrolled. Oral thioridazine was administered at three dose levels including 25 mg (n=6), 50 mg (n=4) and 100 mg (n=3) every 6 hours (q6h) from days 1 to 22 inclusive. Intermediate-dose (ID) cytarabine was administered at 1 g/m^2 intravenously for 5 consecutive days between days 6-10 of the study. Dose-limiting toxicity (DLT) was observed at the 25 mg thioridazine dose in one patient who developed grade 3 QTc interval prolongation. Two patients at the 100 mg thioridazine dose experienced neurological DLTs including gait disturbance, depressed level of consciousness and dizziness. Other grade 2 or greater thioridazine-related adverse events included moderate QTc interval prolongation (n=10), urinary incontinence (n=1) and fatigue (n=1). Steady-state concentrations of thioridazine and 4 metabolites were dose-dependent. *CYP2D6* genotype contributed to inter-patient variation of plasma drug concentrations. At the 50 mg dose, the sum of circulating thioridazine and two metabolites harbouring DRD2 antagonistic properties (2-sulfoxide and 2-sulfone) approached the target concentration of 10 μM, considered to be active against human AML based on previous *in vitro* studies. Eleven of 13 patients completed an initial 5-day lead-in treatment with thioridazine monotherapy. Comparative peripheral blood and marrow samples

obtained at day 1 and day 5, demonstrated a reduction in absolute blasts ranging from 19-55% (N=8 patients). Three patients showed a progressive disease trend. Expression of thioridazine target receptor DRD2 was associated with the extent of blast reduction observed while on thioridazine monotherapy. We suggest that DRD2 blockade offers a novel approach to AML therapy with preliminary evidence of an anti-leukemic effect. Oral thioridazine at a dose of 50 mg q6h can be safely administered to elderly patients with relapsed/refractory AML over 55 years of age in combination with intermediate dose cytarabine.

Introduction

Therapeutic options for older patients with acute myeloid leukemia (AML) remain limited. Intensified chemotherapies do not improve survival and are poorly tolerated due to age-related comorbidities (Dohner et al., 2017; Dohner et al., 2010; Stone et al., 2001). For older patients with relapsed/refractory disease, effective treatment options are even more limited and therapy responses are less favourable, resulting in complete remission (CR) rates of 16-21% and median survival times of 6-9 months (Dohner et al., 2017; Ivanoff et al., 2013; Ritchie et al., 2013). These outcomes highlight the urgent need for novel therapeutic strategies in older AML patients with relapsed or refractory disease.

Thioridazine, a dopamine receptor (DRD) antagonist, was identified to harbour potent anti-leukemic properties in a large-scale chemical screen(Sachlos

et al., 2012). In these pre-clinical studies, monotherapy with thioridazine exhibited potent suppression of leukemic clonogenicity and leukaemia initiation (Sachlos et al., 2012). Moreover, combination of thioridazine with a standard chemotherapy agent cytarabine resulted in a 100-fold reduction in the effective dose of cytarabine required *in vitro*, highlighting the efficacy of thioridazine as a potentially synergistic with cytarabine (Sachlos et al., 2012). Importantly, healthy hematopoiesis remained unharmed after exposure to thioridazine + cytarabine combination *in vitro*, revealing thioridazine's selectivity for cancer (Sachlos et al., 2012).

Informed by the prescribed dosage and associated adverse effects of thioridazine as a former antipsychotic (Harrigan et al., 2004; Kemper et al., 1983; Menkes and Knight, 2002; Reilly et al., 2000), we designed a phase 1 clinical trial to evaluate the safety of oral thioridazine at 25-100 mg q6h alone and in combination with intermediate dose cytarabine ($1g/m^2$)(Faderl et al., 2012) in AML patients 55 years and older with relapsed/refractory disease. In addition to the primary outcome measure of safety, we also investigated whether a clinically tolerable dose of thioridazine achieves adequate plasma thioridazine levels required for an anti-leukemic activity, as established by *in vitro* studies (Sachlos et al., 2012).

Methods

Patient eligibility criteria

Following informed consent, patients at 55 years of age or older with relapsed/ refractory AML, including patients diagnosed with RAEB-2 and evolving myelodysplastic syndrome on hypomethylation therapy were enrolled. Exclusion criteria included: (i) concomitant use of any other standard or other investigational anti-leukemic therapies, (ii) having received more than 3 prior chemotherapy lines for AML, (iii) chemotherapy within 4 weeks from the trial, excluding hydroxyurea (HU), (iv) impaired renal function (estimated glomerular filtration rate < 60 ml/min/1.73m^2) or abnormal liver function (serum bilirubin >1.5xUNL; aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase > 2.5x upper limit of normal (ULN), (v) acute or chronic graft versus host disease (GVHD) or (vi) on-going systemic uncontrolled infection.

Other specific exclusion criteria included a left ventricular ejection fraction less than 45%, history of uncontrolled cardiac arrhythmias, diagnosis of prolonged QTc interval at ≥470ms for males and ≥ 480ms for females or presence of conditions that prolong the QT interval, known severe hypotensive or hypertensive heart disease.

Study design and treatment schema

Trial design was a traditional 3+3 dose escalation phase I study(Storer, 1989). Up to 3 dose levels of oral thioridazine were assessed sequentially, including 25 mg q6h (dose level I), 50 mg q6h (dose level II) and 100 mg q6h (dose level III). Thioridazine was administered for a total of 21 days and cytarabine was

administered at an intermediate dose ($1g/m^2$) (Faderl et al., 2012) as a 2-hour infusion on 5 consecutive days (days 6-10) of the trial. Hydroxyurea (optional) was permitted in the first 5 days of study treatment prior to the planned administration of cytarabine on day 6 (Figure 1).

Toxicity evaluation

Toxicity criteria were assessed according to NCI-CTCAE version 4.03. A DLT was defined as the presence of any toxicity that was possibly, probably or definitely related to thioridazine. Toxicities included ≥grade 2 neurosensory toxicity-vision, ≥grade 3 gastrointestinal toxicity, ≥grade 3 neurosensory toxicity, ≥grade 2 renal toxicity, ≥grade 2 hepatic toxicity, ≥grade 2 pulmonary toxicity, ≥grade 2 neurological toxicity (other than neurosensory toxicity) and ≥grade 2 cardiac toxicity. For this study, ECG was measured on a daily basis. QTc prolongation to >500ms on at least 2 separate ECGs was considered a DLT (grade 3 as per the NCI-CTCAE version 4.03). The second ECG was completed within 1 hr from the initial ECG showing QTc prolongation. If a patient experienced a DLT, the study drug was permanently discontinued. Drugs known to prolong the QTc including specific anti-emetics and anti-fungal agents were not permitted.

Response evaluation

As a secondary measure, bone marrow (20ml) and peripheral blood samples (50ml) were obtained to evaluate potential treatment efficacy. Bone marrow and

peripheral specimens were collected prior to the start of thioridazine treatment (Day 1), after treatment with thioridazine as a monotherapy and prior to the start of cytarabine (Day 5), on the last day of thioridazine treatment (Day 22) and on the last day of the trial, coinciding with day 14 after the last thioridazine dose and day 26 after the last cytarabine dose (Day 36). Previously defined criteria for complete remission (CR), complete remission with incomplete count recovery (CRi), partial remission (PR), treatment failure (TF) or as not evaluable (NE) were employed to evaluate leukemia response (Dohner et al., 2017).

Pharmacokinetic analysis

Plasma trough concentration of TDZ and its 4 metabolites(Wojcikowski et al., 2006) including thioridazine 2-sulfoxide (mesoridazine), thioridazine 2-sulfone (sulforidazine), thioridazine 5-sulfoxide and N-desmethyl-thioridazine (Northiordiazne) were measured in samples collected on days 1 (prior to the 1st dose and approximately 5 hours after the 1st dose of TDZ), days 5, 8, 10, 17, 22, 29 and 36. Plasma concentrations of TDZ and metabolites were determined using liquid chromotography-tandem mass spectrometry. Briefly, plasma was spiked with internal standard (d3-TDZ, Toronto Research Chemicals) and proteins precipitated by addition of acetonitrile. Solutes were separated from the resulting plasma extracts by reverse phase chromatography (Hypersil Gold C18 column 3 x 50 mm, 5 μm, ThermoFisher Scientific; Agilent 1100 chromatograph) using gradient elution with 0.1% formic acid in water and acetonitrile. Compounds were

detected after electrospray ionization and detection in positive mode on a triple quadruple mass spectrometer (Thermo Vantage) with multiple reaction monitoring (TDZ m/z 371.2 → 126.2, d3-TDZ m/z 374.2 →129.2, Northioridazine mz 357.1 → 112.2, mesoridazine and 5-sulfoxide m/z 387.2 → 98.1, Sulforidazine m/z 403.2 → 98.1). The lower limits of quantitation were 3.5 nmol/L for TDZ, 15 nmol/L for mesoridazine, 7 nmol/L for 5-sulfoxide, 0.2 nmol/L for Northioridazine and 7.5 nmol/L for Sulforidazine.

Pharmacogenetic analysis

Genomic DNA was isolated from blood sample preparations with Gentra Puregene extraction kit (Qiagen Alameda) and quantified by Quant-iT PicoGreen dsDNA (Invitrogen). *CYP2D6* genotyping was performed by TaqMan Drug Metabolism Genotyping and Copy Number Assays (Applied Biosystem), using a 7500 Real-time PCR (Applied biosystems). The following inactive or reduced function alleles were analyzed: *CYP2D6*4* (g.1846G>A; rs3892097), *CYP2D6*3* (g.2549A>del; rs1057910), *CYP2D6*9* (g.2613_2615delAGA; rs5030656), *CYP2D6*10* (g.100C>T; rs1065852) and *CYP2D6*41* (g.2988G>A; rs28371725). *CYP2D6* (Hs04502391_cn) copy number variation, deletion or multiplications of the gene were also assessed.

Based on *CYP2D6* genotypes, the patients were categorized into the following phenotypes: poor metabolizers if carrying 2 inactive alleles, intermediate metabolizers if carrying 2 decreased function alleles or one

active/decreased function allele and one inactive allele. Ultrarapid metabolizers if carrying a gene duplication in the absence of inactive or decreased function alleles, and as extensive metabolizers if they were carrying 2 wild type alleles.

Primary patient sample processing

Mononuclear cells (MNCs) were prepared from clinical bone marrow and peripheral blood samples by density gradient centrifugation (Ficoll-Paque Premium, GE Healthcare), followed by red blood cell lysis using ammonium chloride solution (Stemcell technologies).

Flow Cytometric Analysis

Cell surface immunopheontyping for DRD2 was carried out using mouse anti-human dopamine receptor DRD2 (clone B-10, sc-5303) sourced from Santa Cruz Biotech. Anti-mouse Alexa-Fluor-647 (Life Technologies) was used as the secondary antibody. Samples were blocked with donkey serum (Jackson ImmunoResearch Laboratories) plus human FC block (eBioscience) and/or human IgG (Sigma) prior to staining. 7-aminoactinomycin D was used for live/dead cell discrimination.

Statistical analysis

Significant differences between groups were determined via unpaired two-tailed Student's t-test. Prism (version 5.0a; GraphPad, San Diego, CA, USA) software

was used for all statistical analyses, and the criterion for statistical significance was $p \leq 0.05$.

Results

Trial overview

Between July 2014 and September 2016, a total of 13 AML patients aged 55 years or older with relapsed/refractory disease (n=9) or AML with myelodysplastic changes (AML-RAEB-2, N=4) were enrolled. Baseline patient characteristics are summarized in Table 1. Patient 3 in dose level I experienced grade 3 QTc interval prolongation on day 7 to 535 ms. This DLT led to discontinuation of thioridazine. This patient was removed from the study after completion of the cytarabine regimen on day 10. As a result of this DLT, an additional 3 patients were enrolled in dose level I as per protocol(Storer, 1989). Patient 8 left the study on day 9 on personal request. For Patient 11, thioridazine dose was temporarily de-escalated from 100 mg q6h to 50 mg q6h on day 10 after a number of consecutive grade 1-2 QTc prolongation events. This patient later developed severe sepsis on day 16, which was considered unrelated to thioridazine but led to permanent discontinuation of thioridazine as the patient required intensive care. Patient 12 in dose level III experienced a grade 3 depressed level of consciousness on day 3. Patient 13 in dose level III suffered from grade 2 dizziness and gait disturbance on day 3. These neurological symptoms were considered DLTs and subsequently defined the maximum tolerated dose (MTD) of 50mg q 6h of thioridazine in this elderly AML patient population. Thus, in total, 3 (23%) participants developed DLT, and 8 patients completed the treatment regimen that included thioridazine on days 1-22 in combination with intermediate dose cytarabine on days 6-10.

Safety profile

Major toxicities are summarized in Tables 2 and 3. Consistent with previous reports, QTc prolongation was the most frequent adverse event(Buckley and Sanders, 2000; Buckley et al., 1995; Ewer and Ewer, 2015; Kemper et al., 1983; Reilly et al., 2000). Notably, the median age in all 3 cohorts was greater than 65, which is an independent predictor of QTc prolongation (Reilly et al., 2000). From a baseline QTc of 445 ms, Patient 3 developed severe (grade 3) QTc prolongation to 553 ms on day 7 of thioridazine treatment, coinciding with the second day of cytarabine treatment. This was considered a DLT, leading to a permanent discontinuation of thioridazine for this patient. However, it is noteworthy that following thioridazine discontinuation, Patient 3 continued to show significant QTc prolongation. Plasma thioridazine and thioridazine 5-sulfoxide (the metabolite associated with thioridazine-induced cardiotoxicity (Gottschalk et al., 1978; Hale and Poklis, 1984; Hale and Poklis, 1986; Hartigan-Go et al., 1996) concentrations were not detected despite frequent QTc prolongation on serial ECGs (Supplementary Figure 2A). These QTc changes in Patient 3 could not be explained by fluctuations in magnesium or potassium levels. Patients 7 and 9 in dose level II, as well as Patient 11 in dose level III also experienced grade 2 QTc prolongations, defined as QTc between 481-500 ms (NCI-CTCAE version 4.03). No other cardiac-related DLTs were reported in the additional three patients recruited in cohort I as per the phase I design (Storer, 1989), or in any of the patients in dose level II and III.

Following thioridazine dose escalation, we noted a modest increase in the average frequency of QTc prolongation events per cohort, as previously reported (Supplementary Figure 2B) (LLerena et al., 2002). However, patients' QTc intervals at baseline appeared to provide a more robust predictor of QTc prolongation during the trial (Supplementary Figure 2C). Specifically, Patient 3 with a grade 3 QTc elongation and Patient 9 with the highest frequency of grade 2 QTc prolongations exhibited baseline QTc levels that exceeded 440 ms(Hartigan-Go et al., 1996) and were greater than all other patients.

In addition to cardiac adverse events, neurological DLTs were noted in 2 patients at dose level III (100mg q6h). Patient 12 experienced grade 3 depressed level of consciousness and Patient 13 experienced grade 2 dizziness and gait disturbance (Table 3). Other non-hematologic grade ≥ 2 adverse events that were not dose-limiting included fatigue and urinary incontinence, affecting less than 10% of all patients (Table 3).

Grade 3 or greater adverse events recurring in ≥ 20% of patients predominantly included myelosuppression and associated complications (Table 2). These adverse events were observed across all 3 cohorts, independent of the dose of thioridazine and included anemia, reduced platelet, neutrophil and leukocyte counts. Patients were managed with standardized transfusions and administration of anti-microbial agents according to institutional standard of care.

There were 4 incidents of grade ≥ 3 sepsis in Patients 8, 11-13. All 4 cases of sepsis were considered unrelated to thioridazine. There was one on-study death

(Patient 12), which was associated with disease progression. Based on dose-related neurological DLTs observed in dose level III, we concluded that oral thioridazine at 50mg q6h in combination with intermediate dose cytarabine was safe and feasible in older patients with relapsed/refractory AML.

Pharmacokinetics and pharmacogenetics

Pharmacokinetic analyses were carried out to examine whether a safe dose of thioridazine achieves the putative target plasma concentrations of thioridazine (10μM), previously reported to display anti-leukemic activity *in vitro*(Sachlos et al., 2012). Blood samples were collected to measure plasma trough concentration of thioridazine and its 4 metabolites on days 1, 5, 8, 10, 17, 22, 29 and 36. As previously reported, the concentration of thioridazine and metabolites accumulated upon repeated dosing(Ng and Crammer, 1977) and steady-state plasma thioridazine was achieved approximately by day 5, prior to the administration of cytarabine (Fig, 2A, Supplementa ry Figure 1). At dose level III, Patient 11 achieved steady-state plasma thioridazine levels. However for this patient, thioridazine was de-escalated to 50 mg q6h on day 10 after a series of grade 1-2 QT prolongation events. On day 16, thioridazine was completely discontinued due to the development of sepsis, which was considered unrelated to thioridazine. Thioridazine did not reach steady state for Patients 12 and 13 as thioridazine was discontinued early on days 4 and 2, respectively. Detectable levels of thioridazine and its 2 principle metabolites including 2-sulfoxide and 5-

sulfoxide(Berecz et al., 2003) were sustained for approximately 1 week after the last dose of thioridazine (day 29) (Figure 2A). By day 36, thioridazine and metabolites were no longer detectable in plasma (Figure 2A).

Steady-state plasma thioridazine concentrations were dose-dependent and did not reach the target concentration of 10μM (Figure 2B). However, the sum of thioridazine and its two metabolites exhibiting DRD2 antagonism including 2-sulfoxide and 2-sulfone(Bylund, 1981; Daniel et al., 2000; Kilts et al., 1984; Vanderheeren and Muusze, 1977) approached 10μM in cohorts II and III (Figure 2C). Thioridazine is metabolised by Cytochrome P450 2D6 enzyme (CYP2D6) and *CYP2D6* polymorphism has been associated with variability in drug metabolism(Bertilsson et al., 2002; Dorado et al., 2009; Eap et al., 1996). *CYP2D6* genotyping of our patients indicated intermediate and extensive metabolizing phenotypes (Table 4, Figure 2D), which may explain the inter-patient heterogeneity in plasma drug concentrations(Berecz et al., 2003; LLerena et al., 2002; Ng and Crammer, 1977). As previously reported, patients with predicted extensive metabolizer phenotypes exhibited lower plasma concentrations for thioridazine and metabolites compared to intermediate metabolizers within the same cohort (Bertilsson et al., 2002; Linnet and Wiborg, 1996) (Figure 2D).

Response rates

To evaluate the biology of human AML exclusively to thioridazine alone, we incorporated a lead-in treatment period with thioridazine as a monotherapy. This treatment design provided an opportunity to evaluate treatment effects with thioridazine alone (days 1-5) and then subsequently in combination with cytarabine (days 6-10). We collected bone marrow and peripheral samples to evaluate a potential therapeutic efficacy on day 5 in response to thioridazine alone, on day 22 after termination of the thioridazine and cytarabine combination, and finally on day 36 for end point response assessment, coinciding with 14 days after the last dose of thioridazine and 26 days after the last dose of cytarabine (Figure 1A). Disease responses on Day 36 are summarized in Supplementary Table 1. Briefly, of the 13 recruited patients, Patients 3, 8, 12 and 13 received less than a third of the scheduled dose of thioridazine, and Patients 8 and 12 missed the cytarabine treatment and were therefore excluded from this analysis. Of the 9 evaluable patients, one patient (Patient 2, 11%) achieved partial remission as defined by a greater than 50% reduction of the bone marrow blast relative to pre-treatment levels(Dohner et al., 2017). Patient 7 showed no reduction of bone marrow blast % but a 66% reduction of peripheral blast was achieved. The 7 remaining patients showed resistant disease patterns on day 36.

Patient outcomes after the lead-in period with thioridazine alone revealed a reduction of peripheral leukemic burden, ranging from 19% to 55%, in 8 of the 11 patients (72%) who completed the 5-day portion of the study (Patients 1-

11)(Figure 3A). These outcomes with thioridazine alone were noteworthy given a brief exposure period(Dohner et al., 2017). Moreover, efficacy of thioridazine monotherapy was further highlighted when 5-day blast fluctuations were compared with the patients' respective blast trajectories prior to the start of the trial and in the absence of thioridazine (Figure 3A). Peripheral blast levels continued to decline with the addition of cytarabine (day 6-10) and up to day 22 when thioridazine was discontinued (Supplementary Figure 3A).

Importantly, despite the notable reduction of leukemic blast levels after only a 5-day exposure to thioridazine, residual neutrophil and platelet counts on day 5 were not dramatically reduced from baseline levels (Figure 3B-C). Also, addition of thioridazine regimen to intermediate dose cytarabine did not appear to increase the number of required red blood cell or platelet transfusions relative to disease- and age-matched AML patients receiving cytarabine-based chemotherapy (Figure 3D).

Thioridazine has been historically administered as an antipsychotic drug whose properties were mediated through DRD2 antagonism(Seeman and Lee, 1975). Previous *in vitro* assays with thioridazine suggested that DRDs also mediate thioridazine's anti-leukemic properties, whose preferential expression in leukemic versus healthy hematopoietic cells affords a selective response to thioridazine (Sachlos et al., 2012). Accordingly, we found that baseline DRD2 expression in patient mononuclear cells (MNCs) was positively associated with the gradient of blast fluctuations after the 5-day treatment with thioridazine

(Figure 3E). These blast level changes could be partitioned into three categories: (i) greater than 30% reduction, (ii) less than 30% reduction and (ii) greater than 20% increase in blast levels (progressive disease) (Figure 3A). When we compared DRD2 protein levels across these three categories, we found that DRD2 was minimally expressed in the MNCs of patients that exhibited progressive disease trends relative to those with a greater than 30% blast reduction (Figure 3E). Patients with a less than 30% blast reduction showed an intermediate DRD2 level relative to patients that showed progressive disease or over 30% blast reduction (Figure 3E). These findings suggest a potential on-target effect for thioridazine in a clinical setting and validate the relevance of DRD2 antagonism as a cancer-selective therapeutic approach against AML.

Discussion

Current therapeutic options for older AML patients with relapsed/refractory disease are associated with median survival times of 6-9 months(Dohner et al., 2017). In patients at second or third relapse, median overall survival is reduced to approximately 3 months(Dohner et al., 2017). Given that AML occurs predominantly in older adults as well as the strikingly high rate of relapse, this patient population in fact makes up a significant fraction of AML cases that are in urgent need of novel therapies.

In pursuit of novel compounds with anti-leukemic activity, we previously carried out a high throughput chemical screen that led to the identification of

thioridazine, an antipsychotic with selectivity for cancer(Sachlos et al., 2012). *In vitro*, thioridazine at 10μM suppressed the clonogenic growth of primitive leukemia cells within AML patient samples(Sachlos et al., 2012). The function of these cells has been associated with leukemic blast production and therefore provides a clinically relevant measure for evaluation of novel therapeutics (Bonnet and Dick, 1997; Browman et al., 1983; Griffin and Lowenberg, 1986; Preisler, 1980; Tehranchi et al., 2010). Thioridazine inhibited leukemia initiation capacity of primary patient samples *in vivo* whereas the repopulation capacity of healthy donor samples remained intact (Sachlos et al., 2012).

These findings motivated the current phase I clinical trial to evaluate the safety and feasibility of oral thioridazine at routine therapeutic doses(Kane, 1996), administered in combination with intermediate dose cytarabine in older AML patients with relapsed/refractory disease. The trial was structured to include a 5-day lead-in with thioridazine alone, as well as a combination phase with cytarabine. This design provided us with a unique opportunity to interrogate the safety as well as the biology of human AML in response to thioridazine alone and together with cytarabine within a single trial. We found that despite the presence of poor cytogenetic features that are associated with adverse therapy responses(Dohner et al., 2017), leukemic burden was alleviated in 8 of 11 patients following a 5-day exposure to thioridazine monotherapy (Figure 1, 3A). The effect of thioridazine appears to be irrespective of hydroxyurea, which was administered to 4 of the 8 of the patients that displayed reduced blasts levels, as

well as 2 of the 3 patients with progressive disease trends (Supplementary Table 2). Peripheral leukemic burden continued to decline with the addition of cytarabine (day 6-10) and up to day 22, coinciding with termination of thioridazine (Supplementary Figure 3). After day 22, we noted an increase in blast levels as patients approached the end point on day 36 (Supplemental table 1). While this pattern of failed re-induction is common in this patient population, an extended thioridazine treatment period beyond day 21 may afford a more durable anti-leukemic effect and potentially attenuate the rate of disease regeneration.

The variability in blast level changes could be explained by DRD2 expression across all doses (Figure 3E), indicating an on-target effect for thioridazine's anti-leukemic activity. Given that flow cytometry for cell surface proteins is a routine component of AML diagnostics (Grimwade and Freeman, 2014; Ivey et al., 2016; Terwijn et al., 2013), inclusion of DRD2 in this analysis may offer a bio-marker for patient pre-screening for more guided therapies in the future. While thioridazine as a monotherapy showed a level of efficacy against leukemic blasts, it did not appear to adversely affect residual healthy hematopioetic cell counts up to day 36, including residual neutrophil and platelet counts (Figure 3B-D). This observation indicates that thioridazine regimen did not induce significant leukopenia, an observation previously reported with a more chronic use of thioridazine (Ferguson et al., 1977; King and Wager, 1998; Weiden and Buckner, 1973). Other adverse event data were monitored and documented on a daily basis throughout the study. As previously reported with several

antipsychotic drugs, thioridazine has been associated with adverse effects, the most serious of which include neuroleptic symptoms and cardiotoxicity(Menkes and Knight, 2002). In our study, neurological adverse events were noted in 2 patients at dose level III with thioridazine at 100 mg q6h, equivalent of 400 mg/day. While it is likely that gradual dose escalation towards an equivalent dose of 400 mg/day may improve thioridazine tolerability in future trials (Abbate et al., 2012; Huang et al., 2015), dose level III of the current trial was considered toxic using the proposed treatment regimen. As a consequence of neurological symptoms at dose level III, the recommended phase II dose of thioridazine for this specific patient population was determined at 50 mg po q6h (dose level II). Neurological events at this dose were not reported and the other adverse events included moderate QTc elongation in 2 patients (481-500 msec). This observation was in line with previous studies reporting that QTc intervals rarely exceed 500 milliseconds at comparable therapeutic doses (Harrigan et al., 2004).

DRD2 targeting represents a novel approach for AML therapy, originated in a screening platform, evaluated in pre-clinical assays and ultimately tested in a clinical setting. The primary outcome measure of this study was to evaluate safety and we conclude that DRD2 antagonist thioridazine at 50 mg q6h can be safely administered to older AML patients with relapsed/refractory disease. However, this safe dose of drug did not achieve the projected therapeutic levels of plasma thioridazine (Sachlos et al., 2012) following detailed PK analysis. Alternative treatment regimens, including intra-patient dose escalation may enable achieving

tolerability at higher doses than tested in this study. Altered formulations or analogues of thioridazine, or alternative DRD2 antagonists that do not cross the blood-brain barrier may also be considered for future studies. In addition to clinical toxicity and PK studies, serial blood and marrow analysis provided evidence of an anti-leukemic effect. This effect was associated with the level of dopamine receptor expression. We suggest that less urgent cases of leukemic disease, or myelodysplastic syndrome (MDS) patients that are not eligible for chemotherapy may also be a target population for future studies evaluating thioridazine as a monotherapy.

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Figure 1

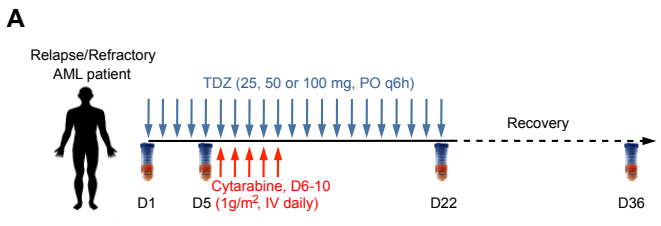


Figure 1. Study design. (A) Patients received thioridazine daily at 3 dose levels including 25 mg q6h (dose level I), 50 mg q6h (dose level II) and 100 mg q6h (dose level III) for 21days. Intermediate dose cytarabine at 1g/m^2 was administered as a 2-hour infusion for 5 consecutive days between days 6-10 of the study.

Figure 2

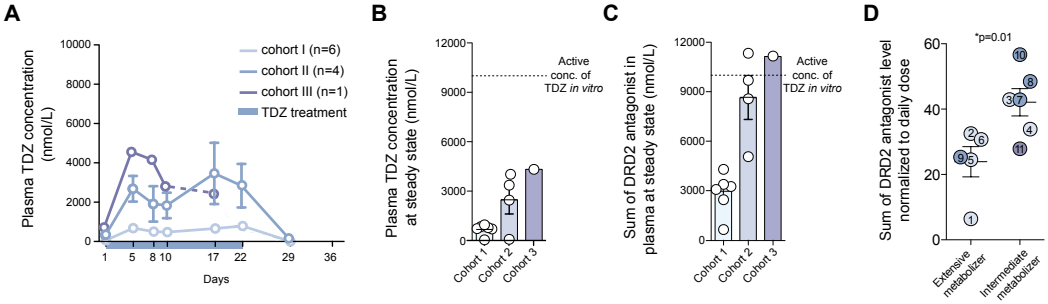


Figure 2. Pharmacokinetic analysis. (A) Thioridazine trough concentrations were measured on days 1, 5, 8, 10, 17, 22, 29 and 36 using LC-MS/MS for each study cohort. Plasma thioridazine concentrations reached a steady state by day 5 and thioridazine remained detectable up to day 29. In cohort III, thioridazine was discontinued early for patients 12 and 13 and only patient 11 received doses to achieve steady-state plasma thioridazine concentrations. This patient received thioridazine at dose level III (100mg q6h) for the first 10 days (solid line), after which thioridazine was de-escalated to 50 mg q6h (dotted line) up to day 16 and discontinued thereafter. (B) Plasma thioridazine levels at steady state in relation to the target 10μM concentration, previously shown to be effective against human AML *in vitro*(Sachlos et al., 2012). (C) Sum of plasma thioridazine and its 2 active metabolites (2-sulfoxide and 2-sulfone) with DRD2 antagonistic effects in relation to the target concentration of 10 μM. (D) Plasma DRD2 antagonist levels for individual patients normalized to daily dose of thioridazine. Patient variation in plasma DRD2 antagonist level was associated with *CYP2D6* genotype and its predicted phenotypes. P=0.01 by t-test. All summarized data are expressed as mean ± sem.

Figure 3.

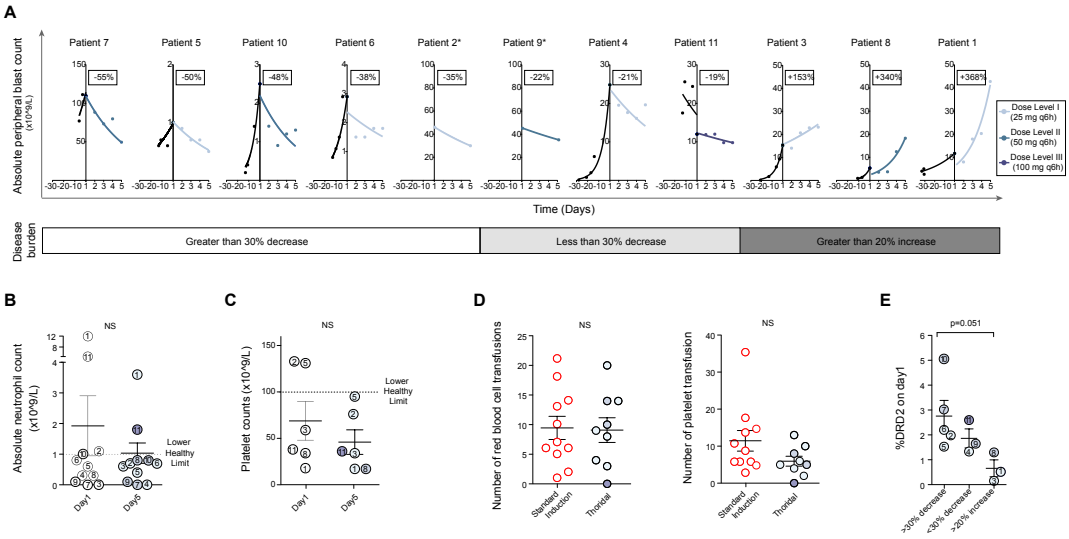
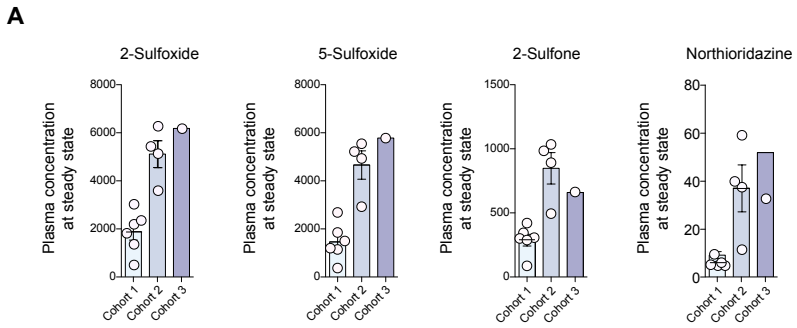


Figure 3. Response. (A) Disease burden in response to a 5-day treatment with thioridazine as a monotherapy was monitored at the level of peripheral blood absolute blast counts (ABC). Response for Patients 2 and 9 was determined by %bone marrow blast due to a lack of circulating blasts as indicated by the asterik. Patients 12 and 13 were excluded from this analysis as they did not complete the 5-day treatment with thioridazine. Black curves indicate disease progression based on ABC prior to the start of thioridazine treatment. Blue color coding indicates thioridazine dose level. Blast level fluctuations were grouped into 3 categories including patients that exhibited a greater than 30% reduction (n=5), patients that exhibited a less than 30% reduction (n=3) and patients that showed a greater than 20% increase of the disease burden (n=3). (B) Absolute neutrophil counts are not significantly reduced after a 5-day exposure to thioridazine for up to 100 mg q6h. 1.0x10^9/L was considered as the lower limit for healthy neutrophil count(Dohner et al., 2010). Patients 12 and 13 were excluded from this analysis as they did not complete the 5-day treatment with thioridazine. Each dot represents an individual patient (N=11). p= 0.3 by unpaired t-test. (C) A 5-day treatment with thioridazine does not significantly compromise platelet counts. 100x10^9/L was considered as the lower limit for healthy platelet count(Dohner et al., 2010). Data points include patients that completed the 5-day thioridazine treatment and did not receive platelet transfusions within the first 5 days of thioridazine, or up to 4 days prior to the start of the trial. p=0.3 by unpaired t-test. (D) Thoridal patients receiving thioridazine at up to 100mg/day q6h together with

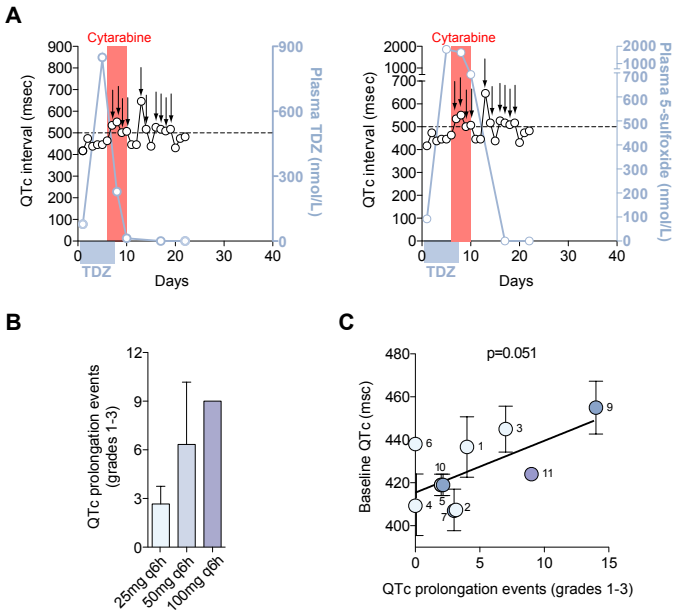
intermediate dose cytarabine did not require additional red blood cell or platelet transfusions compared to age- and disease- matched AML patients receiving standard re-induction chemotherapy with HiDAC, FLAG-IDA or Idarubicin + cytarabine. N=9 for Thoridal, day 1 versus 36. Patients 3, 8, 12 and 13 were excluded from this analysis as they missed a significant portion of the 21-day thioridazine treatment. N=11 for standard re-induction chemotherapy, day 1 versus 28. All summarized data are expressed as mean ± sem. (E) Comparison of DRD2 protein level on day 1 MNCs across the 3 response groups defined in 3A.

Supplementary Figure 1



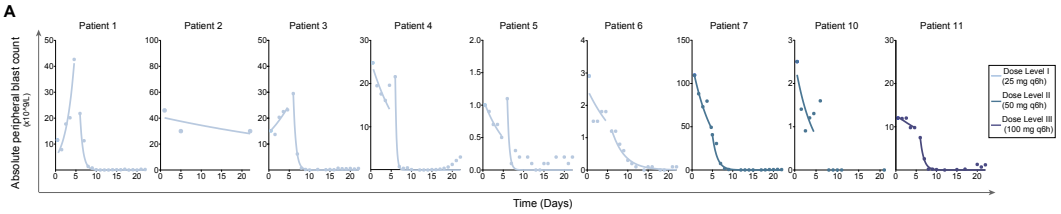
Supplementary Figure 1. Plasma levels of thioridazine metabolites. (A) Plasma concentration of thioridazine metabolites at steady state across the 3 dose levels. Dots represent individual patients. Data are expressed as mean ± sem.

Supplementary Figure 2



Supplementary Figure 2. QTc interval in response to thioridazine. (A) Daily ECG analysis and plasma measurements of thioridazine and 5-sulfoxide up to day 22 for Patient 3. This patient experienced a grade 3 QTc interval on day 7. Additionally, QTc exceeded 500 milliseconds (dotted line) on multiple other occasions as indicated by the arrows. Thioridazine was not detectable in the plasma on days 17 and 22. (B) Average QTc prolongation events of grades 1-3 (NCI-CTCACE, version 4.03) per cohort. ECG was recorded daily for all patients with the exception of Patients 8, 12 and 13 due to early termination. These Patients are not included in the analysis. (C) Average of 3 independent QTc measurements at baseline versus total QTc prolongation events of grades 1-3. ECG was recorded daily for all patients with the exception of Patients 8, 12 and 13 due to early termination. These Patients are not included in the analysis.

Supplementary Figure 3.



Supplementary Figure 3. Leukemic blast response to Thioridazine alone and in combination with intermediate dose cytarabine. (A) Clinical response to a 5-day treatment with thioridazine as a monotherapy and in combination with intermediate dose cytarabine in ABCs. The response for Patient 2 was determined by %bone marrow blast due to a lack of circulating blasts. Patient 8 was removed from the study early and was not included in this analysis. Patient 9 Day 22 blast values were unavailable and were therefore not included in this analysis.

Table 1. Patient characteristics at baseline

	Dose Level I (n=6)	Dose Level II (n=4)	Dose Level III (n=3)
Median Age (range)	66 (58-75)	71.5 (67-79)	71 (58-75)
Sex (M:F)	4:2	1:3	2:1
ECOG performance			
0	2	0	2
1	4	4	1
2	0	0	0
Diagnosis			
Relapse/refractory AML	4	3	2
AML-MRC	2	1	1
Cytogenetics risk group*			
Favorable	0	0	0
intermediate	4	2	2
Adverse/High	2	2	1
No. of prior chemotherapy regimens			
≤3	5	1	0
≥4	1	3	2
NA	0	0	1
BM blast % at base line (mean ± SEM)	50.1±10.8	57.6±6.1	31.6±6
ABCx10^9/L at base line (mean ± SEM)	8.1±3.8	28.5±27.6	12.2±12.2 (Pt 13 NA)

• Risk stratification was evaluated based on ELN (Dohner et al. 2017), EPI (Breems et al. 2005) and refractory score (Giles et al. 2005) criteria

Table 2. On-study grade ≥ 3 adverse events reported by ≥ 20% of patients

Adverse event	AE relation	Dose Level I (n=6)		Dose Level II (n=4)		Dose Level III (n=3)		Total frequency of occurrence
		Freq. per cohort, n (%)	Freq. of occurrence	Freq. per cohort, n (%)	Freq. of occurrence	Freq. per cohort, n (%)	Freq. of occurrence	
Sepsis	Unrelated	0 (0%)	0	1 (25%)	1	3 (100%)	3	4
Anemia	Unrelated	5 (83%)	14	4 (100%)	16	3 (100%)	9	39
Febrile neutropenia	Unrelated	4 (66%)	4	3 (75%)	3	2 (66%)	4	11
Lymphocytopenia	Unrelated	5 (83%)	15	3 (75%)	8	3 (100%)	17	40
Neutropenia	Unrelated	6 (100%)	22	4 (100%)	13	2 (66%)	5	40
Thrombocytopenia	Unrelated	6 (100%)	25	4 (100%)	27	3 (100%)	16	68

Table 3. Adverse events grade ≥ 2 possibly-related to thioridazine

Adverse event	Grade	Dose Level I (n=6)		Dose Level II (n=4)		Dose Level III (n=3)		Total frequency of occurrence
		Freq. per cohort, n (%)	Freq. of occurrence	Freq. per cohort, n (%)	Freq. of occurrence	Freq. per cohort, n (%)	Freq. of occurrence	
General								
Fatigue	2	1 (16%)	1	0 (0%)	0	0 (0%)	0	1
Urinary incontinence	2	0 (0%)	0	1 (25%)	1	0 (0%)	0	1
Neurological								
Gait disturbance	2	0 (0%)	0	0 (0%)	0	1 (33%)	1	1
Dizziness	2	0 (0%)	0	0 (0%)	0	1 (33%)	1	1
Depressed level of consciousness	3	0 (0%)	0	0 (0%)	0	1 (33%)	1	1
Cardiovascular								
QTc Interval prolongation (481-500 ms)	2	0 (0%)	0	2 (50%)	7	1 (33%)	2	9
QTc Interval prolongation (≥ 501 ms on at least 2 separate ECGs)	3	1 (16%)	1	0 (0%)	0	0 (0%)	0	1

Table 4. Patient pharmacogenetic profile

Patient ID	Cyp2D6 genotype	Copy number variation	Predicted phenotype
1	*1/*41	>2	Extensive metabolizer
2	*1/*1	2	Extensive metabolizer
3	*1/*9	>2	Intermediate metabolizer
4	*1/*4	2	Intermediate metabolizer
5	*1/*41	2	Extensive metabolizer
6	*1/*41	2	Extensive metabolizer
7	*1/*4	2	Intermediate metabolizer
8	*1/*4	2	Intermediate metabolizer
9	*1/*1	2	Extensive metabolizer
10	*1/*4	2	Intermediate metabolizer
11	*1/*4	2	Intermediate metabolizer
12	*10/*10	>2	Intermediate metabolizer
13	*1/*1	2	Extensive metabolizer

Supplementary Table 1. Study termination and disease response on Day36

Patient No.	Termination / reason	CG/Mol	Disease stage	Prognosis/ Classification	Day1 PB blast count	Day36 PB blast count	Day1 BM Blast %	Day36 BM Blast %
1	Normal	46,XY,del(7)(q11.2)	Refractory	High/ Refractory Score	11.6	14.6	54	69
2	Normal	46,XY/ Normal	Relapse	Intermediate/ EPI Score	0	0.4	46	7
3	Early/DLT	46,XX, t(8;14)(p11.2;q32.1)	Relapse-2	Intermediate-1/ Refractory score	15.1	0.6*	89	91*
4	Early/Disease progression	46,XY, del(9)(q13q22)/ FLT3-ITD+	AML-MRC Diagnosis	Intermediate-2/ ELN	24.8	135.3	87	78*
5	Normal	46,XX/Normal	Relapse	High/ EPI Score	1	1.8	61	70
6	Normal	46,XY/ FLT3-ITD+	AML-MRC Diagnosis	Intermediate-1/ ELN	2.9	8.3	25	80
7	Normal	46,XX, del(5)(q13q33)/ FLT3-D835+	Relapse	High/ EPI Score	109.7	37.1	94	71*
8	Early/Patient request	46,XX, t(4;6)(q23;p25), del(7)(q22q34), del(20)(q11.2q13.1)	Relapse	Intermediate/ EPI Score	0.9	18.4	81	NA
9	Normal	46,XY/Normal	AML-MRC Diagnosis	Intermediate-2/ ELN	0	0	45	34
10	Normal	46,XX/Normal	Relapse	High/ EPI Score	2.5	1.2	64	74
11	Normal	46,XY, der(22) t(1;22)(q11;p11.2)	Relapse	Intermediate/ EPI Score	12	16.6	73	69#
12	Early/DLT	Complex Karyotype	AML-MRC Diagnosis	Poor/ ELN	0	NA+	22	NA+
13	Early/DLT	46,XY/Normal	Relapse	Intermediate/ EPI Score	0.2	0	56	6

* Day22 BM blast% indicated as day36 value was unavailable
\# Value reported in a follow up later than day36
\+ Value unavailable as patient expired

Supplementary Table 2. Hydroxyurea and blood transfusions during the first 5 days

Patient ID	Hydroxyurea	Number of red blood cell transfusions	Number of platelet transfusions
1	Yes	2	0
2	No	0	0
3	Yes	0	0
4	Yes	2	2
5	No	0	0
6	No	0	0
7	Yes	2	0
8	No	2	0
9	No	2	0
10	Yes	2	1
11	Yes	0	0

CHAPTER 5: DISCUSSION

5.1 Thesis Overview

AML is one of the deadliest adult cancers (Ferrell et al., 2016) with poor survival rates and treatment options that fail to produce long-term outcomes. Identification of LSCs 20 years ago (Bonnet and Dick, 1997; Lapidot et al., 1994) offered the promise of leukemia eradication upon LSC targeting. Nevertheless, several barriers exist towards the integration of LSC-based approaches into clinical management of AML: Conceptually, examples of other leukemias that were durably managed with non-LSC directed therapies, such as CML, questioned the necessity of targeting LSC populations (Bhatia et al., 2003). Practically, targeting rare and quiescent LSC fractions that have not been demonstrated to participate in immediate leukemogenesis may not provide the urgent interventions needed for patients, especially in cases where leukemia disseminates into the blood and other organs, resembling the emergency states of metastasis already at diagnosis. As a result, cytotoxic chemotherapies that achieve immediate cytoreduction remain indispensable as first line of treatment. Finally, while LSCs have long been associated with major clinical challenges such as chemoresistance and relapse, concrete evidence that supports this theory has not been provided. To date, the biological basis of therapy failure remains ambiguous, and strong experimental foundations that elucidate the underlying mechanisms of therapy failure have not been established.

We developed an *in vivo* relapse model to formally characterize the cellular events that give rise to leukemic relapse for the first time, which allowed us to define a unique transcriptional bio-marker for relapse prior to overt regeneration of the disease. Through this in-depth molecular characterization of relapse, we were also able to identify a set of druggable targets that can be modulated to prevent disease recurrence.

5.2 Surrogacy of the *in vivo* relapse model

Residual leukemia subsets post-chemotherapy are rare and cannot be unambiguously discriminated from their healthy counterparts due to overlapping molecular and phenotypic properties (Eppert et al., 2011; Klco et al., 2014; Levine et al., 2015). As a result, it has not been possible to isolate and characterize the cellular fractions that contribute to therapy resistance directly from AML patients. In contrast, species-specific antigens between mouse and human provide unambiguous resolution even under states of low disease burden. As a result, we developed a xenograft-based relapse model to trace these residual cells after chemotherapy and investigate their involvement in relapse. In addition to the benefit of enhanced resolution, monitoring of vehicle-control treated xenografts in parallel to the AraC-treated group allowed us directly evaluate the causal effects of AraC on AML regeneration, providing a unique internal control not otherwise available in the clinic.

To mimic clinical induction chemotherapy *in vivo*, we focused on optimizing the tolerable dose and duration of AraC as it presents the backbone of chemotherapy and serves as the only cytoreductive agent that is routinely administered as a monotherapy (Reese and Schiller, 2013). AraC treatment *in vivo* resulted in reduction of human leukemia levels similar to that observed in AML patients in the clinic after exposure to AraC (**Chapter 2**). A similar observation was made by a comparable dose of AraC *in vivo* administered by a different group that developed a xenograft model of leukemic chemoresistance (Farge et al., 2017). This validated the surrogacy of the *in vivo* chemotherapy regimen that we employed to induce clinically meaningful levels of cytoreduction in our xenograft model of leukemia regeneration. Importantly, global transcriptional shifts of xenografted AML cells after *in vivo* exposure to AraC alone resembled those observed with an AML patient sample after clinical exposure to standard chemotherapy, which includes daunorubicin in addition to AraC. This highlighted that LRC development was not restricted to xenografting or AraC monotherapy (**Chapter 2**). Collectively, these observations indicated that our xenograft model provided a strong experimental foundation to explore the cellular dynamics that occur between remission and relapse.

Accurate surrogacy of the xenograft model in reflecting the biology of human AML has been challenged due to a recent study reporting that only mono-clonal xenografts could be established from poly-clonal parent AML tumors (Klco et al., 2014). This is critical as clonal evolution has been theorized to

contribute to leukemia recurrence and therapy resistance (Ding et al., 2012; Klco et al., 2014). However, a recent study contradicted these findings as they reported successful derivation of polyclonal xenografts with at least 4 of 5 distinct sub-clones detected in the de novo sample (Shlush et al., 2017). Application of more lenient xenografting methodologies such as IF injection or pre-conditioning may help avoid skewing of clonal architecture by reducing the selective pressures that are endured upon xenotransplantation.

While genetic alterations are proposed to give rise of therapy failure, next generation sequencing has not been able to explain the mechanisms of therapy resistance or the basis of clonal survival, growth advantage or dominance in a straight forward manner. In fact, only modest alterations have been found in the composition of clones or in mutations that consistently occur across patients from diagnosis to relapse (Ding et al., 2012; Ho et al., 2016). A separate study corroborated few changes in clonal architecture over time. However, they described dramatic alterations in surface, intracellular and signaling profiles of leukemic cells using high dimensional mass cytometry (Ho et al., 2016). As a result of these observations, we applied global gene expression analysis as our method of choice to characterize the molecular basis of leukemic recurrence. This approach successfully led to the identification of a number of druggable gene products. Proof-of-concept functional assays demonstrated the validity of our approach and the therapeutic value of our unique gene signature **(Chapter 2)**.

5.3 A preventative therapy approach against leukemic relapse

Relapsed disease becomes increasingly difficult to manage and therapy options become even more limited at relapse (Dohner et al., 2010). This highlights the value of alternative approaches that offer a *preventative* measure against relapse, instead of treating at relapse. Our study uncovered early cellular and transcriptional dynamics that give rise to leukemic regeneration, prior to the occurrence of overt disease. The observed transcriptional shifts were transient at early phases of regeneration and were not detectable at diagnosis or overt relapse, suggesting a transient window of therapeutic opportunity. Proof-of-concept suppression of one of the identified molecular targets afforded suppression of leukemia regeneration and relapse prevention. While it may be argued that current consolidation therapies also provide a preventative measure against relapse, these therapies are predominantly AraC-based (Dohner et al., 2017) and may in turn provoke accelerated leukemic re-growth according to our findings (**Chapter 2**). Other means of consolidation therapy with allo-HSCT are associated with high rates of morbidity and mortality (Dohner et al., 2010; Lowenberg, 2008), resulting in allo-HSCT to be considered only for a subset of patients.

Ultimately, while LRC-targeted approaches may not eradicate AML, they offer durable suppression of aggressive disease features. This not only presents a novel therapy approach with a focus on relapse prevention, but it also offers an

alternative perspective on therapy goals to keep disease levels under control under circumstances where aggressive leukemic disease cannot be eradicated.

5.4 Investigation of LSCs as the origin of leukemic relapse

While relapse has long been associated with LSCs that persist through chemotherapy, a recent study reported that only a fraction of relapse cases originate from sub-clones with LSC capacity. However, in some patients, relapse-fated clones did not harbor functional LSC properties (Shlush et al., 2017), suggesting that some instances of relapse may arise independently of LSCs. Additionally, LSCs have been suggested to persist through anti-proliferative chemotherapies due to their dormant state. However, these interpretations originated from early studies that applied acute chemotherapy regimens *in vivo* (Saito et al., 2010) that are unlike clinical regimens of chemotherapy. In contrast to these findings, our observations as well as others (Farge et al., 2017) demonstrated that LSC subsets did not preferentially persist through clinically-relevant chemotherapy regimens that include repeated exposure to cytotoxic agents. As previously suggested for other CSCs (Kurtova et al., 2015), dormant CSCs may become recruited in active cycling after repeated doses of chemotherapy to compensate for the loss of leukemic blasts. Concurrently, cycling CSCs become increasingly sensitive to S-phase targeting chemotherapy (Saito et al. 2010), similar to their healthy counterparts (Wilson et al., 2008; Jorgensen et al., 2006, Morrison et al., 1997).

This dynamic response of LSCs to chemotherapy-induced damages suggests that LSCs are neither inert nor invincible as previously presumed. Emerging concepts in other cancer types support that chemotherapy may reshape the tumor hierarchy and induce CSCs to adopt a dynamic role to overcome the cytotoxic consequences of chemotherapy (de Sousa e Melo et al., 2017; Ebinger et al., 2016).

Consistent with the exit of CSC pools from dormancy into cycling post-chemotherapy (Kurtova et al., 2015), our findings indicated that cellular subsets that actively contribute to leukemia recurrence harboured highly clonogenic properties. This highlights the direct and immediate role of clonogenic fractions in regulating clinically relevant features of the disease. It also suggests that an alternative therapy approach to focus on clonogenic cancer fractions may provide a more tangible benefit than LSC-directed strategies that pursue long-term goals, especially in the case of aggressive cancers such as AML where immediate therapeutic interventions are required.

5.5 Interrogation of the signaling basis of chemotherapy-induced regeneration

Our observations indicate that exposure to cytotoxic-based chemotherapy directly reshapes the properties of the AML hierarchy. However, little is known about the nature of cell intrinsic and extrinsic signals that are induced by chemotherapy. Studies in human ALL have recently described the participation of

niche-related factors in the dynamic response of leukemia cells after exposure to chemotherapy (Duan et al., 2014; Ebinger et al., 2016). Consistently, our preliminary observations indicated that the regenerative effects of AraC on human AML cannot be recapitulated *in vitro*. However, incubation of de novo AML patient samples with serum obtained from *in vivo* AraC-treated mice reproduced the clonogenic effects of AraC, similar to that observed *in vivo* **(Chapter 2)**. This implied that the *in vivo* microenvironment may be involved in conveying the AraC-induced regenerative signals. Additionally, these findings imply that the niche involvement in communicating regeneration cues does not occur through BM geographic coordinates, but rather through soluble signals generated by the niche. Moreover, these regeneration signals demonstrated a temporal pattern, consistent with the transient nature of LRC features *in vivo* **(Chapter 2)**. Specifically, maximal clonogenic effect was observed with serum collected early post-AraC whereas serum samples obtained at a later time point were unable to elicit a significant clonogenic response. In line with these observations, Quach et al reported a transient elevation of several inflammatory cytokines including TNF-a, IL-6 and the monocyte chemoattractant protein 1 (MCP1) in mice exposed to cytotoxic injury (Quach et al., 2015). Concurrently, TNFa and IL-6 have been shown to stimulate the regeneration of various tissues such as liver, epithelia (Ando et al., 2003; Yamada et al., 1997) and injured intestinal mucosa (Becker et al., 2004; Chen et al., 2003; Grivennikov et al., 2009). It is likely that the cytokine cascade may also coordinate the regrowth of leukemia populations

after chemotherapy-induced cytotoxic injury and is therefore valuable to investigate in future studies. In addition to cytokines, other signaling mediators such as catecholamines may also be involved in the chemotherapy-induced leukemic regeneration. Dopamine levels may rise after exposure to chemotherapy given that emesis is a common side effect associated with chemotherapy and is partly regulated by elevated dopaminergic signals (Grunberg, 1989). Concurrently, our observations demonstrated that DA promoted the clonogenic capacity of AML patient samples *in vitro* **(Chapter 3)**. Furthermore, analysis of BM cellularity post-chemotherapy revealed additional morphological changes to the BM architecture including dynamic changes to the BM adipogenic content, which may take part in orchestration of the niche-mediated regenerative response to chemoetharpy.

The nature of these signals can be interrogated using cutting-edge methodology with matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS), which enables characterization of a variety of molecules including proteins, peptides, lipids, etc. (Wildburger et al., 2015; Woods and Jackson, 2006). Notably, MALDI-IMS can be customized to determine the spatial distribution of signals while maintaining the cellular integrity of the tissue (Caldwell and Caprioli, 2005; Fujino et al., 2016; Walch et al., 2008). Overlaying the chemotherapy-induced molecular map of the niche with the detailed cellular map of the BM will help unravel the nature and the source of the regenerative signals that may be involved in coordinating leukemic relapse.

5.6 DRD biology in regulating features of neoplasticity

Dopamine receptors have been classically associated with the functions of the nervous system such as memory, learning, locomotion and regulation of the sympathetic tone (Beaulieu et al., 2011). Recently, the biological processes mediated by DRDs were re-evaluated as they were found to be expressed in transformed cells of various tissues. Neoplastic cells of hematopoietic, breast, ovarian and brain origins displayed an upregulation of DRD members (Borcherding et al., 2016; Bowen et al., 2009; Robinson et al., 2012; Sachlos et al., 2012). In glioblastomas, patients with higher levels of DRD4 and tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis, showed inferior survival outcomes compared to those with lower DRD4 levels (Dolma et al., 2016). In addition to their recurring presence in multiple cancer cells, DRDs were found to be involved in mediation of proliferation (Lu et al., 2015), cell motility (Spiegel et al., 2007), invasion (Borcherding et al., 2016) and metastasis (Borcherding et al., 2016; Lu et al., 2015). These findings suggest that in addition to their classic roles as mediators of the nervous system, these cell surface antennae may sense environmental cues and instruct key neoplastic properties.

While the presence of DRDs in tumors of various tissue origins may suggest that a unifying paradigm exists across different cancers, the mechanisms downstream of DRDs appear to rely on the presence of specific DRD subtypes and their engagement in various signaling circuitries in a cell context dependent

manner (Beaulieu et al., 2011; Beaulieu and Gainetdinov, 2011; Perez et al., 2006). In human AML cells, we found an intact DRD-cAMP-CREB signaling axis, which is the predominant signaling pathway associated with DRDs as previously described in neuronal cells. cAMP elevation downstream of DRD led to a suppression of leukemic clonogenicity and induction of cellular maturation **(Chapter 3)**. The role of cAMP in promotion of hematopoietic maturation has been previously reported (Brodsky et al., 1998; Tortora et al., 1989). In a large-scale screen of chemical compounds, 5 of the 8 lead candidates capable of inducing functional myeloid cell maturation were known inducers of cAMP (Stegmaier et al., 2004). Moreover, cAMP elevation downstream of DRD led to the activation of transcription factor CREB in AML cells. Preliminary data validated an upregulation of CREB target genes including dFOSB in TDZ- versus control-treated leukemic xenografts. dFOSB is a highly stable molecule that participates in durable epigenetic remodeling following acute molecular stimuli (Nestler et al., 2001). This may explain how short-term exposure to DRD-directed therapy with a specific antibody and small molecules was able to sustain durable suppressive effects on leukemic progenitor function **(Chapter 3)**. Future studies are required to investigate the mechanism by which DRD signaling participates in the epigenetic memory within the context of leukemia cells.

5.7 Concluding remarks

For decades, academic discoveries in AML research have suggested that LSCs are the predominant driving force of chemoresistance and relapse whereas for the clinical side, the underlying mechanisms of therapy failure span beyond the LSC concept. This has resulted in divergent targeting ideologies and therapy approaches between the two camps. However, regardless of the belief system in LSCs, there is unity that therapy failure remains a major clinical challenge for AML and novel therapeutic approaches are required to improve the unsatisfactory survival rates for AML patients.

The studies within this thesis were designed to unravel the biological basis of therapy failure, focusing on leukemia cell vulnerabilities that can be targeted to overcome clinical challenges of therapy resistance and relapse. These studies offer a xenograft-based relapse model that can be used to evaluate the efficacy of novel compounds in conjunction with AraC towards a durable suppression of leukemic relapse. Using this model, we were able to characterize the cellular, transcriptional and chronological events of leukemia recurrence post-chemotherapy. Unlike the traditional views of LSC involvement, our observations suggest that alternate cellular mechanisms, that are directly instructed by chemotherapy, coordinate leukemia recurrence. These newly described pathways of regeneration provided druggable targets, including a member of the DRD family. Follow-up functional studies defined a unique role for DRD in human AML biology and suggests DRDs a leukemia-selective therapy target.

The compelling pre-clinical data using DRD-targeted approaches by our group motivated a phase 1 clinical trial, to evaluate the safety and efficacy of DRD targeting with TDZ in a clinical context. A safe dose of TDZ was able to reduce leukemic blast levels in the majority of patients. Higher doses of TDZ that could potentially induce clinical level remission could not be safely administered due to treatment-related side effects (**Chapter 4**). Future efforts are required to investigate whether alternate means of DRD targeting including analogues of TDZ will produce superior safety and efficacy, particularly towards a more durable management of AML. Overall, this thesis suggests that sophisticated experimental surrogates provide an authentic understanding of the leukemia cell biology, which can be used to devise informed therapies to ultimately reduce the gap between academic discoveries and clinical application (**Figure 1**).

Figure 1

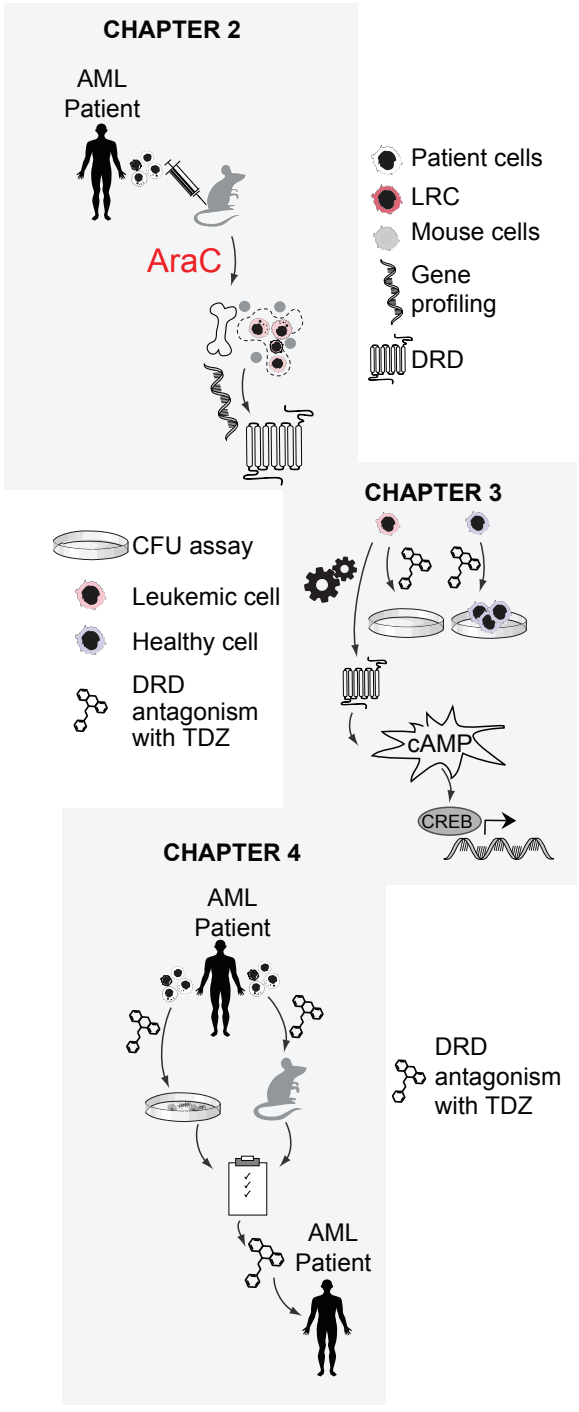

CHAPTER 2
AML Patient
AraC
Patient cells
LRC
Mouse cells
Gene profiling
DRD
CHAPTER 3
CFU assay
Leukemic cell
Healthy cell
DRD antagonism with TDZ
cAMP
CREB
CHAPTER 4
AML Patient
DRD antagonism with TDZ
AML Patient

Figure 1. Summary of the experimental approaches
The diagram illustrates the experimental approaches used in the thesis. In Chapter 2, xenograft-based assays coupled with transcriptional profiling provided druggable targets against human AML. In Chapter 3, we unraveled the mechanism of action downstream of DRDs in human AML cells. Also, we employed *in vitro* assays to characterize the functional consequences of DRD targeting on healthy versus leukemic hematopoiesis. Our *in vitro* and *in vivo* pre-clinical findings demonstrated the efficacy of DRD-targeted approaches and motivated a phase 1 clinical trial (**Chapter 4**).

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