Leukemic Stem Cells and Therapy Resistance

Master thesis
T.C. Kubbenga
Student nr. 3453626

Cancer Genomics and Developmental Biology
Coordinator: Dr. P.H. Steenbergh
Utrecht University

July - August 2011

Supervisor: Dr. Linda Smit
VU Amsterdam
Examinator: Dr. Menno Creyghton
Hubrecht Institute

Abbreviations

5-FU	5-fluorouracil
ABC	Adenosine Triphosphate-Bindings Cassette
ALDH	Aldehyde Dehydrogenase
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
Ara-C	Cytosine Arabinoside
As_2O_3	Arsenic Trioxide
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Triphosphate
AZA	5'aza-deoxycytidine
CFU	Colony Forming Units
CML	Chronic Myeloid Leukemia
CNDAC	2'-C-cyano-2'-deoxy-1-β-D-arabino-pentofuranosylcytosine
COX-2	Cyclooxygenase-2
CS-682	N4-palmitoyl
CSC	Cancer Stem Cell
CY	Cyclophosphamide
DNR	Daunorubicin
EMSA	Electrophoretic Mobility Shift Assay
ERCC	Excision Repair Cross-Complementing rodent repair deficiency
G-CSF	Granulocyte Colony-Stimulating Factor
HIF	Hypoxia-Inducible Factor
HSC	Hematopoietic Stem Cell
IGF	Insulin-like Growth Factor
IκB-α	Inhibitory κB-α
IL	Interleukin
INF	Interferon
LAA	L-ascorbic Acid
LSC	Leukemic Stem Cell
MDR	Multi Drug Resistance
MDR1	Multi Drug Resistance Transporter 1 gene
MGMT/AGT	O6-Methylguanine DNA methyltranferase / O6-Alkylguanine DNA alkyltransferase
MMP	Matrix Metalloproteinase
MNP	Magnetic Nano Particle
MRD	Minimal Residual Disease
NER	Nucleotide Excision Repair
NF-κB	Nuclear Transcription Factor κB
NOD/SCID	Non-obese Diabetic mouse with Severe Combined Immunodeficiency disease
NSG	NOD/SCID;IL 2Rγ chain null mouse
ODN	Oligodeoxynucleotide
PGP	Permeability-related glycoprotein
PML	Promyelocytic Leukemia
RISC	RNA-induced Silencing Complex
RNAi	RNA interference
ROS	Reactive Oxygen Species
SC	Stem Cell
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SL-IC	SCID Leukemia-Initiating Cell
SP	Side Population
STAT	Signal Transducer and Activators of Transcription
TC-NER	Transcription Coupled-Nucleotide Excision Repair
VLA	Very late Antigen

Index

ABBREVIATIONS **2**

INDEX **3**

1 | INTRODUCTION **4**

1.1 CANCER STEM CELLS 4
1.2 LEUKEMIC STEM CELLS 6

2 | THERAPY RESISTANCE OF THE LEUKEMIC STEM CELL **9**

2.1 QUIESCENCE 9
2.2 ABC TRANSPORTERS PUMP 12
2.3 DNA REPAIR MECHANISMS 15
2.4 THE HYPOXIC MICROENVIRONMENT 17

3 | CONCLUDING REMARKS **19**

LITERATURE **21**

1 | Introduction

1.1 Cancer Stem Cells

Treatment of cancer is challenging because this pathology involves dysregulation of endogenous and essential cellular processes [1]. The development and maintenance of cancer can be explained by two different models. One model is the so called stochastic model, in where all cancer cells are able to form and maintain the tumor mass (figure 1a). The other model, the hierarchical model, is a model in where a small group of cells is susceptible for transformation and forms and maintains the tumor (figure 1b). The hierarchical model is also called the cancer stem cell (CSC) model because the group of cells responsible for this maintenance of the tumor has stem cell like characteristics [2-4].

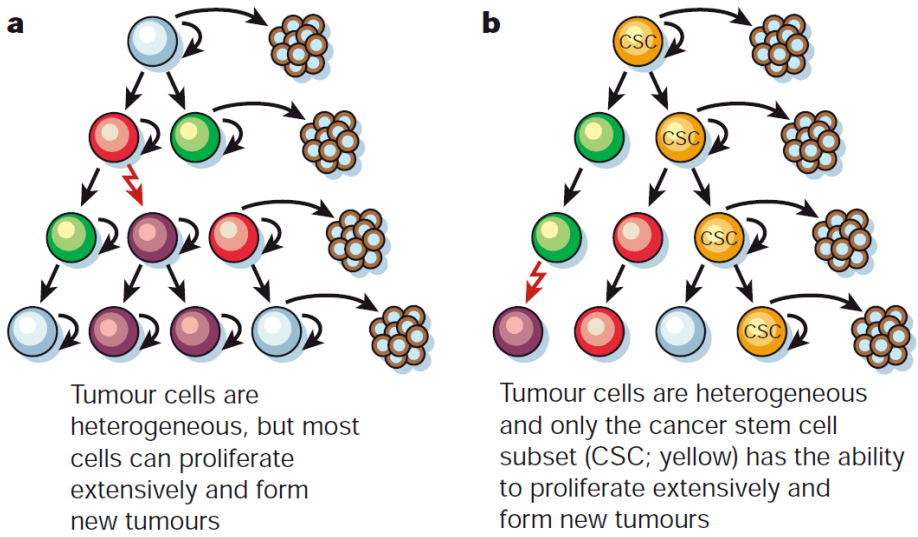


Figure 1. **Two general models of heterogeneity in cancer cells. a)** Cancer cells of many different phenotypes have the potential to proliferate extensively, but any one cell would have a low probability of exhibiting this potential in an assay of clonogenicity or tumorigenicity. **b)** Most cancer cells have only limited proliferative potential, but a subset of cancer cells consistently proliferate extensively in clonogenic assays and can form new tumors upon transplantation. The model shown in b predicts that a distinct subset of cells is enriched for the ability to form new tumors, whereas most cells are depleted of this ability. Existing therapeutic approaches have been based largely on the model shown in a, but the failure of these therapies to cure most solid cancers suggests that the model shown in b may be more accurate [5].

Increasing evidence is found that supports the CSC hypothesis and the overlap between SCs and CSCs are found to be very close [5]. Normal SCs have the ability to proliferate life-long, are immortal and are mostly resistant to drugs by multiple mechanisms. SCs can divide asymmetrically and produce two cells: a daughter SC and a progenitor cell that can differentiate into different lineages but cannot self-renew. SCs have specific markers and are able to differentiate into certain tissues and cells due to the microenvironment and other factors. CSCs are quite similar to these criteria. CSCs have the ability to proliferate and self-renew and are heterogeneous. The CSC develops along the differentiation path similar as normal SCs and finally the tumor comprises of tumor initiating cells (CSCs) and of abundant non-tumor initiating cells. CSCs express specific markers, often also found on SCs and importantly CSCs are often more resistant to drugs then the bulk of the tumor [5-7]. Other

evidence for the existence of CSC is found by *in vitro* and *in vivo* experiments. When myeloma cells were extracted from mouse ascites, separated from normal hematopoietic cells and put in clonal colony-formation assays, only 1-10,000 to 1-100 cells were able to form colonies [8]. When leukemic cells were transplanted in mice assays, only 1-4% of cells could form spleen colonies[5, 9]. This *in vivo* assay does not separate two possible causes of only small percentages of cells forming colonies: either these 1-4% cells are the only cells that have clonogenic capacity, or the probability of proliferation was low and these cells were the only cells that did proliferate however in theory all cells could proliferate. Bonnet *et al.* proves that the first possibility is the most plausible one by showing that cells with the CD34+/CD38- phenotype are the cells that are able to proliferate and initiate leukemia. This population of cells represent 0.2% of the human leukemia population [4].

Zou postulates the definition of the CSC as follows:

"Cancer stem cells can be defined as the specific cell population inside cancer which has the capacity for self-renewal, the potential to develop into any cells in the overall tumor population, and the proliferative ability to drive continued expansion of the population of malignant cells." [10].
These CSCs can also be formulated as tumor initiating cells, but because of the properties these cells possess are similar to SCs, in this report they will be referred to as CSCs.

A study in 1964 has shown that cancer cells develop more or less according to normal development. Kleinsmith and Pierce have shown that single teratocarcinoma cells can develop into different types of tissues and differentiate into tumorigenenic and non-tumorigenic cells [11]. Another study has shown that cancer cells are derived from the differentiation of malignant initiating cells, later to be called CSCs, in order to develop a tumor [12]. It is not known whether CSCs really arise from SCs however it is possible that deregulation of the normal SC gives the development of cancer [10]. Tumorigenesis starts either with transformation of a multipotent SC which leads to uncontrolled self-renewal or transformation of a more downstream progenitor cell leading to acquired self-renewal of a cell that did not have self renewal capacity [5, 13].

In 1988, Pierce and Speers define the CSC concept as follows:

"A concept of neoplasms, based upon developmental and oncological principles, states that carcinomas are caricatures of tissue renewal, in that they are composed of a mixture of malignant stem cells, which have a marked capacity for proliferation and a limited capacity for differentiation under normal homeostatic conditions, and of the differentiated, possibly benign, progeny of these malignant cells." [14]

1.2 Leukemic Stem Cells

The first one to identify the leukemic stem cell (LSC) were Bonnet and Dick in 1997 [4]. Until then, there was much conflict in reasoning about the identity of the initiating cell in leukemia [13]. It was not clear whether the stochastic model or the hierarchical model was applicable, and within the hierarchical model it is still not clear whether the normal SC is targeted and transformed and/or a progenitor cell initiates leukemia. By using non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) it was shown that cells that are able to initiate leukemia (the SCID leukemia-initiating cells or SL-ICs) have the ability to proliferate, self-renew, and differentiate via asymmetrical division. The cells identified by Bonnet as the leukemia initiating cells reside in the CD34+CD38- immunophenotypic compartment [4].

Acute myeloid leukemia (AML) is initiated and maintained by a small population of cells that have SC-like characteristics. These cells proliferate, divide asymmetrically and are able to self-renew and are called LSCs [6, 15]. Most cancer centers use therapy based on the combination of anthracyclin and cytarabine chemotherapy to treat AML patients. Between 70% - 80% of adult AML patients achieve complete remission after combined chemotherapy [16]. However, in many cases, a seemingly successful treatment ends in disease relapse which is thought to be caused by the outgrowth of dormant chemotherapy resistant LSCs [4, 17].

Several relapse related factors have been identified such as certain cytogenetic and molecular aberrations at diagnosis, lack of early treatment response and high level of minimal residual disease (MRD, small number of leukemic cells that remain in the patient) after treatment. In particular, high frequencies of LSCs at diagnosis and after treatment have been shown to predict relapse in AML [17]. It is therefore essential to develop new therapeutic strategies to eradicate LSCs. This will be important for the prevention of relapse and improving long-term outcomes in AML. LSCs are located at the endosteal region of the bone marrow and are mainly non-cycling [15, 18-20]. This quiescent property could be the reason for their escape from therapy and releasing them from this quiescence state might be an opportunity to lead the LSCs into cell death.
LSCs and normal hematopoietic stem cells (HSC) share several characteristics, they both reside in the AML bone marrow and they share self-renewal potential, dormancy and similar cell surface markers. The success of anti-LSC therapy relies on functional manipulation of genes that lead to specific killing of LSC while saving normal HSC. This way LSCs could be an effective target to treat leukemia [7]. After treating AML with chemotherapy, it is common for patients to have a relapse. Most cells are killed using this therapy but due to its phenotype just discussed, it seems that the LSC is the reason for relapse [10].

AML is clinically, cytogenetically and molecularly a heterogeneous disease, which makes it challenging to classify it properly. AML is a cancer of the myeloid line of blood cells. Blocking differentiation at the myeloid lineage results into growth of abnormal white blood cells called blasts, which disrupt the growth of normal blood cells, white and red blood cells. This leads to a default in the immune system resulting in infections, bleeding or organ infiltration and a defect in the formation of red blood cells resulting in anemia [10, 21]. AML patients outcome are

very poor and without treatment patients acquire these symptoms within one year of diagnosis [21].

The AML phenotype is cell-autonomous, meaning that the disease develops by genetic and/ or epigenetic events leading to the transformation of the HSC [3, 22] or progenitor in such a way that leukemia is generated. In the case of transformation of the progenitor, LSCs maintain the identity of the progenitor from which they arose, while acquiring SC-like features such as self-renewal [23]. The phenotype of the LSC is much more heterogeneous than expected and can vary even within a single AML sample [24]. It has been suggested that LSCs can exist, besides the CD34+CD38- compartment, as well in the CD34+CD38+ or CD34- blast compartments, at least in some types of AML. Apart from these CD34/CD38 phenotypes, functional phenotypes have been associated with stem cell properties [25].

Increasing evidence was found for a group of cells now known as the Side Population (SP). These cells were able to efflux the Hoechst 33342 dye which is a characteristic profile during fluorescent activated flow cytometric analysis (figure 2). Normal mouse bone marrow and neuroblastoma cell line SK-N-SH were stained with Hoechst 33342. Flow cytometry has shown that 0.027% of the bone marrow population and 0.05% of the SK-N-SH cell line is SP [26]. Research done by Wulf *et al.* shows that SP cells were found in more than 80% of investigated patients, were mostly CD34low/- and carried cytogenic markers of AML in all cases of active disease. These cells actively force out cytostatic (lipophilic) anti-leukemic drugs [27]. SP cells were only found in the normal bone marrow, not in peripheral blood what may say something about the bone marrow niche (microenvironment) where these stem cell-like cells locate [27]. SP cells are able to reconstitute the bone marrow of lethally irradiated mice and 3 of 28 transplanted leukemic SP cells were able to generate AML in NOD/SCID mice. From these results, Wulf *et al.* concluded that these SP cells could consist of LSC that are responsible for relapse in AML patients [27].

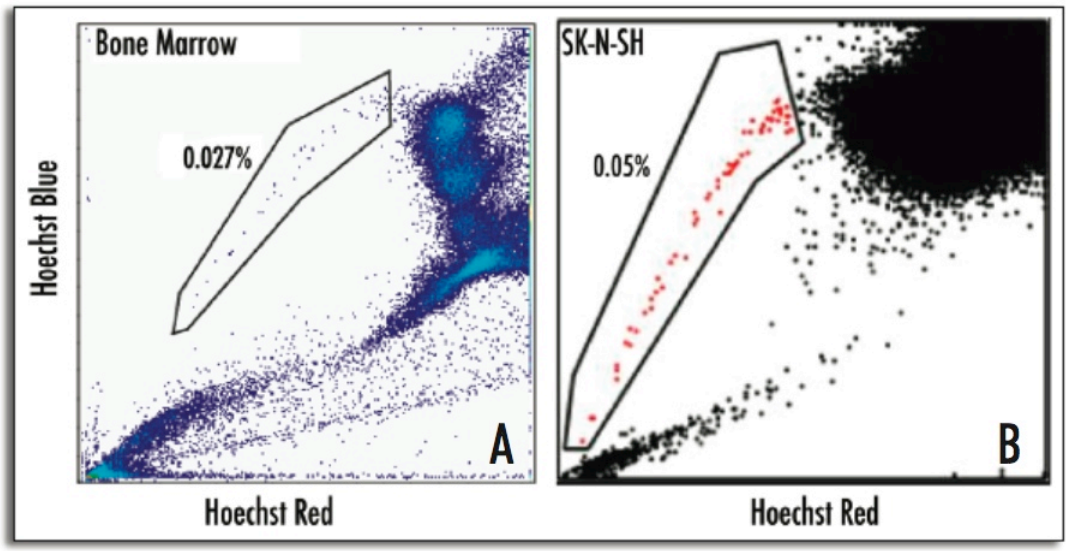


Figure 2. **"Side-population" phenotype in normal bone marrow and a neuroblastoma cell line.** Both **A**: normal bone marrow (mouse) and **B**: the neuroblastoma cell line SK-N-SH were stained with Hoechst 33342 and analyzed by flow cytometry [26].The SP population is indicated by the gate and in normal bone marrow 0.27% and in SK-N-SH 0.05%.

The family of non-conventional ATP-binding cassette (ABC) is evidently connected to the malignant phenotype of SP cells. The ABCG2 transporter was shown to efflux the Hoechst 33342 dye and is expressed in AML cells [26, 28]. A different class transporter is the intracellular transporter. Such a transporter like ABCA3 is highly expressed in leukemia, but not in normal peripheral blood or bone marrow [28, 29]. All these results lead to the conclusion that transporter proteins contribute to the SP phenotype. Besides the SP phenotype, the functional phenotype defined by high aldehyde dehydrogenase (ALDH) activity is a stem cell characteristic. ALDH, a cytosolic enzyme responsible for oxidation of intracellular aldehydes, has been implicated in cyclophosphamide resistance [30] and is now suggested to play a role in overall chemotherapy resistance [31, 32]. ALDH1 regulates resistance against cyclophosphamide in normal SCs and resistance has been correlated to high expression of *ALDH1* in leukemic cells [33], implicating that ALDH1 might be responsible for chemotherapy resistance [34].

Irrespective of whether the LSC is defined by an immunophenotypic or a functional characteristic, leukemic engraftment (or tumor initiating potential) in NOD/SCID is a characteristic of all phenotypes that are associated with LSC properties.
AML is normally treated by chemotherapy but regarding heterogeneity, it may be necessary to consider personalized medicine targeting the mutation of the transformed cells in order to increase the survival rate. By using personalized medicine, even the LSCs may be targeted.

2 | Therapy Resistance of the Leukemic Stem Cell

AML is a heterogeneous disease that is believed to develop from a rare group of disease initiating cells, termed LSCs. These LSCs are mostly unresponsive to drugs, presumably because of intrinsic resistance. The heterogeneous population of cells evolve by acquiring defects such as genetic mutations, which leads to genetic instability and SC plasticity. Populations of mutated cells evolve through a step-wise process with variable latency periods, such that the small LSC populations are able to stay small, therefore undetectable, and expand during or after therapy to a dominant clone and clinically overt relapsing disease [35]. Within the CD34+CD38- LSC population in AML patients, there is a tendency for cycle-dependent chemotherapy resistance. This resistance is associated to quiescence of the LSCs in the endosteal region of the bone marrow and therefore could be a target for therapy [18]. Finally, interaction between LSC and microenvironment may be important in the behavior of the LSC and therapy resistance [36]. Taking all these considerations together, targeted drug therapy or combination therapy has been proposed in order to suppress or even eliminate LSC's in AML. A few mechanisms will be described where LSC may acquire therapy resistance and how therapy resistance can be inhibited.

2.1 Quiescence

Quiescence of LSCs, also called dormancy, is probably the most important reason for treatment failure of AML cells because these cells are not in cycle. Most conventional chemotherapy triggers cell death by inducing DNA damage which leads to interference with cell growth and keeping the cells in S-phase resulting in the induction of cell death. Rapid dividing cells, such as tumorcells, are most affected by chemotherapy [37]. The LSC, however, are non-cycling and most chemotherapeutics do not trigger cell death in these cells as easy as in rapidly dividing cells. The LSC escapes the induction of cell death and relapse is likely. After treatment of AML using chemotherapy, quiescent LSC's often survive because of their dormant properties. There are a few agents that can bring normal SCs into cell cycle and awake the quiescent cells. Cytokines like Interferon (INF)α and granulocyte colony-stimulating factor (G-CSF) are able to activate HSCs. The compound arsenic trioxide (As_2O_3) targets the promyelocytic leukemia (PML) protein resulting in degradation of the protein and the increase of mRNA [15]. This results, like INFα and G-CSF, in cells getting into cycle.

2.1.1 Interferon

INFs are cytokines that are responsible for transcriptional activation of genes important for the innate immunity. They have antiviral, anti-proliferative and immunomodulatory properties in many types of cell lines and provide fundamental defense against virus infections and cancer [38-40]. INF functions by activation of the JAK pathway which involves the JAK kinases and the signal transducer and activators of transcription (STAT). This pathway activates INF stimulated genes [39, 40]. Essers *et al.* have shown that, treatment of mice with INFα results in the transient proliferation and activation of HSCs [41]. INFα does not actively take the HSCs out of their niche. They stay in their microenvironment. However, it is likely that INFα interacts with the niche via membrane proteins [15]. Because of this, it is probable that this explains the difference in results between *in vitro* [38] and *in vivo* data. *In vitro* data, where cells lack niche,

show INFα is found to be anti-proliferative. *In vivo*, where HSCs exist in their niche, show data in which INFα has anti-quiescent properties [15]. However this could also be due to differences between cell types and function. INFα acts anti-proliferative in order to eliminate infections and bulk of tumor cells. INFα acts proliferative to activate HSCs and produce new immune cells.

The way INFα treatment results in cell proliferation is by binding to HSCs, subsequent phosphorylation of STAT-1 and then finally the transcription and activation of INFα target genes. Treatment with IFNα might be used in addition to chemotherapy treatment in order to release the LSC from their dormant condition and subsequently kill them by chemotherapeutics [15]. For this combination therapy it will be necessary to use a two-step model, first administrating INFα followed by the treatment of chemotherapy e.g. 5-fluorouracil (5-FU). Additionally, the simultaneous treatment of INFα and 5-FU did not lead to sensitization of HSCs in mice. A two-step model is crucial for elimination of LSCs [41]. Applying the two-step model for leukemia and specifically to eradicate LSCs might be a promising therapeutic option. Activation of quiescent LSCs by INFα is not yet proven. However, that this approach might work is shown by a French chronic myeloid leukemia (CML) trial. Within this trial 6 patients stopped the usual Imatinib treatment and showed long term remission. Normally patients take lifelong Imatinib in order to prevent the LSCs awakening and the development of a relapse. Prominent, all of these 6 patients were treated with INFα before they were treated with Imatinib suggesting that the LSCs have been getting out of their dormancy by INFα treatment. It seems that the subsequent Imatinib treatment has eliminated the LSCs [42].

2.1.2 Granulocyte colony-stimulation factor

G-CSF is a cytokine that is able to drive HSCs into cycle. This was first shown in a study where both cyclophosphamide (CY) and G-CSF was injected into mice. This resulted into the activation of HSCs, more than 12 fold expansion of HSCs, and finally mobilization of HSCs. After 2 days of treatment the number of bone marrow HSCs decreased but the remaining HSCs continued to proliferate [43]. The mechanism behind activation of the cell cycle by G-CSF is still unknown. However it is known that mobilization of HSCs from the bone marrow is induced by matrix metalloproteinases (MMPs), elastase or cathepsin G. These enzymes are responsible for cleaving the adhesion proteins between the HSC and the osteoblasts in the bone marrow niche and the HSC is able to mobilize out of the bone marrow niche into the bloodstream. MMP-9 not only mobilizes the HSCs from the niche but also results in the release of Kit-ligand which transfers HSCs from a quiescent to a proliferative niche, affecting the state of the HSC [43-45].

As discussed before, within the CD34+CD38- LSC population in AML patients, there is a tendency for cycle-dependent chemotherapy resistance. This quiescence associated resistance could be the target for additional therapy. Besides the activation of HSCs by G-CSF, it also has been shown that LSCs can get out of their quiescent state by G-CSF. In a study by Saito *et al.*, G-CSF has been used to change the LSCs from a quiescent to a proliferative state. Induction of the cell cycle was measured by RNA and DNA synthesis (PyroninY and Hoechst33342, relatively). More RNA and DNA synthesis was seen upon G-CSF induction. In a xenograft experiment in the NOD/SCID;Interleukin (IL) 2Rγ chain null

(NSG) mice model were recipients of human LSCs, the amount of LSCs in the G0 phase of the cell cycle was reduced after 5 days of treatment with G-CSF. Combination therapy of human G-CSF and cytosine arabinoside (Ara-C) led to activation and elimination of the human AML LSCs and an increased survival of secondary recipients after re-transplantation of leukemia cells as compared to treatment with chemotherapy alone [18]. In G-CSF and cytarabine combination therapy treated NSG mice that were successfully transplanted with human AML LSCs, the human AML LSCs were eliminated. Importantly the mouse normal HSCs were not affected by the human G-CSF treatment and stayed quiescent [18]. This suggests a distinction between the response of normal HSC and LSC on G-CSF treatment or a distinction between the (human) G-CSF response on mouse and human SCs.

2.1.3 Arsenic trioxide

As_2O_3 has been implicated in apoptosis and proliferation in both HSCs and LSCs [46]. As_2O_3 causes the proteosomal-dependent degradation of the nuclear factor PML [47] which encodes a tumor suppressor highly expressed in HSCs. Loss of PML results into enhanced cycling of HSCs and increased mTOR activity [48]. Increased mTOR activity due to loss of PML by As_2O_3 was tested in a CML mouse model and it was shown that the LSCs escaped quiescence and got into cell cycle. As_2O_3 increased the efficacy of Ara-C treatment. Using As_2O_3, both human and mouse LSCs acquired increased sensitivity to apoptotic drug stimuli like cytarabine [48].

As_2O_3 also has additional effects on treatment with the anti-oxidant L-ascorbic acid (LAA). LAA acts as a pro-oxidant in certain conditions and to certain tumors [49] and is widely known for its anti-tumorcell properties. As_2O_3 triggers apoptosis of acute promyelocytic leukemia (APL, a subtype of AML characterized by blockage of granulocytic differentiation at the promyelocytic stage) cell line NB4 [50] and induces complete remission and a long-term relapse free period in AML patients [51]. In an electrophoretic mobility shift assay (EMSA) performed by Han *et al.*, nuclear transcription factor κB (NF-κB) was proven to be suppressed by LAA, and additionally suppressed in combined use with As_2O_3 what led to apoptosis. As_2O_3 induces *in vitro* growth inhibition and apoptosis of different cancer cell lines such as the AML cell line HL-60, malignant lymphocytes and myeloma cells by inhibiting glutathione which is a proton acceptor in the breakdown of H_2O_2. This leads to accumulation of H_2O_2 and enhances the ability of LAA to suppress NF-κB activity and the expression of NF-κB target cyclooxygenase-2 (COX-2), needed for the induction of cell death. Concluded from this, As_2O_3 can lead to cell death by accumulation of intracellular H_2O_2 [52]. As_2O_3 could be very useful to activate dormant LSCs. Inducing exit from quiescence increases the efficacy of chemotherapy to eliminate all cancer cells.

2.1.4 The microenvironment of the LSC

Normal hematopoeisis requires bi-directional signaling between the bone marrow niche and the HSC. A SC niche is believed to be located close to the endosteal region of trabecular bone marrow cavities [19, 20] and is stated as a molecular or cellular environment that regulates the SC functionality together with the SC autonomous mechanism. The regulation of the SC is to control quiescence, self-renewal and differentiation [53]. LSCs have characteristics of normal SCs and hijack these processes. Homing to the microenvironment, such as the normal SCs, have been proven to be important for LSCs to survive [3]. The mobilization of LSCs away from the bone marrow niche is associated with the expression of very late antigen

(VLA)-4. VLA-4 might therefore be a good target for the induced transport of LSCs from the bone marrow to the bloodstream. Releasing the LSC form bone marrow might overcome their quiescent state and therefore might be a possible target for the treatment of AML LSCs [3]. Proof of this is presented by a U937- (monocytoid cell line) inoculated MRD mouse model treated with therapeutic VLA-4 antibody. These mice show MRD by inoculation of U937 cells. Treatment results in mobilization of bone marrow LSCs to the peripheral blood [3,36]. When these mice were treated with a combination of VLA-4 antibody and Ara-C, a 100% survival was achieved in the 62 day observation period and, where Ara-C treatment alone only achieved a mean survival of 37 days [3,36]. In addition, 10 VLA-4 negative patients had a 100% survival rate after 5 years, where overall survival for 15 VLA-4 positive patients only was 44.4% after 5 years [3] suggesting a negative effect of VLA-4 expression on the chemotherapy response. Therapeutic targeting of VLA-4 using a neutralizing antibody in xenograft transplantation model prevented the development of AML [3].Therefore, up regulation of VLA-4 on the LSC seems to induce resistance to chemotherapy. All together, these results suggest that inhibition of VLA-4 mobilizes LSCs away from the bone marrow niche thereby making the LSC susceptible to chemotherapy [3].

The microenvironment might be an important target when designing strategies to eliminate LSCs and cure AML. It is believed that by activating dormant LSCs, they move out of their quiescent niche at the endosteal region, they go into cycle and become more sensitive to chemotherapy. Taking into account what was discussed before on INFα, G-CSF and AS_2O_3, addition of these compounds and breaking the interaction of the LSC with the microenvironment leads to mobilization of LSCs from the bone marrow niche into the bloodstream resulting into awakening and more sensitivity to chemotherapy [19]. An overview is shown in figure 3 where conventional or targeted therapy is compared to firstly activation of dormant LSCs using e.g. INFα and after sensitizing the LSCs, using the targeted chemotherapy approach and ultimately cure on long-term.

2.2 ABC Transporters Pump

Leukemic SP cells have increased ability to pump out drugs. This ability most likely contributes to chemotherapy resistance seen in LSCs because most stem cells have increased expression of the responsible transporters [27, 54]. Drug transporters are expressed in normal SCs to efflux toxic products and protect the cell from apoptosis. Over expression of these ABC transporters lead to increased expelling of drugs, related to resistance against drugs. If by any way the intracellular chemotherapeutic concentration can be increased, it is believed to enhance the therapy success [55].
Multi drug resistance (MDR) is a phenomenon seen when a tumor is resistant to a cytotoxic drug and also resistant to a large amount of other structurally unrelated chemotherapeutic drugs.

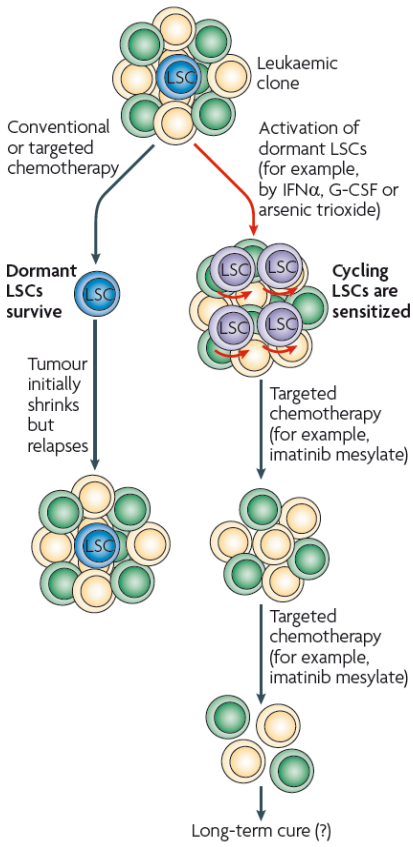


Figure 3. **Leukemic stem cells may be targeted by a two-step strategy.** Leukemic stem cells (LSCs), which may be a minority within the leukemic clone, show significant resistance to anti-proliferative chemotherapy regimes that are thought to be the cause of frequently observed tumor relapse. In analogy to normal dormant hematopoietic stem cells (HSCs), resistance of LSCs may, at least in part, be mediated by a state of deep dormancy. Thus to specifically target dormant LSCs one may postulate a two-step therapy model. First, dormant LSCs would be activated by factors, such as Interferon α (IFNα), granulocyte colony-stimulating factor (G-CSF) or arsenic trioxide (As_2O_3) and exit from their niche. Once they are cycling, treatment would be continued through administration of targeted chemotherapy, such as Imatinib or cytarabine [19].

Permeability-related glycoprotein (PGP) is such a plasma membrane pump able to extrude drugs. PGP is a member of the superfamily of adenosine triphosphate (ATP)-bindings cassette (ABC) transporters [56] and a product of the *multidrug resistance transporter 1* (*MDR1*) gene [54, 57]. Over expression of the *MDR1* gene is correlated with the MDR phenotype. One possibility to enhance drug sensitivity in MDR-1 over expressing LSCs is the downregulation of these transporters. A study done by Marthinet *et al.* has shown a strategy to modulate the MDR phenotype by the development of oligodeoxynucleotides (ODNs) targeting PGP. After downregulation of PGP, the leukemic cancer cells were sensitized efficiently to vinblastine [58]. Another efficient strategy to downregulate MDR is the combination of short hairpin RNAs (shRNA) targeting PGP with magnetic nano particles (Fe_3O_4, MNPs). Using shRNAs targeting MDR1 mRNA, an increase in the accumulation of drugs within the cell resulting in inhibition of drug resistance is seen [55, 59].

Chen *et al.*, showed that daunorubicin (DNR) loaded MNPs effectively lowers MDR in K562-n/VCR cells (MDR leukemic cells with the expression of the *MDR1* gene). Tumor weight of these nude mice treated with a combination of MNPs and DNR was significantly lower, and the transcription of *MDR1* was lower. DNR has been reported to be able to interact with DNA in precense of Fe_3 ions, so lower transcription of *MDR1* is probably due to DNR interacting

with the *MDR1* gene. From this was concluded that DNR loaded MNPs were able to overcome MDR *in vivo* [60].

Besides MNPs as Fe_3O_4, other methods were found to overcome MDR. Carbon nanotubes are excellent intracellular carriers with a large adsorption capacity and prolonged release ability [61]. This could be an addition to drug delivery therapy for targeting CSCs by using these carbon nanotubes in combination with chemotherapeutics. Carbon nanotubes is advantaged by its large inner volume compared to the dimensions of the tube, but very importantly cell membrane penetrability and the capacity of not being ejected from the cell and therefore having the ability to bypass MDR [62]. Such drug delivery methods can be made very specific, for example if the bonds of the particles are acid-labile, the nanotubes will dissolve in a acid environment such is found in tumor cells which makes it possible to deliver very site-specific [63, 64]. This technique could be used to target LSCs by using a LSC marker conjugated to the carbon nanotubes e.g. CD34 [65].

Inhibiting the production of PGP and delivering drugs site-specific to tumor cells are mechanisms to avoid drug resistance by efflux of drugs. This combination have been used by Wang *et al.*. They synthesized new compounds from fangchinoline and tetrandrine, pharmacotherapeutics, normally used as anti-inflammatory and analgesic medicines, which have been shown to increase the cytotoxicity of anticancer drugs in PGP-dependent tumor cells *in vitro* [66, 67]. One compound (named Compound #9, 5,14-dibromotetrandrine [67]) specifically increased the intracellular concentration of chemotherapeutic drug vinblastin. *In vivo* experiments showed a prolonged life-span of mice inoculated with MDR leukemic P388/ADR cells after Compound #9 treatment in combination with vinblastin [67]. This Compound #9 could give promising results when tested in LSCs in combination with chemotherapy.

2.3 DNA Repair Mechanisms

DNA repair mechanisms are linked to drug resistance in cancer patients by the existence of errors in these repair mechanisms. These mistakes can lead to transformation of cells acquiring malignant properties leading to cancer. In healthy mammalian cells, the response and repair mechanisms that act upon DNA damage, sustain the cells ability to transcribe genes and inhibit formation of mutations, which is crucial to life. Two major repair systems of human cells are homologous recombination and non-homologous end joining [68]. The most simple repair mechanism is the non-homologous end joining machinery. This is used to repair the breaks in two-ended DNA double strand breaks (figure 4A). This machinery is termed 'non-homologous' because during repair, a small 1-6bp region of homologous sequence often facilitates the repair [68]. Homologous recombination is a mechanism facilitated by 100bp or more DNA sequence that is homologous and is used to repair one-ended DNA double strand breaks (figure 4B). This is an error free process, where non-homologous end joining is not [68].

Activation of the DNA repair mechanism is often correlated with resistance for chemotherapeutics however defects in the mismatch repair system has also been correlated with drug resistance [69]. The chemotherapeutic drug nemorubicin acts through a mechanism which requires an intact DNA repair system called the nucleotide excision repair (NER) system [69]. In this repair system DNA is removed around the damaged section and is replaced by DNA polymerases [1]. Methylation of a NER gene, *XPG*, is a mechanism to induce therapy resistance in cancer cells by decreasing the activity of the DNA repair system, proven by *in vitro* experiments where cells were made resistant against nemorubicin and showed increased sensitivity to UV damage [69]. If *XPG* could be demethylated, the rate of therapy resistance could be lowered. Experiments where nemorubicin-resistant cells were incubated in the demethylating agent 5'aza-deoxycytidine (AZA), showed an increased sensitivity to nemorubicin [69], and promises reverting the resistant phenotype of cancer.

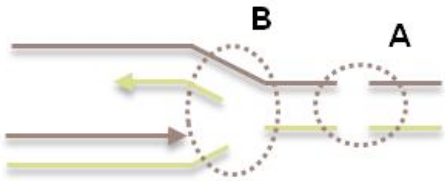


Figure 4. **Three major systems of recombination. A**) Two-ended DNA double-strand break, created by direct fracture of a DNA duplex. **B**) One-ended DNA double-strand break, created when a replication fork encounters a DNA single-strand break. Adapted from Helleday *et al.* [68].

DNA methylation is a clever epigenetic mechanism to silence genes heritably without change of the DNA sequence. The DNA repair protein O6-Methylguanine DNA methyltransferase, also termed O6-alkylguanine DNA alkyltransferase (MGMT/AGT), protects healthy cells against mutagenesis by removing alkylating lesions at position O6 of guanine [1, 70]. This same protein is overexpressed in leukemia [71] and is implicated in resistance against chemotherapeutic alkylating agents: low MGMT/AGT expression in cancer cells is correlated with higher survival rate [1, 70]. It would be important to consider inhibition of the MGMT/AGT protein to sensitize cancer cells for therapeutics. Direct targeting of cancer cells would solve the toxicity problem of MGMT/AGT inhibition. Research has been done on other compounds inhibiting MGMT/AGT such as 8-aza- and 4-bromoethyl derivatives. These are more efficient than benzylguanine-based inhibitors of MGMT/AGT, but are not tumor specific. Another compound 4-(benzyloxy)2,6-diamino-5-nitrosopyridine was promising but had a very short half-life *in vivo*. A combination of O6-benzylguanine-based inhibitors of MGMT/AGT with alkylating agents, however, improved the efficacy of treatment of cancer sufficiently [1].

Nucleoside analogs are a class of therapeutics (e.g. cytarabine) that is effective against hematological malignancies. An antileukemic drug such as the nucleoside analog 2'-*C*-cyano- *al.* have done research on the resistance against therapeutic drugs targeting cancer, specifically on repairing CNDAC induced double strand breaks. Ataxia telangiectasia mutated (ATM) kinase, the activator of homologous recombination, was essential for cell survival after CNDAC treatment [73]. If ATM is crucial for surviving CNDAC treatment, targeting ATM might be a solution to this type of drug resistance.

Cancer cells repair their DNA damage by the transcription coupled NER (TC-NER). In excision repair cross-complementing rodent repair deficiency (ERCC) 6 deficient cells, which initiates the TC-NER pathway, clonogenic selection occurs for sensitivity to CNDAC. Endonuclease XPF and XPF partner protein ERCC1 deficient cell lines showed a 2.6- and 4.6-fold sensitivity in response to CNDAC [73]. From this could be concluded that targeting XPF and ERCC1 leads to lower drug resistance and that the TC-NER pathway causes resistance against CNDAC.

Resistance against chemotherapy is also implicated by enhancement of non-homologous end joining repair capacity [74]. Anti-proliferation drug D-501036 induces double strand breaks in various types of cancer. Yang *et al.* has performed experiments to show induced resistance against D-501036. KB-derived (human cervical carcinoma) cells resistant to D-501036 exhibited increased DNA rejoining ability through upregulation of non-homologous end joining repair. In conclusion, epigenetics, TC-NER and non-homologous end joining repair plays an important role in cancer therapeutics resistance. It would be interesting to target methylation and non-homologous end joining repair proteins to revert the resistant phenotype.

Altogether, DNA repair can contribute to resistance against chemotherapy by repairing DNA damage induced by cancer therapy, protecting the cell against mutagenesis (MGMT/AGT), homologous recombination and end joining DNA repair. Targeting these systems could lead to eliminating all LSCs.

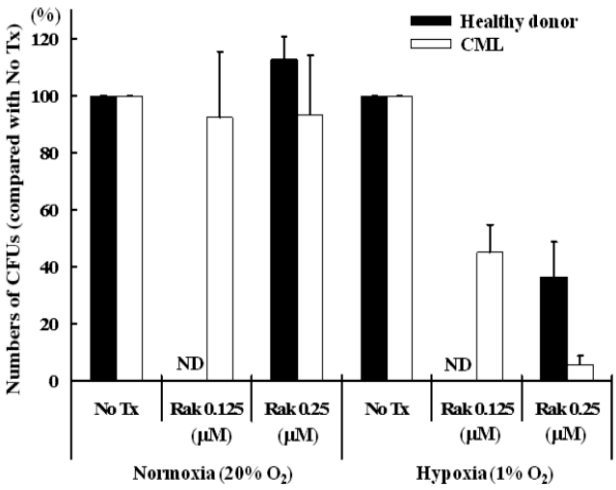
2.4 The Hypoxic Microenvironment

Hypoxia, low levels of oxygen, is a known characteristic of solid tumors and leukemia resulting in the increase in drug resistance. Hypoxia influences the metabolism of the cell, changes the cell cycle and enhances resistance to death stimuli. High level of hypoxia in cancer patients is correlated with poor prognosis. The bone marrow microenvironment is known to be hypoxic which allows leukemic cells to become insensitive to death stimuli, but also protects HSCs from DNA damage via reactive oxygen species (ROS) and keeping the cells in resting phase [75].

There are several reasons why hypoxia induces drug resistance. Some chemotherapeutics need oxygen in order to be active because hydroxyl radicals are produced to induce DNA damage [76]. The hypoxic environment results in a G1 arrest [77]. This G1 arrest or quiescent state leads to resistance against drugs that target proliferating cells. Lastly, PGP, discussed before, is proven to be regulated by hypoxia [78, 79]. PGP is induced by a hypoxic environment because the *MDR1* gene has a hypoxia-inducible factor (HIF)-1 bindings site. In the hypoxic environment there is more production of HIF-1. Inhibition of the expression of HIF-1 showed significant inhibition of the MDR1 protein PGP [78] and lowers therapy resistance.

In order to target LSCs that are responsible for relapse in patients, a hypoxia-selective cytotoxic compound that is produced naturally by the *Micromonospora* bacteria strain, Rakicidin A [80], was tested in CML cells with SC-like features. CML cells continuously cultured at 1% O_2 and the subclones (hypoxia adapted cells) that survived these hypoxic conditions were treated with Rakicidin A. These cells showed shrinkage, nuclear condensation and fragmentation which indicates that these cells are in apoptosis. Hypoxia adapted cells are less viable when treated with up to over 7.5 μM Rakicidin A compared to parental cells that are not hypoxia adapted. Apoptosis by Rakicidin A was induced via caspase dependent and mitochondria dependent pathways, as proved by caspase-3 inhibition, and the resulting delay in apoptosis [75]. The effect of combination therapy of Rakicidin A and Imatinib was tested in hypoxia adapted cells and in non hypoxia adapted cells. Combined chemotherapy using Rakicidin A and Imatinib was synergistic in hypoxia adapted cells, meaning cell death was induced in LSCs. This combined chemotherapy was rather antagonistic than synergistic in non-hypoxia adapted cells showing that Rakicidin A and Imatinib specifically induces cell death in hypoxia adapted cells [75].

The ideal therapeutic will eliminate the LSC while saving the HSC. A way to achieve this might be to develop a drug that is inactive until it reaches a hypoxic area and then specifically targets LSCs. The hypoxic cytotoxin tirapazamine is a drug that is inactive until it reaches hypoxic area and has been proven to work in lung cancer [81]. However, Rakicidin A seems to specifically target LSCs while protecting HSCs. The influence of Rakicidin A on normal HSCs compared to CML LSCs was tested by Takeuchi *et al.* (figure 5), and it seems that Rakicidin A specifically targets CML cells.



Normal HSCs treated with 0.25μM Rakicidin A in normoxic conditions (20% O_2) had 113% colony forming units (CFU) against 36% CFU in hypoxic conditions (1% O_2). This seems a big difference, although in the hypoxic condition there is a high survival of normal HSCs (36% CFU) compared to the survival of CML cells (6%) when treated with 0.25μM Rakicidin A [75]. It is not known yet in which pathway Rakicidin A works. It has been proven that it does not influence HIF-1 transcriptional activity [79].

Figure 5. **Colony Formatting Units of normal HSCs and CML cells treated with Rakicidin A (Rak).** The inhibitory effect of Rak on normal HSCs (healthy donor, black bars) is low compared to the effect on CML cells (white bars) in hypoxia. In normoxia Rak does not seem to have any influence on HSCs or CML cells, proving the specific mechanism of Rak in hypoxia. ND, not done [75].

It does not seem that Rakicidin A has been tested on LSCs cells derived from AML patients yet, or even tested in AML patients. It is also not known how Rakicidin A works on the molecular level. This anti-cancer drug gives high expectance on eliminating LSCs since these are in a hypoxic environment and more research should be done on this topic.

The HIF pathway may be a molecular target for the elimination of LSCs. HIFs are transcription factors that mediate the response to hypoxia [82] and HIF2α is found to be critical for tumorigenicity of glioma SCs [83]. HIF1 transcriptional activity is increased in hypoxic cells [82] and specifically HIF1α is expressed in cancer types, believed to protect the cancer cells against hypoxia, and induces angiogenesis [39]. Hypoxia induces drug resistance because of a lack of hydroxyl radicals needed for certain chemotherapeutics [76], a block in cell cycle and the induction of quiescence [77] and because HIF-1 induces transcription of the *MDR1* gene and production of PGP [78]. HIF-1α is highly expressed in proliferating cancer cells such as lung, colon, breast, gastric, skin, ovarian, pancreatic, prostate, and renal carcinomas [84]. In contrast to the finding that hypoxia induces upregulation of HIF-1 and quiescence [77], is the finding that cells that are induced to proliferate by growth factors such as insulin-like growth factor (IGF)-1 and IGF-2 [85] and by plating cells low-density [82] have an upregulated HIF-1α expression.
It was discussed that hypoxia is believed to inhibit proliferation by inducing G1 arrest [77], but found by several research groups hypoxia promotes proliferation [82, 84, 85]. These are opposite findings but could be explained by the various cell types used. Where HIF-1α was increasingly expressed in the proliferating cancer cells, these are possibly not CSCs. It might be that hypoxia induces drug resistance by inducing quiescence in LSCs while HIF-1α induces proliferation in the bulk of the tumor cells. HIF-1a might be expressed in both celltypes however with different functions.

3 | Concluding remarks

Therapy resistance is an important issue in the treatment of leukemic patients. However the bulk of the cancer cells are usually efficiently targeted by chemotherapy, a rare population of cells often survives. CSCs have been thought to be the cells that are more resistant to conventional chemotherapy within tumors as compared to non, more differentiated CSCs. These rare and quiescent CSCs can be resistant due to many mechanisms of functional cellular dysregulation and LSCs are responsible for the formation of relapse in AML patients [1]. Most chemotherapy treatments target the cancer cells and induce death by disruption of the process of mitosis in highly proliferative cells. Various studies studied the dormancy of LSCs and the involvement of dormancy in chemotherapy resistance [54]. Quiescence is a feature of LSCs and responsible for part of the therapy resistance seen by treatment with cell-cycle dependent drugs.

In this report, different mechanisms are discussed in order to possibly activate quiescent LSCs. The most important characteristics of LSCs are that they are much like normal SCs: they have the ability to self-renew, proliferate, differentiate and sustain the (tumor) population [4]. INFα, G-CSF and As_2O_3 are compounds able to awaken SCs from dormancy and have been successfully brought LSCs into cell cycle. Two-step combination therapy could lead to elimination of LSCs in AML patients, first the activation of LSCs with either INFα, G-CSF or As_2O_3 and subsequently treating the cancer with chemotherapeutics [15, 18, 41].
Membrane pumps able to efflux toxic products out of the cell are normally used to protect healthy cells from apoptosis. However cancer cells use this system to bypass cancer therapeutics induced cell death. PGP is such a pump and can be targeted by using shRNA against the *MDR1* mRNA, coupled to MNPs to aim specific to the cancer cells [55, 58, 59].
Lastly, the DNA repair mechanism and the hypoxic microenvironment contribute to the MDR phenotype as well. When DNA repair is disorganized and not functioning properly, some chemotherapeutics are not able to work. DNA damage induces by chemotherapeutics can be repaired by the DNA repair system [69].
In the bone marrow, hypoxia keeps HSCs and LSCs quiescent and as a consequence drug resistant. The overcoming of the hypoxic microenvironment by specific therapeutics would be interesting for LSCs specific therapy [75]. Figure 6 states an overview of the various possible causal mechanisms of LSCs therapy resistance as discussed in this report.

Due to the heterogeneity of AML, it is very important to consider personalized targeted therapy in a two-step way. The targeting of the mutated cells responsible for relapse is the best therapeutic option. The two-step model is important because by first activating dormant LSCs, conventional chemotherapy is able to eliminate all cancer cells. By using personalized medicine, in every patient targeting the resistant causing mechanism, will be the most promising therapy eliminating LSCs. Because there are various mechanism for LSCs to become resistant and cause relapse it will be most promising to use combination therapies targeting the various resistant mechanism.

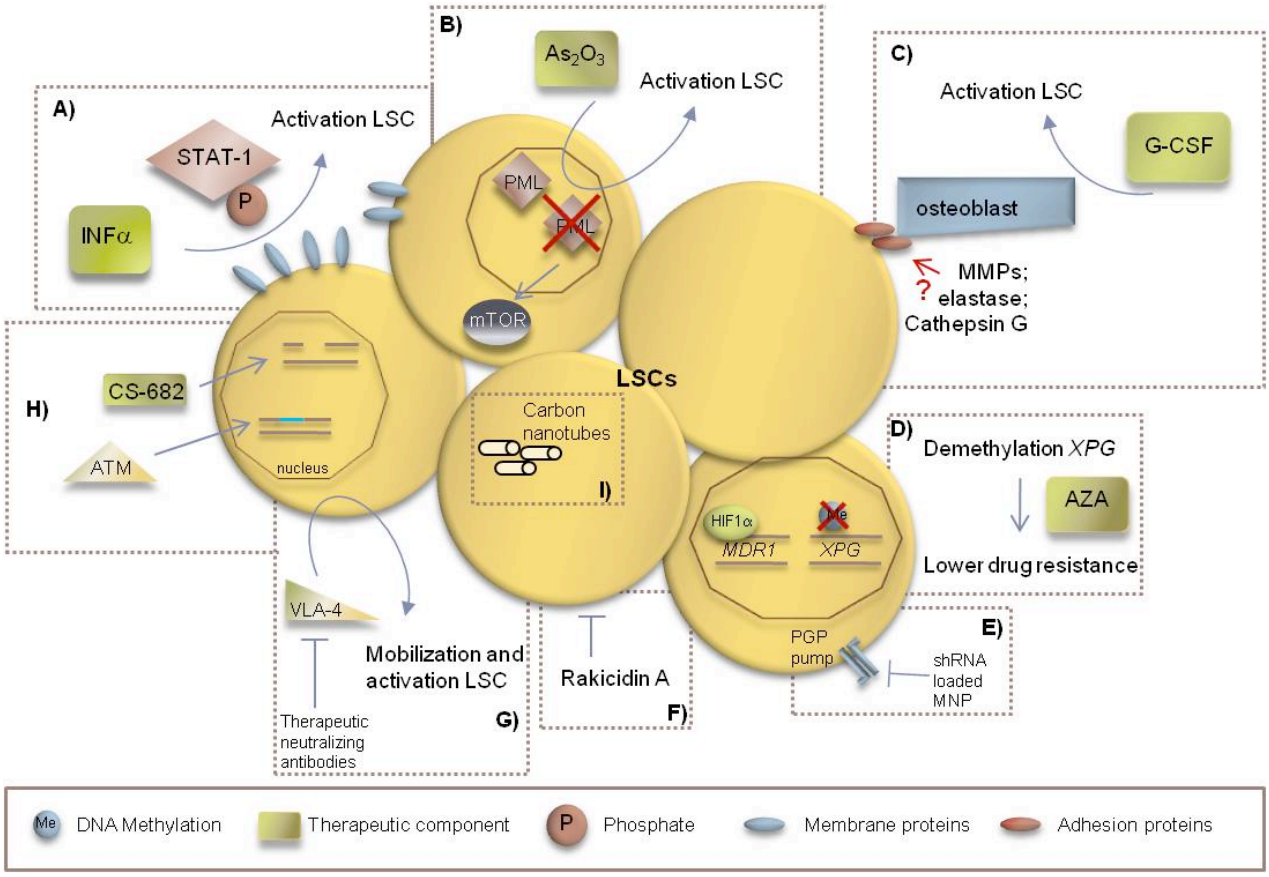


Figure 6. **Targeting the drug resistant LSC. A)** INFα is responsible for the phosphorylation of STAT-1 and transcription and activation of INFα target genes. Also, it seems that INFα interacts with the niche via membrane proteins. **B)** As_2O_3 causes the degradation of the nuclear factor PML, which encodes a tumor suppressor highly expressed in HSCs. The degradation of this factor leads to increased mTOR activity and escape from quiescence. **C)** It is not clear how G-CSF influences the activity of the LSC, but it is seen in HSCs that MMPs, elastase and cathepsin G are able to activate the cell by interacting with adhesion proteins between the LSC and osteoblasts in the micro environment [43-45]. **D)** The DNA repair system is required for the chemotherapeutic drug nemorubicin to work. Methylation of the *XPG* gene is resistance mechanism by decreasing the activity of the DNA repair system. By demethylation of *XPG*, drug resistance could be inhibited. AZA is a compound known to demethylate the *XPG* gene, which has been proven to lead to decreased drug resistance [69]. **E)** The PGP pump is able to efflux toxic components out of the cell to protect the cell from unnecessary apoptosis. Leukemic SP cells misuse the PGP pump to increase chemotherapy resistance [27, 54]. The PGP pump originates from *MDR1*, regulated by HIF1α [78]. In order to sensitize the resistant cancer cells, oligodeoxynucleotides or shRNA loaded magnetic nano particles (MNPs) targeting the PGP-mRNA could be used to down regulate the PGP pump, and has been proved to sensitize the cells for vinblastine [55, 58, 59]. Also triggering HIF1α regulates the expression of the PGP pump [78]. Besides using shRNA loaded MNPs to trigger the PGP pump, also Daunorubicin loaded MNPs seemed to overcome multi drug resistance *in vivo* [60]. **F)** Rakicidin A is a cytotoxic compound, found to target cells in hypoxic environment. Rakicidin A is responsible for apoptosis of CML cells with SC-like features, but does not harm HSCs. Therefore it is attractive to state that Rakicidin A may also target AML LSCs. **G)** It has been found that VLA-4 targeting, by e.g. therapeutic antibodies, LSCs are mobilized and activated from quiescence. **H)** CS-682 is a nucleoside analog, therapy used for the treatment of leukemia by inducing single strand DNA breaks [72]. However, ATM kinase seems to be responsible for survival of leukemic cells by apparently activation of homologous recombination and DNA repair [73]. When ATM could be triggered, CS-682 might work more effectively against leukemia through DNA damage. **I)** Another way to bypass drug pumps, is using carbon nanotubes loaded with chemotherapeutics. The carbon nanotube could be conjugated with a LSC specific marker in order to trigger the LSCs. The advantage is that the cell is not able to eject the nanotubes [61, 62, 65].

Literature

1. Juillerat, A. & Juillerat-Jeanneret, L. S-alkylthiolation of O6-methylguanine-DNA-methyltransferase (MGMT) to sensitize cancer cells to anticancer therapy. *Expert Opin. Ther. Targets* **11**, 349-361 (2007).

2. Schwarz-Cruz-y-Celis, A. & Melendez-Zajgla, J. Cancer stem cells. *Rev. Invest. Clin.* **63**, 179-186 (2011).

3. Lane, S. W., Scadden, D. T. & Gilliland, D. G. The leukemic stem cell niche: current concepts and therapeutic opportunities. *Blood* **114**, 1150-1157 (2009).

4. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* **3**, 730-737 (1997).

5. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111 (2001).

6. Soltysova, A., Altanerova, V. & Altaner, C. Cancer stem cells. *Neoplasma* **52**, 435-440 (2005).

7. McCulloch, E. A. & Till, J. E. Perspectives on the properties of stem cells. *Nat. Med.* **11**, 1026-1028 (2005).

8. Park, C. H., Bergsagel, D. E. & McCulloch, E. A. Mouse myeloma tumor stem cells: a primary cell culture assay. *J. Natl. Cancer Inst.* **46**, 411-422 (1971).

9. Bergsagel, D. E. & Valeriote, F. A. Growth characteristics of a mouse plasma cell tumor. *Cancer Res.* **28**, 2187-2196 (1968).

10. Zou, G. M. Cancer stem cells in leukemia, recent advances. *J. Cell. Physiol.* **213**, 440-444 (2007).

11. Kleinsmith, L. J. & Pierce, G. B.,Jr. Multipotentiality of Single Embryonal Carcinoma Cells. *Cancer Res.* **24**, 1544-1551 (1964).

12. Pierce, G. B. & Wallace, C. Differentiation of malignant to benign cells. *Cancer Res.* **31**, 127-134 (1971).

13. Wang, J. C. & Dick, J. E. Cancer stem cells: lessons from leukemia. *Trends Cell Biol.* **15**, 494-501 (2005).

14. Pierce, G. B. & Speers, W. C. Tumors as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation. *Cancer Res.* **48**, 1996-2004 (1988).

15. Essers, M. A. & Trumpp, A. Targeting leukemic stem cells by breaking their dormancy. *Mol. Oncol.* **4**, 443-450 (2010).

16. Lowenberg, B., Griffin, J. D. & Tallman, M. S. Acute myeloid leukemia and acute promyelocytic leukemia. *Hematology Am. Soc. Hematol. Educ. Program.*, 82-101 (2003).

17. van Rhenen, A. *et al.* High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. *Clin. Cancer Res.* **11**, 6520-6527 (2005).

18. Saito, Y. *et al.* Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nat. Biotechnol.* **28**, 275-280 (2010).

19. Trumpp, A., Essers, M. & Wilson, A. Awakening dormant haematopoietic stem cells. *Nat. Rev. Immunol.* **10**, 201-209 (2010).

20. Ishikawa, F. *et al.* Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat. Biotechnol.* **25**, 1315-1321 (2007).

21. Estey, E. & Dohner, H. Acute myeloid leukaemia. *Lancet* **368**, 1894-1907 (2006).

22. Schoofs, T. & Muller-Tidow, C. DNA methylation as a pathogenic event and as a therapeutic target in AML. *Cancer Treat. Rev.* (2011).

23. Krivtsov, A. V. *et al.* Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* **442**, 818-822 (2006).

24. Taussig, D. C. *et al.* Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* **115**, 1976-1984 (2010).

25. Cavalcanti, G. B.,Jr *et al.* Coexpression of p53 protein and MDR functional phenotype in leukemias: the predominant association in chronic myeloid leukemia. *Cytometry B. Clin. Cytom* **61**, 1-8 (2004).

26. Hirschmann-Jax, C., Foster, A. E., Wulf, G. G., Goodell, M. A. & Brenner, M. K. A distinct "side population" of cells in human tumor cells: implications for tumor biology and therapy. *Cell. Cycle* **4**, 203-205 (2005).

27. Wulf, G. G. *et al.* A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood* **98**, 1166-1173 (2001).

28. Norwood, K. *et al.* An in vivo propagated human acute myeloid leukemia expressing ABCA3. *Leuk. Res.* **28**, 295-299 (2004).

29. Wulf, G. G. *et al.* ABC transporter ABCA3 is expressed in acute myeloid leukemia blast cells and participates in vesicular transport. *Haematologica* **89**, 1395-1397 (2004).

30. Kastan, M. B. *et al.* Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood* **75**, 1947-1950 (1990).

31. Pearce, D. J. *et al.* Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. *Stem Cells* **23**, 752-760 (2005).

32. Ginestier, C. *et al.* ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell. Stem Cell.* **1**, 555-567 (2007).

33. Hilton, J. Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Res.* **44**, 5156-5160 (1984).

34. Magni, M. *et al.* Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer. *Blood* **87**, 1097-1103 (1996).

35. Valent, P. Targeting of leukemia-initiating cells to develop curative drug therapies: straightforward but nontrivial concept. *Curr. Cancer. Drug Targets* **11**, 56-71 (2011).

36. Matsunaga, T. *et al*. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat. Med.* **9**, 1158-1165 (2003).

37. Suwalsky, M., Hernandez, P. L., Villena, F. & Sotomayor, C. P. The anticancer drug cytarabine does not interact with the human erythrocyte membrane. *Z. Naturforsch. C.* **58**, 885-890 (2003).

38. Borden, E. C. *et al*. Interferons at age 50: past, current and future impact on biomedicine. *Nat. Rev. Drug Discov.* **6**, 975-990 (2007).

39. de Veer, M. J. *et al*. Functional classification of interferon-stimulated genes identified using microarrays. *Journal of Leukocyte Biology* **69**, 912-920 (2001).

40. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227-264 (1998).

41. Essers, M. A. *et al*. IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).

42. Rousselot, P. *et al*. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* **109**, 58-60 (2007).

43. Morrison, S. J., Wright, D. E. & Weissman, I. L. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 1908-1913 (1997).

44. Petit, I. *et al*. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat. Immunol.* **3**, 687-694 (2002).

45. Heissig, B. *et al*. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* **109**, 625-637 (2002).

46. Ito, K., Bernardi, R. & Pandolfi, P. P. A novel signaling network as a critical rheostat for the biology and maintenance of the normal stem cell and the cancer-initiating cell. *Curr. Opin. Genet. Dev.* **19**, 51-59 (2009).

47. Lallemand-Breitenbach, V. *et al*. Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. *J. Exp. Med.* **193**, 1361-1371 (2001).

48. Ito, K. *et al*. PML targeting eradicates quiescent leukaemia-initiating cells. *Nature* **453**, 1072-1078 (2008).

49. Bram, S. *et al*. Vitamin C preferential toxicity for malignant melanoma cells. *Nature* **284**, 629-631 (1980).

50. Chen, G. Q. *et al*. In vitro studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR alpha/PML proteins. *Blood* **88**, 1052-1061 (1996).

51. Zheng, X. *et al*. Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARalpha-positive leukemic stem cells. *Haematologica* **92**, 323-331 (2007).

52. Han, S. S. *et al*. L-ascorbic acid represses constitutive activation of NF-kappaB and COX-2 expression in human acute myeloid leukemia, HL-60. *J. Cell. Biochem.* **93**, 257-270 (2004).

53. Ehninger, A. & Trumpp, A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J. Exp. Med.* **208**, 421-428 (2011).

54. Eyler, C. E. & Rich, J. N. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J. Clin. Oncol.* **26**, 2839-2845 (2008).

55. Chen, B. A. *et al*. Reversal of multidrug resistance by magnetic Fe3O4 nanoparticle copolymerizating daunorubicin and MDR1 shRNA expression vector in leukemia cells. *Int. J. Nanomedicine* **5**, 437-444 (2010).

56. Leonard, G. D., Polgar, O. & Bates, S. E. ABC transporters and inhibitors: new targets, new agents. *Curr. Opin. Investig Drugs* **3**, 1652-1659 (2002).

57. Hegewisch-Becker, S. MDR1 reversal: criteria for clinical trials designed to overcome the multidrug resistance phenotype. *Leukemia* **10 Suppl 3**, S32-S38 (1996).

58. Marthinet, E., Divita, G., Bernaud, J., Rigal, D. & Baggetto, L. G. Modulation of the typical multidrug resistance phenotype by targeting the MED-1 region of human MDR1 promoter. *Gene Ther.* **7**, 1224-1233 (2000).

59. Lin, B. L., Shen, X. D. & Cui, S. Application of nanosized Fe3O4 in anticancer drug carriers with target-orientation and sustained-release properties. *Biomed. Mater.* **2**, 132-134 (2007).

60. Chen, B. A. *et al*. Daunorubicin-loaded magnetic nanoparticles of Fe3O4 overcome multidrug resistance and induce apoptosis of K562-n/VCR cells in vivo. *Int. J. Nanomedicine* **4**, 201-208 (2009).

61. Li, R., Zou, H., Xiao, H. & Wu, R. Carbon nanotubes as intracellular carriers for multidrug resistant cells studied by capillary electrophoresis-laser-induced fluorescence. *Methods Mol. Biol.* **625**, 153-168 (2010).

62. Beg, S. *et al*. Advancement in carbon nanotubes: basics, biomedical applications and toxicity. *J. Pharm. Pharmacol.* **63**, 141-163 (2011).

63. Martin, C. R. & Kohli, P. The emerging field of nanotube biotechnology. *Nat. Rev. Drug Discov.* **2**, 29-37 (2003).

64. Murthy, N., Thng, Y. X., Schuck, S., Xu, M. C. & Frechet, J. M. A novel strategy for encapsulation and release of proteins: hydrogels and microgels with acid-labile acetal cross-linkers. *J. Am. Chem. Soc.* **124**, 12398-12399 (2002).

65. Wang, C. H. *et al*. Photothermolysis of glioblastoma stem-like cells targeted by carbon nanotubes conjugated with CD133 monoclonal antibody. *Nanomedicine* **7**, 69-79 (2011).

66. Choi, S. U. *et al*. The bisbenzylisoquinoline alkaloids, tetrandine and fangchinoline, enhance the cytotoxicity of multidrug resistance-related drugs via modulation of P-glycoprotein. *Anticancer Drugs* **9**, 255-261 (1998).

67. Wang, F. P., Wang, L., Yang, J. S., Nomura, M. & Miyamoto, K. Reversal of P-glycoprotein-dependent resistance to vinblastine by newly synthesized bisbenzylisoquinoline alkaloids in mouse leukemia P388 cells. *Biol. Pharm. Bull.* **28**, 1979-1982 (2005).

68. Helleday, T., Lo, J., van Gent, D. C. & Engelward, B. P. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst)* **6**, 923-935 (2007).

69. Sabatino, M. A. *et al*. Down-regulation of the nucleotide excision repair gene XPG as a new mechanism of drug resistance in human and murine cancer cells. *Mol. Cancer.* **9**, 259 (2010).

70. Gerson, S. L. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat. Rev. Cancer.* **4**, 296-307 (2004).

71. Liu, L. & Gerson, S. L. Targeted modulation of MGMT: clinical implications. *Clin. Cancer Res.* **12**, 328-331 (2006).

72. Hanaoka, K. *et al*. Antitumor activity and novel DNA-self-strand-breaking mechanism of CNDAC (1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl) cytosine) and its N4-palmitoyl derivative (CS-682). *Int. J. Cancer* **82**, 226-236 (1999).

73. Liu, X. *et al*. Homologous recombination as a resistance mechanism to replication-induced double-strand breaks caused by the antileukemia agent CNDAC. *Blood* **116**, 1737-1746 (2010).

74. Yang, Y. N. *et al*. Enhancement of non-homologous end joining DNA repair capacity confers cancer cells resistance to the novel selenophene compound, D-501036. *Cancer Lett.* **309**, 110-118 (2011).

75. Takeuchi, M. *et al*. Rakicidin A effectively induces apoptosis in hypoxia adapted Bcr-Abl positive leukemic cells. *Cancer. Sci.* **102**, 591-596 (2011).

76. Doroshow, J. H. Role of hydrogen peroxide and hydroxyl radical formation in the killing of Ehrlich tumor cells by anticancer quinones. *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4514-4518 (1986).

77. Brown, J. M. & Giaccia, A. J. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res.* **58**, 1408-1416 (1998).

78. Comerford, K. M. *et al*. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. *Cancer Res.* **62**, 3387-3394 (2002).

79. Yamazaki, Y., Kunimoto, S. & Ikeda, D. Rakicidin A: a hypoxia-selective cytotoxin. *Biol. Pharm. Bull.* **30**, 261-265 (2007).

80. McBrien, K. D. *et al*. Rakicidins, new cytotoxic lipopeptides from Micromonospora sp. fermentation, isolation and characterization. *J. Antibiot. (Tokyo)* **48**, 1446-1452 (1995).

81. Brown, J. M. The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. *Cancer Res.* **59**, 5863-5870 (1999).

82. Semenza, G. L. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit. Rev. Biochem. Mol. Biol.* **35**, 71-103 (2000).

83. Wang, Y., Liu, Y., Malek, S. N., Zheng, P. & Liu, Y. Targeting HIF1alpha eliminates cancer stem cells in hematological malignancies. *Cell. Stem Cell.* **8**, 399-411 (2011).

84. Zhong, H. *et al*. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.* **59**, 5830-5835 (1999).

85. Feldser, D. *et al*. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res.* **59**, 3915-3918 (1999).