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Exceptional Stability of Artemin Neurotrophic Factor Dimers: Effects of Temperature, pH, Buffer and Storage Conditions on Protein Integrity and Activity

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Abstract Artemin (ARTN) is a neurotrophic growth factor of the GDNF ligand family that signals through the specific GFRα-3 coreceptor/cRet tyrosine kinase-mediated signaling cascade. Its expression and signaling action in adults are restricted to nociceptive sensory neurons in the dorsal root ganglia. Consequently, Artemin supports survival and growth of sensory neurons and has been studied as a possible treatment for neuropathic pain. We have developed a robust and sensitive cellular assay to measure ARTN biological activity. Using recombinant Artemin produced in *Escherichia coli* bacteria together with this specific assay, we demonstrate that ARTN is an exceptionally stable polypeptide. Multiple freeze–thaw cycles, incubation at elevated temperatures (up to 90 °C) for 0.5 h, prolonged storage at 4 °C, and exposure to conditions of different pH, salt concentration, and additives had no measurable effect on the biological activity of ARTN. In some of the tested conditions, partial removal of nine NH_2-terminal amino acids of the ARTN protein occurred, but this truncation had no important effect on the ARTN signaling response. Consequently, we postulate that formulation and storage for in vivo testing of ARTN in neuropathic pain paradigms in animals and humans should be straightforward.

Keywords Cellular assay · ERK · Growth factor · Neurological disease · Neurotrophic factor · Protein stability

Abbreviations

ARTN	Artemin
DRG	Dorsal root ganglia
FLISA	Fluorescence-linked immunosorbent assay
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF receptor alpha
MAPK1	Mitogen-activated protein kinase 1
NRTN	Neurturin
PSPN	Persephin

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Introduction

Neurotrophic growth factors of the glial cell line-derived neurotrophic factor (GDNF) family, comprising of GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) [1], have recently been studied as attractive therapeutic targets for the treatment of different neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis (GDNF and NRTN), stroke (PSPN), and peripheral neuropathies (ARTN) [2].

GDNF family receptor alpha 3 (GFRα-3) is the extracellular glycosyl phosphatidyl-inositol (GPI)-linked membrane receptor for the GDNF family member Artemin, also known as Enovin or Neublastin [3–5]. All GDNF-family ligands (GFL) signal through the cRet receptor tyrosine kinase. Different models have been suggested to describe the sequence and stoichiometry of the different steps in the activation process [2, 6–9]. A model whereby receptor activation involves the initial low-affinity binding of Artemin as a covalent homodimer to monomeric GFRα-3, followed by recruitment of one Ret molecule into a ternary complex that then recruits, in a highly cooperative fashion, an additional molecule each of GFRα-3 and Ret to form a pentameric signaling complex ARTN-$(GFR\alpha\text{-}3)_2$-$(Ret)_2$ [6], has recently been substantiated using a combination of quantitative cell-based experiments and mathematical modeling [10]. cRet is a single-pass membrane protein that autophosphorylates upon dimerisation after activation. Once phosphorylated, the tyrosine residues in the intracellular domain serve as high-affinity binding sites for various intracellular signaling proteins, initiating multiple downstream signaling cascades.

Artemin has been shown to play a role in the embryonal development of neurons in the sympathetic ganglia, such as the superior cervical ganglion and stellate ganglion, as well as in dorsal root ganglia (DRG) [11]. In adults, GFRα-3 expression is largely restricted to a specific subpopulation of sensory neurons in the peripheral nervous system [12–14]. Consequently, Artemin signaling supports survival of cultured sensory neurons in vitro [3, 15]. ARTN has been shown to have beneficial effects on nerve regeneration in several rat and mouse models [16–18].

Since Artemin supports the survival of sympathetic sensory neurons in the peripheral nervous system, it is being investigated for the possible treatment of neuropathic pain [19, 20]. Clinical trials with recombinant GDNF have been hampered by the limited bioavailability of the protein due to its inability to penetrate the blood–brain barrier and its binding to heparan sulfates of the extracellular matrix. Side effects due to pleiotropic action, induction of inflammation and formation of antibodies have also been reported [21, 22]. Artemin is less promiscuous than GDNF, and its peripheral action might result in a better bioavailability and less side effects.

In this paper, we describe a robust and sensitive cellular assay to measure ARTN activity and use this assay to demonstrate and characterize the exceptional stability of this neurotrophic factor. Our results should greatly facilitate the formulation of ARTN for future in vivo studies.

Materials and Methods

Materials

SUMO-protease 1 (SUMO-hydrolase/UD-1/ULP) was purchased from LifeSensors Inc., Malvern, PA, USA. All other chemical reagents were analytical grade.

Expression and Purification of Artemin

The DNA sequence coding for the mature Artemin protein (NCBI reference sequence NP_476431, amino acid residues 116–228) was expressed as an NH_2-terminal fusion protein with yeast SUMO as described [23]. Briefly, the 6×His-SUMO-ARTNmat/pSUMO expression plasmid is transformed in BL21(DE3) competent *Escherichia coli* cells. Protein expression is induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside. After induction, cells are grown for an additional 3 h prior to harvesting the cell pellet. The pellet is stored at −70 °C until purification.

In order to prepare washed inclusion bodies, the cell pellet is thawed and resuspended at 4 °C in 5 volumes (*w*/*v*) of resuspension buffer (20 mM Tris–HCl pH 7.2, 200 mM NaCl) supplemented with 0.4% Triton X-100, 0.4% Na-deoxycholate, and one tablet of Complete protease inhibitor cocktail (Roche Diagnostics, Vilvoorde, Belgium) per 50 ml buffer (buffer A). The suspension is mixed for 30 min, cells are sonicated, centrifuged (30 min at 10,000×*g*), and resuspended in 5 volumes of buffer A. Cells are again centrifuged and resuspended in 5 volumes of resuspension buffer with 0.8% Triton X-100 and 0.8% Na-deoxycholate. After another centrifugation step, cells are resuspended in 5 volumes of resuspension buffer, mixed well, centrifuged, and the final inclusion body pellet is stored at −70 °C.

For metal-chelating affinity chromatography purification under denaturing conditions, the cell pellet is resuspended in 20 volumes of a buffer containing 6 M guanidine-HCl, 20 mM Tris–HCl pH 8.0, 200 mM NaCl and 2 mM β-mercaptoethanol (guanidine buffer), and incubated for 1 h with mixing. The mixture is subsequently centrifuged for 30 min at 10,000×*g*. The supernatant is collected and supplemented with 1 ml of prewashed (guanidine buffer) Ni-Sepharose FF resin (GE Healthcare, Diegem, Belgium) for each 20 mg of SUMO-ARTN fusion protein. After mixing (4 h) and centrifugation, the resin is washed twice with 5 volumes of buffer B (10 mM Tris–HCl, 100 mM NaH_2PO_4, 8 M urea, 150 mM NaCl, pH 8.0). The resin is resuspended in 2 volumes of buffer B and packed on a XK16/20 column (GE Healthcare). After equilibration with buffer B, the protein is eluted using a gradient from buffer B pH 8.0 to pH 4.5. Fractions are collected and immediately diluted with an equal volume of a buffer containing 10 mM Tris–HCl, 100 mM NaH_2PO_4, 6 M urea, 150 mM NaCl, 6 mM cysteine, 0.02% Tween-20, 10% glycerol, pH 8.0. The SUMO-ARTN containing fractions are pooled and stored (−70 °C) or immediately used for refolding.

The affinity-purified SUMO-ARTN is diluted to a concentration of about 1.2 mg/ml with buffer C (10 mM Tris–HCl, 100 mM NaH_2PO_4, 6 M urea, 150 mM NaCl, 3 mM cysteine, 0.02% Tween-20, 10% glycerol, pH 8.0). The sample is transferred to a dialysis membrane (3,500 or 6,000 Da MWCO Snakeskin dialysis membrane, ThermoFisher Scientific). Dialysis is performed in several steps, and each step lasts for at least 16 h against at least 15 volumes of dialysis buffer. In each dialysis step, the urea concentration in buffer C (pH 8.0) is lowered from 6 to 4 to 2 M. Finally, the sample is dialyzed twice against 10 mM Tris–HCl, 100 mM NaH_2PO_4, 250 mM NaCl, 10% glycerol, pH 8.0 in the absence of urea. After dialysis, protein precipitated during the process is removed by centrifugation.

The refolded SUMO-ARTN solution is diluted with buffer to a protein concentration of about 0.6 mg/ml and the protein solution is supplemented with NaCl to a final concentration of 300 mM. SUMO-protease 1 is then added to a final concentration of 50 U/1 mg of SUMO-ARTN protein. The mixture is incubated at room temperature for 2 to 4 h until about 95% or more of the fusion protein has been cleaved. Precipitation formed during the cleavage process is removed by centrifugation and the mixture is filtered through a 0.45-μm polyethersulfone (PES) membrane (Thermo Fisher Scientific).

The filtered ARTN protein solution is diluted with at least 1 volume of cation exchange dilution buffer (50 mM bis-tris propane pH 8.3, 10% glycerol). After dilution, the pH is readjusted if required. For cation exchange chromatography, 12 ml of Source 15S resin is packed in a Tricorn 10/150 column (both from GE Healthcare). The column is pre-equilibrated with equilibration buffer (50 mM bis-tris propane pH 8.3, 50 mM NaCl, 10% glycerol). The ARTN is applied to the column, washed with several volumes of equilibration buffer and eluted from the column in a gradient from equilibration buffer to elution buffer (50 mM bis-tris propane pH 8.3, 800 mM NaCl, 10% glycerol). Elution fractions containing pure ARTN are pooled.

The pooled fractions from the cation exchange chromatography are concentrated to a final concentration of about 1.5 mg/ml and the sample is dialyzed overnight against 100 volumes of dialysis buffer (10 mM Tris–HCl, 100 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 8.0) at 4 °C. Finally, the sample is filter-sterilized (0.2 μM PES membrane filtration unit) and the protein concentration is determined. The purified Artemin is stored at −70 °C in 20% glycerol.

Cellular Activity Assay

A cellular fluorescence-linked immunosorbent assay (FLISA) was developed based on detection of the phosphorylation of the mitogen-activated protein kinase 1 (MAPK1; also referred to as ERK) upon stimulation of GFRα-3 with Artemin. GFRα3-cRet-NIH3T3 cells are maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum, 110 μg/ml pyruvic acid, 300 μg/ml L glutamine, and antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin sulfate and 1 μg/ml puromycin). The cells are grown at 37 °C in a 5% CO_2 environment. Twenty-four hours prior to an experiment, cells are seeded in black poly-D-lysine coated 384-multiwell plates (Greiner Bio One, Frickenhausen, Germany) at a density of 3,000 cells per well (30 μl volume) in the same medium containing 0.5% serum and in the absence of puromycin. After stimulation of the cells for 10 min at 37 °C with ARTN (30 μl volume), cells are fixed by adding 45 μl of a 4.67% formaldehyde solution and incubated for 30 min at room temperature. Cells are then permeabilized by addition of 30 μl 100% methanol for 10 min. Permeabilized cells are incubated with 30 μl of a specific rabbit antibody directed against phosphorylated MAPK1 (Cell Signaling Technology, Danvers, MA, USA) (dilution 1:800 in PBS supplemented with 1% protease-free BSA) for 1 h at 37 °C. After washing of the cells with PBS containing 0.01% Triton X-100, cells are incubated for 45 min at 37 °C with a 1:800 dilution of AlexaFluor 647 goat anti-rabbit secondary antibody (Invitrogen, Paisley, UK) in PBS with 1% protease-free BSA. After washing, fluorescence is measured with an FMAT 8,200 fluorescence reader (Applied Biosystems, Carlsbad, CA, USA) with excitation at 633 nm.

Detection of cRet and MAPK1 (ERK) Phosphorylation by Western Blot Analysis

GFRα3-cRet-NIH3T3 cells are maintained as described above. After seeding in 10 cm^2 poly-L-lysine-coated petri dishes at 60,000 cells per square centimeter, cells are grown in medium containing 10% serum. After 24 h, the medium is replaced with medium containing 0.5% serum and the cells are incubated for 3 h prior to addition of 10 ng/ml ARTN. After different incubation periods, cells are harvested on ice in radioimmunoprecipitation assay buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate) supplemented with protease inhibitor mix (Roche Diagnostics, Vilvoorde, Belgium). After brief sonication (5 s),

insoluble matter is removed by centrifugation (20,000×*g*, 4 °C). Volumes equivalent to 15 μg protein are run on 4–12% NuPAGE gels in MOPS running buffer. Proteins are transferred to a PVDF membrane by semi-dry blotting according to the manufacturer's instructions (BioRad, Hercules, CA, USA). All primary antibody incubations are performed overnight at 4 °C. Triplicate blots are developed with phosphorylation-state-specific antibodies p-cRet (Tyr-905) or p-ERK 1/2 (p44/42) (Thr202/Tyr204) (Cell Signaling Technology) diluted 1:500 in PBS+0.1% Tween-20 (PBS-T) and 5% BSA or with the Lamin A/C primary antibody (Cell Signaling Technology) diluted 1:750 in PBS-T+3% non-fat dry milk. HRP-linked secondary anti-rabbit antibodies (GE Healthcare) are diluted 1:3,000 in PBS-T+3% non-fat dry milk. Secondary antibody incubation is for 1 h at room temperature. Blots are developed using the SuperSignal West Dura Extended Duration substrate (ThermoFisher Scientific, Erembodegem, Belgium).

Protein Stability Analysis

Samples for the analysis of the stability of Artemin were prepared by thawing a stock solution of 1 mg/ml of the protein in storage buffer (10 mM Tris–HCl, 100 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, pH 8.0) on ice and removing precipitate by centrifugation (20,000×*g* for 5 min at 4 °C). Equal amounts of the resulting protein solution (0.25 ml in a 1.5 ml eppendorf tube) were then subjected to different treatments: (1) multiple (three or six) freeze–thaw cycles (at least 1 h at each temperature), (2) incubation for 30 min at 37, 60, or 90 °C and (3) prolonged storage (1, 2, or 21 days) at 4 °C. Tubes were incubated at the required temperature for the indicated durations and were then transferred to a cooled water bath (15 °C). Precipitate was removed by centrifugation (20,000×*g*, 5 min, 4 °C), and the soluble protein was analyzed for degradation and cellular activity. Purified ARTN stored at −80 °C and thawed immediately prior to analysis was used as positive control.

After each treatment, samples were tested for their ability to induce MAPK1 phosphorylation in the GFRα-3/cRet receptor expressing cell line grown in 384-multiwell plates. Each sample was tested in a concentration range of 1,000–0.001 ng/ml (42 nM to 0.042 pM). Samples were additionally analyzed by SDS/PAGE on NuPAGE 4–12% gels with MES running buffer (Invitrogen) and stained with InstantBlue stain (Expedion, Harston, UK). Samples were run under reducing conditions and also under non-reducing conditions (to evaluate the dimerisation state).

To monitor degradation of Artemin over time in different buffers at different temperatures, purified ARTN in storage buffer was diluted four-fold (to about 0.25 mg/ml) with the selected buffer. The diluted solution was subsequently dialysed overnight against 100 volumes of the same dilution buffer (Slide-A-Lyzer dialysis cassettes, ThermoFisher Scientific). After dialysis, precipitate was removed by centrifugation (20,000×*g*, 5 min, 4 °C).

Equal amounts of the resulting protein (concentration around 0.1–0.2 mg/ml) were then submitted to different treatments. Each eppendorf tube was filled to 90% of its maximal volume in order to limit oxidation during incubation. Tubes were incubated at the required temperature for the set duration and then transferred to a cooled water bath (15 °C). Precipitate was removed by centrifugation (20,000×*g*, 5 min, 4 °C) and soluble protein was analyzed for degradation. Samples were analyzed under reducing conditions by SDS/PAGE on NuPAGE 4–12% gels with MES running buffer and staining with SyproRuby protein gel stain (Invitrogen) to maximize sensitivity and dynamic range. Stained gels were scanned (Typhoon Imager, GE Healthcare or Chemigenius 2 imager, Syngene, Cambridge, UK) and densitometrically analyzed (TotalLab software, GE Healthcare or GeneTools software, Syngene), and the relative amount of the formed degradation products was quantified.

Truncation Analysis

The exact molecular masses of purified Artemin and of its degradation product were determined using high-resolution mass spectrometric analysis on a Waters Q-Tof-2 instrument using high-performance liquid chromatography (Waters Alliance 2695) electrospray ionization mass spectrometry in the positive ionization mode. NH_2-terminal amino acid sequencing was done at Eurosequence, Leiden, The Netherlands.

In order to compare the biological activity of the truncated ARTN-104 variant with the mature ARTN-113 form, an expression construct for recombinant expression of SUMO-ARTN-104 in *E. coli* was made, and ARTN-104 was expressed and purified using the same methods as described above for ARTN-113.

Results

In order to study the formulation of recombinant Artemin, information on the physical as well as the biological stability of the protein under different conditions is needed. We have developed a functional assay to measure activation of GFRα-3/cRet in a cellular (NIH-3 T3) background. ARTN has been shown to specifically interact with GFRα-3 and to lead to cRet activation and autophosphorylation and downstream intracellular signaling [3, 4]. The assay measures the formation of phosphorylated MAPK1 kinase in GFRα-3/cRet-transfected cells. Treatment of the cells with 10 ng/ml purified recombinant ARTN leads to phosphorylation of cRet and MAPK1 within 5 min, with a maximal response between 15 and 45 min (Fig. 1a). Using this assay, purified ARTN batches reproducibly showed an EC_{50} around 100–150 pM (Fig. 1b). In the buffer used, the protein can be stored at 1 mg/ml for at least 1 year at −80 °C without detectable degradation or loss of biological activity.

To evaluate the stability of Artemin under different conditions which may occur during experiments and during general storage and handling, an ARTN protein solution was subjected to a variety of temperatures and storage conditions. Samples were analyzed for visual precipitation, for functional activity in the cellular assay and for protein degradation and dimerisation status by SDS/PAGE (Figs. 2 and 3). In a first set of experiments, the influence of repeated freeze–thawing (once, thrice, six times), of exposure for 30 min to 37, 60, or 90 °C, and of prolonged exposure (24 h, 48 h, 21 days) to 4 °C on ARTN integrity was investigated (Fig. 2). None of the tested conditions seem to induce precipitation as no pellets were observed after centrifugation. Results from the cellular FLISA assay (table in Fig. 2) demonstrate that no significant reduction in cellular activity in terms of EC_{50} value could be detected. Some variation was observed in the maximal level of MAPK1 phosphorylation as compared to background levels (S/N ratio). On SDS/PAGE gels, the treated ARTN samples yield protein bands of the expected apparent sizes of monomeric ARTN (12 kDa under reducing conditions) and dimeric ARTN (24 kDa under non-reducing conditions). However, the sample which was stored for 21 days at 4 °C contains a slightly shorter, truncated fragment as well. Mass spectrometric analysis (data not shown) of a partially degraded sample identified two products with exact molecular masses of 11,960 Da (compared to a calculated expected value of 11,958.6 for the 113 amino acid monomer) and 11,150 Da. The difference of 810 Da corresponds to the predicted mass of the nine NH_2-terminal amino acid residues (*Ala–Gly–Gly–Pro–Gly–Ser–Arg–Ala–Arg*) of the ARTN monomer. NH_2-terminal sequencing of the truncation product confirmed the absence of these nine amino acid residues and yielded *Ala–Ala–Gly–Ala–Arg–Gly–(Cys)–Arg–Leu–Arg* as the sequence (an expected cysteine residue is put between brackets because

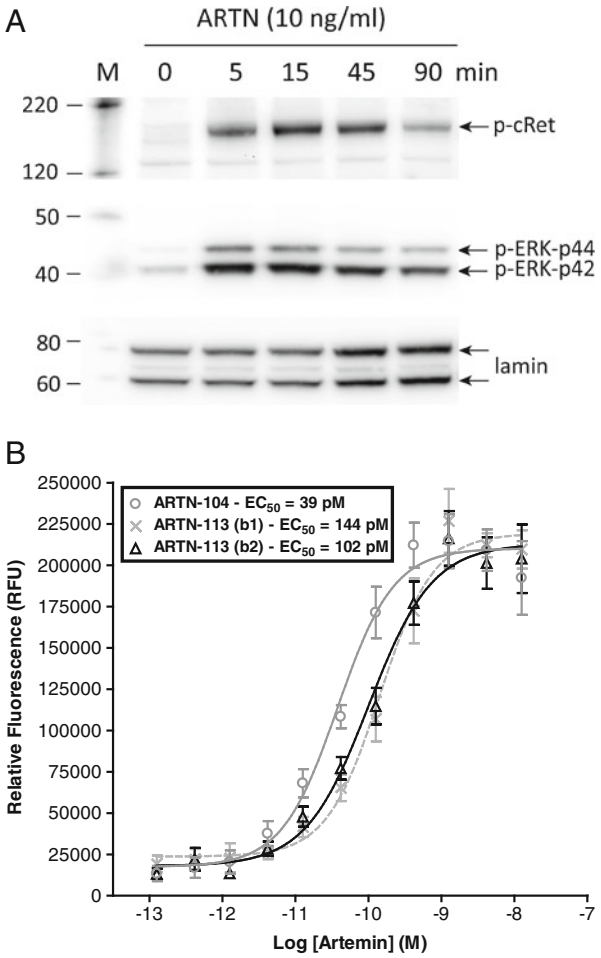


Fig. 1 Cellular activity assay for Artemin. **a** GFRα3–cRet–NIH3T3 cells were treated with ARTN (10 ng/ml) for 0, 5, 15, 45, or 90 min and phosphorylated cRet (p-cRet) and MAPK1 (p-ERK-p42 and p-ERK-p44) were detected using Western blotting as described in the "Materials and Methods" section. Detection of Lamin A/C (lamin) expression was used as an internal control for sample loading. A molecular mass standard (M; sizes in kDa) is included at the left-hand side. **b** Two different batches of purified ARTN (b1 and b2) were analyzed in an in vitro fluorescence-linked immunosorbent assay (FLISA) based on detection of MAPK1 phosphorylation upon GFRα-3/cRet stimulation with different dilutions of ARTN as described in the "Materials and Methods" section. The measured fluorescence (in relative fluorescence units; RFU) is plotted against log[ARTN] and curve fitting and data calculation are performed using GraphPad Prism v4.02 software. The EC_{50} for both Artemin batches is about 100–150 pM. The activity of the truncated purified recombinant ARTN-104 form was also determined (39 pM). The data points are means and the error bars show the standard deviations for five independent measurements

cysteine residues are not detected by the method used for the amino acid sequence analysis). Consequently, the protein band appearing below the intact monomer on SDS/PAGE after 21 days at 4 °C corresponds to a 104 amino acid residue truncation product. In order to be able to compare the biological activity of the truncated ARTN-104 variant with the mature ARTN-113 form, we recombinantly produced purified ARTN-104 in *E. coli* using the same methods as described for mature ARTN-113. As shown in Fig. 1b, ARTN-104 has similar biological activity in our cellular FLISA assay, with an EC_{50} around 40 pM.

	1x freeze/ thaw (control)	3x freeze/ thaw	6x freeze/ thaw	30 min 37°C	30 min 60°C	30 min 90°C	24 h 4°C	48 h 4°C	21 days 4°C
Artemin pEC_{50}	10.40	10.28	10.56	10.54	10.36	10.46	10.28	10.45	10.51
S/N ratio	6.2	9.9	9.5	8.9	7.2	6.8	8.1	7.1	5.7

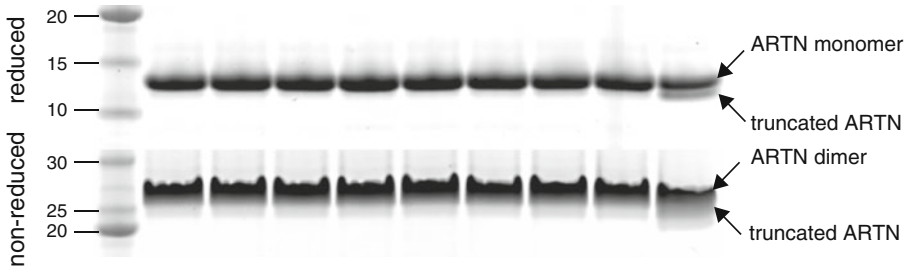


Fig. 2 Stability analysis of ARTN under different conditions. Aliquots (250 µl) of purified ARTN (1.0 mg/ml) in storage buffer (50 mM Bis-Tris propane, pH 8.3, 400 mM NaCl, 20% glycerol) were subjected to different treatments: 1 freeze/thaw cycle (control), 3 or 6 freeze/thaw cycles, incubation for 30 min at 37, 60, or 90 °C and prolonged incubation at 4 °C for 1, 2, or 21 days. Aliquots are subsequently analyzed by SDS/PAGE (4–12% NuPAGE gel in MES buffer) in the presence (reduced) or absence (non-reduced) of DTT. In addition, the treated ARTN samples were tested in dose–response in an activity assay, and the pEC_{50} and signal-to-noise ratio (S/N) were compared between all treatments (table at top). A molecular mass standard (in kilodalton) is included at the left-hand side. The position of the ARTN monomer (12 kDa) and dimer (24 kDa) are indicated at the right. A truncated ARTN product can be observed after incubation at 4 °C for 21 days

Interestingly, Artemin seems very stable upon repeated freeze/thawing and for a short period of time at elevated temperature, even up to 90 °C. Also at 4 °C, ARTN is stable for at least a few days. The stability of Artemin was further investigated over time in different formulations and at different temperatures (Fig. 3). Figure 3a shows, as a representative example of such experiments, the SDS/PAGE analysis gels of ARTN stored at 40 °C for 1 to 21 days in simple buffers with different salt concentrations (150 or 300 mM NaCl) and different pH values (4.5 versus 6.5). The relative amount of the 104 amino acid truncation product in the samples after treatment was determined by scanning densitometry of the SyproRuby stained gels and is plotted in Fig. 3b. No visual precipitation of protein could be observed in any of the tested conditions. In some conditions, the 113 amino acid residue mature Artemin protein (ARTN-113) is partially degraded to the 104 amino acid residue product (ARTN-104), but no further degradation occurred in any of the test conditions. Since the 104 amino acid product shows cellular activity comparable to the 113 form (Fig. 1b), the total amount of active ARTN remains the same under all test conditions. The formation of the 104 truncation product increases with increasing pH (more degradation at pH 6.5 then at pH 4.5), higher temperature, and longer incubation time. No significant ARTN-104 formation occurred within the first 24 h in any of the tested conditions. At pH 4.5 and in the presence of low salt (150 mM), no ARTN degradation occurs, even at elevated temperature (40 °C) and after prolonged incubation (up to 21 days). An additional set of buffer compositions was tested starting from the optimal conditions identified in the first experiment (10 mM Na-citrate, pH 4.5, 150 mM NaCl; condition F5 in Fig. 3b). Additional components were added to this buffer in order to try to increase ARTN stability (conditions F9 to F12 in Fig. 3c). Dimerisation and solubility were not affected by any of the treatments. No significant influence of the additions (sucrose, trehalose, or hydroxypropyl-β-cyclodextrin) was observed. The ARTN-113 form was stable at 4 °C for up to 90 days, but at 25 or 40 °C, more ARTN-104 was formed upon prolonged incubation (with about 75% of the protein converted to the 104 form at 40 °C after 90 days).

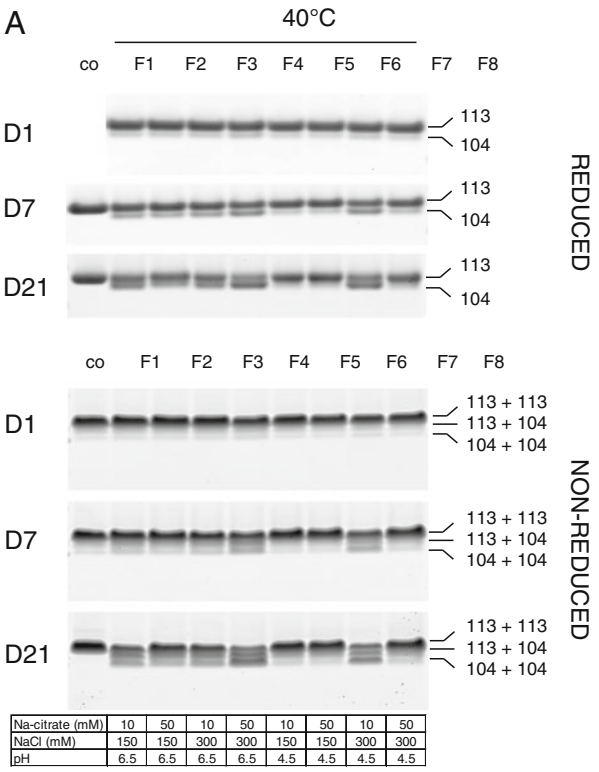
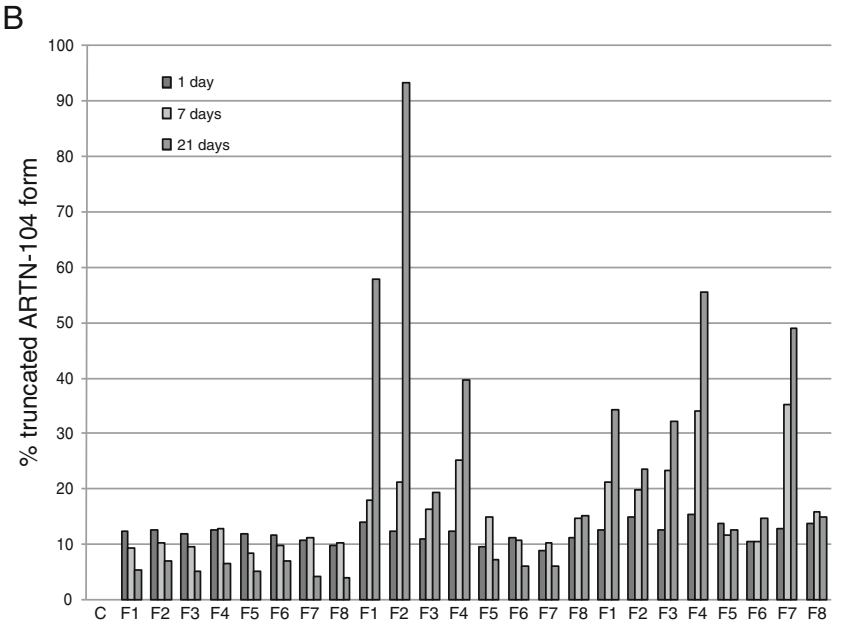


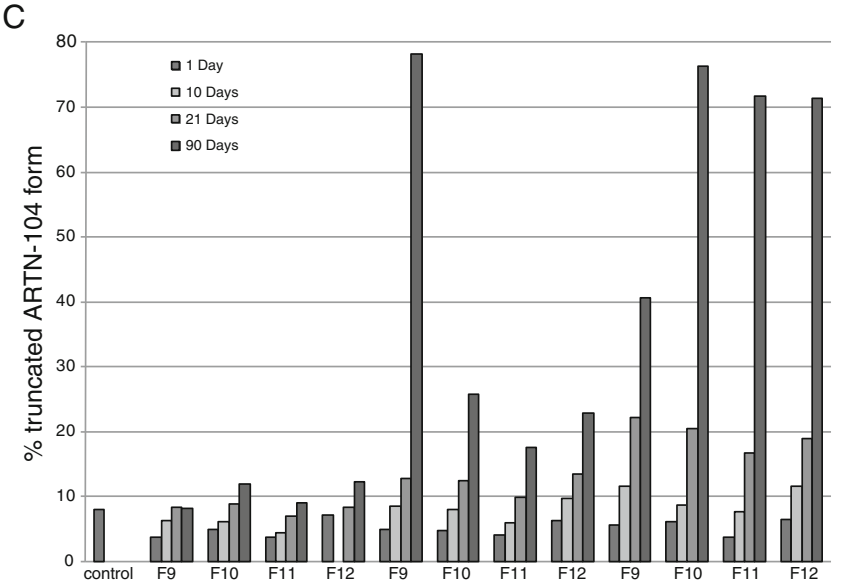
Fig. 3 Analysis of ARTN in different buffers after prolonged incubation at different temperatures. Aliquots of purified ARTN (0.15 mg/ml) were prepared in different buffers. *F1* 10 mM Na-citrate pH 6.5, 150 mM NaCl; *F2* 50 mM Na-citrate pH 6.5, 150 mM NaCl; *F3* 10 mM Na-citrate pH 6.5, 300 mM NaCl; *F4* 50 mM Na-citrate pH 6.5, 300 mM NaCl; *F5* 10 mM Na-citrate pH 4.5, 150 mM NaCl; *F6* 50 mM Na-citrate pH 4.5, 150 mM NaCl; *F7* 10 mM Na-citrate pH 4.5, 300 mM NaCl; *F8* 50 mM Na-citrate pH 4.5, 300 mM NaCl; *F9* 10 mM Na-citrate pH 4.5, 150 mM NaCl, 50 mg/ml sucrose; *F10* 10 mM Na-citrate pH 6.5, 150 mM NaCl, 50 mg/ml sucrose; *F11* 10 mM Na-citrate pH 6.5, 150 mM NaCl, 50 mg/ml trehalose; *F12* 10 mM Na-citrate pH 6.5, 150 mM NaCl, 150 mg/ml hydroxypropyl-β-cyclodextrin. **a** Aliquots were subjected to incubation for 1 (*D1*), 7 (*D7*), or 21 (*D21*) days at 40 °C in buffers *F1–F8*. Samples were subsequently analyzed by SDS/PAGE (4–12% NuPAGE gel in MES buffer) in the presence (reduced) or absence (non-reduced) of DTT. The positions of the three different protein bands on the non-reduced gels are tentatively labeled at the right as 113+113, 113+104, and 104+104 dimers. The positions of the mature (113 amino acid residues) and the truncated (104 amino acid residues) ARTN monomers on the reduced gels are indicated at the *right*. **b** Aliquots were subjected to incubation for 1, 7, or 21 days at 4, 25, or 40 °C in buffers *F1–F8*. Samples were subsequently analyzed by SDS/PAGE (4–12% NuPAGE gel in MES buffer) in the presence of DTT (reduced). The gels (as exemplified in **a**) were scanned and densitometrically analyzed, and the % of truncated 104 amino acid residues monomer in each sample was determined. *C* control. **c** Aliquots were subjected to incubation for 1, 10, 21, or 90 days at 4, 25, or 40 °C in buffers *F9–F12*. Samples were subsequently analyzed by SDS/PAGE (4–12% NuPAGE gel in MES buffer) in the presence of DTT (reduced). The gels (as exemplified in **a**) were scanned and densitometrically analyzed, and the % of truncated 104 amino acid residues monomer in each sample was determined

Discussion

GDNF was the first member of the GFL family of neurotrophic factors to be described [24], and has therefore been investigated in most detail. The use of GDNF therapy in humans, however, has been hampered by the emergence of unwanted side effects, such as weight



Na-citrate (mM)	10	50	10	50	10	50	10	50	10	50	10	50	10	50	10	50	10	50	10	50	10	50	10	50
NaCl (mM)	150	150	300	300	150	150	300	300	150	150	300	300	150	150	300	300	150	150	300	300	150	150	300	300
pH	6.5	6.5	6.5	6.5	4.5	4.5	4.5	4.5	6.5	6.5	6.5	6.5	4.5	4.5	4.5	4.5	6.5	6.5	6.5	6.5	4.5	4.5	4.5	4.5
temperature	4°C								25°C								40°C							



Na-citrate (mM)	10	10	10	10	10	10	10	10	10	10	10	10
NaCl (mM)	150	150	150	150	150	150	150	150	150	150	150	150
pH	4.5	6.5	6.5	6.5	4.5	6.5	6.5	6.5	4.5	6.5	6.5	6.5
additive	sucrose	sucrose	trehalose	cyclodextrin	sucrose	sucrose	trehalose	cyclodextrin	sucrose	sucrose	trehalose	cyclodextrin
mg/ml	50	50	50	150	50	50	50	150	50	50	50	150
temperature	4°C				25°C				40°C			

Fig. 3 (continued)

loss, paresthesia, allodynia, and hyponatremia [25]. Artemin, as well as its specific coreceptor GFRα-3, are predominantly expressed in nociceptive sensory neurons (which also express VR1 receptor, peripherin, cRet, TrkA, and CGRP) in the adult DRG [15]. In view of the limited expression of ARTN in adult peripheral sensory nociceptors, the potential adverse effects of Artemin treatment may be more limited than the ones observed for GDNF [26].

An early in vivo study did not provide evidence to support the use of ARTN as a neuroregenerative agent, but on the contrary warned for undesirable side effects upon ARTN administration to adult animals, such as hyperplasia or neuronal metaplasia at the adrenal corticomedullary junction [27]. This study also mentioned the occurrence of tactile hyperestesia after spinally or systemically administered ARTN in a spinal nerve ligation rodent model, but later publications showed that loss of this tactile allodynia begins to appear 7 days after the beginning of Artemin injections [19]. These later studies did show positive effects of Artemin administration in several animal models of neuropathic pain, without significant adverse side effects. For example, in a spinal nerve ligation rat model inducing tactile and thermal hypersensitivity, systemic, intermittent administration of ARTN produced dose- and time-related reversal of nerve injury-induced pain behavior and partial to complete normalization of associated morphological and neurochemical features. Artemin effects lasted for at least 28 days and did not elicit sensory or motor abnormalities [19]. Systemic administration of ARTN after crush injury of the dorsal spinal nerve roots in rats resulted in re-entry of multiple classes of sensory fibers into the spinal cord, re-establishment of synaptic function, and even recovery of simple and complex behavior [28]. Essentially complete and persistent restoration of nociceptive (thermal and mechanical thresholds) and sensorimotor functions were observed after intermittent systemic administration of ARTN over a period of 2 weeks, and these effects persisted for at least 6 months following treatment. Therefore, ARTN may have potential for the clinical treatment of traumatic nerve injury [19].

In view of these positive in vivo results with ARTN, we have developed a novel method to produce gram quantities of highly pure and biologically active ARTN [23]. In the present paper, we investigated the stability of the ARTN protein under different physical conditions and in different formulations. The effects of these conditions on the biological activity of ARTN were studied using a sensitive cellular assay measuring receptor activation upon ARTN stimulation of GFRα-3/cRet overexpressing cells. We found that ARTN treatment of GFRα-3/cRet cells resulted in MAPK1 activation with an EC_{50} of 0.1 nM. Our study provides information which will be of use in preparing ARTN for human clinical trials. We have found that Artemin is an extremely stable protein as no significant precipitation, degradation, loss of functional activity, or change in dimerisation state was observed under any of the tested conditions (including a 30-min incubation at 90 °C in the buffer used to store the purified protein). Only storage at 4 °C for periods longer than 2 days should be avoided as this may eventually result in the formation of a truncated form of 104 amino acid residues. This truncated form retains full receptor-activating activity, but it has been shown that ARTN-104 has a ten-fold lower affinity for heparin binding than ARTN-113 [29]. This is important when considering in vivo application of ARTN, since heparin binding can have a profound effect on the pharmacokinetic and pharmacodynamic properties of proteins. Besides of having a good overall stability, the tested Artemin sample displays an unusually high thermostability which is due to the cysteine knot present in the mature dimeric growth factor (our incubation results and [23]).

Our expression and purification protocol for the production of high-quality, active human ARTN, together with the finding that the active ARTN dimer is exceptionally stable in simple buffer systems and under different environmental conditions will allow straightforward formulation of Artemin for in vivo application.

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