0026-895X/07/020385-07$3.00/0

MOLECULAR PHARMACOLOGY, **57**:385–391 (2007).

A Designed Peptidomimetic Agonistic Ligand of TrkA Nerve Growth Factor Receptors

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Received September 2, 1999; accepted November 2, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

A proteolytically stable small molecule β-turn peptidomimetic, termed D3, was identified as an agonist of the TrkA neurotrophin receptor. D3 binds the Ig-like C2 region of the extracellular domain of TrkA, competes the binding of another TrkA agonist, affords selective trophic protection to TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. Agents such as D3 that bind the extracellular domain of Trk receptors will be useful pharmacological agents to address disorders where Trk receptors play a role, by targeting populations selectively.

TrkA is a transmembrane tyrosine kinase receptor with high selectivity for the neurotrophin nerve growth factor (NGF). Related neurotrophins include brain-derived neurotrophic factor (BDNF), which binds TrkB receptors, and neurotrophin-3 (NT-3), which prefers binding to TrkC receptors (Barbacid, 1994).

Docking of TrkA with NGF initiates receptor dimerization, catalytic phosphorylation of cytoplasmic tyrosine residues on the receptor, and a cascade of cell-signaling events (Kaplan and Stephens, 1994). These signals lead to prevention of apoptotic cell death (Maliartchouk and Saragovi, 1997), promotion of cellular differentiation and axon elongation, and up-regulation of choline acetyl transferase (ChAT) (Hefti et al., 1985).

Several neuronal cell types that are implicated in important disease states express TrkA and therefore respond to NGF, including sensory, sympathetic, and cholinergic neurons. It has been suggested that NGF therapy may delay the onset of Alzheimer's disease (Barinaga, 1994; Lindsay, 1996) and ameliorate peripheral diabetic neuropathies (Ebadi et al., 1997). Other applications proposed for NGF include treatment of neuronal damage (Hughes et al., 1997) and targeting of neuroectoderm-derived tumors (Cortazzo et al., 1996; LeSauteur et al., 1996a). For a review of disease targets, see Saragovi and Burgess (1999).

Despite the therapeutic potential of NGF, clinical trials featuring this protein have been disappointing (Verrall, 1994; Saragovi and Burgess, 1999). There are several reasons for this: inherent drawbacks associated with the use of polypeptides applied as drugs (Saragovi et al., 1992), in vivo instability (Barinaga, 1994), and pleiotrophic effects due to activation of signals that were not intentionally targeted (e.g., those mediated via the low-affinity NGF receptor p75 (Carter and Lewin, 1997)). Moreover, the NGF protein is relatively expensive to produce for medicinal applications.

For these reasons, we aimed to design small, proteolytically stable molecules with neurotrophic activity, selective for cells expressing TrkA. However, strategies that result in agonists of tyrosine kinase receptors have not been well established. Previously, we used ligand mimicry and antibody mimicry strategies (Saragovi et al., 1991; Saragovi et al., 1992) to generate peptide analogs of two agonists directed to the extracellular domain (ECD) of TrkA: the natural ligand NGF (LeSauteur et al., 1995; LeSauteur et al., 1996a; Debeir et al., 1999), and monoclonal antibody (mAb) 5C3 (LeSauteur et al., 1996b). TrkA binding is mediated by discrete β-turn regions of these ligands. Only certain cyclic

Supported by a grant of the Medical Research Council of Canada (to H.U.S.) and by National Institutes of Health Grants GM 50772 and CA 82642 (to K.B.), the Advanced Texas Research Program, and The Robert Welch Foundation. H.U.S. is a Scholar of the Pharmaceutical Manufacturer's Association of Canada-Medical Research Council of Canada.

ABBREVIATIONS: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; BOC, tert-butoxycarbonyl; ChAT, choline acetyl transferase; DMF, dimethylformamide; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; FACScan, fluorescent activated cell scanner; FITC, fluorescein isothiocyanate; FMOC, fluorenyloxycarbonyl; MCF, mean channel fluorescence; MTT, 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; NT-3, neurotrophin-3; RIA, radioimmunoassay; TFA, trifluoroacetic acid; Trt, trityl; ECD, extracellular domain; mAb, monoclonal antibody; HEK, human embryonic kidney; BB, binding buffer; OD, optical density.

β-turn analogs were active (Beglova et al., 1998), and other conformers or linear peptides were inactive.

Based on the pharmacophores of the mAb 5C3 and NGF peptide analogs described above, we synthesized a focused β-turn peptidomimetic library of ~60 members. We report the identification of compound D3, a small, selective, and proteolytically stable agonist of the TrkA receptor. Furthermore, the docking site of D3 onto TrkA was studied. Our findings support the notion that a small peptidomimetic ligand can agonize a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. These agents may offer an alternative therapeutic strategy with pharmacological agents that selectively target neuronal populations expressing specific receptors on the cell surface.

Materials and Methods

Preparation of D3 and D3-Biotin. Compound D3 was prepared according to methods previously outlined for related compounds (Feng et al., 1998). fluorenyloxycarbonyl (FMOC)-Gly, FMOC-Hse-(Trt), FMOC-Lys(*tert*-butoxycarbonyl (BOC)), FMOC-Glu(OtBu), then 2-fluoro-5-nitrobenzoyl chloride were coupled (di-iso-propylcarbodiimide activation, 20% piperidine in dimethylformamide (DMF) to remove FMOC groups) to TentaGel S PHB resin at 0.18 mmol/g loading. The supported peptide was treated six times with 1% trifluoroacetic acid (TFA)/4% $HSiiPr_3$ in CH_2Cl_2 for 5 min to remove only the trityl (Trt) protection. Cyclization was effected by treatment with 5.0 equivalents of K_2CO_3 in DMF for 40 h. After 90% TFA/5% H_2O/5% $HSiiPr_3$ cleavage, the final product was purified by reversed-phase HPLC. D3 and its derivatives were soluble in water to 5 mg/ml (the highest concentration tested).

D3-biotin was prepared in the same way as D3, except that after the cyclization the nitro group was reduced by treatment with 10 equivalents of $SnCl_2 \cdot 2H_2O$ in DMF for 20 h. After reduction, FMOC-Gly, then biotin-*N*-hydroxysuccinimide was coupled to the newly formed arylamine. The product was then cleaved from the resin. The final product was purified by reversed-phase HPLC.

Cell Lines. B104 rat neuroblastomas express p75 receptors but do not express any of the Trks ($TrkA^-$ $p75^+$). The 4-3.6 cells are B104 cells stably transfected with human TrkA cDNA and express equal levels of p75 and TrkA ($TrkA^+$ $p75^+$) (kindly provided by Dr. E. Bogenmann) (Maliartchouk and Saragovi, 1997). Surface expression of each of NGF receptor was routinely controlled in all cells by quantitative fluorescence-activated cell sorter scanner (FACScan) assays (Becton Dickinson, CA) (data not shown) using anti-TrkA mAb 5C3 and anti-p75 mAb MC192.

Generation of Human TrkA-Rat TrkB Chimeras in Human Embryonic Kidney (HEK) 293 Cells. The IgG-C2 domain of human TrkA was generated by PCR as described (Perez et al., 1995) using unique restriction sites in the primers to allow exchange with the corresponding rat TrkB domain. The chimeric receptors were constructed by subcloning the human TrkA IgG-C2 domain into the corresponding restriction sites of the rat *trkB* cDNA reported in a previous work (Perez et al., 1995). Chimeric constructs (kindly provided by Dr. P. Perez) were confirmed by sequencing and were cloned into the pCDNA3 expression vector that contains a selection gene providing resistance to neomycin (G418; Life Technologies, Rockville, MD). HEK293 cells were transfected using the lipofectamine plus method (Life Technologies), selected with neomycin (0.5 mg/ml), and at least three independent subclones were obtained by limiting dilution techniques (293-TrkB/A-IgC2 chimera). Western blot analysis with polyclonal antibody 203 directed to the Trk intracellular domain (a gift of Dr. D. Kaplan) and cell-surface FACScan analysis with polyclonal antibody 1001 directed to the TrkA-ECD (our unpublished data) indicated that all stable subclones express comparable levels of chimeric receptors (data not shown).

Dissociated Neuronal Dorsal Root Ganglia (DRG) Cultures. Fetal rat DRG primary cultures were established essentially as described (Kimpinski et al., 1997) from Sprague-Dawley day 17 rat embryos. All ganglia were dissected and dissociated first enzymatically with trypsin and then mechanically. Dissociated cells were cultured (10^5 cells/well) in 96-well plates precoated with collagen and grown for a total of 8 days in Neuro Basal Medium containing N2 supplement (Life Technologies), antibiotics, and L-glutamine. These DRG cultures are ~85% TrkA-expressing and are heavily dependent on TrkA signals for survival (Vogelbaum et al., 1998).

Septal Neuronal Cultures. Cell cultures were established from the septal area of 17-day-old rat embryos as described (Hefti et al., 1985). In brief, tissue was incubated in PBS containing trypsin and DNase. Tissue pieces then were mechanically dissociated. After centrifugation, the pellet was suspended in Leibovitz's L-15 medium. Cells were plated onto 96-multiwell Nunc dishes (10^5 cells/well) coated with poly-D-lysine (5 μg/ml). Pure cultures of septal neurons were treated 1 day after plating. Drugs, prepared in medium, were added directly to the cells without changing the initial medium. The incubation continued for 8 days, at which time ChAT activity was evaluated.

D3·TrkA Binding Assays

Direct Binding Studies. Direct binding studies were done as described (Saragovi et al., 1998) using 6 ng/well of recombinant baculovirus TrkA-ECD or control BSA (Fraction V; Boehringer Mannheim, Mannheim, Germany) immobilized onto 96-well microtest plates. Wells were blocked with binding buffer (BB: PBS with 1% BSA) for 1 h. Then, 50 ng/well of biotinylated D3 was added as primary reagent in BB for 40 min in the absence or presence of excess nonbiotinylated D3 as competitor. Wells were washed five times with BB, and horseradish peroxidase-coupled avidin (Sigma, St. Louis, MO) was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity was determined colorimetrically using 2,2-azinobis (3-ethylbenzthiazoline sulfonic acid) (Sigma). The optical density (OD) was measured at 414 nm in a Microplate reader (Bio-Rad, Richmond, CA). Assays were repeated at least three times ($n = 4$).

FACScan binding assays. 4-3.6 cells (2×10^5) in FACScan binding buffer (PBS, 0.5% BSA, and 0.1% NaN3) were immunostained as described (LeSauteur et al., 1996; Saragovi et al., 1998). Saturating anti-TrkA mAb 5C3, or anti-p75 mAb MC192, or control nonbinding IgGs were added to cells for 1 h at 4°C, in the presence or absence of D3 as competitor. Excess primary antibody was washed off, and cells were immunostained with fluorescinated goat-anti-mouse IgG secondary antibody. Cells were acquired on a FACScan, and mean channel fluorescence (MCF) of bell-shaped histograms were analyzed using the LYSIS II program.

Binding competition. Binding competition studies were as described for direct binding assays to TrkA-ECD, except that as primary reagent 50 ng anti-TrkA mAb 5C3/well were added in BB, in the presence or absence of D3 or controls as competitors as described (Saragovi et al., 1998). Wells were washed five times with BB, and horseradish peroxidase-coupled goat anti-mouse was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity were determined. Assays were repeated at least three times ($n = 4$).

Cell Survival Assays

Primary DRG Cultures. After a total of 8 days of culture with the indicated test or control ligands, cell survival were studied using the 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide colorimetric (MTT) assay and by microscopic observation as described (Debeir et al., 1999).

Cell lines. A total of 5,000 cells/well in protein-free media (PFHM-II; Life Technologies) containing 0.2% BSA (crystalline fraction V; Sigma) were seeded in 96-well plates (Falcon, Mississauga, Ontario,

Canada). The cultures were untreated or treated with the indicated test or control ligands. Cell viability was quantitated using the MTT assay after 56 to 72 h of culture, as described (Maliartchouk and Saragovi, 1997). Percent protection was standardized from OD readings relative to optimal NGF (1 nM) = 100%. The OD of untreated cells were subtracted. The higher OD of untreated primary cultures is likely due to cellular heterogeneity and to endogenous production of limiting amounts of growth factors.

Measurement of ChAT Activity. At day 8 of culture, the medium was aspirated, and ice-cold lysis buffer (10 mM sodium phosphate, pH 7.4/0.1% Triton X-100) was added. ChAT activity assays were performed directly in the wells using Fonnum's method (Fonnum, 1975).

Detection of Putative TrkA·TrkA Homodimers. Live 4-3.6 cells suspended in PBS were treated with the indicated ligand(s) for 40 min at 4°C to allow binding. Cells were then washed in PBS, cross-linked with the membrane impermeable cross-linker disuccinimidyl suberate (Pierce, Rockford, IL; 1 mM, 15 min at 15°C). Unreacted disuccinimidyl suberate was quenched with 5 mM ammonium acetate. Then cells were either lysed directly in SDS sample buffer (whole-cell lysate) or lysed in nonionic detergent Nonidet P-40 and immunoprecipitated with anti-Trk or anti-p75 antibodies as described (LeSauteur et al., 1996b). Similar results were obtained with either method. For Western blot analysis, equal amounts of protein or cell equivalents for each sample were resolved in a 5 to 10% SDS-polyacrylamide gel electrophoresis gradient, transferred to nitrocellulose membranes (Xymotech Biosystems, Montréal, Quebec, Canada), and blotted with anti-Trk polyclonal antibody 203 that recognizes the intracellular domain of Trk. Blots were visualized using the enhanced chemiluminescence system (New England Nuclear, Boston, MA).

Results

Synthesis of Focused β-Turn Peptidomimetic Libraries. A solid-phase synthesis was developed to yield a macrocyclic ring with the i + 1 and i + 2 residues of a β-turn in the appropriate conformation. Approximately 60 compounds of this type were prepared (Feng et al., 1998), with amino acid side chains incorporated to correspond to β-turns of NGF and mAb 5C3 implicated in docking to TrkA (LeSauteur et al., 1995; LeSauteur et al., 1996a,b; Debeir et al., 1999). TrkA binding is mediated by discrete β-turn regions of these ligands. Cyclic peptide β-turn analogs of NGF and of mAb 5C3 were active only in the appropriate conformation (Beglova et al., 1998).

Figure 1 shows the molecular structure of D3 and that of a similar, but inactive, molecule called C59. C59 was used as a negative control. A biotinylated form of D3, termed D3-biotin, was synthesized to carry out direct binding studies to TrkA. All ligands were highly soluble in physiological buffers and did not require organic solvents.

D3 Is a Selective Ligand of TrkA. FACScan analysis featuring the secondary fluorescent agent avidin-fluorescein isothiocyanate (FITC) was used to detect binding of D3-biotin to the cell surface (Table 1). The 4-3.6 cells ($p75^+$ $TrkA^+$) had fluorescence approximately four times greater for D3-biotin than for a background control peptide-biotin. Moreover, a 10-fold molar excess of D3 abolished binding of D3-biotin. In contrast, no specific binding was measured for B104 cells ($p75^+$ $TrkA^-$). Because 4-3.6 cells are B104 cells stably transfected with *TrkA* cDNA and these cell lines are otherwise identical, the data indicate that D3-biotin and D3 bind cell-surface TrkA.

Similar binding data for D3-biotin was obtained by enzyme-linked immunosorbent assay (ELISA) using pure soluble TrkA-ECD produced in baculovirus (data not shown, also see Table 3). These data further indicate that D3 binds to the ECD of TrkA and that membrane lipids are not required.

D3 Binds Within an Agonistic Site of TrkA. Previously, mAb 5C3 was shown to act as a full TrkA agonist. Monoclonal antibody 5C3 binds with K_d 2 nM (LeSauteur et al., 1996b) at an epitope within the IgC2 domain of TrkA near the NGF binding site. This site is postulated to define a receptor "hot spot" (Wells, 1996). We tested whether D3 and mAb 5C3 bind to overlapping receptor sites.

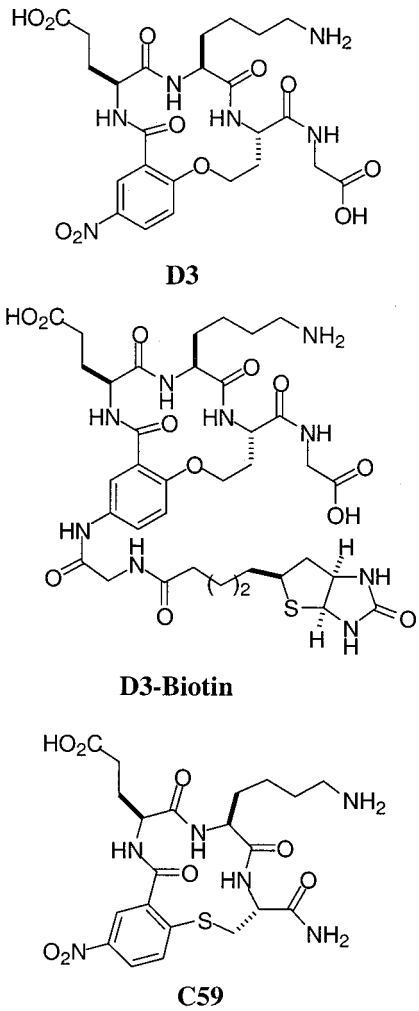

Fig. 1. Structures of D3, D3-biotin, and C59.

TABLE 1

D3 and D3-biotin bind TrkA

Binding of D3-biotin to B104 cells ($p75^+$ $TrkA^-$) or 4-3.6 cells ($p75^+$ $TrkA^+$) was quantitated by FACScan analysis. Ligands are control-biotin (an inactive biotinylated peptide) (row 2), D3-biotin (row 3), or D3-biotin with a 10-fold molar excess of D3 (row 4). All ligands were followed with avidin-FITC as a fluorescent label. Data shown are MCF of bell-shaped histograms, 5000 events acquired. MCF data ± S.E.M. are averaged from three independent experiments.

Ligand	MCF	
	B104	4-3.6
Untreated	10 ± 3	13 ± 2
Control-biotin 20 μM	11 ± 1	10 ± 3
D3-biotin 20 μM	10 ± 4	53 ± 4
D3-bio 20 μM + D3 200 μM	11 ± 2	17 ± 7

Two related assays tested the ability of D3 to compete for the binding of the full TrkA agonist mAb 5C3. In the first test, a FACScan-based assay using intact cells, D3-induced a dose-dependent competitive decrease of mAb 5C3·TrkA interactions (Table 2, rows 2–5). On average, D3 exhibited an IC_{50} of 4 μM. From experimental conditions, we estimate a K_d ~2 μM for D3·TrkA interactions. Blocking of 5C3·TrkA interactions by D3 is selective because the binding of mAb MC192 directed to the p75 NGF receptor subunit was not blocked (Table 2, rows 7 versus 8). Furthermore, inactive control C59 peptidomimetic did not inhibit the binding of either mAb 5C3 (Table 2, row 6) or mAb MC192 (data not shown).

The second test used purified recombinant TrkA-ECD immobilized onto ELISA plates to assay competitive blocking of 5C3·TrkA-ECD by D3. D3 exhibited a dose-dependent inhibition of 5C3·TrkA-ECD interactions, but control inactive C59 peptidomimetic had no effect (Table 3). Because a K_d ~2 nM was measured for 5C3·TrkA interactions, from the experimental IC_{50} a K_d ~2 μM was calculated for D3·TrkA-ECD interactions. This calculation is consistent with the data shown in Table 2. Interestingly, similar ELISA and radioimmunoassay (RIA) binding assays revealed that D3 did not substantially block NGF·TrkA-ECD interactions (data not shown).

D3 Affords Trophic Activity Selectively via TrkA, and Is Proteolytically Stable. Because D3 binds at or near an agonistic site of TrkA, trophic effects were probed in cell survival assays using the quantitative MTT method (Maliartchouk and Saragovi, 1997). Several doses of D3 were tested. However, for clarity only near optimal concentrations are shown, which approximate the estimated K_d.

Dissociated primary neuronal cultures from fetal DRG are dependent on TrkA agonists for survival (Vogelbaum et al., 1998). Exogenous NGF showed a dose-dependent trophic effect (Table 4, rows 2–4). D3 alone had a significant protective effect on DRG cultures (Table 4, row 5), but control C59 did not (Table 4, row 6). Primary cultures are heterogeneous and low levels of neurotrophins are made endogenously (Kimpinski et al., 1997), which explains a relatively high OD for untreated cultures (Table 4, row 1).

Because D3 does not block NGF binding, potential synergy between NGF and D3 was assessed. D3 combined with different concentrations of exogenous NGF demonstrated an additive or potentiating effect on DRG survival (Table 4, rows 7–9).

Similar results were obtained with other neuronal cell lines, wherein D3 potentiated the effect of low NGF concentrations (Table 5). Optimal protection of 4-3.6 cells ($p75^+$ $TrkA^+$) and HEK293-TrkB/A-IgC2 chimeras corresponded to treatment with 1 nM NGF (Table 5, row 2), whereas 10 pM NGF gave significantly less protection (Table 5, row 3). D3 alone afforded low but significant protection (Table 5, row 4), and protection was enhanced with a combination of 10 pM NGF plus 10 μM D3 (Table 5, row 6). The negative control C59 compound had no effect alone or in enhancing 10 pM NGF (Table 5, rows 5 and 7).

In other controls (data not shown), neither D3 nor NGF protected B104 cells, wild-type HEK293 cells, or TrkB-expressing HEK293 cells from apoptosis. Hence the trophic

TABLE 2

D3 specifically blocks mAb 5C3 binding to cell-surface TrkA

4-3.6 cells were analyzed by FACScan for binding of anti-TrkA mAb 5C3 or anti-p75 mAb MC192. Cells exposed to control primary mouse IgG with or without 40 μM D3 afford identical background staining (data not shown). For each condition, 5000 cells were acquired. Percentage maximal bindings were calculated from the MCF of bell-shaped histograms, using the formula ($TEST_{MCF}$ − $background_{MCF}$) × 100/($MAXIMAL_{MCF}$ − $background_{MCF}$). MCF ± S.E.M. are averaged from three independent experiments.

	mAb (1 nM)	Competitor	Dose (μM)	% Maximal Binding
1	5C3	None	0	100 ± 0
2	5C3	D3	0.20	95 ± 4
3	5C3	D3	1	80 ± 3
4	5C3	D3	5	53 ± 5
5	5C3	D3	40	33 ± 4
6	5C3	C59 control	40	97 ± 6
7	MC192	None	0	100 ± 0
8	MC192	D3	40	101 ± 2

TABLE 3

D3 inhibits 5C3 · TrkA interactions in vitro

The binding of mAb 5C3 (at constant 2 nM) to purified TrkA-ECD immobilized onto ELISA plates was measured in the absence or presence of competitors. Background (<2%) was the OD of wells with all reactants except immobilized TrkA-ECD. Data are averaged from three experiments (n = 1).

	Competitor Added	Concentration (μM)	% Binding ± S.E.M.
1	–	–	100 ± 3
2	D3	0.05	100 ± 14
3	D3	0.2	89 ± 8
4	D3	1	64 ± 10
5	D3	5	43 ± 12
6	D3	20	38 ± 7
7	D3	40	31 ± 4
8	C59	40	96 ± 9

TABLE 4

D3 protects TrkA-expressing primary neurons from apoptosis and potentiates NGF

NGF-dependent primary neuronal cultures from embryonic rat DRGs were treated with the indicated ligands for a total of 8 days. Cell survival was measured by MTT assays. Protection was calculated relative to optimal NGF (1 nM, 100% protection) with subtraction of the OD of untreated cells. Shown is the OD from one experiment, mean ± S.E.M. (n = 1). Percentage protection was averaged from three experiments.

Treatment	OD	% Protection
1 Untreated	256 ± 15	0 ± 2
2 NGF 1 nM	823 ± 28	100 ± 4
3 NGF 20 pM	316 ± 11	9 ± 1
4 NGF 500 pM	535 ± 19	68 ± 3
5 D3 10 μM	405 ± 22	38 ± 2
6 Control C59 10 μM	271 ± 8	0 ± 1
7 D3 10 μM + NGF 20 pM	471 ± 28	48 ± 3
8 D3 10 μM + NGF 500 pM	603 ± 26	84 ± 3
9 D3 10 μM + NGF 1 nM	977 ± 38	120 ± 7

TABLE 5

D3 potentiates NGF in protecting TrkA-expressing cell lines from apoptosis by binding to the IgC2 domain of the receptor

4-3.6 cells or HEK293 cells expressing TrkB/TrkA IgG-C2 chimeric receptor were treated with the indicated ligands for a total of 72 h. Survival was measured by MTT assays. Percentage protection was calculated as in Table 4. Shown is the OD from one experiment, mean ± S.E.M. (n = 4). Percent protection was averaged from six (4-3.6 cells) or three (293-IgG-C2 chimera) independent experiments.

Treatment	4-3.6 Cells		HEK293-TrkB/TrkA Chimera	
	OD	% Protection	OD	% Protection
1 Untreated	64 ± 7	0 ± 2	32 ± 5	0 ± 4
2 1 nM NGF	412 ± 24	100 ± 6	350 ± 12	100 ± 4
3 10 pM NGF	205 ± 19	40 ± 5	88 ± 8	18 ± 5
4 10 μM D3	95 ± 9	8 ± 2	69 ± 7	9 ± 3
5 10 μM C59	76 ± 4	2 ± 1	30 ± 7	−1 ± 2
6 10 μM D3 + 10 pM NGF	255 ± 14	55 ± 3	165 ± 11	42 ± 5
7 10 μM C59 + 10 pM NGF	209 ± 17	41 ± 4	90 ± 9	21 ± 6

activity of NGF and D3 require TrkA expression, or at least the IgG-C2 domain of TrkA. Additionally, D3 did not enhance the trophic effect of epidermal growth factor, suggesting that it may be NGF selective. Lastly, D3 enhanced NGF protection of NIH3T3 cells stably transfected with *TrkA* cDNA (data not shown), but did not enhance NT-3 protection of NIH3T3 cells stably transfected with *trkC* cDNA. These data indicate that D3 selectively accentuates the trophic effect of NGF, and that p75 expression is not required.

The proteolytic stability of D3 versus trypsin and papain was assessed. D3 was first exposed to enzymatic treatment as described previously (Saragovi et al., 1991; Saragovi et al., 1992), followed by gauging its biological activity on 4-3.6 cells. Compound D3 remained fully active in trophic assays even after 1 h of exposure to trypsin or pepsin, whereas NGF lost all activity within minutes under the same conditions (data not shown).

D3 Induces Differentiation of Primary Cultures of Fetal DRG and Fetal Septal Neurons. The effect of D3 on TrkA-mediated cellular differentiation was assessed using two independent assays: morphometric analysis of DRG dissociated neurons and induction of ChAT activity in septal neuronal cultures. In the first of these assays, data indicate that DRG neuronal cultures undergo neurite outgrowth in response to D3, and that D3 potentiates the effect of NGF (Fig. 2). In the second assay, ChAT activity was found to increase in response to NGF (Table 6, rows 1 and 2) and to D3 alone (Table 6, rows 3–5), whereas C59 control had no effect (Table 6, row 6). Increases in ChAT activity in response to 2 μM D3 alone were comparable with 10 pM exogenous NGF. Moreover, combinations of 2 μM D3 plus 10 pM NGF markedly increased ChAT activity and were more effective than 400 pM NGF (Table 6, rows 8–10).

D3 Enhances or Stabilizes Putative TrkA·TrkA Homodimers. Based on the data above, it was expected that D3 would induce or stabilize TrkA·TrkA interactions. This hypothesis was studied biochemically in 4-3.6 cells exposed to ligands, followed by cell-surface chemical cross-linking (Fig. 3).

The expected doublet consistent with previously reported TrkA monomers of p110 and p140 were seen in all samples (Fig. 3, thick arrow). Bands of ~300 kDa, consistent with the molecular mass of TrkA·TrkA homodimers (Fig. 3, thin arrow), were seen in samples from cells treated with TrkA ligands 1 nM NGF, 10 pM NGF, or 10 pM NGF plus 10 μM D3 and was also detected (albeit very more weakly) in cells treated with 10 μM D3 alone. The intensity of the 300-kDa band, presumed to be TrkA dimers, was analyzed densitometrically from four independent experiments standardized to 1 nM NGF (100%). There was a consistent increase in dimers after treatment with D3 alone (21 $\pm$ 4%) or 10 pM NGF alone (52 $\pm$ 6%), which was higher after treatment with 10 pM NGF plus 10 μM D3 (77 $\pm$ 7%). Control cells cross-linked in the absence of ligand or cells exposed to ligand but not-cross-linked (data not shown) did not have putative dimers.

TrkA homodimers are stable to SDS denaturation because of covalent cross-linking. Given that the efficiency of chemical cross-linking is ~1 to 4% of the total TrkA pool, we were precluded from further biochemical characterization of the complexes, other than the fact that they contain TrkA. The complexes may contain cross-linked NGF. However, it is unlikely that the bands comprise p75 because immunoprecipitations with anti-p75 antibodies did not reveal any material in the molecular mass of TrkA homodimers (data not shown). Furthermore, material of 215 kDa that would comprise p75-TrkA heterodimers was not seen consistently.

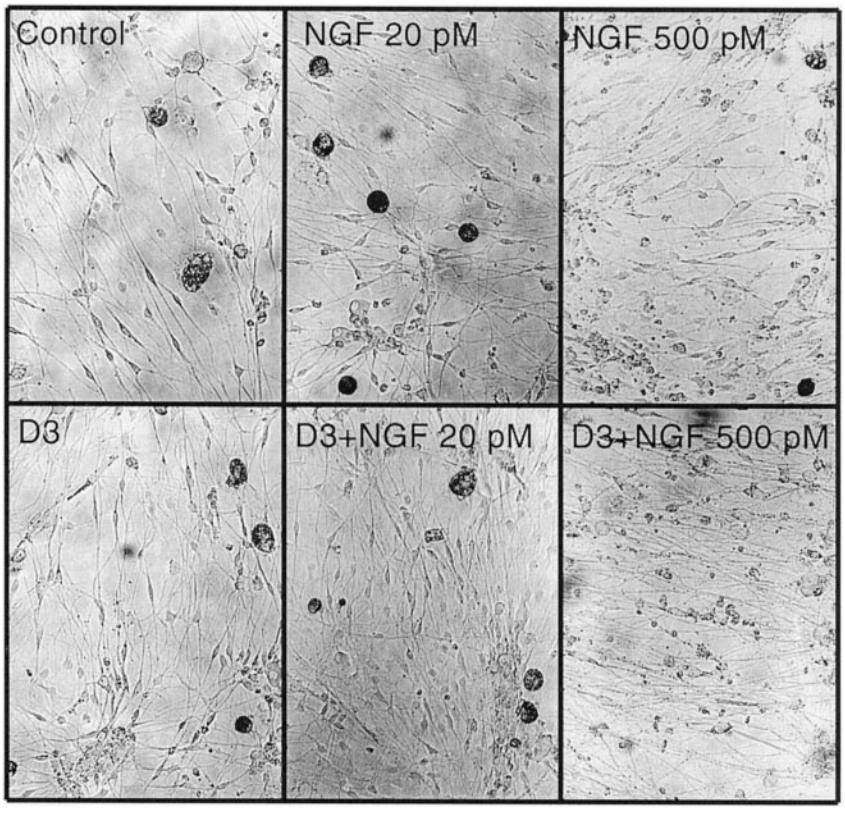

Fig. 2. D3 induces the differentiation of embryonic DRG cultures. Primary neuronal DRG cultures were treated as indicated for 8 days, and cell differentiation was studied morphometrically. Magnification, 60×. Pictures representative of three independent experiments.

Discussion

We report on a proteolytically stable β-turn peptidomimetic small molecule agonist of the TrkA neurotrophin receptor. We showed that D3 binds TrkA, competes the binding of the TrkA agonist mAb 5C3, selectively potentiates trophic protection of TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand.

Recent advances in ligand mimicry have resulted from screening large phage or peptide libraries (Reddy et al., 1996; Wrighton et al., 1996), natural products (Zhang et al., 1999), or chemical libraries (Owolabi et al., 1999). However, most of the ligands described are antagonists, or otherwise require the dimerization of relatively large peptides, have a 2-fold axis of symmetry that resemble a dimer, or are poorly soluble in physiological buffers. In contrast, D3 is a small, nonsymmetrical, proteolytically stable, highly water soluble peptidomimetic that binds the ECD of TrkA.

Recently, a symmetrical alkaloid-like molecule screened from fungi was found to potentiate, at micromolar concentrations, the action of insulin presumably by binding near the catalytic domain of insulin receptors (Zhang et al., 1999). Hence, with regards to agonistic activity and optimal concentration our compound D3 is analogous to the insulinomimetic ligand. In addition, binding and ligand competition studies demonstrate selective interaction of D3 with the ECD of TrkA rather than the catalytic domain. Hence, the water solubility and extracellular targeting of D3 mean that toxic organic solvents are not required to permeate the cell membrane.

Mechanism of Action of D3. It is surprising that D3 is an agonist because the natural ligand NGF is a symmetrical dimer known to activate TrkA via homodimerization (Kaplan and Stephens, 1994). D3 is not a dimer and, from NMR studies, it has no detectable propensity to dimerize even at high millimolar concentrations in solution (data not shown). Then, why does D3 behave as an agonist of TrkA? One hypothesis is that D3 stabilizes the signaling conformation of preformed TrkA homodimers without per se inducing efficient receptor dimerization. This hypothesis is supported by the data because, as would be predicted, exposure to low levels of NGF enhanced D3 activity in bioassays and in receptor cross-linking assays.

TABLE 6

D3 induces ChAT synthesis

Septal neuronal cultures were treated as indicated for a total of 8 days. ChAT activity (pmol Ach/min/well ± S.E.M.) was measured at day 8. Average ± S.E.M. Data averaged from three independent experiments (n = 4).

	Treatment	ChAT Activity	Fold Increase
1	10 pM NGF	0.42 ± 0.07	1.4
2	400 pM NGF	0.72 ± 0.10	2.41
3	0.2 μM D3	0.37 ± 0.05	1.23
4	2 μM D3	0.44 ± 0.02	1.47
5	20 μM D3	0.48 ± 0.06	1.56
6	20 μM C59 control	0.30 ± 0.05	1
7	Untreated	0.31 ± 0.07	1
8	0.2 μM D3 + 10 pM NGF	0.60 ± 0.04	2.00
9	2 μM D3 + 10 pM NGF	0.76 ± 0.03	2.53
10	20 μM D3 + 10 pM NGF	0.79 ± 0.04	2.63

What is the role of picomolar concentrations of NGF? Given the low concentrations used in synergy with D3, it is unlikely that the effect of NGF was mediated by docking with the low-affinity receptor p75. We speculate that NGF acts by increasing TrkA·TrkA interactions whereas D3 stabilizes the homodimers or reduces the rate of separation of receptor homodimers by inducing conformational changes. Indeed, there are precedents for ligands of serpentine receptors acting in this manner (Milligan et al., 1995), and recent models of single transmembrane receptor dimerization and activation are compatible with this view (Tian et al., 1998; Livnah et al., 1999; Remy et al., 1999).

In the present study, the biological data shown are with low micromolar concentrations of D3, which are optimal. As expected from the affinity estimated for TrkA·D3 interactions, lower D3 concentrations afford lower efficacy. It is noteworthy that whereas NGF·TrkA affinity is $\sim 10^{-11}$ M, optimal activity requires 2 nM NGF concentrations. Hence, D3 is optimal at concentrations that approximate its K_d, whereas NGF is optimal at concentrations ~100-fold over its K_d. We interpret this difference to mean that D3 is more stable in solution, and this notion is supported by D3 resistance to proteolysis.

Ligand Binding Sites. D3 competitively blocks the binding of mAb 5C3, but it does not block NGF. Moreover, the optimal agonistic activity of mAb 5C3 (Maliartchouk and Saragovi, 1997) was inhibited by D3 in a dose-dependent manner (data not shown), whereas the agonistic effect of NGF was enhanced. These results are intriguing because previously we reported that mAb 5C3 can block ~50% of the NGF binding sites on a cell expressing TrkA, whereas NGF can block ~25% of the mAb 5C3 binding sites (LeSauteur et al., 1996b). It is unlikely that D3 does not block NGF because of affinity differences, because NGF·TrkA-ECD and 5C3·TrkA-ECD interactions are both in the nanomolar range.

Two factors could account for this result. First, both mAb 5C3 and D3 dock onto a single and continuous epitope within the IgG-C2 domain of TrkA, whereas NGF binds a discontin-

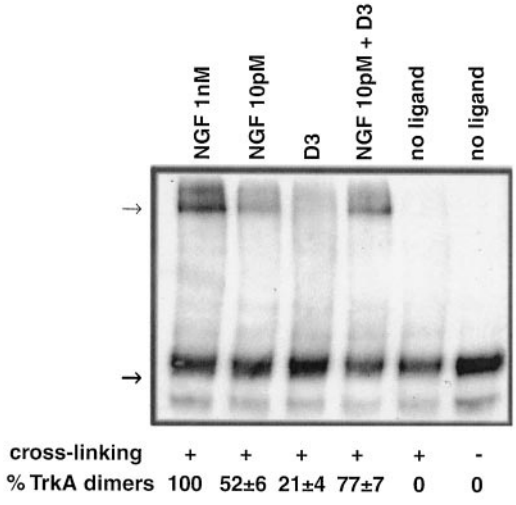

Fig. 3. D3 enhances cell-surface TrkA·TrkA homodimers. 4-3.6 cells were exposed to TrkA ligands as per Table 5 (*lanes 1–4*) or no ligand (*lanes 5 and 6*) and chemically cross-linked (*lanes 1–5*) or not cross-linked (*lane 6*). Cell lysates were Western blotted with anti-TrkA 203 antisera. The intensity of the 300-kDa band was analyzed densitometrically from four experiments standardized to 1 nM NGF.

uous epitope within the IgG-C1 and IgG-C2 domains of TrkA (Perez et al., 1995), and at least one other domain (Windisch et al., 1995). This would facilitate mAb 5C3 blocking by D3, whereas NGF could bind via its second docking site. Second, mAb 5C3 and NGF bind TrkA at sites partially overlapping but not identical (LeSauteur et al., 1996b). Hence, the data suggest that D3 binds TrkA at an epitope overlapping the agonistic mAb 5C3 "hot spot" of the IgG-C2 domain of TrkA, near the NGF docking site. These observations may account for D3 synergizing with NGF and blocking mAb 5C3.

The fact that D3 is bioactive and was selected from a relatively small pool of β-turn-based compounds has broad implications for many research initiatives involving protein-protein interactions. Other small molecules with neurotrophic activity have been reported (Steiner et al., 1997; Maroney et al., 1998). However, the molecular targets of these ligands are ubiquitous intracellular proteins, and the mechanisms of action are often unclear. Thus, these other molecules are not Trk ligands, and are not defined as peptidomimetics of known ligands.

In contrast, we report a small molecule peptidomimetic that binds and activates TrkA. In the present report of D3, we show that a hybrid of a peptide and a small organic molecule designed to hold key amino acid residues in a turn conformation within a small framework offers a means to transform a peptide lead into an active organic small molecule. Hence, D3 represents the validation of the peptidomimetic concept for the Trk family of tyrosine kinase receptors. This small molecule peptidomimetic ligand of TrkA that has neurotrophic activity may be useful to address neurodegenerative disorders, pain, neoplasias, and other pathologies (reviewed by Saragovi and Burgess, 1999) where TrkA receptors play a role.

Acknowledgments

We are grateful to Drs. E. Bogenmann (Children's Hospital of Los Angeles), D. R. Kaplan (McGill University, Canada), P. Perez (Universidad de Salamanca, Spain), and K. Mearow (Memorial University, Canada) for reagents and discussions; and to Y. Mawal, and M. C. Zaccaro for technical help.

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