Pharmacology & Therapeutics 177 (2017) 23–31

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Associate editor: B. Teicher

Fatty acid synthase – Modern tumor cell biology insights into a classical oncology target

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ARTICLE INFO

Available online 12 February 2017

Keywords:
Fatty acid synthase
Lipogenesis
Palmitoylation
Oncoantigen

ABSTRACT

Decades of preclinical and natural history studies have highlighted the potential of fatty acid synthase (FASN) as a bona fide drug target for oncology. This review will highlight the foundational concepts upon which this perspective is built. Published studies have shown that high levels of FASN in patient tumor tissues are present at later stages of disease and this overexpression predicts poor prognosis. Preclinical studies have shown that experimental overexpression of FASN in previously normal cells leads to changes that are critical for establishing a tumor phenotype. Once the tumor phenotype is established, FASN elicits several changes to the tumor cell and becomes intertwined with its survival. The product of FASN, palmitate, changes the biophysical nature of the tumor cell membrane; membrane microdomains enable the efficient assembly of signaling complexes required for continued tumor cell proliferation and survival. Membranes densely packed with phospholipids containing saturated fatty acids become resistant to the action of other chemotherapeutic agents. Inhibiting FASN leads to tumor cell death while sparing normal cells, which do not have the dependence of this enzyme for normal functions, and restores membrane architecture to more normal properties thereby resensitizing tumors to killing by chemotherapies. One compound has recently reached clinical studies in solid tumor patients and highlights the need for continued evaluation of the role of FASN in tumor cell biology. Significant advances have been made and much remains to be done to optimally apply this class of pharmacological agents for the treatment of specific cancers.

Contents

Abbreviations: HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; 3-V Bio, 3-V Biosciences, Inc.; AMPK, AMP activated protein kinase; AR, androgen receptor; ChREBP, carbohydrate-activated transcription factor response element binding protein; CPT-1, carnitine palmitoyltransferase-1; DNL, de novo lipogenesis; DH, dehydrase; DAG, diacylglycerol; ER, enoyl reductase; FASN, fatty acid synthase; Her2, human epidermal growth factor receptor 2; IC_{50}, half-maximal inhibitory concentration; iPrECs, immortalized prostate epithelial cells; KR, β-ketoacyl reductase; KS, β-ketoacyl synthase; KO, knock-out; LXRα, liver X receptor α; MAT, malonyl/acetyl transferase; mTOR, mammalian target of rapamycin; PARP, poly ADP ribose polymerase; SREBP-1c, sterol regulatory element binding protein-1c; TE, thioesterase.

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http://dx.doi.org/10.1016/j.pharmthera.2017.02.021

1. Introduction

Fatty acid synthase (FASN) is a homodimeric protein; each monomer is a large polypeptide comprised of six separate enzymatic domains working in concert to synthesize the 16-carbon saturated fatty acid palmitate from building blocks of acetyl-CoA and malonyl-CoA. Often considered a cytoplasmic protein, FASN also can localize to intracellular membranes; the specific impact of this partitioning on the cell's function remains a field of active exploration. FASN can be phosphorylated by kinases such as mammalian target of rapamycin (mTOR) and human epidermal growth factor receptor 2 (Her2) and this regulation may be important for both activity of the enzyme and its subcellular localization (Jensen-Urstad et al., 2013; Jin et al., 2010). Additionally, the FASN enzyme may be regulated by protein-protein interactions. FASN has been shown to co-localize with caveolin-1 on the membranes of prostate cancer cells; the presence of these antigens correlates with poor prognosis, notwithstanding that the functionality of this co-localization has not been elucidated (Di Vizio et al., 2008). Recently, FASN has been shown to interact physically with the protein, designated as FAMIN, on perioxosomes, driving the flux of lipid synthesis; and the thyroid hormone responsive protein Spot14 has been shown to enhance the activity of the FASN enzyme (Cader et al., 2016; Di Vizio et al., 2008; Rudolph et al., 2014).

The concept of targeting FASN for cancer therapy has been described in the scientific literature for over 20 years starting with the identification of onco-antigen OA-519, a breast cancer tumor antigen that correlated with poor prognosis, as FASN (Kuhajda et al., 1994). A large number of preclinical studies over the years have demonstrated that this enzyme and de novo lipogenesis (DNL), its cognate pathway, are critical components of tumor cell survival and proliferation for a wide range of cancers. Studies on the mechanistic role of FASN in tumor biology have illuminated effects ranging well beyond what one typically classifies as cellular energy metabolism; FASN integrates with signaling pathways, contributes to post-translational palmitoylation of proteins and impacts membrane structure and function. FASN is an example of a classic oncology drug target by meeting the following criteria. First, it is an enzyme, amenable to traditional small molecule inhibitor drug discovery methodologies; second, it is differentially expressed in tumor cells compared to normal cells, enabling the potential for tumor-selective activity; and third, its sustained activity is requisite for tumor cell growth and survival. This review will focus on the foundational observations supporting the concept of FASN as an oncology target; for reviews detailing the mechanisms of FASN action in tumor cell biology, signaling pathways or early attempts at generating inhibitory compounds refer to the following excellent reviews: (Flavin, Peluso, Nguyen, & Loda, 2010; Heuer, 2016; Jones & Infante, 2015; Liu, Liu, Wu, & Zhang, 2010; Menéndez & Lupu, 2007; Mullen & Yet, 2015; Röhrig & Schulze, 2016).

2. The role of FASN and DNL in non-tumor tissues

Examining the role of FASN in humans or animal models outside the context of tumor biology enables one to build a foundation upon which to design and interpret potential toxicological outcomes associated with inhibiting this target. Using both human biopsy and autopsy materials, Weiss concluded that FASN-mediated DNL was a pathway of minor importance in humans, in contrast to rats where counterpart tissues have a 10–50 fold higher flux through this pathway (Weiss et al., 1986). A review of in vivo stable isotope studies concluded that DNL (both hepatic and whole body) in humans was inconsequential whereas hepatic and adipose DNL in rodents accounted for more than 50% of fatty acids (Murphy, 2006); the molecular features driving these differences require further investigation. This species-specific difference may become relevant when attempting to model pharmacological and toxicological outcomes from an extrapolation of drug levels in a more sensitive species (i.e. rats) when compared to humans.

An additional source of information comes from murine FASN gene knock-out (KO) models in mice. While these studies are informative, they imperfectly reflect inhibition by a drug. Gene KO completely ablates all enzymatic activity associated with the target as well as any potential scaffolding, localization, or protein-protein interaction roles; in contrast, pharmacological inhibition rarely achieves 100% continuous inhibition of the enzyme, reduces protein associations and localization, or causes significant reduction in the quantity of the protein. A classic example of this difference can be found with statin drugs. These drugs are used by millions of people to safely and chronically lower cholesterol levels; however, a murine KO of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the target of statin therapy, is embryonically lethal (Ohashi et al., 2003). In addition, mice with a liver-specific KO of HMG-CoA reductase develop steatosis and die soon thereafter (Nagashima, Yagyu, Ohashi, & Tazoe, 2012). The earliest publication of a germline murine KO of FASN showed that this gene was also essential for the development of the embryo (Chirala et al., 2003). This observation aligns with the role that FASN plays in highly proliferative tissues, such as those found in the developing embryo and many tumor cells. Following this report, several tissue-specific FASN KO models were developed, including liver, intestine, skeletal muscle, adipose tissues, endothelial cells and cardiomyocytes (Chakravarthy et al., 2005; Funai et al., 2013; Lodhi et al., 2012; Razani et al., 2011; Wei et al., 2011, 2012). In all cases, these animals were viable. In some of these models, such as the liver-specific KO (FASKOL) mouse, there was no observable phenotype under normal conditions; placing FASKOL animals on a zero-fat, high carbohydrate diet resulted in significant and paradoxical hepatic steatosis and hypoglycemia (all of which were reversed by PPARα agonist) but did not lead to death (Chakravarthy et al., 2005). The other models demonstrated roles for FASN in the integrity of specific cell-to-cell barriers, muscle and fat performance under disrupted metabolic conditions, and response to cardiac stress as well as a dependence of adult neural stem cells on FASN to regenerate (Knobloch et al., 2012). A recent study utilizing a whole animal inducible KO of FASN showed that animals died 10 days following induction; death was likely a consequence of a disrupted mucosal layer in the intestine giving rise to sepsis (Lodhi et al., 2015). While it is unlikely that pharmacological levels of a FASN inhibitor would produce these phenotypes, these studies can be helpful in interpretation of adverse events that may be observed during routine animal toxicity testing of FASN inhibitors.

Early studies using small molecule FASN inhibitors shed light on some interesting FASN biology; however, the off-target effects of these molecules also clouded the direct attribution of effect to FASN. One molecule widely used in preclinical studies, C-75, not only inhibits FASN irreversibly, but binds to several other cellular proteins and is a CPT-1 agonist, stimulating fatty acid oxidation (Chen et al., 2014). In animal studies, this drug can cause significant weight loss, anorexia and other issues that complicate interpretation of results (Thupari, Landree, Ronnett, & Kuhajda, 2002). Studies using C-75 showed the impact of FASN on the regulation of feeding behavior in mice. By delivering C-75 by direct intracerebroventricular administration and monitoring malonyl-CoA levels, investigators showed that inhibition of FASN in the hypothalamus led to an increase in malonyl-CoA, a reduction in neuropeptide Y, and a cessation of feeding (Hu, Cha, Chohnan, & Lane, 2003; Loftus et al., 2000); the direct observation of FASN in this circuit was later confirmed using a KO model in which FASN was deleted from the hypothalamus (Chakravarthy et al., 2007). Based on these results, FASN inhibitors that cross the blood-brain barrier should be monitored for an impact on feeding and weight loss.

3. FASN, a cancer antigen portending poor prognosis

Studies from a large range of different tumor types including breast, prostate, colorectal, bladder, and lung show that FASN protein can be detected in sections of tumor tissue, and that the intensity of FASN

staining increases with stage of the disease. In contrast, normal, non-tumor tissues adjacent to the tumor rarely express significant amounts of FASN protein (Bhatt et al., 2012; Cai et al., 2014; Di Vizio et al., 2008; Gelebart, Zak, Anand, Belch, & Lai, 2012; Kuhajda et al., 1994). In addition, studies in breast, pancreatic and colorectal cancers demonstrated that these patients can have elevated levels of FASN in their sera; again correlating to later disease states (Alo et al., 2007; Notarnicola et al., 2011; Walter et al., 2009; Wang et al., 2001). 3-V Bio performed a small study on banked sera from patients with different cancers as well as healthy controls; this study showed a significant increase of FASN antigen in the blood of patients with different cancers compared to control subjects lacking a known cancer (Fig. 1). This study helps identify tumor types that are more frequently associated with higher FASN levels, and follow up studies with better annotated samples (e.g. number of prior therapies, metastatic state, etc.) would be useful in determining the optimal utility of this approach.

Several mechanisms may be responsible for increasing the level of FASN protein in tumor tissues. The expression of FASN mRNA is regulated by the transcription factors sterol regulatory element binding protein-1c (SREBP-1c) and carbohydrate-activated transcription factor response element binding protein (ChREBP) (Ishii, Iizuka, Miller, & Uyeda, 2004), both of which are modulated by cellular energy sensing mediators such as mTOR, AMP-activated protein kinase (AMPK), and liver X receptor α (LXRα) (Hansmannel, Mordier, & Iynedjian, 2006). Additional factors such as NAC1, the acetyltransferase P300, and certain microRNAs have also been shown to regulate expression of FASN, especially in tumor cells (Gang et al., 2016; Ueda et al., 2010; Wang et al., 2016). It is likely that increased activity of energy sensing pathways, such as mTOR, in many tumors drives increased transcription of FASN via activation and nuclear localization of SREBP-1c. Other mechanisms can also lead to the dysregulated expression of FASN activity. The FASN protein itself can be stabilized by expression of the USP2a isopeptidase, itself often overexpressed in tumors, particularly those of prostate origin (Graner et al., 2004). Finally, perhaps less common, an examination of the Catalogue of Somatic Mutations in Cancer (COSMIC) and Cancer Cell Line Encyclopedia (CCLE) datasets show that some tumors, notably ovarian tumors, have increased copy numbers of the FASN gene.

Three natural history studies have extended the observation that FASN expression is a correlate of poor prognosis. A small study of patients with early stage non-small cell lung cancer followed the patients for mortality. Patients with low amounts of FASN in their tumor sections that were prepared from specimens collected at lobectomy or pneumectomy tended to have a higher rate of survival than those with high levels of FASN, although the difference was not statistically significant (p-value = 0.1) (Visca et al., 2004). A larger observational study was performed in 424 overweight men with prostate cancer; there was a statistically significant correlation between high levels of FASN in their tumors and a shorter time to death by prostate cancer than in men with low levels of FASN (Nguyen et al., 2010). Recently, low levels of tumor FASN expression in women with Her2+ breast cancer predicted better relapse free survival than women with high levels of FASN; this antigen offered no differentiation in women with other subtypes of breast cancer (basal, luminal A or luminal B) (Corominas-Faja et al., 2016).

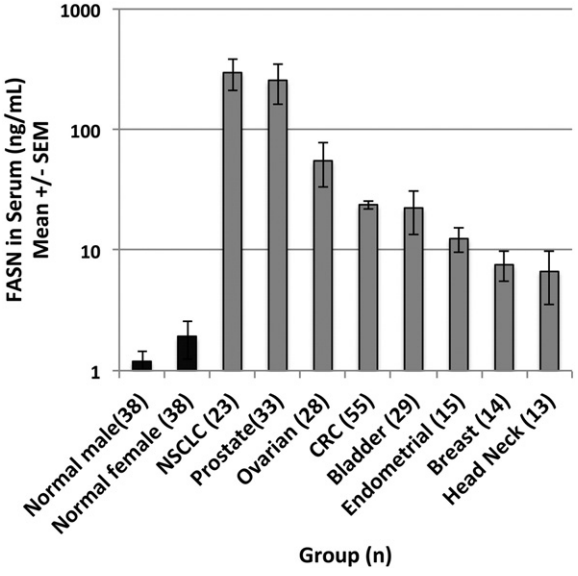

Fig. 1. FASN levels in the serum from normal subjects (black bars) and patients with different solid tumors (grey bars) were acquired from a tissue bank and FASN antigen evaluated by ELISA. SEM: standard error of the mean.

4. FASN as an oncogene in experimental systems

The correlation of FASN expression with disease and disease state does not address the question of whether FASN is directly causing the tumorigenic properties or whether it is a downstream consequence of other changes in the tumor tissue. In vitro experiments have been used to show that FASN overexpression can be a significant contributor to transformation and tumorigenic potential. An in vitro study showed that ecotopic overexpression of FASN in previously non-tumorigenic human breast cells led to enhanced lipogenesis, cellular growth, proliferation, Her2 kinase activity and other indications of tumorigenic potential that included anchorage-independent growth in culture (Vazquez-Martin, Colomer, Brunet, Lupu, & Menendez, 2008). More recently, these studies were extended; shutting off the expression of FASN in tumorigenic breast cancer cells led to a reversal of tumorigenicity and a return to a stable, non-malignant state and architecture (Gonzalez-Guerrico et al., 2016). In addition, immortalized prostate epithelial cells (iPrECs) overexpressing FASN led to invasive tumors (90%) with a much higher frequency than those lacking overexpressed FASN (30%) (Fiorentino et al., 2008). These invasive tumors appeared to have increased palmitoylation of Wnt-1 and stabilized β-catenin. Further work in this model showed that co-expression of the androgen receptor (AR) was required for the tumor to establish itself as an invasive adenocarcinoma, suggesting FASN was essential but not sufficient to elicit the complete tumorigenic potential (Migita et al., 2009). AR activation induces SREBP dependent transcription raising the possibility that the AR requirement is linked to further driving FASN overexpression.

These studies show that the overexpression of FASN is a key element in the pathway to establishing tumorigenicity in previously normal cells and its inhibition can restore some tumor cells to non-tumorigenic phenotypes. However, simply correlating FASN quantity, or activity, to dependence of the tumor cell on this enzyme has not yielded a consistently positive trend. Tumor cell lines of different sensitivity to a FASN inhibitor were assessed for FASN enzymatic activity, protein quantity and mRNA abundance; none of these parameters strongly correlated with sensitivity (Benjamin et al., 2015). It remains unclear how much FASN overexpression and, possibly quite importantly, at what stage(s) of normal-to-tumor cell progression this overexpression is critical to drive the changes forward. It is possible that timing of FASN overexpression is as, or more, important than the degree of overexpression. One might speculate a rapidly amplifying cycle in the transition of normal to tumorigenic cells via receptor tyrosine kinase (RTK) pathways. Upregulation of RTK signaling via mutation or aberrant ligand binding leads to increased activity of the Akt/mTor axis. This increased activity leads to increased expression of positive regulators of FASN mRNA expression including SREBP-1c. The increased expression of FASN would lead to the remodeling of plasma membranes, enrichment of microdomains that increase the efficiency of signaling creating a cycle that leads to an increased activity of important tumor survival and proliferation pathways (see Fig. 2).

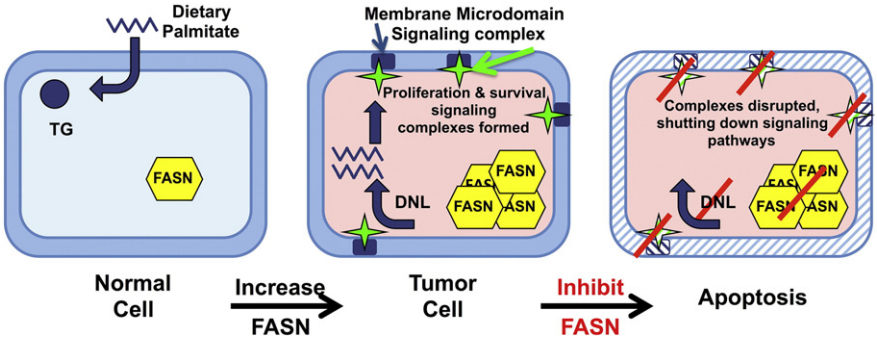

Fig. 2. Model for establishing dependence on FASN for tumor cell survival. Normal cells take up palmitate from dietary sources and convert it rapidly to triglyceride (TG) pools for storage; relying very little on FASN for production of palmitate. Overexpression of FASN is a critical step in the transformation to a highly proliferative tumorigenic cell type. These tumor cells become reliant on FASN catalyzed synthesis of palmitate for membrane formation; membrane microdomains (darkened membrane regions), enriched in saturated fatty acids, enable signaling complexes (stars) to efficiently assemble bringing the integral membrane receptor proteins together with their perimembranous, cognate intracellular partners. These signaling pathways (eg Her2/PI3K/Akt/mTOR, and PKC) are likely different among different tumor cells types. Inhibition of FASN reduces the level of palmitate required to build new membranes and maintain the microdomain enrichment. Once the microdomains breakdown, the signaling complexes are disrupted and lose their ability to maintain the tumor cell's proliferation and survival pathways, resulting in apoptosis.

5. FASN inhibition kills many tumor cell types but not normal cells

Normal, non-cancerous cells typically acquire the majority of their palmitate from dietary sources and rapidly store it in triglycerides. Palmitate itself along with other long-chain, saturated fatty acids has an inherent toxicity (lipotoxicity). To avoid this problem, most normal cells rapidly convert free saturated fatty acids to triglycerides, thereby storing the energy and neutralizing the deleterious effect (Listenberger, Ory, & Schaffer, 2001). In contrast, most cancerous cells actively synthesize their own fatty acids and typically store very little in triglyceride pools, rendering them dependent on, or addicted to, FASN; tumor cells often use these fatty acids to produce phospholipids to build membranes supporting cellular growth and proliferation (Li & Cheng, 2014). The lipid composition of many tumor cells typically is enriched for mono-unsaturated/saturated 16–18C fatty acids making them more resistant to reactive oxygen species and altering the fluidity of the plasma membrane. This enrichment can be reversed to more normal levels by inhibiting FASN (Rysman et al., 2010). Normal cells typically have longer chain and polyunsaturated lipids dominating the membrane composition. One interesting observation made at 3-V Bio using a small set of cell lines ranging from non-transformed, diploid human cells to tumorigenic cells, showed that the sensitivity to cell killing by a selective small molecule inhibitor of FASN correlated with the quantity of saturated fatty acids stored in the cell; high levels of fatty acids in the normal cells correlated with insensitivity to FASN inhibition (Fig. 3).

In addition to these structural uses, palmitate is used to covalently modify certain tumor-promoting polypeptides at specific sites. Wnt, tubulin, HRAS, NRAS and the KRAS-A isoform among others all require posttranslational palmitoylation in order to localize and function properly (Fiorentino et al., 2008; Gao & Hannoush, 2014; Heuer et al., 2016; Song et al., 2013; Zambito & Wolff, 2001; Zhao et al., 2016). FASN inhibition was shown to reduce the palmitoylation of Wnt in prostate cancer cells leading to a loss of activity in the tumor-critical Wnt/β-catenin signaling pathway. Signaling pathways including the PI3K/Akt/mTOR, Wnt/β-catenin and protein kinase C pathways in tumor cells become dependent on FASN for survival (for recent reviews see (Heuer, 2016; Jones & Infante, 2015; Röhrig & Schulze, 2016).

Her2 and FASN are often found overexpressed in breast cancer cell lines and inhibiting either activity can effectively result in apoptosis of the tumor cell. In a study using lipidomic analyses, Benjamin, et al. showed that FASN inhibition led to a decrease in diacyl glycerols (DAGs), particularly those in which at least one acyl chain was palmitate. The reduction in this class of DAGs resulted in a decline in protein kinase C stimulation and signaling, again leading to death of the tumor cell (Benjamin et al., 2015).

Inhibition of FASN by pharmacologic and genetic moieties (e.g. RNAi) can kill many different tumor cell types yet have little direct effect on many normal cells, again related to the observation that few non-cancerous cells rely on FASN to produce palmitate (Li & Cheng, 2014; Ventura et al., 2015). Apart from a vast literature showing that C-75 and other small molecule FASN inhibitors can kill tumor cells, a select number of studies used siRNAs to FASN. One model showed that the FASN siRNA not only killed prostate tumor cells in vitro, but direct injection into a tumor xenograft showed a reduction in tumor growth (Chen, Chang, Chuang, Tai, & Hwang, 2012). In addition, tumor cells killed by FASN inhibition go through a bona fide apoptotic process as shown by activation of cleaved poly ADP ribose polymerase (PARP) and staining by annexin V (Ventura et al., 2015). Exposing non-tumorigenic cells to a FASN inhibitor typically leads to either reduced proliferation, or no effect at all, but no cell killing. Since tumor cells typically store much less energy in the form of triglycerides, it is intriguing to speculate that they rely much more heavily on DNL to supply their various needs for

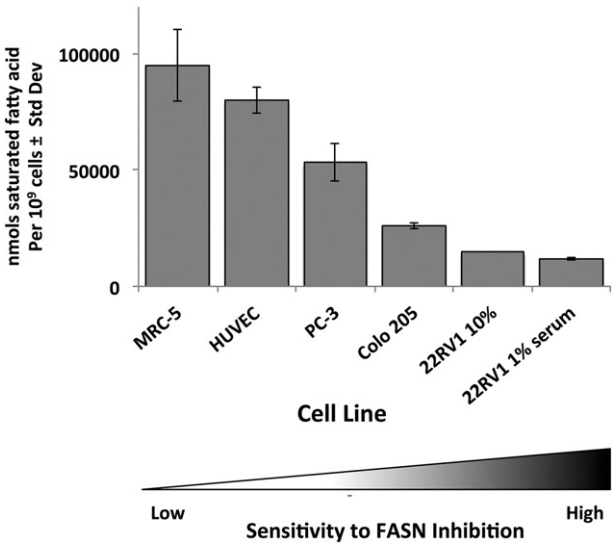

Fig. 3. The indicated cell lines were seeded into T-175 flasks and incubated in 1% charcoal-stripped fetal bovine serum (FBS) or 10% charcoal-stripped FBS (22RV1 only) for 2 days. Cells were rinsed with cold phosphate buffered saline, scraped from the dish, collected, counted, centrifuged and pellets frozen in liquid nitrogen. Cell pellets were hydrolyzed, lipids saponified and total fatty acids were esterified. The esterified fatty acids were quantitated by gas chromatography/mass spectrometry at Metabolon, Inc. The quantities of C-14 to C-24 saturated fatty acids were combined and plotted.

survival and proliferation, therefore making them more sensitive to FASN inhibition.

One hurdle that needed to be addressed in the field was the potential for dietary sources of palmitate to overcome killing of tumors in vivo. Animal studies have shown clearly that an anti-tumor effect can be elicited in the face of adequate levels of dietary fat. The concept that dietary palmitate is utilized differently than the pool synthesized by FASN-mediated DNL has been shown in a number of different studies. In vitro experimental systems examining the impacts of FASN inhibition often utilize the addition of high levels of exogenous palmitate in the presence of a FASN inhibitor to nullify the inhibitor's effect, thereby demonstrating the on-mechanism activity of the compound (Ventura et al., 2015). However, elegant in vivo studies have highlighted the different utility of the pools of dietary palmitate and newly synthesized palmitate. FASN gene KO studies showed that fat made by DNL in the liver was critical for signaling and controlling specific metabolic processes (Chakravarthy et al., 2005; Razani et al., 2011). More recently using ^{14}C acetate or ^{14}C palmitate tracers, Wei and colleagues showed that the palmitate required for the formation of membrane microdomains, critical for macrophage function, was supplied by DNL; exogenous palmitate was unable to substitute despite the addition of high levels (Wei et al., 2016).

Other features of the tumor microenvironment may be important for FASN dependent tumors. Vascularization of tumors, especially colorectal tumors and gliomas appear to depend on FASN for appropriate integrity of the vessels (Zaytseva et al., 2014). Additionally, exosomes from tumor cells appear to carry FASN and can stimulate surrounding cells to begin actively synthesizing lipids (Sano et al., 2014). Finally, FASN in the tumor stroma may impact other critical factors including inflammatory responses (Moon et al., 2015).

6. The FASN enzyme – a druggable target

The physical attributes of FASN along with its multiple, enzymatic domains enable a broad range of drug discovery efforts, as there is no a priori rationale for prioritizing one of these features over another from the perspective of efficacy. Each of the subunits in the FASN multifunctional complex offers opportunities for engineering a selective small molecule inhibitor to intervene including acetyl-CoA priming, malonyl-CoA loading, cofactor binding and chain elongation steps. One approach to drug design includes designing analogs to these substrates as inhibitors. However, since many of these substrates are used in other anabolic processes, selectivity for FASN would be very difficult to achieve. Uncompetitive or allosteric inhibition of the FASN chain elongation steps in which a small molecule binds to areas outside of the common fatty acid intermediate binding pockets offers an attractive avenue for engineering inhibitor specificity. The X-ray crystal structure of mammalian FASN was revised in 2008 revealing a new quaternary X-shaped homodimer architecture in which the condensing enzymatic domains β-ketoacyl synthase (KS) and malonyl/acetyl transferase (MAT) are separated from the dehydrase (DH), enoyl reductase (ER) and β-ketoacyl reductase (KR) modifying domains, and all subunits are spaced by flexible linkers which appear to aid substrate transfer (Leibundgut, Maier, Jenni, & Ban, 2008; Maier, Leibundgut, & Ban, 2008; Maier, Leibundgut, Boehringer, & Ban, 2010). Most of FASN's enzymatic domains, with the exception of KS, retain function as isolated subunits thereby supporting the use of independent enzymes for screening and structure-base design (S. Smith, Witkowski, & Joshi, 2003). For example, the co-crystal structure of orlistat (Xenical®), primarily a lipase inhibitor with FASN-inhibitor activity, in the active sites of the thioesterase (TE) dimer has been solved elucidating both a covalent and hydrolyzed product binding interaction (Pemble, Johnson, Kridel, & Lowther, 2007; Ritchie et al., 2016). Starting from a high throughput screening campaign using human FASN, optimization of a triazolone series resulted in potent compound, GSK2194069 (FASN IC_{50} 7.7 nM), which was the first reported inhibitor co-crystal with the KR domain (Hardwicke et al., 2014). The recent advances in FASN structure and enzyme domain co-crystal structures open the way for new structure-based drug design, targeted virtual screening and subunit screening programs.

Early examples of small molecule FASN inhibitors include cerulenin, a FASN inhibitor isolated from fungal extract (Vance, Goldberg, Mitsuhashi, & Bloch, 1972), and C-75, a covalent inhibitor based on the binding mode of cerulenin; both have been used extensively as tool compounds to probe the biology of FASN. Originally identified as an antifungal, cerulenin is an irreversible inhibitor of both bacterial and mammalian FASN (Kuhajda et al., 2000; Omura, 1976). While active against a variety of tumor cell lines and xenograft models, the highly reactive nature of the cysteine-reactive epoxide group and off-target activities prevented clinical development of cerulenin (Angeles & Hudkins, 2016; Liu et al., 2010). A routine tool compound used in many published in vitro and animal studies, C-75 was shown to interact with 3 different enzymatic functions of FASN including the TE, KS and ER activities (Rendina & Cheng, 2005). This breadth of reactivity along with its irreversible nature and reactivity with sulfhydryl groups may underlie the overall promiscuous nature of this particular compound, which has been shown to interact with and agonize carnitine palmitoyltransferase-1 (CPT-1) as well as bind to a number of different cellular proteins (Cheng, Li, Uttamchandani, & Yao, 2014).

A reduced form of the natural product lipstatin, orlistat, is intended to treat obesity, has been found to irreversibly inhibit the TE domain of FASN and has been employed to evaluate FASN activity in vitro and in vivo (Kridel, Axelrod, Rozenkrantz, & Smith, 2004). Orlistat blocks absorption of free fatty acids form the gastrointestinal tract by inhibiting pancreatic and gastric lipase which hydrolyze triglycerides (Borgström, 1988; Guerciolini, 1997; Hadváry, Lengsfeld, & Wolfer, 1988). Orlistat contains a highly reactive beta-lactone that covalently captures reactive serine residues such as Ser^{2308} in the TE domain of FASN (Pemble et al., 2007). Exploring its FASN-inhibitor activity, several studies have reported inhibition of proliferation in multiple tumor cell lines, tumor cell apoptosis and mediation of tumor growth in murine models (Kridel et al., 2004; Liu et al., 2010) However due to its chemically unstable beta-lactone, inhibition of multiple enzymes, poor water solubility and poor GI absorption, orlistat is not well suited for clinical development as an orally-delivered systemic anti-tumor agent (Mullen & Yet, 2015; Zhi, Melia, Eggers, Joly, & Patel, 1995).

Recent efforts to identify FASN inhibitors with improved pharmaceutical properties over early tool compounds and natural products have focused on molecules that are selective, nonreactive and reversible inhibitors. Rational, systematic design efforts to find selective inhibitors have recently shown promising results. A screening effort directed at the KR domain yielded selective and reversible compounds; GSK2194069 was optimized from these early compounds and shown to be a specific inhibitor of the KR activity with potent anti-tumor activity against proliferation of A549 non-small-cell lung cancer cells (Hardwicke et al., 2014; Vázquez et al., 2008). A series of 3-aryl-4-hydroxyquinolin-2(1H)-ones identified from screening were empirically optimized using human FASN activity to produce a potent compound, designated compound 16 (IC_{50} 19 nM). The series was hypothesized to mimic the intermediate formed in the KS domain, although substantiating data was not reported (Rivkin et al., 2006).

Fasnall, a thiophenopyrimidine, was recently discovered using a target-agnostic chemoproteomic screening strategy which reports release of proteins bound to cibacron blue sepharose beads upon exposure to small molecule libraries (Alwarawrah et al., 2016). Calibrated toward identifying inhibitors of FASN cofactor NADPH, the small molecule library was selected for structural similarity to purine. Screening hits were then assayed for inhibition of functional FASN activity by acetate and glucose incorporation into total lipids in HepG2 cells. Several molecules of similar structure were identified; Fasnall being the most potent (IC_{50} 147 nm ^{3}H acetate and 213 nM ^{3}H glucose), although no data were presented for cofactor competition or identifying the target domain

inhibited. Fasnall was shown to reduce proliferation of several breast cancer cell lines and reduce tumor progression in a MMTV-Neu/Her2 + breast cancer mouse model.

A series of imidazopyridines were discovered by screening against human FASN activity and optimized for in vivo stability and pharmacokinetic properties. A potent example, compound 19 (IC_{50} 17 nM human) was used to demonstrate dose-dependent pharmacodynamic reduction of de novo palmitate synthesis in cells and in vivo in rat livers after oral dosing (Oslob et al., 2012). Another analogue from this series, TVB-3166, was assessed for anti-tumor activity in preclinical models. Evaluated against a panel of 90 diverse tumor cell lines, TVB-3166 showed dose-dependent induction of cell death in all lines; this oral once-daily drug was well tolerated in mice and showed anti-tumor growth properties in multiple xenograft tumor models (Ventura et al., 2015).

FASN offers drug developers a number of approaches for the design of small molecule inhibitors. To date, the TE and KR domains of FASN have been established as targets for small molecules inhibitors, however several programs have yielded potent and selective compounds derived from FASN functional screening and recent reviews of these efforts can be found in the literature (Angeles & Hudkins, 2016; Mullen & Yet, 2015) and the development status of a select number of these inhibitors is shown in Table 1. Modern, advanced, potent FASN inhibitors are typically well tolerated in animal studies. This represents a significant advance in the field from older compounds described in the literature and increases the confidence of FASN as a viable, widely applicable therapeutic target. Tools for evaluating independent FASN domains and utilizing constructs for structure-based design will help elucidate the specific subreaction and molecular interactions of recent inhibitors; as well as potentially fuel the next generation of selective FASN inhibitor discovery.

7. Combining FASN inhibitors with other anti-tumor agents

Combination therapy can be thought of in 3 broad categories: a) agents that both work on a tumor but provide no synergy (additive), b) bona fide synergistic activities and c) agents that restore tumors' sensitivity to other drugs. FASN inhibitors may be combined with other drugs to achieve all 3 classes of effect; however, this review will focus on synergy and chemoresensitization approaches. FASN inhibition and taxanes have been shown to be synergistic in tumor cell killing. Combining a FASN inhibitor with a taxane resulted in cell killing beyond that which either compound could achieve on its own and exceeded the degree of killing expected by simply adding together that of the individual drugs (Menéndez, Vellon, Colomer, & Lupu, 2005). A thorough evaluation of the mechanisms of action of FASN inhibitors and taxanes was necessary in order to understand how bringing these two compounds together elicits such a strong anti-tumor effect. First, a portion of tubulin is directly modified by palmitoylation (Wolff, 2009). Second, it was recently shown that FASN treated tumor cells have significantly rearranged microtubules and a reduction in the posttranslational palmitoylation of tubulin (Heuer et al., 2016). The consequence of removing palmitate likely weakens or removes the anchoring of the microtubule to the membrane. This combination of separating microtubules from the membrane (FASN inhibition) and destabilizing the filaments by interfering with microtubule depolymerization (taxane) delivers two separate yet potent hits to the cellular microtubule network, thereby eliciting a synergistic interaction (Heuer et al., 2016). Other potentially synergistic FASN inhibitor combinations have been suggested by preclinical studies. The proteasome inhibitor, bortezomib, augmented suboptimal FASN inhibition in preclinical models; the crosstalk of cellular pathways induced by the combination of agents made cells highly sensitive to stress mechanisms and killing (Little, Wheeler, Koumenis, & Kridel, 2008).

Early studies on FASN demonstrated that increased expression in cells led to resistance to previously effective drugs (reviewed in Wu, Qin, Fako, & Zhang, 2013). Elegant studies showed that tumor cells that became resistant to drugs had elevated FASN levels and a commensurate enrichment of saturated fatty acids in their membrane phospholipids. This change in membrane composition gave rise to the cells with less fluid membranes resulting in reduced permeability to agents such as doxorubicin, reducing chemotherapeutic access to the intracellular compartment. Following inhibition of FASN in these cells, the membranes were returned to a more normalized and fluid lipid composition and doxorubicin was again able to cross the membrane to cause cell killing (Rysman et al., 2010).

8. Identifying tumors susceptible to FASN killing

As expected, not all tumor cells are killed by FASN inhibition. Identifying those tumors susceptible to this class of inhibitors is a critical component of a personalized or precision medicine therapeutic approach to therapy. As already stated, simple examination of FASN protein levels or activity in the tumor is not sufficient to identify susceptible tumor types. Gene expression signatures, encompassing broader pathway analyses, may provide an orthogonal approach to defining susceptible tumors. Gene sets identified by two groups showed that tumor cell types could be segregated into broad metabolic categories and that these categories were enriched for susceptibility to certain agents (Collisson et al., 2011; Daemen et al., 2015)

Daemen and colleagues classified pancreatic tumor cells as either “glycolytic” or “lipogenic” based on the selected gene set. The glycolytic cells were almost all sensitive to an inhibitor of glycolysis whereas a small molecule FASN inhibitor had virtually no effect on this class of cell. In contrast, cells types with the lipogenic signature clearly enriched for sensitivity to a FASN inhibitor although several lipogenic cell types remained unaffected (Daemen et al., 2015). Further work is necessary to refine this signature to identify the subset of lipogenic cells that are dependent on FASN. Further refinement of this approach could be very useful for the field such as looking for tissue of origin, genetic drivers or combinations of all these criteria, and which may be helpful in identifying those tumors that are absolutely dependent on FASN for survival. These studies may enable patient selection and personalized medicine approaches with FASN inhibitors in the future.

FASN fills the criteria of a bona fide cancer target. Work spanning two decades from a large number of laboratories has provided an

Table 1
Development status of select FASN inhibitors.

Compound	Status	Original sponsor	Reference
G28UCM	Preclinical	Univ. Girona, Spain	Puig et al. (2011)
GSK2194069	Preclinical	GlaxoSmithKline	Hardwicke et al. (2014)
Fasnall	Preclinical	Duke Univ. Ohio State Univ.	Alwarawrah et al. (2016)
JNJ-54302833 JNJ-54302833	Preclinical	Janssen Research & Development, LLC	Lu, Alexander, Bignan, Bischoff, and Connolly (2014)
IPI-9119	Preclinical	Infinity Pharmaceuticals	Brophy, Conley, O'Hearn, Douglas, and Cheung (2013)
Platensimycin	Preclinical	Merck Research Laboratories	Wu et al. (2011)
TVB-2640	Clinical phase 1/2	3-V Biosciences	Dean et al. (2016), OFarrell et al. (2016)

excellent foundation for interest and investment to develop drugs to inhibit this target. FASN is critical for the oncogenic process, its overexpression portends poor outcome in patients and the toxicological profile of inhibition is acceptable for pharmacological manipulation of this target safely in patients. One inhibitor, TVB-2640, an oral, once-daily dosed, inhibitor of FASN is the first to enter the clinic. TVB-2640 has demonstrated prolonged stable disease when given in monotherapy and confirmed Response Evaluation Criteria in Solid Tumors (RECIST) partial responses when combined with weekly paclitaxel. Responses were seen across multiple tumor types, including $KRAS^{mut}$ non-small cell lung, ovarian, and breast cancer. Further exploration in specific tumor types is ongoing (Dean et al., 2016).

The dysregulation of FASN is also important in a number of other diseases including hepatitis C and liver diseases such as NASH. Many of the underlying ideas presented here can form a framework for applying FASN inhibitors in these diseases; however, the pharmacological application of a drug to these applications will require very different approaches depending upon the indication. In most oncology applications, the common practice is to establish a maximally tolerated dose for use in early, and often later, studies. This approach assumes that target engagement is maximized for a particular compound and often comes with a downside of relatively high rates of adverse events. In fact early phase clinical trial designs in oncology (i.e. classical 3 + 3 approach) are based on increasing the dose of the drug until an unwanted adverse event profile is reached, regardless of where the dose lies on the target modulation curve that this occurs; it is reasonable to expect that some drugs are dosed at levels substantially beyond where saturation of inhibitory activity has been achieved, although this cannot always be measured in early trials. In the first-in-human study with TVB-2640, novel biomarker readouts of FASN inhibition have been identified using metabolomics and lipidomics of serum and sebum respectively, which provide a framework for dose selection and pharmacodynamic monitoring in future clinical studies for this class (OFarrell et al., 2016). For other diseases, ablation of FASN activity may not be necessary for active therapy and lower doses may be sufficient to achieve efficacy. These will be important parameters for basic scientists, pharmacologists and clinicians to weigh as this important target is developed further.

Conflict of interest

D. Buckley, G.Duke, G.Kemble, W.McCulloch, and A. Wagman are current employees and equity holders of 3-V Biosciences, Inc. T. Heuer and M. O'Farrell are former employees of 3-V Biosciences, Inc.

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