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Scientific Research

Affinity Crosslinking of Y1036 to Nerve Growth Factor Identifies Pharmacological Targeting Domain for Small Molecule Neurotrophin Antagonists

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ABSTRACT

Classically, small molecule antagonists have targeted membrane bound receptors and intracellular enzyme targets. While this drug discovery strategy is extremely successful, the number of new chemical entities in the pharmaceutical pipeline is diminishing and complementary strategies are in need. A particularly attractive therapeutic strategy is to neutralize soluble signalling proteins using small molecules. Small molecule-based technologies have the potential to sufficiently alter the molecular topology of a given ligand and inhibit ligand/receptor interactions—effectively neutralizing the ligand's signalling capacity. Recent technical advances in the field of structural biology have enabled the elucidation of ligand/receptor complexes at atomic resolution enabling a detailed appreciation of the molecular interactions governing ligand-mediated receptor activation. Exploiting molecular modeling techniques to study these signalling complexes allows for a paradigm shift from "receptorcentric" to "ligandcentric" screening strategies. Nerve growth factor (NGF) is a prototypical protein signalling ligand, which binds two receptors, TrkA and $p75^{NTR}$. We first explore the molecular landscape governing the ligand/receptor interactions of NGF/TrkA and NGF/p75 structures. Next, we use the recently reported NGF neutralizing small-molecule, Y1036, as an affinity probe to determine residues in proximity to the pharmacological targeting domain of NGF and perform theoretical docking experiments to predict the residues which comprise distinct pharmacological targeting domains on the surface of NGF. Exploiting such strategies may facilitate "ligandcentric" drug discovery and could further the development of a trophic-factor-selective compound such as a BDNF-selective antagonist.

Keywords: NGF; BDNF; Inhibitor; Antagonist; Pain

1. Introduction

Historically, small molecule-based therapeutics have generally focused on compounds directed towards membrane bound receptors, channels and intracellular enzyme targets. However, the physiological role of several of these targets is governed by soluble protein ligands such as cytokines or growth factors. Traditional "receptorcentric" screening strategies yielded clinically effective therapeutics for most of the past century [1]. Obvious examples in the field of pain research include targets such as cyclooxygenase (Cox1/2), canabinoid and opioid receptors, and a variety of ion-channel inhibitors [2]. However, in the last decade, the success of these strategies has not kept pace with the demand for new chemical entities and, complementary approaches may help to revitalize lead identification in many drug discovery programs.

In an attempt to counteract declining productivity, research and development in the pharmaceutical industry are moving towards unprecedented targets and novel technologies [3]. By definition, unprecedented targets are proteins or enzymes for which a pharmacological (or biological) therapy has yet to be approved in a clinical setting [3]. In the field of neuropharmacology, a prime example of an unprecedented target is nerve growth factor (NGF).

NGF is a soluble ~26 kDa homodimeric protein which is known to be critical for the development and maintenance of the central and peripheral nervous system [4]. NGF is a member of the neurotrophin family of proteins and mediates its physiological role by binding the com-

mon low-affinity receptor $p75^{NTR}$ [5] and the selective high-affinity receptor TrkA [6]. Interestingly, the dysregulation of NGF has also been implicated in several disease states of the nervous system [4,7]. Thus, identifying mechanisms to inhibit pathological signalling may have significant therapeutic potential in the field of neuropharmacology.

The therapeutic efficacy of targeting the ligand in a ligand/receptor system has been demonstrated by the neutralizing effect of monoclonal antibodies in the clinic [8]. However, antibody-mediated therapies have several technical, practical, and economical limitations that restrict their widespread application in many clinical settings. A similar strategy using small-molecules to neutralize soluble peptide ligands in a pathological setting is just starting to be explored.

NGF neutralizing small-molecules (which bind NGF rather than the TrkA or p75NTR receptors) has been described by our group and others. For example, ALE-0540 [9], Ro 08-2750 [10], PD90780 [11] and Y1036 [12] are known to effectively neutralize NGF activity. Interestingly, Y1036 is reported to also bind the related neurotrophin brain-derived neurotrophic factors. Better understanding the high resolution of structural biology governing the mode-of-action of these molecules may facilitate the identification and optimization of such compounds in drug discovery programs directed at unprecedented targets.

In this study, we describe the identification of a putative pharmacological targeting domain on the structure of NGF. Beginning with high-resolution structural biology data, we dissect the molecular interactions governing the NGF/$p75^{NTR}$ and NGF/TrkA interface. Next, we use the NGF-binding antagonist Y1036 as an affinity probe to identify the putative pharmacological targeting domain. From these data, we perform theoretical docking experiments which demonstrate that the binding of Y1036 can substantially alter the molecular topology of NGF. The results of this study provide valuable information towards the identification of a pharmacological targeting domain on the surface of a soluble signalling ligand and may prove usefulness in rationally modifying small molecules directed towards the neutralization of NGF.

2. Methods

2.1. Molecular Modeling

All molecular modeling was performed in the Sybyl 8.0 environment (Tripos; St. Louis, MO). The 3D coordinates of the protein backbone were kept fixed at their X-ray geometry, and a water environment was not included in the model. Ribbon structures of NGF [RCSB ID-1BET [13]], NGF/TrkA [RCSB ID-2IFG [6]] and NGF/$p75^{NTR}$ [RCSB ID-1SG1[5]] were generated using the Molcad rendering suite of Sybyl 8.0. External surfaces were calculated using the Fast Connoly approximation of the MolCad suite. Surface to charge ratios were approximated using the MMFF94 molecular mechanical force field [14].

2.2. NGF Crosslinking and Digest

NGF was purchased from Cedarlane Laboratories (Burlington, ON, Canada). NGF (1 mg/mL) and Y1036 (50 μM) were incubated for 1 hour in 25 mM phosphate buffer (pH 7.4) at room temperature in a volume of 100 μl. Covalent crosslinking of NGF to Y1036 was performed via 1-ethyl-3-(3-dimethyl amino-propyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS)-mediated reaction (Pierce; Rockford, IL). 5 mM EDC and 2 mM NHS were added to the solution and incubated at 25˚C temperature for 30 minutes. Crosslinked NGF was reduced in 200 mM DTT in 100 mM ammonium bicarbonate buffer (pH = 8.0) for 1hr at room temperature. The reaction mixture was then acetylated with iodoacetamide (1 M) via 1hr incubation at room temperature in the dark. Excess iodoacetamide was neutralized using dithiotheritol (DTT) for 1hr at room temperature. A protein digest was then performed by incubating 1 μg of sequencing grade trypsin (Madison, WI, USA) with 50 μg of NGF or crosslinked NGF-Y1036 at 37˚C for 18 hrs.

2.3. Mass Spectroscopy

Peptide analysis was performed at the Biological Mass Spectrometry Laboratory at the University of Western Ontario (London, ON, Canada). Peptides were separated on a CapLC high-performance liquid chromatography system using a Nano-Acquity C18 column and analyzed using Q-TOF Micro mass spectrometer (Waters, Mississauga ON, Canada). NGF-peptides were identified via the MASCOT analysis (Matrix Science; Boston MA). Analysis was then used to identify peptides which exactly matched the m/z of a given NGF peptide + Y1036 – H_2O.

2.4. Theoretical Docking

Molecular modeling and *in silico* docking of Y1036 to NGF [RCSB ID-1BET (Wehrman *et al*., 2007)] was carried out using the software program Sybyl 8.0 (Tripos, St. Louis, MO). The structure of NGF was prepared for docking using the Biopolymer suite of Sybyl 8.0. Co-structures were deleted and hydrogens were added, the appropriate formal charges were applied to the N- and C-termini and the structure was optimized using the MMFF94 molecular mechanical force field [14]. Flexible docking of Y1036 was performed using the Surflex-Doc suite [15] incorporated into Sybyl 8.0. The docking

protmol (molecular space) was generated to include the residues using a 5Å radius from the lysines of interest (K57) with a bloat factor = 0 and a threshold value of 0.5. For the Surflex-Doc function, the angstroms to expand search grid was set at 6 and the maximum confirmations per fragment was set to 20.

2.5. HPLC Analysis

High-performance liquid chromatography (HPLC) was carried out using a System Gold Microbore HPLC with 32 Karat Software from Beckman Coulter including a delivery module pump and a diode array detector. An Inertsil ODS-3 column (150 × 4.6 mm, 5 µm) with an Inertsil ODS-3 guard cartridge (10 × 4 mm, 5 µm) was used for separation. A mixture of two solvents constituted the mobile phase: solvent A [0.1% concentrated trifluoroacetic acid (TFA) in deionized water] and solvent B [acetonitrile (MeCN) with 0.1 % TFA]. The flow rate was 1 mL/min. Samples (100 µL) were injected and separated using the following gradient system: 5% B to 55% B (over 40 min) then to 100% B in 10 min. The eluent was monitored by photodiode array detection at 280 nm and 450 nm.

3. Results

3.1. Molecular Modeling Identifies Putative NGF Targeting Domains

Recently, atomic resolution structures have been reported which describe the interactions between NGF and its receptors, TrkA [6] and p75NTR [5]. Using structural modeling techniques, we examined the molecular topology of the NGF/p75NTR-dependent and NGF/Trk-dependent binding. With respect to ligand/receptor binding mode, it is important to note that the orientation in which p75NTR binds NGF is opposite to that of TrkA (**Figure 1**).

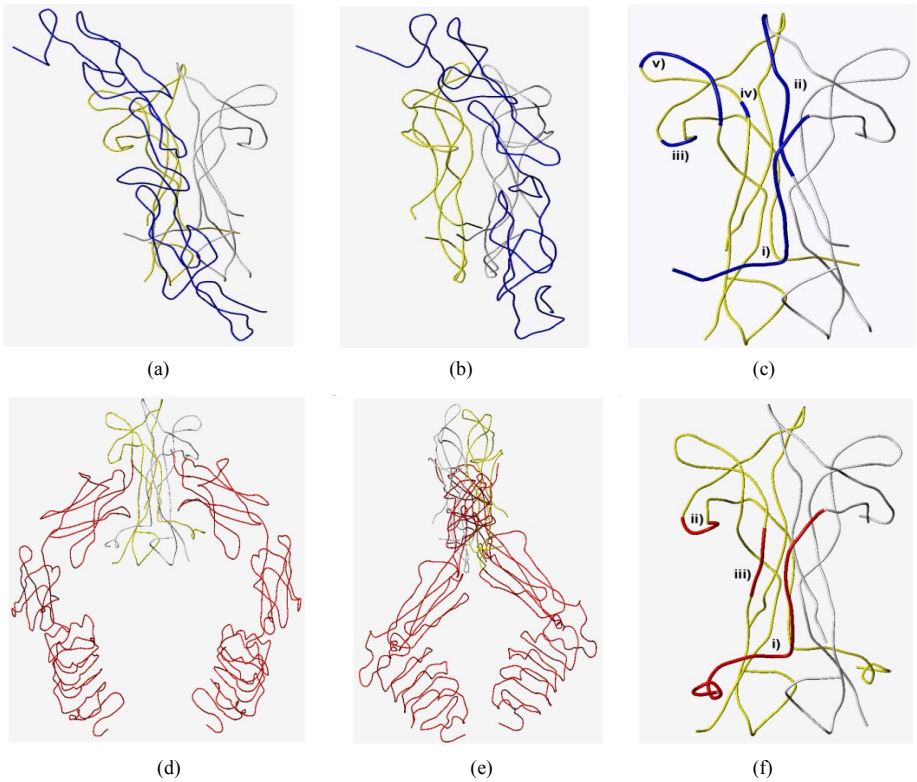

Figure 1. Footprinting of the molecular interactions governing NGF/receptor binding. (a) The asymmetrical NGF/p75^{NTR} complex is presented as ribbon structure (RCSB ID-1S1G). Two NGF dimers (NGF monomer presented in white; NGF' monomer presented in yellow) are bound by p75^{NTR} (blue). Panel (b) illustrates the NGF/p75^{NTR} orientation rotated by approximately 90˚. (c) Residues of NGF which participate directly in NGF/p75^{NTR} complex are highlighted in blue. Region i) is composed of residues 9 - 23 and region ii) is composed of residues 48 - 55 of the NGF monomer. Region iii) is comprised of residues 30 - 32; region iv) is comprised of residue 88 and region v) is comprised of 95 - 100 of the NGF' monomer. (d) The symmetrical NGF/TrkA complex is illustrated as ribbon structure (RCSB ID – 2IFG). Two NGF dimers (NGF monomer presented in white; NGF' monomer presented in yellow) are bound by TrkA (red). Panel (e) illustrates the NGF/TrkA orientation rotated by approximately 90˚. (f) Residues of NGF which participate directly in NGF/TrkA complex are highlighted in Red. Region i) is comprised of residues 1 - 23 of the NGF monomer. Region ii) is composed of residues 30 - 32 and region iv) is composed of residues 83 - 86 of the NGF' monomer.

Interestingly, select regions participating in $p75^{NTR}$ binding of NGF are shared with TrkA binding while other regions are primarily distinct from those involved in TrkA interactions. For instance, the N-terminal residues of NGF participate in binding with both receptors, but the loop I/IV region is of critical importance to $p75^{NTR}$ interactions. The NGF/TrkA complex reported by Wehrmann *et al.* [6] involves the residues of loop IV, but it is unclear if loop I participates in binding. NGF/TrkA interactions also occur at the C-terminal domain of NGF which are outside the contact region of $p75^{NTR}$. **Figure 1** illustrates the NGF ligand/receptor footprint with respect to $p75^{NTR}$ and TrkA. The molecular topology of NGF's ligand/receptor complex suggests that an inhibiting small molecule would likely bind in proximity to these regions of interaction.

3.2. Proximity Cross-Linking Identifies Pharmacological Targeting Domains

Y1036 is a small molecule which neutralizes the signalling activity of NGF and inhibits NGF interaction with the $p75^{NTR}$ and TrkA receptors [12]. The minimized energy structure of Y1036 is presented in **Figure 2**. The free carboxyl group of Y1036 has the potential to form H-bonds with the ε-amine of a given lysine residue on the surface of NGF. Importantly, this pharmacological property may allow Y1036 to be used as chemical probe to identify a functional pharmacological targeting domain(s) on the surface of NGF. Thus, we performed a zero-distance EDC/NHS crosslinking proximity assay. Recombinant NGF was incubated in the presence of excess Y1036 at room temperature for 1 hr. Pre-activation of Y1036 or NGF with EDC/NHS did not affect the efficiency of the crosslinking reaction. Crosslinking was then performed via an EDC/NHS reaction. Native NGF and crosslinked NGF-Y1036 then underwent tryptic digest.

Using time-of-flight mass spectrometry, we were able to resolve the full sequence coverage of NGF via Mascot analysis of tryptic peptides as summarized in **Figure 3(a)**. Similar analysis of NGF crosslinked to Y1036 identified a principal peak corresponding in mass to the Y1036-modified peptide QYFFETK(-Y1036) (**Figures 3(b)** and **(c)**). As EDC/NHS crosslinking occurs between the carboxyl group of Y1036 and primary amines of lysine residues these data suggests that Y1036 crosslinked with residues K57 (**Figure 3(c)**). **Figure 4** illustrates the spatial orientation of the QYFFETK(-Y1036) on the structure of NGF.

Theoretical docking experiments support the involvement of K57 in pharmacological targeting domains.

To evaluate the potential contribution of K57 to the binding mode of Y1036, we created an *ab initio* docking

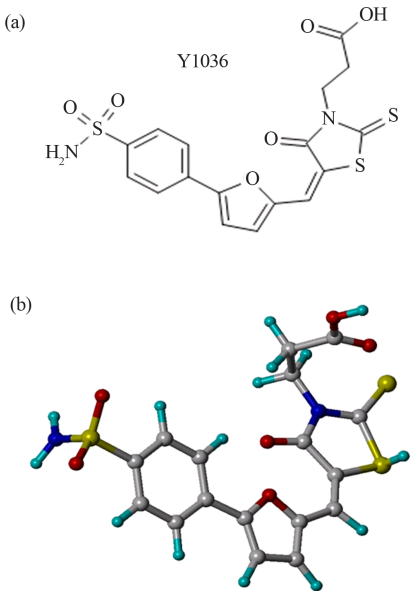

Figure 2. Structure of Y1036. (a) The structure of Y1036, 3-[(5E)-4-oxo-5-[[5-(4-sulfamoylphenyl)-2-furyl]methylene]-2-thioxo-thiazolidin-3-yl]propanoic acid. (b) The minimized energy conformation of Y1036.

site biased to the residues within a 5Å radius of the K57 crosslinking site. We then performed flexible docking experiments to obtain a theoretical binding mode for Y1036 in the proximity to the dimer interface (K57 site). A favorable docking mode for Y1036 was obtained with a consensus score of 4.

In the case of the pharmacological targeting domain near the hydrophobic interface, the docking results predicted the formation of three stabilizing H-bonds: the first H-bond is formed between the ε-amine of Lys57 and the carboxyl group of Y1036; another H-bond is shared by the aminosulfonyl group of Y1036 and the α-carbonyl group of Trp21; and a third H-bond with the double bonded oxygen of the sulfonamyl group of Y1036 and the α-amide of Gly23 (**Figure 5(a)**). Accordingly, the docking mode also demonstrates a favorable pose with little conformational strain consistent with the minimized energy conformation of Y1036.

Importantly, the results of the docking experiments suggest the formation of an H-bond between the carboxyl group of Y1036 and the ε-amine of the lysine (K57). Thus, these theoretical docking experiments are consistent with the mass spectra data obtained the Y1036 proximity crosslinking assay. These results narrow the possible pharmacological targeting domains of NGF to the hydrophobic interface with involvement of K57.

In order to rule out non-specific crosslinking/binding activity, we attempted to crosslink a non-active analogue of Y1036 (**Figure 6(a)**). Y410 (**Figure 6(b**) shares structural homology to Y1036, but is not an active NGF an-

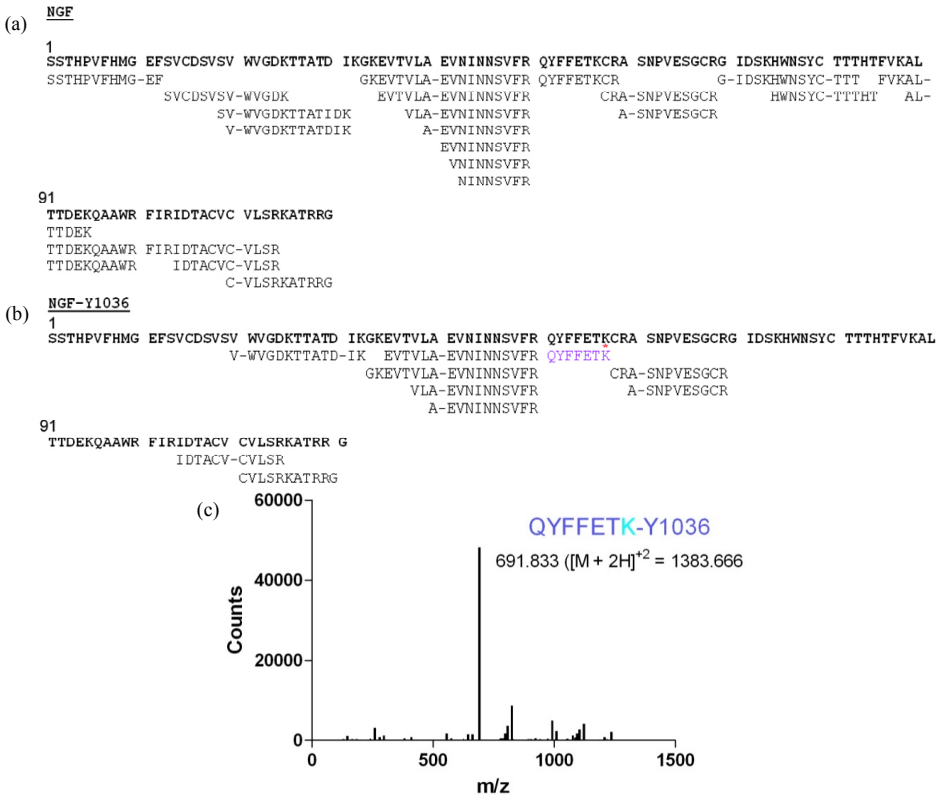

Figure 3. Proximity cross-linking of Y1036 identifies putative docking site(s) on NGF. LCMS identified peptides of (a) trypsin digested NGF and (b) trypsin digested NGF-Y1036 are aligned to the consensus sequence of NGF. Mass spectra of the peptide corresponding to (c) QYFFETK(-Y1036). The peptide sequence represented in violet corresponds to the peptide which cross-linked at K57 (colored cyan).

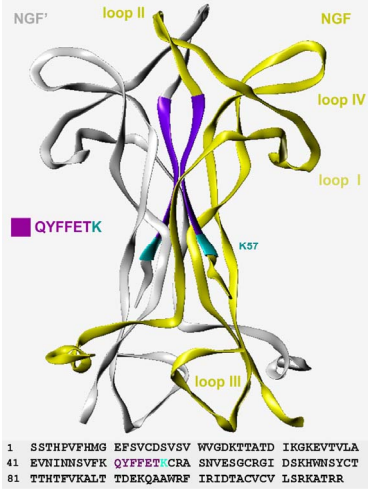

Figure 4. Landmarking of pharmacological targeting domains on the structure of NGF. The symmetrical ribbon structure of the NGF dimer is composed of two monomers (NGF, white; NGF', yellow). The results of Y1036 proximity assay suggest crosslinking occurs at K57. The regions corresponding to the QYFFETK(-Y1036) crosslinked peptide is highlighted in violet or green, respectively. The location of K57 is highlighted in cyan. The structure and amino acid sequence of NGF were obtained from (RCSB ID-2IFG).

tagonist (IC_{50} > 50 μM; unpublished data). Following EDC/NHS crosslinking and tryptic digestion, we obtained evidence for crosslinked peptides of Y1036 and NGF by HPLC-UV analysis (**Figure 6(c)**). Using a similar experimental approach, no crosslinking was observed between Y410 and NGF (**Figure 6(d)**). Similarly, Y410 also did not yield a favorable docking mode at the K57 site with consensus score < 2 (data not shown). The results of this control experiment provide further support for the proposed crosslinking site and docking model.

4. Discussion

NGF-dependent signalling has been identified as a potential pharmacological target for therapeutic intervenetion in several neurological disorders, including chronic pain, Parkinson's disease, and Alzheimer's disease [4]. In the past, small molecule kinase inhibitors, such as K252a [16,17] and the isothiazole family of compounds [18], have functionally limited TrkA signalling in experimental models, but their partial specificity has prevented clinical translation.

An alternative strategy which shows much promise is the use of therapeutic biologicals. For example, mono-

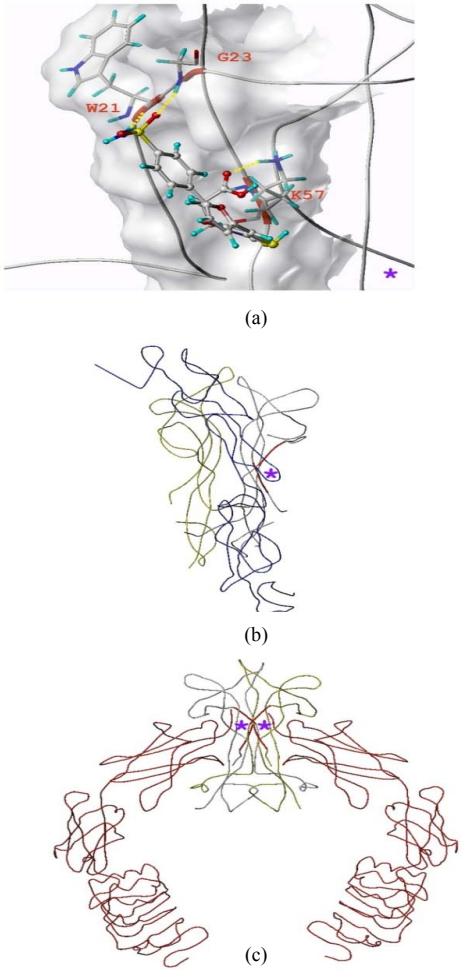

Figure 5. Theoretical docking experiments are consistent with the involvement of K57 in the binding mode of Y1036 to NGF. Schematic representations of Y1036 docked to NGF as determined by molecular modeling. (a) Flexible theoretical docking experiments predict that Y1036 is able to bind at the K57 pharmacological targeting domain in a favorable mode by forming three H-bonds with Trp21, Gly23 and K57. Y1036 is rendered as a ball and stick formation. Residues of the NGF pharmacological targeting domains are rendered as capped stick formations. The relative location of K57 (purple asterisk) is illustrated on the crystal structure of (b) NGF/p75^{NTR} (1SG1) and (c) NGF/TrkA (2IFG). Atoms are colored according to their types: C, gray; N, blue; O, red; H, cyan. Hydrogen bonds are illustrated as dashed yellow lines. The location of residues participating forming H-bond are highlighted on the ribbon structure in red.

clonal antibodies directed towards NGF effectively neutralize its signalling activity [8,19]. Despite the efficacy of such antibody mediated therapies, technical, practical and economical issues may limit their application in many clinical settings. To this end, several groups have been exploiting peptidomimetic approaches directed towards modulating neurotrophin receptors [21-23].

A complementary approach to receptor-centric strategies is to develop ligand-centric small molecule antagonists which are bound to and sufficiently alter the molecular topology of the ligand (NGF) effectively neutralizing its signalling activity. Our group has previously shown that Y1036, a small molecule which are bound to NGF can effectively neutralize NGF's signalling activity [12]. In this study, we use available high-resolution structural data to identify the pharmacological targeting domain of NGF's signal neutralizing small molecules. Such findings may facilitate the rational development of small molecules directed towards NGF and other soluble signalling proteins.

The elucidations of the NGF/TrkA and the NGF/p75^{NTR} complexes now allow for the evaluation and prediction of molecular interactions which govern efficient neurotrophin signalling. However, it is also important to understand the caveats of these structures. For example, **Figure 1** nicely illustrates the location, proximity, and footprint of ligand/receptor interaction of both TrkA and p75^{NTR} binding to NGF. However, some regions of the NGF/TrkA crystal proved difficult to resolve, and data are not available for NGF residues 61-68 [5,6]. Importantly, in order to facilitate crystallization of the NGF/TrkA complex, Wehrman truncated the structure of TrkA by a 32 residue sequence which joins the Ig-C2 domain TrkA to the cell surface [6]. This sequence likely plays a very important role for TrkA's ability to resolve one neurotrophin ligand from another. Thus, the contribution of loop domains I and II of NGF cannot be analyzed with respect to TrkA binding from crystal structure analysis. It is also important to note that the binding epitope of αD11 NGF-neutralizing antibody has been demonstrated to bind at the loop II and loop IV regions of NGF [24].

In the case of the NGF/p75^{NTR}, the ligand/receptor binding mode more completely encompasses the whole of NGF, but some very important questions still surround the structure of the NGF/p75^{NTR} complex. For instance, the structure put forth by He and Garcia [5] proposes an asymmetrical binding mode with one p75^{NTR} receptor bound to an NGF dimer. Follow-up work demonstrated the importance of a disulfide bond in arranging symmetrical p75^{NTR} receptor [25], and the related neurotrophin NT-3 indeed binds NGF in a symmetrical 1:1 manner [26]. Similarly, p75^{NTR} has been found to also symmetrically bind the pro-NGF [27]. Understanding these subtleties may aid in the rationalization and design of novel ligandcentric inhibitors.

It may also be worth considering the orientation of the NGF ligand with respect to the membrane in binding to p75^{NTR} or TrkA. NGF is believed to bind p75^{NTR} with the N- and C-terminal domains oriented towards the membrane. When bound to TrkA, the loop regions of NGF are

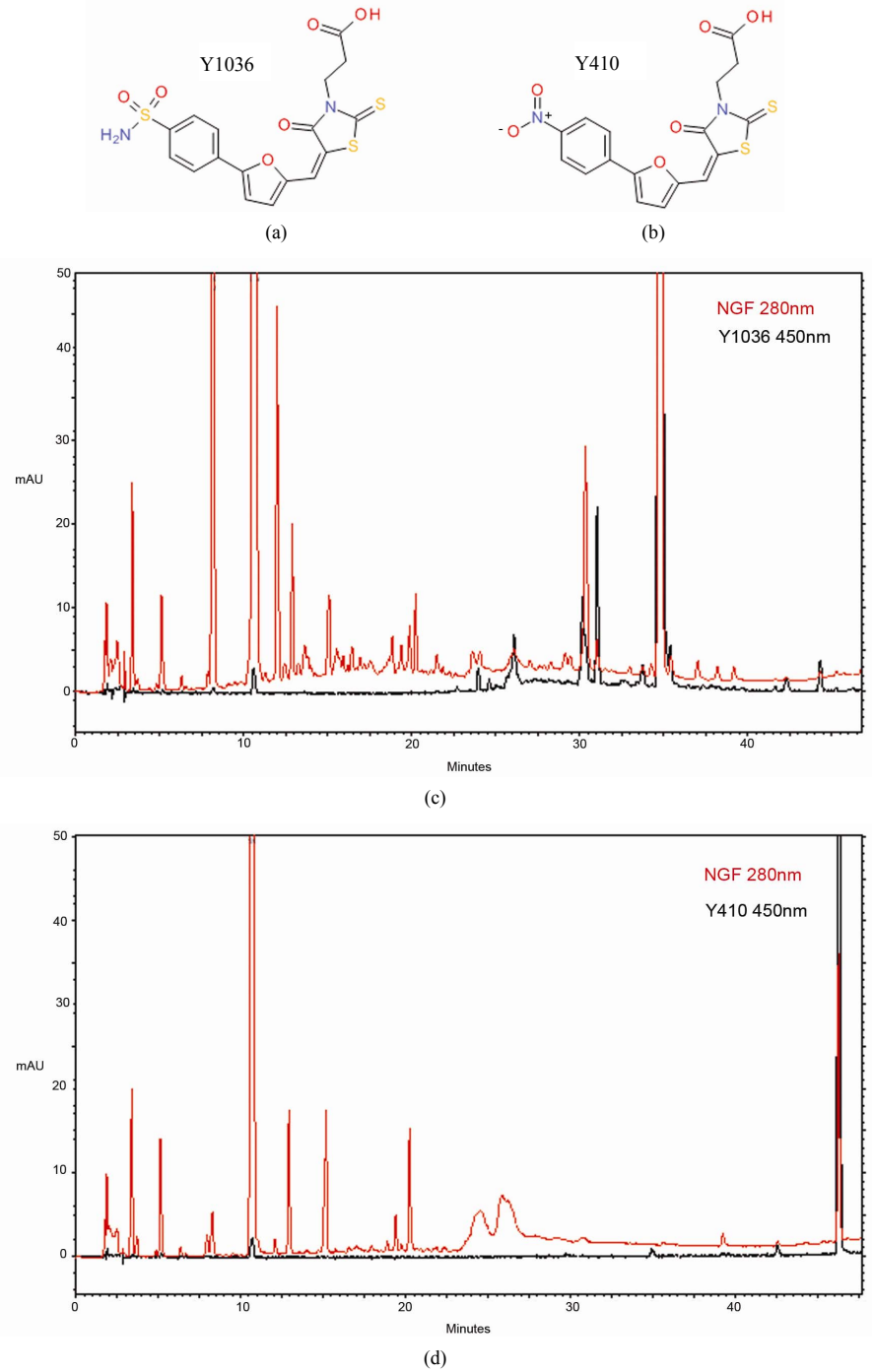

Figure 6. Y410 does not crosslink to NGF. (a) The structure of Y1036 and (b) Y410. (c) HPLC-UV analysis confirms NGF-Y1036 crosslinked peptides following tryptic digest. Crosslinked peptides were monitored at 280 nm (red trace) and 450 nm (black trace). Chromatograms are zoomed such that larger peaks may exceed the 50 m Au displayed on the Y-axis. The large peak at 34.5 minutes corresponds to the elution of free Y1036. (d) No peptides eluted with a signal at 280 nm and 450 nM following incubation and crosslinking of NGF and Y410. The large peak eluting at 46.5 min corresponds to free Y410.

oriented towards the plane of the membrane with the N- and C-terminal domains interacting with the ectodomain. Currently, two hypotheses exist to reconcile this spatial phenomenon. First, it has long been hypothesized that the $p75^{NTR}$ and TrkA form "high-affinity" binding complex whereby both receptors interact with NGF simultaneously [28]. Analysis by Wehrman *et al.* [6], demonstrated that the footprints of $p75^{NTR}$ and TrkA in NGF may al-

low for simultaneous binding. Cross-linking experiments have demonstrated that a NGF/$p75^{NTR}$/TrkA complex forms [29], but it's not clear if this is a stable or transient complex. Alternatively, a "hand-over" mechanism has also been proposed whereby $p75^{NTR}$ first binds NGF and a hand-to-hand exchange from $p75^{NTR}$ to TrkA occurs [28]. It is thought that this mechanism may account for the binding of NGF in opposite orientation. For the purposes of rational drug design, it is important to consider the dynamics of such receptor interplay. With respect to the pharmacological targeting domains put forward in this study, the potential mechanism at the hydrophobic interface (K57 site) is not obvious from crystallography data. The molecular topology altered by Y1036 binding at the K57 site is in proximity, but outside of the static interaction surfaces of NGF/$p75^{NTR}$ or NGF/TrkA. The static nature of crystallography provides very useful insights, but further real-time high-resolution techniques will undoubtedly refine our current understanding of these events.

5. Conclusion

The results of our molecular modeling suggest that the binding of Y1036 to NGF is sufficient to alter the molecular topology such that the resultant change in surface charge density may alter the receptor: neurotrophin interface and prevent neurotrophin mediated signalling. However, there are limitations to the interpretation of our data. In order to have definitive proof of binding mechanism of Y1036, co-crystallization experiments will be required. The docking model presented in this study is consistent with our experimental observations. Understanding the structural and molecular features of the residues in proximity to the NGF crosslinking site now gives researchers the ability to modify chemical scaffolds in a rational manner. Taking advantage of similar high resolution structural biology, it may be possible to apply this strategy to soluble protein ligands more generally. Adopting such emerging high resolution strategies may ultimately enhance drug discovery activities and aid in revitalizing the collective pharmaceutical pipeline.

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