

The identification and characterization of small molecules for the inhibition of NGF and proNGF-p75^{NTR} interactions using surface plasmon resonance spectroscopy

by

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Thesis Abstract

The survival of neurons in both the central and peripheral nervous systems depend on a balance between pro-survival and pro-death signaling pathways. Neurotrophins and their precursors, proneurotrophins, are a unique family of soluble signaling proteins, which act to preserve this balance. Dysregulation of these proteins, however, has been implicated in several pathologies. Specifically, both nerve growth factor (NGF) and pro-nerve growth factor (proNGF) have been implicated in the development and progression of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, through p75^{NTR}-mediated apoptotic signaling. Therefore, the identification of small molecule inhibitors capable of binding to and modulating NGF and proNGF may be of therapeutic interest. This thesis aims to characterize the inhibitory action of previously reported small molecule-based NGF inhibitors, ALE-0540, PD90780, Ro 08-2750, and PQC 083, as well as novel derivatives of these compounds, using surface plasmon resonance (SPR) spectroscopy. Of the established inhibitors, PD90780 offered the most effective inhibitory action for both NGF and proNGF binding to p75^{NTR}, suggesting that this compound may offer multipotent inhibition. Further, several novel ligand specific molecules were identified, which selectively inhibited either NGF or proNGF-p75^{NTR} interactions. Specifically, BVNP 1, BVNP 6, and BVNP 7 were shown to be effective inhibitors of NGF-p75^{NTR} interactions, while BVNP 3 and BVNP 4 were found to be selective for the inhibition of proNGF binding to p75^{NTR}. The findings of this thesis offer new insight into the use of small molecule inhibitors to

block both neurotrophin and proneurotrophin-mediated signaling. In addition, the compounds described herein may be used for future lead development.

Keywords: nerve growth factor, pro-nerve growth factor, surface plasmon resonance, p75^{NTR}, TrkA, biosensor, inhibition

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List of Abbreviations

ALS	Amyotrophic lateral sclerosis
BDNF	Brain-derived neurotrophic factor
CI	Confidence interval
CNS	Central nervous system
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FDA	Food and drug administration
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IC ₅₀	Half-maximal inhibitory concentration
K _D	Affinity constant
NGF	Nerve growth factor
NHS	N-hydroxysuccinimide
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
p75 ^{NTR}	p75 neurotrophin receptor
PNS	Peripheral nervous system
ProNGF	Pro-nerve growth factor
RU	Response unit
SPR	Surface plasmon resonance
TNF	Tumour necrosis factor
Trk	Tropomyosin-receptor kinase

Chapter 1: Introduction

Nerve growth factor (NGF) was first described by Rita Levi-Montalcini in 1951 as a result of a series of experiments examining the response of sensory and sympathetic ganglia of chick embryos upon exposure to tumor tissue (Levi-Montalcini & Hamburger, 1951). It was observed that a soluble signaling protein (later named NGF) released by the tumor promoted the development of neuronal projections from the sensory ganglia (Fig. 1.1) (Levi-Montalcini & Hamburger, 1951; Levi-Montalcini, 1952; Cohen et al., 1954). For this discovery, Levi-Montalcini was awarded the Nobel Prize in Physiology or Medicine in 1986 (Bartowska et al., 2010).

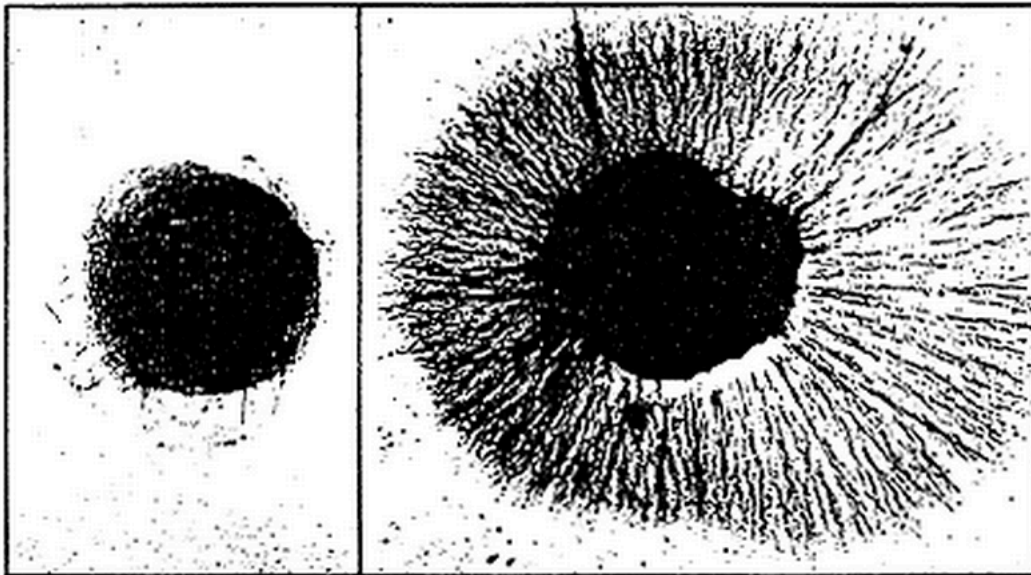


Figure 1.1: The effect of NGF on an eight-day-old chick embryo. Left panel: Ganglia were cultured in a medium containing no NGF. Right panel: Ganglia were cultured in a medium containing 10 ng/mL. This figure was originally published in *Scientific American* (Levi-Montalcini & Calissano, 1979).

In collaboration with Stanley Cohen, Levi-Montalcini developed several NGF-antibodies with which they were able to demonstrate the functional significance of NGF in the development of sensory and sympathetic ganglia (Bartowska et al., 2010). Further work by Cohen led to the discovery of other soluble signaling

proteins, including epidermal growth factor (EGF), for which he was a co-winner of the Nobel Prize in Physiology or Medicine in 1986 (Cohen, 2008).

In the following decades, several novel, yet structurally homologous proteins, were discovered and termed neurotrophins. Members of the neurotrophic factor family include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Barde et al., 1982; Philips et al., 1990; Ibanez et al., 1993).

The four members of the neurotrophin family modulate a diverse range of functions and signal with exceptional specificity. In general, neurotrophins regulate cell proliferation, differentiation, and survival in both the central and peripheral nervous systems (Cai et al., 2014). The unique signaling properties of each neurotrophin are a result of differential tropomyosin-receptor kinase (Trk) activation (Hempstead, 2014). For instance, NGF signals through TrkA, BDNF and NT-4 exert their cellular effects through TrkB, and NT-3 binds specifically to TrkC (Chao et al., 2006). In addition, each neurotrophin is capable of binding the common neurotrophin receptor, p75^{NTR}, a member of the tumor necrosis factor (TNF) receptor superfamily (Chao et al., 2006).

Neurotrophins are initially synthesized from larger precursors known as proneurotrophins, which consist of an N-terminal prodomain and a C-terminal mature domain (Hempstead et al., 2006). Proneurotrophins have been found to act as distinct ligands with biological functions beyond their roles as precursors; including the ability to oppose the actions of mature neurotrophins (Fahnestock et

al., 2004; Hempstead, 2014). Further, these proteins bind a receptor complex consisting of p75^{NTR} and sortilin, a member of the Vps 10p-domain receptor family (Nykjaer et al., 2004).

The dysregulation of most widely studied member of the neurotrophin family, NGF, in addition to its precursor, proNGF, have been implicated in several pathologies. For instance, NGF levels are elevated in several painful conditions, such as arthritis, cystitis, and chronic headaches (Aloe et al., 1992; Halliday et al., 1998; Lowe et al., 1997; Oddiah et al., 1998; Sarchielli et al., 2001). In addition, NGF administration has been shown to produce allodynia, which is defined as pain resulting from a stimulus that does not normally produce pain, in addition to causing hypersensitivity (Dyck et al., 1997). Further, NGF dysregulation has been associated with neurodegenerative diseases, such as Alzheimer's disease, as it has been shown that NGF signaling through the p75^{NTR} receptor induces apoptosis leading to neuronal death (Chao et al., 2006; Kenchappa et al., 2010).

ProNGF also acts as an apoptotic ligand through p75^{NTR}-mediated signaling and has been reported to induce apoptotic signaling in neurons, smooth muscle cells, and oligodendrocytes (Beattie et al., 2002; Lee et al., 2001; Song et al., 2010). Interestingly, proNGF has also been shown to cause apoptosis in basal forebrain cholinergic neurons, suggesting involvement in the development and/or progression of Alzheimer's disease (Al-Shawi et al., 2008). In support of this, elevated levels of proNGF have been found in both Alzheimer's diseased brains and in animal models of Alzheimer's disease (Fahnestock et al., 2001; Pedraza et al.,

2005; Perez et al., 2011). Furthermore, other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's and Huntington's diseases, have been associated with both NGF and proNGF dysregulation (Chen et al., 2008; Allen et al., 2013; Cai et al., 2014). Therefore, therapeutic strategies to inhibit both NGF and proNGF p75^{NTR}-mediated signaling may be of significant clinical interest.

Currently, there are various strategies for the inhibition of NGF and proNGF. One approach to the inhibition of proNGF focuses on modulating the p75^{NTR} receptor, as opposed to the precursor protein itself. For example, a small, nonpeptide p75^{NTR} ligand, LM11A-31, has been reported to prevent proNGF-induced death and reverse the cholinergic neurite dystrophy in Alzheimer's disease mouse models through binding the p75^{NTR} receptor (Simmons et al., 2014). Another strategy aimed at blocking proNGF-mediated signaling involves reducing the amount of proNGF synthesized in the central nervous system. Studies have shown that the administration of minocycline, a derivative of tetracycline, significantly reduces proNGF production in microglia through its ability to inhibit the phosphorylation of p38MAPK (Yune et al., 2007).

Historically, drug discovery efforts to inhibit NGF-mediated signaling have focused on inhibiting its interaction with the TrkA receptor. One example of this is the use of NGF-mimetic peptides, which competitively bind to the TrkA receptor; however, they do not elicit downstream NGF-mediated signaling. While these peptide-based strategies show biochemical promise, they have not yet been successful in clinical settings (Eibl et al., 2012). Another approach to the inhibition

of NGF is the use of humanized monoclonal antibodies. Of these antibody-mediated therapeutics, Tanezumab, developed by Pfizer, is perhaps the most successful with initial clinical trials demonstrating efficacy in the reduction of pain and improvement in joint function (Lane et al., 2010). Although Tanezumab initially demonstrated promising therapeutic potential for the treatment of chronic pain, there have been a number of safety concerns, including adverse changes to the sympathetic nervous system and osteonecrosis of joint tissue. Due to the unfavorable side effects associated with the use of this therapeutic, the Food and Drug Administration (FDA) suspended further clinical investigations of this compound (Cattaneo, 2010; Garber, 2011). Earlier this year, however, partial clinical holds on this antibody-mediated therapy were lifted.

While antibody-mediated therapies, such as Tanezumab, have become of growing clinical interest, these therapeutics remain both technically and economically challenging to produce. Furthermore, these therapies remain highly specific and are unable to cross the blood-brain barrier. Conversely, small molecules, an increasingly popular approach to the inhibition of NGF-receptor interactions, have several pharmacological advantages, including that they are generally easy to synthesize, are cost-effective, and benefit from oral activity.

Small molecule inhibitors have been previously described and have shown therapeutic potential for the inhibition of NGF-mediated signaling (Jaen et al., 1995; Owolabi et al., 1999; Niederhausser et al., 2000; Colquhuon et al., 2004; Hefti et al., 2006; Eibl et al., 2010; Eibl et al., 2013). However, there exists a gap in the scientific

research such that these inhibitors have only been investigated for their ability to inhibit NGF-TrkA interactions. Interestingly, the characterization of the ability of these molecules to block NGF-p75^{NTR} interactions has not been performed.

Furthermore, with regards to the inhibition of proNGF, no small molecule-based approaches have been investigated.

In this thesis, I completed a series of studies with the objective of characterizing the ability of both known and novel compounds to inhibit NGF and proNGF-p75^{NTR} interactions using surface plasmon resonance (SPR) technology, which is a biosensing tool that offers highly sensitive, label-free, real-time analysis; characteristics which make it an extremely suitable tool for the characterization of small molecule-based NGF inhibitors (Willander & Al-Hilli, 2009; Piliarik et al., 2009). I begin by evaluating the ability of previously reported small molecule inhibitors (ALE-0540, PD90780, Ro 08-2750, and PQC 083) to block NGF binding to both p75^{NTR} and TrkA receptors and present inhibition profiles for each compound in the form of half-maximal inhibitory concentrations (IC₅₀). Next, I explore the ability of these known compounds to block proNGF-p75^{NTR} interactions, in addition to characterizing the inhibition of both NGF and proNGF-p75^{NTR} interactions by novel bivalent naphthalimide derivatives.

Chapter 2: Hypothesis and Objectives

Both NGF and proNGF bind the common neurotrophin receptor p75^{NTR}. The dysregulation of each of these proteins has been implicated in several pathological disease states, including neurodegenerative diseases linked to p75^{NTR}-mediated apoptotic signaling. Therefore, the identification of mechanisms to modulate both NGF and proNGF signaling through the p75^{NTR} receptor may be of therapeutic interest.

Small molecule-based inhibitors which bind to and modulate NGF have been described previously. These compounds, however, have never been investigated for their ability to block either NGF or proNGF-p75^{NTR} interactions. Therefore, the central aim of this thesis is to characterize and explore the mechanisms by which established small molecule-based NGF inhibitors, as well as novel bivalent naphthalimide derivatives of these compounds, modulate both neurotrophin and proneurotrophin signaling using surface plasmon resonance (SPR) spectroscopy.

Hypothesis: Both established and novel small molecule chemical entities can inhibit NGF and proNGF from binding to the p75^{NTR} receptor.

This hypothesis will be evaluated by investigating the following experimental objectives:

Objectives:

- 1) Determine the affinity of NGF and proNGF for the p75^{NTR} receptor.
- 2) Assess the selectivity of the small molecules for NGF by examining whether or not they bind to the p75^{NTR} receptor.
- 3) Evaluate percent inhibition of binding between NGF/proNGF and p75^{NTR} by the small molecules.
- 4) Determine the half-maximal inhibitory concentration (IC₅₀) values of the small molecules for both NGF and proNGF binding to the p75^{NTR} receptor.

Chapter 3: Characterizing nerve growth factor-p75^{NTR} interactions and small molecule inhibition using surface plasmon resonance spectroscopy

Characterizing nerve growth factor-p75^{NTR} interactions and small molecule inhibition using surface plasmon resonance spectroscopy

(Original Research)

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Abstract

Nerve growth factor (NGF) is critical for the proliferation, differentiation, and survival of neurons through its binding to the p75^{NTR} and TrkA receptors. Dysregulation of NGF has been implicated in several pathologies including neurodegeneration (i.e. Parkinson's and Alzheimer's disease) and both inflammatory and neuropathic pain states. Therefore, small molecule inhibitors that block NGF-receptor interactions have significant therapeutic potential. Small molecule antagonists ALE-0540, PD90780, Ro 08-2750, and PQC 083 have all been reported to inhibit NGF from binding the TrkA receptor. Interestingly, the characterization of the ability of these molecules to block NGF-p75^{NTR} interactions has not been performed. In addition, the inhibitory action of these molecules has never been evaluated using surface plasmon resonance (SPR) spectroscopy, which has been proven to be highly useful in drug discovery applications. In the present study, we use SPR biosensors to characterize the binding of NGF to the p75^{NTR} receptor, in addition to characterizing the inhibitory potential of the known NGF antagonists. The results of this study provide the first evaluation of the ability of these compounds to block NGF binding to p75^{NTR}. In addition, only PD90780 was effective at inhibiting the interaction of NGF with p75^{NTR}, which suggests receptor selectivity between known NGF inhibitors.

Introduction

Neurotrophins are a unique family of soluble signaling proteins, which act to influence the proliferation, differentiation, and survival of neurons in the central and peripheral nervous systems (Cai et al., 2014). Nerve growth factor (NGF), the most widely studied member of this protein family, interacts with two separate receptor classes; the selective tropomyosin-receptor kinase, TrkA, and the common neurotrophin receptor, p75^{NTR} (Pattarawarapan & Burgess, 2003).

The interaction of NGF with TrkA demonstrates affinity in the high picomolar range and results in autophosphorylation of the receptor leading to a downstream signaling cascade promoting neuronal survival (Pattarawarapan & Burgess, 2003; Teng & Hempstead, 2004; Dray, 2008). Conversely, NGF-p75^{NTR} interactions are characterized by low nanomolar affinity and may lead to both pro-apoptotic and pro-survival signaling (Pattarawarapan & Burgess, 2003). While NGF is capable of binding both receptors, when both receptors are expressed, its affinity for TrkA is influenced by the presence of the p75^{NTR} receptor. For instance, p75^{NTR} has been shown to enhance the expression of TrkA, in addition to increasing its binding affinity for NGF (Massa et al., 2006; Mochetti & Brown, 2008).

NGF dysregulation has been implicated in several pathologies. For instance, both inflammatory and neuropathic pain states have been associated with NGF dysregulation and increased TrkA-mediated signaling (Chao et al., 2006; Dray, 2008; Pezet & McMahon, 2006). Likewise, increased levels of NGF have been detected in the cerebrospinal fluid of patients suffering from Parkinson's disease (Nagatsu et al,

2000) and improvements in the delivery of NGF have been shown to ameliorate cognitive function in patients with Alzheimer's disease (Tuszynski et al., 2005). It follows, then, that therapeutic strategies to inhibit NGF-receptor interactions have become of clinical interest.

One such approach to blocking NGF from binding to its receptors is small molecule inhibitors. These inhibitors bind to and modulate NGF, as opposed to binding to the TrkA and p75^{NTR} receptors. Several of these antagonists have been discovered and described in the literature (Jaen et al., 1995; Owolabi et al., 1999; Niederhauser et al., 2000; Colquhoun et al., 2004; Hefti et al., 2006; Eibl et al., 2013). Interestingly, while these molecules are known NGF-TrkA inhibitors and have been reported to inhibit TrkA phosphorylation and TrkA-mediated downstream signaling cascades, their ability to block NGF from interacting with p75^{NTR} has not yet been assessed. Further, of the previously described NGF-binding agents, none have ever been investigated using surface plasmon resonance (SPR) technology, which is a tool designed to investigate biomolecular interactions and has been proven to be useful in drug discovery applications (Myszka & Rich, 2000; Huber & Mueller, 2006; Minunni & Bilia, 2009). SPR biosensors offer highly sensitive, label-free, real-time analysis, which makes them extremely suitable for the characterization of small molecule-based NGF antagonists (Willander & Al-Hilli, 2009; Piliarik et al., 2009).

In the present study, we use SPR technology to characterize the binding of NGF to both the TrkA and p75^{NTR} receptors, in addition to characterizing the inhibitory potential of known NGF antagonists PD90780, ALE-0540, Ro 08-2750, and PQC 083.

We present affinities of NGF to each of its receptors determined through steady-state affinity analysis using SPR spectroscopy. Furthermore, we assess the receptor selectivity of the known NGF antagonists by examining their ability to inhibit NGF from binding both the p75^{NTR} and TrkA receptors. Inhibition profiles for each inhibitor in the form of specificity for NGF and half-maximal inhibitory concentrations (IC₅₀) obtained through novel methodology are presented.

Materials and Methods

Materials

Series S CM5 sensor chips, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline with EDTA (ethylenediaminetetraacetic acid) and surfactant P20 (HBS-EP) buffer (0.001 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% v/v surfactant P20), immobilization buffers (sodium acetate, pH 4.5 and pH 5.5), amine coupling reagents (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1.0 M ethanolamine-HCl, pH 8.5, and regeneration solutions (glycine-HCl, pH 2.0) were purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). Carrier-free TrkA and p75^{NTR} were obtained from R&D Systems (Minneapolis, MN, USA). Mouse NGF was obtained from Cedarlane Labs (Burlington, ON, Canada). PD90780, ALE-0540, Ro 08-2750, and PQC 083 were synthesized by Sussex Research (Ottawa, ON, Canada). Pierce elution buffer and sodium chloride were purchased from Fisher Scientific (Ottawa, ON, Canada).

SPR and preparation of sensor surfaces

All experiments were carried out using a Biacore T200 SPR spectrometer purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). Prior to immobilization, pH scouting experiments were conducted in order to determine the optimal pH for the immobilization of both the p75^{NTR} and TrkA receptors. It was concluded that 10 mM sodium acetate pH 4.5 and 5.5 yielded the greatest pre-concentration effect for p75^{NTR} and TrkA immobilizations, respectively.

Immobilization experiments investigating the optimal binding levels for both receptors were conducted, allowing for the selection of an immobilization level between 850-950 RU; increased protein loading interfered with binding kinetics, as dissociation constants were inconsistent. The CM5 sensor chip was activated by injecting a mixture of 0.2 M EDC and 0.05 M NHS at a flow rate of 5 $\mu\text{l}/\text{min}$ for 7 minutes. p75^{NTR} was diluted to 10 $\mu\text{g}/\text{mL}$ in 10 mM sodium acetate buffer pH 4.5 and immobilized until a level between 850 and 950 relative response units (RU) ($\sim 15 \text{ fmol}/\text{mm}^2$) was reached. Excess reactive esters on the sensor chip surface were deactivated with 1 M ethanolamine pH 8.5 at a flow rate of 5 $\mu\text{l}/\text{min}$ for 7 minutes. The TrkA receptor was immobilized following the above procedure; however, 10 mM sodium acetate pH 5.5 immobilization buffer was used. In the TrkA immobilization, a level between 850 and 950 RU ($\sim 11 \text{ fmol}/\text{mm}^2$) was reached. Flow cells used for reference were activated and blocked as described for the p75^{NTR} and TrkA immobilizations; however, remained uncoupled. Binding was expressed as relative response units (RU), which is defined as the response obtained from the flow cells containing the immobilized receptors minus the response obtained from the reference flow cells.

Affinity assays for NGF binding to p75^{NTR} and TrkA receptors

Affinity of NGF to p75^{NTR} and TrkA was determined using a serial dilution series. NGF was diluted in HBS-EP buffer with concentrations ranging from 0.0125 nM to 50 nM and allowed a 60 second contact time followed by a 120 second dissociation phase. For experiments involving p75^{NTR}, the sensor chip surface was

regenerated with a 15 second injection of a salt cocktail (2:1 v/v Pierce elution buffer and 4 M NaCl) previously described by Abdiche et al. (2008). In experiments with TrkA, a 15 second injection of glycine-HCl pH 2.0 was used for sensor surface regeneration.

Interactions of small molecules with p75^{NTR} and TrkA receptors

Small molecules were diluted in HBS-EP buffer at a concentration of 50 μ M and injected over the immobilized p75^{NTR} and TrkA receptors for 60 seconds. All compounds were allotted a 120 second dissociation time. Receptor binding was determined by assessing the binding response of each compound in RUs (1 RU= 1pg/mm²). The sensor chip surfaces were regenerated as described above.

Inhibition of NGF binding p75^{NTR} and TrkA receptors by small molecules

Small molecules were diluted in HBS-EP buffer at a concentration of 50 μ M and pre-incubated for 1 hour with 10 nM NGF before injection over the immobilized receptors. Control samples (with no added inhibitor) were used and yielded the maximal binding response. Percent inhibition of the protein-receptor interaction was determined by assessing the compound binding response in relation to the control sample response. The sensor chip surfaces were regenerated as described above.

IC₅₀ determination

Small molecules were diluted in HBS-EP buffer at varying concentrations ranging from 320 μ M to 3.2 nM, corresponding to equal spacing on a logarithmic

scale. Compounds were pre-incubated for 1 hour with 10 nM of NGF before injection over either the immobilized p75^{NTR} or TrkA. Dose-response curves were generated and were used for the determination of the half-maximal inhibitory concentrations (IC₅₀) for all compounds.

Data analysis

Transformation of data for NGF-receptor affinity analyses, small molecule receptor-interactions, and inhibition experiments were performed with BIA evaluation software from GE Healthcare Life Sciences (Mississauga, ON, Canada). The generation of dose-response curves and determination of IC₅₀ values were conducted using Prism GraphPad 6.0 (La Jolla, CA, USA).

Results and Discussion

Determination of the affinity of NGF to p75^{NTR} and TrkA receptors

The affinity of NGF for both p75^{NTR} and TrkA receptors was determined through steady-state analysis. A concentration series ranging from 0.0125 nM to 50 nM was run over each immobilized receptor (Figs. 3.1A, 3.1B). The concentrations chosen for this determination were based on results from screening experiments in which NGF saturated the immobilized receptors on the sensor surface. Using the BIA evaluation software, the response of NGF was plotted against concentration for each concentration of NGF used in the dilution series (Figs. 3.1C, 3.1D). Data for both p75^{NTR} and TrkA was evaluated using a 1:1 binding model. Binding of NGF to p75^{NTR} yielded a higher response than that observed with TrkA (Figs. 3.1A, 3.1B). This may be due to the fact that through the amine coupling procedure described previously, TrkA was immobilized in such a way that prevented the same amount of NGF binding. In addition, the affinity (K_D) of NGF for p75^{NTR} (12.9 ± 0.5 nM) was determined to be similar to the affinity of NGF for TrkA (15.0 ± 3.7 nM). Previously, the affinities of NGF for its receptors have been determined using kinetic studies and SPR spectroscopy, and were reported to be 1 nM for NGF-p75^{NTR} interactions and 2 nM for NGF-TrkA interactions (Nykjaer et al., 2004). While the affinities obtained through kinetic studies are lower than those obtained through our steady-state analyses, our reports are consistent with the previously reported affinity constants in that the affinity of NGF for p75^{NTR} and TrkA interactions were in a similar range. The differences in the magnitude of values may be due to the differing

methodology used for their determination. Further, our data supports the notion that the affinities of NGF to p75^{NTR} and TrkA are similar unless p75^{NTR} is co-expressed with TrkA to elicit higher affinity binding of NGF and TrkA (Hempstead et al, 1991; Ross et al., 1998; Toni et al., 2014).

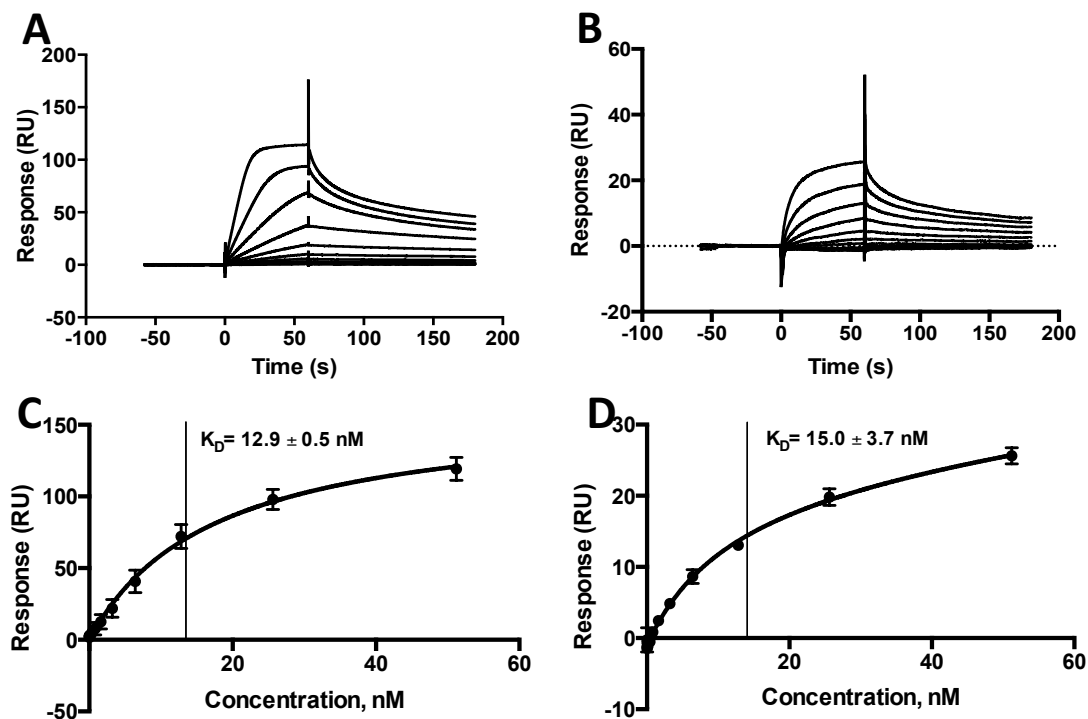


Figure 3.1: Affinity of NGF for p75^{NTR} and TrkA receptors. Concentration series of NGF (0.0125 nM to 50 nM) were run over p75^{NTR} (A) and TrkA (B) and used to calculate the affinity of NGF for each receptor through steady-state analysis. Response of NGF binding to both p75^{NTR} (C) and TrkA (D) was plotted versus concentration for each concentration used in the dilution series. The affinity (K_D) of NGF for p75^{NTR} and TrkA was determined to be 12.9 ± 0.5 nM and 15.0 ± 3.7 nM, respectively. Data points are presented as mean values of triplicate measures. Error bars are represented as standard error of the mean with an n-value=3.

Interactions of small molecules with p75^{NTR} and TrkA receptors

Previously described small molecule-based NGF antagonists, ALE-0540, PD90780, Ro 08-2750, and PQC 083, have been suggested to share conserved molecular features (Eibl et al., 2013). For instance, these inhibitors share a planar

conjugated ring system, incorporating common amine, methyl, carbonyl, and carboxylic acid functional groups (Eibl et al., 2013). The loop I/IV cleft of NGF is the proposed binding site for these small molecules inhibitors, with high importance placed on the lysine 32, phenylalanine 101, and arginine 100 residues (Eibl et al., 2013). The structures of these compounds are presented in Figure 3.2.

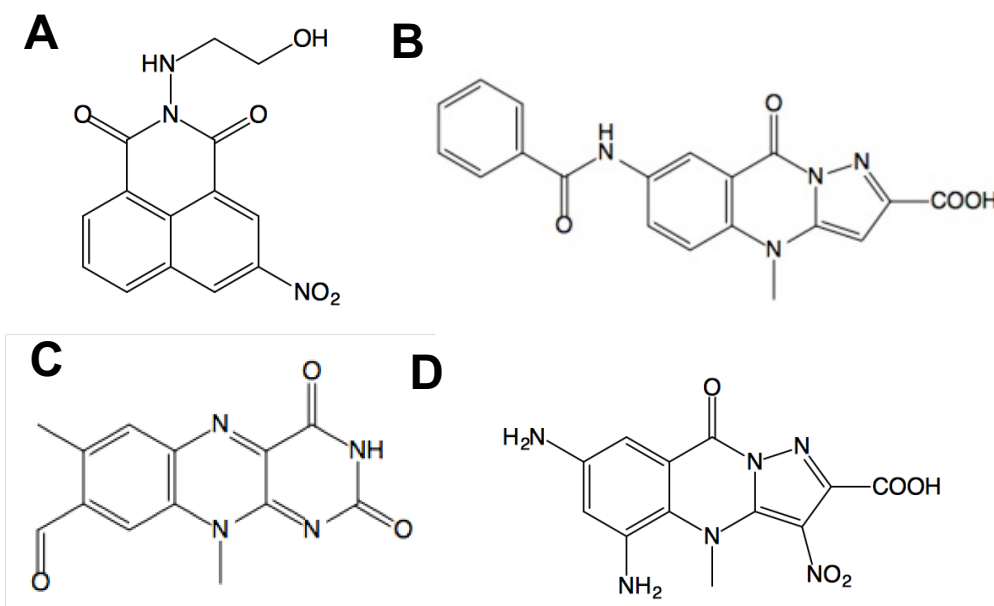


Figure 3.2: Previously reported small molecule NGF antagonists share conserved molecular features. The structures of previously reported NGF-binding antagonists (A) ALE-0540 (B) PD90780 (C) Ro 08-2750 and (D) PQC 083.

To determine the specificity of the small molecules for NGF, we assessed whether they also bound to the p75^{NTR} and TrkA receptors (Figs. 3.3A, 3.3B). After injection of 50 μ M of each compound over both the p75^{NTR} and TrkA sensor surfaces, it was found that ALE-0540 (0.2 RU), PD90780 (0.3 RU), and Ro 08-2750 (0.1 RU) bound neither the p75^{NTR} nor the TrkA receptor in comparison to a blank injection of buffer with no added small molecule ($P > 0.05$). PQC 083, on the other

hand, was found to bind significantly to both p75^{NTR} (18 RU; P=0.0006) and TrkA (42 RU; P=0.0003). We attribute the positive binding of PQC 083 to multiple proteins to the hydrophobic nature of the compound, making it unsuitable for use in SPR assays. These findings suggest that ALE-0540, PD90780, and Ro 08-2750 exhibit specific binding to NGF as opposed to the p75^{NTR} and TrkA receptors, whereas PQC 083 displays non-specific binding.

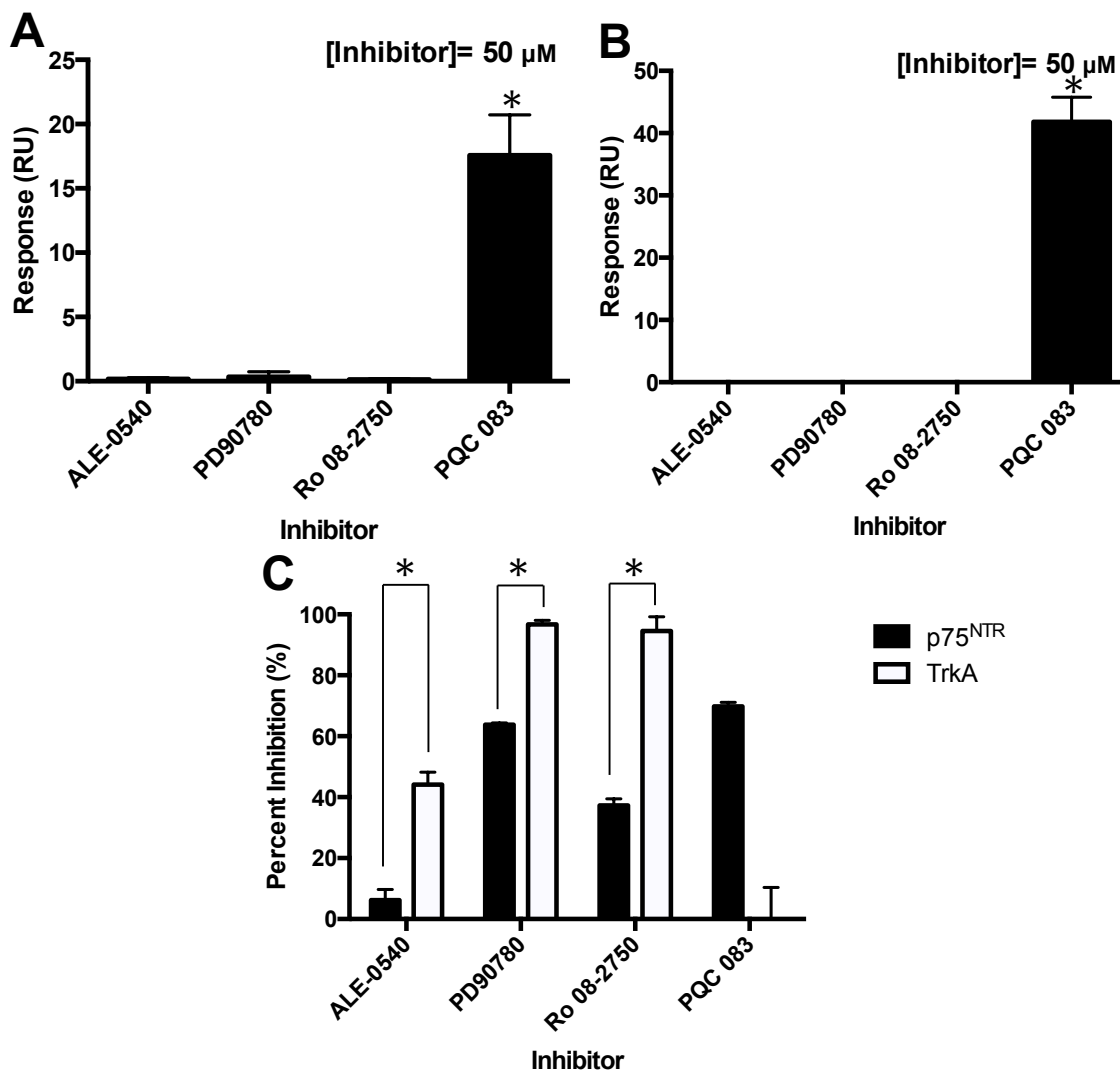


Figure 3.3: NGF antagonist receptor binding and inhibition. The known NGF antagonists were run over p75^{NTR} (A) and TrkA (B) to determine if binding is specific to NGF. The antagonists were then incubated with 10 nM of NGF and run over p75^{NTR} (C) and TrkA (D) to assess the inhibitory potential of each. Data are presented as mean values of triplicate measures with standard error of the mean bars with an n-value=3. Significant difference is denoted by an asterisk.

Inhibition and determination of half-maximal inhibitory (IC₅₀) concentrations

An evaluation of the inhibitory potential of each small molecule was conducted by incubating each compound at 50 μM with 10 nM of NGF for one hour (Figs. 3.3C, 3.3D). The binding responses were then assessed using SPR and compared to control samples of 10 nM of NGF with no added inhibitor to generate percent inhibition of maximal binding values for each small molecule. Following this, we generated a series of dose-response curves in order to determine the half-maximal inhibitory concentrations (IC₅₀) of each compound (Fig. 3.4). The IC₅₀ values are presented in Table 3.1 for ease of use and interpretation. Combined, these studies offer a novel approach to the characterization of small molecule antagonists, in addition to allowing for the determination of the specificity and selectivity of the known NGF inhibitors for both NGF-p75^{NTR} and NGF-TrkA interactions.

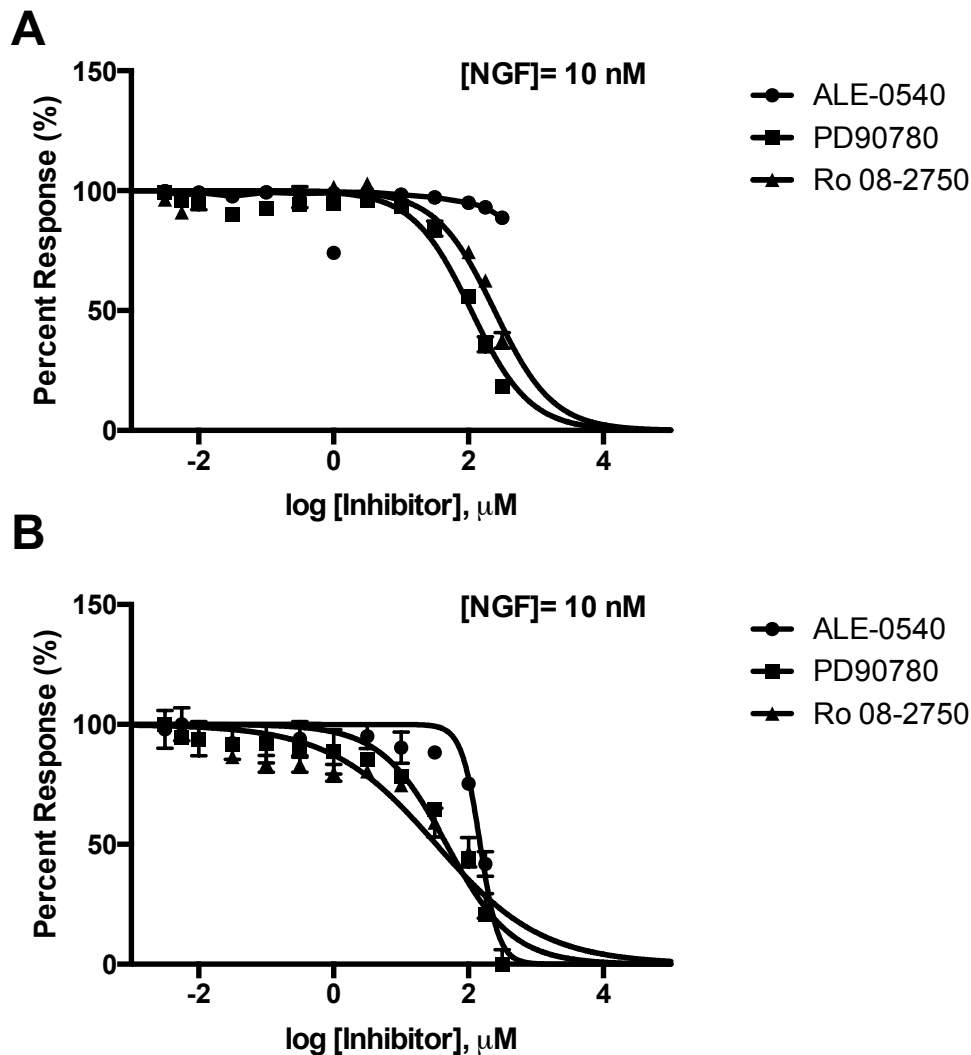


Figure 3.4: Inhibition of NGF binding to p75^{NTR} and TrkA by known NGF antagonists PD90780, ALE-0540, Ro 08-2750, and PQC 083. (A) Detailed concentration response demonstrates that ALE-0540 ($\text{IC}_{50} = > 300 \mu\text{M}$; $R^2 < 0.5$), PD90780 ($\text{IC}_{50} = 110 \mu\text{M}$; $\text{CI} = 93\text{-}129 \mu\text{M}$; $R^2 = 0.95$), and Ro 08-2750 ($\text{IC}_{50} = 244 \mu\text{M}$; $\text{CI} = 203\text{-}293 \mu\text{M}$; $R^2 = 0.87$) inhibits NGF-p75^{NTR} interactions in the low-to-high micromolar range. (B) Detailed concentration response demonstrates that ALE-0540 ($\text{IC}_{50} = 149 \mu\text{M}$; $\text{CI} = 128\text{-}175 \mu\text{M}$; $R^2 = 0.88$), PD90780 ($\text{IC}_{50} = 47 \mu\text{M}$; $\text{CI} = 35\text{-}64 \mu\text{M}$; $R^2 = 0.94$), and Ro 08-2750 ($\text{IC}_{50} = 33 \mu\text{M}$; $\text{CI} = 19\text{-}57 \mu\text{M}$; $R^2 = 0.87$) inhibits NGF-TrkA interactions in the mid-to-high micromolar range. Data points are presented as mean values of triplicate measures. Error bars are represented as standard error of the mean with an n-value=3. IC_{50} values are presented with 95% confidence intervals (CI). R^2 is defined as the coefficient of determination and used to assess goodness of fit.

Our studies indicate that ALE-0540 has a moderate inhibitory effect on NGF-TrkA interactions (44%) and that it is not a strong inhibitor of NGF binding to p75^{NTR} (6.2%); this differential receptor inhibition is statistically different ($P=0.0002$). Whereas accurate inhibition curves could not be plotted for NGF-p75^{NTR} interactions ($IC_{50}>300\mu\text{M}$), dose-response assays demonstrate that ALE-0540 has inhibitory action in the high micromolar range for TrkA ($149\ \mu\text{M}$; $CI= 128-175\ \mu\text{M}$). The previously reported value for NGF-TrkA interactions is $3.7\ \mu\text{M}$ (Owolabi et al., 1999); the discrepancy between values is likely due to the differences in the assays used for determination. For instance, experiments conducted by Owolabi et al. (1999) utilized PC12 whole cell studies, whereas the assay presented here eliminates certain possibilities, such as receptor interactions. There is no reported IC_{50} for NGF-p75^{NTR} interactions; however, an IC_{50} of $5.8\ \mu\text{M}$ has been reported for NGF-TrkA-p75^{NTR} (Owolabi et al., 1999). The low micromolar inhibition reported here might be due to the presence of the TrkA receptor and, as such, is not comparable to the NGF-p75^{NTR} data reported in this study.

PD90780 was found to be an effective inhibitor of NGF binding to TrkA (100%) and a moderate inhibitor in blocking NGF-p75^{NTR} interactions (63%). The inhibitory action of PD 90780 on NGF-TrkA interactions was shown to be significantly greater than for the interaction of NGF and p75^{NTR} ($P=0.0001$). Inhibitory binding assays revealed mid-micromolar inhibition of NGF-TrkA ($47\ \mu\text{M}$; $CI= 35-64\mu\text{M}$) and high micromolar inhibition of NGF-p75^{NTR} ($110\ \mu\text{M}$; $CI= 93-129\ \mu\text{M}$). Colquhoun et al. (2004) have previously described the inhibitory effects of

PD90780 on NGF-TrkA and NGF-p75^{NTR} interactions, and report 1.8 μM and 23.1 μM , respectively. Our data is consistent with PD90780 having a greater inhibitory effect on NGF binding TrkA in comparison to p75^{NTR}. In comparison to the low micromolar values reported previously, our data fall in the mid-to-high micromolar range, which could be due to the differences in experimental procedures and assays used.

Our studies demonstrate that Ro 08-2750 blocks NGF from interacting with TrkA (99%) significantly more than p75^{NTR} (37%) ($P=0.0001$). Our dose-response studies indicate that Ro 08-2750 has inhibitory action in the low-to-mid micromolar range (33 μM ; CI= 19-57 μM) for NGF-TrkA and high micromolar range (244 μM ; CI= 203-293 μM) for NGF-p75^{NTR}. Although Ro 08-2750 has been described previously (Niederhauser et al., 2000), there are no reported IC₅₀ values for the inhibition of NGF-receptor interactions in the literature.

Interestingly, PQC 083 demonstrated moderate inhibitory effects of NGF binding p75^{NTR} (70%); however, mixed inhibition of NGF-TrkA interactions was observed. For instance, PQC 083 demonstrated high non-specific binding (bound the receptor in the absence of inhibiting NGF receptor binding). Further, the inhibitory binding assays proved to be difficult to perform with PQC 083 due to the incompatibility of the compound with SPR assays. We were, therefore, unable to analyze dose-response data when evaluating the inhibitory action of PQC 083 on both NGF-p75^{NTR} and NGF-TrkA interactions; no IC₅₀ values could be determined for this inhibitor. However, in previous studies conducted by Eibl et al. (2012), low

micromolar (7.0 μM) inhibition of NGF binding to TrkA by PQC 083 has been reported. The inhibitory capacity of PQC 083 to block NGF from interacting with p75^{NTR}, though, has not been assessed.

Table 3.1: IC₅₀ data for small molecule inhibition of NGF-p75^{NTR} and NGF-TrkA interactions.
Data are presented as mean values of triplicate measures with n=3.

	p75 ^{NTR}	TrkA
ALE-0540	> 300 μM	149 μM (CI= 128-175 μM)
PD90780	110 μM (CI= 93-129 μM)	47 μM (CI= 35-64 μM)
Ro 08-2750	244 μM (CI= 203-293 μM)	33 μM (CI= 19-57 μM)
PQC 083	Not Determined	Not Determined

Conclusions

Through the utilization of SPR technology, the effectiveness of small molecule-based NGF inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083 was assessed with regards to their ability to inhibit binding to both the p75^{NTR} and TrkA receptors. PD90780 was found to offer the most potential for the inhibition of both NGF-TrkA and NGF-p75^{NTR} interactions. ALE-0540 offered inhibitory action for only NGF binding to TrkA, while Ro 08-2750 was shown to be an effective inhibitor of NGF-TrkA binding and only mildly blocked NGF from interacting with p75^{NTR}. Conversely, PQC 083 yielded experimental artifacts (demonstrated non-specific binding) making it difficult to characterize with our assays. Using SPR biosensors to characterize NGF-binding agents offers highly sensitive, label-free, real-time analysis. This technology has been proven to be highly effective in drug discovery applications and may be useful for further identification of novel therapeutics that inhibit NGF-p75^{NTR} interactions, which may have implications in the treatment of neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases.

Chapter 4: Using surface plasmon resonance spectroscopy to characterize the inhibition of NGF-p75^{NTR} and proNGF-p75^{NTR} interactions by small molecule inhibitors

Using surface plasmon resonance spectroscopy to characterize the inhibition of NGF-p75^{NTR} and proNGF-p75^{NTR} interactions by small molecule inhibitors

(Original Research)

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Abstract

Nerve growth factor (NGF), a member of the neurotrophin family, acts to influence the survival and differentiation of neurons in both the central and peripheral nervous systems via its binding to the p75^{NTR} and TrkA receptors. Its precursor, proNGF, has been shown to be the dominant form of NGF in the central nervous system, suggesting a biological function beyond its role as a precursor. Like NGF, proNGF is known to bind the p75^{NTR} receptor. The dysregulation of both NGF and proNGF have been implicated in several pathologies, including neurodegenerative diseases linked to p75^{NTR}-mediated apoptotic signaling. Therefore, the identification of small molecule inhibitors capable of inhibiting both NGF and proNGF-p75^{NTR} interactions may be of therapeutic interest. In the present study, we examine the inhibitory action of known small molecule-based inhibitors PD90780, ALE-0540, Ro 08-2750, and PQC 083, as well as novel derivatives of these compounds, using surface plasmon resonance (SPR) spectroscopy.

Introduction

Neurotrophins are a unique family of soluble signaling proteins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). These proteins act to influence the survival and differentiation of neurons in the central and peripheral nervous systems, in addition to being critical to both developing and mature nervous systems (Chao et al., 2006). While the four members of the neurotrophin family share remarkable structural homology, these proteins modulate a diverse range of functions and signal with exceptional specificity (Pattarawarapan & Burgess, 2003). The unique signaling properties of each neurotrophin are a result of differential tropomyosin-receptor kinase (Trk) activation (Huang & Reichardt, 2003). For instance, the most widely studied member of the neurotrophin family, NGF, signals through the selective tropomyosin-receptor kinase, TrkA, in addition to the common neurotrophin receptor, p75^{NTR} (Kaplan & Miller, 2000).

NGF-TrkA interactions demonstrate picomolar affinity, which results in the autophosphorylation of the receptor leading to a downstream cascade of Trk-mediated signaling and the promotion of neuronal survival (Pattarawarapan & Burgess, 2003; Teng & Hempstead, 2004; Dray, 2008). Conversely, NGF signaling through the p75^{NTR} receptor results in a low nanomolar interaction and is recognized to be complex. For example, NGF-mediated p75^{NTR} signaling has been implicated in both neuronal survival and neuronal death, in addition to axonal pruning and apoptosis (Colquhoun et al., 2004; He & Garcia, 2004). While NGF binds to both the TrkA and p75^{NTR} receptors, the high affinity nature of NGF-TrkA binding

is mediated by the presence of p75^{NTR} (Massa et al., 2006; Mochetti & Brown, 2008). The mechanism by which p75^{NTR} mediates the NGF-TrkA high-affinity state is still not fully understood. Currently, there are two theories; the first suggests that p75^{NTR}, TrkA, and NGF form a ternary complex resulting in a higher affinity in comparison to when either receptor is expressed alone, while the other theory proposes that p75^{NTR} acts to cluster the NGF together, facilitating binding to TrkA (Barker, 2007).

All neurotrophins are synthesized from larger precursors known as proneurotrophins, which consist of an N-terminal prodomain and a C-terminal mature domain (Wehrman et al., 2007). The precursor of NGF, proNGF, while initially thought to be biologically inactive, has been shown to be the dominant form of NGF in the central nervous system, suggesting a biological function beyond its role as a precursor (Fahnestock et al., 2004). ProNGF, like NGF, binds the p75^{NTR} receptor; however, proNGF also binds sortilin, a member of the Vps 10p-domain receptor family (Nykjaer et al., 2004).

The interaction of proNGF with p75^{NTR} has been shown to demonstrate low nanomolar affinity, whereas its interaction with sortilin is of much higher affinity (Nykjaer et al., 2004). ProNGF has been shown to induce apoptosis through the activation of the p75^{NTR} receptor, which further supports the notion that the precursor is a distinct and biologically active ligand capable of opposing the actions of the mature neurotrophin, NGF (Lee et al., 2001; Song et al., 2010).

Dysregulation of both NGF and proNGF have been implicated in several pathologies. For example, NGF levels are elevated in several painful conditions, such as arthritis, cystitis, and chronic headaches (Aloe et al., 1992; Lowe et al., 1997; Halliday et al., 1998; Oddiah et al., 1998; Sarchielli et al., 2001). In addition, a subcutaneous injection of NGF into the forearm of healthy adults was found to produce allodynia, which is defined as pain resulting from a stimulus that does not normally produce pain, in addition to causing hypersensitivity (Dyck et al., 1997). Further, NGF dysregulation has been implicated in neurodegenerative disease states as NGF-p75^{NTR} mediated signaling induces apoptosis leading to neuronal death (Kenchappa et al., 2010). Likewise, proNGF has been associated with neurodegeneration. For instance, proNGF-p75^{NTR} interactions induce apoptotic signaling in neurons, smooth muscle cells, and oligodendrocytes at subnanomolar concentrations (Lee et al., 2001; Beattie et al., 2002; Song et al., 2010). In addition, increased levels of proNGF have been found in Alzheimer's diseased brains (Fahnestock et al., 2001). Therefore, therapeutic strategies to inhibit both NGF and proNGF signaling may be of significant clinical interest.

While the inhibition of NGF has been widely studied, investigations regarding the inhibition of proNGF are limited. One therapeutic approach gaining increased attention is the use of small molecule inhibitors to block NGF from binding the TrkA and p75^{NTR} receptors. These compounds bind NGF (as opposed to the receptors) in order to modulate signaling and have been described previously (Jaen et al., 1995; Owolabi et al., 1999; Niederhauser et al., 2000; Colquhoun et al., 2004; Hefti et al., 2006; Eibl et al., 2010; Eibl et al., 2013; Sheffield et al., 2015). Conversely, proNGF

inhibitory investigations have focused on modulating the p75^{NTR} receptor (as opposed to proNGF itself), as well as reducing the production of proNGF in the central nervous system (Massa et al., 2006; Yune et al., 2007; Al-Gayyar et al., 2011). Interestingly, therapeutics that bind to and modulate proNGF have not yet been investigated.

In the present study, we use surface plasmon resonance (SPR) spectroscopy to characterize proNGF-p75^{NTR} interactions. In addition, we examine the inhibitory potential of known small molecule-based inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083, as well as novel derivatives of these compounds. We assess the ligand selectivity of the small molecules through the examination of their ability to block both NGF and proNGF from binding the p75^{NTR} receptor.

Materials and Methods

Materials

Series S CM5 sensor chips, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline with EDTA (ethylenediaminetetraacetic acid) and surfactant P20 (HBS-EP) buffer (0.001 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% v/v surfactant P20), immobilization buffer (sodium acetate, pH 4.5), amine coupling reagents (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1.0 M ethanolamine-HCl, pH 8.5, and regeneration solutions (glycine-HCl, pH 2.0) were purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada). Carrier-free p75^{NTR} was obtained from R&D Systems (Minneapolis, MN, USA). Mouse NGF and recombinant human proNGF were obtained from Cedarlane Labs (Burlington, ON, Canada). All small molecule inhibitors were synthesized by Sussex Research (Ottawa, ON, Canada).

SPR and sensor surface preparation

All experiments were carried out using a Biacore T200 SPR spectrometer from GE Healthcare Life Sciences (Mississauga, ON, Canada). Prior to immobilization, pH scouting experiments were conducted in order to determine the optimal pH for the immobilization of the p75^{NTR} receptor and it was concluded that 10 mM sodium acetate pH 4.5 yielded the greatest pre-concentration effect. Immobilization experiments investigating the optimal binding levels for both receptors were conducted, allowing for the selection of an immobilization level

between 850-950 RU, as increased protein loading interfered with binding kinetics. The CM5 sensor chip was activated by injecting a mixture of 0.2 M EDC and 0.05 M NHS at a flow rate of 5 $\mu\text{l}/\text{min}$ for 7 minutes. p75^{NTR} was diluted to 10 $\mu\text{g}/\text{mL}$ in 10 mM sodium acetate buffer, pH 4.5 and immobilized until a level between 850 and 950 relative response units (RU) ($\sim 15 \text{ fmol}/\text{mm}^2$) was reached. Excess reactive esters on the sensor chip surface were deactivated with 1 M ethanolamine, pH 8.5 at a flow rate of 5 $\mu\text{l}/\text{min}$ for 7 minutes. Flow cells used for reference were activated and blocked as described above, but remained uncoupled. Binding was expressed as relative response units (RU), which is defined as the response obtained from the flow cell containing the immobilized receptor minus the response obtained from the reference flow cell.

Affinity assays for NGF and proNGF- p75^{NTR} interactions

The affinity of NGF and proNGF to p75^{NTR} was determined using a serial dilution series. NGF was diluted in HBS-EP buffer with concentrations ranging from 0.0125 nM to 50 nM and allowed a 60 second contact time followed by a 120 second dissociation phase. Similarly, proNGF was diluted in HBS-EP buffer with concentrations ranging from 0.025 nM to 100nM and allowed a 120 second contact time followed by a 900 second dissociation phase. The sensor chip surface was regenerated with a 15 second injection of a salt cocktail (2:1 v/v Pierce elution buffer and 4 M NaCl) and a 15 second injection of glycine-HCl, pH 2.0 for NGF and proNGF experiments, respectively. The response of NGF and proNGF obtained from

each concentration in the dilution series was plotted against concentration value using the BIA evaluation software and was evaluated using a 1:1 binding model.

Interactions of small molecules with the p75^{NTR} and receptor

Small molecules were diluted in HBS-EP buffer to a concentration of 100 μM and injected over the immobilized p75^{NTR} receptor for 60 seconds. All compounds were allotted a 120 second dissociation time. Receptor binding was determined by assessing the binding response of each compound in RUs (1 RU= 1pg/mm²). The sensor chip surface was regenerated as described above.

Inhibition of NGF and proNGF binding the p75^{NTR} receptor by small molecules

Small molecules were diluted in HBS-EP buffer at a single concentration (100 μM) and pre-incubated for 1 hour with either 10 nM NGF or 10 nM proNGF before injection over the immobilized receptor. Control samples (with no added inhibitor) were used to determine the maximal binding response. Percent inhibition of both NGF-p75^{NTR} and proNGF-p75^{NTR} interactions were determined by assessing the compound binding response in relation to the control sample response. The sensor chip surfaces were regenerated as described above.

The accuracy of our assay system is typically 3% standard deviation, and antagonists of interest demonstrate ligand selectivity through a 30% or greater difference between the inhibition of NGF and proNGF-p75^{NTR} interactions. Power calculations suggested triplicate measurement would allow for the detection of greater than 20% inhibition with confidence of greater than 95%.

IC₅₀ determination

Half-maximal inhibitory concentrations (IC₅₀) were determined for small molecules exhibiting 50% or greater percent inhibition of maximal binding. Small molecules were diluted in HBS-EP buffer at varying concentrations ranging from 320 μM to 3.2 nM and 100 μM to 10 nM (corresponding to equal spacing on a logarithmic scale) for NGF and proNGF experiments, respectively. Compounds were pre-incubated for 1 hour with either 10 nM NGF or 10 nM proNGF before injection over the immobilized p75^{NTR} receptor. Dose-response curves were generated and were used for the determination of the half-maximal inhibitory concentrations (IC₅₀).

Data analysis

Transformation of data for NGF and proNGF-p75^{NTR} affinity analysis, small molecule receptor-interactions, and inhibition experiments were performed with BIA evaluation software from GE Healthcare Life Sciences (Mississauga, ON, Canada). The generation of dose-response curves and determination of IC₅₀ values were conducted using Prism GraphPad 6.0 (La Jolla, CA, USA).

Results

The affinity of proNGF-p75^{NTR} interactions

The affinity of both NGF and proNGF for the p75^{NTR} receptor was determined using steady-state analysis. For NGF experiments, a concentration series ranging from 0.0125 nM to 50 nM was run over the immobilized receptor and allowed a 60 second contact time followed by a 120 second dissociation phase (Fig. 4.1A). Similarly, proNGF was flowed over the immobilized receptor for 120 seconds at varying concentrations (ranging from 0.025 nM to 100 nM) and allotted a 900 second timeframe before regeneration of the sensor surface due to the slow dissociation rate of the protein-receptor interaction (Fig. 4.1B). The concentration values used in these dilution series were determined based on results from preliminary experiments in which NGF and proNGF reached near-saturation levels of the immobilized p75^{NTR} on the sensor surface. The response of NGF and proNGF obtained from each concentration in the dilution series was plotted against concentration value using the BIA evaluation software and was evaluated using a 1:1 binding model (Fig. 4.1C, 4.1 D). The affinity (K_D) of NGF for p75^{NTR} was determined to be 12.9 ± 0.5 nM, while the affinity of proNGF for p75^{NTR} was determined to be 23.5 ± 0.4 nM. As reported by Nykjaer et al. (2004), the reduced affinity of the proNGF-p75^{NTR} interaction, in comparison to the interaction between NGF and p75^{NTR}, is due to lack of processing of the precursor protein.

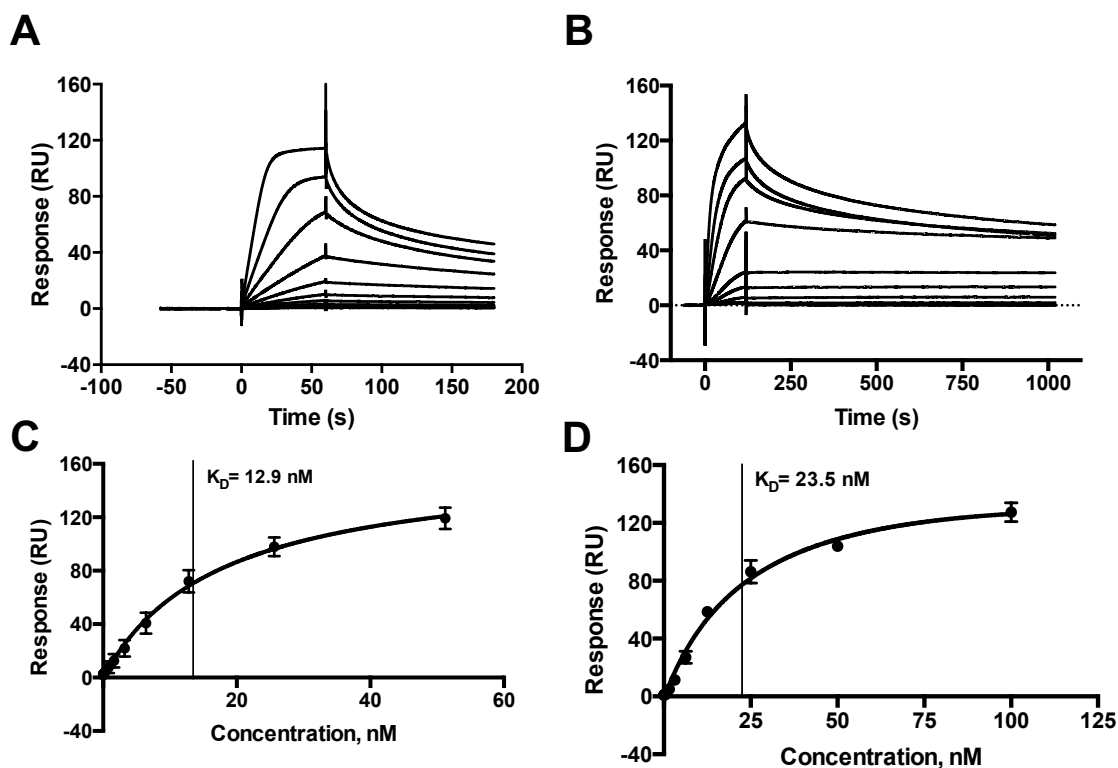
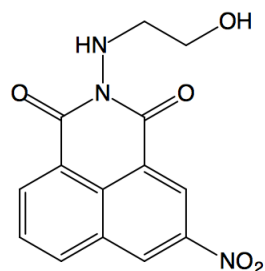


Figure 4.1: Affinity of NGF and proNGF for the p75^{NTR} receptor. Concentration series of NGF (0.0125 nM to 50 nM) (A) and proNGF (0.025 nM to 100 nM) (B) were run over p75^{NTR} and used to calculate the affinity of the ligand-receptor interactions through steady-state analysis. Response of NGF (C) and proNGF (D) binding to p75^{NTR} was plotted versus concentration. The affinity (K_D) of NGF and proNGF for p75^{NTR} was determined to be 12.9 ± 0.5 nM and 23.5 ± 0.4 nM, respectively. Data points are presented as mean values of triplicate measures. Error bars are represented as standard error of the mean with an n-value=3.

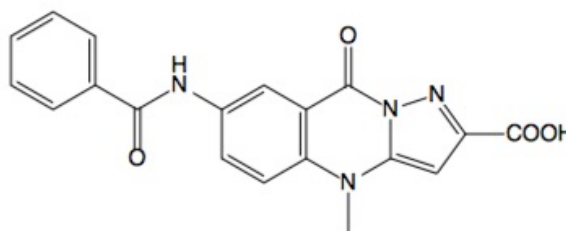
Small molecule and p75^{NTR} receptor interactions

It has been suggested that previously reported small molecule-based inhibitors, ALE-0540, PD90780, Ro 08-2750, and PQC 083, share structural homology (Fig. 4.2) (Eibl et al., 2013). In this study, the known inhibitors, in addition to novel derivatives of ALE-0540, were evaluated for their ability to inhibit both NGF and proNGF from binding the p75^{NTR} receptor. The structures of these novel compounds are presented in Figure 4.3.

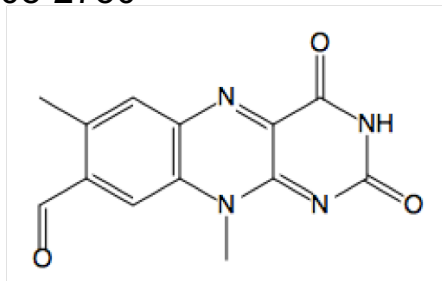
ALE-0540



PD90780



Ro 08-2750



PQC 083

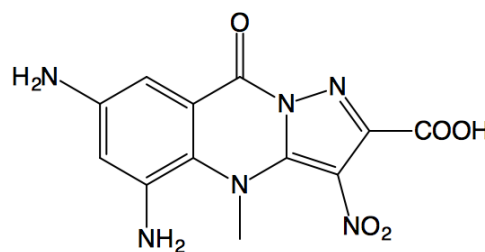


Figure 4.2: Previously reported small molecule NGF inhibitors share remarkable structural homology. The structures of previously reported NGF-binding inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083.

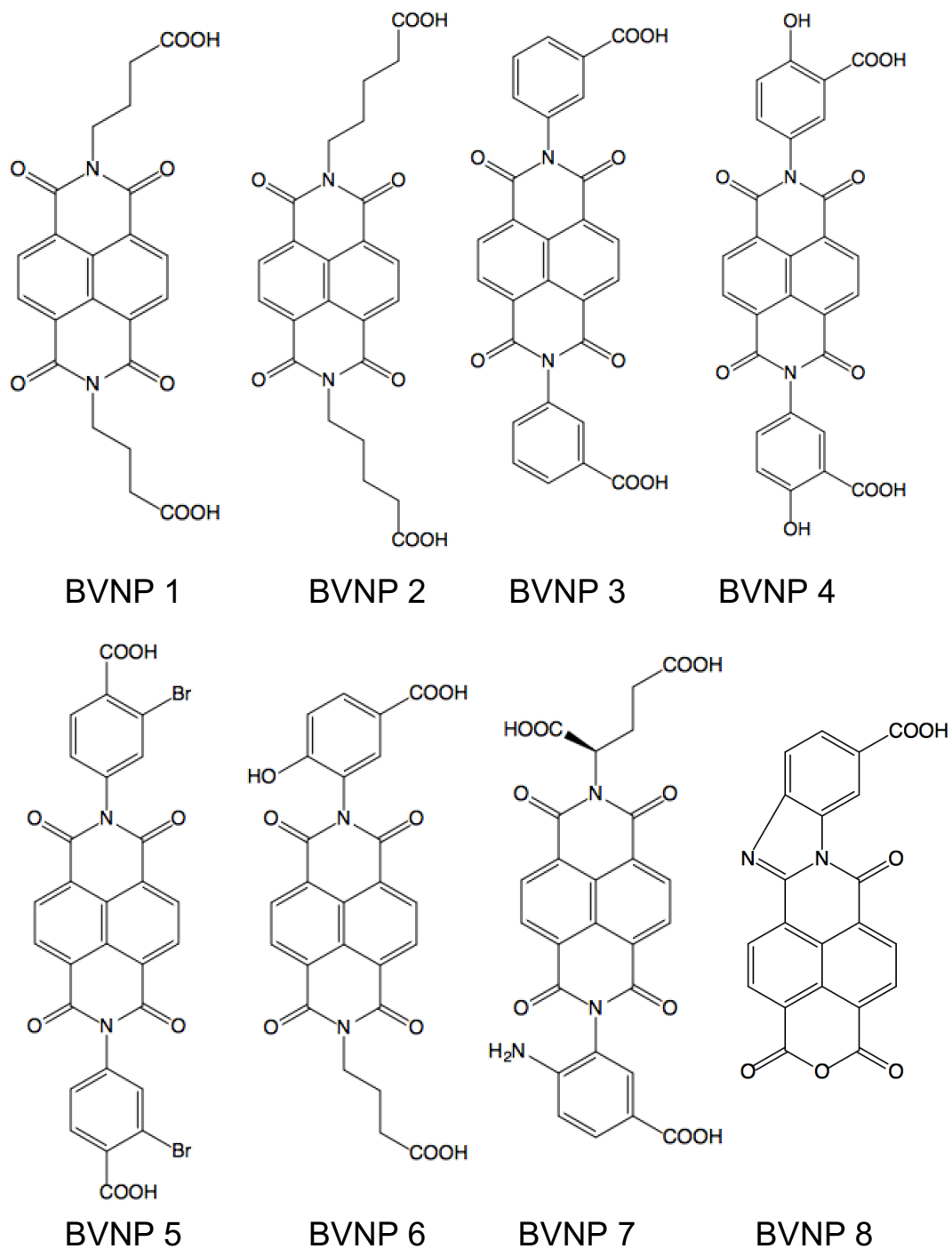


Figure 4.3: Novel bivalent naphthalimide derivatives of ALE-0540. The structures of novel inhibitors BVNP 1, BVNP 2, BVNP 3, BVNP 4, BVNP 5, BVNP 6, BVNP 7, and BVNP 8. Compounds were custom synthesized by Sussex Research (Ottawa, ON).

In order to ensure that the inhibitors were ligand-specific and did not bind the p75^{NTR} receptor, we injected the compounds (100 μ M) over the immobilized p75^{NTR} with no added NGF or proNGF (Fig. 4.4A). Of the known inhibitors, PQC 083 was found to show significant binding to the p75^{NTR} receptor (72 RU; P=0.0131), while PD90780, ALE-0540, and Ro 08-2750 showed minimal or no binding in comparison to a blank injection of buffer with no added small molecule (P>0.05). Our evaluation of the novel derivatives revealed that BVNP 1, BVNP 2, BVNP 3, BVNP 4, BVNP 6, and BVNP 7 did not significantly bind the p75^{NTR} receptor (P>0.05). Conversely, BVNP 5 (34 RU; P=0.014) and BVNP 8 (59 RU; P=0.0004) demonstrated significant binding to the receptor. Interestingly, PQC 083 (P=0.0014), BVNP 5 (P=0.0053), and BVNP 8 (P=0.005) also displayed significant binding to the blank, uncoupled reference flow cell, in which there is no immobilized protein, in comparison to the remaining compounds (P>0.05) (Fig. 4.4B), supporting the notion that these compounds demonstrate non-specific binding. Due to the non-specific nature of the binding demonstrated by these inhibitors, PQC 083, BVNP 5, and BVNP 8 are difficult to characterize using SPR spectroscopy and were concluded to be unsuitable for use in this assay system.

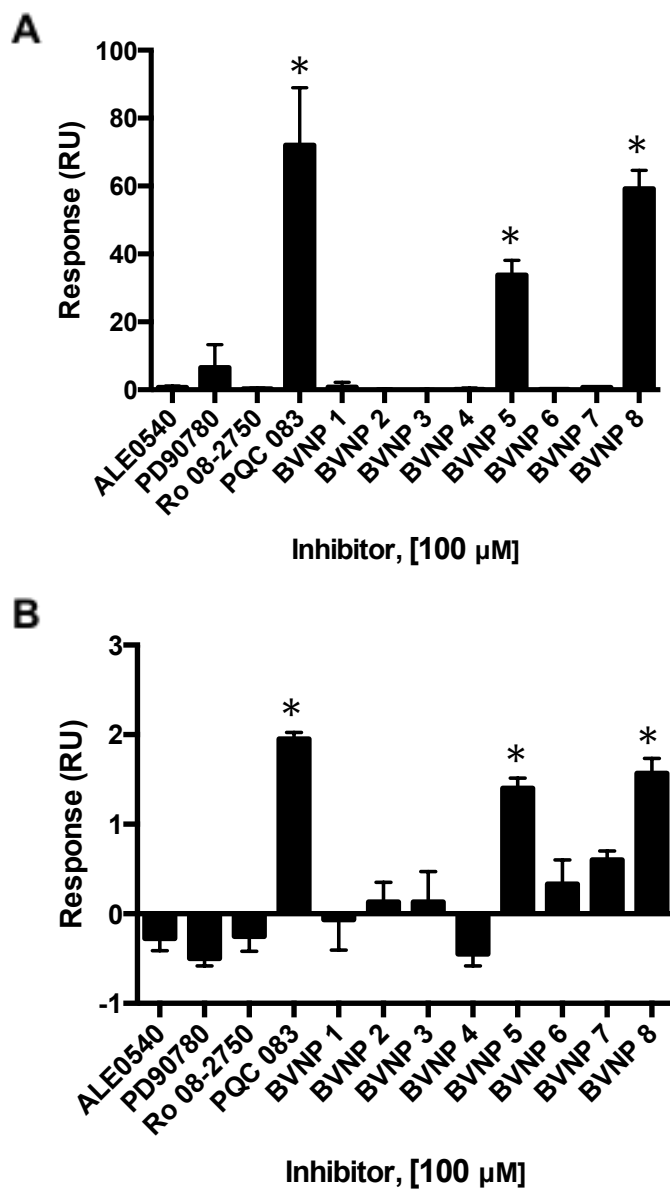


Figure 4.4: Small molecule binding to the p75^{NTR} receptor and uncoupled reference flow cell. The inhibitors (100 μM) were run over p75^{NTR} (A) and the uncoupled reference flow cell (B) in order to determine the specificity of compounds. Data are presented as mean values of triplicate measures with standard error of the mean bars with an n-value=3. Significant difference is denoted by an asterisk.

Inhibition of NGF-p75^{NTR} and proNGF-p75^{NTR} interactions by small molecules

The inhibitory potential of known and novel small molecule inhibitors was assessed for the inhibition of both NGF-p75^{NTR} and proNGF-p75^{NTR} interactions. Compounds (100 μ M) were incubated for one hour with either 10 nM NGF and 10 nM proNGF before injection over the immobilized p75^{NTR} on the sensor surface (Fig. 4.5). It should be noted that the concentration (10 nM) of NGF and proNGF used in these inhibitory assays were below the determined K_D values to minimize potential non-specific binding. At this concentration, reasonable binding responses were obtained, such that accurate IC_{50} values were determined. Next, using SPR spectroscopy, the binding responses of each compound were assessed and compared to a control sample of either 10 nM NGF or 10 nM proNGF in which no inhibitor was added. Percent inhibitions of maximal binding values were then generated for each small molecule. Due to the unsuitability of PQC 083, BVNP 5, and BVNP 8 for use with SPR spectroscopy, the evaluation of these compounds yielded experimental artifacts and were therefore not included in this analysis.

Our studies of the known inhibitors indicate that at a concentration of 100 μ M, ALE-0540 had minimal inhibitory action on both NGF-p75^{NTR} and proNGF-p75^{NTR} interactions (7.5% and 13%, respectively) ($P > 0.05$). Ro 08-2750, on the other hand, was found to be significantly more effective at blocking NGF from interacting with the p75^{NTR} receptor (39%), in comparison to its ability to block proNGF (17%) ($P = 0.0235$). Finally, PD90780 was found to inhibit proNGF from

binding p75^{NTR} (91%), whereas it had meaningfully less inhibitory action for NGF-p75^{NTR} interactions (59%) (P=0.0023).

Using a single screening concentration for each of the compounds (100 μ M), studies of our novel inhibitors revealed that BVNP 1 displayed moderate inhibition of both NGF (54%) and proNGF (34%) binding to the p75^{NTR} receptor (P>0.05). Likewise, BVNP 2 offered similar inhibitory action for both NGF (38%) and proNGF (31%) receptor interactions (P>0.05). BVNP 3, on the other hand, was found to be highly effective for proNGF-p75^{NTR} interactions (91%), but was not a strong inhibitor of NGF binding to p75^{NTR} (28%); this differential receptor inhibition was found to be statistically significant (P=0.0001). Similarly, BVNP 4 was more effective at inhibiting proNGF (54%) from binding to p75^{NTR} than NGF (28%); however, the difference in receptor inhibition was not significant (P>0.05). Conversely, BVNP 6 demonstrated effective inhibition of NGF-p75^{NTR} binding (86%), whereas it offered meaningfully less inhibition for the interaction of proNGF and p75^{NTR} (32%) (P=0.0183). Finally, BVNP 7 displayed strong inhibition of NGF-p75^{NTR} binding (89%), while it offered significantly less inhibitory action for proNGF-p75^{NTR} interactions (4%) (P=0.0001).

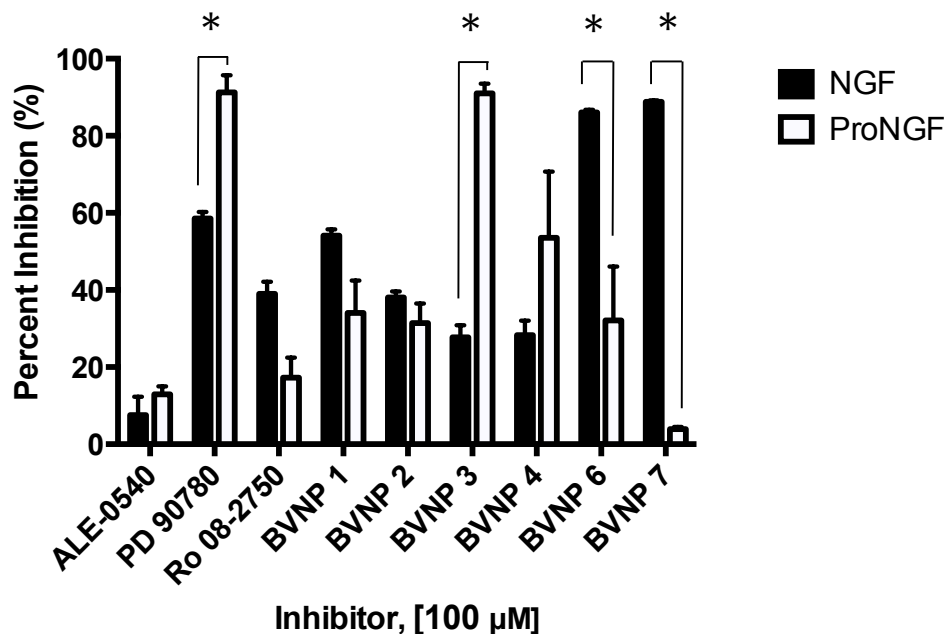


Figure 4.5: The inhibition of NGF-p75^{NTR} and proNGF-p75^{NTR} interactions by known and novel inhibitors. The compounds (100 μM) were incubated with 10 nM NGF and 10 nM proNGF to assess their ability to inhibit both NGF-p75^{NTR} and proNGF-p75^{NTR} interactions. Data are presented as mean values of triplicate measures with standard error of the mean bars with an n-value=3. Significant difference is denoted by an asterisk.

Determination of half-maximal inhibitory (IC₅₀) concentrations

Dose-response curves were generated for small molecules exhibiting 50% or greater percent inhibition of maximal binding by incubating varying concentrations of small molecule with either 10 nM NGF or 10 nM proNGF (Fig. 4.6). Using these curves, half-maximal inhibitory (IC₅₀) concentrations were determined and are presented in Table 4.1.

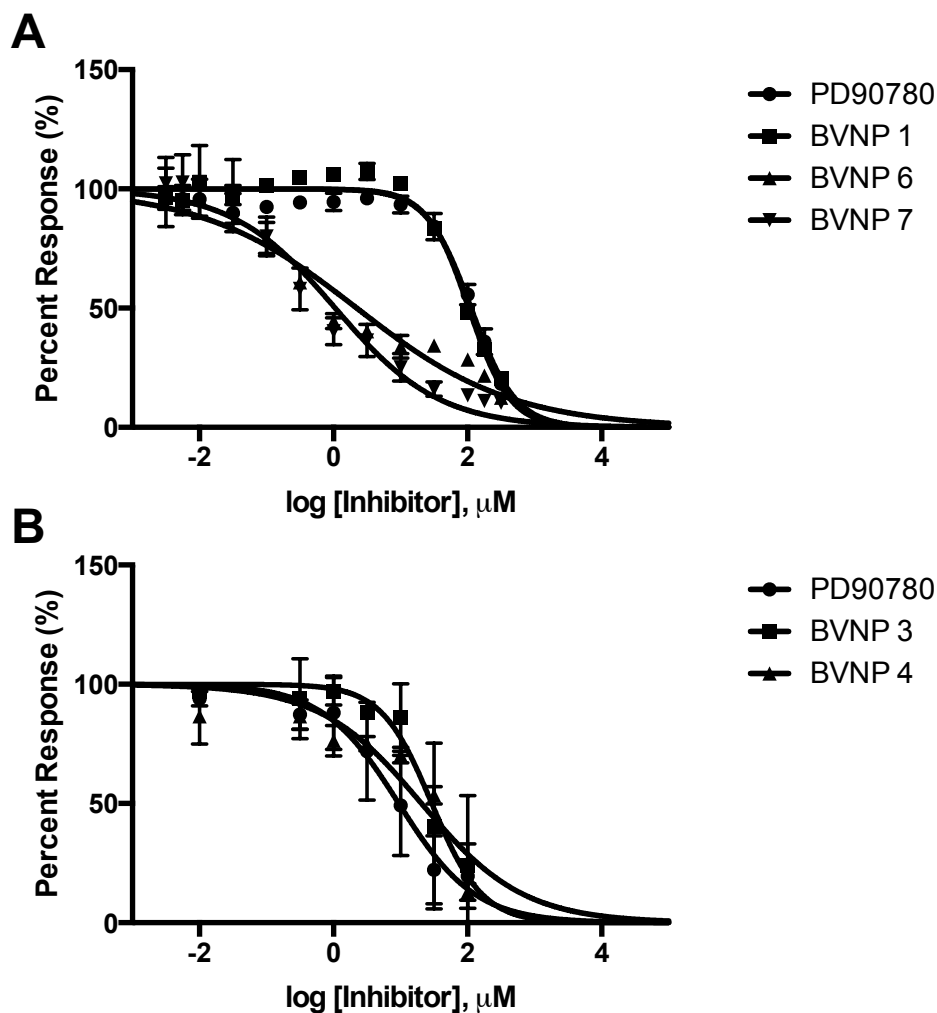


Figure 4.6: The inhibition of NGF and proNGF binding to p75^{NTR} by both known and novel inhibitors. (A) Concentration response analysis demonstrates that PD90780 (IC_{50} = 110 μM ; CI= 93-129 μM ; R^2 =0.95), BVNP 1 (IC_{50} = 104 μM ; CI= 93-117 μM ; R^2 =0.97), BVNP 6 (IC_{50} = 2.2 μM ; CI= 1.5-3.3 μM ; R^2 =0.93), and BVNP 7 (IC_{50} = 1.1 μM ; CI= 0.7-1.6 μM ; R^2 =0.93) inhibit NGF-p75^{NTR} interactions in the low-to-high micromolar range. (B) Concentration response analysis demonstrates that PD90780 (IC_{50} = 9.4 μM ; CI= 5.7-16 μM ; R^2 =0.83), BVNP 3 (IC_{50} = 29 μM ; CI= 17-50 μM ; R^2 =0.73), and BVNP 4 (IC_{50} = 21 μM ; CI= 12-36 μM ; R^2 =0.79) inhibit proNGF-p75^{NTR} interactions in the low-to-mid micromolar range. Data points are presented as mean values of triplicate measures. Error bars are represented as standard error of the mean with an n-value=3. IC_{50} values are presented with 95% confidence intervals (CI). R^2 is defined as the coefficient of determination and used to assess goodness of fit.

For the inhibition of NGF-p75^{NTR} interactions, our dose-response assays revealed that both PD90780 and BVNP 1 have inhibitory action in the high micromolar range (110 μ M; CI: 93-129 μ M and 104 μ M; CI= 93-117 μ M, respectively). Conversely, both BVNP 6 and BVNP 7 have low micromolar inhibition of NGF binding p75^{NTR} with IC₅₀ values of 2.2 μ M (CI= 1.5-3.3 μ M) and 1.1 μ M (CI= 0.7-1.6 μ M), respectively.

Dose-response curves generated for the inhibition of proNGF and p75^{NTR} binding indicate that PD9070 has inhibitory action in the low micromolar range (9.4 μ M; CI= 5.7-16 μ M). The inhibitory effects of BVNP 3 and BVNP 4, 29 μ M (CI= 17-50 μ M) and 21 μ M (CI= 12-36 μ M), respectively, were similar and offered low-to-mid micromolar inhibition for the interaction of proNGF and p75^{NTR}.

Table 4.1: IC₅₀ data for small molecule inhibition of NGF-p75^{NTR} and proNGF- p75^{NTR} interactions. Data are presented as mean values of triplicate measures with n=3.

	NGF	ProNGF
PD90780	110 μ M (CI= 93-129 μ M)	9.4 μ M (CI= 5.7-16 μ M)
BVNP 1	104 μ M (CI= 93-117 μ M)	Not Determined
BVNP 3	Not Determined	29 μ M (CI= 17-50 μ M)
BVNP 4	Not Determined	21 μ M (CI= 12-36 μ M)
BVNP 6	2.2 μ M (CI= 1.5-3.3 μ M)	Not Determined
BVNP 7	1.1 μ M (CI= 0.7-1.6 μ M)	Not Determined

Discussion

In the present study, we determined the affinity of proNGF for the p75^{NTR} receptor using novel methodology. In addition, we have characterized the ability of both known and novel inhibitors to block proNGF-p75^{NTR} interactions. Further, we assessed the ligand specificity of the inhibitors through the characterization of their ability to inhibit NGF from interacting with p75^{NTR}.

Several pathological conditions exist where dysregulation of both neurotrophins and proneurotrophins have been implicated. More specifically, signaling of both NGF and proNGF through the p75^{NTR} receptor has been shown to induce apoptosis leading to neuronal death, which may result in neurodegenerative disease, depending on the neuronal population affected (Kenchappa et al., 2010).

Historically, proNGF inhibition strategies have focused on modulating the p75^{NTR} receptor, as opposed to proNGF (Massa et al., 2006). For instance, LM11A-31, a small, nonpeptide p75^{NTR} ligand has been shown to induce survival signaling and inhibit proNGF-induced death (Massa et al., 2006). More recently, this small molecule has been found to reverse the cholinergic neurite dystrophy in Alzheimer's disease mouse models through its binding and modulation of the p75^{NTR} receptor (Simmons et al., 2014). Another approach to the inhibition of proNGF-mediated apoptotic signaling is through the reduction of proNGF synthesis in the central nervous system. Studies have found that minocycline, a derivative of tetracycline, is able to offer neuroprotective effects in experimental models of neurodegenerative diseases (Simmons et al., 2014). Specifically, it has been shown that minocycline

significantly reduces the production of proNGF in microglia both *in vitro* and *in vivo* through the inhibition of the phosphorylation of p38MAPK (Simmons et al., 2014). While these therapeutic strategies show early promise, their clinical relevance has not yet been investigated.

Drug discovery efforts to inhibit NGF-mediated signaling have focused on inhibiting its interaction with the TrkA receptor. For example, the use of NGF-mimetic peptides, which bind the TrkA receptor in the same manner as NGF, but do not elicit downstream signaling, have been described previously (Eibl et al., 2012). Unfortunately, peptide-based strategies have not proven effective in clinical settings (Eibl et al., 2012). More recently, the humanized monoclonal antibody, Tanezumab, has demonstrated promising therapeutic potential for the treatment of chronic pain, including conditions such as osteoarthritis (Cattaneo, 2010). While there have been a number of safety concerns, such as adverse changes to the sympathetic nervous system and osteonecrosis of joint tissue, partial clinical holds on this antibody-mediated therapy were lifted earlier this year (Cattaneo, 2010; Garber, 2011).

While antibody-mediated therapies have shown promise in clinical settings, these therapeutics remain highly specific and are unable to cross the blood-brain barrier. Further, the production of these therapies is both technically and economically challenging. Conversely, small molecules may be of high clinical interest as they are generally easy to synthesize, are cost-effective, and benefit from oral activity.

Previously, five small molecule inhibitors known to block NGF-TrkA interactions have been described (ALE-0540, PD90780, Ro 08-2750, PQC 083, and Y1036) (Jaen et al., 1995; Owolabi et al., 1999; Niederhauser et al., 2000; Colquhoun et al., 2004; Hefti et al., 2006; Eibl et al., 2010; Eibl et al., 2013). Recently, the ability of four of these compounds (ALE-0540, PD90780, Ro 08-2750, and PQC 083) to inhibit both NGF- p75^{NTR} and NGF-TrkA interactions has been evaluated using SPR spectroscopy (Sheffield et al., 2015). These small molecules bind NGF, as opposed to the TrkA and p75^{NTR} receptors, in order to modulate NGF dysregulation. Of these small molecules, PD90780 was found to be the most effective at inhibiting both NGF-TrkA and NGF-p75^{NTR} interactions (Sheffield et al., 2015). To date, no small molecule-based approaches to the inhibition of proNGF-p75^{NTR} interactions have been performed.

Our study provides the first characterization of the ability of known NGF inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083 to inhibit the binding of proNGF to the p75^{NTR} receptor. Our findings indicate that of the previously reported inhibitors, PD90780 offered the most inhibitory action for proNGF binding p75^{NTR}, while ALE-0540 and Ro 08-2750 only mildly blocked this interaction. The ability of these compounds to inhibit proNGF from binding p75^{NTR} is similar to their inhibitory action of NGF-p75^{NTR} interactions. For example, of the known compounds, PD90780 was found to be the most effective inhibitor of NGF-p75^{NTR} interactions, while both ALE-0540 and Ro 08-2750 were found to offer only mild inhibition of this interaction (Sheffield et al., 2015). The enhanced inhibition of PD90780 on proNGF-p75^{NTR} interactions, in comparison to its inhibitory action on

NGF-p75^{NTR} interactions, may likely be due to the structural differences between NGF and proNGF, which allows for altered binding of the small molecule to the ligand. Further, consistent with previous findings, we found that PQC 083 yielded experimental artifacts in the form of non-specific binding and was difficult to characterize using SPR spectroscopy. Of particular interest, our results suggest that PD90780 may offer multipotent inhibition, as it is able to inhibit both neurotrophin and proneurotrophin-receptor interactions.

The characterization of the ability of the novel derivatives of ALE-0540 to inhibit both NGF and proNGF binding to p75^{NTR} revealed several ligand specific molecules. For example, BVNP 1, BVNP 6, and BVNP 7 were shown to be effective inhibitors of NGF-p75^{NTR} interactions, while these compounds only mildly blocked proNGF from binding p75^{NTR}. Of these compounds, both BVNP 6 and BVNP 7 offered low micromolar inhibitory action (2.2 μ M and 1.1 μ M, respectively), while the inhibition of BVNP 1 was found to be in the high micromolar range (104 μ M). Conversely, BVNP 3 and BVNP 4 were found to be specific for the inhibition of proNGF-p75^{NTR} interactions. Both BVNP 3 and BVNP 4 were found to have inhibitory effects in the low-to-mid micromolar range with IC₅₀ values of 29 μ M and 21 μ M, respectively.

Finally, through steady-state analysis, the affinity of proNGF for the p75^{NTR} receptor was determined to be 23.5 ± 0.4 nM, which is consistent with previous reports in the literature. Using a combination of kinetic studies and SPR spectroscopy, Nykjaer et al. (2004) reported an affinity of 15 nM for proNGF-p75^{NTR}

binding. Though the methodology between studies varies, both analyses conclude that the interaction between proNGF and p75^{NTR} is low affinity in nature. Further, previous steady-state analyses have revealed that binding between NGF and p75^{NTR} has an affinity of 13 nM (Sheffield et al., 2015). The reduced affinity of the proNGF-p75^{NTR} interaction, in comparison to the interaction between NGF and p75^{NTR}, is due to lack of processing of the precursor protein (Nykjaer et al., 2004).

Conclusions

Known small molecule inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083 were assessed on their ability to block proNGF from binding p75^{NTR} using SPR spectroscopy. In addition, novel bivalent naphthalimide derivatives of ALE-0540 were evaluated for their effectiveness to inhibit binding of both NGF and proNGF to the p75^{NTR} receptor. Of the known inhibitors, PD90780 was found to offer the most effective inhibitory action for proNGF-p75^{NTR} interactions, which suggests that this compound offers multipotent inhibition, as it is capable of blocking both neurotrophin and proneurotrophin-receptor interactions.

The analysis of novel compounds revealed several ligand specific molecules. BVNP 1, BVNP 6, and BVNP 7 were shown to be effective inhibitors of NGF-p75^{NTR} interactions, while BVNP 3 and BVNP 4, on the other hand, were found to be selective for the inhibition of proNGF binding to p75^{NTR}. The identification of novel compounds capable of inhibiting p75^{NTR}-mediated apoptotic signaling may have implications for the treatment of neurodegenerative diseases, such as Alzheimer's disease. Furthermore, these molecules may serve as a starting point for the development of NGF and proNGF specific inhibitors. To develop the promising outcomes of this work, investigations with both primary cell culture and animal models are now needed to assess the suitability of these bivalent naphthalimide compounds for potential lead development.

Chapter 5: Conclusions and Future Directions

Neurotrophins are a unique family of soluble signaling proteins, which act to influence the proliferation, differentiation, and survival of neurons in the central and peripheral nervous systems (Cai et al., 2014). These proteins are initially synthesized as precursors known as proneurotrophins, which have been reported to act as distinct ligands with biological functions beyond their role as precursors (Fahnestock et al., 2004; Hempstead, 2014).

The dysregulation of both neurotrophins and proneurotrophins have been implicated in several pathologies. For instance, NGF, the most widely studied member of the neurotrophin family, has been reported to have involvement in both inflammatory and neuropathic pain states through increased TrkA-mediated signaling (Chao et al., 2006; Pezet & McMahon, 2006; Dray, 2008). In addition, NGF dysregulation has been implicated in the development and progression of neurodegenerative disease states (i.e. Parkinson's and Alzheimer's diseases) through increased binding to the p75^{NTR} receptor, which has been shown to induce apoptosis leading to neuronal death (Kenchappa et al., 2010). Similarly, dysregulation of the NGF precursor protein, proNGF, has been implicated in neurodegeneration through p75^{NTR}-mediated apoptotic signaling (Fahnestock et al., 2001; Lee et al., 2001; Beattie et al., 2002; Song et al., 2010). Further, several studies have reported that proNGF dysregulation is implicated in breast cancer, following myocardial infarction, and psoriasis (Hempstead, 2014). Therefore,

therapeutic strategies to inhibit both NGF and proNGF signaling may be of significant clinical interest.

Drug discovery efforts to modulate NGF and proNGF signaling vary. Several approaches to the inhibition of NGF have been investigated, including both NGF-mimetic peptides and human monoclonal antibody therapeutics. While NGF-mimetics have shown initial biochemical promise, they have not yet been successful in clinical settings (Eibl et al., 2012). The most successful anti-NGF antibody, Tanezumab, is the first NGF inhibitor to advance to clinical trials. Unfortunately, due to the number of safety concerns associated with the use of this therapeutic, clinical investigations of this compound were suspended. Earlier this year, however, partial clinical holds on this antibody-mediated therapy were lifted, resulting in a sizeable advancement in the field of neurotrophin-related therapeutics (Cattaneo, 2010; Garber, 2011).

Investigations into the inhibition of proNGF-mediated signaling are limited. One such approach utilizes a small, nonpeptide p75^{NTR} ligand, LM11A-31. This compound has been reported to prevent proNGF-induced death and reverse the cholinergic neurite dystrophy in Alzheimer's disease mouse models (Simmons et al., 2014). Another strategy for the inhibition of proNGF-mediated signaling involves reducing the amount of proNGF synthesized in the central nervous system through the administration of minocycline, a derivative of tetracycline (Yune et al., 2007). While each of these strategies have shown early potential, no therapeutics directed at the inhibition of proNGF have made it to clinical trial.

Traditionally, drug discovery efforts have focused on binding to and modulating the receptors, as opposed to the ligand. The use of small molecules to target a ligand, such as NGF, rather than a receptor, such as p75^{NTR} or TrkA, is a relatively novel pharmacological strategy. To date, five of these inhibitors have been discovered and described in the literature (Jaen et al., 1995; Owolabi et al., 1999; Niederhauser et al., 2000; Colquhoun et al., 2004; Hefti et al., 2006; Eibl et al., 2013). However, while these small molecule-based NGF binding agents are known NGF-TrkA inhibitors, their ability to block NGF from interacting with p75^{NTR} had not yet been assessed. Further, these compounds had never been investigated using SPR spectroscopy, a tool which has been proven to be extremely suitable for use in drug discovery applications and the characterization of small molecules, such as the NGF inhibitors discussed previously (Willander & Al-Hilli, 2009; Piliarik et al., 2009). This thesis provides the first evaluation of the ability of these compounds to block NGF binding to p75^{NTR}. Of the previously reported NGF inhibitors, only PD90780 was effective at inhibiting the interaction of NGF with p75^{NTR}, which suggests receptor selectivity between known NGF inhibitors. The pharmacological advantages of receptor-selective inhibition are obvious, as signaling through each receptor, either p75^{NTR} or TrkA, results in markedly different outcomes, which are associated with distinct pathologies.

Therapeutics that bind to and modulate proNGF had not yet been investigated. Compounds which are able to block the interaction of proNGF and p75^{NTR} offer potential for the treatment of several conditions, including neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases.

Interestingly, the ability of the known NGF inhibitors to also block proNGF-mediated signaling has not been evaluated previously. To this end, a study investigating the ligand selectivity of these compounds was conducted. As with its inhibitory action on NGF-p75^{NTR} interactions, PD90780 was found to offer the most effective inhibition of proNGF binding to p75^{NTR}. These results suggest that this compound offers multipotent inhibition, as it is capable of blocking both neurotrophin and proneurotrophin-receptor interactions. Multipotent compounds such as this may have potential clinical application for the treatment of neurodegenerative diseases, such as Alzheimer's disease, where dysregulation of both NGF and proNGF have been implicated.

Furthermore, there is a potential need for compounds with the ability to selectively inhibit either NGF or proNGF-mediated signaling. As such, novel bivalent naphthalimide derivatives of known NGF inhibitor, ALE-0540, were evaluated for ligand selectivity through the examination of their ability to block both NGF and proNGF from binding the p75^{NTR} receptor. Investigations revealed several ligand specific compounds. For instance, BVNP 1, BVNP 6, and BVNP 7, which were shown to be effective inhibitors of NGF-p75^{NTR} interactions, while BVNP 3 and BVNP 4 were found to be selective for the inhibition of proNGF binding to p75^{NTR}.

The studies presented in this thesis offer new insight into the use of small molecule inhibitors to block both neurotrophin and proneurotrophin-mediated signaling. Several opportunities exist for the use of these compounds in the

treatment of various pathologies, including both neuropathic and inflammatory pain states, and neurodegenerative disease states.

While, in recent years, progress has been made in the understanding of the vast utility of these compounds for experimental purposes, several studies are needed before these molecules undergo clinical investigation. For example, primary cell culture studies will be needed in order to assess the toxicity and bioavailability of these inhibitors, in addition to pre-clinical trials using animal models before investigations on human participants can be established. Finally, investigations of the ability of both the known and novel small molecule inhibitors to block proNGF-sortilin interactions may act to further expand the knowledge basis surrounding proNGF inhibition and the use of these bivalent naphthalimide compounds for potential lead development.

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