Identification and Validation of a Selective Small Molecule Inhibitor Targeting the Diacylglycerol Acyltransferase 2 Activity

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Diacylglycerol acyltransferase 2 (DGAT2) is one of two distinct DGAT enzymes that catalyze the last step in triacylglycerol (TG) synthesis. Findings from previous studies suggest that inhibition of DGAT2 is a promising strategy for the treatment of hepatic steatosis and insulin resistance. Here, we identified compound 122 as a potent and selective inhibitor of human DGAT2, which appeared to act competitively against oleoyl-CoA in vitro. The selective inhibition of DGAT2 was also confirmed by the reductions in enzymatic activity and de novo TG synthesis in DGAT2-overexpressing HEK293 cells and hepatic cells HepG2. Compound 122, as a newly identified inhibitor of DGAT2, will be useful for the research on DGAT2-related lipid metabolism as well as the development of therapeutic drug for several metabolic diseases.

Key words metabolic disease; triacylglycerol; diacylglycerol acyltransferase 2; small molecule inhibitor; isatin

Triacylglycerol (TG), a class of neutral lipids, is the most representative storage form of energy in eukaryotic cells. However, an imbalance between energy intake and expenditure can lead to the excessive accumulation of TG in tissues, which is pathologically associated with metabolic diseases such as obesity, hyperlipidemia, hypertension, hepatic steatosis, and insulin resistance. Therefore, inhibition of TG biosynthesis has been suggested to be one of therapeutic strategies to treat these diseases.

The final and only committed step in the synthesis of TG is catalyzed by diacylglycerol acyltransferase (DGAT) enzymes, DGAT1 and DGAT2, which are encoded by two distinct genes and share limited sequence homology. Both genes are ubiquitously expressed, but with the highest levels of expression found in tissues that are active in TG synthesis such as adipose tissue, liver, small intestine, and mammary gland.

Studies in mice have shown that DGAT2 is responsible for the majority of TG synthesis. DGAT2 knockout mice are severely deficient in TG (ca. 90% TG reduction) and have impaired skin barrier function, leading to early death, while DGAT1 knockout mice are still viable with a moderate reduction in TG (ca. 50% TG reduction). In addition, hepatic suppression of DGAT2 with antisense oligonucleotides reduced hepatic TG content, increased fatty acid oxidation, and thereby reversed diet-induced hepatic steatosis and insulin resistance in rodents. Conversely, upregulation of DGAT2 expression induced increase of cytoplasmic TG content and lipid droplets (LDs) in rat hepatocytes and adipocytes, and similarly liver-directed DGAT2 overexpression caused hepatic TG accumulation and promoted hepatic insulin resistance in mice. Therefore, inhibition of DGAT2 enzyme, particularly by small molecule, is expected to be a feasible therapeutic strategy for hepatic steatosis and its complications, such as insulin resistance.

Even though DGAT1 and DGAT2 take part in the same reaction in TG biosynthetic pathway, their biochemical properties seem to be somewhat different. In order to define the distinct role of each protein, it is crucial that DGAT2 is pharmacologically differentiated from DGAT1 by using a selective inhibitor. Therefore, a selective inhibitor of DGAT2 is needed not only to evaluate its therapeutic potential but also to provide a tool for pharmacological study on the role of DGAT2 in TG synthesis.

Recently, a couple of DGAT2 inhibitors have been reported. However, the pharmacological validation of its use in cells and animal models remains to be examined. Still, DGAT2 inhibitors with different modes of action or pharmacophores will be useful to advance the understanding of DGAT2 biology and the development of therapeutic drug for several metabolic diseases.

The aim of this study is to discover a potent DGAT2 inhibitor and to validate its use in cells. In this study, using a high-throughput assay for human DGAT2 activity, we identified compound 122 as a potent and selective DGAT2 inhibitor with a competitive mode of inhibition with regard to the oleoyl-CoA. Further cell-based assays firmly confirmed the inhibitory effect of compound 122 on TG biosynthesis in DGAT2-overexpressing cells and hepatic cells.

MATERIALS AND METHODS

Materials Fatty acid-free bovine serum albumin (BSA) and sn-1,2-dioleoylglycerol were obtained from Sigma. [3H]Oleoyl-CoA, [14C]oleoyl-CoA, and [14C]glycerol were purchased from PerkinElmer. The Bac-to-Bac Baculovirus Expression System was purchased from GIBCO (U.S.A.). An antibody against DGAT2 was purchased from Abcam (U.K.). The DGAT1 inhibitor, a class of oxazoles, was domestically synthesized (Supplementary Figure 1).

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Expression and Preparation of Enzyme Sources

Recombinant human DGAT1, DGAT2, and glycerol-3-phosphate acyltransferase 1 (GPAT1) were overexpressed in SF-9 insect cells using the Bac-to-Bac Baculovirus Expression System, according to the manufacturer’s instructions. The DGAT1, DGAT2, and GPAT1 cDNA clones were amplified by polymerase chain reaction (PCR) and inserted into pFastBac1 donor vector to obtain the recombinant baculoviruses. SF-9 cells infected with the baculovirus at 10 multiplicity of infection (MOI) for 72 h were harvested and homogenized in sucrose solution (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM ethylenediamine-tetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT)). For DGAT assay, the homogenates were centrifuged at 600 × g for 15 min, and the resulting supernatants were further centrifuged at 100000 × g for 1 h to collect the total membrane fraction. The same procedure was applied for preparation of total membrane fractions from HEK293 Tet-on cells inducibly overexpressing DGAT1 or DGAT2. In case of GPAT1 assay, cells were homogenized and nuclei were removed by centrifuging at 600 × g for 15 min. The resulting supernatants were further centrifuged at 8000 × g for 15 min to collect crude mitochondrial fraction. The collected proteins were resuspended and the protein concentration was measured using the Bradford protein assay.

Screening of Human DGAT2 Inhibitors

The high-throughput screening to discover small-molecule inhibitors of human DGAT2 was achieved using basic FlashPlate (PerkinElmer), which is based on scintillation proximity assay. DGAT2 activity was assayed in a solution containing 20 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.4), 5 mM MgCl2, 1 mg/ml BSA, 100 µM diacylglycerol, 10 µM [3H]oleoyl-CoA, 8 µM DGAT2-containing membrane, and 10 µM compound in a final reaction volume of 100 µL in 96-well format Basic FlashPlate. The reaction mixture was incubated for 2 h at 37°C, and the enzymatic reaction was stopped by adding 100 µL of isopropanol. The plates were sealed and incubated for overnight at room temperature. The newly synthesized [3H]-labeled TG is in close proximity to the coated scintillant in the FlashPlate during incubation, and then we quantified the plates by scintillation counting with a Micro-beta (PerkinElmer) plate reader.

Conventional Extraction-Based in Vitro DGAT Assay

DGAT activities in total membranes prepared from DGAT2- or DGAT1-overexpressing SF-9 cells and HEK293 Tet-on cells were determined by measuring the formation of [14C]-triacylglycerol from [14C]oleoyl-CoA. The reaction mixture for DGAT2 assay contains 175 mM Tris (pH 7.5), 5 mM MgCl2 (100 mM MgCl2 for DGAT1 assay), 200 µM sn-1,2-diacylglycerol, 20 µM [1-14C]oleoyl-CoA (5.5 µCi), 2 mg/ml BSA, and 32 µg of the membrane protein. The mixture was incubated for 20 min at 37°C, and then the reaction was stopped by the addition of 1.5 mL of stop solution [2-propanol–heptane–water (80:20:2, v/v/v)] and vortexed with 1 mL of heptane and 0.5 mL of water. The top heptane phase was collected and washed with 2.0 mL alkaline ethanol solution [ethanol–0.5 N NaOH–water (50:10:40, v/v/v)]. The radioactivity of the top phase was determined by liquid scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer, PerkinElmer).

In Vitro GPAT Assay

The assay buffer contained 75 mM Tris, pH 7.5, 4 mM MgCl2, 8 mM NaF, 100 µM palmitoyl-CoA, 1.8 µM [1-14C]glycerol-3-phosphate, and 2 mg/mL BSA. The reaction was started by mixing 2 µL of mitochondrial membrane fraction with the assay buffer in a final volume of 200 µL and shaking the samples for 20 min at 26°C. The reaction was stopped by the addition of 1 mL of water-saturated butanol and then adds 0.5 mL of butanol-saturated water to extract lysophosphatic acid (LPA). After vortexing, 0.8 mL of the organic phase was transferred to a tube and washed once with 0.8 mL of butanol-saturated water. The radioactivity of the top phase was determined by liquid scintillation counting (Tri-Carb 2900TR Liquid Scintillation Analyzer, PerkinElmer).

Establishment of Stable Cell-Lines in HEK293 Tet-On Cells

The pTRE2-hygro (Clontech) was used to tightly regulate the expression of DGAT1 and DGAT2. Each cDNAs was amplified by PCR and inserted into the pTRE2-hygro vector. Stable HEK293 Tet-on cells expressing DGAT1 or DGAT2 were established by transfection of pTRE2-hygro-DGAT1 or pTRE-hygro-DGAT2, followed by selection with hygromycin for 2 weeks. Clonal cell lines were tested for the expression of DGAT1 or DGAT2 in the presence and absence of doxycycline by using Western blot analysis. Cells were fixed with 4% formaldehyde for 20 min, and lipid droplets (LDs) were stained with a Bodipy 493/503 dye (Invitrogen). Images were analyzed using the Cellomics ArrayScan instrument (Cellomics Inc.) and software module, known as the Spot Detector BioApplication.

Determination of Isotope-Labeled TGs in Mammalian Cells

To examine intracellular de novo TG synthesis, HEK293 Tet-on cells inducibly overexpressing DGAT1 or DGAT2 were incubated with dimethyl sulfoxide (DMSO), compound 122, or DGAT1 inhibitor at the indicated doses (2.5, 5, 10 µM) in the presence of [14C]glycerol (0.6 µCi) for 6 h. At the end of the incubation, intracellular lipids were extracted with mixture of hexane–isopropanol (3:2, v/v) and separated on TLC plate using the hexane–diethyl ether–acetic acid (80:20:1, v/v/v) as a developing solvent. The isotope-labeled TG was detected and quantified with a bio-imaging analyzer (FLA-7000, Fuji). To differentiate DGAT2-mediated TG synthesis from DGAT1-mediated one in intact hepatic cells, HepG2 cells were treated with DMSO, compound 122, or DGAT1 inhibitor at the indicated doses (2.5, 5, 10 µM) in the presence of [14C]glycerol (0.6 µCi) or [14C]oleate (1.25 µCi) for 6 h. The following procedure is the same as described above.

RESULTS

Identification of a DGAT2 Inhibitor by High-Throughput Screening

In order to identify human DGAT2 inhibitors, we first established an in vitro assay for DGAT2 activity which is compatible to high-throughput screening. Human DGAT2 was overexpressed in insect cells SF-9 using baculovirus-mediated expression system, and total membranes containing human DGAT2 were prepared and used for screening. The high-throughput screening to discover small-molecule inhibitors of human DGAT2 was achieved using a scintillation proximity assay on FlashPlate, as described in Materials and Methods. In this assay, the newly synthesized [3H]-labeled TG is in close proximity to the coated scintillant in the FlashPlate, resulting in the increase of scintillation, which reflects the activity of human DGAT2 enzyme in total membranes. DGAT2 overexpression resulted in a marked increase of DGAT2 activity in a dose-dependent manner, indicating a sufficient assay
window to differentiate the activity in DGAT2-overexpressing cells from basal activity in intact cells (data not shown). With this robust assay, we had performed a primary screen of 20000 compounds derived from ChemBridge chemical library at a final concentration of 10 µM and identified 15 compounds that significantly inhibited DGAT2 activity in vitro (>3SD, three times higher than standard deviation, data not shown). In the following assays, compound 122 consistently exhibited the strongest inhibitory activity, and its inhibitory activity was further confirmed by using a conventional extraction-based in vitro DGAT assay, in which the newly synthesized isotope-labeled TG was extracted by a series of combination of organic solvents and the radioactivity was quantified (Figs. 1B, 2C). The inhibition of DGAT2 was dependent on the concentration of compound 122, with an estimated IC₅₀ of 5 µM (Supplementary Figure 2).

Structurally, compound 122 is composed of isatin core with a side chain of two consecutive benzyl groups (Fig. 1A). Interestingly, other compounds with a similar structure to compound 122, existing in the chemical library and used for primary screening, did not have any inhibitory efficacy against DGAT2 activity at 10 µM (Figs. 1A, B). Consequently, this preliminary structure–activity relationship (SAR) study showed us that the distal benzyl in the side chain and carbonyl group(s) in isatin core, both of which are present only in compound 122, are required for full inhibitory activity against DGAT2.

**Compound 122 Is a Selective and Acyl-CoA-Competitive Inhibitor**

To gain a better understanding of the inhibitory mechanism of compound 122, we measured the DGAT2 activity (hereinafter referred to as activity measured by using a conventional extraction-based in vitro DGAT assay) with varying concentrations of each of two substrates (oleoyl-CoA and diacylglycerol) in the presence of different doses of the compound in DGAT2-containing total membranes. As shown in Figs. 2A and B, compound 122 appeared to behave as a competitive inhibitor with regard to oleoyl-CoA but not to diacylglycerol.

Many enzymes in TG biosynthetic pathway including DGAT2 commonly involve acyl-CoA as a substrate, which raised the possibility that compound 122 may not be selective to DGAT2. To determine the selectivity of this compound, we examined its effect on the activity of DGAT1 using total membrane fraction of human DGAT1-overexpressing SF-9 cells. DGAT1 is the other isozyme of DGAT2, and both enzymes particularly share similar specific activity with respect to substrates for TG synthesis. As shown in Fig. 2C, compound 122 had little inhibitory activity against DGAT1.
at the concentration of 10 µm, while showing a marked effect on DGAT2. Moreover, compound 122 had a marginal effect (less than 10%) on GPAT1, which is one of major player in TG biosynthetic pathway (Fig. 2C). These data suggest that compound 122 is a selective inhibitor of DGAT2 activity in vitro.

**Compound 122 Selectively Inhibits DGAT2 Enzyme in Mammalian Cells**

To examine whether compound 122 selectively inhibits DGAT2 in mammalian cells, we first established HEK293 cell systems inducibly overexpressing DGAT2 or DGAT1 by addition of doxycycline, and then lipid droplets (LDs) were visualized by staining with a fluorescent Bodipy dye and quantified by using Cellomics BioApplication analysis software. As expected, DGAT2 or DGAT1 overexpression alone drastically increased the fluorescent signal of LDs by more than 50-fold, as compared with control cells that showed a faint basal signal (Figs. 3A, B). Increased fluorescence of each cell system mostly represents induction of intracellular TG occurred by additionally expressed DGAT2 or DGAT1, indicating that both DGAT1 and DGAT2 play an important role in intracellular TG biosynthesis.

Next, we examined the selective effect of compound 122 on DGAT2 by measuring the enzymatic activities (using conventional extraction-based in vitro DGAT assay) in total membranes isolated from both DGAT2- and DGAT1-overexpressing cells. Compound 122 showed a strong inhibitory activity, with IC₅₀ value of 9.7 µm, in DGAT2-containing total membranes, but a weak inhibitory activity in DGAT1-containing total membranes with much higher IC₅₀ of 91.8 µm (Fig. 3C). Conversely, DGAT1 inhibitor strongly affected the activity of DGAT1-containing total membranes but not that of DGAT2-containing total membranes (Fig. 3C). In addition, the selective inhibition of DGAT2 by compound 122 was further confirmed by examining intracellular de novo TG synthesis, which is measured by incorporation of isotope-labeled substrate into newly synthesized TG in DGAT2- and DGAT1-overexpressing cells, as described in Materials and Methods (see the section ‘determination of isotope-labeled TG in mammalian cells’). As expected, overexpression of DGAT2 or DGAT1 alone increased de novo TG synthesis (Figs. 3D, E). Compound 122 significantly suppressed de novo TG synthesis induced by overexpression of DGAT2 but not that by DGAT1 (Fig. 3D). Conversely, DGAT1 inhibitor only affected de novo TG synthesis induced by DGAT1 (Fig. 3E).

It has been proposed that DGAT1 and DGAT2 mediate distinct hepatic functions in spite of catalyzing the same biochemical reaction.6 According to recent reports,13,14 DGAT2 preferentially esterifies exogenously added glycerol to endogenously synthesized fatty acids, while DGAT1 preferentially esterifies exogenously added oleic acid to already existing diacylglycerol. Therefore, in order to test if compound 122, as an inhibitor of DGAT2, also support this observation, we measured the newly synthesized isotope-labeled TG in hepatic cells HepG2, using two types of isotope-labeled substrates [14C]glycerol and [14C]oleate that would be subject to preferentially DGAT2- and DGAT1-mediated TG synthesis, respectively. As a result, compound 122 decreased incorporation of [14C]glycerol into TG by 40%, while it had little effect on incorporation of [14C]oleate into TG (Fig. 4A). These data agree well with the previous reports, abovementioned, and also support that compound 122 selectively inhibits DGAT2 in cells. It is noteworthy that treatment of 10 µm compound 122 scarcely shows any toxic effect on HepG2 cells (Fig. 4B). Taken together, these data obviously demonstrate that compound 122 is an inhibitor selectively working to DGAT2 in...
In this study, we identified a DGAT2 inhibitor by extensive HTS and provided a comprehensive assessment of this compound by examining the effect on DGAT2 in a variety of assays involving mammalian cells and cell-free systems.

One of the most important requirements for DGAT2 inhibitor is how selectively it works on DGAT2 over other enzymes in lipid metabolism, particularly isozyme DGAT1. High selectivity of compound 122 to DGAT2 was demonstrated by many experimental settings. First, compound 122 strongly inhibits DGAT2 but marginally DGAT1 and GPAT1 in cell-free enzymatic assays (Fig. 2C). Second, compound 122 inhibits DGAT2 enzymatic activity (Fig. 3C) and incorporation of [14C]glycerol into TG (Fig. 3D) in DGAT2-overexpressing cells, but to a much lesser extent those in DGAT1-overexpressing cells (Figs. 3C, E). Third, compound 122 affected the incorporation of [14C]glycerol into TG in hepatic cells HepG2, while it did not show any effect on the incorporation of exogenous fatty acid [14C]oleate (Fig. 4A), as recently suggested that DGAT2 is mainly responsible for TG synthesis involving endogenously originated fatty acids. Still, we could not completely exclude the possibility that compound 122 may work on other untested enzymes in lipid metabolism that have acyl-CoA as their substrate.

Compound 122 appears a little hydrophobic, and this property raises a concern about a possible promiscuous binding to many proteins including DGAT2. However, several lines of evidence are not in accord with this possibility. First, accord-
ing to a competition assay (Fig. 2A), compound 122 seems to work by binding directly to acyl-CoA binding site on DGAT2. Second, compound 122 selectively inhibits DGAT2 but not DGAT1 and GPAT1 (Fig. 2C), even though they all require acyl-CoA as a substrate, indicating differential effects on the similar kind of acyltransferases. Third, preliminary SAR identified at least two moieties (distal benzyl and carbonyl groups) that are required for full inhibitory activity of compound 122. Collectively, these data suggest that compound 122 is not working promiscuously like simply forming lipid-like substance. Rather, compound 122 seems to act in a specific way. Given that distal benzyl and carbonyl group(s) are required for full inhibitory activity, these two moieties presumably enable compound 122 to fit well to a binding pocket on DGAT2, by forming hydrophobic interaction with aromatic residue or hydrogen bond with amide N–H, respectively. Biochemical studies in a deeper way will improve our understanding on the mode of action of compound 122. Further derivatization of compound 122 will determine more precisely which part of compound 122 is critical for activity and modifiable. Currently, we are exploring compounds that would be more potent and have improved properties in terms of solubility and stability, which will be required for further validation in animal model.

Compound 122 is a member of isatin family, which has been reported to exhibit a wide range of effects, including antiviral, antibacterial, antifungal, antipototic, antimtumor, and antiangiogenic activity. Unfortunately, there is little publication with regard to compound 122. The sole report suggests that compound 122 has an antiviral activity against human immunodeficiency virus (HIV). It is not simple to correlate the effect of compound 122 on DGAT2, one of lipid metabolism enzyme, with that on HIV. One possible scenario is that there might be a certain involvement of lipid metabolism in the life cycle of HIV in host cells. In that sense, recent reports on the role of DGAT1 in the maturation and budding-out of hepatitis C virus (HCV) particle is definitely worthy of further consideration.

In the viewpoint of therapeutics, DGAT2 inhibitor would be considered for treatment of hepatic steatosis and hyperlipidemia, since considerable reports support the critical contribution of DGAT2 in the progression of those diseases. In addition, DGAT2 inhibitor may be worth being tested for Type II diabetes. To date, most of therapeutic approach to deal with Type II diabetes has been targeting glycemic control, exemplified by DPP4 inhibitors. In contrast, relatively little has been tried with the concept controlling lipid metabolism, particularly inhibiting TG biosynthesis. Even though DGAT1 inhibitor has been tried intensively in vivo, the efficacy in controlling glucose level was not proved sufficiently. In that sense, it is noteworthy that an improved glycemic control was shown only in DGAT2-knockdowned rodents but not in DGAT1-knockdowned rodents. Consequently, compound 122, an inhibitor of DGAT2, is anticipated to be improved to provide a new therapeutic option for treating type II diabetes as well as hepatic steatosis and hyperlipidemia.

In conclusion, we newly identified compound 122 as a potent and selective inhibitor of DGAT2. This compound would be a useful tool for pharmacological study of DGAT2-related biology. Further validation in disease models remains to suggest another therapeutic option for the treatment of several metabolic diseases.

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