

Targeting Lipid Metabolism in Cancer

Taylor R. Kavanagh¹ and Carmen Priolo^{1*}

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA

***Corresponding author:** Carmen Priolo, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Thorn Building 8th floor, Boston, MA 02115, USA, Tel: (857) 307 0783, Email: cpriolo@partners.org

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INTRODUCTION

Recent large-scale metabolomic and lipidomic profiling studies of human tumors and patient blood support the theory that the metabolic phenotype of a tumor reflects its genomic pattern and signaling pathway status [1-3]. This is leading to new opportunities in precision medicine: the use of tumor metabolic fingerprinting as a diagnostic tool to improve selection of patients for targeted therapies, and the identification of tumor-specific metabolic vulnerabilities for novel therapeutic approaches in cancer.

This chapter will provide an overview of ongoing attempts to develop novel therapeutics that target enzymes of lipid metabolism, including *de novo* fatty acid synthesis, phospholipid synthesis, and fatty acid catabolism. Figure 1 illustrates key regulatory steps of lipid synthesis and metabolism, including the candidate therapeutic targets here discussed.

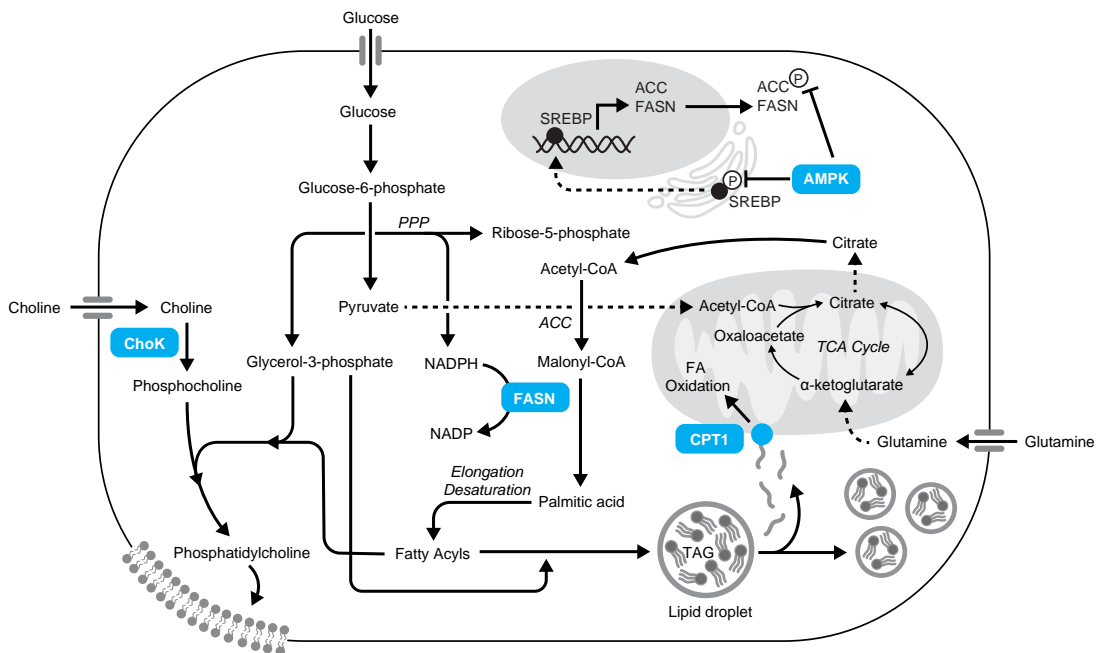


Figure 1: Graphical representation of lipid biosynthesis and fatty acid oxidation pathways. The therapeutic targets discussed in this chapter are highlighted in blue. **ACC**, Acetyl-CoA Carboxylase; **AMPK**, 5-AMP Activated Kinase; **ChoK**, Choline Kinase; **CPT1**, Carnitine Palmitoyltransferase I; **FASN**, Fatty Acid Synthase; **PPP**, Pentose Phosphate Pathway; **SREBP**, Sterol Regulatory Element-Binding Protein; **TAG**, Triacylglycerol; **TCA**, Tricarboxylic Acid Cycle.

DE NOVO FATTY ACID SYNTHESIS

Fatty acids, the major component of the cellular polar and neutral lipids, are generated from acetyl-CoA through reactions catalyzed by two enzymes: **acetyl-coA carboxylase (ACC)** and **fatty acid synthase (FASN)**. This process takes place in the cytoplasm. FASN is a homodimeric, 250-270 kDa enzyme that synthesizes a 16-carbon fatty acid product, palmitate, using acetyl-coA, malonyl-coA, and NADPH [4-6]. FASN is constituted by four C-terminal catalytic domains (acyl carrier protein, β -ketoacyl reductase, enoyl reductase, and thioesterase) and three catalytic domains in the N-terminal (β -ketoacyl synthase, dehydrase, and malonyl/acetyltransferase) [4]. FASN became of particular interest in cancer biology in the early 1990's when it was discovered as oncogenic antigen- 519 (OA-519), a protein expressed at high levels in breast cancer patients with a poor prognosis [7,8]. FASN was thus found to be overexpressed in many epithelial cancers and their pre-neoplastic lesions, including breast, colorectal, prostate, bladder, ovary, esophagus, stomach, and endometrial cancers. In several of these cancers, FASN overexpression was associated with tumor recurrence and poor prognosis [9-15], becoming an appealing target for molecular and therapeutic studies in oncology.

In a genetically engineered mouse model, FASN overexpression under the control of prostate-specific ARR2 probasin promoter led to prostate intraepithelial neoplasia [16]. In the same study, overexpression of FASN in immortalized, non-transformed prostate epithelial cells (iPRECs), together with androgen receptor, induced oncogenic transformation and *in vivo* orthotopic tumor growth. These results suggest a potential role for FASN as an oncogene in prostate cancer [16].

More recently, *in vitro* and *in vivo* studies of genetic (short-hairpin RNA, shRNA) downregulation of FASN in T24 Ha-ras-transformed human mammary epithelial cells serially passaged in immunodeficient mice, namely MCF10A-CA1d cells [17], led to suppression of tumor growth in an orthotopic model. FASN-knockdown MCF10A-CA1d cells injected into the fat pad of the mouse mammary gland formed dramatically smaller tumors with pathological features of low-grade lesions, unlike their highly invasive and proliferative control counterparts expressing FASN [18]. In keeping with these results suggesting a role for FASN in tumor cell “differentiation”, FASN was shown to be implicated in maintaining stemness of glioma stem cells (GSCs) [19]. High levels of FASN expression were found in the cytoplasm of human glioblastoma cells that expressed Sox2, a marker of GSCs. In addition, *de novo* lipogenesis and FASN expression were higher in patient-derived GSCs compared to their serum-differentiated non-GSCs. FASN inhibition by **cerulenin** decreased proliferation, invasiveness, sphere formation, and expression of stemness markers in human GSCs [19].

Cerulenin is a small fungal metabolite and specific inhibitor of FASN that binds to the active site of the condensing region of FASN, covalently inactivating its β -ketoacyl synthase site and resulting in inhibition of fatty acid synthesis. A pro-apoptotic activity of cerulenin was also shown in breast cancer cell lines [20].

Orlistat, otherwise known as tetrahydrolipstatin and marketed as Xenical, is a U.S. Food and Drug Administration (FDA) - approved drug used in the treatment of obesity. In addition to inhibiting the pancreatic and gastric lipases to prevent absorption of dietary fat [21-24], orlistat inhibits the thioesterase domain of FASN, disrupting fatty acid synthesis. In preclinical models of cancer, this results in inhibition of tumor growth and tumor progression, and apoptosis. Specifically, orlistat anti-cancer activity has been shown in prostate and breast cancer cells [25]. Interestingly, this has implications for the activity of the Her2/*neu* oncoprotein. Treatment of SK-Br3 and BT-474 breast cancer cell lines (which overexpress both FASN and Her2/*neu*) with orlistat reduced the expression of FASN and Her2/*neu*, suggesting that FASN is implicated in the regulation of Her2/*neu* in breast cancer cells [26]. It is convincing that orlistat has anti-tumoral activity; however, due to the hydrophobicity of FASN active site, the FDA-approved inhibitor orlistat has low solubility and bioavailability, making FASN a more complex molecular target [27].

Similar to orlistat, the FASN inhibitor **C75**, a synthetic molecule in the family of α -methylene- γ -butyrolactones, was discovered to induce weight loss in mice [28,29]. C75 was first identified in a screen of α -methylene- γ -butyrolactones, which were predicted through molecular modeling and

structure-based searches to inhibit the β -ketoacyl synthase subunit of FASN, similar to cerulenin [28]. α -Methylene- γ -butyrolactones lack the epoxide that cerulenin contains, which helps decrease the reactivity of the compounds while increasing their specificity and chemical stability [28]. *In vivo*, treatment of MCF-7 human breast cancer xenografts with C75 led to significant inhibition of both fatty acid synthesis and tumor growth, with no evidence of toxicity seen in other tissues beside the tumor [30]. *In vitro*, C75 inhibited fatty acid synthesis in HL60 cells, as monitored by the inhibition of radio-labeled acetate incorporation into triglycerides and phospholipids [28].

Most recently, **Fasnall**, a novel FASN inhibitor, was identified. Fasnall is a thiophenopyrimidine that targets the nucleotide co-factor binding sites of FASN. Inhibition by Fasnall led to significant changes in the lipidome of BT474 Her2+ breast cancer cells, inducing an increase in neutral lipids, unsaturated fatty acids, and pro-apoptotic lipids such as ceramides, and a decrease in the incorporation of exogenous palmitate into phospholipids [31]. *In vivo*, treatment of MMTV-Neu mice with Fasnall showed efficacy in decreasing tumor volume and increasing mouse survival rates due to anti-proliferative activity. Combined treatment with Fasnall and carboplatin, a chemotherapeutic agent used in the treatment of breast cancer, showed a greater response against tumor growth with a lessened toxic effect as seen in carboplatin treatment alone [31]. These results demonstrate the potential for Fasnall to be used in combined treatments with chemotherapeutic agents, enabling dose reduction for these agents while increasing therapeutic efficacy. Importantly, Fasnall did not induce weight loss in mice.

These results implicate the promise of developing small molecule inhibitors of FASN as anti-cancer agents. However, since most of the FASN inhibitors induce weight loss in small animal studies, none of the discussed compounds was brought to the clinic.

Indirect ways of targeting FASN activity have been extensively tested. **5' AMP-activated kinase (AMPK)** is a major regulator of cellular metabolism and signaling pathways, including lipogenesis and the mTOR pathway [32]. AMPK is a heterotrimeric serine/threonine kinase made of a catalytic α subunit and regulatory β and γ subunits that is regulated by the cellular AMP/ATP ratio [33]. An increase in AMP/ATP ratio, caused by metabolic stress, signals AMPK to turn off ATP-consuming pathways while turning on ATP-generating pathways, switching a cell from an anabolic to a catabolic state [33,34]. This switch occurs through the phosphorylation of key metabolic targets including the lipogenic transcription factor sterol regulatory-element binding protein 1 (SREBP1, which regulates the transcription of ACC and FASN), ACC, and the glucose-sensitive transcription factor ChREBP [35].

Recent data suggest that sustained activation of AMPK may suppress tumor growth [32,36]. Consistent with this, lack of AMPK activation by phosphorylation at the Th172 residue, due to inactivating mutations of the serine/threonine kinases responsible for this phosphorylation [37], has been associated with certain cancers, including genetic syndromes. One such example is the serine/threonine kinase LKB1 (Liver Kinase B1), one of the key upstream activators of AMPK

[38,39]. LKB1 acts as a tumor suppressor gene, and mutations in the gene cause the inherited Peutz-Jeghers cancer syndrome (PJS), as well as sporadic human lung cancer, including some types of non-small cell lung cancer (NSCLC) [40-43].

AMPK has thus become a multifaceted target in cancer. Interestingly, orlistat inhibits the AMPK/mTOR pathway in the endometrial cancer cell lines ECC-1 and KLE, and decreases the expression of lipogenic enzymes such as FASN, allowing for the inhibition of tumor cell proliferation [44].

Metformin became one of the first studied AMPK activators in cancer research after its use in the treatment of type II diabetes mellitus. Metformin is an oral biguanide that functions in sensitizing patients with diabetes to insulin and inhibiting hepatic gluconeogenesis [45]. Indirectly, metformin also functions as an AMPK activator that increases the AMP/ATP ratio by inhibiting complex I of the mitochondrial respiratory chain [46]. Associations between metformin treatment and lower risk of cancer, particularly prostate cancer, or cancer-related mortality in patients with type II diabetes were found in comparison to patients under other anti-diabetic treatments [47-49]. These epidemiological studies have increased the enthusiasm for the use of metformin in various cancers, including breast and prostate cancer.

In cellular models, metformin has been shown to target and remove cancer stem cells in breast cancer cell lines [50]. More relevant, the use of metformin in combination with chemotherapy in women with breast cancer led to a higher response *versus* chemotherapy alone [51].

The hypothesis that metformin activates AMPK in an LKB1-dependent manner was confirmed in mouse studies using wild-type and LKB1-deficient livers: metformin activated AMPK only in the wild-type livers and not in the livers deficient for LKB1. Blood glucose levels similarly declined by 50% in wild-type mice fed with a high fat diet for 6 weeks, while no decrease in blood glucose levels were observed in LKB1-deficient mice [52].

An ongoing phase II clinical trial at the Dana-Farber Cancer Institute (Boston, MA, USA) aims at determining whether metformin and exercise, either alone or in combination, can decrease fasting insulin levels in patients who completed standard therapy for stage I-III colorectal or breast cancer (NCT01340300). Another ongoing study at the M.D. Anderson Cancer Center (Houston, TX, USA) is testing the effects of metformin on molecular pathways such as glucose metabolism and mTOR signaling in the endometrium of women with endometrial cancer who do not have diabetes (NCT01205672). Other clinical trials are currently recruiting participants to study the effects of metformin in combination with other therapies in various cancers such as prostate cancer (NCT02153450), non-small cell lung cancer (NCT02285855), bladder cancer (NCT02360618), and breast cancer (NCT01980823).

Due to the indirect mechanistic nature of metformin, and the fact that AMPK may not be its only target [53-56], it has become critical to develop direct activators of AMPK.

5-amino-imidazol-4-carboxamide-1- β -D-ribofuranoside (**AICAR**) is a direct activator of AMPK that mimics AMP [32] and demonstrated anti-tumor activity in prostate cancer models [53,57]. While metformin did not inhibit proliferation of colon cancer cells, AICAR showed promising results [58]. Combined therapy with AICAR and 5-fluorouracil (5-FU) in the HCT116, HT29 and RKO colorectal cancer cell lines showed the ability of AICAR to activate the AMPK pathway of the mTOR signaling pathway and inducing apoptosis, which were all enhanced by the 5-FU treatment. These results suggest the ability of AICAR to work synergistically with a current colorectal cancer treatment to facilitate apoptosis and an overall more effective treatment [58]. AICAR induces cell death in chronic myelogenous leukemia (CML) cells, B-cell chronic lymphocytic leukemia cells, childhood acute lymphoblastic leukemia (ALL), and BCR-ABL-expressing cells [59-62]. Unfortunately, the oral bioavailability of AICAR is limited and treatment with AICAR often induces side effects such as increase in blood levels of uric and lactic acid [32].

Abbot Laboratories has identified a thienopyridone AMPK activator, **A-769662**, through a chemical library screen of over 700,000 compounds using purified rat liver AMPK. A-769662 reversibly binds the β 1 subunit of AMPK and was found to inhibit fatty acid synthesis in primary rat and mouse hepatocytes with no evidence of cellular stress or cytotoxicity reported. *In vivo*, A-769662 acts to decrease levels of malonyl-coA, lower glucose levels, and alter lipid metabolism in Sprague Dawley (SD) rats and ob/ob mice [63]. The ability of A-769662 to alter both glucose and lipid metabolism has clinical implications not only for diabetes and other metabolic disorders but also for cancer. Treatment with A-769662 decreased tumor growth in PTEN \pm mice with a hypomorphic LKB1 allele [64]. This opens up the possibility for the molecule to be used in the treatment of other cancers that may result from hyperactivation of the PI3K/Akt/mTOR pathway.

MT 63-78 inhibited cell growth in both androgen-dependent LNCaP and androgen-independent PC3 prostate cancer cells at higher potency than A-769662 and induced mitotic arrest and apoptosis in these cell lines. *In vivo*, 14 day-treatment of LNCaP tumor xenografts with MT 63-78 inhibited tumor growth by 33% [36].

DE NOVO CHOLINE PHOSPHOLIPID SYNTHESIS

Enhanced choline metabolism is emerging as a novel metabolic hallmark in cancer. Specifically, an increase in the synthesis of phosphatidylcholine, a major phospholipid of cellular membranes, can be detected in different cancers [65-67], and choline uptake by tumor cells can be monitored using positron emission tomography (PET)-based metabolic imaging [68]. **Choline kinase (ChoK)** is an enzyme encoded by two genes, CHKA and CHKB, which catalyzes the phosphorylation of free choline in the cytoplasm using ATP to generate phosphocholine. Three isoforms of ChoK are known in mammalian cells: ChoK α -1, ChoK α -2, and ChoK β , and the active form of the enzyme consists of a hetero- or homo-dimer [69]. ChoK is the first enzyme in the Kennedy pathway, which is responsible for the synthesis of phosphatidylcholine and phosphatidylethanolamine. ChoK α expression and activity have been found upregulated in several types of cancer, including

lung, prostate, colorectal, breast, ovarian, and bladder [70-76], becoming an attractive potential therapeutic target in oncology [77].

Hemicolinium-3 (HC-3) is an inhibitor of ChoK that cannot be used clinically as an anticancer agent due to its high toxicity on the cholinergic nerve terminals; however, modifying its structure can reduce toxicity. This has been implemented as an approach for the development of novel anticancer compounds that target ChoK. Two HC-3 derivative compounds, MN58b and TCD-717, act as competitive inhibitors, preventing binding of choline to the choline kinase active site [78].

MN58b is a first generation HC-3 derivative that shows high specificity for ChoK α . The two isoforms ChoK α and ChoK β may have similar homology; however, ChoK α has the ability to act as a choline kinase and an ethanolamine kinase, whereas ChoK β preferentially acts as an ethanolamine kinase. Treatment of tumor cells with MN58b has been shown to induce severe cellular metabolic changes, including a decrease in *de novo* phosphatidylcholine synthesis and an increase in ceramides that selectively trigger apoptosis in cancer cells [79,80].

The effects of MN58b on the growth of pancreatic ductal adenocarcinoma (PDAC) cell lines (SK-PC-1, Suit2 008, IMIM-PC-2 and RWP-1) were investigated. At the dose of 5 μ mol/L, MN58b halted colony formation in all of the tested cell lines. The IC₅₀ across a panel of 12 PDAC cell lines ranged between 0.23 and 3.2 μ mol/L, and a correlation between ChoK α protein expression and sensitivity to MN58b was shown. Consistent with this result, a direct correlation between ChoK α expression and apoptotic rate after 48-hr treatment was found in dose-response experiments [81].

Although MN58b shows antitumoral effects, its toxic profile is a limiting factor to move into clinical testing [82]. Thus, MN58b became a good model for generating new, second generation HC-3 derivative compounds.

Second generation ChoK inhibitors were synthesized to obtain a lower toxicity profile and enhanced antitumoral activity [78]. **RSM-932A** belongs to this second generation of HC-3 derivative inhibitors. Four-five athymic mice were treated with increasing concentrations of ten second generation compounds, for 5 consecutive days. Treatment was followed by a 9-day rest period, concluded with a second 5-day cycle of treatment. Maximum tolerated dose (MTD) was determined for each of the compounds based on lethality and toxicity (weight loss, changes in behavior, fur appearance). Safe and effective doses of these new compounds were then tested *in vivo* in of an aggressive, well-differentiated human colon cancer model (HT-29 cell line).

RSM-932A showed the highest efficacy in inhibiting tumor growth (77% decrease) and the lowest toxicity to the mouse (no liver toxicity). Thus, RSM-932A was chosen for further preclinical studies. A panel of 27 human cancer cell lines was used to test IC₅₀ (50% inhibitory concentration of a substance) and other parameters of drug efficacy for RSM-932A, showing a significant antiproliferative effect at doses in the low micromolar range in most cancer cell types, including brain, breast, melanoma, lung, colon, liver, ovary, cervix, bladder, kidney, bone, and pancreas [78].

Using ChoK inhibitors, particularly RSM-932A, in combination with antineoplastic agents such as 5-fluorouracil (5-FU), enhanced drug efficacy compared to single drug treatments was shown in various cancer models, including DLD-1, HT29, and SW620 colorectal cell lines, *in vitro* and *in vivo*. Mechanistically, this synergistic effect could be due to downregulation of thymidylate synthase (TS) and thymidine kinase (TK1), key enzymes involved in 5-FU metabolic activity, by the ChoK inhibitors [77].

RSM-932A, also named TCD-717, is the first ChoK α inhibitor to be tested in humans in a phase I clinical trial conducted by TCD Pharma (Valladolid, Spain) in two U.S. clinical centers (NCT01215864) in patients with advanced solid tumors. The primary endpoints of this study were to assess the safety of the drug and to determine the maximum tolerated dose and appropriate dose for phase II studies. Secondary endpoints were to measure the efficacy of TCD-717 and to test the potential correlation between choline levels in the tumors and tumor response using magnetic resonance spectroscopy. Results of this clinical trial are not yet available.

Vertex Pharmaceuticals developed a novel choline kinase inhibitor, **V-11-0711**, aiming to test whether ChoK α possesses non-metabolic functions in addition to its enzymatic activity. Specifically, Falcon et al. assessed the levels of phosphocholine in HeLa cells in response to treatment with V-11-0711 or transfection with small interfering RNA (siRNA) inhibiting ChoK α expression. A 68% decrease in ChoK α protein levels by siRNA was associated with a significant reduction in phosphocholine. Consistent with this, V-11-0711 induced a similar decrease in phosphocholine at relatively low doses (IC₅₀ < 1 μ M). However, while ChoK α siRNA induced apoptosis in HeLa cells, V-11-0711 rather halted cell growth inducing very low levels of apoptosis [83]. Interestingly, it emerged that ChoK α may function as a protein scaffold [83] associating with other oncogenic proteins such as Epidermal Growth Factor Receptor (EGFR) to promote carcinogenesis [84].

These results may have interesting implications on the role of ChoK α in cancer, suggesting the possibility that mechanisms other than the metabolic activity of ChoK can participate in tumorigenesis. Another implication of these results is that novel approaches targeting ChoK may take into account the protein scaffold function and possible interactions of ChoK with oncogenic proteins.

INHIBITORS OF FATTY ACID OXIDATION

Carnitine Palmitoyltransferase I (CPT1) is responsible for the transfer of long chain fatty acids across the mitochondrial membrane; therefore, it is considered an important rate limiting step and regulator of fatty acid oxidation [85,86]. The isoform of CPT1 found in the human brain, CPT1c, has been implicated in cancer. An overexpression of CPT1c has been shown to increase fatty acid oxidation and ATP production, and could induce resistance to metabolic stressors such as nutrient deprivation or hypoxia. Consistent with this, CPT1c-overexpressing tumors were found to be more resistant to treatment with the mTORC1 inhibitor rapamycin [87]. CPT1c may therefore contribute to the metabolic reprogramming and enhanced growth of tumor cells despite

the often hostile microenvironment, representing a new therapeutic target for tumors displaying CPT1c overexpression. Compounds that target CPT1 have been developed in an effort to treat human diseases such as chronic congestive heart failure and cancer.

Etomoxir, a clinically available small molecule inhibitor of CPT1, has previously been tested in clinical trials of chronic congestive heart failure. By not permitting the entry of long chain fatty acids into the mitochondria, etomoxir prevents fatty acid oxidation, forcing a cell to use glucose oxidation as an alternative [88,89]. Studies of etomoxir in prostate cancer [90], leukemia [91], colon, and breast cancer models [92] have shown promising results for the inhibition of fatty acid oxidation as a therapeutic approach. Mouse xenografts of VCaP prostate cancer cells treated with etomoxir for 21 days showed a decline in tumor growth with no toxicity [90]. Etomoxir sensitized MOLM13 human leukemia cells to ABT-737 [91], a known BH3 mimetic and BCL-2 protein family inhibitor [93]. The combined therapy decreased leukemia burden in mice and prolonged median survival by 33%, showing superiority to treatment with either drug alone. The same results were observed combining etomoxir treatment with the leukemia chemotherapeutic drug cytosine arabinoside (Ara-C) [91]. These studies report dependence of different types of cancer on lipid catabolism and fatty acid oxidation for generating critical ATP levels, highlighting the need for novel CPT1 inhibitors to be tested as single agents or in combination with standard therapies.

CONCLUSIONS

Lipid metabolism is involved in the regulation of key properties of cancer cells such as uncontrolled proliferation, stemness, and resistance to apoptosis. A few compounds that directly or indirectly target metabolic processes are being tested in clinical trials, and many more of these compounds are being developed in pre-clinical studies. The ultimate goal is to identify or generate compounds against metabolic targets that are fundamental for cancer cells but only have marginal relevance for normal cells in the human body. This involves the ability to clinically profile tumor metabolism using metabolic imaging or other molecular pathology methodologies to identify ideal patient candidates, and to identify a critical therapeutic window assessing optimal safe doses and maximal efficacy of these next-generation therapies.

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