CHARACTERIZATION AND TARGETING OF METABOLIC ALTERATIONS IN THE LEUKEMIC MICROENVIRONMENT

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ABSTRACT

The bone marrow microenvironment is an important determinant for normal and malignant hematopoiesis. Bone marrow mesenchymal stromal cells (BM-MSCs), endothelial cells, osteoclasts and osteoblasts promote the maintenance and survival of quiescent leukemia initiating cells via cell contact and paracrine signaling pathways. However, other components of this microenvironment such as platelets have not been studied and their precise role remains incompletely understood.

In contrast, one of the most studied interactions have been between BM-MSCs and leukemia cells, which promote mitochondrial uncoupling—a disconnection between the electrochemical gradient of the mitochondrial membrane and the oxidative phosphorylation (the major source of cellular ATP); characterized by an increase of resistance to intrinsic apoptosis, decrease of entrance of pyruvate into the Krebs cycle presumably to use glucose carbon skeletons for the generation of biomass, and a shift to the metabolism of fatty acids to support oxygen consumption. Previous evidence demonstrated that pharmacological inhibition of fatty acid oxidation (FAO) sensitizes leukemia cells to intrinsic apoptosis, suggesting that targeting carbon utilization in the context of mitochondrial uncoupling may be a valid therapeutic strategy. However, inhibition of fatty acid oxidation may not be a feasible clinical strategy due to chronic toxicity. Whether other metabolic parameters can be targeted with clinically available drugs for the therapy of leukemia remains to be determined.

Based on these antecedents, we decided to investigate if platelets play a role in promoting leukemia cell survival. Our work indeed demonstrates that platelets promote survival of leukemia cells, in part by promoting mitochondrial uncoupling and increased reliance on FAO in much the same way as BM-MSC. Given that FAO relies on increased electron transport we also investigated if the antidiabetic drug Metformin, which has been shown to partially inhibit the electron transport chain (ETC), could overcome the metabolic reprogramming of leukemia cells and sensitize them to the induction of apoptosis. Lastly, given that increased FAO results in depletion of intracellular oxygen and very likely promotion of glycolysis and accumulation of lactate as a consequence, we
questioned if hypoxia activated pro-drugs (PR-104, TH-302) and pH sensitive peptides (pHLIP) would be effective therapeutic agents for the treatment of leukemia.

Our results evidenced the resistance to targeted therapy induced by platelets through mitochondrial uncoupling, which could be potentially overcome by the use of Metformin and other agents (PR-104, TH-302, pHLIP) affecting several of the metabolic re-arrangements found in leukemia cells.

The results generated from these experiments will advance our understanding of leukemia cell survival and metabolism in its microenvironment, and potentially provide scientific rationale for the use of Metformin; Hypoxia activated pro-drugs and pH sensitive peptides for the treatment of the leukemic bone marrow.
CHAPTER 1

PROBLEM STATEMENT-RATIONALE

Even though the five-year relative survival rate for leukemia has significantly improved compared with 50 years ago increasing from 14% to approximately 80% (depending on the type of leukemia), due to the development of new and more efficient therapies, 20% of the patients develop a chemorefractory disease. In the particular case of Colombia the remission rates differ from developed countries in approximately 30% due to delays in the diagnostic and treatment.

Refractory disease is characterized for the presence of leukemia cells that were not completely eliminated during chemotherapy period due to resistance, increasing the possibilities of remaining blasts that could acquire new mutations. Such characteristics accompanied by a protective bone marrow microenvironment, promote the survival and maintenance of the leukemic blasts (minimal residual disease).

Components of this microenvironment included several type of cells and cellular fragments such as platelets, which tend to be decreased in leukemia patients perhaps due to competition for the hematopoietic niche with the malignant clone; and while platelets play a role in solid tumor metastasis and maintenance, the fundamental mechanisms by which platelet contents regulate leukemia cell survival continue under investigation, most importantly it is not known if platelets promote chemoresistance of leukemia cells.

Several mechanisms of chemoresistance have been described. Some of them involve upregulation/dowregulation of antiapoptotic and pro-apoptotic proteins respectively, increase of signal transduction through different axis (CXCR4/SDF-1, FLT3, BCR-ABL, AKT, Integrin linked kinase, etc.), metabolic adaptations and immune regulation (Tabe Y, 2014). Nevertheless, the exact cellular and molecular dynamics of these mechanisms have not yet been completely defined.
Amongst metabolic adaptations, it has been demonstrated that mesenchymal stromal cells promote mitochondrial uncoupling – an alteration in the electrochemical gradient of the mitochondrial membrane- in leukemia cells, promoting oxygen consumption supported by fatty acids and inducing resistance to intrinsic apoptosis, in part inhibiting bax and bak oligomerization.

Moreover, recent evidence suggests that the bone marrow microenvironment is hypoxic – presumably in part due to increase oxygen consumption by leukemic blasts – and this microenvironmental characteristic may favorably support glycolysis and contribute to extracellular acidity. To date, agents targeting mitochondrial respiration or activated just under hypoxic conditions are being tested in preclinical leukemia models, phase I/II studies in combination or not with other compounds.

This project aimed to elucidate the role of Platelets and Metformin in the metabolism and survival of leukemia cells; determine if pH sensitive peptides target leukemia cells and if Hypoxia activated pro-drugs (TH-302-PR-104) are able to reverse hypoxia and improve survival in vivo. All of these with the purpose to answer the followed questions:

- Do platelets regulate the metabolism or survival of leukemia cells?
- If there is high oxygen consumption by leukemia cells, can we target it with mitochondrial inhibitors as Metformin or Phenformin?
- Can we target hypoxic conditions generated presumably by leukemia cells -through a high respiration rate- with hypoxia activated prodrugs (TH-302) or sensitive to pH peptides (pHLIP)?
CHAPTER 2
BACKGROUND LITERATURE REVIEW

2.1 Leukemia

Hematologic malignancies defined as the uncontrolled proliferation of any blood forming tissue (Cooper GM, 2000) could be divided in two different large groups: Lymphoid and Myeloid disorders depend on the origin of the disease. These in tum have other subgroups according to the stage of the initial disease: Acute or Chronic (Attar E, 2010). For years research has been done trying to determine the causes of these diseases and it has been concluded they are instigated by the influence of different factors: genetic and environmental, as has been proposed in other types of cancer (Belson M, 2007).

Leukemia is defined as a malignant progressive disease of any type of blood cell: Red Blood Cells, White Blood Cells and Platelets (Cellular Fragment) that could occur as a consequence of acquired mutations in the DNA conferring advantages in the rate of proliferation and decreasing the threshold of apoptosis induction. This uncontrolled proliferation could lead to a displacement of other types of blood cells in the bone marrow, the normal physiologic and initial niche in the process of blood formation. As a consequence of this shift, normal cells die or can't be generated from their respective progenitors causing in the patients several cytopenias that are evidenced due to usual symptoms related to leukemia: Frequent or severe infections, easy bleeding or bruising, presence of petechiaes etc. (Estey EH, 2012).

The diagnostic of Leukemia is performed through several exams that are complementary and include bone marrow aspiration and biopsy to determine morphologically the presence of abnormal cells, analysis by flow cytometry to determine different surface markers that could be aberrantly expressed, cytogenetics to define the presence of any change in the chromosomes and molecular analysis to determine possible gene fusions (Mason J, 2012).
Worldwide and according to statistics of 2012, leukemia contributes 2.5% of the total number of newly diagnosed cancer (Ferlay J, 2012). In the United States the number of new cases (age-adjusted) during 2008-2012 was 13.3 per 100,000 men and women per year, and a number of deaths of 7 per 100,000 men and women per year. The 5 years survival was 58.5% during 2005 and 2011. For the year 2015 were estimated 54,270 new cases (3.3% of all new cancer cases) and an estimated of deaths of 24,450 (4.1% of all cancer deaths) (National Cancer Institute). In Colombia’s case, during the period of 2007-2011 were estimated 29,734 new cases of cancer in men and 33,084 new cases of cancer in women with an average of 16,081 deaths per year in men and 16,572 deaths per year in women. In the pediatric population the cancer with major incidence was leukemia with 319 estimated cases in boys and 263 in girls with an estimated number of deaths of 148 cases in boys and 108 cases in girls (Pardo C, 2015). Accordingly, developed countries present approximately 33,000 new cases of cancer in children per year while developing countries 180,000, where the first ones have access to the treatment in the 100% of the cases and the second ones just in the 20% of the cases. This reflects why the cure rate in rich countries is 80% while in the less-advantaged countries is just 35% (Howard S, 2004). The principal causes of this outcome are the lack of an early diagnosis and access to the treatment, abandonment of therapy which could lead to relapses, death as a consequence of treatment and suboptimal conditions of care etc. (Metzger ML, 2003).

2.2 Treatment and outcomes of Leukemia

The treatment of leukemia is prescribed according to the age, general health and presence or not of gene mutations. In general most of the treatments have 3 different phases with well-defined goals: Induction (to kill leukemia cells and induce remission), consolidation (intensification of the treatment to kill any remaining leukemia cells) and maintenance (lower doses of chemotherapy to prevent the growth of any remaining leukemia cells). General protocols for the treatment of Acute Lymphoid Leukemia (ALL) could include Prednisolone, Dexamethasone, Cyclophosphamide, Asparaginase,
Vincristine, Etoposide, Mercaptopurine, Methotrexate and Anthracyclines; while for the treatment of Acute Myeloid Leukemia (AML), the use of Anthracyclines only is predominant. In any of the cases (AML and ALL) adjustments or additions are made depending on the risk factors of each patient. The complementary treatments may include other therapies as radiation, targeted therapy (tyrosine kinase inhibitors, monoclonal antibodies) or stem cell transplantation, contingent to the alterations that are found in the leukemia cells (Mayo Clinic). It has been seen that the use of these complementary therapies has led to better responses in the long term (American Cancer Society).

Responses to therapy could vary according to the type of leukemia, access to health care system and inner patient characteristics. In the United States and for the case of Acute Myeloid Leukemia the response rates are between 50% and 70% while for Acute Promyelocytic Leukemia is more than 90% (American Cancer Society); for the case of Acute Lymphoid Leukemia the rates are 80% but the event-free survival at 5 years is only 40-50%. In the particular case of Colombia, patients with AML have a global survival at 2 years of 90% for a low risk group, 61% for intermediate risk and 30% for high risk (Combariza J, 2015). The principal factors that were defined to affect the global survival were the lack of complete remission and transplant. For ALL, it has been seen that the 10 years event free survival is 94% for patients with standard risk while for patients with high risk is just 36% (Buendia MT, 2008); Suggesting that numerous factors, including supportive care and socioeconomic conditions can affect the final outcome seen in adult and pediatric patients (Boer J, 2015).

2.3 Bone marrow microenvironment

Normal and malignant hematopoiesis occurs under the regulation of two different bone marrow niches: endosteal (osteoblastic) and vascular niche (Calvi LM, 2003) (Kiel MJ, 2005), (Yilmaz, OH 2006). The endosteal niche is located close to the bone and is composed of osteoblasts, osteoclasts and mesenchymal stromal cells (MSC). The vascular niche is proximal to vascular
structures (Nilsson SK, 2005) and formed by sinusoidal, perivascular stromal cells and immune cells (Nwajei F, 2013).

Each compartment provides different signals to normal and leukemia cells to promote maintenance, survival, quiescence or proliferation. In the case of endosteal niche ligand-receptor interactions, Jagged 1 on osteoblasts and its receptor NOTCH on HSC (hematopoietic stem cells) promote HSC expansion (Varnum-Finney B, 1998); while Ang-1 in osteoblasts interacts with its receptor Tie2 on HSC to induce quiescence and maintenance through activation of β1 integrin and N-Cadherin (Arai, 2004). It is noteworthy that CXCL12 a major chemo-attractant for hematopoietic stem and progenitor cells (HSPCs) (Christopher MJ, 2009), that is produced by cellular component in both compartments (osteoblasts, stromal cells and endothelial cells) has been reported to mediate not only migration, but also growth, differentiation (Kondo M, 2003), upregulation of pro-survival signals and quiescence of both HSCs and leukemic stem cells through its receptor CXCR4 (Riether C, 2015). For instance, disruption of this principal axis CXCR4/CXCL12 is currently being tested in several clinical trials (Phase I and II) and the FDA has approved one CXCR4 agonist (Plerixafor) already, to use in hematopoietic stem cell mobilization in patients with non-Hodgkin’s lymphoma and multiple myeloma (Debnath B, 2013).

Further studies in the endosteal niche, have found that alterations in the mesenchymal osteoprogenitor cells (knockout of the RNAse III endonuclease Dicer 1) (Raaijmakers MH, 2010) and osteoblasts (mutation of β-catenin) (Kode A, 2014) can result in myelodysplasia or AML respectively; suggesting that changes or mutations in the cells surrounding the tumor could affect direct or indirectly the development of disease. On this basis, it was showed that BM MSCs derived from healthy donors or AML patients express similar phenotypic markers as CD29, CD44, CD73, CD90 and CD324, which were comparable with normal stromal cell lines, HS5 and HS27a. However, and after gene expression profiling there were found differences in collagen expression and extracellular matrix genes, upregulation of gene expression by transforming growth factor beta (TGF-β) 1, TNF (Tumor Necrosis Factor), tissue transglutaminase 2 (TGM2), CCAAT/enhancer
binding protein alpha (C/EBPA) and others (Huang JC, 2015). For instance, the production of TGF-
β by a variety of BM cells has been shown to be regulated for glial nonmyelinating Schawn cells
(Yamazaki S, 2011), where the axis TGF-β/Smad plays an important role in the maintenance of
HSC in a hibernation state (not entrance to the cell cycle) (Yamazaki S, 2009).

In the particular case of hematology malignancies and knowing that bone and platelets are the main
reservoirs of TGF-β (Sing GK, 1988), has been proposed that platelet-derived TGF-β could possibly
inhibit the growth of leukemia cells by modulating cell cycle protein expression and/or
phosphorylation status, investigated by Hux X et al in 1999 and reported for synthetic TGF-β. Likewise, an apoptotic role of TGF-β (Transforming growth factor-beta) mediated by Smad4
mitochondrial translocation and cytochrome c oxidase subunit II interaction, was reported in cancer
cells (Pang L, 2011), suggesting a possible direct mitochondrial regulation. Another factor that is
widely released by platelets is PDGF, which was found to be released in conjunction with soluble
CD62P (Platelets Activation Indicator) after the exposition to AML blasts from certain patients
(Bruserud OB, 1998), suggesting the possibility that AML blasts could promote platelet activation to
release multiple factors that could perhaps promote their proliferation and survival (Foss B et al
2008). In addition, PDGF was reported to induce mitochondrial fragmentation, decrease mitofusin 2
(fission protein) and glucose oxidation, as to increase fatty oxidation in vascular smooth muscle
cells (Salabei J, 2013), proposing a direct mitochondrial effect that could mediate cell survival.
Nevertheless, the essential mechanisms by which platelet content control leukemia cell survival
remain still under investigation and it is not known if platelets contribute to chemoresistance.

2.4 Mitochondrial Uncoupling

BM-MSCs are able to interact directly with leukemia cells upregulating important anti-apoptotic
proteins as Bcl-2 and Bcl-xL (Konopleva M, 2002), promoting resistance to starvation (serum free),
chemotherapeutic agents as Cytarabine, and supporting the survival of quiescent leukemia stem
cells. Additional to this upregulation, the exposure of leukemia cells to MSCs can induce
mitochondrial uncoupling (loss of coupling between the rate of electron transport and ATP production) (Mitchell P, 1968) in the leukemia cells mediated by the expression of UCP2 (uncoupling protein 2), increasing proton conductance and loss of mitochondrial membrane potential (MMP), promoting fatty acid oxidation (FAO) to support oxygen consumption, decreasing ROS production and finally exacerbating chemo-resistance (Samudio I, 2008). Moreover the inhibition of FAO improved the effect of chemotherapy in mouse models of human leukemia, suggesting a new possible target (Samudio I, 2010). In other cancer models (Pancreas, Colon) have been demonstrated that the expression of UCP2 can reduce the production of ROS presumably increasing the conductance of protons back to the matrix and subsequently decreasing electron escape; promoting as a consequence resistance in cancer cells due to the dependence of ROS production to induce cell death (Pozza E, 2012). Additional to these properties, UCP2 has been reported to inhibit caspase 3 activation and its activity in isolated mitochondria is enhanced by palmitic acid (Mattiasson G, 2003), supporting the notion that proton conductance associates with the fatty acyl composition of inner membrane phospholipids (Divakaruni A, 2011).

2.5 Mitochondrial Recoupling

Even though mitochondrial uncoupling have been described as a mechanism of resistance in several cancer models, the theory of recoupling as a therapeutic approach is just starting to surge. In a recent report, Rial et al described how chromane derivatives can bind deep inside the α-helical bundle core of UCPs, presumably displacing purine nucleotides (ADP and guanosine 5′-diphosphate) and inhibiting proton conductance promoted by UCP1 and UCP2, sensitizing as a consequence HT29 colon cancer cells to common chemotherapeutic agents (Cisplatin and Doxorubicin). Still, fair to remember that UCPs have important and normal functions in different tissues especially in brown fat cells, which could be a limitation for their targeting (Arsenijevic D, 2000) in cancer cells.
2.6 Apoptosis

Defined as programmed cellular death, is a process tightly regulated through the expression of multiple proteins: pro and antiapoptotic; Can be differentiated from other types of cell death due to the characteristics that presents after activation of its program: Cell reduction, nuclear condensation and fragmentation, plasmatic membrane blebbing, loss of adhesion to other cells or extracellular matrix, cleavage of chromosomal DNA and externalization of phosphatidylserine (Ouyang L, 2012).

Two different pathways to induce apoptosis have been described: extrinsic and intrinsic (Galluzzi L, 2015) depending on the stimuli, the former depends on a death receptor located in the plasmatic membrane that needs to interact with its ligand (Fas, Tumor necrosis factor 1 and others) to activate the cleavage of initiators and effector enzymes (caspases); while the intrinsic one is initiated by stress signals to prompt the release of apoptotic factors (Cytochrome c, Apoptosis Inducing Factor) from the mitochondrial intermembrane space, process strictly dependent on mitochondrial outer membrane permeabilization (MOMP). In the latest, the release of given factors requires several mitochondrial proteins (Bcl-2 family proteins) that keep a balance between pro (Bax, Bak, Bad, Bid, Bim, Bmf, NOXA, PUMA) and anti-apoptotic (Bcl-2, Bcl-XL, Mcl1) signals (Testa U, 2007); Between these proteins, PUMA, BIM and BID are important regulators of the physical and functional interactions of the other proteins (Galluzzi L, 2015). Although both pathways differ in the manner they are activated, they can converge through the cleavage of Bid (tBid) by caspase 8 causing its subsequent translocation to the mitochondria, where it can interact with pro apoptotic factors as Bax to trigger the intrinsic pathway (Fulda S, 2006). However, other mechanisms independent of caspase activation have been described. One of the first ones was seen in a leukemic model (Jurkat cells) where the inhibition of caspases didn’t inhibit Bax-induced cell death (Xiang et al); later on it was demonstrated that in vivo caspase inhibition didn’t relieve toxicity induced by TNF-α in mice (Cauwels A, 2003) indicating that caspase-independent cell death is not limited to in vitro models. Even more, “mitochondrial permeability transition” (MPT), a sudden increase in the
permeability of the inner mitochondrial membrane doesn’t require caspase activation either (Galluzzi L, 2015).

Alterations in both of the pathways have been described and due to the high frequency of evasion of programmed cell death found in several types of cancer, this characteristic has been defined as a hallmark of the disease itself (Hanahan D, 2000). In leukemia the acquisition of mutations during the progression of the disease have conducted to the over or lack of expression of anti and pro apoptotic proteins respectively. For example it has been shown that fusion proteins as AML1/ETO can activate the transcription of Bcl-2 (Klampfer L, 1996) and that PML/RAR-α can modulate the p53 pathway, an essential tumor suppressor (Pandolfi PP, 2001). Most commonly, the internal tandem duplication (ITD) in Flt3 (Receptor Tyrosine Kinase) that affects 20% of the patients with Acute Myeloid Leukemia, has been related with Bcl-XL hyperexpression (Minami Y, 2003).

Targeting of any of both pathways has been explored during the past 10 years and with regard to the extrinsic pathway it has been shown that even though numerous clinical trials (Phase I and II) to test TRAIL (TNF-related apoptosis-inducing ligand) receptor agonists have been opened, significant survival benefit has not been detected so far. In contrast, the intrinsic pathway has shown to be targetable with promising evidence in several cancer models, solid and liquid tumors. As the overexpression or unchanged levels of Bcl-2 family proteins and particularly Bcl-2 protein before and after chemotherapy has been correlated with the response and the prognosis of patients with breast cancer and Acute Myeloid Leukemia (Buchholz TA, 2003; Campos L, 1993; Tothova E, 2002); multiple clinical trials have aimed to target the latest protein (Bcl-2) showing so far better results with single agents (ABT-737, 263,199) in hematological malignancies than solid tumors (Vogler M, 2014).

The first of the Bcl-2 inhibitors to be developed was ABT-737, which through nuclear magnetic resonance was identified to bind into the hydrophobic pocket of Bcl-XL as Bcl-2 and Bcl-w due to their similar structure; causing the displacement of pro apoptotic proteins Bim, Bax and Bak to form
a pore in the outer mitochondrial membrane and promote the release of cytochrome c and AIF (Apoptosis Inducing Factor). Remarkably, proteins as Mcl1, Bcl-B and Bcl2a1 are not inhibited by this compound due to the lack of homology, allowing them to keep sequestering pro apoptotic proteins and subsequently inhibiting their function. Most importantly the absence of Mcl1 inhibition has been characterized as the principal mechanism of resistance to Bcl-2 inhibitors (Vogler M, 2014).

Later in the development of such inhibitors, couples of leads ABT-263 and ABT-199 were generated aiming to improve ABT-737 availability. For instance ABT-263 is orally bioavailable, giving an advantage to the treatment of chronic diseases and demonstrating to have activity as a single agent at least in hematological malignancies (Roberts AW, 2012). Nonetheless, its high affinity for Bcl-XL has generated side effects expressly in platelets, where the apoptosis pathway depends primordially on the mentioned protein (Mason KD, 2007). Accordingly, ABT-263 was reengineered and ABT-199 was generated as an inhibitor of Bcl-2 but not Bcl-XL or Bcl-w. Currently numerous clinical trials particularly in hematological malignancies, have focused in the use of ABT-199 as a single agent or in combination due to its impressive reported antitumor activity in vivo in humans. Investigation in other types of cancer as SCLC (Small Cell Lung Cancer) and Breast Cancer are in preclinical studies still showing very encouraging outcomes (Vaillant F, 2013).

2.7 Cancer Cell Metabolism and Alterations

As shown in Diagram 1, under normal conditions, glucose and other substrates (Fatty acids, glutamine, etc.) are oxidized in the cytoplasm and/or mitochondria through two different processes: glycolysis and the Krebs cycle respectively; producing metabolic intermediates including NADH and FADH2 that are substrates of the electron transport chain and oxidative phosphorylation (OXPHOS) pathways, which power the ATP synthase to generate energy (Lodish H, 2000) The transport of electrons from electron donors (NADH-FADH) to electron acceptors as complexes embedded in the inner mitochondrial membrane and oxygen, releases energy that allows the flow of protons through
the mitochondrial membrane generating potential energy (difference in electric charges and pH gradient) which is used by the ATP synthase to form ATP (Adenosine Triphosphate) from ADP (Adenosine diphosphate) (Mitchell P, 1967). This final reaction is driven primordially by the proton flow, which induces the counter-rotation of the A and C subunits in the Fo motor of the enzyme, making of this an essential process for the synthesis of ATP (Dimroth P, 2000).

Given that mitochondria potential is the main driving force for the ATP production (Dimroth P, 2000), alterations in any of the described stages could lead to an “uncoupling” of OXPHOS and the electron transport chain, having as a consequence a “futile” cycle that just produces heat instead of energy; without mentioning the possible accumulation of intermediates that could inhibit several steps of the metabolism, cytoplasmic and mitochondrial. Examples of these events, include several enzymes participating in glycolysis or the Krebs cycle, that are inhibited by their immediate products as NADH and Acetyl CoA (Pyruvate Dehydrogenase) or ATP, NADH (Isocitrate Dehydrogenase), and succinyl CoA (α-ketoglutarate dehydrogenase) (Berg JM, 2002). The last ones being the principal points of regulation of the Krebs cycle due to its essential function: ATP production. Although oxygen is not a direct regulator of the Krebs cycle, this process functions only under aerobic conditions, because NAD+ and FAD cannot be regenerated by the electron transport chain in the absence of oxygen which needs to be reduced as well. Without oxygen, NADH and FADH2 accumulate and the cycle cannot continue as it needs NAD+ and FAD to keep running (Berg JM, 2002).

For years the use of PET (Positron emission tomography) for the detection and follow up of different solid cancers, has supported Otto Warburg’s observations (from over 80 years ago) that malignant cells depend on glycolysis despite the presence of adequate oxygen levels (Aerobic Glycolysis). Warburg hypothesized that mitochondria in tumor cells (Warburg O, 1927) contained an “irreversible” damage characterized by the absence of pyruvate entrance into the Krebs cycle and subsequent accumulation of lactate, a characteristic that is considered a hallmark of tumor transformation (Hanahan D, 2011). The exact mechanisms of how the Warburg effect confers
advantages to cancer cells are not completely understood. Despite there is an increasing amount of data suggesting that changes in energy metabolism can modulate oncogenic signalling pathways to allow cancer cells to adapt to several conditions (Hsu PP, 2008; Vander Heiden MG, 2009): lack of nutrients, oxidative stress, apoptosis, survival under acidic microenvironments (generated as a consequence of their accelerated glycolysis activity) and evasion of immune responses to facilitate invasive growth (Gatenby RA, 2004; DeBerardinis RJ, 2008; Hsu PP, 2008).

This paradigm has been challenged due to recent findings about the functioning of mitochondria and oxidative phosphorylation in tumor cells. Examples of adaptation in response to chemotherapeutic agents or essential activities of OXPHOS (Oxidative Phosphorylation) have been described in different types of tumors including Melanoma, Adenocarcinoma, Breast, Pancreatic Cancer and Leukemia. Specifically, the weakening of oxidative phosphorylation through down regulation or deletion of peroxisome proliferator-activated receptor γ (PPARGC1A) caused sensitization of Melanoma cells to agents that induce apoptosis by accumulation of ROS (Vasquez F, 2013). Alterations in the expression of mitochondrial transcription factor A (Tfam) compromised the growth and frequency of oncogenic Kras-driven lung adenocarcinomas in mice (Weinberg F, 2010). High levels of glycolysis in breast cancer were not enough to sustain the growth of xenografts in mice when knockdown of p32 mitochondrial protein was done (Fogal V, 2010). These and other reports sustain the notion that oxidative phosphorylation is active in malignant cells of diverse origins and that could be a renewed potential target (Wei A, 2013).

In support of OXPHOS activity in cancer cells, several reports have described further function of the Bcl-2 protein besides regulating apoptosis, proposing and demonstrating new functions as regulation of oxidative phosphorylation increasing oxygen consumption, COX (Cytochrome Oxidase) activity (Chen ZX, 2007), regulating ROS (Reactive Oxygen Species) levels in leukemic stem cell (LSC) populations and possibly making them more dependent on oxidative phosphorylation instead glycolysis (Lagadinou ED, 2013). Moreover, stem cells from glioblastoma have been shown to depend more on oxidative phosphorylation nurturing the possibility that cancer
stem cells may not always utilize the Warburg effect to meet energy demands (Vlashi E, 2011). Therefore, the studies of Lagadinou et al demonstrated the importance of Bcl-2 protein in oxidative phosphorylation through the use of specific inhibitors (ABT-263) or RNAi, where there was a quick diminishing in the mitochondrial respiration, a decrease in ATP concentrations and subsequently apoptosis induction. Importantly, these effects were seen in both populations (LSC and bulk) with the difference that the bulk cells were able to compensate by increasing glycolysis while the LSCs were not. Thus providing a possible metabolic target (oxidative phosphorylation) specially in the leukemia initiating cells.

Backing up this data and suggesting that abnormal HSCs display and increased consumption of oxygen, several reports provide evidence of the presence of an oxygen gradient inside the bone marrow (from vascular to osteoblastic domain), where the most primitive and quiescent hematopoietic stem cells (normal and leukemic) are sequestered in hypoxic regions (Parmar K, 2007; Winkler IG, 2010), perhaps suggesting that somehow hypoxia may sustained stem cells. Chow et al. anticipated as well that stem cells need to be located in a region with very low oxygen concentrations preventing them of being exposed to oxidative stress, in agreement with the recent findings made by Lagadinou et al.

Recent studies in murine and human leukemia models, showed noticeable expansion of hypoxic areas in the bone marrow of immunodeficient mice engrafted with Acute Lymphoid Leukemia cells (ALL) in contrast to healthy control mice, consistent with chemoresistance in various cell lines and sensitivity to the use of hypoxia-activated prodrug PR-104 (Benito J, 2011); a phosphate ester that is hydrolyzed in vivo to the corresponding alcohol PR-104A and that under low oxygen concentrations is reduced mainly to the amine and hydroxyl-amine nitrogen mustards able to induce DNA cross-linking in hypoxic cells (Patterson A, 2007; Singleton RS, 2009). To date, several phase III/II and I trials are being evaluated: Phase III: Soft tissue sarcoma (in combination with Doxorubicin), Pancreatic Cancer (in combination with gemcitabine), Phase II: non-squamous non-small cell lung cancer and Phase I: other solid tumors and hematological malignancies (Threshold
Pharmaceuticals). Furthermore, an update in a phase I/II study in refractory/relapsed Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia with PR-104 alone, reports responses of 32% in AML and 20% in ALL; and although the number of patients was small (40), the existence of hypoxic leukemic cells was validated and their reduction after PR-104 administration was corroborated. Concluding that leukemia microenvironment has hypoxic features that could be targeted with hypoxia-activated prodrugs that need to be further evaluated alone or in combination with chemotherapy in acute leukemia (Konopleva M, 2015).

TH-302 or evofosfamide is an investigational second-generation hypoxia activated prodrug (HAP), designed to overcome some of the potential limitations of previous HAPs, including regions of activation compared with normal tissue. TH-302 is activated after a process of 1-electron reduction mediated by ubiquitous cellular reductases, after this process a radical anion prodrug is generated, which under normal oxygen conditions returns to initial pro-drug state and superoxide; however under hypoxic conditions (0.5% O₂) the radical anion can be fragmented and/or undergo further reduction leading to the release of the active drug Br-IPM (Bromo Isophosphoramide Mustard), which can alkylate DNA and induce its cross-linking (Weiss G, 2011). The exploration of this agent as a chemotherapeutic alone or in combination is on-going, with several clinical trials in solid and liquid tumors being performed.

On the other hand, indirect measurements of hypoxia have been done through the use of different techniques as proteomics, gene expression of hypoxia inducible factor-1 α (Hif-1α) and other genes, as staining with hypoxic markers sensitive to low concentrations of oxygen (Pimonidazole). Recent examinations using two-photon phosphorescence lifetime microscopy revealed absolute numbers of oxygen pressure inside the bone marrow, determining that its general pressure is low (<32 mmHg) compared with arterial (75 mmHg-100 mmHg) or venous (30 mmHg-40 mmHg) blood. Even more, despite the very high vascular density, there was found heterogeneity in the local oxygen pressures, having an area close to the peri-sinusoidal region with the lowest pressure (~9.9 mmHg) (Spencer JA, 2014).
Other approaches have been taken for multidisciplinary groups combining physic and chemical methods, where peptides sensitive to changes in the pH (Potential Hydrogen) due to the presence of protonable residues as Aspartate, increase their hydrophobicity and insert themselves reversibly into the plasmatic membrane of acidic cells to label, target and kill them. pHLIP (pH low insertion peptide) as these peptides are called, can be attached to impermeable molecules via its C terminus with a non-cleavable bond to translocate them through the surface of cells located in an acidic environment. Such peptides have been used in different models of inflammation and cancer (Breast and Pancreatic) demonstrating higher targeting and co-localization compared with healthy tissue. Of notice, tissues as kidney showed accumulation of the peptide, very likely due to the catabolism of low molecular weight proteins and the presence of acidic regions (Andreev OA, 2007).

Thereby, is enticing to propose that a dynamic and functional TCA cycle, requiring different sources of energy (glucose, fatty acids, glutamine), is necessary to keep an active mitochondrial respiration and produce energy supplies to sustain the high demand of cell division in tumor cells; thus opening new opportunities to affect cancer cells given its metabolic alterations.

2.8 Targeting leukemic mitochondria

Even though the concept of aerobic glycolysis was initially based on the notion that cancer cell mitochondria was unable to efficiently oxidize carbon skeletons, multiple metabolic cancer alterations has been described during the last 10 years suggesting that carcinogenic tissues could oxidize other metabolic substrates different from glucose, including glutamine and fatty acids. The above observations suggested that – contrary to Warburg’s original hypothesis- cancer cell mitochondria are not irreversibly damaged and in fact may be highly active to support Krebs cycle function and the generation of biomass via cataplerotic reactions (Wise D, 2008; Gao P 2009; Samudio I, 2010).
The antidiabetics and mitochondrial complex 1 inhibitors, Metformin (Owen MR, 2000) and Phenformin (Stumvoll M, 1995), have chemopreventive and direct antitumor properties (Dowling RJ, 2012; Segal E, 2011); however Phenformin was withdrawn from the markets in the majority of the world for safety reasons (lactic acidosis) (Luft D, 1978) while Metformin is being tested in several ongoing clinical trials, alone or in combination with chemotherapeutic agents to determine prospectively its safety and efficacy in the treatment of human cancer (Bost F, 2012).

It has been reported that Metformin activates the AMP-dependent kinase (AMPK) in vitro and in vivo, via the tumor suppressor kinase LKB1 (Xie Z, 2008) or by promoting an increase in the AMP: ATP ratios (Foretz M, 2010). Although is yet unclear how Metformin activates LKB1, it has been shown that Metformin can increase the AMP: ATP ratio as a result of moderated inhibition of the electron transport chain at the mitochondrial complex I (Foretz M, 2010; Owen MR, 2000); activating AMPK and in tum phosphorylating and activating tumor suppressor TSC2 (Tuberous Sclerosis Complex 2), a negative regulator of the mammalian target of rapamycin (mTOR) (Sun Y, 2008). The subsequent reduction in mitochondrial bioenergetics impedes hepatic gluconeogenesis and in most tissues promotes catabolism trying to balance the lack of energy. Interestingly, albeit activation of AMPK and the resulting inhibition of the mammalian target of rapamycin (mTOR) signaling have been suggested to facilitate the antitumor effects of Metformin (Martelli AM, 2012), AMPK-independent growth inhibitory effects of this compound in tumor cells have also been described (Vazquez-Martin A, 2009; Kalender A, 2010), proposing that inhibiting electron transport per se may be cytostatic or cytotoxic to cancer cells.

In leukemia cells, as reported recently (Scotland S, 2013) Metformin markedly blocked cell cycle, suppressed cell proliferation and colony formation, however the apoptotic response varied depends on AKT (Protein Kinase B) phosphorylation status. Although Metformin induced early and transient activation of AMPK, abrogation of AMPKα 1/2 didn’t revoke anti-proliferative or pro apoptotic effects of Metformin, suggesting that other pathways could be possible affecting viability and survival of leukemia cells. Metformin decreased mitochondrial activity, oxygen consumption and mitochondrial
ATP synthesis, while presumably stimulated glycolysis for ATP and lactate production, pentose phosphate pathway for purine biosynthesis, fatty acid metabolism, as anaplerotic and mitochondrial gene expression. Altogether this data proposes that this compound is generating different and deep changes in leukemia metabolism and that could be more effective in early stages of the disease, when seemingly the cells are consuming more oxygen and are more mitochondrial active.

The complex interactions between leukemia cells and their microenvironment cause multiple changes in the cancer cells specially at the metabolic level, generating a great need for the development of new drugs or the repurposing of old ones to target such alterations. The use of single agents and the combination with chemotherapeutics can improve the responses of leukemia patients to reach a complete and stable remission.
CHAPTER 3

OBJECTIVES

GENERAL OBJECTIVES:
Characterize metabolic alterations induced by the leukemia microenvironment to expand our knowledge of the disease and permit the discovery of new targets as the development of new therapies.

SPECIFIC OBJECTIVES

- Characterize the metabolic effects of platelets in leukemia cells and their role in cell survival.
- Characterize the metabolic effects of Metformin in leukemia cells and determine how this agent alone or in combination affects leukemic cell survival.
- Determine if agents targeting hypoxic or acidic leukemia cells can induce apoptosis or improve mice survival.
HYPOTHESIS

- Leukemia cells can interact with platelets and induce platelet activation.

- Factors released by platelets or platelets themselves can induce leukemia cell survival by promoting mitochondrial uncoupling in leukemia cells.

- If fatty acid dependent mitochondrial uncoupling and increased electron transport is a common pro-survival mechanism in leukemia cells, pharmacologic inhibition of electron transport ought to sensitize leukemia cells to cell death.

- If there is an increase in O2 consumption (presumably due to high electron transport activity) and consequently the generation of intracellular hypoxia and glycolysis, we could target the hypoxic and acidic leukemic microenvironment with hypoxia activated pro-drugs and/or pH sensitive peptides.
CHAPTER 4

MATERIALS AND METHODS

4.1 Cell lines, Chemicals, and Biochemicals.

Different leukemia cell lines were provided by the Section of Molecular Hematology and Therapy of the University of Texas MD Anderson Cancer Center to develop the present work. The cells lines were pathogen tested and authenticated through STR (Short Tandem Repeat) method on 2010 at the same center. The cell lines kindly provided were OCI-AML3 (Human Acute Myeloid Leukemia), MOLT4 (Acute Lymphoblastic Leukemia), REH (Acute Lymphocytic Leukemia, non-T, non-B), NALM-6 (Human B cell precursor Leukemia) and KBM5 (Chronic Myeloid Leukemia). All culture reagents were obtained from Gibco (Life Technologies, Grand Island NY). Cells were maintained in RPMI that was supplemented with 10% fetal calf serum (FCS), 1% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mg/L amphotericin B in a 37°C incubator containing 5%CO2. Mesenchymal stromal cells (MSC) were obtained from lipoaspirates of patients undergoing cosmetic liposuction as previously described (Bieback K, 2008). BM-MSCs were isolated from healthy donors who were undergoing BM harvest for use in allogeneic BM transplantation (Jacamor R, 2014). In some experiments 2 U/ml sodium enoxaparin (Clexane, Sanofi Aventis, France) was used to supplement the culture medium prior to addition of PL (Platelet lysates) or PRP (Platelet Rich Plasma) to prevent clot formation. The antibodies Anti-CD62 conjugated to FITC, anti-CD90 conjugated to APC, and Annexin V conjugated to FITC were obtained from EBiosciences (San Diego, CA). LipidTox Green for neutral lipids and LipidTox Red for charged lipids, Tetramethyl Rhodamine Methyl Ester (TMRM), dyhydroethidium (DHE), JC-1, CellTracker Green (CTG) and nonyl acridine-orange (NAO) were obtained from Invitrogen (Carlsbad, CA). Rotenone, sodium cyanide, Metformin and Phenformin were obtained from Sigma-Aldrich and dissolved in DMSO and water respectively. ABT-737 was synthesized at the University of Texas MD Anderson Cancer center based on the published structure and dissolved in DMSO. CDDO-ME was kindly provided by Dr Michael Andreeff from the same cancer center.
4.2 Preparation of Platelet rich plasma (PRP), platelet lysate (PL), and platelet supernatant (SP).

Whole citrated blood was centrifuged and PRP was prepared with minor modifications as previously described (Arguelles, 2006). Healthy volunteers signed an informed consent to donate blood into an ACD (solution A) vaccutainer tube (Beckton Dickinson, Franklin NJ). Right after extraction, tubes were centrifuged at room temperature 800xg for 8 minutes, and the bottom 50% of plasma volume was removed with a plastic transfer pipette taking care to avoid the plasma/blood interface, this plasma was considered rich in platelets (PRP). PRP routinely contained 2.6 +/- 0.6 x 10^6 platelets/µL (Woodell-May JE, 2005). In some experiments where heparin (anticoagulant) was not used, PRP was exposed to leukemia cells (1x10^5/mL) until clot formation occurred and culture media was centrifuged, filtered, and used as platelet supernatant (SP) to resuspend fresh leukemia cells. Lastly and to obtain PL, pooled units (n=8) of platelets submitted to 3 cycles of freeze/thaw were centrifuged at 2000xg for 5 minutes, and the subsequent extraction of buffy coat derived platelets was performed. In general buffy coat derived platelet bags contained an average of 1 x 10^6 platelets/µL. The pooled units, recently (<24 h) expired bags were a kind gift of the Colombian Red Cross.

4.3 Determination of platelets activation through CD62P expression and Annexin V staining.

2.5% PRP was added in 300 µl of RPMI medium, exposed to 1% Calcium gluconate (Inductor of Platelets Activation) or increasing numbers of MOLT4 or OCI-AML3 cells (50, 100, or 250 x 10^3/mL) for 1 hour under previous described standard culture conditions. After incubation time, cultures were stained with CD62P (Lu Q, 2011) or Annexin V-FITC (1:100 dilutions) (Tzima E, 2000) for 30 minutes on ice; washed with PBS 1X and the expression of the different markers was determined by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) using a 488-nm argon ion excitation laser gating on platelets by FSC and SSC parameters.
4.4 Determination of apoptosis and mitochondrial damage by flow cytometry.

After determined treatments, cells were harvested, washed two times in PBS and resuspended in 100 μl Annexin binding buffer containing a 1:100 dilution of Annexin V-FITC and 50 nmol/L tetramethyl-rhodamine methyl ester (TMRM). For those experiments including MSCs, a dilution (1:100) of anti-CD90 APC-conjugated antibody was added. CD90 is a marker of MSC and was used to distinguish them from leukemia cells that are negative for the marker. Cells were analyzed by flow cytometry in a FACS Calibur flow cytometer or a Guava EasyCyte 6-2L capillary cytometer (Merck, Millipore) using a 488-nm argon ion and 633-nm HeNe excitation lasers. Annexin-FITC (+) cells were considered apoptotic and TMRM (-) cells were considered with mitochondrial damage. Both parameters were quantified as percentage. The percentage (%) cell death was determined as 

\[
\text{Percentage of cell death} = \left( \frac{\text{TMRM}^- \text{cells}}{\text{TMRM}^- \text{cells} + \text{TMRM}^+ \text{cells}} \right) \times 100.
\]

4.5 Determination of mitochondrial membrane potential (ΔΨM) and flurometric oxygen consumption.

Mitochondrial membrane potential was determined using the ratiometric probe: JC-1. A solution of 5 mg/mL JC-1 in DMSO was diluted 1:100 in pre-warmed RPMI medium with vigorous vortexing and added directly to the cell culture to further dilute 1:100. The cells were incubated for 15 min under standard previously described culture conditions. After incubation time, the cells were harvested and washed once in PBS and the mitochondrial membrane potential was determined by flow cytometry as previously described (Cosarrizza A, 1993). For oxygen consumption determination, OCI-AML3 or MOLT4 cells (2x10^5 cells in 100μl of RPMI medium) were seeded in 96-well BD oxygen biosensor plates (BD Biosciences, Bedford MA) and treated with different concentrations of PRP or PL for 30 mins. The wells were covered with 2 drops of mineral oil and incubated at 37°C for 4 hours preceding to record fluorescence (Ex485, Em590; bottom optic) in a Fluostar Optima plate reader (BMG, Germany). Extra-mitochondrial (background) oxygen consumption was
corrected by subtracting the signal intensity from wells treated with 2 mM NaCN (Inhibitor of respiration). Results were expressed as arbitrary fluorescent units (AU).

**4.6 Determination of oxygen consumption by flow cytometry.**

Microfuge (1.5 mL) tubes were used to seed leukemia cells (2.0 x 10⁶/ml) in complete RPMI medium and expose them to Rotenone or Metformin (mitochondrial complex I inhibitors) at the indicated doses for 2 h at 37°C, 5% CO₂. Untreated samples were used as controls and sodium cyanide (5 mM) as a positive control of respiration inhibition. Hypoxia marker Pimonidazole (Chemicon International, Temecula, CA) was added (100 μmol/L) to all samples, except to control (untreated) sample which was used to assess background staining of the anti-pimonidazole antibody. After addition of pimonidazole, cell suspensions were covered with 500 μl of mineral oil and further incubated for 2 h at 37°C. After incubation time, a micropipetor was inserted through the mineral oil, taking care to eject any stuck oil, and the cells were recuperated slowly, being careful not to collect any oil. The cells were transferred to new microfuge tubes and centrifuged (1200 g, 5 min) to be washed once in PBS, resuspended in 250 μl of 1.6% formaldehyde (in PBS), and incubated at room temperature for 15 min. After fixation, the cells were centrifuged (1200 g, 5 min), resuspended in 1000 μl of 100% methanol, and incubated at -20°C for 1 h to overnight. 1 hr later or the next day, the cells were centrifuged (1200 g, 5 min), washed twice in PBS supplemented with 2% FCS, and incubated for 1 hr at room temperature with 100 – 1,000 μl of a 1:200 - 1:500 dilution of anti-pimonidazole antibody (Chemicon International, Temecula, CA) in PBS supplemented with 2% FCS and 0.002% sodium azide. The cells were washed twice in PBS and analyzed by flow cytometry in a Guava EasyCyte 6-2L capillary cytometer or a FACSCalibur cytometer, using a 488-nm argon ion excitation laser and acquiring the fluorescent emission at 525 nm. Results were expressed as mean fluorescent intensity (MFI).
4.7 Determination of oxygen consumption and extracellular acidification rate by Seahorse system.

KBM5 and REH cells (0.4X10^6 cells/ml in 10 ml) were seeded in T75 flasks during the time of treatment (2 hrs) in substrate-limited medium as suggested by the manufacturer. After incubation time the cells were harvested, transferred to 15 ml tubes and spun down, substrate-limited medium discarded and the pellet re-suspended in FAO (Fatty Acid Oxidation) medium. The cells were seeded in XF96e Seahorse Biosciences plates at a concentration of 0.2-0.3 million of cells/well. Fifteen minutes before the initial measurement, Etomoxir (40 μM) was added to the control wells and immediately before the start of data acquisition, substrates were added to the specific wells. Oxygen Consumption (OCR) and extracellular acidification rate (ECAR) were determined per the manufacturers’ instructions.

4.8 Determination of superoxide production.

After determined treatments, cells were stained with 500 nmol/L of DHE (Dihydroethidine) during 30 minutes in a 37°C incubator containing 5%CO2. After this time, 1 μmol/L rotenone was added or not as a control of oxidative stress induction and the samples were incubated during additional 30 minutes. At the end of the incubation time, the cells were washed twice in PBS, and fluorescent emission at 590 nm analyzed by flow cytometry in a FACS Calibur or a Guava EasyCyte 6-2L capillary cytometer using a 488-nm argon ion excitation laser. Results were expressed as mean fluorescent intensity (MFI).

4.9 Determination of neutral or charged lipid content.

After appropriate treatments, cells were stained with LipidTox neutral (green) or charged (red) stains for 45 minutes in a 37°C incubator containing 5%CO2, followed by two washes in PBS.
Fluorescent emission at 520 nm and 590 nm was analyzed by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) or a Guava EasyCyte 6-2L using a 488-nm argon ion and 633-nm HeNe excitation lasers. Results were expressed as mean fluorescent intensity (MFI).

4.10 Determination of mitochondrial phospholipid mass.

After determined treatments, cells were stained with 100 nmole/L of nonyl-acridine-orange (NAO) during 30 min in a 37°C incubator containing 5% CO2, followed by two washes in PBS. Fluorescent emission at 525 nm was analyzed by flow cytometry in a Guava EasyCyte 6-2L capillary cytometer (Merck, Millipore) using a 488-nm argon ion excitation laser. Results were expressed as mean fluorescent intensity (MFI).

4.11 Determination of reduced glutathione (GSH) content.

After determined treatments, cells were loaded with 200 μmole/L of CellTracker Green for 30 min on ice, followed by two washes in cold PBS. Fluorescent emission at 525 nm was analyzed by flow cytometry in a Guava EasyCyte 6-2L capillary cytometer (Merck, Millipore) using a 488-nm argon ion excitation laser. Results were expressed as (MFI).

4.12 Bax and Bak crosslinks.

Bax and Bak crosslinks were investigated as previously described (Samudio, 2010). After exposure to the treatments, mitochondrial extracts were generated by hypotonic lysis and resuspended in 150 mM NaCl, 10 mM HEPES (pH 7.4) and 1% CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) at 1 mg/ml of protein. Treatment with 0.4 mM bismaleimidohexane (Thermo Scientific) was done during 1h at room temperature. Lysates (12.5 μg of protein per well) were separated in a 12% polyacrylamide gel using electrophoresis, followed by protein transfer to polyvinyl difluoride (PVDF) membranes (Thermo Scientific). The membranes were then incubated with Bax (clone D2E11 Rabbit mAb; Cell Signaling Technology) or Bak antibody (clone D2D3 Rabbit mAb, Cell Signaling Technology) and the signals were detected by immunofluorescence,
using fluorochrome conjugated anti-rabbit secondary antibodies and the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

4.13 Bcl-2 and Mcl1 detection.

After exposure to the treatments, cells were harvested, counted and 1 million of them re-suspended in 2X Laemmli Buffer (4% SDS, 20% Glycerol, 10% 2-Mercaptoethanol, 0.004% Bromophenol Blue and 0.125M Tris HCL). Afterwards, denaturation was done at 95°C during 15 min. 20µl of protein were separated in a 12% polyacrylamide gel using electrophoresis, followed by protein transfer to polyvinyl difluoride (PVDF) membranes (Thermo Scientific). The membranes were then incubated with mouse monoclonal anti Bcl-2 antibody (Dako, Carpantaria, CA), rabbit monoclonal anti Mcl-1 antibody (D35A5, #5453, Cell Signaling Technology) and mouse monoclonal anti-β-actin (Sigma-Aldrich). Signals were detected by immunofluorescence, using fluorochrome conjugated anti-rabbit and anti-mouse secondary antibodies and the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

4.14 Primary samples.

For in vitro studies bone marrow or peripheral blood samples were obtained from patients with AML or ALL. Informed consent was obtained and samples collected during routine diagnostic procedures. Mononuclear cells were separated by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation and used immediately. Frozen samples were obtained from the hematology cell bank at the British Columbia Cancer Agency under protocol H12-00727. All experiments with primary samples were performed in triplicate.

4.15 Animal Studies.

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.
For *in vivo* hypoxia determination and efficacy study of TH-302, NSG (NOD/Scid/IL2Rγ-KO) mice were injected with one million (per mouse) of primary AML cells and the progression of the disease was followed through the detection of human CD45+ cells in peripheral blood by flow cytometry. After engraftment (~5% human CD45+ cells at Day 37), the mice were treated with TH-302 (50 mg/kg intraperitoneally 3 times a week for 3 weeks) or PBS. The absolute number of circulating AML cells was determined at week 2 and 3. At the end of the treatment and 3 hours prior sacrifice, two mice per group were injected via i.p with a solution of pimonidazole to reach a final concentration into the mice of 100mg/kg. After this time the mice were humanly euthanized and further immunohistochemistry was done in the bone marrow to establish hypoxia levels. The remaining number of mice were followed till each mouse reached a 25% of human CD45+ cells in peripheral blood or till they became morbid or had a weight loss of 20% or greater; In this moment the mice were euthanized.

For metabolic studies *in vivo*, C57Bl/6 mice were injected with a million of AML1/ETO-luc-GFP cells. The progression of the disease was followed by bioluminescence imaging in a Xenogen IVIS-200 System machine able to detect luminescence and fluorescence. After 3 weeks and prior to image, each mice was anesthetized, distributing 2% isoflurane in oxygen and circulation warm water; and catheterized via tail vein to deliver a solution of 80 mM hyperpolarized [1-13-C] pyruvate. The images were taking in a 7T Bruker MR Scanner using a dual tuned 1H/13C volume coil. Slice selective 13C spectroscopy was utilized to capture the 13C signal in the two femurs after hyperpolarized pyruvate injection. Vital signs were carefully monitored throughout the experiments.

For the blast crisis in a lethal CML model; inducible, transgenic Scl-tTA-BCR-ABL mice in the FVB (Friend Virus B)/N background (Huettnner CS, 2003; Koschmieder S, 2005) were crossed with transgenic GFP-expressing mice (FVB.Cg-Tg [ACTB-EGFP] B5Nagy/J, Jackson Laboratories), in which the characteristic expression of the fusion BCR-ABL gene is controlled by a tetracycline (tet-off system) regulated enhancer of the murine stem cell leukemia (Scl) gene (Huettnner CS, 2003; Koschmieder S, 2005), allowing to target the fusion gene expression in stem/progenitor cells.
Withdrawal administration of tetracycline allows the expression of BCR-ABL1 and its addition reverses the expression, providing evidence of the dependence on BCR-ABL1 to develop leukemia (Huettner CS, 2000).

For the aggressive MOLM 13 AML model, NSG mice were irradiated (250 cGy) one day before intravenous injection of a million of leukemic cells. The progression of the disease was followed by flow cytometry detecting human CD45+ cells. Due to the aggressiveness of the model the experiments with these mice were started at day 10 of leukemic cells injection.

4.16 Immunohistochemistry.

The diverse organs were extracted and fixed in 4% of formalin and sent to the histology core of the MD Anderson Cancer Center to get numerous sections 5μm thick. These sections were stained with hematoxylin eosin or DAPI (4',6-diamidino-2-phenylindole) depends on the type of microscopy that was going to be done, light microscopy or fluorescence microscopy respectively.

For hypoxia detection in formalin-fixed, paraffin-embedded bone marrows, the sections were deparaffinized in xylene (3 times for 5 minutes) and rehydrated through graded concentrations of alcohol for 5 minutes each. For antigen retrieval, tissue sections were heated in EDTA (Biocare Medical, Concord, Calif) for 30 minutes in a Decloaking Chamber (Biocare Medical, Concord, Calif) and incubated with Biocare Medical peroxidase blocking reagent for 5 minutes. After washing with buffer, tissue sections were incubated with primary antibodies for 60 minutes at room temperature, to detect pimonidazole adducts. Afterwards, sections were washed and incubated with secondary antibodies using a Mach 4 AP Universal Polymer kit (Biocare Medical) according to manufacturer's instructions. To visualize the signals, Vulcan Fast Red chromogen (Biocare Medical) was used for 10 minutes. All sections were counterstained with hematoxylin (Dako, Carpintera, Calif) for 3 minutes. Slides were then air dried and cover-slipped. Slides were analyzed under a 60x/1.40 PlanApo objective lens on an Olympus FV500 confocal microscope with Fluoview version 4.3 software (Olympus) (Benito J, 2011).
4.17 pH sensitive peptides (pHLIP) studies.

All the variants used in this study were kindly provided by Dr Yana Reshetnyak from the University of Rhode Island (Kingston, RI, USA). The constructs that were used; WT-pHLIP -Alexa 647, WT-pHLIP-Alexa 750 and pHLIP-Amanitin share the same WT-pHLIP sequence: ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT (Var0-WT) (Weerakkody D, 2013; Moshnikova A, 2013). Each marker (Alexa 647- Alexa 750) or toxin (Amanitin) was conjugated with Cys of WT-pHLIP and purified using HPLC (High Performance Liquid Chromatography). The peptides were stored at -20°C till used. The concentration for in vitro experiments varied according to the parameter to measure: (Colocalization or viability and apoptosis) and the concentration for animal experiments was always 40 uM in PBS injected via I.V.

For in vitro detection of the peptide a Gallios flow cytometer was used. Fluorescent emission at 650 nm was acquired with a 635 nm Red Diode excitation laser. Results were expressed as (MFI).

For in vivo imaging and detection of the peptides we used a Xenogen IVIS-200 System which is able to detect several fluorescence signals including Alexa Fluor 647 and 750 with its filter set 2 (Ds Red) and 3 (Cy5.5) respectively.

For tissue visualization and peptide detection, the diverse organs were extracted, fixed in 4% of formalin and sent to the histology core of the MD Anderson Cancer Center to get numerous sections 5μm thick. Formalin-fixed, paraffin-embedded bone marrows were deparaffinized in xylene (3 times for 5 minutes) and rehydrated through graded concentrations of alcohol for 5 minutes. Slides were then air dried and stained with a solution of 300 nM DAPI during 5 minutes protecting the slides from light. After the incubation time the stain solution was removed and the slides were washed 3 times with PBS. Slides were then air dried and one drop of ProLong Gold Antifade Mountant (ThermoFisher Scientific) was added. The slides were cover slipped for further co-
detection of pHLIP and DAPI signals under a Vectra 3.0 automated quantitative pathology imaging system, with an inForm\textsuperscript{®} analysis software.

4.18 Statistics.

Results were expressed as mean ± SD of 3 independent experiments, unless otherwise indicated. $P$ values were determined by 1-way ANOVA followed by $F$ statistics, or unpaired student t-test. A $P$ value less than 0.05 was considered significant.
5.1 Leukemia Cells Promote Platelet Activation. (Velez J et al, 2014).

To explore and validate previous reports suggesting the interaction of platelets with leukemia cells in vitro (Bruserud OB, 1997; 1998) and their subsequent activation, we decided to culture and expose OCI-AML3 (acute myeloid) and MOLT4 (acute T lymphoblastic) leukemia cell lines to increasing doses (%v/v) of platelet rich plasma (PRP); using as a control of platelets activation calcium gluconate (1 % v/v). The exposition of PRP to leukemia cells ended up producing clot formation even in the presence of heparin (Anticoagulant-Inhibitor clot formation) and independently of the addition of calcium gluconate, leading us to explore the possibility that leukemia cell lines alone were able to induce platelets activation. To corroborate this hypothesis we performed flow cytometry to determine CD62P (P-Selectin) expression and phosphatidyl serine externalization, reported markers of platelets activation (McEver RP, 1989; Tzima E, 2000). In a cell dose-dependent manner, MOLT4 cells significantly ($p<0.05$) promoted the expression of CD62P in live platelets and OCI-AML3 promoted externalization of phosphatidyl serine in live platelets (Figure 1A and 1B respectively), supporting the previous work stated by Bruserud et al; where normal platelets released soluble P selectin when cultured alone or in the presence of AML blasts (Bruserud Ø, 1998), suggesting that cell-platelet interactions and/or soluble factors in leukemia cells culture activated platelets. To further examine if platelet derived factors released after activation can influence the proliferation of leukemia cells, OCI-AML3 and MOLT4 were exposed during 96 hrs to increasing doses of PRP or platelet lysate (1, 2.5, and 5 %) in the presence of heparin (to avoid interference from clots during cell counting and or flow cytometry); and viable or dead cells were counted in a Neubauer chamber at the end of the incubation time. Contrary to the effect of live platelets reported for AML blasts (Bruserud Ø,1998), and platelet lysates (PL) for MSC (Schallmoser K, 2009) and solid tumors (Cowan DH, 1983), neither PRP (from 3 healthy individuals) nor PL significantly affected the viability of OCI-AML3 or MOLT4 cells (Data not shown).

Due to the mitochondrial reported effects of soluble mediators (TGF-β and PDGF) that are abundantly released by active platelets, we decided to measure several mitochondrial parameters in the leukemia cells after being exposed to platelet components. These parameters included: mitochondrial membrane potential ($\Delta \Psi M$), oxygen consumption, neutral and charged lipid content and superoxide production. The mitochondrial membrane potential was monitored by flow cytometry utilizing the ratiometric potential sensitive dye JC-1 as previously described (Samudio I, 2008). OCI-AML3 and MOLT4 cell lines were exposed during 2 hrs to 2.5% PL, stained with JC-1 as described in methods and analyzed by flow cytometry. Representative contour plots are shown. The JC-1 aggregates fluorescence (FL2:H) is shown in Y axis and JC1 monomers fluorescence (FL1:H) is shown in X axis (Figure 1C). Intriguingly, the exposure to 2.5 % PL reduced $\Delta \Psi M$ by ~80 % in both MOLT4 and OCI-AML3 cells, resembling of the decrease observed in OCI-AML3 cells cultured on MSC feeder layers (Figure 1D), suggesting that platelet contents – like MSC feeder layers – directly and strongly modulate mitochondrial metabolism. Further experiments demonstrated that the effect seen with PL was reproducible with SP and induced a significant ($p < 0.001$), dose and time dependent decrease in $\Delta \Psi M$ (Figure 1E and 1F), again as observed for OCI-AML3 cells cultured on MSC feeder layers (Samudio I, 2008). The significant ($p < 0.0001$) loss of $\Delta \Psi M$ occurred rapidly, within 30 min of exposure to platelet supernatants (SP; $p < 0.001$), inducing a deep mitochondrial depolarization at 18 h (Figure 1F). Similar dose-dependent effects on $\Delta \Psi M$ were observed in MOLT4 cells treated with increasing doses of PL for 4 h (Figure 1G). Although Harrison et al, have previously reported the platelets release significant amounts of TGF-β1, the effects of SP on $\Delta \Psi M$ were not reversed by pharmacological treatment with the potent and specific inhibitor of the TGF-β receptor superfamily, SB-431542 (Figure 1H). Suggesting the presence of an additional abundant factor able to induce critical changes in the $\Delta \Psi M$. For further characterization of the possible factor involved in such change in the $\Delta \Psi M$, we filtered (0.2 μm) and heat inactivated (65 °C for 1 h) SP hoping to inhibit or decrease the effect over the, nevertheless and after the mentioned interventions;
SP retained its $\Delta \Psi M$ reducing effect $\Delta \Psi M$, indicating that platelets contain or release a small and heat insensitive mitochondrial depolarizing factor (Figure 1H).

To then investigate if the loss in $\Delta \Psi M$ is a consequence of reduced electron transport, we examined oxygen consumption in OCI-AML3 cells exposed to increasing doses of PL for 30 min. As shown in Figure 2A, OCI-AML3 cells significantly ($p < 0.005$) increase their oxygen consumption capacity after exposure to PL, supporting the notion that instead to induce a damage in the oxidative capacity of leukemia cells mitochondria, platelet contents could be promoting mitochondrial uncoupling. Similar results were observed in MOLT4 cells (data not shown). To further investigate if the increase in oxygen reducing capacity in OCI-AML3 cells exposed to PL was dependent on the oxidation of fatty acids (Substrate that utilized high amounts of oxygen to be oxidized), we measured basal oxygen consumption, with SP and with the fatty acid inhibitor Ranolazine (200 µM) (Figure 2B). As expected, SP increased oxygen consumption, which was sensitive to Ranolazine; reducing significantly ($p < 0.005$) the oxygen consumption capacity to control levels. In support of this data and consistent with previous reports that uncoupled mitochondria depend on fatty acids to support oxygen reduction capacity (Samudio I, 2010); the exposure of leukemia cells to several concentrations of PL (1-2.5-5%) induced a decrease in phospholipid and triglyceride levels, being significant ($p < 0.005$) at the highest concentrations (2.5-5%) for neutral lipids only (Figure 2C). As mitochondrial uncoupling has been reported to reduce the production of reactive oxygen species (Barreiro E, 2009; Oelkrug R, 2010), we examined superoxide production in OCI-AML3 cells by flow cytometry. As showed in Figure 2D, PL promoted a decrease in basal (maximal reduction of 36 %, $p < 0.005$) and rotenone-induced (maximal reduction of 27 %, $p < 0.05$) superoxide levels in a dose dependent manner. All together this data support the notion that platelet contents promote mitochondrial uncoupling.

5.3 Platelet Contents Promote Resistance to Intrinsic Apoptosis in Leukemia Cells and Primary Samples. (Velez J et al, 2014).
As previous reports evidenced the capacity of platelets derived factors to promote solid tumor progression (Brockmann MA, 2011; Ringvall M, 2011, Demers M, 2011) and mitochondrial uncoupling has been correlated with an impairment in the induction of oxidative stress, necessary to trigger cell death in certain tumor models (Pancreas and Colon) (Pozza E, 2012); we decided to determine if PL could affect the response of leukemia cells to chemotherapeutics or mitochondriotoxics. The exposure of leukemia cells to 2.5 % PL compromised the effect of mitochondriotoxic agents ABT-737 or CDDO-ME but not the effect of traditional leukemia chemotherapeutics doxorubicin and AraC (Cytarabine). 2.5 % PL mediated significant (p<0.05) resistance to apoptosis (Figure 3A and 3B) and mitochondrial damage (TMRM-negative cells; Figure 3C and 3D) induced by ABT-737 in both MOLT4 and OCI-AML3 cells, proposing the notion that platelet contents specifically oppose activation of the intrinsic apoptotic pathway in leukemia cells. To verify that the protection effect was dependent of platelet derivatives we exposed OCI-AML3 to increasing doses of PRP and treat them with a single dose of ABT-737 (500 nmol/L). As evidenced in Figure 3E, PRP protected OCI-AML3 from apoptosis induction in a dose dependent manner, confirming our proposed hypothesis. This data was reproducible when we used CDDO-ME, a synthetic triterpenoid able to induce direct permeabilization of the inner mitochondrial membrane to induce swelling and release of apoptogenic factors (Samudio I, 2006). MOLT4 and OCI-AML3 cells were exposed to 2.5 % PL and CDDO-ME, and as shown in figure 4A and 4B, PL significantly (p<0.0005) protected leukemia cells from apoptosis induction and mitochondrial damage (Figure 4C and 4D) induced by CDDO-ME; supporting the hypothesis that platelet derived factors also antagonize mitochondrial permeability transition-induced apoptosis. Again and to verify that the protection effect was dependent of platelet derivatives we exposed OCI-AML3 cells to increasing doses of PRP and treat them with a single dose of CDDO-ME (500 nmol/L). As evidenced in Figure 4E, PRP conferred similar antiapoptotic effects in a dose dependent manner, confirming our proposed hypothesis. To investigate if platelets could promote resistance to apoptosis induction in primary leukemia blasts we tested 4 ALL and 2 AML CD34(+) blasts in response to ABT-737 or CDDO-ME (Figure 5A-5F), having as a result that prosurvival effects conferred by platelet lysates are not limited to immortalized cell lines. The clinical characteristics of
the samples analyzed are summarized in Table 1, and suggest that the observed effects are not specific of a particular phenotype, molecular or cytogenetic group. We theorize as well that the less impressive effects observed on all primary samples compared to cell lines, could be due to an increase in sensitivity of cell lines to platelet derived components and/or as a consequence that primary samples have been already exposed to platelet components in the in vivo setting.

To further elucidate the mechanism by which platelets derivatives support resistance to mitochondriotoxic agents, we exposed OCI-AML3 cells to 2.5% PL and ABT-737 (500 nmol/L) during 16 h. After incubation time, mitochondrial extracts were obtained, exposed to bismaleimidohexane (reported croslinker) and Bax crosslinking was determined by Western Blot. The reduced formation of higher molecular weight Bax immunoreactive bands in response to ABT-737 after the exposition to 2.5% PL (Figure 6A), support the notion that platelet derivatives affect directly the formation of the mitochondrial pore to allow the release of apoptogenic factors (AIF, Cytochrome C). This data was in accordance with previous observations demonstrating that fatty acid oxidation and mitochondrial uncoupling counteracted oligomerization of Bax in leukemia cells.

Taken together, our results propose that platelet contents contribute to resistance to activation of the intrinsic apoptotic pathway via mitochondrial uncoupling, an alteration that was previously described to be induced by MSCs (Samudio I, 2008) and that is characterized for the loss of mitochondrial membrane potential, increase in oxygen consumption dependent of fatty acid oxidation, decrease in superoxide production and acquisition of resistance to mitochondriotoxic agents.

Knowing then that fatty acid oxidation and mitochondrial respiration are active processes in leukemia cells and that can promote survival and resistance to mitochondriotoxic agents, we decided to explore the possibility that the use of mitochondrial inhibitors as Metformin could affect directly (respiration) or indirectly (fatty acid oxidation) processes to sensitize leukemia cells to apoptosis.
5.4 Metformin inhibits mitochondrial electron transport in leukemia cell lines and primary samples. (Velez J et al 2016).

Metformin has been described to be able to inhibit mitochondrial respiration in hepatocytes and leukemia cell lines (Foretz M, 2010; Scotland S, 2013), however the effect in the respiration of primary leukemia samples have not yet been described. To validate the targeting of the mitochondria by Metformin, we developed a novel flow cytometric method employing the oxygen sensitive probe pimonidazole to determine mitochondrial respiration. We reasoned that if we limited air exchange by covering cell cultures with mineral oil, oxygen consumption would result in cellular and local hypoxia which could be quantitated by measuring pimonidazole adducts -which were formed due to the decrease in oxygen tension <10mmHg as a consequence of its consumption- by immunofluorescence flow cytometry. To test this reasoning, NALM-6 and REH leukemic cell lines were exposed to 10 mmole/L Metformin (Mitochondrial complex 1 inhibitor), 1 μmole/L rotenone (Mitochondrial complex 1 inhibitor) or 4 mmole/L sodium cyanide (Mitochondrial complex IV inhibitor) for 1 hr, followed by addition of 100 μmole/L pimonidazole and processed as described in Materials and Methods. As presented in Figure 7A, a short (1 hr) exposure to Metformin inhibited the accumulation of pimonidazole adducts (p<0.05) in both REH and NALM-6 cells to a similar degree as rotenone or sodium cyanide (>90%), indicating that our methodology satisfactorily monitor the effects of mitochondrial inhibitors on the molecular reduction of oxygen in leukemia cell lines. To further investigate the pharmacology of Metformin and if its effect was expandable to other cell lines, KBM5, OCI-AML3, NALM-6, and REH cells were exposed to increasing concentrations (1, 5, and 10 mmol/L) of Metformin for 1 hr and oxygen consumption determined as above. As evidenced in Figure 7B, Metformin rapidly inhibits oxygen consumption, showing different IC50 values dependent of the cell line 1.5 mM (NALM-6), 2.5 mM (REH), 3.2 mM (OCI-AML3) and 6 mM (KBM5). This data corroborated previous reports where a different system was used to measure oxygen consumption (OxygenBiosensor plates) (Samudio I, 2008) (Supplemental Figure 1). Likewise, the effect caused by Metformin was also observed in 3 primary leukemia samples (Fig 7C; gating in sample #2 is on CD34 (+) blasts), implying that this biguanide can inhibit mitochondrial
oxidative metabolism even in primary leukemia cells. Since milli-molar concentrations of Metformin may not be achievable \textit{in vivo}, we questioned if micromolar concentrations of this agent and the related biguanide Phenformin on oxygen consumption in leukemia cells was similar. As evidenced in Figure 7D, Phenformin (50 \mu mols/L) was 10-fold more potent inhibiting \~40\% of oxygen consumption, while Metformin significantly inhibited oxygen consumption just when it reached a concentration of 500 \mu mols/L; implying that biguanides inhibit molecular reduction of oxygen in leukemia cells with different potency.

5.5 \textbf{Inhibition of electron transport by Metformin is associated with accumulation of triglycerides. (Velez J et al 2016).}

As previously described the electron transport chain in leukemia cells is largely dependent upon FAO (Samudio I, 2010). We then hypothesized that inhibition of electron transport chain would promote accumulation of triglycerides in these cells. To assess this we treated NALM-6 and REH cells with Metformin (10 mmole/L) and rotenone as a control (1 \mu mole/L) for 16 h and determined accumulation of triglycerides with the neutral lipid sensitive stain LipidTox via flow cytometry. Metformin and rotenone significantly \((p<0.05)\) increased neutral lipids in both cell lines (Figure 8A). In support of this data Seahorse XF96 measurements demonstrated that Metformin partially inhibited the oxidation of palmitate in KBM5 and REH cells (Supplemental Fig 2 and 3), suggesting that this fatty acid could be accumulating inside leukemia cells. To additionally determine how early the accumulation of neutral lipids was evident, we exposed KBM5, NALM-6 and REH cell lines to Metformin and measured the concentration of neutral lipids as described. The accumulation of neutral lipids was seen as 4hr and in a dose-dependent manner (Figure 8B), However, OCI-AML3 cells didn’t accumulate neutral lipids at 4 h or 16 h (data not shown) indicating that Metformin could be activating lipolysis (Via Acetyl CoA Carboxilase inhibition) in addition to electron transport inhibition. Taken together, these observations support the notion that inhibition of electron transport in leukemia cells may antagonize FAO.
Metformin causes alterations in mitochondrial mass and promotes superoxide generation in leukemia cells. (Velez J et al 2016)

Since inhibition of mitochondrial respiration by Metformin caused what it seems to be a blockage of FAO, we predicted an increase flux of fatty acids into phospholipid synthesis what could affect mitochondrial lipid mass directly due to its principal phospholipid component: Cardiolipin. In a dose dependent manner and after treatment with Metformin for 4 h there was a significantly increased in the mitochondrial mass in leukemia cell lines as monitored by flow cytometry using the fluorescent dye nonyl-acridine orange (NAO) (Figure 8C). This data was reinforced by the use of rotenone, which similarly increased NAO accumulation (Figure 8D), supporting the notion that inhibition of electron transport may be associated with structural changes in the mitochondrial membranes of leukemia cells. As fatty acid accumulation has been reported to increase production of superoxide anions (Kohli R, 2007; Koppers AJ, 2010) and because Metformin inhibits complex I of the respiratory chain (a described major site of superoxide production when inhibited) (Owen MRE, 2000), we inquired if Metformin could promote reactive oxygen species (ROS) generation in leukemia cells. As evidenced in Figure 9A, Metformin significantly increased superoxide production in KBM5, REH and OCI-AML3 cells. However, Phenformin was again more potent than Metformin inducing superoxide production from OCI-AML3 cells (Figure 9B) even at doses as low as 50 μmoles/L. Notably and although Metformin inhibited O2 consumption of NALM-6 cells, these cells were resistant to the pro-oxidant effects of both rotenone and Metformin, alone or in combination (Figure 9C); implying that mitochondria in these cells do not produce superoxide in response to complex I inhibition, fatty acid accumulation, or that their superoxide dismutase activity could be remarkably high. Lastly and since Metformin increased superoxide production and has been reported to antagonize one-carbon metabolism (required for the de novo synthesis of Glutathione, the principal cellular antioxidant) (Corominas FB, 2012), we rationalized that the treatment with Metformin should diminish reduced Glutathione (GSH) levels in leukemia cells. As shown in Figure 9D, Metformin significantly (p < 0.05 for 5 and 10 mmol/L as compared to control) reduced GSH levels by 40% in REH cells, which augmented superoxide production in response to Metformin.
However NALM-6 cells ($p < 0.05$ for 5 and 10 mmol/L as compared to control) which did not produce superoxide in response to Metformin treatment, still decreased its concentration of GSH (30% reduction at 10 mmol/L); suggesting that in addition to a pro-oxidant activity, Metformin may also disrupt one-carbon metabolism to reduce GSH pools in leukemia cells. Together, the above data support the notion that Metformin promotes rapid increase in mitochondrial lipid mass, generation of superoxide, and decrease of GSH levels.

5.7 Metformin, Phenformin, and rotenone potentiate the cytotoxic effects of the bcl-2 inhibitor ABT-737. (Velez J et al 2016).

As previously reported, pharmacological inhibition of FAO sensitizes leukemic cells to cell death induced by the bcl-2 inhibitor ABT-737 (Samudio I, 2010), and since GSH has been shown to disrupt the cytotoxicity of ABT-737 (Howard, 2009); we hypothesized that Metformin, Phenformin, and rotenone – via blockage of electron transport, subsequent inhibition of FAO, and/or reduction in antioxidant capacity – would also potentiate the cytotoxic effects of ABT-737. Exposure of KBM5 and REH cells to Metformin reduced the IC50 for ABT-737 by 75% and 92% respectively (Figure 10A and 10B). Rotenone similarly potentiated the effects of ABT-737 at inducing cell death in leukemia cells (Figure 10C). As evidenced in Figure 10D, the reduction in the IC50 dose was mediated by increased apoptosis as showed in the plots of Annexin V and TMRM staining in KBM5 cells. Remarkably, low micromolar doses of Phenformin but not Metformin synergized with ABT-737 in inducing cytotoxicity in OCI-AML3 cells (Figure 10E), demonstrating the higher potency of this biguanide. Nonetheless, millimolar concentrations of Metformin synergized with ABT-737 to induce cell death in various leukemia cell lines (Figure 10F, and Supplemental Figures 4-6) with average combination index (CI) of 0.15.

To advance our knowledge in the mechanism of action of Metformin and if the improvement in sensitivity to ABT-737 was mediated by alterations in Bcl-2 or Mcl-1 protein levels, we treated KBM5, REH, OCI-AML3 and U937 cells with Metformin (10 mmoles/L) for 16 hrs, and the protein expression was evaluated by immunoblot. Interestingly Mcl-1 levels were decreased only in REH
cells, whereas Bcl-2 levels changed in two cell lines only after Metformin treatment (Figure 11A). KBM5 evidenced an increase in the protein levels while U937 displayed a decrease in the protein levels; suggesting that alterations in antiapoptotic proteins may not be an absolute requirement for the detected synergy between Metformin and ABT-737. However confirmation through messenger RNA expression should be performed.

Notably and although KBM5 or REH cells cocultured with bone marrow-derived MSC were significantly \((p<0.01)\) protected from the cytotoxic effects of ABT-737, both rotenone and Metformin overcome the protective effect of MSC (Figure 11B and C), supporting the notion that inhibition of FAO (Samudio I, 2010), in this case as a consequence of mitochondrial respiration impairment can reverse the chemoprotective effects of the leukemic microenvironment.

To further elucidate the mechanism by which Metformin was able to sensitize leukemia cells to the effects of ABT-737, we added palmitoleate – a reported cytoprotective monounsaturated fatty acid (Welters HJ, 2004) – to leukemia cells exposed to both agents and we found that also sensitized them to the cytotoxicity of ABT-737 (Figure 11D); providing more evidence that accumulation of intracellular fatty acids may be associated with the sensitizing effects of Metformin. Moreover and as shown in Figure 11E, addition of diethylmaleate (DEM) or ethacrynic acid (EA) reported depleting GSH agents (Weber CA, 1990; Awasthi S, 1993) also sensitized OCI-AML3 cells to the cytotoxic effects of ABT-737, suggesting that the reduction in GSH levels induced by Metformin may also contribute to the synergistic interaction of this biguanide with ABT-737. As previously reported for Etomoxir (FAO inhibitor) (Samudio I, 2010), Metformin potentiated oligomerization of Bak in leukemia cells treated with ABT-737 (Fig 11F), suggesting that the observed synergy is partially mediated by enhanced outer mitochondrial pore formation to release apoptogenic factors. Taken together, the above data support the hypothesis that pharmacological inhibition of electron transport chain, the subsequent accumulation of intracellular fatty acids and/or the reduction of GSH levels sensitizes leukemia cells – cultured alone or on bone marrow-derived MSC feeder layers – to apoptosis induction by ABT-737.
5.8 Metformin and Phenformin potentiate the cytotoxicity of ABT-737 in primary leukemia samples. (Velez J et al, 2016).

To validate our findings of synergy between biguanides and ABT-737 in human primary leukemic cells, we exposed 10 primary acute leukemia samples (8 AML and 2 ALL) to increasing doses of Metformin, ABT-737, or the combination of these two agents using a fixed dosing schedule for 24 h. After the incubation time, we analyzed viable CD34 (+) cells or total blasts according to their detection. Two primary acute myeloid leukemia samples shown in Figure 12A and B, evidenced synergy between Metformin and ABT-737 (averaged CI <0.8) to induce apoptosis in the CD34 (+) compartment. The additional samples demonstrated a synergy of ~80% with the combination of ABT-737 and Metformin (Figure 12C). As observed in leukemic cell lines, micromolar doses of Metformin didn’t significantly sensitize primary leukemic cells to apoptosis induced by ABT-737. Instead, Phenformin in combination with ABT-737 (50 nmoles/L) significantly (p<0.05) induced apoptosis in 3 of 5 additional primary samples (Figure 12D and E). Taken together, the above results support the notion that biguanides can potentiate the toxicity of ABT-737 not only in immortalized cell lines but in primary leukemic samples.

Although we partially elucidated the multiple mechanisms by which Metformin was able to sensitize leukemia cells to the effects of ABT-737, the fact that Metformin was able to reverse mitochondrial uncoupling through inhibition of mitochondrial respiration, could prevent the leukemia cells from reaching hypoxic points where further metabolic adjustments are needed (shift from oxidative to glycolytic metabolism) (Yang C, 2014); accompanied of proliferation, differentiation, chemo-resistance and leukemia progression. Recent evidence demonstrated that leukemic bone marrow has an enlargement of hypoxic areas compared with healthy marrow (Benito J, 2011); however the exact causes of this finding have not been clarified. Thus, two hypotheses have been introduced: the first one proposed that leukemia propagation causes structural changes in the blood vessels decreasing blood perfusion and subsequently oxygen distribution (Schaefer, 2008). The second one suggested that the intense blast proliferation would eventually decrease the oxygen availability due to high consumption (Deynoux M, 2016), implying that leukemic cells have a functional electron
transport chain able to reduce oxygen and produce energy, contrary to previous theories about mitochondrial damage in cancer cells (Warburg, 1924). This last hypothesis is also in accordance with our findings that inhibition of oxygen consumption sensitizes leukemia cells to targeted therapy, demonstrating the important role that oxidation of Krebs cycle intermediates plays in this type of cancer.

5.9 TH-302 reverses bone marrow hypoxia and prolongs survival in a primary AML xenograft murine model. (Benito J et al, 2016).

Hypoxia-activated prodrugs (HAPs) have been studied during the last 20 years; however till recently they have started to be included in clinical protocols due to their high therapeutic potential in diseases as cancer, specially myeloma and leukemia. It has been previously described that PR-104 a HAP can be effective reversing bone marrow hypoxia and improving survival of mice engrafted with ALL, indicating that hypoxia environment is targetable and represents an opportunity to treat acute leukemias (Benito J, 2011). Here we explored the in vivo activity of a newer HAP: TH-302, a 2-nitroimidazole triggered bromo-isophosphoramide mustard (Br-IPM) able to induce DNA-crosslinking, disabling tumor cells to replicate and divide causing as final consequence cell death (Duan JX, 2008). Furthermore, TH-302, as an optimized HAP has demonstrated to be efficacious in multiple myeloma models in vitro and in vivo (Hu J, 2010).

Human AML cells (1X10⁶/ml) were injected into irradiated (250 cGy) NOD/Scid/IL2Rγ-KO (NSG) mice and the progression of the disease was followed by flow cytometry detecting human CD45+ cells. After engraftment (~5%) of human CD45+ cells was detected in peripheral blood, the mice were randomized and treated with TH-302 (50 mg/kg intraperitoneally 3 times a week for 3 weeks) or PBS. At the end of the treatment and 3 hrs prior to being humanly euthanized, two mice per group were injected (via i.p) with a solution of pimonidazole to reach a final concentration of 100mg/kg per mouse. After this time the mice were sacrificed to detect hypoxia levels in the bone marrow and the remaining mice were followed to determine if TH-302 conferred any prolonged survival. The extraction and staining of bone marrow sections with an antibody detecting the
hypoxic sensitive agent pimonidazole, evidenced a decrease in the hypoxic areas of treated mice compared to control mice; 20X magnification (Figure 13A).

The percentage and absolute number of circulating AML cells was determined and the survival of the mice was followed. At week three, 2 or 3 mice were bled and the number of human CD45+ cells was calculated: Controls (17.3 and 9.2x10⁶ cells/ml) and TH-302 (5.5, 2.3 and 0.53x10⁶ cells/mL) (Fig. 13B). As seen in figure 13C, the decrease in the number of human CD45+ cells in peripheral blood was correlated with increase of survival in the TH-302 treated group (Median Survival time=75 days) compared to control mice (Median Survival Time=56 days). P=0.003, n=8 mice/group.

5.10 C57Bl/6 mice engrafted with AML1/ETO cells produce more lactate than control mice. (Benito J et al, 2016).

To further explore the possibility of a shift from oxidative to glycolytic metabolism in the leukemic BM presumably as a consequence of mitochondrial uncoupling and subsequent hypoxia, we decided to measure glycolysis rate in vivo through High Hyperpolarization Magnetic Resonance Spectroscopy (HP-MRS). C57Bl/6 mice were injected with one million of AML1/ETO-luc-GFP cells and the progression of the disease was followed by bioluminescence imaging (Figure 14A). After 3 weeks the mice were injected with a solution of 80 mM hyperpolarized [1-¹³-C] pyruvate and imaged in a 7T Bruker MR Scanner using a dual tuned ¹H/¹³C volume coil. ¹³C surface coil was placed on the femur of the animal and the resonance frequency signal was detected (Figure 14B). Data was processed and dynamic curves characterizing the arrival of hyperpolarized pyruvate and its chemical –through lactate dehydrogenase- conversion into lactate were generated (Figure 14C). Normalized lactate, defined as the ratio of total cumulative lactate to the total HP-MR signal, was calculated for each ¹³C scan. As evidenced in figure 14D normal animals produced less lactate compared with animals bearing AML, supporting the notion that leukemic hypoxic microenvironment generates a shift from oxidative to glycolytic metabolism in vivo possible suggesting that leukemia cells are uncoupled in the bone marrow microenvironment.
5.11 pH sensitive peptides (pHLIP) target leukemia cells in vitro and in vivo.

Given ours and others findings that leukemic bone marrow is hypoxic and reprograms its metabolism, we decided to test the hypothesis that accumulation or increase of lactate –one of the final products of glycolysis- could be decreasing the pH and promote tropism of peptides sensitive to pH.

To determine in vitro the specificity of pHLIP and their activity under different pHs specially acidic ones, we exposed OCI-AML3 to RPMI medium adjusted with aliquots of 0.1M HCL to different pHs (7.4-7.6-5-6-5-5) and incubated for 48 hrs with a single concentration (250 nM) of WT-pHLIP labeled with the fluorochrome Alexa Fluor 647 for their detection. As observed in figure 15A-15B, WT-pHLIP significantly (p<0.02) accumulated better in the cells that were cultured in a low pH media (6.5-5-6-5.5) without affecting their viability or induce apoptosis (Figure 15C-15D). Representative dot plots of leukemia cells incubated in medium with different pHs and a single dose of WT-pHLIP are shown, demonstrating a higher accumulation (almost 3 times more) of the peptide when the cells were cultured in a media with pH 6.0 (Figure 15E).

To define if the specificity seen in vitro with the pH sensitive peptides was reproducible in vivo, we used an inducible transgenic Chronic Myeloid Leukemia (CML) mouse model in which the characteristic expression of the fusion BCR-ABL gene is controlled by a tetracycline regulated enhancer of the murine stem cell leukemia (Scl) gene (Huettner CS, 2003), allowing to target the fusion gene expression in stem/progenitor cells. As an exploratory experiment, 4 mice that were previously characterized as leukemic due to tetracycline withdrawal for 4 weeks, leukocytosis with neutrophilia primordially, and thrombocytosis, were studied to determine affinity of pHLIP. In this experiment the conditions were as followed: A BCR-ABL mouse without injection of the peptide, a FVB (Friend Virus B) WT normal mouse with injection of the peptide and two BCR-ABL mice with injection of the peptide. Three (One WT normal mouse and two BCR-ABL mice) of the 4 mice were injected via IV (Intravenous) with 100 µl of a solution of 40 µM WT-pHLIP-Alexa 750 in PBS while the forth mouse was not. Two, four and twenty four hrs after injection, the mice were imaged with an
IVIS 200 imaging system (PerkinElmer) using ICG bkg/excitation and ICG/emission channels. As evidenced in figure 16, mice bearing CML (Number 3 and 4) had a higher accumulation of the peptide in each time point, being present even at the longest time (24 hrs). Suggesting a possible acidic microenvironment especially in the lower part of the body where femurs, the principal niche of leukemia are located. To further explore the timeline and distribution of the peptide in CML mice model we exposed or not transgenic mice to drinking water with 2 mg/ml of doxycycline (a more stable tetracycline analog) and 1% of sucrose (Cawthorne C, 2007) changing the water once a week during a month. The presence of thrombocytosis and neutrophilic leukocytosis was detected in peripheral blood by flow cytometry and was considered a sign of disease. As shown in figure 17A the mice that were exposed to doxycycline present a lower number (25.6%) of neutrophils (Ly6G+ cells) compared with the mice that were not exposed to doxycycline (54.98%). The mice were then injected with a solution of 40 µM WT-pHLIP-Alexa 647 in PBS and imaged 4 and 24 hrs later using an IVIS 200 imaging system (PerkinElmer) and the filter set 2 (DsRed) with 500-550 and 575-650 excitation and emission wavelengths respectively. As seen in figure 17B, at 4 hrs WT-pHLIP-Alexa 647 accumulated primordially in the upper legs of the mice and the signal was higher in the mice not exposed to doxycycline. After the images were taken, the mice were sacrificed and the distribution of WT-pHLIP-Alexa 647 in different organs (Upper legs, Femur, Kidney, Spleen, and Liver) was determined. The strong signal coming from the upper legs was confirmed in both mice (Dox OFF and ON) while a new signal coming from the femur was detected (Figure 17C). Kidney, Spleen or Liver didn't present accumulation of the peptide (Figure 17D). Twenty four hours after injection, the same procedure was performed evidencing a similar pattern of accumulation in the upper legs of both mice (Dox OFF and ON), being stronger in mice where doxycycline was removed (Figure 17E). This data was confirmed after organ extraction (Figure 17F). Kidney, Spleen or Liver didn't present accumulation of the peptide even at this longest time point (Figure 17G)

To further detect the peptide at an intracellular level, pieces of the organs were sent to the histopathology core, slides for microscopy were generated and additional staining with DAPI (4',6-diamidino-2-phenylindole) was made to detect nucleus. The slides were analyzed in a Vectra 3
automated quantitative pathology imaging system and representative images of upper legs and femurs are shown in Figure 17H. As seen in both cases (Dox OFF and ON) the peptide accumulated in cells of the bone marrow. However in the case of Dox ON the accumulation was higher in the tissue surrounding the bone marrow while in Dox OFF the signal was prominent in the cells that are part of the bone marrow itself. All together this data suggested that WT-pHLIP-Alexa 647 is able to target -in vivo- different type of cells located in the bone marrow microenvironment.

To test if this effect was seen in other leukemia models we used the aggressive MOLM 13 AML model. One million of leukemic cells were injected into 5 irradiated (250 cGy) NSG mice and the progression of the disease was followed by flow cytometry. At day 10, mice were injected with 100 μl of WT-pHLIP-Alexa 647 (40 μM in PBS) via IV and imaged after 2, 4 and 24 hrs. As observed in Figure 18A, mice bearing or not AML accumulated the peptide similarly at the different time points; Data supported by organ (Spleen and Femur) extraction at the last time point (Figure 18B). Trying to determine possible differences in the tissues we ran flow cytometry in cells extracted from them but we were not able to detect the peptide or evidence any difference (Data not shown); suggesting possible variations between animal models (immunocompromised xenograft vs immunocompetent genetic).

To establish if peptides bound to toxins as Amanitin (Moshnikova A, 2013) are able to target leukemia cells under a range of pHs (7.4-7-6.5-6-5-5), we exposed OCI-AML3 to RPMI medium adjusted with aliquots of 0.1M HCL to the different pHs and incubated for 48 hrs with a single concentration (1μM) of pHLIP-SPDP (Linker) or pHLIP-Amanitin. As seen in figure 19A pHLIP-SPDP didn’t affect significantly the number of viable cells or apoptosis rate (Annexin V + Cells) in any of the pH conditions, while pHLIP-Amanitin did decrease the number of viable cells and increase apoptosis rate at pH 7 and 6.5. To further investigate the minimum dose of pHLIP-Amanitin required to see an effect in viability or apoptosis of leukemia cells, we seeded OCI AML3 in RPMI medium adjusted to pH 6 or 7.4 and exposed them to several doses of pHLIP-SPDP or pHLIP-Amanitin (0.25, 0.5, 1 μM) during 96 hrs. After the incubation time the cells were harvested and stained with Annexin V and DAPI for 15 min before running the flow cytometry. As observed in
figure 19B pHLIP-Amanitin decreased the number of viable cells in a dose and pH dependent manner, being more efficient at pH 6.0 compared with pH 7.4 and increasing the percentage of apoptosis at the higher dose (1 μM); On the other hand, pHLIP-SPDP independent of the the pH didn’t alter the number of viable cells or apoptosis rate.

All together this data indicates the potential of pH sensitive peptides to target leukemia cells in vitro and in vivo, however further studies are needed to demonstrate the efficacy of the peptides and improvement of survival in vivo.
CHAPTER 6

DISCUSSION

Given the microenvironment where platelets and hematopoietic cells are developed, interactions between normal and malignant cells occur and as previously described by several authors and us, platelets can promote resistance (Velez J, 2014) and tumor metastasis (Gupta GP, 2004) in different cancer models; however the exact mechanisms by which these events take place have not been elucidated. Studies by Foss and Bruserud (1997, 1998) have demonstrated that platelets can interact with normal and leukemic myeloid cells in vitro, inducing soluble P-Selectin (CD62P) release from platelets and promoting blast proliferation. Although in our studies we did observe platelets activation (as assessed by expression of CD62P and Annexin V) in response to coculture with leukemia cells and in a concentration dependent manner, we didn’t see an increase in the number of leukemia cells that were seeded initially, implying a possible dual proliferative effect (promoter or suppressor) of factors released by activated platelets as TGF-β or PDGF-BB (Lebrun JJ, 2012).

TGF-beta has been involved in pro and antitumor activities depending on the stage of the disease; in normal and early carcinoma for example the factor behaves as a tumor suppressor, while its cytostatic effects are frequently lost during the progression of the disease (Lebrun JJ, 2012). In our case and due to previous reports suggesting TGF-β activity in the mitochondria directly, we aimed to determine if this factor was responsible for the mitochondrial uncoupling we found. However and even though we used a high specific inhibitor of TGF-beta receptor kinase we were unable to reverse the loss of mitochondrial potential -one of the principal characteristics of mitochondrial uncoupling- in the leukemia cells after the exposition to platelets or platelets derivatives; inferring that TGF-β was not mediating such effect.

On the other hand PDGF-BB has been known for its role in cell growth and division, despite, very little is known about its function or effects in organelles as mitochondria. Recently, the effects of PDGF over vascular smooth muscle cells (VSMCs) have been characterized, finding that this factor
induces mitochondrial fragmentation and a change in mitochondrial substrates utilization from glucose to fatty acids (Salabei JK, 2013); in agreement with the metabolic changes we described in our system (Platelets and Leukemia cells), nevertheless two different mechanisms seem to be involved, mitochondrial fission and mitochondrial uncoupling respectively. Although in both cases loss of mitochondrial membrane potential could be seen and related to autophagy processes, our results utilizing the autophagic vacuole marker Monodansylcadaverine didn’t evidence the presence of autophagic vacuoles (data not shown); indicating differences between both mechanisms.

Furthermore, PDGF has been found to be released in conjunction with soluble CD62P (Bruserud OB, 1998) by platelets but remained to be studied as an uncoupling agent due to their described effects on mitochondria, including metabolism of glucose and specially fatty acids (Salabei JK, 2013); which have been determined as the principal source of protons for the electron transport chain and oxygen reduction under an uncoupling state mediated by uncoupling proteins 2 and 3 (Pecqueur C, 2008; Wang S, 2003), supporting the notion that the increase in the oxygen consumption seen during mitochondrial uncoupling is sustained by fatty acids.

Here we demonstrated that platelets or platelets derivatives are able to reduce the mitochondrial membrane potential of leukemic cells while paradoxically increasing oxygen consumption, which is sustained through the oxidation of fatty acids as triglycerides and was sensitive to the use of a fatty acid oxidation inhibitor (Ranolazine). These findings are consistent with mitochondrial uncoupling, an alteration in the potential difference across the mitochondrial membrane, which was initially described in normal brown adipose tissue and connected to a thermogenic function (Ricquier D, 2000). Despite its normal function, mitochondrial uncoupling has been associated with increased of human life span, as improvement of diabetes and Parkinson’s disease due to the decrease of insulin release by pancreatic β cells (Brand, 2005) and ROS levels respectively (Ho PW, 2012). Conversely, its role in cancer has been linked to chemo resistance (Samudio I, 2008; Pozza E, 2012) as could be evident in our studies.
More recently mitochondrial uncoupling has been linked to lipid catabolism, Akt inhibition and carcinogenesis reduction in a skin cancer model, demonstrating that mitochondrial uncoupling increases fatty acid oxidation and membrane phospholipid catabolism impairing the recruitment of Akt to the plasma membrane; revealing in this way a novel mechanism of crosstalk between mitochondrial metabolism and growth signaling (Nowinski SM, 2015). This data proposes mitochondrial uncoupling —mediated by UCP3— as an effective strategy to diminish proliferation and tumorigenesis, contrary to our studies that evidenced a promotion of resistance in leukemia cells by an uncoupling agent released by platelets; indicating that the effects of mitochondrial uncoupling could be cell context or protein dependent, this due to previous reports demonstrating UCP2 to be involved in chemo-resistant processes (Samudio I, 2008; Pozza E 2012). Even more, the resistance seen in our model is specific to mitochondriotoxics as when we used current approved leukemia therapies (Doxorrubicin and or cytarabine) the effect was not observed, proposing a very precise role for platelets to selectively protect against agents that could affect mitochondria and subsequently the intrinsic apoptotic pathway. Here we determined that platelets partially inhibited the oligomerization of Bax-Bak (at the outer mitochondrial membrane) and the direct permeabilization of the inner mitochondrial membrane, effects generated by ABT-737 and CDDO-ME respectively (Konopleva M, 2006; Samudio I, 2006). Data concordant with previous reports demonstrating that MSCs are able to induce mitochondrial uncoupling in leukemia cells, increasing their oxygen consumption dependent of fatty acid oxidation and sensitizing leukemia cells to the proapoptotic agent, ABT-737 (Samudio I, 2010).

Although we couldn’t characterize completely the uncoupling agent released by platelets, we determined pair of its characteristics as size (<0.2 μm) possibly ruling out microvesicles (1 μm), and heat resistance due to the inactivation at 65°C and maintenance of the uncoupling activity. CAPs or cationic antimicrobial peptides are released by thrombin stimulated human platelets and include microbial chemokines as platelet factor 4, RANTES and CTAP-3 (Connective tissue activating peptide 3), together with fibrinopeptide B and thymosin β-4 (Tang YQ, 2002); which also require the presence of cardioliopin to permeabilize bacterial membranes and accomplish their function
These features suggest that CAPs could affect mitochondria directly due to their affinity for negative charged spaces (matrix) and cardiolipin enriched membranes; however this hypothesis requires further investigation.

All together these findings have generated a new paradigm where cells (MSCs) or cell fragments (Platelets) of the bone marrow microenvironment are able to induce and sustain metabolic changes as mitochondrial uncoupling to support a malignant disease. Although in both cases the majority of the biochemical characteristics are present: loss of mitochondrial membrane potential, increase in oxygen consumption, fatty acid oxidation dependence and promotion of chemo-resistance; the use of FAO inhibitors in the clinic has been limited to heart failure or angina due to liver toxicities. Even more, to our knowledge there are not current cancer clinical trials with these or other FAO inhibitors. Despite, recent advances have been made to develop compounds able to affect indirectly and through the mitochondria given process (FAO). One of them is the ME-344, developed by MEI Pharma and which has been found to be a mitochondrial inhibitor of complexes I and IV causing a reduction in the oxygen consumption, loss of mitochondrial membrane potential and destabilization of the complexes with subsequent degradation (Lim SC, 2015). Although this study didn't explore the effect of this compound over fatty acid oxidation, we hypothesize that this process should be inhibited due to the accumulation of reducing equivalents (NADH and FADH\textsubscript{2}) and the lack of oxidized co-factors (NAD-FAD) which are required for the proper functioning of enzymes as 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA dehydrogenase respectively (Eaton S, 2002). Early studies in isolated mitochondria demonstrated that the limited rate of oxidation of NADH induces accumulation of 3-hydroxyacyl- and 2-enoyl- CoA intermediates, inhibiting subsequently enoyl-CoA hydratases and acyl-CoA dehydrogenases (Eaton S, 2002); however if these events occur in a leukemia model remains to be probed. Meanwhile the compound has been used in a phase I human study of patients with refractory solid tumors, demonstrating that 35% of the patients achieved a stable or durable disease with an ongoing partial response in one patient with small cell lung cancer (Bendell JC, 2015). Data allowing us to reason that old or new mitochondrial inhibitors could be used as cancer therapies alone or in combination with chemotherapy.
As we had shown here mitochondrial inhibitors as Metformin and/or Phenformin are able to inhibit mitochondrial respiration (oxygen consumption) and fatty acid oxidation, sensitizing leukemia cells to the effects of bcl-2 inhibitors (ABT-737); however multiple compounds have shown to have similar properties without making it successfully to the clinic, principally due to their induced toxicities. In our particular case Metformin could have better chances to progress clinically as have been used for several years in diabetic patients, is inexpensive, well tolerated and has been linked to a decrease in the risk of occurrence of various types of cancer especially pancreas, colon and hepatocellular carcinoma (Kasznicki J, 2014). Furthermore, recent leukemia papers have described the therapeutic potential of Metformin due to its antiproliferative, apoptotic (Bruno S, 2015) and metabolic (Scotland, 2013) effects in some cases being dependent or independent of signaling pathways as AMPK or Akt. Different groups have reported alternative pathways including RagG GTPase, which is activated rapidly after changes in the energy status to maintain homeostatic mTORC1 signaling (Kalender A, 2010), or REDD1 (RTP801/Dig2/DDIT4) a factor that was initially identified as a target gene of HIF1 and that lately has been linked to the regulation of cell survival, responding to DNA damage, nutrient depletion, glucocorticoid and insulin; and being described as a negative regulator of mTOR (Sahra, 2011), a protein ruling cell growth, proliferation, motility, protein synthesis, autophagy and transcription (Hay N, 2004). Even more, it has been reported that Metformin increases the expression of REDD1, implying a possible mechanism for inhibition of mTOR signaling (Sahra IB, 2011. Concordantly, our studies suggest that the effects exert by Metformin were very likely caused as a consequence of mitochondrial respiration inhibition instead AMPK activation, this due the lack of AMPK phosphorylation at Thr172 (catalytic domain) in our western blots. However and due to we didn’t measure AMP levels we cannot rule out the possibility that AMP is not being accumulated and subsequently not activating AMPK. However, even in cell lines as NALM-6 where the oxygen consumption was deeply compromised with an IC50 of 1.5 mM the % of cell death with Metformin, ABT-737 or the combination was less than 10%, indicating that other mechanisms as oxidative stress, NADH and/or fatty acids accumulation could be mediating the sensitizing effect of Metformin. In this regard and contrary to our findings, Metformin has been reported to inhibit the production of reactive oxygen species in AMPKα+/+ and AMPKα−/− MEFs
exposed to an organic compound (paraquat) being known for being an oxidative stress inductor (Algire C, 2012) reducing further inflammatory events. However and as suggested for our data, Metformin induced superoxide production and diminished GSH (Glutathione) levels even in cell lines (NALM-6) where superoxide production was not observed. Thus, these results are in agreement with a recent report that suggests this biguanide can antagonize one-carbon metabolism which is required for the synthesis of GSH (Corominas FB, 2012), even more our experiments with diethylmaleate and ethacrynic acid demonstrated that GSH depletion may also contribute to the synergic interaction of Metformin with ABT-737.

Another mechanism is the possible accumulation of NADH due to inhibition of the electron transport chain that could repress FAO and redirect FAO substrates to neutral lipid stores, which could perhaps induce lipotoxicity affecting signal transduction pathways and/or acting directly on mitochondria to prompt transitory permeabilization and cytochrome C release (Furuno T, 2001). In our studies we showed that accumulation of intracellular triglycerides follows the inhibition of oxygen consumption and that could cause the subsequent sensitization of Metformin to the effect of ABT-737. Moreover, we validated this hypothesis using palmitoleate in our experiments and resembling sensitization. However we didn't detect accumulation in p53-wild-type OCI-AML3 cells, perhaps in agreement with previous observations that p53 activity accompanies increased lipolysis (Liu Y, 2014). Likewise we found alterations in mitochondrial phospholipid content as monitored by NAO staining, data supported for previous publications by Garcia-Fernandez demonstrating higher affinity of NAO for state 3 (Coupled; ADP dependent) respiring mitochondria, suggesting a re-coupling effect by Metformin (Garcia Fernandez MI, 2004). Together with our Bak crosslinking experiments, the above may also lend support to a paradigm in which increased exposure of cardiolipin (or other phospholipid) molecules favors interaction with caspase cleaved bid (t-bid) (Gonzalvez F, 2010), Bax (Sani MA, 2009), or Bak (Landeta O, 2011), and facilitates the permeabilization of the outer mitochondrial membrane and subsequent release of proapoptotic proteins (Figure 11F).
A final mechanism that could explain Metformin’s sensitizing properties is the downregulation of Mcl1, one of the principal resistant factors that have been described for bcl-2 inhibitors (Van Delft, 2006). Recent studies in CLL (Chronic Lymphocytic Leukemia) demonstrated that Metformin impedes the up-regulation of Mcl-1 occurring in response to CLL activation stimuli, even more; Metformin induced an increase in Noxa (Pro-apoptotic protein) expression neutralizing Mcl-1 protein and promoting its proteasomal degradation (Bruno S, 2015). Although we explored the possibility of alterations in the expression of Bcl-2 or Mcl-1 prompted by Metformin after 16 hrs of treatment, we found that Bcl-2 levels were unchanged and that Mcl-1 levels were decreased only in REH cells; indicating that mechanisms independent to the regulation of antiapoptotic machinery could be mediating synergy between Metformin and ABT-737.

The orally bioavailable derivative of ABT-737, ABT-263 (Navitoclax) and its more recent lead ABT-199 (Venetoclax) that inhibit specifically Bcl-2 protein, is currently being evaluated in several clinical trials for its safety and efficacy against human leukemia, specially CLL (Roberts AW, 2016; Walensky LD, 2012). A recently completed study with relapsed/refractory chronic lymphocytic leukemia patients reports a 79% of responses ranging from 71 to 79% among patients in subgroups with an adverse prognosis as resistance to fludarabine, chromosome 17p deletions and unmutated IGVH. Complete remissions were reported in 20% of the patients and the 15 month progression-free survival estimate for the 400 mg dose groups was 69% (Roberts AW, 2016). In agreement with basic observations that leukemia cells from AML models, at least in vitro, are exquisitely sensitive to ABT-199 (Pan R, 2014). Still, any strategy that could potentiate the antileukemic effects of Venetoclax in other types of leukemia or cancer is of utmost interest. While rotenone is an unthinkable component of any chemotherapeutic scheme due to its toxicity, Metformin has been positioned as a new companion in the treatment of different types of cancer, especially solid tumors; being combined with traditional chemotherapy, radiation and targeted therapy due to its safety demonstrated at least in breast cancer (Jiralerspong S, 2009). This last study was published in JCO (Journal of Clinical Oncology) and established in a wide (2529 patients) cohort that Metformin given to diabetic patients with breast cancer and neoadjuvant chemotherapy generated a
higher pCR (Partial Complete Response) rate than in diabetics that didn’t receive Metformin or in the nondiabetic group. It is also tempting to speculate that the greater potency of Phenformin over that of Metformin may be more desirable in a possible combination strategy with Venetoclax, although the toxicity of Phenformin may also be limiting in this scenario. Nonetheless, our findings suggest that pharmacologic inhibition of electron transport maximizes the antileukemic effects of ABT-737, and lend support to the use of Metformin or Phenformin in combination with Venetoclax as a potential therapeutic strategy for the treatment of leukemia.

Even though the approach to inhibit mitochondrial respiration could be applicable to early stages of leukemia development, where the high oxygen consumption rate could lead to later stages where oxygen decreases profoundly and alternative metabolic pathways not involving mitochondria (glycolysis etc.) are taken; there is a need for the use of compounds that are active even under hypoxic conditions. As we show here and previous reports support, a primary AML xenograft murine model developed highly hypoxic areas in the bone marrow in advanced stages of leukemia. While mechanisms responsible for this change have not been completely elucidated, different explanations have been proposed. One is the possible high oxygen consumption by leukemic blasts that lowers the steady state oxygen concentrations, hypothesis supported by time course studies in a blast crisis CML model where the progression of the disease was marked by the appearance of hypoxic areas (Benito J, 2011). Interestingly enough, not only leukemic cells were hypoxic if not contiguous stroma expressed HIF-1α, a principal hypoxic marker; indicating a complete reprogramming in the leukemia microenvironment. Secondary causes of this hypoxia could be structural and functional alterations of microcirculation and/or diffusion conditions, notion supported by Schaefer et al, demonstrating that tumor progression is associated with microvascular permeability, an initial angiogenic wave and a final decrease in vessel density and reduced tissue perfusion. Further alterations in the release of activators or inhibitors of angiogenesis have been described in pancreatic ductal adenocarcinoma as well.

Numerous approaches to target hypoxia have been taken, from directly inhibiting HIF-1α to compounds that are activated as a consequence of changes in the microenvironment (HAPs,
pHLIPs). Here we report that TH-302, a HAPs that releases the DNA alkylator bromo-

isosiphophoramide under hypoxic conditions is able to reduce hypoxic areas and improve mice survival in an in vivo primary AML xenograft murine model. Data concordant with previous reports of preclinical human acute myeloid leukemia models evidencing a decrease in proliferation, HIF 1α expression, cell cycle arrest and enhanced double-stranded DNA breaks in hypoxic AML cells treated with TH-302; besides inhibition of disease progression, prolonged overall survival and reduction in the number of hypoxic cells in human AML xenografts (HEL, HL60) (Portwood S, 2013). These results have allowed the execution of several clinical trials in different types of cancer and more recently the granting of FDA (Food and Drug Administration) fast track designation for Soft Tissue Sarcoma.

The HIF hydroxylase system is the most characterized key transcription factor mediating responses to hypoxia, being the HIF1 α subunits (at a protein level) the principal regulators of HIF activity (Masson N, 2014). Between the multiple functions that this factor has is to regulate angiogenesis, cell proliferation, survival, apoptosis and metabolism, which can confer advantages for cancer cells in a determined microenvironment. These alterations have conducted to a prominent search for a “hypoxia signature” through gene expression profiling to identify other possible alterations and predict resistance or poor prognosis in cancer patients (Harris BH, 2015). Among the genes that have been differentially expressed are CA9, GLUT1 and VEGF (Winter IG, 2007). Even more recently other genes closely related to glycolysis (LDHA and PGK1) have been described (Benito J, 2016) and were in agreement with findings that AML subtypes could be identified given common aberrant transcriptional programs including low cycle activity, combined with elevated activities of inflammatory response, hypoxia and signaling (Rapin N, 2014).

Hypoxia has been known for inducing adaptive changes in cancer metabolism including switching from oxidative phosphorylation to glycolysis, increased glycogen synthesis and switch from glucose to glutamine as the major substrate for fatty acid synthesis (Semenza GL, 2013). However in vivo validation of glycolysis activity has been difficult due to technical and anatomical issues. Here, using HP (Hyperpolarized)-13C pyruvate which amplifies the signal improving its detection, we
demonstrated that leukemic BM presents a higher conversion rate from pyruvate to lactate compared with normal BM; further providing insight about the metabolic rearrangements made at the microenvironment level due to reduced oxygen availability.

A consequence of glycolysis up-regulation and subsequent lactate output is the intracellular titration of acid with bicarbonate and the involvement of the pentose phosphate shunt to release CO2 from cells, promoting a decrease in the pH which could favor tumor growth, invasion and development. Additionally, the accumulation of CO2 could cause the expression of the enzyme carbonic anhydrase 9 (CAIX) on the tumor cell surface hydrating the CO2 and generating HCO3− and H+ which will decrease the pH further (Swietach P, 2007). These particular characteristics allow a unique opportunity to target cancer cells that overexpress CAIX or that are surrounded by a hypoxic/acidic microenvironment. For this reason we decided to test if pHLIPs (pH low insertion peptides) were able to target leukemia cells in vitro and or in vivo. As evidenced here WT-pHLIP was able to target leukemia cells in an in vitro setting under pH controlled conditions validating previous reports evidencing the properties of pH low insertion peptides to bind to liposomes at low pH 5 (Weerakkody D, 2013). Although this last study used more specific techniques to determine partitioning and intrinsic peptide fluorescence to demonstrate insertion, both studies corroborate the affinity of these peptides for bilayers at low pHs.

To confirm that the effect seen in vitro was reproducible in vivo we used a CML mouse model which evidenced overall a higher accumulation of WT -pHLIP -Alexa 750 at 2.5, 4 and 24 hrs; And to further explore the kinetics and dynamics of the peptide we used WT-pHLIP -Alexa 647 in the same model detecting the signal in several organs after extraction. Conversely to the normalization required due to kidney accumulation of the peptide in previous reports (Weerakkody D, 2013), here was not necessary as we didn’t detect any signal in the same organ. Instead the peptide accumulated significantly in the upper legs and femurs at 4 and 24 hrs, suggesting a bone marrow (cancer niche) affinity. Tissue slides seen under a fluorescence microscope evidenced the presence of the peptide inside cells from the bone marrow. Interestingly, the distribution of the peptide around the bone marrow seems to be greater in mice exposed to doxycycline which we expect to don’t
have leukemia compared with mice with doxycycline withdrawal. A possible explanation of that is the presence of bone lining cells and osteocytes which are involved in resorption processes, where low pH is one of the principal requirements needed for the dissolution of mineral components (Everts V, 2011); without mentioning that bone lining cells regulate ions exchange, possible controlling pH as well. As today, several groups have reported bone-lining osteolineage alterations in hematological disorders and/or malignancies, demonstrating that mutations in mouse collagen-expressing osteoblastic cells could lead to MDS or AML (Schajnovitz A, 2014) (Kode A, 2014). Likewise, leukemia bone marrow is characterized for the presence of fibrosis which has been correlated with biological factors, treatment response and outcome of children with acute lymphoblastic leukemia (ALL) (Norén-N, 2008). Even more, recent studies have demonstrated the partial recovery of the leukemia bone marrow architecture during induction chemotherapy in children with ALL, indicating that the morphological changes seen in the bone marrow were a consequence of the disease itself (Nguyen TV, 2015). Thus, although the accumulation of the peptides seem overall to be higher in mice with signs of the disease (Thrombocytosis, Leukocytosis and Neutrophilia), the presence of a strong signal in animals without apparent signs of disease call to a careful analysis, interpretation and validation of the data.

Lastly, as evidenced here and in recent papers (Moshnikova A, 2013; Burns K, 2015) new peptides are being bound to different toxins or inhibitors as Amanitin and Monomethyl Auristatin E (microtubule inhibitor) to demonstrate in vitro an in vivo activity. In the first study, pHLIP-Amanitin demonstrated to inhibit cell growth and induce cell death in a dose and pH dependent manner. However and as Amanitin is a very toxic agent other groups have decided to conjugate pHLIP to validated compounds as Monomethyl Auristatin E (MMAE), which is currently being linked to monoclonal antibodies showing potency in vitro and in vivo against solid and liquid tumors (Dosio F, 2011). In the latest, pHLIP–MMAE induced >90% inhibition of cell growth in a concentration and pH dependent manner, after 2 hrs of incubation and without altering plasma membrane, besides exhibiting higher antiproliferative effect at low pH than at physiological pH or the free drug.
Altogether this data indicates that pHLIPs conjugated should be studied further to determine their efficiency, potency, and toxicity in preclinical studies.

Our studies demonstrated that leukemia cells can interact with platelets and induce their activation promoting beneficial effects in the leukemia cells through mitochondrial uncoupling; supporting the notion that several type of interactions could happen at the leukemic bone marrow microenvironment contributing to the pathogenesis of the disease. Platelets in leukemia could be decreased due to an ineffective production of thrombocytes (Izak M, 2014) and/or accelerated destruction in some cases as a consequence of the treatment; however recent studies have found that children with acute lymphocytic leukemia have an elevated relative immature platelet fraction (IPF%) (Strauss G, 2011), suggesting that thrombopoiesis is still being stimulated and that somehow platelets are being destructed or consumed before they reach peripheral blood. This evidence in conjunction with the findings about MSCs inducing mitochondrial uncoupling (Samudio I, 2008) led us to think that this phenomenon could be happening in the bone marrow itself, maintaining the very well described protective microenvironment with a profound metabolic alteration that could conduce to hypoxia and to a reprogramming of metabolism from oxidative to glycolytic. The fact that these two important cells or fragments part of the bone marrow microenvironment are inducing the same event and promoting resistance, open wider therapeutic opportunities to use compounds able to affect any of the principal characteristics of mitochondrial uncoupling: loss of mitochondrial membrane potential, oxygen consumption and/or fatty acid oxidation; as the subsequent conditions generated for metabolic reprogramming, hypoxia and acidosis. Here we demonstrated that the inhibition of the oxygen consumption by Metformin or Phenformin sensitizes leukemia cells to the effects of ABT-737 in vitro, that hypoxia activated prodrugs (HAPs) have a therapeutic potential in vivo and that pH sensitive peptides have activity in vitro. Despite in vivo validation and studies of pharmacokinetics and pharmacodynamics are required to determine the safety and toxicity profile of each compound or their combination, except for the HAPs that are already part of phase I clinical trials.
CHAPTER 7

CONCLUSIONS

In summary, we identified a new interaction between platelets and leukemia cells in vitro that promotes platelets activation, as mitochondrial uncoupling and resistance to apoptosis induced by mitochondriotoxics in leukemia cells. Having established the characteristics involved in given process, we explored the possibility to target couple of the mitochondrial alterations seen during mitochondrial uncoupling: high respiration rate and fatty acid oxidation, as reduction of oxidative stress and crosslinking of proapoptotic proteins (Bax and Bak), needed for mitochondrial pore formation and subsequent cytochrome C release. With this approach, we decided to use two broadly reported mitochondrial inhibitors; Metformin and Phenformin, which reverse mitochondrial uncoupling reducing respiration rate as fatty acid oxidation, and increasing oxidative stress as crosslinkings between proapoptotic proteins, to sensitize leukemia cells to the effect of mitochondriotoxics, specifically ABT-737. On the other hand, we propose a further approach for later stages of leukemia where mitochondrial uncoupling could induce local hypoxia and subsequent acidosis. In this case the use of compounds as hypoxia activated prodrugs (HAPs) and pH sensitive peptides (pHLIP) could improve the responses in in vivo models and in subsequent human studies, providing evidence to develop better, specific and more efficacious therapies for leukemia.
CHAPTER 8

PERSPECTIVES

Although we proposed three different alternatives to reverse the metabolic reprogramming caused by leukemia in the bone marrow microenvironment: treatment with mitochondrial inhibitors as Metformin or Phenformin in combination with pro-apoptotic agents as ABT-737, treatment with hypoxia activated prodrugs TH-302 and treatment with pH sensitive peptides conjugated to toxins; several studies remained to be performed to secure specific targeting, safety and efficacy of the compounds separately and in combination. Pharmacodynamic and pharmacokinetic studies are needed to determine effective doses except for TH-302 that is currently being tested in phase I clinical trials.
LIST OF DIAGRAMS AND FIGURES

DIAGRAM 1: Reduced schema of normal metabolism (Glycolysis and Krebs Cycle).

FIGURE 1: Leukemia cells promote platelets activation in a concentration dependent manner (A-B). (A) RPMI medium was inoculated with 2.5% Platelets Rich Plasma and different concentrations of MOLT4 cells (50, 100, 250 ×10^3/mL) or OCI-AML3 cells (B), for 1 h under standard culture conditions. As positive control for platelets activation, 1% Calcium Gluconate was used. After incubation time, expression of CD62P on platelets exposed to MOLT4 cells and externalization of phosphatidyl serine on platelets exposed to OCI-AML3 cells was determined by flow cytometry as described in Materials and Methods. *=p<0.05; **=p<0.001. Platelet derivatives induce loss of mitochondrial membrane potential (similar to the effect induced by Mesenchymal Stromal Cells) in a concentration and time dependent manner (C-G). (C) MOLT4 and OCI-AML3 cells (1x10^5/ml) were exposed to 2.5% Platelet Lysates during 2 h. After the incubation time, the cells were stained with JC1 dye to determine mitochondrial membrane potential by flow cytometry. Shown are representative contour plots of JC1 aggregate fluorescence (FL2:H) vs JC1 monomer fluorescence (FL1:H). (D) OCI-AML3 cells (1x10^5/ml) were seeded on MSC feeder layers (1x10^4/cm²) for 16 hrs, after the incubation time cells were stained with CD90 (MSCs are positive for this marker while leukemia cells are not) and JC1 to determine mitochondrial membrane potential by flow cytometry. (E) OCI-AML3 cells (1x10^5/ml) were exposed to increasing doses (0, 0.5, 1 and 2.5%) of Supernatant of Platelets for 18 hrs Mitochondrial membrane potential was determined by flow cytometry as described in Materials and Methods. (F) OCI-AML3 cells (1x10^5/ml) were exposed to 5% of Supernatant of Platelets for 0, 0.5, 4 and 18 h. Mitochondrial membrane potential was determined by flow cytometry as described in Material and Methods. (G) MOLT4 cells (1x10^5/ml) were exposed to increasing doses (0, 0.5, 1 and 2.5%) for 4 hrs. Mitochondrial membrane potential was determined by flow cytometry. (H) OCI-AML3 cells (1x10^5/mL) were exposed to SB-431542 (1 μmol/L) for 30 min, prior to the addition of 5% Supernatant of Platelets for 3 h. In parallel, cells were also exposed to Supernatant of Platelets that had been heated to 65 °C for 1 h (SP H) or filtered through a 0.2 μm mesh (SP F). Mitochondrial membrane potential was determined by flow
cytometry as described in Materials and Methods. *= p<0.05; **= p<0.001; ***= p<0.0001 from untreated controls. The loss of mitochondrial potential is independent of TGF-β. The factor responsible for the loss of mitochondrial potential is smaller than 0.2 μM and is heat insensitive.

FIGURE 2: Loss of mitochondrial membrane potential is accompanied for an increase in oxygen consumption (A), sensitivity to fatty acid oxidation inhibitors (B); decrease of charged, neutral fatty acids (C) and superoxide production (D). (A) OCI-AML3 cells (100 μl; 2×10^5 cells in 100 μl) were seeded in Oxygen Biosensor plates and exposed to increasing doses of PL for 4 h. Oxygen consumption was determined by fluorometry as described in the materials and methods. (B) OCI-AML3 cells (100 μl; 2×10^5 cells in 100 μl) were seeded in Oxygen Biosensor plates and exposed, or not, to 200 μmol/L ranolazine (RAN) for 30 min prior to addition of 2.5 % PL for 4 h, oxygen consumption was determined as above. (C) OCI-AML3 cells (1×10^5/mL) were exposed to increasing doses of PL (0, 0.5, 1, and 2.5 %) for 18 h and phospholipid and triglyceride content was determined by flow cytometry with Lipidtox and as described in the materials and methods. (D) OCI-AML3 cells (1×10^5/mL) were exposed to increasing doses of PL (0, 0.5, 1, and 2.5%) for 3 h followed by the addition, or not of 1 μmol/L rotenone. Superoxide levels were determined with Dihydroethidine and as described in the materials and methods. (A-C) *= p<0.05; **= p<0.005; from untreated control; #= p<0.005 from RAN control. (D) *= p<0.05; **= p<0.005; from rotenone control without PL; # = p<0.005 from untreated control without PL.

FIGURE 3: Platelet lysates protect leukemia cells from apoptosis (A-B) and mitochondrial damage (C-D) induced by ABT-737. Platelets rich plasma protects leukemia cells from apoptosis in a concentration dependent manner (E). (A) and (C) MOLT4 and (B) and (D) OCI-AML3 cells (1×10^5/mL) were exposed, or not, to 2.5 % PL for 30 min prior to the addition of increasing doses of ABT-737 for 16 h. Apoptosis (A and B) and mitochondrial damage (C and D) were determined as described in the materials and methods. (E) OCI-AML3 cells (1×10^5/mL) were exposed to increasing doses of PRP (0, 0.5, 1, and 2.5 %) 30 min prior to the addition of 500 nmoles/L ABT-737 for 16 h. Apoptotic cells were quantified as above.
FIGURE 4: Platelet lysates protect leukemia cells from apoptosis (A-B) and mitochondrial damage (C-D) induced by CDDO-ME. Platelets rich plasma protects leukemia cells from apoptosis in a concentration dependent manner (E). (A) and (C) MOLT4 and (B and D) OCI-AML3 cells (1×10^5/mL) were exposed, or not to 2.5 % PL for 30 min prior to the addition of increasing doses of CDDO-ME for 16 h. Apoptosis (A and B) and mitochondrial damage (C and D) were determined as described in the materials and methods. **= p<0.0005 from untreated RPMI without platelet components; # = p<0.0005 from CDDO-ME treated RPMI without platelet components. (E) OCI-AML3 cells (1×10^5/mL) were exposed to increasing doses of PRP (0, 0.5, 1, and 2.5 %) 30 min prior to the addition of 500 nmoles/L CDDO-ME for 16 h. Apoptotic cells were quantified as above. *= p<0.0005 from untreated RPMI without platelet components; **= p<0.0005 from CDDO-Me treated RPMI without platelet components.

FIGURE 5: Platelet lysates protect primary leukemia cells from apoptosis induced by CDDO-ME and ABT-737. (A-F) Peripheral blood mononuclear cells (1×10^6/mL) from AML or ALL patients were exposed, or not, to 2.5 % PL for 30 min prior to the addition of increasing doses of CDDO-ME or ABT-737 for 16 h. Cells were harvested, stained with CD34-FITC and propidium iodide, and viable cell numbers were determined by flow cytometry as described in the materials and methods. **= p<0.0005 from untreated RPMI without platelet components; #= p<0.0005 from CDDO-ME treated RPMI without platelet components. *= p<0.05 from RPMI only control.

FIGURE 6: Platelet lysates decrease Bax crosslinkings induced by ABT-737. OCI-AML3 cells (1×10^5/mL) were exposed, or not to 2.5 % PL and treated with 500 nmol/L ABT-737 for 16 h. Mitochondrial extracts from leukemic cells were obtained and exposed to bismaleimidohexane (BMH), and Bax crosslinking was determined by Western blot as described in the materials and methods.

DIAGRAM 2: Platelets induce mitochondrial uncoupling: Loss of mitochondrial membrane potential, increase of oxygen consumption, decrease of superoxide production, fatty acids concentration, Apoptosis and Bax Crosslinkings
FIGURE 7: Mitochondrial inhibitors (Metformin, Rotenone and NaCN) decrease oxygen consumption (A). Metformin in a dose dependent manner inhibits oxygen consumption in different cell lines (B). Metformin inhibits oxygen consumption in primary leukemia cells (C). Metformin and Phenformin inhibit oxygen consumption, Phenformin being more potent at a lower concentration range (D). (A) REH and NALM-6 cells were seeded at 2×10⁶ cells/mL in 100 µl of RPMI medium in microfuge tubes and treated with 10 mmol/L Metformin (MET), 1 µmole/L rotenone or 4 mmole/L sodium cyanide (NACN) for 1 hr and processed to determine oxygen consumption by flow cytometry as described in Materials and Methods. Untreated cells were used as controls. ** = p < 0.05 compared to control. (B) KBM5, OCI-AML3, NALM-6, and REH cells (2×10⁶ cells/mL in microfuge tubes) were exposed to increasing concentrations (0, 1, 5, and 10 mmol/L) of Metformin for 1 hr and oxygen consumption determined as above. IC50 values for Metformin are indicated next to each cell line. ** = p < 0.05 compared to 0 mmole/L Metformin for all lines tested. (C) Three primary samples (#1, #2, and #3) were seeded in 100 µl of RPMI medium in microfuge tubes and treated with 0, 5, or 10 mmol/L Metformin for 1 h and processed to determine oxygen consumption by flow cytometry. Sample #2 was stained with anti-CD34 APC prior to fixation, and results are derived from CD34-positive leukemia blasts. (D) OCI-AML3 cells were treated with 4 mmole/L sodium cyanide (as a control for inhibition of oxygen consumption) or increasing micromolar concentrations of Metformin or Phenformin for 1 hr and oxygen consumption determined as above. * = p < 0.05 compared to control. The data was normalized by substracting the MFI values of OCI-AML3 cells treated with 4 mmole/L sodium cyanide.

FIGURE 8: Metformin and rotenone induce accumulation of fatty acids (A) in a dose dependent manner (B). Metformin induces accumulation of the fluorescent dye nonyl-acridine orange (NAO) in a dose dependent manner (C), effect reproduced in cells treated with Rotenone (D). (A) REH and NALM-6 cells were seeded at 2×10⁵ cells/mL (300 µl in 48-well plates) and treated with 10 mmol/L Metformin (MET) or 1 µmole/L rotenone for 16 hr and processed to determine neutral lipid content with Lipidtox staining as described in Materials and Methods. Untreated cells were used as controls. (B) KBM5, NALM-6, and REH cells (2×10⁵ cells/mL in 48-well plates) were exposed to increasing
concentrations (0, 1, and 10 mmol/L) of Metformin for 4 hr and accumulation of neutral lipids determined as above. (C) KBM5, OCI-AML3, NALM-6, and REH cells (2x10^5 cells/mL in 48-well plates) were exposed to increasing concentrations of Metformin as above, and mitochondrial phospholipid content assessed via NAO staining as described in Materials and Methods. (D) REH and KBM5 cells (2x10^5 cells/mL in 48-well plates) were seeded as above and treated with Metformin (10 mmol/L) or rotenone (1 μmol/L) for 6 h and mitochondrial phospholipid content determined as above. * = p < 0.05 when compared to control; # = p < 0.005 when compared to control; $ = p < 0.0005 when compared to control.

**FIGURE 9:** Metformin induces superoxide production in a dose dependent manner (A). Metformin and Phenformin induce superoxide production in a dose dependent manner, Phenformin being more potent at a lower concentration range (B). Metformin and Rotenone induce superoxide production in a similar fashion (C). Metformin induces GSH reduction in a dose dependent manner (D). (A) KBM5, REH, and OCI-AML3 cells were seeded at 2x10^5 cells/mL (300 μl in 48-well plates) and treated with Metformin (MET; 5 or 10 mmol/L) for 2 h and superoxide generation determined via dihydroethidine (DHE) staining as described in Materials and Methods. (B) OCI-AML3 cells were seeded at 2x10^5 cells/mL (500 μl in 24-well plates) and treated with increasing micromolar concentrations of Metformin or Phenformin for 2 h and superoxide generation determined via DHE staining as above. (C) REH and NALM-6 cells (2x10^5 cells/mL in 48-well plates) were exposed to 10 mmol/L Metformin (MET) or 1 μmole/L rotenone for 2 h and superoxide generation assessed as above. (D) REH and NALM-6 cells (2x10^5 cells/mL in 48-well plates) were exposed to increasing concentrations (0, 1, 5, and 10 mmol/L) of Metformin for 2 h and reduced GSH levels determined by flow cytometry as described in Materials and Methods. * = p < 0.05 when compared to control; ** = p < 0.005 when compared to control.

**FIGURE 10:** Metformin sensitizes leukemia cells to the effects of ABT-737 (A-B). Rotenone as Metformin sensitizes leukemia cells to the effects of ABT-737 (C). Leukemia cells treated with Metformin and ABT-737 present mitochondrial damage and an increase in apoptotic rate (D). Phenformin sensitizes leukemia cells to the effects of ABT-737 at a lower concentration range than
Metformin (E). Combination index between Metformin and ABT-737 in different cell lines (F). (A) KBM5 cells (2×10^5 cells/mL in 48-well plates) were exposed to increasing concentrations of ABT-737 (0, 2, 4, and 6 μmol/L), alone or in the presence of 10 mmol/L Metformin for 16 h and % cell death was determined via TMRM staining as described in Materials and Methods. (B) REH cells (2×10^5 cells/mL in 48-well plates) were exposed to increasing concentrations of ABT-737 (0, 250, 500, and 750 nmol/L), alone or in the presence of 10 mmol/L Metformin for 16 h and % cell death was determined as above. (C) REH cells seeded as above were treated with 250 nmol/L ABT-737, alone or in the presence of 10 mmol/L Metformin or 1 μmol/L rotenone for 16 h and cell death was determined as above. (D) KBM5 cells (2×10^5 cells/mL in 48-well plates) were exposed to 750 nmol/L ABT-737, alone or in the presence of 15 mmol/L Metformin and TMRM and Annexin V staining was quantitated by flow cytometry and as described in Materials and Methods. (E) OCI-AML3 cells were seeded at 2×10^5 cells/mL (500 μl in 24-well plates) and treated with increasing micromolar concentrations of Metformin or Phenformin, ABT-737, or combinations of biguanides and ABT-737 in an isobologram design. Viable cells were determined by propidium iodide staining and flow cytometry as described in Materials and Methods. * = p < 0.05 when compared to ABT-737 alone; ** = p < 0.005 when compared to ABT-737 alone. (F) Leukemia cells (2×10^5 cells/mL in 48-well plates) were treated with Metformin (5, 10, or 15 mmol/L) or ABT-737 (250, 500, 750 nmol/L for REH and KBM5; 1, 2, 3 μmol/L for OCI-AML3; 1, 2, 3 μmol/L for NALM-6; and 250, 500, and 750 nmol/L for U937), and the fixed ratio combinations of Metformin + ABT-737 for 16 h and cell death was determined as described in Materials and Methods. Isobologram analysis and CI were determined using CalcuSyn version 2.1. Averaged CI values are shown for each cell line.

**FIGURE 11:** Expression of Mcl-1 and Bcl-2 proteins after treatment with Metformin (A). Metformin and rotenone sensitizes leukemia cells to the effects of ABT-737 under co-culture conditions (B-C). Palmitoleate (PO), a monounsaturated fatty acid, synergizes with ABT-737 to induce leukemia cell death (D). Diethylmaleate (DEM) and ethacrynic acid (EA), reported depleting GSH agents, synergize with ABT-737 to induce apoptosis in leukemia cells (E). Metformin increases Bak crosslinkings induced by ABT-737 (F). (A) KBM5, REH, OCI AML3, U937 (0.2×10^6 cells/ml) were
seeded in T25 flasks and treated or not with Metformin (10 mM) for 16 hrs. After incubation, the cells were counted and lysed to perform immunoblot detection of Bcl-2 and Mcl-1 proteins. (B) KBM5 cells were cultured alone (2×10^5 cells/mL in 300 μl in 48-well plates), or on a feeder layer of 1×10^4 bone marrow derived MSC, and exposed to 4 μmol/L ABT-737 +/- 10 mmol/L Metformin or 1 μmol/L rotenone. Cells were incubated for 16 h and % cell death was determined as described in the Materials and Methods in the CD90 (-) compartment. (C) REH cells were cultured alone (2×10^5 cells/mL in 300 μl in 48-well plates), or on a feeder layer of 1×10^4 bone marrow derived MSC, and exposed to 500 nmol/L ABT-737 +/- 10 mmol/L Metformin. % cell death was determined as above.* = p < 0.05 when compared to cells cultured alone; # = p < 0.05 when compared to ABT-737 alone in coculture. (D) REH and NALM-6 cells (2×10^5 cells/mL in 48-well plates) were cultured in RPMI or RPMI supplemented with 200 μmol/L palmitoleate (PO), and treated with ABT-737 (500 nmol/L for REH and 4 μmol/L for NALM-6) for 16 h and cell death determined by flow cytometry as described in Materials and Methods. * = p < 0.01 when compared to RPMI without PO. (E) OCI-AML3 cells (2×10^5 cells/mL in 48-well plates) were treated with 250 nmol/L ABT-737, +/- 200 μmol/L ethacrynic acid (EA) or 2 mmol/L diethylmaleate (DEM) for 16 h and apoptosis was determined by Annexin V staining as described in Materials and Methods. $ = p < 0.0005 when compared to ABT-737 alone. (F) U937 cells were seeded at 2×10^5 cells/mL (15 mls in T-75 culture flasks) and treated with 500 nmol/L ABT-737 in the presence or absence of 10 mmol/L Metformin for 24 h. Bak crosslinking was determined as described in Materials and Methods.

**FIGURE 12:** Metformin sensitizes primary leukemia CD34 (+) cells to the effects of ABT-737 (A-B). Samples information: DX (Diagnostic), S (Source), WBC (White Blood Count), G (Gender), CI (Combination index) (C). Phenformin sensitizes primary leukemia cells to the effects of ABT-737 at a lower concentration range than Metformin (D-E). (A and B) Two AML primary samples were exposed to increasing concentrations of Metformin, ABT-737, or a combination of each agent using a 1:10 fixed dose increase schedule. Apoptosis was determined by flow cytometry, gating on CD34 (+) cells as described in Materials and Methods. (C) Summary of CI values (10 samples) and
patient characteristics for the primary leukemia samples used in this study. Acute lymphoblastic leukemia samples were analyzed by gating on leukemic blasts by FSC/SSC. (D) Two primary AML samples were exposed to micromolar concentrations (50 μmoles/L) of Metformin or Phenformin, +/- 50 nmoles/L ABT-737 for 24 h and apoptosis determined by flow cytometry as above. * = p<0.05 from ABT-737 alone. (E) 3 primary AML samples were exposed to 20 μmoles/L Phenformin, +/- 20 nmles/L ABT-737, and apoptosis determined by flow cytometry, gating on CD34 (+) cells as in A and B. * = p<0.05 from ABT-737 alone

**DIAGRAM 3:** Metformin inhibits oxidative phosphorylation (OXPHOS), decreases oxygen consumption and reduced glutathione concentration (GSH), increasing Fatty acids and Cardiolipin concentration as promoting superoxide production, Apoptosis and Bak crosslinkings.

**FIGURE 13:** TH-302 (Hypoxia activated pro-drug) reverses hypoxia in the bone marrow of mice bearing AML (A). TH-302 reduces the number of circulating human CD45+ AML cells (B). TH-302 prolongs the survival of AML-bearing mice compared to the vehicle treated mice (C). (A) Human AML cells (1X10^6/ml) were injected into irradiated (250 cGy) NOD/Scid/IL2Rγ-KO (NSG) mice and the progression of the disease was followed by flow cytometry detecting human CD45+ cells in peripheral blood. After engraftment (~5%) of human CD45+ cells was detected in peripheral blood the mice were randomized and treated with TH-302 (50 mg/kg intraperitoneally 3 times a week for 3 weeks) or PBS. At the end of the treatment and 3 hrs prior to being humanly euthanized, two mice per group were injected (via i.p) with a solution of pimonidazole to reach a final concentration of 100mg/kg per mouse. After this time the mice were sacrificed to detect hypoxia levels in the bone marrow and the remaining mice were followed to determine if TH-302 conferred any prolonged survival. The extraction and staining of bone marrow sections with an antibody detecting the hypoxic sensitive agent pimonidazole, evidenced a decrease in the hypoxic areas of treated mice compared to control mice; 20X magnification. (B) The percentage and absolute number of circulating AML cells was determined and the survival of the mice was followed. At week three, 2 or 3 mice were bled and the number of human CD45+ cells was calculated: Controls (17.3 and 9.2x10^6 cells/ml) and TH-302 (5.5, 2.3 and 0.53x10^6 cells/mL). (C) The decrease in the number of
human CD45+ cells in peripheral blood was correlated with increase of survival in the TH-302 treated group (Median Survival time=75 days) compared to control mice (Median Survival Time=56 days). P=0.003, n=8 mice/group.

**FIGURE 14:** Bioluminescence imaging of C57Bl/6 mice injected with AML1/ETO cells. (A) C57Bl/6 mice were injected with one million of AML1/ETO-luc-GFP cells and the progression of the disease was followed by bioluminescence imaging. After 3 weeks the mice were injected with a solution of 80 mM hyperpolarized [1-13-C] pyruvate and imaged in a 7T Bruker MR Scanner using a dual tuned 1H/13C volume coil. 13C surface coil was placed on the femur of the animal and the resonance frequency signal was detected. (B) Axial slice showing the location analyzed in several mice by hyperpolarized magnetic resonance. (C) Axial slices and frequency signals from [1-13C]-pyruvate and HP [1-13C]-lactate in normal control and AML mice. (D) Data was processed and dynamic curves characterizing the arrival of hyperpolarized pyruvate and its chemical –through lactate dehydrogenase- conversion into lactate were generated. Normalized lactate, defined as the ratio of total cumulative lactate to the total HP-MR signal, was calculated for each 13C scan.

**FIGURE 15:** WT-pHLIP targets leukemia cells more efficiently at lower pHs (6.5-6-5.5) (A-B). WT-pHLIP does not affect number of viable OCI-AML3 cells or apoptotic rate (C-D). WT-pHLIP accumulates more in cells seeded in media adjusted to pH 6.0 (E). (A-B) OCI-AML3 (0.2×10^6 cells/ml) were seeded in RPMI medium adjusted with aliquots of 0.1M HCL to different pHs (7.4-7-6.5-6-5-5) and incubated for 48 hrs with a single concentration (250 nM) of WT-pHLIP labeled with the fluorochrome Alexa Fluor 647 for their detection. WT-pHLIP significantly (p<0.02) accumulated better in the cells that were cultured in a low pH media (6.5-6-5.5) without affecting their viability or induce apoptosis (C-D). Representative dot plots of leukemia cells incubated in medium with different pHs and a single dose of WT-pHLIP-Alexa Fluor 647 are shown (E).

**FIGURE 16:** WT-pHLIP -Alexa 750 accumulates better in mice with CML compared with the respective control. Imaging of (1) BCR-ABL mouse without injection of the peptide, (2) FVB (Friend Virus B) WT normal mouse with injection of the peptide and two (3-4) BCR-ABL mice with injection.
of the peptide. Three (One WT normal mouse and two BCR-ABL mice) of the 4 mice were injected via IV (Intravenous) with 100 μl of a solution of 40 μM WT-pHLIP-Alexa 750 in PBS while the forth mouse was not. Two, four and twenty four hrs after injection, the mice were imaged with an IVIS 200 imaging system (PerkinElmer) using ICG bkg/excitation and ICG/emission channels.

**FIGURE 17:** Transgenic mice were exposed or not to drinking water with 2 mg/ml of doxycycline and 1% of sucrose. The water was changed once a week during a month. The presence of thrombocytosis and neutrophilic leukocytosis was detected in peripheral blood by flow cytometry and was considered a sign of disease (CML). (A) Representative dot plots demonstrating an increase in the number of neutrophils in mice with doxycycline withdrawal. The mice were then injected with a solution of 40 μM WT-pHLIP-Alexa 647 in PBS and imaged 4 and 24 hrs later using an IVIS 200 imaging system (PerkinElmer) and the filter set 2 (DsRed) with 500-550 and 575-650 excitation and emission wavelengths respectively. (B) pHLIP-Alexa 647 accumulates in the upper legs of mice with doxycycline withdrawal (Time point: 4 hrs). After the images were taken, the mice were sacrificed and the distribution of WT-pHLIP Alexa 647 in different organs (Upper legs, Femur, Kidney, Spleen, and Liver) was determined. (C) Organ extraction evidenced the intensity of WT-pHLIP Alexa 647 signals and even uncovered signals not previously detected (Time Point: 4 hrs). (D) No signals were detected in other organs as spleen, liver or kidney (Time Point: 4 hrs). Twenty four hours after injection, the same procedure was performed evidencing a similar pattern of accumulation in the upper legs of both mice (Dox OFF and ON), being stronger in mice where doxycycline was removed (E) WT-pHLIP-Alexa 647 accumulates in the upper legs of mice with doxycycline withdrawal (Time point: 24 hrs). (F) Organ extraction evidenced the intensity of WT-pHLIP-Alexa 647 signal (Time Point: 24 hrs). (G) No signals were detected in other organs as spleen, liver or kidney (Time Point: 24 hrs). Pieces of the organs were sent to the histopathology core and slides for microscopy were generated. Additional staining with DAPI (4',6-diamidino-2-phenylindole) was made to detect nucleus as well. (H)

**FIGURE 18:** (A) Distribution of WT-pHLIP-Alexa 647 in mice injected or not with AML cells (MOLM-13). (Time points 2-4 and 24 hrs). One million of leukemic cells (MOLM-13) were injected into 5
irradiated (250 cGy) NSG mice and the progression of the disease was followed by flow cytometry. At day 10, mice were injected with 100 µl of WT-pHLIP -Alexa 647 WT (40 µM in PBS) via IV and imaged after 2, 4 and 24 hrs. (B) Distribution of WT-pHLIP -Alexa 647 in different organs of mice injected or not with AML cells (MOLM-13) (Spleen and femur).

**FIGURE 19:** (A) OCI-AML3 cells were seeded in RPMI medium adjusted with aliquots of 0.1M HCl to different pHs (7.4-7-6.5-6-5-5) and incubated for 48 hrs with a single concentration (1µM) of pHLIP-SPDP (Linker) or pHLIP-Amanitin. After the incubation time the cells were harvested and stained with Annexin V and DAPI for 15 min before running the flow cytometry. pHLIP-Amanitin decreases viability and increases apoptosis levels under lower pH conditions (7-6.5). (B) OCI AML3 cells were seeded in RPMI medium adjusted to pH 6 or 7.4 and exposed to several doses of pHLIP-SPDP or pHLIP-Amanitin (0.25, 0.5, 1 µM) during 96 hrs. After the incubation time the cells were harvested and stained with Annexin V and DAPI for 15 min before running the flow cytometry. pHLIP-Amanitin decreases viability in a concentration dependent manner and increases apoptosis levels under low pH conditions.

**DIAGRAM 4:** As platelets promote mitochondrial uncoupling, an increase in oxygen consumption and fatty acid oxidation could lead to an eventual decrease in oxygen abundance, reducing Krebs Cycle activity and promoting glycolysis instead. These events could open new therapeutic opportunities for the use of hypoxia activated prodrugs and pH sensitive peptides.
LIST OF SUPPLEMENTAL FIGURES

Supplemental Figure 1: Oxygen consumption measured by a different method (OxygenBiosensor plates) evidenced the effect of Metformin on respiration rate. OCI-AML3 cells were seeded in 100 µl (2×10^5 cells/mL) in oxygen biosensor plates, treated with the indicated doses of Metformin or 4 mM NaCN, overlaid with mineral oil, and incubated for 4 h at 37°C. Fluorescence was read (ex 485, em 520) in a Fluostar Optima fluorescent plate reader. Fluorescent intensities were corrected for NaCN values (background substraction) and results expressed as % control. * = p < 0.001.

Supplemental Figure 2: Oxygen Consumption Rate measured by Seahorse System. Palmitate (Fatty acid) partially recovered the oxygen consumption inhibited by Metformin and Etomoxir in KBM5 cells. KBM5 cells (0.4×10^6 cells/ml) were seeded in T75 flasks and treated with Metformin for 2 hrs. After treatment, cells were transferred to XF96e Seahorse Biosciences plates and exposed to different substrates (Bovine Serum Albumin (BSA), Palmitate) or inhibitors (Etomoxir). Oxygen consumption (OCR) was determined and expressed as values normalized to BSA-only treated cells (BSA W/O/E; defined as 100%). * = p<0.05 from BSA-only treated cells; ** = p < 0.001 from palmitate W/O/E, and no metformin treatment.

Supplemental Figure 3: Oxygen Consumption Rate measured by Seahorse System. Palmitate (Fatty acid) partially recovered the oxygen consumption inhibited by Metformin and Etomoxir in REH cells. REH cells (0.4×10^6 cells/ml) were seeded in T75 flasks and treated with Metformin for 2 hrs. After treatment, cells were transferred to XF96e Seahorse Biosciences plates and exposed to different substrates (Bovine Serum Albumin (BSA), Palmitate) or inhibitors (Etomoxir). Oxygen consumption (OCR) was determined determined and expressed as values normalized to BSA-only treated cells (defined as 100%). * =p<0.05 from BSA-only treated cells; ** = p < 0.001 from palmitate W/O/E, and no metformin treatment.

Supplemental Figure 4: Isobologram OCI-AML3. Fixed dose ratio analysis of the combination of Metformin and ABT-737 in OCI-AML3 cells. OCI-AML3 cells (2×10^5 cells/mL in 48-well plates) were treated with the indicated doses of Metformin or ABT-737 and the fixed ratio combinations of
Metformin + ABT-737 for 16 h and cell death determined as described in Materials and Methods. * = p < 0.05 when compared to ABT-737 alone. ** = p < 0.01 when compared to ABT-737 alone.

Supplemental Figure 5: Isobologram NALM-6. Fixed dose ratio analysis of the combination of Metformin and ABT-737 in NALM-6 cells. NALM-6 cells (2×10^5 cells/mL in 48-well plates) were treated with the indicated doses of Metformin or ABT-737, and the fixed ratio combinations of Metformin + ABT-737 for 16 h and cell death determined as described in Materials and Methods. * = p < 0.05 when compared to ABT-737 alone.

Supplemental Figure 6: Isobologram U937. Fixed dose ratio analysis of the combination of Metformin and ABT-737 in U937 cells. U937 cells (2×10^5 cells/mL in 48-well plates) were treated with the indicated doses of Metformin or ABT-737, and the fixed ratio combinations of Metformin + ABT-737 for 16 h and cell death determined as described in Materials and Methods. * = p < 0.05 when compared to ABT-737 alone.
DIAGRAMS AND FIGURES

DIAGRAM 1

Glucose → Glucose 6P → NAD+ NADH → GAD-3-P → 3-PG → Pyruvate → Lactate+NAD

Fatty Acids → Acetyl-CoA → Citrate → FADH2 → NADH

Lipids + Phospholipids + Glutamine

O2 + H2O → OXPHOS → MITOCHONDRIA
FIGURE 2

A

O₂ consumption (A.U.)

30000
25000
20000
15000
10000
5000
0

0 1 2.5 5

PL (%)

** * *

B

O₂ consumption (A.U.)

35000
30000
25000
20000
15000
10000
5000
0

control RAN SP RAN + SP

** *

C

MFI (% control)

120
100
80
60
40
20
0

phospholipids triglycerides

0 1 2.5 5

PL (%)

* **

D

ethidium/DNA (MFI)

120
100
80
60
40
20
0

OCI OCI + Rot

0 1 2.5 5

PL (%)

* **

# # #
FIGURE 3

A. MOLT4

% Annexin V (+) cells

B. OCI-AML3

% Annexin V (+) cells

C. MOLT4

% TMRM (-) cells

D. OCI-AML3

% TMRM (-) cells

ABT-737 (µM)

ABT-737 (nM)
FIGURE 6
FIGURE 9

A

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<td>10</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

D

% CONTROL GSH

- REH
- NALM

MET (mM)
FIGURE 10

A. Graph showing cell death (% CD57) vs. ABT (nM).
   - Dashed line: RPMI
   - Solid line: MET

B. Graph showing cell death (% CD57) vs. ABT (nM).
   - Line: RPMI
   - Dotted line: MET

C. Bar graph showing cell death (% CD57) vs. DMSO, ABT, and ROT.
   - Met: 70, 60, 50, 40, 30, 20, 10, 0
   - ROT: 70, 60, 50, 40, 30, 20, 10, 0

D. Flow cytometry plots comparing annexin V and TMRM.

E. Graph showing cell death (% CD57) vs. Met (µM), Phen (µM), ABT-737 (nM), Met+ABT-737, and Phen+ABT-737.

F. Table showing cell line sensitivity.
   - NALM: 0.47
   - REH: 0.50
   - KBM5: <0.1
   - OCI: <0.1
   - U937: <0.1
FIGURE 13

A

B

Total MN cells

Total human AML cells

C

Percent survival

P=0.0027

Median survival

TH-302=75

Control=56
FIGURE 14

A

B
C

Normal Control

AML

D

Conversion of HP Pyruvate to Lactate

P < 0.01
% POSITIVE CELLS (pHLIP-ALEXA FLUOR-647)

OCI-AML3

WT-pHLP 250 nM

100

90

80

70

60

50

40

30

20

10

0

10

20

30

40

50

60

70

80

90

100

pH

5.5

6

6.5

7

7.4

B
Control

pHLIP 250 nM

E

pH 7.4

Gate Y-AMean
All 0.65

pH 6.0

Gate Y-AMean
All 0.68

Gate Y-AMean
All 11.83

Gate Y-AMean
All 30.82
**FIGURE 16**

1. BCR-ABL no injection
2. FVB WT
3. BCR-ABL #1
4. BCR-ABL #2
FIGURE 17

A

DOX ON

[CD45+] GFP / MsCD45-PB

LY6G+ : 25.17%  
GFP LY6G+ : 0.43%

LY6G GFP- : 74.25%  
GFP + : 0.14%

DOX OFF

[CD45+] GFP / MsCD45-PB

LY6G+ : 4.50%  
GFP LY6G+ : 50.48%

LY6G GFP- : 25.74%  
GFP + : 19.21%

GFP
1. BCR-ABL no injection
2. BCR-ABL no injection
3. BCR-ABL+pHLIP
4. BCR-ABL+pHLIP
5. BCR-ABL+pHLIP
C

Upper leg
Femur
Kidney

BCR-ABL+pHLIP

DOX OFF DOX ON

Radiant Efficiency
\( \text{p/sec/cm}^2/\text{sr}, \ \mu\text{W/cm}^2 \)
Min = 7.22e7
Max = 2.53e8

D

Spleen
Liver
Kidney

BCR-ABL+pHLIP

DOX OFF DOX ON

Radiant Efficiency
\( \text{p/sec/cm}^2/\text{sr}, \ \mu\text{W/cm}^2 \)
Min = 4.84e7
Max = 5.76e7
1. BCR-ABL no injection
2. BCR-ABL no injection
3. BCR-ABL+pHLIP
4. BCR-ABL+pHLIP
F

Upper leg

Femur

Spleen

---

BCR-ABL

No Inj  pHILIP

DOX ON  DOX OFF

---

Epi-fluorescence

Radiant Efficiency (p/sec/cm²/sr, μW/cm²)

Min = 5.13e8
Max = 6.17e8

G

Liver

Kidney

---

BCR-ABL

No Inj  pHILIP

DOX ON  DOX OFF

---

Epi-fluorescence

Radiant Efficiency (p/sec/cm²/sr, μW/cm²)

Min = 8.25e7
Max = 8.25e7
1. AML no injection
2. NO AML+pHLIP
3. AML+pHLIP
4. AML+pHLIP
5. AML+pHLIP

B

Spleen

Femur

No Inj  +pHLIP
FIGURE 19

A

![Graph showing viable cells per mL at different pH levels for pHLIP-SPDP and pHLIP-Amanitin](image)

B

![Graph showing viable cells and annexin V-positive cells at different pH levels for pHLIP-SPDP and pHLIP-Amanitin](image)
DIAGRAM 4

Glucose
  ↓
Glucose 6P

GAD-3-P → 3-PG → Pyruvate → Lactate NAD+

Fatty Acids → Acetyl-CoA

FADH2 → NADH → Citrate → α-KG

O2 ↔ H2O

OXPHOS

Platelets
  ↓
Oxygen Consumption Fatty Acid Oxidation

[O2]↑
Krebs Cycle Activity

Hypoxia

Glycolysis Lactate
SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1

SUPPLEMENTAL FIGURE 2
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APPENDIX

PEER REVIEWED PAPERS


BOOK CHAPTERS


POSTER PRESENTATIONS


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