Multipotent Neurotrophin Antagonist Targets Brain-Derived Neurotrophic Factor and Nerve Growth Factor

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are members of the neurotrophin family that normally play a role in the development and maintenance of the nervous system. However, neurotrophin dysregulation has been implicated in several neurodegenerative diseases and psychiatric disorders including Alzheimer’s disease, Parkinson’s disease, neuropathic pain, depression, and substance abuse. Despite their central role in the nervous system, neurotrophins have proved to be an elusive pharmacological target. Here, we describe a novel multipotent neurotrophin antagonist, 3-[[5-(4-sulfamoylphenyl)-2-furyl]methylene]-3-methyl-5-oxo-pyrazol-1-yl]benzoic acid (Y1036). Y1036 binds BDNF (K_D = 3.5 ± 0.3 μM) and NGF (K_D = 3.0 ± 0.4 μM) preventing either BDNF or NGF from interacting with their obligate receptors. Y1036 prevents both BDNF- and NGF-mediated trk activation, downstream activation of the p44/42 mitogen-activated protein kinase pathway, and neurotrophin-mediated differentiation of dorsal-root ganglion sensory neurons. Identification of a BDNF- and NGF-specific antagonist is of considerable interest in the study and treatment of diseases where dysregulation of multiple neurotrophins has been implicated.

Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) regulate the survival, differentiation, and growth of neurons both centrally and peripherally (Lu et al., 2005; Chao et al., 2006). BDNF and NGF are neurotrophins that interact with two distinct receptor classes. The common neurotrophin receptor, p75^NTR, binds both BDNF and NGF; whereas the structurally unrelated and selective trkB and trkA receptors bind BDNF and NGF, respectively (Barker, 2007; Hu and Russek, 2008).

Neurodegenerative diseases are commonly found to have associated BDNF and NGF dysregulation. For example, BDNF and NGF levels are elevated in the cerebrospinal fluid of patients with Parkinson’s disease (Nagatsu et al., 2000). Dysregulation of these neurotrophins have also been found in Alzheimer’s disease, Huntington’s disease, and amyotrophic lateral sclerosis (Hu and Kalb, 2003; Chao et al., 2006; Pehar et al., 2007). Both neurotrophins have been implicated in exaggerated pain states such as inflammatory and neuropathic pain (Pezet and McMahon, 2006; Schulte-Herbrüggen et al., 2007). Likewise, common psychiatric disorders such as depression and substance abuse have also been associated with BNDF and NGF dysregulation (Chao et al., 2006). Thus, identifying a small-molecule inhibitor capable of modulating BDNF and NGF may be of broad therapeutic interest.

Small-molecule-based NGF antagonists (which bind NGF rather than the trkA or p75^NTR receptors) have been described previously (Hefti et al., 2006). For example, ALE-0540 interacts with NGF inhibiting binding to trkA or p75^NTR (Owolabi et al., 1999). Ro 08-2750 is also an effective NGF-binding agent and prevents NGF from interacting with either receptor, but higher concentrations are required to affect trkA (Niederhauser et al., 2000). PD90780 also binds NGF, but it is more effective at blocking NGF/p75^NTR interaction than NGF/trkA (Spiegel et al., 1995; Colquhoun et al., 2004). However, none of these NGF-interacting antagonists are reported to be active against BDNF.

Efforts to antagonize the effects of BDNF by use of anti-BDNF antiserum or a trkB-IgG construct have been
successful in models of neuropathic pain and inflammatory hypersensitivity (Matayoshi et al., 2005). However, to date, no small-molecule antagonists of BDNF have been described.

In this study, we describe Y1036, a small-molecule neurotrophin-interacting antagonist that is active against BDNF and NGF. We demonstrate that Y1036 antagonizes either BDNF or NGF, preventing neurotrophin-induced receptor activation and downstream signaling via the p44/42 MAPK pathway.

Materials and Methods

Molecular Modeling and In Silico Docking. Molecular modeling and in silico docking of Y1036 to NGF [RCSB Protein Data Bank ID 1bet (McDonald et al., 1991)] and BDNF [RCSB Protein Data Bank ID 1bnd (Robinson et al., 1995)] were carried out by use of the software program Sybyl 7.3 (Tripos, St. Louis, MO). Neurotrophin structures were prepared for docking by use of the Biopolymer suite of Sybyl 7.3. Costructures were deleted and hydrogens added, the appropriate formal charges were applied to the N and C termini, and the structure was optimized by use of the MMFF94 molecular

Fig. 1. Identification of Y1036 as a potential multipotent neurotrophin antagonist. a, the protein sequences of BDNF and NGF are highly conserved [50% fully conserved (blue), 16% functionally conserved (green), and 11% weakly conserved (black); nonconserved residues are unmarked]. Yellow-highlighted residues were used to construct the putative neurotrophin antagonist targeting domain of a given protomer. b, the neurotrophin molecular surfaces are colored according to local electrostatic potential: red, positive; green, neutral; and blue, negative. Dashed-red box indicates location of the neurotrophin-targeting domain. c, the chemical structure of Y1036 (left); minimized energy state of Y1036 (right). d, red and blue arrows are landmarks in the schematic representation of Y1036 docked to BDNF or NGF as determined by molecular modeling. Y1036 is rendered as a ball-and-stick formation. Residues of the neurotrophin-targeting domain are rendered as capped stick formations. Atoms are colored according to their types: C, gray; N, blue; O, red; H, cyan.
mechanical force field (Halgren, 1990). Flexible docking of Y1036 was performed by use of the Surflex-Doc suite (Jain, 1996) incorporated into Sybyl 7.3. The docking protomol (molecular space) was generated to include the residues highlighted in Fig. 1a with a bloat factor of 0 and a threshold value of 0.5. For the Surflex-Doc function, the angstroms to expand the search grid was set at 6 and the maximum confirmations per fragment was set to 20.

**Analytical Ultracentrifugation.** Analytical ultracentrifugation was performed by use of a Beckman XL-A according to Niederhauser et al. (2000). Equimolar concentration of recombinant BDNF (Peprotech; Rocky Hill, NJ), NGF (Serotec, Raleigh, NC), or lysozyme control (Sigma-Aldrich, St. Louis, MO) was incubated with 20 μM Y1036 for 1 h at 25°C in 10 mM Tris-HCl buffer, pH 7.3. Sedimentation equilibrium was established after centrifugation for 17 h at 17,000 rpm (10°C) by use of a Proteome Lab XL-A analytical ultracentrifuge with a four-place rotor (Beckman Instruments, Palo Alto, CA).

**Size Exclusion Chromatography.** PD10 chromatography columns (Millipore Corporation, Billerica, MA) were pre-equilibrated in 10 mM HEPES, pH 7.35, + 0.1% bovine serum albumin. Either BDNF (10 μg) or NGF (10 μg) was incubated in 100 μl of 10 μM Y1036 for 1 h at room temperature. After incubation, the reaction mixture was loaded on the column, and 250-μl fractions were collected. Fractions were analyzed for NGF or BDNF via SDS-PAGE electrophoresis followed by fluorescent staining densitometry (Fluorothene; ChemAdvance, Ottawa Canada) by use of a FX Pro fluorescent scanner (Bio-Rad Laboratories, Hercules, CA). Fractions were also analyzed for Y1036 by use of a UV/vis-1700 spectrophotometer (Shimadzu, Kyoto, Japan) at 450 nM.

**Equilibrium Dialysis.** Affinity determination for binding to neurotrophins was performed with use of the rapid equilibrium dialysis system (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Varying concentrations of Y1036 in 10 mM HEPES buffer, pH 7.35, + 0.01% bovine serum albumin were added to individual sample and equilibrium compartments. Ten

**TABLE 1**

<table>
<thead>
<tr>
<th>Chemical properties of Y1036</th>
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<tr>
<td>Molecular formula C_{17}H_{14}N_{2}O_{6}S_{3}</td>
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<tr>
<td>LogP</td>
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<td>LogSW</td>
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<td>Rotatable bonds</td>
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<tr>
<td>HAcc</td>
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<td>tPSA</td>
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<tr>
<td>K_D, μM BDNF</td>
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<td>NGF</td>
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*3PSA, topographical polar surface area.

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**Fig. 2.** Y1036 binds to BDNF and NGF. The sedimentation equilibrium profiles of BDNF (a), NGF (c), and lysozyme (e) are illustrated at 280 nm after centrifugation for 17 h at 17 krpm. A clear sedimentation profile is observed for Y1036 at 370 nm in the presence of BDNF (b) and NGF (d) but not lysozyme (f). All experiments were performed in triplicate. A schematic representation of an equilibrium sedimentation profile can be found in the supplementary information.
micrograms of recombinant NGF (Serotech) or BDNF (Peprotech) were included the sample compartment. The compartments were allowed to equilibrate overnight at 37°C, and Y1036 was quantified via UV/vis spectrophotometry (Shimadzu UV/vis-1700). Scatchard analysis was performed to determine $K_i$ values.

**Cell Culture.** PC12 and PC12*trkB* cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); CATH.a cells were grown in RPMI-1640 medium supplemented with 4% FCS and 8% equine serum; NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 5% FCS. Cells were harvested by replacing the medium with a 0.25% trypsin-0.55 mM EDTA solution and incubating at 37°C for 5 min. All cell cultures were incubated at 37°C in 5% CO$_2$.

**Cell Transfections.** The full-length trkB cDNA was cloned into the cytomegalovirus plasmid vector. Transient expression of trkB in PC12*trkB* cells was achieved by transfecting the cells with the trkB expression vector with use of Lipofectamine Plus reagent (Invitrogen Canada, Burlington, ON). PC12*trkB* cells were first passed through a syringe fitted with a 21-gauge needle several times to prevent clumping, and then $10 \times 10^6$ cells were plated in a 100-mm dish and used for transfection the next day. A solution of 12 $\mu$g of DNA and 30 $\mu$l of Plus reagent diluted in 750 $\mu$l of OptiMEM medium (Invitrogen Canada) was incubated at room temperature for 15 min. The mixture was then added to 60 $\mu$l of Lipofectamine reagent in 750 $\mu$l of OptiMEM medium and incubated for another 15 min at room temperature. Cells were rinsed once with serum-free RPMI 1640 medium and then placed in 5.0 ml of the medium. The DNA mixture was added directly to the plated cells, which were subsequently incubated at 37°C. After 3 h, 6.5 ml of RPMI 1640 medium containing 20% FCS was added to the dish, restoring serum levels to 10%. Cells were used 24 h after transfection.

**Neurotrophin Radiolabeling, Receptor Binding, and Receptor Cross-Linking Assays.** Iodination of mouse 2.5S (-)NGF and recombinant human BDNF and receptor binding and receptor cross-linking were performed as described in Ross et al., (1997). In brief, Y1036 was incubated in the presence of NGF or BDNF for 1 h at room temperature before addition to the cells. Cell treatments were performed for 2 h at 4°C before direct gamma counting. Agonist radioligand receptor-binding assays for EGF and PDGF were conducted by Cerep (Poitiers, France). The PDGF and EGF assays were performed in triplicate at 50 D values.

**Immunoprecipitation.** Cell samples were solubilized in 1 ml of lysis buffer and incubated for 30 min at 4°C, and immunoprecipitations were conducted by use of the Sieze kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions. Polyclonal anti-trk antibody 545 (generated as described in Ross et al., 1997) and polyclonal anti-p75$^{NTR}$ antibody 9992 (Promega, Madison, WI) were used in the respective assays.

**trk and p44/42 Phosphorylation Assay.** Phosphorylation of trk was assessed via Western blot analysis after immunoprecipitation of trkA or trkB with the antiphosphotyrosine antibody 4G10 (Millipore, Billerica, MA) according to the manufacturer’s instructions. Likewise, p44/42 phosphorylation was performed with either p44/42 MAPK antibody or phospho-p44/42 MAPK (thr202/tyr204) antibody (Cell Signaling Technology, Danvers, MA).

**Neurite Outgrowth Assay.** Embryonic day 6 dorsal-root ganglion neurons were cultured according to vendor specifications (Lonza; Shawingan, QC, Canada). Cells were plated at a density of 500 cells/well and grown on poly(d-lysine)-coated terasaki plates (Greiner Bio-One, Longwood, FL) at 37°C and 5% CO$_2$. Neurons were cultured in the presence of mitotic inhibitors (17.5 $\mu$g/ml uridine and 7.5 $\mu$g/ml 5-fluoro-2-deoxyuridine; Sigma-Aldrich). Seven days after plating, neurons were treated with 10 nM BDNF or NGF and varying concentrations of Y1036. Cells were then allowed to grow for 48 h before scoring. Cells on the entire lower horizontal surface of the well were scored by use of a phase-contrast microscope. A neurite was scored if its caliber from the origin to the terminal was constant and its length was equal to or greater than 1.5 cell body diameter.

**Results**

**Identifying Y1036 as a Neurotrophin Antagonist.** The neurotrophins BDNF and NGF have highly conserved structures, and basic sequence alignment illustrates the degree of similarity (∼50% fully conserved, ∼16% functionally conserved, and ∼11% weakly conserved) (Fig. 1a). In an effort to identify a multipotent neurotrophin antagonist, we explored the recently described NGF/p75$^{NTR}$ (He and Garcia, 2004) and NGF/trkA (Wehrman et al., 2007) crystal structures and created an ab initio docking site comprising highly conserved residues that participate in both p75$^{NTR}$ and trk interactions (Fig. 1, a and b). By use of a structural similarity search based on known NGF antagonists (ALE-0540 (Owolabi et al., 1999); Ro 08-2750 (Niederhauser et al., 2000), and PD 90780 (Colquhoun et al., 2004)), a combinatorial panel of furan-ring derivatives was identified and screened in silico. Flexible docking experiments identified compound Y1036 (Fig. 1c) as having a similar and favorable docking mode for both BDNF and NGF (Fig. 1d). The pharmacological properties of Y1036 are listed in Table 1.

The resultant docking modes are consistent between BDNF and NGF with respect to the spatial orientation of Y1036 in the docking site. The theoretical binding mode obtained for Y1036 and BDNF is stabilized by four H bonds:
The aminosulfonyl group of Y1036 interacts with the carbonyl group of Glu18 and the /H9251-carbonyl group of Trp19, and the terminal carboxyl group is also stabilized by a pair of H bonds that interact with Glu55 and Lys57.

Likewise, the obtained theoretical binding mode for Y1036 and NGF is predicted to also be stabilized by terminal H-bonding of Y1036 (Fig. 1d). However, only three H bonds are predicted in this docking mode. Again, the aminosulfonyl group of Y1036 interacts with /H9251-carbonyl group of Trp21 and the /H9251-amide of Gly23. In addition, an H bond is formed between the ε-amine of Lys57 and the terminal carboxyl group of Y1036. We attribute the fact that Glu55 does not participate in the NGF docking mode because it is slightly removed from its contiguous location in BDNF.

Y1036 Binds BDNF and NGF. To validate our theoretical docking results, we performed a series of experiments to confirm binding between Y1036 and BDNF or NGF. We first used equilibrium analytical ultracentrifugation to confirm that Y1036 interacts with NGF or BDNF, but not lysozyme control. Equilibrium centrifugation of Y1036 in the presence of either neurotrophin demonstrated a clear sedimentation profile (BDNF, Fig. 2a and b; NGF, Fig. 2c and d), whereas no sedimentation was observed for Y1036 in the presence of lysozyme (Fig. 2e and f). To further support the assertion that Y1036 binds NGF and BDNF, we performed size exclusion chromatography experiments that demonstrated the coelution of Y1036/BDNF (Fig. 3a) and Y1036/NGF (Fig. 3b) from a PD10 column. Finally, to determine the affinity of Y1036 for either BDNF or NGF we used the technique of equilibrium dialysis. The affinity of Y1036 for BDNF (K_D = 3.5 ± 0.3 μM) and NGF (K_D = 3.0 ± 0.4 μM) was found to be in the low micromolar range. Taken together, multiple lines of evidence support the finding that Y1036 binds BDNF and NGF.

Next, we determined the efficiency at which Y1036 inhibits neurotrophin receptor binding. To assess the effect of Y1036 on BDNF, we used PC12<sup>r</sup> cells that express p75<sup>TR</sup>, but
tor, p75NTR immunoprecipitation yielded a band similar to PC12nnr5 cells after transfection with a trkB expression vector. No signal was detected after immunoprecipitation with a pan-trk antibody. In accordance with trkA's specificity for NGF, NGF but not BDNF successfully competed with 125I-NGF for the receptor. The lower molecular mass band, at approximately 100 kDa, has a molecular mass consistent with a p75NTR-NGF complex. This band was immunoprecipitated with an antibody against p75NTR, and, in agreement with this receptor's affinity for both BDNF and NGF, either ligand successfully competed with 125I-NGF for the receptor.

With use of the trkB-expressing PC12nnr5 cells, the ability of varying concentrations Y1036 to inhibit 125I-BDNF cross-linking to its receptors was assessed. Y1036 was found to impede 125I-BDNF cross-linking to both p75NTR and trkB in a concentration-dependent manner with IC50 values (mean ± S.E.M.) of 6.2 ± 1.0 μM and 3.7 ± 1.1 μM, respectively (Fig. 5a). Likewise, in trkA-expressing PC12 cells, Y1036 inhibited 125I-NGF cross-linking to p75NTR and trkA with IC50 values of 5.7 ± 1.1 μM and 8.8 ± 1.1 μM, respectively (Fig. 5b).

To assess the general specificity of Y1036, we performed an agonist radioligand-binding assay using EGF and PDGF as representative non-neurotrophin growth factors. In the presence of 50 μM Y1036, no evidence of agonist inhibition was detected for EGF or PDGF. Residual binding for EGF was found to be 98%, and residual binding for PDGF was ≥100%.

**Y1036 Inhibits Receptor Activation, Signaling, and Neurotrophin-Mediated Differentiation.** We confirmed the efficacy of Y1036 as neurotrophin antagonist by assessing the phosphorylation state of the trk receptors after BDNF or NGF stimulation. To test trkB phosphorylation, we chose the trkB expressing CATH.a cell line (Fig. 6a). Unstimulated cells demonstrated little basal trkB phosphorylation. Upon stimulation with 40 pM BDNF, there was an induction of phosphorylated trkB, which was attenuated by treatment with 30 μM Y1036.

However, PC12nnr5 cells transfected with trkB expression vector demonstrated nonspecific/artifactual basal phosphorylation, and this assay system could not be used for evaluation of Y1036. In untreated PC12 cells, no detectable basal phosphorylation of trkA was observed. After treatment with NGF, a strong phosphotyrosine signal was detected for trkA, consistent with a trkA-NGF complex. In accordance with p75NTR and trkA in wild-type PC12 cells (Fig. 4a).

Neither trkB nor trkA. PC12nnr5 cells were transiently transfected with a trkB expression vector. Likewise, to assess the effect of Y1036 on NGF we used PC12 cells, which intrinsically express both p75NTR and trkA. Appropriate receptor expression was confirmed via 125I-BDNF or 125I-NGF receptor cross-linking experiments followed by immunoprecipitation of p75NTR or trkB. When 125I-BDNF was cross-linked to PC12nnr5 cells, a single band of approximately 100 kDa, consistent with the molecular mass of a p75NTR-BDNF complex, was detected after immunoprecipitation with an antibody against p75NTR. In accordance with the affinity of p75NTR for both BDNF and NGF, either ligand successfully competed with 125I-BDNF for the receptor. In PC12nnr5 cells, no signal was detected after immunoprecipitation with a pan-trk antibody. When 125I-BDNF was cross-linked to PC12nnr5 cells after transfection with a trkB expression vector, p75NTR immunoprecipitation yielded a band similar to that obtained with nontransfected cells. This band was also abolished by either NGF or BDNF. trkB immunoprecipitation yielded a higher-molecular mass band of approximately 170 kDa, consistent with a trkB-BDNF complex. In keeping with trkB's selectivity for BDNF, BDNF but not NGF successfully competed with 125I-BDNF for the receptor (Fig. 4a).

A similar strategy was used to ensure appropriate expression of p75NTR and trkA in wild-type PC12 cells (Fig. 4a). When 125I-NGF was cross-linked to PC12 cells, two bands were detected on an autoradiograph after SDS-PAGE (Fig. 4b). The higher molecular mass band immunoprecipitated with a pan-trk antibody, and at approximately 170 kDa, its size is consistent with a trkB-NGF complex. In accordance with trkA's specificity for NGF, NGF but not BDNF successfully competed with 125I-NGF for the receptor. The lower molecular mass band, at approximately 100 kDa, has a molecular mass consistent with a p75NTR-NGF complex. This band was immunoprecipitated with an antibody against p75NTR, and, in agreement with this receptor's affinity for both BDNF and NGF, either ligand successfully competed with 125I-NGF for the receptor.
the p44/42 MAPK pathway (Fig. 6e). NIH 3T3 cells do not express the p75\textsuperscript{NTR} or trk receptor, and in this model Y1036 did not alter basal p44/42 activation, nor did it affect EGF-stimulated p44/42 signaling. Thus, these results support the antagonist effects of Y1036 on neurotrophin signaling.

To assess the inhibitory effect of Y1036 in a cell culture model, we chose to evaluate the ability of Y1036 to inhibit the neurotrophin-mediated differentiation of dorsal-root ganglion neurons. Embryonic day six rat neurons were exposed to 40 pM concentrations of either BDNF (Fig. 7a) or NGF (Fig. 7b) in the presence of varying concentrations of Y1036. After a 48-h incubation, Y1036 inhibited neuronal differentiation in a dose-dependent manner. These results provide functional evidence that Y1036 is an effective multipotent neurotrophin antagonist.

**Evaluating the Validity of the Proposed Y1036 Docking Model.** In an effort to validate the theoretical docking mode of Y1036, we chose to test a series analog, Y1370, in both the docking model and receptor-binding assays. The structures of Y1036 and Y1370 are displayed in Fig. 8a. In silico docking results suggest that Y1370 binds BDNF in a favorable conformation making key H-bonding interactions with Asp24, Glu55, and Lys57 and an overall orientation similar to that used for Y1036. By use of trkB-expressing M cells, the ability of varying concentrations Y1036 to inhibit \textsuperscript{125}I-BDNF cross-linking to its receptors was assessed. Y1370 was found to impede \textsuperscript{125}I-BDNF cross-linking to both p75\textsuperscript{NTR} and trkB in a concentration-dependent manner with IC\textsubscript{50} values (mean ± S.E.M.) of 6.7 ± 1.1 \mu M and 7.6 ± 1.2 \mu M, respectively (Fig. 8d). These binding results are consistent with the docking mode suggested for Y1036. Alternatively, according to molecular modeling, Y1370 is predicted to bind less favorably to NGF. Accordingly, in trkA-expressing PC12 cells, Y1370 inhibited \textsuperscript{125}I-NGF cross-linking to p75\textsuperscript{NTR} with IC\textsubscript{50} values of 5.8 ± 1.1 \mu M but was found ineffective against inhibiting NGF/trkA interactions (Fig. 8e). Therefore, the predicted docking Y1370 to BDNF and NGF are supported by these experimental results lending further confidence to the proposed docking mode of Y1036.

**Discussion**

The therapeutic strategy of targeting the ligand in a ligand/receptor system has realized success with the approval of anti-TNF antibodies for the treatment of rheumatoid arthritis (Feldmann and Maini, 2008). Yet, similar strategies using small molecules to target peptide ligands in a pathological setting are just starting to be realized. In the present study we identify and describe Y1036, a multipotent small-molecule antagonist of BDNF and NGF. Using a variety of in silico and in vitro molecular techniques, we demonstrate that Y1036 binds to BDNF and NGF, preventing receptor interaction, activation, signal transduction, and neurotrophin-induced differentiation.

Work from our laboratory and others have demonstrated that small molecules are able to bind and inhibit NGF from interacting with its receptor (ALE 0540, Owalabi et al., 1999; Ro 08-2750, Niederhauser et al., 2000; PD90780, Colquhoun et al., 2004). Similar to previously reported NGF antagonists, Y1036 demonstrates low micromolar inhibitory concentrations toward NGF. Inhibitory binding assays demonstrate that Y1036 is slightly less effective at preventing trkA/NGF interactions (8.8 \mu M) than either PD90780 (1.8 \mu M; Colquhoun et al., 2004) or ALE-0540 (5.8 \mu M; Owalabi et al., 1999). Y1036 also demonstrated low-micromolar inhibition of NGF/p75\textsuperscript{NTR} (5.7 \mu M) which is lower than PD90870 (23.1 \mu M) and comparable with ALE-0540 (3.7 \mu M; Owalabi et al., 1999). In affinity-based assays, Ro 08-2750 (K\textsubscript{D} = 1.0 \mu M; Niederhauser et al., 2000) performed similarly to Y1036 (K\textsubscript{D} = 3.0). Unlike previously reported NGF antagonists, Y1036 also demonstrates low micromolar inhibitory action toward BDNF for both trkB (3.7 \mu M) and p75\textsuperscript{NTR} (6.2 \mu M).

Clinically, there are several indications that could benefit from the actions of a multipotent neurotrophin antagonist. Indications such as neuropathic pain, inflammatory pain, and diabetic neuropathy, where concurrent dysregulation of BDNF and NGF have been implicated (Pezet and McMahon, 2006) and where current therapeutic strategies are lacking (Max and Stewart, 2008), could take advantage of the actions of Y1036. There are other indications where a small-molecule inhibitor of BDNF may be of therapeutic interest. For example, recent studies have implicated a role for BDNF in the progressive loss of motoneurons in amyotrophic lateral sclerosis (Fryer et al., 2000; Hu and Kalb, 2003; Mojsilovic-Petrovic et al., 2006).

Because Y1036 targets both BDNF and NGF, attempting to specifically target either NGF or BDNF alone may present a challenge, and systemic administration may result in unwanted side effects. By use of emerging liposomal drug delivery strategies, it may be possible to target delivery of Y1036 to locations where BDNF is dysregulated providing NGF is not serving a crucial role at the delivery site.

The chemical properties of Y1036 abide by Lipinski’s Rule of Five (Lipinski et al., 2001), suggesting that Y1036 has properties lending to oral drugability. In addition, topographical polar surface area obeys the criteria for penetration of the blood-brain barrier set forth by Clark (1999). Thus, it is possible that Y1036 may possess in vivo activity.

Consistent with our theoretical and biochemical data, we suggest that the binding of Y1036 to either BDNF or NGF is sufficient to alter the molecular topology of both neurotrophins. The resultant change in surface charge density is
To confirm that receptor activation was indeed inhibited, the effect of Y1036 on trkB activation and neurotrophin-induced signaling was investigated. After neurotrophin-mediated stimulation, trk receptors undergo dimerization and trans-autophosphorylation, which initiates intracellular signaling cascades, including the p44/42 MAPK pathway (Lu et al., 2005). Y1036 inhibited BDNF-induced trkB phosphorylation in CATH.a cells, and NGF-induced trkA phosphorylation in PC12 cells. Treatment of trkB-expressing PC12<sup>trans</sup> cells with BDNF did lead to an increase in phosphorylated p44/42 MAPK. In these cells, Y1036 was able to inhibit BDNF-dependent p44/42 activation. Y1036 was also effective at reducing NGF-induced p44/42 activation, which was measured in PC12 cells.

To test the specificity of Y1036 for neurotrophin-induced p44/42 MAPK phosphorylation, its effects on EGF-stimulated p44/42 activation were tested in alternate NIH 3T3 fibroblast cells that express neither trk nor p75<sup>NTR</sup>. EGF is a growth factor unrelated to the neurotrophin family that activates p44/42 MAPK via the EGF receptor tyrosine kinase (Yamada et al., 1997). Y1036 was unable to prevent EGF-induced p44/42 activation, demonstrating its antagonist function on spinal nociceptive transmission during inflammation in the rat. J Physiol 569:655–695.


