ATP citrate lyase (ACLY) inhibitors: an anti-cancer strategy at the crossroads of glucose and lipid metabolism

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Abstract: ATP citrate lyase (ACLY) is a cytosolic homotetrameric enzyme that catalyzes the conversion of citrate and coenzyme A (CoA) to acetyl-CoA and oxaloacetate, with the simultaneous hydrolysis of ATP to ADP and phosphate. Interestingly, ACLY is a strategic enzyme linking both the glycolytic and lipidic metabolism. In tumour cells characterized by an altered energetic metabolism, an increased glucose uptake and an accelerated glycolytic flux lead to an intensified production of mitochondrial citrate. Once transported to the cytosol, citrate is here converted by ACLY to acetyl-CoA, an essential biosynthetic precursor for fatty acid synthesis and mevalonate pathway. ACLY expression and activity proved to be aberrantly expressed in many types of tumours, and its pharmacological or genetic inhibition significantly inhibited cancer cell proliferation and induced apoptosis. Increasing evidences highlight the central role of ACLY, conferring a great therapeutic potential to this enzyme as a key target for the treatment of cancer. ACLY inhibitors, previously developed for metabolic disorders, have recently attracted interest as promising anti-cancer agents. After a brief introduction to the structure and the pathophysiological role of ACLY, this review article provides an overview of the main ACLY inhibitors reported in the literature.

Keywords: ATP citrate lyase, inhibitors, cancer, lipid metabolism

1. Introduction

1.1. ATP citrate lyase structure and function

Human ATP citrate lyase (ACLY) protein is a homotetramer of four identical subunits of about 120 KDa, with each polypeptide chain containing 1101 residues, and it shares a high homology level with the rat isoform (about 98% identity) [1,2]. ACLY is the cytosolic enzyme that catalyzes the Mg-ATP-dependent synthesis of oxaloacetate and acetyl-coenzyme A (acetyl-CoA) from citrate and coenzyme A (CoA, Figure 1) [3]. Despite the prominent cytosolic localization of ACLY, which is bound to endoplasmic reticulum in mammalian cells, ACLY was also detected in the nucleus, for example in mouse embryonic fibroblasts, murine pro-B-cell lymphoid cells, human glioblastoma and colon carcinoma cells [4,5].

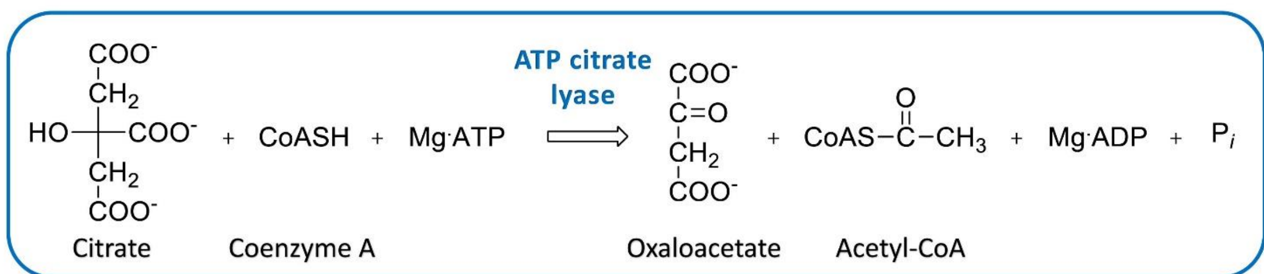

Figure 1. The reaction catalysed by ATP citrate lyase.

Many studies were performed to fully understand the ACLY catalytic mechanism, which is based on three different subsequent steps (Figure 2): 1) the initial binding of Mg-ATP to the free enzyme which catalyzes the phosphorylation of a conserved histidine residue (His760 is the phosphate acceptor in the human isoform), thus provoking the subsequent release of Mg-ADP; 2) the just formed phospho-enzyme catalyzes the formation of an enzyme-bound citryl phosphate, which is then attacked by the thiol group of coenzyme A, thus forming a thioester bond between citrate and CoA and triggering the release of inorganic phosphate; 3) at this point, the enzyme-bound

intermediate, citryl-CoA, is cleaved by a retro-Claisen reaction that leads to the formation of acetyl-CoA and oxaloacetate, which are released from the enzyme active site [6–8].

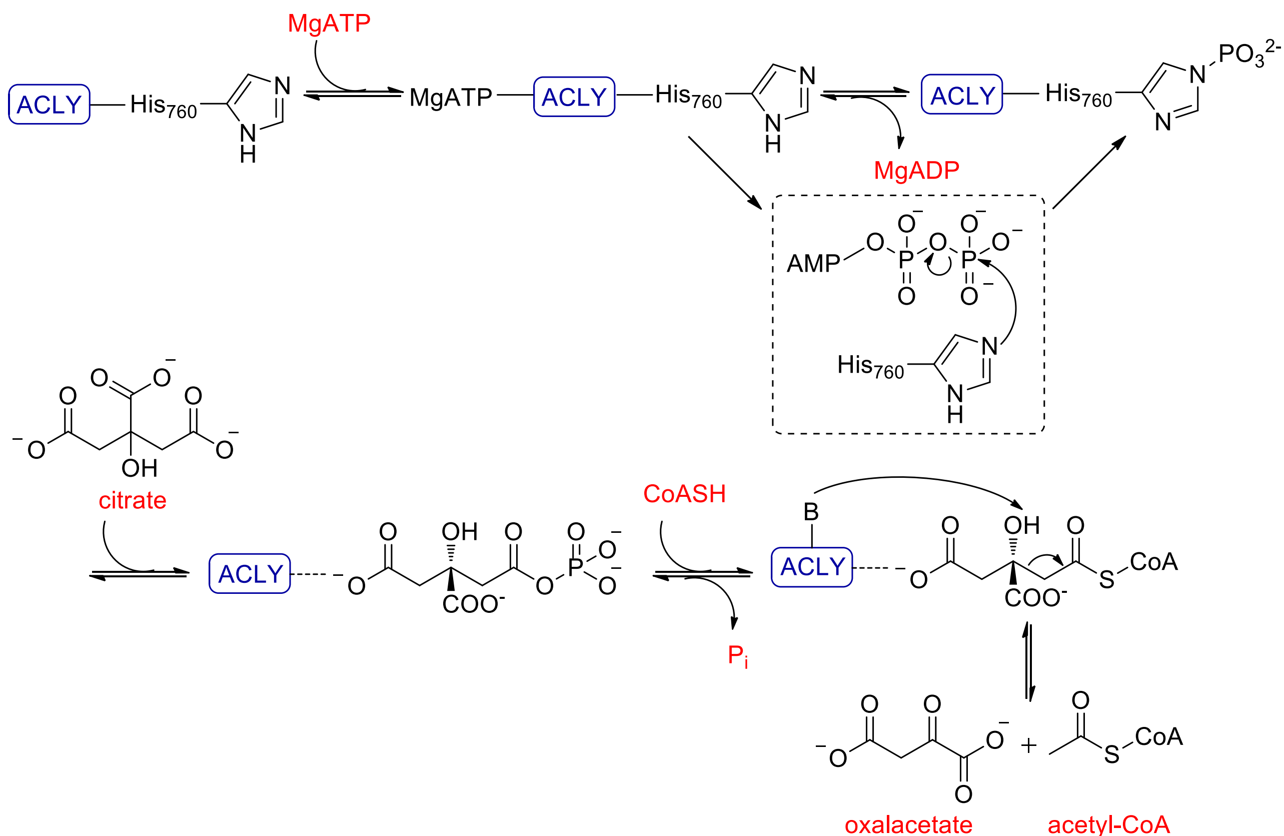

Figure 2. Mechanism of the ACLY-catalyzed reaction. The initial phosphorylation of ACLY by ATP-Mg^{2+} occurs at His760, then phosphate is transferred from His760 to citrate. Citryl-phosphate which is bound to the enzyme is then attacked by the thiol group of CoA to form the S-CoA ester and release inorganic phosphate (P_i). The dashed line between citryl-phosphate and ACLY indicates that they are not covalently bound, differently from His760 and ACLY for which the solid line highlights that His760 belongs to ACLY amino acid sequence. Lastly, the intermediate citryl-CoA undergoes a retro-Claisen reaction, promoted by the attack of an active site basic residue (B), and citryl-CoA is cleaved to oxaloacetate and acetyl-CoA, that are released from the enzyme active site.

ACLY belongs to the acyl-CoA synthetase (nucleoside diphosphate-forming) superfamily and it has five functional domains typical of all the members of this superfamily. The α-subunit has two

domains: domain 1 that binds CoA and domain 2 that contains the phosphorylated histidine residue. Domains from 3 to 5 are in the β-subunit: domains 3 and 4 adopt an ATP-grasp fold and bind ATP, whereas domain 5 interacts with domain 2 forming one of the two "power helices" containing the phosphorylated histidine residue at the N termini and it also hosts the binding site of citrate. The first attempt to obtain a crystal structure of human ACLY was made by Sun and co-workers who crystallized a truncated form of the enzyme. They identified a loop formed by residues Ser343–Thr348 that was defined as the citrate binding site. Citrate is a small organic acid that contains three carboxylic groups, one hydroxyl group, and in order to distinguish between the two terminal carboxylic groups, they are referred to as *pro*-R and *pro*-S, since citrate contains a prochiral centre (Figure 3). A strong salt bridge was present between the *pro*-R COOH moiety and Arg379, the central COOH group established hydrogen bonds with the side chain of Thr348 and the backbone nitrogen of Asn346 and Thr348, while the hydroxyl group interacted with the side chain of Thr348 and the backbone nitrogen of Gly309 (Figure 3). The *pro*-S carboxylic group was free to interact with CoA, without being involved in any interaction with the protein [9].

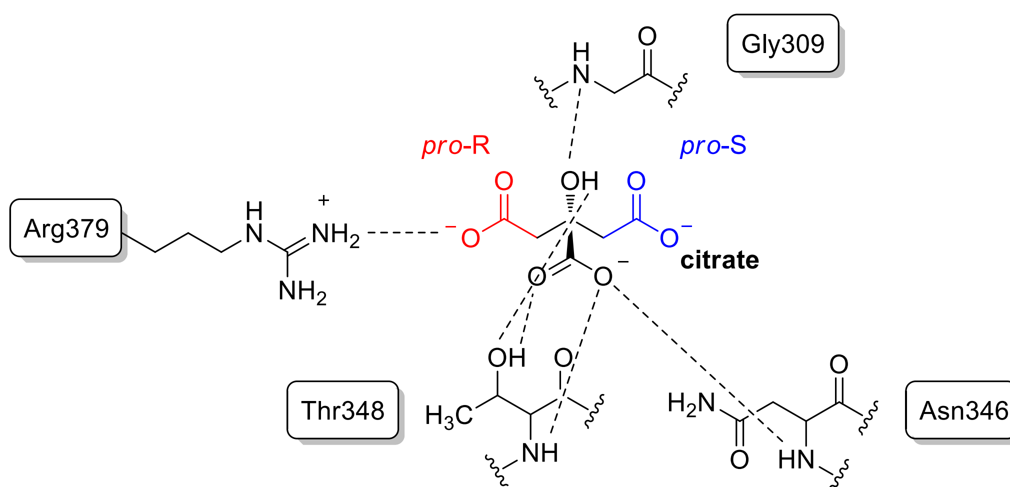

Figure 3. Main interactions of citrate with surrounding residues of the citrate binding site. *Pro*-R (in red) and *pro*-S carboxylates (in blue) are highlighted.

One year later, the amino-terminal portion of the enzyme was crystallized in the presence of the inhibitor tartrate, ATP and magnesium ions. The crystal structure showed the complex ADP-Mg^{2+} bound to the domain possessing the ATP-grasp fold and the adenine ring, the ribose moiety and the phosphate groups that established interactions with the protein residues [10]. In these first two crystal structures, information about some regions were missing, such as the loop containing His760 (phosphohistidine loop) and the residues 426–486 (termed as the linker), which are phosphorylated in the regulation of ACLY by kinases. New insights in the structure of ACLY were recently obtained by crystallizing the complex of ACLY with the inhibitor (–)-hydroxycitrate (see section 2.2). In particular, in this complex the protein was modified and the region comprising His760 was identified, but it was observed that (–)-hydroxycitrate, that was bound to the amino terminal portion of the enzyme, was not phosphorylated as expected, because the active site was crystallized in an open conformation [11].

1.2. ATP citrate lyase and cancer metabolism

Considering the position of ACLY in the cellular metabolism network, this enzyme plays a key role since it links the glucose metabolism to that of fatty acids (Figure 4). Most normal cells prefer to use dietary lipids for their need, differently cancer cells display an upregulated *de novo* fatty acid synthesis [12]. The *de novo* synthesis of fatty acids is activated by high levels of carbohydrates and low level of fatty acids: in these conditions the glycolytic process converts huge amounts of glucose to pyruvate by several enzymatic steps in the cytosol (glycolysis). The end product pyruvate is mostly excreted out of the cells as lactate, but part of the produced pyruvate enters the mitochondrial Krebs cycle and is converted to acetyl-CoA by the pyruvate dehydrogenase enzymatic complex. However, fatty acid synthesis occurs in the cytosol, but acetyl-CoA cannot exit out of the mitochondrial membrane to give its acetyl group that is necessary to start the process of lipid formation. This problem is bypassed by citrate that is formed by acetyl-CoA through citrate

synthase in the mitochondria: the two-carbon acetyl group of acetyl-CoA is donated to the four-carbon acceptor oxaloacetate to form the six-carbon compound citrate. When citrate is present at high levels, it is transported to cytosol through the tricarboxylate transporter (also known as citrate carrier) and once in the cytosol citrate acts as the substrate of ACLY, which cleaves citrate regenerating acetyl-CoA and oxaloacetate. Both these molecules - acetyl-CoA and oxaloacetate - are precursors in important metabolic pathways. Oxaloacetate can be used in the gluconeogenesis in the liver or after being reduced to malate it can return to mitochondria. Acetyl-CoA is a highly versatile building block because it represents a carrier of acyl groups: it is carboxylated by acetyl-CoA carboxylase into malonyl-CoA, and this reaction represents the first step in the synthesis of fatty acids. Acetyl-CoA is also necessary in the mevalonate pathway in which two molecules of acetyl-CoA condense and form acetoacetyl-CoA, leading to the synthesis of cholesterol. Moreover, the mevalonate pathway leads to the formation of farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, which are required in the modifications of proteins. Finally, acetyl-CoA is also required for acetylation reactions that modify proteins such as histone acetylation [13].

Considering the physiological role of ACLY in lipogenesis, historically this enzyme has been considered a drug target for the treatment of hyperlipidemia and hypercholesterolemia, since it affects both lipid and cholesterol synthesis and many compounds were specifically designed to inhibit ACLY in order to treat these pathological conditions. Nowadays, ACLY is still a valid target in this field, since ACLY inhibitors have reported positive results in human clinical trials as cholesterol-lowering drugs [14,15].

However, it is well known that many aggressive tumours present a dysregulated metabolism, for what concerns both the glycolytic pathway and the fatty acid synthesis. The altered glycolytic metabolism is a hallmark of cancer cells: it is based on a marked preferential dependence on glycolysis to produce energy with respect to the mitochondrial oxidative phosphorylation ("Warburg effect") [16]. This aspect was observed for the first time in cells with a reduced oxygen concentration, seeming dependent on the oxygen status of the cell, but further studies demonstrated

that this condition was maintained even under normoxic conditions. The modest efficiency of glycolysis in the production of ATP molecules is counterbalanced by an acceleration of the glycolytic process, since invasive cancer cells need a continuous and rapid supply of nutrients. Therefore, many tumour cells are characterized by an increased uptake of glucose, thus resulting in an augmented production of lactate in the final conversion of the glycolytic end product pyruvate by the enzyme lactate dehydrogenase (LDH) [17]. This strategy provides the sufficient energy to highly proliferating cells, and the acidification of the extracellular environment promotes the formation of metastasis. Moreover, metabolic intermediates of glycolysis are useful for macromolecular biosynthesis to meet the demand of rapidly dividing cells. The alteration of fatty acid metabolism in tumour cells is less known by the scientific community: many tumour types showed a greater "addiction" to lipids than to sugars [18,19]. Lipid uptake and storage are increased in malignant tumours to satisfy the demand of growing cells. Many cancer cells, in which the vascular supply of nutrient is limited due to the irregular growth of tumour mass, mainly rely on *de novo* fatty acid synthesis and most of the produced fatty acids are stored as monoacylglycerols inside the cell and they are used by the catabolic process of β-oxidation to produce energy on demand. Nomura and co-workers identified the enzyme monoacylglycerol lipase (MAGL) as the protein regulating the fatty acid network in cancer cells [20]. MAGL hydrolyzes monoacylglycerols into fatty acids and glycerol and the so produced fatty acids are incorporated into new cellular membranes for proliferating cells, they are used to regulate signal transduction and motility and they are also used for the synthesis of protumorigenic lipid modulators.

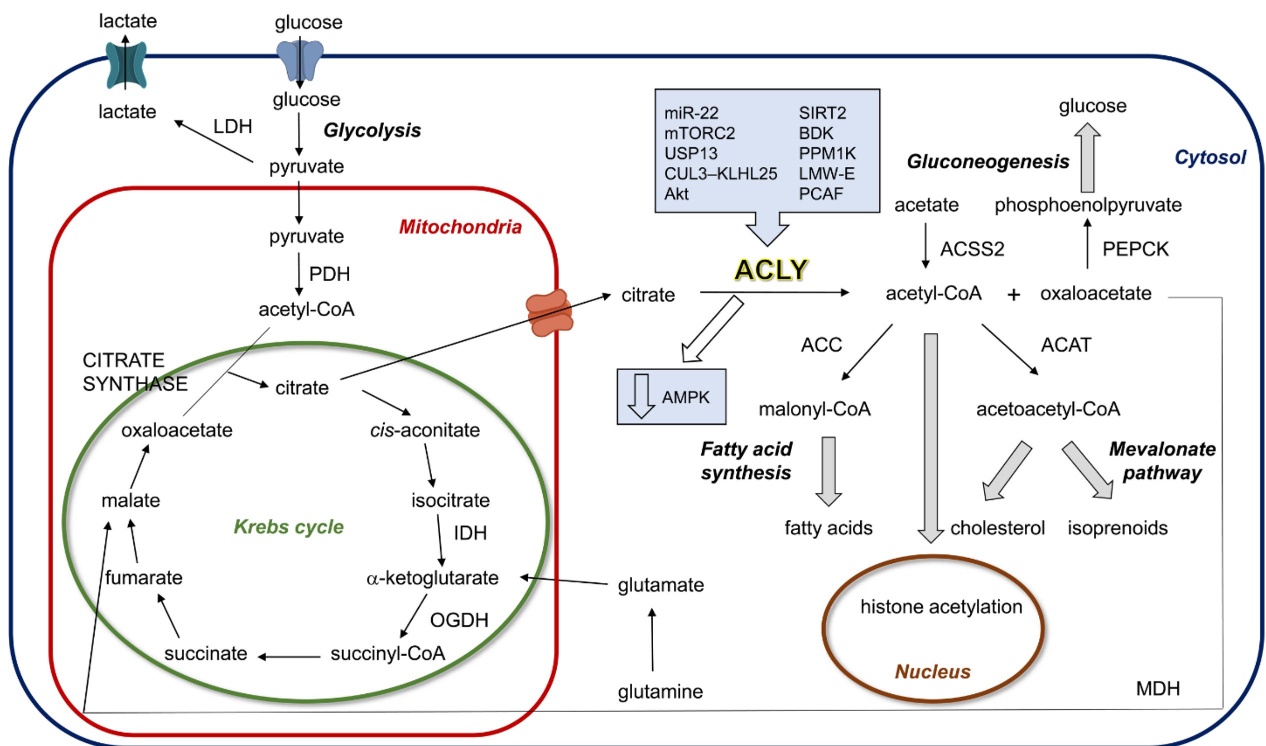

Figure 4. Schematic overview of mechanism of action of ACLY. Increased glucose uptake leads to an increased flux of glucose carbons into the mitochondria in the form of pyruvate (after glycolysis), finally leading to an increase in the mitochondrial concentration of citrate. Alternatively, pyruvate is transformed to lactate by LDH and then lactate is extruded out of the cell. Citrate is then transported to the cytosol, the cleavage of citrate by ACLY generates acetyl-CoA and oxaloacetate. Acetyl-CoA is the precursor for the synthesis of fatty acids, cholesterol, and isoprenoids. ACLY is also involved in histone acetylation in the nucleus. Oxaloacetate is reduced to malate, which is converted back to oxaloacetate in mitochondria, and is otherwise used in gluconeogenesis. Glutamine enters the Krebs cycle being converted to α-ketoglutarate. If glucose is not sufficient to produce acetyl-CoA, an alternative pathway for the generation of acetyl-CoA is from acetate by the catalytic action of ACSS2. The blue boxes show factors influencing ACLY (upper box) or that are influenced by ACLY (lower box, ACLY decreases AMPK activity). Legend: IDH = isocitrate dehydrogenase; OGDH = oxoglutarate dehydrogenase; ACSS2 = acetyl-CoA synthetase short-chain family member 2; PDH = pyruvate dehydrogenase; ACC = acetyl-CoA

carboxylase; ACAT = acetyl-CoA acetyltransferases or thiolase; MDH = malate dehydrogenase; PEPCK = phosphoenolpyruvate carboxykinase; LDH = lactate dehydrogenase.

In this multifactorial context ACLY is the interconnection between the two main metabolic alterations typical of tumours: the aberrant glucose and lipid metabolism. ACLY is the final enzyme of the glycolytic cascade, and at the same time it represents the starting point for the lipid synthesis. Coupling glucose metabolism with lipogenesis is a potential effective way to attack cancer and it could offer a novel therapeutic target: inhibition of ACLY leads to tackle both the glucose metabolism (by targeting the downstream effector) and also the lipid synthesis (by targeting the upstream effector).

ACLY was shown to be a potential anticancer therapeutic target in 2005, when it was demonstrated that its inhibition by genetic methods or a chemical inhibitor (SB-204990, see section 2.2) negatively affected the proliferation and survival of tumour cells. The genetic depletion of ACLY or its pharmacological inhibition determined a reduction in the total acetyl-CoA level as well as the glucose-dependent lipid synthesis and these two effects were accompanied by a reduction of cell proliferation. Genetic or pharmacological ACLY inhibition led to inhibition of tumour growth in xenograft tumour models, but the effect was only cytostatic, and no tumour regression was observed. Moreover, histological studies revealed that the cells in which ACLY was inhibited had a more differentiated morphology and a more organized cellular architecture, in contrast with the poorly differentiated phenotype of tumour cells. A different sensitivity to ACLY inhibition was observed in the different types of cancer cell lines: the sensitivity varied with the cellular rate of glucose utilization and lactate production, therefore highly glycolytic cells proved to be more dependent on ACLY activity for their survival. Cancer cells characterized by a high glycolytic flux need glucose for lipid synthesis, relying on ACLY activity for their growth and proliferation. This preferential addiction to the glucose-to-lipid metabolism makes ACLY a relatively safe target, since

cells with a physiological metabolism and a normal growth rate are significantly less influenced by its inhibition [21,22]. Some years later in 2012, it was confirmed that ACLY genetic silencing in several types of cancer cells exerted an antiproliferative effect, but it was observed only in cells grown with reduced lipid content. Considering that exogenous supplementation with fatty acids and/or cholesterol partially reversed this effect, it is likely that the deprivation of fatty acids and/or cholesterol led to an impaired cell growth. Zaidi *et al.* hypothesized an alternative metabolic way to produce cytosolic acetyl-CoA in order to overcome the absence of ACLY: the use of acetate as substrate and its conversion to acetyl-CoA by the cytosolic enzyme acyl-CoA synthetase short chain family member 2 (ACSS2), whose expression was increased in ACLY-deprived cells [23]. Very recently, important findings confirmed the compensation mechanism in ACLY-deficient cells: ACLY deficiency caused upregulation of ACSS2, which utilizes exogenous acetate to provide acetyl-CoA by using ATP. Cells showed a sort of metabolic flexibility to adapt to different conditions and to obtain acetyl-CoA from different sources. This glucose-to-acetate switch consequent to the absence of ACLY allowed cells to survive, however they showed an impaired proliferation rate and a low histone acetylation level, highlighting that despite the compensatory mechanism ACLY remained necessary for cellular proliferation [24]. Additionally, it was found that ACLY knockdown or inhibition has an important impact on cancer stem cells. Cancer stem cells were detected before and after ACL knockdown or inhibition by (–)-hydroxycitrate, by using stem cell markers or observing cell morphology. The number of cancer stem cells diminished after treatment in human NSCLC cell lines (A549, H1975 and H1650) and in human breast cancer cell lines (HMLE and HMLER), suggesting that ACLY activity was fundamental for stem cell maintenance. The influence of ACLY on cancer stemness was mediated by Snail (a transcription factor inducing epithelial to mesenchymal transition), since Snail was found to be reduced by ACLY knockdown. The authors of this study hypothesized that the reduced stemness could be mediated by ACLY effect on glycolysis. Citrate accumulation, resulting from ACLY inhibition or knockdown, inhibits glycolysis by affecting phosphofructokinase-1, therefore stem cells that are

principally located in hypoxic regions of tumours and rely on glycolysis are strongly affected by ACLY activity [25].

ACLY is mainly present in the liver and white adipose tissue, whereas lower levels were detected in brain, small intestine and muscle tissues. Abnormally increased levels of ACLY were observed in several tumour tissues, and ACLY represents a negative prognostic factor in several types of cancer such as non-small cell lung, colorectal, renal, epithelial ovarian, prostate, breast, bladder cancer, hepatocellular carcinoma and glioblastomas, thus reflecting the elevated activity of this lipogenic enzyme [26–35].

It was found that ACLY knockdown by small interfering RNAs caused apoptosis and growth suppression in cancer cells by generating mitochondrial ROS and cells characterized by low basal ROS levels were more sensitive to ACLY depletion. Unfortunately, the mechanism underlying the ACLY inhibition-mediated ROS production is unknown. The authors hypothesized that ACLY-induced citrate accumulation led to internalization of citrate into mitochondria to maintain the chemical equilibrium, and then the citrate could be oxidized, thus provoking ROS generation. An alternative explanation was that an increased influx of pyruvate into mitochondria promoted by ACLY inhibition could generate ROS. This concept was in agreement with the finding by Thompson research group that glycolytic cancer cells, possessing low basal ROS levels, were highly sensitive to ACLY inhibition. On the contrary, cells showing high basal ROS levels were characterized by strong antioxidant capabilities, and therefore they were able to adapt to high concentrations of ROS. This effect led to the phosphorylation of AMP-activated protein kinase (AMPK), a crucial regulator of lipid metabolism. Moreover, the ACLY induced antiproliferative effect was due to the induction of triglyceride accumulation with altered fatty acid composition [36,37].

In A549 non-small cell lung cancer ACLY knockdown led to cellular apoptosis and differentiation, with a resulting inhibition of tumour growth *in vivo*. A synergistic effect with statins, that are inhibitors of 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase (a hepatic enzyme

that plays a central role in the cholesterol biosynthesis) was observed: tumour growth was markedly reduced in ACLY-deficient cells treated with statins. The inhibition of phosphatidylinositol-3-kinase (PI3K)/Akt signalling and mitogen-activated protein kinase (MAPK) pathway resulted to be involved in the anticancer effect exerted by ACLY downregulation-statin administration. ACLY knockdown cells showed diminished phosphorylation of Akt, despite previous reports demonstrated an opposite effect, as it was postulated that ACLY was a target of Akt by phosphorylation on Ser454 [38,39]. An analysis of glucose transporter 1 (GLUT1) and ACLY expression in patients with non-small cell lung cancer revealed that high expression of these two prognostic proteins was associated with poor overall survival. This fact may be explained considering the high connection of these two proteins in the metabolic reprogramming of cancer cells: when there is a great availability of glucose, ACLY activity is increased in order to promote the glucose-to-lipid synthesis, thus conferring a growth advantage to tumour cells [40].

A recent paper reported that CUL3–KLHL25 (Cullin 3-Kelch-like family member 25) ubiquitin ligase behaves as a negative regulator for ACLY: CUL3 interacted with ACLY through its adaptor protein KLHL25 and promoted ACLY degradation. This negative regulation led to diminished lipid synthesis and cell proliferation in lung cancer cells and to reduced xenograft tumour growth. Inversely, low CUL3 expression involved high ACLY expression and poor prognosis in human lung cancer [41].

A link between pancreatic β cells and ACLY activity was observed when it was found that the prolonged exposure to the fatty acid palmitate reduced ACLY activity, since ACLY expression is present not only in tissues such as adipose tissue and liver, but also in pancreatic β cells. This effect provoked an increase in pancreatic β cell apoptosis and endoplasmic reticulum stress, and both of these effects contributed to the onset of type 2 diabetes. However, the acute inhibition of this enzyme did not impair glucose-stimulated insulin release [42].

A special focus on ovarian cancer is necessary to mention the key role played by ACLY in the highly aggressive high-grade serous ovarian cancer, characterized by the overexpression of

ubiquitin-specific peptidase 13 (USP13), that is correlated to poor clinical outcome. USP13 led to the upregulation of ACLY and oxoglutarate dehydrogenase (OGDH), which is one of the subunit of α-ketoglutarate dehydrogenase complex, an enzymatic complex involved in glutaminolysis. The roles in the cellular metabolism of ACLY and OGDH are connected by citrate, since glutamine enters the Krebs cycle being converted to α-ketoglutarate, that can be either oxidized by OGDH to succinate or it can go back to isocitrate by isocitrate dehydrogenase and then to citrate. Inhibition of USP13 reduced cell proliferation and reduced the tumorigenic potential of this lethal kind of tumour by impairing oxidative phosphorylation and glucose oxidation for lipid synthesis [43].

In breast cancer cyclin E is overexpressed and is correlated with poor prognosis. In particular, cyclin E is post-translationally cleaved into low molecular weight (LMW-E) isoforms, that are tumorigenic cytoplasmatic proteins. It was found that LMW-E proteins interact with ACLY by increasing its expression, and therefore promoting the abnormal lipid metabolism typical of tumours. ACLY inhibition by *sh*RNA reduced the invasiveness of breast cancer cells *in vitro* and xenograft tumour growth *in vivo* [44]. A further relevant study concerning breast cancer was focused on the relationship between miR-22 (a short non-coding RNA molecule able to post-transcriptionally regulate gene expression) and ACLY: miR-22 inhibited the growth and metastasis of MCF-7 cells by down-regulating the expression of ACLY [45]. Similar effects were previously investigated in osteosarcoma, prostate, cervical and lung cancer: miR-22 negatively regulated ACLY. I*n vivo* studies stressed the importance of this discovery, as miR-22-treated mice developed smaller tumours, less metastasis and were characterized by longer survivals [46]. In breast cancer, ACLY proved to be regulated by the kinase mTOR by phosphorylation at Ser455 and thus promoting the conversion of glucose to lipids. In particular, mTOR Complex 2 (mTORC2) proved to be necessary for ACLY activity since mTORC2-ACLY axis promoted an enhanced lipogenic metabolism [47].

A role of ACLY in cellular senescence was identified: ACLY knockdown in normal cells led to cellular senescence and activation of tumour suppressor p53, however supplementation of acetyl-

CoA did not rescue cells from p53 activation. The mechanism underlying this effect is the ACLY interaction and the subsequent inhibition of AMPK activity. Therefore, in ACLY deprived cells, AMPK was activated and led to the phosphorylation and activation of p53, which ultimately promoted DNA damage and the consequent apoptosis or cellular senescence in both normal and cancer cells [48]. Furthermore, lipogenesis is an event involved in senescence, because most membranous subcellular organelles increase their mass and membrane lipids as well as expression of lipogenic enzymes are enhanced, including ACLY. The expression of lipogenic enzymes was found to be regulated by sterol regulatory element-binding protein (SREBP-1), that was also up-regulated during senescence. SREBP-1 activation is involved in lipogenesis during cellular senescence, being a clear indicator of the lipogenesis status of the cell. siRNA-mediated ACLY or SREBP-1 knockdown diminished cellular senescence, confirming that lipogenesis initially activated by SREBP-1 regulation and then supported by ACLY activity was fundamental for senescence [49,50].

ACLY can be regulated by acetylation at three lysine residues (Lys540, Lys546 and Lys554) and this process was found to be stimulated by P300/calcium-binding protein (CBP)-associated factor (PCAF) acetyltransferase in conditions of high levels of glucose. Acetylation promoted ACLY stability by blocking its degradation and consequently it increased fatty acid synthesis from glucose source and cell proliferation. Conversely, the enzyme deacetylase sirtuin 2 (SIRT2) deacetylated and destabilized ACLY [51].

An important discovery was made by the research group of C. B. Thompson: histone acetylation is regulated by ACLY in response to growth factor stimulation and during cellular differentiation. The expression of the glucose transporter GLUT4 and three glycolytic enzymes, hexokinase 2 (HK2), phosphofructokinase-1 (PFK-1) and lactate dehydrogenase A (LDH-A), were suppressed upon ACLY silencing, confirming that ACLY-dependent changes in histone acetylation affected glucose metabolism [5].

Branched-chain amino acids (BCAA) are strongly associated with metabolic diseases, in particular with the aberrant lipid and glucose metabolism of cancer. Inhibition of the branched-chain ketoacid dehydrogenase kinase BDK and overexpression of the phosphatase PPM1K lowered circulating BCAA, improving the metabolic profile (glucose tolerance and triglyceride levels) of fatty rats in *in vivo* studies. ACLY was found to be a substrate of these two enzymes: BDK phosphorylated ACLY at Ser455 thus activating it; conversely PPM1K overexpression led to a decrease in phosphorylation of ACLY [52].

This review will provide an overview of ACLY inhibitors, both of synthetic and natural origin (Tables 1 and 2), with the aim of summarizing the present status of published ACLY inhibitors, and focusing the attention on their relevance as potential anti-cancer agents.

1.3. ATP citrate lyase enzymatic assays

ACLY activity is usually measured to test the inhibitory potency of ACLY inhibitors. There are three main methods: 1) an indirect coupled enzymatic assay in the presence of the enzyme malate dehydrogenase (MDH) or chloramphenicol acetyltransferase (CAT), 2) a homogeneous assay by using [^{14}C]citrate and 3) an ATP-based bioluminescent method.

The first method is based on an indirect measurement of ACLY enzymatic activity, since a second enzyme is introduced in the assay to generate a quantifiable response. In the MDH coupled enzymatic assay, once the product oxaloacetate is formed, it is then converted to malate by MDH, with the simultaneous oxidation of NADH to NAD^+. Therefore, the initial ACLY activity was correlated with NADH level, by detecting the change in absorbance observed at 340 nm [53]. A variation of this kind of assay involves the use of CAT, which catalyzes the acetylation of its substrate, chloramphenicol. [^{14}C]citrate was converted by ACLY to radiolabeled acetyl-CoA, whose acetyl group is then transferred to chloramphenicol by CAT. Extraction with an organic solvent, lyophilisation and resuspension allow the deposition of [^{14}C]acetyl-chloramphenicol on a thin-layer chromatography (TLC) plate. The resulting radioactive spots are scraped and the radioactivity is

quantified in a scintillation counter. This more laborious technique correlates the radioactivity with ACLY activity, through several procedural steps [54,55].

The second method was developed by Ma *et al.* and is based on the detection of [^{14}C]acetyl-CoA, which is the product of the ACLY catalyzed reaction, starting from [^{14}C]citrate. This direct homogenous assay is able to specifically detect [^{14}C]acetyl-CoA thanks to the use of an appropriate reagent (MicroScint-O), without interferences with [^{14}C]citrate. Moreover, it proves to be suitable to high-throughput screening and allows the use of less amount of enzyme [56].

The third method, initially developed for kinase assays, is also applied to ACLY enzymatic tests, due to the involvement of ATP in the reaction. The ADP-Glo™ is a bioluminescent assay based on different steps: firstly, the enzymatic reaction is terminated and the remaining ATP is depleted, then the formed ADP is converted back to ATP and the newly formed ATP is used in a luciferase/luciferin reaction to generate a light signal. The generated luminescent signal resulting from the assay is thus correlated with the amount of produced ADP and is therefore proportionally correlated with the level of ACLY activity [57]. This last method has some advantages: it is eco-friendly compared to previous radioactive assays, it shows a high sensitivity and it is suitable for a wide range of ATP concentrations. For all these reasons, the ADP-Glo™ is predominantly adopted for testing the more recent ACLY inhibitors [58].

2. ACLY inhibitors

2.1. Natural inhibitors

The first discovered and the most well-known ACLY inhibitor is (–)-hydroxycitric acid or (2*S*,3*S*)-2-hydroxycitrate [59] (compound **1**, HCA, Figure 5), which was isolated for the first time from fruit rinds of *Garcinia cambogia*, but it is also present in other species, that are *Garcinia indica* and *Garcinia atroviridis* [60]. HCA showed a K_i value of 8 μM on ACLY from rat liver, however it was also found capable of inhibiting several other targets, such as the enzymes acetyl-coA carboxylase,

isocitrate dehydrogenase, aconitase, citrate synthase, pyruvate dehydrogenase and phosphofructokinase [61,62]. Compound **1** is more active than its lactone form, that showed a K_i value in the range 50-100 μM on rat liver ACLY [62]. On the ACLY isoform extracted from human liver, HCA proved to be a weak competitive inhibitor with respect to citrate (K_i = 0.3 mM) [63]. Many studies demonstrated the ability of this inhibitor to reduce lipogenesis and suppress fatty acid synthesis. HCA tested at high concentrations (in the millimolar range) in human liver HepG2 cells proved to affect cholesterol homeostasis, by decreasing its biosynthesis, thus resulting in an increase of low-density-lipoprotein (LDL) receptors and HMG-CoA reductase levels. These two effects were triggered by the decreased rate of the cholesterol synthetic pathway [64]. The hypolipidemic effects of HCA were confirmed in animal models, in which HCA reduced triglyceride levels and lipogenesis [65]. HCA has been extensively studied both *in vitro* and *in vivo* for its promising effects for weight loss and regulation of lipogenesis and it was included, alone or in combination with other agents, in many formulations for these purposes [66]. Just to cite an example, the highly bioavailable calcium and potassium salt of HCA [67] was combined with other two components that regulate appetite and weight, that are niacin-bound chromium and a *Gymnema sylvestre* extract, and the effects of this combination on weight loss were monitored in moderately obese subjects. This combination was able to regulate lipid profile and improve weight control [68]. It was conceived a combination of HCA calcium salt with α-lipoic acid (namely METABLOC™). α-Lipoic acid is a cofactor of the pyruvate dehydrogenase complex, a multi-enzymatic system linking glycolysis to Krebs cycle, since it promoted the transformation of pyruvate to acetyl-CoA. In particular, α-lipoic acid is an inhibitor of pyruvate dehydrogenase kinase (PDHK1), that is able to inhibit pyruvate dehydrogenase, therefore α-lipoic acid activates pyruvate dehydrogenase. This combination found its utility in cancer models, thus shifting the traditional use of HCA combinations as weight reducing formulas towards anti-cancer applications. This combination was evaluated on the growth of tumours obtained from bladder, melanoma and lung cancer cells transplanted into mice. HCA and α-lipoic acid reduced tumour growth without any toxicity signs,

thus confirming that the synergistic inhibition of metabolic pathways that contribute to tumour maintenance and development is a useful approach to tackle cancer [69]. A further development was accomplished by the insertion of a third drug, such as the well-known chemotherapeutics cisplatin or methotrexate: HCA and α-lipoic acid combination potentiated the efficacy of the tested standard anticancer drugs for what concerns a reduced tumour growth and an increased survival in both lung carcinoma and bladder cancer animal models [70].

A screening of natural compounds, aimed at finding new agents able to block lipogenesis and to lower serum triglyceride and cholesterol levels, led to the identification of 2-chloro-1,3,8-trihydroxy-6-methylanthracen-9(*10H*)-one **2** (Figure 5), a metabolite isolated from a soil fungus. Anthrone **2** was a potent inhibitor of rat liver ACLY, with an IC_{50} value of 283 nM, calculated by the malate dehydrogenase (MDH) coupled enzymatic assay [71]. Enzymatic characterization determined its competitive behaviour with respect to the substrate citrate and mixed non-competitive inhibition against CoA and ATP. Chemical structure of **2** closely resembles anthraquinone emodin **3** (Figure 5), since emodin differs only for the absence of the chlorine atom between the two phenolic hydroxyls and the additional carbonyl group in the central ring of the scaffold (Figure 5). This slight different substitution pattern caused a complete loss of inhibition on the target and different hypotheses of the binding mode of **2** were postulated to justify the notable inhibition activity compared to **3**, but no experimental data were reported to confirm them.

The same research group identified some antibiotics of the chemical class of Antimycins from *Streptomyces* bacteria, by using a bioassay directed isolation procedure, that led to the identification of natural components showing an inhibitory activity on rat liver ACLY. Among the isolated compounds, Antimycins A2 and A8 (compounds **4** and **5**, respectively, Figure 5) were the most potent ACLY inhibitors with K_i values of about 4 μM. Each compound was identified as a couple of closely related isomers, possessing a structure that differs only for the alkyl chain on the ester substituent present on the nine-membered bis-lactone cycle: R_1 in Figure 5 is a propyl or an *i*-propyl chain for compound **4** and it is a *i*-butyl or *s*-butyl moiety for compound **5** [72].

A good ACLY inhibitory activity was observed in an aqueous ethanol extract isolated from the marine sponge *Iotrochota* sp. during a screening of natural compounds aimed at finding new ACLY inhibitors at SmithKline Beecham Pharmaceuticals. This preliminary result led to the subsequent separations of further fractions, but the pure compound Purpurone (**6**, Figure 5), responsible of such inhibitory activity, was isolated only after a mild acid hydrolysis of one of these fractions, thus leading to hypothesize that this compound was present in the form of a conjugate to sugars or proteins [73]. This polyphenolic compound showed an IC_{50} value of 7 μM and it was able to reduce fatty acid synthesis, but it failed to be cytotoxic in human liver HepG2 cancer cell line at the dose of 0.1 mg/mL.

The ACLY inhibitory activity of the 14-membered macrolide Radicicol (**7**, Figure 5), originally isolated from *Monosporium bonorden*, was discovered in 2000, when a synthesized Radicicol derivative biotinylated at the phenolic group at C17 was able to bind to a 120-kDa protein, that exactly corresponded to rat and human ACLY, as confirmed by immunoblotting studies [74]. Radicicol is a natural compound with many biological activities and its main known target is heat shock protein 90 (Hsp90). The identification of ACLY as a Radicicol target should give a more exhaustive explanation of the several biological activities of this biologically relevant macrolide. In particular, compound **7** inhibited ACLY in a non-competitive manner, showing K_i values for citrate and ATP of 13 and 7 μM, respectively. Radicicol proved to block of about 50% the glucose-induced insulin secretion in pancreatic β cells in the presence of 5 and 10 mM glucose, and this effect was mediated by ACLY inhibition, since similar effects were observed after ACLY genetic inhibition, suggesting an important role of this enzyme in the regulation of the glucose-induced stimulation of insulin [75,76].

Cucurbitacin B (**8**, Figure 5) is a natural compound belonging to the family of Cucurbitacins, one of the main classes of bioactive compounds found in cucumber. The anticancer effects of Cucurbitacin B are widely known; however, the exact mechanism has not been fully elucidated. Cucurbitacin B reduced the proliferation of PC-3 and LNCaP prostate cancer cells (IC_{50} value of about 0.1 μM) by

inducing apoptosis, without significantly affecting normal prostate epithelial PrEC cells. This tetracyclic triterpenoid derivative demonstrated its efficacy *in vivo*, since pre-treatment by oral administration inhibited tumour growth in PC-3 xenografts, without provoking any side effect. The molecular mechanism underlying this cytotoxic effect was investigated and ACLY was identified as the main molecular target, since it resulted to be downregulated after Cucurbitacin B treatment both *in vitro* and *in vivo* assays. ACLY pharmacological and genetic inhibition not only reduced its levels, but it also affected cell viability and increased apoptotic cell death in human prostate cancer cells. On the contrary, ectopic expression of ACLY led to the abrogation of apoptotic cell death induced by compound **8** [77].

A Japanese patent reported two ACLY inhibitors produced as secondary metabolites by *Penicillium Islundicum* for hyperlipidemia treatment. These compounds (**9** and **10**, Figure 5) have the same scaffold, differing only for the substituents on one of the ring (ring A, Figure 5, configuration at stereocenters was not specified). They showed similar 50% inhibitory concentration, that was of 4.0 μg/ml for compound **9** and 6.4 μg/mL for compound **10** in enzymatic assays on rat liver ACLY [78].

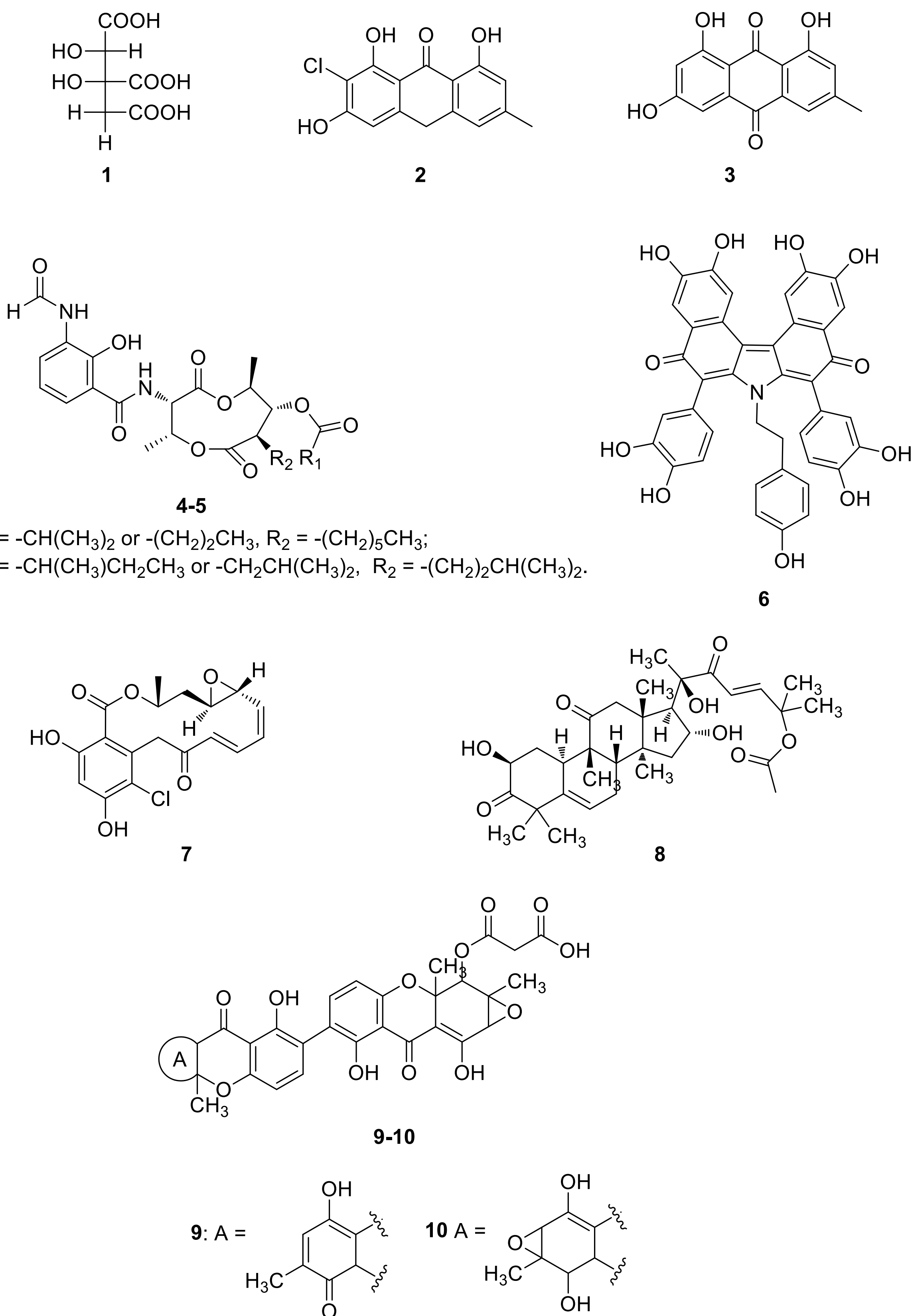

Figure 5. Natural ACLY inhibitors.

Table 1. Summary table for natural ACLY inhibitors.

Compounds	Biological activities	Used methods	Ref.
(–)-Hydroxycitric acid (**1**)	K_i = 8 µM (ACLY from rat liver) K_i = 300 µM (ACLY from human liver)	enzymatic assays	[61–70]
	reduction of lipogenesis, suppression of fatty acid and cholesterol synthesis	*in vitro* and *in vivo* studies	
	combination with other agents, to reduce weight or cancer growth	*in vivo* studies	
2-Chloro-1,3,8-trihydroxy-6-methylanthracen-9(*10H*)-one (**2**)	IC_{50} = 283 nM (ACLY from rat liver)	enzymatic assays	[71]
Antimycins A2 (**4**) and A8 (**5**)	K_i = 4 µM (ACLY from rat liver)	enzymatic assays	[72]
Purpurone (**6**)	IC_{50} = 7 µM (not specified isoform)	enzymatic assays	[73]
Radicicol (**7**)	K_i = 13 or 7 µM (ACLY from rat liver)	enzymatic assays	[74–76]
	decrease of the glucose-induced insulin secretion	*in vitro* assays	
Cucurbitacin B (**8**)	IC_{50} = 0.1 µM (antiproliferative activity in prostate cancer cells)	*in vitro* assays	[77]
	tumour growth inhibition	*in vivo* assays	
Compounds (**9**) and (**10**)	IC_{50} = 4.0 or 6.4 µg/mL (ACLY from rat liver)	enzymatic assays	[78]

2.2. Synthetic inhibitors

Dolle *et al.* at SmithKline Beecham Pharmaceuticals identified a sulfoximine ACLY inhibitor with the aim of finding potential therapeutic agents for hypolipidemic disorders. This discovery was guided by considering the reaction mechanism of the ACLY catalyzed reaction and observing that a similar behaviour was displayed by the enzyme glutamine synthetase. Compound **11** (Figure 6), a compound resembling the well-known glutamine synthetase inhibitor methionine sulfoximine [79], was synthesized and it was considered as a citrate analogue in which a terminal carboxylic group was replaced by a sulfoximinoyl moiety [80]. Once phosphorylated by the enzyme in the first step of the enzymatic reaction, compound **11** should mimic the intermediate citrate phosphate anhydride, and therefore behave as a tight-binding inhibitor. This hypothesis was confirmed by experimental

studies on rat liver ACLY: sulfoximine **11** displayed a weak and reversible binding mode with a K_i value of 250 μM.

Insertion of fluorine atoms in the citrate structure led to the synthesis of enantiomers (3*R*)- and (3*S*)-2,2-difluorocitrate (**12a** and **12b**, respectively, Figure 6) as ACLY inhibitors. The choice to use fluorine was made considering the nature of this atom that was considered a good replacement of hydrogen for its small size and similar C-F bond distance, and for the fluorine ability to act as a hydrogen bond acceptor. Moreover, considering that fluorine atom could be eliminated during the reaction course, it should lead to the formation of alkylating species, acting by an irreversible mechanism. However, neither of the two compounds acted with an irreversible binding mode, and (+)-2,2-difluorocitrate was a more potent competitive inhibitor against citrate, with a K_i value of 0.7 μM, compared to (-)-2,2-difluorocitrate (K_i = 3.2 μM). Conversely, (-)-2,2-difluorocitrate was a stronger inhibitor of the enzyme aconitase (K_i = 1.5 μM), which uses citrate as the substrate. A correlation with the absolute configuration of the compounds was not reported by the authors [81].

Three years later, the same research group developed a second ACLY inhibitor, the *cis*-expoxide **13** (Figure 6), taking into account that the mechanism for catalysis involves the participation of an active site nucleophile thiol group that directly attacks the intermediate anhydride and leads to the formation of a citrate-enzyme adduct by a covalent bond [82]. The design of the epoxide derivative was planned on the base of its high similitude with the substrate citrate and on its potential ability to covalently modify the active site nucleophile thiol group by way of opening the epoxide ring and therefore to irreversibly inactivate the enzyme. Other compounds were designed with a similar aim, but in enzymatic experiments on rat ACLY compound **13** revealed to be the most potent inhibitor, with a K_i value of 18 μM. However, the observed mechanism was reversible without a time-dependent inactivation, typical of irreversible inhibition. This unexpected mechanism led the authors to agree with a revised catalytic mechanism [83], in which the step of the active site nucleophile attack was eliminated, and it was suggested that the anhydride was directly attacked by the thiol group of CoA.

The work on ACLY inhibitors as potential lipid lowering agents was further developed by Gribble *et al.* who based their design strategy on the similarity between the CoA sites of ACLY and HMG-CoA reductase, an NADH-dependent enzyme of the mevalonate pathway. Most of HMG-CoA inhibitors contain a hydrophobic moiety, which anchors the compound in the active site, linking the two most polar portions. After the enzymatic reaction of HMG-CoA, CoA product remained bound only to the hydrophilic regions and the repulsive effects with the hydrophobic part of the enzyme facilitate the expulsion of the product. A similar situation was envisaged for ACLY, in which acetyl-CoA was released in the last step of reaction. Consequently, the authors developed compounds based on a hydrophilic "citrate-like head" linked to a lipophilic group, in the place of one of the carboxylic groups of citrate, such as the most promising compounds **14a-d** (Figure 6) [84]. All these compounds possess a 2,4-dichlorophenyl ring, linked by a chain composed of six methylene groups and a spacer, which was a sulphur atom (**14a** and **14b**), a further methylene group (**14c**) or a carbonyl moiety (**14d**), to the "citrate head". In the case of **14b** the tertiary hydroxyl group was removed. Inhibition constant values of rat ACLY were similar (K_i = 3.3, 2.6 and 2.9 μM for **14a**, **14b** and **14c**, respectively), demonstrating that the nature of the spacer was not relevant in terms of inhibition potency, as well as the presence of the tertiary hydroxyl group. Competition experiments of **14a** highlighted mixed type behaviour with respect to citrate and non-competitive binding with respect to CoA. The most potent compound **14d** (K_i = 1.2 μM), which differs from **14a** and **14c** only for the carbonyl spacer, was also able to act as an ACLY substrate, since in the absence of all the physiological substrates of the enzyme, the enzymatic reaction with **14d** produced oxaloacetate. The obtained results suggested that these compounds bound as citrate with their butanedioic acid moiety and that their affinity was modulated by interactions between their lipophilic portion and an adjacent hydrophobic binding region. Unfortunately, butanedioic acids did not reach their initial goal that was the reduction of cholesterol or fatty acid synthesis in human hepatoma HepG2 cell line, since their carboxylic groups strongly limited their cell membrane permeation.

Later, the lack of cell penetration by diacid derivatives was solved by developing their prodrugs [85]. Compound **15** (SB-201076, Figure 6), closely resembling previous derivative **14c** with an additional hydroxyl group in the 5-position of the citrate moiety, was a potent human and rat ACLY inhibitor with the same K_i value of 1 μM, but devoid of any activity in cell-based assays. The lactonization of the hydroxy-acid derivative **15** generated the thermodynamically favoured five-membered ring prodrug **16** (SB-204990, Figure 6). The cell-penetrant lactone **16** reduced cholesterol and fatty acid synthesis of 91% and 82%, respectively, when tested at the maximum concentration of 30 μM in HepG2 cells, however at higher concentrations it resulted to be toxic to cells. Oral administration of **16** to rats confirmed its *in vivo* bioactivation to the corresponding active compound **15**: after 7 days of treatment it efficiently reduced plasma cholesterol (up to 46%) and triglycerides (up to 80%). Similar results were obtained in dogs, but after longer times (15 days) and to lesser extent, since cholesterol concentration was reduced up to 23% and triglyceride levels up to 38% [86,87]. The same prodrug strategy was applied to the best inhibitor, the tricyclic derivative **17** (Figure 6), that displayed a K_i value of 220 nM on human ACLY, but its lactone prodrug failed to show activity in *in vivo* models. SB-204490 **16** tested at 100 μM markedly decreased acetyl-CoA content in platelet cytoplasm and suppressed platelet aggregation in diabetic patients, in which blood platelet activity is usually increased, thus emphasizing a therapeutic role of ACLY in diabetes [88]. Further subsequent studies were performed by Thompson research group to demonstrate that similar effects were produced by pharmacological or genetic inhibition of ACLY and led to evident anticancer effects, as anticipated in the introduction of the present review [21]. Interleukin-3-dependent cell lines were used to study the effect of the lactone **16** on cancer cell growth since interleukin-3 stimulates glycolysis and cell proliferation. Pre-incubation of cells with **16** provoked a dose-dependent decrease in D-[6-^{14}C]glucose-dependent lipid synthesis, and treatment of cells with **16** for 24 hours determined an inhibition of proliferation and treated cells displayed a dose-dependent G1 cell cycle arrest. Moreover, human lung cancer A549, prostate cancer PC3 and ovarian carcinoma cell SKOV3 cell lines proved to be time- and dose-dependent

sensible to treatment with **16**, with IC_{50} values of 24, 26 and 53 μM, respectively. SKOV3 cells were the most resistant to **16**, since they showed a reduced glucose utilization and lactate production compared to the other two cell lines, due to a lower rate of glycolysis. Very interesting results derived from the use of a genetic model of cytochrome c null mouse embryonic cell lines, in which the tricarboxylic acid cycle is not functional, thus these cells depend only on glycolysis for ATP production. Cytochrome c null cells proved to be highly resistant to compound **16**, demonstrating that these cells had a reduced dependence on glucose-dependent lipid synthesis and ACLY activity. *In vivo* evaluation of **16** revealed that tumour growth was inhibited in both A549 and PC3 xenografts, however without manifesting a tumour regression, and in agreement with the results obtained *in vitro* no effects were observed after treatment of SKOV3 xenografts.

Thompson *et al.* patented the application of an ACLY inhibitor and/or a tricarboxylate transporter inhibitor as an effective anticancer strategy able to induce apoptosis in cancer cells. This strategy was planned considering that highly glycolytic cells produce large amounts of pyruvate, that can enter the Krebs cycle after transformation to acetyl-CoA and that cells manage this increased production of acetyl-CoA by exporting it, after its conversion to citrate. This synergistic combination might inhibit the exit of citrate from mitochondira to the cytosol and at the same time its conversion to oxaloacetate. However, the patent was focused on the biological effects provoked by ACLY inhibitors in tumour cells. The ACLY inhibitor (–)-hydroxycitrate **1** inhibited cell survival at millimolar concentrations, whereas compound **15** was effective at lower concentrations in the micromolar range. The lactone prodrug **16** reduced cell viability by increasing mitochondrial hyperpolarization and thus inducing apoptosis. A particular focus was made on the relationship between cells that have undergone oncogenic transformations and efficacy of ACLY inhibitors: cells in which the oncogenic kinase Akt is activated or cells deprived of the tumour suppressor PTEN were more sensitive to treatment with ACLY inhibitors and decreased their glycolytic rates [89].

In 1985 it was observed the ability of the dicarboxylic acid derivative Medica 16 (β,β'-methyl-substituted a,ω-dicarboxylic acid, compound **18** in Figure 6) to inhibit lipid synthesis in the liver of mice, with a concomitant reduction of cytosolic acetyl-CoA, resulting in a marked hypocholesterolemic effect. The synthesis of this long-chain acid was planned on the base of the similarity with long-chain fatty acids, since it behaves as a normal fatty acid except for the dimethyl substitution in position 3 of the carbon chain that prevents the β-oxidation, in order to act as a non-metabolizable fatty acid mimic [90]. After the initial *in vivo* evaluation, the mode of action of compound **18** was investigated *in vitro:* it reduced fatty acids and cholesterol synthesis in cultured rat hepatocytes, by inhibiting rat liver ACLY competitively to citrate, with a K_i value of 16 μM [91,92]. However, compound **18** was not selective for ACLY, because both the diacid form and the CoA-monothioester of **18** proved to inhibit also the hepatic enzyme acetyl-CoA carboxylase, thus contributing to the observed hypolipidemic effect [93,94]. Compound **18** administered in an animal model for obesity-induced insulin resistance sensitized liver, muscle and adipose tissue to insulin, moreover the insulin-induced hepatic glucose production decreased and the insulin-induced total-body glucose disposal increased [95].

A similar dicarboxylic acid, 3-thiadicarboxylic acid (1,10 bis(carboxymethylthio)decane, compound **19** in Figure 6), in which the geminal dimethyl substitution in β position was replaced by a sulphur atom, reduced plasma levels of triglycerides and cholesterol in a nephrotic hyperlipidemic animal model, due to its inhibitory activity on ACLY, and on the enzymes fatty acid synthase and HMG-CoA reductase [96,97].

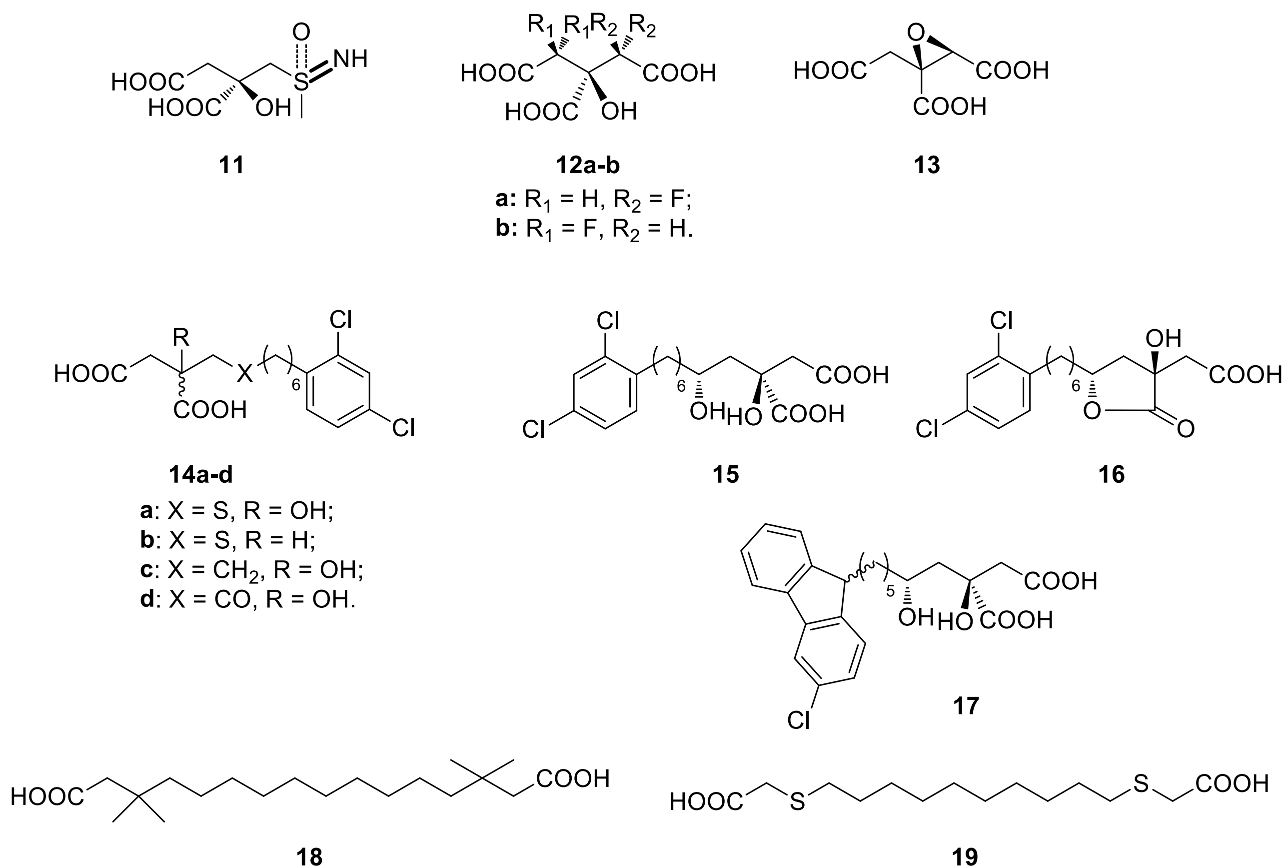

Figure 6. Synthetic ACLY inhibitors **11-19.**

Esperion Therapeutics, Inc. company developed compound ETC-1002 also named bempedoic acid (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid, compound **20** in Figure 7) as a therapeutic agent for patients with elevated low-density lipoprotein cholesterol (LDL-C). At present this investigational drug has completed Phase 1 and Phase 2 clinical studies and has entered Phase 3 trials [98,99]. ETC-1002 is a prodrug that needs activation by very long-chain acyl-CoA synthetase-1 (ACSVL1) to the active form ETC-1002-CoA (compound **21**, Figure 7). This activation occurs mainly in liver in which ACSVL1 is highly expressed, thus limiting the effects of ETC-1002 only in this organ [100]. The active thioester derivative **21** was able to modulate both ACLY and AMPK. ACLY was inhibited and AMPK was allosterically activated by the active compound, nevertheless genetic and pharmacological inhibition of ACLY as well as *in vivo* studies highlighted that only ACLY was responsible of the LDL-C decrease, LDL receptor upregulation and attenuation of

atherosclerosis, independently from AMPK activity. The great advantage of using this compound instead of statins for the treatment of dyslipidemia was the absence of myalgia typical of statin administration. Myalgia derives from the inhibition of the muscular isoform of HMG-CoA reductase. Considering that ACSVL1 is absent in skeletal muscle, the consequent activation of ETC-1002 does not take place, thus avoiding the myotoxicity associated with statins. Kinetic studies revealed that ETC-1002-CoA conjugate was a competitive inhibitor of ACLY with respect to CoA, showing a K_i value of 2 μM, and non-competitive with respect to citrate and ATP. More recent *in vivo* studies confirmed the therapeutic potential of compound **20**, since it ameliorated dyslipidemia, hyperinsulinemia and obesity in mice fed with a high-fat, high-cholesterol diet and it displayed anti-atherogenic and anti-inflammatory effects [101].

In 2017 the use of combinations of compounds for the treatment of cardiovascular pathologies due to hyperlipidemic conditions was patented. In particular, the authors claimed the possibility to use a combination of an ACLY inhibitor, such as ETC1002, with an AMPK inhibitor. The important role of AMPK in fatty acid oxidation, lipolysis, lipogenesis and glucose uptake may lead to a beneficial synergistic action with an ACLY inhibitor, thus making the use of two anti-hyperlipidemic compounds a less toxic alternative to statins. Moreover, it was envisaged a further therapeutic improvement by the addition of a cholesteryl ester transfer protein inhibitor, that increased high density lipoprotein-cholesterol (HDL-C) and decreases LDL-C concentration [102].

In a screening effort of an internal compound collection at the Bristol-Myers Squibb Pharmaceutical Research Institute to find cell-permeable ACLY inhibitors as potential agents able to reduce cholesterol and triglyceride levels, 2-hydroxy-*N*-arylbenzenesulfonamide **22** (Figure 7) showed an IC_{50} value of 130 nM in enzymatic assay on recombinant human ACLY, measured by the malate dehydrogenase-coupled enzymatic assay [103]. Compound **22** inhibited total lipid synthesis without exerting any cytotoxic action in human liver HepG2 cancer cells. Pharmacokinetic studies evaluated its oral bioavailability that was about 55% and its half-life that was relatively short (2.1 hours). This preliminary evaluation was finalized to *in vivo* studies: compound **22** was administered for 34 days

to high-fat fed mice at the daily dose of 10 or 100 mg/Kg and it lowered plasma cholesterol and triglyceride levels and also fasting plasma glucose. Treated mice exhibited a reduction in body weight without any change in food consumption or other toxicity signs, and a reduction of fat tissues. Unfortunately, the improved glycemic and lipid profile was accompanied by a certain inhibition activity (IC_{50} values in the low micromolar range) on acetyl-CoA carboxylase (ACC), therefore this collateral activity could contribute to the effects showed by compound **22**. It was found that ACLY is involved in the lipopolysaccharide-induced B lymphocyte differentiation: ACLY inhibition by compound **22** blocked the synthesis of fatty acids and cholesterol in B lymphocytes and reduced viability and differentiation of these cells [104].

In 2017 Koerner *et al.* developed a series of inhibitors inspired by the promising inhibitory activity on ACLY shown by the natural anthrone derivative **2** (Figure 5) [105]. Compound **23** (Figure 7), characterized by a chemical structure similar to **3** (Figure 5), with two carbonyl groups on the central ring and two bromine atoms on the resorcinol ring, was the most potent compound of this series, with an IC_{50} value of 2.9 μM, obtained by the enzymatic ADP Glo assay [57]. It is important to consider that **3** proved to be completely inactive despite the high similarity with **23**. Docking studies performed both on parent compound **2** and the newly synthesized inhibitor **23** suggested that these compounds occupied an allosteric hydrophobic cleft adjacent to the substrate binding domain, thus blocking the entry of citrate to the binding site and confirming the previously observed competitive mechanism of action of **2**. Moreover, compound **2** established a π-π stacking interaction between one of the two phenyl rings and Phe347 and hydrogen bonds involved the OH group in position 8 with Asp346, and the carbonyl group with Gly664. Both the chlorine atom and the methylene linker did not interact with any residue. Compound **23** adopted a similar binding pose, sharing the same interactions of **2**, with the additional carbonyl group located in a region exposed to the solvent. Compound **23** was evaluated in antiproliferative studies in A549 non-small cell lung cancer cell line: it reached a total growth inhibition at the concentration of 10 μM, as achieved by the ACLY knockdown. Moreover, **23** reduced the spheroid size of cancer stem cells and induced

apoptosis in A549 cells and HMLE-Snail breast cancer cells. The authors raised the question if other targets of **23**, as in the case of compound **3**, which has a broad spectrum of biological properties by targeting proteins different from ACLY [106], may contribute to the exerted anticancer effect *in vitro*.

Jernigan *et al.* from Harvard Medical School performed an *in silico* screening of a compound library characterized by a common furoic acid portion, prompted by previous studies [107] in which furoic acid derivatives inhibited ACLY [58]. About 2000 2- or 3-furoic acid derivatives from ZINC database were screened by a dual docking protocol and the resulting 24 compounds were purchased and screened by ADP-Glo enzymatic assay at 10 and 100 μM to determine their inhibition percentages. The resulting compounds belonged to four different chemical scaffolds and among them we mention **24a-b** (Figure 7) that are 4-substituted-2-furoic acids with a differently substituted 2-chromenone moiety, and **25** (Figure 7) that is a 5-sulfonamido-naphtofuran-3-carboxylic acid. Compounds **24a** and **24b** reached about a total inhibition at 100 μM, resulting in IC_{50} values of 4.1 and 11.9 μM, respectively, while **25** showed an IC_{50} value of 13.8 μM. For these three compounds, calculated logP values were determined and resulted to be in the range 4.4-5.1, thus correlating with good drug-like properties. Docking studies of **24a** and **25** suggested that they bound mainly in the citrate binding domain, partially overlapping with the hydrophobic allosteric domain. Their carboxylic groups formed important interactions; in particular, compound **24a** established a hydrogen bond with Ser343 and a salt bridge with Arg379, whereas **25** formed H-bonds with Asn346, Phe347 and Thr348. Both compounds were able to establish lipophilic interactions: the furan and the chromenone rings of **24a** with the side chain of Phe347 and the phenyl ring of **25** with the side chains of Phe347 and Ile597. Moreover, the ether bridge of **24a** was involved in a hydrogen bond with Gly309. Inhibition of ACLY as a strategy to reduce cancer cell stemness found a further confirmation thanks to these furoic derivatives: they were tested in E-snail cells and surface markers distinguishing cancer stem from non-stem cells were detected. Treatment with compound **25** at 10 or 25 μM for 48 hours determined a dose-dependent decrease of cancer stem cells and an increase

in the cancer non-stem cell population, thus supporting the important role of ACLY in cancer stemness.

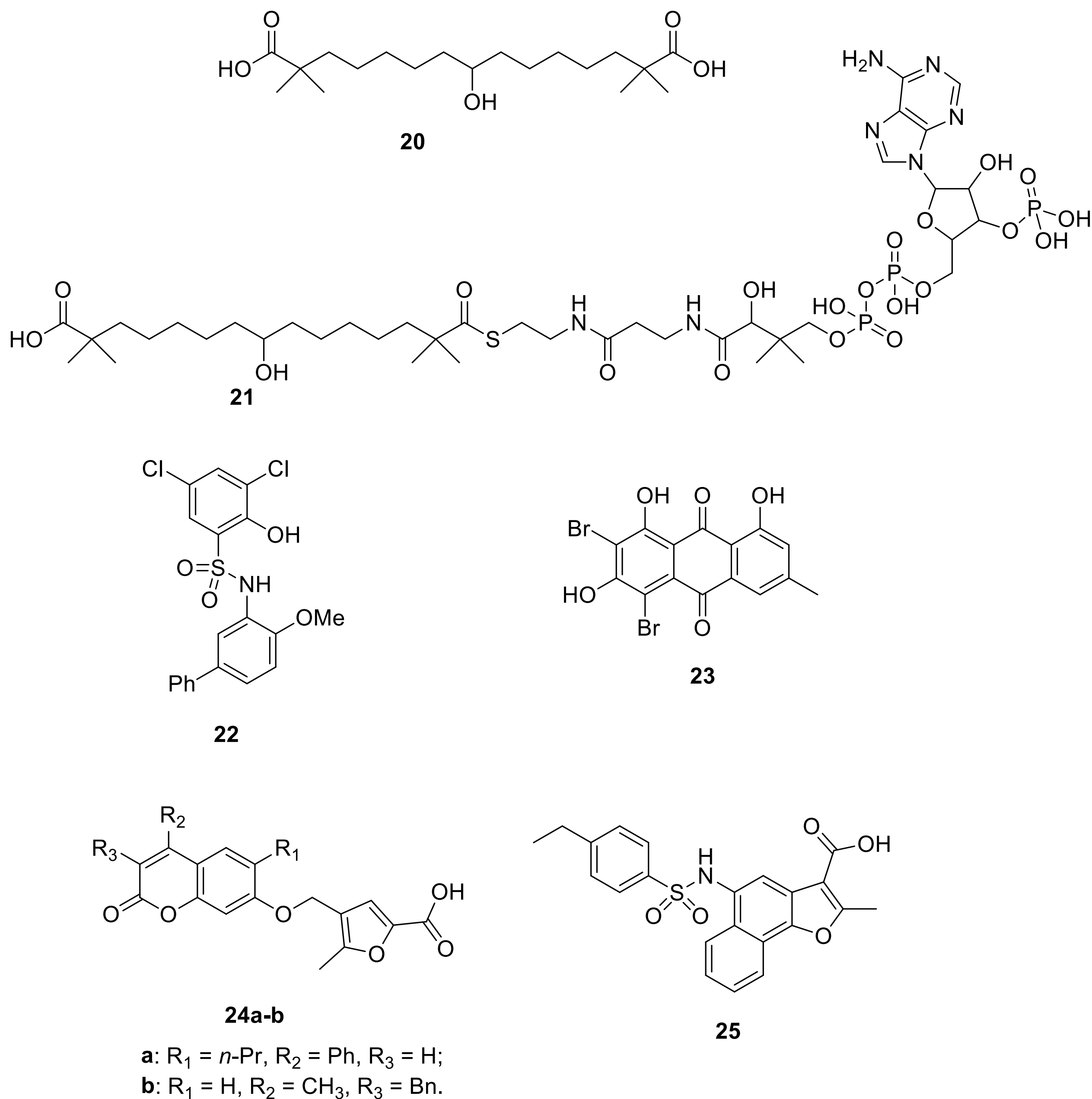

Figure 7. Synthetic ACLY inhibitors **20-25.**

The mentioned synthetic ACLY inhibitors can be roughly grouped into two different classes, on the base of their chemical structures: 1) the first group includes the "citrate-based" compounds **11-21**, since they resemble to different extent the structure of the substrate citrate, however some of them,

such as compounds Medica 16 (**18**) or thiadicarboxylic derivative **19**, only maintain the alkyl linear skeleton linking the two terminal carboxylic groups, whereas other compounds more strictly resemble citrate, such as sulfoximine **11** or difluorocitrates **12a-b**; 2) compounds from **22** to **25** belong to the second class, since they possess structures highly different from citrate, although some of them still have polar moieties like carboxylic, phenolic or sulfonamido groups. This last feature can be justified considering that the presence of polar moieties may represent a structural requirement necessary for an optimal interaction with ACLY, which normally hosts polar substrates such as citrate, ATP and CoA.

Table 2. Summary table for synthetic ACLY inhibitors.

Compounds	**Biological activities**	**Used methods**	**Ref.**
Sulfoximine (**11**)	K_i = 250 µM (ACLY from rat liver)	enzymatic assays	[80]
2,2-Difluorocitrate (**12a**-**b**)	K_i = 0.7-3.2 µM (ACLY from rat liver)	enzymatic assays	[81]
Expoxide (**13**)	K_i = 18 µM (ACLY from rat liver)	enzymatic assays	[82]
Compounds (**14a-d**)	K_i = 1.2-3.3 µM (ACLY from rat liver)	enzymatic assays	[84]
SB-201076 (**15**)	K_i = 1 µM (rat and human ACLY)	enzymatic assays	[85,86]
SB-204990 (**16**)	reduction of cholesterol and fatty acid synthesis	*in vitro* and *in vivo* assays	
	suppression of platelet aggregation in diabetic patients	*in vivo* assays	[88]
	anti-proliferative activity in several cancer cell lines	*in vitro* assays	[21]
	tumour growth inhibition	*in vivo* assays	
Compound (**17**)	K_i = 220 nM (human ACLY)	enzymatic assays	[85]
Medica 16 (**18**)	inhibition of lipogenesis and cholesterogenesis in rats	*in vivo* assays	[90–95]
	reduction of fatty acids and cholesterol synthesis in cultured rat hepatocytes	*in vitro* assays	
	K_i = 16 µM (ACLY from rat liver)	enzymatic assays	
	regulation of insulin-induced hepatic glucose production and utilization	*in vivo* assays	
3-Thiadicarboxylic acid	reduction of plasma triglycerides	*in vivo* assays	[96,97]

(**19**)	and cholesterol		
ETC-1002 (**20**) and ETC-1002-CoA (**21**)	improvement of hypercholesterolemia, type 2 diabetes	clinical studies	[98–101]
	reduction of circulating proatherogenic lipoproteins, hepatic lipids, and body weight	*in vivo studies*	
	attenuation of hypercholesterolemia, hypertriglyceridemia, hyperglycemia, hyperinsulinemia; prevention of atherosclerotic lesion	*in vivo studies*	
	K_i = 2 µM (**21**, human ACLY)	enzymatic assays	
2-Hydroxy-*N*-arylbenzenesulfonamide (**22**)	IC_{50} = 130 nM (human ACLY)	enzymatic assays	[103]
	inhibition of lipid synthesis	*in vitro* studies	
	decrease of plasma cholesterol and triglycerides, reduction of body weight	*in vivo* studies	
	involvement in glucose-dependent de novo lipogenesis in B lymphocytes	*in vitro* studies	[104]
Compound (**23**)	IC_{50} = 2.9 µM (human ACLY)	enzymatic assays	[105]
	antiproliferative activity in cancer cells	*in vitro* studies	
	reduction of cancer stemness	*in vitro* studies	
Furan carboxylate derivatives (**24a-b**) and (**25**)	IC_{50} = 4.1-13.8 µM (human ACLY)	enzymatic assays	[58]
	reduction of cancer stemness	*in vitro* studies	

2.3. Other compounds interfering with ACLY activity

Other compounds interact with ACLY by different mechanisms, for example compounds **26** and **27** (Figure 8) inhibited rat liver ACLY by modifying the arginine residues of the protein in the CoA binding site. It is known that α,β-dicarbonyl compounds are able to react with the guanidine side chain of arginine with the consequent formation of cyclic derivatives. Phenylglyoxal **27** was more rapid and potent (85% inhibition) in the inhibition of the enzyme, compared to 2,3-butanedione **26** (35% inhibition), and incubation for long times led to a complete inactivation, thus demonstrating a

behaviour typical of irreversible inhibitors. The pre-incubation with the substrates citrate and CoA protected ACLY from the subsequent inactivation by **26** and **27** [108].

The aminoacid L-glutamic acid **28** (Figure 8) inhibited rat liver ACLY (K_i value of about 0.3 mM), and this inhibitory effect was potentiated at long pre-incubation times in the presence of ATP and $MgCl_2$, although high concentration of ATP could reverse this effect. The supposed mechanism of action involved the formation of a complex of L-glutamic acid, Mg^{2+} and the enzyme [109].

The sodium salt of a bile acid, sodium deoxycholate **29** (Figure 8), reduced ACLY levels in animal models [110]. Similarly, the polychlorinated biphenyl Aroclor 1254 **30** (Figure 8), a toxic compound characterized by a biphenylic scaffold substituted with chlorine atoms, inhibited ACLY (69% inhibition) when administered in the rat diet (0.01% w/v) [111].

Krivanek *et al.* found that the vanadium element when present at the highest oxidation state (vanadate, oxidation state +5) inhibited the phosphorylation of histidine in ACLY from rat liver, thus reducing its activity [112].

Finally, Nateglinide **31** (Figure 8), a drug for the treatment of type 2 diabetes whose function is to stimulate pancreatic β cells to secrete insulin, was found to reduce the expression of ACLY by *in vivo* administration. Therefore, Nateglinide was patented for its potential therapeutic use in liver diseases related to an abnormal lipidic metabolism [113].

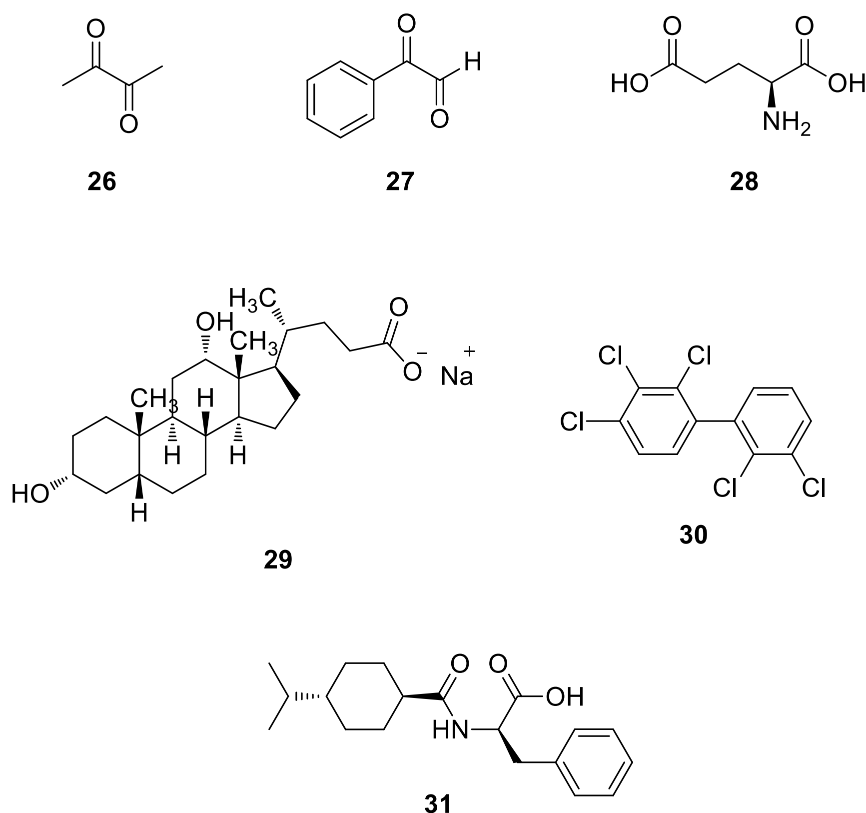

Figure 8. Other compounds influencing ACLY activity.

3. Conclusions

It has been clearly established that increased expression of ACLY in many tumour types and the antiproliferative activity deriving from ACLY inhibition both *in vitro* and *in vivo* provide the evidence supporting the role of this enzyme as an attractive therapeutic target for cancer treatment. According to many studies reported in literature, ACLY deprivation led to tumour cell death by fatty acid starvation. ACLY has the advantage of being upstream, compared to other lipogenic enzymes and therefore it is the preferential target to block the glucose dependent lipid synthesis. Considering the studies performed by the Thompson group, the glycolytic status of cancer cells needs to be considered when targeting ACLY, since cell lines characterized by a low rate of glycolysis are mainly unaffected by this type of treatment, thus confirming that glucose and lipid networks are closely correlated for the vitality of tumours and ACLY is the enzyme linking these two metabolic pathways. Moreover, ACLY is involved in several highly interconnected metabolic

pathways and it would be important to identify which of these pathways is the most important for the energetic support of tumour cells, in order to consider the exact role of ACLY, since some compensatory mechanisms may occur after ACLY inhibition. For example, a reduced de novo lipid synthesis deriving from ACLY inhibition may be counteracted by an increased internalization of fatty acids into cancer cells, in addition acetate is converted to acetyl-CoA by the ACSS2 enzyme, providing another source for lipid synthesis. ACLY is at the upstream position with respect to the lipid and cholesterol synthesis, thus it is the first enzyme triggering these two biosynthetic processes. Therefore, many ACLY inhibitors were initially evaluated for their potential use in therapies in which a block or a reduced synthesis of fatty acids or cholesterol would be desirable, such as obesity and hyperlipidemia. Nowadays, the interest of the scientific community is gradually shifting towards the repurposing of existing ACLY inhibitors as anti-cancer agents [114]. Additional studies for the existing ACLY inhibitors are still necessary to verify that their mechanisms of action and their observed pharmacological effects can be translated towards a fruitful application in the fight against cancer. Moreover, the development of more potent and selective ACLY inhibitors is the goal to achieve in this quite unexplored field. Nevertheless, the obtainment of a dual inhibitor targeting both ACLY and another protein covering a pivotal position in the deregulated glucose and lipid metabolism cannot be excluded for the development of effective anti-cancer drugs. A potential dual inhibitor should be able to simultaneously target two enzymes or receptors covering relevant positions in cancer metabolism. For example, it would be interesting to induce a block of glucose catabolism - by inhibiting a glycolytic enzyme - and to stop *de novo* lipogenesis - by inhibiting ACLY - to induce a lethal starvation in tumours. This goal could be reached by developing “hybrid” compounds possessing chemical portions able to inhibit two different targets. Otherwise, a specific drug design strategy should be pursued to design compounds that could interact with more than one target. However, this goal is not easily achievable, due to the different structural requirements for each single target and the complexity of the metabolic pathways. Another option could be the use of combination therapies in which an ACLY inhibitor is

administered with other therapeutic agents. This strategy has already been adopted for ETC1002 (**20**) in combination with cholesterol-lowering agents, such as the drug Ezetimibe, Atorvastatin or the monocolonal antibody Evolocumab, for the treatment of hyperlipidemic and hypercholesterolemic pathologies [115]; however, to date no similar combination approaches with **20** have been considered as anti-cancer therapies. Differently, we have previously discussed that HCA (**1**) was successfully used in combination therapies not only for the regulation of lipid levels and body weight, but also to counteract cancer. Overall, as our knowledge of tumour metabolism continuously evolves, the development of ACLY inhibitors will undoubtedly play an increasingly important role in the anticancer field.

Acknowledgments

Dr. Fiammetta Granchi is gratefully acknowledged for helpful suggestions and corrections.

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