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## A monovalent agonist of TrkA tyrosine kinase receptors can be converted into a bivalent antagonist

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### Abstract

**Background**—Receptor tyrosine kinases (RTK) act through dimerization. Previously it was thought that only bivalent ligands could be agonistic, whereas monovalent ligands should be antagonistic. This notion changed after the demonstration that monovalent ligands can be agonistic, including our report of a small molecule monovalent ligand “**D3**” that is a partial agonist of the NGF receptor TrkA. A bivalent “**D3-linker-D3**” was expected to increase agonism.

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**Methods**—Dimeric analogs were synthesized and tested in binding, biochemical, and biological assays.

**Results**—One analog, **1-ss**, binds TrkA with higher affinity than **D3** and induces or stabilizes receptor dimers. However, **1-ss** exhibited antagonistic activity, through two mechanisms. One mechanism is that **1-ss** blocks NGF binding, unlike **D3** which is non-competitive. Inhibition of NGF binding may be due to the linker of **1-ss** filling the inter-receptor space that NGF traverses before docking. In a second mechanism, **1-ss** acts as a pure antagonist, inhibiting NGF-independent TrkA activity in cells over-expressing receptors. Inhibition is likely due to **1-ss** “freezing” the TrkA dimer in the inactive state.

**Conclusions**—Dimerization of an RTK can result in antagonism, through two independent mechanisms.

**General Significance**—we report a small molecule monovalent agonist being converted to a bivalent antagonist.

## Keywords

neurotrophin; ligand; tyrosine kinase receptor; linker; agonist; antagonist

## 1. Introduction

The high affinity receptor for Nerve Growth Factor (NGF), TrkA, is a member of the receptor tyrosine kinase (RTK) family. TrkA, and RTKs in general, act through receptor dimers where each receptor trans-activates the juxtaposed intracellular tyrosine kinase domain of its neighbor [1]. Because NGF is a dimeric ligand, it is thought to induce ligand-dependent TrkA receptor dimerization or to stabilize preformed receptor dimers. This lead to the generally accepted notion that only dimeric ligands could be agonistic [2,3]. Indeed modification of a ligand from a monovalent to a bivalent form could generate agonists: this was documented for TrkA [4,5] and for other RTKs [6,7].

However, bivalency is not absolutely required. Indeed, monovalent and monomeric agonists have been reported for several RTKs [8,9], including TrkA [4,10,11]. Activation by monovalent ligands can take place through conformational changes in pre-formed receptor dimers [12]. However, to date structural analyses have not demonstrated conformational changes in TrkA even after the binding of protein ligands such as NGF [13]. Thus, the correlation between ligand-induced RTK dimerization, the activation state of a receptor, and agonistic activity is questionable for the natural protein ligands; and it is even less certain for receptor activation caused by small molecule ligands.

Here, we tested whether dimerization of an agonistic monovalent ligand could generate an antagonist. We generated homobivalent analogs of a previously reported NGF peptidomimetic D3 (hereafter termed “A”). Mimetic D3 is a monovalent and monomeric ligand of TrkA, and acts as an agonist to activate this receptor. D3 has intrinsic agonistic activity at TrkA, in the absence of NGF. Moreover, D3 binding to TrkA is non-competitive with respect to NGF. Thus, NGF and D3 can bind TrkA concomitantly, and in fact D3 potentiates NGF activity [4,10].

The bivalent analogs generated are two D3 units (**A-A**) linked through a series of progressively longer and relatively rigid linkers, resulting in various lengths that approximate the geometry of the NGF dimer. Unlike the parental D3 molecule, the dimeric **A-A** antagonizes NGF binding and NGF-dependent function. Moreover, although dimeric **A-A** induces or stabilizes TrkA dimers, it actually antagonizes receptor function in TrkA over-expressing cells. Thus, non-covalent cross-linking of the receptor pair by a bivalent ligand could “freeze” the receptor dimer in an inactive state, resulting in an antagonist.

Switching the action of a small molecule ligand from an agonist to an antagonist can provide strategies to discover a new class of inhibitors or activators of NGF receptors, and the concept could be expanded to other members of the RTK family.

## 2. Methods

### 2.1 Preparation of the Bivalent Derivatives

The chemical synthesis and purification of non-tagged and the dye-tagged peptidomimetics is described in the Supplemental methods. A scheme of the reactions is given in Supplemental Figure 1. A scheme of the reactions for homodimerization is given in Supplemental Figure 2. All the final purified compounds were analyzed by analytical HPLC again and MALDI-MS as shown in Supplemental Table 1.

### 2.2 Cell Lines

NIH-TrkA cells are NIH3T3 fibroblasts transfected with human *trkA* cDNA. NIH-TrkC cells are NIH3T3 fibroblasts transfected with human *trkC* cDNA. NIH-IGF-1R cells are NIH3T3 fibroblasts transfected with human *IGF-1R* cDNA. All cells are stably transfected subclones that express high levels of their receptor. Cells are grown under drug selection (0.5 mg/ml G418) and are routinely screened for receptor expression by FACScan using monoclonal antibodies directed to the receptor extracellular domains. PC12 cells are a rat pheochromocytoma expressing low levels of endogenous TrkA (~2,000 receptors/cell) and high levels of endogenous p75 (~200,000 receptors/cell). 4-3.6 cells are stably transfected rat neuroblastoma expressing endogenous p75 (~40,000 receptors/cell) and human TrkA (~30,000 receptors/cell) [4,10].

### 2.3 Antibodies

mouse anti-TrkA mAb 5C3, mouse anti-TrkC mAb 2B7, mouse anti-p75 mAb MC912, and mouse anti-IGF-1R mAb alphaIR3 were purified using protein G-Sepharose (Pharmacia, Baie d'Urfe, Quebec, Canada). They have all been described [4,14].

### 2.4 FACScan analysis

Cells ( $2 \times 10^5$ ) in 100  $\mu$ l of FACScan binding buffer (BB: phosphate-buffered saline, 0.5% BSA, and 0.1%  $\text{NaN}_3$ ) were immunostained as described. Saturating mAbs, or control non-binding mouse IgGs were added to cells for 20 min at 4 °C, excess primary antibody was washed off, and cells were immunostained with fluoresceinated goat anti-mouse IgG (FITC-G- $\alpha$ -M) secondary antibody. As cellular controls NIH-3T3 cells not expressing NGF receptors were used (e.g. NIH-IGF-1R). Cells were acquired on a FACScan, and bell-shaped histograms were analyzed using LYSIS II and the CellQuest-pro program as described [4].

### 2.5 FACScan analysis of peptidomimetics

Peptidomimetics labeled with fluorescein were used in FACScan binding studies, as described in section 2.4. The assay was slightly modified to extend the incubation of the “primary” reagent to 40 minutes, followed by two washes to remove unbound material. There was no secondary reagent added, as the compounds are directly labeled.

### 2.6 FACScan binding competition assays

Blocking to the binding of FITC-labeled peptidomimetics were studied by pre-incubation of the cells with increasing concentrations of TrkA ligands anti-TrkA mAb 5C3, for 30 min at 4 °C. Then, the FITC-labeled peptidomimetic was added, without previous washing, and the study progressed as described in section 2.5.

## 2.7 Ligand Binding

$^{125}\text{I}$ [NGF] (73.1 mCi/mg; NEN Life Science Products) binding assays were done on cells as described [15]. NIH-TrkA cells ( $1 \times 10^6$  per point) were added to serial dilutions of  $^{125}\text{I}$ [NGF] in BB at  $4^\circ\text{C}$ , that was prepared in the presence or absence of a molar excess of test peptidomimetic or control cold NGF. During the serial dilutions, the test peptidomimetic or control cold NGF remain at the indicated constant fold-molar excess over  $^{125}\text{I}$ [NGF]. NIH-3T3 wild type cells not expressing NGF receptors were used to assess nonspecific background (<15% of total binding). Scatchard plot analysis of the data was performed.

In related binding assays, increasing concentrations of the test peptidomimetic or control cold NGF were added to a constant dose of  $^{125}\text{I}$ [NGF].

## 2.8 Survival Assays

Cells (5,000–10,000 cells/well) were added to 96-well plates and cultured either in media containing 5% fetal bovine serum or in serum free media with 0.1% BSA (SFM). Ligands consisted of serial dilutions of neurotrophins or peptidomimetics were then added. FITC-tagged and TEG-tagged mimetics were tested, but only the data for TEG-tagged mimetics are shown. A suboptimal dose of neurotrophins (0.1 – 0.2 nM, affording ~25% of survival) was used to test the effect of combination of NGF with peptidomimetics. The proliferative/survival profile of the cells was quantitated using the tetrazolium salt reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) and optical density (OD) readings as described. Assays were done 4-7 times, each assay  $n$  4–8.

## 2.9 Receptor Dimerization Assays

Receptor cross-linking assays were carried out as reported elsewhere [10]. Here, NIH-TrkA cells ( $10^6$  cells/ml each group) were exposed to the indicated ligands (untreated control, NGF 10 nM, 1-ss-fluorescein (20  $\mu\text{M}$ ), or 1-ss-TEG (20  $\mu\text{M}$ )) for 30 min at  $4^\circ\text{C}$ . Following washing, cells were chemically cross-linked with 1 mM final disuccinimidyl suberate (DSS, Pierce) for 7 min. Un-reacted DSS was quenched with a 5-molar excess of ammonium acetate and the cells were washed two times with HBSS at  $4^\circ\text{C}$ . Then each cell pellet was detergent solubilized (1% NP40 containing protease inhibitors). Protein concentration for each of the cleared lysates were determined using a detergent-compatible BioRad kit. Equal protein (20  $\mu\text{g}$ /lane) of each sample were resolved by SDS-PAGE, and after Western transfer the membranes were analyzed by western blotting with anti-TrkA mAbs. Equal loading was further verified by Coomassie blue staining of the gels.

## 2.10 Receptor Activation Assays

NIH-TrkA cells were cultured at  $37^\circ\text{C}$  in SFM for 12 hours to reduce their baseline tyrosine phosphorylation. Then the cells were exposed to the indicated ligand (NGF, 1-ss, untreated control) at  $37^\circ\text{C}$  for 20 minutes or for 2 hours. After washing in PBS, cells were detergent solubilized, and equal protein (20  $\mu\text{g}$ /lane) of each of the clear lysates were resolved by SDS-PAGE, and after Western transfer the membranes were analyzed by western blotting with anti-pTyr mAb 4G10, or with anti-phospho-Akt as previously reported [16]. As controls, TrkA loading was verified using anti-TrkA mAb 5C3 and protein with anti-actin [17,18].

## 3. Results

### 3.1 Synthesis of D3-homodimers

Previously we reported the design, synthesis, and activity of small molecule mimics of NGF [10,19-22]. One of the early leads identified, originally reported as peptidomimetic D3 is herein termed compound “A” for simplicity. Biochemical and cellular assays showed that

peptidomimetic “A” is a monovalent TrkA agonist binding at the D5 domain of TrkA [10], and *in vivo* is effective to activate TrkA and afford neuroprotection [23-25].

The precursor to peptidomimetic “A” (Figure 1A) was assembled to make bivalent molecules through a modification of the literature procedure [26]. Assembly of the monovalent molecules into the bivalent ones was achieved using a triazine-based linker (Figure 1B) [27]. The monovalent building blocks “A” represent the pharmacophore and were purposely not modified, other than the nitro-aryl moiety which is not relevant for binding. However, the triazine and the linker parts were varied in two ways.

*First*, we used a combinatorial approach to attach mimetic A to a short (s), a medium (m), or a long linker (l) (Figure 1C). These were then paired on the triazine scaffold to yield a family of homobivalent compounds **1** representing combinations of linkers: **1-ss**, **1-sm**, **1-sl**, **1-mm**, **1-ml**, and **1-ll** (see structures in Figure 2). *Second*, the triazine had one of two different “tags”. Fluorescein isothiocyanate (FITC) was used to add a single fluorescein label to compounds to facilitate screening via fluorescence activated cell scanning (FACScan). A flexible triethylene glycol (TEG) tag was also added, and had no function except to substitute the fluorescein and to increase the water solubility of the mimics (Figure 1B). The purity and the composition of the bivalent molecules were confirmed by HPLC/MS (see supplemental Table 1).

### 3.2 Binding studies with labeled peptidomimetics

Direct FACScan-based binding assays were performed using fluorescein-labeled compounds, and NIH-3T3 cells stably transfected to express the neurotrophin receptor TrkA (NIH-TrkA), or controls expressing the p75 neurotrophin receptor (NIH-p75), or the IGF-1R receptor (NIH-IGF-1R) to test for selectivity (Figure 3), or the TrkC neurotrophin receptor (NIH-TrkC) (data not shown).

Peptidomimetic **1-ss** bound TrkA selectively, and better than any other dimer. Using linkers based on the same triazine backbone but which are of longer length resulted in molecules with lower efficacy of TrkA binding. Progressively longer peptidomimetics **1-sm**, **1-mm**, **1-sl**, **1-ml**, and **1-ll** exhibited significantly lower TrkA binding than **1-ss**. These studies were replicated using at least two independent syntheses of the compounds, with each synthesis being tested independently at least three times.

### 3.3 1-ss inhibits the binding of NGF

Binding experiments tested **1-ss** effects on the binding of  $^{125}\text{I}$ [NGF] to TrkA-expressing NIH-3T3 cells (Figure 4). These cells do not express the p75 co-receptor, thus TrkA accounts for all the specific binding. Unlabeled NGF was used as control competitor. Scatchard plot analyses of  $^{125}\text{I}$ [NGF] binding data showed the expected  $\sim 150,000$  receptors/cell on the surface (Figure 4A). A constant 500-fold excess of peptidomimetic **1-ss** reduced the binding of  $^{125}\text{I}$ [NGF] by  $\sim 50\%$ , without changing the affinity of the residual  $^{125}\text{I}$ [NGF] bound to TrkA. In a positive control, 100-fold excess unlabeled NGF as competitor blocked nearly all the binding of  $^{125}\text{I}$ [NGF]. The background cpms were determined by using the same concentration range of  $^{125}\text{I}$ [NGF] binding to NIH-wild type cells (not expressing TrkA). In all assays, background ranged from 5-15% of the total binding, and in each case these background cpms were subtracted.

Similar binding assays were carried out using a constant  $^{125}\text{I}$ [NGF] concentration (2 nM, resulting in maximal 16,500 cpms bound); and a dose-range of the inhibitors **1-ss** or unlabeled NGF as control. The inhibitors reduced  $^{125}\text{I}$ [NGF] binding in a dose-dependent manner (Figure 4B). Averaged from three independent assays  $\pm$  sem the inhibition of  $^{125}\text{I}$ [NGF] by cold NGF is  $\text{IC}_{50} 9 \pm 5$  nM; and the **1-ss**  $\text{IC}_{50}$  is  $5,000 \pm 420$  nM (e.g.  $\sim 550$ -fold higher than NGF

competing itself). We estimate that **1-ss** has a  $K_d \sim 105$  nM, which is improved compared to the affinity estimated for the monovalent parental compound **A** ( $K_d \sim 10$   $\mu$ M). This  $\sim 100$ -fold improved affinity was expected from **1-ss** bivalency.

### 3.4 NGF does not block the binding of 1-ss

We attempted the converse experiment, to block the binding of **1-ss-fluorescein** by pre-incubation of cells with NGF. In these FACScan-based binding assays NGF did not affect the binding of **1-ss-fluorescein** (data not shown), even at 200 nM NGF concentrations, known to saturate TrkA. These data suggest that **1-ss** binds to site of TrkA that is not overlapping with that of NGF. This is consistent with previous reports showing that parental compound **A** (from which **1-ss** is made) binds TrkA in a way that does not compete with NGF binding [10]. It is therefore possible that NGF and **1-ss** could bind a TrkA receptor at the same time.

### 3.5 1-ss Induces or Stabilizes Receptor Dimers

The increased affinity in **1-ss** (compared to parental monovalent **A**) is likely due to its bivalent binding. Thus, we performed chemical cross-linking experiments to address the hypothesis that **1-ss** binds to a TrkA-TrkA homodimer. NIH-TrkA cells were exposed to test ligands or controls, followed by chemical cross-linking to stabilize receptor dimers on the cell surface. Then cell lysates were resolved in denaturing SDS-PAGE and analyzed by western blotting with highly specific anti-TrkA antibodies (Figure 5).

Lysates from unliganded cross-linked NIH-TrkA cells show a band at p140 (cell surface TrkA monomer) and a less intense p110 band previously reported to be intracellular TrkA with a lower degree of glycosylation. A band at  $\sim 280$  kDa is also detected. This is consistent with ligand-independent pre-formed homodimers in receptor over-expressing cells [12]. Exposure of NIH-TrkA cells to ligands **1-ss** or NGF significantly increased detection of the 280 kDa band using highly specific anti-TrkA mAbs. This would be consistent with a ligand-mediated increase in TrkA dimers. Standardization of the TrkA dimers detected after exposure of cells to 10 nM NGF as 100% show that **1-ss-fluorescein** affords  $75 \pm 8\%$ , and **1-ss-TEG** affords  $77 \pm 5\%$ . Previous cross-linking data reported for the parental monovalent **A** compound showed that it affords  $21 \pm 4\%$  of TrkA dimers relative to 10 nM NGF [10]. Controls performed without chemical cross-linking result is no detection of  $\sim 280$  kDa bands, because pre-formed homodimers are non-covalent and fall out in denaturing SDS-PAGE (data not shown).

Overall, the data indicate that NGF and **1-ss** have the ability to induce or to stabilize TrkA dimers at the cell surface, and that **1-ss** has improved ability to induce TrkA dimers compared to the parental monovalent agent.

### 3.6 1-ss Antagonizes NGF-promoted cell survival

The parental monovalent **A** compound is a partial TrkA agonist. Thus, the bioactivity of the bivalent peptidomimetics was tested in quantitative MTT assays testing receptor-mediated cell survival, using NIH-3T3 cells expressing TrkA, or as controls NIH-3T3 cells expressing TrkC, or IGF-1R.

When cells are cultured in serum-free media (SFM) they undergo apoptosis. In these conditions, cells can be protected by their appropriate growth factor (NIH-TrkA is protected by NGF, NIH-TrkC is protected by NT-3, NIH-IGF-1R is protected by IGF-1). Growth factor protection from apoptosis is dose-dependent, and suboptimal doses of growth factor can be used that result quantitatively and consistently in reduced survival (25-50% of maximal).

In initial assays the fluoresceinated peptidomimetic **1-ss** was used. These assays proved that **1-ss** antagonizes NGF (data not shown). However, using a fluorescein-labeled compound in



bioassays is not desirable. Thus, the assays were repeated using analogs of **1-ss**, **1-mm**, and **1-ll** that contain a TEG derivative to substitute the fluorescein moiety. This was done to verify that the fluorescein moiety was not relevant for antagonistic function.

Mimetic **1-ss-TEG** was antagonistic of NGF bioactivity, measured as a reduction of NGF-mediated protection of NIH-TrkA cells undergoing apoptosis after serum deprivation (Figure 6A). Antagonism was selective because mimetic **1-ss-TEG** did not antagonize the protective function of NT-3 upon NIH-TrkC cells (Figure 6B), nor the protective function of IGF-1 upon NIH-IGF-1R (data not shown). In control assays, mimetics **1-mm-TEG**, and **1-ll-TEG** had no significant effect on the survival-promoting effect of any growth factor, NGF (Figure 6A), NT3 (Figure 6B), or IGF-1 (data not shown). These inactive mimetics are highly related to mimetic **1-ss-TEG** and are therefore crucial controls.

Antagonism of NGF action by **1-ss-TEG** was dose-dependent. The survival promoted by 0.2 nM NGF (~40% of maximal survival) was reduced by **1-ss-TEG**. The mimetic at 5  $\mu$ M, 20  $\mu$ M, and 50  $\mu$ M respectively reduced the survival promoted by NGF from ~40% to ~25% (non-significant), and to a statistically significant ~15%, and ~0% survival (Figure 6C).

In the converse experiment, increasing doses of NGF could partially overcome the antagonism of 20  $\mu$ M **1-ss** (Figure 6D). In the NIH-TrkA cells that over-express TrkA, 20  $\mu$ M **1-ss** inhibited NGF-mediated survival with efficacy ranging from ~100% inhibition (0.08 nM NGF) to ~70% inhibition (0.4 nM NGF) to ~50% inhibition (2 nM NGF) to ~25% inhibition (10 nM NGF).

Lastly, we tested **1-ss-fluorescein** and **1-ss-TEG** antagonism of NGF activity in PC12 cells because they express very low levels of TrkA. Mimetics **1-ss-fluorescein** and **1-ss-TEG** (20  $\mu$ M) antagonized ~50% of the survival promoted by 10 nM NGF in serum-free media (Figure 6E). Similar antagonism of NGF activity was obtained using the 4-3.6 cell line that expresses medium levels of TrkA (data not shown).

Together, these data demonstrate that **1-ss** is an antagonist of NGF-dependent TrkA function, and that antagonism is dependent on the stoichiometry of **1-ss**/NGF as well as on the relative levels of cell surface TrkA.

### 3.7 **1-ss** Antagonizes NGF-independent TrkA activity

Over-expressed TrkA in NIH-TrkA cells has spontaneous ligand-independent trophic functions. Indeed, high baseline ligand-independent activity has been reported in cells over-expressing TrkA as well as for many other RTKs, and this activity is oncogenic [2,3]. Indeed, in the NIH-TrkA cells TrkA-dimers were observed in the absence of ligand (e.g. see Figure 5). For that reason, we hypothesized that mimetic **1-ss** could *accelerate* the death of NIH-TrkA cells in SFM, in the absence of NGF. This would reflect antagonism of baseline receptor activity, which is ligand-independent.

Mimetic **1-ss-TEG** significantly *accelerated* the death of NIH-TrkA cells in SFM, in the absence of NGF (Figure 7A). This effect was TrkA-selective, as it was not observed in control NIH-TrkC cells (Figure 7A) or NIH-IGF-1R cells (data not shown). Both of these control cell lines over-expressing RTKs also have high baseline receptor activity. In assays that control for compound, mimetics **1-mm-TEG**, and **1-ll-TEG** did not have a significant effect on ligand-independent activity of any of the three receptors (Figure 7A).

Accelerated death of NIH-TrkA cells in SFM by **1-ss-TEG** was dose dependent and significant at concentrations of 20  $\mu$ M and 50  $\mu$ M (Figure 7B). The actual OD<sub>490</sub> readings from MTT are shown in Table 1.

These data show the relatively high resistance of NIH-TrkA and NIH-TrkC cells to death in SFM, because they over-express a tyrosine kinase receptor and have a degree of ligand-independent activity. This relatively high background survival is not seen in cells that express lower TrkA density (e.g. PC12 cells) (Table 1). It is noteworthy that there was no detectable toxicity in MTT assays for any cell type (over-expressing NIH transfectants, PC12, or 4-3.6 cells) when they were cultured in serum containing media + the mimetics (see Table 1).

In sum, cells over-expressing RTKs exhibit a relative resistance to death in SFM. In NIH-TrkA cells the resistance is reduced by **1-ss-TEG**, resulting in the accelerated death of these cells. In contrast, the NIH-TrkC resistance is unaffected by **1-ss-TEG**. In addition, as reported in Figure 6, the survival promoted by NGF in NIH-TrkA and PC12 cells is reduced by **1-ss-TEG**. In contrast, the survival promoted by NT-3 in NIH-TrkC is unaffected by **1-ss-TEG**.

### 3.8 Antagonism of biochemical signals by 1-ss

We further characterized ligand-dependent and ligand-independent antagonism. We carried out biochemical analyses of receptor-tyrosine phosphorylation (pTyr) in untreated or ligand treated cells by western blotting using anti-phosphotyrosine antibodies (Figure 7). To show equal receptor loading the membranes were stripped and re-blotted with anti-TrkA mAb 5C3.

Untreated NIH-TrkA cells have significant basal TrkA-pTyr (Figure 7C **lane 1**), consistent with the ligand-independent receptor dimers reported in Figure 5 and ligand-independent survival activity reported in Figure 7 and Table 1. Ligand-independent TrkA-pTyr decreases significantly after culture with **1-ss-TEG** (20  $\mu$ M) for 2 hours, and decreases even when treated for a relatively short 20 minutes (Figure 7C **lane 1 versus 2; lane 5 versus 6**).

Treatment with NGF (2 nM) results in significant and sustained increases in TrkA-pTyr lasting longer than 2 hours (Figure 7C **lane 3, lane 7**). In these conditions, **1-ss** was able to reduce the level of TrkA-pTyr even in the continuous presence of NGF over a 2 hour period (Figure 7C, **lane 4**). Similar data were obtained when a downstream adaptor of activated TrkA, phospho-Akt (p-AKT), was studied (Figure 7D). NGF induces rapid p-Akt over baseline, which is inhibited by **1-ss**.

The biochemical data are consistent with the biological data, and indicate that **1-ss** has the ability to inhibit NGF-dependent, as well to inhibit the NGF-independent, activation of TrkA and signals downstream.

## Conclusions

Here we address whether and how dimerization of an agonistic monovalent ligand could generate an antagonist. We rationalized that if an agonistic ligand must induce conformational changes in a receptor, a relatively rigid bivalent small molecule ligand could “freeze” the receptor dimer by non-covalently cross-linking a receptor pair, and in actuality prevent conformational changes that may be necessary for activation. This is important because conformational changes in TrkA take place upon the binding of its protein ligand NGF have not yet been demonstrated through structural analyses and this is the matter of current debate (e.g. see letters to editor in [13]).

Both the monovalent parental ligand D3 and the bivalent ligand **1-ss** have high selectivity towards TrkA. While **1-ss** has higher affinity and efficacy at inducing or stabilizing TrkA-homodimers, **1-ss** also gained the ability to block NGF binding and to inhibit TrkA function. Inhibition of NGF binding is peculiar because the parental D3 molecule and NGF do not block each other, even though both bind to the D5 domain of TrkA [10]. In fact D3 potentiates NGF function [10]. For those reasons we expected that **1-ss** and NGF should not block each other.



On the other hand, we found that while pre-bound **1-ss** blocks NGF binding, pre-bound NGF does not block **1-ss** binding. How does **1-ss** block NGF binding to TrkA? Speculative *in silico* molecular docking (presented in the Supplemental Figure 3) suggest that the extended triazine linker bridge of **1-ss** “fills” the inter-receptor space where NGF would access its docking sites on the TrkA dimers. This would conceivably result in NGF and **1-ss** being able to bind simultaneously to TrkA, provided that NGF is bound first prior to **1-ss** blocking its access to the receptor. The biological outcome of concomitant binding by NGF and **1-ss** could result either in an overactive TrkA (i.e. NGF is “trapped”) or in an inactive TrkA (i.e. the dimer is “frozen” in the inactive state). Our biological and biochemical data supports the latter possibility, as **1-ss** antagonizes ligand-dependent TrkA-pTyr, and prevents the survival signals arising from TrkA activation.

The simplest way to explain the differences in the intrinsic function of **1-ss** (antagonist) compared to the parental **D3** mimetic (agonist and potentiator of NGF) would be in their ability to affect the activation state of the receptor. For example, receptor topology and inter-domain interactions have been suggested to be critical for TrkA receptor activation [18,28-30]. The specific receptor-receptor assembly required for activation may be affected by the linker of **1-ss**, and this may be key for changing an agonist into an antagonist. This notion is further supported by the fact that **1-ss** inhibits the ligand-independent (oncogenic) TrkA activation in receptor over-expressing cells, even though its binding results in increased detection of TrkA-TrkA dimers which are generally presumed to be the active form of receptor tyrosine kinases.

In sum, **1-ss** docks onto TrkA and acts as an antagonist of NGF binding. Logically, blocking NGF binding results in antagonism of the trophic activity of NGF. However, it is important to emphasize that antagonism by **1-ss** is not exclusively the result of blocking NGF binding, because functional antagonism can also take place in bioassays over long periods (48-72 hours) under conditions where the much higher affinity NGF ligand can bind to TrkA. The **1-ss** mimetic also acts as a dimer-promoting receptor antagonist, reducing the baseline ligand-independent TrkA activity and tyrosine phosphorylation in TrkA over-expressing cells.

Ligand-independent trophic functions in RTK over-expressing cells is oncogenic [2,3]. It would seem unusual that an antagonist would increase TrkA-TrkA dimers, because dimeric receptors are thought to be in the active state. There is a plausible albeit more complex explanation with precedents in the ErbB2 family of receptors. In that family, growth factor-induced dimerization causes receptor phosphorylation, then the phosphorylated receptors dissociate and each phosphorylated monomer can interact with a new (nonphosphorylated) receptor to form a secondary dimer that amplifies signals [31]. It is possible that **1-ss** inhibits the monomerization of phosphorylated TrkA dimers, thereby inhibiting signal amplification. Also, because one ErbB2 monomer can interact with one of two possible complementary surfaces on a second ErbB2 monomer [32], differential juxtapositioning of the dimer can alter the signaling; and it is attractive to speculate that this may also apply to TrkA.

We report on an agonistic ligand being converted to an antagonist through dimerization. This model can provide strategies to discover a new class of inhibitors or activators of NGF receptors, and the concept could be expanded to other members of the receptor tyrosine kinase family.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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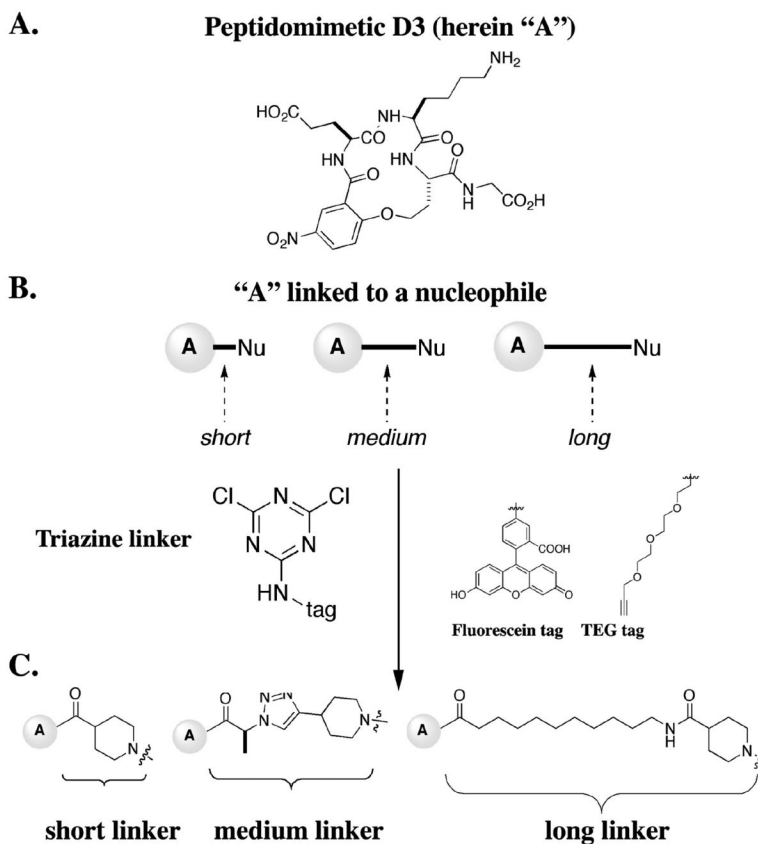
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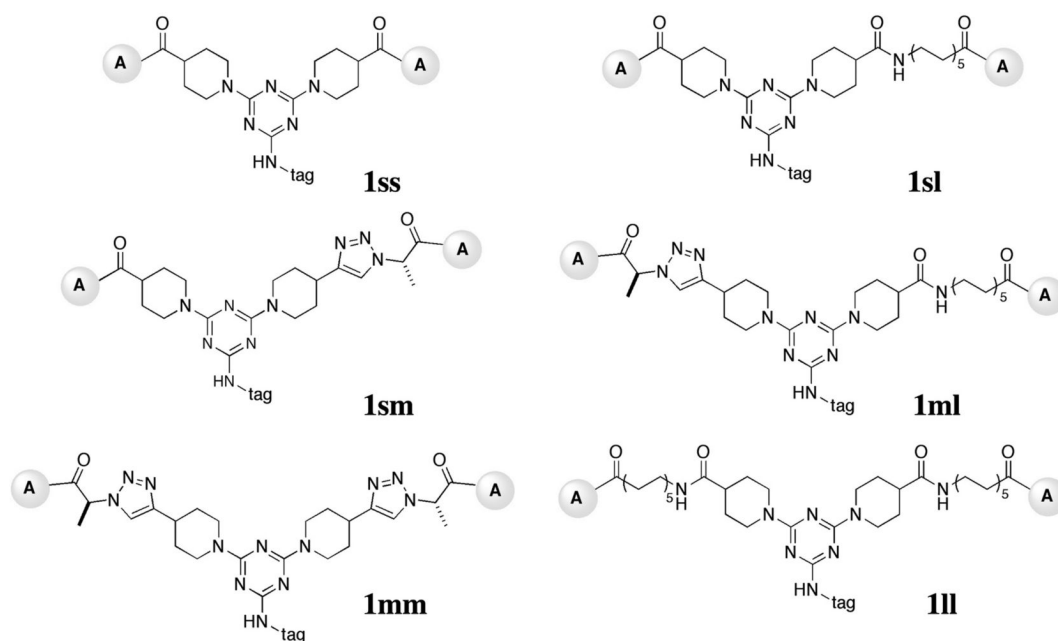
### Non-standard abbreviations used

FACScan	Fluorescence Activated Cell Scanner
FITC	Fluorescein isothiocyanate
TEG	Triethylene Glycol
Trk	Tropomyosin receptor kinase family
NGF	Nerve Growth Factor
p75	Neurotrophin Receptor
IGF-1	Insulin-like growth Factor-1
IGF-1R	Insulin-like growth Factor-1 Receptor

pTyr	Phosphotyrosine
RTK	Receptor Tyrosine Kinase

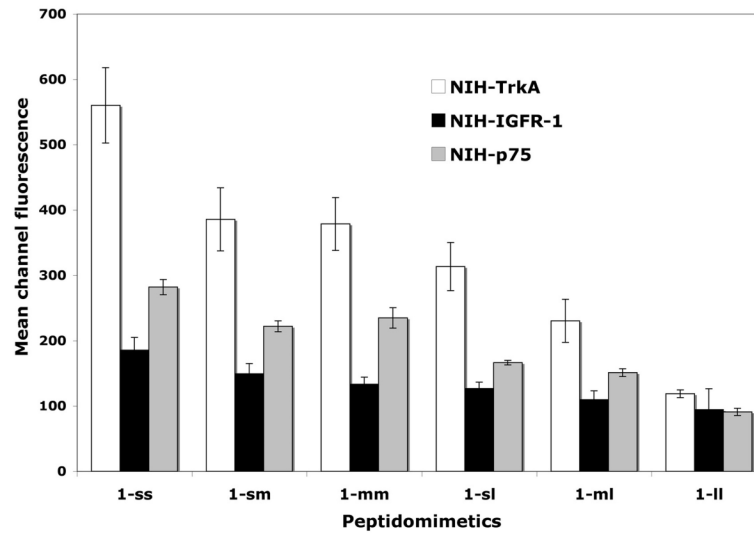


**FIGURE 1. Synthetic scheme for a homobivalent library**  
 (A) Lead compound D3 compound (herein **A** for simplicity) was used to make a set of bivalent molecules using a combinatorial approach. (B) Mimetic **A** was attached to a short **s**, medium **m**, or long linker **l** bearing a nucleophile. (C) These were then paired on a triazine scaffold to which a tag (either a fluorescein (FITC) or a TEG) were incorporated.

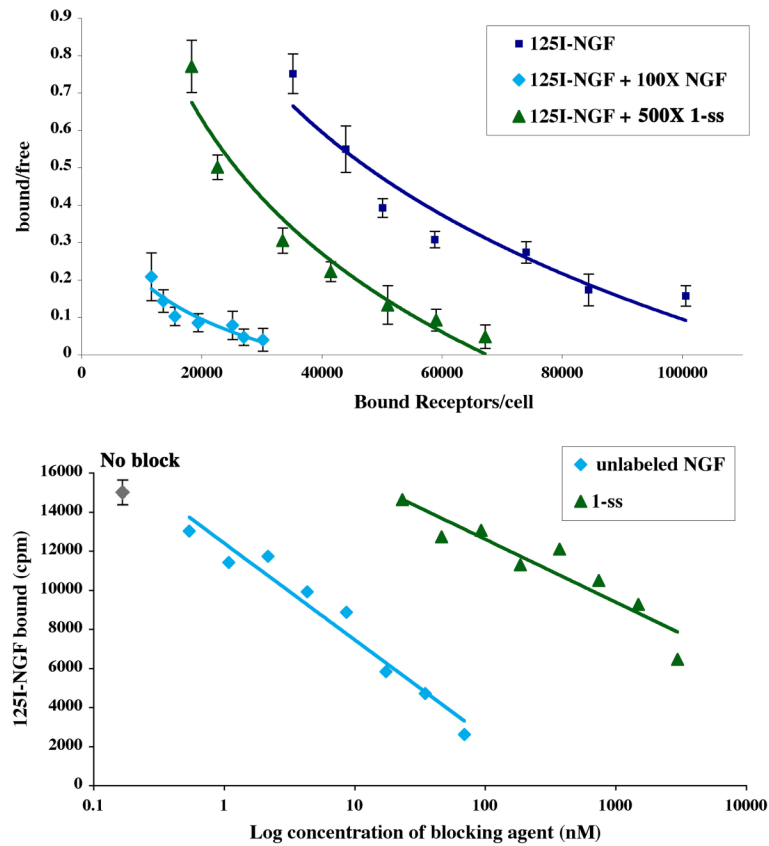


**FIGURE 2. Structure of the compounds 1-ss, 1-sm, 1-sl, 1-mm, 1-ml, and 1-ll.** The “ss” indicates a short-short linker length combination, “sm” a short-medium linker length combination, “sl” indicates a short-long linker length combination etc. Each of these structures can have a FITC tag or a TEG tag. Compounds were purified and their correct masses were verified.



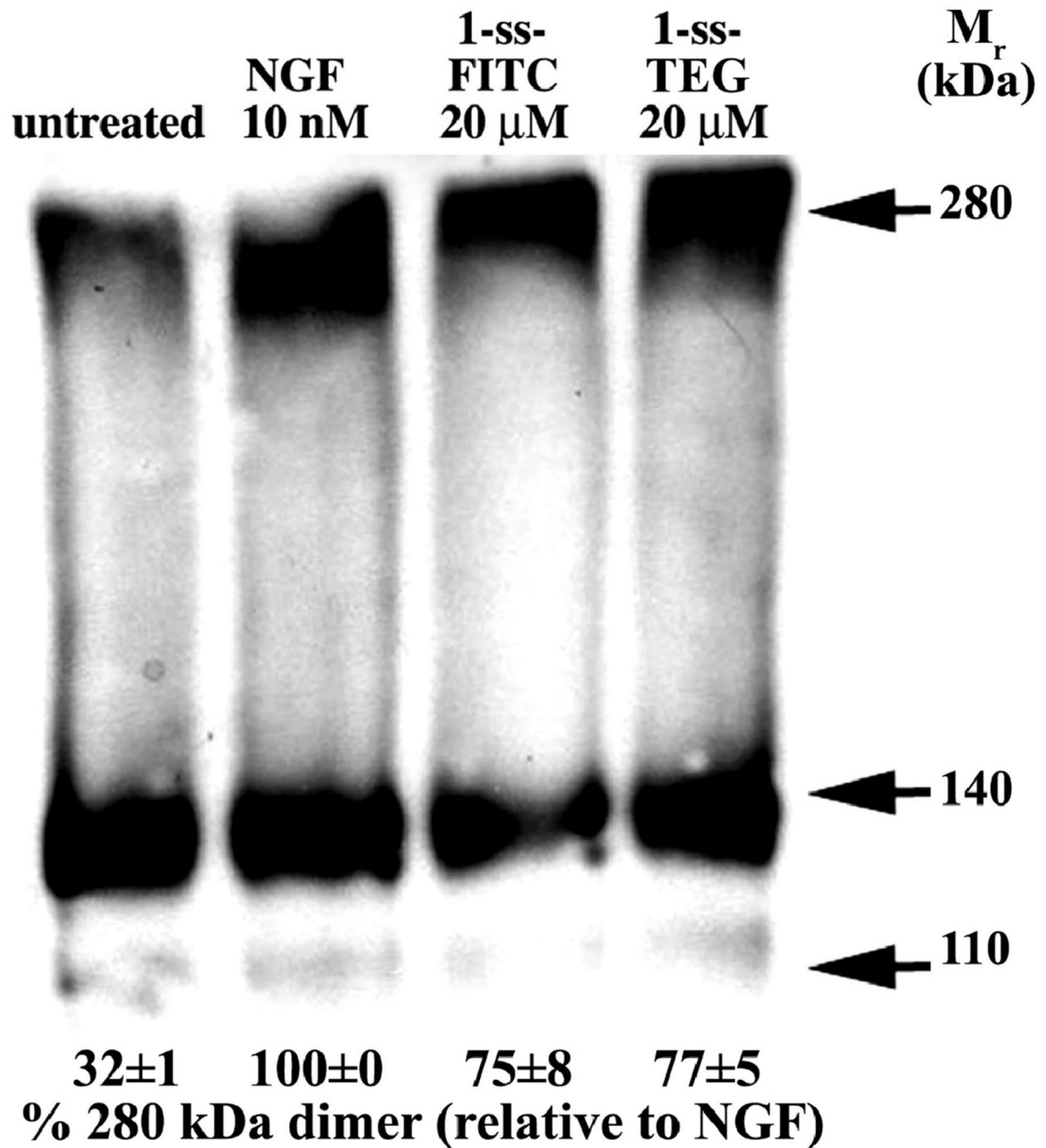


**FIGURE 3. Direct binding FACSscan assay with FITC-peptidomimetics**  
Cells expressing the indicated receptor were bound with test ligand (20  $\mu$ M) at 4°C. After washing, data was acquired and analyzed by FACSscan/CellQuest with background subtracted. Mean channel fluorescence  $\pm$  sem, n =3-6 independent experiments.



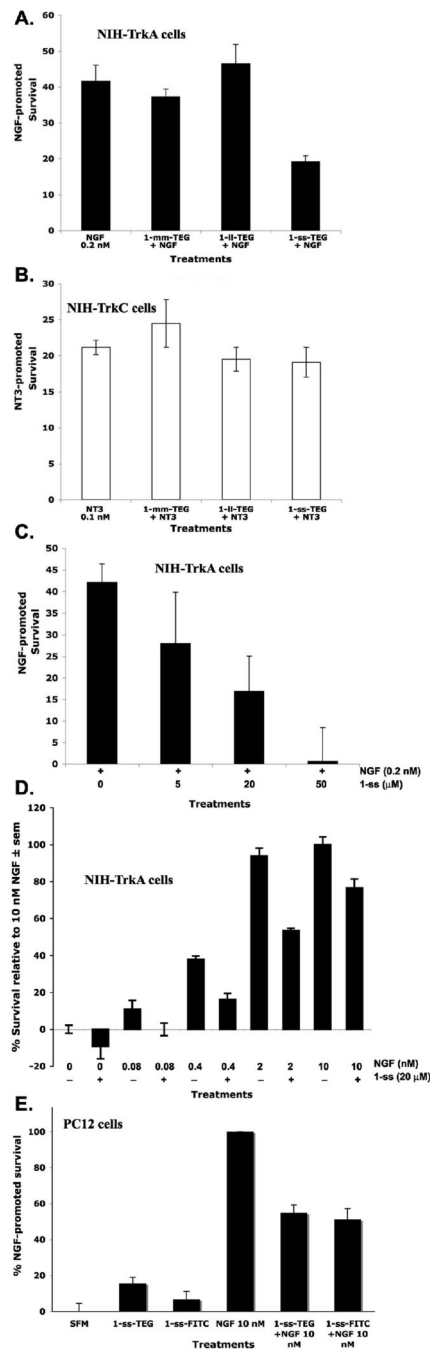
#### FIGURE 4. Inhibition of NGF binding to TrkA

NIH-TrkA cells were studied. (A) Scatchard plot analysis of high affinity  $^{125}\text{I}[\text{NGF}]$  binding data. Binding assays were carried out using a range of  $^{125}\text{I}[\text{NGF}]$  and a 100-fold excess of unlabeled NGF or a 500-fold excess of mimetic **1-ss** as inhibitors. (B) Displacement of a constant concentration of  $^{125}\text{I}[\text{NGF}]$  (~15,000 cpm) by increasing doses of unlabeled NGF or by mimetic **1-ss**. In all assays NIH-3T3 wild type cells were used to assess nonspecific background (<15% of total binding).



**FIGURE 5. NGF and 1-ss induce/stabilize cross-linking of TrkA dimers**

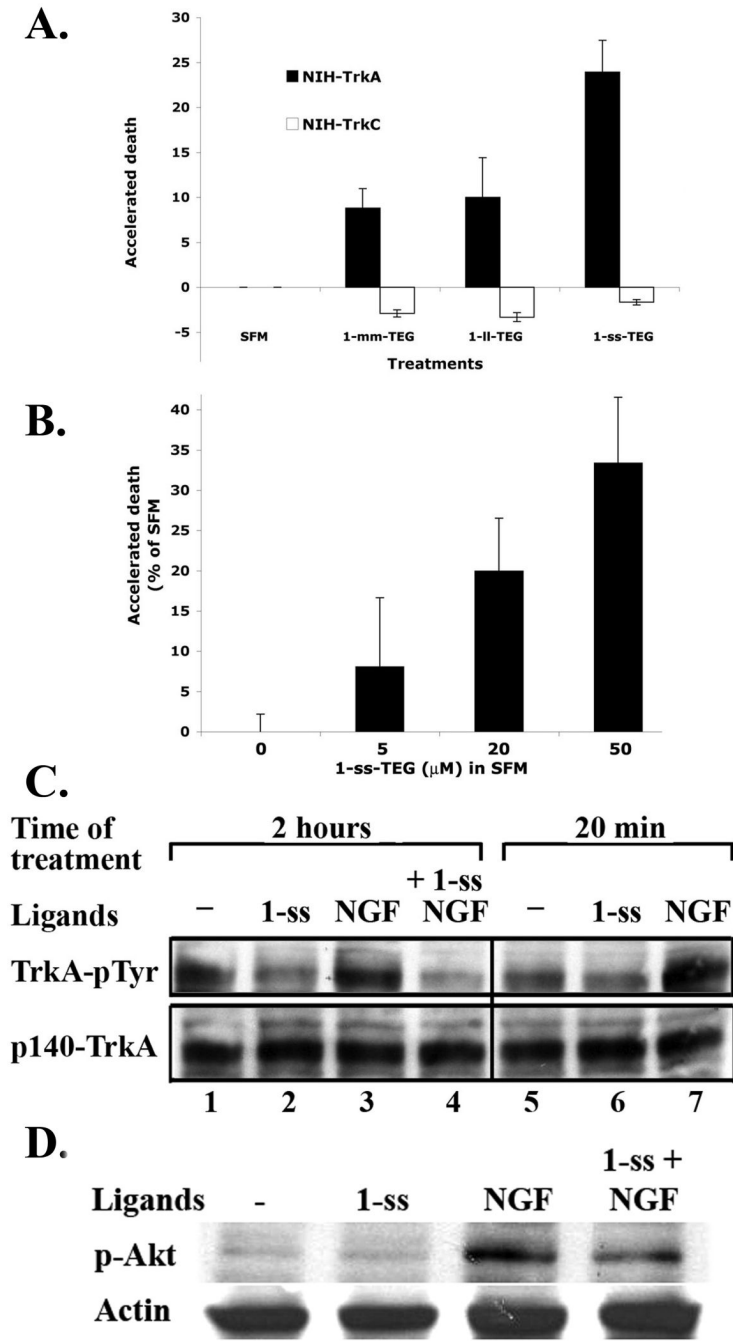
NIH-TrkA cells were studied. After exposure to the indicated ligand (30 min at 4°C), or to no ligand, cells were chemically cross-linked with DSS. After washing, cells were detergent solubilized and samples (20 μg protein/lane) were studied by western blotting with a highly specific anti-TrkA mAb 5C3. The amount of putative TrkA dimer (280 kDa) relative to optimal NGF-induced dimer (100%) is indicated. Note that untreated NIH-TrkA cells have ~32% of the level of cross-linked TrkA dimers found in NGF-treated cells.



**FIGURE 6. Mimetic 1-ss-TEG and 1-ss-fluorescein antagonize NGF and TrkA in cell survival assays**

NIH-3T3 cells expressing TrkA or TrkC, or PC12 cells were cultured in SFM alone or supplemented with growth factor (NGF for TrkA-expressing cells, NT-3 for TrkC-expressing cells). Survival was measured by MTT assays, and was calculated relative to optimal neurotrophin (100% protection). Suboptimal concentrations of growth factor (0.2 nM or 0.1 nM) were used to achieve limited survival. Results shown are average  $\pm$  SEM, from at least three independent experiments ( $n = 4$  per experiment). (A) Mimetic **1-ss-TEG** (20  $\mu$ M) antagonizes 0.2 nM NGF; but not (B) 0.1 nM NT-3. (C) Dose-dependent antagonism of TrkA-NGF by increasing doses of **1-ss-TEG** in NIH-TrkA cells over-expressing TrkA. (D)

Increasing doses of NGF oppose the antagonism of a constant dose of **1-ss-TEG** (20  $\mu$ M) in NIH-TkA cells over-expressing TrkA. **(E)** Antagonism of optimal (10 nM) NGF-induced survival in PC12 cells, expressing low levels of TrkA, using 20  $\mu$ M **1-ss-TEG** or **1-ss-fluorescein**.



**FIGURE 7. Mimetic 1-ss-TEG antagonizes baseline (ligand-independent) and ligand-dependent TrkA activity**  
**(A)** selective inhibition of ligand-independent TrkA baseline activity, but not TrkC baseline activity, accelerates the death of NIH-TrkA in SFM, **(B)** dose-dependent inhibition of TrkA baseline activity. **(C)** NIH-TrkA cells were cultured in SFM  $\pm$  the indicated ligands at 37°C in a cell incubator (NGF 2 nM; **1-ss** 20  $\mu$ M). After detergent solubilization the phosphotyrosine levels of the samples were studied by western blotting with anti-pTyr mAb 4G10. Total TrkA loading was verified on the same membranes with a highly specific anti-TrkA mAb 5C3. **(D)** Same as in (C) except that samples were studied by western blotting with anti-p-Akt mAb,



standardized versus actin loading control, after treatment with the indicated ligands for 20 minutes.

**TABLE 1**

Representative 3-day MTT assay of cell survival. The indicated cells were cultured in SFM in 96-well plates (n=6 wells/condition). The indicated wells were supplemented with serum (normal culture: growth + survival), or neurotrophin (NTF: NGF for TrkA-expressing cells, NT-3 for TrkC-expressing cells); with or without **1-ss-TEG** (20  $\mu$ M). The relative resistance to death in SFM is reduced by **1-ss-TEG** in NIH-TrkA but not in NIH-TrkC. Ligand-independent survival is not seen in PC12 cells, as these cells do not over-express TrkA. Furthermore, the survival promoted by NGF is reduced by **1-ss-TEG** in NIH-TrkA and PC12 cells, but the survival promoted by NT-3 is not reduced in NIH-TrkC.

TREATMENT	OD <sub>490</sub> ( $\times 1000$ ) values		
	TrkA-NIH	TrkC-NIH	PC12
5% serum	609 $\pm$ 28	706 $\pm$ 61	403 $\pm$ 13
5% serum + 1-ss-TEG	611 $\pm$ 22	700 $\pm$ 34	398 $\pm$ 10
NTF optimal (10 nM)	464 $\pm$ 12	535 $\pm$ 16	309 $\pm$ 8
NTF suboptimal (0.2 or 0.1 nM)	243 $\pm$ 17	269 $\pm$ 23	145 $\pm$ 25
SFM	127 $\pm$ 7	153 $\pm$ 4	29 $\pm$ 4
1-ss-TEG	59 $\pm$ 5	156 $\pm$ 5	32 $\pm$ 5
NTF suboptimal + 1-ss-TEG	155 $\pm$ 11	274 $\pm$ 19	42 $\pm$ 6