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HU0622: A small molecule promoting GAP-43 activation and neurotrophic effects

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Abstract

During the course of neuronal development or regeneration, the axonal growth cone protein growth-associated protein 43 (GAP-43) is expressed in a great majority of differentiating neurons, suggesting that the regulation of this gene is tied to important differentiation signals common to many neurons. In order to discover non-peptide molecules capable of mimicking the effects of NGF, we developed a reporter gene assay system based on measurement of light production in PC12 cells stably transfected with the luciferase reporter gene, the expression of which depends on the transcriptional activation of GAP-43. High throughput screening of the proprietary compound collection using this system revealed (*E,E*)-1-[5-(3,4-dihydroxyphenyl)-1-oxo-2,4-pentadienyl]piperidine (HU0622), a piperine derivative, to be an activator of GAP-43 transcription. HU0622 strongly induced neurite outgrowth and extension in PC12 and sensory neuronal cultures of chick dorsal root ganglia. The compound induced sustained extracellular signal-regulated kinase (ERK) activation that is crucial for neurite outgrowth activity without activating NGF receptor, TrkA. Furthermore, HU0622 as well as NGF promoted PC12 survival under serum-free conditions and activated Akt/protein kinase B downstream from phosphatidylinositol 3-kinase (PI3K). HU0622 also promoted survival of rat dorsal root ganglion neurons deprived of NGF. HU0622, a small non-peptidyl molecule, may be a novel promising lead compound for the stimulation of nerve regeneration.
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Keywords: Nerve growth factor; Growth-associated protein 43; Extracellular signal-regulated kinase

1. Introduction

Neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are critical for the development, differentiation and maintenance of distinct populations of neurons (Bibel and Barde, 2000). In light of their demonstrated beneficial effects, neurotrophic factors have been suggested as potential therapeutic agents for the treatment of such neurological diseases as Alzheimer's and Parkinson's diseases. However, as these factors are polypeptides of high molecular weight, they cannot cross the blood–brain barrier and are easily proteolyzed when administrated peripherally. The therapeutic use of neurotrophic factors therefore necessitates intracranial injections, transplantation of cells secreting neurotrophic factors, or gene therapy. Such approaches have resulted in promising results in several animal models of neuronal degeneration (Knusel et al., 1992; Emerich et al., 1994; Kordower et al., 2000). Unfortunately procedures involving the administration of compounds directly into the brain or cerebrospinal fluid would not be acceptable for human patients. A useful strategy for addressing this drug delivery problem is the use of small organic

Abbreviations: NGF, nerve growth factor; GAP-43, growth-associated protein 43; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; DRG, dorsal root ganglion; Trk, tyrosine kinase receptor.

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compounds, which themselves either can directly maintain neuronal function or up-regulate neurotrophic factors. Much effort has been made in the search for non-peptidyl small molecule promoting neurotrophic activity, but most of the compounds that have been found either lack specificity for the desired mechanism and effects or lack efficacy in appropriate in vivo models, or both (Skaper and Walsh, 1998; Swain et al., 1999; Saragovi and Gehring, 2000).

As a molecular target to isolate neurotrophic non-peptide small molecule, we focused on the growth-associated protein-43 (GAP-43), a presynaptic protein whose expression is largely restricted to the nervous system. Because GAP-43 is maximally expressed in most differentiating neurons during neuronal development and regeneration, it is frequently used as a marker for sprouting (Oestreicher et al., 1997). Accordingly we developed a reporter gene assay for evaluating GAP-43 promoter activity in PC12 cells. In this paper, we report on the identification of a neurotrophic non-peptide small molecule and its mechanism during neurite outgrowth.

2. Materials and methods

2.1. Reagents

Rat pheochromocytoma PC12 cells was purchased from ATCC. The antibodies for phospho-ERK1/2, ERK1/2, phospho-Akt, Akt were from Santa Cruz Biotechnology. Anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase were purchased from Amersham, K252a, anti-GAP-43 antibody, anti-β-tubulin antibody, human recombinant BDNF and NT3 from Sigma, and mouse NGF (7S) from Chemicon. Stock solutions of the compounds (HU0622, KU1215) were prepared in dimethyl sulfoxide (DMSO) at 10 mM for the experiments. The same final concentration of DMSO (0.1%) was present in all of the conditions tested.

2.2. Cell culture

PC12 cells were maintained at 37 °C in 5% CO_2 on tissue culture flasks which had been coated with mouse collagen type IV (BD Biosciences). The growth medium consists of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated horse serum.

2.3. Generation of stable reporter gene transfectants and luciferase assays

A DNA fragment encompassing human GAP43-P2 promoter (located within the −207 bp form the translational start codon) was amplified by PCR and ligated into the pGL3-basic vector (Promega) to obtain pGL3-GAP-43-P2 vector. pGL3-GAP-43-P2 vector and pWL/neo were co-transfected in PC12 cells by FuGENE6 transfection reagent (Boehringer Mannheim), and stable clones were selected after being kept in 250 μg/ml Geneticin (G418, Invitrogen) for 2 weeks. Individual clones were picked out at that time and screened for their ability to induce luciferase in response to treatment with 50 ng/ml NGF. Based upon the outcome of this preliminary screening, clone 5 (PC12#5) was chosen for the further experiments. For luciferase assays, 4000 cells were seeded in each well of a 384-well plate and were allowed at least 6 h to become attached then were exposed to the drug overnight (approximately 18 h). The luciferase activity was determined according to the instructions of the manufacturer, using a Pica Gene luciferase assay system (Toyo Ink). Relative light units (RLU) were measured with a Lumat LB 9501 luminometer (EG & G Berthold, Wildbad, Germany). All experiments were done in triplicate or quadruplicate with results presented as mean ± S.E.

2.4. Western blot analysis

Cells were lysed in lysis buffer [25 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 5 mM NaF, 1 mM vanadate, 1% Nonidet P-40, EDTA-free CompleteTM protease inhibitor mixture (Roche Molecular Biochemicals)]. Protein extracts transferred to nitrocellulose membranes were incubated with primary antibodies followed by horseradish peroxidase-coupled secondary antibodies. Signals were detected by the ECL PLUS system (Amersham) using Hyperfilm (Amersham) for exposure.

2.5. Determination of neurite outgrowth

PC12 cells, 2×10^4 cells, were seeded in each well of a 24-well plate which had been coated with mouse collagen type IV in DMEM containing 1% heat-inactivated FBS and 0.5% heat-inactivated horse serum with or without the compound or NGF. After incubation for 24 h, process-bearing neurites were counted under microscopic observation, with scoring as possible processes those with the length being greater than the cell's diameter. Each value is the mean ± S.E. for about 100 PC12 cells sampled from three independent experiments.

2.6. Confocal microscopy

PC12 cells were allowed to grow on poly-D-lysine/mouse laminin coated culture slides (BD Biosciences) and were incubated with or without the compound or NGF for 24 h. At the day of the experiment, cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 100% methanol for 30 min, and blocked with 5% donkey serum/PBS for 30 min. After incubation with primary antibodies (anti-GAP-43 mAb, anti-β-tubulin mAb) for 1 h, cells were rinsed with PBS and incubated with Alexa 488-labeled secondary antibody (Molecular Probes) for 1 h. Propidium iodide (Molecular Probes) was used to visualize nucleus. Fluorescent images were obtained by FV300 laser scanning confocal microscopy (OLYMPUS).

2.7. Neurite extension in chick sensory neuronal culture

Dorsal root ganglia were dissected from E9 day embryos and washed in PBS. The tissue explants were plated 1 per well in 24-well tissue culture plates coated with collagen type IV in DMEM supplemented with 10% heat-inactivated FBS and 5% heat-inactivated horse serum. 24 h later, the sensory neuronal cultures were treated with compound. At 96 h after drug treatment, 3 or 4 photomicrographs were taken for each explant.

2.8. Cell survival assay

PC12 cells were seeded on mouse collagen type IV coated 96-well plates at 5×10^3 cells/well. At 24 h after plating, the cells were washed three times with PBS, and were grown in serum-free DMEM treated with or without compound or NGF at the indicated concentration. Cell survival was determined using the [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay-based Cell Counting Kit-8 (Dojindo), and relative cell numbers were determined in triplicate by estimating the value of day 0 as 100.

2.9. Detection of apoptosis

PC12 cells were grown in 6-well plates that had been coated with mouse collagen type IV until they reached ~50% confluence. The medium was then replaced with complete or serum-free medium in the presence or absence of indicated ligand (4 μM HU0622, 100 ng/ml NGF). After 24 h incubation, the cells attached to the plates and floating (detached) dead cells were harvested in PBS/EDTA (5 mM EDTA in PBS). The cells were then centrifuged and washed twice with PBS and fixed by 70% ethanol at −20 °C for a minimum of 2 h. Ethanol-fixed cells were then centrifuged and washed with PBS. Finally, the cell pellet was suspended in 1 ml of staining solution (20 μg/ml PI, 0.2 mg/ml RNase in PBS) and incubated for 30 min at room temperature. Flow

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