**SUPPORTING INFORMATION**

# Identification and Preliminary Characterization of a Potent, Safe and Orally Efficacious Inhibitor of Acyl-CoA:Diacylglycerol Acyltransferase 1

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Contents:

## 1. General

Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further purification. All reactions were performed under nitrogen atmosphere unless specifically noted. Normal-phase flash chromatography was performed using Merck silica gel 60 (230-400 mesh) from E.M. Science, or on a hybrid system employing Gilson components and Biotage prepacked columns. Following work-up, reaction mixtures were dried over $MgSO_4$ or $Na_2SO_4$, filtered through a fritted glass funnel or a plug of cotton, and concentrated with a rotary evaporator at *ca*. 15 mm Hg, warming when necessary. Thin layer chromatography systems were the same as those used for column chromatography, with $R_f$ approximately = 0.3. Analytical reversed-phase chromatography was performed using a Zorbax SP-C18 5μM 4.6x250 mm column with UV detection analyzed at 254 nM. Analytical method: (water with 0.1% trifluoracetic acid and $CH_3CN$ gradient) 0-100% $CH_3CN$ over 18 minutes at 1.5 mL/min. Analytical LC-MS was performed on a Shimadzu HPLC system with a Zorbax SB-C8, 5 μm 2.1 × 50 mm (Agilent technologies) column equipped with a PE Sciex, API 150EX single quadropole mass spectrometer, at a flow rate of 1 mL/min (0.05% $NH_4OAc$-buffer: $CH_3CN$ and 0.05% HCOOH in $H_2O$:$CH_3CN$). UV analysis was performed at 220 and 254 nM. $^1$H-NMR spectra were recorded at 300 MHz; all values are referenced to tetramethylsilane as internal standard and are reported as shift (multiplicity, proton count, coupling constants). Mass spectral analysis is accomplished using fast atom bombardment (FAB-MS), electrospray (ESI-MS) or direct chemical ionization (DCI-MS) techniques.

**Synthetic procedures and characterization**

**General Procedure for the preparation of compounds 3-11.** *t*-BuOCH($NMe_2$)$_2$ (2 equiv.) was added dropwise to a stirred and heated (120$^o$C) solution of the corresponding benzylnitrile (1 equiv.) in dry toluene (0.2 M). The resulting solution was heated for another 3 hours before it was concentrated to dryness. The resultant material was then taken up in a 2:1 mixture of toluene and acetic acid (0.33 M) and transferred to a microwave reaction tube. 1H-pyrazol-5-amine (1 equiv) was then added and the mixture heated to 150 $^o$C for 20 minutes (Personal Chemistry Microwave 150 $^o$C). After this time, the solvents were evaporated and the mixture separated via reverse phase HPLC to afford the final compounds.

**6-(4-tert-butylphenyl)pyrazolo[1,5-a]pyrimidin-7-amine (3).** $^1$H NMR (300 MHz, DMSO) δ 6.45 (d, *J* = 2.3 Hz, 1H), 7.40 (s, 2H), 7.49 (td, *J* = 8.6, 6.4 Hz, 4H), 8.11 (s, 1H), 1.34 (s, 9H), 8.12 (d, *J* = 2.3 Hz, 1H). MS (ESI) m/z 267.0 $[M+H]^+$.

**6-phenylpyrazolo[1,5-a]pyrimidin-7-amine (4).** $^1$H NMR (500 MHz, DMSO-$D_6$) δ ppm 6.46 (d, *J*=2.44 Hz, 1 H), 7.38 - 7.42 (m, 1 H), 7.46 - 7.54 (m, 6 H), 8.10 - 8.15 (m, 2 H). MS (ESI) m/z 211.0 $[M+H]^+$.

**6-(4-bromophenyl)pyrazolo[1,5-a]pyrimidin-7-amine (5).** $^1$H NMR (500 MHz, DMSO- $D_6$) δ ppm 6.55 (d, *J*=2.14 Hz, 1 H), 7.41 - 7.51 (m, 2 H), 7.64 - 7.75 (m, 2 H), 8.20 - 8.30 (m, 2 H), 8.61 (s, 2 H). MS (ESI) m/z 289.2 $(M+H)^+$

**6-(4-bromophenyl)pyrazolo[1,5-a]pyrimidin-7-amine (6).** $^1$H NMR (300 MHz, DMSO- $D_6$) δ ppm 6.56 (d, *J*=2.37 Hz, 1 H), 7.42 - 7.55 (m, 2 H), 7.61 - 7.68 (m, 1 H), 7.71 (m, *J*=1.70 Hz, 1 H), 8.27 - 8.33 (m, 2 H), 8.77 (s, 2 H). MS (ESI) m/z 290.0 $[M+H]^+$.

**6-(2-bromophenyl)pyrazolo[1,5-a]pyrimidin-7-amine (7).** $^1$H NMR (500 MHz, DMSO- $D_6$) δ ppm 6.58 (d, *J*=2.14 Hz, 1 H), 7.40 - 7.47 (m, 1 H), 7.49 - 7.55 (m, 2 H), 7.81 (d, *J*=7.63 Hz, 1 H), 8.23 (s, 1 H), 8.31 (d, *J*=2.14 Hz, 1 H), 8.73 (s, 2 H). MS (ESI) m/z 288.9 $[M+H]^+$.

**6-(4-isopropylphenyl)pyrazolo[1,5-a]pyrimidin-7-amine (8).** $^1$H NMR (500 MHz, DMSO- $D_6$) δ ppm 1.26 (d, *J*=7.02 Hz, 6 H), 2.89 - 3.03 (m, 1 H), 6.55 (d, *J*=2.44 Hz, 1 H), 7.34 - 7.49 (m, 4 H), 8.23 - 8.33 (m, 2 H), 8.68 (s, 2 H). MS (ESI) m/z 253.0 $[M+H]^+$.

**6-(4-cyclohexylphenyl)pyrazolo[1,5-a]pyrimidin-7-amine (9).** $^1$H NMR (300 MHz, DMSO- $D_6$) δ ppm 1.26 - 1.54 (m, 4 H), 1.67 - 1.78 (m, 1 H), 1.77 - 1.90 (m, *J*=7.12 Hz, 6 H), 6.54 (d, *J*=2.03 Hz, 1 H), 7.32 - 7.49 (m, 4 H), 8.24 - 8.33 (m, 2 H), 8.70 (s, 2 H). MS (ESI) m/z 293.0 $[M+H]^+$.

**4-(7-aminopyrazolo[1,5-a]pyrimidin-6-yl)benzoic acid (10).** $^1$H NMR (400 MHz, DMSO- $D_6$) δ ppm 6.55 (d, *J*=2.15 Hz, 1 H), 7.65 (d, *J*=8.29 Hz, 2 H), 8.06 (d, *J*=8.29 Hz, 2 H), 8.23 - 8.32 (m, 2 H), 8.50 (s, 2 H). MS (ESI) m/z 252.9 $[M+H]^+$.

**6-(4-(methylsulfonyl)phenyl)pyrazolo[1,5-a]pyrimidin-7-amine (11).** $^1$H NMR (300 MHz, DMSO- $D_6$) δ ppm 3.25 - 3.27 (m, 3 H), 6.49 (d, *J*=2.37 Hz, 1 H), 7.71 - 7.83 (m, 4 H), 7.96 - 8.05 (m, *J*=8.14 Hz, 2 H), 8.14 – 8.19 (m, 2 H). MS (ESI) m/z 288.9 $[M+H]^+$.

**2. In Vitro Compound Evaluation**

**Cellular triglyceride synthesis assay.** To evaluate *in cellulo* compound efficacy, an assay was developed with HeLa cells, which have been shown to express mRNA for DGAT-1. These cells accumulate lipid when cultured with exogenous fatty acid. Overnight serum starvation of the cells followed by addition of exogenous [1-$^{14}$C] oleic

acid results in the time-dependent formation of $^{14}$C-labeled triolein, which can be visualized and quantitated by TLC. Briefly, HeLa cells were plated at a density of 50,000 cells/well (100 µl in TC Isoplates, Perkin Elmer) in MEM media (Invitrogen) + 10% FBS + 50 µg/mL gentamicin. The next day, the media was replaced with 100 µl serum-free MEM (no FBS, no antibiotics) and starved overnight. The next morning, the media was removed and replaced with 75 µl fresh serum-free MEM, followed by 1 µl of a dilution of drug stock in DMSO to each well, and the plates incubated at 37 ºC for 30 min. After this time, 14 µl of oleic acid-albumin (Sigma, O3008) containing 28 µl of H-3 oleic acid [9,10-3H(N)] (ARC, ART198) per mL of oleic acid-albumin was added, and the plates incubated for 60 min, 37 ºC. After the 60 min incubation, the reactions were quenched with icewater followed by heptane:isopropanol (3:2, v:v), and separated into aqueous and organic phases. The reaction mixtures were applied to Merck Silica-60 TLC plates which were developed in an 80:20:1 (v:v:v) heptane, ethyl ether, acetic acid solvent system to separate the final triglyceride product from starting reactants. Following development, TLC plates were dried and exposed to phosphor screens overnight, followed by quantitation in a Molecular Dynamics Storm PhosphorImager. Metabolite quantitation was performed using ImageQuant software. For a relevant reference describing a similar assay, see Qian, Y.; Wertheimer, S. J.; Ahmad, M.; Cheung, A. W.; Firooznia, F.; Hamilton, M. M.; Hayden, S.; Li, S.; Marcopulos, N.; McDermott, L.; Tan, J.; Yun, W.; Guo, L.; Pamidimukkala, A.; Chen, Y.; Huan, K.; Ramsey, G. B.; Whittard, T.; Conde-Knape, K.; Taub, R.; Rondinone, C. M.; Tilley, J.; Bolin, D. Discovery of orally active carboxylic acid derivatives of 2-phenyl-5-trifluoromethyloxazole-4-carboxamide as

potent diacylglycerol acyltransferase-1 inhibitors for the potential treatment of obesity and diabetes. *J. Med. Chem.* **2011**, *54*, 2433-2446.

**Microsomal stability.** Liquid handling was carried out on a Tecan EVO robotic system. Triplicate incubations were carried out at a final test compound concentration of 1 μM with 0.5 mg/ml microsomal protein, and 1 mM NADPH. Pooled human liver microsomes (1 mg/ml protein) and NADPH (2 mM) were prepared in 50 mM phosphate buffer at pH 7.4. Stock solutions (10 mM) of the compounds were prepared in DMSO and then diluted to 100 μM in 1:1 acetonitrile/water. The compounds were added into cofactor in a 2 ml 96-well plate. Cofactor was added to the microsomes (1:1) that had been pre-incubated for 10 minutes at 37°C. Samples (0.1 ml) were incubated in 96-well plates at 37°C for 0, 10, 20 and 30 min in a Tecan 4-slot incubator for mouse liver microsomal stability. A two-point assay at 0 and 30 minutes was used to determine the percentage remaining in human microsomal stability. At each time points, the robotic arm removed one of the replicate plates and the reactions were stopped by adding 1 volume (100 μl) of acetonitrile with internal standard (0.05 μM buspirone) to each well. All plates were centrifuged at 3500 rpm for 30 min, and the supernatant was transferred to a 96-well injection plate. The plates were stored at 4°C until analyzed.

LC-MS/MS analysis. The samples were analyzed in positive mode using the turbospray ion source of PE/Sciex API 4000 Q-Trap mass spectrometer with Shimadzu HPLC system. Samples were injected (5 μL) onto a Lancer C18 column (5 μm, 30 x 2.1 mm) from Analytical Sales and Services Inc. (Pompton Plains, NJ) and separation occurred *via* a gradient: The flow rate was 0.5 mL/min; starting conditions of 7.5% B, 2.5%C, increasing to 30% B and 10 % C at 0.4 min. The percentage of B and C were rapidly

increased to 74 and 21%, respectively, over 0.5 min and held for 0.7 min, then decreased back to the initial conditions over 0.1 min, and held for 0.4 min, for a total run time of 2.5 min. Mobile phase A was 95/5 water/methanol (v/v) with 10 mM ammonium acetate and 60 μL/L acetic acid. Mobile phase B was methanol containing 10 mM ammonium acetate and 60 μL/L acetic acid. Mobile phase C was acetonitrile.

**Caco-2 Permeability assay.** Caco-2 (Human Adenocarcinoma Colon Cells from DKFZ Heidelberg) passage# 45 - 55 and 20 – 25 days in culture (seeding day is day 0); 24 Transwell Polycarbonate Filter (Corning Costar #3397); HBSS buffer (Gibco Cat#15630-080); HEPES (Gibco Cat#15630-080); Lucifer Yellow (LY) (Sigma Cat#L0259); DMEM (Gibco Cat#41965-047); L-Glutamine 200mM 100x (Gibco Cat#25030-024); MEM NEAA (100X) (Gibco Cat#11140-035); Sodium Pyruvate 100mM (Gibco Cat#11360-039). Complete DMEM medium: 1000 ml DMEM + 10 ml L-Glutamine + 10 ml MEM + 10 ml Sodium Pyruvate + 100 ml FBS. Rat tail collagen, Roche Cat#11179179001 coating according to the protocol of the supplier.

Compounds are tested at a concentration of 5 μM unless otherwise specified. The compounds are prepared as a 10 mM DMSO stock solution diluted in HBSS (Hank's Balanced Salt Solution) buffer with 4 % BSA. Caco-2 seeding density is 300000 cells/cm2 and cells are fed on Monday, Wednesday and Friday on both sides with DMEM supplemented with 10 % FBS. The apical volume is 250 μL and the basolateral volume 1 mL. Cells are cultured for 20-25 days. For each well, the cell monolayer membrane potential and resistance is measured with a chopstick electrode (Millicell ERS/see Millicell ERS User Guide). Cell monolayers are rinsed with DMEM without

phenol red (250 µL apical and 1 mL basolateral). Following the first rinse and prior to dosing of Caco-2 cells with compound, the monolayers are rinsed with HBSS containing 4 % BSA (200 µL apical and 400 µL basolateral) and pre-incubated (30 min) in HBSS with 4 % BSA. Cell monolayers are dosed on the apical side (for A-to-B direction measurements) or basolateral side (B-to-A direction measurments) with test compound (5 µM + 4 % BSA + 0.5 mg/mL Lucifer Yellow in HBSS ). Apical volume = 200 µL, basolateral volume = 400 µL. Incubations (37 °C/5%$CO_2$/95% air 50 rpm) are performed in duplicate for each time point. Incubation time points are 0 and 2 hours, and donor well samples (100 µL sample volume) for 0 and 2 hours and receiver well 2 hours. Aliquots taken from the receiver chambers (20 µL sample + 180 µL HBSS buffer) at the end of the incubation period are used to evaluate monolayer integrity. Aliquots are analyzed and Lucifer Yellow quantified on a fluorescent plate. Background fluorescence (HBSS buffer without LY) must be subtracted. Result: LY = % of total/hour

LC-MS/MS sample preparation: Quench solution is delivered at a 4:1 v/v ratio quench to samples. Quench solution: cold Ethanol with Ketoprofen (positive ion mode) and Chloroxazone (negative ion mode) as ISTD. Samples are stored in a freezer (–20° C). Samples are thawed, vortexed and centrifuged for 15 minutes at 2000 g and 20 µL aliquots are transferred to a 96 well microtiter plate using a 96 channel device (Liquidator Fa. Steinbrenner). Dilution of supernatant of the samples 1:10 with water + 0.1 % formic acid. The standard curve samples are prepared and extracted in the same manner as the experimental samples. Marker control compounds are run in every assay; atenolol (low perm), propranolol (high perm), digoxin (P-gp efflux), sulfasalazine (MRP2/BCRP efflux).

**Data Analysis**. Apparent permeability ($P_{app}$) values are calculated from the area-under-the-curve quantitation of the LC-MS/MS analysis. The calculation is:

$$P_{app}=(\Delta Q/\Delta t)*(1/(A*C_o))$$

Where delta Q is amount of drug solute transported (μmole), delta t is incubation time (sec), A is filter surface area in $cm^2$, and $C_o$ is mean of starting-end drug concentration in the donor well (μM). Drug velocity is expressed as apparent permeability, $P_{app}$, at the units 1 x $10^{-6}$ cm/sec.

**3. In vivo characterization**

**Pharmacokinetic analysis of plasma exposure in rodent.** Compounds are dosed in mice (10 mg/kg) in a vehicle containing 1% Tween-80 and water. Plasma samples are drawn at 0.5, 1, 2, 4, 6, 8, 12, and 24 h after the dose, and drug-concentrations are determined by mass spectroscopy analysis in comparison with a standard curve. The three mice with highest concentrations were averaged to provide the peak plasma concentration ($C_{max}$) ± SEM; the time for these three samples was averaged to provide the time to peak plasma or brain concentration ($T_{max}$) ± SEM. The mean plasma concentration data were submitted to multi-exponential curve fitting using WinNonlin. The area under the mean plasma concentration-time curve from 0 to t hours (time of the last measurable plasma concentration) after dosing ($AUC_{0-t}$) was calculated using the linear trapezoidal rule for the plasma concentration-time profile. The residual area was extrapolated to infinity, determined as the final measured mean plasma concentration ($C_t$)

divided by the terminal elimination rate constant (β), and added to $AUC_{0-t}$ to produce the total area under the curve ($AUC_{0-\infty}$).

Compounds are dosed in Sprague-Dawley rats (5 mg/kg) in a vehicle containing 1% Tween-80 and water. The same protocol was used with the exception of animal number, in which 3 rats were used for the iv dose and 3 for the po dose. Plasma sample analysis was determined from each rat throughout the course of the studies in a longitudinal fashion.

Table S1. Pharmacokinetics at two hours post lipid challenge in CD-1 mice.

Mouse Results:

| Moue # | Dose (mg/kg, PO) | Time (hour) | Plasma Conc. (µg/mL) |
|---|---|---|---|
| 21 | 0.03 | 3 | 0.030 |
| 22 | 0.03 | 3 | 0.041 |
| 23 | 0.03 | 3 | 0.038 |
| 24 | 0.03 | 3 | 0.025 |
| Mean | | | 0.033 |
| SEM | | | 0.004 |
| 31 | 0.3 | 3 | 0.25 |
| 32 | 0.3 | 3 | 0.36 |
| 33 | 0.3 | 3 | 0.42 |
| 34 | 0.3 | 3 | 0.39 |
| Mean | | | 0.36 |
| SEM | | | 0.04 |
| 41 | 3 | 3 | 3.57 |
| 42 | 3 | 3 | 3.55 |
| 43 | 3 | 3 | 3.18 |
| 44 | 3 | 3 | 2.10 |
| Mean | | | 3.10 |
| SEM | | | 0.35 |