Targeting Nutrient Transporters in Acute Myeloid Leukemia

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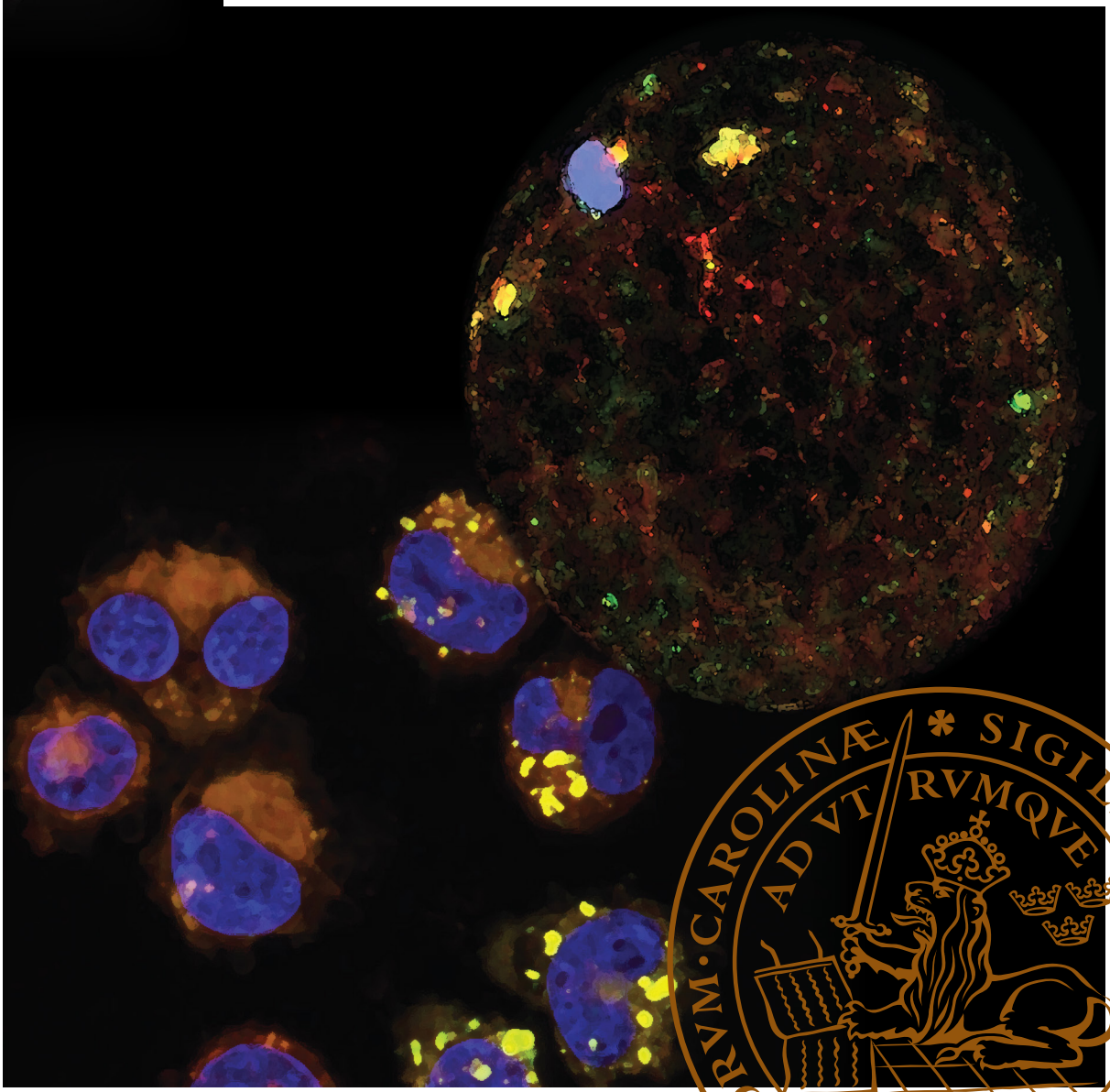

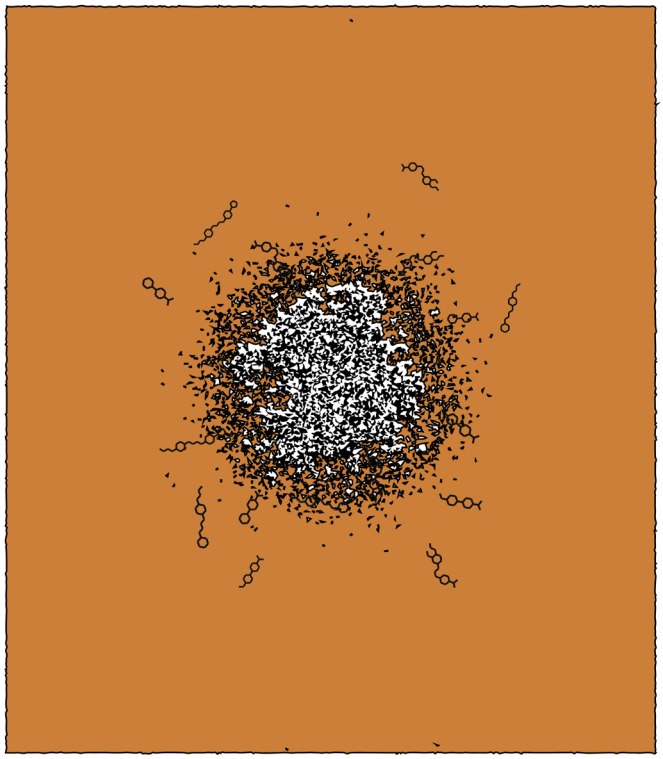

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Targeting Nutrient Transporters in Acute Myeloid Leukemia

Hannah Åbacka

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DOCTORAL DISSERTATION

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Abstract:

Acute myeloid leukemia (AML) leads to the accumulation of immature myeloid blasts in the blood and bone marrow. It is the most common acute leukemia in adults and second most common in children. Standard treatment has not changed substantially since the 1970s and new therapies are needed that are effective and manageable for the patients. In this thesis cell surface transporters have been studied that are involved in the transport of glucose, fatty acid (FA) or glycerol across the cell membrane. The aim has been to target them with new small-molecule inhibitors in AML cells.

One type of transporters studied are the glucose transporters (GLUTs). The insulin-sensitive translocation of GLUT4 was investigated and its expression and co-localization with its tethering protein ASPL in human primary adipocytes determined. Another transporter of glucose, GLUT1, was studied in AML cell lines. Two new GLUT1 inhibitors, PGL-13 and PGL-14, were found to have negative effects on cell viability in an AML cell line with high GLUT1 expression. This effect was synergistic with the standard chemotherapy treatment Cytarabine (Ara-C).

Moreover, transporters involved in lipid transport have been investigated. The inhibitor SMS121 was found to bind to CD36, a receptor responsible for lipid uptake, and shown to reduce the uptake of a fluorescent FA analogue in a cell line with high protein expression of CD36. The inhibition also led to decreased AML cell viability. Lastly, the compound Z433927330 has been evaluated for its inhibition of the glycerol channel aquaporin 7 (AQP7). Z433927330 lowered the proliferation of an acute promyelocytic (APL) cell line confirmed to express AQP7.

In conclusion, transporters involved in the uptake of nutrients are potential future targets in AML. The inhibitors tested show promising results by blocking the transport of glucose, FA or glycerol and they all led to detrimental effects in AML cell lines. The inhibitors serve as a framework for assessing the potential of metabolic inhibitors in sensitizing existing cancer thearapies.

Key words: Acute myeloid leukemia, metabolism, transporters, GLUT1, GLUT4, CD36, AQP7, ASPL, small-molecule inhibitor, glucose, fatty acid, glycerol

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Hannah Åbacka

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Paper IV

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[*] Authors contributed equally

[#] Shared last authors

Abbreviations

2DG	2-Deoxy-D-glucose
3-KAT	3-Ketoacylthiolase
Acetyl-CoA	Acetyl Coenzyme A
ADMET	Absorption, distribution, metabolism, excretion, toxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
AQP	Aquaporin
Ara-C	Cytarabine
ASPL	Alveolar soft-part sarcoma locus
ATP	Adenosine triphosphate
BM	Bone marrow
BODIPY	4,4-Difluoro-3a,4a-diaza-s-indacene
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
DHODH	Dihydroorotate dehydrogenase
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
e^-	Electron

ECAR	Extracellular acidification rate
ETC	Electron transport chain
FA	Fatty acid
$FADH_2$	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FAT	Fatty acid translocase
FLT3	FMS-like tyrosine kinase 3
GLUT	Glucose transporter
Glycerol-3-P	Glycerol-3-phosphate
GMP	Granulocyte-macrophage progenitor
GSV	GLUT4 storage vesicle
GTP	Guanosine triphosphate
H^+	Proton
HCT	Hematopoietic cell transplantation
HMA	Hypomethylating agent
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HTS	High throughput screening
IC	Inhibitory concentration
ICH	Intracellular helical domain
IDAC	Intermediate dose cytarabine
LCFA	Long-chain fatty acid
SCLSC	Leukemic stem cell
MAT	Marrow adipose tissue
MEP	Megakaryocyte-erythrocyte progenitor
MFS	Major facilitator superfamily
MPP	Multipotent progenitor
NAD	Nicotinamide adenine dinucleotide

NHEJ	Non-homologous end joining
OCR	Oxygen consumption rate
OS	Overall survival
oxLDL	Oxidized low density lipoprotein
OXPHOS	Oxidative phosphorylation
PDX	Patient derived xenograft
PET	Positron emission tomography
PLA	Proximity ligation assay
PLIN1	Perilipin 1
PMA	Phorbol myristate acetate
PPARγ	Peroxisome proliferator activated receptor gamma
PPP	Pentose phosphate pathway
RARA	Retinoic acid receptor alpha
RCA	Rolling circle amplification
ROS	Reactive oxygen species
SCARB3	Scavenger receptor B3
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLC2	Solute carrier 2
SPR	Surface plasmon resonance
SSO	Sulfo-N-succimidyl ester of the long chain FA oleate
SVF	Stromal vascular fraction
T2D	Type 2 diabetes
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TIRF	Total internal reflection fluorescence
TSP-1	Thrombospondin-1
TUG	Tether containing a UBX domain for GLUT4

VLCAD	Very long chain-acylCoA dehydrogenase
WHO	World Health Organization
α-KG	α-Ketoglutarate

Cancer

Cancer – the abnormal, uncontrolled expansion and dissemination of cells. This disease leads to deleterious consequences for the affected and their family. The word itself, cancer, stems from the Greek word for crab, *karkinos*, describing the excessive mass of over proliferating cells spreading into its surroundings. Cancer has always surrounded humanity. It is thought to first have been described in 3000 BC, Egypt[1,2]. Given its long history, much work remains in the effort of getting it eradicated. It is one of the top causes of premature mortality worldwide, leading to 10 million deaths each year[3].

The onset of leukemia

Leukemia (from greek. *Leukos*: white, *haima*: blood) is cancer of the white blood cells, the leukocytes. It is a result of acquired genetic changes that affect the blood cells resulting in their abnormal development[4]. Initiation of over-proliferation and differentiation arrest leads to their outnumbering of normal blood cells[5]. Leukemia can broadly be categorized into myeloid and lymphoid leukemia, depending on which blood cell lineage it affects. It can also be divided according to how fast the disease progresses, being acute or chronic[4].

Normal hematopoiesis

Hematopoiesis is the development of blood cells (Figure 1). All blood cells originate from hematopoietic stem cells (HSCs) which in human adults predominantly are located in the bone marrow (BM)[6,7]. HSCs are defined by having ability to self-renew and being multipotent. Self-renewal makes them able to produce more HSCs while multipotency means that they can be differentiated into any hematopoietic cell type. The HSCs can be divided into two sub-types, long-term HSCs with high self-renewal capacity, which are giving rise to short-term HSCs with lower self-renewal capacity. Next in the differentiation stage are the multipotent progenitor cells (MPPs), which have lost the self-renewal capacity but still have the potency of becoming cells of both myeloid and lymphoid lineages. If an MPP becomes a common lymphoid progenitor cell (CLP) it can be further differentiated into T cells, B cells, natural killer cells or

dendritic cells. If it instead becomes a common myeloid progenitor (CMP) it can further be differentiated into megakaryocyte-erythrocyte progenitor cells (MEPs) and granulocyte-macrophage progenitors (GMPs). From MEP stems the megakaryocyte, thrombocytes and erythrocytes. From GMP one gets the granulocytes (basophil, neutrophil, eosinophil) as well as monocytes, macrophages and dendritic cells[8,9] .

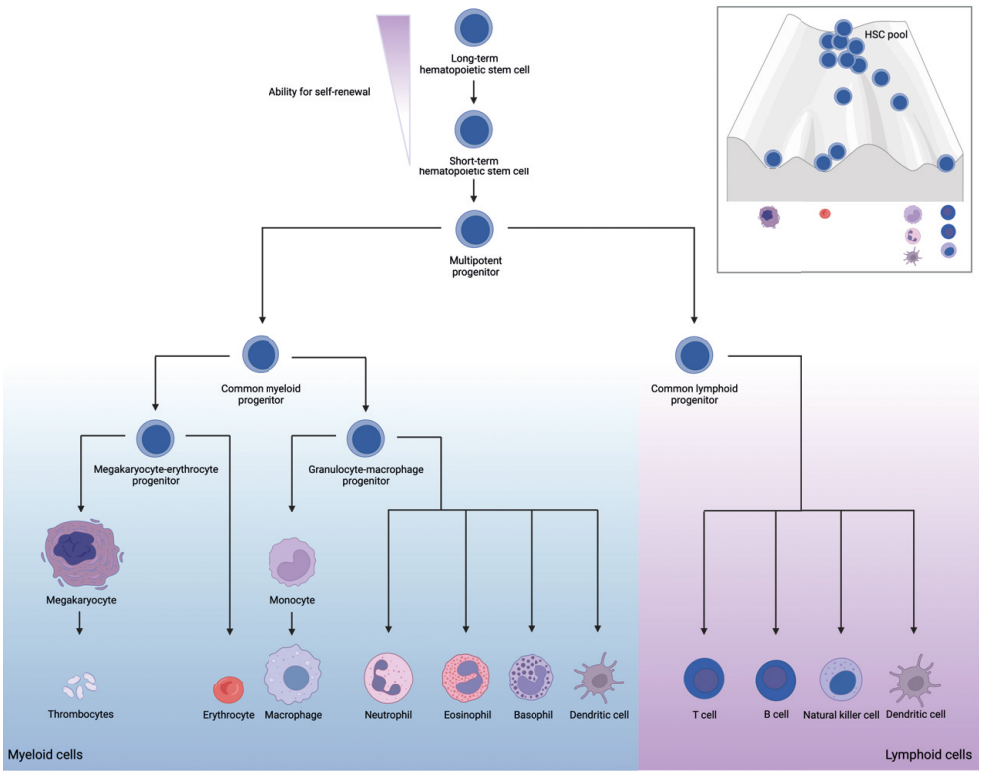


Figure 1 | Hematopoiesis. The differentiation of blood cells from the hematopoietic stem cell to its progenitors, displayed as a traditional hierarchical tree with step-wise differentiation. In the small box the more recent continuum model is showed. (Created with BioRender.com). Modified from:[7–9].

This hematopoiesis model has the shape of a tree with hierarchies and strict differentiations routes. In this context, if a cell is selected for a particular developmental route it commits to that trajectory. For instance, committing to becoming either a myeloid or a lymphoid cell. However, the more information we get from new technologies, especially from single cell data, this hematopoiesis tree is becoming more of a shapeshifter. Inputs are changing all the time giving additional subdivisions of cells and more complex trees. It is questioned if cells follow the steps of a hierarchical tree or if they instead evolve from a pool of HSCs and have more of a continuous differentiation with less strict boundaries, for example as portrayed by the continuum model (Figure 1, box)[7,9].

Leukemic hematopoiesis

Leukemia is caused by chromosomal abnormalities, gene mutations and alterations in chromatin structure due to epigenetic modifications in the blood cells[10]. Additional insights have emerged regarding that the onset of these mutations could arise already in the HSC, affecting their self-renewal, proliferation and differentiation into progenitors[5]. Their quiescent state reduces stress stemming from active cell cycle DNA replication and metabolism. Nevertheless, this condition also generates a preference towards the more error prone non-homologous end joining (NHEJ) DNA repair, resulting in the accumulation of mutations and chromosomal aberrations[11]. The long-lived nature of HSCs also increases the likelihood of mutations to accumulate with age[12].

If a mutation occurs in a HSC that disrupts its control over its self-renewal, it may undergo excessive renewal making it dominate the cell population and have increased propensity for acquiring additional mutations that ultimately may lead to leukemia. The presence of a clonally expanded HSC population can lead to a so-called pre-leukemic state. This has been described as the HSCs obtaining mutations that increases production of progenitors or mutations that inhibit progenitor differentiation. Collective pressure of these mutations and accumulation of further mutations can give rise to a stem cell or stem cell progenitor that have increased production of immature cells. These immature blasts will take over the normal blood cells of the BM and blood[5]. The awareness about the potential existence of a leukemia initiating cell which has the ability of a stem cell to self-renew, has over-proliferative capability and can differentiate into leukemic blasts has given rise to the concept of leukemic stem cells (LSCs)[13]. LSCs are spreading fear due to their enhanced ability to survive cancer therapy and cause relapse[14].

Leukemic cells in their microenvironmental niche

In the bone marrow the HSC is surrounded by the stromal cells. These are for example the endothelial cells lining the BM blood vessels, the osteoblasts generating the bone, the T cells of the immune system and the fat containing cells, called adipocytes[15]. The BM stromal cells release factors that impact HSC proliferation and differentiation[16]. They release signals to support the quiescence and stemness of the HSC (Figure 2)[5]. It is also speculated that the niche cell signals decide if a stem cell should self-renew or produce a more differentiated progeny[17].

The acquired mutations in the pre-leukemic HSC can make it better than its fellow stem cells at meeting the requests of the niche cells, making it have increased self-renewal and clonally expand[5]. The leukemic blasts can release exosomes with signals to change the BM environment, making it more leukemia friendly. They can also affect

the niche signals, making them support leukemic hematopoiesis instead of normal hematopoiesis[18].

Chemotherapy targets the expanding blasts with a purpose of eradication to stop the leukemia progression. However, a big threat lies within the LSCs. They are not as active in the cell cycle and are concealed in the BM as chemotherapy resistant cells, with the possibility of giving rise to leukemia relapse[16]. Additionally, pre-leukemic HSCs remaining after treatment can also be a cause of relapse by acquiring additional mutations[5]. Better treatments are needed to combat the chemo-resistant cells residing in the BM without harming the non-leukemic stromal cells[15]. Instead of targeting the leukemia cells another alternative is to target the niche signals that are supporting the leukemic environment[17].

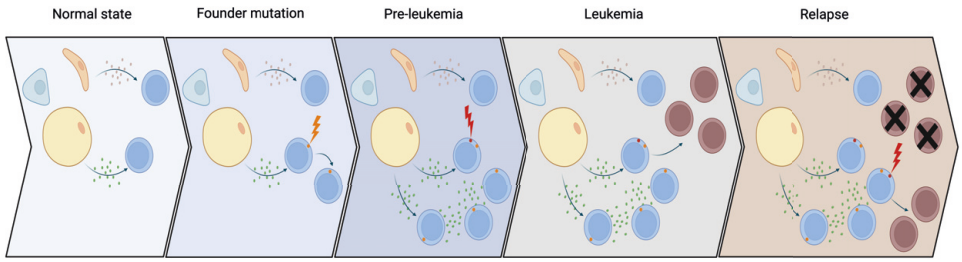


Figure 2 | Leukemia development in the bone marrow niche. In the normal state the microenvironment niche cells are releasing supporting signals for normal development of blood cells (shown in blue). A mutation occurs (shown in orange) and there is a progress to a pre-leukemic hematopoietic stem cell (HSC) with increased self-renewal. The mutation enhances the ability of the pre-leukemic cell and its progeny in coping with the niche signals, giving them advantage to expand more than other stem cells. An additional mutation (shown in red) alters the proliferation rate or differentiation patterns of a pre-leukemic HSC and it can now be considered a leukemic stem cell (LSC). The LSC produces immature blast cells (shown in brown) that take over the bone marrow and starts altering and effecting the other cells leading to a full leukemia outbreak. Chemotheraphy might terminate the blast cells (shown as cross) but hiding in the niche are resistant pre-leukemic HSCs and LSCs that can cause new outbreaks and relapse. (Created with BioRender.com). Modified from:[5].

Adipocytes supporting the microenvironment niche

Included in the BM stroma there are the adipocytes[16]. Adipocytes in the BM increase with age and can occupy more than 70 % of the BM cavity[19,20]. The adipocytes in the marrow adipose tissue (MAT) resemble white adipocytes but with some alterations in gene expression, lipid profile and metabolism. They are for example not affected by calorie restriction. The adipocytes provide HSCs with energy and stem cell factor like growth factors, hormones, cytokines, chemokines, proteins and metabolites that all might support the development and maintenance of leukemic cells[21]. Conversely, the leukemic cells are also thought to influence the adipocytes, imposing them to secrete desirable molecules that can be used as energy by the leukemic cells[22].

Acute myeloid leukemia (AML)

In this thesis the focus is on targeting cell surface receptors in acute myeloid leukemia (AML). In AML, mutations leading to increased proliferation and arrest in differentiation of HSCs or myeloid progenitor cells makes immature myeloid blasts accumulate in the BM[23]. This leads to a deficiency of normal mature cells and symptoms of cytopenia such as increased risk of bleeding, fatigue and infections as consequence. The leukemic cells can also invade other tissues such as in the spleen and central nervous system, with complications related to the affected organs[24].

The challenges of AML

The incidence of AML has increased substantially over the last decades. Globally, around 120000 people are diagnosed with AML every year[25]. The global annual death by AML is almost 100 000 [25], and the numbers are believed to intensify each year[26]. In Sweden approximately 14 out of 100 000 individuals have the disease[27]. AML is the most common acute leukemia in adults and in children it is the second most common after acute lymphoblastic leukemia (ALL)[28]. AML is mostly affecting elderly people, with the median age at diagnosis in Sweden being 72 years[29]. The reason for higher incidence by age is possibly due to the accumulating number of mutations[5]. Main risk factors of AML are smoking, high body mass index and having an occupation where carcinogens like benzene and formaldehyde are used[25].

In 1973 it was reported that a combination treatment of seven days of Cytarabine (Ara-C) and three days of daunorubicin had promising effect against AML[30]. This combined therapy involving Ara-C in conjunction with an anthracycline is commonly referred to as the 7 + 3 regimen. Today, 50 years later, this protocol is still seen as the standard care for AML[31]. Most adult AML patients, 80 %, now reach complete remission. The problem is that half of them will experience relapse. The 5-year overall survival (OS) rate after relapse is only 10 %[15]. The performance status before starting treatment is very important for the outcome[29,32]. Older patients have a worse prognosis. Their health status already before the leukemia diagnosis is generally worse than in younger patients. They often suffer from additional diseases, multidrug resistance and have unfavorable cytogenetics[32]. Pediatric AML has a higher chance of successful outcomes with a 5-year OS of 70 %[33]. Still, the cytotoxic chemotherapy can result in life-long side-effects on multiple organ systems. These children may face future challenges, such as having increased risk of secondary cancers, heart diseases and infertility[34.]

More studies must be conducted to find new, better treatments that can eliminate all leukemic cells and at the same time be gentle on the patient. Adipocytes in the microenvironment niche and leukemic cell metabolism are possible future targets focused on in this thesis.

Cell lines of AML

AML is a heterogenous disease[27] with an expanding array of genomic mutations continuously being added to the list[35]. To increase the accuracy of diagnosis and prognosis the World Health Organization (WHO) started to classify AML by incorporating genetic findings to existing classifications based on cell morphology and maturation level[36,37]. The classifications have evolved over the years with several revised versions where increasing numbers of genetic verdicts have been included. Today many AML cases are classified based on genetic abnormalities[36,38,39,40].

In this thesis I have used the leukemia cell lines THP-1, KG-1 and NB4 to study their protein expression of transporters involved in metabolism and explore their susceptibility to get targeted by small-molecule inhibitors. The cell lines show evidence of AML diversity, harboring different genetics and coming from various types of AML with diverse cell maturity.

The THP-1 cell line was first cultured from the peripheral blood of a 1-year-old boy with acute monocytic leukemia[41]. These cells can be differentiated into macrophage-like cells with stimulation of phorbol myristate acetate (PMA)[42], which is a reason why they often are used for differentiation studies[42]. THP-1 cells have a genetic translocation of chromosomes 9 and 11 leading to the fusion gene *KMT2A::MLLT3*[42], which is common in monocytic leukemia[43]. Other genetic alterations have also been found including several genetic deletions and trisomy of chromosome 8[41,43].

The KG-1 cells were initially obtained from the bone marrow of a 59-year-old male that suffered from erythroleukemia which had evolved into AML. Earlier cytogenetic studies have shown a complex karyotype with several chromosomal translocations and deletions[44,45]. The KG-1 cells are less mature than THP-1 since they resemble myeloblasts and promyelocytes, which are early precursors in the myeloid lineage[46]. Like THP-1 they have ability to be differentiated with PMA to macrophages but are mostly reported to differentiate into cells resembling dendritic cells[47,48].

NB4 is a cell line of acute promyelocytic leukemia (APL). It was acquired from a 20-year-old woman with a translocation of chromosomes 15 and 17, which is characteristic for APL[49]. This translocation fuses the promyelocytic leukemia (*PML*) gene to retinoic acid receptor alpha (*RARA*) gene which results in a PML::RARA fusion protein. This can in turn inactivate myeloid cell differentiation[50,51]. A combination of retinoic acid together with arsenic trioxide can re-initiate cell differentiation by affecting the transcription of *PML::RARA* and enhancing its degradation[51].

The specific genetic changes seen at diagnosis is used to diagnose patients with AML and affect the treatment and prognosis. For example, rearrangements of the *KMT2A* gene, as seen in THP-1 cells, are correlated with intermediate prognosis while the *PML::RARA,* as in the APL cell line, currently has very good prognosis[52]. For the APL subgroup the genetic alteration has made it possible to invent a treatment shown to be

effective. The treatment with retinoic acid and arsenic trioxide has led to remission rates of up to 95 % and more than 80 % of patients are being cured[53]. The genetic background and differentiation level of AML cells can also have impact on the metabolic pathways activated by leukemic cells[54].

Metabolism in normal state and leukemogenesis

From the food we consume, the cells obtain both energy and building materials to sustain their existence and growth. These are tightly regulated processes.

Normal metabolism

The normal, differentiated cell generally uses oxidative phosphorylation (OXPHOS) to acquire its fuel. Here, substrates are converted into energy in the mitochondria in the form of adenosine triphosphate (ATP)[55]. The anabolism and katabolism of nutrients both within and outside the mitochondria involve series of intertwined chemical reactions (Figure 3). A brief introduction will follow to give more insight regarding these events.

Glycolysis

The initial breakdown of glucose, glycolysis, is a chain of reactions in the cell cytoplasm which require ATP, adenosine diphosphate (ADP) and nicotinamide adenine dinucleotide (NAD^+). The net end products of glycolysis from one glucose molecule are two molecules each of pyruvate, ATP and reduced NAD (NADH). The pyruvate is transported into the mitochondrial matrix where it is converted into acetyl Coenzyme A (acetyl-CoA) which enters the tricarboxylic acid (TCA) cycle [55,56.]

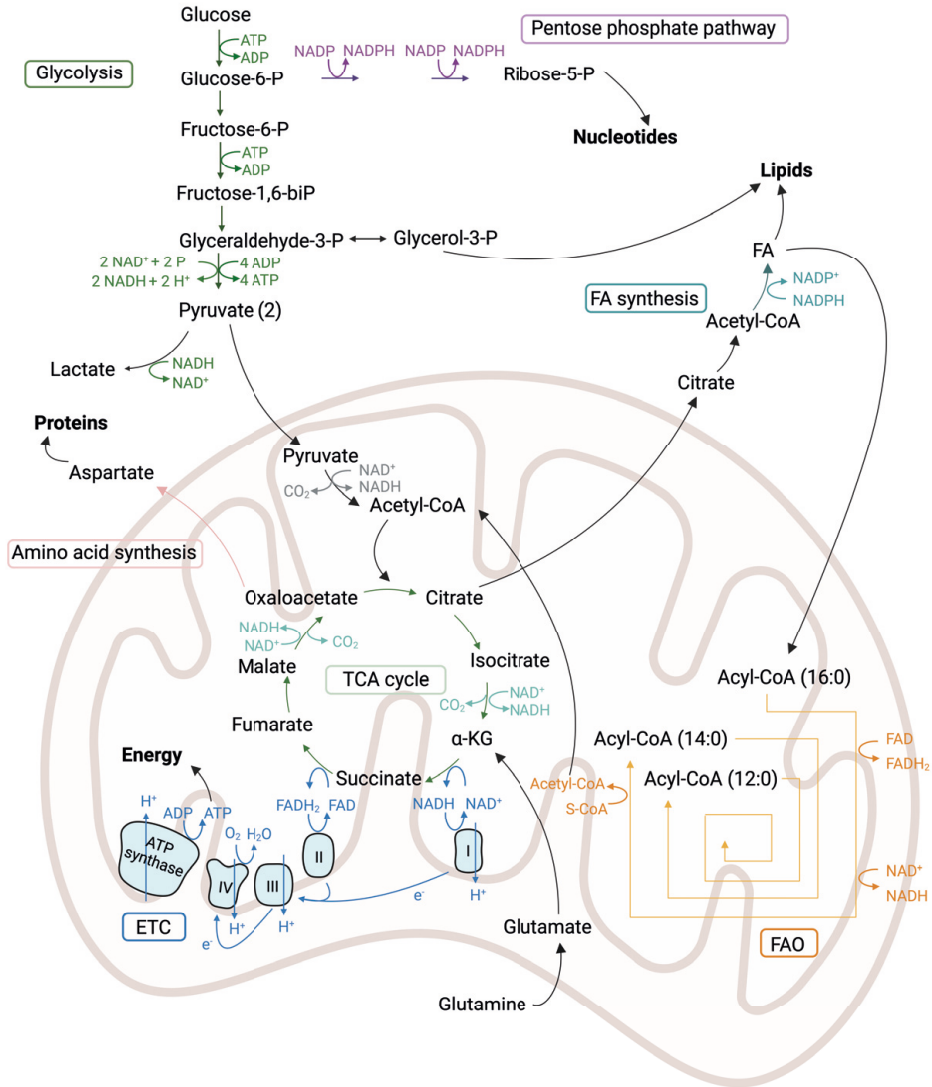


Figure 3 | Metabolism of the cell. These are the core metabolic processes in the mammalian cell and how they are associated with energy, lipid, nucleotide and protein production. The most important substrates, products and steps of each pathway are shown. TCA: tricarboxylic acid, FAO: fatty acid oxidation, ETC: electron transport chain, FA: fatty acid, S-CoA: free coenzyme A. (Created with BioRender.com). Modified from: [55–61].

Tricarboxylic acid cycle

In the TCA cycle, also called the citric acid cycle or Krebs cycle after its founder Sir Krebs[62], acetyl-CoA and oxaloacetate form citrate[55]. This is followed by several oxidation steps which produces NADH, reduced flavin adenine dinucleotide ($FADH_2$), guanosine triphosphate (GTP) and the biproduct CO_2[58,63]. Intermediate products in the TCA cycle can be further processed to form macromolecules[55]. One example is citrate that can be exported from the mitochondria and be converted back to acetyl-CoA and made into fatty acids (FAs) in a process called fatty acid synthesis[64]. Another is the amino acid aspartate, which can be used for both protein and nucleotide synthesis[58]. Various amino acids can be made from the intermediates[65] and can sustain the continuation of the TCA cycle. For example, glutamine is an important amino acid for cell metabolism by being converted to glutamate, which can transfer an amino group to α-ketoglutarate (α-KG) in the TCA cycle[59].

The electron transport chain and OXPHOS

The electron transport chain (ETC) is linked to the TCA cycle and is an important part of cell respiration. ETC is made up of four complexes (I-IV) positioned at the inner membrane of the mitochondrial cristae. Complexes I and II are interconnected with the TCA cycle. Here, electrons (e^-) are transferred from NADH and $FADH_2$ during the production of succinate and fumarate. They are further moved via the complexes until complex IV transfers them to O_2 to make H_2O. This process is thus oxygen dependent. The electrons travelling through the complexes induce translocation of protons (H^+) to the mitochondrial inner membrane space, thereby creating a proton gradient. This gradient is re-balanced by H^+ being transported back through ATP synthase. Simultaneously, ADP is phosphorylated to ATP, yielding its nomenclature oxidative phosphorylation[60].

Fatty acid oxidation

Fatty acids are broken down by mitochondrial fatty acid oxidation (FAO), also known as β-oxidation. First, the FA is converted into a long-chain acyl-CoA which is assisted into the mitochondria by transporting proteins CPTI, CACT and CPTII[64]. Once there, the FAO takes place which is a continuous reaction where the FA will lose two carbons per cycle, resulting in sequential shortening of the FA chain. For example, palmitic acid, which has 16 carbons (16:0), will have 14 carbons (14:0) after one reaction. Every round gives one molecule each of $FADH_2$ and NADH that can be used to produce ATP in the electron transport chain. Each cycle also produces acetyl-CoA, which can become citrate and be a part of the TCA cycle. Very long chain fatty acids

are pre-processed to become shorter by FAO already in the peroxisome outside of the mitochondria[61].

Cancer metabolism

In 2011 Hanahan and Weinberg added "deregulating cellular energetics" to the infamous list of hallmarks of cancer, started roughly a decade earlier[66,67]. This inclusion certainly expressed the importance of metabolism in cancer development and simultaneously highlighted the prospect of cancer metabolism as a drug target.

Each time the leukemic cell divides it must duplicate its cell content; lipids, proteins, nucleic acids etc. Because malignant cells tend to grow excessively, they need to import and metabolize more nutrients[82]. Multicellular organisms, such as humans, usually have unlimited access to nutrients. Our cells therefore need control systems to not over-proliferate. Growth factors have an influence on the uptake of nutrients from the environment. However, cancer cell mutations can make these factors hyperactive, giving over-activation of nutrient uptake and metabolism[59].

Glucose metabolism in cancer

Normal and quiescent cells, such as differentiated leukocytes, have a basal rate of glycolysis[55,59], as they get their main source of energy from OXPHOS in the mitochondria[55]. From one mole of glucose they acquire around 36 moles of ATP (Figure 4)[55]. However, OXPHOS is oxygen dependent. If oxygen is scarce the cell can obtain its energy from anaerobic glycolysis. This is for example common for quiescent HSCs residing in a hypoxic BM environment[68,69]. Here, the pyruvate generated from glycolysis is converted into lactate instead of being utilized for ATP production in the mitochondria. No oxygen is needed, but the drawback is a substantial decrease in number of ATP being produced, about 2 mol ATP/mol glucose[55]. In rapidly dividing cells, and especially in highly proliferating leukemic blasts, there appears to be a preference for generating lactate instead of using mitochondria, even though oxygen is present. This is called aerobic glycolysis or the Warburg effect [55,59]. Because oxygen is present a small share of the pyruvate will still go through the TCA cycle. Yet, the ATP production is kept low with about 4 mol ATP/mol glucose[55].

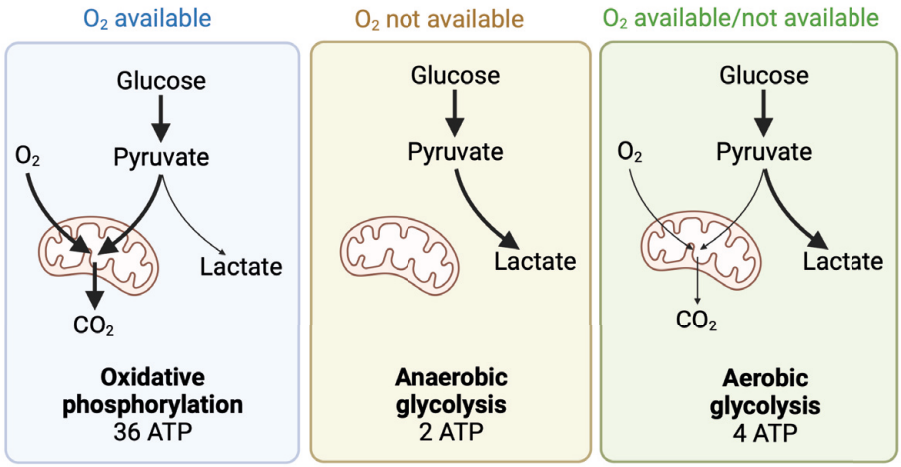


Figure 4 | Glucose metabolism with and without oxygen and involvement of mitochondria. The difference between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg effect) in mammalian cells. The ATP values are approximate numbers of mol ATP obtained from one mol of glucose for each system. (Created with BioRender.com). Modified from:[55].

It was already in the 1920s that Warburg described how cancer cells appeared indifferent to the sufficiency of oxygen supply. In most instances they tended to break down glucose to lactate without the use of oxygen[70]. It seemed bizarre to him that they squandered the opportunity to gain a lot of ATP, which ought to be important for highly dividing malignant cells. Almost 100 years later it is still debated why cancer cells would prefer this type of glycolysis. Warburg and others have postulated that cancer cells might have malfunctioning mitochondria accompanied with defective OXPHOS[71]. However, this hypothesis seems less plausible given that studies have demonstrated the mitochondria to be functional[55,72]. It should also not be due to lack of oxygen, as proven by the presence of leukemic blasts in oxygen-filled blood vessels[55].

There are some better proposals for this puzzle. First, the ATP produced by aerobic glycolysis seem to give enough energy for the cancer cell to survive[55]. An important contribution is that malignant cells can gain the ability to take up more glucose than normal, leading to substantial amounts of ATP being produced in the end[59]. Second, massive production of ATP might not be the main goal. Instead, the proliferating cell is using the glucose molecules to make biomass for growth and division[57]. Malignant mutations promote high proliferation instead of maximum production of ATP. The leukemia cells need non-essential amino acids to make protein, nucleotides to make DNA and lipids to use for new cell membranes. From one glucose molecule six carbons can be obtained, which is an essential element in all of the above[55]. The glucose can also be used to form intermediates in the TCA cycle. For example, citrate can be used

for lipid synthesis. From the TCA cycle as well as from intermediates in the glycolysis pathway there are also amino acids produced to be used for protein and nucleotide synthesis[55]. The carbons from glucose can be moved into the pentose phosphate pathway (PPP), an anabolic pathway to gain ribose for DNA synthesis. This pathway is also a big producer of NADPH which will help donating electrons and neutralizing reactive oxygen species (ROS) which can be prominent in metabolically and chemotherapy stressed cells. Several oncogenes are thought to control the flux of glucose into PPP and in that way make the cancer cell adjust and survive better in the malignant environment. NADPH is also needed for FA synthesis[73]. The glucose molecule can also be used to make glycerol via glycerol-3-phosphate, which in turn can be used for production of lipids[74].

In the shadow of the Warburg effect, some pyruvate still contributes to ATP production by mitochondrial respiration. However, the aerobic glycolysis is a fast process and produces so much pyruvate that the majority is converted into lactate that is excreted from the cell. This switch to make lactate in cancer can be linked to oncogenes. The production of lactate also generates NAD^+ which helps continue fuel glycolysis[59].

The plasticity of cancer metabolism

Cancers are recognized for their heterogeneity, and consequently, their metabolic profiles exhibit diversity as well. Recent reports show that the Warburg effect is not as prominent in the stem cells of cancer, which prefer OXPHOS instead. This has been noticed in many cancers and is especially prominent in the malignant cells resisting chemotherapy[75,76]. This concept has also been proved for LSCs of AML, emphasizing the importance of finding ways to target additional components of metabolism than glycolysis[77,78].

Thus, several parts of metabolism should be targeted to be able to eradicate all malignant cells, both blasts and stem cells. Cancer cells are utilizing their metabolic plasticity to obtain material from metabolic intermediates. They are surrounded by high levels of ROS and therefore must have antioxidant systems to survive[55]. Cancer stem cells like LSCs also often use amino acids to fuel their OXPHOS. Especially glutamine, glutamate and proline could be important targets[79]. Another notable strategy is to target lipid metabolism.

Lipid metabolism in cancer

Cancer cells obtain lipids by uptake from the environment. They can also be produced by *de novo* synthesis in which acetyl-CoA is converted into malonyl-CoA and by FA synthesis goes through numerous elongations by fatty acid synthase[61]. For this process NADPH is needed. The end product is palmitic acid with 16 carbons (16:0). Lipid

metabolism research in cancer has primarily been focused on fatty acid synthesis. The breakdown of fatty acids, by FAO, needs more attention[61].

Leukemia cells are strongly dependent on lipid utilization and FAO[68,80–82]. As in malignant glycolysis, it is not necessarily the maximal production of ATP that is important[61]. Acetyl-CoA from FAO can be used for other things than energy production in the TCA cycle, making useful bi-products for protein and nucleotide synthesis. The citrate from FAO can be used for NADPH production, which protects the cell from ROS[61,64]. Additionally, FAO is believed to be involved in the protection of leukemic cells from mitochondrial-driven apoptosis[80]. Just like in glucose metabolism, the malignant cell can upregulate systems to take up more lipids from the environment to be used for its proliferation[83].

Transporters

As mentioned previously, the leukemia cell niche contains adipocytes from where fatty acids can be acquired (Figure 5). The adipocytes are thought to originate from mesenchymal stem cells. They are developed through a process called adipogenesis, which includes a first differentiation step into a preadipocyte that will be filled with lipids to become a mature adipocyte in a second differentiation step[84]. The bone marrow adipocyte has the same morphology as the white adipocyte. It mainly consists of a large lipid droplet surrounded by a very thin cytoplasm holding all the essential organelles such as Golgi, mitochondria and nucleus. The cell can expand due to increased lipid content and become very large with diameters ranging from 30 to 160 µm[85,86]. After a meal the level of insulin increases and accelerates the uptake of lipids from the diet[87,88], which can be released and used as energy during fasting[74].

The lipid droplet in adipocytes consists of triacylglycerols (TAGs), also known as triglycerides. In circulation, the TAGs are coupled to lipoproteins and must be hydrolyzed to FAs before being taken up by the adipocyte[89]. Shorter FAs can diffuse rapidly through the cell membrane while transporters are utilized to mediate the uptake of longer FAs. The receptor-mediated uptake of FAs is regulated by insulin signaling at fed state[89]. Insulin will also enhance the uptake of glucose to the adipocyte. Glucose is taken up by glucose transporters (GLUTs) and can be converted into glycerol-3-phosphate (glycerol-3-P)[74]. During lipogenesis, esterification of FAs to a backbone of glycerol-3-P produces TAG that can be stored in the lipid droplet[74]. When needed, TAG can be converted back into FA and glycerol in a process called lipolysis[89]. These two molecules can subsequently be transported out of the adipocyte and be taken up by neighboring cells. Cancer cells are thought to promote lipolysis in surrounding adipocytes, to get more FA and glycerol for their own metabolism[90]. To enhance the impact, they also upregulate the number of transporters for lipid and glucose uptake[91,92].

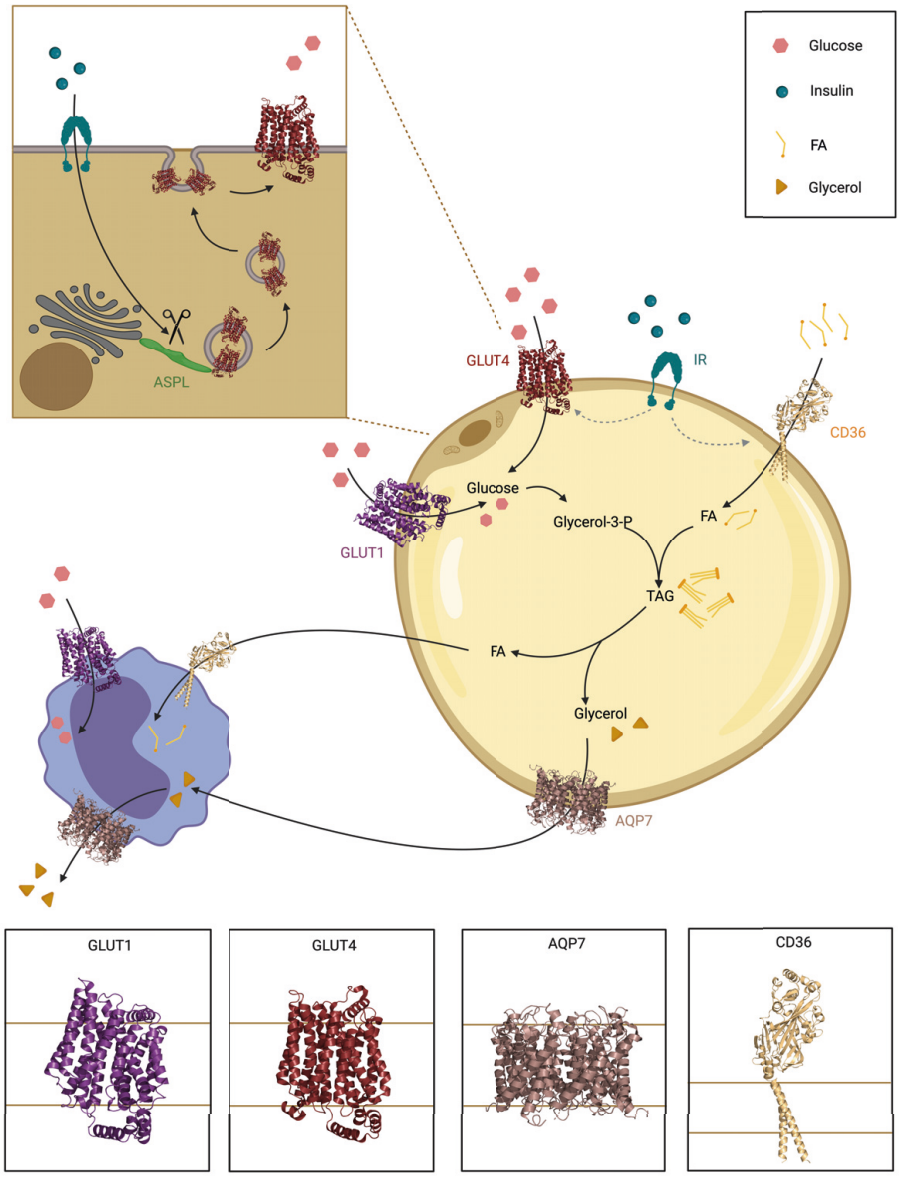


Figure 5 | Receptors involved in interplay and nutrient uptake of adipocytes and leukemia cells. Schematic figure of uptake, release and transfer of glucose, fatty acids and glycerol in the adipocyte and leukemia cell and the transporters involved. The main steps for lipogenesis and lipolysis in the adipocyte are included as well as the involvement of insulin. Zoomed in shows how alveolar soft-part sarcoma locus (ASPL) is tethering glucose transporter 4 (GLUT4) intracellularly to Golgi and how insulin signalling can cleave this bond leading to traslocation of GLUT4 to the cell surface in vesicles. IR: insulin receptor, FA: fatty acid, TAG: triacylglyceride. (Created with BioRender.com). PDB IDs: 5EQI, 7WSN, 8C9H, 5LGD.

Glucose transporters (GLUTs)

The glucose transporters are a family of 14 human transporters decoded by the Solute carrier 2 (SLC2) gene. They belong to the major facilitator superfamily (MFS) representing their importance in facilitating sugar transport over cell membranes[93]. The common GLUT structure consists of 12 transmembrane helices with both termini intracellularly and with an intracellular helical domain (ICH)[94] Even though there are structural similarities within the GLUT family, there are some differences in substrate selectivity and the cell types they are expressed in. The GLUTs facilitate passage of mainly hexose molecules, such as glucose, over the cell membrane. The GLUTs can be divided into three groups based on their sequence similarity. Class I includes GLUT1-GLUT4, which are most abundantly expressed and have been most comprehensively examined, as well as GLUT14. They perform passive transport of glucose down a concentration gradient[93] and can transport glucose to the inside of the cell by undergoing conformational changes from outward open to inward open state[95]. Some of them can also regulate glucose uptake by being translocated from inner compartments to the cell membrane[93].

GLUT4

GLUT4 has a special function in the body as it is regulated by insulin. It is essentially expressed in insulin sensitive cells of adipose tissue, skeletal muscle and heart muscle[93]. Figure 6 shows the expression of GLUT4 in human adipocytes (Figure 6, left). In adipocytes GLUT4 plays a major role for instant influx of glucose from the blood to the adipocytes after a meal. When eating a meal, there is an increase in blood glucose levels and subsequent release of insulin. Insulin-stimulated uptake of glucose by GLUT4 gives a rapid uptake, within minutes, of glucose into adipocytes and muscle cells to be metabolized or stored [96].

It was found that GLUT4 at basal state is localized in the cytoplasm and that insulin stimuli can impact translocation of GLUT4 through different inner compartments of the adipocyte to the cell surface[97]. This can increase the GLUT4 expression at the adipocyte surface up to 20-30 times[98,99]. To be able to rapidly take up glucose, there is a pre-existing pool of membrane vesicles with GLUT4[100]. These GLUT4 storage vesicles (GSVs) are kept at the matrix of golgi and are released after insulin stimulation and transported to the cell surface. Here they can fuse with the plasma membrane to enable GLUT4 expression[101].

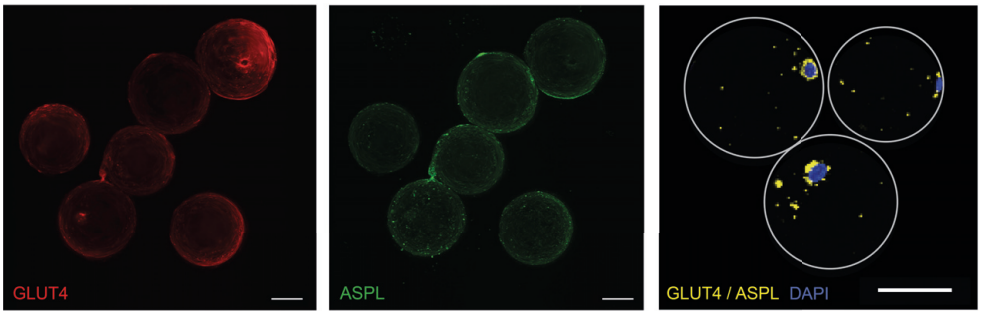


Figure 6 | Proteins important for glucose transport in adipocytes. Protein expression of (left) GLUT4 and (center) ASPL in human primary adipocytes. (Right) Yellow colour shows protein interaction of GLUT4 and ASPL. Nuclei are stained with blue DAPI. Scale bars show 50 µM.

In rodents, a protein responsible for keeping GLUT4 intracellularly was identified and named tether containing a UBX domain for GLUT4 (TUG). TUG can bind to GLUT4, but this interaction can be disrupted in the presence of insulin[102]. In **paper I** we have studied this phenomenon in a human context. The human analogue of TUG, with a ~75 % sequence identity, is alveolar soft-part sarcoma locus (ASPL)[102,103]. The expression of ASPL on the human primary adipocyte surface was confirmed (Figure 6, center). At basal state ASPL was co-localized with GLUT4 (Figure 6, right). Many of the co-localizations were close to the nuclei, suggesting that ASPL is keeping GLUT4 around the nucleus which has also previously been seen in mice adipocytes[104]. Structural and biochemical studies further proved that ASPL makes complex with GLUT4, and it is believed to carry out the same function as TUG in rodents. The hypothesis is that ASPL can bind to golgi matrix proteins and at the same time bind to vesicles with GLUT4 to keep them in the cytosol (Figure 5, zoom in)[101]. Insulin stimuli leads to proteolytic cleavage of ASPL, enabling the translocation of GLUT4 to the cell membrane where it can take up glucose, which has been shown before with TUG and GLUT4 in mice adipocytes[104], and muscle[105].

GLUTs in cancer

Glucose transporters are frequently upregulated in cancer leading to an increase in glucose uptake and metabolism. This concept is so prominent that it can be used for grading the invasiveness of solid tumors by ^{18}F-FDG PET scanning. ^{18}F-FDG is a glucose analogue that can be transported into tumor cells by GLUTs. Lacking the ability to be completely metabolized, it can start accumulating in the tumor which can be visualized by positron emission tomography (PET)[106]. Over-expression of the different GLUT isoforms have been found in different malignancies. The type of GLUTs expressed in different cells and tissues varies, and likewise will the isoforms differ depending on the malignant tissue type[107,108].

Within all GLUT classes, GLUT1 and GLUT3 exhibit the most prominent expression across cancer types[107]. They are both upregulated in poorly differentiated breast and endometrial carcinoma cells[109]. GLUT3 is expressed in brain tumor initiating cells and is accompanied with poor survival[110] and could be a good target for GLUT3-dependent glioblastoma[111]. This transporter has also been associated with worse prognosis in gastric cancer[112]. Because GLUT1 is the most ubiquitously expressed in the body it is also expressed in a lot of different malignancies, where it has been associated with poor overall survival and metastasis[108]. GLUT1 shows possibilities of being a good target in hepatocellular carcinoma[113]. A meta-analysis showed that low expression of GLUT1 led to better prognosis in several malignancies such as cancer of the pancreas, breast, gallbladder and lung[114]. Another meta-analysis including over 2600 patients with lung cancer indicated that GLUT1 over-expression could be a good biomarker for prognosis in lung cancer, due to high expression being associated with shorter survival[115]. GLUT1 expression led to radiotherapy resistance in cervical cancer[116] and its pharmacological inhibition in AML patient samples gave synergistic antileukemic effects together with inhibition of OXPHOS[117].

Another example of GLUTs in cancer is GLUT5 whose expression in AML is upregulated to enable increased fructose uptake to compensate for scarce glucose levels in the environment. This overexpression is associated with bad prognosis in AML patients and by inhibiting GLUT5 with a fructose analog, AML cells and an AML mouse model could be sensitized to Ara-C[118]. Other examples are GLUT2 that has been found to have prognostic significance in hepatocellular carcinoma[119], and that multiple myeloma cells are dependent on GLUT4 expression for glucose uptake[120].

GLUT1

GLUT1 is in comparison to GLUT4 expressed at the cell surface in more similar quantities both in the presence and absence of insulin (Figure 5)[121]. It is found in most cell types with especially high numbers in erythrocytes and blood brain barrier endothelial cells[93]. In adipocytes GLUT1 keep the basal flux of glucose in complement with GLUT4[122]. In context of the leukemic cell, GLUT1 is interesting because of the prospect of malignant cells upregulating glucose transporters for enhanced glycolysis. Malignant mutations can increase growth factor signaling to increase number of GLUT1 at the cell surface. One important example is mutations in the PI3K/Akt/mTOR pathway that can lead to more GLUT1 expression and subsequent more glycolysis and lactate production[59].

High glycolysis has been correlated with worse outcome in a subset of AML patients. Markers of enhanced glycolysis could be used for precision medicine and prognosis prediction in patients[123]and GLUT1 could be a suitable marker for this [123]. In **paper II** we sought to explore the GLUT1 protein expression in AML cell lines. Of four cell

lines tested two showed opposing results, THP-1 with high expression levels of GLUT1 and KG-1 with much less (Figure 7).

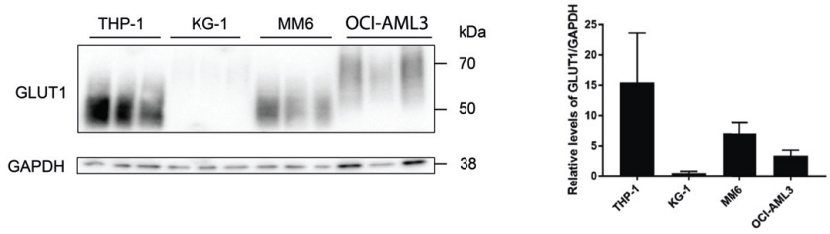


Figure 7 | GLUT1 expression in AML cell lines. (Left) Western blot of GLUT1 protein expression in three samples each of THP-1, KG-1, MM6 and OCI-AML3. GAPDH was used as loading control. (Right) The expression levels of GLUT1 compared to GAPDH control. Bars show mean + SD, n = 3.

Shift to using high glycolysis has also been correlated with less sensitivity to Ara-C in AML patients[123]. Therefore, I tested the effects of different concentrations of Ara-C in an ATP-based cell viability assay. All cell lines studied were affected by Ara-C, with THP-1 showing less sensitivity compared to KG-1 having inhibitory concentrations IC_{25} of 611 nM and 139 nM respectively (Figure 8).

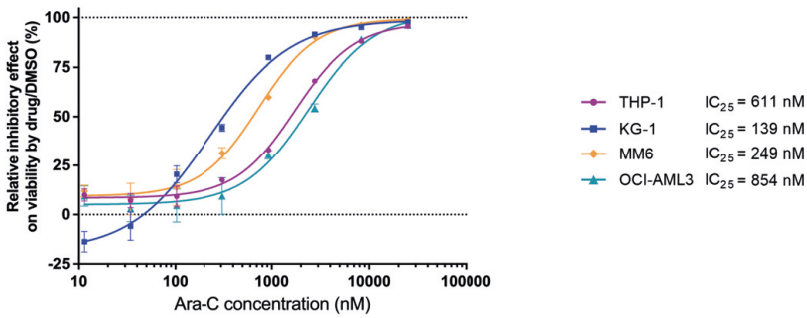


Figure 8 | Effect of cytarabine (Ara-C) in AML cell lines. The inhibitory effect on viability by 72 hours of treatment with Ara-C in various concentrations. Cell lines analysed are THP-1, KG-1, MM6 and OCI-AML3. Values show mean of n = 3. Error bars show SD.

Fatty acid transporter CD36

Another protein whose expression at the cell surface might be regulated by endosomal vesicle transport and insulin is cluster of differentiation 36 (CD36) (Figure 5)[124]. This is a transmembrane protein responsible for fatty acid uptake in cells. It has showed

ability to translocate from intracellular compartments to the cell membrane in heart muscle when FAO was stimulated by muscle contraction[125]. This event could be reversed by presence of glucose[126]. Except for FAs CD36 can also interact with other substrates like oxidized low density lipoproteins (oxLDL)[127]. It was first found in platelets[124], and can bind to angiogenesis inhibitor thrombospondin-1 (TSP-1)[128]. Due to its many ligands, one of them being FAs, CD36 has many additional names such as scavenger receptor B3 (SCARB3) and fatty acid translocase (FAT)[124].

CD36 is ubiquitously expressed but has enhanced expression in tissues with high FA metabolism like adipose tissue and muscles[129]. It is an 88 kDa[130], heavily glycosylated protein with two helices spanning the cell membrane that are connected by a ~47 kDa domain on the extracellular side[131]. The two intracellular termini are short[132]. It has an internal cavity which is hydrophobic, making it suitable for accommodating fatty acids. This is shown in the available CD36 structure where two palmitic acids are occupying the cavity (PDB ID: 5LGD)[131].

If fatty acids can easily diffuse through the plasma membrane or if an active transporter is needed to help them has long divided the experts. A possibility is that CD36 is not a tunnel for fatty acid transfer but rather an acceptor of FAs. It is attracting the fatty acids to the hydrophilic membrane surface where they can use the CD36 cavity to progress to the outer leaflet of the lipid bilayer and use a flip-flop mechanism to reach the inner leaflet. CD36 is spanning the membrane to help FAs from the inner leaflet to another lipid protein, such as fatty acid-binding protein (FABP), to take over on the inside[133]. In this way, amount of CD36 at the plasma membrane is increasing the speed of fatty acid uptake and release, even though it is the fatty acid gradient on each side of the plasma membrane that is defining the actual speed[126]. FAs stimulating endocytosis of CD36 together with the FA has been described in adipocytes and could also lead to enhanced lipid uptake rate[124,134].

CD36 in cancer

CD36 is a potential novel drug target for leukemic stem cells[135]. It has also been shown to be expressed on sarcoma and breast cancer cells[136]. Its upregulation in prostate cancer leads to worse prognosis and is linked to metastasis[137]. Overexpression of CD36 in human oral carcinoma cells also gives increased metastasis that can be reduced by CD36 deletion. Based on this it was hypothesized that for metastasizing cells to survive in a new environment, CD36 might be crucial by supplying FAO with lipids[92]. This was supported by a study where an upregulation of the CD36 gene in acute monocytic leukemia cells was detected when co-cultured with adipocytes, together with upregulation of surface CD36 expression[138]. Moreover, leukemic stem cells were found residing in gonadal adipose tissue in a murine model that induced lipolysis of adipose tissue. Expression of CD36 also increased FAO and protected cells from chemotherapy

which could be reversed with loss of CD36[83]. CD36 was also upregulated on primitive chronic myeloid leukemia (CML) cells and were less vulnerable to CML drug imatinib and could be killed by selective targeting of CD36[139]. Additionally, LSCs resistant to venetoclax, an OXPHOS inhibitor, were found to have increased CD36 expression[82].

In **paper III** we have investigated how the expression of CD36 affects uptake of fatty acids in AML. In the chosen AML cell lines, I found that KG-1 had higher CD36 protein expression than THP-1 (Figure 9).

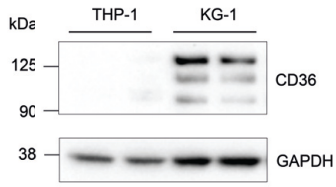


Figure 9 | Protein expression of CD36 in AML cell lines. Western blot of CD36 expression in THP-1 and KG-1 cell lines. Two cell lysates were used for each cell line. GAPDH was the loading control.

The leukemic cells with high expression of CD36, KG-1, took up lipids that had been released from human primary adipocytes, in a similar manner as has previously been noted in ovarian cancer cells (Figure 10, left)[90]. This phenomenon was not as prominent in the THP-1 cells. When comparing the uptake of a green, fluorescent FA analogue directly from the media, the high CD36 expression in KG-1 gave a great advantage regarding uptake while low FA uptake was seen in THP-1 (Figure 10, right).

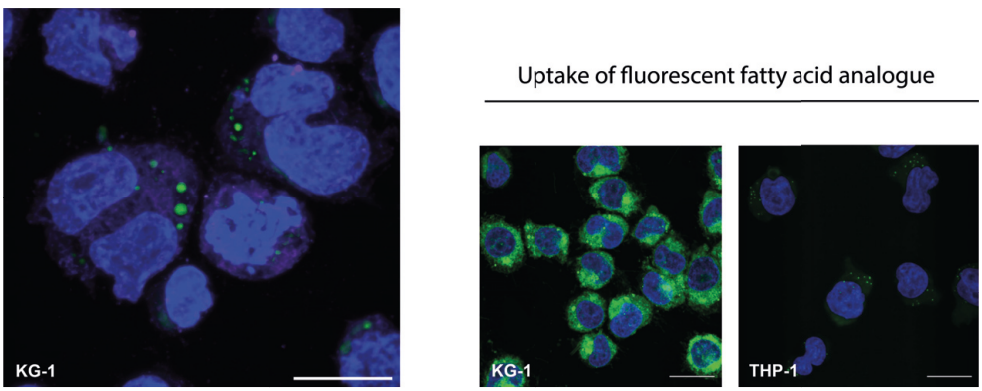


Figure 10 | CD36 expression affects fatty acid (FA) uptake in AML cell lines. (Left) Lipid droplets with green FAs in KG-1 cells taken up from human adipocytes during 22 hours of co-culture. Cell nucleus is shown in blue and cell body in violet. (Right) Comparison between KG-1 and THP-1 cells of their uptake of green FA analogue added to the cell media for 10 minutes. Scale bars show 20 µm.

Aquaporins (AQPs)

Aquaporins (AQPs) are transporters that facilitate water transport over the cell membrane dependent on the osmotic pressure (Figure 5). There are 13 AQPs in mammals, and they are divided into three groups; the orthodox aquaporins which are water permeable channels (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8), the aquaglyceroporins (AQP3, AQP7, AQP9, AQP10) that in addition to water also are permeable to small molecules like glycerol, and lastly the unorthodox/super aquaporins (AQP11, AQP12), whose functions are still to be established but are thought to operate at intracellular membranes instead of the plasma membrane[140,141]. The aquaporins share a common structure with six transmembrane helices connected with five loops, A-E, and intracellular termini. Loop B and E each connects to one of two additional half helices. The protein is a tetramer of monomers where each monomer has a pore for transport of solute and/or water. In the center of the tetramer there is an additional pore, the central pore. The type of AQP expressed varies between tissues and organs[140]. Especially the aquaglyceroporins are thought to have big impact on diseases connected to metabolism like obesity, diabetes and cancer[141]

AQPs in cancer

AQPs contribute to the pathogenesis of several cancers, acting as channels for nutrient uptake to be used as fuel for proliferation, promoting metastatic processes and influencing the transport of ROS, as they can facilitate the transport of hydrogen peroxide[142]. Due to different isoform expression in different tissues, the AQP isoforms are connected to different malignancies. The majority of the AQPs have been found upregulated and connected to various types of cancers such as in the liver, breast, colorectal and lung[142]. Therefore, they are possible future markers for prognosis and treatment susceptibility[141].

Several articles emphasize the significance of AQPs in cancer. For example, high AQP1 expression is correlated with worse survival in breast cancer patients[143]. AQP3 is overexpressed in gastric cancer cells and when reduced with siRNA the glycerol uptake and metabolism was affected, which could induce apoptosis[144]. AQP5 expression in bone marrow samples has been correlated with occurrence of CML and resistance to imatinib while targeting of AQP5 with siRNA could lower proliferation of CML cells[145]. AQP7 expression in relation to malignancy has so far mainly been studied in breast cancer. It has been correlated with lower overall survival in breast cancer patients from The Cancer Genome Atlas. Additionally, it causes more invasive metastasis in mice models[146]. AQP7 has also been mentioned in the context of altered metabolism in cancer. Samples from patients with thyroid carcinomas harboring a genetic alteration affecting peroxisome proliferator activated receptor gamma (PPARγ) had upregulation

of AQP7 simultaneously as showing different expression in genes involved in glucose, lipid and AA metabolism[147].

AQP7

AQP7 is not as efficient as the orthodox aquaporins in water transport. Instead, it is an effective glycerol channel (Figure 5)[148]. It is recognized for its expression in adipose tissue[149] where perilipin 1 (PLIN1) has been reported to keep AQP7 intracellularly at the lipid droplet of adipocytes during insulin signaling[149,150]. During fasting when glycerol is needed as an energy source by the surrounding cells, AQP7 is important for efflux of glycerol[151,152], and it will consequently be translocated to the cell surface[149,150]. Thus, AQP7 deficiency is associated with expansion of adipocytes due to accumulation of glycerol and TAG. However, it is important to note that AQP7 also is expressed in tissues beyond adipose and might exert systemic metabolic effects[153].

In addition to glycerol, AQP7 can also transport arsenic trioxide[148]. Because patients with APL are treated with arsenic trioxide, we hypothesized that an APL cell line would have AQP7 expressed. In **paper IV** I thus studied AQP7 in relation to the APL cell line, NB4. The expression of AQP7 in NB4 was confirmed with western blotting (Figure 11).

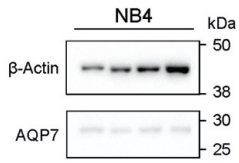


Figure 11 | Expression of AQP7 in NB4. Western blot confirming protein expression of AQP7 in the APL cell line NB4. Four cell lysates of NB4 was used with β-actin serving as the loading control.

Targeting metabolism in leukemia

Due to new genetic findings, there has been alterations on the treatment protocols for AML to some extent, but most things remain the same. For patients considered adequately fit there will be an induction therapy, the so called 7 + 3 regimen, with high dose Ara-C for days 1-7 and daunorubicin or idarubicin for day 1-3[31]. Ara-C is a nucleoside analogue which by mimicking nucleosides gets incorporated into the DNA and blocks DNA synthesis. It also inhibits enzymes in nucleotide base synthesis which leads to apoptosis[50]. When complete remission is reached, consolidation therapy with intermediate dose cytarabine (IDAC) can be given, followed by maintenance therapy with the hypomethylating agent (HMA) azacytidine[31]. FMS-like tyrosine kinase 3 (FLT3) mutations are common, occurring in about 30 % of AML patients, and gives less favorable prognosis[154]. This patient group should receive the kinase inhibitor midostaurin on top of the 7 + 3 regimen and as maintenance therapy[31,155]. Part of the treatment plan can also be to perform an allogenic hematopoietic cell transplantation[31].

Patients with inferior clinical outcome are often of old age and have age-related comorbidities. These patients are not suitable candidates for the intensive high-dose 7 + 3 chemotherapy. Instead, low dose Ara-C or HMAs are typically considered. However, is has been noted that these alternative treatments have shown limited effect[31,155]. Because AML is a disease mostly affecting the elderly this subgroup represents a substantial portion of AML cases. There is consequently an urgent need of finding new therapy strategies for these patients. The increased understanding of AML heterogeneity is paving the way for individualized precision medicine. AML subtype information and classification could help tailor the treatment based on individual patient profiles. One possible approach is to gather information about the different ways cancer cells de-regulate their metabolism. This not only enhances our understanding of the disease but also offers new markers of malignant metabolism that can become specific targets for new more effective therapies. Already, there has been a lot of effort made in targeting different parts of cancerous metabolism[154].

Inhibiting OXPHOS

A lot of focus has been on targeting mitochondrial metabolism. LSC in AML have enhanced mitochondrial mass in comparison to regular hematopoietic cells which is a sign of increased mitochondrial OXPHOS[156]. Additionally, AML cells with high OXPHOS phenotype are more resistant to Ara-C[157]. Venetoclax is a drug that inhibits BCL-2 which is involved in the mitochondrial apoptotic pathway and was proven important for AML cell survival[158]. Ventoclax has now become an important addition to the treatment of elderly AML patients and others not suitable for high-dose Ara-C[31]. Together with an HMA venetoclax lead to long remissions with better results than conventional therapies in these patients[158,159]. It selectively targets the LSCs which are OXPHOS dependent[160], but simultaneously are thought to generate low ROS levels. These cells are specifically dependent on uptake and metabolism of Aas, like glutamate, for their OXPHOS and venetoclax is thought to reduce this AA-dependent mitochondrial respiration[79].

One early reason for targeting mitochondria in cancer was due to metformin[57]. This is a drug usually used for treatment of type 2 diabetes (T2D) because it targets AMPK, which affects the glucose uptake from blood to muscle[161]. Its main target though is in the mitochondrial ETC complex I[56,162]. In epidemiological studies, diabetic patients taking metformin had reduced risk of cancer[161]. It has since been tested in several cancer types, albeit with suboptimal outcomes. The use of high concentrations in *in vitro* studies gave promising results that could not be replicated in humans. Another complicating matter is that metformin led to a compensatory increase in glucose uptake and glycolysis. Furthermore, safety concerns arose due to metformin inducing lactic acidosis which could be dangerous for cancer patients. Within leukemia it has only shown effect against relapse in ALL. In AML an attempt on testing the effect on metformin in combination with Ara-C was done but succumbed premature termination because of slow patient recruitment[56].

There have also been other studies on how to target OXPHOS. For example, 20 % of AML patients have mutated genes encoding the production of IDH1 and IDH2[50], which are enzymes needed for production of TCA cycle-dependent α-KG. There have been two drugs approved by FDA, ivosidenib and enasidenib, which inhibits mutant IDH1 or IDH2 respectively[50]. Another drug is CPI-613, or devimistat, which inhibits PDH that is important for conversion of pyruvate to acetyl-CoA. This results in acetyl-CoA not entering the TCA cycle[56]. It first had promising results together with chemotherapy in Phase I and II clinical trials specifically in elderly patients but came in short with low effect in a phase III study with high-dose chemotherapy, which led to the trial being stopped[50,163].

A trend has emerged of OXPHOS inhibitors being promising in pre-clinical testing, yet their efficacy in clinical trials is low or hampered with toxicity. This pattern suggests that OXPHOS inhibitors have narrow therapeutic windows, emphasizing the balance between achieving therapeutic benefits but avoiding adverse effects[164]. There are problems with drug resistance and the urge for testing these inhibitors in combination with other drugs. Even with the successful venetoclax there is a 30 % change of relapse due to resistance. One reason for this resistance is the leukemic upregulation of FAO as a defense mechanism[79,82].

Inhibiting fatty acid metabolism

Ara-C resistant AML cells show upregulated FAO[157], which emphasizes the need to target FA metabolism. Because inhibition of FAO has been tested and used for treatment in heart diseases there are inhibitors available approved for human use. One of these is etomoxir, which inhibits CPT1, an enzyme that helps long chain FA-derived acyl-CoA transport into the mitochondria. This is an early rate-limiting step of FAO and it its thus a good target[64]. Etomoxir has been proven to reduce proliferation and increase the sensitivity of an apoptosis drug in AML cells[80]. However, when tried in patients it had to be withdrawn due to hepatoxicity[61]. New CPT1 inhibitors are being tested and show promising pre-clinical cytotoxic effects in AML cells[165]. Other drugs used for heart diseases that could be tested are angina drugs against 3-ketoacylthiolase (3-KAT), which is an enzyme in the final step of FAO[61].

In pre-clinical investigations notable advancements have also been observed by inhibiting the first step in mitochondrial long chain FA metabolism, the very long chain-acyl-CoA dehydrogenase (VLCAD). This enzyme is upregulated in AML and its inhibitor led to altered FAO resulting in AML cells incapable of proliferation and engraftment. Notably, this method selectively killed AML cells without affecting normal cells[81]. At present there are two FAO inhibitors being tested in clinical trials, for hepatocellular carcinoma and prostate cancer. Nevertheless, not many FAO inhibitors have made it into clinical trials which underscores the importance of more effort in enhancing the potency of drugs targeting lipid metabolism[164].

Inhibiting glucose metabolism

There have also been drugs tested that directly inhibit glycolysis. 2-Deoxy-D-glucose (2DG) is a competitive inhibitor of glucose metabolism. In pre-clinical assessments the drug demonstrated notable anti-cancer effects but when tested in patients with

glioblastoma the dose had to be so high that it led to adverse toxic effects. A concern is that a competitive inhibitor like 2DG might have to be used in high concentrations to outcompete blood glucose levels[166].

Another possibility is to make the use of aerobic glycolysis in cancer cells a vulnerability. By targeting the enzyme that makes pyruvate to lactate, LDH-A, there is a decrease in MYC-driven tumors in xenograft models and it can also slow down the progression of myeloid leukemia[57] . Again, the problem has been to show that the effect is potent enough to function in humans. These inhibitors have shown toxicity or low effect in malignancies. Another promising strategy could be to inhibit the lactate transport instead[58].

There is a clear demand for better inhibitors and co-treatment strategies. One approach is to recognize the fact that AML patients with FLT3 mutations have increased aerobic glycolysis. Hence, inhibitors of glycolysis alone or in combination with an FLT3 inhibitor is thought to be effective in patients with this mutation[167].

Testing new inhibitors

This thesis contains the experimental validation of novel small-molecule inhibitors that target transporters involved in cancer metabolism. Specifically, the focus encompasses receptors GLUT1, CD36 and AQP7. Being located on the cellular surface, they were postulated to be available targets for inhibition. The inhibitors have previously been found through either high throughput screening (HTS) or virtual screening. In HTS libraries with a large number of compounds are tested either directly at the target or in cell-based assays where the readout will change in case of an activity downstream of the target[168]. In virtual screening the library of molecules tested are virtual. They can be tested against the protein structure of the target and the substances are scored according to how well they are docking to the target[169]. The inhibitors used here have then been validated, either by us or others, to target transporters connected to metabolism. The aim for this thesis has been to test if these inhibitors have any affect in AML cell lines.

What makes a good inhibitor?

During my work I have focused on small-molecule inhibitors. They are renowned for their economic efficiency, easy storage and that they generally can be administered orally[170]. A good compound needs to be drug-like, meaning that it should possess favorable properties of absorption, distribution, metabolism, excretion and toxicity (ADMET). These underscore the significance of considering the drugs' pharmacokinetic behaviors such as the ability of traversing barriers like the gastrointestinal membranes, being distributed to the correct tissue holding the target, hepatic metabolism and proper elimination by the liver and kidneys. At the same time as the inhibitor must be non-toxic for safe usage, it is also very important that the compound has good activity at the target. This activity can for example be calculated by the half-maximal inhibitory concentration (IC50)[171]. This is the concentration of a compound required to inhibit 50 % of the targeted activity. The drug's efficacy is based on the activity combined with the exposure, such as concentration and duration. Further desirable properties are good solubility and chemical stability[171]. A valuable guideline can be to consider the Lipinski rule of 5, which is based on the structure of compounds making it to clinical trials. To have good absorption and permeability if given orally the structure should have ≤ 5 H-bond donors (sum of OH and NH), ≤ 10

H-bond acceptors (sum of N and O), a MW ≤ 500 and LogP ≤ 5[172]. Hydrogen bonds increase solubility in water but are a disadvantage if the drug is to pass lipid bilayer membranes by passive diffusion. The size is important because bigger molecules are less soluble, less absorbed at the intestinal epithelium and have worse passive diffusion through membranes. LogP measures lipophilicity; how a compound distributes between a hydrophobic and a hydrophilic solvent, which can affect ADMET[173]. Target binding, lipophilicity and solubility are all three important parameters that must be considered when choosing a candidate inhibitor[171].

The goal is for a compound to have good target activity without off-target effects. Utilizing cell lines offers an approach to evaluate the activity downstream of the target and test the efficacy of the candidate compound before being tested in animal models. Based on these results structural modifications can be applied to the compound to enhance its potency, thereby refining its therapeutic potential.

Inhibition of GLUTs

As GLUTs are promising targets in cancer, inhibition of them have already been tested with various drugs. The first natural inhibitor of GLUTs was Phlorizin, which hindered the transport of D-glucose over the renal proximal tubule in rats[174]. Additional natural inhibitors have been extracted since, and their effect in malignancies established. One example is Cytochalasin B which is a mycotoxin that by co-crystallization has shown binding to GLUT1[175]. Together with an antimycin it had synergistic suppression of ATP production in a cell assay of lung carcinoma[176]. However, it also affects the cytoskeleton which might negatively affect the tumor microenvironment stromal cells[107]. There have also been several small-molecule inhibitors to GLUTs developed. WZB 117 inhibits GLUT1 and diminishes cancer cell proliferation in lung and breast cancer cells as well as lung tumor growth in a mice model[177]. WZB 117 can also re-sensitize doxorubicin resistant breast cancer cells[178]. Another inhibitor showing very selective inhibition of the GLUT1 isoform is BAY-876 which also has high oral bioavailability[179]. When tested in hepatocellular carcinoma tissues the tumor cells had slower proliferation and less expression of epithelial-mesenchymal transition (EMT) related factors[180]. The compound DRB18 is a pan-GLUT inhibitor meaning it can inhibit several GLUTs, in this case GLUT1-4[181]. It has demonstrated efficacy against approximately 60 different cancer cell lines, however, no effect has been confirmed in leukemia so far[107]. Another small-molecule pan-GLUT inhibitor is Glutor, which has effect on GLUT1-3. It affects proliferation in 44 out of 94 different cancer cell lines tested, with an IC50 < 100 nM, and synergistically targets cell growth in colon cancer cells when co-administered with a glutaminase inhibitor[182]. However, Glutor still needs to be tested *in vivo*[107].

In general, more information is needed on compounds that can inhibit GLUTs and information on where they bind, how they affect different cancers and how they can be used together with other drugs in treatment of malignancies[107].

In **paper II** we have analyzed inhibitors for GLUT1. Different variants of them had previously been tested in a lung cancer cell line showing inhibition of glucose uptake and antiproliferative effects[183,184]. Two of the compounds, PGL-13 and PGL-14, performed inhibition of glucose uptake into giant vesicles expressing human GLUT1 and were chosen for further studies (Figure 12). I tested the inhibitors in cell lines THP-1 and KG-1 using a viability assay based on ATP measurements. Both PGLs affected the viability after 72 hours of incubation, yielding an IC_{25} for each compound that could be utilized in combination studies. This was a sign that the PGLs could become effective in targeting AML cells.

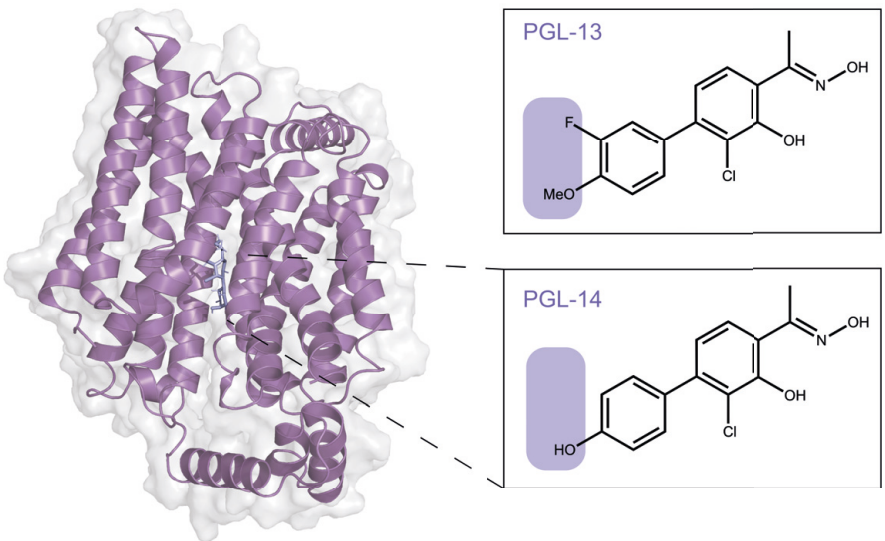


Figure 12 | Inhibitors of GLUT1. Protein structure of GLUT1 (PDB ID: 5EQI) with inhibitor PGL-14 at the predicted transmemebrane binding site. PGL-13 is believed to bind in a similar way. Molecules of PGL-13 and PGL-14 are shown with their structural differences highlighted.

Co-treatment with IC_{25} concentrations of PGL-13 and Ara-C lead to synergistic detrimental effects on THP-1 cell viability (Figure 13, left). Synergism means that the combined effect is greater than the sum of their individual effects and is a sign of PGL-13 causing a negative effect on THP-1 cell survival that is not competing with the Ara-C target, but enhancing its effect. When repeating the assay on KG-1 cells the synergistic effect did not occur. Instead, the combination was not even reaching

additive potential (Figure 13, left). The same results were seen when adding PGL-14 in combination with Ara-C in the two cell lines (Figure 13, right).

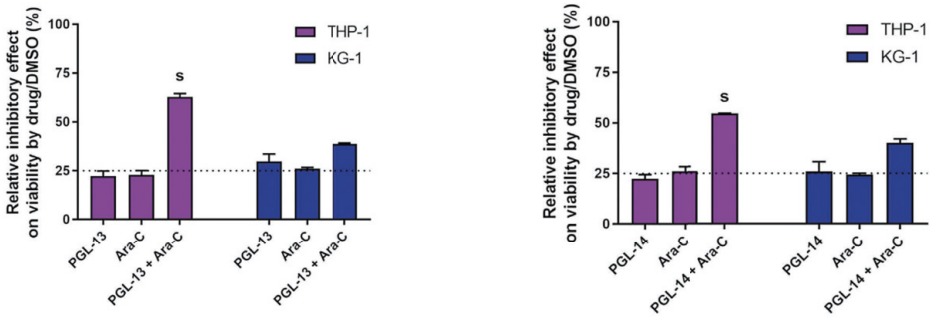


Figure 13 | Effect by PGLs together with Cytaraine (Ara-C) in AML cells. Relative inhibition of cell viability in THP-1 (magenta) and KG-1 (blue) by IC_{25} concentrations of Ara-C and (left) PGL-13 or (right) PGL-14, alone or in combinaition. 100 % means complete inhibition. Theoretical IC_{25} values are shown with dotted lines and synergistic effect is marked with "S". Bars show mean + SD, n = 3-5.

Because THP-1 showed more GLUT1 expression than KG-1 (Figure 7) we concluded that the synergistic co-treatment effect is dependent on GLUT1 expression. This was also an indication that the PGLs selectively targeted GLUT1. To further confirm that the PGLs inhibit glucose transport the experiment was repeated with Ara-C in combination with maltose, a disaccharide known to bind to GLUT1 but without ability to get transported[185]. This showed that even maltose, typically considered a common sugar, in combination with Ara-C results in synergistically adverse effect in THP-1 cells (Figure 14A). However, the IC25 of maltose was roughly 10 000 times higher than that of PGLs, confirming that PGLs are much more potent inhibitors of GLUT1.

The effect by GLUT1 inhibition by PGLs were also tested in combination with doxorubicin (DOX). DOX is an anthracycline that has been used to treat many types of cancer, including leukemia, and is pharmacologically related to daunorubicin, commonly used in the 7 + 3 regimen[186] . These combinations also had synergistic effect in THP-1 cells (Figure 14B-C). Thus, blocking glucose uptake in THP-1, and consequently blocking glycolysis, shows synergistic effect in combination with drugs targeting DNA synthesis.

Because LSCs have indicated to be highly dependent on OXPHOS[77,78], I tried combination therapies with PGLs and Ara-C together with dihydroorotate dehydrogenase (DHODH) inhibitor Brequinar, known for its anti-OXPHOS effects[187]. Interestingly, there was no synergistic effect with Brequinar in co-treatment with either Ara-C, PGL-13 or PGL-14 (Figure 14 D-E). Also, when combinations of Brequinar was analyzed together with both Ara-C and PGL no additional synergy was

obtained (Figure 14 D-E). The absence of Brequinar effect can be due to the possibility of THP-1 cells being disassociated from OXPHOS and instead are using aerobic glycolysis. The dependence on OXPHOS has also mainly been seen in LSCs, while THP-1 cells have a more blast like phenotype. However, it would be interesting to test the PGLs in combination with other OXPHOS inhibitors like venetoclax.

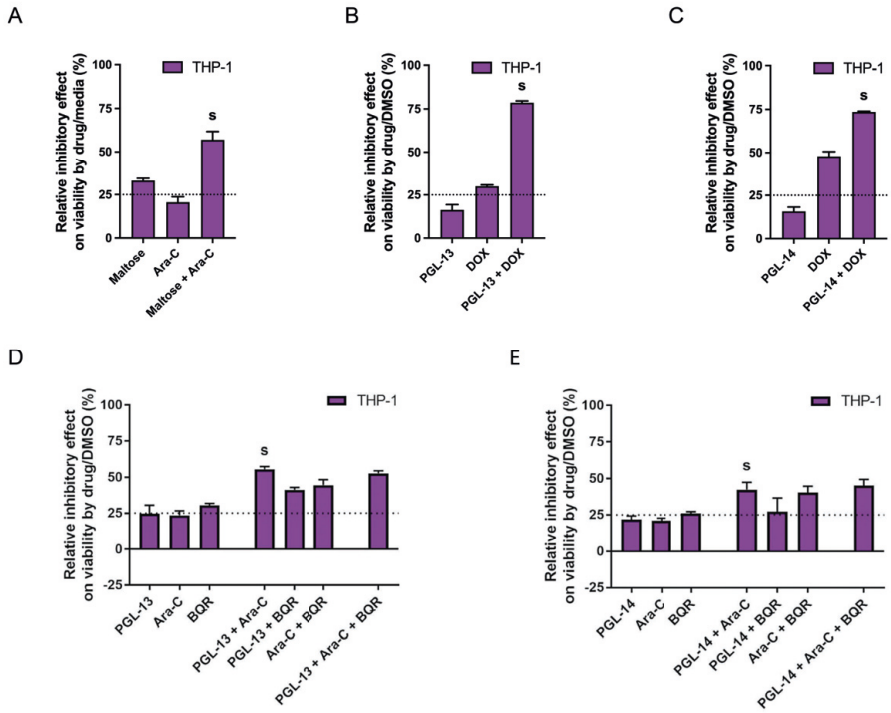


Figure 14 | Further validation of PGLs in THP-1 cells. IC_{25} concentrations were used for various inhibitors and drugs, alone or in combination. Graphs show results of (A) Maltose and Cytarabine (Ara-C), (B) PGL-13 and doxorubicin (DOX), (C) PGL-14 and DOX, (D) PGL-13, Ara-C and Brequinar (BQR), (E) PGL-14, Ara-C and BQR. Bars show mean + SD, n = 3. Synergistic effect is marked with "S".

Both PGLs were validated to be potent GLUT1 inhibitors with ability to decrease viability of AML cells. Their synergistic effect with Ara-C demonstrates that glucose uptake and utilization could be targeted to overcome Ara-C resistance, a common drawback in AML. Also, by sensitizing the cells to Ara-C the chemotherapy dose administered could be lowered, a desired entity in elderly patients. It would be motivating to try these inhibitors in additional drug combinations and test if the effects are similar in other types of cancer. It would also be of value to further analyze the PGLs selectivity for GLUT1 over other GLUT isoforms. This would give information

about if they could be used to specifically target cancer cells with high GLUT1 expression or if their effect is more general, affecting cells with high glucose uptake.

Inhibition of CD36

As previously noted, CD36 serves not only as a receptor for FAs but also for various other substances. Majority of anti-cancer treatments targeting CD36 in clinical trials have been mimicking the binding of TSP-1 to CD36, which has apoptotic and anti-angiogenic effects in various cancers[132]. Inhibition of lipid transport by CD36 on the other hand has been inhibited by antibodies and small-molecule inhibitors believed to bind to CD36 and block the FA binding and uptake. One antibody that has been used in several assays is JC63.1. It lowered metastasis of oral squamous cell carcinoma to lymph nodes and lungs in a mice model with no toxic side effects noticed[92]. It also showed inhibition of lung metastasis initiation from gastric cancer in mice[188] and increased the radio-sensitizing effect of a fatty acid synthase inhibitor in prostate cancer cell lines[189].

A common molecular inhibitor tested in CD36 FA uptake experiments is the sulfo-N-succimidyl ester of the long chain FA oleate (SSO), first confirmed to inhibit fatty acid transport in rat adipocytes[190]. It has since been confirmed to block long-chain fatty acid (LCFA) uptake also in other cell types of rat[191], like pneumocytes[192], and sarcolemmal vesicles of the heart[193]. In human context, SSO has been studied for its effect on cancers. It was shown to block FA-enhanced cell viability and migration of lung adenocarcinoma[194], and lowered migration of liver cancer cells in a cell assay[195]. However, it has been debated whether SSO affect the FA transport by CD36 or if it instead can cross the cell membrane and intracellularly affect proteins involved in FA metabolism[196].

There are some small-molecule inhibitors tied to CD36 function being tested with promising effects. However, a more comprehensive understanding is still needed on how they structurally bind to CD36 and more effort must be made to increase inhibitor affinities to CD36 and lowering their off-target effects[197].

In **paper III** we evaluated a small-molecule inhibitor of CD36 (Figure 15). The inhibitor, named SMS121, was first confirmed to bind the CD36 protein in a surface plasmon resonance (SPR) assay before processing to subsequent cell studies.

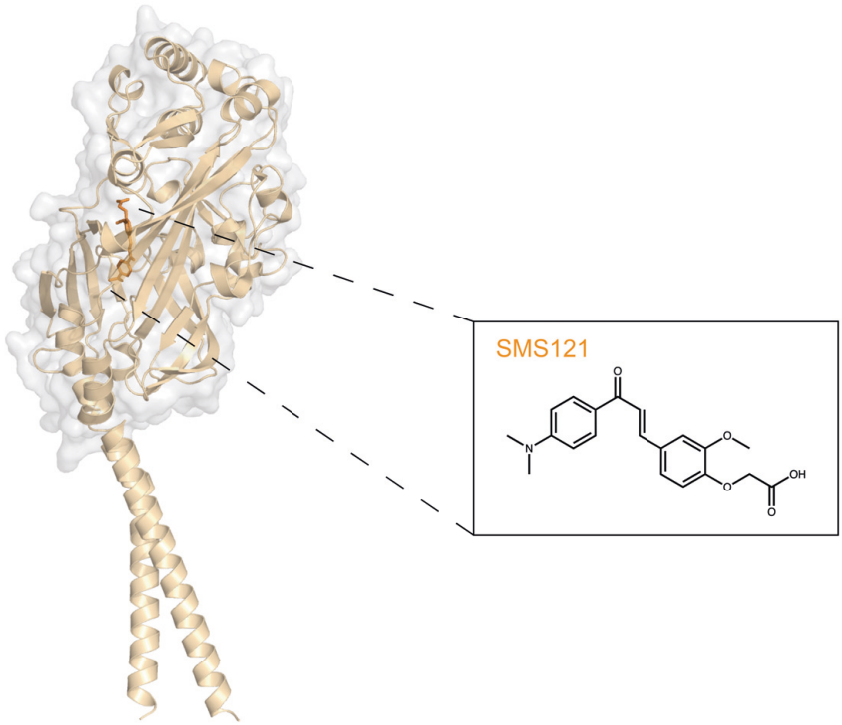


Figure 15 | CD36 inhibitor SMS121. Structure of CD36 (PDB ID: 5LGD) with inhibitor SMS121 in its putative binding site as predicted by docking. The transmembrane domain was created with AlphaFold.

Utilizing the information that KG-1 cells express more CD36 and has more lipid uptake than THP-1 made these cell lines suitable for a comparative study (Figure 9 and Figure 10). The inhibition of FA uptake by CD36 was evaluated with a fluorescent long-chain FA analogue by microscopy. Pre-incubation of KG-1 cells with SMS121 lead to decreased uptake of the fluorescent FA (Figure 16, left). This was observed in a concentration dependent manner showing that the compound inhibits FA uptake in cells.

The low impact of glucose inhibition with PGLs together with chemotherapy suggests that KG-1 is more dependent on FAO than glycolysis. This was further supported by its high CD36 expression and FA uptake. Certainly, incubation of KG-1 with SMS121 for 72 hours leads to effect on cell viability as measured by ATP assay (Figure 16, right). The IC_{50} value calculated for viability (156 µM) was similar as for lipid uptake (164 µM), implying that the viability is affected by the lowered lipid uptake.

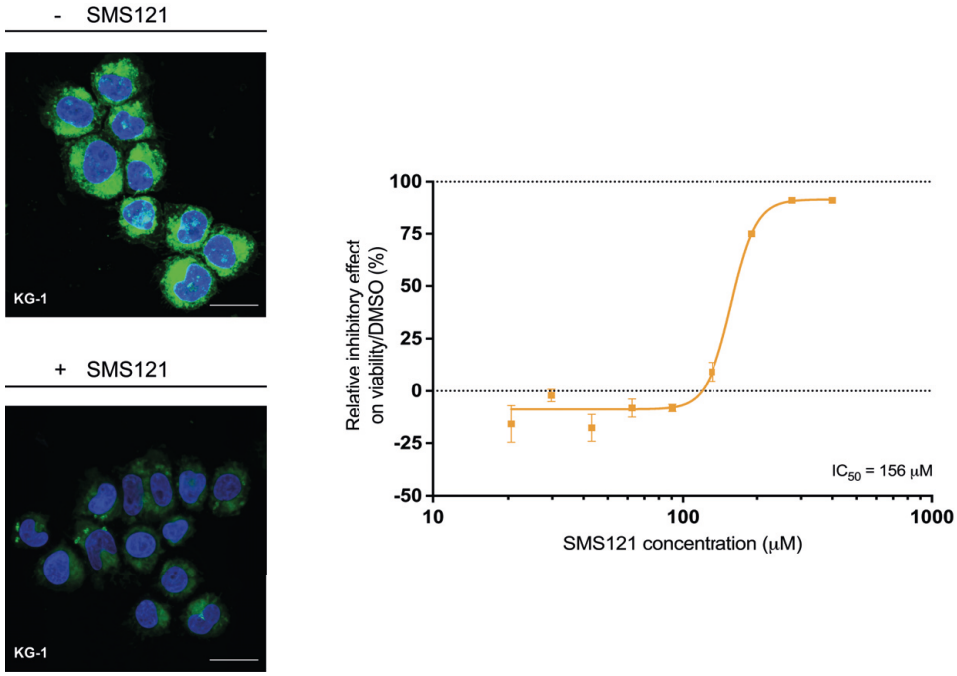


Figure 16 | SMS121 effect in KG-1 cells. (Left) The effect of SMS121 on uptake of a green fatty acid (FA) analogue in KG-1 cells. SMS121 (200 μM) or a DMSO negative control was added for 50 minutes before additional 10 minutes of uptake of FA analogue. Scale bars show 20 μm. (Right) Inhibition of KG-1 cell viability by SMS121 in various concentrations after 72 hours. Values show mean of n = 3. Error bars show SD.

To gain more information on the potential of SMS121 and FA uptake inhibition in a cancer treatment setting, I treated both KG-1 and THP-1 cells with SMS121 for 96 hours and calculated the number of remaining live cells. Interestingly, both cell lines were affected by the inhibitor (Figure 17A, Figure 17D), however with more effect on KG-1. This might argue that the amount of CD36 expressed is not the determent of the outcome by long-term inhibition of CD36 and that cells with low expression, like THP-1, also are affected. Nevertheless, it could also be a sign of off-target toxic effects. This was, however, ruled out by rescue experiments, by adding either the long chain FA oleic acid (OA) or bovine serum albumin (BSA) to the cells prior to SMS121 addition. Added oleic acid was able to compensate for the diminished FA uptake in both cell lines (Figure 17 B, Figure 17 E). Also, BSA, capable of binding to CD36 and thus competing with SMS121[198], could rescue both cell lines (Figure 17 C, Figure 17 F).

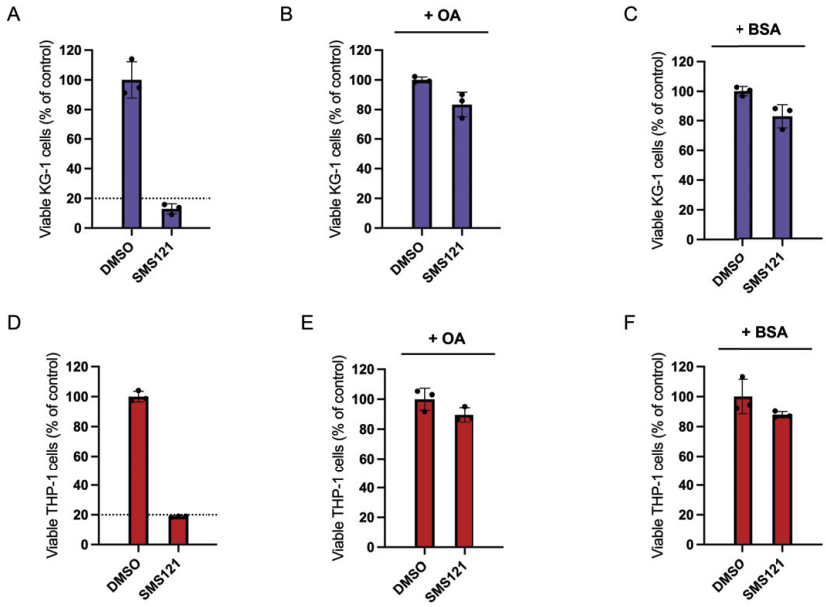


Figure 17 | Effect on AML cells by SMS121 and rescue experiments. Live cell count by tryphan blue exclusion was analysed after 96 hours of incubation with SMS121 (150 µM) in KG-1 (blue) and THP-1 (red) cells compared to a DMSO control. The incubation was done (A, D) without additatives or with rescue of (B, E) oleic acid (+ OA) or (C, F) bovine serum albumin (+ BSA). Bars show mean of n = 3. Error bars show SD.

Generally, SMS121 is a promising new inhibitor to CD36 with proper binding to the target and effect on both lipid uptake and cancer cell viability. The many associations of CD36 to malignancy, both in AML[82,138] and other cancers[83,199], makes it an intriguing target to study further across cancer cell types. The introduction of SMS121 provides a valuable mechanism for evaluating and understanding these relations. Lipid metabolism is a big part of cancer metabolic rewiring and upregulation of fatty acid transporters and FAO can be a way for cancer cells to defeat chemotherapy[157]. Further studies should be done on how SMS121 effects conventional AML therapy and if it has potentiating effects in combination with other drugs targeting cancer metabolism. Given the substantial influence of the microenvironment on LSC growth, inhibition of FA uptake from adipocytes by SMS121 has good potential.

Inhibition of AQP7

AQPs are considered challenging for drug development. Because different isoforms are expressed in different tissues an inhibitor needs to be selective to diminish off-target effects[200]. The inhibition of water transport in AQP1 has been blocked with inhibitors based on heavy metals like mercury and gold. These compounds are usually toxic and cannot be implemented in the clinic. Other small-molecule inhibitors have been analyzed, initially demonstrating effectiveness in early *in vitro* assays but without the ability to block water flux in later studies[200]. Instead, the effect on AQPs in cancers has been studied by targeting AQP expression. For example, knock down of AQP7 in mouse breast cancer cells reduced proliferation and metastasis[146]. Also, AQP3 siRNA silencing or mercuric chloride inhibition made prostate cancer cells more sensitive to cryo treatment[201].

Regarding AQP7, a gold (III) compound named Auphen has showed ability to block glycerol flux of both human and mouse homologue of AQP7 expressed in an adipocyte cell model[202]. Three acyl glycerol derivates were also displayed to inhibit water and glycerol flux of human AQP7 when expressed in canine kidney cells[203]. Due to its impact on glycerol flux, the potential of inhibiting AQP7 has mainly been considered for evaluation in the context of treating obesity[200,204]. Concerning AQP7 inhibition effect by small molecules in cancer cells there has not been much progress yet.

One drug-like small molecule called Z433927330 was identified and showed ability to inhibit water permeability in Chinese hamster ovary (CHO) cells expressing mouse AQP7, with selective inhibition of AQP7 over AQP3 and AQP9. Additionally, it also inhibits glycerol flux of mouse AQP7[205]. In **paper IV** I studied the impact on blocking AQP7 with this inhibitor in the APL cell line NB4. Z433927330 was confirmed to selectively bind and block also human AQP7 by combining structural and functional data before being subjected to NB4 cell studies (Figure 18). It was compared to 9016645, another candidate AQP inhibitor with similar molecular structure lacking the pyrazole ring (Figure 18).

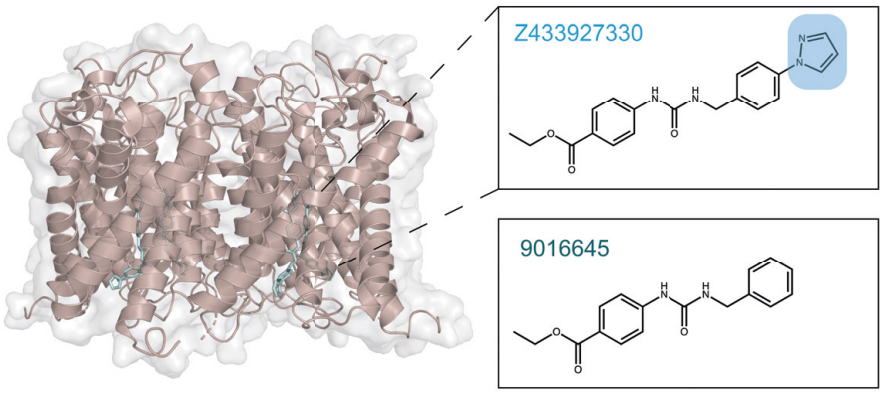


Figure 18 | Aquaporin (AQP) inhibitor Z433927330. Cryo-EM structure of AQP7 in complex with Z433927330 (PDB ID: 8C9H). Molecules show Z433927330 with the pyrazole group higlighted in comparison to another candidate AQP inhibitor 9016645.

The absence of the pyrazole ring makes 9016645 have fewer potential interactions in the glycerol pore of AQP7 and could explain why it does not show as good inhibitory effect on mouse AQP7 as Z433927330[205]. The inhibitor was tested in the NB4 cell line, confirmed to express AQP7 (Figure 11). A concentration of 10 µM affected the proliferation of NB4 cells over four days showing a reduction in the number of live cells compared to a control (Figure 19). This effect could also be seen at a lower concentration, although less prominent. Decrease in proliferation after addition of 9016645 was not detected, confirming that the effect by Z433927330 is due to its strong binding to AQP7 (Figure 19).

The scarcity of inhibitors targeting AQP7 makes the discovery of Z433927330 exhibiting inhibitory effects on human AQP7 both notable and promising. The ability to influence leukemia cell viability provides bright prospects for further studies in AML but also in other types of cancer. Because AQP7 is a glycerol channel[148], and has been shown to have superior glycerol transport than the other aquaglyceroporins AQP3 and AQP9[148], blocking it with Z433927330 possibly have big impact on glycerol flux in the cell. Glycerol is closely related to lipid metabolism[66] and inhibition of glycerol uptake could be the reason behind the detrimental effect on NB4 cell viability. However, AQP7 has previously been described as a transporter for glycerol efflux and blocking glycerol efflux could also lead to potential accumulation of intracellular glycerol, which is toxic[123,124].

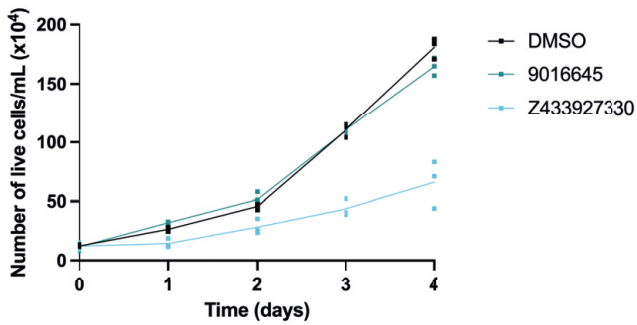


Figure 19 | Effect on proliferation of Z433927330 in APL cells. Inhibitor Z433927330 (light blue) was added to the APL cell line NB4 in concentrations of 10 µM for 4 days. The number of live cells/mL was calculated each day and compared to inhibitor 9016645 and a DMSO control, n = 3.

The effect by Z433927330 on NB4 is useful knowledge for future studies targeting metabolism in various malignancies, especially those with known AQP7 dependence, like breast and thyroid cancers[146,147]. The selectivity for AQP7 raises the potential of avoiding off-target effects. Yet, APL treatment is uniquely dependent on AQP7 for arsenic transport[51,53]. Hence, blocking AQP7 in these patients is not optimal. Rather, the effect by Z433927330 on NB4 cells serves as a positive indicator that the inhibitor is affecting human cancer cells. This gives Z433927330 potential to have similar effects in other cancer cells. It encourages further exploration and testing across various cancer cell lines to ascertain the inhibitor's efficacy and possible therapeutic implications. Because AQP7 also is important for glycerol efflux from adipocytes[151,152], and adipocytes are affecting the maintenance of cancer cells[21], it would furthermore be interesting to see the effect on Z433927330 in samples with adipocytes in co-culture with AML cells. Due to AQP7 implications in obesity[200,204], this could be another noteworthy route to discover further. Since AQP7 is affecting glycerol flux and lipid metabolism, Z433927330 have potential in increasing the effect of other metabolism inhibitors or chemotherapy.

General discussion and future perspectives

Cancer metabolism has received increased attention in recent years, emerging as a promising target in the treatment of AML and other cancer types. Metabolic drugs are seen as potential contributors for reducing the dosage levels required in standard treatment. However, a notable gap becomes apparent when reading published literature, revealing a significant shortage of metabolic inhibitors. Not only have very few drugs advanced to clinical trials, but even fewer have achieved success to the extent that they are implemented in the clinic. There is also not that many inhibitors targeting metabolic processes that have been synthesized and tested in pre-clinical models. Nevertheless, this field within cancer drug research is relatively new and there is anticipation for future advancements.

In this thesis three kinds of new inhibitors have been described along with their implementations in AML cell studies. Each of these inhibitors target distinct aspects of cell metabolism. The PGLs target glucose uptake of GLUT1, SMS121 impacts FA uptake by CD36 and Z433927330 affects glycerol flux via AQP7. At the same time as they target metabolism substrates, these inhibitors also show detrimental effect on viability of various cell lines of AML. The inhibitors already show promising druggability adhering to the Lipinski rules[172,206]. However, with the inhibitory concentrations applied now being in the µM-range, there can still be improvements on their effect of activity at their respective targets. This can be pursued in future studies by making small changes to their molecular structures, such as altering side-groups. We already show cryo-EM data of Z433927330 in complex with its target protein AQP7. Extending this approach to obtain similar cryo-EM or X-ray crystallography structures of the other inhibitors and their targets would be preferable in future investigations when making decisions on how to improve the affinities. On top of efficacy also solubility, toxicology and potential off-target effects should be further evaluated before progressing to *in vivo* testing. Yet, the analyzed compounds harbor promising characteristics. Being small-molecule inhibitors they could, for example, ease administration compared to antibody-based drugs. By targeting transporters on the cell surface, they can aim at early steps of cancer metabolism. This is an appealing approach that is relatively uncommon amongst other inhibitors for metabolism on the market.

There is a need for more compounds to be tested for their anti-cancer activity and to further evaluate their mechanisms and downstream effects. The demonstrated effects in human cell lines shown in this thesis opens possibilities for further studies regarding downstream effects of transporter inhibition. This encompasses investigating downstream effects of metabolic activities such as mitochondrial respiration. Considering the shared metabolic pathways effected in various cancers, the inhibitors could undergo testing in a wide range of cancer types beyond AML.

Due to the heterogeneity of AML, it might not be enough to target just one metabolic pathway. Previous *in vivo* and clinical studies in cancer have shown that relying on inhibition of a single metabolic pathway is insufficient because the drugs often display narrow therapeutic windows, and the cancer cells have propensity to develop compensation mechanisms[164]. For example, attempts by others to target the ETC lead to the activation of FAO instead[61], highlighting the adaptability of cancer cells and their propensity to escape therapeutic interventions. Combination therapy targeting several metabolic pathways together with conventional chemotherapy is a potential approach to overcome resistance.

In my research I have assessed the PGL-13 and PGL-14 effect by targeting glucose uptake with GLUT1 in combinations with chemotherapy drug Ara-C. Both PGLs could sensitize AML cells to Ara-C, which proofs that combination therapy works. For the future it would be beneficial to test all the inhibitors, PGLs, SMS121 and Z433927330, in different combinations and also together with chemotherapy and other available cancer treatments. This could offer a comprehensive strategy, targeting multiple pathways. While the PGLs did not show any improved effect of an OXPHOS inhibitor in my studies, others have showed that CD36 knockdown could re-sensitize venetoclax resistant primary AML cells[82]. Thus, CD36 inhibitor SMS121 should be tested for its potential capability of re-sensitizing these resistant cells.

Certainly, for clinical applications, it is important to not target all metabolic pathways of the healthy cell. A precision medicine approach is essential to avoid adverse effects. It will be crucial to evaluate the specific metabolic rewiring of the patient's cancer cells. To achieve this a thorough investigation into how various mutations in different patients contribute to distinct metabolic upregulations and resistance mechanisms is highly relevant. In this study, AML cell lines and adipocytes from subcutaneous fat have been used as model systems to provide evidence of the various inhibitors' efficacy. While these models have served their purpose well it would be of interest to evaluate how they perform in a more systemic and leukemic environment setting. The data presented in this thesis clearly shows that different cell lines can have different transporters expressed, which can influence their response to the inhibitors. Therefore, it would be interesting to further analyze the effect in primary AML cells and further investigate if the compounds would affect leukemic blast cells and LSCs differently.

The microenvironment can affect cancer cells in various ways, and it can also impact drug resistance. For example, stromal cells, including adipocytes, can reduce the effect of FAO inhibitors by augmenting their FA uptake or redirecting cellular metabolism towards glycolysis instead[207]. BM stromal cells are thought to by transfer of mitochondria to AML cells restore drug induced mitochondrial respiration and rescue leukemic cells from chemotherapy[208,209]. It was also suggested that FA from adipocytes can be transferred to the nucleus of AML cells where they can activate PPARγ and induce transcription of genes coding for proteins involved in lipid transport such as CD36[138]. AML cells can in turn also affect adipocyte signaling and induce adipocyte lipolysis to increase the availability of fatty acids in the environment for their own metabolic needs[210,22,83]. In the CD36 project the interplay between AML cells and adipocytes have been of particular focus. We detected a potential signaling molecule between AML cells and adipocytes in co-culture called Inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1). This protein has potential to induce insulin resistance in adipocytes and could thus effect glucose levels and lipolysis[211]. ITIH1 should be further investigated to clarify if it is a signal released by AML cells to enhance nutrient acquisition from adipocytes. Moreover, the lipid transfer and signaling dynamics between leukemic cells and microenvironmental adipocytes presents an interesting opportunity for future studies. Discovering means to interfere in the communication signals between cells early on holds potential to lead to new innovative targeted therapeutics.

In the context of adipocytes, it is relevant to mention obesity in AML. Obesity has in some studies been connected to increased incidence and worse prognosis in leukemia[212,213]. However, bone marrow adipose tissue appears to expand instead of shrink with calorie restriction[85]. Also, body mass wasting due to cachexia and reduced appetite, commonly occurring as side effects of chemotherapy, is coupled to malnutrition that not only extends hospital stays but also elevates risk of mortality in AML[214]. Certainly, it is crucial to emphasize that calorie restriction is not a recommended treatment for leukemia and that inhibitors to metabolic processes should have specific effects on cancer cell metabolism, not systemic metabolism. On another note, obesity is often linked to type 2 diabetes (T2D) and some solid tumors, including colorectal, endometrial and breast cancers, are more prominent in patients with T2D[215,216]. Leukemia has also shown intermediate increase in risk for T2D patients[217,218] and diabetes drug metformin has been considered preventive against cancers[161]. Thus, with a systemic viewpoint, it would be advisable to ensure proper insulin signaling when targeting metabolic pathways.

Previous data showed that leukemic mice with *KMT2A* rearrangement, like THP-1, displayed leukemia induced insulin resistance in adipose tissue and muscle which led to reduced glucose uptake in these tissues and increased leukemic burden[210]. It is believed that leukemia cells might induce insulin resistance to redirect nutrients from

cells highly affected by insulin, like muscle and adipocytes, to support their own growth. Reduced insulin signaling in these cells could potentially affect all transporters discussed in this thesis. Less insulin sensitivity would lead to more GLUT4 being tethered intracellularly of adipocytes and not at the surface to take up glucose. This would in turn lead to more blood glucose that the AML cells can take up with, for example, upregulated GLUT1. CD36 has also been confirmed to be more expressed at the surface of muscle and adipocyte when insulin is present[124,219]. Moreover, insulin resistance is associated with adipocyte lipolysis[220]. If insulin resistance decreases CD36 expression in these tissues, there will be more FA in the environment for the malignant cells to utilize. After food intake when insulin levels are high, AQP7 is kept in the cytosol of the adipocyte[149,150]. Hence, insulin resistance in the adipocyte could cause higher expression of AQP7 at the adipocyte surface, and subsequently more glycerol being released to the surrounding that AML cell can use for lipid synthesis. In conclusion, there is an opportunity to perform a more extensive study of systemic effects, particularly investigating downstream insulin-signaling in both AML cells and adipocytes after co-culture, and further examine the potential of all the new inhibitors on these processes.

Methodology

This thesis comprises research based on cellular and protein studies. The focus has been to examine the function and expression patterns of transporters in adipocytes and leukemic cells to later establish their drug target potential. The experimental approach utilized new inhibitors that were analyzed for their capacity in affecting these transporters, aiming to limit malignant progression.

Cell methods

The studies have been done using cell lines, primary adipocytes or a combination of them. Different types of cell models have distinct advantages and can contribute to the acquirement of valuable scientific information.

Cell lines

Immortalized cell lines originate from cancerous tissues and are important tools in research. They are highly proliferative which can give them different metabolic profile than quiescent cells making it resemble the metabolism of a blast more than of an LSC[59]. To study cells even closer to human pathology and e.g. select for LSCs, one can use primary cancer cells. These are directly derived from tissue of either a patient or an animal model[221]. For studying human metabolism, human cells are preferred over animal cells. However, human primary cells are not as readily accessible as commercial cell lines. The metabolic phenotype and use of nutrients can also differ in a cell in culture compared to a malignant cell in the human body or in an animal model[58]. One can use patient derived xenograft (PDX) models where human cancer cells are injected and analyzed in mice[57]. This gives the opportunity to study systemic effects after a drug treatment on for example metabolism pathways. It also offers an advantage in using human cells surrounded by a tumor microenvironment, a factor known to exert significant influence on metabolism[59]. However, PDX models are usually performed in immunocompromised mice, hence their immunological microenvironment will be affected[57]. Additionally, the microenvironment is not of human origin.

In **paper II**, **paper III** and **paper IV** commercial AML cell lines were used. Despite having some limitations in comparison to primary cells and animal models, they offer greater accessibility, ease of long-term maintenance, and involve fewer ethical considerations. The selected cell lines derived from the cancer type studied and could be verified to express the proteins of interest. These types of cell lines are very useful for evaluating direct effects of protein inhibition in the AML cell, without having to be transported through tissues and excluding confounding impact of other cells in the microenvironment. They give a platform for high throughput analysis of inhibitor effect on systems like metabolism and proliferation, which can verify and further evolve results that previously have been noticed in chemical and structural assays. When studying inhibitors targeting human proteins, human cell lines expressing the protein of interest in its natural form are particularly well-suited. For pre-clinical investigations, especially in the early stages of assessing the inhibitory effect of compounds, cell lines can be valuable models. This preliminary testing constitutes a crucial step before advancing to animal models and clinical trials.

Human primary adipocytes

We had the opportunity to have access to patient derived adipose tissue from which human primary adipocytes could be isolated and used for experiments in **paper I** and **paper III**. In the isolation process fat lobules were excised from the adipose tissue and digested with collagenase. The adipocytes were then washed several times to obtain clean cells. Because adipocytes mainly consist of one large lipid droplet, they float on top of the washing buffer. This makes it easy to remove contaminating adipocyte stromal vascular fraction (SVF) from underneath with a syringe[222]. It also simplifies the process of separating them from other cells in co-culture. The primary adipocytes floating makes them exhibit a more inherent nature than most human and animal adipocyte cell lines, which usually lack this ability. Additionally, they are from human origin, making them natively express human proteins. This gives them advantage over animal primary cells or the widely used mouse 3T3-L1 cell line. Restrictions are that human primary adipocytes begin to undergo decay and cannot be used after 48 hours. Their use is also constrained by their limited accessibility.

Protein detection methods

This thesis includes a comprehensive analysis of protein expression patterns in various cell lines. Several methods were utilized to confirm protein expressions and interactions.

Western blot

To detect proteins and compare their expression levels in different cell lines western blot was used. For this technique the cells are lysed and their proteins extracted and separated by size with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After, the proteins are transferred to a membrane with the help of a perpendicular electric field. Once on the membrane, primary antibody specific to the protein of interest can attach. By using a secondary antibody connected to horseradish peroxidase (HRP), the protein can be visualized with chemiluminescence[223]. Western blot is considered a semi-quantitative method because it is not possible to exactly quantify the expression of a protein levels. One can however do comparisons of expression[224], e.g. between two cell lines. This method is dependent on using highly specific antibodies[223]. Two antibodies can have different specificity which is also the reason why one should not compare two different protein expressions from each other. The localization of the protein band on a western blot can be affected by post-translational modifications. For example, the GLUT1 protein in **paper II** comes out more like a smear than a distinct band, which is common due to having heterogenous glycosylation[225].

Microscopy

To show the expression and localization of the proteins in cells laser-scanning confocal microscopy was used. In fixed and mildly permeabilized cells the proteins were labelled with specific primary antibodies. Fluorophores on secondary antibodies were then excited and could be detected. Confocal microscopy has better signal-to-noise ratio and gives better resolution images than epifluorescence microscopy. It also has the advantage of depth visualization in samples, reaching up to 200 µm depth into the sample[226]. This makes it possible to visualize the localization of a protein in a middle section of a cell. It also makes it possible to take images in several focal planes of the cell and put them together into a three-dimensional image. This was useful in **paper I** where adipocytes even being large, could be visualized. Another microscopy type that could be considered to further explore differences in movement of GLUT 4 and ASPL in adipocyte membranes during insulin stimulation is total internal reflection fluorescence (TIRF). TIRF is not used for making stack images of whole cells but is superior in imaging proteins activities the plasma membranes of live cells[227]. As in western blot imaging fluorescently labelled proteins is dependent on specific antibodies which makes it important to use negative controls where no primary antibody is used.

Proximity ligation assay

Because the adipocyte cytoplasm is so thin in comparison to the lipid droplet, it can make co-localization analysis by simply targeting two proteins with different fluorescent probes and looking at overlap with microscope difficult. In **paper I**, GLUT4 and ASPL was verified to bind to each other by a pull-down assay and to be expressed in adipocytes. But, to verify that GLUT4 and ASPL co-localize in their natural state in the human adipocyte *in situ* proximity ligation (PLA) was applied. PLA is a method for protein-protein interaction studies in fixed cells where proximity of the two proteins is needed to obtain a signal[228,229]. Two proteins to be studied, e.g. GLUT4 and ASPL, are labelled with their specific primary antibodies. The primary antibodies are produced in two different species to enable distinct recognizing by two secondary antibodies. The secondary antibodies, also called PLA probes, each have a single-stranded oligonucleotide sequence. If the two proteins are in proximity the PLA probes will connect and form a circular DNA. Rolling-circle amplification by polymerase amplifies the DNA which can be detected in a microscope by many fluorescent complementary probes binding to it[229,230]. Because of the signal being amplified by rolling circle amplification (RCA) the method is sensitive and makes it easier to detect also single events of protein-protein interaction. It is not a complete proof that two proteins form a complex, however, it verifies that they are within 40 nm distance from each other. An advantage of PLA is that it allows you study the cell's endogenous proteins with no genetic manipulation needed.

Inhibitor assays

In **paper II**, **paper III** and **paper IV** small-molecule compounds have been tested for their inhibitory effect on cell surface receptors in AML cells. The inhibitors used must be dissolved in dimethyl sulfoxide (DMSO). DMSO itself can be toxic to cells, especially if used in doses over 1 % but the lower concentration used the better[231]. The inhibitors have been diluted in cell media 1000 times to make the end concentration of DMSO in contact with cells as low as possible. Most of the time the end concentration has been 0.1 % and the highest concentration used was 0.4 % for a short time experiment. To account for potential DMSO effects, all tests were conducted using negative controls with same DMSO concentration. When adding an inhibitor to cells it is therefore important that it is soluble enough to be able to be diluted in media. Because the inhibitor must be diluted 1000 times it is preferred that it has proper effect on the target already at this stage to avoid the demand of high starting concentrations. The starting concentrations used for the compounds were differently selected. For GLUT1 concentration curves were applied to the ATP assay and the IC25 value was used for subsequent combination assays. AQP7 inhibitor was utilized based on previous

concentration range used in CHO cells[205] and availability of already diluted compound. The inhibitor of CD36 was first prepared based on concentrations used for CD36 inhibitor SSO[191] which was later confirmed to be a proper concentration to use by IC50 values obtained from dilution series microscopy and an ATP assay. For chemotherapy drugs used for evaluation of GLUT1 inhibitors, Ara-C and Doxorubicin were used, based on that Ara-C and the doxorubicin precursor and structurally similar daunorubicin often are used for patients as the 7 + 3 regimen. These drugs were also added in IC25 concentrations based on dilution curve response in an ATP assay.

The effect by inhibitors were evaluated by different techniques based on what receptor was inhibited. These were uptake of a fluorescent FA, viability assay and proliferation assay. For future analysis of inhibitor effect on metabolism flow cytometry would be a useful technique. This could be used to for example do cell cycle analysis and measure the effect on apoptosis by Annexin V[232]. Interesting information about effect on mitochondrial respiration and glycolysis could be gained by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)[233]. Additionally, it would be noteworthy to study activation and upregulation of proteins downstream of the receptors by for example western blot.

Fluorescent fatty acid uptake

BODIPY (4,4-difluoro-3a,4a-diaza-s-indacene) is a lipophilic fluorophore which is available either as dies, to stain intracellular natural lipids, or connected to lipids to make lipid analogues[234]. Different types of fatty acids connected to BODIPY or added together with BODIPY stain has been used in lipid tracking studies[134,235,236]. This thesis contains experiments performed with C_1-BODIPY 500/510 C_{12}. This is a fatty acid analogue which in total resembles an 18-carbon fatty acid making it a long-chain fatty acid. The fatty acid analogue was used in **paper III**. First it was shown to be taken up by human adipocytes and transferred to AML cells. Later it was used for testing the performance of SMS121 blocking CD36 fatty acid uptake. This BODIPY fatty acid analogue is comfortable to use, has good chain length for studying a lipid uptake receptor and its readout is not affected by the natural lipids of the cell.

ATP assay

To evaluate viability of the cancer cells after addition of inhibitors, an ATP-assay was used in a 96-well format. This was used in both **paper II** and **paper III**. ATP production is a measure of metabolically active and alive cells. In this assay the cells are lysed which leads to the release of intracellular ATP. Luciferase is used to catalyze a reaction between luciferin and ATP to produce light which can be detected by a luminometer. The luminescence is proportional to the amount of ATP in a sample which in turn

represents the number of viable cells it contains[232]. The difference in luminescence readout between viable and dead cells are 200-fold[237]. This is a sensitive yet straightforward method with high potential for scaling up. One must be careful with temperature fluctuations, light sensitive and ATP contaminations of the reagent which can alter the result.

Trypan blue exclusion

Trypan blue exclusion is another user-friendly method to determine cell health. Trypan blue not being able to enter a cell is a mark that its membrane is intact and viable, while if the cell is dead its inner compartments will be stained blue[237]. This method was used in **paper II**, **paper III** and **paper IV** to be able to calculate live cell numbers during several days after inhibitor and chemotherapy addition. These numbers were used to measure the effect on cell proliferation.

Summary

The articles and manuscripts featured in this thesis are the result of a collaborative endeavor involving structural, biochemical and cell studies. Notably, a comprehensive overview mainly containing the cell-related aspects have been previously provided. The following are concise summaries of the complete content of each paper, incorporating additional information beyond what has previously been discussed to offer a more comprehensive outlook.

Paper I

The intracellular helical bundle of human glucose transporter GLUT4 is important for complex formation with ASPL

GLUT4 is carrying out the glucose transport into cells, mainly muscle and adipocytes, in an insulin-sensitive fashion. Upon insulin stimulation GLUT4 is translocated to the cell membrane where it can take up glucose. In rodents, this translocation has been seen to be dependent on cleavage of a protein, TUG. Here, we studied the human equivalent of this protein, ASPL, in relation to GLUT4.

Main results:

GLUT4 and ASPL were proven to be expressed in the cytoplasm of human adipocytes. The two proteins were demonstrated to establish contact and form a complex. The interaction was shown to be between the C-terminal helical lariat of ASPL and the intracellular helical bundle (ICH) of GLUT4. These results indicate that ASPL is important for keeping GLUT4 intracellularly when the adipocyte is not stimulated by insulin.

Paper II

Targeting GLUT1 in acute myeloid leukemia to overcome cytarabine resistance

In cancer cells a common phenomenon involves a metabolic switch toward high dependency on aerobic glycolysis. In AML this dependency on glucose metabolism is associated with chemotherapy resistance and worse prognosis. In this study we established new inhibitors to a glucose transporter, GLUT1, and analyzed their effect in combination with conventional chemotherapy in AML cells.

Main results:

Two new GLUT1 inhibitors, PGL-13 and PGL-14, were tested and validated to bind to GLUT1 and inhibit transport of glucose. Two potential binding sites for the inhibitors were found, one at the transmembrane and one intracellularly of GLUT1. Both inhibitors were able to synergistically sensitize AML cells with high GLUT1 expression, THP-1, to chemotherapy drugs Ara-C and doxorubicin. Their IC_{25} was 13 µM for PGL13 and 4 µM for PGL14 in these cells. The synergistic effect was not observed when targeting OXPHOS instead and synergy could not be increased by targeting OXPHOS simultaneously as inhibiting GLUT1 in combination with Ara-C.

Paper III

CD36 inhibitor impairs fatty acid uptake and viability of acute myeloid leukemia

Lipid metabolism is crucial for the highly proliferating cancer cells. CD36 is a cell membrane receptor responsible for fatty acid uptake. Its expression level is correlating with worse prognosis in several cancers, among them AML. With this information we set out to discover and validate an inhibitor to CD36.

Main results:

It was confirmed that fatty acids can be taken up by AML cells from adipocytes in co-culture. We showed that an AML cell line with high CD36 expression, KG-1, had increased uptake of a fluorescent fatty acid analogue. A new inhibitor to CD36, SMS121, was evaluated. It was validated to bind to CD36 by surface plasmon resonance and structural analysis. It decreased the amount of fluorescent fatty acid uptake into cells with an IC_{50} of 164 µM. SMS121 was able to reduce viability of KG-

1 cells in an ATP assay with an IC_{50} of 156 µM. Additionally, proliferation of KG-1 cells was affected which could be avoided by pre-treatment with BSA or oleic acid. SMS121 requires further development to enhance its efficacy but is a promising potential future drug targeting fatty acid uptake and metabolism in AML.

Paper IV

Molecular basis for human aquaporin inhibition

Aquaporin 7 (AQP7) is a membrane protein that facilitates glycerol transport. It is important for glycerol efflux in adipocytes to prevent build-up of glycerol intracellularly. Its expression has been linked to breast and thyroid cancer. In this paper, a new inhibitor to AQP7 was tested for the first time in human leukemic cells.

Main results:

A cryo-EM structure of human AQP7 with the inhibitor Z433927330 was solved, showing that the inhibitor blocks the glycerol channels of the aquaporin. The inhibitor was determined to specifically target AQP7 over AQP3 and APQ9 and was confirmed to affect AQP7 dependent arsenic transport. An APL cell line, NB4, was confirmed to express AQP7. When incubated with Z433927330, at both 5 µM and 10 µM concentrations, the inhibitor could slow down the proliferation and affect live cell counts of NB4 cells.

Popular science summary

Cancer is a devastating illness spreading fear all over the world, upsetting everyone that comes in its way. Acute myeloid leukemia, or AML, is a specific type of blood cancer where the treatment has not changed a lot in the past 40 years. Drugs used against AML are not that effective in distinguishing cancer cells to the body's normal cells. This leads to patients having awful drug side-effects and a lot of the time never fully recovering from the disease. The problem with these cancer cells is that they use up a lot of energy from the body to be able to grow and spread in a crazy manner. We are trying to flip this problem and make it into our advantage, by making the cancer cells starve.

In this thesis, transporters sitting on the surface of cells which are affecting the uptake of nutrients like sugar and fat have been analyzed. In **paper I** a sugar transporter in fat cells is studied. It can be held inside the fat cell when you are starving by a protein called ASPL. After a meal, the transporter is moved to cell surface where it can help take up the sugar you just ate.

Another sugar transporter was found to be in elevated numbers on the surface of some AML cells in **paper II**. This gives them the opportunity to increase their uptake of sugar, which provides more energy and a better shield against cancer drugs. We have tested two new drugs that hinder the increased uptake of sugar. By blocking the uptake of sugar into cancer cells I managed to make them more vulnerable to the standard cancer treatment as well.

As for sugar, there is increasing evidence that cancer cells also increase the uptake of fatty lipids from their surroundings. This will make them stronger at evading drugs and give them a good energy source to rely on when spreading to new parts of the body. The data in **paper III** supports this, showing the transfer of lipids from human fat cells to AML cells. One type of AML cell was more prone to take up lipids by having more transporters for lipids on their surface. These transporters could later be blocked with a new inhibitor. Less lipids were taken up but also, as when blocking sugar intake, depriving the cancer cells of energy from lipids made them weak and vulnerable.

In **paper IV**, instead of blocking the uptake of energy I trapped glycerol inside of leukemia cells. This was accomplished with a new drug against a channel for outward flow of glycerol. When the cells cannot get rid of the accumulating glycerol, they become stressed. To our excitement, this did also affect the liveliness of the cancer cells.

We need to target the AML cells on several levels to get effective treatments. This includes targeting their nutrient intake (Figure 20). By finding ways to target the transport of nutrients in and out of the cells and deprive cancer cells of their most used energy sources we can increase the effect of already used treatments and more strongly attack the disease.

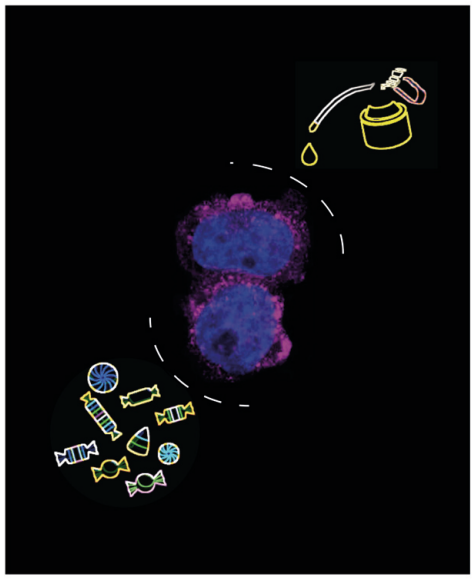


Figure 20 | Blocking uptake of nutrients in acute myeloid leukemia (AML). Drugs targeting fatty lipids and sugar uptake have potential as treatments against leukemia. The two cells in the center have been stained with their nucleus in blue. On their surface they have transporters for glucose uptake that have been stained in pink.

Populärvetenskaplig sammanfattning

Cancer är en förödande sjukdom som sprider rädsla över hela världen genom att påverka alla som kommer i dess väg. Akut myeloisk leukemi, eller AML, är en specifik typ av blodcancer där behandlingen inte har förändrats mycket under de senaste 40 åren. Läkemedel som används mot AML är inte så bra på att skilja cancerceller från kroppens normala celler. Detta leder till att patienter får hemska biverkningar och att de många gånger aldrig helt återhämtar sig från sjukdomen. Problemet med dessa cancerceller är att de tar mycket energi från kroppen för att kunna växa och sprida sig helt okontrollerat. Jag har utnyttjat detta i min avhandling och gjort det till en fördel, genom att svälta cancercellerna.

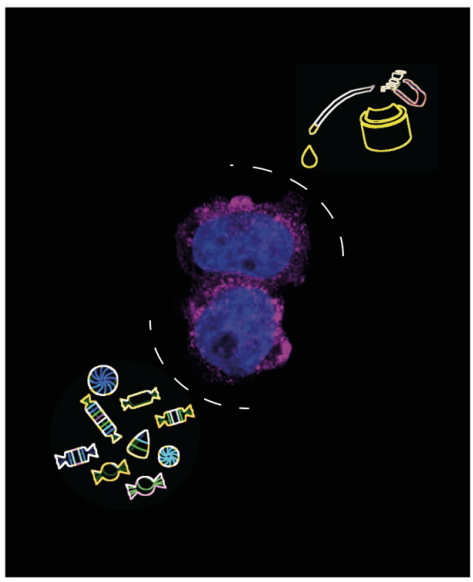
I denna avhandling har transportörer som sitter på utsidan av celler som är ansvariga för upptaget av näringsämnen som socker och fett analyserats. I **artikel I** studeras en sockertransportör i fettceller. Den kan hållas inne i fettcellen när du är hungrig av ett protein som kallas ASPL. Efter en måltid flyttas transportören till cellytan där den kan hjälpa till att ta upp det socker du just åt.

En annan sockertransportör visade sig ha ökat antal på ytan av vissa AML-celler studerade i **artikel II**. Detta gav cellerna möjlighet att öka sitt upptag av socker, vilket gav mer energi och ett bättre försvar mot cellgifter. Jag har testat två nya läkemedel som hindrar det ökade upptaget av socker. Genom att blockera sockerupptaget i cancercellerna lyckades jag också göra dem mer sårbara för den vanliga cancerbehandlingen med cellgifter.

Liksom för socker finns det allt fler bevis på att cancerceller också ökar upptaget av fett (lipider) från sin omgivning. Detta gör cellerna bättre på att överleva läkemedelsbehandlingar och ger dem en bra energikälla att förlita sig på för att kunna sprida sig till nya delar av kroppen. Min data i **artikel III** stöder detta, genom att visa att överföringen av lipider från mänskliga fettceller till AML-celler är möjlig. En typ av AML-celler var mer benägna att ta upp fett genom att ha fler transportörer för lipider på deras cellyta. Dessa transportörer kunde senare blockeras med en ny inhibitor som jag testade. Detta gjorde också att mindre antal lipider togs upp och precis som när jag blockerade sockerintaget kunde jag göra cancercellerna svaga och sårbara genom att beröva dem på energi från fett.

I stället för att blockera upptaget av näringsämnen, kunde jag i **artikel IV** fånga glycerol inuti leukemicellerna. Detta åstadkoms med ett nytt läkemedel mot en kanal viktig för utflöde av glycerol. När cellerna inte kunde bli av med glycerolen blev de stressade. Även här påverkades cancercellernas möjlighet till överlevnad.

För att få mera effektiva behandlingar måste vi slå mot cancercellerna på flera plan, inklusive deras näringsintag (Figur 21). Genom att hitta sätt att påverka transporten av näringsämnen in och ut ur cellerna, samt beröva cancerceller deras mest använda energikällor, kan vi öka effekten av de behandlingar som redan finns och mera kraftfullt angripa sjukdomen.



Figur 21 | Blockering av upptaget av närinsämnen i akut myeloisk leukemi (AML). Läkemedel som påverkar upptag av lipider och socker har potential som behandlingar mot leukemi. De två cellerna i mitten har fått sin cellkärna färgad blå. På dess yta har de transportörer för sockerupptag som har färgats rosa.

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