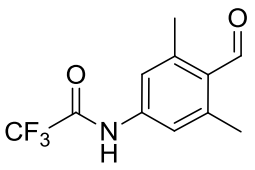
Intestinally-targeted Diacylglycerol Acyltransferase 1 (DGAT1) Inhibitors Robustly Suppress Postprandial Triglycerides

Michael H. Serrano-Wu,[a] Gary M. Coppola,[a] Yongjin Gong,[a] Alan D. Neubert,[a] Ricardo Chatelain,[b] Kevin B. Clairmont,[b] Renee Commerford,[b] Theresa Cosker,[c] Thomas Daniels,[b] Ying Hou,[a] Monish Jain,[c] Marlene Juedes,[d] Lisha Li,[b] Tara Mullarkey,[b] Erik Rocheford,[b] Moo Je Sung,[a] Andrew Tyler,[b] Qing Yang,[b] Taeyoung Yoon,[a] and Brian K. Hubbard[b]*

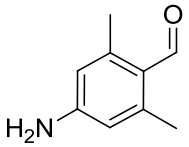
Departments of [a]Global Discovery Chemistry, [b]Cardiovascular and Metabolism, [c]Metabolism and Pharmacokinetics, and [d]Translational Sciences, Novartis Institutes for Biomedical Research, 100 Technology Square, Cambridge Massachusetts 02139

*To whom corresponding should be addressed: michael.serrano-wu@novartis.com; phone (617) 871-7523; fax (617) 871-7043

Synthesis of New Compounds

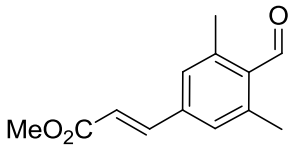


2,2,2-Trifluoro-N-(4-formyl-3,5-dimethylphenyl)-acetamide (8b). To a solution of N-(4-bromo-3,5-dimethylphenyl)-2,2,2-trifluoroacetamide[1] (**8a**) (14.0 g, 47.3 mmol) in THF (200 mL) at -78 ^{o}C under a nitrogen atmosphere was added dropwise MeLi/LiBr (44.1 mL of a 1.5M solution in ether, 66.2 mmol). The mixture was stirred at -78 ^{o}C for 5 min then s-BuLi (47.3 mL of a 1.4M solution in cyclohexane, 66.2 mmol) was added dropwise. After 5 min of stirring at -78 ^{o}C, DMF (22.5 mL, 331 mmol) was added dropwise. After the addition was complete, the mixture was allowed to warm to ambient temperature and stirred for 30 min. The mixture was quenched with water and was extracted with methylene chloride. The organic phase was washed with water and brine and was dried over $MgSO_4$. The solvent was removed under reduced pressure to give 9 g (76% yield) of the product as a light yellow solid: ms (m/z) 246 $(M+H)^+$. This product was used directly in the next reaction.

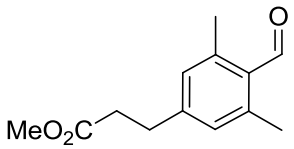


4-Amino-2,6-dimethylbenzaldehyde (9). To a suspension of **8** (9 g, 36.7 mmol) in MeOH (30 mL) was added 1N NaOH (30 mL) and the mixture was stirred at ambient temperature for 18 h. Water (100 mL) was added and the solid was filtered, washed with water and dried. The aqueous phase was extracted with EtOAc and the organic phase was washed with water and brine and

was dried over $MgSO_4$. The solvent was removed under reduced pressure to furnish additional product. The combined solids were purified by flash chromatography (heptane/EtOAc, 4:1) to give 5 g (91% yield) of the product as a yellowish solid: mp = 177-180 ^oC; ms (m/z) 150 $(M+H)^+$.

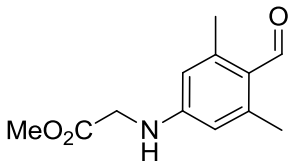


3-(4-Formyl-3,5-dimethylphenyl)-acrylic acid methyl ester (10). To aldehyde **9** (1.0 g, 6.71 mmol) was added 48% aqueous HBF_4 until the suspension stirred freely. The mixture was cooled to 0 ^oC then a solution of $NaNO_2$ (463 mg, 6.71 mmol) in water (5 mL) was added slowly. The mixture was stirred at 0 ^oC for 30 min then MeOH (20 mL) was added followed by $Pd(OAc)_2$ (229 mg) and methyl acrylate (1.155 g, 13.42 mmol) then the mixture was heated at 80 ^oC for 30 min. The suspension was filtered through Celite and was washed with methylene chloride. The filtrate was extracted with methylene chloride and the combined organic solutions were dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (gradient of heptane/EtOAc, 10:1→5:1) to give 900 mg (62% yield) of the product as a pale-yellow oil: ^{1}H-NMR (DMSO-d6): δ 10.51 (,s 1H), 7.60 (d, J = 16.17 Hz, 1H), 7.53 (s, 2H), 6.77 (d, J = 16.04 Hz, 1H), 3.74 (s, 1H).

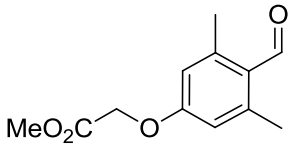


3-(4-Formyl-3,5-dimethylphenyl)-propionic acid methyl ester (11). A solution of **10** (900 mg, 4.1 mmol) in methylene chloride (20 mL) was hydrogenated over 10% Pd/C (90 mg) at 1 atm for

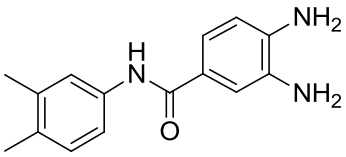
18 h. The catalyst was filtered through Celite and the filtrate was evaporated under reduced pressure. The residual oil was purified by flash chromatography (heptane/EtOAc, 5:1) to give 750 mg (83% yield) of the product as a pale-yellow oil: ^{1}H-NMR ($CDCl_3$): δ 10.65 (s, 1H), 7.00 (s, 2H), 3.76 (s, 3H), 2.99 (t, 2H), 2.72 (t, 2H), 2.67 (s, 6H).



(4-Formyl-3,5-dimethylphenylamino)-acetic acid methyl ester (13). To a solution of **9** (600 mg, 4 mmol) and methyl bromoacetate (0.4 mL, 4.2 mmol) in DMF (20 mL) was added K_2CO_3 (1.4 g, 10.1 mmol) and the mixture was stirred at 80 ^{o}C for 1 h. An additional 0.4 mL of methyl bromoacetate was added and the mixture was stirred at 80 ^{o}C for 1 h. This process was continued until reaction was complete. The mixture was cooled then water was added and extracted with EtOAc. The organic phase was washed with water (3x) and was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue purified by flash chromatography (hexanes/EtOAc, 2:1) to give 725 mg (82% yield) of the product as a yellowish solid: ^{1}H-NMR (DMSO-d6): δ 10.19 (s, 1H), 6.90 (t, 1H), 6.27 (s, 1H), 4.02 (d, J = 6.44 Hz, 2H), 3.66 (s, 3H), 2.45 (s, 6H); ms (m/z) 222 $(M+H)^+$. *Anal.* Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.84; H, 6.64; N, 6.17.

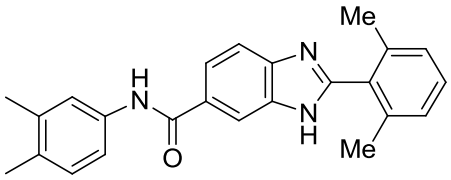


(4-Formyl-3,5-dimethylphenoxy)-acetic acid methyl ester (14). To a mixture of 2,6-dimethyl-4-hydroxybenzaldehyde[3] (1.5 g, 10 mmol) and K_2CO_3 (2.07 g, 15 mmol) in acetone (30 mL) was added methyl bromoacetate (1.14 mL, 12 mmol) and the mixture was stirred at ambient temperature for 3 h. Water was added and the mixture was extracted with EtOAc. The organic phase was washed with water and brine then was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue purified by flash chromatography (heptane/EtOAc, 4:1) to give 2.0 g (90% yield) of the product as a yellowish solid: mp = 73-75 ^oC; ^1H-NMR ($CDCl_3$): δ 10.49 (s, 1H), 6.59 (s, 2H), 4.68 (s, 2H), 3.83 (s, 3H), 2.61 (s, 6H). *Anal.* Calcd for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35. Found: C, 65.14; H, 6.08.



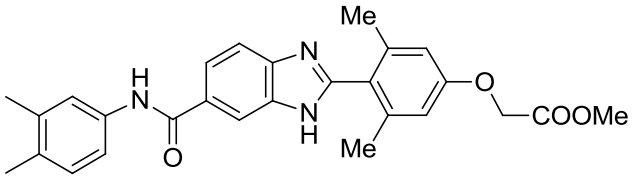
3,4-Diamino-N-(3,4-dimethylphenyl)-benzamide (15). To a solution of 4-amino-3-nitrobenzoic acid (1.82 g, 10 mmol) in DMF (20 mL) was added HOBt (1.49 g, 11 mmol) and EDCI (2.1 g, 11 mmol). The mixture was stirred at ambient temperature for 10 min then 3,4-dimethylaniline (1.2 g, 10 mmol) and DIEA (5.3 mL, 32 mmol) were added. The mixture was stirred at ambient temperature for 18 h. Water was added and the mixture was extracted with EtOAc. The organic phase was washed with water and brine and was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residual solid was recrystallized from

EtOAc to give 2.0 g (70% yield) of the product: mp = 190-191 ^{o}C; ^{1}H-NMR (DMSO-d6): δ 10.04 (s, 1H), 8.71 (s, 1H), 7.97 (dd, J = 8.97 and 2.15, 1H), 7.84 (s, 2H), 7.51 (s, 1H), 7.47 (d, J = 8.08 Hz, 1H), 7.08 (m, 2H), 2.21 (s, 3H), 2.18 (s, 3H); ms (m/z) 286 $(M+H)^{+}$. *Anal.* Calcd for $C_{15}H_{15}N_3O_3$: C, 63.15; H, 5.30; N, 14.73. Found: C, 63.17; H, 5.05; N, 14.74. A solution of 4-amino-N-(3,4-dimethylphenyl)-3-nitrobenzamide (2.0 g, 7 mmol) in EtOH (40 mL) was hydrogenated over PtO_2 (200 mg) for 18 h. The catalyst was filtered through Celite and the filtrate evaporated under reduced pressure to give 1.6 g (89% yield) of the product as a light yellow solid, mp = 140-142 ^{o}C; ms (m/z) 256 $(M+H)^{+}$. This product was used directly in the next step.

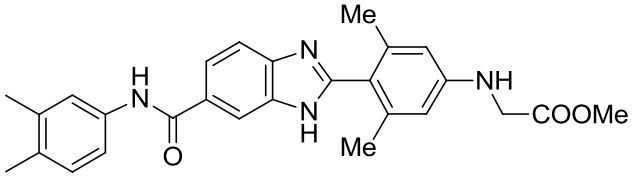


2-(2,6-Dimethylphenyl)-3H-benzoimidazole-5-carboxylic acid (3,4-dimethylphenyl)-amide (2). A mixture of **15** (10.2 mg, 0.04 mmol), 2,6-dimethylbenzaldehyde (5.9 mg, 0.044 mmol) and $FeCl_3$ (150 μL of a 0.02M solution in THF, 5 equiv) in DMSO (300 μL) was stirred at ambient temperature for 18 h. The mixture was loaded on a benzenesulfonic acid-bonded silica gel SPE column and was washed with 5 mL MeOH then with 5 mL of EtOAc/MeOH/Et_3N (20:2:1). Concentration of the eluent furnished 13.6 mg (92% yield) of the product. ^{1}H-NMR (MeOD): δ 8.15 (s, broad, 1H), 7.80 (d, J = 8.08 Hz, 1H), 7.61 (d, J = 8.08 Hz, 1H), 7.37 (s, 2H), 7.33 (d, J = 8.08 Hz, 1H), 7.23 (t, 1H), 7.10 (d, J = 7.58 Hz, 2H), 7.02 (d, J = 8.08 Hz, 1H), 2.19 (s, 3H), 2.16 (s, 3H), 2.06 (s, 6H). HRMS: calcd for $C_{24}H_{24}N_3O$, 370.1919 $(M+H)^{+}$; found 370.1913.

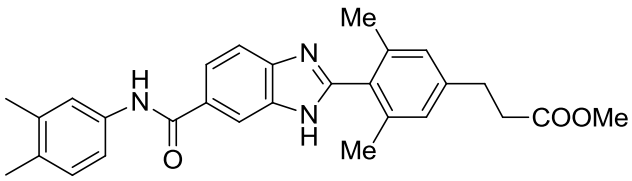
General procedure for benzimidazole formation using $Yb(OTf)_3$. To a solution of 0.75 mmol of **15** and 0.75 mmol of aldehyde **11**, **13** or **14** in DMSO (10 mL) was added 0.15 mmol of $Yb(OTf)_3$ and the mixture was stirred at ambient temperature for 60 h. Water was added and the mixture was extracted with EtOAc. The organic phase was washed with water and brine and was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (heptane/EtOAc, 1:2) to give the product **16**, **17** or **18**.



{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenoxy}-acetic acid methyl ester (16). ^{1}H-NMR (MeOD): δ 8.25 (s, broad, 1H), 7.91 (d, J = 8.08 Hz, 1H), 7.71 (s, broad, 1H), 7.49 (s, 1H), 7.45 (d, J = 8.08 Hz, 1H), 7.13 (d, J = 8.08 Hz, 1H), 6.79 (s, 2H), 4.84 (s, 3H), 4.76 (s, 2H), 2.30 (s, 3H), 2.27 (s, 3H), 2.16 (s, 6H); ms (m/z) 458 $(M+H)^+$.

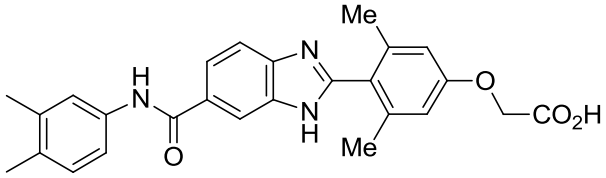


{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenylamino}-acetic acid methyl ester (17). ^{1}H-NMR (MeOD): δ 8.20 (s, broad, 1H), 7.87 (d, J = 8.34 Hz, 1H), 7.67 (s, broad, 1H), 7.47 (s, 1H), 7.43 (d, J = 8.08 Hz, 1H), 7.12 (d, J = 8.08 Hz, 1H), 6.42 (s, 2H), 3.97 (s, 2H), 3.75 (s, 3H), 2.29 (s, 3H), 2.26 (s, 3H), 2.09 (s, 6H); ms (m/z) 457 $(M+H)^+$.

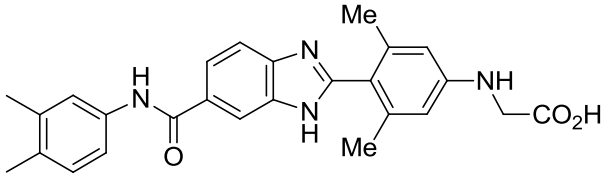


3-{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenyl}-propionic acid methyl ester (18). ^{1}H-NMR (MeOD): δ 8.15 (s, broad, 1H), 7.90 (d, J = 8.08 Hz, 1H), 7.64 (s, broad, 1H), 7.47 (s, 1H), 7.43 (d, J = 8.08 Hz, 1H), 7.12 (d, J = 8.21 Hz, 1H), 7.06 (s, 2H), 2.94 (t, 2H), 2.67 (t, 2H), 2.29 (s, 3H), 2.26 (s, 3H), 2.14 (s, 6H); ms (m/z) 456 $(M+H)^+$.

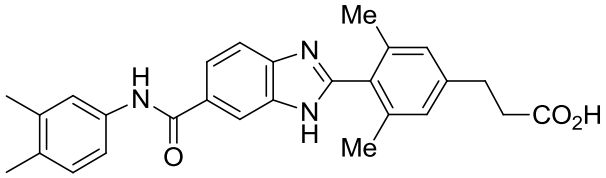
General procedure for the hydrolysis of esters 16, 17 and 18. To a solution of 1.5 mmol of the appropriate ester in MeOH (10 mL) was added 1N NaOH (10 mL) and the mixture was stirred at ambient temperature for 2 h. The mixture was acidified to pH 4 with 1N HCl and the resulting precipitate was filtered, washed with water and dried under reduced pressure to give the carboxylic acids **4**, **5** or **6**.



{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenoxy}-acetic acid (4). ^{1}H-NMR (MeOD): δ 8.24 (s, 1H), 7.91 (d, J = 8.46 Hz, 1H), 7.70 (d, J = 8.46 Hz, 1H), 7.47 (s, 1H), 7.43 (d, J = 8.08 Hz, 1H), 7.12 (d, J = 8.34 Hz, 1H), 6.79 (s, 2H), 4.68 (s, 2H), 2.29 (s, 3H), 2.26 (s, 3H), 2.15 (s, 6H). ms (m/z) 444 $(M+H)^+$. HRMS: calcd for $C_{26}H_{25}N_3O_4$, 443.18451; found 443.18499.



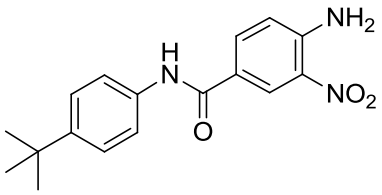
{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenylamino}-acetic acid (5). ^{1}H-NMR (MeOD): δ 8.23 (s, 1H), 7.91 (d, J = 8.59 Hz, 1H), 7.70 (d, J = 8.34 Hz, 1H), 7.47 (s, 1H), 7.43 (d, J = 8.08 Hz, 1H), 7.12 (d, J = 8.08 Hz, 1H), 6.45 (s, 2H), 3.92 (s, 2H), 2.29 (s, 3H), 2.26 (s, 3H), 2.11 (s, 6H); ms (m/z) 443 $(M+H)^+$. *Anal.* Calcd for $C_{26}H_{26}N_4O_3 \cdot 0.5\ H_2O$: C, 69.16; H, 6.03; N, 12.41. Found: C, 69.26; H, 5.76; N, 12.35.



3-{4-[6-(3,4-Dimethylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenyl}-propionic acid (6). ^{1}H-NMR (MeOD): δ 8.23 (s, broad, 1H), 7.90 (d, J = 8.59 Hz, 1H), 7.70 (d, J = 8.59 Hz, 1H), 7.47 (s, 1H), 7.42 (d, J = 8.34 Hz, 1H), 7.12 (d, J = 8.08 Hz, 1H), 7.08 (s, 2H), 2.93 (t, 2H), 2.61 (t, 2H), 2.29 (s, 3H), 2.26 (s, 3H), 2.14 (s, 6H); ms (m/z) 442 $(M+H)^+$. *Anal.* Calcd for $C_{27}H_{27}N_3O_3 \cdot 0.4\ H_2O$: C, 72.27; H, 6.24; N, 9.36. Found: C, 72.36; H, 5.98; N, 9.30.

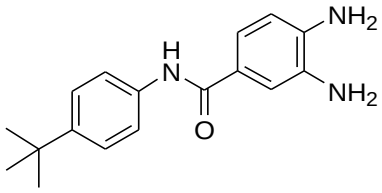
Preparation of compound 7.

4-Amino-N-(4-tert-butylphenyl)-3-nitro-benzamide



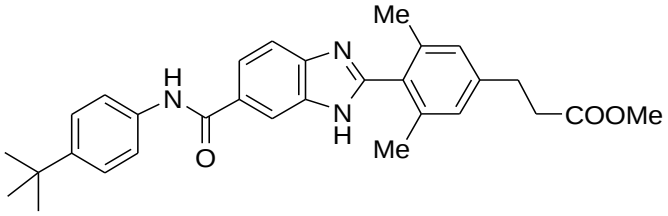
To a solution of HATU (3.61 g, 9.5 mmol) in DMF (10 mL) was added 4-amino-3-nitrobenzoic acid (1.15 g, 6.32 mmol), Et_3N (0.958 g, 9.47 mmol) and 4-tert-butylaniline. The mixture was stirred at ambient temperature for 18 h then was poured into EtOAc. The mixture was washed with water (1x) and brine (5x) and the organic phase was dried over $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by chromatography using a gradient of 30→70% heptane/EtOAc to give the product which was used directly in the following step. ^{1}H-NMR ($CDCl_3$): δ 8.62 (s, 1H), 7.98 (dd, J = 8.72 and 2.15 Hz, 1H), 7.74 (s, broad, 1H), 7.55 (d, J = 8.59 Hz, 2H), 7.40 (d, J = 8.59 Hz, 2H), 6.91 (d, J = 8.72 Hz, 1H), 1.33 (s, 9H);); ms (m/z) 314 $(M+H)^+$.

3,4-Diamino-N-(4-tert-butylphenyl)-benzamide



A solution of the above compound in 1:1 EtOAc/MeOH (50 mL) was hydrogenated over 5% Pt/C at 1 atm for 3 h. The catalyst was filtered through Celite and the solvent was removed under reduced pressure to give 1.37 g (76% yield for two steps) of the product which was used directly in the following step.

3-{4-[6-(4-tert-Butylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenyl}-propionic acid methyl ester



To a solution of the above diamine (200 mg, 0.71 mmol) and **11** (190 mg, 0.86 mmol) in DMF/water (5 mL/ 0.5 mL) was added oxone (286 mg, 0.47 mmol) and the mixture was stirred at ambient temperature for 1 h. The mixture was poured into EtOAc and was washed with water (1x) and brine (5x). The organic phase was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by chromatography using a gradient of 50→90% heptane/EtOAc to give 310 mg (90% yield) of the product. ^{1}H-NMR ($CDCl_3$): δ 8.51 (s, broad, 1H), 8.07 (s, broad, 1H), 7.8 (d, J = 8.5 Hz, 1H), 7.53 (d, J = 8.1 Hz, 2H), 7.31 (d, J = 8.6 Hz, 2H), 6.82 (s, 2H), 3.63 (s, 3H), 2.89 (t, 2H), 2.63 (t, 2H), 1.92 (s, 6H), 1.30 (s, 9H); ms (m/z) 484 $(M+H)^+$.

3-{4-[6-(4-tert-butylphenylcarbamoyl)-1H-benzoimidazol-2-yl]-3,5-dimethylphenyl}-propionic acid (7). A mixture of the above ester (310 mg, 0.64 mmol) and 1N NaOH (3.2 mL) in MeOH (1.6 mL) was stirred at ambient temperature for 18 h. The pH of the solution was adjusted to 4-5 with 1N HCl and the resulting precipitate was filtered, washed with water and dried under reduced pressure to give 229 mg (76% yield) of the product. ; ^{1}H-NMR (DMSO-d6): δ 12.17 (s, broad, 1H), 10.22 (s, 1H), 8.29 (s, broad, 1H), 7.89 (d, J = 8.5 Hz, 2H), 7.74 (d, J = 9.0 Hz, 2H), 7.72 (m, 1H), 7.37 (d, J = 9.0 Hz,2H), 7.10 (s, 2H), 2.85 (t, 2H), 2.59 (t, 2H), 2.10 (s, 6H); ms (m/z) 470 $(M+H)^+$. *Anal.* Calcd for $C_{29}H_{31}N_3O_3$: C, 74.18; H, 6.65; N, 8.95. Found: C, 74.13; H, 6.56; N, 8.88.

Primary assay for the inhibition of human DGAT1 enzyme activity

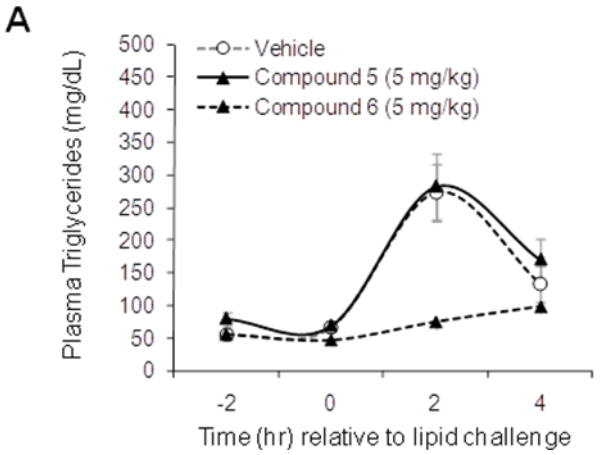
The enzyme preparation used in this assay is a membrane preparation from Sf9 cells over-expressing human $(His)_6$DGAT1. The protein concentration of the membrane preparation was quantified using BCA protein assay with 1% SDS. The membrane preparation was aliquoted, frozen on dry ice, and stored at -80ºC. Test compounds were dissolved in the appropriate volume of DMSO to a final concentration of 10 mM. A 10-point, 3-fold dose response was used to evaluate compound potency.

Cellular assay for inhibition of intracellular TG synthesis

C2C12 cells were seeded 18 hours prior to assay in 96-well plates in Dulbecco's Modified Eagle Medium (DMEM) containing 5 mM glucose and 10% fetal bovine serum. Following 18 hours incubation at 37°C, medium was replaced with DMEM containing 5 mM glucose), 250 μM oleate complexed to BSA, and DGAT1 inhibitors at the specified concentrations in dimethylsulfoxide to a final concentration of 0.5% for 2 hrs at 37 °C. The medium was removed at the end of the incubation and 150 μl/well of 1-butanol containing the internal standard, tripalmitolein at 0.2 μM, added. The plates were sealed and left at room temperature for at least 10 min. Butanolic extracts (85 μl/well) were transferred to 384-well LC/MS plates and centrifuged at 209 *x g* for 5 min prior to loading on the LC/MS. Samples were analyzed by LC/MS using an Agilent 1100 Series LC and Micromass Quattro Micro API mass spectrophotometer with an ESI+ source. For dose-response determinations, the LC/MS/MS was run in the MUX mode, using a Gilson Quad-Z 215 autosampler. Compound potency was determined by an 11-point, 3-fold dose response.

Rat lipid challenge model

All procedures were in compliance with the Animal Welfare Act Regulations 9 CFR Parts 1, 2 and 3, and other guidelines (Guide for the *Care and Use of Laboratory Animals*, 1995). Male Sprague Dawley rats from Charles River Laboratories, weighing 225-250 g (~8 weeks old), were allowed to acclimate at least 1 week prior to study. Animals were assigned to experimental groups on the basis of pre-study body weight (n = 8/group) and were maintained on normal light cycle and allowed *ad libitum* access to standard chow. Following an overnight fast, baseline plasma samples were collected by tail nick. Rats were dosed with vehicle (0.5% methyl cellulose/0.1% Tween-80, 4 mL/kg) or test compound suspended in vehicle (5 mg/kg). Two hours later (considered time = 0 hr), the rats were orally gavaged with Intralipid containing 20% lipid (soybean oil) at 10 mL/kg to induce a plasma TG excursion. Post-dosing blood samples were collected into EDTA-lined tubes at 2 and 4 hrs to assess plasma TG concentrations. Blood samples remained on ice following collection for no longer than 30 min prior to centrifugation. Following centrifugation (10 min, 4 ^{o}C, 1000 *x* g), plasma was aliquoted for TG analysis. Plasma TG's (corrected for glycerol) were compared on an absolute basis and as area above baseline over the baseline to 4 hr time point (AABtg) using the Trapezoidal rule. One-way analysis of variance (ANOVA) was used to compare groups when data were expressed as AABtg. To assure that the Intralipid bolus produced a significant increase in plasma TG's, a repeated measures (RM) one-way ANOVA was used with Tukey's post-hoc test applied to compare means at 2 and 4 hrs vs. baseline TG concentrations in the vehicle group only.



Supplementary Figure A: Plasma triglycerides following oral administration of compounds **4-6** (5 mg/kg) followed by Intralipid (10 mL/kg) to Sprague-Dawley rats.

Enterocyte PK/PD model

Following an overnight fast, male Sprague Dawley rats were dosed with either vehicle or compound **7** in vehicle at 5.0 mg/kg in a 4 mL/kg dosing volume. Thirty minutes following the provision of drug, rats were dosed orally with Intralipid (10 mL/kg) to induce a plasma triglyceride excursion. At either 2, 6, or 17 hrs post-Intralipid, rats were anesthetized with Euthasol (pentobarbital sodium, 100 mg/kg, i.p.) and upon absence of pedal reflex, the abdominal cavity was opened, the intestines reflected to the right, and blood (portal vein and vena cava), intestine and enterocytes were collected. The entire intestine was removed and divided into three segments of approximately equal length. The most distal third (considered the ileum) was disposed of at this time. The remaining two segments (the duodenum and jejunem) were processed as follows: the first (most proximal) 10 cm of each segment was opened to expose the intestinal lumen and was thoroughly rinsed in 50 mL of cold phosphate buffered saline to assure complete removal of unbound compound. This was repeated two more times for each segment in cold normal saline for the second and third washings. The enterocytes were

then scraped into aluminum foil, were placed in liquid nitrogen, and were stored at -80°C until later analysis.

For quantification of **7** in enterocyte samples, enterocyte tissue was first homogenized using a TissueLyser instrument. To each sample a 3 mm bead was added and phosphate buffered saline (PBS) at 5 x volume of the tissue weight. Samples were then homogenized in 30 sec intervals for up to 5 minutes. Aliquots (50 µL) of homogenized enterocytes were extracted by precipitation of proteins with acteonitrile (300 µL) containing 100 ng/mL of internal standard (glyburide). Samples were subsequently vortexed for 5 min followed by centrifugation at approximately 3000 *x g* for 5 min. The resulting supernatant (~150 µL) was transferred into a clean 96 well plate, 50 µL of water was added and samples were vortexed for 2 min prior to LCMS analysis.

	Duodenum	
Treatment	TGs (µg/mg tissue)	% from Veh
Vehicle	82.3 ± 17.4	N/A
Compound 7	26.5 ± 5.9	67.8%

Dog lipid challenge model

Male Beagle dogs (Marshall Farms, North Rose, NY) weighing 7-13 kg were used in this study. Animals were fed a standard chow diet (Harlan 8653 diet [fat: 9.95 grams %; 19% kcal from fat; 4.02 kcal/gram]) and water was provided *ad libitum*. Dogs were maintained in an environment with temperature and humidity appropriate for the species and kept on a 12-hour light/dark cycle.

All procedures were performed in accordance with the standards of the US Department of Health and Human Services and were approved by the Novartis Animal Care and Use Committee (Protocol DB 9018 07G). Animal husbandry and maintenance were in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

A colony of Beagles was screened to determine the plasma triglyceride response to a bolus of heavy cream administered via gavage (10 mL/kg body weight; Heavy cream Shop-Rite Brand: total fat 0.33 g/mL, saturated fat: 0.23 g/ml, cholesterol: 1.33 mg/mL; sodium 0.33 mg/mL; potassium: 1 mg/mL; carbohydrate: 0; protein: 67 mg/mL). Four dogs were fasted for 20 hrs and a baseline blood sample was obtained at 30 min before administering the cream (-30 min). Immediately thereafter the vehicle (0.5% carboxymethylcellulose, 5 mL/kg) was administered via gavage and, after 30 min, a blood sample was drawn (0 time). The heavy cream was given and subsequent blood samples were obtained after 1, 2, 4, 6 and 24 hrs from the ingestion of the cream. A bowl with standard diet (Harlan 8653) was returned to the cages after taking the 6^{th} hour sample. Compound **7** was tested using the same four dogs but both studies were separated by a washout period lasting for 30 days. Compound **7** was administered orally at 1 mg/kg suspended in 0.5% carboxymethylcellulose vehicle (5 mL/kg) and the same protocol used to test the vehicle was performed. Blood samples (3 mL) were drawn from the cephalic vein, collected in tubes containing EDTA and placed on ice until centrifugation at 3,000 rpm for 20 min at 4 ^{o}C. Triglycerides were analyzed in fresh plasma by an enzymatic method (Pointe Scientific, Canton, MI 48188, U.S.A.). Plasma was also apportioned into aliquots and stored at –70 ^{o}C for the measurements of other parameters.

The time course of triglyceride changes after ingestion of the heavy cream meal were analyzed by repeated measures ANOVA followed by Dunnett's post-tests between each time period

versus baseline. Effects of compound **6** versus vehicle were analyzed using paired t-tests comparing the incremental area under the curve ($AUC_{0\text{-}6h}$).

References

(1) Baroudy, B. M.; Clader, J. W.; Josien, H. B.; McCombie, S. W.; McKittrick, B. A.; Miller, M. W.; Neustadt, B. R.; Palani, A.; Smith, E. M.; Steensma, R.; Tagat, J. R.; Vice, S. F.; Gilbert, E.; Labroli, M. A. Piperazine Derivatives useful as CCR5 antagonists, US Patent 6,391,865, 2002.

(2) Burgess, D. A.; Rae, I. D. Oxidation of alkyl groups accompanying the Zinin reduction of nitroarenes, *Aust. J. Chem*. **1977**, *30*, 927-931.

(3) Coppola, G. M.; Gong, Y. A reliable, high-yielding preparation of 2,6-dimethyl-4-hydroxybenzaldehyde, *Org. Prep. Proced. Int*. **2007**, *39*, 199-202.