Lipid Synthesis Is a Metabolic Liability of Non–Small Cell Lung Cancer

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The renaissance in the study of cancer metabolism has refocused efforts to identify and target metabolic dependencies of tumors as an approach for cancer therapy. One of the unique metabolic requirements that cancer cells possess to sustain their biosynthetic growth demands is altered fatty acid metabolism, in particular the synthesis of de novo fatty acids that are required as cellular building blocks to support cell division. Enhanced fatty acid synthesis that is observed in many tumor types has been postulated to open a therapeutic window for cancer therapy and, correspondingly, efforts to pharmacologically inhibit key enzymes of fatty acid synthesis are being pursued. However, despite these efforts, whether inhibition of fatty acid synthesis stunts tumor growth in vivo has been poorly understood. In this review, we focus on the recent evidence that pharmacologic inhibition of acetyl-CoA carboxylase, the enzyme that regulates the rate-limiting step of de novo fatty acid synthesis, exposes a metabolic liability of non–small cell lung cancer and represses tumor growth in preclinical models.

ALTERED FATTY ACID SYNTHESIS IN CANCER

Although most normal cells and tissues satisfy their requirement for fatty acids by importing them from the circulation, the observation more than 60 years ago that tumors undergo high rates of de novo lipogenesis suggested that cancer cells satisfy their lipid demand differently compared with their normal counterparts (Medes et al. 1953). Surprisingly, the rate of lipogenesis measured in tumors was comparable to that of the liver, an organ that requires a high rate of fatty acid (FA) synthesis. Since then, enhanced FA synthesis has been documented in a number of tumors (reviewed in Swinnen et al. 2006; Abramson 2011). The elevated de novo FA synthesis observed in tumors is reflected by the increased expression of lipogenic enzymes that has been described in a number of cancers (Szutowicz et al. 1979; Witters et al. 1994; Milgraum et al. 1997). A seminal finding in this respect was the identification of the oncoprotein OA-519 as fatty acid synthase (FASN), which was overexpressed in aggressive breast cancer (Kuhajda et al. 1994) and now confirmed to be up-regulated in a wide number of other cancers such as those of the prostate, ovary, colon, lung, and bladder (Menendez and Lupu 2007).

Lipogenic enzymes in the FA synthesis pathway are regulated at various levels. Strikingly, many of the genes encoding these enzymes are controlled transcriptionally by the sterol regulatory element-binding proteins (SREBPs) (Fig. 1). SREBPs are basic helix–loop–helix transcription factors that are crucial for maintaining cellular lipid homeostasis (Horton 2002). There are three SREBP isoforms in mammals: SREBP1a and SREBP1c,

which are products of the *SREBF1* gene, and SREBP2, a product of the *SREBF2* gene. Although SREBP1c mainly regulates genes in the FA synthesis pathway and SREBP2 regulates genes involved in cholesterol synthesis, redundancy among the SREBPs allows each isoform to regulate genes within each pathway (Horton 2002). Inhibition of SREBP1 and SREBP2 in cancer cells led to altered cellular lipid composition, which triggered endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) (Griffiths et al. 2013), demonstrating that SREBPs play a critical role in maintaining cellular lipid homeostasis in cancer. Emerging evidence also indicates that SREBPs provide a crucial link between oncogene signaling and altered fatty acid metabolism. SREBP1 activity is induced by AKT in a manner that is dependent on mTORC1 (mammalian target of rapamycin complex 1), demonstrating that lipogenesis and cell growth are intimately connected through the AKT–mTORC1 pathway (Fig. 1; Porstmann et al. 2008; Duvel et al. 2010).

In addition to transcriptional regulation, the activity of lipogenic enzymes is modulated posttranslationally by phosphorylation. For example, AKT directly phosphorylates and stimulates ATP citrate lyase (ACLY) (Bauer et al. 2005) contributing to the control of histone acetylation in response to growth factor stimulation (Lee et al. 2014). In contrast to this phosphorylation stimulating ACLY by oncogenic signaling, in response to low energy levels, the AMP activated protein kinase (AMPK) phosphorylates and inactivates key regulators of FA synthesis, including acetyl-CoA carboxylase 1 and 2 (ACC1 and ACC2) as well as SREBP1c itself (Fig. 1; Carling et al. 1989; Li et al. 2011). Thus, during periods of energy deprivation, AMPK dominantly inhibits lipogenesis. The acti-

Figure 1. Overview of fatty acid biosynthesis. Glucose- or glutamine-derived citrate is converted into acetyl-CoA by ATP-citrate lyase (ACLY). Acetyl-CoA is also generated from exogenous uptake of acetate by acetyl-CoA synthetase short-chain family member 2 (ACSS2). Acetyl-CoA carboxylase (ACC) carboxylates acetyl-CoA to form malonyl-CoA, which is then used as a substrate for fatty acid synthase (FASN) to form the 16 carbon saturated fatty acid palmitate (C16:0). Palmitate is then elongated and desaturated to generate pools of monounsaturated and polyunsaturated fatty acids. Essential fatty acids that cannot be endogenously synthesized have to be taken up from the bloodstream. Cellular pools of fatty acids can then be synthesized into additional fatty acid and lipid species that are used by tumor cells for a number of essential processes. Lipogenic enzymes in the fatty acid synthesis pathway are regulated at multiple levels. The AMP-activated protein kinase (AMPK) phosphorylates and inactivates Raptor (mTOR), ACC, sterol regulatory binding protein 1c (SREBP1c), and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) during periods of energy deprivation to eliminate ATP consumption by fatty acid synthesis. AKT directly phosphorylates ACLY and indirectly stimulates SREBP. SREBP activation promotes the transcriptional induction of several lipogenic enzymes in a manner that is also dependent on activation by mTOR. Proteins inactivated in cancer shown in orange and those hyperactivated in cancers shown in yellow. α -KG, α -ketoglutarate; ELOVL, elongation of very-long-chain fatty acids; GLS, glutaminase; HK, hexokinase; IDH1, isocitrate dehydrogenase 1; LKB1, liver kinase B1; PDH, pyruvate dehydrogenase; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; TCA, tricarboxylic acid; TSC1/2, tuberous sclerosis complex.

vation of AMPK is induced by phosphorylation by the tumor suppressor serine threonine kinase 11 (STK11), otherwise known as liver kinase B1 (LKB1), suggesting that ACC activity and lipogenesis itself may be hyperactivated in the broad variety of cancers lacking this tumor suppressor (Shackelford and Shaw 2009), paralleling the hyperactivation of ACLY predicted in the many cancers mutated for AKT, PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), or PTEN (phosphatase and tensin homolog).

Generally, the importance of FA synthesis in cancer cell proliferation and survival *in vitro* has now been described in a number of studies (Rohrig and Schulze 2016). However, whether autochthonous tumors *in vivo* possess a similar requirement is less understood. Because of the complex nature of tumors and the availability of lipids from both the circulation and the tumor microenvironment, the effects of *de novo* FA synthesis inhibition *in vitro* may not be translatable *in vivo*. Furthermore, the need to rigorously evaluate the anticancer effects of FA synthesis inhibitors is required to understand whether the enhanced synthesis observed in tumors is truly a metabolic dependency of cancer or rather simply a by-product of altered oncogene signaling and metabolism (Kim and DeBerardinis 2016).

FASN: THE FIRST TARGETED COMPONENT OF THE FA SYNTHESIS PATHWAY

Given elevated FASN was the first molecular discovery linking fat metabolism to cancer (Kuhajda et al. 1994), many studies have since shown increased FASN expression and enhanced activity in a wide variety of tumors (Menendez and Lupu 2007). Correspondingly, efforts focused on pharmacologically inhibiting FASN activity have been pursued for a number of years, and several FASN inhibitors have been described to date. The mycotoxin cerulenin inhibits the activity of FASN and has shown the ability to inhibit proliferation and induce apoptosis of cancer cells and xenograft tumors *in vivo* (Pizer et al. 1996; Menendez et al. 2004). Similarly, the more stable analog, C75, displayed antitumor activity in xenograft models of breast, prostate, and lung cancer (Pizer et al. 2000; Chen et al. 2012; Relat et al. 2012). C75 was also shown to significantly delay breast cancer progression in HER2 transgenic mice (Alli et al. 2005). TVB-3166, a potent and orally bioavailable inhibitor of FASN, induced apoptosis in breast and prostate cancer cell lines and displayed antitumor activity in several tumor xenograft models (Ventura et al. 2015). The thiophenopyrimidine-based FASN inhibitor Fasnall recently showed potent antitumor activity in the mouse mammary tumor virus (MMTV)-Neu model of HER2⁺ breast cancer, where it also synergized with carboplatin (Alwarawrah et al. 2016). Collectively, the preclinical activity of FASN inhibitors has been encouraging, and recently the first FASN inhibitor was entered into clinical trials for cancer. However, the mechanism underlying the antitumor efficacy of various FASN inhibitors is poorly understood, as FASN inhibition was shown in some settings to increase malonyl-CoA

levels (Thupari et al. 2001), which led to reduced food intake and body weight in mice (Loftus et al. 2000). Newer studies have also shed light on multiple mechanisms underlying sensitivity to FASN inhibitors (Benjamin et al. 2015; Ventura et al. 2015).

ACLY AND ACSS2: MORE THAN ONE WAY TO MAKE ACETYL-CoA

Other enzymes within the FA synthesis pathway have been hypothesized to be attractive therapeutic targets in cancer. In the cytoplasm, glucose- or glutamine-derived citrate is converted into acetyl-CoA by ACLY (Fig. 1). Acetyl-CoA is an essential substrate for FA synthesis; hence, ACLY inhibition should result in depleted acetyl-CoA pools and offer a way to shut down FA synthesis in cancer. Inhibition of ACLY by either RNAi or pharmacological inhibitors results in cell cycle arrest and induction of apoptosis *in vitro* and *in vivo* (Hatzivassiliou et al. 2005). However, whether these effects are specifically due to loss of FA synthesis in cancer cells is questioned, as fatty acid supplementation did not rescue the effects of ACLY inhibition (Migita et al. 2008). Moreover, given that acetyl-CoA contributes to the mevalonate pathway as well as to histone acetylation (Wellen et al. 2009), ACLY activity may modulate cancer growth on multiple levels.

Importantly, ACLY activity does not provide the only source of cellular acetyl-CoA. The enzyme acetyl-CoA synthetase short-chain family member 2 (ACSS2) produces acetyl-CoA using acetate as a substrate (Fig. 1; Yoshii et al. 2009; Comerford et al. 2014). Thus, ACSS2 may compensate for ACLY inhibition if cellular pools of acetate are not limiting. To this end, exogenous treatment of cancer cells with acetate was able to rescue the effects of ACLY inhibition on proliferation arrest and histone acetylation (Wellen et al. 2009; Hanai et al. 2012). Furthermore, ACSS2 expression is up-regulated in ACLY-deficient cells *in vitro* and *in vivo*, suggesting that acetate metabolism underlies a crucial mechanism for ACLY deficiency (Zhao et al. 2016). The contribution of acetate to acetyl-CoA production plays a fundamental role in intermediary metabolism and proliferation of cancer cells (Lyssiotis and Cantley 2014). Exogenous acetate is readily captured and metabolized by cancer cells to fuel cell growth and interestingly was found to contribute a greater proportion of carbon to the acetyl-CoA pool than glucose or glutamine (Mashimo et al. 2014). ACSS2 has been shown to be essential in this process, as ACSS2 deletion in mouse models of hepatocellular carcinoma led to a significant reduction in tumor burden (Comerford et al. 2014). Additionally, ACSS2 contributes to acetyl-CoA generation during hypoxia and is essential for cancer cell survival in tumor xenografts (Schug et al. 2015). Taken together, the pivotal roles of ACLY and ACSS2 in generating acetyl-CoA pools that are required for cancer cell growth make them attractive therapeutic targets for cancer. However, because of their relative redundancies in maintaining these pools and the ability of cancer cells to readily uptake exogenous sources of acetate *in vivo*, whether pharmacological inhibition of either ACLY or

ACSS2 will provide a maximal therapeutic benefit still needs to be fully analyzed.

ACC: A CANCER TARGET WITH A DISTINCT ADVANTAGE?

An alternative approach to inhibit FA synthesis in cancer is the pharmacological inhibition of ACC, which controls the enzymatic step in between ACLY and FASN; the first-committed step of the lipogenesis pathway. ACC inhibition may offer a favorable advantage over other lipogenic enzymes because of its critical role in controlling the rate-limiting reaction of FA synthesis, the production of malonyl-CoA, which serves as the substrate for the *de novo* synthesis of all endogenously made fatty acids (Fig. 1). ACC uses acetyl-CoA for malonyl-CoA production regardless of the source from which it was derived. Thus the generation of malonyl-CoA pools that are essential for FA production depends entirely on the activity of ACC and is not confounded by functional redundancies among other lipogenic enzymes such as ACLY and ACSS2. Furthermore, inhibition of ACCs should lead to greater lipid depletion than inhibition of any other lipogenic enzyme in the pathway, because of loss of FA synthesis and simultaneous induction of fatty acid oxidation (FAOxn) that occurs when ACC is inhibited (see below). Correspondingly, efforts to target ACC have been pursued in the bio-pharmaceutical industry for the past two decades; however, until recently the development of potent and specific ACC inhibitors with favorable drug-like properties has been difficult (reviewed in Tong and Harwood 2006; Abramson 2011). Here we focus on the recent development of a novel small-molecule allosteric ACC inhibitor that has shown promise as an anticancer compound in preclinical models of non-small cell lung cancer (NSCLC) (Svensson et al. 2016).

ACC REGULATION OF FATTY ACID METABOLISM

Eukaryotic ACCs are large, single-chain multidomain enzymes with a biotin carboxylase (BC) domain, a biotin carboxyl carrier protein (BCCP) domain, and a carboxyltransferase (CT) domain (Fig. 1). ACC catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is the first committed step in fatty acid synthesis and the rate-limiting reaction in the pathway (Kim 1997). The carboxylation of acetyl-CoA to malonyl-CoA is catalyzed by the BC domain of ACC and involves the ATP-dependent carboxylation of biotin with bicarbonate serving as the donor of CO₂. CT then transfers the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA. In mammals, ACC exists as two isoforms, a cytoplasmic 265-kDa ACC1 isoform that is expressed in lipogenic tissues such as the liver and a 280-kDa ACC2 isoform expressed in oxidative tissues such as heart and muscle, where it is localized exclusively to the outer mitochondrial membrane (Bianchi et al. 1990; Kim 1997).

Dimerization of ACC is required for enzymatic activation, and the active form of mammalian ACC is a large linear polymer. Elegant studies have shown that ACC forms multiple dimers within the BC domain of the enzyme, which promotes its activity as a carboxylase to enable the generation of malonyl-CoA (Fig. 1; Shen et al. 2004; Cho et al. 2008). The original model in the field was that the malonyl-CoA produced from ACC1 serves as a substrate for FASN and results in the formation of fatty acids, and the malonyl-CoA produced by ACC2 at the outer mitochondrial membrane functions to allosterically inhibit carnitine palmitoyltransferase 1 (CPT-1) to prevent fatty acid entry into mitochondria to undergo β -oxidation (McGarry et al. 1978). However, recent findings from genetic knockout and knock-in mice with point mutations resulting in mild gain-of-function alleles of ACC reveal that this strict division of ACC isoforms in regulation of FA synthesis and FAOxn is probably not accurate, as the evidence suggests that there are substantial redundancies between each ACC isoform (Savage et al. 2006; Harada et al. 2007; Fullerton et al. 2013). Thus, the ability of malonyl-CoA to promote fatty acid synthesis or to inhibit FAOxn independent of the ACC isoform may be an important feature for tumor cell adaptation. However, regardless of the acetyl-CoA and malonyl-CoA source, the net effect of ACC activation in a tumor results in enhanced fatty acid synthesis and reduced fatty acid degradation via FAOxn inhibition (Fig. 2A). The *de novo* synthesis of intracellular fatty acids results in the production of fatty acids and lipids that can be used by tumor cells for a number of purposes—including membrane biogenesis, posttranslational modification of oncoproteins, to function as signaling molecules, or stored for energy production—all of which favor the growth of a tumor (Fig. 1).

Physiological inhibition of ACC activity is controlled by phosphorylation by AMPK (Steinberg and Kemp 2009). AMPK is activated under conditions of low ATP, such as following nutrient deprivation or mitochondrial inhibitors, but also modulated by metabolic hormones and whole-body effects from exercise and dietary restriction. AMPK phosphorylates conserved serine residues Ser79 in mouse ACC1/Ser212 in ACC2 to inhibit its dimerization that is required for activation. In addition, ACC is activated allosterically by citrate, which provides the signal that acetyl-CoA pools are sufficient to enable FA synthesis. Citrate promotes dimerization of ACC and dephosphorylation of the AMPK phosphorylated serines, which serves to induce ACC activity and malonyl-CoA formation. The stimulatory effect of citrate on ACC is reversed by palmitoyl-CoA, which is abundant when there is an excess of fatty acids and therefore functions to promote the inactive conformation of ACC.

ACC AS AN ANTICANCER DRUG TARGET

Several lines of evidence support the rationale for targeted ACC inhibition in oncology. Short interfering RNA (siRNA) knockdown of ACC1 expression induced apo-

Figure 2. Model of acetyl-CoA carboxylase (ACC) regulation of fatty acid synthesis and inhibition by ND-646. (A) Eukaryotic ACC is a multidomain enzyme. ACC is inactive in its monomeric form and requires dimerization at the biotin carboxylase (BC) domain for activation. Activated dimeric ACC produces malonyl-CoA in a reaction that requires biotin, ATP, and CO₂. Malonyl-CoA promotes induction of fatty acid synthesis and inhibition of fatty acid oxidation. (B) ACC inhibition by ND-646 mimics physiologic inhibition of ACC by AMP-activated protein kinase (AMPK). (Right) AMPK phosphorylates conserved serine residues of the A-domain tail in the BC domain of ACC1 and ACC2 to disrupt enzyme dimerization and inhibit ACC activity. Upon phosphorylation the tail undergoes a conformation change and the phosphorylated serine binds to the dimer interface of the BC domain. Upon binding ACC dimers are disrupted into monomers and inactivated. Phosphorylated serine is protected from dephosphorylation while bound to the BC domain, and phosphorylated ACC (P-ACC) is detected. (Left) ND-646 binds to the dimer interface of the BC domain to disrupt dimerization and promote monomeric ACC. The AMPK phosphorylated serine of ACC is unable to bind into the BC domain because of the presence of ND-646 and subsequently becomes accessible to phosphatases that dephosphorylate ACC. Phosphorylated ACC is not detected when ND-646 is bound to ACC. CT, carboxyltransferase; BCCP, biotin carboxyl carrier protein.

ptosis in prostate (Brusselmans et al. 2005) and breast (Chajes et al. 2006) cancer cells and in cisplatin-resistant lung cancer cell lines (Wangpaichitr et al. 2012), and chemical inhibition of ACC1 and ACC2 by the macrocyclic myxobacterial natural product Soraphen A led to growth arrest in breast cancer cells (Beckers et al. 2007) and induction of apoptosis in prostate cancer cells (Corominas-Faja et al. 2014). Constitutively active ACC mutants also protected head and neck squamous cancer cells from cetuximab-induced growth inhibition (Luo et al. 2016). However, although these observations provide compelling data that ACC mediates cellular proliferation, these studies were exclusively *in vitro* and did not address whether ACC activation was required for tumor growth *in vivo*, as the genetic role of ACC in tumor growth had not been investigated. In an attempt to understand this and also to determine whether loss of cell proliferation and survival that had been observed with ACC inhibition was a direct result of FA synthesis inhibition and lipid depletion, our laboratory recently analyzed the role of ACC in NSCLC cells using CRISPR–Cas9 (Svensson et al. 2016). Consistent with the lipogenic phenotype that has been observed in tumors, we found that ACC1 expression levels were high in the panel of NSCLC cells that we analyzed and interestingly were significantly higher than ACC2, which had expression ranging from low to undetectable. CRISPR–Cas9 deletion of ACC1 led to pronounced proliferation defects and cell death, which was completely rescued by the exogenous addition of the fatty acid palmitate to the media, suggesting that the FA synthesis was impaired by ACC1 deletion. Metabolic tracing experiments to quantitate fatty acid production revealed that ACC1 deletion led to a complete loss of *de novo* FA synthesis, consistent with the critical role of ACC1 in lipogenesis. When we implanted ACC1-deleted NSCLC cells subcutaneously into mice, we found that tumor growth was severely impaired relative to the growth of cells bearing endogenous ACC1 expression. However, tumor growth in ACC1-deleted cells was completely rescued by stable re-expression of ACC1 into deleted cells. Taken together, our findings revealed that ACC1 mediates a metabolic liability of NSCLC, providing the rationale for targeted pharmacologic inhibition of ACC.

Consistent with the important role of fatty acid biosynthesis in cancer and consistent with our RNA-sequencing profiling, the expression of ACC1 has been shown to be frequently up-regulated in several tumor types (Witters et al. 1994; Milgraum et al. 1997; Swinnen et al. 2000; Yahagi et al. 2005). Conversely, and consistent with our studies describing low expression levels of ACC2 mRNA in NSCLC cells, the expression or activation of ACC2 has been found to be down-regulated in tumors (Corbet et al. 2016; German et al. 2016). This may be due to the important role of FAOxn in tumors to support tumor growth and survival (Carracedo et al. 2013). ACC2 inactivation in tumors can occur via phosphorylation by AMPK and recent evidence suggests that ACC2 activity is also modulated by hydroxylation (German et al. 2016). Hydroxylation of ACC2 by prolyl hydroxylase 3 (PHD3)

was required for ACC2 activation to repress FAOxn and cancer cell proliferation, and it was found that cancer cells down-regulate expression of PHD3, which directly correlated with loss of ACC2 activity. Additionally, ACC2 expression may be regulated by the acetylome, providing an epigenetic mechanism for ACC2 down-regulation in tumors (Corbet et al. 2016). Ultimately, despite the fact that ACC1 and ACC2 function can be redundant, certain conditions may still allow for concomitant activation of FA synthesis and FAOxn to support tumor growth.

FAOxn inhibition itself is a focus of cancer therapy and inhibition of FAOxn has shown therapeutic efficacy in cancer models (Samudio et al. 2010; Camarda et al. 2016). This may present a conundrum when discussing the value of ACC inhibition as a strategy for cancer therapy as an ACC2 inhibitor would be expected to promote FAOxn, which in theory may enhance tumor growth rather than inhibit it. However, this conundrum can be solved by development of compounds that allow the simultaneous inhibition of ACC1 and ACC2. In principle, a dual ACC1/-2 inhibitor would result in FA synthesis inhibition and induction of FAOxn, which should result in severe lipid depletion in tumors by shutting off fatty acid production and enhancing fatty acid degradation. Thus, if tumors require the mass abundance of lipids to support cell growth, then the induction of FAOxn that may support tumor growth would be negated by the severe lipid depletion achieved by ACC1 and ACC2 inhibition, which ultimately should repress tumor growth.

ACC: OLD TARGET, NEW DRUG

Because of the pivotal role of ACC in regulating fatty acid metabolism, ACC has been under intense investigation as a clinical drug target in several metabolic diseases, including nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), and, correspondingly, ACC has been the focus of several clinical pipelines in the pharmaceutical industry. Inhibition of FA synthesis and stimulation of FAOxn via ACC1/-2 inhibition may favorably affect the morbidity and mortality associated with metabolic syndromes (Wakil and Abu-Elheiga 2009). To this end, several isoform nonselective ACC inhibitors have been described to date. However, because of the high degree of hydrophobicity in the regions of ACC that these inhibitors interact, they lack the optimal drug-like properties that are required for their clinical use. Recently, however, the development of small-molecule ACC inhibitors with more favorable drug-like properties was described by Harriman et al. (2016). In an effort to identify potent inhibitors of ACC1/-2 that display enhanced pharmacokinetics (PK) and pharmacodynamics (PD), Harriman et al. targeted the dimerization site in the BC domain of the enzyme in an attempt to identify novel allosteric protein–protein interaction inhibitors of ACC. Using state-of-the-art structure-based drug design, Harriman et al. identified a series of allosteric ACC1/-2 dimerization inhibitors and reported

the ability of the liver selective compound ND-630 to reduce hepatic steatosis, improve insulin sensitivity, and reduce weight gain in experiment animal models of metabolic disease. Our own laboratory recently described the ability of another member of this series, ND-646, a more broadly distributed ACC inhibitor, to repress FA synthesis and tumor growth of NSCLC in genetically engineered preclinical models (Svensson et al. 2016).

MECHANISM OF ACTION OF ND-646

ND-646 and other members of the ACC inhibitor series developed by Harriman et al. function as allosteric inhibitors of ACC1 and ACC2, by preventing the dimerization that is required for activity (Harriman et al. 2016; Svensson et al. 2016). The site that ND-646 binds to in the BC domain of ACC is of importance, as it is the exact same site that the AMPK phosphorylated serine of ACC interacts to inhibit dimerization and activity of ACC. Thus, ACC inhibition by ND-646 mimics physiological inhibition of ACC by AMPK. Phosphorylation of conserved serine residues by AMPK in ACC1 (mouse serine 79, human serine 117) and ACC2 (mouse serine 212, human serine 222) in the A-domain tail of the BC domain promotes a conformational change of the tail and binding of the phospho-peptide to the BC dimer interface (Fig. 2). Binding of the phosphorylated serine to the BC domain disrupts dimerization of ACC and promotes the formation of the inactive monomer. Given that ND-646 binds to the same site in the BC domain of ACC to analogously disrupt enzyme dimerization, it also fortuitously facilitates analysis of the phosphorylation state of ACC as a biomarker to determine whether ND-646 is bound. When ND-646 binds to the BC domain, it prevents binding of the phosphorylated serine to the same site, which results in constitutive dephosphorylation of ACC as the phosphate is no longer protected from protein phosphatases (Fig. 2). Thus, phosphorylated ACC is not detected when ND-646 is bound and can be used as an efficient biomarker in cells and tissues (Svensson et al. 2016).

ACC REGULATION OF FATTY ACID SYNTHESIS AS A METABOLIC LIABILITY OF NSCLC

The development of ND-646 as a broadly distributed ACC inhibitor with good drug-like properties enabled us to characterize the *in vivo* PD properties of ND-646 and to determine whether ACC inhibition in tumors *in vivo* would be therapeutically beneficial. To determine whether ACC inhibition by ND-646 would lead to decreased *de novo* fatty acid synthesis in autochthonous tumors *in vivo*, our laboratory, in collaboration with the laboratory of Christian Metallo, performed metabolic labeling analyses in genetically engineered mouse models of NSCLC using deuterated water ($^2\text{H}_2\text{O}$). $^2\text{H}_2\text{O}$ is incorporated into a number of biosynthetic intermediates that are required for fatty acid production and only labels fatty acids that are newly synthesized (Lee et al. 2000). Thus, quantitation of

deuterium-labeled fatty acids in harvested tumors provides a powerful method to measure the synthesis rates of newly made fatty acids. The ability to administer deuterated water directly into the drinking water of mice may offer advantages over alternative approaches to quantitate metabolic flux *in vivo*, as it is a facile procedure and well tolerated. Consistent with the studies of Medes et al. more than 60 years ago, we found that lung tumors from $\text{Kras}^{\text{G12D}/+}$; $\text{Trp53}^{-/-}$ (KP), and $\text{Kras}^{\text{G12D}}$; $\text{LKB1}^{-/-}$ (KL) mouse models were undergoing high rates of *de novo* fatty acid synthesis (Svensson et al. 2016). In our unpublished studies, we also directly compared the synthesis rates in tumors of KP mice to livers in the same animals and found that the rate of fatty acid synthesis of the most predominant saturated fatty acid palmitate was higher in the tumor tissue, supporting the original conclusions of Medes et al. and suggesting that this high rate of synthesis may be required to support the fast growth of these tumors. We found that treatment of lung tumors with ND-646 led to a significant decrease in the rate of fatty acid synthesis. Furthermore, plasma levels of free fatty acids were also significantly reduced in ND-646-treated mice, demonstrating the ability of ND-646 to inhibit FA synthesis in other tissues. To the best of our knowledge, our study describing these effects is the first published report of an ACC inhibitor with these pharmacodynamic properties *in vivo*.

Given that ND-646 was able to significantly inhibit fatty acid synthesis in lung tumors in these autochthonous models, it led us to investigate whether this inhibition would affect the growth of the tumors after chronic dosing. We set up large-scale preclinical trials in KP and KL mice to test the therapeutic efficacy of ND-646, as a single agent and also in combination with the standard of care chemotherapeutic agent carboplatin. We recently reported the results from these trials, where we found that KP mice were particularly sensitive to ND-646 monotherapy and that the combination of ND-646 and carboplatin led to striking suppression of lung tumor growth in both KP and KL mouse models. In summary, our study showed that ACC inhibition in biologically relevant preclinical mouse models of NSCLC led to suppression of fatty acid synthesis and tumor growth, revealing that ACC mediates a metabolic liability for this particular subset of cancers (Fig. 3).

CONCLUSION AND FUTURE PERSPECTIVES

Medes et al. concluded more than 60 years ago that the elevated rates of *de novo* lipogenesis they measured in tumors was probably required to support the rapid proliferation of cancer cells. Our work further supports this model and demonstrates the therapeutic viability of targeting this pathway (Svensson et al. 2016).

An open question that remains from our study is understanding whether decreased levels of plasma free fatty acids that is caused by ND-646 treatment contributes to the antitumor efficacy evoked by ACC inhibition (Fig. 3). We found that chronic 6 weeks of dosing of ND-646 led to significant reductions in the levels of plasma free fatty

Figure 3. Model depicting the therapeutic mechanism of action of ND-646 in non-small cell lung cancer. (A) Lung tumors with active acetyl-CoA carboxylase (ACC) undergo high rates of de novo FA synthesis to provide fatty acids and lipids to support tumor growth. (B) ND-646 treatment inhibits ACC activity and suppresses fatty acid synthesis and tumor growth and also lowers plasma levels of fatty acids. AMPK, AMP-activated protein kinase; FASN, fatty acid synthase.

acids and given the ability of exogenous palmitate to rescue the effects of ACC inhibition *in vitro* suggests that the levels of fatty acids that are taken up by autochthonous tumors are limiting and unable to compensate for the loss of de novo synthesis. This leads to the possibility that the antitumor effects of ND-646 occur by at least a two-prong mechanism: (1) direct inhibition of FA synthesis in tumors, and (2) inhibition of FA synthesis in the tumor microenvironment and other tissues such as the liver, which is a major contributor to plasma fatty acid levels, thus reducing the ability of circulatory and micro-environmental fatty acids to rescue the effects of ND-646. The availability of the liver-directed ACC inhibitor, ND-630, may aid in determining whether the reduction in plasma free fatty acids levels alone has any effect on *in vivo* lung tumor growth. Additionally, whether diet modulates fatty acid bioavailability to tumors and the response to ND-646 will need to be determined.

Our study warrants further investigation of the utility of ACC inhibitors in oncology. Identification of specific tumor types and subsets of cancers that respond to ND-646 will be of great interest. Furthermore, whether the mutational status of the tumors dictates the therapeutic response to ACC inhibitors will need to be examined. Our work showed that LKB1 deletion in *Kras*^{G12D} lung tumors led to increased rates of de novo fatty acid synthesis compared with p53 deletion, consistent with hyperactivation of ACC activity due to LKB1 loss; however, KL tumors were less sensitive to ND-646 as a single agent. This may be related to the absolute levels of fatty acids in the tumors and the degree of suppression by ND-646, which may dictate the therapeutic efficacy of ACC inhibition. However, KL lung tumors were exquisitely sensitized to the combined effects of ND-646 and carboplatin. Because of the genetic integrity of p53 in the KL tumors, we found that carboplatin induced nuclear p53 accumulation in these tumors, which might explain the striking synergy that we observed with this combination treatment, as p53 activation is known to promote FAOxn via its induction of mRNAs for *Cpt1c*, *Lipin1*, and *Acad11* (Assaily et al. 2011; Zaugg et al. 2011; Jiang et al. 2015).

Thus, the ability of ND-646 to inhibit FA synthesis, combined with carboplatin-induced p53-dependent FAOxn, would be expected to significantly deplete the cellular pools of fatty acids that are needed for cellular division. Given that ND-646 inhibits both ACC1 and ACC2, ND-646 by itself would be expected to inhibit FA synthesis and promote FAOxn; however, the majority of tumor cell lines that we have analyzed express little to no endogenous ACC2.

Although not directly assessed in our study with ND-646, ACC inhibition with ND-630 led to potent suppression of malonyl-CoA production (Harriman et al. 2016). However, whether the intracellular levels of acetyl-CoA are affected by decreased conversion into malonyl-CoA has not been analyzed. Although we showed that non-FA synthesis pathways that use acetyl-CoA, such as cholesterol synthesis, were unaffected, we did not determine whether acetylation of histones were affected, as has been described with genetic deletion of ACC in yeast and mouse liver (Galdieri and Vancura 2012; Chow et al. 2014). It will be of great interest to determine these effects and whether ACC provides an additional link between fatty acid metabolism and epigenetic control of transcriptional regulation.

Finally, given that many oncogenic proteins are modulated by lipidation, it raises the intriguing possibility that the antitumor activity of ND-646 might also be explained in part by decreased posttranslational modification of *Kras*, which is the oncogenic driver of NSCLC growth in our models. *Kras* undergoes extensive posttranslational modification, including farnesylation and palmitoylation (Ahearn et al. 2012); thus, whether loss of FA synthesis through ACC inhibition affects these modifications and the signaling ability of *Kras* should be investigated.

In conclusion, the development of ND-646 as a potent and specific ACC inhibitor has revealed that ACC mediates a metabolic liability of cancer. The continued discovery of compounds targeting other members of the FA synthesis pathway and the elucidation of new targets will shed light on whether inhibition of FA synthesis will translate from bench to bedside in oncology.

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