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A novel role for DGATs in cancer

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Introduction

Dysregulation of lipid metabolism is pathologically associated to metabolic diseases such as atherogenic dyslipidemia, hepatic steatosis, obesity and type 2 diabetes. Up-regulation of lipid metabolism has been demonstrated to result in lipid droplets biogenesis within the cells. Furthermore, there is increasing evidences linking elevated numbers of lipid droplets with cancer biology.

Acyl-CoA:diacylglycerol acyltransferase (DGAT1 and DGAT2) proteins control lipid synthesis by catalyzing the last step involved in the formation of triacylglycerol (TAG) synthesis. Our group has recently unveiled cross-talk between glycerolipid and sphingolipid metabolism through DGAT2-dependent synthesis of 1-O-acylceramide from ceramide and fatty acyl-coA in the endoplasmic reticulum (ER)- lipid droplet interface (LD). Sequestration of pro-apoptotic ceramide by 1-O-acylceramide formation was associated with chemotherapy resistance in colon cancer cells.

Here, we review the function of DGAT enzyme and focus on the role of DGAT in ceramide homeostasis, as well as the potential therapeutic of pharmacological inhibition of DGAT protein in the treatment of metabolic diseases and cancer; LD biogenesis and its relationship with DGAT proteins. We explore the emerging roles of DGAT enzymes and LDs in tumor carcinogenesis, cancer aggressiveness, chemotherapy resistance and cancer stem cells invasiveness.

1. Sphingolipid metabolism

Sphingolipids, are fundamental constituents of all eukaryotic membranes and their metabolism is carried out by a broad array of anabolic and catabolic reactions with ceramide as their hub (for a review, (1)). Ceramide can be formed by multiple pathways: the *de novo* pathway where ceramide is synthesized from non-sphingolipid precursors, the multi-step

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hydrolysis of complex sphingolipids and the salvage pathway where sphingosine can be re-acetylated to form ceramide.

The *de novo* biosynthesis of sphingolipids begins with the condensation of serine and palmitoyl-CoA, a reaction catalyzed by the ER-located enzyme serine palmitoyltransferase (SPT) (reactions are depicted in Figure 1). Its product, 3-ketodihydrosphingosine, is converted to dihydrosphingosine by the enzyme 3-ketodihydrosphingosine reductase (KDHR). Ceramide synthases (CerS1-6) *N*-acetylate the dihydrosphingosine backbone to form dihydroceramide, which can be then transformed to ceramide by the enzyme dihydroceramide desaturase (DES). Interestingly, ceramide can be further modified at the 1-hydroxyl position to form more complex sphingolipids (Figure1). For instance, ceramide kinase (CERK) catalyzes its phosphorylation to generate ceramide-1-phosphate. Sphingomyelin is the product of the sphingomyelin synthases activity (SMS1-2), which transfer a phosphocholine moiety from phosphatidylcholine to the 1-O-position. Glucosylceramides are produced by the addition of UDP-galactose (galactosyltransferase, CGT) or UDP-glucose (glucosylceramide, GCS) at the ceramide backbone. More recently, we discovered the formation of O-acylceramides by the addition of a fatty acyl (FA) chain at the 1 or 3-O-hydroxyl and catalyzed by the ceramide O-acyltransferase activity of diacylglycerol acyltransferase, DGAT 1 or 2 enzymes (see in detail below) (2). Interestingly, CerS1-6, fatty acyl-CoA synthase (ACSL) and DGAT2 were found to form a multi-enzyme complex on the ER-LD interface (2) (Figure 2).

The salvage pathway is regulated by several enzymes, such as sphingomyelinases (neutral, acid or alkaline; SMase) or by the cleavage of glycosphingolipids, controlled by β-glucosidases (GCases) and galactosidases (GalC). Likewise, ceramidases (CDases) breakdown ceramide generating sphingosine, which can either be phosphorylated to sphingosine-1-phosphate by the action of sphingosine kinases (SK1-2) or re-acylated to ceramide by ceramide synthases (CerS1-6). Hydrolysis of acyl-ceramides may also contribute to the generation of ceramides. However, the enzyme(s) that catalyze(s) this reaction remains unknown.

2. Diacylglycerol acyltransferase (DGAT) enzymes

Triacylglycerides (TAG) are the principal source of energy storage in eukaryotic cells. TGs are neutral lipids formed by a glycerol backbone and three molecules of fatty acids (FA) covalently linked by ester bonds. FAs need to be activated to acyl-CoA FAs by the ACSL enzyme. Because of their hydrophobic nature, TGs are stored in plasma lipoproteins or in cytosolic LDs. TGs can be synthesized within the cell by two pathways: the glycerol phosphate or Kennedy pathway and the monoacylglycerol pathway. Both pathways converge into the last reaction of TAG synthesis, where diacylglycerol (DG) is converted to TAG by the action of the acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) enzyme (3).

Mammals have two DGAT enzymes, DGAT1 and DGAT2. However, they are members of two different gene families and differ in their tissue distribution. Recent studies have concluded that DGAT1 and DGAT2 differ in their substrate affinities, topology, protein partners and cellular functions.

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Several lines of evidence suggested that DGAT2 is involved in the bulk of TAG synthesis. For example, overexpression of DGAT2 in rat hepatoma cells showed significantly more TAG mass accumulation than DGAT1 overexpressing cells (4). Conversely, deletion of the yeast ortholog of DGAT2 (*Dga1*) resulted in higher reduction of TAG than the ortholog of DGAT1 (*Are1* or *Are2*) (5) (6). Likewise, DGAT2-deficient mice (*Dgat2(–/–)*) had deficient lipid body (lipopenia) and died soon after birth due to impaired permeability barrier function in the skin (4), whereas DGAT1-deficient mice (*Dgat1(–/–)*) showed less TAG in the liver and mammary gland (7). Mutations in *DGAT1* associated with decreased levels of DGAT1 protein were identified in patients with congenital diarrheal disorders. Patient-derived organoids showed altered TAG metabolism, such as reduced TAG levels and lack of LD formation when cells were loaded with oleic acid. In addition, DGAT1-deficient cells were sensitized to lipid stress upon oleate treatment which resulted in increased caspase3/7 activation as compared to control. Overexpressed DGAT1 or DGAT2 reversed the phenotype observed in the organoids derived from mutant DGAT1 patients (8).

2.1. Identification of human DGAT genes

Identification of DGAT1 gene—The enzymatic synthesis of TG was first described in 1960 (9). Nevertheless, the genes encoding murine and human acyl-CoA:diacylglycerol acyltransferase (DGAT1) were first identified by the Farese laboratory in 1998 (10). In this study, human and murine expressed sequence tags (EST) with similarity to acyl-CoA: cholesterol acyltransferase (ACAT) were identified. One cDNA was found to encode a 500-aminoacids protein with a 20% sequence similarity to mouse ACAT. Then, H5 insect cells were infected with baculovirus containing this cDNA. The authors found that infected cells highly expressed an approximately 50Kda protein in the membrane fraction, as well as showed increased TAG mass. In addition, DGAT activity was more than 5-fold higher in membranes isolated from insect cells expressing the cDNA as compared to wild-type virus-infected cells. Taken together, these data confirmed the identification of a DGAT cDNA clone.

The human DGAT1 gene (*Dgat1*) has been located to chromosome 8 and comprises 17 exons (3,10). DGAT1 protein is ubiquitously expressed, with the highest expression levels in the small intestine, followed by testis, adipose tissue, mammary gland, pancreas and liver. Orthologues of DGAT1 have been found in plants and yeast (3).

Identification of human DGAT2 gene—Several lines of evidence pointed at the existence of more than one DGAT activity. Smith et al. reported that DGAT1-deficient (*Dgat* –/–) mice shown decreased body mass, whereas they had normal plasma TG levels (7). At the same time, Lardizabal et al. purified and identified a couple of peptides with DGAT enzymatic activity (36 and 36.5 kDa, respectively) from the lipid bodies of the fungus *Mortierella ramanniana* (11). Sequencing of the peptides and later cloning of the cDNA, showed that the novel DGAT (designated as DGAT2) expressing genes were unrelated to the previously identified DGAT1.

Farese's group cloned and characterized the mammalian DGAT2 gene thorough its homology to the fungal DGAT2 identified in *M. ramanniana* (12). DGAT2 homologs were

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identified in fungi, plants, and mammals. In 2002, the acyl-CoA:monoacylglycerol acyltransferase (MGAT, EC 2.3.1.22) was identified as a DGAT2 gene family member through its homology (13).

The human DGAT2 gene (*Dgat2*) has been located to chromosome 11 and comprises 8 exons (3). In most species, the DGAT2 gene encodes a 350–400 amino acid protein, with a predicted molecular weight of 40-45 kDa. The DGAT2 protein is expressed majorly in liver and adipose tissue, followed by mammary gland and testis (12,14).

2.2. Biochemistry of DGAT enzymes

Multiple lines of evidence have concluded that both DGAT1 and DGAT2 enzymes catalyze the synthesis of TAG. First, isolated membranes from insect cells overexpressing mouse DGAT1 or DGAT2 cDNA resulted in elevated TAG mass. Second, increasing *in vitro* synthesis of TAG in those membranes correlated with increasing substrate concentration of either DAG or (C18:1) oleoyl-CoA (10,12). Third, mice lacking DGAT1 or DGAT2 show defective tissue-specific synthesis of TAG.

Because of the challenges associated to the purification and crystallization of membrane proteins, DGAT enzymes have not been successfully purified yet. Nevertheless, Cases et al utilized similar amounts of overexpressed DGAT1 and DGAT2 enzymes in insect cells to study substrate specificity of DGAT1 and DGAT2 enzymes. The two enzymes showed similar maximal rates of TAG synthesis when incubated with DAG and oleoyl-CoA up to 200μM. However, DGAT1 overexpressing cells retained the enzyme activity at higher oleoyl-CoA than DGAT2, suggesting different K_m values, with DGAT2 enzyme being more active at lower oleoyl-CoA concentrations. In addition, *in vitro* competition assays of distinct unlabeled FA-CoAs with [^{14}C]oleoyl-CoA were performed. Interestingly, (C18:2) linoleyl-CoA, (C16) palmitoyl-coA and (C20:4) arachidonyl-coA competed similarly. Taken together, the authors concluded that both DGAT1 and DGAT2 enzymes have comparable specificity for FA-CoA substrates (12).

2.3 Non-canonical acyltransferase activities

DGAT1 catalyzes the synthesis of diacylglycerols, retinyl esters and waxes— Additional acyltransferase activities have been probed for both DGAT1 and DGAT2 enzymes. DGAT1 has been reported to possess acyl-CoA:retinol acyltransferase (ARAT) activity both *in vitro* assays with DGAT1-overexpressing insect cells and *in vivo* assays in murine and mammalian cells (15-17). Additionally, DGAT1 also possesses acyl-CoA:monoacylglycerol acyltransferase (MGAT) activity (17), suggesting that the DGAT1 enzyme might be able to catalyze the two-step esterification of MAG to TAG. Overexpressed DGAT1 in COS-7 cells exhibited wax synthase activity, whereas the control cells did not (17).

DGAT2 catalyzes the synthesis of 1-O-acylceramide—As mentioned above, our group discovered that human DGAT2 displays *in vitro* acyl-CoA:ceramide acyltransferase activity resulting in the formation of O-acylceramide (2). By using a proteomic approach, Senkal et al. found that ACSL5 interacts with several CerS (CerS 1, 2, 4, 5 and 6) in

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HCT-116 colon cancer cells. The authors hypothesized that the FA-CoA generated by ACSL5 would be utilized by CerS to generate ceramide. However, ACSL5 down-regulated resulted in an increase in total ceramide mass although CerS activity remained unchanged. Inhibition of SPT by myriocin or CerS by fumonisin B1 abrogated the accumulation of ceramide in cells lacking ACSL5; thus, suggesting that ACSL5 down-regulation accumulates ceramide synthesized by the *de novo* pathway at the level of CerS. On the other hand, overexpression of ACSL5 decreased the cellular levels of ceramide, implying that ACSL5 enzyme might direct ceramide to the formation of O-acylceramide (2).

To test the new hypothesis, cells were subjected to a base hydrolysis reaction that resulted in the selective hydrolysis of ester-linked FAs at carbon 1 position and therefore producing ceramide and free FA. Levels of O-acylceramide can be quantified by measuring the different in ceramides levels upon base hydrolysis (18). Interestingly, the levels of C16-O-acylceramide were significantly increased in ACSL5-overexpressing cells. Conversely, downregulation of ACSL5 resulted in the reduction of C16-O-acylceramide. Thus, ACSL5 modulates the cellular levels of ceramide and O-acylceramide (2).

Synthesis of O-acylceramides was first characterized *in vivo* in rat brain in 1977 (19). Three different radio-labeled (^{3}H and ^{14}C-labeled) ceramides were injected into the brains of 18-day-old rats. After 2h, the animals were sacrificed, and the sphingolipids were isolated and purified, which led to the discovery of an unknown group of lipids that were identified as ceramide fatty acid esters. Almost twenty years later, it was found that a novel enzyme catalyzed the esterification of ceramide at the hydroxyl group at the C1-position in Madin-Darby canine kidney (MDCK) cells and mouse tissue homogenates (20). In 2002, a novel enzyme with lysosomal phospholipase A2 and transacylase activity was purified and characterized from bovine brain (21). *In vitro* activity assays unveiled its capability to transfer the acyl group at the sn-2-position from glycerophospholipids (such as, phosphatidylethanolamine (PE) and phosphatidylcholine (PC)) to ceramide. The new enzyme was named 1-O-acylceramide synthase (ACS).

Most recently, two yeast acyl-CoA:sterol acyltransferases (Lro1p and Dga1p) were found to acylate ceramide at the C1 hydroxy group (22). *LRO1* is homologous with the mammalian LCAT (lecithin cholesterol acyltransferase) (6). Lro1p enzyme was known to transfer a FA from PE (phosphatidylethanolamine) or PC (phosphatidylcholine) to DAG. Voynova et al demonstrated that NBD–ceramides were acylated *in vitro* and *in vivo* by Lro1p in the presence of oleic acid. Likewise, purified Lro1p–GFP enzyme catalyzed the O-acylation of NBD–ceramide to produce O-acylceramides. Conversely, *S. cerevisiae lro1* cells showed reduced levels of O-acylceramides (22). Interestingly, when the capability of Dga1p to acylate ceramides with different acyl-CoAs as a donor was tested *in vitro* (microsomal fraction), no changes in O-acylceramide levels were observed. However, when metabolic labelling experiments were carried out *in vivo*, Dga1p-dependent synthesis of O-acylceramides was detected, suggesting that the Dga1p may act in a different location than in the ER. The authors speculated that synthesis of O-acylceramides might occurr at the LDs, where Dga1p is known to catalyze the formation of TAG. Furthermore, mice lacking DGAT2 do not survive and show impaired permeability barrier function in the skin associated to reduced levels of acylceramide (4).

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Considering the above evidence, our group tested whether modulation of DGAT2 activity could affect ceramide levels in HCT-116 colon cancer cells. Several approaches were examined. First, silencing of DGAT2 resulted in increased ceramide levels and concomitant decrease of O-acylceramides. On the other hand, gain-of-function experiments induced accumulation of O-acylceramide within the cells. Second, acyl-CoA:ceramide acyltransferase activity was assayed *in vitro* from DGAT2 overexpressing cells. Thus, microsomal fraction was incubated with palmitoyl-CoA and NBD-C12-ceramide which resulted in the formation of NBD-acylceramide, Interestingly, the levels of acylceramide remained unchanged when catalytically inactive DGAT2 was used. Third, *in vitro* synthesis of O-acylceramide was significantly reduced by the DGAT2 specific inhibitor PF-06424439 in a dose-dependent fashion (2). Taken together, these results suggested that DGAT2, in collaboration with CerS and ACSL5, is involved in the formation of cellular O-acylceramides.

The bioactive role of ceramide in cell death has been extensively studied since we demonstrated that exogenous ceramide treatment induced apoptosis in leukemic cells (23). Thus, Senkal et al focused on the biological significance of the formation of O-acylceramide from ceramide in the context of cancer cell death. As expected, the levels of ceramide were increased in HCT-116 cells treated with the chemotherapeutic 5-fluorouracil (5-FU). Interestingly, knock-down of ACSL5 basally decreased cell viability and induced caspase3/7 activation (measured by activity assay and by western blot), resembling the effects of CerS1 enzyme overexpression. When cells lacking ACSL5 or DGAT2 were challenged with 5-FU, caspase activation was synergistically elevated and ceramide increase was also observed. On the other hand, overexpression of ACSL5 protected cells from 5-FU induced cell death. Thus, inhibition of O-acylceramide generation resulted in accumulation of ceramide levels and sensitized cells to chemotherapy.

2.4 Topology of DGAT enzymes

Both DGAT1 and DGAT2 enzymes are integral membrane proteins that localize mainly to the microsomal fraction, hence ER localization, where TAG synthesis takes place. Shockey et al investigated the production of TAG by DGAT1 and DGAT2 enzyme in seeds of the tung tree (*Vernicia fordii*). Myc epitope-tagged tung DGAT1 or DGAT2 was overexpressed in tobacco (*Nicotiana tabacum*) suspension cells and then examined their immunofluorescence staining patterns by confocal laser-scanning microscopy (CLSM). As previously published, both DGAT1 and DGAT2 proteins localized to the ER and were enriched in subdomains of the ER. When myc-DGAT1 and GFP-DGAT2 were co-expressed in tobacco cells, the authors found that DGAT1 and DGAT2 do not co-localize but rather were juxtaposed at different subdomains of the ER, thus, suggesting that DGAT1 and DGAT2 might be involved in different subcellular pools of TAG (24).

In the yeast *Saccharomyces cerevisiae*, the highest DGAT (homolog of DGAT) enzyme activity was found in lipid particles, followed by the microsomal and mitochondrial factions, respectively (5). DGAT2 but not DGAT1 translocates to LDs when cells are treated with oleate (a known inducer of LD formation) in *Drosophila* S2 cells (25), *Saccharomyces cerevisiae* (26) as well as mammalian cells (27). Furthermore, overexpressed murine DGAT2

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partially co-localized with mitochondria in COS-7 cells. When cells were loaded with oleate, mitochondria re-organized around LDs which did not occur in DGAT1-overexpressing cells. Subcellular fractionation showed DGAT2 enrichment in the mitochondria-associated-membranes (MAMs), correlated with higher DGAT2 enzymatic acyl-CoA:diacylglycerol acyltransferase activity as compared to the microsomal fraction. A mitochondrial targeting sequence was identified in the N terminus between amino acids 61 and 66 of murine DGAT2 (28). Taken together, these findings suggest that reorganization of organelles such as LDs, ER and MAMs might maximize lipid synthesis, and that DGAT2 protein (through its N-terminus) may favor this reorganization by localizing to the contact sites between the different organelles (bridging function) and promoting more efficient lipid synthesis.

2.5. Inhibition of DGAT enzymes as a therapeutic target

DGAT1 Inhibitors—Pharmacological inhibition of DGAT protein has been proposed to have therapeutic potential in the treatment of metabolic diseases such as atherogenic dyslipidemia, hepatic steatosis, obesity and type 2 diabetes. Small-molecules inhibitors for DGAT1 and DGAT2 enzymes have been developed by several pharmaceutical companies and have entered clinical trials (29). For instance, JTT-553 compound trans-5'-(4-amino-7,7-dimethyl-2-trifluoromethyl-7H-pyrimido[4,5-b][1,4]oxazin-6-yl)-2',3'-dihydrospiro(cyclohexane-1,1'-inden)-4-yl]acetic acid monobenzenesulfonate, a selective inhibitor of DGAT1, was shown to increase glucose uptake in adipose tissues, reduce body weight gain and fat weight in diet-induced obesity (DIO) mouse models. It also suppressed plasma TAG in a dose-dependent fashion upon olive oil loading in Sprague-Dawley (SD) rats (30,31). The small molecule T863 2-((1,4-trans)-4-(4-(4-Amino-7,7-dimethyl-7H-pyrimido[4,5-b][1,4]oxazin-6-yl)- phenyl)cyclohexyl)acetic acid, has been tested *in vitro* and *in vivo*. When orally administered, T863-treated mice showed delayed fat absorption. Likewise, T863 compound caused weight loss, improved insulin sensitivity and lessened hepatic steatosis in a diet-induced obese C57/BL6 mouse model. T863 enhanced insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes (32).

Several DGAT1 inhibitors have been evaluated in clinical trials. The compound LCQ908 2-[4-[4-[5-[[6-(trifluoromethyl)pyridin-3-yl]amino]pyridin-2-yl]phenyl]cyclohexyl]acetic acid has been extensively study *in vitro* and *in vivo*. Also, named Pradigastat, this selective and potent DGAT1 inhibitor entered a Phase 2 evaluation for the treatment of diabetes mellitus 2 in 2010 (www.clinicaltrials.gov identifier NCT00901979). Although no results have yet been published in a peer-reviewed journal. Pradigastat is currently in Phase 3 evaluation for the treatment of familial chylomicronemia syndrome, where it showed to decreased TAG in patients (www.clinicaltrials.gov identifier NCT01514461). Pradigastat has been demonstrated to reduced postprandial TAG levels and glucose after high-fat meal in a single-dose study in overweight or obese but healthy subjects. Mild gastrointestinal adverse effects were reported, such as diarrhea, nausea, and abdominal pain (33,34) (35).

The compound PF-04620110 trans-4-[4-(4-Amino-7,8-dihydro-5-oxopyrimido[5,4-f][1,4]oxazepin-6(5H)-yl)phenyl]-cyclohexaneacetic acid is an orally bioactive, selective DGAT1 inhibitor. *In vivo* experiments showed decreased TAG synthesis in HT-29 human

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cells as well as rats (36). In 2009, PF-04620110 was selected for clinical studies for the treatment of obesity as reported on www.clinicaltrials.gov (identifier NCT00959426). To our knowledge, the results of the Phase 1 Clinical Trials have not been reported in the literature. Likewise, PF-04620110 entered a Phase 1 Clinical Trials for the treatment of type 2 diabetes patients (www.clinicaltrials.gov identifier NCT01298518). The results of the study are publicly available on the website. AstraZeneca developed the compound AZD7687, which showed a potent and selective inhibition against recombinant DGAT1 as well as the enzyme isolated from human liver microsomal, adipose and intestinal tissues (29,37). A single-dose clinical study in humans with AZD7687 reported decreased levels of postprandial TAG levels following a high-fat meal challenge in a dose-dependent manner. AZD7687 can be administered orally and no major safety concerns were observed. However, gastrointestinal adverse events (such as nausea, diarrhea and vomiting) was reported in the subjects treated with the highest concentrations of AZD7687, thus, limiting the dose escalation (38). Nevertheless, several Phase 1 clinical trials with AZD7687 have been reported in www.clinicaltrials.gov for the treatment of obesity (identifier NCT01119352) and Type 2 Diabetes (identifier NCT01217905).

DGAT2 Inhibitors—The use of DGAT2 tissue-specific antisense oligonucleotides in rodents has unveiled the beneficial effects of regulating DGAT2 enzymatic activity. Suppression of DGAT2 expression, but not DGAT1, reduced TAG levels in the liver and improved hepatic insulin sensitivity in a diet-induced hepatic steatosis rat model (39). Knockdown of DGAT2 in mouse liver showed reduced TAG synthesis, VLDL TAG, Apo B secretion and plasma cholesterol as compared to wild-type animals (40). In addition, liver and adipose knockdown of DGAT2 reduced hepatic lipogenesis, hepatic steatosis, and attenuated hyperlipidemia in both high-fat diet-induced obese and *ob/ob* mouse models (41). These data have led to the development of DGAT2 inhibitors (14) (42), whereby several specific DGAT2 inhibitors are commercially available for research purposes, such as JNJ DGAT2-A (chemical name: 3-Bromo-4-[2-fluoro-4-[[4-oxo-2-[[2-(pyridin-2-yl)ethyl]amino]-1,3-thiazol-5-(4H)ylidene]methyl]phenoxy]benzonitrile) and JNJ DGAT2-B (chemical name: 5-Fluoro-2-(3-phenoxyphenyl)-1,2-benzisothiazol-3(2H)-one that were tested *in vitro* with purified recombinant DGAT2 expressed in insect cells or HepG2 cells lysates (43). To our knowledge, these compounds have not been tested in animals. An imidazopyridine-based inhibitor, namely PF-06424439 (chemical name: [(3R)-1-[2-[1-(4-Chloro-1H-pyrazol-1-yl)cyclopropyl]-3H-imidazo[4,5-b]pyridin-5-yl]-3-piperidinyl]-1-pyrrolidinyl-methanone methanesulfonate), selectively inhibits DGAT2 and has been demonstrated to decrease circulating and hepatic lipids when orally administered to dyslipidemic rodent models (44,45).

Most recently, our group has reported the effects of PF-06424439 on the levels of ceramide and O-acylceramide in the liver and LDs from 8-week old mice fed a high-fat diet enriched with oleate. Mice loaded with oleate had increased LDs and steatosis as compared to the control group. Furthermore, accumulation of ceramide and O-acylceramides were significantly increased in both the liver and LDs from oleate high-fat diet fed mice. However, when PF-06424439 was added to the drinking water, levels of total ceramides and O-acylceramides were unchanged in the control mice group, but decreased in the high-fat

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diet fed mice group. These data suggested that DGAT2 is indeed responsible for synthesis of O-acylceramide in mice (2).

Unpublished data from our group point at DGAT2 as a new therapeutic target in the treatment of breast cancer. We found that the percentage of dead cells (as quantified by the lactate dehydrogenase (LDH) released in the media assay) were synergistically increased in MCF-7 cells co-treated with the DGAT2 inhibitor PF-06424439 and cisplatin. Furthermore, whereas cisplatin alone increased total levels of ceramide 2-fold, the combination of PF-06424439/cisplatin resulted in a 5-fold elevation of ceramides as compared to untreated cells. PF-06424439 alone did not significantly affect ceramide levels or cells death, although it did decrease basal levels of O-acylceramide by half. Moreover, we also found synergistic effects in cell death using DGAT2 inhibition in several other conditions we tested, as depicted in Table1.

3. DGAT proteins, lipid droplets and cancer cells

3.1 Lipid droplets accumulate in distinct cancers

Lipid droplets biogenesis—For a long time, LDs were solely considered as fat storage compartments and only recently has the scientific community begun to appreciate the pathobiological role of LDs. Currently, LDs are considered dynamic and functional organelles involved in a myriad of cellular processes such as lipid metabolism, cellular membrane biosynthesis, cell signaling, inflammation and cancer (for a review, see (46)). LDs are found in almost all human cells, but are especially abundant in hepatocytes, enterocytes, and adipocytes (47). Ultrastructural analysis demonstrated that LDs are cytoplasmic organelles formed from the budding of the ER outer lipid monolayer, and are often in contact with mitochondria and the ER. LDs have also been reported to be present in nuclei (48).

Due to FA lipotoxicity (excessive FAs within the cells triggers β-oxidation, which in turn is associated with increased ROS levels, ER stress, mitochondria failure and subsequent cell death), increased FAs levels within the cells are stored in the LDs as neutral lipids (TAG), where DGAT proteins catalyze the last step of the TAG synthesis (49). Several models for the formation of LDs have been reported. Wilfling et al, proved that two distinct populations of LDs coexist in the cell. Smaller LDs resulted from ER budding (named growing LDs), where DGAT1 is involved in TAG synthesis. Larger LDs (named expanding LDs) result from the *in situ* enzymatic activity of DGAT2, that translocate to the LDs from the ER (25,50). The Walther group also showed that downregulation of DGAT1 greatly affects the abundance of smaller LDs, whereas the lack of DGAT2 affects the larger population of LDs in oleate-induced lipid droplet formation in *Drosophila* cells. In agreement with these results, our group has observed a dramatic decrease in the formation of LDs when a specific DGAT1 (T863 or PF-04620110) inhibitor was used in MCF-7 breast cancer cells loaded with oleate, stained with BODIPY-493/503, and visualized using Leica confocal microscope. However, a DGAT2 (PF-06424439) inhibitor modestly affects the number of formed LDs, suggesting that DGAT2 function may not be at the level of LD synthesis but rather at the expansion level. We also observed that formation of LDs is completely blocked when DGAT1 and DGAT2 inhibitors are simultaneously added to the cells (unpublished data).

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Besides TAG, the LDs are enriched in a variety of lipids such as FAs, DAG, cholesterol, ceramides and O-acylceramides (49). Proteins derived from the cytosol of the ER are also present in the LDs, which play an important role in the formation, growth, trafficking and catabolism of the LDs (46). The protein coating of the LDs varies between different LDs in the same cell, in response to metabolic conditions or between different cell types (49). Proteomic analysis has concluded that the proteins present in the LDs can be classified into three groups: 1) proteins involved in the metabolism and catabolism of the LDs, such as DGAT2, adipose tissue triacylglycerol lipase (ATGL), monoacylglycerol lipase (MGL) and hormone-sensitive lipase (HSL) (51); 2) structural proteins, such as the PAT family formed by 5 different members (perilipin, adipose differentiation-related protein (ADRP), tail-interacting protein of 47 kiloDaltons (TIP47), S3-12, and OXPAT). Perilipins are the most abundant proteins in the LD surface and control the access of lipases to the lipid core (52); 3) proteins involved in membrane trafficking, such as Rab GTPases (for instance, Rab8a), caveolins, cavins or the CIDE family proteins (including Cidea, Cideb, and Fsp27). Fsp27 has been reported to be enriched at LD-LD contact site and be involved in LD fusion (53,54).

Lipid droplets and cancer cell proliferation and aggressiveness—Cancer cells are characterized by energy metabolism reprogramming that favors an uncontrolled growth, which represents the basis of neoplasia (55). Increased glucose uptake followed by glycolysis and lactate acid fermentation in aerobic conditions (Warburg effect) favor the synthesis of ATP and *de novo* generation of nucleotides, FAs, and proteins necessary as building blocks for cell proliferation (46,56,57). In 1963, Aboumrad et al reported a class of mammary carcinoma characterized by abundant intracytoplasmic neutral lipid storage stained with Sudan IV (58). A decade later, Ramos et al clinically and morphologically examined several lipid-rich carcinomas of the breast and concluded that those tumors had a more aggressive behavior (59). Since then, increased numbers of LDs were observed in various cancers such as breast (60-65), colon adenocarcinomas (66-69), pancreatic cancer (70-72), prostate cancer (73), glioblastoma (74,75), liver tumor (76,77), cervical carcinoma cells (78,79), lung carcinoma cells (80,81) and ovarian cancer (82,83).

Accioly et al, reported increased numbers of lipid bodies (LDs) in human colon adenocarcinoma cell lines (CACO-2, LOVO, HT-29, and HCT-116 cells) and in a H-rasV12-transformed intestinal epithelial cell line (IEC-6 H-rasV12) as compared with non-transformed IEC-6 cells. COX-2 and prostaglandin E2 (PGE2) synthase (which is believed to promote tumor growth) were increased in the CACO-2 cells as compared to the IEC-6 cells. Furthermore, they were found to localize to the LDs. Inhibition of LDs formation by either aspirin or FAS inhibitor, correlated to inhibition of PGE2 generation and cell proliferation (67).

Nieva et al. used Raman microspectroscopy (RS) to examine the lipid phenotype associated with cancer malignancy in several breast cancer cell lines: MDA-MB-435 (lung metastasis), MDA-MB-468 (non-metastatic breast cancer cell line), MDA-MB-231 (bone metastasis), SK-BR-3 (non-metastatic, epithelial morphology cell line), MCF-7 (breast ductal carcinoma) and MCF-10A (benign breast tumor) cells. The Raman spectra allowed the quantification of total fatty acid (TFA, 2845 cm^{-1} band) and total unsaturated fatty acid

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(TUFA, 3015 cm^{-1} band) levels in the different cell lines. The TFA band intensity was significantly higher in the MDA-MB-435 as compared to the MCF-10A cell line, in agreement with the Ramos' observation that lipid accumulation is a characteristic of aggressive cancer cells. No significant differences were observed in the TUFA bands. Interestingly, the authors developed an algorithm to predict the metastatic ability of breast cancer cells (84). Most recently, Abramczyk et al. utilized RS to analyze the composition of the LDs in non-malignant and malignant human breast epithelial cell lines. It was found that the number of LDs in MCF-10A cancer cells was 2-fold lower than in MCF-7 cells and 4-fold lower than in MDA-MB-231 cancer cells. Thus, increased levels of LDs correlated with increased aggressiveness (85).

Lipid droplets and chemotherapy resistance—Multidrug resistance is a major impairment for the treatment of cancer cells. Several lines of evidence have pointed at LDs as a factor involved in the survival of cancer cells upon a cell stressor such as chemotherapy.

When human colorectal cancer (CRC) cell lines were treated with 5-FU or oxaliplatin alone or in combination, increased number of LDs were present within the cells, as stained with Nile red. LD formation was observed to depend on acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT2) activity. When LPCAT2 was overexpressed, higher number of LDs was present within cells, which conferred chemotherapy resistance as compared to wild-type cells. Conversely, inhibition of LD biogenesis reversed the resistance phenotype (86). Moreover, progesterone receptor (PR)-positive cells T47D showed increased lipid accumulation and LD formation upon progestin treatment, whereas PR-negative MDA-MB-231 cells did not. Increased number of LDs correlate with docetaxel resistance (65). Differences in metabolism were studied in two ovarian cancer cell lines: cisplatin-sensitive (2008) and cisplatin-resistant (C13) cells. Higher lipid accumulation mainly in the LDs were found in the C13 cells (87).

Lipid droplets and cancer stem cells—Altered lipid metabolism has also been reported in cancer stem cells. For example, FA synthase was found to be upregulated in glioma stem cells (GSCs) as compare to non- GSCs. The expression of fatty acid synthase (FASN) was upregulated in human glioblastoma cells. The same study demonstrated that inhibition of FASN activity by cerulenin decreased cell survival of GSCs, as well as lipogenesis and invasiveness. Together, these data suggested that *de novo* FA synthesis is essential for the GSC stemness (88).

Kashuba et al, reported that overexpression of mitochondrial ribosomal protein MRPS18-2 (S18-2) resulted in immortalization of primary rat embryonic fibroblast (REFs) with stem cell phenotype (89). Years later, the same group observed that LD formation was increased in the tumorigenic S18-2 clone as compared to REFs. S18-2 clone but not REFs showed anchorage-independent growth in soft agar and formed tumors in SCID mice, suggesting that LDs may play a role in cancer tumorigenicity and cancer stem cell phenotype (90).

Other lines of evidence that outline the role of LDs and cell in stem-like cells are: 1) resveratrol-induced cell death in breast cancer stem-like cells by decreasing the levels of FASN upstream of apoptosis (91), 2) circulating tumor cells are an indicator of metastasis

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and are associated with a poor prognosis. However, their detection presents technical difficulties. By RS microscopy, lipid-rich prostate circulating tumor cells were detected in peripheral blood of patients with metastatic prostate cancers, which could serve as a more sensitive biomarker for prostate metastasis (92).

3.2 DGATs are overexpressed in cancer cells

Numerous genes involved in normal function of the cell, play a role in cancer. When dysregulated, due to up or down-regulation, these genes are involved in uncontrolled growth. When performing cross-cancer queries at the cBio Cancer Genomics Portal (http://cbioportal.org) for the DGAT gene expression (93,94), our group found that both DGAT1 and DGAT2 were overexpressed. As depicted in Figure 3A, DGAT1 is overexpressed in liver, colon, breast, bladder and ovarian cancers. DGAT2 (Figure 3B) is highly expressed in liver, breast, thyroid, prostate and pancreatic cancers.

As stated above, metabolic reprogramming and subsequent lipid accumulation favor cancer cell proliferation and play a role in chemotherapy resistance. Elucidating and identifying cellular modifications that favor lipid storage pathways, such as overexpression of DGAT proteins, can open new therapeutic targets. Although very few peer-review articles have been published on this matter, their results look promising. For instance, DGAT1, ABHD5, ACAT1 and ATGL genes were found to be overexpressed in prostate cancer cells. Knock-down of DGAT1 in prostate cancer LNCaP cells resulted in decreased cell growth and autophagy (73). Moreover, in breast cancer studies overexpression of the protooncogene HER2 in the normal breast cell line HB4a led to lipogenic reprogramming and overexpression of DGAT and other lipid related genes upon treatment by trustuzumab (Herceptin) (95). These studies are beginning to implicate DGAT enzymes and lipogenesis in oncogenic transformation in cancer.

Conclusions and future directions.

Our and others studies are giving us novel insights into the role of DGATs, LDs, and acylated ceramides in the regulation of cancer biology. Interestingly DGATs, their inhibitors and LDs albeit extensively studied in metabolic and cardiovascular related diseases, have not previously been significantly implicated in cancer biology. New insights into fat storage and relative resistance to cell death pathways have begun to advance our knowledge about altered lipid metabolic pathways as potential targets for cancer therapeutics. Our work on the role of DGATs in ceramide metabolism to acylceramide and its sequestration into LDs thus making it “inaccessible” for cell death opened up a novel avenue of investigation for us. We show for the first time that DGATs are overexpressed in many cancers and that inhibiting DGATs appears to synergize with chemotherapy to increase ceramide levels and enhance cancer cell death. These observations will hopefully lead to future preclinical and clinical studies on targeting DGATs as potential novel approaches to cancer chemotherapy.

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Abbreviations:

DGAT1 and DGAT2	Acyl-CoA:diacylglycerol acyltransferase
MGAT	Acyl-CoA:monoacylglycerol acyltransferase
ACAT	acyl-CoA:cholesterol acyltransferase
ARAT	Acyl-CoA:retinol acyltransferase
LPCAT2	Acyl-CoA:lysophosphatidylcholine acyltransferase
CerS	Ceramide synthase
DAG	Diacylglycerol
FASN	Fatty acid synthase
HSL	Hormone-sensitive lipase
LDs	Lipid droplets
MAG	Monoacylglycerol
MGL	Monoacylglycerol lipase
TAG	Triacylglycerol
ATGL	Triacylglycerol lipase

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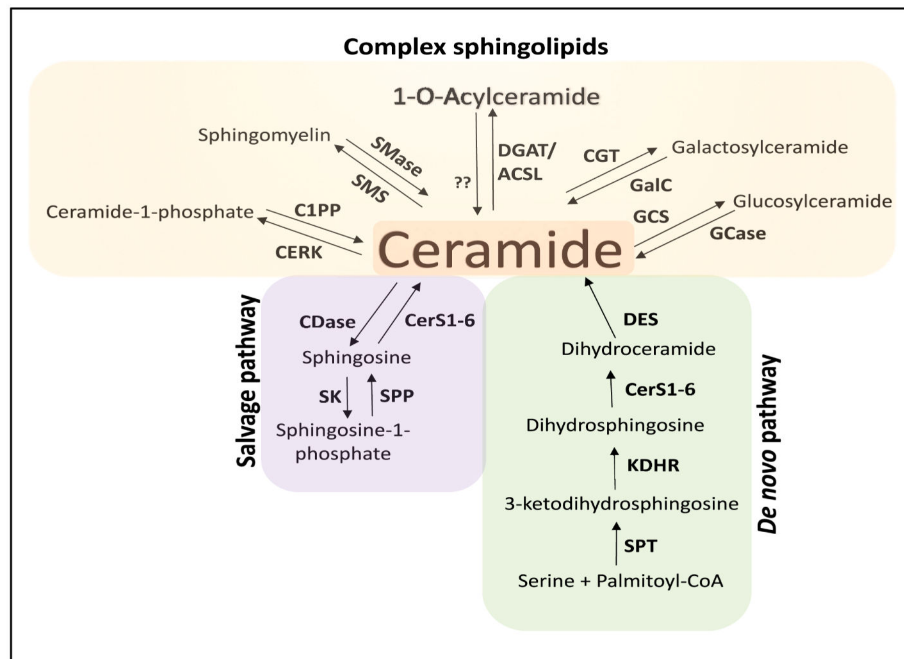

Figure 1. Sphingolipid metabolism

Ceramide is the hub of the sphingolipid metabolism and can be synthesized by several pathways. Ceramide can be generated by the *de novo* pathway (green box) from the condensation of serine and palmitoyl-CoA to generate 3-ketodihydrosphingosine, which is then reduced to dihydrosphingosine and further N-acylated to form dihydroceramide by (dihydro)ceramide synthases (CerS). Dihydroceramide desaturase (DES) then forms ceramide.

Ceramide can be hydrolyzed to sphingosine and then re-acylated back to ceramide in the salvage/recycling pathway (purple box) or phosphorylated to sphingosine-1-phosphate by sphingosine kinase (SK).

Ceramide can be also modified to form more complex sphingolipids (yellow box) such as ceramide-1-phosphate, sphingomyelin, hexosylceramdies (glucosylceramide and galactosylceramide) and O-acylceramides. Serine palmitoyltransferase (SPT); 3-ketodihydrosphingosine reductase (KDHR); ceramidase (CDase); sphingosine kinase (SK); sphingosine-1-phosphate phosphatases (SPP); ceramide kinase (CERK); ceramide-1-phosphate phosphatase (C1PP) sphingomyelinases (SMase); sphingomyelin synthase (SMS); glucosylceramide synthase (GCS); glucosylceramidase (GCase); ceramide galactosyltransferase (CGT); galactosylceramidase; fatty acyl-CoA synthase (ACSL); acyl-CoA:diacylglycerol acyltransferase (DGAT2)

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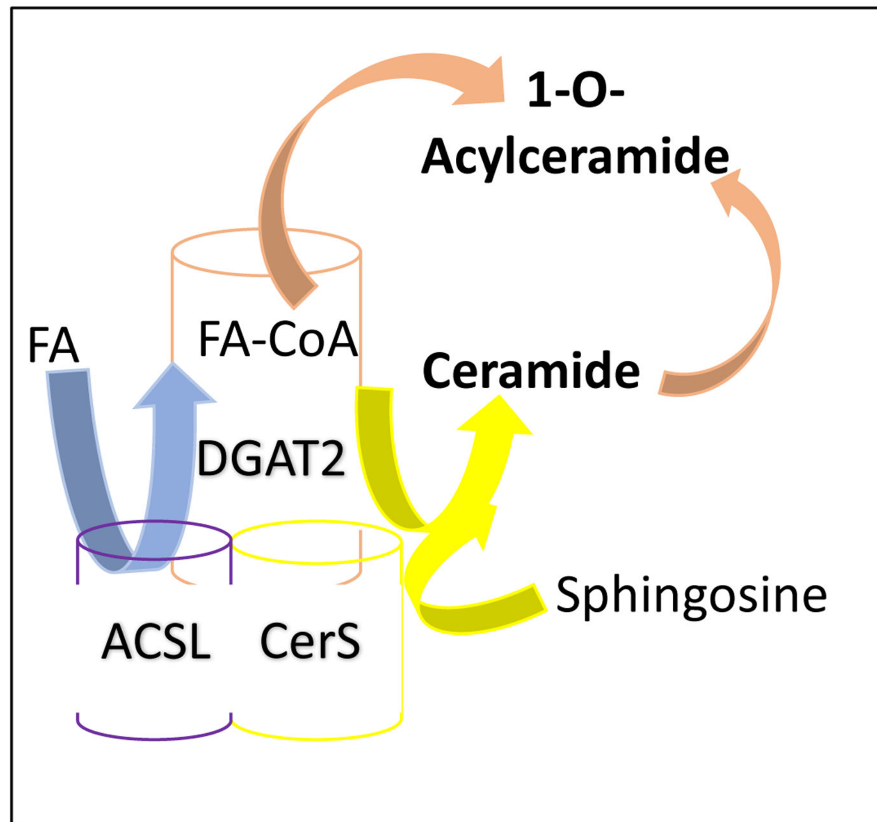

Figure 2. Synthesis of O-acylceramide by an ACSL-CerS-DGAT2 enzymatic complex

Synthesis of O-acylceramide from ceramide is carried out by the enzymatic complex ACSL-CerS-DGAT2 localized to the ER/lipid droplet contact sites. Fatty acyl-CoA synthase (ACSL) catalyzes the synthesis of the activated fatty acid (FA) as fatty acyl-CoA (FA-CoA). Ceramide synthase (CerS) synthesize ceramide by N-acylating the sphingosine backbone. Acyl-CoA:diacylglycerol acyltransferase (DGAT2) transforms ceramide to O-acylceramide by transferring a FA group from FA-CoA to the hydroxyl group likely in the carbon-1 of ceramide.

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Figure 3. DGAT proteins are overexpressed in multiple cancers

Analysis of the genetic alterations of DGAT1 (A) and DGAT2 (B) by cBioPortal (http://cbioportal.org). Every spot represents a single study. Purple spots represent fusion genes, grey sports represent truncating genes, green dot represent missense mutations, blue spots represent normal results of gene sequencing of DGAT1 or DGAT2 and white spots represent those analyzed without gene sequencing. Gene expression is represented as a logarithm in base 2.

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Table 1.

Various breast cancer cell lines were either left untreated or treated with PF-06424439 (100μM) alone or with the indicated chemotherapeutic drug after 1 hour of PF-06424439. Treatments were performed as follows: MCF-7 cells were treated with either 20μM of cisplatin (24h) or 1μM of doxorubicin (48h). MDA-MB-231 cells were treated with 1μM of doxorubicin or 25μM of cisplatin (48h). SK-BR-3 cells were treated with 25μM of cisplatin (24h). At the end of the treatment period, plasma membrane permeabilization was measured by LDH release into the medium, using a colorimetric assay kit commercially available (Biovision, Milpitas, CA) following the manufacturer's instructions. Briefly, 50μL of reaction mixture was transferred to a 96-well plate. Same volume of sample medium was added to the wells. The plate was incubated for 30min at 37°C. Absorvance was measured at 490nm and 680nm. Cell death was quantified by subtracting the absorbance at 680nm from the absorbance at 490nm. Percentage of cell death was calculated as compared to total cell lysis. Data are the average of three independent experiments.

Cancer Cell Line	Percentage of cell death (as compared to total lysis)			
	Untreated	Cisplatin	PF-06424439	PF-06424439+ Cisplatin
MCF-7	**6**	**46**	**5**	**82**
MDA-MB-231	**10**	**40**	**11**	**55**
SK-BR-3	**21**	**40**	**29**	**63**
Cancer Cell Line	**Percentage of cell death (as compared to total lysis)**			
	DMSO	Doxorubicin	PF-06424439	PF-06424439+ Doxorubicin
MCF-7	**15**	**39**	**25**	**63**
MDA-MB-231	**13**	**44**	**18**	**55**