Chemistry & Biology, Vol. 12, 1015–1028, September, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.06.015

Selective Small Molecule Peptidomimetic Ligands of TrkC and TrkA Receptors Afford Discrete or Complete Neurotrophic Activities

Maria Clara Zaccaro,[1] Hong Boon Lee,[2] Mookda Pattarawarapan,[2,7] Zebin Xia,[2] Antoine Caron,[1] Pierrre-Jean L'Heureux,[3] Yoshua Bengio,[3] Kevin Burgess,[2] and H. Uri Saragovi[1,4,5,6,*]

[1]Department of Pharmacology and Therapeutics
McGill University
Montreal, Quebec
Canada

[2]Department of Chemistry
Texas A&M University
Box 30012
College Station, Texas 77843

[3]Department d'Informatique et de Recherche Operationalle
University of Montreal
Montreal, Quebec
Canada

[4]Oncology/Cancer Center
University of Montreal
Montreal, Quebec
Canada

[5]Bloomfield Center for Cognitive Disorders
McGill University
Montreal, Quebec
Canada

[6]Lady Davis Institute—Jewish General Hospital
3755 Cote St. Catherine, Room F-223
Montreal, Quebec H3T 1E2
Canada

Summary

We designed a minilibrary of 55 small molecule peptidomimetics based on β-turns of the neurotrophin growth factor polypeptides neurotrophin-3 (NT-3) and nerve growth factor (NGF). Direct binding, binding competition, and biological screens identified agonistic ligands of the ectodomain of the neurotrophin receptors TrkC and TrkA. Agonism is intrinsic to the peptidomimetic ligand (in the absence of neurotrophins), and/or can also be detected as potentiation of neurotrophin action. Remarkably, some peptidomimetics afford both neurotrophic activities of cell survival and neuronal differentiation, while others afford discrete signals leading to either survival or differentiation. The high rate of hits identified suggests that focused minilibraries may be desirable for developing bioactive ligands of cell surface receptors. Small, selective, proteolytically stable ligands with defined biological activity may have therapeutic potential.

*Correspondence: Uri.Saragovi@mcgill.ca
[7]Present address: Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand.

Introduction

Neurotrophins are dimeric polypeptide growth factors that regulate the peripheral and central nervous systems and other tissues [1, 2]. Neurotrophins and their receptors are validated targets for therapeutics in a variety of pathologies, ranging from cancer to neurodegeneration [1, 3–5]. However, therapeutic uses of the neurotrophins have failed because they cause full receptor activation, which leads to toxicity and pleiotrophic effects, short half-lives, and inability to reach target tissues [6, 7].

Neurotrophic activities arise from ligand binding to two types of cell surface receptors. The Trk family of receptors are tyrosine kinases. Ligand binding induces phosphorylation (pTyr) of TrkA and associated signaling partners, and activation of the "traditional" neurotrophic biological signals. These signals promote growth or survival under stress (trophic activity) and cellular differentiation (neuritogenic activity).

The Trk receptors are selective and of high affinity for neurotrophins ($\sim K_d$ 10^{-11} M). Nerve growth factor (NGF) docks with TrkA, neurotrophin-3 (NT-3) interacts with TrkC, but can also bind TrkA, and brain-derived neurotrophic factor (BDNF) interacts with TrkB [8]. The Trk extracellular domain consists of 5 subdomains: the *N*-terminal leucine-rich motif (LRM), two cysteine-rich clusters, and two imunoglobulin (Ig)-like subdomains, IgC1 and IgC2 (also respectively known as D4 and D5) [9]. The D5 subdomain is the main site for NGF/TrkA, BDNF/TrkB, or NT-3/TrkC binding and functionality [10]. The D4 subdomain regulates the levels of receptor autophosphorylation, and its mutagenesis can result in oncogenic autoactivation [10–13]. The LRM subdomain is another activation site that mediates NGF/TrkA functionality [10]. These regulatory or activation sites define functional "hot spots" of the receptors, and are targets for the development of ligands [14–18] that bind a defined activation or regulatory hot spot (e.g., only the D5 or only the LRM subdomains). We reasoned that ligands binding these hot spots could afford partial receptor activation or regulation, as opposed to full receptor activation.

The p75 receptor is a member of the tumor necrosis factor (TNF) receptor superfamily. All the neurotrophins bind the p75 receptor, but with lesser affinities, varying from low (10^{-9} M) to high (10^{-11} M) [19–21]. Depending on the cellular environment and the ligand available, the p75 receptor can transduce signals that are either prosurvival, proapoptotic, or prodifferentiation [22, 23]. The p75 receptor also regulates Trk function and Trk ligand docking preference [9, 10]. These features make p75 biology extremely complex and the behavior of p75 ligands difficult to predict. Consequently, we reasoned that it would be desirable to develop peptidomimetic ligands that target Trk receptors and exclude p75.

Previously, we used protein mimicry to develop disulfide-linked cyclic peptides that bind TrkA as agonists or antagonists [24–27]. The peptides adopt β-turn conformations similar to regions of the proteins from which

they were designed [28, 29]. One early lead β-turn peptidomimetic ligand, termed D3, is a partial agonist selective for TrkA. D3 is proteolytically stable, it induces TrkA-TrkA homodimers, potentiates the effect of NGF in activating the receptor [26], and induces TrkA tyrosine phosphorylation. However, D3 has low intrinsic agonistic activity at TrkA, and it could be best described as an NGF potentiator, synergistically enhancing NGF action. Thus, we sought to design improved agents and applied the mimicry concepts that evolved from developing earlier NGF mimetics [30] to the neurotrophin family member NT-3.

We designed a library of peptidomimetics based on β-turn NT-3 structure, with the rationale of targeting the extracellular domain of the TrkC and TrkA receptors, and perhaps excluding p75. The design also aimed to enhance the intrinsic agonistic activity of the peptidomimetics and to reduce the size and the peptidic character of the compounds.

The resulting new peptidomimetics are less peptidic than D3 and express relevant sequences from the β-turn pharmacophores. Conformational and molecular dynamic studies have shown that these new peptidomimetics adopt the desired β-turn configurations and are more rigid than D3 [31–33]. Binding and biological screens lead to the identification of water-soluble, small molecule (mass ~600 Da) agonistic ligands of TrkC and TrkA receptors. A subset of peptidomimetics are selective agonists of TrkC, and another subset are agonists of both TrkC and TrkA. Direct binding assays using fluorescinated peptidomimetics demonstrated binding specificity toward Trk receptors. Also, we show that some peptidomimetics competitively block the binding of other agonists that bind to the Ig-C2 (D5) extracellular hot spot of TrkA or TrkC. Interestingly, some agonistic peptidomimetics can activate both trophic survival and neuritogenic differentiation, and some can activate only one pathway.

Our findings demonstrate that a small molecule peptidomimetic can selectively bind and activate Trk receptors, and that it is possible to transduce either single or multiple signal pathways by engaging hot spots of wild-type receptors. This work may help us understand how the neurotrophins function via Trk receptors, and may be a step forward in the search for small, proteolytically stable molecules with possible therapeutic potential in cognitive (TrkA) or motor neuron (TrkC) disorders. The high rate of success of this approach suggests that similar focused library approaches designed specifically to target receptor hot spots may be useful for identifying functional ligands.

Results

Design and Synthesis of a D3-like Library of Ligands for TrkC and TrkA

We prepared three β-turn backbones, coded **1–3** (Figure 1A), each with four amine substituents X, coded **A–D** (Figure 1B), where **A** = NH_2 (amine); **B** = NH-C(=NH)NH_2 (guanidine); **C** = $NHSO_2CH_3$ (methyl sulfonamide); and **D** = NH-Arg, for a total of 12 possible β-turn templates. In these 12 templates, we incorporated selected dipeptide sequences, coded **a–k** (Figure 1C).

The complete dipeptide library, using the 20 natural protein amino acids, would have comprised 4800 possible combinations (12 × 400), but this was not attempted. Instead, a focused library comprising 11 dipeptides was prepared based on the sequences of the NT-3 (and partly from NGF) β-turn regions [24] (Figure 1B, substituent **C**).

Modeling was based on X-ray crystallographic studies of the free neurotrophins [34–37], and neurotrophins coupled with a Trk receptor fragment [38–40]. Most of the dipeptides were modeled after *i* + 1 and *i* + 2 residues of β-turns (Figure 1C, dipeptides **a–f**). However, a few were also prepared corresponding to *i* to *i* + 1 residues (Figure 1C, dipeptides **h**, **i**, and **j**) and *i* + 2 to *i* + 3 residues (Figure 1C, dipeptides **g** and **k**). The reason for this was 2-fold. First, in solution, β-turns are dynamic (e.g., NGF crystals examined in two different studies show different turn conformations). Second, divergence of solution structures from the solid-state structure are likely when the ligands are docked with the Trk receptors [41]. For this study, there were 55 compounds prepared: **1Aa**, **1Ad**, **1Ba**, **1Bd**, **1Da**, **2Aa**, **2Ab**, **2Ac**, **2Ad**, **2Ae**, **2Af**, **2Ag**, **2Ah**, **2Ai**, **2Ak**, **2Aj**, **2Ca**, **2Cb**, **2Cc**, **2Cd**, **2Ce**, **2Cf**, **2Cg**, **2Ch**, **2Ci**, **2Cj**, **2Ck**, **3Aa**, **3Ab**, **3Ac**, **3Ad**, **3Ae**, **3Ah**, **3Aj**, **3Ak**, **3Ba**, **3Bb**, **3Bc**, **3Bd**, **3Be**, **3Bg**, **3Bh**, **3Bi**, **3Bj**, **3Bk**, **3Ca**, **3Cb**, **3Cc**, **3Cd**, **3Ce**, **3Cg**, **3Ch**, **3Ci**, **3Cj**, and **3Ck**. For comparison, the structure of the first reported [26] peptidomimetic TrkA ligand **D3** is shown (Figure 1D).

Agonism in Trophic Survival Assays

The 55 peptidomimetics were tested for agonism in assays of cell survival, using the quantitative MTT method. Cells cultured in serum-free media (SFM) die by apoptosis. The survival-inducing effect can be measured by the ability of peptidomimetics to protect cells from apoptosis selectively in a receptor-dependent and dose-dependent manner. Also, peptidomimetics were tested in combination with 0.1 nM of the corresponding neurotrophin. This suboptimal concentration of neurotrophins affords suboptimal protection (~25%), and therefore it is possible to measure potentiation of activity where the peptidomimetics enhance survival above 25%. We identified 14 compounds that, at 50 μM or lower concentrations, afford significant ($p \leq 0.01$) intrinsic trophic activity (in the absence of neurotrophin) and/or potentiate the trophic action of 0.1 nM neurotrophin (Table 1).

Peptidomimetics **1Ad** (row 2), **3Ce** (row 9), **3Ck** (row 11), **1Aa** (row 12), and **1Ba** (row 13) have intrinsic trophic activity selective for TrkC. All of these trophic agents, except **1Ad**, can also potentiate the survival-promoting effect of 0.1 nM NT-3 on TrkC. Peptidomimetics that have no intrinsic trophic activity, but which selectively potentiate the survival-promoting effect of 0.1 nM NT-3 on TrkC, include **3Aa** (row 3), **3Ba** (row 5), **3Bg** (row 6), **3Bi** (row 7), and **3Cg** (row 10).

None of the peptidomimetics afford selective trophic protection to TrkA-expressing cells, and none potentiate the effect of 0.1 nM NGF on TrkA.

Some peptidomimetics are trophic for *both* TrkA- and TrkC-expressing cells. These include **3Ak**, **3Ca**, **3Ac**, and **3Ae**. All these peptidomimetics also potentiate the

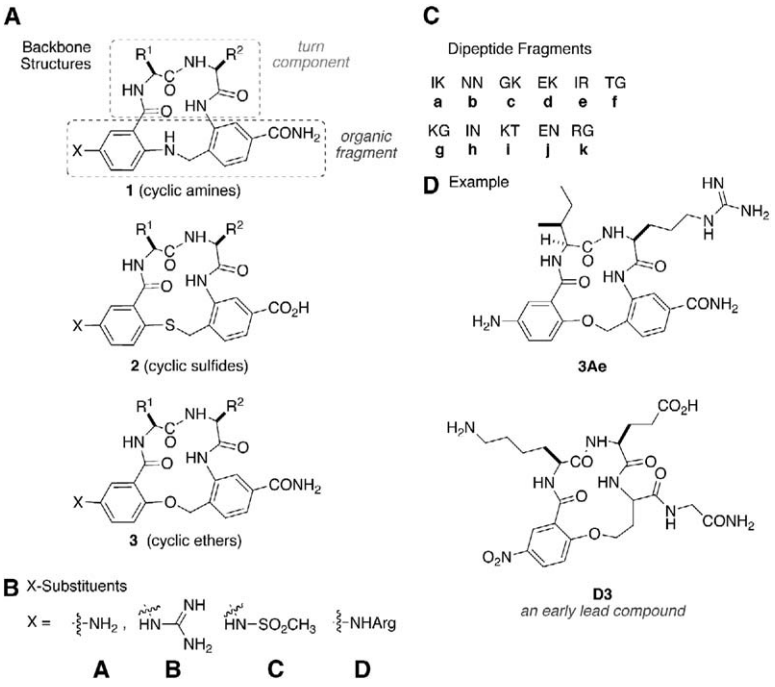


Figure 1. Library and Library Code

(A) Code for numbered β-turn backbones **1**, **2**, and **3**.

(B) Code for capital letter X substituents **A**, **B**, **C**, **D**.

(C) Code for dipeptide (R^1, R^2) substituents.

(D) Example **3Ae**, and published mimetic **D3**.

survival-promoting effect of 0.1 nM NT-3 on TrkC. In control assays, we demonstrated that cell survival is Trk receptor-dependent because wild-type NIH-3T3 cells are not protected from death, and the survival-promoting effect of unrelated growth factors EGF and β-FGF are not affected either (data not shown).

Overall, the activity of the 14 agonistic peptidomimetics could be divided into the following four classes: (1) trophic selectivity toward TrkC, but do not potentiate 0.1 nM NT-3 (**1Ad**); (2) trophic selectivity toward TrkC, and which synergistically potentiate 0.1 nM NT-3 (**3Ce**, **3Ck**, **1Aa**, and **1Ba**); (3) trophic selectivity toward TrkC *only* as potentiators of NT-3 (**3Aa**, **3Ba**, **3Bg**, **3Bi**, and **3Cg**); and (4) trophic agonists of both TrkC and TrkA (**3Ak**, **3Ca**, **3Ac** and **3Ae**). These peptidomimetics potentiate NT-3, but do not potentiate NGF.

In some cases, the potentiation is synergistic and enhances suboptimal NT-3 from ~25% to ~100%, which is optimal and equivalent to 2 nM NT-3 (e.g., **3Ac**, **3Ae**). Four of the 14 agonistic peptidomimetics (**1Aa**, **1Ba**, **3Ac**, and **3Ae**) afford statistically significant trophic survival at concentrations of 10 μM or lower (Table 1).

Signal Transduction and Receptor Activation

We performed receptor pTyr assays after exposing cells to each single ligand for 20 min (peptidomimetics at 50 μM, and NTFs at 0.1 nM and 2 nM) to confirm the agonistic activity and the selectivity of the peptidomimetics toward TrkC or TrkA (Figure 2). Peptidomimetics **1Aa** and **1Ba** induce significant ($p \leq 0.05$) TrkC-pTyr, and do not activate TrkA. Peptidomimetic **3Ac** induces significant ($p \leq 0.05$) TrkC-pTyr and TrkA-pTyr. The data for these three compounds are consistent with the preceding survival assays.

Peptidomimetic **3Ae** induces robust TrkC-pTyr, and does not activate TrkA. These data are inconsistent with the preceding survival assays, in which **3Ae** supports TrkA-mediated survival (Table 1). Likely, the 20-min time point selected for the pTyr assay is not appropriate for detecting TrkA-pTyr induction by **3Ae** (see Kinetics of Activation, below).

Peptidomimetics **1Ad** and **3Ba** also induce significant TrkC-pTyr, as expected. Unexpectedly, **1Ad** and **3Ba** *also* induce significant and robust TrkA-pTyr. TrkA activation was unexpected because **1Ad** and **3Ba** are selectively trophic for TrkC-expressing cells (Table 1).

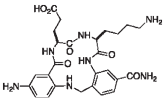
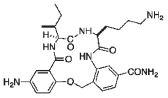
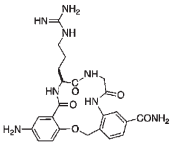
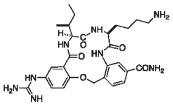
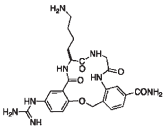
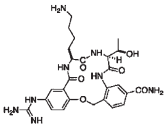
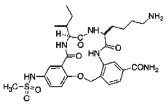
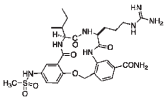
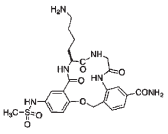
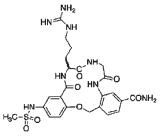
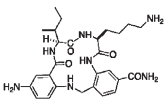
Differentiation Assays

The finding that **1Ad** and **3Ba** induce TrkA-pTyr without affording TrkA-mediated trophic survival leads to the hypothesis that signals could result in neuritogenic differentiation. The rationale for this prediction was that NT-3 activation of TrkA preferentially results in neuritogenesis in some cells [42]. Neuritogenesis by agonistic peptidomimetics (at 50 μM concentrations, in the absence of neurotrophins) was tested in differentiation assays, using PC12 cells ($TrkA^+p75^{+++}$) or nnr5-TrkC ($TrkC^+p75^{+++}$) cells (Figure 3A; summarized in Figure 3B).

Untreated nnr5-TrkC cells are somewhat more differentiated than untreated PC12 cells, but NT-3 can induce their differentiation in a dose-dependent manner. Peptidomimetics **1Aa**, **1Ad**, **3Aa**, **3Ac**, **3Ae**, **3Ba**, and **3Ca** induced significant ($p < 0.01$) differentiation of nnr5-TrkC cells.

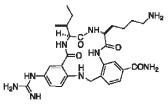
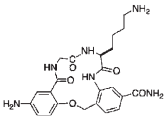
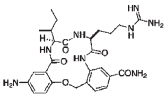
Untreated PC12 cells are undifferentiated, and NGF induces differentiation in a dose-dependent manner. Peptidomimetics **1Ad**, **1Ba**, **3Ac**, and **3Ba** induced significant ($p < 0.01$) differentiation of PC12 cells compared to untreated cells. These data indicate that the TrkA-pTyr induced by **1Ad** and **3Ba** affords neuritogenic differentiation without affording trophic survival.

Table 1. Trophic Protection by Peptidomimetics

	Row	Code	Dose (μM)	NIH-3T3 TrkA Cells		NIH-3T3 TrkC Cells	
				SFM	+NGF low	SFM	+NT-3 low
	1	Untreated		0	**24 ± 2**	0	**26 ± 3**
	2	**1Ad**		–1 ± 1	23 ± 1	**20 ± 1**	38 ± 2
	3	**3Aa**		–4 ± 2	7 ± 2	4 ± 1	**54 ± 1**
	4	**3Ak**		**9 ± 1**	30 ± 1	**16 ± 4**	**90 ± 5**
	5	**3Ba**		2 ± 2	33 ± 2	–1 ±1	**54 ± 4**
	6	**3Bg**		–5 ± 3	19 ± 2	–3 ± 1	**39 ± 1**
	7	**3Bi**		–1 ± 3	2 ± 1	–2 ± 1	**52 ± 1**
	8	**3Ca**		**7 ± 1**	30 ± 2	**12 ± 3**	**76 ± 7**
	9	**3Ce**		2 ± 1	18 ± 1	**7 ± 2**	**69 ± 8**
	10	**3Cg**		1 ± 1	23 ± 1	2 ± 1	**59 ± 3**
	11	**3Ck**		2 ± 1	19 ± 1	**9 ± 2**	**68 ± 5**
	12	**1Aa**	0.4	–5 ± 5	20 ± 4	1 ± 0	29 ± 5
			2	–5 ± 4	20 ± 3	8 ± 1	29 ± 2
			10	–4 ± 5	21 ± 3	13 ± 1	35 ± 3
			50	1 ± 3	23 ± 1	17 ± 2	71 ± 5

(*continued*)

Table 1. Continued

	Row	Code	Dose (μM)	NIH-3T3 TrkA Cells		NIH-3T3 TrkC Cells	
				SFM	+NGF low	SFM	+NT-3 low
	13	**1Ba**	0.4	–3 ± 2	19 ± 2	0 ± 2	32 ± 2
			2	–5 ± 1	22 ± 2	1 ± 3	35 ± 2
			10	8 ± 5	32 ± 1	6 ± 2	44 ± 3
			50	4 ± 3	15 ± 1	11 ± 1	54 ± 5
	14	**3Ac**	0.4	6 ± 3	29 ± 3	0 ± 1	**37 ± 3**
			2	5 ± 1	30 ± 4	–1 ± 1	43 ± 9
			10	7 ± 2	30 ± 8	8± 2	61 ± 26
			50	13 ± 3	33 ± 7	15 ± 3	97 ± 6
	15	**3Ae**	0.4	3 ± 2	27 ± 2	2 ± 1	27 ± 4
			2	6 ± 2	29 ± 1	4 ± 1	34 ± 3
			10	9 ± 1	27 ± 3	10 ± 1	51 ± 7
			50	10 ± 2	27 ± 1	20 ± 3	111 ± 10

Cells were cultured in SFM supplemented with control or peptidomimetics (the concentrations indicated, or 50 μM where not indicated), either with no neurotrophins (SFM) or with 0.1 nM of the corresponding neurotrophin (NGF for TrkA cells; NT-3 for TrkC cells). Survival was measured in MTT assays, and was calculated relative to 2 nM neurotrophin (100% protection). Results shown are average ± SEM (n = 4), from at least three independent experiments. **Bold numbers** indicate statistically significant cell survival compared with the corresponding untreated cells ($p \leq 0.01$).

None of the other agonists in the trophic survival assays induced significant differentiation (e.g., **3Ak**). Only one compound, **3Cg**, was not tested in the differentiation assay.

Kinetics of Activation

We hypothesized that the 20 min time point selected for the pTyr assay may not be always appropriate for detecting TrkA-pTyr by peptidomimetics, although it is an appropriate time point for NGF. For example, **1Ba** affords differentiation of PC12 cells (Figure 3B), but does not afford detectable TrkA-pTyr in NIH-TrkA cells after 20 min of exposure (Figure 2).

Thus, a time course of Trk phosphorylation kinetics was done for **1Ba** (50 μM), using NIH-TrkA ($TrkA^{+++}$) (summarized in Figure 3C), and using NIH-TrkC ($TrkC^{+++}$) and PC12 ($TrkA^{+}p75^{+++}$) cells (Figure 3D). NT-3 and NGF (10 nM) were used as positive controls and to standardize the assay versus untreated (no ligand) negative controls.

On NIH-TrkA cells, **1Ba** activates TrkA with rapid and transient kinetics, whereas NGF activates with rapid and sustained kinetics and with higher potency. **1Ba** exposure induces TrkA-pTyr after 5 min; the activity is still significant after 10 min, but is insignificant after 20 min. Interestingly, on PC12 cells, **1Ba** activates TrkA with rapid and *sustained* kinetics, and with a potency ~35% that of NGF (Figure 3D). On NIH-TrkC cells, both **1Ba** and NT-3 activate TrkC with rapid and sustained kinetics, although the potency of **1Ba** is much lower than that of NT-3 (Figure 3D).

Thus, **1Ba** seems to be a better TrkA agonist for PC12 cells than for NIH-TrkA cells, even though the latter express higher density of TrkA receptors. Because it is possible that coexpression of p75 receptors in PC12 cells may be key in agonism by **1Ba**, this was tested in preliminary studies using NIH-TrkA cells infected with p75-expressing adenoviruses [10]. FACScan phenotyping showed that NIH-TrkA+p75 cells expressed high density of both receptors ($TrkA^{+++}p75^{+++}$) (data not shown). On NIH-TrkA+p75 cells, **1Ba** activates TrkA with rapid and sustained kinetics, and with relatively higher potency (data not shown).

Together, these data suggest that **1Ba** is a TrkA agonist independent of p75 coexpression (e.g., **1Ba** by itself induced TrkA-pTyr in NIH-TrkA cells), but that p75 expression can affect the kinetics of the TrkA signaling by **1Ba**. This could conceivably happen either because p75 is a known regulator of TrkA activity [10, 43], because **1Ba** binds to p75, or it binds to a putative heteromeric TrkA-p75 receptor complex. Additionally, these data suggest that TrkA activation by **1Ba** leads to cellular differentiation only. Indeed, further survival assays using PC12 cells confirmed that **1Ba** did not afford cellular survival (data not shown). Similar kinetic studies were not carried out with **3Ae**, but it is likely that, in analogy to **1Ba**, differential kinetics account for its ability to afford TrkA-mediated survival (Table 1) without detectable TrkA-pTyr after 20 min of exposure (Figure 2).

Direct Receptor Binding Studies

Analogs of some agonistic peptidomimetics were synthesized in their fluorescinated form (peptidomimetic-FITC). These labeled analogs were used in quantitative FACScan assays measuring direct binding to NIH-TrkC cells, NIH-TrkA cells, or negative-control wild-type NIH-3T3 cells to determine Trk-receptor specificity. In addition, NIH-3T3 fibroblasts expressing p75 receptors (NIH-p75) and nnr5 cells were also studied to address the possibility that the labeled peptidomimetics may bind to p75 (data not shown). Anti-TrkA mAb 5C3 and anti-TrkC mAb 2B7 were used as positive controls.

Two examples of FACS profiles (**3Ac-FITC** and **3Aa-**

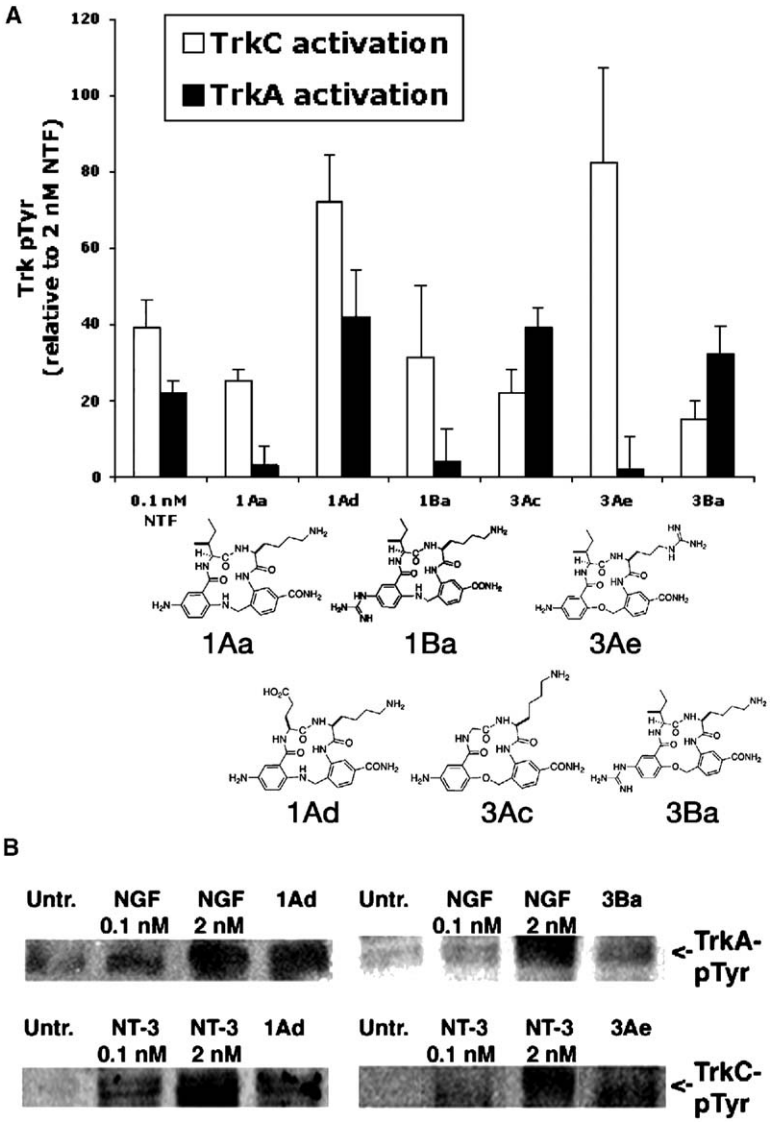


Figure 2. Receptor Tyrosine Phosphorylation Induced by Peptidomimetics

(A) NIH-TrkC or NIH-TrkA cells were exposed to the indicated ligand (peptidomimetics alone at 50 μM or NTFs alone as indicated), and detergent lysates were analyzed by Western blotting with anti-pTyr mAb 4G10. Data were quantified by densitometry, relative to 2 nM NTF, and are presented as average (n = 3) ± SD. NTF = neurotrophin (NT-3 for NIH-TrkC and NGF for NIH-TrkA).

(B) Representative examples of compounds inducing Trk-pTyr.

FITC) are shown in Figure 4. Compound **3Ac-FITC** bound to NIH-TrkA and NIH-TrkC cells, but wild-type NIH-3T3 cells did not bind **3Ac-FITC** (Figure 4A), nor did they bind antireceptor mAbs (data not shown). This compound is bioactive at both TrkA and TrkC, hence the binding data are consistent with the bioassays. Compound **3Aa-FITC** binds only to NIH-TrkC cells and does not bind to NIH-TrkA (Figure 4B) or to wild-type NIH-3T3 cells (data not shown). This compound is bioactive only on cells expressing TrkC, hence binding data are consistent with bioassays.

Table 2 summarizes the direct binding assays with fluoresceinated peptidomimetics. **3Ai-FITC** has no significant TrkA or TrkC binding. This compound was made as a negative control because **3Ai** does not activate TrkA or TrkC in bioassays. **3Aa-FITC** binds only to TrkC. This binding is consistent with TrkC-selective activity in bioassays using the unlabeled parental compound. **3Ac-FITC**, **3Ae-FITC**, **1Ad-FITC**, and **3Ak-FITC** bind TrkC and TrkA. Binding data are consistent with activity in bioassays using the unlabeled parental compounds. **1Aa-FITC** also binds NIH-TrkA and NIH-TrkC cells. In bioassays using the unlabeled parental **1Aa** compound, only TrkC is activated. It is possible that **1Aa** binds TrkA without activating it efficiently, much like NT-3 does [42, 44]. However, we cannot rule out that the FITC adduct could have altered the binding properties of **1Aa** or other compounds.

Other FACScan binding assays versus NIH wild-type, nnr5, and NIH-p75 cells demonstrated that the FITC-labeled peptidomimetics did not bind p75-expressing cells significantly above background (data not shown). Together, these data suggest that the FITC-labeled peptidomimetics (and likely the corresponding unlabeled parental peptidomimetics) do not bind the p75 receptor. If the unlabeled compounds do bind p75, then it must be that the FITC label otherwise blocks their binding. Given the possibility that the FITC label may have prevented p75 binding in the FACS assays, and because there are no reliable and robust functional assays for p75, we could not correlate lack of p75 binding with lack of p75-mediated activity.

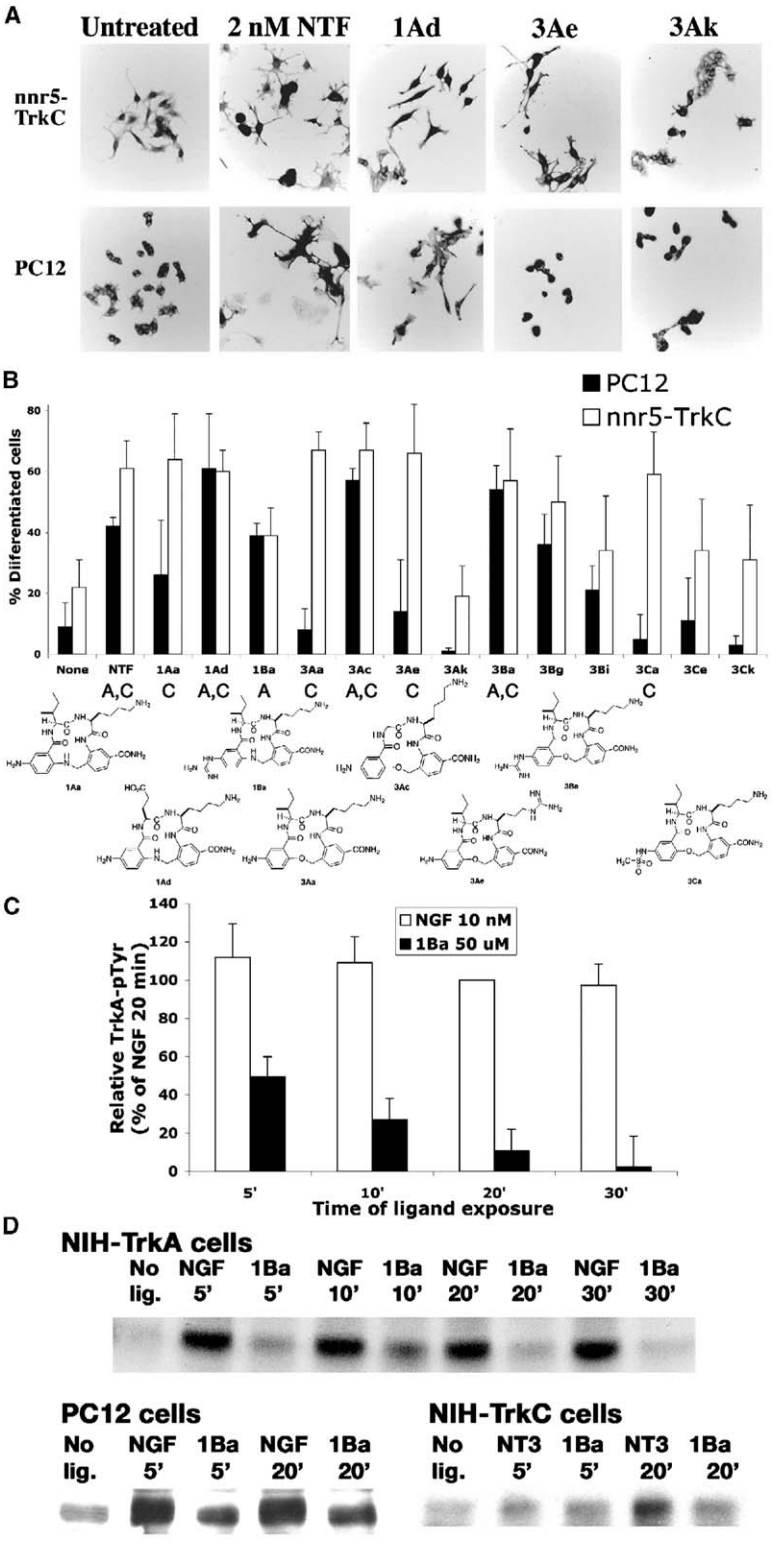


Figure 3. Cellular Differentiation Induced by Peptidomimetics

(A) Representative assays of PC12 (TrkA+) or nnr5-TrkC cells (TrkC+) differentiation. NTF = 0.1 nM NT-3 or NGF.

(B) Quantitative summary (±SD) and statistical analyses. The letters under the graph (A; C; and A,C) indicate statistically significant differentiation compared with untreated ($p \leq 0.01$). Note that the untreated group (labeled "none") in each cell line has a different background. Cells were plated with ligands or controls, and differentiation 3 days after plating was determined as percent of cells with neurites (>2 cell bodies long). Four independent plates were scored, with an average cell count of 203 ± 33 per condition.

(C) Summary of kinetic studies of TrkA-pTyr by **1Ba** in NIH-TrkA cells (n = 7 independent gels standardized to protein loading ±SD).

(D) Examples of TrkA-pTyr or TrkC-pTyr Western blots for NIH-TrkA cells, PC12 cells, or NIH-TrkC cells.

Indirect Receptor Binding Studies

Ligand competition assays were used to further define peptidomimetic binding to Trk receptors. MAb 5C3 is an agonistic antibody directed to the TrkA-IgC2 hot spot, and mAb 2B7 is an agonistic antibody directed to the TrkC-IgC2 hot spot. The agonistic peptidomimetics were tested for competitive block of mAb·hot spot interactions in binding assays.

Cells were treated with neurotrophins or peptidomimetics, followed by labeling with fluorescein-tagged 5C3 or 2B7 mAbs. Analyses were done by FACScan, and percent inhibition of binding (average ± SD, n =

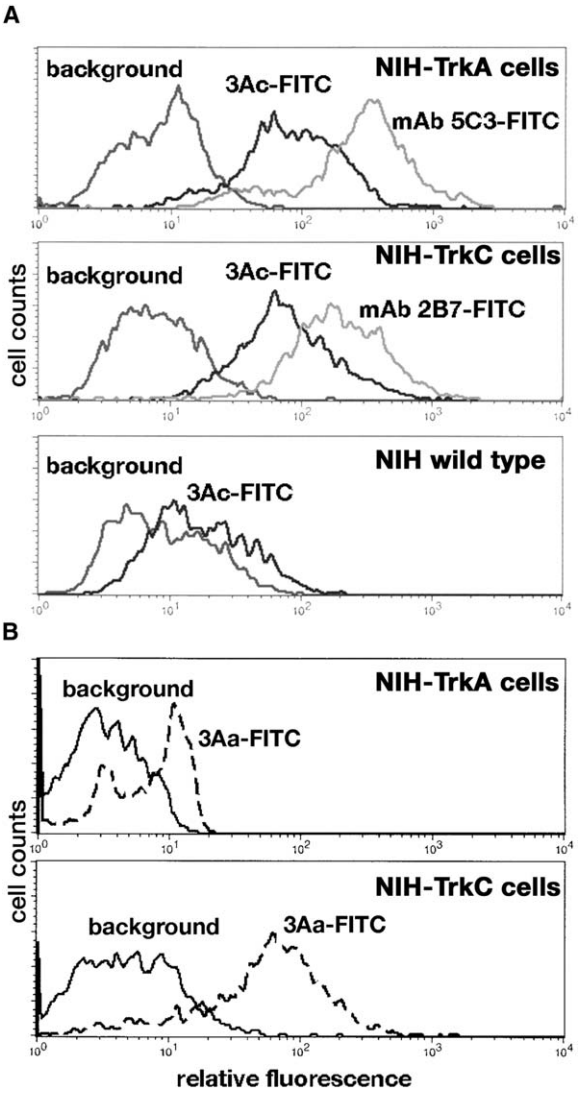


Figure 4. Receptor Binding Specificity Data

(A and B) FACScan binding assays with FITC-peptidomimetic. The indicated cells were bound with ligand (50 μM), and washed. Data are representative of three independent assays.

3) were calculated as a change in mean fluorescence channel (MCF) with respect to untreated cells. The positive-control NGF blocked 5C3·TrkA by 27 ± 4%, and NT-3 blocked 2B7·TrkC interactions by 37 ± 3% (significant, $p \leq 0.05$).

Peptidomimetics **3Ba**, **3Bg**, and **3Bi** blocked 2B7·TrkC binding by 57% ± 1%, 27% ± 3%, and 30% ± 2%, respectively. Blocking was significant ($p \leq 0.05$) and selective for TrkC because 5C3·TrkA binding was not affected. We estimate a K_d of ~4 μM for **3Ba**, based on its IC_{50} toward mAb 2B7 (where ~1500-fold molar excess **3Ba** competes 50% of mAb 2B7 [K_d ~6 nM]). Although it is possible that inhibition is steric rather than competitive, these data further support the notion that at least **3Ba**, **3Bg**, and **3Bi** are true receptor ligands binding TrkC at or near the IgC2 hot spot.

In built-in controls, none of the other agonistic peptidomimetics blocked 2B7·TrkC binding or 5C3·TrkA binding significantly. Lack of competition by other agonistic peptidomimetics may simply indicate that they bind the receptor elsewhere outside the mAb hot spots, or that the peptidomimetic affinity is too low to compete with a high-affinity bivalent mAb.

Discussion

In this study, we expand on the rational development of the first reported peptidomimetic small molecule TrkA agonist, termed D3. While D3 is a partial agonist of TrkA, its intrinsic activity is relatively low and it is best revealed when acting as a synergistic potentiator of NGF [26]. We therefore completed a study for peptidomimetic analogs related to D3 and the neurotrophin β-turns.

Functional assays showed that peptidomimetics reported here have intrinsic partial agonistic activity in the absence of exogenous neurotrophins, and are therefore improved agents compared to D3. Agonism can be selective toward TrkC only, or both TrkC and TrkA. Agonism can comprise full neurotrophic signals (survival and differentiation) or discrete neurotrophic signals (either survival or differentiation).

Binding assays showed that the functional peptidomimetics are bona fide artificial ligands and bind to Trk receptors. Some peptidomimetics are specific for TrkC and some bind both TrkC and TrkA. Some of the peptidomimetics bind at Trk receptor epitopes defined by agonistic antireceptor mAbs [33, 45], and these domains are also defined as "hot spots" by reported receptor mutagenesis, neurotrophin binding studies and functional studies [9, 10].

The FITC-labeled peptidomimetics did not bind p75 receptors. However, we cannot rule out that the FITC adduct could have altered the binding properties of the compounds. If the unlabeled compounds do bind p75, then it must be that the FITC label blocks their binding. Given the possibility that the FITC label may have prevented p75 binding in the FACS assays, and because there are no reliable and robust functional assays for p75, we could not correlate lack of p75 binding with lack of p75-mediated activity. However, we noted that **1Ba** affords sustained and more robust Trk-pTyr in cells coexpressing TrkA and p75. Thus, while overall the data suggest that expression of p75 is not necessary for peptidomimetic binding and activity (e.g., NIH-TrkC and NIH-TrkA do not express p75), and expression of p75 does not interfere with function (e.g., PC12 cells and nnr5-TrkC cells expressing p75 respond in bioassays), we cannot rule out that expression of p75 could regulate binding or function (see below for more discussion on this issue). Indeed, previous reports have shown that cryptic Trk "hot spots," where NGF docks and activates, are unmasked when p75 is coexpressed [9, 10]. Thus, the definitive answer to whether the compounds bind p75 may require further formal proof.

Table 3 summarizes the biological, receptor selectivity, and binding data. This table highlights that, generally, the binding specificity and the biological data correlate well, and presents some interesting findings. For example, binding can occur without activation (e.g.,

Table 2. Summary of Direct Binding Studies

Structure	Compound	Mean Channel Fluorescence	
		NIH-TrkA	NIH-TrkC
	5C3 or 2B7, control mAb	364 ± 7	175 ± 31
3Aa-FITC	**3Aa**-FITC	42 ± 15	73 ± 11
3Ac-FITC	**3Ac**-FITC	52 ± 11	79 ± 10
3Ae-FITC	**3Ae**-FITC	41 ± 10	66 ± 5
3Ak-FITC	**3Ak**-FITC	44 ± 26	62 ± 18
1Ad-FITC	**1Ad**-FITC	52 ± 24	90 ± 31
1Aa-FITC	**1Aa**-FITC	56 ± 5	65 ± 7
3Ai-FITC	**3Ai**- FITC	9 ± 5	18 ± 9

FACScan binding assays with FITC-peptidomimetic. Cells were bound with ligand (50 μM), washed, and studied. Data was acquired and analyzed by FACScan/CellQuest, with backgrounds subtracted. Mean channel fluorescence (MCF) ± SD, n = 3.

1Aa binds TrkA but does not activate it). Also activation (pTyr) of a receptor can lead to either or both differentiation and survival (e.g., **1Ba** induces TrkA-pTyr and leads to cellular differentiation but not to survival). In part, the biological outcome depends on the kinetics of receptor pTyr (e.g., **1Ba**), and likely also depends on ligand-dependent receptor internalization, as was reported elsewhere for other TrkA agonists [46, 47].

The Screening Process

A total of 26 out of the 55 designed peptidomimetics were biologically active (14 agonists described in this report, and 12 antagonists [unpublished data]). The high efficacy of a focused library suggests that, for some targets (such as cell surface receptors), this approach may be more desirable than random screening of larger libraries.

The main advantages of a focused library are: (1) compounds insoluble in physiological buffers and compounds that are toxic are quickly discarded; (2) medium-throughput trophic assays can use live cells, and can include several cell types to account for receptor selectivity; (3) potentiation and antagonism of neurotrophins can be assayed simultaneously; (4) direct binding and competitive binding assays can be performed using labeled ligands; and (5) more elaborate neuritogenic assays can be performed in a reduced pool of candidates. A typical high-throughput screen (e.g., based on receptor pTyr or based on competition of ligand binding) would have missed compounds, such as **3Ba**, that potentiate neurotrophin action.

One of our binding screens was based on peptidomimetic competition of mAbs directed to Trk hot spots. This high-throughput screen is desirable because it selects compounds that likely bind to functional receptor ectodomains, and it can assess target selectivity because anti-TrkA and anti-TrkC mAbs can be screened simultaneously. While the overall efficacy of this particular screen is very high, we should note that simple docking at a receptor hot spot is not necessarily sufficient for activation, and that ligands generally must also induce a receptor conformational change.

Electrostatic and Side Chain Considerations

Please see Supplemental Data for structures and models and the electrostatic maps of some compounds.

Table 3. Summary of Results

Code	aa	Scaffold	X	Survival[a]	pTyr[b]	Differentiation[c]	Binding[d]	Antibody Displacement
1Aa	IK	amine	amine	Cpt	C	nnr5-TrkC	AC	–
1Ad	EK	amine	amine	Ct	CA	PC12; nnr5-TrkC	AC	nt
1Ba	IK	amine	guanadine	Cpt	C	PC12	nt	nt
3Aa	IK	ether	amine	Cp	nt	nnr5-TrkC	C	nt
3Ac	GK	ether	amine	AC	AC	PC12; nnr5-TrkC	AC	nt
3Ae	IR	ether	amine	AC	C	nnr5-TrkC	AC	nt
3Ak	RG	ether	amine	AC	nt	–	AC	nt
3Ba	IK	ether	guanadine	Cp	AC	PC12; nnr5-TrkC	nt	C
3Bg	KG	ether	guanadine	Cp	nt	–	nt	C
3Bi	KT	ether	guanadine	Cp	nt	–	nt	C
3Ca	IK	ether	$MeSO_2NH$	AC	nt	–	nt	nt
3Ce	IR	ether	$MeSO_2NH$	Cpt	nt	–	nt	nt
3Cg	KG	ether	$MeSO_2NH$	Cp	nt	–	nt	nt
3Ck	RG	ether	$MeSO_2NH$	Cpt	nt	–	nt	nt

"Survival" refers to studies on NIH-TrkC or NIH-TrkA cells; "pTyr" refers to studies on NIH-TrkA and NIH-TrkC, except **1Ba**, which was studied in other cells; "Binding" refers to studies using FITC-analogs, thus the "X" substituents of these labeled peptidomimetics are different than the parental peptidomimetics used in the other studies; "Antibody Displacement" assays were done with parental peptidomimetics and mAbs 5C3 or 2B7 directed to IgC2 (D5) receptor hot spots of TrkA and TrkC, respectively.

Abbreviations: aa, amino acid; AC, the compound has activity for both receptors but no preference for TrkA versus TrkC; C, selective activity at TrkC; CA, compound has activity for both receptors but stronger activation of TrkC (e.g., pTyr **1Ad** and **3Ba**); nt, not tested; p, potentiation; t, intrinsic trophism; –, no effect.

[a] See Table 1.

[b] See Figures 2 and 3.

[c] See Figure 3.

[d] See Figure 4.

Electrostatic or conformational mechanisms may account for the following observations highlighted below: (1) the apparent negative influence of the sulfur or the appended carboxylic acid (group 2 compounds are not agonistic); (2) the possible role of the "X" substitution/modification in affecting ligand selectivity; and (3) the possible role of the amine backbone in affecting biological activity.

The sequence "IK" is present in 5/14 agonistic peptidomimetics. The sequences "IR," "GK," and "RG" are present in five of the remaining nine agonists, and "EK," "KT," and "KG" comprise the rest.

With respect to "IK" side chains, **1Aa** and **3Aa** display the dipeptide "IK" but in different backbones: amines (**1Aa**) or ethers (**3Aa**). Functionally **1Aa** and **3Aa** are specific activators of TrkC, although the derivative **FITC-1Aa** does bind to TrkC. Two related compounds are **1Ba** and **3Ba**, which display "IK" in the same respective backbones as **1Aa** and **3Aa** but with a different "X" substituent (a guanidine instead of an H) (Figure 1). **1Ba** and **3Ba** activate both TrkC and TrkA. Either the "X" substitution in **1Ba** and **3Ba** or the FITC-adduct at the "X" position in **FITC-1Aa** may be responsible for their reduced receptor specificity. Likewise, the "X" substitution of **3Ca** may be responsible for reduced receptor specificity. **3Ca** is similar to **3Aa**, and displays the dipeptide "IK" with an ether backbone, but has a methyl sulfonamide "X" substituent. **3Ca** activates TrkC and TrkA. These data suggest that while the nature of the cyclic backbone allows some modifications with no loss of activity or selectivity, the "X" substitution or modification may regulate selectivity.

With respect to "EK" and "GK" side chains, compound **1Ad** is similar in structure to **1Aa** (both backbone and "X" substituent) but displays the dipeptide "EK" instead of "IK". Likewise, compound **3Ac** is related to **3Aa** but displays the dipeptide sequence "GK" instead of "IK." Both **1Ad** and **3Ac** activate TrkC and TrkA, whereas **1Aa** and **3Aa** are TrkC-selective. These data suggest that the "EK" dipeptide may be less receptor-specific than "IK." Again, the importance of the "X" substituent may be demonstrated with the inactive compounds **1Bd** and **3Bc** (related to **1Ad** and **3Ac**, but with X = guanidine). On the other hand, when the "X" position is derivatized with the larger FITC moiety (e.g., **FITC-1Ad**), the compound still binds to TrkA and TrkC, suggesting that this substitution is not absolutely critical.

Other related compounds, **2Ad**, **2Cd**, **3Ad**, **3Bd**, and **3Cd**, also display the dipeptide "EK" but on different backbones (sulfide or ether, instead of amine), and they are not agonistic. These data suggest that the amine backbone may be relevant to biological activity.

It may also be significant that the aromatic substituents in the active peptidomimetics in the **A** and **B** series are also cationic (amine and guanidine). In contrast, the series **2** compounds were relatively less active in all the assays performed, and all have a pendant carboxylic acid. Series **2** compounds also all have endocyclic *S*-atoms, so lack of activity could be coincidental. Because compounds segregating the influence of endocyclic *S*-atoms versus the pendant carboxylic acid are lacking, the influence of each change cannot be fully assessed. However, it does appear that positively charged peptidomimetics were more active in the binding assays.

It is informative to compare the structures of the active peptidomimetics with the sequences of the amino acids around the turn regions of human and murine NGF and NT-3. NGF turn 1 has sequence $D^{30}IKG^{33}$

and $D^{30}EKG^{33}$, NGF turn 2 has sequence $I^{44}NNS^{47}$, and NGF turn 3 has sequence $D^{93}EKQ^{96}$, $D^{93}GKQ^{96}$, or $D^{93}NKQ^{96}$, depending on species. For NT-3, turn 1 is DIRG, turn 2 is KTGN, and turn 3 is ENNK. Most of the active compounds identified tend to contain lysine or, to a lesser degree, arginine. This correlates with the sequences of the 31–32 and 94–95, $i + 1/i + 2$, residues of the turn regions of NGF, both of which contain lysine. Thus, one neurotrophin pharmacophore could be defined as "IKGK" or "IRG" side chains displayed on a β-turn backbone. It is noteworthy that another pharmacophore, "DEK," is found *both* in the first and in the fourth loop regions of NT-3 and NGF. However, this observation partially reflects the loop sequences targeted, and does not per se exclude other regions of the neurotrophins. The Arg^{103} of NGF is present in all neurotrophins as part of the so-called "conserved patch" [48], and is thought to increase binding affinity without significantly influencing the specificity of binding. Consequently, it is conceivable that some or all of the peptidomimetics may not be mimicking the roles of the NGF turn regions. They might instead tend to bind to the regions of the TrkA receptors that would normally interact with Arg^{103}, and the data do not allow this possibility to be discounted.

Several compounds (**3Bg**, **3Bi**, **3Ce**, and **3Ck**) afford only survival (no differentiation), and only through TrkC receptors. These compounds encompass the dipeptide sequences "KG," "KT," "IR," and "RG," respectively. **3Ak** also affords only survival, but can act through both TrkA and TrkC. **3Ak** displays the sequence "RG," as does the related compound **3Ck**. Hence, the amine in **3Ak** reduces receptor specificity (**3Ck** is TrkC-specific). **3Ae** activates TrkA and TrkC, whereas **3Ce** is TrkC-specific. Both **3Ae** and **3Ck** display the sequence "IR." As above, the methyl sulfonamide substitutent in **3Ae** reduces receptor specificity. None of the methyl sulfonamide substituents afford differentiation, but they do afford survival. While the backbone is less sensitive to substitutions and accepts amines or ethers, the sulfide backbone (which closely mimics the ether backbone) always resulted in inactive compounds.

Signal Transduction and the Role of p75 Coreceptors

It is impressive that a small molecule ligand can induce receptor pTyr and activate *both* trophic and neuritogenic signals, in the absence of neurotrophins. We also show that a peptidomimetic with both trophic and neuritogenic signals can be modified to generate an analog that affords discrete signals such as trophic survival only (e.g., **3Ak**, **3Bg**, **3Bi**, **3Ca**, **3Ce**, and **3Ck**). By comparison, both NT-3 and NGF bind TrkA but do so at relatively nonoverlapping sites. NGF binding to TrkA activates survival and differentiation signals, whereas NT-3 binding to TrkA as a heterologous ligand activates differentiation but does not activate strong trophic signals [42]. The potency of TrkA activation also depends on coexpression of p75 receptors, with NGF signaling being enhanced and NT-3 signaling being inhibited [49].

Peptidomimetics **1Ad** and **3Ba** induce TrkA phosphorylation leading only to differentiation and, therefore, behave much like NT-3. However, unlike NT-3, **1Ad** and **3Ba** seem to be unaffected by coexpression of p75 in PC12 cells. In addition, other data demonstrate that peptidomimetic binding and activity are not dependent on p75 (e.g., some compounds are active on NIH-TrkC cells lacking p75).

As summarized in Table 3, initial studies of receptor binding and biological activity did not correlate well for compounds **1Ba** and **3Ae**. Kinetic studies were carried out for **1Ba**, but not for **3Ae**. Compound **1Ba** signals through TrkA and TrkC in a manner that is independent of, but can be regulated by, p75 coexpression. **1Ba** activates TrkC with fast and sustained kinetics leading to survival, independent of p75 coexpression (e.g., NIH-TrkC cells). However, in NIH-TrkA cells, **1Ba** activates TrkA with transient kinetics that do not afford survival, whereas in cells coexpressing TrkA and p75, it activates TrkA with fast and sustained kinetics and higher potency; activity that, in PC12 cells, result in differentiation.

Whether or not these data mean that **1Ba** binds/activates both TrkA and p75 is inconclusive. We favor the interpretation that p75 acts as a regulator of peptidomimetic-dependent Trk activity because both the potency of activation and the type of biological response to TrkA activation by NGF and NT-3 are known to depend on coexpression of p75 receptors [49]. Additionally, there is no evidence that ligand-bound p75 is a prodifferentiation receptor in PC12 cells. Rather, the simplest explanation is that these peptidomimetics have distinct kinetics of receptor activation (or internalization), leading to distinct biological outcomes of survival or differentiation. Indeed, ligand-dependent Trk receptor internalization is known to determine the biological outcome [46, 47]. Also, we cannot rule out that the compounds induce or stabilize receptor dimerization or clustering [50], as has been shown elsewhere [27], or that the compounds interact with two independent receptor hot spots.

Conclusions

Altered function of the neurotrophins or their receptors is relevant to pathologies [1, 3–5], but clinical trials have been disappointing [7] due to in vivo instability, side effects produced by the activation of signals that were not intentionally targeted, and drawbacks inherent to proteins when used as drugs. Thus, small molecule peptidomimetics of the neurotrophins that have better pharmacokinetic properties may be useful therapeutics. Another potentially attractive feature is that small molecules could target receptors at single activation or regulatory hot spots (e.g., only Trk IgC2 or only LRM subdomains), thus affording agents with high Trk selectivity, partial Trk agonism, and potentially excluding binding to p75. From a drug development perspective, it may be desirable to have small molecules that uncouple trophic and neurotigenic signals because they can be used as selective therapeutics in conditions where a single activity is required. For example, in stroke or Alzheimer's disease, it may be desirable to support existing neurons from death without inducing connections de novo that might be dysfunctional. The screening approaches we describe here can be easily adapted to other cell surface receptors.

Significance

Our findings demonstrate that a small molecule peptidomimetic ligand of TrkA Trk tyrosine kinase receptors can activate selectively by binding at the ectodomain, without apparent interactions with p75 coreceptors. We also show that engaging a receptor "hot spot" is sufficient to induce a functional response, and that small molecule ligands can activate discrete or multiple signal transduction pathways. Some peptidomimetics induce both neurotrophic activities of survival and differentiation, while other peptidomimetics afford either survival or differentiation. These data suggest that certain receptor ectodomain "hot spots" may be responsible for specific signals. Of conceptual interest is the high rate of success of the design and screening approach, which suggests that a focused library designed specifically to target receptor hot spots may be a useful generic method for identifying functional ligands. This work helps us to understand how the neurotrophins function through Trk receptors, and is a step forward in the search for small, stable molecules with selective agonistic activity and possible therapeutic potential in cholinergic/cognitive (TrkA) or motor neuron (TrkC) disorders.

Experimental Procedures

Preparation of Template Compounds 1–3 and Their Derivatives

The β-turn backbone (Figure 1) comprises an organic moiety and a dipeptide moiety R^1 R^2 within a turn. The backbone has a functionality to generate diversity with any of three substituents: cyclic amines, cyclic sulfides, or cyclic ethers (Figure 1A). Compounds **1–3** can be made on a solid phase, in parallel, to give small libraries. The "X" functionality can be derivatized with four substituents to generate diversity: amino, guanidine, *N*-methyl sulfonamide, and arginine (Figure 1B). The amino (X = NH_2) backbone derivatives **1A**, **2A**, and **3A** were prepared on resin bearing the side chain-protected cyclic peptide with nitro groups, as previously described [30, 33, 51], and purified by preparative HPLC. The guanidine (X = $C(=NH)NH_2$) backbone derivatives **1B**, **2B**, and **3B** were prepared on resin containing side chain-protected cyclic peptide. The resin was swelled in CH_2Cl_2 for 30 min and treated with 10 Eq of *N,N-bis*-BOC-1-guanylpyrazole in DMF (for **1** and **2**) or 10 Eq of *N,N-bis*-BOC-thiourea and 10 Eq of *N*-methyl-2-chloropyridinium chloride in CH_2Cl_2 (for **3**) for 1 day. After washing, the peptide was cleaved from the resin by treatment with a mixture of 90% CF_3CO_2H, 5% $HSiEt_3$, and 5% H_2O for 2 hr, filtered, and the crude peptide was purified by preparative HPLC. The *N*-methyl sulfonamide (X = SO_2CH_3) backbone derivatives **1C**, **2C**, and **3C** were prepared on resin containing side chain-protected cyclic peptide with amino group. The resin was swelled in CH_2Cl_2 in a fritted syringe for 30 min and treated with 10 Eq of methanesulfonyl chloride, 10 Eq of pyridine, and 1 Eq of 4-dimethylaminopyridine in CH_2Cl_2 for 1 day. After washing, the peptide was cleaved from the resin as above, filtered, and the crude peptide was purified by preparative HPLC. The arginine-substituted (X = Arg) backbone derivative **1D** was prepared on resin containing side chain-protected cyclic peptide. The resin was swelled in CH_2Cl_2 in a fritted syringe for 30 min and treated with 4 Eq of FmocArg(Pmc)OH, 4 Eq of PyBrop, and 15 Eq of 2,6-lutidine in CH_2Cl_2 for 20 hr. After washing, Fmoc protecting group was removed with 20% piperidine. The peptide was cleaved from the resin by treatment with a mixture of 90% CF_3CO_2H, 5% $HSiEt_3$, and 5% H_2O for 10 hr, the crude peptide was triturated using anhydrous ethyl ether and purified by preparative HPLC. All compounds were analyzed as homogeneous single peaks by analytical HPLC (2%–40% B in 30 min), by MALDI MS, and by 1H NMR (300 MHz, dimethylsulfoxide [DMSO]-d_6) [33, 51], confirming the expected structures.

Preparation of Fluorescinated Compounds

Resin supporting the side chain-protected cyclic peptidomimetics with amino group was swelled in CH_2Cl_2 for 30 min and treated with 4 Eq of Fmoc-β-Ala-OH, 4.6 Eq of PyBrOP and 15 Eq of 2,6-lutidine in CH_2Cl_2. After 20 hr of gentle shaking, the resin was washed and Fmoc was removed with 20% piperidine in DMF. The resin was then treated with fluorescein 5-isothiocyanate (i.e., "isomer I," 3 Eq), 5 Eq DIEA in CH_2Cl_2/DMSO (2:1) for 3 hr, washed again, and finally cleaved with a mixture of 90% TFA, 5% Et_3SiH, and 5% H_2O. After most of the cleavage cocktail was evaporated in a stream of nitrogen. Anhydrous ethyl ether was added to precipitate the product, which was purified by preparative HPLC. Not all FITC compounds were prepared, only agonists of interest.

Peptidomimetic Solubility

Most of the compounds were soluble in physiological buffers, but some required up to 1.5% DMSO as solvent, which did not interfere with the binding assays or the bioassays. Peptidomimetics requiring higher concentrations of DMSO were excluded because of possible toxicity. Vehicle was always used as control.

Cell Lines

Cell lines used were wild-type NIH-3T3 fibroblasts, NIH-3T3 stably transfected with human p140 *trkC* cDNA (NIH-TrkC), or NIH-3T3 fibroblast stably transfected with human p140 *trkA* cDNA (NIH-TrkA cells). NIH-p75 fibroblasts were produced by infection with adenoviruses constructed to express *p75* [10]. Rat PC12 pheochromocytoma cells express the p75 neurotrophin receptor and low levels of TrkA ($TrkA^+$ $p75^{+++}$), and can be induced to differentiate into neurite-bearing cells by TrkA activation. The nnr5 cells are a variant of PC12 cells that have lost TrkA expression ($TrkA^-$ $p75^{+++}$). The nnr5-TrkC cells are nnr5 cells stably transfected with human p140 *trkC* cDNA. The nnr5-TrkC cells express low levels of TrkC ($TrkC^+$ $p75^{+++}$), and can be induced to differentiate into neurite-bearing cells by TrkC activation.

Cell Survival Assays

Cell survival was measured quantitatively by the MTT assay and optical density (OD) readings, as previously described [46, 52], after culture in SFM. Cells in SFM die by apoptosis, but can be rescued if supplemented with the appropriate neurotrophin if they express functional receptors. Wells were not supplemented (negative control), or were supplemented with suboptimal (0.1 nM) or optimal (2 nM) concentrations of the indicated neurotrophins. Test peptidomimetics or vehicle were added to the two conditions above. Lack of effect on NIH-TrkA with effect on NIH-TrkC is evidence of selectivity. Trk receptor selectivity and lack of toxicity was assessed in wild-type NIH-3T3 cells in SFM surviving to fibroblast growth factor (βFGF) or NIH-3T3 cells under normal growth conditions (5% serum). Lack of effect on βFGF or 5% serum-mediated survival is evidence of Trk specificity. All assays were repeated independently at least three times (n = 4–6 for each assay). MTT data are standardized to: 2 nM neurotrophin = 100% survival, and SFM = 0% survival, using the formula $[(OD_{test} - OD_{SFM}) \times 100/(OD_{2\ nM\ NTF} - OD_{SFM})]$.

Neurite Outgrowth Assays

Peptidomimetics were tested for their ability to induce neurite outgrowth in PC12 and nnr5-TrkC cells. Cells were incubated in complete media without or with peptidomimetics alone (50 μM) or neurotrophins alone (0.1 nM or 2 nM). Neurite outgrowth was measured every 24 hr for 3 days. After 3 days, cells were fixed for 20 min with 4% paraformaldehyde, washed two times with PBS, incubated at room temperature for 20 min with permeabilization solution (2% BSA, 0.3% TritonX, in PBS), and incubated for 1 hr at room temperature with 1:200 dilution of FITC-anti-CD90 (Thy-1) (Pharmingen). Quantification was done by image analysis, from at least three independent assays, scoring an average of 203 ± 33 cells per each condition. Differentiation was scored morphologically, neurites being processes longer than two cell bodies.

Antibodies

mAb 5C3 is an agonistic anti-TrkA IgG-C2 (D5) domain antibody [45]. The mAb 2B7 [33] is an agonistic anti-TrkC IgG-C2 (D5) domain antibody. The docking site of these mAbs on TrkA and TrkC overlaps, respectively, with the binding site of NGF and NT-3 on the receptor IgG-C2 (D5) domains.

Direct FACScan Binding Assays

The FITC-peptidomimetics were tested by quantitative FACScan for direct binding to NIH-TrkA cells, NIH-TrkC cells, NIH-p75 cells, nnr5 cells ($p75^{+++}$ Trk^{-}), or wild-type NIH-3T3 control cells. Cells were resuspended in binding buffer (PBS, 0.5% BSA, and 0.1% NaN_3) and 40 μM of FITC peptidomimetics were added for 1 hr. Cells were washed three times, acquired on a FACScan, and mean channel fluorescence (MCF) of bell-shaped histograms was analyzed using the CellQuest program. MAbs 5C3 (anti-TrkA), 2B7 (anti-TrkC), and MC192 (anti-p75) were used as positive binding controls, and mouse IgG-FITC was used as negative binding control. Nonbinding FITC-peptidomimetics were also made and used as negative controls.

Competitive FACScan Binding Assays

Peptidomimetics were tested for their ability to block the binding of mAbs 5C3 or 2B7 toward their respective receptors in quantitative FACScan assays. Cells were resuspended in binding buffer (PBS, 0.5% BSA, and 0.1% NaN_3) and saturating anti-TrkA mAb 5C3, or anti-TrkC mAb 2B7 ($\sim$60 nM) or control nonbinding IgGs, were added in the absence or presence of peptidomimetics (100 μM) or controls, as previously described [26]. Cells were then washed and immunostained. Cells were acquired on a FACScan, and MCF of bell-shaped histograms was analyzed using the CellQuest program.

pTyr Assays

Assays were performed, as previously described [46, 52], by Western immunoblotting with antiphosphotyrosine (α-pTyr) antibody 4G10 (Upstate Biotechnology, Lake Placid, NY). After ligand treatment (0.1 nM or 2 nM neurotrophins alone, or 50 μM peptidomimetics alone) at 37°C for 20 min, cells were solubilized and protein concentrations were determined. Equal protein loading was confirmed by Coomassie blue staining of gels, and by stripping and reblotting membranes with anti-pan Trk polyclonal antibody 203. Quantification was done by densitometric analysis relative to optimal (2 nM) neurotrophin.

Statistical Analyses

The data were analyzed with one-way ANOVA followed by t tests with Bonferroni corrections; statistical significance is indicated in some figures by an asterisk (*).

Supplemental Data

Supplemental Data, including structures and models and the electrostatic maps of some compounds, are available at http://www.chembiol.com/cgi/content/full/12/9/1015/DC1/.

Acknowledgments

Supported by grants to H.U.S. from the Canadian Institutes of Health Research (MT-13265), and to K.B. from the National Institute of Health (CA82642), the Texas Advanced Technology Program, and the Robert A. Welch Foundation. M.C.Z. received a scholarship from the McGill Faculty of Medicine. We thank Dr. H. Nedev (McGill University), the TAMU/LBMS-Applications Laboratory (Dr. S. Stichy), and the Laboratory for Molecular Simulation (Dr. L. Thompson) for aid with reagents, assays, modeling, synthesis, and characterization. The NMR instrumentation in the Biomolecular NMR Laboratory at Texas A&M University was supported by a grant from the National Science Foundation (DBI-9970232) and the Texas A&M University System.

Received: January 23, 2004
Revised: June 28, 2005
Accepted: June 28, 2005
Published: September 23, 2005

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