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A peptidomimetic of NT-3 acts as a TrkC antagonist

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Abstract

Neurotrophins are a family of growth factors that regulate the peripheral and central nervous system. We designed and tested a mini-library of small molecules peptidomimetics based on β -turns of the neurotrophin growth factor polypeptides NT-3, which is the natural ligand for TrkC receptors. Biological studies identified a peptidomimetic **2CI** that exhibited selective antagonism of TrkC. **2CI** reduces TrkC-activation and signaling promoted by NT-3, and selectively blocks ligand-dependent cell survival. **2CI** also blocks ligand-independent TrkC activation and signals that take place when the receptor is over-expressed. This work adds to our understanding of how the neurotrophins function through Trk receptors, and demonstrates that peptidomimetics can be designed to selectively disturb neurotrophin-receptor interactions, and receptor activation.

Introduction

Neurotrophins are dimeric polypeptide growth factors that regulate the peripheral and central nervous systems and other tissues. Neurotrophins (Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), and Neurotrophin-3 (NT-3)), as well as their cell surface receptors (p75, TrkA, TrkB, and TrkC) are validated targets for therapeutics in a variety of pathologies ranging from cancer to neurodegeneration [19,20].

All the neurotrophins bind the p75 receptor [13], a member of the tumor necrosis factor (TNF) receptor superfamily. The p75 receptor has multiple functions depending on the cells in which it is expressed, whether a ligand engages it, and many other factors [3,4]. This makes its function difficult to predict and control pharmacologically.

On the other hand, many neurotrophic activities arise from ligand binding preferentially and with selectivity to the Trk family of receptors. For example, NGF docks with TrkA, BDNF binds preferentially to TrkB [1,21], whereas NT-3 interacts preferentially with TrkC but can also bind to TrkA [5]. Trk receptors are typical receptor tyrosine kinases (RTKs), with an ectodomain, a single transmembrane region, and an intracellular tyrosine kinase catalytic

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domain. The extracellular domain of Trk binds the ligand, leading to activation of the tyrosine kinase, phosphorylation (pTyr) of the Trk intracellular domain, and signal transduction cascades involving associated partners such as MAPK and Akt [6].

However, over-expression of Trk receptors can also cause activation of signals in a ligand-independent manner [23]. This has been reported for Trks and for many other RTKs, and over-expression is thus pro-oncogenic [19,20]. Thus it would be important to develop Trk antagonists as tools to better understand how these receptors function.

Previously we used protein mimicry of neurotrophin β -turns to develop β -turn cyclic peptides and organic peptidomimetics of neurotrophins [2,11,12,14,15]. To date, we have reported on selective agonists for TrkA [15] or TrkC [24]. Here, we sought to develop antagonists by mimicry of NT-3. We designed a library of peptidomimetics with an organic backbone, and a pharmacophore based on β -turn NT-3 structure. The aim was to target the extracellular domain of the TrkC and TrkA receptors, because NT-3 can bind to both TrkC and TrkA [5].

The resulting new agents were tested in biological screens which lead to the identification of a small molecule NT-3 peptidomimetic **2CI** that is a selective antagonist of TrkC receptors, but which does not affect TrkA receptors. This antagonist blocks ligand-dependent as well as ligand-independent TrkC activity. This reagent may help us understand how the neurotrophins function via Trk receptors.

1. Materials and methods

1.1 Synthesis of peptidomimetics

For a view of the peptidomimetic structures see Figure 1. The pharmacophores are described in Table 1. The syntheses protocols of the compounds are published [8,9,24], and are described below in sections 1.1.1, 1.1.2, and 1.1.3; and schematically in scheme 1 and scheme 2.

1.1.1 Procedure for Coupling Compound 1 to Polystyrene Wang Resin—

Polystyrene resin functionalized with a 4-hydroxybenzyl linker (800 mg, 1.30 mmol/g, 1.04 mmol), template **1** (500 mg, 1.1 mmol), diisopropylcarbodiimide (172 mg, 1.1 mmol), HOBt (148 mg, 1.1 mmol), and *N,N*-diisopropylethylamine (348 μ L, 2.0 mmol) in 8 mL of 4:1 dichloromethane/*N,N*-dimethylformamide were gently stirred at 25 °C for 12 h. The resin was filtered, washed, and treated with 10 equiv of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in DMF for 1 day. After washing and drying, the loading of the resin was determined to be 0.78 mmol/g by UV analysis (405 nm, $\epsilon = 37000$) of the trityl content of a known amount of resin in 10% trifluoroacetic acid in dichloroethane. Subsequent couplings of amino acids were performed following the general procedure given for peptidomimetics below.

1.1.2 Procedure for Coupling Compounds 4 to Polystyrene-Rink Resin—

Rink resin (1.4 g, 1.0 mmol/g) was swelled in CH_2Cl_2 (10 mL/g) in a fritted syringe for 30 min. The Fmoc group on the resin was removed by treating the resin with 20% piperidine in DMF (2 $\times$ 5 mL, 10 min and then 15 min). The resin was washed, after which templates **4** (3 equiv), HBTU (3 equiv), HOBt (3 equiv), and DIEA (5 equiv) in DMF (2.0 mL) were added. After 1 h of gentle shaking, a ninhydrin test on a small sample of beads gave a negative result. The reaction mixture was drained, and the resin was subjected to the above washing cycle. The loading of the resin was determined to be 0.46 mmol/g by UV analysis.

1.1.3 General Experimental Procedure for Preparation of the Macrocylic

Peptidomimetics—The resin containing template **1** was treated with Fmoc-Lys-(Boc)-OH (4 equiv), PyBroP (4.8 equiv), and 2,6-lutidine (15 equiv) in CH_2Cl_2 (1.5 mL) for 24 h. After washing and Fmoc deprotection, the resin was then treated with Fmoc-Gly-OH (3 equiv),

PyBOP (3 equiv), HOBt (3 equiv), and DIEA (5 equiv) in DMF (3 mL) for 5 h. The washing cycle and Fmoc deprotection were repeated, and 2-fluoro-5-nitrobenzoic acid moiety was introduced by treating the resin with 2-fluoro-5-nitrobenzoic acid (3 equiv) PyBOP (3 equiv), HOBt (3 equiv), and 2,6-lutidine (15 equiv) in CH₂Cl₂/DMF (1:1, 3 mL) for 5 h. The side chain protecting group (Trt) of the template was removed by treatment with 1% TFA and 5% HSiPr₃ in CH₂Cl₂ (7×2 min, or until color disappeared). After the resin was washed, the macrocyclization step was carried out by treating the supported peptide with K₂CO₃ (10 equiv) in DMF at 25 °C. After gentle shaking for 2 d, the peptide-resin was washed then dried in vacuo for 4 h. The peptide was cleaved from the resin by treatment with a mixture of 90% TFA, 5% HSiPr₃, and 5% H₂O for 3 h. The cleavage solution was separated from the resin by filtration. After most of the cleavage cocktail (about 90%) was evaporated in vacuo, the crude peptide was triturated using anhydrous ethyl ether, dissolved in H₂O, and then lyophilized to give the crude product. Preparative HPLC (Beckman System, 10–80% MeCN in H₂O + 0.1% TFA in 40 min) was carried out to provide a yellowish powder of **2Ag** (5 mg, 43%) obtained as an ammonium salt. In general, all the products were in their protonated forms.

1.2 Cells

NIH-3T3 cells are mouse fibroblasts that do not express any neurotrophin receptors. Parental NIH-3T3 cells were stably transfected with the indicated receptors, using plasmids also encoding for neomycin resistance. After long-term neomycin drug selection and sub-cloning, the cells were characterized to express the appropriate functional cell surface receptor. Stable clones of NIH-TrkC express ~100,000 TrkC receptors/cell, NIH-TrkA express ~200,000 TrkA receptors/cell, and NIH-IGF-1R express ~100,000 IGF-1R receptors/cell. These cells, and their functional responses to the appropriate growth factor have been reported [24].

1.3 Cell Survival Assays

Cell survival was measured quantitatively by the MTT assay and optical density (OD) readings, as previously described [14,15], in 96-well plates. Cells in SFM die by apoptosis, but can be rescued if supplemented with the appropriate growth factor if they express functional receptors. Wells were not supplemented (negative control), or were supplemented with suboptimal or optimal concentrations of the indicated growth factor. NIH-TrkA cells respond to NGF, NIH-IGF-1R cells respond to IGF-1, and NIH-TrkC cells respond to NT-3. Test peptidomimetics or vehicle control were added to the two culture conditions above, namely, in SFM or SFM supplemented with growth factor. Cellular controls are provided by lack of effect on NIH-TrkA or NIH-IGF-1R with effect on NIH-TrkC, as evidence of selectivity. Lack of general toxicity was assessed by testing compounds on all cells growing in normal serum conditions (data not shown). All assays were repeated independently at least three times (n = 4 for each assay). MTT data are standardized to optimal dose of neurotrophin = 100% survival, and SFM = 0% survival, using the formula $[(OD_{\text{test}} - OD_{\text{SFM}}) \times 100 / (OD_{\text{optimal NTF}} - OD_{\text{SFM}})]$.

1.4 Signal Transduction Assays

Cells were stimulated with solvent (negative control), optimal concentrations of growth factor alone (positive control) or growth factor plus **2Cl** at 50 μM as indicated. Detergent lysates were resolved in SDS-PAGE and analyzed by Western blotting with anti-phosphotyrosine (pTyr) antibody 4G10 (Upstate Biotechnology, Lake Placid, NY), or anti-phospho-MAPK (New England Biolabs), or anti-phospho-Akt (Ser473) antibody (New England Biolabs). After stripping, membrane was reblotted with anti-actin (Sigma) to gauge protein loading. Quantification was done by densitometric analysis [16]. For receptor pTyr assays the stimulation was for 10 min. For phospho-Akt assays stimulation was for 5 or 15 min as indicated. For phospho-MAPK assays ligand stimulation was for 15 min.

2 Results

2.1 Synthesis of peptidomimetics

We designed a library of 12 peptidomimetics based on the β -turn structure of NT-3 (Figure 1). Their synthesis [8,9] and the codes [24] used to name these compounds were based on our previous literature. The code contains a number, and two letters.

The number of the code corresponds to the ring closure (containing an S or an O), the first letter of the code corresponds to the appendix ($-\text{NH}\text{SO}_2$ or $-\text{NH}_2$), and the second letter of the code corresponds to the dipeptide side chains displayed at the β -turn. For an abbreviated summary of the dipeptide see Table 1. The compounds were purified, and characterized by 1D-NMR and LC-MS (data not shown).

2.2 Selective inhibition of TrkC-mediated cell survival

12 peptidomimetics were tested in assays of cell survival, using the quantitative MTT method. Cells undergo apoptotic death when cultured in serum-free medium (SFM). NGF protects TrkA-expressing cells, IGF-1 protects IGF-1R-expressing cells, while NT-3 protects TrkC-expressing cells from apoptotic death in SFM. Growth-factor protection in SFM is dose-dependent and time-dependent. Optimal concentrations of growth factor afford maximal protection (2 nM, 100%). Suboptimal concentrations of growth factor (0.2 nM) protect at ~30% of maximal (in a 3 day bioassay) or at ~60 % of maximal (in a 2 day bioassay).

First, the peptidomimetics were tested for their effect on NGF or NT-3-mediated survival. From the 12 compounds, **2CI** significantly lowered the survival induced by NT-3 (Figure 2A). In selectivity controls, **2CI** had no effect on the survival of TrkA-expressing cells responding to 2 nM NGF, and did not even affect sub-optimal doses of NGF either (Figure 2B).

Further survival assays were performed to test dose-dependent effects of **2CI** (5, 10, 20 μM) on the metabolism of NIH-TrkC, NIH-TrkA or NIH-IGF-1R (Figure 3). These assays were performed in the presence of growth factor to test for antagonism of ligand-dependent signals (as above), and also in the absence of growth factor to test the effects of the compounds on ligand-independent receptor activity.

In the absence of neurotrophins **2CI** at 10 μM or at 20 μM significantly reduced the metabolism of NIH-TrkC cells ($p < 0.05$ versus untreated control); but had no effect on NIH-TrkA or NIH-IGF-1R (Figure 3). These data suggest that **2CI** antagonizes ligand-independent TrkC activity selectively. Lack of effects on the growth of NIH-TrkA or NIH-IGF-1R, and lack of general toxicity when cells are grown in normal serum conditions (data not shown) suggest that the reduced metabolic activity of NIH-TrkC may be due to reduced TrkC signaling.

The same dose-dependent assays were performed in the presence of neurotrophins; to further test the effects of the compounds on ligand-dependent receptor activity. The NT-3 induced survival of NIH-TrkC cells was also blocked significantly by **2CI** at 10 μM or at 20 μM ($p < 0.05$ versus untreated control). These doses of **2CI** had no effect on the NGF-induced survival of NIH-TrkA or on the IGF-1-induced survival of NIH-IGF-1R (Figure 3). These data suggest that **2CI** antagonizes ligand-dependent TrkC signaling selectively.

2.3 Selective inhibition of TrkC-mediated signal transduction

To confirm the antagonistic activity of **2CI** we analyzed signal transduction biochemically (Figure 4). The tyrosine phosphorylation of the receptors (Figure 4A), the phosphorylation of Akt (Figure 4B), and the phosphorylation of MAPK (Figure 4C) were studied in western blots of cell lysates, after stimulation of cells with the appropriate growth factor $\pm$ **2CI**.

In NIH-TrkC cells NT-3 induces strong TrkC-pTyr, and the phosphorylation of Akt and MAPK; indicating activation. **2CI** decreases these signals. Inhibition was significant for all treatments ($p < 0.05$ versus NT-3 stimulated control). The only exception was that the inhibition of phospho-Akt was significant versus control for the 15 minute of stimulation with NT-3, but not for the time point of 5 minute of stimulation with NT-3. In selectivity control assays, **2CI** does not reduce the activating signals that NGF affords through TrkA, or the activating signals that IGF-1 affords through IGF-1R. These data indicate that an NT-3 derived peptidomimetic **2CI** can inhibit NT-3-dependent activation and tyrosine phosphorylation of TrkC and of signals downstream of TrkC; and these data are consistent with the effects reported on ligand-dependent survival bioassays.

As well, the inhibition of ligand-independent receptor pTyr by **2CI** was tested in biochemical assays (Figure 4D). A fraction of the TrkC or the TrkA receptors in transfected NIH-3T3 cells are tyrosine phosphorylated in the absence of ligand activation. Treatment of the cells with **2CI** for 15 minutes at 37°C reduced the pre-existing levels TrkC-pTyr, but did not affect the pre-existing levels of TrkA-pTyr; relative to actin control. These data were reproduced in three independent assays. However, these biochemical data are not quantified by densitometry because to reveal the ligand-independent Trk-pTyr the X-ray films are generally over-exposed and outside the linear range. Nevertheless, these data does show qualitatively that **2CI** selectively antagonizes ligand-independent TrkC activation; and the data are consistent with the effects reported on ligand-independent survival bioassays.

3 Discussion

Here we report on the biological characterization of a library of twelve small molecule peptidomimetics designed to mimic NT-3, one of which termed **2CI**, acts as a selective TrkC antagonist in bioassays and in biochemical assays. Antagonism of TrkC by **2CI** was evident in inhibiting ligand-independent activity (which is often oncogenic) as well as a more conventional antagonism of NT-3-dependent activity.

The easiest interpretation is that **2CI** antagonizes receptor signals by binding to TrkC selectively. However, we have no direct binding data to TrkC, as we failed to obtain labeled ligands (either **2CI** or NT-3) for binding studies. Thus, we can not formally rule out that **2CI** could potentially bind to NT-3 itself and neutralize it, therefore causing antagonism. Against that possibility, however, we show that **2CI** selectively antagonizes ligand-independent TrkC activity (e.g. in the *absence* of growth factor); thus at least some of the activity takes place at the TrkC receptor. Antagonism of ligand-dependent activation can be most easily explained in terms of competitive antagonism, meaning that the small molecule prevents the binding of the natural ligand to the receptor. This could be anticipated from an NT-3 mimetic.

However, in previous work we made NT-3 mimetics with agonistic activity [8,9,24]. These mimetics have a different pharmacophore than **2CI**, and can bind to and activate both TrkC and to TrkA. While this is consistent with the ability of NT-3 to bind to and activate both receptors [5], the selectivity of **2CI** suggests that it interacts functionally only with TrkC. Hence, it seems that the unique **2CI** pharmacophore is more restricted compared to the other mimetics. In addition, **2CI** is an antagonist, whereas the other agents are partial agonists.

The mechanism by which small molecule **2CI** may antagonize the ligand-independent oncogenic activation of a RTK is intriguing. It is speculated that over-expressed RTKs, including TrkC, may require lateral or rotational mobility within the membrane to become activated [10,17,18,22]. It is possible that **2CI** restricts this mobility thereby causing antagonism. It is also conceivable that **2CI** prevents events that are required for receptor

activation such as receptor-receptor dimerization, or dimer stabilization, or receptor conformational changes [6,7].

This compound is further proof that small molecule mimetics can be developed from neurotrophin β -turns. While previous work using this strategy resulted in peptidomimetics with partial agonistic activity, the present work is the first example of a TrkC antagonist.

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Figure 1.
Structures of NT-3 peptidomimetics, following the codes reported in [24].

Figure 2. 2Cl antagonizes TrkC in cell survival assays

NIH-TrkC cells (A) or NIH-TrkA cells (B) were cultured in SFM supplemented with optimal or sub-optimal concentrations of the appropriate growth factor (NGF for TrkA, NT-3 for TrkC). Test compounds were added (50 μ M) to these conditions. Survival was measured in MTT assays, and was calculated relative to optimal neurotrophin (100% protection). Results shown are average $\pm$ SEM, from at least three independent experiments ($n = 4$ per experiment). (C) the structure of 2Cl.

Figure 3. 2Cl antagonizes TrkC in a ligand-dependent and ligand-independent manner

NIH-3T3 cells expressing the indicated receptor were tested by culture in SFM supplemented with or without the appropriate growth factor (NGF for TrkA, NT-3 for TrkC, IGF-1 for IGF-1R). The indicated concentrations of **2Cl** were added $\pm$ growth factor. Results shown are average $\pm$ SEM, from at least three independent experiments ($n = 4$ per experiment). Cell survival in serum-free media is set to 0%, and optimal growth factor to 100%. **2Cl** selectively accelerates cell death through TrkC (in the absence of NT-3) by antagonizing ligand-independent TrkC activity, and also reduces NT-3-mediated cell survival. * indicates $p < 0.05$ versus control.

Figure 4. 2Cl antagonizes TrkC-mediated signal transduction

NIH-TrkC or NIH-TrkA or NIH-IGF-1R cells were exposed to vehicle control, or optimal concentrations of the appropriate growth factor with or without 2Cl (50 μ M) as indicated. Detergent lysates were analyzed by Western blotting with (A) anti-pTyr mAb 4G10 (the M_r of the receptors in reducing gels are indicated by arrows), (B) anti-phospho-Akt, or (C) anti-phospho-MAPK. After stripping, membranes were re-blotted with anti-actin. Data were quantified by densitometry. (D) NIH-TrkC or NIH-TrkA cells were exposed to vehicle control or to 2Cl (50 μ M) for 15 min. Detergent lysates were analyzed by Western blotting with anti-pTyr mAb 4G10. After stripping, membranes were re-blotted with anti-actin. Data were not

quantified by densitometry due to over-exposure of films to reveal ligand-independent Trk-pTyr.

Scheme 1.

Scheme 2.

Table 1**Structure Codes and Corresponding Amino Acids**

The codes listed correspond to those used previously. Compound **2CI** has the combination of amino acids (Arg-Val), given the code “I”.

Code	Amino <i>i</i> + 1	Acid <i>i</i> + 1
2Ah	Asn	Ile
2Ag	Lys	Gly
2Af	Gly	Thr
2Ac	Gly	Lys
2Ak	Gly	Arg
2Aj	Asn	Glu
2Ca	Lys	Ile
2Cg	Lys	Gly
2CI	Arg	Val
2Ai	Thr	Lys
2Cb	Asn	Asn
3Cb	Asn	Asn