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

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ABSTRACT

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

Neurotrophins are a unique family of soluble signaling proteins that act to influence the proliferation, differentiation, and survival of neurons in the central and peripheral nervous systems [1]. Nerve growth factor (NGF), the most widely studied member of this protein family, interacts with two separate receptor classes: the selective tyrosine kinase receptor, TrkA, and the common neurotrophin receptor, p75^{NTR} [2].

The interaction of NGF with TrkA demonstrates affinity in the high picomolar range and results in autophosphorylation of the receptor, leading to a downstream signaling cascade promoting neuronal survival [2–4]. Conversely, NGF–p75^{NTR} interactions are characterized by low nanomolar affinity and may lead to both pro-apoptotic and pro-survival signaling [2]. Although NGF is capable of binding both receptors, when both receptors are expressed, its affinity for TrkA is influenced by the presence of the p75^{NTR} receptor. For instance, p75^{NTR} has been shown to enhance the expression of TrkA in addition to increasing its binding affinity for NGF [5,6].

NGF dysregulation has been implicated in several pathologies. For instance, both inflammatory and neuropathic pain states have been associated with NGF dysregulation and increased TrkA-mediated signaling [4,7,8]. Likewise, increased levels of NGF have been detected in the cerebrospinal fluid of patients suffering from Parkinson's disease [9], and improvements in the delivery of NGF have been shown to ameliorate cognitive function in patients with Alzheimer's disease [10]. Although it is understood that NGF and its precursor protein, pro-nerve growth factor (proNGF), play a crucial role in neurodegeneration, there are still significant details to be worked out with respect to signaling mechanisms [11]. Nevertheless, research suggests that therapeutic strategies to inhibit NGF–receptor interactions are of significant clinical interest.

One such approach to blocking NGF from binding to its receptors is small molecule inhibitors. These inhibitors bind to and modulate

Abbreviations used: NGF, nerve growth factor; SPR, surface plasmon resonance; HBS–EP, Hepes-buffered saline with EDTA and surfactant P20; EDTA, ethylenediaminetetraacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; RU, response units.

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NGF, as opposed to binding to the TrkA and $p75^{NTR}$ receptors. Several of these antagonists have been discovered and described in the literature [12–17]. Interestingly, although these molecules are known NGF–TrkA inhibitors and have been reported to inhibit TrkA phosphorylation and TrkA-mediated downstream signaling cascades, their ability to block NGF from interacting with $p75^{NTR}$ has not yet been assessed. Furthermore, of the previously described NGF-binding agents, none have ever been investigated for $p75^{NTR}$ interactions using surface plasmon resonance (SPR) technology, which is a tool designed to investigate biomolecular interactions and has been proven to be useful in drug discovery applications [18–20]. SPR biosensors offer highly sensitive, label-free, real-time analysis, which makes them extremely suitable for the characterization of small molecule-based NGF antagonists [21,22].

In the current study, we used SPR technology to characterize the binding of NGF to both the TrkA and $p75^{NTR}$ receptors in addition to characterizing the inhibitory potential of known NGF antagonists PD90780, ALE-0540, Ro 08-2750, and PQC 083. We presented affinities of NGF to each of its receptors determined through steady-state affinity analysis using SPR spectroscopy. Furthermore, we assessed the receptor selectivity of the known NGF antagonists by examining their ability to inhibit NGF from binding both the $p75^{NTR}$ and TrkA receptors. Inhibition profiles for each inhibitor in the form of specificity for NGF and half-maximal inhibitory concentrations (IC_{50} values) obtained through novel methodology are presented.

Materials and methods

Materials

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

SPR and preparation of sensor surfaces

All experiments were carried out using a Biacore T200 SPR spectrometer purchased from GE Healthcare Life Sciences. Prior to immobilization, pH scouting experiments were conducted to determine the optimal pH for the immobilization of both the $p75^{NTR}$ and TrkA receptors. It was concluded that 10 mM sodium acetate at pHs 4.5 and 5.5 yielded the greatest pre-concentration effect for $p75^{NTR}$ and TrkA immobilizations, respectively. Immobilization experiments investigating the optimal binding levels for both receptors were also conducted, allowing for the selection of an immobilization level between 850 and 950 RU; increased protein loading interfered with binding kinetics because dissociation constants were inconsistent. The CM5 sensor chip was activated by injecting a mixture of 0.2 M EDC and 0.05 M NHS at a flow rate of 5 μl/min for 7 min $p75^{NTR}$ was diluted to 10 μg/ml in 10 mM sodium acetate buffer (pH 4.5) and immobilized until a level between 850 and 950 relative response units (RU) (~15 fmol/mm^2) was reached. Excess reactive esters on the sensor chip surface were deactivated with 1 M ethanolamine (pH 8.5) at a flow rate of 5 μl/min for 7 min. The TrkA receptor was immobilized following the above procedure; however, 10 mM sodium acetate (pH 5.5) immobilization buffer was used. In the TrkA immobilization, a level between 850 and 950 RU (~11 fmol/mm^2) was reached. Flow cells used for reference were activated and blocked as described for the $p75^{NTR}$ and TrkA immobilizations; however, they remained uncoupled. Binding was expressed as relative response units, which is defined as the response obtained from the flow cells containing the immobilized receptors minus the response obtained from the reference flow cells.

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

Affinity of NGF to $p75^{NTR}$ and TrkA was determined using serial dilution series. NGF was diluted in HBS–EP buffer with concentrations ranging from 0.0125 to 50 nM and was allowed a 60-s contact time followed by a 120-s dissociation phase. For experiments involving $p75^{NTR}$, the sensor chip surface was regenerated with a 15-s injection of a salt cocktail (2:1 [v/v] Pierce elution buffer and 4 M NaCl) previously described by Abdiche and coworkers [23]. In experiments with TrkA, a 15-s injection of glycine–HCl (pH 2.0) was used for sensor surface regeneration.

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

Small molecules were diluted in HBS–EP buffer at a concentration of 50 μM and injected over the immobilized $p75^{NTR}$ and TrkA receptors for 60 s. All compounds were allotted a 120-s dissociation time. Receptor binding was determined by assessing the binding response of each compound in response units (1 RU = 1 pg/mm^2). The sensor chip surfaces were regenerated as described above.

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

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

IC_{50} determination

Small molecules were diluted in HBS–EP buffer at varying concentrations ranging from 320 μM to 3.2 nM, corresponding to equal spacing on a logarithmic scale. Compounds were pre-incubated for 1 h with 10 nM NGF before injection over the immobilized $p75^{NTR}$ or TrkA. Dose–response curves were generated and were used for the determination of the half-maximal inhibitory concentrations for all compounds.

Data analysis

Transformation of data for NGF–receptor affinity analyses, small molecule receptor–interactions, and inhibition experiments was performed with BIA evaluation software from GE Healthcare Life Sciences. The generation of dose–response curves and the