The use of surface plasmon resonance spectroscopy in characterizing small molecule-based nerve growth factor inhibitors

by

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Thesis Abstract

Neurotrophins are well known for their effects on neuronal survival and growth. Over the past two decades, considerable evidence has accumulated from both human and animal models that one neurotrophin, nerve growth factor (NGF), is a peripheral pain mediator, particularly in inflammatory pain states. NGF is upregulated in a wide variety of inflammatory conditions, and NGF-neutralizing molecules are effective analgesic agents in many models of persistent pain. Such molecules are now being evaluated in clinical trials and although seemingly effective in early clinical studies, many deteriorating side effects are now being brought to light. Thus, a need still remains for novel NGF-quenching therapeutics. In the past two decades, a handful of small molecule NGF-inhibitors have been described, however, their effects are still quite minimal compared to other inhibitory agents. The central aim of this thesis was to use novel screening strategies to identify small molecule NGF-inhibitors capable of modulating NGF signalling with greater efficiency than previously reported compounds for the purpose of therapeutic development. Using surface plasmon resonance (SPR) spectroscopy, established NGF-inhibitors and a series of novel compounds were analyzed for their specificity to NGF and their inhibitory properties of NGF binding to the TrkA receptor. *In vitro* techniques confirmed the high nanomolar inhibitory properties of a novel compound, BVNP-0197. Molecular modeling techniques also described a putative binding domain for BVNP-0197 to NGF at the loop II/IV cleft, an alternative domain than previously described by small molecule compounds. This binding domain describes an area

with higher specificity for TrkA over binding for $p75^{NTR}$. Serum albumin was also described as having a role in the binding events that occur between small molecule compounds to NGF *in vitro*. SPR kinetic analysis established that the addition of serum albumin into solution with small molecule compounds increased their specificity for NGF, as well as decreased their potential toxicity. The findings presented in this thesis will contribute to the future development of small molecule NGF-inhibitors.

Keywords: NGF, neurotrophin, SPR, biosensor, pain therapeutic, inhibition, binding.

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Kennedy AE, Scott JA and Ross GM (2016) Optimization of surface plasmon resonance measured binding affinity of small molecule-based nerve growth factor inhibitors with the addition of bovine serum albumin. *Anal. Biochem.* (Submitted)

Kennedy AE, Ross MS, Laamanen CA, Vohra R, Boreham DR, Scott JA and Ross GM (2016) Nerve growth factor inhibitor with novel binding domain demonstrated in both cell-based and cell-free assay systems. *Mol Pharm* (Submitted)

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Kennedy AE, Sheffield KS, Eibl JK, Murphy MB, Vohra R, Scott JA and Ross GM (2016) A surface plasmon resonance spectroscopy method for characterizing small-molecule binding to nerve growth factor. *J. Biomol. Screen* 21: 96-100.

Eibl JK, Abdallah Z, **Kennedy AE**, Scott JA and Ross GM (2013) Affinity crosslinking of Y1036 to nerve growth factor identifies pharmacological targeting domain for small molecule neurotrophin antagonists. *Neurosci Med* 4: 290-8.

List of Abbreviations

^{125}I – iodine-125

AD – Alzheimer's disease

AGE – advanced glycation end product

ATP – adenosine triphosphate

BDNF – brain derived neurotrophic factor

BFC – basal forebrain complex

BSA – bovine serum albumin

BVNP – bivalent naphthalimide

CIPA – congenital insensitive to pain with anhidrosis

CNS – central nervous system

DMSO – dimethyl sulfoxide

DRG – dorsal root ganglion

EDC – 1-ethyl-3-(3-dimethylaminoproryl) carbodiimide hydrochloride

EDTA – ethylenediaminetetraacetic acid

FDA – Food and Drug Administration

Grb2 – growth factor receptor-bound protein 2

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HSA – human serum albumin

HSAN – human sensory and autonomic neuropathy

HSV – herpes simplex virus

IC_{50} – half maximal inhibitory concentration

IL-1β – interleukin 1 beta

k_a – kinetic rate of association

k_d – kinetic rate of dissociation

K_D – equilibrium dissociation constant (binding affinity)

MEK – MAPK/Erk kinase

NGF – nerve growth factor

NHS – N-hydroxysulfosuccinimide

NO – nitric oxide

NSAID – nonsteroidal anti-inflammatory drug

NT-3 – neutrophin-3

NT-4/5 – neurotrophin-4/5

$p75^{NTR}$ – low affinity nerve growth factor receptor

PC12 – pheochromocytoma cell line derived from the rat adrenal medulla

PDVF – polyvinylidene difluoride

PKC – protein kinase C

PLC-γ – phospholipase C-γ

PNS – peripheral nervous system

RAGE – receptor of advanced glycation end product

ROS – radial oxygen species

RU – response unit

SCG – superior cervical ganglion

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPR – surface plasmon resonance

TNFα – tumour necrosis factor alpha

TrkA – tropomyosin receptor kinase A

TRP – transient receptor potential

Chapter 1: Introduction to Nerve Growth Factor

In 1949, a young researcher by the name of Rita Levi-Montalcini, observed that upon transplanting mouse sarcoma tissue into a chick embryo, the tumor cells stimulated the growth of fibers originating from the neurons of the sensory ganglia into the tumour (Levi-Montalcini 1987). She hypothesized that transplanted tumour tissue released a diffusible agent that was able to stimulate the growth of developing nerve cells in the internal chick organs (Levi-Montalcini et al. 1996; Aloe 2011). She continued *in vitro* analysis to show the direct stimulating actions of this extract on sensory and sympathetic nerve cells (Manca et al. 2012). She called this unknown molecule nerve growth-stimulating factor, which was later renamed nerve growth factor (NGF) (Cohen 2008). The impact of Levi-Montalcini's work was recognized in 1986 with the Nobel Prize in Physiology and Medicine (Aloe 2011).

Since the discovery of NGF, a family of neurotrophic factors, all with similar homology, have been identified. These include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), all of which play a role in regulating the growth and differentiation of neurons in the peripheral and central nervous systems (Aloe et al. 2012; Watson et al. 2008). Each neurotrophin binds to a common receptor, $p75^{NTR}$, and to their respective receptor tyrosine kinases (TrkA, TrkB, TrkC) (Huang and Reichardt 2001; Mendell et

al. 2014). The trophic actions of NGF are mainly attributed to the activation of TrkA expressed on both peripheral and central neurons (Skaper 2001; Barker 2007).

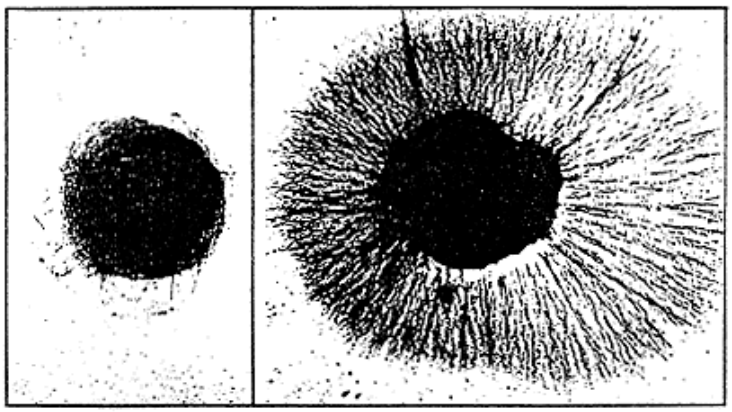

Figure 1.1: The effects of NGF on sensory ganglion of a chick embryo. The left panel demonstrated a sensory ganglion in the absence of NGF stimulation. The right panel depicts the sensory ganglion in the presence of NGF. This figure was originally published in (Levi-Montalcini and Calissano 1979).

TrkA activation from NGF results in internalization of the NGF-TrkA complex to regulate gene expression (Ginty and Segal 2002). Transgenic mice lacking NGF or TrkA have sensory and sympathetic deficiencies and usually do not survive long after birth (Chao et al. 2006; McKelvey et al. 2013). In addition, human genetic studies reveal that conditions with TrkA mutations, where restricted NGF binding occurs, impair the normal development of sensory neurons (Capsoni et al. 2011). However, a large body of evidence suggests that in adults, the main role of NGF is to act as a mediator in pain signaling (Pezet and McMahon 2006; Marchand et al. 2005; Salat et al. 2014).

Binding of NGF to cellular membrane TrkA, results in dimerization and cross-phosphorylation of intracellular tyrosine residues, forming binding sites for scaffolding proteins (Reichardt 2006). Upon TrkA activation two simultaneous signalling events occur leading to hypersensitivity and pain signalling. First, the binding of scaffolding proteins, Shc, growth factor receptor-bound protein 2 (Grb2), and guanine-nucleotide exchange factor SOS, to phosphorylated tyrosine residues activates the small G-protein Ras, leading to the subsequent activation of Raf, the MAPK/Erk kinase (MEK) and in turn Erk (Kaplan and Miller 2000; Chao 2003). Activation of Erk in sensory neurons has been associated with hyperalgesia and sensitivity (Nicol and Vasko 2007). The phosphorylated tyrosine residues can also bind phospholipase C-γ (PLC-γ) leading to the activation of protein kinase C (PKC), a regulator in the sensitivity of sensory neurons to noxious stimuli (Malik-Hall et al. 2005). This triggered transduction system of NGF-TrkA signalling (Figure 1.2) transmits pain signals either to the nucleus, altering gene expression, or through dorsal root ganglion (DRG) to the brain for pain perception (Marchand et al. 2005; Hirose et al. 2016).

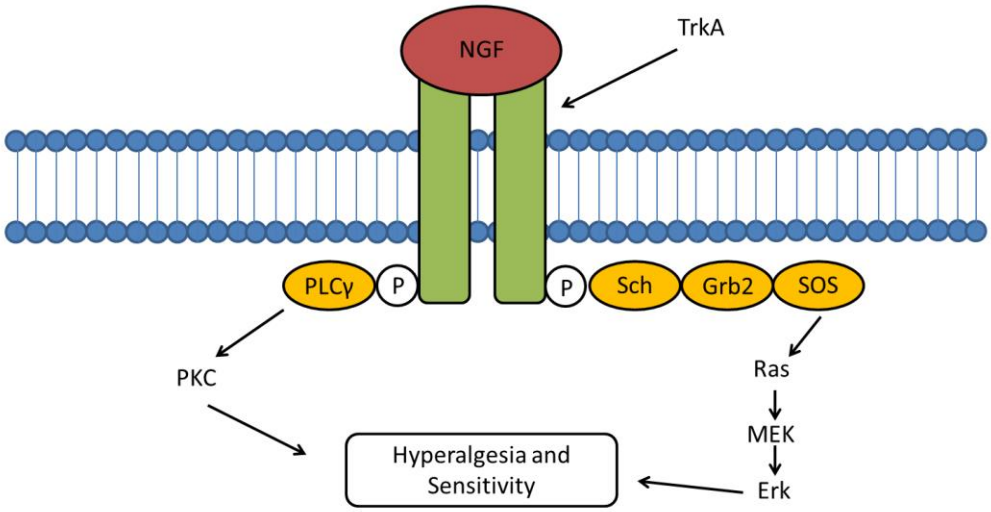

Figure 1.2: Intracellular signalling pathways associated with NGF activated TrkA leading to hyperalgesia and sensitivity in sensory neurons.

Neurotrophins were thought to hold a great therapeutic potential in treating pathologies related to abnormal sensory development and homeostasis; including neurodegeneration, chronic inflammation and nerve injury (Dray 2008; Watson et al. 2008; Ghilardi et al. 2010; Hefti et al. 2006). The first clinical trial conducted using NGF as a therapeutic agent aimed to treat patients suffering from diabetic neuropathy (Apfel et al. 1994; Apfel 2002). NGF-mediated therapy was identified as a candidate for treatment in these clinical trials due to its growth stimulating effects on small fiber pain sensory neurons affected during diabetic neuropathy (Pittenger and Vinik 2004; Verge et al. 2014). Clinical trials were abandoned quite early due to recurring side effects of severe pain localized at the injection site (Apfel 2002). Following these studies, further biochemical and physiological data emerged identifying NGF as a critical mediator in pain signalling (Hefti et al. 2006; Pezet and McMahon 2006; Max and Stewart 2008;

Gerber et al. 2011). Since then, elevated levels of NGF have been associated with other painful pathologies such as osteoarthritis, fibromyalgia and interstitial cystitis (Giovengo et al. 1999; Seidel et al. 2010; Lowe et al. 1997; Chen et al. 2016). These findings have led to the strategy of inhibiting NGF for the treatment of pain.

Thus far, the only NGF-inhibiting strategy that has entered clinical trials is the use of anti-NGF antibodies. The forefront of this field is Tanezumab, a fully humanized monoclonal antibody; however, its journey through clinical trials has not been without challenges. The FDA first placed Tanezumab on partial clinical hold for allegations of peripheral effects leading to osteonecrosis and complete joint failure (Sałat et al. 2014; Chang et al. 2016). The hold was lifted once further investigation into these claims revealed that only two of the alleged cases were in fact osteonecrosis; both of which were not linked to the administration of Tanezumab. These cases were rather diagnosed as osteonecrosis triggered by the overuse of an already deteriorating joint (Hochberg et al. 2016; FDA 2012). Only months after the first hold was lifted, a second clinical hold was placed on all anti-NGF antibodies due to suspected peripheral nerve effects. This hold was lifted in early 2015 and Tanezumab is currently underway in Phase III clinical trials to determine its safety profile during long-term administration (Chang et al. 2016). Despite the apparent effectiveness of antibody therapeutics in blocking the signal of NGF-related pain, many remaining obstacles must be overcome prior to reaching market.

Other strategies have been investigated for the inhibition of NGF as a potential therapeutic target for pain. These approaches include synthetic mimetic peptides, small molecule TrkA-inhibitors and small molecule NGF-inhibitors. Mimetic peptides are derived to resemble the structure of loops I and IV of mature NGF and competitively bind to TrkA (Beglova et al. 2000; LeSauteur et al. 1996). Alternatively, small molecule TrkA-inhibitors prevents NGF-induced phosphorylation by binding in the catalytic domain for ATP (Berdún et al. 2015; Tapley et al. 1992). Both of these strategies were proven effective in inhibiting NGF-TrkA signalling *in vitro*, however, lack the specificity required for use as a therapeutic agent.

A series of small molecule NGF-inhibitors have been described in the literature with the ability to bind NGF and alter the molecular topology in a manner that prevents NGF-docking to TrkA. Small molecules ALE-0540 (Niederhauser et al. 2000), Ro 08-2750 (Colquhoun et al. 2004), PD 90780 (Colquhoun et al. 2004) and PQC-083 (Eibl et al. 2013a) are described as binding at the loop I/IV cleft on mature NGF. This area is of high variability among neurotrophin family members thus allowing for binding specificity to NGF (Eibl et al. 2012). Another small molecule, Y1036, has been described as a multi-neurotrophin inhibitor (Eibl et al. 2013b). It is hypothesized that Y1036 binds at the hydrophobic interface of mature NGF monomers, which is an area highly conserved among neurotrophin family members (Eibl et al. 2013b). Small molecule NGF-strategies are advantageous due to their specificity for NGF, however, their therapeutic potential is still described as quite low since they bind to NGF with micromolar affinity.

Recent advancements in the sensitivity of surface plasmon resonance (SPR) spectroscopy have allowed for drug binding properties to be explored for larger target proteins (Myszka and Rich 2000; Gopinath and Kumar 2014; Geschwindner et al. 2012). SPR is a label-free detection system with the ability to measure biomolecule interaction in real-time (Ahmad et al. 2003; Papalia et al. 2006). SPR is advantageous over other methods for investigating the strength and rate of binding interactions as it eliminates the need for radioisotope tags or reporter molecules; which have the ability to alter the molecule interaction (Patching 2014). Real-time analysis also allows for pharmacokinetics of drug compounds to be analyzed for tailored therapeutic development. In addition, the cell-free technology measures true binding events which occur between a drug compound and their target protein, eliminating the influence of other factors found during *in vitro* analysis. The ability to remove variables which are present during *in vitro* allows for the identification of potential mediators of described binding events.

In this thesis, a series of studies were completed with the objective of characterizing the ability of established small molecule and novel compounds in their inhibition of NGF-TrkA interactions using SPR technology. To begin, the ability of SPR to characterize NGF-binding with therapeutic agents was established (Chapter 3). Next, binding events for each existing NGF-inhibitor to immobilized NGF and their ability to block NGF binding to TrkA was determined (Chapter 4). In addition, the identification of a novel bivalent naphthalimide compound with nanomolar inhibitory efficiency in both cell-free and cell-based assays with a putative binding domain in

the loop II/IV cleft was completed using SPR (Chapter 5). Finally, the role of serum albumin protein may play in the selectivity and pharmacokinetics of small molecule-inhibitors for NGF and optimization strategies for screening small molecule compounds was explored (Chapter 6).

Chapter 2: Thesis hypothesis and objectives

Nerve growth factor (NGF) is best characterized as the tropic factor responsible for the development and maintenance of the central and peripheral nervous system during development. In the adult, NGF is known to be a potent mediator of pain resulting from inflammatory and neuropathic pain conditions by activating the high affinity receptor TrkA. The recognition that NGF has a central role in pain signalling mechanisms in adults provides a unique opportunity to develop a novel class of pain therapeutics.

Recent clinical trials with selective antibodies for inhibiting NGF have been shown to be effective in quenching NGF pain signalling. Although seemingly effective, these anti-NGF antibodies are limited in their application due to significant side effects with their administration. Small molecule NGF-inhibitors also have been described as holding potential therapeutic potential in NGF-inhibition as they have been shown to modulate NGF activity *in vitro*. However, the effects of small molecules have only been previously explored using cell-based assays. This holds the possibility that fluorescent probes required to measure the binding, alter the biomolecular interaction, or that unknown binding mediators are at play. Therefore, the central aim of this thesis is to characterize the binding of previously reported small molecules to NGF using the cell-free system presented with surface plasmon resonance

(SPR) spectroscopy, as well as to identify novel small molecule NGF-inhibitors which hold a higher binding affinity and inhibitory potential than previously reported compounds.

Hypothesis: Binding of established and novel small molecule compounds, and the inhibitory properties they hold in TrkA signalling, can be determined using surface plasmon resonance spectroscopy.

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

Objectives:

1) Determine binding affinity, specificity and stoichiometry for established NGF-inhibitors and immobilized NGF using SPR spectroscopy (Chapter 4).
2) Govern methods for screening practises for characterizing binding events between novel small molecule compounds and NGF using a cell-free system (Chapter 4).
3) Identify novel chemical structures which inhibit NGF *in vitro* with higher binding affinity and inhibitory efficiency than established entities (Chapter 5).
4) Evaluate the influence that serum albumin, a known drug carrier present in cell-based assays, may have on binding events between small molecule based-inhibitors and NGF (Chapter 6).

Chapter 3: Advances in identifying potential therapeutics targeting nerve growth factor for pain and other pathologies using surface plasmon resonance spectroscopy

(Review)

Allison E. Kennedy, John A. Scott, Gregory M. Ross

[Submitted to *Sensors*]

Abstract

Characterization of potential therapeutic compound kinetics and binding affinity to a target receptor is key in the development of new drugs. Surface plasmon resonance (SPR) spectroscopy has emerged as a label-free, real time technique for measuring the rate and strength of biomolecular interactions. Recent advances in SPR sensitivity allows for the characterization of nerve growth factor (NGF) specific binding to potential therapeutic drugs, proteins or target receptors for use in chronic pain management, herpes simplex virus antiviral treatments, or as potential therapeutics for Alzheimer's disease. These potential markets represent significant value as it is estimated that 1 in 5 Canadians suffer from chronic pain, one third of which do not experience relief from current pain management strategies. There remains a large need to develop NGF-specific pain therapeutics that do not have the associated side effects, tolerance and dependence issues seen with current NSAID and opioid drug. Reviewed here are current novel drug discovery targets for inhibiting NGF-induced pain as well as recent advancements in other NGF binding events with the use of SPR technologies.

SPR technology

SPR is a widely used optical detection technique that is ideal for investigation of the strength and rate of biomolecular interactions in real time. SPR is also a label-free technique advantageous over other detection techniques because it eliminates the need for radioisotope tags or for fluorescent reporter molecules. SPR has shown to be versatile in studies involving a wide-range of molecular interactions that range from simple proteins, lipids, small molecule compounds, and carbohydrates to large complex proteins and cells (Wittenberg et al. 2014; Cooper 2002). These studies also looked at binding affinities ranging from picomolar to millimolar (Cooper 2002). By eliminating the labeling step, the possibility of altering the molecular interaction by means of the tag is eliminated as well as time is saved during an often complex process with expensive materials (Maynard et al. 2009; Myszka and Rich 2000). Real-time techniques, like SPR, are also advantageous since it allows for the calculation of association and dissociation kinetic rate constants (k_a and k_d) of the binding interaction (Jason-Moller et al. 2006; Wittenberg et al. 2014). By calculating these two kinetic rate constants, the equilibrium dissociation constant (K_D) can be determined. Finally, SPR techniques are advantageous due to the small amount of reagents required in comparison to equilibrium assays, especially when investigating low affinity binding mechanisms (Papalia et al. 2006).

Since the first commercial instrument release in 1991, SPR technology has been predominantly developed by BIAcore technology (Patching 2014; Jongerius-Gortemaker et al. 2002). One of the newer SPR instruments (BIAcore T200) monitors analyte binding to the immobilized

receptor and subtracts the refractive index changes monitored by a reference flow cell with no immobilized receptor. More accurate measurements are obtained by subtracting the background refraction associated with the buffered solution in which the analyte is suspended. Most SPR devices are not equipped for allowing high throughput assays, however computer automated systems do allow for numerous sampling from multiwell plates allowing for a higher throughput than other hand held assays, as well as reduced error by limiting user influence (Myszka 2004; Cannon et al. 2004). For example, the BIAcore 4000 is able to run uninterrupted and unsupervised for 60 hours allowing for approximately 1000 interactions per hour (Wittenberg et al. 2014).

When investigating molecular interactions for drug discovery programs, the ability to calculate kinetic rate constants is advantageous over an endpoint-only measurement (such as ELISA or radioisotope binding assays) by their ability to characterize mechanisms of how a drug may interact (Jongerius-Gortemaker et al. 2002). In some pathologies where drug toxicity is of concern with therapeutics, a drug may be needed to quickly dissociate from its target receptor (high k_d) (Copeland et al. 2006; Núñez et al. 2012). Alternatively, other therapeutics require a drug to remain bound to its target receptor for a long period of time (low k_d) in order to reduce the necessary dosage and prolonging the biological effect of the drug (Copeland et al. 2006; Núñez et al. 2012). Depending on the kinetics required by a drug, discovery efforts can be modified to follow drug leads described by measuring rate constants. Kinetic rate constants can also aid in determining specificity of a drug to a target receptor. A short association phase of a

drug (low k_a) to its target receptor compared to a long association phase to other receptors (high k_a) would translate into higher specificity and likely reduced side effects (Copeland et al. 2006; Núñez et al. 2012).

SPR experiment

SPR experiments involve immobilization of a target receptor on to a flow-cell's sensor surface and monitoring the sensor signal change (by refractive index) when a flowing analyte solution is exposed to the sensor surface. Each flow cell is approximately 1 mm wide and contains a sensor surface which is covered in a thin metal, usually made of gold or silver. To measure binding, a laser light is directed onto the metal film and the refractive index of the laser is measured. When the photons of the light ray hit the metal surface, they couple with the free electrons in the metal to cause an oscillation, or plasmon, at the surface of the sensor surface. This plasmon is confined to the metal surface and changes the interfacial refractive index. This results in a change in refractive index as the analyte binds to the receptor immobilized on the sensor surface (Figure 3.1). The magnitude of the shift in refractive index is directly proportional to the amount of bound analyte and is measured in Response Units (RU). Each RU is equal to an angle shift of 10^{-4} degrees.

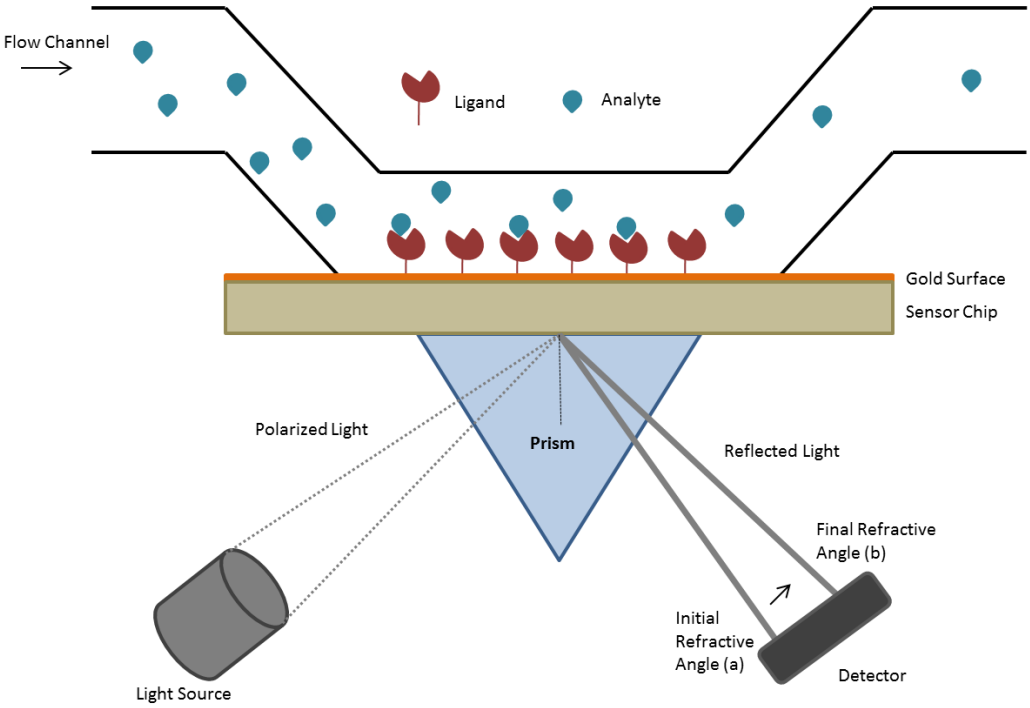

Figure 3.1: Basic flow cell diagram used during surface plasmon resonance spectroscopy. Protein is immobilized on the sensor surface and interactions with a flowing analyte is analysed. Changes in refractive index (a and b) generate a response associated with specific binding of the analyte to the immobilized protein on the sensor surface.

At the start of any binding experiment, all immobilized receptors have not been exposed to the analyte solution and the RU value corresponds to the initial refractive angle (a). As the analyte solution is injected over the sensor surface, analyte binds to the immobilized receptor molecules, where the rate of association can be measured by the curve related to the binding of analyte to the receptor (k_a). When a steady-state is achieved, the RU value is considered to be the final refractive angle (b) which is related to the concentration of analyte and immobilized receptor and can be used to calculate the binding affinity (K_D). A rate of dissociation is measured by the curve created when the analyte is removed from the immobilized receptor and binding sites are no longer occupied (k_d). The sensor surface is then

regenerated to return to the initial refractive angle (a) such that the experiment can start again with a different analyte sample (Figure 3.2).

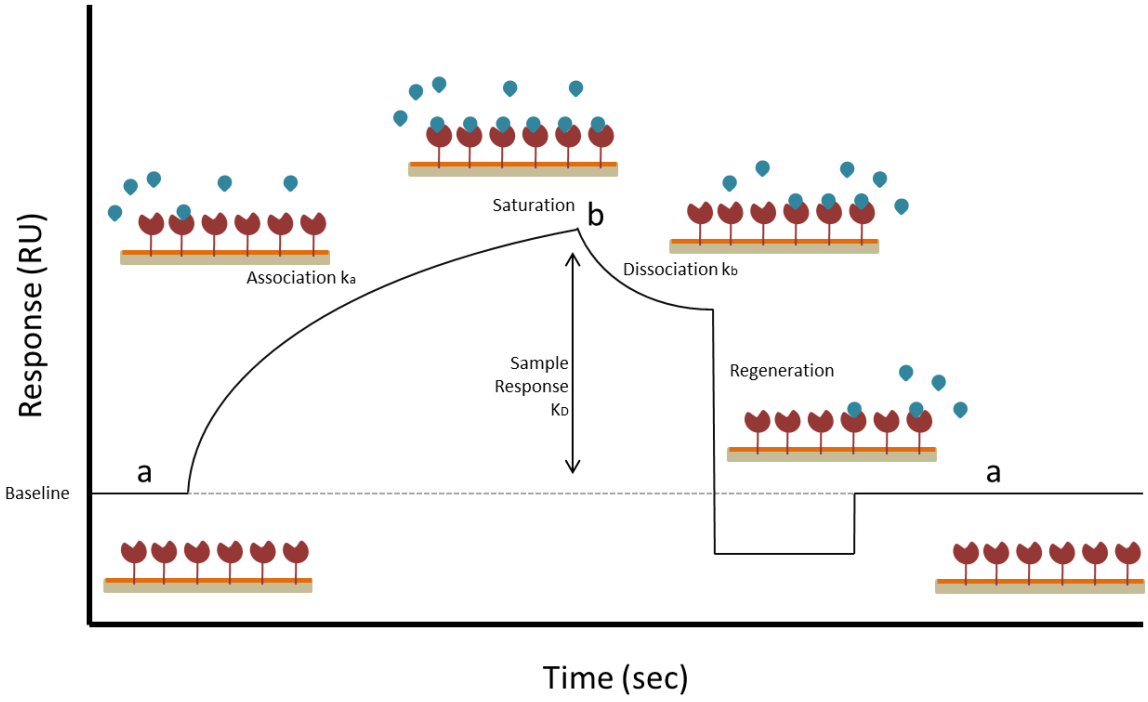

Figure 3.2: Basic real-time binding curve during analyte binding to immobilized protein on sensor surface, forming a sensogram. When the flowing analyte binds to immobilized protein, the refractive index shifts, which is seen as an increase in Response signal. At the start of any experiment the initial critical angle (a) is measured. As analyte molecules are injected into the flow channel, the response increases during analyte binding until the injection time is complete. The shape of this curve is used for measuring the rate of association (k_a). At this time a final critical angle (b) is measured which relates to that analyte concentration's steady-state affinity (as many occupied binding sites as possible). When analyte molecules are removed from immobilized receptors from the steady flow, the signal begins to decrease. This curve is used for calculating a rate of dissociation (k_d). Finally, regeneration removes the remaining analyte molecules from the immobilized protein and returns the critical angle to (a) prior to the next injection start time.

The lowest detectable concentration in an SPR experiment depends on various factors including the molecular weight of the immobilized receptor, the surface coverage of the receptor on the sensor surface, the molecular weight of the analyte, the optical property, and

the binding affinity of the analyte molecule. The response measured by the SPR is directly correlated with the change in mass bound to the sensor surface. The response measured depends, therefore, on the molecular weight of the analyte molecule and the number of receptor sites on the sensor surface. A theoretical maximum response (RU_{max}) describes the maximum binding capacity of the sensor bound receptor. The upper limit response that can be measured with a specific sensor chip is determined from:

$$RU_{max} = (MW_{analyte}/MW_{receptor}) \text{ X } RU_{receptor} \text{ X Stoichiometric Ratio}$$

where $MW_{analyte}$ is the molecular weight of the analyte, $MW_{receptor}$ is the molecular weight of the receptor, $RU_{receptor}$ is the response measured from the sensor chip with immobilized receptor and Stoichiometric Ratio is the proposed binding of the analyte-receptor interaction (Altintas et al. 2012; *Biacore Assay Handbook* 2016). Achieving an optimal RU_{max} is important for determining accurate binding kinetics of an interaction. A crowded receptor bound sensor chip would allow for non-specific binding to the sensor surface, where as a sparsely bound sensor chip would require the sensitivity of the instrument to be high in order to measure the kinetics behind a binding event.

Nerve growth factor

A variety of trophic factors are necessary for the development and maintenance of neurons in both the central nervous system (CNS); including the brain and spinal cord; as well as the peripheral somatic, autonomic and sensory nervous systems. One class of trophic factors,

neurotrophins, have critical roles in central and peripheral neuronal homeostasis (Lu et al. 2013; Aloe et al. 2012). The founding neurotrophin, nerve growth factor (NGF), is critical during development for the growth and survival of sympathetic and sensory neurons (Levi-Montalcini 1987; Ritter et al. 1991). Other neurotrophin family members include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). However, NGF is not necessary for neuronal survival in adults, but rather has been associated with the transmission of pain signals resulting mainly from inflammatory and neuropathic pain pathology (Dray 2008).

The trophic actions of NGF are accredited to the activation of its high affinity (K_D = 10^{-11} M) troponin-like receptor kinase A (TrkA) which is expressed on peripheral and central neurons (Hefti et al. 2006). Once activated there is an auto-phosphorylation of the TrkA intracellular domain (Wehrman et al. 2007) which results in activation of a number of signalling pathways to internalize the NGF-TrkA complex, altering gene expression (Goedert et al. 1981; Delcroix et al. 2003). Interestingly, the affinity of TrkA to NGF is altered by the expression of NGF's low affinity $p75^{NTR}$ receptor which binds all neurotrophin family members with similar low affinity (K_D~ 10^{-9} M) (Hefti et al. 2006). When $p75^{NTR}$ and TrkA are co-expressed on the cell surface, the affinity of TrkA for NGF increases by two orders of magnitude from K_D = 10^{-9} M to K_D = 10^{-11} M suggesting cross-talk between the two receptors (Hempstead et al. 1991). It has been suggested that $p75^{NTR}$ plays a critical role in maintaining TrkA's high affinity for NGF as well as increases TrkA-mediated phosphorylation and downstream signalling (Lachyankar et al. 2003;

Epa et al. 2004; Wehrman et al. 2007). However, it has been also found that the affinity of $p75^{NTR}$ for NGF does not change in the presence or absence of TrkA (Barker 2007).

The greatest amount of NGF production in the CNS occurs in the cortex, the hippocampus and the pituitary gland (Jiang and Salton 2013). Although significant amounts of NGF are also found in other CNS areas, including the basal ganglia, thalamus and the retinas (Mcallister 2001). NGF has been shown to pay a crucial role in cholinergic neuron homeostasis in the basal forebrain complex (BFC) (Iulita and Cuello 2016; Dreyfus 1989) which is responsible for functions such as attention, arousal, memory, consciousness and motivation. Interestingly, NGF has been investigated as a potential protective agent in neurological disorders where the BFC neurons are highly affected, such as in Alzheimer's disease (AD) (Allen and Dawbarn 2006; Ferreira et al. 2015; Tuszynski et al. 2015). NGF has been also found to play a key role in CNS stress response by regulating features of the noradrenergic nuclei of the hypothalamus and brain stem (Gioiosa et al. 2016; Manni et al. 2008).

It has been well documented that cells of the immune system in the peripheral nervous system (PNS) also produce and utilize NGF. Work dating back to the 1970's provided evidence of NGF effects on mast cells (Aloe and Levi-Montalcini 1977; Leon et al. 1994). Since then the effect of NGF on immune cell function has been characterized. NGF receptors are expressed on immune cells populations which allows for NGF to alter cell differentiation and regulate immune response (Aloe et al. 2012). NGF affects the survival and differentiation of lymphocytes (Torcia

et al. 1996; Bracci-Laudiero et al. 2005; Brodie and Gelfand 1992; Otten et al. 1989), monocytes (Noga et al. 2007; Caroleo et al. 2001; Bracci-Laudiero et al. 2005; Prencipe et al. 2014) granulocytes such as basophils (Takafuji et al. 1992; Gibbs et al. 2005), neutrophils (Beigelman et al. 2009), eosinophils (Takafuji et al. 1992) and leukocytes (Boyle et al. 1985), as well as stem cells (Auffray et al. 1996; Chevalier et al. 1994). It has also been noted that during tissue damage, inflammatory mediators induce NGF production by a variety of cells to elicit a painful response (Kawamoto and Matsuda 2004; Chao et al. 2006).

Pain signalling and therapeutic options

As a protective agent for behaviours which may result in harm to the body, strong stimulus of bodily tissues elicits a painful response. Most pain signals are sent from thermal, mechanical or chemical stimuli activating primary sensory neurons by means of nociceptive receptors. Such nociceptive signals are dependent on the activation of several transient receptor potential (TRP) channels and G-protein-coupled receptors found at the nerve endings (Lewin and Moshourab 2004). During tissue damage lysed cells also release ATP, which has the ability to increase the activation of nociceptive receptors (Cook et al. 1997). Immune cells which release NGF are not active players in pain signalling during acute noxious stimulus. Alternatively, pain sensitivity related to NGF-meditated responses is usually related to inflammation.

During an inflammatory response, a variety of immune cell types are recruited to the site of inflammation and contribute to pain sensitivity. As a result of tissue damage mast cells, neutrophils and macrophages are activated and mediate pain signals by releasing a series of inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO), and NGF (Pezet and McMahon 2006; Marchand et al. 2005). These mediators act directly on their respective receptors found on nociceptive nerve terminals. The algesic effect of NGF is a consequence of binding to its high affinity receptor TrkA located on approximately 50% of peripheral nociceptors. TrkA activation leads to the phosphorylation and sensitization of TRPV1 receptors, suggesting a mechanism behind NGF-induced heat and mechanical hyperalgesia (Shutov et al. 2016; Nishigami et al. 2013). Upon TrkA activation, TRPV1 gene expression is also altered following the intracellular transport of NGF-TrkA complex to the nucleus which assists in increased long-term thermal and mechanical sensitivity (Mills et al. 2013; Mizumura and Murase 2015).

Pharmacological methods commonly used for managing pain symptoms include non-opioid analgesics or non-steroidal anti-inflammatory drugs (NSAIDs) for mild to moderate pain and opioid drugs for moderate to severe pain (Katz and Barkin 2010). Pharmacological options offered for mild to moderate pain do provide significant benefit in the relief of painful symptoms, but with long-term usage there is an increased risk of serious side effects and complications. Long-term NSAID administration has been associated with gastrointestinal bleeding and ulcers, hyperkalemia and acute renal failure (Wolfe et al. 1999; Whelton 2000). In

addition, they have a ceiling effect where after a certain dose their efficacy does not increase (Fishman and Teichera 2003). It has been estimated that 7,600 deaths and a number more of hospitalizations occur each year in the United States as a result of NSAID complications, usually resulting from gastrointestinal bleeding (Fries 1992; Tamblyn et al. 1997).

Long-term opioid use has a long list of complications including nausea, vomiting, cognitive impairment, dizziness, and respiratory depression. In addition to these risks, there also exists a high rate of dependence and misuse by patients (Cherny 1996; Barkin and Barkin 2001; Fishman and Teichera 2003). It is important to note that despite the alarming number of hospitalizations and deaths related to the current pain therapeutic options, there have not been any recent novel pharmaceutical pain management agents which have made it to market. NSAIDs were the most recent advancement in pain therapeutics and these date back to the 1950's. It is also estimated that one in five Canadians suffer from chronic pain (Moulin et al. 2002). There remains, therefore, a large unmet need to develop novel classes of pain therapeutics as a third of these patients do not experience relief from current pain management strategies (Kalso et al. 2004).

Novel drug discovery relating to NGF mediated pain

Neurotrophin dysregulation has been associated with a variety of pathologies including chronic pain, neurodegeneration, fibromyalgia, and psoriasis (Chao et al. 2006; Xu et al. 2016; Truzzi et

al. 2011). Pain is, however, in the forefront of therapeutic development targeting NGF dysregulation. Currently there are three strategies on targeting NGF signalling: humanized monoclonal anti-NGF antibodies which bind and sequester free NGF; NGF memetic peptides which competitively bind to NGF receptors; and small molecule inhibitors which bind to either NGF or its receptors for inhibition of signal transduction.

Antibody NGF inhibitors

Since the approval of using anti-TNFα monoclonal antibodies for the treatment of rheumatoid arthritis (Maini et al. 1999), an increase in drug discovery efforts toward targeting signalling proteins with the use of monoclonal antibodies have been observed. Humanized monoclonal antibodies have been proven effective the treatment of various pathologies including psoriasis (Lebwohl et al. 2015; Salinger et al. 2014), multiple sclerosis (Rudick and Miller 2008; McCormack 2013) and juvenile idiopathic arthritis (De Benedetti et al. 2012; Brunner et al. 2015; Barone et al. 2016). Similarly, several pharmaceutical companies began monoclonal anti-NGF antibody clinical trials in 2009 for the treatment of chronic pain. Thus far the most clinically advanced of the anti-NGF monoclonal antibody therapies is Tanezumab (RN 624) by Pfizer (now Pfizer and Lilly). Tanezumab has demonstrated to have a picomolar binding affinity for NGF (K_D = 10 pM) and with a dissociation constant measured using surface plasmon resonance of over 100 hours (Abdiche et al. 2008). Tanezumab functions by sequestering free NGF to induce analgesia by decreasing the number of nerve fibers and expression of proteins related to nociception (Hoffman et al. 2011).

Initial clinical trials of Tanezumab were found effective for treatment of osteoarthritis in reducing pain and increasing joint function compared to placebo (Lane et al. 2010). Phase II clinical trials concluded that two doses of Tanezumab (10-200µg/kg) administered 8 weeks apart were able to alleviate osteoarthritic knee pain by 45-62% compared to baseline, whereas the placebo patients reported a 22% in pain reduction (Lane et al. 2010). Following phase II clinical trials in 2010, the FDA placed a partial clinical hold on Tanezumab and other similar class drugs due to reports of worsening osteoarthritis leading to bone necrosis requiring joint replacement (Lane et al. 2010; Garber 2011). It was theorized that Tanezumab interfered with blood vessel formation which led to avascular necrosis of the joint in which Tanezumab was injected. This partial clinical hold was lifted in 2012 following an external examination of the clinical information surrounding the allegations. It was determined that in 249 reviewed cases of reported osteonecrosis or total joint replacement from various clinical trials, only 2 cases were reviewed as primary osteonecrosis (Hochberg et al. 2016). 200 cases were reviewed as worsening osteoarthritis and the remaining 47 cases were alternatively diagnosed or lacked information leading to a diagnosis (Hochberg et al. 2016). The FDA Arthritis Advisory Committee released an explanation that the two cases of osteonecrosis were due to the anti-NGF therapy being overly effective in relieving chronic pain symptoms (FDA 2012). They suspected that patients experienced relief from their osteoarthritic pain allowing them to overuse the joint which accelerated damage leading to joint failure (FDA 2012). Months later a second partial clinical hold was placed on anti-NGF antibodies when animal studies showed peripheral nervous system effects (Bannwarth and Kostine 2014). During this clinical hold it was

determined that treatment with a high dose of Tanezumab co-administered with another NSAID class of therapy had an associated increased risk of osteonecrosis (Schnitzer et al. 2014; Ekman et al. 2014) and a potential increase in peripheral sensory abnormalities (Lane et al. 2010). As a result, safety precautions were implemented with the clinical use of Tanezumab and high doses were not to be administered with NSAID drugs.

It was estimated that when the second clinical hold was lifted in March 2015, over 11, 000 patients had been treated with Tanezumab since 2009. The second clinical hold was lifted when investigation into allegations of PNS neural changes were found not to be associated with neuronal death or apoptosis.

A number of new clinical trials are currently underway to establish an efficacy and safety profile of Tanezumab in long-term administration studies and to determine the optimal dose to maximize benefit to risk associated with this class of drug (Schnitzer and Marks 2015; Chang et al. 2016). One recent study on toxicity effects of Tanezumab in cynomolgus monkeys determined that during pregnancy, a dose of Tanezumab (0.5-30 mg/kg) showed no material toxicity, but was associated with an increased number in still births and post birth infant mortality (Bowman et al. 2015). All maternal doses of Tanezumab were also associated with a decrease in infant growth accompanied by changes in sensory neurons in the peripheral nervous system (Bowman et al. 2015). Therefore, although Tanezumab has demonstrated to

be efficient in relieving NGF-induced pain, there still remain several safety concerns and toxicity effects which need to be resolved.

NGF mimetics

Mimetic peptides have been investigated in several studies which focus on developing synthetic peptides to mimic protein structure as a competitive binder. NGF inhibitory mimetic peptides were first introduced in the mid-1990's as peptides which resemble loops I and IV of mature NGF. Constrained peptides which resembled loop I of NGF (residues C28-35) were able to competitively bind and inhibit both TrkA and $p75^{NTR}$ receptor binding of radiolabelled NGF and effectively inhibit neurite outgrowth of PC12 cells (LeSauteur et al. 1995; Beglova et al. 1998). Loop IV mimetic peptides (residues C92-96) were only able to inhibit TrkA receptor binding of radiolabelled NGF (LeSauteur et al. 1995). These results are consistent with regions of NGF which are responsible for individual receptor binding.

While trying to make the agonistic effects of NGF mimetic peptide D3 (NGF residues 95 and 96) more effective, Brahimi and colleagues unexpectedly created a bivalent compound called 1ss with an antagonist effect (Brahimi et al. 2010). 1ss consist of two D3 residues with a short chemical linker which is effective in inhibiting TrkA receptor binding of radiolabelled NGF (Brahimi et al. 2010). Unfortunately developments of NGF inhibitory mimetic peptides have not

progressed significantly further, and even though these peptides show promise in inhibiting NGF-receptor binding, they remain far from clinical use.

Small molecule inhibition

K252a is one of the first small molecules to be described inhibiting NGF-induced TrkA tyrosine phosphorylation by preventing ATP binding in the catalytic domain (Berg et al. 1992). K252a is an alkaloid-like compound which has been proven efficient in inhibiting NGF-TrkA signalling and alter hypersensitivity in animal models of pancreatitis (Winston et al. 2003) and mast cell degranulation associated with postoperative ileus (Berdún et al. 2015). *In vitro*, K252a is effective in inhibiting NGF-TrkA signalling and neurite outgrowth, but it does not directly interfere with NGF-$p75^{NTR}$ signalling (Tapley et al. 1992). Unfortunately K252a binds to many kinases and interferes with other signalling pathways, which limit its potential as an NGF-induced pain therapeutic (Tapley et al.1992; Eibl et al. 2012).

A number of small molecule inhibitors have been developed to bind NGF and inhibit its action on TrkA and $p75^{NTR}$. Compounds such as ALE-0540, Ro 08-2750, PD 90780, PQC-083 and Y1036 have been described to bind NGF, and non-covalently alter the molecular topology and electrostatic potential of the surface of NGF to inhibit receptor binding (Eibl et al. 2013a; Eibl et al. 2012). Small molecule inhibitors may have an advantage over other strategies of inhibiting the response of NGF since they can be tailored to respond as a specific NGF inhibitor or as a

general neurotrophin inhibitor by designing molecules to bind to specific areas of the neurotrophin structure. Small molecules may also be advantageous since they are generally less expensive and timely to produce, as well as having a possibility of being orally active.

ALE-0540 was identified through screening of commercial compounds libraries as the first small molecule NGF inhibitor. ALE-0540 showed efficacy in inhibiting neurite outgrowth in PC12 cells at a half maximal inhibitory concentration (IC_{50}) of 5.88 µM ± 1.87 µM (Owolabi et al. 1999). The IC_{50} was not altered by the presence or absence of BDNF, suggesting that ALE-0540 has a specificity for NGF (Owolabi et al. 1999). Unfortunately, ALE-0540 required relatively high doses during rat models of nerve ligation and thermal sensitization suggesting a lack of efficacy *in vivo* (Owolabi et al. 1999).

Other small molecules such as PD 90780, Ro 08-2750 and PQC-083 have since been described as specific NGF inhibitors, although the mechanism by which they interact with NGF has not been definitively resolved. Molecular modeling of PD 90780 binding to NGF identified the loop I/IV cleft as a potential docking domain, and suggested 2:2 stoichiometry (Colquhoun et al. 2004). Screening through a series of analogues with a scaffold similar to PD 90780 identified PQC-083, which was also shown using molecular modeling to bind to NGF at the loop I/IV cleft (Eibl et al. 2013a). The structure of loop I/IV varies among the neurotrophin family members and plays a critical role in discrimination of receptor binding (Wehrman et al. 2007; He and Garcia 2004), suggesting a mechanism for the NGF specificity of these inhibitors. Due to the

similar molecular features; including a ridged conjugated ring structure and functional groups which are similarly oriented; it is hypothesized that ALE-0540 and Ro 08-2750 bind and inhibit NGF in a similar fashion as described by PD 90780 and PQC-083 (Eibl et al. 2012).

Finally, a small molecule Y1036 was identified as a multipotent neurotrophin inhibitor. Y1036 is a furan derivative and was demonstrated to be efficient in inhibiting both NGF and BDNF *in vitro* (Eibl et al. 2010). Molecular modeling suggests that rather than binding to the loop I/IV cleft like other small molecule NGF inhibitors, Y1036 binds to the hydrophobic dimer interface, which is highly conserved among all neurotrophin family members (Eibl et al. 2010; Eibl et al. 2013b). This binding interaction between Y1036 and NGF makes it a candidate for pathologies involving multiple neurotrophin dysregulation.

It is possible that these small molecule inhibitors have the ability to inhibit acute NGF-nociceptive stimulation and chronic pain models. However, more *in vivo* analysis is still required prior to introduction into clinical trials. These compounds could serve as lead agents for drug development as they have the potential to avoid side effects currently observed with the use of anti-NGF antibodies. Furthermore, due to the size of these small molecules, they have the potential to cross the blood brain barrier and assist in treatment of neurodegenerative pathologies in the CNS.

NGF related therapeutic advancements due to SPR

Recently, advancements in the binding properties of NGF have been brought to light by using SPR technologies. SPR allows for interactions to be identified when more than one potential ligand is at question. Specific binding to receptors, proteins or a virus have been identified using a number of different instruments to identify mechanisms for potential therapeutic applications (Table 3.1)

Table 3.1: Summary of SPR binding experiments involving NGF specific binding. Binding affinities (K_D) of each interaction are described. n.d. = not determined n.r. = not reported

Immobilized on sensor chip	Concentration of NGF in flow analyte	K_D Measured	Reference
Alzheimer's potential therapeutics			
ADAM10	80 – 320 nM	n.r.	(Wijeyewickrema et al. 2010)
TrkA	0.06 – 50 nM	0.147 ± 0.02 nM	(Bennmann et al. 2015)
Glycated TrkA	0.06 – 50 nM	0.457 ± 0.09 nM	
NGF-induced pain			
TrkA	4 – 500 nM	0.94 nM	(Covaceuszach et al. 2010)
	(R100W NGF)	1.44 nM	
$p75^{NTR}$	4 – 500 nM	1.53 nM	
	(R100W NGF)	125 nM	
TrkA	0.0125 – 50 nM	15 nM	(Sheffield et al. 2016a)
$p75^{NTR}$	0.0125 – 50 nM	13 nM	
Herpes antiviral			
SgG1	50 – 750 nM	n.d.	(Cabrera et al. 2015)
SgG2	50 – 750 nM	14 nM	

NGF metalloproteinase inhibitor

High levels of NGF were found in snake venom with remarkable similarity in structure and function to mammalian NGF (Cohen 2008). The snake venom also contained high levels of metalloproteinase-disintegrins which have been previously reported to have unique probes for platelet receptors initiating thrombus formation (Andrews et al. 2004; Wijeyewickrema 2016). It was a serendipitous discovery by Wijeyewickrema and colleagues who originally were interested in isolating metalloproteinases from cobra venom using Ni^{2+} -agarose. By using this technique, they isolated NGF from the venom leading to the hypothesis that NGF may regulate metalloproteinase activity (Wijeyewickrema et al. 2010). SPR technology was utilized for determining the binding of human NGF with recombinant human metalloproteinase ADAM10. ADAM10 was immobilized on a CM5 chip and NGF (0.8-3.2 μM) was passed over the ADAM10 immobilized surface. An association followed by a rapid dissociation of ADAM10 and NGF compounds were reported, although no binding affinity measured from this interaction was reported (Wijeyewickrema et al. 2010). ADAM10 has a critical role in proteolytic processing of the amyloid precursor protein, which is found to be elevated in the hippocampal neurons of patients with Alzheimer's disease (Haass et al. 2012; Marcello et al. 2013). These results may suggest a novel therapeutic approach to preventing or delaying the symptoms associated with the advancement of amyloid plaques in the brains of patients.

Glycation and neurodegeneration

Glycation is a process involving the metabolism of sugars in aging systems. In this process, a protein or lipid molecule covalently binding to a sugar molecule leads to protein denaturing and protein accumulation (Stirban et al. 2014). Glycation impairs the function of biomolecules and leads to the synthesis of advanced glycation endproducts (AGEs) through a series of slow reactions, including Amadori, Schiff base and Maillard reactions (Stirban et al. 2014). Radical oxygen species (ROS) are also produced as a side product during AGE production leading to further cell damage and apoptotic processes associated with aging (Uribarri et al. 2007). AGEs are implicated with age-related chronic diseases such as Alzheimer's (amyloid proteins are side products of AGEs processes), stroke (weakening of the collagen in blood vessels causing aneurisms) and peripheral neuropathy (AGE myelin attack causing demyelination) (Uribarri et al. 2007; Münch et al. 2002). It has been demonstrated that an increase in AGEs reduces cell adhesion and NGF-mediated neurodifferentiation in PC12 cells (Bennmann et al. 2014), as well as inhibits NGF-mediated neuroprotection (Espinet et al. 2015).

Bennmann and colleagues utilized SPR technology to determine the role of receptor glycation in binding of NGF to TrkA and in AGE binding to its multi-ligand receptor of advanced glycation endproducts (RAGE) (Bennmann et al. 2015). In their first set of experiments, TrkA was immobilized by a C-terminal His-tag onto a Ni-NTA sensor chip to allow for directed binding of NGF to the free N-terminal binding domain of TrkA. A binding affinity for NGF (0.06 nmol/L-50 nmol/L) from the immobilized TrkA was measured at $K_D = (1.47 \pm 0.2) \times 10^{-10}$ M (Bennmann et

al. 2015). Glycation of TrkA was completed directly on the chip by exposing immobilized TrkA to methylglyoxal (0.1 mM) for 20 hours. Following glycation, the binding affinity of NGF for TrkA was measured again at a threefold reduction $K_D = (4.57 \pm 0.9) \times 10^{-10}$ M (Bennmann et al. 2015). This was also supported with a threefold decrease in neurodifferentiation using PC12 cells.

Similar inhibition was also observed in a second set of experiments where BSA linked to fructose or glucose was exposed to immobilized RAGE. RAGE was immobilized using standard amine coupling techniques to a CM5 sensor chip. BSA on its own did not show any affinity for RAGE, but when linked to fructose or glucose showed binding affinities of $K_D = (1.95 \pm 0.7) \times 10^{-5}$ M and $K_D = (1.39 \pm 0.2) \times 10^{-5}$ M respectively (Bennmann et al. 2015). Once RAGE was glycated with a 10 hour exposure of methylglyoxal (0.1 mM) binding of BSA-fructose was completely inhibited (no K_D could be measured) and BSA-glucose was strongly inhibited $K_D = (7.62 \pm 0.4) \times 10^{-5}$ M (Bennmann et al. 2015).

These results demonstrate a mechanism by which AGE accumulation as a normal function of ageing could influence the differentiation of neuronal cells by the TrkA receptor. NGF activates cell survival and the reduction in binding affinity for glycated TrkA could be one factor that contributes to the reduced function of ageing neuronal cells (Sofroniew et al. 2001).

“Painless” mutations

Physiological evidence of the crucial role that NGF plays in pain signalling is made evident in rare genetic conditions such as congenital insensitivity to pain, also known as human sensory and autonomic neuropathy (HSAN) type IV and V. These conditions are caused by mutations in the NTRK1 gene which codes for TrkA (Indo 2001) or the NGFB gene coding for NGF (Einarsdottir et al. 2004). HSAN IV mutation completely abolishes the ability for TrkA to respond to NGF and patients suffer from neuro-developmental effects including mental retardation, and a lack of sensory response (Indo 2012). More recently the HSAN V mutation was found to include a R to W substitution at position 100 of the mature NGF protein (Einarsdottir et al. 2004). Patients with this substitution, unlike HSAN IV, do not show cognitive impairments, but rather impaired temperature sensations and pain perception (de Andrade et al. 2008). Covaceuszach and colleagues utilized SPR technology to determine the mechanisms by which NGF was inhibited to cause these differences in development (Covaceuszach et al. 2010). The extracellular domains of TrkA and $p75^{NTR}$ were immobilized on a CM5 sensor surface using standard amine coupling techniques. Humanized NGF bound to immobilized TrkA and $p75^{NTR}$ with binding affinities of K_D = 0.94 nM and K_D = 1.53 nM respectively (Covaceuszach et al. 2010). Alternatively the R100W mutated NGF showed an unaltered binding affinity for TrkA (K_D = 1.44 nM) and a significantly lower affinity for $p75^{NTR}$ (K_D = 125 nM) (Covaceuszach et al. 2010). This evidence supports physiological symptoms of patients with HSAN V who suffer from loss of pain perception, but show no mental retardation or delay in other neurological functions (Capsoni et al. 2011; Verpoorten et al. 2005; Dyck et al. 1983). This mutation allows for TrkA binding to remain unaltered and allows maintaining of neurotrophic signalling,

although it hinders signalling involved with nociceptive sensitization. These findings support a growing body of evidence that implicates $p75^{NTR}$ signalling with pain transmission and sensitization (Nicol and Vasko 2007). The mutation described could provide insight to designing NGF therapeutics for the treatment of Alzheimer's disease which would not be associated with painful symptoms (Cattaneo et al. 2008).

Development in NGF inhibitors

Sheffield and colleagues also explored the relationship between NGF and $p75^{NTR}$ with SPR technology (Sheffield et al. 2016a). A large body of evidence suggest that pain signalling not only constitutes NGF-TrkA signalling, but also NGF-$p75^{NTR}$ signalling (Nicol and Vasko 2007). $p75^{NTR}$ and TrkA were immobilized on their respective CM5 sensor surfaces using amine coupling. NGF (0.0125-50 nM) was injected over immobilized $p75^{NTR}$ and TrkA yielding binding affinities of K_D = 13 nM and K_D = 15 nM respectively (Sheffield et al. 2016a). Previously reported NGF-TrkA inhibitors (ALE-0540, PD 90780, Ro 08-2750 and PQC-083) were examined for their inhibitory effect on NGF-$p75^{NTR}$ signalling. It was determined that PD 90780 was able to inhibit NGF-$p75^{NTR}$ signalling more effectively (IC_{50} = 110 μM) than ALE-0540 (IC_{50} > 300 μM), Ro 08-2750 (IC_{50} = 244 μM) and PCQ 083 (IC_{50} not determined). However, no *in vitro* evidence was provided to support the biological significance of these findings. NGF dysregulation is not only implicated in chronic pain pathologies but also in neurodegenerative diseases such as Parkinson's disease (Nagatsu et al. 2000). The identification of inhibitors which can inhibit both NGF-TrkA signalling and NGF-$p75^{NTR}$ signalling could hold great therapeutic potential.

Human pathogen affecting neurotrophin function

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) are neurotropic human pathogens which originate in epithelial cells in the skin and mucosa of the oral tract and genitalia (Sauerbrei 2016). HSV-1 and HSV-2 are highly adaptive and modify several aspects of the immune and nervous system of their host (Groves 2016). NGF is located in skin and mucosal epithelial cells for axonal growth and neuronal survival (Nakamura et al. 2007). Cabrera and colleagues determined that HSV-2 secreted glycoprotein G (SgG2) binds directly to NGF and increases neurotrophin function at the site of infection. SgG1 and SgG2 were immobilized on a CM4 sensor chip through amine coupling, and analyte NGF demonstrated a binding response to SgG1 (Cabrera et al. 2015). However, this binding was found not to be specific due to unattainable saturation of the sensor surface (Cabrera et al. 2015). Alternatively, NGF was found to have specific binding for immobilized SgG2 with a measured binding affinity of K_D = 1.4 x 10^{-8} M (Cabrera et al. 2015). To determine if SgG2 altered NGF function, superior cervical ganglion (SCG) neurons were cultured and exposed to NGF in the presence of SgG2. The presence of SgG2 augmented axonal growth of SCG neurons compared to a control (Cabrera et al. 2015). The SgG2 interactions observed with neurotrophic factors sheds light on the very complex network between the HSV virus and the host. By understanding the mechanisms that allow HSV-1 and HSV-2 to modify the nervous system, it will allow for targeted approaches for synthesizing new antiviral strategies.

Conclusion

Current therapeutic options for pain are not showing to be effective in quenching NGF-induced chronic and inflammatory painful pathologies. Even though NSAIDs and opioids have a long list of side effects and limitations associated with their use, no novel pain therapeutic options have made it to market to replace them. Unfortunately, current NGF inhibitory drug discovery programs have exposed limitations with therapeutic options in terms of specificity for their target and effectiveness in inhibiting NGF-induced pain. SPR technology has been very useful in characterizing interactions between potential therapeutic drug strategies and their receptors over the last three decades. Due to advancements in sensitivity, SPR technology has recently been effective in characterizing NGF binding interactions with possible inhibitors or with receptors of interest to aid in the development of therapeutics. With the advancements reviewed, mechanisms of NGF specific binding have been identified as possible drug therapeutic options for Alzheimer's disease, chronic pain and herpes antiviral strategies.

Chapter 4: A surface plasmon resonance spectroscopy method for characterizing small molecule binding to nerve growth factor

(Original Research)

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Abstract

Small molecule inhibitors have been previously investigated to identify possible therapeutics for the treatment of chronic pain. In the present study, known nerve growth factor (NGF) inhibitors identified by ^{125}I-NGF binding were characterized using affinity and binding evaluations by surface plasmon resonance (SPR) spectroscopy. A novel strategy for characterizing NGF inhibitors was used to determine the binding affinity (K_D) and saturation ability of each compound with immobilized NGF. 74% of compounds screened demonstrated a positive binding event to NGF. A K_D less than 10µM and a percent saturation of over 50% were used as thresholds to identify inhibitors which would warrant further investigation. This study details for the first time a methodology which can be used to directly characterize the binding event between small molecule inhibitors and NGF.

Introduction

Neurotrophins play a crucial developmental role in regulating the survival and differentiation of neurons in both the peripheral and central nervous systems (Bennett 2001). Nerve growth factor (NGF) is a member of the neurotrophin family and has been well established with a role in development in sympathetic and sensory neurons (Nicol and Vasko 2007). NGF has also been associated with pain-signalling systems in adult animals and in humans by binding and activating high affinity tyrosine-kinase receptor TrkA (Hefti et al. 2006).

Inhibiting the activity of NGF may have significant therapeutic potential for pathologies related to neuropathic pain, including congenital insensitivity to pain with anhidrosis (CIPA) or hyperalgesia, where a dysfunction of the NGF-TrkA signalling pathway is observed (Watson, Allen, and Dawbarn 2008). One method of inhibiting the effect of NGF is with the use of the anti-NGF monoclonal antibody Tanezumab. Clinically, Tanezumab has demonstrated efficiency in the treatment of pain in patients with osteoarthritis, chronic lower back pain and diabetic peripheral neuropathy (Brown et al. 2014). However, there remains limitations with the use of Tanezumab with respect to autoimmune responses, variability in pharmacokinetics, as well as drawbacks in safe administration of the antibody and production cost (Samaranayake et al. 2009). Tanezumab was also under several clinical holds during its phase studies due to evidence of peripheral nerve effects. Utilizing an approach to inhibiting NGF with the use of small molecules may have significant pharmacological, practical and economic advantages over monoclonal antibodies. Small molecules such as ALE-0540 (Owolabi et al. 1999), PD 90780

(Colquhoun et al. 2004), PQC-083 (Eibl et al. 2013a), Ro 08-2750 (Niederhauser et al. 2000) and Y1036 (Eibl et al. 2010) have previously demonstrated an ability to inhibit NGF activity *in vitro* and several of these have been suggested to bind the ligand NGF, rather than the receptor.

The mature form of NGF is a symmetrical dimer consisting of two identical monomers (Eibl et al. 2012) which associate by hydrophobic interactions (McDonald et al. 1991). The structure of NGF proposed by McDonald and colleagues, describes a 118 amino acid sequence which forms a monomer with four distinct loop regions and two β-pleated sheet strands (McDonald et al. 1991). Molecular modeling of PD 90780 and PQC-083 binding to NGF suggests a binding site at the loop I/IV cleft of NGF (Colquhoun et al. 2004; Eibl et al. 2013a), which would suggest a 2:1 stoichiometry (two small molecules : one NGF dimer). Originally, ALE-0540 was thought to bind to the binding sites on TrkA and p75 receptors (Owolabi et al. 1999) (rather than to NGF) and Ro 08-2750 was suggested to inhibit NGF at the hydrophobic dimer interface (Niederhauser et al. 2000). Others have suggested by using molecular modeling evidence, that this is not likely the mechanism of inhibition and that ALE-0540 and Ro 08-2750 bind similarly to NGF as PD 90780 (Eibl et al. 2012). Y1036 has been suggested to bind near the hydrophobic dimer interface (Eibl et al. 2010), more specifically at Lysine-57 (Eibl et al. 2013b), suggesting a 2:1 stoichiometry.

Previously used screening strategies for NGF inhibitors required the use of radioisotopes; such as ^{125}I (Colquhoun et al. 2004; Eibl et al. 2010; Owolabi et al. 1999; Eibl et al. 2013a). Inhibition constants were calculated based on the amount of radioactive decay detected as labelled NGF bound to receptors; a process which would be inhibited when NGF was previously exposed to active small molecules. However, using radioisotopes has the potential to alter the molecular function of NGF, as well as has the limitation that the small molecules may be interacting with the receptor of rather than NGF, which would give similar inhibitory results.

The present work deals with the application of biosensor technology to observe the direct interaction between small molecules and NGF without the use of radiolabels. Having an alternative label-free method for measuring the small molecule-NGF binding event rather than the downstream binding, signalling or functional effect of the event would be of great benefit to understanding the mechanism of these inhibitors.

Materials and methods

Binding of ^{125}I-NGF to PC12 cells

Full length (1-118) mouse NGF purified by HPLC from 2.5S NGF (purity greater than 95%) was obtained from Cedarlane Laboratories (Toronto, ON). The iodination of NGF was performed as previously described (Ross et al. 1997). The ^{125}I-NGF obtained (typically 80-120 c.p.m./pg) was purified by size exculsion chromatography on a PD10 column (Pharmacia) preequilibrated with

HKR buffer (10 mM HEPES [pH 7.35] containing 125 mM NaCl, 4.8 mM KCl. 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 g/l glucose, 1 g/l BSA). PC12 cells were grown in RPMI with 10% fetal calf serum. Each data point set up in a single tube containing ^{125}I-NGF (at the required concentration), 400,000 cells (for a final concentration of 10^6/ml) and NGF (at 10 nM for nonspecific binding) as required. The tubes were incubated for 2 h at 4˚C, and 100 µl aliquots (providing triplicate data points for each sample) were transferred to 400 µl microcentrifuge tubes containing 200 µl of 10% glycerol in HKR. Tubes were centrifuged for 2 min at 5,000 rpm, the tip containing the cell pellet was cut off, and radioactivity bound to the cells was determined.

Surface plasmon resonance spectroscopy

A Biacore™ T200 spectrometer was obtained from GE Healthcare Life Science (Piscataway, NJ, USA). Synthetic organic compounds were synthesized by Sussex Research (Ottawa, ON, Canada). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Oakville, ON, Canada). A Biacore Amine Coupling Kit was obtained from GE Healthcare Life Sciences (Piscataway, NJ, USA). Other analytical grade compounds were obtained from Fisher Scientific (Ottawa, ON, Canada). Deionized water from a Milli-Q Water Purification Systems was used for making all solutions. A series S Sensor Chip CM5 was used. HBS-EP buffer containing 0.5% DMSO was used as the running buffer pH 7.5 (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.05% v/v Tween 20). The running buffer was degassed using a vacuum chamber. Sensor Chip CM5 was docked

and normalized with a 70% w/w glycerol solution for stabilization. The instrument was primed with running buffer twice before use.

Surface preparation with immobilization of NGF

The NGF protein was coupled using the amine coupling method. Sensor Chip CM5 was activated using a 7 minute contact time of 0.4 M EDC/0.1 M NHS from Biacore Amine Coupling Kit, at a flow rate of 10 µL/min. A solution of NGF in acetic acid was first made by dissolving 1 mg of NGF into 50 µL of 0.02% acetic acid solution. The NGF-acetic acid solution was then mixed with HBS-EP buffer to yield 10 µg/mL solution. Following activation, the described NGF solution was injected over the activated sensor chip surface for 9 minutes at a flow rate of 10 µL/min. Immobilization was measured at 6645 Response Units (RU) (1 RU = 1 pg/mm^2). After the injection, the surface was washed with 1M NaCl to remove any uncoupled or non-covalently bound material from the surface. The excess hydroxysuccinimidyl groups on the surface were deactivated with 1M ethanolamine hydrochloride, pH 8.5 for 7 minutes at a flow rate of 10 µL/min. The surface of a reference flow cell was activated with 0.4 M EDC/0.1 M NHS for 7 minutes with a flow rate of 10 µL/min, and then deactivated with a 7 minute exposure of 1 M ethanolamine at a flow rate of 10 µL/min. With no ligand bound to the flow path, the control flow cell was used to detect non-specific binding of the small molecules to the sensor chip surface during screening affinity assays.

Small molecule sample preparation

Measureable amounts of each small molecule were obtained with an analytical balance and dissolved in an appropriate amount of DMSO to give a 10 mM solution. The samples were diluted with the running buffer to yield small molecule solutions for the assay of concentrations that varied from 0.7 µM to 200 µM.

Analyte injection

Prior to analyte injection, the series S CM5 chip was conditioned with three 30 second cycles of running buffer followed by three start up cycles, allowing the response to stabilize before analyte injection. Data was collected at a temperature of 25 °C. Individual compound samples were tested from lowest to highest concentrations, separated by a 15 second stabilization period after each sample in each compound series. During each sample cycle, analyte was injected for 60 seconds at a flow rate of 30 µL/min. A dissociation period was monitored for 30 seconds after analyte injection before regeneration with 1.0 M NaCl for 120 seconds at a flow rate of 30 µL/min to wash any remaining analyte from the sensor chip before running the next sample.

Two-site steady state binding affinity analysis

The Biacore T200 was programmed to run an automated assay with the various small molecule samples. Once the responses were measured, they were processed using Biacore T200

Evaluation Software Version 1.0. The responses measured in the blank flow cell (control) were subtracted from the response measured in the flow cell with protein immobilized. The binding affinities (K_D) of each small molecule were obtained by plotting the subtracted responses against the concentration and fitting the curve with a two-site steady state affinity fit. Each maximal response (Rmax) was compared to the theoretical Rmax calculated for each compound to determine percent saturation of available NGF on the chip. Percent saturation was calculated using the following equation; where density of ligand refers to the immobilization response of NGF:

$$Percent\ Saturation = \frac{Rmax}{Density\ of\ Ligand\ (RU) x \frac{Molecular\ Weight\ of\ Small\ Molecule}{Molecular\ Weight\ of\ Protein\ Immobilized}\ x\ Stoichiometric\ Ratio}\ x\ 100$$

Results and discussion

Fifty novel compounds of an analogue series and four previously reported compounds (PQC-083, PD 90780, ALE-0540 and Ro 08-2750) were screened using the described protocol. ^{125}I analysis identified 21 novel NGF inhibitors. However with ^{125}I-NGF being an estimation of the binding event, SPR analysis was required to identify NGF-binding inhibitors as opposed to receptor inhibitors.

Figure 4.1 represents the blank-subtracted sensogram for the concentration dependent binding of PQC-083 to the NGF immobilized on the sensor chips. Figure 4.2 represents the two site steady state affinity plot for the binding of varying concentrations of PQC-083 to NGF. The sensograms and affinity plots of the remaining 53 compounds binding to the immobilized NGF were of similar quality to that of PQC-083. Binding affinities were calculated from the affinity plots using the Biacore T200 Evaluation Software.

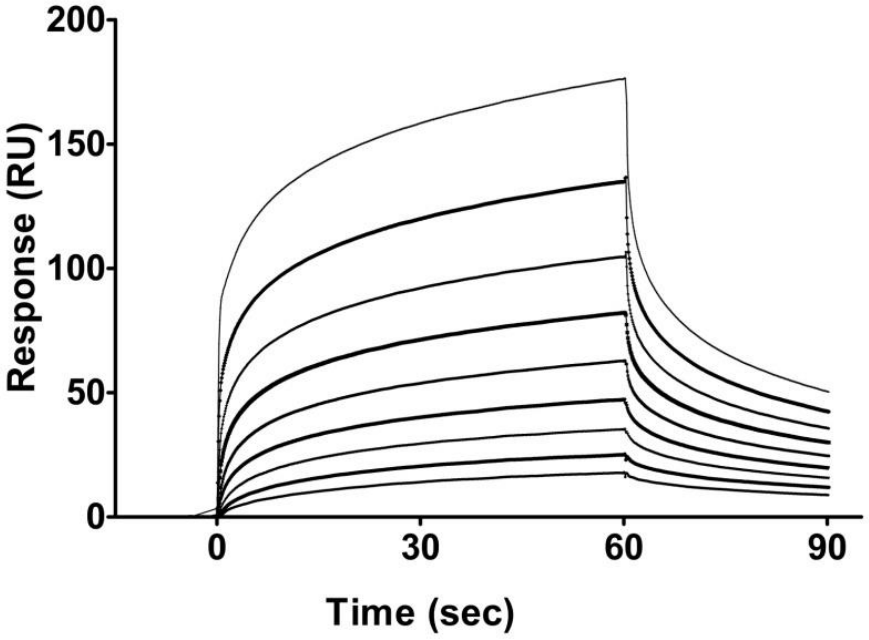

Figure 4.1*:* Blank subtracted sensogram which describes the association and dissociation of each concentration of PQC-083 tested in the assay. Time 0-60 seconds represents the association of PQC-083 to NGF; at 60 seconds the analyte flow stops. A dissociation period from 60-90 seconds is monitored before regeneration of the chip occurs before the next sample is run. Top line: 200 µM. Second line from top: 100 µM. Third line from top: 50 µM. Forth line from top: 25 µM. Fifth line from top: 12.5 µM. Forth line from bottom: 6.25 µM. Third line from bottom: 3.13 µM. Second line from bottom: 1.56 µM. Bottom line: 0.78 µM.

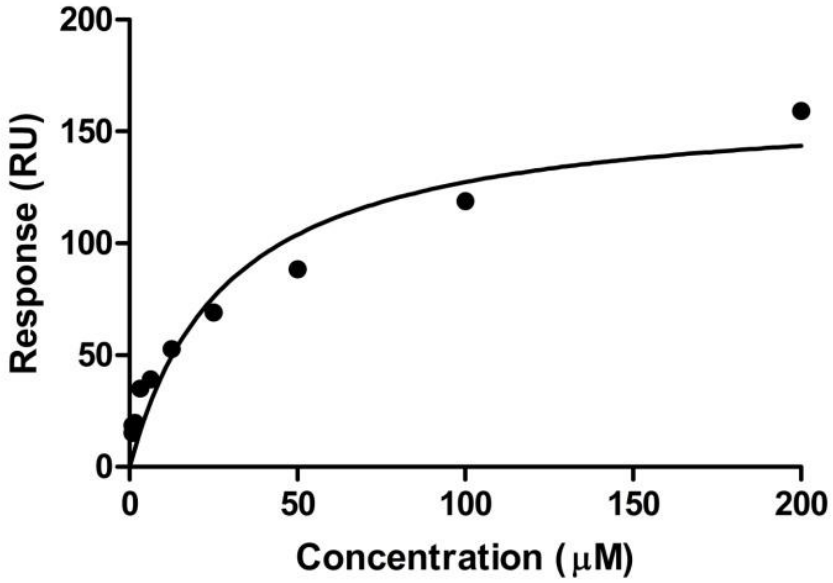

Figure 4.2: Two site steady state binding model describing PQC-083 binding to NGF (x-axis: concentration (μM); y-axis: subtracted response (response from active flow cell minus response from reference flow cell); each data point represents the subtracted response from the saturation (last 10 seconds of the association phase) of PQC-083 at varying concentrations (Figure 4.1).

Of the fifty four compounds analyzed, 74% of compounds resulted in a response characterized as direct binding to NGF while using SPR analysis. It was determined that the novel analogues that bound to NGF and the previously reported compounds, fit best to a 2:1 stoichiometry (two small molecules : one NGF dimer) and were analysed using a two-site binding model with the Biacore T200 Evaluation Software. 18% of the investigated compounds displayed a K_D lower than the previously reported compounds (<10μM). However, the degree to which the compounds were able to saturate the available NGF immobilized varied (0.3% – 150% saturation, Figure 4.3). When calculating the percent saturation, a 2:1 stoichiometry was considered, and of the 54 compounds screened, 16% of them were able to saturate the

available NGF immobilized to a level of over 50%. Of these compounds, two had a K_D lower than 10 μM (circled in Figure 4.3) and therefore, are good candidates for further investigation.

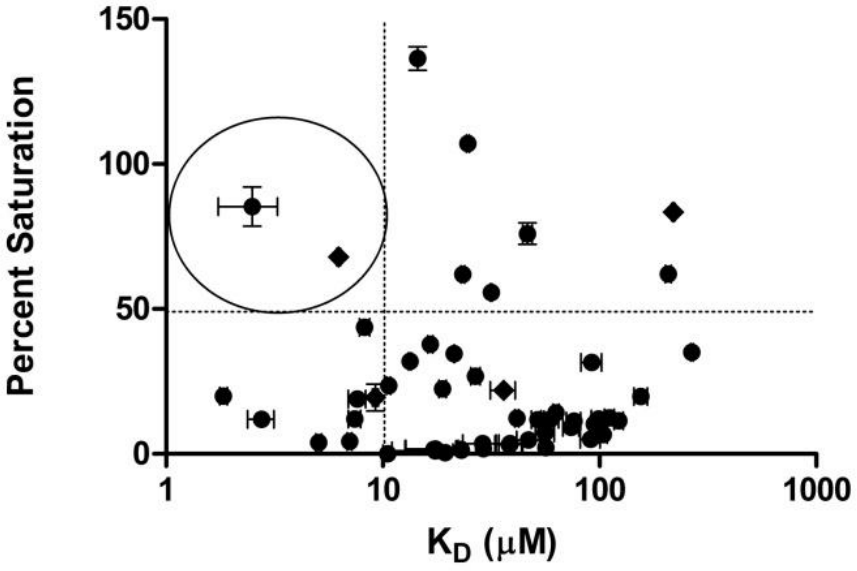

Figure 4.3: Ranking of small molecules screened using percent saturation and binding affinity (log[M]) to NGF. Diamond: previously reported small molecule inhibitors. Circles: small synthetic analogue series. Circled: two compounds which were able to saturate the available NGF >50% and bind with an affinity <10μM. Each compound was measured n=4.

By integrating the SPR analysis into the results determined from ^{125}I-NGF binding analysis, 14 novel compounds and three of the previously reported small molecules were identified as NGF-binding inhibitors (Figure 4.4). It was also determined that there is a relationship between the high affinity binding of the two site binding analysis from the novel compounds to the IC_{50} of the same compounds determined by ^{125}I-NGF (Figure 4.4) ($F(1,16) = 8.381$, $p = 0.0111$, $R^2 = 0.3585$).

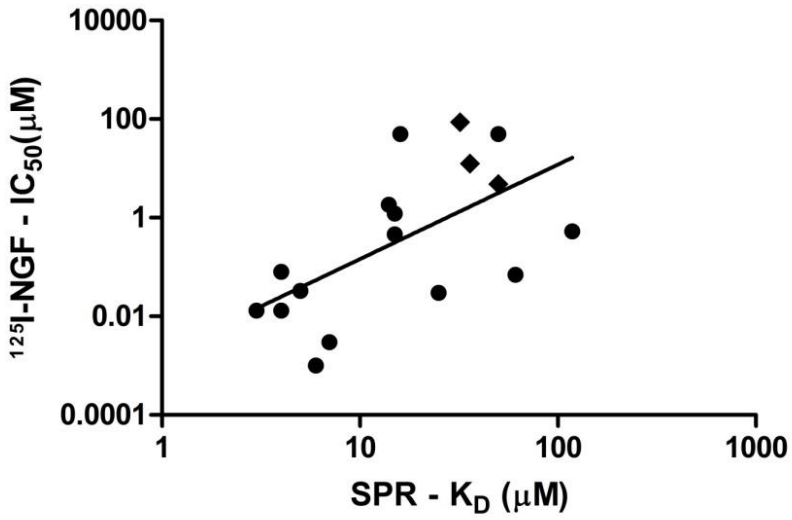

Figure 4.4: Demonstrates the relationship of 14 novel compounds and 3 previously reported compounds between their IC_{50} measured using ^{125}I-NGF, and high affinity KD measured using SPR two site analysis. Diamond: previously reported small molecule inhibitors. Circles: Small synthetic analogue series. ($F(1,16) = 8.381$, $p = 0.0111$, $R^2 = 0.3585$).

Previous methods for screening small molecule inhibitors used radioisotopes and have the limitation of altering the molecular function of NGF in an assay. By using biosensor technology sensitive enough to analyse the biomolecular interactions between small molecules (~300 Dal) and NGF (~26 kDal) we can identify further characteristics about these small molecules inhibitors including identifying whether the molecules are NGF-binding inhibitors, or bind elsewhere in the complex.

This paper describes the ability to characterize novel small molecule inhibitors by determining their ability to bind and saturate NGF. By being able to identify the stoichiometry of this relationship, the ability for these small molecules to saturate NGF can be measured. A 2:1 stoichiometry (two small molecules : one NGF dimer) is hypothesized, which confirms previous

work with molecular modeling techniques (Eibl et al. 2013a; Eibl et al. 2010; Colquhoun et al. 2004). Small molecules can bind to proteins at a relatively low affinity to non-specific sites as well as to a high affinity at specific sites, which can be measured using a two-site affinity model. This model groups together the low affinity as one weak binding site that provides a much more accurate K_D value for the high affinity site, which is usually the site of interest. By using the two-site affinity model to describe the binding of these small molecules to NGF, we provide evidence that when binding occurs to one of the available sites on a NGF monomer, a conformational change alters binding affinity for the secondary binding site on the adjacent monomer. However it is also a possibility that immobilization of NGF to the sensor chip for screening occupies one of the potential binding sites, altering the measured affinity.

Surface plasmon resonance affinity assays are demonstrated to be useful in the characterization of compounds which bind and inhibit NGF. Without the use of radiolabels, we have provided further evidence of a theoretical 2:1 stoichiometry and a model by which these small molecules are able to saturate the available NGF in assay. Identifying small molecules with the ability to highly saturate the available NGF, in addition to having a high binding affinity would be ideal for the use of a therapeutic. These properties allow for smaller dosing yet still yield therapeutic effects in patients displaying symptoms associated with a dysregulation in NGF.

Chapter 5: Nerve growth factor inhibitor with novel binding domain demonstrates nanomolar efficacy in both cell-based and cell-free assay systems

(Original Research)

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Abstract

Nerve growth factor (NGF), a member of the neurotrophin family, is known to regulate the development and survival of a select population of neurons through the binding and activation of the TrkA receptor. Elevated levels of NGF have been associated with painful pathologies such as diabetic neuropathy and fibromyalgia. However, completely inhibiting the NGF signal could hold significant side effects, such as those observed in a genetic condition called congenital insensitivity to pain and anhidrosis (CIPA). Previous methods of screening for NGF-inhibitors used labeling techniques which have the potential to alter molecular interactions. The present study utilizes molecular modeling flexible docking to identify a novel binding domain in the loop II/IV cleft of NGF. SPR spectroscopy and NGF-dependent cellular assays were utilized to identify a novel NGF-inhibitor, BVNP-0197 (IC_{50} = 90 nM), the first NGF-inhibitor described with a high nanomolar NGF inhibition efficiency.

Introduction

Nerve growth factor (NGF) is a member of the neurotrophin family of proteins, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). These proteins are necessary for the developmental growth and maintenance of the central and peripheral nervous systems (Levi-Montalcini 1987). Not only is NGF critical for the developmental differentiation of sympathetic and sensory neurons (Chao et al. 2006), but during adult life, NGF is also an important mediator in pain signalling (Capsoni et al. 2011). The actions of NGF are largely attributed to the activation of a receptor tyrosine kinase TrkA that is expressed on central and peripheral neurons (Huang and Reichardt 2001). When the NGF-TrkA complex is activated, a number of signalling pathways are internalized altering gene expression (Max and Stewart 2008). Genetic studies in patients with congenital insensitivity to pain with anhidrosis (CIPA) have shown mutations in TrkA which impair normal development of sensory neurons (Indo 2001). In addition, transgenic mice lacking NGF (Crowley et al. 1994) or TrkA (Smeyne et al. 1994) have sensory and sympathetic deficiencies and do not survive long after birth. NGF also binds with lower affinity to a common neurotrophin receptor $p75^{NTR}$. Activation of $p75^{NTR}$ by NGF mediates cell growth, apoptosis and modulated neuronal excitability by augmenting the sensitivity of TrkA (Khan and Smith 2015).

Elevated levels of NGF have been implicated in several chronic pain syndromes such as osteoarthritis (Kc et al. 2016), diabetic neuropathy (Malerba et al. 2015), and interstitial cystitis/pelvic pain syndrome (Watanabe et al. 2011). Experimental evidence has shown that

NGF is released by several cell types including mast cells (Skaper et al. 2001; Bienenstock et al. 1987), lymphocytes (Torcia et al. 1996), monocytes/macrophages (Bracci-Laudiero et al. 2005), and Schwann cells (Créange et al. 1997) in response to tissue inflammation. Interestingly, NGF has also been found to produce hyperalgesia when administered in several animal species (Cahill et al. 2003; Hao et al. 2000; Brodie 1995). These pain-related behavioural responses to NGF in animals manifest within minutes, and can last anywhere from several hours to days depending on the dose (Zahn et al. 2004; Lewin et al. 1994). Subcutaneous injection of NGF into the forearm of healthy human adults induced localized allodynia and hypersensitivity within minutes, lasting for several hours (Dyck et al. 1997). In addition, small intravenous NGF doses in healthy human adults are responsible for widespread deep pain and tenderness which persists for several days (Svensson et al. 2003). The evidence of upregulated NGF in painful pathological conditions, in addition to the evidence that NGF causes pain in humans and in animals, have led to the rational for developing therapeutics based on the inhibition of NGF activity.

A growing body of evidence suggests that an anti-hyperalgesia effect can be observed with pharmacological interference of NGF-TrkA interactions in several neuropathic pain models (Beglova et al. 2000; Hefti et al. 2006; Gwak et al. 2003; Wild et al. 2007). Monoclonal anti-NGF antibodies, such as Tanezumab, have been used as NGF sequestering therapy. Tanezumab binds to NGF with high affinity and selectivity thus blocking NGF-TrkA interactions and inhibiting the signalling of sensory neurons for the perception of pain (Schnitzer et al. 2011).

During a Phase II trial, individuals with knee osteoarthritis where administered two Tanezumab injections eight weeks apart reducing knee pain by a mean of 45-62% from a baseline, compared to a 22% reduction with placebo (Lane et al. 2010). Although seemingly effective, Tanezumab was placed on clinical hold during Phase III trials when several individuals developed joint damage, which progressed to a stage where joint replacement was necessary. Despite the apparent successes in the antibody therapeutics, there are still potential drawbacks such as delivery challenges, potential for autoimmune responses, capacity for production and financial considerations (Samaranayake et al. 2009). Therefore, the generation of small molecule antagonists which have the ability to selectively disrupt NGF-TrkA interactions may have significant therapeutic advantage.

A series of novel nonpeptidic small molecules have been demonstrated to inhibit binding of NGF to TrkA. Compounds such as ALE-0540 (Owolabi et al. 1999), PD 90780 (Colquhoun et al. 2004), Ro 08-2750 (Niederhauser et al. 2000) and PQC-083 (Eibl et al. 2013a) have been shown to inhibit NGF-TrkA signal transduction pathways *in vitro*. However, the mechanisms by which these small molecules exert their inhibitory effect remains speculative (Eibl et al. 2012). Historically, the identification of small molecule NGF-inhibitors resulted from high-throughput receptor binding assays. However recent advances in our understanding of the structural biology of NGF-TrkA interactions have allowed for rational development of novel small molecules (Eibl et al. 2013a). With newly identified crystal structures explaining the

interactions during NGF-TrkA binding (Wehrman et al. 2007), small molecules have been developed to alter the molecular topology of NGF to inhibit TrkA binding.

Determining how potential therapeutic drugs modulate analyte-ligand interactions and bind to target molecules will help determine strategies for developing future therapeutics. One such technique for investigating the strength and rate of biomolecular interactions is surface plasmon resonance (SPR) spectroscopy (Cooper 2002). SPR is advantageous over other techniques because it monitors biomolecular interactions in real time and is label-free, eliminating the need for fluorescent reporter molecules or radioisotope tags (Mir and Shinohara 2013). Not only is this advantageous in saving time during labeling and reducing resources, but more importantly it eliminates tags which can alter the molecular interactions (Fraser et al. 2014). Real time measurements allow for calculation of kinetic rates during the association and dissociation phases, in addition to the equilibrium dissociation constants.

SPR has been used to study molecular interactions between a wide variety of molecules; from simple proteins, carbohydrates, lipids, nucleic acids and small molecule compounds, to larger protein complexes and even cells (Wittenberg et al. 2014). Interactions have also been studied across a wide range of affinities from millimolar to picomolar interactions (Cooper 2002). More recently, SPR techniques have be utilized to characterize interactions between novel small molecule inhibitors to NGF and proNGF (Sheffield et al. 2016a) and their interactions with the receptor $p75^{NTR}$ (Sheffield et al. 2016b).

In the present study, we use a combination of molecular modeling and SPR to identify a series of novel small molecule analogues with specificity for NGF that inhibit binding to TrkA. Our theoretical flexible docking experiments revealed a novel binding domain in the loop II/IV cleft of NGF where a series of analogues bind to inhibit NGF signalling. SPR spectroscopy was also utilized to characterize novel small molecule interactions with NGF and their inhibitory effect on TrkA interactions. Analogues also demonstrated efficient inhibitory activity of NGF *in vitro* using a NGF-dependent PC12 assay. Guided by receptor docking, we were able to identify BVNP-0197 as a small molecule capable of inhibiting NGF *in vitro* with greater potency than previously reported NGF inhibitors.

Materials and Methods

Molecular modeling

Molecular modeling and in silico docking of bivalent naphthalimide compounds to NGF (RCSB Protein Data Bank ID 1BET) (McDonald et al. 1991) were completed using Sybyl-X 2.1.1 software. The structure of NGF was prepared for docking using the Biopolymer suite of Sybyl-X 2.1.1. Co-structures were deleted, hydrogens were added and the appropriate formal charges on the C- and N- termini were added. The structure was optimised using the MMFF94 molecular mechanical force field. Flexible docking of each compound was performed using the Surflex-Doc suite incorporated into Sybyl-X 2.1.1 (Jain 1996). The docking protomol was generated to include the residues of the loop II/IV cleft (Glu41-Phe49 and Gln96-Trp99) with a bloat factor of 0 and a threshold value of 0.5. For the Surflex-Doc function, the angstroms to

expand the search grid was set at 6 and the maximum confirmations per fragment was set to 20.

SPR spectroscopy

Mouse NGF (Burlington; Canada) was coupled using the amine coupling method. Sensor Chip CM5 was activated using 0.4 M EDC/0.1 M NHS at a flow rate of 10 µL/min for 7 minutes. NGF was dissolved in a HBS-EP buffer, pH 4.5, to yield a 10 µg/mL solution. Following activation, the NGF solution was injected over the activated sensor chip surface at a flow rate of 10 µL/min for 9 minutes. After the injection, the surface was washed with 1M NaCl to remove any uncoupled or non-covalently bound material from the surface. The excess hydroxysuccinimidyl groups on the surface were deactivated with 1M ethanolamine hydrochloride, pH 8.5, at a flow rate of 10 µL/min for 7 minutes. The surface of a reference flow cell was activated with 0.4 M EDC/0.1 M NHS and then deactivated with 1 M ethanolamine, with respective flow rates and times.

Bivalent naphthalimide chemical synthesis

All chemicals and solvents were obtained from commercial sources without further purification. Mass spectra were recorded using Waters (Micromass ZQ) equipped with a Thermo Betasil C18 (150 x 2.1mm, 3µ). ^{1}H NMR spectra were recorded on a Varian AS500 instrument. Chromatography on silica gel columns were performed using Merck silica gel 60

(230-400 mesh), and analytical thin layer chromatography (TLC) was conducted on glass plate coated with a 0.25cm thin layer of silica gel $60F_{254}$.

General procedure for naphthalimide derivatives

Under a nitrogen atmosphere, the primary amine (1 equiv) and sodiym acetate (1 equiv) were added to a stirred solution of 1,4,5,8-naphthalene tetracarboxylic dianhydride (1 equiv) in glacial acetic acid was added. The reaction mixture was refluxed and the progress of the reaction was monitored by TLC. Upon completion of the reaction, if the resulting solution was clear, the solvent was evaporated under reduced pressure and the residual solid was reprecipitated and/or recrystallized from appropriate solvent(s). If the reaction mixture contained precipitate then the mixture was cooled to room temperature and the solid was removed by filtration. The filtrate was concentrated and then diluted with water to precipitate the crude intermediate product. The crude product was dissolved in ethanol and heated to reflux for 10 minutes, cooled to room temperature, and filtered to remove the solid. The filtrate was concentrated under reduced pressure to get intermediate product A/B, which was dried in vacuum for 24 hrs and used for next steps (Figure 5.1).

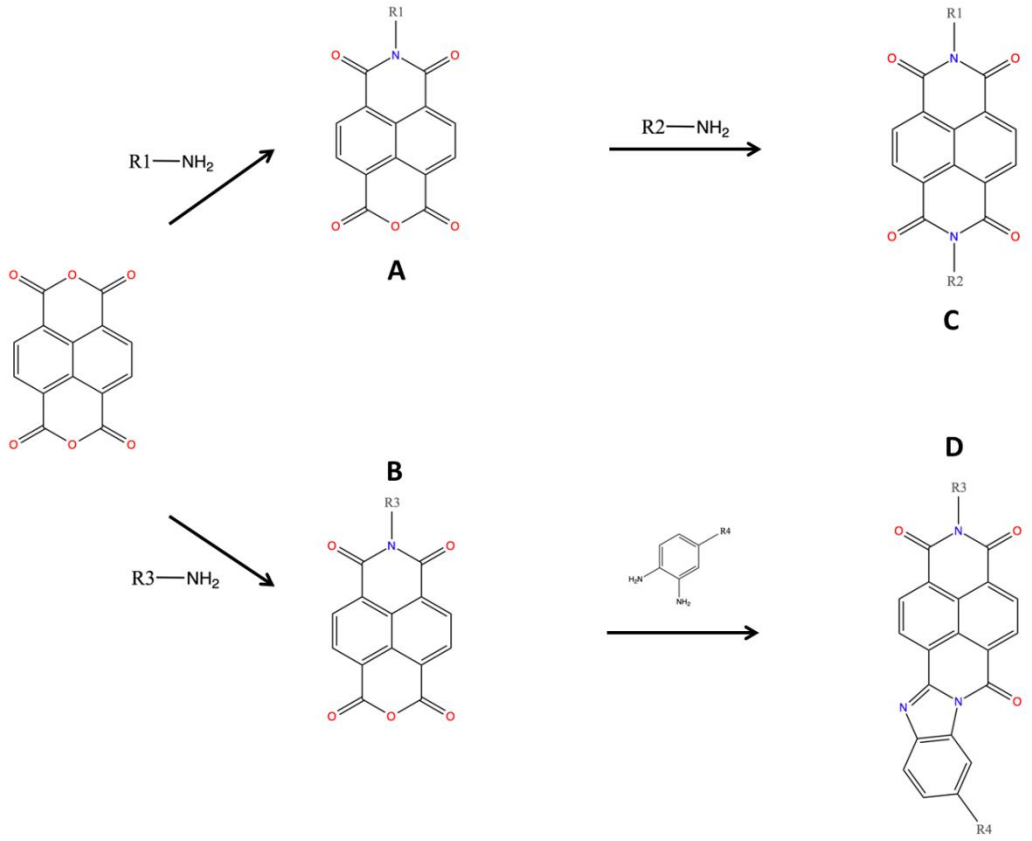


Figure 5.1: Synthesis of bivalent naphthalimide analogue series. Final products (C/D) were screened for NGF inhibitory properties.

Under a nitrogen atmosphere, the appropriate amine (1 equiv) and sodium acetate (1 equiv) or substituted diaminobenzene (1 equiv) was added to a stirred solution of intermediate compound A (1 equiv) in glacial acetic acid was added. The reaction was refluxed for 10 hrs and then cooled to room temperature. The solid was collected by filtration, which was further washed with distilled water or dilute acid. It can be reprecipitaed and/or recrystallized from the appropriate solvents. The solid final product C/D was dried under a vacuum (Figure 5.1).

Compound sample preparation for SPR analysis

Each compound of interest were weighed on an analytical balance and dissolved in the appropriate amount of DMSO to give a 10 mM solution. The samples were diluted with running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.05% v/v Tween 20; pH 7.5) to yield solutions for the binding experiments with concentrations varying from 0.7 µM to 200 µM and for IC_{50} binding experiments with concentrations varying from 3.16 nM to 316 µM.

Analogue binding affinity to immobilized NGF

Binding affinities of the bivalent naphthalimide analogues to immobilized NGF were determined as previously described using a Biacore T200 SPR (Kennedy et al. 2016). Prior to analyte injection, the series S CM5 chip was conditioned with three 30 second cycles of running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.05% v/v Tween 20; pH 7.5) followed by three start up cycles, allowing the response to stabilize before analyte injection. Data was collected at a temperature of 25 °C. Individual compound samples were tested from lowest to highest concentrations, separated by a 15 second stabilization period after each sample in each compound series. During each sample cycle, analyte was injected for 60 second at a flow rate of 30 µL/min. A dissociation period was monitored for 30 seconds after analyte injection before regeneration was induced with 1.0 M NaCl for 120 seconds at a flow rate of 30 µL/min to wash any remaining analyte from the sensor chip before running the next sample.

PC12 cell culture

PC12 cells were obtained from the American Type Culture Collection (ATCC; USA). Cell passage 18-21 were used for all experiments in the manuscript. PC12 cells were maintained in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum (FBS) (Gibco; USA). Media was supplemented with working stock concentration of penicillin (0.1 U/mL) and streptomycin (0.1 U/mL). PC12 cells were seeded at 50% confluence and were passaged at 90% confluence in T25 cm^2 tissue culture treated flasks (Corning; USA). PC12 cells were incubated at 37˚C in 5% CO_2.

PC12 toxicity assay

PC12 toxicity assay was performed by plating cells at a density of 200 cells/well on Terasaki plates (Greiner Bio-One; USA) at 37˚C and 5% CO_2. Cells were exposed to 100 µM of compound of interest in the Terasaki well for 72 hours in the absence of NGF. Cells on the lower horizontal surface of the well were imaged using an EVOS XL Core cell imaging system at 20 x phase observation. The total cells per well were counted and compared to control. Three runs of each experiment were performed, and each condition was tested in quadruplicate.

PC12 NGF-dependent neurite outgrowth assay

For purposes of the neurite outgrowth assay, PC12 cells were plated 24 hours prior to treatment at a density of 200 cells/well and were grown on Terasaki plates (Greiner Bio-One;

USA) at 37˚C and 5% CO_2. PC12 cells were exposed to 500 pM of NGF (Life Technologies; Canada) pre-incubated with varying concentrations of each compound of interest (31.6 nM – 100 µM). Terasaki wells were imaged 72 hours after exposure to NGF. Cells on the lower horizontal surface of the well were imaged using an EVOS XL Core cell imaging system at 20 x phase observation. Differentiation events were manually scored when a cell developed a process with a constant calibre from the origin to the terminal and its length was equal or greater than 1 x cell body diameter. Wells with fewer than 50 cells or greater than 350 cells were excluded from analysis. Three trials of each experiment were performed, and each condition was tested in quadruplicate.

IC_{50} determination using SPR spectroscopy

TrkA was immobilized to a CM5 sensor surface as previously described (Forsell et al. 2013). Prior to analyte injection, the Series S CM5 chip was conditioned with three 30 second cycles of running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.05% v/v Tween 20; pH 7.5) followed by three start up cycles, allowing the response to stabilize before analyte injection. Data was collected at a temperature of 25 °C. Individual compound samples were tested from lowest to highest concentrations, separated by a 15 second stabilization period after each sample in each compound series. During each sample cycle, analyte was injected for 60 seconds at a flow rate of 30 µL/min. A dissociation period was monitored for 120 seconds after analyte injection. Regeneration occurred with a 10 mM Glycine solution at pH 2.0 for 15

seconds at a flow rate of 30 µL/min to wash any remaining analyte from the sensor chip before running the next sample.

Statistical analysis

Statistical analysis was performed using Prism Graphpad 5.0 (San Diego; USA). Error bars are expressed as standard error of the mean with a sample size of n = 3. Inhibition curves were fit using a non-linear regression model.

Results

Molecular modeling flexible docking

Previous studies have suggested that loop I, II and IV of each NGF monomer are responsible for binding to TrkA (Barker 2007; Wehrman et al. 2007), whereas loop I and IV are necessary for NGF binding to $p75^{NTR}$ (Barker 2007; He and Garcia 2004) (Figure 5.2). Previous drug discovery efforts have been focused on a putative binding domain for small molecule NGF inhibitors in the loop I/IV cleft of the NGF monomer (Colquhoun et al. 2004; Eibl et al. 2013a). Our flexible docking identified a novel binding domain located in the loop II/IV cleft of NGF for a series of bivalent naphthalimide compounds which were derived using similar molecular features to previously reported small molecule NGF inhibitors. This novel binding domain demonstrated higher binding capabilities than previously suggested docking areas. The pharmacological targeting domain included NGF monomer residues Glu41, Val42, Asn43, Ile44, Asn45, Asn46,

Ser47, Val48, Phe49, Gln96, Ala97, Ala98, and Trp99. Figure 5.3 demonstrates the protomol which was derived from the described residues of NGF.

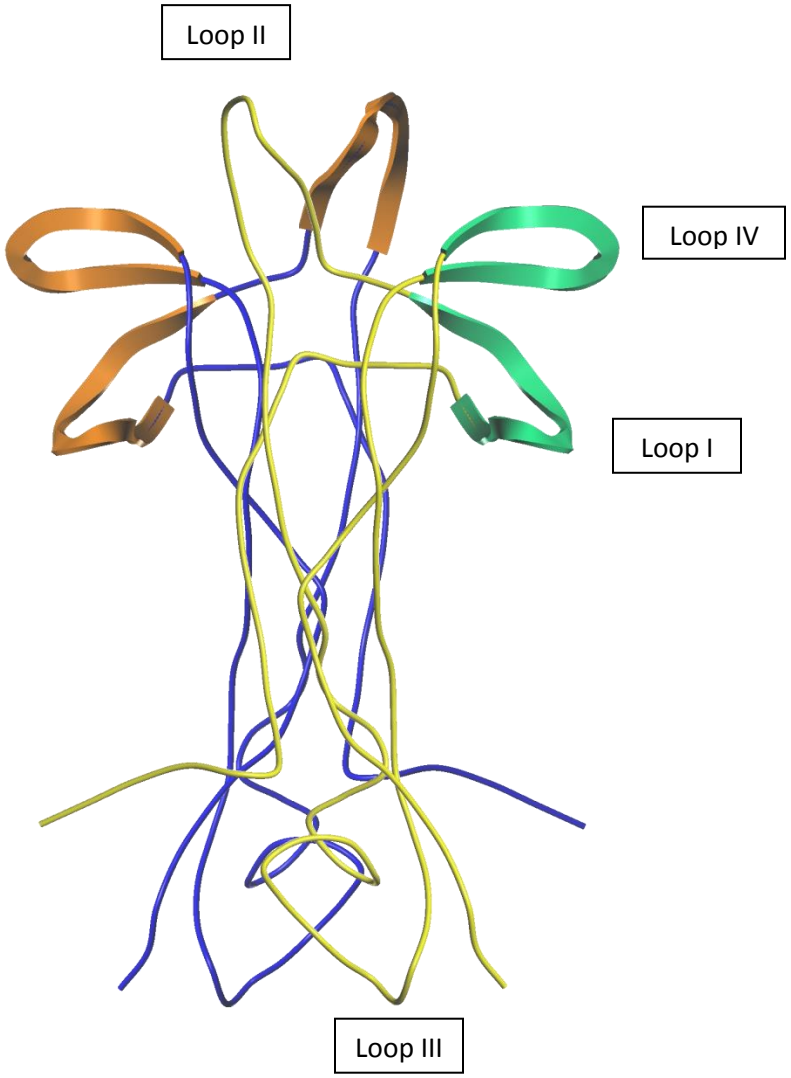


Figure 5.2: The dimeric structure of NGF (RCSB: 1BET) (McDonald et al. 1991) is presented in ribbon format. NGF monomers are coloured blue and yellow. Structural features of the yellow NGF monomer are labeled. Orange highlights represent structural features of the blue NGF monomer which are responsible for TrkA binding. Green structural features highlighted represent areas responsible for $p75^{NTR}$ binding of the yellow NGF monomer.

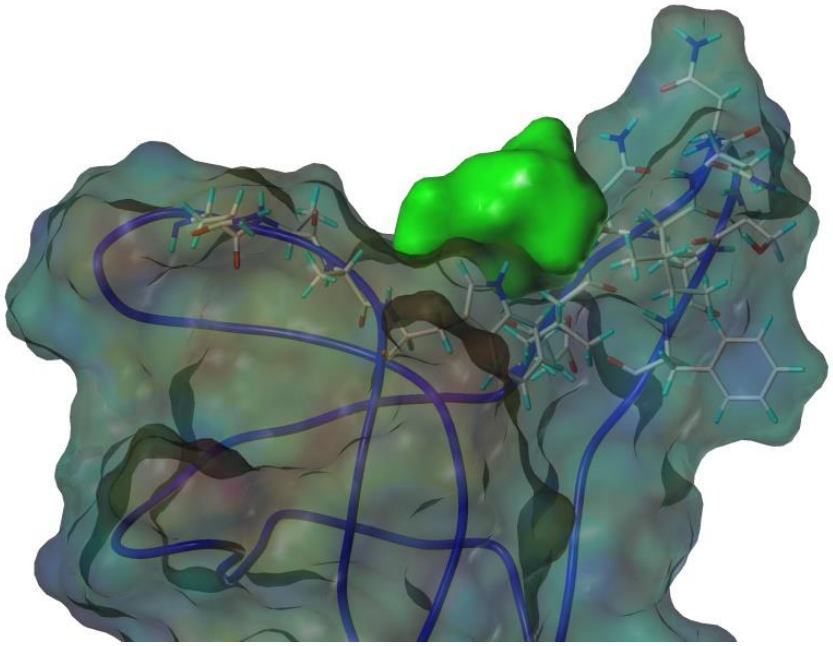


Figure 5.3: Theoretical docking experiments identified a docking site for bivalent naphthalimide scaffold at loop II/IV domain of NGF. NGF is represented as a ribbon structure (RCSB: 1BET) (McDonald et al. 1991). Resides Glu41, Val42, Asn43, Ile44, Asn45, Asn46, Ser47, Val48, Phe49, Gln96, Ala97, Ala98, and Trp99 were selected to create a protomol binding domain for bivalent naphthalimide analogues represented in green.

The results of compound BVNP-0187 were chosen as a representative bivalent naphthalimide scaffold analogue following flexible docking at the loop II/IV cleft of NGF (Figure 5.4). BVNP-0187, like previously reported small molecule NGF inhibitors, has a ridged backbone structure comprised of a bivalent naphthalimide motif containing 2-hydroxy-5-benzoic acid residue occupying –R1 and butanoic acid residue in place of –R2 (Figure 5.5). Flexible docking determined that BVNP-0187 had the highest theoretical docking score of all analogues screened, with a total score of 5.6086. Flexible docking suggested that four hydrogen bonds are formed between BVNP-0187 and NGF residues Asn43, Asn45 and Trp99 to stabilize within the loop II/IV cleft and measured differences of electrostatic potential were found at the BVNP-0187 docking domain (Figure 5.4).

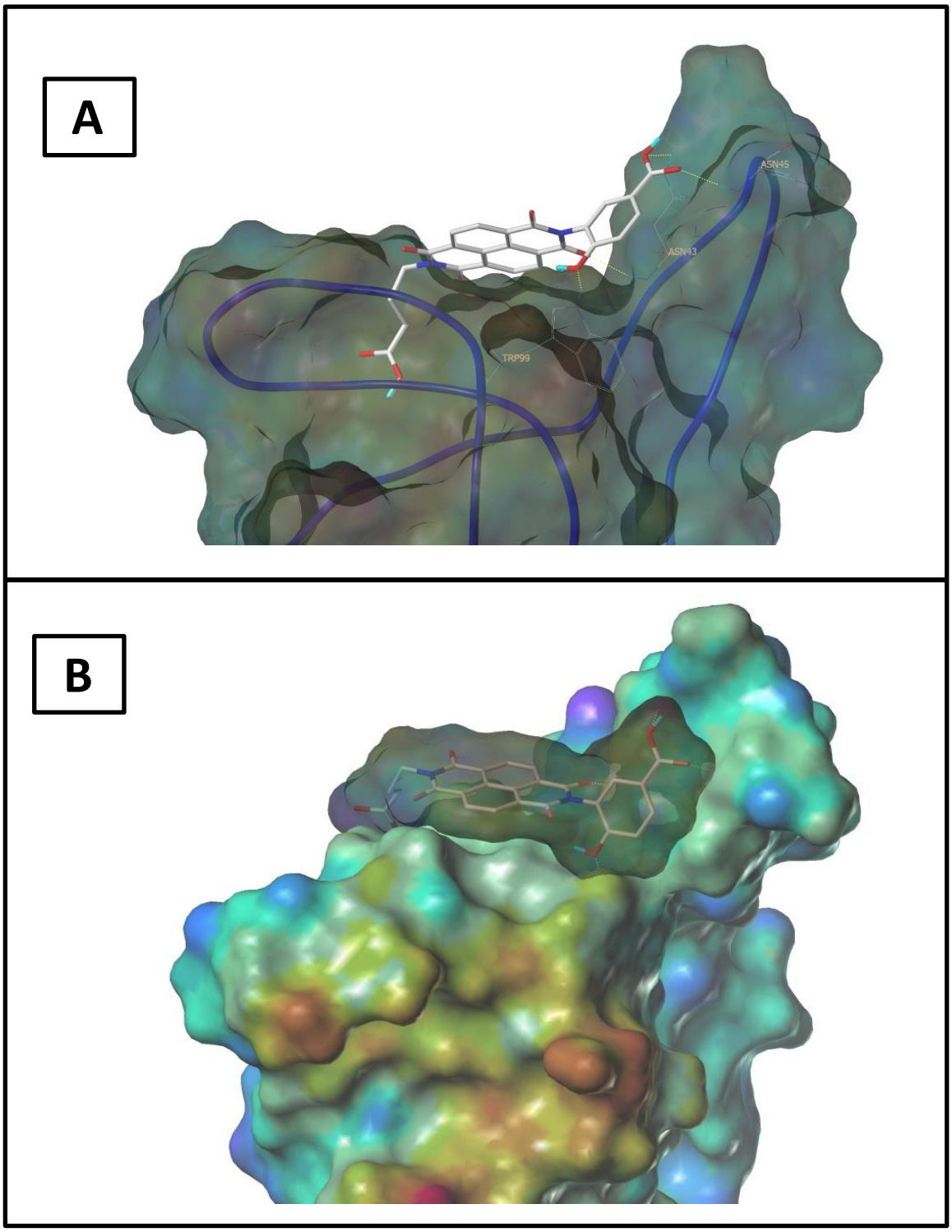
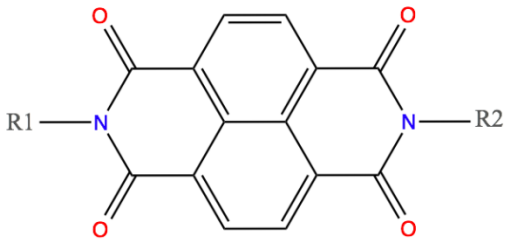


Figure 5.4: Schematic representation of BVNP-0187 docked to NGF as determined by molecular modeling. Flexible theoretical docking experiments predict that the bivalent naphthalimide scaffold is suited to bind at the loop II/IV cleft of NGF. A) Hydrogen bonds are represented at residues Asn43, Asn45 and Trp99 of NGF to stabilize BVNP-0187 within the loop II/IV cleft. B) Differences in electrostatic potential at the docking domain. Blue areas represent electron-poor regions where as a gradient to red represents electron-rich regions.



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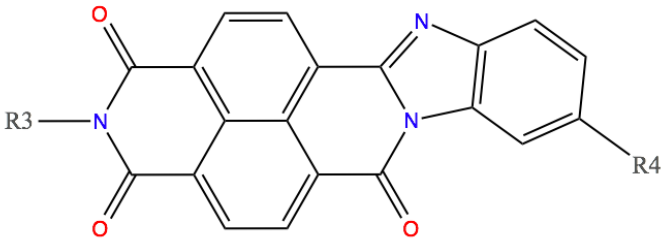
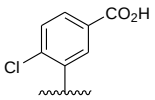
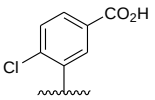
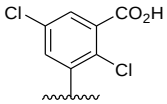
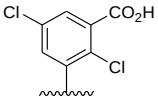
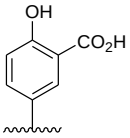
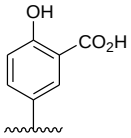
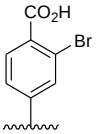
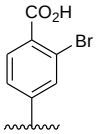
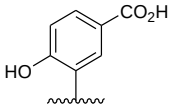
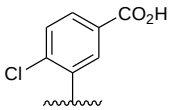
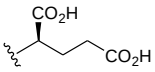
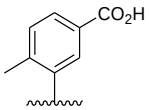
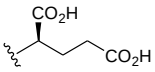
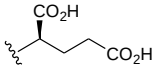
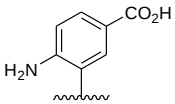
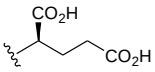
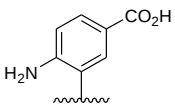
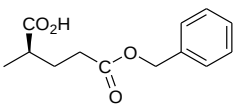


D

Compound	-R1	-R2	-R3	-R4
BVNP-0182	$(CH_2)_3CO_2H$	$(CH_2)_3CO_2H$		
BVNP-0183				
BVNP-0184				
BVNP-0185				
BVNP-0186				
BVNP-0187		$(CH_2)_3CO_2H$		
BVNP-0188				
BVNP-0189				
BVNP-0190				CO_2H
BVNP-0191				
BVNP-0192				

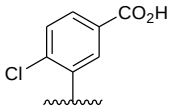
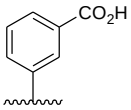
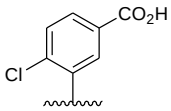
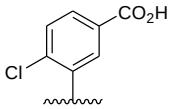
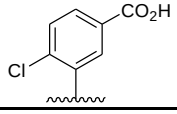
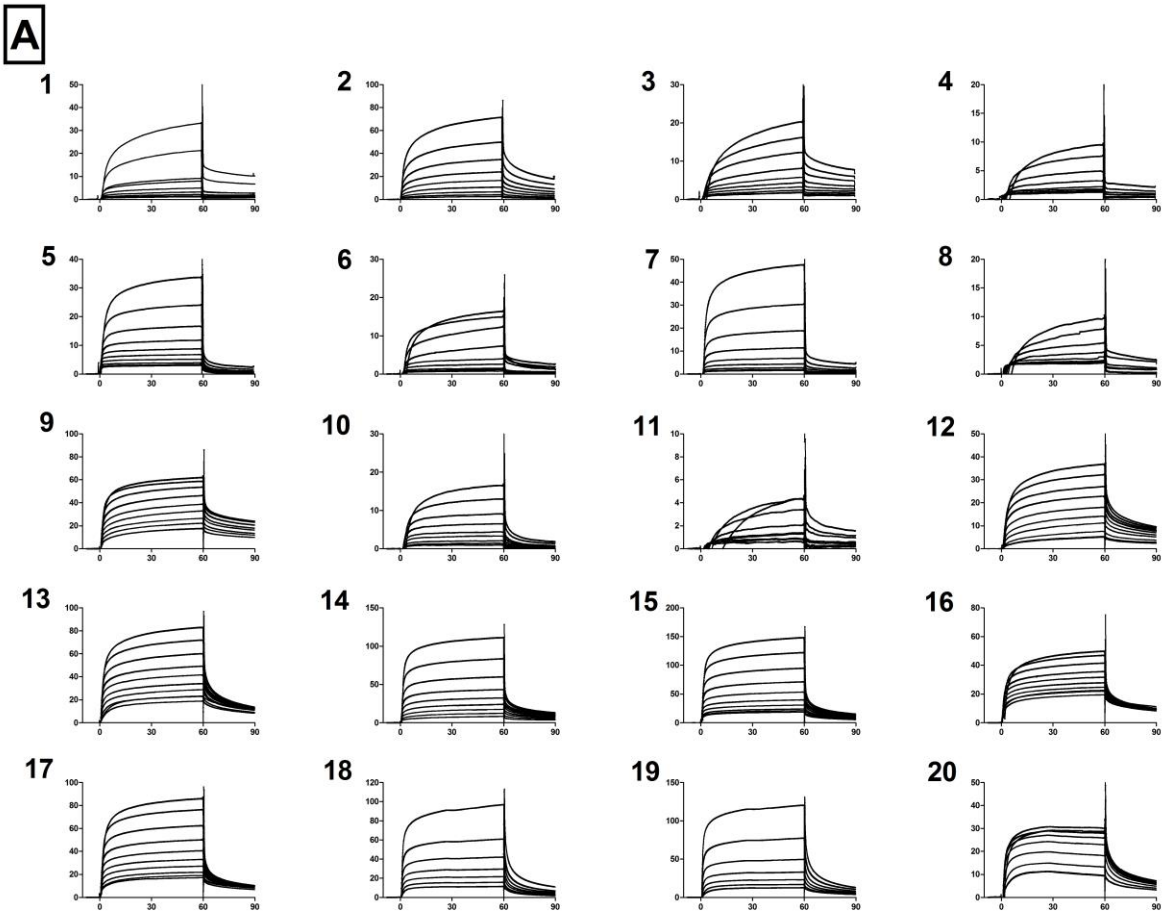
BVNP-0193		CO_2H
BVNP-0194	$(CH_2)_3CO_2H$	CO_2H
BVNP-0195		CO_2H
BVNP-0196		Cl
BVNP-0197		NO_2
BVNP-0198		CO_2Me

Figure 5.5: Residue description of bivalent naphthalimide analogues which were identified as specific binders of NGF using SPR analysis, and were shown to exhibit inhibitory properties in NGF-TrkA mediated signalling. Analogue series were synthesized around a bivalent naphthalimide scaffold with varied side chains substitutions at –R1, and –R2 or –R3 and –R4.

Affinity of bivalent naphthalimide compounds to immobilized NGF

The affinity of bivalent naphthalimide analogues binding to NGF were determined using a two-site steady-state analysis. A concentration series ranging from 0.70 µM to 200 µM was injected over immobilized NGF for a 60 second contact time followed by a 30 second dissociation phase. Concentration values were determined based on results from preliminary binding experiments in which bivalent naphthalimide compounds reached near-saturation levels of the immobilized NGF on the sensor surface. The response obtained from each bivalent naphthalimide sample was plotted against concentration using the BIA evaluation software (Version 2.0) and was evaluated using a two-site binding model as previously described (Kennedy et al. 2016). 17

bivalent naphthalimide analogues were identified as specific binders of NGF (Figure 5.6) with binding affinities ranging from 2.63 µM to 118.10 µM. The 17 analogues showed no significant binding to immobilized TrkA on a sensory surface and are therefore described specific for NGF. Structures of these analogues are summarized in Figure 5.5. Three previously reported compounds, ALE-0540, PD 90780 and Ro 08-2750 were also screened to determine the binding affinites (K_D) for NGF. The K_D of ALE-0540, PD 90780 and Ro 08-2750 were determined to be 49.71 µM, 35.83 µM and 32.39 µM respectively, and none showed binding to immobilized TrkA. The total binding score measured during flexible docking was related to the binding affinity measured with SPR binding (Figure 5.7).



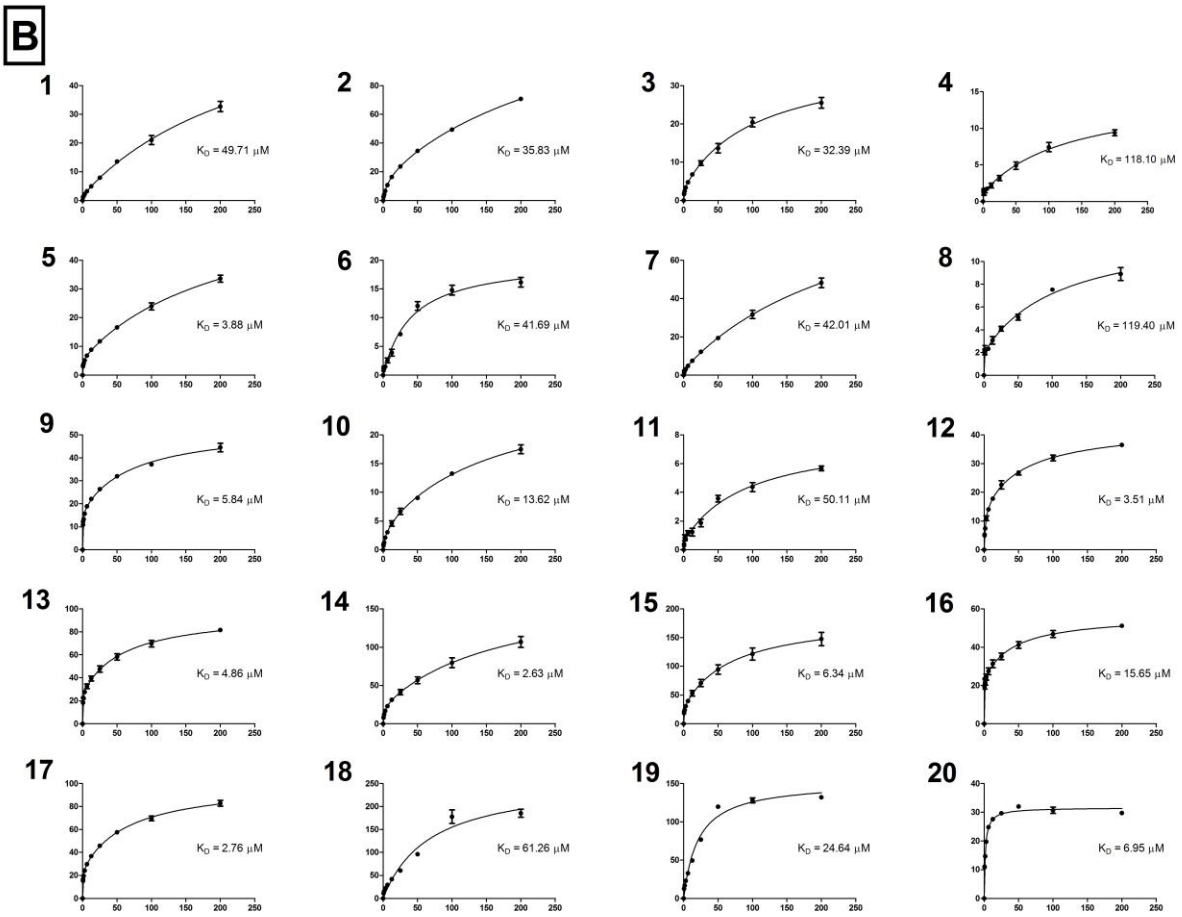


Figure 5.6: SPR subtracted sensograms and specific binding curves for the 17 bivalent naphthalimide analogue found to be specific binders of NGF, as well as three previously reported compounds – ALE-0540, PD 90780 and Ro 08-2750 (1-3 respectively). 4-20 represent BVNP-0182-BVNP-0198 respectively. Data points represent the mean of triplicate measure. Error bars represent the standard error of the mean with an n=3. A) Blank subtracted sensograms which describe the association and dissociation of each compound. In each sensogram, the x-axis represents time. 0-60 seconds represents the association of the compound to NGF; at 60 seconds the analyte flow stops and a dissociation period is represented from 60-90 seconds prior to chip regeneration at 90 seconds. Y axis represents the response of binding in response units (RU). 1 RU = 1 pg/mm^2. The lines represent small molecule concentrations, from top to bottom: 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.13 μM, 1.56 μM and 0.78 μM respectively. B) Two-site steady-state binding models describing the specific binding of each compound to NGF. X axis represents concentration (μM) and Y axis represents subtracted response (response from active flow cell minus response from reference flow cell) (RU). Each data point represents the subtracted response from the saturated association phase (last 10 seconds) of the subtracted sensograms from each compound concentration (refer to A). Binding affinity (K_D) for each compound was calculated using the BIA evaluation software using a 2:1 binding model.

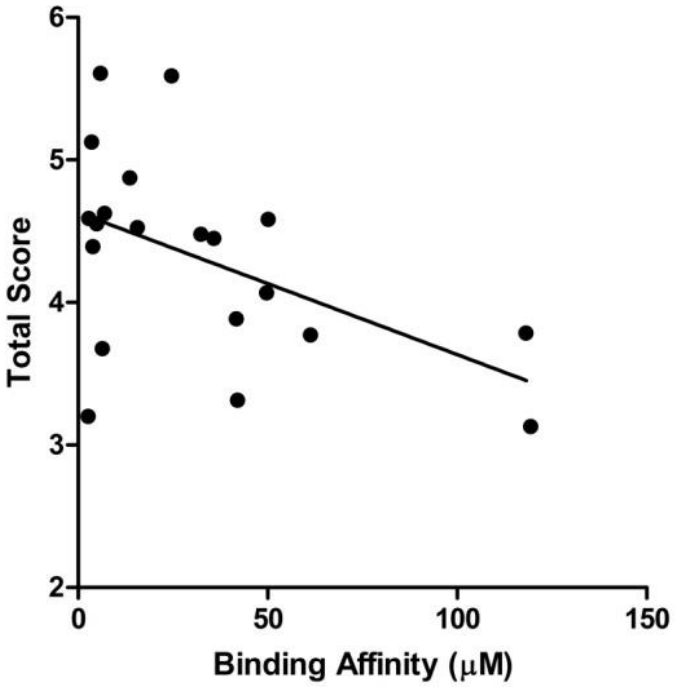


Figure 5.7: The relationship between total score calculated during molecular modeling flexible docking and the K_D measured by SPR spectroscopy for the 17 bivalent naphthalimide analogues and three previously reported compounds (ALE-0540, PD 90780 and Ro 08-2750). X-axis: K_D (μM) measured using SPR – Data points represent the mean of triplicate trials. A concentration range (0.70 μM – 200 μM) of each analogue was injected over immobilized NGF. Y-axis: total score measured during flexible docking. ($F(1,18) = 5.807$; $p=0.0269$; $R^2=0.2439$).

Inhibitory effects of bivalent naphthalimide analogues on NGF-dependent PC12 cell differentiation

In order to assess the toxicity of the bivalent naphthalimide analogues *in vitro*, PC12 cells were exposed to 100 μM of each compound for 72 hours in the absence of NGF. The cell count did not significantly alter from the control conditions (Figure 5.8) and the morphology of PC12 cells was also unaltered following the 72 hour exposure.

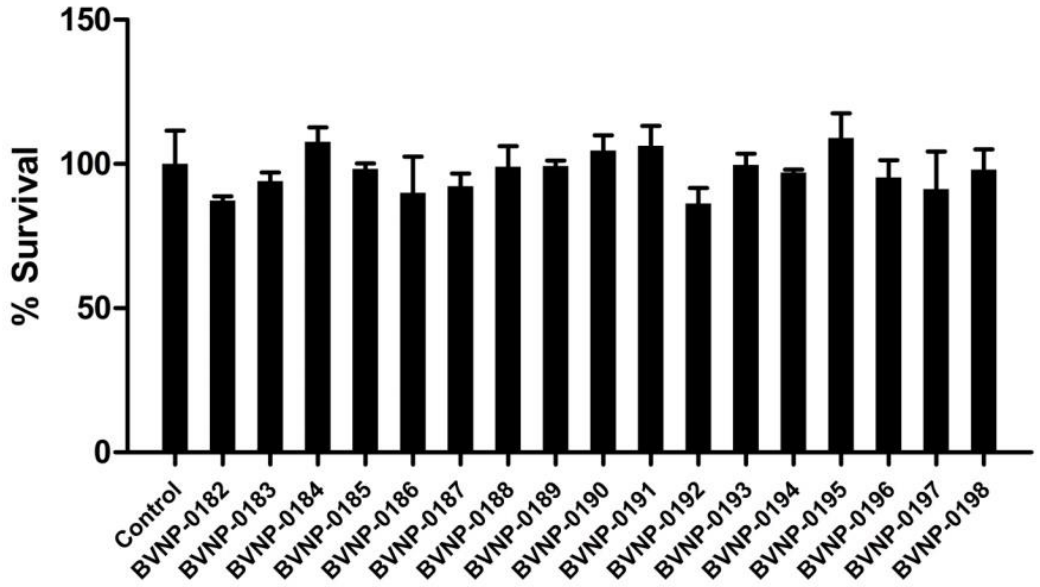


Figure 5.8: *In vitro* toxicity of bivalent naphthalimide analogue series. PC12 cells were incubated in the absence (control) or presence of 100 µM of each analogue for 72 hours. The average control cell count per well was considered 100% viability. Each analogue treated sample is represented by the average percentage of the control cell count. Standard error of the mean count is shown with error bars (n = 3). Treatment of PC12 cells with the analogues shown no significant difference in cell count ($p>0.05$) implicating no toxic effect of the analogues *in vitro*.

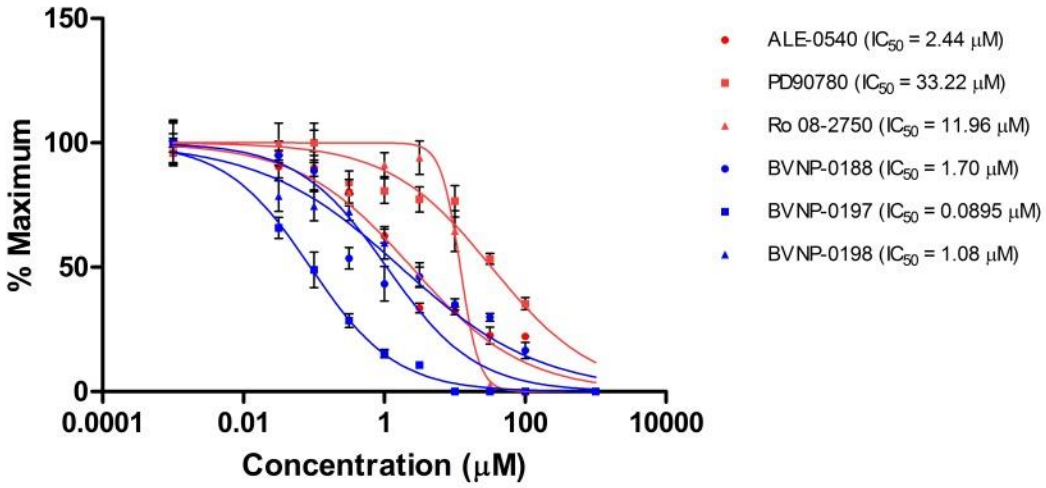
The inhibitory effects of the 17 bivalent naphthalimide analogues which were found to be specific NGF binders were tested in the NGF-dependent PC12 cell differentiation assay along with ALE-0540, PD 90780 and Ro 08-2750 (Table 5.1). The three previously reported compounds demonstrated IC_{50} values of 2.44 µM, 33.22 µM, and 11.96 µM respectively (Table 5.1). Of the 17 bivalent naphthalimide analogues, three compounds demonstrated inhibitory effects more efficient than previously reported NGF-inhibitors, which we defined as an $IC_{50} < 2$ µM. BVNP-0188 and BVNP-0198 demonstrated IC_{50} values of 1.70 µM and 1.08 µM, respectively. The most efficient analogue in NGF-dependent PC12 cell differentiation inhibition was BVNP-0197 which demonstrated an IC_{50} value of 89.5 nM (Figure 5.9). Flexible docking

molecular modeling experiments predicted four hydrogen bonds at NGF residues Asn43, Thr92 and Trp99 to stabilize BVNP-0197 within the loop II/IV cleft, as well as measured electrostatic potential differences at the docking domain (Figure 5.10). A total binding score of 5.5898 was measured for BVNP-0197 docking to the loop II/IV cleft.

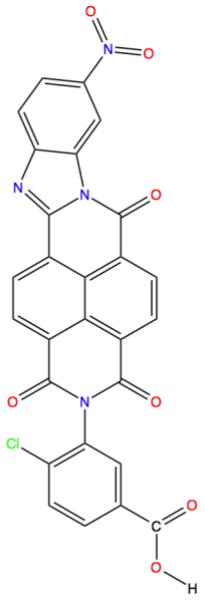
Table 5.1: A PC12 cell based neurite outgrowth assay was used to score the relative inhibitory effects of each bivalent naphthalimide analogue and three previously reported compounds (ALE-0540, PD 90780 and Ro 08-2750) in the presence of 500 pM NGF for 72 hours. PC12 cells were treated with a concentration range from 100 µM – 31.6 nM. Each condition was completed in quadruplicate, with an n = 3.

Compound	IC_{50} PC12 neurite outgrowth
ALE-0540	2.44 µM
PD 90780	33.22 µM
Ro 08-2750	11.96 µM
BVNP-0182	>100 µM
BVNP-0183	16.86 µM
BVNP-0184	>100 µM
BVNP-0185	11.08 µM
BVNP-0186	15.18 µM
BVNP-0187	5.45 µM
BVNP-0188	**1.70 µM**
BVNP-0189	>100 µM
BVNP-0190	4.27 µM
BVNP-0191	4.33 µM
BVNP-0192	2.46 µM
BVNP-0193	7.09 µM
BVNP-0194	5.35 µM
BVNP-0195	6.50 µM
BVNP-0196	5.37 µM
BVNP-0197	**0.089 µM**
BVNP-0198	**1.08 µM**

A


150
100
50
0
% Maximum
0.0001
0.01
1
100
10000
Concentration (μM)
ALE-0540 (IC50 = 2.44 μM)
PD90780 (IC50 = 33.22 μM)
Ro 08-2750 (IC50 = 11.96 μM)
BVNP-0188 (IC50 = 1.70 μM)
BVNP-0197 (IC50 = 0.0895 μM)
BVNP-0198 (IC50 = 1.08 μM)

B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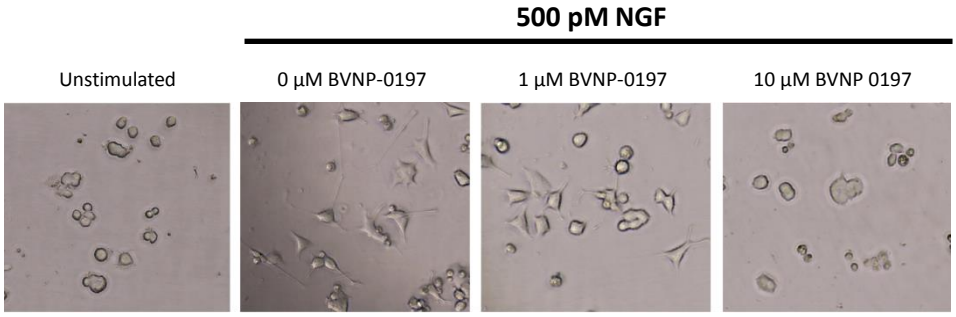

Figure 5.9: BVNP-0197 demonstrated inhibitory effects in the nanomolar range in the PC12 differentiation assay. A) Detailed dose response experiments were conducted for all 17 bivalent naphthalimide compounds and three previously reported compounds. Previously reported compounds ALE-0540, PD 90780 and Ro 08-2750 were determined to have IC_{50} values of 2.44 µM, 33.22 µM and 11.96 µM respectively. Three bivalent naphthalimide compounds were shown to have inhibitory effects more efficient than the previously reported compounds. BVNP-0188, and BVNP-0198 were determined to have IC_{50} values of 1.70 µM and 1.08 µM respectively whereas BVNP-0197 was found to have an IC_{50} value of 89.5 nM. Previously reported compounds are depicted in red where novel compounds are in blue. Data points represent a mean value of quadruplicate results with an n = 3. Error bars are represented as standard error of the mean with an n=3. B) The chemical structure of BVNP-0197. C) BVNP-0197 functionally inhibits NGF-mediated PC12 cell process formation. In the presence of 500 pM NGF, PC12 cells were treated with varying concentrations of BVNP-0197 (100 µM – 0 µM). Images from 0, 1 and 10 µM are shown. In the presence of NGF and the absence of inhibitor, PC12 cells underwent cell process formation. Process formation significantly decreased with the addition of increasing concentrations of BVNP-0197. An unstimulated (0 pM NGF) sample was prepared to demonstrate the NGF-dependence in cell process formation.

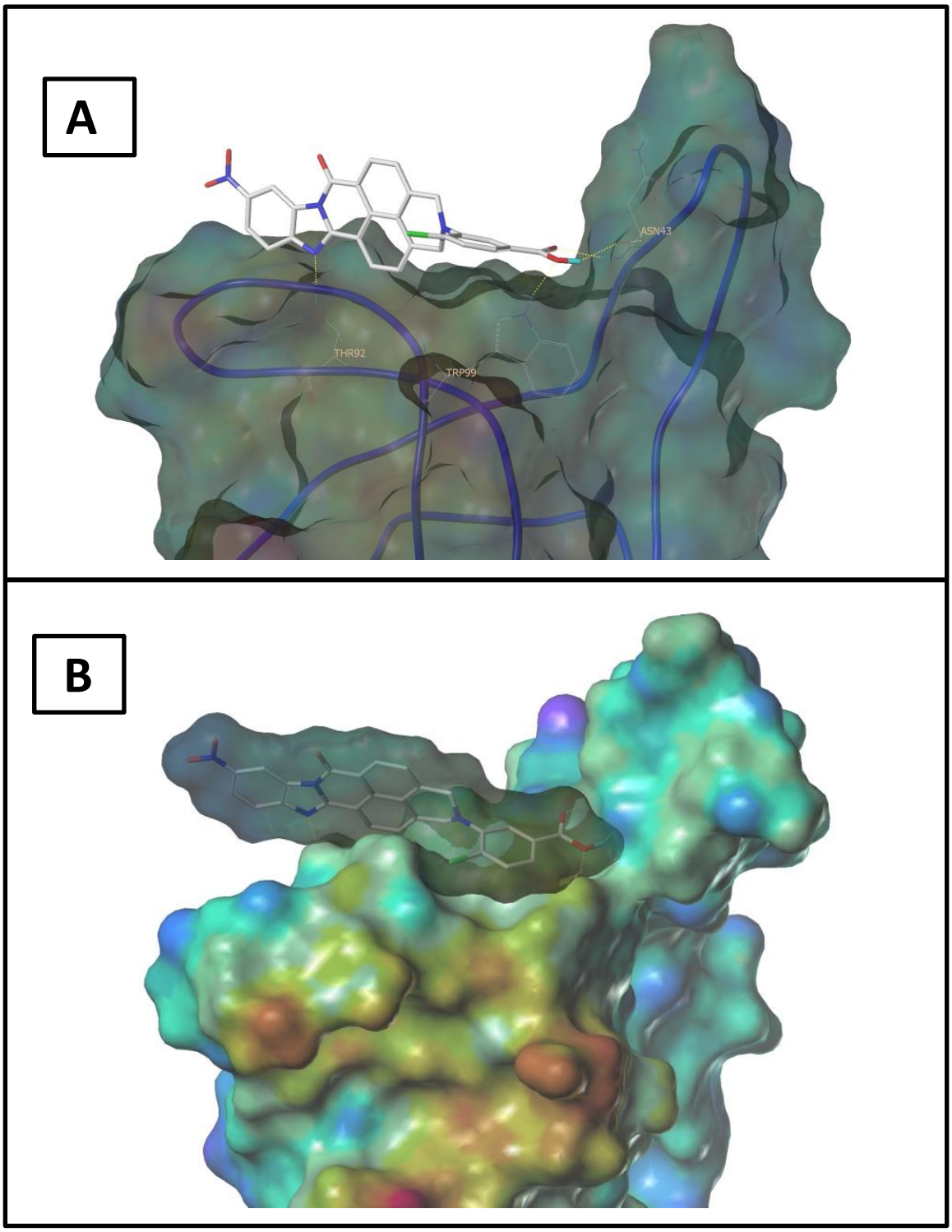

Figure 5.10: Schematic representation of BVNP-0197 docked to NGF as determined by molecular modeling. Flexible theoretical docking experiments predict that the bivalent naphthalimide scaffold is suited to bind at the loop II/IV cleft of NGF. A) Four hydrogen bonds are represented at residues Asn43, Thr92 and Trp99 of NGF to stabilize BVNP-1097 within the loop II/IV cleft. B) The differences in electrostatic potential at the docking domain. Blue areas represent electron-poor regions where a gradient to red represents electron-rich regions.

Inhibitory effects of bivalent naphthalimide analogues

To determine the inhibitory properties of the 17 bivalent naphthalimide analogues using SPR spectroscopy, a dilution series of each analogue (3.16 nM to 316.23 µM) was incubated for 1 hour with 10 nM NGF prior to a 60 second injection over immobilized TrkA on the sensor surface. Each injection over immobilized TrkA was followed by a 120 second dissociation phase prior to regeneration before the next injection. A response for each sample was assessed using SPR spectroscopy and compared to a control sample of 10 nM NGF with no analogue present. Dose response curves were generated to determine IC_{50} values using the BIA evaluation software (Version 2.0) and were evaluated using a one-site steady-state binding model. The 17 analogues demonstrated IC_{50} values ranging from 18.5 nM to 81.40 µM which were related to the IC_{50} values calculated from neurite outgrowth assays (Figure 5.11).

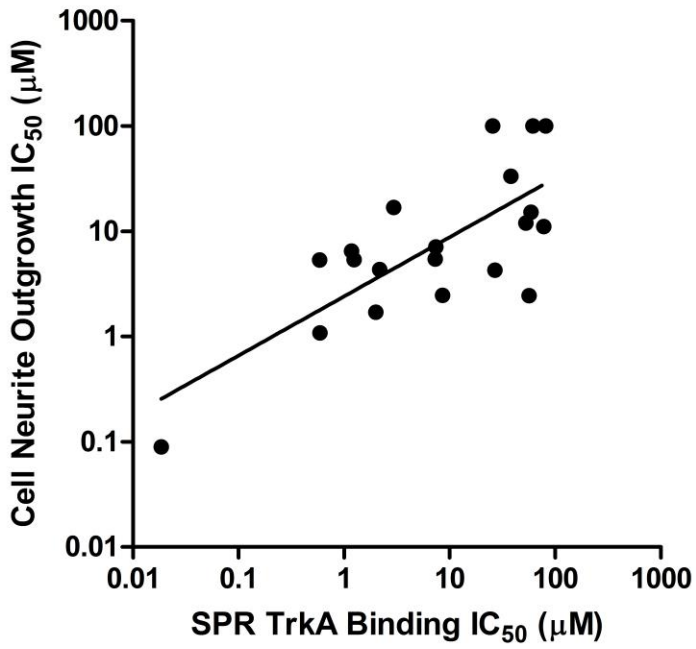

Figure 5.11: The relationship of the IC_{50} measured by SPR spectroscopy for the 17 bivalent naphthalimide analogues and the three previously reported compounds (ALE-0540, PD 90780 and Ro 08-2750) and the IC_{50} measured using the PC12 cell NGF-dependent differentiation assay. X-axis: IC_{50} (μM) measured using the SPR – Various concentrations of analogue were incubated for 1 hour with 10 nM NGF prior to injection over immobilized TrkA. Y-axis: IC_{50} (μM) measured following PC12 cell treatment of increasing analogue concentration and 500 pM NGF for 72 hours. Each data point represents the mean of triplicate results. ($F(1,18) = 22.69$; $p=0.0002$; $R^2=0.5576$).

Discussion

In the present study, we identified a novel bivalent naphthalimide compound, BVNP-0197, with a higher NGF-inhibitory efficiency than previously described compounds utilizing cell-free SPR technology and cell-based PC12 neurite outgrowth assays**.** BVNP-0197 is also predicted to bind to NGF in a novel binding domain found in the loop II/IV cleft.

Historically, most drug discovery efforts in NGF-mediated pain signalling therapeutics have been directed towards identifying small molecules which inhibit signalling through the receptor TrkA. Small molecules such as K252a have been reported, however, pharmacological specificity has limited the advancement of such compounds into clinical trials (Watson et al. 2008). NGF-mimetic peptides have also been evaluated, however no peptide based strategies has advanced into clinical trials or proven effective (Eibl et al. 2012).

Success of targeting a protein in a protein-receptor system was first demonstrated with the approval of anti-tumour necrosis factor (TNF) antibodies for the therapeutic treatment of rheumatoid arthritis (Elliot et al. 2008) and Crohn's disease (Deželak et al. 2016; Tolentino et al. 2016). Similar strategies have been explored with anti-NGF antibodies, such as Tanezumab, for the treatment of NGF-mediated pain. Although seemingly effective, anti-NGF antibodies are still facing several therapeutic drawbacks with potential serious side effects involving autoimmune responses. Antibody-mediated pain management strategies are also highly specific and are restricted from crossing the blood brain barrier to the central nervous system (Bannwarth and Kostine 2014).

Similar strategies of NGF inhibition have been introduced using small molecule NGF-inhibitors. Small molecules such as ALE-0540 (Owolabi et al. 1999), Ro 08-2750 (Niederhauser et al. 2000), PD 90780 (Colquhoun et al. 2004), and PQC-083 (Eibl et al. 2013a) have demonstrated efficiency in binding and inhibiting NGF from interacting with its receptors. Another small

molecule Y1036 (Eibl et al. 2010), has been introduced as a multipotent neurotrophin inhibitor by binding to not only NGF, but also BDNF and inhibiting their action on receptors. Compounds such as ALE-0540, Ro 08-2750, PD 90780 and PQC-083 have been shown to bind to the loop I/IV cleft and sufficiently alter the molecular topology in a manner such that NGF can no longer bind efficiently to its receptors (Eibl et al. 2013a; Eibl et al. 2010). This loop region of NGF has the highest degree of variance among all the neurotrophins and is responsible for the selectivity in receptor selection among the neurotrophin family members. Alternatively Y1036 has been described to bind at the neurotrophin hydrophobic interface, the region conserved among all neurtrophin family members, and therefore loses the specificity for NGF (Eibl et al. 2013b).

SPR spectroscopy has been used to study biomolecular interactions and has proven effective in determining how a potential drug therapeutic will interact with their target receptor(s). Previously used screening strategies for NGF inhibitors included the use of radioisotopes such as ^{125}I (Owolabi et al. 1999; Colquhoun et al. 2004; Eibl et al. 2013a). SPR is advantageous over labeling strategies because it eliminates the need of fluorescent reporter molecules or radioisotope tags which adds the concern of altering the molecular interactions being examined.

The present study introduces SPR spectroscopy for screening of a novel bivalent naphthalimide analogue series to determine a novel NGF-inhibitor, BVNP-0197, with a higher potency than

previously reported molecules. SPR screening revealed 17 novel compounds which specifically bind to NGF with binding affinities between 3 µM - 119 µM. This study also provides novel evidence that the bivalent naphthalimide analogues described, as well as the three historic compounds examined (ALE-0540, Ro 08-2750, and PD 90780) are true NGF-inhibitors as they do not bind to TrkA to inhibit NGF-TrkA mediated signalling. The *in vitro* cell count and morphology of PC12 cells treated with the bivalent naphthalimide analogue series, including BVNP-0197, did not significantly differ from untreated cells after 72 hours in culture. The NGF-dependent cell assay determined that three analogues had higher efficiency in NGF- inhibition than previously reported molecules (IC_{50} < 2 µM). BVNP-0188, BVNP-0197 and BVNP-0198 demonstrated IC_{50} values calculated from neurite outgrowth assays at 1.70 µM, 89.5 nM and 1.08 µM respectively. IC_{50} values determined using PC12 neurite outgrowth assays were also related to the IC_{50} values determined for each analogue using SPR binding assays (Figure 5.11) demonstrating that SPR could be utilized in future therapeutic screening strategies prior to entering *in vitro* studies.

It is expected that BVNP-0197 may have several potential applications in the field of neurotrophin research. For example, BVNP-0197 may serve as a therapeutic with the ability to inhibit NGF-TrkA mediated pain signalling since the predicted docking domain interferes with TrkA binding. BVNP-0197 and the other bivalent naphthalimide analogues examined in this study may also serve as evidence for the development of future compounds that inhibit neurotrophin mediated pain and other NGF related disorders. SPR analysis of the bivalent

naphthalimide analogue-NGF interaction may allow for the development of compounds with varied binding kinetics thus leading to more efficient therapeutic options for NGF dysregulation. Further analysis in primary cell culture and animal models will be required to validate BVNP-0197 as a potential therapeutic in clinical use for pathologies associated to NGF dysregulation, such as osteoarthritic pain and neurodegenerative diseases.

Chapter 6: Optimization of surface plasmon resonance measured binding affinity of small molecule-based nerve growth factor inhibitors with the addition of bovine serum albumin

(Original Research)

Allison E. Kennedy, John A. Scott, Gregory M. Ross

[Submitted to *Analytical Biochemistry*]

Abstract

The study of the interactions between a drug and plasma serum proteins is necessary in determining pharmacological and toxicological properties for the investigated compound. Small molecule NGF-inhibitors have been investigated for their abilities to inhibit NGF binding to TrkA as a therapeutic option for potential treatment of neuropathic and inflammatory pain. In this study, surface plasmon resonance (SPR) spectroscopy and ^{125}I-NGF radioisotope tags were carried out to better understand the interaction between small molecule NGF-inhibitors and bovine serum albumin (BSA) *in vitro*. Serum albumin has been characterized as a universal drug carrier with up to seven binding domains on its surface to transport drug molecules to targeted tissues. Here, we use SPR kinetic analysis to demonstrate the change in specificity small molecule NGF-inhibitors have for NGF with the presence of BSA. The binding affinity to NGF with BSA in solution corresponds to an average 10-fold increase, matching *in vitro* analysis. Our results suggest a crucial role for serum albumin in the pharmacokinetics of small molecule NGF-inhibitors which require attention when developing therapeutic agents.

Introduction

Nerve growth factor (NGF) functions not only in prenatal nerve growth, but also has a significant role in pain and immune function in adults (Aloe et al. 2012; Gerber et al. 2011). During tissue damage, NGF, interleukins and tumour necrosis factor-α are secreted by immune cells resulting in painful pathology (Chao et al. 2006; Skaper 2001). NGF in particular leads to hyperalgesia and allodynia by triggering peripheral and central neuronal sensitization by activating the receptor TrkA (McKelvey et al. 2013; Max and Stewart 2008). In such conditions, having an analgesic which can block NGF binding to TrkA and the resulting signal would be extremely useful.

It is estimated that 20-30% of adults suffer from chronic pain, many of which whom are not adequately alleviated from the painful symptoms with current therapeutic options (Hirose et al. 2016; Schopflocher et al. 2016). Therefore, the development of novel therapeutics would hold a great benefit. Targeting NGF-TrkA signalling for the development of novel therapeutic agents has been the focus in several studies. Currently the humanized monoclonal anti-NGF antibody Tanezumab is in Phase III clinical trials for sequestering free NGF for the treatment of arthritic joint pain (Chang et al. 2016). Despite being highly selective for NGF, there are many downfalls to antibodies as a therapeutic agent including autoimmune responses, delivery challenges and production capacity (Samaranayake et al. 2009).

Small molecule inhibitors have emerged as a possible therapeutic option for inhibiting NGF. Small molecule NGF-inhibitors such as PD 90780 (Colquhoun et al. 2004), ALE 0540 (Owolabi et al. 1999), Ro 08-2750 (Niederhauser et al. 2000) and PQC-083 (Eibl et al. 2013a) have demonstrated their efficacy in inhibiting NGF *in vitro*. Flexible docking experiments suggest that these small molecule inhibitors bind to NGF at the loop I/IV cleft to alter the molecular topology in a manner which alters binding capacity to TrkA (Eibl et al. 2012). More recently, a series on naphthalimide analogues were screened using surface plasmon resonance (SPR) spectroscopy to identify a compound called BVNP-0197, which has a nanomolar half maximal inhibitory concentration (IC_{50}) for NGF binding to TrkA (Kennedy et al. in press).

Human serum albumin (HSA) is an abundant plasma protein in the circulatory system that functions as the main transport vehicle for fatty acids (Curry et al. 1998; Simard et al. 2006). HSA is also capable of transporting a wide variety of drug molecules, which affects the pharmacokinetics and pharmacodynamics of therapeutic agents (Buchholz et al. 2002; Esmaeilzadeh et al. 2016). Binding to HSA alters the plasma half-life of a drug, significantly lowers the rate of clearance and changes the volume and the pattern of drug distribution (Benet and Hoener 2002). HSA had a complex binding capacity which makes it difficult to study in terms of the interactions which occur with varying ligands. At least seven hydrophobic binding pockets on the surface of HSA act as universal receptors for drug molecules to modulate cell delivery (Reichenwallner and Hinderberger 2013; Pal et al. 2016). The high ligand binding capacity of HSA has become an interest to pharmaceutical companies for drug

development although yet to be investigated in respect to small molecule NGF-inhibitor binding.

Herein, we report on the results of small molecule NGF inhibitors binding to bovine serum albumin (BSA) for receptor docking. In this study BSA was used as a model protein because of its low costs, availability and structural homology with human serum albumin (Huang et al.2004). The binding affinity associated with receptor binding was found to have an approximate 10 fold increase with the introduction of BSA to solution. These findings may provide valuable information necessary for drug delivery and drug design of pain therapeutics.

Materials and Methods

Surface plasmon resonance spectroscopy

A BIAcore T200 spectrometer was obtained from GE Healthcare Life Science (Piscataway, NJ, USA). Full length (1-118) mouse NGF purified by HPLC from 2.5S NGF (purity greater than 95%) was obtained from Cedarlane Laboratories (Toronto, ON) and coupled to a CM5 sensor chip using standard amine coupling methods. Data was collected at a temperature of 25 °C. Individual compound samples were tested from lowest to highest concentrations, separated by a 15 second stabilization period after each sample in each compound series. During each sample cycle, analyte was injected for 60 second at a flow rate of 30 µL/min. A dissociation period was monitored for 30 seconds after analyte injection before regeneration occurred with

a 1.0 M NaCl for 120 seconds at a flow rate of 30 µL/min to wash any remaining analyte from the sensor chip before running the next sample. Synthetic organic compounds were synthesized by Sussex Research (Ottawa, ON, Canada) and diluted to concentrations ranging from 0.78 µM to 200 µM using HBS-EP buffer with 0.5% DMSO (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA and 0.05% v/v Tween 20; pH 7.5). To determine binding affinity, the response obtained from each sample run in a series was plotted against concentration using the BIA evaluation software version 2.0 and was evaluated using a two steady-state site binding model as previously described (Kennedy et al. 2016). Data was also analyzed using a heterogeneous ligand model to determine kinetic rate constants.

Binding of ^{125}I-NGF to PC12 cells

Full length (1-118) mouse NGF purified by HPLC from 2.5S NGF (purity greater than 95%) was obtained from Cedarlane Laboratories (Toronto, ON). The iodination of NGF was performed as previously described (Ross et al. 1997). The ^{125}I-NGF obtained (typically 80-120 c.p.m./pg) was purified by size exculsion chromatography on a PD10 column (Pharmacia) preequilibrated with HKR buffer (10 mM HEPES [pH 7.35] containing 125 mM NaCl, 4.8 mM KCl. 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 g/L glucose, 1 g/L BSA). PC12 cells were grown in RPMI with 10% fetal calf serum. Each data point set up in a single tube containing ^{125}I-NGF (at the required concentration), 400,000 cells (for a final concentration of 10^6/mL) and NGF (at 10 nM for nonspecific binding) as required. The tubes were incubated for 2 h at 4˚C, and 100 µL aliquots (providing triplicate data points for each sample) were transferred to 400 µL microcentrifuge

tubes containing 200 µL of 10% glycerol in HKR. Tubes were centrifuged for 2 min at 5,000 rpm, the tip containing the cell pellet was cut off, and radioactivity bound to the cells was determined.

Results

A series of small molecule NGF inhibitors were screened using SPR spectroscopy for their binding affinity to immobilized NGF. A two-site binding model was applied using BIAevaluation software (Version 2.0). The response obtained due to binding from each analogue concentration was plotted against concentration to determine binding affinity (K_D). To determine the effect of BSA on small molecule binding, samples were suspended in HBS-EP buffer with 0.5% DMSO with and without 1% w/v BSA. Figure 6.1 represents the two-site steady-state affinity plot for the binding of varying concentration of BVNP-0197 to immobilized NGF. The binding affinity for NGF without BSA in solution was measured at 21.0 µM. The addition of 1% BSA increased the binding affinity of BVNP-0197 for NGF to 0.428 µM.

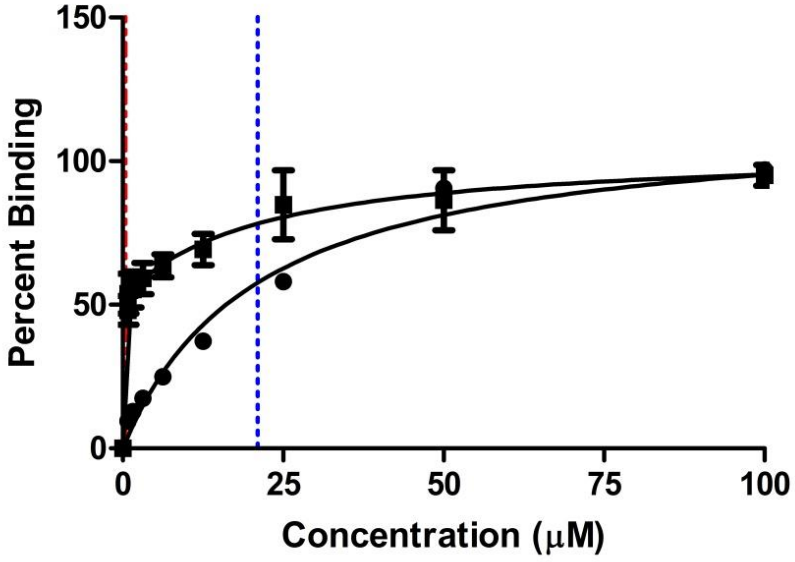

Figure 6.1: Two-site steady-state binding curve describing the binding of small molecule NGF-inhibitor BVNP-0197 binding to immobilized NGF. X-axis: concentration of sample (μM); y-axis: percent binding subtracted response (active flow cell minus response measured from reference flow cell). Samples containing 0% BSA are represented in circles; samples containing 1% BSA are represented in squares. Standard error of the mean is shown with error bars (n=3). K_D vales for 0% and 1% BSA samples are shown in blue and red broken lines respectively (21.0 μM and 0.428 μM).

Kinetic rates were determined with the heterogeneous ligand model in the BIAevaluation software (Version 2.0). The addition of 1% BSA decreased the rate of association during analyte injection during all small molecule compounds. There was also an observed increase of rate of dissociation in all small molecule compound concentrations prior to regeneration. The subtracted sensogram of BVNP-0197 (200 μM) was chosen to represent the changes in kinetic rates (Figure 6.2).

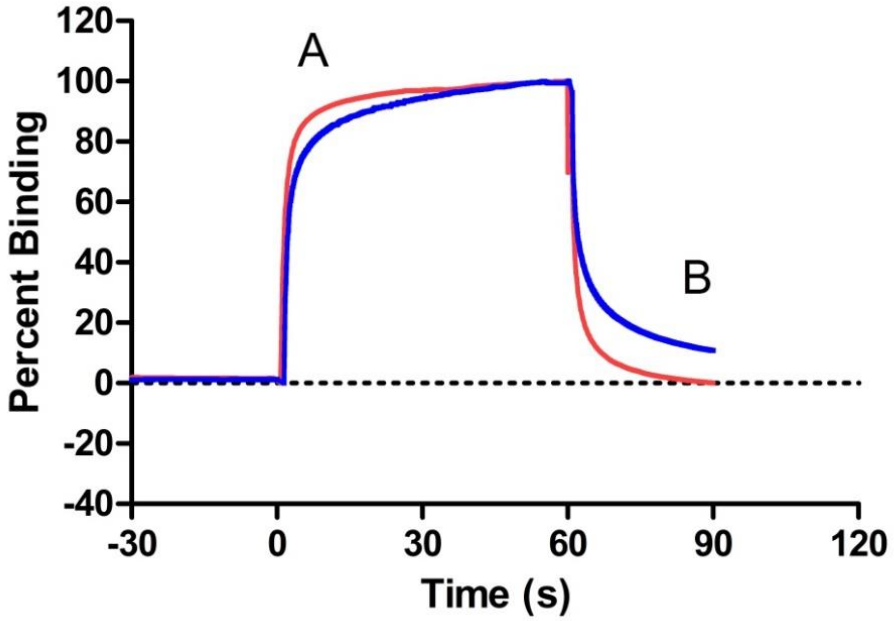

Figure 6.2: Subtracted sensogram of BVNP-0197 (200 µM) demonstrating the changes in kinetic rates of small molecule binding to immobilized NGF. Time 0-60 seconds represents the association of BVNP-0197 to NGF; at 60 seconds the analyte flow stops. A dissociation period from 60-90 seconds is monitored before regeneration of the chip occurs before the next sample is run. Blue: BVNP-0197 binding to NGF with 0% BSA in solution. Red: BVNP-0197 binding to NGF with 1% BSA in solution. A) Decreased rate of association with 1% BSA in solution. B) Increased rate of dissociation with 0% BSA in solution

Binding saturation studies with ^{125}I-NGF using PC12 cells were completed to measure inhibition of compounds. A relationship was determined between the high-affinity binding of the two-site binding analysis from the NGF-inhibitor to the IC_{50} measured from the same compound using ^{125}I-NGF (Figure 6.3) without BSA ($F(1,17)=5.620$; $p=0.0316$; $R^2=0.2726$) and with the addition of 1% BSA ($F(1,10)=5.070$;$p=0.0480$;$R^2=0.3364$) to solution. The addition of 1% BSA did not alter the slope of the relationship ($F(1,25)=0.0322$;$p=0.8588$) but rather increased affinity for NGF by an average 10 fold ($F(1,26)=44.63$;$p<0.0001$) (Figure 6.3).

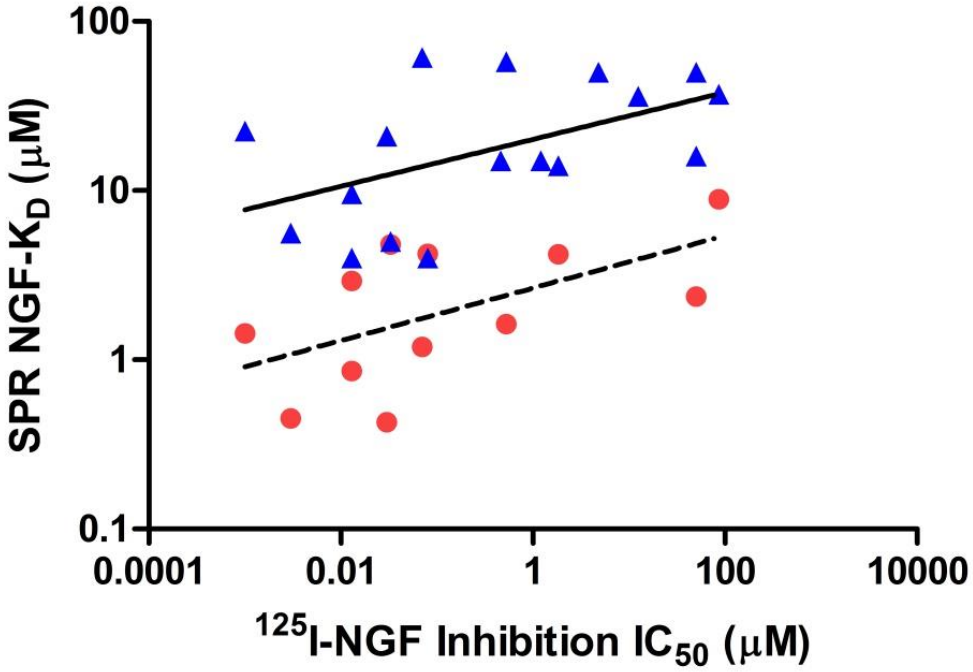

Figure 6.3: Demonstrates the relationship between the IC_{50} measured from ^{125}I-NGF PC12 cell assays and the high-affinity K_D measured using SPR two-site analysis. No difference in slope is seen from the addition of BSA into solution, however a 10 fold increase in K_D is measured with the addition of BSA. Triangle data points represent 0% BSA added to solution. Circle data points represent 1% BSA added to solution.

Discussion

Early phase drug discovery is focused on optimizing the affinity and selectivity for the drug target. Target affinity is often complete in cell-free systems which can accurately measure direct binding to a drug target without the influence of other identities in a system (Núñez et al. 2012; Copeland et al. 2006). SPR is one method available to measure the specificity of a drug to its target protein using label-free technology and with high enough sensitivity to look at small molecule binding (Wittenberg et al. 2014; Gopinath and Kumar 2014).

Drug interactions are often analysed for a IC_{50} or K_D value, however more emphasis has been placed on drug kinetics to identify compounds which have a shorter association periods and longer dissociation periods (Copeland et al. 2006; Myszka 2004; Geschwindner et al. 2012). A shorter association phase translates into higher specificity and likely reduced side effects from a drug. Alternatively, a longer dissociation period is targeted since the drug occupies the receptor for longer which is necessary for reducing doses and extending the effects from the drug. However, a longer dissociation period has drawbacks as it is also associated with drug toxicity and significant side effects.

Small molecule NGF-inhibitors have been an area of focus for nearly two decades as a therapeutic option for pain. In conditions where responses of inflammatory pain persist; such as in autoimmune diseases and arthritic pain; NGF plays a large role in pain signalling through the TrkA receptor (Bennett 2001; Capsoni et al. 2011; Barker 2007). Recently, small molecule NGF-inhibitor screening has been introduced to the label free SPR techniques which have identified novel small molecules with high specificity for NGF (Kennedy et al. 2016; Sheffield et al. 2016a; Sheffield et al. 2016b). However, with this introduction to SPR analysis, a discrepancy in K_D and IC_{50} measurements are found when comparing to *in vitro* measurements to the direct binding results (Kennedy et al. in press). These discrepancies lead to the idea that there may be a carrier protein involved during *in vitro* assays altering IC_{50} and K_D determination.

HSA has been identified by several others as a universal drug carrier to aid drug delivery to targeted tissues (Belatik et al. 2012; Ma et al. 2015; Buchholz et al. 2002). BSA has also been studied as a drug carrier as it shares 76% homology to HSA (Huang et al. 2004). Cell culture protocols, such as ^{125}I-NGF binding experiments, require the addition of serum albumin to media to increase the productivity of cells (Peters 1995). Serum albumin has the ability to deliver important nutrients to cells as well as to bind toxins and free radicals reducing cell damage (Peters 1995). This study has determined that serum albumin in cell culture media may also plays a role in the binding of small molecule NGF-inhibitors to TrkA receptors on PC12 cells. SPR analysis of small molecules binding to immobilized NGF with the presence of 1% BSA has identified an average 10 fold increase in binding affinity (Figure 6.3) compared to no BSA in solution. This fold change in binding affinity could explain the discrepancies previously observed in SPR analysis when comparing binding events to *in vitro* analysis. Kinetic rate analysis determined that with the addition of 1% BSA, there is an increase in rate of association during small molecule inhibitor binding to NGF and a decrease in rate of dissociation (Figure 6.2). These changes in kinetic rate would translate to an increased specificity for NGF and a decreased potential in toxic effect with the introduction of BSA as a drug carrier.

Conclusion

Identifying carrier proteins which participate in optimizing small molecule binding to NGF may prove to be beneficial in the development of future pain therapeutics. In a closed system, serum albumin has shown an effect in K_D determination of small molecules binding to NGF. This increase

in affinity is similar to affinities determined through *in vitro* assays where serum albumin is added to media for cell health. By understanding all mechanisms involved in drug target binding, efficacy of future therapeutics can be optimized by tailoring developmental strategies to account for all contributing components of the binding event.

Chapter 7: Conclusions and future directions

Chronic pain presents a huge economic and social burden, with existing therapeutic options largely unable to satisfy the medical needs of patients. Nerve growth factor (NGF) has emerged as a major mediator of inflammatory and neuropathic pain providing a new therapeutic target. Originally, NGF was discovered as a trophic factor for sympathetic and sensory neurons during development, although it now appears that in adults, elevated levels of NGF are associated with many acute and chronic pain conditions (Pezet and McMahon 2006; Wiesmann et al. 2001). Furthermore, preclinical animal models of inflammatory and neuropathic pain also show increased NGF levels, while sequestering the free NGF alleviates the associated hyperalgesia (Lewin et al. 1993; Lewin et al. 1994; Capsoni et al. 2011).

Current clinical strategies for the isolation of free NGF include the use of antibody therapies such as Tanezumab, a humanized monoclonal anti-NGF antibody. Early clinical trials involving Tanezumab demonstrated its efficiency in alleviating hip and knee osteoarthritic pain in comparison to the placebo (Lane et al. 2010). Although seemingly effective, two independent partial clinical holds were placed on anti-NGF antibodies for allegations of peripheral nerve effects including osteonecrosis leading to joint replacement (Bannwarth and Kostine 2014; FDA

2012). Recently, the partial clinical holds were lifted and Tanezumab, along with other anti-NGF antibodies, have begun Phase III clinical trials (Chang et al. 2016; Bowman et al. 2015).

Despite the effective therapeutic properties of anti-NGF antibodies, there are still a number of drawbacks and side effects to overcome in clinical application (Sanga et al. 2013; Samaranayake et al. 2009). Other approaches sought out for inhibiting NGF include the use of small molecule NGF-inhibitors. Several small molecules have been described as advantageous over antibody strategies due to their size and their economic value (Eibl et al. 2012). However, their inhibitory effects are still quite minimal in comparison to other clinical tactics as their specificity for NGF is only described in the micromolar ranges (Eibl et al. 2013a; Colquhoun et al. 2004; Niederhauser et al. 2000; Owolabi et al. 1999).

Surface plasmon resonance (SPR) spectroscopy has been well established as a label-free technique capable of investigating biomolecular interactions in real-time. The studies presented in this thesis utilize SPR technology to investigate small molecule-NGF interactions at a higher throughput rate than other cell-based techniques. Historically, the efficiency of small molecule binding to NGF was analyzed using radioisotope tags, which have the ability to alter the biomolecular interaction being investigated. Similarly, by exploiting a cell-free system, other key players which could impact cell-based binding are eliminated. For the first time, SPR has allowed the direct binding between established small molecule entities and NGF to be

evaluated. Furthermore, this strategy allows novel compounds to be rapidly screened for higher specificity and inhibitory properties, targeted for therapeutic application.

Characterizing the binding properties of novel compounds to NGF proved valuable in identifying a lead compound with a higher binding affinity and inhibitory efficiency than previously reported small molecules. BVNP-0197 was described, using SPR screening, to have specificity for NGF, with a measured binding affinity of 21 μM and a TrkA inhibitory efficiency in the high nanomolar range. Cell-based neurite outgrowth assays were used to determine a cell-based half maximal inhibitory property (IC_{50}) of 90 nM. This inhibitory potential was significantly higher than any other established small molecule NGF-inhibitors, which were measured to be between 2-33 μM. Flexible docking also identified a novel docking domain for BVNP-0197 to NGF. Previous small molecule-NGF inhibitors have been described with a docking domain at the loop I/IV cleft which is necessary for TrkA/$p75^{NTR}$ binding (Eibl et al. 2012; Eibl et al. 2013a). Our analogue series identified a docking domain in the loop II/IV cleft, an area more specialized for NGF binding to TrkA rather than for $p75^{NTR}$ docking (Barker 2007; Wehrman et al. 2007).

Finally, this thesis describes the role that serum albumin has during cell-based NGF-TrkA binding. Serum albumin has been identified by many as a universal drug carrier to aid in drug delivery to targeted tissues (Esmaeilzadeh et al. 2016; Simard et al. 2006). Cell-based assays require the addition of serum albumin in media for the delivery of nutrients to cells in culture

and to bind free radicals, thus reducing cell damage. The shift to a cell-free system presented with SPR, although effective when compared to cell-based assays, presented a discrepancy in reporting binding affinities of small molecule compounds. The addition of serum albumin to buffer solutions during small molecule binding experiments to immobilized NGF, was associated with an average 10 fold increase in binding affinity. This increase in magnitude corresponds to measurements acquired using cell-based assays, inferring that serum albumin plays a role during NGF-TrkA binding *in vitro*. In addition, the kinetic rate analysis of BVNP-1097 binding to NGF with serum albumin demonstrated a decrease in the association phase and an increase in the dissociation phase when compared to NGF binding in the absence of serum albumin. These changes in kinetics translate to a higher specificity and likely reduced side effects. It would also be expected that drug toxicity would be reduced with the addition of serum albumin since the small molecule is not occupying the docking domain for as lengthy of a period of time.

The data presented in this thesis confirms key components of established small molecule NGF-inhibitors: ALE-0540, PD 90780, Ro 08-2750 and PQC-083. These small molecules were, for the first time, evaluated on their specificity for NGF as opposed to the TrkA receptor. Data presented also reveals a method to quickly and efficiently screen small molecules, measure their binding affinity for NGF and evaluate their inhibitory potential for NGF binding to TrkA. Finally, we suggested a role for serum albumin in small molecule-NGF docking, proposing that the presence of serum albumin increases the specificity for NGF by an approximate 10 fold.

SPR methods detailed in this document have the potential to be extended to investigate the therapeutic binding properties of other neurotrophin family members. For example, inhibiting BDNF may be relevant in targeting neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease or other diseases such as obesity (Lu et al. 2013; Cao et al. 2009). Identifying compounds that can bind several neurotrophins simultaneously may also have therapeutic potential in multi-neurotrophin related dysfunctions, for instance, in deafness caused by a dysregulation of neurotrophin-3 (NT-3) and BDNF (Atkinson et al. 2012).

The function of serum albumin during small molecule-NGF binding could also be extended to determine specific binding domains for small molecules. Several studies have suggested between four and seven binding domains on surface pockets of serum albumin for transport of a wide range of drugs and fatty-acids. Binding analysis of small molecule NGF-inhibitors to immobilized serum albumin (using SPR) may provide further insight into serum albumin's role in cell-based assays.

Progress has been made in recent years to develop an understanding of the capabilities small molecule-based therapeutics have for experimental purposes, however, several stages of investigation are still required prior to these compounds entering clinical investigation. For example, the putative binding mechanisms presented would have to be evaluated via X-ray

crystallography or NMR studies. *In vivo* studies would also have to be completed to evaluate toxicity and bioavailability of compounds. Additionally, pre-clinical animal models of neuropathic or inflammatory pain would be necessary prior to establishing a potential for the described compound to enter human clinical trials. Ultimately, the completion of clinical evaluation is required to establish therapeutic application for small molecule-based NGF inhibitors.

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