

# *Supporting information for:*

## **Rotationally-constrained 2,4-diamino-5,6-disubstituted pyrimidines: A New Class of Histamine H<sub>4</sub> Receptor Antagonists With Improved Drug-likeness and Broad In Vivo Efficacy in Pain and Inflammation Models**

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**Combustion analysis data for new compounds.**

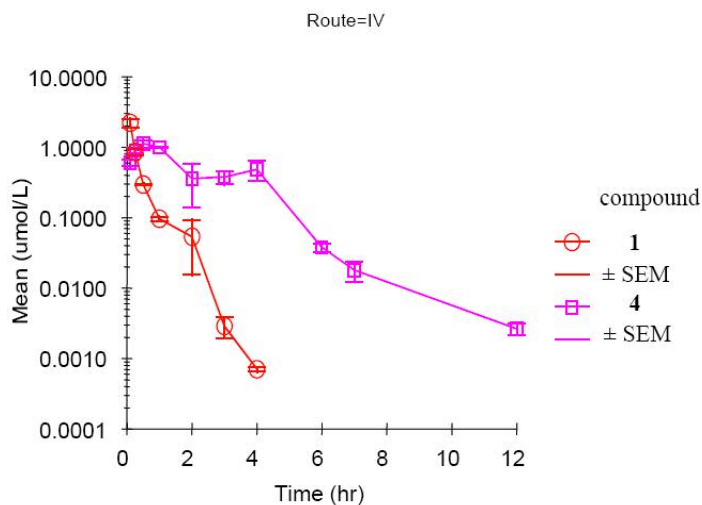
Compound	Composition	Combustion Analysis Results
<b>6</b>	$C_{17}H_{21}N_5 \cdot 0.05CH_2Cl_2$	Calcd: C, 68.35; H, 7.10; N, 23.37 Found: C, 68.30; H, 7.06; N, 23.37
<b>7</b>	$C_{16}H_{19}N_5 \cdot 0.45CH_2Cl_2$	Calcd: C, 61.83; H, 6.28; N, 21.91 Found: C, 61.87; H, 6.51; N, 21.85
<b>8</b>	$C_{16}H_{19}N_5 \cdot 0.2CH_3OH \cdot 0.2CH_2Cl_2$	Calcd: C, 63.62; H, 6.31; N, 24.09 Found: C, 63.4; H, 6.14; N, 23.85
<b>9</b>	$C_{17}H_{21}N_5 \cdot C_4H_4O_4 \cdot 0.25H_2O$	Calcd: C, 60.64; H, 6.18; N, 16.84 Found: C, 60.71; H, 5.84; N, 16.48
<b>10</b>	$C_{17}H_{21}N_5 \cdot C_4H_4O_4 \cdot \text{acetone} \cdot 0.75H_2O$	Calcd: C, 59.68; H, 6.78; N, 14.50 Found: C, 59.72; H, 6.88; N, 14.71
<b>11</b>	$C_{18}H_{23}N_5 \cdot C_4H_4O_4 \cdot 0.2\text{acetone} \cdot 1.5H_2O$	Calcd: C, 58.49; H, 6.67; N, 15.09 Found: C, 58.73; H, 6.41; N 14.71
<b>12</b>	$C_{17}H_{21}N_5 \cdot 1.05C_4H_4O_4$	Calcd: C, 61.02; H, 6.09; N, 16.78 Found: C, 61.17; H, 6.04; N 16.49
<b>13</b>	$C_{17}H_{20}FN_5 \cdot 2HCl \cdot 1.5H_2O$	Calcd: C, 49.40; H, 6.10; N, 16.94 Found: C, 49.56; H, 6.04; N, 16.62
<b>14</b>	$C_{19}H_{25}N_5 \cdot 2HCl \cdot 0.75H_2O$	Calcd: C, 55.68; H, 7.01; N, 17.09 Found: C, 55.80; H, 7.17; N, 17.22

## In vitro selectivity data

Table of in vitro selectivity in binding assays, relative to human H<sub>4</sub> Ki, expressed as a ratio of Ki values.

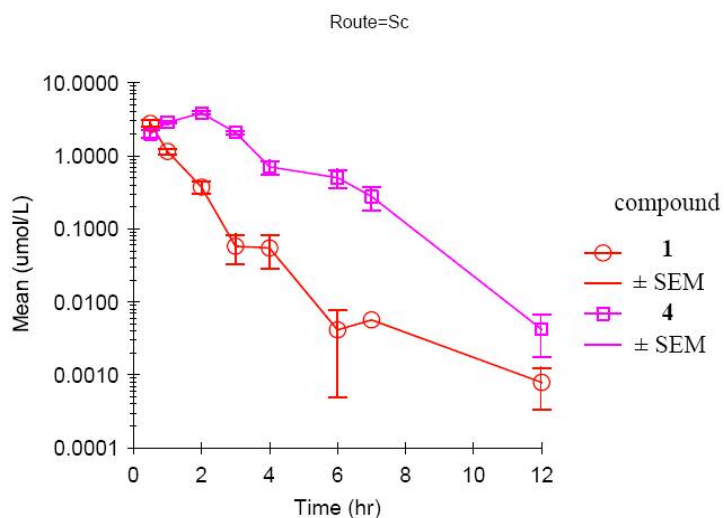
Compd	human H <sub>4</sub> Ki (nM)	Fold selectivity for hH4 versus other human receptors							
		H <sub>1</sub>	H <sub>2</sub>	H <sub>3</sub>	5-HT <sub>1a</sub>	5-HT <sub>1d</sub>	5-HT <sub>2a</sub>	D <sub>2</sub>	alpha <sub>1</sub> adrenergic
<b>1</b>	12	230x	>760	190x	>830x	>830x	410x	74x	444x
<b>2</b>	35	>170x	>260x	30x	1.2x	3.5x	120x	>140x	330x
<b>9</b>	71	>93x	>130x	52x	13x	17x	>185x	>140x	110x
<b>10</b>	4.7	>1400x	>1470x	>640x	610x	470x	4020x	1920x	190x
<b>11</b>	3.5	>1890x	1300x	570x	460x	235x	2030x	1820x	150x
<b>12</b>	11	>610x	>860x	93x	940x	310x	235x	123x	215x
<b>13</b>	5.1	>1290	>1820	470x	3070x	850x	765x	>2000x	1215x
<b>14</b>	39.8	>160x	>230x	56x	11x	81x	2.2x	480x	125x

**PK profile in mice:** JNJ-7777120 (**1**) is rapidly metabolized by demethylation to an H<sub>4</sub>-active metabolite (**4**) in mouse



IV Dose (5 mg/kg)*							
compound	C <sub>max</sub> (uM)	T <sub>max</sub> (hr)	t <sub>1/2</sub> (hr)	AUC <sub>0-t</sub> (uM*h)	AUC <sub>0-INF</sub> (uM*h)	V <sub>β</sub> (L/kg)	CL <sub>p</sub> (L/hr/kg)
<b>1</b>	2.22	0.10	0.38	0.90	0.90	11.04	20.01
<b>4</b>	1.12	0.50	1.63	3.03	3.04	0.00	NA

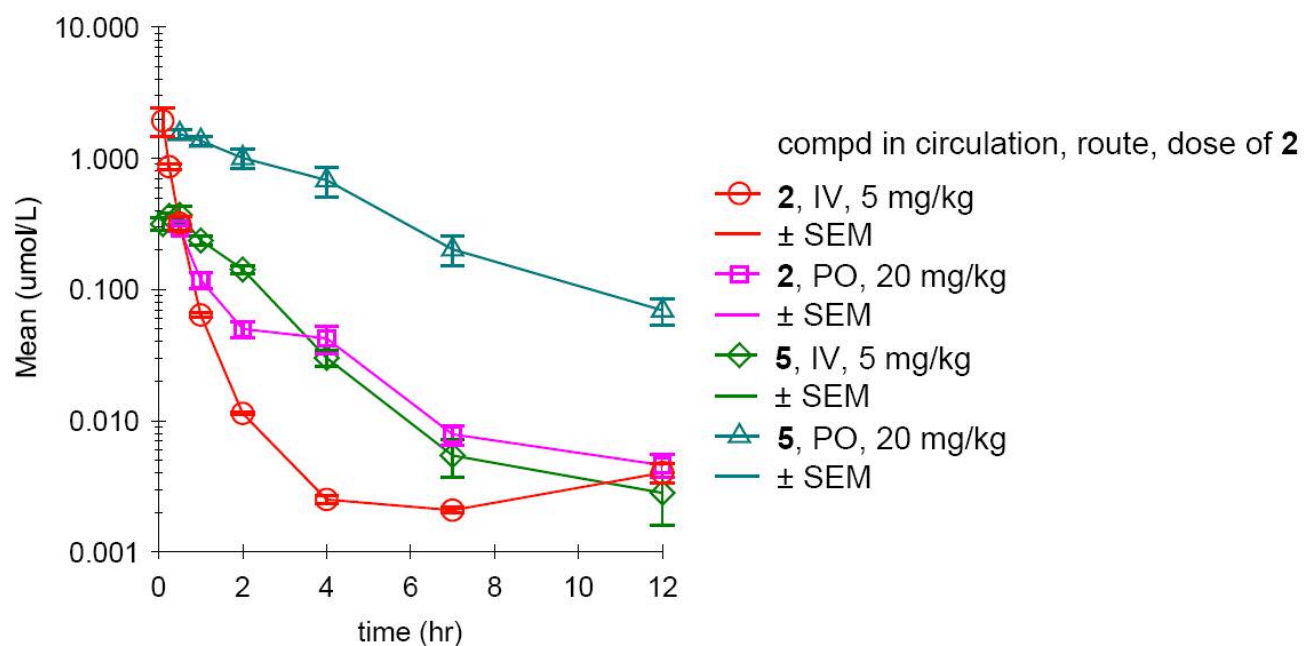
\* vehicle = 22.5% (w/v) hydroxypropyl-beta-cyclodextran, q.s. with D5W pH to 7.



Sc Dose (20 mg/kg)*						
compound	C <sub>max</sub> (uM)	T <sub>max</sub> (hr)	t <sub>1/2</sub> (hr)	AUC <sub>0-t</sub> (uM*h)	AUC <sub>0-INF</sub> (uM*h)	F (%)
<b>1</b>	0.50	0.50	0.97	2.79	2.79	77.61
<b>4</b>	2.00	2.00	0.86	11.77	11.77	0.00

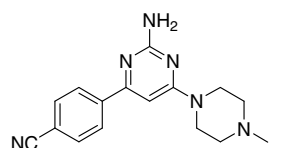
\* vehicle = 22.5% (w/v) hydroxypropyl-beta-cyclodextran, q.s. with D5Wp

**PK profile in mice for compound 2.** Determination of circulating drug levels of parent compound **2** and the des-methyl metabolite **5** over after dosing of compound **2** to mice, expressed as mean ( $\pm$  SEM) plasma levels. IV dosing of **2** was 5 mg/kg, and oral (PO) dosing of **2** was 20 mg/kg, to female Balb/C mice.

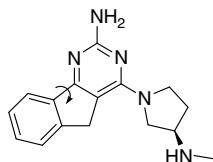


### Three-dimensional minimized structures with dihedral angles and in vitro functional data

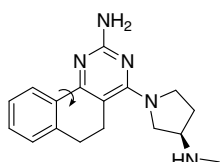
It is tempting to look to three-dimensional structural differences among the analogs to rationalize the difference in in vitro functional performance in the rodent FLIPR assays, and broader SAR supports that it is the dihedral angle between the pyrimidine and the phenyl ring of **6-13** that controls whether compounds can induce the rat H<sub>4</sub> receptor to activate the chimeric G-protein (G $\alpha_{qi5}$ ) in HEK cells the FLIPR assay in vitro. Among a broad series of analogs, it was a general finding that the five- and six-membered ring compounds fully or partially activated rat H<sub>4</sub> receptor (but not human H<sub>4</sub>) in the FLIPR assay, whereas seven-, eight-, and nine-membered rings did not activate rodent H<sub>4</sub> receptors in the FLIPR assay. One of the most obvious structural differences between these is dihedral angle between the carbons of the pyrimidine ring and the phenyl ring. As illustration of this, among analogs of **12**,<sup>ref. US patent application number.</sup> the 5- and 6-membered ring analogs have dihedral angles of 0.2° and 21.6° and activate rat H<sub>4</sub> to 104% and 70%, whereas the 7-, 8-, and 9-membered rings have dihedral angles of 49.6°, 62.5°, and 98.4° and did not activate rat H<sub>4</sub> in the FLIPR assay. The acyclic analog **2** (A-846714) also shows 64% activation of the rat H<sub>4</sub> receptor in the FLIPR assay,<sup>ref-Altenbach</sup> and the minimized structure has a dihedral angle of 14.2°, similar to that of the 5- and 6-membered ring compounds **S2**, **S3**, and **6-8**. The dihedral angles were calculated for the structures using MM2 minimization in ChemDraw 3D.



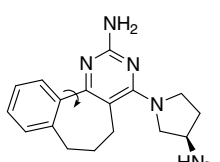
Compound **2**  
dihedral angle 14.2°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 8.53  
rat H<sub>4</sub> pEC<sub>50</sub> 7.23 (64%)



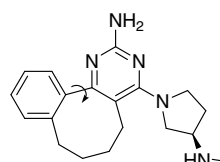
Compound **S2**  
dihedral angle 0.2°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 8.50  
rat H<sub>4</sub> pEC<sub>50</sub> 7.72 (104%)



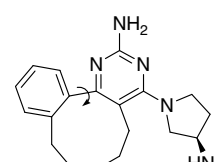
Compound **S3**  
dihedral angle 21.6°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 8.29  
rat H<sub>4</sub> pEC<sub>50</sub> 7.66 (70%)



Compound **12**  
dihedral angle 49.6°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 8.21  
rat H<sub>4</sub> pK<sub>b</sub> 8.18



Compound **S4**  
dihedral angle 62.5°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 8.15  
rat H<sub>4</sub> pK<sub>b</sub> 7.50



Compound **S5**  
dihedral angle 98.4°  
FLIPR profile  
hum H<sub>4</sub> pK<sub>b</sub> 7.60  
rat H<sub>4</sub> pK<sub>b</sub> 7.25

**Preparation of biological reagents and materials, and Method for the in vitro assay for the histamine H<sub>4</sub> receptor FLIPR Ca<sup>+2</sup>-flux functional assay:**

**In vitro histamine assay methods for the FLIPR Ca<sup>+2</sup>-flux functional assay:** Histamine H<sub>4</sub> receptors were cloned and stably expressed in HEK-293 (human embryonic kidney) cells coexpressing a G-protein (G $\alpha_{q15}$ ) as previously described in Esbenshade (Esbenshade, T. A.; Fox, G. B.; Krueger, K. M.; Baranowski, J. L.; Miller, T. R.; Kang, C. H.; Denny, L. I.; Witte, D. G.; Yao, B. B.; Pan, J. B.; Faghieh, R.; Bennani, Y. L.; Williams, M.; Hancock, A. A. Pharmacological and behavioral properties of A-349821, a selective and potent human histamine H<sub>3</sub> receptor antagonist. *Biochem. Pharmacol.* **2004**, 68, 933–945). Before testing, cells are loaded with a Ca<sup>+2</sup> sensitive fluorescent dye, in this case Fluo-4. In the case of partial agonist or agonist ligands, addition of compound to the cells leads to the increase in intracellular Ca<sup>+2</sup> which is detected by FLIPR (Fluorescence Imaging Plate Reader; Molecular Devices, Sunnyvale, CA) technology. In a similar manner, compounds that are antagonists, inverse agonists, block the increase in fluorescence induced by the full histamine H<sub>4</sub> agonist histamine, and partial agonists reduce the amount of fluorescence induced by the full histamine H<sub>4</sub> agonist histamine. The fluorescence intensities measured before addition of the test compound are subtracted from the fluorescence intensities at later time points. Peak response values determined at each concentration of ligand are expressed as a percentage of the response obtained with the full agonist histamine. Concentration versus response data are analyzed to obtain compound potency as K<sub>b</sub> values for antagonists and inverse agonists and as EC<sub>50</sub> values for partial agonists.

**Method for the in vitro assay for the histamine H<sub>4</sub> receptor competition binding assay:** The potency of compounds of the invention in displacing <sup>3</sup>H-histamine in competition binding assays is assessed by methods described in Esbenshade (Esbenshade, 2004). In this assay, membranes were prepared from HEK-293 cells transiently transfected with the pCINeo expression vector harboring the histamine H<sub>4</sub> receptor by homogenization of the cells on ice in TE buffer (50 mM Tris-HCl buffer, pH 7.4, containing 5 mM EDTA), 1 mM benzamidine, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml

pepstatin. The homogenate was centrifuged at 40,000g for 20 minutes at 4 °C. This step was repeated, and the resulting pellet was resuspended in TE buffer. Aliquots were frozen at -70°C until needed. On the day of assay, membranes were thawed and diluted with TE buffer. Competition radioligand binding assays were performed with increasing concentrations of test compound in the presence of [<sup>3</sup>H]-histamine incubated at 25°C for 1 h in a total volume of 0.5 ml of 50 mM Tris, 5 mM EDTA, pH 7.4. All binding reactions were terminated by filtration under vacuum onto polyethylenimine (0.3%) presoaked Unifilters (PerkinElmer Life Sciences) or Whatman GF/B filters (Whatman, Clifton, NJ) followed by three brief washes with 4 ml of ice-cold TE buffer. Bound radiolabel was determined by liquid scintillation counting. For all of the radioligand competition binding assays, IC<sub>50</sub> values and Hill slopes were determined by Hill transformation of the data and K<sub>i</sub> values were determined by the Cheng–Prusoff equation.

**In vitro histamine assay methods the native cell assay for histamine induced shape change in bone marrow derived mast cells (BMMCs):**

**Bone Marrow Mast Cell (BMMC) Culture:** Cells were collected from femurs from Balb/c female mice (age 8-10 weeks) were flushed with cold RPMI 1640, filtered through a 100 micron filter and centrifuged (1200 r.p.m., 5 minutes). After treatment with PharmLyse (Pharmingen) to lyse red blood cells, the cells were washed twice with cold RPMI-1640 medium, and plated at  $1 \times 10^5$ - $10^6$  cells/ml in complete RPMI medium (RPMI-1640 contains 5mM glutamine, 0.1 mM non-essential amino acids, 160 IU/mL Penicillin, 160 ug/mL Streptomycin, 50  $\mu$ M  $\beta$ -mercaptoethanol and 10 ng/ml recombinant murine interleukin IL-3). After each week, non-adherent cells were removed, centrifuged (1200 r.p.m., 5 minutes), fresh media added and transferred to a new dish for further culture. After four weeks, cells were analyzed by flow cytometry for expression of target biomarkers (Fc $\epsilon$ RI and c-kit (CD117)). Mast cells from 4-7 week cultures were found to be >99% positive for both biomarkers Fc $\epsilon$ RI and c-kit and were used in GAFS experiments.



**Assessment of Shape change by Gated Autofluorescence Forward Scatter (GAFS):** Cells prepared as described above were centrifuged (1200 r.p.m., 5 minutes) and resuspended at  $2 \times 10^6$  cells/ml in RPMI-1640 media containing 0.1% bovine serum albumin. 140  $\mu$ L samples of cells were incubated in the presence or absence of antagonists for 5 minutes at 37 °C. Cells were stimulated with histamine (10  $\mu$ M final concentration) for 10 minutes at 37 °C, then fixed with 1.5% paraformaldehyde on ice to maintain cell shape. Fluorescence was measured by flow cytometry (Becton Dickinson FACSCaliber system). BMMCs were gated based on their autofluorescence and 10000 events were acquired for sample. Results were calculated as percentage increase in forward scatter (FSC) compared to unstimulated cells. Raw data was analyzed using GraphPad Prism® 4 software (GraphPad Software, Inc.). IC<sub>50</sub> values for antagonists were calculated using the non-linear regression and Kb values calculated using a modified Cheng-Prusoff equation (Leff and Dougall, Trends Pharmacol. Sci. 14 (4), 110-2 (1993)).

**Animal Care:** All animals were housed in AAALAC-approved facilities in accordance with international standards of animal care, at Abbott Laboratories in a temperature-regulated environment under a controlled 12-h light-dark cycle, with lights on at 6:00 a.m. Food and water were available *ad libitum* at all times, except during actual testing. All testing was done following procedures outlined in protocols approved by Abbott's Institutional Animal Care and Use Committee.

**In vivo methods for pharmacologic blockade of H<sub>4</sub> agonist-induced itch:** The method of Bell (Bell, J. K.; McQueen, D. S.; Rees, J. L. Involvement of histamine H<sub>4</sub> and H<sub>1</sub> receptors in scratching induced by histamine receptor agonists in balb C mice. *Br. J. Pharmacol.* **2004**, *142*, 374–380) was used. Animals (male CD-1/ICR mice weighting 25-35 grams, Charles River Labs) were acclimated in an observation cage for 20 minutes. Then the histamine H<sub>4</sub> agonist clobenpropit (10 nmoles in 100  $\mu$ L water) was injected intradermally by syringe with a 30-gauge needle into the rostral part of the back. After injection, the animals were put back into the cage (with one mouse per cage), and itching was assessed by counting the number of scratches during the 25-minute period immediately following

injection of clobenpropit. In this method, only the scratching made with the hind paw was recorded, with scratching by the forelimbs were disregarded. The injection site was chosen to be accessible by the hind paws. A 'bout' of scratching was defined as three or more individual rapid scratch movements with the hind paws to the area around the injection site (e.g. the back of the neck). Each mouse was used for only one determination. To determine the ability of compounds to block clobenpropit induced scratching, drug was administered (i.p.) 30 min before the injection of the clobenpropit. Data are presented as mean  $\pm$  S.E.M., where treatments are compared to control clobenpropit-induced scratch responses.

**In vivo methods for blockade of peritonitis inflammation assay: Mouse Zymosan-Induced Peritonitis Model.** Mice (male Balb/C, Charles River Laboratories, Wilmington, MA) were used to study the effects of H<sub>4</sub> antagonists on zymosan-induced peritonitis. The H<sub>4</sub> antagonist was dosed s.c. in a vehicle containing 2%DMSO/3% Tween-80/95% (v/v, 10 ml/kg) 30 minutes before i.p. administration of the inflammogen zymosan (0.25 mg in saline at 0.5 mL/mouse, Sigma-Aldrich, St. Louis, MO). After 2 hours, the peritoneal cavities were washed with 3 ml of ice-cold phosphate buffered saline (pH 7.2), and the lavage fluid was collected in an EDTA-coated tube. The number of migrated PMNs (polymorphonuclear cells) was determined with a CellDyn analytical cell counter (Abbott Laboratories, Abbott Park, IL) on an aliquot (10 ul) of the lavage fluid. As a control, indomethacin was also included, dosed at 3 mg/kg (in 2% DMSO/3% Tween-80/95% HPMC, v/v, 10 ml/kg s.c.). The samples that were visibly red were not included in the analysis (Rao T.S.; Currie, J. L.; Shaffer, A. F.; Isakson, P. C. In vivo characterization of zymosan-induced mouse peritoneal inflammation. *J. Pharmacol. Exp. Ther.* **1994**, 269, 917-925.

**PMNL Myeloperoxidase (MPO) Activity.** The lavage samples (1 ml) were centrifuged at 2000g for 5 minutes in refrigerated micro-centrifuge tubes. The cell pellets were re-suspended in 1 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, sonicated for 30 seconds, freeze-thawed three times, and centrifuged at 20,000 x g for 10 min a refrigerated microfuge to collect supernatants which were used in MPO assays. MPO assays were performed as per

the procedures previously (Bradley et al., 1982) adapted to a 96 well plate format. The reaction was initiated by the addition of assay buffer containing o-dianisidine (0.2 mg/ml) and hydrogen peroxide (0.001%). The rate of change of absorbance was monitored at 450 nanometer wavelength in kinetic mode by a plate reader. At ED<sub>50</sub>, the responses of drug-treated animals were significantly ( $p < 0.05$ ) from the responses of vehicle-treated animals.

**In vivo methods assessment of inflammatory pain:** To assess the effectiveness of representative compounds of the invention against acute model inflammatory pain, animals were tested in an acute model of carrageenan-induced thermal hyperalgesia (see for example, Honore, et al. *Behavioural Brain Research* 167 (2006) 355–364; Porreca, et al. *Journal of Pharmacology and Experimental Therapeutics* (2006) vol. 318 pp. 195–205). Carrageenan was injected into the test paw of the animal, and after 90 minutes, the test drug was administered by intraperitoneal dosing. The effect on thermal hyperalgesia was assessed in a hotbox assay which was done 30 minutes after the intraperitoneal dosing of the test drug, and the MPE (maximal percent effect) reported by comparison to the control paw (not injected with carrageenan), according to 100 times the withdrawal latency of the carrageenan injected paw (in seconds) divided by the withdrawal latency of the control (not injected with carrageenan) paw. At ED<sub>50</sub>, the responses of drug-treated animals were significantly ( $p < 0.05$ ) from the responses of vehicle-treated animals.

**In vivo methods assessment of neuropathic pain:** Animals were prepared for testing, by use of a surgical procedure that induces neuropathic pain in one paw. Male Sprague Dawley rats were purchased from Charles River (Portage, MI). Prior to surgery, animals were housed in groups and maintained in a temperature-regulated environment. Following nerve ligation surgery, animals were housed in groups, and had access to food and water ad libitum.

The L5 and L6 spinal nerves of anesthetized rats were tightly ligated in a manner described previously (see Kim and Chung, *Pain* (1992) vol. 50 pp. 355-363). An incision was made on the dorsal portion of the hip and the muscle was blunt-dissected to reveal the spinal processes. The L6 transverse

process was removed, and the left side L5 and L6 spinal nerves were tightly ligated with 5.0 braided silk suture. The wound was cleaned, the membrane sewn with 4.0 dissolvable Vicryl suture and the skin closed with wound clips. The paw affected by the surgical procedure (the left paw) develops an allodynic response, a hypersensitivity to mechanical and other stimuli; neuropathic pain is assessed as an increased sensitivity in the surgically affected (left) allodynic paw compared to the control paw on the right side, and measured by comparing the response of the (left side) allodynic paw to the response of the unaffected right side control paw.

Mechanical allodynia in the affected paw of animals that had undergone spinal nerve ligation was evaluated using testing with von Frey filaments. As described previously by S.R. Chaplan, et al ("Quantitative assessment of tactile allodynia in the rat paw" J. Neurosci. Meth. (1994) vol. 53 pp. 55-63), two weeks following surgery rats were acclimated to a testing box constructed of plexiglass with a wire mesh floor which allowed access to the plantar surface of the animal's hindpaws. Using an Up-Down method (Dixon, Annual Rev. Pharmacol. Toxicol. (1980) vol. 20, pp. 441–462; Chaplan et al. "Quantitative assessment of tactile allodynia in the rat paw" J. Neuroscience Methods (1994) vol. 53 pp. 55–63), von Frey filaments of increasing stiffness were applied to the plantar surface of the hindpaws and the withdrawal response of the animals was observed; for the surgically affected paw with neuropathic pain (the left side paw) the baseline level of allodynia has a withdrawal threshold of  $\leq 4$  g of pressure. By comparison, for the control paw without allodynia (in this case the right side paw), the typical withdrawal pressure is around 15 g. Representative compounds of the invention, administered intraperitoneally 30 minutes before testing, are able to reduce the symptoms of neuropathic pain and induce a dose-dependent increase in the withdrawal threshold for allodynic (left side) limb, up to a maximum effect of 15 g. The efficacy of the compound in reducing neuropathic pain at different doses is determined by comparing response in the surgery-affected paw versus the response in the control paw. This is expressed as the MPE (maximum percent effect), or 100 times the withdrawal threshold of the allodynic (left side) divided by the withdrawal threshold of the control (right side).

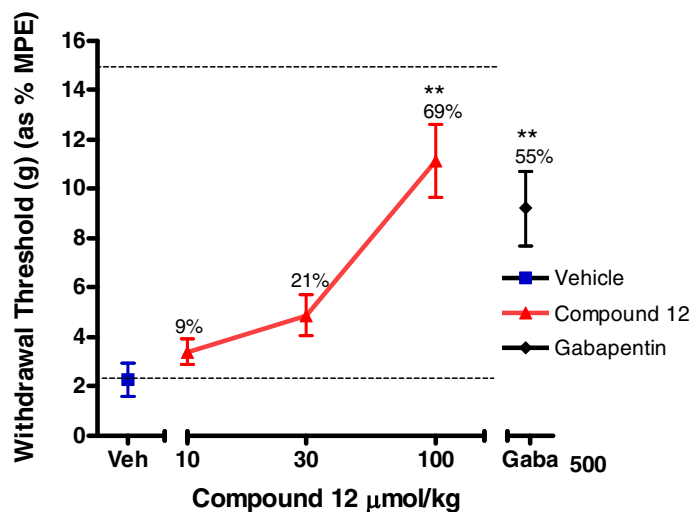
**In vivo method for assessment of efficacy in a post-surgical pain model:** The surgical skin incision model described by Joshi, et al. (Pain 123 (2006) 75–82) was used. Animals (rats) were prepared for testing by subjecting them to a surgical procedure carried out under sterile conditions, where the plantaris muscle was elevated and incised longitudinally with the origin and insertion of the muscle remaining intact. The skin was then closed with two mattress sutures (e.g. 5-0 nylon sutures). After surgery, animals were allowed to recover on a warming plate and housed individually in cages with soft bedding. After this surgery, the animals develop a hypersensitivity called allodynia; allodynia is pain due to a stimulus that does not normally provoke pain. Animals were tested for mechanical allodynia using von Frey hair mechanical stimulation 2, 24, and 48 h after surgery as described for the Chung model.

**In vivo method for assessment of efficacy against CFA-induced hyperalgesia:** To assess the effectiveness of representative compounds of the invention against hyperalgesia, animals were tested in an acute model of adjuvant-induced thermal hyperalgesia (see for example, Honore, et al. *Behavioural Brain Research* 167 (2006) 355–364; Carroll, et al. *US Patent application US 2006/0025614A1*). To prepare animals for the model, a solution of Complete Freund's adjuvant (150 microliters of a 50% aqueous solution, Sigma Chemical Company, St. Louis, MO) was injected into the plantar surface of test paw of the animal (rat). Rats so injected develop a hyperalgesia to thermal stimulation which is assessed 48 hours after injection of the Complete Freund's adjuvant using a commercially available paw thermal stimulator (UARDG, Dept. of Anesthesiology, Univ. California at San Diego, La Jolla, CA). For testing, rats were placed individually in a so-called 'hot box' (i.e. Plexiglass cubicles mounted on a glass surface) maintained at 30 °C, and allowed a 30 minute habituation period. A thermal stimulus, in the form of radiant heat emitted from a focused projection bulb, was then applied to the plantar surface of each hind paw. The stimulus current was maintained (e.g. at 4.5 Amp) and the time of exposure set to limit the extent of possible tissue damage (e.g. maximum of 20 seconds exposure to thermal heating from the projection bulb). The time (in seconds) for the withdrawal of the paw that was injected with the Complete Freund's adjuvant (CFA) and subjected to the thermal stimulus was recorded, and this was compared to the time (in seconds) for withdrawal of the uninjected paw subjected to the same thermal

stimulus. The paw injected with CFA develops a thermal hyperalgesia which is expressed as a shorter time (typically around 6 seconds) before paw withdrawal under thermal stimulus; uninjected paws do not show thermal hyperalgesia and have a normal withdraw time (typically around 10-12 seconds). To test the effect of a compound on the hyperalgesia, the test compound is dissolved in a vehicle (e.g. water or saline) and administered by i.p. (intraperitoneal) injection 30 minutes before the testing of the animal's response to the thermal stimulus to the paws. The antihyperalgesic activity of the compound in blocking the pain is shown by an increase in the time to withdraw the CFA-injected paw subjected to thermal stimulus. Experiments are done with single-dose and escalating-dosages of the test compound, and dose response curves constructed to determine ED<sub>50</sub> values.

### Anti-nociceptive efficacy data on compound **12** in additional models (a, b):

(a) Dose response of the anti-nociceptive efficacy of compound **12** expressed as the maximum percent effect (data as mean and SEM) in a model of post-surgical pain after skin incision (n = 6, dosed i.p.)



(b) Dose response of the anti-nociceptive efficacy of compound **12** expressed as the maximum percent effect (data as mean and SEM) after administration of complete Freund's adjuvant CFA (n = 6, dosed i.p.).

