SUPPLEMENTARY INFORMATION for "Tetrahydroisoquinoline CXCR4 Antagonists Adopt a Hybrid Binding Mode within the Peptide Subpocket of the CXCR4 Receptor"

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I. Supplementary Figures, Charts and Schemes

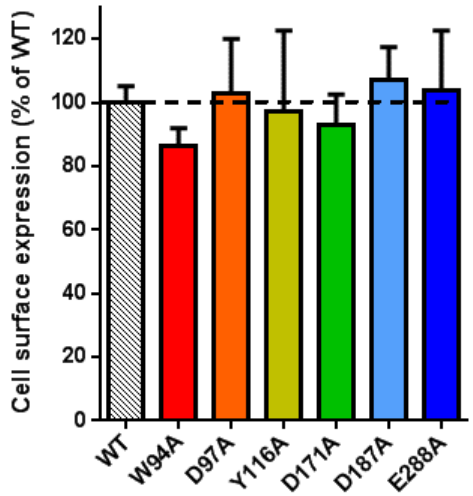


Figure S1. Cell surface expression of CXCR4 mutants. Surface expression of CXCR4 mutants transiently expressed in HEK293 cells was determined by FACS analysis using anti-CXCR4 antibody staining. Data are expressed as percent of WT surface expression and represents average ± S.D. from two independent experiments.

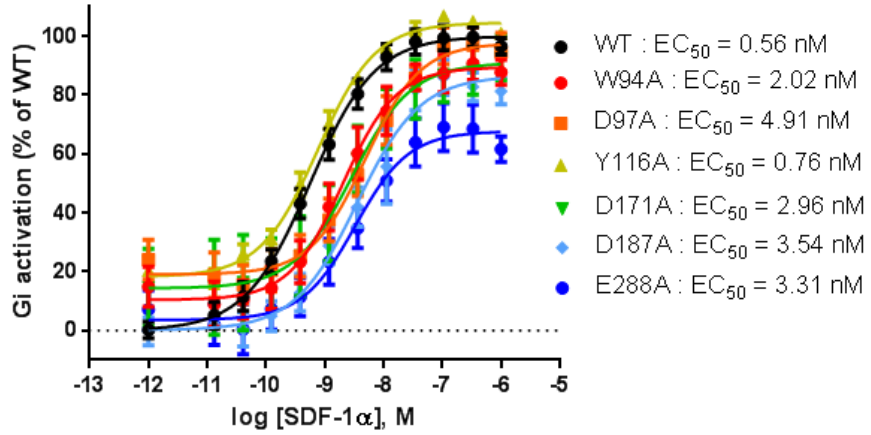


Figure S2. Functionality of CXCR4 mutants. The functionality of CXCR4 mutants was determined as their ability to activate Gi signaling, following stimulation with the natural ligand SDF-1α. The Gi BRET biosensor was used to assess Gi signaling, by measuring the decrease in the BRET signal between Gαi2-*R*luc and GFP-Gγ1 upon ligand treatment. Data are expressed as percent of WT response and represents average ± S.E.M. from three independent experiments.

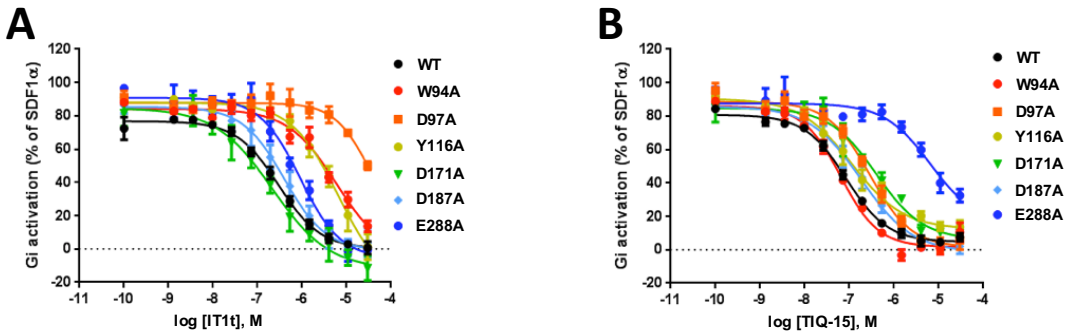


Figure S3. The antagonist activity of IT1t (**1**) and TIQ-15 (**5**) on CXCR4 mutants. The ability of IT1t (**1**) and TIQ-15 (**5**) to block SDF-1α response on different CXCR4 mutants was assessed using the Gi BRET biosensor assay. HEK293 cells expressing the CXCR4 receptor as well as Gαi2-*R*luc, Gβ1 and GFP-Gγ1, were stimulated with either IT1t (Panel A) or TIQ-15 (Panel B), in addition to SDF-1α. Mutants are listed by color code. Data are expressed as percent of SDF-1α response and represents average ± S.E.M. from three independent experiments. The IC_{50} values are listed in Table 1.

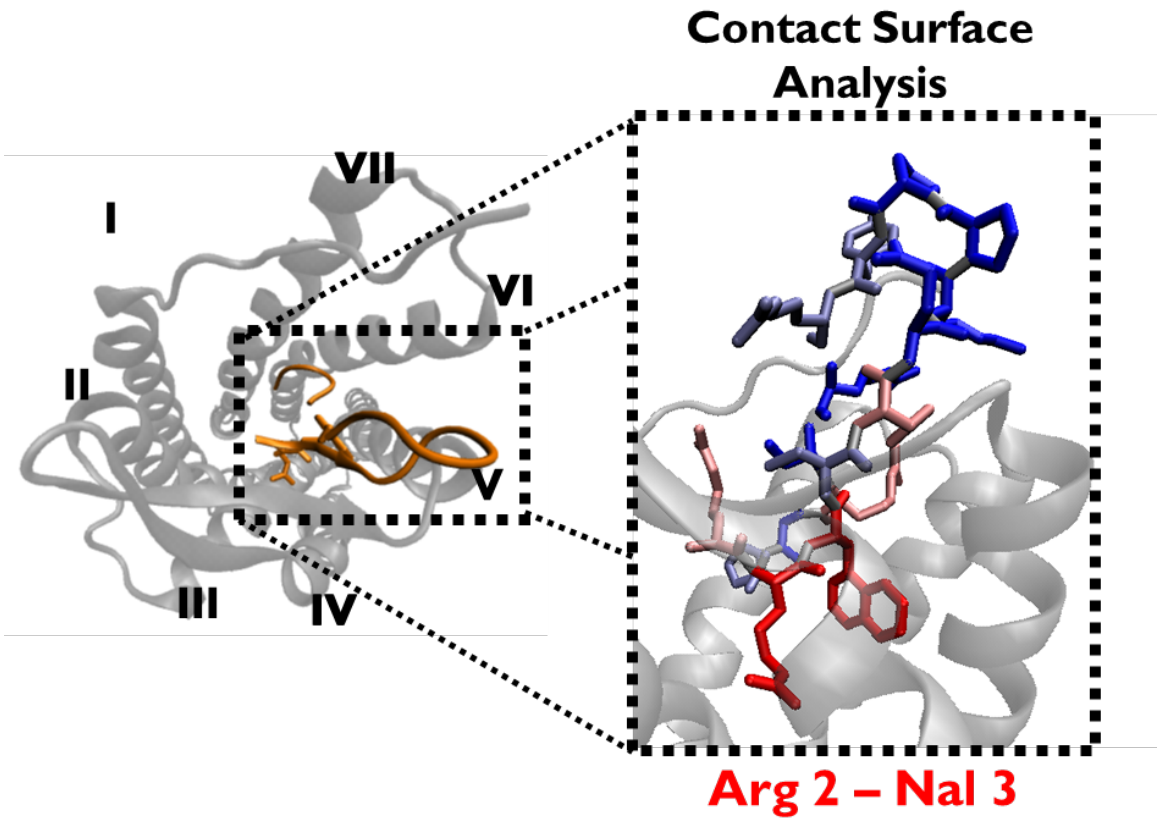


Figure S4. Contact Surface Analysis of the Crystal Structure of CXCR4 with CVX15. Highlights the location of the Arg_2-Nal_3 residue sequence (in red) that forms the most stabilizing interactions with the receptor.

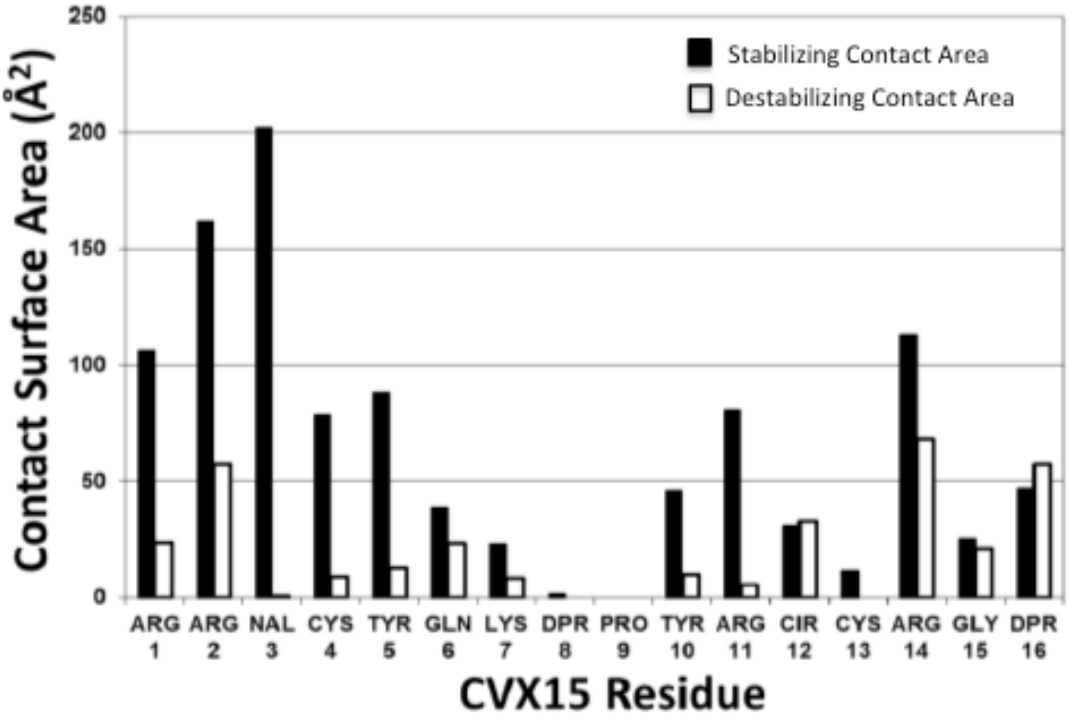


Chart S1. Contact surface analysis of the CVX15 peptide binding to CXCR4. Values of both Stabilizing (black bars) and Destabilizing (white bars) Contact Surface Areas of CVX15 per residue within the CXCR4 receptor calculated from electron densities within the crystal structure.

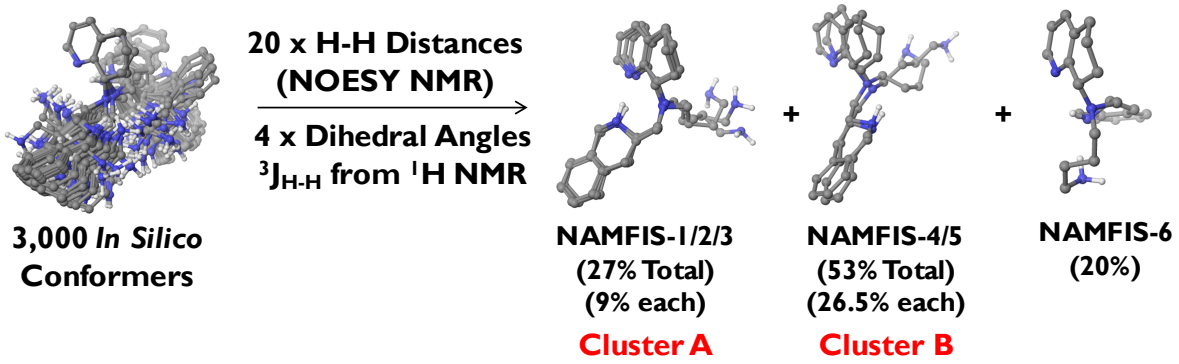
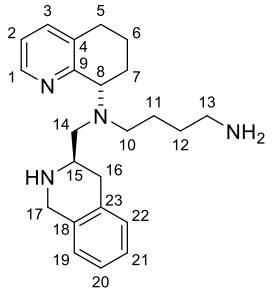


Figure S5. Description of the NAMFIS method, generation and de convolution of the six main solution conformers (NAMFIS-1 thru 6) of TIQ-15 (**5**).

II. NMR Peak Assignments for Compound **5** (TIQ-15).



Atom position map for NMR assignments.

Table S1. ^{1}H and ^{13}C NMR Spectral Assignments of **5**.

Atom Number	**^{1}H**	**^{13}C**
1	8.46 (dd, *J* = 4.4, 0.9 Hz, 1H)	146.9
2	7.05-7.02 (m, 1H)	121.7
3	7.35-7.32 (m, 1H)	136.9
4	--	134.7
5 up	2.79 (m, 1H)	29.7
5 down	2.67 (app. dt, *J* =16.8, 5.0, 5.0 Hz, 1H)	29.7
6 up	1.75-1.67 (m, 1H)	22.2
6 down	2.02-1.96 (m, 1H)	22.2
7 up	2.14-2.06 (m, 1H)	28.6
7 down	1.90 (dddd, *J* = 15.7, 13.0, 10.2, 3.1 Hz, 1H)	28.6
8 up	4.08 (dd, *J* = 9.6, 6.6 Hz, 1H)	62.0
9	--	158.8
10	3.02 (dt, *J* = 13.0, 5.8, 5.8 Hz, 1H)	54.6
10'	2.61-2.60 (m, 1H)	54.6
11	1.61-1.48 (m, 2H)	27.6
12	1.61-1.48 (m, 1H)	31.1
12'	1.48-1.40 (m, 1H)	31.1
13	2.76-2.74 (m, 2H)	42.2
14	2.98 (dd, *J* = 13.2, 3.2 Hz, 1H)	58.5
14'	2.38 (dd, *J* = 12.9, 10.5 Hz, 1H)	58.5
15 down	2.77-2.75 (m, 1H)	52.6
16 up	2.43 (dd, *J* = 15.9, 11.0 Hz, 1H)	34.0
16 down	2.63-2.61 (m, 1H)	34.0
17 up	4.07 (d, *J* = 15.1 Hz, 1H)	48.7
17 down	3.92 (d, *J* = 15.1 Hz, 1H)	48.7
18	--	135.7
19	7.09-7.08 (m, 1H)	126.2
20	7.08-7.07 (m, 1H)	125.8
21	7.02-7.01 (m, 1H)	126.6

22	7.03-7.07 (m, 1H)	129.3
23	--	134.3

Table S2. NOE-derived Distances for **5**.

NOE Contact	**Distance (Å)**
2-3	2.48*
5 down-6 up	3.26
5 down-5 up	1.98
10-12	2.85
6 down-7 up	2.50
7 down-14 down	2.79
16 up-17 up	2.58
7 up-7 down	1.79
7 down-15 down	2.81
17 up-17 down	2.00
10-10’	1.83
14 up-14 down	1.75
7 up-8 up	2.31
6 up-8 up	2.77
7 down-8 up	3.35
10’-12’	3.18
3-5 down	2.63
3-5 up	2.69
6 up-6 down	1.72
1-2	2.42
16 up-16 down	1.88
6 up-7 up	2.42

*Internal standard used to calculate distances.

III. Supplemental Experimental Procedures

III.A. Mutational studies with CXCR4 and Functional Analysis of TIQ-15 and IT1t in key mutants.

III.A.1 Plasmid DNA

The CXCR4 WT plasmid construct encoding the wild-type receptor was generated by optimizing the coding sequence of human CXCR4 (GeneOptimizer from ThermoFisher), and adding an artificial cleavable signal peptide from bovine prolactin (MDSKGSSQKGSRLLLLLVVSNLLLCQGVVS) in N-terminus of the receptor to increase cell surface expression. The different mutants of the receptor, W94A, D97A, Y116A, D171A, D187A and E288A were then obtained by site-directed mutagenesis using CXCR4 WT as the template. The Gαi2-*R*luc construct used for BRET studies was generated by inserting the *Renilla* luciferase II at the position 100 in the Gαi2 protein (Quoyer J., Janz J.M., Luo J, Ren Y, Armando S, Lukashova V, Benovic JL, Carlson KE, Hunt SW 3rd, Bouvier M. Pepducin Targeting the C-X-C Chemokine Receptor Type 4 Acts as a Biased Agonist Favoring Activation of the Inhibitory G Protein. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E5088-5097). The Gγ1 protein was tagged with GFP10 to obtain GFP-Gγ1, using the same strategy described previously for GFP-Gγ2 (Galés C., Rebois R.V., Hogue M., Trieu P., Breit A., Hébert T.E., Bouvier M. Real-Time Monitoring of Receptor and G-Protein Interactions in Living Cells. *Nat. Methods* **2005**, *2*, 177-184).

III.A.2 Quantification of receptor surface expression by flow cytometry

HEK293 cells were cultured in DMEM supplemented with penicillin-streptomycin and 10% Fetal Bovine Serum (FBS). Two days before the FACS experiment, HEK293 cells were transfected with the different receptors using linear polyethylenimine 25 kDa (PEI, Polysciences) at a PEI:DNA ratio of 3:1. Transfected cells were directly seeded in 6-well plates, at a density of 700 000 cells per well, and maintained in culture for the next 48 h. FACS experiments were carried out subsequently using the following procedures: cells were washed once with

phosphate-buffered saline (PBS) buffer, then detached and re-suspended in Tyrode's buffer. After cell counting, 50 000 cells per condition were re-suspended in Tyrode's buffer with 1% BSA containing PerCP Cy5.5 antibody anti-human CD184, directed against CXCR4 (Biolegend # 306516; 1 µl of antibody for 500 000 cells). After 45 min of incubation at 4°C, cells were centrifuged 1min at 1000 x g and washed using Tyrode's buffer with 1% BSA. A subsequent washing step in Tyrode's buffer was performed, before cell surface expression of CXCR4 was finally analyzed through iQue flow cytometer (IntelliCyt) set to detect PerCP/Cy5.5.

III.A.3 BRET assays

HEK293 cells were cultured in DMEM supplemented with penicillin-streptomycin and 10% Fetal Bovine Serum. Two days before the BRET experiment, HEK293 cells were transfected with the CXCR4 WT or mutants, as well as Gαi2-*R*luc, Gβ1 and GFP-Gγ1, using linear polyethylenimine 25 kDa (PEI, Polysciences) at a PEI:DNA ratio of 3:1. Transfected cells were directly seeded in 96-well plates pre-treated with poly-D-lysine (CulturPlate, PerkinElmer), at a density of 35 000 cells per well, and maintained in culture for the next 48 h. BRET experiments were carried out subsequently using the following procedures: cells were washed once with Tyrode's buffer (137 mM NaCl, 1 mM $CaCl_2$, 0.9 mM KCl, 1 mM $MgCl_2$, 3.6 mM NaH_2PO_4, 5.5 mM glucose, 12 mM $NaHCO_3$, and 25 mM HEPES, pH 7.4). Tyrode's buffer was then added to each well and cells were equilibrated in their new buffer at room temperature for at least 30 minutes. Following the equilibration period, prolume purple coelenterazine at a final concentration of 2 µM was distributed to each well (Nanolight Technology) and the test compounds were added using the HP D300 digital dispenser from Tecan (12 singlicate concentrations). Cells were incubated with the compounds for 5 min at room temperature, and BRET readings were first collected to record agonist response, using a Synergy Neo Multi-Mode

reader from BioTek with BRET2 filters 410 ± 80 nm / 515 ± 30 nm. In order to measure the antagonist responses of the compounds, cells were further incubated at room temperature for an additional 25 min (for a total of 30 min pre-incubation period with the compounds), before addition of a fixed dose of SDF-1α (Recombinant Human Stromal Cell-Derived Factor-1α; Cedarlane) corresponding to the EC_{80} of the receptor. A second reading was collected 5 min after this second addition, using the Synergy Neo Multi-Mode reader from BioTek with BRET2 filters 410 ± 80 nm / 515 ± 30 nm, to record antagonist response. The BRET signal was determined by calculating the ratio of the light emitted by GFP (515 nm) over the light emitted by the Rluc (410 nm). BRET signal values were converted into percentage of activation using the non-stimulated control as 0% and SDF-1α maximal response as 100%. Sigmoidal concentration-response curves were generated with those normalized values using a 4-parameter logistic equation (GraphPad; Prism software), to determine IC_{50} of the two different compounds.

III.B Computational Modeling Procedures

III.B.1 CXCR4 X-ray Structures Used for Molecular Docking Studies

Similarly to our previous study, we examined both the CVX15:CXCR4 crystal structure (PDB ID 3OE0) and the IT1t:CXCR4 crystal structure (PDB ID 3ODU) as templates for predictive docking. The 3ODU IT1t:CXCR4 structure was selected due to superior resolution (2.5 Å) when compared to the other three IT1t:CXCR4 structures (~3.1 Å). Unless otherwise stated, default parameters were chosen for the software packages.

III.B.2 Receptor GRID Preparation

The CXCR4 crystal structures were treated and prepared in Schrodinger's Maestro (2012, v9.1). Since the crystal structures did not share common locations of crystallographic waters and fatty acids, only the protein – ligand complexes were considered. Hydrogens were added to the

proteins using the Protein Preparation Wizard, and the ionization states were optimized using Epik. Receptor GRIDs were constructed using Glide with a 10 x 10 x 10 Å boundary box centered on either the IT1t ligand (for 3ODU) or Arg 2 – Nal 3 (for 3OE0). This box was large enough to cover the entirety of the binding site.

III.B.3 Modeling Methods

Compound **5** was prepared in Maestro using the 2D sketcher in the di-cation protonation state with a positive charge on the butyl-amine and the tetrahydroisoquinoline (THIQ) nitrogens. The di-cation protonation state for compound **5** is the dominant state in solution based on pKa calculations (ACD labs, SI) and experimental ^{1}H NMR chemical shift perturbation measurements (SI). First, 88 Low-energy conformers of compound **5** were predicted using MacroModel with the OPLS-2005 force field. All low-energy conformers were docked flexibly into the IT1t:CXCR4 and CVX15:CXCR4 receptor GRIDs using Glide with Standard Precision. The top ten poses for each receptor model were subjected to a post-docking Prime MM-GBSA refinement using 15 Å cut-off. The top-scoring poses by MM-GBSA ΔG Bind are presented in Table 1. Optimized CXCR4 receptor GRIDs were prepared from the top-scoring Prime MM-GBSA complexes with compound **5** using Glide. The 10x10x10 Å boundary box was centered on the docked ligand, which was large enough to span the extracellular binding pocket. Ligands were prepared in the di-cation protonation state with the exception of the aniline THIQ compounds (**8**, **10**) as a mono-cation. Low-energy conformers of all ligands were generated using MacroModel, and all conformers were docked flexibly into the optimized CXCR4 GRIDs using Glide at Standard Precision. The top 10 poses for each ligand were re-scored using Prime MM-GBSA with a 0.0 Å cut-off.

III.C NMR Procedures

III.C.1 NMR Experiments with Compound **5** and NAMFIS Analysis

NMR experiments were performed on a Varian Unity Inova 600 equipped with either a 5 mm TRES-ZPFG (1H/13C/15N) probe or a 5 mm ID-ZPFQ (1H/X) probe operating at 600 MHz (^{1}H) and 150 (^{13}C). Both spectrometers use VNMR 6.1C software. For all 2-D experiments, the probe was tuned to the sample, and the 90° pulse width (^{1}H pw90) was calibrated at a specific pulse power (tpwr) by identifying the 180° pulse which gave a null spectrum and dividing the result by two. The inversion recovery method was utilized to measure the spin-lattice relaxation times (T1) of the various ^{1}H nuclei.

A sample for 1-D and 2-D NMR experiments was prepared by dissolving 5.0 mg of compound **5** in 0.65 mL of $CDCl_3$ (Cambridge Isotope Laboratories) and then the sample was subsequently degassed and sealed. In order to assist in structural proton assignments, an HMQC spectrum (nt = 48, ni = 256, experiment time ~15 hours) and a COSY spectrum (nt = 32, ni = 512, experiment time ~13 hours) was acquired. All ^{1}H and ^{13}C NMR peak assignments for **5** are listed in Table S1 in the Supporting Information.

A NOESY spectrum was recorded at 300 ms mixing time, which is expected to be in the linear range for small molecules. Inter-proton distances were calculated from cross-peak volumes using an internal calibration distance between H-2 and H-3 of 2.48 Å. The relaxation delay (d1) was set to 6 s (~3 times the longest T1 of ~2.5 s), nt = 32, and ni = 256 (experiment time = 29.5 hours). The 22 NOE-derived distances are presented in the supplemental material section (SI, Table S2).

Macromodel was used to generate ~3000 unique *in silico* conformers of compound **5** using 50,000 step MCMM sampling searches with OPLS-2005, AMBER*, and MMFFs force

fields *in vacuo*, $CHCl_3$, and water. Maximum allowed energy was 21 kJ/mol (~5kcal/mol) to filter out high-energy conformations. Twenty-two inter-proton distances measured from the 2-D NOESY NMR spectrum were used to deconvolute approximately 3000 *in silico* conformers. The NAMFIS conformers and docked conformations were compared using the RMSD Trajectory Tool available from Visual Molecular Dynamics (VMD).

III.D Synthetic/Medicinal Chemistry

III.D.1 General

All reagents were obtained from commercial suppliers and used without further purification unless specified otherwise. Reaction progress was monitored by thin layer chromatography (TLC) on pre-coated glass plates (silica gel 60 F254, 0.25 mm) or liquid chromatography-mass spectrometry (LC-MS) using an analytical column (Agilent, ZORBAX Eclipse XDB-C18, 50 x 4.6 mm, 3.5 μm). When noted, flash chromatography was performed manually or on a Teledyne ISCO Combiflash Companion with prepackaged Teledyne Redisep or Silicycle disposable normal phase or amine functionalized silica columns. Proton and carbon NMR spectra were recorded on an INOVA-400 (400 MHz), VNMRS 400 (400 MHz), INOVA-600 (600 MHz), or Unity-600 (600 MHz). All chemical shifts are reported in parts per million and referenced to the residual solvent peak. Liquid chromatography/mass spectrometry (LCMS) data was obtained to verify molecular mass and analyze purity of products. The specifications of the LCMS instrument are the following: Agilent 1200 HPLC coupled to a 6120 quadrupole mass spectrometer (ESI-API), UV detection at 254 and 210 nm, Agilent Zorbax XDB-18 C_{18} column (50 mm x 4.6 mm, 3.5 μm), gradient mobile phase consisting of MeOH/water/0.1 % formic acid buffer, and a flow rate of 1.00 mL/min. The chemical purity of all final compounds was

determined by LCMS and confirmed to be ≥ 95%. High resolution mass-spectra (HRMS) were acquired on a VG 70-S Nier Johnson or JEOL mass spectrometer. The compounds IT1t (**1**) and TIQ-15 (**5**) were prepared according to published methods.[15,29]

III.D.2 Synthetic Procedures for Compounds **11**, **17** and **23**-**25** in Scheme 1.

tert-Butyl (*R*)-3-((((*S*)-1,2,3,4-tetrahydronaphthalen-1-yl)amino)methyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate *(***8**). To a solution of 0.25 mL of (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthyl amine (**6**, Aldrich, 1.7 mmoles) and 0.27 g (1.03 mmoles) of aldehyde **7**[15] in 8 mL of 1,2-dichloroethane was added 0.63 g (2.97 mmoles) of sodium triacetoxy borohydride and the resulting mixture was stirred at room temperature overnight. The reaction was diluted with dichloromethane and washed with $NaHCO_3$ (aq.) and NaCl (aq.) solutions. The organic layer was separated and dried over Na_2SO_4(s). Filtration, solvent removal and column chromatography (Hexanes/EtOAc gradient) provided 0.224 g of compound **8** as a light orange, viscous oil (55% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (s, 9H); 1.68 (m, 2H), 1.82 (m, 1H), 1.92 (m, 2H), 2.68 (m, 2H), 2.77 (m, 1H), 3.05 (dd, 1H, J=2 Hz, J=14 Hz), 3.04 (dd, 1H, d=5 Hz, J=16 Hz), 3.72 (bs, 1H), 4.26 (d, 1H, J=16 Hz), 4.45 (m, 1H), 4.72 (quart, 1H, J=22 Hz), 7.04 (t, 1H, J=4 Hz), 7.12 (m, 3H), 7.16 (m, 2H), 7.34 (m, 1H); ^{13}C NMR (95 MHz, $CDCl_3$): δ 19.46, 28.79, 29.64, 31.39, 43.36, 48.23, 55.72, 80.08, 126.02, 126.43, 126.85, 129.06, 129.26, 133.42, 137.59, 139.45, 155.71; MS (e/z): 393.2 ($M+H^+$). HRMS (ESI) *m*/*z* $[M+H]^+$ calcd. for $C_{25}H_{33}N_2O_2$: 393.25420; found 393.25464.

tert-Butyl (*R*)-3-(((4-(Bis-(tert-butoxycarbonyl)amino)butyl)((*S*)-1,2,3,4-tetrahydronaphthalen-1-yl)amino)methyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (**10**). To a solution of 0.135 g (0.34 mmoles) of compound **8** and 0.28 g (0.98 mmoles) of aldehyde **9**

(ref.) in 5 mL of dichloromethane was added 0.1 mL of acetic acid and 212 mg (1 mmole) of sodium triacetoxy borohydride. The reaction was stirred at room temperature overnight. The mixture was then extracted with dichloromethane and washed with $NaHCO_3$ (aq.) and NaCl (aq.) solutions. The organics were separated and dried over Na_2SO_4(s). Solvent removal and column chromatography on silica gel with a Hexanes/EtOAc gradient gave 0.134 g of a white foam (59% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H), 1.46 (s, 9H), 1.48 (s, 9H), 1.86 (m, 2H), 2.03 (m, 1H), 2.21 (t, 1H, J=11 Hz), 2.3 (m, 1H), 2.53 (d, 1H, J=10 Hz), 2.66 (bs, 2H), 2.93 (dd, 1H, J=6 Hz, J=16 Hz), 3.12 (m, 1H), 3.36 (m, 2H), 3.46 (m, 1H), 3.59 (m, 1H), 3.94 (m, 1H), 4.27 (m, 1H), 4.63 (d, 1H, J=17 Hz), 6.98 (m, 2H), 7.07 (m, 2H), 7.11 (m, 3H), 7.65 (bs, 1H); MS (m/z): 664.4 ($M+H^+$). MS (e/z): 393.2 ($M+H^+$). HRMS (ESI) *m*/*z* $[M+H]^+$ calcd. for $C_{39}H_{58}N_3O_6$: 663.42474; found 663.42462

N^1-(((*R*)-1,2,3,4-Tetrahydroisoquinolin-3-yl)methyl)-N^1-((*S*)-1,2,3,4-tetrahydronaphthalen-1-yl)butane-1,4-diamine (**11**). To a solution of 0.134 g of compound **10** dissolved in 2 mL of dichloromethane was added 2 mL of trifluoroacetic acid. The mixture was stirred at room temperature overnight, followed by removal of volatiles in vaccuo. The residue was then extracted with dichloromethane and washed with 1 M NaOH (aq.) and NaCl (satd., aq.) solutions. The organics were separated and dried over Na_2SO_4(s). Solvent removal and column chromatography on silica gel with DCM/MeOH/NH_4OH (90:10:0.1) gave 0.042 g of a pale brown viscous oil (58% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.46 (m, 4H), 1.68 (m, 2H), 1.98 (m, 2H), 2.47 (m, 2H), 2.63 (m, 4H), 2.77 (m, 2H), 3.86 (d, 1H, J=16 Hz), 3.96 (t, 1H, J=6 Hz), 4.01 (d, 1H, J=16 Hz), 6.99 (m, 2H), 7.08 (m, 4H), 7.16 (t, 1H, J=7 Hz), 7.7 (d, 1H, J=8 Hz); ^{13}C NMR (95 MHz, $CDCl_3$): δ 22.36, 22.89, 27.19, 30.07, 30.81, 34.01, 41.76, 48.39, 52.25, 52.95, 57.89, 61.04, 110.0, 125.57, 125.62, 126.01, 126.17, 126.22, 127.93, 128.86, 129.26, 134.56,

135.52, 138.39, 139.07; MS (m/z): 364.2 ($M+H^+$), 234.2, 131.2. HRMS (ESI) *m/z* $[M+H]^+$ calcd. for $C_{24}H_{34}N_3$: 364.27472; found 364.27464.

tert-Butyl (*R*)-2-((((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)piperidine-1-carboxylate (**14**). A 250 mL round bottom flask was charged with STAB-H (1.181 g, 5.57 mmol) and (*S*)-5,6,7,8-tetrahydroquinolin-8-amine (**12**, 0.688 g, 4.64 mmol) dissolved in half the 1,2-dichloroethane (37.1 ml) . Then to this stirred solution (R)-tert-butyl 2-formylpiperidine-1-carboxylate (**13**, 0.792 g, 3.71 mmol) dissolved in the other half of the solvent was added. The reaction was allowed to stir for 2.5 hours before being quenched with $NaHCO_3$. Brine and 10% NaOH were added until the two layers had clear distinction. The DCE layer was still alittle murky so ample amounts of drying agent were necessary at the end of the extraction. The water layer was extracted with DCM 3 times. The reaction mixture was purified on a 40 gram column with a gradient going from 3-15% MeOH in DCM. 704 mg (55% yield). ^{1}H NMR (400 MHz, Chloroform-d): δ 8.26 (dd, *J* = 4.6, 1.8 Hz, 1H), 7.27 – 7.23 (m, 1H), 6.94 (dd, *J* = 7.7, 4.7 Hz, 1H), 4.27 (s, 1H), 3.92 (d, *J* = 13.7 Hz, 1H), 3.69 (dd, *J* = 7.3, 5.2 Hz, 1H), 2.87 – 2.74 (m, 2H), 2.74 – 2.59 (m, 3H), 2.06 (ddd, *J* = 12.9, 8.4, 5.0 Hz, 1H), 1.97 – 1.85 (m, 1H), 1.76 (dd, *J* = 9.2, 3.5 Hz, 1H), 1.64 (tdq, *J* = 8.0, 5.5, 2.5 Hz, 1H), 1.55 – 1.42 (m, 5H), 1.37 (s, 9H); ^{13}C NMR (101 MHz, cdcl3) δ 157.62, 155.40, 146.87, 136.89, 132.34, 121.90, 79.42, 57.63, 50.66, 46.52, 39.70, 28.91, 28.63, 26.52, 25.64, 19.73, 19.40. HRMS calcd. for $C_{20}H_{32}O_2N_3$ 346.24890; found 346.24889 [M+H].

tert-Butyl (*R*)-2-(((4-(1,3-dioxoisoindolin-2-yl)butyl)((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)piperidine-1-carboxylate (**16**). (R)-tert-Butyl 2-((((S)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)piperidine-1-carboxylate (**14**, 0.650 g, 1.881 mmol) was dissolved in 1,2-DCE (18.81 ml) in a 100 mL round bottom flask. 4-(1,3-dioxoisoindolin-2-

yl)butanal (**15**, 0.450 g, 2.070 mmol) was then added and the reaction was allowed to stir for 1 hour. At which point STAB-H (0.598 g, 2.82 mmol) was all added as one batch. The reaction was then allowed to stir for an additional 3 hours checked by LCMS (reaction complete) and then quenched with 5 mL $NaHCO_3$. Brine and 10% NaOH were added until the two layers had clear distinction. The DCE layer was still alittle murky so ample amounts of drying agent were necessary at the end of the extraction. The water layer was extracted with DCM 3 times. The crude mixture was purified on a 24g ISCO column with an eluent from 0-15% MeOH in DCM. 800 mg (78% yield). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.24 (dd, *J* = 4.7, 1.6 Hz, 1H), 7.67 – 7.61 (m, 3H), 7.54 (td, *J* = 5.3, 3.0 Hz, 3H), 7.13 (dd, *J* = 7.7, 1.7 Hz, 1H), 6.83 (dd, *J* = 7.7, 4.6 Hz, 1H), 4.01 (dd, *J* = 15.4, 8.2 Hz, 1H), 3.87 (dd, *J* = 8.9, 5.9 Hz, 1H), 3.74 (d, *J* = 13.7 Hz, 1H), 3.56 (t, *J* = 6.8 Hz, 1H), 3.49 (t, *J* = 7.1 Hz, 2H), 2.80 – 2.43 (m, 4H), 2.43 – 2.26 (m, 4H), 1.94 – 1.77 (m, 2H), 1.69 – 1.40 (m, 3H), 1.29 (s, 9H), 1.15 – 1.04 (m, 6H), 0.73 – 0.64 (m, 2H). ^{13}C NMR (101 MHz, cdcl3) δ 200.81, 168.34, 155.12, 147.04, 136.35, 134.37, 133.89, 132.24, 123.13, 121.36, 78.94, 60.71, 51.73, 51.04, 41.15, 38.08, 37.16, 34.74, 31.66, 29.78, 29.40, 28.64, 26.50, 26.23, 25.49, 25.36, 25.20, 22.74, 21.51, 21.25, 19.04, 14.25.

HRMS calcd. for $C_{20}H_{32}O_2N_3$ 547.32843; found 346.24889 [M+H].

N^1-(((*R*)-Piperidin-2-yl)methyl)-N^1-((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)butane-1,4-diamine (**17**). Step 1: (*R*)-tert-Butyl 2-(((4-(1,3-dioxoisoindolin-2-yl)butyl)((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)piperidine-1-carboxylate (**16**, 0.750 g, 1.372 mmol) was dissolved in DCM (10.29 ml)and then TFA (3.43 ml) was added with stirring.

The reaction was stirred overnight. The reaction was then partitioned between DCM and 10% NaOH. Base was added until the aqueous layer was basic on pH paper. The crude material was then dried with sodium sulfate, concentrated in vaccuo and re-dissolved in MeOH. In methanol

solid crashed out which was filtered away, the mother liquor was concentrated. This crude material was taken on to the next step.

Step 2: The material from the previous step was then dissolved in 8mL of MeOH and hydrazine (0.995 ml, 10.97 mmol) was added with stirring. The mixture was stirred overnight and then checked by HPLC in the morning (reaction complete). At this point the crude mixture was concentrated in vacou and then diluted with 10mL of 1N HCl. The water layer was extracted with DCM twice (both times pulling off alittle bit of yellow coloration). Then the water layer was bascified with 10% NaOH until blue by pH paper and extracted into DCM twice. The organic phase was dried with sodium sulfate and then concentrated to afford the pure material in the above spectra. This material was then ran through a short plug of silica to get rid of any inorganic impurities. Yield 190 mg (48%) over two steps.

^{1}H NMR (400 MHz, Chloroform-d) δ 8.37 (dd, *J* = 4.8, 1.6 Hz, 1H), 7.28 (dd, *J* = 7.7, 1.7 Hz, 1H), 6.99 (dd, *J* = 7.7, 4.7 Hz, 1H), 4.00 (dd, *J* = 10.1, 6.3 Hz, 1H), 3.04 (dt, *J* = 12.0, 2.6 Hz, 1H), 3.01-2.90 (m, 1H), 2.79 – 2.56 (m, 6H), 2.54 – 2.39 (m, 2H), 2.35 – 2.16 (m, 2H), 2.15-1.85 (m, 4H), 1.80 (tdd, *J* = 12.5, 10.0, 2.6 Hz, 1H), 1.73 – 1.60 (m, 2H), 1.54 – 1.33 (m, 5H), 1.21 (qt, *J* = 12.7, 4.3 Hz, 1H), 1.02 – 0.85 (m, 1H). ^{13}C NMR (101 MHz, cdcl3) δ 159.26, 146.78, 136.58, 134.18, 121.43, 61.29, 58.70, 56.01, 54.12, 47.13, 42.45, 31.80, 30.65, 29.66, 29.60, 27.47, 26.44, 25.11, 22.24. HRMS calc'd for $C_{19}H_{33}N_4$ 317.26997; found 317.26975 [M+H].

LCMS 75% MeOH Isocratic >95% pure rt= 0.996 min.

tert-Butyl (*R*)-3-(((2-((tert-butoxycarbonyl)amino)ethyl)((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (**20**). To a mixture of compound **18** (0.366 g, 0.93 mmol)[add ref] dissolved in 3.0 mL of DCM was added a solution of *tert*-butyl (2-oxoethyl)carbamate (**19a**, 0.14 mL, 0.93 mmol) in 2.0 mL of DCM. To the

stirring mixture was added sodium triacetoxyborohydride (0.296 g, 1.40 mmol). The reaction stirred at room temperature overnight. An additional 0.5 eq (0.07 g) of aldehyde was added to reaction and allowed to stir for 1 hours. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with DCM (3x). The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated to a brown/orange oil (0.57 g). The crude material was purified by flash column chromatography (ISCO, 12 g silica gel, liquid load, 0-25% DCM-95:5 DCM/MeOH over 6 minutes, hold 3 minutes, 25-50% over 5 minutes, hold 3 minutes, 50-100% over 4 minutes, hold 4 minutes) resulting in an off-white foam (0.262 g, 53%). ^{1}H NMR [600 MHz, $CDCl_3$] δ 8.65-8.45 (m, 1H), 7.49-7.28 (m, 1H), 7.22-6.95 (m, 5H), 4.69-4.53 (m, 2H), 4.36 (dt, *J* = 33.3, 14.6 Hz, 2H), 4.17-3.99 (m, 1H), 3.33 (dd, *J* = 39.0, 15.6 Hz, 1H), 2.95 (dd, *J* = 15.4, 5.5 Hz, 1H), 2.86 (ddt, *J* = 13.1, 6.7, 3.5 Hz, 1H), 2.81-2.64 (m, 3H), 2.53 (dt, *J* = 25.1, 11.6 Hz, 1H), 2.45-2.15 (m, 4H), 2.09-1.96 (m, 1H), 1.83-1.57 (m, 2H), 1.56 (s, 18H). HRMS (ESI) $[M+H]^+$ calc'd for $C_{31}H_{45}N_4O_4$ 537.34408, found 537.34422.

tert-Butyl (*R*)-3-(((3-((tert-butoxycarbonyl)amino)propyl)((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (**21**). To a mixture of compound **17** (0.206 g, 0.52 mmol) dissolved in 2.0 mL DCM was added a solution of propionaldehyde **19b** (0.091 g, 0.52 mmol) in 1.0 mL of DCM. To the stirring mixture was added sodium triacetoxyborohydride (0.166 g, 0.79 mmol). The reaction stirred at room temperature overnight and was quenched with sat. aq. $NaHCO_3$ and extracted with DCM (3 x). The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated to a light brown oil (~0.3 g). The crude material was purified by flash column chromatography (ISCO, 12 g silica gel, liquid load, 0-50% DCM-95:5 DCM/MeOH over 15 minutes, then 100% 95:5

DCM/MeOH over 7 minutes) resulting in a white foam (0.211 g, 73%). HRMS (ESI) $[M+H]^+$ calc'd for $C_{32}H_{47}N_4O_4$ 551.35918, found 551.35903.

tert-Butyl (*R*)-3-(((5-((tert-butoxycarbonyl)amino)pentyl)((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)amino)methyl)-3,4-dihydroisoquinoline-2(1H)-carboxylate (**22**). To a mixture of compound **17** (0.150 g, 0.38 mmol) dissolved in 3.0 mL of DCM was added a solution of pentyl aldehyde **19c** (0.077 g, 0.38 mmol) in 2.0 mL of DCM. To the stirring mixture was added sodium triacetoxyborohydride (0.121 g, 0.57 mmol). The reaction stirred at room temperature for 48 hours. The reaction was quenched with sat. aq. $NaHCO_3$ and extracted with DCM (3 x). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated to a brown/orange oil (0.225 g). The crude material was purified by flash column chromatography (ISCO, 12 g silica gel, liquid load, 0-25% DCM-95:5 DCM/MeOH over 11 minutes, hold 4 minutes, 25-50% over 6 minutes, hold 4 minutes, 50-100% over 4 minutes, hold 4 minutes) resulting in an off-white foam (0.155 g, 70%). HRMS (ESI) $[M+H]^+$ calc'd for $C_{34}H_{51}N_4O_4$ 579.39048, found 579.39048.

General Procedure for Boc Deprotection. Preparation of **N^1-(((*R*)-1,2,3,4-Tetrahydroisoquinolin-3-yl)methyl)-N^1-((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)ethane-1,2-diamine** (**23**). A flask containing compound **20** (0.246 g, 0.46 mmol) in 3:1 DCM (2.4 mL) and TFA (0.8 mL) mixture was stirred at room temperature for 3 hours. The reaction was quenched with aq. sat. $NaHCO_3$. The aqueous layer was extracted with DCM (3x). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated to an off-white foam (~0.15 g). The crude material was purified by flash column chromatography (silica gel, 9:1:0.1 DCM/MeOH/aq. NH_4OH) to obtain the title compound as an off-white foam (0.077 g, 50%). ^{1}H NMR [600 MHz, $CDCl_3$] δ 8.47 (dd, J = 4.8, 1.7 Hz, 1H), 7.41-7.33 (m, 1H), 7.10 (td,

J = 7.2, 3.4 Hz, 3H), 7.04 (dd, *J* = 7.0, 2.1 Hz, 1H), 6.97 (d, *J* = 6.3 Hz, 1H), 5.51-5.02 (br s, 3H), 4.03 (d, *J* = 16.0 Hz, 1H), 3.99 (dd, *J* =1 0.9, 5.9 Hz, 1H), 3.76 (d, *J* = 15.9 Hz, 1H), 3.11 (dd, *J* = 13.6, 3.5 Hz, 1H), 3.07-2.74 (m, 6H), 2.74-2.70 (m, 2H), 2.62 (dd, *J* = 13.5, 10.1 Hz, 1H), 2.50 (dd, *J* = 16.2, 10.1 Hz, 1H), 2.28-2.10 (m, 1H), 2.03 (dtt, *J* = 13.3, 5.3, 2.8 Hz, 1H), 1.86-1.78 (m, 1H), 1.78-1.69 (m, 1H); ^{13}C NMR [150 MHz, $CDCl_3$] δ 159.0, 146.9, 136.8, 135.2, 134.6, 134.2, 129.3, 126.6, 126.2, 125.8, 121.6, 61.7, 59.2, 57.8, 54.2, 52.7, 48.4, 42.3, 33.6, 29.8, 29.6, 29.0, 24.7, 22.2; HRMS (ESI) $[M+H]^+$, calc'd for $C_{21}H_{29}N_4$ 337.23867, found 337.23886.

N^1-(((*R*)-1,2,3,4-Tetrahydroisoquinolin-3-yl)methyl)-N^1-((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)propane-1,3-diamine (**24**). A flask containing compound **21** (0.199 g, 0.36 mmol) in 3:1 DCM (2.4 mL) and TFA (0.8 mL) mixture was stirred at room temperature. After 4 hours, the reaction showed consumption of starting material. The reaction was quenched with aq. sat. $NaHCO_3$. The aqueous layer was extracted with DCM (3x). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated to afford an off-white foam (0.15 g). The material was purified by flash column chromatography (ISCO, 15.5 g amine functionalized gold column, 0-5% DCM-MeOH over 15 minutes). The fractions appeared to contain impurities, so the fractions were concentrated and the material (0.1 g) was repurified (ISCO, 4 g silica gel, 0-25% DCM-DCM/MeOH/NH_4OH 80:20:2 over 9 minutes, hold 3 minutes, 25-50% over 5 minutes, hold 4 minutes, and 50-100% over 5 minutes, hold 3 minutes) resulting in a brown oil (0.065 g, 51%). ^{1}H NMR [600 MHz, $CDCl_3$] δ 8.45 (d, *J* = 4.6 Hz, 1H), 7.32 (d, *J* = 7.6 Hz, 1H), 7.10-6.99 (m, 5H), 4.08 (dd, *J* = 10.2, 6.3 Hz, 1H), 4.04 (d, *J* = 15.0 Hz, 1H), 3.91 (d, *J* = 15.0 Hz, 1H), 3.09 (dt, *J* = 13.3, 6.7 Hz, 1H), 2.94 (dd, *J* = 13.2, 3.3 Hz, 1H), 2.79-2.74 (m, 3H), 2.73-2.64 (m, 3H), 2.62 (dd, *J* = 16.0, 3.6 Hz, 1H), 2.47-2.26 (m, 3H), 2.09 (dddd, *J* =

10.9, 8.0, 5.0, 2.2 Hz, 2H), 1.99 (dh, *J* = 12.9, 4.6, 4.0 Hz, 2H), 1.90 (tdd, *J* = 13.1, 10.2, 3.1 Hz, 2H), 1.75-1.64 (m, 2H); ^{13}C NMR [150 MHz, $CDCl_3$] δ 156.8, 147.1, 138.2, 134.6, 134.2, 133.4, 129.5, 126.7, 126.5, 126.4, 122.7, 66.0, 58.4, 52.8, 51.8, 46.5, 40.0, 32.9, 29.9, 29.2, 26.2, 21.9. HRMS (ESI) *m*/*z* $[M+H]^+$ calcd. for $C_{22}H_{31}N_4$: 351.25487; found 351.25495.

N^1-(((*R*)-1,2,3,4-Tetrahydroisoquinolin-3-yl)methyl)-N^1-((*S*)-5,6,7,8-tetrahydroquinolin-8-yl)pentane-1,5-diamine (**25**). A flask containing compound **22** (0.15 g, 0.26 mmol) in 3:1 DCM (2.4 mL) and TFA (0.8 mL) mixture was stirred at room temperature. After 3 hours, the starting material was completely consumed. The reaction was quenched with sat. $NaHCO_3$ (aq.). The aqueous layer was extracted with DCM (3x). The organic extracts were combined, dried over Na_2SO_4, filtered, and concentrated to a brown oil (0.1 g). The crude material was purified by flash column chromatography (silica gel, 9:1:0.1 DCM/MeOH/NH_4OH) to obtain the title compound as a yellow oil (0.047g, 48%). ^{1}H NMR [600 MHz, $CDCl_3$] δ 8.45 (dd, *J* = 4.6, 1.6 Hz, 1H), 7.33 (dd, *J* = 7.6, 1.7 Hz, 1H), 7.20-6.91 (m, 5H), 4.17-4.04 (m, 2H), 3.92 (d, *J* = 15.0 Hz, 1H), 3.00 (ddd, *J* = 13.6, 6.9, 4.4 Hz, 2H), 2.78 (dtd, *J* = 16.6, 10.9, 6.5 Hz, 2H), 2.73-2.57 (m, 9H), 2.54-2.42 (m, 2H), 2.08 (dtd, *J* = 15.6, 5.3, 2.3 Hz, 1H), 1.98 (dtd, *J* = 13.4, 5.4, 3.3 Hz, 1H), 1.90 (tdd, *J* = 13.0, 10.1, 3.1 Hz, 1H), 1.77-1.67 (m, 1H), 1.59-1.42 (m, 4H), 1.42-1.28 (m, 2H); ^{13}C NMR [150 MHz, $CDCl_3$] δ 159.0, 146.9, 136.8, 135.2, 134.6, 134.2, 129.3, 126.6, 126.2, 125.8, 121.6, 61.7, 59.2, 57.8, 54.2, 52.7, 48.4, 42.3, 33.6, 29.8, 29.6, 29.0, 24.7, 22.2; HRMS (ESI) $[M+H]^+$ calc'd for $C_{24}H_{35}N_4$ 379.28562, found 379.28579.

III.E MAGI HIV-1_{IIIB} Assay Method

III.E.1 Cell Preparation

MAGI-CCR5/CXCR4 cells (obtained from the NIH AIDS Research and Reference Reagent Program) are passaged in T-75 flasks prior to use in the antiviral assay. MAGI-CCR5/CXCR4 cells are derived from HeLa-CD4-LTR-b-gal cells. The cells have been engineered to express high levels of CD4 and CXCR4 and contain one copy of the HIV-1 LTR promoter driving expression of the b-galactosidase gene upon HIV-1 Tat transactivation. On the day preceding the assay, the cells are plated at 1 X 10^4 well and incubated at 37° C overnight. Total cell and viability quantification is performed using a hemacytometer and trypan blue exclusion. Cell viability is greater than 95% for the cells to be utilized in the assay.

III.E.2 Virus Preparation

The virus used for these tests is the CXCR4-tropic strains HIV-1IIIB. This virus was obtained from the NIH AIDS Research and Reference Reagent Program[24] and was grown in Ghost Hi5/MAGI-CCR5/CXCR4 co-cultures for the production of stock virus pools. For each assay, a pre-titered aliquot of virus is removed from the freezer (-80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus is re-suspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 mL is approximately ten TCID50/well (~0.001 TCID50/cell).

III.E.3 Assay Setup

Compounds are evaluated at one or two concentrations (e.g., for initial screening) or in dose-response at six concentrations (triplicate wells/concentration). On the day of assay setup, compound dilutions are prepared at two-times (2X) the final required concentrations. Media used for plating the cells the day before assay setup is aspirated from the plates and replaced with 50 mL of the 2X compounds, followed by the addition of 50 mL of virus, which dilutes the compounds to the final 1X concentrations. Cell control wells (cells only) and virus control wells

(cells plus virus) are included on each assay plate. Identical uninfected assay plates (virus replaced with media) are prepared for parallel cytotoxicity testing. The cultures are incubated for 48 hours or 6 days (depending on compound or client requirements) after which antiviral efficacy is measured as the inhibition of b-galactosidase reporter expression and cytotoxicity is monitored by MTS staining.

III.E.4 β-galactosidase Chemiluminescent Endpoint Analysis

A chemiluminescent endpoint is used to determine the extent of b-galactosidase expression as a measure of HIV-1 infection of the cells. Once HIV-1 has attached and entered the MAGI-CXCR4 cells, HIV-1 Tat transactivates the LTR dependent b-galactosidase enzyme to express higher than normal levels of b-galactosidase. Thus there is a direct relationship between the level of HIV-1 infection and the level of β-galactosidase detected in the cells. At 48 hours or 6 days post infection, plates are aspirated and PBS is added to each well. Gal-screen reagent (Tropix, Bedford, MA) is then added per the manufacturer's instructions for chemiluminescent detection of b-galactosidase activity and incubated at room temperature for 90 minutes. The resulting chemiluminescence signal is then read using a Microbeta Trilux luminescence reader (PerkinElmer/Wallac).

III.E.5 MTS Staining for Cell Viability

At assay termination, the cytotoxicity assay plates are stained with the soluble tetrazolium-based dye MTS (CellTiter Reagent, Promega) to determine cell viability and quantify compound toxicity. MTS is metabolized by the mitochondrial enzymes of metabolically active cells to yield a soluble formazan product, allowing the rapid quantitative analysis of cell viability and compound cytotoxicity. The MTS is a stable solution that does not require preparation before use. At termination of the assay, 15 mL of MTS reagent is added per well. The microtiter plates

are then incubated 1.5-2 hrs at 37^{o}C; the incubation interval was chosen based on empirically determined times for optimal dye reduction. The plates are read spectrophotometrically at 490/650nm with a Molecular Devices Vmax plate reader.

III.E.6 Data Analysis

Percent inhibition of virus replication and percent cell viability at each concentration are calculated using an in-house computer program. For dose-response testing, IC_{50} (50% inhibition of virus replication), IC_{90} (90% inhibition of virus replication), TC_{50} (50% cytotoxicity), and therapeutic index values ($TI = TC_{50}/IC_{50}$; also referred to as Antiviral Index or AI) are provided. Raw data for both antiviral activity and cytotoxicity with analyzed/tabulated data are provided in a printout summarizing the individual compound activity. For dose-response testing, a graphical representation of the data is also provided. An IC_{50} of 2 to 5 nM was obtained for AMD3100.