Online Supplement:

Novel peptide isomer strategy for stable inhibition of catecholamine release: Application to hypertension.

Short title: Retro-inverso catestatin.

By

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METHODS.

Catestatin synthetic peptides. Human catestatin ($hCHGA_{352-372}$) was synthesized in 4 isomeric versions: wild-type (W-T); inverso (all D-amino acids); retro (reversing sequence from amino→carboxyl, to carboxyl→amino), and retro-inverso (R-I, reversing sequence, and inverting chirality to all D-amino acids). Peptides were synthesized by the solid-phase method, using 9-fluorenylmethoxycarbonyl (Fmoc) protection chemistry (Table-1). The peptides were purified at a scale of 20 mg, and then purified to ~95% homogeneity by preparative reverse-phase (C-18) high-performance liquid chromatography (RP-HPLC). Authenticity and purity of each peptide was further verified by repeat RP-HPLC and electrospray ionization mass spectrometry.

Nicotinic cholinergic catecholamine secretion pathway. Rat PC12 pheochromocytoma cells were grown at 37ºC with 6% CO_2, in 12-well plates, in Dulbecco's modified Eagle's medium (high glucose) supplemented with fetal bovine serum, horse serum and penicillin/streptomycin. Secretion assays were performed as described previously [1]. The cells were labeled for 3 hr with 1 µCi/ml of [^{3}H]-norepinephrine (catalog number NET678, Perkin-Elmer, Waltham, MA), then washed three times with basal medium and one time with secretion buffer. Subsequently, the cells were incubated for 30 min with or without the nicotinic agonist nicotine (60 µmol/L), in the presence or absence of peptide antagonists (0.1 to 16 µmol/L). In a separate experiment, another nicotinic agonist, epibatidine (1 µmol/L), was used to stimulate secretion. The release medium and cell lysates were assayed for [^{3}H]-norepinephrine by liquid scintillation counting. Results were expressed as percent secretion: [amount released/(amount released+amount in lysate)]x100. Net secretion was calculated as agonist-stimulated release minus basal release.

To establish the specificity of secretory inhibition by catestatin peptides, in some experiments catecholamine release was triggered with other classes of secretagogues that bypass the nicotinic cholinergic pathway: membrane depolarization (55 mmol/L KCl); ATP P2x purinergic receptor stimulation (100 µmol/L ATP); K^+ (repolarizing) channel blockade (2 mmol/L $BaCl_2$ in the absence of extracellular Ca^{2+}); or a Ca^{2+} ionophore (1 µmol/L ionomycin).

Actions of catestatin peptides were compared with the classical non-competitive nicotinic antagonist chlorisondamine, wherein labeled cells were incubated with different doses of nicotine in the presence or absence of peptide antagonists or chlorisondamine (10 µmol/L).

Desensitization of nicotinic cholinergic-stimulated catecholamine release. PC12 cells were labeled with [^{3}H]-norepinephrine, washed and exposed to nicotine (30 µmol/L) for 10 min (incubation I) in the presence or absence of catestatin peptides as described [2]. Cells were then washed twice (5 min each) in secretion buffer and re-exposed to nicotine (30 µmol/L) for another 10 min (incubation II), after which the cells were harvested for measurement of norepinephrine in intracellular lysate versus secretion medium.

Nicotinic cholinergic-stimulated transcription of CHGA. PC12 cells were plated one day before transfection on 24-well poly-L-lysine coated polystyrene plates. The cells were then transfected with plasmid pHJLD5 [3], containing a functional 1133-bp mouse CHGA promoter driving expression of the firefly luciferase reporter, using TransFectin^{TM} lipid reagent (Bio-Rad, Hercules, CA). After transfection, nicotinic cholinergic effects on the transfected CHGA promoter were established by adding nicotine (1 mmol/L) in the presence or absence of peptide antagonists (10 µmol/L). Cells were harvested after 18 to 20 hr, and lysed (125 µl/well) for subsequent luciferase and protein assays. Protein concentration was determined using a coomassie blue reagent (Bio-Rad).

Nicotinic cholinergic signal transduction: $^{45}Ca^{+2}$ uptake by PC12 cells. $^{45}Ca^{+2}$ uptake was performed as described previously with minor modifications [4]. Briefly, PC12 cells were grown on poly-L-lysine coated 6-well culture dishes, and were washed with 1 mL release buffer (150 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 10 mmol/L Hepes buffer, pH 7.4) every 15 minutes for 1 hour at 37°C. The cells were then pre-incubated for 1 min in release buffer in the presence or absence of catestatin peptide. After that, the cells were incubated for 2 minutes with 1 mL of Ca^{2+}-free release buffer containing 2 µCi of $^{45}Ca^{2+}$ (catalog number NEZ01300, Perkin-Elmer), 60 µmol/L nicotine in the presence or absence of catestatin. Thereafter, Ca^{2+} uptake was terminated by the addition of 2 mL of ice-cold Ca^{2+}-free secretion medium containing 2 mmol/L EGTA and 1 mmol/L $LaCl_3$, and further washed six times with 2 mL of the same buffer. Cell lysis buffer (secretion medium containing 0.1% Triton X-100, 1 ml) was then added, and the lysate was collected for liquid scintillation counting.

Peptide stability: Pronase digestion and mass spectrometry. Synthetic catestatin peptides (284 ng/10 µl reaction) were incubated with 5.7 or 0.57 ng of pronase (catalog number 53702, activity 61676.6 PUK/g, Calbiochem) in 10 mmol/L Hepes, pH 7.4 at 37 ºC for 1 hr. In controls, the peptides were incubated without pronase under identical conditions. The reactions were terminated by acidification with trifluoroacetic acid (0.5%). The reaction products were purified using a C-18 Zip-Tip (Millipore, Billerica, MA), mixed with matrix α-cyano-4-hydroxy-cinnamic acid at a ratio of 1: 4, and spotted onto a MALDI plate. Mass spectra were acquired on a Voyager DeSTR mass spectrometer at the UCSD biomolecular and proteomics mass spectrometry facility as described [1]. Peptide masses (MH+) were analyzed by the program Protein Prospector <http://prospector.ucsf.edu> to identify proteolytic cleavage.

Structural biology: Molecular modeling and circular dichroism (CD). Peptide primary structures were created in ChemDraw (CambridgeSoft, Cambridge, MA), and then chirality was inverted for D-amino acids, in Chem-3D, followed by energy minimization (molecular mechanics, MM2). The CD spectra on W-T and R-I catestatin peptides (0.125 mg/ml in 98% 2,2,2-trifluoroethanol) were obtained at 25ºC in a 2-mm path length cuvette over 190-260 nm wavelength, using an Aviv model 400 CD spectrometer (Aviv Biomedical,

Lakewood, NJ) as described previously [1]. From the ellipticity data, the percent contribution of α-helix was computed using K2D2 <www.ogic.ca/projects/k2d2/>.

Telemetric continuous intra-arterial measurement of blood pressure (BP). Telemetric measurement of blood pressure BP in conscious hypertensive mice: monogenic *Chga*-/- (knockout) and polygenic hypertensive BPH/2J mice [5] were achieved using a Data Sciences International (DSI; Transoma Medical) Physiol Tel telemetry system, implanting a catheter coupled to a TA11PA-C20 (DSI) transmitter, as described [6]. We then waited 10 days after surgery for stabilization of the animals. Chga-/- mice (n=7/group) were treated with catestatin peptide (W-T or R-I; 2 µg/g body weight, given intraperitoneally). BPH/2J polygenic hypertensive mice (n=7/group) were treated with catestatin peptide (W-T or R-I; 5 µg/g body weight). BP and heart rate were measured continuously over 24 hours, before and after peptide administration.

Statistical analyses. Experiments in cultured cells with catestatin peptides were repeated at least 3 times, with 2-3 wells per condition in each experiment. Curve fitting, slopes and intercepts were computed using Kaleidagraph (Synergy Software, Reading, PA). The results were expressed as mean ± SEM. Multiple comparisons were made using one-way ANOVA followed by Bonferroni post hoc tests, or by two-way ANOVA using Kaleidagraph. Statistical significance was concluded at $p<0.05$.

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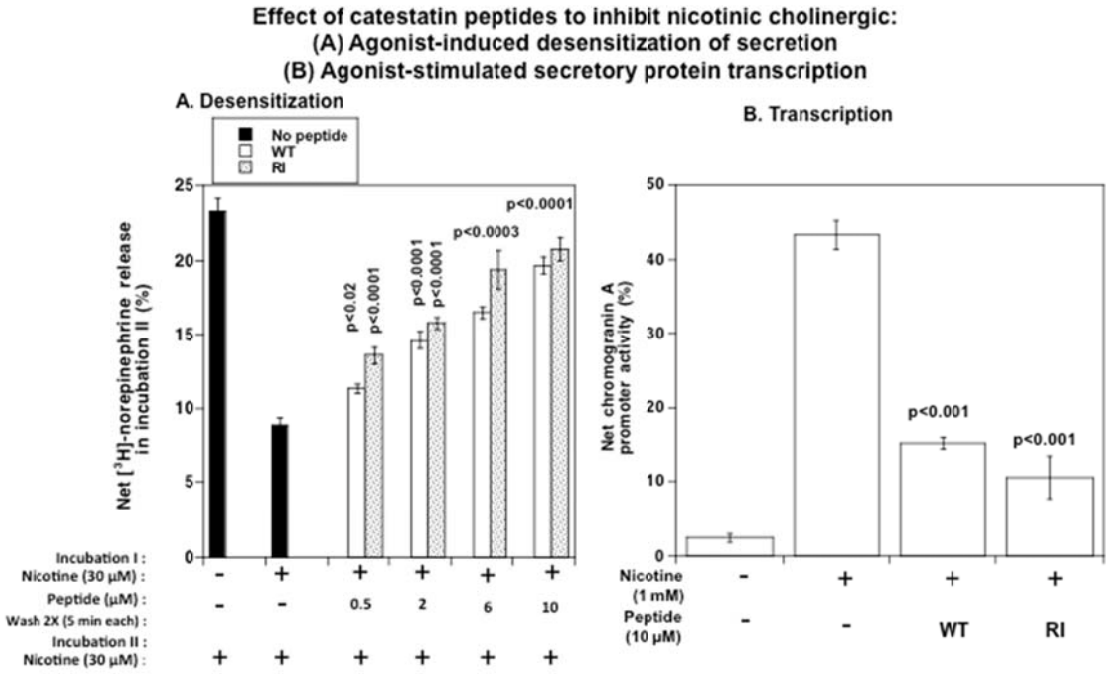


Figure S1. Inhibition of additional nicotinic cholinergic processes: Desensitization and transcription.

(A) Desensitization of nicotinic cholinergic triggered catecholamine release; effect of catestatin peptides. PC12 cells were pretreated (Incubation I) with an initial dose of nicotine (30 µmol/L for 10 min), in the absence or presence of ascending doses (0.5, 2, 6, 10 µmol/L) of catestatin peptide, W-T or R-I. Cells were washed twice (5 min each) with buffer and then exposed to a second secretory challenge (incubation II) with nicotine (30 µmol/L for 10 min) alone. After that, medium and cells were harvested for measurement of [^{3}H]-norepinephrine release. Each data point represents the mean data from two separate wells. Results were analyzed by two-way ANOVA evaluating the effect of peptide ($F=13.2$, $p<0.0003$), dose ($F=95.9$, $p<0.0001$). p values compare each peptide point to the desensitized value by nicotine alone. The IC_{50} values were 0.61 and 0.25 µmol/L for the W-T and R-I peptide respectively.

(B) Effect of catestatin peptide on the secretory protein transcription after nicotinic cholinergic stimulation. PC12 cells were transfected with a chromogranin A promoter/luciferase reporter plasmid and after 5 hours the cells were exposed to 1 mmol/L nicotine in the presence or absence of catestatin peptides (10 µmol/L each). Cells were then harvested after 18-20 hour for measurement of luciferase activity and protein. Control (100 %) net chromogranin A promoter activity represents the activity in the absence of peptide. Results are expressed as percentage of luciferase activity stimulated by nicotine alone, minus blank (no nicotine). Results were analyzed by one-way ANOVA evaluating the effect of peptide $F=81.8$, $p<0.0001$. P values compare each peptide point to the no peptide control

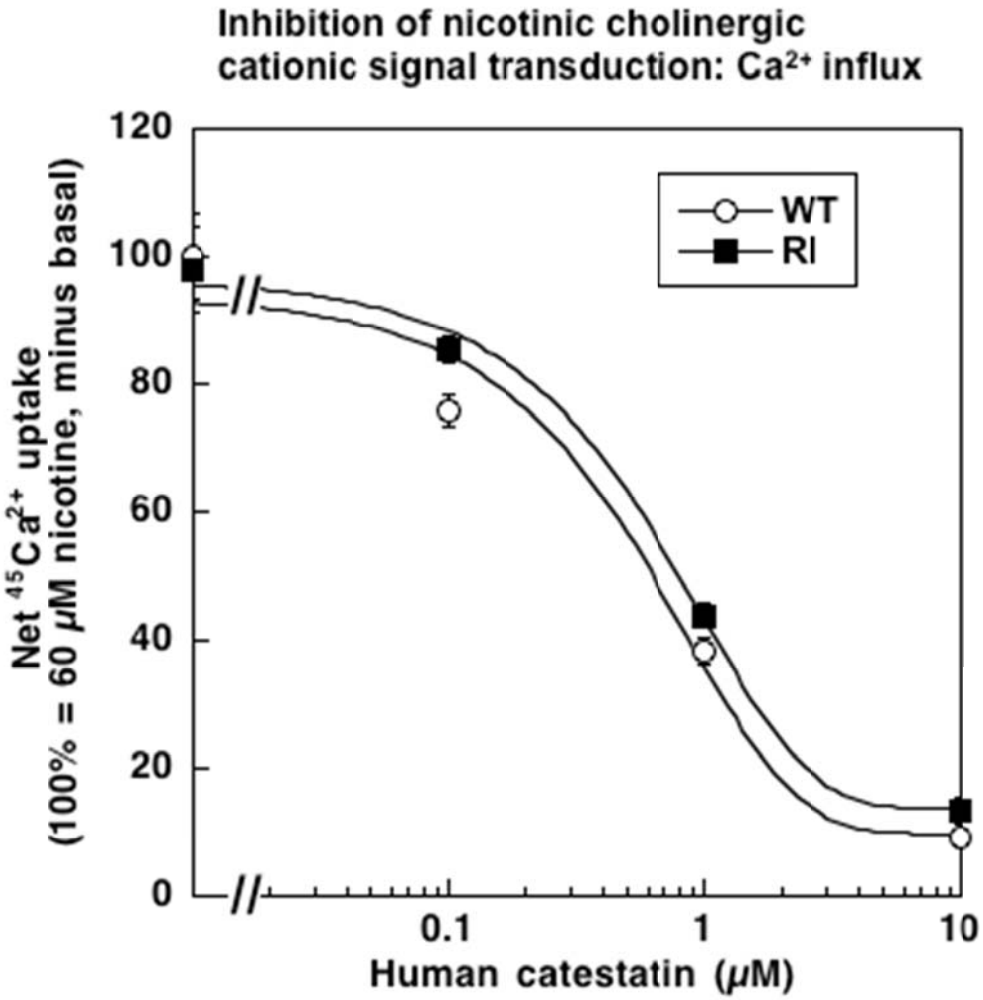


Figure S2. **Inhibition of nicotinic cholinergic cationic signal transduction: Ca^{2+} influx**. Effect of W-T and R-I peptides on nicotine-induced uptake of $^{45}Ca^{2+}$ in PC12 cells. Cells were grown in 6-well dish, incubate with $^{45}Ca^{2+}$ plus nicotine (60 μmol/L) in presence or absence of 0.1, 1 and 10 μmol/L of respective peptide for 1 min, followed by removal of the medium and cell lysis for measurement of $^{45}Ca^{2+}$ uptake. Control (100 %) net calcium uptake represents the uptake in the presence of 60 μmol/L nicotine alone minus basal. Results were analyzed by 2-way ANOVA evaluating the effect of dose ($F=219.2$, $p<0.0001$). The IC_{50} values were 0.62 and 0.81 μmol/L for the W-T and R-I peptide respectively.

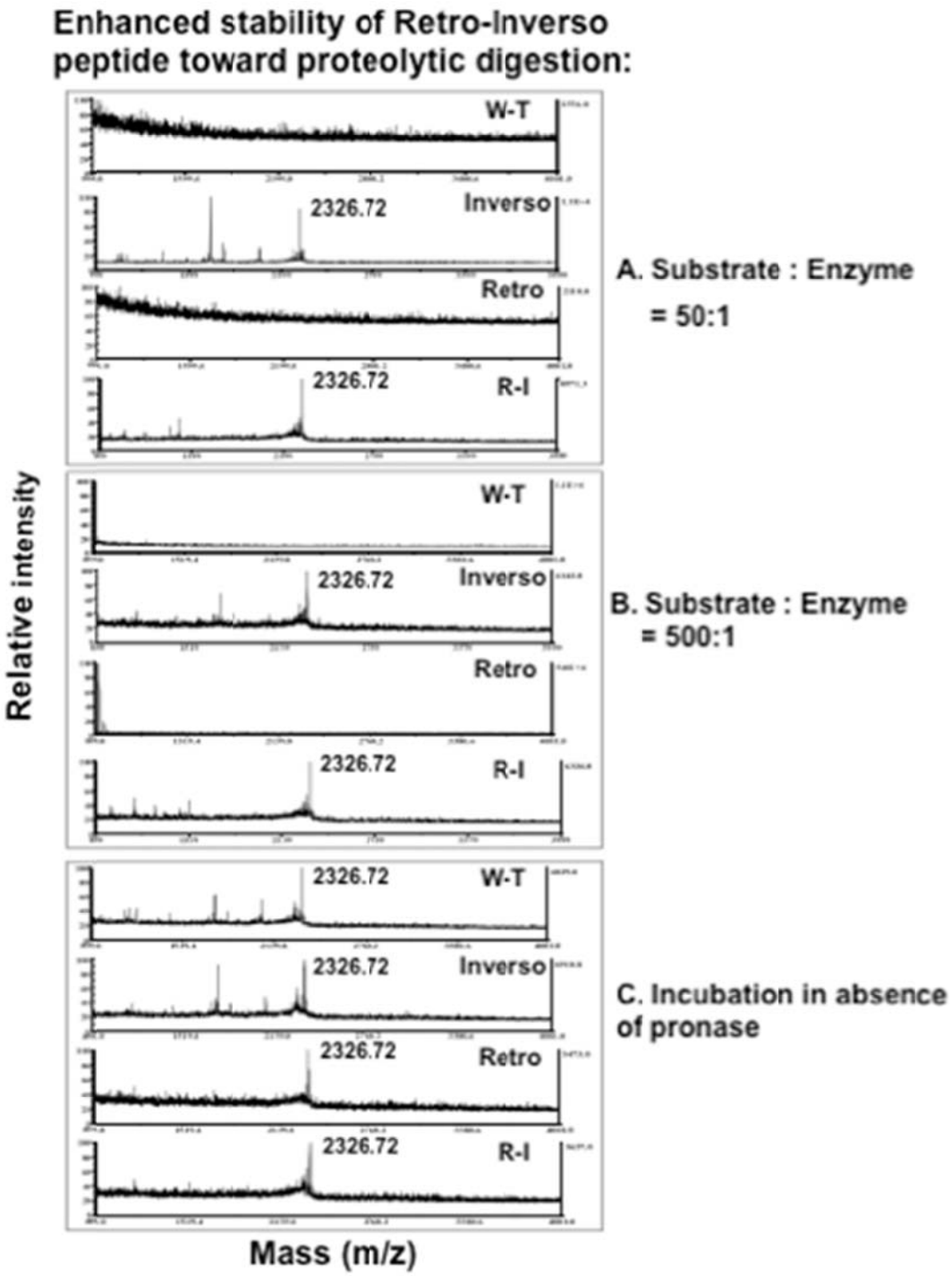
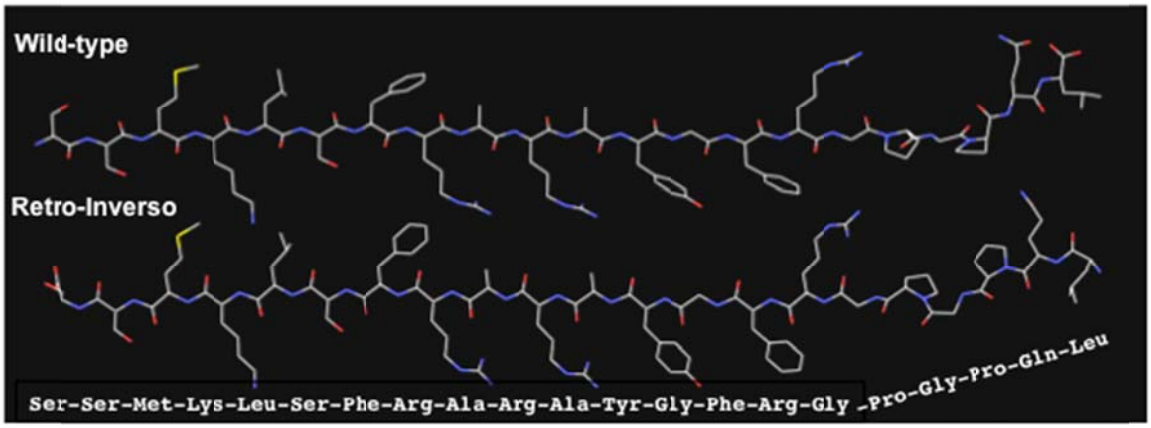


Figure S3. **Enhanced stability of retro-inverso peptide toward proteolytic digestion**. Catestatin peptides (W-T, Inverso, Retro, R-I) were digested (284.6 ng/10 µl) with pronase 5.7 ng (substrate : enzyme = 50:1, panel A) and 0.57 ng (substrate : enzyme = 500:1, panel B) in 10 mmol/L Hepes, pH 7.4 for 1 hr at 37 ºC. Panel C represents the reaction in the absence of pronase. The reaction was terminated by acidification with TFA. The resulting peptides were purified by C-18 Zip-Tip and analyzed by MALDI-TOF.

A. **Molecular model of W-T and R-I catestatin peptides**



B. **Circular dichroism spectra of W-T and R-I catestatin peptides in the far UV**

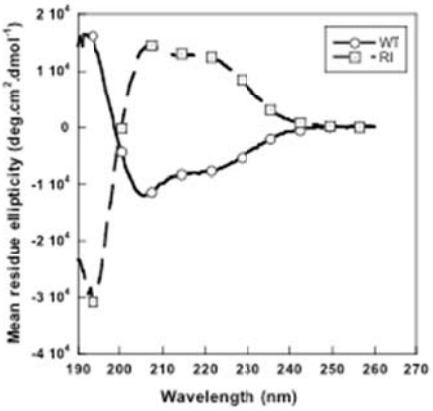


Figure S4. Structural biology. **(A)** Molecular modeling of W-T and R-I peptides. The structures were created in ChemDraw and the inversion of chirality was achieved in Chem3D, followed by energy minimization (MM2 force field). **(B)** Circular Dichroism (CD) spectroscopy of W-T vs. R-I synthetic catestatin regions from human CHGA. CD spectra of 21-amino acid catestatin peptides ($hCgA_{352-372}$) at 0.125 mg/ml in 98% 2,2,2-trifluoroethanol were taken over 190-260 nm.