Facoltà di Farmacia e Medicina

Corso di dottorato in Scienze Immunologiche, Ematologiche e Reumatologiche.

Curriculum Ematologico.

TARGETING LEUKEMIA CELL METABOLISM

Tesi di Dottorato

Relatore:	**Candidato:**
Prof. Agostino Tafuri	**Simone Mirabilii**
Correlatore:	**Matricola:**
dott.ssa Maria Rosaria Ricciardi	**1181948**

Ciclo XXVII   Anno Accademico 2013/2014

Content

Chapter III – Targeting Glutaminolysis in AML Cells: Aminooxyacetate

Chapter IV – Targeting FAO in Leukemia Cells: ST1326

Chapter V – Conclusions, *p.58*

Bibliography, *p.61*

Abstract

Recent evidences show that malignant cell undergo a metabolic reprogramming, acquiring new bioenergetic phenotypes. These new metabolic features grant them the capability of sustaining expensive processes such as cell growth and proliferation. Among the metabolic pathways involved, an increased glycolytic flux, the exploitation of alternative carbon source like glutamine, and an increase in fatty acid metabolism may be included. Metabolic reprogramming is strictly related to aberrant activation of signal transduction pathways, but the causal relationship between altered signalling and redesigned metabolism is currently under review, based on recent works showing a feedback mechanism, in which metabolites are involved in controlling signal fluxes through specific sensor kinases which monitor the cell bioenergetic status. Therefore, cancer cell metabolism represents a potential target for therapeutic intervention that may be integrated with conventional chemotherapy and molecularly targeted agents. In solid tumors, a great number of studies supports the potential of this approach while only few are published in hematologic malignancies.

With the aim of extending the knowledge of leukemia cell metabolic phenotypes, we studied the role of the main catabolic pathways, such as glycolysis, glutaminolysis and fatty acid oxidation.

This study involved the use of metabolic inhibitors, in order to identify novel molecular therapeutic strategies in hematological malignancies.

Dichloroacetate (DCA) is a pyruvate-mimetic molecule, which acts as pyruvate dehydrogenase kinase (PDHK) inhibitor, thus activating pyruvate dehydrogenase (PDH). This activation causes a shift in leukemic cell metabolism, from aerobic glycolysis to glucose oxidation. Our results documented that acute myeloid leukemia (AML) cells are characterized by higher rates of glycolysis compared to normal cells and that the use of DCA, an inhibitor of glycolysis, acts in a short time (30-60 minutes), increasing the levels of oxidative phosphorylation and, in a longer time, reducing cell growth and inducing apoptosis in cells of AML.

Aminooxyacetic Acid (AOA) is a cellular transaminase inhibitor. This compound is able to inhibit the conversion from glutamate to α-ketoglutarate, second step of the glutaminolytic process.

ST1326 is an aminocarnitine derivative, able to block the activity of CPT1a, the enzyme which catalyze the rate-limiting step of fatty acid oxidation. The results obtained in our in vitro study showed a high pro-apoptotic activity of this inhibitor in models of cell lines and primary cells from acute leukemia.

Overall, these preclinical results demonstrate the role of cell metabolism as a potential therapeutic target in hematologic malignancies, warranting further extension of this strategy.

CHAPTER I

Introduction

1.1 Cancer cell metabolism: Introduction

It is currently known that the transformation from normal to neoplastic cells (tumorigenesis) is the result of a multistep process that starts with the emergence of genetic alterations [1]. These changes involve the acquisition of several functional advantages, allowing cancer cells to survive, proliferate and disseminate within tissues and organs.

In 2000 Hanahan and Weinberg observed that the vast repertoire of genetic alterations present in cancer cells is manifested through the acquisition of six major functional characteristics: independence from growth factors, insensitivity to anti-proliferative stimuli (e.g. contact inhibition), the ability to escape the mechanisms of programmed death (e.g. apoptosis), a practically unlimited replicative potential, increased angiogenesis, and the ability to invade and metastasize tissues [2].

In more recent years, several authors have shown that many of the Signal Transduction Pathways (STPs) aberrantly activated in cancer cells actually converge on the deregulation of common metabolic mechanisms, responsible for cell growth and survival [3-6]. In light of these findings, it has been proposed that alterations in cellular metabolism are further distinctive feature of cancer [7].

In neoplastic cells alterations of metabolic processes are necessary to fulfill three main basic needs: rapid production of ATP, an increased biosynthesis of macromolecules and an adequate cellular redox state [8]. To this end, tumors activate complex mutagenesis programs that induce drastic changes in levels and utilization of all biological macromolecules (carbohydrates, lipids, proteins, nucleic acids) [8]. The alterations of the metabolic processes, however, differ from tumor to tumor, because of the close interaction between the cells and the tumor microenvironment, where the concentrations of nutrients such as glucose and glutamine, or gas such as oxygen, are spatially and temporally heterogeneous [9].

In recent years a large number of studies have focused on the analysis of metabolic aspects that distinguish cancer cells compared to normal counterpart, highlighting a central role for glycolytic and glutamine metabolism, not only in the maintenance of STPs but also, and above all, their direct contribution in the process of tumorigenesis [10].

It is clear therefore that the study of metabolic changes that characterize neoplastic cells represent a very promising field of research for development of new therapeutic strategies capable of selectively targeting tumor cells [11]. The inhibition of metabolic processes, by blocking the catalytic activity of the enzymes involved, is configured as a strategy to reverse the neoplastic phenotype by inhibiting the growth, inducing apoptosis and inhibiting the neoangiogenic processes in invasive tumor cells [12].

The most successful example of antineoplastic strategy based on metabolic therapies is certainly represented by the class of antifolate drugs [13], such as 5-fluorouracil, methotrexate, gemcitabine and fludarabine, widely used in the treatment of several cancers. Another example is represented by the use of L-asparaginase in the treatment of Acute Lymphoblastic Leukemia (ALL) [14] and lymphomas [15]. In more recent years, thanks to the elucidation of the molecular basis of metabolic reprogramming, it has been possible to define new therapeutic strategies based on the administration of glycolytic inhibitors [16], compounds capable of reducing the excess lactic acid produced by the tumor cells. However, other strategies can be adopted, focused on alternative carbon source, such as glutamine [17] and fatty acids [18].

1.1.1 Glycolysis

In the beginning of the last century, Warburg's work demonstrated that cancer cells consume more glucose and generate more lactate when compared to normal cells. This lactic fermentation, distinctive of an anaerobic process, is independent from oxygen availability [19]. This anaerobic glycolysis upregulation, called "Warburg Effect", has subsequently been confirmed in most cancer types [20], with few exceptions. Ramanathan and colleagues showed that the process of carcinogenesis increases cellular dependence on glycolytic, while decreasing its dependence on mitochondrial energy production [21]. By using a fibroblast model transfected with the telomerase catalytic subunit (hTERT) in combination with simian virus 40 large T antigen (LT), small T antigen (ST), and with an oncogenic allele of H-ras, and exposing these transfected fibroblasts to different metabolic inhibitors, they observed an increase in glycolysis inhibition sensitivity and a parallel loss in sensitivity to OXPHOS targeted agents, thus suggesting a shift from an oxidative to a more glycolytic metabolism during carcinogenesis [21].

For each glucose molecule the glycolytic process produces through lactic acid production 2 molecules of ATP and re-generates redox intermediates: 2 NAD^+ [22]. Conversely, the complete oxidation of glucose, though OXPHOS, has a theoretical yield of 38 ATP per molecule of glucose. This inefficiency in energy generation by cancer cells that appears as a paradox, given the high

energy demand, can be overcome by increasing the glycolytic flux: GLUT1 glucose transporter has been reported as overexpressed in a variety of tumors, such as bladder, lung, colon, breast, ovarian and gastric cancers [23]. Glycolytic enzymes, such as hexokinase, phopshofructokinase and pyruvate kinase, are as well upregulated in most cancer histotypes [24]. It has been suggested that, in non-limiting glucose condition, lactate production grants a higher energy yield compared to OXPHOS [25, 26]. Moreover, coupling glycolysis to ATP generation would leave Tricarboxylic Acid cycle (TCA) cycle intermediates available for incorporation in biosynthetic pathways, increasing the anaplerotic functions of the latter process.

Furthermore, glucose itself can be used for biosynthesis of amino acids, lipids and nucleotides [27]. This observation is supported by data obtained by Nuclear Magnetic Resonance (NMR) focused on the carbon isotope 13 (^{13}C), performed on glioblastoma cells, confirming Warburg effect and indicating that 10% of total glucose utilization is devolved to synthesis of fatty acids, amino acids and nucleotides [28]. In addition, the same work, along with others, underlines the importance of glutamine in tumor cell metabolism.

1.1.2 Glutaminolysis

Glutamine is a conditionally essential amino acid: normal cells are able to synthesize it, but in stress condition, such as sepsis, the increased demand is not met by biosynthesis [29]. Similarly to glucose, glutamine is fundamental in cancer cell metabolism. It acts as a carbon source, nitrogen donor, and as redox buffer, being the precursor of glutathione [30]. Glutamine catabolic process start with its deamination operated by the glutaminase enzyme, to form glutamate (Glu) and NH_4. Glutamate is then converted by a deamination step to α-ketoglutarate (α-KG), a TCA cycle intermediate. The conversion of Glu to α-KG is catalyzed by Glu dehydrogenase or one of several transaminases, which convert α-keto acids into their corresponding amino acids [31]. Importantly, the flux of glutamine provide high rate of NADPH production used by cell for lipid and nucleotide biosynthesis [17].
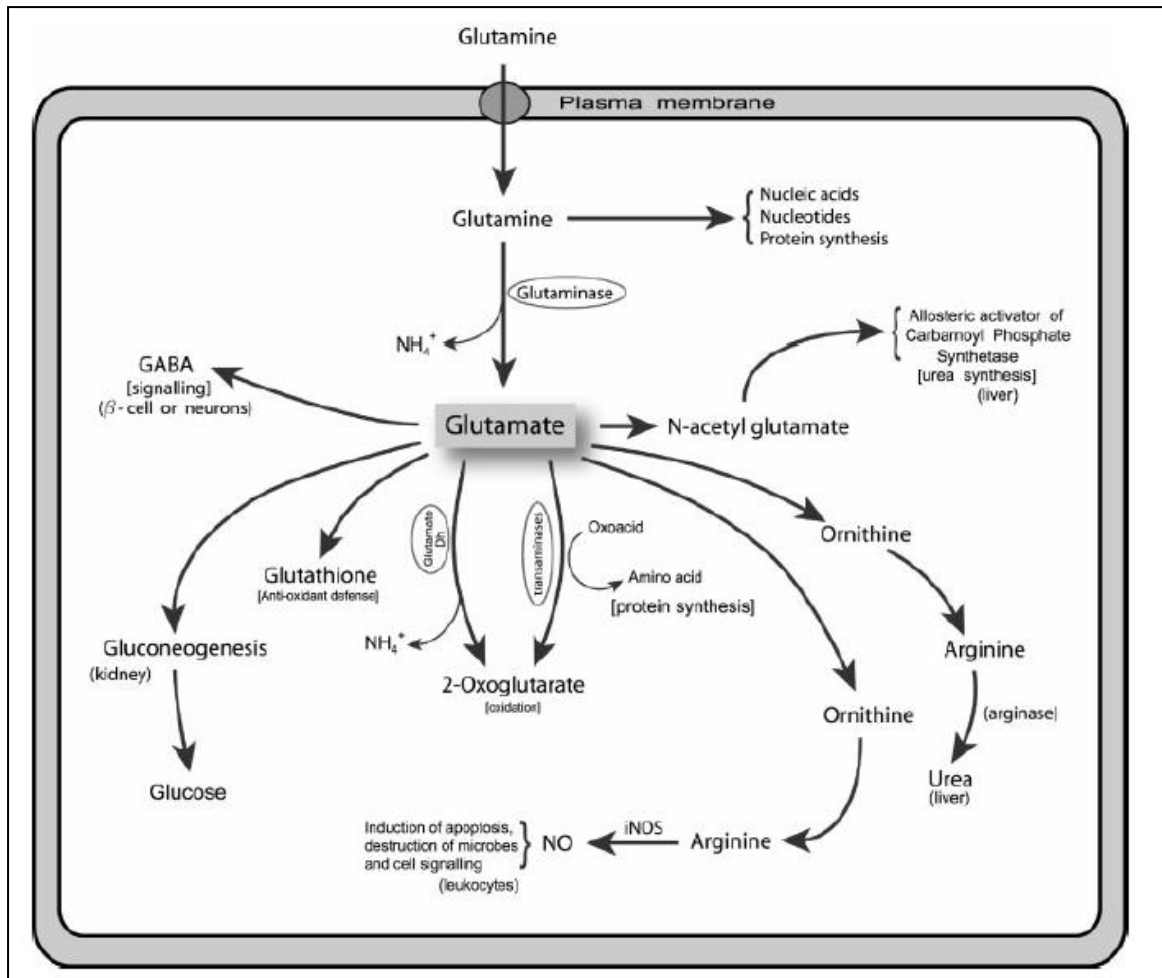
Figure 1 – Metabolism of glutamine in cancer cells (from [32])

Thus, Glutamine can enter the TCA cycle directly (anaplerosis), or being diverted in peculiar series of reactions, termed reductive carboxilation: glutamine-derived α-ketoglutarate can be reductively converted into isocitrate, which is then isomerized to citrate to supply acetyl-CoA for *de novo* lipogenesis [33]. However, ^{13}C tracking analysis on glioblastoma showed that 60% of glutamine is oxidized to lactate and alanine, while the remaining is used as an anaplerotic source, to replenish TCA cycle flux impaired by the diversion of glucose-derived pyruvate into lactic fermentation [28].

1.1.3 Fatty Acid Metabolism

The metabolic reprogramming of cancer cells leads to a re-arrangement that involves also the third major carbon sink, fatty acids (FA). Similarly to glycolysis and glutaminolysis, it's long known that cancer cells up-regulates the synthesis of these molecules [34]. Since these early observations, most groups focused their efforts on dissecting the *de novo* synthesis of FA (FAS), as a way for cancer cells to produce cell membranes, signaling lipid molecules, protein post-

translational modifications and as energy supply to support rapid cell proliferation [35]. The overexpression of the Fatty Acid Synthase enzyme (FASN), the key enzyme of FAS, has been in fact reported in prostate, ovarian, breast, endometrial, thyroid, colorectal, bladder, lung, thyroid, oral, tongue, esophageal, hepatocellular, pancreatic and gastric carcinomas, malignant melanoma, mesothelioma, nephroblastoma and retinoblastoma, soft tissue sarcoma, gastrointestinal stromal tumor, Paget's disease of the vulva and multiple myeloma (MM), as well as in some benign and pre-invasive lesions of prostate, breast, lung, stomach, colon and cutaneous nevi [36]. Interestingly, cancer cells prefer to synthesize new FA, instead of using available FA from bloodstream or from extracellular environment: Ookhtens and colleagues, using ^{14}C labeled FA, demonstrated that 93% of FA is built *de novo* [37]. Moreover, a recent report from Scott and colleagues assessed the significant contribution of glutaminolysis, through the above mentioned process of reductive carboxilation, in providing the building blocks for FAS [38]. The established perturbations in FAS partially eclipsed the role of the oxidation process of these molecules (Fatty Acid Oxidation, FAO) in the bioenergetic balance of cancer cells, given that these two pathways are always been considerate as mutually exclusive. In fact, relatively few papers investigated FAO process in the tumor setting. However, a series of recent works focused on this process pinpointed the importance of FAO in supporting cancer cell survival and proliferation. Thompson's group demonstrated that FAO can rescue cancer cells from glucose deprivation-induced apoptosis [39]. Nomura and colleague reported the overexpression of monoacylglycerol lipase (MAGL), a lipolytic enzyme that catalyzes the hydrolysis of monoacylglycerols, is highly expressed in aggressive tumors and controls the levels of free fatty acids (FFA) in cancer cells [40]. This mobilization of FA can then be exploited by tumor cell through β-oxidation. This process has been reported to be of high importance in glioblastoma [41, 42], prostate [43] and breast cancer [44]. Moreover, in breast cancer cells FAO appears to be activated by prolactin [45] and in recovering from stress conditions, such as loss of matrix attachment [46] and fluctuation of oxygen levels [47]. Combined, these evidences point thus toward a protective role for FAO in cancer cell metabolic stresses induced by microenvironment.

1.2 Targeting Leukemia Cell Metabolism

While in most solid tumors metabolism has been well characterized, few evidences have been reported on cell metabolism in hematological malignancies. However, in the last years the number of publications in this field increased. Results indicate that perturbations in metabolic

pathways are similar to what happen in solid tumors, paving the way for a therapeutic exploitation of these phenomena.

1.2.1 Glycolysis

Among the first metabolic alterations reported, an increase in glycolytic flux has been observed in leukemia cells. Using Akt-transfected immortalized hematopoietic cells, Thompson's group demonstrated that Akt exerts a direct influence on glucose metabolism, inducing a dose-dependent stimulation of glycolysis that correlates with a more aggressive phenotype [48]. The increase in glycolysis is not correlated to an increase in oxygen consumption rates, i.e. a complete glucose oxidation through the TCA cycle and OXPHOS, indicating that Akt hyper-activation promotes the Warburg effect [48].

An increased glycolytic flux has been then reported in Acute Lymphoblastic Leukemia (ALL) cells [49]. The gene expression profile of ALL blasts from newly diagnosed pediatric patients shows an up-regulation of genes related to glycolysis, and a down-regulation of genes related to TCA cycle, when compared to normal $CD34^+$ [49]. Moreover, ALL blasts show a higher protein expression of GLUT-1 transporter [49]. Importantly, GLUT-1 appears to be fundamental in maintaining cell growth and apoptosis resistance in Bcr-Abl positive B-ALL [50]. Additionally, glycolysis rate has been strongly linked to chemotherapeutic drugs resistance in ALL [51-54].

Aberrant signaling by Bcr-Abl seems implicated in increasing the glycolytic flux in Chronic Myeloid Leukemia (CML) cells, via the PI3K-Akt axis [55], and higher glycolysis in CML cell lines is related to imatinib resistance [56].

Evidences have been reported also in Acute Myeloid Leukemia (AML) cells. The aerobic glycolysis appears to be exacerbated in these cells by stromal interactions [57], and the extent of glycolytic flux seems to inversely correlate with patient prognosis [58]. More recently, Chen and colleague confirmed the enhanced glycolysis in AML cells, using mass spectrometry-based metabolomics [59]. In particular, they individuated a panel of 6 metabolites related to glucose metabolism (Lactate, 2-Oxo-glutarate, Pyruvate, 2-Hydroxy-Glutarate, Glycerol-3-phosphate, Citrate) having an independent prognostic value in cytogenetically normal AML patients [59].

Perhaps the most convincing proof of the importance of aerobic glycolysis in leukemogenesis is presented in a very recent work by Wang and colleagues [60]. They used genetically modified murine models to investigate the effects of deletion in the two last enzymes of aerobic glycolysis, pyruvate kinase (PK) and lactate dehydrogenase (LDH), in normal hematopoietic stem cells and in normal murine BM cells transfected with either BCR-ABL or MLL-AF9 genes, in order to simulate a CML-like or an AML-like disease, respectively. After serial transplantations, they

observed that the conditional deletion of PK or LDH profoundly affected viability of leukemic-like cells, even following the first transplantation, while the effects on normal hematopoietic stem cells were detectable only after multiple transplants, thus individuating a "therapeutic window" for glycolysis interfering in leukemogenesis [60].

Taken together, these results show that an increase in glycolytic flux is nearly universal in the different leukemia subtypes, pointing at the Warburg effect as a promising target for therapeutic intervention.

Given that aerobic glycolysis is the best characterized metabolic alteration in leukemia cells, it is not surprising that the vast majority of metabolic inhibition attempts has been aimed at this pathway. The most used inhibitor has been 2-deoxyglucose (2-DG), a glucose analog that is phosphorylated by hexokinase but cannot be further processed, and accumulates in the cytosol inducing a decrease in ATP production, cell cycle blockage, inhibition of cell growth and apoptosis [61]. As single agent, 2DG proved effective in inducing apoptosis in ALL cells [49] and in suppressing xenotransplanted AML tumor growth [62].

However, being a glucose agonist, 2-DG is active only at high concentrations (millimolar range), difficultly achievable in patients due to serious side effects, such as induction of hyperglycemia [63]. Nonetheless, the use of 2-DG appear promising when used in combination therapy, showing a high degree of synergism with conventional chemotherapeutic agents, such as glucocorticoids in ALL [52], ATO [64] and AraC [59] in AML.
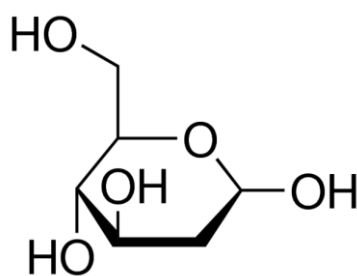

Figure 2 – Chemical structure of 2-Deoxyglucose (from www.sigma-aldrich.com)

Given the well-established impact of aberrant cell signaling on glycolytic flux, researchers focused on the possibility to target simultaneously the upstream kinase activity and the downstream effect on metabolism, in a two-level intervention aiming to overcome the resistance caused by bypassing mechanisms. Indeed, 2-DG is reported to sensitize leukemia cells to Bcl2 agonists [65, 66], Akt targeting agents [67] and death receptor activators [68, 69].

The promising results obtained with 2-DG, together with its mentioned limitations, has led to the development of more potent glycolysis inhibitors, such as 3-bromopyruvate (3-BrPA) and its derivative, 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP). 3-BrPA acts on Hexokinase, and it is reported to be effective in the micromolar range on AML cells, showing synergism with the main chemotherapeutic drugs [70, 71].
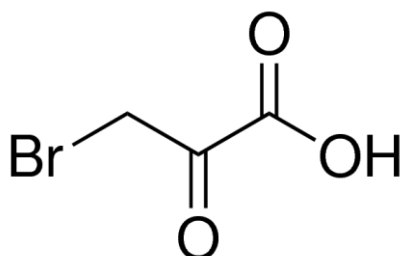

Figure 3 – Chemical structure of 3-bromopyruvate (from www.sigma-aldrich.com)

However, authors reported an instability for this compound, that led to the generation of a more stable form, 3-BrOP. 3-BrOP is an ester of 3-BrPA, which can permeate cell membrane and undergoes hydrolyzation by cellular esterases, releasing the active moiety of the compound, the 3-BrPA [72]. 3-BrOP has been reported to be more potent than its native form, inducing a reduction of cell proliferation in a panel of 15 different acute leukemia cell lines and primary blasts of myeloid and lymphoid origin, a drop in intracellular ATP levels and massive apoptosis; moreover, 3-BrOP showed high synergism when combined with an mTOR inhibitor, rapamycin [73].

1.2.2 Glutaminolysis

It's long known that leukemia cells, as solid tumors cells, require glutamine as substrate. Some hints derive from lesson learned from cell culture: without the addition of this amino acid, leukemia cells show impaired nucleotide synthesis [74], reduction of proliferation and of DMSO-induced differentiation [75], decrease in ATP content [76], cell growth arrest and apoptosis [77]. Similarly to solid tumors, glutamine has been reported as anaplerotic substrate: diverting pyruvate into lactate, leukemia cells exploit glutamine to replenish TCA cycle and keep it active, in order to maintain the availability of this cycle intermediates for biosynthetic purposes [78].

Glutamine addiction assumes a particular meaning in AML cells. In fact, mutation in metabolic genes has been recently reported in approximately 20% of AML [79]. In particular, using massive parallel genomic DNA sequencing, mutation in Isocitrate dehydrogenase (IDH) genes has been

found, with a peculiar association to cytogenetically normal patients. These genes encode for proteins involved in the TCA cycle, catalyzing the conversion from isocitrate to α-ketoglutarate [80]. Mutations in the active sites of these proteins leads to a gain of function: the enzymes catalyze an inverted reaction, starting from α-ketoglutarate to produce 2-hydroxy-glutarate (2HG). It is still unclear how this metabolite contributes to the carcinogenesis; however, evidences suggest that it can compete with α-ketoglutarate, blocking the activity of α-ketoglutarate-dependent enzymes (i.e. dyoxigenases), or that 2HG can influence cellular epigenetic status interfering with histone demetylases, thus activating oncogenes or silencing tumor-suppressor [80].
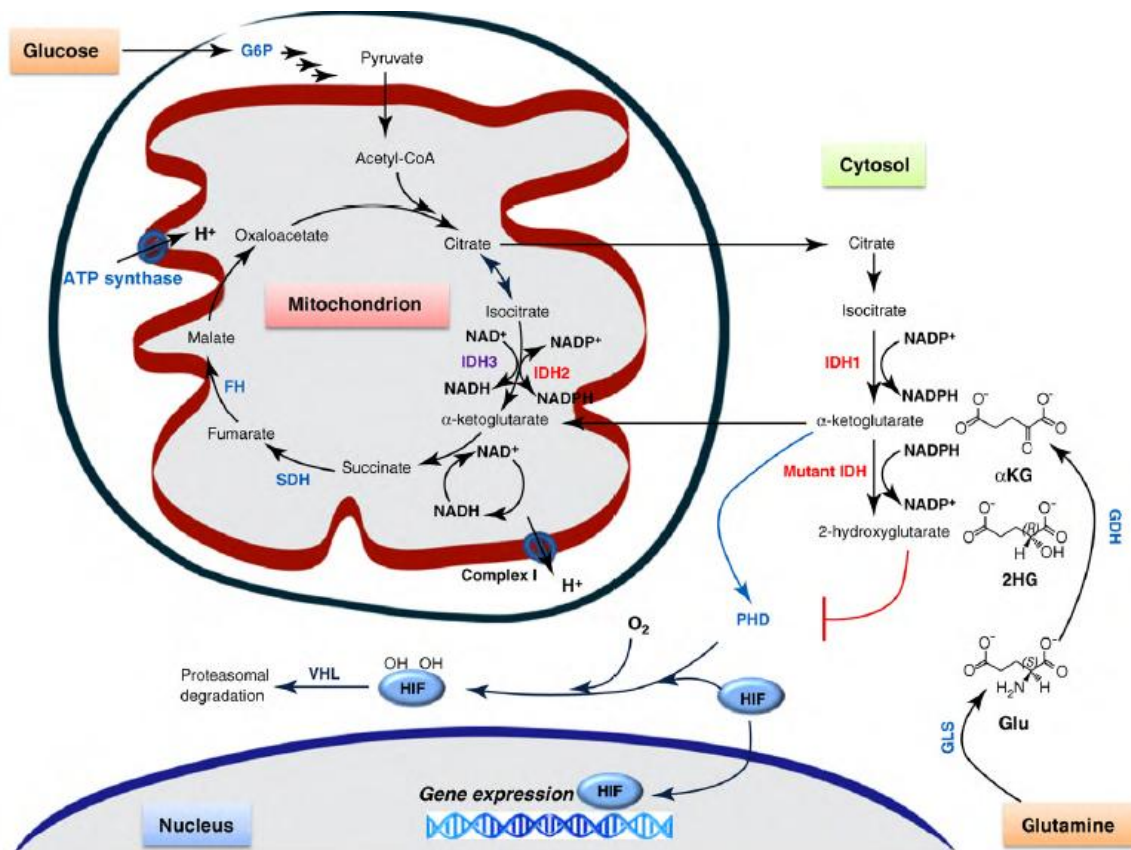

Figure 4 – Impact of 2-hydroxyglutarate on cellular processes (from [80])

The α-ketoglutarate used for 2HG production derives almost entirely from glutamine: through ^{13}C-labeled substrates and a liquid chromatography coupled to mass spectrometry (LC-MS)

approach, Dang and colleague demonstrated that cells transfected with mutant IDH produces high level of 2HG, using the carbon skeleton from glutamine [81].

Glutamine antagonists exert cytotoxic effects on leukemia cells. Lyons and colleague showed in 1990 that azaserine, acivicine and 6-diazo-5-oxo-L-norleucine block glutamine-dependent purine synthesis in murine leukemia cells, inbalancing the UTP/ATP ratio and causing accumulation of intermediate metabolites [82]. However, only recently additional reports underlined the promising effectiveness of targeting glutamine catabolism in leukemia cells.

Bis-2-[5-(phenylacetamido)-1,3,4-thiadiazol-2-yl]ethylsulfide (BPTES) is a small molecule inhibitor that acts on glutaminase, the enzyme that catalyze the conversion from glutamine to glutamate, releasing ammonia ions [83]. This compound has been reported to be effective on Burkitt's Lymphoma cells, reducing their proliferation in normoxic condition while inducing apoptosis in hypoxic condition [84]. Moreover, it significantly slows tumor progression in xenografted mice [84]. More recently, BPTES has been applied to primary AML cells with IDH mutation, since their mentioned reliance on glutamine [85]. The high sensitivity of IDH1 mutant cells to this compound [85] prompts further study on the therapeutic role of glutamine metabolism targeting in this particular subclass of patients.
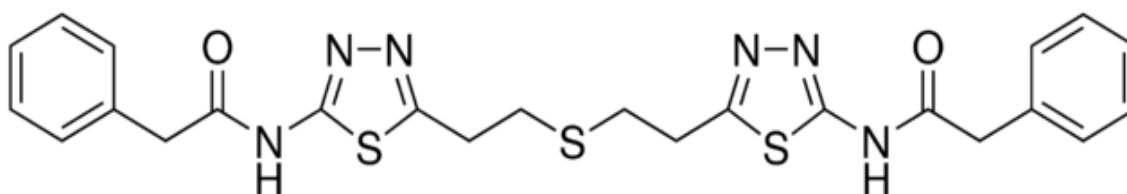

Figure 5 – Chemical structure of BPTES (from www.sigma-aldrich.com)

1.2.3 L-asparagine

Strictly biochemically related to glutamine, asparagine (Asn) is a non-essential amino acid. In the 1960s, the activity of asparagine synthetase (ASNS), the enzyme responsible for its synthesis from aspartate and glutamine, has been found impaired in some leukemia subtype, especially in ALL [86]. This reduced activity cause a dependence of these cells from exogenous source of Asn, and this dependence has been promptly exploited as a therapeutic target: treatment with L-asparaginase (L-ase) started in 1967, and since then L-ase has become of fundamental importance in childhood ALL treatment, inducing 40-60% of complete remissions as a single

agent, and nearly 95% of complete remissions when combined with vincristine and prednisone [88].

Figure 6 – E. coli L-asparaginase tetramer

Interestingly, a debate is currently ongoing in the scientific community about the actual mechanism of action of L-ase, since this enzyme has also a glutaminase activity. While some authors attribute to this activity the insurgence of serious side-effects [89], prompting the development of new L-ases with undetectable glutaminase activity [90, 91], others suggest that the effectiveness of this therapy is also due to glutamine depletion [92, 93]. Recent studies support this latter hypothesis: using an LC-MS approach on adherent cells, Purwaha and colleagues showed that the induction of apoptosis by L-ase exposure is more related to glutamine levels than to asparagine concentration [94].

Ehsanipur and colleagues reported that a possible resistance mechanism of ALL cells to L-ase may be caused by adipocytes releasing glutamine [95]. Bouscary's group suggests that L-ase treatment may be useful in AML, since they reported mTORC1 inhibition, protein synthesis suppression and apoptosis induced by the glutaminase activity of L-ase in AML cell lines and

primary blasts [96]. A way to overcome resistance may be thus selecting for L-ases that can metabolize glutamine with higher affinity.

1.2.4 Fatty Acid Metabolism

While solid tumors often show an upregulation of FAS, evidences in leukemia for higher rates of this process are thus far very few. Some authors described an upregulation of FASN in AML [97, 98], but the vast majority described a system in which both FAS and FAO works in a nearly physiological manner. An early report noted that leukemia cells tend to incorporate free fatty acids (FFAs) into phospholipids without altering them, while part of this FFAs are β-oxidized [99]. Importantly, nearly no carbon atoms from glucose are used by leukemia cells to de novo synthesis of lipids [99]. Supporting these observations, Skrede and Iversen reported an increase in oxidation and incorporation into phospholipids of labeled palmitate in a murine model of Acute Promyelocytic Leukemia (APL) [100]. More recently, two works from Nitta's group suggested that, in some AML subtype, represented by THP-1 and HL60 cell lines, FAO can be important in replenish TCA cycle, providing the acetyl-CoA to feed this central metabolic hub [101, 102]. Taken together, these results suggest a higher rate of FA metabolism in leukemia cells, with a balance between oxidation and synthesis. In effect, some author suggested that to meet bioenergetic and biosynthetic needs of cancer cells, these two mutually exclusive processes can occur at the same time, by compartmentalizing them in two different cellular environment (namely cytosol for FAS, mitochondria for FAO) [103]. Alternatively, it can be speculated that leukemia cells alternate cycles of FAS and FAO, based on different time points in the cell cycle, in order to satisfy both the need for replenish TCA cycle and the demand for membrane phospholipids.

Interfering with FA metabolism is a relatively new approach of targeting leukemia cell metabolism. As mentioned, the lack of extensive proofs on FAS leads researchers to focus on the β-oxidation process. Etomoxir is a well known FAO inhibitor: it blocks the import of FA in mitochondria, thus irreversibly inhibiting the first step of this process, catalyzed by carnitine-palmitoyl-transferase 1 [104]. Samudio et al. in 2010 reported that etomoxir can reduce AML cell proliferation, alone or in co-culture with stromal cells; moreover, it synergizes with ABT-737 in apoptosis induction [105]. More recently, etomoxir has been shown to potentiate the apoptotic effects of ATO and glycolysis inhibitors, such as 2DG, on HL60 cell line [106].
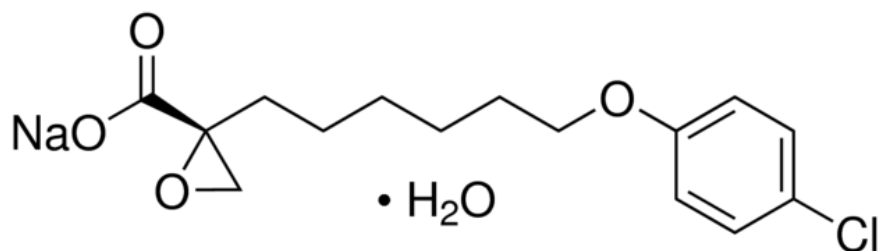
Figure 7 – Chemical structure of etomoxir (www.sigma-aldrich.com)

Following studies on MM confirmed the effectiveness of etomoxir-induced FAO inhibition in hematological malignancies [107]. Interestingly, in the same study FAS inhibition by orlistat seems to induce apoptosis as well [107], thus indicating that the whole machinery involved in FA can be a promising therapeutic target.

1.2.5 Pentose Phosphate Pathway

Early observations by Lengle and colleagues reported that pentose phosphate shunt (PPP) is upregulated in leukemic cells from a murine model [108]. However, authors concluded that, whether the flux was higher, the percentage of glucose metabolized by this pathway was comparable to that of normal thymocytes [108]. This high flux through the non-oxidative branch of PPP has been confirmed in Jurkat cells through ^{13}C-NMR analysis: labeled glucose is partially diverted in PPP and metabolized for de novo synthesis of ribose-5-phosphate, an essential precursor of nucleotides [109]. Chemoresistance has been linked to high flux in PPP [110] and to overexpression of glucose-6-phosphate dehydrogenase, the key enzyme of this process [111]. Moreover, a recent report from Yamamoto and colleague showed an upregulation of PPP flux in U937 cell line when exposed to oxidative stress, and a concomitant reduction in glycolytic flux mediated by methylation of phopshofructokinase/fructose bisphosphatase 3 [112]. Some evidence of a hyperactivity of 6-phoshogluconate dehydrogenase, involved in the oxidative branch of PPP, has been reported in leukemia blasts [113], indicating that both the oxidative and non-oxidative branches of PPP may represent a potential target.

The effect of 6-Aminonicotinamide (6-AN), a 6-phoshogluconate dehydrogenase inhibitor, has been extensively investigated, showing a cytotoxic effect as single agent [114] and a sensitizing action when used in combination with chemotherapeutic drugs [115, 116]. Furthermore, 6-AN is reported to synergize with the Bcl2 inhibitor ABT-263 in inducing apoptosis in leukemia cells [117]. Additional studies are needed to fully explore the potentiality behind PPP interfering.
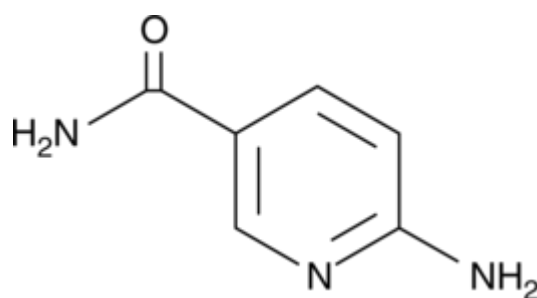
Figure 8 – Chemical structure of 6-Aminonicotinamide (from www.caymanchem.com)

1.2.6 Oxidative Phosphorylation (OXPHOS)

Recently, alterations of mitochondrial gene expression have been reported in CML stem cells. In particular, an upregulation of genes encoding for complex I, II, IV and V, and a deregulation of complex III gene transcription [118] which the authors correlated with the high amount of ROS usually found in those cell has been reported [119]. Several groups detected mutations on mitochondrial DNA in genes coding for complex I [120, 121], and complex IV [122] in AML patients. Although these studies lack of extensive functional characterizations, their results may partially explain the dependence of leukemic cells from glycolysis, at least in some subgroups.

On the contrary, different evidences are emerging about Chronic Lymphoblastic Leukemia (CLL) cell metabolism, as these cells seem to rely more on oxidative phosphorylation than glycolysis. Jitschin and colleagues observed in CLL cells high levels of ROS derived by an enhancement in OXPHOS, caused by higher functional mitochondria biogenesis and increased respiratory rates [123]. If confirmed, these results place CLL in a different metabolic category compared to the other hematological malignancies, as these cells are not subjected to the Warburg effect.

The upregulation of glycolysis, and the diversion of pyruvate into lactate, does not completely shut down the OXPHOS. Thus, mitochondrial respiration could be a reliable target. Since the appearance of a report, observing a reduced incidence of cancer in metformin-treated diabetic patient [124], this molecule has been extensively studied as anti-neoplastic agent. Metformin is a biguanide derivative with a double action: it inactivates mTORC, through an AMPK dependent mechanism, and inhibits electron transfer chain complex I [125].
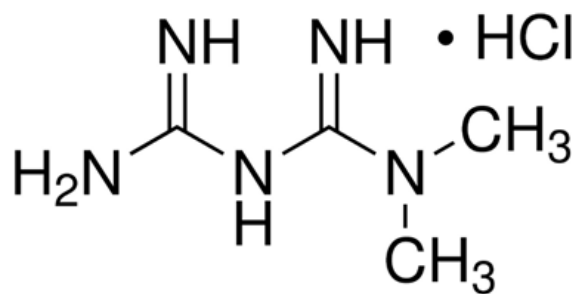
Figure 9 – Chemical structure of Metformin (from www.stressmarq.com)

This molecule appear to be effective in induce reduction of cell proliferation and apoptosis in CML [126], AML [127, 128] and ALL [126, 129, 130]. However, it is still unclear if metformin exerts its pro-apoptotic action through the mTORC inhibition or its effect on cell metabolism, prompting further study to understand and enhance its properties.

1.3 Traditional and Novel Technologies for Studying Cell Metabolism in Hematological Malignancies

Cell metabolism has been investigated through a variety of instrumentation, from Clarke-type oxygen sensor to measure oxygen consumption, to spectrophotometric detection of metabolites through generation of fluorescent intermediates. However, three are the current technologies that allow the most comprehensive view of cell metabolism. While Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) are nowadays well established and widespread, Seahorse XF Analyzer is rapidly expanding as a cost-effective way to monitor in real-time metabolic rates of live intact cells.

NMR exploits nuclear spin of magnetic-field immersed atom nuclei: the emission of radiofrequencies allows to interact and detect specific atom groups, distinguishing up to 75 metabolites in a sample, with a sensitivity lower limit around 5µM [131].

Mass spectrometry is based on fragmentation of molecules and their separation based on mass/charge ratio. Typically, to increase resolution power, MS is coupled to purification system, such as liquid chromatography (LC) [132]. Combining these two technologies, LC-MS is able to identify from 200 to 500 metabolites, with a limit of detection in the picomolar order [131].

Usually, there are two kind of strategies related to both types of technology: an untargeted approach, aimed at the detection of all possible metabolites and in putative differences in

metabolite distributions between paired samples (i.e. normal vs. cancer, control vs. treated, time-series), and a targeted approach, in order to detect specific pathways and to quantify their fluxes [133]. Both these technology have been successfully applied to the biological characterization of hematological malignancies: NMR metabolomics on patient sera proved effective in distinguish between IGHV-mutated and unmutated in early-stage untreated CLL patients [134], while MS-based metabolomics has been able to show that glucocorticoids act by inhibiting glucose metabolism in ALL cells [53]. An untargeted approach, and a combination of NMR and MS, has been adopted by Tiziani and colleagues to detect differences between the bone marrow and peripheral blood compartments in pediatric ALL primary blast metabolism as well as the metabolic response to L-ase treatment, confirming high rates of aerobic glycolysis in these cells and that L-ase partially acts by depleting glutamine pool [135].

However, the main limitation of NMR and MS is the need for a metabolite extraction, which means that cells have to be destroyed in order to perform experiments.

On the other hand, Seahorse XF Analyzer is a new instrument able to simultaneously measure, in real time and on live cells, extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). ECAR is proportional to lactate derived from glycolysis and excreted by cells in the extracellular medium [136]. OCR is proportional to mitochondrial respiration, and thus to OXPHOS [136]. The presence of drug injection ports (4 per well) allows to interact with cell metabolism, adding specific inhibitors to further dissect metabolic fluxes, or investigating metabolic response to a specific drug.

A typical XF experiment proceeds through a seeding phase, where cells are moved in the special multiwell designed for the XF; a switch in culture medium, replacing it with a non-buffered medium, in order to allow the acidification rate measurement; loading of chosen compound in the drug injection ports; calibration of XF fluorimetric sensors. Once started, the XF measures OCR and ECAR for about 120 minutes.

To measure glycolysis rate, only glutamine is added to the assay medium. The first three round of measurement gives the basal state. The first injection is composed of glucose 10mM, followed by 3 rounds of measurements to detect glycolytic rates. Measurements following injection of oligomycin A, an ATP-synthase inhibitor that shuts down mitochondrial energy production, reveal the maximal glycolytic rates. Finally, 2-DG is injected to block glycolysis and detect the lactate-independent acidification, to be subtracted during data processing. Fig 10 shows a representative glycolysis experiment on the XF analyzer.
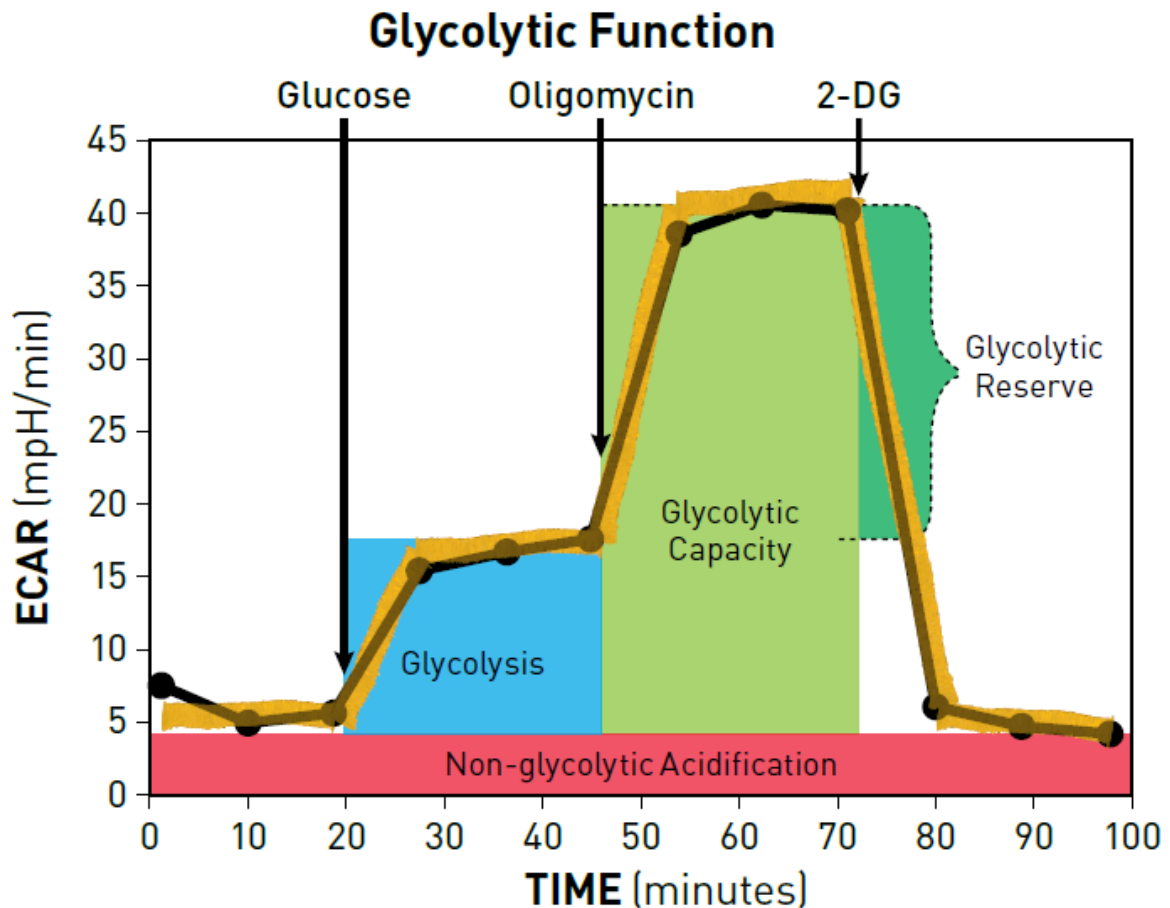
Figure 10 – Typical experiment to measure cellular glycolytic capacity (from www.seahorsebio.com)

For mitochondrial respiration experiments, the assay medium is supplemented with glucose, pyruvate and glutamine. As for the measurement of glycolysis rate, the first three rounds of measurements give the basal respiration rate. Injection of oligomycin A and following measurements reveal the portion of oxygen consumption coupled to ATP synthesis. Then, the injection of FCCP, an agent that discharge the across membrane electrochemical gradient, allow the detection of maximal respiration rates. Finally, a mixture of complex I and complex III inhibitors, rotenone and antimycin respectively, completely shuts down the electron transfer chain and reveals the non-mitochondrial oxygen consumption. Fig 11 shows a representative mitochondrial respiration experiment with the XF analyzer.
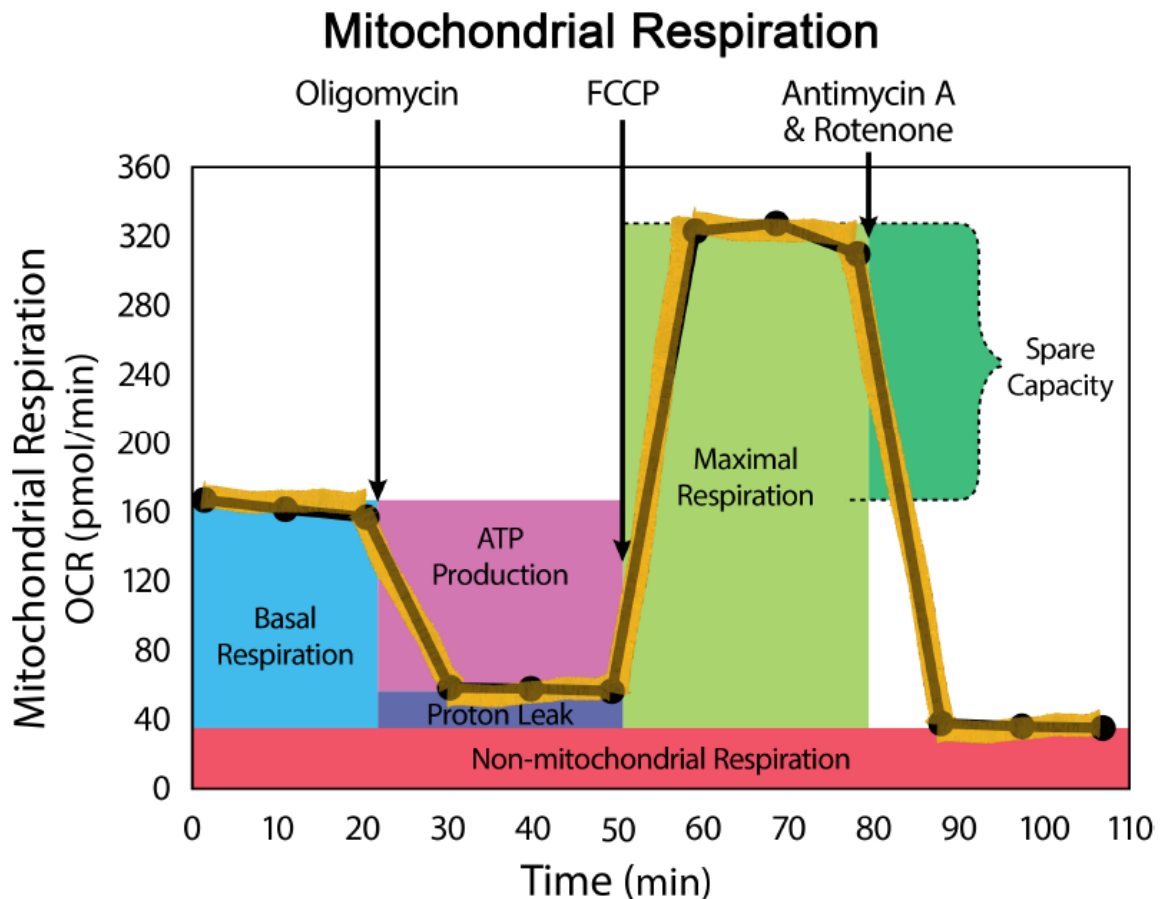
Figure 11 – Typical experiment to measure cellular mitochondrial respiration (from www.seahorsebio.com)

By varying substrate composition in assay medium, it is possible to investigate the various contribution of each substrate to cell metabolism. For example, in order to study FAO the assay medium is supplemented with BSA-conjugated palmitate and L-carnitine [137]. The mitochondrial respiration experiment, conducted in this assay buffer, reveals the oxygen consumption only due to β-oxidation. In the same way, it is possible to measure glutamine contribution to cellular metabolism.

Seahorse XF analyzer has already been successfully applied in hematological malignancies, to investigate metabolic dependence [123, 138, 139] and in drug discovery [140-142]

1.4 Aim of the study

In recent decades a large number of studies have shown that genetic alterations acquired by tumors deeply reprogram basal metabolism, allowing these cells to acquire proliferative advantages, combining the energy demands of growth with the biosynthetic needs imposed by proliferation, and to significantly increase the cell survival even in adverse environmental conditions.

Initially described by the German scientist Otto Warburg, these alterations in cancer cells induce a purely glycolytic metabolic phenotype through increasing at both transcriptional and protein level the expression and the activity of enzymes involved in the glucose catabolism. At the same time, this metabolic program is able to strongly reduce the oxidative processes of tumor cells deflecting the normal carbon fluxes from the catabolic pathways to those biosynthetic. This effect appears as a result of the reduction of the expression and/or activity of mitochondrial enzymes, or following the expression of specific isoforms, differently regulated than those expressed in healthy cells. In addition, in certain tumor contexts amino acids such as glutamine or lipids such as FA are metabolized intensively for energetic and biosynthetic purposes, thus participating in the growth, proliferation and survival of cancer cells. In other tumor models (e.g. breast cancer, pancreatic tumors, glioblastoma, and acute leukemias) the metabolic program described above is associated with the use of β-oxidation process for energy purposes. The fatty acid oxidation in these cells may in fact represent an alternative source of carbon, capable of maintaining the Krebs cycle and other mitochondrial oxidative metabolism fully functional.

Thus, the purpose of this work is to investigate the therapeutic potential of a metabolism-targeted approach, aimed at the three main catabolic pathways (glycolysis, glutaminolysis, and fatty acid oxidation) in leukemia models.

To this end we evaluated the effect of three specific inhibitors: i) Dichloroacetate, an agent able to reverse the Warburg effect and restore an oxidative phenotype; ii) Aminooxyacetate, a transaminase inhibitor that interfere with the glutaminolytic process; iii) ST1326, a FAO inhibitor that selectively and reversibly targets CPT1a, the key enzyme of this process.

The effects induced by the aforementioned compounds have been investigated with both traditional/functional approach (cell counts, flow cytometry) and cutting-edge technology (Seahorse XF Analyzer), in order to elucidate the mechanisms behind the action of these metabolic inhibitors on cell lines and primary samples from hematological malignancies.

Chapter II

Targeting glycolysis in AML cells: Dichloroacetate

2.1 Introduction

As reported by Chen and colleagues, AML cells actively uses aerobic glycolysis in order to produce the energy needed for survival and proliferation [59]. Thus, we chose to target this process with a metabolic modulator, the dichloroacetate (DCA).
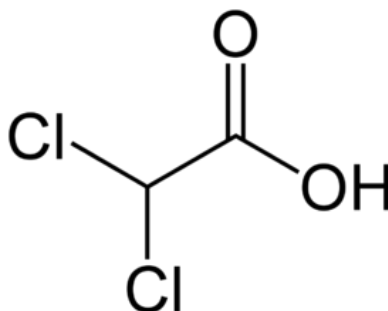
Fig 12 – Chemical structure of DCA (from [143])

DCA is a pyruvate-mimetic molecule, which acts as pyruvate dehydrogenase kinase (PDHK) inhibitor, thus activating pyruvate dehydrogenase (PDH) [144]. DCA binds a site located in the N-terminal domain of PDHK, promoting a conformational change that inactivates this kinase [143].Thus, pyruvate derived from glucose catabolism is no longer diverted towards lactic acid generation, but it's re-directed toward complete oxidation in mitochondrial TCA cycle. The net effect is the reversal of the Warburg effect, and a modulation of cell metabolism to adopt a more oxidative phenotype (fig. 13).
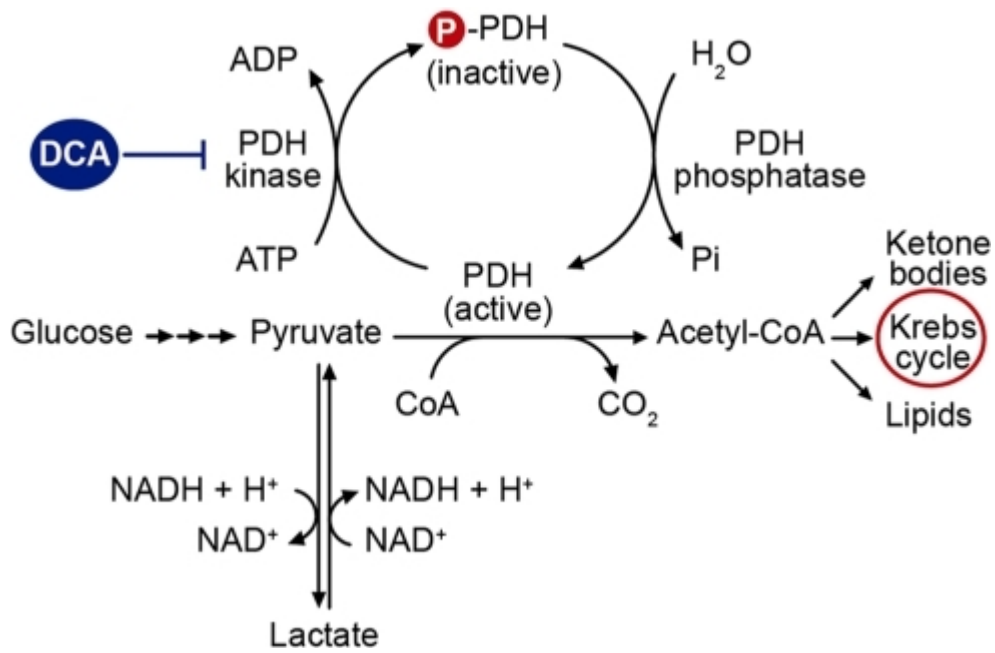
Figure 13 – DCA mechanism of action and metabolic repercussions (from [145])

For its action, dichloroacetate has been used to treat lactic acidosis in children [146], generally demonstrating a good tolerability and safety [147].

In the attempt to reverse Warburg effect, in 2007 Michelakis and colleagues reported that DCA induces a mitochondrial-dependent apoptosis in cancer cells [148]. Since then, DCA has been investigated in cancers with different origins: the anti-neoplastic effects of this molecule as single agent have been reported in glioblastoma [149], metastatic breast [150], endometrial [151], colorectal [152] and ovarian cancers [153]. Additionally, DCA has been shown to synergize with conventional chemotherapy [154-156] and with metformin [157, 158].

In hematological malignancies, the pro-apoptotic activity of DCA has been demonstrated in T-cell lymphoma [159], MM [160] and in chronic lymphocytic leukemia [161].

Chen and colleagues reported a DCA-induced apoptosis in a small panel of cell lines and in 3 primary samples of AML [59].

Here, we extend these observations, evaluating the effects of cell metabolism modulation, by DCA exposure, on a larger panel of AML cell lines and primary samples.

2.2 Materials

2.2.1 Reagents

DCA was purchased from Sigma-Aldrich (Gillingham, UK) and stored at +4°C until use.

2.2.2 Cell culture and primary samples

AML cell lines (HL60, KG1, MOLM-13, OCI-AML2, OCI-AML3, U937) were cultured under standard conditions (37°C in 5% CO_2 humidified atmosphere), were harvested in log-phase growth for all experiments, and exposed for 24, 48, 72 hours to scalar concentrations of DCA (1 to 7.5mM). Normal mononuclear cells were obtained from peripheral blood (NPBLs) samples of volunteer donors. PB and/or bone marrow (BM) aspirates samples were obtained from 8 AML patients (Table 2), who provided written informed consent, in accordance with regulations and protocols sanctioned by the Human Subjects Committee of Helsinki and approved by the Institutional Review Board of the Sapienza University of Rome. Cells were suspended in complete medium to a starting concentration of $1.0x10^6$/mL and exposed to scalar concentration of DCA (1 to 7.5mM).

Following DCA exposure, cell lines and primary cells were evaluated for cell counts, viability, induction of apoptosis and ROS production

2.3 Methods

2.3.1 Apoptosis analysis

Induction of apoptosis was assessed by measuring Annexin V binding to externalized phosphatidylserine, as previously described. Briefly, cells were washed twice with PBS and resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2, Sigma Chemical Co.). FITC conjugated Annexin V (Roche Diagnostic Corp., Indianapolis, Indiana, USA) was added at a final concentration of 1µg/ml. The mixture was incubated at room temperature for 15 min in the dark prior to flow cytometry analysis. Membrane integrity was simultaneously assessed by PI (0.25µg/ml) exclusion.

2.3.2 ROS production

For the analysis of ROS content, cells were incubated with 4 µM dihydroethidium (DHE; Molecular Probes, Eugene, OR) for 15 minutes at 37°C. After incubation, the cells were analyzed

by flow cytometry. ROS production was evaluated at different time points after treatment, as indicated.

2.3.3 Real-time Metabolic Analysis

Basal and DCA-affected glycolytic and mitochondrial metabolism were measured by Seahorse XF24 Analyzer (Seahorse Bioscience, MA, USA). Briefly, XF24 plates were coated with Celltak (BD Bioscience) to ensure cell adherence, following manufacturer's protocol. Cells were moved into minimal DMEM assay medium (Seahorse Bioscience, MA, USA), with no glucose, no glutamine, no phosphate buffer, added with 2mM glutamine (Euroclone, Milan, Italy) and adjusted to pH 7.35, then transferred in XF microplates (500k cells per well). Microplates were briefly centrifuged (1000 rpm, 5') and then transferred in a CO_2-free incubator.

For glycolysis rate experiments, drug injector ports in the disposable sensor-containing cartridge were loaded with glucose 100mM (Sigma Aldrich), dissolved in the assay medium, adjusted to pH 7.35, for a final concentration in the well of 10mM, in port A. Port B were loaded with oligomycin (Seahorse Bioscience, MA, USA), dissolved in assay medium, at concentration of 10µM, for a final concentration in the well of 1µM. Port C were loaded with 2-DG 1M (Seahorse Bioscience, MA, USA), dissolved in assay medium, adjusted to pH 7.35, for a final concentration in the well of 100mM. After a 30' equilibration in a CO_2-free incubator, cartridges were inserted into the XF analyzer for the calibration step. After calibration, cell-containing plates were moved in the XF analyzer. The experiments were composed of: a) three cycles of measurements, b) injection of glucose (port A), c) three cycles of measurements, d) injection of oligomycin (port B), e) 3 cycles of measurements, f) injection of 2-DG (port C), g) 3 cycles of measurements.

For DCA experiments, drug injector ports in the disposable sensor-containing cartridge were loaded with glucose 100mM (Sigma Aldrich), dissolved in the assay medium, adjusted to pH 7.35, for a final concentration in the well of 10mM, in port A. Port B were loaded with DCA, dissolved in assay medium, adjusted to pH 7.35, at concentration of 50 and 100mM, for a final concentration in the well of 5 and 10mM, respectively. After a 30' equilibration in a CO_2-free incubator, cartridges were inserted into the XF analyzer for the calibration step. After calibration, cell-containing plates were moved in the XF analyzer. The experiments were composed of: a) three cycles of measurements, b) injection of glucose (port A), c) three cycles of measurements, d) injection of DCA or vehicle (port B), e) 6 cycles of measurements.

For mitochondrial repiration experiments, drug injector ports in the disposable sensor-containing cartridge were loaded with oligomycin 10µM (Sigma Aldrich), dissolved in the assay medium, adjusted to pH 7.35, for a final concentration in the well of 1µM, in port A. Port B were

loaded with FCCP 2µM, dissolved in assay medium, adjusted to pH 7.35, for a final concentration in the well of 0.2µM. Port C were loaded with Antimycin A (10µM) and Rotenone (10µM), dissolved in assay medium, adjusted to pH 7.35, for a final concentration in the well of 1µM for both compounds. After a 30' equilibration in a CO2-free incubator, cartridges were inserted into the XF analyzer for the calibration step. After calibration, cell-containing plates were moved in the XF analyzer. The experiments were composed of: a) three cycles of measurements, b) injection of Oligomycin (port A), c) three cycles of measurements, d) injection of FCCP (port B), e) 3 cycles of measurements, f) injection of Antimycin and Rotenone (port C), g) 3 cycles of measurements. Cell viability after XF experiments were assessed by Trypan Blue exclusion. When needed, data from XF analyzer were normalized for cell counts.

2.3.4 Data analysis and statistics

For all in vitro results, at least three independent experiments were performed to determine mean and SD values. IC50s were calculated using the CalcuSyn software (Biosoft, Cambridge, MA) based on the number of Annexin V positive cells at 72h. Statistical comparisons were performed using the paired Student's t test.

2.4 Results & Discussion

First, we investigated the glucose metabolism in AML cell lines, by using the XF Analyzer. All cell lines tested showed remarkably higher rate of lactate excretion than NPBLs: for example, MOLM13 showed a 4.6 fold increase in glycolytic rates when compared to NPBLs [18.9±2.6 *vs.* 4.1±0.2 (mpH/min) 10^6cells, respectively, p<0.001] (fig 14).
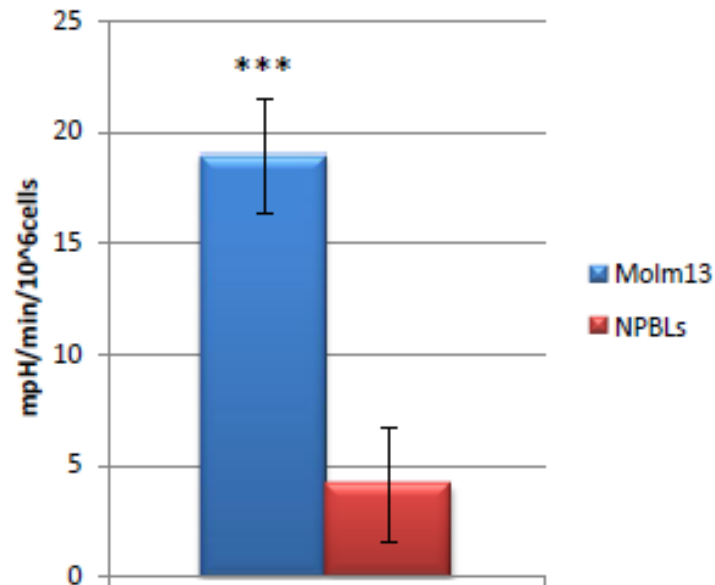
Figure 14 – AML cell line show higher rate of glutaminolysis compared to NPBLs. Glycolytic levels of NPBLs and Molm13 were determined in real time with the Seahorse XF analyzer, as indicated in the method section.

Then, we assessed DCA effects on leukemia cell metabolism. The injection of DCA (5mM, fig.16) on U937 cells caused a temporary dose-dependent drop in lactate production (-36.5%, p=0.0014, compared to vehicle), followed by a rapid recovery in ECAR value, returning to control levels after 30 minutes of exposure (fig. 15a). On the other hand, mitochondrial respiration was positively affected: DCA induced an increase in O_2 consumption (45%, p=0.014, compared to vehicle) (fig. 15b). These enhanced mitochondrial respiration remained stable throughout the entire experiments (120 mins).
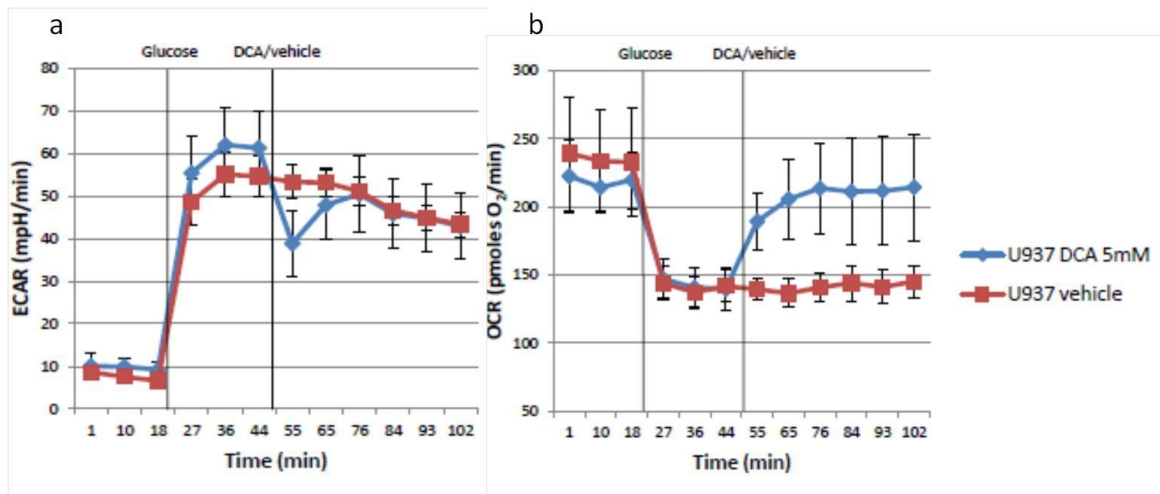
Figure 15 – DCA induces a metabolic shift in AML cells. Real time metabolic measurement was conducted on U937, as described in the method section. Data from the same experiment show: a) glycolytic rate and b) mitochondrial respiration rate, obtained by the Seahorse XF analyzer, before and after the injection of glucose and DCA.

To confirm this mitochondrial metabolism enhancement, we evaluated the effects on mitochondrial respiration after 1 hour of DCA (10mM) exposure, compared to glycolysis inhibitor 2DG (10mM) (fig.17). As mentioned, 2-DG is a "canonical" glycolysis inhibitor, a glucose analog that cannot be metabolized, blocking the activity of hexokinase. The comparison has been made to exclude a compensatory effect, i.e. an upregulation of oxidative phosphorylation to face the reduced energy income from glycolysis. Indeed, 2DG showed little activity on mitochondrial metabolism, indicating that AML cells do not compensate a reduced glycolysis with an increased OXPHOS. On the contrary, DCA was able to significantly enhance both basal and maximal respiration rate (measured after the injection of an electron chain accelerator, FCCP) (fig. 16). These data are in line with the reported DCA mode of action [144], with a shift toward a more oxidative and less glycolytic metabolism by cells when exposed to this molecule.
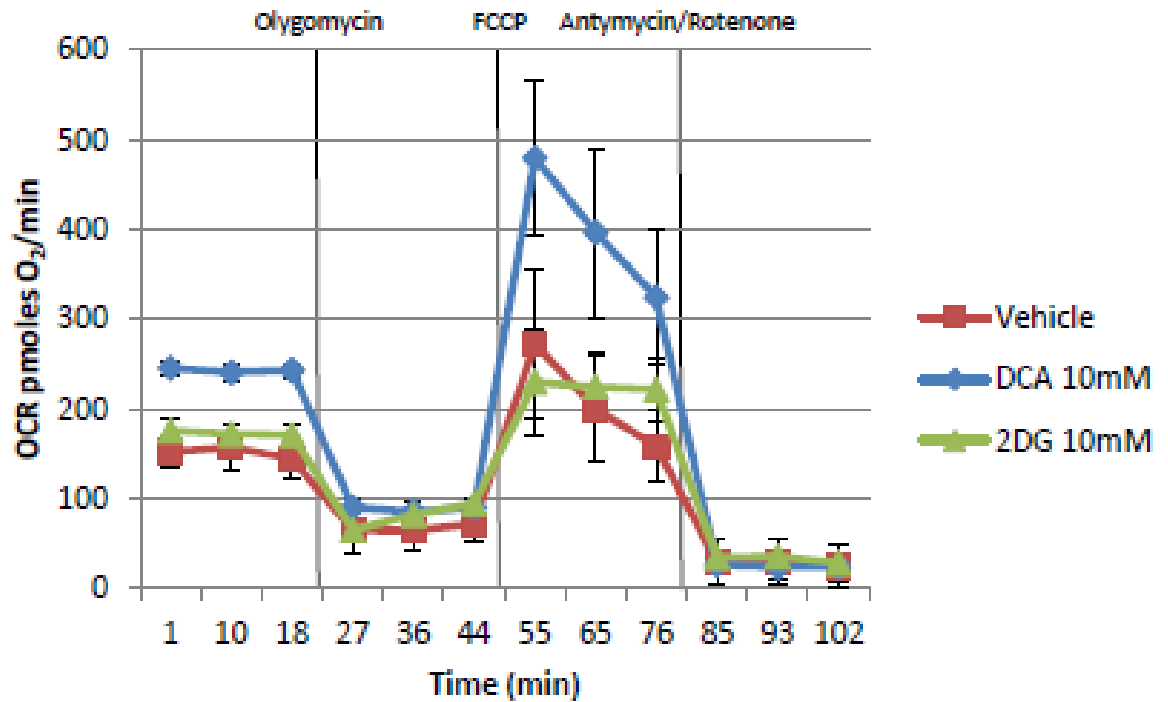
Figure 16 – DCA mediates a reversal of Warburg effect. U937 cell lines were exposed for 1h to DCA or 2DG. Mitochondrial respiration was measured through the Seahorse XF Analyzer, as described in the methods section.

We then tested the long-term effects of DCA exposure on AML cell lines (HL60, KG1, MOLM-13, OCI-AML2, OCI-AML3, U937). In all lines, DCA induced a reduction of cell growth in a dose- and time-dependent fashion, as indicated in fig 17.

Cell growth reduction was associated with apoptosis, as confirmed by Annexin V analysis (fig 18). While U937 appeared to be the most sensitive (72h IC50=2.62mM), two cell lines, HL60 and OCI-AML2, proved resistant to this compound (table 1).
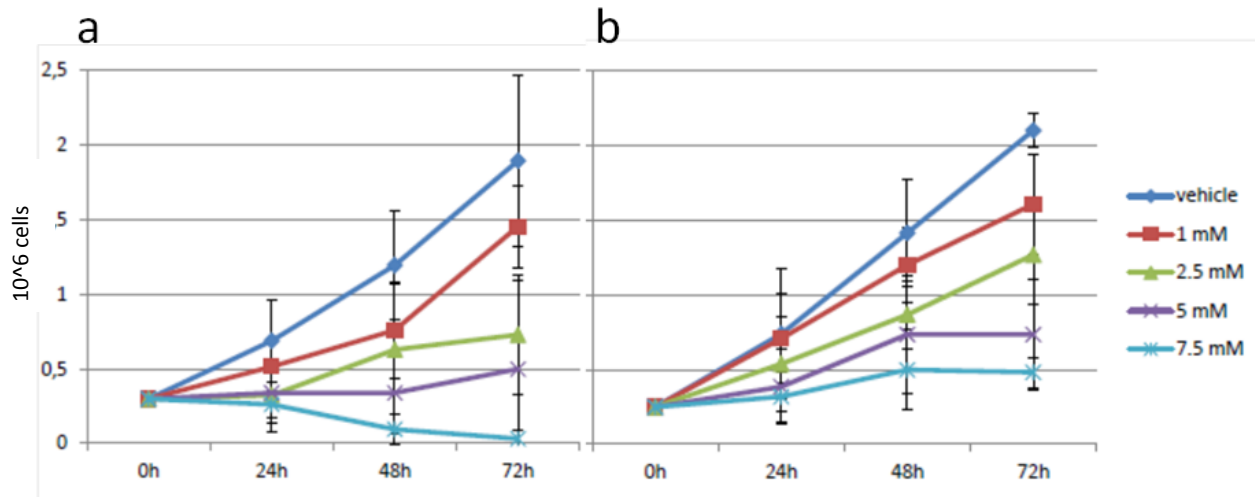
Figure 17 - DCA induces a reduction in AML cell proliferation: MOLM13 (a) and HL60 (b) cell line were exposed to the indicated concentrations of DCA. Cell counts and viability were assessed at the times indicated by Trypan blue exclusion counting.
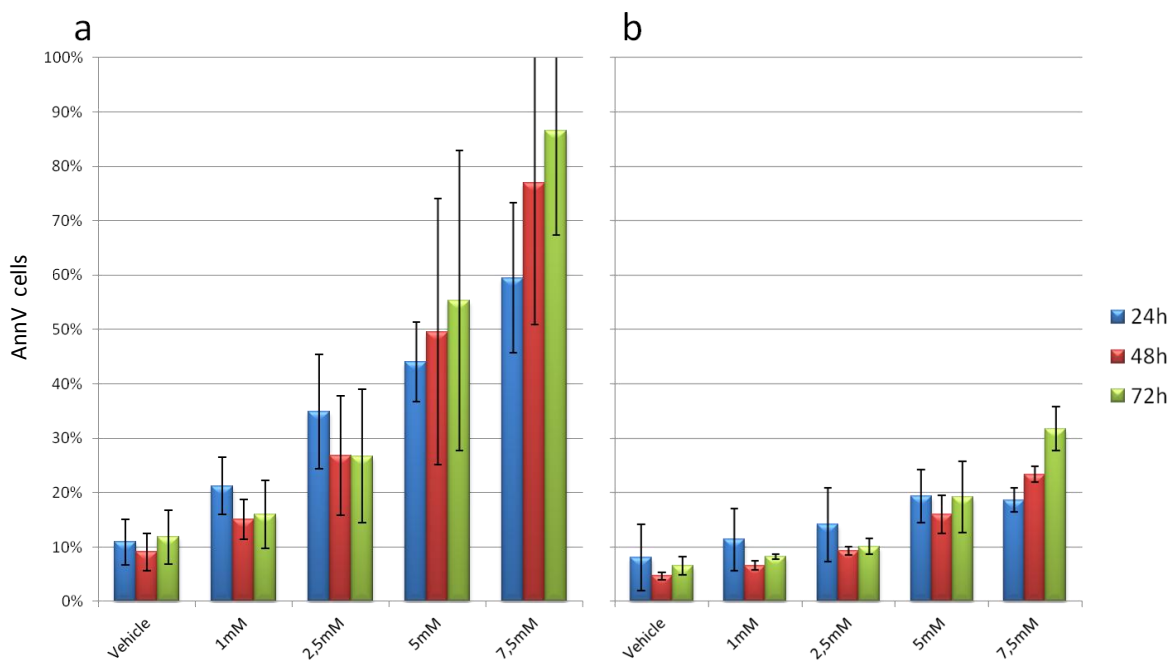

Figure 18 – DCA exerts a dose- and time-dependent pro-apoptotic effect on AML cell lines. MOLM13 (a) and HL60 (b) cells were exposed to the indicated concentrations of DCA. Apoptosis induction was assessed by Annexin V/PI staining (see "Material and Methods" section). Percentage of Annexin V positive cells are shown in the figure.

Cell Line	DCA IC$_{50}$ (mM)
U937	2.62
OCI-AML3	5.81
MOLM13	5.1
KG1	6.61
HL60	nd
OCI-AML2	nd

Table 1 – DCA induces apoptosis in AML cell lines. IC50 was calculated after 72h of DCA exposure

We found no correlation between basal cell line glycolytic rates and DCA sensitivity (fig 19). Thus, we suggest that this heterogeneous effect may be probably related to an inability by the sensitive cell lines to cope with the shift toward a more oxidative metabolism.
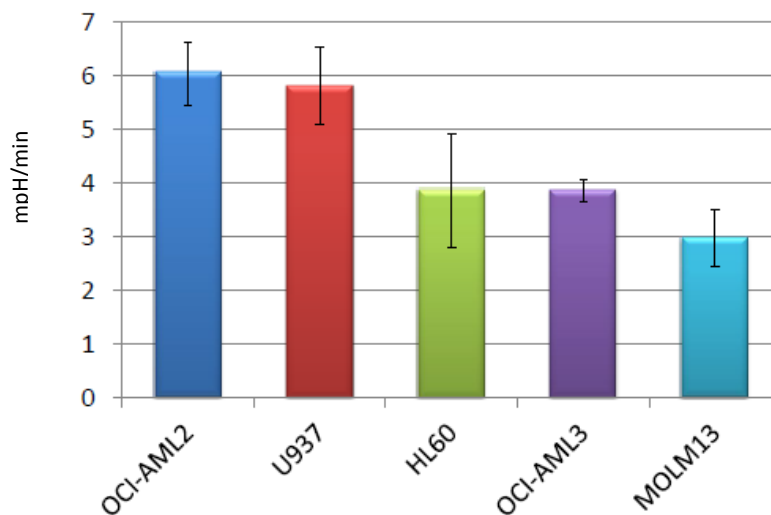
Figure 19 – DCA effect has no correlation with the glycolytic rate. Maximal glycolytic rates of AML cell line panel were measured by Seahorse XF Analyzer as described in the method section.

It has been suggested that DCA exerts pro-apoptotic effect through ROS accumulation [162, 163]. To confirm this evidences, we evaluated ROS production in cell lines after 24h of DCA (1-7.5mM) exposure by DHE staining. At 24h, percentage of DHE-positive U937 cells exposed to

DCA increased from 9.2±1.7% (vehicle) to 12.6±0.5% (1mM, p=0.002), 20.3±4.5% (2.5mM, p=0.043), 30.2±5.2% (5mM, p=0.018) and 39.6±4.7% (7.5mM, p=0.011) (fig. 20). This observation partially supports our hypothesis of a leukemia cell subset that cannot sustain high rate of oxidative phosphorylation.
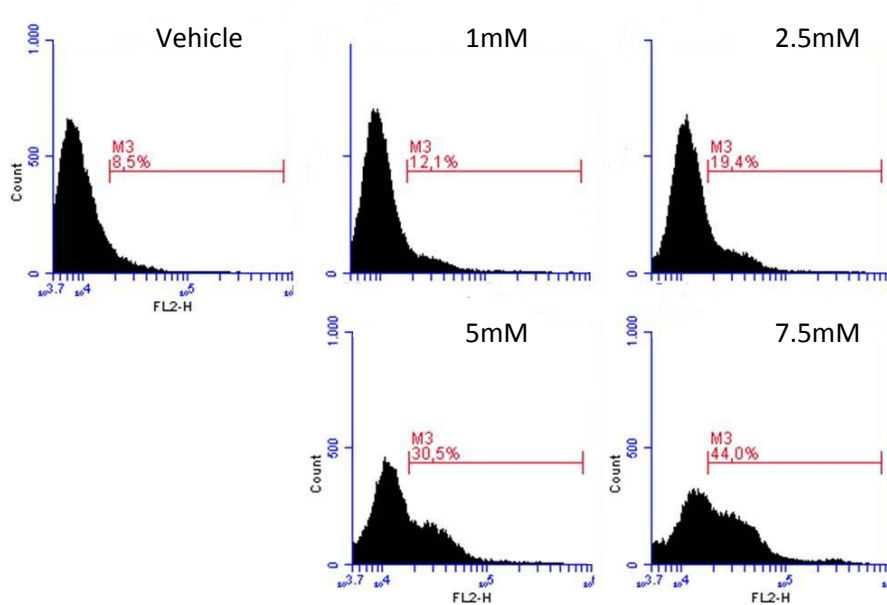
Figure 20 – DCA acts through a dose-dependent ROS over-production. U937 were grown in the absence or presence of scalar concentrations of DCA for 24h. ROS levels were determined at 24h by monitoring DHE uptake (see methods).

DCA is reported to be well tolerated in subjects with metabolic diseases, even in long-term administration [164]. In order to confirm DCA safety on our normal controls, we exposed NPBLs to DCA 5 and 7.5mM for up to 72h. No significant effects were observed on both normal and activated NPBLs: at 72h, Annexin V positive cells ranged from 26.2±11.9% (vehicle), to 27.8±7% (5mM) and 35,5±5,3% (7.5mM) for NPBLs (p>0.05) (suppl.fig.2a); and from 57±6.7% (vehicle), to 59.1±3.8% (5mM) and 64.4±3.8% (7.5mM) for PHA-activated NPBLs (p>0.05) (Fig. 21a,b).
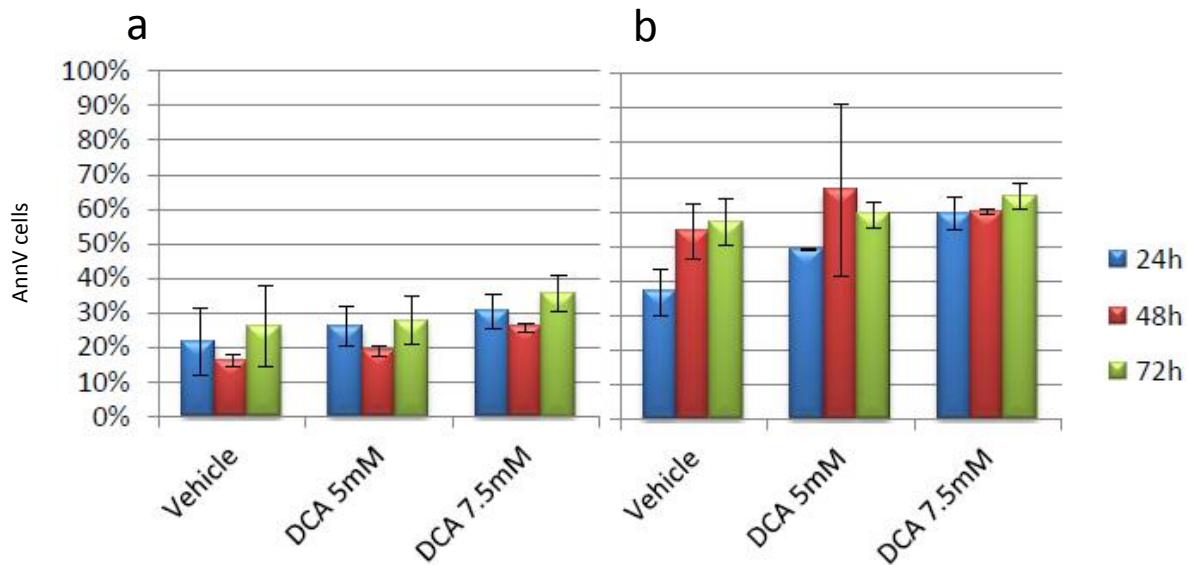
Figure 21 – DCA has no effects on NPBLs. Normal (a) and activated (b) NPBLs were incubated with DCA for up to 72 hours. Apoptosis induction was assessed by Annexin V/PI staining (see "Material and Methods" section). Percentage of Annexin V-positive cells is shown in the figure.

Finally, we investigated the effects of DCA exposure (1-7.5mM) on primary blasts from 8 patients (table 2). Cell growth reduction and induction of apoptosis were observed: after 72h of exposure to scalar concentration of DCA, Annexin V positive cells ranged from 21.1±9.4% (vehicle) to 33.6%±15,3% (1mM, p=0.044), 44.9±19,9% (2,5mM, p=0.0046), 61.2±25.7% (5mM, p<0.001), 69.7±19.1% (7,5mM, p<0.001) (fig.22).

pt #	Sex	BM Blasts (%)	FAB
1	F	100	-
2	M	90	M1/M2
3	M	80	M1
4	M	64	M2
5	M	99	M2
6	F	90	M5
7	M	90	M5
8	F	100	M1

Table 2 – Patient Population

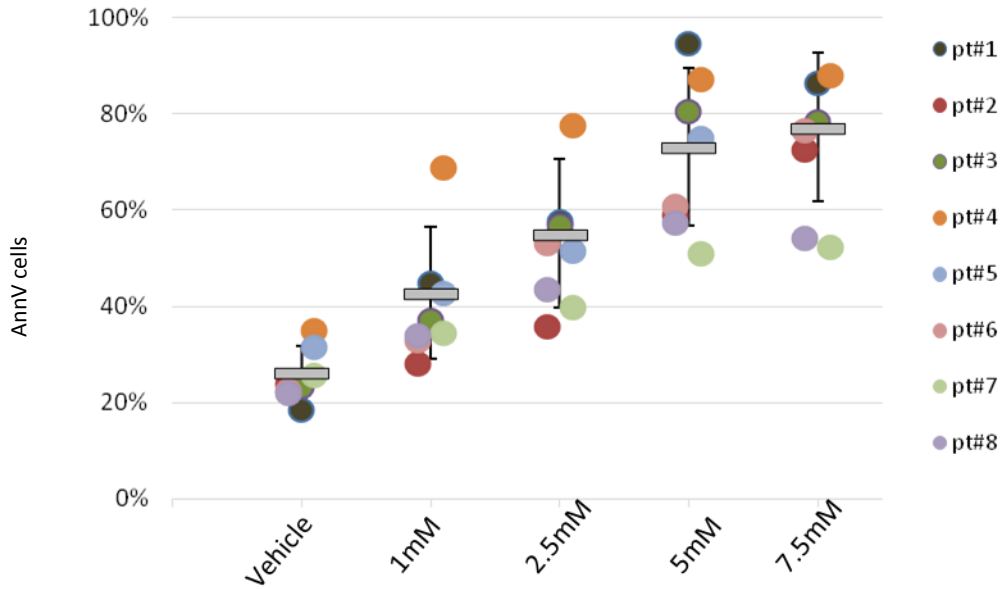**Figure 22 – DCA induces a dose-dependent apoptosis in AML primary cells.** AML primary cells were exposed to the indicated concentrations of DCA for 72h. Apoptosis induction was assessed by Annexin V/PI staining (see Methods section). Percentage of Annexin V-positive cells is shown in the figure.

In agreement with results obtained by Chen and colleagues [59] indicating a DCA-induced anti-neoplastic effect in AML cells, our analysis confirm the effectiveness of this agent in inducing cell growth reduction and apoptosis induction on these cells. Moreover, concentrations used were sufficient to modulate cellular glycolysis in our model, forcing leukemic cells to adopt a more oxidative metabolism. The shift to the oxidative phenotype is not sustainable by these cells, as suggested by the accumulation of intracellular ROS briefly after DCA exposure. Thus, together with results obtained in MM and CLL [160, 161], these evidences underline the promising role for DCA in modulating cell metabolism in hematologic malignancies, prompting further studies about its possible clinical role in conjunction with standard chemotherapy.

Chapter III

Targeting glutaminolysis in AML cells: Aminooxyacetate

3.1 Introduction

Glutamine deprivation has been demonstrated to induce cell growth reduction and apoptosis on AML cells. Thus, we targeted this process, by using a chemical inhibitor of cellular transaminases, Aminooxyacetic Acid (AOA) [165]. Among this class of enzymes, glutamate-dependent transaminases contribute to glutaminolysis by converting glutamate into the TCA cycle intermediate α-ketoglutarate [166].
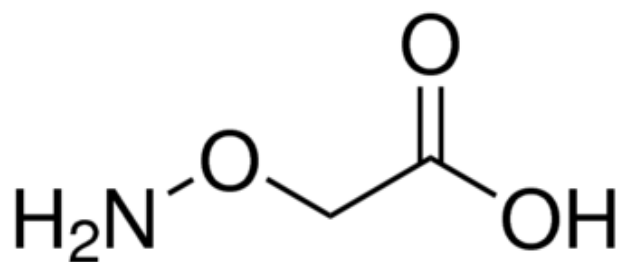
Figure 23 – Chemical structure of AOA (from www.sigma-aldrich.com)

AOA proved particularly effective in inhibiting glutaminolysis, as shown by Wise and colleagues in an important work about the role of c-Myc in driving glutamine catabolism in cancer cells [167]. Using c-Myc transfected fibroblast, they demonstrated that AOA is able to selectively induce apoptosis in glutamine-addicted Myc-expressing cells [167]. To further demonstrate AOA specificity, they observed that the addition of α-ketoglutarate, the final product of glutamate-dependent transaminases, fully prevented AOA-induced apoptosis [167].
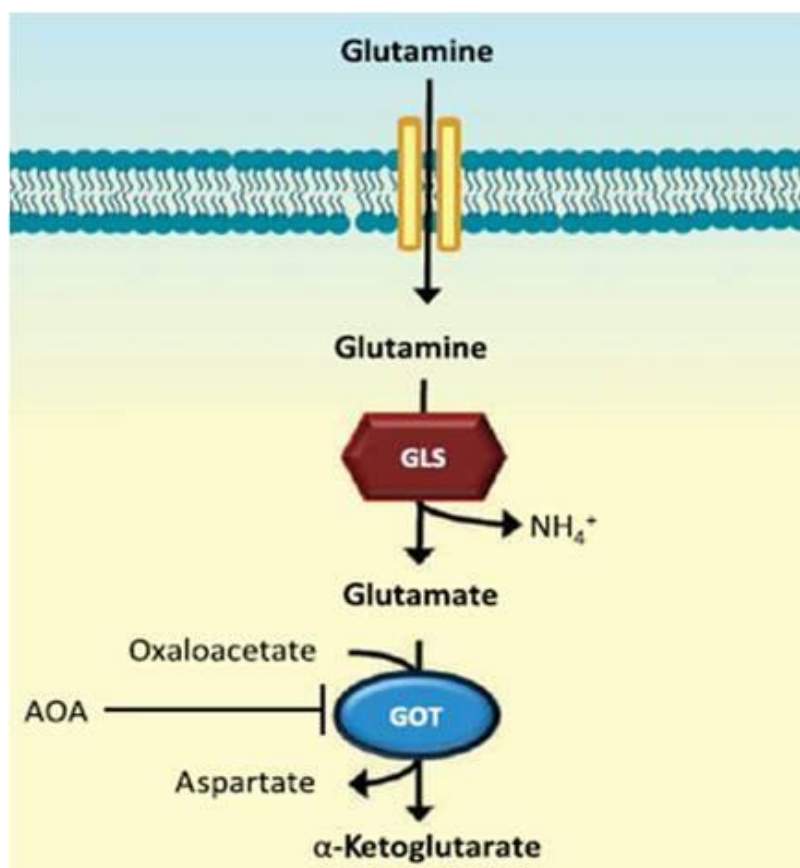
Figure 24 - AOA inhibition of glutaminolysis

Thornburg and colleagues reported an AOA-induced reduction of cell proliferation, in cell lines and in xenograft murine models of breast cancer, through mitochondrial metabolism impairment [168]. Moreover, Qin and colleagues reported a sensitivity of melanoma cells to AOA, particularly when combined with death receptor ligands [169]. Additionally, a similar AOA pro-apoptotic activity, through reduction of cellular respiration, has been reported in osteosarcoma [170].

These results, along with the pronounced glutamine addiction of cancer cells, prompted us to investigate the effect of glutaminolysis inhibition, by AOA exposure, in AML cells.

3.2 Materials

3.2.1 Reagents

AOA was purchased from Sigma-Aldrich (Gillingham, UK). The compound was stored as dry powder at 0-4°C and dissolved in PBS immediately before use.

3.2.2 Cell culture and primary samples

AML cell lines (HL60, MOLM-13, OCI-AML2, OCI-AML3, U937) were cultured under standard conditions (37°C in 5% CO_2 humidified atmosphere), were harvested in log-phase growth for all experiments, and exposed for 24, 48, 72 hours to scalar concentrations of AOA (from 100 to 1000μM).

Normal mononuclear cells were obtained from peripheral blood (NPBLs) samples of volunteer donors. PB and/or bone marrow (BM) aspirates samples were obtained from 8 AML patients (Table 2), who provided written informed consent, in accordance with regulations and protocols sanctioned by the Human Subjects Committee of Helsinki and approved by the Institutional Review Board of the Sapienza University of Rome. Cells were suspended in complete medium to a starting concentration of $1.0x10^6$/mL and exposed to scalar concentration of AOA (100 to 1000μM).

Following AOA exposure, cell lines and primary cells were evaluated for cell counts, viability, and induction of apoptosis as reported in Chapter II.

3.3 Methods

3.3.1 Glutaminolytic Rates

Ammonia is the byproduct of the transamination reaction that converts glutamate in α-ketoglutarate. Once produced, it is rapidly excreted in the extracellular environment [28]. Glutaminolysis rates in cell lines and NPBLs, resting or PHA-activated, were detected by measuring ammonia levels in culture medium, as reported in literature [101]. Briefly, cells were centrifuged (1200 rpm, 5'), the supernatant were collected, and stored at -20°C until analysis. Ammonia levels were measured through Ammonia Colorimetric Assay Kit (Biovision, CA, USA), following manufacturer's instruction.

3.4 Results & Discussion

First, we measured glutaminolytic rates of AML cell lines, compared to NPBLs. After 24h, the ammonia level in cell line medium were significantly ($p<0.05$) higher than both normal and activated NPBLs (fig.25): OCI-AML3 excreted 3 times more ammonia compared to normal NPBLs ($p=0.02$), thus confirming a sustained glutaminolytic rate in our models.
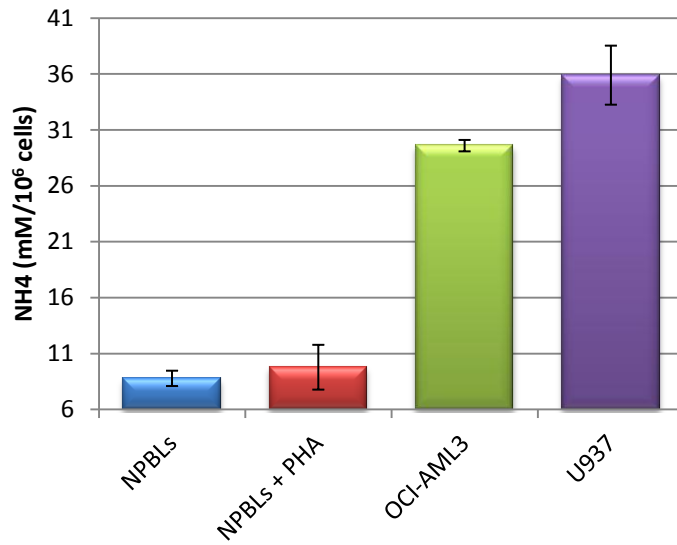
Figure 25 - AML cell line show higher rate of glutaminolysis compared to NPBLs. Normal or activated NPBLs, OCI-AML3 and U937 were cultured for 24h. NH4 levels on culture medium were determined as indicated in the method section.

We then measured ammonia levels after 24h of AOA exposure. In OCI-AML3 cells, we found a trend toward a dose-dependent inhibition of glutaminolysis, although not statistically significant (Fig 26). Minor effects were seen in AOA-exposed normal or activated NPBLs (fig 27a, b).
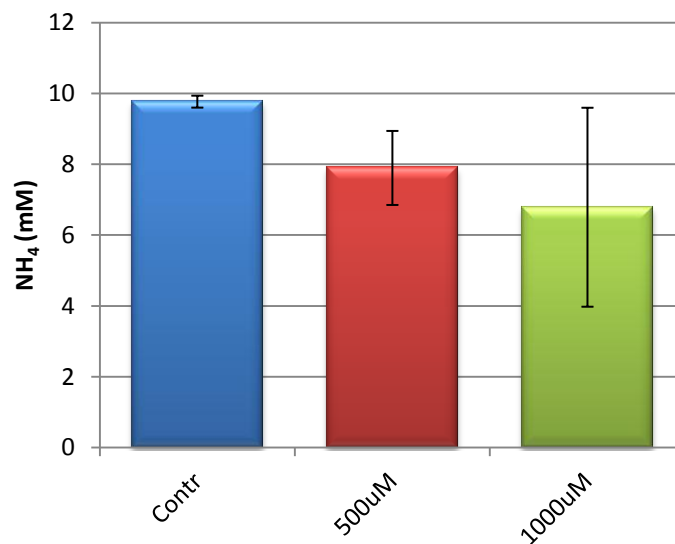
Figure 26 – AOA inhibits glutaminolysis in AML cell lines. OCI-AML3 cells were exposed to the indicated concentrations of AOA for 24h. NH_4 levels on culture medium were determined as indicated in the method section.
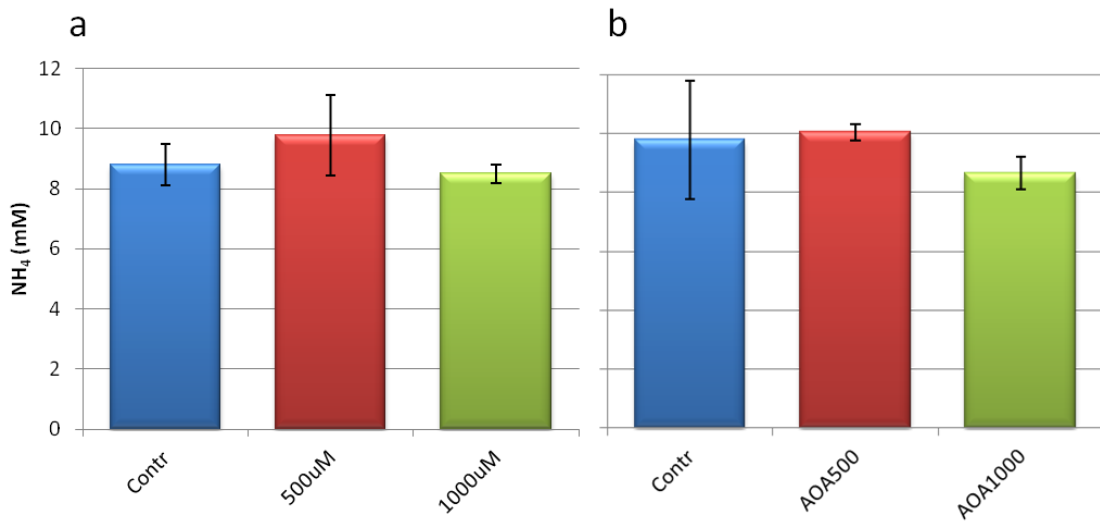
Figure 27 – AOA exposure induces only minor effects on NPBLs' glutaminolytic rate. Resting (a) or PHA-activated (b) NPBLs were exposed to the indicated concentrations of AOA for 24h. NH_4 levels on culture medium were determined as indicated in the method section.

Moreover, we evaluated the effect of long-term AOA exposure on cell growth. All cell lines tested showed a marked sensitivity to this compound, with a dose- and time-dependent reduction of cell count (fig. 28)
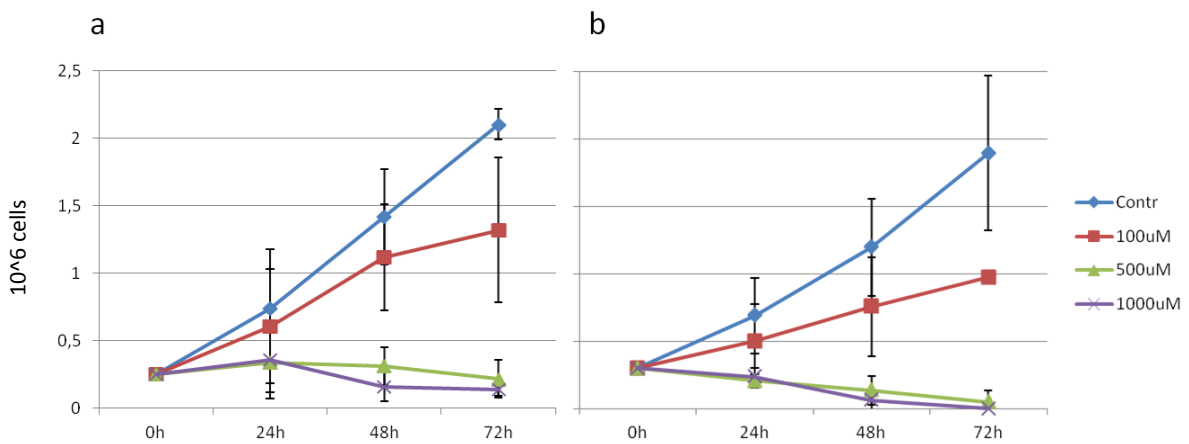
Figure 28 - AOA induces a dose- and time-dependent cell growth reduction on AML cell lines. OCI-AML2 (a) and MOLM13 (b) were exposed to the indicated concentrations of AOA. Cell counts and viability were assessed at the times indicated by Trypan blue exclusion counting.

The AOA-induced cell growth reduction was associated to an apoptotic process, as demonstrated from Annexin V analysis (fig 29). Table 3 reports the IC50, calculated after 72h of AOA exposure, for our cell line panel, with OCI-AML3 being the most sensitive (136.9µM) and HL60 the least (978,6µM). This HL60 mild sensitivity to AOA, together with the resistance to glycolysis modulator DCA, suggests that this leukemia model has a peculiar metabolism, that diverge from the other cell lines tested, confirming previous observation from other groups [101].
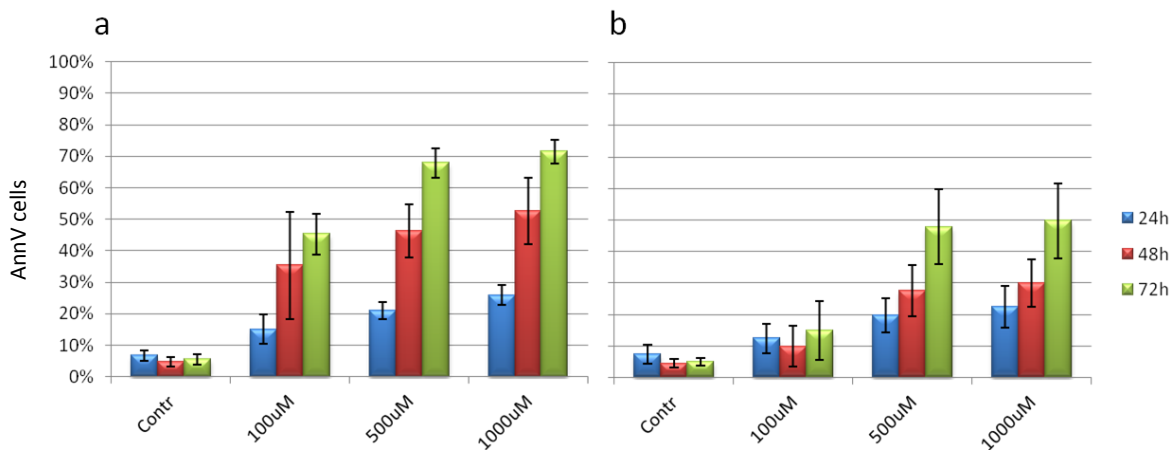
Figure 29 – AOA exerts a dose- and time-dependent pro-apoptotic effect on AML cell lines. OCI-AML3 (a) and HL60 (b) cells were exposed to the indicated concentrations of AOA. Apoptosis induction was assessed by Annexin V/PI staining (see "Material and Methods" section). Percentage of Annexin V-positive cells is shown in the figure.

Cell Line	IC50
U937	270,7 uM
OCI-AML3	136,9 uM
HL60	978,6 uM
MOLM13	268,1 uM

Table 3 - AOA induces apoptosis in AML cell lines. IC50 was calculated after 72h of DCA exposure

To evaluate the effects of this inhibitor on normal cells, we exposed normal and activated NPBLs to scalar concentrations of AOA. Results obtained demonstrated that AOA did not exert pro-apoptotic effect in NPBLs (fig. 30).
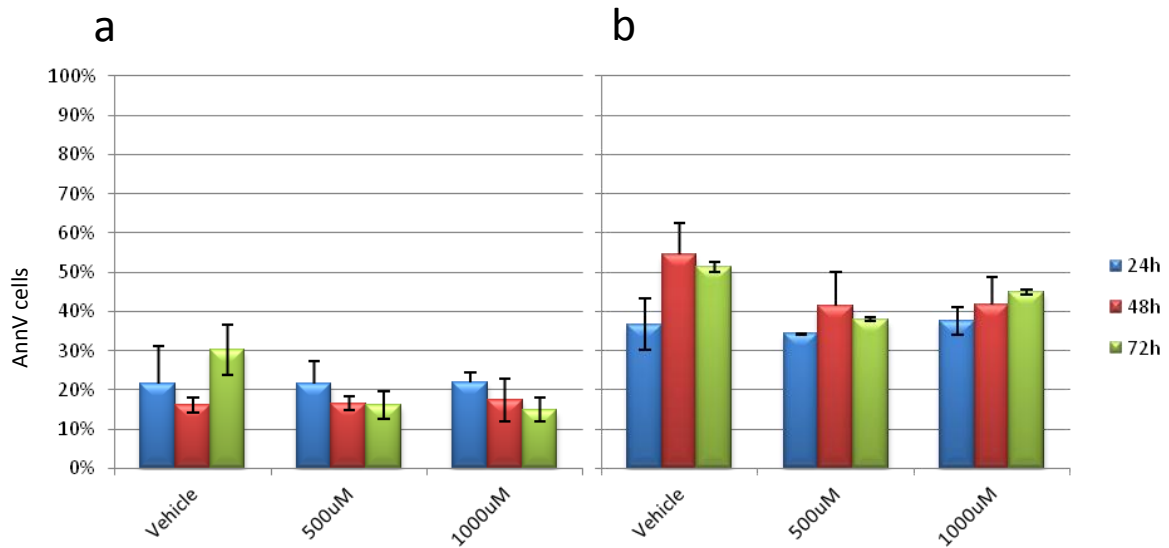
Figure 30 – AOA shows no effects on NPBLs. Normal (a) and activated (b) NPBLs were incubated with AOA for up to 72 hours. Apoptosis induction was assessed by Annexin V/PI staining (see "Material and Methods" section). Percentage of Annexin V-positive cells is shown in the figure.

Finally, we evaluated the effect of AOA exposure on primary blasts from 8 AML patients (table 2). 72h of AOA exposure exerted a statistically significant apoptotic effect, ranging from 25±4.9% (control) to 28.6±4.8% (100µM, p=0.04), 32.1±11.4% (500µM, p=0.03) and 35.7±13.5% (1000µM, p=0.01) (fig. 31)
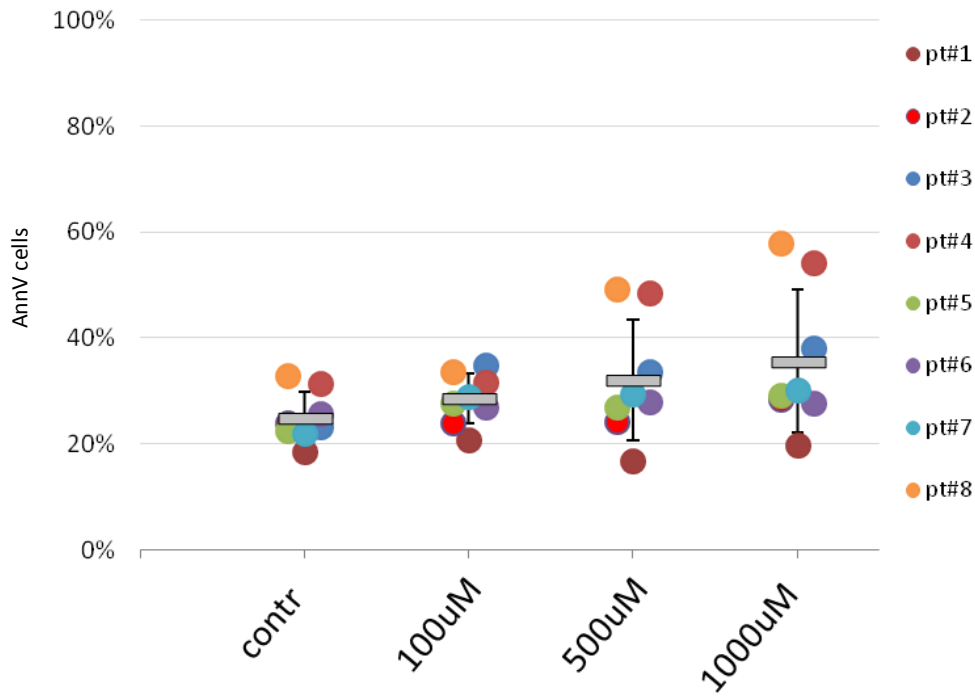
Figure 31 - AOA induces a dose-dependent apoptosis in AML primary cells. AML primary cells were exposed to the indicated concentrations of AOA for 72h. Apoptosis induction was assessed by Annexin V/PI staining (see Methods section). Percentage of Annexin V-positive cells is shown in the figure.

To our knowledge, this is the first report of AOA pro-apoptotic effects on AML cells. In line with recent results in literature, we show that glutamine is a fundamental substrate for AML cells. Bouscary's group demonstrated that glutamine uptake, by l-ase administration, can be considered a promising target in AML treatment [96]. However, they reported the sudden insurgence of a resistance mechanism, through up-regulation of glutamine synthase, the enzyme responsible for its synthesis [96]. Thus, we propose a different approach, targeting the downstream process of glutamine incorporation in the TCA cycle, thus preventing its use as anaplerotic substrate for leukemic cells. Interesting results has been reported in AML with other glutaminase inhibitors, such as BPTES [85], Zaprinast [171], and a molecule developed by Calithera Biosciences (CA, USA), named CB-839 [172], which recently entered in phase I clinical trials (NCT02071927).

Taken together, these evidences underline the role of glutamine in AML cell metabolism, and provide the rationale for targeting this metabolic process in AML treatment.

Chapter IV

Targeting Fatty Acid Oxidation in Leukemia Cells: ST1326

4.1 Introduction

Recent studies have focused attention on the key role of fatty acid oxidation (FAO), as a way to produce ATP, NADPH and acetyl-CoA in neoplastic cells [173]. The carnitine palmitoyl transferase 1a (CPT1a) catalyzes the first step of FAO, by loading long-chain fatty acyl-groups onto carnitine, transporting them through the mitochondrial membrane (fig 32). CPT1a has been previously demonstrated to interact with members of the apoptotic machinery, such as Bcl-2 [174] and t-Bid [175], and its inhibition can cause an accumulation of the toxic metabolite palmitate, resulting in mitochondrial damage and cell death [105]. The inhibition of CPT1a has already been applied in leukemia with positive results [105]
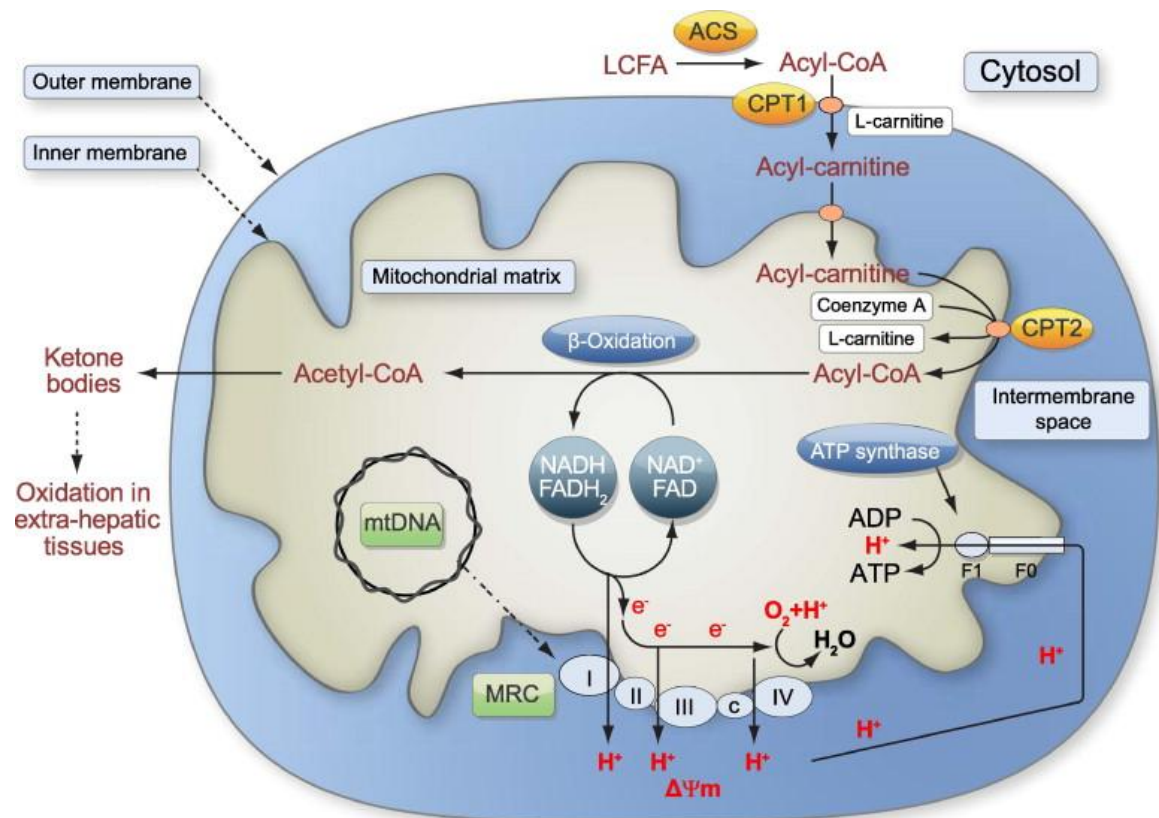
Figure 32 - Lipid transport across mitochondrial membrane and beta-oxidation, from [176]

.

ST1326 is an aminocarnitine derivative characterized by a selective reversible inhibitory activity towards the liver isoform of the enzyme CPT-1a. ST1326 was initially selected by Sigma-Tau laboratories as a candidate for the oral therapy of diabetes and for the treatment and prevention of ketoacidosis [177], being the liver CPT1 inhibition considered an efficacious strategy in the therapy of diabetes [178].
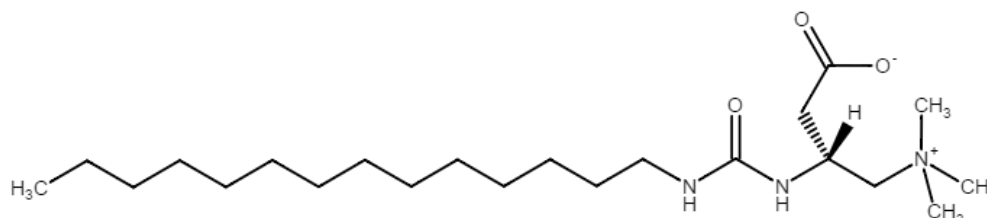
Figure 33 – Chemical structure of ST1326 (from www.http://chem.sis.nlm.nih.gov/chemidplus/rn/250694-07-6)

Results recently reported by Pacilli and colleagues [179] demonstrated that the pharmacological inhibition of FAO by ST1326 strikingly impairs cancer cell survival and inhibits tumor cell proliferation in vitro and in vivo models of Burkitt's lymphoma. Therefore, we aimed in this study to expand this approach, investigating the in vitro anti-leukemic activity of ST1326 on both human leukemia cell lines and primary cells obtained from patients with different haematological disease.

4.2 Materials

4.2.1 Reagents

The CPT-1 inhibitor, ST1326, kindly provided by Sigma-Tau Pharmaceutical Industries S.p.A. Italy, was stored as dry powder at 0-4°C and dissolved in PBS immediately before use.

4.2.2 Cell culture and primary samples

Cell lines originating from hematological malignant disorders of myeloid (U937, KG-1, HL60, *Hl60MTX*, K562) and lymphoid (Raji, Jurkat, *Molt4, CEM S, CEM R*) origin, have been used as cell models. Cell lines cultured under standard conditions were harvested in log-phase growth for all experiments, washed and seeded with scalar concentrations of ST1326 (1 to 50µM). Wash out experiments were also performed by washing the cells exposed for 24-72h to ST1326 twice with PBS.

Normal mononuclear cells were obtained from peripheral blood (NPBLs) samples of volunteer donors. Normal CD34+ cells were obtained by immunomagnetic separation (Miltenyi Biotec, Bologna, Italy) from leukapheretic product of a normal volunteer (PBSC). PB and/or bone marrow (BM) aspirates samples were obtained from 24 leukemia patients (Table 4), who provided written informed consent, in accordance with regulations and protocols sanctioned by the Human Subjects Committee of Helsinki and approved by the Institutional Review Board of the Sapienza University of Rome. Cells were suspended in complete medium to a starting concentration of 1.0x106/mL and exposed to scalar concentration of ST1326 (1 to 50μM), and for selected experiments, to Etomoxir (5 to 50μM).

4.3 Methods

4.3.1 Cell cycle and apoptosis analysis

Cell cycle distribution changes were evaluated using the Acridine Orange (AO) technique as previously described [180, 181]. The percentage of cells in the G0, G1, S, and G2M were determined by measuring simultaneously the DNA and RNA total cellular content. The percentage of apoptotic cells was measured based on the decreased stainability of apoptotic elements in DNA green fluorescence (sub-G1 peak on DNA-frequency histograms) coupled with a higher RNA red fluorescence (which is common to chromatin condensation) [180]; cell debris were excluded from the analysis on the basis of their forward light scatter properties. Cell-cycle distribution was analyzed using the ModFit LT software (Verity Software House, Topsham, ME). Induction of apoptosis was also assessed by measuring Annexin V binding to externalized phosphatidylserine, as previously described. Briefly, cells were washed twice with PBS and resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2, Sigma Chemical Co.). FITC conjugated Annexin V (Roche Diagnostic Corp., Indianapolis, Indiana, USA) was added at a final concentration of 1μg/ml. The mixture was incubated at room temperature for 15 min in the dark prior to flow cytometry analysis. Membrane integrity was simultaneously assessed by PI (0.25μg/ml) exclusion.

4.3.2 Mitochondrial Membrane Potential

To measure loss mitochondrial membrane potential (ΔΨm), cells were loaded with 300nM Chloromethyl-X-Rosamine (CMXRos) and 100μM MitoTracker Green (MTGreen), (Molecular Probes®, Invitrogen, Eugene, OR) for 1 hour at 37°C. The ΔΨm was then assessed by measuring

CMXRos retention (red fluorescence) while simultaneously adjusting for mitochondrial mass (green fluorescence).

4.3.3 Functional FAO Assay

FAO rates were measured in real time by the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, MA, USA). Cells were suspended in unbuffered DMEM medium supplemented with 2.5mM glucose and 0.5mM L-carnitine (all from Sigma-Aldrich) adjusted to pH 7.35, transferred in Celltak (BD Bioscience) coated plates and incubated for 30 minutes at 37°C in a CO2-free incubator. ST1326 or Etomoxir (Sigma-Aldrich) were added 15 minutes before the assay to a final concentration of 50µM. BSA or BSA-Palmitate (Seahorse Bioscience, MA, USA) were added to each well just prior the assay, to a final concentration of 33 and 200µM, respectively. Oxygen Consumption Rates (OCR) were measured for basal state and following the sequential injection of oligomycin (1µM), FCCP (0.4µM), and a mix of Antimycin A (1µM) and Rotenone (1µM) (all from Seahorse Bioscience, MA, USA) in each well. After the assay, cells were detached and manually counted to assess cell number and viability. OCR data were normalized for cell number.

4.3.4 Histomorphological analysis

Mitochondria were stained by incubating cells with 500nM MitoTracker Red CMXRos (Molecular Probes, Milan, Italy) in complete medium for 30 min, followed by 30 min in complete medium without MitoTracker. For CPT1A staining, cells were fixed by using 3% paraformaldehyde in 100mM phosphate buffer and 60mM sucrose for 15 min at room temperature (RT), and then washed twice in PBS. Cells were permeabilized with 1% (w/v) Triton X-100 in PBS and 20 mM glycine for 10 min at RT and then washed twice in PBS. Before the addition of the CPT1A antibody, cells were incubated with PBS containing 10% pre-immune goat serum at RT for 30 min. Afterwards, cells were stained with the rabbit anti-CPT1A polyclonal antibody (Novus Biological, Milan, Italy) for 1h at 37°C following the manufacturer's procedure. After washing twice in PBS/20mM glycine, cells were incubated with goat anti-rabbit Alexa Fluor® 594 (Molecular Probes) for 1h at 37°C, and then washed twice in PBS. Fluorescent staining was viewed on a Zeiss LSM 710 confocal laser-scanning microscope equipped with 63x objective at excitation wavelengths of 561 nm for Alexa Fluor 594.

4.4 Results & discussion

We initially confirmed in a collaborative study the presence of the target, CPT1a, in Raji cells by immunofluorescence analysis that demonstrated the mitochondrial localization of CPT1a (Fig. 34, courtesy of G. Peluso).
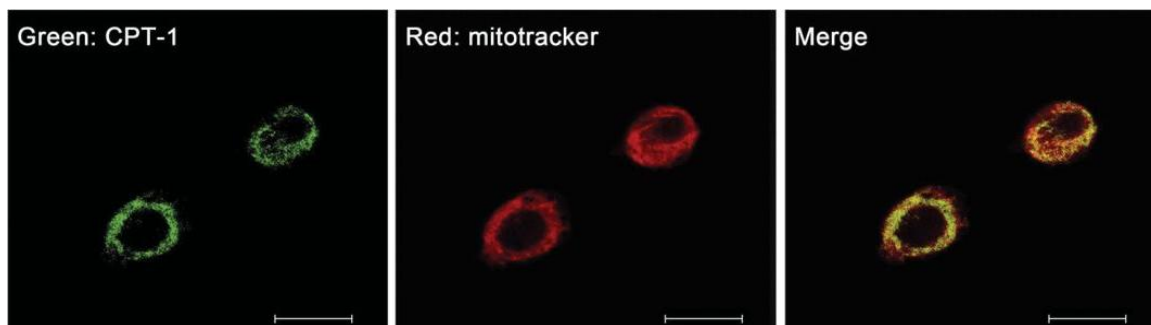
Figure 34– Leukemia cells express CPT1 isoform A. Raji cells were stained as described in the method section

Then, we documented that ST1326 was effective in inducing FAO inhibition as demonstrated by real-time metabolic analysis. The exposure for 75 minutes to ST1326 at 50µM caused a significant impairment of fatty acid (FA)-based maximal respiratory capacity: U937 cells in fact showed a 45.2% (p<0.001) reduction of this parameter when compared to palmitate-only control, while 50µM of Etomoxir induced only minor effects (4% reduction) (Figure 35).
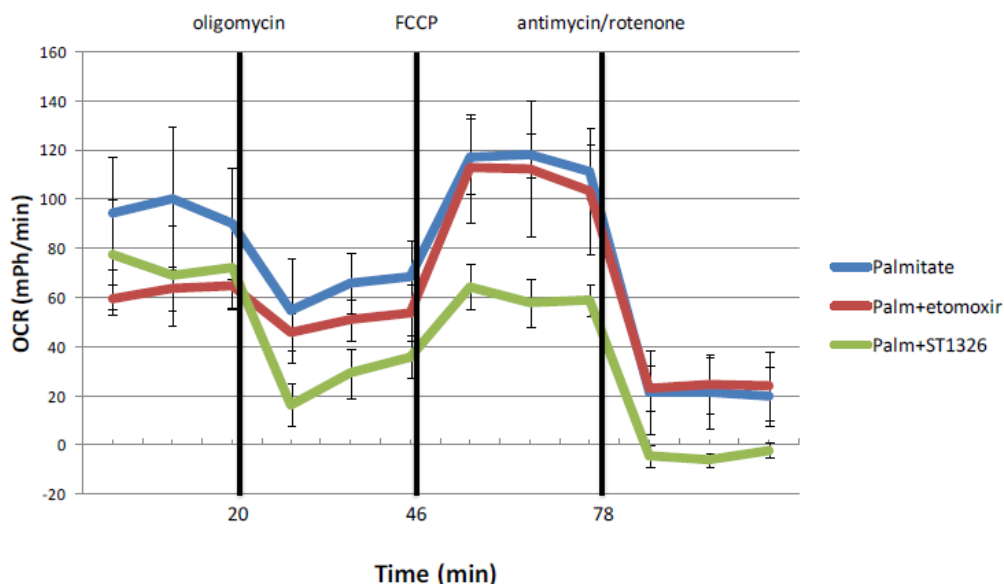
Figure 35 - ST1326 induces FAO decrease in AML cells. Time course of a FAO experiment on U937 cells, exposed to ST1326 or etomoxir

Moreover, ST1326 affected both basal and maximal FA-based respiratory capacity in HL60-MX2 cells (Figure 36a): the reduction was 82% (p<0.01) and 67.2% (p=0.01), respectively, when compared to palmitate-only condition. The effectiveness of ST1326 (50μM) in inhibiting FAO process was confirmed on primary acute myeloid leukemia (AML) cells (Figure 36b): an impairment of FA-based mitochondrial activity was obtained on both basal (-59.4%, p<0.01) and maximal respiration (-53.9%, p=0.037).
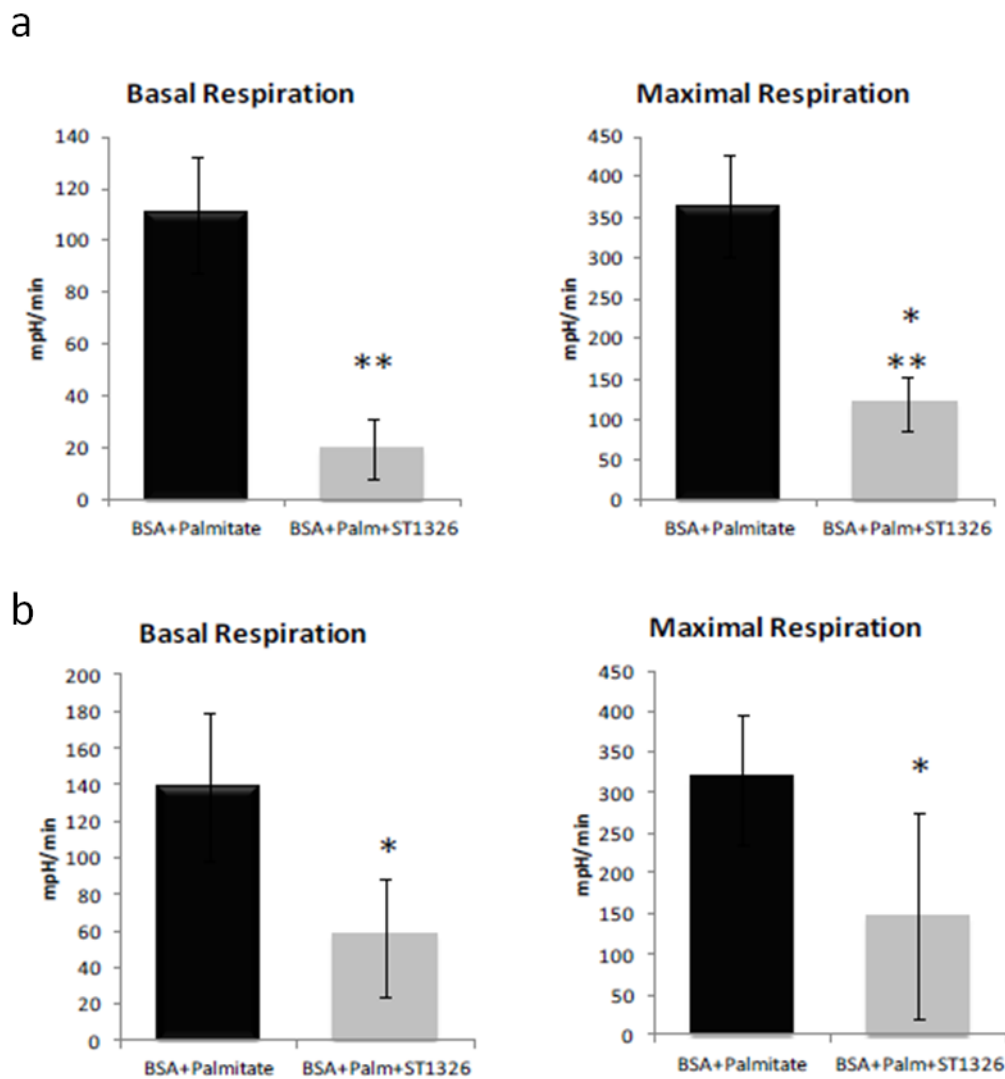
Figure 36 - ST1326 induces FAO decrease in AML cells. Basal and maximal respiration rates for (a) HL60-MX2 cells and (b) AML primary blasts exposed to ST1326. Basal rates are calculated at 19 minutes from the beginning of the experiment (34 minutes of ST1326 exposure); maximal respiration rates are calculated after the electron transport chain-accelerator (FCCP) injection, 55 minutes after the beginning of the experiment (70 minutes of ST1326 exposure).

We then analyzed anti-proliferative and cytotoxic activity of ST1326 demonstrating that the CPT1a inhibitor induced dose- and time-dependent cell growth arrest, cell cycle modulation and apoptosis (Figure 37, 38, 39). In particular, a marked depletion of the S phase, associated with an increase in both G2M phase and sub-G0/1 peak, was documented after 24 hours of exposure to 50μM of ST1326. The same effects occurred also at low ST1326 concentrations over longer times of exposure (Figure 39). Etomoxir failed to reproduce similar significant results (Figure 38). The cytotoxic activity of the molecule was also confirmed by using Annexin V (data not shown).
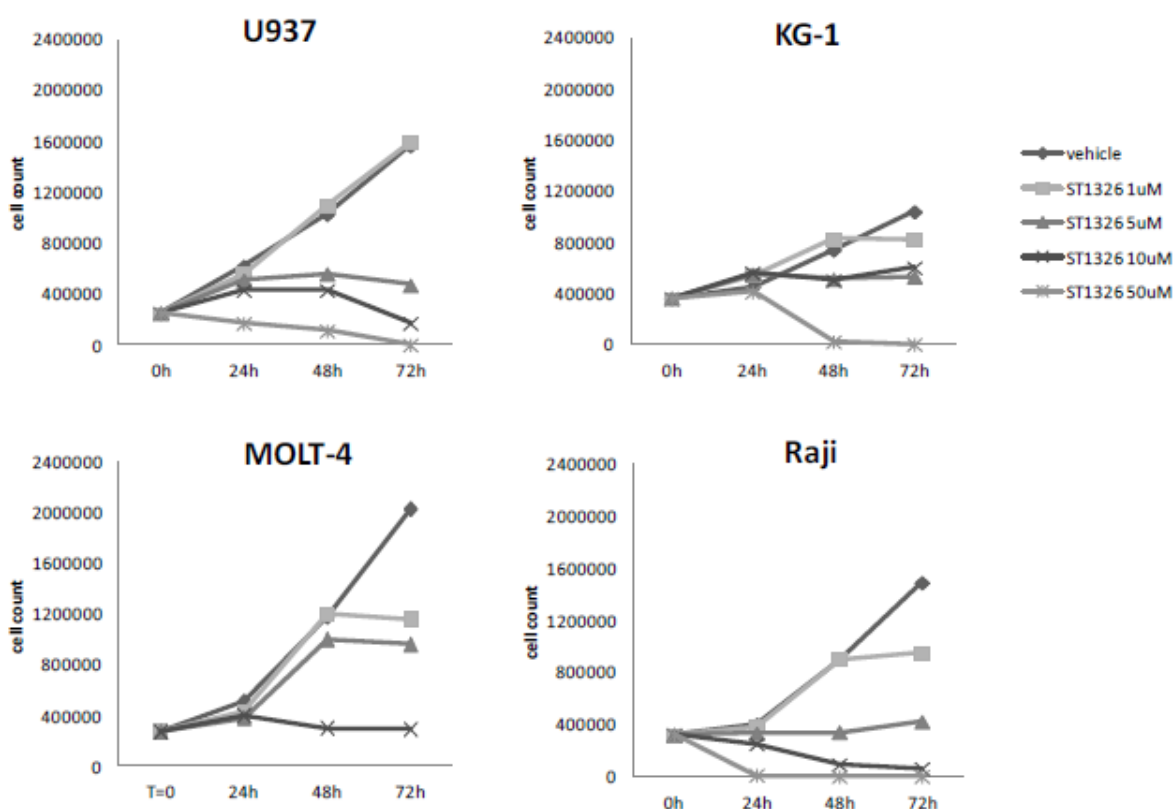
Figure 37 - ST1326 induces cell growth arrest in dose and time-dependent fashion in hematopoietic cell lines with different ontogenesis. Dose-response growth curves: several hematopoietic cell lines were exposed to increasing concentrations of ST1326 for the indicated periods of time. Cell counts and viability were then assessed by Trypan blue exclusion counting.
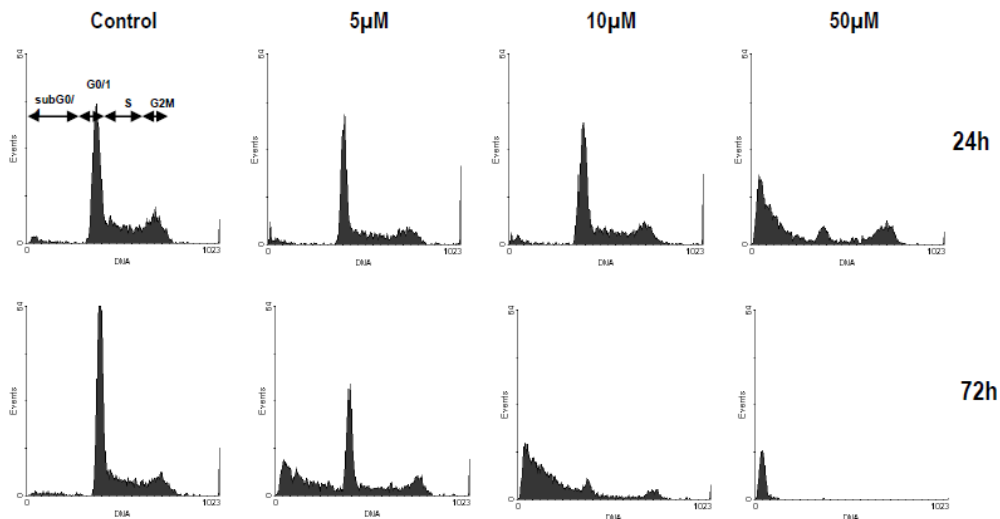
Figure 38 - ST1326 induces a dose and time-dependent cell cycle modulation and apoptosis induction in U937 cells. U937 cells were exposed to the indicated concentrations of ST1326 or vehicle control for up to 72 h. Distribution of cells in the different phases of cell cycle and in the sub-G0/1 peak was assessed by Acridine orange DNA/RNA staining as described in methods section. DNA histograms show results of one representative experiment performed on U937 cells.
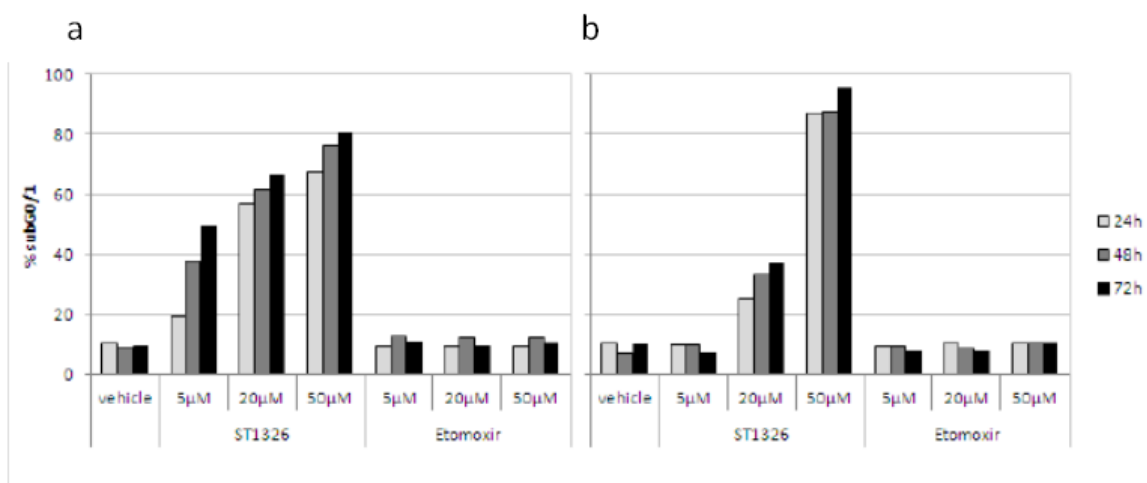
Figure 39 - ST1326 induces a higher apoptotic effect compared to Etomoxir in hematopoietic cell lines. HL60 (a) and MOLT4 (b) were treated with ST1326 (1 to 50µM) or with Etomoxir (1 to 50µM) for up to 72 hours. At the indicated time points, cells were harvested, counted by Trypan blue dye-exclusion under a light microscope, and distribution of cells in the different phases of cell cycle was assessed as described in methods. Percentages of cells in subG0/1 peak are shown in the figure.
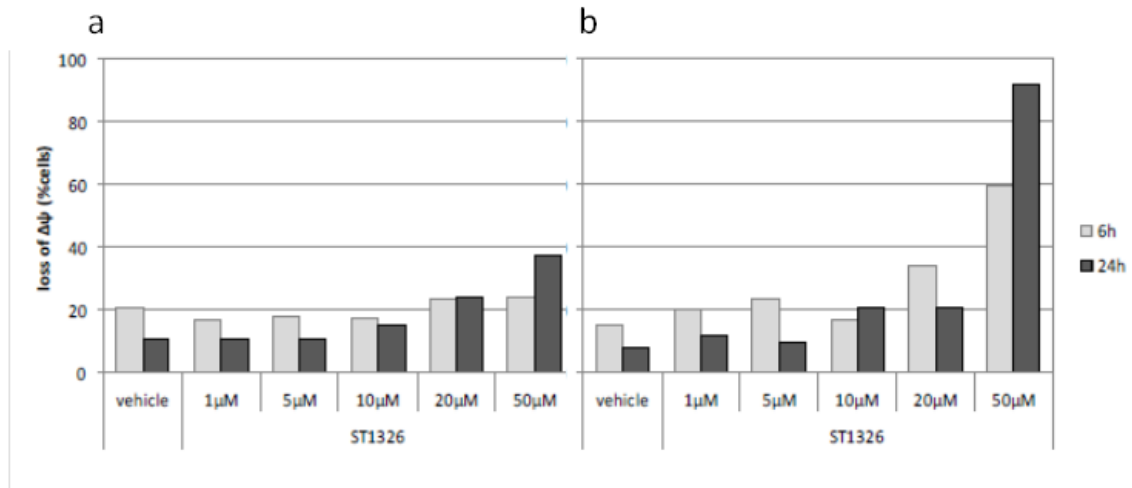
Figure 40 - ST1326 induces ΔΨm decrease in hematopoietic cell lines. HL-60/MX2 (a) and MOLT4 (b) were grown in the absence or presence of scalar concentrations of ST1326 for up to 72 h. Loss of ΔΨm was determined at the indicated time points by monitoring CMXRos uptake.

The experiments of wash-out of the drug carried on the U937 cell line have shown that ST1326, when administered for 24 hours at a concentration of 50µM, exerted an irreversible cytotoxic activity (Fig. 41a). In contrast ST1326 displayed reversible drug effects on cell cycle and apoptosis at the doses of 5, 10 and 20µM. In fact, cell cycles rate was re-established after 10 days from the drug removal at 20µM. Prolonged exposure (72 hours) of the ST1326 concentration exerted an irreversible cytotoxic activity also at 20µM (Fig. 41b).
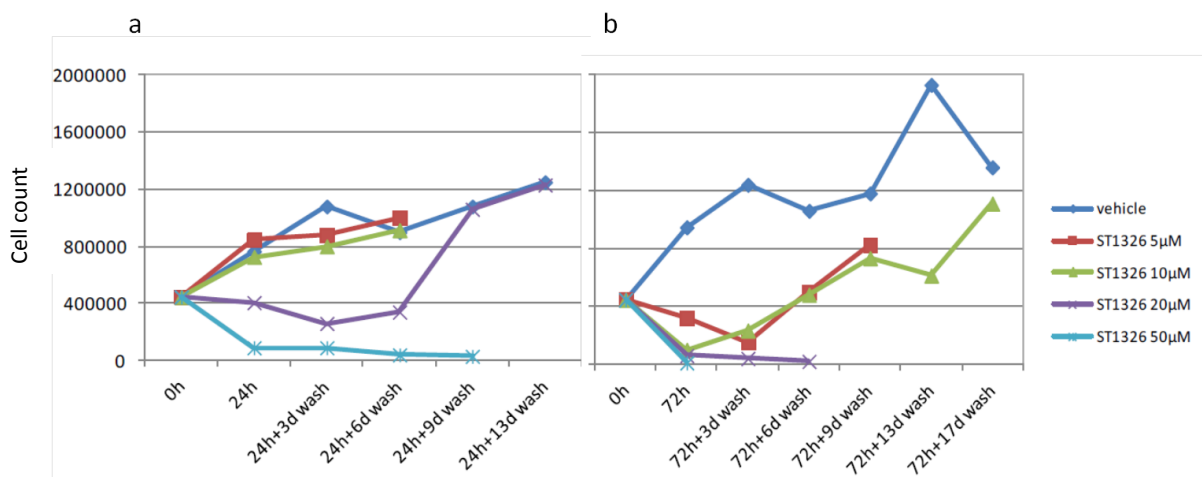
Figure 41 - ST1326 exerts an irreversible cytotoxic effect at the higher concentrations in U937 cell. U937 cells were exposed to the indicated concentrations of ST1326. The compound was removed after (a) 24 h and (b) 72h, the U937 cells were then re-suspended in fresh medium. Cell counts and viability were then assessed at the times indicated by Trypan blue exclusion counting.

The results obtained from the analysis of AML samples indicated that ST1326 exerts antiproliferative and cytotoxic activity in a dose- and time-dependent fashion in all (12/12) samples studied (Figure 42a). Induction of apoptosis was associated with a significant reduction of cell counts (Figure 42b). ST1326 induced a significant increase in apoptosis also in samples from two relapsed AML: Annexin V+ cells increased from 21.18%±7.75 to 25.23%±6.53 (p=n.s.), 30.20%±3.27 (p=n.s.), 73.09%±4.11 (p=0.00018) in the presence of 10, 20, 50μM ST1326, respectively.

A time and dose-dependent increase in the levels of apoptosis was observed in 5/5 samples from acute lymphoblastic leukemia (B-ALL) (Figure 42c) and in 6/6 samples from chronic lymphoblastic leukemia (CLL) (Figure 42e) analyzed after exposure to ST1326. As already observed for AML samples, the apoptosis increase was associated with a significant (at 50μM) cell count reduction (Figure 42d, f). The sample obtained from a relapsed B-ALL patient was equally sensitive to ST1326 cytotoxic activity.

#	Diagnosis	Sex	Age	% leukemia cells
1	AML-M4	M	66	90
2	AML-M0	M	77	80
3	AML-M2	M	39	79
4	AML-M4	F	29	70
5	AML-M5a	M	55	88
6	AML-M5a	F	50	86
7	relapsed AML-M5a	F	32	80
8	AML-M1	F	53	80
9	AML-M4	M	39	>70
10	AML-M1	M	32	93
11	relapsed AML-M2	M	51	>70
12	AML-M5b	F	41	90
13	B common ALL	F	3	89
14	B ALL	F	8	99
15	B ALL	M	54	100
16	B ALL	F	13	97
17	relapsed B ALL	M	70	65
18	B ALL	F	64	80
19	CLL 0	F	70	73
20	CLL 0	M	49	66,7
21	CLL 0	M	45	75
22	CLL 0	M	55	80,9
23	CLL 0	M	55	67,2
24	CLL 0	M	83	73,2

Table 4 - Patient population
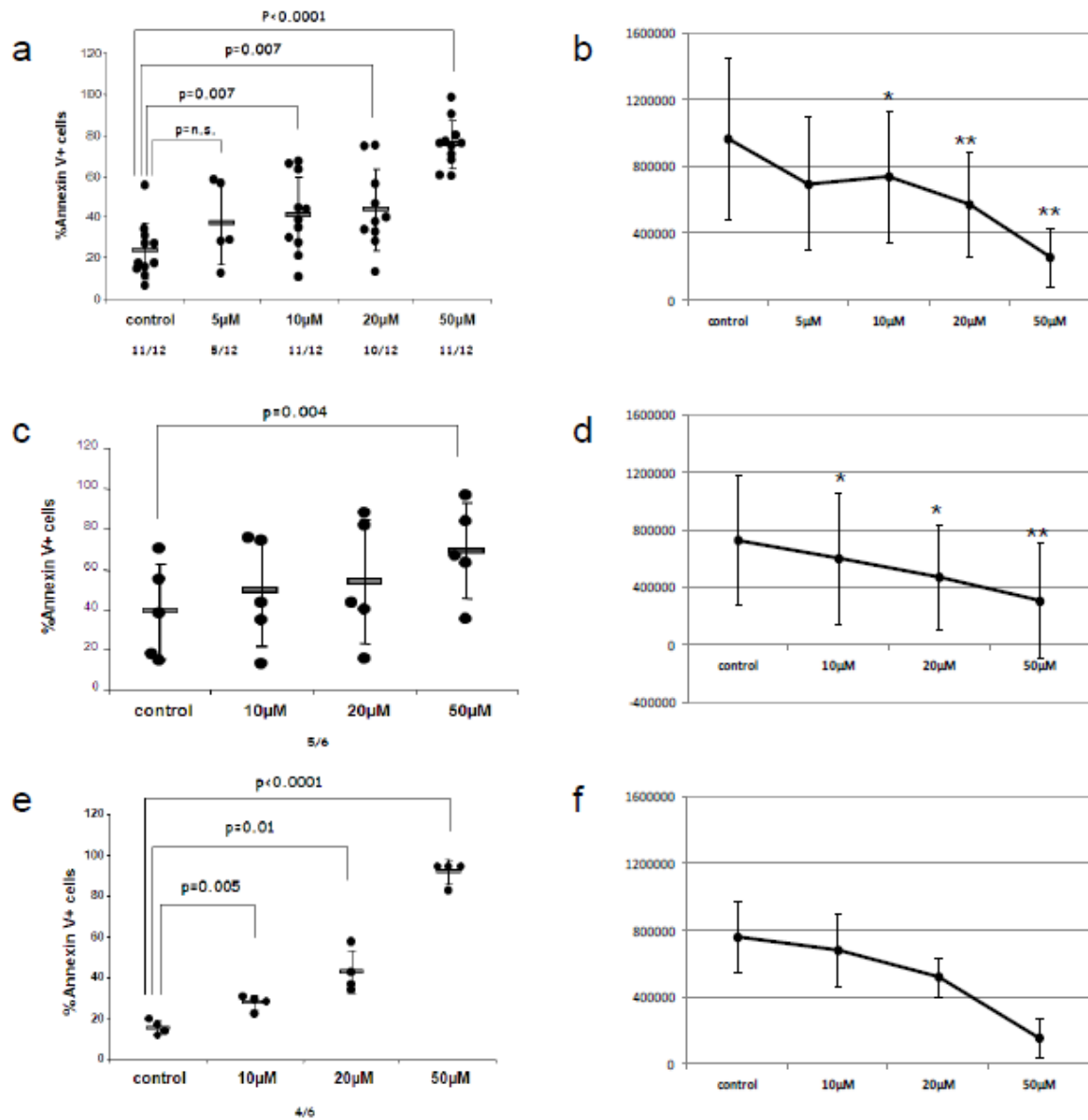
Figure 42 - ST1326 induces a dose and time-dependent apoptosis in primary cells from hematopoietic disease. AML (a/b) ALL (c/d) and CLL (e/f) primary cells were exposed to the indicated concentrations of ST1326. (a, c, e) Apoptosis induction was assessed by Annexin V/PI staining (see "Material and Methods" section). Percentage of Annexin V-positive cells and Annexin V-positive/PI cells are shown in the figure. (b, d, f) Cell counts and viability were assessed at the times indicated by Trypan blue exclusion counting.

With the aim of analyze the activity of ST1326 on normal hematopoietic cells, NPBLs obtained from healthy donors have been studied in liquid culture +/- ST1326 in the absence and in the presence of a proliferative stimulus (PHA). The results showed that ST1326 induced an increase in the levels of apoptosis only after stimulation with PHA at a dose of 50µM after 72 hours of culture (Fig. 43b). No cytotoxic effects were detected on resting NPBLs (Fig. 43a). Moreover, we tested the effects of ST1326 exposure on PBSCs from a normal donor demonstrating the absence of significant changes in the viability of CD34+ cells, as reported in figure 44.
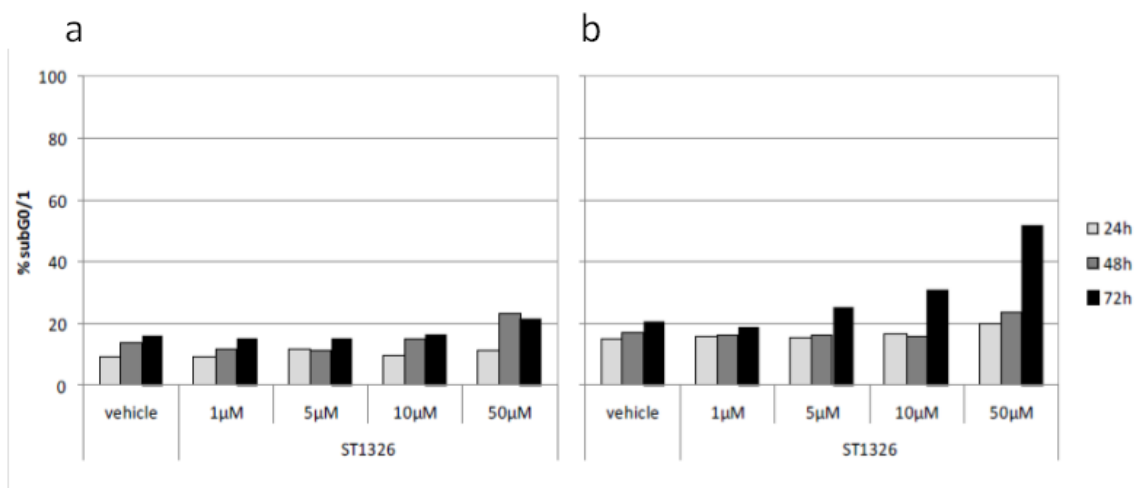
Figure 43 - ST1326 induces apoptotic effect only at the highest concentration in PHA-stimulated normal PBL. Normal and activated NPBLs were incubated with ST1326 for up to 72 hours. Percentages of cells in subG0/1 peak, assessed as described in methods section, are shown in the figure.
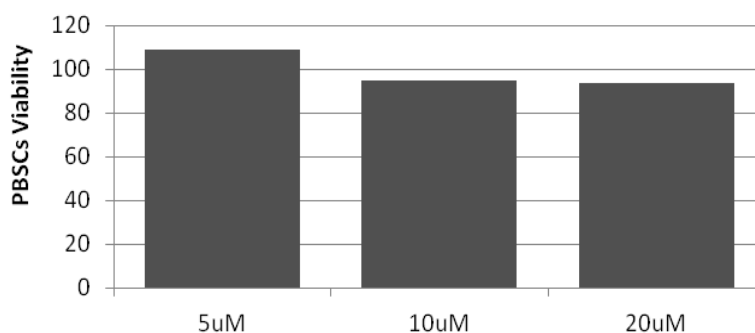
Figure 44 - ST1326 has no effect on PBSCs. Normal PBSCs were incubated with ST1326 for up to 72 hours. Percentages of viable CD34+ cells are shown in the figure, assuming the viability of untreated CD34+ as 100%.

So far, lipid metabolism in cancer cells has been investigated mainly from the anabolic perspective, being Fatty Acid Synthase (FASN) overexpressed in many cancers [182]. Conversely, the importance of FAO in cancer cell bioenergetic balance has been poorly established. Our results agree with the previously data reported by Samudio and colleagues [105], reinforcing the idea that β-oxidation represents a crucial metabolic pathway for leukemic cells, not only for

energy production but also for controlling cell growth, survival and chemoresistance. Moreover, these observations have been strengthened by following works on other haematological malignancies, such as MM [107], Diffuse Large B Cell Lymphoma [183] and CLL [184]. In particular, Tung S et al. [184] suggested that FAO plays a role in resistance of CLL to glucocorticoids and that the co-administration of glucocorticoids and FAO inhibitor may overcome this resistance.

Taken together, these findings emphasize the role (i) of FAO as a potential target for leukemia treatment and (ii) of ST1326 as a promising agent able to effectively inhibit FAO on leukemia cells, leading to cell growth reduction and apoptosis.

Chapter V

Conclusions

Despite the recent advances in understanding the biology of leukemia cells, introduced by the use of next generation techniques, the arising of chemoresistance up front or, more frequently, during the course of the disease, remains "the problem" to be solved in order to improve the outcome, especially of adult and elderly patients.

In fact, the overall long term survival rate is, in adult ALL patients, less than 35-45% [185]. Similarly, among the adult patients affected by AML, only 40% remains in remission 5 years after diagnosis [186].

The use of novel targeted therapies, so far entered as standard treatment only in few selected diseases, did not erase the later onset of resistance to these agents [187].

These observations represent the background of our studies addressed at searching for therapeutic agents, targeted toward novel specific and pathogenetic alterations of leukemia cells.

The metabolic reprogramming is currently recognized as one of the main feature that differentiates cancer cells from normal cells [7]. Thus, it represents a promising target for innovative treatment. The reason for the interest lies in the fact that metabolism is downstream of genomic and proteomic alterations. So, it constitutes a sort of a "bottleneck" in which activated oncogenes and aberrant signaling converge [188].

Progress has been made, with several clinical trials exploiting this metabolism targeted-approach in solid tumors (table 5). However, to our knowledge, only few of these have been recently opened in hematological malignancies: Metformin in CLL (NCT01750567), Metformin in combination with Temsirolimus in Lymphoma (NCT00659568), CPI-613 (a mitochondrial inhibitor) in combination with bendamustine in T-cell Lymphoma (NCT02168140) and CB-839 (glutaminase inhibitor) in Acute Leukemias (NCT02071927)

Our work, together with others, attempts to shed light on preclinical features of leukemia cells, by targeting the three major catabolic pathways that contributes to cellular bioenergetic status, glycolysis, glutaminolysis and FAO.

DCA is directed at the most characterized metabolic alteration of tumor cells, the aerobic glycolysis. Our results suggest that it is possible to reverse this alteration, forcing leukemia cells to adopt a metabolism that cannot be sustained by them, inducing ROS accumulation and apoptosis. Indeed, resistance showed by some cell lines suggests that at least some AML subtype

may not respond to this kind of therapy. Nevertheless, results from DCA-exposed primary blasts show high rates of apoptosis in all patients included in the study, reaffirming the potentiality of this molecule.

Compound	Target	Effect	Stage as anti-tumour therapy	Tumour types targeted	Study number(s)
2-deoxyglucose	Hexokinase	Inhibits glycolysis	Phase I/II	Advanced solid tumours (e.g. lung, breast, prostate and gastric)	• NCT00633087 • NCT00096707 • NCT00247403
Lonidamine	Hexokinase	Inhibits glycolysis	Phase III	Benign prostatic hyperplasia	• NCT00435448 • NCT00237536
3-bromopyruvate	Hexokinase	Inhibits glycolysis	Preclinical	N/A	N/A
TLN-232	Pyruvate kinase	Inhibits glycolysis	Phase II	Metastatic melanoma and renal cell carcinoma	NCT00735332
Dichloroacetate	PDK1	Reactivates PDH	Phase I/II	Metastatic solid tumours, glioma and GBM	• NCT00540176 • NCT00566410 • NCT00703859
Phenylacetate	Glutamine	Reduces plasma glutamine levels	Phase II	Brain tumours (e.g. glioma, astrocytoma and medulloblastoma)	• NCT00003241 • NCT00006450 • NCT00001565
Asparaginase and Pegasparaginase	Asparagine	Reduces plasma asparagine levels	Phase II/III	ALL, TCL and BCL	• NCT00400946 • NCT00004034 • NCT00165178 • Others
Arginine deiminase	Arginine	Reduces plasma arginine levels	Phase I/II	Metastatic melanoma and hepatocellular carcinoma	• NCT00450372 • NCT00029900 • NCT00056992
Acetazolamide, Indisulam and other sulfonamides	Carbonic anhydrases	pH regulation	Phase II	Solid tumours (e.g. pancreatic, lung, melanoma and metastatic breast)	• NCT00060567 • NCT00165594 • NCT00165880 • Others
Cariporide	NHE1	pH regulation	Preclinical	N/A	N/A
SB-204990	ATP-citrate lyase	Inhibits fatty acid synthesis	Preclinical	N/A	N/A
Orlistat, GSK837149A and C75	FASN	Inhibits fatty acid synthesis	Preclinical	N/A	N/A

Table 5 – Metabolic inhibitors in clinical trials, from [189]

Glutaminolysis inhibition, by AOA, directly targets an anaplerotic substrate, able to provide energy, nitrogen, and redox buffering to cancer cells. TCA cycle is the central core of cellular metabolism, acting as energy source and biosynthetic intermediate provider. A failed entrance into TCA cycle of glutamine may determine in leukemia cells an inability in keeping this process active, depriving them of such a fundamental bioenergetic hub. Our work indicates that this approach can be feasible. Moreover, other author reported [85, 171] that glutaminolysis inhibition can be particularly effective in the IDH-mutated AML patient subclass.

Targeting FAO represents an innovative approach among this strategy, being so far little investigated in both solid tumors and hematological malignancies. However, evidences are accumulating stating the importance of this process in the bioenergetic balance of cancer cells, especially in leukemias [105]. FAO process can be the factor explaining the observed link between obesity and cancer incidence [190]. Thus far, the mostly used inhibitor, etomoxir, failed to show the selectivity required to target cancer cells [179]. Moreover, it has been reported that etomoxir is active only against long-chain fatty acid oxidation [191].

Results obtained by Pacilli et al., using the novel FAO inhibitor ST1326, proved the effectiveness of this compound against both long and short-chain fatty acid oxidation [179]. Additionally, it demonstrated a selective activity against the CPT1a isoform, expressed by lymphoblastoid cells [179]. In our models the same molecule, ST1326, proved to induce a pro-apoptotic effect in AML, in the low micromolar range, and at 50μM in ALL and in CLL while etomoxir failed to show any activity.

In conclusion, these results prompt further studies aimed to characterize metabolic features associated with different hematological diseases, and to establish correlations between clinical, immunophenotype, molecular genetic, signaling features and metabolic alteration. Hence, it would be possible to select for metabolic inhibitors effective, alone or in combination with standard therapy, in the hematological malignancies characterized by the correspondent metabolic alteration.

Bibliography

1] Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett. 2008 Jul 18;266(1):6-11.

2] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70.

3] Menon S, Manning BD. Cell signalling: nutrient sensing lost in cancer. Nature. 2013 Jun 27;498(7455):444-5.

4] Dang CV. MYC on the path to cancer. Cell. 2012 Mar 30;149(1):22-35.

5] Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature. 2006 May 25;441(7092):424-30.

6] Li B, Simon MC. Molecular Pathways: Targeting MYC-induced metabolic reprogramming and oncogenic stress in cancer. Clin Cancer Res. 2013 Nov 1;19(21):5835-41.

7] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

8] Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. Curr Opin Genet Dev. 2008 Feb;18(1):54-61.

9] Obre E, Rossignol R. Emerging concepts in bioenergetics and cancer research: Metabolic flexibility, coupling, symbiosis, switch, oxidative tumors, metabolic remodeling, signaling and bioenergetic therapy. Int J Biochem Cell Biol. 2014 Dec 24. pii: S1357-2725(14)00400-2.

10] Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. Cancer Cell. 2012 Mar 20;21(3):297-308.

11] Cuperlovic-Culf M, Culf AS, Touaibia M, Lefort N. Targeting the latest hallmark of cancer: another attempt at 'magic bullet' drugs targeting cancers' metabolic phenotype. Future Oncol. 2012 Oct;8(10):1315-30.

12] Zhang Y, Yang JM. Altered energy metabolism in cancer: a unique opportunity for therapeutic intervention. Cancer Biol Ther. 2013 Feb;14(2):81-9.

13] Visentin M, Zhao R, Goldman ID. The antifolates. Hematol Oncol Clin North Am. 2012 Jun;26(3):629-48, ix.

14] Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, Goekbuget N, Schrappe M, Pui CH. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. Cancer. 2011 Jan 15;117(2):238-49.

15] Tse E, Kwong YL. How I treat NK/T-cell lymphomas. Blood. 2013 Jun 20;121(25):4997-5005.

16] Elf SE, Chen J. Targeting glucose metabolism in patients with cancer. Cancer. 2014 Mar 15;120(6):774-80.

17] Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci. 2010;35:427–433

18] Currie E, Schulze A, Zechner R, Walther TC, Farese RV Jr. Cellular fatty acid metabolism and cancer. Cell Metab. 2013 Aug 6;18(2):153-61.

19] Warburg O. On the origins of cancer cells. Science. 1956 Feb 24;123(3191):309-14.

20] Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer. 2011 Feb;11(2):85-95.

21] Ramanathan A, Wang C, Schreiber SL. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. Proc Natl Acad Sci U S A. 2005 Apr 26;102(17):5992-7.

22] Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000.

23] Medina RA, Owen GI. Glucose transporters: expression, regulation and cancer. Biol Res. 2002;35(1):9-26.

24] Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. Front Pharmacol. 2011 Aug 25;2:49.

25] Aledo JC, Pérez-Claros JA, Esteban del Valle A. Switching Between Cooperation and Competition in the Use of Extracellular Glucose. J Mol Evol. 2007 Sep;65(3):328-39.

26] Vazquez A, Liu J, Zhou Y, Oltvai ZN. Catabolic efficiency of aerobic glycolysis: The Warburg effect revisited. BMC Syst Biol. 2010 May 6;4:58.

27] Jones NP, Schulze A. Targeting cancer metabolism -- aiming at a tumour's sweet-spot. Drug Discov Today. 2012 Mar;17(5-6):232-41.

28] DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19345-50.

29] Andrews FJ, Griffiths RD. Glutamine: essential for immune nutrition in the critically ill. Br J Nutr. 2002 Jan;87 Suppl 1:S3-8.

30] Matés JM, Pérez-Gómez C, Núñez de Castro I, Asenjo M, Márquez J. Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. Int J Biochem Cell Biol. 2002 May;34(5):439-58.

31] Eng CH, Abraham RT. Glutaminolysis yields a metabolic by-product that stimulates autophagy. Autophagy. 2010 Oct;6(7):968-70.

32] Newsholme P, Procopio J, Lima MM, Pithon-Curi TC, Curi R. Glutamine and glutamate--their central role in cell metabolism and function. Cell Biochem Funct. 2003 Mar;21(1):1-9.

33] Zhang J, Ahn WS, Gameiro PA, Keibler MA, Zhang Z, Stephanopoulos G. 13C isotope-assisted methods for quantifying glutamine metabolism in cancer cells. Methods Enzymol. 2014;542:369-89.

34] Medes G, Thomas A, Weinhouse S. Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. Cancer Res. 1953 Jan;13(1):27-9.

35] Zhang F, Du G. Dysregulated lipid metabolism in cancer. World J Biol Chem. 2012 Aug 26;3(8):167-74.

36] Flavin R, Peluso S, Nguyen PL, Loda M. Fatty acid synthase as a potential therapeutic target in cancer. Future Oncol. 2010 Apr;6(4):551-62.

37] Ookhtens M, Kannan R, Lyon I, Baker N. Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. Am J Physiol. 1984 Jul;247(1 Pt 2):R146-53.

38] Scott DA, Richardson AD, Filipp FV, Knutzen CA, Chiang GG, Ronai ZA, Osterman AL, Smith JW. Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. J Biol Chem. 2011 Dec 9;286(49):42626-34.

39] Buzzai M, Bauer DE, Jones RG, Deberardinis RJ, Hatzivassiliou G, Elstrom RL, Thompson CB. The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. Oncogene. 2005 Jun 16;24(26):4165-73.

40] Nomura DK, Long JZ, Niessen S, Hoover HS, Ng SW, Cravatt BF. Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. Cell. 2010 Jan 8;140(1):49-61.

41] Pike LS, Smift AL, Croteau NJ, Ferrick DA, Wu M. Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. Biochim Biophys Acta. 2011 Jun;1807(6):726-34.

42] Cirillo A, Di Salle A, Petillo O, Melone MA, Grimaldi G, Bellotti A, Torelli G, De' Santi MS, Cantatore G, Marinelli A, Galderisi U, Peluso G. High grade glioblastoma is associated with aberrant expression of ZFP57, a protein involved in gene imprinting, and of CPT1A and CPT1C that regulate fatty acid metabolism. Cancer Biol Ther. 2014 Jun 1;15(6):735-41.

43] Liu Y. Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. Prostate Cancer Prostatic Dis. 2006;9(3):230-4.

44] Carracedo A, Weiss D, Leliaert AK, Bhasin M, de Boer VC, Laurent G, Adams AC, Sundvall M, Song SJ, Ito K, Finley LS, Egia A, Libermann T, Gerhart-Hines Z, Puigserver P, Haigis MC, Maratos-Flier E, Richardson AL, Schafer ZT, Pandolfi PP. A metabolic prosurvival role for PML in breast cancer. J Clin Invest. 2012 Sep 4;122(9):3088-100.

45] Linher-Melville K, Zantinge S, Sanli T, Gerstein H, Tsakiridis T, Singh G. Establishing a relationship between prolactin and altered fatty acid β-oxidation via carnitine palmitoyl transferase 1 in breast cancer cells. BMC Cancer. 2011 Feb 4;11:56.

46] Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, Gao S, Puigserver P, Brugge JS. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. Nature. 2009 Sep 3;461(7260):109-13. doi: 10.1038/nature08268. Epub 2009 Aug 19.

47] Bensaad K, Favaro E, Lewis CA, Peck B, Lord S, Collins JM, Pinnick KE, Wigfield S, Buffa FM, Li JL2, Zhang Q, Wakelam MJ, Karpe F, Schulze A, Harris AL. Fatty acid uptake and lipid storage induced by HIF-1α contribute to cell growth and survival after hypoxia-reoxygenation. Cell Rep. 2014 Oct 9;9(1):349-65.

48] Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res. 2004 Jun 1;64(11):3892-9.

49] Boag JM, Beesley AH, Firth MJ, Freitas JR, Ford J, Hoffmann K, Cummings AJ, de Klerk NH, Kees UR. Altered glucose metabolism in childhood pre-B acute lymphoblastic leukaemia. Leukemia. 2006 Oct;20(10):1731-7.

50] Liu T, Kishton RJ, Macintyre AN, Gerriets VA, Xiang H, Liu X, Abel ED, Rizzieri D, Locasale JW, Rathmell JC. Glucose transporter 1-mediated glucose uptake is limiting for B-cell acute lymphoblastic leukemia anabolic metabolism and resistance to apoptosis. Cell Death Dis. 2014 Oct 16;5:e1470.

51] Beesley AH, Firth MJ, Ford J, Weller RE, Freitas JR, Perera KU, Kees UR. Glucocorticoid resistance in T-lineage acute lymphoblastic leukaemia is associated with a proliferative metabolism. Br J Cancer. 2009 Jun 16;100(12):1926-36.

52] Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJ, Evans WE, Pieters R, Den Boer ML. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood. 2009 Feb 26;113(9):2014-21.

53] Buentke E, Nordström A, Lin H, Björklund AC, Laane E, Harada M, Lu L, Tegnebratt T, Stone-Elander S, Heyman M, Söderhäll S, Porwit A, Ostenson CG, Shoshan M, Tamm KP, Grandér D. Glucocorticoid-induced cell death is mediated through reduced glucose metabolism in lymphoid leukemia cells. Blood Cancer J. 2011 Jul;1(7):e31.

54] Samuels AL, Heng JY, Beesley AH, Kees UR. Bioenergetic modulation overcomes glucocorticoid resistance in T-lineage acute lymphoblastic leukaemia. Br J Haematol. 2014 Apr;165(1):57-66.

55] Kim JH, Chu SC, Gramlich JL, Pride YB, Babendreier E, Chauhan D, Salgia R, Podar K, Griffin JD, Sattler M. Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species. Blood. 2005 Feb 15;105(4):1717-23.

56] Klawitter J, Kominsky DJ, Brown JL, Klawitter J, Christians U, Leibfritz D, Melo JV, Eckhardt SG, Serkova NJ. Metabolic characteristics of imatinib resistance in chronic myeloid leukaemia cells. Br J Pharmacol. 2009 Sep;158(2):588-600. doi: 10.1111/j.1476-5381.2009.00345.x. Epub 2009 Aug 6.

57] Samudio I, Fiegl M, McQueen T, Clise-Dwyer K, Andreeff M. The warburg effect in leukemia-stroma cocultures is mediated by mitochondrial uncoupling associated with uncoupling protein 2 activation. Cancer Res. 2008 Jul 1;68(13):5198-205.

58] Herst PM, Howman RA, Neeson PJ, Berridge MV, Ritchie DS. The level of glycolytic metabolism in acute myeloid leukemia blasts at diagnosis is prognostic for clinical outcome. J Leukoc Biol. 2011 Jan;89(1):51-5.

59] Chen WL, Wang JH, Zhao AH, Xu X, Wang YH, Chen TL, Li JM, Mi JQ, Zhu YM, Liu YF, Wang YY, Jin J, Huang H, Wu DP, Li Y, Yan XJ, Yan JS, Li JY, Wang S, Huang XJ, Wang BS, Chen Z, Chen SJ, Jia W. A distinct glucose metabolism signature of acute myeloid leukemia with prognostic value. Blood. 2014 Sep 4;124(10):1645-54.

60] Wang YH, Israelsen WJ, Lee D, Yu VW, Jeanson NT, Clish CB, Cantley LC, Vander Heiden MG, Scadden DT. Cell-state-specific metabolic dependency in hematopoiesis and leukemogenesis. Cell. 2014 Sep 11;158(6):1309-23.

61] Zhang D, Li J, Wang F, Hu J, Wang S, Sun Y. 2-Deoxy-D-glucose targeting of glucose metabolism in cancer cells as a potential therapy. Cancer Lett. 2014 Dec 28;355(2):176-83.

62] Tsunekawa-Imai N, Miwa H, Shikami M, Suganuma K, Goto M, Mizuno S, Takahashi M, Mizutani M, Horio T, Komatsubara H, Gotou M, Yamamoto H, Wakabayashi M, Watarai M, Hanamura I, Imamura A, Mihara H, Nitta M. Growth of xenotransplanted leukemia cells is influenced by diet nutrients and is attenuated with 2-deoxyglucose. Leuk Res. 2013 Sep;37(9):1132-6.

63] Shanmugam M, McBrayer SK, Rosen ST. Targeting the Warburg effect in hematological malignancies: from PET to therapy. Curr Opin Oncol. 2009 Nov;21(6):531-6.

64] Estañ MC, Calviño E, de Blas E, Boyano-Adánez Mdel C, Mena ML, Gómez-Gómez M, Rial E, Aller P. 2-Deoxy-D-glucose cooperates with arsenic trioxide to induce apoptosis in leukemia cells: involvement of IGF-1R-regulated Akt/mTOR, MEK/ERK and LKB-1/AMPK signaling pathways. Biochem Pharmacol. 2012 Dec 15;84(12):1604-16. doi: 10.1016/j.bcp.2012.09.022. Epub 2012 Oct 5.

65] Coloff JL, Macintyre AN, Nichols AG, Liu T, Gallo CA, Plas DR, Rathmell JC. Akt-dependent glucose metabolism promotes Mcl-1 synthesis to maintain cell survival and resistance to Bcl-2 inhibition. Cancer Res. 2011 Aug 1;71(15):5204-13.

66] Yamaguchi R, Janssen E, Perkins G, Ellisman M, Kitada S, Reed JC. Efficient elimination of cancer cells by deoxyglucose-ABT-263/737 combination therapy. PLoS One. 2011;6(9):e24102.

67] DeSalvo J, Kuznetsov JN, Du J, Leclerc GM, Leclerc GJ, Lampidis TJ, Barredo JC. Inhibition of Akt potentiates 2-DG-induced apoptosis via downregulation of UPR in acute lymphoblastic leukemia. Mol Cancer Res. 2012 Jul;10(7):969-78.

68] Muñoz-Pinedo C, Ruiz-Ruiz C, Ruiz de Almodóvar C, Palacios C, López-Rivas A. Inhibition of glucose metabolism sensitizes tumor cells to death receptor-triggered apoptosis through enhancement of death-inducing signaling complex formation and apical procaspase-8 processing. J Biol Chem. 2003 Apr 11;278(15):12759-68.

69] Pradelli LA, Bénéteau M, Chauvin C, Jacquin MA, Marchetti S, Muñoz-Pinedo C, Auberger P, Pende M, Ricci JE. Glycolysis inhibition sensitizes tumor cells to death receptors-induced apoptosis by AMP kinase activation leading to Mcl-1 block in translation. Oncogene. 2010 Mar 18;29(11):1641-52.

70] Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res. 2005 Jan 15;65(2):613-21.

71] Calviño E, Estañ MC, Sánchez-Martín C, Brea R, de Blas E, Boyano-Adánez Mdel C, Rial E, Aller P. Regulation of death induction and chemosensitizing action of 3-bromopyruvate in myeloid leukemia cells: energy depletion, oxidative stress, and protein kinase activity modulation. J Pharmacol Exp Ther. 2014 Feb;348(2):324-35.

72] Xu RH, Pelicano H, Zhang H, Giles FJ, Keating MJ, Huang P. Synergistic effect of targeting mTOR by rapamycin and depleting ATP by inhibition of glycolysis in lymphoma and leukemia cells. Leukemia. 2005 Dec;19(12):2153-8.

73] Akers LJ, Fang W, Levy AG, Franklin AR, Huang P, Zweidler-McKay PA. Targeting glycolysis in leukemia: a novel inhibitor 3-BrOP in combination with rapamycin. Leuk Res. 2011 Jun;35(6):814-20.

74] Raivio KO, Andersson LC. Glutamine requirements for purine metabolism in leukemic lymphoblasts. Leuk Res. 1982;6(1):111-5.

75] Dass PD, Murdoch FE, Wu MC. Glutamine promotes colony formation in bone marrow and HL-60 cells; accelerates myeloid differentiation in induced HL-60 cells. In Vitro. 1984 Nov;20(11):869-75.

76] Kitoh T, Kubota M, Takimoto T, Hashimoto H, Shimizu T, Sano H, Akiyama Y, Mikawa H. Metabolic basis for differential glutamine requirements of human leukemia cell lines. J Cell Physiol. 1990 Apr;143(1):150-3.

77] Petronini PG, Urbani S, Alfieri R, Borghetti AF, Guidotti GG. Cell susceptibility to apoptosis by glutamine deprivation and rescue: survival and apoptotic death in cultured lymphoma-leukemia cell lines. J Cell Physiol. 1996 Oct;169(1):175-85.

78] Goto M, Miwa H, Shikami M, Tsunekawa-Imai N, Suganuma K, Mizuno S, Takahashi M, Mizutani M, Hanamura I, Nitta M. Importance of glutamine metabolism in leukemia cells by energy production through TCA cycle and by redox homeostasis. Cancer Invest. 2014 Jul;32(6):241-7.

79] Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, Fulton LA, Locke DP, Magrini VJ, Abbott RM, Vickery TL, Reed JS, Robinson JS, Wylie T, Smith SM, Carmichael L, Eldred JM, Harris CC, Walker J, Peck JB, Du F, Dukes AF, Sanderson GE, Brummett AM, Clark E, McMichael JF, Meyer RJ, Schindler JK, Pohl CS, Wallis JW, Shi X, Lin L, Schmidt H, Tang Y, Haipek C, Wiechert ME, Ivy JV, Kalicki J, Elliott G, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson MA, Baty J, Heath S, Shannon WD, Nagarajan R, Link DC, Walter MJ, Graubert TA, DiPersio JF, Wilson RK, Ley TJ. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009 Sep 10;361(11):1058-66.

80] Dang L, Jin S, Su SM. IDH mutations in glioma and acute myeloid leukemia. Trends Mol Med. 2010 Sep;16(9):387-97.

81] Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2009 Dec 10;462(7274):739-44.

82] Lyons SD1, Sant ME, Christopherson RI. Cytotoxic mechanisms of glutamine antagonists in mouse L1210 leukemia. J Biol Chem. 1990 Jul 5;265(19):11377-81.

83] Robinson MM, McBryant SJ, Tsukamoto T, Rojas C, Ferraris DV, Hamilton SK, Hansen JC, Curthoys NP. Novel mechanism of inhibition of rat kidney-type glutaminase by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES). Biochem J. 2007 Sep 15;406(3):407-14.

84] Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, Tsukamoto T, Rojas CJ, Slusher BS, Zhang H, Zimmerman LJ, Liebler DC, Slebos RJ, Lorkiewicz PK, Higashi RM, Fan TW, Dang CV. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab. 2012 Jan 4;15(1):110-21.

85] Emadi A, Jun SA, Tsukamoto T, Fathi AT, Minden MD, Dang CV. Inhibition of glutaminase selectively suppresses the growth of primary acute myeloid leukemia cells with IDH mutations. Exp Hematol. 2014 Apr;42(4):247-51.

86] Horowitz B, Madras BK, Meister A, Old LJ, Boyes EA, Stockert E. Asparagine synthetase activity of mouse leukemias. Science. 1968 May 3;160(3827):533-5.

87] Oetgen HF, Old LJ, Boyse EA, Campbel HA, Philps FS, Clarkson BD, Talal L, Leper RD, Schwartz. MK, Kim JH. Inhibiton of leukemias in man by L-asparaginase. Cancer Res. 27: 2619- 2631, 1967

88] Richards NG, Kilberg MS. Asparagine synthetase chemotherapy. Annu Rev Biochem. 2006;75:629-54.

89] Duval M, Suciu S, Ferster A, Rialland X, Nelken B, et al. Comparison of Escherichia coli–asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organization for Research and Treatment of Cancer–Children's Leukemia Group phase 3 trial. Blood. 2006 99(8): 2734–2739

90] Chan WK, Lorenzi PL, Anishkin A, Purwaha P, Rogers DM, Sukharev S, Rempe SB, Weinstein JN. The glutaminase activity of L-asparaginase is not required for anticancer activity against ASNS-negative cells. Blood. 2014 Jun 5;123(23):3596-606.

91] Mahajan RV, Kumar V, Rajendran V, Saran S, Ghosh PC, Saxena RK. Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from Bacillus licheniformis: in vitro evaluation of anti-cancerous properties. PLoS One. 2014 Jun 6;9(6):e99037.

92] Offman MN, Krol M, Patel N, Krishnan S, Liu J, Saha V, Bates PA. Rational engineering of L-asparaginase reveals importance of dual activity for cancer cell toxicity. Blood. 2011 Feb 3;117(5):1614-21.

93] Avramis VI. Is glutamine depletion needed in ALL disease? Blood. 2014 Jun 5;123(23):3532-3.

94] Purwaha P, Lorenzi PL, Silva LP, Hawke DH, Weinstein JN. Targeted metabolomic analysis of amino acid response to L-asparaginase in adherent cells. Metabolomics. 2014;10(5):909-919.

95] Ehsanipour EA, Sheng X, Behan JW, Wang X, Butturini A, Avramis VI, Mittelman SD. Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. Cancer Res. 2013 May 15;73(10):2998-3006.

96] Willems L, Jacque N, Jacquel A, Neveux N, Maciel TT, Lambert M, Schmitt A, Poulain L, Green AS, Uzunov M, Kosmider O, Radford-Weiss I, Moura IC, Auberger P, Ifrah N, Bardet V, Chapuis N, Lacombe C, Mayeux P, Tamburini J, Bouscary D. Inhibiting glutamine uptake represents an attractive new strategy for treating acute myeloid leukemia. Blood. 2013 Nov 14;122(20):3521-32.

97] Pizer ES, Wood FD, Pasternack GR, Kuhajda FP. Fatty acid synthase (FAS): a target for cytotoxic antimetabolites in HL60 promyelocytic leukemia cells. Cancer Res. 1996 Feb 15;56(4):745-51.

98] Pardee T, DeFord-Watts LM, Peronto E, Levitan DA, Hurd DD, Kridel S. Altered lipid and mitochondrial metabolism are viable targets in acute leukemia [abstract]. Blood. 2011;118(21):1546.

99] Burns CP, Welshman IR, Spector AA. Utilization of long-chain free fatty acids and glucose by human leukemic blast cells. Cancer Res. 1977 May;37(5):1323-7.

100] Skrede S, Iversen PO. Enhanced oxygen consumption and fatty acid metabolism in rat bone marrow with acute promyelocytic leukaemia. Leuk Res. 1995 Jul;19(7):463-7.

101] Suganuma K, Miwa H, Imai N, Shikami M, Gotou M, Goto M, Mizuno S, Takahashi M, Yamamoto H, Hiramatsu A, Wakabayashi M, Watarai M, Hanamura I, Imamura A, Mihara H, Nitta M. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation. Leuk Lymphoma. 2010 Nov;51(11):2112-9.

102] Miwa H, Shikami M, Goto M, Mizuno S, Takahashi M, Tsunekawa-Imai N, Ishikawa T, Mizutani M, Horio T, Gotou M, Yamamoto H, Wakabayashi M, Watarai M, Hanamura I, Imamura A, Mihara H, Nitta M. Leukemia cells demonstrate a different metabolic perturbation provoked by 2-deoxyglucose. Oncol Rep. 2013 May;29(5):2053-7.

103] Carracedo A, Cantley LC, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. Nat Rev Cancer. 2013 Apr;13(4):227-32.

104] Kaikaus RM, Sui Z, Lysenko N, Wu NY, Ortiz de Montellano PR, Ockner RK, Bass NM. Regulation of pathways of extramitochondrial fatty acid oxidation and liver fatty acid-binding protein by long-chain monocarboxylic fatty acids in hepatocytes. Effect of inhibition of carnitine palmitoyltransferase I. J Biol Chem. 1993 Dec 25;268(36):26866-71.

105] Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, Kaluarachchi K, Bornmann W, Duvvuri S, Taegtmeyer H, Andreeff M. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. J Clin Invest. 2010 Jan;120(1):142-56.

106] Estañ MC, Calviño E, Calvo S, Guillén-Guío B, Boyano-Adánez Mdel C, de Blas E, Rial E, Aller P. Apoptotic efficacy of etomoxir in human acute myeloid leukemia cells. Cooperation with arsenic trioxide and glycolytic inhibitors, and regulation by oxidative stress and protein kinase activities. PLoS One. 2014 Dec 15;9(12):e115250.

107] Tirado-Vélez JM, Joumady I, Sáez-Benito A, Cózar-Castellano I, Perdomo G. Inhibition of fatty acid metabolism reduces human myeloma cells proliferation. PLoS One. 2012;7(9):e46484.

108] Lengle EE, Gustin NC, Gonzalez F, Menahan LA, Kemp RG. Energy metabolism in thymic lymphocytes of normal and leukemia AKR mice. Cancer Res. 1978 Apr;38(4):1113-9.

109] Miccheli A, Tomassini A, Puccetti C, Valerio M, Peluso G, Tuccillo F, Calvani M, Manetti C, Conti F. Metabolic profiling by 13C-NMR spectroscopy: [1,2-13C2]glucose reveals a heterogeneous metabolism in human leukemia T cells. Biochimie. 2006 May;88(5):437-48.

110] Ferretti A, Chen LL, Di Vito M, Barca S, Tombesi M, Cianfriglia M, Bozzi A, Strom R, Podo F. Pentose phosphate pathway alterations in multi-drug resistant leukemic T-cells: 31P NMR and enzymatic studies. Anticancer Res. 1993 Jul-Aug;13(4):867-72.

111] McBrayer SK, Yarrington M, Qian J, Feng G, Shanmugam M, Gandhi V, Krett NL, Rosen ST. Integrative gene expression profiling reveals G6PD-mediated resistance to RNA-directed nucleoside analogues in B-cell neoplasms. PLoS One. 2012;7(7):e41455.

112] Yamamoto T, Takano N, Ishiwata K, Ohmura M, Nagahata Y, Matsuura T, Kamata A, Sakamoto K, Nakanishi T, Kubo A, Hishiki T, Suematsu M. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nat Commun. 2014 Mar 17;5:3480.

113] Shan C, Elf S, Ji Q, Kang HB, Zhou L, Hitosugi T, Jin L, Lin R, Zhang L, Seo JH, Xie J, Tucker M, Gu TL, Sudderth J, Jiang L, DeBerardinis RJ, Wu S, Li Y, Mao H, Chen PR, Wang D, Chen GZ, Lonial S, Arellano ML, Khoury HJ, Khuri FR, Lee BH, Brat DJ, Ye K, Boggon TJ, He C, Kang S, Fan J, Chen J. Lysine acetylation activates 6-phosphogluconate dehydrogenase to promote tumor growth. Mol Cell. 2014 Aug 21;55(4):552-65.

114] Ogata S, Takeuchi M, Fujita H, Shibata K, Okumura K, Taguchi H. Apoptosis induced by nicotinamide-related compounds and quinolinic acid in HL-60 cells. Biosci Biotechnol Biochem. 2000 Feb;64(2):327-32.

115] Budihardjo II, Walker DL, Svingen PA, Buckwalter CA, Desnoyers S, Eckdahl S, Shah GM, Poirier GG, Reid JM, Ames MM, Kaufmann SH. 6-Aminonicotinamide sensitizes human tumor cell lines to cisplatin. Clin Cancer Res. 1998 Jan;4(1):117-30.

116] Berger NA, Catino DM, Vietti TJ. Synergistic antileukemic effect of 6-aminonicotinamide and 1,3-bis(2-chloroethyl)-1-nitrosourea on L1210 cells in vitro and in vivo. Cancer Res. 1982 Nov;42(11):4382-6.

117] Stathem M, Marimuthu S, O'Neal J, Rathmell JC, Chesney JA, Beverly LJ, Siskind LJ. Glucose availability and glycolytic metabolism dictate glycosphingolipid levels. J Cell Biochem. 2015 Jan;116(1):67-80

118] Flis K, Irvine D, Copland M, Bhatia R, Skorski T. Chronic myeloid leukemia stem cells display alterations in expression of genes involved in oxidative phosphorylation. Leuk Lymphoma. 2012 Dec;53(12):2474-8.

119] Nieborowska-Skorska M, Kopinski PK, Ray R, Hoser G, Ngaba D, Flis S, Cramer K, Reddy MM, Koptyra M, Penserga T, Glodkowska-Mrowka E, Bolton E, Holyoake TL, Eaves CJ, Cerny-Reiterer S, Valent P, Hochhaus A, Hughes TP, van der Kuip H, Sattler M, Wiktor-Jedrzejczak W, Richardson C, Dorrance A, Stoklosa T, Williams DA, Skorski T. Rac2-MRC-cIII-generated ROS cause genomic instability in chronic myeloid leukemia stem cells and primitive progenitors. Blood. 2012 May 3;119(18):4253-63.

120] Damm F, Bunke T, Thol F, Markus B, Wagner K, Göhring G, Schlegelberger B, Heil G, Reuter CW, Püllmann K, Schlenk RF, Döhner K, Heuser M, Krauter J, Döhner H, Ganser A, Morgan MA. Prognostic implications and molecular associations of NADH dehydrogenase subunit 4 (ND4) mutations in acute myeloid leukemia. Leukemia. 2012 Feb;26(2):289-95.

121] Piccoli C, Ripoli M, Scrima R, Stanziale P, Di Ianni M, Moretti L, Biscottini B, Carella M, Boffoli D, Tabilio A, Capitanio N. MtDNA mutation associated with mitochondrial dysfunction in megakaryoblastic leukaemic cells. Leukemia. 2008 Oct;22(10):1938-41.

122] Silkjaer T, Nyvold CG, Juhl-Christensen C, Hokland P, Nørgaard JM. Mitochondrial cytochrome c oxidase subunit II variations predict adverse prognosis in cytogenetically normal acute myeloid leukaemia. Eur J Haematol. 2013 Oct;91(4):295-303.

123] Jitschin R, Hofmann AD, Bruns H, Giessl A, Bricks J, Berger J, Saul D, Eckart MJ, Mackensen A, Mougiakakos D. Mitochondrial metabolism contributes to oxidative stress and reveals therapeutic targets in chronic lymphocytic leukemia. Blood. 2014 Apr 24;123(17):2663-72.

124] Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. BMJ. 2005 Jun 4;330(7503):1304-5.

125] Rosilio C, Ben-Sahra I, Bost F, Peyron JF. Metformin: a metabolic disruptor and anti-diabetic drug to target human leukemia. Cancer Lett. 2014 May 1;346(2):188-96.

126] Vakana E, Altman JK, Glaser H, Donato NJ, Platanias LC. Antileukemic effects of AMPK activators on BCR-ABL-expressing cells. Blood. 2011 Dec 8;118(24):6399-402.

127] Green AS, Chapuis N, Maciel TT, Willems L, Lambert M, Arnoult C, Boyer O, Bardet V, Park S, Foretz M, Viollet B, Ifrah N, Dreyfus F, Hermine O, Moura IC, Lacombe C, Mayeux P, Bouscary D, Tamburini J. The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. Blood. 2010 Nov 18;116(20):4262-73.

128] Scotland S, Saland E, Skuli N, de Toni F, Boutzen H, Micklow E, Sénégas I, Peyraud R, Peyriga L, Théodoro F, Dumon E, Martineau Y, Danet-Desnoyers G, Bono F, Rocher C, Levade T, Manenti S, Junot C, Portais JC, Alet N, Récher C, Selak MA, Carroll M, Sarry JE. Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells. Leukemia. 2013 Nov;27(11):2129-38.

129] Leclerc GM, Leclerc GJ, Kuznetsov JN, DeSalvo J, Barredo JC. Metformin induces apoptosis through AMPK-dependent inhibition of UPR signaling in ALL lymphoblasts. PLoS One. 2013 Aug 23;8(8):e74420.

130] Pan J, Chen C, Jin Y, Fuentes-Mattei E, Velazquez-Tores G, Benito JM, Konopleva M, Andreeff M, Lee MH, Yeung SC. Differential impact of structurally different anti-diabetic drugs on proliferation and chemosensitivity of acute lymphoblastic leukemia cells. Cell Cycle. 2012 Jun 15;11(12):2314-26.

131] Lutz NW, Sweedler JV, Wevers RA. Methodologies for Metabolomics: Experimental Strategies and Techniques. Cambridge University Press 2013.

132] Milne SB, Mathews TP, Myers DS, Ivanova PT, Brown HA. Sum of the parts: mass spectrometry-based metabolomics. Biochemistry. 2013 Jun 4;52(22):3829-40.

133] Halama A. Metabolomics in cell culture-A strategy to study crucial metabolic pathways in cancer development and the response to treatment. Arch Biochem Biophys. 2014 Dec 15;564C:100-109.

134] MacIntyre DA, Jiménez B, Lewintre EJ, Martín CR, Schäfer H, Ballesteros CG, Mayans JR, Spraul M, García-Conde J, Pineda-Lucena A. Serum metabolome analysis by 1H-NMR reveals differences between chronic lymphocytic leukaemia molecular subgroups. Leukemia. 2010 Apr;24(4):788-97.

135] Tiziani S, Kang Y, Harjanto R, Axelrod J, Piermarocchi C, Roberts W, Paternostro G. Metabolomics of the tumor microenvironment in pediatric acute lymphoblastic leukemia. PLoS One. 2013 Dec 13;8(12):e82859.

136] Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am J Physiol Cell Physiol. 2007 Jan;292(1):C125-36.

137] Wang D, Green MF, McDonnell E, Hirschey MD. Oxygen flux analysis to understand the biological function of sirtuins. Methods Mol Biol. 2013;1077:241-58.

138] Sharma A, Janocha AJ, Hill BT, Smith MR, Erzurum SC, Almasan A. Targeting mTORC1-mediated metabolic addiction overcomes fludarabine resistance in malignant B cells. Mol Cancer Res. 2014 Sep;12(9):1205-15.

139] Jhas B, Sriskanthadevan S, Skrtic M, Sukhai MA, Voisin V, Jitkova Y, Gronda M, Hurren R, Laister RC, Bader GD, Minden MD, Schimmer AD. Metabolic adaptation to chronic inhibition of mitochondrial protein synthesis in acute myeloid leukemia cells. PLoS One. 2013;8(3):e58367.

140] Pardee TS, Lee K, Luddy J, Maturo C, Rodriguez R, Isom S, Miller LD, Stadelman KM, Levitan D, Hurd D, Ellis LR, Harrelson R, Manuel M, Dralle S, Lyerly S, Powell BL A phase I study of the first-in-class antimitochondrial metabolism agent, CPI-613, in patients with advanced hematologic malignancies. Clin Cancer Res. 2014 Oct 15;20(20):5255-64.

141] Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, Ashton JM, Pei S, Grose V, O'Dwyer KM, Liesveld JL, Brookes PS, Becker MW, Jordan CT. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell. 2013 Mar 7;12(3):329-41.

142] Adekola KU, Dalva Aydemir S, Ma S, Zhou Z, Rosen ST, Shanmugam M. Investigating and targeting chronic lymphocytic leukemia metabolism with the human immunodeficiency virus protease inhibitor ritonavir and metformin. Leuk Lymphoma. 2014 Aug 4:1-10.

143] Kato M, Li J, Chuang JL, Chuang DT. Distinct structural mechanisms for inhibition of pyruvate dehydrogenase kinase isoforms by AZD7545, dichloroacetate, and radicicol. Structure. 2007 Aug;15(8):992-1004.

144] Whitehouse S, Randle PJ. Activation of pyruvate dehydrogenase in perfused rat heart by dichloroacetate (Short Communication). Biochem J. 1973 Jun;134(2):651-3.

145] Miquel E, Cassina A, Martínez-Palma L, Bolatto C, Trías E, Gandelman M, Radi R, Barbeito L, Cassina P. Modulation of astrocytic mitochondrial function by dichloroacetate improves survival and motor performance in inherited amyotrophic lateral sclerosis. PLoS One. 2012;7(4):e34776.

146] Stacpoole PW, Kerr DS, Barnes C, Bunch ST, Carney PR, Fennell EM, Felitsyn NM, Gilmore RL, Greer M, Henderson GN, Hutson AD, Neiberger RE, O'Brien RG, Perkins LA, Quisling RG, Shroads AL, Shuster JJ, Silverstein JH, Theriaque DW, Valenstein E. Controlled clinical trial of dichloroacetate for treatment of congenital lactic acidosis in children. Pediatrics. 2006 May;117(5):1519-31.

147] Kankotia S, Stacpoole PW. Dichloroacetate and cancer: New home for an orphan drug? Biochim Biophys Acta. 2014 Dec;1846(2):617-629.

148] Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Bonnet S, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B, Michelakis ED. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell. 2007 Jan;11(1):37-51.

149] Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, Maguire C, Gammer TL, Mackey JR, Fulton D, Abdulkarim B, McMurtry MS, Petruk KC. Metabolic modulation of glioblastoma with dichloroacetate. Sci Transl Med. 2010 May 12;2(31):31ra34.

150] Sun RC, Fadia M, Dahlstrom JE, Parish CR, Board PG, Blackburn AC. Reversal of the glycolytic phenotype by dichloroacetate inhibits metastatic breast cancer cell growth in vitro and in vivo. Breast Cancer Res Treat. 2010 Feb;120(1):253-60.

151] Wong JY, Huggins GS, Debidda M, Munshi NC, De Vivo I. Dichloroacetate induces apoptosis in endometrial cancer cells. Gynecol Oncol. 2008 Jun;109(3):394-402.

152] Madhok BM, Yeluri S, Perry SL, Hughes TA, Jayne DG. Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. Br J Cancer. 2010 Jun 8;102(12):1746-52.

153] Saed GM, Fletcher NM, Jiang ZL, Abu-Soud HM, Diamond MP. Dichloroacetate induces apoptosis of epithelial ovarian cancer cells through a mechanism involving modulation of oxidative stress. Reprod Sci. 2011 Dec;18(12):1253-61.

154] Xuan Y, Hur H, Ham IH, Yun J, Lee JY, Shim W, Kim YB, Lee G, Han SU, Cho YK. Dichloroacetate attenuates hypoxia-induced resistance to 5-fluorouracil in gastric cancer through the regulation of glucose metabolism. Exp Cell Res. 2014 Feb 15;321(2):219-30.

155] Zheng MF, Shen SY, Huang WD. DCA increases the antitumor effects of capecitabine in a mouse B16 melanoma allograft and a human non-small cell lung cancer A549 xenograft. Cancer Chemother Pharmacol. 2013 Nov;72(5):1031-41.

156] Kumar A, Kant S, Singh SM. Antitumor and chemosensitizing action of dichloroacetate implicates modulation of tumor microenvironment: a role of reorganized glucose metabolism, cell survival regulation and macrophage differentiation. Toxicol Appl Pharmacol. 2013 Nov 15;273(1):196-208.

157] Haugrud AB, Zhuang Y, Coppock JD, Miskimins WK. Dichloroacetate enhances apoptotic cell death via oxidative damage and attenuates lactate production in metformin-treated breast cancer cells. Breast Cancer Res Treat. 2014 Oct;147(3):539-50.

158] Choi YW, Lim IK. Sensitization of metformin-cytotoxicity by dichloroacetate via reprogramming glucose metabolism in cancer cells. Cancer Lett. 2014 May 1;346(2):300-8.

159] Kumar A, Kant S, Singh SM. Novel molecular mechanisms of antitumor action of dichloroacetate against T cell lymphoma: Implication of altered glucose metabolism, pH homeostasis and cell survival regulation. Chem Biol Interact. 2012 Jul 30;199(1):29-37. doi: 10.1016/j.cbi.2012.06.005. Epub 2012 Jun 15.

160] Sanchez WY, McGee SL, Connor T, Mottram B, Wilkinson A, Whitehead JP, Vuckovic S, Catley L. Dichloroacetate inhibits aerobic glycolysis in multiple myeloma cells and increases sensitivity to bortezomib. Br J Cancer. 2013 Apr 30;108(8):1624-33.

161] Agnoletto C, Melloni E, Casciano F, Rigolin GM, Rimondi E, Celeghini C, Brunelli L, Cuneo A, Secchiero P, Zauli G. Sodium dichloroacetate exhibits anti-leukemic activity in B-chronic lymphocytic leukemia (B-CLL) and synergizes with the p53 activator Nutlin-3. Oncotarget. 2014 Jun 30;5(12):4347-60.

162] Niewisch MR, Kuçi Z, Wolburg H, Sautter M, Krampen L, Deubzer B, Handgretinger R, Bruchelt G. Influence of dichloroacetate (DCA) on lactate production and oxygen consumption in neuroblastoma cells: is DCA a suitable drug for neuroblastoma therapy? Cell Physiol Biochem. 2012;29(3-4):373-80.

163] Lin G, Hill DK, Andrejeva G, Boult JK, Troy H, Fong AC, Orton MR, Panek R, Parkes HG, Jafar M, Koh DM, Robinson SP, Judson IR, Griffiths JR, Leach MO, Eykyn TR, Chung YL. Dichloroacetate induces autophagy in colorectal cancer cells and tumours. Br J Cancer. 2014 Jul 15;111(2):375-85.

164] Abdelmalak M, Lew A, Ramezani R, Shroads AL, Coats BS, Langaee T, Shankar MN, Neiberger RE, Subramony SH, Stacpoole PW. Long-term safety of dichloroacetate in congenital lactic acidosis. Mol Genet Metab. 2013 Jun;109(2):139-43.

165] Rej R. Aminooxyacetate is not an adequate differential inhibitor of aspartate aminotransferase isoenzymes. Clin Chem. 1977 Aug;23(8):1508-9.

166] Sookoian S, Pirola CJ. Alanine and aspartate aminotransferase and glutamine-cycling pathway: their roles in pathogenesis of metabolic syndrome. World J Gastroenterol. 2012 Aug 7;18(29):3775-81.

167] Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, Thompson CB. Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci U S A. 2008 Dec 2;105(48):18782-7.

168] Thornburg JM, Nelson KK, Clem BF, Lane AN, Arumugam S, Simmons A, Eaton JW, Telang S, Chesney J. Targeting aspartate aminotransferase in breast cancer. Breast Cancer Res. 2008;10(5):R84.

169] Qin JZ, Xin H, Nickoloff BJ. Targeting glutamine metabolism sensitizes melanoma cells to TRAIL-induced death. Biochem Biophys Res Commun. 2010 Jul 16;398(1):146-52.

170] Anso E, Mullen AR, Felsher DW, Matés JM, Deberardinis RJ, Chandel NS. Metabolic changes in cancer cells upon suppression of MYC. Cancer Metab. 2013 Feb 4;1(1):7.

171] Elhammali A, Ippolito JE, Collins L, Crowley J, Marasa J, Piwnica-Worms D. A high-throughput fluorimetric assay for 2-hydroxyglutarate identifies Zaprinast as a glutaminase inhibitor. Cancer Discov. 2014 Jul;4(7):828-39.

172] Gross MI, Demo SD, Dennison JB, Chen L, Chernov-Rogan T, Goyal B, Janes JR, Laidig GJ, Lewis ER, Li J, Mackinnon AL, Parlati F, Rodriguez ML, Shwonek PJ, Sjogren EB, Stanton TF, Wang T, Yang J, Zhao F, Bennett MK. Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. Mol Cancer Ther. 2014 Apr;13(4):890-901.

173] Carracedo A, Cantley C, Pandolfi PP. Cancer metabolism: fatty acid oxidation in the limelight. Nat Rev Cancer. 2013;13(4):227-32.

174] Paumen MB, Ishida Y, Han H, Muramatsu M, Eguchi Y, Tsujimoto Y, Honjo T. Direct interaction of the mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2. Biochem Biophys Res Commun. 1997 Feb 24;231(3):523-5.

175] Giordano A, Calvani M, Petillo O, Grippo P, Tuccillo F, Melone MA, Bonelli P, Calarco A, Peluso G. tBid induces alterations of mitochondrial fatty acid oxidation flux by malonyl-CoA-independent inhibition of carnitine palmitoyltransferase-1. Cell Death Differ. 2005 Jun;12(6):603-13.

176] Begriche K, Massart J, Robin MA, Borgne-Sanchez A, Fromenty B. Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver. J Hepatol. 2011 Apr;54(4):773-94.

177] Giannessi F, Pessotto P, Tassoni E, Chiodi P, Conti R, De Angelis F, Dell'Uomo N, Catini R, Deias R, Tinti MO, Carminati P, Arduini A. Discovery of a long-chain carbamoyl aminocarnitine derivative, a reversible carnitine palmitoyltransferase inhibitor with antiketotic and antidiabetic activity. J Med Chem. 2003;46(2):303-309.

178] Anderson RC. Carnitine palmitoyltransferase: a viable target for the treatment of NIDDM? Curr Pharm Des. 1998;4(1):1–16.

179] Pacilli A, Calienni M, Margarucci S, D'Apolito M, Petillo O, Rocchi L, Pasquinelli G, Nicolai R, Koverech A, Calvani M, Peluso G, Montanaro L. Carnitine-acyltransferase system inhibition, cancer cell death, and prevention of myc-induced lymphomagenesis. J Natl Cancer Inst. 2013;105(7):489-98.

180] Ricciardi MR, Petrucci MT, Gregorj C, Ariola C, Lemoli RM, Fogli M, Mauro FR, Cerretti R, Foà R, Mandelli F, Tafuri A. Reduced susceptibility to apoptosis correlates with kinetic quiescence in disease progression of chronic lymphocytic leukaemia. Br J Haematol. 2001;113(2):391-399.

181] Tafuri A, Meyers J, Lee BJ, Andreeff M. DNA and RNA flow cytometric study in multiple myeloma. Clinical correlations. Cancer. 1991;67(2):449-54.

182] Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis Nat Rev Cancer 2007;7(10):763-77.

183] Caro P, Kishan AU, Norberg E, Stanley IA, Chapuy B, Ficarro SB, Polak K, Tondera D, Gounarides J, Yin H, Zhou F, Green MR, Chen L, Monti S, Marto JA, Shipp MA, Danial NN. Metabolic signatures uncover distinct targets in molecular subsets of diffuse large B cell lymphoma. Cancer cell. 2012;22(4):547-60.

184] Tung S, Shi Y, Wong W, Zhu F, Gorczynski R, Laister RC, Minden M, Blechert AK, Genzel Y, Reichl U, Spaner DE. PPARa and fatty acid oxidation mediate glucocorticoid resistance in chronic lymphocytic leukemia. Blood. 2013;122(6):969-980.

185] Bassan R: Evolving strategies for the management of high-risk adult acute lymphoblastic leukemia. Haematologica 2005, 90(10):1299.

186] Löwenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999 Sep 30;341(14):1051-62.

187] Wei G, Rafiyath S, Liu D. First-line treatment for chronic myeloid leukemia: dasatinib, nilotinib, or imatinib. J Hematol Oncol. 2010 Nov 26;3:47.

188] Leni Z, Parakkal G, Arcaro A. Emerging metabolic targets in the therapy of hematological malignancies. Biomed Res Int. 2013;2013:946206.

189] Tennant DA, Durán RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. Nat Rev Cancer. 2010 Apr;10(4):267-77.

190] Ligibel JA, Alfano CM, Courneya KS, Demark-Wahnefried W, Burger RA, Chlebowski RT, Fabian CJ, Gucalp A, Hershman DL, Hudson MM, Jones LW, Kakarala M, Ness KK, Merrill JK, Wollins DS, Hudis CA. American Society of Clinical Oncology position statement on obesity and cancer. J Clin Oncol. 2014 Nov 1;32(31):3568-74.

191] Zhou W, Simpson PJ, McFadden JM, Townsend CA, Medghalchi SM, Vadlamudi A, Pinn ML, Ronnett GV, Kuhajda FP. Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. Cancer Res. 2003 Nov 1;63(21):7330-7.