JPET Fast Forward. Published on January 12, 2004 as DOI: 10.1124/jpet.103.060772

Histogranin-like antinociceptive and anti-inflammatory derivatives of o-phenylenediamine and benzimidazole

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Short running title: histogranin-like non-peptides

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Number of text pages: 30

Number of Tables: 4

Number of Figures: 4

Number of References: 50

Number of Words: Abstract: 237; Introduction: 780; Discussion: 2086

ABBREVIATIONS: AcOH, acetic acid; AD_{50}, the concentration that produces 50% analgesia; Boc, t-butyloxycarbonyl; DCC, N,N'-dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, N,N'-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; HF, hydrogen fluoride; HOBt, N-hydroxybenzotriazole; i.c.v., intracerebroventricular; i.t., intrathecal; i.v., intravenous; i.p., intraperitoneal; NMM, N-methylmorpholine; NMP, 1-methyl-2-pyrrolidone; PyBOP, benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid; PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; PBS, phosphate buffer saline; FBS, foetal bovine serum; lipopolysaccharide, LPS.

Recommended Section Assignment: Behavioral Pharmacology

ABSTRACT

Histogranin (HN)-like non-peptides were designed and synthesized using benzimidazole (compound **1**) and o-phenylenediamine (compounds **2-7**) as scaffolds for the attachment of phenolic hydroxyl and basic guanidino pharmacophoric elements present in HN. The benzimidazole derivative N-5-guanidinopentanamide-(2R)-yl-2-(p-hydroxybenzyl)-5-carboxybenzimidazole (**1**) and the o-phenylenediamine derivative N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl) phenylenediamine (**2**) were more potent analgesics than HN in both the mouse writhing (5.5 and 3.5 as potent as HN, respectively) and tail-flick (11.8 and 8.0 as potent as HN, respectively) pain assays. Improvements in the potencies and times of action of compound **2** in the mouse writhing test were obtained by attaching carboxyl (**6**) or p-Cl-benzoyl (**7**) groups at position 4 of the (2R) o-phenylenediamine derivative (**5**). In rats, compounds **2** (80 nmol; i.t.), **6** (36 nmol; i.t.) and **7** (18 nmol; i.t.) were effective in blocking both persistent inflammatory pain in the formalin test and hyperalgesia in the complete Freund adjuvant (CFA) assay. Compounds **2**, **6** and **7**, but not compound **1** at 10 nmol (i.c.v.) also mimicked the HN (60 nmol; i.c.v.) blockade of NMDA-induced convulsions in mice. Finally, in primary cultures of rat alveolar macrophages, HN and compounds **1**, **2**, **6** and **7** (10^{-8} M) significantly blocked lipopolysaccharide (LPS)-induced COX-2 induction and PGE_2 secretion. These studies indicate that both derivatives of benzimidazole and o-phenylenediamine mimic the *in vivo* antinociceptive and *in vitro* antiinflammatory effects of HN; but the HN protection of mice against NMDA-induced convulsions is mimicked only by the o-phenylenediamine derivatives.

Histogranin (HN; Fig. 1), a pentadecapeptide 80% homologous to the C-terminal portion 84 to 100 of histone H4 (H4-(86-100)), was originally isolated from bovine adrenal medulla (Lemaire et al., 1993). In the rat, immunoreactive HN was found to be present in various tissues including the pituitary, adrenal glands, spleen, lungs and brain (Lemaire et al., 1995). *In vivo* experiments in mice indicated that HN and its chemically stable analogue, [Ser^{1}]HN, are potent blockers of convulsions induced by NMDA, but not AMPA, kainate and bicuculline (Lemaire et al., 1993; Shukla et al., 1995). Recently, HN and related peptides were also found to display non-opioid analgesic activities. Thus, intracerebroventricular (i.c.v.) administration of these peptides in mice dose- and structure-dependently blocked writhing induced by intraperitoneal (i.p.) administration of acetic acid and tail-flick induced by radiant heat (Ruan et al., 2000). In the rat, intrathecal (i.t.) administration of [Ser^{1}]HN blocked the second phase of pain induced by the injection of formalin into the hind paws (Siegan and Sagen, 1997) and hyperalgesia induced by constriction of the sciatic nerve (Siegan et al., 1997) or administration of NMDA into the spinal cord (Hama et al., 1999). Finally, [Ser^{1}]HN and related peptides were also demonstrated to block long-term hyperalgesia induced by hind paw administration of complete Freund's adjuvant (CFA) (Hama and Sagen, 2002; Le et al., 2003). Although the role of prostaglandins in spinal inflammation and hyperalgesia has already been well documented (Seybold et al., 2003), the possible modulatory effect of HN and related compounds on the production of these proinflammatory agents has not yet been reported.

Among various analogues and fragments of HN, HN-(7-15) was observed to be significantly more potent analgesic than HN itself in the mouse writhing pain assay

(Ruan et al., 2000). In this C-terminal fragment of HN, the side-chains of three amino acids, i.e. Arg^{10}, Tyr^{13} and Phe^{15}, protrude outside of the backbone of the molecule and were suggested to play an important role in the analgesic activity of the peptide (Le et al., 2003). Herein, centrally constrained cores were designed as templates to create non-peptidic HN mimetics. Benzimidazole and o-phenylenediamine were used as scaffolds for the attachment of phenolic hydroxy and basic guanidino groups present in HN, the benzene ring of Phe^{15} in HN being comprised in the structures of these support molecules. The benzimidazole scaffold (Brase et al., 2002; Akamatsu et al., 2002; Vourloumis et al., 2003) has been used for the production of various drug libraries, including mimics of bradykinin B_2 (Heitsch, 2002) and enkephalin δ (Balboni et al., 2002) receptors and antagonists of substance P (NK_1; Khan, et al., 1996), angiotensin II (Kubo et al., 1993) and neuropeptide Y (Zarrinmayeh et al., 1999) receptors. On the other hand, the less rigid molecule of o-phenylenediamine, which possesses a hydrophilic skeleton, can also be used as a linker of pharmacophoric elements via attachment on adjacent free amino groups. Ortho-phenylenediamine has been successfully used as a precursor for the synthesis of tetrahydroquinoxaline-2-ones (Lee et al., 1997), benzopiperazinones (Morales et al., 1998), benzimidazoles and quinoxalines (Wu and Ede, 2001).

Herein, a building block was produced on solid-phase by nucleophilic substitution of the halogen atom in o-fluoro-nitroarenes with the basic N_α amino atom of Arg(Tos)-MBHA resin (Fig. 1). Attachment of a phenolic hydroxy group to this building unit was accomplished by reaction with p-hydroxybenzaldehyde or p-hydroxyphenylacetic acid to provide derivatives of benzimidazole and o-phenylenediamine, respectively. The choice

of the substitutions that were made on the structures of the non-peptide HN mimics was aimed at four specific goals: 1. to verify the importance for analgesic activity of the stabilization of the aromatic rings in the scaffold and attached phenolic hydroxyl group via the introduction of electron-withdrawing groups (Bean, 2002) (i.e. small lipophilic CF_3 group in compounds **3**, polarizable NO_2 group in compound **4**; Fig. 1); 2. to verify if the hydrogen in position 4 of o-phenylenediamine could be substituted with a carboxyl group (compound **6**) in analogy with that introduced in the benzimidazole derivative compound **1** (Fig. 1); 3. to verify if the addition of a p-chlorobenzoyl group at position 4 of o-phenylenediamine (compound **7**) would enhance its HN-like analgesic activity as it did for the potent HN-like cyclic tetrapeptide cyclo-(-Gly-pCl-Phe-Tyr-D-Arg-) (Le et al., 2003); and finally, 4. to verify the importance of the L-configuration (compound **2**) and corresponding D-isomer (compound **5**) of attached Arg pharmacophoric element for the analgesic potency, time of action and possible peripheral use of o-phenylenediamine derivatives. The results provided clues as to the relative importance of the scaffolds and attached pharmacophoric elements in the production of non-peptide compounds endowed with the *in vivo* analgesic and *in vitro* anti-inflammatory effects of HN as well as HN-like protection against NMDA-induced convulsions.

Materials and Methods

Materials. Chemicals and reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON L6H 9Z9). Boc-(L/D)-Arg(Tos)-OH, MBHA resin and all the reagents for solid-phase synthesis were purchased from Bachem Bioscience Inc. (King of Prussia, PA 19406) or Calbiochem-Novabiochem (San Diego, CA 92121). High purity solvents suitable for chemical synthesis were obtained from VWR Canlab (Mississauga, ON L5N 5Z7). Analytical HPLC separations were performed on a Waters model 600E, operating at a flow rate 1 ml/min, using a μ-Bondapak C18 (125 Å, 10 μm) column (3.9 x 300 mm) and monitoring at 310 nm and 254 nm with a variable wavelength absorbance detector (Waters 484). The crude products were purified by gel filtration using Sephadex G-10 (Amersham Pharmacia Biotech, QC H9X 3VI) and a preparative μ-Bondapak C18 (125 Å, 10 μm) column (25 x 100 mm), at a flow rate 5 ml/min. TLC was performed on precoated silica gel plates 60 F_{254} (Merck KGaA, Darmstadt, Germany) with the solvent system, (v/v) 1-butanol-acetic acid-water-pyridine (15:3:10:12). The compounds were visualized by the three following procedures: 1. UV; 2. iodine vapor (Choi et al., 2002), 3. Pataki spray reagent (Pataki, 1965). Mass spectra were recorded by the Centre Régional de Spectrométrie de Masse (Chemistry Department, University of Montreal, QC H3C 3J7) and the University of Ottawa Mass Spectrometry Center (Ottawa, ON K1N 6N5) using fast atom bombardment mass spectrometry (FAB-MS) and electrospray ionization (ESI) techniques.

Animals. Mice (male 20-25 g, Swiss Webster) were obtained from Charles River, St-Constant, Quebec. They were housed five per cage in a room with controlled

temperature (22 ± 2^{o}C), humidity and artificial light (06.30-19h). The animals had free access to food and water and were used after a minimum of 4 days of acclimation to housing conditions. Experiments were carried out between 10:00 a.m. and 4:00 p.m. in an air-regulated and soundproof laboratory (23 ± 1^{o}C, 40 % humidity), in which mice were habituated at least 30 min before each experiment. Rats (male 225-250 g, albinos Sprague Dawley) were obtained from Charles River, St-Constant, Quebec and housed individually for 7 days prior to experiments. The housing room was maintained at 23 ± 0.5 ^{o}C with a 12 h light/dark cycle. Food and water were available *ad libitum.* The experiments were authorized by the animal care committee of the University of Ottawa and Sherbrooke in accordance with the guidelines of the Canadian Council on Animal Care.

Drug Synthesis

The compounds were prepared manually on solid phase utilizing the para-methylbenzhydrylamine (MBHA) resin (Matsueda and Stewart, 1981) following reported procedures (Tumelty et al., 1999; Smith, et al., 1999) with modifications (Fig 1).

N-5-guanidinopentamide-(2R)-yl-2-(p-hydroxybenzyl)-5'-carboxybenzimidazole (compound 1) was synthesized in 4 steps beginning with Boc-D-Arg(Tos)-OH (1.1 g, 2.68 mmol) which was loaded onto to the MBHA resin (1 g, 0.67 mmol, Novabiochem) in the presence of the coupling agent PyBOP (1.4g, 2.68 mmol), HOBt, H_2O (0.2 g, 1.34 mmol) and DIEA (0.9 ml, 5.36 mmol) in 50 ml of DMF/DCM (1:1). After Boc-deprotection, H-D-Arg(Tos)-MBHA resin (1g, approximately 0.67 mmol) was converted to 2-nitroaniline derivative using 4-fluoro-3-nitrobenzoic acid (1.1 g, 6.7 mmol) and of DIEA (0.6 ml, 3.35 mmol) in DMSO (20 ml). The completion of the coupling reaction was monitored by the Kaiser test (Kaiser et al., 1970). Reduction of the

nitro group by the treatment with $SnCl_2$, $2H_2O$ (1 M, 4.5g) and NMM (1 M, 2.2 ml) in NMP (20 ml) overnight at room temperature gave resin-bound aminoaniline. The resulting resin was immediately condensed with p-hydroxybenzaldehyde (0.8 g, 6.7 mmol) in NMP (50 ml) by stirring the mixture for 8 hr at room temperature, followed by heating at 50^0 C for 8 hr to produce the resin-bound benzimidazole. Cleavage from 1g of resin was achieved using 15 ml of anhydrous liquid HF and 1 ml of anisole as scavenger for 1 hr at 0^o C to give compound **1**. The crude compound was precipitated and washed using anhydrous diethyl ether (200 ml), dissolved in DMF (4 x 50 ml), and then concentrated *in vacuum.* It was purified by gel filtration on Sephadex G-10 followed by semipreparative RP-HPLC using a 25 x 200 mm column (μBondapak C18, Waters), operating at a flow 5 ml/min. The chromatography (gradient system: 15% to 65% of acetonitrile in 1 hr) was carried out to provide a lyophilized white powder (94 mg, 34%-based on the substitution of the starting resin). HPLC k'= 2.50 (analytical C_{18}, 15-80% of acetonitrile in 50 min), Rf = 0.59, ES MS for compound **1** ($C_{20}H_{22}N_6O_4$): calcd 411.33; found $[M+H^+]$ 411.2 (Table 1).

N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl)phenylenediamine (compound 2) was prepared in a similar fashion as described above. Boc-Arg(Tos)-OH (1.1 g, 2.68 mmol) and 1-fluoro-2-nitrobenzene (0.7 ml, 6.7 mmol) were used instead of Boc-(D)Arg(Tos)-OH and 4-fluoro-3-nitrobenzoic acid for coupling into the MBHA resin (1g, 0.67 mmol) in the 1^{st} and 2^{nd} step (Fig. 1). In the final step, the resin-bound aminoaniline was immediately acetylated with 10 equiv of p-hydroxyphenylacetic acid in the presence of DCC/ HOBt overnight at room temperature. The completion of the coupling reaction was monitored

by the Kaiser test (Kaiser et al., 1970). Finally, the cleavage and purification steps were accomplished using the same conditions as those described for compound **1**. Yield: 120 mg (45%) as the lyophilized white powder. HPLC k'= 2.63 (analytical C_{18}, 15-65% of acetonitrile in 50 min), Rf = 0.66, ES MS for compound **2** ($C_{20}H_{26}N_6O_3$): calcd 399.46; found [$M+H^+$] 399.6 (Table 1).

N-5-guanidinopentanamide-(2S)-yl-2-N-(4'-hydroxy-3'-nitrophenylacetyl) phenylenediamine (compound 3) was prepared as described for compound **2** with the exception that 4-hydroxy-3-nitrophenylacetic acid (0.4 g, 2.01 mmol) was used for acetylation of the o-aminoaniline resin (24hrs; Fig. 1). Yield: 74 mg (25%) as the lyophilized white powder. HPLC k'= 2.41 (analytical C_{18}, 15-65% of acetonitrile in 50 min), Rf = 0.69, ES MS for compound **3** ($C_{20}H_{25}N_7O_5$): calcd 444.46; found [$M+H^+$] 444.5 (Table 1).

N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl)-4-trifluoromethyl-phenylenediamine (compound 4) was prepared in a similar fashion as described for compound **2**. 4-fluoro-3-nitrobenzotrifluoride (0.9 ml, 6.7 mmol) was used instead of 1-fluoro-2-nitrobenzene to load into the deprotected H-L-Arg(Tos)-MBHA resin (1g, approximately 0.67 mmol) in the 2nd step (Fig. 1). Yield: 96 mg (31%) as the lyophilized white powder, HPLC k'= 2.18 (analytical C_{18}, 15-65% of acetonitrile in 50 min), Rf = 0.70, ES MS for compound **4** ($C_{21}H_{26}N_6O_3F_3$): calcd 467.2; found [$M+H^+$] 467.2 (Table 1).

N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)phenylenediamine (compound 5) was obtained following the same procedure as the one described for the preparation of compound **2** except that Boc-

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D-Arg(Tos)-OH was used for the first coupling step to MBHA resin (1 g, 0.67 mmol; Fig. 1). Yield: 56 mg (21%) as the lyophilized powder. HPLC k': 2.64 (analytical C_{18}, 15%-65% of acetonitrile in 50 min). R_f: 0.66, ES-MS for compound 3 ($C_{20}H_{26}N_6O_3$). Calculated 399.46; found [$M+H^+$] 399.6 (Table 1).

N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)-4-carboxy-phenylenediamine (compound 6) was prepared in a similar fashion as described for compound **2**. 4-fluoro-3-nitrobenzoic acid (1.2 g, 6.7 mmol), was used instead of 1-fluoro-2-nitrobenzen to load into the deprotected H-D-Arg(Tos)-MBHA resin (1g, approximately 0.67 mmol) in the 2^{nd} step (Fig. 1). Yield: 90 mg (30%) as the lyophilized white powder, HPLC k'= 0.70 (analytical C_{18}, 15-80% of acetonitrile in 50 min), Rf = 0.68, ES MS for compound **6** ($C_{21}H_{27}N_6O_5$): calcd 443.20; found [$M+H^+$] 443.20 (Table 1).

N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)-4'-(p-chloro-benzoyl)phenylenediamine (compound 7) was a sub-product derived from the synthesis of compound **6.** O-(N-acyl) phenylenediamine-resin (1g, approximately 0.67) was treated with 1,1'–carbonyldiimidazole (1.1 g, 6.7 mmol) and DMAP (0.4 g, 3.35 mmol) in THF (20 ml) overnight at 4^0C then immediately coupled with 4-chlorophenylmagnesium bromide (6.7 ml of 1.0 M solution in diethyl ether, 6.7 mmol) in THF (20 ml) overnight at 4^0C. Yield: 49 mg (14%) as the lyophilized white powder. HPLC k'= 1.54 (analytical C_{18}, 15-80% of acetonitrile in 50 min), Rf = 0.70, ES MS for compound **7** ($C_{27}H_{29}ClN_6O_4$): calcd 537.01; found [$M+H^+$] 537.22 (Table 1).

***In Vivo* assays**

Mouse writhing test. Male swiss webster [(SW)f BR] mice were injected i.p. with 1.0 % acetic acid (10ml/kg) 5 min after i.c.v. (or i.p., as indicated) injection of 0 (vehicle), 0.25, 0.5, 1, 10, 25 and 50 nmol of HN or related non-peptides. Freehand i.c.v. injection of the tested compounds were made into the left lateral ventricle of the conscious mouse by the method of Clark et al. (1988) with a no 27 gauge, p.25-in needle attached to a 500 µl Hamilton syringe and an automatic dispenser (PB 600; Hamilton Co, Reno, NV) as described by Shukla et al. (1995). The number of writhes displayed by each mouse was counted for a period of 10 min after the injection of the acetic acid solution as described by Le et al. (2003). Groups of 10 mice were used for each dose. Antinociceptive activity was expressed as % analgesia as calculated by the formula: [(mean number of writhes in control group - mean number of writhes in the test group)/(mean number of writhes in control group)] x 100. The percent analgesia for various effective doses was then used to calculate the AD_{50} and potency ratio by the method of Lichfield and Wilcoxons (1949) using procedure 47 of the computer program of Tallarida and Murray (1986). The times of action of the compounds were determined by injection of 1 % acetic acid at various times after the administration of the peptides. Data are analyzed by the Wilcoxon's paired non-parametric test. The criterion for statistical significance was $P < 0.05$.

Mouse tail flick assay. The latency to withdraw the tail from a focused light stimulus was determined using a photocell (D'Amour and Smith, 1941) as described by Le et al. (2003). Experiments were performed between 10:00 and 15:00 hr. Mice were lightly restrained under paper wadding and their tails were placed gently on a beam radiation window. Noxious stimulation was provided by a beam of high density light focused on the tail. The light intensity was set at 40 to give a control reading of about 3 s. The response time latency was measured automatically and was defined as the interval between the onset of the thermal stimulus and the abrupt flick of the tail. Each

determination was performed in at least 10 animals. The mean score was taken as the response latency. A cut-off latency of 12 s was employed to prevent the possibility of tissue damage. The antinociceptive effect of the compounds (i.c.v.) was expressed as the percentage of the maximum possible effect, as calculated by the formula: %MPE = [(post-injection latency-baseline latency) / (cut off latency-baseline latency)] x 100. Group %MPE means were compared using one-way ANOVAs and $P \leq 0.05$ was considered significant.

Rat pain assays. Experimental procedures for *in vivo* rat pain assays were similar to those previously described by our group (Le et al., 2003). Briefly, prior to surgery, rats were pre-medicated with atropine sulfate (0.05 mg/kg) injected intramuscularly (i.m.) and, 30 min later, were anesthetised with a mixture of ketamine (90 mg)/xylazine (6 mg) i.m. Animals were first placed into the stereotaxic apparatus to isolate the caudal part of the skull and to pierce the occipital membrane. Then, animals were removed from the stereotaxic apparatus and a polyethylene tubing (PE 10, 11 cm) was slid down 7.5 cm (to the level of the lumbar enlargement). The tubing was secured onto the skull by a drop of dental cement. A piece of tubing (PE50, 1 cm) was inserted onto the extremity of the catheter to avoid clogging. Rats were allowed at least 7 days to recover from the surgery before testing began.

Rat formalin assay. To avoid stress-induced analgesia, rats were habituated to the formalin boxes and testing environment 30 min per day for four consecutive days. On testing day, the protective tube at the end of the i.t. catheter was removed. Saline or one of the compounds were administered i.t. in a volume of 10 µl using a 50 µl Hamilton syringe attached to a polyethylene tubing (PE20) through a 30g needle. Five min later, 50 microliters of diluted formalin (2.5%) was injected subcutaneously into the plantar

surface of the hindpaw using a 0.3 ml disposable syringe. Testing started 25 min after formalin injection and animals were observed for a period of 30 min. Behaviours were rated using a BASIC program which calculates the time spent in 4 mutually exclusive categories of behaviour (BASIC program K.B.J. Franklin, McGill University). Categories of behaviours were defined according to the description of Dubuisson and Dennis (1977).

Rat CFA assay. Baseline paw withdrawal latencies from the hot plate were first obtained (two trials per rat). The hot plate consisted of a 30 x 30 cm metal plate heated at 49^{o}C. This low intensity of thermal stimulation was chosen to allow the observation of the hyperalgesic effects induced by Complete Freund adjuvant (CFA). Once baseline latencies were obtained, 100 µl of CFA (50 µg) was administered into the plantar surface of one hind paw and animals were returned to their home cage. Twenty-four hours later, animals were brought to the testing room. Baseline latencies were obtained again to verify that CFA injection had effectively induced a hyperalgesic response. Each rat received an i.t. injection of saline or HN analogue. Testing started 10 min after injection. Paw withdrawal latencies were obtained at 10 min intervals for 70 min. A cut-off of 20 sec was imposed in order to avoid tissue damage. The experiments were repeated at 48 h, using the same animals. Data were analyzed by means of Analyses of Variance (ANOVA) followed by planned post-hoc comparisons using the Fisher procedure. In all cases, differences were considered significant if they had a probability of random occurrence of less than 5%.

Anticonvulsive activity in mice. Synthetic HN (60 nmol) and related non-peptides (10 nmol except for compound **2**: 25 nmol) were injected (i.c.v.) in mice 5 min prior to the administration of NMDA (1 nmol/mouse, 10 µl, i.c.v.). Control experiments were injected with saline (10 µl) 5 min prior to NMDA. The animals were observed for

30 min for the signs of convulsions and death (Lemaire et al., 1993). Groups of 10 animals were tested. In each group, the number of animals showing the behavioural signs of convulsion was recorded. Statistical calculation were made using Student t- test.

***In vitro* bioassays**

Measurement of prostaglandin E_2 (PGE_2) from rat alveolar macrophages (AM). Male Wistar rats weighing 250 to 300 g were purchased from Harlan Sprague Dawley Inc. (Indianapolis). These animals were derived from a pathogen-free colony, shipped behind filter barriers, and housed in a horizontal laminar flow isolator (Johns Scientific Inc., Toronto). Bronchoalveolar cells were obtained by bronchoalveolar lavage as described (Lemaire, 1991). Briefly, after the animals were killed, the abdominal aorta was severed and the trachea cannulated. A total volume of 49 ml of PBS (pH 7.4) in 7-ml aliquots was infused in each animal, 93% (45 ml) of which was recovered. The bronchoalveolar cells were obtained by centrifugation at 200g at 4^{o}C for 5 minutes and resuspended in RPMI-1640 medium containing 0.5% dialysed FBS (Wisent Inc., St-Bruno, Quebec) and 0.8% Hepes (Sigma Chemical Co., St-Louis, MO), which will henceforth be referred to as tissue culture media. Differential cellular analysis, made from cytocentrifuged smears (4 x 10^4 cells) stained with Wright-Giemsa, indicated that the bronchoalveolar cells represent a pure population of alveolar macrophage (AM, 99%). Alveolar macrophages (0.2 x 10^6) were incubated in 0.2 ml tissue culture media for 20 h at 37^{o}C in a humidified 95% air-5% CO_2 atmosphere alone or with LPS (1 μg/ml; Sigma chemical Co., St-Louis, MO) in the presence and absence of HN or the various synthetic compounds at 10^{-8} M. The culture supernatants were collected, centrifuged and frozen at -80^{o}C. The following day, PGE_2 was determined in cell-free supernatants using a competitive enzymeimmunoassay (EIA) system (Biotrak, Amersham Biosciences, Little Chalfont, England).

COX-1 and COX-2 Immunoblotting. Macrophages were cultured at 10^6/ml in 24-wells for 20 h at 37°C in a humidified 95% air-5% CO_2 atmosphere alone or with LPS (1 μg/ml; Sigma chemical Co., St-Louis, MO) in the presence and absence of HN or the various synthetic compounds at 10^{-8} M. Cells were collected with a rubber policeman, pooled and centrifuged (5 min, 200 x g). The pellet was washed with PBS (pH 7.4) and frozen at -80°C. The cell pellet from each sample was resuspended in 100mM Tris, pH 7.4 and sonicated for 15 sec twice with an Ultrasonics cell disrupter to lyse the cells. Cell lysates were assayed for protein content by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Protein from each sample (5 - 20 μg) was denatured in Laemmli buffer for 5 min and resolved by SDS-gel electrophoresis on a polyacrylamide gel (4% stacking and 10% resolving layer) using an apparatus for minigels (Hoefer Scientific Instruments, San Francisco, CA). After electrophoresis, the proteins were transferred to nitrocellulose membranes with a Transfor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The membranes were blocked overnight at 4°C in Tris-buffered saline-0.1% Tween 20 (TBS-T) supplemented with 3% fat-free dried milk. After rinsing away the blocking solution with TBS-T-1% milk, the membranes were incubated for 90 min with primary antibody against COX-2 (1:1000, Cayman, Ann Arbor, MI) or COX-1 (1:100, Cayman Ann Arbor, MI) and against actin (1:250 or 1:2000 for COX-2 and COX-1 detection respectively, Sigma, St-Louis, MO). The specificity of the COX isoform-specific antibodies was tested by Western blotting of purified COX-2 (50 ng) and COX-1 (500 ng) electrophoresis standards per lane (Cayman, Ann Arbor, MI). After washes with TBS-T-1% milk, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz, CA) (1:1000 for COX-2 and 1:100 for COX-1) for 1 hr at

room temperature. Excess secondary antibody was washed away with TBS-T-1% milk (3X) followed by TBS (5X). The results were visualized after developing with BM chemiluminescence blotting POD substrate (Roche Diagnostics, Laval, Quebec) according to the manufacturer's instructions. Scanning densitometry was performed using a Kodak digital science Image Station and software. COX-2 and COX-1 signal density was normalized to actin density. Results were expressed as percent of control and represent mean ± SEM of at least 3 different experiments.

Results

Synthesis. For the synthesis of HN-like non-peptides, the basic amino acid Arg, the phenolic hydroxyl group and the benzene ring in positions 10, 13 and 15 of HN, respectively, were included in the structures of benzimidazole and o-phenylethylenediamine derivatives (Fig. 1). These compounds were synthesized by solid-phase on MBHA resin starting with the attachment of Boc-(D or L) Arg(Tos)-OH using PyBOP as the coupling agent. The introduction of aromatic nitro groups on Arg(Tos)-resins was accomplished by reaction with o-fluoro-nitroarenes and reduction with $SnCl_2.2H_2O$ in NMP. The MBHA-supported o-phenylenediamine was then treated with p-hydroxybenzaldehyde to provide the benzimidazole derivative (**1)**. The preparation of the o-phenylenediamine derivative compound **2** and analogues (compounds **3**-**7**) was performed as described above except for the last step wherein p-hydroxyphenyl acetic acids were used for the acetylation of the o-phenylenediamine-(+/-)Arg(Tos)-resin. Various modifications were made to the structure of the starting compound **2** (N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl)phenylenediamine; Fig. 2) by introducing a nitro group ortho to the phenolic hydroxyl group (**3**), changing the orientation of the guanidino group from the S to the R configuration (**4**) and attaching CF_3 (**5**), carboxyl (**6**) or p-chlorobenzoyl (**7**) groups at position 4 of the benzene ring of the R-configuration. The completed synthetic compounds were deprotected and cleaved from the resin with liquid HF and purified by gel filtration on Sephadex G-10 and HPLC on μ-Bondapak C18. All synthetic compounds were shown to be pure by various analytical criteria including thin-layer chromatography (a single spot, iodine vapor, UV

and ninhydrin detection), analytical HPLC (a single peak, 240 nm detection) and mass spectrometry, with yields varying between 14% and 45% (Table 1).

Antinociceptive activities. HN and related peptides (i.c.v.) were already shown to produce dose-dependent analgesic activity in the mouse writhing and tail-flick assays (Ruan et al., 2000). HN-like non-peptides displayed similar dose-dependent analgesic effects with significant increased potency as compared with HN or H4-(86-100) (Tables 2 and 3). Thus, compounds **1**, **2**, **6** and **7** were 5.5, 3.5, 7.3 and 8.8 times as potent as HN, respectively, in the mouse writhing test and 11.8, 8.0, 3.6 and 8.7 times as potent as HN, respectively, in the mouse tail-flick assay. Compounds **4** and **5** also showed the full intrinsic analgesic activity of HN, but their increased potency as compared with HN was not significant. In the mouse writhing test, compounds **1**, **5**, **6** and **7** displayed somewhat longer analgesic effects than HN with half-maximal decays observed at 36, 35, 58 and 36 min, respectively, as compared with 22 min for HN (Table 2). Interestingly, the HN-like peptides, H4-(86-100) showed analgesic potencies and times of action that were comparable to that of HN. In this respect, the C-terminal HN fragment HN-(7-15) was 2.5 times as potent as HN but its half-maximal response decay was smaller than 10 min, indicating the importance of the N-terminal portion of the molecule for its time of action. Among the various non-peptides, compounds **1**, **2** and **5** displayed significant analgesia (52.5%, 42.0% and 43.5% analgesia, respectively) in the mouse writhing test after peripheral (i.p.; 20 μmol/kg) administration while the other synthetic non-peptide compounds were ineffective. On the other hand, the analgesic effects of the synthetic non-peptides in the mouse tail-flick assay were slightly shorter lasting than that of HN (half-maximal decays of 16.7 to 28.9 min as compared with 45 min for HN; Table 3).

The HN-like peptide H4-(86-100) and HN-like non-peptides were also evaluated for their ability to modulate persistent inflammatory pain in the rat formalin test (Fig. 2) and reverse thermal hyperalgesia induced by intraplantar administration of CFA (Fig. 3). Intrathecal (i.t.) administration of H4-(86-100) (Fig. 2A) and compounds **2**, **6** and **7** (Fig. 2B) in rats decreased formalin pain in a dose-dependent fashion although the dose-response relation was biphasic and disappeared with larger doses. The maximal analgesic effects were obtained with 80 nmol of compound **2**, 36 nmol of compound **6,** 18 nmol of compound **7** and 0.25 nmol of H4-(86-100). In the rat CFA test, H4-(86-100) (0.25 nmol; i.t.) and compounds **2** (80 nmol; i.t.), **6** (36 nmol; i.t.) and **7** (18 nmol; i.t.) significantly blocked CFA-induced hyperalgesia 24 h (Fig. 3A) and 48 h (Fig. 3B) after the administration of CFA into a hind paw.

Blockade of NMDA-induced convulsions and death. HN and related non-peptide compounds also blocked convulsions in mice induced by the administration of NMDA (Table 4). Intracerebroventricular preadministration of HN (60 nmol) or compounds **2**, **6** or **7** (10 nmol) five min prior to NMDA (1 nmol) reduced the percentage of mice that showed signs of convulsions from 80% (NMDA alone) down to 53%, 53%, 60% and 40%, respectively. The mortality rate observed within 30 min after the administration of NMDA was either not affected or reduced by the preadministration of HN or compounds **2**, **6** or **7** (Table 4). On the other hand, compound **1** did not significantly block convulsions induced by NMDA, but instead caused a marked increase in NMDA-induced mortality from 20% to 60% (Table 4).

Blockade of LPS-induced PGE-2 release and COX-2 expression in macrophages. Prostaglandins are known to play an important role in the transmission of pain. Isolated rat alveolar macrophages stimulated with LPS (the archetype of bacterial antigen) produce significant amounts of PGE_2. LPS-stimulated release of PGE_2 from primary cultures of rat alveolar macrophages was reduced by HN and related peptides and non-peptides (Fig. 4A). At 10^{-8} M, the inhibitory effect of HN was comparable to that H4-(86-100) and compounds **1**, **2**, **6** and **7** and slightly less pronounced than that of HN-(7-15). Cyclooxygenase (COX), the enzymatic system responsible for the formation of PGE_2 exists under the two isoforms COX-1 and COX-2. In macrophages, COX-1 is expressed constitutively while COX-2 expression needs to be induced by appropriate stimuli such as LPS to be observed. The effects of HN and related compounds were determined on both isoenzymes. HN and related compounds **1**, **2**, **6** and **7** at 10^{-8} M did not alter the basal level of constitutively expressed COX-1 (not shown), but significantly inhibited LPS induction of COX-2 protein as assessed by immunoblot analyses of the ratio between the abundance of COX-2 and actin in protein extracts of control and LPS-stimulated alveolar macrophages (Fig. 4B).

DISCUSSION

Design of HN-like compounds. In previous studies, various analogues and fragments of HN have been synthesized and shown to inhibit NMDA-induced convulsions in mice (Prasad et al., 1995) and cause analgesia in various animal models of pain (Ruan et al., 2000; Le et al., 2003). Major improvement in the analgesic activity of the peptide was made by cyclization of the minimal active core HN-(7-10) and its modification of positions 8, 9 and 10 to provide cyclo-(-Gly-pCl-Phe-Tyr-D-Arg-) as a very potent and long-lasting analgesic (Le et al., 2003). The enhanced analgesic activity of this cyclic tetrapeptide as compared with that of HN was first suggested to be due to the presence in its structure of three pharmacophoric elements present at positions 10 (Arg), 13 (Tyr) and 15 (Phe) of HN (Fig. 1). It is also interesting to note that both N- and C-terminal fragments of HN, i.e. HN-(1-8) and HN-(8-15), contain Tyr and basic amino acids at positions that are symmetric in regard with the central amino acid Gln^8 (Fig. 1). Both C- and N-terminals portions in HN may have a role to play in the biological activity of the parent peptide, thus explaining the significant analgesic efficacies of HN-(1-10) and the short lasting analgesic effects of the potent peptide HN-(7-15) (Ruan et al., 2000; Table 2). Therefore, an ideal non-peptide mimic of HN may be constituted of a centrally located neutral scaffold that holds together the above mentioned pharmacophoric elements with some symmetrical spatial orientation between each other. The aim of the present study was to design and synthesize non-peptide models for the attachment or inclusion of HN pharmacophoric elements and assess the *in vitro* and *in vivo* HN-like activities of the synthetic compounds.

Choice of benzimidazole and the o-phenylenediamine as scaffolds for the design of HN mimics. The proposed non-peptide models, i.e. the benzimidazole and the o-phenylenediamine derivatives, in analogy with Phe[15] in HN, contain a benzene ring in their core structure (Fig. 1). However, benzimidazole provides a more rigid structure than o-phenylenediamine for the attachment of HN basic guanidino and phenolic hydroxyl pharmacophoric elements. The choice of these scaffolds for the attachment of HN pharmacophoric elements was based on several criteria. The benzimidazole and o-phenylenediamine scaffolds were first selected on the basis of previous reports which indicated that these types of molecules could be used as effective non-toxic peptide mimics and/or activators of specific membrane receptors (Heitsch, 2002; Balboni et al., 2002; Khan, et al., 1996; Kubo et al., 1993; Zarrinmayeh et al., 1999; Morales et al., 1998; Wu and Ede, 2001).

The next criteria was based on the principle that these molecules contain sites on which guanidino and phenolic hydroxy groups can be attached and still conserve enough freedom for proper orientation and interaction with the HN receptor(s). In this respect, the benzimidazole derivative (compound **1**) was created by the nucleophilic substitution of a fluorine atom within o-fluoro-nitroarene with the N_{α} amino group of Arg, followed by intramolecular cyclization to simplify the synthetic procedure. However, the resulting nitrogen atom in the benzimidazole ring was not ideally positioned to incorporate Arg in its natural free 3D conformation, possibly explaining why this compound did not display the complete biological profile of HN, being an effective analgesic and anti-inflammatory agent but not protecting mice against NMDA-induced convulsions. On the other hand, o-

phenylenediamine may be a scaffold that allows all pharmacophoric elements to find, in their search for global minima rather than just local minima (Menzler et al., 1998; Menzler et al., 2000), the best fit for binding with the HN receptor(s) and thus produce mimics with larger profiles of HN activities. The construction of the o-phenylenediamine derivatives resulted from the exploration of a simple modification of the designed δ-opioid non-peptide ligand [(2S,3R)TMT[1]]DPDPE to incorporate the HN pharmacophoric elements in its 1,4-piperazine backbone structure (Liao et al., 1998; Hruby, 2001). In this structure, the distance between the two nitrogen atoms was calculated to average that of a two amino acids spacer i.e. 7Å. At such a distance on o-phenylenediamine, the phenolic hydroxy and basic cationic groups may be well positioned to interact with their respective binding pocket on the HN receptor(s).

The various changes introduced on the scaffolds or attached pharmacophoric elements induced specific changes in the analgesic activity of the compounds in the mouse writhing assay, a pain model that was shown to be highly sensitive to i.c.v. administration of HN (Ruan et al., 2000). Thus, it was noted that the stabilization of the aromatic ring in o-phenylenediamine (compound **4)** or its attached phenolic hydroxyl group (compound **3)** markedly decreased or did not affect their analgesic potency, respectively, as compared with that of the parent compound **2.** On the other hand, the introducion of a carboxylic group at position 4 of the aromatic ring markedly enhanced both analgesic potencies and times of action of compound **6** as compared with compound **2.** Modification of the o-phenylenediamine ring on this position by the incorporation of a more hydrophobic and bulky group, i.e. the p-chlorobenzoyl group, slightly increased the analgesic potency and decreased the time of action of compound **7** in comparison with

compound **6**. However, the analgesic activity of compound **7** in the rat formalin test was more pronounced and it was observed with a larger dose range than that of compound **6**, indicating the importance of the type of pain assay and route of administration for the assessment of the biological activity of HN mimics. Finally, compounds **1**, **2** and **5** were the sole non-peptide compounds that displayed significant analgesic activity after peripheral administration (i.p.) in the mouse writhing test. This latter data indicates that among the various changes that were made at position position 4 of benzymidazole or o-phenylenediamine derivatives, the sole atoms and group of atoms that were tolerated for passage of the molecule through the blood brain barrier were the hydrogen atom or the carboxylic group. Furthermore, the switch from L- to D-configurations of the guanidino group in compounds **2** and **5**, respectively, slightly decreased the potency and enhanced the time of action, but it did not affect their peripheral analgesic activity (42.0% and 43.5% analgesia, respectively, at 20 μmol/kg, i.p.) in the mouse writhing test.

Histogranin-like activities of benzimidazole and o-phenylenediamine derivatives. Various *in vivo* and *in vitro* criteria indicate that the designed compounds are good mimics of HN. First, compounds **1**, **2**, **3**, **6** and **7** display improved analgesic potency and times of action as compared with HN in the mouse writhing test. Secondly, like HN, all synthetic compounds are more potent analgesics in the mild persistent mouse writhing pain assay than the acute radiant heat tail-flick test (Ruan et al., 2000; Tables 2 and 3). Thirdly, the analgesic effects of the non-peptidic HN-like compounds in the rat formalin assay are observed within a small range of concentrations and only during the second (tonic) phase of pain. Fourthly, the compounds completely abolish hyperalgesia in rats 24 hrs and 48 hrs after hindpaw administration of CFA, i.e. times when hyperalgesia is firmly established. Finally, in primary cultures of rat alveolar macrophages, the non-

peptides at 10^{-8} M are as efficient as HN in blocking the induction of COX-2 and reducing the release of PGE_2. All the above mentioned properties of the synthetic non-peptides are common to those of HN (Ruan et al., 2000; Siegan et al., 1997; Siegan and Sagen, 1997; Hama et al., 1999; Hama and Sagen, 2002). However, while o-phenylenediamine derivatives are as effective as HN in blocking NMDA-induced convulsions in mice, the benzimidazole derivative (compound **1**) does not block convulsions but instead, increases NMDA-induced mortality (Table 4). Therefore, the o-phenylenediamine derivatives represent here the sole group of non-peptides that can mimic all the known *in vivo* and *in vitro* effects of HN.

Possible mechanism of action of HN and related compounds. We have previously shown that HN and related compounds interact with the dopaminergic system (Ruan et al., 2000; Ruan and Lemaire 2001; Le et al., 2003). For instance, HN and derivatives compete with the binding of the D2 receptor antagonist [H3]raclopride to rat brain membranes and the analgesic effects of HN in the mouse writhing test can be reversed by central administration of raclopride (Ruan et al., 2000). These data suggest that HN acts as an agonist at the dopamine D2 receptor. Interestingly, dopamine agonists, mostly those with high affinity for the D2 subtype, are potent and efficacious analgesics (Morgan and Franklin, 1991). However, it is not known how HN produces its analgesic/anti-inflammatory effects in the spinal cord, but its effects could be attributed to inhibition of pro-inflammatory mediators. In the present paper, we show that HN blocks the LPS-induced expression of COX-2 and release of PGE_2 in primary cultures of rat alveolar macrophages.

PGE_2 is one of the various oxygenated metabolites of arachidonic acid known to mediate inflammatory responses associated with nociception. Nociception is the

downstream result of activation of primary afferent C fibers, which enter the spinal cord where they make synapses with interneurons and second order neurons projecting to the brain. Spinal prostaglandin release has been shown following administration of formalin in the hindpaw (Freshwater et al., 2002). Among the enzymes involved in the formation of prostaglandins, spinal COX-2 but not COX-1 has been implicated in the antihyperalgesic action of nonsteroidal anti-inflammatory drugs (NSAIDS) (Yaksh et al., 2001). Interestingly, HN and related non-peptides are potent blockers of LPS-induced COX-2 but not constitutive COX-1 in rat alveolar macrophages, an effect that is accompanied by a decrease in the LPS-evoked release of PGE_2 (Fig. 4). The spinal action of HN may be to inhibit the induction of COX-2 resulting from peripheral or central inflammation and thereof decrease the production and release of PGE_2. In support of this idea, the D2 receptor agonist quinpirole has recently been shown to inhibit the release of PGE_2 from pulmonary C fibers (Lin et al., 2003), the same type of fibers that encodes and transmit nociceptive information from the periphery to the spinal cord. Lending further support to the possible involvement of the dopamine D2 receptor in the antihyperalgesic action of HN is the fact that D2 receptor agonists, like HN, are usually far more efficacious in relieving inflammatory pain and hyperalgesia than altering basal pain threshold (Gilbert and Franklin, 2001).

In animals, spinal administration of NMDA produces marked thermal hyperalgesia (Tao and Johns, 2000) and NMDA receptor antagonists such as MK-801 relieve thermal and mechanical hyperalgesia induced by either peripheral nerve damage (Wegert et al., 1997) or peripheral inflammation (Ren et al., 1992, Leem et al., 2001). Despite their great efficacy in various animal models of inflammatory pain, the clinical

use of NMDA antagonists has been impeded by the compound toxicity as well as motor and cognitive side-effects. The interaction of HN and related peptides with the NMDA receptor was previously demonstrated by the ability of the peptides to partially inhibit the specific binding of the NMDA antagonist [^{3}H]CGP-39653 to rat brain membranes and to block NMDA-induced convulsions in mice (Lemaire et al., 1993; Shukla et al., 1995). The anticonvulsive effect of HN and related peptides correlated well with their ability to inhibit the binding of [^{125}I][Ser^{1}]HN to rat brain membranes (Rogers and Lemaire, 1993; Prasad et al., 1995) but not to cause analgesia in the mouse writhing test (Ruan et al., 2000). Herein, the fact that the benzimidazole derivative displays the analgesic and anti-inflammatory effects of HN, but not its anticonvulsive activity, further supports the concept that the interaction of HN with the NMDA receptor is not linked to its antinociceptive and anti-inflammatory activities. It is interesting to note that even high doses of HN injected centrally produced analgesia and antihyperalgesic effects without affecting locomotor activity (Ruan et al., 2000; Hama and Sagen, 2002). HH and related compounds may thus constitute a new class of therapeutic agents for the management of pain that fails to respond to conventional treatment.

Conclusions. The present results demonstrate that the designed o-phenylenediamine derivatives, which are constituted of basic guanidino and phenolic hydroxyl groups attached to a benzene ring, mimic the *in vivo* antinociceptive and NMDA-modulatory effects of HN by a mechanism that may involve a modulation of COX-2 induction and PGE_2 production. On the other hand, similar derivative of benzimidazole can also mimic the *in vivo* analgesic and *in vitro* anti-inflammatory effects of HN, but not the HN

protection against NMDA-induced convulsions, suggesting that the effect of HN on the NMDA receptor may be independent from its analgesic and anti-inflammatory activities. The possible role of the dopamine D2 receptor in the analgesic and anti-inflammatory effects of HN and related compounds remains to be clarified.

Acknowledgements

We wish to thank Mrs. Yanmei Wu and Mr. Hong Ruan for their skillful technical assistance.

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Footnotes

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This work was supported by the Canadian Institutes of Health Research (CIHR) and University Medical Discoveries Inc. (UMDI). Reprint requests should be addressed to Simon Lemaire.

Legend for Figures

Figure 1. Synthesis route of histogranin-like non-peptides. Bioactive histogranin-like derivatives of benzimidazole (**1**) and phenylenediamine (**2**, **3**, **4**, **5**, **6**, **7**) were made in four consecutive steps, first by the attachment of Boc-(L/D)Arg(Tos)-OH to MBHA resin using the PyBOP activation procedure (a) followed by the incorporation and reduction of nitroarene elements (b) and formylation /cyclization (c) or acylation of o-aminoaniline groups of resin-products (d). The synthetic compounds were cleaved from the resin and deprotected with liquid HF.

Figure 2. Analgesic effects of various concentrations of H4-(86-100) (A) and compounds **2**, **6** and **7** (B) in the second (tonic) phase of pain of the rat formalin test. Formalin assay in rats was performed as described under "Materials and Methods". For each value, n= 7 and *P $\leq$ 0.05 is considered significant as compared with the saline control assay.

Figure 3. Antihyperalgesic effects of H4-(86-100) (0.5 nmol, i.t.) and compounds **2** (80 nmol, i.t.), **6** (36 nmol, i.t.) and **7** (18 nmol; i.t.) in the rat CFA assay 24 h (A) and 48 h (B) after the administration of CFA into one hind paw. CFA assay in rats was performed as described under "Materials and Methods". For each value, n= 7 and *P $\leq$ 0.05 indicates significantly shorter paw withdrawal latencies compared to Pre-CFA latencies, suggesting that CFA induced significant thermal hyperalgesia. **P $\leq$ 0.05 indicates significantly longer latencies compared to saline-treated rats, meaning that histogranin and related compounds reversed thermal hyperalgesia induced by CFA.

Figure 4. Effects of HN, related peptides and non-peptides on LPS-evoked stimulations of PGE_2 secretion (A) and COX-2 induction (B) in primary cultures of rat alveolar macrophages. Isolated rat alveolar macrophages (1×10^6 cells) were incubated for 24

hours at 37^{o} C with LPS (1 μg/ml) in the absence (control) or presence of 10^{-8} M of the indicated compound. Their release of PGE_2 and content in COX-2 were monitored by radioimmunoassay and immunoblot analyses, respectively, as described under "Materials and Methods". * $P < 0.05$ is considered as significant as compared with control.

Table 1. Physicochemical characteristics of synthetic non-peptidic histogranin-like compounds

Compound	Structure	Purification Yield[a] mg (%)	Purity (%)	K' (HPLC)	Rf[d] (TLC)	ES-MS $(M+H)^+$
1	N-5-guanidinopentanamide-(2R)-yl-2-(p-hydroxybenzyl)-5-carboxybenzimidazole	94 (34)	98[b]	2.50[b]	0.59	411.2
2	N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl)phenylenediamine	120 (45)	98[c]	2.63[c]	0.66	399.6
3	N-5-guanidinopentanamide-(2S)-yl-2-N-(4'-hydroxy-3'-nitrophenylacetyl)phenylenediamine	74 (25)	98[c]	2.41[c]	0.69	444.5
4	N-5-guanidinopentanamide-(2S)-yl-2-N-(p-hydroxyphenylacetyl)-4-trifluorometylphenylenediamine	96 (31)	98[c]	2.18[c]	0.70	467.2
5	N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)phenylenediamine	56(21)	98[c]	2.64[c]	0.66	399.6
6	N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)-4-carboxyphenylenediamine	90 (30)	98[b]	0.70[b]	0.68	443.2
7	N-5-guanidinopentanamide-(2R)-yl-2-N-(p-hydroxyphenylacetyl)-4-(p-chlorobenzoyl)phenylenediamine	49 (14)	98[b]	1.54[b]	0.70	537.2

[a]Product was purified as described under the Experimental Section. [b]Analytical RP-HPLC (Bondapak C-18) with the gradient of 15-80% acetonitrile in 0.1% TFA over 50 min. [c]Analytical RP-HPLC (Bondapak C-18) with the gradient of 15-65% acetonitrile in 0.1% TFA over 50 min. [d] B:A:W:P (n-butanol: acetic acid: water: pyridine; 15:3:10:12).

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Table 2. Comparison of AD_{50}, potency ratio and half-maximal response decays of HN and related non-peptides in the mouse writhing pain assay.

Compound	AD_{50} (nmol/mouse)[a] (95% CL)	Potency ratio[b] (95 % CL)	Time of half-maximal response decay[c] (min)
HN	23.0 (12.5 - 47.0)	1.0	22
H4-(86-100)	23.5 (14.0 -39.4)	0.98	22.5
HN-(7-15)	8.5 (1.9 - 15.4)	2.7 (0.81 – 24.7) *	<10
1	4.1 (3.2 - 7.4)	5.5 (1.7 - 14.7)*	36
2	6.5 (4.5 - 9.3)	3.5 (1.3 - 10.4)*	15
3	5.6 (3.5 - 8.8)	4.1 (1.4 - 13.4)*	-
4	16.1 (9.9 - 26.3)	1.4 (0.5 - 4.7)	19
5	12.8 (10.0 - 16.3)	1.8 (0.8 – 4.7)	35
6	3.2 (1.8 - 5.6)	7.3 (2.2 - 26.1)*	58
7	2.6 (1.5 - 4.5)	8.8 (2.8 – 31.3)*	36

[a] 95% confidence limit. [b] Relative to HN. [c]Tested at 50 nmoles, i.c.v. for HN and 10 nmoles i.c.v. for the non-peptides. * $P \leq 0.05$ as compared with HN.

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Table 3. Comparison of AD_{50}, potency ratio and half-maximal response decays of HN and related non-peptides in the mouse tail-flick pain assay.

Compound	AD_{50} (nmol/mouse) (95% CL) [a]	Potency ratio [b] (95 % CL)	Time of half-maximal response decay [c] (min)
HN	114 (92-141)	1.0	45
1	9.6 (2.1-50)	11.8 (1.8-67.1)*	28.5
2	14.2 (11.5-17.4)	8.0 (5.3-12.3)*	21.3
4	98.6 (70-139)	1.1 (0.7-2.0)	18.5
6	31.7 (22.9-43.8)	3.6 (2.1-6.1)*	16.7
7	13.1 (10.6-16.1)	8.7 (8.6-13.3)*	28.9

[a] 95% confidence limit. [b] Relative to HN. [c]Tested at 50 nmoles, i.c.v. for HN and 10 nmoles i.c.v. for the non-peptides. * $P \leq 0.05$ as compared with HN.

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Table 4. Effect of HN and related non peptides on NMDA induced convulsion and mortality in mice.

Compound[a]	Convulsed/tested (n ratio)	% Convulsed	% Protection against convulsions	Mortality[b] (n ratio)
Saline	8/10	80	-	2/10
HN (60 nmol)	8/15	53*	34	ND
1 (10 nmol)	11/15	73	9	9/15*
2 (10 nmol)	8/15	53*	34	2/15
6 (10 nmol)	6/10	60*	25	2/10
7 (10 nmol)	4/10	40*	50	1/10

[a] Saline, HN and related compounds were administered i.c.v. 5 min prior to NMDA (1 nmol; i.c.v.). Convulsions were generally observed within 3 min after the administration of NMDA and mice were allowed to recover for a period of 30 min within which time mortality was monitored.

[b]The saline pretreated group had a mortality rate of 17.5%. ND: not determined.

* $P \leq 0.05$ as compared with the saline pretreated group.

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Figure 1

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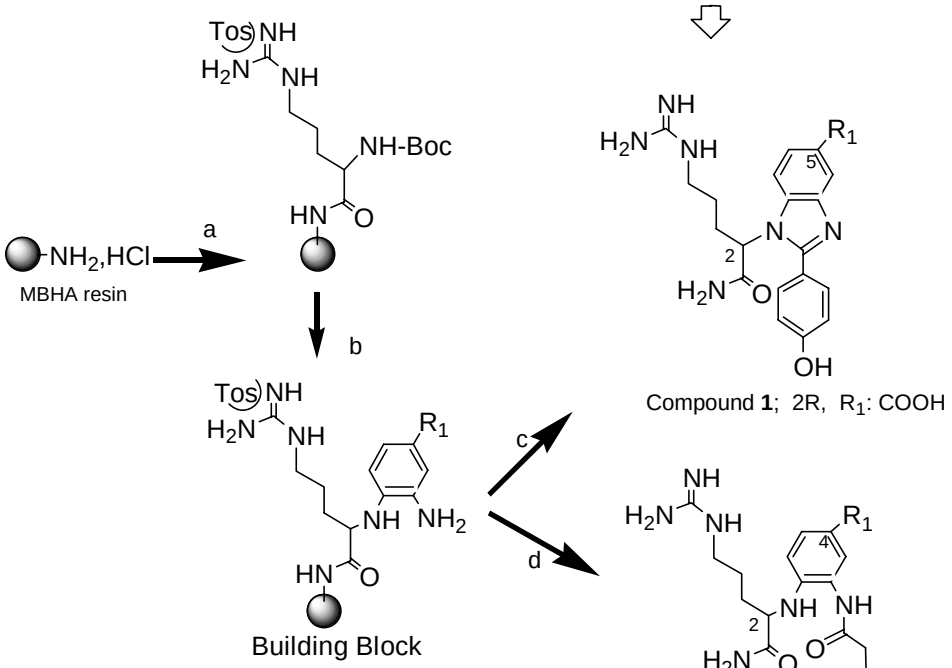
H-Met-Asn-**Tyr**-Ala-Leu-**Lys**-Gly-**Gln**-Gly-**Arg**-Thr-Leu-**Tyr**-Gly-Phe-OH
Histogranin (HN)



(a) 5%DIEA; Boc-(L/D)-Arg(Tos)-OH, PyBOP, DIEA, DCM/ DMF
(b) 40%TFA; o-fluoro-nitroarenes, DIEA, DMSO; Tin(II) chloride dihydrate, NMP
(c) p-hydroxybenzaldehyde, NMP; HF
(d) p-hydroxyphenylacetic acid, DCC, DCM; HF

Compound **1**; 2R, R_1: COOH

Compound **2:** 2S, R_1: H; R_2: H
Compound **3:** 2S, R_1: H; R_2: NO_2
Compound **4:** 2S, R_1: CF_3; R_2: H
Compound **5:** 2R, R_1: H; R_2: H
Compound **6:** 2R, R_1: COOH; R_2: H
Compound **7:** 2R, R_1: p-chlorobenzoyl; R_2: H

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Figure 2

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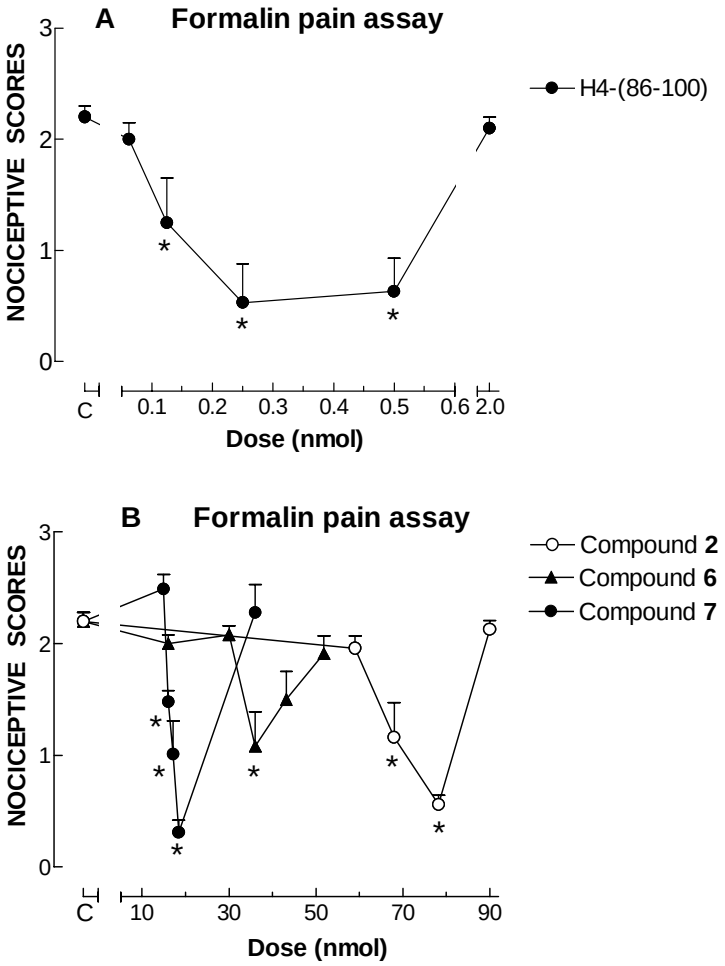

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Figure 3

JPET #60772

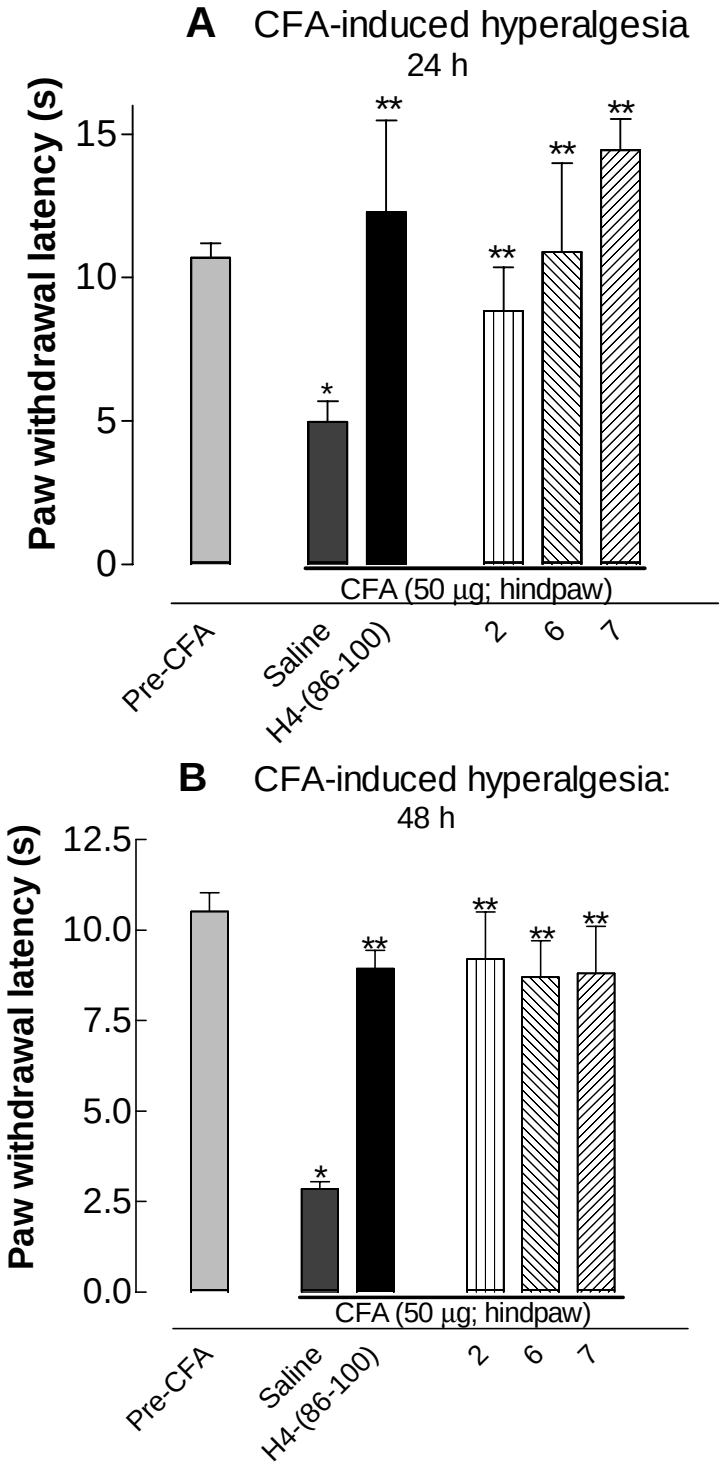

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Figure 4

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