Supporting Information

Discovery of Bispecific Antagonists of Retinol Binding Protein 4 That Stabilize Transthyretin Tetramers: Scaffolding Hopping, Optimization, and Preclinical Pharmacological Evaluation as a Potential Therapy for Two Common Age-Related Comorbidities

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Table of Contents

RBP4 *In Vitro* Assay Information

***In vitro* binding of compounds to RBP4.** Compound binding to RBP4 was assessed in the radiometric scintillation proximity (SPA) assay that was previously described.[18, 19, 26] The assay measured competitive displacement of radiolabeled retinol from native RBP4 purified from human urine (Fitzgerald, 30R-AR022L). The protein was biotinylated using the EZ-link Sulfo-NHS-LC-Biotinylation kit from ThermoFisher (Cat #21335) as recommended by the manufacturer. Binding assays were implemented in a final volume of 100 µL in SPA buffer (1 X PBS, pH 7.4, 1 mM EDTA, 0.1% BSA, 0.5% CHAPS). The assay reaction included a radioligand, 10 nM ^{3}H-retinol (48.7 Ci/mmol; PerkinElmer, Waltham, MA), along with the 0.3 mg/well Streptavidin-PVT beads (PerkinElmer, RPNQ0006) and 50 nM biotinylated human RBP4. Unlabeled retinol (Sigma, cat # 95144) at 20 µM was added to control wells to assess a nonspecific binding. Radioactivity counts were measured using CHAMELEON plate reader (Hidex Oy, Turku, Finland) after 16 h of incubation at rt with mild shaking.

Assessment of antagonistic activity in the HTRF RBP4-TTR interaction assay. The ability of analogues to act as antagonists of all-*trans*-retinol-dependent RBP4-TTR interaction was measured in the HTRF (Homogenous Time-Resolved Fluorescence) assay as we described previously.[18, 19, 26] Untagged TTR (Calbiochem, cat #529577) and Maltose-Binding Protein-tagged RBP4 expressed in *E. coli* were used in this assay. HTRF Cryptate labeling kit from CisBio (Cisbio, cat #62EUSPEA, Bedfored, MA) was used to label TTR with Eu^{3+} Cryptate. The assay was performed in a final assay volume of 16µl in the buffer that contained 10 mM Tris-HCl pH 7.5 , 1 mM DTT, 0.05% NP-40, 0.05% Prionex, 6% glycerol, and 400 mM KF. Other components of the reaction mix included 60 nM MBP-RBP4, 5 nM TTR-Eu, 26.7 nM of anti-MBP antibody conjugated with d2 (Cisbio, cat #61MBPDAA), and 1 µM all-*trans* retinol (Sigma, cat # 95144). All the reactions were performed under dim red light in the dark. The plates were read in the SpectraMax M5e Multimode Plate Reader (Molecular Devices, Sunnyvale, CA) after the overnight incubation at 4 ^{o}C. Fluorescence was excited at 337 nm; emission was measured at 668 and 620 nm with 75 µs counting delay. The HTRF signal was expressed as the ratio of fluorescence intensity: Flu_{668}/Flu_{620} X 10,000.

TTR *In Vitro* Assay Information

Fluorescence Polarization TTR Tetramer Binding Assay. Compound binding to TTR was assessed in the fluorescence polarization assay. The assay measured competitive displacement of the fluorescent probe, FITC-diclofenac, from TTR isolated from human plasma (Clabiochem-Millipore, cat. No. 52957). FITC-diclofenac was synthesized at LeadGen Labs, LLC following the published procedure.[59] Each well contained 200 nM TTR and 100 nM FITC-diclofenac in the FP buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.01% CHAPS, 0.01%Prionex) along with test compounds. Nonspecific binding was determined in the presence of 500 µM unlabeled diclofenac (Sigma-Aldrich). Reactions with test compounds were incubated overnight at +4 ^{o}C and FP was measured on SpectramaxM5e plate reader (Molecular Devices).

TTR Aggregation Assay. The ability of test compounds to prevent TTR aggregation was evaluated under the acidic conditions that favor TTR aggregation and fibril formation. A 2 µl solution of 167 µM human TTR (ACROBiosystems #H5223) was incubated with 7 µl 50 mM sodium acetate pH 4.0 (Sigma # S7545), 100 mM KCl (Sigma # S5405) in the presence or absence of 1 µl TTR inhibitor for 72 h at 37 °C. At the end of the incubation, 3.5 µl 500 mM sodium phosphate (Sigma #S5136) buffer pH = 8.0 was added to each sample for neutralization and 0.6 µl 5% CHAPS (Sigma #C5070) as a detergent to prevent reassociation of protein. The cross-linking was performed by adding 1.5 µl 5% glutaraldehyde solution (Sigma# G6257). After 4 min, the reaction was stopped by the addition of 2.5 µl freshly made 5% NaBH4. Samples were subjected to TTR western blotting with prealbumin antibodies (1:500; Dako #A0002). Band intensity for TTR monomer and TTR aggregates was quantified from scanned images of the blots.

Mouse PK Study Information and Data

Drug naïve adult male CD-1 mice were administered a single dose administration of the test article by intravenous (IV) or oral gavage (PO) dose routes.

Testing Facility and Test Site: Absorption Systems, LLC, 436 Creamery Way, Suite 600, Exton, PA 19341–2556.

TEST ARTICLE AND VEHICLE INFORMATION:

IV dosing vehicles: 3% DMA/45% PEG300/12% ethanol/40% sterile water

PO dosing vehicle: 2% Tween 80 in 0.9% saline

Dose formulation: The dose formulation was prepared by the step-wise addition (in the order listed) of the individual components of the vehicle to a weighed quantity of test compound in a volume that yielded the desired final concentration. Each formulation was prepared by mixing a weighed quantity of test compound with the appropriate volume of vehicle.

Dosing Solution Analysis: The dosing solutions were analyzed by LC-MS/MS. The measured dosing solution concentrations are shown in Table 1. The dosing solutions were diluted into mouse blood and analyzed in triplicate. All concentrations are expressed as mg/mL of the free base. The nominal dosing level was used in all calculations for Group 1.

TEST SYSTEM:

Species and strain: mouse; male CD-1

Mean weight: 0.034 kg for the IV arm; 0.027 kg for PO arm

Number: 3 animals total (same three animals used for each dosing group (Group 1 (IV) and Group 2 (PO))

COMPLIANCE: This non-clinical study followed established practices and standard operating procedures of Absorption Systems as well as the study protocol. This study was exploratory in nature and was not conducted in accordance with the principles set forth in the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 Code of Federal regulations (CFR) Part 58. The report is archived in a validated scientific data management system. Electronic signatures comply with the regulation 21 CFR Part 11.

EXPERIMENTAL DESIGN:

Blood was collected from mice at pre-dose and at 5, 15 and 30 min, and 1, 2, 4, 8, 12, 24, and 48 h post-dose. Hemolyzed blood samples were extracted by protein precipitation using acetonitrile. Following protein extraction with acetonitrile, compound levels were measured by LC-MS/MS. Pharmacokinetic parameters were calculated from the time course of the blood concentrations. Pharmacokinetic parameters were determined with Phoenix WinNonlin (v8.0) software using a non-compartmental model. The maximum blood concentrations (C_0) after IV dosing were estimated by extrapolation of the first two time points back to t = 0. The maximum blood concentration (C_{max}) and the time to reach maximum blood concentration (t_{max}) after PO dosing were observed from the data. The area under the time concentration curve (AUC) was calculated using the linear trapezoidal rule with calculation to the last quantifiable data point, and with extrapolation to infinity if applicable. Blood half-life ($t_{1/2}$) was calculated from 0.693/slope of the terminal elimination phase. Mean residence time, MRT, was calculated by dividing the area under the moment curve (AUMC) by the AUC. Clearance (CL) was calculated from dose/AUC. Steady-state volume of distribution (V_{ss}) was calculated from CL*MRT.

Bioavailability was determined by dividing the individual dose normalized PO AUC∞ values by the average dose-normalized IV AUC∞ value. Any samples below the limit of quantitation (1.00 ng/mL) were treated as zero for pharmacokinetic data analysis.

Individual and average blood concentrations are shown in Supplementary Table 1 and Table 2. All data are expressed as ng/mL of the free base. Samples that were below the limit of quantification (1.00 ng/mL) were not used in the calculation of averages. Average concentrations versus time data are plotted in Figure 1 and Figure 2.

Supplementary Table S-1. Individual and average blood concentrations (ng/mL) and pharmacokinetic parameters for (±)-**44** (ACPHS-52-3) after intravenous (IV) administration at 2 mg/kg in male CD-1 mice.

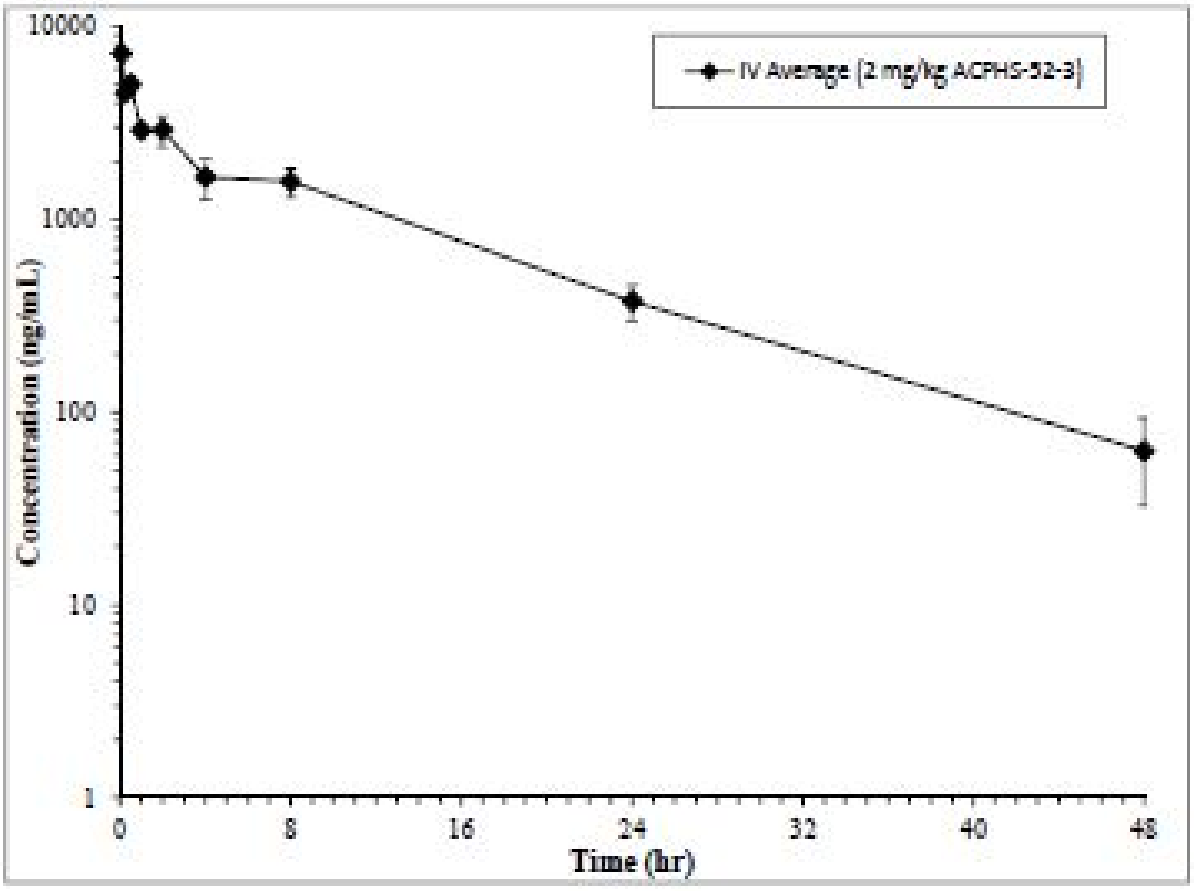
Intravenous (2 mg/kg)					
Time (hr)	Mouse #				
	507	508	509	Mean	SD
0 (pre-dose)	BLOQ	BLOQ	BLOQ	ND	ND
0.083	7030	7680	6980	7230	391
0.25	4610	4620	4350	4527	153
0.50	4720	5430	4890	5013	371
1.0	2950	3030	2690	2890	178
2.0	2950	3380	2340	2890	523
4.0	**1930**	**1830**	1200	1653	396
8.0	**1280**	**1790**	**1640**	1570	262
24	**310**	**354**	**461**	375	77.7
48	**28.8**	**73.3**	**86.4**	62.8	30.2
Animal Weight (kg)	0.033	0.032	0.036	0.034	0.002
Volume Dosed (mL)	0.07	0.06	0.07	0.07	0.01
C_0 (ng/mL)[1]	8670	9887	8829	9129	661
$t_{1/2}$ (hr)	7.29	9.06	9.46	8.60	1.15
MRT_{last} (hr)	8.48	9.08	10.7	9.43	1.17
CL (L/hr/kg)	0.0555	0.0454	0.0488	0.0499	0.00511
V_{ss} (L/kg)	0.494	0.464	0.596	0.518	0.0692
AUC_{last} (hr-ng/mL)	35743	43062	39764	39523	3665
AUC_{∞} (hr-ng/mL)	36046	44020	40943	40336	4021
Dose-normalized Values[2]					
AUC_{last} (hr-kg-ng /mL/mg)	17871	21531	19882	19761	1833
AUC_{∞} (hr-kg-ng /mL/mg)	18023	22010	20471	20168	2011

C_0: maximum blood concentration extrapolated to t=0; t_{max}: time of maximum blood concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; CL: clearance; V_{ss}: steady state volume of distribution; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity; ND: not determined; BLOQ: below the limit of quantitation (1.00 ng/mL).

[1]Extrapolated to t=0.

[2]Dose-normalized by dividing the parameter by the nominal dose in mg/kg.

Figure S-1. Average blood concentrations of (±)-**44** (ACPHS-52-3) after IV administration at 2 mg/kg in male CD-1 mice.



Supplementary Table S-2. Individual and average blood concentrations (ng/mL) and pharmacokinetic parameters for (±)-**44** (ACPHS-52-3) after oral (PO) administration at 5 mg/kg in male CD-1 mice.

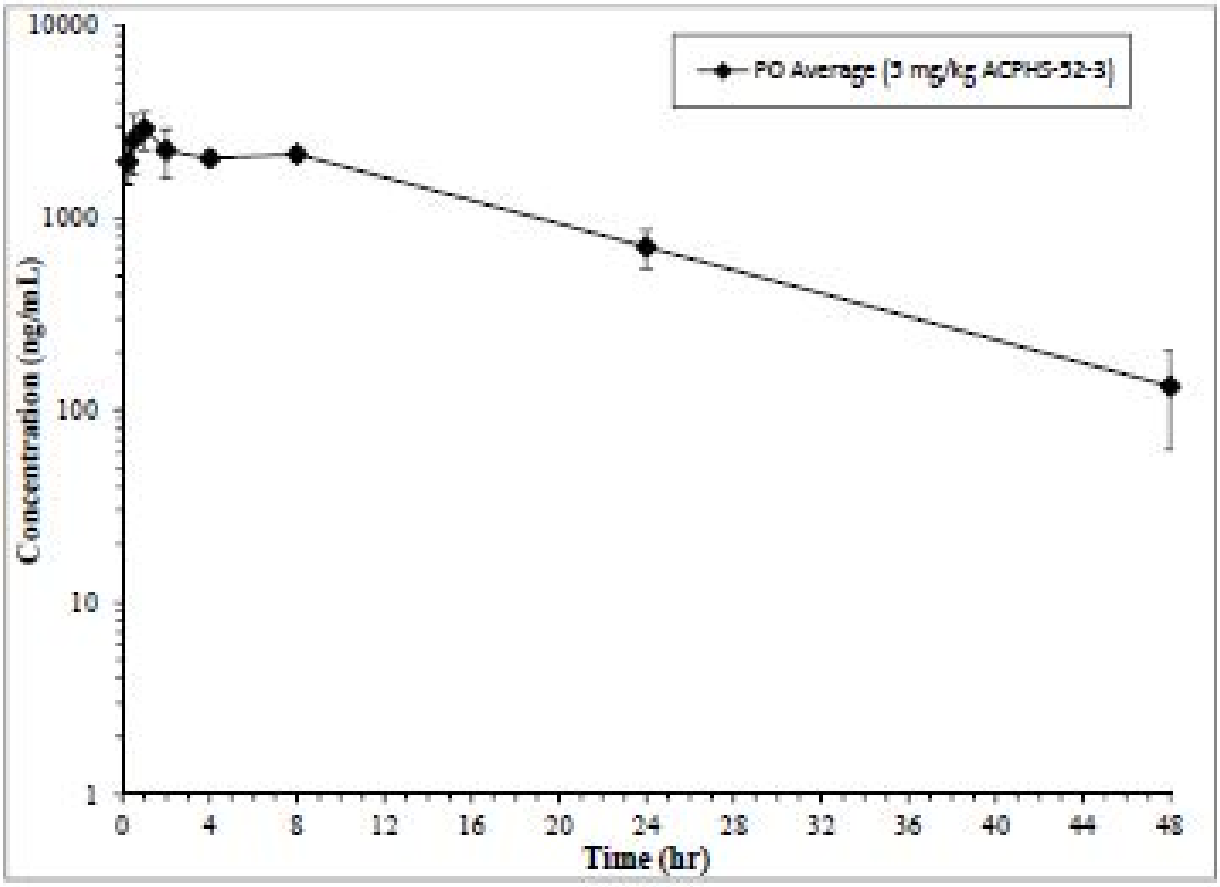
Oral (5 mg/kg)					
Time (hr)	Mouse #				
	510	511	512	Mean	SD
0 (pre-dose)	BLOQ	BLOQ	BLOQ	ND	ND
0.25	1730	2500	1650	1960	469
0.50	1550	2920	3210	2560	887
1.0	2270	3620	2860	2917	677
2.0	2260	2880	1610	2250	635
4.0	2050	2220	1870	2047	175
8.0	**1990**	**2140**	**2320**	2150	165
24	**634**	**891**	**597**	707	160
48	**120**	**210**	**70.1**	133	70.9
Animal Weight (kg)	0.027	0.027	0.027	0.027	0.000
Volume Dosed (mL)	0.14	0.14	0.14	0.14	0.00
C_{max} (ng/mL)	2270	3620	3210	3033	692
t_{max} (hr)	1.0	1.0	0.50	0.83	0.29
$t_{1/2}$ (hr)	9.88	11.9	7.91	9.90	2.00
MRT_{last} (hr)	12.2	13.3	11.4	12.3	0.957
AUC_{last} (hr·ng/mL)	46276	57155	47767	50400	5898
AUC_{∞} (hr·ng/mL)	47987	60763	48567	52439	7214
Dose-normalized Values[1]					
AUC_{last} (hr·kg·ng /mL/mg)	9255	11431	9553	10080	1180
AUC_{∞} (hr·kg·ng /mL/mg)	9597	12153	9713	10488	1443
Bioavailability (%)[2]	47.6	60.3	48.2	52.0	7.15

C_{max}: maximum blood concentration; t_{max}: time of maximum blood concentration; $t_{1/2}$: half-life, data points used for half-life determination are in bold; MRT_{last}: mean residence time, calculated to the last observable time point; AUC_{last}: area under the curve, calculated to the last observable time point; AUC_{∞}: area under the curve, extrapolated to infinity; ND: not determined; BLOQ: below the limit of quantitation (1.00 ng/mL).

[1]Dose-normalized by dividing the parameter by the nominal dose in mg/kg.

[2]Bioavailability determined by dividing the individual dose-normalized oral AUC_{∞} values by the average dose-normalized IV AUC_{∞} value.

Figure S-2. Average blood concentrations of (±)-**44** (ACPHS-52-3) after PO administration at 5 mg/kg in male CD-1 mice.



Animal Care and Use Statement: All procedures in this protocol are in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 1996; and the National Institutes of Health, Office of Laboratory Animal Welfare. Whenever possible, procedures in this study are designed to avoid or minimize discomfort, distress, and pain to animals.

Serum RBP4 Measurement Information

Blood samples were collected from a tail vein. Whole blood was drawn into a centrifuge tube and was allowed to clot at rt for 30 min followed by centrifugation at 2000*g* for 15 min at 48 °C to collect serum. Aliquots of plasma samples collected in the mouse pharmacokinetic study were analyzed for the RBP4 concentration using the RBP4 (mouse/rat) dual ELISA kit (AdipoGen, San Diego, CA) following the manufacturer's instructions. In adi-hRBP4 transgenic mouse experiments, blood samples were collected from a tail vein. Whole blood was drawn into a centrifuge tube and was allowed to clot at rt for 30 min followed by centrifugation at 2000*g* for 15 min at +4 ˚C to collect serum. Mouse serum RBP4 (produced predominantly in the liver) was measured using the RBP4 (mouse/rat) dual ELISA kit (AdipoGen, San Diego, CA; catalog number AG-45A-0012YTP-KI01)

Animal Care and Use Statement: All procedures in this protocol are in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (9 CFR Parts 1, 2, and 3); the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Academy Press, Washington, D.C., 1996; and the National Institutes of Health, Office of Laboratory Animal Welfare. Whenever possible, procedures in this study are designed to avoid or minimize discomfort, distress, and pain to animals.

In Vitro ADME Assay Information

Solubility

Kinetic Solubility in PBS:
Kinetic aqueous solubility determination for compound (±)-**44** in PBS (pH 7.4) was conducted by Eurofins using UV detection (230 nm). Aqueous solubility (μM) was determined by comparing the peak area of the principal peak in a calibration standard (200 μM) containing organic solvent (methanol/water, 60/40, v/v) with the peak area of the corresponding peak in a buffer sample. In addition, chromatographic purity (%) was defined as the peak area of the principal peak relative to the total integrated peak area in the HPLC chromatogram of the calibration standard. A chromatogram of the calibration standard of each test compound, along with a UV/VIS spectrum with labeled absorbance maxima, was generated.

Standards for the kinetic solubility study:

Metoprolol - 192.6 μM

Rifampicin - 200 μM
Ketoconazole - 152.8 μM
Phenytoin - 101.8 1 μM
Simvastatin - 14.2 μM
Diethylstilbesterol - 7.0 μM
Tamoxifen - 1.9 μM

CYP450 Inhibition

Inhibition potential (IC_{50} values) results for compound (±)-**44** against the human cytochrome P450 (CYP) isoforms 2C9, 2C19, 2D6, and 3A4. Each recombinant human CYP isoform was tested with a standard positive and negative control, using fluorometric detection for measuring CYP activity. The measured IC_{50} values for the respective standard inhibitors were all within expected ranges for each isoform (see below).

IC_{50} Concentrations of Standard CYP Inhibitors:
CYP Inhibitor IC_{50} (μM):
2C9 Sulfaphenazole IC_{50} = 3.4 μM
2C19 Tranylcypromine IC_{50} = 2.8 μM
2D6 Quinidine IC_{50} = 0.058 μM
3A4 Ketoconazole IC_{50} = 0.0084 μM

Pre-formulated NADPH regenerating solutions, recombinant CYP isoforms 2C19 and 3A4 (Lot # 3007790 and 2276593 respectively), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), 3-cyano-7-ethoxycoumarin (CEC) and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) were obtained from Corning Life Sciences (Bedford, MA). Recombinant CYP isoform 2D6 (Lot # 49242) was obtained from Invitrogen (Carlsbad, CA). CYP isoform 2C9 (Lot # 0446966-1) was obtained from Cayman Chemical (Ann Arbor, MI). 7-methoxy-4-trifluoromethylcoumarin (MFC), trans-2-phenylcyclopropylamine HCl (TCP), sulfaphenazole (SFZ), ketoconazole (KTZ) and quinidine (QDN) were obtained from Sigma (St. Louis, MO). All solvents and buffers were obtained from commercial sources and used without further purification.

Methods:
Test compound was prepared as a 10 mM stock solution in acetonitrile. Four human P450 isoforms cDNA-expressed in insect cell microsomes (CYP2C9, CYP2C19, CYP2D6, and CYP3A4) were tested for inhibition by test compound using fluorescence-based assays. Nine serial dilutions (concentrations from 0–100 μM) using each test compound stock solution were prepared in black microtiter plates, in duplicate. This dilution series was incubated at 37 °C with the individual CYP isoforms and a standard fluorogenic probe substrate for each respective isoform. The concentration of the probe substrate added was at or near the Km value for each CYP isoform. Reaction mixtures contained potassium phosphate buffer, pH 7.4 and the NADPH-

regenerating system. The final reaction volume was 0.20 mL and the reaction was terminated with 75 µL of stop solution (0.5 M Tris base in acetonitrile) after the appropriate incubation time (15-45 min). Fluorescence measurements were made at the appropriate excitation and emission wavelengths. Duplicate control wells with no test compound, duplicate blank wells containing stop solution prior to adding isoform, and a dilution series in duplicate containing a standard inhibitor for each isoform were also conducted. IC50 values were calculated using a non-linear regression of the data using the four-parameter logistic model (dose response equation) fit with XLFit 5.2 from IDBS Software (Emeryville, CA), supported by linear interpolation of data points at concentrations indicating inhibition levels approximately 50% of the uninhibited rate.

% Plasma Protein Binding

Plasma protein binding (PPB) for compounds determination for compound (±)-**44** in PBS (pH 7.4) was conducted by Eurofins using equilibrium dialysis of plasma with HPLC-UV/Vis detection.

Mean Plasma Protein Binding of Control Propranolol in Human, Rat (Sprague Dawley), Mouse (CD-1), and Dog (Beagle) Plasma

The peak areas of the test compound in the buffer and test samples were used to calculate percent binding and recovery according to the following formulas:

$$\text{Protein binding}(\%) = \frac{\text{Area}_p - \text{Area}_b}{\text{Area}_p} * 100$$

$$\text{Recovery}(\%) = \frac{\text{Area}_p + \text{Area}_b}{\text{Area}_c} * 100$$

Where:
Area_p = Peak area of analyte in protein matrix
Area_b = Peak area of analyte in buffer
Area_c = Peak area of analyte in control sample

Metabolic Stability

Metabolic Stability in Microsomes
The results of metabolic stability determinations for novel compounds and testosterone (positive control) were conducted in the presence of human, rat, mouse, and monkey liver microsomes.

Values shown are percent of parent remaining after a 30 min incubation. All measurements were done in duplicate. Assay results for testosterone were within an acceptable range.

Metabolic Clearance in Microsomes

Mixed-gender human liver microsomes (Lot# 1710084), male Sprague-Dawley rat liver microsomes (Lot# 1610290), male CD-1 mouse liver microsomes (Lot# 1710069), and male Cynomolgous liver microsomes (Lot# 1510193) were purchased from XenoTech. The reaction mixture, minus NADPH, was prepared as described below. The test article was added into the reaction mixture at a final concentration of 1 µM. The control compound, testosterone, was run simultaneously with the test article in a separate reaction. An aliquot of the reaction mixture (without cofactor) was equilibrated in a shaking water bath at 37 °C for 3 min. The reaction was initiated by the addition of cofactor, and the mixture was incubated in a shaking water bath at 37 °C. Aliquots (100 µL) were withdrawn at 0, 10, 20, 30, and 60. Test article and testosterone samples were immediately combined with 400 µL of ice-cold 50/50 acetonitrile (ACN)/H2O containing 0.1% formic acid and internal standard to terminate the reaction. The samples were then mixed and centrifuged to precipitate proteins. All samples were assayed by LC-MS/MS using electrospray ionization. The peak area response ratio (PARR) to internal standard was compared to the PARR at time 0 to determine the percent remaining at each time point. Half-lives were calculated using GraphPad software, fitting to a single-phase exponential decay equation.

Supplementary Table S-3. Metabolic Clearance of (±)-**44** in Human, Rat, Mouse, and Monkey Microsomes

Compound	Human		Rat		Mouse		Monkey	
	$t_{1/2}$ (min)	Microsomal CL_{int} (µL/min/mg)	$t_{1/2}$ (min)	Microsomal CL_{int} (µL/min/mg)	$t_{1/2}$ (min)	Microsomal CL_{int} (µL/min/mg)	$t_{1/2}$ (min)	Microsomal CL_{int} (µL/min/mg)
(±)-**44**	>60	<0.0231	>60	<0.0231	>60	<0.0231	>60	<0.0231
Testosterone	19	0.0742	1.4	0.987	4.9	0.285	6.4	0.217

Intrinsic clearance (CL_{int}) was calculated based on $CL_{int} = k/P$, where k is the elimination rate constant and P is the protein concentration in the incubation.

General Chemistry Information

General Chemistry. All reactions were performed under a dry atmosphere of nitrogen unless otherwise specified. Indicated reaction temperatures refer to the reaction bath, while room temperature (rt) is noted as 25 °C. Commercial grade reagents and anhydrous solvents were used as received from vendors and no attempts were made to purify or dry these components further. Removal of solvents under reduced pressure was accomplished with a Buchi rotary evaporator at approximately 28 mm Hg pressure using a Teflon-linked KNF vacuum pump. Thin layer chromatography was performed using 1" x 3" AnalTech No. 02521 silica gel plates with fluorescent indicator. Visualization of TLC plates was made by observation with either short wave UV light (254 nm lamp), 10% phosphomolybdic acid in ethanol or in iodine vapors. Preparative thin layer chromatography was performed using Analtech, 20 × 20 cm, 1000 micron preparative TLC plates. Flash column chromatography was carried out using a Teledyne Isco CombiFlash Companion Unit and a Biotage® Selekt System with Teledyne Isco RediSep Rf and Biotage Sfär silica gel columns. If needed, products were purified by reverse phase chromatography, using a Teledyne Isco CombiFlash Companion Unit and a Biotage® Selekt System with a RediSep Gold C18 reverse phase column. Proton NMR spectra were obtained on a 400 MHz Varian nuclear magnetic resonance spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constant (*J*) values are given in Hz, with the following spectral pattern designations: s, singlet; d, doublet; t, triplet, q, quartet; quint, quintet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets; dq; doublet of quartets; br, broad signal. Tetramethylsilane was used as an internal reference. Peak listing, multiplicity designations, and coupling constant calculations were conducted using Mnova v.14 software (Mestrelab Research). Carbon NMR spectra were obtained on a 500 MHz Bruker AV III nuclear magnetic resonance spectrometer and tetramethylsilane was used as an internal reference. Fluorine NMR spectra were obtained on a 400 MHz Bruker AV III nuclear magnetic resonance spectrometer. Any melting points provided are uncorrected and were obtained using a Stanford Research Systems OptiMelt melting point apparatus (MPA100) with an automated melting point system. Mass spectroscopic analyses were performed using ESI ionization on a Waters AQUITY UPLC MS triple quadrapole mass spectrometer. High pressure liquid chromatography (HPLC) purity analysis was performed using a Waters Breeze2 HPLC system with a binary solvent system A and B using a gradient elusion [A, H_2O with 0.1% formic acid; B, CH_3CN with 0.1% formic acid] and flow rate = 0.5 mL/min, with UV detection at 254 nm (system equipped with a photodiode array (PDA) detector). An ACQUITY UPLC BEH C18 column, 130 Å, 1.7 µm, 2.1 mm × 50 mm was used. High resolution mass spectrometry (HRMS) analysis was was performed using an Agilent 6530 Accurate-Mass Q-TOF. All final compounds tested for *in vitro* and *in vivo* biological testing were purified to ≥95% purity, and these purity levels were measured by both ^{1}H NMR and HPLC.

Figure S-3. ^{1}H NMR (400 MHz, DMSO-d_6) Spectrum and Proton Assignment of Analogue (±)-**44**

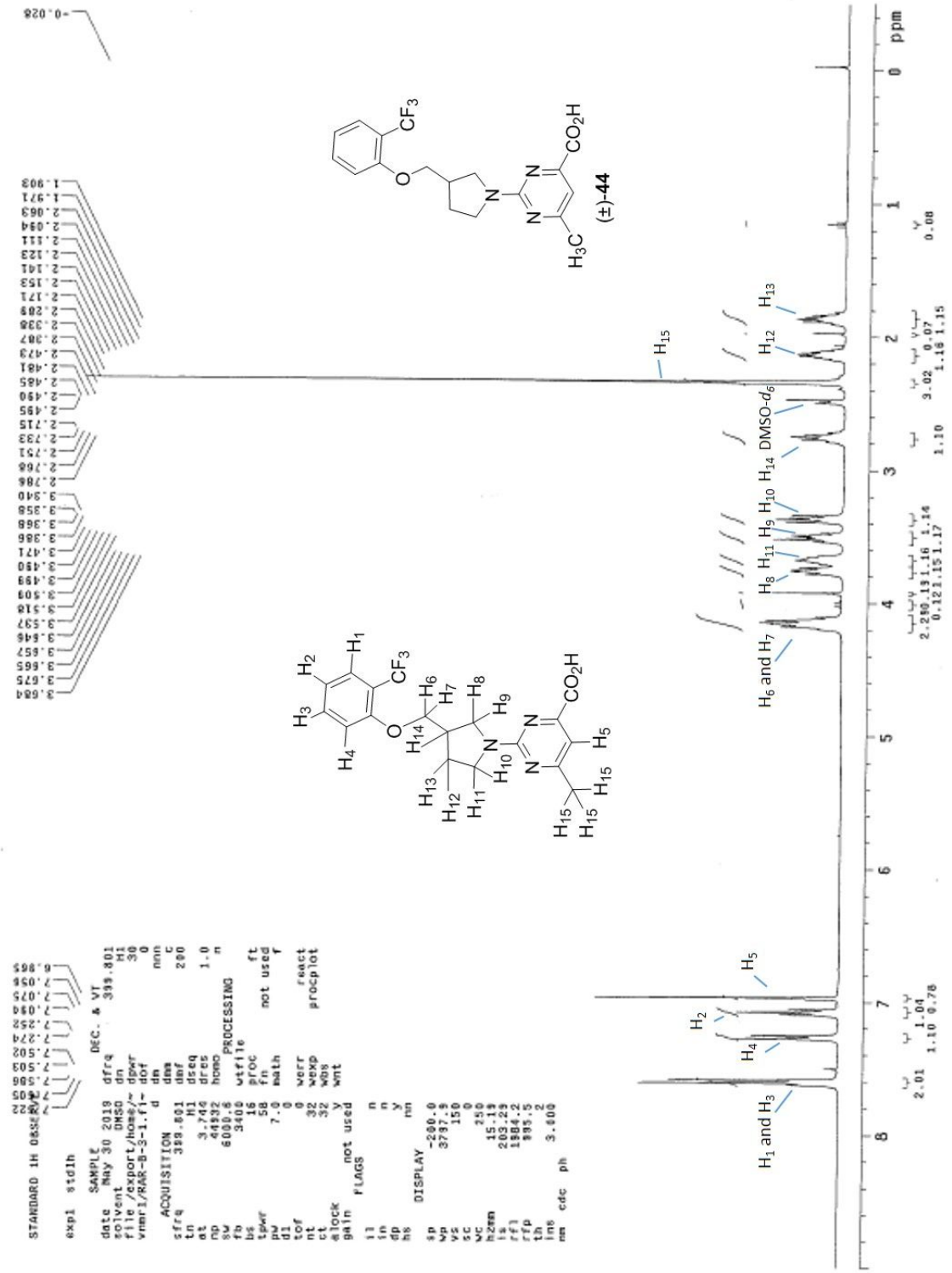


Figure S-4. ^{13}C NMR (500 MHz, DMSO-d_6) Spectrum of Analogue (±)-**44**

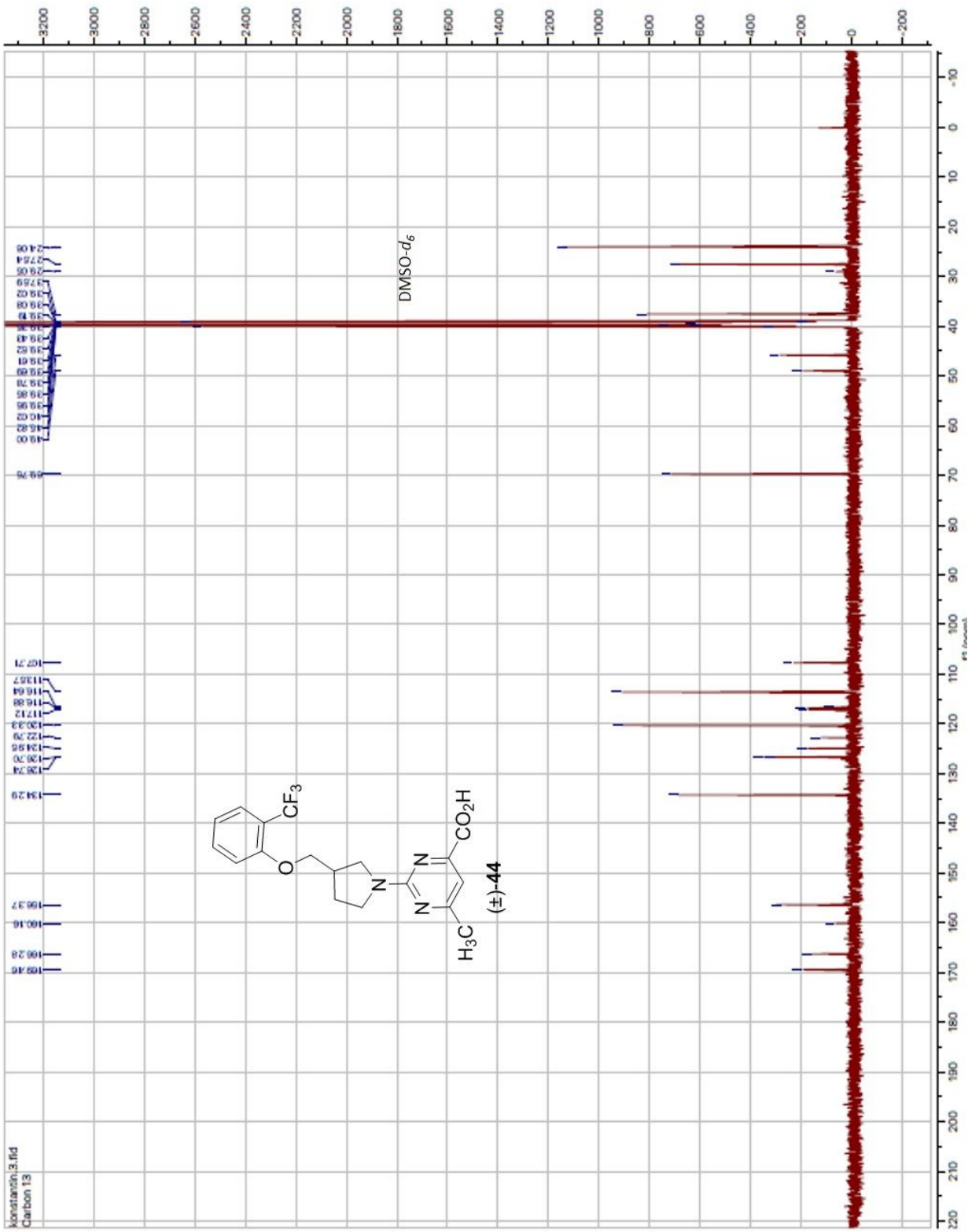


Figure S-5. ^{19}F NMR (400 MHz, DMSO-d_6) Spectrum of Analogue (±)-**44**

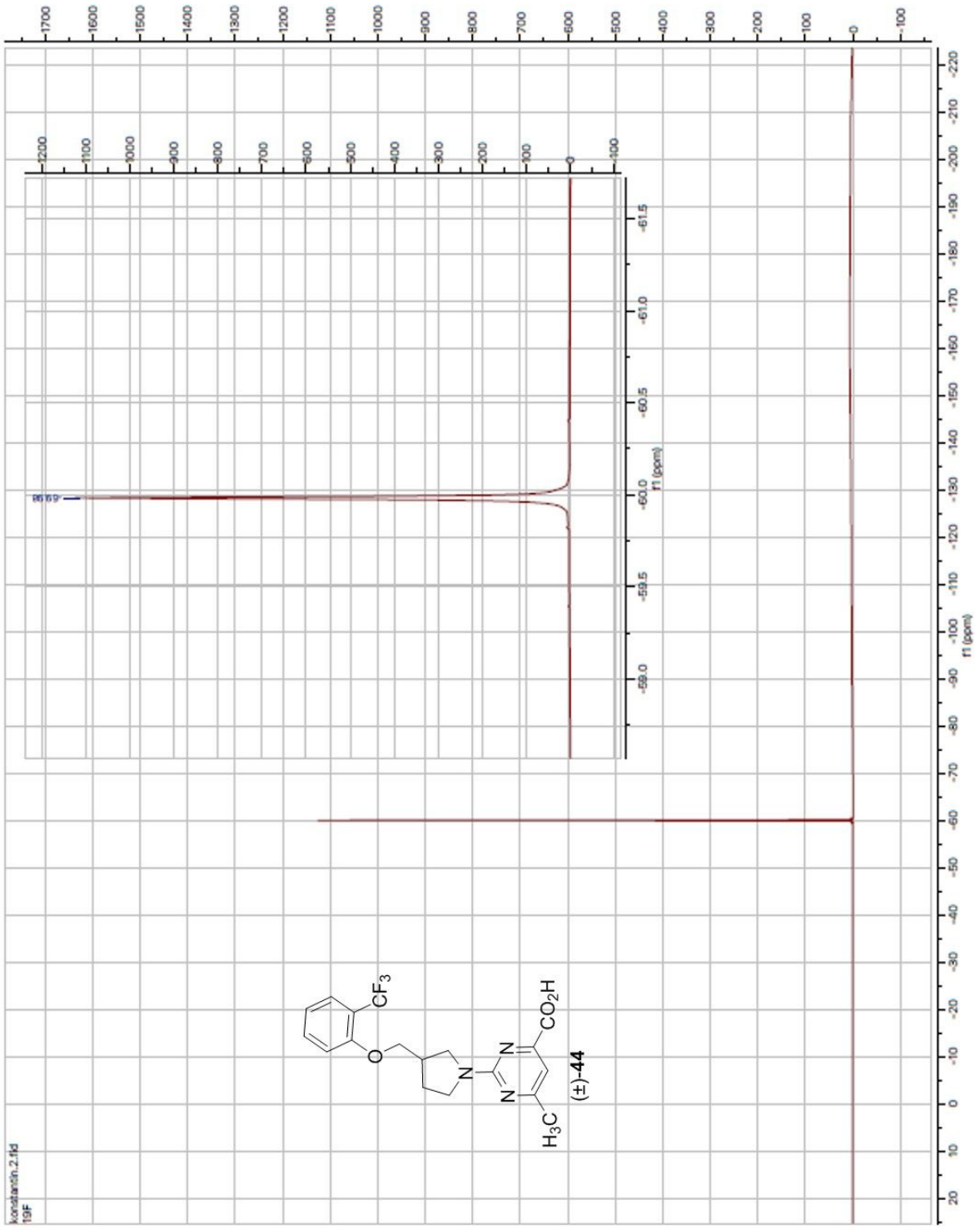
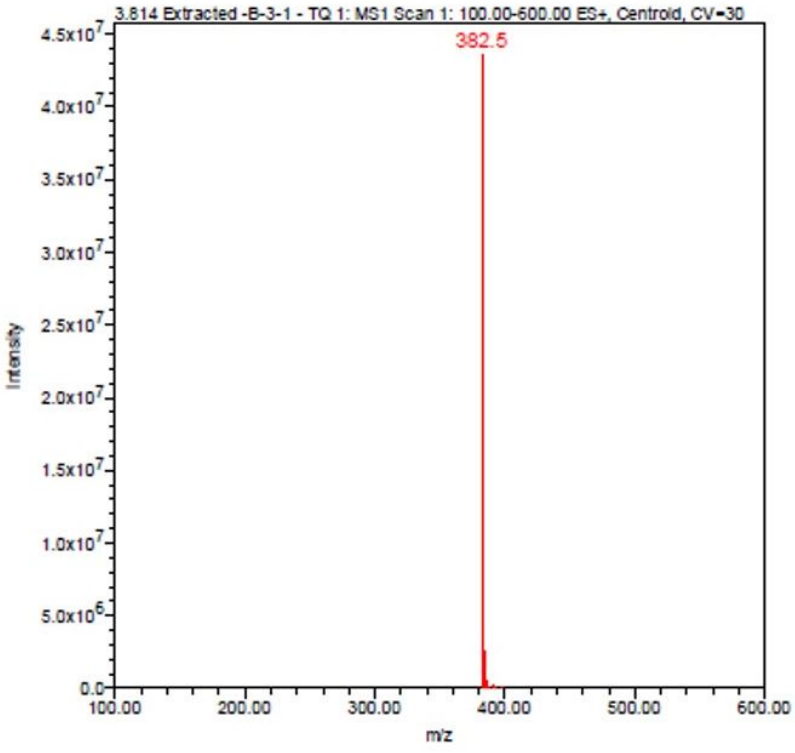
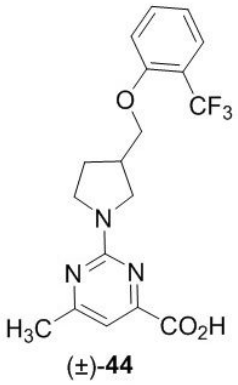


Figure S-6. ESI MS Analysis for Analogue (±)-**44**

Instrument Model	ACQUITY UPLC®
Column	ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm
Mobile Phase :	
Aqueous Reservoir (A)	0.1% Formic acid in Water
Organic Reservoir (B)	0.1% Formic acid in ACN
Flow rate	0.5 mL/min
Injection volume	5 µL
Run Time	5 min
Wave length	UV= 254 nm

Mass Spectra (ES+):





(±)-**44**

Figure S-7. ESI HRMS Analysis for Analogue (±)-**44**

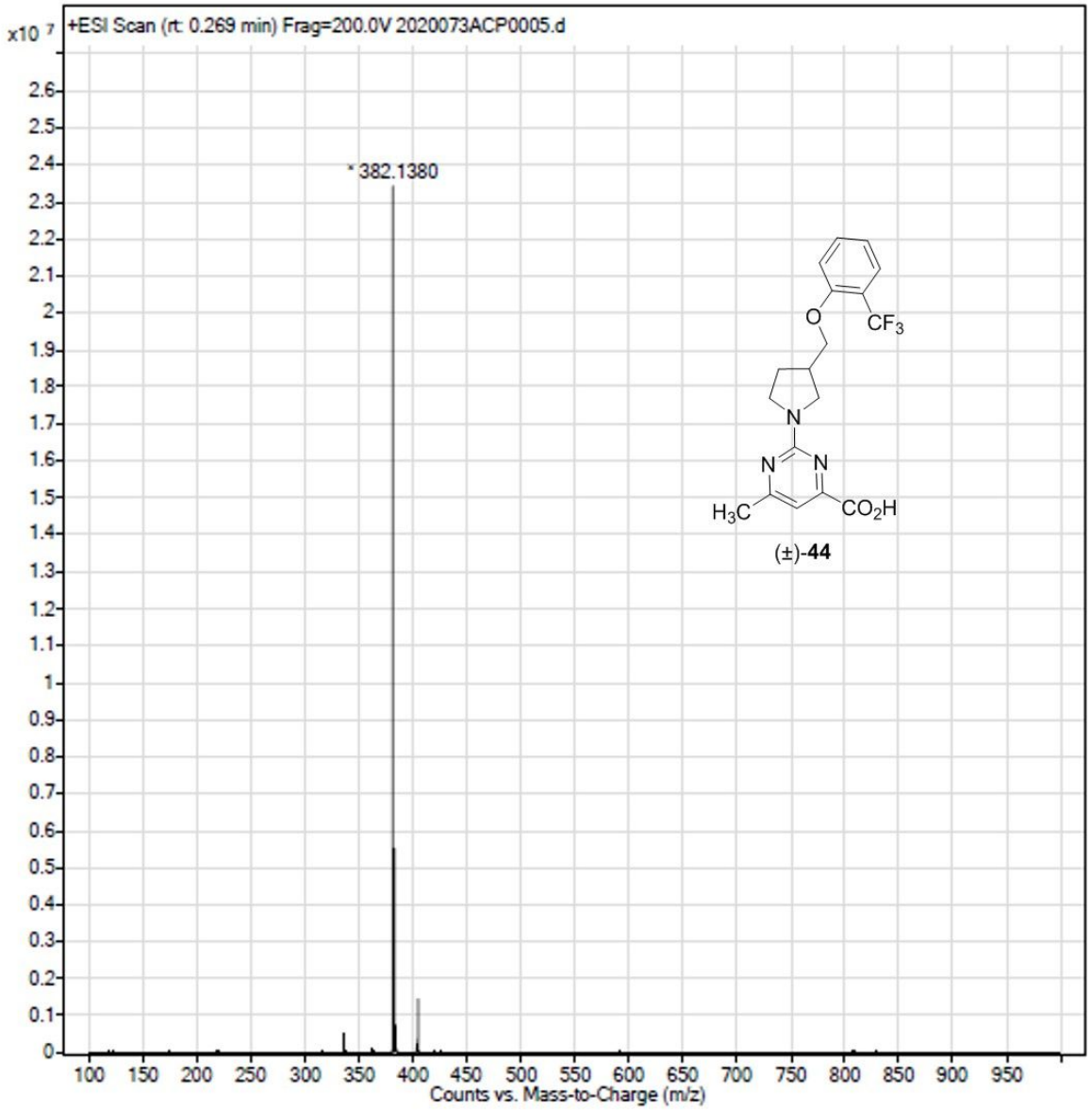


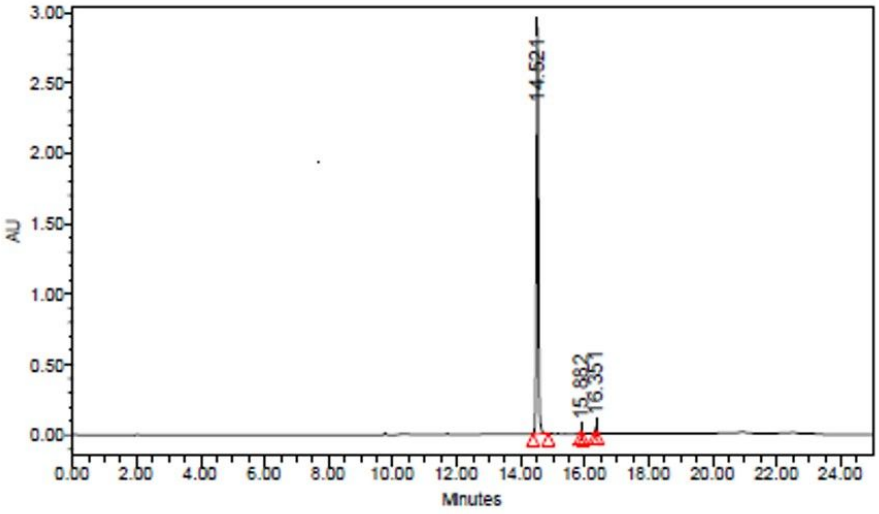
Figure S-8. HPLC Chromatogram Analysis of Analogue (±)-**44**

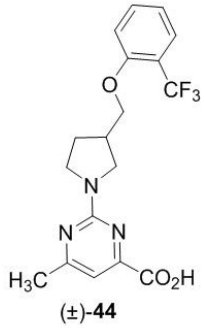
Instrument Model	Breeze™ 2 HPLC
Column	XBridge BEH HILIC C18, 4.6 X 150 mm, 5 µm
Mobile Phase :	
Aqueous Reservoir (A)	0.25% TFA in Water
Organic Reservoir (B)	0.25% TFA in ACN
Flow rate	1.0 mL/min
Injection volume	5 µL
Run Time	30 min
Wave length	UV= 220 nm

HPLC gradient program:

Time (min)	%A	%B
0.01	95	5
3.0	95	5
13.0	5	95
18.0	5	95
22.0	95	5
25.0	Stop	

LC chromatogram:





(±)-**44**

	Name	Retention Time	Area	% Area
1		14.521	12267238	98.70
2		15.882	36412	0.29
3		16.351	125385	1.01

Supplementary Table S-4. SMILES Strings for Biologically Tested Compounds

Compound	SMILES
21	CC1=CC(C(O)=O)=NC(N2CCC(OC3=CC=CC=C3C(F)(F)F)CC2)=N1
27	CC1=CC(C(O)=O)=NC(N2CCC(OC3=C(C(F)(F)F)C=CC=C3)C2)=N1
32	CC1=CC(C(O)=O)=NC(N2CC(OC3=CC=CC=C3C(F)(F)F)C2)=N1
38	CC1=CC(C(O)=O)=NC(N2CC(COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
44	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
(R)-50	CC1=CC(C(O)=O)=NC(N2CC[C@@H](COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
(S)-56	CC1=CC(C(O)=O)=NC(N2CC[C@H](COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
62	CC1=CC(C(O)=O)=NC(N2CC(COC3=C(C(F)(F)F)C=CC=C3)CCC2)=N1
67	CC1=CC(C(O)=O)=NC(N2CCC3(CN(C4=C(C(F)(F)F)C=CC=C4)CC3)C2)=N1
71	CC1=CC(C(O)=O)=NC(N2CCC(CSC3=C(C(F)(F)F)C=CC=C3)C2)=N1
76	CC1=CC(C(O)=O)=NC(N2CCC(CNC3=C(C(F)(F)F)C=CC=C3)C2)=N1
80	CC1=CC(C(O)=O)=NC(N2CCC(OCC3=C(C(F)(F)F)C=CC=C3)C2)=N1
83	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(C)(C)C)C=CC=C3)C2)=N1
84	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C4CCCC4)C=CC=C3)C2)=N1
85	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C4CCCCC4)C=CC=C3)C2)=N1
86	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C(Cl)=CC=C3)C2)=N1
87	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=C(F)C=C3)C2)=N1
88	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC(F)=C3)C2)=N1
89	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3F)C2)=N1
90	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC(OC)=C3)C2)=N1
91	CC1=CC(C(O)=O)=NC(N2CCC(COC3=CC(C(F)(F)F)=CC(C(F)(F)F)=C3)C2)=N1
92	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CN=C3)C2)=N1
93	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)N=CC=C3)C2)=N1
94	FC(C(C=CC=C1)=C1OCC2CN(C3=NC=CC(C(O)=O)=N3)CC2)(F)F
95	CC1=CC(C(O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2)=C1
96	FC(C(C=CC=C1)=C1OCC2CN(C3=CC=CC(C(O)=O)=N3)CC2)(F)F
97	FC(C(C=CC=C1)=C1OCC2CN(C3=NC=CC=C3C(O)=O)CC2)(F)F
98	FC(C=CC(C(O)=O)=C1)=C1N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2
99	CC1=CC(C(NS(=O)(C)=O)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
100	CC1=CC(C(N)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
101	CC1=CC(C(NC)=O)=NC(N2CCC(COC3=C(C(F)(F)F)C=CC=C3)C2)=N1
102	CC1=CC(C(NC2CC2)=O)=NC(N3CCC(COC4=C(C(F)(F)F)C=CC=C4)C3)=N1
103	CC1=CC(C2=NNN=N2)=NC(N3CCC(COC4=C(C(F)(F)F)C=CC=C4)C3)=N1