Role of hydrophobic substituents on terminal nitrogen of histamine in receptor binding and agonist activity: development of orally active histamine type 3 receptor agonist and evaluation of its anti-stress activity in mice

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Supporting Information

Contents:

Synthesis of **7a**-**7g**, **7i** and **7j**. Experimental details of receptor binding assay, [^{35}S]GTPγS binding assay, measurement of N^{τ}-methylhistamine level in mouse brain, mouse resident-intruder test, pharmacokinetic test in rats and in vitro human CYP inhibition test.

4-(2-(4-*tert*-Butylphenylamino)ethyl)-1H-imidazole (7e)

4-*tert*-Butylaniline (292 mg, 2.0 mmol) was dissolved in DMF (10 ml), then

4-(2-Bromoethyl)imidazole (**6**, 100 mg, 0.39 mmol) was added and the mixture was stirred for 16 h at 80 °C. The reaction was quenched with Ethyl acetate (50 ml) and the resulting solution was washed with sat $NaHCO_3$ aq (20 ml). The organic layer was washed with water (20 ml) twice, dried over $MgSO_4$ and concentrated. The crude residue was purified by means of silica-gel column chromatography (chloroform/methanol = 1/1). The residue was taken up in 5 N HCl aq (1 ml), and the solution was evaporated. The residual yellow oil was slowly solidified with ether and recrystallized from methanol/ether to afford **7e** (105 mg, 2HCl salt) as a pale red powder (85.1%). mp 190 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.31 (9H, s), 3.19 (2H, m), 3.30 (2H, t, J = 7.8 Hz), 7.35-7.50 (3H, m), 7.59 (2H, d, J = 8.8 Hz), 8.84 (1H, s). MS (EI) m/z: 243 (M^+). Anal. Calcd for $C_{15}H_{21}N_3$ 2HCl : C, 56.96; H, 7.33; N, 13.29. Found: C, 57.10; H, 7.53; N, 13.56.

4-(2-(Cyclohexylamino)ethyl)-1H-imidazole (7a)

Using the procedure described under **7e, 7a** was obtained as a white solid (73.2%, 2HCl salt). mp 223 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.25–1.45 (5H, m), 1.73 (1H, m), 1.91 (2H, m), 2.16 (2H, m), 3.10-3.30 (3H, m), 3.30-3.40 (2H, m), 7.50 (1H, s), 8.87 (1H, s). MS (EI) m/z: 193 (M^+). Anal. Calcd for $C_{11}H_{19}N_3$ 2HCl 5/4H_2O : C, 45.75; H, 8.20; N, 14.55. Found: C, 45.89; H, 8.36; N, 14.63.

4-(2-(Phenylamino)ethyl)-1H-imidazole (7b)

Using the procedure described under **7e, 7b** was obtained as a pale yellow solid (81.2%, 2HCl salt). mp 174-175 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.20-3.40 (2H, m), 3.70-3.90 (2H,

m), 7.40-7.60 (5H, m), 8.86 (1H, s). MS (FAB+) m/z: 188 (M^++H). Anal. Calcd for $C_{11}H_{19}N_3$ 2HCl 1/4H_2O : C, 49.91; H, 5.90; N, 15.88. Found: C, 49.93; H, 5.92; N, 15.91.

4-(2-(4-Methylphenylamino)ethyl)-1H-imidazole (7c)

Using the procedure described under **7e, 7c** was obtained as a pale yellow solid (72.4%, 2HCl salt). mp 181 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 2.44 (3H, s), 3.21 (2H, t, J = 8.0 Hz), 3.73 (2H, t, J = 8.0 Hz), 7.42 (4H, m), 7.48 (1H, s), 8.87 (1H, s). MS (EI) m/z: 201 (M^+). Anal. Calcd for $C_{11}H_{15}N_3$ 2HCl H_2O : C, 49.32; H, 6.55; N, 14.38. Found: C, 49.37; H, 6.82; N, 14.59.

4-(2-(3,4-Dimethylphenylamino)ethyl)-1H-imidazole (7d)

Using the procedure described under **7e, 7d** was obtained as a white solid (59.4%, 2HCl salt). mp 204-205 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 2.32 (3H, s), 2.35 (3H, s), 3.24 (2H, t, J = 7.82Hz), 3.74 (2H, t, J = 7.82 Hz), 7.25–7.45 (3H, m), 7.48 (1H, s), 8.87 (1H, s). MS (FAB+) m/z: 216 (M^++H). Anal. Calcd for $C_{13}H_{17}N_3$ 2HCl : C, 54.17; H, 6.64; N, 14.58. Found: C, 53.89; H, 6.70; N, 14.42.

4-(2-(3-*tert*-Butylphenylamino)ethyl)-1H-imidazole (7f)

Using the procedure described under **7e, 7f** was obtained as a pale blue solid (74.1%, 2HCl salt). mp 173-174 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.36 (9H, s), 3.27 (2H, m), 3.78 (2H, t, J = 7.8 Hz), 7.35-7.60 (5H, m), 8.88 (1H, s). MS (EI) m/z: 243 (M^+). Anal. Calcd for $C_{15}H_{21}N_3$ 2HCl : C, 56.96; H, 7.33; N, 13.29. Found: C, 56.79; H, 7.44; N, 13.02.

4-(2-(4-Cyclohexylphenylamino)ethyl)-1H-imidazole (7g)

Using the procedure described under **7e, 7g** was obtained as a pale yellow solid (72.9%, 2HCl salt). mp 196 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.45 (5H, m), 1.86 (5H, m), 2.60 (1H, m), 3.22 (2H, t, J = 7.82 Hz), 3.74 (2H, t, J = 7.82 Hz), 7.43 (4H, m), 7.48 (1H, s), 8.87 (1H, s). MS (EI) m/z: 269 (M^+). Anal. Calcd for $C_{17}H_{23}N_3$ 2HCl : C, 59.65; H, 7.36; N, 12.28. Found: C, 59.65; H, 7.49; N, 12.28.

4-(2-(4-Trifluoromethylphenylamino)ethyl)-1H-imidazole (7i)

Using the procedure described under **7e, 7i** was obtained as a white solid (60.8%, 2HCl salt). mp 129-130 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.06 (2H, t, J = 6.95 Hz), 3.54 (2H, t, J = 6.95 Hz), 6.81 (2H, d, J = 8.54 Hz), 7.37 (1H, s), 7.62 (2H, d, J = 8.54 Hz), 8.81 (1H, s). MS (EI) m/z: 255 (M^+). Anal. Calcd for $C_{12}H_{12}N_3F_3$ 2HCl 1/4H_2O : C, 43.32; H, 4.39; N, 12.63. Found: C, 43.03; H, 4.13; N, 12.58.

4-(2-(4-Methoxyphenylamino)ethyl)-1H-imidazole (7j)

Using the procedure described under **7e, 7j** was obtained as a pale yellow solid (47.0%, 2HCl salt). mp 197-198 °C ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.21 (2H, t, J = 7.80 Hz), 3.71 (2H, t, J = 7.80 Hz), 3.84 (3H, s), 7.09 (2H, d, J = 9.75 Hz), 7.43 (2H, d, J = 9.75 Hz), 7.47 (1H, s), 8.87 (1H, s). MS (EI) m/z: 217 (M^+). Anal. Calcd for $C_{12}H_{15}N_3O$ 2HCl : C, 49.67; H, 5.90; N, 14.48. Found: C, 49.51; H, 5.99; N, 14.61.

Receptor binding assay

Membranes prepared from CHO cells stably expressing human recombinant histamine H_3 receptors were incubated with [^{3}H]N-α-methylhistamine (1 nM, PerkinElmer Life Sciences) and

test compounds in a buffer containing 50 mM Tris-HCl (pH 7.4) and 5 mM EDTA at 25 °C for 1 h. Non-specific binding was assessed with thioperamide (10 μM). Radioligand binding was terminated by filtration through GF/B filters (PerkinElmer Life Sciences) and the amount of bound radiolabel was determined by liquid scintillation counting.

[^{35}S]GTPγ[S] Binding assay

Membranes prepared from CHO cells stably expressing human recombinant histamine H3 receptors were incubated with [^{35}S]GTPγ[S] (200 pM, GE Healthcare Bio-Sciences) and test compounds alone or in the presence of R-α-methylhistamine (10 nM, Sigma-Aldrich) in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT and 10 μM GDP at 25 °C for 1 h. Radioligand binding was terminated by filtration through GF/B filters and the amount of bound radiolabel was determined by liquid scintillation counting.

Measurement of N$^{\tau}$-methylhistamine level in mouse brain

Male ddY mice (20-25 g, Japan SLC) were killed by decapitation after having been treated with test compounds (1-30 mg/kg, p. o.) and pargyline (65 mg/kg, i. p.). The cerebral cortex was dissected out and homogenized in 10 vol. (w/v) of 0.1 N $HClO_4$. The clear supernatant (19,000 x g for 20 min) was made alkaline, extracted with *n*-butanol/chloroform (1:1), and back-extracted with HCl (0.01 N), and the solution was evaporated to dryness. The residue was derivatized with heptafluorobutyric anhydride and pyridine. Derivatives were extracted into toluene and assayed by selected ion monitoring at *m/e* 304 and 307 for N$^{\tau}$-methylhistamine

and its internal standard (trideuteromethylhistamine, Euriso-top), respectively. Ions 517 and 520 were also monitored for confirmation. Gas chromatography was performed with a GCMS system (QP2010, Shimadzu).

Resident-intruder test

The test was performed as described by Sanchez and Hyttel.[24] After an acclimation period, the resident mice (male ICR mice, 40-48 g, Japan SLC) were kept isolated for 6 weeks in a metal cage (32 × 22 × 18 cm). All of these mice underwent a pretest to evaluate their levels of aggressiveness. In this pretest, an intruder mouse, which had been housed in a group, was placed in a resident home cage and the duration of attacks by the resident on the intruder mouse was measured for 10 min by observer blinded as to the treatment status. Attack was defined as biting or an attempt to bite the intruder mouse. Only mice with a total attack time of longer than 10 sec were included in drug administration tests. These mice were randomly assigned to 2 or 3 groups based on their pre-test values. In the test session, isolated mice were given i. p. fluvoxamine (7.5 and 30 mg/kg) and **7h** (3 and 30 mg/kg) or vehicle 30 min prior to the test. Then, the resident mice were confronted with the intruder mice in the residents' home cages, and the total attack time was measured for 10 min.

Rat S9 Metabolic Activity[a]

Preparation of Microsomes. S7

Rats were anesthetized with diethyl ether, and livers were removed, perfused with ice-cold saline, and weighed. Then a 20% (w/v) homogenate was prepared using 100 mM potassium

phosphate buffer containing 1.15% potassium chloride (pH 7.4). The homogenate was centrifuged at 600*g* for 10 min. The supernatant was centrifuged at 12500*g* for 20 min, and the resulting supernatant was recentrifuged at 105000*g* for 60 min. The microsomal pellet was suspended in the same buffer and centrifuged again at 105000*g* for 60 min. The washed pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4). Microsomal protein concentration was measured by the method of Lowry et al. (1951). The microsomes were stored at -80 °C until use.

In Vitro Metabolism by Rat Liver Microsomes.

All incubations [rat liver microsomes, substrate, glucose-6-phosphate, β-$NADP^+$, glucose-6-phosphate dehydrogenase, $MgCl_2/6H_2O$, phosphate buffer (pH 7.4), and EDTA Na_2] were performed on a gently shaking platform maintained at 37 °C. Incubations were started by the addition of substrate and were stopped after 0, 15, 30, and 60 min by addition of DMF. Precipitated proteins were removed by centrifugation and supernatants were injected into the HPLC system to determine the remaining amount of each compound. HPLC analysis was performed with an Inertsil C4 (ϕ 4.6 x 250 mm) column (GL Science Inc., Tokyo, Japan), which was attached to an Inertsil C4 (ϕ 4.0 x 10 mm) guard column cartridge. The column was developed with a linear gradient from acetonitrile/50 mM sodium acetate (pH = 6.5) 35:65 to 45:55 from 0 to 20 min, and then with a linear gradient to 80:20 from 20 to 35 min, at a flow rate of 1 mL/min. The column temperature was kept at 40 °C, and the eluate was monitored at 250 nm.

(*a*: Mie, K.; Hisashi, S.; Izumi, K. Studies on the cytochrome P450-mediated metabolic properties of miocamycin. *Drug Metab. Dispos.* **2000**, *28*, 409-417.)

In Vivo Rat Pharmacokinetic Test

Intravenous and peroral formulations of compound **7h** were prepared by dissolving the compound in 1 vol% Tween 80 (2 mg/ml) and 0.5 w/v% methylcellulose (2.5 mg/ml), respectively. The compound was administered intravenously (2 mg/kg) or orally (5 mg/kg) to non-fasted 10-week-old male Wistar rats. Blood samples were collected at 2, 5, 15, 30 minutes, 1, 2, 4, 6 h for intravenous dosing, and 5, 15, 30 minutes, 1, 2, 4, 6, 8, 10 and 24 h for oral dosing after the administration. To determine the brain concentration of **7h**, some of the rats that had received the compound orally were sacrificed just after the collection of a blood sample at 1 h after the administration. The concentration of **7h** in plasma and brain homogenate was measured using liquid chromatography quadrupole tandem mass spectrometry (LC-MS/MS). Analysis software (WinNonlin Version 3.1m Scientific Consulting, Inc.) was used to perform the pharmacokinetic analysis.

In Vitro Human CYP Inhibition Test

The reagents and enzymes were commercial products from the following sources: insect cell microsomes containing cDNA-expressed human CYP1A2, CYP2C19, CYP2D6 and CYP3A4 (Gentest Corporation), $NADP^+$ (Oriental Yeast Co. Ltd.), Glucose-6-phosphate (G-6-P, Oriental Yeast Co. Ltd.), glucose-6-phosphate dehydrogenase (G-6-P dehydrogenase, Oriental Yeast Co. Ltd.), 3-cyano-7-ethoxycoumarin (CEC, Gentest Corporation), dibenzylfluorescein (DBF,

Gentest Corporation), 3-[2-(*N*,*N*-diethylamino) ethyl]-7-methoxy-4-methylcoumarin (AMMC, Gentest Corporation), 7-benzyloxy-4- (trifluoromethyl)-coumarin (BFC, Gentest Corporation).

The assay was performed in 96-well microtiter plates according to a protocol reported by Gentest Corporation (http://www.gentest.com). The conditions are summarized in Table I.

Test compound was serially diluted with DMSO and added to the prepared NADPH regenerating system in KPO_4 buffer (pH 7.4). After preincubation at 37 ℃, microsomes and substrate were added to the mixtures, and then incubation was carried out for the appropriate period. The reaction was stopped with stop solution and fluorescence was measured using a multi label counter (WALLAC 1420 ARVO). The IC_{50} values were calculated by linear interpolation.

Table I Reagents and conditions.

Reagents and conditions				
P450	CYP1A2	CYP2C9	CYP2D6	CYP3A4
NADPH regenerating system				
$NADP^+$	1.3 mM	1.3 mM	8.2 □M	1.3 mM
G-6-P	3.3 mM	3.3 mM	0.41 mM	3.3 mM
$MgCl_2$	3.3 mM	3.3 mM	0.41 mM	3.3 mM
Ge-6-P dehydrogenase	0.4 units/ml	0.4 units/ml	0.4 units/ml	0.4 units/ml
Other reagents				

KPO_4 (pH 7.4)	100 mM	25 mM	100 mM	200 mM
Substrate	CEC 5 μM	DBF 5 μM	AMMC 1.5 μM	BFC 50 μM
Enzyme	0.5 pM	2.0 pM	1.5 pM	1.0 pM
Incubation time	15 min	30 min	30 min	30 min
Measurement of fluorescence				
Fluorescent product	CHC	Fluorescein	AHMC	HFC
Excitation (bandwidth of filter)	409 nM (20 nM)	485 nM (20 nM)	390 nM (20 nM)	409 nM (20 nM)
Emission (bandwidth of filter)	460 nM (40 nM)	538 nM (25 nM)	460 nM (40 nM)	530 nM (25 nM)
Stop solution	80% CH_3CN / 20% 0.5 M Tris base	2 N NaOH	80% CH_3CN / 20% 0.5 M Tris base	80% CH_3CN / 20% 0.5 M Tris base

Abbreviations:

CHC, 7-hydroxy-3-cyanocoumarin

AHMC, 3-[2-(*N*,*N*-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin

HFC, 7-hydroxy-4-trifluoromethylcoumarin