Carboxyl-directed Pegylation of Brain-derived Neurotrophic Factor Markedly Reduces Systemic Clearance with Minimal Loss of Biologic Activity

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Purpose. Brain-derived neurotrophic factor (BDNF) was modified by carboxyl-directed protein pegylation in order to both retain biologic activity of the neurotrophin and reduce the rate of systemic clearance of this cationic protein *in vivo*. Since the modification of surface lysine residues of neurotrophins results in loss of biologic activity, the present studies examine the feasibility of placing polyethyleneglycol (PEG) polymers on carboxyl residues of surface glutamate or aspartate residues of BDNF.

Methods. PEG molecules with terminal hydrazide (Hz) moieties of molecular weight 2,000 (PEG^{2000}-Hz) or 5,000 (PEG^{5000}-Hz) Daltons were coupled to BDNF carboxyls using carbodiimide.

Results. The systemic clearances of the BDNF-PEG^{2000} and BDNF-PEG^{5000} were reduced 67% and 91%, respectively, compared to unconjugated BDNF. The brain volume of distribution (V_D) of BDNF-PEG^{5000} was not significantly different from the cerebral plasma volume. Cell survival studies and TrkB auto-phosphorylation assays showed that the biologic activity of BDNF was not changed following pegylation with PEG^{2000}, and was minimally impaired following pegylation with PEG^{5000}.

Conclusions. These experiments describe the first carboxyl-directed pegylation of a neuropeptide, and show this formulation substantially reduces the systemic distribution and elimination of the neurotrophic factor. The biologic activity of the neurotrophin is retained with carboxyl-directed pegylation.

KEY WORDS: pegylation; pharmacokinetics; blood-brain barrier.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) was cloned in 1989 (1) and subsequently shown to have neurotrophic effects for the central nervous system (CNS) (2,3). BDNF is a potential neuropharmaceutical that could be used in different neurodegenerative conditions in humans. However, BDNF does not cross the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) in vivo (4). Neuropeptides not capable of transport through the BBB may be transported into brain by conjugating these molecules to BBB delivery vectors (5). The latter are proteins or monoclonal antibodies that undergo receptor-mediated transcytosis through the BBB. The BBB transferrin receptor mediates the transcytosis of both transferrin and the OX26 murine monoclonal antibody (MAb) to the rat transferrin receptor (6–9). The BBB transport of BDNF was increased following conjugation to the OX26 delivery system; however, the maximal brain uptake of the BDNF conjugate was only 0.07% of injected dose per gram brain (4), a value that is approximately 6-fold lower than the maximal brain uptake of the unconjugated OX26 MAb (5). The reduced brain delivery of the BDNF/OX26 conjugate was due to the marked decrease in the plasma area under the concentration curve (AUC) of the BDNF/OX26. The suboptimal pharmacokinetic properties of the BDNF/OX26 conjugate arises from the cationic nature of BDNF and the very active hepatic transport of this cationic protein in vivo (4). The rapid hepatic uptake of the BDNF, the low plasma AUC, and the suboptimal pharmacokinetics, make it difficult to deliver effective amounts of BDNF to brain using BBB vector-mediated delivery systems.

The pharmacokinetics of BDNF may be optimized by protein pegylation, wherein the surface of the protein is modified by covalent attachment of 2,000–5,000 Dalton strands of polyethyleneglycol (PEG). Previous studies show that the rate of plasma clearance of peptides and proteins is markedly reduced following protein pegylation (10–12). Proteins are generally pegylated by attachment of the PEG polymer to ϵ-amino groups on surface lysine residues (10–12). However, BDNF is a member of the nerve growth factor (NGF) family, and modification of surface lysine residues of NGF-like neurotrophins results in loss of biologic activity (13). Similarly, there is loss of receptor affinity of cytokines following amino-directed pegylation (14). BDNF pegylation with retention of biologic activity may be achieved through carboxyl-directed pegylation, wherein surface glutamate or aspartate residues are the sites of attachment of the PEG strands. Therefore, the present studies report on methods for carboxyl-directed pegylation of BDNF and demonstrate retention of biologic activity of the neurotrophin following this modification. The PEG-BDNF is shown to have markedly reduced rates of plasma clearance following carboxyl-directed protein pegylation, which results in optimized pharmacokinetic properties.

MATERIALS AND METHODS

Materials

Human recombinant BDNF (Lot number BW#2135E5) was produced in *E. coli* and was obtained from Dr. James Miller (Amgen, Thousand Oaks, CA) (15). [^{125}I]-iodine was obtained from Amersham Corp. (Chicago, IL). Methoxy-PEG-hydrazide (Hz) (MW = 2,000 Da), designated PEG^{2000}-Hz, was custom synthesized by Shearwater Polymers, Inc. (Huntsville, AL). Methoxy-PEG-Hz, (MW 5,000 Daltons), designated PEG^{5000}-Hz, was purchased from Shearwater Polymers, Inc. [^{125}I]-BDNF (2,200 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). Adult male Sprague-Dawley rats (275–300 g) were purchased from Harlan-Sprague Dawley, Inc. (Indianapolis, IN). N-methyl-N′-3-(dimethylaminopropyl)carbodiimide (hydro-

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ABBREVIATIONS: BBB, blood-brain barrier; BNDF, brain-derived neurotrophic factor; RSA, rat serum albumin; RHB, Ringers-Hepes buffer; TCA, trichloroacetic acid; V_D, organ volume of distribution; ID, injected dose; D, dose; V_{SS}, steady state volume of distribution; AUC, area under the plasma concentration curve; Cl, systemic clearance; K_i, organ clearance; V_o, organ plasma volume of distribution; NGF, nerve growth factor; PEG, polyethyleneglycol; FCS, fetal calf serum; Hz, hydrazide.

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chloride) (EDAC) and all other reagents were obtained from Sigma Chemical Corp. (St. Louis, MO). Hi-Trap columns (1ml volume) were obtained from Pharmacia (Piscataway, NJ). 3T3 cells transfected with the TrkB gene, designated TrkB-FL#14/NIH3T3, were provided by Dr. Andrew Welcher, Amgen Corp. The CellTiter 96™ Aq_{ueous} Non-Radioactive Cell Proliferation Assay was obtained from Promega Corp. (Madison, WI).

Radioiodination with Lactoperoxidase

BDNF was radioiodinated with lactoperoxidase as previous studies have demonstrated retention of BDNF biologic activity following this form of radiolabeling (17). To a 500 μl microfuge tube was added the following: 3.4 μl of BDNF (50 μg, 2.1 nmol of monomer), 5 μl of lactoperoxidase (0.03 units, 0.15 μg), 2 mCi [^{125}I]-I (1 nmol), 3 μl of 0.00075% H_2O_2 (0.022 μg, 0.6 nmol). After 1 minute of incubation, another 3 μl of H_2O_2 was added, and this was repeated two more times. The reaction was stopped by the addition of 200 μl of stop solution (2 mg/ml L-tyrosine, 0.5 M NaCl, pH = 7) and the mixture was applied to a 0.7 × 28 cm column of Sephadex-G25 that was pre-equilibrated with 0.5 M NaCl (pH = 6). The radiolabeled BDNF was eluted with 0.5 M NaCl (pH = 6) and eluted from the column as a single peak with a specific activity of 11 μCi/μg and a trichloroacetic acid (TCA) precipitability of 99%. The [^{125}I]-BDNF eluted from a C4 reverse phase HPLC column as a singe peak using the gradient system of Rosenfeld et al (18), wherein labeled BDNF elutes at a slightly longer retention time than unlabeled BDNF (18).

BDNF Pegylation

BDNF was pegylated with either PEG^{2000}-Hz or PEG^{5000}-Hz. A 25 μl aliquot of BDNF (0.37 mg) was added to 200 μCi of [^{125}I]-BDNF (0.03 mg) and 275 μl of 0.01M pyridine (pH = 4.8). To this solution, either 90 mg of PEG^{5000}-Hz or 18 mg of PEG^{2000}-Hz was added followed by the addition of either 50 μl (3.5 mg, 18 μmol) or 25 μl (1.75 mg, 9μmol) of fresh EDAC, respectively. The solution was mixed and set overnight at room temperature, and the next day buffer A (0.05 M Na_2HPO_4, 0.5 M NaCl, pH = 7.0) was added to 1 ml, and the solution was applied to a 1 ml Hi-Trap copper affinity column. The unreacted PEG migrated in the void volume of the column and the BDNF-PEG was eluted with imidazole. Prior to application of the BDNF, the 1 ml column was washed with 5 ml of water, 0.5 ml of 0.1 M $CuSO_4$, and 5 ml of water. After application of the 1.0 ml sample, the column was washed with 10 ml of buffer A, and the PEG-BDNF was eluted with the 5 ml of 50 mM imidazole. The column was eluted at a rate of 1 ml/min. The ratio of PEG-Hz/EDAC/BDNF carboxyls was 50/50/1 and 25/25/1 for pegylation with PEG^{5000}-Hz and PEG^{2000}-Hz, respectively. The final protein content was measured with the bicinchoninic acid assay from Pierce Chemical Company (Rockford, IL). The molecular size of the BDNF, the BDNF-PEG^{2000}, and the BDNF-PEG^{5000} was measured with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% minigels from BioRad Corp (Richmond, CA). The gels were run at constant voltage and were stained with Coomassie blue, followed by destaining and scanning. SDS-PAGE molecular weight standards were obtained from BioRad.

Survival, Radioreceptor, and Auto-Phosphorylation Assays

3T3 cells transfected with the TrkB gene (16) strongly expressed immunoreactive TrkB, particularly at confluency, when fixed in 100% methanol and immunostained with a rabbit polyclonal antiserum (#51-809, provided by Dr. Andrew Welcher, Amgen, Inc.) at a 1:500 dilution using the avidin-biotin immunoperoxidase method (Vector Labs, Burlingame, CA). The 3T3-TrkB cells were plated in 96-well culture dishes and grown in Dulbecco's modified Eagle medium with 10% fetal cat serum (FCS) and 400 μg/ml G418. To each well was added 200 μl (75,000 cells per ml or 15,000 cells per well). On day 2, the cells were rinsed with assay medium (RPMI 1640 medium containing 0.3 mg/ml L-glutamine and no serum) and then 150 μl of assay medium was added to the well followed by the addition of 50 μl BDNF solutions (19). On day 6, each well was supplemented with 40 μl of the MTS-PMS solution of the CellTiter 96™ AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega); PMS = phenazine methylsulfate, and MTS = (3-(4, 5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfylphenyl)-2H-tetrazolium, inner salt. The cells were incubated for 2 hours at 37°C followed by reading of absorbance at 490 nm. The quantity of formazan product is measured by the amount of 490 nm absorbance, and is directly proportional to the number of living cells in tissue culture.

The radioreceptor assay was performed by plating the 3T3-TrkB cells in 24-well culture dishes and the cells were grown in the DMEM/10% FCS medium. Four days later, the medium was aspirated, and the wells were washed with HBSB buffer (0.01 M Hepes, 0.15 M NaCl, 0.1% bovine serum albumin). To each well was then added 50 μl of [^{125}I]-BDNF (0.3 μCi/ml, obtained from DuPont-NEN), which was dissolved in buffer B (0.01 M Hepes, 0.5 M NaCl, 1% BSA, pH = 7.4) followed by addition of 50 μl of BDNF dilutions and the volume of each well was brought to 500 μl with HBSB buffer. The wells were incubated for 45 minutes at room temperature, as initial experiments showed that binding equilibration was reached at this time period. At the end of the incubation, the medium was removed and cells washed with 2 ml of cold PBS, followed by the addition of 0.5 ml of 1N NaOH per well. The plates were heated to 50 °C to solubilize the protein, and 300 μl was removed for [^{125}I]- counting, and a 100 μl aliquot was removed for protein content using the Pierce protein assay. The data were reported as percent [^{125}I]-bound per mg of 3T3 protein.

BDNF-induced auto-phosphorylation of the 3T3-TrkB cells was performed by incubating serum-starved cells (0.5% FCS overnight) for 15 minutes with 0, 1, 10, or 100 ng/ml BDNF or BDNF-PEG^{2000} using the method of Arakawa *et al.* (14). The cells were collected in lysis buffer and TrkB was immuno-precipitated overnight with an anti-TrkB antiserum (provided by Dr. Andrew Welcher, Amgen, Inc.), followed by incubation with protein G-sepharose and centrifugation. The pellet was dissolved in SDS sample buffer and applied to 7.5% minigels (BioRad) followed by electro-blotting to Immobilon-P (Millipore) in 10% methanol. The filter was probed with 2 μg/ml anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), and stained with an avidin-biotin peroxidase technique (Vector Labs, Burlingame, CA). The filter was scanned, and quantitated by NIH Image and Adobe Photoshop software on a Power Macintosh computer. The size of the TrkB

immuno-precipitate was estimated with biotinylated molecular weight standards (BioRad).

Plasma Pharmacokinetics

Plasma pharmacokinetics and organ uptake were measured exactly as described previously in ketamine-xylazine anesthesized male Sprague-Dawley rats (4), except the dose of [^{125}I]-BDNF (either free or pegylated) was 3-fold higher at 10 μg/rat (equivalent to 5 μCi/rat). These methods adhere to the "Principles of Laboratory Animal Care" (NIH Publication #85-23, revised 1985).

RESULTS

The pegylation scheme is depicted in Figure 1, which shows formation of the amide bond between surface carboxyl moieties on the BDNF and the hydrazide functional group on the PEG molecule. The PEG may be either 2,000 or 5,000 Daltons in size, and the terminal residue of the PEG (R) may either be a methoxy residue or a biotin moiety. In the latter case, the BDNF-PEG could be coupled to avidin/MAb delivery systems (4). The copper affinity chromatographic procedure proved to be an effective and simple means for separating the unconjugated PEG, which eluted with phosphate buffer, from the BDNF-PEG, which eluted with imidazole (Methods). Following purification, the BDNF-PEG was subjected to SDS-PAGE (Figure 2). The BDNF-PEG^{2000} had an average molecular size of 28 kDa with two predominant species, indicating an average of 6 to 7 PEG residues were added per BDNF monomer. The BDNF-PEG^{5000} was comprised of 3 principle forms and had an average molecular size of 50 kDa, indicating approximately 5–7 PEG moieties were applied per BDNF monomer.

The pharmacokinetic analysis is shown in Figure 3. The unconjugated BDNF was rapidly removed from the plasma compartment with a systemic clearance of 4.2 ± 0.1 mL/min/kg (Table 1). The BDNF plasma radioactivity decreased monoexponentially with a half-time of 9.7 ± 0.3 minutes (Figure 3, Table 1). The unconjugated BDNF was rapidly degraded following intravenous injection as the plasma TCA precipitability at 60 minutes after injection was less than 40% (Figure 3B). The metabolic stability of the BDNF-PEG^{2000} was increased as the plasma TCA precipitability 60 minutes after IV injection was in excess of 80% (Figure 3B). The metabolic stability of the PEG-BDNF^{5000} was increased further and the TCA precipitability was in excess of 95% at 60 minutes after IV injection (Figure 3B). Similarly, the rates of removal of BDNF from the plasma compartment were progressively decreased following pegylation with either PEG^{2000} or PEG^{5000} (Figure 3A). The rate of plasma clearance of the BDNF-PEG^{2000}, 1.4 ± 0.1 mL/min/kg (Table 1), was decreased 67% compared to the unconjugated BDNF. The rate of plasma clearance of the BDNF-PEG^{5000}, 0.37 ± 0.01 mL/min/kg (Table 1), was decreased 91% compared to the unconjugated BDNF. An analysis of the organ V_D and organ clearance values (Table 2) showed that the principle organ responsible for the rapid removal of unconjugated BDNF from the plasma compartment was the liver (Table 2). The organ clearance of the BDNF-PEG^{2000} was decreased 67% in liver, which paralleled the comparable decrease in plasma clearance of BDNF-PEG^{2000} compared to unconjugated BDNF. The hepatic clearance of BDNF-PEG^{5000}, 5.1 ± 0.7 μL/min/g, was decreased 92% compared to the hepatic clearance of the unconjugated BDNF and this also paralleled the decrease in plasma clearance of BDNF following conjugation of PEG^{5000}. The clearance of BDNF by peripheral tissues, such as heart or lung, was also proportionally decreased by pegylation (Table 2). In contrast, the renal clearance of BDNF-PEG^{2000} was actually more than two-fold increased compared to the renal clearance of the unconjugated BDNF, whereas the rate of renal clearance of BDNF-PEG^{5000} was decreased 61% compared to the unconjugated BDNF (Table 2).

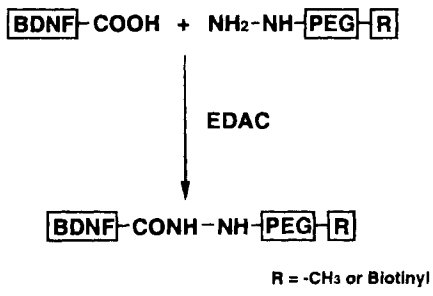

Fig. 1. Scheme for chemical coupling of polyethyleneglycol (PEG) polymers to brain-derived neurotrophic factor (BDNF) surface carboxyl residues using PEG hydrazide derivatives and carbodiimide (EDAC). If bifunctional PEG reagents are used, then a biotin residue may be placed at the terminus (R) of the PEG strand.

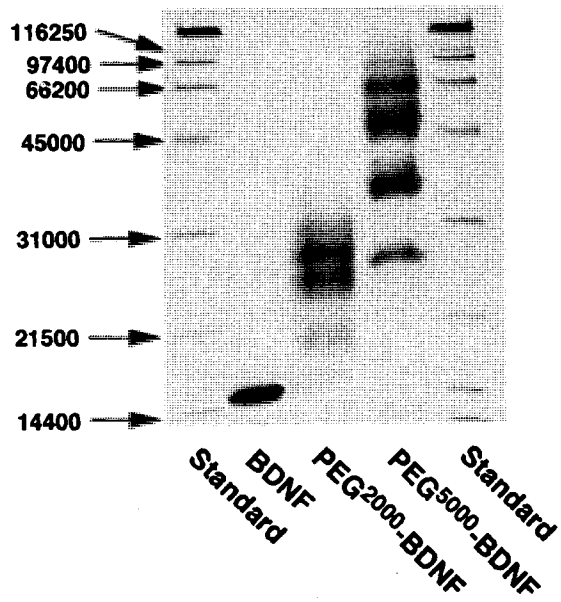

Fig. 2. SDS-PAGE of molecular weight standards, BDNF, BDNF-PEG^{2000}, and BDNF-PEG^{5000}. The gel was stained with Coomassie blue.

The brain volume of distribution of BDNF was progressively decreased following pegylation with PEG^{2000} or PEG^{5000} (Figure 4). The organ volume of distribution of the BDNF-PEG^{5000} was not significantly different from the brain plasma volume, 11 ± 2 μL/g (Figure 4).

The biologic activity of the BDNF in tissue culture was retained following conjugation with PEG^{2000} as shown by the survival assay (Figure 5B). The biologic activity of BDNF-PEG^{5000} was partially retained based on the studies of the survival assay (Figure 5A). The affinity of unconjugated BDNF and

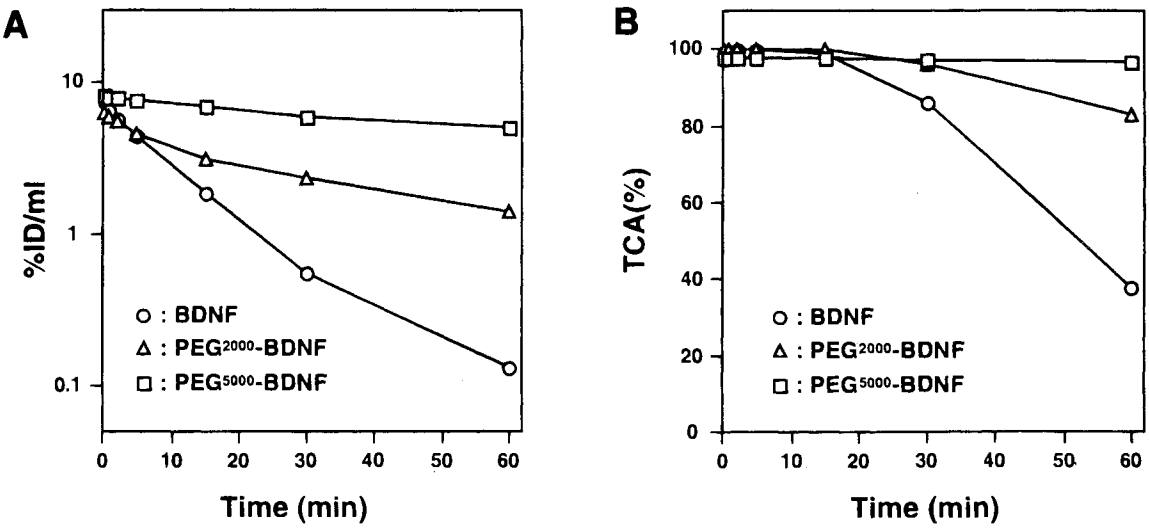

Fig. 3. (A) The plasma percent of injected dose (ID)/ml TCA-precipitable radioactivity is shown versus the time after intravenous injection for BDNF, BDNF-PEG^{2000}, and BDNF-PEG^{5000}. Data are mean of three rats at each point. Standard errors vary < 10% of the mean. (B) The percent of plasma radioactivity that is trichloroacetic acid (TCA) precipitable is shown relative to the time after intravenous injection for the three different forms of BDNF.

BDNF-PEG^{2000} were comparable based on the radio-receptor assay (data not shown), and the bio-activity of the BDNF and BDNF-PEG^{2000} were equivalent based on TrkB autophosphorylation assays (Figure 6).

DISCUSSION

The results of these studies are consistent with the following conclusions. First, carboxyl-directed pegylation of a protein, such as BDNF, is possible using PEG-hydrazide derivatives (Figures 1,2); the chromatographic separation of the unconjugated PEG from the BDNF-PEG is facilitated by copper affinity chromatography (Results). Second, pegylation has a profound effect on the rate of plasma clearance of the BDNF (Figure 3A, Table 1) and the metabolic stability of the neurotrophin (Figure 3B). Third, the enhanced metabolic stability of the BDNF is paralleled by a decrease in the brain volume of distribution (Figure 4), indicating the brain uptake of radioactivity following the intravenous injection of radiolabeled unconjugated BDNF is artifactual and reflects the brain uptake of metabolites. Fourth, the biologic activity of BDNF was retained following carboxyl-directed pegylation (Figures 5,6), which was consistent with previous results, indicating NGF-like neurotrophins may be modified at surface carboxyl residues (13).

Protein pegylation is well known to decrease systemic clearance of proteins, and recent studies have demonstrated an inhibition of the plasma clearance of tumor necrosis factor (TNF) α, interleukin (IL)-2, and growth hormone (GH) (10–12). The decrease in plasma clearance caused by BDNF pegylation parallels exactly the decrease in hepatic clearance (Figure 3, Table 2). For example, the hepatic uptake of unconjugated BDNF is 5.2 ± 0.3 %ID/g (Table 2). Since the weight of the liver in a 300 g rat is 10 g, more than 50% of the injected dose

Table 1. Pharmacokinetic Parameters

Parameter	BDNF	BDNF-PEG^{2000}	BDNF-PEG^{5000}
A_1 (%ID/mL)	6.1±0.2	2.6±0.6	7.9±0.8
A_2 (%ID/mL)	—	3.8±0.5	—
k_1 (min^{-1})	0.072±0.002	0.17±0.03	0.0083±0.0004
k_2 (min^{-1})	—	0.016±.0003	—
$t^1_{1/2}$ (min)	9.7±0.3	4.6±0.9	84±4
$t^2_{1/2}$ (min)	—	45±8	—
V_{ss} (mL/kg)	59±1	87±9	45±3
$AUC\|_0^{60}$ (%ID·min/mL)	84±5	157±7	374±32
$AUC\|_0^{\infty}$ (%ID·min/mL)	86±5	205±18	951±51
Cl_{ss} (mL/min/kg)	4.2±0.1	1.4±0.1	0.37±0.01
MRT (min)	14±1	62±11	121±5

Parameters estimated by fitting the data in Figure 4 to either a single-exponential (BDNF, BDNF-PEG^{5000}) or a double-exponential (BDNF-PEG^{2000}) model. A_1 and A_2 and k_1 and k_2 are the intercepts and slopes derived from fitting the plasma data (Figure 3A) to either a mono-exponential (BDNF or BDNF-PEG^{5000}) or to a bi-exponential function (BDNF-PEG^{2000}), depending on which function gave a best fit to the data based on an analysis of the residual sum of squares. The $t_{1/2}$'s were derived from the k's. Data are mean ±SE (n = 3 rats). The injected dose was 5 uCi/rat (10 ug/rat).

Table 2. Organ Volume of Distribution (V_D), Clearance (K_i), and % Injected dose (ID) Per Gram Organ

Organ	Parameter	BDNF	BDNF-PEG^{2000}	BDNF-PEG^{5000}
heart	V_D(μL/g)	1,267±62	277±28	77±6
lung		8,895±227	592±50	280±44
liver		39,958±2,375	2,540±122	506±55
kidney		4,164±165	1,979±75	308±26
brain	K_i (μL/min/g)	0.29±0.04	0.19 ±0.01	0
heart		1.7±0.1	1.1±0.2	0
lung		13±1	3.2±0.5	0
liver		61±2	22±3	5.1±0.7
kidney		6.2±0.3	16±1	2.4±0.3
brain	%ID/g	0.024±0.002	0.029±0.002	0
heart		0.15±0.01	0.17±0.03	0
lung		1.1±0.1	0.50±0.05	0
liver		5.2±0.3	3.4±0.3	1.9±0.1
kidney		0.53±0.04	2.5±0.1	0.86±0.06

Data are mean±S.E. (n = 3). Ogans sampled 60 minutes after IV injection. The K_i and %ID values for BDNF-PEG^{5000} in brain, heart, and lung are 0, because the organ V_D values are not significantly different from the organ plasma volumes of distribution (V_o) reported previously (4).

is rapidly removed by the liver with a half-time of 10 minutes (Table 1). Conversely, only 34% or 19% of the injected dose is removed by the liver following IV injection of BDNF-PEG^{2000} or BDNF-PEG^{5000}, respectively (Table 2). With respect to renal clearance of BDNF, modification by PEG^{2000} has a paradoxical increase in the renal clearance (Table 2). Since the BDNF is pegylated in these studies at carboxyl residues, this neurotrophin actually becomes more cationic following pegylation. The kidney selectively extracts cationic substances from the bloodstream (20), and the increased cationic nature of the BDNF-PEG^{2000} may account for the paradoxical increase in renal uptake of the neurotrophin. However, when BDNF is pegylated with PEG^{5000}, renal clearance is decreased (Table 2), and this may reflect the fact that the effective size of the BDNF is increased to an extent that glomerular filtration of the protein is significantly inhibited.

The data in Figure 5 show that the apparent brain volume of distribution (V_D) of BDNF is progressively decreased with pegylation, such that the brain V_D of BDNF-PEG^{5000} is not

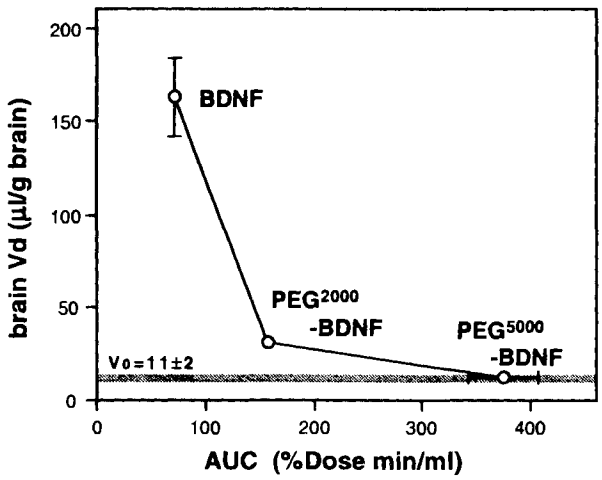

Fig. 4. The brain volume of distribution (V_D) for BDNF, BDNF-PEG^{2000}, and BDNF-PEG^{5000} is plotted against the corresponding plasma AUC at 60 minutes after IV injection for each preparation. The shaded horizontal bar is the brain plasma volume of distribution (V_O). Data are mean ± S.E. (n=3). The brain V_D of the BDNF-PEG^{5000} is not significantly different from the brain plasma volume.

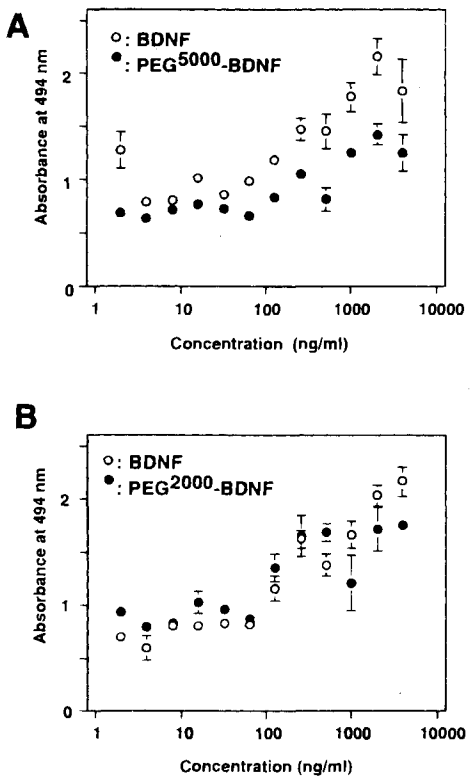

Fig. 5. The survival of NIH 3T3-TrkB cells that are grown in serum free medium is plotted relative to the medium concentration of either BDNF and BDNF-PEG^{5000} (A), or BDNF and BDNF-PEG^{2000} (B). Data are mean ± S.E. (n=3) and in some cases the error bars are too small to be visible.

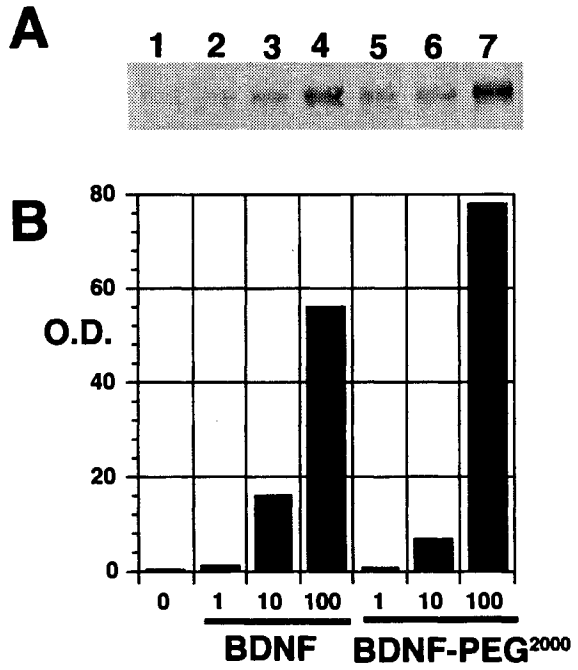

Fig. 6. The induction of TrkB autophosphorylation by BDNF or BDNF-PEG^{2000} is comparable in serum-starved 3T3-TrkB cells. (A) Western blot after staining with an anti-phosphotyrosine antibody. The immunoreactive TrkB migrated at a molecular weight of 144 kDa. (B) Optical density (O.D.) of the scanned film at each concentration of BDNF or BDNF-PEG^{2000}.

significantly different from the brain plasma volume. This study has implications to the general field of neuropeptide transport across the BBB. There are several studies in which a metabolically labile, radioactive neuropeptide is injected intravenously, and an apparent brain volume of distribution above the plasma volume (V_O) is recorded (21,22,23). This is interpreted as evidence for BBB transport of the neuropeptide, although there is rapid peripheral metabolism of the radiolabeled neuropeptide with the concomitant release of radiolabeled metabolites, such as iodotyrosine. The latter may undergo carrier-mediated transport across the BBB in vivo, and lead to the situation where the brain V_D exceeds the brain V_O. However, in this setting there has been no actual transport of the neuropeptide across the BBB, and the increase in brain V_D is an artifact, owing to uptake of radiolabeled metabolites. Prior to the present study, there has been no attempt to inhibit the peripheral degradation of an intravenously injected neuropeptide. However, neuropeptide pegylation can reduce peripheral metabolism, and in this setting more accurate estimates of neuropeptide transport through the BBB may be obtained. The results in Figure 4 emphasize the need to exercise caution in interpreting brain radioactivity data following intravenous injection of a metabolically labile, radiolabeled neuropeptide.

The survival assay and the auto-phosphorylation assay (Figures 5 and 6) show that the biologic activity of BDNF was retained following pegylation when the PEG moieties are attached to surface carboxyl residues. This study is apparently the first reported demonstration of protein pegylation on surface carboxyl residues, although Zalipsky (24) has outlined methods for carboxyl-directed or carbohydrate-directed protein pegylation. Carboxyl-directed pegylation was employed in these studies, because previous results have shown that NGF-like neurotrophins cannot be modified at ε-amino groups on surface lysines and retain biologic activity (13). In most studies involving protein pegylation, surface lysine residues are used as the target for pegylation (10–12), although surface sulfhydryls or carbohydrate moieties have also been pegylated (24,25). However, BDNF lacks a free sulfhydryl, and carbohydrate is not available on recombinant BDNF produced in *E. coli*. Certain proteins, such as neurotrophins (13) or IL-15 (14), loose biologic activity when surface amino groups are modified. Amino-directed pegylation of growth hormone (GH) results in an impairment in GH affinity for its cognate receptor (11). However, the decrease in growth hormone binding to the growth hormone receptor is less than the increase in the plasma AUC. Accordingly, the GH-PEG is a more pharmaceutically active preparation than the unconjugated GH (11).

In summary, these studies demonstrate that carboxyl-directed pegylation of BDNF is a satisfactory way of optimizing the plasma pharmacokinetics of this potential neuropharmaceutical. Moreover, the use of bifunctional PEG residues, such as that used recently to prepare pegylated immunoliposomes (26), allows for the placement of a biotin moiety at the tip of the PEG tail. Thus, the pegylated BDNF may then be attached to an MAb/avidin delivery system, which has been used in previous studies to facilitate neuropeptide delivery through the BBB (5). These results suggest that neurotrophins may be converted into more effective neuropharmaceuticals through drug delivery strategies that place importance on the dual task of optimizing both plasma pharmacokinetics, (with the use of pegylation technology), and transcellular membrane transport, (with the use of vector-mediated drug delivery systems).

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