Leukemia treatment: studies exploring bone marrow microenvironment, drug resistance and cannabidiol

Jaramillo Mantilla, Christopher Adrian

2021

document version
Publisher's PDF, also known as Version of record

LEUKEMIA TREATMENT: STUDIES EXPLORING BONE MARROW MICROENVIRONMENT, DRUG RESISTANCE AND CANNABIDIOL

Christopher Adrian Jaramillo Mantilla

This research was performed at the Department of Hematology of the Erasmus MC, Cancer Institute, Rotterdam, the Netherlands, and at the Laboratory of Hematology, Amsterdam University Medical Centers (AUMC), location VUMC, Amsterdam, the Netherlands. Some work was performed within the Laboratory Medical Oncology of the AUMC, Cancer Center Amsterdam, location VUMC, Amsterdam, the Netherlands. Financial support was obtained from Secretariat of Science and Technology of Ecuador SENESCYT (Ecuador) and from funds from the Laboratory Hematology, AUMC, location VUMC, Amsterdam, the Netherlands.

All previously published papers were reproduced with permission from the publisher.

Published by Amsterdam UMC

VRIJE UNIVERSITEIT

LEUKEMIA TREATMENT: STUDIES EXPLORING BONE MARROW MICROENVIRONMENT, DRUG RESISTANCE AND CANNABIDIOL

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op maandag 21 juni 2021 om 13.45 uur
in de online bijeenkomst van de universiteit,
De Boelelaan 1105

door
Christopher Adrian Jaramillo Mantilla
geboren te Quito, Ecuador

promotoren:

prof.dr. G. J. Peters

prof.dr. J. Cloos

Copromotoren:

dr. G. Jansen

dr. R. W. Stam

To my wonderful ladies: Jenny, Carol, Diana and Arwen and Saba

CONTENTS

CHAPTER 1

Introduction to hematopoiesis, bone marrow niche, leukemia and drug resistance

GENERAL INTRODUCTION

Acute leukemias are a diverse group of blood cell malignancies that arise from the clonal expansion of myeloid (acute myeloid leukemia, AML) or lymphoid (acute lymphoid leukemia, ALL) leukemic hematopoietic progenitor cells. Leukemic cells are characterized by aberrant intracellular processes that lead to a block in cellular differentiation and abnormal cell proliferation, resulting in accumulation of immature cells in the bone marrow (BM) and peripheral blood (PB)[1–4]. Leukemic cells are non-functional and interfere with normal blood cell production, resulting in weakness, predisposition to bleeding and infection, among other symptoms and life-threatening complications[5,6].

In the healthy/non-malignant cell, a series of synchronized checkpoints must be passed before cell division can occur. Leukemic cells fall away from this tightly orchestrated progression and acquire new capabilities which allows them to reprogram nutrient metabolism, evade cell cycle checkpoints and achieve replicative immortality[7–10]. By resisting apoptosis, cells proliferate continuously, loose function, and destroy healthy tissue architecture[7].

Emerging evidence suggests that the surrounding extracellular BM microenvironment is also relevant in leukemic transformation and progression by stimulating cellular proliferation and angiogenesis[11–13]. From this perspective, leukemia, similar to many types of malignancies can be seen as a process that takes place in two basic compartments: an intracellular and extracellular compartment, composed by the or BM niche or microenvironment (BMM). Under normal conditions the BMM, composed by stromal cells, endothelial cells, osteoblasts/osteoclasts, nervous fibers, and pericytes among other cell types[14–17] create a protective and nurturing niche for the hematopoietic stem cells (HSC)[7,14]. For example, specialized BM endothelial cells provide paracrine signalling that promote stem cell proliferation and BM regeneration after injury[14,18–20]. Leukemic stem cells (LSC) compete with healthy HSC for the BM niche, to further support proliferation[7,15,16,21,22].

The HSC and BMM constitute two compartments separated by the cellular membrane, through which they interact (either via cell-cell adhesion, microvesicle packaging or ligand-receptor interaction). Malignant cells are able to highjack and manipulate these intracellular and extracellular compartments to promote their own proliferation and survival[7,8], while expressing membrane proteins, such as ATP-binding cassette transporters (ABC transporters), among others, that restrict control of drug transport from one compartment to another. This tighter control over transport systems of xenobiotics allows leukemic cells to become multidrug resistant[7,8,23,24].

The dynamic interaction between these compartments contributes to leukemogenesis by stimulating cell self-renewal, manipulating nutrient/drug metabolism and transport, supporting developmental arrest, and cell cycle dysregulation[1,7,22].

Under normal conditions the BM microenvironment, composed by stromal cells, endothelial cells, osteoblasts/osteoclasts and pericytes among other cell types[14–17] creates a protective and nurturing niche for the hematopoietic stem cells (HSC)[7,14].

For example, specialized BM endothelial cells provide paracrine signalling that promote stem cell proliferation and BM regeneration after injury[14,18–20]. Leukemic stem cells (LSC) compete with healthy HSC for the BM niche, to further support proliferation[7,15,16,21,22].

The dynamic interaction between these compartments contributes to leukemogenesis by stimulating cell self-renewal, manipulating nutrient/drug metabolism and transport, supporting developmental arrest, and cell cycle dysregulation[1,7,22].

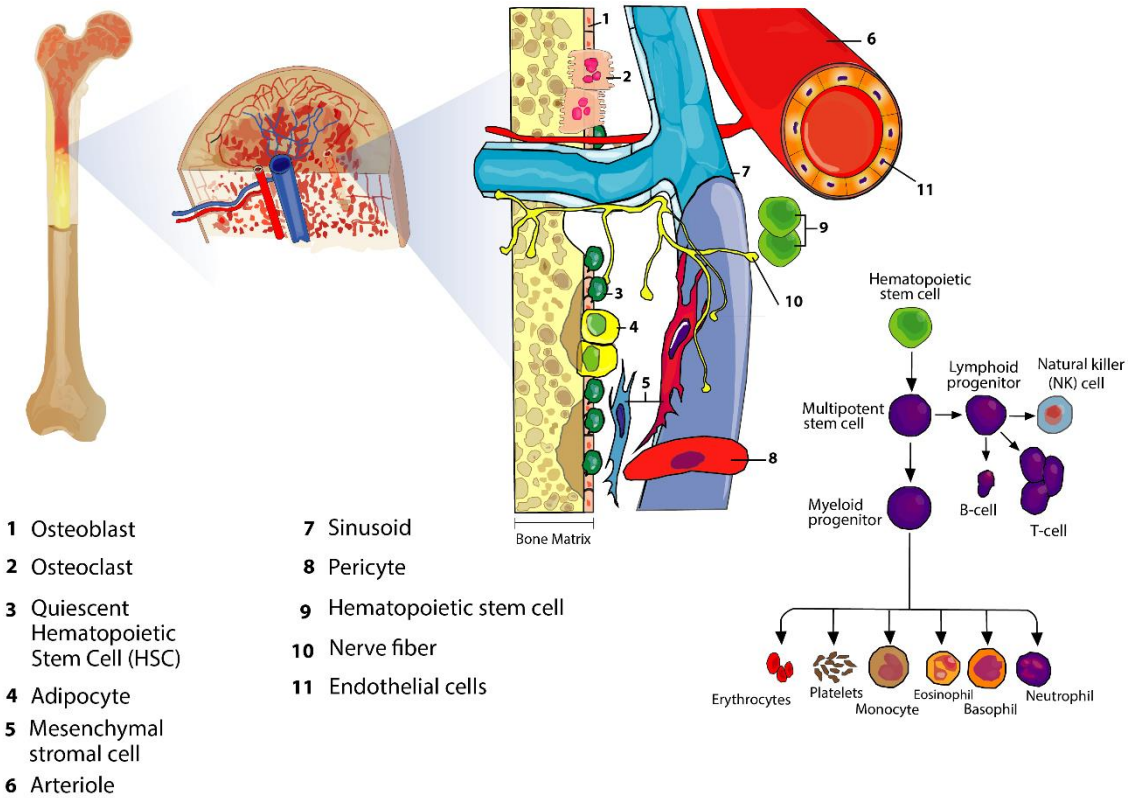


Figure 1: *Schematic representation of hematopoiesis. The bone marrow niche is a complex microenvironment composed of structures such as arterioles, sinusoids and nerve fibers, along with diverse cell types such as bone cells, (osteoblasts and osteoclasts), adipocytes, endothelial cells (composing arterioles and sinusoids), pericytes, mesenchymal stromal cells among others, all of which sustain HSCs and hematopoiesis.*

Bone marrow, hematopoiesis and leukemia

Hematopoiesis takes place within the BM, which is a highly innervated and vascularized tissue[25]. HSCs reside within the endothelial sinusoids and nerve fibers of the BM, and are responsible for all blood cell restitution. Under healthy/normal conditions, HSC divide in a highly regulated manner to produce myeloid or lymphoid progenitors which in turn will generate the different lineages of blood cells, on physiological demand[15]. The BM microenvironment cells supports hematopoiesis by secretion of proliferative factors (such as IL-33 by endothelial cells) which stimulates hematopoiesis and BM regeneration after injury[14,18] (Figure 1).

Epidemiology of leukemia

In 2018, leukemia was ranked as the 11th most common cancer worldwide and accounting for 3.5% of all cancers[26,27]. In the Netherlands, it is the 14th most common cancer and its yearly incidence in 2018 of around 1600 cases[27]. Even though adult and childhood-onset leukemia have distinct epidemiological differences, many common environmental risk factors (RF) have been identified (radiation and chemical exposure, as well as certain retroviral infections). Genetic conditions such as Down syndrome, Li-Fraumeni, and neurofibromatosis are also well known RF. Still, most patients with leukemia have no previous history of genetic or environmental RF[3,28].

Epidemiology of ALL in children and adults

Overall, ALL is more common in children and AML is more common in adults. ALL has a bimodal distribution in the population, affecting both children and the elderly, yet it is much more frequent in childhood[2]. In fact, childhood ALL is the most common type of cancer in childhood, with a prevalence of up to 25% of all diagnosed cancers in patients <15 years old[29]. Most childhood ALL cases are of B-cell lineage (85%), followed by T-cell ALL (10-15%) while NK (<1%) cell leukemias are very rare[5]. The therapy outcome of pediatric ALL has improved outstandingly in the last decades. Fifty years ago, the survival of children with ALL was as low as 10%–20%[30] but with current treatment regimes, 8 to 9 out of every ten children with ALL are cured in high-income countries[31]. Nonetheless, there are still 10-20% of high risk group of children with ALL who have a poor outcome, mainly due to multidrug resistance, such as in t(4;11) *MLL*-rearranged leukemias, Philadelphia chromosome positive cases t(9;22) *BCR/ABL,* or hypoploidy (≤44 chromosomes)[32,33]. On the other hand, ALL is uncommon in adults where patients face a poor long-term survival (30–40%) which worsens with age, even with an initial positive response to induction therapy[34,35].

Epidemiology of AML in children and adults

AML is much more common in adults and it becomes more frequent as the population ages (accounting for ~ 80% of leukemia cases)[3,26]. Adult-onset AML has a worse prognosis than pediatric AML, with a 5-year overall survival (OS) of around 40% which gradually declines with age, until it reaches around 4% for patients >75 years[36,37]. In contrast, childhood-onset AML only accounts for 15% of pediatric leukemia cases, yet, the long-term OS is around 60-70%, which warrants new treatment strategies[38].

Treatment and Prognosis in ALL

Treatment of ALL is also risk-stratification dependant. The current treatment protocol of adult ALL has been adapted from pediatric protocols[34]. In pediatric patients, current chemotherapeutic treatment, consists of induction, consolidation and long-term maintenance and central nervous system (CNS) prophylaxis. This treatment strategy results in high cure rates in children (~ 90%)[2,34]. The backbone of induction therapy typically includes vincristine, corticosteroids and an anthracycline. The goal of

induction therapy is to achieve complete remission and to restore normal hematopoiesis[34,39]. Unfortunately, the 5-year OS ranges between 30- 40% in patients > 75 years old, and in children with ALL, approximately 20% will relapse and face a poor survival with current treatment options[2,28,34]

Treatment and prognosis in AML

Treatment of leukemia depends on risk stratification. Childhood AML treatment often includes two courses of intensive induction chemotherapy followed by either a cytarabine-based consolidation therapy or hematopoietic cell transplantation (HCT). For standard induction chemotherapy, an anthracycline (for example, daunorubicin or idarubicin) plus cytarabine (Ara-C) is frequently used[38,40]. The cure rate with current treatment protocols reaches between 60-70% and depends on risk stratification[38,40]. Still, 30-40% of pediatric AML patients relapse and have a poor prognosis[41].

For induction therapy in adults, the “7+3” regime is frequently used, which includes cytarabine (administered continuously for seven days) plus anthracycline on days 1 to 3 of treatment. This is followed by a consolidation phase comprising one or more high-dose Ara-C (HiDAC) courses or HCT[6].

Multidrug resistance and leukemia: where treatment fails

Even though the development of new drugs and treatment combinations has improved the cure rate of patients with leukemia, a major challenge that cancer patients face is drug resistance. Multidrug resistance (MDR), defined as the pharmacological resistance to multiple chemically unrelated drugs, remains one of the contributing factors in chemotherapy failure in leukemia[42]. Both intrinsic (primary) or acquired MDR in leukemic cells can lead to low intracellular drug concentration, resulting in ineffective treatment[24,43].

One of the most studied mechanisms of drug resistance is the expression of ATP-binding cassette (ABC) transporters, usually expressed at the cellular membrane. This superfamily of drug efflux transporters includes 49 genes identified in the human genome- encoding for transmembrane transporters[44,45]. while expressing membrane proteins, such as ATP-binding cassette transporters (ABC transporters), that restrict control of drug and nutrient traffic, which allows them to become multidrug resistant[7,8,23,24].

P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2), and multidrug resistance proteins 1-5 (MRP1-5, ABCC1-5) represent the most relevant ABC transporters in the context of drug resistance. The physiological function of ABC transporters is to detoxify cells from toxic xenobiotics. Not surprisingly, they are highly expressed in the gut, at the blood-brain barrier, kidney and lungs. Due to their broad transport capacity, ABC transporters allow the active extrusion of hydrophobic, hydrophilic, conjugated or unconjugated drugs, positively or negatively charged, across the cell membrane. Drugs targeted by ABC transporters include important drugs such as methotrexate (MTX)[46,47], tyrosine kinase inhibitors (TKIs), vincristine,

cytarabine (Ara-C), dexamethasone (Dex) among many others used in leukemia[24,43,44,48]. Some ABC transporters seem to have a higher expression in malignant cells of multiple cancer types, including leukemia, where it can impact survival[23,24,49,50]

The clinical significance of the expression of these transporters singularly, in cancer cells, is still controversial[51], even though many of them have been found to have higher expression in malignant cells of multiple cancer types, including leukemia[24,49]. Nevertheless, the orchestrated expression of multiple ABC transporters has been shown to can affect intracellular MTX concentration ex vivo and lower patient survival, as seen in childhood leukemia[50]. Another example in childhood AML is the expression of nucleoside transporters hENT1 and hCNT1, which contribute 80% of cytarabine uptake and are widely expressed at diagnosis[45,52,53,54]. Notably, BCRP and P-gp expression have been identified previously in drug-resistant LSC in AML and chronic myeloid leukemia (CML)[55,56]. Furthermore, pharmacological strategies have been studied to bypass drug efflux in LSC "side populations", as a strategy to overcome MDR in leukemia[55,57,58].

Extracellular contributions to MDR are also important, as the BM niche has a key role in oncogenesis and multidrug resistance[12,59]. Proliferative factors, (such as IL-33) have been identified as important contributors to BM and bone regeneration post-chemotherapy[14,60]. This suggests that if LSC persist after myeloablation, they can potentially sequester the proliferative capacity of the BM niche and exploit it to further leukemogenesis.

Experimental therapeutics in leukemia: Cannabidiol as an emerging anti-cancer drug

Cannabidiol (CBD) is a non-psychoactive plant-derived phenol cannabinoid, which possesses remarkably low toxicity. Due to its ability to penetrate the blood-brain barrier and its potent anti-inflammatory[61–63], anti-oxidative[61,64] and anti-ischemic protective effects[65,66], CBD has emerged as an interesting pharmaceutical target in CNS diseases such as epilepsy[67] and Alzheimer's disease[68]. CBD interacts with the endocannabinoid system (ECS), which is composed of endocannabinoids (such as anandamide and 2-arachidonoyglyerol) and cannabinoid receptors CN1/CN2 and putative receptors GPR55, GPR119 and TRPV1 among others. The ECS is involved in sleep, attention, appetite, lipid metabolism, regulation and immunomodulation. CBD has a series of pleiotropic non-toxic effects in all of these physiological functions[69], and thus represents a potential pharmaceutical target in a myriad of diseases[70–72] (Table 1, Figure 2 and Figure 3).

It has been shown that CBD possesses anti-tumor effects against various cancer cells (Figure 3) that express a wide array of resistance mechanisms (such as CNS tumors, lymphoma, thyroid, breast, uterus, ovary, prostate, colon and lung cancer, as well as malignant leukemic cell lines such as HL-60 and Jurkat[102–104]). Leukemic cells exposed to CBD undergo oxidative stress via up-regulation of NAD(P)H oxidases, $p22^{phox}$ and Nox 4 resulting in apoptosis[103] .

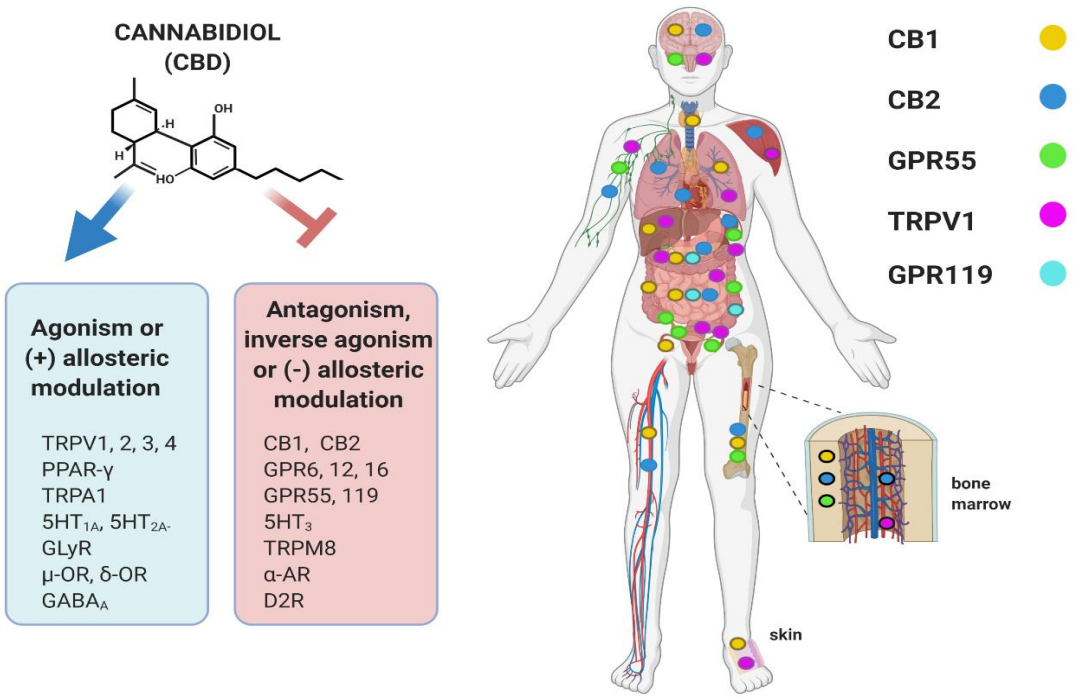


Figure 2: *Pleiotropic effects of cannabidiol (CBD) on various receptors and receptor localization. Left: CBD interacts with wide range of receptors localized in all systems and tissues. This pleiotropic activity explains the complex pharmacodynamic profile of CBD and its multiple therapeutic uses. Right: the diverse localization of various putative and canonical cannabinoid receptors (CB1,CB2) in multiple tissues and systems. Abbreviations: transient receptor potential cation channel subfamily V member 1-4 (TRPV1-4), peroxisome proliferator-activated receptor gamma (PPAR-γ), transient receptor potential ankyrin 1 (TRPA1), serotonin receptors (5HT), glycine receptor (GLyR), Mu and delta opioid receptors (μ-OR, δ-OR), gamma-aminobutyric acid receptor type A ($GABA_A$), cannabinoid receptor type 1 and 2 (CB1, CB2), G-protein coupled receptor (GPR), transient receptor potential channels type 8 (TRPM8), adrenoreceptor alfa (α-AR), dopamine receptor D2 (D2R). This figure was created with Biorender.com*

CBD exposure also down-regulates pivotal pathways involved in leukemic transformation, such as MAPK/ERK, PI3K/AKT/mTOR and Id-1[105–107]. Intrinsic and extrinsic pathways of apoptosis were found to be activated upon CBD treatment in Jurkat, colon cancer, glioma and breast cancer cell lines[102,103,105,106,108]. CBD is able to inflict selective mitochondrial membrane damage ensuing cytochrome-c furthering stimulating apoptosis activation[103,109,110]. Notably, a few studies reported the CBD may exert cell proliferative effects and the concentration range between toxicity and proliferation is very small, suggesting that it should be handled carefully in an oncological setting[111,112].

Furthermore, MDR efflux proteins, such as P-gp/MDR1, ABCG2/BCRP are negatively modulated by CBD at the protein and mRNA level in chemo-resistant cells *in vitro*. Sub-toxic concentrations of CBD sensitized MDR-protein overexpressing cells to cytotoxic effects of chemotherapy and increased the intracellular concentration of chemotherapeutic agents[116,117]. Moreover, in the malignant microenvironment, CBD reduces inflammation and blocks angiogenesis by inhibiting inflammatory factor HIF-

1α (hypoxia-inducible transcription factor) resulting in reduced tumorigenesis[13,108]. The anti-inflammatory and immunomodulating properties of CBD in the BM are currently being explored in the clinical setting to prevent graft-versus-host disease post HCT [118]. The potential anti-cancer properties of CBD are currently being studied in several clinical trials (clinical trial #: NCT01812616 & NCT01812603 for glioblastomas, NCT02255292 for solid tumors, NCT02432612 for pharmacokinetics of CBD in advanced cancer, NCT02423239 for hepatocellular carcinoma & pancreatic cancer).

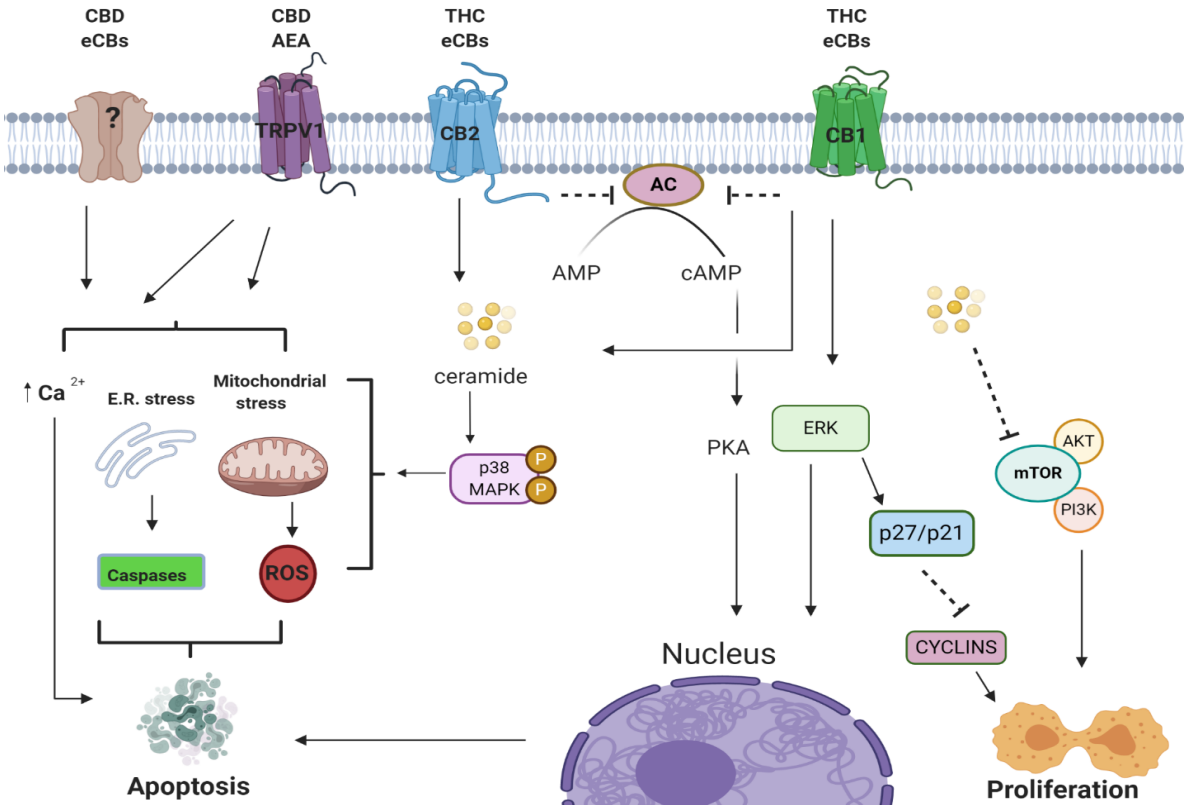


Figure 3: *Known signaling pathways involved in (endo)cannabinoids induced apoptosis in cancer cells. Activation of cannabinoid receptors (CB1 and CB2) induces inhibition of intracellular cAMP, resulting in reduced PKA activity. This in turn alters gene expression by multiple mechanisms, including transcription inhibition via the reduced cAMP response binding element (CREB)[113] and protein tyrosine phosphatase activation which also initiates the ERK1/2 signaling cascade[114]. CBD stimulation of TRPV1 mobilizes Ca2+ and alters mitochondrial Ca2+ homeostasis (independently of membrane Ca2+ channels) leading to apoptosis in T cell leukemia[115]. Activation of CB2 leads to accumulation of proapoptotic sphingolipid ceramide, which is able to induce ER stress and inhibit mTOR pathway and activating apoptosis[69]. However, CBD is also capable of generating ROS in many types of cancer cells, thereby inducing cytotoxicity or apoptosis and autophagy[69]. Many CBD induced activities are independent of canonical cannabinoid receptor activity (CB1/CB2) and known non-canonical receptors. Abbreviations: adenylyl cyclase(AC), cannabidiol (CBD), endocannabinoids (eCBs), reactive oxygen species (ROS), endoplasmic reticulum (ER), 9-tetrahydrocannabinol (THC); protein kinase A (PKA), protein kinase B (AKT), phosphatidylinositol 3-kinase (PI3K), extracellular regulated kinase (ERK), mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), factor 4E (eIF4E)-binding protein 1 (4E-BP1), myristoylated alanine-rich C-kinase substrate (MARCKS), cyclin-dependent kinase inhibitor proteins (p27/p21). Figure modified and adapted from Ligresti et al, 2016[69]. This figure was created with BioRender.com*

Table 1: Clinical effects of CBD and their effector receptors

System/Organ	*Action*	*Possible receptors*	*Refs*
Nervous system			
CNS	Anxiolytic	5-$HT_{1A,}$ CB1, TRPV1	73,74
	Anti-epileptic/ antioxidant/neuroprotective	TRPV1, δ OR, GPR55, $GABA_A$, CB2, 5-HT_{1A}	67,75–77
	Anti-psychotic	CB1, D2, 5-$HT_{1A,}$ 5-HT_{2A}	78,79
PNS	Analgesia, (neuropathic pain)	TRPV1, δ OR, GLyR	80–82
Respiratory system			
Lungs	Anti-inflammatory, anti-fibrotic	CB1, CB2	83,84
Cardiovascular			
Heart	Cardioprotective (antioxidant in ischemia and arrhythmia models)	Adenosine A_1, CB2	85–87
Arteries	↓SBP, ↓ arterial stiffness	5-$HT_{3,}$ 5-HT_{1A}	88
Gastrointestinal			
Gut	anti-inflammatory, analgesic	PPAR-γ, GPR55	89–91
Pancreas	anti-inflammatory, antioxidant, anti-neoplastic	GPR55, CB1, CB2, PPAR-γ	91–93
Liver	anti-inflammatory, hepatosteatosis	GPR55	92,94
Skin			
Subcutaneous tissue	Antioxidant, Analgesic, anti-inflammatory, antibacterial	TRPV1-4, TRPA1, TRMP8, PPAR-γ, CB1, CB2	95,96
Immune system			
Immune cells	hENT inhibition, adenosine modulation, immunomodulation	$A_{2A,}$ TRPV1, CB2	97,98
Musculoskeletal			
Bones/joints	↑ fracture healing, ↑ collagen production ↓ bone resorption, Anti-inflammatory Anti-arthritic	GPR55, CB1, CB2	99,100
Muscles	Analgesic, myorelaxant	CB1, CB2	100,101

Abbreviations: Central nervous system (CNS), peripheral nervous system (PNS), systolic blood pressure (SBP), adenosine receptor A1 and A2A ($A_{1,}$ A_{2A}), cannabinoid receptors (CB1,CB2), transient receptor potential cation channel subfamily V member 1-4 (TRPV1-4), peroxisome proliferator-activated receptor gamma (PPAR-γ), transient receptor potential ankyrin 1 (TRPA1), serotonin receptors 1A, 3 (5-$HT_{3,}$ 5-HT_{1A}), glycine receptor (GLyR), delta opioid receptors (μ-OR, δ-OR), gamma-aminobutyric acid receptor type A ($GABA_A$), cannabinoid receptor type 1 and 2 (CB1, CB2), G-protein coupled receptor (GPR).

Even though, backbone drugs for leukemia treatment such as MTX which have been around for over 70 years, and contributed to an increased life span of patients, toxicity issues and development of chemoresistance development, warrant continuous research for novel treatment strategies, for which CBD may be attractive candidate.

INTRODUCTION TO THE CHAPTERS

Chapter 2 describes the identification and molecular characterization of endothelial cells associated with hematopoietic niche formations in humans. Notably, CD105+ endothelial cells showed the ability to secrete regenerative factors (such as IL-33) and were associated with bone marrow recovery after chemotherapy in AML in adults and during early fetal bone marrow development.

Chapter 3 covers a review on how to overcome ATP-binding cassette (ABC) drug efflux transporter mediated drug resistance. This review discusses the clinical impact of the main ABC transporters BCRP (ABCG2), MRP1 (ABCC1) and P-gp (ABCB1) in chemotherapy resistance and summarizes the current mechanisms and strategies of transporter inhibition to reverse this resistance modality.

In **chapter 4,** we describe correlations between the expression of multiple ABC transporters - BCRP (ABCG2), MPR1 (ABCC1), MRP4 (ABCC4) and MRP5 (ABCC5) - and the intracellular accumulation of polyglutamate forms of the anti-leukemic methotrexate polyglutamate accumulation as contributors to *ex vivo* resistance in primary childhood acute lymphoblastic leukemia cells. In particular, overexpression of MRP4 appeared to be related to *ex vivo* methotrexate resistance, while a combination of MRP4 and BCRP overexpression was associated with low intracellular accumulation of methotrexate polyglutamates and poor survival in children with acute lymphoblastic leukemia.

In **chapter 5,** we report on the immunohistochemical expression of the nucleoside transporter hENT1 (SLC29) and hCNT1 (SLC28) in primary pediatric acute myeloid leukemia samples at diagnosis and correlated these parameters with in vitro drug sensitivity for nucleoside analogs such as cytarabine.

Chapter 6 describes the effect of dexamethasone on the antileukemic effect of cytarabine, and in particular the role of deoxycytidine kinase as cytarabine-activating enzyme. Studies in a rat model of acute myeloid leukemia showed that dexamethasone did not affect the efficacy of cytarabine treatment, even though it had a down-regulatory effect on deoxycytidine kinase activity.

In **chapter 7,** experimental therapeutic studies were performed with cannabidiol (CBD) to assess its potential effect as anti-leukemic drug and its ability to target drug-resistant leukemia cells. CBD could inhibit growth of leukemia cells *in vitro* at concentrations round 10 µM, but it conferred growth stimulatory effects at around 1 µM concentrations. Intriguingly, chronic exposure of leukemia cells to CBD induces addiction to CBD for their growth. CBD displayed cross-resistance to leukemia cells with acquired resistance to the proteasome inhibitor bortezomib. These beneficial and adverse effects of CBD warrant further investigations to position its role in leukemia treatment.

Chapter 8 provides a summary, general discussion and future perspectives of the results described in this thesis.

References

1. Zuckerman T, Rowe JM. Pathogenesis and prognostication in acute lymphoblastic leukemia. *F1000Prime Rep*. 2014;6.
2. Paul S, Kantarjian H, Jabbour EJ. Adult Acute Lymphoblastic Leukemia. *Mayo Clin Proc*. 2016;91(11):1645-1666.
3. De Kouchkovsky I, Abdul-Hay M. 'Acute myeloid leukemia: a comprehensive review and 2016 update.' *Blood Cancer Journal*. 2016;6(7):e441-e441.
4. Saultz JN, Garzon R. Acute Myeloid Leukemia: A Concise Review. *J Clin Med*. 2016;5(3).
5. Horton TM, Steuber, Philip C., Aster, John C. Overview of the clinical presentation and diagnosis of acute lymphoblastic leukemia/lymphoma in children. In: *UpToDate*. ; 2019. Accessed July 23, 2020. https://www.uptodate.com/contents/overview-of-the-clinical-presentation-and-diagnosis-of-acute-lymphoblastic-leukemia-lymphoma-in-children
6. Kolitz, Jonathan E. Overview of acute myeloid leukemia in adults. In: Larson RA, ed. *UpToDate*. ; 2020. Accessed July 20, 2020. https://www.uptodate.com/contents/overview-of-acute-myeloid-leukemia-in-adults
7. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011;144(5):646-674.
8. Fouad YA, Aanei C. Revisiting the hallmarks of cancer. *Am J Cancer Res*. 2017;7(5):1016-1036.
9. Chen C, Liang C, Wang S, et al. Expression patterns of immune checkpoints in acute myeloid leukemia. *Journal of Hematology & Oncology*. 2020;13(1):28.
10. Campos-Sanchez E, Toboso-Navasa A, Romero-Camarero I, Barajas-Diego M, Sanchez-Garcia I, Cobaleda C. Acute lymphoblastic leukemia and developmental biology. *Cell Cycle*. 2011;10(20):3473-3486.
11. Sarkaria SM, Decker M, Ding L. Bone marrow microenvironment in normal and deranged hematopoiesis: Opportunities for regenerative medicine and therapies. *Bioessays*. 2018;40(3).
12. Das M, Law S. Role of tumor microenvironment in cancer stem cell chemoresistance and recurrence. *The International Journal of Biochemistry & Cell Biology*. 2018;103:115-124.
13. Elbaz M, Nasser MW, Ravi J, et al. Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: Novel anti-tumor mechanisms of Cannabidiol in breast cancer. *Molecular Oncology*. 2015;9(4):906-919.
14. Kenswil KJG, Jaramillo AC, Ping Z, et al. Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor. *Cell Rep*. 2018;22(3):666-678.
15. Wang M, Zhao J, Zhang L, et al. Role of tumor microenvironment in tumorigenesis. *J Cancer*. 2017;8(5):761-773.
16. Schepers K, Campbell TB, Passegué E. Normal and Leukemic Stem Cell Niches: Insights and Therapeutic Opportunities. *Cell Stem Cell*. 2015;16(3):254-267.
17. Sá da Bandeira D, Casamitjana J, Crisan M. Pericytes, integral components of adult hematopoietic stem cell niches. *Pharmacology & Therapeutics*. 2017;171:104-113.
18. Kobayashi H, Butler JM, O'Donnell R, et al. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol*. 2010;12(11):1046-1056.
19. Ramasamy SK, Kusumbe AP, Adams RH. Regulation of tissue morphogenesis by endothelial cell-derived signals. *Trends Cell Biol*. 2015;25(3):148-157.
20. Ramasamy SK, Kusumbe AP, Itkin T, Gur-Cohen S, Lapidot T, Adams RH. Regulation of Hematopoiesis and Osteogenesis by Blood Vessel-Derived Signals. *Annu Rev Cell Dev Biol*. 2016;32:649-675.
21. Whiteside T. The tumor microenvironment and its role in promoting tumor growth. *Oncogene*. 2008;27(45):5904-5912.
22. Asada N, Takeishi S, Frenette PS. Complexity of bone marrow hematopoietic stem cell niche. *Int J Hematol*. 2017;106(1):45-54.
23. Li W, Zhang H, Assaraf YG, et al. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. *Drug Resistance Updates*. 2016;27:14-29.
24. Jaramillo AC, Saig FA, Cloos J, Jansen G, Peters GJ. How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance? *Cancer Drug Resistance*. 2018;1(1):6-29.
25. Hays K. Physiology of normal bone marrow. *Seminars in Oncology Nursing*. 1990;6(1):3-8.
26. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin*. 2018;68(1):7-30.
27. World Health Organization. *Cancer Today, Global Initiative for Cancer Registry Development.* World Health Organization Accessed July 22, 2020. https://gco.iarc.fr/today/home

28. Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. *N Engl J Med*. 2015;373(16):1541-1552.
29. Kakaje A, Alhalabi MM, Ghareeb A, et al. Rates and trends of childhood acute lymphoblastic leukaemia: an epidemiology study. *Scientific Reports*. 2020;10(1):6756.
30. George P, Hernandez K, Hustu O, Borella L, Holton C, Pinkel D. A study of “total therapy” of acute lymphocytic leukemia in children. *J Pediatr*. 1968;72(3):399-408.
31. Rivera GK, Ribeiro RC. Improving treatment of children with acute lymphoblastic leukemia in developing countries through technology sharing, collaboration and partnerships. *Expert Review of Hematology*. 2014;7(5):649-657.
32. van der Linden MH, Boer JM, Schneider P, et al. Clinical and molecular genetic characterization of wild-type MLL infant acute lymphoblastic leukemia identifies few recurrent abnormalities. *Haematologica*. 2016;101(3):e95-e99.
33. Stam RW. MLL-AF4 driven leukemogenesis: what are we missing? *Cell Research*. 2012;22(6):948-949.
34. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer J*. 2017;7(6):e577.
35. Jabbour E, O’Brien S, Konopleva M, Kantarjian H. New insights into the pathophysiology and therapy of adult acute lymphoblastic leukemia. *Cancer*. 2015;121(15):2517-2528.
36. Pulte D, Redaniel MT, Jansen L, Brenner H, Jeffreys M. Recent trends in survival of adult patients with acute leukemia: overall improvements, but persistent and partly increasing disparity in survival of patients from minority groups. *Haematologica*. 2013;98(2):222-229.
37. Pulte D, Jansen L, Brenner H. Changes in long term survival after diagnosis with common hematologic malignancies in the early 21st century. *Blood Cancer J*. 2020;10(5).
38. Gamis AS, Alonzo TA, Perentesis JP, Meshinchi S, COG Acute Myeloid Leukemia Committee. Children’s Oncology Group’s 2013 blueprint for research: acute myeloid leukemia. *Pediatr Blood Cancer*. 2013;60(6):964-971.
39. Gottlieb AJ, Weinberg V, Ellison RR, et al. Efficacy of daunorubicin in the therapy of adult acute lymphocytic leukemia: a prospective randomized trial by cancer and leukemia group B. *Blood*. 1984;64(1):267-274.
40. Masetti R, Vendemini F, Zama D, Biagi C, Pession A, Locatelli F. Acute Myeloid Leukemia in Infants: Biology and Treatment. *Front Pediatr*. 2015;3:37.
41. Kaspers GJL. Pediatric acute myeloid leukemia. *Expert Rev Anticancer Ther*. 2012;12(3):405-413.
42. Baguley BC. Multiple drug resistance mechanisms in cancer. *Mol Biotechnol*. 2010;46(3):308-316.
43. Choi YH, Yu A-M. ABC Transporters in Multidrug Resistance and Pharmacokinetics, and Strategies for Drug Development. *Curr Pharm Des*. 2014;20(5):793-807.
44. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. *Hum Genomics*. 2009;3(3):281-290.
45. Peters GJ, van der Wilt CL, van Moorsel CJA, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacology & Therapeutics*. 2000;87(2):227-253.
46. Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy*. 2012;15(4):183-210.
47. Hooijberg JH, Broxterman HJ, Kool M, et al. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res*. 1999;59(11):2532-2535.
48. Gupta SK, Singh P, Ali V, Verma M. Role of membrane-embedded drug efflux ABC transporters in the cancer chemotherapy. *Oncol Rev*. 2020;14(2):448.
49. Gottesman MM, Fojo T, Bates SE. Multidrug Resistance in Cancer: Role of Atp-Dependent Transporters. *Nature Reviews Cancer*. 2002;2(1):48-58.
50. Jaramillo AC, Cloos J, Lemos C, et al. Ex vivo resistance in childhood acute lymphoblastic leukemia: Correlations between BCRP, MRP1, MRP4 and MRP5 ABC transporter expression and intracellular methotrexate polyglutamate accumulation. *Leukemia Research*. 2019;79:45-51.
51. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat Rev Cancer*. 2018;18(7):452-464.
52. Jaramillo A, Hubeek I, Broekhuizen R, et al. Expression of the nucleoside transporters hENT1 (SLC29) and hCNT1 (SLC28) in pediatric acute myeloid leukemia. *Nucleosides, Nucleotides & Nucleic Acids*. 2020;39(10-12):1379-1388.
53. Clarke ML, Mackey JR, Baldwin SA, Young JD, Cass CE. The role of membrane transporters in cellular resistance to anticancer nucleoside drugs. *Cancer Treat Res*. 2002;112:27-47.

54. Català A, Pastor-Anglada M, Caviedes-Cárdenas L, et al. FLT3 is implicated in cytarabine transport by human equilibrative nucleoside transporter 1 in pediatric acute leukemia. *Oncotarget*. 2016;7(31):49786-49799.
55. Raaijmakers MHGP, de Grouw EPLM, Heuver LHH, et al. Breast cancer resistance protein in drug resistance of primitive CD34+38- cells in acute myeloid leukemia. *Clin Cancer Res*. 2005;11(6):2436-2444.
56. Huang F-F, Zhang L, Wu D-S, et al. PTEN regulates BCRP/ABCG2 and the side population through the PI3K/Akt pathway in chronic myeloid leukemia. *PLoS ONE*. 2014;9(3):e88298.
57. Wang F, Wang X-K, Shi C-J, et al. Nilotinib enhances the efficacy of conventional chemotherapeutic drugs in $CD34^{+}CD38^{-}$ stem cells and ABC transporter overexpressing leukemia cells. *Molecules*. 2014;19(3):3356-3375.
58. Gao C, Zhang J, Wang Q, Ren C. Overexpression of lncRNA NEAT1 mitigates multidrug resistance by inhibiting ABCG2 in leukemia. *Oncol Lett*. 2016;12(2):1051-1057.
59. Straussman R, Morikawa T, Shee K, et al. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature*. 2012;487(7408):500-504.
60. Sivan U, De Angelis J, Kusumbe AP. Role of angiocrine signals in bone development, homeostasis and disease. *Open Biol*. 2019;9(10):190144.
61. Atalay S, Jarocka-Karpowicz I, Skrzydlewska E. Antioxidative and Anti-Inflammatory Properties of Cannabidiol. *Antioxidants (Basel)*. 2019;9(1).
62. Lowin T, Tingting R, Zurmahr J, Classen T, Schneider M, Pongratz G. Cannabidiol (CBD): a killer for inflammatory rheumatoid arthritis synovial fibroblasts. *Cell Death & Disease*. 2020;11(8):1-11.
63. Burstein S. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg Med Chem*. 2015;23(7):1377-1385.
64. Booz GW. Cannabidiol as an Emergent Therapeutic Strategy for Lessening the Impact of Inflammation on Oxidative Stress. *Free Radic Biol Med*. 2011;51(5):1054-1061.
65. Durst R, Danenberg H, Gallily R, et al. Cannabidiol, a nonpsychoactive Cannabis constituent, protects against myocardial ischemic reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2007;293(6):H3602-3607.
66. Hayakawa K, Mishima K, Fujiwara M. Therapeutic Potential of Non-Psychotropic Cannabidiol in Ischemic Stroke. *Pharmaceuticals (Basel)*. 2010;3(7):2197-2212.
67. Silvestro S, Mammana S, Cavalli E, Bramanti P, Mazzon E. Use of Cannabidiol in the Treatment of Epilepsy: Efficacy and Security in Clinical Trials. *Molecules*. 2019;24(8).
68. Watt G, Karl T. In vivo Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease. *Front Pharmacol*. 2017;8.
69. Ligresti A, De Petrocellis L, Di Marzo V. From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex Pharmacology. *Physiological Reviews*. 2016;96(4):1593-1659.
70. Laezza C, Pagano C, Navarra G, et al. The Endocannabinoid System: A Target for Cancer Treatment. *Int J Mol Sci*. 2020;21(3).
71. Malfitano AM, Ciaglia E, Gangemi G, Gazzerro P, Laezza C, Bifulco M. Update on the endocannabinoid system as an anticancer target. *Expert Opin Ther Targets*. 2011;15(3):297-308.
72. Wu J. Cannabis, cannabinoid receptors, and endocannabinoid system: yesterday, today, and tomorrow. *Acta Pharmacol Sin*. 2019;40(3):297-299.
73. Shannon S, Lewis N, Lee H, Hughes S. Cannabidiol in Anxiety and Sleep: A Large Case Series. *Perm J*. 2019;23.
74. Blessing EM, Steenkamp MM, Manzanares J, Marmar CR. Cannabidiol as a Potential Treatment for Anxiety Disorders. *Neurotherapeutics*. 2015;12(4):825-836.
75. Reddy DS. The Utility of Cannabidiol in the Treatment of Refractory Epilepsy. *Clin Pharmacol Ther*. 2017;101(2):182-184.
76. Pazos MR, Mohammed N, Lafuente H, et al. Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: role of 5HT(1A) and CB2 receptors. *Neuropharmacology*. 2013;71:282-291.
77. Campos AC, Fogaça MV, Sonego AB, Guimarães FS. Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacological Research*. 2016;112:119-127.
78. Rohleder C, Müller JK, Lange B, Leweke FM. Cannabidiol as a Potential New Type of an Antipsychotic. A Critical Review of the Evidence. *Front Pharmacol*. 2016;7.
79. Devinsky O, Cilio MR, Cross H, et al. Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia*. 2014;55(6):791-802.
80. Xiong W, Cui T, Cheng K, et al. Cannabinoids suppress inflammatory and neuropathic pain by targeting α3 glycine receptors. *J Exp Med*. 2012;209(6):1121-1134.

81. Matarazzo AP, Elisei LMS, Carvalho FC, et al. Mucoadhesive nanostructured lipid carriers as a cannabidiol nasal delivery system for the treatment of neuropathic pain. *Eur J Pharm Sci*. 2021;159:105698.
82. Capano A, Weaver R, Burkman E. Evaluation of the effects of CBD hemp extract on opioid use and quality of life indicators in chronic pain patients: a prospective cohort study. *Postgrad Med*. 2020;132(1):56-61.
83. Vuolo F, Abreu SC, Michels M, et al. Cannabidiol reduces airway inflammation and fibrosis in experimental allergic asthma. *Eur J Pharmacol*. 2019;843:251-259.
84. Turcotte C, Blanchet M-R, Laviolette M, Flamand N. Impact of Cannabis, Cannabinoids, and Endocannabinoids in the Lungs. *Front Pharmacol*. 2016;7.
85. Gonca E, Darıcı F. The effect of cannabidiol on ischemia/reperfusion-induced ventricular arrhythmias: the role of adenosine A1 receptors. *J Cardiovasc Pharmacol Ther*. 2015;20(1):76-83.
86. Mach F, Montecucco F, Steffens S. Cannabinoid receptors in acute and chronic complications of atherosclerosis. *British Journal of Pharmacology*. 2008;153(2):290-298.
87. Rajesh M, Mukhopadhyay P, Bátkai S, et al. Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, inflammatory and cell death signaling pathways in diabetic cardiomyopathy. *J Am Coll Cardiol*. 2010;56(25):2115-2125.
88. Kicman A, Toczek M. The Effects of Cannabidiol, a Non-Intoxicating Compound of Cannabis, on the Cardiovascular System in Health and Disease. *International Journal of Molecular Sciences*. 2020;21(18):6740.
89. De Filippis D, Esposito G, Cirillo C, et al. Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis. *PLoS One*. 2011;6(12):e28159.
90. Irving P, Iqbal T, Nwokolo C, et al. PTH-056 Trial to assess cannabidiol in the symptomatic treatment of ulcerative colitis. *Gut*. 2015;64(Suppl 1):A430-A430.
91. Schicho R, Storr M. A potential role for GPR55 in gastrointestinal functions. *Curr Opin Pharmacol*. 2012;12(6):653-658.
92. Romero-Zerbo SY, García-Fernández M, Espinosa-Jiménez V, et al. The Atypical Cannabinoid Abn-CBD Reduces Inflammation and Protects Liver, Pancreas, and Adipose Tissue in a Mouse Model of Prediabetes and Non-alcoholic Fatty Liver Disease. *Front Endocrinol (Lausanne)*. 2020;11:103.
93. Sharafi G, He H, Nikfarjam M. Potential Use of Cannabinoids for the Treatment of Pancreatic Cancer. *J Pancreat Cancer*. 2019;5(1):1-7.
94. Silvestri C, Paris D, Martella A, et al. Two non-psychoactive cannabinoids reduce intracellular lipid levels and inhibit hepatosteatosis. *J Hepatol*. 2015;62(6):1382-1390.
95. Baswan SM, Klosner AE, Glynn K, et al. Therapeutic Potential of Cannabidiol (CBD) for Skin Health and Disorders. *Clin Cosmet Investig Dermatol*. 2020;13:927-942.
96. Sheriff T, Lin MJ, Dubin D, Khorasani H. The potential role of cannabinoids in dermatology. *J Dermatolog Treat. 2020 Dec;31 (8):839-845*
97. Carrier EJ, Auchampach JA, Hillard CJ. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci U S A*. 2006;103(20):7895-7900.
98. Peyravian N, Deo S, Daunert S, Jimenez JJ. Cannabidiol as a Novel Therapeutic for Immune Modulation. *Immunotargets Ther*. 2020;9:131-140.
99. O'Connor CM, Anoushiravani AA, Adams C, Young JR, Richardson K, Rosenbaum AJ. Cannabinoid Use in Musculoskeletal Illness: a Review of the Current Evidence. *Curr Rev Musculoskelet Med*. 2020;13(4):379-384.
100. Madden K, van der Hoek N, Chona S, et al. Cannabinoids in the Management of Musculoskeletal Pain: A Critical Review of the Evidence. *JBJS Reviews*. 2018;6(5).
101. Nitecka-Buchta A, Nowak-Wachol A, Wachol K, et al. Myorelaxant Effect of Transdermal Cannabidiol Application in Patients with TMD: A Randomized, Double-Blind Trial. *Journal of Clinical Medicine*. 2019;8(11):1886.
102. Massi P, Solinas M, Cinquina V, Parolaro D. Cannabidiol as potential anticancer drug. *Br J Clin Pharmacol*. 2013;75(2):303-312.
103. McKallip RJ, Jia W, Schlomer J, Warren JW, Nagarkatti PS, Nagarkatti M. Cannabidiol-induced apoptosis in human leukemia cells: A novel role of cannabidiol in the regulation of p22phox and Nox4 expression. *Mol Pharmacol*. 2006;70(3):897-908.
104. Bergamaschi MM, Queiroz RHC, Zuardi AW, Crippa JAS. Safety and side effects of cannabidiol, a Cannabis sativa constituent. *Curr Drug Saf*. 2011;6(4):237-249.
105. Giacoppo S, Pollastro F, Grassi G, Bramanti P, Mazzon E. Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis. *Fitoterapia*. 2017;116:77-84.

106. Sultan AS, Marie MA, Sheweita SA. Novel mechanism of cannabidiol-induced apoptosis in breast cancer cell lines. *Breast*. 2018;41:34-41.
107. Shrivastava A, Kuzontkoski PM, Groopman JE, Prasad A. Cannabidiol Induces Programmed Cell Death in Breast Cancer Cells by Coordinating the Cross-talk between Apoptosis and Autophagy. *Molecular Cancer Therapeutics*. 2011;10(7):1161-1172.
108. Solinas M, Massi P, Cinquina V, et al. Cannabidiol, a non-psychoactive cannabinoid compound, inhibits proliferation and invasion in U87-MG and T98G glioma cells through a multitarget effect. *PLoS ONE*. 2013;8(10):e76918.
109. McAllister SD, Murase R, Christian RT, et al. Pathways mediating the effects of cannabidiol on the reduction of breast cancer cell proliferation, invasion, and metastasis. *Breast Cancer Res Treat*. 2011;129(1):37-47.
110. Powles T, te Poele R, Shamash J, et al. Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway. *Blood*. 2005;105(3):1214-1221.
111. Derocq JM, Ségui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett*. 1995;369(2-3):177-182.
112. Derocq JM, Bouaboula M, Marchand J, Rinaldi-Carmona M, Ségui M, Casellas P. The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines. *FEBS Lett*. 1998;425(3):419-425.
113. Bosier B, Muccioli GG, Hermans E, Lambert DM. Functionally selective cannabinoid receptor signalling: therapeutic implications and opportunities. *Biochem Pharmacol*. 2010;80(1):1-12.
114. Howlett AC, Abood ME. CB1 & CB2 Receptor Pharmacology. *Adv Pharmacol*. 2017;80:169-206.
115. Olivas-Aguirre M, Torres-López L, Valle-Reyes JS, Hernández-Cruz A, Pottosin I, Dobrovinskaya O. Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell Death Dis*. 2019;10(10):779.
116. Holland ML, Panetta JA, Hoskins JM, et al. The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochem Pharmacol*. 2006;71(8):1146-1154.
117. Holland ML, Allen JD, Arnold JC. Interaction of plant cannabinoids with the multidrug transporter ABCC1 (MRP1). *Eur J Pharmacol*. 2008;591(1-3):128-131.
118. Yeshurun M, Shpilberg O, Herscovici C, et al. Cannabidiol for the Prevention of Graft-versus-Host-Disease after Allogeneic Hematopoietic Cell Transplantation: Results of a Phase II Study. *Biol Blood Marrow Transplant*. 2015;21(10):1770-1775.
119. Sharom FJ. The P-glycoprotein multidrug transporter. *Essays Biochem*. 2011;50:161.

CHAPTER 2

Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 as an Anabolic Factor

Cell Reports. 22, 666–678, 2018

Keane Jared Guillaume Kenswil**[*1]**, Adrian Christopher Jaramillo**[*1]**, Zhen Ping[1], Si Chen[1], Remco Michiel Hoogenboezem[1], Maria Athina Mylona[1], Maria Niken Adisty[1], Eric Moniqué Johannes Bindels[1], Pieter Koen Bos[2], Hans Stoop[3], King Hong Lam[3], Bram van Eerden[4], Tom Cupedo[1], and Marc Hermanus Gerardus Petrus Raaijmakers[1]

[1] Department of Hematology, Erasmus MC Cancer Institute, Rotterdam, the Netherlands

[2] Department of Orthopaedics, Erasmus MC, Rotterdam, the Netherlands

[3] Department of Pathology, Erasmus MC Cancer Institute, Rotterdam, the Netherlands

[4] Department of Internal Medicine, Erasmus MC Cancer Institute, Rotterdam, the Netherlands

***** Shared first authorship

SUMMARY

Bone marrow formation requires an orchestrated interplay between osteogenesis, angiogenesis, and hematopoiesis that is thought to be mediated by endothelial cells. The nature of the endothelial cells and the molecular mechanisms underlying these events remain unclear in humans. Here, we identify a subset of endoglin-expressing endothelial cells enriched in human bone marrow during fetal ontogeny and upon regeneration after chemotherapeutic injury. Comprehensive transcriptional characterization by massive parallel RNA sequencing of these cells reveals a phenotypic and molecular similarity to murine type H endothelium and activation of angiocrine factors implicated in hematopoiesis, osteogenesis, and angiogenesis. Interleukin-33 (IL-33) was significantly overexpressed in these endothelial cells and promoted the expansion of distinct subsets of hematopoietic precursor cells, endothelial cells, as well as osteogenic differentiation. The identification and molecular characterization of these human regeneration-associated endothelial cells is thus anticipated to instruct the discovery of angiocrine factors driving bone marrow formation and recovery after injury.

INTRODUCTION

Endothelial cells (ECs) govern tissue development and regeneration by signaling molecules on their cell surface and the release of factors, such as cytokines and extracellular matrix proteins. This angiocrine function of the endothelium drives tissue development and regeneration in multiple organs. In the hematopoietic system, studies in mice have revealed pivotal contributions of ECs to the formation and regeneration of bone and bone marrow (BM) (Hooper et al., 2009; Ramasamy et al., 2016). Coordinated activation of osteogenesis, angiogenesis, and hematopoiesis is required for BM regeneration after tissue injury (Rafii et al., 2016; Ramasamy et al., 2015) induced by irradiation or chemotherapy. ECs support the proper regeneration of the hematopoietic system after myeloablation (Butler et al., 2010; Hooper et al., 2009; Kobayashi et al., 2010). Engraftment and repopulation of hematopoietic stem and progenitor cells (HSPCs) in mice is dependent on the regeneration of sinusoid ECs, which are vulnerable to toxic injury (Hooper et al., 2009). Sinusoid regeneration is mediated through vascular endothelial growth factor receptor 2 (VEGFR2) signaling, the blockage of which resulted in delayed reconstitution of peripheral blood values in irradiated mice (Hooper et al., 2009). ECs do not seem to be created equally in their ability to drive or contribute to bone and hematopoietic development and regeneration. Recent studies in mice have identified a specialized endothelial subset that controls the number of HSPCs in addition to coupling angiogenesis and osteogenesis (Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). This endothelial subtype, dubbed type H endothelium for its high expression of endomucin and CD31, is enriched in the bone metaphysis at the endosteal surface, where it is adjacent to osteoprogenitor cells and gives rise to sinusoidal endothelial vessels. Importantly, insight into the molecular programs underlying the capacity of this endothelial subtype to drive angiogenesis and osteogenesis enabled its pharmacologic manipulation in mice, resulting in increased bone formation (Kusumbe et al., 2014; Ramasamy et al.,

2015). Taken together, various studies indicate that specified EC-derived signals can orchestrate complex multicellular network interactions in the mammalian marrow, driving bone formation and regeneration under stress conditions. These findings in murine models open the perspective of EC-instructed strategies to regenerate bone and marrow in humans, both in degenerative conditions as well as after injury, such as chemotherapy and irradiation. Translation of these important findings to human regenerative medicine, however, will be critically dependent on our ability to identify and interrogate molecularly equivalent human ECs driving ontogeny and regeneration. Here, we describe the identification of a human EC subtype that is strongly associated with human fetal BM development and regeneration after chemotherapeutic injury. This EC displays striking immunophenotypic and molecular commonalities with type H endothelium in mice, including transcriptional activation of programs and angiocrine factors previously related to BM recovery in mice. Interleukin 33 (IL-33) is identified as a putative regenerative factor facilitating hematopoietic expansion and bone mineralization ex vivo, thus supporting the notion that the transcriptome of this human EC may serve as an important resource instructing discovery as well as validating the relevance of findings in murine models to human regenerative medicine.

RESULTS

Identification of Endoglin (CD105)-Expressing ECs Associated with BM Regeneration after Chemotherapeutic Injury.

To identify niche cells potentially implicated in the regeneration of the hematopoietic system in humans, we interrogated the composition of the hematopoietic niche during recovery after chemotherapeutic injury. Chemotherapeutic exposure causes damage to endothelial, hematopoietic, and osteolineage cells within the BM (Hooper et al., 2009; Kopp et al., 2009; Lerner and Harrison, 1990; Xian et al., 2006), resulting in a prolonged neutropenia associated with considerable morbidity and mortality in humans. Flow cytometric assessment of the niche (7AAD- CD45- CD235a-; Figure 1A) composition in the regenerating BM of acute myeloid leukemia (AML) patients (see Experimental Procedures) revealed an unaltered frequency of ECs (CD31+CD9+) (Barreiro et al., 2005) in comparison to marrow under homeostatic conditions (healthy donors) (22.1% ± 2.24% versus 31.57% ± 9.66%; $p = 0.15$ by unpaired Student's t test) (Figures 1A and 1B). To identify putative immuno-markers of EC subtypes emerging during regeneration, we performed massive parallel RNA sequencing (RNA-seq) of the fluorescence-activated cell sorting (FACS)-purified endothelial compartment (CD31+CD9+) (Figure 1A). A total of 903 transcripts were found to be significantly differentially expressed in ECs in the regenerative marrow in comparison to ECs from normal, steady-state marrow (glmLRT function, EdgeR; false discovery rate [FDR] < 0.05). The top 200 overexpressed transcripts contained 16 genes encoding for cluster of differentiation (CD) molecules, among which CD105 (endoglin) (Table S1), a co-receptor for transforming growth factor b (TGF-b) promoting angiogenesis (Cheifetz et al., 1992; Duff et al., 2003) (Miller et al., 1999) and previously associated with tissue injury (Wang et al., 1995).

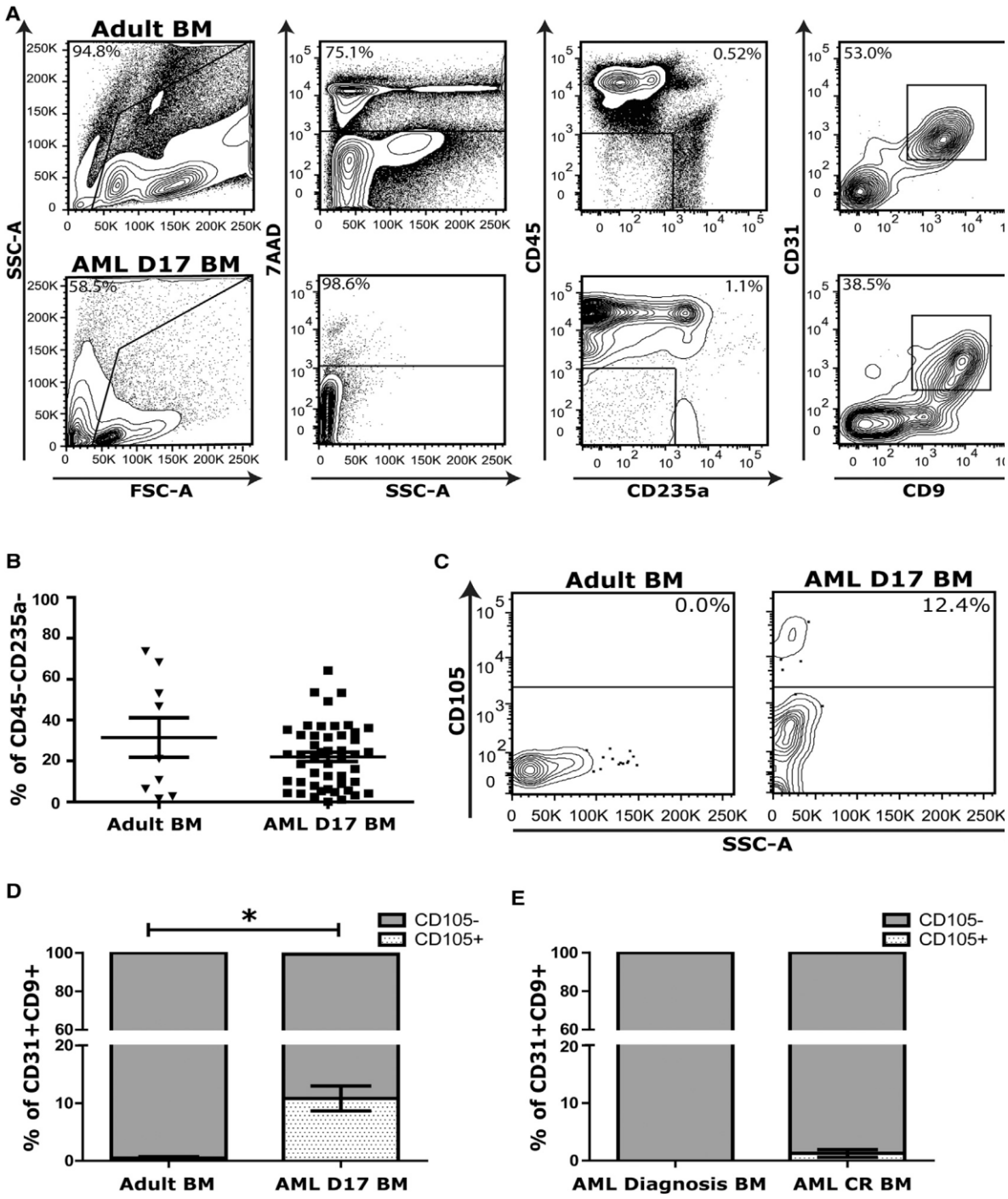


***Figure 1**. Identification of CD105 (Endoglin)-Expressing ECs Associated with BM Regeneration after Chemotherapeutic Injury BM obtained by aspirates on recovery after chemotherapeutic injury (AML D17) was compared with normal adult BM. (A) Gating strategy for identification and isolation of ECs. After doublet exclusion, 7AAD- mononuclear cells (MNCs) were gated based on low/negative CD45 and CD235a expression to identify niche cells. ECs were identified as CD31+CD9+ cells within the niche. (B) Frequency of ECs within the niche in adult steady-state (n = 9) and regenerative (n = 48) BM. (C) Representative FACS plots revealing the existence of a CD105-expressing endothelial subset in regeneration. (D) The frequency of CD105+ cells in the endothelial niche in normal BM (n = 9) and during regeneration after chemotherapy (n = 48). (E) The frequency of CD105+ cells in the endothelial niche in BM aspirates of AML patients at diagnosis (n = 4) and after recovery in complete remission (n = 3). Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. AML, acute myeloid leukemia; CR, complete remission; D17, day 17.*

CD105 protein levels, assessed by FACS analysis (Figure 1C), identified a distinct subset of CD105-expressing ECs strongly enriched in the regenerative marrow compared with normal BM (10.81% ± 2.14% versus 0.48% ± 0.24% of CD31+CD9+; p = 0.043) (Figure 1D). The presence of this endoglin-expressing subset was temporally restricted, as it was (virtually) absent in AML patients at diagnosis (0% ±0%of CD31+CD9+ cells) or after full recovery of peripheral blood values (in complete remission) (1.27% ± 0.66% of CD31+CD9+ cells) (Figure 1E).

Endoglin-Expressing ECs Are Enriched in the Mouse BM after Chemotherapeutic Myeloablation

To confirm our observation that the CD105-expressing subset of ECs is enriched during BM regeneration and establish a broader relevance for mammalian biology, we next translated our observations. to an experimental setting in which we exposed C57BL/6 wild-type mice to a myeloablative dose of the chemotherapeutic agent 5-fluorouracil (5FU) or PBS (vehicle control). The endoglin expressing subset constituted a rare subpopulation of endothelial (CD31+) cells in the steady-state adult BM niche (0.05% ± 0.02% of CD45- Ter119- cells) and in the collagenased bone niche (0.42% ± 0.12% of CD45-Ter119- cells) (Figures 2A, 2B, S1A, and S1B). This fraction increased significantly after administration of 5FU in the BM (0.65% ± 0.15% of CD45-Ter119- cells, fold-change (FC) increase of 13.6 ± 3.2, p = 0.026) and collagenased bone (8.18% ± 1.43% of CD45-Ter119- cells, FC increase of 19.6 ± 3.4, p = 0.007) (Figures 2A, 2B, S1A, and SB), confirming a relative increase of this specific subset in the regenerative phase after myeloablation. Of note, the absolute number of CD31+CD105+ ECs in the BM did not increase after exposure to 5FU (Figure 2C), suggesting that selection of these cells under chemotherapeutic pressure (rather than absolute expansion) may be implicated.

CD105 (Endoglin)-Expressing ECs Are Enriched in the Human BM during Fetal Development

The temporally restricted enrichment of CD105-expressing ECs during recovery after chemotherapeutic injury suggests that this cell type could potentially be implicated in regeneration and hematopoietic niche formation. To corroborate this notion, we sought to define other conditions in human biology where angiogenesis, osteogenesis, and hematopoiesis are synergistically activated. In human fetal bone development, hematopoiesis shifts from the fetal liver to the bones starting at week 10 after gestation. During this process, angiogenesis, osteogenesis, and hematopoiesis are tightly coupled to allow coordinated bone and hematopoietic development (Cosxkun et al., 2014; Jagannathan-Bogdan and Zon, 2013; Medvinsky et al., 2011). Invasion of blood vessels into the mesenchymal condensate is crucial for the coordinated activity of chondrocytes and osteoblasts, and each of these cell types stands in spatial and molecular interaction with ECs (Maes, 2013; Salazar et al., 2016). Flow cytometric dissection of the endothelial composition of fetal BM at gestational week 15–20 revealed a striking predominance of CD105-expressing cells within the endothelial

compartment (62.8% ± 5.9% of CD31+CD9+ cells) (Figures 3A and 3B). Massive parallel sequencing of these cells confirmed overexpression of genes encoding CD markers identified in the ECs in regenerating BM (Figure S2A), supporting the notion that the enrichment of these transcripts in ECs in the regenerative marrow is caused by overexpression in the subset of CD105+ ECs. The frequency of CD105-expressing ECs within the endothelial compartment was even higher when examining collagenased fetal bone fractions (average 82.65% ± 2.5% of CD31+ CD9+ cells) (Figures 3A and 3B), suggesting that this endothelial subpopulation might preferentially localize to the endosteal surface of fetal long bones. In line with this, CD105-expressing ECs were identified, albeit at a considerably lower frequency, in collagenased bone fractions of human adult postnatal bone (average 25.9% ± 5.0% of CD31+CD9+ cells) (Figures 3A and 3B).

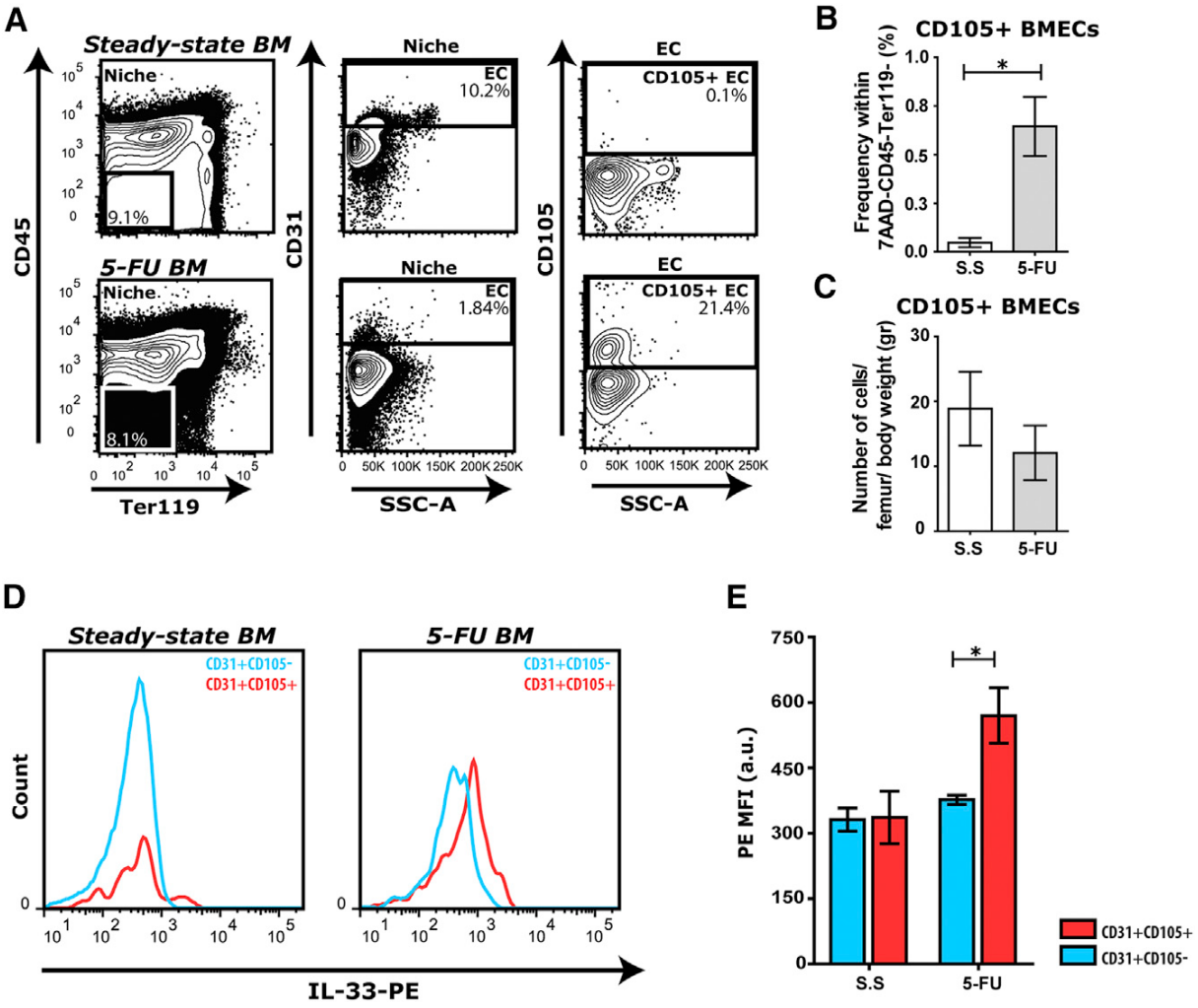


Figure 2. *CD105-Expressing ECs Are Enriched in Mice during Regeneration after Chemotherapeutic Injury (A) Representative FACS plots for identification of murine BM ECs. After doublet exclusion, 7AAD- mononuclear cells (MNCs) were gated based on low/negative CD45/ Ter119 expression to select for BM niche cells. BM ECs were identified by CD31 and CD105 expression. (B) Frequency of CD105+ ECs within the murine BM niche during S.S (n = 3 mice) and on recovery after 5-FU (n = 5 mice). (C) Numbers of CD105+ BM ECs during S.S (n = 3) and after 5-FU treatment (n = 5). (D and E) CD105+ BM ECs differentially express IL-33 on injury. (D) Representative FACS plots. (E) IL-33 expression in CD105+ BM ECs and CD105- BM ECs in S.S (n = 3) and after 5-FU treatment (n = 5). Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. 5-FU = 5-fluorouracil. S.S = steady-state.*

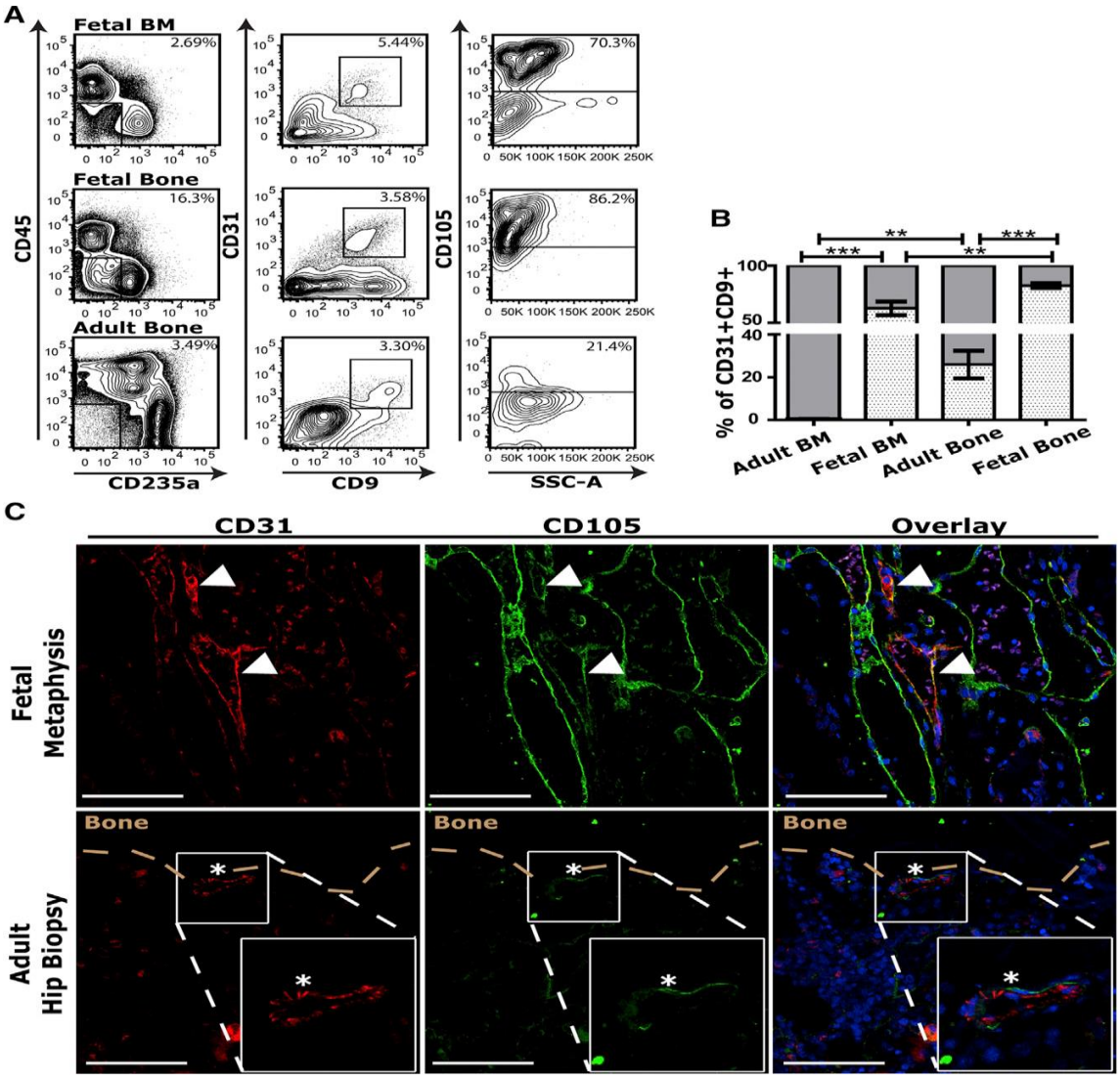


***Figure 3**. CD105-Expressing ECs Are Enriched in Fetal Development and Collagenized Bone Fractions (A) Representative FACS plots identifying CD105- expressing ECs in fetal BM, fetal bone, and adult bone. (B) Frequency of CD105+ cells in the endothelial compartment in healthy adult BM aspirates (n = 9), Fetal BM (n = 15), fetal bone (n = 21), and adult bone (n = 9). Data represent mean ± SEM. **p < 0.01, ***p < 0.001, 1-way ANOVA (p < 0.0001) followed by Bonferroni's multiple comparison test. (C) In situ immunofluorescence demonstrating the existence of capillary structures in the trabecular area of fetal long bone comprised of CD31+CD105+ ECs (arrowheads). Sinusoid structures are CD31lo CD105+. In human adult BM, the majority of CD31+ ECs lacks CD105 expression (asterisks). The area within the dotted line in the adult BM represent bone tissue. Magnification, 20x. White scale bar represents 100 mm.*

To provide anatomical context for this CD31+CD105+ EC subset, we performed in situ immunohistochemistry and immunofluorescence on fetal femurs and core hip bone biopsies obtained from adults. CD31+CD105+ ECs were observed in the trabecular bone area of the metaphysis of fetal femurs (Figures 3C, S3A, and S3B) at a significantly higher frequency than in the trabecular bone of adult BM. The majority of vascular structures in both fetal and adult human bone were lined with CD31-CD105+ ECs, previously shown to be sinusoid ECs. CD31+CD105- cells were present sporadically in adult bone. Collectively, these findings identify a human endothelial

subtype that is enriched in collagenased bone fractions, prevalent during human fetal bone development, declines in frequency on aging, and emerges, in a temporally restricted fashion, in the BM during regeneration after chemotherapeutic injury. Henceforward, we will refer to this EC type as “human regeneration associated ECs” (hRECs).

hRECs Share Immunophenotypic and Molecular Similarities with Murine Type H Endothelium and Express Key Regulators of Angiogenesis and Osteogenesis

Interestingly, similarities exist between hRECs and the recently described specialized endothelium coordinating osteo- and angiogenesis in mice, termed type H endothelium (Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). These include enrichment at the bone surface, reduced frequencies on aging, and an increase in frequency on genotoxic stress, suggesting that hRECs may reflect human equivalents of mouse type H endothelium. To further investigate this, differentially expressed transcripts in CD31+CD9+CD105+ hRECs isolated from human fetal bone (in comparison to steady-state postnatal CD31+CD9+CD105- cells) were related to genes reported to be overexpressed in type H endothelium. This confirmed elevated expression of many transcripts previously reported to be enriched in type H endothelium, including genes encoding the signature markers CD31 (PECAM1) and mucin-like sialoglycoprotein endomucin (EMCN) (Figure 4A) and vessel guidance molecules (Ramasamy et al., 2014) (Figure 4B). Most of these genes were similarly enriched in the CD31+CD9+ fraction of regenerative BM (Figures 4A and 4B) (the limited number of CD105+ cells precluded RNA-seq of this specific subset). In total, 3,718 genes were differentially expressed (glmLRT function, EdgeR; FDR < 0.05) in fetal bone hRECs in comparison to steady-state postnatal BM ECs. Among the overexpressed genes were HSPC niche factors (Figure 4C) and known angiocrine anabolic regulators of osteogenesis and angiogenesis (Figure 4D), further suggesting that hRECs might be involved in hematopoietic niche formation. Next, transcriptional programs and signatures were interrogated in hRECs using gene set enrichment analysis (GSEA) (Subramanian et al., 2005). Activated Notch signaling and stabilization of HIF1a have been identified in type H endothelium as key promotors of the formation of type H capillaries and the release of osteogenic factors that enhance osteogenesis (Kusumbe et al., 2014; Ramasamy et al., 2014). In line with this, key NOTCH regulators, such as Jagged-1, DLL4, NOTCH1, and NOTCH4 (receptors of DLL4), were upregulated in fetal hRECs (Figure 4E), reflected in the activation of downstream transcriptional NOTCH signaling, as demonstrated by GSEA (Figure 4F). Similarly, gene sets related to the HIF1a pathway were enriched in fetal hRECs according to GSEA (Figure S2B). Other relevant molecular signatures that were identified to be significantly enriched in hRECs include ‘‘angiogenesis’’ and ‘‘stemness’’ signatures, among which VEGF and WNT signaling (Figure S2C). Together, the data indicate that hRECs share immunophenotypic and molecular commonalities with murine type H ECs and have a transcriptional wiring that may be congruent with the view that these cells are implicated in the coupling of hematopoiesis, osteogenesis, and angiogenesis in regeneration.

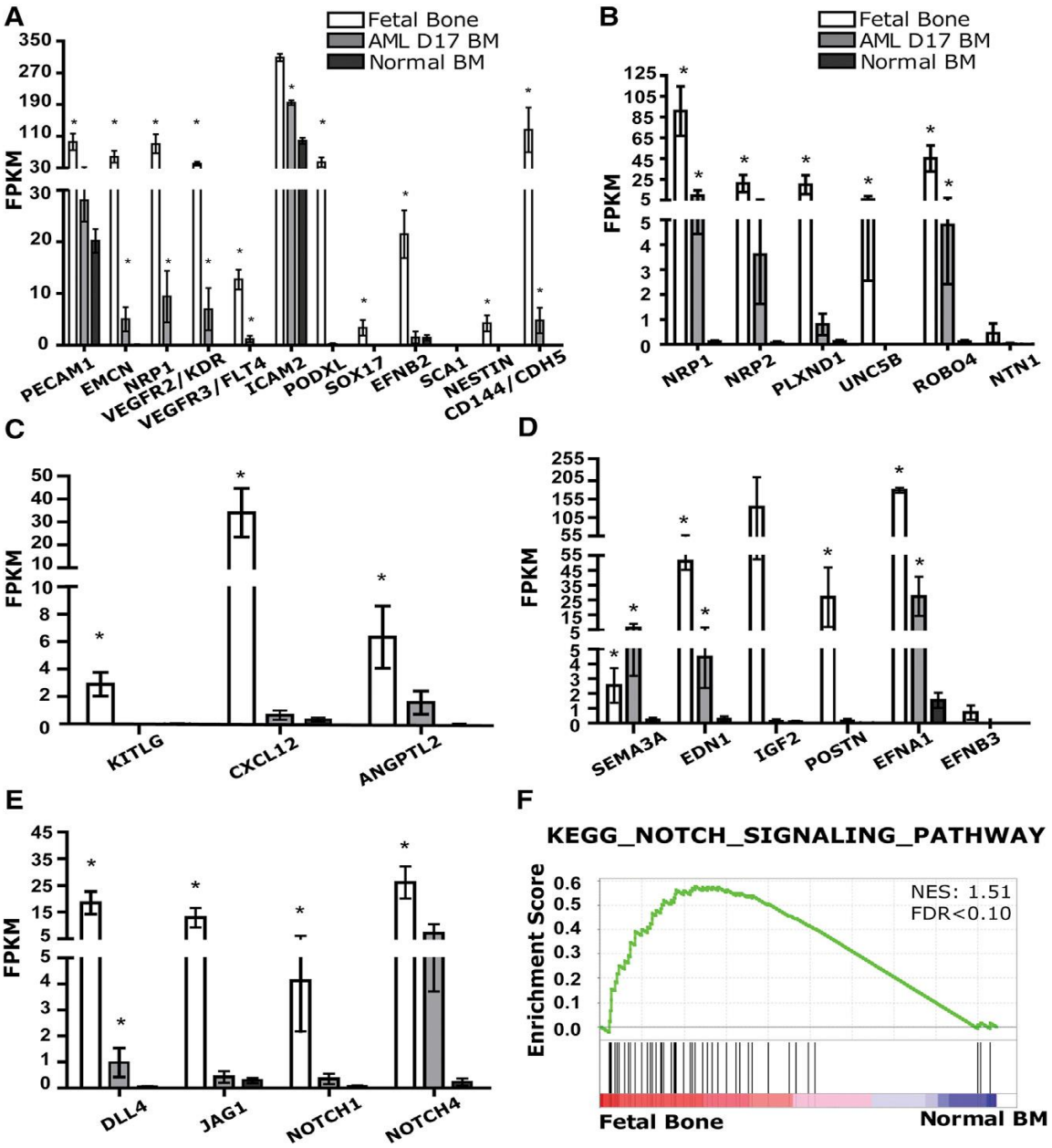


Figure 4. *hRECs Express Transcriptional Programs Defining Niche-Forming ECs in Mice. Transcriptomes of hRECs isolated from human fetal bone (CD45- CD235-CD31+ CD9+ CD105+) (n = 3) and regenerative BM (CD45- CD235- CD31+ CD9+) (n = 3) were compared with postnatal steady-state BM ECs (CD45- CD235- CD31+ CD9+) (n = 7). (A and B) Expression of genes encoding arterial and venous markers (A), and vessel guidance molecules previously reported to be overexpressed in murine type H ECs (B) (Itkin et al., 2016; Kusumbe et al., 2014, 2016; Ramasamy et al., 2014). Note, Sca-1 has no known human homolog (Holmes and Stanford, 2007). (C) Expression of established hematopoiesis supporting cytokines (Broudy, 1997; Sugiyama et al., 2006; Zhang et al., 2006). (D) Expression of genes encoding anabolic regulators of angiogenesis and osteogenesis (Blumenfeld et al., 2002; Clines et al., 2007; Edwards and Mundy, 2008; Oshima et al., 2002; Salani et al., 2000; Salvucci and Tosato, 2012; Serini et al., 2003; Shao et al., 2004; Shigematsu et al., 1999; Tamagnone and Giordano, 2006). (E and F) Expression of genes encoding critical NOTCH pathway components (E) and activation of Notch- signaling (F) as demonstrated by gene set enrichment analysis (GSEA). *FDR < 0.05. FDR, false discovery rate; FPKM, fragments per kilobase million; NES, normalized enrichment score.*

IL-33 Is Expressed by hRECs and Promotes Angiogenesis, Osteogenesis, and the Expansion of Hematopoietic Precursor Cells

We thus hypothesized that elucidation of the transcriptome of ECs related to hematopoietic niche formation might instruct the discovery of pathways or proteins facilitating niche formation. In particular, angiocrine factors may be identified that facilitate angiogenesis, osteogenesis, and hematopoiesis. To provide proof of principle for this assumption, we focused our attention on genes encoding secreted factors that were significantly enriched in both fetal hRECs and regenerative BM ECs. In total, 237 genes were significantly enriched, of which 34 are genes encoding known secreted factors with a strong correlation in levels of expression (Figure 5A). Of interest, the canonical receptors of many of these secreted proteins were overexpressed in fetal hRECs, (MMRN2-CLEC14A, BMP4-BMPR2, EFNA1-EPHA2/EPHA4/ EPHA7, EDN1-EDNRB, SEMA3A-NRP1/NRP2/PLXNA2, and ADM-RAMP2/ CALCRL) (Figure S2D), suggesting the possibility of autocrine signaling. In addition to secreted factors with a known role in regulating HSPC behavior, such as plasminogen activator (PLAT) (Ibrahim et al., 2014) and tissue factor pathway inhibitor (TFPI) (Khurana et al., 2013), we found significant overexpression of IL-33 in hRECs (Figure 5A). IL-33 is a pro-inflammatory cytokine and a chromatin-associated nuclear factor (Carriere et al., 2007). IL-33 protein expression in fetal hRECs was confirmed using fluorescence microscopy (Figure 5B), and IL-33 was differentially overexpressed in murine CD31+CD105+ ECs in comparison to their CD105- counterparts during regeneration (Figures 2D and 2E). The inability to propagate sorted hRECs ex vivo (data not shown) precluded the possibility to perform co-culture blocking experiments to assess the contribution of hREC-derived IL-33 to angiogenesis, hematopoiesis, and osteogenesis. As an alternative strategy, we exposed relevant cell types to recombinant human IL-33 (rhIL-33).

Hematopoiesis

To test if IL-33 facilitates human hematopoiesis in vitro, we exposed cord blood (CB) CD34+ HSPCs to rhIL-33 or vehicle control for one week in serum-free medium containing stem cell factor (SCF). rhIL-33 expanded the total number of hematopoietic cells (total mononuclear cells [MNCs]) (3.4-fold increase ± 0.29; $p < 0.01$) with a concomitant expansion of hematopoietic progenitor cells (HPCs), specifically immunophenotypic multipotent progenitors (MPPs) (2.5-fold increase ± 0.43; $p < 0.05$) and multi lymphoid progenitors (MLPs) (2.7-fold increase ± 0.15; $p < 0.01$) (Figures 5C and S4). Immunophenotypic HSC numbers were not affected by exposure to IL-33. Expansion of myeloid progenitor cells was confirmed in colony-forming unit cell assays (CFU-C assays, demonstrating an increase in granulocyte-macrophage colony-forming units (CFU-GMs) (2.27-fold increase ± 0.21; $p < 0.05$) (Figure 5D).

Osteogenesis

Next, we interrogated a potential role of IL-33 in bone formation. In human fetal bone development (endochondral ossification), vascular invasion of chondrocytes coincides

with expansion of osteoblasts and mineralization of the matrix (Charbord et al., 1996; Ramasamy et al., 2016). To test the role of IL-33 in this process, the effect of rhIL-33 on the osteogenic differentiation of human BM-derived stromal cells (BMDSCs) was assessed. The addition of rhIL-33 to osteogenic induction medium accelerated terminal differentiation of BMDSCs toward matrix-depositing osteoblasts, as suggested by Alizarin red-staining (Figure 6A), indicating increased calcific matrix deposition. To confirm this finding, we performed colorimetric assessment of calcium deposition, demonstrating a striking 3.61-fold ± 0.63 ($p < 0.02$) increase in calcium deposition of IL-33-exposed BMDSCs in comparison to BMDSCs cultured in osteogenic induction medium alone (Figure 6B). The proliferation of BMDSCs was not affected by rhIL-33 (Figures S5A and S5B), suggesting that IL-33 exerted its osteogenic effect by promoting osteoblastic differentiation or the secretion of matrix proteins by osteoblasts rather than by expanding primitive mesenchymal cells.

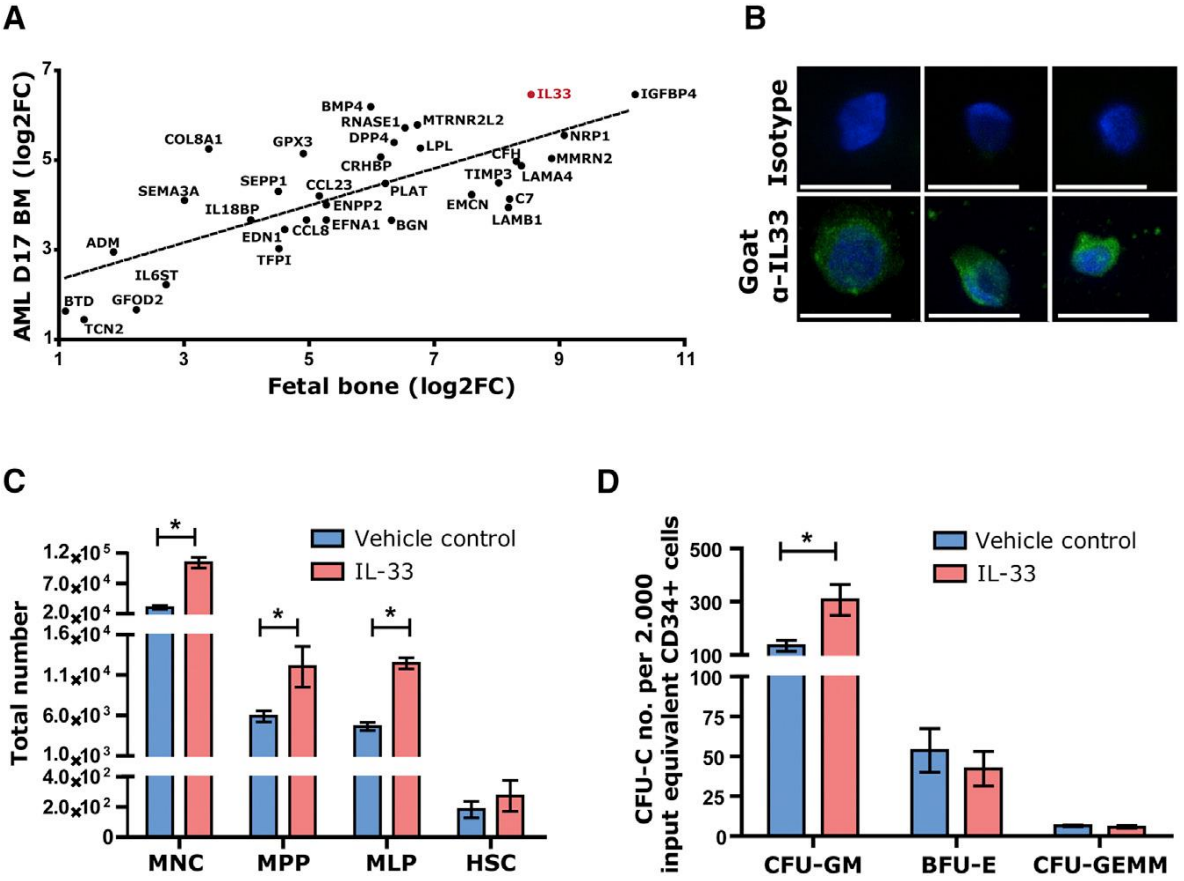


Figure 5. *Identification of IL-33 As a Hematopoietic Niche Factor. (A) Transcript expression of 34 genes encoding secreted factors significantly enriched (FDR <0.05) in fetal bone and regenerating BM ECs (Log2 fold change in comparison to steady-state BM). FDR: false discovery rate. FC: Fold Change. (B) Cropped pictures (magnification, 633) of immunostained fetal hRECs demonstrating protein expression of IL-33. White scale bar represents 20 mm. (C and D) IL-33-mediated expansion of cord blood-derived myeloid and lymphoid progenitor cells as demonstrated by (C) flow cytometric cell counting (n = 4 independent experiments) and (D) CFU-C confirming an increase in CFU-GMs (n = 3 independent experiments). Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test. HSC, hematopoietic stem cell, CD90+CD45RA- ; MLP, multilymphoid progenitor, CD90-CD45RA+; MPP, multipotent progenitor, CD90-CD45RA-*

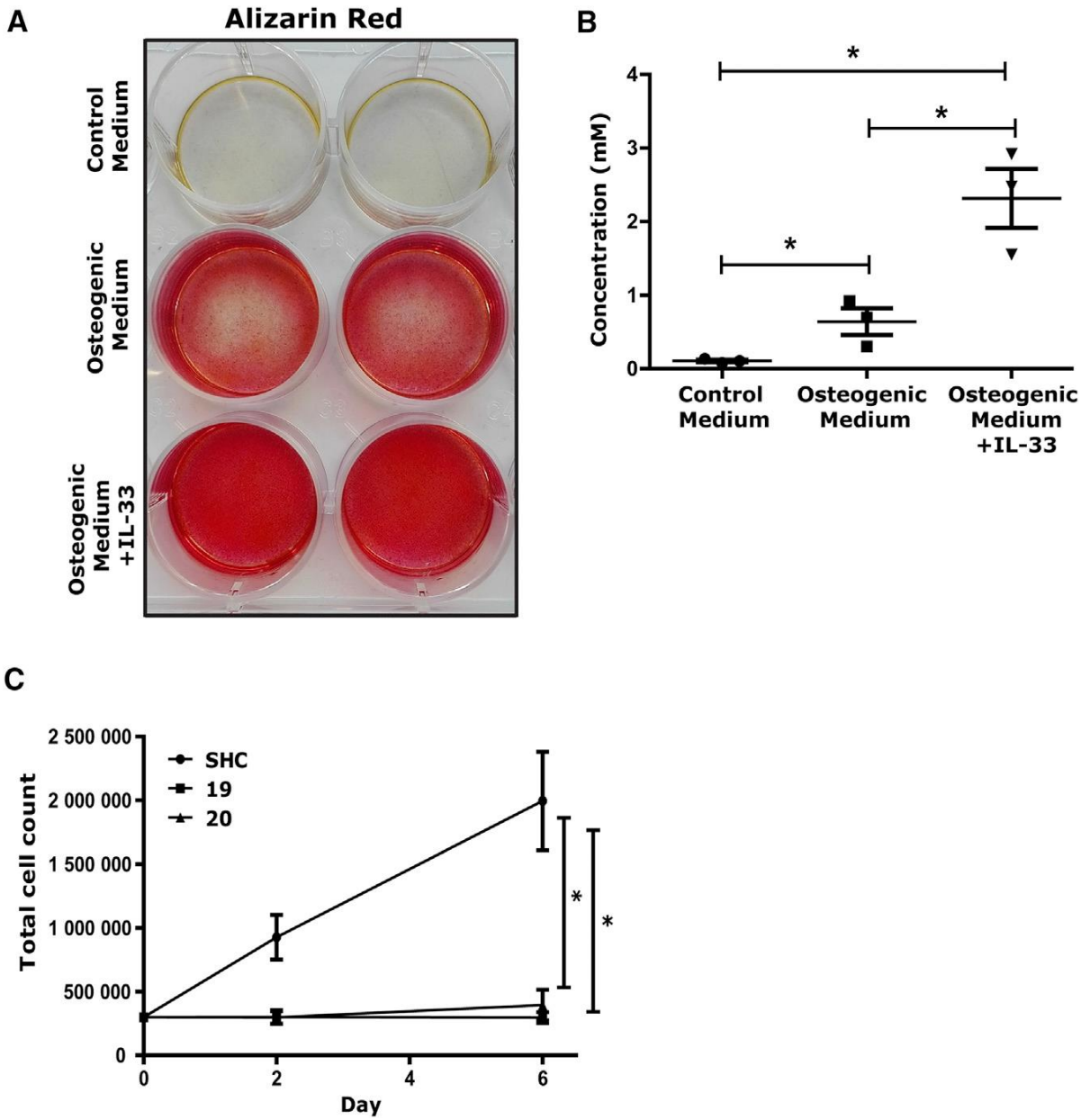


Figure 6. *IL-33 Promotes Osteogenesis and Angiogenesis In Vitro. (A and B) IL-33 accelerates terminal differentiation of mesenchymal cells toward matrix-depositing osteoblasts as demonstrated by (A) Alizarin red staining and (B) colorimetric assessment of calcium deposition (n = 3 independent experiments). (C) Knockdown of IL-33 inhibits expansion of HUVECs. Quantification of the total number of HUVECs over time (combined data of n = 3 independent experiments). Data represent mean ± SEM. *p < 0.05, 2-tailed unpaired Student's t test.*

Angiogenesis

In line with earlier reports (Choi et al., 2009), we confirmed that IL-33 is an angiogenic factor. shRNA-mediated knockdown of IL-33 (Figures S5Cand S5D) from human umbilical vein ECs (HUVECs) dramatically impaired their expansion in culture (Figure 6C), indicating that IL-33 promotes angiogenesis in an autocrine fashion.

IL-33 Promotes Expansion of Hematopoietic Precursor Cells and Alters the Architecture of the BM Niche in Mice

We next studied the in vivo relevance of these effects of IL-33 on distinct cellular components of human BM. The administration of recombinant murine IL-33 (rmIL-33) compared with PBS control resulted in expansion of immature (Lin-) (Figure S6A) and primitive progenitor (Lin-, c-KIT+, Sca-1+ [LKS]) hematopoietic cells, in particular the HPC-1 population (Figures 7A and 7B), earlier shown to contain restricted hematopoietic precursor cells with myeloid and lymphoid lineage potential (Oguro et al., 2013). Expansion of a myeloid progenitor population was confirmed with CFU-C assays (Figure S6B). Total BM cellularity and Lin-Kit+Sca1- cell counts remained unchanged (Figure S6C). In addition, the number of granulocyte-macrophage progenitors (GMPs) and myeloid cells increased after IL-33 administration (Figures 7A, 7B, S6D, and S6E), recapitulating the expansion of human hematopoietic myeloid precursors in vitro. Congruent with our findings in human hematopoietic cells, immunophenotypic HSCs were not numerically affected by IL-33 (Figures 7A and 7B). Interestingly, IL-33 also significantly expanded the population of Lin-Kit-Sca1+ BM cells, previously shown to contain early lymphoid-committed precursors with T cell, B cell, and natural killer (NK) cell potential (Kumar et al., 2008) and innate lymphoid cells (Brickshawana et al., 2011). Hematopoietic changes were accompanied by a relative increase, albeit not reaching statistical significance, in CD31+CD105+ ECs as well as Lin-Ter119-CD51+Sca- cells (earlier shown to contain lineage- committed/ osteoblastic cells) (Schepers et al., 2012) within the niche compartment (Figures S7A and S7B). Collectively, the in vitro and in vivo data indicate that IL-33 modulates distinct cellular components of hematopoietic tissue and has the potential to facilitate angiogenesis, hematopoiesis, and osteogenesis, supporting the view that elucidation of the transcriptome of hRECs may instruct the identification of modulators of these processes.

DISCUSSION

Injury to the hematopoietic system, caused by chemotherapy or irradiation, is a significant cause of morbidity and mortality in the treatment of malignant hematopoietic disease. Studies in mice have demonstrated a pivotal role of specific BM niche cells and secreted molecules in hematopoietic recovery. Translation of these findings to the clinic, however, is hampered, principally by insufficient understanding of the niche cells and molecular programs governing niche formation and hematopoietic recovery in humans. Here, by cellular dissection of the BM niche in humans during fetal development and regeneration after chemotherapeutic injury, we reveal the existence of a specific EC type (hRECs) associated with these conditions. hRECs share phenotypic and molecular similarities with specialized ECs driving hematopoietic niche formation in mice, expressing critical regulators of hematopoiesis, osteogenesis, and angiogenesis. The data comprise, to our knowledge, the first comprehensive molecular characterization of human ECs on tissue regeneration after injury. We identified the TGF-b1 receptor endoglin (CD105) as a marker of endothelium associated with BM (re)generation in fetal development and after chemotherapeutic

injury. Endoglin-expressing ECs have earlier been associated with angiogenesis in tumors and inflammation (Kumar et al., 1996), and loss of endoglin results in defective angiogenesis in mice (Li et al., 1999), supporting the view that it mediates signals governing blood vessel formation. The data indicate that endoglin with concomitant CD31 expression marks a specific subset of angiogenic ECs, which is further supported by observations that endoglin expression is strongly elevated in ECs of small, capillary-like vessels at tumor edges (Miller et al., 1999; Yoshitomi et al., 2008). Endoglin thus likely identifies a subset of endothelium during ontogeny and regeneration that marks an angiogenic subset, in line with observations in different settings. This subset revealed remarkable molecular congruence with EC subsets identified in murine studies driving bone and BM regeneration after injury.

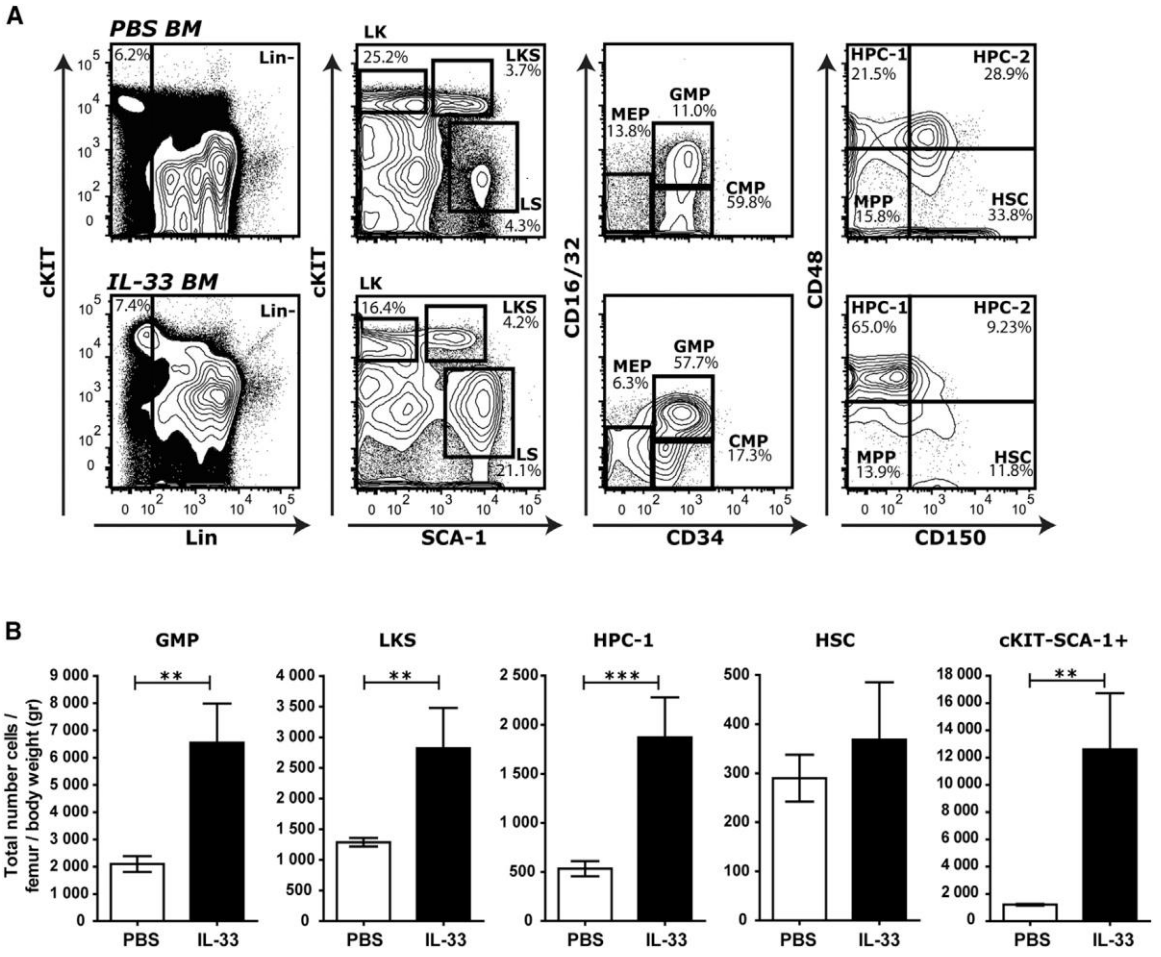


Figure 7*. Recombinant IL-33 Expands Hematopoietic Progenitors In Vivo. (A) Representative FACS plots of PBS versus IL-33-treated mice depicting the distribution of hematopoietic (progenitor) populations. CMP, LK, CD16/32-, CD34+ cells, common myeloid progenitor; GMP, LK, CD16/32+, CD34+ cells, granulocyte-macrophage progenitor; HSC, LKS, CD48- CD150+ cells, hematopoietic stem cell; HPC-1, LKS, CD48+, CD150- cells, hematopoietic progenitor-1; HPC-2, LKS, CD48+, CD150+ cells, hematopoietic progenitor-2; Lin_, Lineage-negative cells; LK, Lin-, c-KIT+, Sca-1- cells; LKS, Lin-, c-KIT+, Sca-1+ cells; LS, Lin-, c-KIT-, Sca-1+ cells. MEP, LK, CD16/32-, CD34- cells, megakaryocyte-erythroid progenitor; MPP, LKS, CD48- CD150- cells, multipotent progenitor. (B) Total BM counts of GMP, LKS, HPC-1, HSC, and LS populations in PBS treated (n = 10) and IL-33-treated (n = 5) mice. Data represent mean ± SEM. *p < 0.05, **p < 0.01, 2-tailed unpaired Student's t test.*

ECs in mice support the regeneration of the hematopoietic system after injury, such as myeloablation (Butler et al., 2010; Hooper et al., 2009; Kobayashi et al., 2010), and in recent years, several markers of specified endothelial subsets exerting this function as well as the underlying mechanisms have been revealed. Interestingly, hRECs share many characteristics with the ECs described in these studies, including expression of the cell surface proteins Tie2/TEK (Figure S2E) (Doan et al., 2013; Kopp et al., 2005), EMCN (Kusumbe et al., 2014; Ramasamy et al., 2014), and Jagged1 and activation of specific signaling pathways, including NOTCH (Butler et al., 2010; Poulos et al., 2013). Also, hRECs express many molecules previously shown to regulate HSPC behavior in mice, such as PLAT (Ibrahim et al., 2014), TFPI (Khurana et al., 2013), E-selectin (ESELE) (Figure S2E) (Winkler et al., 2012), thrombomodulin (TMBD) (Figure S2E) (Gur-Cohen et al., 2015), and tenascin C (TNC) (Figure S2E) (Nakamura-Ishizu et al., 2012). In particular, hRECs displayed striking commonalities with type H endothelium, a murine EC subtype that has recently been functionally implicated in EC-driven formation of the niche through activation of NOTCH and HIF1 signaling (Kusumbe et al., 2014; Ramasamy et al., 2014). Commonalities with the now identified hRECs include enrichment at the bone surface, reduction in frequency on aging, resistance to stress conditions, expression of markers typical for both arterial (Ephn2b, Nestin, Nrp1, Sox17, and VEGFR2), and sinusoidal vessels (VEGFR3 and EMCN), and activation of the NOTCH and HIF1 pathways driving regeneration. Expression of CD105 was not addressed

in the studies on type H endothelium, but our data in mice show that a rare population of endoglin-expressing ECs increases in frequency in the regenerative phase after chemotherapy, likely reflecting increased resistance to myeloablative stress. It is noteworthy that CD105+ ECs associated with elevated HIF-1a expression have been described in the BM of mice on regeneration after 5-flourouracil (5-FU) treatment (Nombela-Arrieta et al., 2013), making it tempting to hypothesize that these represent similar or overlapping cell types. Taken together, these immunophenotypic and molecular similarities between hRECs and murine endothelial subtypes implicated in hematopoietic niche formation point toward evolutionary conservation of these cells between mammalian species. They thus provide human relevance to findings in murine studies, supporting the notion that ECs are implicated in niche regeneration in humans. Providing experimental support for this view is challenging due to limitations inherent to the study of human cells as well as the inability to propagate highly purified hRECs ex vivo, precluding co-culture studies. As an alternative approach, we exploited elucidation of their transcriptome to identify candidate factors driving EC-driven formation of the hematopoietic niche and regeneration of HSPCs. We identified IL-33, a cytokine typically associated with innate immunity and inflammation (Cayrol and Girard, 2014), as a candidate factor. IL-33 was overexpressed in human hRECs, and expression was increased in murine CD31+CD105+ ECs on exposure to 5FU. Hematopoietic niche regenerating properties of IL-33 were demonstrated by its ex vivo capacity to facilitate hematopoiesis (increased numbers of HPCs), osteogenesis (accelerating terminal differentiation of BMDSCs toward matrix-depositing osteoblasts), and angiogenesis (expansion of HUVECs). The data follow recent reports demonstrating IL-33 to predominantly act as an ‘‘alarmin’’ released by cells

undergoing necrosis after tissue damage or active secretion (Kakkar et al., 2012; Lee et al., 2015) and playing anabolic roles in angiogenesis (Choi et al., 2009; Shan et al., 2016) and osteogenesis (Saleh et al., 2011). Our finding that rhIL-33 increased the numbers of HPCs ex vivo seems congruent with recent observations in mice, where administration of IL-33 promoted myelopoiesis (Kim et al., 2014). Of considerable interest, rhIL-33 in our experiments expanded both immunophenotypic MLPs and MPPs, in line with observations in experiments in mice where expansion of splenic lymphoid progenitors after IL-33 administration resulted in enhanced defense against opportunistic infection (Kim et al., 2014). Formal demonstration that secretion by a defined subset of ECs is required for the regenerative actions of IL-33 will have to await in vivo targeted deletion experiments, because it is currently challenging to maintain this particular subset of ECs ex vivo to enable co-culture experiments. The profound effect of IL-33 knockdown on HUVEC proliferation and maintenance precluded the use of this ex vivo system to address this question. The combined findings point to a unique role of IL-33 in mammalian species in facilitating the reconstitution of both hematopoietic lineages, which may be of considerable importance to prepare the hematopoietic system for the extra-uterine environment in ontogeny, but also for immune reconstitution after injury (e.g., hematopoietic stem cell transplantation characterized by long-term lymphocyte depletion and ensuing opportunistic infections). The exact molecular mechanisms by which IL-33 exerts these effects (either direct or indirect) remain to be fully elucidated. IL-33 expression in HUVECs has been associated with a quiescent cellular state (Küchler et al., 2008), and although we did not examine the cell cycle status of CD31+CD105+, IL-33high hRECs, this might help explain the notion that they may be relatively resistant to chemotherapeutic myeloablation. We can speculate that quiescent CD31+CD105+, IL-33high hRECs survive chemotherapy and are ‘‘activated’’ to release IL-33 as an anabolic hematopoietic factor. In this context, it is noteworthy that hRECs display transcriptional activation of DLL4 and the Notch pathway, earlier shown to be an important driver of IL-33 expression (Sundlisaeter et al., 2012). Regardless of the underlying molecular mechanisms of IL-33 expression, the data support the notion that elucidation of the transcriptome of hRECs may instruct the identification of the proteins and pathways driving niche formation after injury. It is conceivable that receptor-ligand interactions allow targeting of these cells to drive regeneration (as previously shown by pharmacologic modulation of NOTCH signaling in mice [Ramasamyet al., 2014]). In this context, it is noteworthy that transcriptional profiling of fetal hRECs revealed overexpression of genes encoding secreted factors as well as their receptors, suggesting the potential relevance of autocrine signaling in the biology of hRECs. Alternatively, it would be worthwhile to test the ability of identified secreted factors to expand human HSPCs ex vivo, either directly or in co-culture settings with mesenchymal elements. Collectively, the identification of human ECs associated with hematopoietic niche formation and the elucidation of their transcriptome are anticipated to provide a valuable resource for the regenerative community to relate findings in animal models to human biology and to instruct in vivo and ex vivo approaches to foster EC-driven regeneration of the hematopoietic system after injury.

EXPERIMENTAL PROCEDURES

Human BM Samples

BM aspirates of AML patients were collected at diagnosis, 17 days after the start of chemotherapy (3+7 schedule of chemotherapy with anthracycline and cytarabin), and on achievement of complete remission (median age: 65 years, range: 28–76 years). The time point of 17 days after the start of chemotherapy represents the neutropenic phase, 10 days after administration of chemotherapy and, on average, 4 days before recovery of neutropenia. Control marrow was obtained by aspiration from donors for allogeneic transplantation (median age: 40 years, range: 39–48 years) after obtaining written informed consent. In addition, trabecular hip bone samples were collected from patients undergoing hip replacement surgery (median age: 55 years, range: 22–71 years). Human fetal long bones (median age: 18 gestational weeks, range: 15–20 gestational weeks) were obtained from elective abortions. Gestational age was confirmed by ultrasonic measurement of skull diameter and femoral length. The use of human samples with informed consent was approved by the Institutional Review Board of the Erasmus Medical Center (the Netherlands) in accordance with the Declaration of Helsinki.

RNA-seq and GSEA Analysis

RNA of sorted cells was extracted according to the manufacturer's instructions for RNA isolation with GenElute LPA (Sigma). cDNA was prepared using the SMARTer procedure (SMARTer Ultra Low RNA Kit, Clonetech). Library preparation and RNA-seq was performed as previously described and validated for low input (Chen et al., 2016). Finally, GSEA was performed on the fragments per kilobase million (FPKM) values using the curated C2 collection of gene sets within the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005).

Mice and In Vivo Procedures

C57BL/6JOlaHsd wild-type mice were purchased from Envigo. Animals were maintained in specific pathogen-free conditions in the Experimental Animal Center of Erasmus MC (EDC). To study the murine niche in regenerative conditions, adult mice (7–12 weeks old) were intraperitoneally administered 250 mg/kg 5-FU and then sacrificed 7 days after 5-FU treatment. To study the effect of rmIL-33 on steady-state hematopoiesis and the BM niche, adult mice (7–14 weeks old) were intraperitoneally injected with 2 mg of recombinant IL-33 (catalog no. 580504, BioLegend) or PBS vehicle control daily for 6 consecutive days and then sacrificed. All mice were sacrificed by cervical dislocation. Mouse BM and bone fraction cells were isolated as previously described (Zambetti et al., 2016). Peripheral blood was collected from the submandibular vein in K2EDTA-coated microtainers (BD) and analyzed using a Vet ABC counter (Scil Animal Care). Animal studies were approved by the Animal Welfare/Ethics Committee of the EDC in accordance with legislation in the Netherlands (approval no. EMC 4015).

Liquid Culture of CD34+ CB Cells

A total of 20,000 CD34+ CB cells in 200 mL per well were cultured in StemSpan SFEM (Stem Cell Technologies, catalog no. 9600) with SCF (50 ng/mL, Cellgenix, Freiburg, Germany), in a flat-bottom, 96-well plate at 37C and 5% CO2. Two mL mQ solution containing only rhIL-33 protein (ProSpec, catalog number CYT-425) or just mQ (vehicle control) was added to the medium for a final concentration of 25 ng/mL rhIL-33 or 1% mQ, respectively. The medium was refreshed every 3 or 4 days, and cells were collected at day 7 for FACS analysis or for hematopoietic colony forming-unit assay.

Culture of Human BMDSCs

Human BMDSCs (catalog no. PT-2501, Lonza) were cultured as described previously (Brum et al., 2015). For osteogenic differentiation, BMDSCs were cultured in osteogenic induction medium (aMEM medium containing 10% heat-inactivated fetal calf serum [FCS] supplemented with 100 nM dexamethasone and 10mMb-glycerophosphate) with rhIL-33 (250 ng/mL) or mQ vehicle control for 3 weeks. Medium was refreshed every 3–4 days.

Culture and shRNA-Mediated Knockdown of IL-33 in HUVECs

HUVECs were expanded in EGM-2 Bulletkit medium (CC-3156 and CC-4176, Lonza). RNAi was achieved by lentiviral transduction. Briefly, shRNAs against IL-33 (sh19: TRCN0000135845 and sh20: TRCN0000135846) and a non-target control (shControl: SHC002 [SHC]) cloned in the pLKO.1 backbones were obtained from the Mission TRC shRNA library (Sigma-Aldrich). Lentiviral shRNAs were produced in HEK293T cells after co-transfection of shControl, sh19, or sh20 together with the packaging plasmids pSPAX2 and pMDG.2. HUVECs were infected with lentivirus for 24 hr and selected for 5 days with 2mg/mL of puromycin.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software). Unless otherwise specified, unpaired, 2-tailed Student’s t test (single test for comparison of 2 means) or 1-way ANOVA followed by Bonferroni correction for multiple comparisons were used to evaluate statistical significance, defined as $p < 0.05$. All results in bar graphs are means ± SEMs.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data derived from human healthy adults, AML day 17 patients, and fetal bone specimens is European Genome-phenome Archive: EGAS00001002736.

REFERENCES

Barreiro, O., Yáñez-Mó, M., Sala-Valdés, M., Gutiérrez-López, M.D., Ovalle, S., Higginbottom, A., Monk, P.N., Cabañas, C., Sánchez-Madrid, F., 2005. Endothelial tetraspanin microdomains regulate leukocyte firm adhesion during extravasation. Blood 105, 2852–2861.

Blumenfeld, I., Srouji, S., Lanir, Y., Laufer, D., Livne, E., 2002. Enhancement of bone defect healing in old rats by TGF-β and IGF-1. Exp. Gerontol. 37, 553–565.

Brickshawana, A., Shapiro, V.S., Kita, H., Pease, L.R., 2011. Lineage(-)Sca1+c-Kit(-)CD25+ cells are IL-33-responsive type 2 innate cells in the mouse bone marrow. J. Immunol. 187, 5795–804.

Broudy, V.C., 1997. Stem cell factor and hematopoiesis. Blood 90, 1345–64.

Brum, A.M., van de Peppel, J., van der Leije, C.S., Schreuders-Koedam, M., Eijken, M., van der Eerden, B.C.J., van Leeuwen, J.P.T.M., 2015. Connectivity Map-based discovery of parbendazole reveals targetable human osteogenic pathway. Proc. Natl. Acad. Sci. 112, 12711–12716.

Butler, J.M., Nolan, D.J., Vertes, E.L., Varnum-Finney, B., Kobayashi, H., Hooper, A.T., Seandel, M., Shido, K., White, I.A., Kobayashi, M., Witte, L., May, C., Shawber, C., Kimura, Y., Kitajewski, J., Rosenwaks, Z., Bernstein, I.D., Rafii, S., 2010. Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells. Cell Stem Cell 6, 251–264.

Carriere, V., Roussel, L., Ortega, N., Lacorre, D.-A., Americh, L., Aguilar, L., Bouche, G., Girard, J.-P., 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc. Natl. Acad. Sci. 104, 282–287.

Cayrol, C., Girard, J.P., 2014. IL-33: An alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr. Opin. Immunol. 31, 31–37.

Charbord, P., Tavian, M., Humeau, L., Peault, B., 1996. Early ontogeny of the human marrow from long bones: an immunohistochemical study of hematopoiesis and its microenvironment. Blood 87, 4109–4119.

Cheifetz, S., Bellón, T., Calés, C., Vera, S., Bernabeu, C., Massagué, J., Letarte, M., 1992. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J. Biol. Chem. 267, 19027–30.

Chen, S., Zambetti, N.A., Bindels, E.M.J., Kenswill, K., Mylona, A.M., Adisty, N.M., Hoogenboezem, R.M., Sanders, M.A., Cremers, E.M.P., Westers, T.M., Jansen, J.H., van de Loosdrecht, A.A., Raaijmakers, M.H.G.P., 2016. Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. Leukemia 30, 1938–42.

Choi, Y., Choi, H., Min, J., Pyun, B., Maeng, Y., Park, H., Kim, J., Kim, Y., Kwon, Y., 2009. ST2 / TRAF6-mediated endothelial nitric oxide production Interleukin-33 induces angiogenesis and vascular permeability through ST2 / TRAF6-mediated endothelial nitric oxide production. Blood 114, 3117–3126.

Clines, G. a, Mohammad, K.S., Bao, Y., Stephens, O.W., Suva, L.J., Shaughnessy, J.D., Fox, J.W., Chirgwin, J.M., Guise, T. a, 2007. Dickkopf Homolog 1 Mediates Endothelin-1-Stimulated New Bone Formation. Mol. Endocrinol. 21, 486–498.

Coşkun, S., Chao, H., Vasavada, H., Heydari, K., Gonzales, N., Zhou, X., de Crombrugghe, B., Hirschi, K.K., 2014. Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. Cell Rep. 9, 581–90.

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B., Chao, N.J., Kirsch, D.G., Chute, J.P., 2013. Tie2 + Bone Marrow Endothelial Cells Regulate Hematopoietic Stem Cell Regeneration Following Radiation Injury. Stem Cells 31, 327–337.

Duff, S.E., Li, C., Garland, J.M., Kumar, S., 2003. CD105 is important for angiogenesis: evidence and potential applications. FASEB J. 17, 984–992.

Edwards, C.M., Mundy, G.R., 2008. Eph receptors and ephrin signaling pathways: A role in bone homeostasis. Int. J. Med. Sci. 5, 263–272.

Gur-Cohen, S., Itkin, T., Chakrabarty, S., Graf, C., Kollet, O., Ludin, A., Golan, K., Kalinkovich, A., Ledergor, G., Wong, E., Niemeyer, E., Porat, Z., Erez, A., Sagi, I., Esmon, C.T., Ruf, W., Lapidot, T., 2015. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. Nat. Med. 21, 1307–17.

Holmes, C., Stanford, W.L., 2007. Concise Review: Stem Cell Antigen-1: Expression, Function, and Enigma. Stem Cells 25, 1339–1347.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.-G., Shido, K., Petit, I., Yanger, K., James, D., Witte, L., Zhu, Z., Wu, Y., Pytowski, B., Rosenwaks, Z., Mittal, V.,

Sato, T.N., Rafii, S., 2009. Engraftment and Reconstitution of Hematopoiesis Is Dependent on VEGFR2-Mediated Regeneration of Sinusoidal Endothelial Cells. Cell Stem Cell 4, 263–274.
Ibrahim, A.A., Yahata, T., Onizuka, M., Dan, T., Van Ypersele De Strihou, C., Miyata, T., Ando, K., 2014. Inhibition of plasminogen activator inhibitor type-1 activity enhances rapid and sustainable hematopoietic regeneration. Stem Cells 32, 946–958.
Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G., Kalinkovich, A., Ludin, A., Kollet, O., Shakhar, G., Butler, J.M., Rafii, S., Adams, R.H., Scadden, D.T., Lin, C.P., Lapidot, T., 2016. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 532, 323–328.
Jagannathan-Bogdan, M., Zon, L.I., 2013. Hematopoiesis. Development 140, 2463–7.
Kakkar, R., Hei, H., Dobner, S., Lee, R.T., 2012. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. J. Biol. Chem. 287, 6941–6948.
Khurana, S., Margamuljana, L., Joseph, C., Schouteden, S., Buckley, S.M., Verfaillie, C.M., 2013. Glypican-3-mediated inhibition of CD26 by TFPI: a novel mechanism in hematopoietic stem cell homing and maintenance. Blood 121, 2587–2595.
Kim, J., Kim, W., Le, H.T., Moon, U.J., Tran, V.G., Kim, H.J., Jung, S., Nguyen, Q.-T., Kim, B.-S., Jun, J.-B., Cho, H.R., Kwon, B., 2014. IL-33–Induced Hematopoietic Stem and Progenitor Cell Mobilization Depends upon CCR2. J. Immunol. 193, 3792–3802.
Kobayashi, H., Butler, J.M., O'Donnell, R., Kobayashi, M., Ding, B.-S., Bonner, B., Chiu, V.K., Nolan, D.J., Shido, K., Benjamin, L., Rafii, S., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. Nat. Cell Biol. 12, 1046–1056.
Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S. V., Ramos, C.A., Zhang, F., Rafii, S., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood 106, 505–513.
Kopp, H.G., Hooper, A.T., Avecilla, S.T., Rafii, S., 2009. Functional heterogeneity of the bone marrow vascular niche. Ann. N. Y. Acad. Sci. 1176, 47–54.
Küchler, A.M., Pollheimer, J., Balogh, J., Sponheim, J., Manley, L., Sorensen, D.R., De Angelis, P.M., Scott, H., Haraldsen, G., 2008. Nuclear Interleukin-33 Is Generally Expressed in Resting Endothelium but Rapidly Lost upon Angiogenic or Proinflammatory Activation. Am. J. Pathol. 173, 1229–1242.
Kumar, P., Wang, J.M., Bernabeu, C., 1996. CD 105 and angiogenesis. J. Pathol. 178, 363–6.
Kumar, R., Fossati, V., Israel, M., Snoeck, H.-W., 2008. Lin-Sca1+Kit- Bone Marrow Cells Contain Early Lymphoid-Committed Precursors That Are Distinct from Common Lymphoid Progenitors. J. Immunol. 181, 7507–7513.
Kusumbe, A.P., Ramasamy, S.K., Adams, R.H., 2014. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 507, 323–328.
Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., Adams, R.H., 2016. Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature 532, 380–384.
Lee, E.-J., Kim, J.W., Yoo, H., Kwak, W., Choi, W.H., Cho, S., Choi, Y.J., Lee, Y.-J., Cho, J., 2015. Single high-dose irradiation aggravates eosinophil-mediated fibrosis through IL-33 secreted from impaired vessels in the skin compared to fractionated irradiation. Biochem. Biophys. Res. Commun. 464, 20–26.
Lerner, C., Harrison, D.E., 1990. 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. Exp. Hematol. 18, 114–8.
Li, D.Y., Sorensen, L.K., Brooke, B.S., Urness, L.D., Davis, E.C., Taylor, D.G., Boak, B.B., Wendel, D.P., 1999. Defective angiogenesis in mice lacking endoglin. Science. 284, 1534–7.
Maes, C., 2013. Role and regulation of vascularization processes in endochondral bones. Calcif. Tissue Int. 92, 307–323.
Medvinsky, A., Rybtsov, S., Taoudi, S., 2011. Embryonic origin of the adult hematopoietic system: advances and questions. Development 138, 1017–31. https://doi.org/10.1242/dev.040998
Miller, D.W., Graulich, W., Karges, B., Stahl, S., Ernst, M., Ramaswamy, A., Sedlacek, H.H., Müller, R., Adamkiewicz, J., 1999. Elevated expression of endoglin, a component of the TGF-beta-receptor complex, correlates with proliferation of tumor endothelial cells. Int. J. cancer 81, 568–72.
Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., Kubota, Y., 2012. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. Blood 119, 5429–5437.
Nombela-Arrieta, C., Pivarnik, G., Winkel, B., Canty, K.J., Harley, B., Mahoney, J.E., Park, S.-Y., Lu, J., Protopopov, A., Silberstein, L.E., 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nat. Cell Biol. 15, 533–543.

Oguro, H., Ding, L., Morrison, S.J., 2013. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell 13, 102–16.

Oshima, A., Tanabe, H., Yan, T., Lowe, G.N., Glackin, C.A., Kudo, A., 2002. A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, Twist. J. Cell. Biochem. 86, 792–804.

Poulos, M.G., Guo, P., Kofler, N.M., Pinho, S., Gutkin, M.C., Tikhonova, A., Aifantis, I., Frenette, P.S., Kitajewski, J., Rafii, S., Butler, J.M., 2013. Endothelial Jagged-1 Is Necessary for Homeostatic and Regenerative Hematopoiesis. Cell Rep. 4, 1022–1034.

Rafii, S., Butler, J.M., Ding, B.-S., 2016. Angiocrine functions of organ-specific endothelial cells. Nature 529, 316–325.

Ramasamy, S.K., Kusumbe, A.P., Adams, R.H., 2015. Regulation of tissue morphogenesis by endothelial cell-derived signals. Trends Cell Biol. 25, 148–157.

Ramasamy, S.K., Kusumbe, A.P., Itkin, T., Gur-Cohen, S., Lapidot, T., Adams, R.H., 2016. Regulation of Hematopoiesis and Osteogenesis by Blood Vessel–Derived Signals. Annu. Rev. Cell Dev. Biol. 32, 649–675.

Ramasamy, S.K., Kusumbe, A.P., Wang, L., Adams, R.H., 2014. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature 507, 376–380.

Salani, D., Taraboletti, G., Rosanò, L., Di Castro, V., Borsotti, P., Giavazzi, R., Bagnato, A., 2000. Endothelin-1 Induces an Angiogenic Phenotype in Cultured Endothelial Cells and Stimulates Neovascularization In Vivo. Am. J. Pathol. 157, 1703–1711.

Salazar, V.S., Gamer, L.W., Rosen, V., 2016. BMP signalling in skeletal development, disease and repair. Nat. Rev. Endocrinol. 12, 203–221.

Saleh, H., Eeles, D., Hodge, J.M., Nicholson, G.C., Gu, R., Pompolo, S., Gillespie, M.T., Quinn, J.M.W., 2011. Interleukin-33, a target of parathyroid hormone and oncostatin m, increases osteoblastic matrix mineral deposition and inhibits osteoclast formation in vitro. Endocrinology 152, 1911–1922.

Salvucci, O., Tosato, G., 2012. Essential Roles of EphB Receptors and EphrinB Ligands in Endothelial Cell Function and Angiogenesis, in: Advances in Cancer Research. pp. 21–57.

Schepers, K., Hsiao, E.C., Garg, T., Scott, M.J., Passegue, E., 2012. Activated Gs signaling in osteoblastic cells alters the hematopoietic stem cell niche in mice. Blood 120, 3425–3435.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391–397.

Shan, S., Li, Y., Wang, J., Lv, Z., Yi, D., Huang, Q., Corrigan, C.J., Wang, W., Quangeng, Z., Ying, S., 2016. Nasal administration of interleukin-33 induces airways angiogenesis and expression of multiple angiogenic factors in a murine asthma surrogate. Immunology 148, 83–91.

Shao, R., Bao, S., Bai, X., Blanchette, C., Anderson, R.M., Dang, T., Gishizky, M.L., Marks, J.R., Wang, X., 2004. Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. Mol. Cell. Biol. 24, 3992–4003.

Shigematsu, S., Yamauchi, K., Nakajima, K., Iijima, S., Aizawa, T., Hashizume, K., 1999. IGF-1 regulates migration and angiogenesis of human endothelial cells. Endocr. J. 46 Suppl, S59–S62.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M. a, Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. 102, 15545–15550.

Sugiyama, T., Kohara, H., Noda, M., Nagasawa, T., 2006. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. Immunity 25, 977–988.

Sundlisæter, E., Edelmann, R.J., Hol, J., Sponheim, J., Kchler, A.M., Weiss, M., Udalova, I.A., Midwood, K.S., Kasprzycka, M., Haraldsen, G., 2012. The alarmin IL-33 is a notch target in quiescent endothelial cells. Am. J. Pathol. 181, 1099–1111.

Tamagnone, L., Giordano, S., 2006. Semaphorin pathways orchestrate osteogenesis. Nat. Cell Biol. 8, 545–547.

Wang, J.M., Kumar, S., Van Agthoven, A., Kumar, P., Pye, D., Hunter, R.D., 1995. Irradiation induces up-regulation of E9 protein (CD105) in human vascular endothelial cells. Int. J. Cancer 62, 791–796.

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., Lévesque, J.-P., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. 18, 1651–1657.

Xian, C.J., Cool, J.C., Pyragius, T., Foster, B.K., 2006. Damage and recovery of the bone growth

mechanism in young rats following 5-fluorouracil acute chemotherapy. J. Cell. Biochem. 99, 1688–1704.
Yoshitomi, H., Kobayashi, S., Ohtsuka, M., Kimura, F., Shimizu, H., Yoshidome, H., Miyazaki, M., 2008. Specific expression of endoglin (CD105) in endothelial cells of intratumoral blood and lymphatic vessels in pancreatic cancer. Pancreas 37, 275–281.
Zambetti, N.A., Ping, Z., Chen, S., Kenswil, K.J.G., Mylona, M.A., Sanders, M.A., Hoogenboezem, R.M., Bindels, E.M.J., Adisty, M.N., Van Strien, P.M.H., van der Leije, C.S., Westers, T.M., Cremers, E.M.P., Milanese, C., Mastroberardino, P.G., van Leeuwen, J.P.T.M., van der Eerden, B.C.J., Touw, I.P., Kuijpers, T.W., Kanaar, R., van de Loosdrecht, A.A., Vogl, T., Raaijmakers, M.H.G.P., 2016. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. Cell Stem Cell 19, 613–627.
Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., Lodish, H.F., 2006. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat. Med. 12, 240–245.

SUPPLEMENTAL INFORMATION

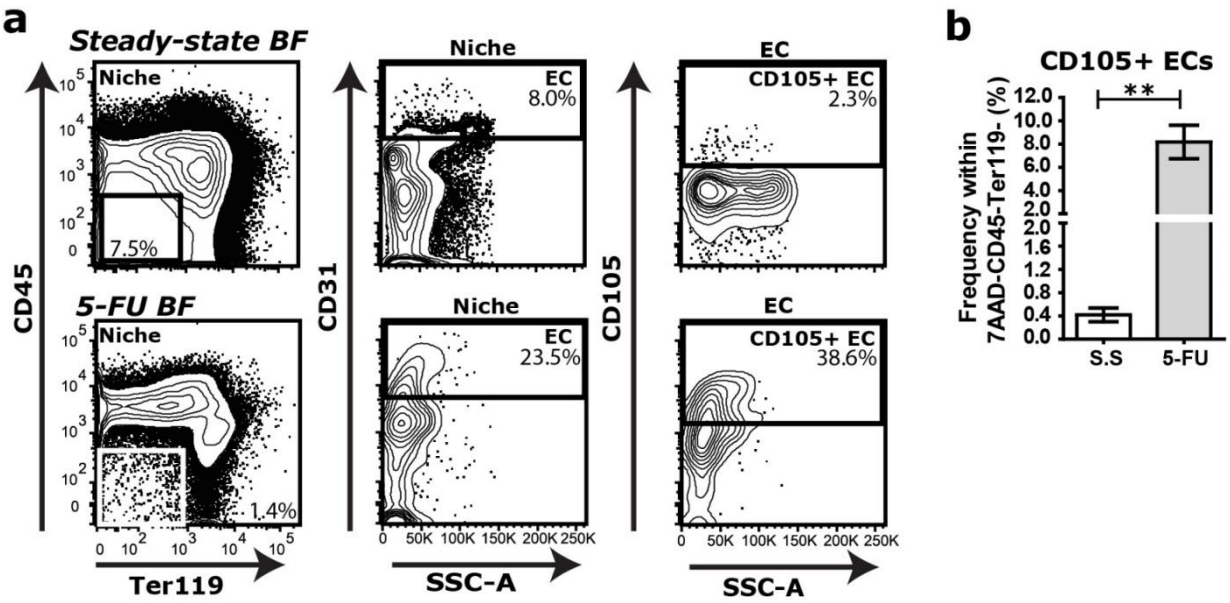
Supplemental Table 1

Supplemental Table 1. Related to Figure 1. Differential expression of candidate membrane markers of endothelial cells associated with regeneration after chemotherapy.

Gene Name	Cluster of differentation	FDR	FC	Avg FPKM AML D17	Avg FPKM Healthy CTRL
DPP4	CD26	4,30E-06	42,06162224	1,447326333	0,018322079
ENG	CD105	1,61E-03	8,55449896	15,62384567	1,547395714
VCAM1	CD106	3,81E-06	50,16296233	88,93116733	0,804735029
LIFR	CD118	2,08E-02	10,1185124	0,1064362	0,016143224
IL1R1	CD121a	1,54E-03	22,29341884	1,835577597	0,040494024
CDH5	CD144	4,26E-03	22,72755944	4,79532	0,072456899
CXCR3	CD183	1,04E-02	6,439581287	4,524633333	1,938153714
CCR2	CD192	1,09E-03	5,554609242	17,7306	4,429592857
PROCR	CD201	1,03E-03	19,40383328	7,131228667	0,224176357
TSPAN7	CD231	2,61E-05	24,32565573	54,17457	1,558463086
TNFSF10	CD253	1,16E-02	5,826945634	10,84400633	1,776891571
CD300LG	CD300g	1,90E-04	38,86795899	14,576502	0,166514471
NRP1	CD304	3,82E-06	47,01754435	9,396109867	0,113237357
KDR	CD309	1,69E-02	18,71674562	6,977216	0,077225637
FZD4	CD344	1,50E-06	45,00592407	2,850081333	0,043208416
S1PR1	CD363	1,33E-03	13,72492829	11,43101533	0,575994457

Genes encoding cluster of differentiation antigens overexpressed in endothelial cells ($CD45^{-}CD235^{-}CD31^{+}CD9^{+}$) in aspirates obtained from regenerative marrow (n= 3) compared to postnatal steady-state BM (n= 7). FC: Fold change. FPKM: fragments per kilobase of exon per million fragments mapped. BM: bone marrow. FDR: false discovery rate.

Supplementary Figure S1. Related to Figure 2. CD105-expressing endothelial cells are enriched in collagenased bone of mice after chemotherapeutic injury

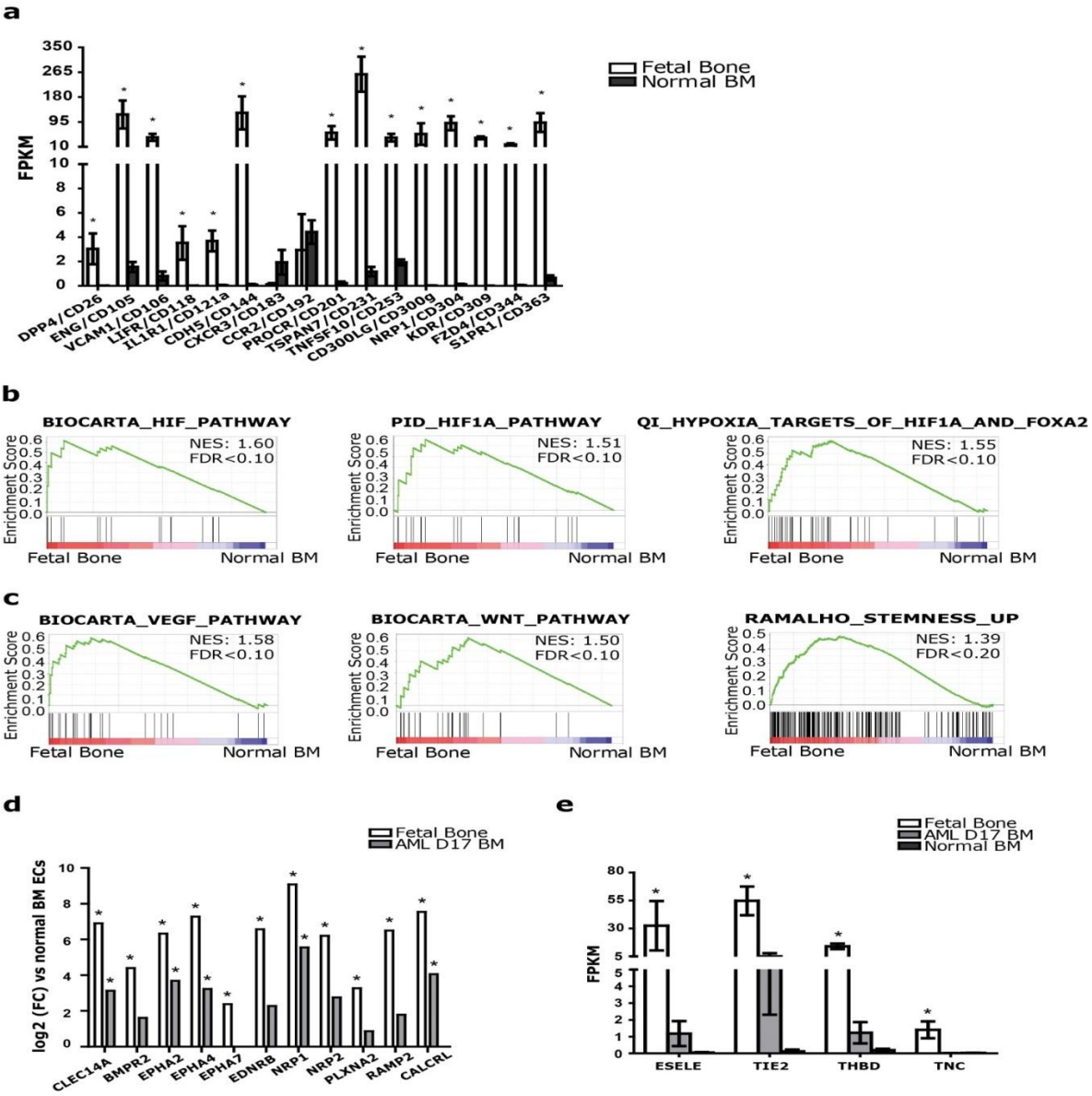


Supplementary Figure S1

(a) Representative FACS plots revealing the enrichment of a CD105-expressing endothelial cells (ECs) in collagenased bone after administration of 5-fluoroucil. Gating strategy for identification of murine ECs is also depicted (see also figure 2). BF=Bone fraction.

*(b) Frequency of CD105^{+} ECs within collagenased bone of mice in steady-state (n=3) and after administration of 5-fluoroucil (n=5). Data represent mean ± s.e.m. **p <0.01, two-tailed unpaired t-test. S.S=Steady-state. 5-FU=5-fluorouracil*

Supplementary Figure S2



Supplementary Figure S2. Related to Supplemental Table 1, Figure 4 and 5. Transcriptional signatures of fetal CD105+ ECs

a. Transcript expression of membrane proteins (CD markers) in fetal bone CD31^{+}CD105^{+} ECs compared to steady-state BM ECs. The overlap with transcripts identified in ECs isolated from D17 BM (Table S1) supports the notion that the enrichment of these transcripts in ECs in the regenerative marrow is caused by overexpression in the subset of CD105^{+} ECs.

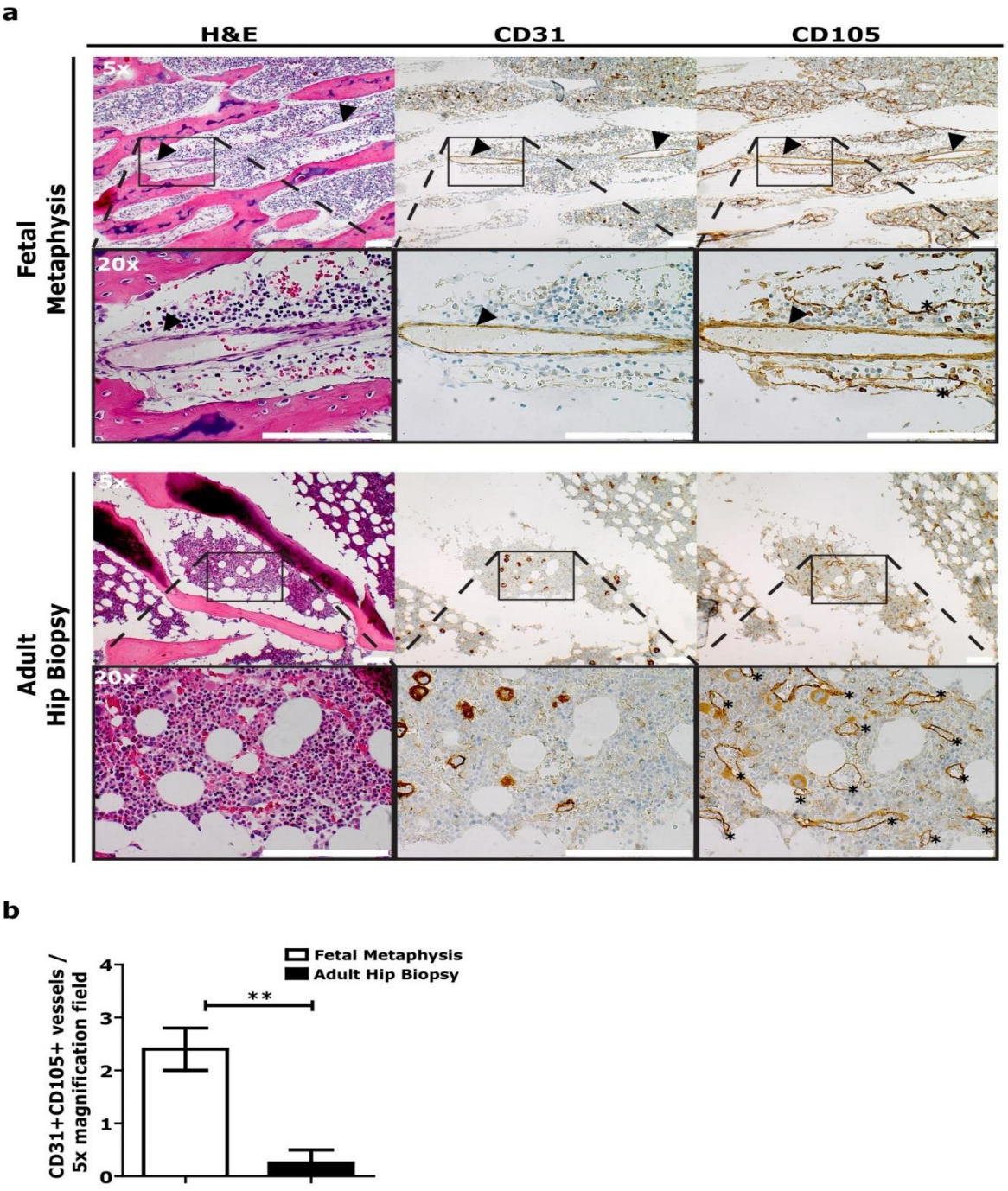
b,c. GSEA plots demonstrating activation of HIF1a signaling (b) and gene sets associated with VEGF, WNT and stem cells (c) in fetal hRECs compared to steady-state BM. GSEA: gene sets enrichment analysis.

d. Transcript expression (Log 2 fold change) of cognate receptors of candidate secreted proteins (Figure 5A) in fetal hRECs and ECs of regenerative marrow compared to steady-state bone marrow.

*e. Transcript abundance of molecules associated with regeneration previously described in murine studies (Doan et al., 2013; Gur-Cohen et al., 2015; Kopp et al., 2005; Nakamura-Ishizu et al., 2012; Winkler et al., 2012). *<FDR 0.05. FDR: false discovery rate.*

**<FDR 0.05. FDR: false discovery rate. FPKM: fragments per kilobase of exon per million fragments mapped. BM: bone marrow. NES: normalized enrichment score. FC: Fold Change.*

Supplementary Figure S3.

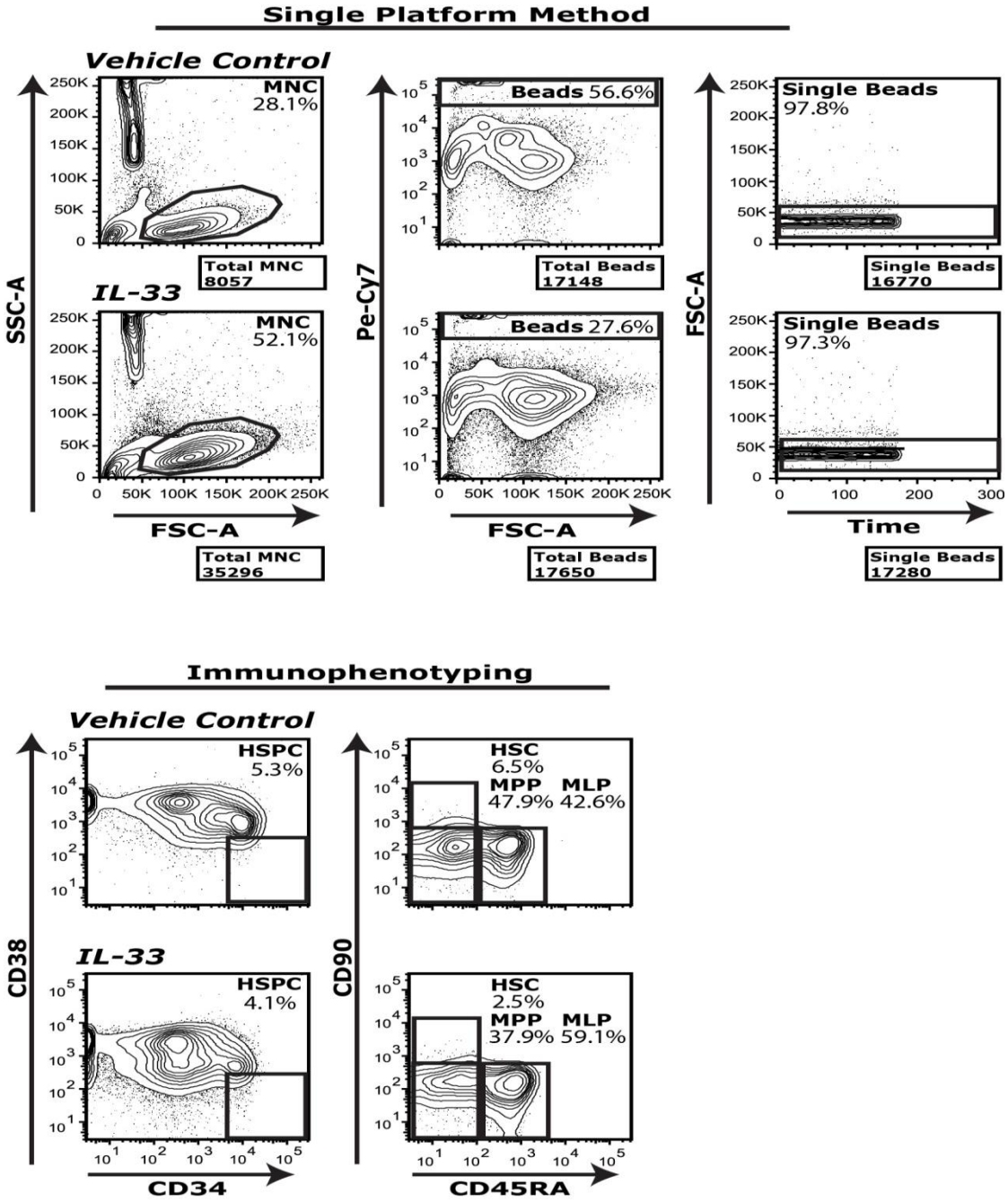


Supplementary Figure S3. Related to Figure 3. In situ immunohistochemistry of CD31⁺CD105⁺ endothelial cells in human fetal and postnatal bone marrow.

a. Hematoxylin and eosin, CD31, and CD105 staining in consecutive serial sections of fetal and adult trabecular bone. $CD31^+CD105^+$ vessels (indicated by black arrowheads) were readily detected in the fetal bone, but at much lower frequency in postnatal trabecular bone. Asterisks highlight sinusoidal endothelium, marked by CD105 expression and concomitant low or lack of CD31 expression. Note: hematopoietic elements with high CD31 expression (indicated by black arrows) represent megakaryocytes. Representative 5x and 20x magnified images. Scale bars represent 100 µm.

*b. Quantified total number of $CD31^+CD105^+$ vessels per 5x magnification field in trabecular areas of human fetal (n=5) and postnatal trabecular bone (n=4). Data represent mean ± s.e.m. **p <0.01, two-tailed unpaired t-test.*

Supplementary Figure S4

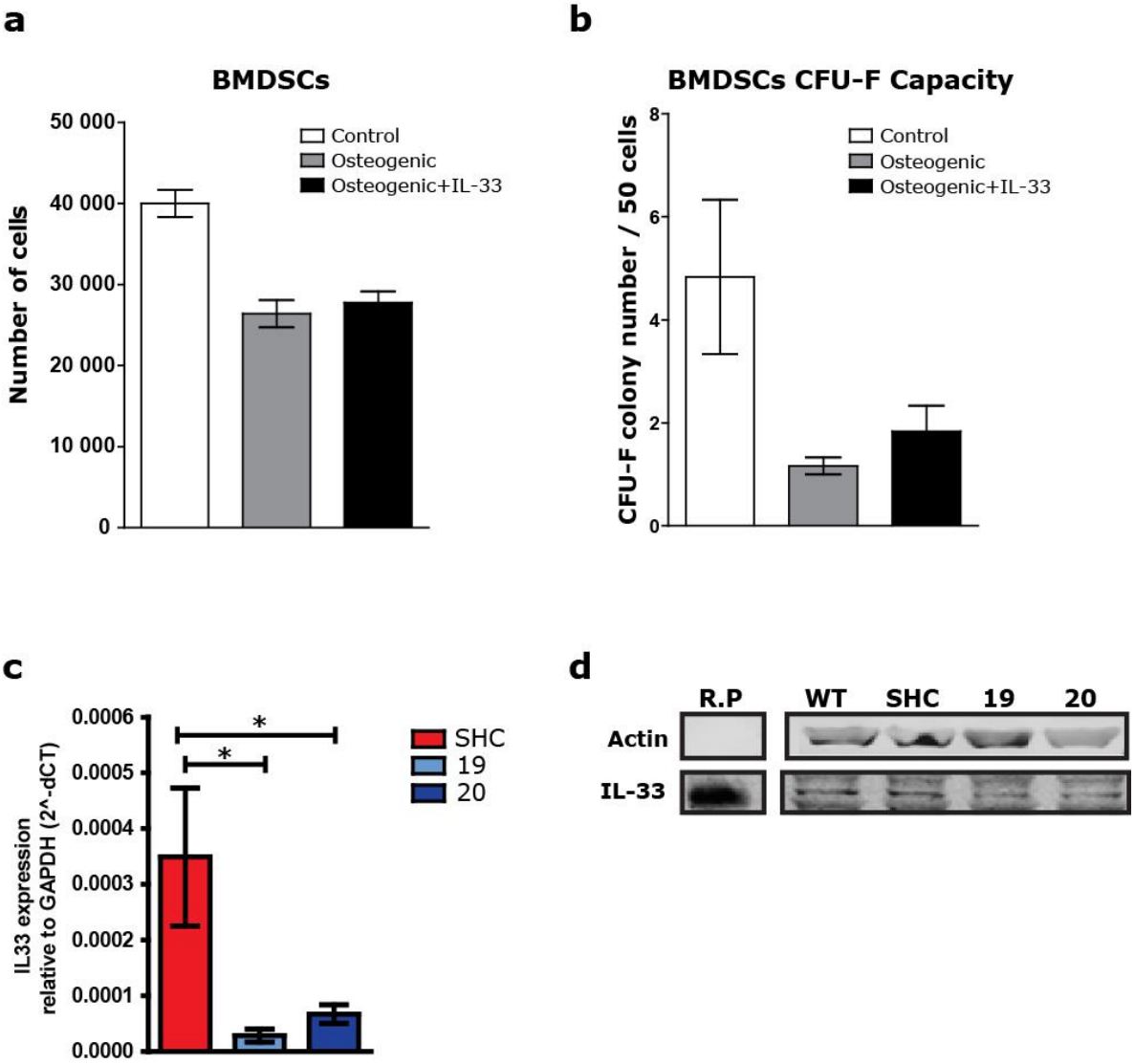


Supplementary Figure S4. Related to Figure 5. Representative FACS plots of cord blood stem/progenitor cells exposed to recombinant IL-33 for one week.

Top, representative example of the single platform flow cytometric assay to quantify total cell number. Total number of mononuclear cells were calculated using the total number of MNC and beads events recorded using the quantification formula described in Experimental Procedures. Note that the MNC/Beads ratio in the vehicle control (VC) sample approximates 0.5, whereas the IL-33 treated sample is 2.0, indicating an a 4-fold increase of MNC in comparison with VC.

Bottom, characterization of the MNCs based on CD marker expression. $CD34^{+}CD38^{lo}$ MNCs were identified as hematopoietic stem and progenitor cells (HSPCs). HSPCs were further subdivided based on CD90 and CD45RA expression. $CD90^{+}CD45RA^{-}$ cells were defined as hematopoietic stem cells (HSCs), CD90-$CD45RA^{-}$ cells classified as multipotent progenitor cells (MPP), and $CD90^{-}CD45RA^{+}$ cells as multilymphoid progenitors (MLP).

Supplementary Figure S5



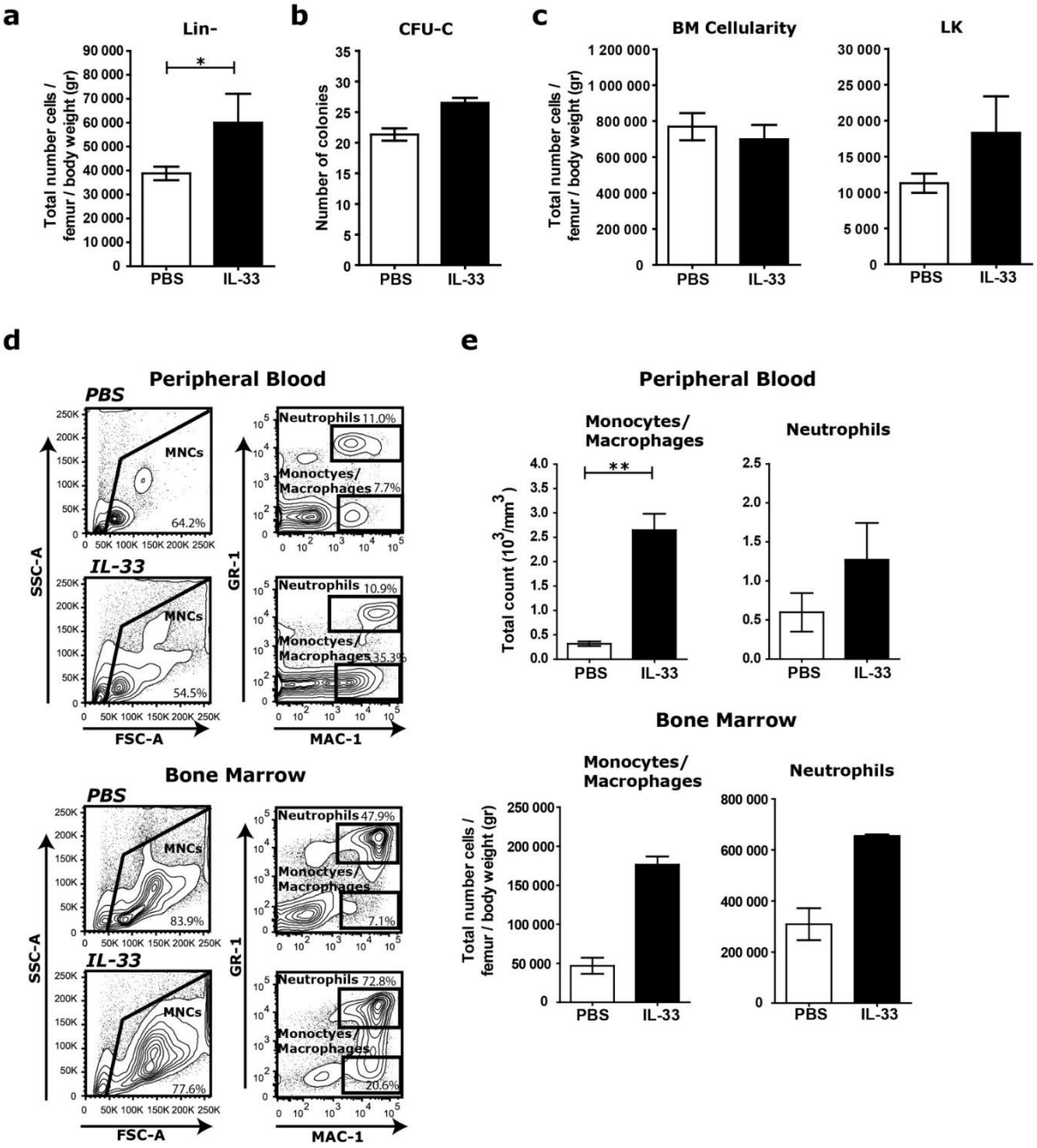
Supplementary Figure S5

a,b. The total number of bone marrow-derived mesenchymal cells (BMDSCs) and their CFU-F capacity is not affected after treatment with recombinant IL-33 in osteogenic conditions. (a) Quantification of the total number of BMDSCs kept in regular culture medium (n=2), osteogenic induction medium (n=2) and osteogenic induction medium containing recombinant IL-33 (n=2). (b) Quantification of the colony-forming unit-fibroblast (CFU-F) capacity of BMDSCs kept in regular culture medium (n=2), osteogenic induction medium (n=2) and osteogenic induction medium containing recombinant IL-33 (n=2).

c,d. Validation of shRNA-mediated IL-33 knockdown in HUVECs. (c) Transcript analysis of IL-33 by quantitative polymerase chain reaction (qPCR) demonstrates efficient knockdown of IL-33 in HUVECs transduced with two independent shRNA (sh19 and sh20) compared to a scramble hairpin control (SHC). (n= 4 independent experiments). (d) Representative Western Blot analysis confirming knockdown of IL33 at the protein level. R.P=recombinant protein IL-33. Protein ladder has been cropped out from both gel figures.

*Data represent mean ± s.e.m. *p <0.05, two-tailed unpaired t-test.*

Supplementary Figure S6



Supplementary Figure S6. Related to Figure 7. IL-33 promotes expansion of myeloid cells.

*a. Total lineage-negative cell counts in PBS (n=10) and IL-33 treated (n=5) mice. Data represent mean ± s.e.m. *p<0.05, unpaired t-test.*

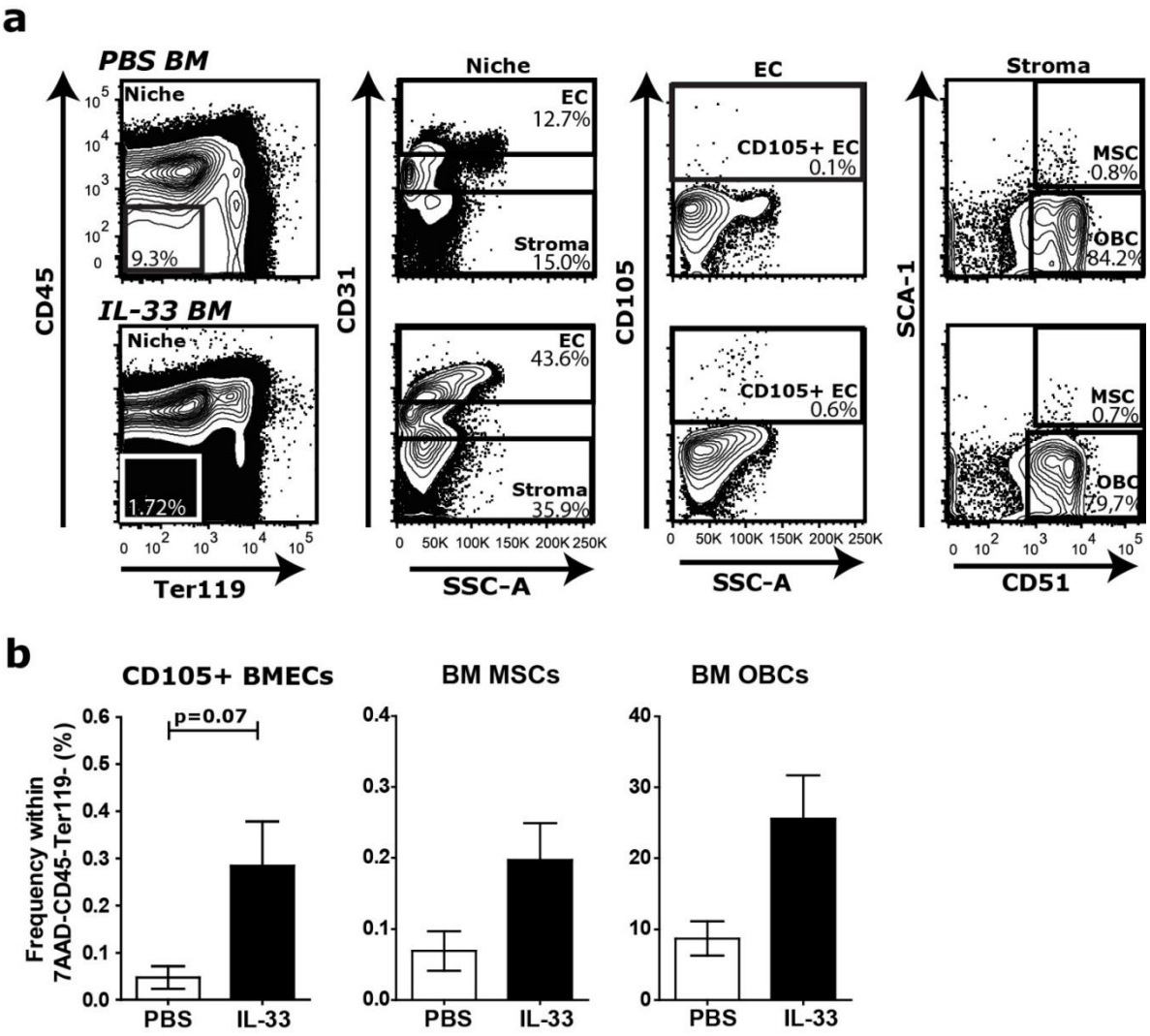
b. Colony-forming assay (CFU-C) on total BM of PBS (n=2) and IL-33 (n=2) treated mice. Data represent mean ± s.e.m. Experiments were performed in triplicate.

c. Total bone marrow cellularity and Lin-, c-KIT+, Sca-1- cell counts in PBS (n=10) and IL-33 treated (n=5) mice. Data represent mean ± s.e.m.

d. Representative FACS plots demonstrating an increased frequency of monocytes/macrophages in the peripheral blood (PB) and bone marrow (BM) of IL-33 treated mice.

*e. Total PB and BM counts of monocytes/macrophages and neutrophils in PBS-treated (n=3 for PB and n=2 for BM) and IL-33 treated (n=3 for PB and n=2 for BM) mice. Data represent mean ± s.e.m. **p<0.01, two-tailed unpaired t-test.*

Supplementary Figure S7



Supplementary Figure S7. Related to Figure 7. Recombinant IL-33 alters the architecture of the BM niche.

*a,b. Representative FACS plots of PBS (n=3) vs IL-33 treated (n=3) mice depicting the distribution of niche ($CD45^-Ter119^-$) cells within the BM. ECs were identified by CD31. Stromal ($CD31^-$) cells were further defined as SCA-1+CD51+ cells (MSCs) or SCA-1-CD51+ cells (OBCs). IL33 administration resulted in a relative increase of $CD31^+CD105^+$, MSC and OBC populations within the niche. Data represent mean ± s.e.m. *p<0.05, two-tailed unpaired t-test.*

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell isolation from human bone (marrow)

Freshly obtained BM aspirates of AML Day 17 patients were diluted 1:25 with red blood cell lysis solution (NH4Cl 0.155 M, KHCO3 0.01 M, EDTA-Na2.2H2O 0.1 M, pH 7.4) and incubated for 10 min at room temperature. Mononuclear cells (MNCs) were collected by centrifugation and washed once with PBS+0.5%FCS.

Fetal BM MNCs were isolated by gently crushing fetal long bones (femora, tibiae, fibulae, humeri, radii and ulna) in PBS+0.5%FCS using a mortar and pestle and passing subsequent cell suspension through a 40-µm filter in a 50 ml collection tube. Simultaneously, bone fragments were digested with 0.25% collagenase type I (Stem Cell. Cat#07902) for 45 minutes at 37°C, vortexing every 15 minutes. Excess PBS was added to the solution and filtered through a 40-µm filter in a collection tube. Next, both BM and bone fragment cell suspensions were washed once with PBS+0.5%FCS and were subsequently cleared from erythrocytes with IOtest3 lysing solution (Beckman Coulter. Cat#A07799), according to manufacturer's instructions.

For adult hip bone cells, trabecular bone was gently crushed using a mortar and pestle and resulting bone fragments were processed as described for fetal bone cells.

Fluorescence active cells-sorting (FACS) of human BM niche cells

Prior to cell sorting, FACS antibody incubations were performed in PBS+0.5%FCS for 20 minutes on ice in the dark with the following antibodies using optimized dilutions: CD45 (clone HI30, 1:100), CD271 (clone ME20.4, 1:100), CD235a (clone HI264, 1:100), CD31 (clone WM59, 1:100), CD9 (clone HI9a, 1:100) from Biolegend, and CD105 (clone SN6, 1:50) from eBioscience. The indicated populations of interest were sorted using a FACS ARIAIII Cell Sorter (BD Biosciences). Dead cells were gated out using 7AAD (Stem-Kit Reagents) after MNC selection and doublets exclusion. For RNASeq, cells were directly sorted in 800µl Trizol (Ambion) for RNA isolation. RNAse free non-stick micro-tubes (Ambion) were used to prevent pre-digestion of RNA.

Flow cytometry on murine peripheral blood and bone (marrow) cells

To identify murine hematopoietic stem and progenitor cells (HSPCs), BM cells were first co-stained with a cocktail of biotin-labelled antibodies against the following lineage (Lin) markers: Gr1 (RB6-8C5), Mac1 (M1/70), Ter119 (TER-119), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7) and B220 (RA3-6B2) (all from BD Biosciences). After washing, cells were incubated with Pacific Orange-conjugated streptavidin (Life Technologies) and the following antibodies: APC anti-c-Kit (2B8), PE anti-CD34 (HM34), Pacific Blue anti-Sca1 (D7), Alexa Fluor 700 anti-CD48 (HM48-1), PE-Cy7 anti-CD150 (TC15-12F12.2) (all from Biolegend), and APC/Cy7 anti-CD16/32 (56054, from BD Biosciences). To analyze murine differentiated cells, we used APC anti-Gr1 (RB6-8C5), PE-Cy7 anti-Mac1 (M1/70), Pacific Blue anti-B220 (RA3-6B2) antibodies

(all from Biolegend). To define murine niche cells, BM and bone fraction cell suspensions were stained with the following antibodies: APC-Cy7 anti-CD45.2 (104), BV510 anti-Ter119 (TER-119), PE-Cy7 anti-CD105 (MJ7/18), PE anti-CD51 (RMV-7), Pacific Blue anti-Sca1 (D7) (all from Biolegend), and PE-CF594 anti-CD31 (MEC 13.3, BD Biosciences). IL-33 expression was assessed in BM cells fixed and permeabilized with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) by incubating cells with PE anti-IL33 (IC3626P, R&D Systems) diluted in 1X Perm/Wash buffer (BD Biosciences).

CD31 and CD105 in situ immunofluorescence and immunohistochemistry

4 µm thick sections of paraffin-embedded BM biopsies were obtained from benign lymphoma patients without evidence of intramedullary localization. Fetal femurs were fixed in 4% formaldehyde for 24 hours and decalcified in 12.5% EDTA overnight. Fetal femurs were subsequently embedded in paraffin and sliced with a Micron HM355Smicrotome (Thermo Scientific) to create 4 µm thick sections.

For in situ immunofluorescence, deparaffinizing and antigen retrieval of the sections were performed with CC1 (Ventana 900-124) at 97oC for 64 minutes in a Ventana BenchMark ULTRA automatic staining system (Ventana Medical Systems, Tuscon, AZ). Next, murine anti-human CD31 (Cell Marque 760-4378) and rabbit anti-human CD105 (Genetex GTX100508_25ul,1:100) primary antibodies were manually added to the sections and incubated in the Ventana BenchMark ULTRA automatic staining system for 60 minutes at 36oC. After thorough washing with PBS/Tween 0.1%, sections were incubated for 30 minutes with a biotinylated rabbit anti-mouse secondary antibody lacking the FC fragment (DAKO E413), followed by washing in PBS/Tween20 0.1% and a 30 minute incubation with Cy™3-streptavidin (Jackson ImmunoResearch 016-0160-084). Next, avidin/biotin blocking was performed (Vector Laboratories P-2001). After thorough washing with PBS/Tween 0.1%, sections were incubated for 30 minutes with a biotinylated swine anti-rabbit secondary antibody (DAKO E431), followed by washing in PBS/Tween20 0.1% and a 30 minute incubation with FITC- streptavidin (Jackson ImmunoResearch 016-010-084). Finally, sections were mounted with DAPI-containing (1:2.000) vectashield (Vector Laboratories H-1000). 20x magnified Z-series images were acquired with a Leica TCS SP5 confocal microscope using the LAS software (Leica Microsystems). Briefly, after deparaffinization sectioned specimens were processed for 64-minute antigen retrieval with CC1. After 20-minute incubation at 36^{o}C with either previously described primary antibodies for CD31 and CD105 (1:500), detection was performed using UltraView DAB IHC Detection Kit (760-500, Ventana). The sections stained with CD31 and CD105 were counterstained with hematoxylin II (Ventana Ref.: 790-2208). Images were acquired with a ZEISS AxioPhot microscope using cellSens Entry 1.9 software (Olympus Corporation).

IL-33 immunofluorescence

FACS-isolated hRECs, obtained from fetal bones and BM, were collected in PBS, cytospun on a glass slide for 3 minutes at 500 rpm using a Cytospin 4 centrifuge (Thermo Scientific), and fixed in 3% PFA/PBS for 15 minutes on ice. After washing with PBS 3 times, cells were permeabilized for 2 minutes in 0.15% Triton-X100/PBS and then incubated in 1%BSA/PBS for 1 hour at room temperature to block aspecific binding sites. Cells were next stained overnight at 4°C with polyclonal goat anti-human IL-33 antibody (AF3625, R&D, 20 µg/ml in 1%BSA/PBS) or goat IgG isotype control antibody(Catalog # AB-108-C). Slides were washed twice with PBS for 5 minutes and incubated for 1 hour at 37°C with Alexa Fluor 488- conjugated donkey anti-goat antibody (A-11055, Invitrogen, 1:200, in 1%BSA/PBS). After 2 washes in PBS, slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). 63x magnified Z-series images were acquired with a Leica TCS SP5 confocal microscope using the LAS software (Leica Microsystems). For *in situ* immunohistochemistry consecutive serial sections of the benign lymphoma patients and fetal femur biopsies were stained for hematoxylin and eosin, CD31 and CD105 separately with the Ventana Benchmark Ultra automated staining system.

Hematopoietic stem progenitor cell isolation from umbilical cord blood

Umbilical cord blood (CB) was collected in a 300 ml bag filled with 20ml of the anticoagulant citrate phosphate dextrose (T2950, Fresenius Kabi), after receiving informed consent from the mother, and retrieved within 24 hours after collection. MNCs were obtained by using a standard Ficoll gradient protocol (1114547, Axis-Shield). CD34+ CB HSPCs were further isolated by magnetic-activated cell sorting (MACS). Briefly, MNCs were incubated with a magnetically coated anti-CD34 antibody (120-000-268, Miltenyi Biotec) and FcR blocking reagent (120-000-265, Miltenyi Biotec) in MACS buffer at 4°C for 30 minutes. After washing, CD34+ CB HSPCs were isolated using a LS column (130-042-401, Miltenyi Biotec) and purity was further enriched by performing an additional MACS selection using two MS columns (130-042-201, Miltenyi Biotec). Purity of the isolated cell population was confirmed with fluorescence-activated cell sorting (FACS).

Quantification and multiparametric immunophenotyping of cultured CB CD34+ cells

Cultured CB cells were thoroughly resuspended and collected for the single platform flowcytometric assay (to determine absolute cell counts) and for immunophenotyping (to specify HSPC subpopulations), respectively. For the single platform flowcytometric assay, cultured cells were incubated with CD34-PE-Cy7 (348811, BD Biosciences, 1:50) and CD45-PE-Cy5 (557075, BD Biosciences, 1:50) antibodies, and a calibrated number of flow-count fluorosphere beads (7547053, Beckman Coulter). In addition, 4',6-diamidino-2-phenylindole (DAPI) (1:5.000) was used to distinguish between living and dead cells. The total number of living MNC per µl in (n) a well was calculated using the following formula: n= ((number of MNC events recorded * bead

concentration)/number of recorded single beads)/ (volume cells/ volume beads). For immunophenotyping, cultured cells were incubated with Lin-FITC (22-7778-72, eBiosciences, 1:25), CD34-PE-Cy7 (348811, BD Biosciences, 1:50), CD38-PerCP-Cy5.5 (561106, BD Pharmingen, 1:60), CD90-PE (12-0909-42, eBiosciences, 1:30), CD45RA-APC-H7 (560674, BD Pharmingen, 1:30) and DAPI. Antibody incubation for FACs took place as previously described. The total number of a specific subpopulations was determined by multiplying the absolute number of living MNCs with the frequencies of the populations, determined with immunophenotyping, of interest. Flowcytometric analysis was performed using a BD LSRII (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Human hematopoietic colony forming-unit assay

To further assess the effect of recombinant human IL-33 on the hematopoietic potential of cultured CB cells, we performed a colony-forming unit assay (CFU). Input equivalent of 2000 7-days cultured CD34+ cells of each condition (rhIL-33 vs vehicle control) was resuspended in 400 µl Iscove's Modified Dulbecco's Medium (IMDM) and transferred to 3.6 ml of methylcellulose (MethoCult H84434, StemCell Technologies). Cells were then plated in triplicate in 1 cm^2 petri-dishes(1 ml/dish) and were kept at 37ºC and 5% CO2. Colonies were counted after 12-14 days.

Osteogenic differentiation and mineralization assays

BMDSC extracts were harvested at the end of culture by scraping the cells in PBS/triton 1% and storing them at −80 °C for biochemical analyses (Bruedigam et al., 2011). After scraping, PBS/triton 1%/0.24 M HCL was added to the wells and kept overnight at 4 °C to release calcium ions from the matrix deposition. Cell extracts were briefly sonicated (Soniprep 150, Sanyo) to produce cell lysates and incubated overnight with 6 M HCL. In short, calcium content was determined colorimetrically by adding o-cresolphthalein complexone to the well contents and cell lysates and measuring the absorbance of the resulting chromophore-complex at 595 nm.

Alternatively, cells were fixed in 70% ethanol (vol/vol) on ice for an hour and after washing with PBS, stained for 10–20 min with alizarin Red S solution (saturated Alizarin Red S (Sigma) in demineralized water adjusted to pH 4.2 using 0.5% ammonium hydroxide).

Human bone marrow fibroblast colony-forming unit assay

BMDSCs were first either cultured in αMEM medium containing 10% heat-inactivated FCS, or osteogenic induction medium containing recombinant human IL-33 (250 ng/ml) or mQ vehicle control for 1 week. After culture, total number of cells per condition were quantified with a Bürker counting chamber. Next, 50 cells per 0.32 cm2 (1 well of a 96-well plate) were seeded in αMEM supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. On day 14, dishes were fixed with 70% ethanol

(vol/vol) and stained with Giemsa. CFU-F colonies were counted as previously described (Chen et al., 2016).

Validation of IL-33 knockdown in HUVECs

For transcriptional analyses, HUVECs were harvested and collected after puromycin selection in TRIzol Reagent (Life Technologies). RNA isolation, conversion to cDNA and qPCR were performed accordingly to previously described methods (Zambetti et al., 2015) using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific). For qPCR, expression levels were obtained using the ddCt method using GAPDH as an internal control. The following primers were used:
GAPDH-Fw: GTCGGAGTCAACGGATT;
GAPDH-Rv: AAGCTTCCCGTTCTCAG;
IL-33-Fw: GGAAGAACACAGCAAGCAAAGCCT;
IL-33-Rv: TAAGGCCAGAGCGGAGCTTCATAA.

For protein analyses, cells were lysed in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2mM NA-vanadate) supplemented with 0.5 mM DTT and the protease inhibitor SigmaFast (Sigma Aldrich). Equal amounts of proteins were denatured and separated on a Novex NuPage 4-12% Bis-Tris Gradient gel (Life Technologies) and transferred to Protran BA83 blotting paper (GE Healthcare Sciences). After blocking with 5% BSA, membranes were incubated overnight at 4°C with a polyclonal goat anti-IL-33 primary antibody (AF3625, R&D systems). Actin was used as a loading control and detected with a mouse anti-actin antibody (A5441 Sigma Aldrich). Western blots were scanned and processed using an Odyssey Infrared Imager (Li-COR Biosciences).

To monitor the expansion of HUVECs after shRNA mediated knockdown of IL-33 after puromycin selection, HUVECs were re-plated and seeded at 300.000 cells in a T-25 flask and grown in EGM-2 medium containing 2µg/ml of puromycin. HUVECs were passaged every 2 to 4 days and total number of cells were quantified using a Casy counter (Roche Innovatis).

Murine hematopoietic colony forming-unit assay

To assess the myeloid progenitor capacity of whole BM from mice treated with PBS or IL-33, BM cells were cultured in methylcellulose containing murine SCF (10 ng/ml), IL-3 (100 ng/ml, purified from supernatant of CHO cells), IL-6 (10 ng/ml) and GM-CSF (10 ng/ml). Cytokines were purchased from PeproTech, if not otherwise mentioned. Specifically, 20000 BM cells per condition were resuspended in 400 µl IMDM and transferred to 3.6 ml of Methocult 3231 (StemCell Technologies). Cells were then plated in triplicate in 1 cm^2 petri-dishes(1 ml/dish) and were kept at 37ºC and 5% CO2. Colonies were counted after 5-7 days.

FIGURE 4 LEGEND REFERENCES

Blumenfeld, I., Srouji, S., Lanir, Y., Laufer, D., Livne, E., 2002. Enhancement of bone defect healing in old rats by TGF-beta and IGF-1. Exp. Gerontol. 37, 553–65.

Broudy, V.C., 1997. Stem cell factor and hematopoiesis. Blood 90, 1345–64.

Clines, G. a, Mohammad, K.S., Bao, Y., Stephens, O.W., Suva, L.J., Shaughnessy, J.D., Fox, J.W., Chirgwin, J.M., Guise, T. a, 2007. Dickkopf homolog 1 mediates endothelin-1-stimulated new bone formation. Mol. Endocrinol. 21, 486–98.

Edwards, C.M., Mundy, G.R., 2008. Eph receptors and ephrin signaling pathways: A role in bone homeostasis. Int. J. Med. Sci. 5, 263–272.

Holmes, C., Stanford, W.L., 2007. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells 25, 1339–1347.

Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G., Kalinkovich, A., Ludin, A., Kollet, O., Shakhar, G., Butler, J.M., Rafii, S., Adams, R.H., Scadden, D.T., Lin, C.P., Lapidot, T., 2016. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 532, 323–328.

Kusumbe, A.P., Ramasamy, S.K., Adams, R.H., 2014. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 507, 323–328.

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mäe, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., Adams, R.H., 2016. Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature 532, 380–384.

Oshima, A., Tanabe, H., Yan, T., Lowe, G.N., Glackin, C.A., Kudo, A., 2002. A novel mechanism for the regulation of osteoblast differentiation: Transcription of periostin, a member of the fasciclin I family, is regulated by the bHLH transcription factor, Twist. J. Cell. Biochem. 86, 792–804.

Ramasamy, S.K., Kusumbe, A.P., Wang, L., Adams, R.H., 2014. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. Nature 507, 376–380.

Salani, D., Taraboletti, G., Rosanò, L., Di Castro, V., Borsotti, P., Giavazzi, R., Bagnato, A., 2000. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Am. J. Pathol. 157, 1703–11.

Salvucci, O., Tosato, G., 2012. Essential roles of EphB receptors and EphrinB ligands in endothelial cell function and angiogenesis. Adv. Cancer Res. 114, 21–57.

Serini, G., Valdembri, D., Zanivan, S., Morterra, G., Burkhardt, C., Caccavari, F., Zammataro, L., Primo, L., Tamagnone, L., Logan, M., Tessier-Lavigne, M., Taniguchi, M., Puschel, A.W., Bussolino, F., 2003. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. Nature 424, 391–397.

Shao, R., Bao, S., Bai, X., Blanchette, C., Anderson, R.M., Dang, T., Gishizky, M.L., Marks, J.R., Wang, X., 2004. Acquired expression of periostin by human breast cancers promotes tumor angiogenesis through up-regulation of vascular endothelial growth factor receptor 2 expression. Mol. Cell. Biol. 24, 3992–4003.

Shigematsu, S., Yamauchi, K., Nakajima, K., Iijima, S., Aizawa, T., Hashizume, K., 1999. IGF-1 regulates migration and angiogenesis of human endothelial cells. Endocr. J. 46 Suppl, S59–S62.

Sugiyama, T., Kohara, H., Noda, M., Nagasawa, T., 2006. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. Immunity 25, 977–988.

Tamagnone, L., Giordano, S., 2006. Semaphorin pathways orchestrate osteogenesis. Nat. Cell Biol. 8, 545–7.

Zhang, C.C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C., Lodish, H.F., 2006. Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat. Med. 12, 240–5.

SUPPLEMENTAL REFERENCES

Bruedigam, C., van Driel, M., Koedam, M., van de Peppel, J., van der Eerden, B.C.J., Eijken, M., van Leeuwen, J.P.T.M., 2011. Basic techniques in human mesenchymal stem cell cultures: Differentiation into osteogenic and adipogenic lineages, genetic perturbations, and phenotypic analyses. Curr. Protoc. Stem Cell Biol. 1–20.

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B., Chao, N.J., Kirsch, D.G., Chute, J.P., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. Stem Cells 31, 327–37.

Gur-Cohen, S., Itkin, T., Chakrabarty, S., Graf, C., Kollet, O., Ludin, A., Golan, K., Kalinkovich, A., Ledergor, G., Wong, E., Niemeyer, E., Porat, Z., Erez, A., Sagi, I., Esmon, C.T., Ruf, W., Lapidot, T., 2015. PAR1 signaling regulates the retention and recruitment of EPCR-expressing bone marrow hematopoietic stem cells. Nat. Med. 21, 1307–17.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S. V., Ramos, C.A., Zhang, F., Rafii, S., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood 106, 505–513.

Nakamura-Ishizu, A., Okuno, Y., Omatsu, Y., Okabe, K., Morimoto, J., Uede, T., Nagasawa, T., Suda, T., Kubota, Y., 2012. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. Blood 119, 5429–5437.

Winkler, I.G., Barbier, V., Nowlan, B., Jacobsen, R.N., Forristal, C.E., Patton, J.T., Magnani, J.L., Lévesque, J.-P., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat. Med. 18, 1651–7.

Zambetti, N.A., Bindels, E.M.J., Van Strien, P.M.H., Valkhof, M.G., Adisty, M.N., Hoogenboezem, R.M., Sanders, M.A., Rommens, J.M., Touw, I.P., Raaijmakers, M.H.G.P., 2015. Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes. Haematologica 100, 1285–1293.

CHAPTER 3

How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance?

Cancer Drug Resistance. 1:6-29; 2018

Adrian C. Jaramillo[1,2], Farah Al Saig[2], Jacqueline Cloos[1], Gerrit Jansen[3], Godefridus J. Peters[2]

[1] Departments of Hematology, VU University Medical Center, Cancer Center Amsterdam, The Netherlands.

[2] Departments of Medical Oncology, VU University Medical Center, Cancer Center Amsterdam, The Netherlands.

[3] Amsterdam Rheumatology and Immunology Center, VU University Medical Center, Cancer Center Amsterdam, The Netherlands.

ABSTRACT

P-glycoprotein (ABCB1), multidrug resistance protein-1 (ABCC1) and breast cancer resistance protein (ABCG2) belong to the ATP-binding cassette (ABC) superfamily of proteins that play an important physiological role in protection of the body from toxic xenobiotics and endogenous metabolites. Beyond this, these transporters determine the toxicity profile of many drugs, and confer multidrug resistance (MDR) in cancer cells associated with a poor treatment outcome of cancer patients. It has long been hypothesized that inhibition of ABC drug efflux transporters will increase drug accumulation and thereby overcome MDR, but until now no approved inhibitor of these transporters is available in the clinic. In this review we present molecular strategies to overcome this type of drug resistance and discuss for each of these strategies their promising value or indicate underlying reasons for their limited success.

Keywords: Breast cancer resistance protein, ATP-binding cassette transporters, multidrug resistance, multidrug resistance protein.

INTRODUCTION

One of the most challenging obstacles in cancer treatment is multidrug resistance (MDR). Despite having achieved lower mortality rates due to recent advances in cancer therapy, the long term survival rate remains poor, primarily due to chemotherapy resistance[1-3]. Resistance can be caused by numerous mechanisms in cancer cells, such as activation of drug-metabolizing enzymes (e.g., cytochrome P450), activation of DNA repair mechanisms, disruptions in apoptotic signaling pathways, reduced drug influx and increased activity of drug efflux pumps[4-6]. Specifically, several members of the ATP-binding cassette (ABC) superfamily mediate the efflux of multiple chemotherapeutic drugs and so contribute to MDR. The ABC superfamily represents one of the largest protein families in biology, including 49 ABC genes identified in the human genome[7,8]. This superfamily is subdivided into seven distinct groups (ABCA through ABCG) and currently at least 15 ABC transporters have been implicated to confer resistance to clinically active drugs, notably P-glycoprotein (P-gp, ABCB1), multidrug resistance-associated protein-1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2)[9].

Structurally, each ABC transporter is composed of at least one hydrophobic membrane-spanning domain (MSD) consisting of 6 α-helices and 1 hydrophilic nucleotide binding domain (NBD). All ABC proteins share a highly conserved region in the ATP binding domain, including the Walker A and Walker B sequences, the ABC signature motif, the H loop and the Q loop[8]. Depending on the clustering of the MSD and the NBD in the transporters, these are grouped into full transporters (e.g., ABCB1 and ABCC1, consisting of 2 MSDs and 2 NBDs), half transporters (e.g., ABCG2, containing only 1 MSD and 1 NBD) and non-transporters, which possess two NBDs, without the MSDs[10]. The subfamily ABCE belongs to the non-transporters, whose proteins play a role in the assembly of the pre-initiation complex and RNAse[11,12] inhibition. ABCF subfamily members also encode non-transporters, they are involved in ribosome assembly and protein translation[13,14].

ABC transporters are present in all living species, in prokaryotes they function as drug importers or exporters, in eukaryotes solely as drugs exporters[15]. By coupling ATP binding and hydrolysis these transporters are able to extrude substrates out of cells or into cellular compartments like cytoplasmic vesicles and lysosomes[8]. This superfamily transports a wide range of substrates, including lipids, bile salts, sugars, amino acids, steroids, peptides, nucleotides, endogenous metabolites, ions and toxins, including antibiotics and chemotherapeutic drugs. Collectively, ABC transporters fulfill both physiological and pharmacological functions impacting drug transport, tumor growth modulation and regulatory pathways (e.g., apoptosis and complement-mediated cytotoxicity[16-18] and immune cell-regulatory pathways[19]. This review focuses on current developments in drug discovery to circumvent MDR induced by P-gp, MRP1 and BCRP in cancer cells.

P-GLYCOPROTEIN

P-glycoprotein (or ABCB1 or MDR1) was originally identified as the first mammalian ABC multidrug transporter in 1976, when Juliano and Ling[20] observed that Chinese hamster ovary cells displayed pleiotropic cross-resistance to a wide range of amphiphilic drugs. This transporter was named P-gp and is also known as ABCB1 or MDR1.

P-gp is encoded by a single polypeptide chain with 2 homologous NBD and 2 homologous transmembrane domains (TMD)[21,22]. The 2 homologous TMD are joined by a linker region of about 60 amino acids[23]. Each TMD contains 6 transmembrane (TM) α-helices and 1 NBD [Figure 1].

Aller et al.[24] reported the X-ray structure of mouse P-gp, which shares 87% sequence identity with the human P-gp. The structure revealed that the inward facing conformation is formed by 2 bundles of 6 TMs arranged as TMs 1-3, 6, 10, 11 and TMs 4, 5, 7-9, 12. This conformation results in a large internal cavity, which is open to the cytoplasm and the inner leaflet. There are 2 portals (formed by TMs 4/6 and 10/12) that allow hydrophobic molecules to enter the cavity directly from the inner leaflet. The drug binding pocket generally contains hydrophobic and aromatic residues and is large enough to accommodate at least two substrate molecules simultaneously[25].

In fact, this was supported by the X-ray structure reported by Aller et al.[24]. Expanding on these observations, Pajeva and Wiese[26] compared the inward facing homology model of human P-gp based on the earlier mentioned mouse structure with the outward facing homology model of human P-gp based on Sav1866 structure. It appeared that residues in TM4 and TM10 face the cavity in the inward facing conformation are completely buried in the outward facing form, while the ligands remain bound to the same residues of TM6 and TM12 in both conformations. This implies that each of the portals has different roles: TM4 and TM10 as “portal keepers” (preventing substrates that entered to escape back to the inner leaflet) and TM6 and TM12 as “portal carriers” (these are mainly responsible for substrate interactions). Together, this model suggests that ligands remain bound to the same residues during the transition from the inward to the outward facing conformation.

The expression pattern of P-gp indicates that its main function is the protection of the body from toxic substances and xenobiotics by excreting them into bile, urine, feces and avoiding their entry into fetus, brain and testes. To this end, P-gp is expressed on the canalicular surface of hepatocytes in the liver, the apical surface of epithelial cells of proximal tubules in the kidneys, columnar epithelial cells of the intestine, epithelial cells of placenta, and the luminal surface of capillary endothelial cells in brain and testes[27,28]. Next to this important physiological function, pharmacologically P-gp constitutes a considerable obstacle to the delivery of various clinically used drugs to their targets, such as anti-cancer drugs, anti-epileptics, immunosuppressive agents, cardiac glycosides, cholesterol-lowering statins, human immunodeficiency virus (HIV) protease inhibitors, anti-hypertensives, calcium channel blockers, anti-histamines and antibiotics[15]. From knockout mice studies it became clear that P-gp plays a major role in determining the drug ADME (absorption, distribution, metabolism, excretion) profile[29,30]. These P-gp knock-out mice showed increased absorption and decreased elimination of drugs, which gave rise to severe toxicity. In addition, P-gp works synergistically with the major phase I drug metabolizing enzyme P450 (CYP3A4), as they share a remarkable similarity between their substrates and regulation, to decrease the oral drug bioavailability.

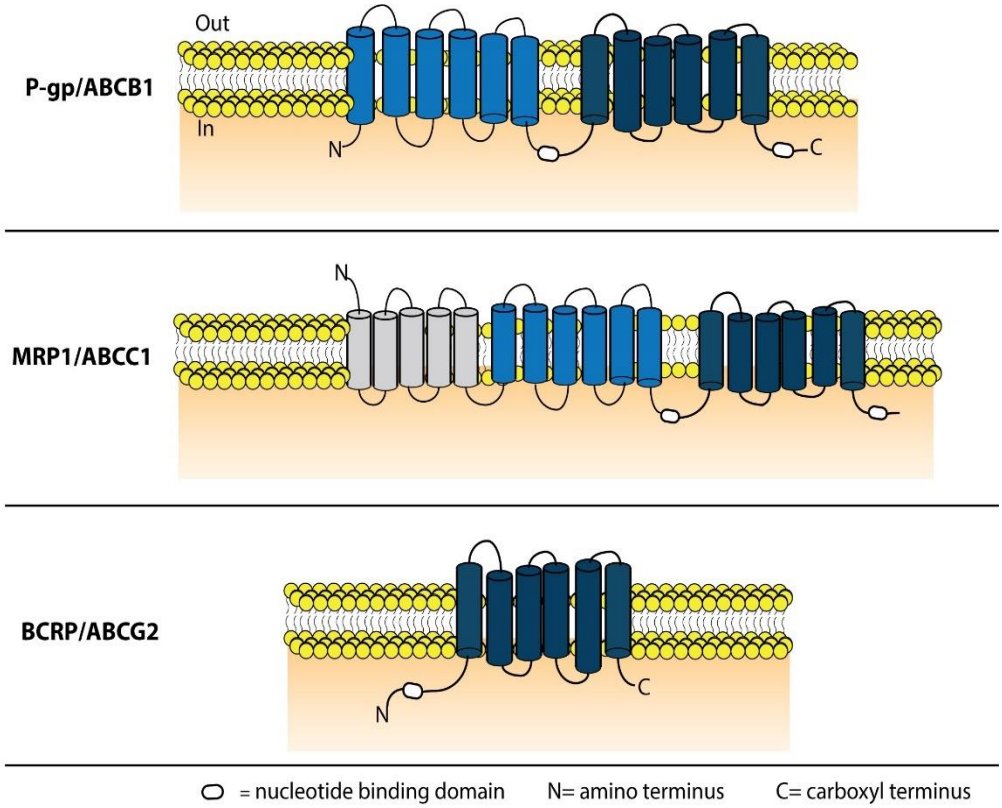


Figure 1: *Schematic representation of the domain structure of P-gp, MRP1 and BCRP. P-gp has 2 TMD consisting of 6 α-helices each and 2 ATP bindings sites. MRP1 also contains 2 ATP-binding regions and 2 TMD containing 6 α-helices and it is extended by 1 additional TMD consisting of 5 α-helices and 1 amino terminus loop (L0). The half transporter ABCG2 contains 1 TMD of 6 α-helices and 1 ATP-binding domain. The ATP-binding site of this transporter is found on the amino-terminal side (N) in contrast to P-gp and MRP1. P-gp: P-glycoprotein; MRP1: multidrug resistance protein-1; BCRP: breast cancer resistance protein; TMD: transmembrane domain*

High expression levels of P-gp have been identified in various solid tumor malignancies, e.g. kidney, colon, liver, ovarian, breast and sarcomas[4] [Table 1]. P-gp overexpression has been reported to be an independent negative prognostic factor in clinical outcome. In high grade osteosarcoma, increased levels of P-gp correlated with a > 3-fold increased risk of adverse events and shorter event-free survival[31]. MDR1 RNA levels increased after lung perfusion with doxorubicin of patients with unresectable sarcoma pulmonary metastases[32]. Furthermore, Gregorcyk et al.[33] reported that P-gp positive breast cancer patients are at significantly higher risk for relapse. In hematological malignancies, P-gp mRNA expression and protein function are increased after chemotherapy treatment. Expression of P-gp in myeloid blasts is correlated with treatment failure and shorter survival in adult acute myeloid leukemia (AML) patients[34,35]. However, childhood acute lymphoblastic leukemia (ALL) results have shown contradictory results, some found no association between high P-gp expression and prognosis[36,37], while other groups showed prognostic impact for P-gp in ALL[38,39]. These differences can at least partially be attributed to different treatment strategies and methods used to detect P-gp[40].

MULTI-DRUG RESISTANCE-ASSOCIATED PROTEIN 1

A decade after the discovery of P-gp, the first member of the C subfamily, MRP1 (systematic name, ABCC1) was discovered[41]. MRP1 is widely expressed in normal tissues, with relatively high levels in the kidney, lung, testis, heart and placenta, while there is a low expression in colon, brain, and small intestine and peripheral blood mononuclear cells[42,43] [Table 1]. Notably, in the above-mentioned tissues, MRP1 expression can vary between different cell types of the same tissue[44]. The highest expression is found in cells with specialized barrier function, e.g., the choroid cells in the blood cerebrospinal fluid barrier[45], or cells with a high proliferative status, e.g., reactive type II pneumocytes in hyperplastic alveoli of the lung[46]. In contrast to P-gp and BCRP, MRP1 is mainly expressed at the basolateral membrane of polarized cells, with the exception of brain capillary endothelial cells. Consequently, it has been suggested that MRP1 functions to protect cell types from xeno- and endobiotics and pumping them into the interstitial space of the body, instead of their expulsion into bile, urine or gut[47]. In addition, studies with mrp1 (-/-) mice revealed other physiologic functions of MRP1 than transporters. For example, MRP1 plays a role in immunological responses by its involvement in leukotriene extrusion[48]. Furthermore, being a transporter of glutathione (GSSG) and glutathione-conjugates, MRP1 plays a crucial role in the elimination of toxic substances formed during oxidative stress[49,50].

Despite the fact that both P-gp and MRP1 can confer resistance to a wide range of drugs, P-gp prefers neutral, or positively charged, hydrophobic compounds, while MRP1 binds and transports anionic substrates, can also be co-transported with free glutathione[50]. Some examples of MRP1 substrates are: various anticancer drugs, antibiotics, pesticides, HIV protease inhibitors, peptides, heavy metals, glutathione conjugates, glucuronide conjugates and sulfate conjugates[15].

Table 1. Characterization of P-gp, MRP1 and BCRP

ABC transporter	Localization	High Expression	Low Expression	Substrates
P-gp (ABCB1)	Apical	Kidney, adrenal glands, liver, pancreas, intestine, lung, BBB, placenta	Prostate, skin, heart/skeletal muscle, ovaries	Neutral, positively charged hydrophobic substrates
MRP1 (ABCC1)	Basolateral (except in placenta and BBB)	Kidney, lung, testis, skeletal/cardiac muscle, placenta	Liver, intestines, brain	Anionic substrates in the form of anionic glutathione, glucoronate or sulfate conjugates
BCRP (ABCG2)	Apical	Placenta, breast, BBB, liver, intestine	Kidney, lung, ovaries, testis, pancreas	+ or – charged, hydrophobic/hydrophilic, conjugated/unconjugated

BBB= blood-brain barrier; P-gp: P-glycoprotein; MRP1: multidrug resistance protein-1; BCRP: breast cancer resistance protein; ABC: ATP-binding cassette

The currently accepted MRP1 topology consists of the following domain arrangement: TMD0-L0-TMD1- NBD1-TMD2-NBD2 [Figure 1]. Compared to P-gp, this transporter is extended by an additional TMD0 containing 5 TM helices and 1 amino terminus loop (L0) consisting of 32 amino acids, which links TMD0 to TMD1. The exact function of the TMD0 still needs to be fully elucidated. It was previously thought that TMD0 had no role in trafficking to the plasma membrane or efflux function, but this view has been revisited by several mechanistic and cell biological reports[52]. It has been shown that TMD0 is important for the processing and trafficking of human MRP1[53]. Additionally, by mutation studies, Yang et al.[54] reported that the amino terminus is required for proper MRP1 function and structure. It has also been suggested by Chen et al.[55] that the amino terminus forms a U-shaped structure which might serve as a gate to regulate the substrate transport activity of MRP1. Finally, TMD0 and L0 seem to contribute to MRP1 homo-dimerization, but whether the functional form of MRP1 is a homodimer or a monomer needs to be determined[56].

Depending on expression levels, MRP1 can confer resistance to a variety of antineoplastic drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins, saquinavir, methotrexate, mitoxantrone, camptothecins, paclitaxel, glucuronide, doxorubicin, epirubicin, and tyrosine kinase inhibitors such as imatinib[57,58]. High MRP1 expression levels have been identified in different cancer types, e.g., lung cancer, breast cancer, prostate cancer, gastrointestinal carcinoma, melanoma, neuroblastoma, ovary and hematological malignancies[44,59] (AML, ALL and chronic lymphoblastic leukemia). In these results, it has been difficult to define statistically significant correlations between high MRP1 expression in the indicated tumors and acquired resistance or prognosis. This was largely due to the fact that tumor samples contained a variable number of MRP1 expressing normal cells, cells, while other drug efflux transporters might contribute as well[44]. However, a prospective study demonstrated that MRP1 is an independent prognostic indicator of outcome in

neuroblastoma patients[60]. In addition, a high expression of MRP1 was associated with shorter tumor-free survival (TFS) and overall survival (OS) in non-small cell lung cancer (NSCLC)[61]. Similarly, in breast cancer MRP1 expression was related to shorter relapse-free survival (RFS) and overall survival[62].

BREAST CANCER RESISTANCE PROTEIN

ABCG2, also known as BCRP, ABCP or MXR, is expressed in a variety of normal tissues, including epithelial cells of the gastrointestinal tract and liver, placental syncytiotrophoblast cells, prostate epithelium, kidney cortical tubules, endothelial cells of the blood-brain barrier (BBB) and hematopoietic stem cells[10,63] [Table 1]. Like P-gp, ABCG2 is expressed on the apical surface of epithelial cells and since it is expressed in tissues with secretory and barrier function, this suggests that its function is similar to P-gp, which is protecting the body from toxic compounds. ABCG2 mRNA expression was higher compared to other efflux transporter in the human small intestine, which is a rate-limiting barrier to oral drug absorption[64]. The significant impact of ABCG2 on the intestinal absorption was further supported by several knockout mice research, which have indicated that ABCG2 affects the pharmacological and toxicological behavior of many drugs[65-67]. Also, polymorphisms might influence the role of ABCG2 in intestinal absorption. For example, ABCG2 c.421C> polymorphism renders BCRP more than 75% less active than its wild-type variant. Since ABCG2 c.421C>A is a common polymorphism, present in more than 30% of the Asian and 15% of European population, decreased drug absorption due to BCRP polymorphisms deserves clinical attention[68]. Additionally, Kruijtzer et al.[69] reported that ABCG2 affect the bioavailability and systemic concentration of topotecan in patients. Inhibition of ABCG2 by elacridar (GF120918), a dual inhibitor of P-gp and ABCG2, increased the bioavailability of topotecan from 40% to 97% after oral administration and reduced the biliary and renal excretion of topotecan after intravenous administration. It has also been noted that the polymorphic variant 421C>A of ABCG2 can alter the pharmacokinetics of several drugs, including diflomotecan, teriflunomide, sulfasalazine, gefitinib, imatinib, and many statins[63]. Furthermore, it has been suggested that ABCG2 plays a role in the maintenance of human pluripotent stem cells in an undifferentiated state and their protection from toxic xenobiotics and hypoxia[10].

BCRP exhibits broad substrate specificity and can transport either positively or negatively charged drugs, hydrophobic or hydrophilic and conjugated or unconjugated substrates[70]. Examples are a wide range of anticancer drugs, sulfate and glucuronide conjugates of xenobiotics, tyrosine kinase inhibitors (TKIs), statins, fluorescent dyes and flavonoids[10,63]. *In vitro* assays showed that ABCG2 might have more than two binding sites, since ABCG2 inhibitors displayed different inhibition profiles, depending on the tested substrate[71-73]. For instance, the inhibitor Ko143 inhibited the transport of all the substrates tested by Giri et al.[72], which suggested its binding to a region that allosterically inhibits the transport of these substrates. However, the ABCG2 inhibitors elacridar, nelfinavir and Pluronic P85 seemed to bind to a different region, which inhibited the transport of the nucleoside analogs abacavir and zidovudine, but showed

no or a partial effect on the transport of prazosin and imatinib. Muenster et al.[73] showed that two different substrates, such as topotecan and albendazole sulfoxide, could simultaneously be transported without hindering each other's transport, which also suggest multiple binding sites in ABCG2. Furthermore, using homology modeling a complete homodimer ABCG2 model was created based on the crystal structure of the bacterial multidrug exporter Sav1866, which also suggested a multiple binding site[74]. ABCG2 is a half transporter consisting of 6 putative transmembrane segments and 1 NBD [Figure 1]. The simplest functional form of ABCG2 is a homodimer, which is bridged by disulfide bridge bonds[75]. Other research has suggested higher order of homo-oligomer on plasma membranes, which could also regulate ABCG2 function by dynamic association and dissociation of ABCG2 monomers[76]. Accordingly, it is currently unknown whether a homodimer or a homo-oligomer is the dominant functional unit of ABCG2 in the plasma membrane[77,78].

Clinically, high expression levels of ABCG2 have been found in many solid tumors, especially adenocarcinomas of the digestive tract, endometrium, lung and melanoma[77]. In addition, Damiani et al.[79] found that ABCG2 was overexpressed in 33% of AML patients and that this feature was significantly associated with shorter disease-free survival and higher risk of relapse. ABCG2 expression is also higher in B-lineage than in T-lineage ALL, while adult ALL patients have a higher expression than infant ALL patients[80,81]. Contradictory results have been published on the correlation between ABCG2 expression and prognosis of solid tumors and on the correlation between ABCG2 expression and prognosis of hematological malignancies[10,39,82]. Overall, even though BCRP activity contributes to MDR and drug pharmacokinetics in cancer, the exact mechanisms of action and interaction remain to be elucidated.

ABC TRANSPORTER INHIBITION

Since overexpression of some of the ABC transporters was correlated with a poor chemotherapeutic response and prognosis of patients with specific cancer types, inhibition of ABC transporters appears a logical approach to circumvent MDR and improve patient's outcome[82,83]. However, other than in a preclinical setting where ABC transporters could reverse MDR, this promise has still not been fulfilled in clinical practice. Inhibition of ABC transporters has been evaluated with rationally designed or natural inhibitors, including competitive and non-competitive inhibitors. Competitive inhibitors exert their function by tightly binding and blocking the substrate binding pockets. Non-competitive inhibitors exert their function by binding to a non-substrate binding site thereby inhibiting the ATPase activity or modulating transporters' function allosterically[15,83-85]. Another strategy to circumvent MDR that will be discussed further is the application of small interfering RNA (siRNA)[86-88] and microRNA (miRNA)[89,90] inoculation to down-regulating ABC transporter expression. Currently, approximately 30 siRNA candidates are being studied in clinical trial, yet unfortunately their high cost, poor stability *in vivo*, delivery challenges and off-target effects still remain a challenge for treatment[89]. A third approach relates to the rational design of new chemotherapeutics which are non-substrates for ABC transporters. TKIs are small

molecules developed to inhibit the uncontrolled activity of various tyrosine kinases (TK) involved in cancer. Many of them can also act as substrates or non-substrates to alter efflux mechanisms mediated by ABC transporters[91]. TKIs have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) since 2001 (such as imatinib, gefitinib and erlotinib and more recently ceritinib, lenvatinib) while several others are under investigation in clinical trials and can potentially be used to inhibit transporter-mediated efflux, as discussed below[92-96]. The main focus of this section of the review will be to discuss modulators which are lead compounds for clinical application.

OVERCOMING P-GP RESISTANCE

P-gp specific inhibition is particularly challenging due to its large binding pocket with low substrate specificity which enables P-gp to interact with over 200 different known substrates[9,97]. Nevertheless, the discovery of new agents to inhibit P-gp is still ongoing, building on encouraging *in vitro* studies and individual clinical trials such as the Southwest Oncology Group (SWOG 9126) study that showed a strong benefit of the use of the P-gp inhibitor cyclosporine A (CSA) in patients with relapsed or refractory AML[98]. To date, three generations of P-gp inhibitors can be distinguished. Both, the first-generation agents (including verapamil, tamoxifen, cyclosporine A, quinidine and dexverapamil), and the second-generation agents [including VX-710 (biricodar), GF120918 (elacridar) and PSC833 (valspodar)] ultimately failed to show improvement in overall drug efficacy in multiple randomized clinical trials due to poor potency and increased toxicity, respectively[99]. Consequently, the third-generation agents were developed featuring a high transporter affinity and a low pharmacokinetic interaction, including XR-9576 (Tariquidar), LY335979 (Zosuquidar) and R101933 (Laniquidar), which will be discussed below.

Inhibition of P-gp

Agents from all the three generations have been tested in clinical trials. Even though some of the early trials showed benefit from P-gp inhibition, results of most large randomized trials have been disappointing[100,101]. As an illustration, a phase I study of tariquidar in combination with vinorelbine showed that tariquidar is a potent P-gp inhibitor, with no noteworthy side effects and pharmacokinetic interactions[102]. Similar findings were observed for zosuquidar. Phase I research with zosuquidar showed that the drug could be administered safely to patients with AML or solid tumors[103,104]. However, a randomized placebo-controlled double-blind trial in 449 patients with newly diagnosed AML or high-risk myelodysplastic syndrome, showed no improvement in OS, or complete remission (CR) rates of drug treatment combined with zosuquidar[105].

The main reason for the initial failures in early clinical trials which started within 10 years after the discovery of P-gp-mediated MDR, was the lack of full understanding of P-gp properties and lack of knowledge on other ABC transporters. A clear example of this is tariquidar, which initially was considered as a specific P-gp inhibitor, but later

results showed inhibitory effects on BCRP and MRP1 leading to the synthesis of more potent tariquidar derivatives[106,107]. Mechanistic studies also showed that tariquidar can act, in a dose-dependent manner, both as a substrate or as a transport inhibitor[106,108,109]. Furthermore, recent 3-dimensional quantitative structure-activity relationship (QSAR) studies of anthranilamide derivatives of tariquidar suggest that a hydrophobic domain is needed for increased P-gp specific inhibitory function[26]. Some tariquidar analogs with sulfonamide groups are P-gp specific, nevertheless tariquidar was toxic to healthy cells[107]. Since its discovery, tariquidar was described as non-competitive and non-transported inhibitor of P-gp[110], while Bankstahl et al [111]*.* have shown that tariquidar is concentration-dependently transported by P-gp. Recent transepithelial drug transport assays using radioactive tariquidar in human and mouse cell lines indicate that tariquidar functions as a high-affinity P-gp substrate rather than a non-competitive inhibitor[112].

The insufficient knowledge of the P-gp transporter led to the design of clinical trials with inadequate inhibitors and patient selection. Agents such as verapamil belonging to the first-generation inhibitors were low in potency, and since dose escalation was limited due to toxicity, ineffective dosages were used[113,114]. Other clinical trials failed due to their choice of compounds that interfered with the physiological role of P-gp and sister proteins. For instance, the dose of the anticancer agents used in combination with the second-generation P-gp inhibitor PSC 833 had to be reduced to prevent toxicities of the anticancer agent because PSC 833 caused a decreased drug clearance via inhibition of its metabolism by cytochrome P450[112]. Regarding patient selection, initial clinical trials enrolled patients irrespective of their P-gp status, whereas rationally patient selection should be based on adequate transporter(s) expression and/or function in tumor specimen and whether the targeted transporter is the dominant mechanism of drug resistance[91]. Moreover, early clinical trial designs did not take into account the role of P-gp polymorphisms. Polymorphic variants of the transporters influence their expression, function and localization and thereby might affect the pharmacokinetics of their substrates[115]. Inclusion of patients with such polymorphic variants might confuse treatment outcome assessments, since these patients might not develop significant MDR via efflux pumps, but face bone marrow toxicity in combination therapy with the inhibitor.

Novel molecules that can successfully interact with P-gp-specific NBDs have been identified through *in silico* and computational studies (compound 19, 29, 45)[116,117]. The aim of such approach is to find molecules that can interfere with the energy harvesting system of a transporter, avoiding its non-specific drug binding domains. Nanayakkara et al.[118] identified through computational studies (compound 29, -34, -45) which, when co-administrated with vinblastine and paclitaxel decreased viability, survival and motility of prostate cancer and other resistant cell lines. Furthermore compound 34 and 45 were found to be P-gp specific while inhibitor 45 also had affinity for ABCG2[117]. These techniques may be a promising approach in the search for P-gp specific NBD inhibitors[119].

Evading P-gp (nanoparticle drug delivery systems)

Focus has now shifted towards downregulating the transcription of P-gp with siRNA[87,120,121] or microRNA[122,123] using nanoparticle (NP) drug delivery systems[124]. Hyaluronic acid based nanoparticles can effectively target CD44+ ovarian cancer cells and downregulate P-gp and increase intracellular concentration of paclitaxel. High CD44+ expression in ovarian cancer cells is related to metastasis and therefore this cell-specific approach has the potential to improve the cell sensitivity in ovarian cancer patients with poor prognosis[120,121]. Encapsulation drugs in liposomal nanoparticles show promising results in the clinical setting. Doxorubicin HCl liposomal injection (Doxil) was approved for the treatment of AIDS related Kaposi's sarcoma (USA in 1995 and Japan in 2007), and for the treatment of ovarian cancer (USA in 1999 and Japan in 2009) after failure of prior systemic chemotherapy or intolerance or after failure of platinum-based chemotherapy[125]. In addition, in 2007 the FDA approved Doxil for the treatment of multiple myeloma in combination with bortezomib. Another candidate is the nanoparticle albumin-bound paclitaxel (nab-paclitaxel, Abraxane), which in 2011 has been approved in 42 countries for the treatment of metastatic breast cancer after failure of combination chemotherapy for metastatic disease or relapse within six months after adjuvant chemotherapy[126]. Additionally, encapsulation of doxorubicin in hydrophobic polymeric micelles cause indirect inhibition of P-gp efflux and enhance intracellular drug concentration without affecting P-gp expression[127]. The BBB is also another target of investigation with NP drug delivery. A recent study analyzed the permeability kinetics of siRNA NPs in a BBB monolayer model using human brain endothelial cells concluded that NP delivery can inhibit P-gp expression by half and increase rhodamine 123 permeability by a third[87]. siRNA-loaded NP carriers could be a promising approach to circumvent the human BBB in order to facilitate drug penetration in the central nervous system. Unfortunately, side effects such as skin and hypersensitivity reactions and neurotoxicity remain common limiting factor[128,129].

The role of microRNAs in P-gp modulation

MicroRNAs are post-transcriptional regulators composed of noncoding RNAs, which can regulate gene expression and have been implicated in drug resistance in many cancers[122,123]. MicroRNA can bind to the 3' untranslated region (UTR) of RNA with high precision, causing RNA degradation and inhibition of gene expression[130]. Several studies report that miRNAs can regulate MDR by modulating the expression of P-gp. For example, it has been shown that miR-451 and miR-27a upregulation was P-gp dependent in cell lines representing ovarian cancer, leukemia and hepatocellular carcinoma[112,114,115]. In contrast, deregulation rather than upregulation of certain miRNAs, such as miR27a and miR 331-5p, can also cause drug resistance reversal and decreased P-gp expression, as seen in doxorubicin resistant leukemia cell lines K562 and HL60[131-133]. Furthermore one study found a possible prognostic value for miR-296, whose expression can distinguish long-term survivors from short-term survivors, while its downregulation sensitized cells to known P-gp and BCRP substrates[134].

MiRNA mediated P-gp inhibition and drug resistance reversal seems to be dose-dependent, as seen with colorectal adenocarcinoma and breast cancer cell lines[135,136]. For example, Bao et al.[136] reported that P-gp expression could be reduced with miR-298 and reversed doxorubicin resistance in breast cancer cells in a dose-dependent fashion. *In vivo* studies also report that miR-873 elicits a dose-dependent response, measured by tumor growth inhibition and cisplatin sensitization in previously resistant cell lines[122]. Other miRNAs with BCRP-modulating capacity include miR-122[137], miR-381, miR-495[138], miR-223[139], miR-9[140], and miR-873[122] [Table 2]. Unfortunately, full understanding of miRNA post-transcriptional modulation is still insufficient and concerns with off target effects on top of which RNA degradation still remains an unresolved issue[89].

Bypassing P-gp: none substrates

Several approaches have been investigated to evade P-gp mediated MDR with novel drugs: (1) decreasing their molecular affinity for P-gp and (2) increasing the affinity to drug targets. This has been challenged for microtubule stabilizing agents (e.g., epothilones, second- and third-generation taxanes), microtubule destabilizing agents (e.g., cryptophycins, halichondrins, hemiasterlins and STX140), inhibitors of topoisomerase I (e.g., lipophilic camptothecins, homocamptothecins and dibenzonaphthyridinones) and inhibitors of topoisomerase II (e.g., lipophilic anthracyclines). Many of these agents have already undergone wide preclinical and clinical investigation, and are comprehensively reviewed[141].

The epothilones including epothilone B (patupilone) and its analogs BMS-310705, sagopilone and ixabepilone, and epothilone D and its derivative KOS-1584, are poor substrates of P-gp. They show a diverse susceptibility profile to P-gp mediated resistance, with ixabepilone the most and sagopilone the less affected by P-gp overexpression[142,143]. Currently, ixabepilone is FDA approved for the treatment of metastatic breast cancer and has been tested in phase III clinical trials in combination with capecitabine[144-146].

TPI-287, a novel microtubule destabilizing drug of the taxane family designed to circumvent MDR, was recently tested in a small group phase I study of children with neuroblastoma and medulloblastoma. TPI 287 was well tolerated half of patients with neuroblastoma who completed one cycle of treatment with TPI 287 alone showed a stable disease although the study was not designed to evaluate treatment response[147]. In the case of epothilones, there are a number of active clinical trials to test its efficacy in a range of advanced solid tumors such as breast cancer, ovary, head and neck, esophagus, lung, gastrointestinal tract cancers and brain tumors (clinical trial reference: NCT00030173, NCT0035516 and NCT00450866). Being a P-gp substrate, ixabepilone might exert its antitumor activity preferentially in tumors where the main resistance mechanism is βIII-tubulin or/and BCRP dependent[148,149]. The exact working mechanism of these novel agents is still unclear. However, bypassing a resistance mechanism such as P-gp-mediated MDR does not imply that patients are not at risk of developing other mechanisms of drug resistance which will lead to intrinsic or acquired tumor refractoriness. For epothilones, expression of some

MRPs, such as MRP7, and β-tubulin mutations has been related to the development of tumor drug resistance to these agents[150,151].

Table 2. Novel approaches to P-gp inhibition

Type	Name	Remarks	Cancer Type	References
P-gp inhibitors	Tariquidar, zosuquidar, laniquidar, cyclosporine, tariquidar derivatives	Zosuquidar shows robust P-gp inhibitoin without affecting survival rates; hydrophobic domains in tariquidar derivatives increase inhibition	AML, high-risk MDS	[26, 98-112]
Computational studies/ *in silico* drug screening	Compound 19, 29, 34, 45	High NBD specificity; compound 34 and 45 are P-gp specific	Prostate cancer and various MDR cell lines	[116-119]
Nanoparticle delivery systems	Encapsulated drugs: doxorubicin, paclitaxel, rhodamine 123	Increases drug concentration and indirectly inhibits P-gp	Ovarian cancer, Kaposi sarcoma	[87, 121, 124-129]
siRNA	siRNA delivered by nanoparticles	Hyaluronic acid based nanoparticles for CD44+ cell, reduces P-gp mRNA by half in BBB model with NP delivery and > 85% in Caco-2 cells without NP delivery, in dose dependent manner	Ovarian cancer, BBB based model, Caco-2 cell line	[87, 120, 121, 135]
MicroRNAs	miR-27a, 451, 873, 381, 101, 229, 495, 9, 296, 298	Inhibits P-gp expression through post-transcriptional modulation; increases drug sensitivity in previously resistant cells	Breast, ovarian cancer, hepatocellular, colorectal carcinoma, leukemia	[122, 123, 130-134, 138, 140]
Non-substrates	Microtubule stabilizing/ de-stabilizing agents, topoisomerase inhibitors	Taxanes such as ixabepilone is FDA approved. B-microtubulin mutations lead to resistance	Breast cancer	[141-149]
Monoclonal antibodies	MRK 16, 17	Currently undergoing phase I clinical trials	Leukemia	[9, 172-175]
TKI's	Alectinib, ibrutinib, neratinib, AG-1393, EKI-785, vandetinib	Sensitized primary resistant cells and cell lines to chemotherapeutics by inhibiting P-gp ATPase activity	AML/ALL, several others	[91-96, 181-183]

P-gp: P-glycoprotein; BBB= blood-brain barrier; TKI: tyrosine kinase inhibitor; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia; MDS: myelodysplastic syndrome; NBD: nucleotide binding domain; MDR: multidrug resistance; NP: nanoparticles

Using P-gp to kill cancer cells

Instead of inhibition of the P-gp function or evading its efflux to overcome P-gp-mediated MDR, an alternative approach is to actually exploit P-gp expression to specifically kill MDR cells. This strategy makes use of the phenomenon of collateral sensitivity (CS). which refers to the fact that some agents show preferential activity toward MDR cancer cells relative to their non-MDR parental cells, as was previously noticed for verapamil, cytosine arabinoside and gemcitabine in MDR small cell lung cancer cells lines[152,153]. Different CS agents have been synthesized from lead compounds such as NSC73306 (1,1-Isatin-4-(4′-methoxyphenyl)-3-thiosemicarbazone and the flavonoid desmosdumotin B[154,155]. It has been hypothesized that CS agents exert their effect by the generation of reactive oxygen species. This strategy is still experimental. Of further interest, within a class of isatins drugs, particularly N-alkylisatins were found not to be susceptible to P-gp mediated efflux, destabilize microtubule formation and induce apoptosis in P-gp overexpressing tumor cells[156].

Another approach to bypass P-gp is to use drugs that show a higher sensitivity to P-gp expressing cells compared to P-gp negative cells. Bergman et al.[153,157] showed that both small cell lung cancer and NSCLC cells with an overexpression of either P-gp or MRP1 were more sensitive to the deoxynucleoside analogs cytarabine and gemcitabine. This was related to an increased activity of the activating enzyme deoxycytidine kinase (dCK) for these nucleoside analogs, leading to an increased accumulation of the active metabolites, Ara-CTP and dFdCTP, respectively. It was argued that in P-gp cells steroids are effluxed efficiently decreasing their intracellular concentration. Since steroids regulate dCK, this may lead to an increased enzyme activity[158]. The increased sensitivity was observed in cells with acquired resistance to P-gp substrates, such as etoposide, doxorubicin, colchicine, but also in cells transfected with the gene for P-gp or MRP1[153,157].

Increase oral bioavailability by increasing gut epithelial uptake

A strategy that can benefit of P-gp inhibition is the increase of the oral bioavailability of drugs, particularly when a drug is a P-gp substrate and is efficiently being effluxed back to the gut leading to a low oral bioavailability. The oral administration of drugs has several advantages over intravenous formulation[159]. First of all, patients prefer oral chemotherapy over intravenous due to the ease of administration, avoiding the discomfort of injection and the related risk of infection[159,160]. Secondly, oral medication is cost saving for hospital and health insurance, since there is no need for hospitalization. Finally, oral administration of drugs enables metronomic therapy with the advantages of chronic exposure of the drugs to its targets and reducing dose-related toxicities, such as the optimizing of anti-angiogenic efficacy of chemotherapeutic agents including paclitaxel and docetaxel[161,162]. Wild-type mice showed limited oral bioavailability of paclitaxel compared P-gp knockout mice[163]. Following this, multiple preclinical mice results have shown that inhibition of P-gp using inhibitors such as cyclosporine, PSC 833 or elacridar improved the oral treatment of paclitaxel in mice[164-166]. Therefore, clinical trials were started to explore the effect of

the oral administration of paclitaxel in combination with cyclosporine. Three phase II trials have tested this combination in patients with NSCLC, advanced gastric cancer and breast cancer. These studies have shown promising results, but long-term oral use of cyclosporine might be complicated by immunosuppressive effects. Therefore, other inhibitors might replace cyclosporine such as elacridar (GF120918) or HM30181, respectively[167,168]. Being a P-gp substrate in addition to the encouraging results of oral paclitaxel, preclinical studies were also initiated with another taxane, namely docetaxel[169]. Different inhibitors were used such as OC144-093 (a P-gp-inhibitor), cyclosporine (a dual inhibitor of P-gp and CYP3A4) and ritonavir (CYP3A4 inhibitor), which have shown increased oral bioavailability of docetaxel[170]. In line with this observation, which indicates that CYP3A4 is the major determinant of the low oral bioavailability of docetaxel, it seems rational that future trials will continue with oral docetaxel in combination with ritonavir[167,170].

Other studies have shown that P-gp restricts the oral bioavailability of the topoisomerase II inhibitor, etoposide, and that P-gp inhibition enhances its oral bioavailability[171]. The appropriate inhibitor and associated dosages (of both drug and inhibitor) still need to be determined to improve the therapeutic outcome with minimal toxicities, since P-gp influences the excretion of drugs in addition to its role in protecting of vital organs such as the brain, the fetus and the testes. Furthermore, attention needs to be paid to specific polymorphisms of P-gp that might impact the drug oral bioavailability.

Monoclonal antibodies against P-gp

Already in the 1986 Hamada and Tsuruo[172] developed two monoclonal antibodies against human P-gp in adriamycin-resistant myelogenous leukemia cells (MRK-16 and MRK-17). Initially, these antibodies were intended to study the membrane changes in MDR cells, but later research demonstrated that MRK-16 was able to inhibit P-gp mediated efflux of vincristine and actinomycin D *in vivo*[172,173]. The addition of a P-gp inhibitor such as cyclosporine A to MRK-16 treatment, further sensitized MDR myelogenous leukemia cells to vincristine and doxorubicin[174,175]. This innovative approach with MRK-16 is currently being investigated again in phase I clinical trials[9].

OVERCOMING MRP1 RESISTANCE

MRP1 was identified as a drug transporter 15 years after P-gp, and therefore its role in MDR in human tumors has not been fully understood and fewer MRP1-specific inhibitors with sufficient potency and efficacy have been identified[176]. Several novel strategies that have been applied to P-gp are being tried on MRP1 overexpressing cells are listed on Table 3. Nanoparticle drug delivery systems have been also applied successfully to inhibit MRP1 mediated resistance[177,178]. For example, Wang et al.[177] used redox-responsive polymeric micelles with a disulfide bond that serves as a linker for delivery of paclitaxel as chemotherapeutic, and indomethacin, as chemosensitizer.

Also, siRNA delivery in porous silicon nanoparticles has shown to effectively downregulate MRP1 mRNA[178] yet more studies are needed, especially *in vivo*.

As with P-gp, numerous TKIs have been also identified to modulate the efflux of MRP1. Cediranib (AZD217) is an orally administered potent small-molecule TKI that inhibits the vascular endothelial growth factor (VEGF) family of receptors (VEGFR 1-3), platelet-derived growth factor receptor (PDGFR) and a stem cell factor receptor, c-Kit[179]. A study by Tao et al.[180] showed that cediranib inhibited the ATPase activity of P-gp in a dose–dependent manner and it also reversed both P-gp and MRP1 mediated MDR, suggesting that cediranib acts as dual inhibitor. Another TKI, vandetanib (ZD6474, Zactima), an inhibitor of VEGFR, epidermal growth factor receptor (EGFR) and rearranged during transfection (RET) tyrosine kinases[181], stimulated P-gp ATPase activity in a dose-dependent manner and reversed MDR in cancer cells by directly inhibiting the function of P-gp, MRP1 and ABCG2[181,182]. Other TKIs such as AG-1393 and EKI-785, have also been found to interact and inhibit P-gp activity[183].

Table 3: Novel approaches to MRP1 inhibition

Type	Name	Remarks	Cancer Type	References
MRP1 inhibitors	Tariquidar, dofequidar, cyclosporin A, biricodar, CBT1 and derivatives of triazolonaphtalimide	Triazolonaphtalimide is antiproliferative in MRP1 overexpressing lung cancer cells	Lung cancer, others	[184-189]
Nanoparticle drug delivery systems	Agents delivered: paclitaxel, indomethacin, and siRNA	Redox-responsive polymeric micelles with a disulfide bond for delivery of paclitaxel and indomethacin. Porous silicon nanoparticles protect siRNA from degradation	Breast cancer, glioblastoma	[177, 178]
TKIs	Alectinib, ibrutinib, neratinib, AG-1393, EKI-785, vandetinib	Sensitized primary resistant cells and cell lines to chemotherapeutics by inhibiting P-gp ATPase activity	AML/ALL, several others	[179-183]

P-gp: P-glycoprotein; MRP1: multidrug resistance protein-1; TKI: tyrosine kinase inhibitor; AML: acute myeloid leukemia; ALL: acute lymphoid leukemia

Among the three generations of P-gp inhibitors, there are inhibitors that also inhibit MRP1 and/or ABCG2. Examples of such inhibitors are cyclosporine A[26,184-187], biricodar (VX-710), dofequidar (MS-209), tariquidar and CBT-1. The novel triazolonaphtalimide derivatives have been reported to achieve an anti-proliferative effect in chemoresistant lung cancer cell lines via MRP1 downregulation without affecting P-gp function[188]. Some of these preclinical studies reached phase III clinical trials, for instance for dofequidar[189] and CBT-1 (NCT00437749). However, inadequate trial design and poor drug specificity might hamper proper assessments

of the full potential of these agents. Further investigation is warranted to assess the effect of these inhibitors on each targeted ABC transporter in clinical trials, to guide selection of the right population of patients to enhance the efficacy and minimize the toxicity.

The scarcity of specific MRP1 inhibitors reflects the fact that there is still no clarity about the benefit of P-gp inhibition in the clinical practice, which retards research efforts to develop MRP1 inhibitors for clinical use[190]. However, the calcium channel blocker verapamil and its derivative NMeOHI2, in addition to the several non-steroidal anti-inflammatory drugs (e.g., indomethacin), have shown collateral sensitivity toward MRP1 overexpressing cells[191,192]. These agents were proposed to trigger apoptosis in cells overexpressing MRP1 via intracellular GSH depletion. Furthermore, through high-throughput screening and chemical modification studies, the tricyclic isoxazole derivatives were identified to exhibit high potency and specificity to MRP1 inhibition. Nevertheless, further studies are needed to address MRP1 inhibition specificity, toxicity and effectivity both *in vivo* and *in vitro*.

The scarcity of specific MRP1 inhibitors reflects the fact that there is still no clarity about the benefit of P-gp inhibition in the clinical practice, which retards research efforts to develop MRP1 inhibitors for clinical use[190]. However, the calcium channel blocker verapamil and its derivative NMeOHI2, in addition to the several non-steroidal anti-inflammatory drugs (e.g., indomethacin), have shown collateral sensitivity toward MRP1 overexpressing cells[191,192]. These agents were proposed to trigger apoptosis in cells overexpressing MRP1 via intracellular GSH depletion. Furthermore, through high-throughput screening and chemical modification studies, the tricyclic isoxazole derivatives were identified to exhibit high potency and specificity to MRP1 inhibition. Nevertheless, further studies are needed to address MRP1 inhibition specificity, toxicity and effectivity both *in vivo* and *in vitro*.

OVERCOMING ABCG2 RESISTANCE

Ahmed-Belkacem et al.[193] divided ABCG2 inhibitors in four groups: (1) ABCG2-specific inhibitors; (2) broad-spectrum inhibitors, including inhibitors that inhibit P-gp and/or MRP1; (3) TKIs; and (4) naturally occurring flavonoids and derivatives.

ABCG2 inhibitors

The mycotoxin fumitremorgin C (FTC) was identified as the first ABCG2 specific inhibitor, but its neurotoxic effect hampered its further clinical development[194,195]. This lead compound was the basis of a new non-toxic tetracyclic analogue of FTC, Ko143, which is a highly potent and specific inhibitor of ABCG2[195]. Ko143 increased topotecan plasma concentration 4-6-fold in *Mdr1a/1b-/-* mice. Pick et al.[196] identified a new class of specific ABCG2 inhibitors which were structurally related to tariquidar, but did not show potency toward P-gp and MRP1. In their research they presented evidence for 3 different binding sites on ABCG2, one occupied by the ABCG2 substrate pheophorbide A (Pheo A), another by the TKI imatinib and a third by the two compounds that specifically inhibit ABCG2. Imatinib showed features of an allosteric

modulator by increasing the inhibitory effect of the inhibitor compounds. Another ABCG2 inhibitor lead compound, PZ-39, was reported by Peng et al.[197]. Interestingly, this compound had a dual mode of action; it could effectively inhibit ABCG2 function and markedly reduced the half-life of ABCG2 protein from approximately 54 to 5 h by accelerating its protein degradation via endocytosis and trafficking to lysosomes. The ABCG2 inhibitor specificity of these compounds was confirmed with respect to P-gp and MRP1, but not for other ABC transporters.

Transcriptional down regulation using siRNA or miRNA are also areas of interest in ABCG2 inhibition. One study shows that combination of siRNA and PI3K/Akt signaling inhibition can reverse chemoresistance in mitoxantrone-resistant BCRP-overexpressing cells lines[198] [Table 4]. Similarly, nanoparticle drug delivery using polyethyleneimine as a carrier for siRNA has been reported to down-regulate ABCG2 and decrease the mitoxantrone IC(50) values by approximately 14-fold[86]. Other efforts to deliver ABCG2 and P-gp inhibitors with nanoparticles show complete reversal of resistance in ABCG2 positive MDR cells[199].

Table 4: Novel approaches to ABCG2 inhibition

Type	Name	Remarks	Cancer Type	References
ABCG2 inhibitors	Tariquidar derivatives, PZ-39, FTC derivatives, Ko- 143, imatinib, gefitinib	Ko-143 is highly specific, PZ-39 reduces half-life of ABCG2	Colon carcinoma, resistant cell lines	[194-197]
TKIs	Imatinib, geftinib	By competitive inhibition, TKIs act as substrates at low concentrations and have inhibitor like properties at high concentrations	CML, NSCLC	[94, 208-214]
Nanoparticle drug delivery systems	Encapsulated agents: mitoxantrone and siRNA	Complete reversal of resistance in ABCG2 positive MDR cells	Resistant cell lines	[86, 198, 199]
siRNA	siRNA delivered by nanoparticles	Polyethyleneimine as a carrier for siRNA inhibits ABCG2 and sensitizes cells to mitoxantrone 14-fold	Resistant cell lines	[86,198]
MicroRNAs	miR-328, -hsa-miR-328, miR -519, 520h, 212, 181a, 487a and -302	Use of microRNA has revealed a more complex role of ABCG2 in drug resistance related signaling pathways: SIRT1/CREB/ABCG2 and Wnt/β-catenin-ABCG2 pathway	Breast, ovarian cancer, other cell lines	[200-207]

TKI: tyrosine kinase inhibitor; MDR: multidrug resistance; CML: chronic myeloid leukemia; NSCLC: non-small cell lung cancer

There are several miRNA that have been identified in a wide range of cancer cells, as potential inhibitors of ABCG2 expression (such as miRNA-328, -hsa-miR-328, -519, -520h, -212, -181a, 487a and miR-302[200-205]. The use of miRNA to interfere with transporter transcription confirmed the role of ABCG2 in drug resistance related signaling pathways. For example, it was seen that miR-132 enhanced cisplatin resistance in gastric cancer cells by targeting SIRT1 which regulates ABCG2 expression by promoting the de-acetylation of the transcription factor CREB (SIRT1/CREB/ABCG2 pathway). Moreover, Kaplan-Meier survival analysis showed that low miR-132 is related to longer survival in chemo resistant gastric cancer[206]. Another example is Wnt/β-catenin-ABCG2 pathway, which is modulated by miR-199a/b in colorectal cancer stem cells, and whose upregulation is related to cisplatin resistance[207].

Dual inhibitors

Some of the broad-spectrum inhibitors of P-gp, including elacridar, reserpine, cyclosporine A, tariquidar and PSC 833 also display ABCG2 inhibitory activity[10]. Such dual inhibitors will be effective in cases where both transporters participate in MDR and the cytotoxic drug is a substrate of both transporters. Conceivably, their inhibition will also increase the oral bioavailability of the drug, in addition to its penetration to the tumor site. However, since these two transporters are also expressed in sanctuary sites, caution for potential toxic side effects should be considered. Moreover, this awareness would also hold for cytotoxic drugs with a narrow therapeutic window, as minor elevations of drug exposure might lead to adverse reactions.

TKIs as ABCG2 inhibitor or substrate

Various TKIs are either a substrate, an inhibitor or both, for P-gp, MRP1 and ABCG2[168]. TKIs such as the BCR-ABL kinase inhibitors (imatinib, nilotinib and dasatinib), EGFRs kinase inhibitors (gefitinib, erlotinib, lapatinib) and others, show substrate-like properties at lower concentration and inhibitor-like properties at higher concentration via competitive inhibition of the transporters function[208]. In addition to the concentration dependent type of interaction, it has been proposed that exposure time to TKIs also plays a role[209]. Short exposure (≤ 24 h) to either gefitinib or vandetanib demonstrated a synergic interaction when SN-38, whereas prolonged exposure (5 days) showed a strong antagonism between gefitinib or vandetanib and SN-38. Gefitinib and EK-785 at a low concentration (0.1 to 1 μmol/L) significantly stimulated the ATPase activity, suggesting that these TKIs are transported substrates of ABCG2 whereas higher concentrations induced strong inhibition of ABCG2[210]. Different types of interactions and corresponding possible effects have been suggested by different research groups, which are demonstrated in a hypothetical model [Figure 2].

Many combination therapies of TKIs with cytotoxic drugs have been performed (e.g., the combination of gefitinib with 5-fluorouracil, leucovorin, and irinotecan in patients with colorectal cancer, the combination of imatinib and cytarabine in newly diagnosed

patients with chronic myeloid leukemia, and the combination of gemcitabine/cisplatin with gefitinib in NSCLC[95,211-214]. TKIs also interact with ABCG2 that can modify ADME-Tox profile of ABCG2 substrates. Gefitinib enhanced the oral bioavailability of irinotecan and topotecan, and increased their apparent bioavailability and decreased systemic clearance in mice[215,216]. Therefore, studies are needed that investigate the exact type of interactions between TKIs and cytotoxic drugs[95,208,209].

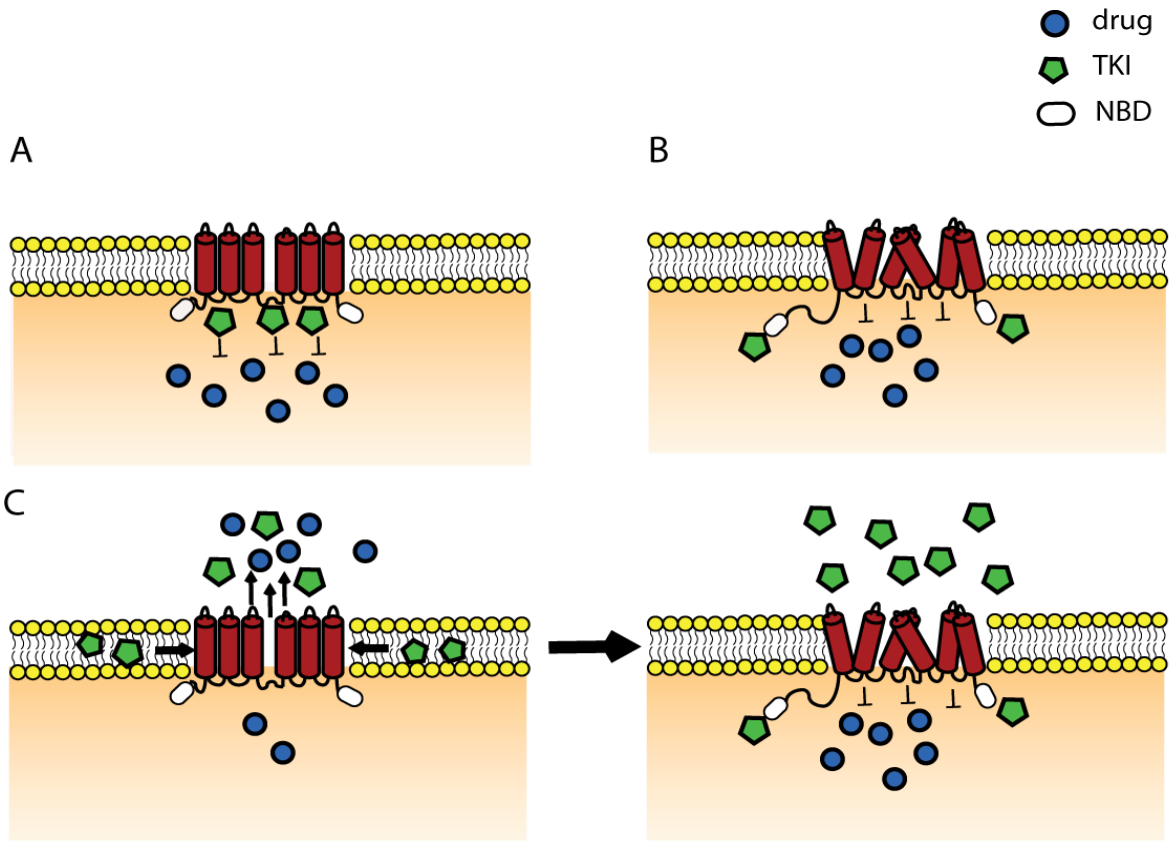


***Figure 2**: Hypothetical model demonstrating the different possible interactions of TKIs with ABC transporters. (A) The competitive binding of TKI at the drug binding site and hereby preventing the binding of the drug and consequently its efflux; (B) the situation when TKI is not a substrate of ABC transporter, but it binds at the NBD or other site inducing allosteric modulation of the transporter which leads to its inhibition; (C) the case when the TKI behaves as a substrate at low concentration, where the "vacuum cleaner" model is applicable[119]. However, when the concentration increases at a given moment saturation takes place and TKIs penetrate into the cell and bind at the NBD and thereby inhibiting the transporter. ABC: ATP-binding cassette; TKI: tyrosine kinase inhibitor; NBD: nucleotide binding domain*

Inhibitors from natural sources

ABCG2 inhibitors originating from natural products have been referred to as the fourth-generation inhibitors. Originally, these compounds showed effects in cancer prevention[217], but later on they were found to exert sensitizing effects to chemotherapeutics. Among them are the flavonoids which are polyphenolic compounds found in foods and herbal products. Different flavonoids are potent modulators of ABC transporters: kaempferol, apigenin and myricetin are inhibitors of MRP1[218], kaempferide, quercetin, diosmin and glabridin are inhibitors of P-gp[219] and

silymarin, hesperetin and daidzein are inhibitors of ABCG2[220]. The bioflavonoids neochamaejasmin B (NCB)[221] and chamaechromone from the root of Stellera chamaejasme L. are able to inhibit the expression and activity of MRP1. Interestingly, chamaechromone is MRP1 specific, while NCB is a substrate for MRP1 and P-gp. Furthermore NCB and chamaechromone co-administration increased chamaechromone bioavailability substantially[166]. However, further investigation is needed to define optimal dose schedules of flavonoids capable of inducing ABC transporter inhibition[219]. Future studies would also have to take into account flavonoid pharmacokinetics, bioavailability and activity of flavonoid metabolites after ingestion, which would reduce active flavonoid plasma concentrations[222,223].

CONCLUSION

Efflux transporters of the ABC superfamily including P-gp, MRP1 and ABCG2 can confer MDR in cancer cells. Additionally, overexpression of these transporters in tumor tissues has been associated with poor therapy outcome of cancer patients. Conceptually, inhibition of these ABC transporters will increase drug accumulation and thereby overcome MDR. However, despite accumulating knowledge of ABC transporters and their complex interplay, clinical trials evaluating the effect of ABC transporter inhibitors did not fulfill their promises. In part, this has also been accounted for by poor patient selection criteria. The design of ABC transporter inhibition research would require several considerations. The choice for a specific inhibitor will increase the likelihood of undesirable side effects when the inhibitor targets multiple ABC transporters, although due to redundancy of ABC transporters, sanctuary sites might be spared from the toxic insults of the cytotoxic drug. When multiple ABC transporters are involved in provoking the MDR phenotype, broad-spectrum inhibitors should be preferred, but as a double-edged sword will also impact physiological functions of these ABC transporters. The evading strategy may serve an alternative for the ABC transporter inhibition dilemma. At this stage, however, the magic bullet that escapes each of the 15 ABC transporters implicated in cancer drug resistance has not been identified and current studies aim to identify those compounds evading the major ABC transporters P-gp, MRP1 and BCRP. Also, MDR evading strategies using encapsulation of drugs have potential downsides such as immune reactions. Lastly, concerning the exploiting strategy, those drugs which cause collateral sensitivity would promote selective targeting of MDR cells, but this concept has not yet been sufficiently explored in animal and clinical studies.

Collectively, each of the MDR reversal strategies has its merits but did not translate in successful clinical application. Future directions should therefore be aimed at combining our current knowledge of clinically relevant ABC transporters in the field of molecular biology (including polymorphic variants), biochemistry, computational biology and sensitive detection and imaging techniques, which in conjunction with state of the art medicinal chemistry can generate new generation inhibitors for future MDR reversal studies. Such an integrated approach may also help to guide personalized therapy in cancer patients to achieve the most optimal treatment outcome.

REFERENCES

1. Binkhathlan Z, Lavasanifar A. P-glycoprotein inhibition as a therapeutic approach for overcoming multidrug resistance in cancer: current status and future perspectives. *Curr Cancer Drug Targets* 2013;13:326-46.
2. Longley DB, Johnston PG. Molecular mechanisms of drug resistance. *J Pathol* 2005;205:275-92.
3. Tiwari AK, Sodani K, Dai CL, Ashby CR Jr, Chen ZS. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. *Curr Pharm Biotechnol* 2011;12:570-94.
4. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48-58.
5. Zahreddine H, Borden KL. Mechanisms and insights into drug resistance in cancer. *Front Pharmacol* 2013;4:28.
6. Cree IA, Charlton P. Molecular chess? Hallmarks of anti-cancer drug resistance. *BMC Cancer* 2017;17:1-8.
7. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001;11:1156-66.
8. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. *Hum Genomics* 2009;3:281-90.
9. Li W, Zhang H, Assaraf YG, Zhao K, Xu X, Xie J, Yang DH, Chen ZS. Overcoming ABC transporter-mediated multidrug resistance: molecular mechanisms and novel therapeutic drug strategies. *Drug Resist Updat* 2016;27:14-29.
10. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. *Int J Biochem Mol Biol* 2012;3:1-27.
11. Zhao Z, Fang LL, Johnsen R, Baillie DL. ATP-binding cassette protein E is involved in gene transcription and translation in Caenorhabditis elegans. *Biochem Biophys Res Commun* 2004;323:104-11.
12. Bisbal C, Martinand C, Silhol M, Lebleu B, Salehzada T. Cloning and characterization of a RNAse L inhibitor. A new component of the interferon-regulated 2-5A pathway. *J Biol Chem* 1995;270:13308-17.
13. Marton MJ, Vazquez de Aldana CR, Qiu H, Chakraburtty K, Hinnebusch AG. Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2alpha kinase GCN2. *Mol Cell Biol* 1997;17:4474-89.
14. Tyzack JK, Wang X, Belsham GJ, Proud CG. ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner. *J Biol Chem* 2000;275:34131-9.
15. Sharom FJ. ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics* 2007;9:105-27.
16. Cinci L, Luceri C, Bigagli E, Carboni I, Paccosi S, Parenti A, Guasti D, Coronnello M. Development and characterization of an in vitro model of colorectal adenocarcinoma with MDR phenotype. *Cancer Med* 2016;5:1279-91.
17. Pallis M, Russell N. P-glycoprotein plays a drug-efflux-independent role in augmenting cell survival in acute myeloblastic leukemia and is associated with modulation of a sphingomyelin-ceramide apoptotic pathway. *Blood* 2000;95:2897-904.
18. Zhang F, Zhang H, Wang Z, Yu M, Tian R, Ji W, Yang Y, Ruifang N. P-glycoprotein associates with Anxa2 and promotes invasion in multidrug resistant breast cancer cells. *Biochem Pharmacol* 2014;87:292-302.
19. van de Ven R, Oerlemans R, van der Heijden JW, Scheffer GL, de Gruijl TD, Jansen G, Scheper RJ. ABC drug transporters and immunity: novel therapeutic targets in autoimmunity and cancer. *J Leukoc Biol* 2009;86:1075-87.
20. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976;455:152-62.
21. Ferry D, Russell M, Cullen M. P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is alloserically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun* 1992;188:440-5.
22. Gottesman MM, Ambudkar SV, Xia D. Structure of a multidrug transporter: crystal structures of a mammalian multidrug efflux pump bound to peptide inhibitors may reveal drug binding sites. *Nat Biotechnol* 2009;27:546-7.

23. Ueda K, Cardarelli C, Gottesman MM, Pastan I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci U S A* 1987;84:3004-8.
24. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* 2009;323:1718-22.
25. Loo TW, Bartlett MC, Clarke DM. Simultaneous binding of two different drugs in the binding pocket of the human multidrug resistance P-glycoprotein. *J Biol Chem* 2003;278:39706-10.
26. Pajeva IK, Wiese M. Structure-activity relationships of tariquidar analogs as multidrug resistance modulators. *AAPS J* 2009;11:435.
27. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci U S A* 1987;84:7735-8.
28. Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 1990;38:1277-87.
29. Anwar-Mohamed A, El-Kadi A. P-glycoprotein effects on drugs pharmacokinetics and drug-drug-interactions and their clinical implications. *Libyan J Pharm Clin Pharmacol* 2012;1:48154.
30. Sharom FJ. The P-glycoprotein multidrug transporter. *Essays Biochem* 2011;50:161.
31. Baldini N, Scotlandi K, Barbanti-Bròdano G, Manara MC, Maurici D, Bacci G, Bertoni F, Picci P, Sottili S, Campanacci M, Serra M. Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *N Engl J Med* 1995;333:1380-5.
32. Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, Scotto KW. Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clin Cancer Res* 1999;5:3352-6.
33. Gregorcyk S, Kang Y, Brandt D, Kolm P, Singer G, Perry RR. P-glycoprotein expression as a predictor of breast cancer recurrence. *Ann Surg Oncol* 1996;3:8-14.
34. McLornan DP, McMullin MF, Johnston P, Longley DB. Molecular mechanisms of drug resistance in acute myeloid leukaemia. *Expert Opin Drug Metab Toxicol* 2007;3:363-77.
35. Xia CQ, Smith PG. Drug efflux transporters and multidrug resistance in acute leukemia: therapeutic impact and novel approaches to mediation. *Mol Pharmacol* 2012;82:1008-21.
36. Choi JH, Lim HY, Joo HJ, Kim HS, Yi JW, Kim HC, Cho YK, Kim MW, Lee KB. Expression of multidrug resistance-associated protein1, P-glycoprotein, and thymidylate synthase in gastric cancer patients treated with 5-fluorouracil and doxorubicin-based adjuvant chemotherapy after curative resection. *Br J Cancer* 2002;86:1578-85.
37. Gao Y, Liao Y, Shen JK, Feng Y, Choy E, Cote G, Harmon D, Mankin HJ, Hornicek FJ, Duan Z. Evaluation of P-glycoprotein (Pgp) expression in human osteosarcoma by high-throughput tissue microarray. *J Orthop Res* 2016;34:1606-12.
38. Brozek J, Bryl E, Płoszyńska A, Balcerska A, Witkowski JM. P-glycoprotein activity predicts outcome in childhood acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* 2009;31:493-9.
39. Steinbach D, Legrand O. ABC transporters and drug resistance in leukemia: was P-gp nothing but the first head of the Hydra? *Leukemia* 2007;21:1172-6.
40. Broxterman HJ, Sonneveld P, Feller N, Ossenkoppele GJ, Währer DC, Eekman CA, Schoester M, Lankelma J, Pinedo HM, Löwenberg B, Schuurhuis GJ. Quality control of multidrug resistance assays in adult acute leukemia: correlation between assays for P-glycoprotein expression and activity. *Blood* 1996;87:4809-16.
41. Cole S, Bhardwaj G, Gerlach J, Mackie J, Grant C, Almquist K, Stewart A, Kurz E, Duncan A, Deeley R. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992;258:1650-4.
42. Flens MJ, Zaman GJ, van der Valk P, Izquierdo MA, Schroeijers AB, Scheffer GL, van der Groep P, de Haas M, Meijer CJ, Scheper RJ. Tissue distribution of the multidrug resistance protein. *Am J Pathol* 1996;148:1237-47.
43. Kourti M, Vavatsi N, Gombakis N, Sidi V, Tzimagiorgis G, Papageorgiou T, Koliouskas D, Athanassiadou F. Expression of multidrug resistance 1 (MDR1), multidrug resistance-related protein 1 (MRP1), lung resistance protein (LRP), and breast cancer resistance protein (BCRP) genes and clinical outcome in childhood acute lymphoblastic leukemia. *Int J Hematol* 2007;86:166-73.
44. Bakos E, Homolya L. Portrait of multifaceted transporter, the multidrug resistance-associated protein 1 (MRP1/ABCC1). *Pflugers Arch* 2007;453:621-41.
45. Rao VV, Dahlheimer JL, Bardgett ME, Snyder AZ, Finch RA, Sartorelli AC, Piwnica-Worms D. Choroid plexus epithelial expression of MDR1 P-glycoprotein and multidrug resistance-

associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. *Proc Natl Acad Sci U S A* 1999;96:3900-5.
46. Bréchot JM, Hurbain I, Fajac A, Daty N, Bernaudin JF. Different pattern of MRP localization in ciliated and basal cells from human bronchial epithelium. *J Histochem Cytochem* 1998;46:513-7.
47. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* 2005;204:216-37.
48. Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, Borst P. Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nat Med* 1997;3:1275-9.
49. Lorico A, Rappa G, Finch RA, Yang D, Flavell RA, Sartorelli AC. Disruption of the murine MRP (multidrug resistance protein) gene leads to increased sensitivity to etoposide (VP-16) and increased levels of glutathione. *Cancer Res* 1997;57:5238-42.
50. Renes J, de Vries EG, Nienhuis EF, Jansen PL, Müller M. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. *Br J Pharmacol* 1999;126:681-8.
51. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res* 1996;56:988.
52. Bakos É, Evers R, Szakács G, Tusnády GE, Welker E, Szabó K, de Haas M, van Deemter L, Borst P, Váradi A, Sarkadi B. Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. *J Biol Chem* 1998;273:32167-75.
53. Westlake CJ, Cole SP, Deeley RG. Role of the NH(2)-terminal membrane spanning domain of multidrug resistance protein 1/ABCC1 in protein processing and trafficking. *Mol Biol Cell* 2005;16:2483-92.
54. Yang Y, Chen Q, Zhang JT. Structural and functional consequences of mutating cysteine residues in the amino terminus of human multidrug resistance-associated protein 1. *J Biol Chem* 2002;277:44268-77.
55. Chen Q, Yang Y, Li L, Zhang JT. The amino terminus of the human multidrug resistance transporter ABCC1 has a U-shaped folding with a gating function. *J Biol Chem* 2006;281:31152-63.
56. Yang Y, Mo W, Zhang JT. Role of transmembrane segment 5 and extracellular loop 3 in the homodimerization of human ABCC1. *Biochemistry* 2010;49:10854-61.
57. Sodani K, Patel A, Kathawala RJ, Chen ZS. Multidrug resistance associated proteins in multidrug resistance. *Chin J Cancer* 2012;31:58- 72.
58. Ween MP, Armstrong MA, Oehler MK, Ricciardelli C. The role of ABC transporters in ovarian cancer progression and chemoresistance. *Crit Rev Oncol Hematol* 2015;96:220-56.
59. Hipfner DR, Deeley RG, Cole SP. Structural, mechanistic and clinical aspects of MRP1. *Biochim Biophys Acta* 1999;1461:359-76.
60. Haber M, Smith J, Bordow SB, Flemming C, Cohn SL, London WB, Marshall GM, Norris MD. Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma. *J Clin Oncol* 2006;24:1546-53.
61. Li XQ, Li J, Shi SB, Chen P, Yu LC, Bao QL. Expression of MRP1, BCRP, LRP and ERCC1 as prognostic factors in non-small cell lung cancer patients receiving postoperative cisplatin-based chemotherapy. *Int J Biol Markers* 2009;24:230-7.
62. Filipits M, Pohl G, Rudas M, Dietze O, Lax S, Grill R, Pirker R, Zielinski CC, Hausmaninger H, Kubista E, Samonigg H, Jakesz R. Clinical role of multidrug resistance protein 1 expression in chemotherapy resistance in early-stage breast cancer: the Austrian Breast and Colorectal Cancer Study Group. *J Clin Oncol* 2005;23:1161-8.
63. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport - an update. *AAPS J* 2015;17:65-82.
64. Taipalensuu J, Törnblom H, Lindberg G, Einarsson C, Sjöqvist F, Melhus H, Garberg P, Sjöström B, Lundgren B, Artursson P. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. *J Pharmacol Exp Ther* 2001;299:164-70.
65. Merino G, Álvarez AI, Pulido MM, Molina AJ, Schinkel AH, Prieto JG. Breast cancer resistance protein (BCRP/ABCG2) transports fluoroquinolone antibiotics and affects their oral availability, pharmacokinetics, and milk secretion. *Drug Metab Dispos* 2006;34:690-5.
66. Seamon JA, Rugg CA, Emanuel S, Calcagno AM, Ambudkar SV, Middleton SA, Butler J, Borowski V, Greenberger LM. Role of the ABCG2 drug transporter in the resistance and oral

bioavailability of a potent cyclin-dependent kinase/Aurora kinase inhibitor. *Mol Cancer Ther* 2006;5:2459-67.
67. Yamagata T, Kusuhara H, Morishita M, Takayama K, Benameur H, Sugiyama Y. Improvement of the oral drug absorption of topotecan through the inhibition of intestinal xenobiotic efflux transporter, breast cancer resistance protein, by excipients. *Drug Metab Dispos* 2007;35:1142-8.
68. Tanaka Y, Kitamura Y, Maeda K, Sugiyama Y. Quantitative analysis of the ABCG2 c.421C>A polymorphism effect on in vivo transport activity of breast cancer resistance protein (BCRP) using an intestinal absorption model. *J Pharm Sci* 2015;104:3039-48.
69. Kruijtzer CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC, Paul EM, Schellens JH. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* 2002;20:2943-50.
70. Staud F, Pavek P. Breast cancer resistance protein (BCRP/ABCG2). *Int J Biochem Cell Biol* 2005;37:720-5.
71. Clark R, Kerr ID, Callaghan R. Multiple drugbinding sites on the R482G isoform of the ABCG2 transporter. *Br J Pharmacol* 2006;149:506-15.
72. Giri N, Agarwal S, Shaik N, Pan G, Chen Y, Elmquist WF. Substrate-dependent breast cancer resistance protein (BCRP1/ABCG2)- mediated interactions: consideration of multiple binding sites in in vitro assay design. *Drug Metab Dispos* 2009;37:560-70.
73. Muenster U, Grieshop B, Ickenroth K, Gnoth MJ. Characterization of substrates and inhibitors for the in vitro assessment of Bcrp mediated drug-drug interactions. *Pharm Res* 2008;25:2320-6.
74. Hazai E, Bikádi Z. Homology modeling of breast cancer resistance protein (ABCG2). *J Struct Biol* 2008;162:63-74.
75. Kage K, Tsukahara S, Sugiyama T, Asada S, Ishikawa E, Tsuruo T, Sugimoto Y. Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization. *Int J Cancer* 2002;97:626-30.
76. Mo W, Zhang JT. Oligomerization of human ATP-binding cassette transporters and its potential significance in human disease. *Expert Opin Drug Metab Toxicol* 2009;5:1049-63.
77. Ni Z, Bikadi Z, Rosenberg MF, Mao Q. Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Curr Drug Metab* 2010;11:603-17.
78. Diestra JE, Scheffer GL, Català I, Maliepaard M, Schellens JHM, Scheper RJ, Germà-Lluch JR, Izquierdo MA. Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J Pathol* 2002;198:213-9.
79. Damiani D, Tiribelli M, Calistri E, Geromin A, Chiarvesio A, Michelutti A, Cavallin M, Fanin R. The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. *Haematologica* 2006;91:825-8.
80. Plasschaert SL, van der Kolk DM, de Bont ES, Kamps WA, Morisaki K, Bates SE, Scheffer GL, Scheper RJ, Vellenga E, de Vries EG. The role of breast cancer resistance protein in acute lymphoblastic leukemia. *Clin Cancer Res* 2003;9:5171-7.
81. Stam RW, van den Heuvel-Eibrink MM, den Boer ML, Ebus ME, Janka-Schaub GE, Allen JD, Pieters R. Multidrug resistance genes in infant acute lymphoblastic leukemia: Ara-C is not a substrate for the breast cancer resistance protein. *Leukemia* 2003;18:78-83.
82. Robey RW, To KKK, Polgar O, Dohse M, Fetsch P, Dean M, Bates SE. ABCG2: a perspective. *Adv Drug Deliv Rev* 2009;61:3-13.
83. Li-Blatter X, Beck A, Seelig A. P-glycoprotein-ATPase modulation: the molecular mechanisms. *Biophys J* 2012;102:1383-93.
84. Maki N, Moitra K, Ghosh P, Dey S. Allosteric modulation bypasses the requirement for ATP hydrolysis in regenerating low affinity transition state conformation of human P-glycoprotein. *J Biol Chem* 2006;281:10769-77.
85. Romsicki Y, Sharom FJ. The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. *Biochemistry* 1999;38:6887-96.
86. Aliabadi HM, Landry B, Mahdipoor P, Hsu CY, Uludağ H. Effective down-regulation of breast cancer resistance protein (BCRP) by siRNA delivery using lipid-substituted aliphatic polymers. *Eur J Pharm Biopharm* 2012;81:33-42.
87. Gomes MJ, Kennedy PJ, Martins S, Sarmento B. Delivery of siRNA silencing P-gp in peptide-functionalized nanoparticles causes efflux modulation at the blood-brain barrier. *Nanomedicine (Lond)* 2017;12:1385-99.
88. Fisher M, Abramov M, Van Aerschot A, Xu D, Juliano RL, Herdewijn P. Inhibition of MDR1 expression with altritol-modified siRNAs. *Nucleic Acids Res* 2007;35:1064-74.

89. Lam JK, Chow MY, Zhang Y, Leung SW. siRNA versus miRNA as therapeutics for gene silencing. *Mol Ther Nucleic Acids* 2015;4:e252.
90. Cuiffo BG, Campagne A, Bell GW, Lembo A, Orso F, Lien EC, Bhasin MK, Raimo M, Hanson SE, Marusyk A, El-Ashry D, Hematti P, Polyak K, Mechta-Grigoriou F, Mariani O, Volinia S, Vincent-Salomon A, Taverna D, Karnoub AE. MSC-regulated microRNAs converge on the transcription factor FOXP2 and promote breast cancer metastasis. *Cell Stem Cell* 2014;15:762-74.
91. Broekman F, Giovannetti E, Peters GJ. Tyrosine kinase inhibitors: multi-targeted or single-targeted? *World J Clin Oncol* 2011;2:80-93.
92. Liu Y, Zhu W, Zhang J, Huang J, Zhao Y, Zhang W, Han B, Yao Y, Jiang L, Li S. The evaluation of efficacy and safety of sunitinib on EGFR-TKI pretreated advanced non-small cell lung cancer patients in China. *Clin Respir J* 2014;8:206-12.
93. Na F, Zhang J, Deng L, Zhou X, Zhou L, Zou B, Yu M, Li Y, Xue J, Liu Y. Continuation of tyrosine kinase inhibitor is associated with survival benefit in NSCLC patients with exon 19 deletion after solitary progression. *J Cancer* 2017;8:3682-8.
94. Zhang J, Yang PL, Gray NS. Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 2009;9:28-39.
95. Wu P, Nielsen TE, Clausen MH. FDA-approved small-molecule kinase inhibitors. *Trends Pharmacol Sci* 2015;36:422-39.
96. Rask-Andersen M, Zhang J, Fabbro D, Schiöth HB. Advances in kinase targeting: current clinical use and clinical trials. *Trends Pharmacol Sci* 2014;35:604-20.
97. Kathawala RJ, Gupta P, Ashby CR, Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Updat* 2015;18:1-17.
98. List AF, Kopecky KJ, Willman CL, Head DR, Persons DL, Slovak ML, Dorr R, Karanes C, Hynes HE, Doroshow JH, Shurafa M, Appelbaum FR. Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology Group study. *Blood* 2001;98:3212-20.
99. Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006;5:219-34.
100. Callaghan R, Luk F, Bebawy M. Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy? *Drug Metab Dispos* 2014;42:623-31.
101. List AF, Kopecky KJ, Willman CL, Head DR, Slovak ML, Douer D, Dakhil SR, Appelbaum FR. Cyclosporine inhibition of P-glycoprotein in chronic myeloid leukemia blast phase. *Blood* 2002;100:1910-2.
102. Abraham J, Edgerly M, Wilson R, Chen C, Rutt A, Bakke S, Robey R, Dwyer A, Goldspiel B, Balis F, Van Tellingen O, Bates SE, Fojo T. A phase I study of the P-glycoprotein antagonist tariquidar in combination with vinorelbine. *Clin Cancer Res* 2009;15:3574-82.
103. Gerrard G, Payne E, Baker R, Jones D, Potter M, Prentice H, Ethell M, McCullough H, Burgess M, Mehta A, Ganeshaguru K. Clinical effects and P-glycoprotein inhibition in patients with acute myeloid leukemia treated with zosuquidar trihydrochloride, daunorubicin and cytarabine. *Haematologica* 2004;89:782-90.
104. Sandler A, Gordon M, de Alwis DP, Pouliquen I, Green L, Marder P, Chaudhary A, Fife K, Battiato L, Sweeney C, Jordan C, Burgess M, Slapak CA. A phase I trial of a potent P-glycoprotein inhibitor, zosuquidar trihydrochloride (LY335979), administered intravenously in combination with doxorubicin in patients with advanced malignancy. *Clin Cancer Res* 2004;10:3265-72.
105. Cripe LD, Uno H, Paietta EM, Litzow MR, Ketterling RP, Bennett JM, Rowe JM, Lazarus HM, Luger S, Tallman MS. Zosuquidar, a novel modulator of P-glycoprotein, does not improve the outcome of older patients with newly diagnosed acute myeloid leukemia: a randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999. *Blood* 2010;116:4077-85.
106. Kühnle M, Egger M, Müller C, Mahringer A, Bernhardt G, Fricker G, König B, Buschauer A. Potent and selective inhibitors of breast cancer resistance protein (ABCG2) derived from the P-glycoprotein (ABCB1) modulator tariquidar. *J Med Chem* 2009;52:1190-7.
107. Li XQ, Wang L, Lei Y, Hu T, Zhang FL, Cho CH, To KK. Reversal of P-gp and BCRP-mediated MDR by tariquidar derivatives. *Eur J Med Chem* 2015;101:560-72.
108. Kakarla P, Inupakutika M, Devireddy AR, Gunda SK, Willmon TM, Ranjana K, Shrestha U, Ranaweera I, Hernandez AJ, Barr S, Varela MF. 3D-QSAR and contour map analysis of tariquidar analogues as multidrug resistance protein-1 (MRP1) inhibitors. *Int J Pharm Sci Res* 2016;7:554-72.

109. Kannan P, Telu S, Shukla S, Ambudkar SV, Pike VW, Halldin C, Gottesman MM, Innis RB, Hall MD. The “specific” P-glycoprotein inhibitor tariquidar is also a substrate and an inhibitor for breast cancer resistance protein (BCRP/ABCG2). *ACS Chem Neurosci* 2011;2:82-9.
110. Martin C, Berridge G, Mistry P, Higgins C, Charlton P, Callaghan R. The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. *Br J Pharmacol* 1999;128:403-11.
111. Bankstahl JP, Bankstahl M, Römermann K, Wanek T, Stanek J, Windhorst AD, Fedrowitz M, Erker T, Müller M, Löscher W, Langer O, Kuntner C. Tariquidar and elacridar are dose-dependently transported by P-glycoprotein and BCRP at the blood-brain barrier: a small animal positron emission tomography and in vitro study. *Drug Metab Dispos* 2013;41:754-62.
112. Weidner LD, Fung KL, Kannan P, Moen JK, Kumar JS, Mulder J, Innis RB, Gottesman MM, Hall MD. Tariquidar is an inhibitor and not a substrate of human and mouse P-glycoprotein. *Drug Metab Dispos* 2016;44:275-82.
113. Mross K, Bohn C, Edler L, Jonat W, Queisser W, Heidemann E, Goebel M, Hossfeld DK. Randomized phase II study of single-agent epirubicin +/- verapamil in patients with advanced metastatic breast cancer. An AIO clinical trial. Arbeitsgemeinschaft Internistische Onkologie of the German Cancer Society. *Ann Oncol* 1993;4:45-50.
114. Leonard GD, Fojo T, Bates SE. The role of ABC transporters in clinical practice. *Oncologist* 2003;8:411-24.
115. Porcelli L, Lemos C, Peters GJ, Paradiso A, Azzariti A. Intracellular trafficking of MDR transporters and relevance of SNPs. *Curr Top Med Chem* 2009;9:197-208.
116. Follit CA, Brewer FK, Wise JG, Vogel PD. In silico identified targeted inhibitors of P-glycoprotein overcome multidrug resistance in human cancer cells in culture. *Pharmacol Res Perspect* 2015;3:e00170.
117. Brewer FK, Follit CA, Vogel PD, Wise JG. In silico screening for inhibitors of p-glycoprotein that target the nucleotide binding domains. *Mol Pharmacol* 2014;86:716-26.
118. Nanayakkara AK, Follit CA, Chen G, Williams NS, Vogel PD, Wise JG. Targeted inhibitors of P-glycoprotein increase chemotherapeutic-induced mortality of multidrug resistant tumor cells. *Sci Rep* 2018;8:967.
119. Zinzi L, Capparelli E, Cantore M, Contino M, Leopoldo M, Colabufo NA. Small and innovative molecules as new strategy to revert MDR. *Front Oncol* 2014;4:2.
120. Yang X, Iyer AK, Singh A, Milane L, Choy E, Hornicek FJ, Amiji MM, Duan Z. Cluster of differentiation 44 targeted hyaluronic acid based nanoparticles for MDR1 siRNA delivery to overcome drug resistance in ovarian cancer. *Pharm Res* 2015;32:2097-109.
121. Ganesh S, Iyer AK, Morrissey DV, Amiji MM. Hyaluronic acid based self-assembling nanosystems for CD44 target mediated siRNA delivery to solid tumors. *Biomaterials* 2013;34:3489-502.
122. Wu D, Li XS, Meng XN, Yan J, Zong ZH. MicroRNA-873 mediates multidrug resistance in ovarian cancer cells by targeting ABCB1. *Tumour Biol* 2016;37:10499-506.
123. Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, Yang JM. Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/ P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* 2008;76:582-8.
124. Da Silva CG, Peters GJ, Ossendorp F, Cruz LJ. The potential of multi-compound nanoparticles to bypass drug resistance in cancer. *Cancer Chemother Pharmacol* 2017;80:881-94.
125. Iwamoto T. Clinical application of drug delivery systems in cancer chemotherapy: review of the efficacy and side effects of approved drugs. *Biol Pharm Bull* 2013;36:715-8.
126. Yamamoto Y, Kawano I, Iwase H. Nab-paclitaxel for the treatment of breast cancer: efficacy, safety, and approval. *OncoTargets Ther* 2011;4:123-36.
127. Jin X, Zhou B, Xue L, San W. Soluplus® micelles as a potential drug delivery system for reversal of resistant tumor. *Biomed Pharmacother* 2015;69:388-95.
128. Win-Shwe TT, Fujimaki H. Nanoparticles and neurotoxicity. *Int J Mol Sci* 2011;12:6267-80.
129. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. *Int J Nanomedicine* 2008;3:133-49.
130. Zhou Y, Hong L. Prediction value of miR-483 and miR-214 in prognosis and multidrug resistance of esophageal squamous cell carcinoma. *Genet Test Mol Biomark* 2013;17:470-4.
131. Li Z, Hu S, Wang J, Cai J, Xiao L, Yu L, Wang Z. MiR-27a modulates MDR1/P-glycoprotein expression by targeting HIPK2 in human ovarian cancer cells. *Gynecol Oncol* 2010;119:125-30.
132. Chen Z, Ma T, Huang C, Zhang L, Lv X, Xu T, Hu T, Li J. MiR-27a modulates the MDR1/P-glycoprotein expression by inhibiting FZD7/β-catenin pathway in hepatocellular carcinoma cells. *Cell Signal* 2013;25:2693-701.

133. Feng DD, Zhang H, Zhang P, Zheng YS, Zhang XJ, Han BW, Luo XQ, Xu L, Zhou H, Qu LH, Chen YQ. Down-regulated miR-331-5p and miR-27a are associated with chemotherapy resistance and relapse in leukaemia. *J Cell Mol Med* 2011;15:2164-75.
134. Hong L, Han Y, Zhang H, Li M, Gong T, Sun L, Wu K, Zhao Q, Fan D. The prognostic and chemotherapeutic value of miR-296 in esophageal squamous cell carcinoma. *Ann Surg* 2010;251:1056-63.
135. Lee SD, Osei-Twum JA, Wasan KM. Dose-dependent targeted suppression of P-glycoprotein expression and function in Caco-2 cells. *Mol Pharm* 2013;10:2323-30.
136. Bao L, Hazari S, Mehra S, Kaushal D, Moroz K, Dash S. Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *Am J Pathol* 2012;180:2490-503.
137. Lin CJ, Gong HY, Tseng HC, Wang WL, Wu JL. miR-122 targets an anti-apoptotic gene, Bcl-w, in human hepatocellular carcinoma cell lines. *Biochem Biophys Res Commun* 2008;375:315-20.
138. Xu Y, Ohms SJ, Li Z, Wang Q, Gong G, Hu Y, Mao Z, Shannon MF, Fan JY. Changes in the expression of miR-381 and miR-495 are inversely associated with the expression of the MDR1 gene and development of multi-drug resistance. *PLoS One* 2013;8:e82062.
139. Yang T, Zheng ZM, Li XN, Li ZF, Wang Y, Geng YF, Bai L, Zhang XB. MiR-223 modulates multidrug resistance via downregulation of ABCB1 in hepatocellular carcinoma cells. *Exp Biol Med (Maywood)* 2013;238:1024-32.
140. Munoz JL, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, Rameshwar P. Delivery of functional anti-miR-9 by mesenchymal stem cell-derived exosomes to glioblastoma multiforme cells conferred chemosensitivity. *Mol Ther Nucleic Acids* 2013;2:e126.
141. Nobili S, Landini I, Mazzei T, Mini E. Overcoming tumor multidrug resistance using drugs able to evade P-glycoprotein or to exploit its expression. *Med Res Rev* 2012;32:1220-62.
142. Stupp R, Tosoni A, Bromberg JEC, Hau P, Campone M, Gijtenbeek J, Frenay M, Breimer L, Wiesinger H, Allgeier A, van den Bent MJ, Bogdahn U, van der Graaf W, Yun HJ, Gorlia T, Lacombe D, Brandes AA. Sagopilone (ZK-EPO, ZK 219477) for recurrent glioblastoma. A phase II multicenter trial by the European Organisation for Research and Treatment of Cancer (EORTC) Brain Tumor Group. *Ann Oncol* 2011;22:2144-9.
143. Lee FY, Borzilleri R, Fairchild CR, Kim SH, Long BH, Reventos-Suarez C, Vite GD, Rose WC, Kramer RA. BMS-247550: a novel epothilone analog with a mode of action similar to paclitaxel but possessing superior antitumor efficacy. *Clin Cancer Res* 2001;7:1429-37.
144. Lechleider RJ, Kaminskas E, Jiang X, Aziz R, Bullock J, Kasliwal R, Harapanhalli R, Pope S, Sridhara R, Leighton J, Booth B, Dagher R, Justice R, Pazdur R. Ixabepilone in combination with capecitabine and as monotherapy for treatment of advanced breast cancer refractory to previous chemotherapies. *Clin Cancer Res* 2008;14:4378-84.
145. Puhalla S, Brufsky A. Ixabepilone: a new chemotherapeutic option for refractory metastatic breast cancer. *Biol Targets Ther* 2008;2:505-15.
146. Sparano JA, Vrdoljak E, Rixe O, Xu B, Manikhas A, Medina C, Ventilari Da Costa SC, Ro J, Rubio G, Rondinon M, Perez Manga G, Peck R, Poulart V, Conte P. Randomized phase III trial of ixabepilone plus capecitabine versus capecitabine in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. *J Clin Oncol* 2010;28:3256-63.
147. Mitchell D, Bergendahl G, Ferguson W, Roberts W, Higgins T, Ashikaga T, DeSarno M, Kaplan J, Kraveka J, Eslin D, Vander Werff A, Hanna GK, Sholler GL. A phase 1 trial of TPI 287 as a single agent and in combination with temozolomide in patients with refractory or recurrent neuroblastoma or medulloblastoma. *Pediatr Blood Cancer* 2016;69:39-46.
148. Dumontet C, Jordan MA, Lee FF. Ixabepilone: targeting betaIII-tubulin expression in taxane-resistant malignancies. *Mol Cancer Ther* 2009;8:17-25.
149. Shen H, Lee FY, Gan J. Ixabepilone, a novel microtubule-targeting agent for breast cancer, is a substrate for P-glycoprotein (P-gp/ MDR1/ABCB1) but not breast cancer resistance protein (BCRP/ABCG2). *J Pharmacol Exp Ther* 2011;337:423-32.
150. Yang CP, Verdier-Pinard P, Wang F, Lippaine-Horvath E, He L, Li D, Höfle G, Ojima I, Orr GA, Horwitz SB. A highly epothilone B-resistant A549 cell line with mutations in tubulin that confer drug dependence. *Mol Cancer Ther* 2005;4:987-95.
151. Wangari-Talbot J, Hopper-Borge E. Drug resistance mechanisms in non-small cell lung carcinoma. *J Can Res Updat* 2014;2:265-82.
152. Hall MD, Handley MD, Gottesman MM. Is resistance useless? Multidrug resistance and collateral sensitivity. *Trends Pharmacol Sci* 2009;30:546-56.
153. Bergman AM, Munch-Petersen B, Jensen PB, Sehested M, Veerman G, Voorn DA, Smid K, Pinedo HM, Peters GJ. Collateral sensitivity to gemcitabine (2',2'-difluorodeoxycytidine) and

cytosine arabinoside of daunorubicin- and VM-26-resistant variants of human small cell lung cancer cell lines. *Biochem Pharmacol* 2001;61:1401-8.
154. Nakagawa-Goto K, Bastow KF, Ohkoshi E, Morris-Natschke SL, Lee KH. Antitumor agents 291 expanded B-Ring modification study of 6,8,8-triethyl desmosdumotin B analogues as multidrug-resistance selective agents. *Med Chem* 2011;1:1000101.
155. Hall MD, Brimacombe KR, Varonka MS, Pluchino KM, Monda JK, Li J, Walsh MJ, Boxer MB, Warren TH, Fales HM, Gottesman MM. Synthesis and structure-activity evaluation of isatin-β-thiosemicarbazones with improved selective activity towards multidrug resistant cells expressing P-glycoprotein. *J Med Chem* 2011;54:5878-89.
156. Vine KL, Belfiore L, Jones L, Locke JM, Wade S, Minaei E, Ranson M. N-alkylated isatins evade P-gp mediated efflux and retain potency in MDR cancer cell lines. *Heliyon* 2016;2:e00060.
157. Bergman AM, Pinedo HM, Talianidis I, Veerman G, Loves WJ, van der Wilt CL, Peters GJ. Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. *Br J Cancer* 2003;88:1963-70.
158. Bergman AM, Pinedo HM, Peters GJ. Steroids affect collateral sensitivity to gemcitabine of multidrug-resistant human lung cancer cells. *Eur J Pharmacol* 2001;416:19-24.
159. Borner MM, Schoffski P, de Wit R, Caponigro F, Comella G, Sulkes A, Greim G, Peters GJ, van der Born K, Wanders J, de Boer RF, Martin C, Fumoleau P. Patient preference and pharmacokinetics of oral modulated UFT versus intravenous fluorouracil and leucovorin: a randomised crossover trial in advanced colorectal cancer. *Eur J Cancer* 2002;38:349-58.
160. Liu G, Franssen E, Fitch MI, Warner E. Patient preferences for oral versus intravenous palliative chemotherapy. *J Clin Oncol* 1997;15:110-5.
161. Grant DS, Williams TL, Zahaczewsky M, Dicker AP. Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). *Int J Cancer* 2003;104:121-9.
162. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 2004;4:423-36.
163. Sparreboom A, van Asperen J, Mayer U, Schinkel AH, Smit JW, Meijer DK, Borst P, Nooijen WJ, Beijnen JH, van Tellingen O. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. *Proc Natl Acad Sci USA* 1997;94:2031-5.
164. van Asperen J, van Tellingen O, van der Valk MA, Rozenhart M, Beijnen JH. Enhanced oral absorption and decreased elimination of paclitaxel in mice cotreated with cyclosporin A. *Clin Cancer Res* 1998;4:2293-7.
165. Bardelmeijer HA, Ouwehand M, Beijnen JH, Schellens JH, van Tellingen O. Efficacy of novel P-glycoprotein inhibitors to increase the oral uptake of paclitaxel in mice. *Invest New Drugs* 2004;22:219-29.
166. Bardelmeijer HA, Beijnen JH, Brouwer KR, Rosing H, Nooijen WJ, Schellens JHM, van Tellingen O. Increased oral bioavailability of paclitaxel by GF120918 in mice through selective modulation of P-glycoprotein. *Clin Cancer Res* 2000;6:4416-21.
167. Koolen SL, Beijnen JH, Schellens JH. Intravenous-to-oral switch in anticancer chemotherapy: a focus on docetaxel and paclitaxel. *Clin Pharmacol Ther* 2010;87:126-9.
168. Shukla S, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. *Curr Drug Targets* 2011;12:621-30.
169. Wils P, Phung-Ba V, Warnery A, Lechardeur D, Raeissi S, Hidalgo IJ, Scherman D. Polarized transport of docetaxel and vinblastine mediated by P-glycoprotein in human intestinal epithelial cell monolayers. *Biochem Pharmacol* 1994;48:1528-30.
170. Jibodh RA, Lagas JS, Nuijen B, Beijnen JH, Schellens JH. Taxanes: old drugs, new oral formulations. *New Front Breast Cancer Treat* 2013;717:40-6.
171. Rezonja R, Knez L, Cufer T, Mrhar A. Oral treatment with etoposide in small cell lung cancer - dilemmas and solutions. *Radiol Oncol* 2013;47:1-13.
172. Hamada H, Tsuruo T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci U S A* 1986;83:7785-9.
173. Pearson JW, Fogler WE, Volker K, Usui N, Goldenberg SK, Gruys E, Riggs CW, Komschlies K, Wiltrout RH, Tsuruo T. Reversal of drug resistance in a human colon cancer xenograft expressing MDR1 complementary DNA by in vivo administration of MRK-16 monoclonal antibody. *J Natl Cancer Inst* 1991;83:1386-91.
174. Naito M, Tsuge H, Kuroko C, Koyama T, Tomida A, Tatsuta T, Heike Y, Tsuruo T. Enhancement of cellular accumulation of cyclosporine by anti-P-glycoprotein monoclonal antibody MRK-16 and synergistic modulation of multidrug resistance. *J Natl Cancer Inst* 1993;85:311-6.

175. Naito M, Tsuruo T. [18]Monoclonal antibodies to P-glycoprotein: preparation and applications to basic and clinical research. *Methods Enzymol* 1998;292:258-65.
176. Cole SP. Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future. *Annu Rev Pharmacol Toxicol* 2014;54:95-117.
177. Wang S, Tan X, Li S, Zhou Y, Geng P, Hua A, Deng A, Yu Z. Indomethacin-based stimuli-responsive micelles combined with paclitaxel to overcome multidrug resistance. *Oncotarget* 2017;8:111281-94.
178. Wan Y, Apostolou S, Dronov R, Kuss B, Voelcker NH. Cancer-targeting siRNA delivery from porous silicon nanoparticles. *Nanomedicine (Lond)* 2014;9:2309-21.
179. Wedge SR, Kendrew J, Hennequin LF, Valentine PJ, Barry ST, Brave SR, Smith NR, James NH, Dukes M, Curwen JO, Chester R, Jackson JA, Boffey SJ, Kilburn LL, Barnett S, Richmond GHP, Wadsworth PF, Walker M, Bigley AL, Taylor ST, Cooper L, Beck S, Jürgensmeier JM, Ogilvie DJ. AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer. *Cancer Res* 2005;65:4389-400.
180. Tao LY, Liang YJ, Wang F, Chen LM, Yan YY, Dai CL, Fu LW. Cediranib (recentin, AZD2171) reverses ABCB1- and ABCC1-mediated multidrug resistance by inhibition of their transport function. *Cancer Chemother Pharmacol* 2009;64:961-9.
181. Zheng L, Wang F, Li Y, Zhang X, Chen L, Liang Y, Dai C, Yan Y, Tao L, Mi Y, Yang A, To KKW, Fu L. Vandetanib (Zactima, ZD6474) antagonizes ABCC1- and ABCG2-mediated multidrug resistance by inhibition of their transport function. *PLoS One* 2009;4:e5172.
182. Mi Y, Lou L. ZD6474 reverses multidrug resistance by directly inhibiting the function of P-glycoprotein. *Br J Cancer* 2007;97:934-40.
183. Hegedus T, Orfi L, Seprodi A, Váradi A, Sarkadi B, Kéri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. *Biochim Biophys Acta* 2002;1587:318-25.
184. Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J Biol Chem* 1994;269:27807-10.
185. Minderman H, O'Loughlin KL, Pendyala L, Baer MR. VX-710 (Biricodar) increases drug retention and enhances chemosensitivity in resistant cells overexpressing P-glycoprotein, multidrug resistance protein, and breast cancer resistance protein. *Clin Cancer Res* 2004;10:1826-34.
186. Katayama R, Koike S, Sato S, Sugimoto Y, Tsuruo T, Fujita N. Dofequidar fumarate sensitizes cancer stem-like side population cells to chemotherapeutic drugs by inhibiting ABCG2/BCRP-mediated drug export. *Cancer Sci* 2009;100:2060-8.
187. Robey RW, Shukla S, Finley EM, Oldham RK, Barnett D, Ambudkar SV, Fojo T, Bates SE. Inhibition of P-glycoprotein (ABCB1)- and multidrug resistance-associated protein 1 (ABCC1)-mediated transport by the orally administered inhibitor, CBT-1®. *Biochem Pharmacol* 2008;75:1302-12.
188. Ji L, Yang S, Li S, Liu S, Tang S, Liu Z, Meng X, Yu S. A novel triazolonaphthalimide induces apoptosis and inhibits tumor growth by targeting DNA and DNA-associated processes. *Oncotarget* 2017;8:37394-408.
189. Saeki T, Nomizu T, Toi M, Ito Y, Noguchi S, Kobayashi T, Asaga T, Minami H, Yamamoto N, Aogi K, Ikeda T, Ohashi Y, Sato W, Tsuruo T. Dofequidar fumarate (MS-209) in combination with cyclophosphamide, doxorubicin, and fluorouracil for patients with advanced or recurrent breast cancer. *J Clin Oncol* 2007;25:411-7.
190. Cole SP. Multidrug resistance protein 1 (MRP1, ABCC1), a "multitasking" ATP-binding cassette (ABC) transporter. *J Biol Chem* 2014;289:30880-8.
191. Trompier D, Chang XB, Barattin R, du Moulinet D'Hardemare A, Di Pietro A, Baubichon-Cortay H. Verapamil and its derivative trigger apoptosis through glutathione extrusion by multidrug resistance protein MRP1. *Cancer Res* 2004;64:4950-6.
192. de Groot DJA, van der Deen M, Le TK, Regeling A, de Jong S, de Vries EG. Indomethacin induces apoptosis via a MRP1-dependent mechanism in doxorubicin-resistant small-cell lung cancer cells overexpressing MRP1. *Br J Cancer* 2007;97:1077-83.
193. Ahmed-Belkacem A, Pozza A, Macalou S, Pérez-Victoria JM, Boumendjel A, Di Pietro A. Inhibitors of cancer cell multidrug resistance mediated by breast cancer resistance protein (BCRP/ABCG2). *Anticancer Drugs* 2006;17:239-43.
194. Rabindran SK, He H, Singh M, Brown E, Collins KI, Annable T, Greenberger LM. Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res* 1998;58:5850-8.

195. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, Schinkel AH. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002;1:417-25.
196. Pick A, Klinkhammer W, Wiese M. Specific inhibitors of the breast cancer resistance protein (BCRP). *Chem Med Chem* 2010;5:1498- 505.
197. Peng H, Dong Z, Qi J, Yang Y, Liu Y, Li Z, Xu J, Zhang JT. A novel two mode-acting inhibitor of ABCG2-mediated multidrug transport and resistance in cancer chemotherapy. *PLoS One* 2009;4:e5676.
198. Komeili-Movahhed T, Fouladdel S, Barzegar E, Atashpour S, Hossein Ghahremani M, Nasser Ostad S, Madjd Z, Azizi E. PI3K/Akt inhibition and down-regulation of BCRP re-sensitize MCF7 breast cancer cell line to mitoxantrone chemotherapy. *Iran J Basic Med Sci* 2015;18:472-7.
199. Singh MS, Juvale K, Wiese M, Lamprecht A. Evaluation of dual P-gp-BCRP inhibitors as nanoparticle formulation. *Eur J Pharm Sci* 2015;77:1-8.
200. Li WQ, Li YM, Tao BB, Lu YC, Hu GH, Liu HM, He J, Xu Y, Yu HY. Downregulation of ABCG2 expression in glioblastoma cancer stem cells with miRNA-328 may decrease their chemoresistance. *Med Sci Monit* 2010;16:HY27-30.
201. Li X, Pan YZ, Seigel GM, Hu ZH, Huang M, Yu AM. Breast cancer resistance protein BCRP/ABCG2 regulatory microRNAs (hsa miR-328, -519c and -520h) and their differential expression in stem-like ABCG2+ cancer cells. *Biochem Pharmacol* 2011;81:783-92.
202. Turrini E, Haenisch S, Laechelt S, Diewock T, Bruhn O, Cascorbi I. MicroRNA profiling in K-562 cells under imatinib treatment: influence of miR-212 and miR-328 on ABCG2 expression. *Pharmacogenet Genomics* 2012;22:198-205.
203. Jiao X, Zhao L, Ma M, Bai X, He M, Yan Y, Wang Y, Chen Q, Zhao X, Zhou M, Cui Z, Zheng Z, Wang E, Wei M. MiR-181a enhances drug sensitivity in mitoxantone-resistant breast cancer cells by targeting breast cancer resistance protein (BCRP/ABCG2). *Breast Cancer Res Treat* 2013;139:717-30.
204. Ma MT, He M, Wang Y, Jiao XY, Zhao L, Bai XF, Yu ZJ, Wu HZ, Sun ML, Song ZG, Wei MJ. MiR-487a resensitizes mitoxantrone (MX)-resistant breast cancer cells (MCF-7/MX) to MX by targeting breast cancer resistance protein (BCRP/ABCG2). *Cancer Lett* 2013;339:107-15.
205. Wang Y, Zhao L, Xiao Q, Jiang L, He M, Bai X, Ma M, Jiao X, Wei M. miR-302a/b/c/d cooperatively inhibit BCRP expression to increase drug sensitivity in breast cancer cells. *Gynecol Oncol* 2016;141:592-601.
206. Zhang L, Guo X, Zhang D, Fan Y, Qin L, Dong S, Zhang L. Upregulated miR-132 in Lgr5+gastric cancer stem cell-like cells contributes to cisplatin-resistance via SIRT1/CREB/ABCG2 signaling pathway. *Mol Carcinog* 2017;56:2022-34.
207. Chen B, Zhang D, Kuai J, Cheng M, Fang X, Li G. Upregulation of miR-199a/b contributes to cisplatin resistance via Wnt/β-catenin ABCG2 signaling pathway in ALDHA1+colorectal cancer stem cells. *Tumour Biol* 2017;39:1010428317715155.
208. Shukla S, Chen ZS, Ambudkar SV. Tyrosine kinase inhibitors as modulators of ABC transporter-mediated drug resistance. *Drug Resist Updat* 2012;15:70-80.
209. Azzariti A, Porcelli L, Simone GM, Quatrale AE, Colabufo NA, Berardi F, Perrone R, Zucchetti M, D'Incalci M, Xu JM, Paradiso A. Tyrosine kinase inhibitors and multidrug resistance proteins: interactions and biological consequences. *Cancer Chemother Pharmacol* 2010;65:335-46.
210. Ozvegy-Laczka C, Hegedus T, Várady G, Ujhelly O, Schuetz JD, Váradi A, Kéri G, Orfi L, Német K, Sarkadi B. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. *Mol Pharmacol* 2004;65:1485-95.
211. Veronese ML, Sun W, Giantonio B, Berlin J, Shults J, Davis L, Haller DG, O'Dwyer PJ. A phase II trial of gefitinib with 5-fluorouracil, leucovorin, and irinotecan in patients with colorectal cancer. *Br J Cancer* 2005;92:1846-9.
212. Deenik W, Janssen JJ, van der Holt B, Verhoef GE, Smit WM, Kersten MJ, Daenen SM, Verdonck LF, Ferrant A, Schattenberg AV, Sonneveld P, van Marwijk Kooy M, Wittebol S, Willemze R, Wijermans PW, Beverloo HB, Löwenberg B, Valk PJ, Ossenkoppele GJ, Cornelissen JJ. Efficacy of escalated imatinib combined with cytarabine in newly diagnosed patients with chronic myeloid leukemia. *Haematologica* 2010;95:914-21.
213. Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP, Baselga J. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. *J Clin Oncol* 2003;21:2237-46.

214. Giaccone G, González-Larriba JL, van Oosterom AT, Alfonso R, Smit EF, Martens M, Peters GJ, van der Vijgh WJF, Smith R, Averbuch S, Fandi A. Combination therapy with gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, gemcitabine and cisplatin in patients with advanced solid tumors. *Ann Oncol* 2004;15:831-8.
215. Stewart CF, Leggas M, Schuetz JD, Panetta JC, Cheshire PJ, Peterson J, Daw N, Jenkins JJ, Gilbertson R, Germain GS, Harwood FC, Houghton PJ. Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. *Cancer Res* 2004;64:7491-9.
216. Leggas M, Panetta JC, Zhuang Y, Schuetz JD, Johnston B, Bai F, Sorrentino B, Zhou S, Houghton PJ, Stewart CF. Gefitinib modulates the function of multiple ATP-binding cassette transporters in vivo. *Cancer Res* 2006;66:4802-7.
217. Nishino H. Cancer prevention by carotenoids. *Mutat Res* 1998;402:159-63.
218. Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SP. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and atpase activities by interaction with dietary flavonoids. *Mol Pharmacol* 2001;59:1171-80.
219. Alvarez AI, Real R, Pérez M, Mendoza G, Prieto JG, Merino G. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. *J Pharm Sci* 2010;99:598-617.
220. Cooray HC, Janvilisri T, van Veen HW, Hladky SB, Barrand MA. Interaction of the breast cancer resistance protein with plant polyphenols. *Biochem Biophys Res Commun* 2004;317:269-75.
221. Pan L, Hu H, Wang X, Yu L, Jiang H, Chen J, Lou Y, Zeng S. Inhibitory effects of neochamaejasmin B on P-glycoprotein in MDCK hMDR1 cells and molecular docking of NCB binding in P-glycoprotein. *Molecules* 2015;20:2931-48.
222. Forester SC, Waterhouse AL. Metabolites are key to understanding health effects of wine polyphenolics. *J Nutr* 2009;139:S1824-31. 223
223. Bohn T, McDougall GJ, Alegría A, Alminger M, Arrigoni E, Aura AM, Brito C, Cilla A, El SN, Karakaya S, Martínez-Cuesta MC, Santos CN. Mind the gap-deficits in our knowledge of aspects impacting the bioavailability of phytochemicals and their metabolites--a position paper focusing on carotenoids and polyphenols. *Mol Nutr Food Res* 2015;59:1307-23.

CHAPTER 4

Ex vivo resistance in childhood acute lymphoblastic leukemia: correlations between BCRP, MRP1, MRP4 and MRP5 ABC transporter expression and intracellular methotrexate polyglutamate accumulation

Leukemia Research. 79:45-51; 2019

Adrian C. Jaramillo[1,2], Jacqueline Cloos[2,3], Clara Lemos[4], Ronald W. Stam[5], Gertjan J.L. Kaspers[3,5], Gerrit Jansen[6], Godefridus J. Peters[1]

[1] Department of Medical Oncology, Amsterdam UMC, Amsterdam, The Netherlands

[2] Department of Hematology, Amsterdam UMC, Amsterdam, The Netherlands

[3] Department of Pediatric Oncology/Hematology, Amsterdam UMC, Amsterdam, The Netherlands

[4] Bayer Pharmaceuticals, Berlin, Germany

[5] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands

[6] Amsterdam Rheumatology and Immunology Center, Amsterdam UMC, Amsterdam, The Netherlands

Abstract

Chemoresistance is an important factor in the treatment failure of childhood acute lymphoblastic leukemia (ALL). One underlying mechanism of chemoresistance involves (over)expression of ATP-dependent drug efflux transporters such as multidrug resistance protein 1-5 (MRP1-5) and breast cancer resistance protein (BCRP), which can extrude the important antileukemia drug methotrexate (MTX). Survival of childhood ALL critically depends on the leukemic blasts' capacity for intracellular retention of MTX and MTX-polyglutamates.

This pilot study assessed whether expression of MRP1, MRP4, MRP5 and BCRP (real-time PCR) in primary childhood ALL blasts (n=23) correlated with ex vivo resistance to MTX (assayed by in situ thymidylate synthase inhibition assay (TSIA)), ex vivo accumulation of (radioactive) MTX polyglutamates, and patient survival. Results show that high MRP4 expression is correlated with ex vivo MTX resistance assayed by TSIA (P=0.01). Moreover, elevated MRP4 and BCRP expression correlated with lower accumulation of MTX-PGs (P= 0.004 and P= 0.03, respectively). Combined high expression of BCRP and MRP4 even further impacted reduced MTX-PG accumulation (P= 0.02). Overall survival was lower (P logrank= 0.04) in children with ALL cells which featured a relatively high expression of both BCRP and MRP4 transporters. These results underscore the impact of high drug efflux transporter expression, notably MRP4 and BCRP, in diminished MTX response in childhood ALL.

Abbreviations

Acute lymphoblastic leukemia (ALL), folylpolyglutamate synthetase (FPGS), multidrug resistance protein 1-5 (MRP1-5), breast cancer resistance protein (BCRP), methotrexate (MTX), methotrexate polyglutamates (MTX-PG), thymidylate synthase (TS), thymidylate synthase inhibition assay (TSIA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent type of childhood malignancy [1]. Current treatment protocols are successful in achieving 5 years overall survival rates of 83-94% [2]. However, approximately 20% of children with ALL will relapse and face a poor survival (30-50%) [3]. One important factor contributing to relapse is the onset of multi-drug resistance (MDR) [4] , mediated by the upregulation of specific drug efflux transporters of the ATP-binding cassette (ABC) transporter family [4-7]. Active extrusion of cytotoxins by ABC transporters results in lower intracellular concentration of chemotherapeutics and confers chemoresistance.

Methotrexate (MTX) is the most widely used anchor drug for central nervous system prophylaxis and in the maintenance therapy for childhood ALL, but emergence of resistance to MTX remains an issue of concern for which several mechanisms have been hold accountable [8, 9]. With respect to ABC transporters, multidrug resistance protein 1-5 (MRP1-5, ABCC1-5) and breast cancer resistance protein (BCRP,

ABCG2) have been implicated in cellular extrusion of MTX, thus constituting a critical factor in the intracellular accumulation of MTX [10-14]. Intracellular accumulation of MTX and its active metabolites, i.e. MTX-polyglutamates (MTX-PGs), is crucial for treatment efficacy in leukemia, as it correlates with event-free survival (EFS) [15-19]. Upon intracellular entry of MTX via the reduced folate carrier (RFC), MTX is metabolized into PG forms via the enzyme folylpolyglutamate synthetase (FPGS) which adds 2-6 glutamate residues to the γ-carboxyl group of MTX [20]. These polyanionic active metabolites are prevented from efflux by ABC transporters [10-14] although BCRP has the ability to extrude short chain polyglutamates (up to MTX-PG3) [11, 21]. *Ex vivo* assessments of MTX sensitivity in primary leukemia samples by commonly used MTT-based cell growth inhibition assays have been hampered by the release of nucleosides from dying cells which rescue leukemic cells from the effects of MTX [22, 23]. To this end, alternative methods based on *in situ* inhibition of TS by MTX-(PGs) have proved useful to distinguish variable MTX sensitivity profiles in primary leukemia samples [23-25]. By examining short-term (3 hrs) versus long-term (21 hrs) MTX drug exposure periods, the *in situ* TS inhibition assay (TSIA) provides insights in the kinetics and dynamics of MTX-PG formation reflected by the capacity to inhibit TS [24, 26], which can be complemented with actual analysis of MTX-PG levels [16, 19, 23].

In the present pilot-study, we explored the association between the *ex vivo* MTX drug sensitivity by TSIA, gene expression of ABC transporters *MRP1, 4, 5* and *BCRP*, and intracellular MTX-PG accumulation with survival in a small cohort of newly diagnosed childhood ALL patients.

2. Materials & Methods:

2.1 Reagents:

RNAzol was purchased from Campro Scientific (Veenendaal, The Netherlands); for reverse transcriptase, Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) and RNase inhibitor were obtained from Promega (Madison, WI, USA). Deoxyribonucleotides (dNTPs), random hexamers and Taq polymerase (5 IU/µl) were obtained from Pharmacia Biotech (Roosendaal, The Netherlands). RESponse Research agarose was purchased from Biozym (Landgraaf, The Netherlands). [3,5,7-[^{3}H]-MTX (20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA).

2.2 Patient samples:

Diagnostic samples were collected from children with B-cell precursor ALL (n=17) and T cell-ALL (n=6) after obtaining informed consent. Patients were enrolled in ALL-8 and ALL-9 study protocols [16], coordinated by the Dutch Childhood Oncology Group. Induction and consolidation regimes with MTX were comparable among the different treatment groups. A detailed description of MTX drug schedules and doses has been reported elsewhere [27]. A description of the patient demographics is presented in Table 1. Because the various assays required relatively large amounts of cells, sample

selection was based on availability white blood cell (WBC) concentration in primary diagnostic bone marrow (BM) or peripheral blood (PB) from patients.

Mononuclear cells were isolated through Ficoll-Paque density gradient centrifugation, following manufacturer's recommendations, within 24 h after collection essentially as described by Rots et al [23]. Samples with <80% leukemic cells were enriched for blasts by removing non-leukemic cells using magnetic bead bound monoclonal antibodies (Dynabeads M-450; Dynal Inc, Oslo, Norway) as described elsewhere [28]. Samples were washed twice with RPMI-1640 medium +2% fetal calf serum (FCS) and resuspended in culture medium consisting of RPMI-1640 (Dutch modification; GIBCO, Uxbridge, UK) supplemented with 20% FCS and antibiotics (all obtained from Flow Laboratories, Irvine, UK). For RNA isolation, 5 x 10^6 cells were lyzed in 1 mL RNAzol and stored at -80°C.

Table 1: Patient Characteristics

	B-cell precursor	T-cell ALL	Total cases
Sample size	18	5	23
Median age (years)	6.1 (0-14)	6.4 (1-11)	6.1
Sex (male/female)	11/7	3/2	14/9
Treatment Protocol:			
ALL 8, 9	10	4	14
Other	3	1	4
N/A	5		5
Relapse rate	8 (44%)	2 (40%)	1 (43%)
Complete remission	10 (56%)	2 (40%)	12 (52%)

2.3 mRNA Extraction and Real Time-PCR:

For RNA extraction procedure and Real Time-PCR methods, please be referred to the supplementary material section.

2.4 Ex vivo drug sensitivity:

Sensitivity of primary leukemia cells was assessed *ex vivo* by the *in situ* TSIA which measures the amount of tritiated water (^3H_2O) released from the conversion of [5-^{3}H]-2'-deoxycytidine to [5-^{3}H]-2'-deoxyuridine monophosphate (^{3}H-dUMP) to deoxythymidine monophosphate (dTMP) in the reaction catalyzed by TS. MTX, and in particular its polyglutamate forms, can effectively inhibit dTMP synthesis and thus block the release of tritiated water from [5-^{3}H]-2'-deoxycytidine. The TSIA has been used previously as a validated tool for *ex vivo* assessment of (inhibition of) intracellular

TS activity in primary cells and cell lines [24-26]. Moreover, TSIA enabled generation of dose-response curves and detected differential MTX sensitivity patterns in T-cell versus common/pre-B cell ALL patient-derived cells, in accordance to clinical observation in patients [23, 29]. To examine the impact of MTX-polyglutamylation, leukemic blast cells were exposed to MTX short-term, i.e. 3 hours followed by an 18 hours drug-free period, or continuously for 21 hours, after which drug sensitivity was evaluated by calculation of the concentration of the drug needed to inhibit 50% of the TS activity, depicted as $TSI_{50,short}$ and $TSI_{50,cont}$, respectively [23].

2.5 MTX accumulation and polyglutamylation analysis.

For this analysis 10^7 cells in 10 ml RPMI-1640 medium supplemented with 10% FCS were incubated for 24 hours at 37ºC with 1 µM [^{3}H]-MTX (2 Ci/mmol). After this period, cells were washed three times with 10 mL of ice-cold phosphate-buffered saline (PBS) and after cell counting and determining total accumulated [^{3}H]-MTX, analysis of intracellular distribution of [^{3}H]-MTX-PGs was performed by high-performance liquid chromatography (HPLC) coupled to a radioactivity detector, as described elsewhere [16, 23].

2.6 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

For assessment of drug sensitivity of drugs other than MTX, the MTT colorimetric assay was performed on cells suspended in 96-well round bottom plates at a concentration of 2 x 10^6 cells/ml in RPMI-1640 cell culture medium containing 20% FCS, 200 µg/ml of gentamycin, 2 mM glutamine, and 0.5ml ITS (insulin: 5µg/ml, transferrin: 5µg/ml, sodium selenite: 5ng/ml) [22]. Treatment wells contained 80 µl of cell suspension and 20 µl of the drugs of interest (concentration ranges are indicated in supplementary Table A.1). Control wells contained 20 µl of RPMI-1640 medium and 80 µl of cell suspension, while blank controls had 100 µl of RPMI-1640 medium. MTT assay was performed in duplicates and plates were incubated at 37°C, in a humidified 5% CO_2 incubator for 4 days. At the end of the incubation period, 10 µl of MTT dye (5 mg/ml) was added to the each well and placed in the 37ºC incubator for an additional 6 hours, followed by the addition of 100 µl isopropanol. After 5 minutes, plates the microplate optical density (OD) was measured at 562 nm. For the analysis, the average OD of the blank wells is subtracted from the average OD of the control wells or the treatment wells. The lethal concentration 50 (LC_{50}) was then determined by calculating cell survival with the following equation [22]: mean OD treatment well (minus blank)/mean OD control well (minus blank) x100.

2.7 Statistical analysis

The Mann-Whitney *U* test was used to analyze differences in the subtypes of childhood ALL (common/B-cell ALL vs. T-ALL, diagnosis vs. remission). The Spearman rank correlation test was used to determine associations between mRNA expression (of MPR1, MPR4, MRP5 and BCRP) with *ex vivo* sensitivity, and to detect

correlations between the mRNA expression-drug sensitivity ratios. Significance level was set at two-sided P value of <0.05. For survival analysis, patients were classified as therapy responders when complete remission (CR) was achieved. The time between the initial treatment date until the end point (death or censoring) was considered as the overall survival (OS). Kaplan-Meier analysis was used to plot OS, and the log-rank test to compare differences between survival curves. Statistical calculation was performed with SPSS software (SPSS, Inc., IL, USA) and GraphPad Prism version 7.0 for Windows (GraphPad Software, CA, USA).

3. Results

3.1 *Patient samples*

Patients were enrolled in several treatment protocols which are listed in Table 1 along with the outcome data, remission rate and relapse rate. Due to logistic reasons and leukemic blast number limitations, not all of the MTX-related parameters could be measured and therefore the number of cases included in the statistical analysis varied between parameters (as listed in the various tables).

3.2 ABC transporter expression in childhood leukemia:

Notwithstanding inter-patient variabilities, mean ABC transporter (*MRP1, MRP4, MRP5* and *BCRP*) expression values did not vary significantly amongst childhood precursor-B cell (n= 18) and T-cell (n= 5) ALL samples. These expressions were also not different between relapsing (n= 10) and non-relapsing patients (n= 11), regardless of ALL subtypes (Figure 1A and 1B). Due to the limited cytogenetic/molecular data available, further risk stratification could not be performed in relation to ABC transporter expression. Spearman rank analysis was performed to detect a pattern of correlated expression amongst ABC transporters in all leukemia samples (n=23). Significant correlations were found between *BCRP* and MRP1 expression (R = 0.44; *P* = 0.035), *BCRP/MRP4* (R = 0.53; *P* = 0.009), *BCRP/MRP5* (R= 0.42; *P* = 0.047) and *MRP5/MRP1* (R=0.51 *P* = 0.013). Based on these observations, we analyzed the intracellular MTX-PG accumulation, *ex vivo* MTX resistance, and survival of patients with a combination of high or low expression of drug efflux transporters.

3.3 Association of intracellular accumulation of MTX polyglutamates and ABC transporter expression ex vivo.

To examine the impact of ABC transporter expression on *ex vivo* MTX-PG accumulation in the cohort of ALL patients (n= 20), we quantified the intracellular accumulation of total MTX-PGs (1–6 glutamate residues) and long-chain MTX-PGs (4–6 glutamate residues) after 24-hour exposure to 1 µM [^{3}H]-MTX (Figure 2 and supplementary Table A2 and A3). High *BCRP* and *MRP4* expression correlated with significantly lower accumulation of total MTX-PGs (MTX-Glu1-6) (*P*= 0.027 and 0.004, respectively, Supplementary Table A.2). Significantly lower accumulation of long chain

MTX-PGs (MTX-Glu4-6) correlated with higher *MRP4* expression (*P*= 0.003) and trended with higher *BCRP* expression (*P*= 0.064, supplementary Table A.3). Spearman rank correlation analysis revealed that *BCRP* and *MRP4* mRNA expression significantly inversely correlated with total MTX-PGs (*P* = 0.04 and 0.02, respectively) (Table 2). In addition, high *MRP4* expression was significantly associated with lower intracellular concentration of long-chain MTX-PGs (*P* = 0.01, Table 2). From all combined correlation analysis, the most significant effect in total and long-chain MTX-PG accumulation was seen with the *MRP4* high /*BCRP* high group (*P*= 0.005). These results concord with previous published results that confirm correlations between transporter mRNA and its correspondent protein levels[30, 31].

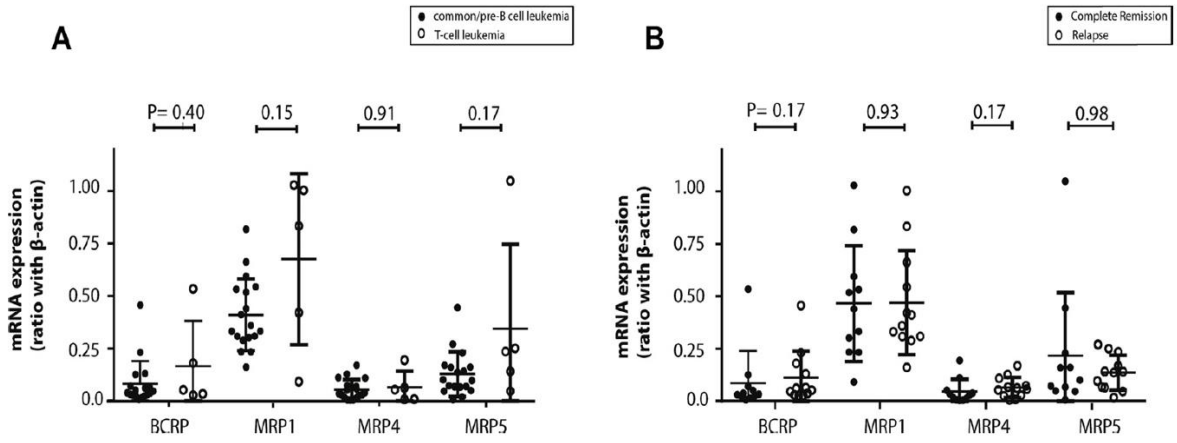


Fig. 1. *(A): mRNA expression levels (relative to β-actin) of BCRP, MRP1, MRP4 and MRP5 in common/pre-B cell (closed symbols) and T-cell childhood leukemia cells (open symbols). (B): mRNA expression levels (relative to β-actin) of BCRP, MRP1, MRP4 and MRP5 in patients who underwent relapse (open symbols) compared to patients who remained in complete remission (CR) during treatment (closed symbols). Each symbol represents an individual patient. Data is depicted as mean ± standard deviation. The median mRNA expression level for ABC transporters relative to the mRNA expression of β-actin was used as cut-off point [BCRP (0.04), MRP1 (0.38), MRP4 (0.04) and MRP5 (0.14)] to differentiate between high and low expression of ABC transporters. Mann Whitney U test was performed to analyze differences between means.*

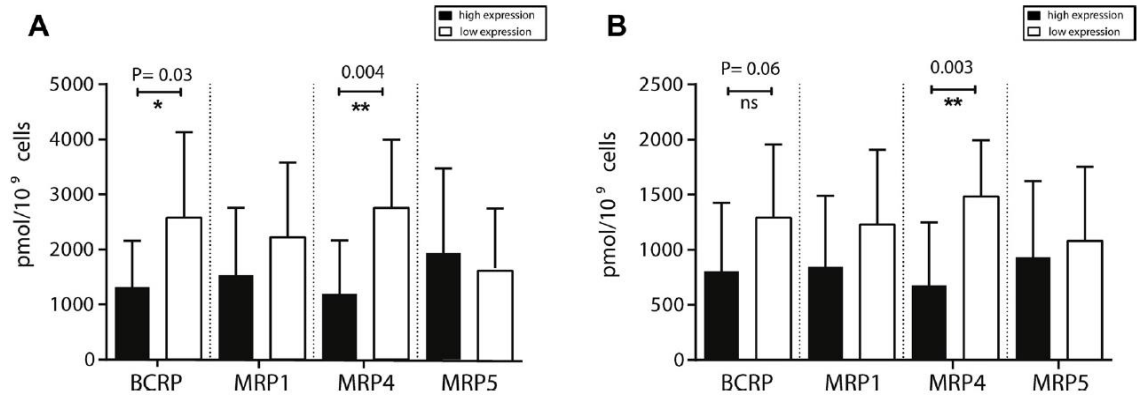


Fig. 2. *(A): Total MTX-PG accumulation and (B) long-chain MTX-PG accumulation in childhood ALL samples with high (black bars) or low (white bars) expression of BCRP, MRP1, MRP4 and MRP5 ABC transporters. Data are depicted as mean ± standard deviation (SD) and expressed as pmol/10^9 cells. Mann Whitney U test was used to analyze differences between means.*

Table 2: Correlation between MTX-PG accumulation and BCRP and MRP4 expression.

(n = 20)	Total MTX-PG	LC MTX-PG
BCRP	-0.5; *P* = 0.04*	-0.4; *P* = 0.1
MRP4	-0.5; *P* = 0.02*	-0.6; *P* = 0.01*

*P- value (P), total (total MTX-PG) and long-chain methotrexate polyglutamates (LC MTX-PG). *= statistically significant (Spearman's correlation coefficient).*

3.4 ABC transporter expression and cellular MTX sensitivity ex vivo

The TSIA has previously served as a suitable method to generate MTX-dose response curves in intact primary leukemia cells. TSIA data available from a former study were used here to correlate MTX sensitivity to ABC transporter expression in childhood ALL specimens [23]. The short-term, 3-hour MTX exposure ($TSI_{50,short}$) and the continuous 21-hour exposure ($TSI_{50,cont}$) are representative of the leukemic cells' capacity to efflux and to accumulate MTX, respectively. These parameters can be used as indirect indicators of the polyglutamylation capacity of leukemic cells and reliably reflect MTX sensitivity *ex vivo,* as shown previously [23].

Mann-Whitney U analysis between high and low expression of drug efflux transporters *MRP1, MRP4, MRP5* and *BCRP* showed that high *MRP4* expression is significantly related to diminished MTX sensitivity in both $TSI_{50,cont}$ (*P* = 0.01) and $TSI_{50,short}$ (*P* = 0.05). Notably, an elevated BCRP expression was not significantly related to MTX efflux (*P* = 0.08) but was related to MTX accumulation, as shown by the $TSI_{50,cont}$ exposure (*P* = 0.01, Figure 3A and 3B).

Next, the impact on MTX resistance was evaluated in relation to all possible paired combinations of ABC transporter expression values. In these analyses, higher LC_{50} values (i.e. increased resistance) were observed in $TSI_{50,cont}$ (*P* = 0.02) and $TSI_{50,short}$ (*P* = 0.03) in leukemia samples with *BCRP*-high/*MRP4*-high expression when compared with *BCRP*-low/*MRP4*-low cells (Figure 3C and 3D).

3.5 Relation between ABC-transporter expression and the sensitivity to other chemotherapeutics

The sensitivity of cells for anti-leukemic drugs other than MTX was measured using MTT analysis. Remarkably, the strong relation between *MRP4* and *BCRP* expression and sensitivity to MTX was not as pronounced for the other anti-leukemic drugs (Table 3). Increased *MRP1* expression showed a significant relationship to resistance to numerous drugs, such as mitoxantrone (*P* = 0.026), doxorubicin (*P*= 0.002), vincristine (*P*= < 0.001), daunorubicin (*P*=< 0.001), teniposide (*P*= 0.001), thiotepa (*P*= 0.033) and cladribine (*P*= 0.049). MRP4 expression only showed a significant relationship to cladribine resistance (*P*= 0.049), while *MRP5* expression was related to thiotepa resistance (*P*= 0.033) and showed a tendency to teniposide resistance (*P*= 0.052). *BCRP* expression was not related to drug sensitivity in the MTT assay (Table 3).

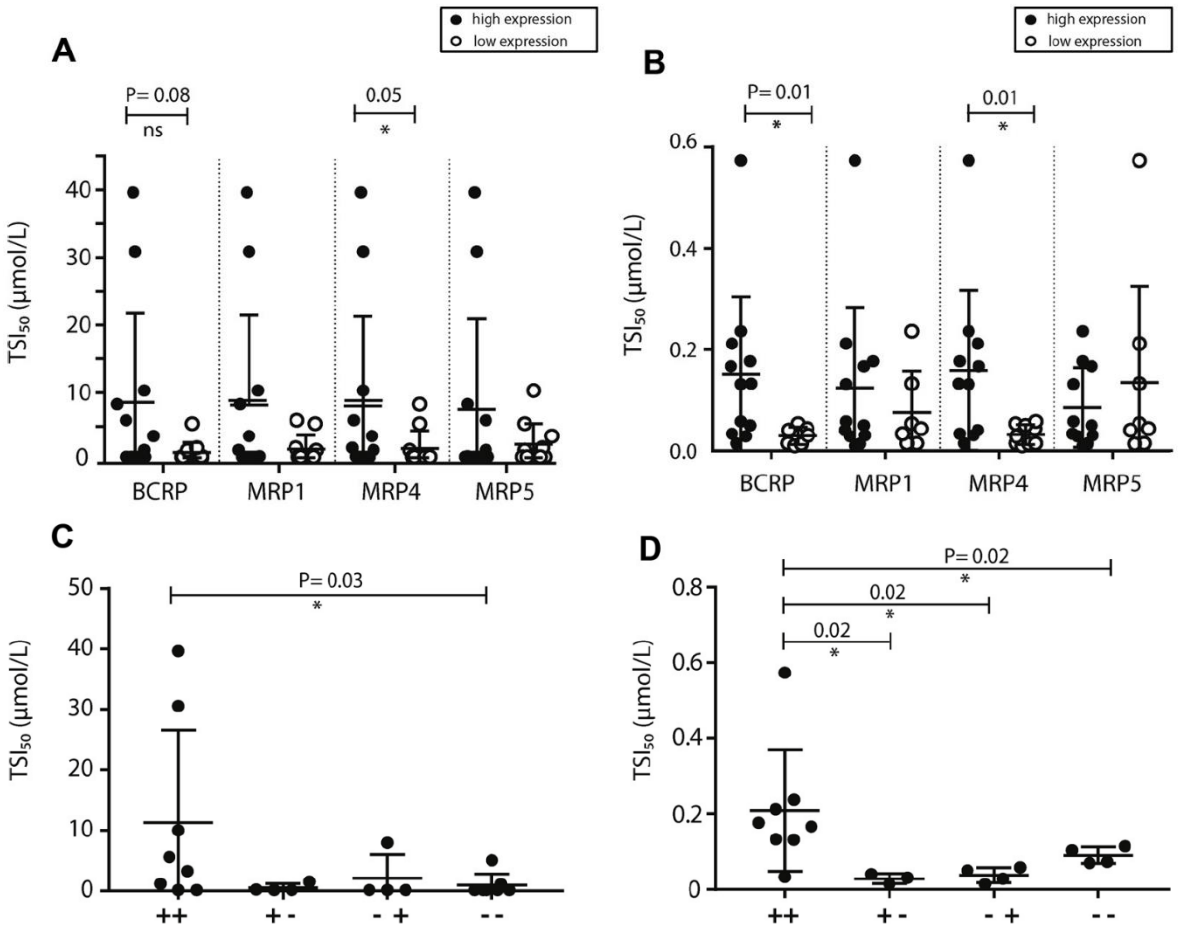


Fig. 3. *MTX sensitivity and ABC transporter expression in childhood ALL samples as represented by the (A) short term (3 h) MTX exposure and (B) continuous (21 h) MTX exposure in the TSIA assay. ABC transporters expression is divided in patient groups with either high (black bars) or low (white bars) expression. TSIA results are represented as TSI_{50} concentration, the concentration of MTX needed to inhibit 50% of thymidylate synthase (TS) activity. (C) TSI_{50} values following the short-term (3-hr) exposure to MTX and (D) continuous (21 h) MTX exposure for patients with MRP4/BCRP double high (++), high/low (+ -) low/high (- +) and low/low (- -) expression. Data are depicted as mean ± SD and expressed as µmol/L. Mann Whitney U test was performed to analyze differences between means.*

3.6 ABC transporter expression and survival

Despite the small sample number, the influence of ABC transporter expression on survival was examined by Kaplan Meier analysis. A relatively poor outcome was observed for patients with elevated *BCRP* expression compared to low expression, which did not reach statistical significance ($P_{logrank}$= 0.30; Figure 4A). Notably, the combined expression of *BCRP* high/*MRP4* high had a negative impact on OS ($P_{logrank}$ = 0.04; Figure 4B) compared with the rest of *BCRP/MRP4* combinations together. Due to the relatively small number of samples and the fact that treatment regimens were comparable, further stratified survival analysis was not performed.

Table 3: Correlates of antileukemic drug sensitivity[a] and ABC transporter expression[b].

DRUG		MRP1		MRP4		MRP5		BCRP	
	n	rs	*P*-value	rs	*P*-value	rs	*P*-value	rs	*P*-value
Asparginase	17	−0.24	0.347	−0.19	0.467	−0.22	0.399	−0.49	0.356
Carboplatin	4	−0.40	0.750	−0.40	0.750	−0.80	0.333	−0.80	0.333
Cisplatin	5	−0.70	0.233	−0.60	0.350	−0.70	0.233	−0.30	0.683
Cladribine	10	−0.65	0.049*	−0.67	0.039*	−0.61	0.067	−0.07	0.865
Cytarabine	16	−0.33	0.213	−0.34	0.200	−0.10	0.704	−0.11	0.680
Daunorubicin	16	−0.78	0.00*	−0.33	0.189	−0.24	0.362	−0.28	0.284
Dexamethasone	16	−0.43	0.100	−0.19	0.471	−0.19	0.489	−0.08	0.768
Doxorubicin	14	−0.73	0.002*	−0.28	0.334	−0.17	0.553	−0.12	0.692
Etoposide	12	−0.52	0.080	−0.22	0.485	−0.08	0.795	−0.22	0.484
Ifosfamide	16	−0.02	0.940	0.08	0.770	−0.07	0.787	−0.09	0.754
Mitoxantrone	11	−0.66	0.026*	−0.18	0.593	−0.15	0.670	−0.03	0.937
Prednisolone	15	−0.39	0.151	0.24	0.398	−0.11	0.685	−0.17	0.541
Teniposide	15	−0.78	0.001*	−0.47	0.076	−0.51	0.052	−0.16	0.576
Thiotepa	6	−0.89	0.033*	−0.77	0.103	−0.89	0.033*	−0.49	0.356
Vincristine	17	−0.78	0.00*	−0.33	0.189	−0.24	0.362	−0.28	0.284
6-Mercaptopurine	16	−0.30	0.264	0.30	0.261	−0.12	0.652	−0.22	0.415
6-Thioguanine	14	−0.18	0.537	0.25	0.396	0.03	0.917	−0.04	0.905

*a= as determined by MTT assay b= as determined by real-time PCR. *= statistically significant (Spearman' s correlation coefficient).*

4. Discussion

This study shows that ABC drug efflux transporters MRP4/BCRP contribute to MTX accumulation and polyglutamylation, and the clinical outcome data suggest an association with response to treatment including MTX in pediatric ALL patients.

Intracellular accumulation of MTX and its conversion to MTX-PGs is well recognized as a predictive factor for response and favorable outcome in childhood leukemia [15-19]. Consequently, deficient MTX-polyglutamylation due to loss of FPGS activity has been associated with MTX resistance [16, 20, 32-34] as non-polyglutamated MTX is prone to be extruded from cells by several family members of the ABC, including MRP1-5 [12-14] and BCRP [10, 11, 21], rather than by P-gp which effluxes primarily non-hydrophilic substrates [10, 14]. To fully unravel the role of MTX polyglutamylation in *ex vivo* assessments of MTX sensitivity in leukemic specimens, short incubation periods (e.g. 3 hrs) are more informative than extended (72-96 hrs) incubation periods [23-25, 29]. Moreover, *ex vivo* testing of leukemia specimens for MTX sensitivity is hampered by the release of nucleosides and bases from dying cells which rescue the cytotoxic effect of MTX [23]. In the present study, these considerations and limitations were met by applying the *in situ* TSIA for MTX in a time and MTX-PG dependent manner. Additional analysis of RNA expression levels of *MRP1, MRP4, MRP5* and *BCRP* mRNA levels, and analysis of (long-chain) MTX-PG accumulation, and outcome allowed assessments of correlating these parameters with *ex vivo* MTX sensitivity.

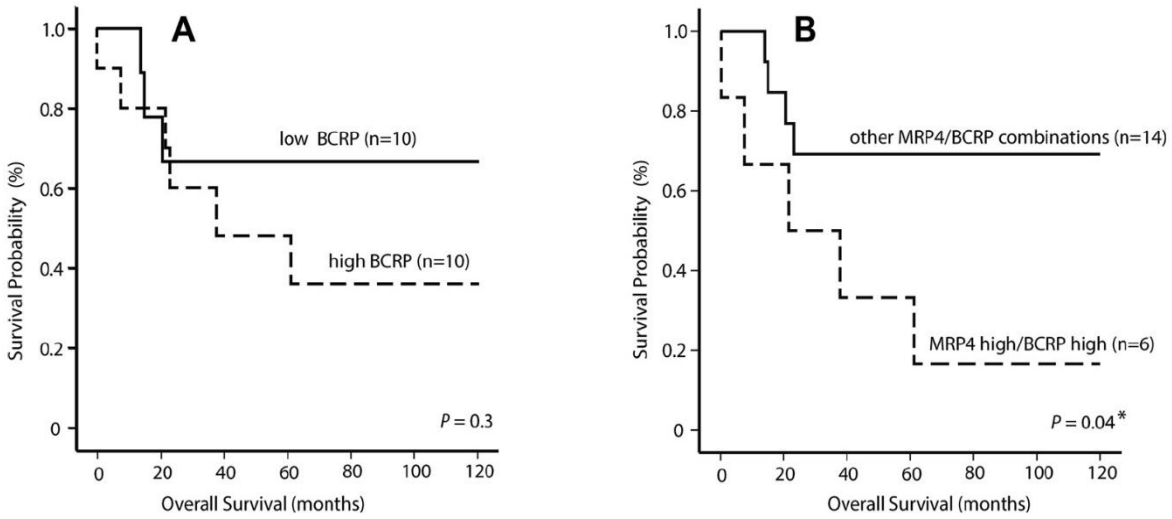


Fig. 4. *(A): Kaplan-Meier analysis of BCRP high (dashed line) and BCRP low (continuous line) expression and overall survival (OS) in childhood ALL. (B): Kaplan-Meier analysis of the combined expression of MRP4/BCRP and OS in childhood ALL. MRP4/BCRP survival curves are labeled with the mean OS estimate (in months), the percentage of censored patients and the number of patients in each subgroup. The continuous line in Fig. 4B represents the Kaplan-Meier OS curve of patients with low/high, high/low, low/ low combinations of MRP4 and BCRP expression, the dashed line represents the MRP4 high/BCRP high group of patients. Statistical evaluation is expressed as P-values analyzed by logrank test.*

A considerable inter-patient variability of *MRP1, MRP4, MRP5* and *BCRP* mRNA expression levels was observed in childhood ALL samples (Figure 1A), being consistent with previously reported data [31, 35-39]. In normal peripheral blood lymphocytes, MRP4 and BCRP expression is rather low as compared to other drug efflux transporters [40, 41]. Elevated expression levels of *MRPs* and *BCRP* in leukemia cells have been reported of clinical relevance and associated with worsened prognosis and relapse of treatment [38, 39, 42-44]. However, various studies employing 3-4 days MTT-based drug testing did not reveal significant correlations with *MRPs* and *BCRP* expression levels in childhood ALL cells and *in vitro* drug resistance to drugs being bona fide substrates for MRPs and BCRP [37, 42, 45, 46]. Of note, these drug testing studies did not include MTX. In the current study we show that *MRP1* expression in childhood ALL cells was associated with in MTT-*in vitro* drug resistance to known MRP1 substrates doxorubicin, daunorubicin, vincristine and mitoxantrone, but not for drugs being substrates for BCRP [7] and MRP4/5 (e.g. thiopurines and cytarabine) [13, 47]. The latter may be explained by the fact that in long term drug testing conditions these prodrugs are converted to their phosphorylated metabolites which are not effluxed by MRP4/5. Previous studies reported monitoring of intracellular accumulation of daunorubicin [48], Ara-CTP [47] and thioguanine nucleotides [49], but sample size limitations in the present study did not allow us to assess intracellular accumulations of drugs other than MTX.

Many in vitro cell lines studies underscored that overexpression of *MRP1-5* and *BCRP* confers MTX resistance after short term drug exposure during which time MTX has not been sufficiently converted to long chain polyglutamates which lack substrate affinity for MRPs and BCRP [11, 21]. Additionally, decreased RFC mediated MTX uptake or diminished FPGS activity can impact to reduced polyglutamylation, facilitating extrusion of non-polyglutamated MTX by MRPs and even short chain MTX-PGs by BCRP [10]. The current study is the first to corroborate that lowered ex vivo accumulation of MTX-PGs in childhood ALL cells is significantly correlated with increased expression of *MRP4 and BCRP*, and *in vitro* MTX drug resistance determined by TSIA. Moreover, combined $MRP4^{high}/BCRP^{high}$ expression in childhood ALL samples proved to be a good indicator of worsened clinical outcome as compared to patients with $MRP4^{low}/BCRP^{low}$ expression. Earlier studies by Bartholomae et al. [50] also showed that in childhood AML dual high expression of *BCRP* and *MRP3* was strongly associated with therapy response, underscoring the impact of coexpression of multiple MDR transporters in drug resistance. Although this study investigated multiple parameters contributing to MTX resistance, one limitation is its small sample size. Confirmatory studies with greater number of patients are warranted and should include transporter protein detection (e.g. with flow cytometry), follow-up at different time points and cytogenetic/molecular phenotypes. Suggested additional analysis may include polymorphic variants of BCRP and MRP4, which impact their functional activity and were implicated in unfavorable clinical outcome [51-55]. Interestingly for MRP4, a specific polymorphic variant was associated with aberrant splicing of *MRP4* pre-mRNA [54] and thus expression. Consistently, recent studies by Wojtuszkiewicz et al [19, 53] identified aberrant pre-mRNA splicing of FPGS in childhood ALL as a molecular basis of reduced FPGS activity and consequently diminished MTX-

polyglutamylation. Altogether, a combined approach of selected ABC transporter gene expression analysis, assessment of aberrant FPGS pre-mRNA splicing, MTX-PG analysis and TSIA-based MTX drug sensitivity monitoring could guide a more personalized medicine of MTX in childhood ALL patients.

5. Conclusions

The thymidylate synthase inhibition assay (TSIA) proved a valuable tool to screen for MTX drug resistance in primary childhood ALL cells. ABC transporters BCRP and MRP4 were identified as determinants associated with reduced MTX accumulation and polyglutamylation, potentially impacting outcome of childhood ALL patients.

References

1 T. Terwilliger, M. Abdul-Hay. Acute lymphoblastic leukemia: a comprehensive review and 2017 update, Blood Cancer J 7(6) (2017) e577

2 C.H. Pui, W.L. Carroll, S. Meshinchi, R.J. Arceci. Biology, risk stratification, and therapy of pediatric acute leukemias: an update, J Clin Oncol 29(5) (2011) 551-565

3 S.P. Hunger, C.G. Mullighan. Acute Lymphoblastic Leukemia in Children, N Engl J Med 373(16) (2015) 1541-1552

4 W. Li, H. Zhang, Y.G. Assaraf, K. Zhao, X. Xu, J. Xie, et al. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies, Drug Resist Updat 27 (2016) 14-29

5 A.C. Jaramillo, F.A. Saig, J. Cloos, G. Jansen, G.J. Peters. How to overcome ATP-binding casette drug efflux transporter-mediated drug resistance?, Cancer Drug Resistance 1(1) (2018) 6-29

6 M.M. Gottesman, O. Lavi, M.D. Hall, J.P. Gillet. Toward a Better Understanding of the Complexity of Cancer Drug Resistance, Annu Rev Pharmacol Toxicol 56 (2016) 85-102

7 G. Szakacs, J.K. Paterson, J.A. Ludwig, C. Booth-Genthe, M.M. Gottesman. Targeting multidrug resistance in cancer, Nat Rev Drug Discov 5(3) (2006) 219-234

8 R. Gorlick, E. Goker, T. Trippett, M. Waltham, D. Banerjee, J.R. Bertino. Intrinsic and acquired resistance to methotrexate in acute leukemia, N Engl J Med 335(14) (1996) 1041-1048

9 M.G. Rots, R. Pieters, G.J. Kaspers, A.J. Veerman, G.J. Peters, G. Jansen. Classification of ex vivo methotrexate resistance in acute lymphoblastic and myeloid leukaemia, Br J Haematol 110(4) (2000) 791-800

10 Y.G. Assaraf. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis, Drug Resist Updat 9(4-5) (2006) 227-246

11 Z.S. Chen, R.W. Robey, M.G. Belinsky, I. Shchaveleva, X.Q. Ren, Y. Sugimoto, et al. Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport, Cancer Res 63(14) (2003) 4048-4054

12 M. Kool, M. van der Linden, M. de Haas, G.L. Scheffer, J.M. de Vree, A.J. Smith, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs, Proc Natl Acad Sci 96(12) (1999) 6914-6919

13 P. Wielinga, J.H. Hooijberg, S. Gunnarsdottir, I. Kathmann, G. Reid, N. Zelcer, et al. The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates, Cancer Res 65(10) (2005) 4425-4430

14 J.H. Hooijberg, H.J. Broxterman, M. Kool, Y.G. Assaraf, G.J. Peters, P. Noordhuis, et al. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2, Cancer Res 59(11) (1999) 2532-2535

15 E. Masson, M.V. Relling, T.W. Synold, Q. Liu, J.D. Schuetz, J.T. Sandlund, et al. Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects in vivo. A rationale for high-dose methotrexate, J Clin Invest 97(1) (1996) 73-80

16 M.G. Rots, R. Pieters, G.J. Peters, P. Noordhuis, C.H. van Zantwijk, G.J. Kaspers, et al. Role of folylpolyglutamate synthetase and folylpolyglutamate hydrolase in methotrexate accumulation and polyglutamylation in childhood leukemia, Blood 93(5) (1999) 1677-1683

17 V.M. Whitehead, D.S. Rosenblatt, M.J. Vuchich, J.J. Shuster, A. Witte, D. Beaulieu. Accumulation of methotrexate and methotrexate polyglutamates in lymphoblasts at diagnosis of childhood acute lymphoblastic leukemia: a pilot prognostic factor analysis, Blood 76(1) (1990) 44-49

18 V.M. Whitehead, J.J. Shuster, M.J. Vuchich, D.H. Mahoney, Jr., S.J. Lauer, C. Payment, et al. Accumulation of methotrexate and methotrexate polyglutamates in lymphoblasts and treatment outcome in children with B-progenitor-cell acute lymphoblastic leukemia: a Pediatric Oncology Group study, Leukemia 19(4) (2005) 533-536

19 A. Wojtuszkiewicz, G.J. Peters, N.L. van Woerden, B. Dubbelman, G. Escherich, K. Schmiegelow, et al. Methotrexate resistance in relation to treatment outcome in childhood acute lymphoblastic leukemia, J Hematol Oncol 8 (2015) 61

20 S. Raz, M. Stark, Y.G. Assaraf. Folylpoly-gamma-glutamate synthetase: A key determinant of folate homeostasis and antifolate resistance in cancer, Drug Resist Updat 28 (2016) 43-64

21 E.L. Volk, E. Schneider. Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter, Cancer Res 63(17) (2003) 5538-5543

22 J. van Meerloo, G.J. Kaspers, J. Cloos. Cell sensitivity assays: the MTT assay, Methods Mol Biol 731 (2011) 237-245

23 M.G. Rots, R. Pieters, G.J. Kaspers, C.H. van Zantwijk, P. Noordhuis, R. Mauritz, et al. Differential methotrexate resistance in childhood T- versus common/preB-acute lymphoblastic leukemia can

be measured by an in situ thymidylate synthase inhibition assay, but not by the MTT assay, Blood 93(3) (1999) 1067-1074
24 R. Mauritz, M.W. Bekkenk, M.G. Rots, R. Pieters, E. Mini, C.H. van Zantwijk, et al. Ex vivo activity of methotrexate versus novel antifolate inhibitors of dihydrofolate reductase and thymidylate synthase against childhood leukemia cells, Clin Cancer Res 4(10) (1998) 2399-2410
25 J.C. Yalowich, T.I. Kalman. Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells in vitro, Biochem Pharmacol 34(13) (1985) 2319-2324
26 R. Mauritz, G.J. Peters, I. Kathmann, H. Teshale, P. Noordhuis, E.M. Comijn, et al. Dynamics of antifolate transport via the reduced folate carrier and the membrane folate receptor in murine leukaemia cells in vitro and in vivo, Cancer Chemother Pharmacol 62(6) (2008) 937-948
27 W.A. Kamps, K.M. van der Pal-de Bruin, A.J. Veerman, M. Fiocco, M. Bierings, R. Pieters. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984 to 2004, Leukemia 24(2) (2010) 309-319
28 G.J. Kaspers, A.J. Veerman, R. Pieters, G.J. Broekema, D.R. Huismans, K.M. Kazemier, et al. Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay, Br J Cancer 70(6) (1994) 1047-1052
29 S. Rodenhuis, J.J. McGuire, R. Narayanan, J.R. Bertino. Development of an assay system for the detection and classification of methotrexate resistance in fresh human leukemic cells, Cancer Res 46(12 Pt 1) (1986) 6513-6519
30 C. Lemos, I. Kathmann, E. Giovannetti, J.A. Belien, G.L. Scheffer, C. Calhau, et al. Cellular folate status modulates the expression of BCRP and MRP multidrug transporters in cancer cell lines from different origins, Mol Cancer Ther 8(3) (2009) 655-664
31 M.G. Rots, J.C. Willey, G. Jansen, C.H. Van Zantwijk, P. Noordhuis, J.P. DeMuth, et al. mRNA expression levels of methotrexate resistance-related proteins in childhood leukemia as determined by a standardized competitive template-based RT-PCR method, Leukemia 14(12) (2000) 2166-2175
32 A.J. Galpin, J.D. Schuetz, E. Masson, Y. Yanishevski, T.W. Synold, J.C. Barredo, et al. Differences in folylpolyglutamate synthetase and dihydrofolate reductase expression in human B-lineage versus T-lineage leukemic lymphoblasts: mechanisms for lineage differences in methotrexate polyglutamylation and cytotoxicity, Mol Pharmacol 52(1) (1997) 155-163
33 R. Mauritz, G.J. Peters, D.G. Priest, Y.G. Assaraf, S. Drori, I. Kathmann, et al. Multiple mechanisms of resistance to methotrexate and novel antifolates in human CCRF-CEM leukemia cells and their implications for folate homeostasis, Biochem Pharmacol 63(2) (2002) 105-115
34 E. Mini, B.A. Moroson, C.T. Franco, J.R. Bertino. Cytotoxic effects of folate antagonists against methotrexate-resistant human leukemic lymphoblast CCRF-CEM cell lines, Cancer Res 45(1) (1985) 325-330
35 M. Kourti, N. Vavatsi, N. Gombakis, V. Sidi, G. Tzimagiorgis, T. Papageorgiou, et al. Expression of multidrug resistance 1 (MDR1), multidrug resistance-related protein 1 (MRP1), lung resistance protein (LRP), and breast cancer resistance protein (BCRP) genes and clinical outcome in childhood acute lymphoblastic leukemia, Int J Hematol 86(2) (2007) 166-173
36 N. Fazlina, A. Maha, R. Jamal, A.L. Zarina, S.K. Cheong, H. Hamidah, et al. Expression of multidrug resistance (MDR) proteins and in vitro drug resistance in acute leukemias, Hematology 12(1) (2007) 33-37
37 R. Pieters, M.L. den Boer, M. Durian, G. Janka, K. Schmiegelow, G.J. Kaspers, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants, Leukemia 12(9) (1998) 1344-1348
38 S.L. Plasschaert, E.S. de Bont, M. Boezen, D.M. vander Kolk, S.M. Daenen, K.N. Faber, et al. Expression of multidrug resistance-associated proteins predicts prognosis in childhood and adult acute lymphoblastic leukemia, Clin Cancer Res 11(24 Pt 1) (2005) 8661-8668
39 S.L. Plasschaert, D.M. Van Der Kolk, E.S. De Bont, E. Vellenga, W.A. Kamps, E.G. De Vries. Breast cancer resistance protein (BCRP) in acute leukemia, Leuk Lymphoma 45(4) (2004) 649-654
40 K. Kock, M. Grube, G. Jedlitschky, L. Oevermann, W. Siegmund, C.A. Ritter, et al. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy, Clin Pharmacokinet 46(6) (2007) 449-470
41 R. van de Ven, R. Oerlemans, J.W. van der Heijden, G.L. Scheffer, T.D. de Gruijl, G. Jansen, et al. ABC drug transporters and immunity: novel therapeutic targets in autoimmunity and cancer, J Leukoc Biol 86(5) (2009) 1075-1087
42 A. Sauerbrey, W. Sell, D. Steinbach, A. Voigt, F. Zintl. Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia, Br J Haematol 118(1) (2002) 147-150
43 D. Steinbach, J. Lengemann, A. Voigt, J. Hermann, F. Zintl, A. Sauerbrey. Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated

proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia, Clin Cancer Res 9(3) (2003) 1083-1086
44 A.I. Svirnovski, T.V. Shman, T.F. Serhiyenka, V.P. Savitski, V.V. Smolnikova, U.U. Fedasenka. ABCB1 and ABCG2 proteins, their functional activity and gene expression in concert with drug sensitivity of leukemia cells, Hematology 14(4) (2009) 204-212
45 K. Swerts, B. De Moerloose, C. Dhooge, G. Laureys, Y. Benoit, J. Philippe. Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia, Eur J Cancer 42(3) (2006) 295-309
46 T. Kakihara, A. Tanaka, A. Watanabe, K. Yamamoto, K. Kanto, S. Kataoka, et al. Expression of multidrug resistance-related genes does not contribute to risk factors in newly diagnosed childhood acute lymphoblastic leukemia, Pediatr Int 41(6) (1999) 641-647
47 R.W. Stam, M.M. van den Heuvel-Eibrink, M.L. den Boer, M.E. Ebus, G.E. Janka-Schaub, J.D. Allen, et al. Multidrug resistance genes in infant acute lymphoblastic leukemia: Ara-C is not a substrate for the breast cancer resistance protein, Leukemia 18(1) (2004)
48 M.L. Den Boer, R. Pieters, K.M. Kazemier, G.E. Janka-Schaub, G. Henze, A.J. Veerman. Relationship between the intracellular daunorubicin concentration, expression of major vault protein/lung resistance protein and resistance to anthracyclines in childhood acute lymphoblastic leukemia, Leukemia 13(12) (1999) 2023-2030
49 A. Wojtuszkiewicz, A. Barcelos, B. Dubbelman, R. De Abreu, C. Brouwer, J.P. Bokkerink, et al. Assessment of mercaptopurine (6MP) metabolites and 6MP metabolic key-enzymes in childhood acute lymphoblastic leukemia, Nucleosides Nucleotides Nucleic Acids 33(4-6) (2014) 422-433
50 S. Bartholomae, B. Gruhn, K.M. Debatin, M. Zimmermann, U. Creutzig, D. Reinhardt, et al. Coexpression of Multiple ABC-Transporters is Strongly Associated with Treatment Response in Childhood Acute Myeloid Leukemia, Pediatr Blood Cancer 63(2) (2016) 242-247
51 M. Ansari, G. Sauty, M. Labuda, V. Gagne, C. Laverdiere, A. Moghrabi, et al. Polymorphisms in multidrug resistance-associated protein gene 4 is associated with outcome in childhood acute lymphoblastic leukemia, Blood 114(7) (2009) 1383-1386
52 Y. Tanaka, A. Manabe, H. Fukushima, R. Suzuki, H. Nakadate, K. Kondoh, et al. Multidrug resistance protein 4 (MRP4) polymorphisms impact the 6-mercaptopurine dose tolerance during maintenance therapy in Japanese childhood acute lymphoblastic leukemia, Pharmacogenomics J 15(4) (2015) 380-384
53 A. Wojtuszkiewicz, Y.G. Assaraf, M. Hoekstra, R. Sciarrillo, G. Jansen, G.J. Peters, et al. The association of aberrant folylpolyglutamate synthetase splicing with ex vivo methotrexate resistance and clinical outcome in childhood acute lymphoblastic leukemia, Haematologica 101(7) (2016)
54 H. Mesrian Tanha, S. Rahgozar, M. Mojtabavi Naeini. ABCC4 functional SNP in the 3' splice acceptor site of exon 8 (G912T) is associated with unfavorable clinical outcome in children with acute lymphoblastic leukemia, Cancer Chemother Pharmacol 80(1) (2017) 109-117.
55 X. Zhai, H. Wang, X. Zhu, H. Miao, X. Qian, J. Li, et al. Gene polymorphisms of ABC transporters are associated with clinical outcomes in children with acute lymphoblastic leukemia, Arch Med Sci 8(4) (2012) 659-671

CHAPTER 5

Expression of the nucleoside transporters hENT1 (SLC29) and hCNT1 (SLC28) in pediatric acute myeloid leukemia

Nucleosides, Nucleotides and Nucleic Acids. 39 (10-12): 1379-1388; 2020

Adrian Christopher Jaramillo[1], Isabelle Hubeek[2], Richard Broekhuizen[1,5], Marçal Pastor-Anglada[6], Gertjan JL Kaspers[7], Gerrit Jansen[3], Jacqueline Cloos[1], Godefridus J Peters[4,8]

[1] Department of Hematology, Amsterdam University Medical Center, Amsterdam, The Netherlands.

[2] Department of Clinical Chemistry, Amsterdam University Medical Center, Amsterdam, The Netherlands.

[3] Department of Rheumatology, Amsterdam University Medical Center, Amsterdam, The Netherlands.

[4] Department of Laboratory Medical Oncology, Amsterdam University Medical Center, Amsterdam, The Netherlands.

[5] Department of Hematology-Oncology[5], Pontifical Catholic University of Chile.

[6] Department de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona, Spain.

[7] Princess Maxima Center for Pediatric Cancer, Utrecht, SKION, and Emma's Children's Hospital, Amsterdam UMC, Location VUMC, Pediatric Oncology, Amsterdam, The Netherlands

[8] Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland

Abstract

Cellular uptake of clinically important deoxynucleoside analogs is mediated by nucleoside transporters including the human equilibrative nucleoside transporter 1 (hENT1) and the concentrative nucleoside transporter-1 (hCNT1). These transporters are responsible for influx of cytarabine and reduced hENT1 expression is a major resistance mechanism in acute myeloid leukemia. We determined hENT1 and hCNT1 protein expression by immunocytochemistry in 50 diagnostic paediatric acute myeloid leukemia patient samples. All samples expressed hENT1 [9/43 (21%) low; 26/43 (60%) medium and 8/43 (19%) high] and hCNT1 [2/42 (5%) low; 35/42 (83%) medium and 5/42 (12%) high] at the cell membrane and cytoplasm. Statistical analysis showed a non-significant relationship between survival and transporter expression and *in vitro* drug sensitivity. In conclusion, the nucleoside transporters hENT1 and hCNT1 are broadly expressed in pediatric acute myeloid leukemia at diagnosis**.**

K**eywords**: acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), human equilibrative nucleoside transporter-1 (hENT1), human concentrative nucleoside transporter-1 (hCNT1), deoxynucleoside analogs (NAs), cytarabine (Ara-C).

1. Introduction

The human equilibrative nucleoside transporter 1 (hENT1; or SLC29A1) and concentrative nucleoside transporter-1 (hCNT1; SLC28A) are transmembrane proteins involved in nucleoside homeostasis and cellular uptake of deoxynucleoside analogs (NAs) by facilitated diffusion or concentrative uptake[1-4]. In leukemic cells, hENT1 is responsible for the uptake of around eighty-percent of cytarabine (Ara-C), a cornerstone NA in the treatment of acute myeloid leukemia (AML) [1, 5-7]. In contrast, gemcitabine, which is used extensively for the treatment of solid tumors, is transported into the cell by both hCNT1 and hENT1[4, 8, 9]

Nucleoside transporters play a role in NA cytotoxicity and chemoresistance. Recent Cox regression analysis of AML patients (n=28) showed that patients with high or baseline hENT1 expression prior to treatment, had a 50% longer median OS (p=0.012) suggesting that hENT1 expression profiling might be of clinical importance[10]. Other studies show inconclusive results, for example, a survival study reported that hENT1 expression did not significantly contribute to Ara-C resistance in acute lymphoblastic leukemia (ALL) patients older than 15 years of age and analysis of 79 B-cell precursor ALL cell lines found no correlation between hENT1 gene expression and Ara-C resistance. Unexpectedly, higher hENT1 expression was seen in cell lines with Ara-C resistance, when compared with cell lines with low expression[11,12].Apparently other parameters also play a role in Ara-C resistance.

However, hENT1 mRNA levels in pediatric and adult AML samples are related to resistance and outcome, suggesting that low transporter expression is a relevant mechanism of Ara-C resistance[13-15]. Micro-array studies performed on Ara-C resistant human ALL cells demonstrated that a decrease in hENT1 expression was linked to

lower Ara-C activity and decreased intracellular drug metabolism[16]. Reconstitution of the hENT1 gene by transfection reversed Ara-C resistance 600-fold in human lymphoid H9 cells[17]. It has been shown that indirect modulation of hENT1 from the cell membrane of leukemic cells resulted in a significant decrease in nucleoside transporter activity and increased Ara-C resistance in AML cells in vitro[18]. Reversal of resistance due to decreased transporter activity has been attempted by bypassing the transporter, e.g. by lipophilic pro-drugs[19].

In addition, elevated hENT1 mRNA, which has been reported previously in infants with *MLL* gene-rearranged ALL (ALL-MLL+) explained the remarkable Ara-C sensitivity in this patient population[20,21]. It is plausible that elevated levels of transporter lead to increased Ara-C influx across the cellular membrane, resulting in increased intracellular drug accumulation and enhanced cytotoxicity[22]. Furthermore, in pediatric ALL-MLL+ indirect modulation of hENT1 expression affects significantly Ara-C cytotoxicity in pediatric AML in vitro[21]. Interestingly, certain genetic variants of hENT1, which may magnify genetic expression, are also associated with treatment outcome in AML[2, 23, 24].

The aim of our present study was to relate the immunohistochemical detection of protein expression of hENT1 and the pyrimidine-preferring hCNT1 transporter, and ex vivo sensitivity to nucleoside analogs.

2. Materials and Methods

2.1 Patient material.

Bone marrow or peripheral blood samples were collected from 50 untreated children diagnosed with de novo AML. The patient population consisted of 32 boys and 18 girls with a median age of 10.7 years (range 0.1-16.8 years). The median white blood cell count (WBC) was 83.8 (range 2.1-524 x 10^6 /ml) and all FAB-types were represented (2 FAB-M0; 5 FAB-M1; 8 FAB-M2, 4 FAB-M3, 18 FAB-M4, 10 FAB-M5, 1 RAEB and 2 unknown). A summary of treatment protocols for these patients (DCOG AML 87, 97, all including Ara-C) is given elsewhere[15].

2.2 Cell isolation and immunocytochemistry staining

Mononuclear cells were separated by density gradient centrifugation (Lymphoprep, density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) and where necessary the percentage of malignant cells was enriched as described previously[25]. All samples contained >80% blasts as determined morphologically by May-Grünwald-Giemsa staining. Immunocytochemical staining was performed on (cryo-preserved) cytospins standard alkaline phosphatase/anti-alkaline phosphatase (APAAP) method. Cells were fixed with acetone (10 minutes at room temperature). Slides were washed with Phosphate-buffered saline (PBS) twice, for 5 minutes) and incubated overnight with the primary antibodies, rabbit-anti human hENT1 and hCNT1 diluted 1:100 and 1:150, respectively, in PBS with 1% bovine serum albumin (BSA) and 0.1% Na-azide as described elsewhere[21, 26]. The following day, slides were washed (2x 5 minutes in

PBS) and incubated with the secondary antibody, biotinylated swine-anti-rabbit (Dako, Glostrup, Denmark) diluted 1:300 in PBS with 1% BSA and 0.1% Na-azide for one hour. After the wash steps, slides were incubated with the alkaline-phosphatase conjugated streptavidine (1:100 in PBS with 1% BSA and 0.1% Na-azide) for 30 minutes (Dako, Glostrup, Denmark). Visualization of AP was performed by incubation in New Fuchsin/naphtol ASBI phosphate solution supplemented with levamisole. Cells were counterstained with using Mayer's Hematoxylin Solution (Merck, Darmstadt, Germany) and embedded in Aquamount. hENT1 and hCNT1 protein expression was evaluated by two independent investigators by scoring the intensity of the staining as either low, medium or high. When compared to positive controls, "low" or weak staining was defined as less than one third of the intensity (<0.3) and "high" staining was defined as the upper fifth of the intensity (>0.8) and medium staining was in between these two intensities [26].

2.3 MTT assay

For assessment of drug sensitivity, the MTT colorimetric assay was performed on cells suspended in 96-well round bottom plates at a concentration of 2 x 10^6 cells/ml in RPMI-1640 cell culture medium containing 20% fetal calf serum (FCS), 200 µg/ml of gentamycin, 2 mM glutamine, and 0.5 ml ITS (insulin 5 µg/ml, transferrin 5 µg/ml, sodium selenite 5 ng/ml[27]. Treatment wells contained 80 µl of cell suspension and 20 µl of the drugs of interest at different concentrations (refer to supplementary table A.4 for concentrations). Control wells contained 20 µl of RPMI-1640 medium and 80 µl of cell suspension, while blank controls had 100 µl of RPMI-1640 medium. MTT was performed in duplicates and plates were incubated at 37°C, in a humidified 5% CO_2 incubator for 4 days. At the end of the incubation period, 10 µl of MTT dye (5 mg/ml) was added to each well and placed in the 37ºC incubator for an additional 6 hours, followed by the addition of 100 µl isopropanol. After 5 minutes, the optical density (OD) was measured at 562 nm. For the analysis, the average OD of the blank wells is subtracted from the average OD of the control wells or the treatment wells. The lethal dose 50 (LC_{50}) was then determined by calculating cell survival with the following equation: mean OD treatment well (minus blank)/ mean OD control well (minus blank) x100, as described elsewhere[27].

2.4 ***mRNA Extraction***

Total mRNA from these primary pediatric AML cells was extracted previously[15] using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and reverse transcribed to cDNA using random hexamers as described elsewhere[15]. For measurement of hENT1 and hCNT1 mRNA expression probes and primers have been described earlier as well[15].

2.5 *Statistical analysis*

Statistical analysis was done using SPSS Version 24.0 (IBM Corp., Armonk, NY, US) software. Overall Survival (OS) analysis was evaluated by constructing Kaplan-Meier curves. For comparisons between means we used Student's *t* test and Spearman's Rho for non-parametric correlations (hENT protein level and outcome). In order to evaluate any significant differences between categorical sets of data (FAB types and immunohistochemistry intensity scores) we used Pearson Chi-square test.

3. Results

HL60 consistently stained positive for both hENT1 and hCNT1 in the cytoplasm (Figure 1A, B, and C). Negative controls were performed by omitting the first antibody and did not have a staining (Figure 1A). Seven samples were non-evaluable due to poor morphology and one sample was lost during the procedure. hENT1 and hCNT1 were localized at the membrane and in the cytoplasm in all AML patient samples (Figure 1 D, E, F, G). There was a relatively uniform staining within one patient, while there was no significant difference between samples obtained from peripheral blood or bone marrow. hENT1 staining intensity was low in 9/43 (21%) samples, medium in 26/43 (60%) samples and high in 8/43 (19%) samples, and appeared to be slightly granular. hCNT1 staining intensity was low in 2/42 (5%) samples, medium in 35/42 (83%) samples and high in 5/42 (12%) samples (Figure 2A, 2B and Table 2). In 8/42 (19%) AML patient samples hCNT1 was located predominantly at the cellular membrane. hENT1 and hCNT1 expression were not associated with age or WBC at diagnosis. Both hENT1 and hCNT1 expression appeared to be higher in (myelo) monocytic (FAB M4/5) AML blasts compared to poorly differentiated/ myeloblastic (FAB M0-3) AML blasts (Table 1) but there was no significant difference (Pearson Chi-square= 10.8, *P*= 0.37). There was no correlation between mRNA expression and immunocytochemistry staining intensity scoring (Spearman's rho= 0.19, two-tailed). Additionally, hENT1 mRNA[15] and corresponding immunohistochemistry intensities, were significantly different from each other (two tailed Student *t*-test, *P*= 0.044) (Figure 2C). There was no significant correlation with cytogenetic markers such as chromosomal rearrangements.

The sensitivity of primary AML samples to Ara-C in the high hENT1 and hCNT protein expression groups tended to be higher (lower LC_{50}) than in the low hENT and hCNT group, but this was not significant (Figure 3A and 3B). We did not observe a difference for the other deoxynucleoside analogs cladribine, gemcitabine, fludarabine and decitabine. The immunocytochemistry staining intensity did not correlate with OS (Spearman's Rho, *P*= 0.67) (Figure 4) and survival analysis for hCNT1 was difficult to determine due to the low number of samples allocated to low and high staining intensity scores (data not shown).

Table 1. hENT1 and hCNT1 staining in different AML FAB types.

		Low	Medium	High
hENT1	FAB M0-3 (n=14)	5 (36%)	8 (57%)	1 (7%)
	FAB M4-5 (n=27)	3 (11%)	17 (63%)	7 (26%)
hCNT1	FAB M0-3 (n=15)	1 (7%)	14 (93%)	-
	FAB M4-5 (n=25)	1 (4%)	76%)	5 (20%)

Table 2. Frequencies of hCNT and hENT samples and staining intensities

	hCNT-Low	hCNT-Medium	hCNT-High
hENT Low	**1**	**8**	**0**
hENT Medium	**1**	**23**	**0**
hENT High	**0**	**3**	**5**

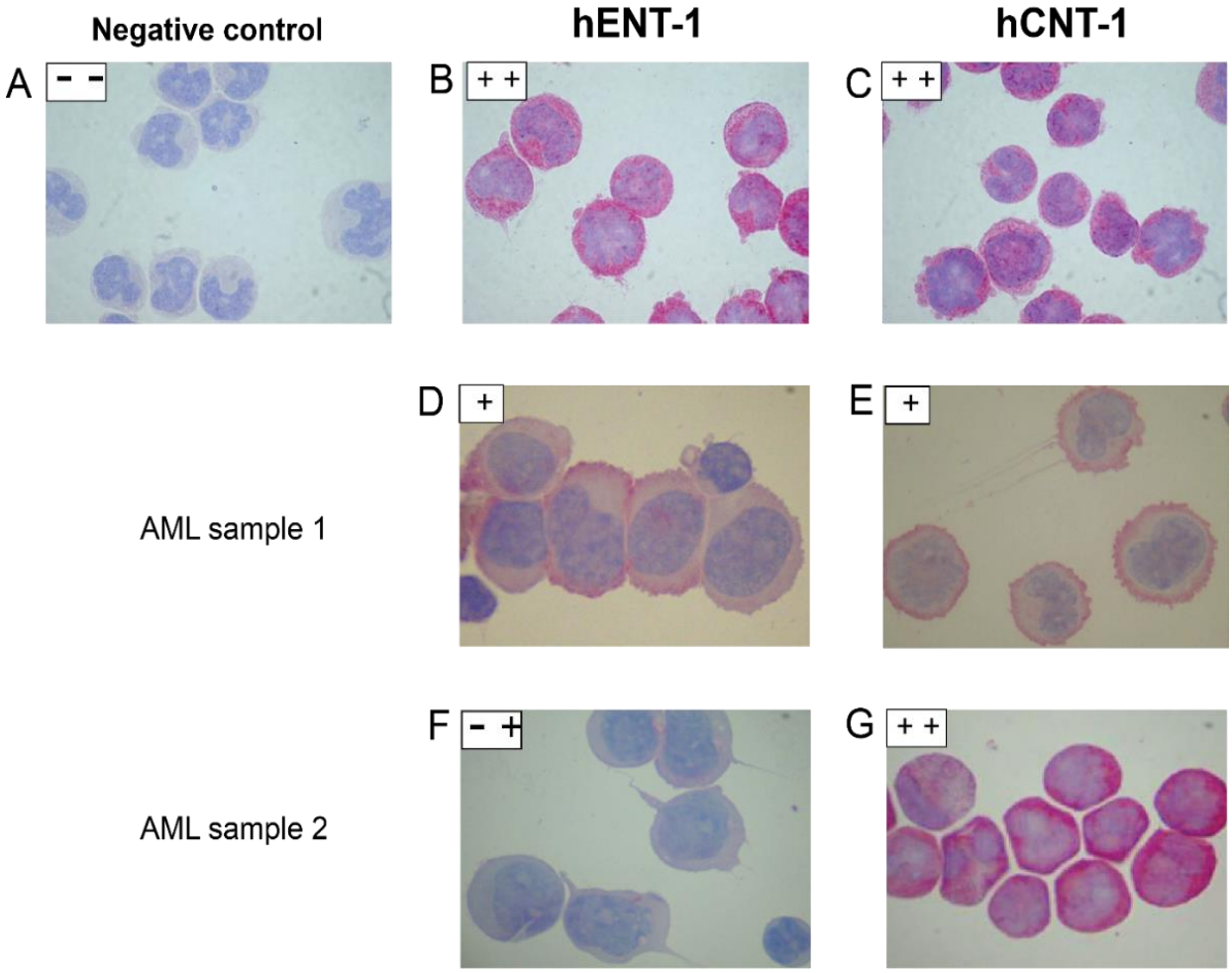


Figure 1. *Immunocytochemistry for hENT1 and hCNT1 proteins, as scored by two independent observers in several representative patient samples. Negative control in HL-60 cells was done by omitting one antibody (**1A**). Positive controls (**1B, 1C**) are compared with AML samples of different color intensity (**1D, E, F, G**). Low staining was defined as less than one third of the intensity (<0.3) and high staining was defined as the upper fifth of the intensity (>0.8) and medium staining was in between these two intensities (- +).*

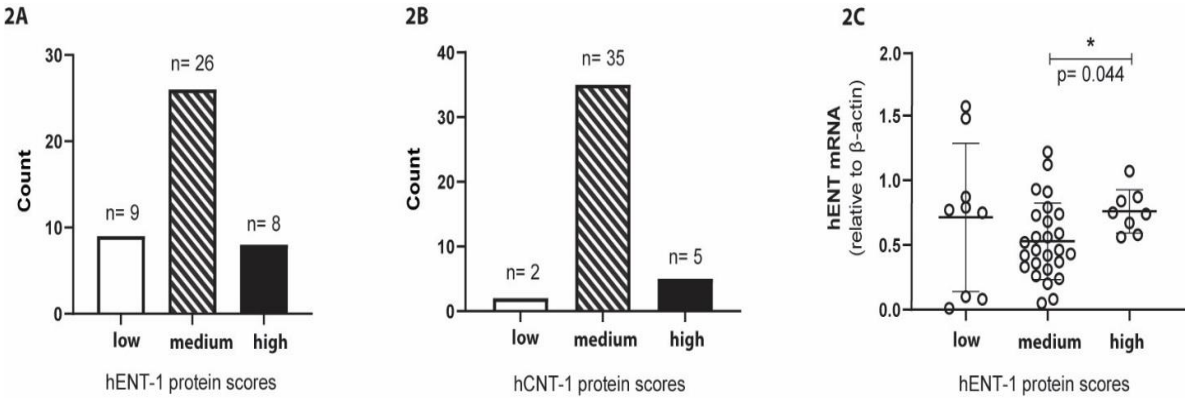


***Figure 2A**: Frequencies (count) of samples in low (- -), medium (- +) and high (+ +) staining intensity for hENT1 and (**2B**) hCNT1. **2C:** hENT1 mRNA levels of samples (from ref [15]) allocated to score groups low, medium and high.*

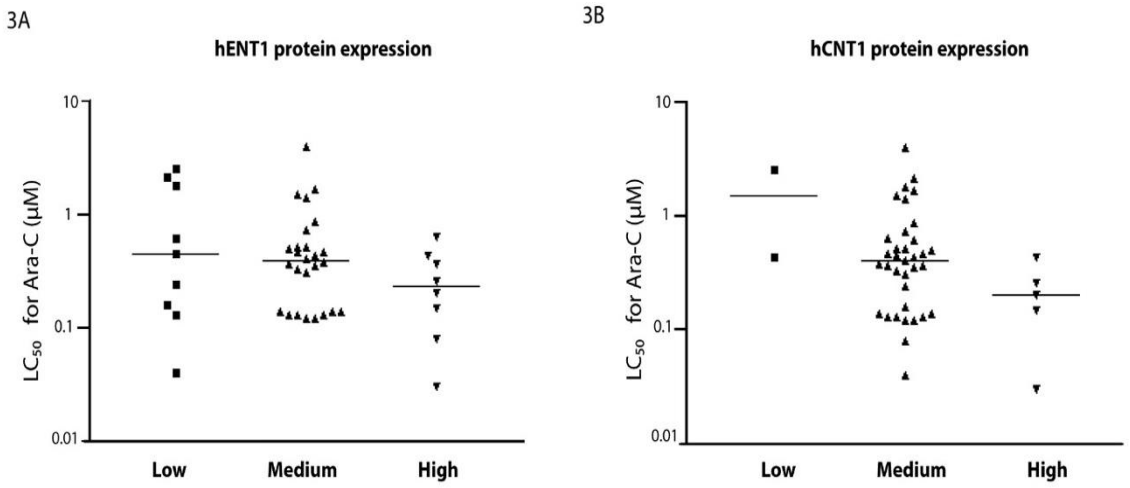


***Figure 3:** Sensitivity of primary AML samples (depicted as Ara-C LC50 values) in hENT1 (**3A**) and hCNT1 (**3B**) immunohistochemistry score groups.*

4. Discussion

The intracellular uptake of antimetabolites such as Ara-C, is transporter dependent. Both hENT1 and hCNT1 are essential for Ara-C uptake[21]. In this study, we find that hENT1 and the pyrimidine-preferring hCNT1 transporters are broadly expressed in the blasts of pediatric AML patients. Since the majority of patients scored medium levels of expression, a potential relation with in vitro sensitivity to deoxynucleoside analogs such as Ara-C was difficult to establish in this study. In addition, it is important to note that the effective concentrations of drugs used in the MTT assay were already so high that Ara-C would be taken up by diffusion, bypassing the transporters. Furthermore, hENT1 and hCNT1 expression are regulated at the posttranslational level, which may explain the lack of correlation between immunocytochemistry protein detection and mRNA levels[2,13,18]. Furthermore, this lack of correlation between hENT1 and hCNT1 expression with OS of these patients, may be related to the multidrug regimens which are commonly used to treat these patients [28].

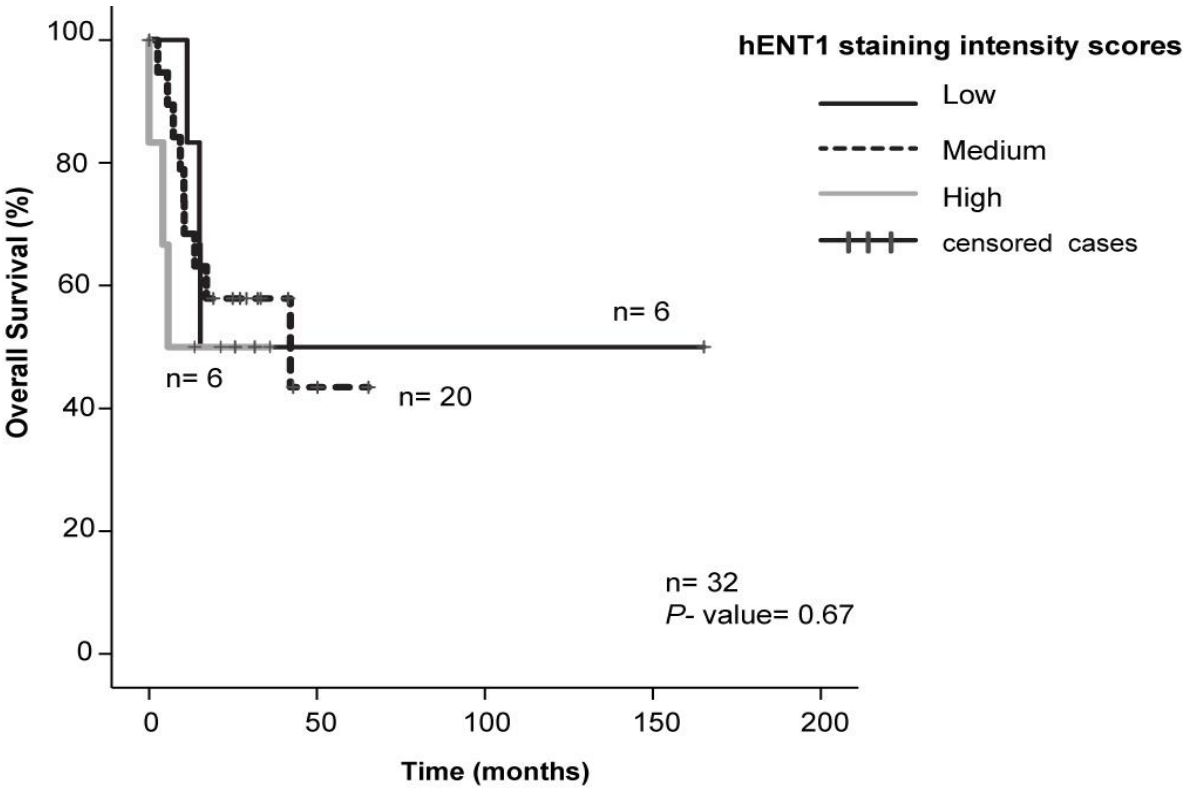


Figure 4: *Kaplan-Meier curves of pediatric AML patients with low (black), medium (dashed) and high (gray) hENT1 immunocytochemistry staining intensities. All patients received induction chemotherapy containing an anthracycline (idarubicin or daunorubicin) in combination with Ara-C and etoposide.*

The cells' functional capacity is characterized by the enzyme activity within its cellular pathways. Proteomic profiling, (i.e. through immunohistochemistry) could be a useful tool to get a glimpse into the cells functional state. Further *ex vivo* cytotoxicity testing (i.e. MTT) can reveal the chemoresistance state of primary AML blasts and can therefore redirect and personalize treatment. In future studies, functional measurements in conjunction with immunocytochemistry might elucidate the precise role of hENT1 and hCNT1 in AML. Recently, a collaborative EORTC-GIMMEA trial has reported increased survival and a positive response rate with high-dose Ara-C in very high-risk setting patients, including AML-FLT3-ITD+ cases, which have notoriously high levels of hENT1 expression levels[21, 29]. This supports the notion that pre-treatment analysis of patient genetic profile might be a useful tool to classify treatment options for patients.

5. Conclusions

The nucleoside transporters hENT1 and hCNT1, which are crucial for the transport of clinically important deoxynucleoside analogs (e.g. cytarabine), can be visualized by immunocytochemistry showing that they are broadly expressed in almost all AML

6. References

1. Clarke ML, Mackey JR, Baldwin SA, Young JD, Cass CE. The role of membrane transporters in cellular resistance to anticancer nucleoside drugs. *Cancer Treat Res* 2002; 112: 27-47.
2. Cao HX, Miao CF, Yan L, Tang P, Zhang LR, Sun L. Polymorphisms at microRNA binding sites of Ara-C and anthracyclines-metabolic pathway genes are associated with outcome of acute myeloid leukemia patients. *J Transl Med* 2017; 15(1): 235
3. Hummel-Eisenbeiss J, Hascher A, Hals PA, Sandvold ML, Muller-Tidow C, Lyko F *et al.* The role of human equilibrative nucleoside transporter 1 on the cellular transport of the DNA methyltransferase inhibitors 5-azacytidine and CP-4200 in human leukemia cells. *Mol Pharmacol* 2013; 84(3): 438-450
4. Pastor-Anglada M, Perez-Torras S. Emerging Roles of Nucleoside Transporters. *Front Pharmacol* 2018; 9: 606.
5. Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol Ther* 2000; 87(2-3): 227-253.
6. Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M *et al.* Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: the AML99 trial from the Japanese Childhood AML Cooperative Study Group. *J Clin Oncol* 2009; 27(24): 4007-4013
7. Murphy T, Yee KWL. Cytarabine and daunorubicin for the treatment of acute myeloid leukemia. *Expert Opin Pharmacother* 2017; 18(16): 1765-1780
8. Ciccolini J, Serdjebi C, Peters GJ, Giovannetti E. Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemother Pharmacol* 2016; 78(1): 1-12
9. Momparler RL. Optimization of cytarabine (Ara-C) therapy for acute myeloid leukemia. *Exp Hematol Oncol* 2013; 2: 20
10. Candelaria M, Corrales-Alfaro C, Gutierrez-Hernandez O, Diaz-Chavez J, Labardini-Mendez J, Vidal-Millan S *et al.* Expression Levels of Human Equilibrative Nucleoside Transporter 1 and Deoxycytidine Kinase Enzyme as Prognostic Factors in Patients with Acute Myeloid Leukemia Treated with Cytarabine. *Chemotherapy* 2016; 61(6): 313-318
11. Rizzieri D, Vey N, Thomas X, Huguet-Rigal F, Schlenk RF, Krauter J *et al.* A phase II study of elacytarabine in combination with idarubicin and of human equilibrative nucleoside transporter 1 expression in patients with acute myeloid leukemia and persistent blasts after the first induction course. *Leuk Lymphoma* 2014; 55(9): 2114-2119
12. Huang M, Inukai T, Miyake K, Tanaka Y, Kagami K, Abe M *et al.* Clofarabine exerts antileukemic activity against cytarabine-resistant B-cell precursor acute lymphoblastic leukemia with low deoxycytidine kinase expression. *Cancer Med* 2018; 7(4): 1297-1316
13. Galmarini CM, Thomas X, Calvo F, Rousselot P, El Jafaari A, Cros E *et al.* Potential mechanisms of resistance to cytarabine in AML patients. *Leuk Res* 2002; 26(7): 621-629.
14. Galmarini CM, Thomas X, Calvo F, Rousselot P, Rabilloud M, El Jaffari A *et al.* In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia. *Br J Haematol* 2002; 117(4): 860-868
15. Hubeek I, Stam RW, Peters GJ, Broekhuizen R, Meijerink JP, van Wering ER *et al.* The human equilibrative nucleoside transporter 1 mediates in vitro cytarabine sensitivity in childhood acute myeloid leukaemia. *Br J Cancer* 2005; 93(12): 1388-1394
16. Takagaki K, Katsuma S, Kaminishi Y, Horio T, Nakagawa S, Tanaka T *et al.* Gene-expression profiling reveals down-regulation of equilibrative nucleoside transporter 1 (ENT1) in Ara-C-resistant CCRF-CEM-derived cells. *J Biochem* 2004; 136(5): 733-740
17. Sarkar M, Han T, Damaraju V, Carpenter P, Cass CE, Agarwal RP. Cytosine arabinoside affects multiple cellular factors and induces drug resistance in human lymphoid cells. *Biochem Pharmacol* 2005; 70(3): 426-432
18. Macanas-Pirard P, Broekhuizen R, Gonzalez A, Oyanadel C, Ernst D, Garcia P *et al.* Resistance of leukemia cells to cytarabine chemotherapy is mediated by bone marrow stroma, involves cell-surface equilibrative nucleoside transporter-1 removal and correlates with patient outcome. *Oncotarget* 2017; 8(14): 23073-23086
19. Bergman AM, Adema AD, Balzarini J, Bruheim S, Fichtner I, Noordhuis P *et al.* Antiproliferative activity, mechanism of action and oral antitumor activity of CP-4126, a fatty acid derivative of gemcitabine, in in vitro and in vivo tumor models. *Invest New Drugs* 2011; 29(3): 456-466. e-pub ahead of print 2010/01/13

20. Stam RW, den Boer ML, Meijerink JP, Ebus ME, Peters GJ, Noordhuis P *et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. *Blood* 2003; 101(4): 1270-1276
21. Catala A, Pastor-Anglada M, Caviedes-Cardenas L, Malatesta R, Rives S, Vega-Garcia N *et al.* FLT3 is implicated in cytarabine transport by human equilibrative nucleoside transporter 1 in pediatric acute leukemia. *Oncotarget* 2016; 7(31): 49786-49799
22. Winters AC, Bernt KM. MLL-Rearranged Leukemias-An Update on Science and Clinical Approaches. *Front Pediatr* 2017; 5: 4
23. Kim JH, Lee C, Cheong HS, Koh Y, Ahn KS, Kim HL *et al.* SLC29A1 (ENT1) polymorphisms and outcome of complete remission in acute myeloid leukemia. *Cancer Chemother Pharmacol* 2016; 78(3): 533-540
24. Amaki J, Onizuka M, Ohmachi K, Aoyama Y, Hara R, Ichiki A *et al.* Single nucleotide polymorphisms of cytarabine metabolic genes influence clinical outcome in acute myeloid leukemia patients receiving high-dose cytarabine therapy. *Int J Hematol* 2015; 101(6): 543-553
25. Kaspers GJ, Veerman AJ, Pieters R, Broekema GJ, Huismans DR, Kazemier KM *et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol-tetrazolium assay. *Br J Cancer* 1994; 70(6): 1047-1052.
26. Hubeek, I., Giovannetti, E., Broekhuizen, A.J.F., Pastor-Anglada, M., Kaspers, G.J.L., Peters, G.J. Immunocytochemical detection of hENT1 and hCNT1 in normal tissues, lung cancer cell lines and NSCLC patient samples. Nucleosides, Nucleotides & Nucleic acids 2008; 27, 787-793
27. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol* 2011; 731: 237-245
28 Hubeek I, Kaspers GJ, Ossenkoppele GJ, Peters GJ. Cytosine arabinoside: Metabolism, mechanisms of resistance and clinical pharmacology. *Cancer Drug Discovery and Development: Deoxynucleoside analogs in Cancer Therapy.* 2006: 119-151.
29. Willemze R, Suciu S, Meloni G, Labar B, Marie JP, Halkes CJ *et al.* High-dose cytarabine in induction treatment improves the outcome of adult patients younger than age 46 years with acute myeloid leukemia: results of the EORTC-GIMEMA AML-12 trial. *J Clin Oncol* 2014; 32(3): 219-228

CHAPTER 6

Effect of dexamethasone on the antileukemic effect of cytarabine: role of deoxycytidine kinase

Nucleosides, Nucleotides & Nucleic Acids. 39 (10-12): 1335-1346; 2020

Adrian C Jaramillo[1], Andries M Bergman[2], Elizabeth M Comijn[3], Gerrit Jansen[4], Gertjan J L Kaspers[5,6], Jacqueline Cloos[1], Godefridus J Peters[3,7]

[1] Laboratory of Hematology, Amsterdam UMC, location VU University Medical Center, Amsterdam, the Netherlands.
[2] Netherlands Cancer Institute, Amsterdam, the Netherlands.
[3] Laboratory Medical Oncology, Amsterdam UMC, location VU University Medical Center, Amsterdam, the Netherlands.
[4] Department of Rheumatology, Amsterdam UMC, location VU University Medical Center, Amsterdam, the Netherlands.
[5] Emma Children's Hospital, Amsterdam UMC, location VUMC, Pediatric Oncology, Amsterdam, The Netherlands.
[6] Princess Maxima Center, Utrecht, the Netherlands.
[7] Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland.

ABSTRACT

Dexamethasone (DEX) is often used in the initial treatment of leukemia. Earlier we demonstrated that DEX decreased the activity of deoxycytidine kinase (dCK) which is essential for the activation of cytarabine (Ara-C). Therefore we investigated the effect of DEX on the in vivo sensitivity of acute myeloid leukemia (AML) to Ara-C and another deoxycytidine analog, gemcitabine, in the Brown Norway Myeloid Leukemia (BNML) rat model for AML, and its Ara-C resistant variant B-araC, in relation to the effects on dCK activity. The antileukemic effect was evaluated as survival of the rats, while dCK activity was measured in leukemic spleen (completely consisting of BNML cells) with liver as representative normal tissue, 24 hr after treatment with Ara-C or DEX with radioactive deoxycytidine (CdR) as a substrate. Treatment with Ara-C increased life-span of BNML by 200%, which was not affected by DEX. Gemcitabine was ineffective. In the liver of BNML bearing rats DEX decreased dCK activity 33%, while Ara-C increased dCK activity slightly (to 129%), but in the combination of Ara-C/DEX dCK activity was also decreased. In the livers of Bara-C bearing rats dCK was 2.7- fold higher compared to BNML rats, which was increased 179% in the gemcitabine-DEX treated rats. In BNML leukemic spleens DEX decreased dCK activity 41% and gem/DEX 46%, but Ara-C increased dCK activity to 123%, but in the combination this effect was neutralized. In Bara-C spleens only Ara-C/DEX decreased dCK activity (32%). In conclusion; in an AML rat model DEX did not affect the antileukemic effect of Ara-C, nor the dCK activity.

1. Introduction

Glucocorticoids such as the synthetic steroid dexamethasone (DEX)[1,2–4] can inhibit the proliferation of several forms of lymphoid cancer [5–8]. Corticosteroids are known to induce changes in gene expression and protein synthesis, yet the molecular pathways involved have not been completely elucidated [9–11]. The interference of glucocorticoids with chemotherapeutics, such as cytarabine (Ara-C), and gemcitabine [2] is not completely clear even though they are commonly used together in the clinic. In pediatric oncology the combination of ondansetron and granisetron with dexamethasone is a well-known anti-emetic combination [12]. The antimetabolite cytarabine (Ara-C), has been the mainstay of chemotherapy in AML for decades.[13–15] In order to exert its effect, Ara-C must be converted into cytosine arabinoside triphosphate (Ara-CTP) by the enzyme deoxycytidine kinase (dCK) [16] Around 30-50% of patients relapse after induction treatment, mostly due to drug resistant leukemia [15,17–19], which might be related to decreased cellular Ara-CTP accumulation and retention, [20,21] or acquired deletion of the DCK gene as found in AML cell lines resistant to Ara-C and in Ara-C refractory patients [22]. Interestingly, previous data showed that AML patient samples with wildtype FLT3 (n= 10) and Ara-C-resistant AML cell lines (n= 4) have increased sensitivity to glucocorticoids compared to mutated FLT3 [23]. Multidrug-resistance cells (both P-gP and MRP1) show collateral sensitivity to gemcitabine and Ara-C [24,25] which has been related to efflux of corticosteroids such as cortisol. It was shown that cortisol and DEX decreased dCK activity [2]. Therefore we hypothesized that DEX may affect Ara-C sensitivity in

AML. DEX use in AML is suggested to be ineffective and can even induce proliferation [12], but there may be a potential use of DEX in AML, [26] since DEX mediated proliferation induction might sensitize AML cells to Ara-C. For these reasons, we explored the effect of DEX on the anti-leukemic effect of Ara-C and gemcitabine, their toxicity and the role of dCK activity in the Brown Norway myeloid leukemia (BNML) rat model, which is considered to be characteristic for human AML [27].

2. Methods

2.1. Materials and cell culture

RPMI-1640 was purchased from Flow Laboratories (Costa Mesa, Ca, USA) and fetal calf serum (FCS) from Gibco (Thermo Scientific, Breda, the Netherlands). Ara-C was obtained from Upjohn (Capelle a/d IJssel, the Netherlands) and gemcitabine was a gift from Eli Lilly (Indianapolis, USA). [5-^{3}H]-deoxycytidine (21.9 Ci/mmol) was from Moravek (Brea, USA). All other chemicals were of analytical grade and commercially available. The *in vitro* variant, BCLO, from the Rat leukemia Brown Norway myeloid leukemia (BNML) and the Ara-C resistant variant (Bara-C) [25] were cultured in RPMI-1640 as previously described, [28] supplemented with 5% fetal calf serum (FCS) and 5% rat serum, 3.2 mM glutamine and 250 ng/ml gentamycin (Gibco) and used to induce *in vivo* leukemia in Brown Norway rats [28]. Cells were regularly screened for mycoplasma contamination and were found to be negative. BCLO and Bara-C were generously provided by Dr A. Hagenbeek (Utrecht, The Netherlands) [27].

2.2. *In vivo* studies

Ara-C was used at its maximum tolerated dose (MTD) as determined earlier [28] and given subcutaneously (s.c.) at 150 mg/kg twice a day for 3 days (q0.5d x 6). The MTD of gemcitabine in these rats was not known and had to be determined; doses of 50, 25, 10 and 5 mg/kg caused an immediate weight loss of > 20%, at 3 mg/kg of about 20%, while at 1 mg/kg (q3dx4) toxicity was acceptable (< 15% weight loss). Since the pharmacokinetics of gemcitabine in rats was also unknown and expected to be different from mice, we also measured plasma concentrations of gemcitabine by taking blood samples via the tail vein. Blood was processed and gemcitabine was measured as determined earlier [29]. The dose of dexamethasone was based on earlier studies and was 3 mg/kg. Body weight was determined on the first day of treatment (day 0) and set at 100%. Rats were weighed at least five times a week (starting on the first day of the treatment). Signs of sickness and mortality were recorded and moribund animals were euthanized and examined macroscopically for side effects. BNML and Bara-C were established as described earlier and maintained by isolation of leukemic cells from the spleen of a Brown Norway rat, as previously described [27]. Death was expected around day 25 after injection, and rats were euthanized when severe weight loss was found indicating that rats became moribund. Treatment was started when the spleen cells were completely replaced by leukemic cells (day 15) [28]. Anti-leukemic effect studies were performed at the MTDs. The randomized control and treatment groups each consisted of 5 rats. Survival times (day

of scarifying plus one) of the animals were used to calculate a median life span of the group (starting with the first day of treatment). The increase in life span (ILS) was determined using the following calculation: % ILS= T/C x 100 (T and C are the median days of death for the treated and control groups, respectively). Efficacy criteria were defined as follows: Inactive= ILS <125%, active= ILS >125% and very active= ILS >200% [28]. Significance of differences between control and treatment groups were calculated by using the non-parametric Mann Whitney U test.

2.2. dCK activity

In order to determine whether the potential interference of dexamethasone was mediated by changes in the activity of the key enzyme dCK, rats were euthanized one day after drug administration and livers (as a normal organ) and spleens (which were completely leukemic at the time of the experiment as was verified by FACS analysis), were immediately removed. Livers (without visible infiltration of leukemic cells) were immediately frozen in liquid nitrogen, while leukemic spleens were gently disrupted on a sieve, washed and the pellet was immediately frozen in liquid nitrogen. In order to determine dCK activity, livers were homogenized using a microdismembrator [30] and the powder was suspended in ice-cold assay buffer at a 1 to 4 ratio (300 mM Tris-HCL, 1 mM EDTA, 50 µM β-mercaptoethanol, pH 8.0). Leukemic spleen cells were suspended in this buffer at 10 x 10^6 cells/ml. The suspension was centrifuged at 600 g and the supernatant subsequently at 20,000 g. This supernatant was used for the enzyme assay, which was performed as described previously [2,31] (using 3H-labeled deoxycytidine (final concentration 230 µM, specific activity 0.04 Ci/mmol) in the presence of 2.5mM MgCl2, 5mM ATP and 1mM thymidine to inhibit thymidine kinase 2 which may also catalyze deoxycytidine phosphorylation [32]. Interference of the reaction by degradation of deoxycytidine by cytidine deaminase (CDA) could be neglected since rats do not express CDA. The enzyme assay was linear in time (assay time 15-60 minutes) and with protein (in the range of 50-500 µg protein per assay). Enzyme activity was expressed as pmol dCMP formed per hour per mg protein (pmol/hr/mg protein). Protein was determined using the Bradford assay. Statistics were performed using the Student's t-test for unpaired samples.

3. Results

3.1. Effect of dexamethasone on the anti-leukemic effect of Ara-C in BNML and Bara-C bearing rats

Rats inoculated with the leukemia cells showed some increase in weight, which was not affected by either Ara-C or gemcitabine, (no weight loss, except for the Ara-C alone of 6% in the Bara-C group and of 7% in the gem-dexamethasone BNML group). The combination of Ara-C/ Dexamethasone had the same significant ($p<0.05$) anti-leukemic effect as Ara-C alone (ILS 200%), while the Dexamethasone monotherapy had a moderate anti-leukemic effect (ILS 140%) (Fig. 1). Gemcitabine did not show an anti-leukemic effect. In the Bara-C group, Ara-C monotherapy was ineffective with an ILS of 73%, which was possibly related to toxicity of Ara-C itself (20% weight loss

at day 7). Although dexamethasone by itself was ineffective, the combination had some non-significant anti-leukemic effect (ILS of 118%) activity, similar to that of the gemcitabine-DEX combination, while gemcitabine alone was ineffective (ILS 91%).

Table 1: Activity of deoxycytidine kinase (dCK) in leukemic spleens and livers from BNML and Bara-C rats

Tissue	BNML rats	Bara-C rats
Leukemic spleen	964 ± 121	611 ± 116
Liver	434 ± 173	1189 ± 215*

*Values (in pmol/hr per mg protein) are means of tissues obtained from 3 rats inoculated with either BCLO cells (BNML rats) or Bara-C cells (Bara-C rats). Value are means ± SEM. * The dCK activity in Bara-C livers was significantly higher than in the BNML rats (p< 0.05)*

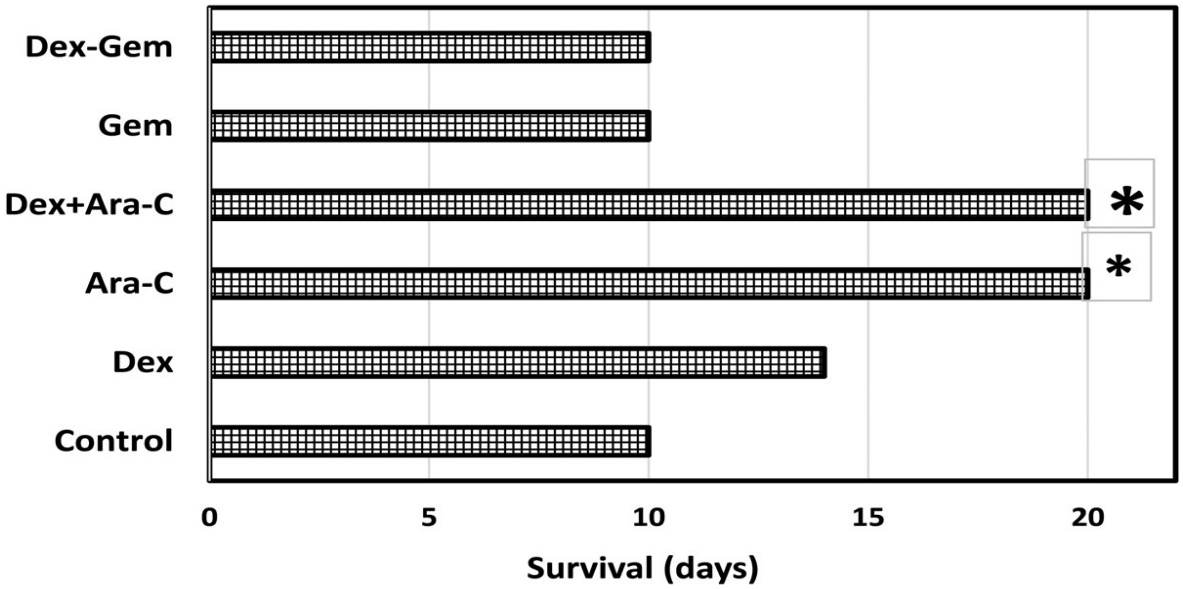


***Figure 1**. Effect of dexamethasone (dex) on the Ara-C and gemcitabine (gem) treatment of BNML rats given as survival time (in days). Values are medians of five animals per group. Drug schedules consisted of Ara-C (s.c.) 150 mg/kg twice a day for 3 days (q0.5d x 6). Dexamethasone was dosed at 3 mg/kg. Only the effect of Ara-C and Ara-C/dex in the BNML rats was significantly different from the controls at p<0.05 (*).*

3.2. Effect of dexamethasone on dCK activity in leukemic spleens from BNML and Bara-C rats

In this study we aimed to investigate the effect of treatment on dCK activity; therefore we measured the activity of dCK in leukemic spleens of control and DEX/Ara-C/gemcitabine treated animals. In BNML leukemic spleens, DEX decreased the activity of dCK significantly (p<0.05), while Ara-C treatment led to a slight increase of 123% (Fig. 2A). Gemcitabine did not affect the dCK activity. DEX decreased the dCK

compared to Ara-C ($p<0.05$) or gemcitabine alone. The dCK activity in leukemic spleens of Bara-C rats was about half of that in BNML rats, possibly explaining the lower Ara-C anti-leukemic effect (Fig. 2B). In these rats DEX, Ara-C and gemcitabine did not significantly affect dCK activity. However, co-treatment with DEX decreased the dCK activity compared to Ara-C and gemcitabine alone by 32% and 21%, respectively.

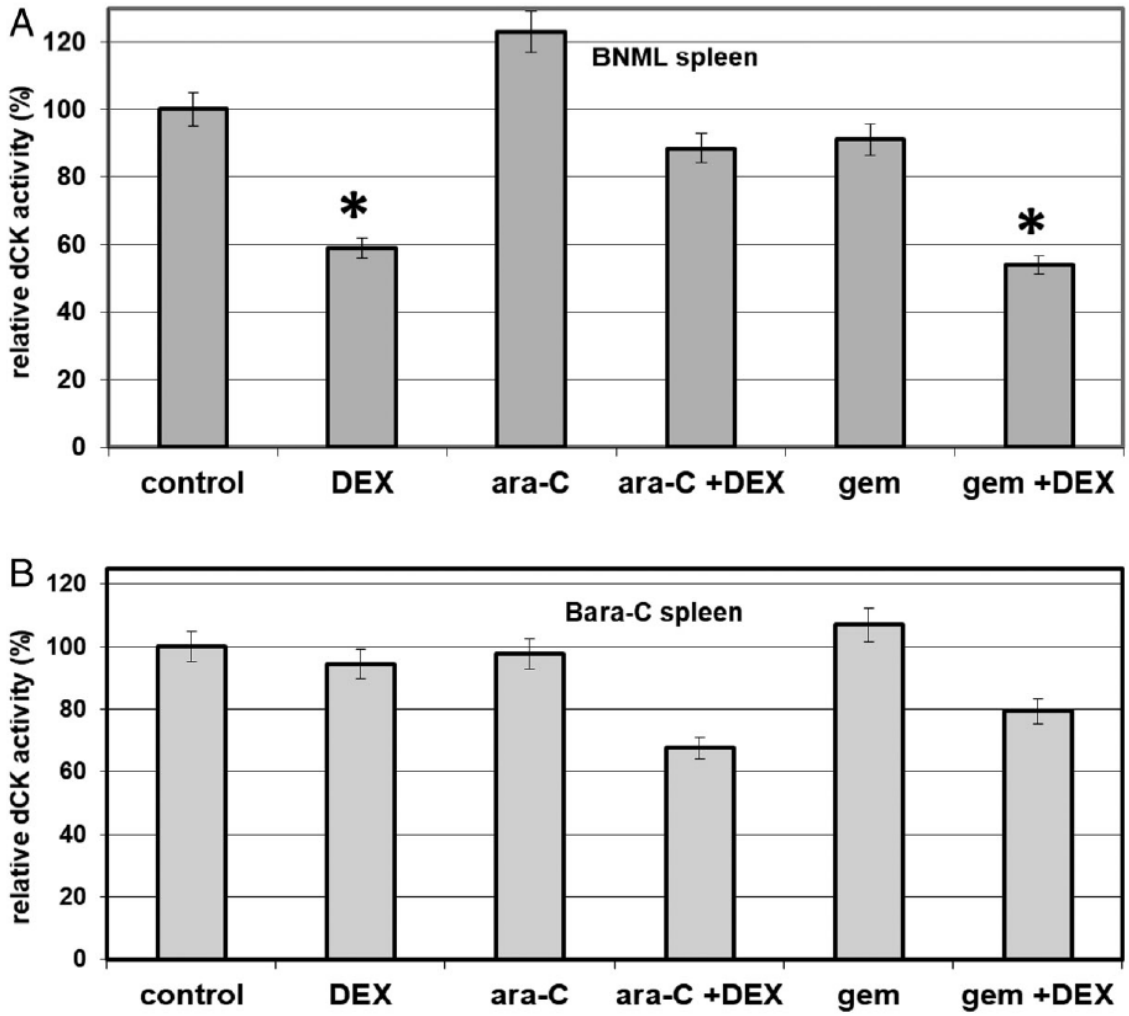


Figure 2. *Relative activity of dCK (in % from untreated animals; the bars provide the relative SEM) in leukemic spleens from BNML (A) and Bara-C (B) rats. dCK activities were measured one day after administration of dexamethasone (DEX), gemcitabine (gem), Ara-C and their combination. The SEM of absolute dCK activities from 3 treated and 3 untreated animals was less than 25%. Controls received saline. The mean value of the untreated animals (from Table 1) was set at 100%. Significance was calculated based on these absolute activities; index and gem/DEX treated animals this activity in BNML spleens was significantly lower than in untreated animals at $p<0.05$ (*).*

3.3. Effect of dexamethasone on dCK activity in normal livers from BNML and Bara-C rats

Livers were included in this study to determine whether treatment would also affect the dCK activity in a normal, non-leukemic organ. At the time of harvesting no visible leukemic cell infiltration in the liver was observed. This would have been visible as

white spots. In livers from BNML rats DEX decreased the dCK by 33%, while Ara-C and gemcitabine increased the dCK to 129 and 179%, respectively, compared to untreated rats (Fig. 3A). In these groups dexamethasone co-treatment decreased the dCK activity in the Ara-C/dexamethasone group by 39% and in the gemcitabine-dexamethasone group even by 23%. In contrast to the leukemic spleens the dCK activity in livers of leukemic Bara-C rats was almost 3.5 fold higher compared to livers from BNML rats (Fig. 3B). In these rats any treatment did not decrease the dCK activity, but both dexamethasone and gemcitabine increased the dCK activity to 119 and 141%, respectively, while dexamethasone co-treatment even led to a further significant ($p<0.05$) increase to 172% in livers of the gemcitabine-dexamethasone treated rats.

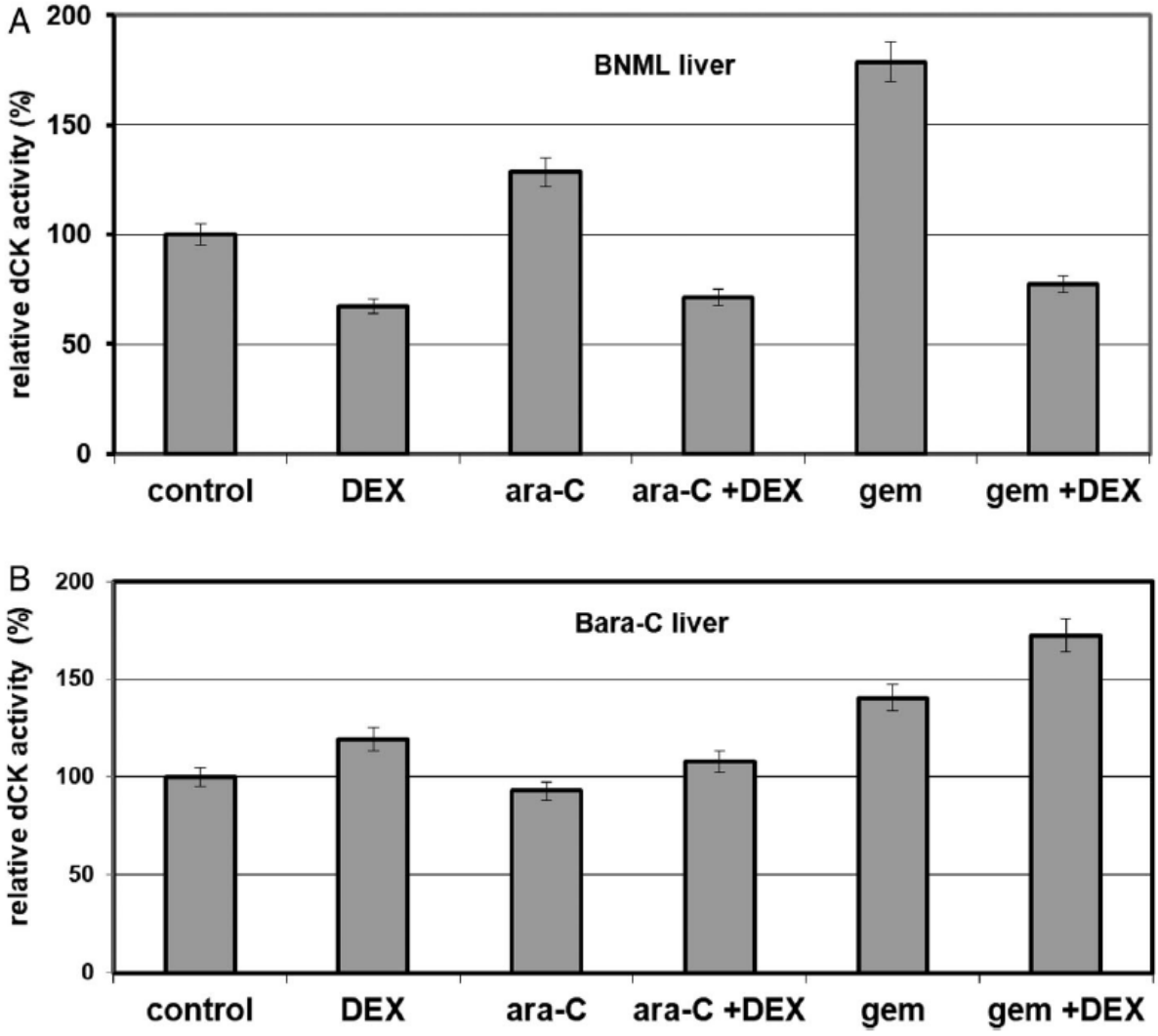


Figure 3. *Relative activity of dCK (in % from untreated animals; the bars provide the relative SEM) in livers from BNML (A) and Bara-C (B) rats. dCK activities were measured one day after administration of dexamethasone (DEX), gemcitabine (GEM), Ara-C and their combination. The SEM of absolute dCK activities from 3 treated and 3 untreated animals was less than 25% (except BNML liver). Controls received saline. The mean value of the untreated animals (from Table 1) was set at 100%. Significance was calculated based on these absolute activities; in GEM/DEX treated animals this activity in BNML livers was significantly higher than in untreated animals at $p<0.05$ (*).*

3.4. Gemcitabine pharmacokinetics

In order to explain the low efficacy of gemcitabine against BNML we determined the plasma concentrations of gemcitabine (Fig. 4); in line with the known absence of CDA in rats, the pharmacokinetics was different from mice [29] and humans [33] with a peak level of 2.9 µM at 60 minutes (which is far below the effective concentration of at least 60 µM), a half-life of more than 4 hours and no formation of the gemcitabine metabolite difluorodeoxyuridine (Fig. 4).

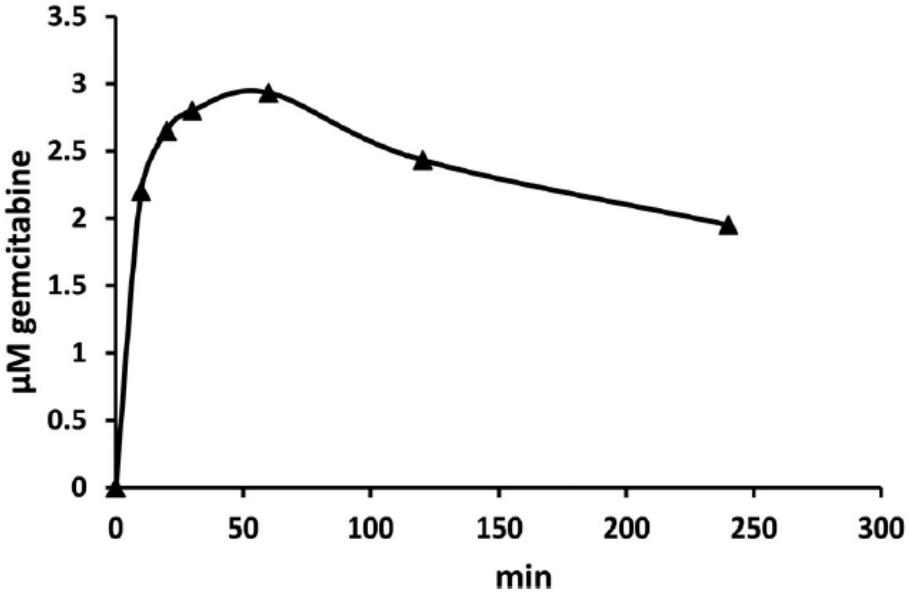


Figure 4. *Plasma concentrations of gemcitabine in healthy Brown Norway rats. Blood was drawn via the tail vein in heparinized tubes, centrifuged and the gemcitabine concentrations in plasma were determined as described in the Methods section. (n=3; SEM less 25%). No 2',2'-difluoro-2'-deoxyuridine could be detected. The half-life could not be determined reliably since at the last sampling the gemcitabine concentration was still more than 50% of the peak level.*

4. Discussion

In this paper we demonstrate that dexamethasone does not affect the antileukemic effect of Ara-C, in a rat model which is considered to be representative for human AML [27]. Since the antileukemic effect of Ara-C is comparable (moderate) to that in humans, we chose this model to study the effect of dexamethasone. The activity of dCK was decreased by dexamethasone treatment. However, treatment with Ara-C or gemcitabine increased the dCK activity. In the combined treatment the effect was neutralized. Gemcitabine did not have any effect, which is possibly due to the observation that cytotoxic plasma levels of gemcitabine were too low in these rats to achieve any antitumor effect. The known deficiency of CDA explains the completely different pharmacokinetics of gemcitabine in these rats, so that plasma concentrations were too low. In humans and mice, the peak at a therapeutic dose is around 40 (humans) and 225 (mice) µM with a half-life of about 15 minutes [29]. However, the low concentration of gemcitabine was maintained for a longer period, which was shown to be cytotoxic in cell culture and expected to be effective in this rat model as

well. However, the inefficacy of gemcitabine is in line with the lack of a clinically relevant antileukemic effect. In childhood acute lymphoblastic leukemia dexamethasone is often used as induction therapy, and in AML it may be used as an anti-emetic, similar to solid tumors. Indeed in the rat model system dexamethasone has some anti-leukemic effect. Interestingly in Bara-C rats, the basal dCK activity was lower than in BNML rats, as was also shown earlier in cultured BNML and Bara-C cells [25]. However, in livers of these rats the dCK activity was 3.5- fold higher in Bara-C rats. Apparently, the presence of the tumor causes an induction of dCK. Earlier it has been published that regulation of dCK activity in both normal and tumor cells is not at the mRNA levels nor at the protein level [34]. Although no leukemic cell infiltration was observed in the livers, we may assume that the extensive leukemic load in the rats may affect dCK activity. Since the Bara-C model seems more aggressive this effect may be more pronounced in the Bara-C rats. Moreover, Csapó et al [35]. showed that a stress like treatment (a drug, radiation) leading to inhibition of DNA synthesis can lead to an increased dCK activity, an effect that was usually higher in normal cells. Earlier we demonstrated a similar effect in tumor cells for radiation, which was not associated with an increased dCK protein level [36]. Smal et al provided evidence that this effect could be explained by phosphorylation of human dCK at the serine site [37]. Unfortunately we were not able to perform similar studies for the rat dCK due to the lack of a suitable anti-rat antibody. Therefore we concentrated in this study on the dCK activity itself. Indeed treatment with drugs (a stress like situation) increased dCK levels, which was most pronounced in gemcitabine and Ara-C treated BNML rats, but less in Bara-C rats. However, in the BNML livers addition of DEX decreased dCK activity compared to Ara-C and gemcitabine alone, which would fit with a protective effects in normal tissues. Only gemcitabine (not used in AML) would increase dCK activity in Bara-C rats. In conclusion: although dexamethasone had some downregulating effect on dCK, it did not affect the anti-leukemic effect of Ara-C.

References

1 Nieman, L. K. Pharmacologic Use of Glucocorticoids. UpToDate: Waltham, MA, 2020.
2 Bergman, A. M.; Pinedo, H. M.; Peters, G. J. Steroids Affect Collateral Sensitivity to Gemcitabine of Multidrug-Resistant Human Lung Cancer Cells. Eur. J. Pharmacol. 2001, 416, 19–24.
3 Herr, I.; Ucur, E.; Herzer, K.; Okouoyo, S.; Ridder, R.; Krammer, P. H.; von Knebel Doeberitz, M.; Debatin, K.-M. Glucocorticoid Cotreatment Induces Apoptosis Resistance toward Cancer Therapy in Carcinomas. Cancer Res. 2003, 63, 3112–3120.
4 Barbour, S. Y. Corticosteroids in the Treatment of Chemotherapy-Induced Nausea and Vomiting. J. Natl. Compr. Canc. Netw. 2012, 10, 493–499.
5 Fietz, E. R.; Keenan, C. R.; López-Campos, G.; Tu, Y.; Johnstone, C. N.; Harris, T.; Stewart, A. G. Glucocorticoid Resistance of Migration and Gene Expression in a Daughter MDA-MB-231 Breast Tumour Cell Line Selected for High Metastatic Potential. Sci. Rep. 2017, 7, 43774.
6 Nakane, T.; Szentendrei, T.; Stern, L.; Virmani, M.; Seely, J.; Kunos, G. Effects of IL- 1 and Cortisol on Beta-Adrenergic Receptors, Cell Proliferation, and Differentiation in Cultured Human A549 Lung Tumor Cells. J. Immunol. 1990, 145, 260–266.
7 Croxtall, J. D.; Flower, R. J. Lipocortin 1 Mediates dexamethasone-induced growth arrest of the A549 lung adenocarcinoma cell line . Proc. Natl. Acad. Sci. USA. 1992, 89, 3571–3575.
8 Lu, Y.-S.; Lien, H.-C.; Yeh, P.-Y.; Yeh, K.-H.; Kuo, M.-L.; Kuo, S.-H.; Cheng, A.-L. Effects of Glucocorticoids on the Growth and Chemosensitivity of Carcinoma Cells Are Heterogeneous and Require High Concentration of Functional Glucocorticoid Receptors. World J. Gastroenterol. 2005, 11, 6373–6380.
9 Ivarie, R. D.; O'Farrell, P. H. The Glucocorticoid Domain: Steroid-Mediated Changes in the Rate of Synthesis of Rat Hepatoma Proteins. Cell 1978, 13, 41–55
10 Greenstein, S.; Ghias, K.; Krett, N. L.; Rosen, S. T. Mechanisms of Glucocorticoid- Mediated Apoptosis in Hematological Malignancies. Clin. Cancer Res. 2002, 8, 1681–1694.
11 Wei, G.; Twomey, D.; Lamb, J.; Schlis, K.; Agarwal, J.; Stam, R. W.; Opferman, J. T.; Sallan, S. E.; den Boer, M. L.; Pieters, R.; et al. Gene Expression-Based Chemical Genomics Identifies Rapamycin as a Modulator of MCL1 and Glucocorticoid Resistance. Cancer Cell. 2006, 10, 331–342.
12 Klein, K.; Haarman, E. G.; de Haas, V.; Zwaan, C. M.; Creutzig, U.; Kaspers, G. L. Glucocorticoid-Induced Proliferation in Untreated Pediatric Acute Myeloid Leukemic Blasts. Pediatr. Blood Cancer 2016, 63, 1457–1460
13 Rowe, J. M. Optimal Induction and Post-Remission Therapy for AML in First Remission. Hematol. Am. Soc. Hematol. Educ. Prog. 2009, 2009, 396–405.
14 Cros, E.; Jordheim, L.; Dumontet, C.; Galmarini, C. M. Problems Related to Resistance to Cytarabine in Acute Myeloid Leukemia. Leuk. Lymphoma. 2004, 45, 1123–1132.
15 Wolach, O.; Itchaki, G.; Bar-Natan, M.; Yeshurun, M.; Ram, R.; Herscovici, C.; Shpilberg, O.; Douer, D.; Tallman, M. S.; Raanani, P.; et al. High-Dose Cytarabine as Salvage Therapy for Relapsed or Refractory Acute Myeloid Leukemia—Is More Better or More of the Same? Hematol. Oncol. 2016, 34, 28–35.
16 Ruiz van Haperen, V. W. T.; Peters, G. J. New Targets for Pyrimidine Antimetabolites for the Treatment of Solid Tumours. Pharm. World Sci. 1994, 16, 104–112. DOI: 10.1007/bf01880661.
17 Kern, W.; Estey, E. H. High-Dose Cytosine Arabinoside in the Treatment of Acute Myeloid Leukemia: Review of Three Randomized Trials. Cancer 2006, 107, 116–124.
18 De Kouchkovsky, I.; Abdul-Hay, M. 'Acute myeloid leukemia: A comprehensive review and 2016 update'. Blood Cancer J. 2016, 6, e441.
19 Thomas, X.; Dombret, H. Timed-sequential chemotherapy as induction and/or consolidation regimen for younger adults with acute myelogenous leukemia. Hematology 2007, 12, 15–28.
20 Plunkett, W.; Liliemark, J. O.; Estey, E.; Keating, M. J. Saturation of Ara-CTP Accumulation during High-Dose Ara-C Therapy: Pharmacologic Rationale for Intermediate-Dose Ara-C. Semin. Oncol. 1987, 14, 159–166.
21 Estey, E.; Plunkett, W.; Dixon, D.; Keating, M.; McCredie, K.; Freireich, E. J. Variables Predicting Response to High Dose Cytosine Arabinoside Therapy in Patients with Refractory Acute Leukemia. Leukemia 1987, 1, 580–583.
22 Peters, G. J.; Jansen, G. Resistance to Antimetabolites. In Principles of Antineoplastic Drug Development and Pharmacology; Schilsky, R. L., Milano, G. A., Ratain, M. P., Eds.; Marcel Dekker, Inc: New York, 1996; pp 543–585.
23 Malani, D.; Murumägi, A.; Yadav, B.; Kontro, M.; Eldfors, S.; Kumar, A.; Karjalainen, R.; Majumder, M. M.; Ojamies, P.; Pemovska, T.; et al. Enhanced Sensitivity to Glucocorticoids in Cytarabine-Resistant AML. Leukemia 2017, 31, 1187–1195.

24 Bergman, A. M.; Pinedo, H. M.; Talianidis, I.; Veerman, G.; Loves, W. J. P.; van der Wilt, C. L.; Peters, G. J. Increased Sensitivity to Gemcitabine of P-Glycoprotein and Multidrug Resistance-Associated Protein-Overexpressing Human Cancer Cell Lines. Br. J. Cancer 2003, 88, 1963–1970.

25 Bergman, A. M.; Pinedo, H. M.; Jongsma, A. P.; Brouwer, M.; Ruiz van Haperen, V. W.; Veerman, G.; Leyva, A.; Eriksson, S.; Peters, G. J. Decreased Resistance to Gemcitabine (20,20-Difluorodeoxycytidine) of Cytosine Arabinoside-Resistant Myeloblastic Murine and Rat Leukemia Cell Lines: Role of Altered Activity and Substrate Specificity of Deoxycytidine Kinase. Biochem. Pharmacol. 1999, 57, 397–406.

26 Hicsonmez, G. The Effect of Steroid on Myeloid Leukemic Cells: The Potential of Short-Course High-Dose Methylprednisolone Treatment in Inducing Differentiation, Apoptosis and in Stimulating Myelopoiesis. Leukemia Res. 2006, 30, 60–68.

27 Martens, A. C.; Van Bekkum, D. W.; Hagenbeek, A. The BN Acute Myelocytic Leukemia (BNML) (a Rat Model for Studying Human Acute Myelocytic Leukemia (AML)). Leukemia 1990, 4, 241–257.

28 Hubeek, I.; Comijn, E. M.; Van der Wilt, C. L.; Merriman, R. L.; Padron, J. M.; Kaspers, G. J. L.; Peters, G. J. CI-994 (N-Acetyl-Dinaline) in Combination with Conventional anti-Cancer Agents is Effective against Acute Myeloid Leukemia in Vitro and in Vivo. Oncol. Rep. 2008, 19, 1517–1523.

29 Veerman, G.; Ruiz van Haperen, V. W. T.; Vermorken, J. B.; Noordhuis, P.; Braakhuis, B. J. M.; Pinedo, H. M.; Peters, G. J. Antitumor Activity of Prolonged as Compared with Bolus Administration of 2',2'-Difluorodeoxycytidine in Vivo against Murine Colon. Cancer Chemother. Pharmacol. 1996, 38, 335–342.

30 Peters, G. J.; Laurensse, E. J.; Leyva, A.; Pinedo, H. M. Tissue Homogenization Using a Micro-Dismembrator for the Measurement of Enzyme Activities. Clin. Chim. Acta 1986, 158, 193–198.

31 Ruiz van Haperen, V. W. T.; Veerman, G.; Braakhuis, B. J. M.; Vermorken, J. B.; Boven, E.; Leyva, A.; Peters, G. J. Deoxycytidine kyinase and deoxycytidine deaminase activities in human tumour xenografts. Eur. J. Cancer 1993, 29, 2132–2137.

32 Eriksson, S.; Kierdaszuk, B.; Munch-Petersen, B.; Oberg, B.; Johansson, N. G. Comparison of the Substrate Specificities of Human Thymidine Kinase 1 and 2 and Deoxycytidine Kinase toward Antiviral and Cytostatic Nucleoside Analogs. Biochem. Biophys. Res. Commun. 1991, 176, 586–592.

33 Peters, G. J.; Clavel, M.; Noordhuis, P.; Geyssen, G. J.; Laan, A. C.; Guastalla, J.; Edzes, H. T.; Vermorken, J. B. Clinical Phase I and Pharmacology Study of Gemcitabine (2', 2'-Difluorodeoxycytidine) Administered in a Two-Weekly Schedule. J. Chemother. 2007, 19, 212–221.

34 Spasokoukotskaja, T.; Sasvári-Székely, M.; Keszler, G.; Albertioni, F.; Eriksson, S.; Staub, M. Treatment of Normal and Malignant Cells with Nucleoside Analogues and Etoposide Enhances Deoxycytidine Kinase Activity. Eur. J. Cancer 1999, 35, 1862–1867.

35 Csapó, Z.; Sasvári-Székely, M.; Spasokoukotskaja, T.; Staub, M. Modulation of Human Deoxycytidine Kinase Activity as a Response to Cellular Stress Induced by NaF. Acta Biochim. Pol. 2001, 48, 251–256.

36 Sigmond, J.; Bergman, A. M.; Leon, L. G.; Loves, W. J. P.; Hoebe, E. K.; Peters, G. J. Staurosporine Increases Toxicity of Gemcitabine in Non-Small Cell Lung Cancer Cells: Role of Protein Kinase C, Deoxycytidine Kinase and Ribonucleotide Reductase. Anticancer. Drugs. 2010, 21, 591–599.

37 Smal, C.; Cardoen, S.; Bertrand, L.; Delacauw, A.; Ferrant, A.; Van den Berghe, G.; Van Den Neste, E.; Bontemps, F. Activation of Deoxycytidine Kinase by Protein Kinase Inhibitors and Okadaic Acid in Leukemic Cells. Biochem. Pharmacol. 2004, 68, 95–103.

CHAPTER 7

Cannabidiol for treatment of (drug-resistant) leukemia cells: a double-edged sword!?

Manuscript in preparation

Adrian C Jaramillo[1,2], Johan van Meerloo[1], Margot S.F. Roeten[1], Fleur Janssen[1], Arwen Stikvoort[1], Ronald W. Stam[3], Godefridus J. Peters[2, 5], Gerrit Jansen[4#], Jacqueline Cloos[1#]

[1] Departments of Hematology and [2] Medical Oncology, Amsterdam University Medical Centers, location VUmc, Amsterdam, the Netherlands

[3] Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

[4] Amsterdam Rheumatology and immunology Center, Amsterdam University Medical Center, location VUmc, Amsterdam, the Netherlands

[5] Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland

[#] shared senior authorship

ABSTRACT

Background: Cannabidiol (CBD) is a popular natural medicine product which has anti-inflammatory and anti-epileptic effects. Beyond this, CBD gained interest as a potential anti-cancer drug given its antitumor effects described both *in vitro* and *in vivo*.

Aim: To investigate the *in vitro* growth inhibitory effects of CBD in human acute lymphoblastic and acute myeloid leukemia cells and drug-resistant sublines, and explore underlying mechanisms of action of CBD.

Methods: Models included CCRF-CEM acute lymphoblastic and THP-1 acute myeloid leukemia cells, a subline of CEM with acquired resistance to the proteasome inhibitor bortezomib (CEM/BTZ), CEM cells adapted for >3 months to 6250 nM CBD (CEM/CBD6250), and lastly normal bone marrow (NBM) cells. Cell growth inhibition was measured with the MTT assay and cell proliferation by flow cytometry using CellTrace™ Violet (Invitrogen, Thermo Fisher Scientific) dye. CBD-induced effects on apoptosis and cell cycle were assessed by flow cytometry. Mechanistic studies included Western blot analyses of CBD receptor 1/2 expression, G protein-coupled receptor GPR55 and GPR119 expression, p-4E-BP1 expression as an indicator of mTOR inhibition, and MARCKS expression as indicator of BTZ resistance and ER stress.

Results: CBD inhibited growth of CCRF-CEM and THP-1 cells in the low micromolar range (IC50: 5.1 and 9.2 µM, respectively), whereas NBM were rather insensitive to CBD (IC50: 43 µM). Drug-resistant CEM/BTZ cells were 2.7 fold cross-resistant to CBD compared with CCRF-CEM cells, and CEM/CBD6250 cells were just two-fold resistant (IC50: 10.7 µM). Intriguingly, at low concentrations of CBD (i.e. 1 µM) CBD showed marked cell-density dependent cell growth stimulatory effects for CEM/BTZ cells (up to 3-fold) and CEM/CBD6250 cells (up to 7-fold). Moreover, CEM/CBD6250 cells displayed a CBD-addiction phenotype requiring CBD for their growth.

CBD also induced apoptosis but induction proceeded slowly (> 48 hrs) as compared to BTZ (< 24 hrs). CBD had minor impact on cell cycle distribution.

CB1 and CB 2 expression was hardly detectable in (drug-resistant) CEM cells. CBD had minor impact on cell cycle distribution.

CEM/BTZ and CEM/CBD6250 cells exhibited a marked down-regulation of GPR55 and GPR119 expression. Interestingly, CEM/CBD6250 cells had a significantly enhanced MARCKs expression to levels observed in CEM/BTZ cells. Finally, inhibition of p-4E-BP1 expression paralleled CBD-induced growth inhibition in CCRF-CEM and drug-resistant sublines.

Conclusions: CBD markedly inhibited growth of leukemia cells at concentrations >5 µM, but induced cell growth stimulatory effects for drug-resistant leukemia cells at low CBD concentrations (~1 µM). CEM/CBD6250 cells acquired a CBD addiction phenotype which may serve as an excellent tool for further investigations into molecular basis for this property.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is an aggressive hematological malignancy, which accounts for 20% of adult leukemia cases and is the most common type of cancer in childhood[1]. Failure of long-term remission occurs in ~20% of children and ~40% of adult patients. Approximately 20% of children with ALL relapse, mainly due to chemotherapy resistance, and have a dismal five-year survival[1,2] . Therefore, novel therapeutic strategies for the treatment of ALL are still needed[2]. In this context, cannabinoids have been proposed as potential anti-leukemic agents given their reported anti-tumor activity against solid tumors[3–8].

Cannabidiol (CBD) is one of over sixty phytocannabinoids derived from the plant *Cannabis sativa*[9,10]. Phytocannabinoids, such as CBD and delta-9-tetrahydro-cannabidiol (THC), along with endogenous endocannabinoids (anandamide, and 2-arachidonoylglycerol [2-AG]), are capable of interacting with endogenous receptors distributed in different cell types. These include cannabinoid receptor CB1 and the ion channel receptor Transient Receptor Potential channel subfamily V member 1 (TRPV1), mostly expressed on nerve cells, and CB2 and G protein-coupled receptor 55 (GPR55) widely expressed on immune cells, together constituting the endocannabinoid system (ECS)[10–13]. In addition, another G protein-coupled receptor, GPR119, was identified as putative cannabinoid receptor[14]

There is accumulating evidence suggesting that the endocannabinoid system is pivotal in modulating basic homeostatic functions such as appetite, metabolism, the immune response, and memory formation[10,13]. Even though cannabis products have been used in palliative care extensively[15], recent data suggest that some cannabinoids exert an important role in both cell proliferation/tumorigenesis[16–19]**.** This has transformed the endocannabinoid receptors into attractive new targets for the treatment of several cancer subtypes. In fact, upregulated expression of various cannabinoid receptors and overexpression of the ECS components is correlated with tumour aggressiveness [20–23]. Either by receptor-dependent or receptor-independent interactions, CBD initiates a signaling sequence including stimulation of *de novo* synthesis of ceramide, which triggers ER-stress, and increases reactive oxygen species (ROS) production, which ultimately leads to autophagy and apoptosis induction[6,24–27]; Furthermore, CBD-induced apoptosis was accompanied by down-regulation of mTOR, cyclin D1 and up-regulation of PPARγ protein expression[28].

CBD has been evaluated for its anti-leukemic activity in leukemic cell lines revealing inhibition of differentiation[29] and apoptosis induction[30–32], usually at CBD concentrations above 10 µM.

In this study we examined the anti-proliferative effects of CBD in leukemia cell lines and subclones of CCRF-CEM leukemia cells with acquired resistance to the proteasome inhibitor bortezomib (BTZ)[33]. Recently CBD was proposed to overcome BTZ resistance and was tested in combination regimens with proteasome inhibitors for treatment of hematological malignancies [24,34]. In addition, we characterized the features of a CCRF-CEM line that was adapted to grow in sub-toxic concentrations of CBD for a period of 4 months after which cells exhibited a drug-addicted phenotype.

MATERIALS AND METHODS

Chemicals

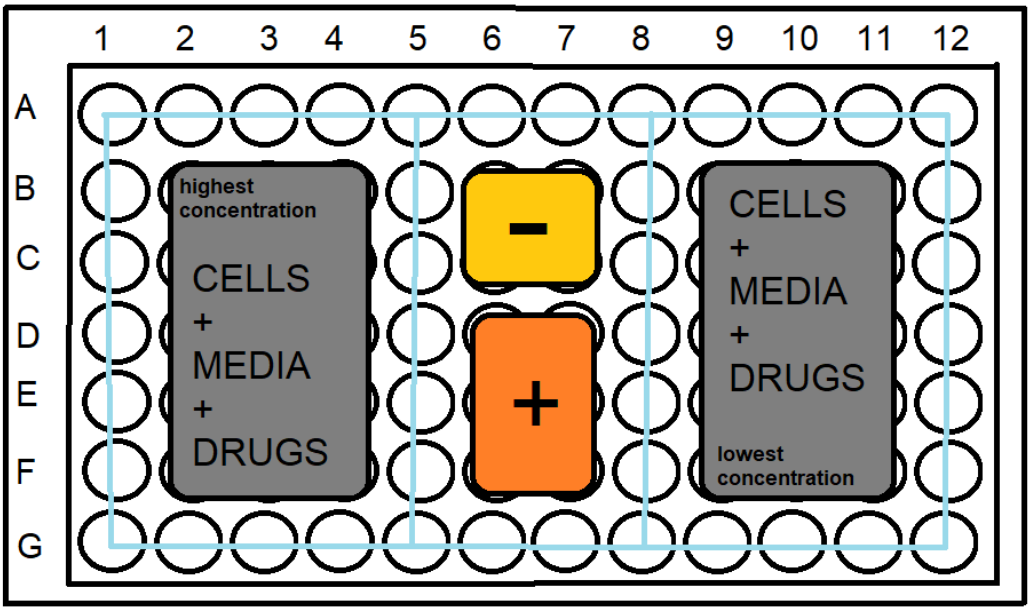
CBD (>98%) (Merck, Germany) was diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) at 25 mM stock solutions and stored at -30°C. Bortezomib (Millennium Pharmaceuticals, USA) was dissolved in DMSO (Sigma-Aldrich, USA) and 1 mM stock solutions were stored at -30^{0}C.

Human cell lines and patient samples

The human myeloid leukemia cell line THP-1 (ATCC® TIB-202™), human T-cell leukemia cell line CCRF-CEM (wild type / WT, ATCC® CCL-119) and its bortezomib (BTZ)-resistant sublines CEM/BTZ7 (10-fold BTZ-resistant) and CEM/BTZ200 cells (170-fold BTZ resistant)[35,36] were used for cell growth inhibition assays. Additionally, we generated a subline of CCRF-CEM (i.e. CEM/CBD 6250) by gradually adapting CEM cells to increasing concentrations of CBD from 1000 to 6250 nM CBD over a period of 4 months. Cells were cultured in RPMI-1640 cell culture medium (Gibco, Thermo Fisher Scientific) containing 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin (5000 U/ml) (Gibco, Thermo Fisher Scientific) and incubated at 37°C in a humidified 5% CO_2 incubator. Cell cultures were refreshed twice weekly at a density of 3 x 10^5 cells/ml. Normal bone marrow (BM) was obtained during cardiological or hip replacement surgeries after written permission according to Dutch law. Mononuclear cells (MNC) were isolated from fresh BM by filtering unprocessed material through a 70 µm strainer followed by centrifugation with Ficoll (Sigma-Aldrich, USA) at 1800 rpm for 20 minutes at room temperature (RT). Remaining red blood cell (RBC) were lysed for ten minutes on ice, with a buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA (pH 7.4), and cells were then washed twice with PBS with 0.1% FCS. Isolated BM MNCs were kept in RPMI-1640 medium supplemented with 20% FCS and were used for analysis immediately thereafter.

MTT drug sensitivity assay

For assessment of drug sensitivity the 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was performed essentially as described by van Meerloo et al[37]. Briefly, this colorimetric assay relies on the conversion of MTT into insoluble formazan crystals by the mitochondria of living cells and is widely used to assay drug sensitivity on primary patient cells or cell lines. For this purpose, cells suspended in culture medium were incubated in 96-wells plates. CEM and THP-1 cell lines were cultured in flat-bottom well plates at a concentration of 3 - 6 x 10^3 cells/well while primary cells were cultured in round bottom well plates at a concentration of 0.16 x 10^6 cells/well. With cell lines each well contained a total volume of 150 µl, composed of 120 µl of cell suspension and 30 µl of culture medium only (control) or culture medium + drug (treatment) at different concentrations, as depicted in the layout below:



For primary cells, a total volume of 100 µl (80 µl cell suspension + 20 µl drug) was used. For blank controls, the wells only contained complete medium, for the control wells, cells were cultured in medium only. The MTT assay was performed in triplicates and plates were incubated at 37°C, in a humidified 5% CO_2 incubator for 4 days. At the end of the incubation period, 15 µl of MTT solution (5 mg/ml in PBS) was added to each well of the cell lines and 10 µl of MTT solution to each well of the primary samples. These plates were incubated for an additional 4 hours at 37ºC, followed by addition of 150 µl acidified isopropanol for cell line plates and 100 µl for the primary cell plates All wells were vigorously mixed until crystals were completely dissolved after 10 minutes and the plates were ready for readout. The optical density (OD) of each well was measured in a Glomax plate reader (Promax, USA) at 540. For the data analysis, the mean OD of the blank wells was subtracted from the mean OD of the control wells and of the treatment wells. The 50% lethal dose (LC_{50}) -for primary cells- or the 50% growth inhibitory dose (IC_{50}) -for cell lines- was then determined by calculating cell survival with the following equation: mean OD treatment well (minus blank)/mean OD control well (minus blank) x100%.

Cell cycle analysis:

To examine the effect of CBD on the cell cycle, CEM/WT, CEM/BTZ200, and CEM/CBD6250 cells were first incubated in individual wells of a 6-wells plate (3 ml cell suspension, initial cell density of 4 x 10^5 cells/ml) in the absence (control) or presence of 1, 10 or 20 µM CBD for different time points (24, 48, 72 and 96 hours). Thereafter, cells were washed once with PBS and fixed with cold 70% ethanol for 24 hours. Cells were then washed twice with PBS, pooled in 100 µl PBS and stained with propidium iodide (PI) solution (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions; 5 x10^4 events were recorded in a LSRFortessa™ flow cytometer (BD, USA). For all flow cytometry analysis, Flowjo™ software v10.5.3 (Treestar, USA) was used.

Apoptosis assay:

To distinguish viable from early and late apoptotic cells after CBD incubations, 7AAD (BD Biosciences) in conjunction with annexin V (Biolegend, San Diego, California) staining was used. For this purpose, cells were plated in 6-well plates with different CBD concentrations and harvested at selected time points (1-4 days) as described above for the cell cycle analysis. After CBD exposure, cells were washed twice and pooled to obtain 10^6 cells in 100 µl PBS. Each fluorochrome was added according to the manufacturer's instructions and incubated for 15 minutes in the dark at room temperature (RT). Samples for annexin V staining had annexin V binding buffer (BD Bioscience) in a dilution of 5:1 in PBS. Thereafter, all samples were measured using flow-cytometry, recording peak emissions in FL1 (488 nm, bandpass 30 nm) for Annexin V-FITC, and FL2 (488 nm, bandpass 40 nm) for 7AAD-PerCP-CY5.5. Cells stained negative for both Annexin V and 7AAD were considered as viable cells, those positive for Annexin V and negative for 7AAD were considered as early apoptotic cells, and those positive for both fluorochromes were considered as late apoptotic cells. Five $x10^4$ events were recorded per incubation condition. Cellular debris and multi-nucleate events were gated out by using forward scatter (FS)/ side scatter (SS).

Cell proliferation assay

For cellular proliferation assessment, cells were stained at the start of the culture with the cell-tracing dye CellTrace™ Violet (Invitrogen, Thermo Fisher Scientific) as follows: cells (max 50 x 10^6) were resuspended in 1 ml PBS and 1 µL of cell dye, after which cells were stored for twenty minutes at RT in the dark, during which cells were mixed frequently. Stained cells were washed twice with PBS and plated at different CBD conditions (0, 1, 10, 20 µM CBD and 100 nM BTZ) in 96-wells plates and stored at 37°C in a humidified 5% CO_2 incubator. Cells were plated at the following densities: 6000 cells/well CEM/WT, 4000 cells/well for CEM/BTZ200 and 7000 cells/well for CEM/CBD6250, based on their proliferation curves. Cells were plated in separate wells per time point and per condition. At each time point, 100 µl cell suspension was put into a FACS tube containing 10 µl of fluorescent counting beads and immediately proceeded to record 1 x 10^4 events/condition in an LSRFortessa™ flow cytometer (BD, USA). Singlet, alive mononuclear cells were gated and sub-gates were placed to trace and analyze cells according to their cell dye fluorescence intensity over time. To standardize gates for the varying passage numbers, each individual file generated was concatenated to create a single file containing the unified data of all time points for a specific condition. These gates were then applied to each separate file for each condition and time point. Additionally, a separate gate was made for fluorescent counting beads in order to normalize data. For this purpose, the number of cells per gate (total or passage specific) was divided by the number of counting beads for each condition. Results are expressed as cells per bead.

Western Blot

Sample processing for western blotting was performed essentially as described previously[33]. Western blot was done on cell lysates, which were prepared by transferring a minimum of 3 x 10^6 cells of interest into 15 ml tubes, and centrifuged for 10 min at 300g. The following antibodies were used: anti-cannabinoid receptor 1 (rabbit-anti human, 1:200, ab23703, Abcam, UK), anti-cannabinoid receptor 2 (rabbit-anti human, 1:500, ab3561, Abcam, UK), anti-GPCR GPR55 (rabbit-anti human, 1:1000, ab203663, Abcam, UK), anti-GPCR119 GPR119 (rabbit-anti human, 1:1000, ab75312, Abcam, UK), Phospho-4E-BP1 (Thr37/46) (236B4) (rabbit-anti human, 1:1000, #2855, Cell Signal Technology, USA), anti-MARCKs (D88D11) XP (rabbit-anti human 1:1000, #5607, Cell signal Technology, USA). After blocking the membrane, the primary antibodies were added to the PVDF membrane and incubated for 16 hours at 4 °C. After incubation the membrane was washed 3x with PBS/0.5% Tween-20 (Sigma-Aldrich, USA) (PBST). The secondary antibody IRDye 800cw (goat-anti rabbit, 1:10000, Li-cor, USA) was added to the membrane after washing and incubated at RT for 1 hour. After incubation the membrane was washed 3x for 15 min at RT with PBST. The membrane was scanned with the Odyssey scanner (Li-cor, USA). β-Actin (mouse-anti human, 1:3000, Cell Signaling, USA) and as secondary antibody IRDye 680RD (goat-anti mouse , 1:10000, Li-cor, USA) was used as reference protein.

Statistical analysis

To determine significant differences between samples, the two-tailed student t-test was performed. Differences between means were considered statistically significant when p-values were < 0.05. Statistical analysis was performed using SPSS® Statistics for Windows, version 26 (IBM Corp. New York, USA). For graphical presentation, GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA) was used.

RESULTS

CBD growth inhibition of (drug-resistant) leukemia cell lines

The potency of CBD to inhibit the growth of the ALL cell line (CEM) and the AML cell line (THP-1) is presented in Figure 1A. IC_{50} values for CBD of CEM/WT were slightly lower (5.1 ± 1.7 µM SEM) than for THP-1 cells (9.2 ± 3.7 µM SEM). Moreover, leukemia cells were markedly more sensitive to CBD than normal BM cells (IC_{50}: 42.9 µM). To investigate whether CBD displays potency against drug-resistant leukemia cells, IC_{50} values were determined for BTZ-resistant and CBD-adapted CEM cells (Figure 1B). Notably, CEM/BTZ200 and CEM/CBD6250 cells were slightly cross-resistant (1.5 -fold) to CBD with IC_{50} values of 13.7 ± 3.7 µM and 10.7 ± 1.1 µM, respectively, as compared to CEM/WT cells. Intriguingly, Figure 1B shows that the CBD dose-response curve for CEM/BTZ200 and CEM/CBD6250 cells showed a growth stimulatory effect at low concentrations of CBD with an apparent 3-fold increase over CEM/WT at CBD concentrations around 1 µM. Together, these results suggest a window of CBD sensitivity for leukemia cells vs NBM cells, moderate cross-

resistance to CBD for drug-resistant cells and a biphasic dose-response curve with growth-inhibitory effects at CBD > 10 µM and growth stimulatory effects at low CBD concentrations.

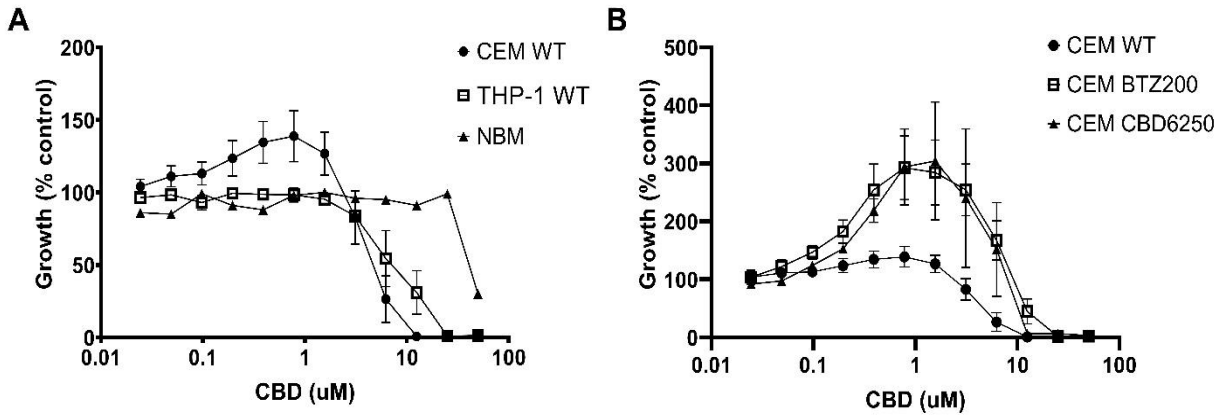


Figure 1. Dose-response curves of CBD in normal and leukemia cells
(A) CEM/WT, THP-1, and normal bone marrow (NBM) MNC cells and (B) CEM/BTZ200 cells and CBD-adapted CEM/CBD6250 cells. Drug exposure time: 96 hr. Growth inhibition was assessed by the MTT assay. Results are the mean ± SEM of at least 3 experiments, except for NBM (n=1).

CBD-induced apoptosis and cell cycle analysis in drug-resistant leukemia cells

To examine whether CBD growth inhibition is associated with induction of apoptosis, CEM/WT, CEM/BTZ200 and CEM/CBD 6250 cells were incubated at a concentration range of CBD (1 – 20 µM) for 24 – 96 hr followed by an analysis of apoptosis induction by Annexin-V/7-AAD staining (Figure 2A). For comparison, apoptosis induction and cell cycle analysis are shown after 24 hr exposure to 100 nM BTZ. For CEM/WT, apoptosis induction was observed at CBD concentrations > 10 µM. At 20 µM CBD the percentage of apoptotic cells gradually increased from 50 % at 24 h drug exposure to 77 % after 96 hr drug exposure. This profile distinguishes from BTZ which induced apoptosis at 100 nM in > 95% of CEM/WT cells after 24 hr drug exposure. Apoptosis induction by 20 µM CBD was markedly lower in CEM/BTZ200 cells than in CEM/WT; 24 % after 24 hr to 30% after 96 hr, which is in line with the low level of cross-resistance to CBD in CEM/BTZ200 cells. Twenty-four hr exposure to 100 nM BTZ did not induce apoptosis in CEM/BTZ200 cells, consistent with their resistant phenotype. The apoptosis induction profile of CEM/CBD6250 largely paralleled the one of CEM/WT; at 20 µM CBD, gradual apoptosis induction was found increasing from 48 % to 72 % for 24 and 96 hr exposure times, respectively, and > 95% apoptotic cells after 24 hr of 100 nM BTZ treatment.

The impact of 24-96 hr CBD exposure on cell cycle distribution in CEM/WT and CEM/BTZ200 cells is depicted in Figure 2B. No major effects were observed other than a slight decrease of cells in S-phase after 96 hr 20 µM CBD exposure along with an increasing fraction in sub-G1 phase consistent with apoptosis induction described in Figure 2A.

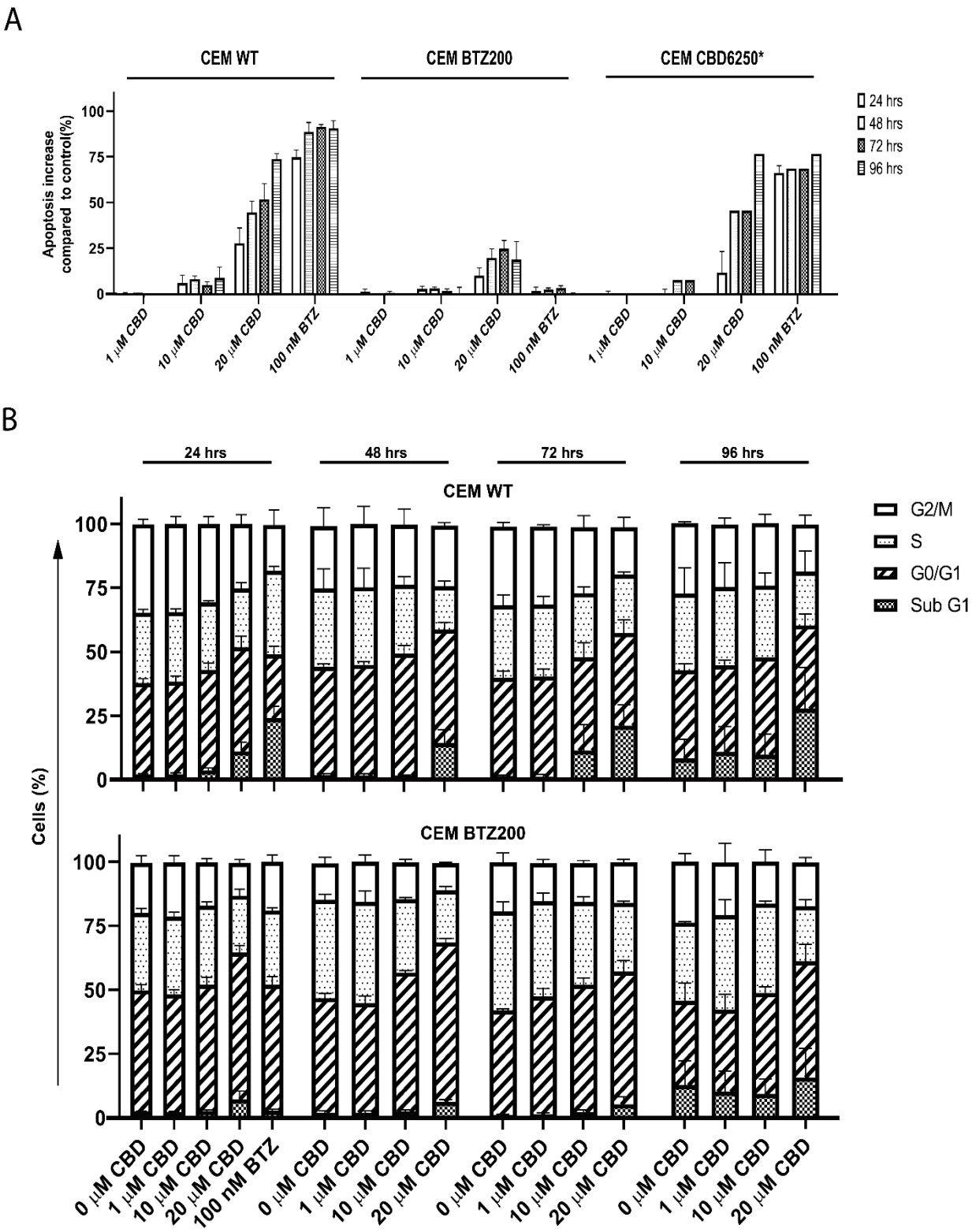


***Figure 2**: Flowcytometric analysis of **(A)** apoptosis induction and **(B)** cell cycle of CEM/WT, CEM/BTZ200 cells following 24-96 hr exposure to the indicated concentrations of CBD. Cells cultured in medium without drugs where considered negative control while incubation with 100 nM BTZ served as a positive control for apoptosis induction. Results depicted are the mean ± SEM of 3 experiments.*

Cell density dependency of cell proliferation induction at low concentrations of CBD

In Figure 1 it is shown that a low concentration of 1 µM CBD stimulated growth in drug-resistant CEM/BTZ200 and CEM/CBD6250 cells and to a lesser extent in CEM/WT cells. We extended these observations by exploring whether this effect was cell density-dependent. Figure 3A shows a representative example of the read-out of the MTT plate for CEM/WT, CEM/BTZ200, and CEM/CBD6250 at initial seeding densities of 4500, 1500 and 4500 cells/well, respectively. Visual inspection revealed a clear peak in colorimetric signal for CEM/BTZ200 and CEM/CBD6250 cells spikes at 1 µM CBD. When the results were analyzed as a function of different cell plating densities (Figure 3B) it became clear that growth-stimulating effects were most pronounced at low cell densities, up to 3-fold for CEM/BTZ200 and 7-fold for CEM/CBD6250 around 1 µM CBD, and dampened with increasing cell densities.

Since the colorimetric read-out of the MTT assay is based on mitochondrial activity in viable cells, we determined with two other assays whether the CBD-growth stimulatory effect indeed involved increased cell numbers rather than increased mitochondrial activity per cell. To this end, we used the Violet trace assay with flow cytometric fluorescent counting beads to measure the proliferation rate of cells cultured at various concentrations of CBD over a 72 hr period. By comparing the ratio of cells/ bead events, absolute numbers of CBD-treated vs non-treated cells can be calculated. As shown in Figure 3C, CEM/BTZ200 and CEM/CBD6250 cells exposed to 1 µM CBD had a two-fold higher ratio of cells/beads when compared to untreated cells. Moreover, results from the Violet trace assay (Figure 3C) recapitulates CBD concentration-effect curves shown in Figure 1 and as a control BTZ resistance in CEM/BTZ200 cells. Lastly, manual counting of viable cells in the MTT plates showed increased cell numbers of up to 4-fold for CEM/BTZ200 at CBD concentrations close to 1 µM and similar effect for CEM/WT cells albeit less pronounced (Figure 3D). Together, by various lines of evidence, these results show that low concentrations of CBD exert growth stimulatory effects, notably for drug-resistant cells.

CBD-mechanistic studies

To gain insight into the mechanism of action of CBD on leukemia cells and their drug-resistant variants, Western blot analyses were performed for various CBD-interacting receptors and down-stream effects. First, since CEM/BTZ200 cells had a low level of cross-resistance to CBD, we examined whether CEM/CBD6250 cells harbor markers of BTZ resistance, such as the myristoylated alanine-rich C-kinase substrate (MARCKS) which has been associated with ER stress and was previously shown to be upregulated in BTZ-resistant leukemia cells[38,39]. This is illustrated in Figure 4A, which also shows that MARCKS expression is markedly upregulated in CEM/CBD6250 cells, suggesting some (in)direct interaction of CBD with the ubiquitin proteasome system. Next, expression levels of CBD interacting CB1 and CB2 receptors were determined in CEM/WT, CEM/BTZ7, CEM/BTZ200 and CEM/CBD6250 cells. CB1 expression in these cells appeared to be below the detection limit (results not shown). CB2 was also barely detectable in these cells and

was not different between the cells (Figure 4B). Expression of two novel CBD receptors, GPR55 and GPR119, was visible in CEM/WT cells, but markedly reduced in drug-resistant CEM/BTZ7, CEM/BTZ200 and CEM/CBD6250 cells (Figure 4C). Other studies indicated that CBD may impact mTOR signaling [25,40], reflected by reduced phosphorylation of downstream 4E-BP1. Baseline p4E-BP1 expression was increased in CEM/BTZ7, CEM/BTZ200 and CEM/CBD6250 cells compared with CEM/WT cells (Figure 4D). All cells exposed to 1 µM CBD showed an upregulation of p4E-PB1 expression. At 10 µM CBD the expression of p4E-PB1 was reduced in CEM/BTZ7 and BTZ200 and almost completely abolished in CEM/WT and CEM/CBD6250 cells. At the highest CBD concentration tested (20 µM), p4E-BP1 expression was still detectable in CEM/BTZ7 and CEM/BTZ200 cells. p4E-BP1 expression was also retained in BTZ-resistant CEM/BTZ7 and CEM/BTZ200 cells exposed to 100 nM BTZ, whereas in BTZ sensitive CEM/WT (IC50 BTZ: 1.3 ± 0.4 nM) and CEM/CBD6250 cells (IC50 BTZ: 2.3 ± 0.7 nM) p4E-BP1 expression was minimized also because of loss of cell viability (reduced β-actin signal) (Figure 4D).

Together, these results show that drug-resistant CEM cells had down-regulated GPR55 and GPR119 expression, which may contribute to diminished CBD sensitivity. Additionally, low concentrations of CBD stimulated mTOR signaling reflected by increased 4E-BP1 phosphorylation.

Discussion

In this study, we demonstrated that the phytocannabinoid CBD, has a biphasic effect on cell vitality and proliferation of leukemia cells, in particular drug-resistant leukemia cells. On the one hand, at 1 µM CBD, the proliferation of BTZ-resistant and CBD-adapted CEM cells were markedly enhanced in association with increased mTOR activation, represented by increased phosphorylation of 4E-BP1. On the other hand, CBD concentrations >10 µM conferred cytotoxicity and induced apoptosis in CEM/WT and resistant subclones but not in healthy NBM, suggesting a therapeutic window for CBD in the treatment of leukemia. Moreover CEM/BTZ200 and CEM/CBD6250 showed a low level of resistance to CBD and expressed MARCKS protein, previously proposed as a marker for BTZ resistance[38], suggesting that CBD and BTZ resistance converge in an acquired common drug-resistance pathway. Finally, the growth of CEM/CBD6250 cells was characterized by a CBD-addiction phenotype which was most pronounced at low cell densities.

CBD has been investigated for its potential anti-cancer activity *in vitro* and *in vivo* in different types of solid tumors and hematological malignancies, including ALL and AML cells. In most *in vitro* studies, anti-tumor effects of cannabinoids were only found when high concentrations of CBD, usually > 10 µM, were used [3–5,7,8,21,23,27,32,41]. Similarly, in CEM and THP cells, apoptosis induction and cell growth inhibition were found at high CBD concentrations (around 10 µM). CBD had a minor impact on cell cycle distribution of CEM/ WT and CEM/BTZ200 cells which is in line with previous reports for Jurkat[41] and CEM cell lines cultured[42] *in vitro* with CBD in combination with other chemotherapeutics, such as doxorubicin[41], vincristine and cytarabine[42].

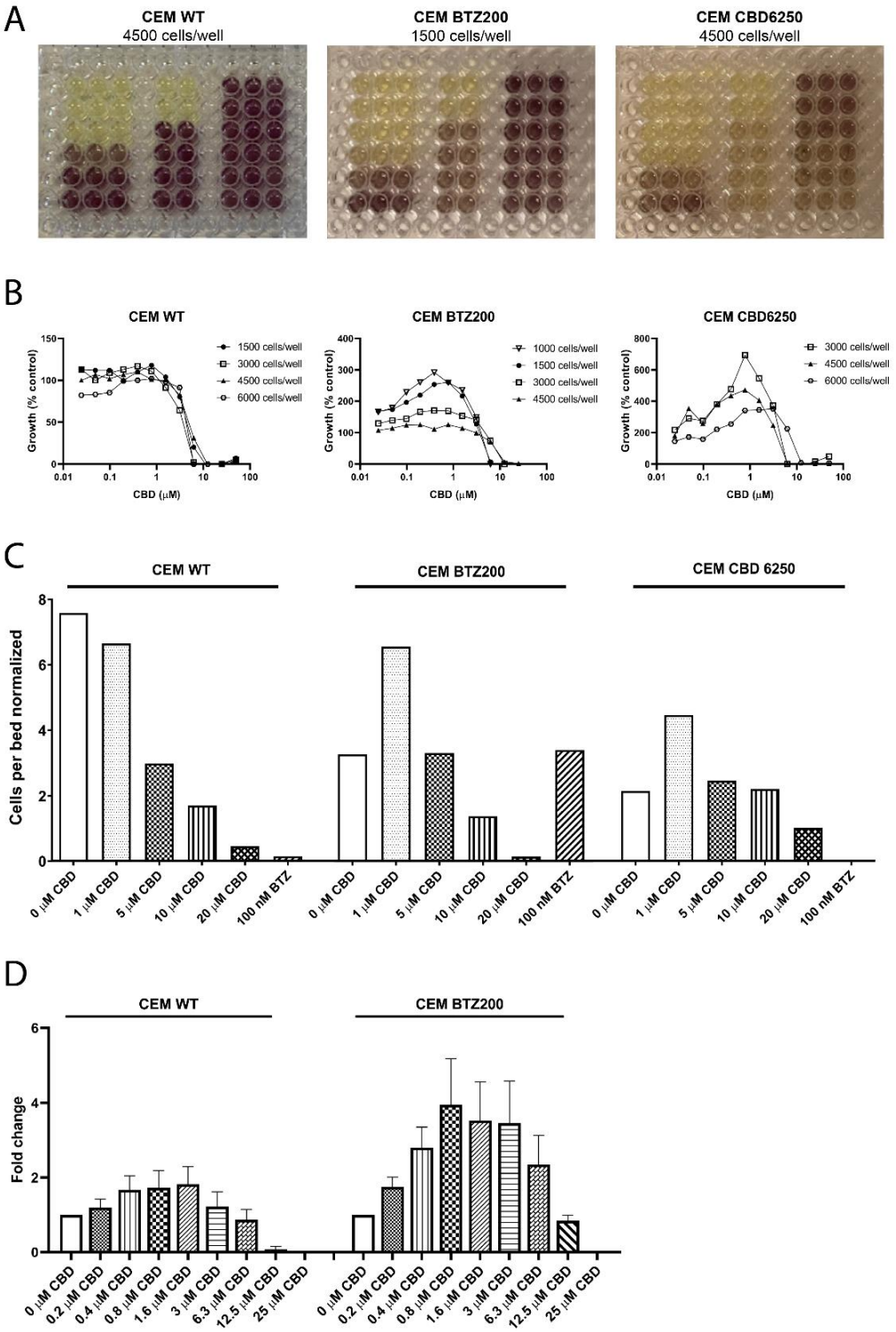


Figure 3. Concentration-dependent effect of CBD in CEM cell lines.

(A) *Representative picture of 96-well MTT plates after 96 hr CBD exposure.* ***(B)*** *Density concentration effect on CBD sensitivity. Results are the mean ± SEM of at least 3 experiments.* ***(C)*** *Violet Cell Trace™ flow cytometric analysis of cell growth stimulation/inhibition with the indicated concentrations of CBD for 72 hr. BTZ (100 nM) was included as control for resistance in CEM/BTZ200 cells and drug sensitivity control for CEM/WT and CEM/CBD6250 cells. Fluorescent beads were used in each condition and relative counts were defined as number of cells per bead.* ***(D)*** *Manual cell counting of cell growth stimulation/inhibition of CEM/WT and CEM/BTZ200 cells incubated with the indicated concentrations of CBD for 72 hr. Results are the mean ± SEM of at least 3 experiments.*

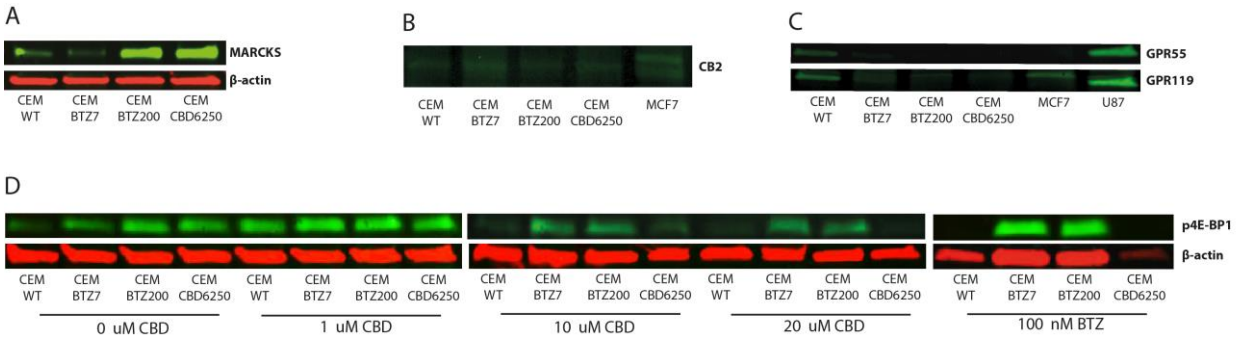


Figure 4. Protein expression of CBD affected pathways

Protein expression analysis of (A) MARCKS (B) cannabinoid receptor CB2, (C) GPR55 and GPR119 cannabinoid receptors, and (D) of phosphorylated 4E-BP1 (p4E-BP1) protein of CEM/WT, CEM/BTZ200 and CEM/CBD6250 cells incubated for 24 hr at the indicated concentrations of CBD or 100 nM BTZ. Expression of β-actin served as protein loading control.

It is not clear whether CBD holds potential against drug-resistant leukemia cells and/or whether leukemia cells are prone to acquire resistance to CBD. With respect to the latter, we exposed CEM cells to stepwise increasing concentrations of CBD over a period of 4 months, but were unable to push CBD concentrations beyond a point of 6250 nM (approximately IC50 values) without introducing delayed cytotoxic effects. Therefore these low level resistant CBD-adapted CEM/CBD6250 cells were further characterized.

One of the major obstacles in cancer treatment is multidrug resistance at the cell level, which is in part mediated by the overexpression of some ATP-binding cassette (ABC) transporters[43]. Previously CBD has been evaluated as a potential reversal agent for multidrug-resistant cells overexpressing common drug efflux ABC transporters, such as ABCB1 (P-glycoprotein, P-gp) and ABCC1 (multidrug resistance-associated protein 1, MRP1). In fact, CBD interacted with ABCB1[44] and ABCC1[45] and reduced expression levels of these transporters, but CBD concentrations required for these effects were at cytotoxic levels, i.e. ~ 10 µM.

In the present study we chose BTZ-resistant CEM cells as a drug resistant model since this proteasome inhibitor is active in multiple myeloma and is also investigated in an acute leukemia setting [46]. Somewhat surprisingly, it was observed that CEM/BTZ200 cells had a low level of cross-resistance to CBD and had an increased expression of a marker of BTZ resistance, MARCKS protein[38]. These results suggest partly overlapping mechanisms of action for BTZ and CBD. Indeed, several studies described that both BTZ and CBD impact mitochondria and calcium homeostasis[32,47]. Moreover, Scuderi et al [48] reported in an Alzheimer Disease model of SHSY5Y neuroblastoma-like cells that CBD promoted the ubiquitination of amyloid precursor protein. Given that one of the proposed functions of MARCKS proteins is to facilitate the extrusion of vesicles containing polyubiquitinated proteins as a mechanism to reduce ER stress in leukemia cells[38], these mechanisms may also hold for CBD-induced accumulation of ubiquitinated proteins in other cell types. This notion warrants further investigations.

Despite overlapping activities, CBD and BTZ have been tested in combination for multiple myeloma cells *in vitro*, revealing synergistic effects[24]. We noted that CBD was unable to reverse BTZ resistance in CEM/BTZ cells (unpublished observation), which may have been anticipated since the underlying resistance mechanism is due to point mutations in the *PSMB5* gene leading to impaired binding of BTZ in the binding pocket of the β5 subunit of the proteasome[33]. Since BTZ resistance may also have other underlying mechanisms[49], combinations of CBD and BTZ deserve further evaluation.

CBD mediates its effects via interactions with either CB1/CB2 receptors, ion channel receptor TRPV1 and or orphan G protein-coupled receptors GPR55 and GPR119[6,14,50,51]. Although CB2 is preferentially expressed in immune-related cells, this receptor was barely detectable in CEM/WT and drug-resistant CEM cells. It cannot be ruled out that is due to a technical shortcoming in the western blot analysis since the proposed positive control of MCF-7 breast cancer cells also showed a faint band and thus this experiment needs re-examination with alternative CB2 antibodies. GPR55 and GPR119 expression were readily detectable in CEM/WT but markedly down-regulated in CEM/BTZ7, CEM/BTZ200 and CEM/CBD6250 cells which were moderately cross-resistant to CBD. To what extent GPR55 and/or GPR119 contribute to CBD-induced growth inhibitory effects is still unexplored and future studies with agonist and antagonists for these receptors may provide insight in their differential relevance compared to other CBD receptors.

One of the downstream effects of CBD receptor interaction involves the downregulation of the phosphorylation of mTOR and 4E-BP1 [6]. Phosphorylation of mTOR and 4E-BP1 are pivotal in promoting cell proliferation and cell survival [52,53]. CBD-induced down-regulation of mTOR and 4E-BP1 phosphorylation has been demonstrated in breast cancer cells and leukemia cells exposed to micromolar concentrations of CBD [25,28,31]. Consistently, we noted the same in CEM leukemia cells exposed to 10 µM CBD, although drug-resistant cells with moderate CBD cross-resistance retained higher p4E-BP1 levels than CEM/WT cells. As such, inhibition of p4E-BP1 may be further explored as a useful indicator of CBD sensitivity in leukemia cells.

The most remarkable observation in this study was that low concentrations of CBD induced marked growth stimulatory effects in drug-resistant leukemia cells. Studies by Derocq et al[16,17] already demonstrated that sub-micro molar concentrations of cannabinoids had growth stimulatory effects on human B-cells and murine lymphoid and myeloblastic cells. This process was cannabinoid receptor-independent and proliferation effects were 30-40% based on [^{3}H]-thymidine incorporation. Later studies by Hart et al[18] demonstrated that nanomolar concentrations of THC (and other synthetic cannabinoids) could accelerate proliferation in glioblastoma cells (1.2-fold) and lung carcinoma cells (2-fold), by a mechanism that involved activation of ERK1/2, MAPK and Akt signaling. Our studies demonstrated a cell density-dependent growth stimulatory effect by CBD up to 3-fold in CEM/BTZ200 and up to 7-fold in CEM/CBD6250 cells. For CEM/CBD6250 cells this growth stimulatory factor is partly due to the fact that these cells acquired a CBD-addiction phenotype. Whether the underlying mechanisms for this involves activation of ERK1/2, MAPK or Akt signaling

is currently under investigation, but the increased 4E-BP1 phosphorylation in cells exposed to 1 µM CBD points in the direction of activation of signaling pathways. Conceivably, the CBD-addiction profile in CEM/CBD6250 cells is reminiscent of cancer drug addiction to MEK, ERK1/2, and BRAF inhibitors which are associated with hyperactivation of the ERK pathway and high levels of pERK1/2[54,55]. In any case, CEM/CBD6250 cells might serve as an interesting *in vitro* model to further investigate molecular aspects of CBD-addiction.

Considering the putative *in vivo* translation of low concentrations of CBD stimulating cancer cell growth, this will depend on the clinical pharmacology of cannabinoids. Bioavailability and pharmacokinetic studies[56–58] documented maximal plasma concentrations (Cmax) of CBD that can be achieved in humans via the various routes of CBD administration. Low plasma levels of CBD, up to 0.04 µM, are monitored after smoking or inhalation, but after CBD intake by oral capsules or iv administration much higher peak plasma levels, 0.5 – 2.5 µM, are achievable[56]. Thus, dependent on the route of administration, plasma levels of CBD may approach concentrations that elicited growth stimulatory effects *in vitro*. There are case reports of cancer patients self- administering high dosing of CBD and reporting documented substantial decrease in symptoms and tumor size when taken together with standard oncological therapy[59,60]. However, caution may have to be exerted with CBD (co)treatment of recurrent/drug-resistant cancers. Currently, several clinical trials are underway to test the safety and efficacy of CBD and other cannabinoids in a cancer therapy setting (NCT03607643, NCT04428203, NCT04283019, NCT01812616, NCT02432612, etc.).The results of these studies will be informative on whether dosing of CBD should be guided and monitored to obtain optimal efficacy and avoid adverse effects of accelerating cancer progression. This also comes with the fact that CBD is a popular alternative-health product that is sold at very low and unregulated levels over the counter and might pose a health risk, especially in drug-resistant/therapy refractory cancer patients. Safety issues for CBD have recently been updated and published in scientific literature and by the World Health Organization which confirms the low toxicity profile of CBD [61–63].

Taken together, our results point to be aware of growth stimulatory effects at low (1 µM range) concentrations of CBD in drug-resistant leukemia cells. CBD can also exert antileukemic effects on (drug-resistant) leukemia cells, but only at high (> 10 µM) CBD concentrations which are hard to reach *in vivo*. Thus, the question arises: is CBD treatment a double-edged sword for drug-resistant leukemia?

REFERENCES

1. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer J*. 2017;7(6):e577.
2. Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. *N Engl J Med*. 2015;373(16):1541-1552.
3. Galve-Roperh I, Sánchez C, Cortés ML, Gómez del Pulgar T, Izquierdo M, Guzmán M. Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat Med*. 2000;6(3):313-319.
4. Velasco G, Sánchez C, Guzmán M. Towards the use of cannabinoids as antitumour agents. *Nat Rev Cancer*. 2012;12(6):436-444.
5. Massi P, Solinas M, Cinquina V, Parolaro D. Cannabidiol as potential anticancer drug. *Br J Clin Pharmacol*. 2013;75(2):303-312.
6. Śledziński P, Zeyland J, Słomski R, Nowak A. The current state and future perspectives of cannabinoids in cancer biology. *Cancer Med*. 2018;7(3):765-775.
7. White CM. A Review of Human Studies Assessing Cannabidiol's (CBD) Therapeutic Actions and Potential. *J Clin Pharmacol*. 2019;59(7):923-934.
8. Hinz B, Ramer R. Anti-tumour actions of cannabinoids. *Br J Pharmacol*. 2019;176(10):1384-1394.
9. Atakan Z. Cannabis, a complex plant: different compounds and different effects on individuals. *Ther Adv Psychopharmacol*. 2012;2(6):241-254.
10. Zou S, Kumar U. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int J Mol Sci*. 2018;19(3).
11. Hu G, Ren G, Shi Y. The putative cannabinoid receptor GPR55 promotes cancer cell proliferation. *Oncogene*. 2011;30(2):139-141.
12. Balenga NAB, Aflaki E, Kargl J, et al. GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. *Cell Res*. 2011;21(10):1452-1469.
13. Wu J. Cannabis, cannabinoid receptors, and endocannabinoid system: yesterday, today, and tomorrow. *Acta Pharmacol Sin*. 2019;40(3):297-299.
14. Brown AJ. Novel cannabinoid receptors. *Br J Pharmacol*. 2007;152(5):567-575.
15. Bridgeman MB, Abazia DT. Medicinal Cannabis: History, Pharmacology, And Implications for the Acute Care Setting. *P T*. 2017;42(3):180-188.
16. Derocq JM, Ségui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett*. 1995;369(2-3):177-182.
17. Derocq JM, Bouaboula M, Marchand J, Rinaldi-Carmona M, Ségui M, Casellas P. The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines. *FEBS Lett*. 1998;425(3):419-425.
18. Hart S, Fischer OM, Ullrich A. Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. *Cancer Res*. 2004;64(6):1943-1950.
19. Elbaz M, Nasser MW, Ravi J, et al. Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: Novel anti-tumor mechanisms of Cannabidiol in breast cancer. *Molecular Oncology*. 2015;9(4):906-919.
20. Chung SC, Hammarsten P, Josefsson A, et al. A high cannabinoid CB(1) receptor immunoreactivity is associated with disease severity and outcome in prostate cancer. *Eur J Cancer*. 2009;45(1):174-182.
21. Caffarel MM, Andradas C, Mira E, et al. Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. *Mol Cancer*. 2010;9:196.
22. Thors L, Bergh A, Persson E, et al. Fatty acid amide hydrolase in prostate cancer: association with disease severity and outcome, CB1 receptor expression and regulation by IL-4. *PLoS ONE*. 2010;5(8):e12275.
23. Malfitano AM, Ciaglia E, Gangemi G, Gazzerro P, Laezza C, Bifulco M. Update on the endocannabinoid system as an anticancer target. *Expert Opin Ther Targets*. 2011;15(3):297-308.
24. Morelli MB, Offidani M, Alesiani F, et al. The effects of cannabidiol and its synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2. *Int J Cancer*. 2014;134(11):2534-2546.
25. Shrivastava A, Kuzontkoski PM, Groopman JE, Prasad A. Cannabidiol induces programmed cell death in breast cancer cells by coordinating the cross-talk between apoptosis and autophagy. *Mol Cancer Ther*. 2011;10(7):1161-1172.

26. Sreevalsan S, Joseph S, Jutooru I, Chadalapaka G, Safe SH. Induction of Apoptosis by Cannabinoids in Prostate and Colon Cancer Cells Is Phosphatase Dependent. *Anticancer Res*. 2011;31(11):3799-3807.
27. McKallip RJ, Jia W, Schlomer J, Warren JW, Nagarkatti PS, Nagarkatti M. Cannabidiol-induced apoptosis in human leukemia cells: A novel role of cannabidiol in the regulation of p22phox and Nox4 expression. *Mol Pharmacol*. 2006;70(3):897-908.
28. Sultan AS, Marie MA, Sheweita SA. Novel mechanism of cannabidiol-induced apoptosis in breast cancer cell lines. *Breast*. 2018;41:34-41.
29. Murison G, Chubb CB, Maeda S, Gemmell MA, Huberman E. Cannabinoids induce incomplete maturation of cultured human leukemia cells. *Proc Natl Acad Sci U S A*. 1987;84(15):5414-5418.
30. Powles T, te Poele R, Shamash J, et al. Cannabis-induced cytotoxicity in leukemic cell lines: the role of the cannabinoid receptors and the MAPK pathway. *Blood*. 2005;105(3):1214-1221.
31. Kalenderoglou N, Macpherson T, Wright KL. Cannabidiol Reduces Leukemic Cell Size - But Is It Important? *Front Pharmacol*. 2017;8:144.
32. Olivas-Aguirre M, Torres-López L, Valle-Reyes JS, Hernández-Cruz A, Pottosin I, Dobrovinskaya O. Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell Death Dis*. 2019;10(10):779.
33. Franke NE, Niewerth D, Assaraf YG, et al. Impaired bortezomib binding to mutant β5 subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells. *Leukemia*. 2012;26(4):757-768.
34. Nabissi M, Morelli MB, Offidani M, et al. Cannabinoids synergize with carfilzomib, reducing multiple myeloma cells viability and migration. *Oncotarget*. 2016;7(47):77543-77557.
35. Niewerth D, Kaspers GJL, Assaraf YG, et al. Interferon-γ-induced upregulation of immunoproteasome subunit assembly overcomes bortezomib resistance in human hematological cell lines. *J Hematol Oncol*. 2014;7:7.
36. Niewerth D, van Meerloo J, Jansen G, et al. Anti-leukemic activity and mechanisms underlying resistance to the novel immunoproteasome inhibitor PR-924. *Biochem Pharmacol*. 2014;89(1):43-51.
37. van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol Biol*. 2011;731:237-245.
38. Franke NE, Kaspers GL, Assaraf YG, et al. Exocytosis of polyubiquitinated proteins in bortezomib-resistant leukemia cells: a role for MARCKS in acquired resistance to proteasome inhibitors. *Oncotarget*. 2016;7(46):74779-74796.
39. Oerlemans R, Franke NE, Assaraf YG, et al. Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood*. 2008;112(6):2489-2499.
40. Giacoppo S, Pollastro F, Grassi G, Bramanti P, Mazzon E. Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis. *Fitoterapia*. 2017;116:77-84.
41. Kalenderoglou N, Macpherson T, Wright KL. Cannabidiol Reduces Leukemic Cell Size - But Is It Important? *Front Pharmacol*. 2017;8:144.
42. Scott KA, Dalgleish AG, Liu WM. Anticancer effects of phytocannabinoids used with chemotherapy in leukaemia cells can be improved by altering the sequence of their administration. *International Journal of Oncology*. 2017;51(1):369-377.
43. Jaramillo AC, Saig FA, Cloos J, Jansen G, Peters GJ. How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance? *Cancer Drug Resistance*. 2018;1(1):6-29.
44. Holland ML, Panetta JA, Hoskins JM, et al. The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells. *Biochem Pharmacol*. 2006;71(8):1146-1154.
45. Holland ML, Allen JD, Arnold JC. Interaction of plant cannabinoids with the multidrug transporter ABCC1 (MRP1). *Eur J Pharmacol*. 2008;591(1-3):128-131.
46. Niewerth D, Kaspers GJL, Jansen G, et al. Proteasome subunit expression analysis and chemosensitivity in relapsed paediatric acute leukaemia patients receiving bortezomib-containing chemotherapy. *J Hematol Oncol*. 2016;9(1):82.
47. Landowski TH, Megli CJ, Nullmeyer KD, Lynch RM, Dorr RT. Mitochondrial-mediated disregulation of Ca2+ is a critical determinant of Velcade (PS-341/bortezomib) cytotoxicity in myeloma cell lines. *Cancer Res*. 2005;65(9):3828-3836.
48. Scuderi C, Steardo L, Esposito G. Cannabidiol promotes amyloid precursor protein ubiquitination and reduction of beta amyloid expression in SHSY5YAPP+ cells through PPARγ involvement. *Phytother Res*. 2014;28(7):1007-1013.
49. Niewerth D, Jansen G, Assaraf YG, Zweegman S, Kaspers GJL, Cloos J. Molecular basis of resistance to proteasome inhibitors in hematological malignancies. *Drug Resist Updat*. 2015;18:18-35.

50. Atalay S, Jarocka-Karpowicz I, Skrzydlewska E. Antioxidative and Anti-Inflammatory Properties of Cannabidiol. *Antioxidants (Basel)*. 2019;9(1).
51. Dobovišek L, Krstanović F, Borštnar S, Debeljak N. Cannabinoids and Hormone Receptor-Positive Breast Cancer Treatment. *Cancers (Basel)*. 2020;12(3).
52. Pópulo H, Lopes JM, Soares P. The mTOR signalling pathway in human cancer. *Int J Mol Sci*. 2012;13(2):1886-1918.
53. Petroulakis E, Mamane Y, Le Bacquer O, Shahbazian D, Sonenberg N. mTOR signaling: implications for cancer and anticancer therapy. *Br J Cancer*. 2006;94(2):195-199.
54. Kong X, Kuilman T, Shahrabi A, et al. Cancer drug addiction is relayed by an ERK2-dependent phenotype switch. *Nature*. 2017;550(7675):270-274.
55. Sale MJ, Balmanno K, Cook SJ. Resistance to ERK1/2 pathway inhibitors; sweet spots, fitness deficits and drug addiction. *Cancer Drug Resistance*. 2019;2(2):365-380.
56. Millar SA, Stone NL, Yates AS, O'Sullivan SE. A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans. *Front Pharmacol*. 2018;9.
57. Ohlsson A, Lindgren JE, Andersson S, Agurell S, Gillespie H, Hollister LE. Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration. *Biomed Environ Mass Spectrom*. 1986;13(2):77-83.
58. Anderson GD, Chan L-N. Pharmacokinetic Drug Interactions with Tobacco, Cannabinoids and Smoking Cessation Products. *Clin Pharmacokinet*. 2016;55(11):1353-1368.
59. Sulé-Suso J, Watson NA, van Pittius DG, Jegannathen A. Striking lung cancer response to self-administration of cannabidiol: A case report and literature review. *SAGE Open Med Case Rep*. 2019;7:2050313X19832160.
60. Dall'Stella PB, Docema MFL, Maldaun MVC, Feher O, Lancellotti CLP. Case Report: Clinical Outcome and Image Response of Two Patients With Secondary High-Grade Glioma Treated With Chemoradiation, PCV, and Cannabidiol. *Front Oncol*. 2018;8:643.
61. Iffland K, Grotenhermen F. An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies. *Cannabis Cannabinoid Res*. 2017;2(1):139-154.
62. Expert Committee on Drug Dependence, World Health Organization. *Cannabidiol Critical Review*. World Health Organization; 2018.
63. Chesney E, Oliver D, Green A, et al. Adverse effects of cannabidiol: a systematic review and meta-analysis of randomized clinical trials. *Neuropsychopharmacology*. 2020;45(11):1799-1806.

CHAPTER 8

General discussion and future perspectives

In this thesis, three topics were investigated in the context of hematopoiesis, onset of acute leukemia and treatment of leukemia; (1) the role of bone marrow microenvironment in hematopoiesis, (2) drug resistance mechanisms for conventional anti-leukemic drugs, and (3) exploring cannabidiol (CBD) as experimental therapeutic agent for leukemia. Each of these topics will be discussed in greater detail hereafter.

1. Bone marrow microenvironment in hematopoiesis

The bone marrow microenvironment constitutes a milieu which supports and regulates normal hematopoiesis. The hematopoietic system, where HSCs in the BM differentiate into all blood cell types, facilitates the supply of billions of newly formed blood cells on a daily basis. Such a regulated and highly productive system requires a reliable and protective support mechanism. The BM microenvironment or niche acts as this support system and as such it can modulate HSC differentiation, quiescence or self-renewal. Under stress or homeostatic conditions, the BM microenvironment is a dynamic and adaptable biological landscape[1–3]. In a leukemia disease setting, the BM environment also contributes to malignant transformation of leukemia cells and drug resistance including mediating pro-survival signaling processes and cell membrane transport systems involved in drug influx or efflux[4,5]. As such, investigating the components of the triad extracellular environment, cell membrane barrier and transporters, and intracellular processes (and their interactions) are highly relevant to uncover their role in hematopoiesis in health and disease.

Endothelial cells (ECs) are key players in transforming the BM microenvironment and HSC behavior are the As described in Chapter 2, human regenerative associated endothelial cells or hRECs, phenotypically identified as CD31+/CD105+/CD9+ cells, originate during developmental (fetal BM) and regenerative (adult BM post-chemo) stages. hRECs are specialized ECs with a transcriptional program related to tissue regeneration, and are able to secrete factors, such as IL-33, which enhance precursor HSC expansion and osteogenesis[2].

The BM microenvironment has also the ability to promote regeneration through paracrine signaling[1,3,6]. When high jacked by LSCs, niche functioning can be redirected to favor malignant expansion and drug resistance, thus becoming a safe haven for leukemic cells[1,3,5,7,8]. Moreover, pro-inflammatory signaling by the microenvironment feeds into this negative feedback-loop, stimulating malignant transformation in an egg-or-chicken type conundrum[9,10].

Examining the capacity of hRECs to modulate the landscape of the BM microenvironment warrants further dissection and expansion in future studies. Relevant questions would be: how do hRECs contribute to osteogenesis and chondrogenesis *in vivo*? What is the transcriptional program responsible for this contribution? How does it direct HSC quiescence or activation? Once these questions have been answered, it would be possible to envision the application of hRECS in two specific fields: (1) regenerative medicine (RM) and (2) cancer therapy. In RM for example, the use of artificial 3D scaffolds has promising potential in tissue engineering for bone and BM renewal. In this approach, stromal cells can be utilized in an artificial

3D environment to recreate the BM niche and promote osteogenesis in order to aid HSCs to graft and reconstitute fresh blood cells[11,12]. Still, these methods have limitations, such as insufficient vascularization, mechanical load and transplantation challenges[12]. Nevertheless, the stimulatory signaling from hRECs could potentially promote vascularization as well as osteo/chondrogenesis which can increase the colonization and output potential of such systems. With respect to cancer therapy, the study of different regenerative capacities of niche cells (hRECs, MSCs, and others) could shed light on how leukemic cells can benefit from proliferation and migration signaling pre- or post-chemotherapy.

In our own studies (Chapter 2), we observed that during 5-fluorouracil (5-FU) myeloablation studies in mice, hRECs were able to survive such treatment and that the absolute number of hRECs did not increase, but that the remainder of cells in the BM died. This finding suggests that under chemotherapeutic pressure, selective survival of hRECs, rather than absolute expansion, could be implicated[2]. It is still unknown if chemotherapy-surviving microenvironment cells influence LSC survival and their role in multidrug resistance in leukemia. It has been proposed that mapping transcriptional and epigenetic signatures in tumor environmental cells may reveal novel markers for malignant transformation and metastasis[13]. It is well known that ECs in the perivascular niche promote a stemness phenotype in cancer cells through e.g. Notch[14,15], sonic hedgehog[16–18] and nitric oxide[19] signaling pathways, which stimulate cell migration[14]. Therefore molecular dissection of the vascular and perivascular niche in leukemia (and cancer in general) could provide a therapeutic opportunity for blocking malignant growth signals and metastasis. In light of our study in Chapter 2, it would be interesting to analyze the contribution of IL-33 to leukemia onset in a leukemic murine model, before and after chemotherapy. Recent studies by Taniguchi *et al*, showed in a murine model of squamous cell carcinoma, a signaling loop involving IL-33 and malignant progression. Under normal conditions IL-33 is stored in the nucleus, and its secretion into the malignant microenvironment by tumor initiating cells promotes invasion and differentiation of myeloid cells into macrophages. These cells release transforming growth factor beta (TGF-β) which further stimulates proliferation of malignant cells[20]. The involvement of IL-33 paracrine loops in leukemia progression is still unknown. It is plausible that additional mechanistic insights in the interactions of LSC and their niche could lead to pharmaceutical targets which can cause a blockade in supportive interactions between LCS and its perivascular niche. It may be critical to focus not only on treating the leukemic blast, but also its perivascular niche signaling loops, in order to develop effective personalized therapeutic strategies in the future[13,21].

Another critical aspect of anti-leukemic therapy and niche biology is to understand its contribution to multi drug resistance (MDR). How hRECs are related to drug resistance is not fully understood, but it is known that ECs in other tightly restricted areas such as the blood-brain barrier (BBB) express drug transporters that tightly regulate xenobiotic accumulation in order to protect the central nervous system (CNS)[22,23].

The ABC drug efflux transporter system is phylogenetically ancient, and well preserved across species. It also constitutes one of the largest superfamily of

transmembrane proteins[24]. These transporters are ubiquitously expressed in all three domains of life: archaea, bacteria and eukaryotes. ABC transporters are responsible for the active transmembrane transport of a broad diversity of xenobiotic substrates, which highlights their physiological and pharmacological importance[25,26]. Active efflux of xenobiotics results in reduced intracellular accumulation of drugs, which can contribute to chemoresistance. In a systems biology view of disease (cells and niche), multiple ABC transporters play a role in MDR since they are expressed on the membrane of both BM microenvironment cells and the HSC/LSCs[22,27,28].

How stromal cells and ECs contribute to MDR via cell membrane drug efflux transporters and how exactly they aid LSCs to tolerate chemotherapy, is still unknown. It has recently been postulated that mesenchymal stromal cells (MSC) can enhance survival of LSC “side populations” via activation of ABC transmembrane transporters in AML. When in contact with leukemic blasts, MSCs can promote the cellular programing required for drug efflux[29]. An important feature in this respect is functional redundancy of drug efflux transporters wherein transporters work in synchrony and can compensate each other. Since the microenvironment conditions promote MDR, future studies should focus on the role of (specific) drug transporters on specialized ECs and MSCs in the BM and how they can protect and detoxify the HSC/LSC niche from noxious drugs (*in vivo,* at regenerative and in steady-states). By this approach, it could be possible to identify sets of transporters in BM ECs, hRECs, MSCs and HSC/LSCs that might have a predictive value to therapy response. Moreover, new pharmacological targets or pathways could be identified in the malignant versus healthy state ECs. If so, it would be of interest to further investigate the relationship between malignant and healthy state compartmental relationship in terms of transporter expression and therapeutic intervention.

2. Drug resistance mechanisms for conventional anti-leukemic drugs

Cornerstone chemotherapeutic drugs such as MTX, Ara-C and glucocorticoids (GC) have been used for decades in clinical oncology. MTX is an essential prophylactic drug used for CNS involvement in childhood ALL[30]. Ara-C and GC are extensively used and continue to be the mainstream agent in the treatment of patients with AML[31] and childhood ALL[32], respectively.

One of the major challenges facing chemotherapy is the onset of drug resistance, which can be either innate or acquired. In drug resistance, there are multiple resistance mechanisms activated. These include: impaired drug influx/receptor binding, enhanced drug efflux, impaired drug activation, increased target enzyme expression and altered downstream effects such as survival pathway modulation and reduced apoptosis induction[33,34]. In this thesis we primarily focused on the role of transport-related resistance mechanism for these conventional anti-leukemic drugs.

Several multidrug resistance-(MDR) related proteins can mediate export of conventional anti-leukemic drugs. For example MRP1-5 (MRP1-5, ABCC1-5) and breast cancer resistance protein (BCRP, ABCG2), which can extrude MTX, and high ABCC4 expression can confer resistance to Ara-C in AML cells *ex vivo*[27(p4),35(p4)].

These results point to functional redundancy of ABC transporters handling the efflux of particular drugs. This was also underscored in our studies (Chapter 4) demonstrating that ABC transporters ABCG2/ BRCP and ABCC4/MRP4 work in conjunction to extrude MTX and reduce the intracellular accumulation of MTX-polyglutamates (MTX-PG). Furthermore, a coupled higher expression of ABCG2/BCRP and ABCC4/MRP4 correlated with *ex vivo* resistance to MTX and to poor survival. It is important to mention that many patients in this study are clinically considered high risk, yet they were selected because they fulfilled all required statistical data points for survival analysis (times of diagnosis, death or remission) plus had the high amount of cells necessary for all the laboratory procedures. Also, based on the limited number of samples and the strict guideline of sequential protocols, it was not possible to collect samples on risk stratification alone. Being aware of the limitations of our sample size and the impact on survival analysis, a large proportion of patients survived, yet valuable survival data was collected for over a decade and this enabled us to perform a Kaplan-Meier curve based on initial ABC transporter expression. We would have liked to analyze Kaplan-Meier curves based on risk stratification, but this process resulted futile since it divided an already small group of patients into several smaller cohorts composed of inconsequential sample numbers. For this reason we focused on the general survival of patients based on the combination of the expression of relevant ABC drug transporters.

Ara-C resistance is seen in conjunction with a lower expression of its main transporter, the equilibrative nucleoside transporter 1(ENT1), in leukemia cells, and a lower expression of ENT1 mRNA has been correlated with a shorter disease-free survival in AML[36,]. Ara-C resistance is not only defined by ENT1 expression controlled by cell intrinsic mechanisms, but also by modulation of the microenvironment. For example it has been reported that BM MSC from AML patients can secrete soluble factors that lower ENT1 activity at the membrane of AML cells and simultaneously activate survival pathways. When this is seen *ex vivo,* it correlated with poor overall survival in AML[38].

2.1 Role of influx and efflux transporters in drug resistance

The cell membrane is a first barrier for drug entry in cancer cells. Depending on the lipophilicity of the drug it can enter by passive diffusion, but active transport via solute carriers (SLCs) commonly applies for many drugs, in particular those with anionic or cationic properties[39]. This was elegantly shown in a recent functional screen for 60 chemically diverse and widely used anticancer drugs in a CRISPR-Cas9 library targeting almost 400 SLC genes[40]. This screen readily confirmed SLC19A (Reduced Folate Carrier, RFC) and SLC46A1 (Proton Coupled Folate Transport, PCFT) as important influx transporters for MTX and confirmed that a knock-down of these SLCs led to MTX resistance. Likewise in this screen, SLC29A1 (human Equilibrative Nucleoside Transporter 1, hENT1) was also confirmed as Cytarabine/Ara-C influx transporter. Moreover, the screen identified functional redundancy in SLCs for multiple cytotoxic drugs.

The role of ABC transporter family members in the efflux of cytotoxic drugs to confer MDR has been well characterized over the past decades[24,27,34,41,42]. ABC transporter

models proved useful at a pre-clinical level for developing molecular models of transporter physiology and pharmacology, determining substrate specificities and the design and testing of inhibitors of specific ABC transporters as potential strategy to overcome drug resistance mediated by ABC transporters (summarized in Chapter 3). Although these MDR-reversal strategies are successful in a laboratory setting, they unfortunately lack efficacy in clinical practice for various reasons[43]. Among others these include low, heterogeneous and interpatient differences in specific ABC transporters, interference with the physiological function of ABC transporters in specific organs, toxicity of the inhibitor and/or drug-drug interactions impacting chemotherapeutic drug pharmacokinetics[42,43].

The current positioning of ABC transporters in cancer drug resistance and underlying reasons for failures of resistance reversal studies in the clinic was recently discussed[44]. The main considerations include the fact of functional redundancy of ABC transporter and that no single efflux pump or transporter is solely responsible for drug resistance. Moreover, although in various types of solid tumors ABC transporter overexpression has been linked to MDR, results vary from sample to sample, underscoring the biological variation in the expression between individuals. Also, the ABC transporter activation pattern is heterogeneous and varies with age and tissue[23,45,46,47,48]. From a leukemia perspective, a diverse pattern of ABC transporter expression has been observed in stromal cells and leukemic blasts from the same (AML) patients[29]. These notions must be taken into account when weighing results from ABC transporter investigation and for personalized treatment considerations of leukemia patients. As future directions for ABC transporter research in leukemia cells, it is worthwhile to examine multiple ABC transporters, preferably in combination with micro environmental cell types (stroma cells and/or ECs). Moreover, even after several decades of MDR research further standardization is required for ABC transporter analysis of protein expression and functional activity. Drug evaluation assays could provide better insights of each individual patient's needs based on actual, real-time, and tissue-specific analysis, and translate this information for precision medicine.

2.2 MTX

A folate analog was first evaluated in an oncological setting in 1948, when Farber et al. reported temporary remission in children with ALL treated with aminopterin[49], which was later replaced by its less toxic analog MTX. Over 70 years MTX has served as an important drug in the chemotherapeutic arsenal of oncology, and later also as an anti-inflammatory drug in auto-immune diseases such as rheumatology[50] and inflammatory bowel disease (IBD). Extensive preclinical research has clarified the mechanism of action of MTX and also identified molecular mechanisms of acquired resistance to MTX, of which some were confirmed in the clinic[51]. The dominant resistance mechanisms to MTX include: (1) impaired cellular uptake via the reduced folate carrier (RFC, SLC19A1) or proton-coupled folate transporter (PCFT, SLC46A1), (2) MTX efflux via ABC transporters ABCC1-5 (MRP1-5) and ABCG2 (BCRP), (3) decreased MTX poly-glutamylation due to decreased Folyl- poly-glutamate synthetase

(FPGS) enzyme activity, and (4) increased expression, mutations and/or lowered MTX affinity of the main target enzyme for MTX; dihydrofolate reductase (DHFR)[51].

In our studies in Chapter 6, we focused on ABC transporters involved in MTX efflux and MTX polyglutamate accumulation in leukemic cells in relation to *ex vivo* MTX resistance. These results corroborated that in leukemia cells the intracellular retention of MTX polyglutamates is dependent on the "teamwork" of at least two efflux transporters (i.e. ABCC4/MRP4 & ABCG2/BCRP)[52]. Since our study included a relative small number of patients, it is conceivable that more relevant correlations exist between additional efflux transporters, which warrant confirmation in future studies in a large cohort of patients.

Conceptually, the future design of a clinical study would incorporate the expression analysis of all relevant MTX influx and efflux transports, MTX-metabolizing enzymes, MTX-target enzymes, MTX-polyglutamate analysis to correlate these parameters with therapy response. Ideally more participants in general under a single treatment or by risk stratification system regimen would be helpful to attain better understanding of the relationship between the expression of these 'MTX-response/resistance' parameters on inter-patient variability, relapse and survival. Since almost all treatment regimens are based on a combination of several drugs, it should also be investigated which limiting factors for the other drug play a role and which parameters are important for the interaction.

For ABC transporters to play a role in MTX efflux and resistance, an excellent efficiency of MTX polyglutamylation by FPGS in leukemia cells is crucial[53–56] as only non-polyglutamated MTX is a substrate for ABC transporters (although ABCG2 can export di- and tri-glutamate forms). Recent research from our laboratory revealed that aberrant splicing of FPGS pre-mRNA is a novel factor accountable for diminished FPGS activity and consequently reduced MTX polyglutamylation and therapy response[53,54,57]. Another recent study revealed that *FPGS* mutations in relapsed leukemia would impact polyglutamylation efficiency[58]). These novel factors deserve further elaboration in studies assessing MTX efficacy/ resistance in leukemia.

Based on the accumulated knowledge of MTX resistance mechanisms, rational approaches have been made to circumvent MTX resistance. One such approach is to prolong MTX infusion time above critical plasma levels, which could be more critical than peak plasma MTX levels alone, especially in childhood acute monocytic leukemia[59]. An alternative approach is to synthesize second generation antifolates aimed to circumvent MTX resistance in leukemias and solid tumors[51,60,61]. In particular, for AML treatment a novel antifolate would be of interest as MTX is less active in AML compared to ALL[62]. Since a might be an alternative as well. In this respect, recent studies pointed to targeting of mitochondrial folate metabolism, rather than cytosolic folate metabolism, by inhibiting a key mitochondrial enzyme methylenetetrahydrofolate dehydrogenase-cyclohydrolase-2 (MTHFD2), which is upregulated in cancer cells, including AML, and could potentially be targeted[63–66]. In fact, MTHDF2 inhibition by shRNA impaired AML progression and induced differentiation *in vivo*[64]. Moreover, suppression of MTHFD2 activity resulted in altered glycine metabolism and affected aerobic respiration by suppressing tricarboxylic acid

cycle *in vitro*, as well as colony formation in treated HSCs. Pre-clinical studies on its safety and toxicity are warranted, since not much is known about the toxicity of MTHFD2 inhibitors *in vivo*. Two interesting approaches worth exploring are (1) using computer modeling systems and (2) novel drug delivery formulations. The first one can aid to identify new antifolate molecules structures which may target folate-dependent enzymes implicated in MTX resistance[65,67]. The second one, could help us deliver MTX more effectively and selectively by using lipid electrically charged nanoparticles[68]. Strategies considering inhibition of specific ABC transporters to reverse MTX resistance are likely to be less successful considering the functional redundancy of ABC transporters.

2.3 Ara-C (and glucocorticoids)

The nucleoside transporters hENT1 and hCNT1 constitute the dominant influx routes for nucleoside analogs Ara-C and gemcitabine. In fact, almost 80% of Ara-C influx is accounted for by these importers[69,70]. For this reason, hENT1 has been proposed as a predictive biomarker of gemcitabine efficacy for various solid tumors[33–35] but clinical data showed conflicting results[74–77]. This inconsistency can be accounted for by using different methodologies in the evaluation of transporter expression (mRNA vs protein analysis) and lack of information on other relevant factors contributing to nucleoside analogue efficacy. Most importantly, crucial clinical studies have been performed using a non-validated antibody SP120, to determine hENT expression; therefore all studies using this antibody should be considered with care[72,78]. Other important resistance mechanisms include increased degradation by cytidine deaminase (CDA) and decreased activation by deoxycytidine kinase (dCK)[79–81]. This enzyme catalyzes phosphorylation of Ara-C and gemcitabine into their mono-, di- and triphosphate metabolites. Phosphorylation of nucleosides to their monophosphates is often a rate-limiting step in the activation of these drugs. In addition, the phosphorylated monophosphate can be inactivated by dCMP deaminase to dFdUMP. All of these intracellular enzymes are limiting factors whose expression has been reported to be potentially involved in gemcitabine resistance[69,82–85].

In the future, molecular research should include standardized methods to study these contributing factors in nucleoside analogue efficacy[78]. A validated analysis may guide personalized treatment with these drugs. One of the challenges that we encountered in our immunohistochemistry study of hENT1 and hCNT1 expression in AML cells (Chapter 5) is the widespread expression of these transporters in *ex vivo* samples. This hampered setting accurate cut-off points to determine an association of high vs low hENT1 and hCNT1 expression with Ara-C sensitivity and resistance, respectively, and/or treatment outcome. Partly due to these limitations, our study did not reveal significant associations between these parameters. Moreover, AML patients are always treated with cocktails of different chemotherapeutics hampering the correlation between these transporter data and the ultimate response to therapy, which is dependent on the combined efficacy. To overcome the drug transporter related resistance, several lipophilic analogs of Ara-C[86] and gemcitabine[87] have been developed; however the gemcitabine prodrug CP-4126 (CO1.01) failed in clinical

studies due to the use of a non-validated antibody to select patients with a low hENT expression. However, a novel liposomal formulation of Ara-C and daunorubicin (at a fixed 5:1 molar ratio) has been investigated clinically[88] and has been registered for treatment. The heterogeneous expression of hENT by *ex vivo* immunohistochemistry was also noted by others for pancreatic cancer cells. hENT1 expression categorized either as “low” or “high” overlapped a large percentage of samples, resulting in mixed survival results, and made cut-off points challenging to determine[77].

3. Cannabidiol (CBD) as experimental therapeutic agent for leukemia

In the search for new anti-cancer drugs with novel mechanisms of action and for overcoming drug resistance, nature has provided (either by serendipity or intentionally) useful drug templates. In fact, most emerging and currently used cancer chemotherapeutic drugs in the twentieth century, come from natural sources. Ara-C, for example, was identified six decades ago in the sea sponge *Cryptotheca crypta*[89], and served as prototype for next generation nucleoside analogues such as its fluorinated analog gemcitabine. Vinca alkaloids are also plant derived (*Catharanthus roseus)*[90]. GCs, such as dexamethasone and prednisolone, are analogs of our steroid hormone cortisol[91]. Cannabinoids, including cannabidiol (CBD) and tetrahydrocannabinol (THC), from the plant *Cannabis sativa,* emerged as novel drugs currently being studied in several clinical trials for its pleiotropic medicinal properties. Due to its low toxicity, CBD is an attractive compound, also because it is not psychoactive and has a high tolerability[92]. *Cannabis sativa* has been used for at least five millennia to treat many ailments, related to pain and inflammation[93,94]. Up-to-date, over 270 clinical trials are registered involving CBD and it is currently finding its niche as a drug in the field of refractory epilepsy, neurodegenerative diseases as well as pain management[95,96], with a few trials involving different types of cancer (for example: NCT03607643, NCT04428203, NCT04283019, NCT01812616, NCT02432612).

It is worth mentioning that in various non-malignant cells, CBD exerts anti-inflammatory properties, inhibition of hENT (blocking adenosine uptake and promoting adenosine receptor binding[97,98]), and elicits mitochondrial-protective properties by modulating intracellular calcium levels and acting as an antioxidant[99,100]. In malignant cells, opposite effects are implicated by CBD treatment, including increasing mitochondrial stress and reactive oxygen species (ROS) levels[101,102]. In tumors with high glycolytic rates, the CBD-induced disruptive mitochondrial activity may add to metabolic stress and growth inhibitory effects[103].

The antitumor effects of various cannabinoids such as THC and CBD in several cancer types have been documented, *in vitro* and *in vivo*[104–108]. These effects may also relate to the fact CBD modulates the tumor microenvironment, inhibits tumor angiogenesis and decreases metastasis by blocking signaling loops and growth stimulation pathways in cancer cells and their niche[104,108–110].

Our *in vitro* studies with leukemic cells (Chapter 7) were aimed to examine the growth inhibitory potential of CBD, including drug resistant leukemic cells (CEM cells with acquired resistance to the proteasome inhibitor bortezomib, BTZ). Moreover, we

explored the effects of long-term exposure of leukemic cells to CBD. The outcome of these studies was that CBD exhibited growth inhibitory effects at concentrations >10 µM by apoptosis induction, and that BTZ-resistant leukemia cells were modestly cross-resistant to CBD. The latter suggests some overlapping mechanisms of action between CBD and BTZ. The most striking observation was that long-term exposure of leukemic cells to CBD resulted in CBD-adapted cells which harboured a CBD-addiction phenotype and displayed a marked growth stimulatory effect at low concentrations of 1 µM CBD. Moreover, CBD-adapted leukemic cells markedly induced MARCKs protein expression which had previously been shown to be involved in vesicular extrusion of polyubiquitinated proteins accumulating and BTZ-induced ER stress[38]. Thus, MARCKS overexpression may be an indicator of ER stress imposed by either BTZ or CBD. The notion that CBD also inhibited mTOR signalling as master regulator of protein synthesis further underscores an impact of CBD on protein synthesis/degradation.

Although any extrapolation of the CBD *in vitro* data for leukemia treatment *in vivo* should be done with caution, the possible implications of our results encourage further research by addressing the following issues:

3.1 CBD plasma levels.

In order to envision whether the reported effects of CBD could hold clinical relevance, it is of importance to put them in perspective of CBD pharmacokinetics, i.e. what plasma and bone marrow microenvironment concentrations of CBD can be achieved and what is the CBD half-life therein? Pharmacokinetic studies in humans demonstrated that CBD smoking or inhalation resulted in plasma levels of up to 0.04 µM. After oral or intravenous administration, much higher peak plasma levels (0.5 – 2.5 µM) can be achieved. CBD plasma half-life vary from 24 hr after iv administration to 31 hr after CBD smoking[92]. No information is available for CBD levels in BM microenvironment. As noted for several other drugs, e.g. trastuzumab, MTX and cetuximab, microenvironment drug concentrations do not always correlate with circulatory plasma drug concentrations. In fact, the tumor microenvironment is enriched with proteins and metabolites (e.g. lactate and adenosine[111]), which can favor tumor growth and decrease drug toxicity[112].

These results would indicate that CBD concentrations > 10 µM are required to induce apoptosis in leukemic cells, but are not readily achievable *in vivo*; therefore anti-leukemic effects may be limited.

3.2 CBD growth stimulatory effects.

Given that maximal growth stimulatory effects by CBD for CBD-adapted cells and BTZ-resistant leukemia cells were observed at pharmacologically achievable CBD concentrations of 1 µM CBD, these effects may be potentially relevant *in vivo*. It still needs to be determined whether these effects are specific for CBD or also induced– and at what concentrations? - by other cannabinoids (e.g. THC) or endogenously secreted endocannabinoids such as anandamide (AEA) and 2-arachidonoylgycerol

(2-AG). These endocannabinoids molecularly resemble plant-derived phytocannabinoids and can also interact and modulate our endocannabinoid system (ECS). The ECS is involved in the regulation of a broad spectrum of physiological functions such as: cognition (pain sensation, memory formation, mood), fertility and pregnancy, sleep, appetite and energy metabolism, immune modulation and inflammation[113,114].

Indeed, one study reported that low concentrations (2 nM) of THC induced proliferation in human tonsillar B-cells *in vitro*[115,116]. Interestingly though in this aspect, is the very high level of proliferation induction that was observed in our studies with CEM leukemia cells *in vitro.* Remarkably, the endogenous cannabinoid anandamide (up to concentrations of 1 μM) lacked the same proliferative effect on B-cells as THC[115]. However, anandamide was able to induce proliferation at low serum concentrations in murine interleukin-dependent lymphoid cell lines[116], suggesting that the level of protein binding is a factor in mediating these effects.

3.3 CBD growth stimulation of drug resistant cells.

The notion that low concentrations of CBD displayed growth stimulatory effects against BTZ-resistant leukemic cells raises the question whether this effect is specific for leukemic cells and/or proteasome inhibitor drugs, or also applies for other classes of anticancer drugs (antimetabolites, anthracyclines, TKIs, etc) and other tumor types. To this end, a survey for a broad array of tumor types and drug-resistant variants thereof would provide insights into the scale of this CBD effect. In case CBD growth stimulatory effects would be a common effect for drug-resistant cancer cells, this would call for awareness to cancer patients to be restrictive to recreative/medicinal CBD use in order not to promote, as a side effect, proliferation of latent drug-resistant clones.

3.4 Dynamics of CBD addiction.

Prolonged exposure of leukemic cells to CBD provoked a CBD-addiction phenotype of the dynamics deserve further investigations. Specifically, experiments to assess whether the addiction is a stable or reversible phenomenon warrant further studies. In case of a reversible effect, how rapidly is it reversed and how rapidly would it be re-introduced upon rechallenge with CBD administration? This information may be relevant considering drug holidays of cancer patients in between therapy courses. In case the reversibility of the CBD addiction takes longer than the drug holiday period, this could negatively impact the efficacy of the next dose of chemotherapy.

3.5 Molecular mechanism of CBD-drug addiction.

Most importantly, further studies should be dedicated to unravel the molecular mechanism underlying CBD-drug addiction and growth stimulatory effects of low CBD concentrations. Drug addiction studies have been reported for solid tumors treated with signalling targeted drugs such as MEK, ERK1/2, BRAF-inhibitors[117,118]. A common feature in these studies appeared to be the involvement of the MAPK/ERK

and mTOR pathways[119 117,118,120]. In fact, hyper activation of the ERK pathway and high levels of pERK1/2[117,118] establish excessive ERK1/2 signaling triggering cell cycle arrest or apoptosis via autophagy by inducing p21 and p53[117,118,121]. During drug addiction, ERK1/2 ensures delicate and specific, yet opposing functions. For example, while ERK2-JUNB-FRA1 pathway activation is observed in several cancer cell lines during treatment addiction, ERK1-JUNB is activated in drug withdrawal-surviving cells. These surviving cells express epithelial-to-mesenchymal transition (EMT)-related genes associated with cell migration genes and proliferation[117,120,122]. Future experiments, including genetic (RNAseq) and pathway analyses under conditions of CBD exposure, should reveal whether CBD-addiction shares overlapping mechanisms with drug addiction to MEK, ERK1/2, BRAF-inhibitors[117,118] and even cross-addiction to these drugs in CBD-adapted leukemic cells. Specifically, a panel of genes deserving particular interest include *MAPK1* (ERK2), *MAP2K1* (encoding MEK1), *JUNB*, *FOSL1* (FRA1) and MARCKS, as well as EMT-related genes such as VCAM. These studies should provide molecular insights on the regulatory programing involving vesicular trafficking (MARCKS), proliferation signaling (mTOR) and drug addiction pathways (ERK). In any case, CBD-adapted CEM/CBD6250 cells serve as an attractive *in vitro* model for future research on cellular CBD-addiction.

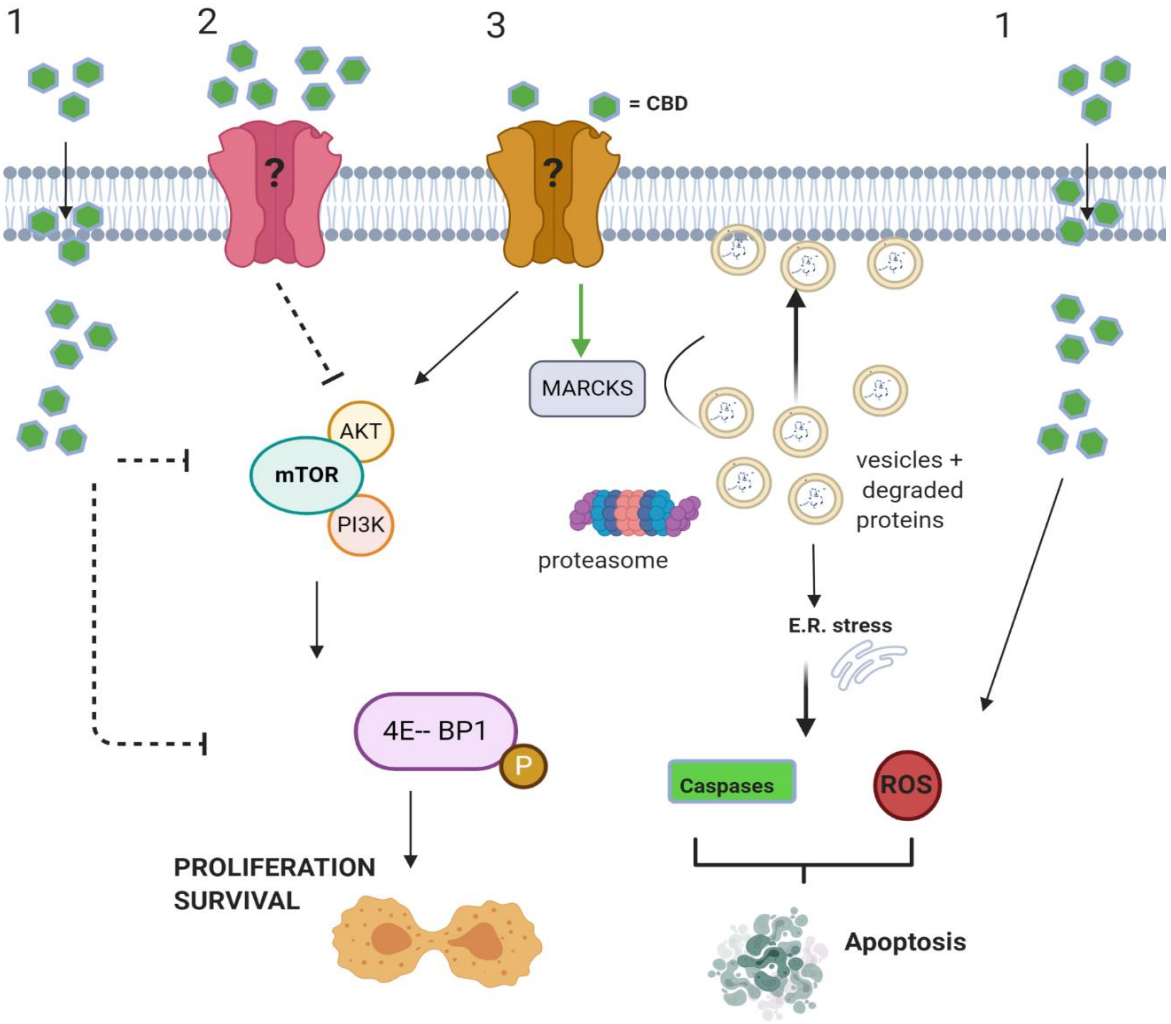
3.6 CBD - leukemia treatment animal studies.

It would be unveiling to examine the *in vivo* effects of CBD in animal models engrafted with leukemic cells or solid tumors to monitor whether *in vitro* effects at low and high dosages of CBD are recapitulated. CBD has been evaluated in a breast cancer model in mice[108,109], in which CBD suppressed EGF/EGFR signaling transduction pathway (and downstream AKT, ERK and NF-kB) at dosages of 10 mg/kg/48 h. CBD treatment decreased metastasis and also inhibited CCL3 and GM-CSF secretion from the cancer cells, resulting in low recruitment of tumor associated macrophages (TAMs) at tumor sites. Thus, CBD is able to modulate the malignant cell - microenvironment loop by inhibition of the adaptive immune response and inhibition of stromal invasion at multiple levels (TAMs, VEGF)[108,109]. Given the dual effects of low vs high CBD concentrations in our leukemia studies *in vitro*, testing different dosages of CBD would be recommended to better understand its activity profile regarding efficacy and adverse effects. A summarizing model of the mechanism of action of CBD in leukemia cells, including membrane receptor proteins and downstream affected pathways, is presented in Figure1.

In summary, the results described in this thesis highlight the role of human regenerative associated endothelial cells (hRECs) in the bone marrow microenvironment and hematopoiesis. By being resistant to 5-FU therapy, hRECs remain active in secreting factors enabling enhanced expansion of precursor HSC. A second part of this thesis provides novel insights into the role of specific influx and efflux transporters implicated in the efficacy/resistance of MTX and Ara-C. Finally, a third part described the results of *in vitro* experimental anti-leukemia therapeutics with cannabidiol, revealing double-edged effects: anti-leukemic effects at high doses of

CBD and cell growth stimulatory effects at low doses of CBD. Together, these results may contribute to improve anti-leukemic chemotherapy efficacy and minimize toxicity.

***Figure 1**: **Possible hypothetical mechanisms of CBD-mediated proliferation or apoptosis in CEM cells in vitro.** CBD at high concentrations (>10 µM) can: (1) diffuse*



through the cell membrane due to its lipophilic characteristics and interrupt signaling pathways vital for cell survival and proliferation (left). CBD is also able to induce ER stress and also mitochondrial stress, resulting in increased ROS production, autophagy and apoptosis. (2) Additionally high CBD concentrations, can activate unidentified GPRs that modulate negatively mTOR activation and 4E-BP1 phosphorylation, leading to reduced survival and apoptosis. (3) At low concentrations (around 1 µM) CBD activates unidentified GPRs that promote 4E-BP1 phosphorylation and stimulate cell proliferation. (4) Sustained, chronic CBD exposure at sub-toxic concentrations, induces CBD resistance, CBD addiction, and MARCKS protein upregulation. MARCKS facilitates the transport of vesicles containing ubiquitinated proteins to overcome proteolytic stress imposed on the ubiquitin/proteasome system and thereby constitutes a well-known mechanism of bortezomib resistance in CEM cells[123]. Abbreviations: G protein-coupled receptors (GPR), cannabidiol (CBD), reactive oxygen species (ROS), endoplasmic reticulum (ER), mammalian target of rapamycin (mTOR), factor 4E (eIF4E)-binding protein 1 (4E-BP1), myristoylated alanine-rich C-kinase substrate (MARCKS). This figure was created with BioRender.com

References:

1. Wang A, Zhong H. Roles of the bone marrow niche in hematopoiesis, leukemogenesis, and chemotherapy resistance in acute myeloid leukemia. *Hematology*. 2018;23(10):729-739.
2. Kenswil KJG, Jaramillo AC, Ping Z, et al. Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor. *Cell Rep*. 2018;22(3):666-678.
3. Sarkaria SM, Decker M, Ding L. Bone marrow microenvironment in normal and deranged hematopoiesis: Opportunities for regenerative medicine and therapies. *Bioessays*. 2018;40(3).
4. Senthebane DA, Rowe A, Thomford NE, et al. The Role of Tumor Microenvironment in Chemoresistance: To Survive, Keep Your Enemies Closer. *Int J Mol Sci*. 2017;18(7).
5. Meyer LK, Hermiston ML. The bone marrow microenvironment as a mediator of chemoresistance in acute lymphoblastic leukemia. *Cancer Drug Resistance*. 2019;2(4):1164-1177.
6. Witkowski MT, Kousteni S, Aifantis I. Mapping and targeting of the leukemic microenvironment. *J Exp Med*. 2020;217(2).
7. Krause DS, Scadden DT. A hostel for the hostile: the bone marrow niche in hematologic neoplasms. *Haematologica*. 2015;100(11):1376-1387.
8. Zhou H-S, Carter BZ, Andreeff M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biol Med*. 2016;13(2):248-259.
9. Munn L. Cancer and Inflammation. *Wiley Interdiscip Rev Syst Biol Med*. 2017;9(2).
10. Vilchis-Ordoñez A, Contreras-Quiroz A, Vadillo E, et al. Bone Marrow Cells in Acute Lymphoblastic Leukemia Create a Proinflammatory Microenvironment Influencing Normal Hematopoietic Differentiation Fates. *BioMed Research International*. 2015;2015:386165.
11. Polo-Corrales L, Latorre-Esteves M, Ramirez-Vick JE. Scaffold Design for Bone Regeneration. *J Nanosci Nanotechnol*. 2014;14(1):15-56.
12. Xu M, Li J, Liu X, et al. Fabrication of vascularized and scaffold-free bone tissue using endothelial and osteogenic cells differentiated from bone marrow derived mesenchymal stem cells. *Tissue Cell*. 2019;61:21-29.
13. Prager BC, Xie Q, Bao S, Rich JN. Cancer Stem Cells: The Architects of the Tumor Ecosystem. *Cell Stem Cell*. 2019;24(1):41-53.
14. Kopan R. Notch Signaling. *Cold Spring Harb Perspect Biol*. 2012;4(10).
15. Meurette O, Mehlen P. Notch Signaling in the Tumor Microenvironment. *Cancer Cell*. 2018;34(4):536-548.
16. Bausch D, Fritz S, Bolm L, et al. Hedgehog signaling promotes angiogenesis directly and indirectly in pancreatic cancer. *Angiogenesis*. 2020;23(3):479-492.
17. Chapouly C, Guimbal S, Hollier P-L, Renault M-A. Role of Hedgehog Signaling in Vasculature Development, Differentiation, and Maintenance. *Int J Mol Sci*. 2019;20(12).
18. Hanna A, Shevde LA. Hedgehog signaling: modulation of cancer properies and tumor mircroenvironment. *Molecular Cancer*. 2016;15(1):24.
19. Somasundaram V, Basudhar D, Bharadwaj G, et al. Molecular Mechanisms of Nitric Oxide in Cancer Progression, Signal Transduction, and Metabolism. *Antioxid Redox Signal*. 2019;30(8):1124-1143.
20. Taniguchi S, Elhance A, Duzer AV, Kumar S, Leitenberger JJ, Oshimori N. Tumor-initiating cells establish an IL-33–TGF-β niche signaling loop to promote cancer progression. *Science*. 2020;369(6501).
21. López de Andrés J, Griñán-Lisón C, Jiménez G, Marchal JA. Cancer stem cell secretome in the tumor microenvironment: a key point for an effective personalized cancer treatment. *Journal of Hematology & Oncology*. 2020;13(1):136.
22. Miller DS. Regulation of ABC Transporters at the Blood-Brain Barrier. *Clin Pharmacol Ther*. 2015;97(4):395-403.
23. Erdő F, Krajcsi P. Age-Related Functional and Expressional Changes in Efflux Pathways at the Blood-Brain Barrier. *Front Aging Neurosci*. 2019;11.
24. Jaramillo AC, Saig FA, Cloos J, Jansen G, Peters GJ. How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance? *Cancer Drug Resistance*. 2018;1(1):6-29.
25. Figueira-Mansur J, Schrago CG, Salles TS, et al. Phylogenetic analysis of the ATP-binding cassette proteins suggests a new ABC protein subfamily J in Aedes aegypti (Diptera: Culicidae). *BMC Genomics*. 2020;21.
26. Xiong J, Feng J, Yuan D, Zhou J, Miao W. Tracing the structural evolution of eukaryotic ATP binding cassette transporter superfamily. *Scientific Reports*. 2015;5(1):16724.

27. Adema AD, Floor K, Smid K, et al. Overexpression of MRP4 (ABCC4) and MRP5 (ABCC5) confer resistance to the nucleoside analogs cytarabine and troxacitabine, but not gemcitabine. *Springerplus*. 2014;3:732.
28. Huang F-F, Zhang L, Wu D-S, et al. PTEN regulates BCRP/ABCG2 and the side population through the PI3K/Akt pathway in chronic myeloid leukemia. *PLoS ONE*. 2014;9(3):e88298.
29. Boutin L, Arnautou P, Trignol A, et al. Mesenchymal stromal cells confer chemoresistance to myeloid leukemia blasts through Side Population functionality and ABC transporter activation. *Haematologica*. 2020;105(4):987-9998.
30. Pui C-H, Mullighan CG, Evans WE, Relling MV. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? *Blood*. 2012;120(6):1165-1174.
31. Reese ND, Schiller GJ. High-dose cytarabine (HD araC) in the treatment of leukemias: a review. *Curr Hematol Malig Rep*. 2013;8(2):141-148.
32. Gao J, Liu W-J. Prognostic value of the response to prednisone for children with acute lymphoblastic leukemia: a meta-analysis. *Eur Rev Med Pharmacol Sci*. 2018;22(22):7858-7866.
33. Alfarouk KO, Stock C-M, Taylor S, et al. Resistance to cancer chemotherapy: failure in drug response from ADME to P-gp. *Cancer Cell International*. 2015;15(1):71.
34. Gillet J-P, Gottesman MM. Mechanisms of multidrug resistance in cancer. *Methods Mol Biol*. 2010;596:47-76.
35. Drenberg C, Hu S, Li L, et al. ABCC4 Is a Determinant of Cytarabine-Induced Cytotoxicity and Myelosuppression. *Clin Transl Sci*. 2016;9(1):51-59.
36. Galmarini CM, Thomas X, Calvo F, et al. In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia. *Br J Haematol*. 2002;117(4):860-868.
37. Levin M, Stark M, Berman B, Assaraf YG. Surmounting Cytarabine-resistance in acute myeloblastic leukemia cells and specimens with a synergistic combination of hydroxyurea and azidothymidine. *Cell Death & Disease*. 2019;10(6):1-14.
38. Macanas-Pirard P, Broekhuizen R, González A, et al. Resistance of leukemia cells to cytarabine chemotherapy is mediated by bone marrow stroma, involves cell-surface equilibrative nucleoside transporter-1 removal and correlates with patient outcome. *Oncotarget*. 2017;8(14):23073-23086.
39. Dobson PD, Kell DB. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat Rev Drug Discov*. 2008;7(3):205-220.
40. Girardi E, César-Razquin A, Lindinger S, et al. A widespread role for SLC transmembrane transporters in resistance to cytotoxic drugs. *Nat Chem Biol*. 2020;16(4):469-478.
41. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nat Rev Cancer*. 2018;18(7):452-464.
42. Choi YH, Yu A-M. ABC Transporters in Multidrug Resistance and Pharmacokinetics, and Strategies for Drug Development. *Curr Pharm Des*. 2014;20(5):793-807.
43. Tamaki A, Ierano C, Szakacs G, Robey RW, Bates SE. The controversial role of ABC transporters in clinical oncology. *Essays Biochem*. 2011;50(1):209-232.
44. Borst P. Looking back at multidrug resistance (MDR) research and ten mistakes to be avoided when writing about ABC transporters in MDR. *FEBS Letters*. 2020, Dec;594(23):4001-4011
45. Efferth T. Adenosine triphosphate-binding cassette transporter genes in ageing and age-related diseases. *Ageing Res Rev*. 2003;2(1):11-24.
46. Kyle-Cezar F, Echevarria-Lima J, Rumjanek VM. Independent Regulation of ABCB1 and ABCC Activities in Thymocytes and Bone Marrow Mononuclear Cells during Aging. *Scandinavian Journal of Immunology*. 2007;66(2-3):238-248.
47. Vetrivel U, Subramanian G. Importance of ABC transporters in different tissues. *Drug Metabolism and Personalized Therapy*. 2014;29(2):65-66.
48. Langmann T, Mauerer R, Zahn A, et al. Real-time reverse transcription-PCR expression profiling of the complete human ATP-binding cassette transporter superfamily in various tissues. *Clin Chem*. 2003;49(2):230-238.
49. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff JA. Temporary Remissions in Acute Leukemia in Children Produced by Folic Acid Antagonist, 4-Aminopteroyl-Glutamic Acid (Aminopterin). *New England Journal of Medicine*. 1948;238(23):787-793.
50. Weinblatt ME. Methotrexate in Rheumatoid Arthritis: A Quarter Century of Development. *Trans Am Clin Climatol Assoc*. 2013;124:16-25.
51. Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy*. 2012;15(4):183-210.
52. Jaramillo AC, Cloos J, Lemos C, et al. Ex vivo resistance in childhood acute lymphoblastic leukemia: Correlations between BCRP, MRP1, MRP4 and MRP5 ABC transporter expression and intracellular methotrexate polyglutamate accumulation. *Leukemia Research*. 2019;79:45-51.

53. Wojtuszkiewicz A, Raz S, Stark M, et al. Folylpolyglutamate synthetase splicing alterations in acute lymphoblastic leukemia are provoked by methotrexate and other chemotherapeutics and mediate chemoresistance. *Int J Cancer*. 2016;138(7):1645-1656.
54. Muller IB, Hebing RF, Jansen G, et al. Personalized medicine in rheumatoid arthritis: methotrexate polyglutamylation revisited. *Expert Review of Precision Medicine and Drug Development*. 2018;3(6):331-334.
55. Whitehead V, Rosenblatt D. Methotrexate metabolism by bone marrow cells from patients with leukemia. *Advances in experimental medicine and biology*. 1983;163:287-303.
56. Synold T, Relling M, Boyett J, et al. Blast cell methotrexate-polyglutamate accumulation in vivo differs by lineage, ploidy, and methotrexate dose in acute lymphoblastic leukemia. *The Journal of clinical investigation*. 1994;94(5):1996-2001.
57. Wojtuszkiewicz A, Assaraf YG, Hoekstra M, et al. The association of aberrant folylpolyglutamate synthetase splicing with ex vivo methotrexate resistance and clinical outcome in childhood acute lymphoblastic leukemia. *Haematologica*. 2016;101(7):e291-294.
58. Yu S-L, Zhang H, Ho B-C, et al. FPGS relapse-specific mutations in relapsed childhood acute lymphoblastic leukemia. *Sci Rep*. 2020;10(1):12074.
59. Rots MG, Pieters R, Jansen G, et al. A possible role for methotrexate in the treatment of childhood acute myeloid leukaemia, in particular for acute monocytic leukaemia. *Eur J Cancer*. 2001;37(4):492-498.
60. Rots MG, Pieters R, Peters GJ, et al. Circumvention of methotrexate resistance in childhood leukemia subtypes by rationally designed antifolates. *Blood*. 1999;94(9):3121-3128.
61. Walling J. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. *Investigational New Drugs*. 2006;24(1):37-77.
62. Bender R. Anti-folate resistance in leukemia: treatment with “high-dose” methotrexate and citrovorum factor. *Cancer treatment reviews*. 1975;2(3):215-224.
63. Zhu Z, Leung GKK. More Than a Metabolic Enzyme: MTHFD2 as a Novel Target for Anticancer Therapy? *Front Oncol*. 2020;10:658.
64. Pikman Y, Puissant A, Alexe G, et al. Targeting MTHFD2 in acute myeloid leukemia. *J Exp Med*. 2016;213(7):1285-1306.
65. Dekhne AS, Hou Z, Gangjee A, Matherly LH. Therapeutic Targeting of Mitochondrial One-Carbon Metabolism in Cancer. *Mol Cancer Ther*. Published online September 2, 2020.
66. Tedeschi PM, Scotto KW, Kerrigan J, Bertino JR. MTHFD2--a new twist? *Oncotarget*. 2016;7(7):7368-7369.
67. Rana RM, Rampogu S, Abid NB, et al. In Silico Study Identified Methotrexate Analog as Potential Inhibitor of Drug Resistant Human Dihydrofolate Reductase for Cancer Therapeutics. *Molecules*. 2020;25(15).
68. Yu W-J, Huang D-X, Liu S, Sha Y-L, Gao F, Liu H. Polymeric Nanoscale Drug Carriers Mediate the Delivery of Methotrexate for Developing Therapeutic Interventions Against Cancer and Rheumatoid Arthritis. *Front Oncol*. 2020;10.
69. Jaramillo A, Hubeek I, Broekhuizen R, et al. Expression of the nucleoside transporters hENT1 (SLC29) and hCNT1 (SLC28) in pediatric acute myeloid leukemia. *Nucleosides, Nucleotides & Nucleic Acids*. 2020:39(10-12):1379-1388.
70. Peters GJ, van der Wilt CL, van Moorsel CJA, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacology & Therapeutics*. 2000;87(2):227-253.
71. Kim J, Kim H, Lee J, et al. Human equilibrative nucleoside transporter 1 (hENT1) expression as a predictive biomarker for gemcitabine chemotherapy in biliary tract cancer. *PLoS One*. 2018;13(12):e0209104
72. Raffenne J, Nicolle R, Puleo F, et al. hENT1 Testing in Pancreatic Ductal Adenocarcinoma: Are We Ready? A Multimodal Evaluation of hENT1 Status. *Cancers (Basel)*. 2019;11(11):1808.
73. Okamura Y, Yasukawa S, Narimatsu H, et al. Human equilibrative nucleoside transporter-1 expression is a predictor in patients with resected pancreatic cancer treated with adjuvant S-1 chemotherapy. *Cancer Sci*. 2020;111(2):548-560.
74. Ormanns S, Heinemann V, Raponi M, et al. Human equilibrative nucleoside transporter 1 is not predictive for gemcitabine efficacy in advanced pancreatic cancer: Translational results from the AIO-PK0104 phase III study with the clone SP120 rabbit antibody. *European Journal of Cancer*. 2014;50(11):1891-1899.
75. Greenhalf W, Ghaneh P, Neoptolemos JP, et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. *J Natl Cancer Inst*. 2014;106(1):djt347.
76. Sinn M, Riess H, Sinn BV, et al. Human equilibrative nucleoside transporter 1 expression analysed by the clone SP 120 rabbit antibody is not predictive in patients with pancreatic cancer

treated with adjuvant gemcitabine - Results from the CONKO-001 trial. *Eur J Cancer*. 2015;51(12):1546-1554.
77. Poplin E, Wasan H, Rolfe L, et al. Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. *J Clin Oncol*. 2013;31(35):4453-4461.
78. Randazzo O, Papini F, Mantini G, et al. "Open Sesame?": Biomarker Status of the Human Equilibrative Nucleoside Transporter-1 and Molecular Mechanisms Influencing its Expression and Activity in the Uptake and Cytotoxicity of Gemcitabine in Pancreatic Cancer. *Cancers (Basel)*. 2020;12(11):3206.
79. Ciccolini J, Serdjebi C, Peters GJ, Giovannetti E. Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemother Pharmacol*. 2016;78(1):1-12.
80. Ruiz van Haperen VWT, Peters GJ. New targets for pyrimidine antimetabolites for the treatment of solid tumours. *Pharm World Sci*. 1994;16(2):104-112.
81. Ruiz van Haperen VW, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2',2'-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res*. 1994;54(15):4138-4143.
82. Heinemann V, Xu YZ, Chubb S, et al. Cellular elimination of 2',2'-difluorodeoxycytidine 5'-triphosphate: a mechanism of self-potentiation. *Cancer Res*. 1992;52(3):533-539.
83. Plunkett W, Huang P, Searcy CE, Gandhi V. Gemcitabine: preclinical pharmacology and mechanisms of action. *Semin Oncol*. 1996;23(5 Suppl 10):3-15.
84. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. *Cancer Res*. 1991;51(22):6110-6117.
85. Bergman AM, Pinedo HM, Peters GJ. Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). *Drug Resist Updat*. 2002;5(1):19-33.
86. Bergman AM, Kuiper CM, Voorn DA, et al. Antiproliferative activity and mechanism of action of fatty acid derivatives of arabinofuranosylcytosine in leukemia and solid tumor cell lines. *Biochem Pharmacol*. 2004;67(3):503-511.
87. Bergman AM, Adema AD, Balzarini J, et al. Antiproliferative activity, mechanism of action and oral antitumor activity of CP-4126, a fatty acid derivative of gemcitabine, in in vitro and in vivo tumor models. *Invest New Drugs*. 2011;29(3):456-466.
88. Alfayez M, Kantarjian H, Kadia T, Ravandi-Kashani F, Daver N. CPX-351 (vyxeos) in AML. *Leuk Lymphoma*. 2020;61(2):288-297.
89. Schwartsmann G, Brondani da Rocha A, Berlinck RG, Jimeno J. Marine organisms as a source of new anticancer agents. *Lancet Oncol*. 2001;2(4):221-225.
90. Cragg GM, Pezzuto JM. Natural Products as a Vital Source for the Discovery of Cancer Chemotherapeutic and Chemopreventive Agents. *Med Princ Pract*. 2016;25(Suppl. 2):41-59.
91. Dexamethasone briefing - its chemistry and history - The Cambridge Crystallographic Data Centre (CCDC). Accessed December 3, 2020. https://www.ccdc.cam.ac.uk/Community/blog/dexamethasone/
92. Millar SA, Stone NL, Yates AS, O'Sullivan SE. A Systematic Review on the Pharmacokinetics of Cannabidiol in Humans. *Front Pharmacol*. 2018;9:1365.
93. Ren M, Tang Z, Wu X, et al. The origins of cannabis smoking: Chemical residue evidence from the first millennium BCE in the Pamirs. *Science Advances*. 2019;5(6):eaaw1391.
94. Zuardi AW. History of cannabis as a medicine: a review. *Brazilian Journal of Psychiatry*. 2006;28(2):153-157.
95. Silvestro S, Mammana S, Cavalli E, Bramanti P, Mazzon E. Use of Cannabidiol in the Treatment of Epilepsy: Efficacy and Security in Clinical Trials. *Molecules*. 2019;24(8):1459.
96. Crippa JA, Guimarães FS, Campos AC, Zuardi AW. Translational Investigation of the Therapeutic Potential of Cannabidiol (CBD): Toward a New Age. *Front Immunol*. 2018;9:2009.
97. Carrier EJ, Auchampach JA, Hillard CJ. Inhibition of an equilibrative nucleoside transporter by cannabidiol: a mechanism of cannabinoid immunosuppression. *Proc Natl Acad Sci U S A*. 2006;103(20):7895-7900.
98. Peyravian N, Deo S, Daunert S, Jimenez JJ. Cannabidiol as a Novel Therapeutic for Immune Modulation. *Immunotargets Ther*. 2020;9:131-140.
99. Garza-Cervantes JA, Ramos-González M, Lozano O, Jerjes-Sánchez C, García-Rivas G. Therapeutic Applications of Cannabinoids in Cardiomyopathy and Heart Failure. *Oxid Med Cell Longev*. 2020;2020.
100. da Silva VK, de Freitas BS, da Silva Dornelles A, et al. Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: implications for neuroprotection. *Mol Neurobiol*. 2014;49(1):222-233.

101. Singer E, Judkins J, Salomonis N, et al. Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma. *Cell Death Dis*. 2015;6(1):e1601.
102. Olivas-Aguirre M, Torres-López L, Valle-Reyes JS, Hernández-Cruz A, Pottosin I, Dobrovinskaya O. Cannabidiol directly targets mitochondria and disturbs calcium homeostasis in acute lymphoblastic leukemia. *Cell Death Dis*. 2019;10(10):779.
103. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*. 2006;9(6):425-434.
104. Giacoppo S, Pollastro F, Grassi G, Bramanti P, Mazzon E. Target regulation of PI3K/Akt/mTOR pathway by cannabidiol in treatment of experimental multiple sclerosis. *Fitoterapia*. 2017;116:77-84.
105. Caffarel MM, Andradas C, Mira E, et al. Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. *Mol Cancer*. 2010;9:196.
106. Massi P, Solinas M, Cinquina V, Parolaro D. Cannabidiol as potential anticancer drug. *Br J Clin Pharmacol*. 2013;75(2):303-312.
107. McAllister SD, Murase R, Christian RT, et al. Pathways mediating the effects of cannabidiol on the reduction of breast cancer cell proliferation, invasion, and metastasis. *Breast Cancer Res Treat*. 2011;129(1):37-47.
108. Elbaz M, Nasser MW, Ravi J, et al. Modulation of the tumor microenvironment and inhibition of EGF/EGFR pathway: Novel anti-tumor mechanisms of Cannabidiol in breast cancer. *Molecular Oncology*. 2015;9(4):906-919.
109. Solinas M, Massi P, Cantelmo A, et al. Cannabidiol inhibits angiogenesis by multiple mechanisms. *Br J Pharmacol*. 2012;167(6):1218-1231.
110. Kisková T, Mungenast F, Suváková M, Jäger W, Thalhammer T. Future Aspects for Cannabinoids in Breast Cancer Therapy. *Int J Mol Sci*. 2019;20(7):1673.
111. Brand A, Singer K, Koehl GE, et al. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. *Cell Metabolism*. 2016;24(5):657-671.
112. Bartelink IH, Jones EF, Shahidi-Latham SK, et al. Tumor Drug Penetration Measurements Could Be the Neglected Piece of the Personalized Cancer Treatment Puzzle. *Clin Pharmacol Ther*. 2019;106(1):148-163.
113. Ligresti A, De Petrocellis L, Di Marzo V. From Phytocannabinoids to Cannabinoid Receptors and Endocannabinoids: Pleiotropic Physiological and Pathological Roles Through Complex Pharmacology. *Physiological Reviews*. 2016;96(4):1593-1659.
114. Hillard CJ. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? *Neuropsychopharmacology*. 2018;43(1):155-172.
115. Derocq JM, Ségui M, Marchand J, Le Fur G, Casellas P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett*. 1995;369(2-3):177-182.
116. Derocq JM, Bouaboula M, Marchand J, Rinaldi-Carmona M, Ségui M, Casellas P. The endogenous cannabinoid anandamide is a lipid messenger activating cell growth via a cannabinoid receptor-independent pathway in hematopoietic cell lines. *FEBS Lett*. 1998;425(3):419-425.
117. Kong X, Kuilman T, Shahrabi A, et al. Cancer drug addiction is relayed by an ERK2-dependent phenotype switch. *Nature*. 2017;550(7675):270-274.
118. Sale MJ, Balmanno K, Cook SJ. Resistance to ERK1/2 pathway inhibitors; sweet spots, fitness deficits and drug addiction. *Cancer Drug Resistance*. 2019;2(2):365-380.
119. Pópulo H, Lopes JM, Soares P. The mTOR signalling pathway in human cancer. *Int J Mol Sci*. 2012;13(2):1886-1918.
120. Bradley CA. Understanding tumour drug addiction. *Nature Reviews Cancer*. 2017;17(11):634-635.
121. Wu P-K, Becker A, Park J-I. Growth Inhibitory Signaling of the Raf/MEK/ERK Pathway. *Int J Mol Sci*. 2020;21(15).
122. Lee RJ, Marais R. Tumours addicted to drugs are vulnerable. *Nature*. 2017;550(7675):192-193.
123. Franke NE, Kaspers GL, Assaraf YG, et al. Exocytosis of polyubiquitinated proteins in bortezomib-resistant leukemia cells: a role for MARCKS in acquired resistance to proteasome inhibitors. *Oncotarget*. 2016;7(46):74779-74796.

CHAPTER 9

Summary

Summary

Leukemia is one of the most common types of hematological malignancies in both adults and children. Some of the fundamental traits of leukemia include the capacity for sustained, uncontrolled proliferation and impaired differentiation of white blood cells. Although chemotherapy remains the cornerstone for curative treatment, some leukemia cells will remain after therapy due to drug resistance, which is an important clinical challenge without a pharmacological solution for all patients at the moment. In addition, cell-specific and microenvironmental factors play a role in both the etiology and progression of leukemia.

Because the bone marrow (BM) and the hematopoietic stem cells (HSC) respond to physiological stimuli, a systems biology perspective of leukemia can help to understand and elucidate the mechanisms underlying sustained, uncontrolled proliferation and drug resistance. This approach allows envisioning leukemia not only as a cell-specific phenomenon, but also as a tissue-based anomaly, in which the dynamic interaction between the leukemic cells and their microenvironment plays a key role in malignant transformation, drug resistance, and dissemination.

In this thesis, we describe leukemia treatment and development through analysis of cell-specific features as well as the microenvironmental influence of endothelial cells in BM regeneration post-chemotherapy. This allows us to identify crucial drug resistance and pro-malignant mechanisms that could be potentially targeted pharmaceutically. Cell-specific factors that we investigated in this thesis include the expression and influence of drug transporters at the membrane level and the metabolic interaction of drugs in cancer therapy. The environmental factors studied include transcriptional expression analysis of specialized endothelial niche cells in the BM.

In **chapter 2** we demonstrate that the BM microenvironment harbors specialized endothelial niches responsible for the maintenance of HSCs and for their ability to provide paracrine signalling to sustain physiological HSC function. Notably, angiocrine factors, such as IL-33, secreted from specialized endothelial cells (human regenerative endothelial cells or hRECs) in the BM, can stimulate hematopoiesis, angiogenesis, and osteogenesis during BM development and regeneration post-chemotherapy in acute myeloid leukemia (AML). Still, the precise role of IL-33 in leukemic transformation and drug resistance remains to be further elucidated.

In **chapters 3** and **4** of the thesis cell-intrinsic mechanisms contributing to drug resistance are described. One of the most studied mechanisms involves the role of ATP-binding cassette (ABC) drug efflux transporter proteins. These diverse and phylogenetically ancient membrane-associated proteins are expressed in all cell types and play a key role in protecting both prokaryotic and eukaryotic cells from toxic xenobiotics. Specifically, many of these transporters can also expel a myriad of chemotherapeutic molecules, including those relevant to leukemia, such as methotrexate (MTX), and therefore are able to impact cellular drug efflux. The clinical relevance of individual ABC transporters for therapy efficacy in leukemia still needs further elucidation in leukemia, but one lesson that is learned from these transporters is their overlapping capability/functional redundancy and working in tandem to complement each other's function. The end result is lowered intracellular drug concentration (in this case MTX), thus leading to drug resistance. In **chapter 3** we explored the molecular characteristics and clinical relevance of various ABC transporters such as P-glycoprotein (P-gp/ABCB1), breast cancer resistance protein

(BCRP/ABCG2), and multidrug resistance-associated protein-1 (MRP1/ABCC1). We further reviewed and discussed recent insights on specific pharmacological mechanisms to overcome ABC transporter-related drug resistance. In **chapter 4** we explored the joined expression of MRP1, MRP4, MRP5, and BCRP in childhood acute lymphocytic leukemia (ALL) samples ex vivo, and how their expression correlated with MTX intracellular concentrations and overall patient survival. Our analysis revealed that while MRP4 expression correlated with ex vivo MTX resistance, it did not correlate by itself with poor survival. Yet, when both MRP4 and BCRP expression is high, not only this was associated with a decreased intracellular MTX accumulation, but also a lower overall survival.

Along this line, we also explored (**chapter 5**) other clinically relevant transporters in pediatric leukemia, such as the expression of the human equilibrative nucleoside transporter 1 (hENT1) and the concentrative nucleoside transporter-1 (hCNT1). These transporters are responsible for three-quarters of the cellular uptake of nucleoside analogs, such as cytarabine (Ara-C). Specifically, immunohistochemistry studies in primary pediatrics AML samples demonstrated that these transporters are widely expressed at the cell membrane and cytoplasm at the time of diagnosis. Interestingly, no significant relationship was found between survival and transporter expression and ex vivo drug sensitivity.

In **chapter 6**, we examined the influence of dexamethasone (DEX) on the in vivo antileukemic effect of Ara-C and gemcitabine in a rat model of AML. Specifically, we studied the effect of DEX administration on the enzymatic activity of deoxycytidine kinase (dCK) . Since dCK catalyses the first step in the conversion of Ara-C into its active metabolite, cytosine arabinoside triphosphate (Ara-CTP) and previous studies showed that DEX administration diminished dCK activity, it was relevant to study this relationship further. It was not surprising to observe an increased life-span in Ara-C-treated AML rats, and concomitant treatment with DEX had no impact on dCK enzymatic activity in leukemic cells. As this challenged results from previously published studies, further in-depth investigations on the impact of DEX in AML patients treated with cytarabine are indicated.

Finally, in **chapter 7**, we explored the proliferating capacity and cytotoxicity profile of cannabidiol (CBD) in vitro in prototypical human (T) ALL cell line CCRF-CEM and its subline (CEM/BTZ) with acquired resistance to bortezomib (BTZ). CBD is an FDA-approved anti-inflammatory, anti-epileptic, and cell-protective lipophilic molecule popularly sold over-the-counter. CBD is also a known modulator of mTOR activity, which is crucial for cell survival and proliferation, both in malignant and healthy cells. Notably, cell growth inhibition/toxicity assays revealed that high concentrations of CBD were needed (around 10 µM) to induce toxicity. Remarkably, low, clinically reachable concentrations of CBD (around 1 µM) were able to induce significant cell proliferation, which was cell-density dependent, which suggests a role for secondary cell-density growth stimulatory effects. Furthermore, chronic stepwise exposition to increasing concentrations of CBD resulted in subline (CEM/CBD6250) with CBD-addiction properties where CBD is required for its growth. The positioning of CBD as a potential treatment option for ALL in vivo still needs to be defined. The role of modulating the microenvironment might be relevant in CBD treatment, as it is also able to inhibit inflammation and angiogenesis in many tumor types. Since we did not yet investigate these features and the mechanisms of action of CBD in leukemia cells was not yet clarified, further research is warranted.

Together, this thesis describes the multifaceted approach of leukemia treatment, BM microenvironment, drug resistance, and the bimodal (proliferative/cytotoxic) properties

of CBD. As new regenerative factors are revealed in specialized endothelial cells during regenerative and developmental stages, new questions arise on how these cells, niches, and factors are involved in leukemia development and protection from drugs and development of drug resistance. Furthermore, in the second part of this thesis, we focused on how cell-intrinsic factors are involved in drug resistance. Such can be conferred by the overexpression of drug-transporter proteins in cancer cells. Even though no single transporter is solely responsible for specific drug resistance, our analyses revealed significant correlations between overexpression of multiple drug efflux transporters and lowered intracellular chemotherapy (MTX) concentration, and poor survival. Some influx transporters (such as hENT1 and hCNT1) are so widely expressed, that this hampers clinically accurate classification of these transporters in leukemia. This implies that a broader approach needs to be applied to fully comprehend the relative distribution of various in- and efflux transporters in optimized and personalized leukemia therapy.

KEY POINTS

- The BM microenvironment harbours specialized human endothelial regenerative cells (hRECs) within endosteal niches. hRECs are active during developmental stages and regeneration and post-chemotherapeutic insult. hRECs overexpress IL-33, which stimulates the expansion of hematopoietic precursor cells, endothelial cells, as well as osteogenic differentiation.

- ABC transporters MRP4 and BCRP are major determinants in regulating the intracellular accumulation of MTX-polyglutamates in leukemic cells, which is associated with overall survival.

- The human equilibrative nucleoside transporter 1 (hENT1) and the concentrative nucleoside transporter-1 (hCNT1) are responsible for cellular uptake of cytarabine (Ara-C). Immunocytochemistry staining revealed that hENT1 and hCNT1 are widely expressed during diagnosis in pediatric AML, hampering accurate clinical classification based on the expression of these transporters.

- Dexamethasone (DEX) and Ara-C are important drugs in childhood ALL treatment. To this end, some studies showed that DEX might affect the activation of intracellular Ara-C by decreasing deoxycytidine kinase (dCK) activity. Our in vivo studies, however, showed that combined DEX-Ara-C administration did not lower dCK activity or affect the anti-leukemic effect of Ara-C in a rat AML model.

- CBD has dual effects on leukemia cells in vitro: it has growth inhibitory effects at concentrations > 10 µM, but growth stimulatory effects, in particular in drug-resistant cells, at concentrations around 1 µM. Given that prolonged CBD exposure also provokes drug-addiction phenomena, this would call for caution for unsupervised use of CBD as an experimental drug in leukemia treatment.

Samenvatting

Leukemie is een van de meest voorkomende hematologische kankersoorten bij zowel volwassenen als kinderen. Enkele belangrijke eigenschappen van leukemie zijn het vermogen tot aanhoudende, ongecontroleerde groei en verminderde differentiatie van witte bloedcellen. Hoewel chemotherapie de hoeksteen blijft voor genezing, zullen sommige leukemiecellen na de therapie overblijven vanwege ongevoeligheid (resistentie) ontwikkeling tegen anti-leukemische geneesmiddelen. Dit is een belangrijke klinische uitdaging, maar helaas nog steeds zonder een farmacologische oplossing waar alle patiëntenbaat bij hebben. Bovendien spelen celspecifieke en micro-omgevingsfactoren een rol bij zowel de etiologie als de progressie van leukemie.

Omdat het beenmerg (BM) en de hematopoëtische stamcellen (HSC) reageren op fysiologische stimuli, kan een systematische en biologische aanpak van leukemie helpen om de resistentie mechanismen te begrijpen die ten grondslag liggen aan de aanhoudende, ongecontroleerde groei en resistentie. Deze benadering maakt het mogelijk om leukemie niet alleen te zien als een celspecifiek fenomeen, maar ook in de context van de weefsels in de omgeving. Hier speelt de dynamische interactie tussen de leukemiecellen en hun micro-omgeving een sleutelrol bij kwaadaardige transformatie, resistentie en verspreiding.

In dit proefschrift beschrijven we de analyse van celspecifieke kenmerken en de invloed van de micro-omgeving op de ontwikkeling van leukemie en op de behandeling en de rol van endotheelcellen bij BM regeneratie na chemotherapie. Dit stelt ons in staat om cruciale resistentie en pro-maligne mechanismen te identificeren die mogelijk farmacologisch kunnen worden aangepakt. Celspecifieke factoren die we in dit proefschrift hebben onderzocht, zijn onder meer de expressie en invloed van transporter eiwitten op membraanniveau en de metabole interactie van medicijnen bij kankertherapie. De bestudeerde omgevingsfactoren omvatten transcriptionele expressie-analyse van gespecialiseerde endotheel cellen in het BM.

In **hoofdstuk 2** laten we zien dat er in de BM-micro-omgeving gespecialiseerde endotheel niches zijn die verantwoordelijk zijn voor het overleven van HSC's en voor hun vermogen om paracriene signalen uit te zenden om de fysiologische HSC-functie te ondersteunen. Angiocriene factoren, zoals IL-33, worden uitgescheiden door gespecialiseerde endotheelcellen (menselijke regeneratieve endotheelcellen of hREC's) in het BM. Deze kunnen hematopoëse, angiogenese en osteogenese stimuleren tijdens de BM-ontwikkeling en regeneratie na chemotherapie bij acute myeloïde leukemie (AML). Toch moet de juiste rol van IL-33 bij leukemische transformatie en geneesmiddelresistentie nog verder worden opgehelderd.

In de hoofdstukken 3 en 4 van het proefschrift worden cel-intrinsieke mechanismen beschreven die een rol spelen in de resistentie tegen geneesmiddelen. Een van de belangrijkste mechanismen betreft de rol van ATP-bindende cassette (ABC) transporteiwitten, die geneesmiddelen naar buiten kunnen pompen. Deze diverse en fylogenetisch oude membraangeassocieerde eiwitten komen tot expressie in alle celtypen en spelen een sleutelrol bij het beschermen van zowel prokaryote als eukaryote cellen tegen toxische xenobiotica. In het bijzonder kunnen veel van deze transporteiwitten ook een groot aantal geneesmiddelen naar buiten pompen, inclusief enkele geneesmiddelen die belangrijk zijn voor leukemie, zoals methotrexaat (MTX) en anthracyclines. De klinische relevantie van individuele ABC-transporters voor de werkzaamheid van therapie bij leukemie is nog steeds een belangrijke onderzoeksvraag. Het is de laatste jaren echter steeds duidelijker geworden dat deze

transporters een overlappende capaciteit en functie hebben en samenwerken om elkaars functie aan te vullen. Het eindresultaat is een verlaagde intracellulaire concentratie van bijv. MTX, wat leidt tot resistentie. In **hoofdstuk 3** hebben we de moleculaire kenmerken en klinische relevantie van verschillende ABC-transporteiwitten onderzocht, zoals P-glycoproteïne (P-gp / ABCB1), borstkankerresistentie-eiwit (BCRP / ABCG2) en multidrug-resistentie-geassocieerd proteïne-1 (MRP1 / ABCC1).). We hebben recente inzichten beschreven over specifieke farmacologische mechanismen om ABC-transporter-gerelateerde resistentie te omzeilen. In **hoofdstuk 4** hebben we ex vivo de gecombineerde expressie van MRP1, MRP4, MRP5 en BCRP onderzocht in monsters van acute lymfatische leukemie (ALL) bij kinderen, en hoe hun expressie correleerde met intracellulaire concentraties van MTX en de algehele overleving van de patiënt. Onze analyse liet zien dat MRP4-expressie correleerde met ex vivo MTX-resistentie, maar niet correleerde met een slechte overleving. In het geval dat zowel MRP4- als BCRP-expressie hoog zijn, was dit niet alleen geassocieerd met een verminderde intracellulaire MTX-accumulatie, maar ook met een lagere algehele overleving.

Langs deze lijn hebben we ook (**hoofdstuk 5**) andere klinisch relevante transporteiwitten bij kinder leukemie onderzocht, zoals de expressie van enkele andere nucleoside transporters zoals hENT1 en hCNT1. Deze transporters zijn verantwoordelijk voor driekwart van de cellulaire opname van nucleoside-analogen, zoals cytarabine (Ara-C). Specifiek, immunohistochemische kleuringen in primaire kinder AML-monsters toonden aan dat op het moment van diagnose deze transporteiwitten op grote schaal aanwezig zijn op het celmembraan en in het cytoplasma. Er werd echter geen significante relatie werd gevonden tussen overleving en expressie van transporteiwitten en ex vivo geneesmiddelgevoeligheid. Wellicht spelen andere factoren een rol. Een van deze factoren, het activeringsenzym.voor Ara-C, deoxycytidine kinase (dCK) werd in **hoofdstuk 6** onderzocht.

In dit hoofdstuk onderzochten we de invloed van dexamethason (DEX) op het in vivo antileukemische effect van Ara-C en gemcitabine in een rattemodel van AML. Daarbij concentreerden we ons op het effect van DEX-toediening op de enzymatische activiteit van dCK. dCK katalyseert de eerste stap in de omzetting van Ara-C naar zijn actieve metaboliet, cytosine arabinoside trifosfaat (Ara-CTP). Omdat we eerder *in vitro* aangetoond hadden dat behandeling met DEX de dCK-activiteit verminderde, hebben we onderzocht of dit ook in een relevant *in vivo* leukemie model het geval was. Zoals verwacht verlengde Ara-C behandeling de levensduur van AML-ratten, maar gelijktijdige behandeling met DEX had geen invloed op het antileukemisch effect en de enzymatische activiteit van dCK in leukemische cellen. DEX had wel een effect of de dCK activiteit in normale lever, terwijl in andere studies met DEX gevonden is dat het de bijwerkingen vermindert.

Ten slotte hebben we in **hoofdstuk 7** het effect van cannabidiol (CBD) *in vitro* onderzocht in de prototypische menselijke (T) ALL cellijn CCRF-CEM en zijn sublijn (CEM / BTZ) met resistentie tegen bortezomib (BTZ). CBD is een door de FDA goedgekeurde ontstekingsremmer, anti-epilepticum en celbeschermende lipofiele molecuul wat ook vrij verkrijgbaar is als cannabis. CBD is ook een bekende modulator van mTOR-activiteit, wat cruciaal is voor celoverleving en celgroei, in kwaadaardige en gezonde cellen. Om de celgroeiremming / toxiciteit te induceren waren echter hoge concentraties CBD nodig (ongeveer 10 µM). Het was opmerkelijk dat lage, klinisch relevante concentraties van CBD (ongeveer 1 µM) significante celgroei konden induceren. Dit was afhankelijk van de celdichtheid, wat een rol suggereert voor secundaire stimulerende effecten van de cellulaire dichtheid. Bovendien resulteerde chronische stapsgewijze blootstelling aan steeds hogere concentraties van CBD in

een “verslaving” van de subliin (CEM / CBD6250). Deze verslavende eigenschappen kunnen verklaren waarom CBD nodig is voor de groei. De mogelijkheid van CBD als een mogelijke behandelingsoptie voor ALL *in vivo* moet nog worden onderzocht. De rol van de micro-omgeving kan relevant zijn bij CBD-behandeling, omdat het ook in staat is om ontsteking en angiogenese bij veel tumortypen te remmen. Aangezien we deze kenmerken nog niet hebben onderzocht en de werkingsmechanismen van CBD in leukemiecellen nog niet zijn opgehelderd, is verder onderzoek nodig.

Samengevat beschrijft dit proefschrift een veelzijdige aanpak van leukemiebehandeling, de BM-micro-omgeving, geneesmiddelresistentie en de bimodale (groei/ cytotoxische) eigenschappen van CBD. De nieuwe regeneratieve factoren die worden gevonden in gespecialiseerde endotheelcellen tijdens regeneratieve en ontwikkelingsstadia, roepen nieuwe vragen voor over de rol van deze cellen, de niches en factoren bij de ontwikkeling van leukemie, de bescherming tegen geneesmiddelen en de ontwikkeling van geneesmiddelresistentie. Daarom hebben we ons in het tweede deel van dit proefschrift gericht op hoe cel-intrinsieke factoren betrokken zijn bij geneesmiddelresistentie. Dit kan worden verklaard worden door de hoge activiteit van transporteiwitten die geneesmiddelen uit de kankercellen pompen. Het bleek echter dat niet een maar meerdere transporteiwitten verantwoordelijk zijnvoor specifieke geneesmiddelresistentie, omdat wij een significante correlatie vonden met niet alleen een lagere intracellulaire concentratie van het geneesmiddel MTX -maar ook met een slechte overleving. Wat betreft enkele transporteiwitten die verantwoordelijk zijn voor de opname bleek dat deze (zowel hENT1 als hCNT1) zo’n hoge expressie hebben dat een klinisch relevante classificatie bij leukemie niet mogelijk was, i.t.t. tot enkele solide tumoren zoals long- en alvleesklierkanker. Dit impliceert dat alleen de relatieve distributie van verschillende in- en efflux transporters niet voldoende is om een geoptimaliseerde en gepersonaliseerde leukemietherapie mogelijk te maken.

BELANGRIJKSTE PUNTEN

• De BM-micro-omgeving heeft gespecialiseerde menselijke endotheel regeneratieve cellen (hREC's) in endostale niches. hREC's zijn actief tijdens ontwikkelingsstadia en regeneratie en na de blootstelling aan chemotherapie. hREC's brengen IL-33 tot overexpressie, wat de expansie van hematopoëtische voorlopercellen, endotheelcellen en osteogene differentiatie stimuleert.

• ABC-transporteiwitten MRP4 en BCRP zijn belangrijke factoren bij het reguleren van de intracellulaire accumulatie van MTX-polyglutamaten in leukemische cellen, wat wordt geassocieerd met de algehele overleving.

• Twee nucleoside transporters (hENT1 en hCNT1 zijn verantwoordelijk voor de cellulaire opname van cytarabine (Ara-C). Immunocytochemische kleuringen toonden aan dat hENT1 en hCNT1 een hoge expressie hebben in kinder AML, wat een nauwkeurige klinische classificatie op basis van de expressie van deze transporteiwitten belemmert.

• Dexamethason (DEX) en Ara-C zijn belangrijke geneesmiddelen bij de behandeling van ALL bij kinderen. Eerder was aangetoond dat DEX de activering van intracellulair Ara-Cdoor deoxycytidine kinase (dCK) zou kunnen verminderen. Onze *in vivo* onderzoeken toonden echter aan dat gecombineerde DEX-Ara-C-toediening de dCK-activiteit niet verlaagde en het anti-leukemische effect van Ara-C in een AML-model bij ratten niet beïnvloedde. De verlaging van dCK in normaal weefsel zou kunnen verklaren waarom DEX wel een beschermend effect op toxiciteit zou kunnen hebben.

• CBD heeft dubbele effecten op leukemiecellen *in vitro*: het heeft groeiremmende effecten bij hoge concentraties (> 10 µM), maar groeistimulerende effecten bij lage concentraties (rond 1 µM) in het bijzonder in resistente cellen, Aangezien langdurige blootstelling aan CBD ook verslavingsverschijnselen veroorzaakt, moet het gebruik van CBD met de nodige voorzichtigheid gepaard gaan en niet zonder toezicht als experimenteel medicijn bij de behandeling van leukemie gebruikt worden

Resumen en Español

La leucemia es una de las neoplasias hematológicas más comunes tanto en adultos como en niños. La proliferación descontrolada las células hemáticas y una diferenciación alterada son rasgos característicos y fundamentales de la leucemia. A pesar de que la quimioterapia sigue siendo de gran importancia en el tratamiento de la leucemia, algunas células malignas son capaces de sobrevivir el tratamiento y perduran debido a su capacidad de fármaco-resistencia. En añadidura, los factores microambientales a nivel tisular y factores celulares intrínsecos, son capaces de contribuir a la malignidad y progresión de la leucemia. Este fenómeno de fármaco-resistencia resulta en un desafío clínico frecuente e importante, sin solución farmacológica actual.

Para abordar este problema clínico, es necesario adoptar una perspectiva de biología de sistemas ya que esto nos puede ayudar a comprender y dilucidar los mecanismos que estimulan la proliferación crónica y la fármaco-resistencia en la leucemia, ya que la médula ósea (MO) y las células madre hematopoyéticas (CMH) responden a varios estímulos fisiológicos provenientes de diferentes partes del organismo (sistema nervioso, sistema renal, etc),y de su medio ambiente. Por tanto, el enfoque desde la biología de sistemas nos permite visualizar la leucemia no solamente como un fenómeno específico de la célula, sino también como una anomalía tisular, donde la interacción dinámica entre las células malignas y su microambiente tisular influye en la transformación cancerígena, la diseminación y la fármaco-resistencia a la quimioterapia.

En la presente tesis, se describe el tratamiento y desarrollo de la leucemia a través de un análisis de las características específicas a nivel celular, así como la influencia microambiental de las células endoteliales en la regeneración de la MO después de la quimioterapia. Este abordaje nos permite identificar factores que influyen en la fármaco-resistencia y a los mecanismos de transformación maligna que podrían potencialmente ser tratados farmacológicamente. Específicamente hemos investigado la expresión e influencia de las proteínas transportadoras de fármacos a nivel membranal y la interacción intracelular de varios fármacos usados la quimioterapia, mientras que los factores microambientales estudiados incluyen el análisis extensivo de la expresión genética de células endoteliales especializadas, que se encuentran localizadas en nichos específicos de la MO durante dos periodos en específico: el desarrollo y la generación de la médula fetal y la regeneración de la médula en adultos (después de la quimioterapia).

En el **capítulo 2** se describe cómo el microambiente de la MO alberga nichos endoteliales especializados responsables de la señalización paracrina necesaria para el funcionamiento fisiológico de las CMH. Específicamente, nos hemos enfocado en la interleucina-33 (IL-33) como factor angiócrino, que siendo secretados por células endoteliales especializadas (células endoteliales regenerativas humanas o hREC) en la MO, es capaz de estimular la hematopoiesis, la angiogénesis y la osteogénesis

durante el desarrollo fetal de la MO pero también *in vitro* e *in vivo*. Además la IL-33 es capaz de promover la regeneración de la MO después de la quimioterapia en la leucemia mieloide aguda (LMA). Sin embargo, el rol específico del factor IL-33 en la transformación leucémica y la fármaco-resistencia es un tema que aún no ha sido esclarecido.

En los **capítulos 3 y 4** de la tesis describimos mecanismos celulares intrínsecos que contribuyen a la fármaco-resistencia en la leucemia. Uno de los mecanismos más estudiados es el de las proteínas transportadoras ABC (del inglés *ATP-binding cassette*). Estas proteínas que se expresan en la membrana celular, forman parte de una familia hiper diversa y se caracterizan por ser filogenéticamente muy antiguas ya que se expresan en todos los organismos procariotas y eucariotas. Desempeñan un rol clave en la protección celular contra moléculas xenobióticas y tóxicas y son capaces de desintoxicar el interior de la célula de una gran cantidad de fármacos, incluyendo quimioterapéuticos, como el metotrexato (MTX), entre muchos otros. Este proceso afecta la concentración intracelular de la droga y por ende, afecta su eficacia. En nuestro estudio, hemos descubierto como grupos específicos de transportadores ABC pueden trabajar en conjunto de una forma coordinada. Esta redundancia funcional y complementaria puede resultar ser importante en cuanto a la desintoxicación celular. La resistencia la quimioterapia puede ser medible desde un punto de vista clínico, como en el análisis realizado de la tasa de supervivencia general en pacientes con expresiones altas de proteínas ABC específicas y el tratamiento con MTX. Cabe destacar que al estudiar el impacto individual de los transportadores ABC (y no en conjunto), su relevancia clínica disminuye. Esto se debe a que el análisis individual de cualquier proteína ABC no nos permite ver el impacto real a nivel de la desintoxicación celular. Por tanto debemos analizar el rol de los transportadores cuando trabajan sincronizadamente en “equipo”. En el **capítulo 3** exploramos las características moleculares y la relevancia clínica de varios transportadores ABC, como la glicoproteína P (P-gp / ABCB1), *breast cancer resistance protein* (BCRP / ABCG2) y la *multidrug resistance protein 1* (MRP1 / ABCC1). Además, revisamos los avances más recientes sobre mecanismos farmacológicos para circunvalar la fármaco-resistencia relacionada a los transportadores ABC. En el **capítulo 4** investigamos *ex vivo* la expresión en conjunto de MRP1, MRP4, MRP5 y BCRP en muestras de leucemia linfocítica aguda (LLA) infantil, y cómo su expresión se correlaciona con las concentraciones intracelulares de MTX y la tasa de supervivencia a largo plazo. Nuestro análisis revela que, si bien la expresión de MRP4 se correlaciona con la resistencia al MTX *ex vivo*, no se correlaciona por sí sola con una baja tasa de supervivencia a largo plazo. Sin embargo, cuando hay una alta expresión de MRP4 y BCRP en conjunto, se da no solo una disminución intracelular del MTX, sino que también disminuye la tasa supervivencia general en pacientes con leucemia.

Igualmente en el **capítulo 5**, también analizamos la expresión tisular de otros transportadores clínicamente relevantes en la leucemia pediátrica, como el *human equilibrative nucleoside transporter-1* (hENT1) y el *concentrative nucleoside transporter-1* (hCNT1). Estos transportadores son responsables de en gran parte de la captación celular de citarabina (Ara-C). Nuestros estudios de tinción inmunohistoquímica en muestras diagnósticas de LMA pediátrica demuestran que estos transportadores se expresan ampliamente en la membrana celular y el citoplasma en muestras de diagnóstico. No se encontró una relación estadísticamente significativa entre la tasa de supervivencia de los pacientes y la expresión *ex vivo* de hCNT1/ hENT1, ni tampoco una relación estadísticamente significativa a la susceptibilidad farmacológica. Esto es seguramente debido a su amplia expresión en casi todas las muestras obtenidas.

En el **capítulo 6**, examinamos si la administración de dexametasona (DEX) *in vivo* influye en la eficacia de Ara-C y gemcitabina dentro de un modelo de LMA en ratas. Específicamente, investigamos el efecto de DEX sobre la actividad enzimática de la desoxicitidina quinasa (dCK) ya que esta cataliza la conversión de Ara-C en su metabolito activo, la citosina arabinósido trifosfato (Ara-CTP). Previamente se ha demostrado que la administración de DEX disminuye la actividad de dCK, por tanto es relevante investigar a fondo esta posible interacción metabólica. En nuestro análisis observamos un aumento de la esperanza de vida en ratas con AML tratadas con Ara-C, como era de esperarse, mientras que el tratamiento en conjunto con DEX y Ara-C no tuvo impacto alguno en la actividad enzimática de dCK en las células leucémicas. Los resultados obtenidos están en contraste con lo que se ha venido observando anteriormente por otros investigadores anteriormente y por tanto, se debería investigar más a fondo el impacto de DEX en la eficacia de la citarabina en la LMA.

Finalmente, en el **capítulo 7**, investigamos *in vitro* la capacidad proliferativa y el perfil citotóxico de la fitomolécula cannabidiol (CBD). Para esto usamos una línea celular CCRF-CEM y la sublínea (CEM / BTZ) que es fármaco-resistente al bortezomib (BTZ). Ambas son líneas celulares prototípicas de LLA de células T. El CBD es una molécula lipofílica con características antiinflamatorias, antiepilépticas y citoprotectoras que ha sido aprobada por la FDA (*food and drug administration* de los Estados Unidos*),* por lo que ha venido ganando notoriedad y que actualmente se la puede adquirir sin receta médica. El CBD también es un modulador de la actividad de la molécula mTOR (*mechanistic targer for rapamycin*), la cuál es crucial para el funcionamiento y proliferación tanto en células malignas como en células sanas. Nuestros análisis en ensayos de citotoxicidad indican que para inducir muerte celular en células leucémicas se necesitan altas concentraciones de CBD (alrededor de 10 µM). Curiosamente, las concentraciones bajas y clínicamente alcanzables de CBD (alrededor de 1 µM) inducen en cambio, una proliferación celular significativa. Esta proliferación es dependiente de la densidad celular *in vitro*, lo que sugiere que la

densidad celular en un espacio determinado posiblemente secretan factores estimuladores del crecimiento de la densidad celular secundaria. Además la exposición a concentraciones incrementales de CBD de manera escalonada y permanente dio como resultado la creación de una nueva sublínea celular (CEM / CBD6250) la cual presenta propiedades de fármaco-adicción al CBD ya que lo requiere para su sobrevivencia y crecimiento. Cabe destacar que las células humanas sanas, no malignas, en cambio no demuestran citotoxicidad alguna incluso a altas concentraciones de CBD. Por tanto, el posicionamiento del CBD como una posible opción de tratamiento para la LLA *in vivo* aún debe definirse más aun.

En resumen, esta tesis describe un enfoque multifacético al tratamiento de la leucemia tomando en cuenta tanto el microambiente de la MO, la fármaco-resistencia a la quimioterapia y las propiedades bifásicas del CBD (proliferación / citotoxicidad). A nivel de medioambiente tisular por ejemplo, a medida que se descubren nuevos factores regenerativos secretados por células endoteliales hREC durante etapas de regeneración y desarrollo, surgen nuevas preguntas sobre cómo estas células, nichos y factores están involucrados en el desarrollo de la leucemia y el desarrollo de la fármaco-resistencia. También nos enfocamos en factores intrínsecos celulares, tales como como las proteínas ABC, involucradas en la fármaco-resistencia, y su expresión amplificada en las células leucémicas. Vale recalcar, que es la acción conjunta de varios transportadores (en contraste a la acción individual) la que influye en la fármaco-resistencia, como se puede ver en las correlaciones obtenidas en durante nuestra pesquisa. Por tanto, concluimos que la sobreexpresión conjunta de varios transportadores está clínicamente ligada a una baja tasa de supervivencia, debido a una la disminución de la concentración intracelular de drogas usadas comúnmente en la quimioterapia (por ejemplo, el MTX). Sin embargo, algunos transportadores fisiológicos, como hENT1 y hCNT1 se expresan tan ampliamente, lo cual dificulta evaluar su verdadero impacto clínico en la leucemia infantil. Por tanto, para obtener un tratamiento personalizado y optimizado en cada paciente con leucemia, se debería aplicar un enfoque más amplio que incluya el análisis de expresión de transportadores de proteínas, durante el diagnóstico y la fase de tratamiento.

PUNTOS CLAVE

• La MO alberga células endoteliales especializadas (hREC) que habitan en nichos endóseos. Las hREC aparecen en gran número durante las etapas de desarrollo fetal y secretan factores regenerativos durante el mismo y también después del tratamiento quimioterapéutico. Las hREC sobre expresan genéticamente IL-33, lo cual estimula la expansión de las células madre hematopoyéticas, las células endoteliales y la diferenciación osteogénica.

• Las proteínas transportadoras ABC MRP4 y BCRP son determinantes en la regulación de la acumulación intracelular de poliglutamatos de MTX en las células leucémicas. La expresión de estas proteínas transportadoras se asocia con una disminución significativa de la tasa de supervivencia general.

• Las proteínas transportadores de nucleósidos *human equilibrative nucleoside transporter-1* (hENT1) y *concentrative nucleoside transporter-1* (hCNT1) son responsables en gran parte de la captación celular de citarabina (Ara-C). Nuestro análisis inmunocitoquímico revela que hENT1 y hCNT1 son ampliamente expresadas en casi todas las células malignas en muestras de diagnóstico de LMA pediátrica, lo que dificulta una clasificación clínica precisa basada únicamente en la expresión de estos transportadores.

• La dexametasona (DEX) y Ara-C son medicamentos importantes en el tratamiento de la LLA infantil. Con este fin, algunos estudios mostraron que DEX podría afectar la disminuir la actividad de la desoxicitidina quinasa (dCK) y por ende la activación del Ara-C intracelularmente. Sin embargo, nuestros estudios *in vivo* mostraron que la administración combinada de DEX y Ara-C no redujo la actividad de dCK ni afectó el efecto antileucémico de Ara-C en un modelo de LMA en ratas.

• El CBD tiene efectos bifásicos en las células leucémicas *in vitro*: por un lado es citotóxico contra células de LLA a concentraciones > 10 µM, y al contrario, a concentraciones de 1 µM estimula el crecimiento celular, en particular en células resistentes a BTZ. Dado que la exposición prolongada al CBD también provoca fenómenos de adicción celular (en el cuál las células malignas necesitan de CBD para sobrevivir), se requiere precaución para el uso del CBD como fármaco experimental en el tratamiento de la leucemia a bajas concentraciones.

CHAPTER 10

List of Publications

Jaramillo AC, Van Meerloo J, Roeten MSF, Janssen F, Stikvoort A, Stam RW, Peters GJ, Jansen G and Cloos J (2021). Cannabidiol for treatment of (drug-resistant) leukemia cells: a double edge sword!? *In preparation*

Kenswil GJK, Pisterzi P, Sánchez-Duffhues G, van Dijk C, Lolli A, Knuth C, Vanchin B, **Jaramillo AC**, Hoogenboezem RM, Sanders MA, Feyen J, Cupedo T, Costa IG, Li R, Bindels EMJ, Lodder K, Blom B, Bos PK, Goumans MJ, ten Dijke P, Farrell E, Krenning G, Raaijmakers MHGP. Endothelium-derived stromal cells contribute to hematopoietic bone marrow niche formation. *Cell Stem Cell*, 2021

Jaramillo AC, Bergman AM, Comijn EM, Jansen G, Kaspers GJ, Cloos J and Peters GJ. Effect of dexamethasone on the antileukemic effect of cytarabine: role of deoxycytidine kinase, *Nucleosides, Nucleotides & Nucleic Acids*, 2020;39:10-12, 1335-1346

Jaramillo AC, Hubeek I, Broekhuizen R, Pastor-Anglada M, Kaspers GJ, Jansen G, Cloos J and Peters GJ. Expression of the nucleoside transporters hENT1 (SLC29) and hCNT1(SLC28) in pediatric acute myeloid leukemia. *Nucleosides, Nucleotides & Nucleic Acids*, 2020;39:10-12, 1379-1388

Jaramillo AC, Cloos J, Lemos C, Stam RW, Kaspers GJ, Jansen G, & Peters GJ (2019). Ex vivo resistance in childhood acute lymphoblastic leukemia: Correlations between BCRP, MRP1, MRP4 and MRP5 ABC transporter expression and intracellular methotrexate polyglutamate accumulation. *Leukemia Research* 2019;79:45-51

Toffalorio F, Santarpia M, Radice D, **Jaramillo AC**, Spitaleri G, Manzotti M, Catania C, Petter Jordheim L, Pelosi G, Peters G.J, Tibaldi C, Funel N, Spaggiari L, de Bruad F, De Pas T, Giovanneti E. 5'-nucleotidase cN-II emerges as a new predictive biomarker of response to gemcitabine/platinum combination chemotherapy in non-small cell lung. *Oncotarget*. 2018; 9:16437-16450

Kenswil GJK*****, **Jaramillo AC***, Ping Z, Chen S, Hoogenboezem RM, Mylona MA, Adisty MN, Bindels EMJ, Bos PK, Stoop H, Lam KH, van Eerden B, Cupedo T *&* Raaijmakers MHGP. Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 as an Anabolic Factor. *Cell Reports* 2018;22: 666–678. *****Shared first authorship.

Jaramillo AC, Al Saig F, Cloos J, Jansen G and Peters GJ (2018). How to overcome ATP-binding cassette drug efflux transporter-mediated drug resistance? *Cancer Drug Resistance* 2018;1:6-29.

CHAPTER 11

Curriculum Vitae

About the Author

C. Adrian Jaramillo Mantilla was born in Quito, Ecuador on December 29, 1978. In 1997 he finished secondary education at the Centro Educativo Integral, in the town of Sangolquí, in Quito. Soon after, he migrated to The United States of America, to work in order to save money for college and worked diverse types of jobs available in a variety of fields (construction, anthropology, food industry, etc) after which enough money was saved to return home. Before returning, he applied and obtained a scholarship to study pre-med at Hamline University, in St. Paul Minnesota, where he got a Bachelor in Science in Neuropsychology in 2003. After working some more time as a social worker with the Federal Emergency Management Agency (FEMA), enough financing was obtained in order to return home to Ecuador and study Medicine. He attended Universidad San Francisco de Quito and Universidad Cristiana Latinoamericana, in Quito, where he obtained his medical degree in 2012. After which he worked in different hospitals in the city of Quito. During all of his medical studies he volunteered at the Hospital Carlos Andrade Marín in Quito in overnight shifts as an extern, and later as a paid intern, in order to gain practical medical experience in different fields. For about six years, also during his medical studies, he volunteered during weekends and holidays at Foundation Human Nature, a non-profit organization dedicated to provide medical assistance and promote sustainable development in rural and marginalized communities in the Esmeraldas province in NW Ecuador tropical forest. Right after graduation he was also awarded a scholarship from the Science and Technology Secretariat of Ecuador (SENESCYT) for a Ph.D. in the Netherlands. He started his Ph.D. at the beginning of 2013 in Erasmus MC (EMC) in Rotterdam, in the Hematology department where he completed the first year of research, after which he did an independent study in the summer of 2014 in the Pediatric Oncology Department of EMC, where the initial CBD studies came about. After a hiatus of a few years due to lack of financing, he was able to continue his Ph.D. track at the Hematology Department in the VUMC in Amsterdam in 2018. There, he also worked as a lab technician which allowed him to acquire extensive lab experience in extracting BM cells for analysis.

Currently, he is living in Sweden with his partner and preparing to continue his medical and research career there, in the field of Oncology.

CHAPTER 12

Acknowledgements

I would like to first give a thanks to my superb team of promoters and co promoters: Jacqueline Cloos, Gerrit Jansen, Frits Peters, and Ronald Stam. Each one has contributed immensely to my growth as a new researcher and supported me in this path in their own way, for that I am very grateful.

I would like to thank and acknowledge the Secretariat for science and Technology, of Ecuador (SENESCYT) for their financial support at the beginning of my PhD. Thank you so much!

Prof. J. Cloos, dear Jac: I would like to thank you personally for taking me into your research team, and making space for me there. Even though I had no funding and things where looking dim, you were a shining light, and a source of scientific and personal wisdom given the circumstances I was in. You are a source of positivity and enthusiasm, and you know how to connect to people, even in the darkest moments. I was not always an easy person to deal with, but you had always so much patience as a person and passion for science and this got us both through.

Dr G. Jansen, dear Gerrit: thank you so much for your support, both with writing, experiment planning and with scientific reviewing. You are a living, walking encyclopedia of molecular biology and a keen source of reason and observation. You always seem to find a way out of a problem. Maybe that is why you are so good at chess. It is an honor to have worked at your side, and I am sure your students and peers appreciate these capacities as much as I do. Thank you, Gerrit. Maybe one day we can play a game of chess, even if its online.

Prof. G.J. Peters, dear Frits: I am very thankful for the opportunity to have me as a PhD student. You have always been not only very kind and appreciative, but also patient, given the circumstances that slowed me down. Your energy shines through your body of work and for me it's a beacon in the path of science. Thanks for helping me connect the dots in the papers we published together and for your honest reviewing. I hope to continue working with you as well in the near future.

Dr. R.W. Stam, dear Ronald: not only am I grateful because you allowed me in your group for a summer, and gave me the opportunity to develop my ideas at your lab, with freedom, but I am also very thankful for your scientific guidance and critical thought. Not only are you an excellent writer but also a good listener and a friend, who it is easy to talk to. It is thanks to your guidance that I first developed the CBD experiments mentioned in this work and that I continue to do so, there for that opportunity for me is a milestone in my personal and professional development, so thank you, Ronald.

Additionally I would like to thank Dr. Elisa Giovannetti, whom worked closely with my team of promoters and co promoters and included me in a research paper of hers and for being part of the thesis defense. Thanks Elisa and sorry for invading your office many times. Best of luck.

Back in Erasmus Medical Center, I would like to thank Noemi Zambetti, Si Chen, Keane again (you lazy bum), Jana, Onno Rovers, Niken Adisty, Eric Bindels, Tom Cupedo, Ronald Stam and his group: Pauline Schneider, Eddy van Roon, Sandra Mimoso, Patricia Garrido and anyone else I might be forgetting.

Importantly, I would like to acknowledge and thank the PhD defense committee members for taking their time to review this thesis during the defense process. For me this is a very fruitful challenge and it is an honor to take questions and comments from you all. Thank you Dr. E. Giovannetti, Dr. A.M. Bergmann, Prof. dr. A.A. van de Loosdrecht , Prof. dr. M.M. van den Heuvel-Eibrink, Prof. Dr. H. Vormoor, Dr. A. Jager.

I would like to express my thankfulness to the team of technicians and researchers in Prof J. Cloos' group. Johan van Meerloo, dear Johan, thanks for sharing with me the knowledge of your technical work and lab magic, not only for our own research but also with the invriesdienst (freezing duty) as part of our work. You are a central part of the hematology lab, I learned a lot from you, and it has been almost always fun to work with you. Long live the capibara king.
Margot S.F. Roeten: dear Margot, it has been very nice to work with you and I am very glad to see that you are taking part of the CBD research and continuing it now that I am not in the lab anymore. It is amazing to see how an idea that started from my own experience continues to live on and create scientific amazement and opportunity in others. This CDB research and, I guess all research is like a relay race, and each participant gets closer and closer to the goal: to find something useful either for regenerative medicine or for cancer treatment, or for both. Thanks for taking the relay and good luck with your medical studies!
I would also like to thank everyone at the hematology department: all the members of the invriesdienst (IVD). Special thanks to all staff whom I worked alongside at the Hematology Department: Maike, Adri, Pauline Inge, Zinia, Arjo, Eline, Paola, Louise, Renée, Donja, Jennemiek, Ruud, Inoka, and all the other wonderful people working hard there.

A big thank you to the friends who became my family during these years in The Netherlands, Señor O Rovers, Alicia, Keane (the crazy Mexican), Ian (maaaa man), Ceylo (ceylove thanks for being there in difficult moments and always) Lena, Emmanu (noki, noki), Mr. Ping (senior P-i-n to the g), Kasia (thanks for all your amazing energy and wisdom), Janita. Josie (thanks for all your support always), Tania and Alex (we love you and will try to be at your wedding).Thanks to my friends in Leiden, Pablo and

Adria (los más guapos) Special thanks also to Niken Adisty, who thought me how to hold a pipette in the first place and how to do a PCR. Thanks to my buddies Hakan, Roberto, Razy Raz (cheesus) and all the Bisons for all the immemorable fun loving time together.
A big thank you to Sodikromo and Kenswill family, whom I consider my family as well: Wilma Sodikromo and Abigayl Kenswill, Bryan, Edzard and Keane!

All my love goes out to my family: whom always supported me with their wisdom and their love. It has been very difficult to be apart for so many years and see things change from a distance. To my father, who is undergoing spinal surgery during the moment of the thesis defence, I wish him quick recovery, limitless love and understanding. I miss you and love you all! This is also for you!

And from the bottom of my heart would like to thank my partner Arwen, who has been my pillar and in whom I found unconditional love. We found each other at the right time and in the right place. Your support and help have been incalculable and I cannot express with words how much I appreciate it. I look forward to live in the present with you and in the future together! Thanks, Arwen!

The end is always the beginning of something else. May this be a fruitful beginning, living in the moment and projecting into the future with love, and hope.

Thank you all!