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Combined targeting of cancer metabolism and PI3K/Akt/mTOR signaling in Primary Effusion Lymphoma

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Abbreviations

PEL: primary effusion lymphoma

NHL: non-Hogkin lymphoma

KSHV/HHV8: Kaposi's sarcoma associated herpesvirus/human herpesvirus 8

IRF4: IFN (interferon) regulatory factor

VEGF: vascular endothelial growth factor

HAART: highly active anti-retroviral therapy

LANA-1: latency associated nuclear antigen

KS: Kaposi's sarcoma

ORF: open reading frame

VHL: von-Hippel Lindau

vGPCR: viral G-protein coupled receptor

vFLIP: viral caspase 8 (FLICE)-like inhibitor protein

CDK6: cyclin-dependent kinase 6

TCA: tricarboxylic acid

OXPHOS: oxidative phosphorylation

ATP: adenosine triphosphate

FDG-PET: [^{18}F]fluorodeoxyglucose positron emission tomography

PPP: pentose phosphate pathway

G6P: glucose-6-phosphate

GLUT-1: glucose transporter 1

HIF-1: hypoxia inducible factor

HK: hexokinase

VDAC: voltage dependent anion channel

PKM: pyruvate kinase

PDH: pyruvate dehydrogenase

PDHK1: pyruvate dehydrogenase kinase

LDH: lactate dehydrogenase

MCT4: mono-carboxylate transporter 4

CA: carbonic anhydrase

R5P: ribose-5-phosphate

ROS: reactive oxygen species

3-PG: 3-phosphoglycerate

OAA: oxaloacetate

ACL: ATP citrate lyase

ACC: acetylCoA carboxylase

FAS: fatty acid synthase

GSH: reduced glutathione

GCL: glutathione cysteine ligase

ME: malic enzyme

IDH1: isocitrate dehydrogenase 1

α-KG: α-ketoglutamate

D-2-HG: d-2-hydroxyglutarate

SDH: succinate dehydrogenase

FH: fumarate hydratase

PFK1: phosphofructokinase 1

SCO2: cytochrome oxidase 2

AMPK: AMP (adenosine mono phosphate)-activate protein kinase

NSCLC: non-small cell lung carcinoma

CAMKK2: calmodulin-dependent protein kinase 2

PFKFB: 6-phosphofructokinase/fructose 2,6 bisphosphatase

HMGCR: HMGCoA reductase

HAT: histone acetyl transferase

HDAC: histone deacetyl transferase

CRTC: CREB-regulated transcription coactivator

2-DG: 2-deoxyglucose

3-BrPa: 3-bromo pyruvate

3-BrOP: 3-bromo-2-oxopropionil-1 propyl estere

DCA: dichloroacetate

AIF: apoptosis inducing factor

PEP: phosphoenolpyruvate

FBP: fructose 1,6 bisphosphate

PGAM1: phosphoglycerate mutase 1

G3P: glycerate-3-phosphate

FGFR1: fibroblast growth factor receptor 1

EGFR: epidermal growth factor receptor

hnRNPA 1 and 2: heterogeneous nuclear ribonucleoprotein

PTB: polypirimidine tract binding protein

PIP_2: phosphatidylinositol-4,5 biphosphate

PIP_3: phosphatidylinositol-3,4,5 triphosphate

RTKs: receptor tyrosine kinase

PI: phosphatidyliositol

Vsp34: vacuolar protein sorting 34

PDK1: phosphoinositide dependent kinase

PP2: protein phosphatase 2

PHLPP: PH-domain leucine-rich repeat phosphatase

FOXO: forkhead family of transcription factor

eNOS: nitric oxide synthase

mTOR: mammalian target of rapamycin

RAPTOR: regulatory associated protein of mTOR

mLST8: mammalian lethal with sec 13 protein 8

Deptor: DEP-domain containing mTOR interacting protein

mSIN1: mammalian stress-activated protein kinase interacting protein

Protor-1: protein observed with Rictor-1

FKBP12: FK506-binding protein

FRB: FKBP12-rapamycin binding domain of mTOR

TSC: tuberos sclerosis complex

GAP: GTP-ase activated protein

Rheb: RAS homolog enriched in brain

REDD1: regulated in developments and DNA damage responses 1

4EBP1: eukaryotic initiation factor 4E (eIF4E)-binding protein-1

SREBP 1/2: sterol regulatory element binding protein 1

IRS1: insulin receptor substrate 1

OMM: outer mitochondrial membrane

G1,6-P_2: glucose 1,6 bisphosphate

F1,6-P_2: fructose 1,6 bisphosphate

HCC: hepatocellular carcinoma

HREs: hypoxia responsive elements

PHDs: prolil-hydroxylase domains

RCC: renal cellular carcinoma

RPPA: reverse phase protein assay

Abstract

PEL is a B-cell non-Hodgkin lymphoma, occurring predominantly as a lymphomatous effusion in body cavities, and characterized by a very aggressive clinical course. The median survival is in the range of 6 months from diagnosis with conventional chemotherapy. Although non conventional drugs have been used anecdotally, with some reports of success, the optimal approach is far from being defined and there is an urgent need to establish a standard therapy.

The shift in glucose metabolism from oxidative phosphorylation to lactate production for energy generation, the so-called Warburg effect, is a well-known metabolic hallmark of tumor cells, that helps generate metabolites, fuel growth, and may also aid in the evasion of apoptosis. How metabolic pathways are regulated to meet the unique needs of tumor cells is not well understood, but mounting evidence suggests that metabolic regulation in cancer cells is intimately linked with the signal transduction pathways that control growth and differentiation, through mutation of key signaling molecules as well as epigenetic changes. Notably, several key signaling pathways, oncogenes, and tumor suppressors, including Akt and mTOR, are linked to the increase in glycolysis seen in tumor cells.

PEL cells are characterized by the Warburg phenotype. The highly hypoxic environment in which they grow *in vivo* makes them even more reliant on glycolysis for energy requirements, and inherently more vulnerable to drugs targeting this metabolic pathway.

In this study, the *in vitro* efficacy of a panel of drugs targeting glycolysis was investigated. We established that PEL cells under hypoxia are sensitive to low concentrations of glycolysis inhibitors such as 2-DG and 3-BrPO, whereas they are resistant to Lonidamine and 3-BrPA. Moreover, a broad analysis of the phosphorylome of PEL cells was carried out. We found that the PI3K/Akt/mTOR pathway is constitutively active in PEL. It is sensitive to well-known pathway-specific inhibitors, though, and can therefore be regarded as a druggable target in this disease. Together, these observations might open a new therapeutic window for PEL. The pharmacologic interaction of drugs blocking glycolysis with pathway-specific inhibitors was explored by comparing the dose-effect curve of single drugs with that of combined treatments, and expressed according to the Chou&Talalay algorithm. We demonstrate that 2-DG combined either to inhibitors of Akt, mTOR or to dual PI3K/mTOR inhibitors, although not toxic to normal lymphocytes from healthy donors, is cytotoxic to PEL cells, with a CI indicating strong synergy, that allows to use lower doses of drugs; a condition that, at least in principle, may reduce adverse reaction *in vivo*.

1. Primary Effusion Lymphoma (PEL)

1.1 Description and Treatment

Primary effusion lymphoma is a rare subtype of B-cell non-Hodgkin lymphoma (B-NHL). In 1995 Cesarman et coworkers (Cesarman E, 1995) described a type of NHL localized in body cavities of AIDS patients and presenting pleural, pericardial and peritoneal lymphomatous effusions, demonstrated to be an highly hypoxic environment. They identified the presence of DNA sequences of the Kaposi's Sarcoma Associated Herpes Virus (KSHV/HHV8) in the lymphoma cells. This subtype of lymphoma, characterized by KSHV infection and lymphomatous effusions, was defined as 'Primary Effusion Lymphoma' in 1996 (Nador RG, 1996) and was included in WHO classification of haematopoietic and lymphoid tissues tumors in 2001 (Jaffe ES, 2001).
From the histological point of view, PEL cells are larger than normal lymphocytes, with marked size heterogeneity, irregular and pleomorphic nuclei, abundant cytoplasm and prominent nucleoli (Schulz TF, 2001) and they also display a high proliferative rate, thus combining morphological aspects of immunoblastic and anaplastic large cell lymphomas (Figure 1.1).

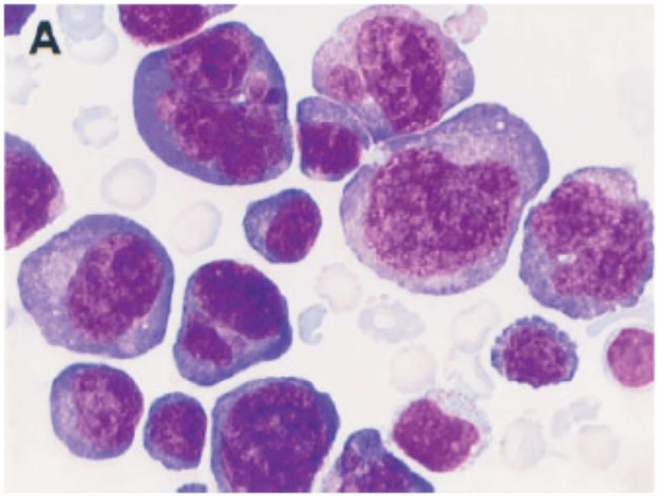

Figure 1.1: PEL cells collected from pleural effusion (May Grünwald-Giemsa) (Boulanger E, 2003)

PEL is derived from clonal expansion of malignant B cells, as demonstrated by clonal immunoglobulin heavy chain rearrangements (Nador RG, 1996), although often does not express classic B-cell associated antigens such as CD19, CD20. In contrast, PEL cells express CD45 and other activation markers, like CD30, CD38, CD71 and epithelial membrane antigen. In 2003 Jenner and coworkers (Jenner RG, 2003) demonstrated that PEL cells display a gene expression profile similar to transformed plasma cells, which is supported by the presence of CD138 (syndecan-1), that mediates the interaction of PEL cells with the extracellular matrix (Gaidano G, 1997), and IRF4 (IFN regulatory factor 4) (Carbone A, 2000). Similar results were

obtained in another study (Klein U, 2003) demonstrating, by means of expression profile analysis of about 12,000 genes, that PEL cells share features of both immunoblastic lymphoma and plasma cells; in particular, they identified upregulation of IRF4, IL10 and VEGF genes and downregulation of B-cell markers (CD19, CD20).

In most cases, PEL cells are both KSHV and EBV positive (Marcelo G, 1997), but the role of EBV in pathogenesis is not clear: in fact, the sole presence of KSHV is sufficient to induce the pathology in immunocompromised mice (Boshoff C, 1998), showing that KSHV is the driving force in the development of PEL.

PEL is a very rare disease, but is more frequent in AIDS patients, accounting for less than 1% of non-AIDS related lymphoma and approximately 3% of all HIV-related NHL (Knowles DM, 2003). PEL can also occur as a consequence of a iatrogenic immunodeficiency following solid organ transplantation (Dotti G, 1999). In some cases (Klepfish A, 2001; Bonaiuto D, 2002) PEL develops in elder patients, usually in the eighth and ninth decades of life and especially in the geographic areas with high prevalence of KSHV infection in the general population, such as the Mediterranean area (Dukers NH, 2003).

The prognosis for PEL is very poor, with a median survival time of around 6 months, as reported by Boulanger et al in 2005 (Boulanger E, 2005), based on the study of 28 cases of PEL diagnosed in six centers during an 11-year period time. Similar results were obtained in other studies, as reviewed by Chen et al in 2007 (Chen YB, 2007), confirming the severity of this pathology. Symptoms derive from accumulation of the malignant effusion: patients commonly present with dyspnea, from pleural and pericardial disease, or abdominal distension, from peritoneal disease. Dissemination to distant sites is not uncommon. The most frequent causes of death are opportunistic infections, HIV-related complications and progression of lymphoma. Given that PEL is a very uncommon disease, there are no clinical trials with high enough number of patients to define an appropriate therapy.

Treatment of PEL patients with conventional chemotherapy (CHOP-cyclophosphamide, doxorubicin, vincristine and prednisolone) results in very poor survival rates, improved to some extent in HIV positive patients by introduction of highly active antiretroviral therapy (HAART) (Boulanger E, 2005; Simonelli C, 2003). Antiretroviral therapy alone is sufficient to achieve prolonged remission in some cases, suggesting the involvement of the immune reconstitution in the control of the lymphoma (Gerard L, 2003). A different strategy, based on the combination of chemotherapy with high doses of methotrexate, produced promising results (Boulanger E, 2003) that are however counterbalanced by the high toxicity of methotrexate (Boulanger E, 2005). In 2005 Luppi et al (Luppi M, 2005) reported complete remission in three HIV negative elderly PEL patients after treatment with intracavitary injections of Cidofovir, a potent antiviral agent. There are no reports of the use of cidofovir in AIDS PEL patients, though. Side effects of intravenous

administration of cidofovir include nephrotoxicity, neutropenia, and decreased intraocular pressure (Moyo TK, 2010).

The inhibition of proteasome function and the NFkB pathway with the proteasome inhibitor bortezomib was also suggested following promising results in an *in vitro* PEL model (An J, 2004), however *in vivo* bortezomib did not exert antitumor activity (Boulanger E, 2008). Furthermore, a number of recent studies described the antitumor effect obtained by blocking the PI3K/AKT/mTOR pathway, which is hyperactivated in PEL cells. These studies are described in detail in section 3.6.

In summary, despite numerous *in vitro* studies, the prognosis of PEL patients remains very poor, confirming that new therapeutic strategies are urgently needed.

1.2 Kaposi's Sarcoma Associated Herpes Virus (KSHV/HHV8)

Kaposi's Sarcoma associated herpesvirus (KSHV), also known as Human Herpesvirus-8 (HHV-8), is a member of the γ-herpesvirus family, with a specific tropism for B and T lymphocytes and for endothelial cells. KSHV is the causative agent of Kaposi's Sarcoma (KS), an endothelial based tumor. KS is grouped into four epidemiological forms: classic KS, affecting elderly men of the Mediterranean area; endemic KS, existing in parts of Central and Eastern Africa; iatrogenic KS, developing in immunocompromised patients; and epidemic or AIDS-associated KS, occurring in AIDS patients. KS lesions consist of spindle cells, derived from endothelial infected cells, and contain numerous infiltrating inflammatory cells and leaky new vessels and extravasated red blood cells. AIDS-associated KS is the most aggressive forms, and is characterized, in addition to the typical cutaneous lesions, by disseminated disease affecting lymph nodes and visceral organs (Mesri EA, 2010). The virus was first characterized in 1994 (Chang Y, 1994) in KS patients and was subsequently identified as the etiologic agent of two different lymphoproliferative disorders: PEL (Cesarman E, 1995) and Multicentric Castelman's Disease (MCD) (Soulier J, 1995).

KSHV is a double strand DNA virus, of approximately 165kbp, with a central low GC-content region called L DNA, flanked by a highly repetitive sequence of high GC content, called H DNA. The L DNA region contains at least 81 ORFs (open reading frames), encoding the viral genes (Russo JJ, 1996). Like other herpesviruses, KSHV establishes a latent infection in cells, as episomal DNA. During latency, viral gene expression is restricted to only a few genes, that are important for the maintenance of the viral episome, avoid antiviral host immune response and provide growth advantage to infected cells. During lytic replication are expressed the majority of viral genes, when virus progeny are produced and the host cell is destroyed (Richard G, 2001).

PEL cells contain multiple copies of episomal KSHV genome (in order of 50-150 copies/cell) (Lallemand F, 2000). (Figure 1.2).

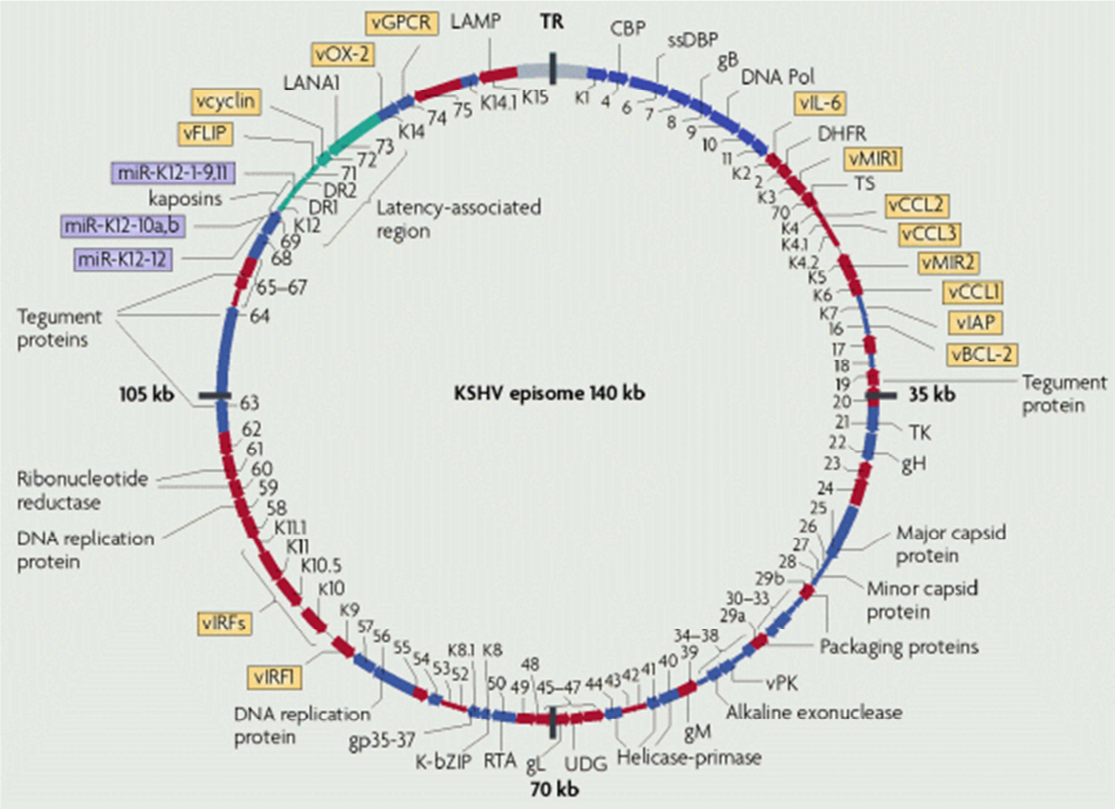

Figure 1.2: KSHV episome: putative latent transcripts are indicated in green and orthologous of cellular genes in yellow (Mesri EA, 2010)

The majority of PEL cells express KSHV genes characteristic of the latent phase (such as LANA-1, vFLIP, vIL-6), while a small percentage express genes of the lytic phase (Judde JG, 2000).

Many KSHV genes are homologous of cellular genes (vIL6, vBcl2, vcyclinD) and are involved in the development of the lymphoma, determining the alteration of numerous cellular pathways involved in proliferation, apoptosis, immune evasion and angiogenesis. The proteins encoded by KSHV and their functions are summarised in Table1 (Wen KW, 2010 (1)).

The latency associated nuclear antigen (LANA-1) is a 222-232 kDa nuclear protein encoded by ORF73. LANA-1 is important for the maintenance of viral DNA in replicating host cells, tethering the KSHV genome to host chromosomes (Cotter MA, 1999). LANA-1 is involved in KSHV tumorigenesis: several studies, the first published in 1999 (Friborg J, 1999), demonstrate that LANA-1 inhibits the activity of p53, inducing chromosomal instability (Si H, 2006), and blocks p53 mediated apoptosis (Katano H, 2001). In addition, LANA-1 was shown to deregulate Wnt pathway, by the inhibition of GSK-3β and the consequent accumulation of β-catenin (Fujimuro M, 2003), a characteristic that occurs in many human cancers (Polakis P, 2000). LANA-1 can also inhibit the anti-proliferative transforming growth factor-β (TGF-β) (Di Bartolo DL, 2008) and

is involved in the stimulation of angiogenesis by inducing the degradation of von Hippel Lindau (VHL) factor with the consequent stabilization of hypoxia inducible factor 1 (HIF1α) (Cai QL, 2006).

The viral G protein-coupled receptor (*vGPCR*) encoded by ORF74, is a seven pass transmembrane protein homologous to the cellular IL-8 receptor and is expressed during the lytic phase (Cesarman E, 1996). vGPCR is a constitutive active receptor (Arvanitakis L, 1997) able to dysregulate several intracellular signaling pathways involved in proliferation and survival. *In vivo* studies show that vGPCR has transforming potential and cells expressing vGPCR can form tumors in nude mice through the activation of JNK and p38MAPK (Bais C, 1998). vGPCR activates NF-kB and induces secretion of proinflammatory cytokine and chemokine that can stimulates proliferation on neighboring cells, in a paracrine manner (Schwarz M, 2001). In addition, vGPCR activates PI3K/AKT (Montaner S, 2001) stimulating the secretion of VEGF, and the mTOR pathway *via* TSC2 (Sodhi A, 2006). Also another viral protein, K1, is implicated in the activation of AKT (Tomlinson CC, 2004), suggesting the importance of this pathway for the tumorigenic potential of KSHV.

vFLIP (viral caspase-8 (FLICE) – like inhibitor protein) is expressed during latency and is involved in the regulation of the NFkB pathway promoting survival of infected lymphoma cells (Guasparri I, 2004).

vBCL2, a Bcl2 homologue acts as an apoptosis inhibitor, promoting survival of infected cells (Flanagan AM, 2008).

vCyclin is a cyclin D homolog which promotes cell cycle progression by associating with cyclin-dependent kinase 6 (CDK6) and promotes cell cycle progression (Godden-Kent D, 1997).

vIL6 is a homolog of cellular IL6; it activates JAK/STAT, MAPK (Osborne J, 1999), and induces the secretion of VEGF, promoting angiogenesis and tumorigenesis (Aoki Y, 1999).

In recent years, some studies gave the evidence that KSHV encodes numerous *miRNA* involved in virus entry and viral replication and in the regulation of cellular immune response and cell proliferation (Qin Z, 2011).

ORF	Alternative name	Functions
K1*	VIP	Transormation: Bcell activation:inhibition of apoptosis:downregulation of surfaces B cellreceptor (BCR): and activation of PI3 K/Akt/mTOR kinases
K2*	viL-6	IL-6homlong; B cell proliferation; andautocrine/paracrine signaling
K3	MIR1	E3 ubiquitin ligase; immune evasion; andinhibition of MHC class 1 and T cell killing
K4	vMIP-II; vMIP-1b ; and vCCL3	MIP-1 homolong; angiogenesis; CCR3 and CCR8 binding; and chemoattraction of TH2 cells and monocytes (immune modulation)
K4.1	vMIP-III; vBCK; and vCCL3	TARC/cotaxin homolog;induction of vEgf-A and angogenesis; CCR4 bindig;chemoattracton of TH2 cells (immune modulation)
K5	MIR2	E3 ubiquitin ligase; immune evasion; and inhibition of MHC classI, B7, and ICAM expression
K6	vMIP-1; vMIP-1a; and vCCL-1	MIP-1 homolong; angiogenesis; CCR3 and CCR8 binding; and chemoattraction of TH2 cells and monocytes
K7	Survivin and vIAP	Inhibitor of apoposis protein (IAP) homolog and inhibition of vGPCR expression and function
K8	K-bZIP	An immediate-early gene that represses Rta transactivation activity and Rta induction of KSHV lytic cycle
K8.1		Viral glycoprotein (structural protein)
K9*	vIRF-1	IRF homolog; inhibition of type 1 interferon, p300, p53 and TGF-β and trasformation
K10	vIRF-4	IRF homolog
K10.1, K10.5, and K10.6	LANA-2 (K10.5) and vIRF-3	IRF homolog of type I interferon production and apoptosis (PKR-and caspase-3 mediated); inhibition of p53 and NFkB and inhibition of Fas-mediated apoptosis via inhibtion of CD95L surface expression
K11, K11.1, and K11.5	vIRF-2 (K11,5)	IRF homolog of type I interferon and NFkB and inhibition of Fas- mediated apoptosis via inhibtion of CD95L surface expression
K12*	Kaposin	Transormation (Kaposin A) and cytokine and AU-rich mRNA stabilization by induction of p38 or MK2 signaling (Kaposin B)
K13*	vFLIP	FLIP homolog transactivator of NFkB; anti-apoptotic function; and transformation
K14	vOx-2	Ox-2 (CD200) homolog; downregulation of myeloid cell activation; and regulatin of inflammatory cytokine production such as IL-Iβ, TNF-α, IL-8, IFN-γ, and IL-6
K15	LAMP	Activation of the intracellular signling pathways (Ras/MAPK, NFkB, and JNK/SAPK), leading to IL-6, IL-8, and Cox-2 induction. A chimeric protein consisting of the CD8 extracellular domain of CD8 and the K15 cytoplasmic domain could inhibit BCR sinaling

Table 1.1: List of proteins encoded by KSHV and their functions (Wen KW, 2010 (1))

2. Metabolism of tumor cells: the ‘Warburg Effect’

2.1 The Warburg Hypothesis

In 1956, Otto Warburg (Warburg O, 1956) discovered for the first time that cancer cells present a very high rate of glycolysis compared to normal cells, preferring to ferment glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation.

Under aerobic conditions, normal quiescent cells metabolize glucose through glycolysis to produce pyruvate which in turn is oxidized in the mitochondrial tricarboxylic acid (TCA) cycle. The TCA cycle reactions generate NADH, used by oxidative phosphorylation (OXPHOS) to produce ATP. This reactions produce 36 molecules of ATP from one molecule of glucose. Under anaerobic conditions, the cells are deprived of the final OXPHOS electron acceptor and cellular respiration is impaired; the pyruvate produced with glycolysis is converted into lactate (anaerobic glycolysis), resulting in a less efficient production of 2 molecules of ATP per molecules of glucose (Fig. 2.1).

Nevertheless, tumor cells or highly proliferative tissues metabolize glucose through glycolysis regardless of the availability of oxygen, producing large amount of lactate. This peculiar metabolism is referred to as ‘aerobic glycolysis’. Thus cancer cells show increased glucose uptake and enhanced glycolytic rates, suggesting that this metabolic alteration may provide a growth advantage for tumor cells.

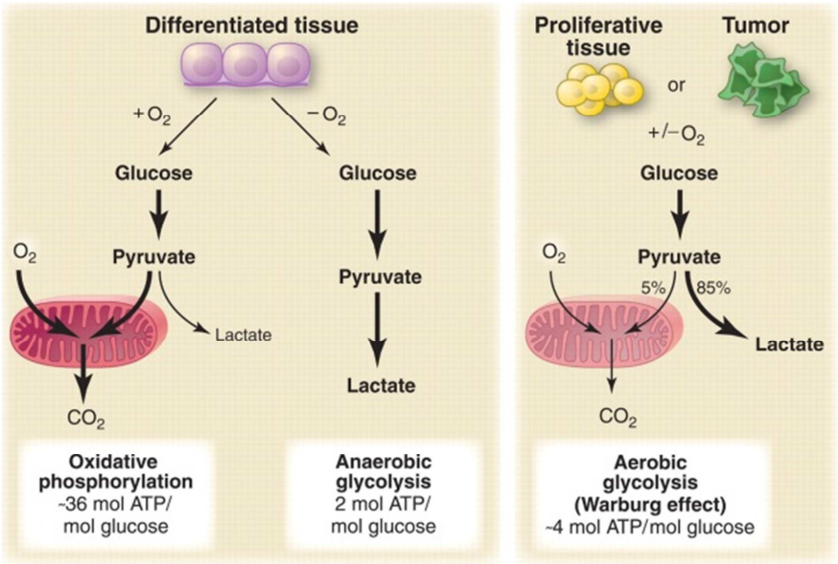

Figure 2.1 Representation of metabolism in differentiated tissues and tumors. In the presence of oxygen differentiated tissues metabolize glucose through glycolysis and then oxidize pyruvate by oxidative phosphorylation. When oxygen is limiting, the pyruvate is converted into lactate. Rapidly proliferating cells and tumor cells convert glucose into lactate despite the presence of oxygen through the so called ‘aerobic glycolysis’ or ‘Warburg effect’ (Vander Heiden MG, 2009)

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The reliance of cancer cells on increased glucose uptake is the basis for clinical [^{18}F]fluorodeoxyglucose positron emission tomography (FDG-PET) imaging. FDG-PET uses a radioactive glucose analogue to detect regions of high glucose uptake, and has proven to be highly effective in the identification and monitoring of many tumor types (Antoch G, 2004), confirming that many tumors presents 'aerobic glycolysis'.

Warburg speculated that this metabolism was due to respiratory injury leading to aerobic fermentation (Warburg O, 1956), but subsequent work demonstrated that most tumor cells had normal mitochondrial functions (Moreno-Sanchez R, 2007), suggesting that other explanations for the onset of aerobic glycolysis were required. Recent work showed that most oncogenes whose activity is altered in cancer (AKT, MYC, p53) have important roles in the regulation of tumor cells metabolism (Levine AJ, 2010).

2.2 Role of glycolysis and advantages of the 'Warburg Effect'.

The high glycolytic rate of cancer cells stems from metabolic needs distinct from those of their normal counterparts. Indeed, while most cells in the adult body are quiescent, and nutrients directed primary towards energy production, cancer cells must divide their incoming nutrients between energy production and macromolecular biosynthesis to support cell growth, DNA replication, and to maintain redox balance. Interestingly, all glycolytic intermediates can support anabolic pathways for the production of the macromolecules necessary for cell growth and proliferation: glucose-6-phosphate can enter the oxidative and non-oxidative pentose phosphate pathway (PPP), that is essential for the synthesis of nucleotides and is also the major source of NADPH, or the ROS detoxification; dihydroxyacetonephosphate is used for the synthesis of triglycerides and membrane lipids; 3-phosphpglicerate provides the carbons for cysteine, glycine and serine synthesis; acetylCoA, derived from pyruvate, can serve as a precursor for fatty acids, cholesterol and non-essential amino acids synthesis; in addition, pyruvate provides the carbons for alanine synthesis (Lunt SY, 2011). Thus, the high glycolytc rate of cancer cells support all the above anabolic processes and provides cells with a growth and proliferation advantage.

2.2.1 Dysregulation of the glycolytic flux in cancer cells

Glycolysis is a ten steps reaction that produces two molecules of ATP from one molecule of glucose; in normal tissues glycolysis is allosterically regulated by its products that inhibit most of the glycolytic enzymes. In tumor cells, many oncogenes can increase the transcription and the activity of glycolytic enzymes causing a major non-controlled glycolytic flux increase (Moreno-Sanchez R, 2007). Glucose transporters, including isoform 1 (GLUT-1) that has high affinity for glucose, are overexpressed in a significant number of human carcinomas (Medina RA, 2002) and enhance glucose uptake. The transcription of GLUT1 is under the control of the oncogenes

AKT (Barthel A, 1999) and c-myc (Osthus RC, 2000) that are frequently altered in tumors, and of the transcription factor hypoxia-inducible factor-1 (HIF1) that is activated under hypoxic conditions (Chen C, 2001).

The first step of glycolysis that converts glucose to glucose-6-phosphate is catalyzed by the enzyme hexokinase. There are four different isoforms of this enzyme (HK1, HK2, HK3 and HK4), with different subcellular localization and kinetics: isoform 1 (HK1) is expressed in all cell types; isoform 2 (HK2) is preferentially over-expressed in tumor cells; isoform 3 (HK3) is found in the cytoplasm of many cell types and, like isoforms 1 and 2, is inhibited by G6P; isoform 4 (HK4), also known as glucokinase, phosphorylates glucose only when its concentration is very high, and is not inhibited by G6P. HK4 is expressed in liver, pancreas, hypothalamus and small intestine (Wilson JE, 2003).

The inhibition of HK2, but not of HK1, in human glioblastoma multiforme results in the restoration of normal oxidative flux (Wolf A, 2011), suggesting the importance of this isoform in tumor metabolism. In addition, HK2 is associated to the outer membrane of mitochondria where it interacts with the voltage-dependent anion channel (VDAC). The interaction of HK2 with VDAC has antiapoptotic activity, as it interferes with VDAC association to the pro-apoptotic protein Bax. Of note, association of HK2 to the mitochondria membrane is promoted by AKT (Majewski N, 2004).

Remarkably, it has been recently demonstrated that tumor cells express high level of the isoform 2 of pyruvate kinase (PKM2), which is less active when compared to PKM1, leading to accumulation of glycolytic intermediates, which can be diverted towards anabolic pathways to sustain cell growth.

2.2.2 Metabolic fate of pyruvate in cancer cells

Pyruvate produced in normal tissues is directed into the mitochondrion to be converted into AcetylCoA by the action of pyruvate dehydrogenase (PDH). On the contrary, in tumor tissues pyruvate is directed away from mitochondrial metabolism and converted into lactate; this reaction is favored by the increased activity of two key enzymes: pyruvate dehydrogenase kinase 1 (PDHK1) and lactate dehydrogenase (LDH).

PDHK1 phosphorylates PDH thereby inhibiting it. Inactivation of PDH prevents pyruvate conversion into AcetylCoA and its entry into TCA. Because TCA is coupled to electron transport, inhibition of PDH blocks mitochondrial respiration (Kim JW, 2006).

LDH converts pyruvate into lactate with concomitant oxidation of NADH into NAD+. Of particular relevance for tumor cell metabolism is the overexpression of the LDH subunit A, which was demonstrated to be important for the growth of different type of tumor cells (Rong Y, 2013), whereas its inhibition impairs cell proliferation (Fantin VR, 2006). The expression of LDHA can

be directly induced by oncogenes, such as c-myc (Shim H, 1997), or indirectly via activation of HIF1α (Dang CV, 1999).

The export of lactate is favored by the overexpression of monocarboxylate transporter 4 (MCT4) (Dimmer KS, 2000). Lactate secretion promotes the acidification of the tumor environment (Koukourakis MI, 2003), a condition that favors tumor invasion (Swietach P, 2007), by influencing the remodeling of the extracellular matrix and permitting blood vessel invasion (Hunt TK, 2007). In addition, the lactate produced by highly glycolytic tumor cells can be used as an energy source by the surrounding stromal cells or by cancer cells with a different metabolic behavior. These cells import lactate trough the MCT1 transporter, and convert it into pyruvate to be used in OXPHOS. This process is therefore able to establish a symbiosis between different types of tumor cells and the cellular environment sustaining their growth (Sonveaux P, 2008). The MCT1-dependent intake of lactate by endothelial cells activates the NFkB/IL-8 pathway, which in turn stimulates angiogenesis and cell migration, and favors tumor cells proliferation (Vegran F, 2011).

On these basis, of cardinal importance to maintain pH homeostasis is the overexpression of carbonic anhydrase (CA), that catalyzes the reversible hydratation of carbon dioxide (CO2) to bicarbonate (HCO3-) and protons (H+). Tumor cells overexpress isoform IX (CAIX) and XII (CAXII), both targets of HIF1, which confer them a proliferative advantage (McDonald PC, 2012). Interestingly, overexpression of CAIX is associated with increased metastatic burden and poor patient survival in several types of malignancies (Hussain SA, 2007).

2.2.3 Anabolic branch off of glycolytic intermediates

As mentioned above, the Warburg shift demands that tumor cells enable a very high rate of glucose uptake to meet their increased requirements for energy, biosynthesis and reducing equivalents. The increased glycolytic flux allows for a fraction of glycolytic intermediates to be spared from ATP generation and be branched off to biosynthetic pathways. The glycolytic intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate can be shunted into the nonoxidative arm of pentose phosphate pathway (PPP) producing ribose-5-phosphate (R5P), fundamental for the production of nucleotides. Glucose-6-phosphate (G6P) enters in the oxidative arm of the PPP generating R5P and NADPH, that is essential for reactive oxygen species (ROS) detoxification. 3-phosphoglycerate (3PG) provides backbone carbons for the synthesis of non-essential amino acids and the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate provides a substrate for the biosynthesis of both phospholipids and triacylglycerols.

The pyruvate produced in the glycolytic pathway can enter a truncated TCA cycle and contribute to the production of mitochondrial citrate, that is then transported back in the cytoplasm where it

is converted to oxaloacetate (OAA) and acetyl-CoA, the precursor for lipid synthesis. The conversion of citrate to acetyl-CoA is catalyzed by the enzyme ATP citrate lyase (ACL), often overexpressed in tumor cells (Bauer DE, 2005). ACL contributes to the maintenance of the Warburg shift in tumor cells by preventing the accumulation of citrate that can inhibit glycolysis. The synthesis of lipids is important for tumor cell proliferation, as inhibition of fatty acid synthesis is an effective chemotherapeutic strategy (Hatzivassiliou G, 2005).

In addition to ACL, tumor cells overexpress two other important enzymes for lipid synthesis, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), whose inhibition has antiproliferative effects (Brusselmans K, 2005). (Fig 2.2)

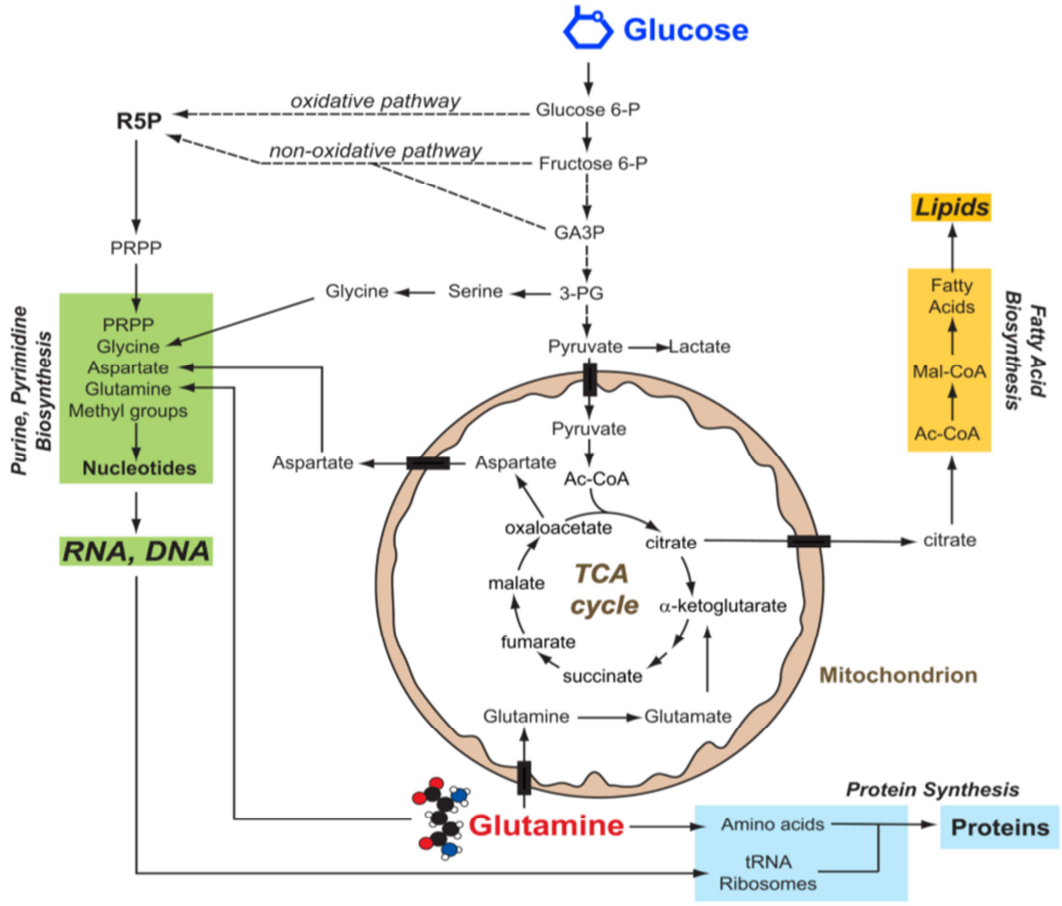


Figure 2.2 Glucose and glutamine as biosynthetic precursor in tumor cells. Glucose and glutamine contribute carbon for the synthesis of amino acid, lipids and nucleic acid. Glucose intermediates act as precursors for amino acids and are metabolized in the oxidative and non-oxidative pentose phosphate pathway (PPP) for the synthesis of nucleotides. AcetylCoA used as precursor for fatty acid biosynthesis derives from the citrate of the TCA cycle. Glutamine functions also nitrogen donor for nucleotide and amino acids biosynthesis. P:phosphate; GA3P: glyceraldehyde 3 phosphate; 3PG: 3-phosphoglycerate; PRPP: phosphoribosyl pyrophosphate; MalCoA: malonylCoA (DeBerardinis RJ, 2008).

2.3 Tumor cell metabolism: not only glycolysis upregulation

2.3.1 Glutamine utilization in cancer cells

As for glucose, it has been known for more than 50 years that tumor cells have increased rates of glutamine uptake and utilization. Besides glycolysis, glutaminolysis is another main pillar for energy production in cancer cells, so much that most cancer cells are addicted to glutamine and cannot survive without an exogenous source of this aminoacid.

The metabolism of glutamine appears essential for the synthesis of nucleotides, non-essential aminoacids, lipids and to maintain the redox balance. Glutamine also plays a regulatory role in a number of cellular processes, such as metabolism, apoptosis, cell proliferation and ROS detoxification (Burgess DJ, 2013).

Glutamine is the most abundant free amino acid in the circulation and in intracellular pools and is imported into the cells through high affinity transporters, such as ASCT2 and SN2 (Wise DR, 2008). Once in the cell, glutamine may donate nitrogen to macromolecular synthesis or be deaminated by glutaminases, which converts glutamine to glutamate (Glu). Glu can be converted directly into glutathione (GSH), one of the most important anti-oxidant molecules in the cells, by glutathione cysteine ligase (GCL), or it can be catabolized to produce α-ketoglutarate, a TCA cycle intermediate. α-ketoglutarate is then converted into OAA, that condenses with Acetyl-CoA to produce citrate. Real-time ^{13}C NMR studies in glioblastoma cell lines demonstrated that glutamine contributes to the majority of the cellular OAA pool (DeBerardinis RJ, 2007), thereby sustaining mitochondrial anaplerosis, fundamental for the maintenance of the mitochondrial membrane potential, and for the synthesis of nucleotides, lipids and proteins.

A significant fraction of glutamine carbon can also be converted into lactic acid and secreted from the cell, a process known as ‘glutaminolysis’. Conversion of glutamine into lactic acid requires the activity of malic enzyme (ME), that oxidatively decarboxylates malic acid, producing carbon dioxide, NADPH and pyruvate. NADPH is an important source of reducing equivalent for the synthesis of lipids and nucleotides.

Several studies demonstrate that the oncogene c-myc plays an important role in the regulation of glutaminolysis (Gao P, 2009); c-myc overexpression upregulates the expression of glutamine transporters, inducing an increase in glutamine uptake and up-regulates the expression of glutaminase in different types of human tumors. Glutamine depletion induces apoptosis in tumor cells in a Myc-dependent manner (Yuneva M, 2007), confirming the importance of glutamine in sustaining tumor growth. Similar results were obtained in a xenograft model. (Lobo C, 2000). In addition to c-myc, RhoGTPase, with a demonstrated role in cell growth and transformation (Vega FM, 2008), is important in the regulation of glutaminase activity, as blocking RhoGTPase activity inhibits the transforming activity of human breast cancer cells.

2.3.2 Alteration of mitochondrial metabolism

The TCA cycle consists of a series of chemical reactions used by aerobic organisms to generate energy through the oxidation of pyruvate, and provides precursors and NADPH for the biosynthetic pathways. Mutations of genes that encode enzymes of the TCA cycle are associated to some types of cancer. Isocitrate dehydrogenase 1 (IDH1) converts isocitrate to α-ketoglutamate (2-KG), with generation of NADH. Mutations of this gene have been identified in glioblastoma tumors (Parsons DW, 2008) and in acute myeloid leukemia (Ward PS, 2010). This mutation seems to confer at the enzyme a new function: the ability to convert 2-KG to d-2-hydroxyglutarate (D-2-HG). Elevated levels of D-2-HG are demonstrated to contribute to formation and progression of different human tumors, for example gliomas (Dang L, 2010). Germline mutations in other two TCA enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH) have been identified in different forms of renal human cell cancer, paraganglioma and pheochromocytoma (Baysal BE, 2000; Pollard PJ, 2003). The effect of these mutations is the accumulation of succinate and fumarate, leading to the stabilization of HIF1 (Selak MA, 2005).

2.4 Regulation of tumor cell metabolism by oncogenes

Many oncogenes, such as PI3K/AKT/mTOR, c-myc and p53, are involved in the regulation of tumor cells metabolism.

The serine/threonine kinase Akt, frequently mutated in cancers, was reported as the 'Warburg kinase', due to its importance in the regulation of different metabolic pathways. The role of Akt in tumor metabolism is described in detail in section 3.4.

The tumor suppressor p53 is frequently mutated in human cancers, and the loss of p53 functions in tumors can lead to Warburg effect. The wild-type p53 protein inhibits the transcription of the glucose transporters GLUT1 and GLUT4, reducing glucose uptake (Dang CV, 1999), and repress the glycolytic enzyme phosphoglycerate mutase (Kondoh H, 2005). p53 induces the expression of TIGAR, that lowers the intracellular concentration of fructose 2,6 bisphosphatase, inhibiting the activity of the glycolytic enzyme phosphofructokinase (PFK1), and thus decreases the glycolytic flux, diverting glucose through the oxidative PPP for production of antioxidant NADPH (Bensaad K, 2006). In addition, p53 transcriptionally induces the synthesis of cytochrome oxidase 2 (SCO2), that is required for the correct assembly of cytochrome c oxidase complex, promoting mitochondrial oxidative phosphorylation (Matoba S, 2006). p53 also induces glutaminase-2 that, unlike glutaminase-1 induced by c-myc, converts glutamine to glutamate, used to enhance the rate of the TCA cycle and oxidative phosphorylation (Suzuki S, 2010). Another important function of p53 in metabolic regulation is the induction of AMPK in stress conditions, inhibiting cell growth (Jones RG, 2005).

One of the principal mechanism by which cells respond to oxygen deprivation is through the activation and stabilization of the transcription factor Hypoxia Inducible Factor 1 (HIF1), which leads to a series of metabolic changes – such as induction of glycolysis and inhibition of mitochondrial metabolism – that are reminiscent of the Warburg effect (Semenza GL, 2003). In tumor cells, the activation of oncogenes, such as c-myc and Akt can induce HIF1 even in normoxic conditions; in some cases most of these oncogenes effects on metabolism occur almost exclusively through HIF1. The oncogene c-myc regulates also the glutamine metabolism, enhancing the transcription of glutaminase-1.

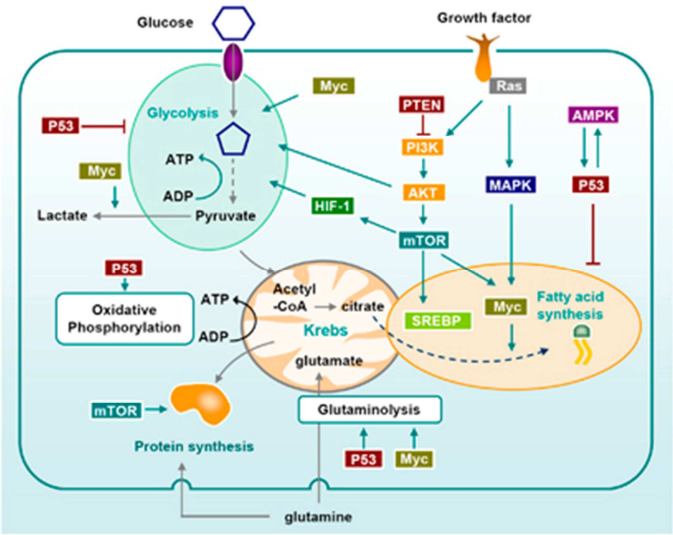


Fig. 2.3 Regulation of tumor cell metabolism by signaling pathways. PI3K/Akt stimulates glycolysis. mTOR stimulates lipogenesis and by the regulation of HIF1 stimulates glycolysis. p53 has opposite effects, blocks glycolysis and stimulates oxidative phosphorylation. C-myc stimulates glutaminolysis. (Munoz-Pinedo C, 2012)

2.5 AMPK pathway and metabolism regulation

AMP-Activated protein kinase (AMPK) is a protein kinase that is conserved in essentially all eukaryotes and is expressed in several tissues, such as skeletal muscle, brain and liver (Oakhill JS, 2012). AMPK plays a fundamental role as an energy sensor in the cells, maintaining energy homeostasis (Hardie DG, 2001).

AMPK is an heterotrimeric complex, with a catalytic α-subunit and two regulatory β- and γ-subunits (Hardie DG, 2012). In mammals there are two α-subunit isoforms (α1 and α2), two β-subunit isoforms (β1 and β2) and three γ-subunit isoforms (γ1, γ2 and γ3), each encoded by distinct genes. The α-subunit contains at N-terminus the catalytic domain, with the conserved threonine residue that must be phosphorylated for the regulation of the kinase activity (Hawley

SA, 1996). The C-terminal region is important for the formation of the complex with the regulatory subunits (Crute BE, 1998). The C-terminal domain of β-subunit tethers α and γ subunit (Iseli TJ, 2005), acting as a scaffold for the formation of the complex. The γ subunit contains the binding sites for AMP/ATP, called Bateman domains (Scott JW, 2004).

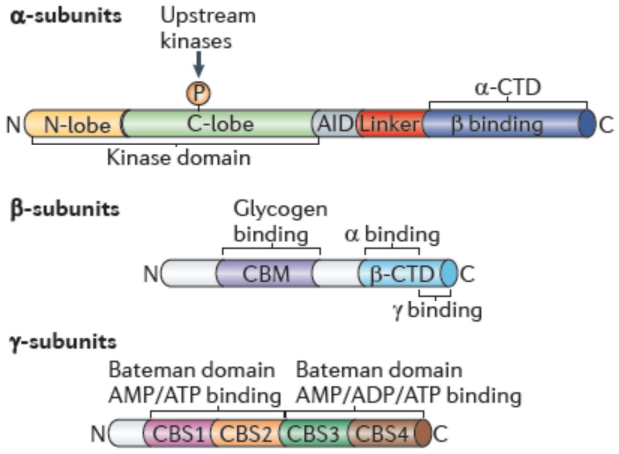


Figure 2.4 AMPK domains. AMPK is an eterotrimer composed of α, β and γ subunits. AID: autoinhibitory domain; CBM: carbohydrate binding module; CBS: repeats in the γ subunit. (Hardie DG, 2012)

AMPK is activated by various type of metabolic stress that cause the reduction of intracellular levels of ATP and increased level of AMP, such as exercise, hypoxia or nutrient deprivation. (Hardie DG, 2007). The activation of AMPK is obtained with the binding of AMP on the γ-subunit and the subsequent phosphorylation at the residue threonine 172 (Thr172). The binding of AMP to APMK γ-subunit promotes the phosphorylation and inhibits dephosphorylation (Suter M, 2006), ATP has opposite effect, promoting dephosphorylation and inhibiting phosphorylation. Recent studies demonstrated that also ADP can regulates phosphorylation of AMPK suggesting a modulation of AMPK activity in response to different levels of stress (Hardie DG, 2012).

LKB1 is the major kinase that phosphorylates AMPK at Thr172 (Carling D, 2008), LKB1 was originally identified as a tumor suppressor gene mutated in Peutz-Jeghers syndrome, a disorder in which patients develop gastrointestinal hamartomas and have an increased risk for developing gastrointestinal, breast and gynecological cancers (Hemminki A, 1998). In addition, LKB1 mutations occur in a large percentage of other sporadic cancers, especially in non small cell lung cancer (NSCLC) (Sanchez-Cespedes M, 2002). The activation of AMPK by LKB1,is probably at the basis of its antitumorigenic activity. In some tissues, like neurons, T lymphocyte and endothelial cells, calcium and calmodulin-dependent protein kinase 2 (CAMKK2) can phosphorylate AMPK at Thr172 resulting in AMPK activation (Woods A, 2005), in response to changes in Ca^{2+} levels. Recently, it was demonstrated that H_2O_2, can mediated the activation of

AMPK thought cysteine oxidation in the α-subunit, suggesting that AMPK is involved also in the regulation of the redox balance in the cell (Zmijewski JW, 2010), also DNA damage can activate AMPK, this effect is mediated by ATM, a kinase that is activated by double-strand breaks in DNA and that phosphorylates LKB1 (Alexander A, 2010).
AMPK activation, in stress condition, determines a variety of effect on cell metabolism, leading to activation of catabolic pathway for the production of ATP, and inhibiting biosynthetic pathway that consumes ATP (Figure 2.5)

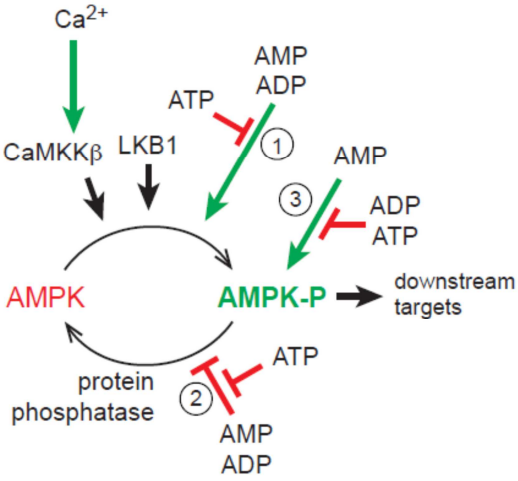


Figure 2.5 Regulation of AMPK. AMPK is activated by the phosphorylation on Thr172 catalyzed by two kinases: LKB1 and CaMKK. CaMKK activates AMPK in response to Ca^{2+} levels. AMP and ADP activates AMPK through 3 different mechanisms: binding of ADP AMP to AMPK causes a conformational change that promotes the phosphorylation by LKB1 (1) and inhibits dephosphorylation by protein phosphatase (2). Binding of AMP causes an allosteric activation of AMPK-P (3) (Hardie DG, 2013).

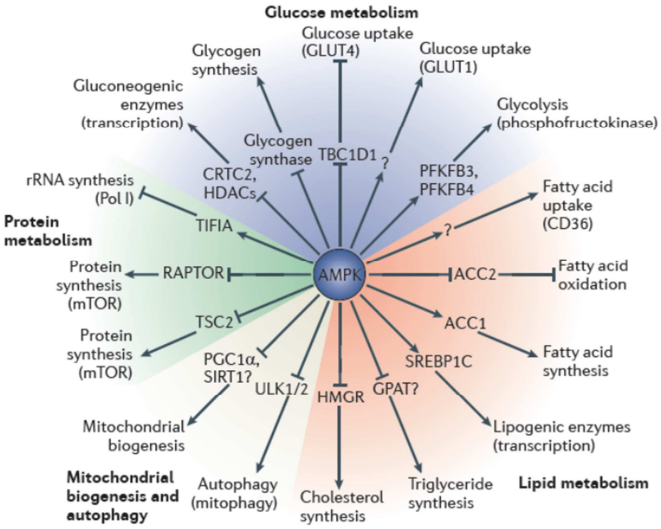
AMPK regulates the expression of glucose transporters, that enhances glucose uptake. In muscle cells, AMPK enhances the expression of GLUT4, during muscle contraction, when glucose is used to generate ATP (Chen S, 2008). AMPK also promotes glucose uptake in cell that express GLUT1, by the activation of the transporter that is already located at the plasma membrane (Barnes K, 2002). AMPK stimulates glycolysis by the phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB), which catalyzes the generation of fructose-2,6 bisphosphate, the allosteric activators of the glycolytic enzyme 6-phosphofructo-1 kinase (PFK1) (Marsin AS, 2000). AMPK inhibits the majority of anabolic pathways, including the biosynthesis of lipids, carbohhydrates, proteins and ribosomal RNA. AMPK inhibits fatty acid and cholesterol synthesis by phosphorylating the enzymes acetyl-CoA carboxylase (ACC1) (Davies SP, 1992) and HMG-CoA redectase (HMGCR) (Clarke PR, 1990), and glycogen synthesis by the interaction with glycogen synthase (Jorgensen SB, 2004). In addition, AMPK directly

phosphorylates PGC1α, the most important activator of mitochondrial biogenesis, increasing the catabolic potential of cells (Jager S, 2007).

AMPK, apart from its function in metabolic regulation, regulates also cell growth and proliferation, this function is mediated by the phosphorylation of p53 and the subsequent accumulation of the cyclin-dependent kinase inhibitor p27 and p21, determining cell-cycle arrest (Jones RG, 2005). In addition, AMPK regulates gene transcription through the phosphorylation of transcriptional coactivators, such as p300 histone acetyltransferase (HAT) (Yang W, 2001), histone deacetyltranferase (HDAC) (McGee SL, 2008) and CRTC (CREB-regulated transcription coactivator) family (Koo SH, 2005), promoting their interaction with 14-3-3 and subsequent cytoplasmic sequestration, this effect mediates the regulation of different metabolic enzymes. Another important transcriptional factor regulated by AMPK is SREBP1, that stimulates fatty acid synthesis, promoting the expression of ACC, AMKP promotes its interaction with 14-3-3 resulting in the inhibition of its function (Li Y, 2011).

One of the most important pathway controlled by LKB1-AMPK is the mTOR pathway: mTOR plays a central role in the regulation of cell growth, protein synthesis, angiogenesis and metabolism (Sabatini DM, 2006). AMPK directly phosphorylates the tumor suppressor TSC2 that inhibits mTORC1 (Shaw RJ, 2004), Moreover, in cells lacking TSC2, AMPK can phosphorylate the regulatory protein associated to mTORC1, Raptor, leading to mTORC1 activity inhibition. (Gwinn DM, 2008). The inhibition of mTORC1 blocks protein synthesis and inhibits cell growth. The effect of APMK on mTORC1 is opposite to the regulation of AKT, that stimulates cell growth. Based on the importance of AMPK in controlling cell growth, the activation of the AMPK pathway may represent a possible antitumor strategy.

Metformin, one of the most widely used drug for the treatment of type 2 diabetes, has been demonstrated to activate AMPK (Musi N, 2002). Recent analysis evidenced that metformin treatment is associated with a decreased incidence of various types of cancer (Decensi A, 2010).



2.6 Effects of AMPK on metabolism. AMPK is involved in the regulation of lipid, protein and glucose metabolism and in the regulation of mitochondrial function and autophagy (Hardie DG, 2012).

In summary, the metabolism of proliferative and tumors cells is different from the metabolism of quiescent cells. Quiescent cells show basal level of glycolysis and the majority of ATP is produced by oxidative phosphorylation in the mitochondria. In proliferating cells, on the contrary, the majority of ATP is produced through glycolysis. These cells show an increased glycolytic flux and most of the resulting pyruvate is converted into lactate, and secreted from the cells; the remaining pyruvate is converted to AcetylCoA and enters the TCA cycle, where it is converted into TCA intermediates, used for macromolecular synthesis (Figure 2.4). As described before, also glycolytic intermediates are used for macromolecular synthesis to sustain the production of building blocks necessary for cell proliferation.

Quiescent Proliferating

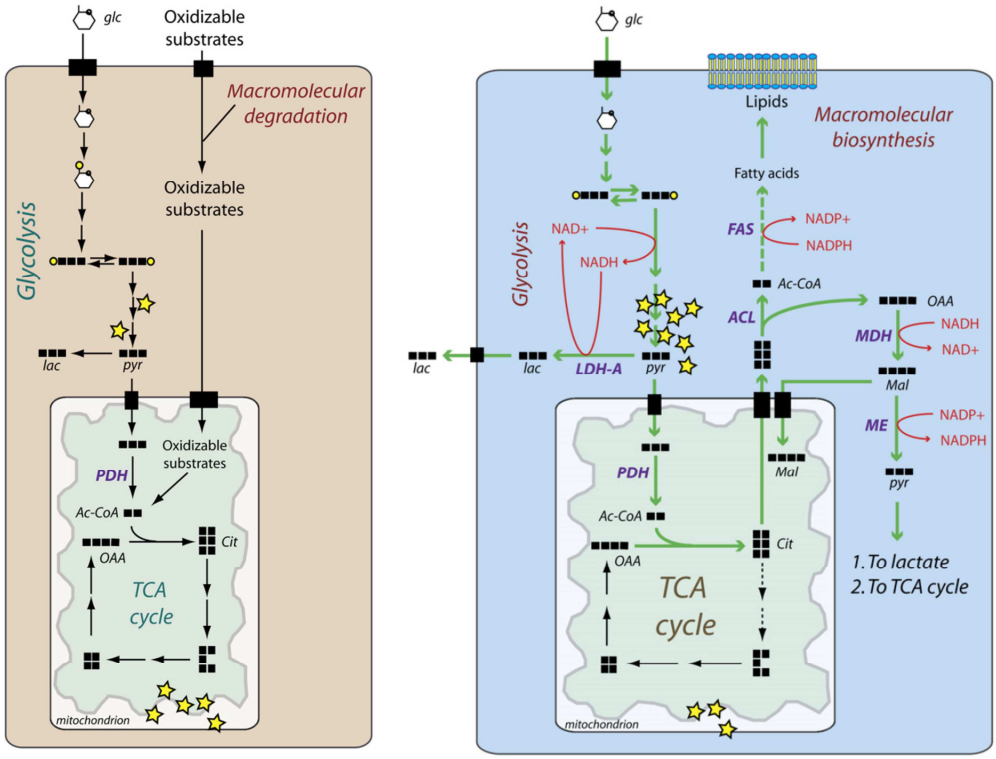


Fig. 2.7 Metabolism of quiescent and proliferating cells. Quiescent cells metabolize glucose through glycolysis and the pyruvate produced enter in the TCA and the majority of ATP (yellow stars) derives from oxidative phosphorylation. Proliferative cells produces ATP through glycolysis. the pyruvate produced is converted into lactate by lactate dehydrogenase A (LDHA) and in AcetylCoA (AcCoA) by PDH (pyruvate dehydrogenase) and enters in the TCA where is converted into citrate that can be used for macromolecular biosynthesis. Citrate is cleaved by ATP-citrate lyase (ACL) in AcCoA used for fatty acid biosynthesis and oxaloacetate (OAA) that is converted to malate (mal) by malate dehydrogenase (MDH). Malate can return in the TCA and can be converted in pyruvate by the malic enzyme (ME). (DeBerardinis RJ, 2008).

In conclusion, 'aerobic glycolysis' may provide many advantages to tumor cell growth:

1. Cancer cells can use glycolytic intermediates for biosynthetic pathway to produce amino acids, lipids and nucleotides for the production of new cells. The high rate of glycolysis can also sustain a sufficient ATP production.

2. Glucose can be diverted to the PPP pathway to generate NADPH that represents the most important antioxidant molecules in cells, thus protecting tumor cells from ROS production

3. Cancer cells secrete large amounts of lactate that alters the extracellular pH, favoring tumor invasion and sustaining the growth of non-Warburg tumor cells in the surrounding stroma.

2.3 Targeting metabolism of tumor cells

The metabolic rewiring which characterizes the Warburg phenotype rendered glycolysis and key glycolytic enzymes new potential drug targets for tumor treatment (Figure 2.5).

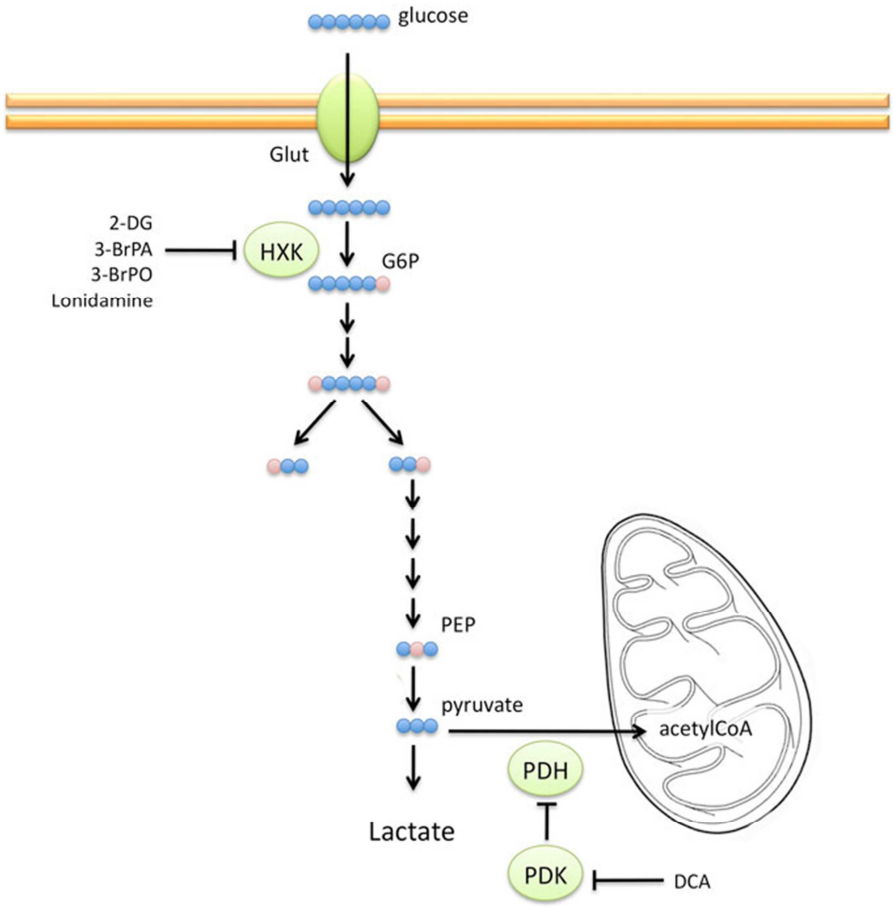


Figure 2.8 Schematic representation of metabolism inhibitors.

2.3.1 2-deoxyglucose

2-deoxyglucose (2-DG) is a glucose analog that blocks glucose metabolism, as 2-DG is phosphorylated by hexokinase to 2-DG-P that cannot be further metabolized by phosphohexose isomerase (Weindruch R, 2001). The accumulation of 2-DG-P blocks glycolysis, probably by feedback inhibition of hexokinase. 2-DG was demonstrated to cause ATP depletion and cell death, especially in cancer cells with mitochondrial respiratory defect (Liu H, 2001). In addition, 2-DG is more effective in hypoxic environment (Maher JC, 2004). 2-DG was also found to block N-linked glycosylation, leading to unfolded protein responses and inducing tumor cells apoptosis, independently from its action on glycolysis (Kurtoglu M, 2007). Another study demonstrated that 2-DG, in combination with ionizing radiation, altered the expression of genes that regulate the cell cycle, apoptosis and cytokine signaling (Heminger K, 2006). Breast cancer cells exposed to high concentration of 2-DG undergo apoptosis, confirmed by the activation of caspase-3 (Aft RL, 2002). Animal studies demonstrated that though 2-DG has low efficacy as single agent, in combination with adriamycin or paclitaxel results in a significant reduction in tumor growth (Masheck G, 2004). Similar results were obtained in a mouse model of retinoblastoma: treatment with 2-DG as adjuvant to chemotherapy increases the efficacy of chemotherapy (Boutrid H, 2008). These results obtained *in vitro* and *in vivo,* in mouse models, suggested to investigate the potential therapeutic effect of 2-DG, especially in combination with canonical chemotherapy.

Safety and feasibility of oral 2-DG administration, as single agent, has been tested in clinical trials in patients with advanced prostate cancer (Stein M, 2010). Furthermore, a phase I/II clinical trial, in 1996, demonstrated the efficacy of 2-DG in combination with radiotherapy in patients with cerebral gliomas, inducing a moderate increase in survival and a better quality of life (Mohanti BK, 1996). In a more recent study, 2-DG is demonstrated to be safe, with no acute toxicity, at doses up to 250mg/kg and effective in combination with radiotherapy in patients with glioblastoma (Singh D, 2005). The side effects observed in patients treated with 2-DG are similar to those of hypoglycemia and are demonstrated to be transient, lasting only a couple of hours (Dwarakanath BS, 2009). Recently a phase I clinical study evaluated the efficacy of 2-DG alone or in combination with docetaxel in patients with advanced solid tumors (Raez LE, 2013). In addition to solid tumors, 2-DG in combination with prednisolone was shown to reverse glucocorticoid resistance in leukemic cells derived from patients (Hulleman E, 2008). The efficacy of 2-DG when used in combination with other agents is also demonstrated in leukemia cells in which 2-DG cooperates with arsenic tioxide to induce apoptosis (Estan MC, 2012). Furthermore, in 2008, Zhong ang colleagues demonstrated that 2-DG can induce AKT phosphorylation, a possible mechanism of resistance, and that the combination of 2-DG with AKT inhibitors may prevent this mechanism (Zhong D, 2008).

2.3.2 Lonidamine

Lonidamine is a derivative of indaxole-3-carboxylic acid that inhibits glycolysis. lonidamine was demonstrated to reduce the oxygen consumption in both normal and neoplastic cells. In contrast, it increased aerobic glycolysis of normal cells but inhibited that of tumor cells, probably due to the inhibition of hexokinase that is .bound to the mitochondrial membrane, which is usually absent in normal differentiated cells (Floridi A, 1981). Lonidamine was reported to cause depletion of ATP in a dose-dependent manner (Floridi A, 1998). Numerous *in vitro* and *in vivo* studies demonstrated the efficacy of lonidamine, in combination with several antineoplastic drugs, in growth inhibition of different types of tumor. The induction of apoptosis is probably due to a direct interaction of lonidamine with Bcl-2, blocking the effect on the mitochondrial permeability pore (Ravagnan L, 1999); furthermore lonidamine is demonstrated to favor mitochondrial membrane permeabilization to a direct effect on adenine nucleotide translocase (Belzacq AS, 2001). Apoptosis induced by lonidamine is independent of p53 (DelBufalo D, 1996). Another important effect of lonidamine is to inhibit endothelial cell proliferation, migration, invasion and vessel formation in a matrigel plug (DelBufalo D, 2004). A recent study reported that lonidamine induces the activation of different prosurvival pathways such as AKT, ERK and mTOR. Combined treatment with lonidamine and arsenic tioxide or specific inhibitors of the hyperactivated pathways enhances apoptosis in human leukemia cells (Calvino E, 2011). Lonidamine enhanced the effect of radiation in human melanoma cell lines (Miyato Y, 2004) and in prostatic carcinoma (Dudak SD, 1996). In addition, post-treatment with lonidamine, after paclitaxel, has synergistic effect when compared with single treatment in hepatocellular carcinoma cell lines (Ricotti L, 2003). Besides, lonidamine potentiate the efficacy of diazepam in human glioblastoma (Miccoli L, 1998). The efficacy of lonidamine was confirmed in human tumor xenografts, were lonidamine has a synergistic effect in combination with cisplatin. (DeCesare M, 1998). Furthermore, lonidamine has been evaluated in clinical trials for the treatment of different solid tumors with encouraging results. In a phase II study of advanced ovarian cancer lonidamine enhances the effect of cisplatin, confirming the effect observed *in vitro* in ovarian cell lines, with reamrkably low toxicity (DeLena M, 2001). In advanced breast carcinoma patients, Lonidamine potentiates the effect of epirubicin (Nistricò C, 1999), and the effect of diazepam in the treatment of recurrent glioblastoma multiforme (Oudard S, 2003). However, a phase III clinical trial reported that addition of lonidamine to a conventional treatment failed to prevent progression of metastatic breast cancer (Berruti A, 2002).

All together, low toxicity and the ability to potentiate the effect of different antineoplastic treatments confirm lonidamine therapeutic potential in the treatment of many tumors.

2.3.3 3-bromopyruvate

3-bromopyruvate (3-BrPA) is an analogue of pyruvate, and an alkylating reagent that can react with the free SH groups of cysteine residues in different proteins. It has been used for the treatment of the parasite *Trypanosoma brucei*, that generates ATP exclusively through glycolysis (Barnard JP, 1993). 3-BrPA inhibits hexokinase II, that is frequently overexpressed in cancer cells, causing a severe depletion of ATP level. The efficacy of 3-BrPA was confirmed in a mouse model of HCC (Ko YH, 2004). In a cell model of human leukemia and lymphoma, 3-BrPA is effective in the inhibition of glycolysis, determining the dephosphorylation of BAD, leading to BAX localization to mitochondria and subsequent apoptosis. Cells that express a multidrug-resistant phenotype still remain sensitive to 3-BrPA. In addition, 3-BrPA is more effective in cells with mitochondrial defects or growing in hypoxic condition, usually less sensitive to conventional chemotherapy (Xu RH, 2005 (1)). This compound causes the dissociation of HKII from mitochondria, leading to the release of apoptosis-inducing factor (AIF) from mitochondria and to apoptosis (Chen Z, 2009). Inhibition of glycolysis and ATP depletion, following 3-BrPA treatment, inactivates ABC transporter restoring the cytotoxic effect of anticancer drug in leukemia, myeloma, prostate carcinoma and breast cancer tumor cell lines (Nakano A, 2011). 3-BrPA acts in synergism with conventional chemotherapeutic agent (Ihrlund LS, 2008). This compound was effective in killing liver cancer cells in a rabbit tumor model (Liapi E, 2011), and in a mouse model of aggressive metastatic lymphoma (Schaefer NG, 2012). In 2012, the treatment with 3-BrPA in a patient with fibrolamellar hepatocellular carcinoma with promising results showed no toxicity (Ko YH, 2012). 3-BrPA is transported inside the cell by the transporter MCT-1, while MCT-1 null cells are resistant to 3-BrPA; forced expression of MCT-1 resensitized tumor cells to the treatment, both *in vitro* and *in vivo*. Expression of MCT-1 is a potential biomarker for 3-BrPA sensitivity (Birsoy K, 2013). 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP) is a cell permeable ester of 3-BrPA, that once inside the cell is hydrolyzed by cellular esterase to release 3-BrPA (Xu RH, 2005 (2)). The preferential target of 3-BrOP is GAPDH, rather than HKII. Moreover, 3-BrPO has been demonstrated to have a synergic effect with the mTOR inhibitor rapamycin in ATP depletion and induction of apoptosis in lymphoma and leukemia cells (Xu RH, 2005 (2)). Similarly, in neurobalstoma cells and xenograft, the combination of 3-BrOP and rapamycin had synergistic effect (Levy AG, 2012). These data demonstrate the potential of these compound as possible new anticancer drug.

2.3.4 Dichloroacetate

Dichloroacetate (DCA) is a small (150Da) molecule that activates pyruvate dehydrogenase (PDH) by inhibiting its regulator pyruvate dehydrogenase kinase (PDHK) (Stacpoole PW, 1989). PDH converts pyruvate to AcetylCoA, controlling the use of metabolites into the citric acid cycle and in turn the generation of ATP by oxidative phosphorylation. PDK phosphorylates and inactivates PDH. Several studies demonstrate that DCA treatment can act as an anticancer drug. DCA reversed the glycolytic phenotype and decreased to normal level the mitochondrial membrane potential, increasing mitochondrial metabolism, promoting apoptosis in cancer cell *in vitro* and significant tumor growth delay in xenografts of lung carcinoma. (Bonnet S, 2007). DCA treatment can also induce apoptosis and cell cycle arrest in colorectal and prostate cancer cells (Madhock BM, 2010; Cao W, 2008). The combination of DCA with cisplatin in HeLa cells has a synergic effect in the inhibition of cell growth (Xie J, 2011), furthermore DCA and bortezomib show an additive effect in multiple myeloma cell line (Fujiwara S, 2013), suggesting that the combination of DCA with other anticancer drugs may result in enhanced antitumor activity. DCA enters the cells by the SLC5A8 transporter and the lack of the transporter makes tumor cells resistant to DCA. SLC5A8 is preferentially expressed in normal cells, and is inhibited in tumors by DNA methylationwhile DCA has no effect on normal cell viability (Babu E, 2011). A breast cancer study (Sun RC, 2011) reported that DCA suppresses expression of HIF1α, c-myc and Bcl-2, inducing apoptosis. The same results were obtained in xenotransplant models of non-small cell lung carcinoma and breast cancer, in which DCA inhibits the expression of HIF1α, resulting in an effective inhibition of angiogenesis and tumor growth. DCA inhibits HIF1α by two different mechanism: one PDH-dependent, increasing the production of α-ketoglutarate, and one PDH-independent, increasing p53 activity (Sutendra G, 2013). The efficacy of DCA was confirmed in other mouse models, such as glioma (Duan Y, 2013) and melanoma (Zheng MF, 2013). DCA has been extensively used for the treatment of mitochondrial diseases and has been tested in clinical trials for the treatment of lactic acidosis in both mitochondrial and non-mitochondrial disorders (Stacpoole PW, 2003). *In vitro* and *in vivo* evidence suggests that DCA might be beneficial in human cancer. In 2010, Michelakis studied the effect of DCA in cells excised from glioblastoma multiforme GBM tumors reportiong DCA depolarized mitochondria of tumor tissues and decreased cell proliferation, but had no effect on normal tissues. Then he administrated DCA orally to five patients with primary GBM reporting evidence of radiologic regression (Michelakis ED, 2010).

2.3.5 Oxamate

Oxamate is a competitive inhibitor of lactic dehydrogenase, that competes with pyruvate and can inhibit aerobic and anaerobic glycolysis as demonstrated by Papaconstantinou in 1961 (Papaconstantinou J, 1961). The importance of LDH-A activity, which catalyzes the transformation of pyruvate to lactate, is demonstrated with shRNA to block LDH-A expression in tumor cells: shRNA impairs tumor growth, inhibits the glycolytic phenotype and stimulate oxidative phosphorylation (Fantin VR, 2006). Lactate is known to stabilize HIF1α, determining an increase of VEGF levels in cultured endothelial cells, and the inhibition of LDH-A prevents the proangiogenic effect of lactate (Hunt TK, 2007). Oxamate resensitizes human breast cancer cells to taxol treatment, supporting the hypothesis that the Warburg effect occurring in cancer cells may play an important role in the resistance of tumor to anticancer drugs (Zhou M, 2010). This results was confirmed in breast cancer xenografts: combination of oxamate and trastuzumab not only synergistically inhibits tumor growth, but also that of trastuzumab resistant cells (Zhao Y, 2011). LDH-A is overexpressed in nasopharyngeal carcinoma cells, and high level of LDH-A in blood serum of patients is associated with poor prognosis. Oxamate suppressed cell proliferation in a dose dependent manner in nasopharyngeal carcinoma cell lines, inducing G2/M cell cycle arrest, and promoting apoptosis by ROS production (Zhai X, 2013). These data underline the therapeutic potential of this compound, especially when used in combination with other drugs.

2.7 The 'Warburg Effect' in PEL cells

The first metabolomic analysis, in viral infected cells, was performed in cells infected with HCMV. The study produced evidence that viral infection can lead to important alterations in the metabolic pathways of the host cells, and that these alterations may provide advantages for viral replication (Munger J, 2006).

KSHV induces aerobic glycolysis in endothelial infected cells: in two different cell model the infection with KSHV results in an increased glucose uptake, the glucose is then converted to lactic acid (Delgado T, 2010). In addition they observed that KSHV induces the expression of the glucose transporter GLUT3 and the rate-limiting glycolytic enzyme HK2, and also that KSHV inhibits oxidative phosphorylation. Treatment with glycolytic inhibitor, 2-deoxyglucose, results in a dose-dependent apoptosis in KSHV infected cells, but not in mock-infected cells, indicating that glycolysis is important for the survival of latent infected endothelial cells. Similar results were obtained in PEL cells: PEL cells when compared with normal primary B-cells showed an increased glycolytic flux and greater concentration of lactate in the medium. As a confirm of this metabolic alteration in PEL cells, compared to their normal counterpart, PEL cells are sensitive to glycolytic inhibitor 2-deoxy-glucose (Bhatt AP, 2012).

Another metabolic alteration observed in PEL cells is the increase of fatty acid synthesis (FAS), probably due to the increased expression of FASN. Glycolysis and FAS are strictly interdependent, the inhibition of glycolysis inhibits also FAS pathway and the inhibition of FAS, blocks glycolysis. This particular metabolism observed in PEL cells depends on PI3K/AKT pathway, the use of an inhibitor of this important pathway, LY294002 (Tomlinson CC, 2004) results in a decrease in the glycolytic flux and in a dose-dependent reduction of FASN expression. In addition the combination of LY294002 and a FAS inhibitor (C75) is most effective in the inhibition of PEL cells proliferation, when compared to single treatment (Bhatt AP, 2012).

These results were confirmed by a metabolomics analysis of KSHV infected cells (Delgado T, 2012), this study is based on the quantification of the metabolites present in KSHV infected cells, compared to mock infected ones. Many of metabolic pathways, glycolysis, amino acid metabolism, pentose phosphate pathway, and fatty acid synthesis, are altered in infected cells. This study confirm also the important role of fatty acid synthesis in the survival of latent infected cells, in fact treatment with an inhibitor lead to apoptosis, this effect is reverted with cotreatment with palmitic acid that is used downstream the block induced with the inhibitor in the FAS pathway.

These studies highlight the importance of glycolysis and fatty acid synthesis in KSHV infected cells and suggest new therapeutic approach based on the inhibition of glycolysis and FAS.

3. PI3K/AKT/mTOR pathway

PI3K/AKT/mTOR signaling is a central hub controlling many biological processes, including proliferation, differentiation, protein synthesis and apoptosis. Growth factor receptors activation recruits PI3K to the membrane where it converts PIP2 to PIP3. Binding of the pleckstrin homology (PH) domain to PIP3 colocalizes Akt and the phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane allowing PDK1 to phosphorylate Akt at T308. Phosphorylation at S473 by kinases such as mammalian target of rapamycin complex 2, mTORC2, or DNA-PK leads to full activation of Akt. mTOR kinase is part of two complexes, mTORC1 and mTORC2, with different biochemical structures and substrate specificity. Akt and mTORC1/2 are linked through a complex relationship, in which each one of the players regulates the other. While mTORC2 phosphorylates Akt at S473, mTORC1 is activated through direct phosphorylation of the tuberous sclerosis complex TSC1/2 by Akt. Moreover Akt phosphorylates the inhibitor of mTORC1, proline-rich Akt-substrate-40 (PRAS40), preventing the ability of PRAS40 to suppress mTORC1 signaling. mTORC1 is an energy sensor and a major regulator of protein synthesis through the phosphorylation and activation of the S6 ribosomal protein kinase (S6K) and the phosphorylation and inactivation of the repressor of mRNA translation initiation factor 4E binding protein 1 (4EBP1).

Aberrant activation of the PI3K/Akt/mTOR axis correlates with poor prognosis or resistance to conventional chemotherapy in several types of cancer, whereas Akt or mTOR inhibitors such as PP242, RAD001, perifosine, Akti 1/2 or its analog MK2206 significantly inhibit cell proliferation and promote apoptosis. As a consequence, a number of specific inhibitors of these signaling molecules are being developed, some of which are currently in clinical trials for solid or blood tumors in which the pathway is constitutively active.

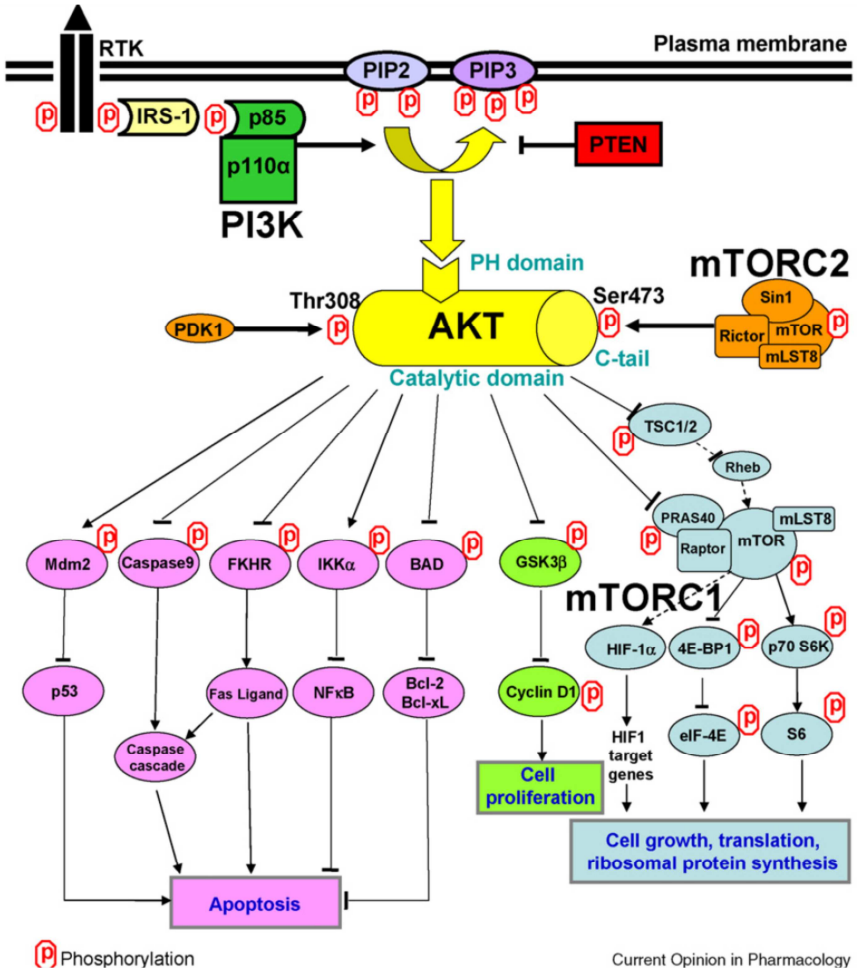


Fig. 3.1 Schematic representation of PI3K/Akt/mTOR pathway and its substrates. PI3K is activated in response to growth factors and promotes the activation of Akt through the phosphorylation of T308 by PDK1. The full activation of Akt is mediated by mTORC2. Akt promotes the activation of mTORC1 and regulates numerous cellular functions, promoting cell proliferation and blocking apoptosis (Yap TA, 2008)

3.1 PI3 Kinase

PI3Ks are a family of lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositols and phosphoinositides. PI3Ks are divided in three different groups (I-III) according to their structure and substrate specificity.

Class I PI3Ks phosphorylate phosphatidylinositol-4,5 bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5 triphosphate (PIP_3), that acts as a second messenger. They are divided in two subfamily, based on their activating receptor. Class IA PI3Ks are activated by growth factor stimulation through receptor tyrosine kinase (RTKs) (Zhao L, 2008) and consist of a p110 catalytic subunit and a p85 regulatory subunit. In mammals there are three different regulatory subunit isoforms, p85α, p85β and p55γ, and three catalytic isoforms p110α, p110β and p110δ. The *PIK3CA* gene that encodes the p110α catalytic subunit is frequently amplified or mutated in several human cancers, such as head and neck, cervical, gastric and lung cancers (Samuels Y, 2004). Class IB PI3Ks are activated by G protein-coupled receptors (GPCRs) and consist of a p101-regulatory subunit and a p110γ subunit. In mammals, class I PI3Ks regulate numerous important cellular pathways involved in glucose homeostasis, growth and survival.

Class II PI3Ks consist of a single catalytic subunit and are probably involved in the regulation of membrane trafficking and receptor internalization (Gaidarov I, 2001). These enzymes phosphorylates PI (phosphatidylinositol) and PI-4-P (phosphatidylinositol-4-phosphate) to generate PI-3-P and PI-3,4-P_2.

Class III PI3Ks, Vsp34 (vacuolar protein sorting 34) was first described as a component of the vacuolar sorting system in *Saccharomyces cerevisiae*. The homologue in mammalian cells, hVsp34 is associated to a regulatory p150 subunit and can act as a sensor in response to amino-acid availability and seems to regulate mTOR signal to regulate cell growth and autophagy in response to nutrient starvation (Nobukini T, 2005). Class III PI3Ks phosphorylate PI to generate PI-3-P.

Class IA have received much research attention as they can be activated in response to a variety of extracellular stimuli, including several growth factors and signaling compounds. These molecules bind to RTKs and induce their autophosphorylation; this event creates docking sites for the SH2 and SH3 domains of the PI3K regulatory subunit which in turn binds the catalytic subunit. In some cases recruitment of an adaptor protein is required. Activated PI3K catalyzes the formation of PIP_3, which is recognized by proteins containing a PH domain, such as Akt and PDK1, allowing them to be anchored to the plasma membrane. (DiNitto JP, 2003; Franke TF, 1997).

The AKT pathway can be inhibited at this step by the action of the tumorsuppressor protein phosphatase PTEN, which dephosphorylates PIP_3, at the 3' position of the inositol ring, which results in the generation of the biphosphate product PIP_2 (PtdIns(4,5)P2). PTEN is frequently

mutated in human cancer; its impaired function leads to PIP_3 accumulation and consequent activation of downstream proteins. (Maehama T, 1999).

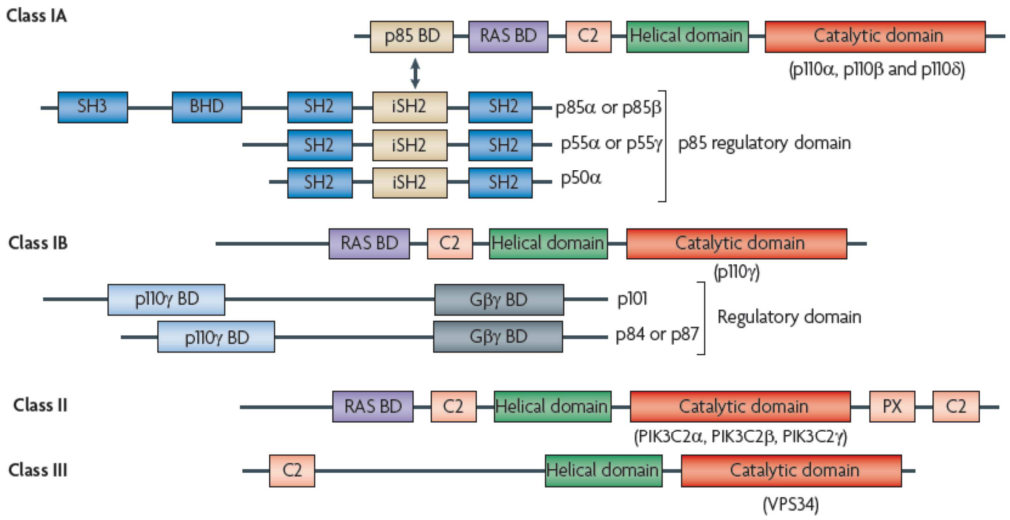


Figure 3.2 PI3K family. There's three different classes of PI3K. Class IA are heterodimers of a p110 catalytic and a p85 regulatory subunit. There are 3 isoforms of p110 (α,β and γ) and 3 isoforms of p85 (α,β and γ). Class IB are heterodimers composed by the p110γ catalytic subunit and p101 regulatory subunit. Class II are monomers with a single catalytic subunit, there's three classes of PI3K II: PI3KC2α, PI3KC2β and PI3KC2γ. Class III consists of a single catalytic subunit, VSP34. RAS BD: RAS GTPase binding domanis; p85 BD: p85 binding domain; BHD: BCR homology domain; C2 putative membrane binding domain; iSH2: inter-Src-homology 2 domain; Px: Phox homology (Liu P, 2009).

3.2 AKT/PKB

Akt (also known as protein kinase B, PKB) was originally discovered as the human homolog of the transforming gene v-Akt of the Akt8 provirus. (Staal SP, 1987). The Akt family comprises three closely related isoforms, Akt1/2/3, which have a highly conserved domain structure: a kinase domain, an N-terminal PH domain and a C-terminal regulatory tail containing a hydrophobic motif. However, despite their structural similarity, the three isoforms differ both in function and in levels of expression in different tissues: Akt1 has a wide tissue distribution and is involved in the regulation of cell growth and inhibition of apoptosis (Cho H, 2001); Akt2 is expressed preferentially in muscle and adipocytes and regulates the insulin signaling pathway (Garofalo RS, 2003); Akt3 is predominantly expressed in the brain, but its function is less clear (Yang ZZ, 2005).

Full Akt activation depends on two phosphorylation sites, one located in the catalytic domain (T308) and the second in the hydrophobic motif (S473). The rate limiting step in the process of Akt activation is the binding of its PH domain to the PIP_3 generated by activated PI3K. As PIP_3 is restricted to the plasma (or nuclear) membrane, this leads to Akt translocation to the plasma membrane. The binding induces a conformational change in Akt, which enables the co-recruited phosphoinositide dependent kinase 1 (PDK1), which also possesses a PH domain, to access

the activation loop and phosphorylate T308 (Calleja V, 2007). The complete activation of Akt occurs with the phosphorylation on S473 by the mTORC2 complex (Sarbassov DD, 2005). Once completely activated, Akt dissociates from the membrane and regulates downstream pathways through phosphorylation of many substrates. In addition to PTEN, other two phosphatases, protein phosphatase 2 (PP2) and PH-domain leucine-rich repeat phosphatase (PHLPP) turn off the Akt activity by dephosphorylating T308 and S473, respectively.

Akt is involved in the regulation of many cellular processes, including proliferation, apoptosis, angiogenesis and metabolism, through the phosphorylation of downstream substrates, which leads to their activation, inhibition, alteration of subcellular localization or protection from degradation.

Akt enhances cell survival by blocking different proapoptotic proteins. It has been reported that, in response to survival factors, Akt directly phosphorylates the pro-apoptotic BH3-only protein BAD, causing its binding to 14-3-3 proteins and its degradation; this prevents BAD interaction with prosurvival Bcl-2 family members, an event that promotes cell survival (Datta SR, 2000). With a similar mechanism, Akt blocks the activity of the Forkhead family of transcription factors (FOXO): Akt phosphorylates FOXO in the nucleus, creating a docking site for 14-3-3 proteins.Binding of 14.3.3 to phosphorylated FOXO leads to its cytoplasmic localization. Through this mechanism, Akt blocks FOXO-mediated transcription of target genes, such as the BH3-only family proteins and Fas-ligand, that promotes apoptosis (Brunet A, 1999). In addition, Akt phosphorylates the E3 ubiquitin ligase MDM2, promoting its translocation to the nucleus where it binds p53 and triggers its degradation (Mayo LD, 2001), blocking its proapoptotic activity. Akt is also demonstrated to directly phosphorylate pro-caspase 9, decreasing its *in vitro* activity (Cardone MH, 1999)

Another important function of Akt is the stimulation of cell proliferation. Akt phosphorylates the $p27^{Kip1}$ cyclin-dependent kinase inhibitor, leading to its cytosolic sequestration and prevention of its cell-cycle inhibiting effects (Viglietto G, 2002). Akt also inhibits the expression of $p27^{Kip1}$ by blocking FOXO (Burgering BM, 2003). Phosphorylation of $p21^{Cip1/WAF1}$ by Akt promotes p21 cytosolic localization and prevents the execution of its antiproliferative effect (Zhou BP, 2001). Akt causes the inactivating phosphorylation of GSK3, a protein that is involved in the regulation of glucose storage and also in cell cycle and apoptosis regulation. Active GSK3 phosphorylates cyclins D1 and E and the transcription factors c-Jun and c-myc, stimulating their translocation to the cytoplasm and proteasomal degradation. Akt-mediated GSK3 inactivation enhances the stability of these proteins, promoting cell-cycle progression (Welcker M, 2003). Furthermore GSK3 directly inhibits a prosurvival Bcl-2 family member, MCL-1, and GSK3 inhibition promotes the activity of this antiapoptotic protein (Maurer U, 2006). Akt is involved in the regulation of angiogenesis, through activation of the endothelial nitric oxide synthase (eNOS) (Fulton D, 1999), that stimulates vasodilation and angiogenesis through NO release. Akt regulation of cell growth and protein

synthesis, through mTORC1, and metabolism are described in detail in sections 3.3 and 3.4. (Figure 3.3)

Akt is one of the most frequently hyperactivated protein kinases in human cancers (Altomare DA, 2005). Due to the involvement of Akt in numerous key cellular pathways, the inhibition of Akt is proposed as a potential therapeutic strategy for human cancers.

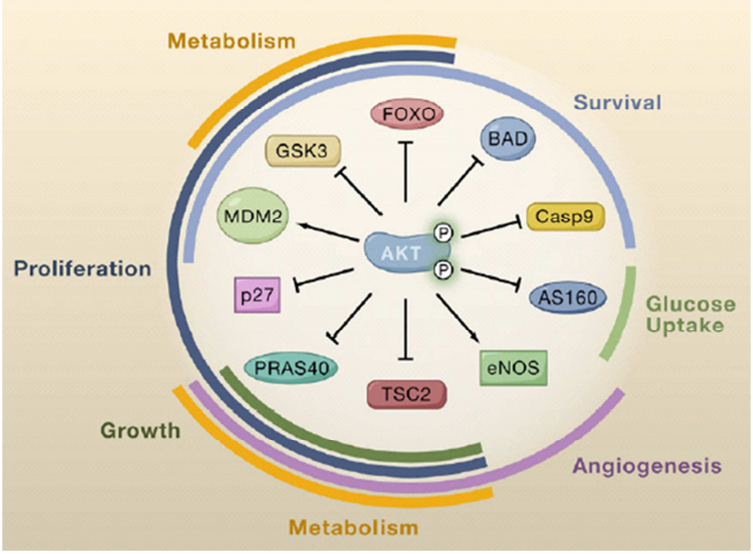


Figure 3.3. Akt pathway. Akt activates and inhibits numerous proteins, promoting proliferation, survival angiogenesis and glucose uptake (Manning BD, 2007)

3.3 mTOR

The target of rapamycin (TOR) was originally discovered in budding yeast, as the mediator of the toxic effect of the bacterial macrolide rapamycin (Kunz J, 1993), and only later the mammalian counterpart, mTOR, was identified (Sabatini DM, 1994). The mTOR protein is a 289-kDa serine/threonine kinase that belongs to the PI3K-related kinase family. The mTOR protein interacts with numerous proteins to form two distinct complexes: mTORC1 and mTORC2, with different functions (Figure 3.4).

mTORC1, also known as the rapamycin-sensitive complex, has five components: mTOR, that is the catalytic subunit, Raptor (regulatory associated protein of mTOR), mLST8 (mammalian lethal with Sec13 protein 8) also known as GβL, PRAS40 (proline-rich rich Akt substrate 40kDa) and Deptor (DEP-domain-containing mTOR interacting protein).

mTORC2 has six components: mTOR, mLST8 and Deptor, also common to mTORC1, Rictor (rapamycin insensitive- companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein) and Protor-1 (protein observed with Rictor-1) (Laplante M, 2013).

mTORC1 regulates multiple signaling pathways in the cell in response to growth factors, stress and amino acids. Once activated, it induces cell growth and stimulates protein and lipids synthesis,

whereas it blocks catabolic processes, such as autophagy. Rapamycin binds to the 12kDa FK506-binding protein (FKBP12) and interacts with the FKB12-rapamycin binding (FRB) domain of mTORC1. On the contrary, FKBP12 does not interact with mTORC2, which is therefore the rapamycin-insensitive complex (Sarbassov DD, 2004). One of the most important regulators of mTORC1 is the tuberous sclerosis complex (TSC). TSC is a heterodimer composed of TSC1 (also known as hamartin) and TSC2 (also known as tuberin), that acts as a GTPase-activating protein (GAP) for the small Ras-related GTPase Rheb (Ras homolog enriched in brain). The GTP-bound form of Rheb interacts with mTORC1 and stimulates its activity. The TSC1/2 complex negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state (Tee AR, 2003). The complex TSC1/2 is regulated in response to growtht factors, such as insulin and IGF1, that stimulates PI3K/Akt and Ras pathways. Activated Akt directly phosphorylates and inactivates TSC1/2, allowing Rheb to accumulate in a GTP-bound state and thus stimulating mTORC1 activity (Inoki K, 2002). Akt can also activate mTORC1 in a TSC1/2 independent manner through phosphorylation of the mTORC1 inhibitor PRAS40, which leads to its dissociation from Raptor. Moreover, PRAS40 is a substrate of mTORC1 itself, and it has been demonstrated that mTORC1-mediated phosphorylation of PRAS40 facilitates the removal of its inhibition on downstream mTORC1 signaling (Sancak Y, 2007). In addition to growth factors, other mechanisms regulate mTORC1 activity. AMPK, activated in response to energy depletion, phosphorylates TSC2, increasing its GAP activity, and reduces mTORC1 activation (Inoki K, 2003). In addition, AMPK can directly phosphorylate Raptor (Gwinn DM, 2008). mTORC1 is also regulated by REDD1 (regulated in developments and DNA damage responses 1) that in response to hypoxia activates TSC1/2 causing the inhibition of mTORC1 (Brugarolas J, 2004). Besides, amino acids, such as leucine and arginine, can activate mTORC1. The activation of mTORC1 in response to amino acids is mediated by the Rag GTP-ase proteins (Kim E, 2008). The Rag GTPase family consists of two subtypes, which associates to form eterodimers, RagA or RagB bound to RagC or RagD (Sekiguchi T, 2001). RagA/B-RagC/D eterodimers bind directly to Raptor in mTORC1 (Sancak Y, 2008). In the absence of amino acids RagA or B is loaded with GDP, preventing the activation of mTORC1. Expression of GTP-bound mutants of RagA or RagB renders mTORC1 resistant to amino acid starvation. In the presence of amino acids, Rag GTP-ases recruit mTORC1 to lysosome surface. In addition to Rag GTPases, the Ragulator complex mediates the binding of mTORC1 to lysosomes and the mTORC1 activation (Sancak Y, 2010).

Upon activation, mTORC1 regulates various cellular processes. One of the best defined is protein synthesis, required for cell growth. mTORC1 directly phosphorylates 4E-BP1 (eukaryotic initiation factor 4E (eIF4E)-binding protein-1), preventing its inhibitory binding to the eukaryotic translation initiation factor eIF4E, thereby promoting the cap-dependent translation of several proteins, such as c-Myc, cyclin D1, Cdk2, Rb protein, p27Kip1, VEGF and STAT3 (Richter JD, 2005). In addition,

mTORC1 phosphorylates S6K1 (S6 Kinase 1), which, through the regulation of numerous proteins, such as the ribosomal protein S6 (rpS6), leads to increased protein synthesis, cell size and cell cycle progression and is also involved in the regulation of glucose homeostasis (Ruvinski I, 2006). mTORC1 regulates lipid synthesis through a family of transcription factors, SREBP1/2 (sterol regulatory element binding protein 1/2), which control the expression of genes involved in fatty acid and cholesterol synthesis (Porstmann T, 2008). In addition, mTORC1 blocks autophagy through the phosphorylation and inhibition of ULK1/Atg13/FIP200, a kinase complex necessary to start autophagy (Ganley IG, 2009). mTORC1 increases mitochondrial DNA content and genes involved in oxidative metabolism, promoting mitochondrial biogenesis and oxidative function (Cunningham JT, 2007). Several studies demonstrate the existence of a regulatory feedback mechanism of the PI3K/Akt/mTOR pathway through inhibition of IRS1 (insulin receptor substrate 1). mTORC1-activated S6K phosphorylates IRS1 (Harrington LS, 2004), reducing its stability and inhibiting its binding to RTKs (insulin/IGF-1 receptor tyrosine kinase), a necessary event for the recruitment and activation of PI3K (Manning BD, 2004).

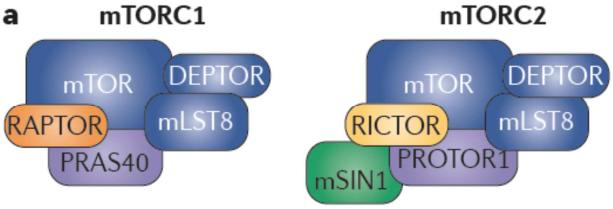


Figure 3.4 mTOR complexes. Both mTOR complexes contain mTOR, DEPTOR (DEP-domain containing mTOR-interacting protein) and mLST8 (mammalian lethal with SEC13 protein 8, also known as GβL). mTORC1 contains RAPTOR (regulatory-associated protein of mTOR) and PRAS40 (proline-rich Akt substrate 40 kDa). mTORC2 contains RICTOR (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated MAP kinase-interacting protein 1) and PROTOR (protein observed with RICTOR) (Bovè J, 2011)

mTORC2 is less characterized than mTORC1. When activated, in response to growth factors, mTORC2 controls the activity of several members of the AGC family, including SGK1, PKCα and Akt. mTORC2 phosphorylates Akt at T308, required for its full activation. (Sarbassov DD, 2005). In addition, knock down of mTORC2 leads to an impairment of cytoskeletal organization, affecting actin polymerization and altering cell morphology (Jacinto E, 2004).

Disregulation of the mTOR pathway is involved in tumor pathogenesis, and rapamycin and many rapamycin analogs (rapalogs) are currently in clinical trials for the treatment of cancer.

3.4 PI3K/AKT/mTOR pathway and metabolism

The PI3K/AKT/mTOR pathway, frequently altered in cancer, is involved in the regulation of tumor cells metabolism. It can regulate cell metabolism by influencing the expression of glucose transporters; the activity of metabolic enzymes, such as PFK2 and HXK-II and the activity of transcription factors that regulate the expression of metabolic enzymes.

3.4.1 Akt Regulation of Glucose Transporters

One of the most important effect of Akt is the stimulation of glucose uptake in response to insulin. It was demonstrated that, in response to insulin, Akt2 binds the GLUT4-trasporting vesicles (Calera MR, 1998). In addition, the constitutive active form of Akt, myr-Akt, stimulates the expression and membrane translocation of GLUT4 (Kohn AD, 1996). The exact mechanism by which Akt stimulates GLUT4 translocation is not well defined. The Rab-GTPase AS160 is involved in this process, in fact it is directly phosphorylated by Akt and is required for GLUT4 translocation to plasma membrane (Mîinea CP, 2005). Also GLUT1, that is the predominant glucose transporter in many cell types is regulated by Akt. In mouse epatoma cells, Akt, in response to insulin or when constitutively active, stimulates the expression of GLUT1 (Barthel A,1999). Akt substrate GSK3β might mediate this effect. Indeed overexpression of GSK3β causes a reduction of glucose uptake due to phosphorylation of TSC2, that in turn inhibits mTORC1. Akt phosphorylates GSK3β causing its inactivation and blocking its inhibitory effect on mTORC1 (Buller CL, 2008).

3.4.2 Akt regulation of glycolysis

3.4.2.1 Hexokinase II (HKII)

Akt stimulates the activity and the association to mitochondrial membrane of hexokinase II (HKII), that catalyzes the first step of glycolysis. This association prevents apoptosis, preserving mitochondrial potential; the effect of Akt is glucose dependent. In the presence of active Akt, there's an increase of intracellular ATP levels (Gottlob K, 2001). HKII, associated to the mitochondrial membrane, can interact with VDAC (voltage-dependent anion channel) blocking the opening of the channel, and maintaining mitochondrial potential (Chiara F, 2008). This effect blocks the release of cytochrome c, preventing the apoptotic cascade. In addition, mtHK competes with Bax and Bak binding and oligomerization at outer mitochondrial membrane (OMM), acting as antiapoptoic signal (Majewski N, 2004). The exact mechanism whereby Akt promotes mtHK association is not well defined. HKII downstream products, glucose 6-phosphate (G6P) and glucose 1,6-bisphosphate (G1,6 P_2), inhibit the association of HKII to mitochondrial membrane with a feedback mechanism: Akt promotes glycolysis and increases the glycolytic flux, preventing the accumulation of G6P and

$G1,6P_2$ and favoring HKII mitochondrial association (Wilson JE, 1995). Furthermore, Akt inhibits GSK3β, thus preventing phsphorylation of VDAC and mtHK binding (Pastorino JG, 2005).

3.4.2.2 Phophofructokinase-2 (PFK-2)

Akt is known to phosphorylate and activate phosphofructokinase-2 (PFK2), favoring the glycolytic rate. Indeed, PFK2 catalyzes the synthesis of fructose 1,6 bisphosphate ($F1,6P_2$), the most potent activator of PFK1 (phosphofructokinase-1), one of the rate limiting enzymes of glycolysis (Deprez J, 1997)

3.4.2.3 Transcription factors (FOXO)

Akt regulates glycolysis also thorugh inhibitory phosphorylation of the family of the transcription factors FOXO. Activated FOXO1 regulates circulating glucose by promoting gluconeogenesis and represses expression of glycolytic enzymes (Zhang W, 2006). Moreover, active FOXO3a promotes the expression of TSC1, inhibiting mTORC1, whereas knockdown of FOXO3a promotes glycolysis. Notably, the same effect was obtained by Akt-mediated inhibition of FOXO3a (Khatri S, 2010).

3.4.3 Akt regulation of lipid synthesis

Akt regulates lipid synthesis through the direct phosphorylation of ACL (ATP-citrate lyase), a cytosolic enzyme that catalyzes the formation of oxaloacetate and acetyl-CoA from citrate in an ATP-dependent. Acetyl-CoA is then converted to malonyl-CoA, and conveyed towards the synthesis of fatty acylCoA by fatty acid synthase (Berwick DC, 2002).

Transgenic mice lacking PKBβ/AKT2, the prevalent AKT isoform in adipose tissue, showed altered glucose tolerance and are insulin-resistant and diabetic, giving the evidence of the importance of AKT in the regulation of metabolism (Cho H, 2001). The expression of myr-Akt can also promote the expression of GLUT1 on plasma membrane, HK activity and PPP shuttle, while prevents the proapoptotic function of the Bax protein (Rathmell JC, 2003).

In pre-B cell lines the conditional expression of Akt or c-myc triggers the Wargburg Effect, as indicated by increased cellular glucose uptake, lactate production and glycolysis. Akt and c-myc seem to regulates different functions: Akt sensitizes cells to apoptosis, when treated with glycolysis inhibitor, whereas c-Myc does not. In contrast, c-myc, but not Akt, sensitizes cells to the inhibition of mitochondrial function (Fan Y, 2010).

In immortalized leukemic cells and in glioblastoma cell lines Akt was clearly demonstrated to play a role in the induction of aerobic glycolysis. In these models, Akt induces a dose-dependent stimulation of glycolysis, that correlates with a more aggressive malignancy in vivo. The expression of the constitutive active form of Akt (myr-Akt) causes an increase of glucose consumption and

lactate production. Furthermore, Akt renders cells dependent on glycolysis: the inhibition of the PI3K pathway, with LY-294002, induces a reduction in glucose metabolism (Elstrom RL, 2004).

3.4.4 Role of mTORC1

The most important target of Akt is mTORC1 that mediates the effect on cell proliferation and metabolism. Based on a genomic approach, was reported that mTORC1 induces genes involved in metabolic pathways, including PPP, glycolysis, fatty acid and cholesterol biosynthesis. $TSC2^{-/-}$ cells show a rapamycin-sensitive increase in glucose uptake, lactate production and de novo lipid synthesis. The effects of mTORC1 on metabolism, are mediated by the stimulation of transcription factors SREBP1, c-myc and HIF1α (Duvel K, 2010). mTORC1 promotes also the transcription of GLUT1, due to its effect on cap-dependent translation (Taha C, 1999). SREBPs (sterol-regulatory element binding proteins), are master transcriptional regulators of genes involvedin de novo lipid and sterol biosynthesis genes (Krycer JR, 2010).

3.5 PI3K/AKT/mTOR pathway inhibitors

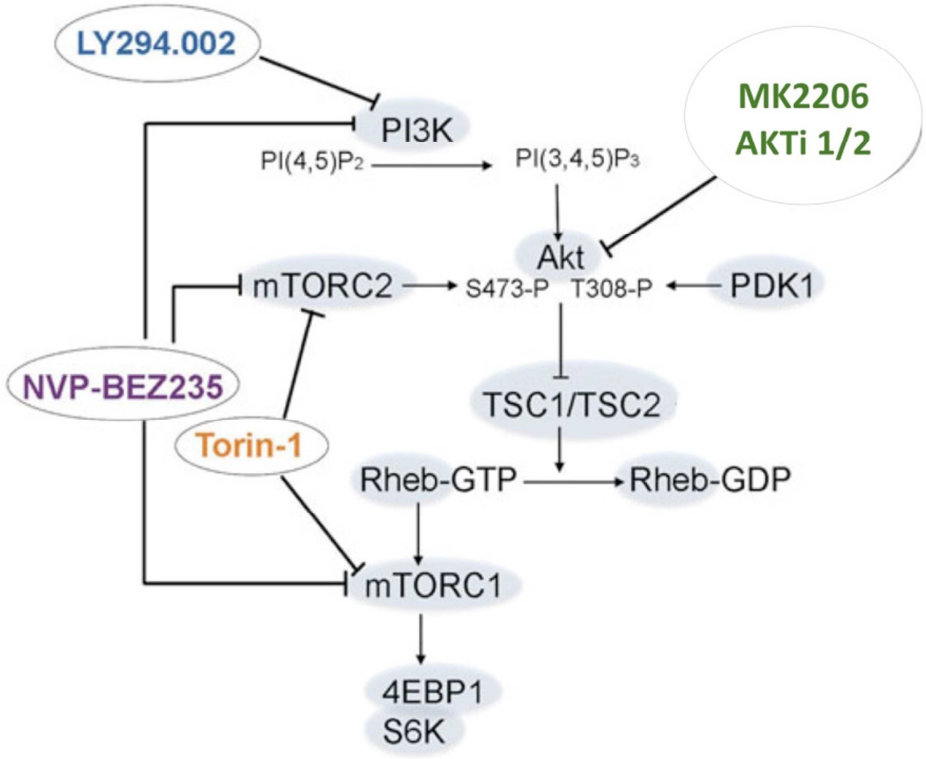


Figure 3.5 Targets of PI3K/Akt/mTOR pathway inhibitors: Akti 1/2 and MK2206, inhibits Akt. Ly294-002 inhibits PI3K; Torin-1 blocks the two mTOR complexes; NVP-BEZ235 is a dual inhibitor of the two mTOR complexes and PI3K.

3.5.1 LY294002

LY294002 is a reversible ATP competitive PI3K inhibitor, which was derived from the flavonoid quercetin (Vlhaos CJ, 1994). LY294002 has been extensively used *in vitro*, over 500 studies in last years, to evaluate the role of PI3K/Akt pathway in normal and transformed cells. Inactivation of PI3K using LY294002 has been demonstrated to inhibit Akt activity. LY294002 treatment abolishes the phosphorylation of S473, and blocks the phosphorylation of the Akt substrates GSK3 and p27 (Shin I, 2002). LY294002 inhibits cell growth and induces apoptosis both *in vitro,* in colon cancer cell lines, and in mouse xenografts, where LY294002 administration resulted in suppression of tumor growth (Semba S, 2002). LY29002 can enhance the efficacy of chemotherapeutic agents in several cell models, such as myeloid leukemia (O'gorman DM, 2001) and pancreatic tumors (Ng S, 2000). Another study demonstrated that LY294002 can enhance the efficacy of paclitaxel in a murine xenograft model of ovarian cancer (Hu L, 2002). Taken together, these studies, indicate the importance of the PI3K/AKT pathway in the regulation of chemotherapy resistance. Despite its widespread adoption as a biochemical tool to block PI3K signaling, LY294002 can also inhibit mTOR (Brunn, GJ 1996), and the caseine kinase 2 (CK2) (Davies SP, 2000) with comparable EC50s.

Although LY294002 is unsuitable for patient use, its backbone structure has been used in the design of novel PI3K inhibitors. SF-1126 is a LY294002 prodrug that is conjugated to a tetra-peptide designed to target tumor vasculature; this compound has demonstrated efficacy in solid tumors xenograft models and is currently in phase I clinical study (Mahadevan D, 2012).

3.5.2 Akti1/2 (Akt inhibitor VIII)

Akti1/2 is a potent, reversible and allosteric inhibitor of Akt1 and Akt2, with minimal activity against Akt3 (Lindsley, CW 2005). Akti1/2 has minimal activity against protein kinases, with the exception of CAMK (Gilot D, 2010). Akti1/2 binds to the Akt PH domain, but not to the ATP binding site (Calleja V, 2009). Akt inhibition is dependent upon a solvent-exposed tryptophan residue (Trp90) in the PH domain; mutation of this residue to alanine makes Akt resistant to Akti1/2 treatment. Akti1/2 treatment abolishes the phosphorylation of Akt in both S473 and T308, and blocks glucose uptake, (Green C, 2008), but does not influence glutaminolysis (Wise DR, 2008).Inhibition of glucose uptake may occur *via* a direct inhibition of glucose transporters, GLUT1 and GLUT4; this effect was found to be independent of the ability of Akti1/2 to inhibit Akt (Tan SX, 2010). LNCaP cells pretreated with Akti1/2 are sensitized to TRAIL treatment with an increase of caspase-3 activity when compared to TRAIL alone (DeFeo Jones D, 2005). Akti1/2 inhibit the phosphorylation of Akt1 and Akt2 *in vivo*, with no effect on Akt3 activity. Akti1/2 enhances apoptosis induced by doxorubicin and camptothecin, and inhibits IGF-1 stimulated Akt phosphorylation in mice (Cherrin C, 2010). The inihibitor is more effective in the induction of caspase-3 activation in tumor cells, compared to

HUVEC, normal prostate epithelial or normal human mammary epithelial cells (DeFeo Jones D, 2005). Akti1/2 induces apoptosis also in lymphocytic leukemia cells (DeFrias M, 2009). This compound is used only in vitro, due to its poor solubility and pharmacokinetics (Barnett SF, 2005) and also for the potential risk of thrombosis, due to the potentiation of platelet aggregation (Hunter RW, 2008).

3.5.3 MK2206

MK2206 is a highly selective allosteric inhibitor of Akt1, Akt2 and Akt3 with IC_{50} values of 8nM, 12nM and 65nM, respectively. MK2206 blocks phosphorylation of Akt and its downstream substrates, including TSC2, PRAS40 and GSK3. MK2206 acts in synergism with different chemotherapeutic agents, both *in vitro* and *in vivo* (Hirai H, 2010). The Akt pathway is frequently hyperactivated in hepatocellular carcinoma (HCC) and treatment with MK2206 of HCC cells, decreased cell viability and induced cell cycle arrest. In addition, in HCC cells MK2206 acts in synergism with doxorubicin (Simioni C, 2013), and is also more effective when used in combination with the mTOR inhibitor RADD1 (Grabinski N, 2012). Similar results were obtained in neuroblastoma, were MK2206 in combination with etoposide caused a more significant decrease in tumor growth and increased murine survival, when compared with MK2206 alone (Li Z, 2012). The Akt pathway is also hyperactivated in hypoxic conditions in head and neck cancer cell lines and, accordingly, MK2206 is more effective in hypoxic conditions in the treatment of this tumor (Stegeman H, 2012). Compared to Akti1/2, MK2206 is more potent and more specific, as it does not block glucose transporters independent from Akt (Tan S, 2011). MK2206 has recently entered phase I clinical trial in patients with advanced solid tumor it is well tolerated with evidence of Akt signaling disruption (Yap TA, 2011).

3.5.4 NVP-BEZ235

NVP-BEZ235 is a dual PI3K/mTOR inhibitor currently under investigation in several phase I/II clinical trials. This inhibitor binds the ATP-binding clefts of PI3K and mTOR kinase, thereby inhibiting their activities (Maira SM, 2008). In breast cancer cells, NVP-BEZ235 blocked the activation of Akt, S6 and 4E-BP1, and reduced the number of viable cells in a dose-dependent manner. NVP-BEZ235 is also more effective compared to the allosteric selective mTOR inhibitor everolimus. NVP-BEZ235 was further studied in a xenograft model of breast cancer, resulting in suppression of tumor growth (Serra V, 2008). The efficacy of NVP-BEZ235 was demonstrated in numerous cancer cells lines, including myeloma (Baumann P, 2009), rabdomiosarcoma, osteosarcoma, Ewing's sarcoma and primary effusion lymphoma. The efficacy of NVP-BEZ235 in sarcoma was demonstrated both *in vitro* and *in vivo*, and the combination with conventional cytotoxic agents, such as vincristine, potentiates its antimetastatic effect (Manara MC, 2010). When

used in combination with melphalan, doxorubicin and bortezomib against multiple myeloma cells, NVP-BEZ235 showed synergistic or additive effect on cell growth inhibition (Baumann P, 2009). In addition, NVP-BEZ235 is effective in the treatment of pancreatic cancer in mouse models (Cao P, 2009) and radiosensitized NSCLC cells harboring K-Ras mutation (Konstantinidou G, 2009). Administration of NVP-BEZ235 strongly inhibits the growth of PEL cells both *in vitro* and in a xenograft model, blocking multiple autocrine and paracrine growth factors required for the survival of lymphoma cells (Bhatt AP, 2010). Through its effects on transcription, NVP-BEZ235 blocks the expression of HIF1α in hypoxic conditions, resulting in a more potent inhibition of cell growth under hypoxia than at normal atmospheric O_2 (Karar J, 2011). Due to the importance of PI3K/AKT/mTOR pathway in the regulation of metabolism, it has been demonstrated that NVP-BEZ235 treatment can alter the whole-body glucose metabolism in mice, indicating that these effects should be monitored in clinical trials (Smith GC, 2012). A phase I, multicenter, single-agent, dose escalation trial of NVP-BEZ235 showed that it is active and well tolerated in patients, especially in those with PI3K pathway dysregulated tumors (Burris H, 2010). These data suggest the potential of this compound in the treatment of numerous tumors, alone or in combination with chemotherapeutic agents.

3.5.5 Torin-1

Torin-1, a pyridonequinoline compound discovered from a biochemical screen for mTOR inhibitors, was identified as a potent and selective ATP-competitive mTOR kinase inhibitors (Liu Q, 2010). Torin-1 inhibits phosphorylation of mTORC1 and mTORC2 substrates, S6K1 and Akt, at concentrations of 2 and 10nM, respectively, as demonstrated in mouse embryonic fibroblasts (MEFs). Torin-1 selectivity towards mTOR has been demonstrated by its evaluation against a broad panel of different kinases (Liu Q, 2012). Torin-1 showed a greater inhibitory effect on cell growth, proliferation and 4E-BP1 phosphorylation than rapamycin, inducing G1/S cell cycle arrest in MEFs (Thoreen CC, 2009). The more potent effect of Torin-1, compared to rapamycin, is probably due to the suppression of rapamicyn insensitive functions of mTORC1 rather than to its ability to inhibit mTORC2, as also mTORC2-deficient MEFs are more sensitive to Torin-1 than rapamycin. Torin-1 inhibits the growth in a U87MG (PTENnull glioblastoma cell line) xenograft model and administration of Torin-1 for 10 days resulted in greater than 99% inhibition of tumor growth. Upon cessation of treatment, however, the tumor resumed growth, suggesting a cytostatic effect of Torin-1 (Liu Q, 2010). The greater efficacy of Torin-1 compared to rapamycin suggests the potential of this compound in the treatment of cancer patients.

3.6 AKT pathway in PEL

The PI3K/Akt pathway is activated in PEL cells and is one of the driving forces responsible for their aggressive phenotype. A major role in the hyperactivation of this pathway is played by KSHV viral proteins such as K1 and vGPCR.

Transgenic K1 mice develop tumors with similar characteristics of KS and PEL (Prakash O, 2002). In B lymphocytes, ectopic expression of K1 activates the PI3K/Akt pathway, inducing the phosphorylation of Akt on T308 and S473 and the inactivation of PTEN, which results in the activation of downstream substrates. Active Akt phosphorylates the FKHR transcription factors, blocking the transcription and expression of FAS-ligand, thus blocking FAS-mediated apoptosis. LY294002 treatment abolishes Akt activation and phosphorylation of its substrates, GSK3β and FKHR, and induces apoptosis. This suggests that hyperactivation of the pathway is mediated by PI3K (Tomlinson CC, 2004) and confirms the importance of PI3K/Akt pathway for PEL cells survival (Uddin S, 2005). K1 also stabilizes Akt through the interaction with the cellular chaperones heat shock protein 90 β (HSP90β), that is important for the sustained activity of Akt; inhibition of HSPs induce apoptosis in cells expressing K1 (Wen KW, 2010 (2)). K1 was demonstrated to activate PI3K/Akt/mTOR pathway also in endothelial cells (Wang S, 2007).

Another viral protein involved in PI3K/Akt activation in PEL is vGPCR. vGPCR has transforming potential, as cells expressing vGPCR induce tumor formation in nude mice (Bais C, 1998). In addition, vGPCR promotes endothelial cell survival though the activation of Akt and promotes the production of VEGF, which protects cells from apoptosis induced by serum-starvation, Akt mediates this effect (Montaner S, 2001). vGPCR phosphorylates TSC2, promoting mTOR activation, and phosphorylates and activates Akt.

The inhibition of mTOR with rapamycin prevents vGPCR induced sarcomagenesis *in vivo*, confirming the importance of the PI3K/Akt/mTOR pathway for KSHV tumorigenic potential and suggests possible therapeutic application of rapamycin in the treatment of KSHV associated tumors (Sodhi A, 2006). In addition, rapamycin is efficacious against PEL cells in cell culture and in a murine xenograft model. Rapamycin treatment inhibits the mTOR signaling pathway, blocks the phosphorylation of S6K, and blocks the production of IL-10, an autocrine signal that sustains PEL cells growth *in vivo*. The addition of exogenous IL-10 or IL-6 reverses rapamycin induced growth arrest (Sin SH, 2007). The efficacy of rapamycin in the treatment of PEL, however, was not confirmed in patients: two renal transplant recipients receiving rapamycin were not protected from PEL occurrence or progression (Boulanger E, 2007). One explanation is probably that rapamycin has a cytostatic rather than cytotoxic effect. Treatment with rapamycin of a PEL xenograft model initially reduces the production of VEGF and the secretion of IL-10, but prolonged treatment causes a new increase in IL-10 levels, although rapamycin remains active, as demonstrated by the absence of phosphorylated S6K. This effect is probably mediated by the hypoxic environment in

PEL ascites, and the increased IL-10 expression represents a resistance mechanism to rapamycin treatment (Gasperini P, 2009). Combination treatment may represent a possible strategy to overcome the induction of resistance. By targeting both PI3K and mTOR, NVP-BEZ235 effectively arrests PEL cells growth *in vitro* and *in vivo* and inhibits the secretion of IL-10 and IL-6, blocking both autocrine and paracrine proliferative loops. NVP-BEZ235 was found to be more effective than perifosine, which blocks only PI3K (Bhatt AP, 2010). The efficacy of the cotreatment was confirmed in another study, where combinations of NFkB and PI3K/Akt pathway inhibitors were reported to have synergistic effect on PEL cells (Hussain AR, 2012).

4. Hypoxia

Hypoxia is a condition characterized by lower oxygen tension in comparison with the normal level. Hypoxia occurs in some physiological processes, such as embryonic development, wound healing and in the maintenance of stem cells pluripotency, and in pathological events, such as ischemia. In addition, most solid tumors contain hypoxic regions because of tumor microvessels impairment (Koh MY, 2012).

4.1 Relevance of hypoxia in tumors

Hypoxia is toxic for both normal and cancer cells, but cancer cells undergo genetic and adaptive changes that allow them to survive and proliferate in hypoxic conditions.

The microenvironment of solid tumors is reported to be hypoxic, and up to 50-60% of locally advanced tumors (breast, head & neck, prostate, pancreas, non-Hodgkin's lymphomas, malignant melanomas, metastatic liver tumors) may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumor mass (Vaupel P, 2007). There are different types of hypoxia in tumors:

- perfusion-related (acute), that is caused by inadequate blood flow in tissues, and is the consequence of functional and structural abnormalities of tumors microvasculature. This type of hypoxia is frequently transient, as demonstrated by the measurement of oxygen tension in human tumor xenografts (Bennewith KL, 2004).
- diffusion-related (chronic) caused by an increased distance from blood vessels due to tumor expansion, cells that are distant (>70μm) from a nutritive blood vessel receive less oxygen and nutrients than required
- anemic hypoxia that is the consequence of reduced oxygen transport capacity of the blood due to tumor-associated or therapy-induced anemia (Vaupel P, 2004).

Tumors hypoxia is highly heterogeneous and occurs with different magnitudes and periodicities in different regions of the same tumor (Bennewith KL, 2004). This heterogeneity may represent an advantage for tumor proliferation: oxygenated and not-oxygenated tumor cells can behave as a metabolic symbionts. Hypoxic tumor cells relay on glycolysis and produce excess of lactate that is secreted through the MCT4 transporter. Lactate can enter the oxygenated tumor cells, through MCT1, and it is then converted into pyruvate that fuels TCA cycle for ATP production. This mechanism allows tumor hypoxic cells the availability of high glucose levels (Figure 4.1)

Tumor hypoxia promotes both tumor progression and resistance to therapy. In addition to promoting tumor cells survival, by shifting cells toward an anabolic metabolism, neovascularization and resistance to apoptosis, hypoxia promotes other responses that contribute to tumor aggressiveness, such as increased tumor instability, invasion and

metastasis; all these effects are largely mediated by the activation of HIF1α (Wilson WR, 2011). Elevated levels of HIF1α, that is induced both in response to hypoxia or in response to oncogenic mutations, are associated with poor prognosis and tumor progression (Zhong H, 1999). Also high levels of HIF2α are associated with tumor proliferation, and inhibition of HIF2α prevents the *in vivo* growth and tumorigenesis of highly aggressive glioblastoma, colorectal, and non-small-cell lung carcinomas (Franovic A, 2009). Another important aspect is that hypoxia affects cellular DNA and chromosomes, promoting cell transformation. Hypoxia has been shown to induce DNA breaks and to disrupt repair of DNA damage (Yuan J, 2000), favoring the accumulation of mutations and promoting tumor development.

Several studies reported that tumor hypoxia is associated with poor prognosis, for example Flyes reported that hypoxia is a statistically significant adverse prognostic factor of disease free-survival (Flyes AW, 1998). Another study, reported a poor outcome associated with low oxygen tension in advanced carcinomas of uterine cervix (Sundfor K, 2000). More recently, it was reported that low oxygen tension in tumors was associated with increased metastasis and poor survival in different types of tumor (Vaupel P, 2007).

In addition to influencing tumor development and progression, hypoxia plays an important role in the response to anticancer treatment. Hypoxic cells are more resistant to classical chemotherapy and radiotherapy. Several studies reported that hypoxia has a negative impact on radiotherapy treatment in tumor cells of various cancers, such as head and neck (Brizel DM, 1999) and uterine cervix (Hockel M, 1996). In addition, *in vivo* experiments demonstrated that hypoxic cells are resistant to chemotherapy (Brown JM, 1999). One possible explanation of chemoresistance is that hypoxic cells proliferate at a slower rate than cells normally targeted by anticancer drugs; a high rate of proliferation is required for many chemotherapeutics to exert their maximum effect. Possible therapeutic strategies are based on the inhibition of the hypoxic status in the tumor, promoting oxygen supply; or on the use of drugs with a more pronounced effect in hypoxic than in normoxic conditions. In 1993 Brown (Brown JM, 1993) demonstrated that the prodrug tirapazamine (TPZ) is 50-200 fold more toxic to hypoxic than normoxic conditions. Under hypoxia TPZ is reduced to a highly reactive radical that produces strand breaks in the DNA; on the contrary, under aerobic conditions, the radical is modified to a non-toxic compound with the concomitant production of less-toxic superoxide radical (Wang J, 1992). Combination of TPZ with radiation has a demonstrated synergistic effect on tumors (Latrigau E, 1996). In addition, TPZ acts in synergism with cisplatin, giving the evidence that TPZ has the ability to chemo-sensitize quiescent cells (Masunaga S, 2000). Phase II clinical trials demonstrated that TPZ can improve outcome in advanced carcinomas when combined with cisplatin (Treat J, 1998). Phase III clinical trials showed contradictory results, indicating the need of further studies on the administration of TPZ in combination with chemo or radiotherapy.

Because hypoxic cells depend on glycolysis the inhibition of glycolysis may represent a possible therapeutic strategy for tumor treatment.

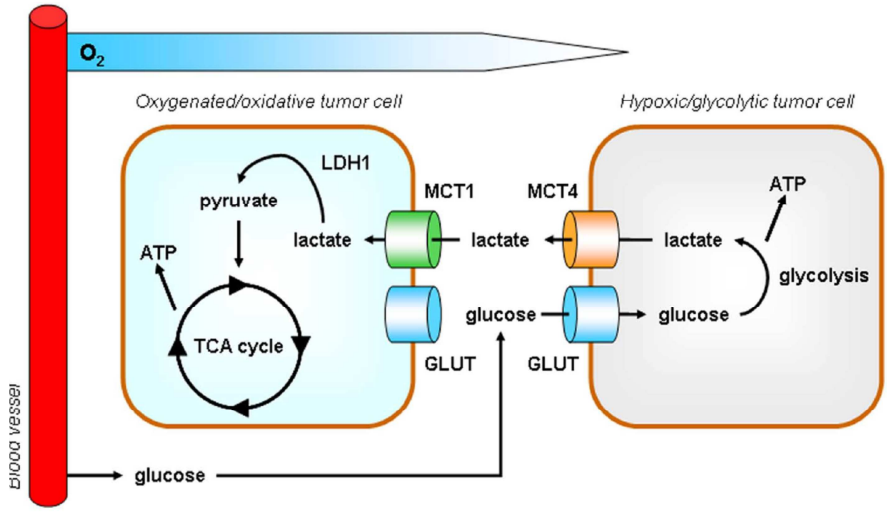


Figure 4.1 Metabolic symbiosis between oxygenated and hypoxic tumor cell. Tumor cells distant from blood vessels grow in hypoxic environment. These cells relay on glycolysis and produced large amount of lactate. Lactate is exported by MCT4. The lactate can then enter in oxygenated cells through MCT1, lactate is oxidized to pyruvate and fuels TCA to produce ATP by oxidative phosphorylation. The use of lactate by oxidative cells allow tumors cells to preserve high levels of glucose that is fundamental for hypoxic tumor cells survival (Porporato PE, 2011).

4.2 Cellular responses to hypoxia: HIF1α and REDD1

4.2.1 HIF1α stabilization

Cells respond to hypoxia through adaptations of cell growth, metabolism and induction of angiogenesis. The most important survival factor expressed by cell in hypoxic condition is the transcription factor hypoxia-inducible factor-1 (HIF1). HIF1 is a heterodimeric transcription factor composed by a constitutively expressed subunit HIF1β, and HIF1α or HIF2α as the O_2 responsive subunit (Jiang BH, 1996). When stable, HIF1α (or HIF2α) dimerizes with HIF1β through their respective N-terminal domains; the heterodimer acts as a transcription factor binding to hypoxia-responsive elements (HREs) in the promoter of target genes, thereby activating the expression of numerous genes, inducing changes in metabolism and proliferation. In well oxygenated cells (21% O_2), HIF1α displays a very short half-life of less than 5 minutes (Berra E, 2001).

At atmospheric O_2 levels HIF1α is not stable, as prolyl hydroxylase domain proteins (PHDs), that are active in the presence of oxygen, hydroxylate prolines 402 and 564 of HIF1α (Bruick RK, 2001). The hydroxylated prolines are recognized by the Von Hippel-Lindau (VHL) factor , which acts as an E3 ubiquitin ligase that targets HIF1α for proteasomal degradation (Maxwell PH,

1999). In hypoxia, PHDs are not active, as for their catalytic activity they require oxygen as co-factor, so HIF1α is not hydrolxylated and becomes stabilized.

Another mechanism of regulation is mediated by the hydroxylase FIH (factor inhibiting HIF1α), that hydroxylates an asparagine residue (A803) of HIF1α and HIF1β in the carboxy-terminal transcriptional activation domain (C-TAD). This modification blocks the interaction of HIF1α with the transcriptional activator CBP/p300 leading to transcription repression (Lando D, 2002). (Figure 4.2)

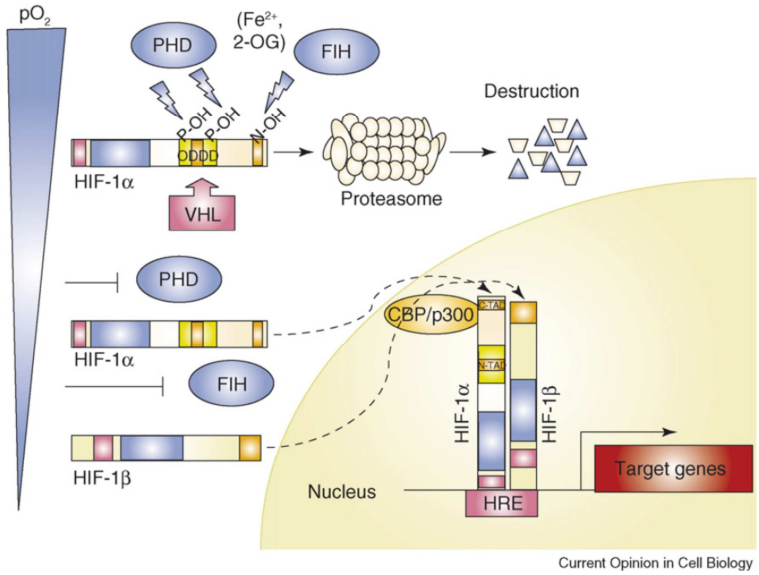


Figure 4.2 O_2 pressure and HIF1α stability. Under normoxic conditions, PHD (prolyl hydroxylase) and FIH (factor inhibiting HIF1α) are functional. PHD hydroxylates two proline residues in the ODD (oxygen-dependent degradation domain) and FIH hydroxylate asparagine in the C-TAD domain (C-terminal activation domain). Prolyl hydroxylation promotes the binding of VHL (Von-Hippel Lindau) promoting HIF1α degradation. Asparagyl hydroxylation promotes the interaction of with CBP/p300 co-activators. With low oxygen concentration PHD and FIH are inactive. HIF1α is stable and can dimerize with HIF1β and binds to HRE (hypoxia responsive element) of target genes (Brahimi-Horn MC, 2007)

4.2.2 HIF1 target genes

The role of HIF1α *in vivo* was evaluated in knockout animals, where the inactivation of HIF1α led to embryonic lethality due to abnormal vascular development (Ryan HE, 1998). In addition HIF1α is important for the functional integrity and antimicrobial defense capacity of the immune system (Peyssonnaux C, 2007) and also for osteoblast development (Wang Y, 2007).

HIF1 promotes the transcription of glucose transporters GLUT1 and GLUT4, increasing glucose uptake (Maxwell PH, 1999), and promotes the expression of glycolytic enzymes (Semenza GL, 2010), enhancing the use of glucose in glycolysis, increasing the glycolytic rate for the production of sufficient energy for the survival of cells in the hypoxic condition. High rates of glycolysis produces a huge amount of pyruvate that needs to be removed; to this purpose HIF1

activates LDHA that catalyzes the conversion of pyruvate and NADH to lactate and NAD^+ (Iyer NV, 1998). In addition, HIF1 promotes the expression of the transporter MCT4 (Ullah MS, 2006) which export lactate to the extracellular matrix, causing acidification of the tumor ambient that may favor tumor invasion (Gatenby RA, 2006). Carbonic anidrase 9 (CAIX) is another HIF1 target; its increased expression contributes to the acidification of the extracellular ambient (Chiche J, 2009). Lactate produced by hypoxic cells can be taken up non-hypoxic cells and used as a respiratory substrate (Sonveaux P, 2008). Another important metabolic enzyme stimulated by HIF1 is the kinase PDHK1, that catalyses the inactivating phosphorylation of PDH, thereby favoring the conversion of pyruvate into lactate, and reducing the TCA flux and oxidative phosphorylation. The mitochondrial function is reduced also by the induction of BNIP3 that promotes mitochondrial autophagy (Zhang H, 2008).

Aside from proteins involved, another important factor upregulated in hypoxia is vascular endothelial growth factor (VEGF), that stimulates angiogenesis and vascular remodeling to improve oxygen perfusion (Rey S, 2010). The expression of VEGF is upregulated in many solid tumors and is correlated with metastasis development and poor prognosis (McMahon G, 2000). HIF1 regulates also nitric oxide synthase (NOS), that catalyzes the production of NO; the latter was shown to increase HIF1α stability in human oral squamous cell carcinoma (Quintero M, 2006), generating a positive feedback loop between HIF1α and NO.

HIF1α mediates G1 cell cycle arrest (Shmaltz C, 1998) through a variety of mechanisms: HIF1α can interfere with Myc function, promoting the transcription of the cyclin-dependent kinase inhibitor p21 (Koshiji M, 2004) and can block β-catenin-dependent transcription (Kaidi A, 2007). HIF1α can block the binding of c-myc to its coactivator, Sp1 and Max disrupting the formation of the myc-Max complex reducing the expression of myc target genes. On the contrary other studies show that HIF1 and c-myc can cooperate to induce the expression of different genes, such as VEGF, HK2 and PDK1, suggesting that the interaction between HIF1 and c-myc is complex and cell type dependent (Kenneth NS, 2008). It was also reported that that the induction of HIF1 or HIF2 in normoxic condition is sufficient to cause cell cycle arrest in NIH3T3 cells,, through the expression of the cyclin-dependent kinase inhibitor p27 (Hackenbeck T, 2009).

4.2.3 HIF1 regulation by oncogenes

In cells defective for VHL, HIF1α and HIF2α proteins expression was observed already in normoxic condition. Mutations in the tumor suppressor gene VHL are responsible for the autosomal dominantly inherited von Hippel-Lindau (VHL) disease, of which the most frequent manifestations are hemangioblastomas. Other VHL gene mutations of VHL are responsible for the development of a variety of tumors, including renal clear cell carcinomas (RCC) and

pheochromocytomas. The inactivation of VHL correlates with a more pronounced production of VEGF and subsequent neoangiogenesis (Krieg M, 2000).

Also, mutations of the two TCA enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH), can affect HIF1α stability. When mutated, FH and SDH causes the accumulation of succinate and fumarate that blocks the function of PHDs. These mutations can cause the accumulation of HIF1α also in normoxic condition (Lu H, 2005).

The tumor suppressor p53, when active, promotes Mdm-2 mediated ubiquitination and proteasomal degradation of HIF1α. Homozygous deletion of p53 in tumor cells enhances HIF1α and increases HIF1-dependent transcriptional activation of VEGF promoting neovascularization and tumor growth in xenografts (Ravi R, 2000).

PKM2, that is frequently expressed in tumors, cooperates with HIF1α in a positive feedback loop: PKM2 gene is a target of HIF1α, as it contains an HRE in its promoter; in addition, when PKM2 is hydroxylated by PHD3, it interacts directly with HIF1α increasing the DNA binding of p300 and HIF1α, resulting in the enhancement of HIF1α target genes transcription (Luo W, 2011). Inactivating PTEN mutations potentiate the induction of HIF1α under hypoxia condition or in response to growth factors stimuli, on the contrary wild type PTEN attenuates hypoxia-mediated HIF1α stabilization (Zundel W, 2000).

Different studies demonstrated that the PI3K/AKT pathway can lead to HIF1 activation even in normoxic conditions. Treatment of HCC cell lines with LY294-002 downregulates HIF1α, and HIF1α depletion decreases the expression of Akt and hypoxia target genes. In addition, treatment with EGF or IGF-II upregulated both HIF1α and phospho-Akt suggesting the existence of a reciprocal activation of PI3K/Akt and HIF1α (Tanaka H, 2006). Similarly, overexpression of constitutively active AKT promotes the expression of HIF1α and its target gene VEGF even in normoxia (Lee BL, 2008). Other studies, however, reported that Akt and HIF1α promotes angiogenesis and tumorigenesis with parallel and independent effects: in murine hepatoma cell lines, with no HIF1 activity, lacking ARNT/HIF1β, the expression of either myrAkt or HIF1 promoted tumor growth with a dramatic increase in tumor size (Arsham AM, 2004). Another study demonstrated that constitutively active PI3K/Akt pathway did not influence HIF1α transcriptional activity either in normoxic and hypoxic conditions (Alvarez-Tejado M, 2002). These results suggested that the effect of Akt on HIF1α is cell type specific, though the studies were not directly comparable, as they differed in the severity and duration of the hypoxic stimulus. In 2009, Shafee (Shafee N, 2009) and coworkers analyzed the expression of two HIF1α substrates CAIX and VEGF, in different cell types and in different growth conditions, and eventulally demonstrated that PI3K/Akt activity has cell-type specific effects on HIF1 activity.

4.2.4 mTOR pathway and REDD1

mTOR can directly enhance the transcriptional activity of HIF1α, probably without increasing HIF1α stabilization. This mechanism was confirmed by the evidence that the mutation of different tumor suppressors - such as LKB1, PTEN and TSC1/2 - induces HIF1 expression by disregulation of mTOR (Semenza GL, 2010). In LKB1 deficient mice, the upregulation of HIF1α and its downstream target genes is inhibited by Rapamycin treatment, suggesting the involvement of mTOR in the regulation of HIF1α (Shackelford DB, 2009). In addition, in TSC1/2 deficient mice HIF1α is upregulated also in normoxic condition and this activation is mTOR dependent and is abolished by rapamycin treatment (Brugarolas J, 2004). Similar results were obtained in PTEN deficient cells, where PI3K/Akt hyperactivation leads to mTOR-dependent HIF1α activation (Majumder PK, 2004).

Hypoxia, on the other hand, blocks mTOR activity by different mechanisms, Arsham and colleagues first showed that brief exposure to modest hypoxia (1%O_2) prevented the insulin-mediated stimulation of mTOR following serum starvation (Arsham AM, 2003). The principal inhibitor mechanism of mTOR in hypoxia involves the activation of the TSC1/TSC2 complex. Hypoxia inhibits mTORC1 also by proteins - such as BNIP3, a proapoptotic protein induced in hypoxia - that interfere with the interaction between mTOR and Rheb. Furthermore, the promyelocitic leukemia (PML) protein was reported to inhibit Rheb-mTOR associations and to promote nuclear accumulation of mTOR, preventing its activation (Bernardi R, 2006). Tumor hypoxia through the inhibition of mTORC1 may act as a barrier against tumor growth, favoring the selection of mutations that overcome this inhibition. On the other hand, the mTOR inhibition in prolonged hypoxia is a survival mechanism, because of the inhibition of cell growth and translation, preserving energy homeostasis (Wouters BG, 2008).

The activation of the TSC1/2 complex in hypoxia, is mediated by two mechanisms: the activation of AMPK and the induction of REDD1 (REgulated in Development and DNA damage responses). The REDD1 gene encodes a protein of 25kDa that was first isolated in a screening for suppressor of insulin signaling. REDD1 that is induced in response to HIF1 stabilization in hypoxia (Reiling JH, 2004), blocks mTOR activity through the release of TSC2 from its inhibitor 14-3-3, opposing the PI3K-Akt-induced TSC2/14-3-3 association (DeYoung MP, 2008).Brugarolas and coworkers (Brugarolas J, 2004), demonstrated that the inhibition of mTOR in hypoxia correlates with the increased expression of REDD1 and that its disruption abrogates the hypoxia-induced inhibition of mTOR, promoting cell proliferation and anchorage-independent growth in hypoxia. In addition, the disruption of the TSC1/2 complex blocks the effects of hypoxia on mTOR and results in an accumulation of HIF1. The importance of REDD1 in the control of cell growth was also demonstrated in a mouse model where REDD1$^{-/-}$, myr-Akt expressing cells exhibited not only an enhanced increase *in vitro* in hypoxia, but they also formed tumors, in immunocompromised mice, more rapidly in comparison with wild-type, myr-Akt cells. These data demonstrated that REDD1, by

the inhibition of mTOR, functions as a suppressor of tumorigenesis. Consistent with this hypothesis, the analysis of REDD1 expression in a subset of primary breast carcinomas specimens demonstrated that REDD1 is significantly down-regulated compared with normal tissues (DeYoung MP, 2008). The down-regulation of REDD1 expression was also observed in an expression profiling study of normal prostate tissues versus invasive prostate carcinomas. (Lapointe J, 2004).

4.3 Hypoxia in PEL

PEL's growth in a hypoxic environment was shown to play an important role in the regulation of KSHV replication and tumor pathogenesis (Houston MC, 1981).

In 2001 Davis et al demonstrated that exposure of PEL cell lines to hypoxia (1% O_2) induces lytic replication of KSHV, as indicated by the increase of lytic protein in infected cells and the presence of virus in cellular supernatants (Davis DA, 2001). Hypoxia also induces the production of vIL6 that is important for PEL cells growth. KSHV promoters are responsive to hypoxia: the RTA gene (ORF50) and the ORF34 promoter region both contain HREs and both can be activated by either HIF1α and HIF2α under hypoxia (Haque M, 2003). RTA is the main lytic switch gene for KSHV, activation of RTA is sufficient to induce lytic replication of KSHV; ORF34 is part of a cluster of lytic genes. Hypoxia regulates KSHV replication and KSHV genes interacts with HIF1α. vGPCR upregulates VEGF trough the activation of MAPK and p38 pathway by the interaction with HIF1α (Sodhi A, 2000). Another latently expressed gene in PEL, vIRF3 (also known as LANA-2) has been shown to interact with HIF1α, increasing its stability, that results in upregulation of VEGF (Shin YC, 2008). In addition, LANA1 activates HIF1α, promoting the expression of VEGFR1 (Carrol PA, 2006), and can cooperate with HIF1α in the induction of RTA expression in hypoxia (Cai QL, 2006). It has been reported that LANA1 expression promotes the nuclear accumulation of HIF1α also in normoxia. It was recently demonstrated that LANA1 is activated in response to hypoxia, suggesting a complex interplay between KSHV genes and HIF1α in the regulation of cell growth (Veeranna RP, 2012).

The most important aspect of the hypoxic environment in PEL pathology is that hypoxia can influence the response to therapy. Rapamycin treatment did not eradicate completely PEL cells in xenograft model, the explanation is that PEL cells, when growing in hypoxic conditions, produce IL-10 that promotes PEL cells proliferation. The hypoxic microenvironment of lymphomatous effusions is important in the development of rapamycin resistance in PEL. (Gasperini P, 2009) and can potentially affect the efficacy of other drugs targeting the PI3K/Akt/mTOR pathway.

5. Materials and methods

5.1. Cell culture

The human PEL cell lines, BCBL1 (HHV-8 positive) and HBL6 (HHV-8 and EBV positive) were cultured in RPMI 1640 medium supplemented with 20% (v/v) of heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, 2mM Glutamine, at 37°C in an humidified atmosphere. Media and supplements were from Euroclone S.p.a. Cells were grown under either normoxic conditions (21% O_2, 5% CO_2) or hypoxic conditions (1% O_2, 5% CO_2) at 37°C for the indicated times and drug concentrations. Low O_2 tension was obtained using either a Hypoxia Chamber (StemCell Technologies) or the INVIVO$_{200}$ hypoxia workstation (Ruskinn Technology Ltd., UK). A mixture of 94% nitrogen, 5% CO_2, and 1% O_2 was used to perfuse the chamber (4 minutes at 20liters/minute) to obtain 1% O_2.

5.2 Transfection

Transfections of BCBL-1 cells were performed using the Amaxa nucleofection system (Amaxa, Cologne, Germany). 5x10 ^{6} cells were resuspended in nucleofector solution V and added of 2.5 µg DNA (either CMV6-HA-myr-Akt or CMV6 empty vector), then transferred to an Amaxa-certified cuvette and transfected using program T-001. Transfected cells were seeded in fresh medium (RPMI 20% FBS) at a concentration of 1x10^6 cells/ml for 24h. Then cells were examined for the expression of transfected genes.

5.3. Cell Lysate

Protein extracts were prepared by lysing cell pellets in RIPA buffer (50mM Tris-HCl pH 8.0, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% SDS, 150mM NaCl, 10mM EDTA, 1mM EGTA, 20mM NaF, 5mM sodium pyrophosphate, 1mM sodium ortovanadate, 1mM para-nitrophenylphosphate and Protease Inhibitor Cocktail (Sigma-Aldrich)) at 4°C for 30 minutes, followed by sonication. The lysates were cleared by centrifugation (10,000g, 4°C, 20 min), separating the total protein (supernatant) from the cellular debris (pellet). Supernatants were transferred to new tubes. Total protein concentration was quantified by Bradford Assay as follow: 1ul of cleared lysates was added to a mixture of 200 µl of Bradford reagent (BioRad) diluted in 800 µl of water, and absorbance was measured at 595nm using a Genesys 10UV spectrophotometer (Thermo Scientific). A standard curve was constructed using bovine serum albumin (BSA, Sigma) between the concentrations of 0.5 and 5 mg/ml; all samples were measured in duplicate.

5.4. Immunoblotting

Cell lysates were denatured by boiling at 100°C for 5 minutes, after the addition of SDS Sample Buffer (Tris-Cl 20mM pH 7.8, 2% SDS, 20% glycerol, 0.6M DTT, bromophenol blue). Equal amounts of protein extract (60ug) were loaded on polyacrylamide gel (7%, 10% or 15%) to resolve proteins, and a molecular weight standard (250kDa-15KDa) was used as reference (BioRad). SDS-PAGE was carried out with on a Mini-PROTEAN® Electrophoresis cell (Biorad) using 1x Tris/Glycine/SDS Running Buffer (BioRad). Gels were run at 120V for approximately 90 minutes, at 4°C. Proteins were transferred to an Immobilon-P PVDF membrane (Millipore) using a semidry apparatus (Thermo Scientific) in Transfer Buffer (25mM Tris, 192mM glycine, 20% methanol). The transfer was performed at 90mA for 1h45'. Once proteins were transferred, the membrane was blocked in 3% BSA in TBS Tween (50mM Tris-HCl pH7.5, 50mM NaCl, 0.1% Tween 20) for 1h. The membranes were then incubated with the relevant primary antibodies (Table 5.1), for 16h at 4°C. The membranes were washed three times for 5 min with TBS-Tween, then incubated with the secondary antibody (30 min, room temperature). Again, the blot was washed for 3 x 10 min in TBS-Tween. The immune-antigen reaction was visualized using either a TurboLuminol (Euroclone) or with 5ml of Luminol Solution (0.8 ml of luminol (4.4mM of luminol, 4.3 mM of 4-iodophenol in Tris 0.1M pH 9.35), 3.9 ml of water; 0.25 ml of Tris 1M pH 9.35; 40μl of BSA 30% and 1μl of H_2O_2) and Kodak Image Station 440CF.

Antibody	Dilution	Company
p-Akt S473	1:1000	Cell Signaling
p-Akt T308	1:1000	Cell Signaling
Akt	1:1000	Cell Signaling
p-PDK1 S241	1:1000	Cell Signaling
p-mTOR S2448	1:1000	Cell Signaling
p-P70S6K T389	1:1000	Cell Signaling
p-AMPKα T172	1:1000	Cell Signaling
AMPK	1:1000	Cell Signaling
p-4EBP1T37/46	1:1000	Cell Signaling
PKM 1/2	1:1000	Cell Signaling
PKM 2	1:1000	Cell Signaling
HIF 1α	1:1000	BD Bioscience
REDD1	1:1000	Protein Tech
β-actin	1:1000	Sigma Aldrich
Hexokinase II	1:500	Santa Cruz Biotechnology

Table 5.1

Antibody	Dilution	Company
Goat Anti-Rabbit IgG, HRP conjugated	1:5000	Thermo Fisher Scientific
Goat Anti-Mouse IgG, HRP conjugated	1:3000	Thermo Fisher Scientific

Table 5.2

5.5 Chemicals and reagents

We have used different metabolism inhibitors (Table 5.3) and PI3K/Akt/mTOR inhibitor (Table 5.4)

Name	Company	MW	Target	Clinical Trial
2-deoxyglucose	Sigma Aldrich	164.16	HKII	Phase I
Lonidamine	LTK Laboratories	321.16	HKII	Phase II/III
3-bromopyruvate	Sigma Aldrich	166.96	HKII	preclninical
Dichloroacetate	Sigma Aldrich	150.92	PDH	approved
Oxamate	Sigma Aldrich	111.03	LDH	preclinical
Metformin	LTK Laboratories	165.62	AMPK	approved

Table 5.3

Name	Company	MW	Target	Clinical Trial
AKTi1/2	BioVision	551.65	AKT	preclinical
MK-2206	SelleckChem	480.39	AKT	Phase I
Torin-1	SelleckChem	607.62	mTOR	preclninical
NVP-BEZ235	SelleckChem	469.55	mTOR/PI3K	preclinical
Ly294002	Sigma Aldrich	307.34	PI3K	preclinical

Table 5.4

5.6 MTT assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. PEL cells were seeded in 96-well plates (10^4 cells/well, 200ul/well), all samples were seeded in triplicate. Cells viability was determined after 24 hours of treatment by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 20 µl of MTT reagent (5 mg/ml in PBS) was added to each well and incubated for 3 h at 37°C. The medium was then removed and 200 µl of isopropanol/HCl 0.1N were added to resuspend formazan crystals . The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570nm, using 650 nm as a reference wavelength, with a Multiwell Plate Reader (nome strumento)

5.7 Cytofluorimetric Analysis

5.7.1 PI staining

Cell cycle and apoptosis (sub-G0G1) were evaluated with Propidium Iodide (PI) staining and subsequent cytofluorimetric analysis. PI is a fluorogenic compound that binds stechiometrically to nucleic acids: fluorescence emission is proportional to the DNA content of a cell (after RNA removal with an RNase treatment). Quiescent (G0) and G1 cells have one copy of DNA and have 1X fluorescence intensity; cells in G2/M phase of the cell cycle have two copies of DNA and 2X intensity; since the cells in S phase are synthetizing DNA and therefore have fluorescence value between 1X and 2X. The sub-G1 peak represents the hypodyploid population, and correspond to the apoptotic population.

$5x10^5$ BCBL-1, after the described treatments, were centrifuged at 1200rpm for 8 minutes, and washed with PBS. The cell pellets were fixed by the resuspension in 100µl of PBS1X and 900µl of cold 70% EtOH. The cellular suspension was conserved at 4°C, for at least 2h. The fixed cells were centrifuged at 1800rpm for 8 minutes to eliminate EtOH and resuspended in 500µl of PBS1X supplemented with 25µg (1µl) of RNAse (Sigma Aldrich, 25 µg/µl). Cells were incubated for 1h at 37°C, 25µl Propidium Iodide (5mg/ml, Sigma Aldrich) were added. The cells were analyzed with a cytofluorimeter (Bechman Culture).

5.7.2 Annexin V/PI assays

For Annexin V/PI assays, cells were stained with Annexin V–FITC and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). Briefly, $1x10^6$ cells were washed twice with phosphate-buffered saline (PBS), and stained with Annexin V–FITC and PI in 1X binding buffer (10mMHEPES, pH 7.4, 140mMNaOH, 2.5mMCaCl2) for 15min at room temperature in the dark. The apoptotic cells were determined using a Becton-Dickinson FACScan cytoflurometer (Mansfield, MA, USA). Both early apoptotic (annexin V-positive, PI-negative) and late (annexin V-positive and PI-positive) apoptotic cells were included in cell death determinations. The Annexin V and PI-negative cells were the viable cells.

5.8 Lactate secretion assay

Cells were rinsed in fresh media and grown for 6 or 24 hours in the presence or absence of inhibitors as indicated. Media samples were then collected, flash-frozen in liquid nitrogen and immediately stored at -80°C until the time of the assay. Lactate levels in media were measured using a L-Lactate Assay Kit I (Eton Bioscience Inc.) according to manufacturer instructions. A standard curve was constructed using L-lactate standard solutions between the concentrations of 0.05 and 3.2mM; all samples were measured at least in triplicate.

5.9 Measurement of cellular ATP content

Intracellular ATP was measured by bioluminescence using a luciferin-luciferase system (ATP bioluminescent assay kit CLS II; Roche) according to manufacturer instructions. The amount of ATP measured was referred to the protein content, determined by the method of Lowry (Lowry et al., 1951), and expressed as nmol/mg protein.

5.10 Real Time PCR

Total RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, Hercules CA, USA) according to manufacturer's instructions. Genomic DNA was removed by DNase treatment. Isoform-specific HXK-2 (NM_000189), PKM1 (NM_182470) and PKM2 (NM_002654) forward and reverse primers were as follows: 5'-TTGACCAGGAGATTGACATGGG-3' and 5'- TTGACCAGGAGATTGACATGGG-3' for HXK-II; 5'- CTGAGGCAGCCATGTTCC- 3' and 5'- CCATGAGGTCTGTGGAGTG- 3' for PKM1; 5'-ACTTGGTGAGGACGATTATG- 3' and 5'- CTGCCATCTACCACTTGC- 3' for PKM2. These PCR primers were designed using Beacon Designer 2.06 (Premier Biosoft International) giving special attention to primer length, annealing temperature, base composition and 3'-end stability, to ensure optimal DNA polymerization efficiency. During preliminary qRT-PCR assays, the optimal primer concentration was determined for each primer pair: it generated the lowest Ct value and a sharp peak, with no amplification of non-specific products or primer-dimer artifacts.

Real-time amplifications, using SYBR Green detection chemistry, were run in triplicate on 96-well reaction plates with the CFX96 machine (Bio-Rad). Reactions were prepared in a total volume of 20 µl containing: 0.8 µl cDNA, 0.6 µl of each 10 µM primer (300 mM each; Invitrogen), 10 µl of iQTM SYBR® Green Supermix (Bio-Rad) and 8 µl RNase/DNase-free sterile water (Qiagen). Blank controls were run in triplicate for each master mix. The cycle conditions were set as follows: initial template denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 s, and combined primer annealing/elongation at 60°C for 30 s. This cycle was followed by a melting curve analysis, ranging from 56°C to 95°C, with temperature increasing by steps of 0.5°C every 10 s.

5.11 Determination of Combination Index (CI)

The effects of the different drug combinations were assessed by calculating the combination index (CI) values using CompuSyn software. Derived from the median-effect principle of Chou and Talalay (Chou TC, 2010), the CI provides a quantitative measure of the degree of interaction between two or more agents. From the general dose-effect equation

$$f_a/f_u = (D/D_m)^m$$

were

f_a: the fraction affected

f_u: the fraction unaffected (1- f_a)= f_u

D: the dose of the drug

D_m: the dose that is required to produce the median effect (e.g. IC_{50}, ED_{50} or LD_{50})

m: the slope of the median-effect signifying the shape of the dose effect curve (m=1: hyperbolic, m>1: sigmoidal, m<1 negative (flat) sigmoidal)

was derived the combination index equation

$$CI= (D)_1/(D_x)_1+(D)_2/(D_x)_2$$

Were $(D_x)_1$ is the dose of Drug1 that inhibit x%, $(D_x)_2$ is the dose of Drug2 that inhibit x%. $(D)_1$ is the portion of Drug1 in combination $(D_1)+(D_2)$ also inhibits x%, likewise $(D)_2$. Thus $(D_1)+(D_2)$ also inhibits x%.

The dimensionless value obtained was termed the combination index (CI)

CI = 1 indicates additive effect

CI < 1 indicates synergism

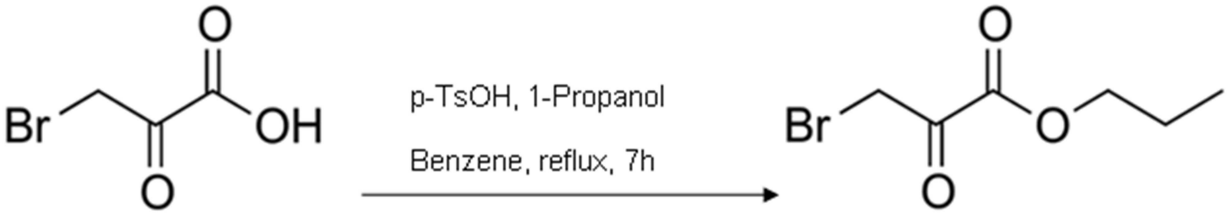
CI > 1 indicates antagonism

In all experiments we have used a constant ratio between the two drugs tested in combination.

The EC50 of the drugs used in our experiments were calculate with the software GrafIt

5.12 Synthesis of Propyl Bromopyruvate (BrOP, (2)).

All reagents and solvents were used as received without further purification: Bromopyruvic acid (≥ 97%) was purchased from Aldrich*, p*-Toluenesulfonic acid monohydrate (98 %), Benzene (99.8 %) and 1-Propanol (≥ 99.5%) were purchased from Sigma-Aldrich.



1 2

Compound **2** was synthesized adapting a reported procedure (Wang B, 2003).

In a 250 mL flamed round bottom flask dried under vacuum, to a solution of bromopyruvic acid (**1**) (200 mg, 1.2 mmol) in benzene (80 mL) was added *p*-toluenesulfonic acid monohydrate (20 mg, 0.1 mmol), 1-propanol (139 µL, 1.9 mmol) and the reaction mixture was heated under reflux for 7 h. After, the reaction mixture was cooled down to room temperature and then concentrated in vacuo to obtain the title compound **2** as white solid (240 mg, yield 96 %).

^{1}H NMR (400 MHz, $CDCl_3$, 25°C) δ (ppm) = 4.40 (2H, s, Br-C*H*$_2$-) and 4.26 (2H, t, *J* = 6 Hz, O-C*H*$_2$CH$_2$CH$_3$) part. overlapped, 1.76 (2H, m, O-CH$_2$C*H*$_2$CH$_3$), 0.98 (3H, t, *J* = 8 Hz, O-CH$_2$CH$_2$C*H*$_3$);

^{13}C-NMR (100 MHz, $CDCl_3$, 25°C) δ = 184.9, 159.5, 68.9, 31.0, 21.8, 10.4.

5.13 Reverse-phase protein arrays (RPPA)

Cells were grown in standard media and 10% fetal bovine serum and then lysed in buffer consisting of a 2.5% solution of 2-mercaptoethanol in loading buffer/TPER plus phosphatase and protease inhibitors. All samples were diluted to a final concentration of 0.5 mg/ml and then 30 µl of each sample, arrayed in a series of 6-fold dilutions, was printed in duplicate on slides. Lysates derived from HeLa cells or HeLa cells treated with pervanadate were also printed on each slide as low and high phosphorylation controls, respectively. The slides were then subjected to immunostaining with a panel of 15 commercially available antibodies primarily directed against specific phosphorylated or cleaved proteins, including PI-3K/Akt, ERK/MAPK, PKC and caspase-dependent apoptosis. Each of these antibodies had previously undergone extensive validation for both phosphorylation and protein specificity using single band detection at the appropriate MW by Western blotting. To estimate the total protein amount, selected arrays were stained with Sypro Ruby protein blot stain (Molecular Probes, Eugene, OR, USA) and visualized on a Fluorchemk imaging system (Alpha Innotech, San Leandro, CA, USA). Slides

were stained on an automated slide stainer (Dako, Carpinteria, CA, USA) using a biotin-linked peroxidase catalyzed signal amplification. Finally, the primary antibodies at concentrations ranging from 1:50 to 1:1000 were applied for 30 min followed by the secondary link antibody for 30 min (concentration 1:10 for anti-mouse antibodies and 1:5000 for anti-rabbit antibodies). To allow normalization of total protein on printed arrays, one to two slides in each print run were stained with Sypro Ruby protein blot stain (Invitrogen) and the value of these stained arrays used for normalization of all end-point values. The intensity value for each end point was determined by identifying spots for each duplicate dilution curve for each sample that were within the linear dynamic range of the staining after background subtraction with each spot (within slide local background and also against a slide stained with secondary antibody only). Single intensity values were obtained by multiplying each spot in the linear range by its dilution factor and averaging candidate linear points. Finally, each value was normalized relative to the total protein intensity value for that sample derived from the Sypro Ruby-stained slide.

6. Aim of the study and results

6.0 Aim of the study

PEL is a B-cell non-Hodgkin lymphoma featured by a very aggressive clinical course and poor prognosis. An optimal pharmacological approach for PEL is far from being defined.

PEL cells are characterized by the Warburg phenotype. Besides, the highly hypoxic environment in which they grow *in vivo* makes them even more reliant on glycolysis for energy requirements, and inherently more vulnerable to drugs targeting this metabolic pathway. Remarkably, in cancer cells metabolic reprogramming is known to be coordinated with proliferative signaling, and regulated by oncogenes and tumor suppressor genes such as the components of the PI3K/Akt/mTOR cascade.

In this study, we asked whether, taken together, the above observations might open a new therapeutic window for PEL.

The broad objective of this study, therefore, was to explore a new approach for PEL, based on dual targeting of metabolic rewiring and activated signaling.

The specific aims were:

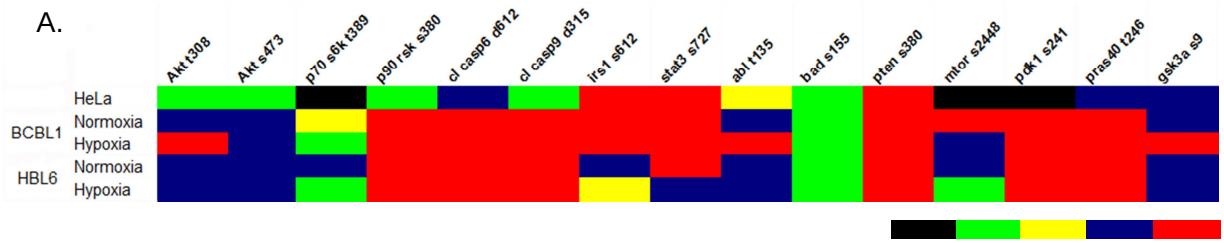
- ✓ to determine the degree of PEL cells sensitivity to a panel of glycolysis inhibitor, already in clinical trial for other types of cancer, and whether the sensitivity is affected by low oxygen environment;
- ✓ to investigate the activation state of the PI3K/Akt/mTOR signaling pathway in two different PEL cell lines by a broad survey with 15 anti-phosphoepitopes by means of reverse phase protein array and Western blotting analysis, and determine its druggability by pathway specific inhibitors;
- ✓ to compare the dose-effect curve of single drugs with that of combined treatments (glycolysis + PI3K/Akt/mTOR inhibitors) in PEL cells *in vitro*, in order to disclose whether the combinations have an additive or even a synergistic effect;
- ✓ to test the cytotoxicity of the combinations with the best CI in normal lymphocytes from healthy donors.

6.1 The hypoxia inducible factor 1, HIF1, is modulated by mTOR in PEL cell lines

The phosphorylome of two different PEL cell lines was profiled by means of reverse phase protein array analysis (RPPA) with a panel of 15 antibodies recognizing primarily phosphorylated epitopes of the PI3K/Akt/mTOR signaling pathway. All antibodies in this study had already been validated for RPPA analysis both by our laboratory and others (Maraldi T, 2011). The result is remarkably clear: the PI3K/Akt/mTOR pathway is constitutively activated in all cell lines tested (Figure 6.1A).

Moreover, since it has been reported that activation of the Akt pathway can induce the expression of HIF1α even in normoxia, although in a cell type dependent manner (Shafee N, 2009), the extent of the interdependence of the Akt pathway and the HIF-mediated hypoxia response in PEL was assessed. HIF1α levels in normoxia and hypoxia were compared, by Western blotting, in the two PEL cell lines BCBL1 and HBL6. As expected, in control cells grown in 21% O2, HIF1α is undetectable, whereas hypoxia stabilizes it (Figure 6.1B). However, in these cells forced expression of a constitutively active form of Akt, namely myr-Akt, (Bellacosa A, 1998), is not sufficient to stabilise HIF1α in normoxia (Figure 6.1C).

Nevertheless, based on the RPPA analysis showing that mTOR activity is in part counteracted by hypoxia, we reasoned that, although hyperactivation of Akt does not protect HIF1 from degradation in normoxia, it might affect it in hypoxia through its effector mTOR. To test this hypothesis, BCBL1 cells were cultured under hypoxia or normoxia, in the presence either of the selective mTORC1 inhibitor rapamycin, or of the mTORC1/2 inhibitor torin-1 (Figure 3.5). The phosphorylation of the mTOR substrate 4EBP1 was used as a readout of drug effectiveness. As expected, expression of HIF1α is undetectable in normoxia, whereas it is induced by 24 hours hypoxia. However, addition of either rapamicyn or torin-1 almost abrogates HIF1α induction in these cells, suggesting that mTOR kinase activity is fundamental for HIF1-dependent response to hypoxia (Figure 6.1D).



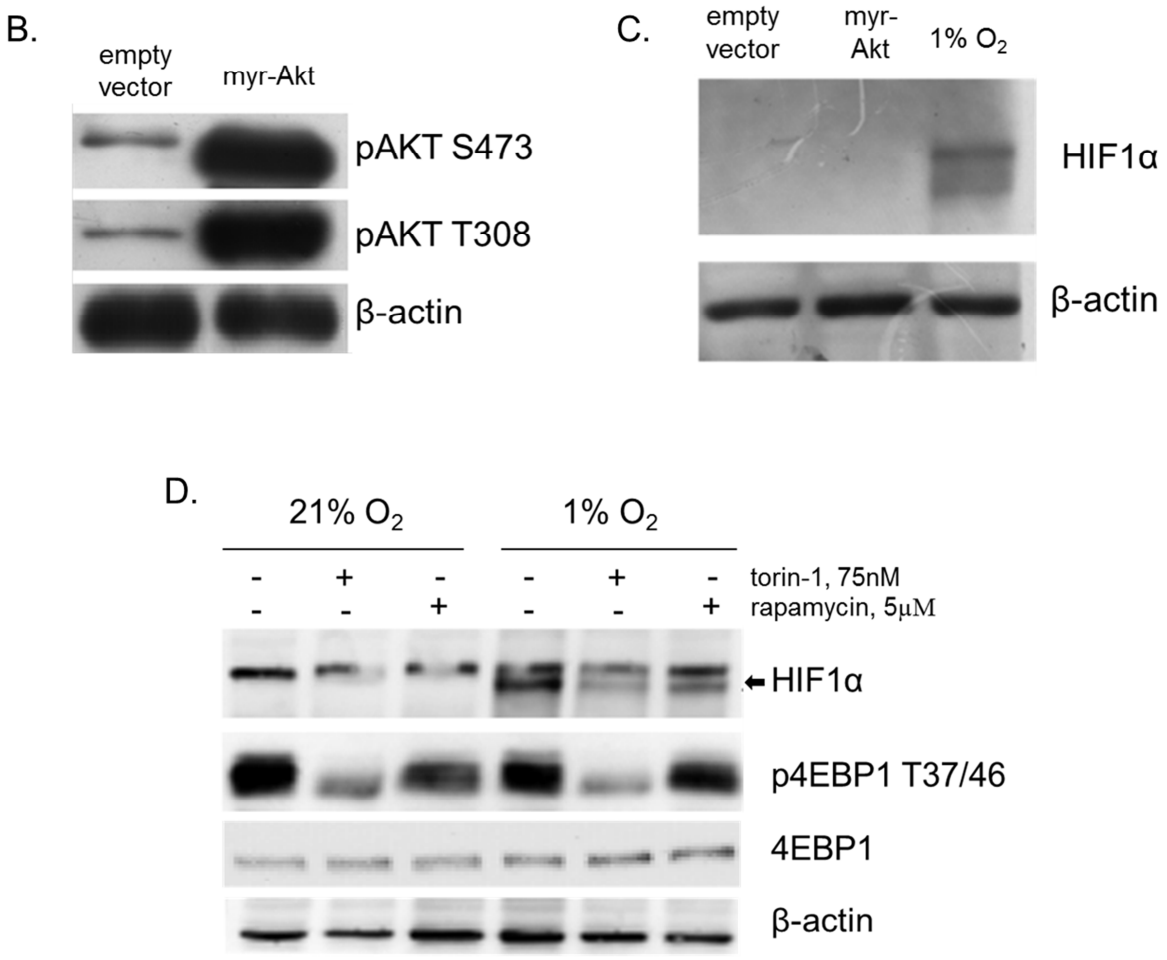


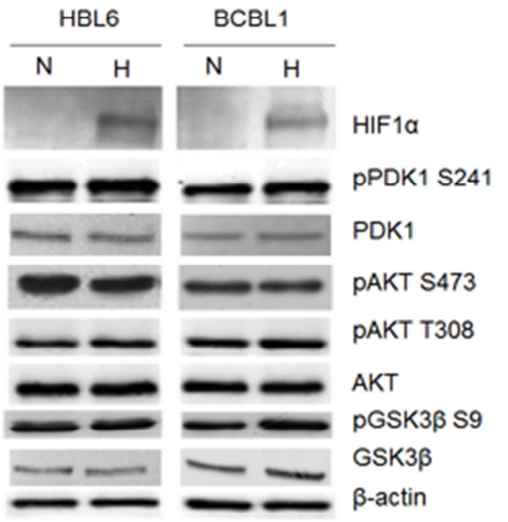
Figure 6.1 Effect of Akt signaling activation on HIF1α expression in BCBL1 cells. (A) Heatmap representation of RPPA analysis showing PEL cells cultured in hypoxia or normoxia compared to HeLa cells. The color is scaled so that black indicates low expression while red indicates high expression. (B) BCBL1 cells were transfected with Caax-Akt to activate Akt signaling, and activation of Akt was confirmed by SDS-PAGE and Western blotting of both phosphoSer473 and phosphoThr308. (C) To investigate the effect of Akt activation on HIF1α expression, the above lysates were run with equal amounts of lysate from BCBL1 cells cultured in hypoxia for 24 hours (positive control), and probed with anti-HIF1α. (D) BCBL1 cells were cultured in hypoxia or normoxia for 24 hours, then treated with the mTOR inhibitors rapamycin (50nM), or torin-1 (75nM), then cellular extracts were resolved by SDS-PAGE and revealed with anti-HIF1α; phosphorylation of 4EBP1 was probed as a readout of mTOR inhibition.

6.2 Hypoxia affects the PI3K/Akt pathway at the mTORC1 level

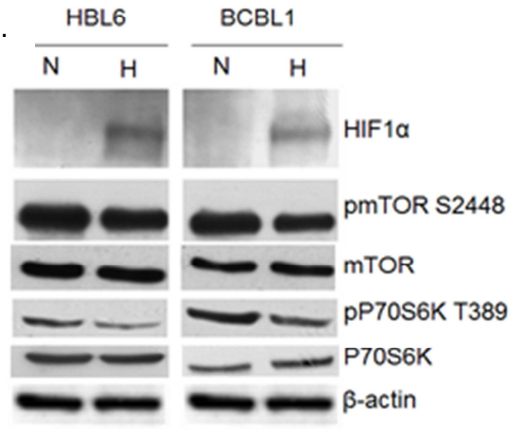
Solid tumors are characterized by hypoxic areas and hypoxia plays an important role in their sensitivity to chemotherapeutic treatments (Brown JM, 1999). Expression of HIF1α is significantly increased in NHL cells, compared to normal B-cells (Giatromanolaki A, 2008). Besides, overexpression of HIF1α is associated with chemoresistance in NHL cell lines through upregulation of the antiapoptotic protein Bcl-XL (Hernandez-Luna MA, 2013). On the other hand, hypoxia is known to activate the Akt pathway in a cell type dependent manner (Alvarez-Tejado M, 2002). Therefore, we investigated the effect of hypoxia on the activation of Akt in BCBL-1 and HBL6 PEL cell lines. Cells were cultured for 24 hours in either hypoxia or normoxia, and the activation of key elements of the PI3K/Akt/mTOR pathway was analyzed by both RPPA and Western blot by means of antibodies against phosphoepitopes directly related to the catalytic activity of the kinase. In spite of a slight increase of Thr308 phosphorylation and of the corresponding Thr308 kinase, PDK1, Akt phosphorylation at Ser473, as well as phosphorylation of Akt direct substrate GSK3β, at the Akt site Ser9, are not significantly modified by hypoxia. Therefore we conclude that hypoxia does not affect Akt in these cells (Fig. 6.1A and 6.2A-B).

Next, the effect of oxygen deprivation on mTOR kinase activity was analyzed. As a readout, the phosphorylation of mTOR itself and that of its substrate, p70S6K, were monitored. As shown in Figure 6.2B, hypoxia induces a reduction of both phospho-mTOR and phospho-p70S6K. This result was not completely unexpected, as hypoxia was already known to affect the activity of mTORC1 in an Akt-independent manner. We reasoned that in this context mTORC1 activity may be modulated either *via* HIF1-dependent upregulation of the development and DNA damage responses 1 protein, REDD1, or *via* activation of the protein kinase AMPK following increased AMP/ATP ratio. Both factors ultimately act through the inhibitory activity of TSC2 on mTORC1. We thus investigated whether one or both mechanisms mediate mTOR modulation. As shown in Figures 6.2C and 6.2D, both mechanisms are activated in PEL cells upon oxygen deprivation, though with a different timing. Indeed, while REDD1 is upregulated as early as 4 hours after the initiation of hypoxic incubation, AMPK is activated at a later stage, compatible with a decrease of ATP levels. To confirm this hypothesis, ATP was quantified by means of a luciferin-luciferase assay kit in normoxia and in hypoxia. After 4 hours hypoxia, the intracellular ATP content is similar to that in normoxia. As expected, however, upon 20 hours the intracellular content decreased significantly (Figure 6.2 E), in good agreement with the activation of AMPK shown above.

A.


HBL6
BCBL1
N
H
N
H
HIF1α
pPDK1 S241
PDK1
pAKT S473
pAKT T308
AKT
pGSK3β S9
GSK3β
β-actin

B.


HBL6
BCBL1
N
H
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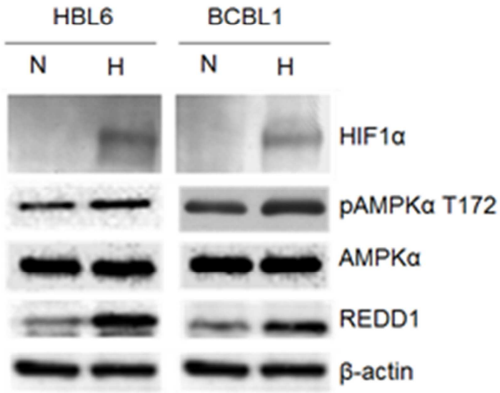
H
HIF1α
pmTOR S2448
mTOR
pP70S6K T389
P70S6K
β-actin

C.


HBL6
BCBL1
N
H
N
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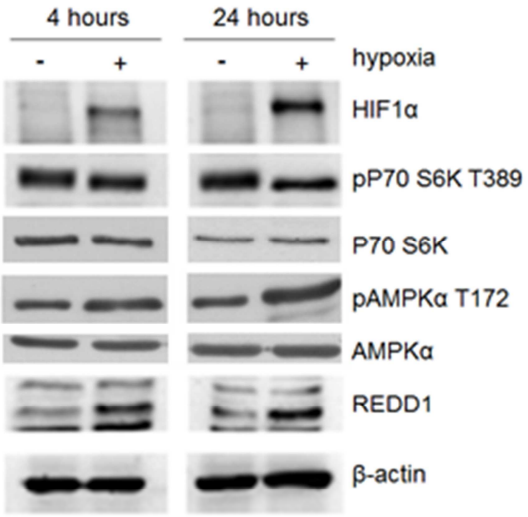
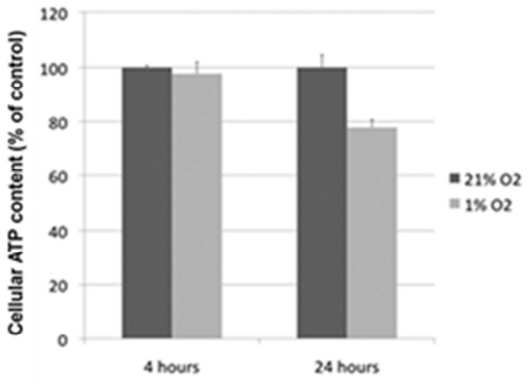
HIF1α
pAMPKα T172
AMPKα
REDD1
β-actin

D.


4 hours
24 hours
-
+
-
+
hypoxia
HIF1α
pP70 S6K T389
P70 S6K
pAMPKα T172
AMPKα
REDD1
β-actin

E.


Cellular ATP content (% of control)
120
100
80
60
40
20
0
4 hours
24 hours
21% O2
1% O2

F.

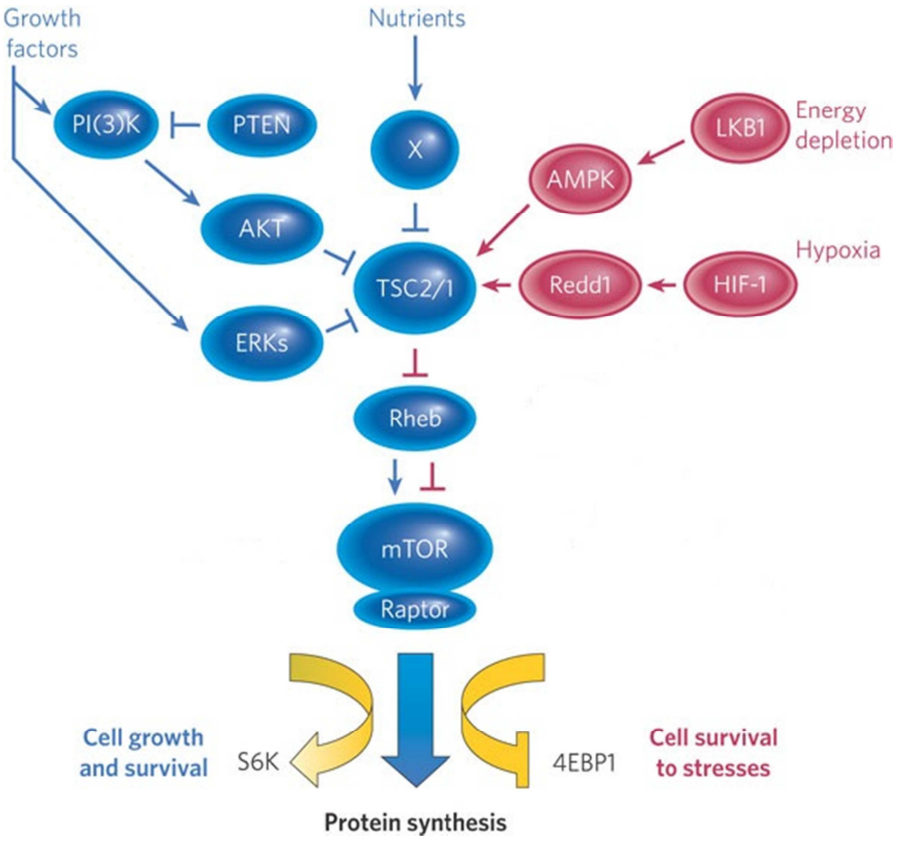


Figure 6.2. The interplay between hypoxia and PI3K/Akt/mTOR pathway in PEL cells. HBL6 and BCBL1 cells were cultured for 24 hours in hypoxia or normoxia conditions. Equal amounts of cellular protein extracts wer e resolved by SDS-PAGE and probed with the indicated antibodies. (A) pPDK1 S241, pAkt T308, pAkt S473 are a readout of Akt downstream signalling activation. (B) mTOR S2448 and P70S6K T389 are a readout of mTOR activation. Equal loading was confirmed by anti-β-actin. (C) In the same lysates of HBL6 and BCBL1 cell, the effect of 24 hours hypoxia on AMPK and REDD1 was monitored by Western blotting. (D) BCBL1 cells were cultured in normoxia or hypoxia for either 4 or 24 hours, then lysates were probed with the indicated antibodies. (E) From the same extracts as in (D), cellular ATP content was assayed by means of a luciferin-luciferase system (ATP bioluminescent assay kit CLS II, Roche). (F) Diagram showing the interplay between PI3K upstream activators, nutrients and hypoxia. Stimulation of PI3K by growth factors inactivates TSC1/2 complex, relieving inhibition over GTP-Rheb, which ultimately activates mTOR (blue arrow). Conversely, depletion of nutrients, ATP or oxygen inhibits mTOR (red arrow). mTOR inhibition under hypoxia requires the transcription of the REDD1 gene, whereas ATP depletion reactivates AMPK, which directly phosphorylates the TSC1/2 complex thus blocking mTOR (modified from Poyssegur J, 2006).

6.3 Hypoxia increases the glycolytic flux in PEL cells

PEL cells display activation of the Warburg effect, reflected in an elevated glycolytic activity under normoxic conditions. Very recently, inhibition of glycolysis was found to be sufficient to reduce PEL cell viability in culture at atmospheric oxygen levels, confirming the importance of this metabolic pathway to sustain the high proliferation rates of transformed cells (Bhatt AP, 2012). Furthermore, the highly hypoxic microenvironment, which PEL cells experience *in vivo* (Funahashi A, 1971; Houston MC, 1981), might render cells even more reliant on glycolysis and inherently more vulnerable to glycolysis inhibitors. We therefore investigated the effect of hypoxia on cell metabolism in the BCBL1 and HBL6 cells by measuring the secretion of lactate in the culturing media and analyzing changes in the expression of key glycolytic enzymes.

Pyruvate, the end product of glycolysis, is converted into lactate by LDH and is secreted through monocarboxylate transporters. The rate of lactate secretion provides a direct indication of the rate of glycolysis. Cells were thus grown in hypoxia for 24 hours, a condition in which we have already shown that cellular ATP is diminished, indicating that a considerable fraction of ATP in normoxic cells is produced through mitochondrial metabolism (Figure 6.2E). Then lactate secretion in the culture medium was assayed. Parallel to the decrease of ATP, a significant increase in the secretion of lactate was observed (Figure 6.3A). Furthermore, it is interesting to note that in hypoxia PEL cells show increased expression of key glycolytic enzymes, such as PKM1, PKM2 and HKII both at the mRNA and protein levels (Fig. 6.3B-E). It can therefore be concluded that hypoxia increases the glycolytic flux in PEL cell.

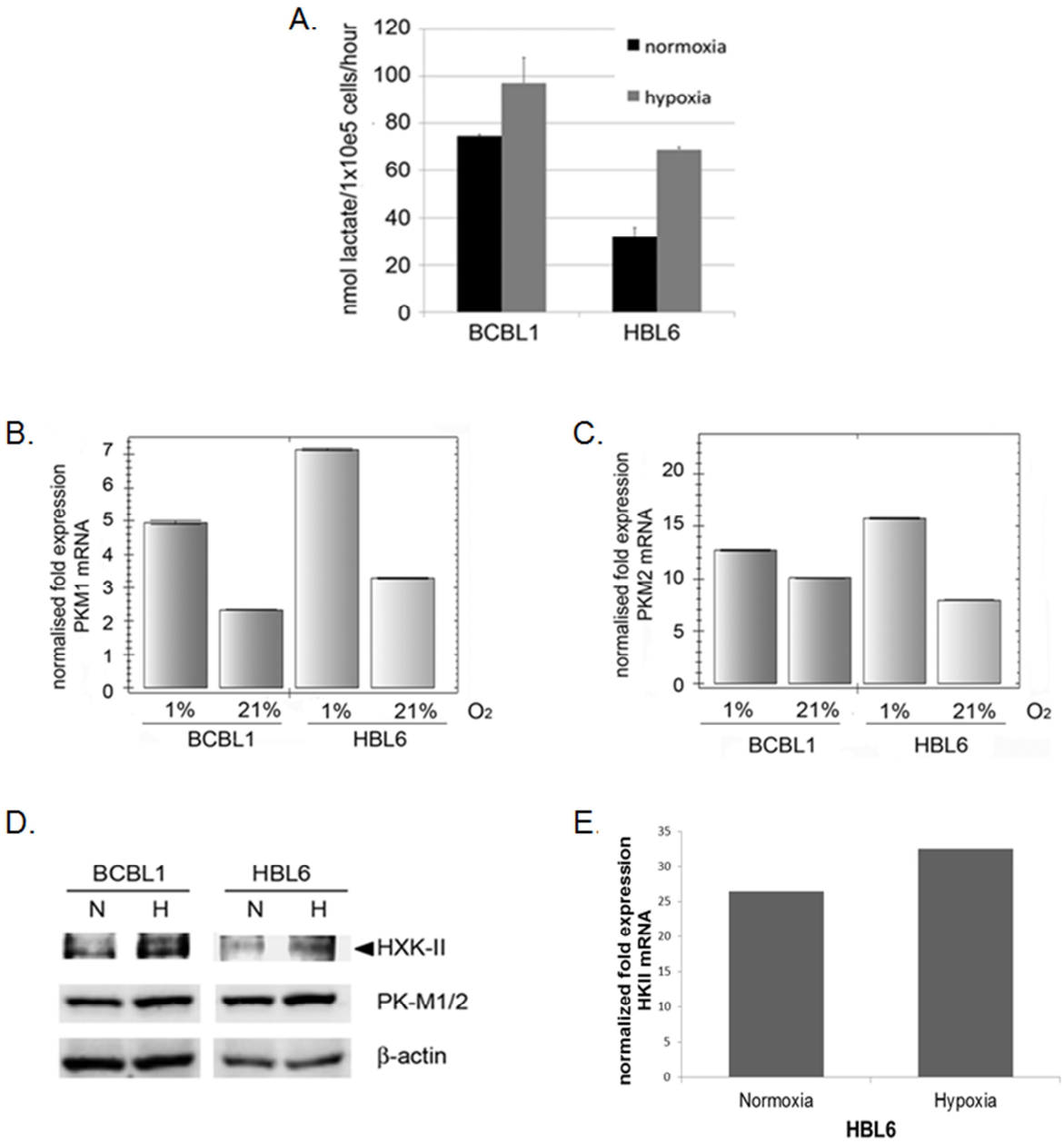


Figure 6.3. Hypoxia increases the glycolytic flux in PEL cells. The level of lactate in the culture media of BCBL1 and HBL6 cells grown in hypoxia or normoxia was measured as described in Methods. The data are expressed as the mean ± S.D. of three different replicates (A). Expression of the glycolytic enzymes PKM1 (B), PKM2 (C) and HKII (E) was monitored by RT-PCR. The results were confirmed by Western blot with the indicated antibodies (D).

6.4 Disruption of the PI3K/Akt pathway in PEL cells inhibits glucose metabolism

Similar to other B-NHL, PEL cell lines in culture display elevated aerobic glycolysis and anabolism, namely the Warburg phenotype (Bhatt AP, 2012). Akt is a converging hub of both metabolic and oncogenic signaling, known to favor and support the Warburg effect in cancer in several ways, both directly and indirectly. It can increase the expression of glucose transporters and glycolytic enzymes. Moreover, Akt upstream activator, PI3K, is a target of multiple growth factors, leading to chronic activation of Akt in cancer cells. Besides, the Akt effector mTORC1 controls aminoacid metabolism and protein translation. Activated Akt is also an essential element of PEL pathogenesis. HIF1-mediated hypoxia response and the PI3K/Akt/mTOR pathway have demonstrated an intimate interdependence in several studies and can act in an integrated way, even though the modality of the interaction is dependent on cell-type and experimental set-up. As discussed above, our results indicate that hypoxia downregulates the PI3K/AKT/mTOR pathway at the level of mTORC1 and its downstream substrates P70S6K and 4EBP1 (Figure 6.2B), suggesting that hypoxia is able to modulate mTOR in an Akt independent manner (Figure 6.2B-E). On these basis, the representative PEL cell line BCBL1 was treated with sublethal doses of a panel of drugs targeting either PI3K/Akt, namely Akti 1/2 and Triciribine, or mTOR, namely rapamycin and torin-1. All the drugs were able to abrogate phosphorylation of direct substrates such as p70S6K, PRAS40 and GSK3, indicating that this pathway can be regarded as a druggable target in PEL (Figure 6.4 A).

Therefore, we next investigated whether modulation of the Akt pathway by the above drugs may be useful in the control of PEL cell metabolic rewiring.

BCBL1 cells were treated as described above for 8 hours, then glucose-to-lactate flux was estimated, based on the rate of secretion of lactate into the culture media. Remarkably, all treatments were able to reduce the rate of lactate secretion, whereas enhancement of the Akt pathway through forced expression of a constitutively active form of Akt, myr-Akt (Bellacosa A, 1998), led to a significant increase in lactate secretion (Fig. 6.4 B,C). The extent of reduction of lactate secretion was very similar to that obtained incubating cells with the non-metabolized glucose analog 2-DG. This observation confirms the involvement of Akt in the regulation of glucose metabolism in PEL cells.

A.

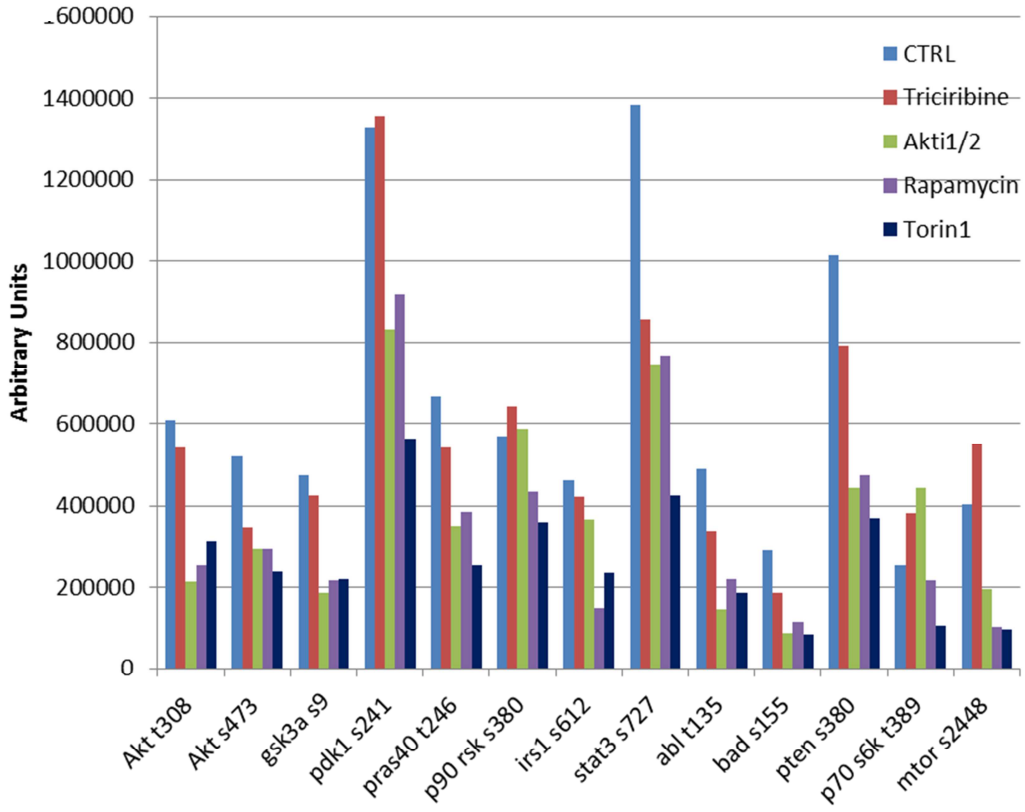
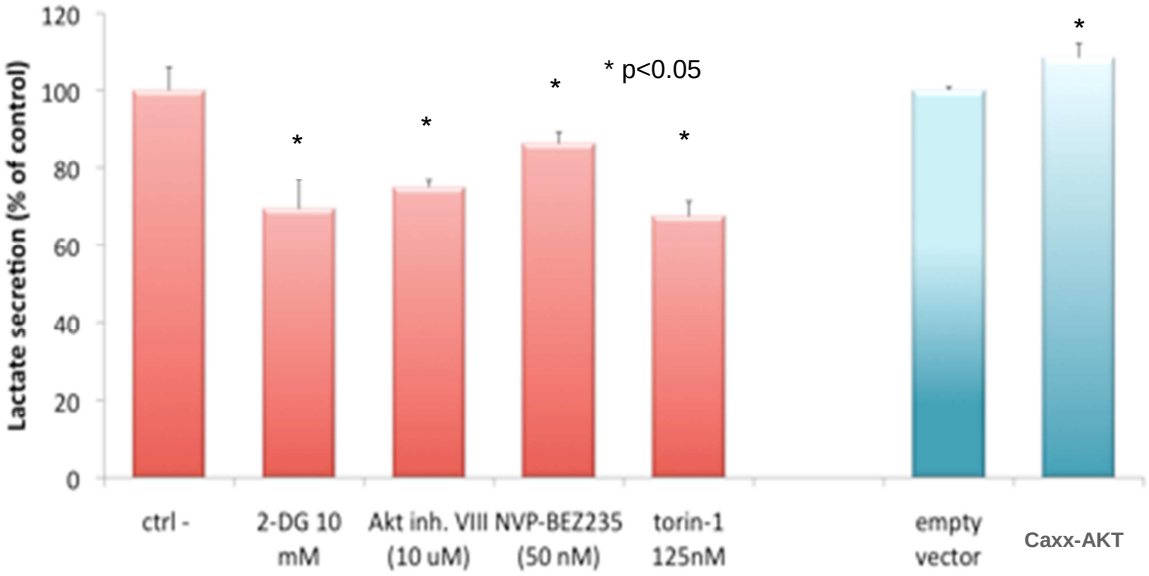


Figure 6.4. (A) The Akt pathway can be regarded as a druggable target in PEL cells. BCBL-1 were treated with Triciribine (1µM), Akti1/2 (1µM), rapamycin (100nM), torin-1 (250nM) for 24 hours. Cell lysates were subjected to RPPA analysis with the indicated antibodies.

B.



C.

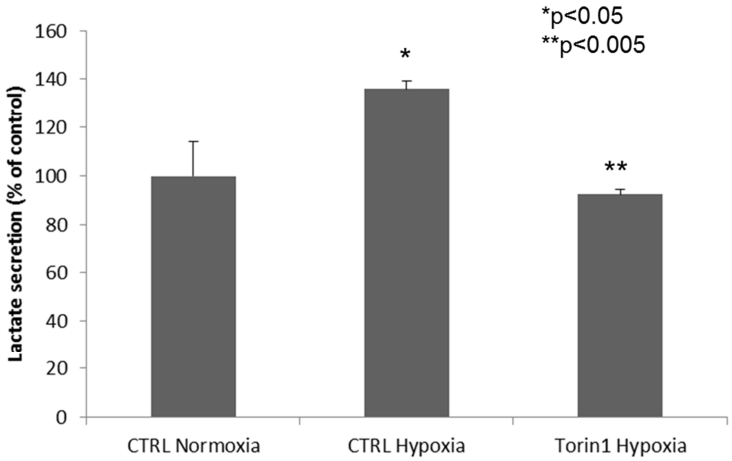


Figure 6.4 B-C. The Akt pathway influences glucose metabolism in PEL cells. (B) BCBL-1 cells were treated as indicated, then lactate secretion was measured after 6 hours in the culture medium. BCBL-1 cells were transfected with a constitutively active form of Akt, followed by extracellular lactate dosage with L-Lactate Assay Kit. (C) BCBL-1 cells were treated with torin-1 in hypoxia or normoxia. Lactate assay was performed in triplicate and values were normalized to cell number by trypan blue counts. Graph shows mean±S.D. *p<0,05, **p<0,005, *t-test*(B).

6.5 PEL cells are sensitive to glycolytic inhibitors

As already underlined, PEL cells are characterized by the Warburg phenotype (Bhatt AP, 2012) and their glycolytic flux is further increased when PEL cells are cultured in low O_2 conditions. The antiproliferative and cytotoxic effects of a panel of drugs targeting cancer metabolism were therefore examined in PEL cell lines. The antiproliferative effects of these compounds were evaluated not only in normoxia, but also under hypoxia, to reproduce the tumor *in vivo* environment. The following glycolytic inhibitors were tested: 2-deoxyglucose, 2-DG (Weindruch R, 2001) and lonidamine (Floridi A, 1981), which inhibit hexokinase; 3-bromo-pyruvate, 3-BrPa, and its pro-drug 3-bromo-pyruvate propyl ester, 3-BrOP, which target hexokinase and GAPDH (Tang Z, 2012); the LDH inhibitor oxamate (Papaconstantinou J, 1961); Dichloroacetate, DCA, acts instead by reversing the cancer-associated suppression of pyruvate dehydrogenase (through inhibition of pyruvate dehydrogenase kinase, PDHK1) thereby promoting the mitochondrial oxidation of pyruvate and its entry into oxidative phosphorylation (Stacpoole PW, 1989). PEL cells were treated with increasing concentrations of the abovementioned drugs and cell viability was estimated after 24 hours, by MTT assay. In addition, the proportion of apoptotic cells was monitored by propidium-iodide staining followed by flow cytometry analysis.

The ratio of the control signals was determined between oxygen deprived and normoxic cells to highlight variations in the proliferating abilities of the cells. At 24 hours the average log2 fold change between the hypoxia and normoxia MTT values was found to be 0.074 ± 2.56 (2-tailed paired t-test of hypoxia vs normoxia values =0.48) and is therefore not statistically significant. A time lapse of 24 hours was therefore suitable for subsequent experiments to exclude a potential alteration of cellular sensitivity to cytotoxic drugs due to a reduction in their proliferation ability.

Lonidamine had no detectable effect on cell viability at the maximum concentration tested (100 μM), even at longer incubation times (48 hours, data not shown) (Fig. 6.5 C). Moreover, cells were sensitive to 3-BrPA only at very high concentrations, of no clinical significance (EC50 values 175-232μM), about 20-fold higher than that of sensitive cell lines, which classifies BCBL-1 and HBL6 cells as resistant to 3-BrPA (Birsoy K, 2012).

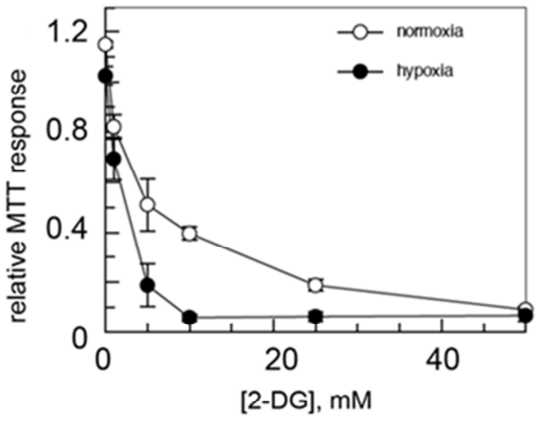
Similar to 3-BrPA, DCA is effective only at high concentrations, and is therefore not relevant for *in vivo* studies.

Conversely, both BCBL-1 and HBL6 cells were sensitive to 2-DG, 3-BrOP, and oxamate (Fig. 6.5 A, B, D, G), with EC50s of the same order of magnitude previously reported for sensitive cell lines (Liu H, 2001; Tang Z. 2012, Zhou M, 2010). In particular, cell viability diminishes following treatment with to 3-BrOP, a cell-permeable ester pro-drug of 3-BrPA (Fig. 6.5 D). Once inside the cell, 3-BrOP is hydrolyzed by cellular esterases releasing 3-BrPA, which then acts directly on its cellular targets. We therefore speculated that the reason for the 7 fold difference in the potency of the two compounds could reside in the inability of 3-BrPA to permeate the cell

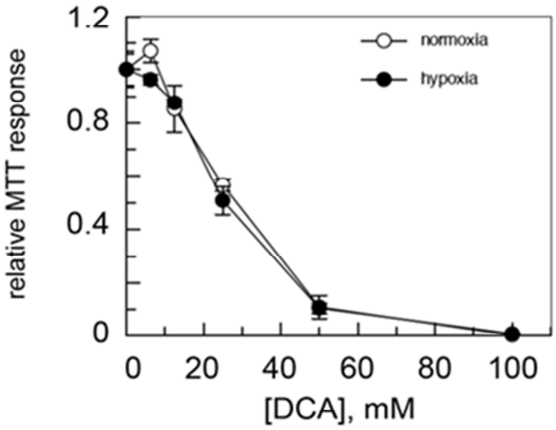
membrane. As both lonidamine and 3-BrPA are transported into the cell through the monocarboxylate transporter 1 (MCT1), and both compounds were found ineffective, we hypothesized that PEL cells express particularly low amounts of the MCT1 protein and would be for this reason inherently resistant to even high concentrations of the drugs. RT-PCR analysis confirmed low expression of the MCT1 transcript, as compared to MCT1 expression in cell lines sensitive to 3-BrPA (Figure 6.5 E).

Of note, 2-DG and oxamate were found to be more effective in hypoxia than in normoxia, whereas 3-BrOP had the same efficacy in either conditions (Fig. 6.5 F and Table1). All compounds were able to induce apoptosis, as measured by the increase in the sub G0-G1 cell population after treatment (Fig. 6.5 G). We conclude that PEL cells are sensitive to several glycolytic inhibitors, both in term of lactate production and apoptosis.

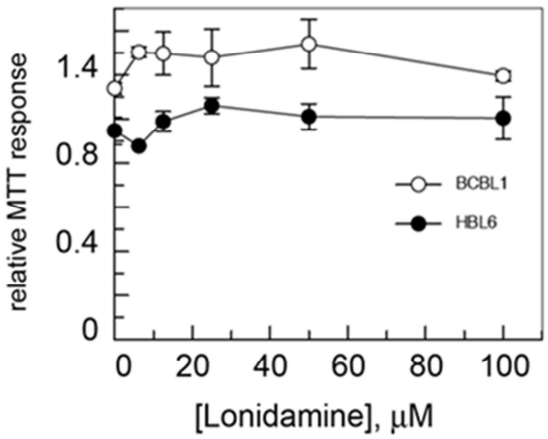
A.


relative MTT response
normoxia
hypoxia
[2-DG], mM

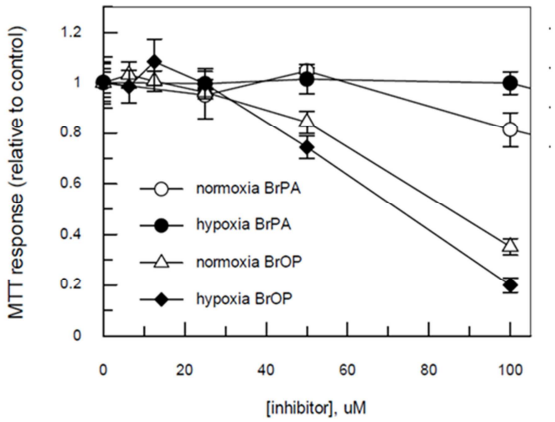
B.


relative MTT response
normoxia
hypoxia
[DCA], mM

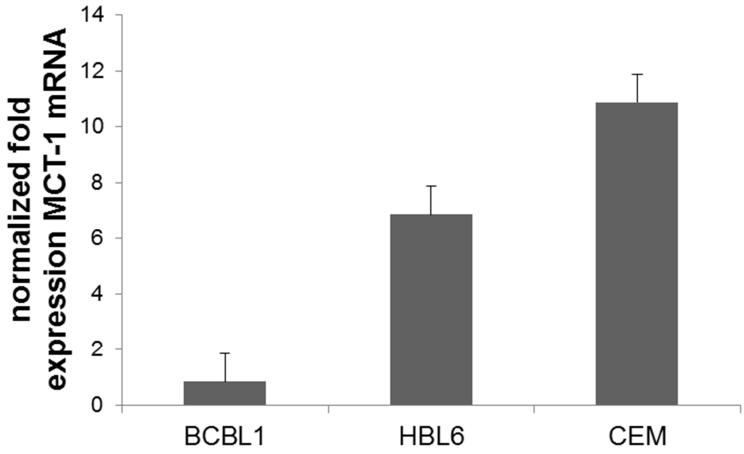
C.


relative MTT response
BCBL1
HBL6
[Lonidamine], µM

D.


MTT response (relative to control)
normoxia BrPA
hypoxia BrPA
normoxia BrOP
hypoxia BrOP
[inhibitor], uM

E.


normalized fold expression MCT-1 mRNA
BCBL1
HBL6
CEM

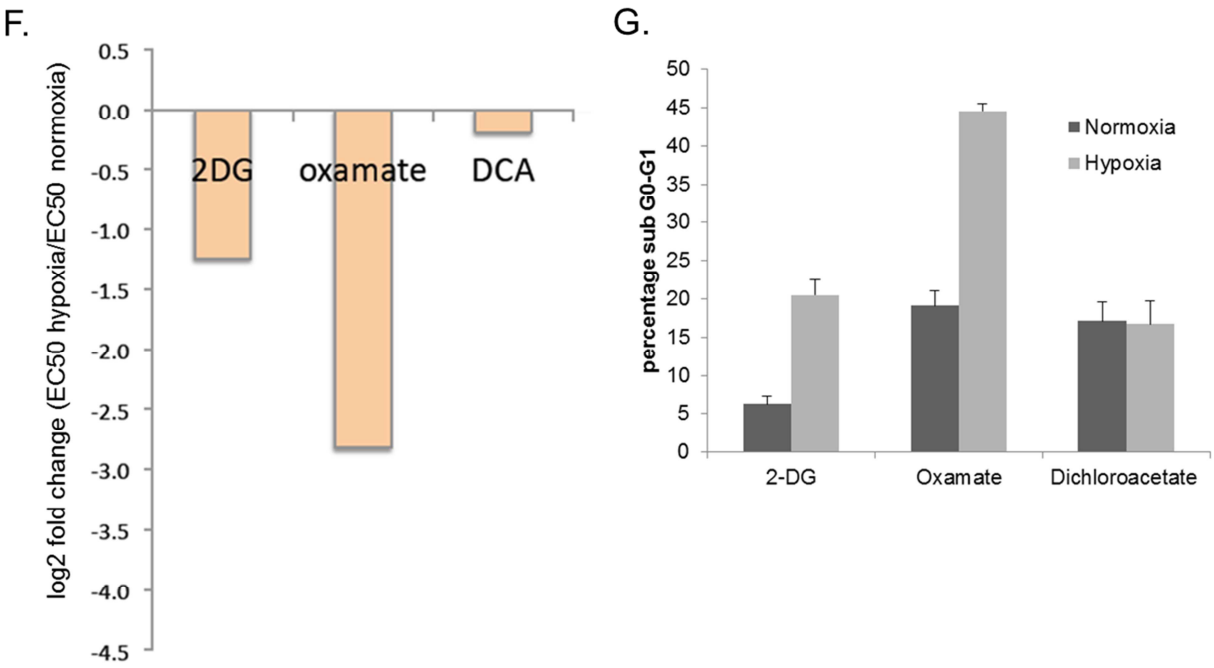


Figure 6.5. PEL cells are sensitive to glycolytic inhibitors. PEL cells were treated with the indicated concentrations of 2-DG (A), dichloroacetate (B), lonidamine (C), 3-BrA and 3-BrOP (D). MTT assays were performed in triplicates. The graphs show the mean ± S.D. (E) mRNA level of the glucose transporter MCT-1 in HBL6, BCBL1 and CEM cell lines. (F) Schematic representation of the log2 fold change of the ratio EC50 hypoxia/EC50 normoxia. (G) PI staining quantification of sub G0-G1 cells after treatment with 2-DG (5mM), oxamate (40mM) and DCA (30mM) for 24 hours under hypoxia or normoxia.

		BCBL1			HBL6		
Drug/compound	**Molecular target**	**EC50 21% O2**	**EC50 1% O2**	**ratio hEC50/nEC50**	**EC50 21% O2**	**EC50 1% O2**	**ratio hEC50/nEC50**
2-deoxyglucose	HXK, protein N-glycosylation	6.63 ± 2.98 mM	1.51 ± 0.16 mM	0.23*	5.10 ± 0.68 mM	2.43 ± 0.24 mM	0.48**
3-bromo-pyruvate	HXK, GAPDH	175.74±11.14 µM	232.39±11.14 µM	1.32**			
3-bromo-pyruvate propyl ester	HXK, GAPDH	34.39 ± 6.13 µM	38.13 ± 3.08 µM	1.11	43.86 ± 1.93 µM	52.61 ± 2.28 µM	1.19**
dichloroacetate	PDK	25.78 ± 1.29 µM	25.29 ± 0.70 µM	0.98	25.31 ± 0.92 µM	24.45 ± 2.88 µM	0.97
lonidamine	HXK-II	N.I. at 100 µM	N.I. at 100 µM	-	N.I. at 100 µM	N.I. at 100 µM	-
sodium-oxamate	LDH-A	76.40 ± 2.42 µM	44.76 ± 4.33 µM	0.58**	62.78 ± 3.64 µM	54.82 ± 3.21 µM	0.87*

Table 1. EC50 values for all drugs targeting cancer metabolism against the PEL cell lines BCBL-1 and HBL-6 cultured in normoxia or hypoxia. *p<0.05, **p<0.01 (*t test*).

6.6 Hypoxia alters PEL cells sensitivity to inhibitors of the PI3K/AKT/mTOR pathway

Based on the previous results, which show that hypoxia perturbates the PI3K/Akt/mTOR pathway in PEL cells and that inhibition of glycolysis by 2-DG and oxamate is favored by low oxygen, we asked whether hypoxia might alter cellular sensitivity to drugs targeting key components of the PI3K/Akt/mTOR cascade.

Therefore PEL cells were grown in hypoxia or in normoxia for 24 hours, upon addition to the culture medium of increasing concentrations of the following drugs: NVP-BEZ235, a dual PI3K/mTOR inhibitor; torin-1, an inhibitor of mTORC1/2; MK-2206 and Akti1/2, allosteric Akt inhibitors; Ly294002, the PI3K inhibitor, which remains a very useful tool for biochemical and pharmacological analysis, although deleterious side effects and low solubility in aqueous solutions have hampered its clinical development (Figure 3.5). Then, cell viability was analyzed by MTT assay. Table 2 summarizes the EC50 values of each drug and the hypoxic *vs* normoxic ratio. Unexpectedly, however, most drugs displayed a similar degree of efficacy both in normoxia and hypoxia (Table2 and Figure 6.6A-B). Surprisingly, although Akti1/2 and MK-2206 belong to the same family of Akt allosteric inhibitors and share most effects both *in vitro* and *in vivo*, MK-2206 is unaffected by low oxygen condition, whereas Akti1/2 efficacy is dramatically increased by hypoxia. This is probably due to the ability of Akti1/2 to block directly the glucose transporters, GLUT1 and GLUT4, independently of the ability of Akti1/2 to inhibit Akt (Tan SX, 2010).

The ability even of the drugs that show less efficacy in a low oxygen environment to downregulate phosphorylation of p70S6K and Akt S473 in either culturing conditions suggests that, at least at this time scale, hypoxia does not promote the onset of feedback mechanisms that restore/sustain the PI3K/Akt/mTOR pathway.

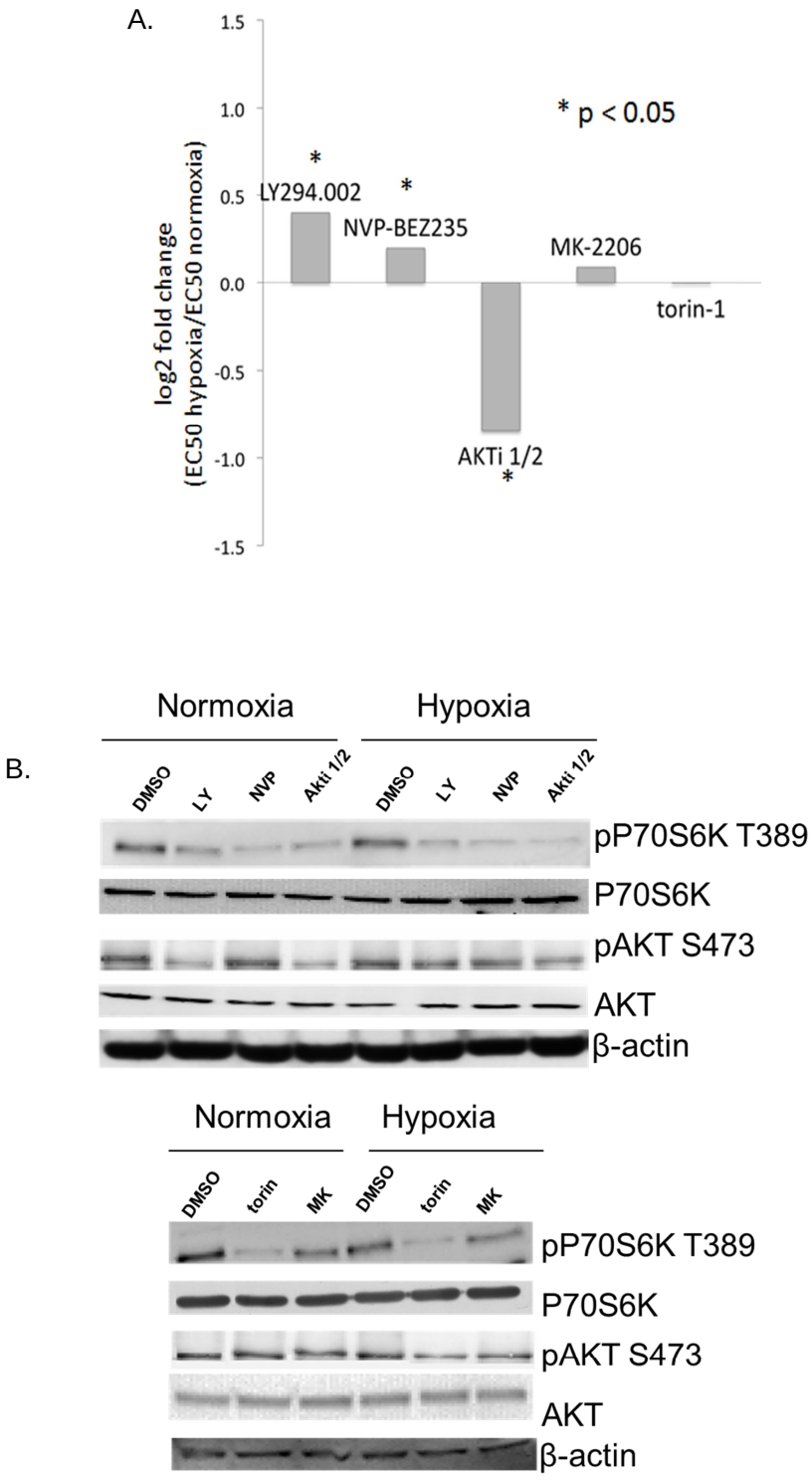


Figure 6.6. Hypoxia influences PEL cells sensitivity to Akti1/2 inhibitor. (A) Schematic representation of the log2 fold change of EC50hypoxia/EC50normoxia. *p <0.05 T test. (B) BCBL-1 cells were cultured for 24 hours in hypoxia or normoxia conditions and treated with Ly294-002 10µM, NVP-BEZ235 50nM, Akti1/2 10µM (upper panel), or torin-1 100nM and MK2206 10µM (lower panel). Equal amounts of cellular protein extract were resolved by SDS-PAGE and probed with the indicated antibodies. Equal loading was confirmed by anti-β-actin Western blotting.

		BCBL1			HBL6		
Drug/compound	Molecular target	EC50 21% O2	EC50 1% O2	ratio hEC50/nEC50	EC50 21% O2	EC50 1% O2	ratio hEC50/nEC50
NVP-BEZ235	PI3K/ mTOR	108.16 ± 2.73 nM	134.46 ± 8.89 nM	1.24*	40.07 ± 1.06 nM	177.10 ± 3.70 nM	4.42**
Akti 1/2	Akt	12.60 ± 0.94 µM	8.35 ± 1.04 µM	0.66*	13.04 ± 2.04 µM	5.33 ± 0.47 µM	0.41*
MK-2206	Akt	11.90 ± 1.14 µM	12.19 ± 0.62 µM	1.02	7.80 ± 1.05 µM	7.91 ± 1.34 µM	1.01
Torin-1	mTORC1 and 2	123.44 ± 12.62 nM	123.08 ± 26.09 nM	0.99			
LY294002	PI3K/mTOR	12.90 ± 0.80 µM	18.10 ± 0.39 µM	1.40**	24.68 ± 4.60 µM	39.42 ± 7.78 µM	1.60*

Table 2. EC50 values for all drugs targeting the PI3K/Akt/mTOR pathway against the PEL cell lines BCBL-1 and HBL-6 cultured in normoxia or hypoxia. *p<0.05, **p<0.01 (*t test*).

6.7 The glycolytic inhibitor 2-DG combined with the PI3K/Akt/mTOR pathway inhibitors display synergistic cytotoxicity in PEL cell lines

Next, due to promising results obtained in clinical studies with these molecules, we decided to investigate only the cytotoxicity of the glycolytic inhibitors 2-DG and 3-BrOP, in combination with drugs targeting the PI3K cascade. Indeed, 3-BrOP has an EC50 in the low micromolar range, and promising results have been reported in preclinical trials in a wide range of solid and hematological malignancies (Xu RH, 2005; Hulleman E, 2008). Thus, we tested the combination of 3-BrOP with the allosteric Akt inhibitor MK-2206 and with the dual PI3K/mTOR inhibitor NVP-BEZ235. Cells were exposed to a range of drug doses spanning the 50% of effective concentration for 24 hours, then cell viability was analyzed by MTT assay. The combination index (CI) values were calculated with the help of the CompuSyn software. According to the theorem of Chou-Talalay, which provides the following quantitative definition: CI = 1, additive effect, CI < 1, synergism, and CI > 1, antagonism in drug combinations, the CI=1 calculated in hypoxia, indicates an additive effect (Figure 6.7 A,B). The results in normoxia are very similar, but the overall EC50 of the combination is higher than in hypoxia (not shown).

In spite of its limited efficacy as a single agent, 2-DG offers the advantage of very low toxicity and high tolerability, as emerged from numerous clinical studies, (Stein M, 2010; Singh D, 2005; Dwarakanath SB, 2009) and potentiate the anticancer activity of conventional chemotherapy and even that of radiotherapy (Boutrid H, 2008; Mohanti BK, 1996). Thus, based on the cytotoxicity observed with single treatments, we next monitored the antiproliferative effects of 2-DG combined to inhibitors of PI3K/Akt/mTOR, in PEL cells cultured in normoxic or hypoxic conditions (Fgure 6.7 C-F).

Remarkably, the combination of 2-DG with the PI3K inhibitor, Ly-294002, displays a synergic effect both in normoxia (CI_{50}=0.52; CI_{75}=0.41; CI_{90}=0.32) and in hypoxia (CI_{50}=0.96; CI_{75}=0.76; CI_{90}=0.64), at all the concentrations tested. The same results were obtained for the combination with the allosteric Akt inhibitor, Akti1/2. Both in normoxia (CI_{50}=0.47; CI_{75}=0.38; CI_{90}=0.33) and in hypoxia (CI_{50}=0.78; CI_{75}=0.69; CI_{90}=0.63) the combinations show a synergic to strong synergic effect. These results were further confirmed by means of flow cytometric determination of the sub G0-G1, indicative of the percentage of cells undergoing apoptosis in response to 2-DG and Akti1/2 (Figure 6.7 F).On the contrary, the other Akt allosteric inhibitor, MK2206, show an antagonistic effect in hypoxia (CI_{50}=1.34; CI_{75}=1.15; CI_{90}=1.10) and only a moderate synergism in normoxia at high doses (CI_{50}=1.26; CI_{75}=0.85; CI_{90}=0.86).

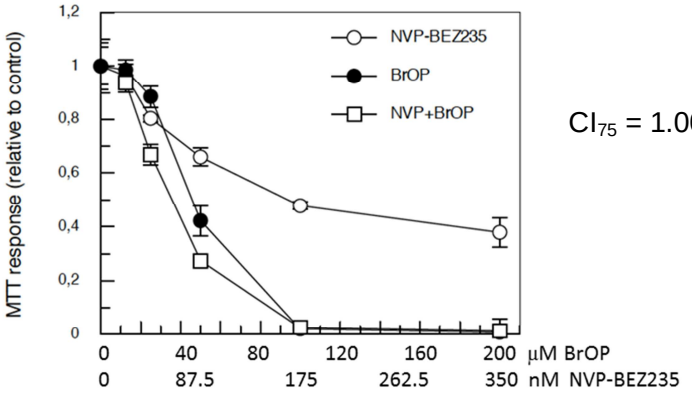
Interestingly, the combination of 2-DG with NVP-BEZ235 shows an increasing efficacy, with a nearly additive effect at lower doses, with a CI_{50}=1.15 in normoxia and CI_{50}=1.04 in hypoxia, and synergism at higher doses. The same trend was observed for the combination of 2-DG with the mTORC1/2 inhibitor, torin-1, in hypoxia: indeed an antagonistic effect was observed at low

doses (CI_{50}=1.31) compared to synergistic one at higher doses (CI_{75}=0.87; CI_{90}=0.59). On the contrary, the combination of 2-DG with torin-1 in normoxia has a synergic effect at all concentrations tested.

All the combinations tested have a synergic effect in HBL6 cells, confirming the promising results obtained in BCBL1 cells.

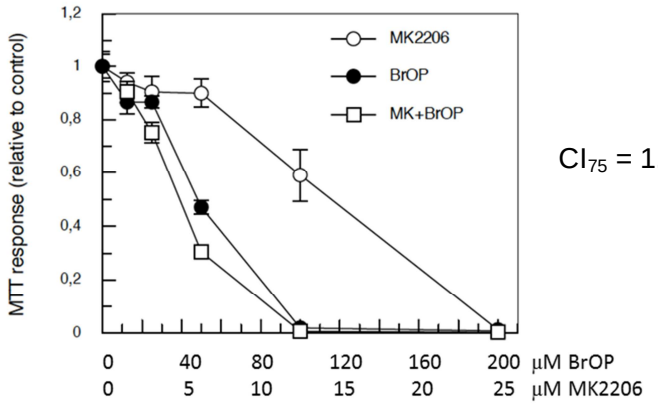
Interestingly, while NVP-BEZ235 and Ly294-002 as single drugs have low anti-proliferative effect in hypoxia, the association with 2-DG sensitizes PEL cells to these drugs, overcoming a possible mechanism of resistance. Notably, drug synergy allows to obtain the same antiproliferative effect at lower doses of drugs, and is therefore expected to reduce toxicity and side effects.

A.



CI_{75} = 1.00

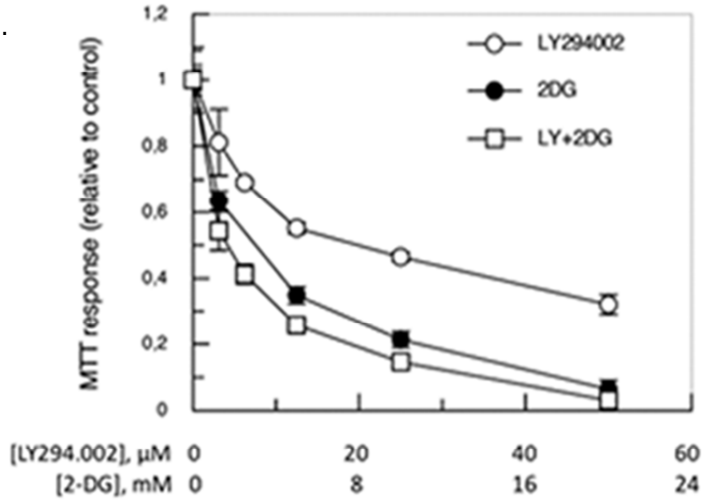
B.



CI_{75} = 1.08

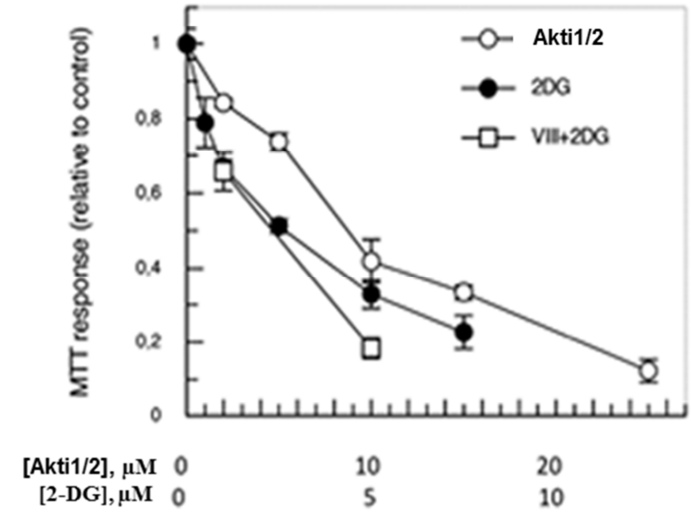
Figure 6.7 A-B. BCBL-1 cells were cultured in hypoxia for 24hours and treated with the indicated concentrations of 3-BrOP in combination with NVP-BEZ235 (A) or MK-2206 (B). The graphs show the MTT response relative to the control. The data are expressed as mean ± S.D. of three different measurements.

C.



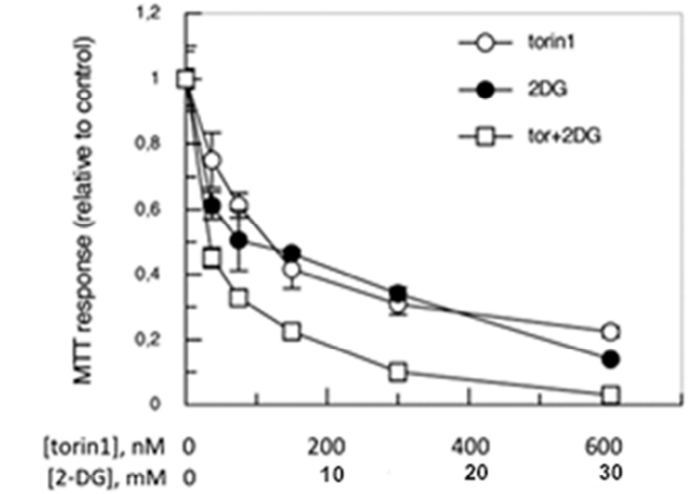
CI_{75} = 0.76

D.



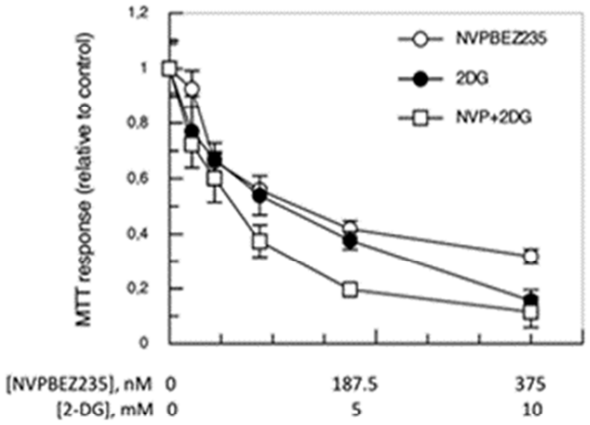
CI_{75} = 0.69

E.



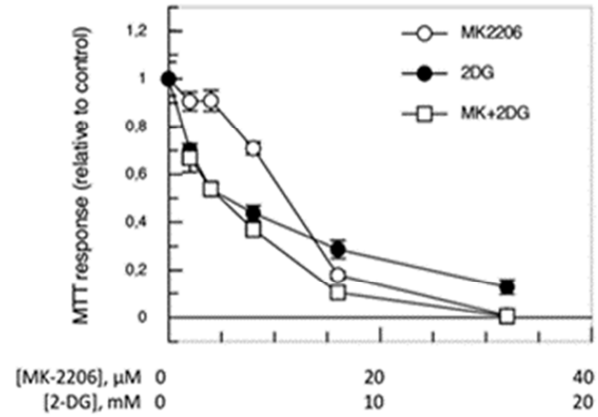
CI_{75} = 0.87

F.



CI_{75} = 0.80

G.



CI_{75} = 1.15

H.

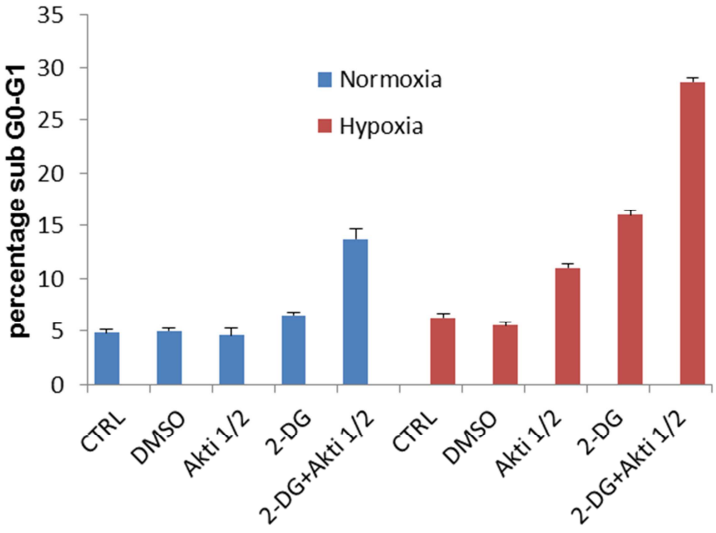


Figure 6.7 C-H. BCBL1 cells were cultured for 24 hours in hypoxia and treated with or without 2-DG, alone (C) or combined to Ly294-002; (D) Akti1/2; (E) torin-1 ;(F) NVP-BEZ235 (G); MK-2206, at the concentrations indicated. Then the response was analyzed by MTT. The results of both single and combined treatments is reported, as mean ± S.D. of three different measurements. (H) BCBL-1 cells treated for 24 hours in hypoxia or normoxia and treated with 2.5mM 2-DG and 7.5µM Akti1/2, alone or in combination. Then, the percentage of sub G0-G1 cells was determined by PI-staining followed by a flow cytometry analysis.

		BCBL1			HBL6		
Drug 1	Drug 2	EC_{75} C.Hyp/ EC_{75} C.Norm	C.I. (50;75;90) normoxia	C.I. (50;75;90) hypoxia	EC_{75} C.Hyp/ EC_{75} C.Norm	C.I. (50;75;90) normoxia	C.I. (50;75;90) hypoxia
NVP-BEZ235	2-deoxyglucose	0.99	1.15;0.64;0.37	1.04;0.80;0.64	1.04	0.61;0.57;0.55	0.56;0.84;1.29
Akti 1/2	2-deoxyglucose	0.35	0.47;0.38;0.33	0.78;0.69;0.63	0.87	0.42;0.34;0.28	0.61;0.61;0.61
MK-2206	2-deoxyglucose	0.85	1.26;0.85;0.86	1.34;1.15;1.10	1.01	0.60;0.41;0.29	0.93;0.48;0.25
Torin-1	2-deoxyglucose	1.05	0.77;0.50;0.33	1.30;0.87;0.59			
LY294002	2-deoxyglucose	1.03	0.52;0.41;0.32	0.96;0.76;0.64	0.83	0.56;0.59;0.65	0.86;0.81;0.82

Table 3: C.I. values calculated with the CompuSyn software for the combinations between 2-deoxyglucose and a panel of PI3K/Akt/mTOR pathway inhibitors (NVP-BEZ235; Akti 1/2; MK-2206; torin-1; LY294002) in HBL6 and BCBL1 cell lines cultured in normoxia or hypoxia.

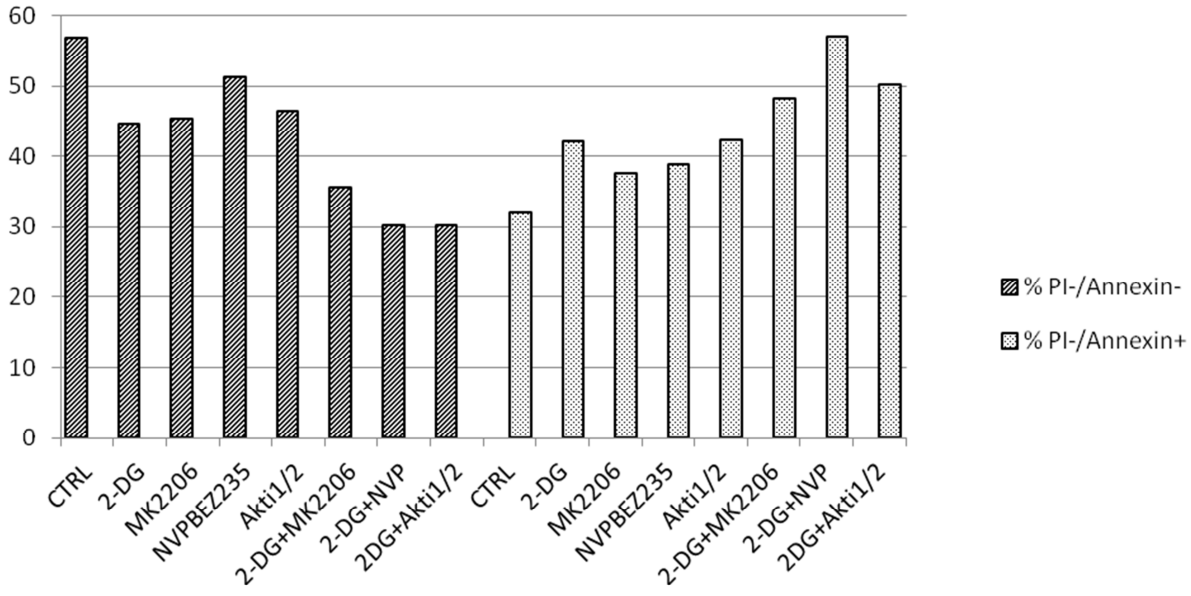
		BCBL1		
Drug 1	Drug 2	EC_{75} C.Hyp / EC_{75} C.Norm.	C.I. (50; 75;90) normoxia	C.I. (50; 75;90) hypoxia
MK-2206	3-bromo-pyruvate propyl ester	0.86	1.04; 1.09; 1.14	1.15; 1.08; 1.02
NVP-BEZ235	3-bromo-pyruvate propyl ester	1.14	1.09; 0.87; 0.94	1.00; 0.95; 0.90

Table 4 C.I. values calculated with the CompuSyn software for the combinations between 3-bromo-pyruvate propyl ester and the PI3K/Akt/mTOR pathway inhibitors MK-2206 and NVP-BEZ235 in BCBL1 cells cultured in normoxia and hypoxia

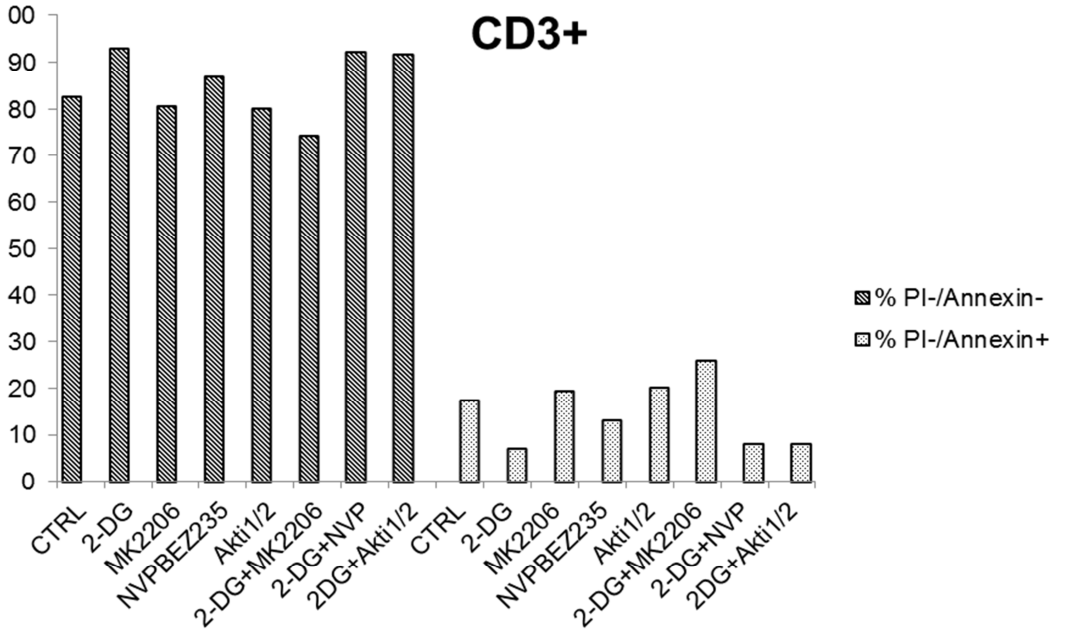
6.8 Combined targeting of glycolysis and PI3K/Akt/mTOR signaling displays low toxicity to normal B and T lymphocytes

To evaluate the suitability of combined inhibition of glycolysis and PI3K/Akt/mTOR signaling as a new pharmacological approach, the combinations showing the more promising results in PEL cell lines were tested *in vitro* in B and T lymphocytes from healthy donors. In agreement with the results shown in figures 6.7 C-H, co-treatment of BCBL-1 cells for 24 hours confirms that 2-DG, though scarcely cytotoxic if added as single drug, becomes much more cytotoxic in combination with either of PI3K/Akt/mTOR pathway inhibitors, resulting in a significant reduction of cell viability (Figure 6.9 A) and increased apoptosis (Figure 6.8 B). On the contrary, all the combinations tested show very low cytotoxicity to normal B lymphocytes (Figure 6.8 C) and no toxicity to normal CD3+ T lymphocytes (Figure 6.8 D). In particular, association of 2-DG with the dual inhibitor NVP-BEZ235 triggers apoptosis in more than 70% of BCBL-1 cells at 24 hours, whereas the same combination fails to evoke a detectable augment of Annexin-stained cells after 24 hours in CD3+ lymphocytes, compared to their untreated counterpart. Moreover, in the same setting the increase of apoptosis is limited to 10% in CD19+ lymphocytes, indicating that this combination indeed warrants further investigation.

A.



B.



C.

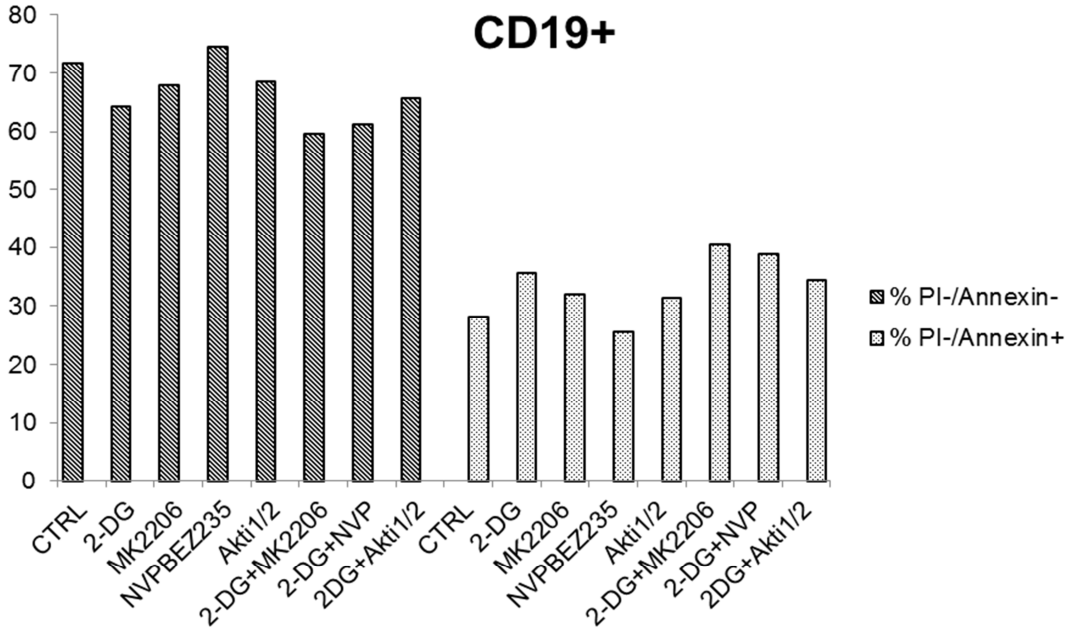


Figure 6.8. BCBL-1 cells and PBMCs from healthy donors were treated for 24h with 2-DG (6mM), MK-2206 (15μM), NVP-BEZ235 (100nM), Akti1/2 (10μM), as single drugs or in combination as indicated. Cells were stained with propidium iodide and Annexin V followed by flow cytometry analysis. (A) double negative PI-/Annexin V- cells represents the percentage of viable BCBL-1 cells. Annexin V positive cells indicate the percentage of apoptotic cells, relative to untreated controls. (B) (C) PBMCs were labeled with anti-CD19 and with anti-CD3 to detect B-lymphocytes (B) and T-lymphocyte (C). The graphs show the percentage of viable (double negative PI-/Annexin V) and apoptotic (PI-/Annexin V positive) CD19 (B) and CD3 (C) cells.

7. Discussion

Decreased oxygenation is a well known factor affecting tumor cells chemosensitivity. Hypoxia adaptation occurs through various biochemical responses such as the activation of cell survival pathways, the regulation of glucose/energy metabolism and the stimulation of angiogenesis and metastasis. These responses are directly or indirectly involved in the mode of action of many chemotherapeutic drugs, often making them less effective against hypoxic tumor cells.

Tumor hypoxia has been more thoroughly studied in the context of solid tumors, which often experience severe hypoxia or anoxia in the unvascularised tumor core (Hockel M, 1996; Vaupel, P 2001). The poor/aberrant vasculature results in a reduced exposure of the anticancer drug to cells growing in those areas, as well as a decrease in cellular proliferation, a high rate of which is required for many chemotherapeutics to exert the maximum effect. PEL is a rare subtype of B-NHLs, caused by the infection of the KSHV (Knowles DM, 2003), that manifests as a liquid effusion which, as it characteristically arises in pleurae and other body cavities, experiences a highly hypoxic environment (Funahashi A, 1971; Houston MC, 1981). Like other B-NHL, PEL is characterized by the Warburg phenotype (Bhatt AP, 2012), a peculiar metabolic rewiring that shifts tumor cells towards elevated glycolytic flux and enhanced lactate production even under normoxia. Based on these evidences, in this study we tested the effect on PEL cells of a panel of six drugs targeting key metabolic enzymes. Our findings established that PEL cells are sensitive only to 3 out of 6 drugs. We observed a dramatic antiproliferative effect upon 24 incubation of cells with 2-DG, a non-metabolized analog of glucose, that blocks the first step of glycolysis. A similar effect was observed with sodium-oxamate, which blocks the LDH-A enzyme, and with 3BrPO. Remarkably, 2-DG and oxamate are more effective in PEL cells cultured in hypoxia.

The dysregulation of many oncogenes (PI3K/Akt, c-myc, Ras) and tumor suppressors (p53, PTEN, LKB1) has been shown to favor the induction of the Warburg phenotype, by affecting the expression and activity of key components of cell metabolism. In particular, the protein kinase Akt is indeed regarded as the 'Warburg kinase' because of its ability to promote aerobic glycolysis and interfere with the mitochondrial function. Besides, the action of the KSHV proteins, K1 and vGPCR, has been suggested to activate the PI3K/Akt/mTOR pathway, and some reports indicate a role of this pathway in PEL pathogenesis and its aggressive phenotype.

By phosphoproteome analysis we confirm here that the abovementioned pathway is indeed highly active in the PEL cell lines utilized in this study and, what's more, that the inhibition of the PI3K/Akt/mTOR pathway influences cell viability, both in hypoxia and in normoxia culturing conditions. Therefore this pathway can be considered a druggable target in PEL. Furthermore, we show that the Akt pathway modulates glucose metabolism, as treatment with pathway-

specific drugs results in a reduction, whereas expression of a constitutively active form of Akt produces a significant increase, of lactate secretion. All together, these results open a therapeutic window for PEL, based on combined targeting of glycolysis and activated signaling. To evaluate this new pharmacological approach, we tested the combination of the glycolytic inhibitors 2-DG and 3-BrOP with a panel of PI3K/Akt/mTOR pathway inhibitors. The combination of the new glycolytic inhibitor 3-BrOP, which blocks the HKII enzyme, with either MK-2206 or NVP-BEZ235, show an additive to synergistic effect in hypoxia. Similarly, combinations of 2-DG with either Ly-294002 and torin-1 show an additive effect. Remarkably, however, 2-DG co-treatments with Akti1/2 or NVP-BEZ235 are highly effective and display strong synergistic action. Thus the same antiproliferative effect should be obtained at lower concentration reducing the overall toxicity of drugs. 2-DG is currently used in clinical trials for the treatment of advanced solid tumors (Stein M, 2010; Mohanti BK, 1996; Raez LE, 2013), and is well tolerated, with no acute toxicity. It is not effective as a single agent, though, but it has been shown to increase the efficacy of radiotherapy and chemotherapy, both *in vitro and in vivo*. Hypoxia is important for tumor progression and is involved in the resistance to antitumor treatment, as shown by association of HIF1α overexpression with poor prognosis. In this study, we present evidence that 2-DG is able to increase the sensitivity of PEL cells, cultured in low oxygen to mimic their natural environment, also both to NVPBEZ235 and Ly294-002, thus preventing resistance to the treatment in hypoxia.

Importantly, we demonstrate that the combination of 2-DG with either Akti1/2, MK-2206 or NVPBEZ235 is not toxic to normal T and B lymphocytes.

In conclusion, PEL is a severe B-NHL with an aggressive clinical course resulting in very poor outcome, and urgent unmet need for improved targeted agents. The results presented in this study pave the way for a new therapeutic strategy, based on the combination of glycolytic inhibitors and PI3K/Akt/mTOR pathway inhibitors. Importantly, since these drugs are very effective in hypoxia as well as in normoxia, this strategy might also be extended to other B-NHL, characterized by the Warburg phenotype, which do not occur under low oxygen, and should therefore be explored not only in PEL but also in B-NHL clinical setting.

8. References

- Aft RL, Zhang FW, Gius D. Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death. Br J Cancer. 2002 Sep 23;87(7):805-12.
- Alexander A, Walker CL. Differential localization of ATM is correlated with activation of distinct downstream signaling pathways. Cell Cycle. 2010 Sep 15;9(18):3685-6
- Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005 Nov 14;24(50):7455-64. Review.
- Alvarez-Tejado M, Alfranca A, Aragonés J, Vara A, Landázuri MO, del Peso L. Lack of evidence for the involvement of the phosphoinositide 3-kinase/Akt pathway in the activation of hypoxia-inducible factors by low oxygen tension. J Biol Chem. 2002 Apr 19;277(16):13508-17.
- An J, Sun Y, Fisher M, Rettig MB. Antitumor effects of bortezomib (PS-341) on primary effusion lymphomas. 2004 Oct;18(10):1699-704.
- Antoch G, Saoudi N, Kuehl H, Dahmen G, Mueller SP, Beyer T, Bockisch A, Debatin JF, Freudenberg LS. Accuracy of whole-body dual-modality fluorine-18-2-fluoro-2-deoxy-D-glucose positron emission tomography and computed tomography (FDG-PET/CT) for tumor staging in solid tumors: comparison with CT and PET. J Clin Oncol. 2004 Nov 1;22(21):4357-68.
- Aoki Y, Jaffe ES, Chang Y, Jones K, Teruya-Feldstein J, Moore PS, Tosato G. Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. Blood. 1999 Jun 15;93(12):4034-43.
- Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. J Biol Chem. 2003 Aug 8;278(32):29655-60.
- Arsham AM, Plas DR, Thompson CB, Simon MC. Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. Cancer Res. 2004 May 15;64(10):3500-7.
- Arvanitakis L, Geras-Raaka E, Varma A, Gershengorn MC, Cesarman E. Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. Nature. 1997 Jan 23;385(6614):347-50
- Babu E, Ramachandran S, CoothanKandaswamy V, Elangovan S, Prasad PD, Ganapathy V, Thangaraju M. Role of SLC5A8, a plasma membrane transporter and a tumor suppressor, in the antitumor activity of dichloroacetate. Oncogene. 2011 Sep 22;30(38):4026-37
- Bais C, Santomasso B, Coso O, Arvanitakis L, Raaka EG, Gutkind JS, Asch AS, Cesarman E, Gershengorn MC, Mesri EA. G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. Nature. 1998 Jan 1;391(6662):86-9.
- Barnard JP, Reynafarje B, Pedersen PL. Glucose catabolism in African trypanosomes. Evidence that the terminal step is catalyzed by a pyruvate transporter capable of facilitating uptake of toxic analogs. J Biol Chem. 1993 Feb 15;268(5):3654-61.
- Barnes K, Ingram JC, Porras OH, Barros LF, Hudson ER, Fryer LG, Foufelle F, Carling D, Hardie DG, Baldwin SA. Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J Cell Sci. 2002 Jun 1;115(Pt 11):2433-42.
- Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, Kahana JA, Kral AM, Leander K, Lee LL, Malinowski J, McAvoy EM, Nahas DD, Robinson RG, Huber HE. Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J. 2005 Jan 15;385(Pt 2):399-408.
- Barthel A, Okino ST, Liao J, Nakatani K, Li J, Whitlock JP Jr, Roth RA. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. J Biol Chem. 1999 Jul 16;274(29):20281-6.
- Bauer DE, Hatzivassiliou G, Zhao F, Andreadis C, Thompson CB. ATP citrate lyase is an important component of cell growth and transformation. Oncogene. 2005 Sep 15;24(41):6314-22.
- Baumann P, Mandl-Weber S, Oduncu F, Schmidmaier R.The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma. Exp Cell Res. 2009 Feb 1;315(3):485-97
- Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN, Richard CW 3rd, Cornelisse CJ, Devilee P, Devlin B. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science. 2000 Feb 4;287(5454):848-51.
- Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene. 1998 Jul 23;17(3):313-25.

- Belzacq AS, El Hamel C, Vieira HL, Cohen I, Haouzi D, Métivier D, Marchetti P, Brenner C, Kroemer G. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene. 2001 Nov 15;20(52):7579-87.
- Bennewith KL, Durand RE. Quantifying transient hypoxia in human tumor xenografts by flow cytometry. Cancer Res. 2004 Sep 1;64(17):6183-9.
- Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell. 2006 Jul 14;126(1):107-20.
- Bernardi R, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, Cordon-Cardo C, Simon MC, Rafii S, Pandolfi PP. PML inhibits HIF-1alpha translation and neoangiogenesis through repression of mTOR. Nature. 2006 Aug 17;442(7104):779-85.
- Berra E, Richard DE, Gothié E, Pouysségur J. HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1alpha degradation. FEBS Lett. 2001 Feb 23;491(1-2):85-90.
- Berruti A, Bitossi R, Gorzegno G, Bottini A, Alquati P, De Matteis A, Nuzzo F, Giardina G, Danese S, De Lena M, Lorusso V, Farris A, Sarobba MG, DeFabiani E, Bonazzi G, Castiglione F, Bumma C, Moro G, Bruzzi P, Dogliotti L; Epirubicin-Lonidamine Group, Orbassano, Torino, Italy. Time to progression in metastatic breast cancer patients treated with epirubicin is not improved by the addition of either cisplatin or lonidamine: final results of a phase III study with a factorial design. J Clin Oncol. 2002 Oct 15;20(20):4150-9.
- Berwick DC, Hers I, Heesom KJ, Moule SK, Tavare JM The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. J Biol Chem. 2002 Sep 13;277(37):33895-900. Epub 2002 Jul 9.
- Bhatt AP, Bhende PM, Sin SH, Roy D, Dittmer DP, Damania B. Dual inhibition of PI3K and mTOR inhibits autocrine and paracrine proliferative loops in PI3K/Akt/mTOR-addicted lymphomas. Blood. 2010 Jun 3;115(22):4455-63
- Bhatt AP, Jacobs SR, Freemerman AJ, Makowski L, Rathmell JC, Dittmer DP, Damania B. Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma. Proc Natl Acad Sci U S A. 2012 Jul 17;109(29):11818-23
- Birsoy K, Wang T, Possemato R, Yilmaz OH, Koch CE, Chen WW, Hutchins AW, Gultekin Y, Peterson TR, Carette JE, Brummelkamp TR, Clish CB, Sabatini DM. MCT1-mediated transport of a toxic molecule is an effective strategy for targeting glycolytic tumors. Nat Genet. 2013 Jan;45(1):104-8.
- Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Bonnet S, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B, Michelakis ED. A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell. 2007 Jan;11(1):37-51.
- Boshoff C, Gao SJ, Healy LE, Matthews S, Thomas AJ, Coignet L, Warnke RA, Strauchen JA, Matutes E, Kamel OW, Moore PS, Weiss RA, Chang Y. Establishing a KSHV+ cell line (BCP-1) from peripheral blood and characterizing its growth in Nod/SCID mice. Blood. 1998 Mar 1;91(5):1671-9.
- Boulanger E, Daniel MT, Agbalika F, Oksenhendler E. Combined chemotherapy including high-dose methotrexate in KSHV/HHV8-associated primary effusion lymphoma. Am J Hematol. 2003 Jul;73(3):143-8.
- Boulanger E, Gérard L, Gabarre J, Molina JM, Rapp C, Abino JF, Cadranel J, Chevret S, Oksenhendler E. Prognostic factors and outcome of human herpesvirus 8-associated primary effusion lymphoma in patients with AIDS. J Clin Oncol. 2005 Jul 1;23(19):4372-80.
- Boulanger E, Meignin V, Afonso PV, Duprez R, Oksenhendler E, Agbalika F, Gessain A. Extracavitary tumor after primary effusion lymphoma: relapse or second distinct lymphoma? Haematologica. 2007 Sep;92(9):1275-6.
- Boulanger E, Meignin V, Oksenhendler E. Bortezomib (PS-341) in patients with human herpesvirus 8-associated primary effusion lymphoma. Br J Haematol. 2008 May;141(4):559-61.
- Boutrid H, Jockovich ME, Murray TG, Piña Y, Feuer WJ, Lampidis TJ, Cebulla CM. Targeting hypoxia, a novel treatment for advanced retinoblastoma. Invest Ophthalmol Vis Sci. 2008 Jul;49(7):2799-805
- Bové J, Perier C. Neurotoxin-based models of Parkinson's disease. Neuroscience. 2012 Jun 1;211:51-76.
- Boxer MB, Jiang JK, Vander Heiden MG, Shen M, Skoumbourdis AP, Southall N, Veith H, Leister W, Austin CP, Park HW, Inglese J, Cantley LC, Auld DS, Thomas CJ. Evaluation of substituted N,N'-diarylsulfonamides as activators of the tumor cell specific M2 isoform of pyruvate kinase. J Med Chem. 2010 Feb 11;53(3):1048-55.

- Brahimi-Horn MC, Chiche J, Pouysségur J. Hypoxia signalling controls metabolic demand. Curr Opin Cell Biol. 2007 Apr;19(2):223-9
- Brizel DM, Dodge RK, Clough RW, Dewhirst MW. Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol. 1999 Nov;53(2):113-7.
- Brown JM. Exploiting tumour hypoxia and overcoming mutant p53 with tirapazamine. Br J Cancer. 1998 Jun;77 Suppl 4:12-4..
- Brown JM. SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. Br J Cancer. 1993 Jun;67(6):1163-70.
- Brown JM. The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. Cancer Res. 1999 Dec 1;59(23):5863-70.
- Brugarolas J, Lei K, Hurley RL, Manning BD, Reiling JH, Hafen E, Witters LA, Ellisen LW, Kaelin WG Jr. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev. 2004 Dec 1;18(23):2893-904. Epub 2004 Nov 15.
- Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001 Nov 9;294(5545):1337-40
- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999 Mar 19;96(6).
- Brunn GJ, Williams J, Sabers C, Wiederrecht G, Lawrence JC Jr, Abraham RT. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. EMBO J. 1996 Oct 1;15(19):5256-67.
- Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem. 2005 Feb 18;280(7):5636-45
- Buller CL, Loberg RD, Fan MH, Zhu Q, Park JL, Vesely E, Inoki K, Guan KL, Brosius FC 3rd. A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. Am J Physiol Cell Physiol. 2008 Sep;295(3):C836-43.
- Buonaiuto D, Rossi D, Guidetti F, Vivenza D, Berra E, Deambrogi C, Ariatti C, Franceschetti S, Conconi A, Ronco M, Valente G, Colombi S. Human herpesvirus type 8-associated primary lymphomatous effusion in an elderly HIV-negative patient: clinical and molecular characterization. Ann Ital Med Int. 2002 Jan-Mar;17(1):54-9.
- Burgering BM, Medema RH. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol. 2003 Jun;73(6):689-701. Review.
- Burgess DJ. Metabolism: Glutamine connections. Nat Rev Cancer. 2013 May;13(5):293
- Burris H, Rodon J, Sharma RS, et al. ASCO. Alexandria, VA: American Society of Clinical Oncology; 2010. First-in-human phase I study of the oral PI3K inhibitor BEZ235 in patients (pts) with advanced solid tumors; p. 3005.
- Cai QL, Knight JS, Verma SC, Zald P, Robertson ES. EC5S ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. PLoS Pathog. 2006 Oct;2(10):e116.
- Calera MR, Martinez C, Liu H, Jack AK, Birnbaum MJ, Pilch PF. Insulin increases the association of Akt-2 with Glut4-containing vesicles. J Biol Chem. 1998 Mar 27;273(13):7201-4.
- Calleja V, Alcor D, Laguerre M, Park J, Vojnovic B, Hemmings BA, Downward J, Parker PJ, Larijani B Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. PLoS Biol. 2007 Apr;5(4):e95.
- Calleja V, Laguerre M, Parker PJ, Larijani B. Role of a novel PH-kinase domain interface in PKB/Akt regulation: structural mechanism for allosteric inhibition. PLoS Biol. 2009 Jan 20;7(1):e17
- Calviño E, Estañ MC, Simón GP, Sancho P, Boyano-Adánez Mdel C, de Blas E, Bréard J, Aller P. Increased apoptotic efficacy of lonidamine plus arsenic trioxide combination in human leukemia cells. Reactive oxygen species generation and defensive protein kinase (MEK/ERK, Akt/mTOR) modulation. Biochem Pharmacol. 2011 Dec 1;82(11):1619-29
- Cao P, Maira SM, García-Echeverría C, Hedley DW. Activity of a novel, dual PI3-kinase/mTor inhibitor NVP-BEZ235 against primary human pancreatic cancers grown as orthotopic xenografts. Br J Cancer. 2009 Apr 21;100(8):1267-76
- Cao W, Yacoub S, Shiverick KT, Namiki K, Sakai Y, Porvasnik S, Urbanek C, Rosser CJ. Dichloroacetate (DCA) sensitizes both wild-type and over expressing Bcl-2 prostate cancer cells in vitro to radiation. Prostate. 2008 Aug 1;68(11):1223-31

- Carbone A, Gloghini A, Cozzi MR, Capello D, Steffan A, Monini P, De Marco L, Gaidano G. Expression of MUM1/IRF4 selectively clusters with primary effusion lymphoma among lymphomatous effusions: implications for disease histogenesis and pathogenesis. Br J Haematol. 2000 Oct;111(1):247-57.
- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. Science. 1998 Nov 13;282(5392):1318-21.
- Carling D, Sanders MJ, Woods A. The regulation of AMP-activated protein kinase by upstream kinases. Int J Obes (Lond). 2008 Sep;32 Suppl 4:S55-9
- Carroll PA, Kenerson HL, Yeung RS, Lagunoff M. Latent Kaposi's sarcoma-associated herpesvirus infection of endothelial cells activates hypoxia-induced factors. J Virol. 2006 Nov;80(21):10802-12.
- Cesarman E, Chang Y, Moore PS, Said JW, Knowles DM. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med. 1995 May 4;332(18):1186-91.
- Cesarman E, Nador RG, Bai F, Bohenzky RA, Russo JJ, Moore PS, Chang Y, Knowles DM. Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. J Virol. 1996 Nov;70(11):8218-23.
- Chang Y, Cesarman E, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994 Dec 16;266(5192):1865-9.
- Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem. 2001 Mar 23;276(12):9519-25. Epub 2000 Dec 18.
- Chen S, Murphy J, Toth R, Campbell DG, Morrice NA, Mackintosh C. Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. Biochem J. 2008 Jan 15;409(2):449-59.
- Chen YB, Rahemtullah A, Hochberg E Primary effusion lymphoma. Oncologist. 2007 May;12(5):569-76. Review.
- Chen Z, Zhang H, Lu W, Huang P. Role of mitochondria-associated hexokinase II in cancer cell death induced by 3-bromopyruvate. Biochim Biophys Acta. 2009 May;1787(5):553-60
- Cherrin C, Haskell K, Howell B, Jones R, Leander K, Robinson R, Watkins A, Bilodeau M, Hoffman J, Sanderson P, Hartman G, Mahan E, Prueksaritanont T, Jiang G, She QB, Rosen N, Sepp-Lorenzino L, Defeo-Jones D, Huber HE. An allosteric Akt inhibitor effectively blocks Akt signaling and tumor growth with only transient effects on glucose and insulin levels in vivo. Cancer Biol Ther. 2010 Apr 1;9(7):493-503
- Chiara F, Castellaro D, Marin O, Petronilli V, Brusilow WS, Juhaszova M, Sollott SJ, Forte M, Bernardi P, Rasola A. Hexokinase II detachment from mitochondria triggers apoptosis through the permeability transition pore independent of voltage-dependent anion channels. PLoS One. 2008 Mar 19;3(3)
- Chiche J, Ilc K, Laferrière J, Trottier E, Dayan F, Mazure NM, Brahimi-Horn MC, Pouysségur J. Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res. 2009 Jan 1;69(1):358-68.
- Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem. 2001 Oct 19;276(42):38349-52. Epub 2001 Aug 31.
- Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010 Jan 15;70(2):440-6
- Clarke PR, Hardie DG. Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver. EMBO J. 1990 Aug;9(8):2439-46.
- Cotter MA 2nd, Robertson ES. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. Virology. 1999 Nov 25;264(2):254-64.
- Crute BE, Seefeld K, Gamble J, Kemp BE, Witters LA. Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. J Biol Chem. 1998 Dec 25;273(52):35347-54.
- Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007 Nov 29;450(7170):736-40.
- Dang CV. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol. 1999 Jan;19(1):1-11

- Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM. Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature. 2010 Jun 17;465(7300):966
- Datta SR, Katsov A, Hu L, Petros A, Fesik SW, Yaffe MB, Greenberg ME. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell. 2000 Jul;6(1):41-51.
- Davies SP, Carling D, Munday MR, Hardie DG. Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. Eur J Biochem. 1992 Feb 1;203(3):615-23.
- Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 2000 Oct 1;351(Pt 1):95-105.
- Davis DA, Rinderknecht AS, Zoeteweij JP, Aoki Y, Read-Connole EL, Tosato G, Blauvelt A, Yarchoan R. Hypoxia induces lytic replication of Kaposi sarcoma-associated herpesvirus. Blood. 2001 May 15;97(10):3244-50.
- De Cesare M, Pratesi G, Giusti A, Polizzi D, Zunino F. Stimulation of the apoptotic response as a basis for the therapeutic synergism of lonidamine and cisplatin in combination in human tumour xenografts. Br J Cancer. 1998;77(3):434-9.
- de Frias M, Iglesias-Serret D, Cosialls AM, Coll-Mulet L, Santidrián AF, González-Gironès DM, de la Banda E, Pons G, Gil J. Akt inhibitors induce apoptosis in chronic lymphocytic leukemia cells. Haematologica. 2009 Dec;94(12):1698-707
- De Lena M, Lorusso V, Latorre A, Fanizza G, Gargano G, Caporusso L, Guida M, Catino A, Crucitta E, Sambiasi D, Mazzei A. Paclitaxel, cisplatin and lonidamine in advanced ovarian cancer. A phase II study. Eur J Cancer. 2001 Feb;37(3):364-8.
- DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab. 2008 Jan;7(1):11-20
- DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19345-50.
- Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B, Gandini S. Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. Cancer Prev Res (Phila). 2010 Nov;3(11):1451-61. doi: 10.1158/1940-6207.CAPR-10-0157. Epub 2010 Oct 12. Review.
- DeFeo-Jones D, Barnett SF, Fu S, Hancock PJ, Haskell KM, Leander KR, McAvoy E, Robinson RG, Duggan ME, Lindsley CW, Zhao Z, Huber HE, Jones RE. Tumor cell sensitization to apoptotic stimuli by selective inhibition of specific Akt/PKB family members. Mol Cancer Ther. 2005 Feb;4(2):271-9.
- Del Bufalo D, Biroccio A, Soddu S, Laudonio N, D'Angelo C, Sacchi A, Zupi G. Lonidamine induces apoptosis in drug-resistant cells independently of the p53 gene. J Clin Invest. 1996 Sep 1;98(5):1165-73.
- Del Bufalo D, Trisciuoglio D, Scarsella M, D'Amati G, Candiloro A, Iervolino A, Leonetti C, Zupi G. Lonidamine causes inhibition of angiogenesis-related endothelial cell functions. Neoplasia. 2004 Sep-Oct;6(5):513-22.
- Delgado T, Carroll PA, Punjabi AS, Margineantu D, Hockenbery DM, Lagunoff M. Induction of the Warburg effect by Kaposi's sarcoma herpesvirus is required for the maintenance of latently infected endothelial cells. Proc Natl Acad Sci U S A. 2010 Jun 8;107(23):10696-701
- Delgado T, Sanchez EL, Camarda R, Lagunoff M. Global metabolic profiling of infection by an oncogenic virus: KSHV induces and requires lipogenesis for survival of latent infection. PLoS Pathog. 2012;8(8):e1002866.
- Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol Chem. 1997 Jul 11;272(28):17269-75.
- DeYoung MP, Horak P, Sofer A, Sgroi D, Ellisen LW. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev. 2008 Jan 15;22(2):239-51.
- Di Bartolo DL, Cannon M, Liu YF, Renne R, Chadburn A, Boshoff C, Cesarman E KSHV LANA inhibits TGF-beta signaling through epigenetic silencing of the TGF-beta type II receptor Blood. 2008 May 1;111(9):4731-40.
- Dimmer KS, Friedrich B, Lang F, Deitmer JW, Bröer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J. 2000 Aug 15;350 Pt 1:219-27.

- DiNitto JP, Cronin TC, Lambright DG. Membrane recognition and targeting by lipid-binding domains. Sci STKE. 2003 Dec 16;2003(213)
- Dotti G, Fiocchi R, Motta T, Facchinetti B, Chiodini B, Borleri GM, Gavazzeni G, Barbui T, Rambaldi A Primary effusion lymphoma after heart transplantation: a new entity associated with human herpesvirus-8. Leukemia. 1999 May;13(5):664-70. Review
- Duan Y, Zhao X, Ren W, Wang X, Yu KF, Li D, Zhang X, Zhang Q. Antitumor activity of dichloroacetate on C6 glioma cell: in vitro and in vivo evaluation. Onco Targets Ther. 2013;6:189-98.
- Dudak SD, Lopez A, Block NL, Lokeshwar BL. Enhancement of radiation response of prostatic carcinoma by lonidamine. Anticancer Res. 1996 Nov-Dec;16(6B):3665-71.
- Dukers NH, Rezza G.Human herpesvirus 8 epidemiology: what we do and do not know. AIDS. 2003 Aug 15;17(12):1717-30. Review. No abstract available.
- Düvel K, Yecies JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellow E, Ma Q, Gorski R, Cleaver S, Vander Heiden MG, MacKeigan JP, Finan PM, Clish CB, Murphy LO, Manning BD. Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol Cell. 2010 Jul 30;39(2):171-83
- Dwarakanath BS, Singh D, Banerji AK, Sarin R, Venkataramana NK, Jalali R, Vishwanath PN, Mohanti BK, Tripathi RP, Kalia VK, Jain V. Clinical studies for improving radiotherapy with 2-deoxy-D-glucose: present status and future prospects. J Cancer Res Ther. 2009 Sep;5 Suppl 1:S21-6
- Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res. 2004 Jun 1;64(11):3892-9.
- Estañ MC, Calviño E, de Blas E, Boyano-Adánez Mdel C, Mena ML, Gómez-Gómez M, Rial E, Aller P. 2-Deoxy-D-glucose cooperates with arsenic trioxide to induce apoptosis in leukemia cells: involvement of IGF-1R-regulated Akt/mTOR, MEK/ERK and LKB-1/AMPK signaling pathways. Biochem Pharmacol. 2012 Dec 15;84(12):1604-16
- Fan Y, Dickman KG, Zong WX. Akt and c-Myc differentially activate cellular metabolic programs and prime cells to bioenergetic inhibition. J Biol Chem. 2010 Mar 5;285(10):7324-33
- Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell. 2006 Jun;9(6):425-34
- Flanagan AM, Letai A. BH3 domains define selective inhibitory interactions with BHRF-1 and KSHV BCL-2. Cell Death Differ. 2008 Mar;15(3):580-8. Epub 2007 Dec 14.
- Floridi A, Bruno T, Miccadei S, Fanciulli M, Federico A, Paggi MG. Enhancement of doxorubicin content by the antitumor drug lonidamine in resistant Ehrlich ascites tumor cells through modulation of energy metabolism. Biochem Pharmacol. 1998 Oct 1;56(7):841-9.
- Floridi A, Paggi MG, Marcante ML, Silvestrini B, Caputo A, De Martino C. Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells. J Natl Cancer Inst. 1981 Mar;66(3):497-9.
- Franke TF, Kaplan DR, Cantley LC, Toker A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science. 1997 Jan 31;275(5300):665-8.
- Franovic A, Holterman CE, Payette J, Lee S. Human cancers converge at the HIF-2alpha oncogenic axis. Proc Natl Acad Sci U S A. 2009 Dec 15;106(50):21306-11.
- Friborg J Jr, Kong W, Hottiger MO, Nabel GJ. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature. 1999 Dec 23-30;402(6764):889-94.
- Fujimuro M, Hayward SD. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3beta. J Virol. 2003 Jul;77(14):8019-30.
- Fujiwara S, Kawano Y, Yuki H, Okuno Y, Nosaka K, Mitsuya H, Hata H. PDK1 inhibition is a novel therapeutic target in multiple myeloma. Br J Cancer. 2013 Jan 15;108(1):170-8.
- Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature. 1999 Jun 10;399(6736):597-601. Erratum in: Nature 1999 Aug 19;400(6746):792.
- Funahashi A, Sarkar TK, Kory RC. PO 2 , PCO 2 , and pH in pleural effusion. J Lab Clin Med. 1971 Dec;78(6):1006.
- Fyles AW, Milosevic M, Wong R, Kavanagh MC, Pintilie M, Sun A, Chapman W, Levin W, Manchul L, Keane TJ, Hill RP. Oxygenation predicts radiation response and survival in patients with cervix cancer. Radiother Oncol. 1998 Aug;48(2):149-56
- Gaidano G, Gloghini A, Gattei V, Rossi MF, Cilia AM, Godeas C, Degan M, Perin T, Canzonieri V, Aldinucci D, Saglio G, Carbone A, Pinto A. Association of Kaposi's sarcoma-associated herpesvirus-

positive primary effusion lymphoma with expression of the CD138/syndecan-1 antigen. Blood. 1997 Dec 15;90(12):4894-900.

- Gaidarov I, Smith ME, Domin J, Keen JH. The class II phosphoinositide 3-kinase C2alpha is activated by clathrin and regulates clathrin-mediated membrane trafficking. Mol Cell. 2001 Feb;7(2):443-9.
- Ganley IG, Lam du H, Wang J, Ding X, Chen S, Jiang X. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. J Biol Chem. 2009 May 1;284(18):12297-305.
- Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature. 2009 Apr 9;458(7239):762-5.
- Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. J Clin Invest. 2003 Jul;112(2):197-208.
- Gasperini P, Tosato G. Targeting the mammalian target of Rapamycin to inhibit VEGF and cytokines for the treatment of primary effusion lymphoma. Leukemia. 2009 Oct;23(10):1867-74
- Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, Gillies RJ. Acid-mediated tumor invasion: a multidisciplinary study. Cancer Res. 2006 May 15;66(10):5216-23.
- Gérard L, Galicier L, Boulanger E, Quint L, Lebrette MG, Mortier E, Meignin V, Oksenhendler E. Improved survival in HIV-related Hodgkin's lymphoma since the introduction of highly active antiretroviral therapy. AIDS. 2003 Jan 3;17(1):81-7.
- Giatromanolaki A, Koukourakis MI, Pezzella F, Sivridis E, Turley H, et al. (2008) Phosphorylated VEGFR2/KDR receptors are widely expressed in B-cell non-Hodgkin's lymphomas and correlate with hypoxia inducible factor activation Hematol Oncol 26: 219–224
- Gilot D, Giudicelli F, Lagadic-Gossmann D, Fardel O. Akti-1/2, an allosteric inhibitor of Akt 1 and 2, efficiently inhibits CaMKIα activity and aryl hydrocarbon receptor pathway. Chem Biol Interact. 2010 Dec 5;188(3):546-52.
- Godden-Kent D, Talbot SJ, Boshoff C, Chang Y, Moore P, Weiss RA, Mittnacht S The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J Virol. 1997 Jun;71(6):4193-8.
- Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev. 2001 Jun 1;15(11):1406-18.
- Grabinski N, Ewald F, Hofmann BT, Staufer K, Schumacher U, Nashan B, Jücker M. Combined targeting of AKT and mTOR synergistically inhibits proliferation of hepatocellular carcinoma cells. Mol Cancer. 2012 Nov 20;11:85
- Green CJ, Göransson O, Kular GS, Leslie NR, Gray A, Alessi DR, Sakamoto K, Hundal HS. Use of Akt inhibitor and a drug-resistant mutant validates a critical role for protein kinase B/Akt in the insulin-dependent regulation of glucose and system A amino acid uptake. J Biol Chem. 2008 Oct 10;283(41):27653-67
- Guasparri I, Keller SA, Cesarman E. KSHV vFLIP is essential for the survival of infected lymphoma cells. J Exp Med. 2004 Apr 5;199(7):993-1003.
- Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008 Apr 25;30(2):214-26.
- Hackenbeck T, Knaup KX, Schietke R, Schödel J, Willam C, Wu X, Warnecke C, Eckardt KU, Wiesener MS. HIF-1 or HIF-2 induction is sufficient to achieve cell cycle arrest in NIH3T3 mouse fibroblasts independent from hypoxia. Cell Cycle. 2009 May 1;8(9):1386-95.
- Haque M, Davis DA, Wang V, Widmer I, Yarchoan R. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains hypoxia response elements: relevance to lytic induction by hypoxia. J Virol. 2003 Jun;77(12):6761-8.
- Hardie DG, Alessi DR. LKB1 and AMPK and the cancer-metabolism link - ten years after. BMC Biol. 2013 Apr 15;11:36
- Hardie DG, Hawley SA. AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays. 2001 Dec;23(12):1112-9.
- Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol. 2012 Mar 22;13(4):251-62.
- Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol. 2007 Oct;8(10):774-85. Review.

- Harrington LS, Findlay GM, Gray A, Tolkacheva T, Wigfield S, Rebholz H, Barnett J, Leslie NR, Cheng S, Shepherd PR, Gout I, Downes CP, Lamb RF. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol. 2004 Jul 19;166(2):213-23
- Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, Hingorani SR, Tuveson DA, Thompson CB. ATP citrate lyase inhibition can suppress tumor cell growth. Cancer Cell. 2005 Oct;8(4):311-21.
- Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem. 1996 Nov 1;271(44):27879-87.
- Heminger K, Jain V, Kadakia M, Dwarakanath B, Berberich SJ. Altered gene expression induced by ionizing radiation and glycolytic inhibitor 2-deoxy-glucose in a human glioma cell line: implications for radio sensitization. Cancer Biol Ther. 2006 Jul;5(7):815-23
- Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Höglund P, Järvinen H, Kristo P, Pelin K, Ridanpää M, Salovaara R, Toro T, Bodmer W, Olschwang S, Olsen AS, Stratton MR, de la Chapelle A, Aaltonen LA. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature. 1998 Jan 8;391(6663):184-7.
- Hernandez-Luna MA, Rocha-Zavaleta L, Vega MI, Huerta-Yepez S. Hypoxia inducible factor-1α induces chemoresistance phenotype in non-Hodgkin lymphoma cell line via up-regulation of Bcl-xL. Leuk Lymphoma. 2013 May;54(5):1048-55
- Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, Ueno Y, Hatch H, Majumder PK, Pan BS, Kotani H. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther. 2010 Jul;9(7):1956-67.
- Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res. 1996 Oct 1;56(19):4509-15.
- Houston MC. Pleural effusion: diagnostic value of measurements of PO2, PCO2, and pH. South Med J. 1981 May;74(5):585-9.
- Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB. Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. Cancer Res. 2002 Feb 15;62(4):1087-92.
- Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJ, Evans WE, Pieters R, Den Boer ML. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood. 2009 Feb 26;113(9):2014-21
- Hunt TK, Aslam RS, Beckert S, Wagner S, Ghani QP, Hussain MZ, Roy S, Sen CK. Aerobically derived lactate stimulates revascularization and tissue repair via redox mechanisms. Antioxid Redox Signal. 2007 Aug;9(8):1115-24.
- Hunter RW, Harper MT, Hers I. The PKB inhibitor Akti-1/2 potentiates PAR-1-mediated platelet function independently of its ability to block PKB. J Thromb Haemost. 2008 Nov;6(11):1923-32.
- Hussain AR, Ahmed SO, Ahmed M, Khan OS, Al Abdulmohsen S, Platanias LC, Al-Kuraya KS, Uddin S. Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. PLoS One. 2012;7(6):e39945
- Hussain SA, Ganesan R, Reynolds G, Gross L, Stevens A, Pastorek J, Murray PG, Perunovic B, Anwar MS, Billingham L, James ND, Spooner D, Poole CJ, Rea DW, Palmer DH. Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer. 2007 Jan 15;96(1):104-9.
- Ihrlund LS, Hernlund E, Khan O, Shoshan MC. 3-Bromopyruvate as inhibitor of tumour cell energy metabolism and chemopotentiator of platinum drugs. Mol Oncol. 2008 Jun;2(1):94-101.
- Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev. 2003 Aug 1;17(15):1829-34. Epub 2003 Jul 17.
- Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 2002 Sep;4(9):648-57.
- Iseli TJ, Walter M, van Denderen BJ, Katsis F, Witters LA, Kemp BE, Michell BJ, Stapleton D. AMP-activated protein kinase beta subunit tethers alpha and gamma subunits via its C-terminal sequence (186-270). J Biol Chem. 2005 Apr 8;280(14):13395-400

- Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. 1998 Jan 15;12(2):149-62.
- Jacinto E, Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, Hall MN. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 2004 Nov;6(11):1122-8.
- Jaffe ES, Harris NL, Stein H, Vardiman JV (eds). World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues IARC Press, Lyon, 2001,
- Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A. 2007 Jul 17;104(29):12017-22
- Jenner RG, Maillard K, Cattini N, Weiss RA, Boshoff C, Wooster R, Kellam P Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. Proc Natl Acad Sci U S A. 2003 Sep 2;100(18):10399-404. Epub 2003 Aug 18
- Jiang BH, Rue E, Wang GL, Roe R, Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem. 1996 Jul 26;271(30):17771-8.
- Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell. 2005 Apr 29;18(3):283-93.
- Jørgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF, Richter EA, Wojtaszewski JF. The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. Diabetes. 2004 Dec;53(12):3074-81.
- Judde JG, Lacoste V, Brière J, Kassa-Kelembho E, Clyti E, Couppié P, Buchrieser C, Tulliez M, Morvan J, Gessain A. Monoclonality or oligoclonality of human herpesvirus 8 terminal repeat sequences in Kaposi's sarcoma and other diseases. J Natl Cancer Inst. 2000 May 3;92(9):729-36.
- Kaidi A, Williams AC, Paraskeva C. Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. Nat Cell Biol. 2007 Feb;9(2):210-7
- Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci. 2011 Dec 2;4:51.
- Katano H, Sato Y, Sata T. Expression of p53 and human herpesvirus-8 (HHV-8)-encoded latency-associated nuclear antigen with inhibition of apoptosis in HHV-8-associated malignancies. Cancer. 2001 Dec 15;92(12):3076-84.
- Kenneth NS, Rocha S. Regulation of gene expression by hypoxia. Biochem J. 2008 Aug 15;414(1):19-29
- Khatri S, Yepiskoposyan H, Gallo CA, Tandon P, Plas DR. FOXO3a regulates glycolysis via transcriptional control of tumor suppressor TSC1. J Biol Chem. 2010 May 21;285(21):15960-5
- Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. Nat Cell Biol. 2008 Aug;10(8):935-45.
- Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006 Mar;3(3):177-85.
- Klein U, Gloghini A, Gaidano G, Chadburn A, Cesarman E, Dalla-Favera R, Carbone A. Gene expression profile analysis of AIDS-related primary effusion lymphoma (PEL) suggests a plasmablastic derivation and identifies PEL-specific transcripts. Blood. 2003 May 15;101(10):4115-21. Epub 2003 Jan 16
- Klepfish A, Sarid R, Shtalrid M, Shvidel L, Berrebi A, Schattner A. Primary effusion lymphoma (PEL) in HIV-negative patients--a distinct clinical entity. Leuk Lymphoma. 2001 Apr;41(3-4):439-43.
- Knowles DM. Etiology and pathogenesis of AIDS-related non-Hodgkin's lymphoma. Hematol Oncol Clin North Am. 2003 Jun;17(3):785-820. Review
- Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS, Hullihen J, Pedersen PL. Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun. 2004 Nov 5;324(1):269-75.
- Ko YH, Verhoeven HA, Lee MJ, Corbin DJ, Vogl TJ, Pedersen PL. A translational study "case report" on the small molecule "energy blocker" 3-bromopyruvate (3BP) as a potent anticancer agent: from bench side to bedside. J Bioenerg Biomembr. 2012 Feb;44(1):163-70
- Koh MY, Powis G. Passing the baton: the HIF switch. Trends Biochem Sci. 2012 Sep;37(9):364-72
- Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem. 1996 Dec 6;271(49):31372-8.

- Kondoh H, Lleonart ME, Gil J, Wang J, Degan P, Peters G, Martinez D, Carnero A, Beach D. Glycolytic enzymes can modulate cellular life span. Cancer Res. 2005 Jan 1;65(1):177-85.
- Konstantinidou G, Bey EA, Rabellino A, Schuster K, Maira MS, Gazdar AF, Amici A, Boothman DA, Scaglioni PP. Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. Cancer Res. 2009 Oct 1;69(19):7644-52
- Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M. The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature. 2005 Oct 20;437(7062):1109-11
- Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, Huang LE. HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. EMBO J. 2004 May 5;23(9):1949-56
- Koukourakis MI, Giatromanolaki A, Sivridis E; Tumour and Angiogenesis Research Group Lactate dehydrogenase isoenzymes 1 and 5: differential expression by neoplastic and stromal cells in non-small cell lung cancer and other epithelial malignant tumors. Tumour Biol. 2003 Aug-Sep;24(4):199-202.
- Krieg M, Haas R, Brauch H, Acker T, Flamme I, Plate KH. Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene. 2000 Nov 16;19(48):5435-43.
- Krycer JR, Sharpe LJ, Luu W, Brown AJ. The Akt-SREBP nexus: cell signaling meets lipid metabolism. Trends Endocrinol Metab. 2010 May;21(5):268-76.
- Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell. 1993 May 7;73(3):585-96.
- Kurtoglu M, Gao N, Shang J, Maher JC, Lehrman MA, Wangpaichitr M, Savaraj N, Lane AN, Lampidis TJ. Under normoxia, 2-deoxy-D-glucose elicits cell death in select tumor types not by inhibition of glycolysis but by interfering with N-linked glycosylation. Mol Cancer Ther. 2007 Nov;6(11):3049-58.
- Lallemand F, Desire N, Rozenbaum W, Nicolas JC, Marechal V. Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. J Clin Microbiol. 2000 Apr;38(4):1404-8.
- Lando D, Peet DJ, Whelan DA, Gorman JJ, Whitelaw ML. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science. 2002 Feb 1;295(5556):858-61.
- Laplante M, Sabatini DM. Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci. 2013 Apr 15;126(Pt 8):1713-9. doi: 10.1242/jcs.125773. Epub 2013 May 2. Review.
- Lapointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K, Ferrari M, Egevad L, Rayford W, Bergerheim U, Ekman P, DeMarzo AM, Tibshirani R, Botstein D, Brown PO, Brooks JD, Pollack JR. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A. 2004 Jan 20;101(3):811-6.
- Lartigau E, Guichard M. The effect of tirapazamine (SR-4233) alone or combined with chemotherapeutic agents on xenografted human tumours. Br J Cancer. 1996 Jun;73(12):1480-5.
- Lee BL, Kim WH, Jung J, Cho SJ, Park JW, Kim J, Chung HY, Chang MS, Nam SY. A hypoxia-independent up-regulation of hypoxia-inducible factor-1 by AKT contributes to angiogenesis in human gastric cancer. Carcinogenesis. 2008 Jan;29(1):44-51.
- Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science. 2010 Dec 3;330(6009):1340-4
- Levy AG, Zage PE, Akers LJ, Ghisoli ML, Chen Z, Fang W, Kannan S, Graham T, Zeng L, Franklin AR, Huang P, Zweidler-McKay PA. The combination of the novel glycolysis inhibitor 3-BrOP and rapamycin is effective against neuroblastoma. Invest New Drugs. 2012 Feb;30(1):191-9
- Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, Gao B, Wierzbicki M, Verbeuren TJ, Shaw RJ, Cohen RA, Zang M. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. Cell Metab. 2011 Apr 6;13(4):376-88.
- Li Z, Yan S, Attayan N, Ramalingam S, Thiele CJ. Combination of an allosteric Akt Inhibitor MK-2206 with etoposide or rapamycin enhances the antitumor growth effect in neuroblastoma. Clin Cancer Res. 2012 Jul 1;18(13):3603-15
- Liapi E, Geschwind JF, Vali M, Khwaja AA, Prieto-Ventura V, Buijs M, Vossen JA, Ganapathy S, Wahl RL. Assessment of tumoricidal efficacy and response to treatment with 18F-FDG PET/CT after intraarterial infusion with the antiglycolytic agent 3-bromopyruvate in the VX2 model of liver tumor. J Nucl Med. 2011 Feb;52(2):225-30.

- Lindsley CW. The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation: a 2009 update. Curr Top Med Chem. 2010;10(4):458-77. Review.
- Liu H, Hu YP, Savaraj N, Priebe W, Lampidis TJ. Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry. 2001 May 8;40(18):5542-7.
- Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov. 2009 Aug;8(8)
- Liu Q, Chang JW, Wang J, Kang SA, Thoreen CC, Markhard A, Hur W, Zhang J, Sim T, Sabatini DM, Gray NS. Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benzo[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. J Med Chem. 2010 Oct 14;53(19):7146-55
- Liu Q, Ren T, Fresques T, Oppliger W, Niles BJ, Hur W, Sabatini DM, Hall MN, Powers T, Gray NS. Selective ATP-competitive inhibitors of TOR suppress rapamycin-insensitive function of TORC2 in Saccharomyces cerevisiae. ACS Chem Biol. 2012 Jun 15;7(6):982-7.
- Lobo C, Ruiz-Bellido MA, Aledo JC, Márquez J, Núñez De Castro I, Alonso FJ. Inhibition of glutaminase expression by antisense mRNA decreases growth and tumourigenicity of tumour cells. Biochem J. 2000 Jun 1;348 Pt 2:257-61.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75.
- Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, Verma A. Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem. 2005 Dec 23;280(51):41928-39. Epub 2005 Oct 13.
- Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol. 2011;27:441-64
- Luo W, Semenza GL. Pyruvate kinase M2 regulates glucose metabolism by functioning as a coactivator for hypoxia-inducible factor 1 in cancer cells. Oncotarget. 2011 Jul;2(7):551-6.
- Luppi M, Trovato R, Barozzi P, Vallisa D, Rossi G, Re A, Ravazzini L, Potenza L, Riva G, Morselli M, Longo G, Cavanna L, Roncaglia R, Torelli G.Treatment of herpesvirus associated primary effusion lymphoma with intracavity cidofovir. Leukemia. 2005 Mar;19(3):473-6. No abstract available.
- Madhok BM, Yeluri S, Perry SL, Hughes TA, Jayne DG. Dichloroacetate induces apoptosis and cell-cycle arrest in colorectal cancer cells. Br J Cancer. 2010 Jun 8;102(12):1746-52.
- Maehama T, Dixon JE. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol. 1999 Apr;9(4):125-8
- Mahadevan D, Chiorean EG, Harris WB, Von Hoff DD, Stejskal-Barnett A, Qi W, Anthony SP, Younger AE, Rensvold DM, Cordova F, Shelton CF, Becker MD, Garlich JR, Durden DL, Ramanathan RK. Phase I pharmacokinetic and pharmacodynamic study of the pan-PI3K/mTORC vascular targeted pro-drug SF1126 in patients with advanced solid tumours and B-cell malignancies. Eur J Cancer. 2012 Dec;48(18):3319-27. doi: 10.1016/j.ejca.2012.06.027. Epub 2012 Aug 23.
- Maher JC, Krishan A, Lampidis TJ. Greater cell cycle inhibition and cytotoxicity induced by 2-deoxy-D-glucose in tumor cells treated under hypoxic vs aerobic conditions. Cancer Chemother Pharmacol. 2004 Feb;53(2):116-22
- Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, García-Echeverría C. Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Mol Cancer Ther. 2008 Jul;7(7):1851-63
- Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB, Hay N. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. Mol Cell. 2004 Dec 3;16(5):819-30.
- Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, Manola J, Brugarolas J, McDonnell TJ, Golub TR, Loda M, Lane HA, Sellers WR. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med. 2004 Jun;10(6):594-601
- Manara MC, Nicoletti G, Zambelli D, Ventura S, Guerzoni C, Landuzzi L, Lollini PL, Maira SM, García-Echeverría C, Mercuri M, Picci P, Scotlandi K. NVP-BEZ235 as a new therapeutic option for sarcomas. Clin Cancer Res. 2010 Jan 15;16(2):530-40
- Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007 Jun 29;129(7):1261-74. Review.

- Manning BD. Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. J Cell Biol. 2004 Nov 8;167(3):399-403. Review. Erratum in: J Cell Biol. 2004 Dec 20;167(6):1255.
- Marcelo G. Horenstein, Roland G. Nador, Amy Chadburn, Elizabeth M. Hyjek, Giorgio Inghirami Daniel M. Knowles, and Ethel Cesarman Epstein-Barr Virus Latent Gene Expression in Primary Effusion Lymphomas Containing Kaposi's Sarcoma – Associated Herpesvirus/Human Herpesvirus-8 . Blood 1997 90: 1186-1191
- Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr Biol. 2000 Oct 19;10(20):1247-55.
- Maraldi T, Bertacchini J, Benincasa M, Guida M, De Pol A, Liotta LA, Petricoin E, Cocco L, Marmiroli S Reverse-phase protein microarrays (RPPA) as a diagnostic and therapeutic guide in multidrug resistant leukemia.Int J Oncol. 2011 Feb;38(2):427-35
- Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, De Young LR, Lampidis TJ. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. Cancer Res. 2004 Jan 1;64(1):31-4.
- Masunaga S, Ono K, Hori H, Suzuki M, Kinashi Y, Takagaki M, Kasai S, Nagasawa H, Uto Y. Change in oxygenation status in intratumour total and quiescent cells following gamma-ray irradiation, tirapazamine administration, cisplatin injection and bleomycin treatment. Br J Radiol. 2000 Sep;73(873):978-86.
- Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F, Hwang PM. p53 regulates mitochondrial respiration. Science. 2006 Jun 16;312(5780):1650-3. Epub 2006 May 25.
- Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell. 2006 Mar 17;21(6):749-60.
- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999 May 20;399(6733):271-5.
- Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A. 2001 Sep 25;98(20):11598-603.
- McDonald PC, Winum JY, Supuran CT, Dedhar S. Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. Oncotarget. 2012 Jan;3(1):84-97. Review.
- McGee SL, van Denderen BJ, Howlett KF, Mollica J, Schertzer JD, Kemp BE, Hargreaves M. AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. Diabetes. 2008 Apr;57(4):860-7
- McMahon G. VEGF receptor signaling in tumor angiogenesis. Oncologist. 2000;5 Suppl 1:3-10.
- Medina RA, Owen GI. Glucose transporters: expression, regulation and cancer. Biol Res. 2002;35(1):9-26
- Mesri EA, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpesvirus. Nat Rev Cancer. 2010 Oct;10(10):707-19. doi: 10.1038/nrc2888. Review.
- Miccoli L, Poirson-Bichat F, Sureau F, Bras Gonçalves R, Bourgeois Y, Dutrillaux B, Poupon MF, Oudard S. Potentiation of lonidamine and diazepam, two agents acting on mitochondria, in human glioblastoma treatment. J Natl Cancer Inst. 1998 Sep 16;90(18):1400-6.
- Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, Maguire C, Gammer TL, Mackey JR, Fulton D, Abdulkarim B, McMurtry MS, Petruk KC. Metabolic modulation of glioblastoma with dichloroacetate. Sci Transl Med. 2010 May 12;2(31):31ra34
- Mîinea CP, Sano H, Kane S, Sano E, Fukuda M, Peränen J, Lane WS, Lienhard GE. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J. 2005 Oct 1;391(Pt 1):87-93.
- Miyato Y, Ando K. Apoptosis of human melanoma cells by a combination of lonidamine and radiation. J Radiat Res. 2004 Jun;45(2):189-94.
- Mohanti BK, Rath GK, Anantha N, Kannan V, Das BS, Chandramouli BA, Banerjee AK, Das S, Jena A, Ravichandran R, Sahi UP, Kumar R, Kapoor N, Kalia VK, Dwarakanath BS, Jain V. Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas. Int J Radiat Oncol Biol Phys. 1996 Apr 1;35(1):103-11.
- Montaner S, Sodhi A, Pece S, Mesri EA, Gutkind JS. The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B.Cancer Res. 2001 Mar 15;61(6):2641-8.

- Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E.Energy metabolism in tumor cells. FEBS J. 2007 Mar;274(6):1393-418
- Moyo T.K., Kristy L. Richards, Blossom Damania. Use of Cidofovir for the Treatment of HIV-negative Human Herpes Virus-8 Associated Primary Effusion Lymphoma. Clin Adv Hematol Oncol. 2010 May; 8(5): 372–374
- Munger J, Bajad SU, Coller HA, Shenk T, Rabinowitz JD. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog. 2006 Dec;2(12):e132.
- Muñoz-Pinedo C, El Mjiyad N, Ricci JE. Cancer metabolism: current perspectives and future directions. Cell Death Dis. 2012 Jan 12;3:e248
- Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljunqvist O, Efendic S, Moller DE, Thorell A, Goodyear LJ. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes. 2002 Jul;51(7):2074-81.
- Nador RG, Cesarman E, Chadburn A, Dawson DB, Ansari MQ, Sald J, Knowles DM. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood. 1996 Jul 15;88(2):645-56.
- Nakano A, Tsuji D, Miki H, Cui Q, El Sayed SM, Ikegame A, Oda A, Amou H, Nakamura S, Harada T, Fujii S, Kagawa K, Takeuchi K, Sakai A, Ozaki S, Okano K, Nakamura T, Itoh K, Matsumoto T, Abe M. Glycolysis inhibition inactivates ABC transporters to restore drug sensitivity in malignant cells. PLoS One. 2011;6(11):e27222.
- Ng SSW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res. 2000 Oct 1;60(19):5451-5.
- Nisticò C, Garufi C, Milella M, D'Ottavio AM, Vaccaro A, Fabi A, Terzoli E. Weekly epirubicin plus lonidamine in advanced breast carcinoma. Breast Cancer Res Treat. 1999 Aug;56(3):233-7.
- Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A. 2005 Oct 4;102(40)
- Oakhill JS, Scott JW, Kemp BE. AMPK functions as an adenylate charge-regulated protein kinase. Trends Endocrinol Metab. 2012 Mar;23(3):125-32
- O'Gorman DM, McKenna SL, McGahon AJ, Cotter TG. Inhibition of PI3-kinase sensitises HL60 human leukaemia cells to both chemotherapeutic drug- and Fas-induced apoptosis by a JNK independent pathway. Leuk Res. 2001 Sep;25(9):801-11.
- Osborne J, Moore PS, Chang Y KSHV-encoded viral IL-6 activates multiple human IL-6 signaling pathways. Hum Immunol 1999 Oct;60(10):921-7.
- Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA, Dang CV. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem. 2000 Jul 21;275(29):21797-800.
- Oudard S, Carpentier A, Banu E, Fauchon F, Celerier D, Poupon MF, Dutrillaux B, Andrieu JM, Delattre JY. Phase II study of lonidamine and diazepam in the treatment of recurrent glioblastoma multiforme. J Neurooncol. 2003 May;63(1):81-6.
- Papaconstantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J Biol Chem. 1961 Feb;236:285-8
- Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA Jr, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. An integrated genomic analysis of human glioblastoma multiforme. Science. 2008 Sep 26;321(5897):1807-12
- Pastorino JG, Hoek JB, Shulga N. Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. Cancer Res. 2005 Nov 15;65(22):10545-54.
- Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V, Johnson RS. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J Clin Invest. 2007 Jul;117(7):1926-32.
- Polakis P.Wnt signaling and cancer. Genes Dev. 2000 Aug 1;14(15):1837-51. Review. No abstract available.
- Pollard PJ, Wortham NC, Tomlinson IP. The TCA cycle and tumorigenesis: the examples of fumarate hydratase and succinate dehydrogenase. Ann Med. 2003;35(8):632-9.

- Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. Front Pharmacol. 2011 Aug 25;2:49
- Porstmann T, Santos CR, Griffiths B, Cully M, Wu M, Leevers S, Griffiths JR, Chung YL, Schulze A. SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. Cell Metab. 2008 Sep;8(3):224-36.
- Pouysségur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature. 2006 May 25;441(7092):437-43
- Prakash O, Tang ZY, Peng X, Coleman R, Gill J, Farr G, Samaniego F. Tumorigenesis and aberrant signaling in transgenic mice expressing the human herpesvirus-8 K1 gene. J Natl Cancer Inst. 2002 Jun 19;94(12):926-35.
- Qin Z, Jakymiw A, Findlay V, Parsons C. KSHV-Encoded MicroRNAs: Lessons for Viral Cancer Pathogenesis and Emerging Concepts. Int J Cell Biol. 2012;2012:603961.
- Quintero M, Brennan PA, Thomas GJ, Moncada S. Nitric oxide is a factor in the stabilization of hypoxia-inducible factor-1alpha in cancer: role of free radical formation. Cancer Res. 2006 Jan 15;66(2):770-4.
- Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, Rocha Lima CM, Schlesselman JJ, Tolba K, Langmuir VK, Kroll S, Jung DT, Kurtoglu M, Rosenblatt J, Lampidis TJ. A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors. Cancer Chemother Pharmacol. 2013 Feb;71(2):523-30
- Rathmell JC, Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. Mol Cell Biol. 2003 Oct;23(20):7315-28.
- Ravagnan L, Marzo I, Costantini P, Susin SA, Zamzami N, Petit PX, Hirsch F, Goulbern M, Poupon MF, Miccoli L, Xie Z, Reed JC, Kroemer G. Lonidamine triggers apoptosis via a direct, Bcl-2-inhibited effect on the mitochondrial permeability transition pore. Oncogene. 1999 Apr 22;18(16):2537-46.
- Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. 2000 Jan 1;14(1):34-44.
- Reiling JH, Hafen E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in Drosophila. Genes Dev. 2004 Dec 1;18(23):2879-92
- Rey S, Semenza GL. Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. Cardiovasc Res. 2010 May 1;86(2):236-42
- Richard G. Jenner, M. Mar Albà, Chris Boshoff, Paul KellamKaposi's Sarcoma-Associated Herpesvirus Latent and Lytic Gene Expression as Revealed by DNA Arrays J Virol. 2001 January; 75(2): 891–902.
- Richter JD, Sonenberg N. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature. 2005 Feb 3;433(7025):477-80
- Ricotti L, Tesei A, De Paola F, Milandri C, Amadori D, Frassineti GL, Ulivi P, Zoli W. Potentiation of antiproliferative drug activity by lonidamine in hepatocellular carcinoma cells. J Chemother. 2003 Oct;15(5):480-7.
- Rong Y, Wu W, Ni X, Kuang T, Jin D, Wang D, Lou W. Lactate dehydrogenase A is overexpressed in pancreatic cancer and promotes the growth of pancreatic cancer cells. Tumour Biol. 2013 Jun;34(3):1523-30
- Russo JJ, Bohenzky RA, Chien MC, Chen J, Yan M, Maddalena D, Parry JP, Peruzzi D, Edelman IS, Chang Y, Moore PS. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc Natl Acad Sci U S A. 1996 Dec 10;93(25):14862-7.
- Ruvinsky I, Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci. 2006 Jun;31(6):342-8.
- Ryan HE, Lo J, Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. 1998 Jun 1;17(11):3005-15.
- Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 1994 Jul 15;78(1):35-43.
- Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006 Sep;6(9):729-34.
- Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human cancers. Cell Cycle. 2004 Oct;3(10):1221-4.

- Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell. 2010 Apr 16;141(2):290-303
- Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008 Jun 13;320(5882):1496-501.
- Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, Carr SA, Sabatini DM. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell. 2007 Mar 23;25(6):903-15.
- Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, Westra WH, Herman JG, Sidransky D. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. Cancer Res. 2002 Jul 1;62(13):3659-62.
- Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004 Jul 27;14(14):1296-302.
- Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol. 2005 Dec;17(6):596-603.
- Schaefer NG, Geschwind JF, Engles J, Buchanan JW, Wahl RL. Systemic administration of 3-bromopyruvate in treating disseminated aggressive lymphoma. Transl Res. 2012 Jan;159(1):51-7
- Schmaltz C, Hardenbergh PH, Wells A, Fisher DE. Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Biol. 1998 May;18(5):2845-54.
- Schulz TF. KSHV/HHV8-associated lymphoproliferations in the AIDS setting. Eur J Cancer. 2001 Jul;37(10):1217-26. Review
- Schwarz M, Murphy PM. Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant. J Immunol. 2001 Jul 1;167(1):505-13.
- Scott JW, Hawley SA, Green KA, Anis M, Stewart G, Scullion GA, Norman DG, Hardie DG. CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J Clin Invest. 2004 Jan;113(2):274-84.
- Sekiguchi T, Hirose E, Nakashima N, Ii M, Nishimoto T. Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. J Biol Chem. 2001 Mar 9;276(10):7246-57. Epub 2000 Nov 9.
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell. 2005 Jan;7(1):77-85.
- Semba S, Itoh N, Ito M, Harada M, Yamakawa M. The in vitro and in vivo effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3'-kinase, in human colon cancer cells. Clin Cancer Res. 2002 Jun;8(6):1957-63.
- Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene. 2010 Feb 4;29(5):625-34.
- Semenza GL.Targeting HIF-1 for cancer therapy. Nat Rev Cancer. 2003 Oct;3(10):721-32. Review.
- Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J, Baselga J. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res. 2008 Oct 1;68(19):8022-30
- Shackelford DB, Vasquez DS, Corbeil J, Wu S, Leblanc M, Wu CL, Vera DR, Shaw RJ. mTOR and HIF-1alpha-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome. Proc Natl Acad Sci U S A. 2009 Jul 7;106(27):11137-42.
- Shafee N, Kaluz S, Ru N, Stanbridge EJ. PI3K/Akt activity has variable cell-specific effects on expression of HIF target genes, CA9 and VEGF, in human cancer cell lines. Cancer Lett. 2009 Sep 8;282(1):109-15
- Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell. 2004 Jul;6(1):91-9.
- Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A. 1997 Jun 24;94(13):6658-63.

- Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J, Arteaga CL. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med. 2002 Oct;8(10):1145-52. Epub 2002 Sep 16.
- Shin YC, Joo CH, Gack MU, Lee HR, Jung JU. Kaposi's sarcoma-associated herpesvirus viral IFN regulatory factor 3 stabilizes hypoxia-inducible factor-1 alpha to induce vascular endothelial growth factor expression. Cancer Res. 2008 Mar 15;68(6):1751-9.
- Si H, Verma SC, Robertson ES.Proteomic analysis of the Kaposi's sarcoma-associated herpesvirus terminal repeat element binding proteins. J Virol. 2006 Sep;80(18):9017-30.
- Simioni C, Martelli AM, Cani A, Cetin-Atalay R, McCubrey JA, Capitani S, Neri LM. The AKT inhibitor MK-2206 is cytotoxic in hepatocarcinoma cells displaying hyperphosphorylated AKT-1 and synergizes with conventional chemotherapy. Oncotarget. 2013 Sep;4(9):1496-506.
- Simonelli C, Spina M, Cinelli R, Talamini R, Tedeschi R, Gloghini A, Vaccher E, Carbone A, Tirelli U. Clinical features and outcome of primary effusion lymphoma in HIV-infected patients: a single-institution study. J Clin Oncol. 2003 Nov 1;21(21):3948-54.
- Sin SH, Roy D, Wang L, Staudt MR, Fakhari FD, Patel DD, Henry D, Harrington WJ Jr, Damania BA, Dittmer DP. Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. Blood. 2007 Mar 1;109(5):2165-73.
- Singh D, Banerji AK, Dwarakanath BS, Tripathi RP, Gupta JP, Mathew TL, Ravindranath T, Jain V. Optimizing cancer radiotherapy with 2-deoxy-d-glucose dose escalation studies in patients with glioblastoma multiforme. Strahlenther Onkol. 2005 Aug;181(8):507-14.
- Smith GC, Ong WK, Rewcastle GW, Kendall JD, Han W, Shepherd PR. Effects of acutely inhibiting PI3K isoforms and mTOR on regulation of glucose metabolism in vivo. Biochem J. 2012 Feb 15;442(1):161-9.
- Sodhi A, Chaisuparat R, Hu J, Ramsdell AK, Manning BD, Sausville EA, Sawai ET, Molinolo A, Gutkind JS, Montaner S. The TSC2/mTOR pathway drives endothelial cell transformation induced by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. Cancer Cell. 2006 Aug;10(2):133-43.
- Sodhi A, Montaner S, Patel V, Zohar M, Bais C, Mesri EA, Gutkind JS. The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. Cancer Res. 2000 Sep 1;60(17):4873-80.
- Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, Kelley MJ, Gallez B, Wahl ML, Feron O, Dewhirst MW. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest. 2008 Dec;118(12):3930-42
- Soulier J, Grollet L, Oksenhendler E, Cacoub P, Cazals-Hatem D, Babinet P, d'Agay MF, Clauvel JP, Raphael M, Degos L, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood. 1995 Aug 15;86(4):1276-80.
- Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A. 1987 Jul;84(14):5034-7.
- Stacpoole PW, Nagaraja NV, Hutson AD. Efficacy of dichloroacetate as a lactate-lowering drug. J Clin Pharmacol. 2003 Jul;43(7):683-91. Review.
- Stacpoole PW. The pharmacology of dichloroacetate. Metabolism. 1989 Nov;38(11):1124-44
- Stegeman H, Kaanders JH, Wheeler DL, van der Kogel AJ, Verheijen MM, Waaijer SJ, Iida M, Grénman R, Span PN, Bussink J. Activation of AKT by hypoxia: a potential target for hypoxic tumors of the head and neck. BMC Cancer. 2012 Oct 10;12:463
- Stein M, Lin H, Jeyamohan C, Dvorzhinski D, Gounder M, Bray K, Eddy S, Goodin S, White E, Dipaola RS. Targeting tumor metabolism with 2-deoxyglucose in patients with castrate-resistant prostate cancer and advanced malignancies. Prostate. 2010 Sep 15;70(13):1388-94.
- Sun RC, Board PG, Blackburn AC. Targeting metabolism with arsenic trioxide and dichloroacetate in breast cancer cells. Mol Cancer. 2011 Nov 18;10:142.
- Sundfør K, Lyng H, Tropé CG, Rofstad EK. Treatment outcome in advanced squamous cell carcinoma of the uterine cervix: relationships to pretreatment tumor oxygenation and vascularization. Radiother Oncol. 2000 Feb;54(2):101-7.
- Sutendra G, Michelakis ED. Pyruvate dehydrogenase kinase as a novel therapeutic target in oncology. Front Oncol. 2013 Mar 7;3:38.

- Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T, Neumann D. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J Biol Chem. 2006 Oct 27;281(43):32207-16
- Suzuki S, Tanaka T, Poyurovsky MV, Nagano H, Mayama T, Ohkubo S, Lokshin M, Hosokawa H, Nakayama T, Suzuki Y, Sugano S, Sato E, Nagao T, Yokote K, Tatsuno I, Prives C. Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. Proc Natl Acad Sci U S A. 2010 Apr 20;107(16):7461-6.
- Swietach P, Vaughan-Jones RD, Harris AL. Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev. 2007 Jun;26(2):299-310. Review.