CANDIDATE THERAPEUTIC TARGETS AGAINST ACUTE MYELOID LEUKEMIA IDENTIFIED VIA SCREENING COMBINATORIAL PEPTIDE AND CHEMICAL LIBRARIES

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Material and Methods

Detailed descriptions of the materials and methods are found in the original publications

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals **I-IV**:

I. Stefanidakis M*, Karjalainen K*, Jaalouk D, Gahmberg CG, O'Brien S, Pasqualini R, Arap W, Koivunen E. **Role of Leukemia Cell Invadosome in Extramedullary Infiltration.** *Blood*, 114(14):3008-3017 (2009). © The American Society of Hematology.

II. Karjalainen K*, Jaalouk DE*, Bueso-Ramos CE, Zurita AJ, Kuniyasu A, Eckhardt BL, Marini FC, Lichtiger B, O'Brien S, Kantarjian HM, Cortes JE, Koivunen E, Arap W*, and Pasqualini R*. **Targeting neuropilin-1 in human leukemia and lymphoma.** *Blood,* 117(3):920-927 (2011). © The American Society of Hematology.

III. Karjalainen K, Jaalouk DE, Bueso-Ramos CE, Bover L, Sun Y, Kuniyasu A, Driessen WHP, Cardó-Vila M, Rietz C, Zurita A, O'Brien S, Kantarjian HM, Cortes JE, Calin GA, Koivunen E, Arap W*, and Pasqualini R*. **Targeting interleukin-11 receptor in leukemia and lymphoma: A functional ligand-directed study and hematopathology analysis of patient-derived specimens.** *Clinical Cancer Research*, In press [Epub ahead of print, PMID: 25779950] (2015).

IV. Karjalainen K, Kornblau S, Lichtiger B, O'Brien S, Kantarjian HM, Cortes JE, Pasqualini R, Arap W, and Koivunen E. **Design, development, and validation of a high-throughput drug-screening assay for targeting of human leukemia.** *Cancer,* 120(4):589-602 (2014).

* Equal contribution

Abbreviations

ADME/Tox	absorption, distribution, metabolism, excretion, and toxicity
Akt/PKB	protein kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ASXL1	additional sex combs like transcriptional regulator 1
ATRA	all-*trans* retinoic acid
ATP	adenosine triphosphate
aUPD	acquired uniparental disomy
B-CLL	B-cell chronic lymphocytic leukemia
BCL-2	B-cell CLL/lymphoma 2
BH3	BCL-2 homology 3
BMPR 1A	bone morphogenetic protein receptor 1A
BRASIL	biopanning and rapid analysis of selective interactive ligands
CAR cells	CXCL12-abundant reticular cells
cDNA	complementary DNA
C/EBPA	CCAAT/enhancer binding protein alfa
CLL	chronic lymphocytic leukemia
CLL-1	C-type lectin-like molecule-1
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CNV	copy number variations
CR	complete remission
CREB	cAMP response element-binding protein
CREBBP	CREB binding protein
CSC	cancer stem cell
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor 4
DNA	deoxyribonucleic acid
ssDNA	signal stranded DNA
DNMT3A	DNA methyltransferase 3 alpha
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	EGF receptor
EP300	E1A binding protein p300
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-macrophage progenitor
FLT3	Fms-like tyrosine kinase 3
FLT3L	FLT3 ligand
FOXO	Forkhead transcription factor
HIF	hypoxia-inducible factor
HSC	hematopoietic stem cell
IDH1	isocitrate dehydrogenase 1
IDH2	isocitrate dehydrogenase 2
IFN-α	interferon alpha
IFN-γ	interferon gamma
IGF-1	Insulin-like growth factor 1
IGFBP2	insulin-like growth factor binding protein 2
IL	interleukin
IL1RAP	interleukin-1 receptor accessory protein
JAK	Janus kinase
JmjC	Jumonji C

LIF	leukemia inhibitory factor
LSC	leukemia stem cell
M-CSF	macrophage colony-stimulating factor
MDS	myelodysplastic syndrome
MEP	megakaryocyte-erythroid progenitor
miRNA	microRNA
MLL	myeloid-lymphoid or mixed lineage leukemia
MLP	multilymphoid progenitor
MMP	matrix metalloproteinase
MOZ	monocytic leukemia zinc finger
MPP	multipotent progenitor cell
MSC	mesenchymal stem cell
NF-kB	nuclear factor kappa B
NOD	non-obese diabetic
NPM1	nucleophosmin 1
NUP98	nucleoporin 98kDA
PDGF-B	platelet derived growth factor B
PD-1	programmed death 1
PD-L1	PD-ligand 1
PGF	placental growth factor
PI3K	phosphatidylinositol-3 kinase
PKC-α	protein kinase C alpha
PML	promyelocytic leukemia gene
PO_2	partial pressure of oxygen
PR-3	human leukocyte proteinase-3
PTH1R	parathyroid hormone receptor
RAGE	receptor for advanced glycation end products
RAPID	RNAi-assisted protein target identification
RARA	retinoic acid receptor-alpha
R-2HG	R-2-hydroxyglutarate
RBC	red blood cell
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
RUNX1	runt-related transcription factor 1
RUNX1T1	runt-related transcription factor 1; translocated to 1
SCF	stem cell factor
SCID	severe combined immunodeficiency
siRNA	short interfering RNA
STAT	signal transducers and activators of transcription
SDF-1	stromal cell-derived factor 1
TET2	ten-eleven-translocation oncogene family member 2
TIF2	transcriptional mediators/intermediary factor 2
TIM-3	T-cell immunoglobulin and mucin domain 3
TIMP	tissue inhibitor of metalloproteinase
TU	transducing unit
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WBC	white blood cell
WT1	Wilms tumor

Abstract

Acute myeloid leukemia (AML), although rare, is highly malignant neoplasms that account for a majority of leukemia-associated deaths. AML results from an over-growth of immature myeloid cells in the bone marrow. These cells are functionally abnormal and interfere with the production of normal hematopoietic cells. Despite apparent phenotypic uniformity, AML is a heterogeneous group of myeloid malignancies, in which multiple genetic and epigenetic aberrations accumulate in hematopoietic stem or progenitor cells leading to disturbed cellular growth, proliferation, and differentiation. The nature and number of these AML-associated molecular abnormalities can vary widely among patients. Accordingly, the survival rate also fluctuates greatly, with an average ~25% overall survival rate.

Chemotherapy is currently the mainstay treatment option for the patient with AML. However, although required to induce initial remission, chemotherapy contributes to new mutations and clonal evolution, which often leads to disease relapse. Consequently, targeted therapies are urgently needed to eradicate AML cells.

One of the major challenges in clinical oncology today is that therapeutic agents cannot be selectively delivered to tumor site without causing toxicity to rest of the body. In addition, tumor microenvironment has a key role in mediating drug efficacy and resistance, which hampers the discovery of clinically relevant drugs. Therefore, functional screening platforms that can identify cancer-specific targets as well as assess the therapeutic relevance of drug candidates within the appropriate disease microenvironment are fundamental in identification of novel targets and therapeutic agents with better attributes than conventional chemotherapy drugs.

In our studies, we have used two discovery approaches: (i) a phage display technology that allows the identification of ligands binding to physiologically relevant targets, and (ii) a novel *ex vivo* screening assay, which allows identification of candidate drugs against leukemia that are effective in the presence of human blood and bone marrow components.

Specifically, our studies have elucidated the function of the leukemia invadosome – a supramolecular complex containing certain $\beta 2$ integrins and matrix metalloproteinases – in the context of extramedullary leukemia. We show that this complex is essential for extravasation of leukemia cells as well as for leukemia cell growth, and that blocking the function of this complex possesses potent anti-leukemia and anti-invasion effects.

We also demonstrate new roles for neuropilin-1 and interleukin-11 receptors in the pathogenesis of leukemia. We show that these membrane-associated proteins are highly expressed in leukemia cell lines as well as in bone marrow samples from leukemia patients. In addition, we show that the ligands binding to these receptors can be utilized in targeted drug delivery.

Finally, we developed a new functional *ex vivo* screening assay to identify candidate anti-leukemia agents in the presence of human blood or bone marrow under hypoxic conditions. Under these conditions, leukemic cells deplete oxygen faster than normal cells causing a shift in the hemoglobin oxygenation state. This shift, detected by measuring the optical density at 600 nm (OD_{600}) after an appropriate incubation time, directly correlates with leukemic cell counts. Thereby, the oxygenation state of native hemoglobin serves as a reliable and reproducible “built-in” indicator of leukemia cell growth and/or viability. Our study showed that this assay is highly amenable for high-throughput screening against leukemia. Our results support the idea that this new methodology could be a tool for the prediction of drug efficacy and/or response in leukemia patients.

Review of the literature

1. The composition of bone marrow

Bone marrow, a hematopoietic organ that is a main site for hematopoiesis, is located within the boundaries of bones. Bone marrow is responsible for maintaining the numbers of different hematopoietic cells in the peripheral blood at a constant level throughout the lifetime of an individual. In effect, approximately one trillion (10^{12}) cells are produced in adult human bone marrow daily, making it one of the most highly regenerative tissues of the human body (Doulatov S, *et al.* 2012). In brief, hematopoiesis is the accumulative result of intricately regulated signaling pathways, which are mediated by soluble factors (cytokines, chemokines, growth factors, and hormones) and their corresponding receptors, adhesion molecules, as well as oxygen and nutrient availability.

1.1. Components

The components of the bone marrow can be broadly separated into hematopoietic cells and bone marrow stroma consisting of mesenchymal-derived cells and extracellular matrix.

1.1.1. Hematopoietic cells

Bone marrow contains a broad range of morphologically different cells that belong to distinctive blood lineages and stages of differentiation (Doulatov S, *et al.* 2012) (**Figure 1**). The major classes of stem and progenitor cells are characterized by cell surface markers, which categorize each cell lineage and differentiation stage with a specific phenotype. The most commonly used cell surface markers for human hematopoietic stem cells (HSCs) are $CD34^+$, $CD38^-$ (Bhatia M, *et al.* 1997), $CD90^+$ (Thy1), $CD45RA^-$ (protein tyrosine phosphatase receptor C or PTPRC), and $CD71^-$ (TfR1) (Mayani H and Lansdrop PM. 1994; Majeti R, *et al.* 2007) with the simultaneous exclusion of mature lineage markers (such as CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and GlyA), collectively referred to as Lin^- markers. In addition, the expression CD49f (integrin α6) is crucial for the HSC activity and multipotent progenitor cells (MPPs) can be identified by the loss of this marker (Laurenti E and Dick JE. 2012). The MPPs give rise to myelo-erythroid progenitor populations, which are common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs), as well as to the population of immature multilymphoid progenitors (MLPs) (Doulatov S, *et al.* 2012). Recent evidence supports the existence of, a “myeloid-based” model, in which lymphoid and myeloid fates remain linked, instead of diverging early in differentiation as described by the “classical” model (Kawamoto H, *et al.* 2010). According to the “myeloid-based” model, the MLP may give rise to all lymphoid lineages, but may also differentiate to certain myeloid lineages. Ultimately, lineage determining cell surface markers (Lin^+) distinguish all fully differentiated populations (B-cell, T-cell, natural killer cell, dendritic cell, monocyte, granulocyte, megakaryocyte, and erythrocyte) from each other (Doulatov S, *et al.* 2012).

HSCs have the capability to differentiate into all myeloid and lymphoid cell lineages and completely reproduce the entire hematopoietic and immune system. Notably, HSCs are self-renewing and are therefore able to maintain the stem cell pools. In effect, transplantation of a single purified HSC to a lethally irradiated mouse can successfully reconstitute the whole hematopoietic system for long-term (Osawa M, *et al.* 1996; Wagers AJ, *et al.* 2002; Matsuzaki Y, *et al.* 2004; Kiel MJ, *et al.* 2005). In principle, when HSCs divide, one daughter cell remains as a HSC with the self-renewal capability, while the other one becomes a progenitor cell that can no longer self-renew but will lead to a production of a specific type of blood cell. The lineage commitment occurs through changes in gene expression, which is driven by growth factors activating signal transduction pathways that alter transcription factors. Epigenetic changes, which mark gene regulatory regions active, silenced, or poised, often proceed and accompany the gene expression changes of stem cell differentiation (Bernstein BE, *et al.* 2006; Doulatov S, *et al.* 2012).

Specific bone marrow microenvironment often referred to as a “stem-cell niche” plays a key role in maintaining and regulating the

self-renewal and differentiation of HSCs. Distinct local microenvironment can create asymmetry between HSCs by exposing two daughter stem cell to different extrinsic signals. Alternatively, asymmetric cell division, which results into differential distribution of the cell fate determinates, may initially coordinate the generation of both self-renewing and differentiating daughter cells. It is plausible, however, that both environmental and divisional mechanisms for achieving asymmetry may be used in parallel by independent HSCs to guide daughter cells to distinct cell fates (Neumüller RA and Knoblich JA. 2009; Wilson A and Trumpp A. 2006).

In steady-state conditions, the bulk of the HSCs exist in the bone marrow while only a limited number of HSCs is circulating in the peripheral blood (Abkowitz JL, *et al.* 2003; Wright DE, *et al.* 2001). In these conditions, the majority of HSCs are slowly cycling (quiescent) cell populations, but can be induced to cycle actively in response to environmental cues. This ensures the proper homeostatic reconstitution of blood cells when needed. In addition, the quiescent state of HSCs protects cells from the damage related to deoxyribonucleic acid (DNA) replication and cellular oxidative metabolism (Orford KW and Scadden DT. 2008).

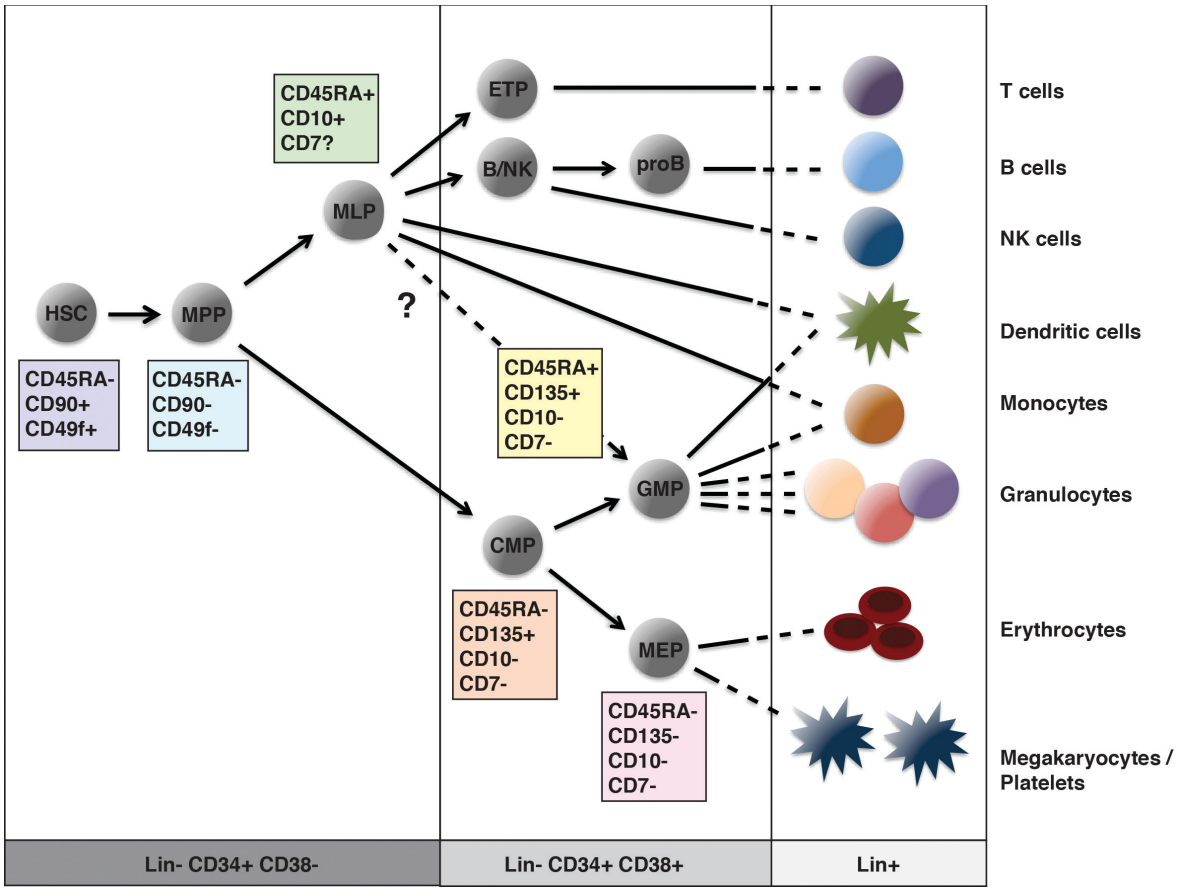

Figure 1. The lineage differentiation of hematopoietic cells in human. Hematopoietic stem cells (HSC) express CD34 and CD49f among other markers. HSC give rise to multipotent progenitor cells (MPP), which are identified by the loss of CD49f marker. Only one population of immature multilymphoid progenitors (MLP) has been described, from which early T-cell precursors (ETP), progenitor B-cells (proB), natural killer cells (NK), as well as dendritic cells and monocytes derive. Several myelo-erythroid progenitors exist, namely: common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP). Lineage marker (Lin) specifies the terminally differentiated populations. This illustration is based on the figure from Doulatov S *et al.* 2012.

1.1.2. Bone marrow stroma

The bone marrow stromal cells are a heterogeneous population of cells, which include mesenchymal stem cells (MSCs), osteoblasts, osteoclasts, adipocytes, vascular endothelial cells, perivascular reticular cells, and smooth muscle cells. They play an essential role in regulating hematopoietic cell development as well as HSC maintenance and localization via the production of cytokines, chemokines, growth factors, and intracellular signals initiated by cellular adhesion (Morrison SJ and Spradling AC. 2008; Konopleva MY and Jordan CT. 2011). For example, MSCs are multipotent stromal cells that provide a microenvironment that is favorable for hematopoiesis. They accomplish this by differentiating into the various cell lineages of the bone marrow stroma including, adipocytes, osteoblasts, osteocytes, fibroblasts, chondrocytes, and myoblasts. MSCs also form a scaffold for cell-cell interactions, and they secrete cytokines and growth factors such as C-X-C motif chemokine 12 (CXCL12) also known as stromal cell-derived factor 1 (SDF-1), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (FLT3L), leukemia inhibitory factor (LIF), as well as many interleukins (Majumdar MK, *et al.* 2000 & 1998; Baggiolini M. 1998; Deans RJ and Moseley AB. 2000; Konopleva M and Andreef M. 2007).

Bone marrow stromal cells are also responsible for the production and deposition of a complex extracellular matrix (ECM), which mainly consist of fibronectin, hyaluronic acid (hyaluronan), collagen types I and IV, laminin, proteoglycans, hemonectin, thrombospondin, heparin sulfate, and chondroitin sulfate. In addition to the stromal cell derived growth factors and interactions between hematopoietic cells and stromal cells, hematopoietic cell interactions with ECM are required for the normal hematopoietic development. The ECM provides structural support as well as participates in controlling cell growth, survival, differentiation, motility, and viability through cell-ECM interactions. Importantly, growth factors, cytokines, and chemokines secreted by stromal cells can be concentrated in specific compartments within the bone marrow due to the differences in local production and through cytokine-binding glycosaminoglycans in the ECM. As such, the ECM serves as a reservoir of many secreted soluble proteins. The availability of these ECM tethered molecules can be regulated via re-arrangements of the matrix usually via degradation by proteases (Bonnans C, *et al.* 2014; Marastoni S, *et al.* 2008).

1.2. Bone marrow niches

Bone marrow is divided into two distinct subcompartments termed “niches”, which are osteoblastic (endosteal) and vascular niches (**Figure 2**). According to their differentiation stage, HSCs and progenitor cells localize into these specific bone marrow microenvironments. These two different niches play an important role in controlling HSC fate by regulating quiescence, survival, self-renewal, and/or differentiation of these cells through interactions between hematopoietic cells and bone marrow stroma. The microenvironment of the niches is interconnected and forms a complex network, however, each niche influences HSCs differently (Tripodo C, *et al.* 2011; Perry JM and Li L. 2007; Konopleva MY and Jordan CT. 2011).

1.2.1. Osteoblastic niche

The osteoblastic niche, also called the endosteal niche or endosteum, is localized at the inner surface of the bone cavity, and is mainly composed of endosteal fibrocytes and osteoblasts. Osteoblasts are bone-forming cells derived from MSCs, and they play a critical role in regulating HSCs. The majority of HSCs in the endosteum are directly in contact with the osteoblasts, and *in vivo* studies in mice have demonstrated a simultaneous increase in bone formation and HSC numbers in bone marrow (Perry JM and Li L. 2007; Arai F and Suda T, *et al.* 2007; Calvi LM, *et al.* 2003; Zhang J, *et al.* 2003; Wilson A and Trumpp A. 2006; Tripodo C, *et al.* 2011).

Specifically, osteoblastic content was increased via activation of parathyroid hormone receptor (PTH1R) (Calvi LM, *et al.* 2003) or bone morphogenetic protein

receptor 1A (BMPR 1A) (Zhang J, *et al.* 2003), which resulted in a simultaneous increase in bone marrow HSC content. As neither PTH1R nor BMPR 1A are expressed on HSC, this indicated that the effect was mediated by osteoblasts. In these studies, the regulation of HSCs by osteoblasts was suggested to occur via Notch ligand and N-cadherin interactions (Calvi LM, *et al.* 2003; Zhang J, *et al.* 2003). However, more recently, it was reported that N-cadherin expression was not necessary for bone marrow HSC maintenance by osteoblasts (Kiel MJ, *et al.* 2009).

The intimate physical associations between HSCs and osteoblasts provide regulatory support to hematopoietic cells. For instance, osteoblasts produce several hematopoietic cytokines such as G-CSF, GM-CSF, and LIF. It has also been demonstrated that osteoblasts are able to support hematopoietic cells *in vitro* and maintain long-term cultures (Porter RL and Calvi LM. 2008), as well as regulate HSC quiescence via Tie2/angiopoietin-1 signaling in the bone marrow (Arai F, *et al.* 2004). Furthermore, osteoblasts can produce osteopontin, an extracellular matrix glycoprotein, during active bone remodeling and the amounts can vary with the activation state of the cell (Porter RL and Calvi LM. 2008). This inducible production of osteopontin has been shown to negatively regulate the HSC pool size *in vivo* (Stier S, *et al.* 2005; Nilsson SK, *et al.* 2005).
It is plausible that osteoblastic precursor cells may also contribute to HSC regulation. In fact, it has been reported that Nestin+ MSCs give rise to osteoblasts, and that depletion of Nestin+ MSCs inhibited HSC homing to the bone marrow as well as attributed to a significant reduction in HSC numbers in bone marrow *in vivo* (Mendez-Ferrer S, *et al.* 2010).

1.2.2. Vascular niche

The vascular niche comprises sinusoidal endothelial cells lining blood vessels as well as other vascular components such as smooth muscle cells and CXCL12-abundant reticular (CAR) cells. Vascular niche stimulates proliferation and differentiation of HSCs by supplying maturation and activation signals to differentiating cells and regulating the intravasation and extravasation of the foreign elements. For example, the vascular niche is responsible for regulating the release of mature hematopoietic cells, such as mature non-nucleated red blood cells (RBCs), platelets, and mature granulocytes, into the circulation (Kopp HG *et al.* 2005; Konopleva M, *et al.* 2009; Wilson A and Trumpp A. 2006; Tripodo C, *et al.* 2011).

Several studies have suggested that bone marrow endothelial cell mediated signaling is required for HSC self-renewal and regeneration after myelosuppression (Butler JM, *et al.* 2010; Salter AB, *et al.* 2009; Hooper AT, *et al.* 2009; Kopp HG, *et al.* 2005). For instance, delayed vascular regeneration and hematopoietic recovery in mice following total body irradiation was observed in vascular endothelial growth factor receptor 2 $(VEGFR2)^{-/-}$ mice (Hooper AT, *et al.* 2009). Similarly, systematic administration of anti-VEGFR2 antibody inhibited the engraftment of HSCs and prevented the hematopoietic reconstitution in irradiated mice (Hooper AT, *et al.* 2009). Significant delay in hematologic recovery was also observed in the studies where vasculogenesis was inhibited via blocking VE-cadherin or Tie2/angiopoietin signaling (Salter AB, *et al.* 2009; Kopp HG, *et al.* 2005). In effect, soluble factors produced by endothelial cells have been suggested to be responsible for HSC self-renewal and regeneration, including angiopoietin-like 5 and insulin-like growth factor binding protein 2 (IGFBP2), cytokines such as stem cell factor (SCF) and FLT3L, as well as a heparin-binding growth factor pleiotrophin (Himburg HA, *et al.* 2010; Kobayashi H, *et al.* 2010; Zhang CC, *et al.* 2008; Chute JP, *et al.* 2006).

1.3. Oxygen levels

Adenosine triphosphate (ATP) synthesis via oxidative phosphorylation is dependent on molecular oxygen (O_2). In the peripheral sites, the partial pressure of O_2 (PO_2) is significantly lower than the atmospheric PO_2, and depending on the cell type can also vary dramatically. HSCs, in particular, are often localized in hypoxic endosteal niches of the bone marrow (Chow DC, *et al.* 2001; Harrison JS, *et al.* 2002). In effect, localization of the HSCs to the compartments with limited O_2 is advantageous to the cells

as O_2 metabolism generates reactive oxygen species (ROS), which can in high levels induce senescence in stem cells. (Jang YY and Sharkis SJ. 2007).

The balance of complex signals in the bone marrow microenvironment control the capacity of HSCs to retain cell cycle quiescence or self-renew and generate different lineages of hematopoietic cells. Undifferentiated states of HSCs are maintained by various factors, among which low PO_2 (hypoxia) is a major player (Mohyeldin A, *et al.* 2010). Gradients of oxygen between 1% in the endosteal niche and 6% in the vascular niche exist in the human bone marrow (Mohyeldin A, *et al.* 2010), and the most primitive HSCs with quiescent state reside in the hypoxic microenvironment (Grassinger J, *et al.* 2010; Winkler IG, *et al.* 2010; Parmar K, *et al.* 2007).

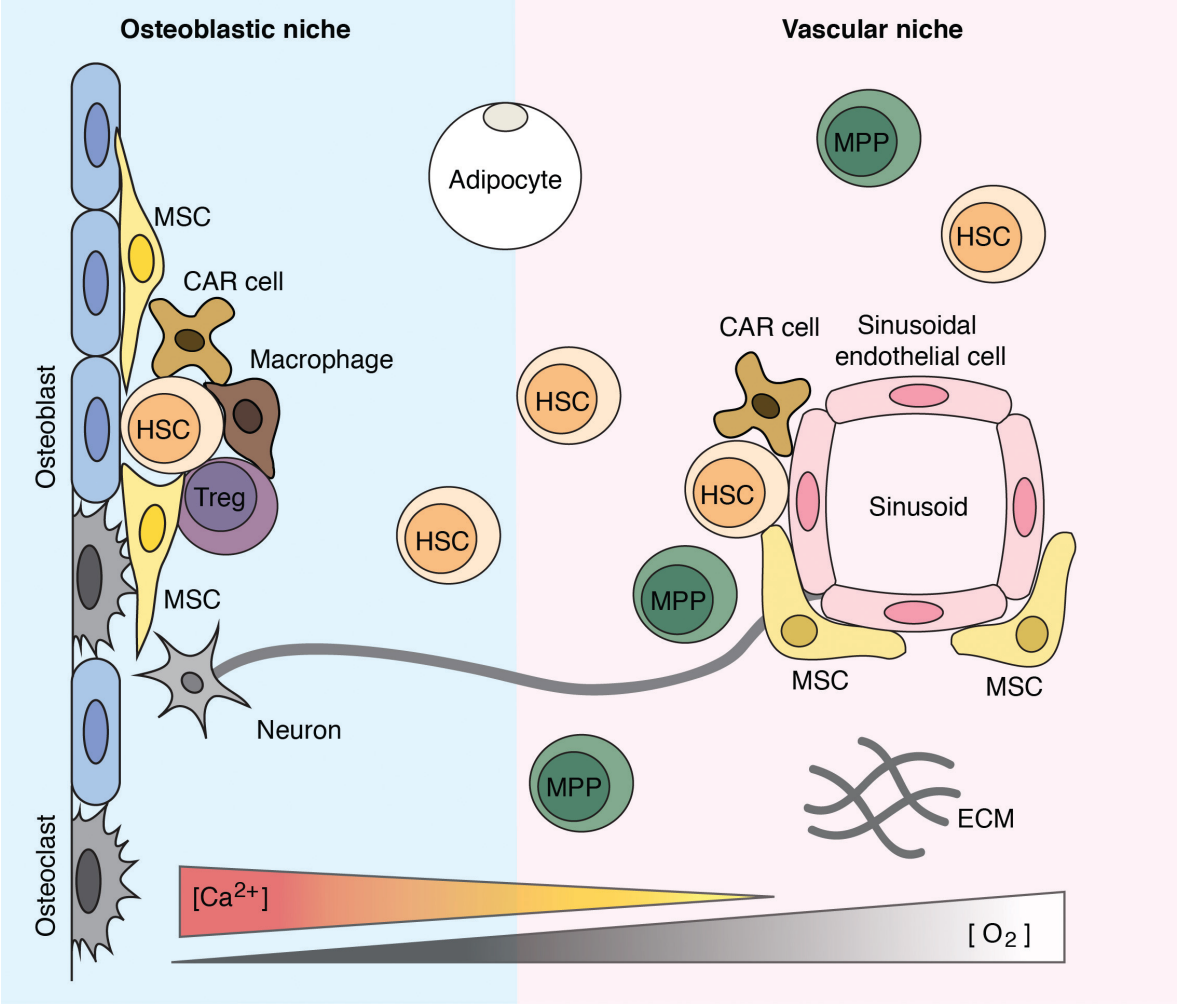

Figure 2. Bone marrow microenvironment. Components of the bone marrow microenvironment include hematopoietic cells such as hematopoietic stem cells (HSC) and multipotent progenitor cells (MPP), osteoblasts, osteoclasts, mesenchymal stem cells (MSC), CXCL12-abundant reticular (CAR) cells, adipocytes, immune cells such as macrophages and regulatory T cells (Treg), neurons, endothelial cells, and extracellular matrix (ECM).

2. Pathogenesis of acute myeloid leukemia

Leukemia is a hematologic malignancy that originates in the bone marrow. It is broadly divided into acute leukemia, which includes acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), and chronic leukemia, which includes chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL). However other subtypes of leukemia also exist. The different types of leukemia may derive from a different cell-of-origin, have different characteristic molecular abnormalities, and have different responses to a therapy. The focus of this literature review is on the pathology of AML, the second most common type of leukemia in adults, and the most frequent cause for leukemia-related deaths.

In AML, molecular abnormalities accumulate in normal hematopoietic stem or progenitor cells leading to neoplastic transformation (Chen J, *et al.* 2010). The cells that have become leukemic appear immature and abnormal, and are commonly referred to as "blasts". They do not function normally and their granulomonocytic differentiation program is impaired.

AML progresses rapidly and the disease burdens the bone marrow and often also the peripheral blood. This interferes with the normal hematopoietic cell production leading to cytopenias. In effect, bone marrow failure and tissue infiltration by circulating leukemic cells account for the presenting clinical symptoms of AML. However, the diversity in clinical symptoms among different patients is mainly due to differences in age and molecular abnormalities (Ghanem H, *et al.* 2012).

2.1. Molecular abnormalities

AML is a heterogeneous group of myeloid malignancies, and the nature and number of the molecular abnormalities can differ broadly between patients. The two most commonly used classification models for AML subtypes are, World Health Organization system (Vardiman JW, *et al.* 2002 & 2008) and French-American-British system (Bennett J, *et al.* 1976)**.**

In principle, leukemia is characterized by recurring gene mutations and chromosomal aberrations that play a key role in leukemogenesis. In addition, epigenetic modifications lead to activation or repression of gene expression and therefore also affect the phenotype of leukemia cells (Chen J, *et al.* 2010). Other mechanisms, such as microRNA (miRNA) deregulation and copy number alterations likewise contribute to this disease (Chen J, *et al.* 2010; Eklund EA. 2010).

Chromosomal translocations that result in the generation of chimeric fusion proteins are frequent in leukemia. They cause transcriptional deregulation thereby directly contributing to leukemogenesis (Testa U. 2011). Common chromosomal translocations in AML include the t(15;17) translocation, which generates the fusion gene with promyelocytic leukemia gene (*PML) and retinoic acid receptor alpha (RARα or RARA)* in acute promyelocytic leukemia (APL), and the t(8;21) translocation, which generates the fusion gene with runt-related transcription factor 1 (*RUNX1*) and *RUNX1* translocated to 1 (*RUNX1T1*) (also known as *AML1-ETO* fusion gene). These fusion genes impair proper differentiation of HSCs thus leading to uncontrolled growth (Watt CD and Bagg A. 2010; Takahaski S. 2011). About 50-60% of newly diagnosed patients with AML have cytogenetic abnormalities (i.e., abnormal karyotype), and cytogenetic analysis at diagnosis is an important prognostic indicator. In general, the cytogenetic abnormalities in AML are classified into three groups: high, intermediate and low risk (Marchesi F, *et al.* 2011; Hong WJ and Medeiros BC. 2011). Patients within the high-risk cytogenetic group are potential candidates for allogeneic HSC transplantation, while patients within the low-risk cytogenetic group will receive only standard chemotherapy (Blum W. 2008). The best treatment strategy for intermediate risk AML patients remains to be defined.

In addition, subkaryotypic alterations, such as gene mutations, are an important factor in risk-group predictions and are now being incorporated into risk assessment categories (Watt CD, Bagg A. 2010). In effect, in a large subset (40-50%) of AML patients it is impossible to identify cytogenetic aberrations via conventional cytogenetics. Patients with

normal karyotype at diagnosis are usually categorized into the intermediate risk group (Ghanem H, *et al.* 2012; Marchesi F, *et al.* 2011). Characteristically, these patients have a notable heterogeneity in clinical outcome, and can have very different responses to treatments. Molecular characterization is particularly important in this subset of AML patients (Marchesi F, *et al.* 2011).

There are many acquired single gene mutations that are crucial to the development of AML (Gaidzik V and Döhner K. 2008). The whole-genome sequencing studies have shown that cytogenetically normal AMLs harbor approximately 750 somatic point mutations (Mardis ER, *et al.* 2009; Ley TJ, *et al.* 2008), however, a large portion of these mutations are predicted to be irrelevant to the pathogenesis of AML. Regardless, the number of genetic aberrations implies that the pathogenesis of AML is a complex multistep process, similarly to other cancers (Hanahan D and Weinberg RA. 2000 & 2011). According to the current model, AML is thought to arise from a series of genetic alterations that confer various types of proliferative and survival advantages (Ghanem H, *et al.* 2012). The absolute number of mutations required to cause AML is unclear, and it remains to be determined which of the mutations are "driver" mutations and which are "passenger" mutations. Several mutations that significantly contribute to leukemogenesis have been identified including the mutations in FMS-related tyrosine kinase 3 (*FLT3*), CCAAT/enhancer binding protein alfa (*C/EBPA*), *RUNX1*, additional sex combs like transcriptional regulator 1 (*ASXL1*), myeloid-lymphoid or mixed lineage leukemia (*MLL*), Wilms tumor (*WT1*) and nucleophosmin 1 (*NPM1*) genes (Takahaski S. 2011; Döhner K and Döhner H. 2008; Mardis ER, *et al.* 2009; Falini B, *et al.* 2005). The different types of molecular abnormalities have been extensively reviewed (e.g. Marchesi F, *et al.* 2011; Watt CD and Bagg A. 2010; Odenike O, *et al.* 2011; de Thé H and Chen Z. 2010).

Furthermore, gene expression can also be deregulated via other types of molecular abnormalities, such as epigenetic chromatin modifications and miRNA regulation (Conway O'Brien E, *et al.* 2014; Agirre X, *et al.* 2012; Plass C, *et al.* 2008). For example, mutations in genes, such as isocitrate dehydrogenase 1 and 2 (*IDH1/2*), ten-eleven-translocation oncogene family member 2 (*TET2*), and DNA methyltransferase 3 alpha (*DNMT3A*) lead to aberrant methylation in AML cells (Figueroa ME, *et al.* 2010; Yan XJ, *et al.* 2011). Moreover, alterations in copy numbers, achieved by insertions, deletions, inversions and translocations, can all contribute to subkaryotypic modifications in genomic content, and subsequently alter gene expression. Several acquired copy number variations (CNV) have been identified in AML (Eklund EA. 2010). According to one report, 24% of patients with cytogenetically normal AML and 40% of those with an abnormal karyotype had CNVs (Walter MJ, *et al.* 2009). Finally, acquired uniparental disomy (aUPD) – a phenomenon caused either by chromosomal non-disjunction or homologous recombination during cell division – contributes to malignant transformation by leading to loss of heterozygosity of mutated genes. It is associated with both loss-of-function mutations of tumor suppressor genes and gain-of-function mutations of proto-oncogenes (Raghavan M, *et al.* 2008; Fitzgibbon J, *et al.* 2005; Sanada M, *et al.* 2009) Notably, aUPD occurs in approximately 15-32% of AML patients (Walter MJ, *et al.* 2009; Akagi T, *et al.* 2009).

The age and presenting white blood cell counts are also important prognostic factors that can be used to predict the clinical outcome in patients (Marchesi F, *et al.* 2011; Ghanem H *et al.* 2012). Temporal differences in leukemia-initiating cells affect the properties of leukemogenic cells as stem cell properties and self-renewal mechanisms alter with age (Levi BP and Morrison SJ, 2008), likely affecting the type of mutations that are capable of initiating cancer. Consistently, the mutation signature of leukemia varies with age, and many driver mutations are only found in elder patients (e.g., in *FLT3*, *NPM1*, and *DNMT3A*), while some are more commonly observed in young patients (e.g., translocations involving *RUNX1*, *MLL*, and nuclear pore complex protein Nup98-Nup96 encoded by the gene *NUP98*. In contrast, mutations that do not have a temporal preference occur throughout all the ages (e.g., in *RAS* genes) (Magee JE, *at al.* 2012).

Notably, somatic mutations in hematopoietic

cells accumulate over time, and clonal hematopoiesis strongly correlates with age, which is associated with increased risk of hematologic cancer and mortality (Jaiswal S, *et al.* 2014; Genovese G, *et al*. 2014). In effect, pre-leukemic clones might be present for years with no indication of a malignancy (Jaiswal S, *et al.* 2014; Genovese G, *et al*. 2014; Welch JS, *et al*. 2012). It has been demonstrated that detectable clonal expansion is most frequently driven by mutations affecting *DNMT3A, TET2,* and *ASXL1* genes (Jaiswal S, *et al.* 2014; Genovese G, *et al*. 2014). The bulk of the somatic mutations, however, are random events, and it has been shown that only one or two cooperating mutations are often sufficient to generate the leukemic clone (Welch JS, *et al*. 2012). In addition, these initial leukemic clones can subsequently acquire more cooperating mutations, which might yield a subclone with a more aggressive phenotype, therefore leading to disease progression and/or relapse (Welch JS, *et al.* 2012). Notably, the pre-leukemic clones have been shown to survive chemotherapy and persist in remission (Shlush LI, *et al.* 2014; Corces-Zimmerman MR, *et al*. 2014), thus providing a potential source for leukemia progression and disease relapse.

Taken together, AML is a highly heterogeneous group of diseases that carry a vast amount of different cytogenetic and molecular abnormalities.

2.2. Intrinsic heterogeneity in leukemia

Beyond the heterogeneity observed among different patients, cancer cells within the same tumor can be highly heterogeneous and differ in morphology, marker expression, proliferation potential, and therapy resistance. These intrinsic differences are caused by genetic and epigenetic changes and they confer heritable phenotypic and functional differences upon cancer cells (Vermeulen L, *et al.* 2012). These changes are subject to selection within tumors – a process referred to as clonal evolution – and it often leads to more aggressive cancers over time (Magee JE, *at al.* 2012; Polyak K, *et al.*, 2009; Bissell MJ and Hines WC, 2011). Whole-genome sequencing of samples from patients with secondary-AML and myelodysplastic syndrome (MDS) revealed that nearly all the bone marrow cells in these patients were clonally derived, and the genetic evolution occurs via multiple rounds of acquisition and clonal selection (Walter MJ, *et al.* 2012). Interestingly, recurrent genetic mutations were found in both the original leukemia-initiating cells as well as in a subsequently evolved subclone selection (Walter MJ, *et al.* 2012). Concomitantly, another study done via whole-genome sequencing demonstrated that accumulation of new mutations and clonal evolution occur in relapsed AML, and that these events are partially induced by the chemotherapy received for the primary AML (Ding L, *et al.* 2012).

The heterogeneity among cancer cells within the same tumor can also arise in response to extrinsic influences, and cancer cells may be phenotypically and functionally different due to dissimilarities in the microenvironment surrounding them. These differences are often reversible, however, environmental changes could also cause irreversible alterations in cancer cell properties (Magee JE, *at al.* 2012; Polyak K, *et al.*, 2009; Bissell MJ and Hines WC, 2011).

The capacity for long-term replication and tumorigenicity also varies among cancer cells. Several studies have shown that, in the majority of cases, only a rare population of cells with immature phenotype has the capacity to replicate long-term, and they can be serially transplanted in mice (Magee JE, *et al.* 2012; Vermeulen L, *et al.* 2012). On the contrary, nontumorigenic cancer cells – although composing the bulk of tumors – are thought to have a limited self-replication potential and therefore have little contribution to cancer progression (Shackleton M, *et al.*, 2009; Dick JE. 2008; Reya T, *et al.*, 2001). As such, the rare tumorigenic cancer cells are commonly referred to as cancer stem cells (CSCs), or cancer initiating cells. Importantly, the CSCs can originate via mutations that de-regulate self-renewal mechanisms in normal stem cells or in progenitor cells and more differentiated cells, and thus the term CSC does not imply the cell-of-origin (Magee JE, *at al.* 2012). Notably, CSCs resemble normal stem cells in that they can self-renew, have the capacity to proliferate long-term as well as have a potential to differentiate to multiple lineages.

This suggests that in cancers that follow the stem cell model, only a small subpopulation of cells can proliferate extensively and are therefore responsible for maintaining the neoplastic process. As such, therapies that only target the bulk of tumors may not be sufficient to cure the patient because they fail to eliminate cancer stem cells (Magee JE, *at al.* 2012).

AML is one such cancer that is thought to follow a stem cell model in which intrinsically different subpopulations of tumorigenic and nontumorigenic cancer cells are hierarchically organized, and tumorigenic cancer cells differentiate into phenotypically diverse nontumorigenic cancer cells.

The pioneering work in cancer stem cell research was carried out by John Dick's laboratory, and was based on transplantation assays of AML cells into non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice, which led to the idea that leukemia stem cells (LSCs) or leukemia initiating cells are rare and exclusively confined in the CD34+CD38- cell population (Lapidot T, *et al.* 1994). Since then, markers that would distinguish the LSCs from the remaining bulk of non-leukemogenic cells have been studied extensively. Moreover, the studies in new and improved xenotransplantation models have shown that cell populations acting as LSCs can have diverse membrane phenotypes in various types of AMLs, and thus LSCs are not only restricted to CD34+/CD38- populations (Testa U. 2011). In effect, several results imply that CSC-affiliated cell surface markers are more heterogeneous than initially comprehended (Testa U. 2011). However, recent results also propose that stem cell expression patterns may exists only in the blast cells at a population level, and that an individual blast cell would not have the complete repertoire or expression level of stem cell markers; therefore the blasts would be different from LSCs (Eppert K, *et al.* 2011).

Nevertheless, xenotransplantation assays may considerably underrate the ratio of human cancer cells that have tumorigenic potential (Vermeulen L, *et al.* 2012; Eppert K, *et al.*, 2011; Adams JM and Strasser A. 2008). In addition, the foreign microenvironment may only support the growth of certain subclones of the cancer. As such, syngeneic transfers of mouse leukemias or lymphomas have been used to shed light on the LSC model without the complexity of xenotransplantation. Interestingly, studies in AML murine models showed that leukemia initiating cells were numerous and rather than resembling a stem cell presented relatively mature phenotypes (Somervaille TC and Clearly ML. 2006; Krivtsov AV, *et al.* 2006).

The leukemia-initiating cells can be very different from each other due to differences in oncogenic mutations that can have profound effects on the frequency and phenotype of these cells (Magee JE, *at al.* 2012). Moreover, recent studies have shown extensive plasticity and dedifferentiation in normal somatic tissue cells that obtain specific environmental inputs or, in an artificial setting, can be induced to become pluripotent stem cells when transduced with the right factors (Chaffer CL, *et al.* 2011; Gupta PB, *et al.* 2011). These results suggest that differentiation of LSCs into non-leukemogenic cells may similarly be reversible, and this plasticity of LSCs may contribute to therapy resistance. However, the identification of markers that characterize the populations with leukemogenic capacity may still be possible if cells in the non-leukemogenic state only transform to the LSCs with low efficiency or under restricted circumstances (Magee JE, *at al.* 2012).

Notably, cancers that develop according to the stem cell model are also subject to clonal evolution and environmental impact that can contribute to heterogeneity. Therefore all the sources of heterogeneity are cumulative rather than mutually exclusive, and different processes may be utilized at variable levels based on the type of cancer (Magee JE, *at al.* 2012).

3. Emerging targeting approaches in acute myeloid leukemia

High-dose chemotherapy has been the standard therapeutic approach for AML patients. This has consisted mainly of cytarabine combined with an anthracycline antibiotic, usually daunorubicin, idarubicin, or the anthracedione mitoxantrone (Burnett A, *et al.* 2011, Tallman MS, *et al.* 2005). In general, treatment of AML with chemotherapy is often initially successful and approximately 60% to 70% of adult patients are expected to achieve complete remission (CR). However, disease relapse occurs in many patients, and duration of CR is shorter in patients older than 60 years (Mayer RJ, *et al.* 1994). Therefore the overall prognosis is poor in AML and the overall 5-year survival rate is only around 25% according to National Cancer Institute. In addition, depending on the AML subtype, survival rates can vary intensely. In particular, successful treatment outcomes have been obtained with APL, in which all-*trans* retinoic acid (ATRA) and arsenic trioxide are used alone or in combination with chemotherapy to target the *PML/RARA* fusion gene (de Thé H and Chen Z. 2010; Sanz MA, *et al.* 2009). However, although differentiation therapy induces remission it is rarely curative. In addition, some improvement in survival rates has been obtained over the past decades even with the conventional chemotherapy drugs; however, this is mostly attributable to advancements in supportive care needed against severe cytopenias caused by effective treatments (Burnett A, *et al.* 2011).

The difficulty of targeting and fully eradicating leukemia cells is a major cause of relapse, and most patients with AML die from progressive disease after relapse (Ding L, *et al.* 2012). Chemotherapy although required to induce initial remission, contributes to new mutations and clonal evolution. In essence, treatment can change the dominance of a clone causing a selection of one or more rare clones that survive the therapy because of new or already existing genetic mutations (Ding L, *et al.* 2012). In addition to acquiring a new set of mutations, the secondary AMLs carry over all the pre-existing mutations from primary clones, thus resulting in subclones with increased numbers of mutations. Secondary AMLs are therefore mixtures of several genomes with unique sets of mutations (Walter MJ *et al.* 2012).

Targeted therapies against different disease-driving molecular aberrations, aberrantly activated signaling pathways as well as alternative compensatory molecular networks are desperately needed to fully eradicate AML (Hopkins AL. 2008).

3.1. Abnormally activated signaling pathways

Targeting of signaling pathways that are aberrantly activated in leukemia are likely to improve therapy outcomes. For instance, several signaling pathways are constitutively activated in leukemia, such as the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway as well as the nuclear factor kB (NF-kB) and the phosphatidylinositol-3-OH kinase (PI3K) – Akt/protein kinase B (PKB), pathways. The constitutive activation can be caused by abnormal autocrine and/or paracrine signaling from the tumor microenvironment, particular genetic aberrations or epigenetic deregulation (Chen E, *et al.* 2012).

For example, a common feature in many hematopoietic malignancies as well as solid tumors is persistent STAT signaling, utilized by several cytokines, including interleukin-6 (IL-6), IL-10, IL-11, IL-15, IL-23, GM-CSF, interferon alpha (IFN- α), and interferon gamma (IFN- γ) (Bromberg J. 2002; Bowman T, *et al.* 2000). STAT3 signaling is constitutively activated in several leukemia cell lines as well as in primary leukemia cells, including AML (Chen E, *et al.* 2012; Redell MS, *et al.* 2011; Zhao J, *et al.* 2011; Ma LD, et al 2010; Ferrajoli A, *et al.* 2006). Persistent STAT3 activation has been reported to increase survival, proliferation, angiogenesis and metastasis, as well as decrease anti-tumor immunity (Sansone P and Bromberg J. 2012; Yu H, *et al.* 2009), and is associated with invasive phenotype (Ranger JJ, *et al.* 2009; Suman P, *et al.* 2009) and poor patient prognosis (Chatterjee D, *et al.* 2008).

The PI3K – Akt/PKB pathway – utilized by growth factors binding to receptor tyrosine kinases, such as insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), and some epidermal growth factor

(EGF) family members (Engelman JA. 2009) – has an essential role in proliferation, survival, metabolism, differentiation and migration of many different cell types, including hematopoietic cells (Polak R and Buitenhuis M. 2012; Cully M, *et al.* 2006; Stiles B, *et al.* 2004). In AML, several different abnormalities, including *FLT3, RAS* or *c-KIT* mutations, as well as autocrine/paracrine secretion of growth factors, such as IGF-1, VEGF, have been shown to upregulate the PI3K/Akt pathway (Martelli AM, *et al.* 2006 & 2009).

NF-κB signaling is often constitutively activated in solid and hematological malignancies (Luo J-L, *et al.* 2005). NF-κB is activated by many different stimuli, such as proinflammatory cytokines including IL-1β, tumor necrosis factor-α (TNF-α), and EGF, T- and B-cell mitogens, viruses and viral proteins, bacteria and lipopolysaccharides, double-stranded RNA, and chemical as well as physical stresses (Aggarwal BB and Sethi G and Aggarwal BB. 2006). In addition, cellular stresses such as ionizing radiation, hypoxia, and chemotherapeutic agents also activate NF-κB (Sethi G and Aggarwal BB. 2006; Sethi G, *et al.* 2008). The NF-κB pathway regulates genes that promote inflammation, proliferation, survival, angiogenesis, invasion, and metastasis (Sethi G, *et al.* 2008). In addition, many cancer cells utilize NF-κB signaling to achieve resistance to anti-cancer drugs, radiation, and cell-death cytokines (Luo J-L, *et al.* 2005). Specific mutations in *c-KIT, FLT3* and *RAS*, which lead to constitutive activation of the NF-κB pathway, have been reported in AML. Increased IL-1β production has also been reported to activate NF-κB signaling in AML (Estrov Z, *et al.* 2003 & 1999). Atypical NF-κB pathway also exists, which can be activated through IKK-independent mechanism via stimuli, such as hypoxia, oxidizing radicals, or ultraviolet light radiation, leading to IκB phosphorylation, ubiquitination, and degradation (Perkins ND. 2007).

In addition, several other signaling pathways contribute to leukemogenesis. For example, Notch, Wnt, Hedgehog, and transforming growth factor beta (TGF-β)/Smad signaling play important roles in regulating fate of both normal and malignant HSCs (Pajcini KV, *et al.* 2011; Irvine DA and Copland M. 2012; McCubrey JA, *et al.* 2014; Blank U and Karlsson S. 2011).

3.2. Altered metabolism

Since 1920, altered metabolism has been recognized as one of the major differences in cancer cells and normal cells when Otto Warburg established that the majority of cancer cells produce large amounts of lactate even in the presence of oxygen (Warburg O, *et al.* 1924; Warburg O. 1925). This phenomenon is often referred to as the "Warburg effect". Several subsequent studies have shown the preference of cancer cells to metabolize glucose via aerobic glycolysis (Deberardinis RJ, *et al.* 2008; Hsu PP and Sabatini DM. 2008), which likely enables faster proliferative metabolism (Vander Heiden MG, *et al.* 2009).

It is now known that genetic and epigenetic alterations in oncogenes and tumor suppressor genes trigger changes in several intracellular signaling pathways that take part in tumor cell metabolism, leading to modifications in the cell survival and growth (Cairns RA, *et al.* 2011). In addition, the malignant tumor microenvironment – which includes components such as hypoxia as well as low pH and glucose levels – plays a key role in controlling the metabolic phenotype of tumor cells, consequently driving the tumor cells to adapt their metabolism to these extreme conditions (Cairns RA, *et al.* 2011). Moreover, altering cellular metabolism toward macromolecular synthesis is important in providing sufficient amount of building blocks for a proliferating cell to increase their biomass prior to cell division (Ward PS and Thompson CB. 2012). However, most proliferating cancer cells still gain a substantial portion of their ATP through oxidative phosphorylation (Ward PS and Thompson CB. 2012). Proliferating cancer cells also use mitochondria as a functional biosynthetic organelle, and glutamine is a primary carbon source for this type of mitochondrial metabolism (Ward PS and Thompson CB. 2012).

Many aberrations in metabolic pathways have been reported in AML. For example, the

metabolic enzymes IDH1 and IDH2 are frequently mutated in AML and associated with unfavorable prognosis (Mardis ER, *et al.* 2009; Green A and Beer P. 2010; Thol F *et al.* 2010 & 2010; Wagner K, *et al.* 2010). The mutations cause the normal enzymatic function – conversion of isocitrate to α-ketoglutarate – to be impaired, while resulting into a gain of function – conversion of α-ketoglutarate to R-2-hydroxyglutarate (*R*-2HG or D-2HG) (Gross S, *et al.* 2010**;** Dang L, *et al.* 2009; Ward PS, *et al.* 2010). The 2HG metabolite can competitively inhibit α-ketoglutarate dependent enzymes, such as ten-eleven-translocation oncogene family member 2 (TET2) and the Jumonji C (JmjC) histone demethylases, thus leading to impaired epigenetic regulation and contributing to progression of leukemia (Ward PS and Thompson CB. 2012; Ko M, *et al.* 2010; Xu W, *et al.* 2011). It has recently been shown that pharmacological inhibition of mutant IDH1 efficiently blocked the colony formation of AML cells derived from patients carrying this mutation, while no effect was observed in normal hematopoietic cell progenitors (Chaturvedi A, *et al.* 2013).

A study with hundreds of AML patient samples and healthy control samples revealed an altered glucose metabolism signature in AML, and enhanced glycolysis reduced sensitivity to anti-leukemic therapy *in vitro* (Chen W-L, *et al.* 2014). Importantly, several serum metabolites involved in glucose metabolism were identified to have prognostic value in cytogenetically normal AML (Chen W-L, *et al.* 2014).

Furthermore, the deregulated metabolism of ROS may also have therapeutic value in AML. Excessive production of ROS has been reported in many cancers including leukemia, and shown to be important for the regulation of HSC population (Ito K, *et al.* 2006). It has been demonstrated that the Forkhead transcription factor (FOXO) family of genes plays a critical role in HSCs by mediating resistance to physiological oxidative stress (Tothova Z, *et al.* 2007). Inhibition of the FOXO activity and its downstream constituents was found to diminish leukemogenic potential in MLL-AF9 AML mouse models (Sykes SM, *et al.* 2011).

Taken together, targeting of the altered metabolism is a promising endeavor in the development of new therapies.

3.3. Leukemia stem cells

LSCs contribute to treatment failure and disease relapse due to their reduced sensitivity to chemotherapeutic agents. The mechanisms involved in LSC maintenance and self-renewal are therefore being investigated as targets for therapy (Konopleva MY and Jordan CT. 2011).

However, one of the main obstacles in targeting LSCs is that treatment strategies must take into consideration that the cell cycle status of LSCs is mostly quiescent. Therefore, therapies must be able to target cells independent of their cell cycle status or activate LSCs prior to therapy (Konopleva MY and Jordan CT. 2011). For instance, Tie2/angiopoietin-1 signaling has been shown to suppress cell cycle progression in AML (Ichihara E, *et al.* 2011), and could therefore be a target for quiescent cells.

In addition, targeting should be directed against properties that are most consistently aberrant in LSCs. Notably, several cell intrinsic mechanisms contribute to the LSC maintenance and self-renewal capabilities, such as constitutive activation of the NF-kB and the PI3K – Akt/PKB pathways, which increase reliance on anti-apoptotic mitochondrial proteins via overexpression of several B-cell CLL/lymphoma 2 (BCL-2) family members, and chromatin modifications (Guzman ML and Allan JN. 2014; Konopleva MY and Jordan CT. 2011). For example, NF-κB pathway is constitutively activated in primary human AML stem cells, indicating that this pathway is important for overall LSC survival as well as for AML cells in general (Guzman ML, *et al.* 2001; Guzman ML, *et al.* 2002). Interestingly, most of the compounds that have shown success to eradicate LSCs function as NF-κB pathway inhibitors (Hassane DC, *et al.* 2008). Therefore, this pathway is a promising target for the development of LSC-specific therapies (Konopleva MY and Jordan CT. 2011). Alternatively, the leukemogenic translocations, such as *RUNX1-RUNX1T1* and monocytic leukemia zinc finger-transcirptional mediators/intermediary factor

2 (*MOZ-TIF2)*, can enhance or confer self-renewal properties to progenitor cells, and may therefore, be utilized for targeting purposes (Steffen B, *et al.* 2011; Huntley BJP, *et al.* 2004).

In addition, LSCs have a unique phenotype and many cell surface markers are selectively expressed on LSCs. For example, it has been demonstrated that CD32 and CD25 markers, specifically expressed in LSC population of most AML patients (Saito Y, *et al.* 2010), have both therapeutic and prognostic value. Other phenotypic markers currently under evaluation include, CD123 markers (Jordan CT, *et al.* 2000), C-type lectin-like molecule-1 (CLL-1) (van Rhenen A, *et al.* 2007; Zhao X, *et al.* 2010), CD96 (Hosen N, *et al.* 2007), T-cell immunoglobulin and mucin domain 3 (TIM-3) (Jan M, *et al.* 2011), CD47 (Majeti R, *et al.* 2009), CD44 (Jin L, *et al.* 2006), interleukin-1 receptor accessory protein (IL1RAP) (Askmyr M, *et al.* 2013), as well as Tie2 and N-cadherin (Qiu S, et al 2014).

A variety of cell-extrinsic mechanisms are also involved in LSC maintenance and self-renewal, such as microenvironmental signaling, cell-cell contact, and oxidative stress. Promising therapeutic strategies include targeting of the mechanisms that mediate LSC adhesion within bone marrow niches as well as the pathways responsible for niche-induced prosurvival and self-renewal signaling in LSCs (Konopleva MY and Jordan CT. 2011).

3.4. Leukemia microenvironment

The normal HSC niche ensures HSC survival and self-renewal, thereby ensuring life-long hematopoiesis. In part, this is accomplished by forming privileged sites that maintain cellular quiescence. Similarly, LSCs can benefit from these sites in order to promote their survival by escaping immune system, evading apoptosis, and acquiring self-renewal capacity (Raaijmakers MHGP. 2011). Leukemia cell growth creates a malignant microenvironment that has the capacity to suppress normal hematopoiesis by impairing normal HSCs and MPPs. For instance, leukemic cells can take over existing bone marrow niches and subsequently utilize the signaling mechanisms of these niches for their maintenance and survival. Leukemia cells were demonstrated to specifically disrupt the niches of normal HSCs in the experiments utilizing *in vivo* imaging (Colmone A, *et al.* 2008; Sipkins DA, *et al.* 2005).

Moreover, the disruption of heterotypic signaling between HSCs and their niche may promote the initiation of cancer, and it has been demonstrated that abnormal bone marrow stromal microenvironment on its own can lead to dysfunctional hematopoiesis and even induce secondary leukemia. These data indicate that primary changes in the bone marrow microenvironment can drive leukemogenesis (Raaijmakers MH, *et al.* 2010). Lineage fate can be determined by stromal clues, and thus, the microenvironment may also affect the lineage commitment of acute leukemia (Wei J, *et al.* 2008; Konopleva MY and Jordan CT. 2011). Similarly, stem cell niches have the capacity to provide stem cell characteristics on the cells that occupy them. In effect, increasing evidence from solid tumors suggests that stemness of a cell can be modulated by the microenvironment and therefore is not a rigid feature. It is conceivable that the linear hierarchy in leukemia also has much more plasticity than thus far recognized (Raaijmakers MHGP. 2011). In addition, several studies have shown that pure populations of MSCs isolated from the patients with hematopoietic malignancies frequently carry karyotypic abnormalities, which in some cases are completely different from those observed in leukemic cells (Flores-Figueroa E, *et al.* 2005; Zhang W, *et al.* 1999; Bhatia R, *et al.* 1995; Blau O, *et al.* 2007). Therefore, molecular and genetic changes not only in leukemia cells, but also in stromal cells may contribute to leukemogenesis.

Importantly, the bone marrow stromal cells can protect malignant cells from chemotherapy-induced death by providing resistance to cell-cycle-dependent cytotoxic therapies (Raaijmakers MHGP. 2011). The molecular mechanisms responsible for maintaining quiescence in normal stem cells may also facilitate LSC survival, and both the vascular and osteoblastic niche play a critical role for survival, proliferation, and differentiation of LSCs (Konopleva MY and

Jordan CT. 2011). For instance, after high-dose chemotherapy, remaining leukemia cells that manage to escape the treatment cluster and adhere to the blood vessels as well as to the endosteum, suggesting that leukemia cells obtain anti-apoptotic signals both from osteoblasts as well as from vascular endothelium (Ninomiya M, *et al.* 2007).

Therefore, strategies that block homing and adhesion of LSCs to a bone marrow niche and/or sensitize leukemia cells to a therapy are of high interest. For instance, disruption of adhesion signals such as interaction between CXCL12 and C-X-C chemokine receptor 4 (CXCR4), by interference with CXCR4 molecules, has shown promise in preventing the homing of LSCs (Burger JA and Peled A. 2009). In addition, the survival and proliferation of leukemic blasts are dependent on several adhesive interactions with stromal ligands, such as vascular cell adhesion molecule 1 (VCAM-1) and fibronectin in the ECM. These interactions are mediated by integrins, such as $\beta 1$ and $\beta 2$ integrins in AML cells (Bradstock KF and Gottlieb DJ. 1995; Bendall LJ, *et al.* 1994). It was also shown that when cell-cycle dependent chemotherapy was combined with colony-stimulating factor (G-CSF) treatment, apoptosis and elimination of human primary leukemia initiating cells was greatly enhanced *in vivo* compared with chemotherapy alone (Saito Y, *et al.* 2010). Moreover, drugs that target the abnormally activated pathways within the niche (resulting from paracrine loops) might be able to ”normalize” the cancer-promoting microenvironment, which in turn might render cancer cells susceptible to a therapy (Grivennikov SI, *et al.* 2010).

Taken together, promising treatment options for AML include drugs targeting the stromal components and the interactions within bone marrow microenvironment.

3.5. Angiogenesis

Angiogenesis is known to be a major contributor to cancer progression in solid tumors; however, it is also an important requirement for the development and progression of hematologic malignancies such as leukemias and lymphomas (Ayala F, *et al.* 2009). In effect, increased vascularization is observed in leukemias, including AML and ALL, as well as in various pre-leukemic disorders, such as MDS and myeloproliferative neoplasms (Hussong JW, *et al.* 2005). Both leukemic blasts (Padró T, *et al.* 2002) as well as bone marrow stroma (Litwin C, *et al.* 2002) promote angiogenesis by secreting growth factors and proangiogenic mediators such as VEGF, basic fibroblast growth factor and angiopoietins. For example, VEGFR, one of the key players in angiogenesis, normally expressed by endothelial cells, is also expressed by leukemia cells to support their survival, growth and migration, and often responds to both autocrine as well as paracrine VEGFs (Li ZW and Dalton WS. 2006; Ayala F, *et al.* 2009). Almost two decades ago, clinical studies already suggested that VEGF levels might have potential prognostic value for survival rates in some high-risk AML patients, independent of blast count (Aguayo A, *et al.* 1999). More recently, a meta-analysis confirmed the prognostic significance of VEGF expression in AML (Guo B, *et al.* 2013). Similarly, the expression of angiopoietins and their receptor Tie2 has been shown in leukemic blasts (Loges S, *et al.* 2005), and high anigopoietin-2 levels have been associated with unfavorable prognosis in AML (Friedler W, *et al.* 2008; Hou HA, *et al.* 2008; Schliemann C, *et al.* 2007).

A recent study demonstrated that AML cells are capable of either fusing with the already established endothelium, or differentiating into endothelial-like cells. The localization of AML cells to the vasculature was shown both in patients as well as in a xenograft model (Cogle CR, *et al.* 2014). These fates may increase the AML cell survival, as well as provide a reservoir for AML cells. Endothelium may therefore serve as a site of residual disease in AML (Cogle CR, *et al.* 2014). The data strongly emphasize the importance of also targeting the malignant vasculature in AML bone marrow.

3.6. Hypoxia

The hypoxia response pathway is vital for the proliferation and survival of hematopoietic progenitors during embryonic hematopoiesis but it also plays an important role in both solid and hematological malignancies

(Takubo K and Suda T. 2012). The major regulators of this pathway are the members of α / β heterodimeric transcription factor hypoxia-inducible factors (HIFs) (Harris AL. 2002; Semenza GL. 2003; Semenza GL. 2012). For example, activation of the HIF-1 α signaling pathway up- or down regulates the expression of numerous gene products controlling energy metabolism, glycolysis, angiogenesis, survival/death, proliferation, pH regulation, adhesion, migration, invasion, and immune tolerance (Semenza GL. 2012; Lu X and Kang Y. 2010; Brahimi-Horn MC, *et al.* 2007). Specifically, HIF-1 regulates the expression of several genes encoding angiogenic growth factors, such as VEGF, platelet derived growth factor B (PDGF-B), stromal cell-derived factor 1 (SDF1), angiopoietin 1 and 2, and placental growth factor (PGF) (Semenza GL. 2012). It has been shown that the overexpression of HIF-1 α might have a profound impact on the prognosis of patients with leukemia, including AML (Fiegl M and Spiekermann K. 2011).

Notably, decreased oxygen availability induces drug resistance and favors tumor progression. Hypoxic zones are considered to be less responsive to radiotherapy due to decreased levels of oxygen-free radicals that are required to produce a sufficient amount of DNA damage to induce cell death (Moeller BJ, *et al.* 2007). In addition, cells residing in hypoxic regions are more resistant to therapies due to their mainly quiescent cell cycle status as well as reduced delivery of drugs via the circulation (Brahimi-Horn M *et al.* 2007). Consequently, approaches to target hypoxic tumor microenvironment to prospectively sensitize tumor cells to chemotherapy have been studied extensively. Some approaches have pursued to directly block HIF-1 α activity, while others take advantage of hypoxia-activated prodrugs that are functional only under hypoxic conditions (Benito J, *et al.* 2011). For instance, hypoxia activated nitrogen mustard prodrug was able to efficiently inhibit leukemia cell growth in preclinical mouse models of human AML (Portwood S, *et al.* 2013).

3.7. Modifiers and regulators of chromatin, and non-coding RNAs

Aberrant epigenetics is playing a crucial role in leukemogenesis, including AML (Plass C, *et al.* 2008). An extensive analysis of DNA methylation changes in AML was performed on a large cohort of clinically annotated patient samples, which revealed specific methylation patterns between different AML subtypes and CD34+ CD38- cells from healthy donors (Ley TJ, *et al.* 2013). In addition, altered levels of histone acetylation have been reported in many malignancies, and mutations in genes encoding acetyltransferases, such as CREB binding protein (CREBBP) and EA1 binding protein p300 (EP300) have been found in leukemia and lymphoma (Mullighan CG, *et al.* 2011; Pasqualucci L, *et al.* 2011).

Furthermore, non-coding RNAs, such as miRNAs and long-non coding RNAs, have been demonstrated to affect the phenotype of a cell by regulating the transcription, stability or translation of encoding genes and their role in cancer has clearly been established (Ling H, *et al.* 2013). For example, miR-155 is overexpressed in many solid tumors and hematological malignancies, including AML (Faraoni I *et al*. 2009; Kong *et al*. 2010). In patients with cytogenetically normal AML, high miR-155 expression was associated with shorter disease-free and overall survival as well as with low CR (Marcucci G, *et al.* 2013). In addition, it was demonstrated that locked nucleic acid-based and nanoparticle-based inhibition of miR-155 decreases tumor growth in mouse models of lymphoma (Babar IA, *et al*. 2012; Zhang Y, *et al*. 2012). These data suggest that therapeutic targeting of miR-155, or other aberrantly regulated miRNAs, may also be similarly achievable in AML.

3.8. Mitochondrial proteins and biogenesis

Many cancer cells rely on deregulated apoptotic signaling for their survival, and are often dependent on anti-apoptotic proteins, such as BCL-2 protein (Brunelle JK and Letai A. 2009). The overexpression of anti-apoptotic proteins has been reported to confer drug resistance, and several strategies to inhibit the anti-apoptotic proteins have shown promise in sensitizing AML cells to therapy (e.g. Rahmani M, *et al.* 2012; Konopleva M *et al.* 2012; Vo TT, *et al.* 2012). For instance, mitochondrial “priming” – which referrers to relative mitochondrial readiness

for apoptosis – can be used to evaluate the sensitivity of AML cells to chemotherapy. BCL-2 homology 3 (BH3) domain profiling, which was used as a measure for differential priming between malignant myeloblasts and normal HSCs, identified BCL-2 inhibition as a promising therapeutic target (Vo TT, *et al.* 2012).

In addition, higher rates of mitochondrial biogenesis are observed in leukemic cells. Inhibition of the mitochondrial translation was shown to be a potential therapeutic strategy in mouse models of human AML (Skrtic M, *et al.* 2011). As such, dependence of AML cell survival on mitochondrial proteins or function may have significant clinical implications.

3.9. Genetic vulnerabilities

Driver mutations in cancer have received much attention as they contribute to the "oncogene addiction", a phenomenon that explains how cancer cells, although containing multiple genetic and epigenetic changes, remain dependent only on a few abnormalities for their survival and growth (Weinstein IB and Joe A. 2008). However, recent research has shown that passenger mutations could also in some cases be exploited in therapeutic targeting. Specifically, when tumor-suppressor genes undergo inactivation via homozygous deletion many of the neighboring (i.e. passenger) genes are also often affected, and may as a result expose cancer-specific therapeutic vulnerabilities when the collaterally deleted gene belongs to a family of functionally redundant genes that carry out an essential function. For instance, deletion of the glycolytic gene enolase 1 in glioblastoma is tolerated because of the expression of enolase 2. However, it was demonstrated that a specific silencing of enolase 2 via RNA interference inhibited the survival and growth of cancer cells. Therefore, targeting of the passenger-deleted genes encoding functionally redundant essential activities provide an effective treatment strategy for cancer that contain such collateral vulnerabilities in their genomes (Muller FL, *et al.* 2012). It is likely that similar collateral vulnerabilities that could be exploited for therapies may also exist in AML.

3.10. Immunotherapy

Leukemia cells have the capacity to modulate immune cells in a manner that creates an immunosuppressive bone marrow microenvironment, where both innate and adaptive immune responses are repressed. The presence of several different immunosuppressive mechanisms facilitates leukemia cells to escape from normal immune control, and contributes to proliferation, survival, and drug resistance of AML (Arpinati M and Curti A. 2014; Isidori A, *et al*. 2014). For instance, several studies have confirmed the role of regulatory T cells (Tregs) in promoting cancer immunotolerance (Beyer M and Schultze JL. 2006; Ustun C, *et al.* 2011; Szczepanski MJ, *et al.* 2009; D'Arena G, *et al.* 2011). For example, AML blasts are known to be capable of increasing the number of Treg cells both via direct and indirect mechanisms (Ustun C, *et al.* 2011). In addition, tumor hypoxia has been shown to promote the recruitment of Tregs (Facciabene A, *et al.* 2011).

Modulation of the immune system in a manner that facilitates the immune system to eliminate cancer cells is therefore actively being investigated, and many immunotherapy options have already been demonstrated to control the development of AML both in preclinical and clinical studies. The immunomodulatory receptors (also referred to as checkpoint molecules), such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) receptor that are expressed on T cells, participate in mediating the immunosuppression induced by cancer. Accordingly, inhibition of CTLA-4, PD-1, as well as PD-ligand 1 (PD-L1) receptors has been successfully shown to promote the immune attack against cancer (Martner A, *et al.* 2013). In addition, adoptive cellular therapies – which include isolation of autologous T-cells followed by *ex vivo* modification and/or expansion and ultimately by reinfusion – are emerging effective therapy options (Smith EL, *et al.* 2014; Rambaldi A, *et al.* 2015).

4. Discovery of novel targets and therapeutic agents

4.1. Druggable targets

The druggable genome is a concept that refers to human genome that is susceptible to pharmacological interactions and concurrently participates in disease driving pathological mechanisms (Hopkins AL and Groom CR. 2002).

In general, in biological systems, therapeutic agents can be developed against four types of macromolecules, namely: proteins, polysaccharides, lipids, and nucleic acids. However, it is often difficult to obtain effective therapeutic agents against the latter three macromolecules, as toxicity and non-specificity are major issues with these types. Therefore, compounds that interfere with the activity of proteins are of high interest (Hopkins AL and Groom CR. 2002).

Based on these limitations, the molecular targets against which commercially feasible drugs can be developed has been restricted. This perspective has also lead to the term of "the druggable genome", which accounts for the ~30,000 genes representing the informational content of the human genome that express proteins. However, only a fraction of these distinct targets have been exploited in the current drug therapies, as many of the proteins have no obvious binding or enzymatic activity, or known substrates, and only a limited number of "druggable proteins" are associated with biochemical mechanisms involved in disease (Hopkins AL and Groom CR. 2002). In effect, it has been estimated that only about 2-5% of the human protein-coding genome (approximately 600-1,500 gene products) may serve as potential drug targets based on their relevance to the disease and targetability by drugs (Hopkins AL and Groom CR. 2002; Rask-Andersen M, *et al.* 2014). Current established pharmaceuticals exploit about 555 of these distinct target genes (Hopkins AL and Groom CR. 2002; Rask-Andersen M, *et al.* 2014). However, these estimates are based on studies with small molecules that are compliant with the "rule-of-five" guidelines (see below), thus including only drug targets that are able to bind compounds with appropriate properties (Hopkins AL and Groom CR. 2002). As the collection of pharmaceuticals is expanding to include new biotechnological products and techniques, such as antisense oligonucleotides and adenoviral gene delivery, the druggable genome is broadening tremendously. Nevertheless, the druggable genome is still a finite concept, which has its limits despite the advances (Rask-Andersen M, *et al.* 2014).

Markedly, human cancers display numerous molecular abnormalities compared to normal tissue, and a widespread heterogeneity between different types and subtypes of cancers also exists (Weinstein IB and Joe AK. 2006). These abnormalities are all potentially druggable targets, which may be exploited for therapies. In effect, the increased knowledge of the disease – based on the advances in technologies including next-generation sequencing and other genomic technologies, genome-wide association studies, proteomics analysis, RNA interference studies such as synthetic lethality screens, and chemical biology – has led to the discovery of many new drug targets and drugs (Patel MN, *et al.* 2013).

Nonetheless, development of molecular targeted therapies is challenging and slow due to struggles in discriminating the pathologically relevant targets as well as evaluating the prognostic value of a specific mutation in the midst of all the other coexisting mutations. As such, information obtained from the discoveries in molecular pathogenesis has been difficult to translate into novel therapeutics (Döhner H and Gaidzik VI. 2011). Functional screening approaches that can identify efficient targeted therapy options against clinically significant molecular abnormalities are thus also needed.

4.2. High-throughput screening assays

A high-throughput assay refers to a methodology that is capable of rapidly screening a large number of pharmaceutical agents or therapeutic targets, and relies on automated handling and detection devices.

High-throughput screening assays can be broadly divided into biochemical target-based assays and cell-based assays (**Table 1**.).

High-throughput Screening methods	Advantages	Disadvantages	Example references
Small molecule phenotypic screening	Enables discovery of new therapeutic agents. No prior knowledge of a target is required. Allow exploration of new phenotypes and new areas of biology via discovery of first-in-class drugs as well as provide tools to study complete signaling pathways and molecular networks. Allow oncogenic pathway prediction by identifying pathway dependences: facilitates the identification of functionally important targets. Identification of small molecules interacting with biological molecules that lack small molecule tools. Allow the identification of enzyme inhibitors, receptor agonists and antagonist, as well as, allosteric inhibitors and protein-protein interactions	Diversity of a small molecule compound library is imitated by the "chemical space" of its compounds, Identification of the mechanism of action for a compound can be difficult: target identification is a major challenge. Optimization of molecular properties of a candidate drug can be challenging, Prioritizing with "hits" to follow-up can be challenging. A compound may give different phenotypes depending on the screening platform used. Pathway dependency prediction: The effective drug target assessment large-scale may rely on an algorithm, which could exhibit both false-positive and false-negative targets	Eggert US. 2013 Swinney D and Anthony J. 2011 Hatzis C, *et al.* 2014 Tyner JW, *et al.* 2013
Functional genomic screening via RNA interference (RNAi)	Facilitate oncogenic target identification and validation of therapeutic targets. Identification of molecular vulnerabilities in malignant cells	Reliability of RNAi-based screens can be limited by false-positive hits resulting from off-target effects and false-negative hits resulting from ineffective RNAi agents Druggability of the target may be challenging	Tyner JW, *et al.* 2009 Tyner JW, *et al.* 2008
Phage display random peptide library screening	No prior knowledge of a target is required. Can identify novel ligand-receptor pairs Identifies physiologically relevant targets Enables identification of "druggable" disease markers Validated ligands can subsequently be used in targeted drug delivery Peptides have benefits over antibodies: relatively simple *in vitro* synthesis and quality control, increased tissue penetration and diffusion, and rapid blood clearance	In theory, the amino acid sequence diversity of a phage display peptide library increases exponentially with the number of randomized residues, however the practical diversity of a phage display library is limited by bacterial transformation efficiencies. The higher selection of a certain peptide over the other via the phage displayed peptide library screening could be paradoxical due to preferential coding utilization in bacteria. Libraries may also contain many peptide sequences that have no compatibility in human, therefore, limiting the diversity of physiologically relevant sequences.	Sergeeva A, *et al.* 2006 Pande J, *et al.* 2010 Hanzeh-Mivehround M, *et al.* 2013

Table.1. Examples of cell-based high-throughput screening methods for the discovery of novel targets and therapeutic agents.

In general, biochemical assays are specific to the target of interest and the homogeneous reaction environment provides minimized experimental variability. These types of assays can be used, for instance, to measure enzymatic activity, ion channels, receptor-ligand binding, nuclear receptor and protein-protein interaction. On the contrary, cell-based assays also enable the study of physiologically relevant mechanisms such as regulatory networks and feedback mechanisms. In addition, cell-based assays do not require any prior knowledge of a direct molecular target and they allow for selection of cell permeable agents. Cell-based assays can also provide clues of acute toxicity. Consequently, approximately half of all high-throughput screens are currently performed in cell-based assays (An WF and Tolliday N. 2010).

Several approaches can be used to identify potential anti-cancer agents via cell-based high-throughput screening assays. One strategy is to screen large chemical libraries to identify biologically active compounds that preferentially inhibit *in vitro* cell growth or induce apoptosis in cancer cells. Alternatively, more focused chemical libraries against certain protein families, such as kinases, facilitate the discovery of best available treatment options for a certain pathway dependency in an individual (Tyner JW, *et al.* 2013). Similarly, advances in personalized treatment tailoring with available drugs have been obtained by combining molecular profiling with *ex vivo* drug sensitivity and resistance screens (Pemovska T, *et al.* 2013).

Genes responsible for maintaining the malignant phenotype of specific cancer cells can be identified through using RNA interference (RNAi) such as siRNA libraries. Subsequently, drugs can be designed to target the proteins expressed by these genes (Weinstein IB and Joe AK. 2006). For example, RNAi-assisted protein target identification (RAPID) technology has been developed for the assessment of each member of the tyrosine kinase gene family as a target in leukemia patients (Tyner JW, *et al.* 2009).

In addition, other types of high-throughput display technologies have also been developed, such as phage display technology, which can be used to map ligand-receptor pairs specific to cancer cells (Sergeeva A, *et al*. 2006). The identified ligands can then be potentially used in disease diagnostics, profiling, imaging and therapy. For instance, the ligands can be linked with cytotoxic agents or proapoptotic peptides to design targeted therapy options. The efficacy of the particular ligand as a drug delivery agent will also help to determine the impact of the cancer specific target under study.

4.3. Small molecule compounds

The advantages of using small molecule compounds in the large-scale screening include the cost-effectiveness and ease in production. In addition, they are frequently internalized into cells and can therefore also interact with intracellular targets.

4.3.1 Drug-like properties

Living systems contain several physiological barriers that drugs must encounter in order to reach the therapeutic target, including cell membranes, pH, metabolic enzymes, and transporters. These barriers usually decrease the efficacy of a drug.

Desirable drug qualities include good absorption and distribution, low metabolism, reasonable elimination, and low toxicity, collectively referred to as ADME/Tox (absorption, distribution, metabolism, excretion, and toxicity) properties (Kerns EH and Di L. 2008). Therefore, in addition to the pharmacological (e.g., efficacy and selectivity) properties, it is essential to optimize the physicochemical (e.g., solubility, permeability, chemical stability, and metabolic stability) and toxicological properties of compounds to increase the success in drug discovery (Kerns EH and Di L. 2008). For instance, lipophilicity of a compound is a major factor of many ADME/Tox properties.

Those compounds that have sufficiently acceptable ADME properties as well as toxicity profiles to survive through the Phase I clinical trials are defined to have “drug-like” properties (Lipinski CA, *et al.* 1997). Based on these data, Lipinski and Verber established a set of guidelines – namely the “rule-of-five” – for structural (physico-

chemical) properties of drug-like compounds (Lipinski CA, *et al.* 2001). These guidelines state that good absorption and permeability, and thus oral bioavailability, are likely when the molecular weight of a compound is ≤500 daltons, the number of hydrogen-bond acceptors (i.e., nitrogen and oxygen molecules) is ≤10, the number of hydrogen-bond donors (i.e., OH and NH groups) is ≤5, and the partition coefficient (1-octanol:water), LogP, is ≤5. These guidelines, although not absolute, can be quite effective and efficient in assessing compounds with good absorption and permeability properties (Kerns EH and Di L. 2008).

The rule-of-five has been the only computationally derived filter for drug-likeness acknowledged by both academia and industry (Ursu O, *et al.* 2011), and the commercially available small molecule compound libraries are typically based on these criteria. Nevertheless, the use of the current commercially available small molecule compounds may limit the discovery of druggable targets, as the libraries are still relatively simple and often comprise of similar compounds (Galloway WRJD, *et al.* 2010). The biological activity of a molecule depends largely on its three-dimensional arrangement of chemical features (often referred to as the "*chemical space*") that can interact with a specific target, and is essential to generate a biological response. Therefore, it is likely that as the chemical libraries become more diverse, for instance as the scaffold diversity increases and thus the chemical space, novel druggable targets will emerge rapidly (Galloway WRJD, *et al.* 2010).

Furthermore, poor pharmacokinetics (clearance, half-life, bioavailability, drug-drug interactions) can cause low drug concentration in the plasma and target tissue, thus leading to poor *in vivo* efficacy. For instance, transporters (e.g., P-glycoprotein) can significantly affect the ADME/Tox characteristics of a compound. In addition, compounds may be instable in blood. For example, enzymatic hydrolysis, plasma protein binding, and red blood cell binding can hinder drug efficacy in the blood stream (Kerns EH and Di L. 2008). Other environmental factors, such as stromal cells and hypoxia, also affect drug stability and efficacy (Tabe Y and Konopleva M. 2014). Therefore, drug-like properties alone are not often sufficient to reflect whether a drug will be clinically efficacious.

4.3.2 Target Identification

Target identification of drug-like small molecules is crucial for understanding the biological phenomena at the molecular level as well as elucidating the mechanism of action of bioactive compounds (Takakusagi Y, *et al.* 2010). Nevertheless, targets for some drugs that are in clinical use or development still remain elusive. Comprehensive mechanistical analysis on these functional compounds and their target identification will likely provide new insight into druggable pathways and molecules (Rix U and Superti-Furga G. 2009).

Unfortunately, target discovery of small-molecules remains a challenging and time-consuming process. One factor that hinders target discovery is a lack of versatile methods to elucidate the mechanism of action of small molecules. In addition, bioactive small molecules often strongly or weakly interact with numerous targets, mainly proteins, *in vivo*. It is therefore technically challenging to thoroughly understand the molecular mechanisms of drug-like small-molecules (Takakusagi Y, *et al.* 2010).

However, advancements in the development of experimental methods, as well as the arrangement of the genome database have facilitated the target identification. For instance, associating results from functional assays, such as cell viability and proliferation assays, with alterations in drug-induced transcriptomic profiles can identify cellular processes affected by a given treatment (Hatzis C, *et al.* 2014), therefore narrowing down the candidate targets.

Furthermore, diverse technologies can be combined to act synergistically in order to increase throughput as well as to facilitate identification of targets for small molecules. Of the experimental technologies available, phage display technology enables rapid and extensive screening for peptides and proteins that interact with drugs. The homology studies of the discovered drug-binding peptides can, in turn, help to uncover the natural target for the small molecule under

study (Takakusagi Y, *et al.* 2010). In addition, the phage display technology can be utilized in identification of novel targets, for instance, via screening the phage-display peptide libraries against cancer cells, therefore making it as a valuable methodology in cancer target discovery. Several other display technologies also exist, such as cell-surface display, which may similarly be utilized in target identification for small molecule compounds (Sergeeva A, *et al.* 2006).

4.4 Peptide based therapeutics

Peptide-based therapeutics have many advantages compared to monoclonal antibodies including their smaller size, ease of derivatization, simplicity of *in vitro* synthesis as well as quality control, which reflect the lower cost of manufacturing. The peptides also have increased tissue penetration and diffusion, as well as faster blood clearance compared to antibodies (Sergeeva A, *et al.* 2006). Nevertheless, the therapeutic efficacy of peptides can be limited due to low affinity, potential immunogenicity, proteolytic degradation, and rapid renal filtration.

The pharmacodynamic properties of peptide therapeutics may be improved by several approaches. For instance, enhanced stability of peptides has been obtained by use of scaffold proteins, blocking of N- and C termini, peptide cyclization, and substitution of L-amino acids with D-amino acids, unnatural amino acids, and chemically modified amino acids. Polyethylene glycol and polysialic acids in conjugation with the peptide has led to longer peptide half-life, improved stability and decreased immunogenicity. In addition, secondary biased library screening and peptide multimerization have helped to improve the affinity of peptides (Krumpe LRH and Mori T. 2007). Therefore, development of peptides with enhanced therapeutic efficacy could include an initial screening of a combinatorial random peptide library to identify targeting prototype-moieties followed by subsequent screening of a secondary synthetic combinatorial peptide library that is based on the targeting moiety (Krumpe LRH and Mori T. 2007).

Phage display technology is an excellent screening tool for the identification of lead targeting moieties. For example, the phage displayed random peptide libraries can be used to identify peptide sequences that bind to specific targets. Importantly, the ability of phage display technology to identify peptide leads that can be engineered into efficient therapeutics and targeting moieties has been well established by many research groups (e.g. Sergeeva A, *et al.* 2006; Krumpe LRH and Mori T. 2007).

4.5. Phage display technology

Phage display technology utilizes combinatorial libraries of exogenous peptides (or proteins) that are expressed on the surface of phage particles (**Figure 3**). These libraries can be screened against purified targets as well as live cells. The advantage of the phage display technology is that no prior knowledge about cell surface targets is required, and the target does not need to be immunogenic (Samoylova TI, *et al.* 2006; Sergeeva A, *et al.* 2006; Pande J, *et al.* 2010).

In principle, a phage-display peptide library contains a wide diversity of peptide sequences and those with binding affinity against the desired target (i.e., a specific type of cell or molecule) can be selected via screening, yielding a set of peptide motifs that bind to the target under study. Importantly, the selected peptide motifs usually bind receptors by mimicking biological ligands of these receptors (Sergeeva A, *et al.* 2006).

One of the major challenges in clinical oncology today is that therapeutic agents cannot be selectively delivered to the tumor site without causing toxicity to the rest of the body. Importantly, phage display technology enables the identification of ligands that bind to targets selectively expressed in certain cancers. These tumor-specific receptors and corresponding ligands can subsequently be applied toward rational drug design for cytotoxic agents, gene therapy vectors, and for target-directed delivery of therapeutic agents and imaging agents (Ruoslahti E. 2002; Kolonin MG, *et al.* 2001; Sergeeva A, *et al.* 2006). For instance, it has been demonstrated that phage particles displaying peptide and antibody ligands that bind to internalizing receptors, such as integrins, can undergo efficient uptake by target cells

(Zurita AJ, *et al.* 2004; Gao C, *et al.* 2003). Phage display technology has also been successfully utilized for targeted gene delivery into mammalian cells (Hajitou A, *et al.* 2006; Poul MA and Marks JD. 1999; Larocca D, *et al.* 1999).

Taken together, the phage display technology enables the identification of lead molecules for the development of novel agents that can potentially be utilized to specifically and effectively diagnose and treat cancer, and thus it has the potential needed for development of targeted anti-cancer therapeutics as well as diagnostic applications (Samoylova TI, *et al.* 2006). A number of comprehensive reviews list peptide motifs and their corresponding ligand/receptor systems discovered via phage display technology (e.g. Uchiyama F, *et al.* 2005; Landon LA and Deutscher SL. 2003; Watt PM. 2006).

4.5.1. Filamentous phage

Filamentous bacteriophages (*Inovirus*) are a group of closely related viruses that selectively infect gram-negative bacteria. Contrary to other phages, such as the T4 phage, filamentous bacteriophages are non-lytic and they replicate and assemble without killing the host cells. The best-known filamentous phages are Ff phages, namely: fd, f1, and M13, which are virtually identical strains. Ff refers to those filamentous phages that require the interaction with F pili (surface protein normally used for interbacterial conjugation) to infect the host bacteria, such as *Escherichia coli* (Marvin DA. 1998; Rasched I and Oberer E. 1986).

The structure of the filamentous phage M13 has been investigated extensively. The filamentous phage particle is long and thin (930 nm in length and 6.5 nm in diameter) with a flexible rod shape structure. Their genetic material consists of a circular single-stranded DNA (ssDNA) genome, which is about 6,000-8,000 bases and is enclosed in a coat comprised of five different proteins, specifically: pIII, pVI, pVII, pVIII, and pIX. Each phage contains about 2700 copies of the pVIII, the major coat protein that covers the length of the phage. Five copies of each of the two minor coat proteins, pIII and pVI cap one end of the phage, while 3 to 5 copies of pVII and pIX comprise the other end. In addition, the M13 phage encodes 6 other proteins, which participate to DNA replication and assembly (Kehoe JW and Kay BK. 2005**;** Pande J, *et al.* 2010).

The infection is initiated when the phage's coat protein pIII binds via its N2 domain to F pilus of the host cell. Phage binding causes the pilus to retract until the phage reaches the surface of the bacterium and binds, via the N1 domain of the pIII protein, to the membrane protein TolA of *E.coli*. The phage transfers its genome into the host cell once contact with the bacterial surface has been established. This leads to the disassembly of the infecting phage and insertion of the coat proteins into the bacterial membrane. The phage genome inside the bacterium is converted to double-stranded DNA by bacterial enzymes and the synthesis of phage proteins is initiated. Upon synthesis, the structural coat proteins spontaneously insert into the inner membrane of the bacterium. Following the replication of ssDNA genomes, the proteins assemble into a phage particle, and are extruded from the cells via the protein complex, consisting of the integral membrane proteins pIV, pXI, and pI, commonly referred to as a membrane pore. As the filamentous phage do not kill host cells, infected bacteria continue to grow and divide indefinitely, however, at half the rate of uninfected cells (Kehoe JW and Kay BK. 2005; Pande J, *et al.* 2010).

4.5.2. Phage display random peptide libraries

The original phage display system was used for the affinity selection of protein fragments expressed from complementary DNA (cDNA) fragments (Smith GP. 1985; Parmley SF and Smith GP. 1988). Subsequently, other types of phage display libraries were developed for the affinity selection of peptides and antibodies (McCafferty J, *et al.* 1990; Devlin JJ, *et al.* 1990; Scott JK and Smith GP. 1990**;** Marks JD. *et al.* 1991; Barbas CF, *et al.* 1991; Breitling F, *et al.* 1991; Hoogenboom HR, *et al.* 1991). Since then, a large number of phage displayed peptide and protein libraries have been constructed (reviewed in Hoogenboom HR. 2002, Szardenings M. 2003, Kehoe JW and Kay BK. 2005;

Sergeeva A, *et al.* 2006; Paschke M. 2006; Pande J, *et al.* 2010).

Combinatorial peptide libraries provide a useful tool to identify ligands with different binding potential (Sergeeva A *et al.* 2006). The most frequently utilized phage display libraries are the random peptide libraries (Pande J *et al.* 2010), which can contain about 10^{10} individual phage clones with a unique peptide sequence (Kehoe JW and Kay BK. 2005; Sergeeva A *et al.* 2006). Structurally more constrained phage display random peptide libraries have shown higher affinity to several targets to which more flexible linear random peptide libraries failed to bind (Pande J, *et al.* 2010). Accordingly, the most commonly used peptide libraries comprise of sequences with the general structure CX_NC, which express peptides with random 5 to 9 amino acid residues that are flanked by cyclized cysteines (Sergeeva A, *et al.* 2006). The foreign DNA fragment, consisting of random oligomers, is inserted into the phage genome and the encoded foreign peptide is displayed on the surface of the phage as a fusion protein with one of the coat proteins. Fusion of the foreign peptide sequence is frequently done to the N-terminus of the pIII and pVIII coat protein (Kehoe JW and Kay BK. 2005; Pande J, *et al.* 2010). During the phage assembly process, the fusion proteins insert into the inner cell membrane of the bacterium along with the other phage proteins, and are incorporated into the nascent phage particle containing the ssDNA encoding the displayed fusion protein (Kehoe JW and Kay BK. 2005).

The pIII coat protein is usually used with the cDNA libraries encoding proteins or protein domains as it tolerates the larger insertion better and has fewer copies than pVIII. Monovalent display may also be used in the case of large insertion in order to retain the coat protein function. In this approach, both fusion-proteins as well as wild-type proteins of the pIII are produced, thus forming a mosaic pattern of the pIII coat protein on the phage surface. In a hybrid display system, a phage genome carries both the wild-type and the fusion protein copies. Alternatively, a phagemid vector (i.e. a plasmid) is used to encode the fusion protein and a helper phage produces the wild-type protein as well as all the other proteins required for the phage to function. A polyvalent phage display – in which all the copies of the coat protein (such as pIII) display the foreign sequence – can be used with libraries containing short peptides that do not interfere with the function of the coat protein and thus the function of the phage. In general, polyvalent phage display provides higher avidity (i.e. higher binding strength) due to the higher number of peptide sequences displayed, and can therefore detect lower-affinity binding (Sergeeva A *et al.* 2006).

The most frequently used phage vectors to generate random peptide phage libraries are filamentous phage belonging to the Ff class (fd, f1 and M13). Several fusion-phage vectors with certain modifications to the phage's wild type genome have been constructed for cloning purposes (Zacher AN *et al.* 1980; Parmley SF and Smith GP. 1988; Scott JK and Smith GP. 1990; Smith GP and Scott JK 1993). The first constructed phage vector was called fd-tet, which comprised of the wild-type fd genome and a segment of transposon Tn10 coding for tetracycline resistance (Zacher AN *et al.* 1980). Subsequently, many of the fusion-phage vectors, including the fUSE vectors and fAFF1, have been derived from fd-tet. Notably, these vectors have several advantages. For example, foreign inserts do not incapacitate the pIII protein significantly, and the phage remains capable of infecting the host cells as well as undergoing normal morphogenesis (Scott JK and Smith GP. 1990). In addition, since the vector carries a tetracyclin selection marker and since filamentous phage do not kill their host, a single infection event is sufficient to produce a detectable tetracycline-resistant clone, which allows infective phage to be quantified as transducing units (TU), which measure bacteria colonies, rather than plaque-forming units, which measure regions of host cell destruction (Kehoe JW and Kay BK. 2005; Pande J, *et al.* 2010).

However, the filamentous phage system also has some limitations, especially when trying to display proteins that do not fold properly in the periplasm (Kehoe JW and Kay BK. 2005). Consequently, alternative phage display systems using lytic bacteriophages – such as T4 (Jiang J, *et al.* 1997), T7 (Danner S and Belasco JG. 2001), and P4 (Catagnoli L, *et al.*

2001; Lindqvist BH and Naderi S. 1995) – have been developed, in which the capsid assembly occurs in the cytoplasm and the phage are released via cell lysis. Bacteriophage lambda is yet another vector system for the surface display of peptides and proteins (Sternberg N and Hoess RH. 1995).

4.5.3. Screening phage display random peptide libraries

The advantages of phage display include the convenience with which libraries can be selected for target-specific binders via affinity purification. Notably, phage display typically enables identification of target-specific sequences with the affinity constants in the micromolar-to-nanomolar range. The phage clones that encode high-affinity polypeptides to the molecule or cell type under study are rapidly enriched by incubating the phage library with a target followed by removal of the non-binding phage and amplification of the high-affinity clone in the host bacteria. Usually, three to five rounds of biopanning are adequate to enrich for target-binding peptide sequences. Thus, the selection of target-specific sequences from a phage display library is a cyclic process involving selective enrichment and amplification. Subsequently, the primary structure of the high-affinity peptide displayed on the surface of the phage is determined by sequencing the corresponding encoding DNA (Sergeeva A, *et al.* 2006). Genomic tools have been integrated to make the process quick and affordable (Dias-Neto E, *et al.* 2009).

Phage display has been routinely utilized in the *in vitro* setting to screen peptide or antibody libraries for ligands on purified and immobilized molecules (Kehoe JW and Kay BK. 2005). These applications have been useful in identification of protein-protein interaction pairs and mapping their interaction domains (e.g. Hertveldt K, *et al.* 2009; Voss M, *et al.* 2009; Samoylova TI, *et al.* 2006; Kolonin MG, *et al.* 2006a; Hartley O. 2002; Walter G *et al.* 2001; Romanov VI. 2003). *In vitro* screening has also yielded agents, including peptides specifically binding to caspases (Tamm I, *et al.* 2003), integrins (Koivunen E, *et al.* 1995; Cardó-Vila M, *et al.* 2003), and metalloproteinases (Koivunen E, *et al.* 1999), that have been used in drug delivery approaches.

Methods to screen phage libraries on cultured cells have also been developed (e.g. Barry MA, *et al.* 1996; Ivanenkov VV, *et al.* 1999; Hong FD and Clayman GL 2000). One such method that enables isolation of specific ligands from a small amount of cells quickly and efficiently is the biopanning and rapid analysis of selective interactive ligands (BRASIL) method (Giordano RJ, *et al.* 2001). Examples of the successful phage display screening performed on cells include the identification of a peptide targeting vascular endothelial cells in gastric cancer (Liang S, *et al.* 2006) and a VEGF-mimic peptide targeting neuropilin 1 (NRP-1) and VEGFR-1 (Giordano RJ, *et al.* 2005). In addition, the BRASIL method was used to systematically screen a combinatorial peptide library on tumor cells of the NCI-60 panel (Kolonin MG, *et al.* 2006a). During the selection for cell-surface binders, each NCI-60 cell line was identified with a unique set of peptide motifs, reflecting on differently expressed receptors on their surface. Tumor cells were subsequently grouped based on these profiles. In essence, the ligand-directed surface profiling of tumor cell lines can facilitate the dissection of cancer cell surface proteome, thus providing information that could be exploited in search for “druggable” receptors against specific types of cancers. For example, epidermal growth factor receptor (EGFR), upregulated by many cancers, was validated as a target of a group of tripeptides mimicking the biological EGFR ligands (Kolonin MG, *et al.* 2006a).

Furthermore, *ex vivo* protocols for selection and evaluation of phage display libraries on biopsy specimens have been evolved (Ardelt PU *et al.* 2003; Arap MA, *et al.* 2004, Shukla GS and Krag DN. 2005). For example, peptides binding to human breast tumor specimens have been identified in this fashion (Shukla GS and Krag DN. 2005). The advantage of the *ex vivo* phage display is that it allows the screening of the phage display peptide library on freshly obtained tumor specimens facilitating the identification of peptides that may bind to tumor-specific targets only under certain microenvironmental context.

Ultimately, *in vivo* phage display represents the most valuable tool to obtain physiologically relevant ligand-receptor pairs. The *in vivo* phage display enables the identification of peptide sequences homing to specific organs in an unbiased and internally controlled manner (Sergeeva A, *et al.* 2006), and ligands selectively homing to various tissues in mice have been selected via phage display screening (e.g. Pasqualini R and Ruoslahti E. 1996; Rajotte D, *et al.* 1998; Trepel M, *et al.* 2001; Samoylova TI and Smith BF. 1999; Arap W, *et al.* 2002a; Kolonin MG, *et al.* 2002; Kolonin MG, *et al.* 2004; Kolonin MG, *et al.* 2006b; Liu JK *et al.* 2014; Hyvönen M, *et al.* 2014). These tissue- and tumor-specific peptide motifs identified via *in vivo* phage display can serve as location signatures, often termed as "zip codes", for these sites. Notably, vascular addresses, which allow angiogenesis-related targeting of tumor blood vessels, have been identified via the *in vivo* phage display (e.g. Arap W, *et al.* 1998; Kolinin MG *et al.* 2001; Pasqualini R *et al.* 2000; Arap W, *et al.* 2002a; Arap W et al 2002b; Joyce JA, *et al.* 2003; Hoffman JA, *et al.* 2003; Marchió S, et al 2004; Kolonin MG, *et al.* 2006b).

The information derived from animal studies, however, does not always translate into real clinical applications, mostly due to the species-specific differences in protein expression as well as protein structure. Consequently, *in vivo* phage display has also been performed directly in human, and peptides homing to specific organs were selected after systemic administration of a random peptide library to a brain-dead patient (Arap W, *et al.* 2002b). Appropriately, ethical framework for the *in vivo* phage display in human has also been established (Pentz RD, *et al.* 2003; Pentz RD and Flamm AL. 2003 & 2005). The screening in human represents a significant improvement in the identification of clinically relevant targets via analysis of the distribution of combinatorial peptides in human tissue and subsequently identifying their corresponding receptors (Arap W, *et al.* 2002b; Zurita AJ, *et al.* 2004). Notably, ligands that bind to receptors overexpressed in cancer have been identified, including the interleukin-11 mimic peptide and the interleukin-11 receptor (Zurita AJ, *et al.* 2004), along with the human leukocyte proteinase-3 (PR-3) and receptor for advanced glycation end products (RAGE) (Staquicini FI, *et al.* 2011).

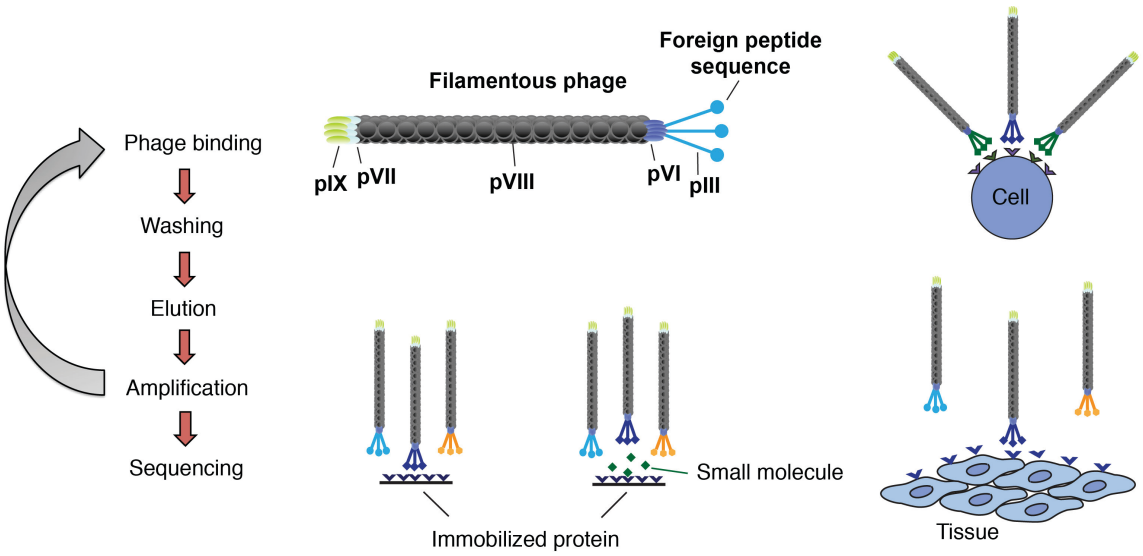

Figure 3. Phage display technology. The principle of the phage display random peptide library screening is demonstrated via a flow chart. A basic structure of the filamentous phage, as well as different screening platforms are shown.

Aims of the study

1. To elucidate the function of the proMMP-9 – αMβ2-integrin complex in migration and growth of leukemia cells and to validate the therapeutic potential of peptides binding to this complex.

2. To profile leukemia cell surface via phage display random peptide libraries and to identify new ligand-receptor pairs for targeted therapy.

3. To study the role of IL-11Rα and NRP-1 as therapeutic targets in leukemia through expression analysis and functional assays.

4. To develop and validate a novel *ex vivo* screening assay for the discovery of drug candidates against leukemia that are effective in the presence of human blood or bone marrow.

Results

1. The pro-MMP9 – αMβ2 integrin complex as a functional target in extramedullary infiltration of AML

1.1. Elucidation of the interaction between pro-MMP9 and αMβ2 integrin and identification of specific inhibitors of this complex

The laboratory of Dr. Erkki Koivunen has previously shown that the proMMP-2 and proMMP-9 gelatinases bind to β2 integrins (Stefanidakis M, *et al.* 2004), and identified peptides that bind to the interfaces of this complex. In brief, a phage display random peptide library (a pool of $CX_{7\text{-}10}C$ and $X_{9\text{-}10}$) was screened against the purified αM I domain of integrin that led to the identification of several peptides with a motif (D/E)(D/E)(G/L)W (Stefanidakis M, *et al.* 2003). It was subsequently found via use of bioinformatic tools that these peptide sequences are naturally found in some of the known αMβ2 integrin ligands as well as in the catalytic domain of MMPs. Correspondingly, the peptide sequences sharing the (D/E)(D/E)(G/L)W motif were shown to mediate the binding between αMβ2 integrin and proMMP-2 or proMMP-9 (Stefanidakis M, *et al.* 2003). From these sequences, the ADGACILWMDDGWCGAAG (from here on referred to as DDGW) peptide was chosen for further studies as it showed the strongest binding to integrin (αM I domain) and was highly soluble in aqueous buffers (Stefanidakis M, *et al.* 2003). To further elucidate the interaction between αMβ2 integrin and proMMP-9, the whole proMMP-9 sequence was synthesized as overlapping 20-mer peptides on a pepspot membrane and binding of the αM I domain was assessed for each of these peptides individually. A single active peptide that located to the MMP-9 catalytic domain, QGDAHFDDDELWSLGKGVVV, was identified (Stefanidakis M, *et al.* 2003) that also contained the (D/E)(D/E)(G/L)W motif, which was initially selected via phage display. Additional screening by the pepspot system, by truncating the 20-mer to overlapping hexapeptides, identified that sufficient integrin-binding activity is achieved by the HFDDDE peptide (Stefanidakis M, *et al.* 2004). Likewise, a novel small molecule, IMB-10, inhibiting the interaction between β2 integrin and proMMP-9 was discovered by allowing a chemical library to compete with the binding of the phage displaying the DDGW peptide to αM I domain of integrin (Björklund M, *et al.* 2006).

In this study, we sought to further elucidate the binding interaction between proMMP-9 and αMβ2 integrins by using two separate truncated MMP-9 domains, (i) the Δ proMMP-9 domain containing the pro-domain and the catalytic domain that contains the collagen binding domain (Overall CM. 2002) composed of three fibronectin type II repeats, and (ii) the C-terminal hemopexin/vitronectin-like domain (PEX), which contains the binding site for tissue inhibitors of MMPs (TIMPs) and is responsible of the dimerization of MMP-9 (Cha H, *et al.* 2002). Both of these domains were able to bind to OCI-AML3 cell surfaces (AML cell line), as shown by ^{125}I-labeled MMP-9 domains, although Δ proMMP-9 domain binding was more prominent (**I, Fig. 6A**). We showed that the cell surface binding of ^{125}I-labeled Δ proMMP-9 domain to OCI-AML3 cells was inhibited with specific α M and α L antibodies (namely TS1/22 and MEM170) as well as with soluble integrin I domain (**I, Fig. 6B**). Similar effects were observed with the peptides HFDDDE and DDGW, as well as CTTHWGFTLC, a peptide that targets the catalytic site of the MMP-9 (Koivunen E, *et al.* 1999), but not with CRVYGPYLLC, a peptide that targets the hemopexin domain of MMP-9 (Björklund M, *et al.* 2004) (**I, Fig. 6B**). Moreover, the anti-MMP9 antibody generated against the sequence YQGDAHFDDDE (referred to as anti-HFDDDE), but not the IgG control, inhibited the Δ proMMP-9 domain binding (**I, Fig. 6B**). Conversely, the cell surface binding of ^{125}I-labeled PEX-domain was inhibited by tissue inhibitor of metalloproteinase (TIMP) – an endogenous MMP inhibitor – and the CRVYGPYLLC peptide, but not by proMMP-9 or α Mβ2 integrin targeting peptides, integrin antibodies, nor I domain (**I, Fig. 6C**). Genetic silencing of the α M with siRNA provided further evidence that α Mβ2 integrin serves as the receptor for the Δ proMMP9 domain (**I, Fig. 6D & 6F**). Furthermore, HFDDDE and DDGW peptides

that had been injected intravenously into Balb/C mice increased the serum levels of MMP-9, suggesting that both peptides prevent cell-surface binding of proMMP-9 also *in vivo* (**I, Fig. 4F**).

These results indicate that both the α M β 2 and α L β 2 serve as receptors for Δ proMMP-9 domain, but not for the PEX domain, and that this interaction is specifically inhibited by the agents targeting the proMMP-9 – β2 integrin complex, such as HFDDDE, DDGW, and IMB-10.

1.2. The expression and co-localization of pro-MMP9 and β2 integrin in AML

In the BRASIL assay (Giordano RJ *et al.* 2001) phage displaying the DDGW peptide bound strongly to OCI-AML3 cells (**I, Fig. 1A**) as well as to bone marrow samples derived from AML patients (**I, Fig. 1B**), suggesting that the interaction between αM β 2 integrin and proMMP-9 also occurs in primary leukemia cells. Flow cytometry was used to assess the expression levels of both MMP-9 and β2 integrin and its subunits. OCI-AML3 cells were treated with phorbol ester, as stimulation of neutrophils by phorbol ester is known to induce the cell surface expression the proMMP9 – αMβ2 integrin complex (Stefanidakis M, *et al.* 2004). In addition, OCI-AML3 cells that had been grown in the presence of an endothelial cell monolayer showed higher levels of both MMP-9 as well as β2 integrin expression as compared to the cells grown in the absence of endothelial cell monolayer (**I, Fig. 1E**). Interestingly, the expression of αL integrin was relatively high on the cell surface even without stimulation, whereas the expression of αM was low and became detectable only after stimulation (**I, Fig. 1E**). Confocal microscopy was subsequently used to analyze the colocalization of MMP-9 and β2 integrin. Colocalization of proMMP-9 was detected with both αL and αM integrin subunits (**I, Fig. 1C**). In addition, when OCI-AML3 cells were cocultured on top of an endothelial cell layer and stimulated with phorbol ester, the colocalization of proMMP-9 and αM β 2 integrin occurred in apparent contact areas between leukemia cells (**I, Fig. 1D**).

These results suggest that the interaction between proMMP9 and αM β 2 integrin may also play a role in primary leukemia cells and that the complex appears to be concentrated at specific interaction sites upon stimulation of leukemia cells.

1.3. Inhibition of the pro-MMP9 – αM β 2 integrin complex exhibits anti-leukemia activity

The ability of DDGW peptide to recognize primary AML cells lead us to study the effects of the proMMP9 – αM β 2 integrin complex in xenograft murine models. When OCI-AML3 cells were grown subcutaneously in nude mice, both the DDGW peptide and HFDDDE peptide, but not the corresponding control peptides, markedly decreased the growth of extramedullary leukemia (**I, Fig. 2A**). The overall tumor volume was significantly reduces with both peptides. When mice were treated with the DDGW peptide the tumor response was significant for upto 20 days, however, with HFDDDE peptide, the tumor response was significant at early (20 days) and later (120 days) time points (**I, Fig. 2B and 2C**). Significant increase in actuarial survival was detected with both peptides (**I, Fig. 2D and 2E**). Markedly, discontinuation of the DDGW treatment, but not the HFDDDE treatment, resulted in leukemia regrowth. Neither of the peptides induces weigh loss or other adverse effects at the doses used. Similar results were obtained in nude mice bearing THP-1 xenografts (**I, Fig. 3A**). Anti-leukemia activity of the proMMP9/αM β 2 integrin complex inhibitors, HFDDDE and IMB-10, was also observed in the long-term *in vitro* cultures of both OCI-AML3 cells and patient derived primary AML cells (**I, Fig. 5E and 5F**).

1.4. Effects of the pro-MMP9 – β2 integrin complex inhibitors on extramedullary invasion and pericellular proteolysis of AML cells

Next, we studied whether the inhibition of proMMP9 – αMβ2 integrin complex would affect transendothelial migration of OCI-AML3 cells. Both the HFFDDE and DDGW peptides as well as the small molecule IMB-10 inhibited the transmigration (**I, Fig. 5A**). Similar results were obtained in primary AML

samples with HFDDDE and IMB-10 in transmigration assay with collagen (**I, Fig. 5C**). Moreover, small interfering RNA (siRNA) against αMβ2 and/or proMMP-9 significantly inhibited the transendothelial migration as compared to the control siRNA (**I, Fig. 5B**), suggesting that the complex may play an important role in extravasation.

To obtain further evidence that the proMMP9 – αMβ2 integrin complex is important for the extravasation of AML cells, we assessed whether the inhibition of the proMMP9 – αMβ2 integrin complex reduces the infiltration of leukemia cells to other commonly invaded extramedullary organ sites, such as liver and spleen *in vivo*. We studied the tissue distribution of the ^{125}I-surface-labeled OCI-AML3 cells (injected intravenously) with or without the HFDDDE and DDGW treatment in Balb/c mice. Treatment with both HFDDDE and DDGW caused an increase in cell pool remaining in the peripheral circulation (**I, Fig. 4A-C**). Notably, HFDDDE was more active than DDGW, and HFDDDE significantly inhibited the accumulation of leukemia cells into bone marrow cavity, liver, and spleen (**I, Fig. 4A**). In addition, both DDGW and HFDDE inhibited leukemia cells infiltration to lungs (**I, Fig. 4C**) Moreover, a treatment with the anti-HFDDDE antibody resulted into similar outcome while the control IgG had no effect (**I, Fig. 4D**).

Next, we sought to assess whether the inhibition of the proMMP9 – αMβ2 integrin complex also affects pericellular proteolysis. The HFDDDE peptide was shown to inhibit gelatinolysis as efficiently as the specific MMP-9 targeting peptides, CTTHWGFTLC and CRVYGPYLLC (**I, Fig. 7A**). Moreover, the endogenous cell surface proteins were biotinylated and their stability was assessed in the presence of the HFDDDE peptide and the control peptide DFEDHD. More biotinylated cell surface proteins were recovered from the cell cultures treated with the HFDDDE peptide compared with the DFEDHD peptide as shown via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**I, Fig. 7B**) as well as via streptavidin-phycoerythrin flow cytometry (**I, Fig. 7C**). Integrin β 2 subunit was among the stabilized proteins with the HFDDDE treatment but not with the DFEDHD treatment (**I, Fig. 7C**). Similar results were obtained with the DDGW peptide (**I, data not shown**). Moreover, the degradation of [^{3}H] proline-labeled endothelial cell layer by OCI-AML3 cells was inhibited by the HFDDDE peptide and the CTTHWGFTLC peptide as measured via release of the radioactive label (**I, data not shown**). The control peptides had no effects.

These data show that the proMMP-9 – αMβ2 integrin complex is required for both extravasation as well as for pericellular proteolysis, and that the inhibition of this complex decreases extramedullary infiltration and gelatinolysis. This results into increased numbers of circulating leukemia as well as increased stability of cell surface proteins.

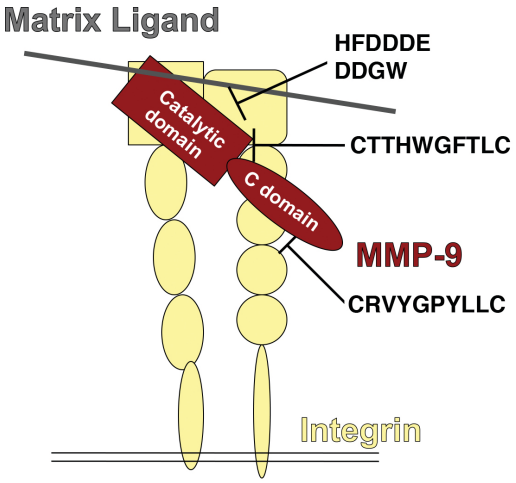

Figure 4. The invadosome complex and its peptide inhibitors.

2. Cell surface profiling of leukemia cells via phage display random peptide libraries: NRP-1 and IL-11Rα as potential therapeutic targets in leukemic bone marrow

2.1. Identification of NRP-1 and IL-11Rα binding peptides

We used phage display random peptide library (CX_7C) for cell surface profiling of leukemia cells (MOLT-4) via BRASIL methodology (Giordano R, *et al.* 2001). Phage selection was performed with an excess of the competing Arg-Gly-Asp (RGD) synthetic integrin-binding peptide motif to

minimize and eliminate the recovery of RGD-containing ligands (Christianson DR, *et al.* 2007; Kolonin MG, *et al.* 2006a). Based on the enrichment of the clones in the samples, a new consensus motif Phe-Phe/Tyr-Any-Leu-Arg-Ser ($F^F/_Y$XLRS) was identified, which accounted for 22% frequency of the selected peptide sequences. Characteristic binding patter of one the $F^F/_Y$XLRS peptide-displaying phages, specifically CGFYWLRSC peptide-displaying phage, on different leukemia cell lines was further studied and the phage was shown to bind to all (n=8) the leukemia/lymphoma cell lines tested (**II, Fig. 1A**). As the CGFYWLRSC peptide-displaying phage was internalized by leukemia cells (**II, Fig. 1B**), thus having a potential to be used in ligand-directed drug delivery approaches, we investigated it further.

The similarity studies via online protein databases (NCBI, BLAST) revealed weak non-linear matches with proteins such as VEGF-165, plexin A1 and B1, all of which are known to interact with neuropilin-1 (NRP-1). We therefore tested the binding of CGFYWLRSC peptide-displaying phage with a recombinant NRP-1 protein, and found it to be significant. (**II, Fig. 2A**). Insertless phage as well as an unrelated peptide-displaying phage served as negative controls. In addition, when NRP-1 was specifically knocked-down in OCI-AML3 cells via RNAi, no binding of the CGFYWLRSC peptide-displaying phage on these cells was observed, while the wild-type cells as well as the cells treated with the control siRNA maintained the binding of this phage (**II, Fig. 2E**). These studies indicate that NRP-1 is a functional target of the CGFYWLRSC peptide sequence, and the enrichment of the CGFYWLRSC peptide-displaying phage on leukemia/lymphoma cells lines suggests that NRP-1 may be a promising candidate for targeted therapy approaches in leukemia.

In another study, a cyclic phage-display random peptide library (CX_7C) was intravenously injected to a brain-dead cancer patient (a 48-year old Caucasian male) who had previously been diagnosed with a B-cell malignancy (Waldenström macroglobulinemia) and treated via splenectomy, systemic chemotherapy, and immunotherapy (Pentz RD, *et al.* 2003 & 2005; Arap W, *et al.* 2002b). The family members signed the informed consent for these studies. After 15 min infusion, tissue biopsies were collected, and phage was subsequently recovered and sequenced from the various tissues (Arap W, *et al.* 2002b). High-throughput pattern recognition analysis of the motif similarities were performed in order to find common sequences shared among multiple selected peptides in a target organ (Arap W, *et al.* 2002b). As expected, both ubiquitously binding motifs as well as organ specific motifs were identified. For instance, AGG was found to be a common tripeptide motif in prostate tissue. One of the selected peptides sharing this common motif was the GRRAGGS peptide that was found to mimic interleukin-11 (IL-11). Further studies validated that this IL-11 mimic peptide interacts with the IL-11 receptor alpha (IL-11Rα), therefore confirming the GRRAGGS peptide and the IL-11Rα form a functional ligand-receptor pair (Arap W, *et al.* 2002b). The follow-up study showed that the GRRAGGS peptide mimicked a new functional protein-binding site within IL-11 (Cardo-Vila M, *et al.* 2008).

A study with a large cohort of human tissues revealed that the expression of IL-11Rα is increased in prostate cancer, particularly in metastatic prostate cancer, and in the associated vasculature (Zurita AJ, *et al.* 2004). Furthermore, the subsequent work done by our group showed that IL-11Rα is strongly expressed by both tumor cells and tumor vasculature in human primary osteosarcoma and pulmonary metastasis, while no expression was observed in normal lung and bone marrow tissues (Lewis VO, *et al.* 2009). The potential of the CGRRAGGSC peptide to be utilized in targeted drug delivery approaches was also demonstrated by therapeutic targeting of prostate cancer via delivery of the pro-apoptotic peptide d(KLAKLAK)2 into the cells (Cardo-Vila M, *et al.* 2008; Zurita AJ, *et al.* 2004; Ellerby HM, *et al.* 1999) .

Given the upregulation of IL-11Rα in bone metastatic prostate cancer and osteosarcoma as well as the fact that the IL-11-mimic peptide was identified via screening a patient with B-cell malignancy, we hypothesized that IL-11Rα might also be expressed by leukemia cells.

2.2. Expression of NRP-1 and IL-11Rα in leukemia cell lines and leukemic bone marrow

To confirm the expression of NRP-1 on leukemia we studied the expression of these proteins on cell lines via flow cytometry. The expression of NRP-1 and IL-11Rα was confirmed in K562, OCI-AML3, and MOLT-4 cell lines (**II, Fig. 2B-D; III, Fig. 1A**).

Consistently with the result obtained in leukemia cell lines (**II, Fig. 1A**), the CGFYWLRSC peptide-displaying phage also bound to patient derived AML and ALL samples (**II, Fig. 5A**) suggesting that NRP-1 is also expressed by primary leukemia cells. Immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded bone marrow samples from patient with AML and ALL confirmed the expression of NRP-1 in the patient derived ALL (n=6) and AML (n=24) bone marrow (**II, Fig. 5B & C**) while only a weak background expression was detected in bone marrow specimens from healthy donors (n=7) (**II, Fig. 5B & C**). A slightly higher NRP-1 expression level was observed in ALL versus AML bone marrow samples (**II, Fig. 5B; Supplemental Fig. 1B**). Similar discrepancy in expression levels of ALL versus AML was subsequently confirmed by others (Younan S, *et al.* 2012). Notably, the NRP-1 expression did not appear to be cell population specific, but rather was generally over-expressed in leukemic bone marrow (**II, Supplemental Fig. 1**), perhaps as a response to hypoxic microenvironment. Notably, bone marrow blast percentage was later shown to correlate with increased NRP-1 expression, with the level being higher in those with bone marrow blast ≥90% (Younan S, *et al.* 2012).

The cell surface expression of IL-11Rα was demonstrated via flow cytometry in a panel of leukemia and lymphoma cell lines (n=12) (**III, Fig. 1A**), as well as in different hematopoietic cells types derived from both healthy and leukemic tissues (**III, Fig. 1B & Supplemental Fig. 1**). All the leukemia and lymphoma cell lines tested stained positive. Also, the expression of IL-11Rα in a total cell population was increased in all the patient AML samples tested (3/3) compared to the normal bone marrow or peripheral blood from healthy donors (3/3) (**III, Fig. 1B & Supplemental Fig. 1**). Analysis of specific cell populations showed that IL-11Rα was mainly expressed on the surface of CD34+, CD33+ and CD14+ cells in the AML patient samples, with the exception of one sample staining positive also with the CD19+ cells (**III, Fig. 1B & Supplemental Fig. 1**). In addition, cell membrane expression of IL-11Rα was detected via cytospin analysis of purified CD34+ cells derived from patients with AML, where one out of two cases stained positive, and from healthy individuals, where both tested cases stained negative (**III, Supplemental Fig. 2 & data not shown)**.

Next, we studied the levels of IL-11Rα in formalin-fixed, paraffin-embedded bone marrow samples from AML patients (n=43) via IHC (**III, Fig. 2 & Table 1**). A strong expression was observed in a CLL bone marrow samples (n=2) (**III, Fig. 2 & Table 1**), which served as positive controls as the IL-11Rα expression in CLL has already been reported (Tsimanis A *et al.* 2001). The leukemic bone marrow from AML patients had a significantly higher overall expression of IL-11Rα, compared to normal bone marrow (Fisher's exact test, p=0.0448). Both leukemic blasts as well as vasculature contributed to the elevated expression levels, and over half of the leukemia blasts (23 of 43, 53%), and practically all the evaluable blood vessels (16 of 17, 94%) – regardless of subtype and corresponding blast cell positivity – stained positively. Importantly, the normal vasculature from healthy bone marrow donors did not have detectable expression of IL-11Rα (Fisher's exact test, p=0.0158). Specifically, 99% of the AML bone marrow samples tested had a significant disease involvement with more than 20% of white blood cells (WBCs) being leukemic blasts, and in most of the AML bone marrow samples tested (24 of 33), the disease involvement was higher than 40%, from which several cases (16) had over 60% blast percentage. In addition, evidence for the specificity of IL-11Rα expression only in bone marrow specimens with significant disease involvement, was also obtained via staining the bone marrow samples with MDS with a partial disease involvement, where most of the tested cases (5 of 6) stained negative. Only two of these cases (cases 12 & 13) had higher than 10% disease

involvement, and from these only one (case 13) stained positive.

Taken together, these results show that both NRP-1 and IL-11Rα are expressed in leukemia cell lines as well as in patient derived leukemic bone marrow samples. Thus both NRP-1 and IL-11Rα could potentially be utilized in targeted drug delivery approaches in leukemia.

2.3. Targeted delivery of pro-apoptotic peptidomimetics to leukemia cells via NRP-1 and IL11Rα binding peptides

To assess the possibility of using NRP-1 and IL-11Rα as targets for systemic delivery of therapeutics into leukemic bone marrow, we evaluated the ability of NRP-1 and IL-11Rα binding peptides to serve as vehicles for targeted drug delivery.

NRP-1 and IL-11Rα binding peptides were synthesized in tandem with a pro-apoptotic moiety, $_D$(KLAKLAK)$_2$ – an amphipathic peptidomimetic that induces apoptosis by physical interference and disruption mitochondrial membranes upon internalization by target cells (Ellerby HM, *et al.* 1999) – and the cytotoxic effect on malignant cells were studied *in vitro*.

Relatively low concentrations (5 μM - 30 μM range) of the NRP-1 targeting peptide, CGFYWLRSC-GG-$_D$(KLAKLAK)$_2$, were sufficient to significantly decreased cell viability in human leukemia and lymphoma cell lines, and in majority of cell lines the maximum inhibition was reached at ≤ 20 μM (**II, Fig. 3**). The control peptides [an admixture of CGFYWLRSC + $_D$(KLAKLAK)$_2$] had no effect at equimolar concentrations. Similar results were obtained with patient derived leukemic bone marrow samples, although with slightly higher IC_{50} values (~30 μM) (unpublished data, AACR Annual Meeting Abstract, 2012). In addition, no apparent cytotoxicity on mononuclear cells from normal bone marrow was observed with the CGFYWLRSC-GG-$_D$(KLAKLAK)$_2$ (upto 30 μM) (unpublished data, AACR Annual Meeting Abstract, 2012).

The IL-11Rα targeting peptide, CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$, also decreased cell viability at relatively low concentrations (40 μM - 100 μM range) in human leukemia and lymphoma cell lines, and in most of the cases the maximum inhibition was reached at 60-80 μM (**III, Fig. 3 & Supplemental Fig. 3**). The control peptides [an admixture of CGRRAGGSC and $_D$(KLAKLAK)$_2$] had no effect at equimolar concentrations. Similarly, no apparent cytotoxicity on mononuclear cells from normal bone marrow was observer with the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide (upto 100 μM) (**III, Fig. 4B**).

To further improve the drug efficacy of the BMTP-11 we designed and evaluated four analogs of this drug with modified structures through Merrifield synthesis and chemical lead-optimization (**III, Supplemental Table 1**). One derivative, BMTP-11 analog #4 (BMTP-11A#4), which was myristoylated on its serine residue, showed an improved anti-leukemia activity as assessed via cell viability and proliferation assays with leukemia cell lines (**III, Fig. 5A**).

Furthermore, the clonogenic potential of patient derived AML cells was assessed via methylcellulose assays and statistically significant inhibition was obtained at 100 μM concentration for CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ and at 30 μM with the analog #4 (**III, Fig. 5C**), while the clonogenic potential of normal bone marrow cells from healthy individuals was not altered at equivalent concentrations (**III, Fig. 5D**). The concentrations used for these peptides in the clonogenic assay were selected based on their approximate maximal inhibition concentration values that were obtained via the cell viability and proliferation assay done with leukemia cell lines (**III, Fig. 5A**).

The cells were further analyzed by AnnexinV and propidium iodide staining to assess the extent of cell death induced by the NRP-1 targeting CGFYWLRSC-GG-$_D$(KLAKLAK)$_2$ peptide as well as with the IL-11Rα targeting CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide and its analog (**II, Fig. 4; III Fig. 4D & 5B**). The OCI-AML3 cells treated with 20 μM of the CGFYWLRSC-GG-$_D$(KLAKLAK)$_2$ peptide for 20 h had undergone a nearly complete cell death induction (> 95% of the cells) (**II, Supplemental Table 1**) while the cells

treated with the control peptides [an admixture of CGFYWLRSC + $_D$(KLAKLAK)$_2$ at equimolar concentrations] had only background levels of cell death, which was similar to those observed in untreated control cells (4-6% of the cells) (**II, Supplemental Table 1**). The concentration-dependent cell death of OCI-AML3 cells was observed with the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ after incubation for 20 h, and nearly a complete cell death induction was observed at 40 μM or higher (>87 % of the cells), while the cells treated with the control peptides [an admixture of CGRRAGGSC and $_D$(KLAKLAK)$_2$ at equimolar concentrations] had only background levels of cell death, which was similar to those observed in untreated control cells (4-6% of the cells) (**III, Fig. 3D**).

These data suggest a potential therapeutic window for our new drug candidates and confirm that they induce targeted cell death upon internalization into leukemia cells. Both of the drug candidates may thus have therapeutic utility in leukemia.

3. Development and validation of a novel *ex vivo* screening assay for leukemia: Identification of new candidate drugs against leukemia

3.1. Optimization of the *ex vivo* assay

Stability and efficacy of a drug candidate can vary significantly depending on its microenvironment. *Ex vivo* models utilizing human tissue as a screening platform are therefore valuable preclinical tools as the species-specific disease microenvironment can be partially recapitulated. Thus, we rationalized that co-culturing leukemia cells with the components of their natural microenvironment, such as blood or bone marrow, would provide beneficial elements for a drug-screening assay. We searched for conditions where leukemia cells could proliferate in the presence of whole blood obtained from healthy volunteer donors. A dilution of 1:10 (vol/vol) of whole blood in a culture medium without serum was optimal for leukemia cell growth for 24 to 48 h. The oxygen level was set to 5%, to mimic the physiological partial pressure of oxygen in peripheral circulation. Several leukemia cell lines were tested (namely: K562, OCI-AML3, Molt-4, THP-1, Kasumi-1), and all of them were able to grow in these conditions. Interestingly, during the optimization, we noticed that leukemia cells induced the color of diluted blood medium to turn from bright scarlet red to dark red during an overnight incubation. Instead, the color change in the cultures without leukemia cells was not significant. Notably, the blood color switch from bright red to dark red was reversible when allowed to stay at normoxia (21% oxygen), and shaking accelerated the process. This suggested that leukemia cells depleted oxygen in the cultures faster than normal cells, causing a shift in hemoglobin's oxygenation state. (**IV, Fig.1**).

Time-dependent increase of white blood cell counts due to proliferation of leukemia cells (OCI-AML3 cells) in the blood-containing cultures was assessed via a blood count analyzer (**IV, Fig. 2A**). In addition, leukemia cell growth was assessed via three independent cell viability and proliferation assays, which measured (i) cellular lactate dehydrogenase activity, (ii) transfected luciferase activity, (iii) BrdU incorporation (**IV, Fig. 2B-D**). Leukemia cell growth above background became detectable after culturing for 20 h or longer. We used whole blood samples from more than 200 individual healthy donors, and all of them supported leukemia cell growth in these conditions.

Since the color change of blood also served as a "built-in" indicator or leukemia cell viability in these cultures, we used hemoglobin's absorption spectra to quantify the change in the oxygenation state. Measurement of absorbance at 600 nm (or optical density 600, OD_{600}) was found to be optimal in these culture conditions, and the OD_{600} measurements were remarkably higher in blood medium with leukemia cells cultured in hypoxia versus normoxia after 40 h (**IV, Fig. 2E**). Specifically, the OD_{600} of cultures without leukemia cells increased less than 0.2 units after incubation for 40 h, while in the cultures with leukemia cells OD_{600} increased by 0.4 to 0.6 units (**IV, Fig. 2E**). Background levels, as determined by measuring the OD_{600} baseline levels at 0 h, varied only slightly (mean 1.8, range 1.7-1.9). Moreover, upon addition of oxygen to the leukemia-cell containing cultures, which had prior been grown under hypoxia for 20 h or more, the

blood's color switched back to bright red and the OD_{600} values were practically identical with the oxygenated cultures grown under normoxia. These results indicated that leukemia cells were responsible of inducing the color change due to leukemia cells depleting the oxygen from the culture medium under hypoxic conditions. Notably, leukemia cell growth rate was similar in both hypoxic and normoxic conditions (**IV, Fig. 2F**), thus excluding the possibility that differences in cell growth rates per se were responsible of the color switch and higher OD_{600} values. In addition, we tested whether leukemia cell growth in human bone marrow aspirates from healthy donors is possible and whether we could detect the color change compared to control bone marrow cultures. We established that similar results to medium containing peripheral blood could be achieved in the cultures containing bone marrow (**IV, Fig. 2G-I**).

To assess whether we could use this assay to detect an anti-leukemia activity, we studied the effects of etoposide – a topoisomerase II inhibitor known to be active against leukemia – and confirmed that sufficient window for detection existed (**IV, Fig. 2J**).

The dependence of OD_{600} values on leukemia cell (OCI-AML3 cells) viability and growth was confirmed by correlating OD_{600} values with WBC counts in two different donor blood samples in the presence and absence of many anti-leukemia compounds. Markedly, the blood count analyzer recognizes leukemic blasts as granulocytes and monocytes, respectively. In accordance, the most robust correlation with OD_{600} was observed with granulocytes (r=0.81 and r=0.79) and monocytes (r=0.66 and r=0.65) (**IV, Fig. 3**). In conclusion, the OD_{600} measurement can thus be reliably used as an initial indicator of leukemia cell viability and/or growth.

We used a large panel of individual whole blood samples (n>200) from healthy donors and all of them supported the growth of index leukemia cell lines (**IV, data not shown**). Under this short-term *ex vivo* assay conditions, we observed no detectable evidence of classical host versus graft reaction on the basis that neither the immunosuppressant cyclosporine A nor the immunostimulator interleukin-2 affected our results (**IV, data not shown**). However, one cannot entirely exclude the possibility that innate immune reactions could occur in the blood samples as leukemia cell growth may perhaps be retarded relative to standard *in vitro* culture conditions. Despite this technical caveat, human leukemia cells were able to overcome this potential obstacle and proliferate (**IV, Fig. 2A-D**).

3.2. Screening of a small molecule compound library

As a proof-of-concept, we screened a chemical library (size ~20,000) against human leukemia cells via the *ex vivo* assay. Specifically, we used the DIVERSet™ chemical library from ChemBridge, which provides a diverse collection of drug-like compounds for primary screening. We identified a total of 70 active compounds ("hit" rate of 0.35%) that decreased OD_{600} by at least 0.2 units (**IV, data not shown**). Further re-examination of an arbitrarily chosen subset of these compounds (n=20, equaling ~30% of the initial "hits") revealed that 18 of 20 of compounds retained their anti-leukemia activity also in the absence of blood, and thus were reproducibly true positives, resulting into a hit-rate of 90% (18/20).

The majority of the identified 70 compounds were novel and had not been previously published. Interestingly, however, some of the compounds selected were identical or structurally similar to compounds described in the literature, and many of these compounds were reported with potential anti-cancer, anti-inflammatory, or anti-microbial activity (**IV, data not shown**). A few examples include N''-{4-[(4-bromo-2,3,5,6-tetramethylbenzyl)oxy]-3-methoxybenzylidene}carbonohydrazonic diamide hydrochloride, a compound that has been listed as an anti-prion agent (USPTO application #: 20100029773), which inhibits protein aggregation involved in diseases linked to protein aggregation and/or neurogenerative diseases, and 1-[2-(4-biphenylyl)-2-oxoethyl]-2,6 dimethylimidazo[1,2-a]pyridin-1-ium bromide, which has also been discovered by others via screening a chemical library for compounds with activity against the intracellular protozoan parasite *Trypanosoma cruzi*

(Bettiol E, *et al.* 2009). Moreover, 5-(2,4-dihydroxybenzylidene)-2-(phenylimino)-1,3-thiazolidin-4-one identified in this study is similar to 5-benzylidene-2-phenylimino-1,3-thiazolidin-4-one (BPT) analogs, which have been reported to selectively induce apoptosis in cancer cell, independently of P-glycoprotein status (Wu S, *et al.* 2006). In addition, three identified compounds were structurally similar to 1-(5-chloro-2-methoxybenzoyl)-4-(3chlorophenyl) piperazine, which has been described as an anti-mitotic agent with anti-tumor activity (Weiderhold KN, *et al.* 2006). Moreover, certain structural moieties were common in the selected compounds, such as piperazine, hydrazide, and hydrazone derivatives. Interestingly, many piperazine derivatives are notable successful drugs, including the tyrosine kinase inhibitor Imatinib, used in the treatment of CML (Quintas-Cardama A, *et al.* 2009). Hydrazone-based coupling methods have been used in medical biotechnology to couple drugs to targeted antibodies (Wu A and Senter PD. 2005). The hydrazone-based bond is stable at neutral pH in the blood, but is rapidly degraded in the acidic environment in lysosomes. In principle, the drug is thereby released inside the cell, where it exerts its function (Wu A and Senter PD. 2005).

3.3. Validation of the discovered anti-leukemia compounds

To study the discovered compounds further, we selected four structurally different test compounds out of the 18 active compounds, namely #1, #2, #3, and #4. The activities of these compounds were detected, in addition to the OD_{600} measurement, in three different standard cell proliferation assay systems (**IV. Fig. 4A**), thus confirming that they were true anti-leukemia compounds. Structure-activity-relationship studies were subsequently performed for each of these four compounds, with the criteria that the similarity of a parent compound to its analog should be at least 50%. First, the compound analogs were screened in the *ex vivo* assay in the presence of blood to identify active analogs (**IV, Fig. 4B**), and subsequently tested in the standard cell proliferation assays (**IV, Fig. 5**). Of the four identified test compounds and their active analogs, the compound #1 (N" - {4 - [(4 - bromo - 2,3,5,6 - tetramethylbenzyl)oxy] - 3 - methoxy-benzylidene} carbono - hydrazonic diamide hydrochloride) and its analogs had the most robust anti-leukemia activity against the variety of leukemia and lymphoma cell lines tested in the standard culture conditions (**IV, Fig. 5**). This group of active carbonohydrazonic diamide-containing compounds (n=7) (**IV, Fig. 6A**) inhibited the increase both in OD_{600} and the leukemia cell counts, and all of them reduced the WBC counts to baseline levels at the 20 h time point with <0.05-0.001% probability (**IV, Fig. 6B**). The activity of the compounds, with the exception of the compound #1A, decreased significantly after the 40 h incubation (**IV, data not shown**). All the compounds, with the exception of the compound #1B, were shown to efficiently induce cell death in leukemia cells (**IV, Fig 6C**). The compound #1N, which does not contain carbonohydrazonic diamide group in its structure, served as a negative control (**IV, Fig. 6B & C**).

We next analyzed the effects of carbonohydrazonic diamide-containing compounds against different blood cell populations via the blood count analyzer at 0, 20, and 40 h time points. The increase in the leukemia cell (OCI-AML3) population in the blood-containing medium was usually ~2 fold after a 40 h incubation, and the leukemic cells were detected as granulocytes and monocytes. Therefore, the compounds that showed activities against granulocytes and monocytes while lacking the effects on other cell population were thought to primarily target leukemia cells. Subsequent analysis of compound activities against normal blood samples lacking leukemia cells may further confirm the specificity towards leukemia cells, however, proper toxicity and safety studies are subsequently needed to validate these compounds. As an example, we show the activities of the most robust compound, the compound #1A, against different cell populations in the *ex vivo* assays (**IV, Fig. 7A & B**). We conclude that the compound #1A prevented the increase in WBC concentration both in the blood and bone marrow microenvironment albeit at different concentrations; and had no clear inhibitory effect against lymphocytes, RBCs, or plates (**IV, Fig. 7A & B**).

Taken together, we showed as an example the initial validation of four arbitrarily selected

compounds that had been identified as "true hits". From these four, the compound #1 and its analogs containing the carbonohydrazonic diamide group were the most effective inhibitors of leukemia cells both in the presence and absence of blood. Markedly, the analogues, which lacked the carbonohydrazonic diamide, were inactive in the presence of blood. These results indicate that carbonohydrazonic diamide is required (Fisher's exact test, p=0.0217) for the anti-leukemia activity of this class of compounds.

3.4. The effects of carbonohydrazonic diamine-containing compounds on primary AML cells.

To validate the *ex vivo* assay in the context of patient samples, we assessed whether the increase in OD_{600} was affected by the initial cell counts, and observed that the measured OD_{600} values were increased in the presence of high blast counts (**IV, Fig. 8A**). In addition, mononuclear WBCs from the peripheral blood of AML patients were isolated and then re-inserted into the *ex vivo* cultures at concentrations which enriched the WBCs (including blasts) ~2-fold. Doubling of the WBC count resulted in a corresponding increase in the OD_{600}, and the higher OD_{600} values were observed with samples containing higher blast counts (**IV, Fig. 8D**). These results indicated that the leukemia blast counts were the major contributor to the OD_{600} values and that this culturing method could also be used with patient samples.

To test the efficacy of the drug candidates in patient samples, we tested the activities of the carbonohydrazonic diamide-containing compounds in the presence of peripheral blood obtained from AML patients. Many of the carbonohydrazonic diamide-containing compounds proved to be active also in the patient sample setting (**IV, Fig 8A**). Certain compounds (namely: #1, #1C, and #1E) had different efficacies depending on the patient sample tested, indicating that individual testing of each patient may be required to identify the best drug candidates for each patient. To confirm that the effects of the carbonohydrazonic diamide-containing compounds are specific to leukemia cells, we assessed the activities of carbonohydrazonic diamide-containing compounds in the presence of normal blood from healthy donors. In two of three normal blood samples the compounds had no statistically significant effects but in the third sample the compound #1A reduced the OD_{600} slightly, suggesting individual-specific differences in susceptibility (**Fig. 8C**).

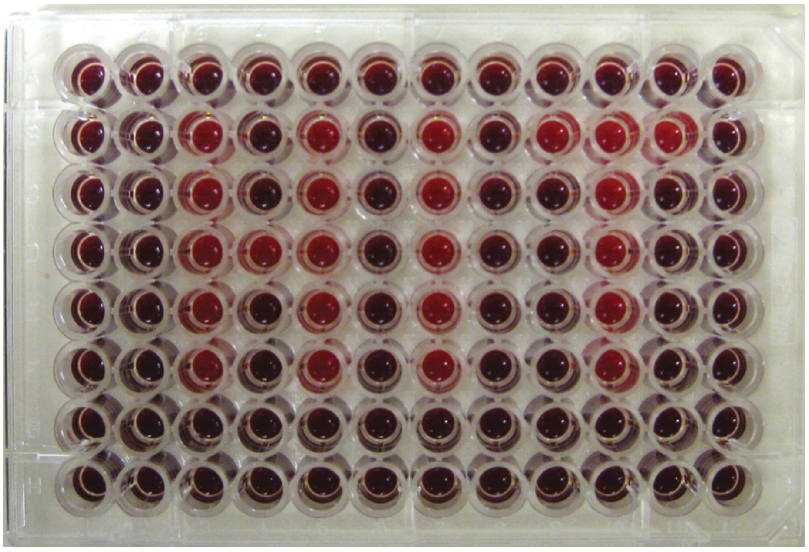

Figure 5. An example screening plate. Leukemia cell viability in a human blood-containing assay is monitored via oxygen consumption of leukemic cells, which is observed as a color change under hypoxic conditions due to depletion of oxygen from hemoglobin. Each well contains a different test compound, and the inhibition of color change from bright red to dark red indicates that the compound may have effective activity against leukemia.

Discussion

1. Identification of new ligand-receptor pairs via phage display technology for therapeutic targeting of leukemia (I-III)

Phage display random peptide libraries offer a high-throughput screening platform for identification of ligand mimics that selectively bind to cell surface markers. These target-specific binders can subsequently be utilized for ligand-directed drug delivery. For example, several ligand mimics that recognize receptors in the cell surface of lymphoma and leukemia cell lines have been reported (e.g. Binder M, *et al.* 2011; Jäger S, *et al.* 2007; McGuire MJ, *et al.* 2006; Takahaski S, *et al.* 2003; Koivunen E, *et al.* 2001). Importantly, many of the selected peptide ligands are internalized by cells and may thus be applicable for ligand-directed drug delivery. For instance, it has been demonstrated that pro-apoptotic peptides guided by the ligand mimics selected by phage display screening against tumor cell surface molecules may be exploited as "drug carriers" that target receptors in specific types of cancer and subsequently deliver the drug inside the cell (Nishimura S, *et al.* 2008; Kolonin MG, *et al.* 2006a; Zurita A, *et al.* 2004; Arap M, *et al.* 2004). Successful development of ligand-directed therapeutic targeting requires a thorough evaluation of the candidate ligand-receptor pairs in the context of the disease. In the work presented here, we report three new leukemia-specific ligand-receptor pairs discovered using the phage display technology, and evaluate their therapeutic potential. Specifically, we identified three new peptide motifs that bind to (i) a αL/αMβ2 integrin - pro-MMP9 complex (termed: the invadosome complex), (ii) NRP-1, and (iii) IL-11Rα.

1.1. Targeting the invadosome complex

The expression of matrix metalloproteinases (MMPs) is increased in several cancers and MMPs are associated in cancer-related processes such as invasion, angiogenesis, and inflammation (Hatfield KJ, *et al.* 2010). However, the broad-spectrum MMP inhibitors have not been very successful in clinical trials (Shi Z-G, *et al.* 2012). Instead, new approaches with more specific mechanisms to target individual MMPs are needed, and selective inhibition of the cell surface associations of individual MMPs, while not interfering with their catalytic activity, offers superior means to target these enzymes.

For instance, MMP-2 and -9 are known to associate with the cell surface, and directly influence cell behavior as well as activate the signaling pathways involved in many biological events such as cell growth, migration and survival (Bauvois B. 2012). Cell surface associations of proteases provide a straightforward mechanism by which cells may direct the proteolytic activity at the right location, greatly controlling cell migration and invasion (Björklund M and Koivunen E. 2005).

Multiple interaction mechanisms of MMP-2 and -9 on the cell surface have been identified. For example, MMP-9 binds with the membrane-associated form of the DNA repair protein Ku in AML cells (Monferran S, *et al.* 2004), and the interaction was shown to inhibit migration and invasion of leukemia cells (Monferran S, *et al.* 2004). In murine mammary carcinoma cells, it has been shown that CD44 provides a cell surface docking site for proteolytically active MMP-9, and that this interaction promotes tumor invasion and angiogenesis as well as cleaves latent TGF-β (Yu Q and Stamenkovic I. 2000). Similarly, MMP-9 binds with the α4β1 integrin and CD44 on the surface of B-cell lymphoma (Redondo-Munoz J, *et al.* 2008), which was shown to lead to Lyn kinase activation, STAT3 phosphorylation and up-regulated expression of the pro-survival protein Mcl-1 (a BCL-2 family member) (Redondo-Munoz J, *et al.* 2010). It has also been shown that the association of MMP-9 with CD44 activates the tyrosine kinase EGFR signaling pathways, and subsequently promotes cell migration (Dufour A, *et al.* 2010). Moreover, in lung cancer cells, binding of MMP-9 to αVβ3 integrin has been shown to induce PI3K/AKT-mediated VEGF expression and related angiogenesis (Chetty C, *et al.* 2010).

A specific association of MMP-2 and MMP-9 with cell surface integrins αLβ2 and αMβ2 has also been reported, and shown to affect the motility of leukocytes (Stefanidakis M, *et al.* 2004). While the αLβ2 integrin (also known as lymphocyte function-associated antigen 1) is expressed on all the leukocytes,

the α M β 2 integrin (also known as macrophage 1 antigen) is expressed on the surface of innate immune system leukocytes such as monocytes, macrophages, granulocytes, and natural killer cells. Importantly, the inhibition of the interaction between α L/ α M β 2 and proMMP-9 appeared to be specific to the leukocytes forming the complex, and does not interfere with the catalytic MMP-9 and MMP-2 activity in the other cell types. Interestingly, bone marrow-derived myelomonocytic cells that were double positive for α M β 2 integrin and MMP-9 were reported to be major contributors to tumor development and vasculogenesis in MMP-9 knockout mice receiving wild-type bone marrow (Ahn G-O and Brown JM. 2008).

In the present study, we show that the HFDDDE peptide, which inhibits the proMMP-9 – α M β 2 integrin complex via competing with proMMP-9 of the integrin binding site, inhibited the leukemia cell migration through endothelial cell layer *in vitro*. Accordingly, the extravasation of leukemia cells that had been injected intravenously into mice was inhibited by the HFDDDE peptide, resulting into a significant reduction of leukemia cell burden in many organs including lung, spleen, liver, and bone marrow. In addition, HFDDDE peptide reduced tumor growth *in vivo*. These data show that the proMMP-9 – α M β 2 integrin complex is important for the migration and growth of leukemia cells, and the inhibition of this interaction may have therapeutic implications.

We also show that the HFDDDE peptide inhibited pericellular proteolysis as efficiently as other previously reported specific gelatinase-inhibiting peptides CTTHWGFTLC and CRVYGPYLLC (Björklund M, *et al.* 2004). Interestingly, the β 2 integrin chain was stabilized by the HFDDDE treatment, suggesting that MMP-9 may also exert protelytic activity on its integrin binding partner. This phenomena, could also explain the low cell surface expression of α M β 2 integrin on OCI-AML3 cells as observed via flow cytometry. Consistently, others have shown that the β 2 integrin subunit is cleaved and shed by MMP-9 and that α M β 2 integrin is a substrate for MMP-9 (Vaisar T, *et al.* 2009). The steady degradation of β 2 integrins may be relevant for mobility and generation of new adhesion sites, and similar proteolytic activity may be required not only for extramedullary infiltration but also within bone marrow.

Association of MMP-9 with its α M β 2 / α L β 2 integrin partners occurs via two main mechanism. The I domain interacts with the MMP-9 catalytic domain, while the β 2 subunit interaction occurs with the C-terminal PEX domain and appears to dynamically mediate MMP9 activation and pericellular gelatinolysis (Björklund M, *et al.* 2004). It is possible that these two different interaction mechanisms between integrin α and β subunit and distinct MMP-9 domains complement each other; for instance by regulating localized proteolysis by directing MMP-9 activity where it is needed. Therefore, inhibition of the PEX domain of MMP-9 may also be required for the efficient anti-tumor effect. Future studies are needed to elucidate these points. As such, targeting the interactions between proMMP-9 and α M β 2 integrin as well as the PEX domain and its binding partners may be viable approaches to inhibit MMP-9-mediated functions.

Taken together, the agents targeting the proMMP-9 – α L/ α M β 2 integrin complex have a potent anti-leukemia effect and may have therapeutic utility in the extramedullary phenotype of AML. The inhibition of the invadosome complex may also be useful for anti-inflammatory effects, as well as for inhibiting bone marrow derived tumor-infiltrating myelomonocytes in solid tumors. However, whether the inhibition of these complexes also interferes with the anti-tumor immune effects needs to be determined.

1.2. Targeting NRP-1

NRP-1 is a transmembrane glycoprotein with a large extracellular domain that interacts with some members of the VEGF ligand family, namely $VEGF_{165}$, VEGF-B, VEGF-E, and placental growth factor (Klagsbrun M, *et al.* 2002), as well as with class 3 semaphorins, a family of secreted polypeptides with key roles in axonal guidance (He Z and Tessier-Lavigne M. 1997; Kolodkin AL, *et al.* 1997). NRP-1 is

mainly expressed by neurons and endothelial cell (Staton CA, *et al.* 2007) and it has an essential role during normal vascular and neuronal development (Kawakami A, *et al.* 1996; Herzog Y, *et al.* 2001; Gu C, *et la.* 2003) by acting as a coreceptor with VEGFR and plexins, the transmembrane receptors of the semaphoring family (Takahaski T, *et al.* 1999; Neufeld G, *et al.* 2002). In addition, NRP-1 has been shown to interact with a number of heparin-binding growth factors (West DC, *et al.* 2005), as well as to be a mediator in the primary immune responses (Wulfing C and Rupp F. 2002; Trodjman R, *et al.* 2002), and up-regulation of the NRP-1 expression is achieved as a response to tissue injury or hypoxic conditions (Brusselmans K, *et al.* 2005; Matthies AM, *et al.* 2002). NRP-1 is highly expressed in diverse solid tumors and increased expression of NRP-1 has been correlated with tumor growth and vascularization *in vivo* and with invasiveness in human cancer (Pan Q, *et al.* 2007; Hansel DE, *et al.* 2004; Latil A, *et al.* 2000; Bachelder RE, *et al.* 2001). Furthermore, beyond its role during normal vascular and neuronal development and tissue repair, NRP-1 expression has also been reported in placenta and heart in normal adult tissues (Soker S, *et al.* 1998). However, targeting of NRP-1 *in vivo* seems to be an appropriate approach for cancer therapies as indicated by studies with mouse models (Sugahara KN, *et al.* 2010; Teesalu T, *et al.* 2009). This may be due to the generally high NRP-1 expression in cancer cells compared to normal tissue, thus resulting in preferential homing of the targeting peptide into tumors.

Correspondingly, the increased anagiogenesis and expression of NRP-1 in CML and AML patients have been reported (Padró T, *et al.* 2000; Hussong JW, *et al.* 2000; Vales A, *et al.* 2007; Yamada Y, *et al.* 2003). In the present study, we found that the CGFYWLRSC phage binding to bone marrow samples derived from leukemia patients correlated with cell-surface expression levels of NRP-1. In addition, the NRP-1 expression was significantly higher in the bone marrow samples obtained from patients with ALL and AML as compared to normal bone marrow samples obtained from healthy donors. In addition, we noticed that bone marrow samples from ALL patients showed a slightly higher median expression level of NRP-1 than samples from AML patients, suggesting that NRP-1 expression might be elevated in ALL as compared to AML. Others have subsequently obtained similar results, confirming that NRP-1 expression is higher in leukemic bone marrow versus normal bone marrow, as well as in ALL versus AML bone marrow (Younan S, *et al.* 2012). To study the expression pattern of NRP-1 in more detail, we analyzed the NRP-1 levels at the cell population level and noticed that NRP-1 expression was increased in several different cell populations obtained from leukemic bone marrow. Thus, NRP-1 appeared to be expressed generally in leukemic bone marrow. As leukemia progresses and leukemia blast numbers increase, the bone marrow microenvironment becomes more hypoxic. Increased hypoxia is known to promote VEGF as well as NRP-1 expression, leading to increased cell survival (Brusselmans K, *et al.* 2005). Accordingly, NRP-1 expression levels have been reported to significantly correlate with the blast percentage higher than 90% in both AML and ALL (Younan S, *et al.* 2012). Also, another study showed that NRP-1 expression correlated with the percentage of blasts both in peripheral blood and bone marrow of AML patients (Lu L, *et al.* 2008). It is therefore feasible that the increased NRP-1 expression correlates with the disease progression in leukemic bone marrow, similarly as reported in invasive solid tumors, in which NRP-1 staining increases progressively in intensity and area of expression during histological progression from low-grade to high-grade lesions (Hansel, DE. *et al.* 2004). Markedly, Younan *et al.* showed that NRP-1 expression was diminished in the ALL and AML patients who had undergone a CR as compared to the prevalent high levels in the patients who did not go into CR (Younan S, *et al.* 2012), suggesting that NRP-1 levels might in fact serve as an indicator for disease progression and severity. Similarly, *in vivo* administration of VEGF was able to induce AML progression in a systemic mouse model, whereas a soluble form of NRP-1 significantly inhibited this progression (Schuch G, *et al.* 2002). In addition, increased NRP-1 expression has been reported to correlate with poor survival in AML patients (Kreuter M, *et al.* 2006). Taken together, these results indicate that NRP-1 is a valid target in leukemia, and that the NRP-1 levels correlate with the disease

progression and severity.

We next evaluated the drug delivery potential of the novel NRP-1-binding sequence CGFYWLRSC. In order to study the cytotoxic effect of NRP-1 targeting against leukemia cells *in vitro*, we synthesized the CGFYWLRSC peptide in tandem to a pro-apoptotic moiety, $_D$(KLAKLAK)$_2$, which is an amphipathic peptidomimetic that induces apoptosis by physical disruption of mitochondrial membranes upon internalization by target cells (Ellerby HM, *et al.* 1999). Importantly, $_D$(KLAKLAK)$_2$ is not able to penetrate into cells on its own and requires an appropriate drug delivery system in order to internalize into the cells and induce apoptosis. In fact, our results show that both leukemia and lymphoma cell viability was inhibited by using the CGFYWLRSC-GG-$_D$(KLAKLAK)$_2$ peptide – in which the glycinylglycine (Gly-Gly) linker was added as a spacer to prevent steric hindrance – while $_D$(KLAKLAK)$_2$ alone did not have such an effect. Moreover, we did not detect any effect on leukemia cells when the CGFYWLRSC peptide was used alone, indicating that the targeting peptide itself did not have any intrinsic anti-tumor activity. On the contrary, another NRP-1 binding peptide reported by others has been shown to induce apoptosis on its own (Barr MP, *et al.* 2005). The advantage of the CGFYWLRSC peptide is, however, that it can be used with several different cytotoxic drug candidates, (KLAKLAK)$_2$ being just an example, thus leaving room for adjustments if any adverse effects were to be obtained with a particular drug candidate. For instance, our ongoing independent pre-clinical studies on (KLAKLAK)$_2$ have shown that the renal clearance of D-residues from the $_D$(KLAKLAK)$_2$ motif is a dose-limiting factor due to nephrotoxicity, however, this toxicity is reversible and is unrelated to the specific targeting peptide (Pasqualini R, *et al.* 2015). In addition, this new ligand-receptor interaction between NRP-1 and the CGFYWLRSC peptide can also be utilized in other drug delivery systems targeting human leukemia. Furthermore, others have recently reported that NRP-1 mediated internalization of peptides is dependent on a C-terminally exposed arginine (or infrequently lysine) residue with a $^R/_K$XX$^R/_K$ motif (Teesalu T, *et al.* 2009; Sugahara KN, *et al.* 2010). However, the CGFYWLRSC peptide does not contain such a motif. Our peptide also does not contain another identified polybasic NRP-1-binding motif, RRXR, likewise selected by phage display (Hong TM, *et al.* 2007), and is distinct from the other known phage-display-derived peptides that bind to NRP-1 (Giordano RJ, *et al.* 2005; Giodano RJ, *et al.* 2010; Giodano RJ, *et al.* 2001). These discrepancies suggest that the CGFYWLRSC peptide may interact with a different NRP-1 binding site than the other described NRP-1 binding peptides.

Taken together, NRP-1 is a promising target in leukemia and lymphoma, and it can be utilized for ligand-directed drug delivery. The NRP-1 binding peptide, CGFYWLRSC in conjunction with a drug candidate may be used for therapeutic targeting of human leukemia and lymphoma. In addition, the NRP-1-binding motif, $F^F/_Y$XLRS offers a promising candidate for lead optimization and targeted drug development. Ultimately, future studies will be needed to address the capability of this new NRP-1 binding peptide motif to facilitate targeted drug delivery *in vivo*.

1.3. Targeting IL-11R α

Many organ-specific molecules are expressed in restricted locations that may not be accessible for therapeutic targeting via circulation. *In vivo* phage display offers an alternative means to profile human tissue and obtain target-specific ligand-mimics that bind to receptors accessible via vasculature. Utilizing this technology by injecting a phage library into a brain-dead cancer patient, we have previously identified a tumor-targeting IL-11 ligand mimic that was demonstrated to target IL-11Rα. We subsequently showed that IL-11Rα is upregulated in bone metastatic prostate cancer (Zurita AJ, *et al.* 2004) and in osteosarcomas (Lewis VO, *et al.* 2009), – both which share a bone marrow microenvironment – but not in healthy tissues, including bone marrow (Lewis VO, *et al.* 2009; Zurita AJ, *et al.* 2004), and successful outcomes were obtained in therapeutic targeting of IL-11Rα in metastatic prostate cancer (Zurita AJ, *et al.* 2004). We therefore rationalized that the IL-11Rα could also be a potential target for therapeutic intervention in leukemia.

Support to this hypothesis also comes from several studies by others, which have reported high expression of the IL-11Rα in different leukemia subtypes, particularly in AML-M5 blast cells and in B-CLL, thus suggesting that IL-11Rα may play a role in the pathogenesis of leukemia (Kimura T, *et al.* 1999; Tsimanis A, *et al.* 2001; Cherel M, *et al.* 1995). For example, it was shown that AML cells, in particular those from M5 subtype expressing high levels of IL-11Rα, exhibited enhanced clonal proliferation in response to co-stimulation by IL-11 and G-CSF (Kimura T, *et al.* 1999). Another study demonstrated that the expression level of IL-11Rα is much higher in B-cell chronic lymphocytic leukemia cells (B-CLL) as compared to peripheral blood lymphocytes from healthy donors (Tsimanis A, *et al.* 2001). The study also showed that the recombinant human IL-11 activates B-CLLs resulting in morphological changes and increased cell proliferation, which suggest that IL-11Rα and its natural ligand might be implicated in the pathogenesis of malignant B-CLL (Tsimanis A, *et al.* 2001). In addition, IL-11Rα transcript expression has been reported in the CML cell line K562, the megakaryocyte cell line Mo7E, and the erythroleukemia cell line TF1 (Cherel M, *et al.* 1995). Moreover, IL-11 signaling has recently been reported to enhance HSC and MPP radioresistance as well as facilitate their recovery from irradiation, subsequently leading to radiation-induced B-cell malignancies (Louria-Hayon I, *et al.* 2013). These data support the idea that IL-11Rα may be a suitable target for leukemia therapies.

In this this study, we evaluate the expression and therapeutic targeting potential of IL-11Rα in the context of human leukemia, with the emphasis on AML. Our results show that IL-11Rα is highly expressed in various leukemia cell lines and that the ligand-directed proapoptotic peptidomimetic, CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ that targets IL-11Rα, significantly reduces leukemia cell viability. Moreover, this peptide exhibited significantly lower toxicity on normal lymphocytes, neutrophils, and monocytes as compared to Molt-4 leukemia cells. Similarly, no toxicity was observed with mononuclear cells isolated from healthy bone marrow. We also show that IL-11Rα is moderately expressed in most of the tested bone marrow samples from AML patients.

Downstream effectors of the IL-11/IL-11Rα signaling pathway include receptor-associated JAKs and STATs, which promote cell survival, proliferation, invasion, angiogenesis, metastasis, as well as immune responses associated with inflammatory diseases and tumor progression. IL-11/IL-11Rα signaling is known to modulate several biological activities, including hematopoiesis and lymphopoiesis. It has been reported that low levels of IL-11 messenger RNA are found ubiquitously, although, the IL-11 protein is rarely detected in the serum of healthy subjects whereas a pathological stimuli such as viral inflammation or many cancers can induce IL-11 expression (Putoczki T and Ernst M. 2010; Schwertschlag US, *et al.* 1999).

Despite the roles of IL-11 during normal biological processes it was previously shown that mice lacking the IL-11Rα gene display normal phenotype (Nandurkar HH, *et al.* 1997). Accordingly, our clinical trial study shows that the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide systemically targets metastatic prostate cancer via targeting the IL-11Rα, without inducing any persistent adverse effects (Pasqualini R, *et al.* 2015). Markedly, we have previously shown that although the CGRRAGGSC peptide mimics the IL-11 ligand, the corresponding location within the native cytokine is not in a characterized interaction site between IL-11 and IL-11Rα; therefore establishing a new interaction mechanism with the IL-11Rα receptor (Cardó-Vila M, *et al.* 2008). This might, in part, contribute to specific tumor targeting attributes of this peptide. As prostate cancer that has metastasized to bone shares a similar microenvironment as leukemia, it is probable that the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide exerts similar specific targeting effects also in the context of leukemia.

Given the successful clinical outcomes with therapies in bone metastatic prostate cancer patients, we initiated the lead optimization efforts for the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide in order to find an

analogue with a superior efficacy profile. To improve its pharmacokinetics, some of the residues where introduced in their d-amino acid forms to the peptide sequence. However, these modifications did not improve the anti-cancer activities of the peptide *in vitro*. Myristoylation of the serine residue, on the contrary, increased the activity significantly in the *in vitro* cultures of both cell lines as well as patient derived primary AML cells. The myristoyl group is highly lipophilic, which enables its incorporation into the phospholipid bilayer of cell membranes, and is likely used to facilitate the internalization of the peptide. In effect, N-myristoylation enables the association and binding of many proteins with cell membranes (Farazi TA, *et al.* 2001). Typically, myristate helps to regulate protein targeting, function, and interaction with cell membranes by serving as a lipid anchor in cell membranes for myristoylated peptides (Farazi TA, *et al.* 2001). For example, N-myristoylated phorbol ester protein kinase C- α (PKC- α) pseudosubstrate peptides potently induced an uptake of chemotherapeutic drugs by human colon cancer cells through a P-glycoprotein-independent mechanism (Bergman PJ, *et al.* 1997). In our study, the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ also achieved improved internalization abilities upon myristoylation of its serine residue as indicated by the induction of apoptosis at earlier time points compared to the original unmyristoylated peptide. Myristoylation of the peptide also improved its efficacy against primary AML cell samples.

Markedly, IL-11 has been reported to play a role in recruiting dormant/quiescent leukemic progenitors into the cell cycle (Kimura T, *et al.* 1999). Accordingly, we have previously shown that the CGRRAGGSC peptide induces STAT3 phosphorylation in TF-1 cells (Cardó-Vila M, *et al.* 2008), in which STAT3 is not constitutively activated naturally, suggesting that IL-11R α may serve as a target that activates quiescent cells thus rendering them susceptible to therapies. In contrast to the studies by others, where leukemia cell mobilizing/sensitizing agents have been used as an initial activator followed by the cytotoxic drugs (Zeng Z, *et al.* 2009; Löwenberg B, *et al.* 2003), our IL-11R α targeting peptide, CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$, both activates the STAT3 and simultaneously induces cell death via the proapoptotic $_D$(KLAKLAK)$_2$ motif, thus making this strategy more specific and potentially more effective in targeting of LSCs. Consequently, the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide and its derivatives may therefore have a dual role in cancer therapy as a cell cycle activator and a cell death inducer. In accordance with this hypothesis, we show that the CGRRAGGSC-GG-$_D$(KLAKLAK)$_2$ peptide and its myristoylated derivative reduced the clonogenic potential of patient-derived AML bone marrow cells, while they did not have a similar effect on normal bone marrow cells at equivalent concentrations. Consistently, IL-11R α expression was also shown in immature CD34 positive cell populations derived from leukemia patients as demonstrated via flow cytometry and cytospin analysis. We speculate that the therapeutic targeting of IL-11R α may be capable of eradicating LSCs. Further studies are needed to evaluate these points.

Taken together, selecting ligand-mimics that target different organs *in vivo* accessible via circulation provide an excellent means to profile the molecular diversity of a human and find clinically relevant targets. Our results show that the IL-11R α receptor system offers a potential targeting approach for therapies in human leukemia and lymphoma.

2. Identification of novel drug candidates against leukemia via screening a small molecule compound library via the *ex vivo* assay (IV)

The microenvironment has an important role in supporting the proliferation and survival of cancer cells, thus contributing to disease progression. In addition, it has a key role in mediating drug efficacy and resistance (Zhou J, *et al.* 2005). Therefore, it is fundamental to develop functional screening platforms that can assess the therapeutic relevance of drug candidates within the appropriate disease microenvironment. Although, *in vitro* screening for anti-cancer agents – for instance, via the NCI60 cell line panel – has been a valuable tool for drug discovery, it fails to address the disease microenvironment such as host-cancer

interactions and the humoral and cellular immune system, all of which can significantly reduce the drug efficacy *in vivo*. Markedly, the compounds selected via the conventional *in vitro* screening methods are often difficult to translate to *in vivo* settings because the activity of a compound is frequently lost or significantly reduced. In effect, the co-existence of the non-malignant host cells (i.e., immune, stromal, mesenchymal) and leukemia stem cells allows complex interactions that are known to affect several biological processes, including response to therapy, drug resistance, self-tolerance, and angiogenesis (Grivennikov SI, *et al.* 2010; Sison EAR and Brown P. 2011; Konopleva M and Andreef M. 2007; Kurtova AV, *et al.* 2009; Roodhart JML, *et al.* 2011; Ustun C, *et al.* 2011). Therefore, mouse models are a traditional preclinical host to assess anti-cancer efficacy of a test compound *in vivo*. However, there are several limitations in mouse modeling, such as species-specific differences, limited recapitulation of *de novo* human tumor development, and differences in drug response as compared to humans (Cheon DJ and Orsulic C. 2011; Testa U 2011). For instance, mice have a tendency to be more resistant to many anti-cancer compounds than patients (Teicher BA. 2009). The lack of functional immune system in these models may also interfere with the outcome, as immune surveillance is known to contribute to several aspects of tumorigenesis. For instance, certain populations of lymphocytes and leukocytes play a tumor-supporting role in cancer (Grivennikov SI, *et al.* 2010; Ustun C, *et al.* 2011). Consequently, immunocompromised mouse models often fail to recapitulate the human microenvironment properly, which might contribute to the fact that the results obtained in xenograft models do not often correlate with clinical outcomes. Furthermore, high-throughput screening in mice is challenging. Therefore, combinatorial approaches that use several model systems are required to further understand the complexity of human cancers (Cheon DJ and Orsulic C. 2011). For instance, assays involving a removal of the diseased tissue from a patient to assess the anti-cancer efficacy of a drug in an *ex vivo* setting prior to the actual treatment can be important preclinical tools.

In this study, we developed a new functional *ex vivo* screening assay for leukemia in the presence of human blood or bone marrow under hypoxic conditions. The advantage of our assay is that it allows for the identification of efficient anti-leukemia agents that are functional in the presence of human blood or bone marrow. Markedly, during the *ex vivo* assay development we observed that leukemic cells deplete oxygen faster than normal cells causing a shift in the hemoglobin oxygenation state. This shift, detected by measuring the optical density at 600 nm (OD_{600}) after an appropriate incubation time, directly correlated with leukemic cell counts. Therefore, we rationalized that this color change could be used as an indicator of leukemia cell viability and to screen for compounds with anti-leukemia activity. Notably, the oxygen-dependent absorbance spectra of hemoglobin is well known (Zijlstra WG, *et al.* 1991; Hoppe-Seyler F. 1866), but to our knowledge, has never before been utilized for drug discovery in blood-containing culture. Importantly, our assay requires no exogenous marker, such an oxygen probe, which has previously been used in the *in vitro* culture systems to monitor oxygen consumption (Hynes J, *et al.* 2009, 2006 & 2003; Arain S, *et al.* 2005; O'Riordan TC, *et al.* 2000 & 2007). The "Z-factor" – a coefficient that is used as a reflective of both the signal dynamic range and the data variation in the assay related to the signal measurements (Zhang JH, *et al.* 1999) – was calculated to be approximately 0.71 for our *ex vivo* assay, which indicates that the results are reproducible and accurate. Nevertheless, the donor blood specific differences may contribute to observed variation in efficacy of drug candidates in different blood samples. Thus, the best application of the *ex vivo* assay is as a tool for personalizing therapy against human leukemia.

Importantly, our *ex vivo* assay provides new means to evaluate drug efficacy in the context of bone marrow microenvironment, in which drug resistance is often encountered. In addition, although AML has its origin in the bone marrow, the leukemic cells can also circulate in large numbers in the peripheral blood and infiltrate many organs, which is associated with poorer prognosis (Chang H, *et al.* 2004). This mobility of AML cells may resemble that of hematopoietic stem and

progenitor cells, which can sustain hematopoiesis in extramedullary sites such as the spleen and liver (Zhu J, *et al.* 2007; Ghinassi B, *et al.* 2010). The circulating levels of hematopoietic stem and progenitor cells also increase in response to stress, inflammation and tissue/organ injury (Rarajczak MZ, *et al.* 2010) and elevated levels of MSCs have also been detected in peripheral blood of cancer patients (Bian Z-Y, *et al.* 2009; Fernandez M, *et al.* 1997). Hematopoietic progenitor cells can be intentionally mobilized into circulation by using mobilizing agents, such as CXCR4 inhibitors, which enhance chemotherapy resulting in markedly reduced leukemia burden and increase in survival (Zeng Z, *et al.* 2009). As such, discovery of leukemia drugs that are effective in the presence of bone marrow as well as blood components is equally important.

Notably, blood and bone marrow are relatively easy to obtained from humans, and thus provide a unique opportunity for the development of preclinical *ex vivo* assays mimicking the human microenvironment. The *ex vivo* assay could also provide information about the toxicity of the drugs in humans, as bone marrow is critically sensitive to many anti-cancer agents (Pessina A, *et al.* 2003). In effect, mouse bone marrow is less sensitive to many cytotoxic agents as compared to human bone marrow, thus allowing blood levels in preclinical efficacy testing that are not achieved in patients (Pessina A, *et al.* 2003; Erickson-Miller C, *et al.* 1997; Kummar S, *et al.* 2006; Kurtzberg LS, *et al.* 2007; Masubuchi N, *et al.* 2004), and therefore rendering mouse models inadequate in addressing the human bone marrow toxicity. In addition, high-throughput screening of small molecules in the *in vivo* model is difficult, and thus chemical libraries are readily amenable to *in vitro* selection only. Therefore, the *ex vivo* assay could be used as an alternative approach for screening of compounds against cancer.

It is expected that any drug that is not a general toxin will likely have varying efficacies depending on the patient sample tested. This could be due to differences in specific molecular abnormalities carried by leukemia cells as well as differences in the microenvironment (i.e. blood or bone marrow). In blood, factors that can be responsible for patient-to-patient variability in response effects include different concentrations of hydrolyzing enzymes, plasma proteins, and red blood cells. All these factors can significantly reduce drug efficacy, inactivating a drug already in the circulation and preventing it from reaching the target tissue. In principle, testing drug candidates in the presence of blood and bone marrow from a leukemia patient should facilitate the selection of a therapeutic strategy for the individual patient. Indeed in the pilot experiments, we were able to optimize the *ex vivo* assay for blood samples directly from AML patients. However, some primary samples contained such a low percentage of blasts that they did not efficiently induce the color shift to dark red (i.e. deoxygenate hemoglobin). However, this obstacle for the assay detection could be overcome by isolating a portion of the mononuclear cells via Ficoll purification and re-implanting them to the remaining donor blood or bone marrow. Moreover, detection of low blast counts may also be possible via utilization of stronger hypoxic condition, such as 1% oxygen levels. Furthermore, similar feasibility studies with human bone marrow aspirates for cell co-cultures showed promise in our preliminary experiments. These results indicate that the *ex vivo* assay introduced here may also be adapted for bone marrow samples from leukemia patients. Our preliminary data suggest that the *ex vivo* assay could potentially be applied for tailoring treatment options for each patient, and may facilitate rapid testing of the available leukemia drugs and new drug candidates, as well as provide guidance regarding a treatment selection or intensification during the course of therapy. Further studies with larger patient sample cohorts and with a variety of subtypes are needed to study the translational value of the *ex vivo* assay.

To establish that our *ex vivo* assay could be utilized in the drug discovery, we screened a small molecule chemical library against leukemia cells in the *ex vivo* assay setting to identify small molecule compounds that had robust activity in the presence of human whole blood.

Notably, the vast majority of the compounds identified via the *ex vivo* assay screening were novel, and many were discovered to be

structurally identical or similar to the anti-cancer, anti-inflammation, or anti-microbial compounds described in the literature. These known mechanisms of the identified compounds support the idea that our *ex vivo* assay is primarily selecting compounds that have potential therapeutic value. However, due to the presence of several cell types and molecules in the blood, the method can in principle also select compounds that affect the oxygen-carrier function of hemoglobin and red blood cells, or some other aspect of host blood cells such as inducing an immune reaction. For instance, a compound could in theory mimic the oxygen binding to hemoglobin, thus inhibiting the observed color shift even in the presence of viable leukemia cells. As a result, some of these compounds could be selected as false positives. However, the initial small-scale analysis with 20 compounds indicates the true-positive hit rate is around 90%, suggesting that the majority of the compounds selected during the screening affected leukemia cells directly. In addition, the "false positives" could be easily discarded early on with the follow up analysis via Coulter counter, which specifically detects the reduction in WBC counts thus confirming that a particular compound is, in fact, a cell viability or growth inhibitor.

Markedly, some of the compounds not showing any activity *in vitro*, could also be "prodrugs" – agents, which are activated by hydrolysis or by biosynthetic reactions, whose metabolites contribute to therapeutic activity – and therefore, may be valid therapeutic agents in the presence of blood. Thus, caution must be used when labeling a compound as a "false positive" based on the *in vitro* validation, as some of the compounds may in reality be "true hits". The use of the Coulter counter system to count the different blood cell populations in a co-culture setting will thus be more informative than a standard cell culture assay as a validation system.

In addition, as an indirect support to our hypothesis that our *ex vivo* assay could identify compounds that are stable in blood, many selected compounds contained amide moieties, which are more stable against plasma hydrolysis than are ester moieties in the same position (e.g., Breitenlechner CB, *et al.* 2004). Other factors such as steric hindrance near a hydrolysable group – which results into differential binding of compounds to plasma enzymes (e.g., Borthwick AD, *et al.* 2003) – the lack of electron-withdrawing groups – which increase the positive charge on the phosphorous atom and increase the rate of hydrolysis (Sawa M, *et al.* 2002) – and the lack of hydrolyzable groups in the compounds may have contributed to increased plasma stability.

In summary, the *ex vivo* assay described here can select compounds that are able to inhibit leukemia cells in complex co-cultures – known to induce drug resistance – as well as estimate the prospective stability of compounds in circulation. Importantly, the *ex vivo* assay demonstrated several key advantages as compared to conventional *in vitro* assays as well as animal models including (i) partial recapitulation of the human leukemia microenvironment, (ii) use of native hemoglobin oxygenation status as a "built-in" indicator of leukemia cell viability/proliferation, (iii) cost-effectiveness, (iv) species-specificity, and (v) high-throughput screening functionality. We anticipate that the *ex vivo* assay can be used to identify new drug candidates for leukemia that are stable in blood and bone marrow microenvironment, and thus potentially have a high translational value. In principle, our assay could hypothetically be used as a tool to predict clinical outcome, as well as facilitate customizing safer and more effective treatment strategies for each patient, by testing available cancer drugs in patient's own blood and bone marrow, and thus potentially selecting the most effective treatment options for each patient prior to administration. Taken together, our *ex vivo* assay provides a novel and unique research tool that can be applied to the discovery of novel drug candidates against leukemia and can function as a clinical tool for personalizing cancer therapy.

Concluding remarks

Although clinical advancements in the systemic therapy of leukemia have been made (Ravandi F. 2011; Ravandi F, *et al.* 2007), non-specific toxicity and drug resistance result in serious long-term side effects, and significant relapse and mortality rates. Targeted combination therapies to specifically and simultaneously block all the disease-driving molecular networks in a patient are needed to overcome the disease and eradicate all the leukemia cells. In addition, customizing a therapy for each patient must go beyond targeting of aberrations intrinsic to leukemia cells and predict how cancer microenvironment affects drug response. Discovery approaches will need to be accordingly directed so that the identification of pathologically important targets and effective therapeutic agents is possible and probable. For example, the phage display technology is a well-established screening tool that can identify new clinically relevant ligand-receptor pairs both *in vitro* and *in vivo*, and provides an excellent tool for identification of cancer-specific targets, toward which drugs can subsequently be developed. Similarly, screening of small molecule compounds in the *ex vivo* assays provides a superior alternative to standard *in vitro* screening, and facilitates the identification of drug candidates effective in the presence of human components.

The work presented in this thesis has utilized both the phage display technology as well as the *ex vivo* screening platform in the discovery of potential therapeutic targets against leukemia. Specifically, we identified and evaluated three new targets for leukemia (namely: the invadosome complex, NRP-1, and IL-11Rα), as well as several candidate therapeutic agents against leukemia, including the inhibitors of the invadosome complex, and proapoptotic ligand-mimics binging to NRP-1 and IL-11Rα, as well as several new small molecule compounds with efficient anti-leukemia activity. In addition, we introduce a novel screening method for leukemia, in which compound stabilities and efficacies can be tested in the presence of human blood or bone marrow, therefore facilitating the identification of drug candidates that may have translational value. This assay may also have future applications in personalized cancer medicine.

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