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SUPPLEMENTAL INFORMATION
Supplemental Information includes 7 figures, Supplemental Experimental Procedures, and Supplemental References.

Author Contributions
SUMMARY

Mitochondrial acetyl-CoA acetyltransferase 1 (ACAT1) regulates pyruvate dehydrogenase complex (PDC) by acetylating pyruvate dehydrogenase (PDH) and PDH phosphatase. How ACAT1 is “hijacked” to contribute to the Warburg effect in human cancer remains unclear. We found that active, tetrameric ACAT1 is commonly upregulated in cells stimulated by EGF and in diverse human cancer cells, where ACAT1 tetramers but not monomers are phosphorylated and stabilized by enhanced Y407-phosphorylation. Moreover, we identified arecoline hydrobromide (AH) as a covalent ACAT1 inhibitor, which binds to and disrupts only ACAT1 tetramers. The resultant AH-bound ACAT1 monomers cannot reform tetramers. Inhibition of tetrameric ACAT1 by abolishing Y407-phosphorylation or AH treatment results in decreased ACAT1 activity, leading to increased PDC flux and oxidative phosphorylation with attenuated cancer cell proliferation and tumor growth. These findings provide a mechanistic understanding of how oncogenic events signal through distinct acetyltransferases to regulate cancer metabolism, and suggest ACAT1 as an anti-cancer target.

Graphical Abstract

INTRODUCTION

Cyclic acetylation/deacetylation plays a key role in regulation of many essential proteins that are involved in diverse cellular processes including cell survival, proliferation, chromatin remodeling and metabolism (Choudhary et al., 2009; Zhao et al., 2010). Recent studies revealed that metabolic enzymes including pyruvate kinase M2 isoform, lactate dehydrogenase A and lysine acetylation of ATP-citrate lyase are regulated by lysine acetylation, providing insights into a mechanistic link between cell signaling pathways and metabolic pathways in cancer cells (Hitosugi and Chen, 2014; Lin et al., 2013; Lv et al., 2011; Zhao et al., 2013). We reported that commonly upregulated acetylation at K76 and K294 of 6-phosphogluconate dehydrogenase (6PGD) promotes NADP+-binding to 6PGD and formation of active 6PGD dimers, respectively. Moreover, we identified DLAT and
ACAT2 as upstream acetyltransferases of K76 and K294, respectively, and HDAC4 as the deacetylase of both sites. Inhibition of 6PGD by expressing acetyl-deficient mutants of 6PGD or treatment with 6PGD inhibitors Physcion or its derivative S3 in cancer cells significantly attenuated cell proliferation and tumor growth (Lin et al., 2015a; Shan et al., 2014). However, how oncogenic events signal through distinct acetyltransferases and deacetylases to regulate metabolic enzymes via cyclic acetylation/deacetylation remains unknown, and metabolism-related protein acetyltransferases and deacetylases as potential anti-cancer targets remain unexplored to date.

The metabolic switch from OXPHOS to glycolysis is partially achieved through upregulation of pyruvate dehydrogenase (PDH) kinase (PDHK) activity by c-Myc and HIF-1α, which phosphorylates and inactivates mitochondrial PDH, leading to attenuated pyruvate dehydrogenase complex (PDC) flux in cancer cells (Kim and Dang, 2006; Papandreou et al., 2006). In addition, we found that lysine acetylation-dependent inhibition of PDH and PDH phosphatase (PDP) is common in EGF-stimulated cells and diverse human cancer cells, contributing to PDC attenuation and consequently promoting cancer cell proliferation and tumor growth. We identified mitochondrial acetyl-CoA acetyltransferase 1 (ACAT1) and SIRT3 as upstream acetyltransferase and deacetylase, respectively, of PDHA1 and PDP1 (Fan et al., 2014). Active ACAT1 is a tetrameric enzyme in ketogenesis that converts two acetyl-CoA molecules to acetoacetyl-CoA and CoA (Balasse and Fery, 1989; Haapalainen et al., 2007; Haapalainen et al., 2006). We reported ACAT1 as a mitochondrial protein acetyltransferase and most importantly, stable knockdown of ACAT1 attenuates tumor growth (Fan et al., 2014). However, how ACAT1 is “hijacked” to contribute to the Warburg effect in human cancer remains unclear. In this study, we demonstrated upregulated Y407 phosphorylation activates ACAT1 by stabilizing tetrameric ACAT1 proteins in cancer cells and validated arecoline hydrobromide (AH) as a tetrameric n ACAT1 in treatment of human cancer cells in vitro and in vivo.

RESULTS

Activation of ACAT1 by EGF stimulation and diverse tyrosine kinases involves tyrosine phosphorylation and tetramer formation

We found that the enzyme activity of ACAT1 is commonly upregulated in diverse human leukemia, lung cancer, head and neck cancer, and prostate cancer cells compared to corresponding control cells that are normal human proliferating cells with same tissue origins, despite comparable protein expression levels of ACAT1 (Figure 1A). In addition, we found that ACAT1 enzyme activity levels are also commonly upregulated in human primary leukemia cells from diverse acute myeloid leukemia (AML), chronic myeloid leukemia (CML), B cell acute lymphoblastic leukemia (B-ALL), and acute promyelocytic leukemia (APL) patients (n=22), compared to control peripheral blood cells from healthy donors (n=4) (Figure 1B). However, ACAT1 protein expression level is not significantly altered in these cancer and leukemia cells compared to normal proliferating cells, or in human primary leukemia cells compared to healthy peripheral blood samples (Figure 1A–1B). Moreover, although ACAT1 was found to be significantly elevated in aggressive prostate cancer (Saraon et al., 2014), ACAT1 gene expression is not significantly upregulated in diverse
human cancers according to Oncomine™ Cancer Genomics Data Analysis (Figure S1A). These results suggest that the underlying molecular mechanism for sustained ACAT1 activation in cancer cells likely does not involve aberrant ACAT1 gene or protein expression levels.

We next found that EGF stimulation activated ACAT1 in NIH/3T3 cells in a time-dependent manner (Figure 1C), which is due to increased ACAT1 tetramer formation (Figure 1D) with increased tyrosine phosphorylation (Figure 1E). We thus performed a series of in vitro kinase assays using purified recombinant tyrosine kinases incubated with ACAT1 protein as an exogenous substrate. We found that puriﬁed EGFR and FGFR1 directly phosphorylate and activate ACAT1 (Figure 1F). Similar results were obtained using puriﬁed recombinant FLT3, ABL and JAK2 (Figure S1B).

**Tetrameric but not monomeric ACAT1 can be phosphorylated at Y407, and such phosphorylation promotes tetramer accumulation**

We next performed mutational analysis and generated four phospho-deﬁcient Y→F mutants of ACAT1 based on public data that identiﬁed ACAT1 as phosphorylated at four tyrosine residues, including Y90, Y214, Y219 and Y407 (Figure 2A), in human cancer cells (http://www.phosphosite.org/proteinAction.action?id=9172&showAllSites=true). We found that substitution of Y407 with phenylalanine resulted in abolishment of FGFR1-dependent activation of ACAT1 (Figure 2B). Further cellular fractionation and Western blot studies revealed that EGF treatment induced Y407 phosphorylation of mitochondrial ACAT1 with detection of a fraction of EGFR along with ACAT1 in the mitochondrial fraction of stimulated NIH/3T3 cells (Figure 2C, upper), while treatment with FGFR1 inhibitor TKI258 in FGFR1-expressing H1299 cells led to decreased Y407 phosphorylation of ACAT1 in the mitochondrial fraction where both ACAT1 and a fraction of FGFR1 were detected (Figure 2C, lower). These data are consistent with our previous ﬁndings of mitochondrial EGFR and FGFR1 (Fan et al., 2014; Hitosugi et al., 2011), and other mitochondrial localized tyrosine kinases including FLT3, ABL1 and JAK2 (Fan et al., 2014; Hitosugi et al., 2011), which may also phosphorylate ACAT1 in mitochondria.

Moreover, we found that Y407 phosphorylation of ACAT1 is commonly upregulated (Figure 2D, upper) in diverse human leukemia, lung cancer, head and neck cancer, and prostate cancer cells with elevated ACAT1 enzyme activity (Figure 1A) compared to corresponding control cells. In addition, treatment with distinct tyrosine kinase inhibitors to target different leukemogenic tyrosine kinases including FOP2-FGFR1 (TKI258), BCR/ABL (imatinib), JAK2-V617F (AG490), FLT3/ITD (TKI258) and FIP1L1-PDGFRα (imatinib) in corresponding leukemia cells resulted in decreased levels of ACAT1 Y407 phosphorylation (Figure 2D, left lower). Treatment with these inhibitors to target tyrosine kinases including JAK2, EGFR, FGFR1 and c-ABL (Figure S1C) in diverse tumor cell lines revealed that FGFR1 is responsible for ACAT1 Y407 phosphorylation in H1299 lung cancer cells (Figure 2D; middle lower), while EGFR represents the upstream kinase of ACAT1 in A549 lung cancer cells, Tu212 and Tu6868 head and neck cancer cells, and PC3 prostate cancer cells (Figures S1D and 2D, right lower). Consistently, we found that FGFR1 promotes tetramer formation and activation of ACAT1 WT and the control Y90F, Y214F, Y219F mutants,
whereas Y407F mutant shows resistance to FGFR1 treatment (Figure 2E). Note that only ACAT1 tetramers but not monomers were phosphorylated. In addition, substitution of each tyrosine residue did not result in a significant decrease in overall tyrosine phosphorylation levels of ACAT1 proteins (Figures 2B and 2E), suggesting that none of these tyrosine residues represents a major phosphorylation site of ACAT1.

We next purified monomeric and tetrameric ACAT1 proteins using a sucrose density ultracentrifugation approach. We found that the majority of recombinant ACAT1 proteins are monomers, while phosphorylation by FGFR1 resulted in a shift to tetramer formation (Figure 2F). Moreover, to determine whether ACAT1 monomers and/or tetramers can be phosphorylated by FGFR1, we separated ACAT1 monomers and tetramers on a native gel. The gel slices containing tetrameric or monomeric ACAT1 proteins were incubated with recombinant FGFR1 in the presence of “cold” ATP or “hot” ATP (γ32P-ATP) in an in vitro kinase assay. The results revealed that only tetrameric ACAT1 proteins were phosphorylated and labeled by 32P (Figure 2G). These data together suggest that Y407 phosphorylation of tetrameric ACAT1 may contribute to accumulation of ACAT1 tetramers. However, Y407 is located distal from the sites of enzymatic cleft and tetramerization, and at the molecular level it is not clear how phosphorylation mediates long-range effects to impact the oligomerization state of ACAT1 (Figure S1E).

**Arecoline hydrobromide (AH) is a covalent ACAT1 inhibitor**

Currently there is no defined ACAT1 inhibitor available. To better understand the role of tetrameric ACAT1 in human cancer, we designed a screening strategy using an in vitro ACAT1 assay and identified arecoline hydrobromide (AH; arecoline•HBr) as an ACAT1 inhibitor from a library of 2,000 FDA approved small molecule compounds (Figure 3A). AH is a salt form of arecoline (methyl 1-methyl-1,2,5,6-tetrahydropyridine-3-carboxylate), which is a nicotinic acid-based alkaloid found in the areca nut that is commonly referred to as betel nut (Gupta and Warnakulasuriya, 2002; Sharan et al., 2012). AH effectively inhibits ACAT1 but not two other human acetyltransferases, acetyl-CoA acetyltransferase 2 (ACAT2) and dihydrolipoamide S-acetyltransferase (DLAT), which were included as negative controls (Figure 3B). AH inhibits purified recombinant ACAT1 with an IC50 value of approximately 11.1±3.9µM, whereas the IC50 values for AH to inhibit ACAT2 and DLAT were not reached (NR; Figure 3B).

Molecular docking study of arecoline based on the crystal structure of ACAT1 (PDB code: 2IB8; (Haapalainen et al., 2007)) revealed that AH binds to ACAT1 at its catalytic site and most of the predicted interaction residues of ACAT1 for AH binding are hydrophobic, indicating the ester group of AH is important (Figure 3C). In particular, we predicted that AH could covalently bind to a nearby cysteine residue (C126) in the ACAT1 catalytic site through a Michael addition reaction (Shiraki et al., 2005) between an α,β-unsaturated ketone group in AH and the thiol group of cysteine (Figure 3C). We thus tested such an addition reaction using purified L-cysteine and AH in water at pH 7 and 5 (to mimic the acidic conditions within tumor). The NMR analysis of the product collected after four hours of the reaction at both pH 7 and 5 showed the C=C bond of AH disappeared after the reaction,
confirming the addition reaction taking place between L-cysteine and AH and thus supporting the predicted model (Figure 3D).

We next generated a C126A mutant of ACAT1 and performed a thermal shift assay. The results suggested that AH directly binds to ACAT1 WT with Kd value determined as 33.8±4.4µM, whereas AH did not bind to the C126A mutant and the Kd value was not reached (NR) (Figure 3E). In addition, AH effectively inhibited purified recombinant ACAT1 wild type (WT) and a control mutant C413A but not ACAT1 C126A mutant (Figure 3F). Moreover, we generated “rescue” H1299 cells with stable knockdown of endogenous ACAT1 followed by rescue expression of ACAT1 WT or distinct C→A mutants. We found that AH treatment effectively inhibited enzyme activity of ACAT1 WT and control C413A mutant, but not AH binding-deficient C126A mutant (Figure 3G). These results together suggest that C126 is important to mediate AH binding and its inhibitory effect on ACAT1.

AH disrupts active ACAT1 tetramers and blocks tetramer reformation

Using purified ACAT1 proteins in the presence or absence of AH, we found that AH inhibits ACAT1 by disrupting ACAT1 tetramers to yield monomers (Figure 3H). Further studies confirmed that purified tetrameric and monomeric ACAT1 proteins represented active and inactive forms of ACAT1, respectively (Figure 3I, left) and ACAT1 activity correlated with increasing levels of tetramer in different compositions of purified monomeric and tetrameric ACAT1 proteins (Figure 3I, right), while incubation of purified monomeric and tetrameric ACAT1 proteins for 12 hours resulted in formation of a small fraction of ACAT1 tetramers and monomers, respectively, accompanied with slightly increased and decreased ACAT1 activity, respectively (Figure 3J). These results suggest an equilibrium between tetrameric and monomeric ACAT1 proteins with spontaneous cycles of formation and disruption of ACAT1 tetramers.

We next sought to determine whether AH promotes disruption of ACAT1 tetramers or blocks monomers from forming tetramers. We separated monomeric and tetrameric ACAT1 WT or C126A proteins on a native gel. The gel was incubated with 3H-labeled AH and the binding ability of 3H-AH to monomeric and tetrameric ACAT1 proteins was assessed by scintillation counting of excised gel bands containing ACAT1 monomers or tetramers. The results of this “overlay” assay suggested that AH binds to ACAT1 tetramers with higher affinity compared to ACAT1 monomers, whereas C126A mutation attenuates AH binding to tetramers (note that C126A monomers still bind less than WT monomers) (Figure 3K), suggesting AH likely binds to and disrupts ACAT1 tetramers to release monomers. Moreover, we found that a small fraction of tetramers were formed from purified ACAT1 monomers in the absence of AH after 12 hours, whereas monomers resulting from AH-dependent disruption of ACAT1 tetramers did not form any tetramers (Figure 3L). Furthermore, we repeated this native gel experiment using 3H-AH-treated purified ACAT1 tetramers compared to purified ACAT1 monomers without AH treatment. The scintillation counting results suggest that after disruption of ACAT1 tetramer, 3H-AH remains bound to ACAT1 monomers (Figure 3M). These data together suggest a “two-step” model where AH binds only to tetrameric ACAT1 and disrupts ACAT1 tetramers to yield monomers, while
although AH cannot bind to ACAT1 monomers, it can bind to monomers resulting from tetramer disruption and prevent reformation of tetramers from monomers.

Y407 phosphorylation activates ACAT1 by stabilizing ACAT1 tetramers but not promoting tetramer formation from monomers

We found that AH treatment not only attenuated EGF-dependent ACAT1 activation and tyrosine phosphorylation of ACAT1 (Figure 4A), but also decreased tetramer formation induced by EGF (Figure 4B). In addition, in an in vitro kinase assay using purified proteins, AH treatment disrupted ACAT1 tetramers that were induced by FGFR1 incubation. Interestingly, the monomeric ACAT1 proteins resulting from FGFR1-phosphorylated ACAT1 tetramers treated with AH were still phosphorylated at Y407 (Figure 4C). This might be due to lack of phosphatase in the in vitro system. In addition, pre-treatment with AH abolished FGFR1-dependent activation of ACAT1 (AH>FGFR1) compared to ACAT1 protein incubated with FGFR1 prior to AH treatment (FGFR1>AH), while AH eventually inhibited phosphorylated and activated ACAT1 (FGFR1>AH) (Figure 4D; left), suggesting that decreased tetramer protein levels due to AH dependent disruption provided less substrate for FGFR1. Indeed, both FGFR1-dependent tetramer formation and Y407 phosphorylation of ACAT1 tetramers pre-treated with AH (AH>FGFR1) were found at lower levels compared to tetramerization and tetramer phosphorylation of ACAT1 incubated with FGFR1 prior to AH treatment (FGFR1>AH), while AH treatment of ACAT1 phosphorylated by FGFR1 resulted in decreased protein levels and phosphorylation of ACAT1 tetramers with increased protein level of monomers (FGFR1>AH) (Figure 4D; right). Note that monomeric ACAT1 was not phosphorylated, with the exception of ACAT1 monomers resulting from disruption of phosphorylated ACAT1 treated with AH (FGFR1>AH) (Figure 4D; right). These data confirm that only tetrameric ACAT1 can be phosphorylated and AH disrupts both phosphorylated and non-phosphorylated ACAT1 tetramers.

We next found that phosphorylation by FGFR1 resulted in a shift to tetramer formation of ACAT1 WT but not Y407F mutant protein, which is predominantly monomeric in the presence and absence of FGFR1 (Figure 4E). In addition, Y407F mutant demonstrated resistance to FGFR1-promoted activation and tetramer formation, while AH treatment inhibited Y407F mutant enzyme activity by disrupting Y407F tetramers (Figure 4F). These data together suggest that Y407 phosphorylation of tetrameric ACAT1 stabilizes ACAT1 tetramers by preventing spontaneous tetramer disruption, which unbalances the equilibrium between tetramer disruption and reformation, leading to a shift to promote spontaneous tetramer formation from monomers that eventually achieves phosphorylation of the majority of ACAT1 proteins (Figures 2F and 4E).

Y407 phosphorylation of ACAT1 is important for cancer cell proliferation and tumor growth, which signals through the PDP1-PDHA1 axis to promote glycolysis

We next generated “rescue” H1299 cells with stable knockdown of endogenous ACAT1 and stable rescue expression of FLAG-ACAT1 WT or Y407F mutant (Figure 5A). Consistent with our previous findings in rescue H1299 cells with stable knockdown of ACAT1 (Fan et al., 2014), expression of catalytically less active ACAT1 Y407F resulted in decreased cell proliferation under hypoxia but not normoxia and made cells more sensitive to ATP synthase
inhibitor oligomycin treatment in terms of attenuated cell proliferation, compared to control cells expressing ACAT1 WT (Figure 5B). In addition, Y407F rescue cells showed decreased phosphorylation and acetylation levels of PDHA and reduced acetylation level of PDP1, which correlated with increased PDH activity (PDC flux rate), decreased glycolytic rate and lactate production, and elevated ROS level (Figure 5C), oxygen consumption rate and intracellular ATP level (Figure 5D), compared to control ACAT1 WT cells. Moreover, Y407F cells were more sensitive to treatment with oligomycin in terms of inhibition of oxygen consumption and ATP production (Figure 5D). Consistent with these findings, we found that decreased cell proliferation under hypoxia in Y407F cells was reversed by expression of acetyl-mimetic PDP1 K202Q mutant but not acetyl-deficient K202R mutant (Figures 5E–5F), which also reversed the decreased lactate production, as well as elevated PDC flux and oxygen consumption rate (Figures 5G–5I, respectively) in Y407F cells, compared to control ACAT1 WT cells. Similar results were obtained by expressing PDHA1 K321Q or K321R mutants in Y407F cells (Figures S2A–S2E). These data together suggest that Y407 phosphorylation is important for activation of ACAT1, which predominantly signals through the PDP1-PDHA1 axis to promote the metabolic switch from oxidative phosphorylation to glycolysis in cancer cells.

Furthermore, in a xenograft experiment, the growth rate and masses (Figure 5J; left two panels) of tumors derived from H1299 cells expressing Y407F were significantly reduced. This was likely due to decreased ACAT1 activity that led to reduced S293 phosphorylation level of PDHA, decreased acetylation levels of PDHA1 K321 and PDP1 K202 (Figure 5J; middle two panels) and consequently attenuated cell proliferation assessed by decreased IHC staining of proliferation marker Ki67 (Figure S2F, and 5J; right panel) in tumors derived from Y407F cells compared to those formed by control ACAT1 WT cells.

AH inhibits glycolysis as well as cancer cell proliferation in vitro and tumor growth in vivo

AH effectively inhibits ACAT1 enzyme activity in a dose-dependent manner in H1299 (Figure 6A) and K562 cells (Figure S3A), as well as cell proliferation of diverse human leukemia and cancer cells compared to control cells (Figure 6B), suggesting selective toxicity of AH in treatment of human cancer cells. In contrast, stable knockdown of ACAT1 conferred resistance to H1299 cells to treatment with increasing concentrations of AH in terms of ACAT1 activity and cell proliferation rate (Figure 6C, left and right, respectively), suggesting that AH as a selective ACAT1 inhibitor has no off-target effect when inhibiting cancer cell proliferation.

In addition, AH treatment effectively inhibits Y407 phosphorylation and tetramer formation of ACAT1 in K562 and H1299 cells (Figure 6D), leading to increased PDH activity (PDC flux rate) with decreased lactate production in H1299 (Figure S3B) and K562 (Figure S3C) cells, as well as decreased glycolytic rate, increased oxygen consumption rate, and elevated intracellular ATP (Figures S3D–S3F, respectively), compared to control untreated cells. AH treated cells are more sensitive to oligomycin treatment in terms of inhibition of oxygen consumption and ATP production (Figures S3E–S3F), suggesting that AH inhibits ACAT1 to attenuate glycolysis and promotes oxidative phosphorylation. Moreover, AH treatment similarly resulted in increased PDH activity, decreased lactate production, increased oxygen consumption rate, and increased oxygen consumption in tumor cells derived from Y407F.
consumption rate with elevated intracellular ATP, as well as decreased cell proliferation rate under hypoxia (Figures S4A–S4D and 6E, respectively) in H1299 “rescue” cells with stable knockdown of PDP1 or PDHA1 and stable expression of PDP1 WT or PDHA WT, respectively. In contrast, “rescue” cells expressing either acetyl-mimetic K→Q or acetyl-deficient K→R forms of PDP1 or PDHA1 demonstrated resistance to AH treatment in terms of these altered metabolic properties (Figures. S4A–S4D, respectively) and cell proliferation rate (Figure 6E). These results are consistent with the observation that AH treatment resulted in reduced S293 phosphorylation level of PDHA as well as decreased acetylation levels of PDHA1 K321 and PDP1 K202 in H1299 and K562 cells (Figure S4E). These data suggest that AH predominantly targets ACAT1, leading to subsequently altered PDC flux that is predominantly important for the changes in cancer cell metabolism and proliferation. Interestingly, inhibition of ACAT1 by AH treatment (Figure S4F) or stable knockdown (Figure S4G) did not affect the intracellular levels of these two major ketone bodies in cells, suggesting that upregulated ACAT1 primarily contributes to the metabolic switch from OXPHOS to glycolysis in cancer cells.

Initial toxicity studies of chronic injection of AH to nude mice for ~4 weeks revealed that treatment with AH at dose up to 50mg/kg/day administered intraperitoneally was well-tolerated, and did not affect complete blood counts (CBC) or hematopoietic properties of nude mice (Figure S5A). Further histopathological analyses revealed no notable differences between PBS as the vehicle and AH-treated groups in tissue samples from different organs (Figure S5B). These results demonstrate that AH treatment has minimal toxicity in vivo. We next performed an in vivo AH treatment experiment using xenograft nude mice with subcutaneous injection of H1299 cells, where AH led to decreased dose-dependent reduction in tumor mass with reduced growth (Figure 6F, left and right, respectively), and did not affect hematopoietic properties of treated nude mice either (Figure S5C). Further drug treatment experiments using the maximum tolerated dose (MTD) of 50mg/kg/day revealed that AH treatment inhibited tumor growth in H1299 xenograft mice (Figure 6G; left two panels) with decreased ACAT1 activity accompanied with reduced S293 phosphorylation level of PDHA and lysine acetylation levels of PDHA1 and PDP1 (Figure 6G; right two panels) in resected tumors from xenograft nude mice, along with reduced Ki67 expression assessed by IHC staining in tumors (Figure 6H). Similar results were obtained using K562 leukemia cell xenograft nude mice treated with AH for 20 days (Figures S5D–S5E). Moreover, AH treatment decreased Y407 phosphorylation level and tetramer formation of ACAT1 in tumors derived from K562 and H1299 cells (Figure 6I) with increased PDH activity and decreased glycolytic rate as well as increased sensitivity to oligomycin treatment in terms of intracellular ATP levels (Figure S5F). These results suggest that AH inhibits ACAT1 in vivo without off-target effect, conferring a specific inhibitory effect on tumor cell proliferation by promoting a metabolic shift from glycolysis to OXPHOS.

AH inhibits Y407 phosphorylation of tetrameric ACAT1, which is commonly upregulated in primary human leukemia cells

Consistently, ACAT1 enzyme activity levels are commonly upregulated in diverse human primary leukemia cells from patients (Figures 1B and 7A; left), which is due to increased Y407 phosphorylation and tetramer formation with comparable ACAT1 protein expression.
levels (Figures 7A; right, 7B and S6A) compared to control peripheral blood (PB) cells from healthy donors. Moreover, AH did not affect cell viability of CD34+ progenitors isolated from bone marrow samples from a healthy donor, or mononucleocytes in PB samples from a representative healthy human donor (Figure 7C, left and right, respectively). In contrast, human primary leukemia cells treated with AH had decreased cell viability (Figures 7D; left, and S6B) with reduced Y407 phosphorylation of ACAT1 (Figures 7D; right, and S6C). Consistent with these results, AH effectively inhibited ACAT1 enzyme activity in diverse primary human leukemia cells (Figures S7A and 7E; right two panels), but not basal level ACAT1 activity in control CD34+ progenitors and mononucleocytes in PB samples from healthy donors (Figure 7E; left two panels). In addition, AH treatment disrupted tetrameric ACAT1 in human primary leukemia cells (Figures 7F and S7B–S7C) but did not affect levels of monomeric ACAT1 in CD34+ BM or PB samples from healthy donors (Figure S7C). Together these data suggest targeting ACAT1 as a promising therapeutic strategy in the treatment of human malignancies, and AH as an ACAT1 inhibitor with minimal toxicity to human blood cells.

DISCUSSION

Our findings reveal a molecular mechanism underlying tetrameric ACAT1 regulation in normal proliferating and highly proliferative cancer cells, and suggest that targeting ACAT1 tetramer stability represents a promising therapeutic strategy in the treatment of human cancer (Figure 7G). In this model, our results suggest a spontaneous equilibrium between monomeric and tetrameric ACAT1 proteins in cells, which represent inactive and active ACAT1 forms, respectively. In normal proliferating cells such as EGF-stimulated cells, only ACAT1 tetramers can be phosphorylated by a fraction of mitochondria-localized EGFR tyrosine kinase, leading to stabilization of active ACAT1 tetramers. Moreover, this mechanism is “hijacked” in human cancer cells, where oncogenic tyrosine kinase signaling is commonly upregulated. These findings suggest a molecular mechanism by which proliferative and oncogenic events signal through distinct acetyltransferases to regulate metabolic enzymes via cyclic acetylation/deacetylation.

Our results also demonstrate that none of the four identified tyrosine residues including Y90, Y214, Y219 and Y407 represents a major phosphorylation site of ACAT1 upon FGFR1 treatment. There might exist unidentified tyrosine residue(s) that, when phosphorylated, represent the majority of tyrosine phosphorylation of ACAT1 by FGFR1. This may be due to such Tyr-modification site(s) being incompatible with the common LC-MS platform, which is possible. It is also possible that phosphorylation of one sister molecule in an ACAT1 tetramer is sufficient to stabilize the tetramer, thus it is not necessary to achieve phosphorylation of all four sister molecules. Nevertheless, although Y407 is not a major phosphorylation site of ACAT1 by FGFR1, Y407 phosphorylation is sufficient to enhance ACAT1 tetramer formation and consequently increase ACAT1 activity to promote the Warburg effect and tumor growth.

AH as a potent ACAT1 inhibitor only binds to and disrupts active ACAT1 tetramers despite its phosphorylation status, while AH-bound monomeric ACAT1 proteins cannot reform active ACAT1 tetramers (Figure 7G). Moreover, AH inhibits cell proliferation of diverse
human cancer cells and primary leukemia cells from human patients as well as tumor growth of xenograft nude mice with no obvious off target effect and minimal toxicity. In addition, Lo et al. recently suggested that ACAT1 may confer doxorubicin resistance to uterine cancer cells (Lo et al., 2015). These data provide “proof of principle” to suggest targeting tetrameric ACAT1 as a promising therapy in the clinical treatment of human cancer patients with elevated ACAT1 activity and glycolysis rate, as well as relapsed cancer patients with chemo-resistance.

One reasonable concern is that targeting ACAT1 might have off-target effects other than impact on PDC flux in cells due to possible additional ACAT1 targets besides reported PDHA and PDP1. However, our results show that rescue cancer cells expressing acetyl-mimetic K→Q or acetyl-deficient K→R mutants of PDHA or PDP1 demonstrated resistance to attenuation of ACAT1 by AH treatment or expression of Y407F mutant in terms of alteration of cell proliferation, PDC flux, or glycolysis and oxidative phosphorylation rates. Another concern is that, although our results showed that inhibition of ACAT1 did not affect intracellular levels of ketone bodies in cancer cells, since ACAT1 is involved in beta-oxidation, targeting ACAT1 may result in similar phenotypes of “beta-ketothiolase deficiency” that is a rare, autosomal recessive metabolic disorder caused by defective mitochondrial ACAT1 due to point mutations, which is characterized by an attenuated ability to process isoleucine amino acid or the products of lipid breakdown with an increased level of cholesterol (Fukao et al., 1992). These concerns warrant further detailed toxicity, pharmacodynamics and pharmacokinetics studies to evaluate the proposed anti-ACAT1 therapies in cancer treatment.

Arecoline hydrobromide is a salt form of arecoline, which is a nicotinic acid-based alkaloid found in the areca nut that is commonly referred to as betel nut (Gupta and Warnakulasuriya, 2002; Sharan et al., 2012). Clinically, low dose intravenous application of arecoline hydrobromide has been used to treat Alzheimer’s disease (Christie et al., 1981; Tariot et al., 1988). Arecoline hydrobromide was reported to induce HA22T/VGH hepatoma cells to undergo anoikis (Cheng et al., 2010) and arecoline causes epigenetic changes in K562 leukemia cells that lead to chemical-mediated cytotoxicity and genotoxicity (Lin et al., 2011). However, arecoline has also been associated with the development of oral premalignant lesions and risk of oral cancer in many areas of Asia, the Pacific region, and in migrant communities in the United Kingdom, United States and South Africa where betel chewing is prevalent (Gupta and Warnakulasuriya, 2002; Sharan et al., 2012). Several animal studies have shown that arecoline administered by oral gavage to male mice induced lung adenocarcinoma and liver hemangioma (Bhide et al., 1984; Bhide et al., 1979), and chronic oral administration of arecoline through drinking water resulted in oral squamous cell carcinoma in mice (Lin et al., 2015b). However, arecoline was found not mitogenic to oral mucosal fibroblasts, and it causes cell cycle arrest of cultured oral KB epithelial cells (Chang et al., 2001; Tsai et al., 1997). Our results also demonstrate the inhibitory effect of intraperitoneal injection with arecoline hydrobromide on tumor growth in xenograft mice. Thus, further detailed studies are warranted to decipher the molecular and pharmacological mechanisms underlying arecoline treatment in vitro and in vivo, optimize dosage, duration and alternative application routes of in vivo treatment with arecoline and related compounds,
and develop second generation of anti-ACAT1 drugs with improved potency and reduced potential cancer risk for future clinical application.

**EXPERIMENTAL PROCEDURES**

**ACAT1 activity assay and inhibitor screening**

An *in vitro* ACAT1 activity assay was performed as described (Haapalainen et al., 2007) to identify lead compounds as ACAT1 inhibitors using a library of 2,000 biologically active compounds (The Spectrum Collection; MicroSource). In brief, 100ng purified ACAT1 protein was added to assay buffer containing 50mM Tris–HCl (pH8.1), 20mM MgCl_2_, 40mM KCl, 10µM acetoacetyl-CoA, and 60µM CoA. The change in absorbance at 303 nm was measured using a SpectraMax Plus spectrophotometer (Molecular Devices).

**Thermal melt shift assay**

In brief, thermal shift assay of compound-protein interaction was performed in 96-well PCR plates with various compound concentrations and 200µg/ml protein in a buffer solution (20mM Tris-HCl, 100mM NaCl, pH 7.4) according to the instruction of Protein Thermal Shift Dye Kit (Thermo Fisher). Small molecules were dissolved in DMSO and added to protein solution. Final DMSO concentration of the solution was 1%.

**Michael addition reaction assay**

The standard NMR spectra of arecoline are available online ([https://orgspectroscopyint.blogspot.com/2014/12/arecoline.html](https://orgspectroscopyint.blogspot.com/2014/12/arecoline.html)). Arecoline hydrobromide (2.36 mg, 0.01 mmol) and L-cysteine (6.06 mg, 0.05 mmol) were mixed before dissolving in deuterium oxide (1 mL) at pH 5 (adjusted by concentrated hydrochloric acid) and 7. The solutions were incubated at 37 °C for four hours. Thin layer chromatography was used to monitor the reaction. The reaction between arecoline and L-cysteine was confirmed by 1HNMR and 13CNMR spectroscopy.

**Arecoline hydroybromide (AH) binding assay**

Purified recombinant 6xHis-FLAG-ACAT1 proteins that are immobilized on anti-Flag beads were treated with or without rFGFR1 in an *in vitro* kinase assay. The ACAT1 proteins were eluted and separated on a native PAGE gel. The gel was incubated with 0.1 µM [³H]-Arecoline hydroybromide (synthesized by American Radiolabeled Chemicals, Inc) for 2 hours at 4 °C and then washed twice with TBS to remove the unbound [³H]-Arecoline hydroybromide. The ACAT1 proteins in monomer and tetramer form were cut from the gel and the retained [³H]-Arecoline hydroybromide on both forms of ACAT1 proteins were measured using a scintillation counter.

**Native PAGE**

Native gels were prepared as described (Zong et al., 2010) in 10% acrylamide, 0.375M Tris-HCl, pH 8.8, without SDS. The cell lysates were mixed with 5x native gel sample buffer (312.5mM Tris-HCl, pH 6.8, 0.05% bromophenol blue, 10% glycerol). The gel was run in native PAGE buffer (25mM Tris-HCl, 192mM glycine) and followed by Western blotting.

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Sucrose density ultracentrifugation

Sucrose gradient centrifugation was previously performed as described (Anastasiou et al., 2012). In brief, purified recombinant 6xHis-FLAG-ACAT1 was laid on a 10–40% sucrose gradient and spun at 50,000 rpm for 12 hours using a Beckman MLS-50 rotor and fractions were analyzed by Coomassie blue staining and Immunoblotting.

Xenograft study and studies using primary human tissue samples from patients

Approvals of mice use and experimental designs were given by the Institutional Animal Care and Use Committee of Emory University. Approval of use of human specimens was given by the Institutional Review Board of Emory University School of Medicine. All clinical samples were obtained with informed consent with approval by the Emory University Institutional Review Board. Clinical information for the patients was obtained from the pathologic files at Emory University Hospital under the guidelines and with approval from the Institutional Review Board of Emory University School of Medicine and according to the Health Insurance Portability and Accountability Act.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. EGF stimulation activates ACAT1 involving tyrosine phosphorylation and tetramer formation of ACAT1

(A) Protein and enzyme activity levels of endogenous ACAT1 in diverse human cancer cell lines. Negative controls include normal human proliferating cells including hematopoietic EL-1 cells, lung epithelia BEAS-2B, oral keratinocytes (HOK), and prostate epithelial PZ-HPV-7 and RWPE1 cells for corresponding group of cancer cells with the same tissue origins.

(B) Human primary leukemia cells isolated from peripheral blood (PB) or bone marrow (BM) samples from representative AML, B-ALL, CML, and APL patients were tested for...
ACAT1 activity (n=22) (upper) and protein expression levels (lower). PB samples from healthy donors were used as normal controls (n=4). Central values represent averages. (C–E) NIH/3T3 cells treated with EGF for increasing time were examined for ACAT1 activity (C), detection of tetrameric and monomeric ACAT1 proteins by native PAGE followed by Western blot (D), and phospho-Tyr (p-Tyr) levels of ACAT1 immunoprecipitates (E). (F) Purified FLAG-ACAT1 incubated with recombinant EGFR (rEGFR, left) or rFGFR1 (right) were tested for phosphorylated ACAT1 (lower panels) and ACAT1 activity (upper panels). Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test. Also see Figure S1.
Figure 2. Y407 phosphorylation is commonly upregulated in human cancer cells and activates ACAT1 by promoting tetramer formation
(A) Schematic representation of reported phosphorylated tyrosine residues in ACAT1.
(B) Purified FLAG-ACAT1 WT, Y90F, Y214F, Y219F and Y407F were incubated with active, recombinant FGFR1, followed by ACAT1 activity assay (left) and Western blot to detect Tyr-phosphorylation of ACAT1 proteins (right).
(C) NIH/3T3 cells with EGF treatment (upper) and H1299 cells with FGFR1 inhibitor TKI258 treatment (lower) were applied to fractionation to separate cytosol and mitochondria, followed by Western blot to show co-localization of ACAT1 and EGFR.
(upper) or FGFR1 (lower) in mitochondria. Cytosolic β-actin and mitochondrial TOM 40 were included as control markers.

(D) *Upper*: Western blot results show Y407 phosphorylation levels of ACAT1 in diverse human leukemia and tumor cells. *Lower*: Cells treated with distinct tyrosine kinase inhibitors including TKI258 (FGFR1, FLT3), imatinib (ABL), AG490 (JAK2) or erlotinib (EGFR) were tested for Y407 phosphorylation of ACAT1 by Western blot.

(E) Purified FLAG-ACAT1 incubated with rFGFR1 were tested for tetrameric and monomeric ACAT1 and their Y407 phosphorylation levels.

(F) Schematic representation of sucrose density ultracentrifugation to purify monomeric and tetrameric FLAG-ACAT1 proteins incubated with or without rFGFR1 (*left*). The collected fractions were examined by Western blot to detect ACAT1 and their Y407 phosphorylation levels (*right*). Fractions 1–4 and 5–8 correlate with monomeric and tetrameric ACAT1 proteins, respectively.

(G) Purified FLAG-ACAT1 was separated by native PAGE (*right*) and the gel slices containing monomeric or tetrameric ACAT1 proteins were incubated with rFGFR1 in the presence of “cold” ATP or “hot” ATP (γ<sup>32</sup>P-ATP) in an *in vitro* kinase assay. Retaining γ<sup>32</sup>P-ATP bound to monomeric and tetrameric ACAT1 proteins was assessed by a scintillation counter (*left*). Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test.

Also see Figure S1.
Figure 3. Arecoline hydrobromide (AH) is a covalent ACAT1 inhibitor, which disrupts active ACAT1 tetramers and blocks tetramer reformation

(A) Schematic representation of an in vitro ACAT1 activity assay-based screening strategy to identify lead compounds including AH (library ID# 21B10) as ACAT1 inhibitors.

(B) Efficacy of AH treatment in inhibition of purified recombinant ACAT1 (rACAT1), rACAT2 and rDLAT proteins; IC\(_{50}\) values were determined; NR: not reached.

(C) Molecular docking study of AH based on the crystal structure of ACAT1 (PDB code: 2ib8). AH (library ID#: 21B10) is docked in catalytic site of ACAT1; predicted binding
residues are indicated (left). Right panel shows the predicted model in which AH covalently binds to C126 of ACAT1 through a Michael addition reaction.

(D) Results of Michael addition reaction determined by NMR spectroscopy. Characteristic peaks for proton on C=C (7.03 ppm) and for C=C (122.9 and 137.8 ppm) in arecoline disappeared in $^1$HNMR (left) and $^{13}$CNMR spectra (right), respectively, in the presence of L-cysteine.

(E) Thermal shift assay shows direct binding of AH to purified rACAT1 WT but not C126A mutant.

(F–G) Effect of AH on purified recombinant ACAT1 variants (F) and “rescue” H1299 cells with stable knockdown of endogenous ACAT1 and expression of FLAG-ACAT1 variants (G).

(H–J) Purified FLAG-ACAT1 treated with or without AH (H), purified tetrameric and monomeric ACAT1, and ACAT1 protein samples containing different compositions of purified monomeric and tetrameric ACAT1 (I; left and right, respectively), or purified tetrameric and monomeric ACAT1 incubated for 0 or 12 hours (J) were applied to ACAT1 activity assay, followed by native PAGE and Western blot.

(K) Purified monomeric and tetrameric ACAT1 proteins were separated in a native gel (upper right), which was incubated with $^3$H-AH prior to excision of bands containing monomeric or tetrameric ACAT1 proteins that were applied to scintillation counting.

(L–M) Purified monomeric ACAT1 proteins (L and M) and purified tetrameric ACAT1 treated with AH (L) or $^3$H-AH (M) were incubated for 0 or 12 hours, followed by native PAGE and Western blot (L), or native PAGE with excision of bands containing monomeric or tetrameric ACAT1 proteins that were applied to scintillation counting (M). Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test.
Figure 4. Only ACAT1 tetramers can be phosphorylated and stabilized by Y407 phosphorylation
(A–B) Detection of ACAT1 activity and Y407 phosphorylation levels (A) and tetrameric and monomeric ACAT1 proteins (B) using NIH/3T3 cells treated with EGF in the presence or absence of AH.
(C) Purified tetrameric FLAG-ACAT1 treated with or without FGFR1 was incubated in the presence or absence of AH for 0 or 12 hours, followed by native PAGE and Western blot to detect tetrameric and monomeric ACAT1 and their Y407 phosphorylation levels.
(D) Purified FLAG-ACAT1 proteins pretreated with AH, followed by incubation with rFGFR1 (AH>FGFR1), or pretreated with FGFR1, followed by AH treatment (FGFR1>AH) were examined for ACAT1 activity (left) and tetrameric and monomeric ACAT1 proteins as well as their Y407 phosphorylation levels (right).

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(E) ACAT1 WT (upper) and Y407F (lower) proteins were incubated with rFGFR1 prior to sucrose density ultracentrifugation. Collected fractions were applied to PAGE, followed by Coomassie Brilliant Blue (CBB) staining.
(F) Purified FLAG-ACAT1 WT and Y407F proteins were treated with AH and/or FGFR1, followed by ACAT1 activity assay (left) as well as crosslinking assay and Western blot (right). Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test.
Figure 5. Y407 phosphorylation of tetrameric ACAT1 signals through the PDP1-PDHA1 axis to promote glycolysis, cancer cell proliferation and tumor growth

(A) Western blot results using "rescue" H1299 cells with stable knockdown of endogenous human ACAT1 and stable "rescue" expression of FLAG-ACAT1 WT or Y407F mutant.

(B–D) ACAT1 Y407F rescue cells and control WT cells were tested for cell proliferation rate under normoxia or hypoxia (B, left) and cell proliferation rate in the presence and absence of ATP synthase inhibitor oligomycin (B, right), phosphorylation and/or acetylation levels of PDP1, PDHA and ACAT1, PDH activity, glycolytic rate, lactate production and...
ROS level (C) and oxygen consumption (D, left) and intracellular ATP level (D, right) in the presence and absence of oligomycin.

(E–F) ACAT1 Y407F rescue cells with transient expression of the acetyl-mimetic form K202Q or acetyl-deficient form K202R of FLAG-PDP1 were tested for cell proliferation under hypoxia (E) and Western blot (F).

(G–I) ACAT1 rescue cells with transient expression of FLAG-PDP1 K202Q or K202R were tested for lactate production (G), PDH activity (PDC flux rate) (H) and oxygen consumption (I).

(J) Xenograft nude mice with injection of H1299 rescue cells expressing ACAT1 WT or Y407F were tested for tumor growth rate and tumor mass (left two panels). Representative tumor samples were tested for ACAT1 enzyme activity (middle), Western blot and cell proliferation assessed by IHC staining of Ki67 (right two panels). Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test. For tumor growth, data are mean ± SEM; p values were obtained by a two-way Anova test.

Also see Figure S2.
Figure 6. AH effectively disrupts tetrameric ACAT1 and inhibits cancer cell proliferation and tumor growth

(A–B) Effect of AH on endogenous ACAT1 in H1299 cells (A) and cell proliferation of diverse human cancer cell lines (color shade lines; B). Negative controls include normal human proliferating EL-1, BEAS-2B, and human keratinocyte HaCaT cells (gray shade lines; B).

(C) Effect of increased concentrations of AH on ACAT1 activity (left) and cell proliferation rate (right panels) in H1299 cells with stable knockdown of ACAT1 or control cells harboring an empty pLKO.1 vector.
(D) Lysates of K562 (left) or H1299 cells (right) with or without AH treatment were tested for levels of Y407 phosphorylation and monomeric and tetrameric ACAT1 proteins.

(E) “Rescue” H1299 cells with stable PDHA1 knockdown expressing FLAG-PDHA1 WT, K321Q, or K321R (left) and “rescue” cells with stable PDP1 knockdown expressing FLAG-PDP1 WT, K202Q, or K202R (right) were tested for cell proliferation rate under hypoxia in the presence and absence of AH.

(F) Effect of intraperitoneal AH administration on tumor mass (left) and growth rate (right) in H1299 xenograft nude mice in a dose-dependent manner.

(G–H) Effect of intraperitoneal injection of AH in H1299 xenograft nude mice on tumor growth rate and mass (G; left two panels, respectively), as well as ACAT1 enzyme activity and phosphorylation and/or acetylation levels of PDP1, PDHA and ACAT1 in representative tumor samples (G; right two panels, respectively). Cell proliferation of tumor samples was assessed by immunohistochemical (IHC) staining of Ki67 (H).

(I) Lysates of tumors from K562 (left) or H1299 xenograft nude mice (right) with or without AH treatment were subject to detection of Y407 phosphorylation and monomeric and tetrameric ACAT1 proteins. Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test. For tumor growth, data are mean ± SEM; p values were obtained by a two-way ANOVA test.

Also see Figures S3–S5.
Figure 7. AH inhibits human primary leukemia cells by disrupting ACAT1 tetramers and blocking Y407 phosphorylation

(A) Primary leukemia cells from bone marrow of a representative AML patient were tested for ACAT1 enzyme activity (left) and Y407 phosphorylation (right). Peripheral blood sample from a healthy donor was included as a control.

(B) Human primary leukemia cells from patients were tested for ACAT1 protein and Y407 phosphorylation levels (left) and tetrameric and monomeric ACAT1 proteins (right). Peripheral blood samples from healthy donors were included as controls.

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(C–E) Effects of AH on cell number of CD34+ progenitors isolated from BM (C; left), mononucleocytes from isolated from PB of representative healthy human donors (C; right), and primary leukemia cells from two representative AML patients (D; left two panels) is shown. Y407 phosphorylation (D; right) and ACAT1 activity (E) were tested at the experimental end point (72 hours).

(F) Primary AML leukemia cells treated with increasing concentrations of AH were tested for cell number (left); monomeric and tetrameric ACAT1 and Y407 phosphorylation (middle), and ACAT1 enzyme activity at the experimental end point (72 hours) (right).

(G) Proposed working model. Data are mean ± s.d; n=3 each; p values were obtained by a two-tailed Student’s test.
Also see Figures S6–S7.