UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
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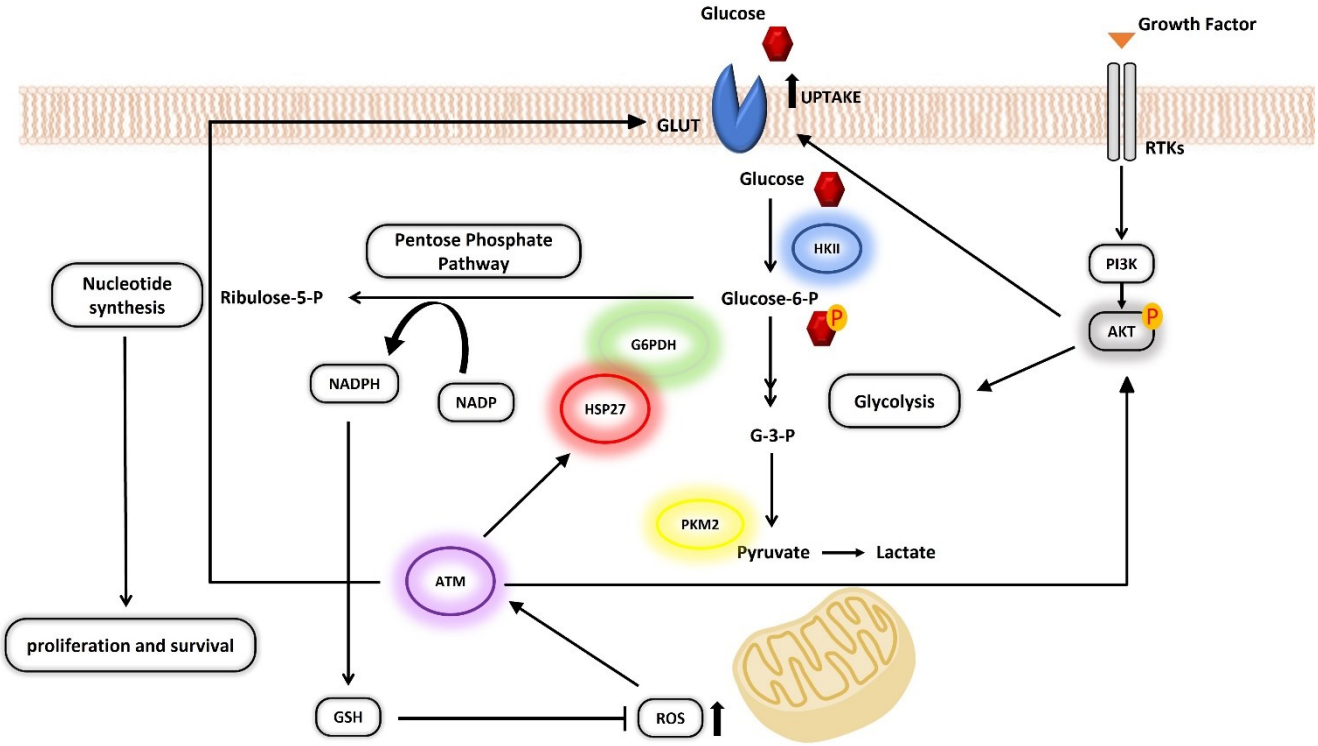
XXXIV CYCLE

Cristina Terlizzi

MODULATION OF GLUCOSE METABOLISM IN ONCOGENE-DRIVEN TUMORS: IMPLICATIONS FOR THERAPY



Academic Year 2020-2021

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MODULATION OF GLUCOSE METABOLISM IN ONCOGENE-DRIVEN TUMORS: IMPLICATIONS FOR THERAPY

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"Happiness can be found, even in the darkest of times, if one only remembers to turn on the light."

Harry Potter and the Prisoner of Azkaban. J.K. Rowling

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Background and rationale: Activation of oncogenes or loss of function of suppressor genes, in addition to induce neoplastic transformation, leads to a reprogramming of glucose metabolism consisting of a shift from oxidative phosphorylation to aerobic glycolysis even in normoxic conditions. A number of evidences indicates that targeting both oncogene signaling and tumor metabolism may affect proliferation and survival of cancer cells. The aim of the present study was to test whether targeting oncogene drivers in Chronic Myeloid Leukemia (CML) and Non-Small cell Lung Cancer (NSCLC) cells can reverse the Warburg effect by reducing glycolysis and upregulating mitochondrial complexes and to test the effects of combined therapy targeting both oncogene drivers and glucose metabolism.

Methods: Oncogene drivers such as EGFR, BCR-ABL and MET were selected as targets along with key proteins regulating glucose metabolism. CML and NSCLC cell lines were subjected to oncogene inhibition by treatment with selective Tyrosine Kinase Inhibitors (TKIs) and tested for glycolytic and mitochondrial protein expression along with glucose uptake, lactate production, oxygen consumption rate and extracellular acidification rate. Co-targeting of oncogene drivers and glucose metabolism was performed by silencing Pyruvate Dehydrogenase Kinase 1 (PDK1), that phosphorylates and inactivates the pyruvate dehydrogenase complex (PDHC), followed by exposure to TKIs. Similarly, the simultaneous inhibition of the oncogene driver and ataxia-telangiectasia mutated protein kinase (ATM), that induces glucose-6-phosphate dehydrogenase (G6PDH) activity, was obtained by exposure to TKIs and ATM inhibitor. Then levels of apoptotic markers were evaluated by Western blot analysis in response to combined treatments and cell viability assays were performed to test toxicity.

Results: EGFR and BCR-ABL driven cells showed a reduction of glycolysis and an upregulation of oxidative phosphorylation in response to treatment with the selective TKIs. In NSCLC cancer cells, silencing of PDK1 combined with TKI treatment caused a more pronounced effects on glycolysis and oxidative phosphorylation as well as higher levels of apoptotic markers as compared to those observed after single treatment. A similar potentiation effects on apoptotic markers and cell viability was observed in the same cell lines exposed to combined treatment with ATM and EGFR inhibitors. Silencing of ATM along with TKI treatment confirmed the results obtained with the ATM inhibitor.

Conclusions: Co-targeting of glucose metabolism and oncogene drivers such as EGFR, BCR-ABL and MET improves tumor response and can be proposed as a suitable strategy for improving the efficacy of TKI treatment.

AML: Acute myeloid leukemia

AMPK: 5' AMP-activated protein kinase

AT: Ataxia-telangiectasia

ATDL: Ataxia-telangiectasia disease like

ATM: Ataxia-telangiectasia protein mutated

ATP: Adenosine triphosphate

AKT: Protein kinase B (PKB)

CML: Chronic myeloid leukemia

DSB: DNA double-strand break

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinases

KAP-1: KRAB-associated protein-1

FGFR: Fibroblast growth factor receptors

FLT3: Fms like tyrosine kinase 3

GLUT: Glucose transporter type

G6PDH: Glucose-6-P dehydrogenase

HKII: hexokinase

HIF-1: Hypoxia-inducible factor 1

LDH: Lactate dehydrogenase

LKB1: Liver kinase B1

mTOR: mammalian Target Of Rapamycin

mTORC: mTOR Complex 1

MET: Hepatocyte growth factor receptor

MRN: Mre11, Rad50 and Nbs1 complex

NAC: N-acetyL-cysteine

NSCLC: Non-Small cell Lung Cancer

OD: Optical density

PDK: Pyruvate dehydrogenase kinase

PDH: pyruvate dehydrogenase complex

PFK: Phosphofructokinase

PK: pyruvate kinase

PI3K: Phosphoinositide 3-kinases

PDGFR: Platelet-derived growth factor receptors

PPP: pentose phosphate pathway

PTEN: Phosphatase and tensin homolog

ROS: Reactive species of oxygen

shRNA: short hairpin RNA

siRNA: small interfering RNA

TCA: tricarboxylic acid

TSC2: Tuberous Sclerosis Complex 2

TKIs: Tyrosine kinase inhibitors

VDAC: Voltage Dependent Anion-selective Channel

1. Hallmarks of cancer and therapeutic strategies

1.1 Oncogene addiction

Tumorigenesis is the acquisition of malignant properties by normal cells. It is widely accepted that neoplastic transformation is a multi-step process driven by the progressive acquisition of activating mutations in oncogenes (gain-of-function) and inactivating mutations in tumor suppressor genes (loss-of-function) (1) . During the last decades more than one hundred oncogenes and several tumor suppressor genes have been identified, and the list is expected to be extended. These genes are involved in cell proliferation, differentiation, cell fate pathways like apoptosis, senescence and autophagy. At the beginning of this century, Hanahan and Weinberg published their pivotal review in which they introduced and summarized shared tumor characteristics now known as hallmarks of cancer (**Fig. 1**). They first described six hallmarks, then few years later they updated the list that now includes: abnormal proliferation, resistance to tumor suppressor signaling, altered apoptotic program, replicative immortality, induction of angiogenesis, metastasis formation, genome instability, tumor-promoting inflammation, reprogramming of energy metabolism and immune evasion (2).

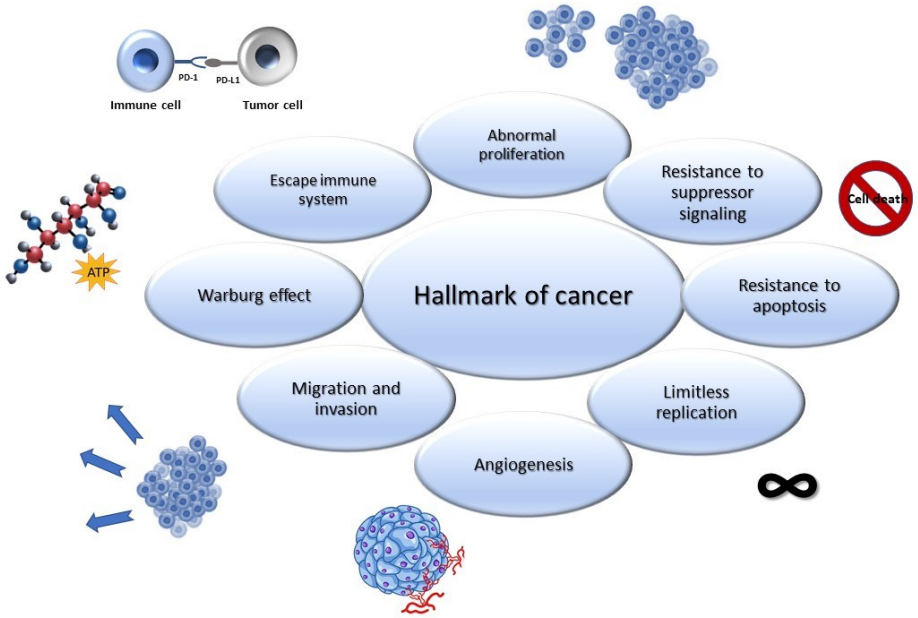

Figure 1. Main hallmarks of cancer including abnormal proliferation, resistance to tumor suppressor signaling, altered apoptotic program, unlimited replication,

neoangiogenesis, metastasis formation, altered energy metabolism and immune evasion.

The first step of tumorigenesis is a stochastic, heritable and irreversible genetic event that decides the commitment of the affected cell and its progeny to the acquisition of the mentioned hallmarks. The acquired characteristics are responsible for the maintenance of malignant phenotype and subsequent tumor progression. Then the unlimited proliferation, the genetic instability, the occurrence of epigenetic events, the unfavorable microenvironmental conditions lead to a high tumor heterogeneity due to presence of different cell subpopulations that vary in growth rate, immunogenicity, drug response and ability to form metastases. Differences among cell subpopulations can be heritable and not heritable since they arise both from genetic and non-genetic events within the tumor.

The greatest challenge faced in the effort to eradicate cancer is represented by the difficulties to discriminate the initial oncogenic events from the subsequent genetic changes acquired during tumor progression. Past and current research as well as therapeutic efforts have been focused on the identification of the so-called "drivers" of cancer process. In fact, the identification of oncogene drivers in a given tumor may provide targets for effective anti-cancer therapy (3).

A confirmation of the validity of this approach is provided by tumors that are “oncogene addicted”, i.e., tumors that depend on one or few genes for the acquisition and maintenance of their malignant phenotype. Oncogene-addiction was firstly described from IB Weinstein and AK Joe (4) to indicate the reliance of several tumors on a single activated oncogenic pathway for the maintenance of their malignant properties. A number of studies provided evidence that the inhibition of the oncogene driver causes growth arrest and apoptosis in tumor cells addicted to that oncogene. Although tumors harbor multiple mutations, targeting a single gene overexpressed in that tumor can result in tumor regression. This concept of oncogene dependence has been reinforced by several studies conducted in different animal tumor models bearing oncogene-driven tumors and successfully treated with driver inhibitors (5-8).

1.2 Targeted therapies and mechanisms of resistance

Unraveling the molecular mechanism of tumorigenesis and tumor progression led to the successful development of new anti-cancer drugs. In fact, cancer treatment strategies have changed over time and we moved from non-specific cytotoxic drugs to selective targeted drugs. By blocking the function of key proteins activated by mutations and driving cancer cell proliferation, invasion and metastasis, targeted agents can inhibit the molecular pathways crucial for tumor growth and progression. Preferred targets are proteins essential

for the establishment or maintenance of the malignant state and since many of these proteins have kinase activity, specific protein kinase inhibitors have been developed (9).

The first example of an oncogene driver targeted with specific protein kinase inhibitors is the chimeric BCR-ABL protein expressed by chronic myelogenous leukemia cells. In this myeloproliferative disorder, the occurrence of a t(9;22)(q34;q11) translocation gives rise to the Philadelphia chromosome (10) and to the BCR-ABL fusion gene that encodes the chimeric BCR-ABL protein. When fused with BCR, ABL tyrosine kinase is constitutively activated and the chimeric protein drives neoplastic transformation by increasing proliferation, impairing transcriptional activity, and enhancing survival. The activity of BCR-ABL can be inhibited by imatinib mesylate, a small-molecule that, by competing with ATP for the binding to the kinase domain of ABL, prevents chimeric protein autophosphorylation and inhibits downstream signaling leading to growth arrest and apoptosis (11). Imatinib was approved for treatment of patients with chronic-phase CML and rapidly became the standard first-line therapy for those patients (12, 13).

Another paradigmatic example of an oncogene driver targeted with specific tyrosine kinase inhibitors is EGFR, a tyrosine kinase receptor that is abnormally activated or expressed in many solid tumors including non-small lung cancer (NSCLC), colorectal carcinoma and glioblastoma. EGFR signaling pathway controls multiple cellular processes including proliferation and survival. Several EGFR tyrosine kinase inhibitors (TKIs) have been developed as targeted therapeutic agents for the treatment of different types of tumors, including NSCLC. Despite the expression of EGFR in a high percentage of NSCLCs, only subgroups of patients benefit from treatment with EGFR TKIs such as gefitinib and erlotinib (14, 15). In fact, previous studies in patients with NSCLC have shown that the occurrence of somatic mutations in the kinase domain of EGFR is a major determinant of the dramatically high responsiveness of subgroups of patients to EGFR TKIs. The most common EGFR mutations are exon 19 deletions and single point mutations in exon 21 and they are referred as activating mutations since they hyperactivate the kinase that continuously transduces signals inside the cell. As a consequence, NSCLC cells become dependent on EGFR signaling for survival and when treated with EGFR TKIs they undergo apoptosis in addition to growth arrest. The presence of activating mutations in EGFR is currently considered as a pre-requisite to candidate NSCLC patients to first-line therapy with EGFR TKIs.

Despite the initial and marked clinical response, most tumors may become resistant to targeted agents. Multiple molecular mechanisms may confer resistance to cancer cells that become refractory to treatment with targeted agents. The chronic exposure to drug induces a strong selective pressure on cancer cells and this may give rise to subpopulations of cells bearing secondary mutations of the target or activate adaptive lateral signaling. For instance, second-site EGFR mutation T790M is often found in EGFR mutant-tumors that initially responded to TKI but subsequently became refractory to this treatment

(16, 17). This secondary mutation is reported to cause resistance to TKI due to steric hindrance at the drug binding site or to a higher affinity of ATP for the drug binding site (**Fig. 2**). Similar mutations have been found in other oncogenic kinases as BCR-ABL, FLT3, PDGFR and FGFR (18). An alternative mechanism of drug resistance found in NSCLC treated with EGFR TKIs is the redundant or lateral signaling through MET amplification (**Fig. 2**). In this case MET induces an EGFR-independent activation of mediators downstream the EGFR receptor thus counteracting the inhibitory effects of EGFR TKIs (19, 20). In general, cancer cells can use the interconnections between signaling pathways and change their signaling network to become resistant. This signaling crosstalk is normally present in cancer cells to ensure the constant functioning of the sustaining pathways. Cancer cells are able to exploit these compensatory mechanisms to phosphorylate the same substrates of the inhibited kinase maintaining active the downstream pathway. For instance, melanoma cells with mutant BRAF can acquire activating mutations in other mediators of the MAP kinase pathway or lose the function of negative regulator of the same pathway (21).

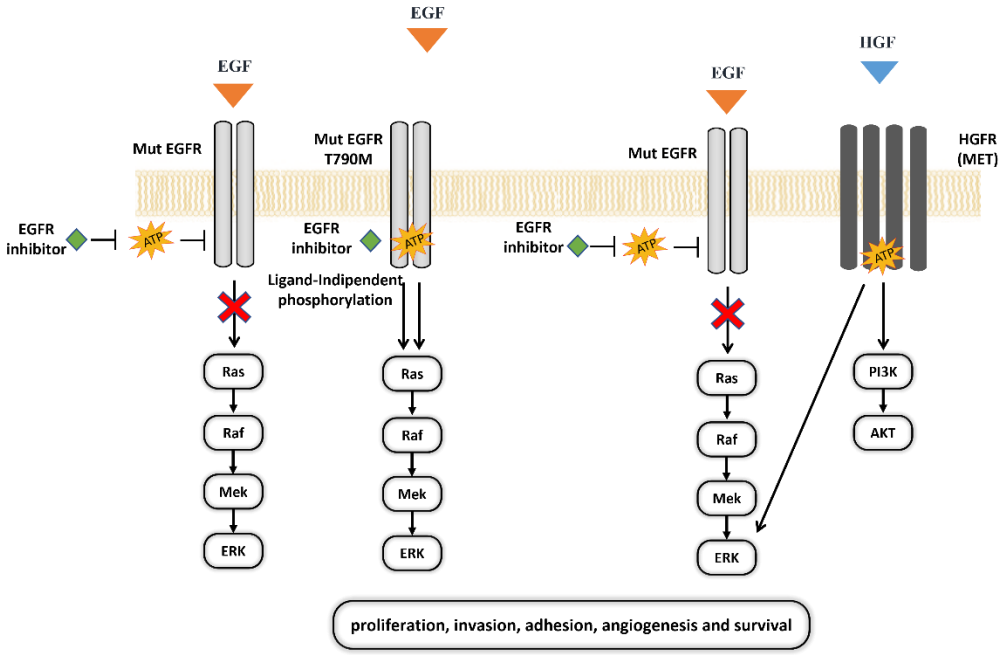

Figure 2. Examples of on-target and off-target resistance to EGFR TKIs due to the occurrence of secondary mutation (T790M) in the EGFR tyrosine-kinase domain or amplification of MET through reactivation of downstream oncogenic pathways.

Once the mechanisms by which cancer cells bypass the inhibition of their own driver have been identified, new therapeutic strategies capable of overcoming the resistance need to be identified. Several strategies have been proposed and the most promising involves a combination of drugs with different targets. Combined therapies have the advantage to reduce the selective pressure of a single drug avoiding the development and expansion of resistant clones and to reduce the doses of each drug (22, 23). Combinations of drugs are proposed based on synergies established in vitro and in vivo. However, targeting the same pathway at multiple points can still leave an escape to the tumor cells. Cross therapies that target independent but closely linked pathways may instead represent a better alternative since they can certainly limit the chances of cancer cells to develop acquired resistance. However, the simultaneous use of two or more drugs poses a clinical problem due to the various dose-limiting toxicities and drug interactions. For this reason, a single drug inhibiting two or more targets appears to be a more attractive strategy. Currently, drugs with multi-pharmacological activities, and in particular double-targeting agents, are increasingly used by researchers. Despite a rapid progress, however, none of those double inhibitors are currently in the clinical trial phase. Therefore, there is still a strong need to find more potent, effective and safer double inhibitors.

2. Glucose metabolism in cancer

2.1 Alterations of glucose metabolism in cancer

Oncogene-driven tumorigenesis implies a reprogramming of energy metabolism in cancer cells that lead to a shift from mitochondrial oxidative phosphorylation (OXPHOS) to aerobic glycolysis. Many years ago, Otto Warburg reported that cancer cells convert glucose to lactic acid through the glycolytic pathway even in the presence of oxygen and this alteration of energy metabolism is termed aerobic glycolysis (24). The first implication of Warburg's observations is that cancer cells are strictly dependent from external glucose supply even in the presence of oxygen, whereas normal mammalian cells in normoxic conditions convert glucose to CO2 and H2O through the tricarboxylic acid cycle with a more efficient production of ATP. The second implication of Warburg's observations is that the glycolytic phenotype is constitutive rather than adaptative in many tumors. Subsequent studies provided consistent evidence that glycolytic phenotype is maintained and regulated by a complex signaling network orchestrated by the oncogene driver (**Fig. 3**) and highly dependent on cellular context and microenvironmental conditions (25, 26). An unresolved question is whether aerobic glycolysis is simply associated with neoplastic transformation or has a causative role in tumorigenesis.

The glycolytic phenotype confers a growth advantage to cancer cells since a high rate of glycolysis provides metabolic intermediates for other biosynthetic pathways that in turn will produce the building blocks such as nucleotides, amino acids and fatty acids needed to sustain abnormal proliferation of cancer cells. In fact, other metabolic pathways branch out from glycolysis including the pentose phosphate pathway (PPP) which contributes to ribonucleotide synthesis by providing pentose phosphates and maintains the redox balance by supplying NADPH, the hexosamine pathway which is essential for the glycosylation of proteins, the glycogen synthesis which generates glucose reserves in the form of glycogen macromolecules, the biosynthesis of serine which supports the synthesis of amino acids. All these pathways are interconnected and dependent from the glucose flux through glycolysis (27).

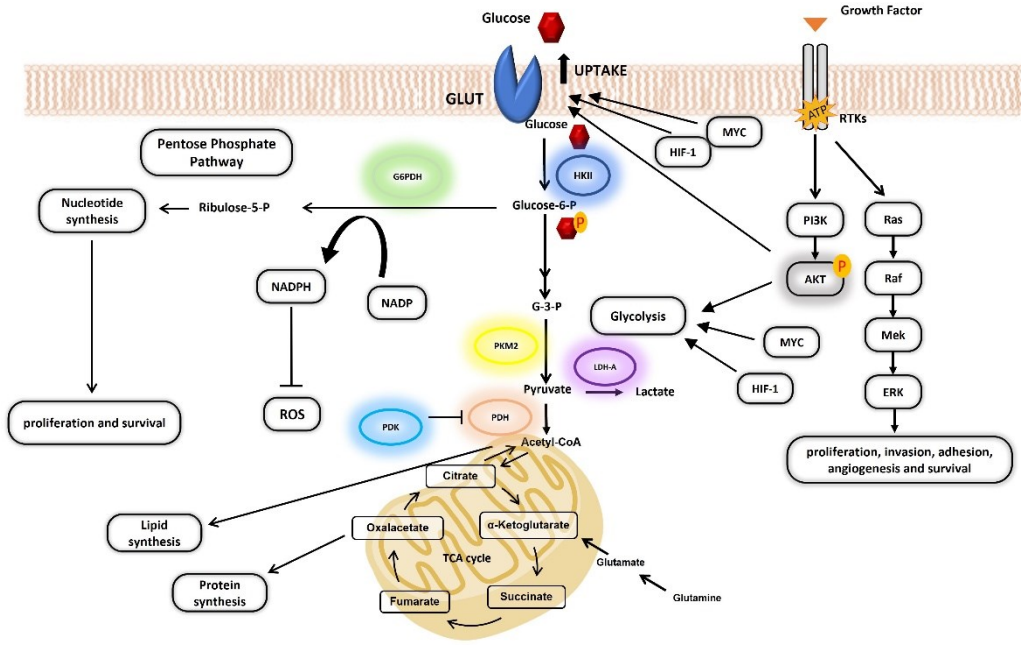

Figure 3. Schematic representation of cell energy metabolism including glycolysis, oxidative phosphorylation and pentose phosphate pathway and their interplay with oncogene signaling. Key enzymes at metabolic crossroads were tested in the present study and are highlighted in colors.

2.2 Rate-limiting enzymes of tumor glycolysis

In order to maintain a high glucose flux through plasma membrane, cancer cells overexpress several glucose transporters and glycolytic enzymes. Among the different isoforms of glucose transporters, Glut1 is reported to be the

major form expressed in cancer cells; hexokinase II and phosphofructokinase are upregulated due to abnormal activity of PI3K/AKT signaling (28); these two enzymes are responsible for two so-called committed step of glycolysis, phosphorylation of glucose and conversion of fructose into fructose-1,6-bisphosphate respectively. Hexokinase II catalyzes an irreversible reaction since, once glucose is phosphorylated, it cannot cross anymore cell membrane. Phosphofructokinase 1 (PFK1) converts phosphorylated fructose into fructose-1,6-bisphosphate, an intermediate that represents a crucial point of glycolysis due to its subsequent transformation in two compounds of three carbons that will generate two molecules of pyruvate. The last irreversible step is the one catalyzed by pyruvate kinase (PK) that converts phosphoenol-pyruvate into pyruvate. The rate of this step is limited in cancer cells by the presence of M2 isoform of PK (29, 30). In fact, PKM2 exists as a low-activity dimeric or high-activity tetrameric form and cancer cells predominantly express the low-activity dimeric form with a reduced ability to convert phosphoenolpyruvate to pyruvate (31). In particular, phosphorylation of PKM2 at Tyr105 is reported to disrupt the active tetrameric form (32, 33) and to reduce its catalytic activity. Preferential conversion of pyruvate to lactate is the result of a complex regulation of pyruvate dehydrogenase kinase (PDK). PDK protein family is composed by four isoforms (PDK1-PDK4) all located into mitochondrial matrix and able to phosphorylate and inactivate the pyruvate dehydrogenase complex that in turn catalyzes the conversion of pyruvate to acetyl-CoA (34, 35). Among the four isoforms, PDK1 has been reported upregulated in a variety of tumors including head and neck cancer and breast and lung cancer (36).
Furthermore, pyruvate is preferentially converted into lactate which is excreted outside the cell. For these reasons the acetyl-CoA levels are limited in cancer cells and to maintain the function of TCA cycle, intermediates of other metabolic pathways may replenish TCA cycle (37).

2.4 Targeting glucose metabolism in cancer

Several strategies have been proposed to modulate glucose metabolism in cancer cells. The first strategy is based on inhibition of signaling mediators and effectors modulating glucose metabolism in cancer cells. For instance, aberrant signaling through PI3K/AKT is reported to increase the expression and membrane translocation of glucose transporters and to phosphorylate key glycolytic enzymes such as hexokinase and phosphofructokinase 2. The PI3K/AKT can be activated by aberrant signaling from EGFR or other receptor tyrosine kinases, loss of function of PTEN or activating mutations in the PI3K complex itself. Inhibition of PI3K/AKT pathway can be achieved by direct targeting PI3K/AKT or indirectly by inhibition of upstream receptor tyrosine kinase or downstream signaling mediators. A large body of evidence indicates that transcription factors such as HIF-1 and MYC can bind to the promoter of

genes encoding most glycolytic enzymes and glucose transporters (38-40). Under hypoxic conditions HIF-1 virtually activates all genes involved in glycolysis from GLUT1 through pyruvate kinase and increases levels of lactate dehydrogenase A and lactate production (39, 41, 42). HIF-1 may also decrease the rate of oxidative phosphorylation through activation of pyruvate dehydrogenase kinases (PDKs) that ultimately results in a decreased conversion of pyruvate to acetyl-CoA thus impairing mitochondrial function (41, 43). In addition to hypoxia, a number of oncogenic events may stabilize HIF-1 resulting in the activation of glycolytic metabolism even in the presence of oxygen. High levels of the oncogenic transcription factor MYC are reported to activate the transcription of several glucose transporters and many glycolytic enzymes including hexokinase 2 (HK2), PDK1 and lactate dehydrogenase A (25, 38, 40). Unlike HIF-1, MYC activates also target genes responsible for mitochondrial biogenesis and function such as metabolism of glutamine that is considered an alternative energy source under glucose deprivation. Although several agents targeting HIF-1 have reached clinical stage and small molecule Myc inhibitors have been recently developed (44) targeting transcription factors for cancer treatment remains challenging.

The second strategy aims at inhibiting glycolytic cascade at one or multiple points. Almost all steps of glycolysis from glucose transport to lactate production can be inhibited by several compounds that are currently available. The major limitation of this approach is the fact that there is no large differential expression of the targets between normal and cancer cells and it is unclear whether a sufficient therapeutic window exists to competitively inhibit glucose transport and glycolytic enzymes in cancer cells. An exception is represented by PKM2 and LDHA that are isoforms differentially expressed in cancer cells and for this reason are considered promising targets for cancer therapy. Several small molecules that inhibit glucose transporters, mainly GLUT1, have been developed and tested. They showed to block glucose transport and to selectively kill cancer cells in vitro (45, 46). However, the wide expression of GLUT1 in normal tissues prevented the clinical use of these compounds. Furthermore, glucose transporters are redundant molecules and the inhibition of one isoform can lead to the expression of other isoforms. An interesting strategy targeting GLUT1 aimed to overexpress GLUT1 to induce an increase of ROS levels inside cells with consequent downregulation of glycolysis (47).

An additional target that has been proposed for tumor starvation, is HKII. Among the most common inhibitors of HKII, 3-bromopyruvic acid (3-BrPA), was reported to induce dissociation of HKII from mitochondria, to reduce ATP production and to cause cell apoptosis (48). Furthermore, 3-BrPA enhanced the effects of sorafenib, a multi-tyrosine kinase inhibitor, in a murine model of hepatocellular carcinoma (49). Metformin that is the first-line treatment for type 2 diabetes, is reported to have anti-tumor effects by inhibiting enzymatic activity of HKII (50). Methyl jasmonate (MJ) has been shown to disrupt VDAC and HKII interactions in the mitochondrial fraction of several murine and human cancer cell lines (51).

PKM2 catalyzes the conversion of phosphoenolpyruvate to pyruvate, elevated levels of PKM2 have been found in active proliferating cells including many tumor cells and not in terminally differentiated cells. The preferential expression of PKM2 over the other isoform PKM1 is achieved by alternative exon splicing modulated by MYC. In addition, the promoter of PKM2 is stimulated by HIF-1 in a mTOR-dependent way (29, 30, 31). When phosphorylated in Ser 37 by EGFR-activated ERK2, PKM2 is able to translocate to the nucleus where it directs transactivation of MYC which in turn upregulates its target genes (32). Knockdown of PKM2 expression in lung cancer cells results in reduced glycolysis and decreased cell proliferation. Previous studies reported that targeting PKM2 protein may improve tumor response to therapies and flavone derivatives and naphthoquinone derivatives were developed for this purpose. In 2009, Vander Heiden and coworkers screened a large library of naphthoquinone derivatives and tested their ability to bind PKM2. Subsequently, these studies led to the identification of shikonin as an effective inhibitor of PKM2 that could downregulate the glycolysis of MCF-7, A549 and HeLa cancer cells (52). Several flavonoid derivatives were also evaluated to be effective against PKM2 along with Cyclosporin A (CsA), Tannic acid (TA), Benserazide (Ben), beta-elemene (β-elemene) and Gliotoxin with different mechanisms of tumor toxicity (53).

Diversion of pyruvate flux from mitochondrial oxidative phosphorylation toward conversion to lactate can be achieved by inhibition of PDK1. Downregulation of PDK1 by short hairpin RNA reverts the glycolytic phenotype, inhibits tumor growth and reduces invasiveness in UM-22A, UM-22B, and JHU-O22 head and neck squamous cell carcinoma cell lines (54).
PDK inhibition by dichloroacetate (DCA) in A549 (non-small-cell lung cancer), M059K (glioblastoma), and MCF-7 (breast cancer) causes a shift of glucose metabolism from glycolysis to oxidative phosphorylation and increases ROS production with subsequent cell death (55). Furthermore, a pan-inhibition of PDKs was also reported to enhance the effects of cytotoxic drugs such as cisplatin and doxorubicin in different cell lines (56-58). Interestingly, the inhibition of PDK1 by using dichloroacetate (DCA) was reported to enhance the anticancer effects of EGFR tyrosine kinase inhibitors (TKIs) in EGFR mutant NSCLC cells and xenografts (59).

The third strategy proposed to target glucose metabolism is the inhibition of OXPHOS. Several drugs including metformin, lonidamin, atovaquone, and arsenic trioxide are used clinically for non-oncological diseases, but emerging data demonstrate their potential use as OXPHOS inhibitors. By differentially inhibiting mitochondrial complexes I-V, they disrupt the respiratory chain causing reduction of OCR, ATP production and alleviating hypoxia (60).

3. Ataxia-telangiectasia mutated protein kinase (ATM)

3.1 Canonical role of ATM

Ataxia-telangiectasia mutated protein kinase (ATM) signaling can be divided into two branches: a canonical pathway, which is involved along with the Mre11-Rad50-NBS1 (MRN) complex in the activation of the DNA damage checkpoint, and several non-canonical activation pathways mainly mediated by the presence of cellular stress (**Fig. 4**) (61).

ATM protein is mainly present in the nucleus where it is found in the form of a catalytically inactive non-covalent homodimer. In the presence of DNA damage, an autophosphorylation of ATM at serine (S)1981 site occurs simultaneously with the monomerization and activation of ATM (62). The MRN complex is composed by the Mre11 protein of the phosphatase family of phosphoesterase, the ATPase Rad50 protein and the Nbs1 protein, which regulates the activities of the first two. The identification of the functional relationship between ATM and the MRN complex is due to the detection of mutations in the MRE11 gene in patients affected by the AT-like disorder (ATLD), i.e. patients with a cellular and clinical phenotype similar to that observed in patients affected by Ataxia-telangiectasia (AT) but with a later onset and a slower progression of ataxia symptoms such as cerebellar ataxia, oculocutaneous teleangiectasia, other neurological disorders and an increased risk of cancer. ATDL cells show reduced ATM activation following double-strand break (DSB) and lower phosphorylation of kinase substrates (63). Furthermore, the addition of the MRN complex to purified dimeric kinase, demonstrating that the activation of ATM signaling is dependent on MRN (64).

3.2 Non-Canonical role of ATM

Over the past two decades, research into the mechanisms underlying the AT phenotype provided strong evidence of the involvement of oxidative stress in the neuronal dysfunction of AT patients. In fact, cells of AT patients and different mouse models of the disease showed high accumulation of reactive oxygen species (ROS) and were particularly sensitive to the administration of phenotypic traits of AT patients could be reduced or delayed by an antioxidant treatment (65-68). Several studies reported the activation of ATM following treatment with hydrogen peroxide in the absence of DNA damage and the phosphorylation of downstream substrates of ATM such as P53 and Chk2 but not KAP-1 protein that plays an essential role in the DNA damage response (69, 70). Recent studies have identified a new mechanism of ATM activation through

direct oxidation instead of MRN stimulation (71, 72). ATM activated in this way is not a monomer but an active dimer that is also phosphorylated in (S)1981 (73). The existence of ATM activation by oxidation raised the need to discriminate between phosphorylated substrates resulting from ATM activation dependent on DNA damage and those phosphorylated by the form of ATM known as oxidized ATM. Unfortunately, separating the two activities is challenging due to the concomitant presence of the two stimuli. In fact, oxidative stress, as well as the production of ROS, induce DNA damage, so ATM is exposed to both damage and oxidation at the same time. On the other hand, the presence of oxidative stress can disrupt MRN binding to DNA and hence MRN-dependent activation of ATM is reduced. Therefore, under stress conditions with ROS accumulation, oxidized ATM is the only activated form (71). ATM also has a role in the activation of AMPK through the phosphorylation of its LKB1 effector. Activated AMPK inhibits mTOR that is considered the main regulator of cellular response to nutrient availability and high mTOR activity leads to oxidative stress through the upregulation of mitochondrial oxygen consumption. Down-regulation of mTOR activity by oxidized ATM could explain the non-canonical role of ATM as redox sensor in cell oxidative homeostasis (74).

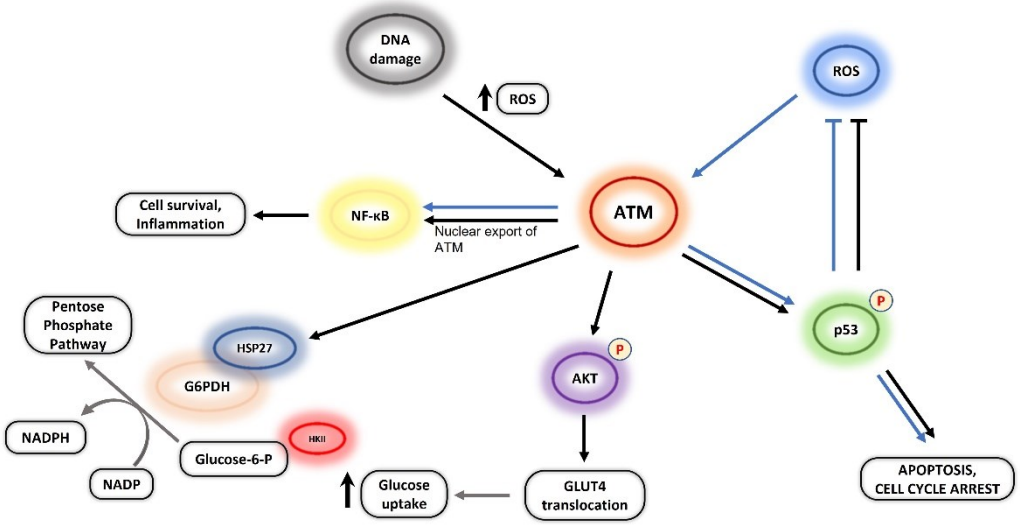

Figure 4. Canonical and non-canonical roles of ATM in response to different stimuli. Canonical ATM signaling enables DNA repair in response to double-strand breaks. Non-canonical ATM signaling modulates the pentose phosphate pathway through activation of G6PDH and glucose uptake through the enhancement of glucose transporters activity in plasma membranes. Different downstream mediators of ATM signaling are highlighted in colors.

3.3 ATM role in glucose metabolism

In addition to its role in ROS balance, ATM is involved in glucose metabolism since it affects glucose uptake by regulating GLUT1 transport activity and GLUT4 translocation. In fact, it is reported that c-terminal domain of GLUT1 contains a known ATM target (S490) through which ATM modulate its activity on cell membrane. Data obtained in a rat skeletal muscle cell line showed that inhibition of ATM causes a decrease of GLUT1 levels whereas ATM upregulation leads to an overexpression of GLUT1 on cell membrane (75). In the same in vitro model, treatment with ATM inhibitor KU55933 completely abolished insulin-dependent transport of glucose and caused a significant decrease of AKT phosphorylation indicating that inhibition of ATM significantly impairs insulin-mediated GLUT4 translocation (76).
The pentose phosphate pathway (PPP) is an alternative mechanism of glucose oxidation which primarily serves for generation of NADPH and ribose-5-phosphate. For these reasons, it is an essential pathway for cellular redox homeostasis and DNA repair. In fact, NADPH is the main reducing agent for quenching oxidative stress pathways whereas ribose-5-phosphate is a precursor of nucleotide biosynthesis needed for both DNA repair and synthesis. ATM is responsible for the metabolic switch of glucose from glycolysis to PPP since it modulates the enzymatic activity of Glucose-6-phosphatedehydrogenase (G6PDH). This enzyme catalyzes the rate-limiting step of the oxidative branch of PPP and it is reported to be overactivated in several tumors. Furthermore, G6PDH enzymatic activity depends on the $NADP^+$ / NADPH ratio. A high activity of this enzyme is observed in cells with a high consumption of NADPH, including cancer cells. Conversely cells at rest and with a low consumption of NADPH have a relatively low NADP + / NADPH ratio, and consequently the activity of G6PDH is reduced despite the high expression levels (77). Interestingly, ATM activates G6PDH both in presence of DNA damage and oxidative stress but with different mechanisms. When ATM exploits its function as a DNA repairer, it activates G6PDH through phosphorylation of HSP27, whereas oxidized ATM is able to directly modulate G6PDH expression (78, 79). Furthermore, recent study demonstrated that ATM is involved in the enhancement of glycolysis and lactate production in cancer associated fibroblasts (CAFs) cultured under hypoxic condition by inducing GLUT1 translocation in cell membrane and by upregulating PKM2 expression through PI3K/AKT pathway (80).

3.4 ATM in cancer therapy

Several studies have shown that ATM is a suitable candidate for cancer therapies, especially for combination strategies. FLT3-driven acute myeloid leukemia (AML) cells exposed to FLT3 inhibitors together with the inhibition of the ATM / G6PDH axis show a higher response to FLT3 inhibitor therapy. In fact, despite FLT3 inhibitors were effective in treatment of patients with AML, they did not cause durable remission. One possible cause could be the hyperactivation of the antioxidant ATM/G6PDH axis. Therefore, co-targeting of FLT3 and ATM/G6PDH axis could improve the efficacy of therapy with FLT3 inhibitors (81). Furthermore, ATM deficiency results in proliferation defect of astrocytes by increasing cellular ROS levels. Proliferation can be partially rescued by the addition of an antioxidant agent such as N-acetyL-cysteine (NAC), suggesting that oxidized ATM may phosphorylate some pathways converging on cell proliferation (82-85). ATM inhibition also caused an enhancement of EGFR-TKI sensitivity by combination therapy in TKI sensitive NSCLC cell lines by causing growth arrest and downregulation of EGFR pathway. The combination of ATM inhibitor (KU55933) and EGFR-TKI (gefitinib) showed a synergistic effect in the inhibition of cell growth and enhancement of apoptosis. These findings suggested that the inactivation of ATM kinase potentiates the anticancer effects of gefitinib (86).

DNA repair and metabolic pathways are vital for maintaining cellular homeostasis in normal cells. However, both pathways are profoundly altered by tumorigenesis and mechanisms for maintenance of the malignant state. A growing body of evidence indicates that DNA repair and metabolic pathways are interdependent and interconnected posing ATM at the crossroad since it serves as DNA repairer and metabolic stress sensor. For these reasons ATM is considered a potential target for innovative cancer therapy especially those based on combination strategies.

Accumulating evidences showed that activation of oncogenes or loss of function of suppressor genes, in addition to cause neoplastic transformation and acquisition of a malignant phenotype, induces a reprogramming of glucose metabolism by upregulating the expression of a pool of genes encoding glucose transporters and most glycolytic enzymes. Furthermore, a number of evidences indicates that targeting both oncogene signaling and tumor metabolism may affect tumor proliferation and survival. We reasoned that the simultaneous targeting of the two pathways may provide more effective tumor response as compared to that of single treatment. The aim of this study was to test whether targeting oncogene drivers in CML and NSCLC cells can reverse the Warburg effect by reducing glycolysis and upregulating mitochondrial complexes and to test the effects of combination therapy targeting both oncogene drivers and glucose metabolism.

Cell Lines and Treatment

Two NSCLC cell lines were obtained from and authenticated by the American Type Culture Collection. In particular, H1993 cells expressing high level of MET due to gene amplification (15 copy numbers) and wild type EGFR and H1975 cells bearing an activating point mutation in exon 21 (L858R) of the kinase domain of EGFR along with T790M secondary mutation which confers resistance upon first line therapies. H1993 and H1975 cells were grown in RPMI culture medium (Gibco, Thermo Fisher, 21875091 and Gibco, Thermo Fisher ATCC modification A1049101) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 50 µg/mL streptomycin in a humidified incubator with 5% CO2 at 37 °C. The CML cell line, K562, was purchased from and authenticated by American Type Culture Collection whereas KCL-22 cells were kindly provided by Prof. B. Izzo and Dr. F. Quarantelli. K562 were grown in IMDM culture medium (Gibco, Thermo Fisher, 12440053) while KCL-22 cells were grown in RPMI 1640 culture medium (Gibco, Thermo Fisher, 21875091), containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 50 µg/mL streptomycin in a humidified incubator with 5% CO2 at 37 ◦C.

The cells were treated with different inhibitors such as erlotinib (1 µM, S1023 Selleck Chemicals) drug against double mutant $EGFR^{L858R/T790M}$, WZ4002 (0.1 or 1 µM, S1173, Selleck Chemicals), cMET specific inhibitors PHA-665,752 (1 µM, S1070 Selleck Chemicals) or Crizotinib (1 µM, S1068, Selleck Chemicals), BCR-ABL inhibitor Imatinib (0.1, 0.5 or 1 µM, PKI-IMTB, Biaffin GmbH &CoKG) and ATM inhibitor KU55933 (10 or 100 nM CAS 587871-26-9 Sigma-Aldrich) or vehicle.

Drug-induced toxicity was assessed by MTS assay (Cell Counting Kit-8 Cell Proliferation/Cytotoxicity Assay Kit CK04 Dojindo). Briefly, CML cell line, K562 and KCL-22 cells were plated at a density of 5000/well in 96-well plates and then treated with increasing concentration (0.1–10 µM) of imatinib or vehicle for 72 hours. H1975 cells were were plated at a density of 5000/well in 96-well plates and then treated for 48 hours with increasing concentrations of KU55933 or WZ4002 (0.01-5 µM) alone and with fixed doses of KU55933 (10 nM and 100 nM) in combination with WZ4002 (0.01-5 µM). The optical density (OD) was measured at 490 nm using microplate spectrophotometer, after 1-2 hours incubation with MTS at 37 ◦C. At least three independent assays were performed, and data are expressed as percentage of viable cells, considering the untreated control cells as 100%.

shRNA and siRNA Interference

PDK1 targeted (shPDK1) and non-targeting (shCTRL) short hairpin RNA were purchased from Sigma-Aldrich (product number TRCN0000006263 and SHC002, respectively). In H1993 and H1975 cells, PDK1 knockdown was achieved using lentiviral vectors (MISSION Lentiviral Packaging Mix, SHP001, Sigma-Aldrich Saint Louis, MO, USA). Lentiviral vectors expressing either a PDK1 targeted shRNA (shPDK1) and non- targeting shRNA (shCTRL) were produced transfecting 293 T cell line according to manufacturer's instructions. Stable transfected cells were selected with puromycin (3 ng/mL, ANT-PR-1, Invivo-Gen, Toulouse, France) for 10 to 14 days. Antibiotic resistant colonies were pooled and expanded for further analysis.

H1993 and H1975 cells were transfected with siRNA targeting ATM (sense CUUAGCAGGAGGUGUAAAU, antisense AUUUACACCUCCUGCUAAG), and control non-targeting siRNA (siCTRL, FE5D0018101005) purchased from Sigma-Aldrich and used according to the manufacturer's instructions. Briefly, H1993 and H1975 cells were plated and allowed to attach for 24 hours. Then, cells were transfected with 100 nM siRNAs using the Dharmafect reagent (T-2001-02, Dharmacon), and after 24 hours EGFR or MET inhibitors were added for further 48 hours. Finally, cells were harvested and subjected to cell lysis to perform Western blot analysis.

Immunoblotting Analysis

Whole cell lysates were prepared after time of exposure to several inhibitors. Untreated and treated cells were lysed on ice in RIPA lysis buffer (R0278, Sigma-Aldrich) with protease (P8340, Sigma-Aldrich) and phosphatase inhibitors (P0044, Sigma-Aldrich) and kept on ice for 30 min. The suspension was homogenized and centrifuged at 13000 g at 4 °C for 30 min then supernatant containing whole cell lysates were collected.

Western blot analysis of proteins from different lysates was performed by using a standard procedure. Antibodies used for Western blotting included phospho-EGFR (2234S, Cell Signaling Technology), EGFR (sc-373746, Santa Cruz Biotechnology), phospho-AKT^{Ser473} (sc-33437, Santa Cruz Biotechnology), AKT (9272S, Cell Signaling Technology), phospho-p42/44 MAP kinase (ERK1/2) (9101S Cell Signaling Technology), p42/44 MAP kinase (ERK 1/2) (4696S Cell Signaling Technology), phospho-MET (3077S Cell Signaling Technology), MET (8198S Cell Signaling Technology), Cyclin D1 (2922, Cell Signaling Technology), α-tubulin (T9026, Sigma-Aldrich), actin (A4700, Sigma-Aldrich), BCR-ABL (MA1-153, Thermo scientific), phospho-STAT3^{Tyr705} (9145S Cell Signaling Technology), STAT3^{ser727} (9134S Cell

Signaling Technology), c-Myc (9402S Cell Signaling Technology), PDHK1 (3820, Cell Signaling Technology), PKM2 (4053, Cell Signaling Technology), cleaved caspase 3 (9661S, Cell Signaling Technology), GAPDH (5174S Cell Signaling Technology), lamin a/c (2032, Cell Signaling Technology), BIM (559685, BD Biosciences), phospho-ATM^{S1981} (ab81292 Abcam), ATM (ab199726, Abcam),) Hexokinase II (2867, Cell Signaling Technology), phospho-PKM2^{Tyr105} (3827, Cell Signaling Technology), phospho-PKM2^{Ser37} (11456, Signalway Antibody), LDH-A (3582, Cell Signaling Technology), phospho-PDH^{Ser293} (ab92696, Abcam), PARP (556494, BD Pharmingen) Glucose-6-Phpsphate Dehydrogenase (sc-373886 Santa Cruz Biotechnology), vinculin (4650, Cell Signaling Technology), phospho-p53^{ser15} (9284S, Cell Signaling Technology), p53 (sc-126, Santa Cruz Biotechnology), and a cocktail of 5 mAbs against OXPHOS (ab110411, Abcam) recognized the following proteins: 20 kD subunit of Complex I (20 kD), COX II of Complex IV (22 kD), 30 kD Ip subunit of Complex II (30 kD), core 2 of Complex III (~50 kD) and F1α (ATP synthase) of Complex V (~60 kD). A commercially available ECL kit (Advansta, San Jose, CA, USA) was used to reveal the reaction.

Glucose Consumption, Intracellular ATP, Citrate and Succinate Levels in Cultured Tumor Cells

In parallel experiments, both NSCLC cell lines transfected with shCTRL and shPDK1, were analyzed for glucose levels in the conditioned media and for intracellular ATP concentrations in basal conditions and in response to TKIs. Briefly, conditioned media were removed, centrifuged at 13000 g at 4 °C for 10 min and then assayed for glucose concentrations using the Glucose Assay Kit (GAG020-1KT, Sigma-Aldrich), following manufacturer's instructions. Intracellular ATP determination was performed using the ATPlite luminescence Assay (6016941, Perkin Elmer) following manufacturer's instructions. Briefly, cells were lysed, incubated with the ATP reaction mixture for 5 min and then subjected to luminescence measurements. Moreover, citrate levels were determined in shCTRL and shPDK1 cells using the Citrate Assay Kit (MAK333, Sigma-Aldrich), following manufacturer's instructions. Absolute glucose, ATP and citrate levels were calculated from the corresponding standard curve and normalized to 10^6 cells. At least three independent experiments were performed, and data were pooled. K562 and KCL-22 cells were subjected to glucose and ATP assays along with establishment of lactate levels by using Lactate Assay Kit (MAK064, Sigma-Aldrich) following manufacturers' instructions. Furthermore, H1993 and H1975 were subjected to glucose and citrate assay along with measurement of pyruvate by using Pyruvate Assay Kit (MAk071, Sigma-Aldrich) and succinate by using Succinate Assay Kit (MAK184, Sigma-Aldrich) following manufacturers' instructions. Cells were seeded in six-well flat-bottomed plates at a density of 3×10^5 cells per well and then treated with

selected inhibitors, then were collected and resuspended in specific substrate assay buffer and incubated with appropriate assay mix. The optical density (OD) was measured using microplate spectrophotometer and metabolites concentrations were calculated from the corresponding standard curve and normalized for 10^6 cells. At least three independent experiments were performed, and data were pooled.

Glucose-6-Phpsphate Dehydrogenase Activity

H1993 and H1975 were subjected to detection of Glucose-6-Phpsphate Dehydrogenase enzymatic activity by using Glucose-6-Phpsphate dehydrogenase assay kit (MAK015, Sigma-Aldrich) following manufacturers' instructions. Briefly, after 48 hours of treatments, cells were rapidly homogenized in ice-cold PBS and centrifugated for 10 minutes at 15.000 x g to remove insoluble materials. Supernatants were added to wells of 96-well plate and incubated with Master reaction mix prepared following instructions. Absorbances were calculated first at 2 minutes of incubation to establish initial time and then multiple measurements were obtained every 5 minutes until the value of must active sample was near to exceed the end of the linear range of the standard curve. Change in measurements from initial to final time were calculated and compared to standard curve to determine the activity during time range. G6PDH activity was reported as nmol/minutes and normalized to 10^6 cells.

Oxygen Consumption and Extracellular acidification rate

The oxygen consumption rate (OCR) was determined using the The Agilent Seahorse XFe96 Analyzer and in particular the Seahorse XF Cell Mito Stress Test Kit which is an assay for measuring mitochondrial function. The protocol involves 3 serial injections: Oligomycin that inhibits ATP synthase (complex V) and is injected first in the assay following basal measurements, the 2nd injection consists of Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) that is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential; the third injection is a mixture of rotenone and antimycin A, a complex I inhibitor, and a complex III inhibitor respectively. Briefly, cells were seeded at 10,000 cells per well Seahorse XF Cell Culture Microplate and allowed to attach overnight. Cells were then treated with selected inhibitors or vehicle for 48 hours. OCR was measured in basal conditions and after the subsequent addition of 1.5 μM oligomycin, 0.5 μM (FCCP), and 0.5 μM rotenone/antimycin A. OCR was monitored over time and each cycle consisted of 3 minutes mixing, 3 minutes waiting, and 3 minutes

measuring. Data were normalized for 10^6 cells and expressed as pmol/min. Extracellular acidification rate (ECAR) was simultaneously measured in basal conditions and after serial addition of 10 mM glucose, 5 μM oligomycin and 100 mM 2-deoxyglucose.

Statistical Analysis

Statistical analysis was performed using the software MedCalc for Windows, version 12.7.0.0, (MedCalc Software, Mariakerke, Belgium). The unpaired Student's t-test was used when appropriate for comparing means. Differences between means were considered statistically significant for $p < 0.05$.

1. Modulation of glucose metabolism through inhibition of EGFR and MET oncogenes in NSCLC cell lines

1.1 Inhibition of EGFR and MET in NSCLC cell lines

The first strategy proposed to test how glucose metabolism can be modulated in oncogene driven tumors was to inhibit in different NSCLC cell lines their driver. In particular, H1975 and H1993 cells are dependent from mutant EGFR (L858R) and amplified MET (15 gene copies), respectively. In addition, they are resistant to EGFR Tyrosine Kinase Inhibitors (TKIs) due to different molecular mechanisms: H1975 cells are resistant to erlotinib due to the presence of secondary T790M mutation in the kinase domain of EGFR ($EGFR^{T790M}$) while H1993 cells harbor wild type EGFR and constitutively active MET receptor. These resistant cells were selected to exclude off-target effects of first generation EGFR TKIs such as erlotinib on glucose metabolism and use erlotinib-treated cells as a negative internal control. Therefore, effective inhibition of EGFR was obtained using third generation TKIs such as WZ4002 that specifically inhibits $EGFR^{T790M}$ whereas MET was selectively inhibited by PHA-665,752. H1975 cells were exposed to erlotinib or WZ4002 whereas H1993 cells were exposed to erlotinib or PHA-665,752 for 48 hours. Then expression levels of phospho-EGFR, and relative downstream mediators such as phospho-AKT, phospho-ERK1/2 and Cyclin D1 were evaluated by Western blot analysis. In response to therapy, H1975 cell line showed a strong reduction of all downstream mediators mentioned above, while erlotinib was not able to affect EGFR signaling. H1993 cells exposed to erlotinib, despite the reduction of phospho-EGFR, did not show any significant change of EGFR signaling mediators whereas phospho-AKT and phospho-ERK1/2 along with Cyclin D1 were strongly reduced in response to PHA-665,752, indicating the addiction of these cells to amplified MET (**Fig. 5**).

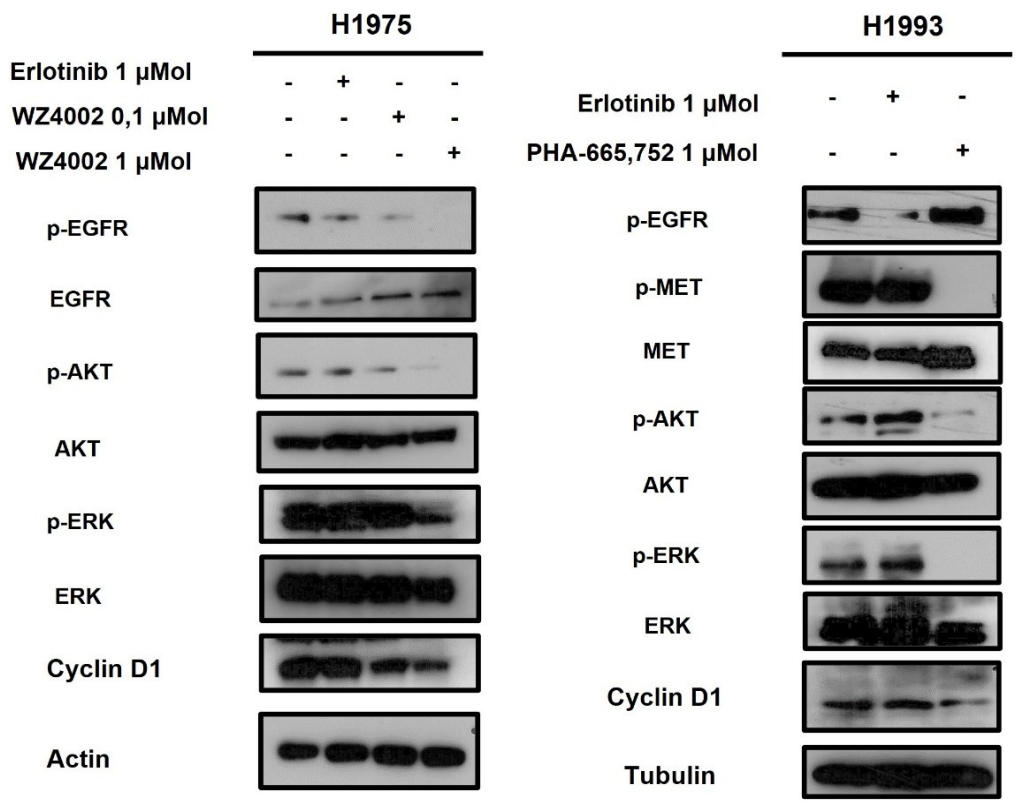

Figure 5. Inhibition of intracellular signaling in H1975 and H1993 NSCLC cells exposed to treatment with WZ4002 and PHA-665,752 at the indicated doses for 48 hours. Proteins were extracted and subjected to immunoblot with the indicated antibodies. H1975 cells showed a dose dependent reduction of phospho-EGFR phospho-AKT, phospho-ERK and Cyclin D1 in response to WZ4002 whereas no changes were observed in cells exposed to erlotinib. H1993 cells showed a reduction of phospho-MET, phospho-AKT and phospho-ERK along with Cyclin D1 levels in response to PHA-665,752 whereas erlotinib, despite the reduced levels of phospho-EGFR, did not cause inhibition of downstream signaling mediators.

1.2 Effects of EGFR and MET inhibition on glucose metabolism

Next, effects of cell exposure to the different TKI inhibitors were evaluated by testing the levels of glycolytic enzymes at the first and last step of glycolysis. H1975 and H1993 cells showed reduction of hexokinase II (HKII) levels after 48 hours treatment with the selective inhibitor **(Fig. 5).** Since it is reported that posttranslational modifications of PKM2 such as phosphorylation at Tyr105 regulate enzymatic activity by enhancing the formation of inactive dimeric instead of active tetrameric form of this protein, the levels of

phosphorylated PKM2 at Tyr105 (phospho-PKM2^{Tyr105}) in whole-cell lysates of H1975 cells exposed to WZ4002 for 48 hours were assessed. A dose-dependent reduction of phospho-PKM2^{Tyr105} levels was observed in response to WZ4002 treatment. A strong reduction of phospho-PKM2^{Tyr105} levels was also observed in H1993 cells after treatment with PHA-665,752 whereas erlotinib caused only a slight decrease of this enzyme. The reduction of PKM2 phosphorylation at Tyr105 site leads to a stabilization of active tetrameric form of the enzyme thus enhancing the conversion of phosphoenolpyruvate to pyruvate that in turn will be converted to lactate or Acetyl-CoA. The next step was to evaluate the effect of EGFR and MET inhibition on oxidative phosphorylation by testing the expression of specific mitochondrial complexes subunits (OXPHOS) in untreated and treated cells. H1975 cells treated with 1 µmol/L WZ4002 showed increased expression of all mitochondrial complex subunits except ATP5A of complex V whose levels remained constant. In H1993 cells treated with 1 µmol/L PHA-665,752, a strong upregulation of all subunits tested was found as compared with untreated control cells **(Fig. 6).**

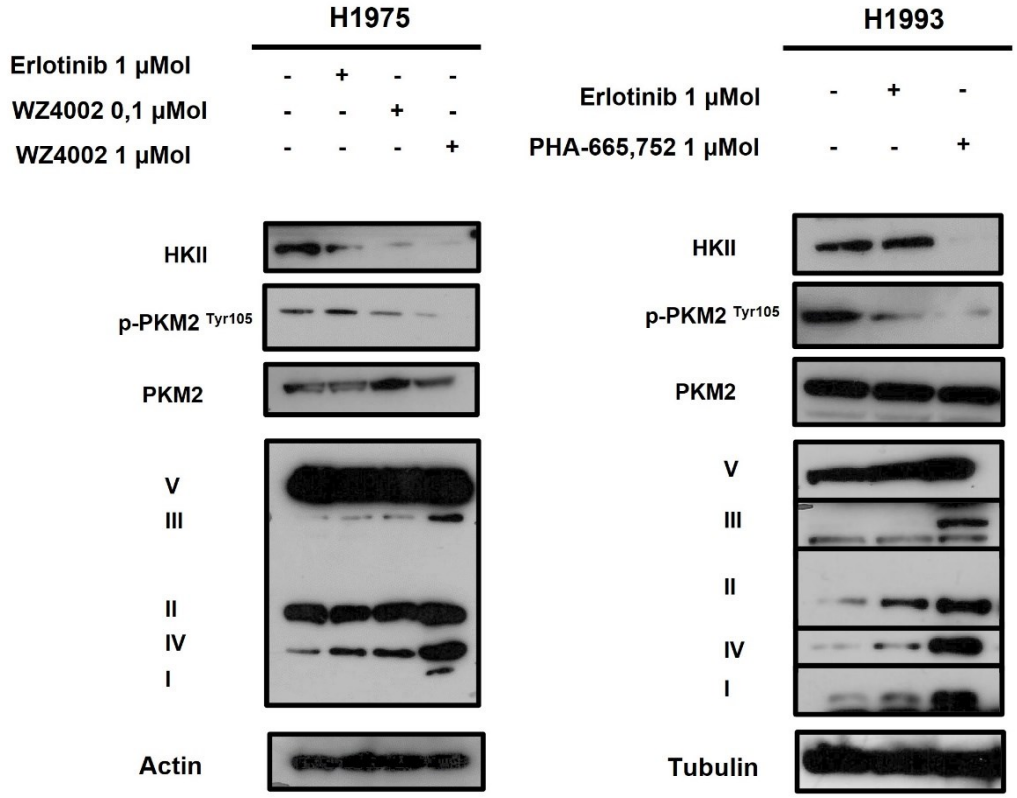

Figure 6. Modulation of HKII, phospho-PKM2 tyr105 and OXPHOS in response to 48 hours treatment with the EGFR inhibitor WZ4002 and MET inhibitor PHA-665,752 in H1975 and H1993 cells, respectively. Both H1975 and H1993 cells

showed a reduction of HKII and phospho-PKM2 tyr105 levels in response to EGFR and MET inhibitors along with a strong upregulation of OXPHOS.

2. *Modulation of glucose metabolism through inhibition of BCR-ABL in chronic myelogenous leukemia (CML) cell lines*

2.1 Inhibition of BCR-ABL tyrosine kinase activity in CML

To confirm the consequences of oncogenes inhibition on glucose metabolism, BCR-ABL-driven K562 and KCL-22 CML cells were exposed to increasing concentrations of imatinib. Imatinib mesylate is a small-molecule inhibitor of BCR-ABL tyrosine kinase activity that by binding to kinase domain of ABL prevents chimeric protein autophosphorylation and downstream signaling leading to growth arrest and apoptosis **(Fig. 7 panel A-B).**

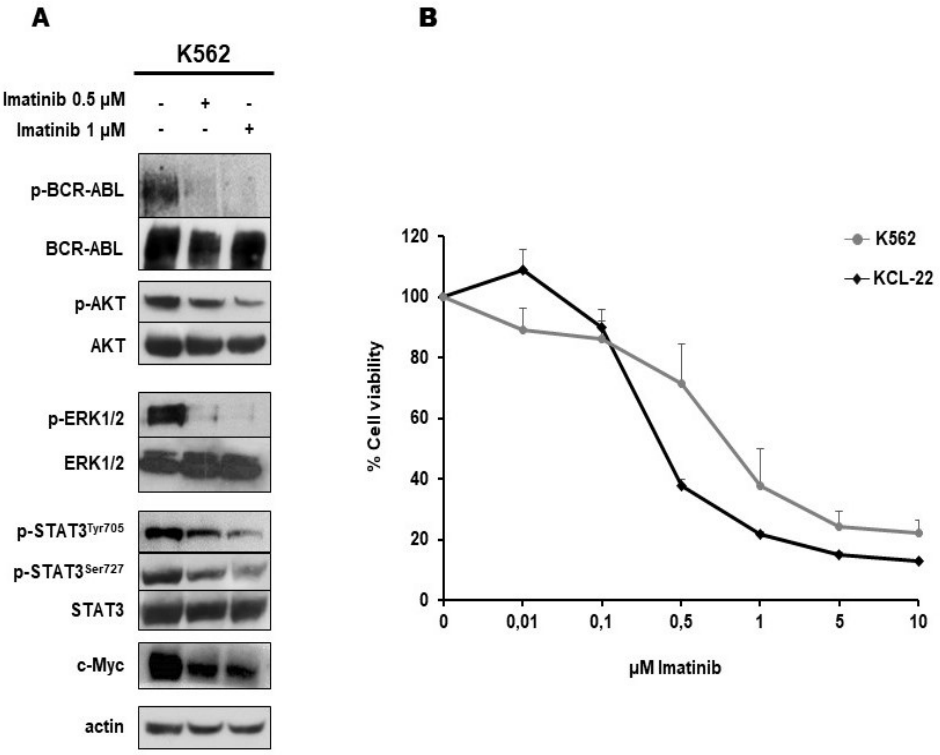

Figure 7. (A) Inhibition of BCR-ABL intracellular signaling in response to imatinib treatment in K562 cells. CML cells showed decreased levels of phospho-BCR-ABL, phospho-AKT, phospho-ERK, phospho-STAT3 tyr705, phospho-STAT3 ser727 and c-Myc after 48 hours treatment with imatinib; (B) Cell viability

assay performed in K562 and KCL-22 cell lines exposed to increasing dose of imatinib. A reduction of cell viability was observed in response to imatinib with an IC50 of approximately 1µMol in both cell lines [adapted from De Rosa V, Monti M, Terlizzi C, Fonti R, Del Vecchio S, Iommelli F. Coordinate Modulation of Glycolytic Enzymes and OXPHOS by Imatinib in BCR-ABL Driven Chronic Myelogenous Leukemia Cells. International Journal of Molecular Sciences. 2019; 20(13):3134. (87)]

2.2 Effects of BCR-ABL inhibition on glucose metabolism

Despite imatinib is widely used for treatment of chronic myelogenous leukemia patients, its effects on glucose metabolism are largely unknown. Therefore, levels of key glycolytic enzymes were determined in untreated and treated K562 and KCL-22 cells. A dose-dependent decrease of HKII and LDH-A expression was observed after 48 hours treatment in both cell lines along with reduced levels of phospho-PKM2^{Tyr105} and phospho-PKM2^{Ser37} **(Fig. 8 panel A).** Conversely, a strong up-regulation of mitochondrial complex subunits (OXPHOS) was observed in both cell lines indicating a concomitant reactivation of mitochondrial oxidative phosphorylation. In agreement with these findings, Seahorse analysis, by measuring oxygen consumption rate (OCR), showed an enhancement of both basal and maximal respiration rate in treated K562 cells as compared to untreated controls **(Fig. 8 panel B).**

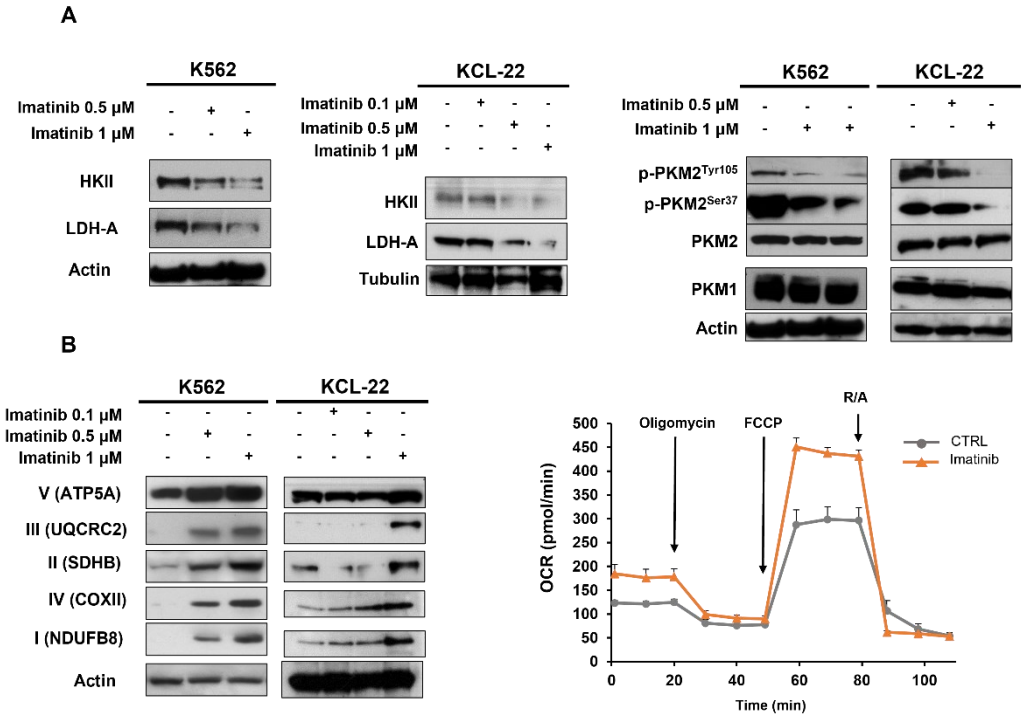

Figure 8. (A) Modulation of HKII, LDH-A and phospho-PKM2 tyr105 glycolytic proteins in response to imatinib treatment in CML cell lines. K562 and KCL-22 cells treated with increasing concentration of imatinib (0.1-1μM) for 48 hours showed a dose-dependent reduction of HKII, LDH-A, phospho-PKM2 tyr105 and phospho-PKM2 ser37. (B) OXPHOS upregulation and Oxygen Consumption Rate (OCR) in response to imatinib treatment. Left: K562 and KCL-22 cells treated as in panel A showed a dose-dependent upregulation of all subunits of mitochondrial complexes. Right: OCR measurements were obtained by Seahorse analysis of untreated and imatinib treated (0.5 μM for 48 hours) K562 cells in basal conditions and after the subsequent addition of 5 μM oligomycin, 1.5 μM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM rotenone/antimycin A (R/A). Treated K562 cells showed an enhancement of both basal and maximal respiration rate as compared to untreated controls [adapted from De Rosa V, Monti M, Terlizzi C, Fonti R, Del Vecchio S, Iommelli F. Coordinate Modulation of Glycolytic Enzymes and OXPHOS by Imatinib in BCR-ABL Driven Chronic Myelogenous Leukemia Cells. International Journal of Molecular Sciences. 2019; 20(13):3134.(87)]

In parallel experiments glucose consumption, lactate secretion and ATP production were assessed in K562 cells exposed to imatinib (**Fig. 9**). A significant increase of glucose concentration was observed at 48 hours in conditioned media of treated cells, indicating a lower glucose consumption, as compared to untreated controls ($p < 0.05$) **(Fig. 9 panel A).** Conversely, a significant decrease ($p < 0.05$) of lactate levels was found at 24 hours in treated cells as compared to untreated controls followed by a reduction of extracellular acidification rate (ECAR) at 48 hours as assessed by Seahorse analysis (**Fig. 9 panel C-D**). Furthermore, intracellular ATP levels were significantly increased after 48 hours of treatment in comparison to untreated cells ($p < 0.01$) **(Fig. 9 panel B).** These findings taken together indicate that imatinib treatment causes a reduction of glucose consumption and an early reduction of lactate production through glycolysis followed by an increase of ATP indicating the reactivation of oxidative phosphorylation.

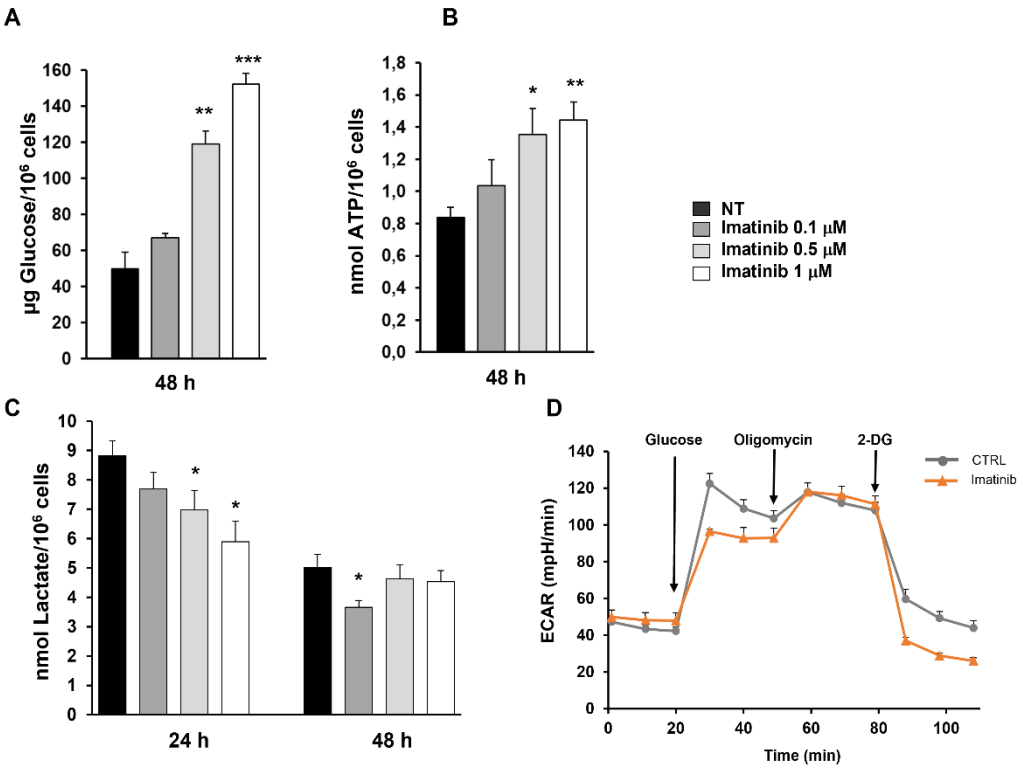

Figure 9. (A) Glucose levels in K562 cell culture medium after 48 hours treatment with increasing concentrations of imatinib; glucose levels were significantly higher in the conditioned media of cells exposed to 0.5 µM and 1 µM imatinib as compared to those obtained in untreated cells indicating a lower glucose consumption. (B) ATP levels were measured in untreated and treated K562 cells and expressed in nmol normalized for 10^6cells. After 48 hours of imatinib treatment ATP levels were significantly higher than those found in untreated cells. (C) Lactate levels were measured after 24 hours and 48 hours of treatment with increasing doses of imatinib and expressed in nmol normalized for 10^6cells. A significant reduction of lactate levels was observed at 24 hours using 0.5 µM and 1 µM imatinib. (D) Extracellular acidification rate was obtained by Seahorse analysis of untreated and treated (0.5 µM for 48 hours) K562 cells in basal conditions and after the subsequent addition of 10 mM glucose, 5 µM oligomycin and 100 mM 2-deoxyglucose. [adapted from De Rosa V, Monti M, Terlizzi C, Fonti R, Del Vecchio S, Iommelli F. Coordinate Modulation of Glycolytic Enzymes and OXPHOS by Imatinib in BCR-ABL Driven Chronic Myelogenous Leukemia Cells. International Journal of Molecular Sciences. 2019; 20(13):3134. (87)]

3. Silencing of pyruvate dehydrogenase kinase 1 (PDK1) by targeted shRNA and inhibition of oncogene driver

To test whether co-targeting of oncogene driver and glucose metabolism may have implications for therapy with TKIs, H1993 and H1975 cells were

simultaneously subjected to downregulation of PDK1 and treatment with crizotinib and WZ4002, respectively. PDK1 is a member of PDK family, that can phosphorylate and inactivate the pyruvate dehydrogenase complex (PDHC), a multi-enzyme complex, that in turn catalyzes the conversion of pyruvate to acetyl-CoA. Therefore, silencing of PDK1 is expected to promote the conversion of pyruvate to acetyl-CoA instead of reduction to lactate. Cells were stably transfected with a lentiviral vector expressing either PDK1 targeted shRNA (shPDK1) and non-targeting shRNA (shCTRL) and levels of glycolytic enzymes and mitochondrial complex subunits were tested by Western blot analysis in cells exposed or not to the specific inhibitors crizotinib or WZ4002. Treatment with crizotinib or WZ4002 in shCTRL cells caused a reduction of HKII, phospho-PKM2^{Tyr105} and LDH-A levels, as expected. In both cell lines, downregulation of PDK1 alone caused a slight decrease in phospho-PKM2^{Tyr105} and LDH-A levels as compared to shCTRL cells whereas treatment with crizotinib and WZ4002 in PDK1 silenced cells caused an enhanced reduction in all selected glycolytic proteins as compared to the effects of the same treatment in shCTRL cells, except for PKM2 and PDH-E1α (**Fig. 10 panel B**). PDK1 silencing in combination with TKI treatment caused a stronger enhancement of OXPHOS in both cell lines than that observed in treated shCTRL cells (**Fig. 10 panel C**)

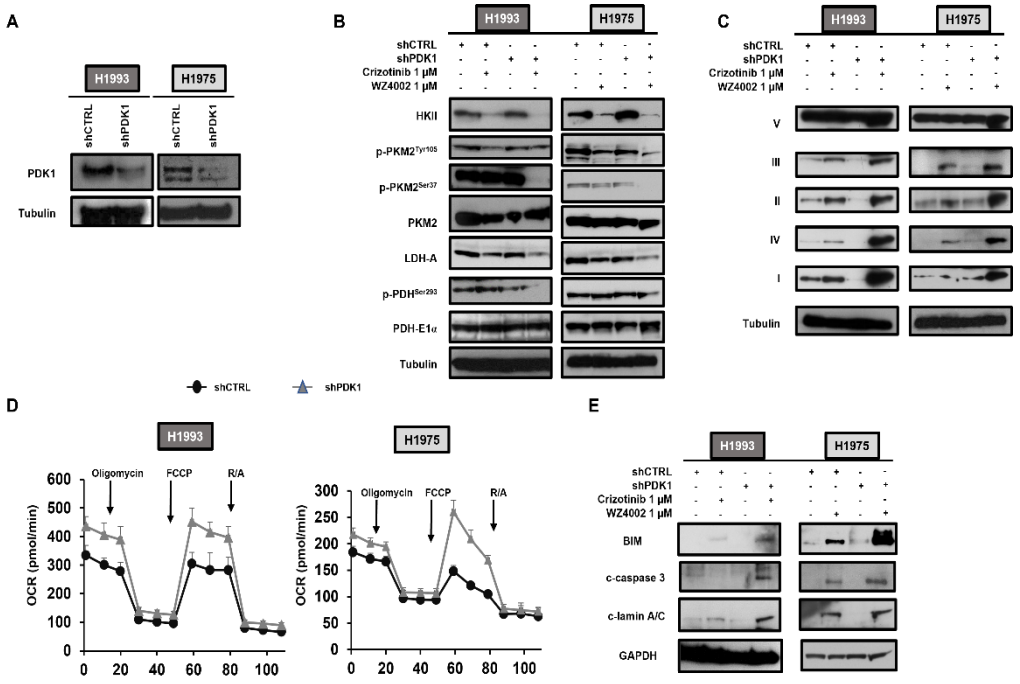

Figure 10. (A) Downregulation of PDK1 protein obtained by stable transfection with lentiviral vector in H1993 and H1975 cells; (B) Effects of downregulation of

PDK1 alone or in combination with TKIs on glycolytic proteins in H1993 and H1975 cells. Both cell lines showed a reduction of HKII, phospho-PKM2 Tyr105 and LDH-A levels in crizotinib or WZ4002 treated shCTRL cells and a slight decrease in phospho-PKM2 Tyr105 and LDH-A levels in untreated shPDK1 cells. Treatment with crizotinib or WZ4002 in shPDK1 cells caused an enhanced reduction of HKII, phospho-PKM2 Tyr105 and phospho-PKM2 ser37. (C) OXPHOS levels of untreated and treated shCTRL and shPDK1 cells. Silencing of PDK1 combined with selective inhibitors caused an upregulation of OXPHOS stronger than that observed in shCTRL cells treated with crizotinib or WZ4002 alone. (D) Oxygen Consumption Rate (OCR) of shCTRL cells and shPDK1 cells. OCR measurements were obtained by Seahorse analysis in basal conditions and after the subsequent addition of 5 μM oligomycin, 1.5 μM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) and 1 μM rotenone/antimycin A (R/A). Both PDK1-silenced cell lines showed an enhancement of maximal respiration rate as compared to shCTRL cells. (E) Effects of combined silencing of PDK1 and 1μM crizotinib or WZ4002 treatments on apoptotic pathway. PDK1 silencing in combination with TKI treatments caused a strong increase of BIM, cleaved-caspase 3 and cleaved-Lamin A/C levels in both cell lines. [adapted from De Rosa, V., Iommelli, F., Terlizzi, C., Leggiero, E., Camerlingo, R., Altobelli, G. G., Fonti, R., Pastore, L., & Del Vecchio, S. (2021). Non-canonical role of pdk1 as a negative regulator of apoptosis through macromolecular complexes assembly at the er–mitochondria interface in oncogene-driven nsclc. Cancers, 13(16), [4133] (88)]

In agreement with these findings, shPDK1 H1993 and H1975 cells exposed to crizotinib or WZ4002 showed a maximal respiration higher than that observed in shCTRL cells exposed to the same inhibitors **(Fig. 10 panel D).**
Along with glycolytic and mitochondrial proteins, selected apoptotic proteins were tested to evaluate cell response to therapies. Both cell lines showed a strong upregulation of BIM, cleaved-lamin A/C and cleaved-caspase 3 indicating activation of the apoptotic pathway **(Fig. 10 panel E).**

These findings taken together indicate that co-targeting of oncogene drivers and glucose metabolism improves response to TKIs in NSCLC cells.

In the present study we showed that by inhibiting oncogene drivers such as EGFR in NSCLC and BCR-ABL in CML, we induced a downregulation of glycolysis and a reactivation of oxidative phosphorylation. As a consequence, cancer cells were not dependent any more from glucose supply from the external environment since the activation of oxidative phosphorylation is more effective than glycolysis in producing energy. In fact, when CML cells were exposed to imatinib glucose concentration in the culture medium was significantly increased indicating a lower consumption whereas lactate levels were significantly decreased due to reduced conversion from pyruvate. These findings were in agreement with reduced LDH levels and decreased extracellular acidification rate as assessed by Seahorse assay in the same cell line.

Activation of oncogenes or loss of function of suppressor genes, in addition to cause neoplastic transformation and acquisition of a malignant phenotype, induces a reprogramming of glucose metabolism by upregulating the expression of a pool of genes encoding glucose transporters and most glycolytic enzymes. In this way cancer cells are more dependent from glycolysis than from oxidative phosphorylation to gain energy and this happens even in the presence of oxygen. What are the advantages for cancer cells to use glycolysis instead of oxidative phosphorylation is not completely elucidated. However, glycolysis is reported to support the uncontrolled growth and survival of cancer cells by favoring the generation of macromolecular building blocks such as nucleotides, amino acids, and lipids through alternative biosynthetic pathways.

Another consideration that can be drawn by our results is that the oncogene drivers such as EGFR or BCR-ABL by activating downstream pathways can reduce the expression of OXPHOS and hence can decrease the oxygen demand by cancer cells. When the oncogene drivers are inhibited, OXPHOS are upregulated and oxidative phosphorylation is restored. This was confirmed also by the enhanced oxygen consumption rate observed in CML exposed to imatinib. The restored function of mitochondria may have important implications for therapy since response to anticancer drugs can be improved by the consumption of oxygen and reduction of extracellular acidification. It is well known that a hypoxic or acidic microenvironment may cause resistance of cancer cells to both chemotherapy and radiation therapy by selecting cancer cells that can survive in adverse microenvironmental conditions having a more aggressive behavior. To test this hypothesis, we decided to simultaneously target PDK1 and EGFR/MET in NSCLC cells. Silencing of PDK1 combined with TKI treatment strongly enhanced oxidative phosphorylation through the upregulation of OXPHOS and reduced glycolysis. More importantly, co-targeting of PDK1 and EGFR/MET caused an enhanced apoptotic response as compared to that of single agent indicating a potentiation of therapeutic effects.

In parallel experiments we focused our attention on ATM and its role in modulating glucose metabolism. The rational to investigate ATM relies on the

reported findings that ATM is a key regulator of pentose phosphate pathway since it activates the glucose-6-P dehydrogenase allowing the diversion of glucose-6-P toward PPP. Through this pathway the metabolic intermediates of glycolysis are employed as precursors of nucleotides and sustain the high proliferation rate of cancer cells. In addition, ATM is reported to be a redox sensor since it become phosphorylated in response to oxidative stress. The first question that we asked was whether the inhibition of the driver could cause changes in the phosphorylation status of ATM and we found increased levels of p-ATM in response to EGFR inhibition. Then we tested the effects of ATM inhibition on glycolysis and OXPHOS and found that reduction of p-ATM levels caused a downregulation of glycolysis and a strong reduction of OXPHOS. Cancer cells exposed to ATM inhibitors do not activate apoptotic program but reduce at minimum glucose consumption. When ATM inhibitors are combined with EGFR TKIs, an enhancement of apoptotic markers could be observed in response to combined treatment even at very low doses of ATM inhibitor (10 nM). Interestingly, EGFR TKIs when used in ATM silenced cells did not cause upregulation of OXPHOS but a reduction of mitochondrial complexes was observed indicating that ATM is required for TKI-dependent modulation of oxidative phosphorylation (**Fig. 14**).

These latter findings seem to be in apparent contradiction with the upregulation of OXPHOS in response to oncogene driver inhibition (**Fig. 6**). However, the increased levels of p-ATM caused by EGFR inhibition (**Fig. 15**) can explain the opposite modulation of OXPHOS in the absence of ATM. In other words, inhibition of EGFR signaling causes increased levels of p-ATM that in turn is required to trigger upregulation of OXPHOS. When p-ATM is inhibited by KU55933, OXPHOS levels are decreased independently from the activation of EGFR pathway. These findings taken together indicate that modulation of OXPHOS and hence oxidative phosphorylation is more dependent from p-ATM levels than from oncogene driver. In agreement with our observations, cells from subjects with Ataxia-telangiectasia and knockout of ATM in mice and cells show mitochondrial dysfunction (89). In particular, lack of ATM causes alterations in total mitochondrial DNA levels and mitochondrial mass and reduction of mitochondrial respiration rates (90).

The simultaneous downregulation of glycolysis and oxidative phosphorylation by ATM inhibition raised the question on which alternative energy source maintains cancer cells alive in these conditions. Cell toxicity assay showed that increasing concentrations of KU55933 up to 5 μM do not cause reduction of cell viability in NSCLC cells indicating that alternative energetic substrates can be obtained by cancer cells from cultured medium or from intermediates of other metabolic pathways. While pyruvate levels are reduced due to downregulation of glycolysis, levels of citrate and succinate are increased (**Fig. 12**). These findings could be explained by anaplerotic replenishment of TCA cycle starting from alternative metabolic substrates such as glutamine, amino acids or fatty acids. This metabolic adaptation can be highly dependent on cellular context and microenvironmental conditions. Several evidence indicate indeed that the

metabolic network of tumors is similar to that of normal tissues in which they take origin (91). In other words, oncogene-driven cancer cells will exploit the same metabolic network of normal parental cells for nutrient supply. Furthermore, differences in microenvironmental conditions may account for metabolic differences among tumors even arising from the same normal tissue.
Our study showed that co-targeting of glucose metabolism and oncogene drivers such as EGFR, BCR-ABL and MET improves tumor response to TKIs. In particular, silencing of PDK1 in oncogene-driven NSCLC enhanced apoptotic response to TKIs as compared to treatment with TKI alone. Similarly, inhibition of ATM in combination with EGFR TKIs showed an additive effect on apoptotic response as compared to that induced by single agents alone. However, in the first case oxidative phosphorylation was improved whereas in the second situation OXPHOS levels were suppressed. These findings raised the question whether it is better to improve or to impair mitochondrial function for a better tumor response to therapies. These different therapeutic approaches are still debate and several clinical trials especially with OXPHOS inhibitors are currently ongoing and will provide a definite answer (60). A pre-requisite to use OXPHOS inhibitors is that mitochondrial complexes are expressed or overexpressed in cancer cells as for instance in leukemias, lymphomas, pancreatic ductal carcinoma and endometrial carcinoma (92). In many oncogene-driven tumors where oxidative phosphorylation is downregulated, treatment with driver inhibitors may be used to increase OXPHOS levels thus sensitizing cancer cells to treatment with OXPHOS inhibitors. Furthermore, since OXPHOS inhibitors would affect respiration of both malignant and normal cells, the occurrence of adverse side effects could not be excluded.
On the basis of these considerations, our study shows that both upregulation and downregulation of OXPHOS can improve tumor response to TKIs. Notably, even very low doses of ATM inhibitor were able to decrease OXPHOS levels with an enhancement of tumor response to TKIs. However, in order to exploit this ability of ATM inhibitors in clinical studies, highly selective agents targeting only the non-canonical function of ATM are required to avoid the impairment of the canonical DNA repair function of ATM. Further studies are needed to unravel the molecular mechanisms mediating ATM-dependent mitochondrial dysfunction. In this way, targets downstream ATM can be safely exploited for therapeutic purposes.

Tumor metabolism was recently included in the list of the hallmarks of cancer. It is widely known that many of the altered metabolic pathways are under the control of aberrant oncogenic signaling. Several strategies have been proposed to modulate glucose metabolism in cancer cells based on inhibition of signaling mediators and effectors, components of glycolytic cascade or oxidative phosphorylation.

In the present study we showed that by inhibiting oncogene drivers such as EGFR in NSCLC and BCR-ABL in CML, we induced a downregulation of glycolysis and a reactivation of oxidative phosphorylation. Therefore, cancer cells were not dependent any more from glucose supply since the activation of oxidative phosphorylation is more effective than glycolysis in producing energy. More importantly, silencing of PDK1 combined with TKI treatment caused a more pronounced effects on glycolysis and oxidative phosphorylation as well as higher levels of apoptotic markers as compared to those observed after single treatment. A similar potentiation effects on apoptotic markers and cell viability was observed in the same cell lines exposed to combined treatment with ATM and EGFR inhibitors. However, the mechanisms underlying the enhanced tumor response to combined treatment seem to be different since one therapeutic approach enhanced OXPHOS and the other inhibited OXPHOS. Although it is debated whether it is better to improve or to impair mitochondrial function for a better tumor response to therapies, our study showed that both upregulation and downregulation of OXPHOS can enhance tumor response to TKIs. Further studies are needed to elucidate how opposite regulation of OXPHOS may sensitize cells to apoptotic stimuli. Interestingly, in tumors with low or undetectable levels of OXPHOS, TKI treatment can be used to enhance levels of mitochondrial complexes providing the rationale to use OXPHOS inhibitors.

Notably, even very low doses of ATM inhibitor were able to decrease OXPHOS levels with an enhancement of tumor response to TKIs. However, in order to exploit this ability of ATM inhibitors in clinical studies, highly selective agents targeting only the non-canonical function of ATM are required to avoid the impairment of DNA repair function of ATM. Further studies are needed to unravel the molecular mechanisms mediating ATM-dependent mitochondrial dysfunction. In this way, targets downstream ATM can be safely exploited for therapeutic purposes.

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