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ATP citrate lyase: a central metabolic enzyme in cancer

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Abstract

ACLY links energy metabolism provided by catabolic pathways to biosynthesis. ACLY, which has been found to be overexpressed in many cancers, converts citrate into acetyl-CoA and OAA. The first of these molecules supports protein acetylation, in particular that of histone, and *de novo* lipid synthesis, and the last one sustains the production of aspartate (required for nucleotide and polyamine synthesis) and the regeneration of NADPH,H⁺ (consumed in redox reaction and biosynthesis). ACLY transcription is promoted by SREBP1, its activity is stabilized by acetylation and promoted by AKT phosphorylation (stimulated by growth factors and glucose abundance). ACLY plays a pivotal role in cancer metabolism through the potential deprivation of cytosolic citrate, a process promoting glycolysis through the enhancement of the activities of PFK 1 and 2 with concomitant activation of oncogenic drivers such as PI3K/AKT which activate ACLY and the Warburg effect in a feed-back loop.

Pending the development of specific inhibitors and tailored methods for identifying which specific metabolism is involved in the development of each tumor, ACLY could be targeted by inhibitors such as hydroxycitrate and bempedoic acid. The administration of citrate at high level mimics a strong inhibition of ACLY and could be tested to strengthen the effects of current therapies.

1. Introduction

ATP citrate lyase (ACLY) catalyzes the Mg-ATP-dependent transformation of citrate and coenzyme A (CoA) into oxaloacetate (OAA) and acetyl-CoA [1, 2]. ACLY is principally located in cytosol and nucleus [3] and is linked to various diseases such as hepatic steatosis, dyslipidemia, diabetes, and numerous malignancies, such as colorectal, breast, prostate, liver, stomach, cervical cancers, osteosarcoma, and glioblastoma [2, 4-6]. Its overexpression correlates with poor differentiation and prognosis of lung adenocarcinoma [7] and acute myeloid leukemia [8].

ACLY makes the connection between the catabolism of nutrients (glucose, glutamine and fatty acid acids), biosynthesis and histone acetylation that are required for DNA transcription and replication [3, 9, 10]. As detailed below, ACLY also plays a pivotal role in cancer metabolism through the potential deprivation of cytosolic citrate, in terms of promoting glycolysis and signaling pathways supporting cancer development [4, 11-13]. Thus, ACLY appears as a key target for specific inhibition [5, 14-16].

2. ACLY structure and function

2.1. Structure

Human ACLY is a 480-k-DA tetramer with a total of 1101 amino acids (aa) (for historic references see [17]). The tetramer comprises four identical subunits, each resulting from the fusion of N-terminal citryl-CoA synthetase (CCS) module and the catalytic C-terminal citryl-CoA lyase (CCL) domain [16, 18, 19]. The N-terminal domain (residues 1-820 aa) contains the ATP, citrate and putative CoA binding sites as well as the catalytic phosphorylated His760 residue [18] (Figure1).

The catalytic reaction involves subsequent steps which have been extensively studied [16, 17]. The reaction is initiated by Mg-ATP with phosphorylation of His760 and a subsequent release of Mg-ADP; an unstable citryl-phosphate enzyme complex is formed which is then attacked by a thiol group of CoA generating citryl-CoA, an unstable complex subsequently cleaved into acetyl-CoA and OAA. Recently, electron microscopy analysis and X-ray crystallography studies have specified the atomic structure of the full-length human ACLY [18, 19]. Wei J et al, have also discovered a powerful mechanism of allosteric inhibition of ACLY activity: the citrate domain contains a hydrophobic cavity close to the citrate-binding site. The binding of the inhibitor to this cavity induces a conformational change of the CS domain of ACLY, resulting in an indirect interruption of the citrate binding [18]. As recently put forward by Verschuere et al, the catalytic C-terminal domain (820-1101 aa) has sequence homology with citrate synthase (CS), the first enzyme of the tricarboxylic acid cycle (TCA) which has a structural analogy with an ancestral tetrameric CCL operating in reverse TCA cycle [19]. This analogy suggests a molecular transition from CCL to CS (and ACLY) likely representing a key process in metabolism evolution, as recently suggested [19].

2.2 The central function of ACLY in cancer cell metabolism

For a better understanding of the key roles played by ACLY, let us first present a comprehensive overview of cancer metabolism.

2.2.1. The Warburg effect sustains cancer metabolism

Cancer cells often rely on the Warburg effect (*i.e.* aerobic glycolysis with lactate production) [20], a reductive metabolism providing carbon molecules for nucleotide synthesis. This enhancement of aerobic glycolysis is related to pyruvate dehydrogenase (PDH) inhibition by pyruvate dehydrogenase kinase 1 (PDK1) [21], with PDK1 being

activated by hypoxia inducible factor 1 (HIF-1) [22] and phosphoglycerate kinase1 (PGK1) [23]. It is important to note that AKT (also named Protein Kinase B) activates PDK1 [24], as well as ACLY [1, 7, 25, 26], thereby concomitantly promoting aerobic glycolysis and lipid synthesis. This linkage is reinforced by phosphorylated forms of sugars, in particular by fructose-6-phosphate (F6P) which can increase ACLY activity up to 60 fold [27], while fructose-1,6-bisphosphate (F1,6BP) activates pyruvate kinase (PK) regulating the end part of the glycolysis (Berg JM, 2011). Basically, beside PKM1, the embryonic isoform PKM2 is frequently expressed in cancer cells. Dimeric PKM2 (which is less active than the tetrameric phosphorylated form) creates a bottle neck at the end of the glycolysis [28], thus leading to an accumulation of upstream intermediates of branched glycolysis sustaining pathways such as: - 1) the pentose phosphate pathways (PPP) providing ribose 5-phosphate (R5-P) for nucleotide synthesis and furnishing nicotinamide adenine dinucleotide phosphate (NADPH,H⁺) to cellular redox state, fatty acid synthesis (FAS) and nucleotide synthesis; -2) the serine-glycine pathway connected with folate and methionine cycles, contributing to nucleotide synthesis, methylation reactions and regeneration of glutathione required for antioxidant defense [29]; - 3) the production of dihydroxyacetone phosphate (DHAP) sustaining glycerol, entering triglycerides and phospholipid metabolism. Monomeric PKM2 translocates into nucleus where it promotes concomitant activation of both c-Myc and cyclin D1, thus enhancing cell cycle progression in G1 phase, glycolytic genes expression and the active transcription of HIF-1 (for references, see [30]).

Pyruvate is preferentially transformed into lactate by lactate dehydrogenase A (LDH-A or LDH-5), an enzyme promoted by HIF-1 and AKT [31]. Lactate is then expelled in the microenvironment by monocarboxylate transporters (MCT), where it suppresses immune response and favors invasiveness. It is noteworthy that PDH inhibition down-regulates the functioning of the TCA cycle, thus reducing mitochondrial synthesis of citrate [4, 11, 29, 32,

33] (Figure 2). Importantly, pyruvate can also be transformed into alanine by glutamate-pyruvate transaminase (GPT) and/or can replenish the TCA cycle in OAA after carboxylation by pyruvate carboxylase (PC). This last pathway is generally linked to glutaminolysis providing nitrogen molecules for biosynthesis as well as carbon molecules - glutaminolysis being especially activated when glucose is lacking [29, 34].

The Warburg effect promotes aggressiveness and resistance to chemotherapy by many processes linked to cell proliferation in case of severe hypoxia, with inhibition of immune response, oxidative stress and apoptosis, extracellular acidity and angiogenesis [35]. In accordance, a high uptake of ^{18}F -fluorodeoxyglucose (^{18}F -FDG) at positron emission tomography (^{18}F -FDG PET scan) is correlated with poor differentiation and lower survival in several hundred patients with cancers of various origins [36]. Therefore, the reliance on the Warburg effect is often considered the Achilles' heel for cancer cells, especially if it turns out to be irreversible because of chronic hypoxia, loss of mitochondria, mutated proteins of respiratory chain (for references see [35]) and/or because it is supported by "robust" oncogenic signals such as HIF-1, RAS and phosphatidylinositol 3-kinase-serine/threonine kinase AKT (PI3K-AKT) [37].

However, cancer metabolism is not unique and many cells are capable to adapt (and are likely to select) the changes occurring in their microenvironment (in O_2 and H^+ concentrations, nutrients, fibroblasts, immune and inflammatory cells) [38, 39]. Therefore, many cancer cells are not inherently glycolytic, preferentially relying on oxidative or intermediate metabolism, while other cells appear capable to shift from one mode of functioning to another: for example, the switch from reductive to oxidative metabolism would favor the mobility of breast cancer cells [40]. Furthermore, some cancer cells can rely on fatty acid oxidation (FAO), in particular those growing in adipose rich environments (like breast tumors or ovarian tumor cells developing in peritoneum) [41]. Accordingly, the inhibition of

OXPPOS and FAO leads to cancer cell death in triple- negative breast cancer cells in *in vitro* and *in vivo* studies [42].

These examples illustrate the plasticity of cancer cell metabolism, which confers to tumor cells many possibilities to adapt to changing conditions. This flexibility is illustrated by cell cycle progression which appears mainly sustained by a reductive metabolism although the oxidation process may temporarily provide more ATP for G1/S transition, S progression and chromosome segregation [30].

2.2.2. The Warburg effect is promoted by oncogenic factors and loss of suppressive controls

The reprogramming of metabolism is supported by oncogenic factors such as HIF-1, c-Myc and various oncogenes, in particular K-RAS promoting aggressive phenotypes in lung, colonic and pancreatic cancers. HIF-1, MYC, and RAS (with their down targets MEK-ERK and PI3K-AKT) promote aerobic glycolysis [37], in particular by up-regulating glucose transporters with high glucose affinity (such as GLUT 1), glycolytic enzymes (such as PFK1, PKM2 and LDH-A). It is noteworthy that MYC also sustains mitochondrial glutamine metabolism [43] which very likely supports an important part of cytosolic citrate synthesis instead of that derived from glucose [44].

All the oncogenic factors aforementioned are not counteracted by active suppressors such as tumor protein 53 (TP53), Phosphatase and TENsin homolog (PTEN) or AMP-activated protein kinase-Liver kinase B1 (AMPK- LKB1) (for review, see [45]). Importantly, the nuclear localization of Forkhead box O (FoxO) - a transcription factor considered as playing a tumor suppressor role in many cancers - is prevented by active AKT stabilized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoting the end part of the

glycolysis' functioning; this inhibition of FoxO nuclear localization precludes Bcl-6 expression leading to Bcl-xL overexpression [46].

2.2.3. How ACLY promotes the Warburg effect

Active ACLY heavily consumes cytosolic citrate deriving from transformation of nutrients, in particular glucose, glutamine and FA [29, 47]. Citrate can be synthesized by CS into mitochondria and it is then exported by the carrier (CiC) or exchanged with malate by the citrate-malate shuttle [9].

The glycolytic flux is regulated by phosphofructokinase1 (PFK1) which itself is inhibited by ATP, citrate and NADH,H⁺, knowing that a high level of these molecules reflects a high energy state. In contrast, low levels and increasing concentration of AMP promote PFK1 activity (for reference [9]).

In cases where the metabolism is reductive and/or high ACLY activity is sustaining biosynthesis, the negative feed-back exercised by ATP and citrate on PFK1 is avoided; furthermore PFK1 activity is strongly enhanced by the intracellular alkaline pH promoted by the down-regulation of mitochondria associated with the Warburg effect (for reference see [35]). Moreover, fructose-2,6-biphosphate (F2,6P) - the product of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK2) - also named PFKFB also "boosts" PFK1 [48]. This bidirectional enzyme is often expressed in cancer as the PFKFB3 isoform. It is induced by hypoxia and HIF-1 [49]. Because of its very high kinase activity, PFKFB3 promotes glycolysis [50]. The inhibition of its phosphatase activity reinforces the Warburg effect since fructose-1,6-bisphosphatase (FBPase) suppresses HIF-1 α activity [51]. It is noteworthy that PFK1 as most glycolytic enzymes and PFKFB3, promotes cancer proliferation by non-metabolic functions involving survival pathways, resistance to apoptosis, and cell cycle progression (for references see [30]).

Therefore, the concomitant activation of the Warburg effect [24] and ACLY by AKT [1, 7, 25, 52, 53] maintains the citrate gauge at low level [4, 11, 25, 33], this being a condition for enhancing PFK1 and PFK2 activities [48, 54] while the inactivity of FBPase promotes HIF-1 α [51]. It is also important to note that, F1,6BP – as the product of PFK1 – binds Son of Sevenless homolog 1 (*Sos1*), thus promoting the activation of Ras and its downstream targets, MEK-ERK and the PI3K-AKT-mTOR axis [55]. Therefore, this initiates a vicious cycle created between PFK1 and the inactive FBPase and the major oncogenic drivers of the Warburg effect (HIF-1 α and AKT) [37], resulting in the enhancement of PFK1 and ACLY in a positive feedback loop (Figures 2 and 3). Of note, PFK1 is also coupled with the oncogenic YAP/TAZ signaling pathway promoting tumor growth, immunosuppression and metastasis [56].

2.2.4. ACLY can also consume citrate derived from glutaminolysis

Instead of glycolysis, citrate can derive from glutamine metabolism because glutaminase 1 (GLS1) transforms glutamine into glutamate, a molecule which is then transformed into α -ketoglutarate (α -Keto) either by glutamate dehydrogenase (GLUD) or by GPT transamination (with concomitant transformation of pyruvate into alanine) [29, 34]. Then, cytosolic carboxylation of α -Keto produces isocitrate which is transformed into citrate by an isocitrate dehydrogenase 1 (IDH1) reverse reaction [44]. Of note, α -Keto is sometimes transformed into 2-hydroxyglutarate (2-HG) by mutant IDH1 (cytosolic) and/or mitochondrial IDH2, in particular in glioma and acute myeloid leukemia (AML), 2-HG altering DNA methylation and stabilizing HIF-1 (for references, see [35]).

The concentration of OAA into the matrix is very low and needs to be regenerated for TCA cycle functioning either by PC [57] or malate dehydrogenase (MDH). In the case of the

Warburg effect, the activity of the malate-aspartate shuttle is reduced [9, 58]. Therefore, ACLY can sustain replenishment of the TCA cycle by sustaining a major part of the cytosolic production of pyruvate: OAA is first transformed by MDH into malate, a molecule which is then transformed into pyruvate by the malic enzyme (ME) with concomitant production of NADPH,H⁺ sustaining cellular redox reactions, FAS and nucleotide synthesis [9]. PC then converts pyruvate into mitochondrial OAA while alanine inhibits PK [59]. As a result, the glutamine metabolism replenishes the TCA cycle either in α -Keto or in OAA [34] while several amino acids and molecules such as fumarate, succinyl-CoA (derived from ketone bodies (KB)) can also participate in mitochondrial replenishment supporting anaplerosis and biosynthesis. It is noteworthy that PC overexpression is associated with aggressive tumors such as KRAS-pancreatic ductal adenocarcinoma (PDAC) [60].

2.2.5. *How ACLY may consume citrate derived from FAO*

Cancer cell metabolism can sometimes rely mainly on FAO which provides high amount of acetyl-CoA, ATP and NADH,H⁺, all molecules promoting PC activity in an allosteric manner [59, 61]. This regulation associated with the inhibition of PDH by acetyl-CoA, promotes gluconeogenesis, whereas glycolysis is nearly switched off by a high level of citrate derived from the increased acetyl-CoA production [59]. This so called “Randle cycle regulation” prevents concomitant activation of glycolysis and gluconeogenesis. However cancer cells may circumvent this rule [47] since the huge lipid synthesis, with more than 90% of triacylglycerol FAs *de novo* being produced, [62] triggers a “leak” in the citrate gauge. This abnormality establishes futile cycles with a possible concomitant activation of catabolic and anabolic pathways such as concomitant glycolysis and gluconeogenesis, whereas a great amount of waste products and/or nutrients are excreted or lost, in particular lactate, NH₄⁺,

alanine and even glutamine [9, 38]. FAO and FAS can also be concomitantly activated by chronic acidosis as demonstrated in *in vitro* and *in vivo* experiments: - while cytosolic reductive carboxylation of α -Keto sustains citrate formation for FAS, acidic pH down-regulates nuclear acetyl-CoA carboxylase (ACC2), thus inducing histone deacetylation resulting in FAO activation [63].

2.2.6. *ACLY produces two crucial molecules for biosynthesis: OAA and acetyl-CoA*

2.2.6.1. *Oxaloacetate sustains the production of aspartate entering in nucleotide and polyamine synthesis*

ACLY reaction produces OAA, a molecule as we have seen, can be converted by two successive reactions into pyruvate. However, OAA can also sustain aspartate synthesis by feeding aspartate transaminase (GOT1). Indeed, this transaminase transforms OAA and glutamate into aspartate and α -Keto. Aspartate is an essential molecule sustaining nucleotide synthesis. It can also replenish the truncated urea cycle sustaining polyamines synthesis involved in many processes of cancer development (gene expression, invasion and metastasis) (for more details and figures, see [29, 38]).

2.2.6.2. *Acetyl-CoA sustains protein acetylation, in particular that of histone*

Acetyl-CoA sustains the acetylation process of histone and of other various proteins. As described below (cf. regulation), glucose metabolism promotes the activation of ACLY at the posttranscriptional level by sustaining its acetylation process.

The acetylation process of histone is performed within the nucleus by histone acetyltransferases (HATs), particularly in phase G1, and opens chromatin structure for DNA transcription. The acetylation process of histone is promoted by alkaline intracellular pH

[64]. It has to be noted that *de novo* FAS seems to be preferentially activated in phase G2 for membrane formation, since, in phase G1, the accumulation of cyclin D1 inhibits lipid synthesis [65].

Glycolysis essentially supports the progression of phase G1, *i.e* the “growth phase”, by consuming nutrients in abundance. At this time, ACLY sustains an enhanced production of acetyl-CoA and OAA. Basically, acetyl-CoA does not cross the nuclear envelope in contrast to citrate and acetate which diffuse freely through the nuclear pore complex. Nuclear ACLY is therefore activated, thus representing the essential determinant of histone acetylation in multiple mammalian cells [3]. However, acetyl-CoA synthase (ACSS1), using acetate as direct substrate, can also participate in providing nuclear acetyl-CoA [66] as well as PDH which may translocate from mitochondria to the nucleus during phases G1 and S [67]. Interestingly, in adipocytes, nuclear ACLY participates in the regulation of the genes involved in glucose metabolism because its silencing suppresses the expressions of GLUT4 (a transporter with very high glucose affinity), HK2, PFK-1, LDH-A, and GOT1 [3]. Further studies are needed to confirm the presence of this regulation process in cancer cells.

~~Glutaminolysis is mainly activated from late G1 to mitosis (for references see because GLS1 is only degraded in late mitosis, in contrast to PFKFB3 (the main activator of PFK1) which is degraded at onset of S phase. In late S phase and G2 phase, *de novo* lipid synthesis sustains membrane formation while histone deacetylation (required for chromosome condensation before mitosis) is promoted by acidic pH and NAD⁺ dependent sirtuin deacetylases (SIRT6). NAD⁺-SIRT1 are stimulated by low nutrient conditions, reflected by an increase of the ratio NAD⁺/NADH,H⁺ .~~

Protein acetylation by cytosolic acetyl-CoA seems to be an ancestral reversible regulation process in life sciences in almost all metabolic enzymes from bacteria to human

[68]. For example, high glucose conditions enhance the acetylation process of PKM2 and ACLY, thus promoting nucleotide and lipid biosynthesis [10, 69]. In such conditions, most of the cytosolic acetyl-CoA is derived from ACLY and sustained by glycolysis, although a significant part can be produced by glutamine, especially when glucose is lacking. However when citrate is lacking, acetyl-CoA can be directly produced in two different ways reflecting a particular aggressiveness: - either from acetate by ACSS2 [66] or from glycolysis through transketolase-like 1 (TKTL1) reaction, with TKTL1 transforming xylulose-5-phosphate (X5P) into glyceraldehyde 3-phosphate (G3P) and acetyl-CoA [70]. It is noteworthy that N-alpha-acetylation of caspase-2, caspase-3, and caspase-9 is reduced in cells overexpressing the anti-apoptotic protein Bcl-xL which also reduces the mitochondrial export of citrate [46, 71]; such process reinforces the Warburg effect.

2.2.6.3. *ACLY sustains lipid and sterol synthesis, isoprenylation and protein glycosylation*

Acetyl-CoA sustains *de novo* lipid synthesis which is dramatically increased in cancer cells [72], This synthesis requires reduced NADPH, H⁺ cofactor regenerated by PPP or ME. *De novo* lipid synthesis is enhanced during phases S and G2 because it is promoted by cyclin D3 and low molecular weight isoforms of cyclin E (LMW-E), LMW-E being associated with ACLY to sustain the production of acetyl-CoA [73]. Acetyl-CoA carboxylase (ACC) catalyzes and regulates the first step of FAS leading to palmitate. Although ACC is physiologically activated by citrate in normal cells [59], in cancer cells however, it is activated by PI3K-AKT even if the citrate level is low [52].

Acetyl-CoA also sustains the mevalonate (MVA) pathway leading to cholesterol, which is required for membrane formation and/or for sustaining isoprenoids synthesis [74]. The first step of MVA pathway is catalyzed by acetyl-CoA acetyltransferase or thiolase (ACAT) that provide acetoacetyl-CoA for hydroxymethylglutaryl CoA (HMGCoA) synthase,

a reaction followed by another reaction catalyzed by HMGCoA reductase and inhibited by statins.

ACLY also sustains the formation of farnesyl pyrophosphate or geranylgeranyl-pyrophosphate. The covalent addition of these molecules to proteins results in their isoprenylation, a process playing a key role in membrane attachment and protein functionality [74].

In addition, acetyl-CoA feeds the glucosamine pathway, which is a branched glycolysis pathway sustaining the glycosylation process of proteins and involved in many processes of cancer development (epigenetics, transcription, signaling, DNA repair, proteins folding, maturation and trafficking, glucose uptake and metabolism) [75].

The regulation orienting acetyl-CoA derived from ACLY towards one of these pathways remains poorly studied. In KRAS-pancreatic ductal adenocarcinoma (PDA), growth factors promote AKT-ACLY signaling: the MVA pathway supports acinar-to-ductal metaplasia (tumorigenesis) while the increasing histone acetylation supports the development of tumoral cells [60].

3. ACLY regulation

Just like glycolytic enzymes [30], ACLY expression likely oscillates during cell cycle, depending on gene expression and epigenome regulation (in particular in relation to histone acetylation/deacetylation), while ACLY enzymatic activity fluctuates based on citrate availability, the activation by phosphorylation and acetylation and the concentration of the enzyme, depending on the degradation by ubiquitin ligases.

3.1. Regulation by citrate concentration

ACLY activity is highly dependent on the cytosolic generation of citrate. Cells with aerobic glycolysis generate less citrate than MYC-positive cancer cells which both stimulate glucose and glutamine metabolism [76, 77]. As we explained, ACLY up-regulation maintains the citrate gauge at a low level. Such process reinforces the Warburg effect through the activation of PFK1 linked to Ras and its down target PI3K-AKT [24, 55] while the inactivity of FBPase promotes HIF-1 [51]. Therefore, a vicious circle promotes the concomitant activation of aerobic glycolysis and ACLY activity while cytosolic citrate is highly consumed.

3.1.1. Transcriptional regulation

Oncogenic signaling pathways deregulate the activity of the sterol regulatory element-binding proteins 1 (SREBP1) while the transcriptional factor regulates the expression of genes encoding ACLY and of many enzymes pertaining to the MVA pathway [78]. ACLY is transcriptionally up-regulated by SREBP-1, thus generating cytosolic acetyl CoA for cholesterol and FAS as shown in human hepatoma [79] and senescent adipocytes [80].

3.1.2. Post-transcriptional regulation

ACLY is activated by phosphorylation and acetylation, and degraded by ubiquitinylation. The balance of these processes most likely establishes the oscillation of enzymatic activity during the cell cycle.

3.1.2.1. Regulation by small interfering RNAs

MicroRNAs (miRNAs) are non-coding small RNAs which negatively regulate gene expression. The miR-22 molecule is involved in the posttranscriptional regulation of ACLY

thus supporting metastasis in breast, osteosarcoma, prostate, cervical and lung cancers growth [6].

~~Of note, ACLY depletion by small interfering RNAs blocks elongation of the fatty acid chain in triglyceride (TG) from C16 to C18, but not in other lipid classes. The accumulation of TG down regulates expression of the mitochondrial transporter of fatty acid carnitine palmitoyltransferase1A (CPT1A), this process likely participating to inhibit FAO when FAS is activated.~~

3.1.2.2. Phosphorylation

ACLY is allosterically activated by covalent phosphorylation induced by phosphorylated sugars, in particular fructose-6-phosphate (F6P) which can increase its enzymatic activity up to 60 fold [27].

ACLY phosphorylation is also promoted by PI3K/AKT [1, 7, 25, 26]. Active AKT phosphorylates ACLY on serine 454 in normal rat adipocytes [53], a site of stimulation being previously recognized for cAMP-dependent protein kinase, insulin, glucagon and β -adrenergic receptor agonist-isoproterenol [81]. ACLY is phosphorylated at Ser455 upon DNA damage [82]. In vascular smooth muscle cells (VSMC), insulin-like growth factor 1 (IGF-1) induces AKT and GSK-3 β phosphorylation as well as that of the IGF-1R β subunit, this activation process being inhibited by curcumin in a dose-dependent fashion [83]. The occurrence of these last mentioned regulations in cancer cells remains to be further investigated.

Interestingly, ACLY physically interacts with the catalytic subunit AMPK and thus inhibits AMPK activity; ACLY knockdown activates AMPK under conditions leading to p53 activation and ultimately to cellular senescence or DNA damage-induced cell death [84].

3.1.2.3. Acetylation and deacetylation

Covalent lysine acetylation has been found to play a broad and critical role in the regulation process of virtually every metabolic enzyme as observed in human leukemia cancer cells [85] and liver tissue, in which the concentration of metabolite fuels (glucose, amino acids and fatty acids) influence the acetylation status of hundreds of metabolic enzymes [68].

The enzymatic activity of ACLY is stabilized by acetylation at lysine residues 540, 546, and 554 (ACLY^{3K}). This process coordinates glucose availability and enhances *de novo* lipid synthesis. Indeed, under high glucose concentration, ACLY lysine acetylation is stimulated by P300/calcium-binding protein (CBP)-associated factor (PCAF) also known as lysine acetyltransferase 2B (KAT2B) [10]. *Such* acetylation process blocks ACLY degradation by ubiquitinylation whereas ACLY^{3K} accumulation promotes lipid synthesis, especially since ACLY^{3K} is not destabilized by deacetylase sirtuin 2 (SIRT2) which competitively modifies the same lysine residues [10]. By these processes demonstrating a rapid turn-over (minutes to hours) [86], a high-glucose condition promotes ACLY activation at post-transcriptional level. In contrast, poor-nutrients conditions promote rapid deacetylation and degradation.

3.1.2.4. Activation by direct binding to the antizyme

ACLY is also regulated by antizyme (AZ), a protein promoting the degradation of polyamines (*i.e.*, of putrescine, spermidine, and spermine) because it targets ornithine decarboxylase (ODC) for proteasome degradation [86]. ODC represents the first enzyme of polyamine synthesis. AZ activates ACLY through direct protein-protein interaction, the binding of which results in an increasing acetyl-CoA production leading to spermidine and

spermine acetylation and to their inactivation [87]. In this way, ACLY functioning is linked to polyamines metabolism and degradation.

3.1.2.5. ACLY degradation by ubiquitination by CUL3–KLHL25 ubiquitin ligase

Cullin3 (CUL3) is a core protein from the CUL3-RING ubiquitin ligase complex which has often been reported to be down-regulated in lung cancer. This complex degrades ACLY through ubiquitination after having been interacting with the ACLY adaptor protein, KLHL25 (Kelch-like family member 25) [88].

Interestingly, ubiquitin-specific peptidase 13 (USP13) specifically inhibits degradation by ubiquitination and thus upregulates ACLY in ovarian cancer. This finding may lead to the development of USP13 inhibitors [89].

4. Therapeutics

There is nowadays a considerable interest to specifically target ACLY in patients with obesity and dyslipidemia because ACLY is positioned upstream of cholesterol and FAS. In contrast, none of the many ACLY inhibitors listed in recent reviews as potential anti-cancer molecules [5, 15, 16] have been tested in human controlled trials. However, this strategy could be interesting, knowing that short hairpin RNA targeting ACLY stopped proliferation and induced apoptosis in a variety of cancer cell lines including prostate, liver and lung cancer cells [14, 90]. *In vitro* and *in vivo* experiments in non-small cell lung cancer cells showed that ACLY knockdown inhibits tumor growth and downregulates the mitogen-activated protein kinase (MAPK) and PI3K/AKT axis promoted by the K-Ras mutation or EGFR gene [25]. The anti-tumor effects of ACLY knockdown were enhanced with citrate or statin treatments [25]. However, ShRNA raises the issue of the use of efficient vectors for clinical effect.

4.1 Natural compounds

Hydroxycitrate (HCA)

HCA is extracted from the rind of the small Indian fruit *Garcinia Cambogia* and popularized as a weight-loss supplement [91, 92]. HCA is a weak competitive inhibitor of ACLY in respect to citrate ($K_i = 0.3$ mM); HCA also inhibits other metabolic enzymes such as ACC, IDH, CS, PDH and PFK [93]. This lack of selectivity could be an advantage to target the plastic metabolism of cancer cells. In three mouse cancer models (B16-F10 melanoma, MBT-2 bladder transitional cell and LL/2 Lewis lung cancers), HCA and α -lipoic acid (a cofactor of the PDH complex) reduced tumor growth with a similar efficacy as cisplatin or 5-fluorouracil [94]. This association combined with cisplatin or methotrexate has proven to be more efficient than cytotoxic drugs or than a metabolic association administered alone [95]. No controlled clinical trials have been found on ClinicalTrials.Gov. This helps confirm these interesting preclinical studies.

A few more natural ACLY inhibitors which have been listed in the literature are derived from antibiotics, such as antimycins (derived from *Streptomyces* bacteria), *Penicillium Islundicum*, or the macrolactone radicicol [16]. Interestingly, cucurbitacin B, which is a bioactive compound of cucumber, reduced the proliferation of prostate cancer cells in both *in vitro* and *in vivo* experiments with a 50% inhibitory concentration ranging about 5.0 $\mu\text{g/ml}$ [96].

A new class of ACLY inhibitors bearing key structural features of the natural product emodin has been reported as blocking the entrance of citrate to its binding site. In a dose-dependent manner, emodin inhibits the proliferation of A549 lung cancer cell line and reduces cancer stemness [97].

4.2 Synthetic compounds

Bempedoic acid (ETC-1002), a small prodrug preventing dyslipidemia in animal models, has been found well tolerated in Phase 2 and 3 *of* clinical trials and as significantly lowering LDL cholesterol levels in patients maximally tolerating statin therapy [98, 99].

SB-204990, a lactone prodrug, has been reported to suppress growth, reduce stemness and promote differentiation of human lung A549 cancer cells in *in vitro* as well as in *in vivo* experiments [14].

NDI-091143 has been recently reported as a compound promoting an extensive conformational change in ACLY, disrupting citrate from its binding site by localizing itself mainly in a hydrophobic cavity next to this binding site [18]. This compound seems to be a very potent ACLY inhibitor because its inhibitory activity is situated in the nanomolar range.

Further studies should be done to test the anti-cancer activities and toxicities of these synthetic molecules.

5. Discussion

As we have seen, ACLY plays a pivotal role in cancer metabolism by linking together glucose catabolism (but also glutamine and fatty acid catabolism) protein acetylation, lipid and nucleotide synthesis. Enhanced nucleotide and lipid synthesis are sustained by ACLY, the upregulation of this enzyme decreasing the cytosolic citrate gauge. As a result, glucose consumption, glycolysis, oncogenic signaling and proliferative pathways (in particular PI3K/AKT) are stimulated, and promote many aspects of cancer growth, as well as ACLY transcription and activity in a feed-back loop (Figure 4). Thus, ACLY appears as a key target for specific inhibition.

However, as we have seen, the metabolism of cancer cells (often plastic metabolism) is not unique. It allows some cloned cells to adapt to adverse conditions. This adaptability renders it difficult to predict the success of ACLY inhibition, considering that the ACLY production of acetyl-CoA can find alternative pathways, using glucose [70] or acetate [66] as sources of molecules instead of citrate. For instance, short hairpin RNA-mediated ACLY silencing resulted in an elevated expression of ACSS2 sustaining acetyl-CoA formation by using acetate as substrate [90].

Thus, the combination of several metabolic inhibitors is surely necessary to produce an anti-cancer effect. For example, patients with non-small lung cancer expressing GLUT1 and ACLY on tumor tissue samples, exhibit poorer overall survival compared to their negative counterpart [100]. Consequently, concomitantly targeting glycolysis with ACLY is namely essential to reinforce the effect of current treatments in these patients. It is noticeable that ACLY inhibition stimulates AKT in a feed-back loop, as observed in metastatic colon cancer [101].

However, the combination of products targeting the metabolism of cancer cells makes it difficult for randomized studies to prove the effectiveness of an add-on product, which can be ineffective as a sole product. Furthermore, choosing which metabolic inhibitory strategy should be tested in priority is also difficult considering that experimental models are far from the same clinical conditions and often give discordant results [102]. Moreover, enzymatic inhibitions can be toxic for normal cells or may inhibit the immune anti-cancer response. ACLY stimulation, for example, appears essential to activate anti-cancer M1-type macrophage [103] and expansion of cytotoxic CD8⁺ relying on enhanced *de novo* lipogenesis [104].

Thus, tailored methods should be developed to determine the different cells phenotypes coexisting within a specific tumor and to find out which metabolism supports

their development. This knowledge appears crucial to target as best as possible the metabolism of cancer cells resisting current therapies and to eliminate stem cells causing relapse and metastasis.

Pending methods capable of determining the metabolic vulnerabilities of tumors, ACLY inhibition by simple drugs such as HCA or bempedoic acid should be tested, optimally associated with glycolytic inhibitors (or glucose starvation diet) and current therapies. Considering that the pivotal role of ACLY in cancer cells is largely based on a decrease cytosolic citrate concentration, a strong ACLY inhibition could be mimicked by “flooding” the cytosol with citrate. Several *in vitro* and *in vivo* experiments have showed that citrate administered at high concentration stops cancer cells proliferation and increases sensitivity to cisplatin [11]. This strategy recapitulates most of the regulations aforementioned, *since* citrate promotes: - the inactivation of PFK1 and decreases ATP production [105]; - the inhibition of PFK2 in ascite cancer cells [54]; - the apoptosis of various cancer cell lines with the deactivation of the anti-apoptotic factor Mcl-1 and the activation of caspases such as caspase 2, 3 and 9 [11, 106]; - the downregulation of the IGF-1R/PI3K/AKT proliferative pathways with a decreased phosphorylation of AKT and ERK in non-small cell lung cancer cells [25]; - with the activation of PTEN suppressor, reversion of dedifferentiation (in particular through Snail inhibition with E-cadherin expression) and stimulation of T lymphocytes response [12]. The intraperitoneal injection of citrate sodium reduced the growth of gastric carcinomatosis [105] and its oral administration regressed various resistant models of mammary, pancreatic and lung cancer in mice, while increasing the sensitivity of tumors to cisplatin [12].

6. Conclusion

ACLY occupies a privileged position at the crossroads of the metabolism of cancer cells, having been found overexpressed in many aggressive cancers. ACLY abundantly consumes citrate from nutrient catabolism (especially glucose and glutamine) to support protein acetylation and intense nucleotide and lipid synthesis. The significant decrease in cytosolic citrate appears to play a central role in cancer metabolism by enhancing the Warburg effect and activating the PI3K / AKT axis promoting ACLY activity in a feedback loop. Thus, the inhibition of factors regulating its expression (such as SREBP1) and its activation (such as AKT) could have an anti-proliferative effect. The better knowledge of its anatomical structure with the recent discovery of a hydrophobic regulatory pocket near the citrate binding site offers hope for the future development of selective allosteric inhibitors that could enhance the effectiveness of current cancer treatments. Pending the development of such specific inhibitors which must be weakly toxic, it seems already possible to test in the light of preclinical studies, natural or synthetic inhibitory molecules currently available (such as hydroxycitrate and bempedoic acid), as well as the "citrate strategy" aimed at submerging the cytosol into citrate to inhibit glycolysis, stop the production of ATP and inhibit the PI3K / AKT proliferative pathway.

P.I. and M.A designed the manuscript, P.I. wrote the text, Z.W. and H. L prepared the figures and contributed to the literature review, A C. and L.F. participated in the writing of the comments and in the editing of the manuscript.

The Authors regret that, due to space and reference limitations, they were unable to cite all of the excellent papers that shaped their modern understanding of ACLY.

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Conflicts of Interest

The authors disclose no conflicts of interest.

Legend for figures

Figure 1. The six domains of human ACLY subunit.

The putative CoA binding site corresponds to domain 1, the catalytic phosphorylated HIS760 residue is located in domain 2, the ATP-grasp domains are formed by domains 3 and 4, the citrate binding site corresponds to domain 5, and the C-terminal domain has sequence homology to citrate synthase (CS). N-terminal citryl-CoA synthetase (CCS) module; catalytic C-terminal citryl-CoA lyase (CCL) domain

Figure 2. The pivotal role of ACLY in promoting cancer metabolism

ACLY transforms citrate into OAA and acetyl-CoA, two crucial molecules sustaining the pathways supporting tumor cell growth. The up-regulation of ACLY decreases the cytosolic citrate level. This process promotes PFK1 activity and thus glycolysis. In a positive feedback, F6P provided by glycolysis promotes ACLY activity. The proteins acetylation (in particular of ACLY and glycolytic enzymes (not figured)) sustains this perverted metabolism linking PFK1 and ACLY activities, glycolysis and biosynthesis. PFK1 also promotes pathway

PI3K/AKT that promotes the Warburg effect by up-regulating HIF-1 and PDK1; while aerobic glycolysis is promoted, TCA functioning is downregulated.

ACLY: ATP citrate lyase, F6P: fructose 6-phosphate, HIF-1 α : Hypoxia-inducible factor-1 α , PDK1: pyruvate dehydrogenase kinase1, PFK1: phosphofructokinase1, PI3K/AKkt: phosphoinositide 3-kinase/AKT also known as Protein kinase B (PKB), TCA: tricarboxylic acid cycle

Figure 3. ACLY promotes cancer cell metabolism by potentially decreasing the cytosolic citrate level.

Glycolysis transforms glucose into pyruvate which is preferentially transformed into lactate since the Warburg effect (promoted by HIF-1a) inhibits PDH. This inhibition results from the PDH phosphorylation by PDK1 (not figured), promoted by HIF-1a and AKT. This process reduces the mitochondrial synthesis of citrate and the molecule can be derived from glutamine metabolism. Citrate is transformed by ACLY into OAA and acetyl CoA. OAA sustains aspartate synthesis entering DNA and protein synthesis, while acetyl-CoA sustains lipid synthesis and protein synthesis. Enhanced de novo lipid synthesis (sustained by ACLY upregulation) maintains a low citrate gauge, which promotes glycolysis because PFK1 and PFK2 are not counteracted by a high level of citrate. In contrast, FBPase is inactivated and this inactivation promotes HIF-1 ∞ . The Warburg effect is also reinforced by the activation of RAS-PI3K-AKT pathway by F1,6BP. ACLY is stabilized by acetylation as other metabolic enzymes, and its activity *is* promoted by AKT. Through these regulations, PFK1 and ACLY are concomitantly activated.

and AKT. Ac: acetylated, ACLY: ATP citrate lyase, AKT also known as Protein kinase B (PKB), F6P : fructose 6-phosphate, F1,6P: fructose-1,6-bisphosphate, F2,6BP: fructose-2,6-biphosphate, GLUT1: membrane glucose transporter 1, HIF-1 α : Hypoxia-inducible factor-1 α ,

α -Keto: α -ketoglutarate, OAA: Oxaloacetate, PDH: pyruvate dehydrogenase, PFK1 : phosphofructokinase1, PKM2: embryonic pyruvate kinase, PDH: pyruvate dehydrogenase, PI3K: phosphoinositide 3-kinase; p110: p110 catalytic subunit of PI3K; Ras: Ras protein family members belonging to a class of small GTPase; Sos1: Son of sevenless homolog 1

Figure 4. A synthetic diagram of ACLY function resulting in the promotion of various aspects of cancer proliferation.

ACLY: ATP citrate lyase, F6P: fructose 6-phosphate, F1,6P: fructose-1,6-bisphosphate, α -Keto: α -ketoglutarate, Mitogen-activated protein kinase: MAPK, OAA: Oxaloacetate, PDK1: pyruvate dehydrogenase kinase1, PFK1 : phosphofructokinase1, PPP: pentose phosphate pathway, phosphatidylinositol-3-kinase (PI3K)/AKT (also named Protein Kinase B), TCA: tricarboxylic acid cycle

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