Stony Brook University

OFFICIAL COPY

The official electronic file of this thesis or dissertation is maintained by the University Libraries on behalf of The Graduate School at Stony Brook University.

© All Rights Reserved by Author.

CPI-613, a non-redox active lipoic acid analog, causes hyper-phosphorylation of BCKDC E1α in H460 lung cancer cells in vitro

A Thesis Presented

by

Michael Dahan

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

August 2014

Stony Brook University

The Graduate School

Michael Dahan

We, the thesis committee for the above candidate for the

Master of Science degree, hereby recommend

acceptance of this thesis.

Zuzana Zachar – Thesis Advisor
Research Assistant Professor – Biochemistry

Shawn Stuart– Second Reader
Postdoctoral Researcher - Biochemistry

This thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Thesis

CPI-613, a non-redox active lipoic acid analog, causes hyper-phosphorylation of BCKDC E1α in H460 lung cancer cells in vitro

by

Michael Dahan

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

2014

The production of lipids and proteins for growth, as well as nucleotides for the replication of the genome, are key requirements that loom as importantly as the need for the production of energy in the transformed metabolic state of cancer cells. The remodeling of the TCA cycle (extensive anaplerosis and cataplerosis) reflects this need for biosynthetic macromolecules. CPI-613, a non-redox active lipoic acid analog that has been shown to selectively increase phosphorylation of the pyruvate dehydrogenase (PDH) E1 α subunit, thereby inactivating the entire complex, was investigated for its role in causing phosphorylation of the branched-chain amino acid dehydrogenase complex (BCKDC) E1 α subunit in H460 lung cancer cells. CPI-613 was shown to cause hyper-phosphorylation of the peptide via western analysis, which triggered further investigation of the role of the drug in causing similar effects in several other epithelial cancer cell lines, as well as one glioblastoma line. At the current time, BCKDC appears to be a third such lipoate-containing mitochondrial enzyme target of CPI-613.

Table of Contents

List of Figures……..v

List of Abbreviations……..vi

Introduction……..1

Transformed Cells Exhibit Altered Metabolism……..1

Carbon from the TCA Cycle Used to Produce Biosynthetic Precursors Needs to be Replenished……..2

Regulation of the Pyruvate Dehydrogenase Complex, the Gatekeeper of Carbon Flux from Glycolysis to the TCA Cycle……..3

Introduction to the metabolism of branched-chain amino acids leucine, valine and isoleucine…….4

Branched-Chain α-Ketoacid Dehydrogenase Structure, Catalysis, and Regulation……5

CPI-613, a non-redox active Lipoic Acid Analog, Increases Phosphorylation of the Pyruvate Dehydrogenase Complex……..6

Results……....10

CPI-613 treatment causes hyper-phosphorylation of the BCKDC E1α subunit in H460 cells……10

CPI-613 increases phosphorylation in BxPC-3 pancreatic tumor cells…….10

Levels of BCKDC E1 α vary across different cell lines……11

Discussion……..20

Materials and Methods..…….23

References…..……25

List of Figures

Figure 1: Schematic of tumor cell metabolism of glucose and glutamine……...……...8

Figure 2: Schematic of the branched-chain amino acid metabolic pathway……...9

Figure 3a: Western detection of phosphorylation of BCDKC E1α in H460 cells……....……13

Figure 3b: Quantification of western anaylsis shown in Figure 3a……...14

Figure 4a: Western Detection of phosphorylation of BCKDC in BxPC-3 Cells……...15

Figure 4b: Quantification of western anaylsis shown in Figure 4a……...16

Figure 5a: Western detection of phosphorylation of BCKDC E1α in survey of epithelial cell lines……17

Figure 5b: Western detection of phosphorylation of BCKDC E1α in MDA 231 cells……18

Figure 5c: Western detection of phosphorylation of BCKDC E1α in HeLa cells……19

Figure 5d: Western detection of phosphorylation of BCKDC E1α in U-87 cells……20

List of Abbreviations

ATP	adenosine 5'-triphosphate
BCAA	branched-chain amino acids i.e., Leucine, Isoleucine, and Valine
BCAT	branched-chain amino acid transaminase
BCKA	branched-chain α-ketoacid
BCKDC	branched-chain α-ketoacid dehydrogenase complex
BDK	branched-chain α-ketoacid dehydrogenase kinase
DMSO	dimethyl sulfoxide
E1	α-ketoacid decarboxylase
E1α	1 of 2 subunits of E1. Site of phosphorylation by BDK
E1β	1 of 2 subunits of E1.
E2	dihydrolipoyl transacylase
E3	dihydrolipoamide dehydrogenase
FAD	flavin adenine dinucleotide
GCS	Glycine Cleavage System
HIF	Hypoxia Inducible Factor
IDH1	Isocitrate dehydrogenase 1
α-KG	α-ketoglutarate
KGDH	α-ketoglutarate dehydrogenase complex
MSUD	Maple Syrup Urine Disease
NADH	nicotinamide adenine dinucleotide
OAA	oxaloacetate
PC	pyruvate carboxylase
PDH	pyruvate dehydrogenase complex

PDK	pyruvate dehydrogenase kinase
R5P	ribose-5-phosphate
TCA cycle	tricarboxylic acid cycle
TPP	thiamin pyrophosphate

Acknowledgments

The diligence and assistance of several people must be recognized. First, is Dr. Zachar, who with her open office, time spent and endless dedication, passion for teaching, profound insight on the science at hand, and experimental help and advice made this endeavor at all possible, both through the entire program and beyond. Moreover, Shawn's approachable nature, expertise (particularly on the whereabouts of the lab), time and additional tips on the "hows" of the assays deserves the utmost praise and thanks. Moises' friendship, intellectually stimulating conversations, willingness to serve as a constant sounding board, and further knowledge as an aspiring scientist requires a special acknowledgement. Lastly, Professor Bingham, since my days as a young undergraduate through this point, evokes special thanks for his role as an educator, scientist, and caring, compassionate individual. His role in my development as an adult has been invaluable

Introduction

Transformed Cells Exhibit Altered Metabolism

Otto Warburg's groundbreaking observation, made in the 1920's, that rapidly proliferating tumors consume glucose at a much higher rate than non-proliferating "normal tissue" and secrete most of the glucose-derived carbon as lactate rather than fully oxidized CO_2 provoked an important question. Why do transformed cells take in excess amounts of nutrients beyond their bioenergetic demands, and why do they use such a "wasteful" form of energy metabolism (glycolysis) even under aerobic conditions, when the tricarboxylic-acid (TCA) cycle produces ATP in a much more efficient manner?

Warburg suggested that the up-regulation of glycolysis resulted from a defect in tumor mitochondria. Work done since then by many different investigators has demonstrated that mitochondria in cancer cells are indeed functional (reviewed in [1]). Additionally, this work has shown that tumor cell mitochondria are deployed very differently than mitochondria in healthy, non-proliferating cells [2]. Specifically, the persistent, proliferative state in which cancer cells exist requires not only ATP but also the doubling of total biomass with the production of two daughter cells per cell cycle. Thus, the production of lipids and proteins for growth, as well as nucleotides for the replication of the genome, are key requirements that loom as importantly as the need for the production of energy (reviewed in [1], [3]).

In the last fifteen years, work done by many investigators has greatly advanced the understanding of the transformed metabolic state of cancer cells. The emerging picture shows that tumor cells globally alter their metabolism to become factories for biosynthetic molecules supporting cell growth and proliferation and generate ATP to support the proliferative state.

Carbon from the TCA Cycle Used to Produce Biosynthetic Precursors Needs to be Replenished

Aside from this high flux of the glycolytic pathway in proliferating cells, which is required to sustain relatively low-flux pathways that diverge off of it to produce biosynthetic precursors (such as production of ribose-5-phosphate (R5P) through the oxidative and non-oxidative arms of the pentose phosphate pathway), the remodeling of the TCA cycle reflects this need for high levels of macromolecular biosynthesis as well. In effect, carbon sources that in normal cells would efficiently be used to produce ATP are diverted towards the production of lipids, nucleotides, and proteins; this draining of TCA intermediates is known as "cataplerosis."

For instance, citrate is required for the synthesis of fatty acids, and cholesterol is needed for the production of lipid membranes in daughter cells, while oxaloacetate (OAA) and α-ketoglutarate (α-KG) are diverted out of the TCA cycle and converted to non-essential amino acids that can be used for the production of nucleotides and proteins.

As a direct consequence, however, intermediates that are drained out of the TCA cycle in the mitochondria require their replenishment, a term known as "anaplerosis," This process ultimately allows the TCA cycle to continue functioning in the face of cataplerosis. For example, pyruvate carboxylase (PC) is the enzyme that regenerates OAA directly from glucose-derived pyruvate. Further, "glutaminolysis," is the metabolism of glutamine (the most abundant amino acid in serum) to produce TCA intermediates like OAA in the mitochondria of proliferating cells, as well as glutamate (an important excitatory neurotransmitter in neuronal cells) and α-KG (another TCA cycle intermediate). Thus glutamine has come to be understood as a carbon source (in addition to glucose) for the regeneration of TCA cycle intermediates,

reinforcing the idea that proliferating cells have a great need for carbon to sustain both bioenergetic and biosynthetic functions (reviewed in [1]). This view of glutamine as a source of carbon for intermediary metabolism, as well as combined effects of bioenergetic and biosynthetic pathways is diagrammed in Figure 1.

Regulation of the Pyruvate Dehydrogenase Complex, the Gatekeeper of Carbon Flux from Glycolysis to the TCA Cycle

The well-characterized pyruvate dehydrogenase complex (PDH), one of four mitochondrial enzyme complexes that uses covalently joined lipoate as a catalytic cofactor, is regulated by four pyruvate dehydrogenase kinases (PDK-1-4). These kinases are responsible for phosphorylating the E1α subunit of PDH, and hence inactivating the entire complex, in response to various conditions including the ratios of ADP to ATP; NAD to NADH; CoA to acetyl CoA and reduced to oxidized lipoamide. I will focus here on the role of the ratio of reduced to oxidized lipoamide. High levels of reduced lipoamide serve as sensors of PDH saturation and lead to activation of the PDKs, and phosphorylation of the E1α subunit and PDH shut down. Two of the PDKs, PDK-1 and PDK-3, are often elevated in tumors and cancer cell lines in response to hypoxia inducible factor (HIF) [2, 4-9], a transcription factor that has been shown to play a large role in cancer cells [7, 10, 11]. Further, HIF has been shown to induce overexpression of PDK-1, the only isoform that is known to phosphorylate three different serine residues on the PDH E1α subunit. Subsequent dephosphorylation of all three sites is required to reactivate the PDH complex (reviewed in [8]).

Introduction to the metabolism of branched-chain amino acids, leucine, valine and isoleucine

The branched chain amino acids, leucine, valine and isoleucine are classified as essential amino acids for mammals because mammals cannot synthesize them and must obtain these amino acids from their diet. These three amino acids collectively account for about 20% of dietary protein and are essential for protein synthesis, branched chain fatty acid synthesis, and neurotransmitter production [12]. The high prevalence of BCAAs in protein structure is largely correlated with their high levels of hydrophobicity, a strong factor that frequently places them in membrane proteins coiled-coiled structures [13].

Maple Syrup Urine Disease (MSUD) [14], is an inborn error of metabolism disease which leads to the accumulation of branched chain amino acids and is characterized by symptoms including convulsions, mental retardation, ketoacidosis, hypoglycemia, and psychomotor delay. This suggests that concentrations of the branched chain amino acids in serum and tissues must be carefully regulated.

Two key enzymes that play a role in the metabolism of branched chain amino acids are the branched-chain amino acid transaminase (BCAT1, cytosolic and BCAT2, mitochondrial) and the branched-chain α-keto acid dehydrogenase complex (BCKDC, mitochondrial). Mutations in the E1α gene of the BCKDC complex have been documented in Maple Syrup Urine disease patients [15].

Branched-Chain α-Ketoacid Dehydrogenase Structure, Catalysis, and Regulation

The breakdown of BCAAs involves two steps using two different enzymes that function as a metabolon [16]. The initial catabolic reaction involves the reversible transamination of the

BCAA amino group to α-ketoglutarate, thereby generating glutamate and the corresponding branched-chain α-ketoacid (BCKA). The branched-chain amino acid transaminase (BCAT) is responsible for catalyzing this step [17]. BCAT will be of interest in a particular context in the discussion section. BCKDC then catalyzes the second catabolic step, the irreversible oxidative decarboxylation of the three BCKAs, sending each to their own degradation pathways [12]. Regardless of pathway, however, all breakdown products catalyzed by BCKDC eventually enter the TCA cycle, serving as carbon sources that sustain the cycle [14]. A schematic of this process is shown in Fig. 2. Thus BCKDC serves a critical role in the regulation of levels of BCAAs.

BCKDC is a member of the conserved lipoate-containing mitochondrial dehydrogenase complexes, including the pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) complexes. The glycine cleavage system (GCS) is a fourth such lipoate-bearing enzyme, though its role in metabolism will not concern us here. PDH, KGDH, and BCKDC each catalyze the oxidative decarboxylation of α-ketoacids to produce acetyl-CoA, succinyl-CoA, or branched-chain acyl-CoAs, respectively [16].

Similar to PDH and KGDH, BCKDC is composed of three distinct catalytic subunits that metabolize different α-ketoacids to TCA cycle intermediates. The branched-chain α-ketoacid decarboxylase is known as E1, E2 is the dihydrolipoyl transacylase, and dihydrolipoamide dehydrogenase is E3 [18]. Furthermore, 24 E2 subunits serve as the core scaffold, with 12 copies of E1 and 6 copies of E3 non-covalently attached to this scaffold. E1, the branched-chain α-ketoacid decarboxylase is made up of two subunits, E1α and E1β. The former is regulated by phosphorylation and dephosphorylation by a dedicated kinase and phosphatase, respectively [19].

Regulation of the PDH complex by phosphorylation of the E1α subunit has long been established and has implications for our study of BCKDC. [20] The PDH complex links glycolysis to the TCA cycle and regulates the flow of glucose-derived carbon into mitochondria in tissues that have high metabolic demands, irreversibly converting glucose-derived pyruvate to acetyl-CoA. Further, the complex serves as the carbon source for fatty acid synthesis in adipose tissue, the mammary glands, and the liver [21]. Similar to BCKDC, the eukaryotic PDH E2 dihydrolipoamide acetyl-transferase core serves as a scaffold to which the E1, pyruvate dehydrogenase (responsible for the oxidative decarboxylation of pyruvate), and E3, dihydrolipoamide dehydrogenase, components are attached [22].

CPI-613, a non-redox active Lipoic Acid Analog, Increases Phosphorylation of the Pyruvate Dehydrogenase Complex

Zachar *et al*. [23] first reported the analysis of CPI-613, a non-redox-active lipoic acid analog that is selectively cytotoxic against tumor cells. The four mitochondrial enzyme complexes PDH, KGDH, BCDKC, and the GCS, use covalently bound lipoic acid (lipoate) as a cofactor for catalysis. Among these enzymes, PDH in particular, however, is known to use ratios of oxidized to reduced lipoamide intermediates (cyclically converted) as a sensor of carbon flux through PDH. When high levels of the acyl and/or reduced lipoamide are present, the pyruvate dehydrogenase kinases (PDKs) are activated to phosphorylate the PDH complex on its E1α subunit, thereby shutting down the complex. Zachar *et al*. have shown that CPI-613 selectively increases phosphorylation of the PDH E1 α subunit, thereby inactivating PDH. Thus, CPI-613, an exogenously supplied lipoic acid-analog, can selectively disrupt tumor cell metabolism and

induce cell death. Moreover, knock down of the PDKs by RNAi protects tumor cells from CPI-613 effects [23].

Among the four lipoate-containing mitochondrial complexes, PDH and BCKDC have been shown to have similar mechanisms of regulation. Five mitochondrial protein kinases have been identified in eukaryotes: the first four are different isoforms of PDKs, all encoded by distinct nuclear genes, while the fifth gene product codes for a mitochondrial kinase that regulates BCKDC [24]. Given this information, I was interested in investigating whether BCKDC regulation by phosphorylation is affected by CPI-613. Secondly, the levels of BCAT, the first enzyme in the catabolism of branched chain amino acids, have been reported to vary among different glioblastomas [25]. In light of this, I investigated whether levels of BCKDC vary among different epithelial tumor lines.

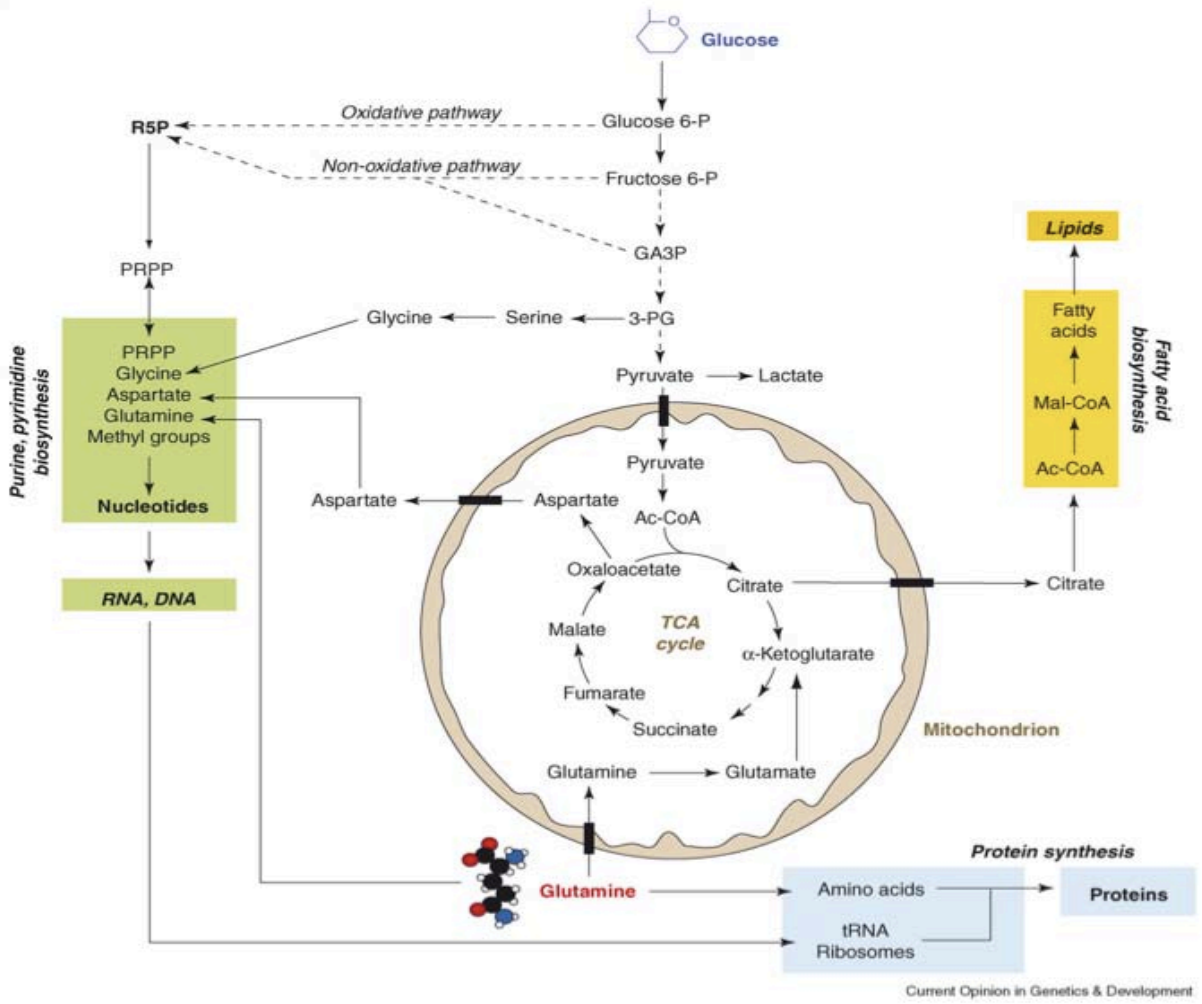

Fig. 1

Tumor cells obtain biosynthetic precursors from glucose and glutamine metabolism. Glucose and glutamine, the two most abundant extracellular nutrients, contribute carbon for the synthesis of the three major classes of macromolecules (nucleic acids, lipids, and proteins) in proliferating tumor cells. Biosynthesis of purines and pyrimidines utilizes ribose 5-phosphate (R5P) produced from diversion of glycolytic intermediates into the oxidative and non-oxidative arms of the pentose phosphate pathway, and nonessential amino acids derived from glucose and glutamine. Fatty acid synthesis, used to produce cellular lipids, requires acetyl-CoA (Ac-CoA), most of which is generated from glucose and transferred from the mitochondria to the cytoplasm via citrate. Protein synthesis requires amino acids, tRNAs and ribosomes (proteins and rRNAs). Both glucose and glutamine are used to generate these molecules. In addition to its role as a carbon source, glutamine also donates nitrogen to nucleotide and amino acid synthesis. Abbreviations: P, phosphate; GA3P, glyceraldehyde 3-phosphate; 3-PG, 3-phosphoglycerate; PRPP, phosphoribosyl pyrophosphate; Mal-CoA, malonyl-CoA.

Reproduced from Deberardinis, R. J., et al. (2008) [3] with permission.

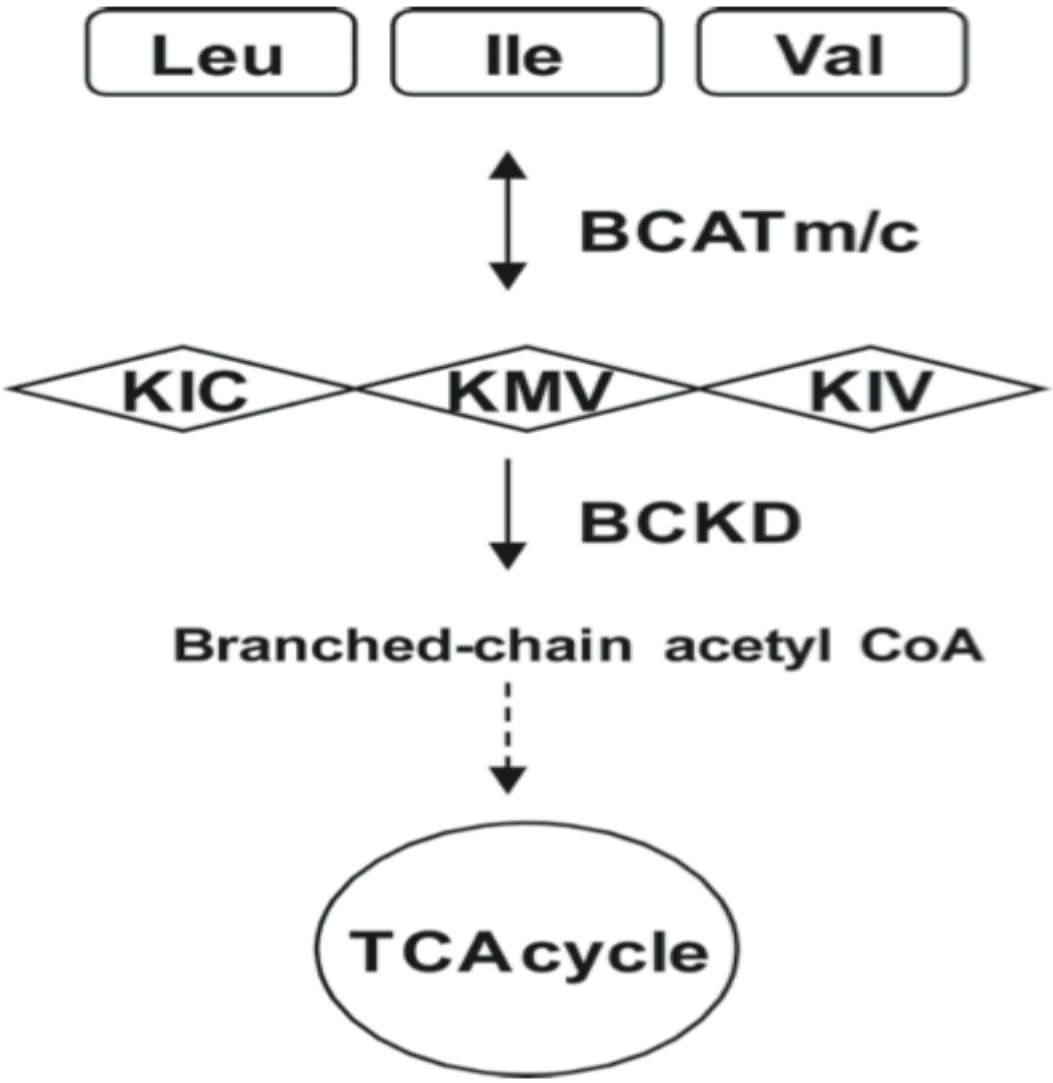

Fig. 2

A schematic of the branched-chain AA (BCAA) metabolic pathway. The first step of BCAA metabolism is the reversible transamination of Leu, Ile, or Val, which is catalyzed by the branched- chain aminotransferase (BCAT) isozymes (BCATm and BCATc). In the second step, the products, α-ketoisocaproate (KIC), α-keto-β- methylvalerate (KMV), or α-ketoisovalerate (KIV), undergo oxidative decarboxylation, which is catalyzed by the branched-chain keto acid dehydrogenase (BCKD) enzyme complex. This irreversible process produces branched-chain acetyl CoA, which can enter the tricarboxylic acid (TCA) cycle.

Reproduced from Suryawan et al. (2011) [26] with permission.

Results

CPI-613 treatment causes hyper-phosphorylation of the BCKDC E1α subunit in H460 cells

Zachar *et al* (2011) showed that CPI-613 treatment of H460 lung tumor cells resulted in hyper-phosphorylation of the PDH E1 α subunit. [23] BCKDC is structurally organized in an analogous fashion to PDH and has a dedicated branched-chain α-ketoacid dehydrogenase kinase (BDK), which has been reported to phosphorylate the BCKDC E1α subunit [27, 28]. I therefore investigated whether CPI-613 treatment would change the phosphorylation status of BCKDC E1α in H460 non-small cell human lung carcinoma cells. H460 cells were treated with solvent (DMSO) or 300uM CPI-613 for three, four or eight hours. Cell lysates were prepared at the end of treatment time and were analyzed by Western blots using an antibody specific for the phospho-epitope of the E1α subunit. Phosphorylation levels were compared to total E1α amounts. Figure 3a shows the results of these experiments. Note that CPI-613 treatment increases phosphorylation of BCKDC E1α up to two fold {Figure 3B).

CPI-613 increases phosphorylation in BxPC-3 pancreatic tumor cells

Given the effects of CPI-613 treatment on phosphorylation of the BCKDC E1 α subunit in H460 cells, I then investigated whether this effect extends to other tumor lines. Pancreatic adenocarcinoma cells, BxPC-3, were treated either with vehicle (DMSO) or 300uM CPI-613 for three, four or eight hours. Whole cells lysate were prepared and analyzed by western blot analysis analogously to the H460 set. Fig. 4a shows the results of this analysis. As with H460, CPI-613 treatment increases phosphorylation of the BCKDC E1α subunit in BxPC3 cells.

Quantifications of the blots shown in Fig. 4a are shown in Fig. 4b. Note that the increase in BCKDC phosphorylation due to CPI-613 treatment is almost two-fold.

Levels of BCKDC E1 α vary across different cell lines

Tonjës et al. [23] have shown that the branched-chain amino acid transaminase 1 (BCAT1; the cytosolic isoform), is upregulated in primary glioblastoma tumors that have a wild-type Isocitrate Dehydrogenase 1 (IDH1; the cytosolic isoform), but is not upregualted in glioblstomas that carry a mutant IDH1. Given that BCAT catalyzes the reaction creating the substrate for BCKDC in the catabolism of branched-chain amino acids, I hypothesized that the levels of BCKDC may vary among different tumor types. I therefore undertook a survey of several epithelial tumor cell lines to see if BCKDC levels varied.

Surveyed epithelial cancer cell lines included H460 (NCI-H460; human non-small cell lung carcinoma), A549 (human small cell lung carcinoma), BxPC-3 (human pancreatic adenocarcinoma), MDA-231 (MDA-MB-231; human metastatic breast cancer), and HeLa cells (human cervical cancer). I also included the human glioblastoma cell line U-87.

A549 cells showed detectable levels of the BCKDC E1α subunit, and a marked increase in phosphorylation of the peptide at the 4 hour time point relative to mock treated cells, as shown in Fig. 5a. Moreover, MDA-231 cells also showed detectable levels of BCKDC E1α, though treatment did not result in such noticeable levels of phosphorylation relative to mock treated cells at either 3, 4 or 8 hours of sample collection (Fig. 5b).

HeLa cell lysates probed for BCKDC E1α showed high levels comparable to those seen in other cell lines, though treatment with 300 μM CPI-613 did not increase levels of phosphorylation to any noticeable extent, as shown in Fig. 5c. Lastly, U-87, the primary glioblastoma cell line, showed highly detectable levels of BCKDC E1α when probed with antibody, however, levels of induced phosphorylation were the least detected relative to control when cells were treated with 300 μM CPI-613 (Fig. 5d).

3A

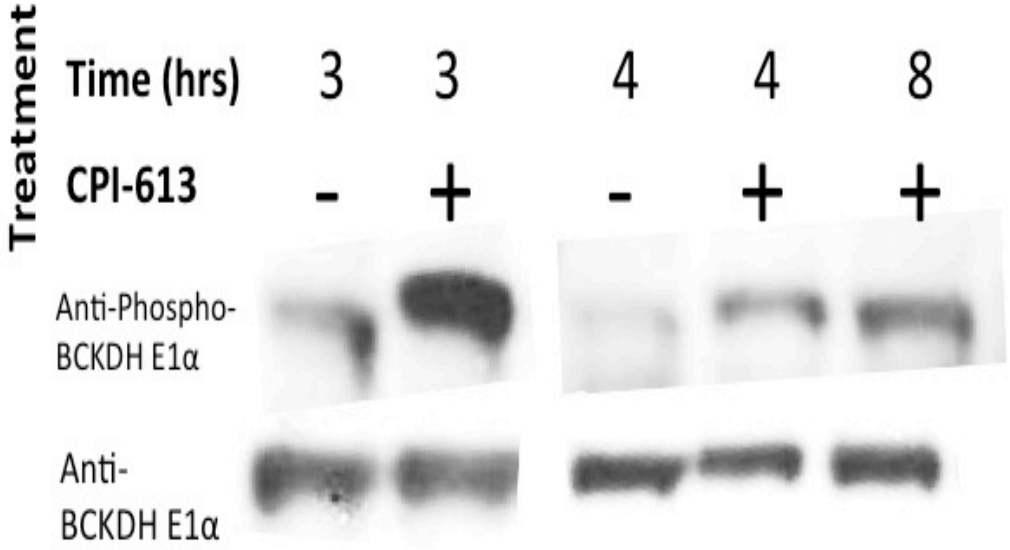

Fig. 3a

Western analysis of two separate experiments involving treatment of H460 lung tumor cells. Cells were treated with either vehicle (DMSO) or 300μM CPI-613 for 3, 4 or 8 hours. Whole Cell lysates were prepared and run on 10% Bis-Tris gels.

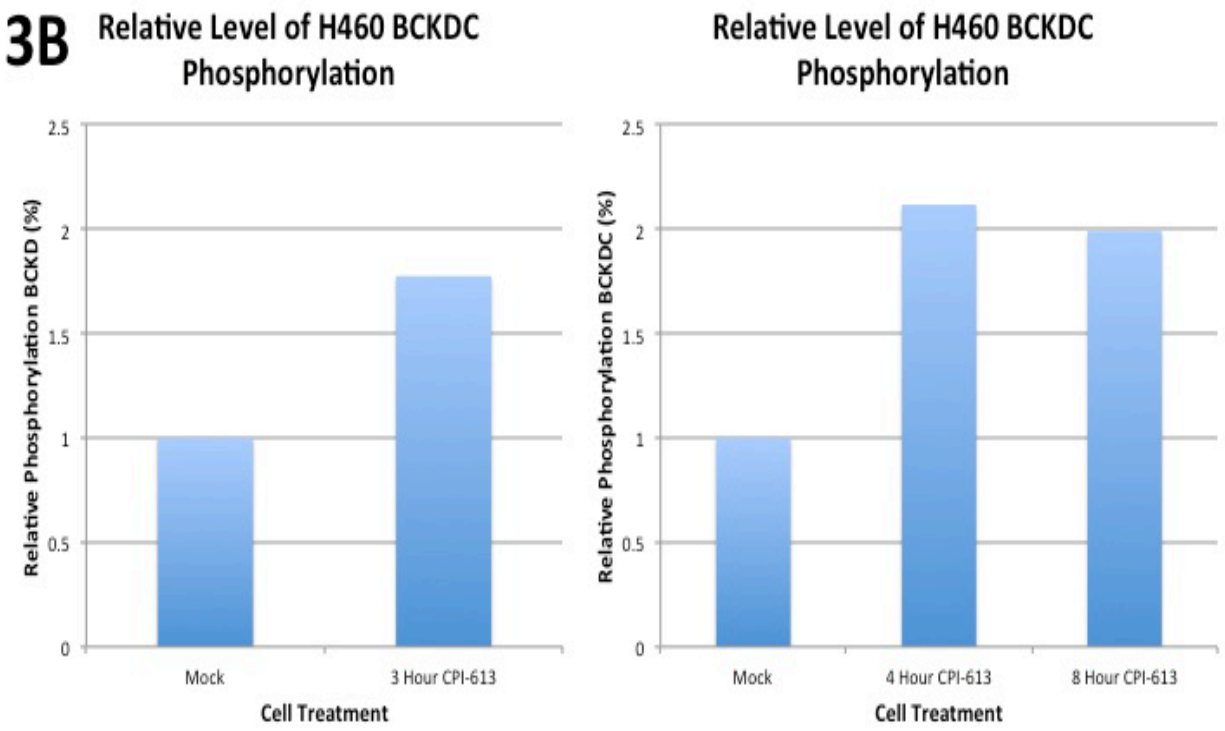

Fig. 3b

Quantification of relative levels of phosphorylation of H460 BCKDC of westerns shown in **Fig. 3a.**

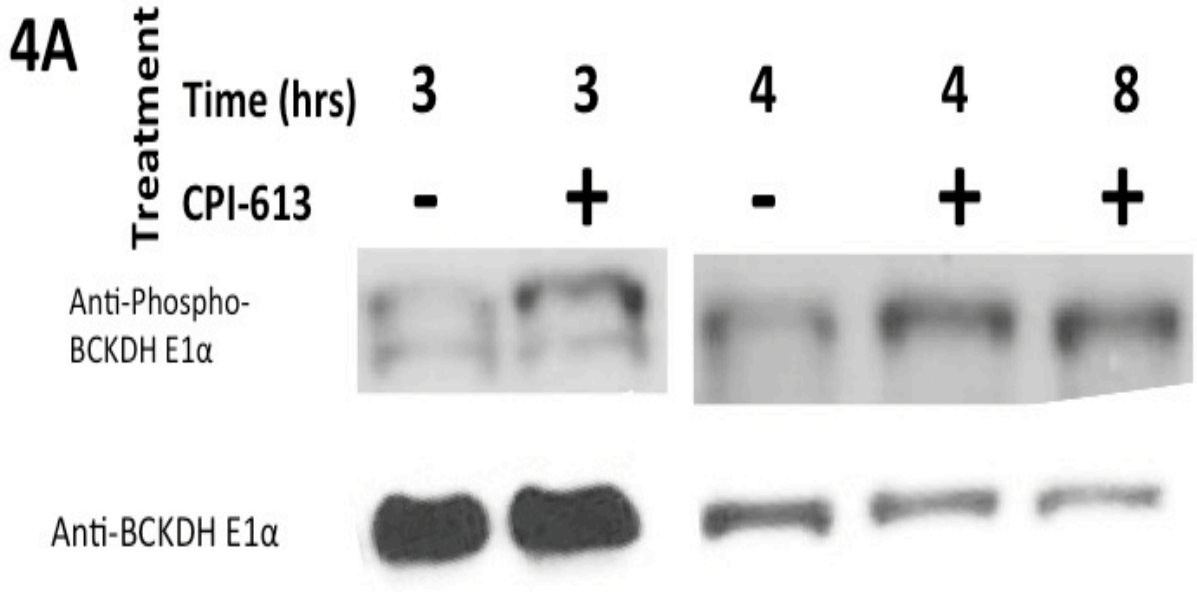

Fig. 4a

Western analysis of two separate experiments involving treatment of BxPC-3 pancreatic adenocarninoma cells. Cells were treated with either vehicle (DMSO) or 300μM CPI-613 for 3, 4 or 8 hours. Whole cell lysates were prepared and run on 10% Bis-Tris gels.

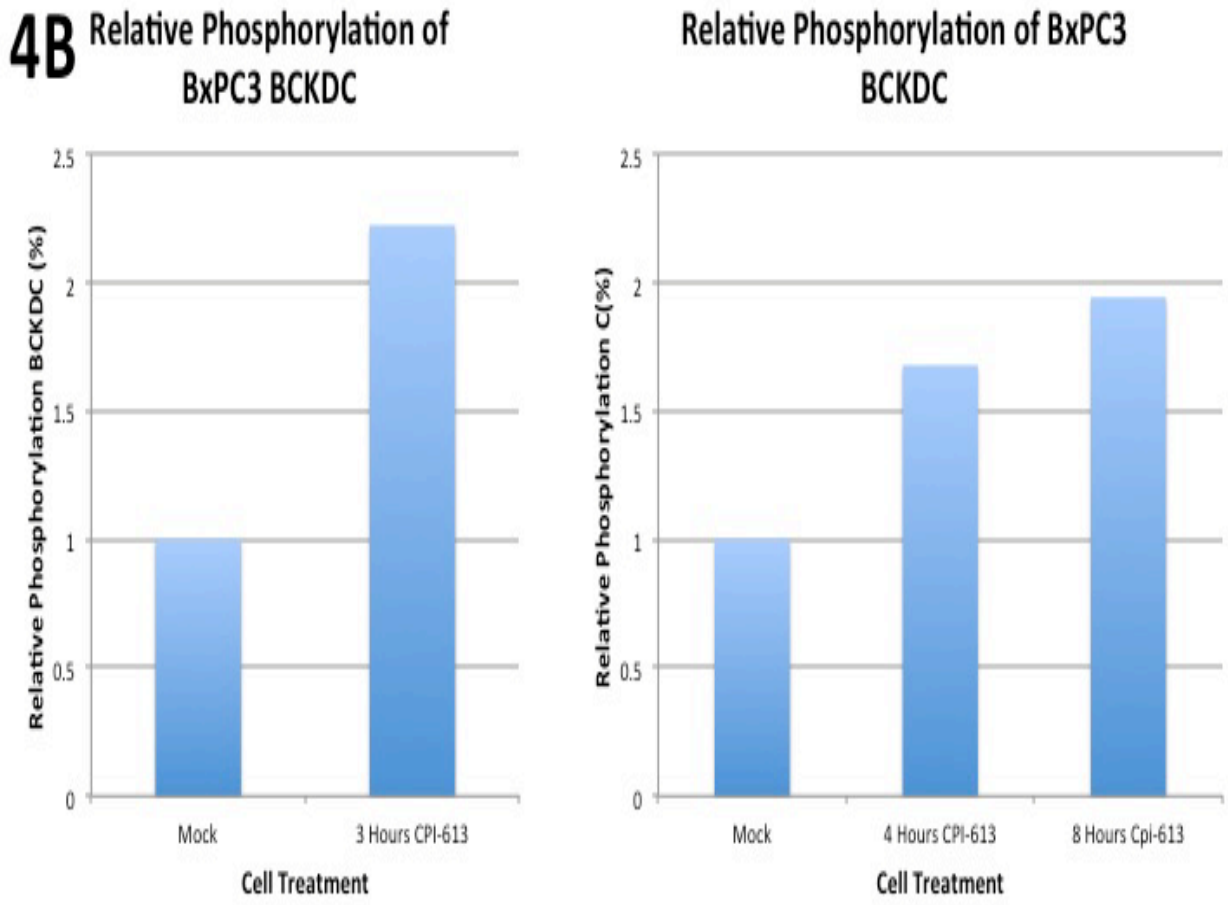

Fig. 4b

Quantification of relative levels of phosphorylation of H460 BCKDC of westerns shown in **Fig. 4a.**

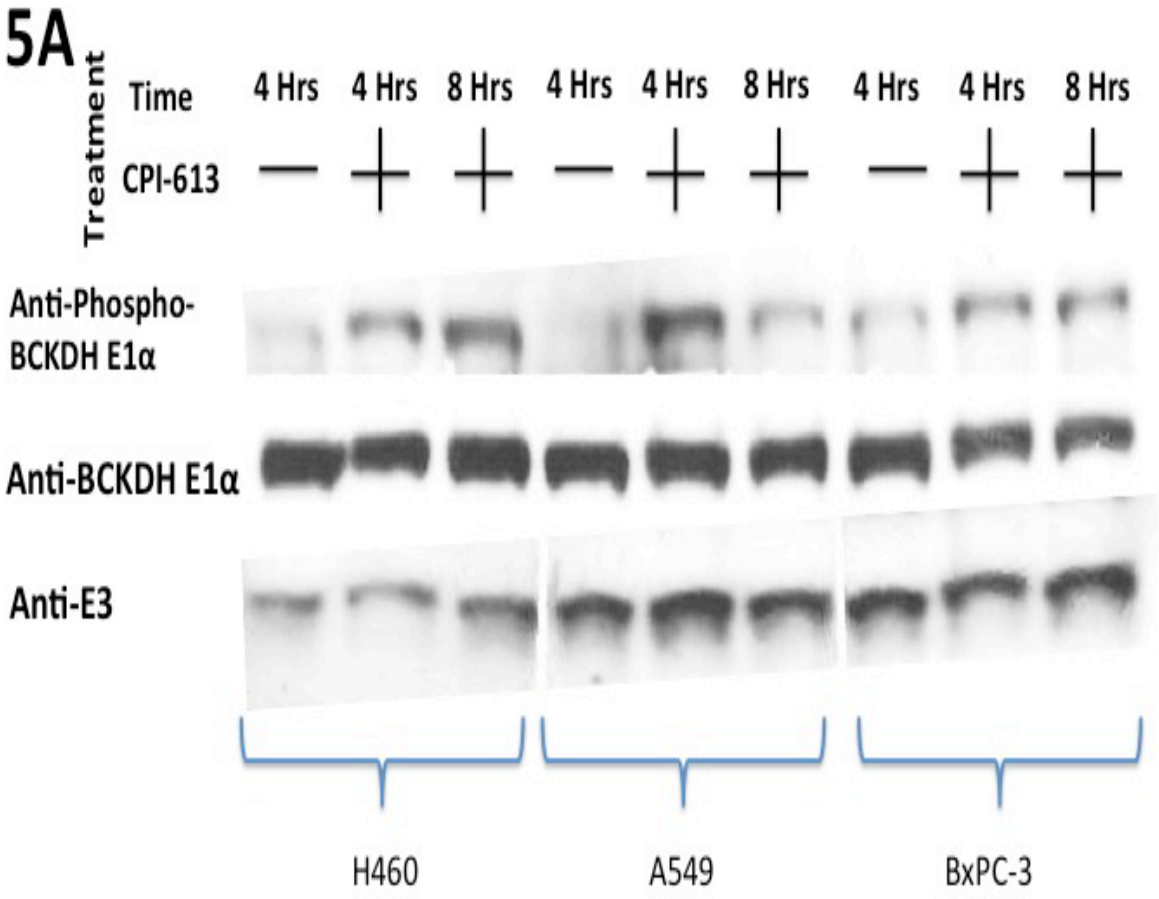

Fig. 5a

Western analysis of survey of epithelial cell lines. H460, A549, and BxPC-3 cells were each treated with either vehicle (DMSO) or 300μM CPI-613 for 4 or 8 hours. Whole cell lysates were prepared and run on 10% Bis-Tris gels.

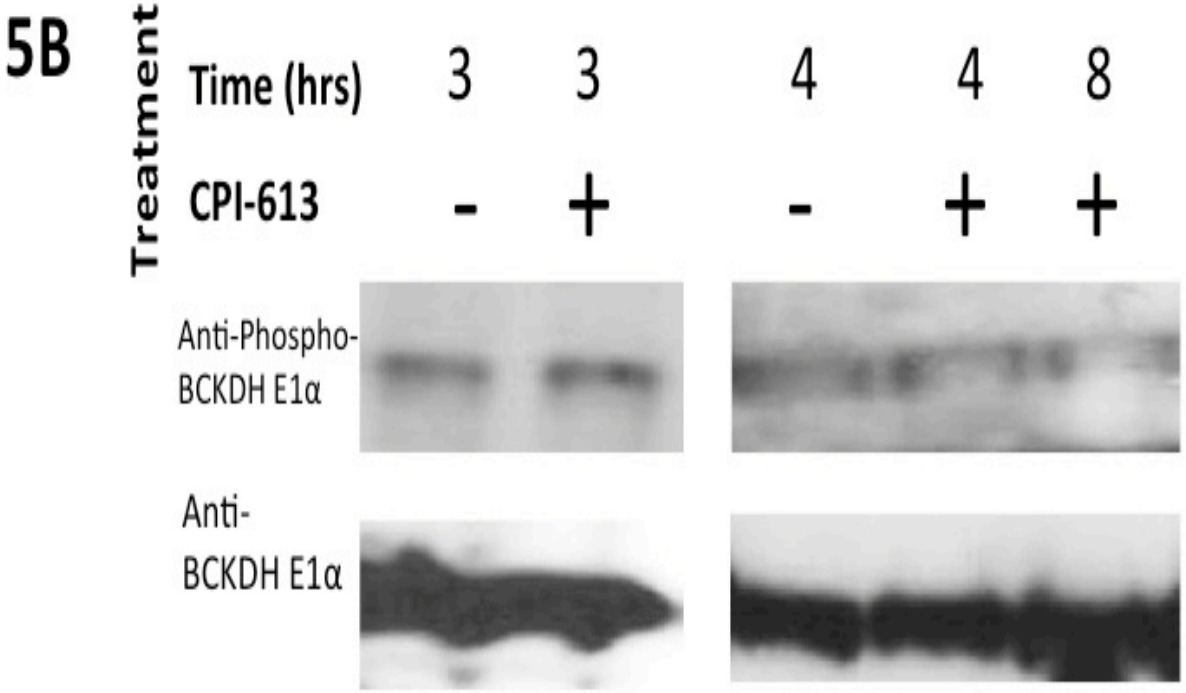

Fig. 5b

Western analysis of two separate experiments involving treatment of MDA-231 metastatic breast cancer cells. Cells were treated with either vehicle (DMSO) or 300μM CPI-613 for 3, 4 or 8 hours. Whole cell lysates were prepared and run on 10% Bis-Tris gels.

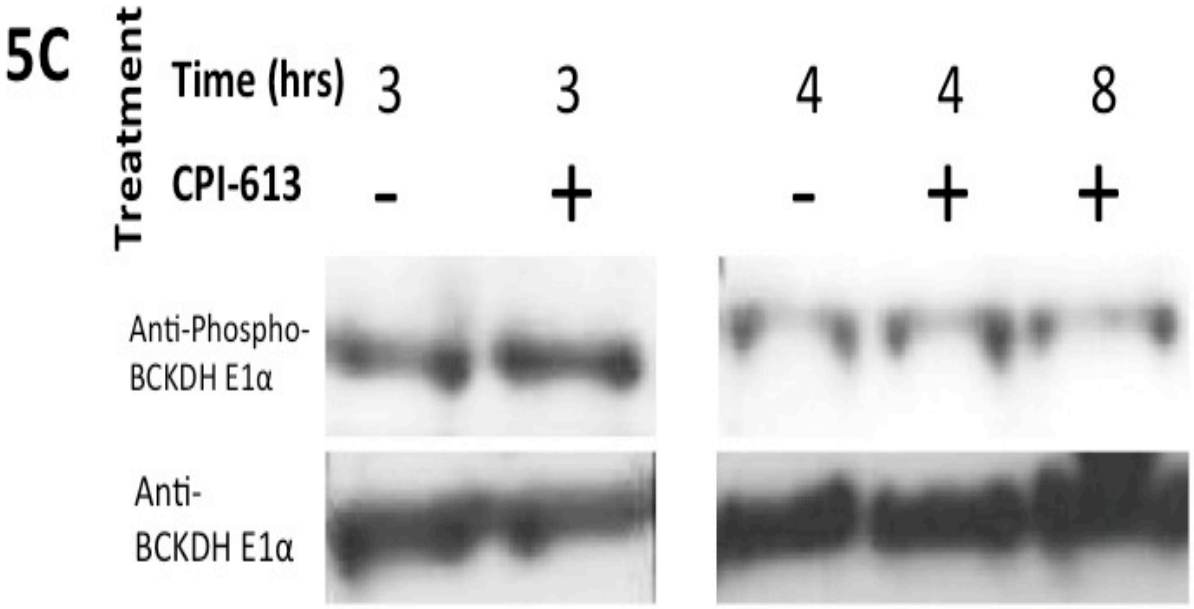

Fig. 5c

Western analysis of two separate experiments involving treatment of HeLa cells. Cells were treated with either vehicle (DMSO) or 300μM CPI-613 for 3, 4 or 8 hours. Whole cell lysates were prepared and run on 10% Bis-Tris gels.

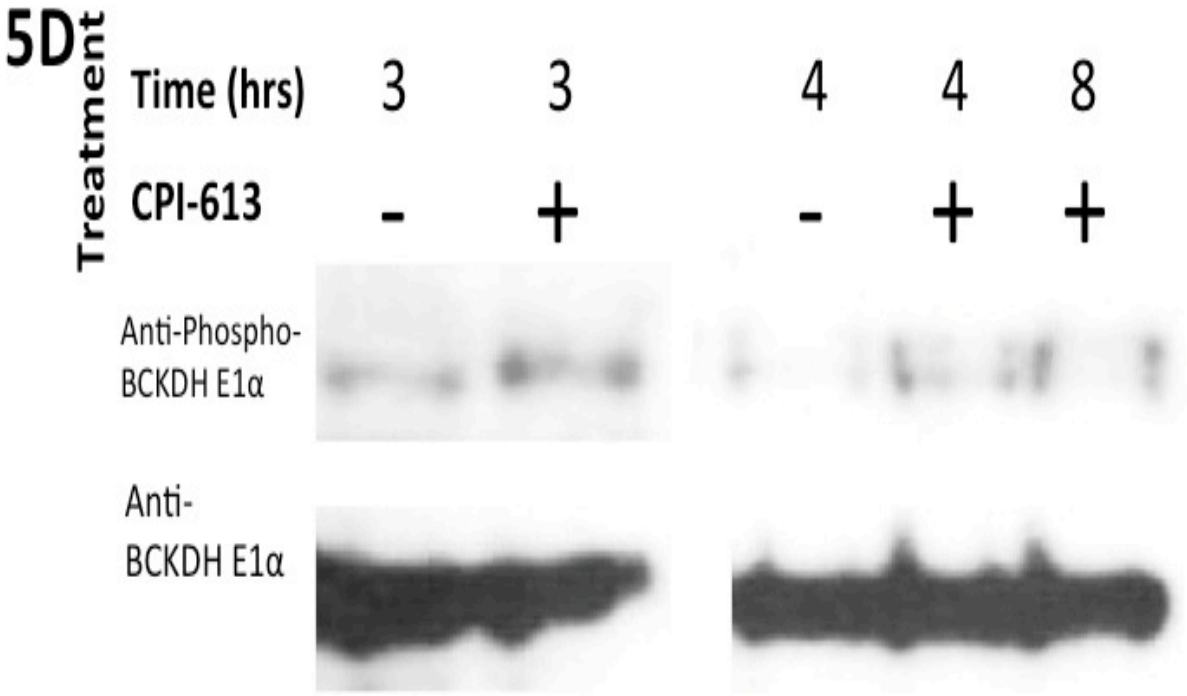

Fig. 5d

Western analysis of two separate experiments involving treatment of U-87 glioblastoma cells. Cells were treated with either vehicle (DMSO) or 300μM CPI-613 for 3, 4 or 8 hours. Whole cell lysates were prepared and run on 10% Bis-Tris gels.

Discussion

The emergent picture of the last two decades of work on the transformed state of cancer cells has placed a large focus on metabolism. Of great appreciation is the idea that tumor cells globally alter their metabolism to become factories for biosynthetic molecules supporting cell growth and proliferation and generate ATP to support the proliferative state. Given this picture, certain cancer therapies look to target mitochondrial enzymes that in the transformed state sustain the TCA cycle for the production of biosynthetic precursor molecules and ATP.

For example, Zachar *et al.* [23] showed that the pyruvate dehydrogenase complex (PDH) is phosphorylated (and inactivated) in human non-small cell lung tumor line H460 in response to treatment with anti-cancer agent CPI-613. Further, RNAi knockdown of the kinases responsible for phosphorylation of PDH E1α protected cells from CPI-613 effects, suggesting the drug's targeting of the kinases to cause inactivation.

The branched-chain α-ketoacid dehydrogenase complex (BCKDC), the enzyme responsible for the final step in catabolism of the branched chain amino acids leucine, isoleucine, and valine, is a member of the four lipoate-containing mitochondrial enzyme complexes including PDH, α-ketoglutarate dehydrogenase (KGDH) complex, and the glycine cleavage system (GCS). BCKDC is, in addition, structurally organized in an analogous manner to PDH, each consisting of three subunits. [20] A kinase responsible for phosphorylating the BCKDC E1α subunit (and inactivating the complex) has been reported. [24] I thus asked whether BCKDC would be phosphorylated in response to treatment with CPI-613, and showed noticeable levels of hyper-phosphorylated BCKDC E1α in several epithelial cell lines. Levels of phosphorylation in H460 cells treated with CPI-613 were about two-fold relative to mock treated cells. I then asked

whether this effect extended to other epithelial cell lines, and a similar effect was observed in BxPC-3 (human pancreatic cancer) cells treated with CPI-613.

Further, when A549 (human lung carcinoma) cells were treated with CPI-613, BCKDC E1α was hyper-phosphorylated after four hours of treatment. MDA-231 cells (MDA-MB-231; human metastatic breast cancer) showed no detectable levels of hyper-phosphorylation of BCKDC E1α at up to 8 hours of treatment with CPI-613, and the same results were seen in HeLa cells (human cervical cancer). Lastly, U-87 cells (U-87 MG; human glioblastoma) showed slight levels of hyper-phosphorylation when treated with drug relative to vehicle. It should be noted that total levels of BCKDC E1α were detected in all cell lines I experimented with. Of course, future studies will involve looking at branched-chain α-ketoacid dehydrogenase kinase (BDK) levels in all of the tested cancer lines.

In addition, when Tönjes *et al*. [25] looked at levels of the branched-chain amino acid transaminase (BCAT) in glioblastoma xenografts, the enzyme just upstream of BCKDC, they found that a subset of tumors that have the wild-type isocitrate dehydrogenase (IDH) enzyme have increased levels of BCAT, while those glioblastomas with a mutant IDH have downregulated levels of BCAT. We would expect that BCAT and BCKDC levels would correlate with one another, as the two enzymes are reported to work as a metabolon to catabolize essential BCAAs [16]. Moreover, it would be interesting to see whether this correlation between the mutational status of IDH and the presence of BCAT holds up in epithelial cell lines.

Future studies on BCKDC will involve siRNA knockdown of the branched-chain α-ketoacid dehydrogenase kinase (BDK). If CPI-613 inactivates BCDKC by inducing the BDK to phosphorylate the E1 α subunit of BCKDC, we expect knockdown of the kinase to result in

lower levels of phosphorylation of the E1α subunit in the presence of drug treatment, relative to scrambled siRNA controls or cells not treated with siRNA. Moreover, flux analysis of radioactive branched-chain α-ketoacids (BCKA) should be used to test whether oxidation of the BCKAs changes as they pass through the BCKDC enzyme. The expectation is that phosphorylation and inactivation of the enzyme due to CPI-613 treatment should results in a lower flux of radioactive BCKAs through the enzyme.

In the grand scheme of things, BCKDC serves as a third potential target of CPI-613 in attacking cancer mitochondrial metabolism as a source of therapy. Future studies on the enzyme hope to show that its modulation by an exogenous agent can result in inactivation, thus shutting down its role in processing BCKAs that serve as carbon sources to support the growth and proliferative state of cancer cells.

Materials and Methods

Cells and cell culture: All cells were obtained from the American Type Culture Collection (ATCC). The human non-small cell lung carcinoma cell line NCI-H460, human lung carcinoma cell line A549, and pancreatic cancer cell line BxPC-3 were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 ug/ml streptomycin (all obtained from Life Technologies). The human metastatic breast cancer cell line MDA-MB-231, human cervical cancer cell line HeLa, and human glioblastoma cell line U-87 MG were each cultured in Dulbecco's Modified Eagle Medium (DMEM), also supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 ug/ml streptomycin.

35mm petri dishes were seeded with 1 million cells of each respective cell line and allowed to grow in their respective growth medium as noted above at 37° C and 5% CO_2 for 18-25 hours. Medium was then replaced with fresh growth medium containing either vehicle (dimethyl sulfoxide, DMSO as a mock treated control) or 300 μM CPI-613 for time intervals as indicated in the results.

Chemicals: CPI-613 was obtained from Cornerstone Pharmaceuticals, Inc. Antibodies to BCKDC E1α and phospho-BCKDC E1α were purchased from Bethyl Laboraties, Inc. Antibody against dihydrolipoamide dehydrogenase (E3) was obtained from Rockland.

Western Analysis: Cell lysates were processed using the Zoom Benchtop proteomics system (Invitrogen Zoom 2D Protein Solubilizer 2) as described in Zachar *et al*. [23] Cells were lysed in

situ with 300 µl Lysis Buffer A [Zoom 2D Protein Solubilizer 2 (Invitrogen), 5.5 mM tris base, 11M protease inhibitor cocktail (Complete min, EDTA free; Roche), 22 mM DTT] and each transferred to a 1.5ml microfuge tube and sonicated for 10 passes at 50% power. To generate samples for Western analysis, 12 µl of each sample was added to 4 µl NuPAGE LDS sample buffer (Invitrogen) and mixed, followed by 15 µl loading of each sample onto NuPAGE 10% Bis-Tris gels (15 well, Life Technologies) for SDS-PAGE. Gels were then transferred to PVDF membranes and detected via chemiluminescence using the WesternBreeze detection kit (Life Technologies).

References

1. DeBerardinis, R.J., et al., *The biology of cancer: metabolic reprogramming fuels cell growth and proliferation.* Cell Metab, 2008. **7**(1): p. 11-20.

2. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science, 2009. **324**(5930): p. 1029-33.

3. Deberardinis, R.J., et al., *Brick by brick: metabolism and tumor cell growth.* Curr Opin Genet Dev, 2008. **18**(1): p. 54-61.

4. Kim, J.W., et al., *HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia.* Cell Metab, 2006. **3**(3): p. 177-85.

5. Koukourakis, M.I., et al., *Pyruvate dehydrogenase and pyruvate dehydrogenase kinase expression in non small cell lung cancer and tumor-associated stroma.* Neoplasia, 2005. **7**(1): p. 1-6.

6. Lu, C.W., et al., *Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance.* J Biol Chem, 2008. **283**(42): p. 28106-14.

7. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell Metab, 2006. **3**(3): p. 187-97.

8. Roche, T.E. and Y. Hiromasa, *Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer.* Cell Mol Life Sci, 2007. **64**(7-8): p. 830-49.

9. Sugden, M.C. and M.J. Holness, *Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs.* Am J Physiol Endocrinol Metab, 2003. **284**(5): p. E855-62.

10. Pouyssegur, J., F. Dayan, and N.M. Mazure, *Hypoxia signalling in cancer and approaches to enforce tumour regression.* Nature, 2006. **441**(7092): p. 437-43.

11. Robey, I.F., et al., *Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors.* Neoplasia, 2005. **7**(4): p. 324-30.

12. Harris, R.A., M. Joshi, and N.H. Jeoung, *Mechanisms responsible for regulation of branched-chain amino acid catabolism.* Biochem Biophys Res Commun, 2004. **313**(2): p. 391-6.

13. Brosnan, J.T. and M.E. Brosnan, *Branched-chain amino acids: enzyme and substrate*

regulation. J Nutr, 2006. **136**(1 Suppl): p. 207S-11S.

14. Harris, R.A., et al., *Overview of the molecular and biochemical basis of branched-chain amino acid catabolism.* J Nutr, 2005. **135**(6 Suppl): p. 1527S-30S.

15. Indo, Y., et al., *Altered kinetic properties of the branched-chain alpha-keto acid dehydrogenase complex due to mutation of the beta-subunit of the branched-chain alpha-keto acid decarboxylase (E1) component in lymphoblastoid cells derived from patients with maple syrup urine disease.* J Clin Invest, 1987. **80**(1): p. 63-70.

16. Islam, M.M., et al., *A novel branched-chain amino acid metabolon. Protein-protein interactions in a supramolecular complex.* J Biol Chem, 2007. **282**(16): p. 11893-903.

17. Mayers, J.R. and M.G. Vander Heiden, *BCAT1 defines gliomas by IDH status.* Nat Med, 2013. **19**(7): p. 816-7.

18. Nobukuni, Y., et al., *Maple syrup urine disease. Complete primary structure of the E1 beta subunit of human branched chain alpha-ketoacid dehydrogenase complex deduced from the nucleotide sequence and a gene analysis of patients with this disease.* J Clin Invest, 1990. **86**(1): p. 242-7.

19. Nellis, M.M., et al., *Relationship of causative genetic mutations in maple syrup urine disease with their clinical expression.* Mol Genet Metab, 2003. **80**(1-2): p. 189-95.

20. Harris, R.A., K.M. Popov, and Y. Zhao, *Nutritional regulation of the protein kinases responsible for the phosphorylation of the alpha-ketoacid dehydrogenase complexes.* J Nutr, 1995. **125**(6 Suppl): p. 1758S-1761S.

21. Harris, R.A., B. Huang, and P. Wu, *Control of pyruvate dehydrogenase kinase gene expression.* Adv Enzyme Regul, 2001. **41**: p. 269-88.

22. Gu, Y., et al., *3D electron microscopy reveals the variable deposition and protein dynamics of the peripheral pyruvate dehydrogenase component about the core.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7015-20.

23. Zachar, Z., et al., *Non-redox-active lipoate derivates disrupt cancer cell mitochondrial metabolism and are potent anticancer agents in vivo.* J Mol Med (Berl), 2011. **89**(11): p. 1137-48.

24. Harris, R.A., et al., *Regulation of branched-chain alpha-keto acid dehydrogenase kinase expression in rat liver.* J Nutr, 2001. **131**(3): p. 841S-845S.

25. Tonjes, M., et al., *BCAT1 promotes cell proliferation through amino acid catabolism in*

gliomas carrying wild-type IDH1. Nat Med, 2013. **19**(7): p. 901-8.

26. Suryawan, A., et al., *Triennial Growth Symposium: leucine acts as a nutrient signal to stimulate protein synthesis in neonatal pigs.* J Anim Sci, 2011. **89**(7): p. 2004-16.

27. Machius, M., et al., *Structure of rat BCKD kinase: nucleotide-induced domain communication in a mitochondrial protein kinase.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11218-23.

28. Shimomura, Y., et al., *Purification and partial characterization of branched-chain alpha-ketoacid dehydrogenase kinase from rat liver and rat heart.* Arch Biochem Biophys, 1990. **283**(2): p. 293-9.