Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Supplemental Information

Non-iminosugar glucocerebrosidase small molecule chaperones

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GC enzyme assay. Assays were conducted using recombinant GC (Cerezyme®, Genzyme Co., Cambridge, MA) and Resorufin-β-*D*-Glucopyranoside (K_m = 28 μM) in an assay buffer composed of 50 mM citric acid/KH_2PO_4, pH 5.9, 10 mM sodium taurocholate and 0.01% Tween-20. GC in assay buffer was added to a 1536-well black plate at 2 μL/well, followed by addition of 23 nL of compound in DMSO with a pin-tool station (Kalypsys, San Diego). After 5 min at RT (~ 21 °C), 1 μL/well of substrate was added and incubated for 20 min at RT. Fluorescence intensity was measured at an excitation of 570 (±10) nm and an emission of 610 (±10) nm. The final concentrations of enzyme and substrate were 1.9 nM and 30 μM, respectively.

Enzyme selectivity assays. Three additional hydrolases and their substrates, α-glucosidase and 4-methylumbelliferyl α-*D*-glucopyranoside (4MU-α-Glc), α-galactosidase and 4-methylumbelliferyl α-*D*-galactopyranoside (4MU-α-Gal), and β-*N*-acetylglucosaminidase from bovine kidney (HEX) and 4-methylumbelliferyl *N*-acetyl-β-*D*-glucosaminide (4MU-β-GSM) were obtained from Sigma-Aldrich. The enzyme assay methods were similar to those previously reported with modification for the miniaturization into 1536-well plates. The buffer for all three enzyme assays consisted of 50 mM citric acid/KH_2PO_4, pH 4.5, 10 mM sodium taurocholate and 0.01% Tween-20. The final enzyme concentrations for α-glucosidase, α-galactosidase and β-*N*-acetylglucosaminidase were 8, 1 and 8 nM, respectively. The substrate concentrations were similar to the K_m values for these related enzymes, at 0.4, 0.16 and 0.2 mM, respectively.

Immunocytochemistry and Laser Scanning Confocal Microscopy. Fibroblasts were seeded in Lab-Tek 4 chamber slides (Fisher Scientific, Pittsburgh, PA). After 5 days of treatment with various concentrations of compound, cells were fixed in 3% paraformaldehyde, permeabelized with 0.1 % Triton-X and blocked in PBS containing 0.1% saponin, 100 μM glycine, 0.1% BSA and 2% donkey serum. This was followed by incubation with mouse monoclonal anti-LAMP-1 (1:100, Developmental Studies Hybridoma bank, University of Iowa, Iowa City, IA), which is the lysosomal marker, and the rabbit polyclonal anti-GCase R386 antibody (1:500). The cells were washed and incubated with secondary donkey anti-mouse or anti-rabbit antibodies conjugated to ALEXA-488 (green) or ALEXA-555 (red), respectively (Invitrogen, Carlsbad, CA), washed, and mounted in VectaShield with DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged with a Zeiss 510 META confocal laser-scanning microscope (Carl Zeiss, Microimaging Inc., Germany) using an Argon (458, 477, 488, 514 nm) 30 mW laser and a HeNe (543 nm) 1 mW laser. Images were taken with the same laser settings and all the images shown are collapsed z-stacks.

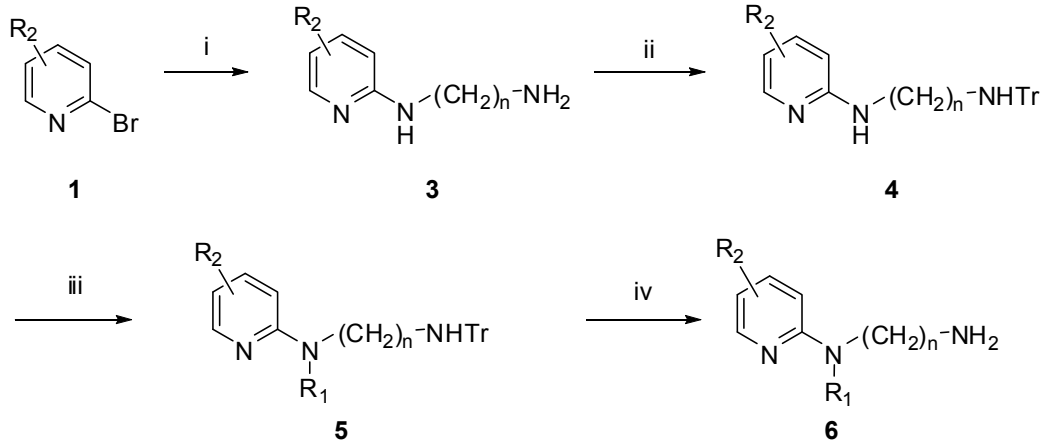
General Materials and Methods: All commercially available reagents and solvents were purchased and used without further purification. All microwave reactions were carried out in a sealed microwave vial equipped with a magnetic stir bar and heated in a Biotage Initiator Microwave Synthesizer. HPLC purification was performed using a Waters semi-preparative HPLC equipped with a Phenomenex Luna® C_{18} reverse phase (5 micron, 30 x 75 mm) column having a flow rate of 45 mL/min. The mobile phase was a

mixture of acetonitrile and H_2O each containing 0.1% trifluoroacetic acid. During purification, a gradient of 30% to 80% acetonitrile over 8 minutes was used with fraction collection triggered by UV detection (220 nM). ^1H spectra were recorded using either an Inova 400 MHz spectrometer (Varian) or an Inova 300 MHz spectrometer (Varian). Two LCMS methods were used to analyze samples' purity. Method 1: Agilent 1200 series LC/MS equipped with a Zorbax™ Eclipse XDB-C_{18} reverse phase (5 micron, 4.6 x 150 mm) column having a flow rate of 1.1 mL/min. The mobile phase was a mixture of acetonitrile and H_2O each containing 0.05% trifluoroacetic acid. A gradient of 5% to 100% acetonitrile over 8 minutes was used during analytical analysis. Method 2: Acquity HPLC equipped with a Waters BEH C18, 1.7 micron, 2.1 x 50 mm column; Column Temperature: 45 °C; Flow: 0.50 mL/min; Solvent A: 0.05% TFA in Water; Solvent B: 0.025% TFA in acetonitrile; Gradient: 2% to 100% Solvent B over 1.3 minutes; Run Time: 3 min.

General Synthetic Procedures

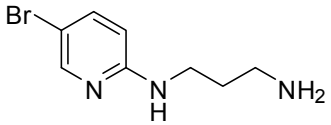
The following general procedures were used to synthesize compounds having different but analogous structures.

General procedure for the synthesis of diamine 6 (Method 1):



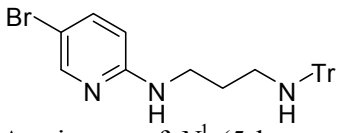
Reagents and conditions: (i) $H_2N(CH_2)_nNH_2$ (**2**), pyridine, reflux; (ii) TrCl, THF, Et_3N, 0 °C-r.t.; (iii) THF, LHMDS, R_1X, r.t.; (iv) TFA, DCM.

***N*^{1}-(5-bromopyridin-2-yl)propane-1,3-diamine (3a)**



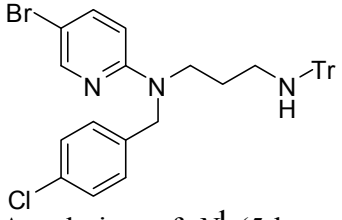
A mixture of 2, 5-dibromopyridine (**1a**, 2.0 g, 8.4 mmol), pyridine (0.80 g, 10.1 mmol), and 1, 3-diaminopropane (10 mL) was refluxed for 18 hours. The reaction mixture was concentrated *in vacuo* and dissolved in 15 mL of anhydrous dichloromethane. The white precipitation was filtered off and the filtrate was concentrated to yield *N*^{1}-(5-bromopyridin-2-yl)propane-1,3-diamine (**3a**, 1.6 g, 83%) as an oil, which was used directly for next reaction without further purification.

N^1-(5-bromopyridin-2-yl)-N^3-tritylpropane-1,3-diamine (4a)



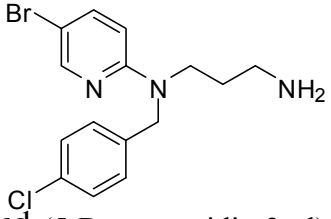
A mixture of N^1-(5-bromopyridin-2-yl)propane-1,3-diamine (**3a**, 5.0 g, 21.7 mmol) and triethylamine (2.6 g, 26 mmol) in anhydrous dichloromethane (50 mL) was slowly treated at 0 °C with trityl chloride (7.3 g, 26 mmol). After stirring at room temperature for 1 hour, the reaction mixture was washed with water (10 mL) for three times, dried over Na_2SO_4, filtered, and evaporated *in vacuo*. The residue was washed with methanol (50 mL×2) to afford N^1-(5-bromopyridin-2-yl)-N^3-tritylpropane-1,3-diamine (**4a**, 9.6 g, 79%) as a solid, which was used directly for next reaction without further purification.

N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-tritylpropane-1,3-diamine (5a)



A solution of N^1-(5-bromopyridin-2-yl)-N^3-tritylpropane-1,3-diamine (**4a**, 0.94 g, 2.0 mmol) in anhydrous tetrahydrofuran (10 mL) was treated at room temperature with LHMDS (6.0 mL, 1.0 M in tetrahydrofuran) under nitrogen atmosphere. After stirring at room temperature for half hour, another solution of 1-(bromomethyl)-4-chlorobenzene (2.05 g, 10 mmol) in 10 mL of anhydrous tetrahydrofuran was added slowly at room temperature to the reaction mixture. The resulting reaction mixture was stirred at room temperature for another hour after the addition was completed. Then, the mixture was poured into ice water (100 mL) and extracted with ethyl acetate (30 mL×3). The combined organic extracts were washed with water (20 mL), dried over Na_2SO_4, filtered, and concentrated. The crude product was further purified by silica column chromatography using hexanes/ethyl acetate (80:1) to yield N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-tritylpropane-1,3-diamine (**5a**, 0.60 g, 50%).

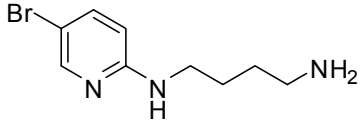
N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)propane-1,3-diamine (6a)



N^1-(5-Bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-tritylpropane-1,3-diamine (**5a**, 597 mg, 1.0 mmol) was dissolved in dichloromethane/TFA (5.0 mL/ 5.0 mL). The reaction mixture was stirred at room temperature for 1 hour. Volatiles were removed under vacuum and the residue was dissolved in methanol (50 mL). The methanol solution was washed by hexanes (10 mL×10); and 1 M NaOH was added to the solution to adjust pH = 7-8. Then the aqueous phase was extracted with dichloromethane (10 mL×3). The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated to yield N^1-

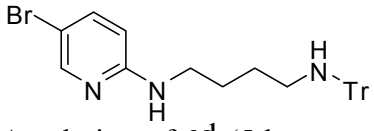
(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)propane-1,3-diamine (**6a**, 300 mg, 85%). ^{1}H NMR (300 MHz, CD_3OD) δ ppm 8.14 (d, J = 2.7 Hz, 1H), 7.43 (dd, J = 2.4 Hz, 9.0 Hz, 1H), 7.27–7.13 (m, 2H), 7.11 (d, J = 5.4 Hz, 2H), 6.33 (d, J = 9.0 Hz, 1H), 4.64 (s, 2H), 3.59 (t, J = 7.2 Hz, 2H), 2.73 (t, J = 6.6 Hz, 2H), 1.78–1.71 (m, 2H); LCMS (ESI): *m*/*z* 355.28 ($M + H^+$).

N^1-(5-bromopyridin-2-yl)butane-1,4-diamine (3e)



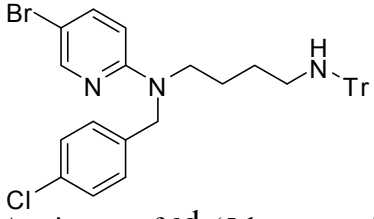
A mixture of 2,5-dibromopyridine (5.0 g, 20 mmol) and butane-1,4-diamine (27.8g, 310 mmol) was stirred at 155 °C for 3 hours. The reaction mixture was concentrated, dissolved in dichloromethane (50 mL), and filtered. The filtrate was evaporated to afford N^1-(5-bromopyridin-2-yl)butane-1,4-diamine (**3e**, 5g, 97%) as an oil which was used directly in the next reaction without further purification.

N^1-(5-bromopyridin-2-yl)-N^3-tritylbutane-1,4-diamine (4e)



A solution of N^1-(5-bromopyridin-2-yl)butane-1,4-diamine (**3e**, 5.0 g, 0.02 mmol) and triethylamine (2.48 g, 0.24 mmol) in tetrahydrofuran (20 mL) was treated at 0 °C with trityl chloride (5.4 g, 0.02 mmol) in several portions. After the addition was completed, the solution was stirred at room temperature for additional 1 hour. The reaction mixture was filtered; and the filtrate was evaporated to give N^1-(5-bromopyridin-2-yl)-N^3-tritylbutane-1,4-diamine (**4e**, 4.0 g, 80%).

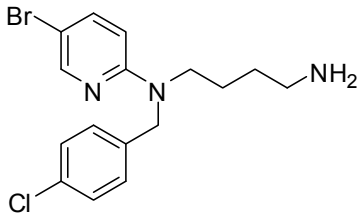
N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-tritylbutane-1,4-diamine (5e)



A mixture of N^1-(5-bromopyridin-2-yl)-N^3-tritylbutane-1,4-diamine (**4e**, 4g, 8.2 mmol) in anhydrous tetrahydrofuran (40 mL) was treated at room temperature with LHMDS (24.6 mL, 1.0 M in tetrahydrofuran) under nitrogen atmosphere. After stirring at room temperature for half hour, another solution of 1-(bromomethyl)-4-chlorobenzene (8.4g, 41.1 mmol) in 10 mL of anhydrous tetrahydrofuran was added slowly at room temperature to the reaction mixture. The resulting reaction mixture was stirred at room temperature for another hour after the addition was completed. Then, the mixture was poured into ice water (50 mL) and extracted with ethyl acetate (30 mL×3). The combined organic extracts were washed with water (20 mL), dried over Na_2SO_4, filtered, and concentrated. The crude product was further purified by silica column chromatography using hexanes/ethyl acetate (80:1) to yield N^1-(5-bromopyridin-2-yl)-N^1-(4-

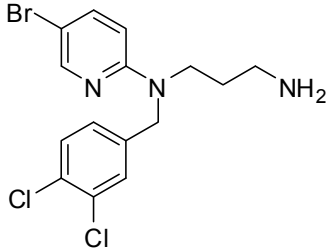
chlorobenzyl)-N^3-tritylbutane-1,4-diamine (**5e**, 3g, 60%) as a solid.

N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)butane-1,4-diamine (6e)



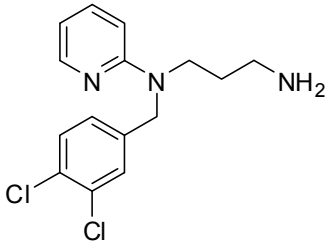
5e (350 mg, 0.57 mmol) was dissolved in dichloromethane/TFA (4.0 mL/ 2.0 mL). The reaction mixture was stirred at room temperature for 2 hour. The pH of the mixture was adjusted to 9 using 4 M NaOH. The aqueous layer was extracted with dichloromethane (5.0 mL×3). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (dichloromethane/MeOH=20:1) to afford **6e** (100 mg, 50%) as an oil.

N^1-(5-bromopyridin-2-yl)-N^1-(3,4-dichlorobenzyl)propane-1,3-diamine (6i)



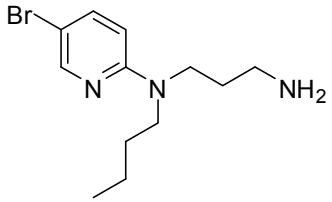
The compound was prepared according to general procedure for the synthesis of diamine **6** (Method 1). ^{1}H NMR (300 MHz, CD_3OD) δ ppm 8.26 (d, J = 2.4 Hz, 1H), 7.39 (dd, J = 2.4 Hz, 9 Hz, 1H), 7.34–7.25 (m, 1H), 7.20 (d, J = 1.5 Hz, 1H), 6.95 (dd, J = 2.1 Hz, 8.4 Hz, 1H), 6.28 (d, J = 9 Hz, 1H), 4.53 (s, 2H), 3.70 (t, J = 7.3 Hz, 2H), 3.04 (t, J = 6.3 Hz, 2H), 2.11–2.07 (m, 2H).

N^1-(3,4-dichlorobenzyl)-N^1-(pyridin-2-yl)propane-1,3-diamine (6b)



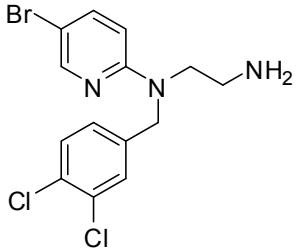
The compound was prepared according to general procedure for the synthesis of diamine **6** (Method 1). ^{1}H NMR (300 MHz, CD_3OD) δ ppm 8.15 (d, J = 2.4 Hz, 1H), 7.41–7.28 (m, 3H), 7.06 (m, 1H), 6.57 (m, 1H), 6.44 (d, J = 8.7 Hz, 1H), 4.70 (s, 2H), 3.59 (t, J = 7.2 Hz, 2H), 2.73 (t, J = 6.6 Hz, 2H), 1.77–1.72 (m, 2H); LCMS (ESI): *m/z* 311.38 (M + H^+).

N^1-(5-bromopyridin-2-yl)-N^1-butylpropane-1,3-diamine (6f)



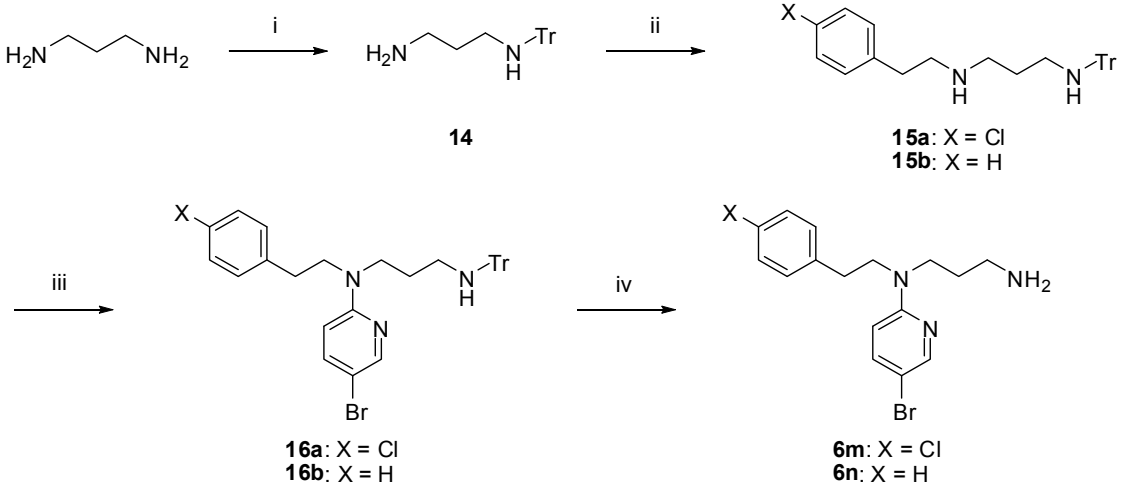
The compound was prepared according to general procedure for the synthesis of diamine **6** (Method 1). ^{1}H NMR (300 MHz, CD_3OD) δ ppm 8.11 (d, J = 2.4 Hz, 1H), 7.43 (dd, J = 3.0 Hz, 9.0 Hz, 1H), 6.37 (d, J = 9 Hz, 1H), 3.54 (t, J = 7.2 Hz, 2H),3.34 (t, J = 7.5 Hz, 2H), 2.71 (t, J = 6.6 Hz, 2H), 1.74-1.53 (m, 4H), 1.38-1.30 (m, 2H), 0.95 (t, J = 7.5 Hz, 2H); LCMS (ESI): *m/z* 287.36 ($M + H^+$).

N^1-(5-bromopyridin-2-yl)-*N*^{1}-(3,4-dichlorobenzyl)ethane-1,2-diamine (6d)



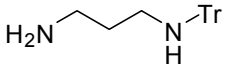
The compound was prepared according to general procedure for the synthesis of diamine **6** (Method 1). ^{1}H NMR (300 MHz, CD_3OD) δ ppm 8.16 (d, J = 2.4 Hz, 1H), 7.47 (dd, J = 2.4Hz, 9 Hz, 1H), 7.36 (d, J = 8.1 Hz, 1H), 7.29 – 7.27 (m, 1H), 7.05–7.02 (m, 1H), 6.39 (d, J = 9 Hz, 1H), 4.72 (s, 2H), 3.57 (t, J = 6.9 Hz, 2H), 2.93 (t, J = 6.9 Hz, 2H); LCMS (ESI): *m/z* 376.29 ($M + H^+$).

General procedure for the synthesis of diamine 6 (Method 2):



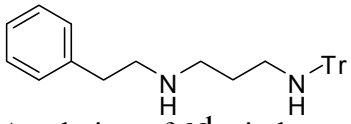
Reagents and conditions: (i) TrCl, 0 ^{o}C-r.t.; (ii) For **15b**: 2-phenylacetaldehyde, $NaBH_3CN$, MeOH, r.t.; For **15a**: 1-(2-bromoethyl)-4-chlorobenzene, DMF, K_2CO_3, 100 ^{o}C; (iii) 2,5-dibromopyridine, Cs_2CO_3, DMF, μW 180 ^{o}C; (iv) TFA, DCM.

N^1-tritylpropane-1,3-diamine (14)



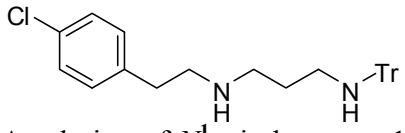
Propane-1,3-diamine (28 g, 0.38 mol) was placed in a flask and cooled with an ice-bath. Trityl chloride (7.0 g, 0.025 mol) was added to the flask in several portions. The reaction mixture was stirred at room temperature for 2 hours. The mixture was concentrated and treated with dichloromethane (50 mL). The suspension was filtered; and the filtrate was evaporated to afford N^1-tritylpropane-1,3-diamine (**14**, 7.9 g, 99%).

N^1-phenethyl-N^3-tritylpropane-1,3-diamine (15b)



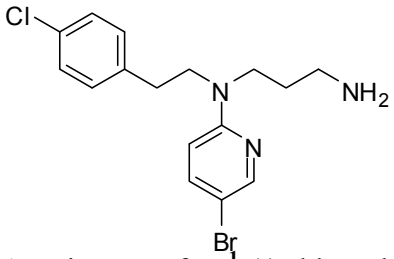
A solution of N^1-tritylpropane-1,3-diamine (**14**, 2.0 g, 6.3 mmol) in methanol (20 mL) was treated with 2-phenylacetaldehyde (0.76 g, 6.3 mmol). The mixture was stirred at room temperature for 2 hours, then treated with $NaBH_3CN$ (0.794g, 1.26 mmol) in portions. The reaction mixture was stirred at room temperature for overnight. After removing the solvent, water was added and the mixture was extracted with ethyl acetate (50 mL x 3). The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by silica-gel column chromatography (dichloromethane/MeOH=120:1 to 100:1) to afford N^1-phenethyl-N^3-tritylpropane-1,3-diamine (**15b**, 800 mg, 30%) as a solid.

N^1-(4-chlorophenethyl)-N^3-tritylpropane-1,3-diamine (15a)



A solution of N^1-tritylpropane-1,3-diamine (**14**, 1.5 g, 7.0 mmol) in DMF (5.0 mL) was treated with 1-(2-bromoethyl)-4-chlorobenzene (2.2 g, 7.0 mmol) and K_2CO_3 (2.0 g, 14 mmol). The reaction mixture was stirred at 100 °C for overnight. The mixture was concentrated and purified by silica-gel column chromatography (dichloromethane/methanol=50:1) to afford N^1-(4-chlorophenethyl)-N^3-tritylpropane-1,3-diamine as solid (**15a**, 1.0 g, 32%).

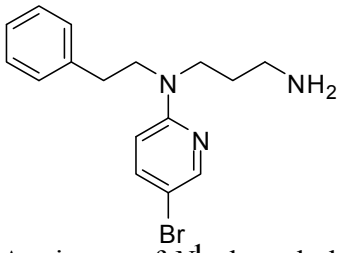
N^1-(4-chlorophenethyl)-N^1-(5-bromopyridin-2-yl)propane-1,3-diamine (6m)



A mixture of N^1-(4-chlorophenethyl)-N^3-tritylpropane-1,3-diamine (**15a**, 1.0 g, 2.2 mmol), 2,5-dibromopyridine (1.0 g, 4.4 mmol), Cs_2CO_3 (1.43 g, 4.4 mmol), and DMF (4 mL) in a sealed tube was heated at 180 °C for 20 min under microwave irradiation. The reaction mixture was diluted with ethyl acetate (30 mL) and washed with water. The organic layer was separated, dried over $MgSO_4$, filtered, and concentrated. The residue

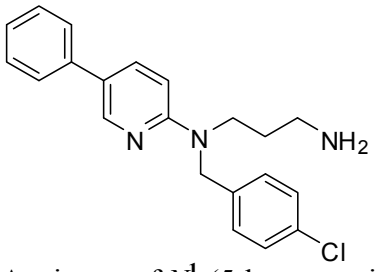
was purified by silica gel column chromatography (hexanes/ethyl acetate = 80:1) to afford **16a** (400 mg, 30%) as a solid.
A solution of **16a** (400 mg, 0.65 mmol) in dichloromethane (3.0 mL) was treated at 0 °C with CF_3COOH (3.0 mL). The reaction mixture was stirred at room temperature for 2 hours. The mixture was neutralized with 4 M NaOH until pH = 9 and extracted with dichloromethane (50 mLx3). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica-gel column chromatography (dichloromethane/methanol = 20:1) to afford N^1-(4-bromophenethyl)-N^1-(5-chloropyridin-2-yl)propane-1,3-diamine (**6m**, 170 mg, 71%) as an oil.

N^1-(5-bromopyridin-2-yl)-N^1-phenethylpropane-1,3-diamine (6n)



A mixture of N^1-phenethyl-N^3-tritylpropane-1,3-diamine (**15b**, 400 mg, 0.95 mmol), 2,5-dibromopyridine (677 mg, 2.85 mmol), Cs_2CO_3 (617 mg, 1.9 mmol), and DMF (4.0 mL) in a sealed tube was heated at 180 °C for 20 min under microwave irradiation. The mixture was diluted with ethyl acetate (30 mL) and washed with water. The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica-gel column chromatography (hexanes/ethyl acetate = 80:1) to afford **16b** (230 mg, 42%) as a solid.
A solution of **16b** (230 mg, 0.4 mmol) in dichloromethane (3.0 mL) was treated at 0 °C with CF_3COOH (3.0 mL). The reaction mixture was stirred at room temperature for 2 hours. The mixture was neutralized with 4 M NaOH until pH = 9. The mixture was extracted with dichloromethane (5.0 mL x 3). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica-gel column chromatography (dichloromethane/methanol = 20:1) to afford N^1-(5-bromopyridin-2-yl)-N^1-phenethylpropane-1,3-diamine (**6n**, 120 mg, 75 %) as an oil.

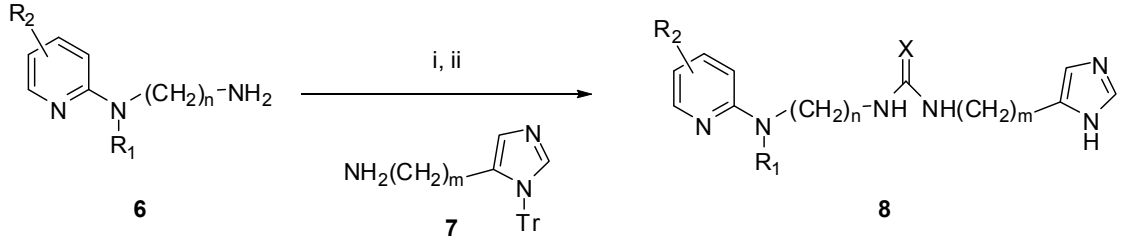
N^1-(4-chlorobenzyl)-N^1-(5-phenylpyridin-2-yl)propane-1,3-diamine (9)



A mixture of N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-tritylpropane-1,3-diamine (**5a**, 700 mg, 1.17 mmol), phenylboronic acid (250 mg, 2.0 mmol), Na_2CO_3 (212 mg, 2.0 mmol), and $Pd(PPh_3)_4$ (70.0 mg) in ethanol/H_2O (7.0 mL/ 2.0 mL) was heated to 100 °C for 2 hours. When the mixture was cooled, the product was precipitated from the reaction solution. The solid was filtered and dissolved in dichloromethane/TFA (10.0 mL/ 10.0

mL). The mixture was stirred at room temperature for 1 hour. Volatile was removed under vacuum and the residue was dissolved in methanol (50.0 mL). The methanol solution was washed by hexanes (10 mL × 3), concentrated to yield N^1-(4-chlorobenzyl)-N^1-(5-phenylpyridin-2-yl)propane-1,3-diamine as a crude product, which was used directly in the next reaction without further purification.

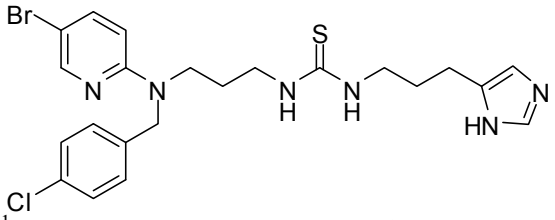
General procedure for the formation of thiourea and urea:



Reagents and conditions: (i) *i*PrNEt$_2$, **7**, 1,1'-thiocarbonyldiimidazole or 1,1'-carbonyldiimidazole, DCM/CH_3CN, μW 120-150 ^oC; (ii) TFA, DCM.

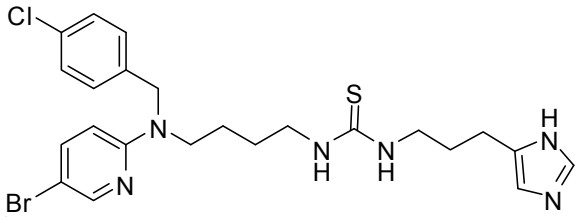
A solution of **6** (0.16 mmol, 1 equiv) in dichloromethane (1.5 mL) was added 1,1'-thiocarbonyldiimidazole or 1,1'-carbonyldiimidazole (0.16 mmol, 1 equiv). The mixture was stirred at room temperature for 3 hours and then treated with another solution of **7** (0.16mmol, 1 equiv.) and DIPEA (0.08 mmol, 0.5 equiv) in CH_3CN (1.5 mL). The reaction mixture was heated at 150 °C for 10 minutes under microwave irradiation. The mixture was concentrated, dissolved in 3.0 mL of dichloromethane, and treated at 0 °C with 3.0 mL of TFA. The mixture was stirred at room temperature for 2 hours. Volatile was removed and the residue was purified by HPLC to afford **8** (10-40%).

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)thiourea (8a)



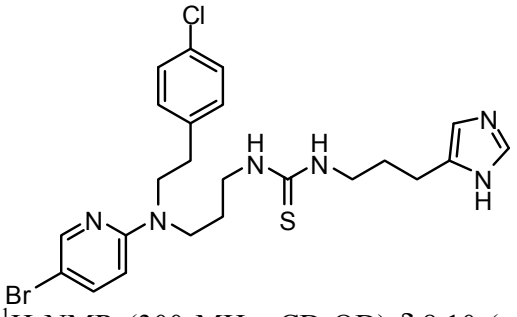
^{1}H NMR (300 MHz, CD_3OD) δ 7.54 (s, 1H), 7.30 (s, 1H), 7.27 (d, J = 9.0 Hz, 2H), 7.20-7.15(m, 4H), 6.79 (s, 1H), 6.59 (d, J = 12 Hz, 1H), 4.51 (s, 2H), 3.46-3.40 (m, 6H), 2.60 (t, J = 6.0 Hz, 3H), 1.91-1.85 (m, 4H); HPLC (method 2): t_R = 2.02 min, UV_{220} = 87%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{27}BrClN_5S$ 520.0926, found 520.0928.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)butyl)thiourea (8e)



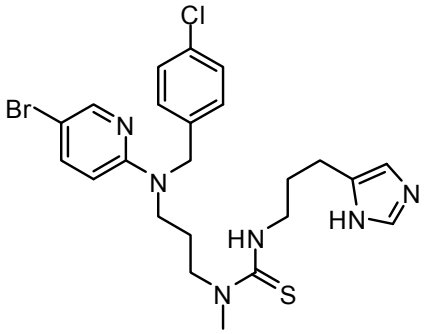
^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (s, 1H), 7.77 (s, 1H), 7.52 (d, J = 3.0 Hz, 1H), 7.27 (d, J = 6.0 Hz, 2H), 7.18 (d, J = 9.0 Hz, 2H), 6.89 (s, 1H), 6.51 (d, J = 9.0 Hz, 1H), 4.71 (s, 2H), 3.55-3.43(m, 6H), 2.63 (t, J = 7.5 Hz, 2H), 1.90-1.64 (m, 2H), 1.61-1.58 (m, 4H); HPLC (method 2): t_R = 1.91 min, UV_{220} = 95%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{28}BrClN_6S$ 534.0968, found 534.0964.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorophenethyl)amino)propyl)thiourea (8m)



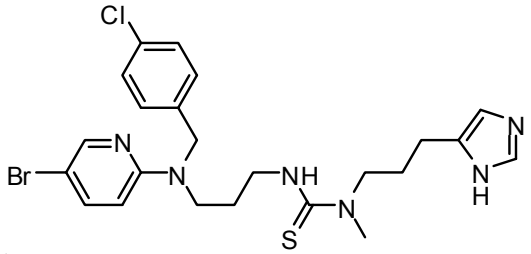
^{1}H NMR (300 MHz, CD_3OD) δ 8.10 (s, 1H), 7.56 (m, 2H), 7.26-7.20 (m, 4H), 6.81(s, 1H), 6.57 (d, J = 9.0 Hz, 1H), 3.65 (t, J = 7.5 Hz, 2H), 3.54-3.38 (m, 6H), 2.87 (t, J = 3.0 Hz, 2H), 2.63 (t, J = 6.0 Hz, 2H), 1.91-1.88 (m, 4H); HPLC (method 2): t_R = 1.86 min, UV_{220} = 100%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{28}BrClN_6S$ 534.0968, found 534.0969.

3-(3-(1H-imidazol-5-yl)propyl)-1-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)-1-methylthiourea (26)



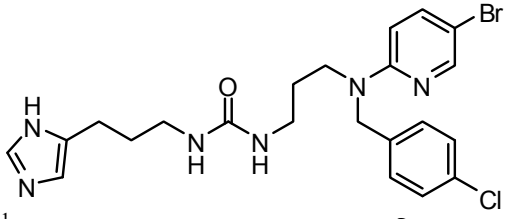
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 8.08(s, 1H), 7.52 (m, 1H), 7.35(s, 1H), 7.28 (d, J = 9.0 Hz, 2H), 7.20 (d, J = 6.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 1H), 4.74(s, 2H), 3.83 (m, 2H), 3.66 (m, 2H), 3.62(m, 2H), 3.07(s, 3H), 2.75 (m, 2H), 1.99-1.92(m, 4H); HPLC (method 2): t_R = 1.96 min, UV_{220} = 95%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{28}BrClN_6S$ 534.0968, found 534.0968.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)-1-methylthiourea (27)



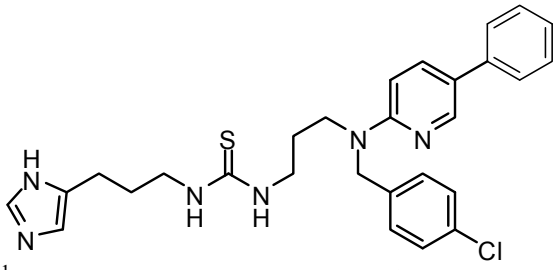
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 8.08(s, 1H), 7.52 (m, 1H), 7.39(s,1H), 7.29 (d, J = 9.0 Hz, 2H), 7.19 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 1H), 4.72(s, 2H), 3.92 (m, 2H), 3.66 (m, 2H), 3.65-3.58(m, 4H), 3.10(s, 3H), 2.75 (m, 2H), 2.05-1.95(m, 4H); HPLC (method 2): t_R = 1.95 min, UV_{220} = 100%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{28}BrClN_6S$ $[M+H^+]$ 534.0966, found 534.0968.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)urea (8k)



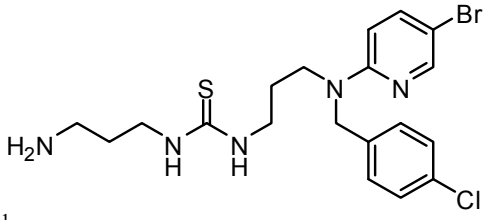
^{1}H NMR (300 MHz, CD_3OD) δ 8.76 (s, 1H), 8.09(s, 1H), 7.52 (m, 1H), 7.29 (d, J= 9.0 Hz, 3H), 7.18 (d, J = 9.0 Hz, 2H), 6.55 (m, 1H), 4.71(s, 2H), 3.56 (m, 2H), 3.19-3.13 (m, 4H), 3.72-3.65 (m, 3H), 1.83-1.76 (m, 4H); HPLC (method 2): t_R = 1.83 min, UV_{220} = 100%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{22}H_{26}BrClN_6O$ 504.1040, found 504.1038.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-phenylpyridin-2-yl)(4-chlorobenzyl)amino)butyl)thiourea (10)



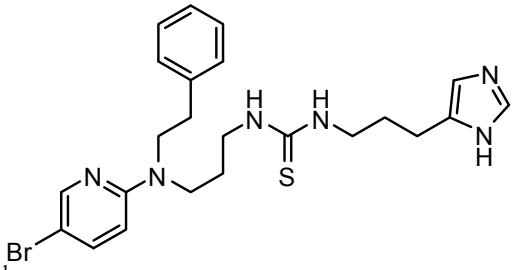
^{1}H NMR (300 MHz, CD_3OD) δ 8.35 (s, 1H), 7.75-7.71 (m, 1H), 7.67 (s, 1H), 7.54-7.49 (m, 2H), 7.40-7.35 (m, 2H), 7.32-7.19 (m, 5H), 6.86 (s, 1H), 6.66 (d, J = 9.0 Hz, 1H), 3.66-3.47 (m, 3H), 3.34-3.30 (m, 4H), 2.63 (t, J = 7.5 Hz, 2H), 1.93-1.87 (m, 4H); HPLC (method 2): t_R = 1.70 min, UV_{220} = 93%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{28}H_{31}ClN_6S$ 518.2019, found 518.2019.

1-(3-aminopropyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)thiourea (13a)



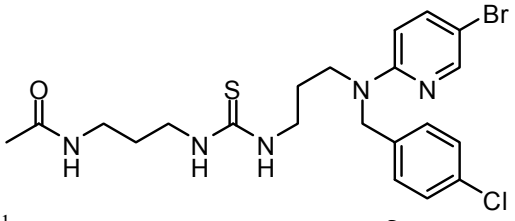
^{1}H NMR (300 MHz, CD_3OD) δ 8.13 (s, 1H), 7.56 (m, 1H), 7.31 (d, J = 6.0 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 1H), 4.73 (s, 2H), 3.66-3.30 (m, 6H), 2.96 (m, 2H), 1.91-1.86 (m, 4H); HPLC (method 2): t_R = 1.87 min, UV_{220} = 100%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{19}H_{25}BrClN_5S$ 469.0703, found 469.0703.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(phenethyl)amino)propyl)thiourea (8q)



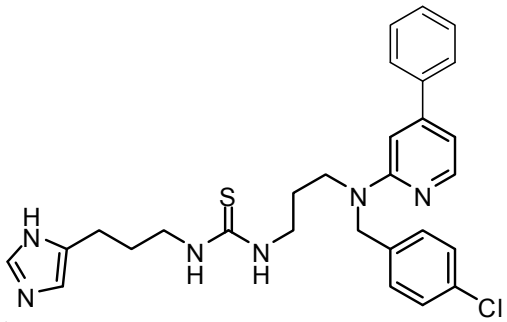
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 8.08 (s, 1H), 7.66-7.62 (m, 1H), 7.34 (s, 1H), 7.28-7.18 (m, 5H), 6.71 (d, J = 9.0 Hz, 1H), 3.70 (t, J = 7.5 Hz, 2H), 3.54-3.52 (m, 2H), 3.46 (t, J = 7.5 Hz, 4H), 2.90 (t, J = 7.5 Hz, 3H), 2.76 (t, J = 7.5 Hz, 2H), 1.97-1.92 (m, 2H), 1.85-1.80 (m, 2H); HPLC (method 2): t_R = 1.77 min, UV_{220} = 100%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{29}BrClN_6S$ 500.1358, found 500.1356.

***N*-(3-(3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)thioureido)propyl)acetamide (13b)**



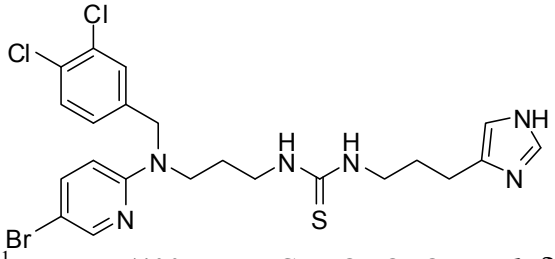
^{1}H NMR (300 MHz, CD_3OD) δ 8.12 (s, 1H), 7.64 (m, 1H), 7.31 (d, J = 6.0 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 6.65 (d, J = 9.0 Hz, 1H), 4.75 (s, 2H), 3.97 (m, 2H), 3.62-3.57 (m, 4H), 3.18 (t, J = 7.5 Hz, 2H), 1.93 (s, 3H), 1.92-1.83(m, 2H), 1.72 (t, J = 7.5 Hz, 2H); HPLC (method 2): t_R = 2.14 min, UV_{220} = 91%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{21}H_{27}BrClN_5OS$ 511.0808, found 511.0803.

1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((4-phenylpyridin-2-yl)(4-chlorobenzyl)amino)butyl)thiourea (8c)



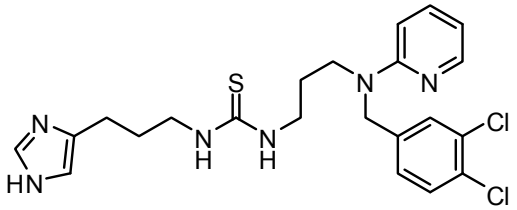
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.78-7.75 (m, 2H), 7.54-7.52 (m, 3H), 7.41-7.38 (m, 3H), 7.31-7.27 (m, 4H), 4.96 (s, 2H), 3.78 (m, 2H), 3.60-3.40 (m, 4H), 2.72-2.65 (m, 2H), 1.93 (m, 2H), 1.91 (m, 2H); HPLC (method 2): t_R = 1.70 min, UV_{220} = 93%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{28}H_{31}ClN_6S$ 518.2019, found 518.2019.

1-[3-[(5-bromopyridin-2-yl)-[(3,4-dichlorophenyl)methyl]amino]propyl]-3-[3-(1H-imidazol-5-yl)propyl]thiourea (NCGC00159568, ML156, 8i)



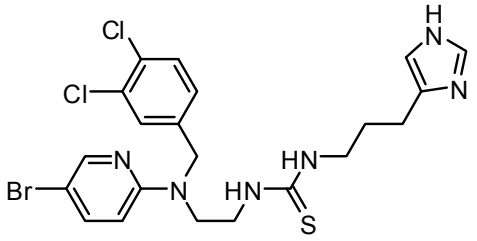
^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 1.85-1.99 (m, 4H), 2.68 (m, 2H), 3.61 (m, 6H), 4.60 (s, 2H), 6.31 (d, J = 9.2 Hz, 1H), 6.81 (s, 1H), 7.00 (dd, J = 8.3, 1.9 Hz, 1H), 7.23 (d, J = 1.8 Hz, 1H), 7.36 (d, J = 8.2 Hz, 1H), 7.44 (dd, J = 9.0, 2.5 Hz, 1H), 7.52 (s, 1H), 8.12 (br. s., 1H); HPLC (method 1): t_R = 5.25 min, UV_{220} = 99%; MS m/z 555.1 ($M+H^+$); HRMS (ESI): *m/z* calcd for $C_{22}H_{25}BrCl_2N_6S$ 554.0422, found 554.0434.

1-(3-(1H-imidazol-4-yl)propyl)-3-(3-((3,4-dichlorobenzyl)(pyridin-2-yl)amino)propyl)thiourea (8b)



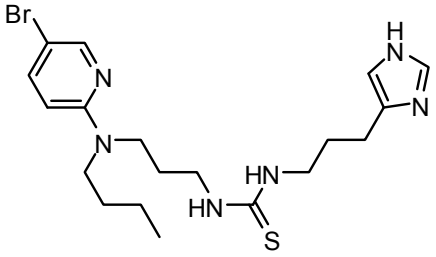
HPLC (method 2): t_R = 1.62 min, UV_{220} = 100%, ELSD = 100%; MS (ESI): *m/z* 476.9 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(2-((5-bromopyridin-2-yl)(3,4-dichlorobenzyl)amino)ethyl)thiourea (8d)



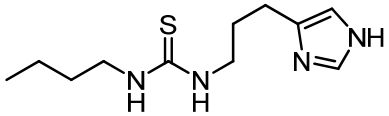
HPLC (method 2): t_R = 1.99 min, UV_{220} = 100%, ELSD = 100%; MS (ESI): *m/z* 540.8 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(butyl)amino)propyl)thiourea (8f)



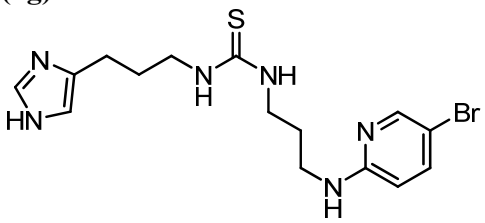
HPLC (method 1): t_R = 3.91 min, UV_{220} = 80%; MS (ESI): *m/z* 453.1 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-butylthiourea



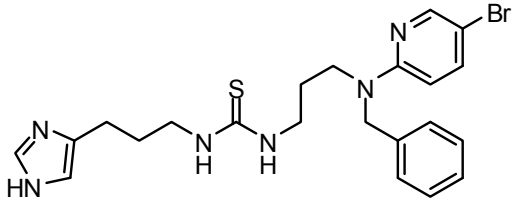
HPLC (method 1): t_R = 3.23 min, UV_{220} = 100%; MS (ESI): *m/z* 241.1 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(3-(5-bromopyridin-2-ylamino)propyl)thiourea (8g)



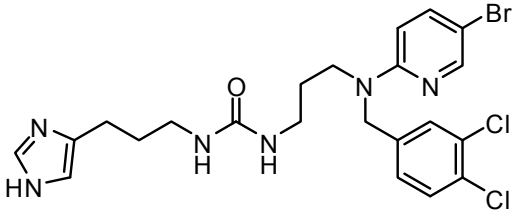
HPLC (method 1): t_R = 2.87 min, UV_{220} = 100%; MS (ESI): *m/z* 397.0 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(3-(benzyl(5-bromopyridin-2-yl)amino)propyl)thiourea (8h)



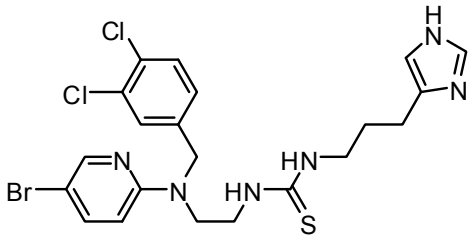
HPLC (method 1): t_R = 4.45 min, UV_{220} = 100%; MS (ESI): *m/z* 487.1 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(3,4-dichlorobenzyl)amino)propyl)urea (8j)



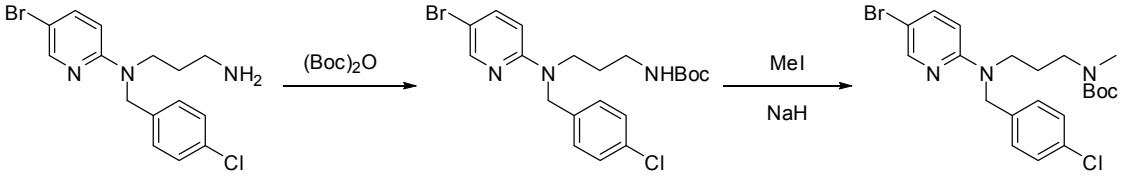
HPLC (method 1): t_R = 4.89 min, UV_{220} = 100%; MS (ESI): *m*/*z* 539.1 ($M+H^+$).

1-(3-(1H-imidazol-4-yl)propyl)-3-(2-((5-bromopyridin-2-yl)(3,4-dichlorobenzyl)amino)ethyl)thiourea (8l)

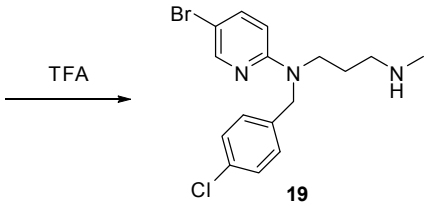


HPLC (method 1): t_R = 5.13 min, UV_{220} = 90%; MS (ESI): *m*/*z* 541.1 ($M+H^+$).

Synthesis of N^1-(5-bromopyridin-2-yl)-N^1-(4-chlorobenzyl)-N^3-methylpropane-1,3-diamine (19)



6a **17** **18**



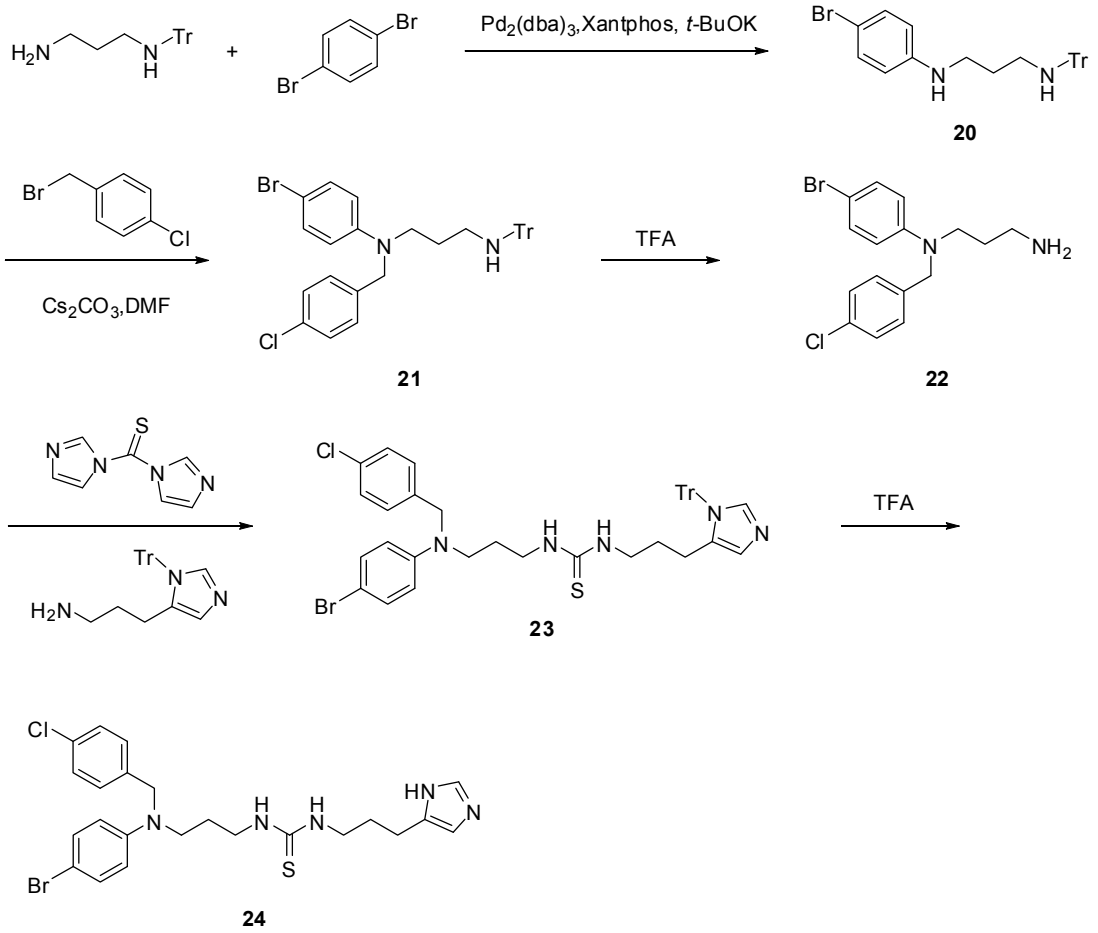
19

A mixture of **6a** (300 mg, 0.85 mmol), $(Boc)_2O$ (220 mg, 1.02 mmol), and triethylamine (103 mg, 1.02 mmol) in tetrahydrofuran (10 mL) was stirred at 30 °C for 3 hours. Volatile was removed and the residue was dissolved in anhydrous tetrahydrofuran and treated with sodium hydride (50.0 mg, 60%, 1.3 mmol) in one portion. The reaction mixture was heated to 60 °C for half hour and treated with iodomethane (600 mg, 4.2 mmol) with caution. The mixture was stirred at 60 °C for overnight, cooled to room temperature and poured into ice-water (20.0 mL). The aqueous phase was extracted with dichloromethane (10.0 mL×3). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to give crude **18** (350 mg).

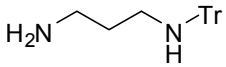
A mixture of crude **18** (350 mg) in TFA/tetrahydrofuran (4.0 mL/ 10.0 mL) was stirred at

30 °C for 5 hours. After the pH value of the reaction solution was adjusted to pH = 7-8 using 1 M of NaOH, the mixture was extracted with dichloromethane (20.0 mL×3). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified by silica-gel column chromatography (dichloromethane/methanol = 40:1) to afford **19** (0.20 g, 54% for 4 steps).

Synthesis of 1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((4-bromophenyl)(4-chlorobenzyl)amino)propyl)thiourea

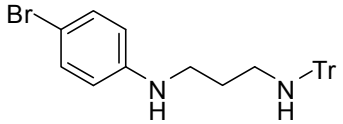


***N*^{1}-tritylpropane-1,3-diamine (20)**



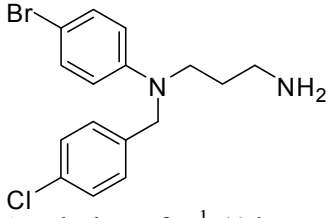
Propane-1,3-diamine (28 g, 0.38 mol) was placed in a flask, cooled with an ice-bath and treated with trityl chloride (7.0 g, 0.025 mol) in several portions. The mixture was stirred at room temperature for 2 hours. After removing solvent, the residue was treated with dichloromethane (50 mL). The mixture was filtered; and the filtrate was evaporated to afford **20** (7.9 g, 99%) as an oil.

***N*^{1}-(4-bromophenyl)-*N*^{3}-tritylpropane-1,3-diamine (21)**



A mixture of **20** (200 mg, 0.63 mmol), 1,4-dibromobenzene (150 mg, 0.63 mmol), $Pd_2(dba)_3$ (58 mg, 0.063 mmol), Xantphos (83 mg, 0.063 mmol), and *t*-BuOK(140 mg, 1.26 mmol) in 1,4-dioxane (3.0 mL) was heated at 125 °C for 30 min under microwave irradiation. After removing volatile, the crude product was purified by silica gel column chromatography (hexanes/ethyl acetate from 80:1 to 50:1) to afford **21** (200 mg, 67%) as an oil.

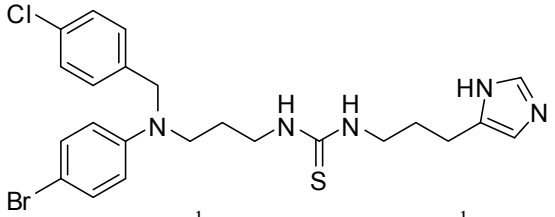
N^1-(4-bromophenyl)-N^1-(4-chlorobenzyl)propane-1,3-diamine (23)



A solution of N^1-(4-bromophenyl)-N^3-tritylpropane-1,3-diamine (370 mg, 0.78 mmol) in DMF (5.0 mL) was treated with 1-bromomethyl-4-chlorobenzene (191 mg, 0.94 mmol) and Cs_2CO_3 (510 mg, 1.57 mmol). The mixture was stirred at 100 °C for overnight. After removing solvent, the crude product was purified by silica gel column chromatography (hexanes/ethyl aceate = 80:1) to afford **22** (310 mg, 70 %).

A solution of **22** (300 mg, 0.5 mmol) in dichloromethane (2.0 mL) was treated at 0 °C with CF_3COOH (2.0 mL). After stirring at room temperature for 2 hours, the reaction mixture was neutralized using 4 M NaOH solution until pH = 9. The mixture was extracted with dichloromethane (5.0 mL×3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 20:1) to afford **23** (150 mg, 84%) as an oil.

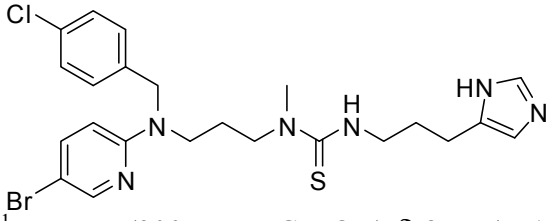
1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((4-bromophenyl)(4-chlorobenzyl)amino) propyl) thiourea (24)



A mixture of N^1-(4-bromophenyl)-N^1-(4-chlorobenzyl)propane-1,3-diamine (50 mg, 0.14 mmol), 4-(1-trityl-1H-imidazol-5-yl)butan-1-amine (50 mg, 0.14 mmol), 1,1'-thiocarbonyldiimidazole (25 mg, 0.14 mmol), DIPEA (9 mg, 0.07 mmol), dichloromethane (1.0 mL) and CH_3CN (1.0 mL) in a sealed tube was heated at 125 °C for 10 min under microwave irradiation. After removing volatile, the crude product was dissolved in dichloromethane (2.0 mL) and treated at 0 °C with CF_3COOH (2.0 mL). The reaction mixture was stirred at room temperature for 2 hours. After removing the solvent, the residue was purified by HPLC to afford **24** (6.0 mg, 8%) as an oil. ^{1}H NMR (300

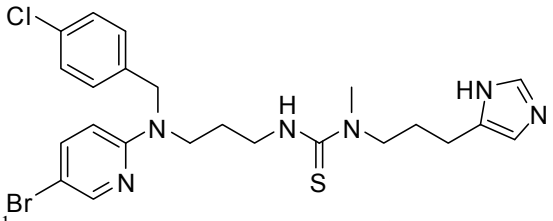
MHz, CD_3OD) δ 7.54 (s, 1H), 7.30 (s, 1H), 7.27 (d, J = 9.0 Hz, 2H), 7.20-7.15 (m, 4H), 6.79 (s, 1H), 6.61-6.57 (m, 1H), 4.51 (s, 2H), 3.46-3.40 (m, 6H), 2.60 (t, J = 6.0 Hz, 3H), 1.91-1.85 (m, 4H); HPLC (method 2): t_R = 2.07 min, UV_{220} = 87%, ELSD = 100%; HRMS (ESI): *m/z* calcd for $C_{23}H_{27}BrClN_5S$ 519.0859, found 519.0855.

3-(3-(1H-imidazol-5-yl)propyl)-1-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)-1-methylthiourea (26)



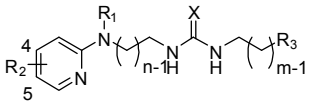
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 8.08 (s, 1H), 7.52 (m, 1H), 7.35 (s, 1H), 7.29 (d, J = 9.0 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 1H), 4.74 (s, 2H), 3.83 (m, 2H), 3.66 (m, 2H), 3.62 (m, 2H), 3.07 (s, 3H), 2.75 (m, 2H), 1.99-1.92 (m, 4H); HPLC (method 2): t_R = 1.97 min, UV_{220} = 100%, ELSD = 100%; MS (ESI): *m/z* 537.1, 535.1 ($M+H^+$).

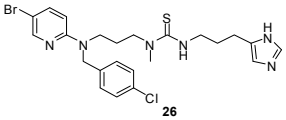
1-(3-(1H-imidazol-5-yl)propyl)-3-(3-((5-bromopyridin-2-yl)(4-chlorobenzyl)amino)propyl)-1-methylthiourea (27)



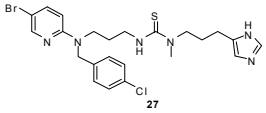
^{1}H NMR (300 MHz, CD_3OD) δ 8.77 (s, 1H), 8.08 (s, 1H), 7.52 (m, 1H), 7.39 (s,1H), 7.29 (d, J = 9.0 Hz, 2H), 7.19 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 1H), 4.72 (s, 2H), 3.92 (m, 2H), 3.66 (m, 2H), 3.65-3.58 (m, 4H), 3.10 (s, 3H), 2.75 (m, 2H), 2.05-1.95 (m, 4H); HPLC (method 2): t_R = 1.96 min, UV_{220} = 100%, ELSD = 100%; MS (ESI): *m/z* 537.1, 535.1 ($M+H^+$).

Compounds Table 1 with key ID

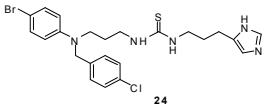




26



27

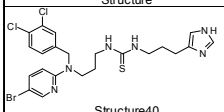
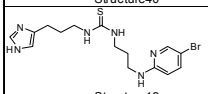
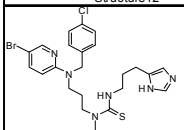
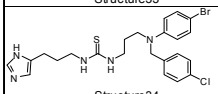
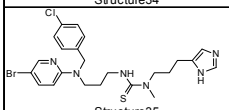
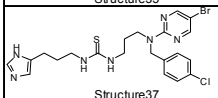
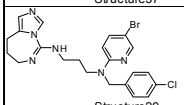
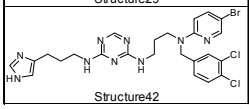


24

Entry	Cmpd. No.	CID	SID	NCGC ID	*	R1	R2	R3	n	m	X	AC50
1	**8i**	9893924	29215544	NCGC00159568-01	P	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	S	0.6±0.1
2	**8i**	9893924	89449177	NCGC00159568-02	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	S	0.6±0.1
3	**8a**	44820550	89449189	NCGC00185835	S	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	S	1.2
4	**8f**	44820562	89449191	NCGC00185837	S	Butyl	5-Br	4-Imidazole	3	3	S	8.2
5	**8h**	44820554	89449193	NCGC00185839	S	Benzyl	5-Br	4-Imidazole	3	3	S	5.8
6	**8g**	44820544	89449192	NCGC00185838	S	H	5-Br	4-Imidazole	3	3	S	46.0
7	**8b**	44820546	89449194	NCGC00185840	S	3,4-Dichlorobenzyl	H	4-Imidazole	3	3	S	7.3
8	**10**	44820557	89449208	NCGC00187953	S	4-Chlorobenzyl	5-Ph	4-Imidazole	3	3	S	1.3
9	**8c**	44820553	89449213	NCGC00187959	S	4-Chlorobenzyl	4-Ph	4-Imidazole	3	3	S	4.0
10	**8d**	10554498	89449195	NCGC00185841	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	2	3	S	2.1
11	**8e**	44820570	89449200	NCGC00187945	S	4-Chlorobenzyl	5-Br	4-Imidazole	4	3	S	0.8
12	**8l**	44820548	89449197	NCGC00185843	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	2	S	18.3
13	**8j**	5311371	89449196	NCGC00185842	S	3,4-Dichlorobenzyl	5-Br	4-Imidazole	3	3	O	0.6
14	**8k**	44820568	89449205	NCGC00187950	S	4-Chlorobenzyl	5-Br	4-Imidazole	3	3	O	1.0
15	**13a**	44820542	89449209	NCGC00187954	S	4-Chlorobenzyl	5-Br	NH2	3	3	S	45.0
16	**13b**	44820564	89449212	NCGC00187957	S	4-Chlorobenzyl	5-Br	NHCOCH3	3	3	S	inactive
17	**25**	44820567	89449198	NCGC00185844	S	3,4-Dichlorobenzyl	5-Br	CH3	3	3	S	inactive
18	**8q**	44820543	89449210	NCGC00187955	S	Phenylethyl	5-Br	4-Imidazole	3	3	S	2.8
19	**8m**	44820565	89449201	NCGC00187946	S	4-Chlorophenylethyl	5-Br	4-Imidazole	3	3	S	2
20	**26**	44820569	89449202	NCGC00187947	S							2.8
21	**27**	44820563	89449204	NCGC00187949	S							18
22	**24**	44820545	89449203	NCGC00187948	S							1.3

* P = purchased S = synthesized

Compounds Table 2 with key ID and SST4 data

Structure	Sample Data Type	Curve Class	AC50(uM)	Cerep ID	ID	sst4 IC50 (M)	sst4 Ki (M)
 Structure40	N370S-spleen	-1.1	0.6352	17998-2	NCGC00159568-03	1.80E-08	1.70E-08
 Structure12	N370S-spleen	-2.2	46.0191	17998-1	NCGC00185838-01	7.00E-07	6.90E-07
 Structure33	N370S-spleen	-1.1	2.5288	17998-4	NCGC00187947-01	1.90E-08	1.90E-08
 Structure34	N370S-spleen	-1.1	1.0067	17998-5	NCGC00187948-01	1.60E-07	1.60E-07
 Structure35	N370S-spleen	-1.1	12.674	17998-6	NCGC00187949-01	2.60E-08	2.60E-08
 Structure37	N370S-spleen	-1.1	1.5956	17998-8	NCGC00187951-01	5.20E-08	5.20E-08
 Structure29	N370S-spleen	-1.1	5.0456	17998-3	NCGC00187958-01	4.80E-08	4.80E-08
 Structure42	N370S-spleen	-1.1	5.0456	17998-7	NCGC00187980-01	3.30E-08	3.30E-08

All animal experiments were performed through CROs based on fee-for-services. Studies are in compliance with the relevant local laws and institutional guidelines.

***In vivo* pharmacokinetic profiles and parameters of NCGC00159568-03 (ML156, 8i) after an IP dose of 30 mg/kg in Male C57BL/6 Mice.**

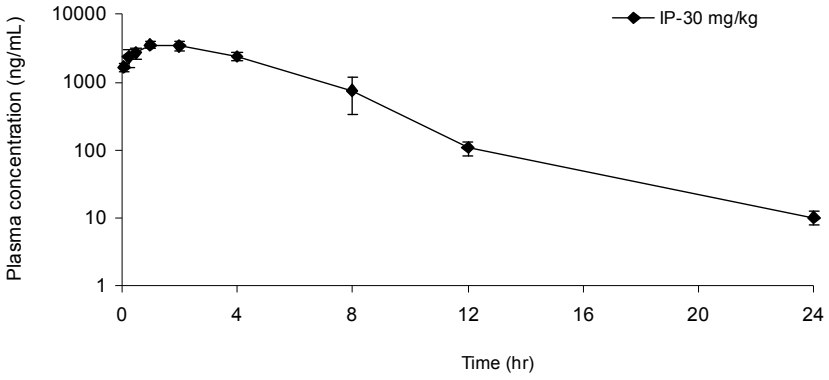
1. SUMMARY

Individual and mean plasma concentration-time data of NCGC00159568-03 after an IP dose of 30 mg/kg in male C57BL/6 mice

Dose (mg/kg)	Dose route	Sampling time (hr)	Concentration (ng/mL) Individual			Mean (ng/mL)	) M	SD	CV(%)
30	IP	0	BQL	BQL	BQL	BQL	BQL	N/A	N/A
		0.083	1820	1820	1410	1683	3.026	237	14.1
		0.25	2420	3010	1640	2357	4.236	687	29.2
		0.5	2530	2270	3270	2690	4.835	519	19.3
		1	3740	3770	3150	3553	6.387	350	9.84
		2	2870	3590	3840	3433	6.171	504	14.7
		4	2560	2030	2570	2387	4.290	309	12.9
		8	1190	357	714	754	1.355	418	55.5
		12	118	127	80.7	109	0.195	24.5	22.6
		24	8.04	12.6	9.98	10.2	0.018	2.29	22.4

PK parameters	Unit	Estimate
T_{max}	hr	1.00
C_{max}	ng/mL	3550 (6.38 mM)
$T_{1/2}$	hr	2.57
AUC_{last}	hr*ng/mL	20600
AUC_{INF}	hr*ng/mL	20700

Mean plasma concentration-time profiles of NCGC00159568-03 after an IP dose of 30 mg/kg in male C57BL/6 mice (N=3)



2. CLINICAL OBSERVATION

In the PK studies of NCGC00159568-03, slight mobility reduction was observed in all 15 animals at 5 minutes after IP administration and lasted for about 15 minutes. The clinical observations were summarized in the Table 1.

Table 1. Summary of Animal Clinical Observation for NCGC00159568-03

Compound ID	Formulation	Dose	Clinical Observations	Duration of Clinical Observation
NCGC00159568-03	5% DMAC 5% Solutol 90% 10 mM Phosphate buffer (pH7.4)	30 mg/kg	Slight mobility reduction	5 – 20 minutes after IP administration

Note: The clinical symptoms described above were observed in all 15 animals.

3. STUDY DESIGN

Treatment Group	Treatment	No. of animals	Route of admin.	Dose Level (mg/kg)	Dose Conc. (mg/mL)	Dose Volume (mL/kg)	Time points
1	NCGC00159568-03	15	IP	30	3	10	Pre-dose, 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hr, plasma only

Test article	NCGC00159568-03
Test system	C57BL/6 mice, 20-21 g, male, N=15, purchased from SLAC Laboratory Animal Co. LTD Qualification No.: SCXK（SH）2007-0005 04056
Food status	Free access to food and water
Administration	IP: 30 mg/kg (10 mL/kg) via lower left abdominal quadrant injection (N=3)
Blood collection	The animals were anesthetized with isoflurane and restrained manually at the designated time points. Approximately 120 μL of blood samples were taken from the animals into K_2EDTA tube via retro-orbital puncture. Blood samples were put on ice and centrifuged to obtain plasma sample (2000 g, 5 min under 4°C) within 15 minutes post sampling.
Sample storage and disposition	Plasma samples were stored at approximately -70 °C until analysis. The backup samples will be discarded after one month unless requested.
Data storage	All raw data will be kept electronically in the computers at ChemPartner for 2 years.

Sampling design after IP administration

Dosing route	Time points	Animal No. (#1~#15)
IP	Pre-dose	#1, #2, #3
	0.083	#4, #5, #6
	0.25	#7, #8, #9
	0.5	#10, #11, #12
	1	#13, #14, #15
	2	#1, #2, #3
	4	#4, #5, #6
	8	#7, #8, #9
	12	#10, #11, #12
	24	#13, #14, #15

4. FORMULATION

IP: 5% DMAC+5% Solutol HS 15+90% 10 mM pH7.4 phosphate buffer

Preparation of IP solution (30 mg/kg, 10 mL/kg)

3 mg/mL solution of 5% DMAC+5% Solutol HS 15+90% 10 mM pH7.4 phosphate buffer

1) Weigh 15.28 mg NCGC00159568-03 to a clean vial.

2) Add 0.255 mL of DMAC into the vial containing NCGC00159568-03, shake it for 1 min and then sonicate for 1 min.

3) Add 0.255 mL of Solutol into the mixture, shake it for 1 min and then sonicate for 3 min.

4) Add 4.584 mL of 10 mM pH9.0 carbonate buffer into the mixture, shake it for 1 min and then sonicate for 1 min.

Note: The formulation of NCGC00159568-03 was prepared just prior to use.

Formulation Checklist		IP		
Process:	no manipulation		sonicate	√
	heat (high)		add EtOH	-
	dilute		heat (low)	
	vortex	√	filtered	-
	add acid / base	-	heat (med)	
	preformulated	√	centrifuge/supernatant	
	other _vehicle ingredients:	5% DMAC+5% Solutol HS 15+90% 10 mM pH7.4 phosphate buffer		
Result:	clear/OK	√	ppt'd out	-
	particulate		color	colorless
	suspension		emulsion	
	cloudy		other	
	homogenous	-		

5. LCMS METHOD

Instrument LC-MS/MS-02 (API4000)

Matrix Male C57BL/6 mouse plasma

Analyte(s) NCGC00159568-03

Internal standard(s) Testosterone

MS conditions

Positive ion, ESI

MRM detection

NCGC00159568-03: $[M+H]^+$ m/z 557.1/390.1

Testosterone: $[M+H]^+$ m/z 289.2/97.2

HPLC conditions

Mobile phase:

Mobile Phase A: H_2O/ 0.025%FA/ 1 mM NH_4OAc

Mobile Phase B: ACN/ 0.025%FA/ 1 mM NH_4OAc

Time (min)	Pump B (%)
0.30	5.0
0.80	98
2.40	98
2.41	5
3.50	Stop

Column: Sepax-BR C18 (2.1*50 mm, 5 μm)

Flow rate: 0.450 mL/min

Retention time:

NCGC00159568-03: 1.88 min

Testosterone: 2.02 min

Injection volume: 5 μL

Sample preparation For plasma samples: an aliquot of 30 μL plasma sample was partitioned by PPT using 0.15 mL ACN which contains IS (Testosterone, 10 ng/mL). The mixture was vortexed for 2 min and centrifuged at 12000 rpm for 5 min. An aliquot of 5 μL supernatant was injected for LC-MS/MS analysis.

For diluted samples: an aliquot of 20 µL plasma sample was added with 80 µL blank plasma to obtain the diluted plasma samples, and the sample dilution factor is 5. An aliquot of 30 µL of diluted plasma sample was partitioned by PPT using 0.15 mL ACN which contains IS (Testosterone, 10 ng/mL). The mixture was vortexed for 2 min and centrifuged at 12000 rpm for 5 min. An aliquot of 5 µL supernatant was injected for LC-MS/MS analysis.

Calibration curve

1.00-10000 ng/mL for NCGC00159568-03 in mouse plasma samples

SD curve of NCGC00159568-03 in mouse plasma

SD sample	Anal. Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)
STD01-01	1.00	0.938	93.8
STD01-02	3.00	3.30	110
STD01-03	10.0	10.3	103
STD01-04	30.0	*34.7	N/A
STD01-05	100	110	110
STD01-06	300	309	103
STD01-07	1000	1150	115
STD01-08	3000	3070	102
STD01-09	10000	11400	114
STD02-01	1.00	*1.84	N/A
STD02-02	3.00	3.40	113
STD02-03	10.0	8.52	85.2
STD02-04	30.0	26.2	87.4
STD02-05	100	86.0	86.0
STD02-06	300	*236	N/A
STD02-07	1000	940	94.0
STD02-08	3000	2650	88.5
STD02-09	10000	9370	93.7

*:The calculated value that was not within 85% to 115% (80% to 120% for LLOQ) of the theoretical value was excluded from the calibration curves.

N/A: not available

QC samples of NCGC00159568-03 in mouse plasma

SD sample	Anal. Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)
QCL-01	3.00	2.85	94.9
QCM-01	1500	*1950	N/A
QCH-01	8000	7510	93.8
QCL-02	3.00	3.08	103
QCM-02	1500	1640	109
QCH-02	8000	*5920	N/A

*:The calculated value that was not within 85% to 115% of the theoretical value.

N/A: not available

Diluted QC samples of NCGC00159568-03 in mouse plasma

SD sample	Anal. Conc. (ng/mL)	Calculated Conc. (ng/mL)	Accuracy (%)
Dilute QC-1	6000	5560	92.7
Dilute QC-2	6000	5650	94.2
Dilute QC-3	6000	6030	100

The *in vivo* pharmacokinetic profiles and parameters of the NCGC00159568-04 (ML156, 8i) in male Swiss Albino mice after twice intraperitoneal administration at 12 hour interval of NCGC00159568-04 (ML156, 8i) at 20 mg/kg dose.

SUMMARY

The objective of this study was to determine the plasma, brain, liver and tail concentration of test article NCGC00159568-04 in male Swiss Albino Mice after twice intraperitoneal administration of NCGC00159568-04 at dose of 20 mg/kg. Second dose was administration after 12 hr of first dose. The pharmacokinetic parameters of NCGC00159568-04 in plasma, brain, live and tail was calculated and $AUC_{(0-t)}$ of plasma, brain, live and tail were used for determination of tissue to plasma ratio. The tissue to plasma ratio of NCGC00159568-04 in male Swiss Albino Mice is found to be 0.08 (brain), 63.31 (liver) and 1.77 (tail.). Animals were found to be lethargi at later time points (15, 17, 20 and 24 hrs). There was ataxia evident in few animals of same groups.

1 FACILITIES

The experiments were performed between 30th August 2010 and 06th September 2010 at the premises of

Animal Facility and Bioanalytical and Pharmacokinetic Analysis Facility
GVK Biosciences Pvt. Ltd.
Biology Division,28 A, IDA, Nacharam,
Hyderabad 500 076
INDIA

2 LIST OF ABBREVIATIONS

µL	microliter
(AUC_{last})	Area under the concentration-time curve from the time of dosing to the last observation.
$AUC_{(0-\infty)}$	Area under the concentration-time curve from the time of dosing extrapolated to infinity.
ΔAUC(%)	% of AUC extrapolated from $AUC_{(0-t)}$ to $AUC_{(0-\infty)}$
BLOQ	Below limit of Quantitation
CAD	Collision associated dissociation
Cl	Clearance
CUR	Curtain gas
DMA	N-N Dimethyl Acetamide
gm	gram
GS1	Gas1
GS2	Gas 2
HQC	Higher Quality Control
h	hours
ihe	Interface heater
ISTD	Internal Standard
k_{el}	Elimination (terminal) rate constant
LC/MS/MS	Liquid Chromatography/Mass Spectrometry/ Mass Spectrometry
LLOQ	Lower Limit of Quantitation
LQC	Low Quality Control
mg	milligram
min	minutes
mL	Milliliter
MQC	Middle Quality Control
MRM	Multiple Reaction Monitoring

MRT last	Mean residence time from the time of dosing to the last observation.
MS	Mass Spectrometer
ng	Nano gram
PK	Pharmacokinetics
QC	Quality control
rpm	Revolutions per minute
Rsq	Correlation coefficient
SD	Standard Deviation
STD	Calibration Curve Standard
$t_{1/2}$	Elimination half life
T_{max}	Time of maximum concentration
DMA	*N*,*N*'-Dimethyl Acetamide
TEM	Temperature
V	Volume of distribution

3 INTRODUCTION

This report describes the plasma, brain, liver and tail pharmacokinetic profiles of the NCGC00159568-04 following itraperitoneal administration after two doses (at interval 12 hours) in male Swiss Albino mice. The analytical procedure was as per Annexure V. Test compound concentrations in plasma, brain, liver and tail were determined by LC/MS/MS.

4 STUDY OBJECTIVE

The objective of this study was to determine the plasma, brain, liver and tail concentration of NCGC00159568-04 in male Swiss Albino mice after two doses (at interval 12 hours) of NCGC00159568-04 through intra-peritoneal route at 20 mg/kg and calculation of tissue to plasma ratio.

5 MATERIALS & METHODS

Study Design

Table 5.1.1 Study design for single dose oral pharmacokinetic study of NCGC00159568-04 in male Swiss Albino mice.

Group	Animal	Test item	Dose (mg/kg)	Dose Conc. (mg/ml)	Dose Volume (mL/kg)	Dose route	No. of animals for each time point	Sample time points (h)
	Swiss Albino Mice (20-35 g)	NCGC00159568-04	20	3	10	Itraperitoneall	3	0 min, 5 min, 15 min, 30 min, 1, 2, 4, 8, 12*, 13, 15, 17, 20 and 24 hours

*Second dosing. 12 hr blood sampling will be done prior to second dosing

Animals

Male Swiss Albino mice used in this experiment were procured from National Institute of Nutrition (NIN), Hyderabad on 12th August 2010. All animals were issued by Animal House Veterinarian to Scientist-in-charge on 27th August 2010. Animals were

acclimatized for three days (27/08/2010 – 29/08/2010) in Animal Holding Room. Full details of Animal Husbandry is provided in Annexure II.

Details of the formulations of test articles used

Test article NCGC00159568-04 was dissolved in DMA, TEG and Water for Injection in the ratio of 20:40:40, and vortexed. Formulation details are provided in Table IIIA in Annexure III.

Test Article Administration

5.1.1.1 Intra-Peritoneal administration

NCGC00159568-04 was administered intra-peritoneally using 1 ml syringes (BD) fitted on top with 26 G needle at a dose level of 20 mg/kg at dose volume of 10 mL/kg. After administration, animals were placed back in their respective cages. After dosing of each animal, the experimenter documented the same in the Dosing and Sample collection sheet. All animals were observed for any abnormal behavioral signs exhibited after drug administration.

Blood samples & Organ Collection

5.6.1 Blood sample collection
Each mouse was anesthized using Isoflurane. Blood was collected through a capillary, guided in retro-orbital plexus. The blood samples were collected in prelabeled Heparin coated tubes (BD, cat. No.365965). 0.3 mL of blood was collected from each mouse at their respective timepoints. After collection of blood samples at each time point, the blood samples were stored on wet ice prior to centrifugation. Blood samples were centrifuged within 15 minutes to separate plasma at 5000 rpm, 4 °C for 10 minutes. The plasma was separated and transferred to pre labelled tubes and promptly frozen at -80 ± 10 °C until bioanalysis.
5.5.2 Organ Collection (Brain, Liver, Tail)
Immediately after blood withdrawal for PK estimation, in situ whole body perfusion was performed using chilled saline. The chest and abdomen of the mouse was exposed, the inferior venacava was cut and Intra-cardiac perfusion was performed through an insertion in the left ventricle. Perfusion for each mouse was followed by decapitation for brain collection. The skin over the cranium was incised and deflected. The head was flexed and a cut was made through the muscles and the spinal cord at the junction of the foramen magnum and atlas vertebra. A circumferential incision was carefully made in the cranium using a pair of small scissors. The roof of the cranium was lifted off to expose the meninges and brain. The meninges were removed carefully. Then holding the head with the nose pointing upward the anterior part of the brain was lifted to separate the brain. After brain collection, liver was excised from the body, carefully all the diaphragmatic and abdominal ligaments were cut using a pair of small scissors and liver was collected. After that, the tail was collected by severing from the point below the tail marking, using a 22 no. surgical blade mounted on 4 no. scalpel. Separated tissues were immediately weighed and instantly freezed at -80 ± 10 °C until homogenization. Individual data and details of dosing and sample collection times are given in Table 1 of the Annexure I section. Individual data of tissue homogenate preparations and test article concentrations

are presented in Annexure I Tables 5 (for each of the tissue separately).

Bio-analytical Study Design

Details of bio-analytical procedure are described in Annexure V.

PK Parameters Analysis

PK parameters are calculated for mean concentration by the non-compartmental model, trapezoid rule (linear interpolation method) using WinNonlin Software Version 4.1. Number of decimal places of plasma concentration used for WinNonlin calculation:-2.0.

Deviations from study protocol

5.9.1 Deviations and changes before the start of the experiment

No deviation or change has been made before the start of the experiment. Bio-analytical: No deviation or change has been made before the start of the experiment.

5.1.2 Deviations and changes that occurred during the experiment

In-life: No deviation or change has occurred during the experiment.
Bio-analytical: No deviation or change has occurred during the experiment.

6 RESULTS

In-Life Observation

Animals were found to be lethargic and listless at later time points (15, 17, 20 and 24 hrs). There was ataxia evident in few animals of same groups. All other animals were found to be normal throughout the study period.

NCGC00159568-04 Intra-peritoneal administration

The pharmacokinetic profiles of the NCGC00159568-04 in male Swiss Albino mice after twice intraperitoneal administration at 12 hour interval of NCGC00159568-04 at 20 mg/kg dose are shown below.

Table 6.2.1. Concentrations of NCGC00159568-04 in plasma, brain, liver and tail. Oral dosing of NCGC00159568-04 at 20.0 mg/kg in male Swiss Albino Mice (mean and SD).

Time (h)	NCGC00159568-04 Concentration (Mean ± SD)							
	Plasma(ng/mL)	µM	Brain(ng/g)	µMol/kg	Liver(ng/g)	µMol/kg	Tail(ng/g)	µMol/kg
0.000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.083	7550.45±4133.97	13.57	156.27±35.04	0.28	114450.60±35495.51	205.72	2382.09±1219.43	4.28
0.25	3718.63±1820.04	6.68	117.00±26.91	0.21	120015.98±31928.46	215.72	1711.93±646.62	3.08
0.50	0.39±0.68	0.00	144.25±59.45	0.26	172131.23±63886.67	309.39	2757.54±1415.49	4.96
1.00	2027.95±1490.37	3.65	249.35±85.99	0.45	188379.50±53862.08	338.60	4515.56±419.31	8.12
2.00	2782.05±2546.66	5.00	264.68±62.48	0.48	197983.68±86771.35	355.86	3948.02±857.57	7.10
4.00	3433.14±323.70	6.17	334.73±9.89	0.60	274815.45±33548.02	493.96	4607.28±480.57	8.28

8.00	3058.29±852.34	5.50	263.30±75.11	0.47	161336.35±97210.11	289.99	4237.98±474.45	7.62
12.00	68.72±21.67	0.12	25.60±27.39	0.05	3178.62±487.48	5.71	1635.27±1112.20	2.94
13.00	4356.09±262.37	7.83	65.50±29.60	0.12	96094.83±50368.63	172.72	3048.19±974.44	5.48
15.00	1332.21±889.37	2.39	114.42±18.10	0.21	121269.48±27551.90	217.97	4746.46±442.57	8.53
17.00	0.00±0.00	0.00	0.00±0.00	0.00	0.00±0.00	0.00	72.02±47.26	0.13
20.00	1242.59±477.67	2.23	78.98±24.56	0.14	101618.15±16886.07	182.65	3217.71±2165.76	5.78
24.00	171.61±126.71	0.31	39.90±23.35	0.07	8024.95±6437.87	14.42	3230.88±1784.48	5.81

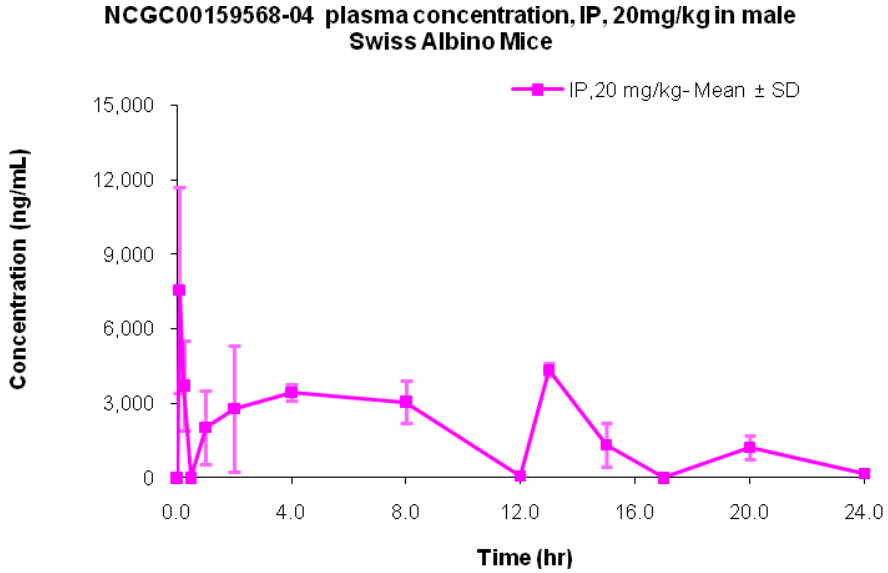


Figure 6.2.1. PK of NCGC00159568-04 in plasma. Intraperitoneal dosing of NCGC00159568-04 at 20 mg/kg in male Swiss Albino mice twice at 12 hr interval (mean and SD).

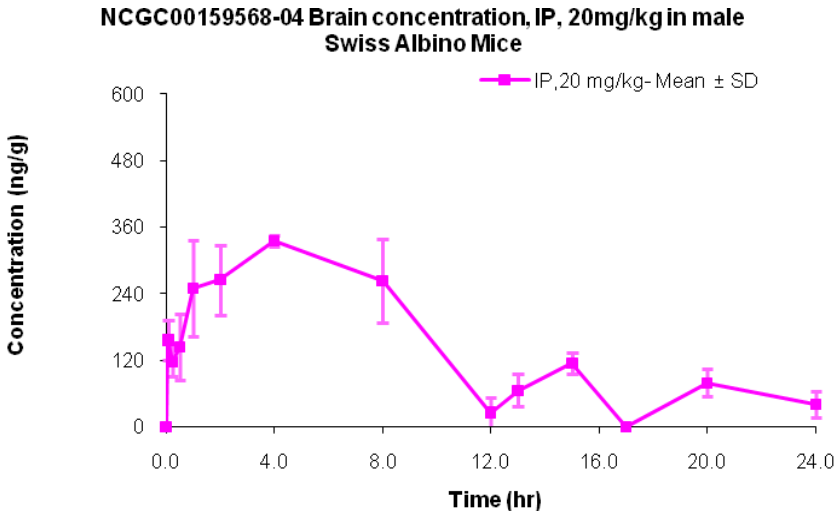


Figure 6.2.2. PK of NCGC00159568-04 in Brain. Intraperitoneal dosing of NCGC00159568-04 at 20 mg/kg in male Swiss Albino mice, twice at 12 hr interval (mean and SD).

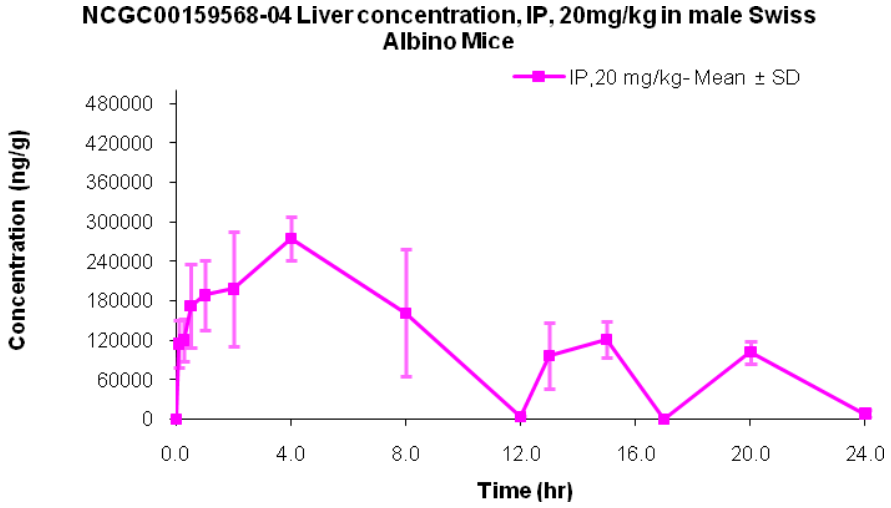


Figure 6.2.3. PK of NCGC00159568-04 in Liver. Intraperitoneal dosing of NCGC00159568-04 at 20 mg/kg in male Swiss Albino mice, twice at 12 hr interval (mean and SD).

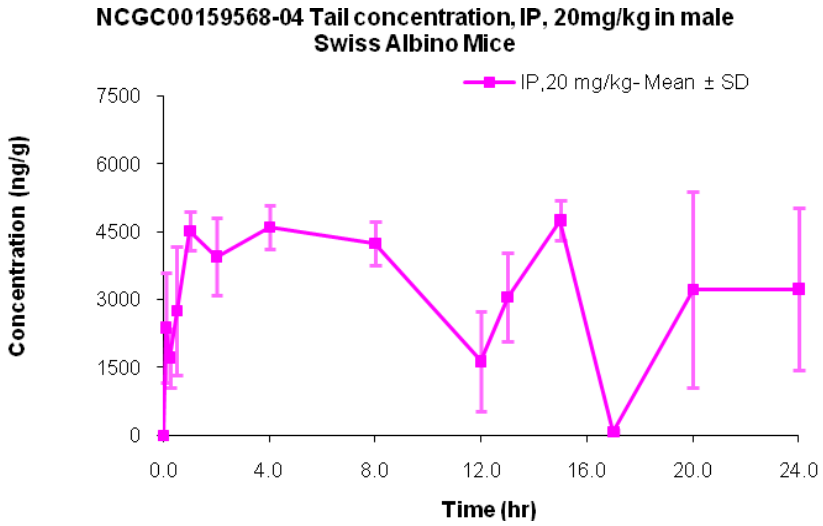


Figure 6.2.3. PK of NCGC00159568-04 in Tail. Intraperitoneal dosing of NCGC00159568-04 at 20 mg/kg in male Swiss Albino mice, twice at 12 hr interval (mean and SD).

PK parameters

6.3.1. Pharmacokinetic parameter of NCGC00159568-04 in plasma following Intraperitoneal administration at dose rate of 20 mg/kg, twice at 12 hours interval.

Analyte	k_{el} (1/h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng·h/ml)	$AUC_{0-\infty}$ (ng·h/ml)	ΔAUC (%)	Cl (ml/hr/kg)	MRT last (h)
NCGC00159568-04 Plasma	0.18	3.80#	0.083	7550.45	44008.54	44950.17	2.09	444.94	8.44

#Rsq:-0.9722

6.3.2. Pharmacokinetic parameter of NCGC00159568-04 in brain following Intraperitoneal administration at dose rate of 20 mg/kg, twice at 12 hours interval.

Analyte	k_{el} (1/h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/g)	AUC_{last} (ng·h/g)	$AUC_{0-\infty}$ (ng·h/g)	ΔAUC (%)	Cl (ml/hr/kg)	MRT last (h)
NCGC00159568-04 Brain	0.12	6.01#	4.00	334.73	3486.64	3832.90	9.03	5217.98	7.57

#Rsq:-0.9465

The brain to plasma ratios of NCGC00159568-04 is 0.08 (AUC_{last} Brain (ng*h/g) / AUC_{last} Plasma (ng*h/ml).

6.3.3. Pharmacokinetic parameter of NCGC00159568-04 in liver following Intraperitoneal administration at dose rate of 20 mg/kg, twice at 12 hours interval.

Analyte	k_{el} (1/h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/g)	AUC_{last} (ng·h/g)	$AUC_{0-\infty}$ (ng·h/g)	ΔAUC (%)	Cl (ml/hr/kg)	MRT last (h)
NCGC00159568-04 Liver	0.18	3.89#	4.00	274815.45	2778271.94	2823313.00	1.59	7.08	8.04

#Rsq:-0.9736

The liver to plasma ratios of NCGC00159568-04 is 63.31 (AUC_{last} liver (ng*h/g) / AUC_{last} Plasma (ng*h/ml).

6.3.4. Pharmacokinetic parameter of NCGC00159568-04 in tail following Intraperitoneal administration at dose rate of 20 mg/kg, twice at 12 hours interval.

Analyte	k_{el} (1/h)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/g)	AUC_{last} (ng·h/g)	$AUC_{0-\infty}$ (ng·h/g)	ΔAUC (%)	Cl (ml/hr/kg)	MRT last (h)
NCGC00159568-04 Tail	NC	NC	15.00	4746.46	77828.41	NC	NC	Nc	10.81

NC-Not calculated

The tail to plasma ratios of NCGC00159568-04 is 1.77 (AUC_{last} Tail (ng*h/g) / AUC_{last} Plasma (ng*h/ml).

In conclusion, on oral administration of NCGC00159568-04, the exposure in liver is found to be more as compare to brain and tail. The tissue to plasma for liver is found to be high, followed by tail and brain. Under the experimental conditions of this study and with the formulations used the tissue to plasma ratio is shown in table below.

	NCGC00159568-04
Brain to plasma ratio	0.08
Liver to plasma ratio	63.31
Tail to plasma ratio	1.77

7. TEST SYSTEM & ANIMAL HUSBANDRY

Animal care was in compliant with the Regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Species/Strain

Adult healthy, male Swiss Albino Mice were procured from the National Institute of Nutrition, Hyderabad, India on 12/08/2010. All animals were acclimatized for a period of three days (27/08/2010 – 29/08/2010) in the Animal House Facility at GVKBIO.

Housing

The mice were housed individually in clean sterilized individually ventilated cages with controlled environmental conditions (Temperature: 22 ± 3 °C, Relative humidity: 30-70%). Autoclaved clean paddy husk was used as bedding material. They were maintained on a 12 hour light / dark cycle.

Animal identification

Each mouse was uniquely identified by an assigned number on tail with permanent marker pen as per SOP-IPH-121 for animal identification.

Feed & Water

The mice were fed with certified rodent pellets ad libitum (VetCare Feeds, Banglore,

India). Potable water was supplied ad libitum.

Body Weight

All animals were weighed on the day of study prior to drug administration.

8. FORMULATION

Formulation of NCGC00159568-04 for intra-peritoneal administration

Formulation for 12 hr dosing of 24 hr timepoint animals:

Study N°	185-10-IPH
Compound ID	NCGC00159568-04
Molecular weight	556.3491
Dose (mg/kg)	20
Dose Volume (mL/kg)	10
Dose Concentration (mg/mL)	2
Administration Route	IP
Species/Strain/Sex	Mouse/Swiss albino/Male
Test Item Weighed: 2.1 mg ~ 1.995 mg (95% pure)	
Total Volume of Vehicle Required: 0.998 ml	
Formulation/Vehicle Used:	**Formulation/Vehicle Used:**
20 % DMA (N-N Dimethyl Acetamide) 40% TEG (Tetra Ethylene Glycol) 40% water	0.200 ml 0.399 ml 0.399 ml
Preparation Procedure and Documentation:	
Procedure	**Observation**
2.1 mg ~ 1.995 mg of NCGC00187872-02 was weighed and 0.200 ml of DMA was added. 0.399 ml of TEG was added and vortexed. 0.399 ml of water was added and vortexed.	- Clear solution. - Clear solution. - Clear solution.

Formulation for 12 hr dosing of 13, 15, 17, 20 hr timepoint animals and 0 hr dosing of 24 hr time point animals:

Study N°	185-10-IPH
Compound ID	NCGC00159568-04
Molecular weight	556.3491
Dose (mg/kg)	20
Dose Volume (mL/kg)	10
Dose Concentration (mg/mL)	2
Administration Route	IP
Species/Strain/Sex	Mouse/Swiss albino/Male
Test Item Weighed: 13.30 mg ~ 12.60 mg (95% pure)	
Total Volume of Vehicle Required: 6.3 ml	
Formulation/Vehicle Used:	**Formulation/Vehicle Used:**
20 % DMA (N-N Dimethyl Acetamide) 40% TEG (Tetra Ethylene Glycol) 40% water	1.26 ml 2.52 ml 2.52 ml
Preparation Procedure and Documentation:	
Procedure	**Observation**
37.48 mg ~ 35.60 mg of NCGC00187872-02 was weighed and 2.374 ml of DMA was added. 4.748 ml of TEG was added and vortexed. 4.748 ml of water was added and vortexed.	- Clear solution. - Clear solution. - Clear solution.

Formulation for 0 hr dosing of all time point animals except 24 hr time point animals:

Study N	185-10-IPH
Compound ID	NCGC00159568-04
Molecular weight	556.3491
Dose (mg/kg)	20
Dose Volume (mL/kg)	10

Dose Concentration (mg/mL)	2
Administration Route	IP
Species/Strain/Sex	Mouse/Swiss albino/Male
Test Item Weighed: 35.10 mg ~ 33.40 mg (95% pure)	
Total Volume of Vehicle Required: 11.14 ml	

Formulation/Vehicle Used:	**Formulation/Vehicle Used:**
20 % DMA (N-N Dimethyl Acetamide) 40% TEG (Tetra Ethylene Glycol) 40% water	2.228 ml 4.456 ml 4.456 ml

Preparation Procedure and Documentation:	
Procedure	**Observation**
37.48 mg ~ 35.60 mg of NCGC00187872-02 was weighed and 2.374 ml of DMA was added. 4.748 ml of TEG was added and vortexed. 4.748 ml of water was added and vortexed.	- Clear solution. - Clear solution. - Clear solution.

9. SAMPLE COLLECTION AND PREPARATION

Blood sample collection

Each mouse was anesthized using Isoflurane. Blood was collected through a capillary, guided in retro-orbital plexus. The blood samples were collected in prelabeled Heparin coated tubes (BD, cat. No.365965). 0.3 mL of blood was collected from each mouse at their respective timepoints. After collection of blood samples at each time point, the blood samples were stored on wet ice prior to centrifugation. Blood samples were centrifuged within 15 minutes to separate plasma at 5000 rpm, 4°C for 10 minutes. The plasma was separated and transferred to pre labelled tubes and promptly frozen at -80 ± 10 °C until bioanalysis.

Organ Collection (Brain, Liver, Tail)

Immediately after blood withdrawal for PK estimation, in situ whole body perfusion was performed using chilled saline. The chest and abdomen of the mouse was exposed, the inferior venacava was cut and Intra-cardiac perfusion was performed through an insertion in the left ventricle. Perfusion for each mouse was followed by decapitation for brain collection. The skin over the cranium was incised and deflected. The head was flexed and a cut was made through the muscles and the spinal cord at the junction of the foramen magnum and atlas vertebra. A circumferential incision was carefully made in the cranium using a pair of small scissors. The roof of the cranium was lifted off to expose the meninges and brain. The meninges were removed carefully. Then holding the head with the nose pointing upward the anterior part of the brain was lifted to separate the brain. After brain collection, liver was excised from the body, carefully all the diaphragmatic and abdominal ligaments were cut using a pair of small scissors and liver was collected. After that, the tail was collected by severing from the point below the tail marking, using a 22 no. surgical blade mounted on 4 no. scalpel. Separated tissues were immediately weighed and instantly freezed at -80 ± 10 °C until homogenization.

Preparation of Tissues Homogenate

Brain, liver and tail samples were weighed and appropriate volume of ice cold homogenizing media (normal saline) was added. Brain, liver and tail samples were homogenized on ice with the Ultra Turrax homogenizer and volume was adjusted with homogenizing media to achieve 1 g of brain per 5 ml of homogenate. After homogenization, the brain, liver and tail homogenates were immediately frozen at -80° C

until analysis.

10. LC/MS/MS PARAMETERS

The parameters of chromatographic conditions and extraction conditions for the NCGC00159568-04 analysis:

Chromatographic Parameters: for Brain Homogenate
Instrumentation : API-4000, agilent RRLC and Pal autosampler
Column : X-Bridge C18, 50x4.6mm, 3.5µ
Mobile Phase :
Aqueous Reservoir (A) : 10mM Ammonium Formate with 0.3% Ammonia
Organic Reservoir (B) : 0.1% Ammonia in Acetonitrile
Flow rate : 1.00mL/min

LC gradient program for analysis of NCGC00159568-04

Time (min)	Gradient Curve	%A	%B
0.01	1	95	5
0.80	1	5	95
3.20	1	5	95
3.30	1	95	5
4.50	1	95	5

Run time : 4.50 min
Column oven temperature : 40 ^{0}C
Auto sampler temperature : 10 ^{0}C
Auto sampler Wash : Wash-1-100% Methanol
Wash-2:0.2%Formic Acid in water
Retention time : NCGC00159568-04 : 2.45 ± 0.05 min.
Metaprolol : 2.10 ± 0.05 min

Mass Parameters:
Mode : MRM
Polarity : Positive
Ion source : Turbo spray
Analyte :NCGC00159568-04(Q1Mass 555.20; Q3 Mass 388.10 225.20,)
ISTD : Metaprolol (Q1 Mass 268.10 ; Q3 Mass 116.10)

Source/ Gas Parameters:
Curtain gas (CUR) 20
Collision gas
(Collision associated Dissociation) CAD 12
Ion Spray Voltage (IS) 5500 V
Temperature (TEM) 550 ° C

GS1	55
GS2	45
Ihe	ON

Source and compound dependent parameters of NCGC00159568-04 and Metaprolol

Parameter	NCGC00159568-04 555.20/388.10	NCGC00159568-04 555.20/225.10	Metaprolol 268.10/116.10
Declustering Potential (V)	50	50	50
Entrance Potential (V)	10	10	10
Collision Energy (eV)	25	30	25
Collision Cell Exit Potential (V)	12	12	12
Dwell Time (millisecond)	100	100	100

Sample dilution-Plasma

Matrix:- Blank plasma

Dilution factor-25

Time points diluted-0.083, 0.25, 0.50 and 1.00 hr:- Group1, 2 and 3

Dilution factor-10

Time points diluted-2.00, 4.00, 8.00, 12.0, 13.0, 15.0, 17.0, 20.0 and 24.0 hr:- Group1, 2 and 3

Sample dilution-Brain

No dilution done

Sample dilution-Liver

Matrix-Blank liver homogenate

Dilution factor-100

All time points- Group-1, 2 and 3

Sample dilution-Tail

Matrix-Blank tail homogenate

Dilution factor-5

All time points- Group-1, 2 and 3

11. EXTRACTION PROCEDURE

Extraction Procedure (Preparation of STD and QC)

To 48µl of Blank plasma sample 2µl of Standard Solution was added, Vortexed

↓

150 µl of Metaprolol was added

↓

Vortexed for 5 min at 2000 rpm

↓

Centrifuged at 13000 rpm for 5 min at 4 °C

↓

130 µl of supernatant was transfered to analysis plate, diluted with 100µL of Methanol:water (50:50,v/v)

↓

Analyzed in LCMS\MS by injecting 25µL

Extraction Procedure (Preparation of Study Samples)

50µl of study sample were taken after vortexing,

↓

150 µl of Metaprolol was added

↓

Vortexed for 5 min at 2000 rpm

↓

Centrifuged at 13000 rpm for 5 min at 4 °C

↓

130 µl of supernatant was transfered to analysis plate, diluted with 100µL of Methanol:water (50:50,v/v)

↓

Analyzed in LCMS\MS by injecting 25µL

Preparation of Internal Standard:-2.68 mg stock of metaprolol was prepared in DMSO. Further 150µL of this stock was diluted in 1000mL of Acetonitrile to get a concentration of 400ng/mL.

12. REAGENT, LABWARE AND INSTRUMENTS

Reagents

Name	Source	Catalog Number	Lot #
Acetonitrile, Lichrosolv (Gradient grade)	Merck	61830025001730	IE01F60262
Dimethyl Sulfoxide, Lichrosolv (DMSO) (HPLC grade)	Sigma	34869	SZE9315S
Methanol, Lichrosolv (Gradient grade)	Merck	61803025001730	SD0SF60354
Water	Elix 3 and Milli-Q Biocel water Purification system	-	-
Heparin vacutainer	BD	365965	
K2 EDTA vacutainer	BD	365974	
Heparin (25000 IU in 5 mL) (Mfd Dt.: Oct. 2009, Exp. Dt: Sept. 2012)	Biological E		Lot # 314
Normal Saline	Nirlife Healthcare Ltd.		Lot # 304212181
Formic Acid	Merck	618009905001730	AC7AF57107
N-N, Dimethyl Acetamide	Sigma	D5511	Batch # 037K3736
Tetraethylene Glycol	Sigma	110175	Batch # 04322BJ
Ammonium Formate	BDH	27169	G222203

Labwares

Microcentrifuge tubes, 1.5 mL	Tarsons	1.5ml-500010
96 deep well plates, 0.5 mL	Eppendorf	0030501.144

Centrifuge tubes (5 mL, 15 mL)	Tarsons	15 ml – 546020
Matrix Screen Mates Tubes (1.4 mL)	Matrix	4247

Instruments

Name	Model	Serial #	Make
Centrifuge	Biofuge fresco	40989744	Kendro
LC- MS/MS	RRLC + API-4000	DE63059111 + V22590803	Shimadzu +AB Sciex.
Liquid Handler	Quadra 4	(4000-10-102)	Tomtec
pH meter	Orion 42 A+	086194	Thermo electron
Pipettes	Research and Research Pro		Eppendorf
Vortexer	Vortex Genie-2 Digital	AH-1022	Scientific Industries
Vortex shaker	Mixmate	535300813	Eppendorf
Water purification system	Combination of Elix 3 and Milli-Q Biocel	BM5NN0140A	Millipore
Weighing balance	CP225D	18110562, 18139173	Sartorius
Ultramicro Balance	UMX2	1129342428	Metler Toledo
Animal weighing Balance	GP5200	22308129	Sartorius
Vortex Mixer	Spinix		Tarson
Magnetic Stirrer	Spinit		Tarson
Refrigerated Centrifuge	5415R	5426XG624295	Eppendorf
Micropipettes (2-20 ul, 20-200 ul, 100-1000 ul, 1-5 mL)			Eppendorf
Minispin	AG22331		Eppendorf
Sonicator	VCX130BP	SOSGS10276	Sonics Vibracell
Homogenizer	IKA T10 Basic		Ultra Turrax
X-Bridge, C18, 4.6x50mm, 3.5µ		013339007136 83	Waters
Autosampler	HTS Pal	113944	CTC Analyticals