Differential Activity of the Nerve Growth Factor (NGF) Antagonist PD90780 [7-(Benzolylamino)-4,9-dihydro-4-methyl-9-oxo-pyrazolo[5,1-b]quinazoline-2-carboxylic Acid] Suggests Altered NGF-p75<sup>NTR</sup> Interactions in the Presence of TrkA

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ABSTRACT

The neurotrophin nerve growth factor (NGF) binds to two receptor types: the tyrosine kinase receptor TrkA and the common neurotrophin receptor p75<sup>NTR</sup>. Although many of the biological effects of NGF (such as neuronal growth and survival) are associated with TrkA activation, p75<sup>NTR</sup> also contributes to these activities by enhancing the action of TrkA when receptors are coexpressed. The NGF antagonist PD90780 [7-(benzolylamino)-4,9-dihydro-4-methyl-9-oxo-pyrazolo[5,1-b]quinazoline-2-carboxylic acid] interacts with NGF, preventing its binding to p75<sup>NTR</sup>. In this study, the actions of this compound are further explored, and it is found that PD90780 is not able to inhibit the binding of either brain-derived neurotrophic factor or neurotrophin-3 to p75<sup>NTR</sup>, consistent with the direct interactions of the antagonist with NGF. In addition, we demonstrate that the ability of PD90780 to inhibit NGF-p75<sup>NTR</sup> interactions is lower when receptors are coexpressed, compared with when p75<sup>NTR</sup> is the only neurotrophin receptor expressed. These results suggest that the interaction between NGF and the p75<sup>NTR</sup> receptor is altered when TrkA is coexpressed. This alteration can be exploited in the development of antagonists that will selectively inhibit the pro-apoptotic actions of p75<sup>NTR</sup> when expressed in the absence of TrkA, although having less effect on the pro-survival effects of p75<sup>NTR</sup> mediated by enhanced TrkA activation.

Neurotrophins function to regulate the survival, growth, and differentiation of neurons. Nerve growth factor (NGF), the first discovered and most extensively researched member of this protein family, selectively binds to a tyrosine kinase receptor, TrkA (Kaplan et al., 1991; Klein et al., 1991). NGF-TrkA interaction results in the phosphorylation of the receptor and initiates an intracellular cascade of reactions that encourage prosurvival events within a neuron (for review, see Kaplan and Miller, 1997).

NGF is also able to bind to a second receptor type, the common neurotrophin receptor p75<sup>NTR</sup>. Like TrkA, this receptor is capable of promoting the trophic actions of NGF (Hempstead et al., 1991; Verdi et al., 1994), generally thought to be mediated through an enhancement of TrkA activation. Alternatively, binding and subsequent activation of the p75<sup>NTR</sup> receptor by NGF is also able to promote pro-apoptotic effects through signaling mechanisms independent of TrkA. This has been noted in mature rat oligodendrocytes expressing p75<sup>NTR</sup>, but not TrkA, where NGF has been found to induce cell death (Casaccia-Bonnefil et al., 1996) and via p75<sup>NTR</sup> activation in mouse ganglion cells (Bamji et al., 1998). In addition, cell death in the developing retina has...
been attenuated by a reduction in NGF (Frade et al., 1996; Frade and Barde, 1998) and was reduced in the embryonic spinal cords of mice with deletions in either NGF or p75NTR genes (Frade and Barde, 1999).

Although NGF is capable of binding to both TrkA and p75NTR, its affinity for each is dependent upon the presence of the alternate receptor. The TrkA receptor possesses a higher binding affinity for NGF when p75NTR is coexpressed compared with when TrkA is expressed in the absence of p75NTR (Hempstead et al., 1991; Dechant et al., 1993; Mahadeo et al., 1994). Consistent with this, it has also been noted that preventing the binding of NGF to p75NTR can reduce binding of NGF to TrkA (Barker and Shooter, 1994).

Recent research has supported the notion that the presence of p75NTR affects high-affinity NGF binding to TrkA, observing that high-affinity binding was dependent upon the ratio of both receptors (Esposito et al., 2001). This binding was not altered by mutations in the extracellular NGF binding domain of p75NTR; however, mutations in either cytoplasmic or transmembrane domains of p75NTR and TrkA revealed that high-affinity binding is likely mediated through interactions within these domains. This supports previous findings that p75NTR and TrkA are associated as a hetero-oligomer on the cell surface (Huber and Chao, 1995; Gargano et al., 1997; Ross et al., 1998). The importance of the p75NTR cytoplasmic domain has also been shown in research that found that overexpression of this region results in substantial neuronal cell death (Majdan et al., 1997). In another study, experiments involving TrkA receptor chimeras revealed that the presence of p75NTR alters the binding site for NGF on TrkA, thereby affecting the high-affinity binding of NGF to this receptor (Zaccaro et al., 2001).

Although previous studies looking at interactions between receptors have focused on the effects of NGF binding to TrkA in the presence of p75NTR, the effects of coexpression upon NGF binding to p75NTR have remained unclear. Our laboratory has previously demonstrated that the affinity of NGF for p75NTR decreases when TrkA is also expressed (Ross et al., 1998). From these findings, it was hypothesized that this receptor-dependent relationship could be elucidated further through comparative concentration effect studies that utilize a NGF antagonist capable of selectively inhibiting the binding of NGF to the p75NTR receptor.

One such compound was characterized in a study where its ability to inhibit the binding of NGF to p75NTR was demonstrated using recombinant extracellular p75NTR binding domains (Spiegel et al., 1995). The antagonist effects of this nonpeptide compound, a pyrazoloquinazolone (PD90780), were demonstrated to result from an interaction between PD90780 and NGF, rather than an interaction between PD90780 and p75NTR. These authors confirmed their observations in the cell-free assay system using an ovary cell line that was transfected with the p75NTR receptor. In both experimental models, the TrkA receptor was not present; therefore, any potential influence it might have had on the ability of PD90780 to inhibit NGF binding to p75NTR was not evaluated.

In the present study, the ability of PD90780 to inhibit NGF-p75NTR binding in the presence of the TrkA receptor was investigated. This was evaluated using rat pheochromocytoma (PC12) cells that express both p75NTR and TrkA receptors, PC12<sup>neo5</sup> cells that express only p75NTR, as well as with truncated p75NTR. These experiments, therefore, permitted a comparison of the antagonist effects of PD90780 on NGF binding with p75NTR when the receptor is coexpressed with TrkA, expressed in the absence of a Trk receptor, and when it is in a soluble state.

Materials and Methods

Radiolabeled Neurotrophin and Receptor Preparation. The iodination of NGF (mouse 2.5s; Cedarlane Labs, Toronto, ON) and rhBDNF (Alomone Labs, Jerusalem, Israel) was performed as described previously for NGF (Sutter et al., 1979) with modification (Ross et al., 1997). The ability of PD90780 to block neurotrophin binding to the p75NTR receptor was evaluated under various receptor conditions. This was accomplished with the use of PC12 cells (TrkA and p75NTR), PC12<sup>neo5</sup> cells (p75NTR only), and truncated p75NTR. The two cell types were cultured in RPMI 1640 medium with 10% fetal calf serum. Recovery of the cells was permitted with the replacement of the medium with calcium-magnesium free balanced salt solution followed by a 15-min incubation at 37°C. Cells were centrifuged, and pellets were suspended in HKR buffer (10 mM HEPES, pH 7.35 containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>P<sub>4</sub>, 1 g/l glucose, and 1 g/l bovine serum albumin). In the case of truncated p75NTR, the culture medium used to grow PC12 cells was removed and centrifuged to ensure it was free of cells. This medium contained p75NTR extracellular domains previously sloughed by the cells (molecular weight approximately 50 kDa; DiStefano and Johnson, 1988).

Chemical Cross-Linking of <sup>125</sup>I-NGF to TrkA and/or p75NTR in the Presence of Antagonists and Immunoprecipitation. <sup>125</sup>I-NGF (0.1 nM) alone or in combination with NGF (100 nM), BDNF (10 nM), or PD90780 (100 μM; Parke-Davis Pharmaceuticals, Ann Arbor, MI) was incubated with PC12 cells at a concentration of 10<sup>6</sup> cells/ml in HKR buffer in 1-ml volume for 2 h at 4°C with rocking. After binding, 20 μl of the cross-linker bis-(sulfosuccinimidyl)suberate (BS<sub>3</sub>) was added (final concentration of 0.4 mM) to each sample and incubated at room temperature for 30 min. The cells were washed three times with TBS, with which reducing SDS sample buffer was added to the pelleted cells to dissolve the proteins, or in the case of immunoprecipitations, prepared as described below. Cell samples undergoing immunoprecipitations for TrkA or p75NTR were solubilized in lysis buffer (TBS containing 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM EGTA) and incubated for 40 min at 4°C. After centrifugation, the lysates were removed to a new tube, and either rabbit polyclonal anti-Trk cytoplasmic domain antibody or rabbit polyclonal anti-p75NTR antibody (9992) (antisera against glutathione S-transferase-fusion protein containing the cytoplasmic domain of p75NTR; obtained from Dr. M. Chao) was added to the soluble proteins to isolate the respective receptors. The samples were left to incubate at 4°C overnight. Antibody complexes were removed through application and incubation with 70 μl of slurry of immobilized Protein G (Pierce Chemical, Rockford, IL) for 2 h at 4°C. The solid phase was washed with lysis buffer three times, with distilled water once, and then the proteins were dissolved in SDS sample buffer. Proteins from cross-linking and immunoprecipitation experiments were separated via 6% SDS-polyacrylamide gel electrophoresis (PAGE).

Chemical Cross-Linking of <sup>125</sup>I-NGF to p75NTR and Concentration Effect Assays. <sup>125</sup>I-NGF was incubated at 4°C for 2 h with or without PD90780. PC12 or PC12<sup>neo5</sup> cells were added at 10<sup>6</sup> cells/ml, and samples were incubated at 4°C for 2 h with rocking. Bound <sup>125</sup>I-NGF and p75NTR proteins were cross-linked with final concentrations of 5 nM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 2 mM sulfo-N-hydroxy-sulfosuccinimide (SNHS) (20 μl of each) and incubated with rocking at room temperature for 30 min. Samples were washed with TBS (10 mM Tris(hydroxymethyl)-aminomethane [pH 8.0] and 150 mM NaCl) three times before...
the addition of reducing SDS sample buffer to dissolve the proteins. The proteins were then separated on a 6% SDS-PAGE gel. In the experiments involving truncated p75NTR-NGF or -BDNF was exposed to the same concentrations of PD90780 and incubated with medium containing truncated p75NTR before cross-linking with EDC/SNHS. The reaction was quenched by adding 15 μl of 1 M glycine followed by 10 min of mixing. The samples were then immunoprecipitated using 192 IgG, which recognizes the extracellular domain of p75NTR (Calbiochem, San Diego, CA).

Neurotrophin Receptor Binding. Neurotrophins NGF, BDNF, and NT-3 were iodinated, and PC12 and PC12nnr5 cells were cultivated and recovered as previously described. Tubes were set up containing single data points that held iodinated neurotrophin (0.5 nM, PD90780 (10 μM), a final concentration of 106 cells/ml and NGF (at 50 nM for nonspecific binding) as required, and were then incubated at 4°C for 2 h. Aliquots (100 μl) were layered on top of 200 μl of 10% glycerol in HKR buffer in 0.4-ml tubes. Samples were then centrifuged at 5000 rpm for 2 min, after which the tip containing the cell pellet was cut off and radioactivity present was determined.

TrkA Phosphorylation Assay. Modification of methods described permitted determination of TrkA phosphorylation (Ross et al., 1998). NGF (40 pM) was incubated with varying concentrations of PD90780 (3, 30, or 300 μM) for 2 h in HKR buffer. PC12 cells used at 106 cells/ml were incubated with NGF and PD90780 solutions for 15 min at 37°C. Samples were washed once with cold PBS and once with cold TBS and then lysed with solutions containing 500 μM orthovanadate and immunoprecipitated with anti-Trk antibody as previously described. An SDS-PAGE run on 6% gel followed by Western blot analysis performed with antiphosphotyrosine antibody (4G10; UBI, Lake Placid, NY) and visualized with ECL (Amersham, Baie d’Urfe, Quebec) permitted the resolution of isolated phosphorylated proteins. The resulting bands were quantified via densitometry analysis.

NGF Protomer Cross-Linking. 125I-NGF (0.1 nM) was incubated for 2 h at 4°C with ZnCl2 (100 μM), PD90780 (30 μM), or ZnCl2 and PD90780 (100 and 30 μM, respectively), along with HKR buffer, for a total volume of 0.1 ml. After incubation, BS3 was added (final concentration of 0.4 mM) in 5-μl volume and set at room temperature for 30 min. Proteins were dissolved with the addition of 50 μl of SDS sample buffer and heating to 95°C for 10 min. Separation of 125I-NGF dimers and 125I-NGF monomers was completed using a 15% acrylamide gel SDS-PAGE.

Analysis of Cross-Linking Experiments. Following SDS-PAGE, gels were fixed and dried, and the radio-iodinated ligands cross-linked to receptors were detected via autoradiography. Receptor ligand bands within SDS-PAGE gels were excised, and radioactivity within each band was detected with a Beckman gamma counter. The concentration-effect curves, S.E.M., IC50 values, and 95% confidence intervals (CIs) described in the concentration effect studies were determined by nonlinear regression analyses and carried out by the program GraphPad Prism, version 3.00 (GraphPad Software Inc., San Diego, CA).

Results

Docking of PD90780 to NGF. Flexible docking of PD90780 (Fig. 1A) to the p75NTR binding site of NGF was carried out by the program FlexiDock incorporated into molecular modeling package SYBYL 6.9 (Tripos Inc., St. Louis, MO). The obtained binding mode is illustrated in Fig. 1B. Stability of the complex is maintained by intermolecular ionic and H-bonding interactions augmented by hydrophobic interaction. This mode has the following features: 1) the carboxylic group is flanked by two basic residues, K34 and K95; 2) the C-4 carbonyl group of the pyridinone ring is H-bonded to three basic residues, K32, K34, and R100; 3) nitrogen of the five-membered ring is H-bonded to K34; 4) amide carbonyl is H-bonded to K32; and 5) the terminal benzene ring forms hydrophobic interactions with F101 and I31. This binding mode is consistent with the observed structure-activity relationships (Jaen et al., 1995), which indicated that the carboxylic group, a polar group on the benzene ring (amide in PD90780), and the C-4 carbonyl group of the pyridinone ring are essential for biological activity.

Effects of PD90780 on BDNF and NT-3. Binding studies using both PC12 and PC12nnr5 cells were completed to determine whether PD90780 was able to inhibit the binding of either BDNF or NT-3 to p75NTR. Neither experiment involving BDNF nor NT-3 resulted in significant inhibition of binding, suggesting that PD90780 does not antagonize these two neurotrophins as effectively as it does NGF (data not shown). This was confirmed in concentration effect cross-linking experiments that analyzed 125I-BDNF binding to truncated p75NTR in the presence of PD90780 concentrations ranging from 0.1 to 100 μM (Fig. 2). Inhibition of cross-linking did not occur until PD90780 concentrations much higher than those required to block 125I-NGF association with truncated p75NTR were used (Fig. 2).

Effects of PD90780 on NGF Cross-Linking to TrkA. To evaluate the ability of PD90780 to block NGF binding to the TrkA receptor, comparative cross-linking studies were conducted. The addition of NGF (100 nM) was able to block the binding of 125I-NGF to both the TrkA and p75NTR receptors of PC12 cells. Inhibition of 125I-NGF cross-linking to p75NTR also occurred with the addition of 100 μM PD90780; however, this concentration only partially inhibited the association of 125I-NGF with the TrkA receptor when compared with total 125I-NGF cross-linking (Fig. 3). This was evaluated further in
a concentration effect TrkA phosphorylation assay. TrkA phosphorylation resulting from NGF binding was decreased only as the concentration of PD90780 approached 300 μM (H9262 M) (Fig. 4).

Conformational Changes of NGF. Previous studies have suggested that zinc alters the conformation of NGF (Ross et al., 1997; Shamovsky et al., 1999a). In doing so, zinc prevents the BS3 cross-linking of the NGF dimer, which, in the presence of reducing SDS sample buffer, dissociates to form monomers. To determine whether this BS3 cross-linking is inhibited in a similar way by PD90780, experiments comparing the maintenance of the NGF dimer in the presence of 100 μM of zinc, 30 μM of PD90780, or combinations of these two compounds were completed under reducing SDS-PAGE conditions. Zinc blocked the cross-linking and subsequent maintenance of dimerized NGF, whereas PD90780 did not appear to alter the dimer/monomer ratio of the control (Fig. 5).

Effects of NGF Receptor Expression on PD90780 Action. To determine whether the presence of TrkA alters the ability of PD90780 to inhibit the binding of 125I-NGF to p75NTR, concentration effect cross-linking studies involving both PC12 and PC12nnr5 cell lines were compared. 125I-NGF was exposed to concentrations of PD90780 ranging from 0.1 to 100 μM and then introduced to either cell line (Fig. 6A). Increasing PD90780 concentration resulted in a greater inhibition of NGF-p75NTR cross-linking, which occurred at a lower concentration of PD90780 in the PC12nnr5 cell line. Quantitative analysis showed that the IC50 value for PD90780 inhibition of NGF-p75NTR association in PC12 cells was 23.1 μM (95% CI; 16.8–31.7 μM). In PC12nnr5 cells, this IC50 value was calculated as 1.8 μM (95% CI; 1.3–2.5 μM). The differential ability of PD90780 to affect NGF binding to p75NTR in PC12 versus PC12nnr5 cells is further demonstrated in Fig. 6B, where the curve representing NGF cross-linking to the p75NTR on PC12nnr5 cells is shifted to the left when compared with cross-linking involving PC12 cells that contain both TrkA and p75NTR. This data were further supported by similar experiments involving truncated p75NTR. 125I-NGF interactions with the soluble extracellular domain of p75NTR were blocked at concentrations of PD90780 more similar to those that affected cross-linking to p75NTR in the PC12nnr5 cells than in the PC12 cell line (Fig. 2). The IC50 value for these experiments was calculated as 0.5 μM (95% CI; 0.4–0.6 μM).

Discussion

Previous studies have attempted to uncover and further classify the roles and actions of NGF and its receptors using NGF antagonists. In a recent study, the presence and combination of receptor types expressed on different cell lines affected the ability of a NGF antagonist, Ro 08-2780, to modify the pro-apoptotic and pro-survival actions of nerve growth factor (NGF) (Ross et al., 1997). In doing so, zinc prevents the BS3 cross-linking of the NGF dimer, which, in the presence of reducing SDS sample buffer, dissociates to form monomers. To determine whether this BS3 cross-linking is inhibited in a similar way by PD90780, experiments comparing the maintenance of the NGF dimer in the presence of 100 μM of zinc, 30 μM of PD90780, or combinations of these two compounds were completed under reducing SDS-PAGE conditions. Zinc blocked the cross-linking and subsequent maintenance of dimerized NGF, whereas PD90780 did not appear to alter the dimer/monomer ratio of the control (Fig. 5).

Fig. 2. The chemical cross-linking (EDC/SNHS) of 125I-BDNF (top panel) and 125I-NGF (bottom panel) to truncated p75NTR receptors, sloughed from PC12 cells, was progressively inhibited with exposure to increasing concentrations of PD90780. The calculated IC50 value for NGF binding to truncated p75NTR is 0.5 μM (95% CI; 0.4–0.6 μM), whereas the IC50 for BDNF binding to truncated p75NTR is 49.5 μM (95% CI; 36.4–67.3 μM).

Fig. 3. The chemical cross-linking of 125I-NGF to TrkA and p75NTR of PC12 cells using BS3. Total observed cross-linking to both receptors (A) was fully inhibited by NGF (100 nM; B). Cross-linking of 125I-NGF to p75NTR was inhibited by the presence of both BDNF (10 nM, C) and PD90780 (100 μM, D). Immunoprecipitations of p75NTR and TrkA are shown in E and F, respectively.

Fig. 4. PC12 cell TrkA receptor phosphorylation levels with 40 pM NGF (+) or in the absence of NGF (−) after exposure to varying concentrations of PD90780. The IC50 of PD90780 is 45.1 μM (95% CI; 11.4–178.5 μM).

Fig. 5. The maintenance of 125I-NGF dimer by the chemical cross-linking of ligands with BS3 in the presence of zinc and PD90780. Without the addition of BS3 cross-linker, 125I-NGF separated into monomers in reducing SDS-PAGE conditions (A). Dimers seen in the sample containing only 125I-NGF and BS3 (B) were inhibited by the presence of zinc (100 μM; C). This inhibition was also noted in the 125I-NGF sample exposed to both zinc (100 μM) and PD90780 (30 μM; E), but not in the sample exposed only to PD90780 (30 μM; D).
The antagonist ALE-0540 prevented NGF binding to both p75NTR and TrkA receptors (Owolabi et al., 1999) and was used in the same study to examine and outline a role for NGF in persistent pain states. Here, we have evaluated the location and selectivity of the association of PD90780 with NGF, clarified the actions of this compound, and used the ability of PD90780 to selectively antagonize NGF binding to the p75NTR receptor to analyze how the presence of TrkA might affect NGF-p75NTR interactions.

It has been previously noted that PD90780 acts through direct interaction with NGF (Spiegel et al., 1995); however, the binding mode has not been elucidated. The theoretically predicted binding mode obtained in this report (Fig. 1B) is consistent with observed structure-activity relationships (Jaen et al., 1995) and with the general location of the PD90780 recognition site predicted by Spiegel et al. (1995), namely the loops I and IV hairpin domains that include Lys-32, Lys-34, and Lys-95. This study provides details of interactions with these key residues and also suggests that additional ionic interactions with Arg-100 take place, as well as hydrophobic interactions with Phe-101 and Ile-31. Several of these residues have been previously demonstrated to participate in the interaction of NGF with p75NTR. Specifically, the direct involvement of NGF residues Lys-32, Lys-34, Lys-95, Arg-100, and Ile-31 in binding with p75NTR and biological activity has been demonstrated (Drinkwater et al., 1991; Ibáñez et al., 1992; Ibáñez, 1994; Shamovsky et al., 1999b). If the predicted binding mode of PD90780 is correct, the binding site for p75NTR is blocked when NGF is bound to PD90780, in which case NGF would not be able to participate in p75NTR interactions, which is consistent with observations.

The selectivity of the association of the antagonist with NGF was displayed in binding studies comparing the effects of PD90780 on this neurotrophin with its effects on BDNF and NT-3. The specific binding of BDNF or NT-3 to receptors did not decrease in the presence of PD90780 as it did for NGF. This was supported by experiments displaying limited inhibition of BDNF cross-linking to truncated p75NTR at concentrations of PD90780 lower than 100 μM (Fig. 2). The p75NTR binding domains for BDNF and NT-3 are spatially and geometrically similar to the NGF binding domain; however, they include different sets of residues (Ryden et al., 1995; Shamovsky et al., 1999b). Given the lack of similarity between primary sequences of BDNF, NT-3, and NGF in this region, PD90780 would not be expected to interact with BDNF or NT-3 nor inhibit their interactions with p75NTR.

To evaluate the specificity of PD90780 antagonism of NGF-p75NTR interactions, the ability of PD90780 to block NGF-TrkA interactions was examined and compared with its ability to inhibit NGF binding to p75NTR. Cross-linking and phosphorylation studies revealed that PD90780 is less effective in the TrkA paradigm. These effects may be indirect and result from the inhibition of NGF-p75NTR interaction, similar to the inhibitory effects of BDNF on TrkA binding and phosphorylation (Barker and Shooter, 1994). PD90780, therefore, does not block NGF binding to TrkA, and it blocks its asso-
ciliation with p75NTR. Further evaluation of the effects of PD90780 on properties of NGF demonstrated that PD90780 does not block the BS6 cross-linking of NGF dimers. These results suggest that it does not act in a similar way to other NGF antagonists, such as Zn(II) or Cu(II), which are thought to inhibit the normal functioning of NGF by inducing a conformational change (Ross et al., 1997, 2001; Shamovsky et al., 1999a).

Cross-linking studies using two different cell lines evaluated the ability of PD90780 to inhibit the binding of NGF to the p75NTR receptor. Differences were found in IC50 values within each cell line. The IC50 value for PD90780 inhibition of NGF-p75NTR interaction in PC12 cells (where both NGF receptors are coexpressed) was 23.1 M, whereas that involving p75NTR within PC12avr5 cells (where only p75NTR is expressed) was 1.8 M. Conventional binding studies would not permit the characterization of multiple affinity states for multiple receptor types (minimum of four affinity states) and, therefore, could not have been used to obtain these data.

It has been shown that the affinity of NGF for p75NTR is decreased when TrkA is coexpressed (Ross et al., 1998). It would be anticipated, therefore, that the effectiveness of PD90780 should increase when TrkA is present. This is not the case, which suggests that TrkA changes the way that NGF binds to p75NTR. This is further confirmed by similar experiments that measured the antagonist action of PD90780 on NGF binding to truncated p75NTR receptors. In these experiments, PD90780 blocked NGF-p75NTR association at a similar concentration to that required for the inhibition of NGF interaction with the p75NTR of PC12avr5 cells, resulting in an IC50 value of 0.5 M. These results are consistent with previous studies, which were based on competitive binding assays that found that the PD90780 IC50 for p75NTR not associated with TrkA was only 220 nM (Spiegel et al., 1995).

Although recent X-ray crystallographic studies of the complex of NGF with the d5 subdomain of TrkA have indicated that the TrkA-d5 domain binds to loop I of NGF (Wiesmann et al., 1999), it does not directly interfere with the binding site of PD90780 illustrated in Fig. 1B. However, it is possible that other TrkA receptor regions can interfere with this binding. Therefore, whether a decreased ability of PD90780 to inhibit binding of NGF to p75NTR in the presence of the TrkA receptor is due to induced conformational changes within p75NTR or a direct competition of TrkA and PD90780 for the same recognition site within NGF remains unclear.

Previous analysis of p75NTR and TrkA interactions has indicated that TrkA functioning is enhanced when the receptors are coexpressed. This is thought to occur either through direct allosteric interactions between the receptors (Mahadeo et al., 1994; Ross et al., 1998; Esposito et al., 2001; Zaccaro et al., 2001) or through an increase in the local concentration of NGF at the cell surface as a result of p75NTR presence (Barker and Shooter, 1994; Ryden et al., 1997). Recent studies have implicated the former model in p75NTR augmentation of TrkA. A current publication has demonstrated that, in the presence of NGF, p75NTR proximity enhances the actions of TrkA (Esposito et al., 2001). This investigation suggests that the transmembrane and cytoplasmic domains of the receptors are responsible for the regulation of high-affinity NGF binding. In addition, the presence of p75NTR has been suggested to alter the binding site for NGF on TrkA (Zaccaro et al., 2001).

Our research supports the claim that direct interactions between receptors can change how NGF binds to a receptor and extends this concept beyond high-affinity binding to the TrkA receptor that has been discussed previously to include differential binding to the p75NTR receptor. The p75NTR- TrkA receptor interactions are, therefore, capable of affecting the binding of NGF to either TrkA or to the p75NTR receptor.

Previous research has also indicated, in addition to modified ligand binding, that coexpression of p75NTR and TrkA can influence TrkA signaling (Barker and Shooter, 1994; Hantzopoulos et al., 1994), perhaps through intracellular signaling mechanisms that lead to downstream modulation (Dostaler et al., 1996; Wang et al., 1998; Yamashita and Toyama, 2003). If TrkA expression also influences the signal of p75NTR, this relationship would have implications for the initiation of apoptosis that has been observed through p75NTR activation under certain circumstances (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Bamji et al., 1998).

To date, the effects of PD90780 have been observed in research where its biological activity in a chick neural retina cell model was noted. The compound was shown to display anti-apoptotic effects by attenuating the NGF-mediated apoptosis of these cells (Allington et al., 2001).

The study presented here demonstrates the potential for specific NGF antagonists that could selectively target and prevent the pro-apoptotic actions of NGF without inhibiting its survival promoting functions mediated through TrkA and p75NTR-enhanced TrkA receptor activation. Such compounds may have utility in preventing neuronal loss in disorders where p75NTR-mediated cell death has been implicated, such as Alzheimer’s disease, amyotrophic lateral sclerosis, stroke, and epilepsy.

Recently, pro-nerve growth factor (pro-NGF), the precursor to NGF, has been observed to bind to the p75NTR receptor with high affinity and to the TrkA receptor with low affinity in comparison with its mature form (Lee et al., 2001). This, and data from the same study revealing that NGF is able to move outside of the cell, suggest that pro-NGF might be not only pro-apoptotic but also capable of acting as a ligand within the extracellular environment. Investigation into how antagonists such as PD90780 might act upon pro-NGF and how they might inhibit its binding to p75NTR may hold great potential for future research.

References


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