Pharmaceutical Significance of the Cyclic Imide Form of Recombinant Human Glial Cell Line Derived Neurotrophic Factor

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Purpose. The purpose of this paper is to determine the significance of cyclic imide formation of an aspartic acid residue during storage on the pharmaceutical quality of a recombinant human glial cell line-derived neurotrophic factor (rhGDNF) formulation.

Methods. A combination of chromatography, peptide mapping, mass spectroscopy, and protein sequencing was used to purify and characterize the degradation product. Circular dichroism, 1,8-ANS and heparin binding, melting temperature determination, bioassays, and preclinical pharmacokinetic and toxicology testing were performed to examine its equivalence to native rhGDNF.

Results. The rhGDNF with cyclic imide at aspartic acid residue 96 showed identical activity, structure, pharmacokinetic profile, and toxicity profile to the native rhGDNF.

Conclusions. Formation of cyclic imide at aspartic acid residue 96 does not affect the pharmaceutical quality of the rhGDNF formulation.

KEY WORDS: rhGDNF; cyclic imide; protein stability; structure.

INTRODUCTION

Recombinant human glial cell line-derived neurotrophic factor, or rhGDNF, is a naturally occurring protein in the developing and adult brain (1). It is a distant member of the transforming growth factor-β (TGF-β) superfamily and a homodimeric protein containing one intermolecular and three intramolecular disulfide bonds (1,2). As the N-terminal methionine is retained in proteins produced in *Escherichia coli,* each rhGDNF monomer is a 135-residue polypeptide (1,3). The rhGDNF is currently under investigation as therapeutic agents for the treatment of neurodegenerative disorders and nerve injury (4,5).

One of important requirements to developing a protein as a therapeutic drug is to maintain the activity and purity of the protein during manufacturing, transportation, storage, and delivery. The protein rhGDNF demonstrates exceptional physical stability, with a T_m of higher than 90°C. However, several chemical modifications of amino acid residues were observed during long-term storage. Optimizing the pH could minimize most chemical modifications, but a single modification discussed in this report cannot be completely inhibited even during refrigerated storage. Although conventional approaches to optimize formulations focus on the use of excipients, preventing the formation of the chief rhGDNF degradation product by adding novel stabilizers proved to be unfeasible because the current route of administration for rhGDNF is intracerebroventricular and the number of excipients known to be safe in this delivery route is currently very small. Additionally, the presence of subtle changes in a protein's side chains may be safer than introducing novel excipients in the formulation to prevent degradation. The study presented here was therefore designed to characterize the modified rhGDNF product and, if possible, demonstrate its bioequivalence to native rhGDNF. This can serve as an alternative approach to making the claim that the protein is stable in the dosage form.

MATERIALS AND METHODS

Materials

Pharmaceutical quality rhGDNF was expressed in *E. coli* and was purified at Amgen, Inc. Tris(2-carboxyethyl) phosphine (TCEP) was purchased from Boehringer Mannheim (Indianapolis, IN). Trypsin and iodoacetamide were purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade water and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI). Sequenal grade TFA and guanidine hydrochloride were products of Pierce (Rockford, IL). All other reagents were of the highest quality commercially available.

Production and Purification of the rhGDNF Degradation Product

A 25 mg/ml solution of rhGDNF in 10 mM sodium citrate, 150 mM sodium chloride, at pH 5.0 was incubated for 1 week at 37°C. The incubated rhGDNF was chromatographed on a TosoHaas (Montgomeryville, PA) SP-5PW semipreparative column (2.15 × 15 cm). A sodium chloride gradient in 20 mM MES buffer at pH 5.0 was used to separate the late-eluting degradation product from native rhGDNF.

Identification of the rhGDNF Degradation Product

The purified degradation product was diluted in 200 μL of 1% acetic acid and reduced with 24 mM TCEP at 45°C for 3 h. The reduced protein was chromatographed on a Vydac (Hesperia, CA) C4 column (0.46 × 25 cm) using an acetoni-

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trile gradient in water and 0.1% TFA. The collected samples were dried to a minimal volume by vacuum centrifugation.

To alkylate the reduced cysteines 50 μL of 0.5 M iodoacetamide in water were added to the reduced protein followed by 50 μL of 6 M guanidine hydrochloride, 0.25 M Tris, 1 mM EDTA, pH 8.5. The reaction mix was incubated in the dark at room temperature for 2 h. After adding 100 μL of 1% TFA the protein solution was immediately chromatographed with a Vydac C4 column and dried to a minimal volume by vacuum centrifugation.

Guanidine hydrochloride in water (10 μL of 8 M) was used to redissolve 100 μg of the reduced/alkylated protein. After addition of 100 μL of 0.1 M Tris-HCl, 1 mM $CaCl_2$, pH 7.0, and 1 μg of trypsin to the resulting solution, the mixture was incubated at 37°C for 16 h. The digest was then chromatographed through a Vydac C18 column (0.21 &mult; 15 cm) and eluted with an acetonitrile gradient in water and 0.1% TFA. Collected peptides were dried prior to subsequent characterization.

Automated Edman degradation of protein and peptide samples was performed using an Applied Biosystems (Foster City, CA) Model 477A sequencer or a Hewlett Packard G1005A sequencer. Each sequencer was equipped with an on-line HPLC analyzer for the identification of phenylthiohydantoin derivatives.

The molecular masses of protein and peptide samples were determined by either electrospray ionization mass spectrometry (ESI) or matrix-assisted laser desorption/ionization mass spectrometry (MALDI). ESI was performed on a Perkin-Elmer Sciex (Thornhill, Ontario, Canada) API 100 mass spectrometer. MALDI was performed with a PerSeptive Biosystems Inc. (Framingham, MA) Voyager Elite time-of-flight mass spectrometer equipped with a nitrogen laser source. Sinapinic acid or α-cyano-4-hydroxycinnamic acid were used as matrices in all experiments. Time to mass conversion was obtained by external and/or internal calibration using bovine pancreatic insulin, *E. coli* thioredoxin, and horse muscle apomyoglobin.

Aspartic Acid (Asp)/Isoaspartic Acid (isoAsp) Quantitation

Relative Asp/isoAsp ratios were obtained by first incubating the degradation product at pH 7.0 at 37°C for 3 h to hydrolyze the cyclic imide ring. Samples were then reduced with 10 mM DTT and chromatographed on a Vydac C-4 column eluted with an acetonitrile gradient in water and 0.1% TFA.

Structural Analyses of the rhGDNF Degradation Product

Near and far Ultraviolet Circular Dichroism (UV CD) spectra were obtained on a Jasco model J715 spectropolarimeter (Easton, MD). Near UV CD spectra were recorded from 340 to 240 nm using a 1 cm path length cylindrical cuvette, whereas far UV CD spectra were recorded from 250 to 190 nm using a cylindrical cuvette with a 0.02 cm path length.

A fluorescence probe, 1-anilinonaphthalene 8-sulfonice acid (1,8-ANS), which binds to hydrophobic patches on the surface of proteins, was titrated against 0.2 mg/ml protein solutions whose intrinsic fluorescence had already been measured. Excitation was at 380 nm, and fluorescence spectra were measured at 400 to 520 nm using a Spectronics (Rochester, NY) SLM Aminco spectrofluorimeter with a 0.5 cm path length.

Thermal stability was monitored by following changes in the far UV CD spectra with increasing temperature. A Jasco model J720 spectropolarimeter equipped with a Peltier JTC 345 thermal control unit was used. The temperature was increased at a rate of 20°C per hour with spectra taken every 4°C and 231 nm readings taken every 0.5°C.

Binding property of rhGDNF species to heparin was examined with a Pharmacia M-trap columns (Upsala, Sweden) equilibrated at 4°C in phosphate-buffered saline (PBS). Columns were eluted with a linear 0 to 2M sodium chloride gradient.

Biological Activity of the rhGDNF Degradation Product

The following *in vitro* bioassays were used to determine both the metabolic activity as well as the capability to bind to the rhGDNF receptor.

The rhGDNF Chicken Embryo Sympathetic Chain Ganglia Survival Assay. rhGDNF was applied to chicken embryo sympathetic ganglia neurons in the presence of fetal bovine serum. Neuronal survival in response to rhGDNF was quantified by measuring the optical density at 570 nm of formazan, the resulting blue product obtained when 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide was reduced by metabolically active cells.

E15 Rat Nigral Bioassay. [^{3}H] dopamine uptake was measured using primary midbrain cultures prepared from rat embryo ventral mesencephalon (6).

The rhGDNF Receptor Binding Assay. Rat rhGDNF receptor-α was expressed on the surface of Neuro-2a cells. The binding of rhGDNF to its receptor was examined by monitoring ligand-induced PTK activation. The cells were treated with rhGDNF, lysed, and the lysates immunoprecipitated with anti-Ret antibody. The immunoprecipitates were analyzed by immunoblotting using an antiphosphotyrosine antibody (7).

The rhGDNF Mitogenic Bioassay. Murine 32Dcl3 cells were transfected with GDNFR-α and a chimeric receptor consisting of the RET extracellular ligand-binding domain and the EPOR transmembrane truncated intracellular signaling domains. Cells were added to plates prepared with rhGDNF and the plates were incubated for 48 h at 37°C with 5.5% carbon dioxide. Alamar blue was then added to each well. The alamar blue assay incorporated a fluorometric/colorimetric growth indicator based on detection of metabolic activity.

Assay variabilities for the chicken embryo sympathetic chain ganglia survival assay, E15 rat nigral bioassay, receptor binding assay, and mitogenic bioassay are 25, 20, 20, and 10%, respectively.

Pharmacokinetics of the rhGDNF Degradation Product

Single intracerebroventricular doses of 18 μg of rhGDNF or the purified degradation product were administered to rats. Plasma and cerebrospinal fluid (CSF) samples were collected by euthanizing the animals at different time-points within 72 h of dosing. At each time-point, samples from a minimum of three animals were collected. Concentrations of rhGDNF in plasma and CSF samples were determined by an

ELISA immunoassay method. Their respective detection limits were approximately 0.313 ng/ml and 0.157 ng/ml.

Toxicology of the rhGDNF Degradation Product

The toxicology of rhGDNF and its degradation product was evaluated after three monthly intracerebroventricular injections of 500 μg of the appropriate protein to rhesus monkeys. Clinical signs, body weights, food consumption, and neuropathology were all examined.

RESULTS AND DISCUSSION

Characterization of the rhGDNF Degradation Product

Figure 1 shows the pH stability profile of rhGDNFs 37°C determined from the cation exchange HPLC following incubation at pHs ranging from 2.0 to 7.0 over a period of 4 weeks. The graph shows that rhGDNF is most stable at a pH around 5.0. At both higher and lower pH, extensive deamidation at asparagines 16, 26, and 39 occurs (data not shown). This observation is consistent with the literature showing that the deamidation reaction is both acid- and base- catalyzed, and therefore, its rate is generally minimized at an intermediate pH (8,9). Further stability studies at pH 5.0 indicated that, although it deamidated only minimally, it did produce a new, late-eluting peak on the cation exchange HPLC that grew at a rate of approximately 2–5% per year under refrigerated storage conditions.

It is well known that the cyclic imide form of aspartic acid is most stable in the intermediate pH range that also minimizes deamidation (6). We therefore thought it likely that the chief degradation product of rhGDNF at pH 5 resulted from cyclic imide formation, which would cause the loss of one negative charge from the protein and thus result in elution as a late peak on cation exchange HPLC. To test this hypothesis, pH of the sample containing the unknown late peak was titrated to 7.0 and incubated for 3 h at 37°C. A sample containing roughly 65% of the unknown species and 35% native rhGDNF was incubated as described above and various time points taken. Figure 2 shows overlays of the cation exchange

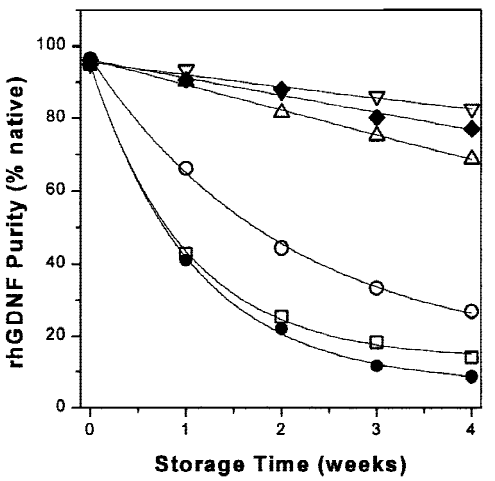

Fig. 1. Effect of pH on the stability of rhGDNF during storage at 37°C. The purity represents the percentage of native rhGDNF determined by the CEX-HPLC analysis (□ - pH 2, ○ - pH 3, △ - pH 4, ▽ - pH 5, ◆ - pH 6, ● - pH 7).

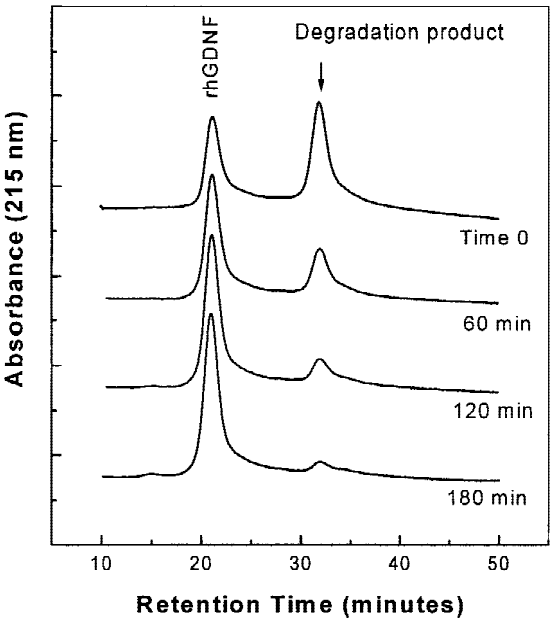

Fig. 2. Conversion of rhGDNF degradation product to native peak at pH 7.0, 37°C, shown by CEX-HPLC chromatograms.

chromatograms generated by these time points. The late peak disappeared under these conditions, a result consistent with the possibility of cyclic imide ring hydrolysis and the resultant return of the aspartic and/or isoaspartic acid negative charge. From these, it is clear that the degradation peak reverted to what seemed to be native rhGDNF after incubation. The first order kinetics expected for this reaction was observed.

Additional rhGDNF degradation product was purified via the techniques described in the Methods section. This material contained only about 5% native rhGDNF, and was therefore suitable for more refined characterization work. MALDI-TOF mass spectrometry on the purified peak gave a mass of 30,367.9 Da, whereas native rhGDNF has a mass of 30,384.8 Da. This 17 Da mass difference implied that the degradation product could have lost a water molecule, consistent with cyclic imide formation from either aspartic or glutamic acid.

Reduction of the rhGDNF degradation product at acidic pH, where a cyclic imide would be stable, led to the formation of a new peak on reverse phase HPLC. This peak was collected and S-alkylated with iodoacetamide as described in the Methods section. At the pH of the alkylation reaction, cyclic imide rings open and form either native aspartic acid or non-native isoaspartic acid (see Fig. 2).

Figure 3 shows a comparison of the relevant sections of the tryptic maps of the reduced/alkylated degradation product peak with reduced/alkylated native rhGDNF. Two new peaks, labeled 3 and 4, appeared in the degradation product maps just before native peaks 1 and 2. Edman degradation and mass spectroscopy were performed on all four of these peaks. Sequencing of peaks 3 and 4 terminated after serine 95, though the peptide masses indicated their lengths were identical to those of peaks 1 and 2. As Edman degradation stops at isoaspartic acid, this provided proof that the rhGDNF degradation product was indeed the result of the formation of a cyclic imide from Asp 96. In the tryptic map, peaks 1 and 2 formed when the cyclic imide ring hydrolyzed

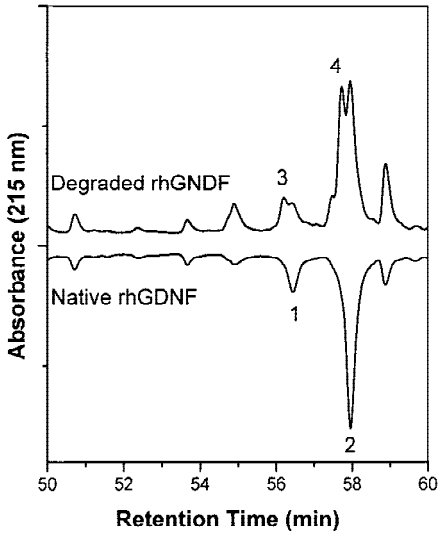

Fig. 3. Comparison of tryptic maps of native and degraded rhGDNF.

into native aspartic acid, and peaks 3 and 4 when it hydrolyzed into non-native isoaspartic acid. These results are summarized in Table I.

Determination of Asp/isoAsp Ratios after Cyclic Imide Ring Opening

The cyclic imide form found in the rhGDNF formulation will be converted to either aspartic form or isoaspartic form after being introduced to the brain because of the neutral pH. Because the Asp form represents the native rhGDNF, it is safe to assume that the fraction of cyclic imide converted to Asp form does not affect the pharmaceutical quality. Typically, when a cyclic imide ring hydrolyzes, more than twice as much isoaspartic acid as aspartic acid is formed (10,11). However, steric factors induced by neighboring residues and overall protein conformations both influence this ratio (12). When the rhGDNF cyclic imide form was treated and analyzed via the protocol described in the Methods section, a surprisingly low 42:58 ratio of isoaspartic acid/aspartic acid was observed. The isoaspartic acid residue, which is generated when cyclic imide form is hydrolyzed at neutral pH, has been observed naturally both *in vitro* and *in vivo* (12). The cyclic imide also can be formed during the deamidation of asparagine or glutamine residues. There is also an enzyme called protein carboxymethyl transferase, which converts isoaspartic acid residues, the side product of the cyclic imide form of aspartic acid, back to the natural aspartic acid (13). This suggests that on delivery, when rhGDNF is exposed to physiological pH and temperature, more than half of the chief degradation product will return to a fully native state.

Rationale for Bioassay Testing and Structural, Pharmacokinetc, and Toxicological Studies

The very limited number of excipients approved for CNS usage severely hampered efforts to eliminate cyclic imide formation by manipulating the formulation. Although a lyophilized formulation that prevented cyclic imide production was developed, proceeding to the clinic with the excipients this formulation contained was considered risky due to their unknown safety profile.

Further, recent surveys of pharmaceutical industries developing protein-based drugs revealed that cyclic imide formation tends to occur in flexible protein domains and that its formation therefore does not necessarily result in a major conformational change (14). This suggests that the isomerization of one rhGDNF residue might have no significant effect on the protein's function, structure, or immunogenicity.

To confirm the safety of the cyclic imide form, it was decided to examine the rhGDNF degradation product for structural variations from the native molecule, to assay its bioactivity, and to establish its pharmacokinetics and toxicity profiles.

Near and Far UV Circular Dichroism

Figure 4a shows the near UV spectra of native rhGDNF, rhGDNF containing cyclic imide, and rhGDNF containing isoaspartic acid (produced by incubating cyclic imide at pH 7.0). Near UV CD reflects the environment of the aromatic amino acids and of disulfides, and provides one way to look at tertiary structure. No differences were seen among the three isoforms.

Figure 4b shows the far UV spectra of the same three

Table I. Sequences and Masses of Native and Degraded rhGDNF Tryptic Peptides Shown in Fig. 3

	Detected sequences[a]	Mass (expected mass)
	R^{92}LVSDKVGQAC*C*RPIAFDDDLSFLDDNLVY^{121}	3503.1 (3502.9)
Peak 1	L^{93}VSDKVGQAC*C*RPIAFDDDLSFLDDNLVYH^{122}	3483.7 (3483.9)
	V^{98}GQAC*C*RPIAFDDDLSFLDDNLVYH^{122}	2941.2 (2941.3)
	L^{93}VSDKVGQAC*C*RPIAFDDDLSFLDDNLVY^{121}	3346.6 (3346.8)
Peak 2		
	V^{98}GQAC*C*RPIAFDDDLSFLDDNLVY^{121}	2804.0 (2804.1)
	R^{92}LVS(*iso*DKVGQAC*C*RPIAFDDDLSFLDDNLVY^{121})	3503.1 (3502.9)
Peak 3		
	L^{93}VS(*iso*DKVGQAC*C*RPIAFDDDLSFLDDNLVYH^{122})	3484.5 (3483.9)
Peak 4	L^{93}VS(*iso*DKVGQAC*C*RPIAFDDDLSFLDDNLVY^{121})	3347.1 (3346.8)

[a] C* is S-carboxymethylated cysteine; *iso*D is isoaspartic acid. Sequences in parentheses were inferred from the peptide masses but not actually sequenced due to isoAsp blocking of Edman degradation.

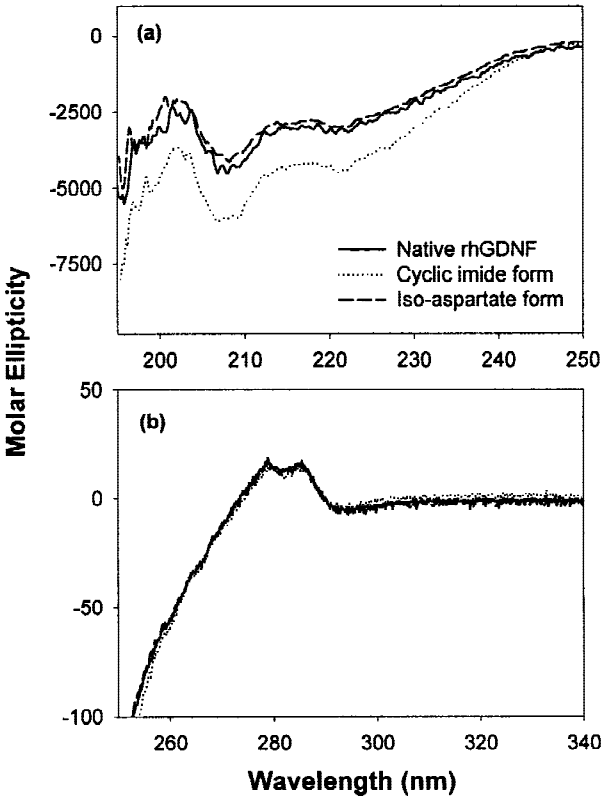

Fig. 4. Near UV **(a)** and far UV **(b)** CD spectra of native, cyclic imide, and isoaspartate forms of rhGDNF.

samples. They all show maxima at 210–220 and 200 nm, characteristic of a protein that contains both β-sheet and disordered secondary structures. However, the rhGDNF containing cyclic imide generated lower signal intensity than the other two. This could be due to a minor local secondary structure change associated with the cyclization of Asp 96.

Far UV CD was also used to compare the thermal melting curves of the three species. No significant differences among them were seen.

ANS and Heparin Binding

1,8-ANS is a fluorescence probe that binds to hydrophobic surfaces of proteins (see Methods). Native rhGDNF is a very hydrophilic molecule that does not bind 1,8-ANS to any significant degree. The cyclic imide and isoaspartate containing molecules also failed to bind 1,8-ANS (data not shown). This was yet another indication that the surface hydrophobicity of the three species was identical.

Heparin binding is another indirect means of partially establishing protein structure. All three forms of rhGDNF eluted from a heparin column at an identical ionic strength of 110 μS^2. Thus, the heparin-binding region of rhGDNF was unaltered by cyclic imide or isoaspartate formation.

Biological Activity of rhGDNF

Four different bioassays of native, cyclic imide, and isoaspartate-containing rhGDNF were performed to ensure that the isoforms have the same bioactivity as the native molecule. Table II summarizes the results. Note that the receptor-binding assay relies on the visualization of immunoblots and

Table II. Biological Activity of Cyclic Imide Form and Isoaspartate Form of rhGDNF Determined by Various *In Vitro* Bioassays

	Activity (% relative to native rhGDNF)	
	Cyclic imide form	Isoaspartate form
Chicken embryo sympathetic ganglia survival assay	106	86
rhGDNF cell line-based bioassay	118	Not done
E15 rat nigral bioassay	112	109
Receptor binding assay	100	100

therefore does not, as currently run, give a numerical result. All activity percentages were normalized to that of native rhGDNF (100%).

Given the inherent variabilities in each of these bioassays, no significant activity difference was seen between the native rhGDNF molecule and its degradation products. The result is consistent with the published structural information that the loop (92RLTSDK97) that connects the α-helix and finger 2 is not critical for the biological activity of rhGDNF (15).

Pharmacokinetics and Toxicology

Comparative pharmacokinetic and toxicology studies were performed on native and cyclic imide rhGDNF to ensure that their distribution, half lives, and toxicology profiles were the same. As cyclic imide would quickly convert to both aspartic and isoaspartic acid *in vivo,* separate administration of the isoaspartic rhGDNF was not performed. Figure 5 shows the CSF concentration profile following intracerebroventricular (ICV) dosing of 18 μg of native and cyclic imide rhGDNF. Results from the immunoassay suggest that their disposition was the same. Terminal half-lives were also very similar, ranging from 12 to 13 h. The area under curve (AUC) values were also essentially identical.

Finally, no difference between native and cyclic imide rhGDNF was observed in the toxicology study. Clinical signs,

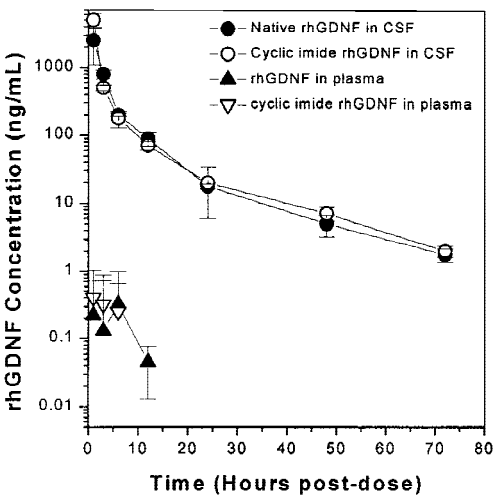

Fig. 5. Pharmachokinetic profiles of different rhGDNF forms in cerebrospinal fluid (CSF) and plasma.

body weights, food intake, and neuropathology were indistinguishable between the two molecules after 3 months of monthly 500 μg doses delivered intracerebroventricularly. The pharmacokinetic and toxicology studies therefore indicate that there was no significant difference between native and cyclic imide rhGDNF *in vivo*.

CONCLUSION

Characterization of the chief rhGDNF degradation product generated during long-term storage showed that it was the result of the formation of cyclic imide at Asp 96. At physiological temperature and pH, this cyclic imide ring rapidly hydrolyzes into both native aspartic acid (58%) and nonnative isoaspartic acid (42%). In four separate bioassays, the degradation product was shown to have identical bioactivity to the native molecule. Structural studies with near and far UV CD, 1,8-ANS binding, heparin binding, and melting temperature determination also showed no significant changes from native rhGDNF. Finally, their pharmacokinetic and toxicological profiles were also essentially identical. These data support the conclusion that the rhGDNF containing cyclic imide can be considered to be equivalent to native rhGDNF in the development of a pharmaceutical dosage form for ICV delivery.

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