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

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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.

1. 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 [1]. 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 [2]. The unique signaling properties of each neurotrophin are a result of differential tyrosine kinase (Trk) receptor activation [3]. For instance, the most widely studied member of the neurotrophin family, NGF, signals through the selective tyrosine kinase receptor, TrkA, in addition to the common neurotrophin receptor, p75^{NTR} [4].

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 [2,5,6]. 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 [2,7,8]. 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} [9,10]. 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 [11,12].

All neurotrophins are synthesized from larger precursors known as proneurotrophins, which consist of an N-terminal prodomain and a C-terminal mature domain [13]. 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 [14]. ProNGF, like NGF, binds the p75^{NTR} receptor; however, proNGF

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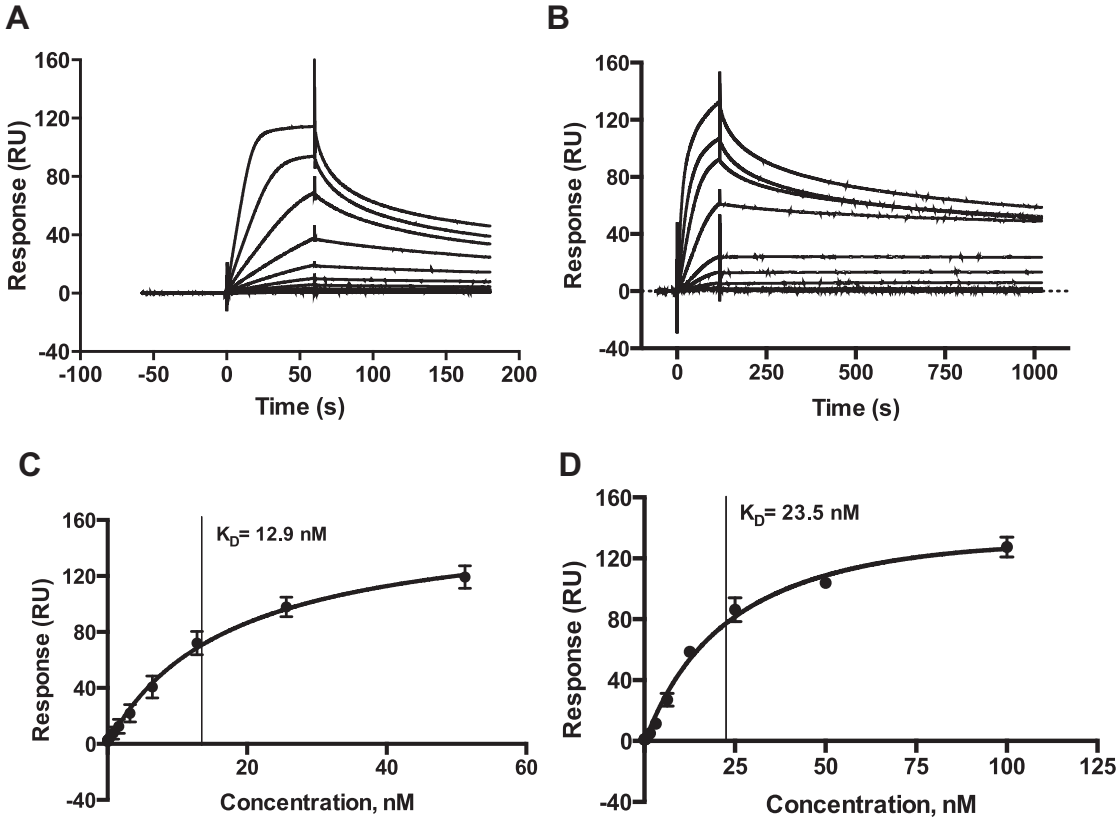

Fig. 1. Affinity of NGF and proNGF for the p75^{NTR} receptor. Concentration series of NGF (0.0125 nM to 50 nM) (A) and proNGF (0.025 nM to 100 nM) (B) were run over p75^{NTR} and used to calculate the affinity of the ligand-receptor interactions through steady-state analysis. Response of NGF (C) and 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 nM and 23.5 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.

also binds sortilin, a member of the Vps 10p-domain receptor family [15].

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 [15]. 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 [16,17].

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 [18–22]. 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 [23]. Further, NGF dysregulation has been implicated in neurodegenerative disease states as NGF-p75^{NTR} mediated signaling induces apoptosis leading to neuronal death [24]. Likewise, proNGF has been associated with neurodegeneration. For instance, proNGF-p75^{NTR} interactions induce apoptotic signaling in neurons, smooth muscle cells, and oligodendricytes at subnanomolar concentrations [16,17,25]. In addition, increased levels of proNGF have been found in Alzheimer's diseased brains [26]. 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 [7,27–33]. Conversely, proNGF inhibitory investigations have focused on modulating the p75^{NTR} receptor (as opposed to proNGF), as well as reducing the production of proNGF in the central nervous system [9,34,35]. 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.

2. Materials and methods

2.1. 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

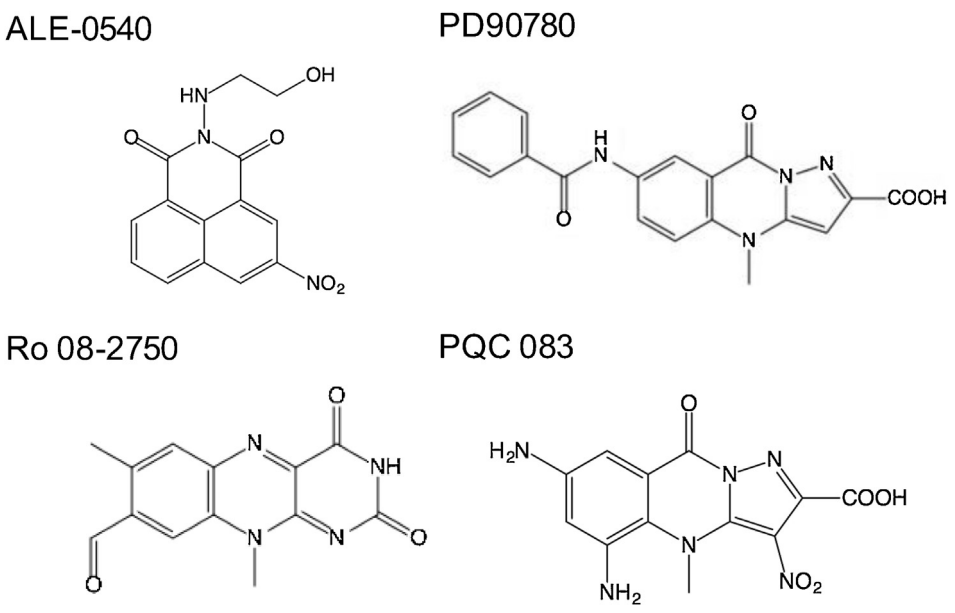

Fig. 2. Previously reported small molecule NGF inhibitors share remarkable structural homology. The structures of previously reported NGF-binding inhibitors ALE-0540, PD90780, Ro 08-2750, and PQC 083.

inhibitors were synthesized by Sussex Research Laboratories Inc. (Ottawa, ON, Canada).

2.2. SPR and sensor surface preparation

All experiments were carried out using a Biacore T200 SPR spectrometer from GE Healthcare Life Sciences (Mississauga, ON, Canada). 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 μl/min for 7 min. $p75^{NTR}$ was diluted to 10 μg/mL in 10 mM sodium acetate buffer, pH 4.5 and immobilized until a level between 850 and 950 relative response units (RU) (~15 fmol/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 μl/min for 7 min. 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.

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

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

2.4. 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 s. All compounds were allotted a 120 s dissociation time. Receptor binding was determined by assessing the binding response of each compound in RUs (1 RU = 1 pg/mm^2). The sensor chip surface was regenerated as described above.

2.5. 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%.

2.6. IC_{50} determination

Half-maximal inhibitory concentrations (IC_{50}) 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 (IC_{50}).

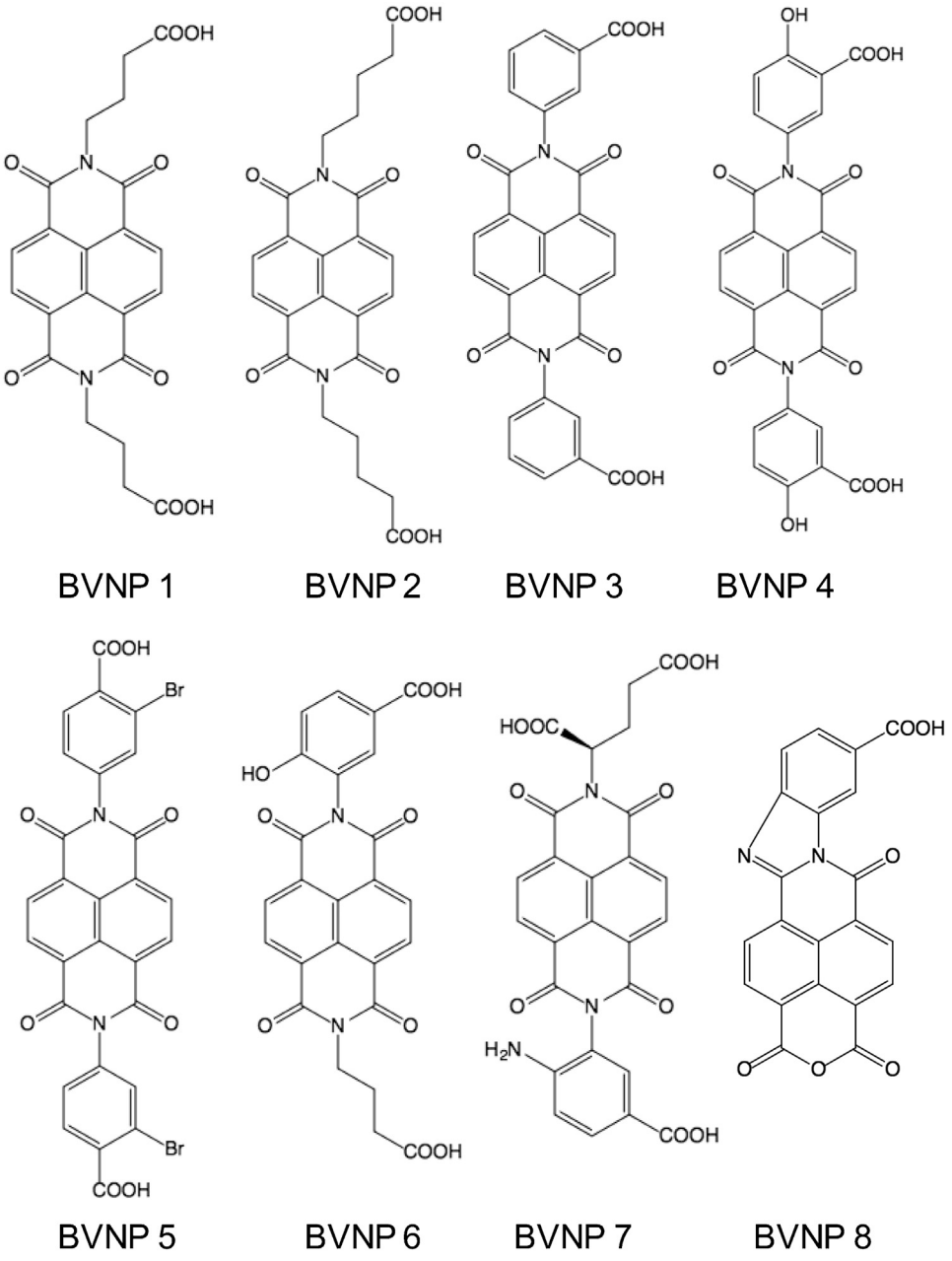

Fig. 3. Novel bivalent naphthalimide derivatives of ALE-0540. The structures of novel inhibitors BVNP 1, BVNP 2, BVNP 3, BVNP 4, BVNP 5, BVNP 6, BVNP 7, and BVNP 8. Compounds were custom synthesized by Sussex Research Laboratories Inc. (Ottawa, ON).

2.7. Data analysis

Transformation of data for 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, determination of IC_{50} values, and statistical analyses were conducted using Prism GraphPad 6.0 (La Jolla, USA).

3. Results

3.1. The affinity of NGF and 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 s contact time followed by a 120 s dissociation phase (Fig. 1A). Similarly, proNGF was flowed over the immobilized receptor for 120 s at varying concentrations (ranging from 0.025 nM to 100 nM) and allotted a 900 s time frame before regeneration of the sensor surface due to the slow dissociation rate of the protein-receptor interaction (Fig. 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 (Figs. 1C,D). The affinity (K_D) of NGF for p75^{NTR} was determined to be 12.9 nM, while the affinity of proNGF-p75^{NTR} interactions was determined to be 23.5 nM. As reported by Nykjaer et al. [15], the reduced affinity of the proNGF-

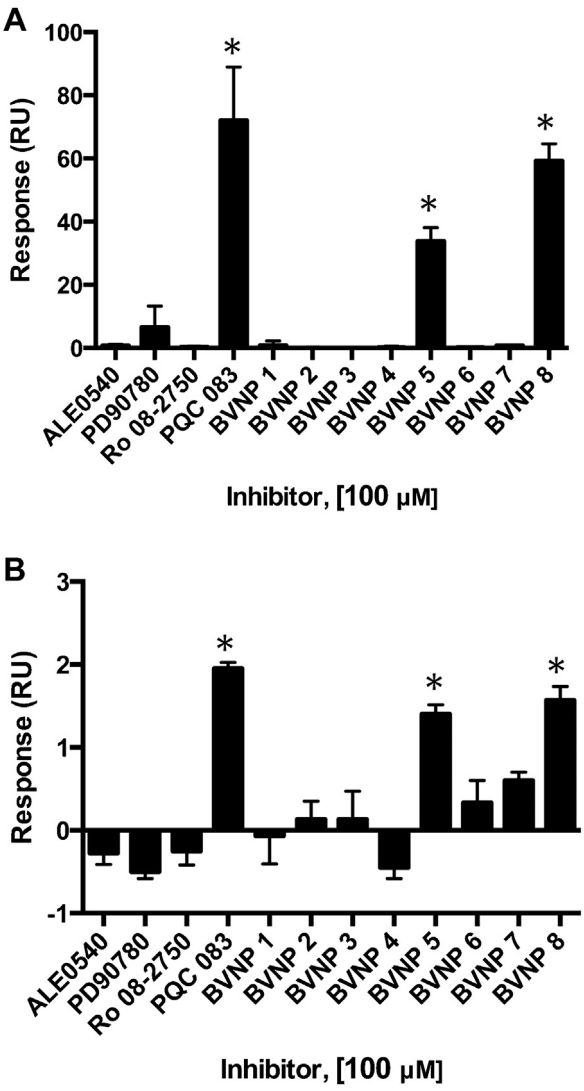

Fig. 4. Small molecule binding to the p75^{NTR} receptor and uncoupled reference flow cell. The inhibitors (100 μM) were run over p75^{NTR} (A) and the uncoupled reference flow cell (B) in order to determine the specificity of compounds. Data are presented as mean values of triplicate measures with standard error of the mean bars with an *n*-value = 3.

p75^{NTR} interaction, in comparison to the interaction between NGF and p75^{NTR}, is due to lack of processing of the precursor protein.

3.2. 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. 2) [32]. 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 Fig. 3.

In order to ensure that the inhibitors were ligand-specific and did not bind the p75^{NTR} receptor, we injected the compounds (100 μM) over the immobilized p75^{NTR} with no added NGF or proNGF (Fig. 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. 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.

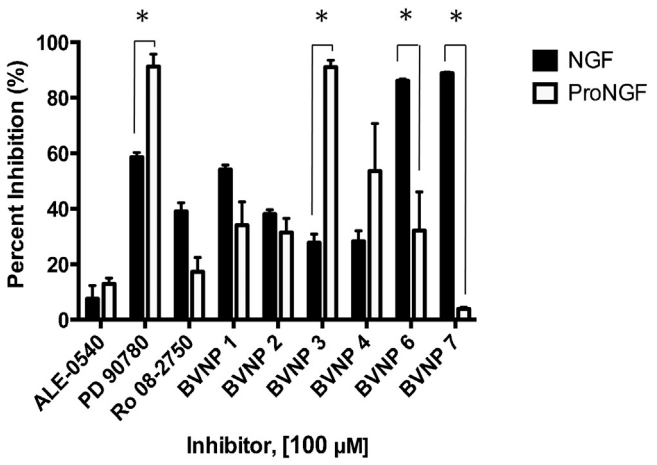

Fig. 5. The inhibition of NGF-p75^{NTR} and proNGF-p75^{NTR} interactions by known and novel inhibitors. The compounds (100 μM) were incubated with 10 nM NGF and 10 nM proNGF to assess their ability to inhibit both NGF-p75^{NTR} and proNGF-p75^{NTR} interactions. Data are presented as mean values of triplicate measures with standard error of the mean bars with an *n*-value = 3.

3.3. 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 μM) 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. 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 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 μM, 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%) ($P = 0.0023$).

Table 1
IC_{50} 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 μM	9.4 μM
BVNP 1	104 μM	Not Determined
BVNP 3	Not Determined	29 μM
BVNP 4	Not Determined	21 μM
BVNP 6	2.2 μM	Not Determined
BVNP 7	1.1 μM	Not Determined

Using a single screening concentration for each of the compounds (100 μM), 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 proNG-p75^{NTR} interactions (4%) ($P = 0.0001$).

3.4. Determination of half-maximal inhibitory (IC_{50}) 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. 6). Using these curves, half-maximal inhibitory (IC_{50}) concentrations were determined and are presented in Table 1.

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 μM and 102 μM, respectively). Conversely, both BVNP 6 and BVNP 7 have low micromolar inhibition of NGF binding p75^{NTR} with IC_{50} values of 2.2 μM and 1.1 μM, 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 μM). The inhibitory effects of BVNP 3 and BVNP 4, 29 μM and 21 μM, respectively, were similar and offered low-to-mid micromolar inhibition for the interaction of proNGF and p75^{NTR}.

4. 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 [24].

Historically, proNGF inhibition strategies have focused on modulating the p75^{NTR} receptor, as opposed to proNGF [9]. For instance, LM11A-31, a small, nonpeptide p75^{NTR} ligand has been shown to induce survival signaling and inhibit proNGF-induced death [9]. 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 [36]. Another approach to the inhibition of proNGF-mediated apoptotic signaling is through the reduction of proNGF synthesis in the central nervous system [34]. Studies have found that minocyline, a derivative of tetracycline, is able to offer neuroprotective effects in experimental models of neurodegenerative diseases [34]. 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 [34]. 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 [37]. Unfortunately, peptide-based strategies have not proven effective in clinical settings [37]. More recently, the humanized monoclonal antibody, tanezumab, has demonstrated promising therapeutic potential for the treatment of chronic pain, including conditions such as osteoarthritis [38]. 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 [38,39].

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) [7,27–32]. 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 [33]. 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 [33]. 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 (9.4 μM) 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 (110 μM) 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 [33]. 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

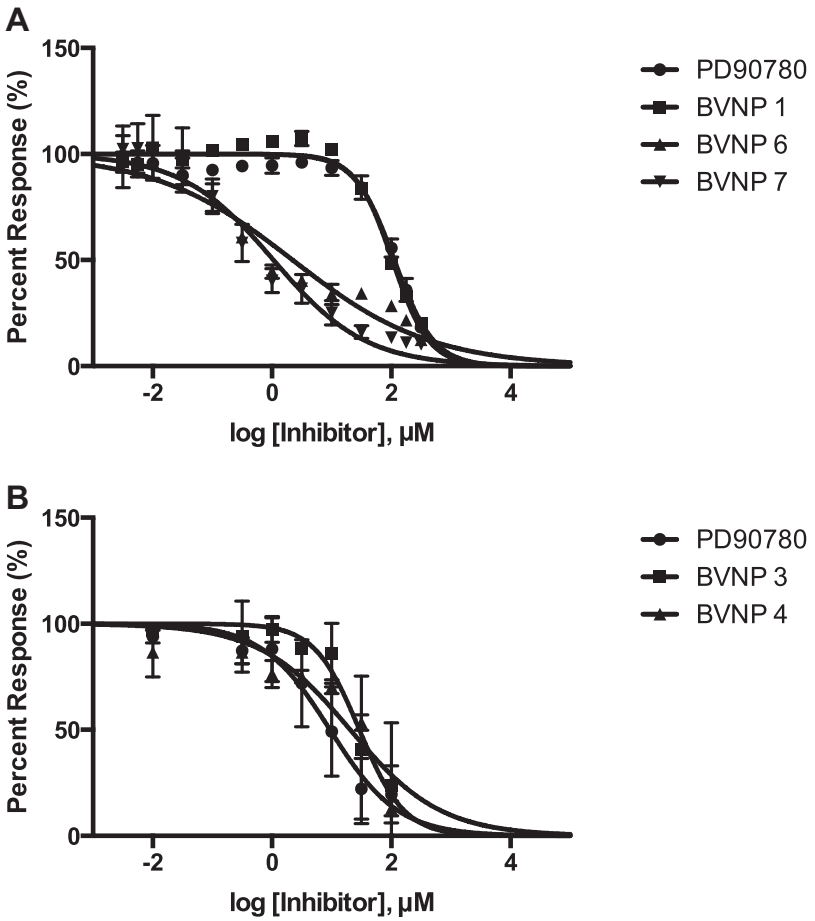

Fig. 6. The inhibition of NGF and proNGF binding to $p75^{NTR}$ by both known and novel inhibitors. (A) Concentration response analysis demonstrates that PD90780 (IC_{50} = 110 μM; CI = 93–129 μM), BVNP 1 (IC_{50} = 104 μM; CI = 93–117 μM), BVNP 6 (IC_{50} = 2.2 μM; CI = 1.5–3.3 μM), and BVNP 7 (IC_{50} = 1.1 μM; CI = 0.7–1.6 μM) 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), BVNP 3 (IC_{50} = 29 μM; CI = 17–50 μM), and BVNP 4 (IC_{50} = 21 μM; CI = 12–36 μM) 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.

PQC 083 yielded experimental artifacts in the form of non-specific binding and was difficult to characterize using SPR spectroscopy. 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 μM and 1.1 μM, respectively), while the inhibition of BVNP 1 was found to be in the high micromolar range (104 μM). 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 IC_{50} values of 29 μM and 21 μM, respectively.

Finally, through steady-state analysis, the affinity of NGF and proNGF for the $p75^{NTR}$ receptor was determined to be 12.9 nM and 23.5 nM, respectively, which is consistent with previous reports in the literature. Using a combination of kinetic studies and SPR spectroscopy, Nykjaer et al. [15] reported an affinity of 1 nM for NGF-$p75^{NTR}$ interactions and 15 nM for the binding of proNGF and $p75^{NTR}$. Though the methodology between studies varies, both analyses conclude that the interaction between proNGF and $p75^{NTR}$ is low affinity in nature and demonstrates reduced affinity in comparison to NGF-$p75^{NTR}$ interactions. The reduced affinity of this interaction is attributed to the lack of processing of the precursor protein [15].

5. 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 proneurotropin-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.

Conflict of interest

The authors declare that there are no conflict of interest.

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