LDHA inhibition deregulates glycolysis intermediates flux in non-oxidative pentose pathway of Mia PaCa 2 cells.

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LDHA inhibition deregulates glycolysis intermediates flux in non-oxidative pentose pathway of Mia PaCa 2 cells.

A Thesis Submitted to the Department of Cancer Biomedical Science in Partial Fulfillment of the Requirements for the Master's Degree of Science

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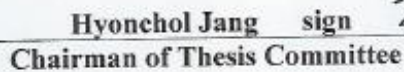

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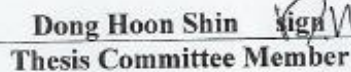

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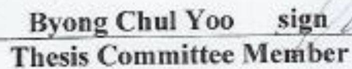

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ABSTRACT

LDHA inhibition deregulates glycolysis intermediates flux in non-oxidative pentose pathway of Mia PaCa 2 cells.

KRAS induces metabolic rewiring in pancreatic cancer cells. Glycolysis intermediates are shunted away from the TCA into the non-oxidative pentose pathway for nucleotide synthesis which are needed for cell proliferation. Cancer cells turn to glutamine to replenish the TCA which undergoes reductive carboxylation to generate citrate and NADPH, the essential intermediates for de novo lipid synthesis to generate building blocks for cellular proliferation. KRAS simultaneously induces fatty acid oxidation for generation ATP used to power the hyper activity of the cancer cell.

The sustainability of all these metabolic alterations in oncogenic KRAS is dependent entirely on the activity of LDHA and MCT 4 transporters to prevent buildup of pyruvate in the cell. Conversion to lactate is an efficient way of removing the potentially toxic end product of glycolysis and glutaminolysis. This makes LDHA an important enzyme during tumor initiation, development, and progression.

A small molecule inhibitor, GSK, was used to study the metabolic deregulations caused by LDHA inhibition. LDHA inhibition causes the cancer cells to increase the expression of the non-oxidative pentose pathway rate determining enzyme, Transketolase in an attempt to prevent accumulation of pyruvate. This excessive

pyruvate cause deregulation of glycolysis intermediates flux within the non-oxidative pentose pathway which may have an effect on the viability of the cancer cells.

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Table of Contents

LIST OF FIGURES 5

LIST OF TABLES 6

ABBREVIATIONS 7

1. INTRODUCTION 9

   1.1 Back ground 9

   1.2 KRAS signaling 10

   1.3 metabolic rewiring in oncogenic KRAS signaling 13

   1.4 Role of LDHA in KRAS induced metabolic rewiring 16

2. PURPOSE OF STUDY 19

3. MATERIALS AND METHODS 19

   3.1 Establishment of cell culture 19

   3.2 Cell culture and extraction of metabolites: 19

   3.3 Preparation of samples for Non-target LC/MS analysis: 21

   3.4 Cell culture for protein expression: 22

   3.5 Protein extraction and western blot analysis: 22

4. RESULTS 24

   4.1 Changes in pyruvate concentration following LDHA inhibition 24

   4.2 Metabolite changes in the non-oxidative pathway resulting from LDHA inhibition 25

   4.3 Expression of the Transketolase enzyme involved in the non-oxidative pentose pathway as a result of LDHA inhibition 28

5. DISCUSSIONS 30

6. FURTHER STUDIES 33

7. REFERENCES 34

ACKNOWLEDGEMENTS 39

LIST OF FIGURES

Figure 1: Downstream signaling pathways and cellular effects following KRAS activation 11
Figure 2: Metabolic pathways altered by oncogenic KRAS signaling....................... 13
Figure 3: Relationship between glycolysis and the non-oxidative pentose pathway. 18
Figure 4: Material and Methods non-targeted metabolite analysis and protein expression analysis 21
Figure 5: Changes in pyruvate concentration 24
Figure 6: Metabolic changes after LDHA inhibition. 25
Figure 7: Expression of Transketolase enzyme in Mia Paca2 cells after exposure to LDHA inhibitor. 28

LIST OF TABLES

Table 1: Details of selected LMI from the Human metabolite Data base................ 27

ABBREVIATIONS

ACC	Acetyl-CoA Carboxylase
ACLY	ATP-Citrate Lyase
ACSL3	Acyl-CoA Synthetase Long Chain Family Member 3
AST	Aspartate Amino Transferase
c-MYC	C-Myelocytomatosis
CDKN2A	Cyclin Dependent Kinase Inhibitor 2a
CPT	Carnitine/Palmitoyl-Transferase
DMEM	Dulbecco's Modified Eagles Media
DMSO	Dimethylsulfoxide
FAO	Fatty Acid Oxidase
FASN	Fatty Acid Synthase
GA	Glycolaldehyde
GAP	GTPase Activating Proteins
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factors
GLS	Glutaminase
GLUD	Glutamate Dehydrogenase
GLUT	Glucose Transtransporter
GOT	Glutamic-Oxaloacetic Transaminase
GTP	Guanosine Triphosphate
HIF	Hypoxia-Inducible Factor 1 Alpha
HMGCoA	Hydroxyl Methylglutaryl-CoA

IDH	Isocitrate Dehydrogenase
KGDH	Keto Dehydrogenase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LDHA	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
MCT	Monocarboxylate Transporter
MDH	Malate Dehydrogenase
ME	Malic Enzyme
MTOR	Mammalian Target Of Rifampicin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFKB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
OAA	Oxaloacetate
PBS	Phosphate Buffered Saline
PDAC	Pancreatic Ductal Adenocarcinoma
PDHC	Pyruvate Dehydrogenase Complex
PDHK	Pyruvate Dehydrogenase Kinase
PFKFB	6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase
PI3K/AKT	Phosphatidylinositol 3 Kinase And Protein Kinase B
SMAD4	Mothers Against Decapentaplegic Homolog 4
SREB	Sterol Response-Elements Binding Protein
TCA	Tricyclic Acid
TP53	Tumor Protein 53

1. INTRODUCTION

1.1 Back ground

Pancreatic cancer is one of the most prevalent and most lethal cancers with over 90% mortality rate worldwide [1]. Pancreatic ductal adenocarcinoma (PDAC) originating from the exocrine gland is the most common type comprising about 90% of all the cases whereas <5% are pancreatic neuroendocrine tumor (PanNET) which arise in the pancreatic endocrine tissue[2].

PDAC is a very aggressive cancer, has a poor prognosis and a dismal five- year survival rate of only 9%, post diagnosis. Its complex etiologic mechanism involves multiple factors including; smoking, alcohol consumption, obesity, diet containing red/processed meat, high-cholesterol and nitrosamine, occupational exposures to elements such as nickel, advanced age >50 years, family history, diabetes mellitus, and infectious agents such as Helicobacter pylori ,hepatitis B and C viruses [3]. Whereas there are genetic variations in genes such as *BRCA1/2, PALB2, ATM, APC, MLH1, MSH2/6, PMS2, PRSS1*, and *STK11* which increase the risk, the greatest risk is posed by mutations in four major genes; a proto-oncogene Kirsten Rat Sarcoma viral oncogene homolog (*KRAS*), and tumor suppressor genes including tumor protein 53 (*p53*), *CDKN2A*, and *SMAD4*, which contribute significantly to the initiation, development and progression of PDAC [4].

The stem cell compartment of pancreatic tissue is composed of acinar cells of the exocrine gland which are characterized by a high degree of plasticity. These are important in regulating homeostasis and regeneration of the pancreatic tissue [5].

These cells are stimulated to undergo acinar-to-ductal metaplasia when exposed to a tissue damaging, inflammatory or stress stimuli. This trans differentiation increases the susceptibility of *KRAS* proto-oncogene to mutations leading to pancreatic intra-epithelial neoplasias (PanINs), the initiation stage in PDAC development [6,7]. Although *KRAS* oncogene is the main driver in PanIN, the evolution into an invasive cancer requires additional and sequential inactivating mutations in tumor suppressor genes, *TP53*, *CDKN2A*, and *SMAD4* accompanied by remodeling of the tumor microenvironment into a distinct desmoplasia characterized by deposition of a dense fibrous tissue. This cause hypovascularisation and the resultant hypoxia plays a big role in augmenting the effects of oncogenic KRAS signaling [8,9].

1.2 KRAS signaling

The product of *KRAS* gene is a small GTPase protein, that normally functions as a transient ON-OFF molecular switch in response to an extracellular stimuli mediated activation of cell surface membrane receptors such as receptor tyrosine kinases (RTKs). The activity of the switch is regulated by intracellular proteins; guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). Upon activation of the membrane receptor, GEFs binds to KRAS causing a conformation change and exchange of GDP for GTP leading to activation of KRAS protein. The active GTP-bound KRAS then binds GAP thereby activating the GTPase activity of KRAS which rapidly hydrolyses the bound GTP to GDP leading to inactivation of KRAS [10]. The activated switch controls the transfer of signals to downstream signaling pathways leading to activation of effector molecules such as MYC and AKT, Figure 1.

In PDAC however, *KRAS* mutations involving substitution of glycine residues at codons 12, 13, or 61 prevent the interaction of the active KRAS protein with GAPs thereby maintaining KRAS in a constitutively active state. This results into persistent, uncontrolled and abnormal signaling through downstream pathways leading to hyper activity of all cellular processes thus increasing its survival [11,12].

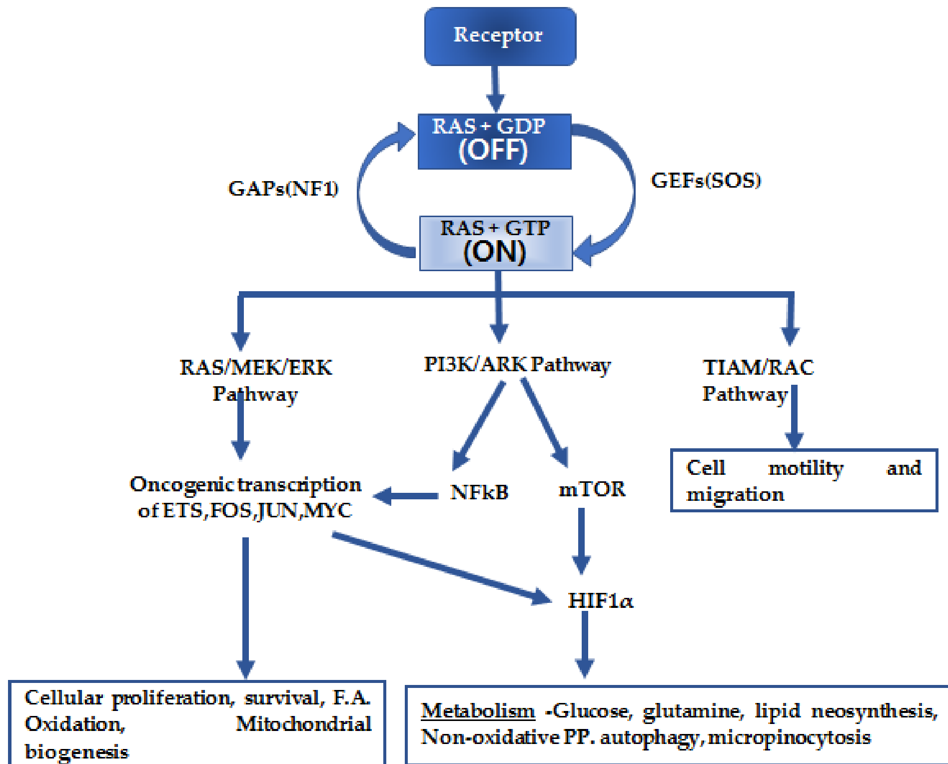


Figure 1: Downstream signaling pathways and cellular effects following KRAS activation

Oncogenic *KRAS* signaling through the MAPK/ERK pathway activates many downstream transcription factors including c-MYC (c-myelocytomatosis oncogene product) leading to increased expression of proliferative genes involved in regulating cell cycle entry, cell proliferation, angiogenesis, and survival of the cell [13]. The KRAS -PI3K-AKT pathway mainly activates HIF-1α factor (hypoxia-inducible factor 1 alpha) through the mTOR complex increasing the expression of genes involved in metabolism and autophagy. This pathway also augments the MAPK oncogenic transcription by activating NF-kB pathway which further enhances cell proliferation and survival [13,14]. KRAS also signals through the TIAM1 pathway which promotes cancer cell motility and migration by activating the Rac-Rho and Rac-PAX dependent network [13].

The persistent and uncontrolled activation of all these downstream signaling cascade accompanied by inactivation of *P53* and other tumor suppressors leads to hyper proliferation, increased survival, local and regional metastatic spread as well as alterations in all the metabolic pathways [15]. The main altered metabolic pathways include; glycolysis, fatty acid oxidation, de novo lipid synthesis, glutaminolysis and hexose amine biosynthesis. These are intended to support the high cellular bioenergy demands as well as providing the building blocks such as nucleotides and lipids necessary to sustain the rapid cell proliferation, survival and metastasis. (*Figure 1*)

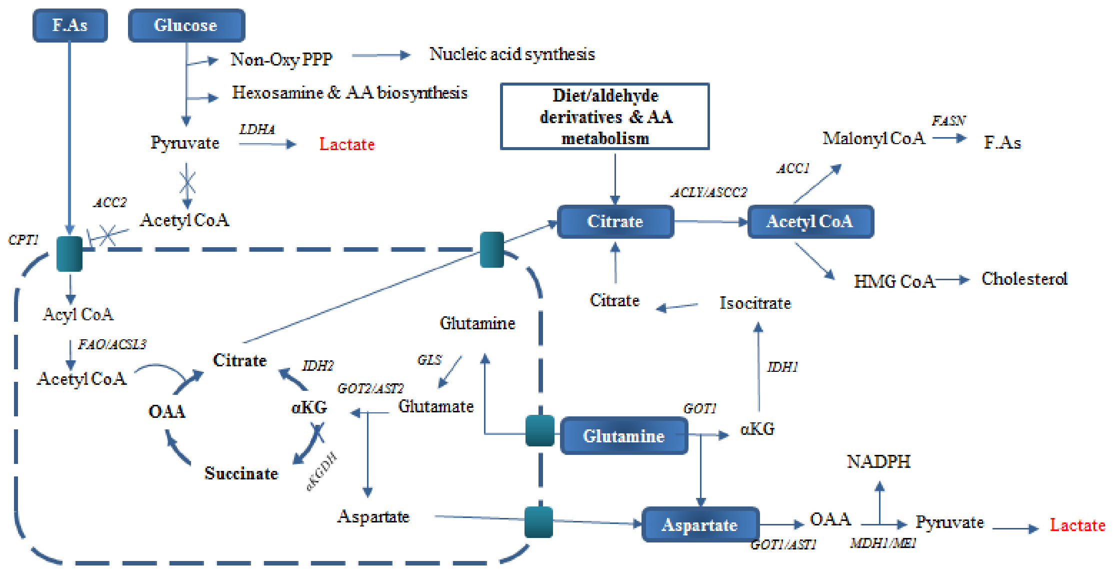


Figure 2: Metabolic pathways altered by oncogenic KRAS signaling

1.3 metabolic rewiring in oncogenic KRAS signaling

HIF-1α activation alters glucose metabolism by increasing the expression several proteins involved in the metabolic pathway including; glucose transporter-1 (GLUT1), glycolysis enzymes such as Hexokinase 2, phosphofructokinase, Phosphoglycerate kinase 1, enolase1, lactate dehydrogenase, as well as lactate transporters, the monocarboxylate transporter 4 (MCT4). All these changes are geared to enhance glucose uptake and metabolism as well as facilitating cancer cell survival even in limited nutrient conditions [16,17]. HIF-1α further strengthens glycolysis by regulating fructose 2,6-bisphosphatase (PFKFB2) expression through phosphorylation at specific serine residues in the C-terminal domain thus increasing its activity [18]. HIF-1α also purposefully inhibits oxidative phosphorylation by reducing the mitochondrial complex IV activity, causing the glucose to be shunted

away from the tricarboxylic acid (TCA) cycle into alternative pathways to the hexosamine biosynthesis, and nucleotide biosynthesis pathways which are vital to sustain proliferation [19–21]. Whereas c-MYC also regulate glycolysis by synergistically augmenting the activity of HIF-1α by binding to the MYC response element of the glycolytic genes [22], it's main function is to specifically regulate glucose metabolism in the alternative pathway by increasing expression of the enzymes involved nucleotide anabolism thus diverting the glycolytic intermediates into the non-oxidative pentose phosphate pathway [23]. Excess glucose however undergoes complete glycolytic process culminating in the formation of pyruvate. The transport of pyruvate by its specific carrier into the mitochondrial matrix is blocked by HIFα induced expression of pyruvate dehydrogenase kinase 1 (PDHK1), which phosphorylates and inhibits pyruvate dehydrogenase complex (PDHC), blocking the formation of acetyl-CoA thus inhibiting glycolysis-mediated oxidative phosphorylation [24]. Instead HIFα induces lactate dehydrogenase (LDH) which converts the pyruvate to lactic acid to prevent the potential negative feedback mechanism or toxicity that can result due to its the accumulation in the cell. Lactate toxicity is also prevented by active secretion into the extracellular matrix through MCT4 transporters. All these changes further enhance glycolysis and nucleic acid synthesis to ensure continuous proliferation and survival of the cell.

The displacement of glucose intermediates into anabolic and lactate pathways induces the cancer cells to resort to glutamine as an anapleurotic compound to replenish the TCA intermediates [25]. KRAS through C-MYC induces the expression of the glutamine transporters SLC1A5 and SLC7A5/SLC3A and

glutaminase enzymes (GLS) to increase its uptake and metabolism [26–28]. Glutamine is metabolized to α-ketoglutarate and aspartate. Aspartate is then exported to the cytoplasm for further conversion to pyruvate with generation of NADPH [29]. HIF-1α reduces the activity α-KGDH resulting in a metabolic shift to carboxylation of α-ketoglutarate (α-KG) to produce citrate which is then shuttled to the cytoplasm where it is used as a precursor for de novo lipid synthesis [30–32].

KRAS mediated up regulation of glutamic-oxaloacetic transaminase 1 (GOT1) and suppression of the glutamate dehydrogenase 1 (GLUD1) expression allows Glutamine metabolism directly in the cytoplasm. The shift of glutamine metabolism from the mitochondrial canonical pathway to the non-canonical pathway further supports NADPH production [33,34]. The pyruvate generated as end-product in both glutaminolysis pathways is converted to lactic acid by the lactate dehydrogenase enzyme.

Oncogenic KRAS signaling also induces continuous de novo lipogenesis which is essential to provide cells with fatty acids for generating membrane building blocks, lipid signaling molecules and for post-translational protein modifications to support the rapid cell proliferation, growth and signaling [35]. HIFα regulates the transcription factor, sterol response-elements binding protein 1 (SREBP1) to increase expression of all lipogenic enzymes [36] including; ATP-citrate lyase (ACLY), fatty acid synthase (FASN), acetyl-coA carboxylase 1 (ACC1) regulated by KRAS [22,37–40]. KRAS also regulates cholesterol de novo synthesis through increasing expression of HMG-CoA reductase by the transcription factor sterol

response-elements binding protein 2 (SREBP2). Citrate and NADPH for de novo synthesis of fatty acids and cholesterol is provided mainly by glutaminolysis [41].

Cells normally regulate fatty acid synthesis and oxidation alternating but oncogenic KRAS simultaneously activate β-oxidation of fatty acids to enable cells to generate ATP to meet the high energy demands of cancer cells by inhibiting the activity of mitochondrial membrane acetyl-CoA carboxylase2 (ACC2) [42]. ACC2 normally generates malonyl-CoA from Acetyl CoA derived from glycolysis which allosterically inhibits the carnitine/palmitoyl-transferase 1 (CPT1) activity at the mitochondrial membrane preventing transport of fatty acyl into the mitochondria for β-oxidation thus the fatty acid and triglyceride (TG) synthesis is increased by using acetyl-coA generated from glycolysis [43]. However in KRAS mutation, HIFα-mediated inhibition of acetyl-coA formation from pyruvate renders this highly active ACC2 unable to generate malonyl-CoA which in turn prevents the inhibition of CPT1 allowing continuous uptake of fatty acids and subsequent formation of acyl-CoA leading to β-oxidation. The oxidation of especially long chain fatty acids generates large quantities of ATP which enables the pancreatic cancer cells to sustain high energy consuming processes such as, cell proliferation, anabolic synthesis, cell signaling and metastasis [44].

1.4 Role of LDHA in KRAS induced metabolic rewiring

The sustainability of all the major metabolic pathways that are altered by oncogenic KRAS depends entirely on the expression and activity of both LDHA and MCT 4 transporters. Production of lactate is an efficient way of removing potentially toxic

end product of glycolysis and glutaminolysis. Therefore LDHA is important during tumor initiation, development, and progression.

KRAS oncogene signaling increases flux of glycolytic pathway intermediates into the non-oxidative pathway by up regulating the related enzymes including transketolase. It also increases conversion of pyruvate to lactate by inhibiting oxidative phosphorylation while increasing expression of lactate dehydrogenase. Inhibition of LDHA may lead to accumulation of pyruvate which may have a negative feedback on glycolysis thus affecting all the other metabolic pathways including the synthesis of nucleic acids. The glycolytic pathway and the non-oxidative pathways are tightly linked with the later acting as the alternative for glucose metabolism and blocking either of them dramatically affects the other[45]. Therefore inhibiting LDHA would favor a reversible glycolytic pathway from pyruvate to increase the flux of the intermediates into the pentose pathway to increase nucleotide synthesis in order to prevent accumulation of cytotoxic pyruvate, *figure 3*. This increased flux would ensure increase nucleotide synthesis and thus increased cellular proliferation. However a study shows that inhibition of LDHA by some small molecules inhibitors prevented the progression of pancreatic and lymphoma xenografts. [46]. It is thus hypothesized that LDHA inhibition disrupts glycolysis intermediate flux into the non-oxidative pentose pathway.

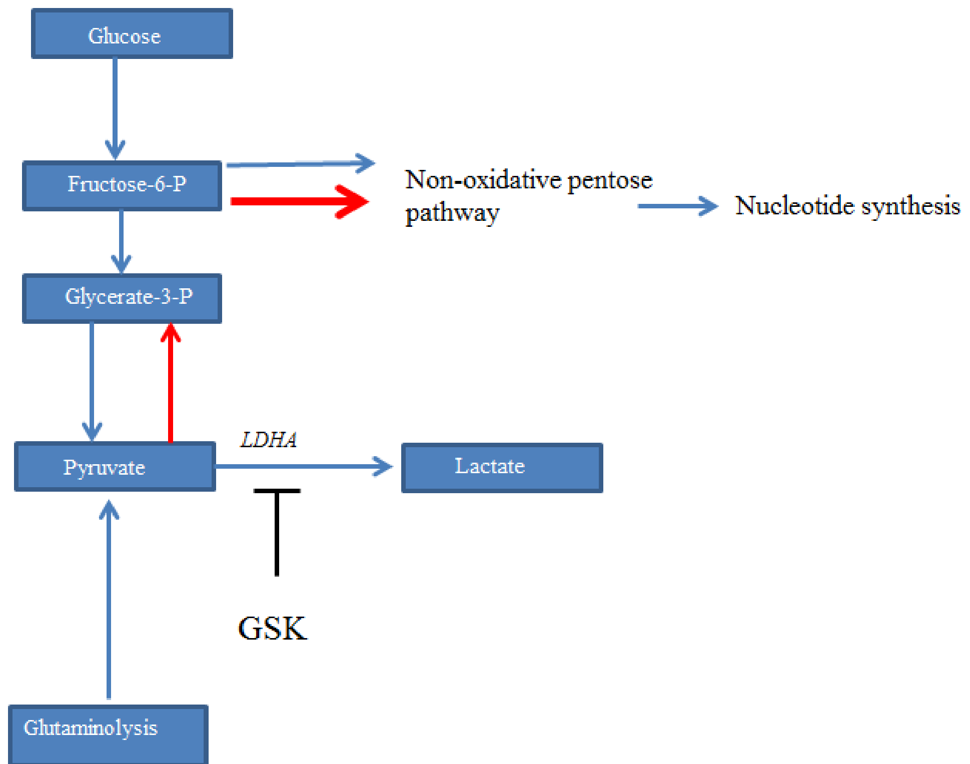


Figure 3: Relationship between glycolysis and the non-oxidative pentose pathway.

2. PURPOSE OF STUDY

1. To determine the metabolite changes resulting from LDHA inhibition in the Mia PaCa 2 cell line.

2. To determine the expression of TKL involved in the non-oxidative pathway as a result of LDHA inhibition in the Mia PaCa 2 cell line.

3. MATERIALS AND METHODS

3.1 Establishment of cell culture

Human pancreatic ductal adenocarcinoma cell line, Mia PaCa 2, was obtained from the stock that being maintained in liquid nitrogen in professor's laboratory (Division of Translational science, NCC Research institute). The cells were first thawed for about 10 minutes then cultured in Dulbecco's Modified Eagles

Media (DMEM, Thermo Fisher Scientific, USA) culture media supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific, USA). Cells were initially sub cultured every week, till they grew at a stable rate and then subculture when they reached 90% confluence using trypsin.

3.2 Cell culture and extraction of metabolites:

Mia PaCa-2 cells from the previously established cell culture were plated at $5x10^4$ /dish (90x20mm) and incubated at 37°C, 5% CO_2 and 95% humidity in DMEM with 10% FBS until they reached 95% confluence. Approximately 1×10^6 cells/ well were then seeded in three 6-well culture plates, labeled A, B, C, and allowed to attach overnight in DMEM media supplemented with 10% FBS, *figure 1(a).* The

following day, the media was changed after which cells in plates B and C treated with 1.9nM GSK (GSK 2837808A, TOCRIS, USA) for 2 and 6 hours respectively whereas cells in plate A were treated with an equal volume of DiMethylSulfoxide (DMSO, Sigma Aldrich) for 2hrs to act as control. The concentration of the LDHA inhibitor used was chosen based on IC50% in published studies. After the respective times the media was removed and the cells immediately washed three times with ice cold PBS. The metabolites were extracted using 1.5ml of ice cold (-48°C) 80% v/v methanol: water solution per well. The cells were then harvested using a disposable cell scrapper into a 2ml eppendorf tube. The extraction of the metabolites was done by submerging the tube into liquid nitrogen for 30 seconds to snap freeze the cells then allowed to thaw for 3 min on dry ice. This was done 3 times with 10 seconds of vortex mixing between cycles. The solution was centrifuged at -9°C, 11500g for 10 minutes. The supernatant was collected into a well labeled 2ml eppendorf tube and stored at -20°C for metabolic profiling.

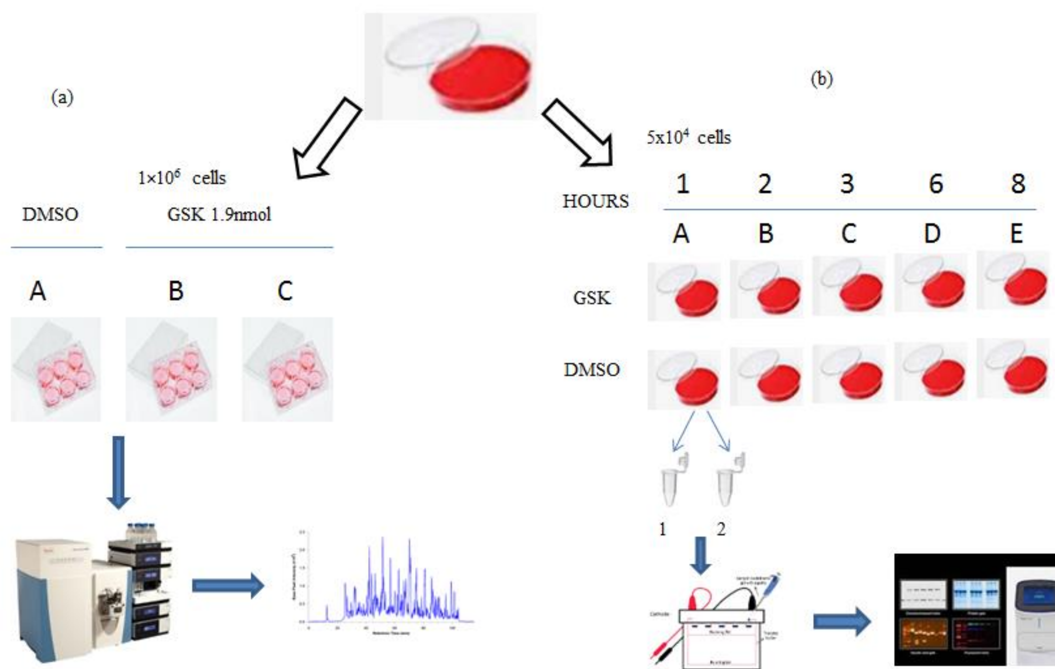


Figure 4: Material and Methods non-targeted metabolite analysis and protein expression analysis

3.3 Preparation of samples for Non-target LC/MS analysis:

Twenty microliter of each sample was mixed with 100µl methanol and vortexed for 1minute. 20µl of the resultant sample then mixed with 80µl of 0.1% formic acid in water. The samples were centrifuged for 10 minutes at 14000rpm at 4°C giving a residual volume of 80µl. These samples were then submitted for LC-MS analysis**.** Only low mass ions with 100% sensitivity and specificity were listed in the LC-MS results.

3.4 Cell culture for protein expression:

Again $5x10^4$ Mia Paca2 cells were sub cultured in two sets of culture plates, labeled A, B, C, D, and E until they reach 95% confluence, *figure 1(b).* The media was changed and one set exposed to 1.9nmol concentration of GSK compound for 1, 2, 3, 6 and 8 hours while the second set was treated with an equal volume of DMSO for time intervals to act as control. At the respective times the media was removed and the cells quickly washed with 10ml of ice cold PBS. Cells were harvested into a 15ml eppendorf tube using a cell scrapper, centrifuged at 15000rpm for 3 minutes and drained of all the PBS. The cells were re-suspend the cells in 2ml ice cold PBS and divided in two equal volumes in 1.5ml eppendorf tubes marked A1 A2, B1 B2, C1 C2 D1 D2, E1 E2 followed by Centrifugation for 3 minutes at 4000rpm in a micro centrifuge to remove all the PBS from the cells.

3.5 Protein extraction and western blot analysis:

Protein extraction was performed using 400μl PRO-PREP lysis buffer (iNtRON Biotechnology, Korea) mixed with 4μl of Halt protease inhibitor(PI, Thermo Fischer Scientific, USA) in samples A1, B1, C1, D1 and E1 whereas 4μl Halt protease-phosphatase inhibitor cocktail(PPI, Thermo Fischer Scientific, USA) was used in samples A2, B2, C2, D2 and E2 group. The samples were incubated on ice for 20 minutes and later centrifuged at 11000 rpm. The supernatant was transferred into new 2ml eppendorff tubes and labeled clearly. The protein concentration in each tube was determined using Pierce BCA protein Assay Kit (Thermo Scientific, USA) and a micro plate reader spectrophotometer. A standardized 10ug/25ml western blot sample prepared 20μl of each protein sample was loaded into the

1.5mm x15 wells Nupage 4-12% Bis Tris gel (Thermo Fischer scientific, USA) in the order (A1,B1,C1,D1,E1,A2,B2.C2,D2,E2) for both the test and control samples, and electrophoresis performed at 100V for 90 minutes. Samples were transferred to PVDF filter membrane (Immobilon-P, Merck Millipore ltd, Ireland) at 100V for 90minutes. After blocking with 3% Albumin for 1 hour and membranes blotted with antibodies. The expression of the Protein determined using primary antibodies (Transketolase (E704M) Rabbit (mAb), Cell Signaling Technology, USA and anti β-Actin, Sigma Aldrich, USA). After washing, the membranes were incubated with secondary antibodies (Goat anti-Rabbit IgG-HRP conjugate, Cell Signaling Technology, USA and anti-mouse-HRP conjugate, Sigma Aldrich, USA). HRP chemiluminiscent substrate, SuperSignal West Pico (Thermo scientific, USA) was added and the membrane imaged using the ibright chemiluminiscence imaging system (Thermo Fischer, USA) to obtain the expression bands

4. RESULTS

4.1 Changes in pyruvate concentration following LDHA inhibition

p=0.01587

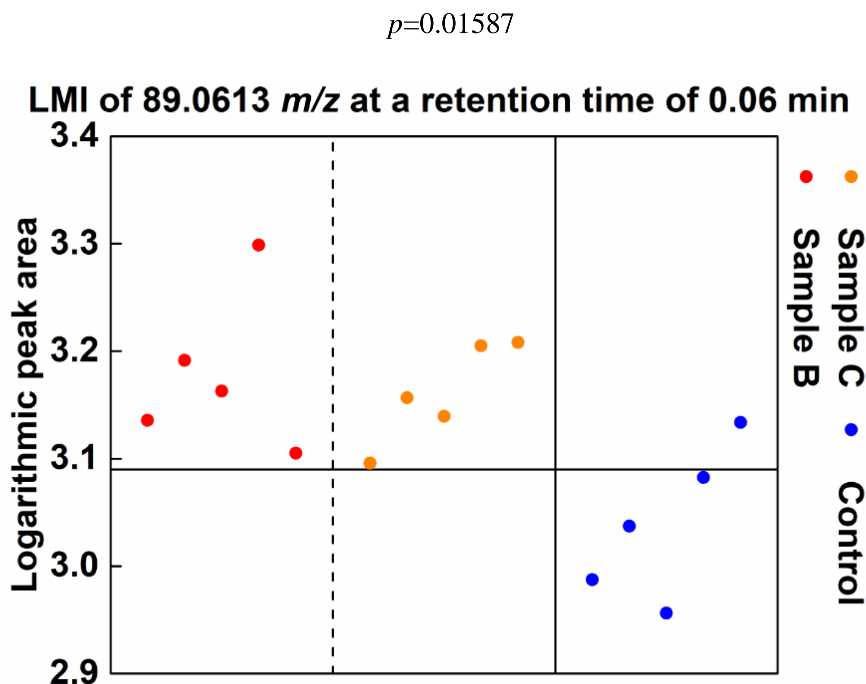


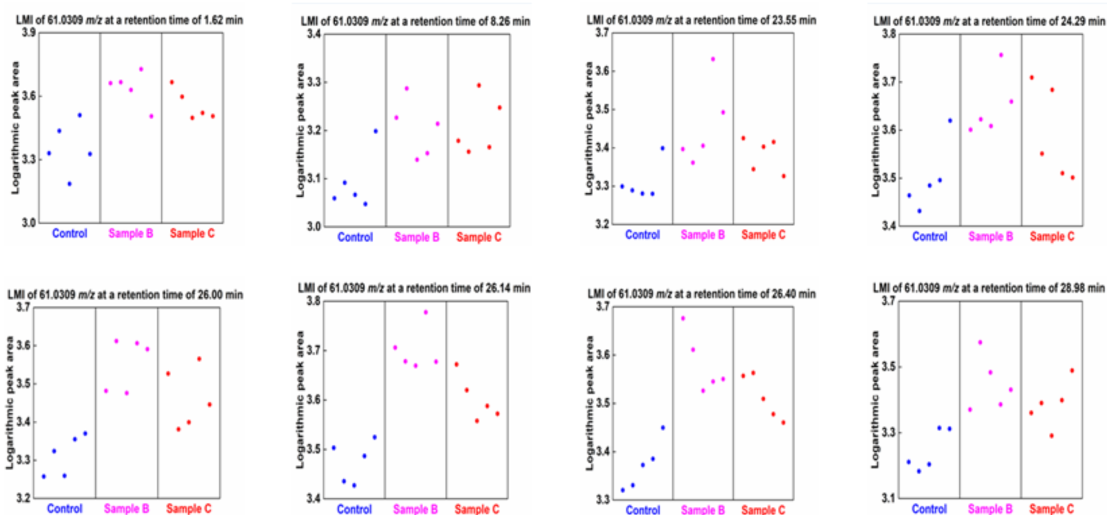
Figure 5: Changes in pyruvate concentration

From the human metabolomics data base we searched for the m/z value of pyruvate which we used to trace the variation in the levels of pyruvate from the LC-MS data. We obtained a low mass ion with a m/z of 89.0613 (delta= 427ppm) which showed an increase in the treated samples compared to the control experiment. This suggested that GSK was effective in inhibiting LDHA enzyme in the Mia PaCa 2 cell lines of our experiments.

4.2 Metabolite changes in the non-oxidative pathway resulting from LDHA inhibition

(a) Sample C > sample B > control

Retention time	1.62	8.26	23.55	24.29	26	26.14	28.98
p-value	0.0045	0.0127	0.008	0.013	0.0007	0.0007	0.0027

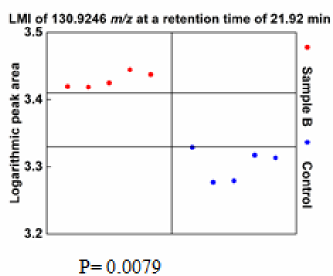


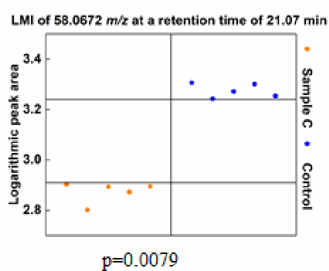
(b)

Sample B > Control

Sample C < Control

Sample BC > Control





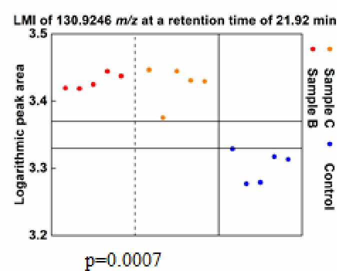


Figure 6: Metabolic changes after LDHA inhibition.

Figure 6(a); Low mass ions of different retention time corresponding to m/z of 61.0309 were found to have a consistent increase between sample B, C and the control. Figure 6(b) the change in the amount of LMI with m/z 130.9246 was not significant between the treated samples and the control whereas LMI of m/z 58.0672 showed a decrease in sample C compared to the control.

We looked up the metabolites in the Human Metabolome Data Base at https://hmdb.ca/metabolites and found the following compounds were identified corresponding to m/z values, table 1.

Table 1: Details of selected LMI from the Human metabolite Data base

Selected LMI (m/z)	Compound	Metabolite Name	Adduct	Adduct MW (Da)	Compound MW (Da)	ppm
58.0672	HMDB0168957	Allylamine	M+H	57.0578	58.0651	36
61.0309	HMDB0003344	Glycolaldehyde	M+H	61.0284	60.0211	41
	HMDB0000042	Acetic acid	M+H	61.0284	60.0211	41
	HMDB0000294	Urea	M+H	61.0396	60.0324	143
	HMDB0062508	N-nitrosomethanamine	M+H	61.0396	60.0324	143
	HMDB0000820	Propyl alcohol	M+H	61.0648	60.0575	555
	HMDB0000863	Isopropyl alcohol	M+H	61.0648	60.0575	555
	HMDB0031225	1,2-Ethanediamine	M+H	61.076	60.0687	739
130.9246	HMDB0011119	Selenite	M+H	129.9169	130.9242	3
	HMDB0029593	Trichloroethylene	M+H	129.9144	130.9217	22
	HMDB0034906	Zinc dithionite	M+H	129.9394	130.9467	169
	HMDB0059919	Dithionous acid	M+H	129.9394	130.9467	169
	HMDB0062193	2,2-dichloro-1,1-ethanediol	M+H	129.9588	130.9661	317

The discovery that the LMI of m/z 61.0309 could possibly be Glycol aldehyde showed that LDHA inhibition in the Mia PaCa 2 cell lines could have an effect on the flux of glycolytic intermediates into the non-oxidative phosphate pentose pathway

4.3 Expression of the Transketolase enzyme involved in the non-oxidative pentose pathway as a result of LDHA inhibition

We were curious to know the effect of LDHA inhibition on the expression of enzymes in the non-oxidative pentose pathway specifically Transketolase, a rate determining enzyme in the pathway leading to synthesis of nucleic acids in pancreatic cancer cells.

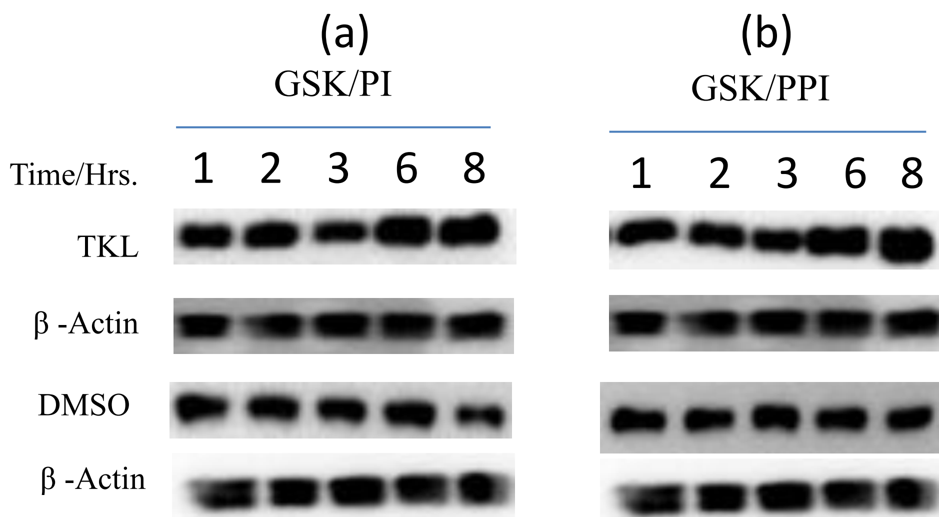


Figure 7: Expression of Transketolase enzyme in Mia Paca2 cells after exposure to LDHA inhibitor. TKL- Transketolase; DMSO- control; GSK- LDHA inhibitor; PI- protease inhibitor; PPI- protease-phosphatase inhibitor cocktail

In figure 7a LDHA inhibition initially does not affect TKL expression even after 2 hours of exposure but the expression starts to decrease at 3 hours of exposure after which it increases again. In figure 7b the total TKL protein also follows the

same trend, decreasing after 3 hours of exposure and then increases 6 hours later and beyond. TKL expression after 6 and 8 hours is even higher than the control.

Taken altogether, the results show that LDHA inhibition increases the expression of Transketolase enzyme, a rate determining enzyme in the non-oxidative pentose phosphate pathway.

5. DISCUSSIONS

Glucose is metabolized mainly through the catabolic glycolytic pathway and alternatively in the anabolic pentose phosphate pathway. These two pathways are closely linked and are used to generate ATP and nucleotides respectively depending on the physiological needs of the cells. When the cells need more ATP it principally metabolizes through the glycolytic pathway but when the cells are actively dividing generation of building blocks is the priority of glucose metabolism and intermediates of glycolysis are channeled to the pentose pathway for synthesis of nucleotides as well as into other anabolic pathways.

Non-oxidative branch of the pentose phosphate pathway is an alternate glycolysis pathway that metabolizes the glycolytic intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate as well as sedoheptulose sugars, yielding ribose 5-phosphate for the synthesis of nucleic acids and sugar phosphate precursors for the synthesis of amino acids. Normally it can supply glycolysis with intermediates derived from ribose 5-phosphate and vice versa, depending on the biochemical demand.

In the case of pancreatic cancer cells, these are highly proliferative and must therefore balance the need for generation of ATP, NADPH for redox control, as well as of building blocks in the form of nucleotides and fatty acids. This is achieved through KRAS induced alteration of the major metabolic pathways through up regulating the expression of the relevant metabolic enzymes.

KRAS increase production of nucleic acids by up regulating enzymes in non-oxidative branch including enzyme transketolase (TKL) that act on fructose-6-bishosphate to form a short chain aldehyde, glycol aldehyde using thiamine pyrophosphate as a co-factor. The compound is transferred by thiamine pyrophosphate during the pentose phosphate pathway. TKL uses a ketone donor (xylose-5-phosphate) and aldose acceptors (Ribose-5-phosphate or erythrose-4-phosphate) to form aldose product (glyceraldehyde-3-phosphate) and ketone products (sedoheptulose-7-phosphate or fructose-6-phosphate), to catalyze the transfer of the two carbons fragment for monosaccharide inter conversion within the non-oxidative pentose pathway[47].

KRAS increase production and excretion of lactate up regulating lactate dehydrogenase enzyme and MCT 4 transporters in order to maintain the activity of these pathways. This LDHA enzyme is very critical in the survival and progression of pancreatic cancer cells. Its inhibition would result in the accumulation pyruvate.

The observed increase in TKL expression after LDHA inhibition is a metabolic re-arrangement to prevent pyruvate toxicity by channeling it to the non-oxidative branch for nucleic acid synthesis. This leads to increased production of an intermediary product, glycol aldehyde. Probably its production exceeds demand for use in downstream processes leading to its accumulation as observed in the non-targeted LC-MS analysis.

GA has been demonstrated to be toxic to the cells. This is because the carbonyl group cannot undergo cyclization which makes it prone to enolization and oxidation

with concomitant production of glyoxal and superoxide. These induce oxidative stress in the cells which affects other metabolic proteins in downstream[48]. This can be due to accumulation of GA in the cells due to reduced utilization in downstream pathway for nucleotide synthesis.

Therefore in conclusion, the increase in expression of Transketolase points to Glycolaldehyde as the main metabolite deregulated in the non-oxidative pentose pathway due to inhibition of LDHA.

6. FURTHER STUDIES

Whereas the results may point to Glycolaldehyde as the main deregulated metabolite from increased TKL expression as a result of LDHA inhibition, this could not be conclusively deduced. A targeted LC-MS analysis is required to confirm that the identity of the low mass compound.

In addition, a study should be carried out to demonstrate the effects of the deregulation of glycolytic intermediate flux on nucleotide synthesis and ultimately on cell viability

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