Supporting Information

Discovery of bisamide-heterocycles as inhibitors of scavenger receptor BI (SR-BI)-mediated lipid uptake

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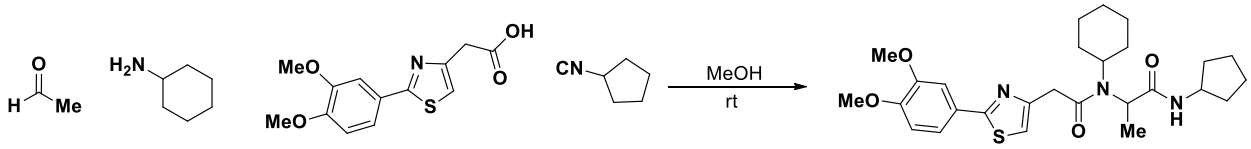
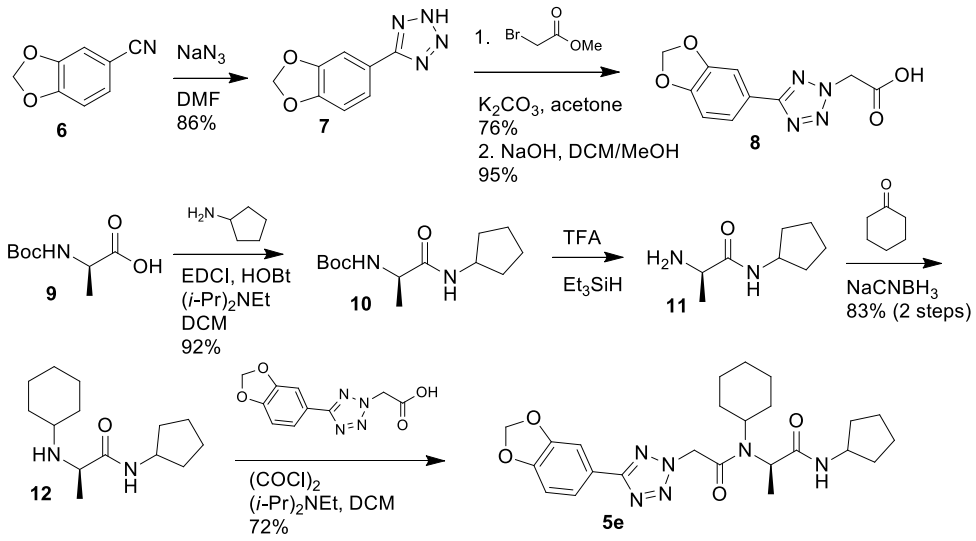
Contents

1. Representative protocol for Ugi reactions



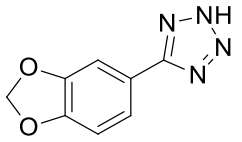
In a round bottom flask, cyclohexylamine (0.041 mL, 0.358 mmol) and acetaldehyde (0.020 mL, 0.358 mmol) were dissolved in dry methanol (2.0 mL) at r.t. under nitrogen and stirred for 10 min. Subsequently, 2-(2-(3,4-dimethoxyphenyl)thiazol-4-yl)acetic acid (100 mg, 0.358 mmol) was added to this mixture in one portion followed by cyclopentyl isocyanide (0.040 mL, 0.358 mmol), and the mixture was stirred for 14 h at r.t. The reaction mixture was subsequently concentrated under reduced pressure and the crude product mixture was purified by column chromatography over 40 g of silica and eluted with ethyl acetate/hexanes to provide the desired Ugi adduct **1a** (47 mg, 26% yield).

2. Synthesis and characterization data for 5e (ML279)



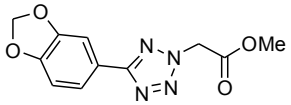
General Details. All reagents and solvents were purchased from commercial vendors and used as received. NMR spectra were recorded on a Bruker 300 MHz or Varian UNITY INOVA 500 MHz spectrometer as indicated. Proton and carbon chemical shifts are reported in parts per million (ppm;δ) relative to tetramethylsilane, $CDCl_3$ solvent, or d_6-DMSO (^{1}H δ 0, ^{13}C δ 77.16, or ^{13}C δ 39.5, respectively). NMR data are reported as follows: chemical shifts, multiplicity (obs. = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, m = multiplet, comp = complex overlapping signals); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25 °C. Flash chromatography was performed using 40-60 μm Silica Gel on a Teledyne Isco Combiflash R_f system. Tandem liquid chromatography/mass

spectrometry (LCMS) was performed on a Waters 2795 separations module and Waters 3100 mass detector. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and aqueous potassium permanganate ($KMnO_4$) stain followed by heating. High-resolution mass spectra were obtained at the MIT Mass Spectrometry Facility with a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform Ion Cyclotron Resonance mass spectrometer. Compound purity and identity were determined by UPLC-MS (Waters, Milford, MA). Purity was measured by UV absorbance at 210 nm. Identity was determined on a SQ mass spectrometer by positive electrospray ionization. Mobile Phase A consisted of either 0.1% ammonium hydroxide or 0.1% trifluoroacetic acid in water, while mobile Phase B consisted of the same additives in acetonitrile. The gradient ran from 5% to 95% mobile Phase B over 0.8 minutes at 0.45 ml/min. An Acquity BEH C18, 1.7 µm, 1.0 x 50 mm column was used with column temperature maintained at 65 °C. Compounds were dissolved in DMSO at a nominal concentration of 1 mg/ml, and 0.25 µl of this solution was injected.



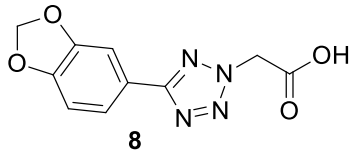
7

5-(Benzo[d][1,3]dioxol-5-yl)-2*H*-tetrazole (7): A round-bottom flask equipped with a magnetic stirbar was charged with benzo[d][1,3]dioxole-5-carbonitrile (2.50 g, 17.0 mmol) and *N*,*N*-dimethylformamide (17.0 mL). Sodium azide (1.22 g, 18.7 mmol) and ammonium chloride (1.00 g, 18.7 mmol) were then added to give a white suspension. This mixture was heated to 150 °C and stirred for 24 hours. The reaction was cooled to ~70 °C, then poured on ice (~50 mL). The mixture was acidified to pH 1 with 1.0 *M* aqueous hydrochloric acid solution. The precipitated solids were collected by filtration, washed with ice-cold water (10 mL), and air-dried on the filter to give the title compound as a white solid (2.79 g, 86%). **^{1}H NMR (300 MHz, CD_3OD):** δ 7.55 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.46 (d, *J* = 1.5 Hz, 1H), 7.02 (d, *J* = 8.1 Hz, 1H), 6.08 (s, 2H); **MS** (ESI^+): 191 (M+H).



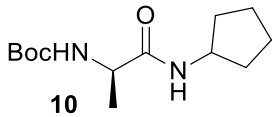
Methyl 2-(5-(benzo[d][1,3]dioxol-5-yl)-2H-tetrazol-2-yl)acetate: A round-bottom flask equipped with a magnetic stirbar was charged with 5-(benzo[d][1,3]dioxol-5-yl)-2*H*-tetrazole (2.79 g, 14.7 mmol) and acetone (49.0 mL). The suspension was cooled to 0 °C before adding anhydrous potassium carbonate. After stirring for 30 minutes at 0 °C, methyl bromoacetate (1.5 mL, 16.2 mmol) was added to the opaque, tan mixture. The reaction was stirred for 18 hours while slowly warming to room temperature. The mixture was filtered through a pad of Celite ®, and the filter was thoroughly washed with additional acetone. The clear, yellow filtrate was

concentrated under reduced pressure to give an orange solid. This mixture of regioisomers (~8:1 ratio) was separated by column chromatography over silica gel (hexanes/ethyl acetate: 100/0 to 40/60) to give the title compound as a white solid (2.93 g, 76 %). **^{1}H NMR (300 MHz, $CDCl_3$):** δ 7.71 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.59 (d, *J* = 1.5 Hz, 1H), 6.91 (d, *J* = 8.1 Hz, 1H), 6.03 (s, 2H), 5.45 (s, 2H), 3.82 (s, 3H); **MS** (ESI^+): 263 (M+H).



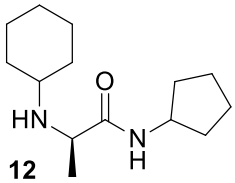
8

2-(5-(Benzo[d][1,3]dioxol-5-yl)-2H-tetrazol-2-yl)acetic acid (8): Methyl 2-(5-(benzo[d][1,3]dioxol-5-yl)-2*H*-tetrazol-2-yl)acetate (2.93 g, 11.2 mmol) was placed in a round-bottom flask equipped with a magnetic stirbar and dissolved with dichloromethane (33.5 mL) and methanol (3.7 mL). Sodium hydroxide pellets (0.89 g, 22.3 mmol) were added to the clear, pale yellow solution. The reaction was stirred at room temperature for 1 hour and the resulting opaque, white mixture was concentrated under reduced pressure to give a white solid. This material was dissolved in water (250 mL) and washed with dichloromethane (2 x 100 mL). The aqueous phase was acidified to pH 1 with concentrated hydrochloric acid to give an opaque white mixture. Extraction with hot ethyl acetate (3 x 100 mL) produced a clear aqueous layer. The combined ethyl acetate extracts were shaken over magnesium sulfate, filtered, and concentrated under reduced pressure to give title compound as a white solid (2.64 g, 95 %). **^{1}H NMR (300 MHz, d_6-DMSO):** δ 7.62 (d, *J* = 8.0 Hz, 1H), 7.52 (s, 1H), 7.11 (d, *J* = 7.9 Hz, 1H), 6.14 (s, 2H), 5.70 (s, 2H); **MS** (ESI^+): 249 (M+H).



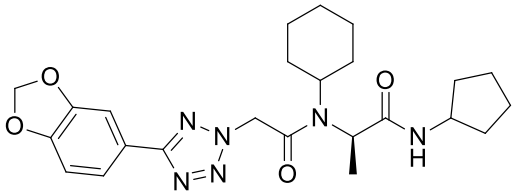
10

(*R*)-*tert*-butyl (1-(cyclopentylamino)-1-oxopropan-2-yl)carbamate (10): *N*-Boc-D-alanine (250 mg, 1.32 mmol) and HOBt (243 mg, 1.59 mmol) were added to a vial with stir bar and dissolved with dry DCM (6.6 mL)). Hunig's base (254 µL, 1.45 mmol) was then added, followed by cyclopentylamine (124 mg, 1.45 mmol), and EDC (304 mg, 1.59 mmol). The reaction was then stirred for 18 h, after which time LC-MS showed complete consumption of starting material and conversion to the desired product. The reaction was diluted with 1 M aq. HCl and EtOAc, then the phases were separated, and the combined organics were washed again with aq. HCl, then twice with half-saturated aq. $NaHCO_3$, then brine. The organics were dried over Na_2SO_4, filtered, and concentrated to yield the title compound as a white solid (311 mg, 92%). **^{1}H NMR (300 MHz, $CDCl_3$):** δ 6.11 (br s, 1H), 5.00 (br s, 1H), 4.19 (app sext, *J* = 6.6 Hz, 1H), 4.08 (m, 1H), 1.93 (m, 2H), 1.61 (m, 4H), 1.47 (s, 9H), 1.39 (m, 2H), 1.32 (t, *J* = 8.0 Hz, 3H); **MS** (ESI^+): 257.37 (M+H).



12

(*R*)-2-(cyclohexylamino)-*N*-cyclopentylpropanamide (12): (*R*)-*tert*-butyl 1-(cyclopentylamino)-1-oxopropan-2-ylcarbamate (310 mg, 1.21 mmol), DCM (6.0 mL), triethylsilane (0.97 mL, 6.1 mmol), and TFA (0.93 mL, 12.1 mmol) were added to a flask with stir bar and stirred for 18 h. The reaction was then quenched by pipetting the reaction solution slowly into a stirred half-saturated aqueous solution of $NaHCO_3$ (50 mL). The mixture was diluted with DCM, the layers were separated, and the organics were washed again with aq. $NaHCO_3$, then brine. The organics were dried over Na_2SO_4, filtered, and concentrated, yielding the intermediate primary amine as an off-white solid. This was immediately dissolved with methanol (6.1 mL) and cooled on ice. Cyclohexanone (0.38 mL, 3.6 mmol) was added, followed by sodium cyanoborohydride (760 mg, 12.1 mmol) added portionwise over 10 min. The reaction was stirred for 24 h, after which time LC-MS analysis showed complete conversion to the desired product. The reaction was concentrated to remove most of the MeOH, then it was diluted with half-saturated aq. $NaHCO_3$ and ether. The layers were separated, then the combined organics were washed again with $NaHCO_3$. Dilute aq. HCl was then added to bring the product into the aqueous layer, which was then washed twice with ether to remove the excess cyclohexanone. The aqueous layer was basified again w/ aq. $NaHCO_3$, then extracted twice with Et_2O. The ether layer was then washed with brine and dried over Na_2SO_4, filtered, and concentrated to provide the title compound as a white solid (239 mg, 83% over two steps). A portion of the material was converted to the hydrochloride salt by dissolving in ether and adding excess HCl in Et_2O, then concentrating. **^{1}H NMR (300 MHz, CD_3OD):** δ 4.13 (app p, *J* = 6.7 Hz, 1H), 3.95 (q, *J* = 6.9 Hz, 1H), 2.97 (m, 1H), 2.15 – 1.80 (comp, 6H), 1.80 – 1.55 (comp, 6H), 1.48 (d, *J* = 6.9 Hz, 3H), 1.40 – 1.15 (comp, 6H); **MS** (ESI^+): 239.68 (M+H).



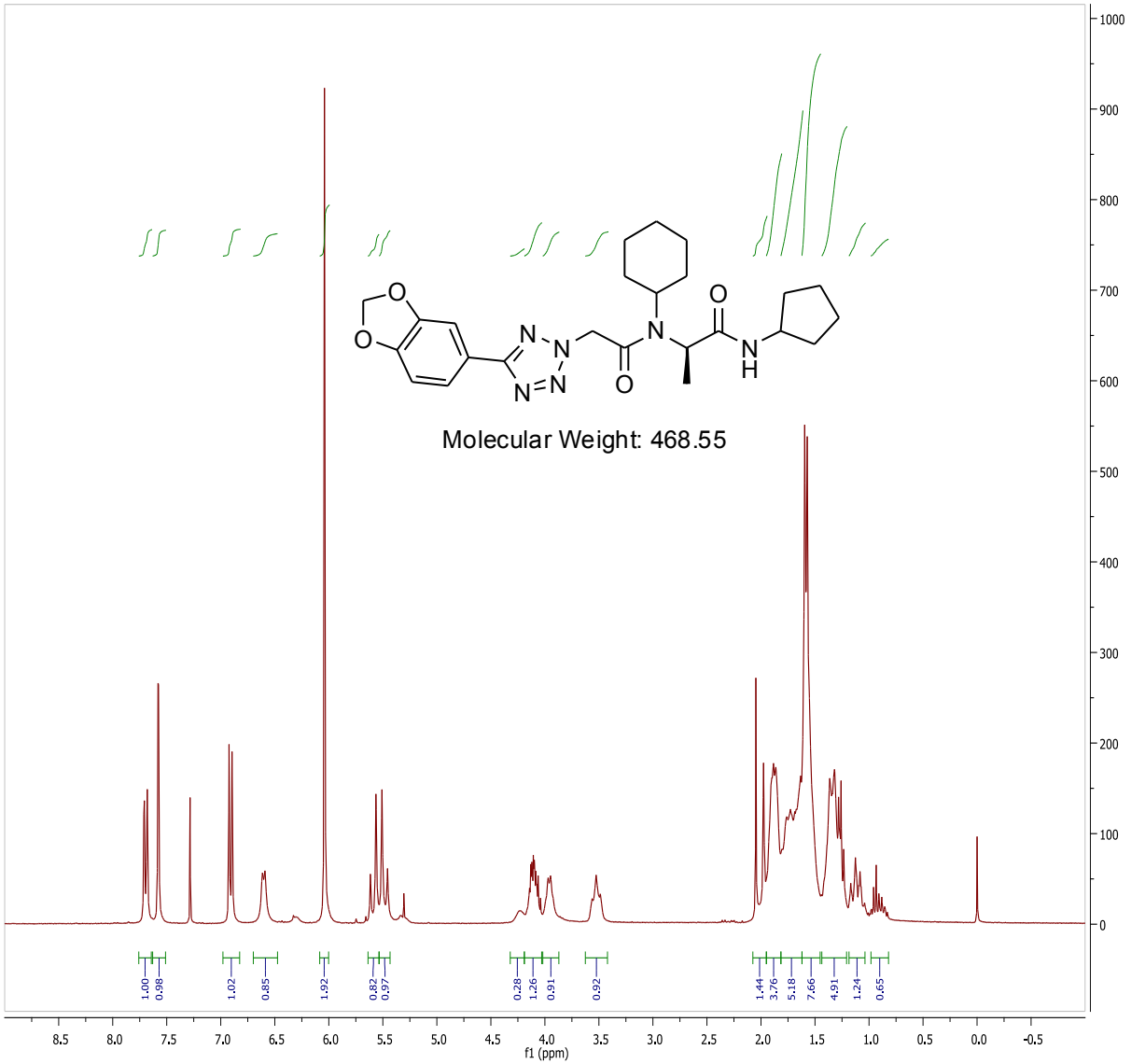
5e
ML279

(*R*)-2-(2-(5-(benzo[*d*][1,3]dioxol-5-yl)-2*H*-tetrazol-2-yl)-*N*-cyclohexylacetamido)-*N*-cyclopentylpropanamide (5e, ML279): 2-(5-(benzo[d][1,3]dioxol-5-yl)-2H-tetrazol-2-yl)acetic acid (92 mg, 0.37 mmol) was sealed under nitrogen in a vial with stir bar. Dry DCM (2.2 mL), oxalyl chloride (32 µL, 0.37 mmol), and DMF (1 drop from a 22-gauge needle) were added, and the reaction was vented to an oil bubbler. The reaction was stirred for 4 h, then (*R*)-2-

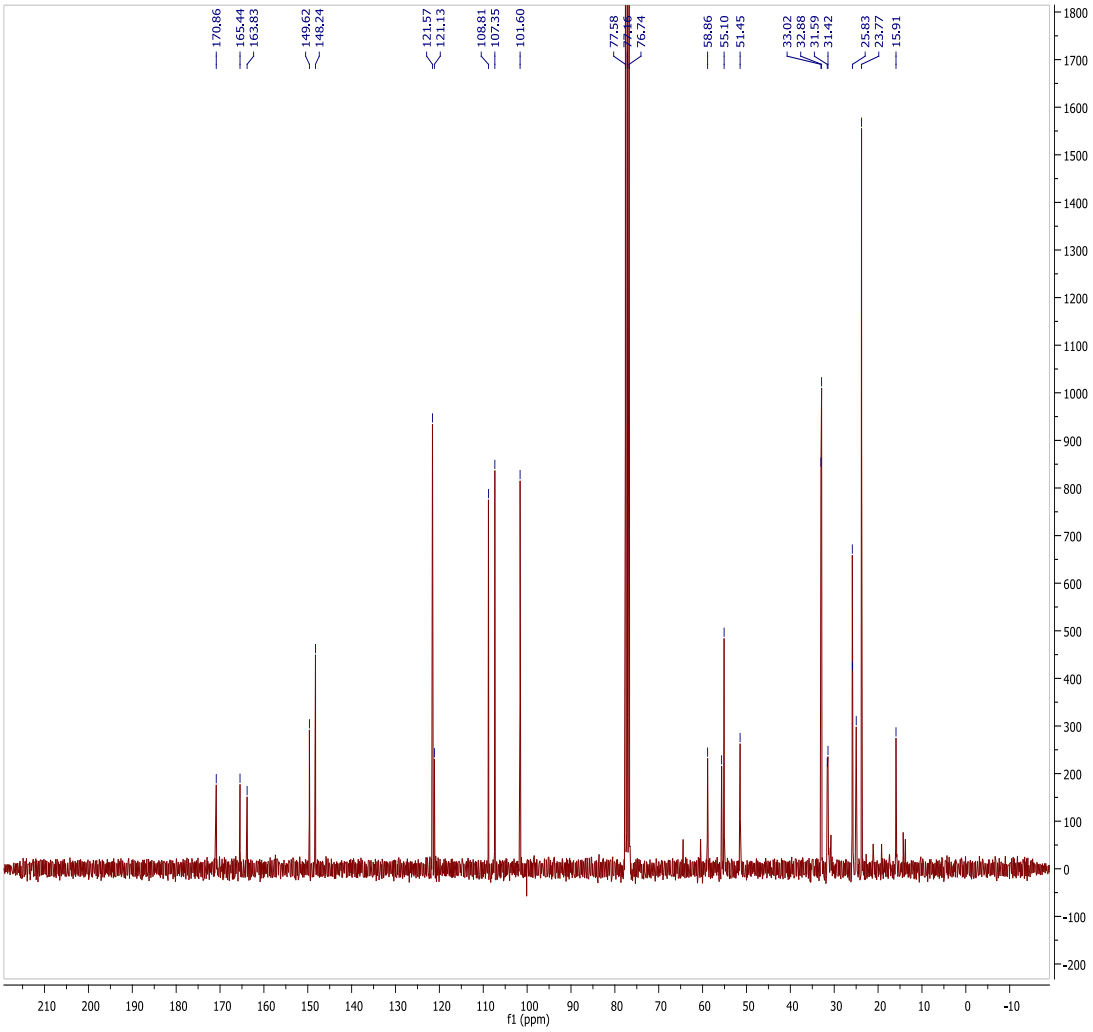
(cyclohexylamino)-*N*-cyclopentylpropanamide (free base, 80 mg, 0.34 mmol) was added to the resulting solution of acid chloride , followed by Hunig's Base (129 µl, 0.74 mmol) and DMAP (4.1 mg, 0.034 mmol). The reaction was stirred for 16 h, after which time LC-MS analysis showed complete conversion to the desired product. The reaction was diluted with 1 M aq. HCl and EtOAc (~25 mL each), the layers were separated, and the combined organics were washed again with aq. HCl, then twice with half-saturated aq. $NaHCO_3$, then brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated to a yellow oil, then purified by column chromatography (0 to 50% EtOAc/hexanes) to yield the title product as a pale yellow solid, after concentrating from an ether solution made cloudy by slowly adding pentane (90 mg, 72%). **^{1}H NMR (300 MHz, $CDCl_3$)** δ 7.70 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.58 (d, *J* = 1.4 Hz, 1H), 6.91 (d, *J* = 8.1 Hz, 1H), 6.60 (br d, *J* = 6.8 Hz, 1H), 6.04 (s, 2H), 5.59 (d, J = 15.4 Hz, 1 H), 5.48 (d, J = 15.4 Hz, 1H), 4.10 (m, 1H), 3.96 (br m, 1H), 3.52 (br app t, *J* = 11.4 Hz), 2.08 – 0.82 (comp, 21H) (peaks are broad due to rotamers); **^{13}C NMR (75 MHz, $CDCl_3$)** δ 170.9, 165.4, 163.8, 149.6, 148.2, 121.6, 121.1, 108.8, 107.4, 101.6, 77.6, 77.2, 76.7, 58.9, 55.7, 55.1, 51.5, 33.0, 32.9, 31.6, 31.4, 25.9, 25.8, 25.0, 23.8, 15.9 (extra peaks are due to the minor rotamer). **HRMS** (ESI^+): calculated for $C_{24}H_{33}N_6O_4$ [M+H] 469.2563, found 469.2552.

^{1}H NMR Spectrum (300 MHz, $CDCl_3$) of Probe 2 (ML279)

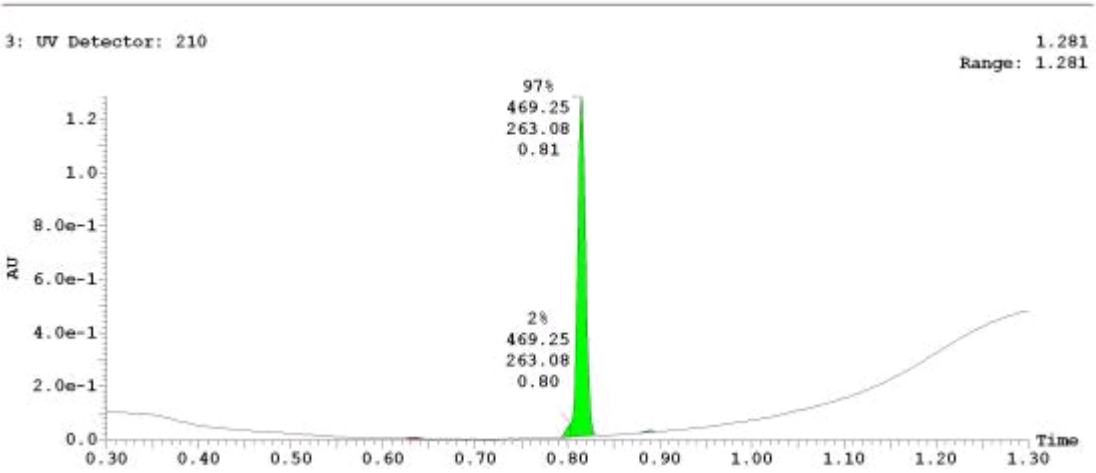
Broad peaks due to rotamers.



^{13}C NMR Spectrum (75 MHz, $CDCl_3$) of Probe 2 (ML279)



UPLC-MS Chromatogram of Probe 2 (ML279)



3. Compound profiling protocols

Solubility. Solubility was determined in phosphate buffered saline (PBS) pH 7.4 with 1% DMSO. Each compound was prepared in duplicate at 100 µM in both 100% DMSO and PBS with 1% DMSO. Compounds were allowed to equilibrate at room temperature with a 250 rpm orbital shake for 24 hours. After equilibration, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer. The DMSO samples were used to create a two-point calibration curve to which the response in PBS was fit.

Plasma Protein Binding. Plasma protein binding was determined by equilibrium dialysis using the Rapid Equilibrium Dialysis (RED) device (Pierce Biotechnology, Rockford, IL) for both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma (0.95% acetonitrile, 0.05% DMSO) and added to one side of the membrane (200 µl) with PBS pH 7.4 added to the other side (350 µl). Compounds were incubated at 37 ºC for 5 hours with a 250-rpm orbital shake. After incubation, samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

Plasma Stability. Plasma stability was determined at 37 ºC at 5 hours in both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma diluted 50/50 (v/v) with PBS pH 7.4 (0.95% acetonitrile, 0.05% DMSO). Compounds were incubated at 37 ºC for 5 hours with a 250-rpm orbital shake with time points taken at 0 hours and 5 hours. Samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

Microsomal Stability. Microsomal stability was determined by measuring degradation of the compound over 1-hour in human and/or mouse liver microsomes (Xenotech LLC, Lenexa, KS). Each compound was prepared in duplicate and added to a microsomal solution in PBS pH 7.4 so that the final concentration was 1 uM compound, 0.3 mg/mL microsomes, 0.5 mM NADPH and 1% DMSO. Compounds were incubated at 37ºC for 1 hour with 250 rpm orbital shake with time points taken at 0 hours and 1 hour. Samples were analyzed by UHPLC-MS (Agilent, Santa Clara, CA) with compounds detected on a time-of-flight mass spectrometer and analyzed using Find By Formula.

4. Representative dose-response curves for ML279

Figure S1. DiI-HDL uptake assay

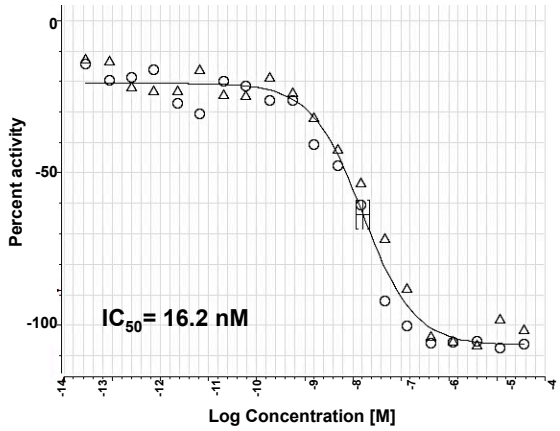
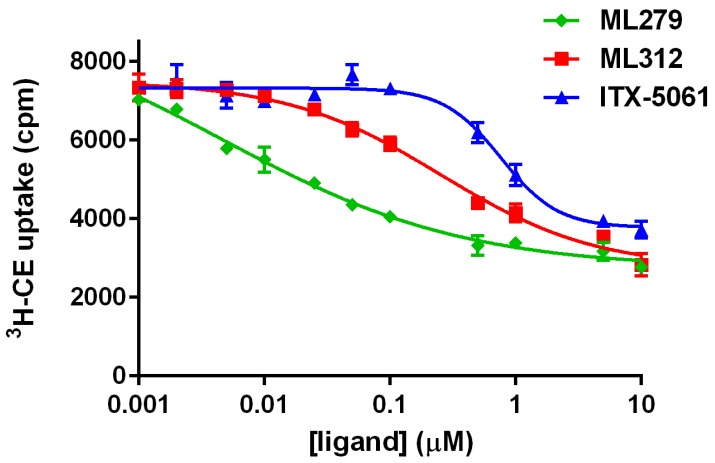
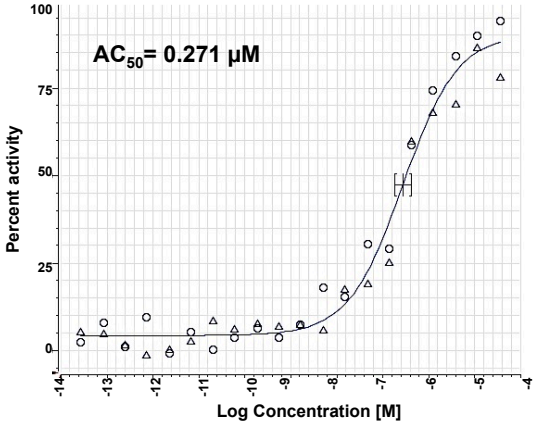


Figure S2. [^{3}H]cholesteryl ester uptake assay



Dose-response for [^{3}H]cholesteryl oleate ester uptake from HDL particles to ldlA[m-SR-BI] cells. Non-selective uptake (~5% of maximal uptake, see assay protocol) was quantified and subtracted from each uptake measurement prior to analysis. Points indicate the mean of n = 3, error bars indicate S.E.M. Uptake with no added inhibitor (ligand concentration = 0) was 8133 ± 134 cpm. IC_{50}s were determined and curves were plotted using non-linear regression analysis (4 parameters), using GraphPad Prism v. 6. IC_{50} = 0.005 μM (ML279); 0.25 μM (ML312); 0.77 μM (ITX-5061) in this assay.

Figure S3. HDL binding assay



5. Assay protocols

Materials and Reagents

- DiI-HDL, custom purified HDL particles derived from human blood were prepared and labeled with 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (DiI; Catalog No. D-282; Invitrogen, Carlsbad, CA). See section 5e) for details.
- Alexa 488 HDL, human HDL particles labeled with the Alexa Fluor® 488 Protein Labeling Kit (Catalog No. A-10235, Invitrogen; Carlsbad, CA) were purified and labeled by the Assay Provider.
- CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega (Catalog No. G7573; Madison, WI).
- Radiolabeled cholesterol [1,2-^{3}H(N)]-, 1 mCi (37 MBq) was obtained from PerkinElmer – NEN (Catalog No. NET139001MC; Waltham, MA).
- Alexa Fluor-594 conjugated human transferrin (Catalog No. T-13343) was obtained from Invitrogen.

Cell Lines

The following cell lines were used in this study:

- ldlA[mSR-BI] is a Chinese hamster ovary (CHO) cell line that overexpresses murine SR-BI, isoform 1 (NP_058021) and lacks the LDL receptor was obtained from the Assay Provider (Krieger Laboratory). This cell line was used for the primary assay and several secondary assays. A variant of this cell line expressing mutant SR-BI, where a cysteine required for interaction with BLT-1 is mutated to serine (C384S SR-BI), was used in several secondary assays.

- [ldlA7] is the parental cell line to ldlA[mSR-BI] cells, and does not overexpresses SR-BI, and can be used to rule out compound activity independent of SR-BI.

a) DiI-HDL Uptake Assay

ldlA[mSR-BI] cells were plated into 384-well plates at 30 µl per well and incubated overnight. As a measurable surrogate for cholesterol uptake, human HDL particles were treated with the lipophilic fluorescent dye DiI and exposed to ldlA[mSR-BI] cells in lipoprotein-free media (Ham's F12/0.5% fatty acid-free Bovine Serum Albumin [BSA]/25 mM HEPES pH 7.4 plus 10 µg protein/ml DiI-HDL). Cells took up the Dil via SR-BI over 3 hours in the presence of compound. After significant uptake of the DiI, the cells became fluorescent. The level of fluorescence correlates with the amount of Dil uptake and can be measured with a standard plate reader. The uptake of lipid (represented by DiI) was inhibited by the compound BLT-1 or with an excess of HDL untreated with DiI.

The ldlA[mSR-BI] cells used in the assay were a CHO cell line lacking expression of the LDL receptors and overexpressing the Scavenger receptor (SR-BI). Inhibitors of SR-BI and HDL-mediated uptake lead to a reduction in fluorescence in this assay. Fluorescence was measured using a PerkinElmer EnVision plate reader. Primary HTS data were analyzed in Genedata Screener Assay Analyzer, and were normalized against DMSO and 1 µM BLT-1 (positive control). For the HTS, the average of two replicates was used to rank order activity and to choose compounds for retests. For dose studies, the curves were generated with Genedata Condeseo and showed percent (%) activity for the individual doses.

Detailed protocol

DiI-HDL Labeling Mix
2200 ml of Ham's/0.5% BSA/25 mM HEPES pH 7.4
DiI-HDL to 10 ug protein/ml final concentration (Stocks vary from 2.81-4 mg/mL)

Ham's/0.5% BSA/25 mM HEPES Assay Media (1 liter)
5 g BSA powder (Sigma fatty acid-free)
1 Liter Ham's F12 base media
25 ml 1 M HEPES pH 7.4

ldlA[mSR-BI] Chinese Hamster Ovary (CHO) cells were maintained in Ham's F12K (Cellgro Catalog No. 10-080-CV)/10% Fetal Calf Serum (Hyclone Catalog No. SH30071.03, Lot No. ATG32533)/ 1x Penicillinstreptomycin-L-Glutamine (Gibco Catalog No.10378-016)/200 ug/mL Geniticin (Gibco Catalog No.10131-027, Lot No.802967). Cells were fluid changed every 2 days and/or split upon reaching 80% confluency. For the primary HTS, cells were thawed at 6 million cells per Falcon T225 flask. After 2 days, the cells were passed to a Corning Hyperflask and plated after 3 days in the Hyperflask with a fluid change the day prior to plating.

On Day 0:

1. Plate 10,000 ldlA[mSR-BI] cells in 30 uL per well in Ham's F12K (Cellgro Catalog No. 10-080-CV)/5% Fetal Calf Serum (Hyclone Catalog No. SH30071.03, Lot No. ATG32533)/ 1x Penicillinstreptomycin-L-Glutamine (Gibco Catalog No. 10378-016) with a Thermo Combi Multi-drop fluid handler.

2. Use Aurora black 384-well, square, clear-bottomed, image-quality plates (Aurora Catalog No. 1022-11330) for the assay.

On Day 1:

1. Remove media with aspirator (Bio-Tek ELX405 plate washer).
2. Add 30 uL Ham's F12/0.5% Bovine Serum Albumin (fatty acid-free) /25 mM HEPES pH 7.4 (Invitrogen) + 10 ug protein/ml DiI-HDL with Thermo Combi fluid handler (slow speed setting).
3. Pin transfer 100 nL compounds and positive control (1 uM BLT-1). **It is recommended that low solubility compounds be sonicated in DMSO to ensure complete dissolution prior to preparing stock solutions.**
4. Incubate 3 hours @ 37°C in humidified cell culture incubator.
5. Remove media with aspirator.
6. Wash twice with 30 uL PBS (+Ca and Mg) using the Thermo Combi on slow speed setting.
7. Analyze DiI-HDL uptake with Perkin-Elmer EnVision plate reader (Bodipy TMR mirror #405, excitation filter is Photometric 550 (#312) and emission filter is Cy3 595 (#229) with bottom read.

b) HDL Binding Assay

HDL binding was assessed using Alexa Fluor 488-labeled HDL particles. For this assay, the Alexa 488 dye was covalently bound to apolipoprotein components of the HDL particle via primary amines; thus, no transfer of the fluorophore to cell membranes occurs. In this manner, direct binding of the HDL particles to SR-BI can be measured. As a positive control, BLT-1 was used at 1 μM, which is known to increase binding of HDL to SR-BI. It is possible that a compound can reduce binding of HDL to the receptor, and this would lead to a decrease in signal. This assay is used to characterize the mechanism of action of a particular compound; therefore, any outcome in the assay is acceptable. Data were normalized against DMSO and BLT-1 (positive control) wells in Genedata Assay Analyzer. Curves were generated with Genedata Condeseo and showed percent (%) activity for the individual doses.

Detailed protocol

ldlA[mSR-BI] Chinese Hamster Ovary (CHO) cells were maintained in Ham's F12K (Cellgro Catalog No. 10-080-CV)/10% Fetal Calf Serum (Hyclone Catalog No. SH30071.03, Lot No. ATG32533)/ 1x Penicillin-streptomycin-L-Glutamine (Gibco 10378-016)/200 ug/mL Geniticin (Gibco Catalog No.10131-027, Lot No.802967). Cells were fluid changed every 2 days and/or split upon reaching 80% confluency.

<u>On Day 0:</u>

1. Plate 10,000 ldlA[mSR-BI] cells in 30 uL per well in Ham's F12K (Cellgro Catalog No. 10-080-CV)/5% Fetal Calf Serum (Hyclone Catalog No. SH30071.03, Lot No. ATG32533)/ 1x Penicillin-streptomycin-L-Glutamine (Gibco 10378-016).

2. Use Aurora black, 384-well, square, clear-bottomed image-quality plates (Aurora Catalog No.1022-11330) for the assay.

On Day 1:

1. Remove media with aspirator (ELX405 plate washer).
2. Add 30 uL Ham's F12/0.5% Bovine Serum Albumin (fatty acid-free) (Sigma Catalog No. A8806-5G) /25 mM HEPES pH 7.4 (Invitrogen) plus 10 ug/mL Alexa 488-HDL with Thermo Combi fluid handler (slow speed setting).
3. Pin transfer 100 nL compounds and positive control (1 uM BLT-1).
4. Incubate 3 hours @ 37°C in a humidified cell culture incubator.
5. Remove media with aspirator.
6. Wash twice with 30 uL PBS (+Ca and Mg) using the Thermo Combi on slow speed setting.
7. Analyze Alexa-488-HDL binding with Perkin-Elmer EnVision plate reader (FITC mirror #403) with bottom read

c) Cholesterol Ester Uptake Assay

The goal of this assay is to verify compounds that disrupt the binding of HDL particles to SR-BI scavenger receptor using an alternative means of labeling cholesteryl esters and avoiding any type of fluorescence measurement. To measure this binding event, HDL particles are loaded with [^{3}H]cholesteryl oleate ester and added to mSR-BI cells. Cells take up HDL and the [^{3}H]cholesteryl ester via the SR-BI scavenger receptor in 2 to 3 hours. After significant uptake of the HDL, the radiolabel can be detected by liquid scintillation counting. The level of radioactivity correlates with the amount of [^{3}H]cholesteryl ester uptake. The uptake can be inhibited by the compound BLT-1 or when co-treated with an excess of unlabeled HDL. The ldlA[mSR-BI] cells utilized in the assay are a CHO cell line lacking expression of the LDL receptors and overexpressing the scavenger receptor, SR-BI. Inhibitors of SR-BI and HDL uptake will have a reduction in liquid scintillation counts.

Detailed protocol

On Day 0:

1. Plate 50,000 ldlA[mSR-BI] cells in 1 mL per well in Ham's F12K/5% Fetal Calf Serum/ 1x Penicillin-streptomycin-L-Glutamine/G418.

On Day 2:

2. Wash cells with prewarmed (37°C) Ham's F12K medium plus 0.5% (wt/vol) fatty acid free BSA prior to adding radioactive lipoprotein.
3. Preincubate cells with 200 uL of inhibitors at indicated concentration or non-labeled HDL (at a 40 fold excess for measurement of non-specific activity) in Ham's F12/0.5% BSA/0.5%DMSO (vol/vol) for 1 hr at 37°C in a humidified cell culture incubator. It is recommended that low solubility compounds be sonicated in DMSO to ensure complete dissolution prior to preparing stock solutions.
4. Add 50 uL Ham's F12/0.5% BSA/0.5%DMSO plus 50 ug/mL [^{3}H]CE-HDL to reach a final concentration of 10 ug/mL and incubate 2 hours at 37°C in a humidified cell culture incubator
5. Remove the medium at the end of incubation at 4C
6. Wash the plates 2X with Tris-BSA buffer and 1X Tris-HCl buffer.
7. Add 1 ml isopropanol, and incubate for 20 min at 4C.
8. Collect 1 ml isopropanol/lysate into counting tube, add 4 ml liquid scintillation buffer, and prepare for counting.
9. Specific uptake is the difference between total and nonspecific activities.
10. Data were analyzed using Prism 6 (GraphPad Software).
11. All calculated errors represent standard errors of the mean.

e) HDL isolation and labeling

Human HDL was isolated from donors by density gradient ultracentrifugation and labeled with [^{3}H]cholesteryl ester ([^{3}H]CE, [^{3}H]CE-HDL) as described previously.[1,2,3,4,5]

6. References

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