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Metabolomic approach to understand the mechanism of metformin-induced PRODH/POX-dependent apoptosis in MCF-7 breast cancer cells.

Doctor of Philosophy's dissertation in Pharmaceutical Sciences

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Contents

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ABBREVIATIONS

AcetyloCoA:	Acetyl coenzyme A
Ala:	Alanine
AMPK:	The AMP-activated protein kinase
α-KG:	α-ketoglutarate
Arg:	Arginine
ATG:	Autophagy- related gene/ protein
ATP:	Adenosine triphosphate
BAX:	Bcl-2-associated X protein
Bcl-2 gene:	B-cell lymphoma 2 gene
BID:	A Bax-like BH3 protein
CaMKKβ:	Calcium/calmodulin-dependent protein kinase
CARM1:	Coactivator-associated arginine methyltransferase 1
Caspase -7:	An effector caspase with important roles in mediating cell death signaling
CRP:	C-reactive protein
Cys:	Cysteine
DD:	Death domain
DED:	Death effector domain
DFMO:	Difuoromethylornithine
DR4/DR5:	TRAIL (TNF-related apoptosis-inducing ligand) death receptors
eIF2:	Eukaryotic initiation factor 2
ETC:	The electron transport chain
F16BP:	Fructose-1,6-biphosphate
FAD:	Flavin adenine dinucleotide
FADD:	Fas Associated via Death Domain
FADH2:	Flavin adenine dinucleotide
Fas/CD95 receptor:	Apoptosis antigen 1
G-6-P:	Glucose 6-phosphate
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GCN2:	General control non-depressible 2
GLC:	Glucose

GLN:	Glutamine
GLS:	Glutaminase
GLU:	Glutamate
GLUD:	Glutamate dehydrogenase
GLYPRO:	Glycyl-proline
GS:	Glutamine synthase
GSA:	Glutamic gamma-semialdehyde
GTP:	Guanosine-5'-triphosphate
HIF-1α:	Hypoxia inducible factor-1 alpha
HPLC:	High-performance liquid chromatography
IFG:	Impaired fasting glucose
IGF1:	Insulin-like growth factor 1
IGT:	Impaired glucose tolerance
Ile:	Isoleucine
InsP3Rs:	Inositol-1,4,5-triphosphate receptors
LA:	Lactic acid
LAMTOR1:	Late endosomal/lysosomal adaptor MAPK and mTOR activator 1
LAT1:	Large Amino Acid Transporter 1
LDH:	Lactate dehydrogenase
Leu:	Leucine
LKB1:	Liver kinase B1
MCF-7 cells:	A human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors
MCF-7^{WT} cells:	Wild-type MCF-7 cells
MCF-7^{crPOX} cells:	PRODH/POX knockout MCF-7 cells
MET:	Metformin
MS:	Mass spectrometry
mTOR:	The mammalian target of rapamycin
MYC:	Regulator genes and proto-oncogenes code for transcription factors
NADH:	Nicotinamide adenine dinucleotide
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$:	Nicotinamide adenine dinucleotide phosphate
NEA:	Non-essential amino acids

OAA:	Carbon oxaloacetate
OAT:	Ornithine δ-aminotransferase
ODC:	Ornithine decarboxylase
ORN:	Ornithine
OXPHOS:	Oxidative phosphorylation
P5C:	Δ^1-pyrroline-5-carboxylate
P5CDH:	Pyrroline-5-carboxylate dehydrogenase
P5CR:	Pyrroline-5-carboxylate reductase
P5CS:	P5C synthase
PAI-1:	Plasminogen activator inhibitor-1
PARP:	Poly (ADP-ribose) polymerase
PEP:	Phospho-enol-pyruvic acid
PHD:	Prolyl hydroxylase domain
PK:	Pyruvate kinase
PKM1/2:	Pyruvate kinase muscle isozyme 1/2
PPARγ:	Peroxisome proliferator-activated receptor gamma
PPP:	Pentose phosphate pathway
PRMTs:	Protein arginine methyltransferases
PRODH/POX:	Proline dehydrogenase/ Proline oxidase
PRO:	Proline
PtdIns3K:	Phosphatidylinositol 3-kinases
PYR:	Pyruvic acid
QqQ:	Triple quadrupole
RB1CC1:	RB1-inducible coiled-coil 1
ROS:	Reactive oxygen species
SAICAR:	Phosphoribosylaminoimidazolesuccinocarboxamide
SDH:	Succinate dehydrogenase
T2DM:	Type II Diabetes
TCA:	Tricarboxylic acid
Thr:	Threonine
TRAIL:	Tumor necrosis factor-related *apoptosis*-inducing ligand
Trp:	Tryptophan
ULK1/2:	Unc-51 Like Autophagy Activating Kinase 1/2

INTRODUCTION

The objective of Ph.D. dissertation is to understand the mechanism of PRODH/POX-dependent apoptosis in MCF-7 breast cancer cells by metabolomic approach. The research hypothesis was presented in the review:

P1. Huynh, T.Y.L., Zareba, I., Baszanowska, W., Lewoniewska S., Pałka, J. Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pox)-dependent apoptosis/autophagy as an approach to targeted cancer therapy. Mol Cell Biochem, 2020, 466, 35–44. DOI: 10.1007/s11010-020-03685-y. Impact Factor ISI: 3.396. MSWiA: 70 points.

The results of this dissertation were presented in the publication:

P2. Huynh, T.Y.L, Ościłowska, I., Sáiz, J., Nizioł, M., Baszanowska, W., Barbas, C., Pałka, J. Metformin Treatment or PRODH/POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells. Biomolecules. 2021 Dec 15;11(12):1888. DOI: 10.3390/biom11121888. Impact Factor ISI: 4.879. MSWiA: 100 points.

Metformin (MET) is the first-line drug in the treatment of type II diabetic patients. The antidiabetic activity of MET is due to its ability to inhibit intestinal glucose absorption, gluconeogenesis, improvement of glycolysis and tissue sensitivity to insulin, leading to hypoglycemia [1]. Several lines of evidence suggest that MET evokes anti-neoplastic activity against different cancers, including breast cancer. The studies on antineoplastic potential of MET were initiated in 2005 by pharmaco-epidemiological analyses that show significantly decreased risk of cancer in diabetic patients treated with MET [2]. Several experimental and clinical trials were recently conducted on the antineoplastic potency of MET [3, 4]. However, the molecular mechanism of the anti-cancer activity of MET is still unknown. It has been considered that complex regulatory mechanisms, including the energetic metabolism of cancer cells, may underlie the process.

The primary energy source is the metabolism of glucose in glycolytic and Tricarboxylic acid (TCA) pathways. However, due to the Warburg effect in cancer cells, the energy shortage is supplemented by the metabolism of lipids, proteins, and amino acids. One of the energy-providing substrates is proline. Although proline could be recovered from protein degradation, mainly collagen, a large amount of this amino acid is derived from proline interconvertible amino acids as glutamine, α-ketoglutaric acid, glutamic acid, and ornithine, linking TCA and urea cycles and indirectly, glycolysis. The interplay between these cycles depends on the cell energy status [5] that is under control of AMP-activated protein kinase (AMPK) [6-9]. When the AMP/ATP ratio is increased, this kinase is activated by phosphorylation mechanism and stimulates oxidative phosphorylation to increase the ATP level and inhibits anabolic and energy consuming processes, such as cell proliferation [10]. Therefore, AMPK is an energy sensor that is induced in conditions of energy deficit [11] for rewiring energetic metabolism, adaptation to microenvironmental changes [12, 13], and restoration of energetic homeostasis during stressful conditions [14, 15].

The above process play a critical role in maintenance balance between autophagy and apoptosis [16]. One of the enzymes involved in regulation of apoptosis/autophagy is proline dehydrogenase (PRODH<, GenBankTM NM_016335), also known as proline oxidase (POX). It is a flavin-dependent mitochondrial enzyme located in the inner mitochondrial membrane in close proximity to electron transport chain (ETC) [17-19]. PRODH/POX catalyzes degradation of proline into Δ1-pyrroline-5-carboxylate (P5C),

during which, electrons are transported to the ETC for ATP synthesis, or they are accepted by oxygen yielding reactive oxygen species (ROS). This unique function of PRODH/POX in regulation of survival and apoptosis is of great interest; however, the mechanism for the switch between PRODH/POX-dependent growth-inhibition and growth-stimulation is not known.

A critical role in PRODH/POX-dependent functions may play proline availability for PRODH/POX. Proline availability for PRODH/POX is regulated by proline providing and proline consuming processes. Collagen biosynthesis is the most effective proline utilizing process. It can also be a "sink" for reducing potential of proline [20]. The important regulator of free proline in cytoplasm is prolidase, the enzyme releasing proline from imidodipeptides [21-25]. However, an increase in proline concentration in the cytoplasm is accompanied by utilization of this amino acid for collagen biosynthesis to remove proline reducing potential and sustain redox balance. Alternatively, reducing potential of proline is utilized in mitochondria by PRODH/POX. However, the most dynamic process supporting proline for PRODH/POX-dependent functions is amino acid metabolism. Proline can be derived from glutamine, glutamate, α-ketoglutarate, and ornithine, linking Krebs and Urea cycles with amino acid metabolism.

Moreover, the TCA cycle is closely correlated with glycolysis and the electron transport chain. Therefore, in publications constituting the doctoral dissertation, I considered that the mechanism of anti-cancer activity of MET could involve the following regulatory processes: i/ prolidase activity, ii/ PRODH/POX expression, iii/ collagen biosynthesis, iv/ amino acids metabolism as well as metabolism in Glycolysis, TCA and Urea cycles. The interplay between these processes may represent a multifunctional interface that switches apoptosis or survival mode in cancer cells depending on the microenvironmental conditions. The introduction below provides a rationale for the undertaken hypothesis that has been explored in the dissertation.

1. Homeostasis and cancer metabolism

Homeostasis is sustained by a cellular metabolic network of balanced processes. Cancer reprogramming occurs in all cell types due to the unmet energy demand. Under starvation, cellular homeostasis is maintained by altering anabolic and catabolic processes. Anabolic processes are regulated by several factors affecting the biosynthesis

of cellular components. Most of the catabolic processes are regulated by ubiquitin-proteasome degradation system and autophagy [16]. Sometimes these processes (apoptosis/autophagy) occur simultaneously in the same cell, or autophagy initiates apoptosis via p53-dependent or AMPK-dependent pathway [16].

Deregulation of metabolism in breast cancer cells involves reprogramming almost all metabolic pathways [16]. Therefore, targeted metabolomics, a cutting-edge approach, was used to investigate the complexity of processes involved in metabolic reprogramming in cancer cells and to evaluate the potential mechanism of antineoplastic activity of Metformin.

2. Metabolic significance of AMP-activated protein kinase (AMPK)

The AMPK is an essential molecule sensor of cellular energy status with highly conserved eukaryotic protein serine/threonine kinases [26]. These proteins are heterotrimeric complexes that comprise a catalytic α subunit and two regulatory β and γ subunits [27-29]. Many isoforms of each subunit differing in mammals (α1, α2, β1, β2, γ1, γ2, and γ3) can form 12 heterotrimer combinations with different subcellular localization and signaling functions [30, 31]. AMPK and its orthologs are mainly activated by phosphorylation of conserved threonine residue at the activation loop located at the kinase domain (Thr172 in rat and Thr210 in S. cerevisiae) [27].

Activation of AMPK connected with binding of 5'-AMP takes place in three different actions. The events include promotion of Thr172 phosphorylation by upstream AMP-related kinases via Liver kinase B1 (LKB1) complex, inactivation of Thr172 dephosphorylation by protein phosphatases as well as allosteric activation [28, 32]. Especially, ADP, as a molecule that mimics AMP, can take part in both phosphorylation or inhibition of dephosphorylation of Thr172. However, ATP enables antagonizing these processes. When cellular AMP/ATP and ADP/ATP ratios increase, the AMPK, as an energy sensor, is activated. AMPK activation is explained in different pathways, which are canonical inputs (adenine nucleotide binding to the AMPK γ subunit), noncanonical inputs (activation by ligands that bind between the α and β subunits or activation by the Ca^{2+}/CaMKKβ pathway), and indirect pathway by inhibiting ATP synthesis, thus increasing intracellular AMP/ATP and ADP/ATP ratios [28] (Figure 1). AMPK activation via an increase in the AMP/ATP ratio leads to stimulating oxidative

phosphorylation to restore normal ATP levels and inhibiting energy expenditure, such as cell proliferation [10, 11]. AMPK is regulated, especially in conditions of energy shortage (e.g., glucose shortage) and hypoxia [11]. It inhibits anabolic processes and stimulates catabolism. There is crosstalk between AMPK pathways and other signaling pathways. In the two-dimensional surface of the lysosome, LKB1 and AMPK are connected by the adapter protein axin and late endosomal/lysosomal adaptor MAPK and mTOR activator 1 (LAMTOR1), revealing a link of the mammalian target of rapamycin (mTORC1) and AMPK. This crosstalk can regulate cell growth and metabolism. The other possible interaction is between AMPK and insulin/ Insulin-like growth factor 1 (IGF1) or Ras-Raf-MEK-ERK [29].

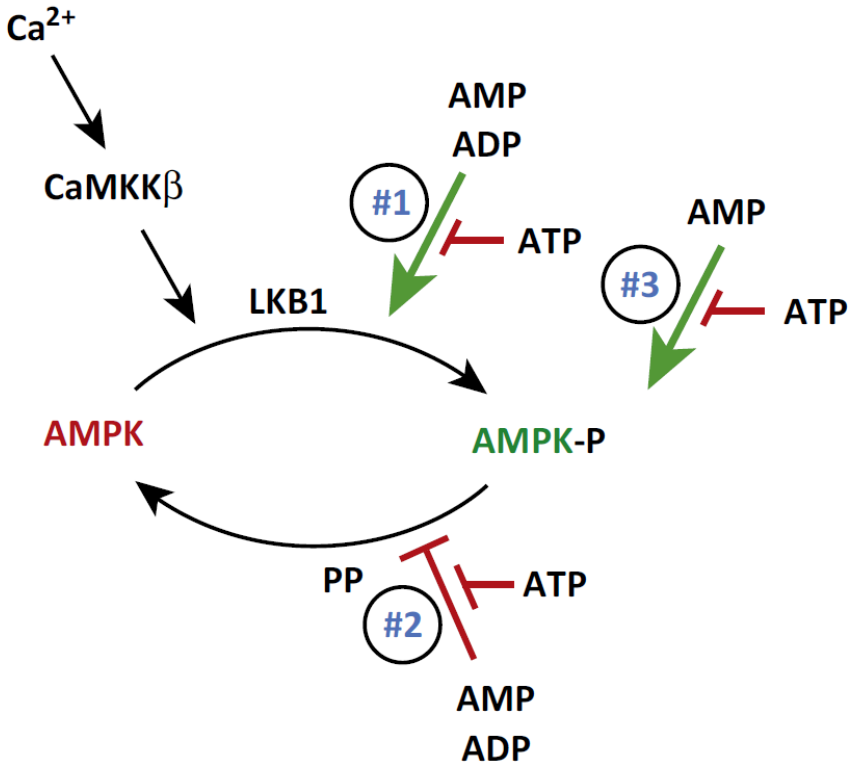

Figure 1. Canonical mechanism of activation of AMPK by adenine nucleotides and the Ca^{2+}-dependent mechanism mediated by CaMKKβ [28].

Interestingly, MET has been found to attenuate the function of mitochondrial complex-I resulting in an increase in AMPK, a decrease in ATP synthesis [33], and inhibition of pyruvate kinase, impairing glucose metabolism [34].

3. Metformin as an antidiabetic and anticancer agent

MET and phenformin are guanidine derivatives of galegine, which are extracted from *Galega Officinalis* known a herbal medicine in Europe [1, 35] (Figure 2). MET (1,1-dimethylbiguanide hydrochloride) was synthesized with two coupled molecules of guanidine but less toxic than galegine. In the 1950s, MET was firstly introduced in clinical studies as diabetes therapy by Jean Sterne [35, 36]. The report mentioned that MET was well-tolerated after prolonged administration and not harmful to the organism. According to Scheen, A. J. 1996 [37], MET was a tautomeric configuration of hydrophilic molecule monoprotonated at neutral pH. MET was used with oral doses of 500 to 1000 mg and reached maximum plasma concentration (2 µg/ml) after administration and a steady-state concentration from 0.3 to 1.5 µg/ml. The half-life of MET was 6-7 h, with about 20% of the active product being excreted.

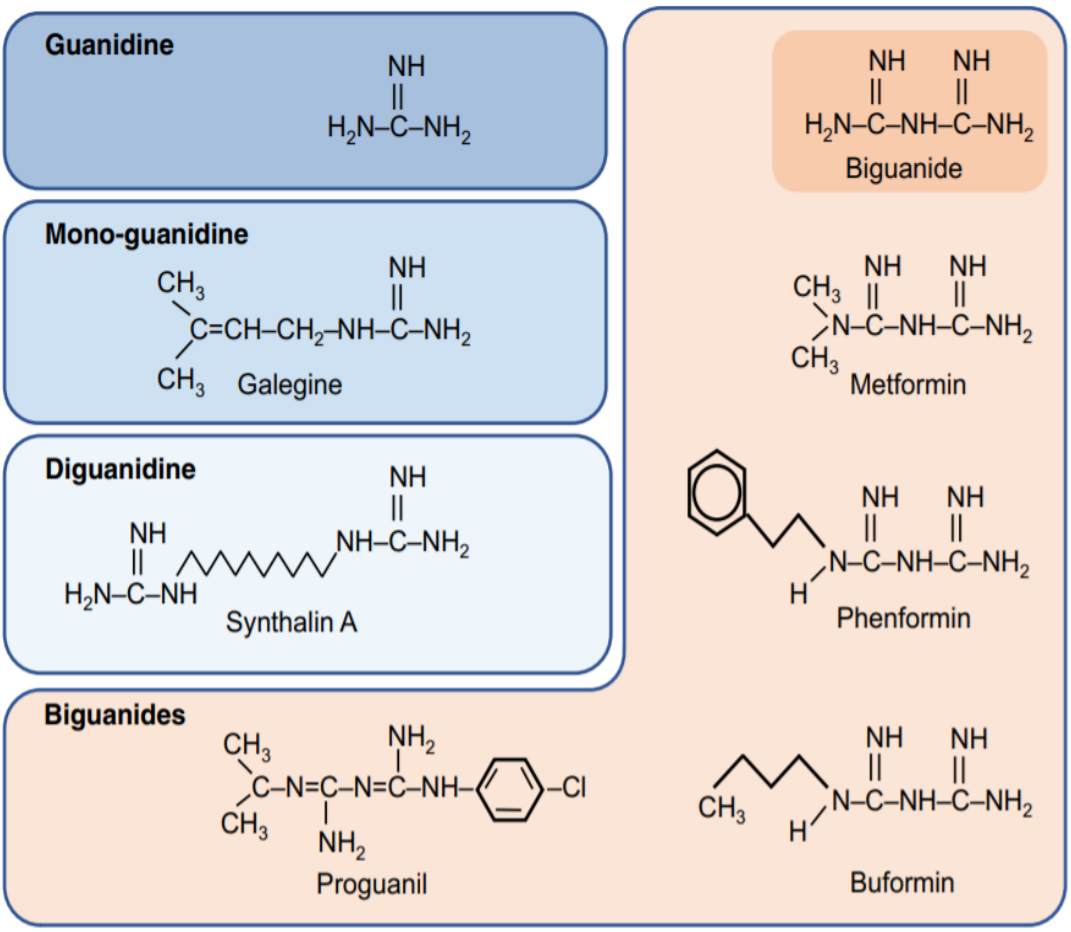

Figure 2. The structure of Metformin and other guanidine compounds [35].

In 1994, MET was widely used to treat Type II Diabetes (T2D) in Europe and USA [38]. MET decreases hepatic gluconeogenesis and increases insulin sensibility via enhancing peripheral glucose uptake in parallel with lowering plasma glucose [1, 35]. Besides the capacity of glucose-lowering, the long-term usage of MET can maintain weight, decrease hypoglycemia risk and significantly reduce mortality and risk of cardiovascular diseases [39-41].

Table 1. Pharmacodynamic effects of metformin in the treatment of type 2 diabetes [35].

Clinical feature	**Effect of metformin**
Hyperglycaemia	Improves glycaemic control in T2D; reduces progression of IGT and IFG to T2D
Insulin resistance	Counters insulin resistance by several insulin-dependent and -independent actions that reduce hepatic glucose output, improve peripheral glucose disposal, increase intestinal anaerobic glucose metabolism and assist endothelial function
Hyperinsulinaemia	Reduces fasting hyperinsulinaemia
Abdominal obesity	Usually stabilises body weight; can facilitate reduction of excess adiposity
Dyslipidaemia	May modestly improve blood lipid profile in some hypertriacylglycerolaemic and hypercholesterolaemic individuals
Blood pressure	No significant effect on blood pressure in most studies but blood pressure control may be improved in overweight individuals achieving weight loss
Proinflammatory state	May reduce CRP and some adipocytokines
Procoagulant state	Some antithrombotic activity, e.g. decrease in PAI-1, fibrinogen and platelet aggregation; improved capillary perfusion
Atherosclerosis	Reduced myocardial infarction and increased survival in T2D: reduced carotid intima-media thickness and reduced levels of adhesion molecules; other evidence for antiatherogenic activity, mostly from animal studies

CRP, C-reactive protein; PAI-1, plasminogen activator inhibitor-1; T2D, type 2 diabetes

MET evokes potent antineoplastic activity [3, 4]. This compound has been proved to decrease the risk of different cancers [42] and prolong the survival of patients with breast cancers [43, 44], hepatocellular carcinoma [45] and lung cancers [46-48]. The anticancer activity of MET (inhibition of cancer invasion and metastasis) undergoes through AMPK dependent signaling pathway [6-9, 49]. However, the molecular mechanism of the antineoplastic activity of MET is unknown. It has been considered that autophagy and apoptosis may represent an underlying mechanism of the anti-cancer activity of MET.

4. Autophagy

Autophagy is intracellular degradation process of dispensable or aberrant proteins and organelles. The products of degradation are usually recycled in cellular metabolic processes. The degradation is initioated by stress conditions, e.g. energy starvation [50-52]. Besides removing useless components retained in the cell, the other function of autophagy is to generate energy for the synthesis of new building blocks in the process of homeostasis and cellular renovation [50, 51]. It may affect cell survival [53] and contribute to inhibition of cancer cell growth. This process could explain the role of autophagy in the mechanism of cancer cell resistance to some therapies. Therefore, several pharmacotherapeutic approach has been undertaken to inhibit autophagy as an approach to supress tumor growth [50, 54]. A variety of proteins have been considered as autophagy markers for the assessment of the autophagy process. Initially, in yeast has been identified about 30 autophagy-related (ATG) genes and among them several are present in higher eukaryotes [55, 56]. Atg proteins are classified according to their function. Autophagosome formation is regulated by Atg1/ULK complex (Atg1, Atg11, Atg13, Atg17, Atg29, and Atg31). Membrane delivery to the expanding phagophore is mediated by Atg9 complex (Atg2, Atg9, and Atg18). PtdIns3P-binding proteins are recruited by PtdIns 3-kinase (PtdIns3K) complex (Vps34, Vps15, Vps30/Atg6, and Atg14). Two ubiquitin-like (Ubl) conjugation systems, the Atg12 complex (Atg5, Atg7, Atg10, Atg12, and Atg16) and Atg8 complex (Atg3, Atg4, Atg7, and Atg8) play a crucial role in vesicle expansion [57, 58]. The mammalian ULK1/2 complex comprises ULK1/2 (mammalian homologs of Atg1), ATG13 (a homolog of yeast Atg13), RB1CC1/FIP200 (a putative Atg17 homolog), and C12orf44/ATG101[59, 60]. The other study showed that ULK1 kinase could be activated by AMPK under conditions of starvation [61]. Of interest is that phosphorylation of mTORC1 inhibits ULK1/2 complex formation, preventing interaction between ULK1 and AMPK. However, down-regulation of mTOR facilitate formation of ULK1/2, ATG13, and RB1CC1 complex, initiating autophagy. The autophagy process is also mediated by Beclin-1 (autophagy-related gene, Atg 6), another gene coding autophagy protein [62, 63]. Some autophagy markers has been linked to the PRODH/POX-dependent apoptosis and autophagy [17, 19, 64-71] and some data provided evidence for crosstalk between both processes (autophagy and apoptosis) [72].

5. Apoptosis

In 1872, Karl Vogt described for the first time apoptosis while the mechanism for the process was presented in 1885 by Walther Flemming. Now this process is known as a programmed cell death that destroys itself to maintain tissue homeostasis [73]. The apoptosis machinery is mediated by a family of proteases, namely caspases, which contain a cysteine at their active site and cleave the target proteins at a residue of aspartic acids [74]. They originate as procaspases in an inactive form. In response to stress signal they are cleaved to active caspases, inducing apoptosis through energy-dependent pathways: the extrinsic pathway, the intrinsic pathway, and the Granzyme B-dependent pathway [74] [75]. The intrinsic apoptosis pathway is activated by pro-apoptotic proteins in the outer membrane of mitochondria, namely Bcl-2-associated X protein (BAX) and Bax-like BH3 protein (BID). They bind to the other protein, BAK, activating cytochrome c by interaction with protease activating factor-1 (Apaf-1) [76]. This cascade activate caspase 9 that triggers a cascade of effector caspases, as caspase 3, caspase 7 and caspase 6, resulting in cell death [77]. The p53 protein is a key factor in activating the intrinsic pathway due to its contribution to activating BAX protein [78]. The extrinsic pathway is initiated extracellularly by ligand binding to plasma membrane death receptors and activating initiator caspase 8 [75]. Death receptors are transmembrane proteins represented by Fas/CD95 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, DR-4 and DR-5 [79, 80]. Fas Associated via Death Domain (FADD) contain a death domain (DD) and a death effector domain (DED) which activate caspase-8 via sequential action of a homotypic DED–DED interaction. Activated caspase-8 initiates downstream effector caspases contributing to cell death. They have the same execution pathway that is initiated by the activation of caspase-3 [75]. Some of the apoptosis biomarkers were linked to PRODH/POX-dependent apoptosis [17, 19, 66-71].

6. Metabolic role of proline dehydrogenase/ proline oxidase (PRODH/POX)

PRODH/POX is a mitochondrial enzyme that converts proline to P5C during which released electrons are transferred to ETC producing ATP or in case they are directly accepted by oxygen, generate ROS [68]. PRODH/POX is coded by two human genes: PRODH1 (chromosome 22q11.21; NCBI Accession NM_016335) and PRODH2 (chromosome 19q13.12; NCBI Accession NM_021232). The enzymes are regulated by

several activation and inhibition factors, however, the enzyme function may depend on the substrate availability, proline.

The important biological role of proline was described by Benjamin List, the winner of Nobel Prize in Chemistry, 2021 who provided evidence that proline can catalyze *aldol reaction*. Proline is the predominant amino acid of collagen. The molecule is degraded extracellularly by tissue collagenases, then intracellularly by non-specific proteases and finally by cytoplasmic imidodipeptidase, prolidase that releases proline that could be utilized for collagen resynthesis or could be degraded by PRODH/POX. Increase in concentration of intracellular proline could be harmful for the cells due to its reducing potential that disturb cellular redox balance. Therefore, collagen biosynthesis could be also a "sink" for reducing potential of proline [20, 71, 74]. In the case of collagen biosynthesis inhibition, proline is degraded by PRODH/POX. After the conversion of proline to P5C, this metabolite could be further metabolized by pyrroline-5-carboxylate dehydrogenase (P5CDH), transforming P5C into glutamate, which is a precursor of α-ketoglutarate (α-KG) involved in TCA cycle. Decreased efficiency of TCA cycle could contribute to the reversible reaction of conversion of P5C into proline by pyrroline-5-carboxylate reductase (P5CR) using NADPH or NADH as a cofactor. The interconversion of proline-P5C/P5C-proline is known as a "proline cycle" [81]. It has been found that the proline, glutamine, ornithine and glutamate are linked to the proline cycle [82] and the process could be important in regulation of apoptosis and survival. Interestingly, the proline cycle is coupled with pentose phosphate pathway through NADPH generated by the pentose phosphate pathway and $NADP^+$ by the proline cycle [18, 67]. Based on this mechanism, the role of PRODH/POX in the regulation of cellular metabolism has recently been studied as an approach to cancer treatment. This cycle is responsible for regulating expression of genes, biosynthesis of nucleotides, redox balance, apoptosis and cell proliferation [66]. Moreover, PRODH/POX has been found to play a variety of regulatory functions, e.g. in regulation of osmotic preasure, response to metabolic stress and signaling in bacteria, plants, and mammals [71]. However, the functional significance of PRODH/POX in apoptosis/autophagy is generation of electrons and their transfer through flavin adenine dinucleotide (FAD) into the ETC for ATP production or in case of disfunction of ETC, generation of ROS. Production of ATP or ROS is dependent on cell environmental conditions [71].

6.1. PRODH/POX-induced apoptosis

Both intrinsic and extrinsic pathways of apoptosis could be induced by PRODH/POX [83]. In the extrinsic pathway (death receptor dependent), PRODH/POX stimulates the expression of TRAIL, DR5, and cleavage of caspase-8 [83, 84], and also indirectly activates caspase-9 and caspase-3 [85, 86]. In cancer cells, PRODH/POX is up-regulated by a variety of factors, e.g. tumor suppressor p53 or inflammatory factor, peroxisome proliferator-activated receptor gamma (PPARγ) [70, 71]. Interestingly, its level in cancer tissues is much lower than in normal tissues [87, 88]. High expression of PRODH/POX can facilitate ROS generation, and the process is linked to p53-dependent mechanisms [68, 89]. Such a mechanism for apoptotic cell death was presented in a variety of cancer cell types [68, 89-93]. Down-regulation of PRODH/POX inhibited p53-dependent apoptosis in cancer cells [83, 90]. The role of PRODH/POX as a driver of apoptosis was established in a model of PRODH/POX knockdown cancer cells [93].

6.2. PRODH/POX-induced autophagy

Silencing of PRODH/POX in MCF-7 breast cancer cells (by shRNA) contributed to an increase in concentration of cytoplasmic proline and induced autophagy, as presented by Zareba et al. (2018). Up to date, only hypoxia or glucose starvation was shown as an environmental conditions that affected PRODH/POX-dependent autophagy and apoptosis [17]. Further studies highlighted the role of proline availability for PRODH/POX-dependent apoptosis/autophagy. The well documented factor involved in the mechanism of proline-dependent autophagy is hypoxia-inducible factor-1 alpha (HIF-1α). In PRODH/POX expressing cells, free proline facilitates generation of α-KG that inhibits the transcriptional activity of HIF-1α. High concentration of α-KG increases the activity of a prolyl hydroxylase domain (PHD) of HIF-1α inducing proteasomal degradation of HIF-1α [84, 86, 94]. In cells with low PRODH/POX activity, proline concentration drastically increases and inhibits the activity of PHD, contributing to a decrease in HIF-1α proteasomal degradation and increase in its transcriptional activity. Moreover, it is well established that glutamine and proline are involved in the onco-metabolism of cancer cells [17]. This process is called as "para-metabolic pathway". Interestingly, the proline biosynthesis was linked to metabolism of glucose (through the TCA cycle and pentose phosphate pathway) and PRODH/POX-dependent

apoptosis/survival. This process was facilitated by oncogene MYC. In summary, proline can be utilized for protein synthesis or oxidized in the mitochondria for energy production. Under starvation or hypoxia, cancer cells may prefer the degradation of proline to produce the energy [64]. Therefore, hypoxia, glucose depletion, or treatment with rapamycin (mTOR inhibitor) stimulated PRODH/POX-dependent degradation of proline and induced autophagy.

7. Glycolysis in complex metabolic pathways

Glycolysis is a biochemical process in which glucose is metabolized into pyruvate, in parallel with ATP synthesis [95]. In 1940 glycolysis was described by Gustav Embden, Otto Meyerhof, and Jakub Karol Parnas as a ten steps reaction. Glycolysis is classified into aerobic glycolysis (the final product as pyruvate) and anaerobic glycolysis (pyruvate is converted to lactate) [96]. Recent views on glycolysis suggest that deregulation of the process may affect allosteric patterns of proteins, their post-translational modifications, transcriptional expression, and cellular localization [97], resulting in enzyme dysfunction [98].

Cellular energy metabolism is regulated in large part in glycolytic pathway that could be affected by enzyme activity and metabolites. The enzyme isoforms characterized by specific allosteric conformations can evoke different functions. Pyruvate kinase (PK) is one of the enzymes involved in the process [97], represented by isoform PKM1 (an active tetramer) and PKM2 (activated by F16BP, serine, and SAICAR) [99, 100]. PKM2 is involved in controlling oxidative flux in terms of protein phosphorylation [101]. PKM2 methylation by coactivator-associated arginine methyltransferase 1 (CARM1) can switch oxidative phosphorylation to aerobic glycolysis for tumorigenesis in breast cancer cells [102]. The aerobic glycolysis is known as the Warburg effect, which is characterized by an increase in lactate concentration generated from glucose in a condition of oxygen depletion [103].

CARM1 is PRMT4, one member of protein arginine methyltransferases (PRMTs) family responsible for protein arginine methylation (a type of posttranslational modification in various cellular processes) [104-107]. CARM1 is the cancer therapy target because the knockout of CARM1 in embryos reveals inhibition of cell differentiation and T cell development [108] and lack of CARM1 in mice leads to lethality

post birth [109]. CARM1 also plays a vital role in promoting gastric cancer cell proliferation and tumorigenesis [110]. There is a link between CARM1 and AMPK in the regulation of autophagy [111, 112]. Methylated PKM2 causes suppression of the expression of inositol-1,4,5-triphosphate receptors (InsP3Rs), resulting in an inhibition of calcium flux from the endoplasmic reticulum to mitochondria (Figure 3). Therefore, the inactivation of PKM2 methylation dramatically affects cell proliferation, migration, and metastasis and alters cellular metabolism [102]. The approaches on PKM2-dependent metabolism, especially post-translational modification of PKM2, have been targeted for anti-cancer therapy [113, 114].

PKM2 induces the expression of lactate dehydrogenase (LDH), contributing to an increase in lactate level [115]. Some lines of evidence suggest that Metformin reduces the energy supply of cancer cells by inhibiting HIF1α/PKM2 pathway [116].

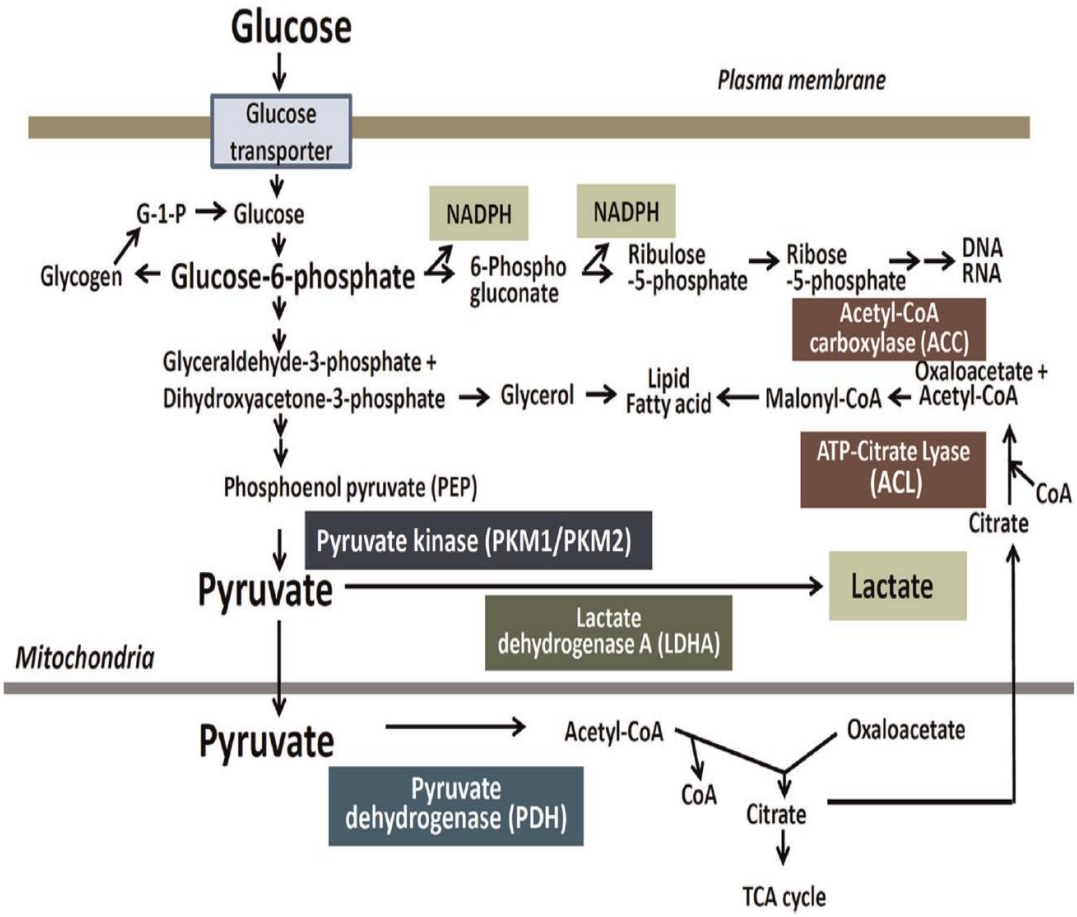

Figure 3. The cellular metabolism under control of PKM1/2 [115].

8. TCA cycle in complex metabolic pathways

Recent findings indicated that the energetic metabolism of mitochondria is tightly correlated with TCA cycle to control cell fate and function. TCA cycle metabolites determine the biosynthesis of nucleotides, lipids, proteins, and control chromatin modifications, DNA methylation, and post-translational modifications of proteins [117].

The TCA cycle (called a citric acid cycle) or the Krebs cycle, located in mitochondria, is the primary source of cellular ATP and plays an essential role in aerobic respiration. It produces NADH that transfers electrons to the ETC, producing ATP by oxidative phosphorylation [117]. TCA metabolites take part in both catabolism and anabolism. When the TCA cycle works appropriately, the metabolites can be involved in the syntheses of nucleotides and lipids. Unlike, anaplerosis occurs if those intermediates are not involved in mentioned biosynthesis.

There are two important anaplerotic mechanisms: the conversion of pyruvate to mitochondrial OAA by pyruvate decarboxylase and the activation of glutaminolysis in which α-ketoglutarate is produced from glutamine. If the ETC is impaired, some TCA cycle metabolites are generated from glutamine-dependent reductive carboxylation [118].

The primary function of TCA cycles is to generate ATP and 3 NADH and 1 $FADH_2$ which are involved in the ETC complex I and II, respectively. Those processes produce ATP through oxidative phosphorylation (OXPHOS). TCA cycle is parallel with OXPHOS because NADH/$FADH_2$ shuttle is needed for both processes. TCA metabolites participate in different signaling pathways [117]. Acetyl-CoA can regulate the chromatin dynamics, immune, cancer, and stem cells functions, while α-KG is responsible for the hypoxic response. When α-KG or Fe^{2+} level is reduced or in limited oxygen conditions, Prolyl-hydroxylases PHD1–3, a critical enzyme in regulating transcription factor HIF-1α, is impaired. As a result, HIF-1α is transferred to the nucleus to alter gene expression, angiogenesis, and immune system [119]. However, an increased level of α-KG in hypoxic cancer cells leads to the reactivation of α-KG, resulting in a sustainable metabolic vulnerability or cell death [120]. Interestingly, α-KG is involved in physiology by regulating epigenetic changes [117]. The other TCA cycle intermediates are succinate, which can act as an oncometabolite because its accumulation leads to alteration of gene expression or mutation, resulting in tumorigenesis [121, 122]. Succinate plays a role in regulating innate immunity by affecting the inflammatory cytokines. Similar to succinate,

fumarate accumulation can promote tumor growth. However, these metabolites can control chromatin modification and regulate protein succination [117] (Figure 4).

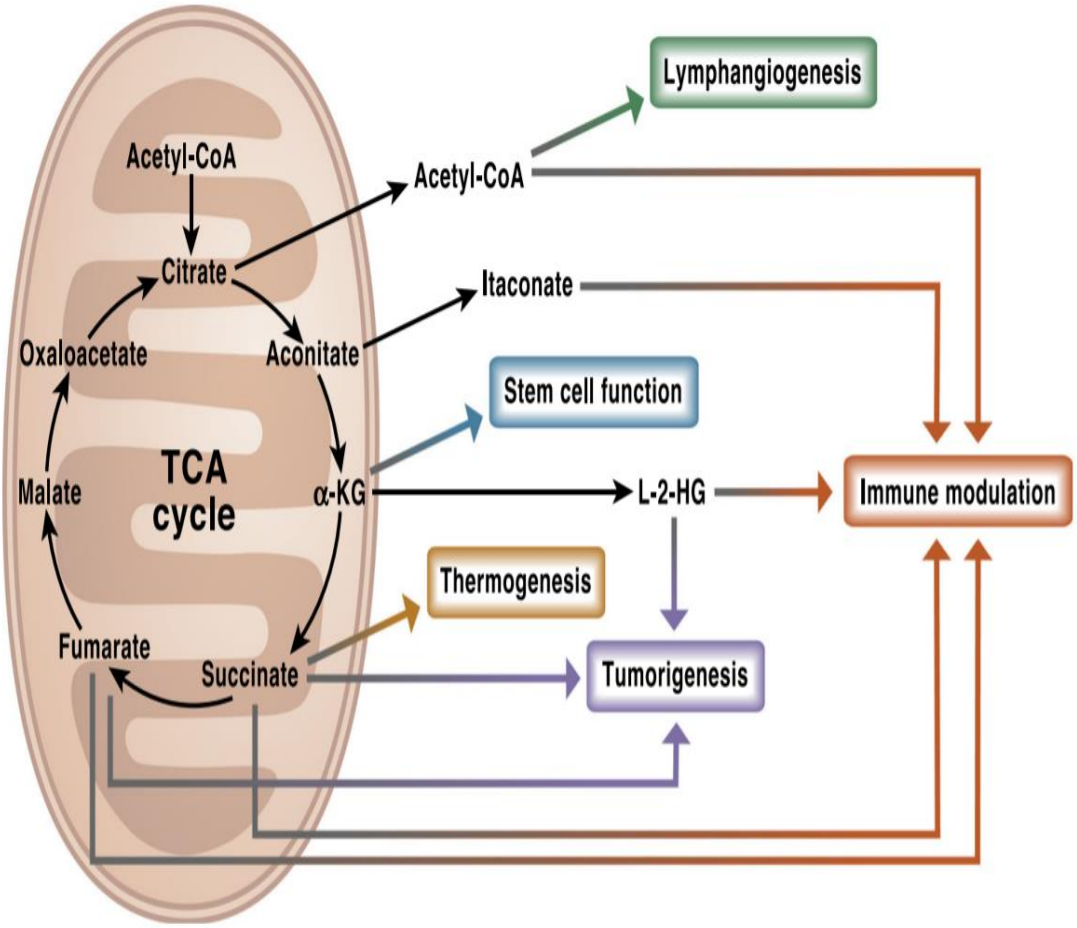

Figure 4. TCA metabolites involved in signaling pathways [117].

9. Amino acids as metabolic regulators

Proteins are multifunctional components of cellular structure and function. They are responsible for cell architecture, catalysis of chemical reactions, gene expression, cell signaling, and several other functions that contribute to inflammation, immune reactions, protection against pathogens, activation of autophagy, apoptosis, and many other processes [123]. The protein structure is composed of more than twenty amino acids. However, free amino acids play an important role in the regulatory processes of cellular metabolism. Among them, proline convertible amino acids as glutamine, glutamate, α-KG, and ornithine play a critical role in complex regulatory mechanisms of the cell.

Several amino acids have been linked to activating or inhibiting apoptosis/autophagy [124]. It is well recognized that they participate in the mTORC1 and GCN2/eIF2 pathways that regulate protein translation and control the cellular demand for amino acids by concomitantly regulating autophagy-dependent catabolism [125-127]. For instance, non-essential amino acids (NEA) as proline in the condition of glucose deprivation activate anti-apoptotic pathways in cancer cells by inducing the expression of anti-apoptotic members of the Bcl-2 gene family and preventing the expression of pro-apoptotic proteins [128]. The study suggested that although apoptosis could be induced in cancer cells under low glucose conditions, the non-essential amino acids may counteract the process by upregulation of large amino acid transporter 1 (LAT1) in the membranes of cancer cells [65, 129, 130].

Glutamine is an important source of energy and an essential metabolite in the proliferation of mammalian cells. This is particularly important for cancer cells due to mitochondrial vulnerability of cancer cells resulting from the altered glycolysis that affect TCA cycle [131]. TCA cycle is the main source of energy for proliferation of cancer cells [132, 133]. This pathway has been linked to several tumor suppressors and oncogenes [103, 132, 134, 135]. The demand for glutamine is 10-fold higher than that for other amino acids [136]. Glutamine affects mTOR kinase, mitochondrial membrane potential, NADPH production [137], is a nitrogen source for purine and pyrimidine synthesis [138, 139] and glutamine-derived glutamic acid continues donating its amine group to accelerate the TCA cycle metabolites for the production of α-ketoglutarate, serine, alanine, aspartate, and ornithine. Glutamine also is a source of carbon and nitrogen for the synthesis of proline, ornithine, and arginine [140]. Lack of exogenous glutamine is one of the major causes of the death of cancer cells [141]. Several cancer cell lines are vulnerable due to glutamine starvation [142]. The study suggested that glutamine derivatives like glutamate, α-ketoglutarate, and glutathione are involved in the apoptotic pathway [143]. Similarly, proline interconvertibility with glutamate and ornithine [66, 144] could play a key role in cell reprogramming and apoptosis/autophagy.

Beside proline, ornithine and glutamate are the main source of P5C. Conversion of ornithine into P5C is catalyzed by mitochondrial vitamin B6-dependent ornithine δ-aminotransferase (OAT), while from glutamate by mitochondrial ATP- and NAD(P)H-dependent P5C synthase (P5CS) [145, 146]. This reaction can be reversed by mitochondrial P5CDH [146]. These reaction has been found to play a role in apoptosis/

autophagy. It has been demonstrated that inhibition of activity of ornithine decarboxylase (ODC) by difuoromethylornithine (DFMO) contributed to accumulation of intracellular ROS and cell arrest and cell death. These findings indicate that the urea cycle contributes to the regulation of apoptosis and autophagy [147]. Since ornithine is P5C convertible amino acid it may affect PRODH/POX-dependent apoptosis/autophagy. These studies allow to present a hypothesis on the regulation of PRODH/POX-dependent apoptosis/autophagy by key amino acids (Figure 5).

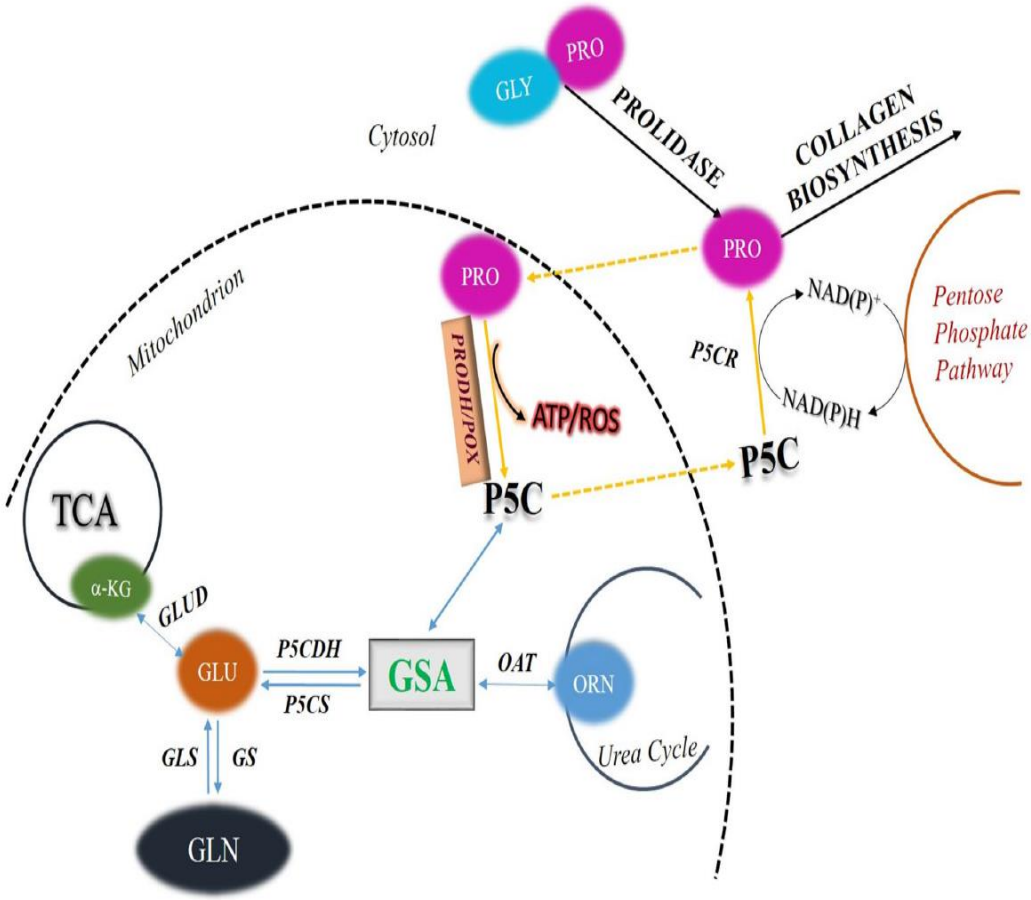

Figure 5. Regulation of PRODH/POX-dependent apoptosis/autophagy by key amino acids. PRO proline; GLU glutamate; ORN ornithine; GLN glutamine; GLYPRO glycyl-proline; PRODH/POX proline dehydrogenase (PRODH)/proline oxidase (POX); ROS reactive oxygen species; P5C pyrroline-5-carboxylate; P5CR pyrroline-5-carboxylate reductase; P5CDH pyrroline-5-carboxylate dehydrogenase; P5CS pyrroline-5-carboxylate synthase; OAT ornithine aminotransferase; GSA glutamic gamma-semialdehyde; αKG α-ketoglutarate; TCA tricarboxylic acid cycle; GS glutamine synthase; GLS glutaminase; GLUD glutamate dehydrogenase.

THE OBJECTIVE OF STUDY

The objective of the Ph.D. dissertation is to evaluate the mechanism of Metformin (MET) induced apoptosis in MCF-7 breast cancer cells.

This Ph.D. dissertation is based on the hypothesis that up-regulation of AMPK by MET can stimulate apoptosis in cancer cells by a cascade of processes involving induction of PRODH/POX-dependent ROS generation under the availability of proline, the PRODH/POX substrate. It has been considered that the processes require specific conditions determined by the complex regulatory machinery of the cell. Proline availability for PRODH/POX-dependent functions is regulated by prolidase activity (proline supporting enzyme), collagen biosynthesis (proline utilizing process), and interconversion of proline, ornithine, glutamate and α-ketoglutarate, linking amino acid metabolism with TCA and Urea cycles. Moreover, the TCA cycle is tightly correlated with glycolysis and electron transport chain. Therefore, I have performed studies on the metabolomic profile of MET-treated MCF-7 cells and PRODH/POX knockout MCF-7 cells to identify key metabolites of the mentioned above processes that facilitate MET-induced POX-dependent ROS generation and apoptosis. The concentrations of intracellular proline, glutamic acids, ornithine, glutamine, α-ketoglutaric acid, glucose, pyruvate, succinate and others were measured by high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS) with a triple quadrupole (QqQ).

The study was carried out by the following experiments:

1. Preparation of MCF-7 breast cancer cells with PRODH/POX knockout by using CRISPR-cas9 technology.
2. Evaluation of the effect of Metformin (AMPK activator) on the cell proliferation and cell cycle in wild-type MCF-7 cells and PRODH/POX knockout MCF-7 cells.
    a) Evaluation of cell proliferation using CyQUANT® Cell Proliferation Assay.
    b) Evaluation of cell cycle phase by using flow cytometry.
3. Evaluation of the effect of Metformin on the expression of AMPK and apoptosis markers by Western Blotting in wild-type MCF-7 cells and PRODH/POX knockout MCF-7 cells.

4. Evaluation of Metformin on metabolic profiles of several key metabolites of glycolysis, TCA cycles, Urea cycles, Pentose phosphate pathway, and several key amino acids in PRODH/POX-dependent pathways using LC-MS/MS/QqQ in wild-type MCF-7 cells and PRODH/POX knockout MCF-7 cells.

The expected results should establish the role of PRODH/POX in the mechanism of MET-dependent apoptosis and might suggest a new molecular target of experimental breast cancer pharmacotherapy.

MATERIALS AND METHODS

To evaluate the role of PRODH/POX in the mechanism of MET induced apoptosis in breast cancer cells we generated a PRODH/POX knock out MCF-7 breast cancer cell line (by CRISPR-Cas9 technology). Cell proliferation was determined by CyQUANT® Cell Proliferation Assay, while cell cycle was analysed by flow cytometry using Nucleo-Counter NC-3000. Expression of pro-apoptotic proteins was evaluated by Western blot. Targeted metabolomics was performed by LC-MS/MS/QqQ. All procedures as well as statistical analysis are described in details in publication entitled “Metformin Treatment or PRODH/POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells”.

RESULTS

1. Generation of PRODH/POX knockout MCF-7 breast cancer cells

PRODH/POX knockout MCF-7 cells (MCF-7^{crPOX} cells) were generated using the CRISPR/Cas9 technology. The knockout of PRODH/POX in MCF-7^{crPOX} cells was confirmed by western blot using anti-PRODH/POX antibody (Santa Cruz).

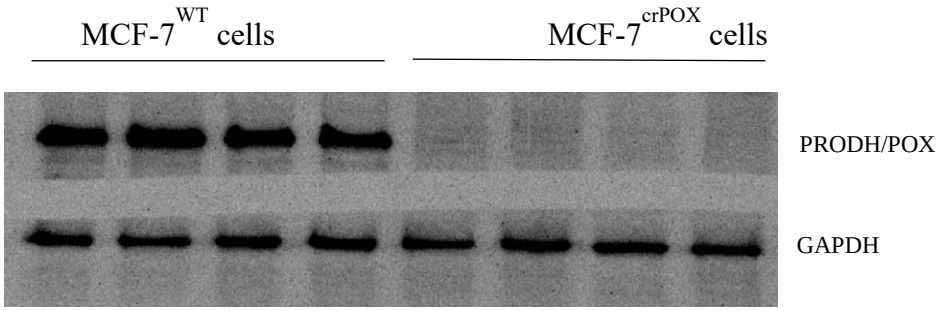

Figure 6. The PRODH/POX expression in wild-type MCF-7 cells and MCF-7^{crPOX} cells by Western Blot using Anti-PRODH/POX antibody (Santa Cruz).

2. The effect of metformin on cell proliferation, cell cycle and apoptosis in MCF-7 breast cancer cells

2.1. The effect of Metformin on the cell proliferation and cell cycle in wild-type MCF-7 cells and PRODH/POX knockout MCF-7 cells

The proliferation of wild-type MCF-7 cells (MCF-7^{WT}) and PRODH/POX knockout MCF-7 cells (MCF-7^{crPOX} cells) treated with 20 mM MET in a medium with or without glutamine for 24 h was investigated. It has been found that MET-treatment of both cell lines contributed to decrease in cell proliferation when incubated in a medium with or without glutamine (Figure 7A). However, the inhibitory effect was more pronounced in the absence of glutamine. The data were corroborated by the ratio of dividing cells to non-dividing cells (the percentage of cells in the G2/M phase to G0/G1 phase). As presented in Figure 7B, both MET-treatment and PRODH/POX knockout strongly suppressed proliferation of MCF-7 cells cultured in a glutamine-free medium. At the same time, there was no effect on the process in the presence of glutamine.

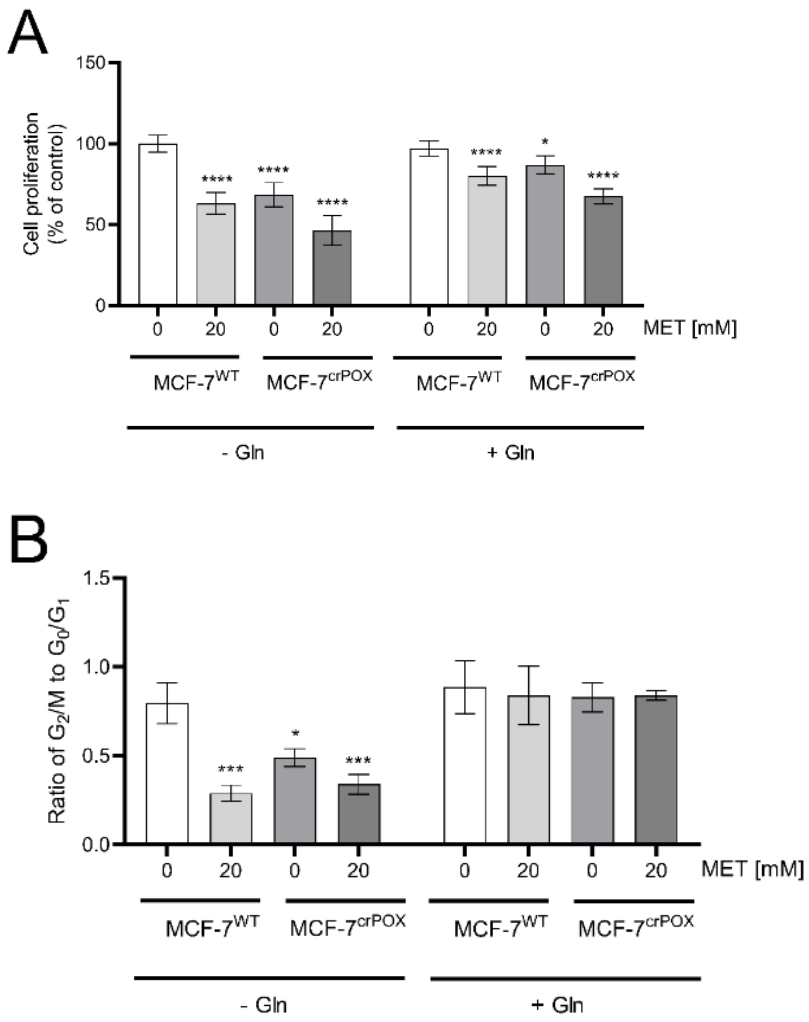

Figure 7. Cell proliferation (A), the ratio of cell percentage in G_2/M to G_0/G_1 phase (B), in metformin (MET, 20 mM) treated MCF-7^{WT} and MCF-7^{crPOX} cells cultured in medium with or without glutamine (Gln) for 24 h. The mean values ± standard error (SEM) from 3 experiments done in duplicates are presented at * $p < 0.05$, and *** $p < 0.001$, **** $p < 0.0001$.

2.2. The effect of Metformin on the apoptosis in wild-type MCF-7 cells and PRODH/POX knockout MCF-7 cells

As presented in Figure 8, MET induced expression of AMPK in both cell lines, however the expression was more pronounced in the absence of glutamine. A significant increase in PRODH/POX expression was also observed in MET-treated MCF-7^{WT} cells cultured in a medium with or without glutamine. In MCF-7^{crPOX} cells, (for obvious reasons due to lack of the PRODH/POX), MET did not affect its expression. However, MET increased the expression of cleaved PARP and Caspase-7 in both cell lines when

cultured in a glutamine-free (-Gln) medium. Interestingly, knockout of PRODH/POX by itself increased expressions of cleaved PARP and Caspase-7 in MCF-7^{crPOX} cells, compared to MCF-7^{WT} cells, when cultured in a glutamine free medium (-Gln). Interestingly, in the presence of glutamine (+Gln), MET had no effect on the expression of the proteins in both studied cell lines.

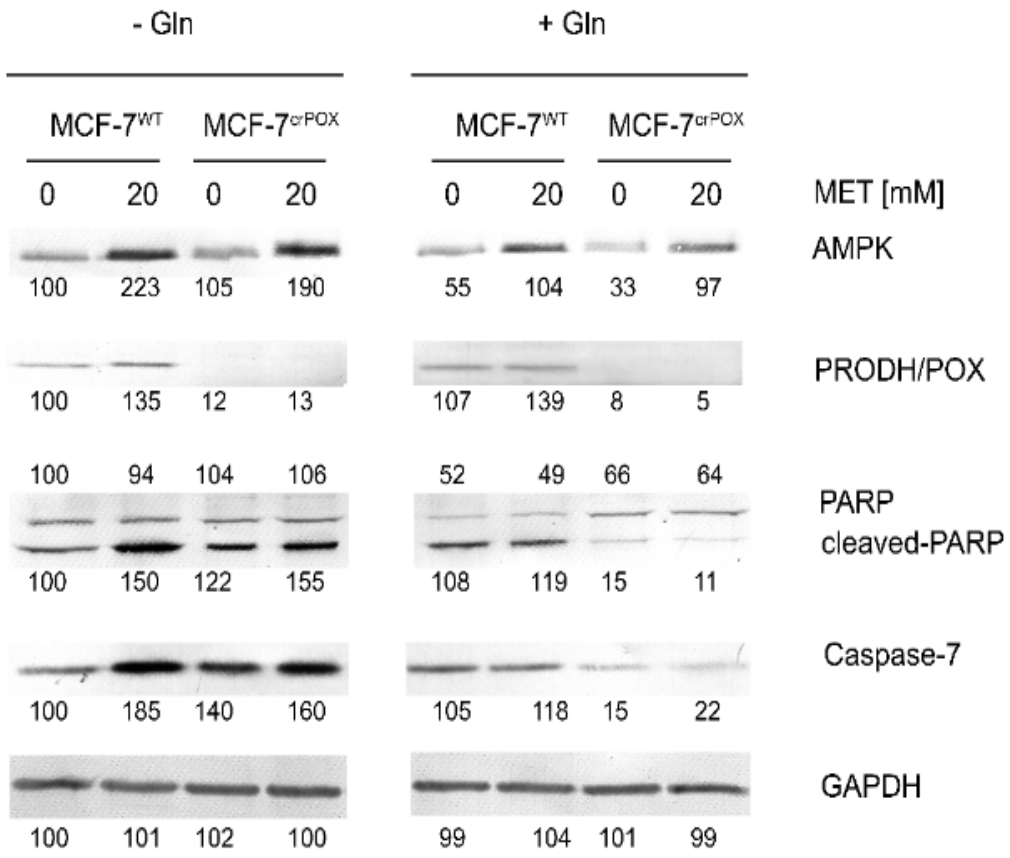

Figure 8. Western blot for AMPK, PRODH/POX, PARP and caspase 7 in metformin (MET, 20 mM) treated MCF-7^{WT} and MCF-7^{crPOX} cells cultured in medium with or without glutamine for 24 h. The mean values ± standard error (SEM) from 3 experiments done in duplicates are presented at * $p < 0.05$, and *** $p < 0.001$, **** $p < 0.0001$. Representative Western blot images were shown.

3. The effect of metformin on metabolic profiles of MCF-7 breast cancer cells

3.1. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and Urea Cycles in MCF-7^{crPOX} and MCF-7^{WT} cultured in glutamine-free (-Gln) medium.

Targeted metabolomics in MCF-7^{WT} and MCF-7^{crPOX} cells cultured in glutamine-free medium showed significant differences in concentration of studied metabolites between the investigated cells. The results in Table 2 show that PRODH/POX-knock out of MCF-7 cells (MCF-7^{crPOX}) contributed to a drastic increase in intracellular glucose (GLC) and pyruvic acid (PYR) concentrations (about 12- and 17-fold, respectively) and about a 2-fold increase in lactic acid (LA) concentration, as compared to MCF-7^{WT}. It was accompanied by a total decrease in the concentrations of phospho-enol-pyruvic acid (PEP) and glucose 6-phosphate (G-6-P), 6-Phospho-gluconic acid, and a significant decrease in the concentrations of all studied TCA cycle and urea cycle metabolites as well as glutamine (Gln) and glutamic acid (Glu), without effect on proline (Pro) concentration in MCF-7^{crPOX} cells. The data suggest that PRODH/POX-knock out contributes to inhibition of GLC, LA, and PYR consumption while PEP and G-6-P and some TCA and urea cycles metabolites are utilized in these conditions. Pro is not significantly affected.

Table 2. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{crPOX} and MCF-7^{WT} cells cultured in (-Gln) medium.

Relevant Metabolic Pathways	**Metabolites**	**Conc. Average (ppb)**		**% Change (MCF-7^{crPOX} vs. MCF-7^{WT})**	***P* Value (Mann–Whitney)**	**MCF-7^{crPOX}**
		MCF-7^{WT}	MCF-7^{crPOX}			
Glycolysis	Pyruvic acid	6.5	117.2	1712.0	0.010	↑
	Glucose	11.2	150.8	1252.0	0.010	↑
	Phospho-enol-pyruvic acid	6449.0	0.0	−100.0	0.010	↓
PPP	Glucose 6-phosphate	328.6	0.0	−100.0	0.010	↓
	6-Phospho-gluconic acid	269.9	10.9	−96.0	0.010	↓
TCA	Malic acid	1126.1	77.4	−93.1	0.010	↓
	Succinic acid	250.5	131.5	−47.5	0.159	
	Fumaric acid	336.2	27.5	−91.8	0.010	↓
	cis-Aconitic acid	43.7	4.2	−90.3	0.010	↓
	Citric acid	6425.6	0.0	−100.0	0.010	↓
	alpha-Ketoglutaric acid	222.3	97.1	−56.3	0.019	↓
Urea Cycle	Citrulline	10.3	4.6	−55.1	0.035	↓
	Arginine	14,526.1	437.6	−97.0	0.010	↓

	Ornithine	2626.1	0.0	−100.0	0.010	↓
AA	Proline	2642.0	2694.3	2.0	0.841	
	Glutamine	31.0	20.3	−34.5	0.010	↓
	Glutamic acid	410.2	155.9	−62.0	0.010	↓
Additional	Lactic acid	4613.3	13,347.5	189.3	0.010	↑
	Fructose	2.3	34.6	1384.5	0.010	↑

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑- significant increase in the concentration of studied compound in MCF-7^{crPOX} cells vs. MCF-7^{WT}, ↓ - significant increase in the concentration of studied compound in MCF-7^{crPOX} cells vs. MCF-7^{WT}.

3.2. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in metformin (MET) treated wild type MCF-7 cells (MCF-7^{WT+MET}) and in MCF-7^{WT} cells cultured in (-Gln) medium.

Metformin treatment of MCF-7^{WT} (MCF-7^{WT+MET}) caused an increase in the intracellular concentration of GLC, PYR and LA associated with a decrease in PEP (insignificantly), G-6-P, and some TCA metabolites, compared to untreated MCF-7^{WT} cells. Of interest is not significant effect on Pro concentration in MCF-7^{WT+MET} cells compared to MCF-7^{WT} cells (Table 3). It suggests that MET significantly decreases consumption of GLC, PYR and LA. At the same time, PEP and G-6-P and some TCA metabolites are utilized in these conditions.

Table 3. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{WT+MET} and in MCF-7^{WT} cells cultured in Gln free medium.

Relevant Metabolic Pathways	**Metabolites**	**Conc. Average (ppb)**		**% Change (MCF-7^{WT+MET} vs. MCF-7^{WT})**	***P* Value (Mann–Whitney)**	**MCF-7^{WT+MET}**
		MCF-7^{WT}	MCF-7^{WT+MET}			
Glycolysis	Pyruvic acid	6.5	227.9	3423.5	0.038	↑
	Glucose	11.2	115.8	938.0	0.038	↑
	Phospho-enol-pyruvic acid	6449.0	417.1	−93.5	0.057	
PPP	Glucose 6-phosphate	328.6	45.5	−86.1	0.038	↓
	6-Phospho-gluconic acid	269.9	607.7	125.1	0.727	
TCA	Malic acid	1126.1	514.0	−54.4	0.260	
	Succinic acid	250.5	168.8	−32.6	0.420	
	Fumaric acid	336.2	179.2	−46.7	0.260	
	cis-Aconitic acid	43.7	5.9	−86.4	0.050	↓

	Citric acid	6425.6	600.3	−90.7	0.050	↓
	alpha-Ketoglutaric acid	222.3	1818.6	718.3	0.483	
Urea Cycle	Citrulline	10.3	14.1	36.8	0.500	
	Arginine	14,526.1	4528.0	−68.8	0.420	
	Ornithine	2626.1	1223.7	−53.4	0.327	
AA	Proline	2642.0	2335.2	−11.6	0.841	
	Glutamine	31.0	25.8	−16.6	0.168	
	L-Glutamic acid	410.2	466.0	13.6	0.841	
Additional	Lactic acid	4613.3	17,831.6	286.5	0.038	↑
	Fructose	2.3	11.5	392.3	0.057	

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑- significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}, ↓ - significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}.

3.3. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{crPOX} treated with MET (MCF-$7^{crPOX+MET}$) and in MCF-7^{WT} cultured in (-Gln) medium

As shown in Table 4, MET treatment contributed to accumulation in intracellular concentration of GLC and PYR (about 26- and 44-fold, respectively) and LA (about 4-fold) in MCF-7^{crPOX} cells (MCF-$7^{crPOX+MET}$), as compared to untreated MCF-7^{WT}. The phenomenon was accompanied by decrease in PEP and G-6-P concentrations and a significant decrease in the concentrations of several TCA cycle metabolites and ornithine in MCF-$7^{crPOX+MET}$ cells, compared to untreated MCF-7^{WT} cells. The data suggest that MET treatment of MCF-7 cells (MCF-$7^{crPOX+MET}$) has an inhibitory effect on GLC, LA, and PYR consumption while PEP and G-6-P and some TCA and urea cycle metabolites are utilized in these conditions.

Table 4. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-$7^{crPOX+MET}$ and in MCF-7^{WT} cultured in Gln free medium.

Relevant Metabolic Pathways	**Metabolites**	**Conc. Average (ppb)**		**% Change (MCF-$7^{crPOX+MET}$ vs. MCF-7^{WT})**	***P* Value (Mann–Whitney)**	**MCF-$7^{crPOX+MET}$**
		MCF-7^{WT}	MCF-$7^{crPOX+MET}$			
Glycolysis	Pyruvic acid	6.5	289.6	4378.6	0.022	↑
	Glucose	11.2	303.1	2618.3	0.022	↑
	Phospho-enolpyruvic acid	6449.0	0.0	−100.0	0.022	↓

PPP	Glucose 6-phosphate	328.6	18.8	−94.3	0.025	↓
	6-Phospho-gluconic acid	269.9	71.9	−73.4	0.104	
TCA	Malic acid	1126.1	133.0	−88.2	0.025	↓
	Succinic acid	250.5	134.1	−46.5	0.169	
	Fumaric acid	336.2	27.6	−91.8	0.025	↓
	cis-Aconitic acid	43.7	1.1	−97.6	0.022	↓
	Citric acid	6425.6	0.0	−100.0	0.022	↓
	alpha-Ketoglutaric acid	222.3	78.2	−64.8	0.132	
Urea Cycle	Citrulline	10.3	10.3	0.4	0.802	
	Arginine	14,526.1	2098.1	−85.6	0.118	
	Ornithine	2626.1	59.4	−97.7	0.025	↓
AA	Proline	2642.0	1244.8	−52.9	0.121	
	Glutamine	31.0	29.1	−6.1	0.578	
	Glutamic acid	410.2	78.6	−80.8	0.025	↓
Additional	Lactic acid	4613.3	21,161.8	358.7	0.022	↑
	Fructose	2.3	18.8	706.0	0.022	↑

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑- significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}, ↓ - significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}.

3.4. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in PRODH/POX-knock out of MCF-7 cells (MCF-7^{crPOX}) and wild type MCF-7 cells (MCF-7^{WT}) cultured in glutamine containing (+Gln) medium.

The result showed that although there was no statistically significant difference in concentration of metabolites between MCF-7^{crPOX} and MCF-7^{WT} cultured in medium containing Gln, PRODH/POX knock out led to an increase in intracellular GLC (insignificantly) and a slight increase in PYR concentration in MCF-7^{crPOX} cells. It was accompanied by decrease in concentrations of PEP, G-6-P, some TCA cycle, urea cycle metabolites, and Gln and Glu, without effect on Pro concentration in MCF-7^{crPOX} cells, compared to MCF-7^{WT} cells (Table 5). The data suggest that the PRODH/POX-knocked out cells cultured in the presence of Gln utilized all studied metabolites but saved consumption of GLC in these conditions. Pro concentration was not significantly affected.

Table 5. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{crPOX} and MCF-7^{WT} cultured in (+Gln) medium.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{crPOX} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{crPOX}
		MCF-7^{WT}	MCF-7^{crPOX}			
Glycolysis	Pyruvic acid	94.3	130.9	38.9	0.653	
	Glucose	10.3	59.5	474.9	0.075	
	Phospho-enol-pyruvic acid	3605.0	78.2	−97.8	0.075	
PPP	Glucose 6-phosphate	184.4	131.9	−28.4	0.660	
	6-Phospho-gluconic acid	794.5	814.3	2.5	1.000	
TCA	Malic acid	1361.2	635.2	−53.3	*0.172*	
	Succinic acid	195.5	158.0	−19.2	0.660	
	Fumaric acid	402.0	184.4	−54.1	*0.172*	
	cis-Aconitic acid	79.6	48.2	−39.5	0.653	
	Citric acid	7462.9	4477.2	−40.0	0.536	
	alpha-Ketoglutaric acid	949.3	684.7	−27.9	0.660	
Urea Cycle	Citrulline	6.1	11.0	81.5	0.377	
	Arginine	10138.1	6694.5	−34.0	0.660	
	Ornithine	3957.4	1510.7	−61.8	*0.172*	
AA	Proline	3288.6	3373.5	2.6	1.000	
	Glutamine	296.2	65.9	−77.7	*0.075*	
	Glutamic acid	369.4	250.6	−32.2	0.543	
Additional	Lactic acid	24,919.0	23,080.4	−7.4	1.000	
	Fructose	12.0	21.4	78.1	0.075	

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle.

3.5. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MET treated wild type MCF-7 cells (MCF-7^{WT+MET}) and in MCF-7^{WT} cells cultured in (+Gln) medium.

It has been found that in MET treated MCF-7^{WT} (MCF-7^{WT+MET}), cultured in the presence of Gln, the concentration of GLC was drastically increased (about 11-fold), as well as concentrations of Gln, Glu (significantly) and PYR (insignificantly) were increased, while concentrations of LA, G-6-P, Orn and some metabolites of the TCA cycle were decreased. Concentrations of Pro and Arg were not much affected, compared to MCF-7^{WT} (Table 6). The data suggest that in the presence of glutamine, MET treatment of MCF-7^{WT} inhibited utilization of GLC, PYR, Gln, and Glu while stimulated

consumption of TCA metabolites and lactic acid and only slightly affected concentration of Pro and some urea cycle metabolites compared to MCF-7^{WT} cells.

Table 6. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{WT+MET} and in MCF-7^{WT} cells cultured in (+Gln) medium.

Relevant Metabolic Pathways	**Metabolites**	**Conc. Average (ppb)**		**% Change (MCF-7^{WT+MET} vs. MCF-7^{WT})**	***P* Value (Mann–Whitney)**	**MCF-7^{WT+MET}**
		MCF-7^{WT}	MCF-7^{WT+MET}			
Glycolysis	Pyruvic acid	94.3	201.7	114.0	0.132	
	Glucose	10.3	124.2	1100.6	0.050	↑
	Phospho-enol-pyruvic acid	3605.0	1502.4	−58.3	0.176	
PPP	Glucose 6-phosphate	184.4	35.7	−80.6	0.165	
	6-Phospho-gluconic acid	794.5	79.2	−90.0	0.050	↓
TCA	Malic acid	1361.2	779.1	−42.8	0.248	
	Succinic acid	195.5	114.5	−41.4	0.165	
	Fumaric acid	402.0	239.1	−40.5	0.248	
	cis-Aconitic acid	79.6	5.7	−92.9	0.050	↓
	Citric acid	7462.9	613.7	−91.8	0.050	↓
	alpha-Ketoglutaric acid	949.3	1576.9	66.1	0.248	
Urea Cycle	Citrulline	6.1	6.3	2.9	0.952	
	Arginine	10,138.1	10963.0	8.1	0.578	
	Ornithine	3957.4	2019.8	−49.0	0.248	
AA	Proline	3288.6	4193.5	27.5	0.165	
	Glutamine	296.2	1666.2	462.4	0.050	↑
	Glutamic acid	369.4	941.7	154.9	0.050	↑
Additional	Lactic acid	24,919.0	15,892.6	−36.2	0.248	
	Fructose	12.0	9.8	−18.3	0.086	

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑- significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}, ↓ - significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}.

3.6. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{crPOX} treated with MET (MCF-$7^{crPOX+MET}$) and in MCF-7^{WT} cultured in the (+Gln) medium.

MET treatment of MCF-7^{crPOX} cells (MCF-$7^{crPOX+MET}$) in the presence of Gln induced a drastic increase in the concentration of GLC (about 18-fold), no significant increase in PYR and Pro, and a total decrease in PEP, G-6-P. However, it led to a

significant decrease in concentration of TCA and urea cycle metabolites, and a slight decrease in LA. Interestingly, Glu concentration was also significantly decreased, compared to untreated MCF-7^{WT} cells (Table 7). The results suggest that in the presence of glutamine, Metformin inhibited GLC utilization while induced utilization of TCA and urea cycle metabolites and LA, without significant effect on Pro concentration in MCF-7^{crPOX} cells, compared to untreated MCF-7^{WT} cells.

Table 7. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-$7^{crPOX+\ MET}$ and in MCF-7^{WT} cells cultured in (+Gln) medium.

Relevant Metabolic Pathways	**Metabolites**	**Conc. Average (ppb)**		**% Change (MCF-$7^{crPOX+MET}$ vs. MCF-7^{WT})**	***P* Value (Mann–Whitney)**	**MCF-$7^{crPOX+MET}$**
		MCF-7^{WT}	MCF-$7^{crPOX+MET}$			
Glycolysis	Pyruvic acid	94.3	131.6	39.6	0.586	
	Glucose	10.3	204.6	1878.0	0.025	↑
	Phospho-enol-pyruvic acid	3605.0	0.0	−100.0	0.025	↓
PPP	Glucose 6-phosphate	184.4	0.0	−100.0	0.025	↓
	6-Phospho-gluconic acid	794.5	0.0	−100.0	0.025	↓
TCA	Malic acid	1361.2	37.0	−97.3	0.025	↓
	Succinic acid	195.5	91.9	−53.0	0.086	
	Fumaric acid	402.0	6.1	−98.5	0.025	↓
	cis-Aconitic acid	79.6	1.4	−98.3	0.025	↓
	Citric acid	7462.9	0.0	−100.0	0.025	↓
	alpha-Ketoglutaric acid	949.3	40.9	−95.7	0.025	↓
Urea Cycle	Citrulline	6.1	1.9	−69.5	0.226	
	Arginine	10,138.1	539.3	−94.7	0.025	↓
	Ornithine	3957.4	0.0	−100.0	0.025	↓
AA	Proline	3288.6	3664.0	11.4	0.905	
	Glutamine	296.2	210.6	−28.9	0.461	
	Glutamic acid	369.4	68.4	−81.5	0.025	↓
Additional	Lactic acid	24,919.0	17,098.2	−31.4	0.226	
	Fructose	12.0	15.8	31.6	0.086	

AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑-significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}, ↓ - significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}.

DISCUSSION

Epidemiological evidence suggests that therapy with the metformin is associated with decreased risk of certain cancers, such as colon, liver, lung, breast as well as decreased cancer mortality [148]. However, there is some discrepancy between these studies. Some data show beneficial effect of metformin in cancer treatment with reduced mortality [149-152], while others fail to document such beneficial effects [148, 152]. It suggests the presence of a specific molecular signature of cancer that increases its susceptibility to the antineoplastic effects of metformin. Therefore, we try to recognize the molecular signature by metabolomic approach.

Metabolomic analyses are promising approaches for identification of specific abnormalities in cancer metabolic pathways that could be considered as a potential target for cancer therapy. Similarly, metabolomic analyses of cancer cells that are treated with compounds of potential antineoplastic activity could identify mechanism of their action. In present study, analysis of some metabolites (targeted metabolomics) of glycolysis, TCA, Urea cycle, pentose phosphate pathway (PPP) and proline convertible amino acids (glutamine, glutamate, ornithine, α-ketoglutarate) was performed in breast cancer cells that have been treated with MET. It has been considered that MET induces reprogramming of energetic metabolism in such a way that instead of glucose facilitate degradation of proline by PRODH/POX, as an alternative source of energy. Therefore, studies on PRODH/POX-knocked out MCF-7 cells were also performed.

Interestingly, in conditions of Gln absence, MET treatment of MCF-7 cells as well as MCF-7 PRODH/POX-knocked out cells contributed to similar inhibition of glycolysis (increased intracellular concentration of GLC, PYR and LA) and utilization (decreased concentration) of PEP, G-6-P and some metabolites of TCA and urea cycle, without significant effect on Pro level, as compared to control MCF-7^{WT} cells. The functional significance of the phenomenon is activation of apoptosis. However, in the presence of Gln, MET treatment of MCF-7 cells as well as MCF-7 PRODH/POX-knocked out cells contributed to utilization of some studied metabolites, (except GLC) and creation of pro survival phenotype of MCF-7 cells cultured in these conditions. It suggests that glycolysis is linked to glutamine and proline metabolism. In fact, glycolysis is quiescent not only in MET treated MCF-7 cells but also in non-treated PRODH/POX-knocked out MCF-7

cells. It seems that in both cases there is metabolic glucose starvation and the cells favor Gln as the source of alternative metabolic energy over glucose. The glucose-independence in these conditions suggests uncoupled glycolysis and TCA cycle that might be the sign of MET-dependent rewiring of energetic metabolism. The possible mechanism of this process could involve MET-dependent inhibition of pyruvate kinase attenuating glucose utilization and subsequently TCA metabolism and P5C synthesis with further consequences on proline cycle and PPP (Figure 9). The link could be also at the level of LDH converting PYR to LA and coupled to redox state by regeneration of NAD for stimulation of glycolysis and simultaneously preventing GLC processing in TCA cycle.

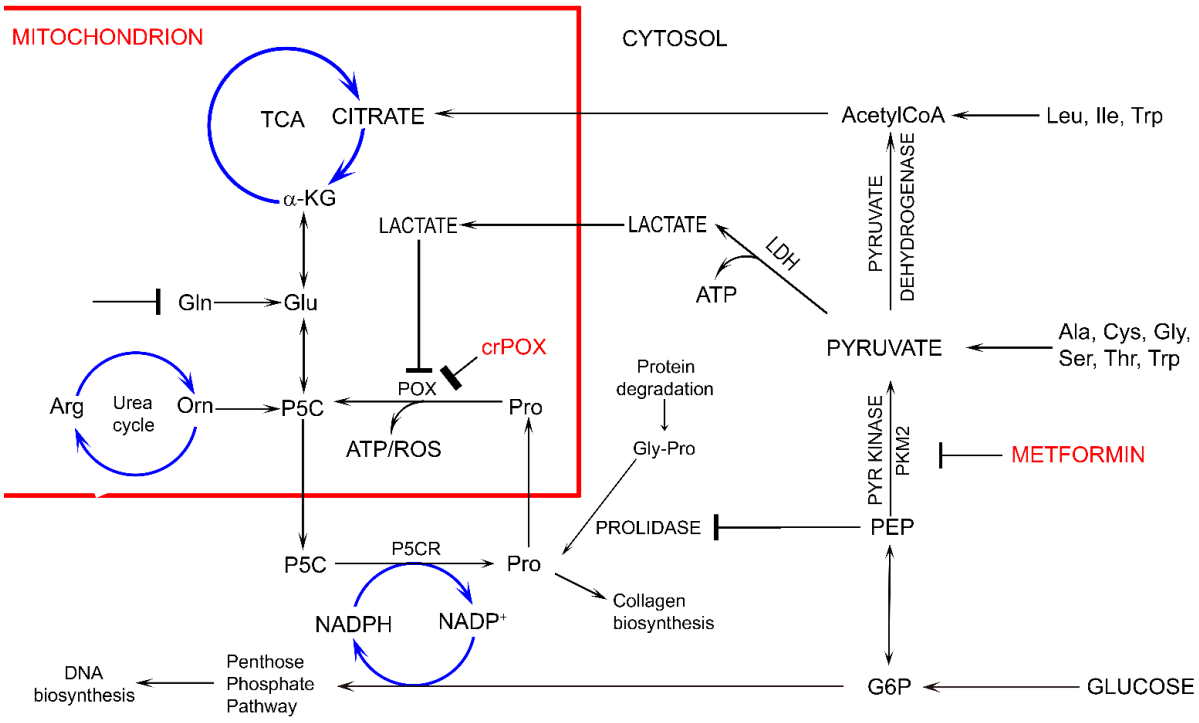

Figure 9. The potential effect of MET on complex regulatory mechanisms of PRODH/POX-dependent apoptosis/survival linking glycolysis, TCA, urea cycles, pentose phosphate pathway, proline cycle (synthesis and degradation), collagen biosynthesis and degradation and prolidase. α-KG—α-ketoglutarate, AcetyloCoA – acetyl coenzyme A, Ala – alanine, Arg—arginine, ATP—adenosine triphosphate, crPOX—CRISPR for POX, Cys – cysteine, Gln-glutamine, Glu- glutamic acid, G6P – glucose-6-phosphate, Gly – glycine, Gly-Pro—glycyl-proline, Ile – isoleucine, LDH—lactate dehydrogenase, Leu – leucine, $NADP^+$ – nicotinamide adenine dinucleotide phosphate, NADPH – reduced form of $NADP^+$, PEP—phosphoenolpyruvate, PYR kinase – pyruvate kinase, Orn—ornithine, PKM2 – pyruvate kinase M2, Pro—proline, POX—proline dehydrogenase/oxidase, P5CR—1-pyrroline-5-carboxylate

reductase, P5C—1-pyrroline-5-carboxylate, ROS—reactive oxygen species, Ser – serine, TCA—tricarboxylic acid cycle, Thr – threonine, Trp - tryptophan.

Cancer cells are characterized by enhanced consumption of glucose-yielding lactate during aerobic glycolysis. The phenomenon known as a Warburg effect ensures rapid production of ATP from glucose to support cancer cell proliferation [153, 154]. Though the process of ATP production from glucose by Warburg effect is less efficient than during mitochondrial oxidative phosphorylation, the conversion of pyruvate into lactate ensures high NAD^+/NADH ratio that accelerate glycolysis. For a long time, Warburg effect has been considered as an effect of impairment of oxidative phosphorylation, but in recent decades it has been documented that the mechanism underlying cancer metabolic reprogramming is much more complex [155]. It is well established that Warburg effect contributes to depletion of TCA cycle and augmentation of glutaminolysis, feeding in this way TCA by glutamine metabolites, as, e.g., α-ketoglutarate [156]. This process is significantly impacted by non-essential amino acids as proline, ornithine and glutamate. They are interconvertible with intermediate of P5C, linking TCA and urea cycles with glutamine metabolism. Particularly, proline could serve as an alternative source of energy. Large quantity of proline comes from protein degradation, mostly from the most abundant extracellular protein, collagen. Deregulation of energetic metabolism in cancer cells due to Warburg's effect facilitates protein degradation as an alternative source of energy.

It has been well established that proline concentration is increased in cancer cells [157, 158]. Increase in the concentration of the amino acid was found in hypoxic cells [159] and glucose starved cells [67] accompanied by increase in the activity of metalloproteinases, MMP-2 and -9, suggesting the mechanism for the increase in intracellular proline concentration. During glucose starvation, cancer cells may select proline as an alternative source of energy, since proline is easily available and do not need to be delivered by circulation. Therefore, proline serves as an energy sensor and energetic substrate. Under glucose starvation, proline interconvertible amino acids: glutamate, α-ketoglutarate and ornithine may serve as alternative sources of energy, providing substrates for production of P5C that links TCA, urea cycles and glutamine metabolism. P5C as a product of proline conversion by PRODH/POX is of special interest. P5C and proline circulate between mitochondria and cytoplasm. Conversion of P5C into proline is catalyzed by P5CR. It is coupled to pentose phosphate pathway for synthesis of

nucleotides. The data presented in this paper suggest tight correlation between glycolysis, proline metabolism by PRODH/POX and PPP. PRODH/POX-knock out of MCF-7 cells or treatment of the cells with MET inhibited glycolysis (increase in intracellular GLC concentration), and attenuated PPP and TCA pathways (decrease in the concentration of metabolites) when cultured in Gln free medium. In the presence of Gln, the cells similarly inhibited GLC utilization however, differentially affected LA utilization. PRODH/POX-knocked out MCF-7 cells utilized LA, while treated with MET inhibited LA utilization in these conditions. It suggests that inhibition of glycolysis in PRODH/POX-knocked out MCF-7 cells and MET treated cells is affected by Gln. Moreover, PRODH/POX-knock out MCF-7 cells that has been treated with MET in Gln free medium inhibited utilization of GLC and LA, while in the presence of Gln induced utilization of LA. It suggests synergistic effects of PRODH/POX-knock out and MET treatment on inhibition of glycolysis and the role of Gln in stimulation of LA utilization in these cells. Therefore, the similar effects of metformin treatment and knockout of PRODH/POX on breast cancer cellular metabolism could be explained at the level of multidirectional regulatory mechanisms including glycolysis, TCA cycle, urea cycle, proline cycle and amino acid metabolism, as shown in Figure 9. It seems that the key metabolite is P5C. Since metformin inhibits pyruvate kinase, it inhibits glucose utilization and subsequently down-regulate TCA cycle and P5C synthesis with further consequences on proline cycle and PPP. The similar effect could be achieved when PRODH/POX is knocked out. The functional significance of the process (activation of apoptosis) was found in MCF-7 cells cultured in glutamine free medium. However, when the cells were cultured in the presence of glutamine (provider of P5C) apoptosis did not occur. The potential mechanism of this processes is outlined in Figure 10.

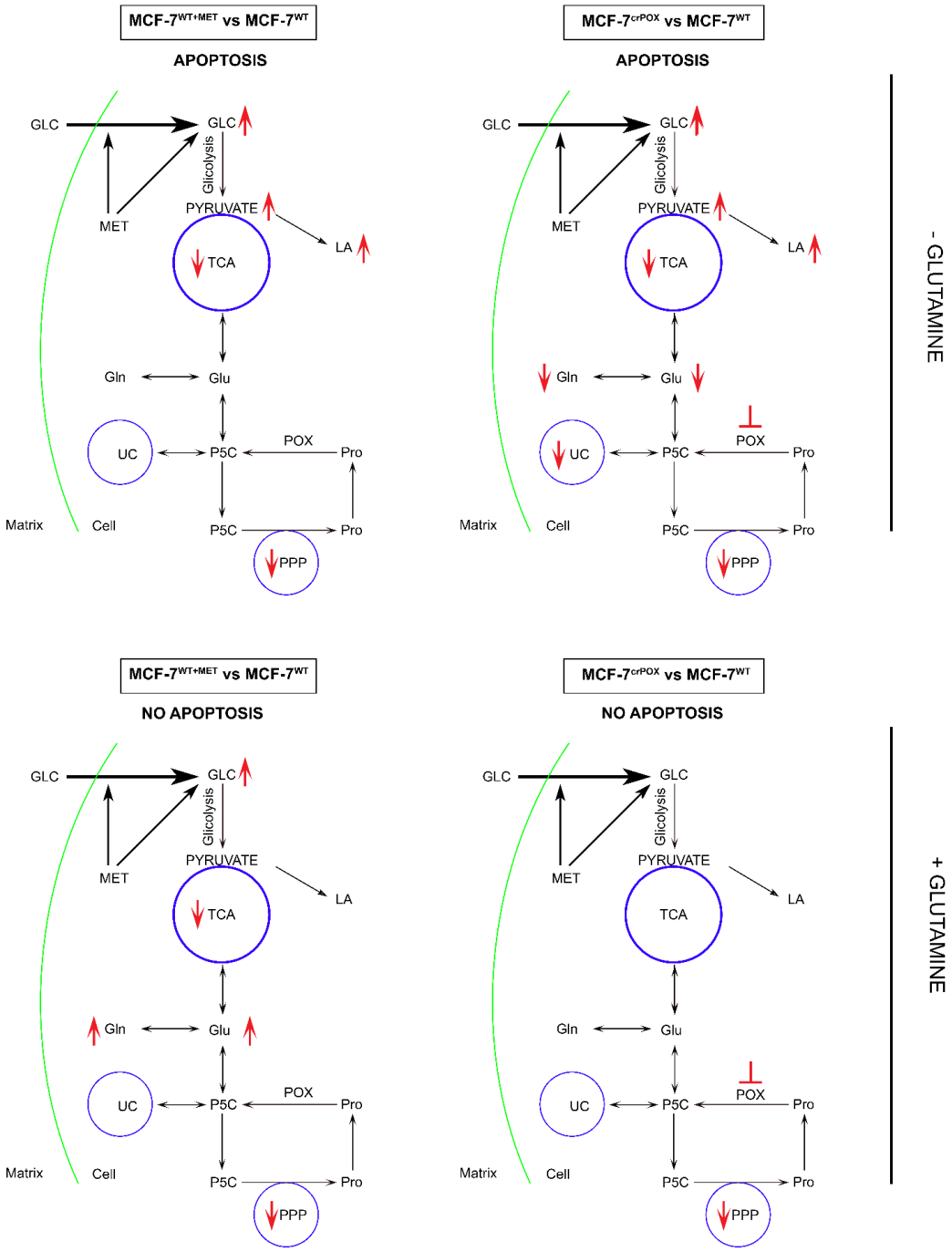

Figure 10. The functional significance of metformin (MET) and PRODH/POX knock-down on complex regulatory mechanisms driving PRODH/POX-dependent apoptosis/survival in wild-type MCF-7 cells (MCF-7^{WT}) and PRODH/POX-knock out MCF-7 cells (MCF-7^{crPOX}), cultured in the presence or absence of Gln. crPOX—CRISPR for POX, GLC—glucose, Gln-glutamine, Glu- glutamic acid, LA—lactate dehydrogenase, Pro—proline, POX—proline dehydrogenase/oxidase, PPP—pentose phosphate pathway, P5C—1-pyrroline-5-carboxylate, TCA—tricarboxylic acid cycle, UC—urea cycle.

Recently we have found that silencing of PRODH/POX induced autophagy while overexpression of prolidase and inhibition of collagen biosynthesis contributed to increase in intracellular proline concentration and PRODH/POX-dependent autophagic cell death in MCF-7 cells [160]. It has been suggested that up-regulation of PRODH/POX by PPAR-gamma ligands could induce apoptosis in cancer cells [161]. Since LA generated in cancer cells due to Warburg effect inhibits PRODH/POX [162], limiting its function (apoptosis/autophagy), it seems that inhibition of Warburg effect (lactate production, e.g., by metformin) contributed to up-regulation of PRODH/POX -induced apoptosis in cancer cells. In fact, inhibiting LA generation in cancer cells by MET attenuated cancer cell growth and survival [163-165]. The data are also supported by studies showing that PRODH/POX is induced by AMPK-dependent pathways [17] and phosphorylated-AMPK was upregulated following glycolysis inhibition by 3-bromopyruvate (3-BP) treatment [166].

We suggest that MET inhibits glycolysis and TCA cycle leading to glucose starvation, ATP depletion, facilitating apoptosis. Similar mechanism was presented for 3-bromopyruvate, inhibitor of pyruvate dehydrogenase [167]. Of great importance is its potential to affect PPP that produce reducing potential and nucleotides for DNA synthesis [168]. Since PPP is directly coupled to glycolysis, any changes in glycolytic pathway may affect NADPH production and DNA biosynthesis. The hypothesis is outlined in Figure 9.

CONCLUSIONS

1. The stable PRODH/POX knockout MCF-7 breast cancer cells were generated using CRISPR-cas9 technology.
2. MET-treatment of both wild type and PRODH/POX knock out cells decreased cell proliferation when incubated in a medium with or without glutamine. However, the inhibition was more pronounced in the absence of glutamine.
3. The percentage of cells in the G2/M phase to G0/G1 phase showed that both MET treatment and PRODH/POX knockout strongly suppressed proliferation of MCF-7 cells cultured in a glutamine-free medium. In the presence of glutamine the effect was not shown.
4. MET induced expression of AMPK (PRODH/POX inducer) in both cell lines regardless of the presence or absence of glutamine. The effect was more pronounced in the cells cultured in glutamine-free medium. In the absence of glutamine, MET induced expression of cleaved PARP and caspase 7 in both cell lines. In the presence of glutamine, the effect was shown only in wild type MCF-7 cells.
5. Metformin treatment of MCF-7 breast cancer cells or PRODH/POX-knock out of the cells induced apoptosis by reprogramming amino acid metabolism, TCA, Urea cycle, and pentose phosphate pathway in the cells. **Metabolomic analyses in the cells cultured with or without glutamine suggest that glycolysis is tightly linked to glutamine and proline metabolism. In the absence of glutamine, MET-treatment or PRODH/POX-knock out contributed to glucose starvation and apoptosis in MCF-7 cells.**
6. **The results of these studies provide insight into mechanism of anticancer activity of MET and suggest that combined treatment of MET with inhibitors of glutamine synthesis may be a new approach to further studies on experimental breast cancer therapy.**

PUBLICATION 1.

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Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pox)-dependent apoptosis/autophagy as an approach to targeted cancer therapy

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Abstract
In stress conditions, as neoplastic transformation, amino acids serve not only as nutrients to maintain the cell survival but also as mediators of several regulatory pathways which are involved in apoptosis and autophagy. Especially, under glucose deprivation, in order to maintain the cell survival, proline and glutamine together with other glutamine-derived products such as glutamate, alpha-ketoglutarate, and ornithine serve as alternative sources of energy. They are substrates for production of pyrroline-5-carboxylate which is the product of conversion of proline by proline dehydrogenase/ proline oxidase (PRODH/POX) to produce ATP for protective autophagy or reactive oxygen species for apoptosis. Interconversion of proline, ornithine, and glutamate may therefore regulate PRODH/POX-dependent apoptosis/autophagy. The key amino acid is proline, circulating between mitochondria and cytoplasm in the proline cycle. This shuttle is known as proline cycle. It is coupled to pentose phosphate pathway producing nucleotides for DNA biosynthesis. PRODH/POX is also linked to p53 and AMP-activated protein kinase (AMPK)-dependent pathways. Proline availability for PRODH/POX-dependent apoptosis/autophagy is regulated at the level of collagen biosynthesis (proline utilizing process) and prolidase activity (proline supporting process). In this review, we suggest that amino acid metabolism linking TCA and Urea cycles affect PRODH/POX-dependent apoptosis/autophagy and the knowledge might be useful to targeted cancer therapy.

Keywords Apoptosis · Autophagy · Proline dehydrogenase/proline oxidase · Proline · Glutamine

Introduction

In stress conditions, cellular homeostasis is maintained by alteration of anabolic and catabolic processes. Anabolic processes are regulated by several factors affecting biosynthesis of cellular components. Major catabolic processes are mediated by the ubiquitin–proteasome system and autophagy [1]. In some cases, autophagy and apoptosis simultaneously occur in the same cell or autophagy precedes apoptosis via p53-dependent pathways or AMP-activated protein kinase (AMPK) [1]. Alternatively, autophagy can directly activate cell death pathway [1, 2]. Both p53 and AMPK are potent stimulators of proline dehydrogenase/proline oxidase (PRODH/POX) that has been implicated in the induction of autophagy and apoptosis [3–10]. Since PRODH/POX is linked to conversion of proline to pyrroline-5-carboxylate (P5C) [11], the availability of proline to this process is of critical importance. Proline and P5C are intermediates of interconversion of glutamine, glutamate, ornithine, and α-ketoglutarate suggesting the key role of these amino acids in the regulation of PRODH/POX-dependent apoptosis/autophagy. Therefore, this review aims to discuss the contribution of proline, glutamine, and its metabolites in regulation of PRODH/POX-dependent apoptosis/autophagy.

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Regulatory mechanism of autophagy and apoptosis

Autophagy

Autophagy is a homeostatic, intracellular degradation process in which dispensable, long-lived, or aberrant proteins and damaged organelles are digested in lysosomes. The

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digestion products are recycled in cellular metabolism. It usually happens under stress conditions such as amino acid starvation [12–14]. Besides the removal of useless components retained in the cell, the other function of autophagy is to generate energy for synthesis of new building blocks in the process of homeostasis and cellular renovation [12, 13]. It suggests that autophagy has a profound impact on cancer cell survival [15]. Autophagy may also contribute to the suppression of cancer cell growth. The activation of autophagy explains a resistance mechanism in the course of cancer therapy. Therefore, the inhibition of autophagy was suggested as a potential pharmacotherapeutic approach for tumor growth suppression [13, 16].

A variety of proteins have been considered as autophagy markers for the assessment of presence or absence of autophagy in the cell. The first autophagy markers were found in yeast and identified more than 30 autophagy-related (ATG) genes, many of which have known orthologs in higher eukaryotes [17, 18]. Atg proteins have been classified into different groups based on their function in autophagy: (1) the Atg1/ULK complex (Atg1, Atg11, Atg13, Atg17, Atg29, and Atg31) regulates the induction of autophagosome formation; (2) the Atg9 complex (Atg2, Atg9, and Atg18), involved in membrane delivery to the expanding phagophore; (3) the PtdIns 3-kinase (PtdIns3K) complex (Vps34, Vps15, Vps30/Atg6, and Atg14) functions to recruit PtdIns3P-binding proteins; (4) two ubiquitin-like (Ubl) conjugation systems including the Atg12 complex (Atg5, Atg7, Atg10, Atg12, and Atg16) and a Atg8 complex (Atg3, Atg4, Atg7, and Atg8) that plays crucial role in vesicle expansion [19, 20] (Table 1). The mammalian ULK1/2 complex comprises ULK1/2 (mammalian homologs of Atg1), ATG13 (a homolog of yeast Atg13), RB1CC1/FIP200 (a putative Atg17 homolog), and C12orf44/ATG101 [21, 22]. The other study provided evidence that ULK1 kinase can be activated by AMP-activated protein kinase (AMPK) under glucose or amino acid starvation [23]. The ULK1/2 complex is inhibited by the phosphorylation of mTORC1 preventing interaction between ULK1 and AMPK. However, during induction of autophagy, the suppression of mTOR occurs and the protein complex of ULK1/2, ATG13, and RB1CC1 is formed to initiate the autophagy. Moreover, the autophagy process is mediated by Beclin-1 (autophagy-related gene, Atg 6) which codes for another autophagy protein [24, 25]. Some of these markers were linked to the PRODH/POX-dependent apoptosis/autophagy [3–10, 26, 27]. Since it has been proved that there is a cross-talk between autophagy and apoptosis [28], it cannot be excluded that the mechanism of this process may involve PRODH/POX.

Apoptosis

A concept of apoptosis was initially reported by Karl Vogt in 1872 then described by Walther Flemming who was the first to explain the mechanism of programmed cell death in 1885. Several studies suggested this mechanism as a program of cellular suicide where the cell destroys itself to maintain tissue homeostasis [29]. The machinery of apoptosis is mediated by a family of proteases, namely caspases which contain a cysteine at their active site and cleave the target proteins at a residue of aspartic acids [30]. Their precursors are called procaspases which are expressed as inactive forms in normal condition. These proteins, however, are cleaved to become active caspases triggering the apoptosis via energy-dependent cascade pathways [30]. The apoptosis is recruited through 3 different pathways: the extrinsic pathway, the intrinsic pathway, and Granzyme B-dependent pathway [31]. Among these pathways, the intrinsic and extrinsic pathways are the major mechanisms of apoptosis.

The intrinsic apoptosis pathway is activated by damages taking place within the cell. This mechanism involves the presence of pro-apoptotic proteins, BAX, and BID in the outer membrane of the mitochondria. They interact with the other protein, BAK to activate cytochrome c that binds to apoptotic protease activating factor-1 (Apaf-1) [32]. This binding activates active caspase 9 that triggers cascade downstream of effector caspases (such as caspase 3, caspase 7, and caspase 6), finally resulting in cell death [33]. The p53 protein is a key factor to activate the intrinsic pathway due to its contribution to activate BAX protein [34].

In contrast, the extrinsic pathway is initiated from extracellular events, triggered by ligand binding to plasma membrane death receptors, leading to activation of initiator caspase 8 [31]. Death receptors such as Fas/CD95 and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors DR-4 and DR-5 are transmembrane proteins that function to detect specific extracellular death signals [35, 36]. For instance, Adapter molecules like Fas Associated via Death Domain (FADD) contain death domain (DD) and a death effector domain (DED) which activate an active caspase-8 via a sequential action of a homotypic DED–DED interaction. Active caspase-8 generates a downstream of effector caspases contributing to cell death. However, they have the same execution pathway which is initiated by the activation of caspase-3 [31]. Typical biomarkers of apoptosis are listed in Table 2. Most of them were linked to PRODH/POX-dependent apoptosis [3–10].

Table 1 Classification of biomarkers of autophagy

ATG complex	Yeast	Mammals	Functions	References
Atg/ULK complex (regulates the class III phosphatidylinositol (PtdIns) 3-kinase complex)	Atg1	ULK1/2	Ser/Thr protein kinase; phosphorylated by M/TORC1; recruitment of Atg proteins to the PAS	[22]
	Atg13	ATG13	Regulatory subunit through phosphorylation by M/TORC1 and/or PKA, linker between Atg1 and Atg17	
	Atg17	RB1CC1/FIP200 (functional homolog)	Scaffold protein, ternary complex with Atg29 and Atg31. Phosphorylation by ULK1; scaffold for ULK1/2 and ATG13	
	C12orf44/Atg101		Component of the complex with ATG13 and RB1CC1	
Atg2-Atg18/Atg9 complex (maintenance of mitochondrial integrity)	Atg2	ATG2	Regulates Atg9 recycling from phagophore assembly site	[79]
	Atg18	WIPI1/2		
	Atg9	ATG9A/B	Required for autophagosome formation; Required for the efficient recruitment of Atg8 and Atg18	
	Atg23		Interaction with Atg9 Required for the biosynthetic cytoplasm to vacuole targeting (Cvt) pathway and efficient autophagy	[80]
PtdIns3K complex (Beclin1-Atg14-Ambra1- Vps15-Vps34)	Vps34	PIK3C3/VPS34	PtdIns 3-kinase	[18]
	Vps15	PIK3R4/VPS15	Ser/Thr protein kinase	
	Vps30/Atg6	BECN 1/Beclin 1	Component of PtdIns3K complex I and II Forms a complex with ER-associated Bcl-2 under nutrient-rich conditions and is released upon phosphorylation of Bcl-2 by JNK1	
		AMBRA1	Interacts with Beclin 1	
	Atg14	ATG14	Component of PtdIns3K complex I	
Atg8 complex (Ubiquitin-like conjugation system)	Atg8	LC3A/B/C, GABARAP, GABARAPL1/2	A unique ubiquitin-like conjugation to phosphatidylethanolamine on the autophagic membrane	[18, 81]
	Atg7	ATG7	E1-like enzyme	
	Atg3	ATG3	E2-like enzyme	
	Atg4	ATG4A-D	Cysteine proteinase LC3/Atg8 C-terminal hydrolase; deconjugating enzyme	
Atg12-Atg5-Atg16 Complex (Ubiquitin-like conjugation system)	Atg12	ATG12	Ubiquitin-like	[18]
	Atg7	ATG7	E1-like enzyme	
	Atg10	ATG10	E2-like enzyme	
	Atg16	ATG16L1	Activate Atg5; Interacts Atg12	
	Atg5	ATG5	Conjugated by Atg12 Directly binds membranes	

PRODH/POX-dependent pathways relevant to apoptosis and autophagy

A variety of approaches to the inhibition of autophagy or activation of apoptosis have recently focused on proline dehydrogenase (PRODH), known also as proline oxidase (POX). PRODH/POX, a mitochondrial enzyme, converts proline to pyrroline-5-carboxylate (P5C) with the concomitant transfer of electrons to cytochrome c producing ATP or directly on oxygen generating reactive oxygen species (ROS) [5]. There are two human genes annotated as PRODH: PRODH1 (chromosome 22q11.21; NCBI Accession

Table 2 Typical biomarkers of apoptosis

Biomarker	Testing sample	Function	Method of detection	References
Activated caspase 2, 3, 7, 8 and 9	Tissue	Primary modulators of apoptosis	IHC, ELISA, flow cytometry, cytometric bead arrays	[82]
Caspase-3	Myocardial injury and cardiovascular disease	Responsible for chromatin condensation and DNA fragmentation	IHC, ELISA, flow cytometry, cytometric bead arrays	[82, 83]
Caspase 3/7	Hypothalamic cell model	Primary modulators of apoptosis	Multiplexing fluorescent and luminescent assays	[84]
Caspase 6	Neurodegenerative disorders (Alzheimer's and Huntington disease)	Primary modulators of apoptosis	Electrochemiluminescence-based ELISA assay	[85]
Cytochrome C	Tissue, serum HL-60 cells and thymocytes	Transfer electrons from the cytochrome bc1 complex to cytochrome oxidase membrane	ELISA, flow cytometry	[82, 86]
CK18	Hepatocellular Carcinoma Treated with Sorafenib		M30- and M65-based sandwich ELISAs	[87]
Cytokeratins	Tissue, serum plasma		IHC, ELISA, flow cytometry,	[82]
Nucleosomal DNA	Tissue, serum		ELISA, DNA array, PCR	[82]
Apo-I/Fas, Fas ligand (sFAsL) Expressed on B and T cells as well as in normal and tumor tissue	Granulomatous disease	Increase the antigen-specific CD8(+) T-cell responses during viral infection	IHC, ELISA, flow cytometry	[82, 88, 89]
Bcl-2/Bcl-xl/Mcl-I	Cells, tissue		IHC, ELISA, flow cytometry	[82]
TRAIL	Inducing the autoimmune inflammation	Induces apoptosis through an extrinsic pathway,		[90]
Tumor protein p53 (TP53)	Colorectal cancer and other cancers	TP53 activation is capable of inducing apoptosis by intrinsic pathway	IHC, ELISA, flow cytometry	[82, 91]

ELISA enzyme-linked immunosorbent assay; *IHC* immunohistochemistry; *PCR* polymerase chain reaction

NM_016335) and PRODH2 (chromosome 19q13.12; NCBI Accession NM_021232). It has been suggested that the function of the enzyme may depend on substrate availability, proline. The main source of this amino acid is collagen which comprises 25% of total protein mass in animals [10, 30].

Briefly, these proteins are classified into major types which are type I in the skin, tendon, and bone, type II in cartilage, and type IV in basal laminae. Up to date, 28 types of collagen with 46 distinct polypeptide chains were found in vertebrates, as well as many other proteins containing collagenous domains [37, 38]. The predominant amino acids in collagen are proline and glycine, which enable triple-helical collagen structure. Extracellular degradation of collagens by tissue collagenases and further intracellular degradation of collagen degradation products in lysosomes release imidopeptides that are cleaved by cytoplasmic prolidase releasing a large amount of proline, the substrate for PRODH/POX.

After the conversion of proline to P5C, further proline metabolism is catalyzed by pyrroline-5-carboxylate dehydrogenase (P5CDH), transforming P5C into glutamate which is a precursor of α-ketoglutarate (α-KG) involved in the tricarboxylic acid (TCA) cycle. When the TCA cycle is overloaded by metabolites, the reversible reaction of conversion of P5C into proline by pyrroline-5-carboxylate reductase (P5CR) may occur, using NADPH or NADH as a cofactor. This interconversion of P5C-proline called proline cycle was first introduced in 1986 [39]. It has been demonstrated that the cellular proline, glutamine, and glutamate are linked to the proline pathway [40] regulating apoptosis/autophagy. The cycle is coupled to pentose phosphate shunt through NADPH from pentose pathway and NADP + from the proline cycle [4, 41]. Base on this mechanism, the role of PRODH/POX in the regulation of cellular metabolism has recently studied as an approach to cancer treatment. This cycle is responsible for the regulation of gene expression, purine biosynthesis, cellular redox state, apoptosis, and cell proliferation [3]. Moreover, PRODH/POX has a variety of regulatory functions, such as osmotic adjustment, protection against metabolic stress, and signaling in bacteria, plants, and mammals [10]. However, the most important function of PRODH/POX is donating electrons through flavin adenine dinucleotide (FAD) into the electron transport chain to generate ROS or ATP depending on environmental conditions [10].

PRODH/POX-induced apoptosis

Both intrinsic and extrinsic pathways of apoptosis may be induced by PRODH/POX [42]. Especially, in the extrinsic pathway (death receptor), PRODH/POX stimulates the expression of tumor necrosis factor-related apoptosis-activated ligand (TRAIL), DR5, and cleavage of caspase-8 [42, 43], and also activates caspase-9 and caspase-3 [44, 45]. In cancer cells, PRODH/POX is upregulated by a variety of factors, for example tumor suppressor p53 and inflammatory factor peroxisome proliferator-activated receptor gamma (PPARγ) [7, 10]. However, its level in cancer tissue is much lower than that in normal tissues from the patients [46, 47]. Regarding the overexpression of POX, the ROS generation is integrated with the p53-dependent mechanisms [5, 48], switching the apoptotic cell death in a variety of cancer cell types [5, 48–51]. The supporting evidence showed that the PRODH/POX coding gene induced the expression of p53 [52]. On the other hand, inactivation of proline oxidase reduced p53-induced upregulation of proline oxidase, a release of cytochrome c from mitochondria, and apoptosis in cancer cells [42, 49]. PRODH/POX acting as a driver of apoptosis was clearly evaluated in a model of PRODH/POX knockdown cancer cells [53].

PRODH/POX-induced autophagy

The recent study of Zareba et al., (2018) showed that in knocked down PRODH/POX MCF-7 breast cancer cells, cytoplasmic proline accumulation induced autophagy. However it was established that environmental conditions such as hypoxia or glucose deficiency may affect PRODH/POX-dependent autophagy/apoptosis [9]. It seems that proline availability may determine PRODH/POX-dependent apoptosis/autophagy. Although the mechanism of this process is not known, it has been suggested that hypoxia-inducible factor-1 alpha (HIF-1α) plays an important role in cancer cell metabolism. The availability of proline in the cell facilitates generation of α-KG that inhibits the transcriptional activity of HIF-1α. An increase in αKG concentration leads to an increase in the activity of a prolyl hydroxylase domain (PHD) of HIF-1α inducing proteasomal degradation of HIF-1α [43, 45, 54]. In contrast, proline through the same mechanism inhibits the activity of PHD, contributing to a decrease in HIF-1α proteasomal degradation and increase in its transcriptional activity.

It is well established that glutamine and proline metabolism, as well as other non-essential amino acids, are involved in oncometabolism of cells [9]. This process is called as "parametabolic pathway". Particularly, the proline biosynthetic pathway was linked to glucose metabolism and POX-dependent apoptosis that is under the regulation of oncogene MYC.

Depending on the metabolic situation, proline can either be used for protein synthesis or oxidized in the mitochondria for energy production. Under nutrient deficiency and hypoxia, cancer cells may adopt to switch a survival mechanism which is the degradation of proline to produce the energy [26]. Therefore, hypoxia, glucose depletion, or treatment with rapamycin stimulated degradation of proline and POX-dependent autophagy.

The impact of amino acids on cell re-programming

Several amino acids have been linked to activation or inhibition of apoptosis/autophagy [55]. It is well recognized that they participate in the mTORC1 and GCN2/eIF2 pathways which function to regulate protein translation and control the cellular demand for amino acids by concomitantly regulating autophagy-dependent catabolism [56–58]. For instance, non-essential amino acids (NEA) as proline in condition of glucose deprivation activate anti-apoptotic pathways in cancer cells by inducing the expression of anti-apoptotic members of the Bcl-2 gene family and preventing the expression of pro-apoptotic proteins [59]. The study suggested that although under low glucose condition apoptosis could be induced in cancer cells, the non-essential amino acids may counteract the process. It was supported by the upregulation of amino acid transporter gene LAT1 in the membranes of cancer cells [27, 60, 61] under glucose stress [59].

Glutamine was proved to be a sustainable source of energy. Early findings indicated that tumor formation is significantly due to the mitochondrial vulnerability through the alteration of glycolysis [62]. The proliferation of cancer cells is mostly maintained by energy products derived from the TCA cycle [63, 64]. A larger majority of tumor suppressors and oncogenes have been linked to metabolic pathways [64–67]. Glutamine is an integral metabolite in the proliferation of mammalian cells. The consumption rate of glutamine in cancer cells is compared to that of other amino acids. However, the demand for glutamine was observed to be tenfold higher than that for other amino acids [68]. Glutamine has profound impact on the functional activity of mammalian target of rapamycin (mTOR) kinase, mitochondrial membrane potential, and NADPH production [69]. Glutamine is a nitrogen source both for purine and pyrimidine synthesis [70, 71]. In the non-essential amino acid synthetic pathways, glutamine-derived glutamic acid continues donating its amine group to accelerate the tricarboxylic acid (TCA) cycle metabolites for the production of

α-ketoglutarate, serine, alanine, aspartate, and ornithine. Glutamine acts as a source of carbon and nitrogen for the synthesis of proline, ornithine, and arginine as well as a donor for the synthesis of asparagine from aspartic acid [69]. Lack of exogenous glutamine is one of the major causes for the death of cancer cells [72]. Several tumor cell lines, generated from pancreatic cancer, glioblastoma multiforme, acute myelogenous leukemia, and small cell lung cancer, are substantially vulnerable due to glutamine starvation [73]. The study suggested that derivatives of glutamine like glutamate, α-ketoglutarate, and glutathione are involved in the apoptotic pathway [74]. Similarly, proline interconvertibility with glutamate and arginine [3, 75] may play an important role in cell programming. However, recent data linked glutamine metabolism and apoptosis/autophagy through P5C to urea cycle.

Ornithine and glutamate are important sources of P5C. Ornithine is converted into P5C in a reaction catalyzed by mitochondrial vitamin B6-dependent ornithine-δ-aminotransferase (OAT), while glutamate through a reduction reaction catalyzed by mitochondrial ATP- and NAD(P)H-dependent P5C synthase (P5CS) [76, 77]. This reaction can be reversed by mitochondrial P5C dehydrogenase (P5CDH) [76]. The role of this metabolic pathway in apoptosis/ autophagy was supported by data showing that degradation of ornithine by ornithine decarboxylase (ODC) play an important role in cell proliferation, differentiation, and cell death. It has been demonstrated that decreasing the activity of ODC by difluoromethylornithine (DFMO) causes accumulation of intracellular reactive oxygen species (ROS) and cell arrest, thus inducing cell death. These findings indicate that urea cycle contributes to the regulation of apoptosis and autophagy [78]. Since ornithine is easily convertible into P5C (products of catalytic activity of PRODH/POX), it may affect PRODH/POX-dependent apoptosis/autophagy. The results of these studies allow us to present a hypothesis on the regulation of PRODH/POX-dependent apoptosis/autophagy by key amino acids (Fig. 1). During conversion of PRO into P5C by PRODH/POX, ATP or ROS is generated inducing autophagy or apoptosis. PRO availability for this process is critical requirement for PRODH/POX-dependent

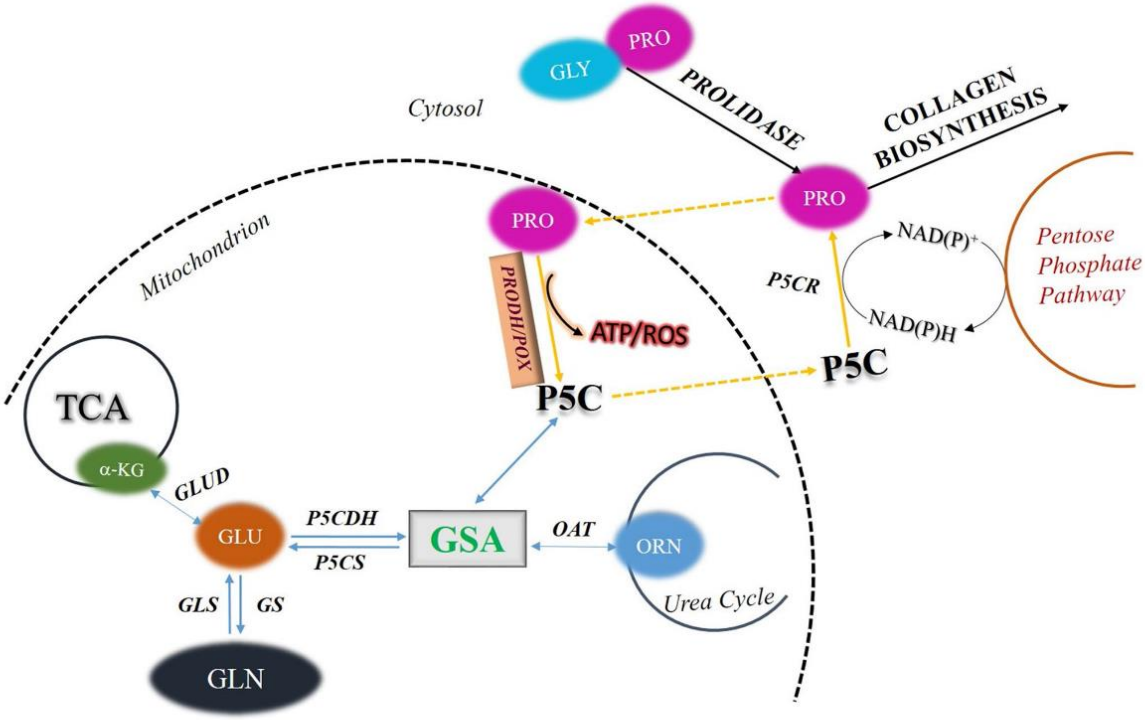

Fig. 1 Regulation of PRODH/POX-dependent apoptosis/autophagy by key amino acids. *PRO* proline; *GLU* glutamate; *ORN* ornithine; *GLN* glutamine; *GLYPRO* glycyl-proline; *PRODH/POX* proline dehydrogenase (PRODH)/proline oxidase (POX); *ROS* reactive oxygen species; *P5C* pyrroline-5-carboxylate; *P5CR* pyrroline-5-carboxylate reductase; *P5CDH* pyrroline-5-carboxylate dehydrogenase; *P5CS* pyrroline-5-carboxylate synthase; *OAT* ornithine aminotransferase; *GSA* glutamic gamma-semialdehyde; *αKG* α-ketoglutarate; *TCA* tricarboxylic acid cycle; *GS* glutamine synthase; *GLS* glutaminase; *GLUD* glutamate dehydrogenase

function. PRO comes from collagen degradation products (last step of the degradation is catalyzed by prolidase) or proline convertible amino acids, mainly GLU and ORN. Conversion of PRO into P5C takes place in mitochondria, while P5C into PRO mainly in cytoplasm. This process is known as a "proline cycle" and is coupled to pentose phosphate pathway generating nucleotides for DNA biosynthesis. Interconversion of PRO, GLU, and ORN through intermediate GSA to P5C may represent an interface regulating PRODH/POX-dependent P5C generation and ATP/ROS for autophagy/apoptosis. The process links TCA and Urea cycles to proline cycle providing complex regulatory mechanism of PRODH/POX-dependent functions. Understanding the interplay between key amino acids and TCA/Urea metabolites and their role in the regulation of PRODH/POX-dependent apoptosis/autophagy might be a promising approach to targeted cancer therapy.

Conclusion

Studies of last decade provided several lines of evidence for the regulatory role of proline availability in PRODH/POX-dependent apoptosis/autophagy in cancer cells. The enzyme expression is often downregulated in various tumors, limiting mitochondrial proline degradation and PRODH/POX-dependent apoptosis. Critical factor for the process is proline availability that depends on the activity of prolidase (enzyme supporting cytoplasmic proline level) and the rate of proline utilization in the process of collagen biosynthesis. However, proline also represents an energy-sensing molecule that reprograms cellular metabolism. Interconversion of proline, glutamate, and ornithine links TCA cycle, urea cycle, and amino acid metabolism to PRODH/POX-dependent apoptosis/autophagy. Deregulation of energetic metabolism in cancer cells due to Warburg's effect facilitates protein degradation as an alternative source of energy. Therefore, when glucose supply is limited, cancer cells may select proline as an alternative energy source. Therefore, amino acid metabolism in specific environmental cellular conditions may represent interface of PRODH/POX-dependent apoptosis and autophagy. The hypothesis is outlined in Fig. 1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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Metformin Treatment or PRODH/POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells

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Abstract: It has been considered that proline dehydrogenase/proline oxidase (PRODH/POX) is involved in antineoplastic activity of metformin (MET). The aim of this study is identification of key metabolites of glycolysis, pentose phosphate pathway (PPP), tricarboxylic acids (TCA), urea cycles (UC) and some amino acids in MET-treated MCF-7 cells and PRODH/POX-knocked out MCF-7 (MCF-7^{crPOX}) cells. MCF-7^{crPOX} cells were generated by using CRISPR-Cas9. Targeted metabolomics was performed by LC-MS/MS/QqQ. Expression of pro-apoptotic proteins was evaluated by Western blot. In the absence of glutamine, MET treatment or PRODH/POX-knock out of MCF-7 cells contributed to similar inhibition of glycolysis (drastic increase in intracellular glucose and pyruvate) and increase in the utilization of phospho-enol-pyruvic acid, glucose-6-phosphate and some metabolites of TCA and UC, contributing to apoptosis. However, in the presence of glutamine, MET treatment or PRODH/POX-knock out of MCF-7 cells contributed to utilization of some studied metabolites (except glucose), facilitating pro-survival phenotype of MCF-7 cells in these conditions. It suggests that MET treatment or PRODH/POX-knock out induce similar metabolic effects (glucose starvation) and glycolysis is tightly linked to glutamine metabolism in MCF-7 breast cancer cells. The data provide insight into mechanism of anticancer activity of MET as an approach to further studies on experimental breast cancer therapy.

Keywords: PRODH/POX; metformin; MCF-7^{crPOX} cells; proline; glutamine; lactic acid

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1. Introduction

Breast cancer is the most frequently diagnosed cancer in woman worldwide and has been a progressively increasing global health problem. The phenotypic characteristics can be attributed to genetic and epigenetic factors, and to nonhereditary mechanisms, such as adaptive responses or fluctuations in the tumor microenvironment signaling pathways [1]. Therefore, optimal methods of treating breast cancer must be developed to effectively cure the malignancy.

Although metformin (MET) is currently used to treat type II diabetes patients, it evokes also antineoplastic potency [2–5]. The molecular mechanism of anti-cancer activity of MET is unknown. One of the effects of MET is activation of adenosine monophosphate (AMP) kinase (AMPK) [6–9]. AMPK is activated when the AMP/ATP ratio rises. This process stimulates oxidative phosphorylation to restore normal adenosine triphosphate (ATP)

levels and inhibit energy expenditure, such as cell proliferation [10,11]. the similar effects of, AMPK is regulated especially in conditions of energy shortage (e.g., glucose shortage) and hypoxia [11]. It inhibits anabolic processes and stimulates catabolism. However, MET was found to attenuate function of mitochondrial complex I resulting in decrease in ATP synthesis [12] and inhibit pyruvate kinase, impairing glucose metabolism [13]. In conditions of energy shortage and under glucose deficiency an alternative source of energy is proline, derived from protein degradation products, mainly collagen. Proline is degraded by proline dehydrogenase/proline oxidase (PRODH/POX). Of great interest is the observation that PRODH/POX is induced by AMPK [14]. Therefore, PRODH/POX could be involved in anti-cancer activity of MET.

The inhibitory role of PRODH/POX in tumor progression is well established. It has been found that PRODH/POX induces apoptosis in several cancer cell types by intrinsic or extrinsic pathway. PRODH/POX-dependent generation of ROS induces mitochondrial apoptosis (intrinsic pathway), while TRAIL (tumor necrosis factor-related apoptosis inducing ligand) and DR5 (death receptor 5) induce extrinsic pathways of apoptosis. Although, the switching mechanism for PRODH/POX-dependent intrinsic/extrinsic apoptosis is unknown, it seems that it is metabolic context dependent [15].

PRODH/POX (PRODH<, GenBank^{TM} NM_016335), also known as proline oxidase, is a flavin-dependent enzyme associated with the inner mitochondrial membrane [16,17]. The enzyme catalyzes conversion of proline into Δ^1-pyrroline-5-carboxylate (P5C). During this process, electrons are transported by the electron transport chain, producing ATP, or they directly reduce oxygen, producing reactive oxygen species (ROS). In the first situation, which usually happens under low glucose stress, AMPK-dependent PRODH/POX activation produces ATP for energy supply and survival [16,18,19]. In the second one, ROS induces apoptotic pathways [20–23]. In the presence of proline, overexpression of PRODH/POX causes cytochrome c release from mitochondria to cytosol and activation of caspase-9 and caspase-3 [21,24]. Therefore, PRODH/POX may play dual role, but the mechanism that switches PRODH/POX from cancer cell growth inhibiting to growth supporting factor is unknown.

PRODH/POX cooperates with P5C reductase (P5CR) participating in proline turnover between mitochondria and cytoplasm. The conversion of proline to P5C that is shuttled between mitochondria and cytosol is coupled to glucose metabolism by pentose phosphate pathway that supports substrates for DNA biosynthesis [14,16–18]. It is also vital in maintenance of redox balance in a cell due to participation of NADPH/NADH in conversion of P5C to proline. Moreover, P5C is converted by P5C dehydrogenase (P5CDH) to glutamate, which is a precursor of α-ketoglutaric acid—a component of tricarboxylic acids cycle (TCA). As a result of PRODH/POX and ornithine aminotransferase (OAT) activity, proline is transformed into ornithine and enters urea cycle (UC) [16]. In view of the inhibitory role of PRODH/POX in tumor progression [19,25,26], the metabolism of proline in neoplastic cells is therefore of great importance.

The conversion of proline into P5C by PRODH/POX is facilitated when P5C is rapidly utilized. In case it cannot be converted in mitochondria into glutamate and α-ketoglutaric acid (that enters TCA cycle), e.g., because of TCA defects, the P5C is converted to proline by P5C reductases (PYCR), mitochondrial PYCR1 or cytoplasmic PYCRL [27]. Cytoplasmic proline could be utilized for collagen biosynthesis [14] or in case of inhibition of collagen biosynthesis enters again mitochondria. Such a cycle of proline/P5C between mitochondria and cytoplasm may be responsible for ROS generation and apoptosis induction [28]. Whether MET is involved in the process requires to be explored. Another possibility is that MET-dependent activation of PRODH/POX and simultaneously inhibition of complex I of respiratory chain contributes to ROS generation instead of ATP production.

We have suggested that MET can stimulate apoptosis in cancer cells by a cascade of processes involving the induction of AMPK, PRODH/POX and ROS generation under proline availability determined by several proline utilization/supporting processes. Proline could be derived from α-ketoglutarate, glutamic acid (Glu), glutamine (Gln) and

ornithine. It links glycolysis, TCA and urea cycles. Therefore, we postulate that complex regulation of glycolysis, TCA, Urea cycles, amino acids metabolism may represent multifunctional interface that switches apoptosis or survival mode in cancer cells depending on the microenvironmental conditions. Therefore, studies on targeted metabolomic profile of MET-treated MCF-7 cells and PRODH/POX-knocked out MCF-7 cells were undertaken. It is tempted to estimate intracellular concentration of some metabolites of glycolysis, TCA, urea and pentose phosphate pathways by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS) with a triple quadrupole (QqQ).

Complex analysis of the effect of MET on the metabolic profile in wild-type MCF-7 (MCF-7^{WT}) breast cancer cells and the cells with knock out PRODH/POX expression (MCF-7^{crPOX}) may contribute to development of knowledge on the mechanism of antineoplastic activity of MET and may help to improve experimental cancer pharmacotherapy.

2. Materials and Methods

2.1. PRODH/POX Knock out CRISPR-cas9 DNA Plasmid Purification

The sgRNAs for PRODH/POX (CRISPR All-In-One Non-Viral Vector with spCas9) were ordered by ABM Company (Richmond, Canada). The vector with expression construct was transformed into Escherichia coli DH5α and grown in Luria–Bertani (LB) media supplemented with 100 $\mu g \cdot mL^{-1}$ ampicillin at room temperature for 24 h. The targeted plasmid was extracted by a plasmid DNA purification kit (Nucleobond Xtra Midi/Maxi, MACHERY-NAREL GmbH, Düren, Germany). After being precipitated by isopropanol, the purified samples were washed by 70% ethanol solution then followed by DNA cleaning-up step by GeneMATRIX Basic DNA Purification Kit (EURX, E3545-01 protocol 1, Gdansk, Poland). The purified DNA concentration was estimated by NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Transfection into MCF-7 Breast Cancer Cell Line

MCF-7 breast cancer cells were cultured in the complete growth medium, DMEM 1X (Gibco) containing 4.5 gL^{-1} glucose, L-glutamine and pyruvate supplemented with 10% Fetal Bovine Serum (FBS) qualified (Gibco), 1% penicillin/streptomycin (Invivogen) at 37 °C in 5% CO_2. The cells were then seeded into 6-well plates to reach 70–90% confluency. The amount of plasmid in the experiment was tested from 1 to 2 μg per well. Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used as a transfection reagent.

Prior to transfection, the plasmid was diluted with 50 μL of medium A, DMEM 1X (Gibco) containing 4.5 gL^{-1} glucose, L-glutamine and pyruvate supplemented with 1% penicillin/streptomycin (Invivogen).

The transfection solution containing 805.4 μL of medium A and 194.6 μL of lipofectamine reagent were gently mixed then incubated at room temperature for 5 min before aliquoting 60 μL of the solution into a vial containing the diluted plasmid solution. The mixture of diluted plasmid and transfection solution was mixed gently then incubated at room temperature for 20 min.

The testing cells were washed by PBS 1X (sterile phosphate buffered saline 1X, Gibco) and freshly added with 1 mL of medium A. After 20-min incubation, the mixture of plasmid and transfection reagent were slowly added to cells then incubated at 37 °C in 5% CO_2 overnight. The following day, the transfected cells were selected in the complete growth medium with 1 $\mu g \cdot mL^{-1}$ of puromycin (Sigma-Aldrich, St. Louis, MI, USA) in the same culture conditions for 10 days. The expression of PRODH/POX in transfected cells was checked by Western blot. Based on the results of expression level between wild-type MCF-7 cells and transfected MCF-7 cells, the PRODH/POX knock out MCF-7 cell line was selected for further stable clone generation. The process of the stable clone generation was manipulated with a serial dilution of the selected cells in the culture media how to obtain 0.7 cell per well in a 96-well plate. The screening steps were done with a random selection of cell clones. The PRODH/POX silencing in cell clones were checked by Western blot using

an anti-PRODH/POX antibody (Santa Cruz, Dallas, TX, USA). The level of PRODH/POX knock down is presented in Supplementary Materials (Figure S1). The PRODH/POX knock out MCF-7 cells defined as MCF-7^{crPOX} cells were banked for further experiments.

2.3. Cell Culture

Wild type MCF-7 (MCF-7^{WT}) cells and PRODH/POX-knocked out cells (MCF-7^{crPOX}) cells were cultured in DMEM 1X (Gibco) containing 4.5 gL^{-1} glucose, L-glutamine and pyruvate supplemented with 10% Fetal Bovine serum (FBS) qualified (Gibco), 1% penicillin/streptomycin (Invivogen) at 37 °C in 5% CO_2. The assay media used in this approach were DMEM 1X containing 4.5 gL^{-1} glucose, L-glutamine and pyruvate supplemented with 1% Penicillin/streptomycin (Gibco); DMEM 1X (Gibco) containing 4.5 gL^{-1} glucose, 0.11 gL^{-1} sodium pyruvate, without L-glutamine supplemented with 1% penicillin/streptomycin (Gibco); DMEM 1X (Gibco) containing 1.0 gL^{-1} glucose, pyruvate, without L-glutamine supplemented with 1% Penicillin/streptomycin (Gibco).

The cells were seeded into Petri dishes to obtain approximately 10 million cells per plate. After that the cells were treated with/without Metformin in 3 different assay media overnight. Fifty testing samples of Metformin untreated/treated wild type MCF-7 cells and MCF-7^{crPOX} cells in different cultured conditions were assigned into 10 different groups were listed in Table S1 (Supplementary Materials). Every group contains 5 replicates of testing samples.

2.4. Metabolite Extraction

After treatment overnight, a sample (approximately 10–20 million cells) was collected in a vial without trypsinizing. The testing cells were washed by PBS 1X (Gibco) before scraping to collect into vial then stored at −80 °C. For extraction, 250 µL of acetonitrile (ACN) (Merck, Darmstadt, Germany) was added into a vial. The cell suspension was sonicated at 60 kHz, for 5 s per time, then place a vial on ice for minute. This step was repeated 4 times. The cell debris was separated by centrifugation (Eppendorf Centrifuge 5415R, Hamburg, Germany) at 16,000× *g* at 4 °C for 15 min. Supernatants (50 µL) was injected to LC-QqQ for targeted approaches.

2.5. Targeted Metabolomics Quantitative Analysis (LC-MS/MS(QqQ))

This study focused on several metabolites involved in Glycolysis, TCA cycles, pentose phosphate pathway, urea cycles and several key amino acids in PRODH/POX-dependent pathways. Testing metabolites are summarized in Table S2 (Supplementary Materials). All stock solution of reference metabolites were prepared in acetonitrile to obtain 1000 ppm ($mg{\cdot}mL^{-1}$). LC-MS/MS analysis was performed using an Agilent 1200 LC coupled to an Agilent 6470 Triple quadrupole (Agilent Technologies, Santa Clara, CA, US) with an InfinityLab Poroshell 120 HILIC-Z column (Agilent Technologies, Santa Clara, CA, US) for (hydrophilic liquid chromatography (HILIC) interaction. The platform was operated in a multiple reaction monitoring (MRM) in negative mode using an electrospray ionization (ESI) source. The optimized transition of amino acid metabolites is listed in Table S3 (Supplementary Materials). The injection volume was 2 µL. Mobile phase A was 10 mM ammonium acetate adjusted to pH = 9 with ammonia, with 2.5 mM InfinityLab deactivator additive (Agilent, P-N. 5191-4506). Mobile phase B was 10 mM ammonium acetate adjusted to pH = 9 in H_2O/ACN (15:85, *v*/*v*) with 2.5 mM of the same deactivator. The flow was constant at 0.250 mL/min. The chromatographic gradient is described in reference [29].

2.6. Data Pre-Treatment

After data acquisition, all chromatograms were inspected in MassHunter Qualitative analysis navigator 8.0 (Figure S7, Supplementary Materials). Accurate peak integration was performed by using Mass Hunter Quantitative analysis (for QqQ) version 8.0 (Figure S8, Supplementary Materials). Stock solutions at different concentrations, ranging from 1 ppb

to 20,000 ppb were prepared and were used to construct calibration curves that covered the range of each metabolite. The quantitation was performed in Excel.

2.7. Cell Lysate Preparation

Cells were cultured in FBS-free DMEM with or without glutamine and MET (20 mM) for 24 h. The procedure for harvesting the cells was performed as previously described [30]. The supernatant was aliquoted and stored at −80 °C. Protein concentration was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Cell Proliferation Assay

The proliferation of MCF-7 and MCF-7^{crPOX} cells was evaluated using CyQUANT® Cell Proliferation Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's procedure. The cells were cultured in glutamine free or glutamine containing DMEM and treated with MET (20 mM) for 24 h. The read was performed on TECAN Infinite® M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) at 480 and 520 nm as excitation and emission wavelengths, respectively. The results were presented as a percent of the control value.

2.9. Cell Cycle Analysis

The cells were trypsinized and centrifuged (5 min, 500× *g*) followed by washing twice with phosphate-buffered saline (PBS). The suspended pellet (500 µL PBS) was fixed in 70% ethanol (4.5 mL) and stored (4 °C) until the day of analysis. After centrifugation (5 min, 500× *g*), ethanol-fixed cells were mixed with Solution 3 (ChemoMetec, Allerod, Denmark), incubated (37 °C, 5 min), and analyzed with an image cytometer (NC-3000, ChemoMetec, Allerod, Denmark).

2.10. Western Immunoblotting

Western blot analysis was carried out as described by Misiura et al. [30]. The membranes were incubated with primary antibodies diluted 1000 times in 5% bovine serum albumin (Sigma Aldrich, Saint Louis, MO, USA) in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6). Anti-PARP, anti-AMPK and anti-caspase-7 and anti-GAPDH, were purchased from Cell Signaling Technology, Danvers, MA, USA; anti-PRODH/POX from St John's Laboratory, London, UK), followed by incubation with alkaline phosphatase-linked goat anti-rabbit or anti-mouse antibodies (dilution: 1:10,000 in 5% non-fat dried milk (Santa Cruz Biotechnology, Dallas, TX, USA) in TBS-T; Sigma Aldrich, Saint Louis, MO, USA). The bands' intensities were semi-quantitatively measured in ImageJ software (https://imagej.nih.gov/ij/, accessed on 27 October 2021). All experiments were run at least in triplicates.

2.11. Statistical Analyses

2.11.1. Targeted Analysis

GRAGHPAD PRISM version 9.0 was used to perform Mann–Whitney tests using the five replicates per group included in this study. Supervised Orthogonal Partial Least Square-Discriminant analysis (OPLS-DA) in SIMCA was applied for multivariate statistics. The volcano plots were built in order to obtain variable importance in the projection (VIP) values and corrected *p*-values (p(corr)). Those metabolites with VIP > 1.00, q ≤ 0.050 and absolute p(corr) ≥ 0.30 were considered as significant. The percentages of change reflecting the difference of each metabolite level between groups were also calculated.

2.11.2. Biological Analysis

All experiments were carried out in duplicates and the experiments were repeated at least three times. Data are shown as a mean ± standard error (SEM). For statistical calculations, one-way analysis of variance (ANOVA) with Dunnett's correction and *t*-test were used. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Soft-

ware, San Diego, CA, USA). Statistically significant differences were marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

3.1. Metformin Inhibits MCF-7 Cell Proliferation and Induces Apoptosis

MCF-7 breast cancer cell line (MCF-7^{WT}) and the corresponding MCF-7 cell line with PRODH/POX-knock out (MCF-7^{crPOX}) were treated with metformin (MET, 20 mM) for 24 h in medium with or without glutamine. MET-treatment of MCF-7^{WT} cells or PRODH/POX-knock out of the cells contributed to decrease in cell proliferation, when incubated in medium with or without glutamine (Figure 1A). MET potentiated inhibition of cell proliferation in both cell lines. However, this process was more pronounced in the absence of glutamine. The data were corroborated by the ratio of dividing cells to non-dividing cells (the percentage of cells in G2/M phase to G0/G1 phase). As presented on Figure 1B, MET-treatment and PRODH/POX knock out strongly inhibited proliferation of MCF-7 cell cultured in glutamine free medium, while in the presence of glutamine there was no effect on the process.

MET induced expression of AMPK in both cell lines (Figure 1C). In the cells cultured in the absence of glutamine this process was more pronounced. The expression of PRODH/POX was also increased in MET-treated MCF-7^{WT} cells cultured in medium with or without glutamine, while in MCF-7^{crPOX} cells, the PRODH/POX was not detected and MET did not affect its expression. MET increased the expression of cleaved PARP and Caspase-7 in both cell lines when cultured in glutamine free (-Gln) medium. Interestingly, PRODH/POX knock out by itself also increased expressions of cleaved PARP and Caspase-7 in MCF-7^{crPOX} cells, compared to MCF-7^{WT} cells, when cultured in glutamine free medium. However, in the presence of glutamine (+Gln), MET did not affect very low expression of the proteins in both studied cell lines (Figure 1 C).

3.2. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in PRODH/POX-Knock out of MCF-7 Cells (MCF-7^{crPOX}) and Wild Type MCF-7 Cells (MCF-7^{WT}) Cultured in Glutamine (Gln) Free Medium

PRODH/POX-knock out of MCF-7 cells (MCF-7^{crPOX}) contributed to drastic increase in intracellular glucose (GLC) and pyruvic acid (PYR) concentrations (about 12- and 17-fold, respectively) and about 2-fold increase in lactic acid (LA) concentration, as compared to MCF-7 wild type cells (MCF-7^{WT}). It was accompanied by total decrease in the concentrations of phospho-enol-pyruvic acid (PEP) and glucose 6-phosphate (G-6-P), 6-Phospho-gluconic acid and significant decrease in the concentrations of all TCA cycle and urea cycle metabolites as well as glutamine (Gln) and glutamic acid (Glu), without effect on proline (Pro) concentration in PRODH/POX-knocked out MCF-7 cells (Table 1).

The data suggest that PRODH/POX-knock out contributes to inhibition of GLC, LA and PYR consumption while PEP and G-6-P as well as some TCA and urea cycles metabolites are utilized in these conditions. Pro is not significantly affected.

3.3. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in Metformin (MET) Treated Wild Type MCF-7 Cells (MCF-7^{WT+MET}) and in MCF-7^{WT} Cells Cultured in Gln Free Medium

Metformin-treatment of MCF-7^{WT} (MCF-7^{WT+MET}) contributed to drastic increase in GLC, PYR, LA compared to control MCF-7^{WT} cells. It was accompanied by decrease in PEP (insignificantly), G-6-P and some TCA metabolites concentrations, compared to MCF-7^{WT}. Of interest is no effect on Pro concentration in MCF-7^{WT+MET} cells, compared to MCF-7^{WT} cells (Table 2).

The data suggest that MET contributes to decrease in GLC, PYR and LA consumption while PEP and G-6-P as well as some TCA metabolites are utilized in these conditions, as compared to MCF-7^{WT} cells. Pro is not significantly affected.

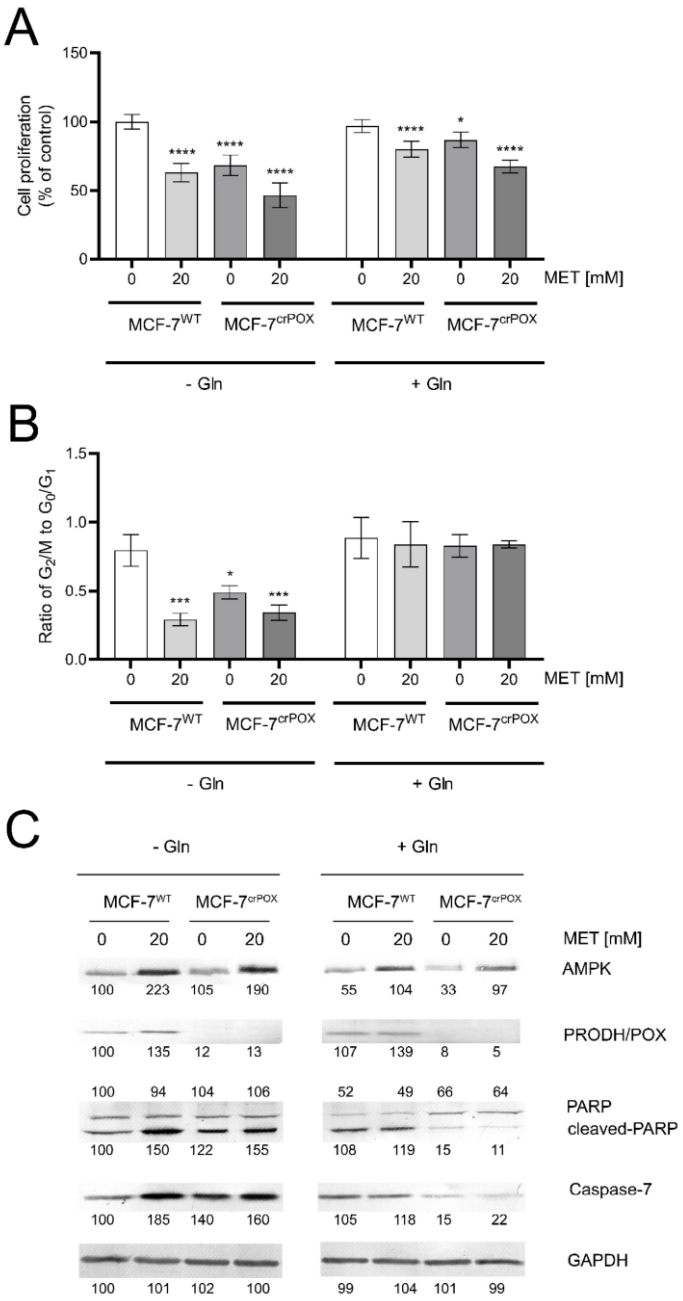

Figure 1. Cell proliferation (**A**) the ratio of cell percentage in G_2/M to G_0/G_1 phase. (**B**) Western blot for AMPK, PRODH/POX, PARP and caspase 7. (**C**) in metformin (MET, 20mM) treated MCF-7^{WT} and PRODH/POX-knock out MCF-7^{crPOX} cells cultured in medium with or without glutamine (Gln) for 24 h. The mean values ± standard error (SEM) from 3 experiments done in duplicates are presented at * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$. Representative Western blot images were shown. Supplementary Materials contain statistical analysis of the evaluated proteins (Figures S3–S6). The percentage of cells in G0/G1, S and G2/M phases of the cell cycle of MCF-7^{WT} and MCF-7^{crPOX} cells treated with metformin with or without Gln (Figure S2).

Table 1. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in PRODH/POX-knock out of MCF-7 cells (MCF-7^{crPOX}) and wild type MCF-7 cells (MCF-7^{WT}) cultured in glutamine (Gln) free medium. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑—significant increase in the concentration of studied compound in MCF-7^{crPOX} cells vs. MCF-7^{WT}, ↓—significant decrease in the concentration of studied compound in MCF-7^{crPOX} cells vs. MCF-7^{WT}.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{crPOX} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{crPOX}
		MCF-7^{WT}	MCF-7^{crPOX}			
Glycolysis	Pyruvic acid	6.5	117.2	1712.0	0.010	↑
	Glucose	11.2	150.8	1252.0	0.010	↑
	Phospho-enolpyruvic acid	6449.0	0.0	−100.0	0.010	↓
PPP	Glucose 6-phosphate	328.6	0.0	−100.0	0.010	↓
	6-Phospho-gluconic acid	269.9	10.9	−96.0	0.010	↓
TCA	Malic acid	1126.1	77.4	−93.1	0.010	↓
	Succinic acid	250.5	131.5	−47.5	0.159	
	Fumaric acid	336.2	27.5	−91.8	0.010	↓
	cis-Aconitic acid	43.7	4.2	−90.3	0.010	↓
	Citric acid	6425.6	0.0	−100.0	0.010	↓
	alpha-Ketoglutaric acid	222.3	97.1	−56.3	0.019	↓
Urea Cycle	Citrulline	10.3	4.6	−55.1	0.035	↓
	Arginine	14,526.1	437.6	−97.0	0.010	↓
	Ornithine	2626.1	0.0	−100.0	0.010	↓
AA	Proline	2642.0	2694.3	2.0	0.841	
	Glutamine	31.0	20.3	−34.5	0.010	↓
	Glutamic acid	410.2	155.9	−62.0	0.010	↓
Additional	Lactic acid	4613.3	13,347.5	189.3	0.010	↑
	Fructose	2.3	34.6	1384.5	0.010	↑

Table 2. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in metformin (MET) treated wild type MCF-7 cells (MCF-7^{WT+MET}) and in MCF-7^{WT} cells cultured in Gln free medium. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑—significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}, ↓—significant decrease in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{WT+MET} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{WT+MET}
		MCF-7^{WT}	MCF-7^{WT+MET}			
Glycolysis	Pyruvic acid	6.5	227.9	3423.5	0.038	↑
	Glucose	11.2	115.8	938.0	0.038	↑
	Phospho-enolpyruvic acid	6449.0	417.1	−93.5	0.057	
PPP	Glucose 6-phosphate	328.6	45.5	−86.1	0.038	↓
	6-Phospho-gluconic acid	269.9	607.7	125.1	0.727	
TCA	Malic acid	1126.1	514.0	−54.4	0.260	
	Succinic acid	250.5	168.8	−32.6	0.420	
	Fumaric acid	336.2	179.2	−46.7	0.260	
	cis-Aconitic acid	43.7	5.9	−86.4	0.050	↓
	Citric acid	6425.6	600.3	−90.7	0.050	↓
	alpha-Ketoglutaric acid	222.3	1818.6	718.3	0.483	

Table 2. *Cont.*

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{WT+MET} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{WT+MET}
		MCF-7^{WT}	MCF-7^{WT+MET}			
Urea Cycle	Citrulline	10.3	14.1	36.8	0.500	
	Arginine	14,526.1	4528.0	−68.8	0.420	
	Ornithine	2626.1	1223.7	−53.4	0.327	
AA	Proline	2642.0	2335.2	−11.6	0.841	
	Glutamine	31.0	25.8	−16.6	0.168	
	L-Glutamic acid	410.2	466.0	13.6	0.841	
Additional	Lactic acid	4613.3	17,831.6	286.5	0.038	↑
	Fructose	2.3	11.5	392.3	0.057	

3.4. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in MCF-7^{crPOX} Treated with MET (MCF-$7^{crPOX+MET}$) and in MCF-7^{WT} Cultured in Gln Free Medium

MET treatment of MCF-7^{crPOX} cells (MCF-$7^{crPOX+MET}$) contributed to increase in intracellular GLC, PYR (about 26- and 44-fold, respectively) and drastic increase in LA (about 4-fold) concentrations, as compared to MCF-7 wild type cells (MCF-7^{WT}). It was accompanied by total decrease in the concentrations of PEP and G-6-P and significant decrease in the concentrations of several TCA cycle and ornithine in MCF-$7^{crPOX+MET}$ compared to MCF-7^{WT}cells (Table 3).

Table 3. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MCF-7^{crPOX} treated with MET (MCF-$7^{crPOX+MET}$) and in MCF-7^{WT} cultured in Gln free medium. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑—significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}, ↓—significant decrease in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-$7^{crPOX+MET}$ vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-$7^{crPOX+MET}$
		MCF-7^{WT}	MCF-$7^{crPOX+MET}$			
Glycolysis	Pyruvic acid	6.5	289.6	4378.6	0.022	↑
	Glucose	11.2	303.1	2618.3	0.022	↑
	Phospho-enolpyruvic acid	6449.0	0.0	−100.0	0.022	↓
PPP	Glucose 6-phosphate	328.6	18.8	−94.3	0.025	↓
	6-Phospho-gluconic acid	269.9	71.9	−73.4	0.104	
TCA	Malic acid	1126.1	133.0	−88.2	0.025	↓
	Succinic acid	250.5	134.1	−46.5	0.169	
	Fumaric acid	336.2	27.6	−91.8	0.025	↓
	cis-Aconitic acid	43.7	1.1	−97.6	0.022	↓
	Citric acid	6425.6	0.0	−100.0	0.022	↓
	alpha-Ketoglutaric acid	222.3	78.2	−64.8	0.132	
Urea Cycle	Citrulline	10.3	10.3	0.4	0.802	
	Arginine	14,526.1	2098.1	−85.6	0.118	
	Ornithine	2626.1	59.4	−97.7	0.025	↓
AA	Proline	2642.0	1244.8	−52.9	0.121	
	Glutamine	31.0	29.1	−6.1	0.578	
	Glutamic acid	410.2	78.6	−80.8	0.025	↓
Additional	Lactic acid	4613.3	21,161.8	358.7	0.022	↑
	Fructose	2.3	18.8	706.0	0.022	↑

The data suggest that MET treatment of PRODH/POX-knock out MCF-7 cells (MCF-$7^{crPOX+MET}$) contributes to inhibition of GLC, LA and PYR consumption while PEP and G-6-P and some TCA and urea cycles metabolites are utilized in these conditions.

3.5. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in PRODH/POX-Knock out of MCF-7 Cells (MCF-7^{crPOX}) and Wild Type MCF-7 Cells (MCF-7^{WT}) Cultured in Medium Containing Gln

The result showed that although differential levels of metabolites between groups were not statistically different, PRODH/POX- knock out of MCF-7 cells (MCF-7^{crPOX}) contributed to increase in intracellular GLC (insignificantly), slight increase in PYR concentrations and decrease in concentrations of PEP, G-6-P, some TCA cycle and urea cycle metabolites as well as Gln and Glu, without effect on Pro concentration in PRODH/POX-knocked out MCF-7 cells as compared to MCF-7^{WT} (Table 4).

Table 4. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in PRODH/POX-knock out of MCF-7 cells (MCF-7^{crPOX}) and wild type MCF-7 cells (MCF-7^{WT}) cultured in medium containing Gln. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{crPOX} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{crPOX}
		MCF-7^{WT}	MCF-7^{crPOX}			
Glycolysis	Pyruvic acid	94.3	130.9	38.9	0.653	
	Glucose	10.3	59.5	474.9	0.075	
	Phospho-enolpyruvic acid	3605.0	78.2	−97.8	0.075	
PPP	Glucose 6-phosphate	184.4	131.9	−28.4	0.660	
	6-Phospho-gluconic acid	794.5	814.3	2.5	1.000	
TCA	Malic acid	1361.2	635.2	−53.3	0.172	
	Succinic acid	195.5	158.0	−19.2	0.660	
	Fumaric acid	402.0	184.4	−54.1	0.172	
	cis-Aconitic acid	79.6	48.2	−39.5	0.653	
	Citric acid	7462.9	4477.2	−40.0	0.536	
	alpha-Ketoglutaric acid	949.3	684.7	−27.9	0.660	
Urea Cycle	Citrulline	6.1	11.0	81.5	0.377	
	Arginine	10,138.1	6694.5	−34.0	0.660	
	Ornithine	3957.4	1510.7	−61.8	0.172	
AA	Proline	3288.6	3373.5	2.6	1.000	
	Glutamine	296.2	65.9	−77.7	0.075	
	Glutamic acid	369.4	250.6	−32.2	0.543	
Additional	Lactic acid	24,919.0	23,080.4	−7.4	1.000	
	Fructose	12.0	21.4	78.1	0.075	

The data suggest that the PRODH/POX-knocked out cells cultured in the presence of Gln utilize all studied metabolites, while saves consumption of GLC in these conditions. Pro is not significantly affected.

3.6. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in MET Treated Wild Type MCF-7 Cells (MCF-7^{WT+MET}) and in MCF-7^{WT} Cells Cultured in Medium Containing Gln

The results of high percentage change indicated that Metformin-treatment of MCF-7^{WT} (MCF-7^{WT+MET}) contributed to drastic increase in GLC (about 11-fold), PYR, Gln, Glu and

decrease in LA, G-6-P, Orn and some metabolites of TCA cycle. However, concentrations of Pro and Arg were not much affected, compared to MCF-7^{WT} (Table 5).

Table 5. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MET treated wild type MCF-7 cells (MCF-7^{WT+MET}) and in MCF-7^{WT} cells cultured in medium containing Gln. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑ —significant increase in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}, ↓ —significant decrease in the concentration of studied compound in MCF-7^{WT+MET} cells vs. MCF-7^{WT}.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-7^{WT+MET} vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-7^{WT+MET}
		MCF-7^{WT}	MCF-7^{WT+MET}			
Glycolysis	Pyruvic acid	94.3	201.7	114.0	0.132	
	Glucose	10.3	124.2	1100.6	0.050	↑
	Phospho-enolpyruvic acid	3605.0	1502.4	−58.3	0.176	
PPP	Glucose 6-phosphate	184.4	35.7	−80.6	0.165	
	6-Phospho-gluconic acid	794.5	79.2	−90.0	0.050	↓
TCA	Malic acid	1361.2	779.1	−42.8	0.248	
	Succinic acid	195.5	114.5	−41.4	0.165	
	Fumaric acid	402.0	239.1	−40.5	0.248	
	cis-Aconitic acid	79.6	5.7	−92.9	0.050	↓
	Citric acid	7462.9	613.7	−91.8	0.050	↓
	alpha-Ketoglutaric acid	949.3	1576.9	66.1	0.248	
Urea Cycle	Citrulline	6.1	6.3	2.9	0.952	
	Arginine	10,138.1	10963.0	8.1	0.578	
	Ornithine	3957.4	2019.8	−49.0	0.248	
AA	Proline	3288.6	4193.5	27.5	0.165	
	Glutamine	296.2	1666.2	462.4	0.050	↑
	Glutamic acid	369.4	941.7	154.9	0.050	↑
Additional	Lactic acid	24,919.0	15,892.6	−36.2	0.248	
	Fructose	12.0	9.8	−18.3	0.086	

The data suggest that MET treated cells (MCF-7^{WT}) cultured in the presence of Gln contributes to inhibition of utilization of GLC, PYR, Gln and Glu, while utilizes TCA metabolites and lactic acid and only slightly affect Pro and some urea cycle metabolites, as compared to MCF-7^{WT} cells.

3.7. Targeted Metabolic Profiles of Some Metabolites of Glycolysis, Pentose Phosphate Pathway, TCA and Urea Cycles in MCF-7^{crPOX} Treated with MET (MCF-$7^{crPOX+MET}$) and in MCF-7^{WT} Cultured in the Medium Containing Gln

MET treatment of MCF-7^{crPOX}cells (MCF-$7^{crPOX+MET}$) in the presence of Gln contributed to drastic increase in concentration of GLC (about 18-fold), not significant increase in PYR and Pro and total decrease in PEP, G-6-P and significant decrease in concentration of TCA, urea cycle metabolites and a slight decrease in LA. Interestingly, Glu concentration was significantly decreased, compared to MCF-7^{WT} (Table 6).

The data suggest that MET treatment of MCF-7^{crPOX} cells (MCF-$7^{crPOX+MET}$) cultured in the presence of Gln contributes to inhibition of GLC utilization while induce utilization of TCA and urea cycle metabolites and LA, without significant effect on Pro concentration, as compared to MCF-7^{WT} cells.

Table 6. Targeted metabolic profiles of some metabolites of glycolysis, pentose phosphate pathway, TCA and urea cycles in MET treated wild type MCF-7 cells (MCF-$7^{crPOX+\ MET}$) and in MCF-7^{WT} cells cultured in medium containing Gln. AA—amino acids, PPP—pentose phosphate pathway, TCA—tricarboxylic acids cycle. ↑—significant increase in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}, ↓—significant decrease in the concentration of studied compound in MCF-$7^{crPOX+MET}$ cells vs. MCF-7^{WT}.

Relevant Metabolic Pathways	Metabolites	Conc. Average (ppb)		% Change (MCF-$7^{crPOX+MET}$ vs. MCF-7^{WT})	*P* Value (Mann–Whitney)	MCF-$7^{crPOX+MET}$
		MCF-7^{WT}	MCF-$7^{crPOX+MET}$			
Glycolysis	Pyruvic acid	94.3	131.6	39.6	0.586	
	Glucose	10.3	204.6	1878.0	0.025	↑
	Phospho-enolpyruvic acid	3605.0	0.0	−100.0	0.025	↓
PPP	Glucose 6-phosphate	184.4	0.0	−100.0	0.025	↓
	6-Phospho-gluconic acid	794.5	0.0	−100.0	0.025	↓
TCA	Malic acid	1361.2	37.0	−97.3	0.025	↓
	Succinic acid	195.5	91.9	−53.0	0.086	
	Fumaric acid	402.0	6.1	−98.5	0.025	↓
	cis-Aconitic acid	79.6	1.4	−98.3	0.025	↓
	Citric acid	7462.9	0.0	−100.0	0.025	↓
	alpha-Ketoglutaric acid	949.3	40.9	−95.7	0.025	↓
Urea Cycle	Citrulline	6.1	1.9	−69.5	0.226	
	Arginine	10,138.1	539.3	−94.7	0.025	↓
	Ornithine	3957.4	0.0	−100.0	0.025	↓
AA	Proline	3288.6	3664.0	11.4	0.905	
	Glutamine	296.2	210.6	−28.9	0.461	
	Glutamic acid	369.4	68.4	−81.5	0.025	↓
Additional	Lactic acid	24,919.0	17,098.2	−31.4	0.226	
	Fructose	12.0	15.8	31.6	0.086	

4. Discussion

Epidemiological evidence suggests that therapy with the metformin is associated with decreased risk of certain cancers, such as colon, liver, lung as well as decreased cancer mortality [31]. However, there is some discrepancy between these studies. Some data show beneficial effect of metformin in cancer treatment with reduced mortality [32–35], while others fail to document such beneficial effects [31,36]. It suggests the presence of a specific molecular signature of cancer that increases its susceptibility to the antineoplastic effects of metformin. Therefore, we try to recognize the molecular signature by metabolomic approach.

Metabolomic analyses are promising approaches for identification of specific abnormalities in cancer metabolic pathways that could be considered as a potential target for cancer therapy. Similarly, metabolomic analyses of cancer cells that are treated with compounds of potential antineoplastic activity could identify mechanism of their action. In present study, analysis of some metabolites (targeted metabolomics) of glycolysis, TCA, Urea cycle, PPP and proline convertible amino acids (glutamine, glutamate, ornithine, α-ketoglutarate) was performed in breast cancer cells that have been treated with MET. It has been considered that MET induces reprogramming of energetic metabolism in such a way that instead of glucose facilitate degradation of proline by PRODH/POX, as an alternative source of energy. Therefore, studies on PRODH/POX-knocked out MCF-7 cells were also performed.

Interestingly, in conditions of Gln absence, MET treatment of MCF-7 cells as well as MCF-7 PRODH/POX-knocked out cells contributed to similar inhibition of glycolysis (increased intracellular concentration of GLC, PYR and LA) and utilization (decreased concentration) of PEP, G-6-P and some metabolites of TCA and urea cycle, without significant effect on Pro level, as compared to control MCF-7^{WT} cells. The functional significance of the phenomenon is activation of apoptosis. However, in the presence of Gln, MET treatment of MCF-7 cells as well as MCF-7 PRODH/POX-knocked out cells contributed to utilization of some studied metabolites, (except GLC) and creation of pro survival phenotype of MCF-7 cells cultured in these conditions. It suggests that glycolysis is linked to glutamine and proline metabolism. In fact, glycolysis is quiescent not only in MET treated MCF-7 cells but also in non-treated PRODH/POX-knocked out MCF-7 cells. It seems that in both cases there is metabolic glucose starvation and the cells favor Gln as the source of alternative metabolic energy over glucose. The glucose-independence in these conditions suggests uncoupled glycolysis and TCA cycle that might be the sign of MET-dependent rewiring of energetic metabolism. The possible mechanism of this process could involve MET-dependent inhibition of pyruvate kinase attenuating glucose utilization and subsequently TCA metabolism and P5C synthesis with further consequences on proline cycle and PPP (Figure 2). The link could be also at the level of lactate dehydrogenase (LDH) converting PYR to LA and coupled to redox state by regeneration of NAD for stimulation of glycolysis and simultaneously preventing GLC processing in TCA cycle.

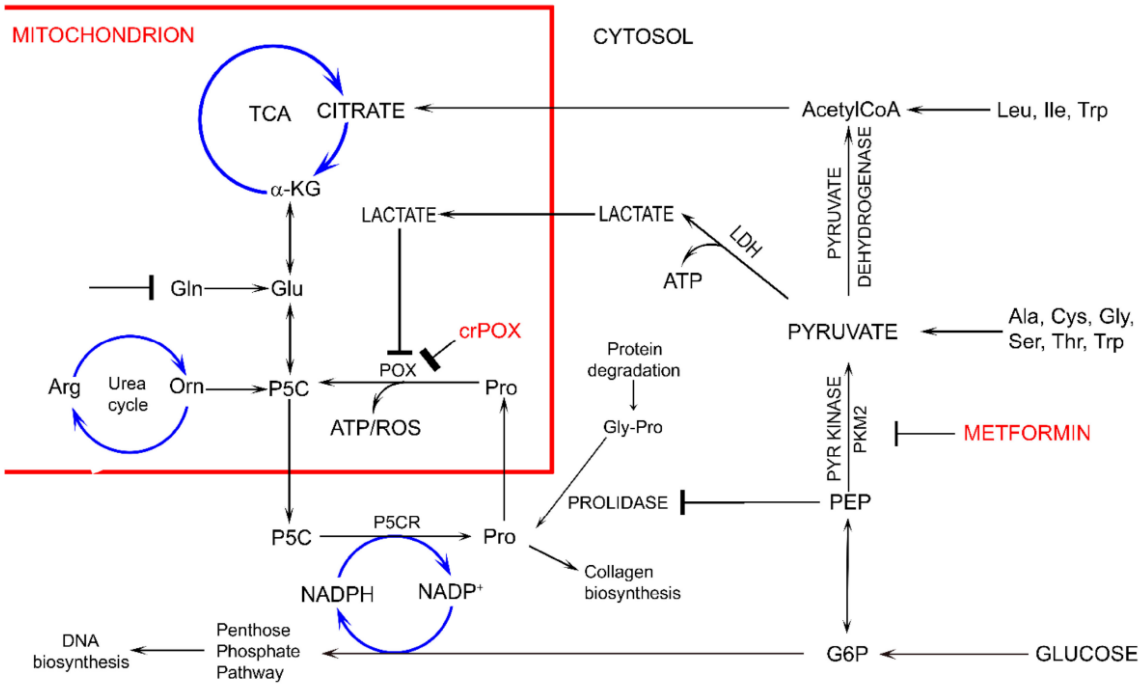

Figure 2. The potential effect of metformin (MET) on complex regulatory mechanisms of PRODH/POX-dependent apoptosis/survival linking glycolysis, TCA, urea cycles, pentose phosphate pathway, proline cycle (synthesis and degradation), collagen biosynthesis and degradation and prolidase. α-KG—α-ketoglutarate, AcetyloCoA—acetyl coenzyme A, Ala—alanine, Arg—arginine, ATP—adenosine triphosphate, crPOX—CRISPER for POX, Cys—cysteine, Gln-glutamine, Glu-glutamic acid, G6P—glucose-6-phosphate, Gly—glycine, Gly-Pro—glycyl-proline, Ile—isoleucine, LDH—lactate dehydrogenase, Leu—leucine, NADP$^+$—nicotinamide adenine dinucleotide phosphate, NADPH—reduced form of NADP$^+$, PEP—phosphoenolpyruvate, PYR kinase—pyruvate kinase, Orn—ornithine, PKM2—pyruvate kinase M2, Pro—proline, POX—proline dehydrogenase/oxidase, P5CR—1-pyrroline-5-carboxylate reductase, P5C—1-pyrroline-5-carboxylate, ROS—reactive oxygen species, Ser—serine, TCA—tricarboxylic acid cycle, Thr—threonine, Trp—tryptophan.

Cancer cells are characterized by enhanced consumption of glucose-yielding lactate during aerobic glycolysis. The phenomenon known as a Warburg effect ensures rapid production of ATP from glucose to support cancer cell proliferation [37,38]. Though the process of ATP production from glucose by Warburg effect is less efficient than during mitochondrial oxidative phosphorylation, the conversion of pyruvate into lactate ensures high NAD+/NADH ratio that accelerate glycolysis. For a long time, Warburg effect has been considered as an effect of impairment of oxidative phosphorylation, but in recent decades it has been documented that the mechanism underlying cancer metabolic reprogramming is much more complex [39]. It is well established that Warburg effect contributes to depletion of TCA cycle and augmentation of glutaminolysis, feeding in this way TCA by glutamine metabolites, as, e.g., α-ketoglutarate [40]. This process is significantly impacted by non-essential amino acids as proline, ornithine and glutamate. They are interconvertible with intermediate of P5C, linking TCA and urea cycles with glutamine metabolism. Particularly, proline could serve as an alternative source of energy. Large quantity of proline comes from protein degradation, mostly from the most abundant extracellular protein, collagen. Deregulation of energetic metabolism in cancer cells due to Warburg's effect facilitates protein degradation as an alternative source of energy.

Several studies showed that proline concentration is increased in cancer cells [41,42]. Both hypoxia [43] and glucose depletion [14] were found to induce activity of metalloproteinases, MMP-2 and -9, suggesting the mechanism for the increase in cellular proline concentration. When glucose supply is limited, cancer cells may select proline as an alternative energy source, since proline has an advantage over fatty acids and glutamine, which like glucose require delivery by the circulation. Therefore, proline may represent energy sense molecule and energy substrate. Especially, under glucose deprivation, in order to maintain the cell survival, proline interconvertible amino acids: glutamate, α-ketoglutarate and ornithine may serve as alternative sources of energy. They are substrates for production of P5C that links TCA, urea cycles and glutamine metabolism. P5C as a product of proline conversion by PRODH/POX is of special interest. P5C and proline circulate between mitochondria and cytoplasm. Conversion of P5C into proline is catalyzed by P5C reductase (P5CR). The shuttle is known as a "proline cycle". It is coupled to pentose phosphate pathway (PPP) producing nucleotides for DNA biosynthesis. The data presented in this paper suggest tight correlation between glycolysis, proline metabolism by PRODH/POX and PPP. PRODH/POX-knock out of MCF-7 cells or treatment of the cells with MET inhibited glycolysis (increase in intracellular GLC concentration), and attenuated PPP and TCA pathways (decrease in the concentration of metabolites) when cultured in Gln free medium. In the presence of Gln, the cells similarly inhibited GLC utilization however, differentially affected LA utilization. PRODH/POX-knocked out MCF-7 cells utilized LA, while treated with MET inhibited LA utilization in these conditions. It suggests that inhibition of glycolysis in PRODH/POX-knocked out MCF-7 cells and MET treated cells is affected by Gln. Moreover, PRODH/POX-knock out MCF-7 cells that has been treated with MET in Gln free medium inhibited utilization of GLC and LA, while in the presence of Gln induced utilization of LA. It suggests synergistic effects of PRODH/POX-knock out and MET treatment on inhibition of glycolysis and the role of Gln in stimulation of LA utilization in these cells. Therefore, the similar effects of metformin treatment and knockout of PRODH/POX on breast cancer cellular metabolism could be explained at the level of multidirectional regulatory mechanisms including glycolysis, TCA cycle, urea cycle, proline cycle and amino acid metabolism, as shown in Figure 2. It seems that the key metabolite is P5C. Since metformin inhibits pyruvate kinase, it inhibits glucose utilization and subsequently down-regulate TCA cycle and P5C synthesis with further consequences on proline cycle and pentose phosphate pathway. The similar effect could be achieved when PRODH/POX is knocked out. The functional significance of the processes (activation of apoptosis) was found in MCF-7 cells cultured in glutamine free medium. However, when the cells were cultured in the presence of glutamine (provider of P5C) apoptosis did not occur. The potential mechanism of this processes is outlined in Figure 3.

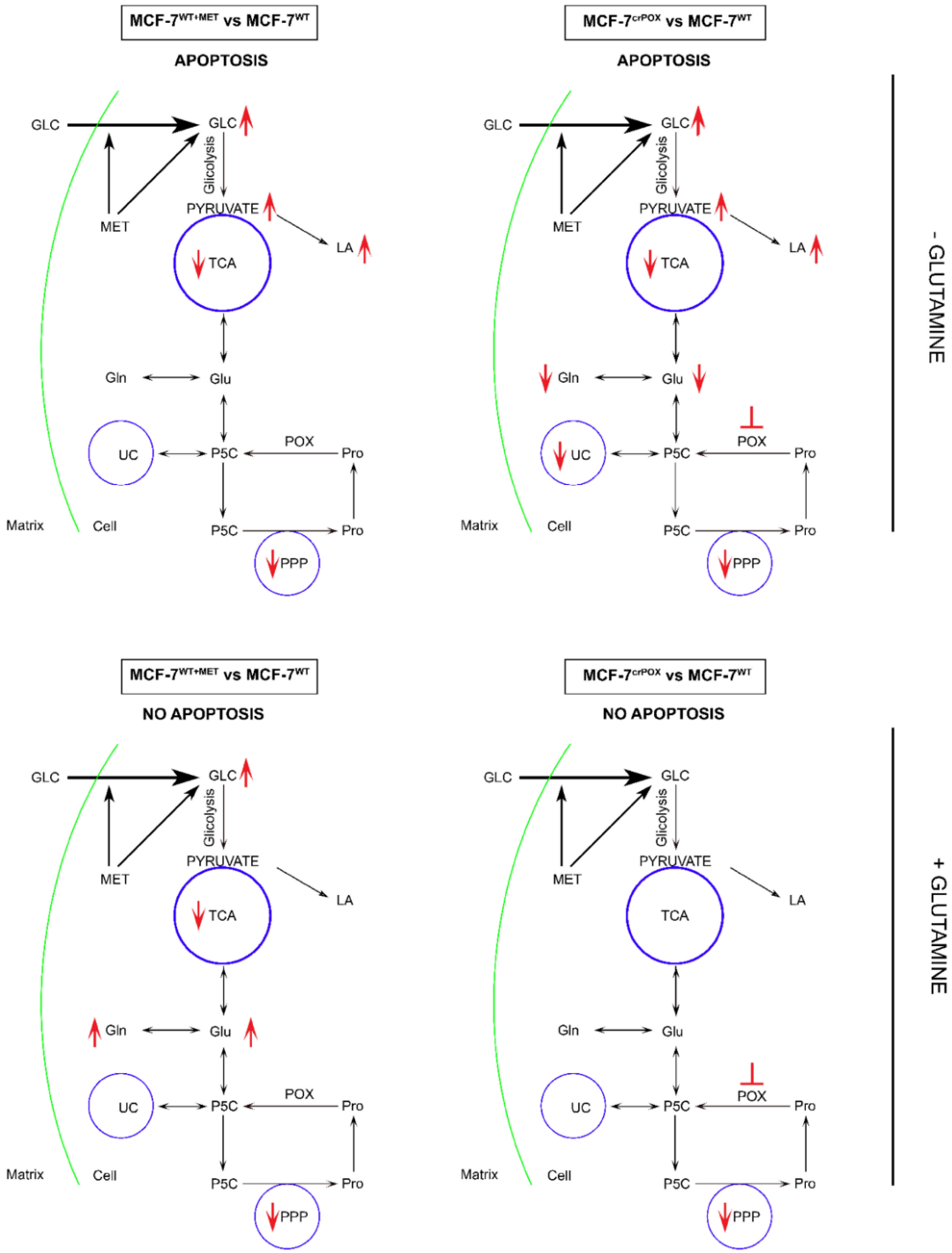

Figure 3. The functional significance of metformin (MET) and PRODH/POX knock-down on complex regulatory mechanisms driving PRODH/POX-dependent apoptosis/survival in wild-type MCF-7 cells (MCF-7^{WT}) and PRODH/POX-knock out MCF-7 cells (MCF-7^{crPOX}), cultured in the presence or absence of Gln. crPOX—CRISPER for POX, GLC—glucose, Gln-glutamine, Glu- glutamic acid, LA—lactate dehydrogenase, Pro—proline, POX—proline dehydrogenase/oxidase, PPP—pentose phosphate pathway, P5C—1-pyrroline-5-carboxylate, TCA—tricarboxylic acid cycle, UC—urea cycle.

Recently we have found that silencing of PRODH/POX induced autophagy while overexpression of prolidase and inhibition of collagen biosynthesis contributed to increase in intracellular proline concentration and PRODH/POX-dependent autophagic cell death in MCF-7 cells [44]. It has been suggested that up-regulation of PRODH/POX by PPAR-gamma ligands could induce apoptosis in cancer cells [45]. Since LA generated in cancer cells due to Warburg effect inhibits PRODH/POX [46], limiting its function (apoptosis/autophagy), it seems that inhibition of Warburg effect (lactate production, e.g., by metformin) contributed to up-regulation of PRODH/POX -induced apoptosis in cancer cells. In fact, inhibiting LA generation in cancer cells by MET attenuated cancer cell growth and survival [47–49]. The data are also supported by studies showing that PRODH/POX is induced by AMP-activated protein kinase (AMPK)-dependent pathways [16] and phosphorylated-AMPK was upregulated following glycolysis inhibition by 3-bromopyruvate (3-BP) treatment [50].

We suggest that MET inhibits glycolysis and TCA cycle leading to glucose starvation, ATP depletion, facilitating apoptosis. Similar mechanism was presented for 3-bromopyruvate, inhibitor of pyruvate dehydrogenase [51]. Of great importance is its potential to affect pentose phosphate pathway (PPP) that produce reducing potential and nucleotides for DNA synthesis [52]. Since PPP is directly coupled to glycolysis, any changes in glycolytic pathway may affect NADPH production and DNA biosynthesis. The hypothesis is outlined in Figure 2.

5. Conclusions

Metformin treatment of MCF-7 breast cancer cells or PRODH/POX-knock out of the cells induces apoptosis by reprograming of amino acid metabolism, TCA, Urea cycle and pentose phosphate pathway in the cells. Metabolomic analyses in the cells cultured with or without Gln suggest that glycolysis is tightly linked to Gln and Pro metabolism. In the absence of Gln, MET-treatment or PRODH/POX-knock out contributed to GLC starvation and apoptosis in MCF-7 cells as outlined in Figure 3. This knowledge provide insight into mechanism of anticancer activity of MET as an approach to further studies on experimental breast cancer therapy.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11121888/s1, Figure S1: The PRODH/POX expression in MCF-7WT cells and MCF-7crPOX cells by Western Blot using Anti-PRODH/POX antibody (Santa Cruz).; Figure S2: The percentage of cells in G0/G1, S and G2/M phases of the cell cycle of MCF-7 and MCF-7crPOX cells treated with metformin with or without glutamine (Gln).; Figure S3: The representatives' blots of AMPK expressions in MCF-7 cells and MCF-7crPOX cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine.; Figure S4: The representatives' blots of PRODH/POX expressions in MCF-7 cells and MCF-7crPOX cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine.; Figure S5: The representatives' blots of PARP and cleaved-PARP expressions in MCF-7 cells and MCF-7crPOX cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine.; Figure S6: The representatives' blots of Caspase-7 expressions in MCF-7 cells and MCF-7crPOX cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine.; Figure S7: Representatives of chromatograms viewed by MassHunter Qualitative analysis navigator post-run LC-QqQ.; Figure S8: The results of lactic acid in Masshunter QQQ Quantitative analysis version 8.0 in reference samples (standard), testing sample and blank.; Table S1: Testing samples for MS-based approaches.; Table S2: The summary of testing metabolites.; Table S3: Optimized transition of targeted metabolites.

Author Contributions: Conceptualization, T.Y.L.H., I.O. and J.P.; data curation T.Y.L.H., I.O., J.S. and J.P.; formal analysis, T.Y.L.H., J.S. and J.P.; funding acquisition, T.Y.L.H. and J.P; investigation, T.Y.L.H., I.O., J.S., M.N. and J.P.; methodology, T.Y.L.H., I.O., J.S., M.N. and W.B.; project administration, J.P.; resources, T.Y.L.H., I.O., J.S., C.B. and J.P.; software, T.Y.L.H., J.S., M.N. and C.B.; supervision, J.S., C.B. and J.P.; validation, T.Y.L.H., M.N. and W.B.; visualization, T.Y.L.H. and I.O.; writing—original draft preparation, T.Y.L.H., I.O., M.N. and J.P.; writing—review and editing, T.Y.L.H., I.O., J.S., C.B. and J.P. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

Supplementary Material

Metformin treatment or PRODH/POX-knock out similarly induces apoptosis by reprograming of amino acid metabolism, TCA, Urea cycle and pentose phosphate pathway in MCF-7 breast cancer cells.

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Supplementary Material

1. Calculation of percentage of change (%)

Due to zero value of several metabolites, the percentage of change (%) were calculated in different ways to avoid the non-valid % change.

- In Group B vs group G, (%) change was calculated by:

Change (%) = [(average of metabolite concentration in MCF-7^{WT} cells group − average of metabolite concentration in MCF-7^{crPOX} cells group)/[average of metabolite concentration in MCF-7^{crPOX} cells group] × 100

- In group D vs group I and group E vs group J, (%) change was calculated by:

Change (%) = [(average of metabolite concentration in MCF-7^{crPOX} cells group − average of metabolite concentration in MCF-7^{WT} cells group)/[average of metabolite concentration in MCF-7^{WT} cells] × 100

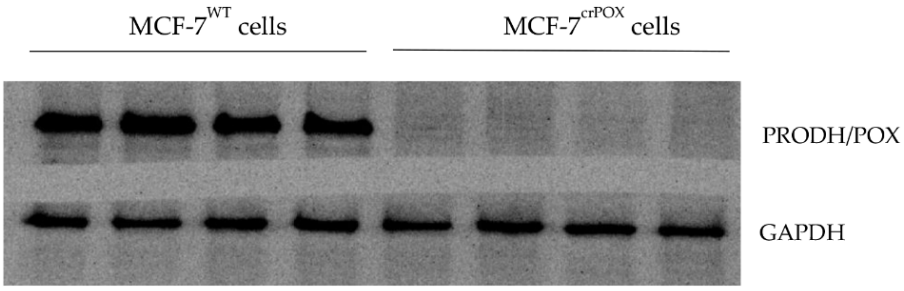


Figure S1. The PRODH/POX expression in MCF-7^{WT} cells and MCF-7^{crPOX} cells by Western Blot using Anti-PRODH/POX antibody (Santa Cruz).

2. Cell cycle phases

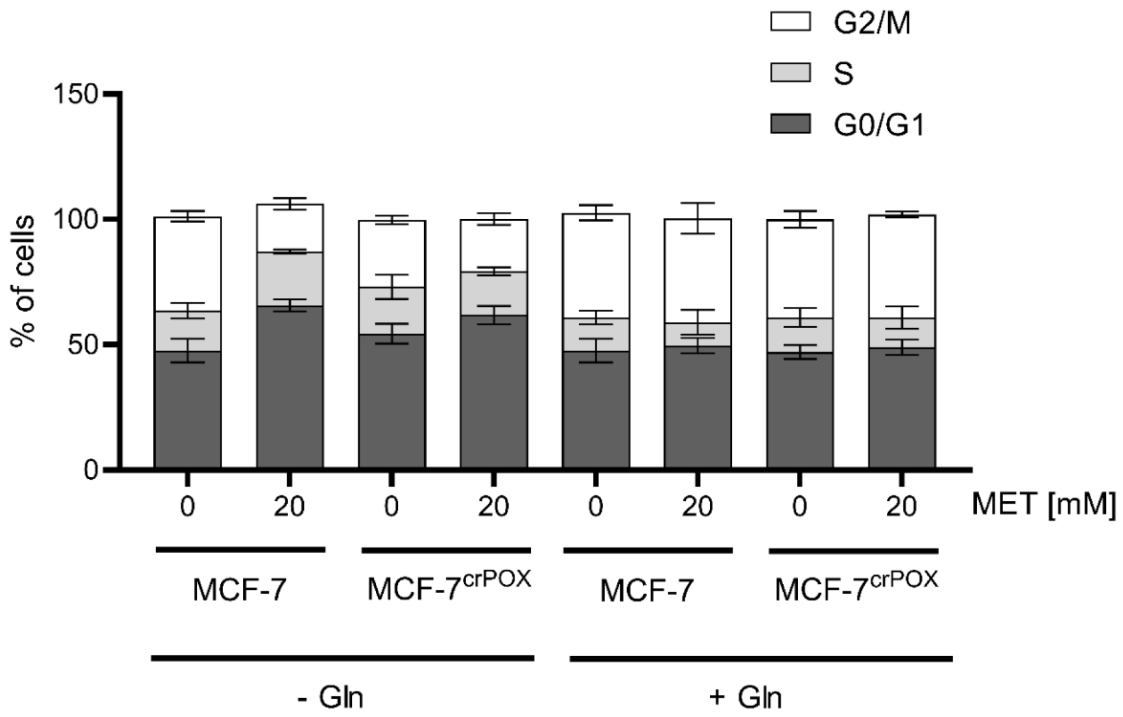


Figure S2. The percentage of cells in G0/G1, S and G2/M phases of the cell cycle of MCF-7 and MCF-7^{crPOX} cells treated with metformin with or without glutamine (Gln).

3. **Expression of AMPPK**

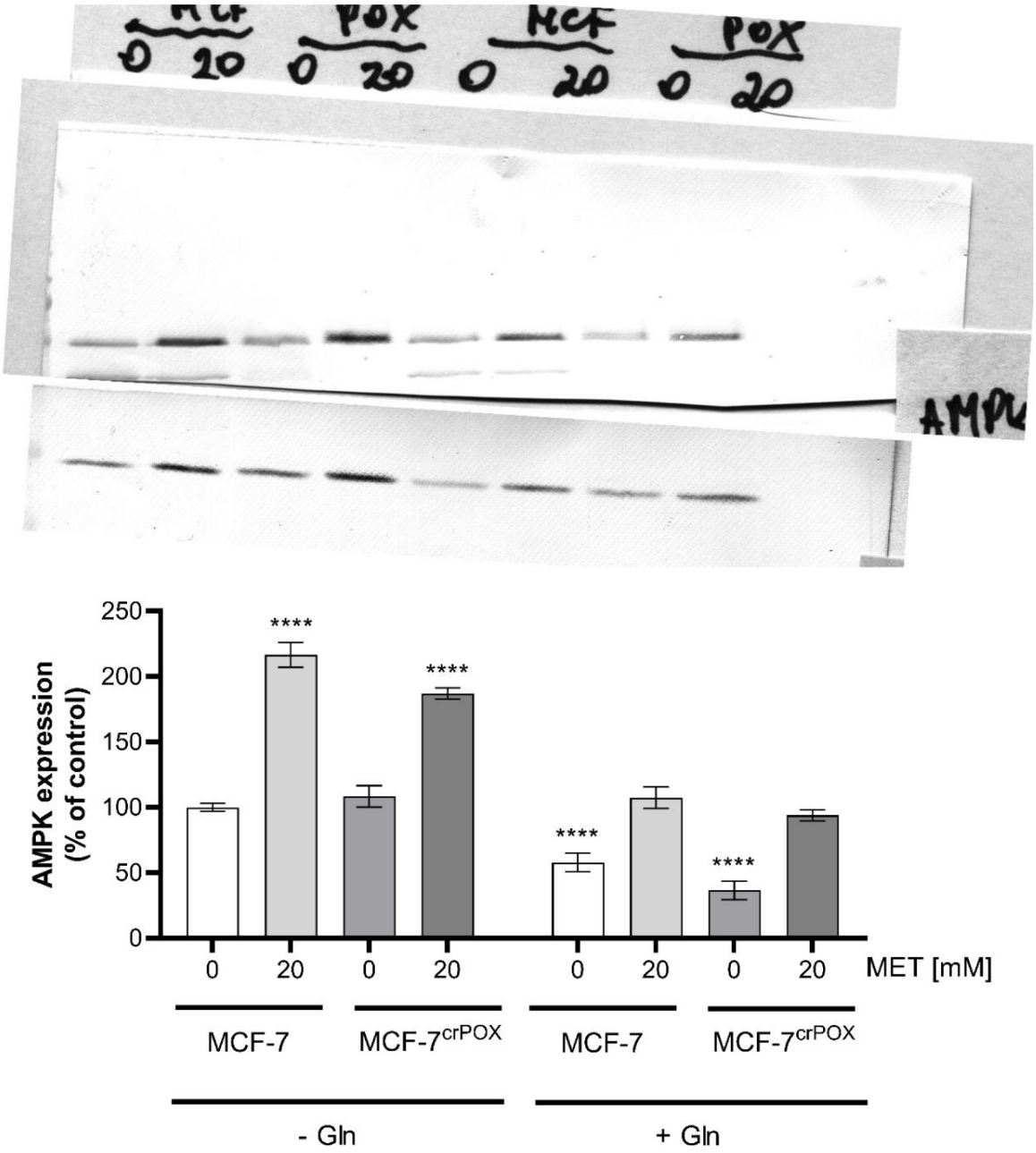


Figure S3. The representatives' blots of AMPK expressions in MCF-7 cells and MCF-7^{crPOX} cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, *P <0.001.

4. **Expression of PRODH/POX**

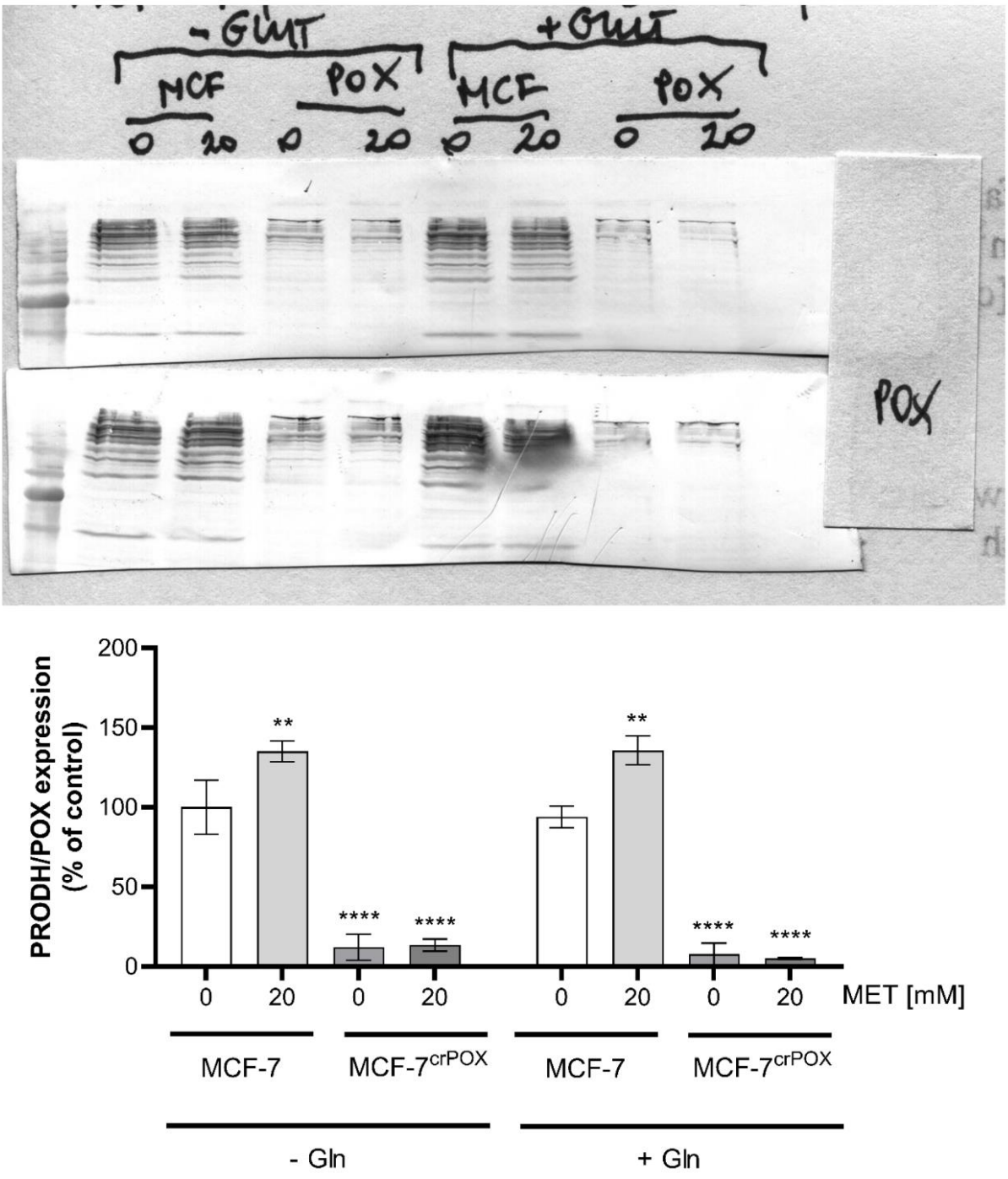
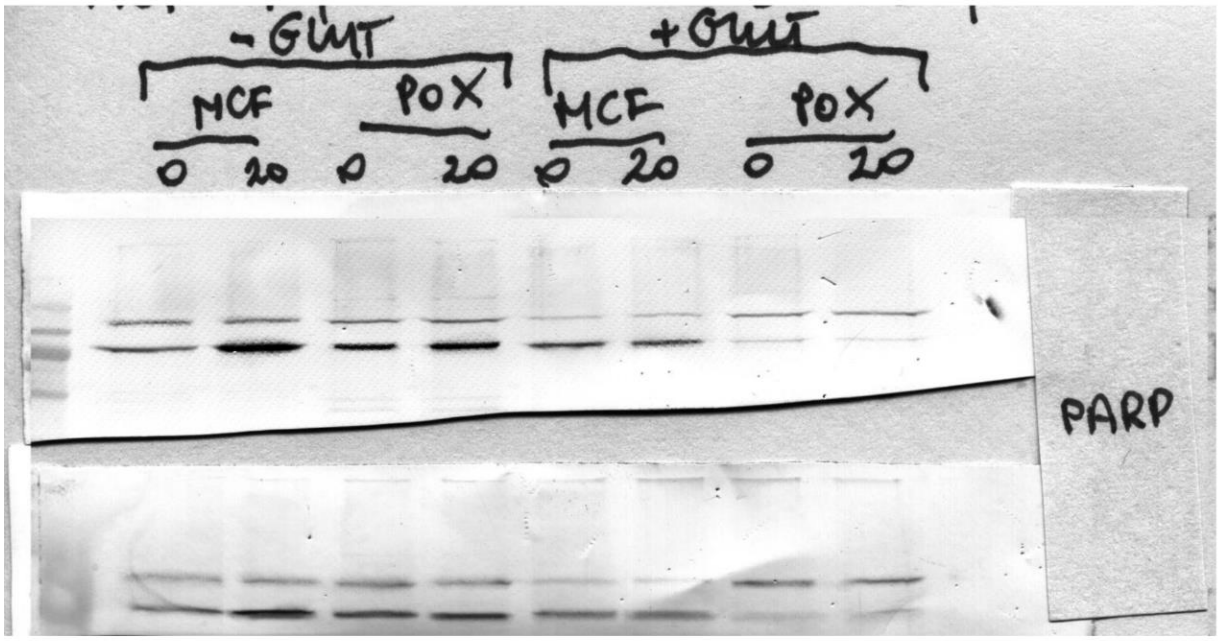
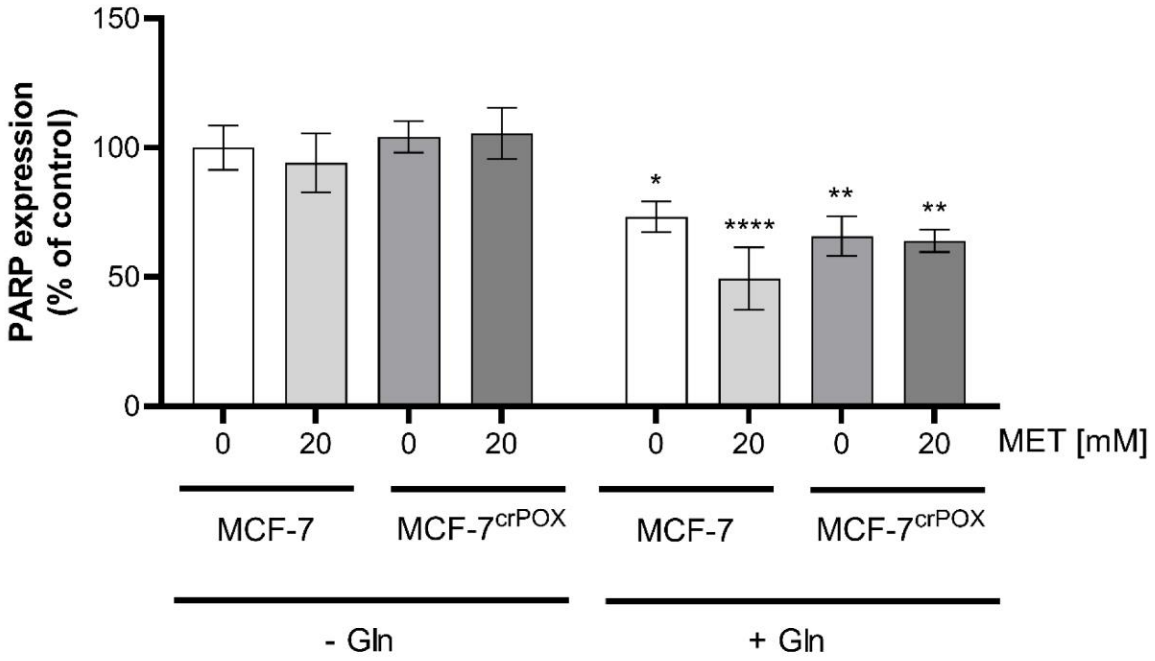


Figure S4. The representatives' blots of PRODH/POX expressions in MCF-7 cells and MCF-7^{crPOX} cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, *P <0.001.

5. **Expression of PARP and cleaved-PARP**





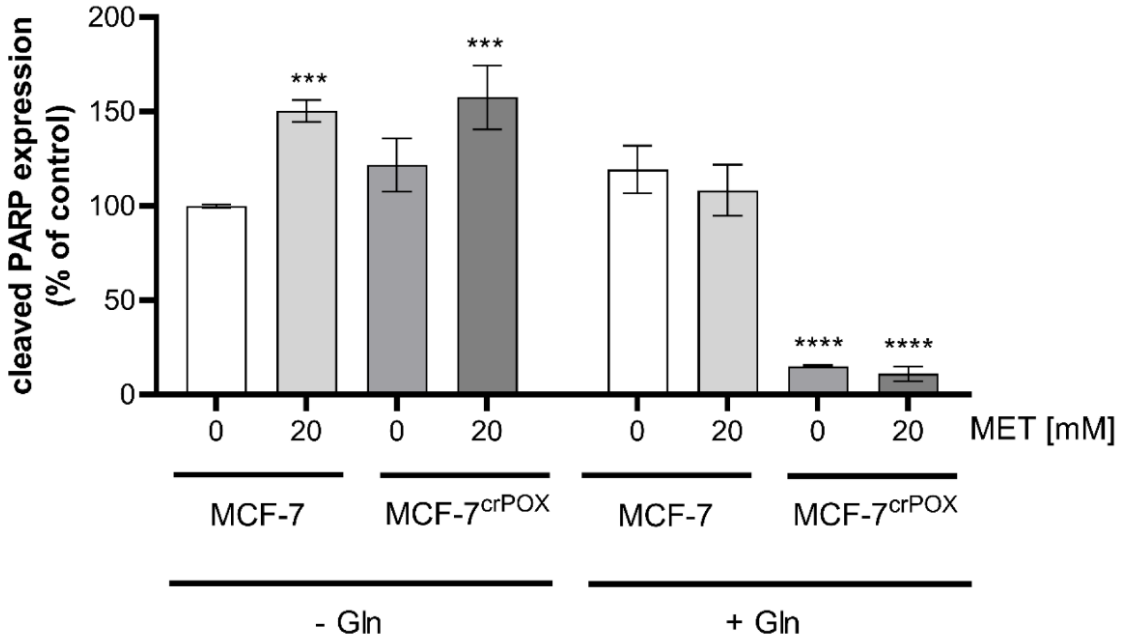
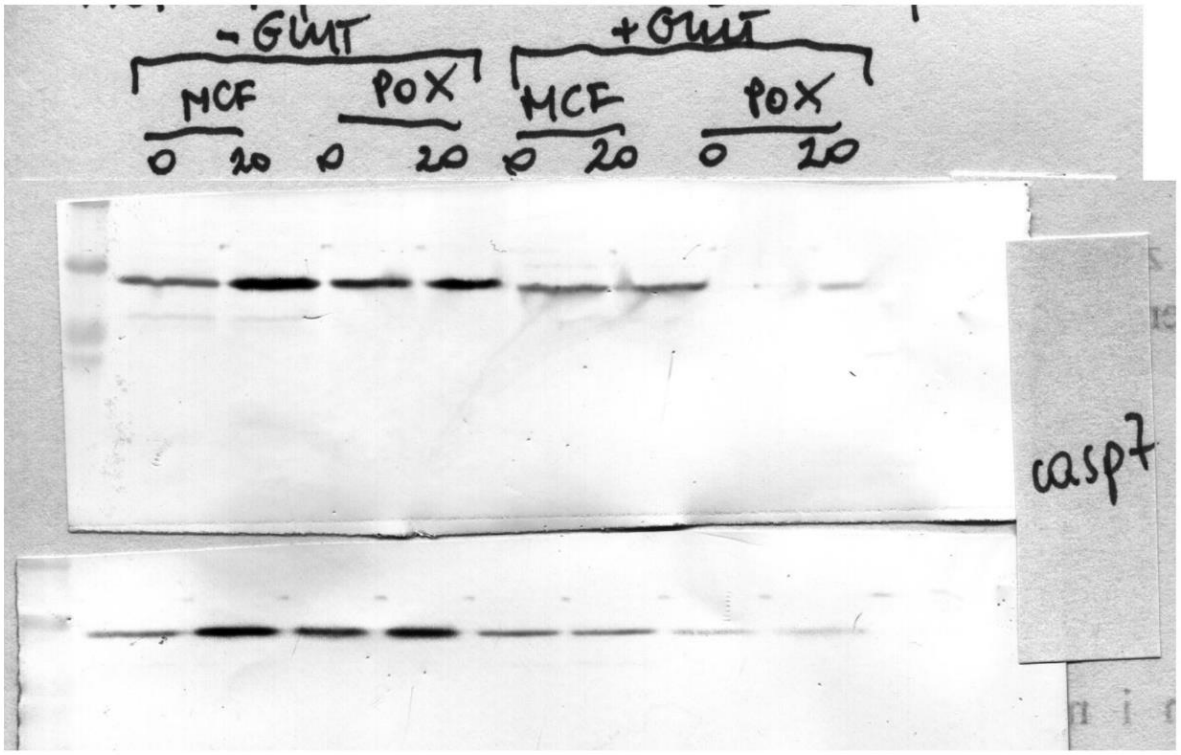


Figure S5. The representatives' blots of PARP and cleaved-PARP expressions in MCF-7 cells and MCF-7^{crPOX} cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, *P <0.001.

6. **Expression of Caspase-7**



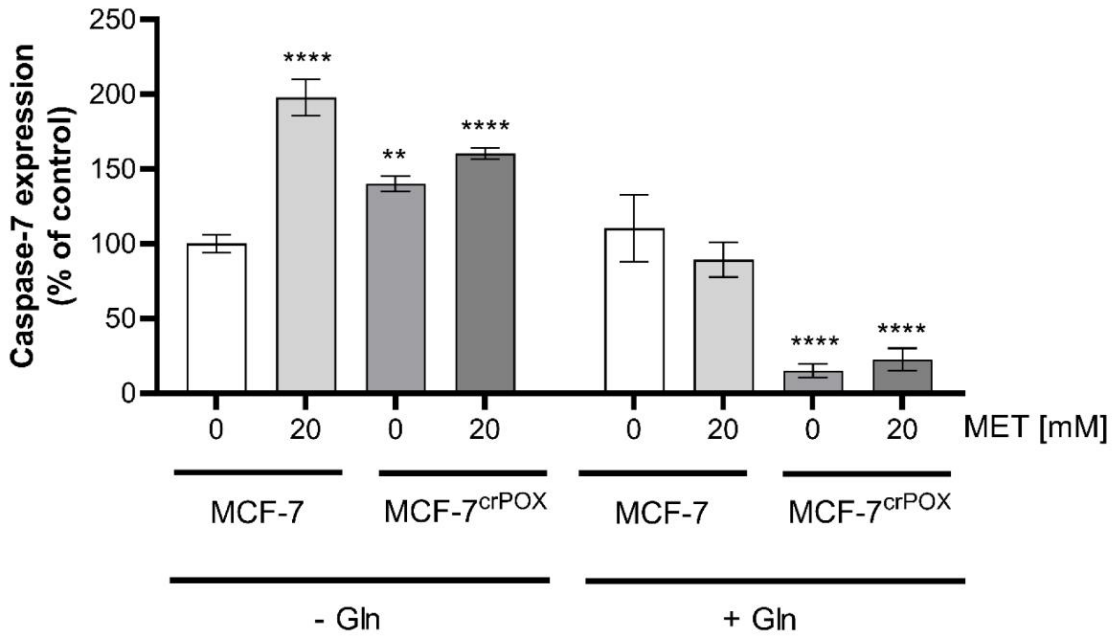


Figure S6. The representatives' blots of Caspase-7 expressions in MCF-7 cells and MCF-7^{crPOX} cells treated with metformin (MET) cultured in DMEM in the presence and absence of glutamine. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, *P <0.001.

Table S1. Testing samples for MS-based approaches

No. Samples	Descriptions	Groups
1-5	MCF-7^{WT} cells in DMEM + glutamine, glucose 4g.l^{-1}, treated with Metformin 20 mM	A
6-10	MCF-7^{WT} cells in DMEM + glutamine, glucose 4g.l^{-1}, untreated	B
11-15	MCF-7^{WT} cells in DMEM - glutamine, glucose 4g.l^{-1}, treated with Metformin 20 mM	C
16-20	MCF-7^{WT} cells in DMEM - glutamine, glucose 4g.l^{-1}, untreated	D
21-25	MCF-7^{WT} cells in DMEM - glutamine, low glucose 1g.l^{-1}, untreated	E
26-30	MCF-7^{crPOX} cells in DMEM + glutamine, glucose 4g.l^{-1}, treated with Metformin 20 mM	F
31-35	MCF-7^{crPOX} cells in DMEM + glutamine, glucose 4g.l^{-1}, untreated	G
36-40	MCF-7^{crPOX} cells in DMEM - glutamine, glucose 4g.l^{-1}, treated with Metformin 20 mM	H
41-45	MCF-7^{crPOX} cells in DMEM - glutamine, glucose 4g.l^{-1}, untreated	I
46-50	MCF-7^{crPOX} cells in DMEM - glutamine, low glucose 1g.l^{-1}, untreated	J

MCF-7^{WT} cells: Wild type MCF-7 cells
MCF-7^{crPOX} cells: PROPDH/POX knockout MCF-7 cells

Table S2. The summary of testing metabolites

Relevant metabolic pathways	Metabolites
Glycolysis	Glucose
	Phosphoenol-pyruvic acid
	Pyruvic acid
Pentose phosphate pathway	Glucose 6-phosphate
	6-phospho-gluconate
Krebs cycle	Fumaric acid
	Alpha-ketoglutaric acid
	Citric acid
	Succinic acid
	Malic acid
	Cis-aconitic acid
Urea cycle	Ornithine
	Arginine
	Citrulline
Amino acids	Proline
	Glutamine
	Glutamic acid
Additional metabolites	Lactic acid
	Fructose

Table S3. Optimized transition of targeted metabolites

No.	Metabolites	Transition	No.	Metabolites	Transition
1	Glucose	259 → 79	11	Phosphoenolpyruvate	167 → 79
2	Fumaric acid	115.1 → 71	12	Pyruvic acid	87 → 43.2
3	Lactic acid	89 → 45.3	13	Succinic acid	117 → 73.1
4	Arginine	173.1 → 131	14	6- phosphogluconic acid	275.1 → 78.9
5	Citrulline	174 → 131	15	Alpha ketoglutaric acid	145 → 101
6	Glutamic acid	146 → 128	16	Cis-aconitic acid	173 → 129
7	Glutamine	145.1 → 127	17	Citric acid	191 → 111
8	Malic acid	133 → 115	18	Fructose	179.1 → 59
9	Ornithine	133 → 133	19	Glucose	179.1 → 59
10	Proline	114 → 68.1			

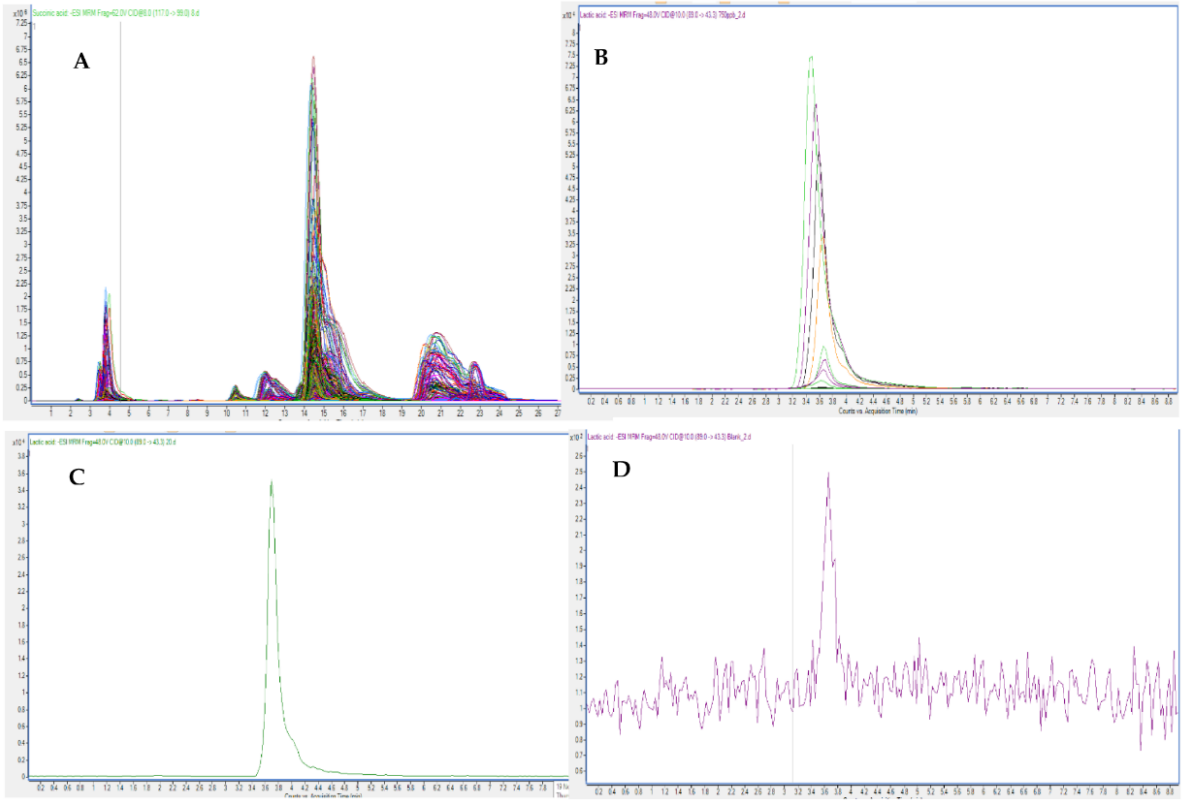


Figure S7. Representatives of chromatograms viewed by MassHunter Qualitative analysis navigator post-run LC-QqQ. A. All extracted profiles. **B.** The standard curve of Lactic acid. **C.** A testing sample with extracted Lactic acid peak. **D.** Blank.

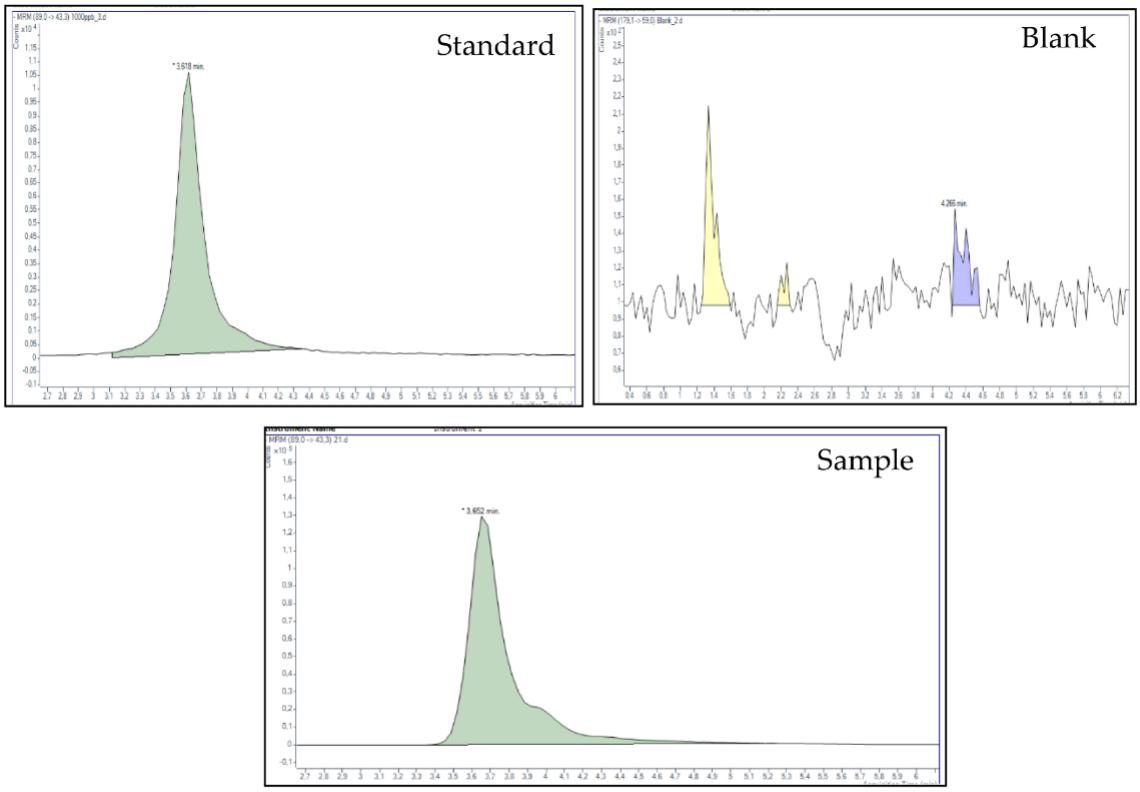


Figure S8. The results of lactic acid in Masshunter QQQ Quantitative analysis version 8.0 in reference samples (standard), testing sample and blank.

AUTHORSHIP/ CO-AUTHORSHIP STASTEMENTS

Bialystok, 03.03.2022

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I declare that my contribution to the preparation of the publication:

1. Huynh, T.Y.L., Zareba, I., Baszanowska, W., Lewoniewska S., Pałka, J. Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pox)-dependent apoptosis/autophagy as an approach to targeted cancer therapy. Mol Cell Biochem, 2020, 466, 35–44. MSWiA: 70 points, Impact Factor ISI: 3.396, DOI: 10.1007/s11010-020-03685-y.

that has been included in my doctoral dissertation consisted: topic of the article, literature review and drafting the manuscript, which I define as 60% participation in the preparation of the above-mentioned publication.

2. Huynh, T.Y.L, Ościłowska, I., Sáiz, J., Nizioł, M., Baszanowska, W., Barbas, C., Pałka, J. Metformin Treatment or PRODH/POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells. Biomolecules. 2021 Dec 15;11(12):1888. doi: 10.3390/biom11121888. MSWiA: 100 points, Impact Factor ISI: 4.879; DOI: 10.3390/biom11121888

that has been included in my dissertation consisted: design of the study, preparation of PRODH/POX knock out cells, biochemical assays, co-participation in performing metabolomic and statistical analyses, interpretation of results and drafting the manuscript content, which I define as 60% of participation in the preparation of the above-mentioned publication.

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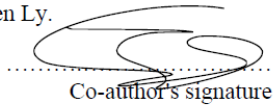

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APPROVAL OF THE BIOETHICS COMMITTEE

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APK.002.4.2022

Sz. P.
prof. dr hab. Jerzy Pałka
Kierownik Zakładu Chemii Leków

W nawiązaniu do pisma z dn. 03.01.2022 r. dotyczącego doktoratu mgr Huynh Thi Yen Ly o tytule: *„Metabolomic approach to understand the mechanism of metformin-induced PRODH/POX-dependent apoptosis in MCF-7 breast cancer cells"* informuję, że nie ma w tym przypadku konieczności uzyskiwania zgody Komisji Bioetycznej.

Przedstawiony certyfikat autentyczności i czystości komercyjnej linii komórkowej gwarantuje dochowanie należytej staranności w przestrzeganiu przepisów pozyskania materiału biologicznego, jak też przestrzegania norm etycznych.

Z poważaniem

prof. dr hab. Otylia Kowal-Bielecka
Przewodnicząca Komisji Bioetycznej
przy Uniwersytecie Medycznym
w Białymstoku

SUMMARY

The objective of the Ph.D. dissertation is to evaluate the mechanism of Metformin (MET) induced apoptosis in MCF-7 breast cancer cells. The Ph.D. dissertation is based on the hypothesis that up-regulation of AMPK by MET can stimulate apoptosis in cancer cells by a cascade of processes involving induction of proline dehydrogenase/proline oxidase (PRODH/POX)-dependent ROS generation under the availability of proline, the PRODH/POX substrate. It has been considered that the processes require specific conditions determined by the complex regulatory machinery of the cell. Under starvation, in order to maintain the cell survival, proline and glutamine together with other glutamine-derived products such as glutamate, alpha-ketoglutarate, and ornithine serve as alternative sources of energy. They are substrates for production of pyrroline-5-carboxylate which is the product of conversion of proline by PRODH/ POX to produce ATP for protective autophagy or to generate reactive oxygen species for apoptosis. Therefore, interconversion of proline, ornithine, and glutamate may regulate PRODH/POX-dependent apoptosis/autophagy. The key amino acid is proline, circulating between mitochondria and cytoplasm. This shuttle is known as proline cycle. It is coupled to pentose phosphate pathway producing nucleotides for DNA biosynthesis. PRODH/POX is also linked to p53 and AMP-activated protein kinase (AMPK)-dependent pathways. Proline availability for PRODH/POX-dependent apoptosis/autophagy is regulated at the level of interconversion of proline, ornithine, glutamate and α-ketoglutarate, linking amino acid metabolism with tricarboxylic acid cycle (TCA) and Urea cycles (UC) and indirectly with electron transport chain. It suggests the presence of a specific molecular signature of cancer cells that could be affected by antineoplastic effects of MET. Therefore, the molecular signature of cancer cells treated with MET was recognized by metabolomic approach. Metabolomic profile was analyzed in MET-treated wild-type MCF-7 (MCF-7^{WT}) cells and PRODH/POX knockout MCF-7 (MCF-7^{crPOX}) cells, generated by CRISPR-Cas9 technology. Cell proliferation was determined by CyQUANT® Cell Proliferation Assay, while cell cycle was analysed by flow cytometry using Nucleo-Counter NC-3000. Expression of pro-apoptotic proteins was evaluated by Western blot. Targeted metabolomics (included the concentrations of intracellular proline, glutamic acids, ornithine, glutamine, α-ketoglutaric acid, glucose, pyruvate, succinate and others) was performed by high-performance liquid

chromatography coupled to tandem mass spectrometry with a triple quadrupole (LC-MS/MS/QqQ).

MET-treatment of both wild type and PRODH/POX knock out cells decreased cell proliferation when incubated in a medium with or without glutamine. However, the inhibition was more pronounced in the absence of glutamine. The percentage of cells in the G2/M phase to G0/G1 phase showed that both MET treatment and PRODH/POX knockout strongly suppressed proliferation of MCF-7 cells cultured in a glutamine-free medium. In the presence of glutamine the effect was not shown. MET induced expression of AMPK (PRODH/POX inducer) in both cell lines regardless of the presence or absence of glutamine. The effect was more pronounced in the cells cultured in glutamine-free medium. In the absence of glutamine, MET induced expression of cleaved PARP and caspase 7 in both cell lines. In the presence of glutamine, the effect was shown only in wild type MCF-7 cells.

In the absence of glutamine, MET treatment or PRODH/POX-knock out of MCF-7 cells contributed to similar inhibition of glycolysis (drastic increase in intracellular glucose and pyruvate) and increase in the utilization of phospho-enol-pyruvic acid, glucose-6-phosphate and some metabolites of TCA and UC, contributing to apoptosis. However, in the presence of glutamine, MET treatment or PRODH/POX-knock out of MCF-7 cells contributed to utilization of some studied metabolites (except glucose), facilitating pro-survival phenotype of MCF-7 cells in these conditions.

It suggests that MET treatment or PRODH/POX-knock out decreased cell proliferation (the inhibition was more pronounced in the absence of glutamine). Metformin treatment of MCF-7 breast cancer cells or PRODH/POX-knock out of the cells induced apoptosis by reprogramming amino acid metabolism, TCA, Urea cycle, and pentose phosphate pathway in the cells. Metabolomic analyses in the cells cultured with or without glutamine suggest that glycolysis is tightly linked to glutamine and proline metabolism. In the absence of glutamine, MET-treatment or PRODH/POX-knock out contributed to glucose starvation and apoptosis in MCF-7 cells. The results of these studies provide insight into mechanism of anticancer activity of MET and suggest that combined treatment of MET with inhibitors of glutamine synthesis may be a new approach to further studies on experimental breast cancer therapy.

STRESZCZENIE

Celem rozprawy doktorskiej jest ocena mechanizmu przeciwnowotworowego działania metforminy (MET) w komórkach raka piersi MCF-7. Praca doktorska oparta jest na hipotezie zakładającej, że MET aktywuje kinazę AMP(AMPK) która indukuje apoptozę w komórkach nowotworowych poprzez kaskadę procesów obejmujących generowanie przez dehydrogenazę prolinową/oksydazę prolinową (PRODH/POX) reaktywnych form tlenu (ROS) w warunkach dostępności proliny, substratu PRODH/POX. Założono, że omawiane procesy wymagają szczególnych warunków metabolicznych, określonych przez złożone mechanizmy regulacyjne komórki. W warunkach niedoboru węglowodanowych i lipidowych substratów energetycznych, w celu przeżycia, komórka wykorzystuje alternatywne źródła energii, głównie prolinę i glutaminę wraz z metabolitami glutaminy, takimi jak glutaminian, alfa-ketoglutaran i ornityna. Są one substratami do produkcji pirolidyno-5-karboksylanu, który jest także produktem degradacji proliny przez PRODH/POX w celu wytworzenia ATP, sprzyjając pro-przeżyciowej autofagii lub generowania ROS indukujących apoptozę. Konwersja proliny, ornityny i glutaminianu może zatem regulować zależną od PRODH/POX apoptozę/autofagię. Kluczowym aminokwasem jest prolina, krążąca między mitochondriami a cytoplazmą w cyklu zwanym cyklem prolinowym. Jest on sprzężony ze szlakiem pentozowo-fosforanowym produkującym nukleotydy niezbędne do biosyntezy DNA. PRODH/POX jest również powiązany ze szlakami zależnymi od p53 i AMPK. Dostępność proliny dla zależnej od PRODH/POX apoptozy/autofagii jest regulowana na poziomie wzajemnych przemian proliny, ornityny, glutaminianu i α-ketoglutaranu, łącząc metabolizm aminokwasów z cyklem kwasów trikarboksylowych (TCA) i cyklem mocznikowym oraz pośrednio z łańcuchem transportu elektronów. Sugeruje to istnienie specyfiki molekularnej komórki nowotworowej, która może ulec zmianie pod wpływem działania MET. W związku z tym, przy użyciu analizy metabolomicznej określono profil metabolomiczny komórek nowotworowych poddanych działaniu MET. Profil ten analizowano w komórkach MCF-7 typu dzikiego (MCF-7WT) poddanych działaniu MET oraz komórkach MCF-7 ze znokautowanym PRODH/POX (MCF-7crPOX), wytworzonym przy użyciu technologii CRISPR-Cas9. Proliferację komórek określano za pomocą CyQUANT® Cell Proliferation Assay, natomiast cykl komórkowy analizowano za pomocą cytometrii przepływowej z użyciem Nucleo-Counter NC-3000. Ekspresję białek proapoptotycznych oceniano metodą

Western blot. Analizę metabolomiczną (obejmującą stężenie wewnątrzkomórkowej proliny, kwasów glutaminowego, ornityny, glutaminy, kwasu α-ketoglutarowego, glukozy, pirogronianu, bursztynianu i innych) przeprowadzono za pomocą wysokosprawnej chromatografii cieczowej sprzężonej z tandemową spektrometrią mas z potrójnym kwadrupolem (LC-MS/MS/QqQ).

Wykazano, że MET hamowała proliferację komórek MCF-7WT jak i MCF-7crPOX inkubowanych zarówno w podłożu zawierającym i w podłożu pozbawionym glutaminy. Hamowanie tego procesu było jednak wyraźniejsze w przypadku braku glutaminy w podłożu hodowlanym. Odsetek komórek w fazie G2/M do komórek w fazie G0/G1 wykazał, że zarówno traktowanie komórek MET, albo nokaut PRODH/POX silnie hamowały proliferację tych komórek hodowanych w podłożu bez glutaminy. Nie wykazano takiego efektu w komórkach hodowanych w obecności glutaminy. MET indukowała ekspresję AMPK (induktor PRODH/POX) w obu liniach komórkowych niezależnie od obecności lub braku glutaminy. Efekt ten był wyraźniejszy w komórkach hodowanych w podłożu bez glutaminy. W przypadku braku glutaminy MET indukowała ekspresję aktywnej formy PARP oraz kaspazy 7 w obu liniach komórkowych. W obecności glutaminy efekt ten był widoczny tylko w komórkach MCF-7 typu dzikiego (MCF-7WT).

W nieobecności glutaminy, traktowanie MET lub nokaut PRODH/POX w komórkach MCF-7 przyczynił się do podobnego zahamowania glikolizy (drastyczny wzrost wewnątrzkomórkowej glukozy i pirogronianu) oraz wzrostu zużytkowania kwasu fosfoenolopirogronowego, glukozo-6-fosforanu i niektórych metabolitów cyklu TCA oraz cyklu mocznikowego (obniżenie wewnątrzkomórkowego stężenia), przyczyniając się do indukcji apoptozy. Jednakże, w obecności glutaminy, traktowanie komórek MCF-7 MET lub nokaut PRODH/POX przyczyniał się do utylizacji niektórych badanych metabolitów (z wyjątkiem glukozy), przyczyniając się do pro-przeżyciowego fenotypu komórek MCF-7 w tych warunkach.

Uzyskane wyniki sugerują, że MET lub nokaut PRODH/POX przyczynia się do hamowania proliferacji komórek (zahamowanie to było wyraźniejsze w przypadku braku glutaminy) oraz indukcji apoptozy poprzez przeprogramowanie metabolizmu aminokwasów, cyklu TCA, cyklu mocznikowego i szlaku pentozowo-fosforanowego w tych komórkach.

Analiza metabolomiczna w komórkach hodowanych z glutaminą lub bez niej sugeruje, że glikoliza jest ściśle powiązana z metabolizmem glutaminy i proliny. W przypadku braku glutaminy, traktowanie MET lub nokaut PRODH/POX przyczynia się do zahamowania glikolizy (głodu glukozowego) i apoptozy w tych komórkach. Wyniki tych badań pogłębiają wiedzę o mechanizmie przeciwnowotworowego działania MET i sugerują, że skojarzone działanie MET z inhibitorami syntezy glutaminy może stanowić przedmiot dalszych badań nad eksperymentalną terapią raka piersi.

SCIENTIFIC ACHIEVEMENTS:

1. List of publications constituting the doctoral dissertation

1. Huynh, T.Y.L., Zareba, I., Baszanowska, W., Lewoniewska S., Pałka, J. Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pox)-dependent apoptosis/autophagy as an approach to targeted cancer therapy. Mol Cell Biochem, 2020, 466, 35–44. DOI: 10.1007/s11010-020-03685-y. Impact Factor ISI: 3.396. MSWiA: 70 points.
2. Huynh, T.Y.L, Ościłowska, I., Sáiz, J., Nizioł, M., Baszanowska, W., Barbas, C., Pałka, J. Metformin Treatment or PRODH/POX-Knock out Similarly Induces Apoptosis by Reprograming of Amino Acid Metabolism, TCA, Urea Cycle and Pentose Phosphate Pathway in MCF-7 Breast Cancer Cells. Biomolecules. 2021 Dec 15;11(12):1888. DOI: 10.3390/biom11121888. Impact Factor ISI: 4.879. MSWiA: 100 points.

2. List of other scientific publications

1. Ościłowska, I., Rółkowski, K., Baszanowska, W., Huynh, T.Y.L., Lewoniewska, S., Nizioł, M., Sawicka, M., Bielawska, K., Szoka, P., Miltyk, W., Pałka, J. Proline dehydrogenase/proline oxidase (PRODH/POX) is involved in the mechanism of metformin-induced apoptosis in C32 melanoma cell line. Int. J. Mol. Sci. 2022, 23(4): 2354. DOI: 10.3390/ijms23042354. Impact Factor: 5.924. MNiSW: 140.000.
2. Ościłowska, I., Huynh, T.Y.L., Baszanowska, W., Prokop, I., Surażyński, A., Galli, M., Zabielski, P., Pałka, J. Proline oxidase silencing inhibits p53-dependent apoptosis in MCF-7 breast cancer cells. Amino Acids. 2021, 53(12), 1943-1956. DOI: 10.1007/s00726-021-03013-8. Impact Factor: 3.520. MNiSW: 100.000.
3. Lewoniewska, S., Ościłowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Pałka, J. Troglitazone-induced PRODH/POX-dependent apoptosis occurs in the absence of estradiol or ERβ in ER-negative breast cancer cells. J Clin Med, 2021, 10(20): 4641. DOI: 10.3390/jcm10204641. Impact Factor: 4.242. MNiSW: 140.000.
4. Baszanowska, W., Lewoniewska, S., Misiura, M., Huynh, T.Y.L., Zaręba, R.,

Baszanowska, H., Prokop, I., Rysiak, E., Ościłowska, I., Rółkowski, K. Kosmetologia onkologiczna. Sytuacje trudne w ochronie zdrowia. T. 6. Red. Agnieszka Lankau, Elżbieta Krajewska-Kułak. Uniwersytet Medyczny w Białymstoku, 2021, 295-302. MNiSW: 20.000.

5. Misiura, M., Baszanowska, W., Lewoniewska, S., Huynh, T.Y.L., Nizioł, M., Zaręba, R., Ościłowska, I., Rysiak, E., Rółkowski, K. Stosowanie produktów naturalnych w pierwotnej profilaktyce chorób nowotworowych. Sytuacje trudne w ochronie zdrowia. T. 6. Red. Agnieszka Lankau, Elżbieta Krajewska-Kułak. Uniwersytet Medyczny w Białymstoku, 2021, 346-353. MNiSW: 20.000.
6. Karna, E., Szoka, Ł.M., Huynh, T.Y.L., Pałka, J. Proline-dependent regulation of collagen metabolism. Cell Mol Life Sci. 2020 May;77(10):1911-1918. DOI: 10.1007/s00018-019-03363-3. Impact Factor: 9.261. MNiSW: 140.000.
7. Zaręba, I., Huynh, T.Y.L., Kazberuk, A., Teul, J., Klupczyńska, A., Matysiak, J., Surażyński, A., Pałka, J.Overexpression of prolidase induces autophagic death in MCF-7 breast cancer cells. Cell Physiol Biochem. 2020 Sep 12;54(5):875-887. DOI: 10.33594/000000275. Impact Factor: 5.141. MNiSW: 140.000.
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9. Lewoniewska, S., Baszanowska, W., Misiura, M., Nizioł, M., Zaręba, R., Huynh, T.Y.L., Zaręba, I., Rysiak, E., Prokop, I. Rola badań profilaktycznych wśród osób pracujących. Problemy promocji zdrowia. Red. Małgorzata Żendzian-Piotrowska, Michalina Krzyżak, Agnieszka Paszko, Mateusz Maciejczyk. Uniwersytet Medyczny w Białymstoku, 2020, 223 – 232. MNiSW: 20.000.
10. Baszanowska, W., Baszanowska, H., Misiura, M., Zaręba, R., Lewoniewska, S., Huynh, T.Y.L., Święcki, A., Rysiak, E., Prokop, I., Zaręba, I. Rola mediów społecznościowych w promocji zdrowia - zalety i zagrożenia. Problemy promocji zdrowia. Red. Małgorzata Żendzian-Piotrowska, Michalina Krzyżak, Agnieszka Paszko, Mateusz Maciejczyk. Uniwersytet Medyczny w Białymstoku, 2020, 181-192. MNiSW: 20.000.
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12. Misiura, M., Nizioł, M., Baszanowska, W., Lewoniewska, S., Zaręba, R., Huynh, T.Y.L., Zaręba, I. Co powinniśmy wiedzieć o interpretacji wyników badań laboratoryjnych? Promocja zdrowia w praktyce klinicznej. Red. Małgorzata Żendzian-Piotrowska, Agnieszka Paszko, Mateusz Maciejczyk, Michalina Krzyżak. Uniwersytet Medyczny w Białymstoku, 2020, 143-154. MNiSW: 20.000.
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3. List of congress reports

1. Lewoniewska, S., Ościłowska, I., Huynh, T.Y.L., Baszanowska, W., Prokop, I., Pałka, J. The impact of estrogen receptor activation on troglitazone - induced apoptosis in breast cancer cells. 4th European Life Sciences | PhD & Postdocs Symposium "Exploring Life Dynamics: in and out of equilibrium", Milan (online), 12-14 May 2021.
2. Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Ościłowska, I., Pałka, J. Proline dehydrogenase/ Proline Oxidase knockdown inhibits p53dependent

apoptosis in MCF-7 breast cancer cells. EMBO Workshop "Systems approaches in cancer". 21-26 September 2021. Split, Croatia. Abstract book (43).

3. Lewoniewska, S., Prokop, I., Huynh, T.Y.L., Baszanowska, W., Zaręba, I. Estrogeny regulują PRODH/POX-zależną indukcję apoptozy. III Poznańska Konferencja "Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia". Poznań, konferencja on-line, 04-05.05.2020, 75-76.
4. Baszanowska, W., Huynh, T.Y.L., Lewoniewska, S., Zaręba, I., Pałka, J. Metformina indukuje PRODH/POX-zależną apoptozę/autofagię w komórkach raka piersi MCF-7. III Poznańska Konferencja "Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia". Poznań, konferencja on-line, 04-05.05.2020, 74.
5. Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Zaręba, I., Pałka, J. The mechanism for proline dehydrogenase/proline oxidase-dependent regulation of apoptosis/autophagy in MCF-7 breast cancer cells. III Poznańska Konferencja "Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia". Poznań, konferencja on-line, 04-05.05.2020, 77.
6. Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Zaręba, I., Pałka, J. Down-regulation of p53 expression in proline dehydrogenase/proline oxidase (PRODH/POX) - dependent pathways in MCF-7 breast cancer cell. 2019 International Conference: Korean Society for Molecular and Cellular Biology (KSMCB), Seoul, Korea. 2019.09.30-10.02, 134.
7. Zaręba, I., Baszanowska, W., Lewoniewska, S., Huynh, T.Y.L., Prokop, I., Rysiak, E., Pałka Jerzy. Down-regulation of estrogen receptor function facilitate proline dehydrogenase/proline oxidase-dependent apoptosis in MCF-7 breast cancer cells. XVIII International Congress of Medical Sciences. Sofia, Bulgaria. 09-12 may 2019, 63.
8. Baszanowska, W., Huynh, T.Y.L., Zaręba, I., Lewoniewska, S., Cywoniuk, A., Petelska, E., Pałka, J. Metformin inhibits collagen biosynthesis facilatating proline availability for PRODH/POX-dependent apoptosis/autophagy in MCF-7 breast cancer cells. XVIII International Congress of Medical Sciences. Sofia, Bulgaria. 09-12 may 2019, 87.
9. Huynh, T.Y.L., Lewoniewska, S., Baszanowska, Weronika, ., Zaręba, I., Pałka, J. POX-dependent inhibition of P53 expression in MCF-7 creast cancer cell. XVIII International Congress of Medical Sciences. Sofia, Bulgaria. 09-12 may 2019, 85.

10. Lewoniewska, S., Baszanowska, W., Huynh, T.Y.L., Zaręba, I., Petelska, E., Surażyński, A., Pałka, J. AMPK induces apoptosis/autophagy in brest cancer MCF-7 cells through proline dehydregenase/proline oxidase (PRODH/POX). XVIII International Congress of Medical Sciences. Sofia, Bulgaria. 09-12 may 2019, 84.

4. List of other scientific activities

4.1. Grants:

- Ph.D. Fellowship of Marie Skłodowska-Curie-Horizon 2020 COFUND:

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 754432 and the Polish Ministry of Science and Higher Education, from financial resources for science in 2018-2023 granted for the implementation of an international co-financed project.

- ImPRESS project 2021-2022, Medical University of Bialystok, Poland:

"Metabolomic approach to understand molecular mechanism of AMPK-inducing agents on PRODH/POX-dependent apoptosis/autophagy in breast cancer cells" (project number MNS/2/H2/21/001/2214)

- ImPRESS project 2020, Medical University of Bialystok, Poland

"Examination of the effect of AMPK-inducing agents on PRODH/POX-dependent apoptosis/autophagy in MDA-MB-231" (project number MNS/2/H2/19/001/2214)

- ImPRESS project 2019, Medical University of Bialystok, Poland

"Proteomic approach to recognize targets of antineoplastic activity of AMPK-inducing agents in breast cancer MCF-7 cells" (project number MNS/1/H2/19/001/2214)

- Annual Doctoral Scholarship 2021 at Medical University of Bialystok
- Pro-quality scholarship 2021 at Medical University of Bialystok
- Annual Doctoral Scholarship 2020 at Medical University of Bialystok
- Annual Doctoral Scholarship 2019 at Medical University of Bialystok

4.2. Scientific internships/ training courses:

- EMBO | EMBL Symposium: Metabolism Meets Epigenetics, virtual meeting,

2021.

- EMBL Conference: Protein Synthesis and Translational Control, virtual meeting, 2021.
- Research Internship in CEMBIO, CEU-San Pablo University, Madrid, Spain. 16th September to 12th December, 2020.
- Training course:
  - Virtual course, "Fundamentals of light microscopy", Cellular Imaging Core Facility, CELLIM, Central European Institute of Technology, Brno, Czech Republic
  - Virtual Summer School in Translational Cancer Research, Cancer Core Europe (CCE), DKFZ.
  - Introduction to RNA-Seq and smallRNA-Seq data analysis (from ideas4biology Company), Medical University of Bialystok.
  - Alternative research methods in searching new biologically active compounds, Medicinal Chemistry Department, Medical University of Bialystok.

List of Figures

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